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NEUROPEPTIDE HORMONES FROM THE EYESTALKS OF *JASUS LALANDII*

by

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I, Heather Gaile Marco, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

June 2000

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Let there be love shared amongst us, and let there be love in our hearts …

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To God be the glory!

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ABSTRACT

The X-organ – sinus gland complex, situated in the eyestalks of decapod crustaceans, are known to be a source of a variety of neuropeptide hormones that regulate a number of diverse physiological processes. This neuroendocrine complex was investigated in 3 crustacean species, viz. the European shore crab *Carcinus maenas*, and 2 spiny lobster species *Jasus lalandii* and *Panulirus homarus* by means of tissue immunocytochemistry and an enzyme-linked immunosorbent assay (ELISA). Positive immunoreactions, associated with the X-organ – sinus gland system only, were obtained with antisera raised against crustacean hyperglycaemic hormone (cHH) of the American lobster (*Homarus americanus*), the Mexican crayfish (*Procambarus bouvieri*) and the edible crab (*Cancer pagurus*), as well as with antisera raised against vitellogenesis-inhibiting hormone (VIH) of the *H. americanus* and moult-inhibiting hormone (MIH) of *C. pagurus*. This is the first time that the immunolocalisation of these 3 hormones have been studied in a single crustacean species. The chief results of this comparative immunocytochemical study showed that (1) neuropeptide hormones of the shore crab and the 2 spiny lobster species were sufficiently homologous in primary structure to be recognised by the heterologous antisera, thus, an indication of conserved peptide structures across the species and infraorder boundaries; (2) preabsorbed complexes of purified peptides and antisera from the edible crab did not produce any immunoreactions in tissue immunocytochemistry, nor in ELISA, thus, indicating the specificity of the anti-cHH and anti-MIH sera; (3) the anti-VIH serum demonstrated the ability to bind epitopes on cHH and MIH peptides and is, thus, not a specific antiserum in this study; (4) there is co-localisation of cHH, MIH, VIH immunoreactivity in the eyestalk neuroendocrine complexes of all 3 species studied which suggests that the different peptide hormones can be synthesized in the same neuronal cell bodies. This co-localisation of neuropeptides in the eyestalk of *J. lalandii* was confirmed by a double-staining immunoflourescence experiment, and finally (5) immunoreactivity of antisera raised against cHH of *H. americanus* and MIH of *C.*

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pagurus was associated with distinct and unique peak fractions, following reverse-phase high pressure liquid chromatographic (RP-HPLC) separation of sinus gland extracts from *J. lalandii*.

A total of 6 neuropeptide hormones belonging to the cHH/MIH/VIH peptide family were isolated, functionally characterised and sequenced from extracts of sinus glands from the South African west coast rock lobster, *Jasus lalandii*. This is the first complete report on these peptides from any species belonging to the Palinuridae infraorder.

The cHH-family peptides were identified after RP-HPLC by ELISA; analyses using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry showed that a single HPLC step yielded sufficiently pure fractions of these peptides for sequencing by automated Edman degradation. A novel strategy was employed to fully elucidate the primary structures of these large, hydrophobic neuropeptides with their 6 cysteine residues and 3 interconnecting disulfide bridges: (1) only native, i.e. non-linearised (non-reduced) peptide material was used for initial N-terminal sequencing and for generating specifically-cleaved peptide fragments; (2) these peptide fragments, arising from digestion with endopeptidases and chemicals, were separated on HPLC, analysed by mass spectrometry and sequenced; (3) interpretation of the sequence information from these fragments was facilitated by knowledge of the N-terminal sequence of the peptide and from overlapping sequence information.

Functional analyses of the cHH-family peptides from *J. lalandii* were carried out by means of biological assays: (1) hyperglycaemia:- a homologous *in vivo* assay in which the concentration of glucose in the haemolymph is measured spectrophotometrically before and after injection of the purified peptides; (2) moult-inhibition:- a homologous *in vitro* assay in which the effect of the purified peptides on the output of moulting hormones (ecdysteroids) from the moulting gland (Y-organ) is assessed by means of a competitive enzyme immunoassay (EIA); and (3) vitellogenesis-inhibition:- a

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heterologous *in vitro* assay in which the effect of purified peptides on the *de novo* synthesis of peptides in the ovary of a penaeid prawn is determined by incorporation of radiolabeled amino acids into ovarian proteins; this was assessed by radioactivity counts of the proteins, as well as by gel electrophoresis coupled with autoradiography.

Two biologically active hyperglycaemic peptides were isolated from *J. lalandii*. Both peptides have: (i) a free N-terminus and an amidated C-terminus, (ii) 72 amino acid residues, and (iii) 6 cysteine residues at positions 7, 23, 26, 39, 43 and 52. The major isoform (code-named *Jala* cHH-I) has a molecular mass of 8380 Da and is present in a ratio of 4:1 relative to the minor isoform (*Jala* cHH-II) which has a mass of 8357 Da. The *Jala* cHHs have 90 % sequence identity. The *Jala* cHH-I fraction seems to consist of homogenous material: the presence of different chiral forms of this peptide, viz. L-Phe^3 versus D-Phe^3 molecules have been investigated with the aid of synthetic peptides, HPLC and mass spectrometry. Only the L-Phe^3 form of *Jala* cHH-I was isolated.

Two other peptides, identified as cHHs by ELISA, demonstrated no hyperglycaemic activity; their full primary sequence revealed that they were C-terminally truncated forms of *Jala* cHH-I and –II. These truncated cHH peptides were also inactive in the moult-inhibition and vitellogenesis-inhibition assays, which suggests that the C-terminal part of the cHH peptide is important for biological activity.

A fifth peak of irregular frequency, with cHH immunoreactivity, a molecular mass of 8389 Da and a less-hydrophobic nature, was partially sequenced up to residue 52; the sequence was 100 % identical to *Jala* cHH-I. It is believed that this peak represents a modified form of *Jala* cHH-I in which the methionine (Met) residue at position 71 has undergone oxidation. This theory was confirmed by carrying out a tryptic digest with subsequent mass analyses.

In a comparative, preliminary study, sinus gland extracts from another South African spiny lobster species, *Palinurus gilchristii*, were passed over HPLC and a putative cHH was identified by ELISA. This peptide of mass 8523 Da, was partially sequenced (residues 1-30) with 100 % sequence identity to *Jala* cHH-I. It is predicted that amino

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acid substitutions are likely to occur in the complete sequence that will account for the mass difference and chromatographic behaviour of the putative *Pagi* cHH. It is interesting to observe that species of the same infraorder share so much sequence identity; this might be a useful indicator of phylogenetic relationships.

Considerable moult-inhibiting activity was detected in a peak that was immunochemically also identified as a peptide with MIH antigenic epitopes. This peptide (code-named *Jala* MIH) has 74 amino acid residues, a mass of 9006 Da, a free N-terminus and an amidated C-terminus. The full sequence reveals 6 cysteine residues in unique positions for an MIH peptide, viz. 6, 23, 26, 39, 43 and 52. *Jala* MIH is 40 % identical to other known MIHs and 30 % identical to the cHHs.

Jala cHH-I also displayed moult-inhibiting activity. The major ecdysteroid in the haemolymph of *J. lalandii* appears to be 20-hydroxyecdysone, as established by normal-phase (NP) HPLC on haemolymph samples and EIA. The profile of the circulating ecdysteroid titre during different stages of the moult cycle of *J. lalandii* is presented; the distinction between intermoult, premoult and postmoult is easily made on this basis. A dramatic decrease in the ecdysteroid titre is evident in the late-premoult period (shortly before ecdysis) in eyestalk-ablated spiny lobsters, as well as in spiny lobsters with the eyestalks intact. This excludes the role of MIH at this stage and argues for negative-feedback from the Y-organ itself, or an extra-eyestalk source of MIH. Crude extracts of sinus glands have no inhibitory effect on Y-organs from postmoult spiny lobsters (at least, 3-21 days post-ecdysis) and the output of ecdysteroids during this time is very low. Again, the level of control over the Y-organ at this stage is not resting with MIH.

VIH activity is not attributable to a unique peptide of *J. lalandii*. *Jala* cHH-II showed the strongest inhibitory effect on *de novo* protein synthesis in the prawn ovary (46% inhibition), followed by the Met-oxidised *Jala* cHH-I (34 %) and *Jala* cHH-I (27 % inhibition). *Jala* MIH had only a slight effect (15 % inhibition) in this assay and the truncated cHHs had no effect. General protein synthesis was inhibited in these assays

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and not exclusively the synthesis of vitellin.

Non-cHH-family peptides were repurified and isolated from the less-hydrophobic HPLC fractions that resulted from separation of hydrophobic peptides from crude extracts of *J. lalandii* sinus glands. A total of 5 peptide sequences were elucidated. One of these is the red pigment-concentrating hormone (RPCH). This octapeptide displayed pigment-concentrating biological activity in a heterologous *in vivo* assay with shrimps. The full elucidation of the primary sequence revealed that this is not a novel sequence; in fact, all the crustacean species studied to date, from a variety of infraorders, have this same, conserved, RPCH peptide structure. The antagonist to RPCH, i.e. pigment-dispersing hormone (PDH), was not detected and isolated in this study. It is believed that this peptide hormone is not preserved during the extraction procedure.

Four structures were completely elucidated that are homologous to crustacean hyperglycaemic hormone precursor-related peptides (CPRPs). The biological function or relevance of these CPRPs have not yet been determined in any species. The 4 CPRPs of *J. lalandii* eluted as 2 sets of peptides, one set being more hydrophobic than the other. The first set of precursor-related peptides (code-named *Jala* CPRP-1 and –2) eluted as an inseparable doublet and consisted of 32 amino acids with an identical amino acid sequence, save for a single substitution at position 18. The second set of precursor-related peptides (code-named *Jala* CPRP-3 and –4) could be completely separated; *Jala* CPRP-3 consists of 29 amino acid residues which are identical to the first 29 residues of *Jala* CPRP-4 which has a total length of 36 amino acid residues. The presence of 4 CPRPs implies that *J. lalandii* must have 4 cHH precursor molecules (preprohormones) and, hence, 4 distinct cHH peptides. Only 2 full-length cHHs have been isolated in this study, however, and it is more likely that *Jala* CPRP-1 and –2 represent microheterogeneity in the spiny lobster populations and that *Jala* CPRP-3 is a C-terminally truncated form of *Jala* CPRP-4, not arising naturally. Thus,

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only 2 CPRPs are present in a *J. lalandii* individual. This theory was not experimentally tested.

A cDNA library was constructed from RNA extracted from *J. lalandii* X-organs. Specific primers were designed to correspond to the N- and C-terminus of *Jala* cHH-I. These primers were used to amplify a 200 bp product by PCR; the sequence of this PCR product is 100 % identical to the partial sequence of *Jala* cHH-I and is, thus, a potential candidate for a probe with which to screen the *Jala* X-organ library for cHH-family precursor molecules.

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1. INTRODUCTION

1.1. *Neurosecretion*

In living organisms, the ability to coordinate the functioning of cells, tissues and organs, and to elicit the appropriate physiological/biological response to ever-changing internal and external factors, is paramount for survival. In arthropods this is achieved in three ways: (i) the nervous system, in which neurons receive and conduct electrical signals to effect a rapid response; (ii) the endocrine system, in which secretory cells are grouped together to form ductless glands from which the synthesised peptide or steroid hormones are directly released into the circulation to effect a relatively slow response in the target organs; (iii) the neuroendocrine system, which is a combination of the first 2 systems and thus, consists of secretory neurons which transport their peptide hormones rapidly along the axonal tract to the nerve terminal endings; these endings are in close proximity to the haemolymph and are collectively referred to as a neurohaemal organ, from which the neurohormones are released into the general circulation.

The neuroendocrine system is not unique to arthropods and is shown to exist in many metazoan groups, being already present in Cnidaria, the first metazoan group with neurons, and in the most recently developed group, the vertebrates (see Gabe, 1966; Berlind, 1977; Morris *et al.*, 1978). Since the concept of neurosecretion was first formulated in 1928 (see Scharrer, 1990), several neuroendocrine organs have been described and studied in vertebrates, as well as in invertebrates.

1.2. *Neurosecretion in decapod crustaceans*

Crustacean preparations have contributed tremendously to the development of concepts of neurosecretion (see Scharrer and Scharrer, 1945). Because of their

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availablity and size, decapod crustaceans have received the most attention in the experimental study of neurosecretion in crustaceans. In decapod crustaceans, neurosecretory cells are located in the cerebral ganglia, eyestalk, sub-oesophageal ganglia and throughout the thoracic and abdominal ganglia, whereas the three main neurohaemal organs are the post-commissural organs, the pericardial organs and the sinus gland (see Cooke and Sullivan, 1982).

The most extensively studied neuroendocrine complex in decapod crustaceans is the so-called X-organ - sinus gland system which is located in the eyestalks. The X-organ (XO) is the collective name given by Bellonci in 1882 to a group of neurons, of then-unknown function (see Gabe, 1966). Situated in one of the four optic ganglia, viz. the medulla terminalis, these neurosecretory neurons synthesise peptide hormones which are stored in the sinus gland (SG), so-named because the nerve endings of the XO abuts onto a large haemolymph sinus. From eyestalk ablation studies, extirpation of the SG and reimplantation studies, early experimental biologists deduced that the XO-SG complex was a source of hormones that influenced major physiological processes in crustaceans (see Kleinholz and Keller, 1979). Despite many of these biologists not taking into account the side-effects brought about by the actual surgery, a number of important conclusions were reached which has set the basis for on-going research into the inventory of neuropeptide hormones from the XO-SG complex. Information about the role of neuropeptides in the eyestalk was also gained from observations following the injection of crude extracts of eyestalks into test animals.

From these early, rather crude ways of experimentation, it became evident that the eyestalk contained factors that affected several physiological processes, such as movement of pigments, glucose metabolism, moulting and reproduction. More recently, however, with increasingly sophisticated technology available, such as high

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pressure liquid chromatography (HPLC), mass spectrometry, improvements in peptide chemistry (especially microsequencing and peptide synthesis), it became easier to purify and sequence neuropeptides from the neuroendocrine organs. Improved biological assays and the sensitivity and ease of immunological methods, also assisted in identifying and characterising neuropeptides and has led to the burgeoning of the field of invertebrate neuroendocrinology. Further, the more recent application of molecular biological techniques has provided important information about the preprohormone structures, given insights into the expression of the hormones during development, and has provided biologists with another set of "tools" with which to manipulate and study the crustacean neuropeptide hormones.

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1.3. Hormones from the XO-SG system

1.3.1. Chromatophorotropins

Many crustaceans are able to change their body colour to adapt to their background surroundings. This adaptation is accomplished by the differential movement of pigment granules in chromatophores (specialised pigment cells). Chromatophorotropins are neuropeptide hormones from the XO-SG complex which affect this pigment migration in the chromatophores of the epidermis and the ommatidia (Josefsson, 1983). The colour change is quickly effected (within minutes), is reversible and under the control of two antagonistic neurohormones.

Red pigment-concentrating hormone (RPCH). RPCH causes the aggregation of pigment in the epidermal cells, which results in an overall blanching effect. RPCH was purified from the prawn *Pandalus borealis* and was the first invertebrate neuropeptide hormone to be fully sequenced; its primary structure was determined as: pQLNFSPGWamide (Fernlund and Josefsson, 1972). This octapeptide seems to be

highly conserved in a range of crustacean species and infraorders with no change in the primary structure of RPCH peptides isolated thus far in species of crab, crayfish, prawn and shrimp (Table 1).

By comparing the structure of RPCH with neuropeptides elucidated from an insect species, it became clear that the crustacean RPCH is homologous to the metabolic neurohormones of insects, the so-called adipokinetic hormones (AKH; Mordue and Stone, 1976; Gäde, 1997). Based on the high degree of structural homology between RPCH and the octapeptide AKHs, especially the pQ^1, F^4 and W^8amide, a family of structurally related peptides was recognised and is called the RPCH/AKH peptide family (see Gäde, 1997). Whereas there is only one RPCH peptide of conserved primary structure in all crustaceans, the AKHs in insects are present as multiple isoforms with variable primary structures (see Table 1).

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Table 1. Primary structures of select members of the RPCH/AKH peptide family from arthropods. Substitutions are indicated in bold letters. For more AKH structures, see Gäde (1997).

SPECIES	SEQUENCE
RPCH*:	
Pandalus borealis[1], *Cancer magister*[2], *Carcinus maenas*[2], *Orconectes limosus*[2], *Penaeus japonicus*[3]	pQLNFSPGW-NH_2
Variants of AKH:	
Locusta migratoria (Lom-AKH-II)	pQLNFS**A**GW-NH_2
Schistocerca gregaria (Scg-AKH–III)	pQLNFS**T**GW-NH_2
Onitis aygulus (Ona-CC)	pQ**Y**NFS**T**GW-NH_2
Melolontha melolontha (Mem-CC)	pQLN**Y**SP**D**W-NH_2

* Peptides with identical amino acid compositions and with RPCH biological activity have been isolated but not sequenced from a further 7 crustacean species (see Gaus *et al.*, 1990).

[1]Fernlund and Josefsson, 1972; [2]Gaus *et al.*, 1990; [3]Yang *et al.*, 1999.

Application of the polymerase chain reaction (PCR) was successfully used to characterise a preproRPCH from a cDNA library constructed from the XO of the

European shore crab, *Carcinus maenas* (Linck *et al.*, 1993) and the blue crab, *Callinectes sapidus* (Klein *et al.*, 1995). The preprohormone structure of RPCH consists of a 25 amino acid signal peptide, the octapeptide RPCH and a RPCH-precursor-related peptide (RPRP) of 74 amino acid residues. This structural organisation (see Fig. 1) of preproRPCH is similar to that of the preprohormone of the insect AKHs, for example, in the locusts *Locusta migratoria* and *Schistocerca gregaria* there is a 22-mer signal peptide, the sequence for the AKH peptide and a 61-63 amino acid AKH-precursor-related peptide (APRP; O'Shea and Rayne, 1992). There is, however, very low homology between the RPRPs and the APRPs (O'Shea and Rayne, 1992; Gäde, 1996).

Pigment-dispersing hormone (PDH). PDH works antagonistically to RPCH to disperse the pigment in the chromatophores and the ommatidial cells of the retina, with a nett darkening effect of the epidermis and the eyes. Fernlund (1976) was the first to isolate and sequence a PDH, then referred to as DRPH (distal retinal pigment hormone), from the prawn *Pandalus borealis* (Table 2). PDH has since been elucidated from a number of crustacean species; a few amino acid substitutions have occurred in the octadecapeptide structure (Table 2). This has given rise to the classification of PDHs into 2 groups, viz. α-PDH which is prevalent in pandalid shrimps, and β-PDH which is more widely distributed (crabs, crayfish, penaeid prawns) (see Rao and Riehm, 1993; Desmoucelles-Carette *et al.*, 1996; Yang *et al.*, 1999).

Neuropeptides with pigment-dispersing activities have also been characterised in insects as pigment-dispersing factors (PDF; Table 2), which are structurally homologous to the crustacean PDHs (Rao *et al.*, 1987). Thus, PDHs and PDFs are considered to be members of a neuropeptide family common to arthropods, viz. the

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PDH/PDF family (Rao and Riehm, 1988). Although the function of PDH has been clearly established as a chromatophorotropin in crustaceans, the role of PDF in insects is not yet known.

Table 2. Primary structures of the PDH/PDF peptide family from arthropods. Substitutions are indicated in bold letters.

SPECIES	SEQUENCE
Variants of α-PDH:	
Pandalus borealis[1], *Pandalus jordani*[2]	NSGMINSILGIPRVMTEA-NH_2
Pandalus jordani[2]	NSGMINSILGIP**K**VM**AD**A-NH_2
Variants of β-PDH:	
Uca pugilator[3], *Cancer magister*[4], *Callinectes sapidus*[5], *Pacifastacus leniusculus*[6]	NSELINSILGLPKVMNDA-NH_2
Procambarus clarkii[7], *Orconectes immunis*[6], *Orconectes limosus*[8]	NSELINSILGLPKVMN**E**A-NH_2
Penaeus aztecus[9], *Penaeus vannamei I*[10]	NSELINS**L**LGI**I**PKVMNDA-NH_2
Pandalus jordani[3]	NSELINS**L**LGLPKVM**T**DA-NH_2
Penaeus vannamei II[10]	NSELINS**L**LGLPKVMNDA-NH_2
Penaeus japonicus I[11]	NSELINS**L**LGI**I**PKVM**T**DA-NH_2
Penaeus japonicus II[11]	NSELINS**L**LGLPK**F**M**I**DA-NH_2
Callinectes sapidus II[5]	NSELINS**L**LGI**SAL**MN**E**A-NH_2
Insect PDFs:	
Periplaneta americana[12]	NSELINS**L**LGLPKV**L**NDA-NH_2
Acheta domesticus[6]	NSE**I**INS**L**LGLPKV**L**NDA-NH_2
Romalea microptera[13]	NSE**I**INS**L**LGLPK**LL**NDA-NH_2
Carausius morosus[14]	NSELINS**LLA**LPKV**L**NDA-NH_2

[1]Fernlund, 1976; [2]Rao *et al.*, 1989; [3]Rao *et al.*, 1985; [4]Kleinholz *et al.*, 1986; [5]Klein *et al.*, 1994; [6]Rao and Riehm, 1993; [7]McCallum *et al.*, 1991; [8]De Kleijn *et al.*, 1993; [9]Phillips *et al.*, 1988; [10]Desmoucelles-Carette *et al.*, 1996; [11]Yang *et al.*, 1999; [12]Mohrherr *et al.*, 1991; [13]Rao *et al.*, 1987; [14]Mohrherr *et al.*, 1994.

Preprohormone structures of crustacean PDHs have been characterised in *C. maenas* (Klein *et al.*, 1992), the crayfish *Orconectes limosus* (De Kleijn *et al.*, 1993), *Callinectes sapidus* (Klein *et al.*, 1994) and the white shrimp *Penaeus vannamei* (Desmoucelles-Carette *et al.*, 1996). All of these preproPDHs consist of a signal peptide, a PDH-precursor-related peptide (PPRP) and the PDH peptide (Fig. 1). As in the case of preproRPCH, the physiological role of the precursor-related peptide of preproPDH is also unknown (De Kleijn and Van Herp, 1995).

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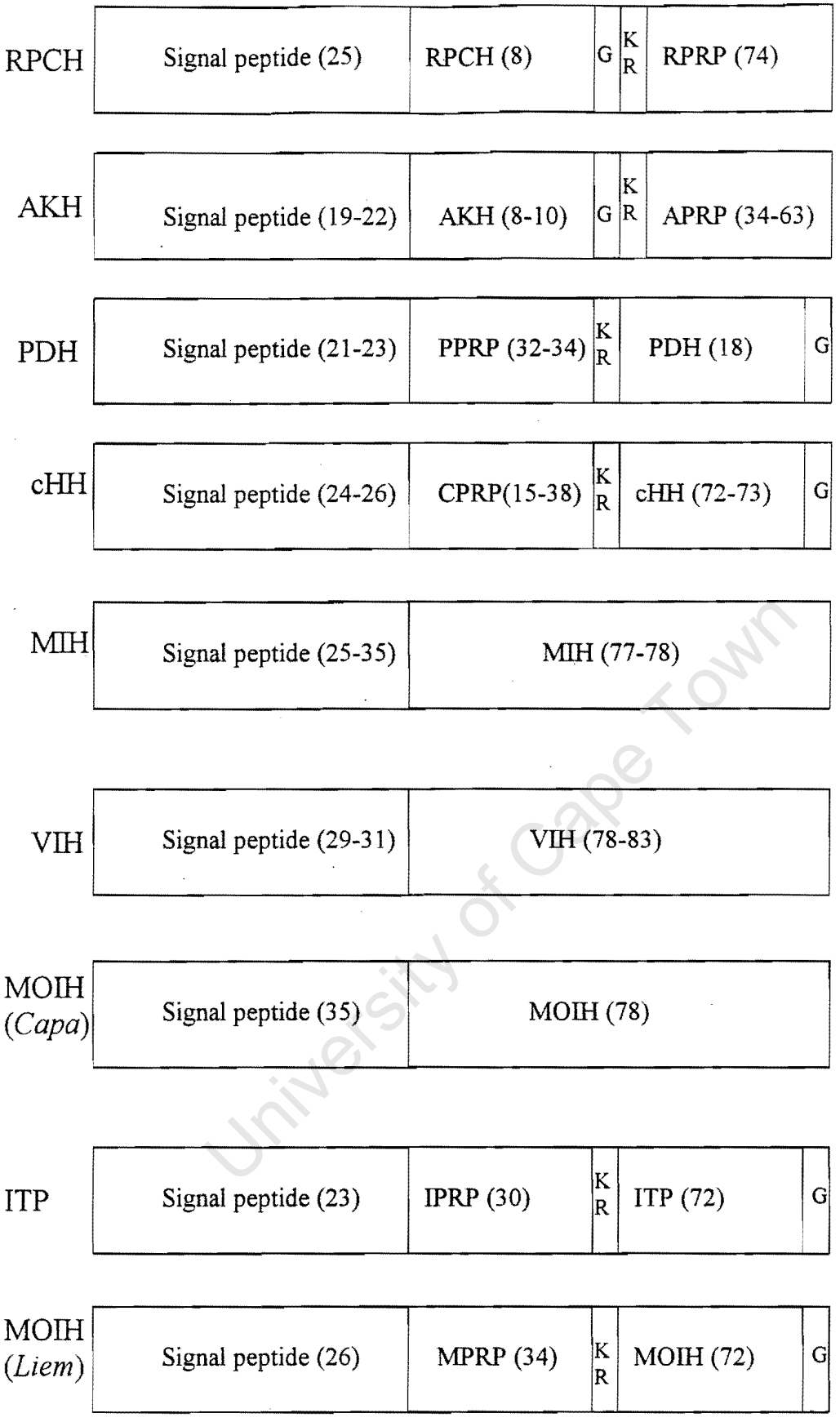

Fig. 1. Schematic diagram of the preprohormone structures of various decapod crustacean neuropeptides from the X-organ, as well as from an isopod and insects. The number of amino acid residues is indicated in brackets. One-letter code for amino acids is used to denote the dibasic cleavage site (K = lysine; R = arginine) and the amidation signal (G = glycine). *Capa* = *Cancer pagurus*; *Liem* = *Libinia emarginata*

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1.3.2. Crustacean hyperglycaemic hormone (cHH)

The injection of crude extracts of eyestalks into *C. sapidus* by Abramowitz *et al.* (1944), gave rise to an increase in blood glucose concentration, and this effect was ascribed to the presence of a "diabetogenic factor" in crustacean eyestalks. This hyperglycaemic factor was later shown to be a peptide neurohormone, which primarily mobilises glycogen from the hepatopancreas and abdominal muscle tissue to result in an elevated blood glucose concentration (Sedlmeier, 1985). Because of the relative ease with which hyperglycaemic bioassays can be conducted *in vivo* and the high abundance of cHH in the sinus gland (see Keller and Wunderer 1978), this peptide became the most extensively studied of the crustacean hormones in a number of different species. Comparative immunological and biological studies of cHH in different decapod crustaceans have provided evidence that this hormone exhibits specificity of activity within certain groups of crustaceans (Kleinholz and Keller 1973). Differences in the electrophoretic mobility of cHH from different taxonomic groups (Keller 1977), and results from immunodiffusion, immunoelectrophoresis and cross-injection studies (Leuven *et al.* 1982) all pointed towards group-specificity of cHH at the level of superfamily. This was further investigated with a more sensitive method, viz. tissue immunocytochemistry (Gorgels-Kallen *et al.* 1982), in which a positive immunoreaction was shown in several astacideans (crayfish and clawed lobsters), a palinuran (spiny lobster) and a caridean (shrimp) with anti-*Astacus leptodactylus* (crayfish) cHH serum. They could, however, not demonstrate a stable and clear immunoreaction in brachyuran (true crab) tissue; but their study nonetheless showed that cHH is not strictly group-specific on an immunological basis.

The primary sequence of a cHH was first determined from the crab, *C. maenas* (Kegel *et al.*, 1989). Since then, the amino acid sequence of cHH has been determined from

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11 decapod crustacean species and one isopod species; a high degree of identity exists between the different cHHs (see Fig. 2). In all the decapod crustacean species studied to date, cHH is present in multiple forms as a 72 amino acid long peptide. The different isoforms of cHH may result either from genetic variation, post-translational modifications, or a combination of both. In the case of brachyurans, the two isoforms differ only by one residue at the N-terminus: a pyroglutamate (pGlu) versus a glutamine (Gln) residue in the other (Chung and Webster, 1996; Chung *et al.*, 1998). In all the astacideans investigated, thus far, the cHH isoforms are N-terminally blocked by pGlu (Kegel *et al.*, 1991; Huberman *et al.*, 1993; De Kleijn *et al.*, 1994a) and, in most species, differ from each other in conformation only; that is, the L-Phe^3 residue undergoes stereoinversion to a D-Phe^3 residue (Aguilar *et al.*, 1995; Van Herp, 1998). Two exceptions are the American lobster, *Homarus americanus*, and the swamp crayfish, *Procambarus clarkii*, where two sets of two isoforms of cHH have been reported. In the former species, two distinct cHHs are 90 % identical to each other and result from two different genes; each of these cHH isoforms undergo stereoinversion to give rise to a further two cHH isoforms (Tensen *et al.*, 1991a; Soyez *et al.*, 1994). In the case of *P. clarkii*, the two cHH isoforms apparently differ only at the N-terminus by pGlu versus Gln (as was observed in brachyurans); each of these isoforms then undergo stereoinversion (as is observed with all other astacurans) (Yasuda *et al.*, 1994; Yasuda, 1997). Information on the cHH sequence from penaeids (prawn) shows the occurrence of 5 distinct cHH isoforms in the Kuruma prawn, *Penaeus japonicus*, each with a different primary sequence and arising from different genes (Yang *et al.*, 1995; 1997); 2 different cHH structures have also been reported in *Metapenaeus ensis* (Gu *et al.*, 2000). All the penaeid cHHs known to date have a free

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Fig. 2. Comparison of the amino acid sequences of cHHs from three different decapod crustacean infraorders, viz. Astacidae: [1]*Homarus americanus* (Tensen *et al.*, 1991a) and [2]*Procambarus bouvieri* (Aguilar *et al.*, 1995); Brachyura: [3]*Carcinus maenas* (Kegel *et al.*, 1989) and [4]*Cancer pagurus* (Chung *et al.*, 1998), and Penaedae: [5]*Penaeus japonicus* (Yang *et al.*, 1997), [6]*Machrobrachium rosenbergii* (Sithigorngul *et al.*, 1999a,b), [7]*Penaeus monodon* (Udomkit *et al.*, 2000), [8]*Metapenaeus ensis* (Gu *et al.*, 2000; # deduced from nucleotide sequence) and [9]*Penaeus schmitti* (Huberman *et al.*, 2000); and an isopod species: [10]*Armidillidium vulgare* (Martin *et al.*, 1993). Aligned with the cHH sequences are related sequences from other non-crustacean arthropods: [11]*Low molecular weight protein* (Gasparini *et al.*, 1994) and [12]*Schistocerca gregaria* ion-transporting peptide (Meredith *et al.*, 1996). Similarities between all the different sequences are underlined. **The cHH sequence of *P. bouvieri* is identical to that of *Procambarus clarkii*, and cHH from *Orconectes limosus* differs from this by only one residue (L^{62}).

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N-terminus and a blocked C-terminus; (Sefiani *et al.*, 1996; Yasuda, 1997; Sithigorngul *et al.*, 1999a; Udomkit *et al.*, 2000; Huberman *et al.*, 2000).

Characteristically, cHHs have (i) 6 cysteine residues at conserved positions with three disulfide bridges assigned between them: C^{7}-C^{43}, C^{23}-C^{39} and C^{26}-C^{52}; (ii) a chain length of 72 amino acid residues and, (iii) a molecular mass of between 8000 and 9000 Da (see Van Herp, 1998). The application of molecular biological techniques, such as cDNA cloning and PCR, has resulted in the elucidation of the preprohormone structure of several cHHs. Reports on preprocHH from *C. maenas* (Weidemann *et al.*, 1989), *O. limosus* (De Kleijn *et al.*, 1994a), *H. americanus* (De Kleijn *et al.*, 1995), *P. japonicus* (Ohira *et al.*, 1997a) have shown a common structural organisation of a signal peptide flanked by a cHH-precursor-related peptide (CPRP), followed by the cHH peptide (Fig. 1). CPRPs were also isolated from sinus glands of *C. maenas*, *O. limosus*, *H. americanus* (Tensen *et al.*, 1991b) and the edible crab *Cancer pagurus* (Chung *et al.*, 1998). Despite attempts to functionally characterise the CPRPs, their physiological relevance is still not understood.

In recent studies, it has been shown that the eyestalk is not the sole source of cHH in decapod crustaceans. cHH gene expression in the ventral nerve cord of *H. americanus* (De Kleijn *et al.*, 1995) and cHH immunoreactivity in the brain, thoracic ganglion and pericardial organs of *C. maenas* (Keller *et al.*, 1985; Dircksen and Heyn, 1998) were reported. More recently, cHH and CPRP immunoreactivity was detected in gut endocrine cells of *C. maenas* (Webster *et al.*, 2000), and in a pair of cells in the suboesophageal ganglion and in the thoracic ganglia of *H. americanus* (Chang *et al.*, 1999). The function of this extra-eyestalk cHH in *C. maenas* appears to be related to ecdysis; a transient surge of cHH (also observed in eyestalk-ablated animals) has been

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recorded during the initial stages of ecdysis and is very likely stimulating the uptake of water (Chung *et al.*, 1999).

1.3.3. Moult-inhibiting hormone (MIH)

From the early, classical physiological experiments, such as eyestalk ablation and implantation, it was observed that eyestalk ablation often but not always resulted in a shortened intermoult period and hence, in a precocious ecdysis (see Chang, 1995). It was thus concluded that the eyestalk, and specifically the XO-SG complex, is the source of a moult-inhibiting factor, which is effective in suppressing the synthesis/secretion of moulting hormones (ecdysteroids) from the moulting glands (Y-organs; Passano, 1953). Despite this early discovery of a putative moult-inhibiting hormone, it took many decades to isolate and characterise an MIH due to the paucity of reliable, convenient and sensitive biological assays. The development of an *in vitro* bioassay, which measured the inhibition of ecdysteroid synthesis by isolated Y-organs after the addition of crude extracts of sinus glands, was a crucial event for progress in the isolation of MIH (Soumoff and O'Connor, 1982). Isolation, characterisation and sequence elucidation of MIH from *C. maenas* was eventually realised (Webster, 1986; Webster and Keller, 1986; Webster, 1991). Before this, a peptide with MIH activity from *H. americanus* was reported (Chang *et al.*, 1987, 1990). This sequence, however, was later found to be incomplete and the "MIH" peptide is now considered to be one of the lobster's hyperglycaemic hormones, viz. cHH A (Tensen *et al.*, 1991a; Chang, 1995).

Because the *H. americanus* cHH also had MIH biological activity and, the absence of a unique peptide with sole MIH activity in the lobster, this led to the idea that only brachyurans possessed distinct cHH and MIH molecules (Chung *et al.*, 1996). During

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the past decade, however, several reports have been published on the primary structure of MIH from different crustacean species and infraorders (Aguilar *et al.*, 1996; Yang *et al.*, 1996; Nagasawa *et al.*, 1996; Chung *et al.*, 1996). The different MIH peptides are homologous in structure to one another, and also to cHH peptides (see Fig. 3). Despite the structural similarities, however, none of the isolated MIHs could induce hyperglycaemia, whereas the corresponding cHH molecules were capable of suppressing ecdysteroid titres in *in vitro* bioassays (Webster and Keller, 1986; Chung *et al.*, 1996; Yang, *et al.*, 1996).

Characteristic of most of the MIHs to date, is (i) the presence of 6 cysteine residues and their connecting disulfide bridges: C^{7}-C^{44}, C^{24}-C^{40}, C^{27}-C^{53}; (ii) a molecular mass of more than 9000 Da and, (iii) a chain length of more than 72 amino acid residues (see Webster, 1998 for review). Of the 5 isolated and sequenced MIH neuropeptides, one, viz. MIH from the Mexican crayfish *Procambarus bouvieri*, has the primary structure which is characteristic of the cHH peptides (compare Fig. 2 with Fig.3). This MIH is 90 % homologous to the cHH from *P. bouvieri*, yet it is claimed not to display any hyperglycaemic activity (Aguilar *et al.*, 1996). Several putative MIH peptide sequences have also been deduced from nucleotide sequences following cDNA cloning, for example MIH from *C. maenas* (Klein *et al.*, 1993b), *P. vannamei* (Sun, 1994), *C. sapidus* (Lee *et al.*, 1995), *P. japonicus* (Ohira *et al.*, 1997b), the sand shrimp *Metapenaeus ensis* (Gu and Chan, 1998), the crab *Charybdis feriatus* (Chan *et al.*, 1998) and the Dungeness crab *Cancer magister* (Umphrey *et al.*, 1998). All of these preproMIHs, except one, showed the same organisation, viz. a signal peptide followed by the MIH peptide, thus lacking a precursor-related peptide (see Fig. 1). The exception to this rule, viz. the MIH of *P. vannamei*, consists of a signal peptide, a precursor-related peptide and the putative MIH sequence (Sun, 1994). Since the MIH

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          1         10        20        30        40        50        60        70        78
Prcl[1]:  RYVFEECPGVMGNRAVHGKVTRVCEDCYNVFRDTDVLAGCRKGCFSSEMFKLCLLAMERVEEFPDFKRWIGILNA-NH2
Casa[2]*: RVINDDCPNLIGNRDLYKKVEWICDDCANIYRSTGMASLCRKDCFFNEDFLWCVRATERSSDLAQLKQWVTILGAGRI
Cama[3]:  RVINDECPNLIGNRDLYKKVEWICEDCSNIFRKTGMASLCRRNCFFNEDFLWCVHATERSEELRDLEEWVGILGAGRD
Capa[4]:  RVINDDCPNLIGNRDLYKKVEWICEDCSNIFRNTGMATLCRKNCFFNEDFLWCVYATERTEEMSQLRQWVGILGAGRE
Chfe[5]*: RVINDDCPNLMGNRDLYKKVEWICDDCANIYRITGMASLCRKDCFFNFDFLWCVRATFRSFDMTQLKQWVRILGAGRI
Camag[6]*:RVINDDCPNLIGNRDLYKRVEWICEDCSNIFRNTGMATLCRKNCFFNEDFLWCVYATERTEEMSQLRQWVGILGAGRE
Peja[7]:  SFIDNTCRGVMGNRDYNKKVVRVCEDCTNIFRLPGLDGMCRNRCFYNEWFLICLKA-NREDEIEKFRVWISILNAGQ
Meen[8]*: SYIENTCRGVMGNRDIYKKVVRVCEDCTNIFRLPGLDGMCRDRCFNNEWFLVCLKAANRDDELDKFKVWISILNPGL
Prbo[9]:  pEVFDQACKG-IYDRAIFKKLELVCDDCYNLYRKPKVATTCRENCYANSVFRQCLDDLLLINVVDEYISGVQIV-NH2
Peva[10]*: DTFDHSCKG-IYDRELFRKLDRVCEDCYNVFREPKVATECKSNCFVNKRFNVCV-ADLRHDVSRFLKMANSALS
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Fig. 3. Comparative alignment of the peptide structure of putative MIHs from the different decapod crustacean infraorders. Astacidae: [1] *Procambarus clarkii* (Nagasawa *et al.*, 1996), and [9]*P. bouvieri* (Aguilar *et al.*, 1996); Penaediae: [7]*Penaeus japonicus* (Yang *et al.*, 1996), [8]*Metapenaeus ensis* (Gu and Chan, 1998) and [10]*P. vannamei* (Sun, 1994); and Brachyura: [2]*Callinectes sapidus* (Lee *et al.*, 1995), [3]*Carcinus maenas* (Webster, 1991), [4]*Cancer pagurus* (Chung *et al.*, 1996), [5]*Charybdis feriatus* (Chan *et al.*, 1998) and [6]*Cancer magister* (Umphrey *et al.*, 1998). Identical amino acid residues, with reference to the brachyuran MIHs, are shown in bold. The (-) indicates the gap(s) introduced into the amino acid sequence to align the Cys residues and to allow for maximal identity. Amidation is shown by: $-NH_2$. Note: not all of the primary structures have been obtained from purified peptides; some have been deduced from nucleotide sequences (*) only.

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peptide of *P. vannamei* was never isolated from the sinus glands and, therefore, has also not been functionally characterised, it may be argued that this preprohormone sequence represents a cHH and not MIH.

The purification of MIH peptides made it possible to generate very specific antisera with which to investigate the neurosecretory system immunocytochemically. Antisera raised against MIH of *C. maenas* localised MIH-producing cells in the X-organ of 5 brachyuran crab species (Dircksen *et al.*, 1988). Double-staining techniques with antisera against MIH and cHH of *C. maenas* did not show co-localisation of these 2 peptides in the brachyuran species studied but demonstrated 2 distinct neurosecretory pathways within the XO-SG complex (Dircksen *et al.*, 1988). This was confirmed by *in situ* hybridisation studies with cRNA probes for *C. maenas* cHH and MIH which clearly showed that cHH mRNA did not co-localise with MIH mRNA in the XO of *C. maenas* (Klein *et al.*, 1993a). In *P. japonicus*, however, some neurons of the XO showed co-localisation of cHH and MIH peptides (Shih *et al.*, 1998). For this immunocytochemical study, the specific cHH and MIH antisera were raised against synthetic partial peptide sequences corresponding to the C-terminal decapeptide sequences of the cHH and MIH, respectively, of *P. japonicus* due to the low sequence identity of the 2 peptides in this region of the molecule (Shih *et al.*, 1998).

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1.3.4. Vitellogenesis-inhibiting hormone (VIH)

The control of reproduction in crustaceans has mostly concentrated on the regulation of female reproduction, specifically, the process of vitellogenesis. Various stages in the development of the oocytes in female crustaceans have been described (see Van Herp and Soyez, 1997). Briefly, previtellogenic oocytes arise from cell divisions (mitotic and meiotic) of undifferentiated gonia. During previtellogenesis (or primary

vitellogenesis), ribosomes accumulate and the rough endoplasmic reticulum develops in the growing oocyte, which is surrounded by follicle cells. Fully-grown previtellogenic oocytes enter a vitellogenesis cycle (secondary vitellogenesis) during the reproductive season by the development of microvilli. Secondary vitellogenesis involves the accumulation of yolk proteins into the oocytes, which will sustain the developing embryo/larva after hatching. The lipoprotein, vitellin (Vt), is a chief component of the yolk (Browdy *et al.*, 1990) and a related protein, vitellogenin (Vg), has also been detected in crustaceans during ovarian development (Shafir *et al.*, 1992); the hepatopancreas is capable of producing this extra-ovarian source of yolk protein (Quackenbush, 1999). At this time, the follicle cells form a network that supports the massive uptake by the oocytes of Vg from the haemolymph via endocytosis. The absorbed Vg is transformed to Vt; in penaeid prawns, there is also an intra-ovarian source of vitellin (Fainzilber *et al.*, 1992; Khayat *et al.*, 1994). The disappearance of the microvilli marks the end of vitellogenesis. This is followed by the breakdown of the germinal vesicle, resumption of meiosis and a period of maturation which culminates with egg-laying (see Van Herp and Soyez, 1997). In many instances, eyestalk-ablation experiments resulted in accelerated secondary vitellogenesis in female crustaceans (see Adiyodi and Adiyodi, 1970). It was, therefore, concluded that the eyestalk housed a vitellogenesis-inhibiting hormone. Like MIH, the isolation and characterisation of VIH has been severely hampered by the absence of suitable, rapid and sensitive biological assays. The first VIH was isolated from sinus glands of *H. americanus* by using a heterologous *in vivo* assay which measured the retardation of oocyte growth in the shrimp *Palaemonetes varians* after injection of HPLC-purified fractions (Soyez *et al.*, 1987). Vitellogenesis is prolonged in most large decapod crustaceans with egg-laying occurring once on an

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annual basis only, it is, therefore, not easy to develop an *in vivo* assay in homologous systems of lobsters and crayfish; a much shorter reproductive cycle occur in prawns and shrimps. The primary structure of VIH from *H. americanus* was elucidated by conventional microsequencing and found to be a peptide with remarkable homology to MIH and cHH from other crustaceans (Soyez *et al.*, 1991). *H. americanus* VIH is a 78 residue peptide with a molecular mass of more than 9000 Da and 6 cysteine residues with at positions 10, 27, 30, 43, 47 and 56 (see Fig. 4). Like *H. americanus* cHH, the lobster VIH was found as 2 isoforms of identical sequence and mass, but only one of these isoforms had VIH biological activity (Soyez *et al.*, 1991). A sinus gland neuropeptide, isolated from *P. bouvieri* also demonstrated VIH capabilities in the prawn *Penaeus setiferus* as measured *in vitro* by the inhibited incorporation of ^{3}H-labeled leucine into yolk proteins after treatment with sinus gland fractions (Aguilar *et al.*, 1992). This bioactivity was, however, obtained with an unphysiologically high dose of 10 SGE; the primary structure of *P. bouvieri* VIH is only partially elucidated (Fig. 4) with a molecular mass of 8388 Da; the position of the Cys residues are identical to the arrangement in the cHH molecules. A VIH peptide has recently been functionally characterised and fully sequenced from the isopod *Armidillidium vulgare* (Greve *et al.*, 1999). The functional characterisation of the isopod VIH was based on (i) an *in vitro* vitellogenin immunoprecipitation assay and, (ii) an *in vivo* bioassay based on the delay of the onset of vitellogenesis as determined by the nature of the moult (normal versus parturial moult, i.e. with egg laying observed in the breeding pouch of the latter moult). The isopod VIH is an 83 residue peptide with a molecular mass of 9485 Da and 6 Cys residues at positions 15, 32, 35, 48, 52 and 61; this peptide did not induce hyperglycaemia *in vivo*, nor did it prolong the intermoult period after conspecific injections (Greve *et al.*, 1999).

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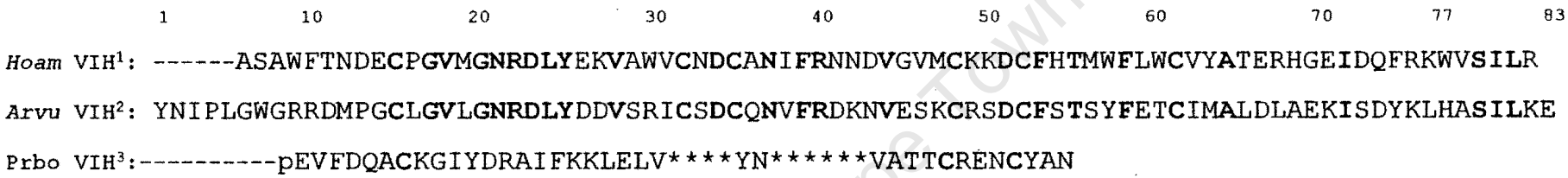

Fig. 4. Comparative amino acid sequences of vitellogenesis-inhibiting hormone (VIH) isolated from [1]*Homarus americanus* (Soyez *et al.*, 1991), [2]*Armadillidium vulgare* (an isopod; Greve *et al.*, 1999) and the partial sequence from [3]*Procambarus bouvieri* (Huberman *et al.*, 1995). The 6 Cys residues are indicated in bold; the asterisk (*) shows the incomplete sequence; gaps (-) have been introduced to align the sequences for maximal sequence identity and identical residues between *Hoam* and *Arvu* VIH are also indicated by bold letters.

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Information on the preprohormone structure of VIH has also been obtained. cDNA which encodes VIH in *H. americanus* has been cloned and sequenced. The structure of preproVIH consists of a signal peptide and the VIH peptide itself (De Kleijn *et al.*, 1994b), thus showing the same organisation as the MIH preprohormones. This organisation of a VIH preprohormone (Fig. 1) was also reported for a putative VIH from *M. ensis*, where the signal peptide consisted of 29 amino acid residues and the mature peptide of 78 residues (Gu and Chan, 1999).

Several immunocytochemical studies have been carried out in crustaceans with antisera against VIH. VIH was shown to be localised in the XO-SG complex of *H. americanus* with a specific antibody to *H. americanus* VIH (Meusy *et al.*, 1987) and also with a cRNA probe (Laverdure *et al.*, 1992). The antibody to *H. americanus* VIH also cross-reacted with sinus gland extracts of the marine shrimps *P. varians* and *Palaemon serratus*, the giant freshwater prawn *Machrobrachium rosenbergii*, and *C. maenas*, and with brain extracts (with the attached sinus gland) of the woodlouse (isopod) *Porcellio dilatatus*, but did not cross-react with sinus gland extracts of the crayfish *A. leptodactylus* and *O. limosus*, nor with the spiny lobster *Jasus paulensis* (Meusy *et al.*, 1987). Co-localisation of cHH and VIH in the XO of *H. americanus* was demonstrated by antisera against *H. americanus* VIH and *A. leptodactylus* cHH (Kallen and Meusy, 1989). Only a small subset of the immunoreactive neurons, however, showed co-localisation of the 2 peptides. A similar partial co-localisation of cHH and VIH peptide, was observed with specific antisera raised against *H. americanus* peptides (Meusy and Soyez, 1991; Rotllant *et al.*, 1993), and was also reflected at the mRNA level in *Homarus gammarus* larvae with cRNA probes directed against *H. americanus* cHH and VIH (Rotllant *et al.*, 1993). The same was found with partial co-localisation of cHH and VIH mRNA in *H. americanus* (De

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Kleijn *et al.*, 1992). In another astacidean, the Norway lobster *Nephrops norvegicus*, no co-localisation was observed with antisera directed against *A. leptodactylus* cHH and *H. americanus* VIH (Guilianini *et al.*, 1998). The physiological relevance of co-localisation and thus, co-secretion of the cHH family peptides is not clear at this stage. Although VIH has been predominantly studied and characterised in female crustaceans, there is evidence that this hormone is also present in male specimens (De Kleijn *et al.*, 1992). VIH is hence, also referred to as GIH (gonad-inhibiting hormone) and is considered to have a different, and as yet unknown, role in the male crustaceans than in their female counterparts.

1.3.5. Mandibular organ-inhibiting hormone (MOIH)

The mandibular organs (MO) of decapod crustaceans synthesise and secrete methyl farnesoate (MF), a sesquiterpenoid (Laufer *et al.*, 1987). The exact physiological relevance of MF, however, is still undefined (see Webster, 1998). The fact that MF is the unepoxidised precursor of the so-called juvenile hormone III (JH III) in insects has led to the assumption that, like JH III, MF also plays a role in reproductive development and growth (Laufer *et al.*, 1993). Indeed, there are some studies that support this hypothesised role of MF in crustaceans, whereas others do not (see Homola and Chang, 1997; Abdu *et al.*, 1998). What has been clearly demonstrated from eyestalk-ablation studies and injection of extracts of sinus glands, is that synthesis of MF by the MO is negatively regulated by a neuropeptide hormone from the XO-SG complex. Using an *in vitro* bioassay to measure the synthesis of MF (by incorporation of ^{3}H-methyl methionine) after incubation with sinus gland fractions, two peptides with MOIH activity were isolated from the edible crab *Cancer pagurus* (Wainwright *et al.*, 1996). The *C. pagurus* MOIHs consisted of 78 residues with

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molecular mass of more than 9000 Da and 6 Cys residues with their connecting disulfide bridges arranged in the characteristic manner of the MIH molecules (Fig. 5). These two *C. pagurus* MOIHs differed from one another by only one substitution at position 33. Three peptides from the sinus gland of the spider crab *Libinia emarginata* were also characterised as MOIHs (Liu and Laufer, 1996). The complete primary structure of one of these peptides have been elucidated (Liu *et al.*, 1997b) and it resembles cHH more than it does MIH or the *C. pagurus* MOIHs. In addition, the *L. emarginata* MOIH candidates displayed significant hyperglycaemic effects (Liu and Laufer 1996). The difference in peptide structure also extends to the preprohormone structure (Fig. 1): preproMOIHs of *C. pagurus* have the same organisation as preproMIHs, i.e. signal peptide and hormone sequence (Tang *et al.*, 1999), whereas *L. emarginata* preproMOIH is organised like preprocHHs with signal peptide, PRP and hormone sequence (Liu *et al.*, 1997a).

1.4. The cHH/MIH/VIH peptide family

As the primary structure of several neuropeptide hormones from the sinus glands of decapod crustaceans became known, it was apparent from sequence homology that the cHH, MIH, VIH, and later also the MOIH molecules, were all members of one peptide hormone family (Keller, 1992; Webster, 1998). For some time, this peptide family was considered to be a novel group of crustacean peptides, until the primary structure of a low molecular weight protein (LMWP) was elucidated from venom of the black widow spider, *Latrodectus mactans tredecimguttatus* (Gasparini *et al.*, 1994). This LMWP is a 70 amino acid residue peptide with 6 Cys residues and 3 disulfide bridges that can be aligned with that of cHH (Fig. 2). Although LMWP co-eluted with the main neurotoxin from the spider venom, it has no toxic effect and its

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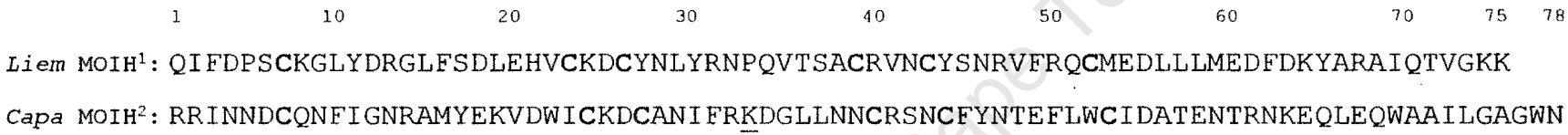

Fig. 5. Amino acid sequences of mandibular organ-inhibiting hormone (MOIH): [1], from *Libinia emarginata* (Liu *et al.*, 1997b) and 2, *Cancer pagurus* (Wainwright *et al.*, 1996). The 6 Cys residues are indicated in bold; note that a second *Capa* MOIH is identical to the one shown here, except for the substitution of K^{33} (underlined) by Q^{33}.

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function is, as yet, unknown (Gasparini, *et al.*, 1994). More recently, the amino acid sequence of an ion transport peptide (ITP), isolated from the neurohaemal organs (corpora cardiaca) of the desert locust *Schistocerca gregaria*, was determined by a combination of microsequencing and molecular biological techniques (Meredith *et al.*, 1996; Philips *et al.*, 1998). The *S. gregaria* ITP consists of 72 amino acid residues, a mass of 8564 Da, with 6 Cys and 3 disulfide bridges at positions typical for cHHs (Fig. 2). The preprohormone of *S. gregaria* ITP has also been investigated and this is organised in the same way as preprocHHs (Philips *et al.*, 1998) (Fig. 1). Comparative information gained about the preprohormones of members of the cHH/MIH/VIH peptide family, leads to the grouping of the peptides into 2 subsets: (i) cHH/ITP and (ii) MIH/VIH (Lacombe *et al.*, 1999). In the first group, the preprohormones are organised as a signal peptide, followed by a precursor-related peptide (PRP) and then the sequence of the mature peptide. The preprohormones of the second group, on the other hand, have only a signal peptide and the mature peptide sequence (see Fig. 1). The arrangement of MOIH into the subgroups is equivocal at this stage, since the primary structures of two MOIH peptides closely resemble that of the MIHs/VIHs (Wainwright *et al.*, 1996), but another putative MOIH has the primary structure of the cHH peptides (Liu *et al.*, 1997b). The preprohormone structure of the former MOIH matches that of the MIH/VIH subgroup (Tang *et al.*, 1999), while the latter preproMOIH is organised in the same manner as peptides belonging to the cHH subgroup (Liu *et al.*, 1997a). Until information becomes available on the sequences of MOIH peptides from other crustaceans, and their preprohormone structures, it is tempting to dismiss the *L. emarginata* MOIH as primarily being a cHH, especially since cHH has been shown to be multifunctional in many crustaceans, e.g. MIH activity (Chang *et al.*, 1990), gonad-stimulating activity (specifically, stimulation of

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oocyte growth; Tensen *et al.*, 1989), MOIH activity (i.e. inhibitory effect on MF synthesis by the MO; Laufer *et al.*, 1994), secretagogue activity (Sedlmeier, 1988), inhibition of protein and mRNA synthesis in ovaries of vitellogenic females (Khayat *et al.*, 1998) and involvement in osmoregulatory processes (Charmantier-Daures *et al.*, 1994).

Although not all the isolated members of the cHH/MIH/VIH peptide family have been tested for different biological activities, it can be expected from the literature to date, that some, if not all, of these peptides are multifunctional. Furthermore, it might be possible to speculate about the evolutionary distance between different crustacean groups, based on the degree of structural homology and functional cross-activities of the cHH/MIH/VIH peptide family.

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1.5. Scope and aims of the present study

Lobsters belonging to the family Palinuridae are called spiny lobsters because of the many spines on the carapace and basal segments of the long second antennae (Fig. 6A,B), and are also referred to as rock lobsters because of their preference to inhabit rock crevices on the sea floor. Unlike clawed (true) lobsters, spiny lobsters lack claws and live gregariously in relatively shallow waters, where rocks are present for shelter (Phillips *et al.*, 1980). Commercially, spiny lobsters are highly valued seafood and form the hub of extremely important fisheries in certain countries (Booth and Phillips, 1994). Due to the ever-increasing demands from the seafood market, however, most fisheries are overexploited and to meet these demands, ventures into aquaculture are being undertaken; this is, however, not an uncomplicated task and the successes are slow in coming (Kittaka and Booth, 1980).

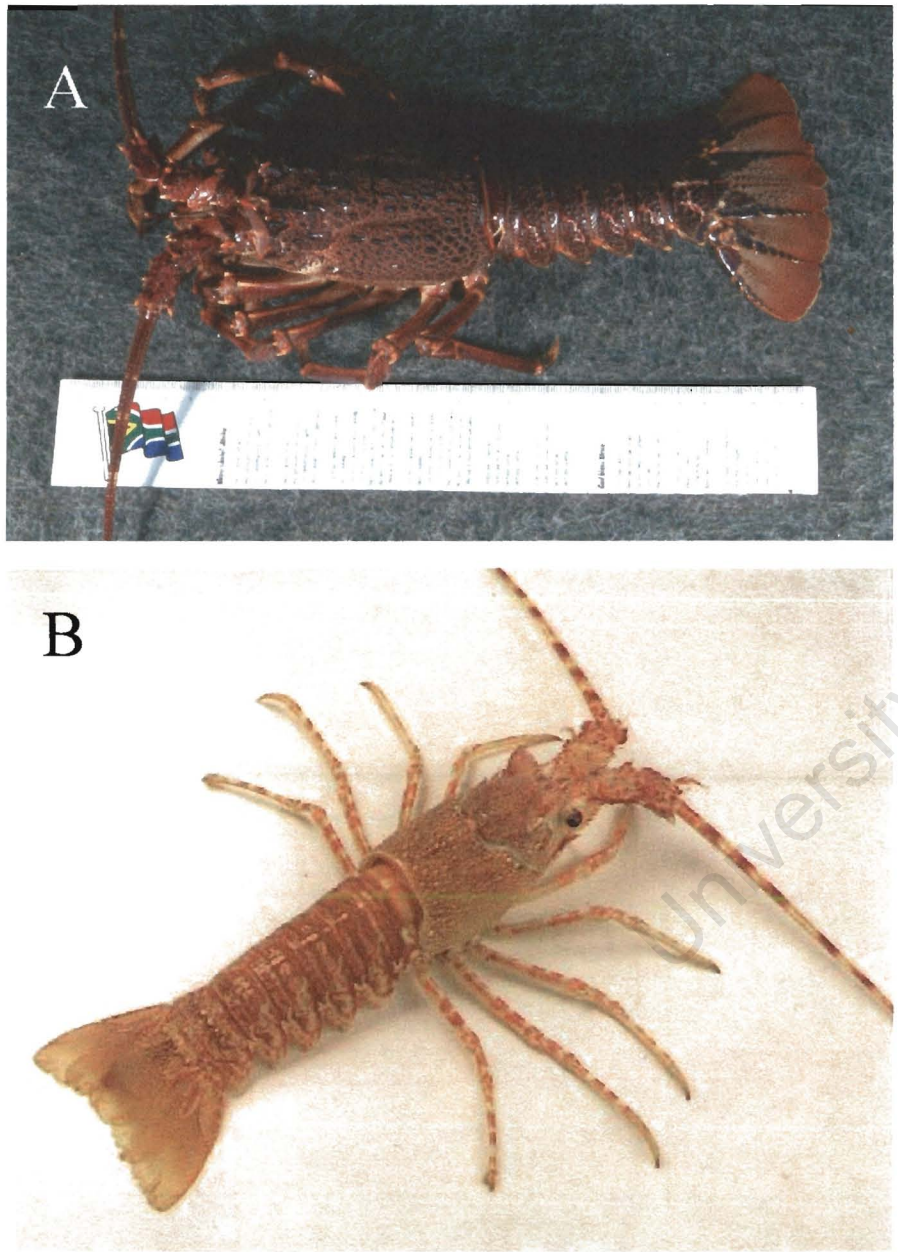

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Fig. 6. Photographs of adult spiny lobsters, **A.** *Jasus lalandii* and **B.** *Palinurus gilchristii*. **C.** Distribution of the 3 genera of spiny lobsters in the oceans surrounding the Republic of South Africa (RSA); 1= *J. lalandii*; 2= *P. gilchristii* and 3= *Panulirus homarus*.

South Africa is one of the countries that supports the commercial and subsistence fishing of spiny lobsters. Here, the rock lobster industry is largely dependent on the exploitation of *Jasus lalandii* (H. Milne-Edwards), which predominates in the cold waters (10-18 ^{0}C) of the Atlantic Ocean along the west coast of South Africa (hence, this species is also known as the west coast rock lobster; Heydorn, 1969). Because of its economic importance (export of frozen tails, whole frozen lobsters, whole cooked lobsters, and live lobsters to overseas markets: *ca* 3700t per annum; Pollock 1986), *J. lalandii* and the associated lobster industry is locally referred to as "red gold". It is believed that the west coast rock lobster fishery is currently valued in excess of R145 million per annum and provides employment for more than 400 people (Mr P. Foley, West Coast Rock Lobster Sea Management Association, pers. comm.).

Two other spiny lobster species occur in South African waters: (i) the east coast rock lobster *Panulirus homarus* (Linnaeus), which is found in the shallow warm waters of the Indian Ocean (20-30 ^{0}C), is not of commercial interest and is only fished recreationally under strict legislation; (ii) the deep water rock lobster *Palinurus gilchristii* (Stebbing; Fig. 6B) is commercially fished, but on a smaller scale than the west coast rock lobster. Because they occur at depths of 100-500 m in the sea, it is not allowed to fish *P. gilchristii* for recreational or subsistence purposes (Heydorn, 1969 and Cockcroft and Payne, 1997). Figure 6C shows the distribution of these three South African spiny lobster species.

Despite the commercial interest in South African spiny lobsters, there is a severe paucity of physiological and biochemical information since most of the research on *J. lalandii* has been conducted at an ecological level (see for example Heydorn, 1969; Griffiths and Seiderer, 1980; Goosen and Cockcroft, 1995; Barkai *et al.*, 1996). It is with this in mind, that the spiny lobster *J. lalandii* has been selected as the

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experimental animal in the present study. It is hoped that the current study will initiate further interest on the cellular, biochemical and molecular levels which will form the cornerstones for more applied aspects of research on this valued decapod crustacean.

The present study has three main objectives. The first is to compile, as complete as possible, an inventory of the neuropeptide hormones found in the XO-SG complex of the South African spiny lobster species *Jasus lalandii*, based on HPLC fractionation and subsequent biological and immunological assays. The second objective is to characterise the isolated peptides by mass spectrometry and to elucidate their primary structures by Edman degradation, in conjunction with enzymatic cleavages and other chemical treatments. The third objective is to lay the groundwork for future research on this topic at the molecular biology level. It is hoped that comparative endocrinologists can use this inventory of XO-SG neuropeptides from *J. lalandii* as a representative of the Palinura (spiny lobster) infraorder, since other studies have focused on Brachyura (true crabs), Astacidea (clawed lobsters and crayfish), Caridea (shrimps) and Penaeidae (prawns; classification is according to Bowman and Abele, 1982). In the present study, brief comparisons with the other South African spiny lobsters are also made, with reference to the histological and biochemical aspects of the neuroendocrine complex in the eyestalk.

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2. MATERIALS AND METHODS

2.1. Animals

Live crustaceans were obtained along the coasts of South Africa, transported on ice to the laboratory where they were held in large tanks containing recirculating sea water. The ambient temperature in the aquarium was 12-14 ^{0}C with a photoperiod of 12 h light:12 h dark. Lobsters and crabs were fed maximally twice a week with pilchards and mussels, whereas shrimps were fed *ad libitum* with dried fish flakes and pieces of mussel tissue. Adult and juvenile specimens of *Jasus lalandii* (*Jala*[1]) (both sexes) were collected from various sites in the Atlantic Ocean off the west coast of South Africa. Adult specimens of *Panulirus homarus* (*Paho*) were collected from the Indian Ocean off the east coast of South Africa and were kindly supplied by Dr. P. Fielding of the Oceanographic Research Institute in Durban. *P. homarus* specimens were not held under the aquarium conditions described above; their eyestalks were immediately ablated on their arrival in Cape Town and prepared for immunocytochemistry (see below), the animals were sacrificed thereafter. *Carcinus maenas* (*Cama*) were collected in baited traps set at the Alfred and Victoria Waterfront harbour in Cape Town. Marine shrimps, *Palaemon pacificus*, were collected with hand-held nets from beds of *Zostera capensis* (eelgrass) in the Langebaan Lagoon in the vicinity of Cape Town. For large-scale preparation of sinus glands to obtain sufficient peptides for

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[1] The nomenclature is according to that proposed by Raina and Gäde (1988) for insect neuropeptides. However, I prefer at least a 4-letter denomination for the Crustacea (2 letters for the genus and 2 or more letters for the species) to prevent confusion with the insect nomenclature. For example, *Cam* denotes the stick insect, *Carausius morosus*, but could also mean *Carcinus maenas*. Therefore, I propose *Cama* for the latter. However, to abbreviate *Cancer magister*, a 5-letter denomination is required for clarity, i.e. *Camag*.

purification and sequencing, eyestalks were ablated from live specimens of *J. lalandii* and *Palinurus gilchristii* (*Pagi*) at lobster factories in Cape Town and Hout Bay. Live prawns, *Penaeus semisulcatus* (*Pese*), were collected from Haifa Bay (Israel). They were kept in 3 m^3 aerated tanks in Haifa, Israel, under natural light conditions with a continuous supply of sea water and a regulated temperature of 25 ^{0}C. The prawns were fed daily with a mixture of frozen shrimp, frozen squid and food pellets.

2.2. Preparation of sinus glands and purification of peptides

Freshly-ablated eyestalks from adult *J. lalandii*, *P. homarus*, *P. gilchristii* and *C. maenas* were excised from the exoskeleton and the sinus gland (SG) was carefully dissected with the aid of a binocular microscope and placed into ice-cold 10 % acetic acid. Batches of 25 SG in 400 µl acetic acid (for preabsorption, immunocytochemical experiments) or 50-100 SG in 800 µl acetic acid (for peptide purification/sequencing) were homogenised in a glass homogeniser for 5 min at 85 ^{0}C. The homogenate was then cooled on ice and centrifuged at 24 200 x g (Sorvall centrifuge) for 15 min at 4 ^{0}C. The resulting supernatant was stored on ice; the pellet was re-extracted with 400 µl 10 % acetic acid on ice and centrifuged. The combined supernatants were dried in a Bachoffer vacuum concentrator.

The dried SG extracts of *J. lalandii*, *P. gilchristii* and *C. maenas* were redissolved in 50 µl 10 % acetic acid and for peptide separation, were injected onto a Nucleosil C-18 column (5 µm particle size, 25 cm length x 0.46 cm internal diameter). The chromatographic equipment was a Gilson high pressure gradient system with UV detection at 214 nm and a flow rate of 1 ml/min. The solvents were: A= 0.11 % trifluoroacetic acid (TFA) in Milli-Q water, B= 0.1 % TFA in 60 % acetonitrile. Peptides of the cHH/MIH/VIH family were separated from the crude extracts by a

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gradient from 20-60 % B in 10 min, followed by a linear increase to 80 % B in 50 min. Peak fractions were collected manually and aliquots were immediately tested in an ELISA (see 2.3.4.), while the remainder was dried *in vacuo* and used either in biological assays (see 2.4.), or pooled for structural elucidation (see 2.5.). To minimise the loss of cHH/MIH/VIH peptides by adhesion to Eppendorf tubes, these peptides were collected into silanated tubes (briefly, the tubes were coated for a few minutes at room temperature with 5 % dimethyl silane in toluene, followed by 3 rinses with toluene and a final rinse with methanol; the tubes were then heated at 50 ^{0}C for 3 h).

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2.3. Comparative immunocytochemical studies

2.3.1. Tissue preparation

Live adult specimens of *J. lalandii*, *C. maenas*, and *P. homarus* were anaesthetised on ice and their eyestalks were ablated. After removal of the exoskeleton, the eyestalks were fixed in Bouin's fixative overnight at room temperature for tissue immunocytochemistry. The fixed material was dehydrated in a graded ethanol series, cleared in xylene and then embedded in Paraplast. Serial sections (5 µm thick) were cut on a Reichert-Jung microtome and every 11th section was routinely stained with haematoxylin and eosin, and viewed with a light microscope to assess the morphology, quality and appropriateness of the tissue area for immunocytochemical staining.

2.3.2. Primary antisera

Primary antisera used in this study were obtained as gifts. Anti-*Homarus americanus* (*Hoam*) cHH and -VIH were provided by Dr. D. Soyez (CNRS, Paris, France) and

were raised in guinea pigs against purified lobster cHH and VIH fractions, respectively (Meusy and Soyez 1991). Antiserum raised in rabbits against *Hoam* VIH was also supplied by Dr. Soyez. Anti-*Cancer pagurus* (*Capa*) cHH and -MIH were provided by Dr. S. Webster (University of Wales, Bangor, Wales) and were raised in rabbits against purified crab cHH and MIH fractions, respectively. Anti-*Procambarus bouvieri* (*Prbo*) cHH was supplied by Dr. A. Huberman (INN, Mexico City, Mexico) and was raised in rabbits against purified crayfish cHH. For each primary antiserum, optimal concentration was tested in a dilution series for each investigated species by tissue immunocytochemistry and by enzyme-linked immunosorbent assay (ELISA, see 2.3.4. below). Anti-*Hoam* cHH serum was tested over a concentration range of 1:2000-1:10000, anti-*Prbo* cHH ranged from 1:2000-1:5000, anti-*Capa* cHH and anti-*Capa* MIH ranged from 1:2000-1:6000, and anti-*Hoam* VIH serum was tested over a concentration range of 1:500-1:2000. The following dilutions were decided upon for use in the indirect peroxidase immunocytochemistry procedure: anti-*Hoam* cHH 1:5000, anti-*Prbo* cHH 1:5000, anti-*Capa* cHH 1:4000, anti-*Capa* MIH 1:2000 and anti-*Hoam* VIH 1:500. Higher concentrations of antisera were selected for use with the immunofluorescence technique (see 2.3.3.2.).

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2.3.3. Tissue immunocytochemistry

2.3.3.1. The indirect peroxidase procedure

Paraplast sections on glass slides were deparaffinised and rinsed in distilled water. To inhibit the activity of endogenous peroxidase, tissue sections were incubated with 1 % H_2O_2 in methanol (30 min at room temperature) followed by a 5 min washing step under running tap water. Before the application of anti-*Hoam* cHH/-VIH to tissue sections, non-specific binding was blocked by incubating the sections with 20 %

normal rabbit serum (NRS, Sigma) for 1 h at room temperature. Likewise, before the application of anti-*Capa* MIH/-cHH and anti-*Prbo* cHH, sections were first incubated with 10 % normal swine serum (NSS, Sigma). Tissue sections were then incubated overnight at 4 ^{0}C with 20 µl primary antiserum diluted in 0.01 M phosphate-buffered saline (PBS, 14 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4), pH 7.6, washed 3 x 5 min in PBS at room temperature and then incubated at room temperature for 1 h with the respective secondary antiserum, viz., 1:150 rabbit anti-guinea pig conjugated to horse radish peroxidase (RAGp-HRP, Dako), or 1:50 swine anti-rabbit conjugated to HRP (SAR-HRP, Dako). This was followed by PBS washes and a final 5 min rinse in Tris-buffered saline (0.1 M NaCl in 0.05 M Tris-HCl, pH 7.6). For visualisation of the immunoreactions, the sections were incubated for 5 min with 2 mg 3,3'-diaminobenzidine (DAB) per 4 ml of 0.05 M Tris-HCl buffer, pH 7.6 and 0.001 % H_2O_2, followed by a tap water rinse. The immunostained sections were mounted in veronal-buffered glycerol (equal amounts of glycerine and veronal buffer [0.1 M NaCl and 0.04 M sodium veronal], pH 8.6) and coverslipped.

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2.3.3.2. Immunofluorescence and double-staining procedure

Dewaxed, rehydrated serial sections of *Jala* eyestalk tissue were double-stained as follows. Non-specific reactions were blocked by incubating the sections with a mixture of 1:5 NRS and 1:10 NSS for 1 h at room temperature. Cocktails of primary antisera, viz. (i) rabbit anti-*Capa* cHH 1:500 + guinea pig anti-*Hoam* VIH 1:250, (ii) rabbit anti-*Capa* MIH 1:500 + guinea pig anti-*Hoam* cHH 1:1000, (iii) rabbit anti-*Capa* MIH 1:500 + guinea pig anti-*Hoam* VIH 1:250, and (iv) rabbit anti-*Hoam* VIH 1:250 + guinea pig anti-*Hoam* cHH 1:1000, were applied to the sections and left to incubate overnight at 4 ^{0}C. Thereafter, the sections were rinsed 3 x 5 min in PBS and

the first secondary antibody, 1:20 SAR conjugated to TRITC (tetramethylrhodamine isothiocyanate, Sigma) with 1:20 normal guinea pig serum (NGpS), was applied to the sections and left to incubate for 1 h at 4 ^{0}C. Excess antisera were removed by 3 x 5 min rinses in PBS. The second secondary antibody was then applied, viz. 1:20 RAGp conjugated to FITC (fluorescein isothiocyanate, Sigma) + 1:10 NRS + 1:10 NSS, and incubated at 4 ^{0}C for 1 h. TRITC- and FITC-labeled secondary antisera were kindly supplied by Prof. B. B. Rawdon (Anatomy and Cell Biology Department., University of Cape Town, Rondebosch, South Africa). The incubation with secondary antisera was followed by PBS washes and the sections were mounted in veronal-buffered glycerol, coverslipped and viewed with a Leitz fluorescent microscope.

2.3.3.3. Specificity controls

Controls for the specificity of the immunocytochemical staining included (1) omission of primary antiserum to check unspecific binding of secondary antibody and other compounds applied later in the procedure, (2) substitution of primary antiserum with normal serum to show specificity of the primary antiserum, (3) application of preabsorbed antisera-peptide complexes to illustrate the specificity of the primary antisera to one particular antigen. Preabsorption of 50 pmol of purified *Capa* MIH or *Capa* cHH (gifts from Dr. S. Webster, University of Wales, Bangor, Wales) per µl of 1:10 anti-*Capa* MIH or 1:10 anti-*Capa* cHH eliminated all specific immunostaining completely.

Preabsorption of antisera with *Hoam* cHH, *Hoam* VIH and *Prbo* cHH could not be carried out because of insufficient amounts of the specific, purified *Hoam* and *Prbo* peptides. As it is known that the anti-*Hoam* VIH recognises *Hoam* cHH to some extent (Meusy and Soyez 1991), specificity tests were carried out by preabsorbing

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putative cHH fractions from *J. lalandii* and putative MIH from *C. maenas* in the following way: sinus gland extracts were prepared from *J. lalandii* and *C. maenas,* the peptide content of the crude extracts was separated on RP-HPLC as described in 2.2. and these peptide fractions were subjected to ELISA using antisera raised against anti-*Hoam* cHH and anti-*Capa* MIH. Those HPLC fractions with strong cHH and MIH immunoreactivity were then used to preadsorp the anti-*Hoam* VIH serum. These peptide-antisera complexes were then tested for immunoreactivity on the putative *Jala* cHH and *Cama* MIH fractions in ELISA studies and on tissue sections of *Jala* eyestalks.

2.3.3.4. Microscopy and photomicrography

Immunostained tissue sections were examined with a Nikon microphot light microscope with bright field transmitted light. Micrographs were also taken on a Wild Photomakroskop M400 (bright field and oblique) and a Leitz Diaplan M400 (Hoffman's modulation contrast). Immunofluorescent sections were examined with a Leitz Labolux K microscope with transmitted fluorescence illumination and filters for rhodamine and FITC. The immunofluorescence was recorded on Fujichrome Sensia (400 ASA, daylight) colour film, whereas the DAB reactions were photographed on Kodak (50 ASA) monochrome film. It was not possible to take double-exposure photographs on the available equipment, therefore, the immunofluorescence results of double-staining were photographed separately with the different filters in place (FITC has an excitation wavelength of 495 nm and emission at 525 nm; TRITC is excitatory at 552 nm and emission occurs at 570 nm).

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2.3.4. Enzymatic-linked immunosorbent assay (ELISA) procedure

Direct ELISA was carried out on Nunc Maxisorp plastic microtiter plates with flat wells (Nunc, Denmark) coated either with 0.25 sinus gland equivalents (SGE) of crude SG extracts from *J. lalandii*, *P. homarus* and *C. maenas* for determining optimal working dilutions of the available primary antisera, or coated with 0.25 SG equivalents of HPLC-purified fractions from *J. lalandii*, *C. maenas* and *P. gilchristii* SG extracts to identify putative cHH, MIH and VIH peptides in these species. Alternatively, 25-50 µl aliquots from 1.5 ml HPLC fractions were coated directly onto the microtitre plate without concentrating the sample beforehand. The ELISA method followed is that of Sefiani *et al.* (1996) with the use of alkaline phosphatase conjugated secondary antisera (Sigma). Briefly, the antigens were dried in the wells either under vacuum or by heating at 37 ^{0}C. Coating buffer (0.1 M sodium carbonate buffer, pH 9.6) was then added in a volume of 100 µl per well, and the plate was incubated for 2 h at 37 ^{0}C or overnight at 4 ^{0}C. The coating buffer was then discarded and the plate rinsed 3x for 5 min with 0.02 M PBS, pH 7.4, containing 0.1 % Tween-20 and 0.02 % sodium azide. The appropriate primary antibody (100 µl per well), diluted in PBS containing Tween, sodium azide and normal goat serum (NGS; Sigma) to a final volume of 1 %, was added to the microtitre wells and were incubated for 1.5 h at 37 ^{0}C. Excess amounts of antibody were washed off by 3 x 5 min rinses in PBS/Tween/sodium azide solution. The appropriate secondary antibody (100 µl per well), also diluted in PBS/Tween/sodium azide/NGS solution was then applied to the wells and incubated for 1.5 h at 37 ^{0}C. The secondary antibodies were 1:10 000 goat anti-guinea pig IgG conjugated with alkaline phosphatase (Sigma) and 1:10 000 goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma). After a 3 x 5 min rinse in PBS/Tween solution, the enzymatic activity was assessed by the addition of para-

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nitrophenyl phosphatase disodium (Sigma) in 0.1 M sodium carbonate buffer, pH 9.6. The optical density was determined every 10-20 min at 405 nm using a Titertek Multiskan Plus reader.

2.4. Functional characterisation of members of the cHH/MIH/VIH peptide family in Jasus lalandii

2.4.1. Biological assay for hyperglycaemic activity

A homologous *in vivo* assay using live juvenile rock lobsters was established. HPLC peak material (peaks 1-5, Fig. 24) which gave positive immunochemical reactions to cHH/MIH/VIH antisera in ELISA, were tested for a hyperglycaemic effect in juvenile specimens of *J. lalandii* (average weight 65 g, carapace length 4.5 cm) which had been starved 2-4 days prior to use. Individual animals were identified by means of numbered tags, which were glued onto the dorsal surface of the tail. The test animals were allowed to move freely in 20 x 50 x 60 cm assay chambers (width x length x depth), which were constructed from transparent perspex material. These chambers were fully submersed, but held afloat, in a large reservoir of recirculating sea water in the aquarium room. In this way, haemolymph sampling and injections could be carried out in the aquarium room under the prevailing temperature regime of 12-14 ^{0}C, and individual animals could easily be identified and removed from the chamber for sampling purposes without submitting them to undue stress. Sampling and injections were usually achieved within 1 min. In pilot experiments, animals were randomly included in the bioassays. However, due to huge variations in the initial glucose concentrations between individual juveniles, the resulting data were not statistically comparable. Therefore, in subsequent experiments, 50 µl haemolymph

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samples were withdrawn from 30 animals and immediately processed to determine the initial glucose concentration (see below). On the basis of these values, individuals were then grouped into subsets and injected with a test substance. For each test substance, the assay was done at least twice with n = 4-10 animals.

Dried HPLC-derived peak material was redissolved in sterile sea water and injected in 50 µl volumes at concentrations of 1-2 SGE through the arthrodial membrane of the fifth walking leg. As control substances, 50 µl sea water and 0.5-1 SGE of crude extracts of *Jala* sinus glands were included in the assays. Two hours after injection, a second haemolymph sample was taken to determine the concentration of glucose.

2.4.1.2. Determination of glucose titres in haemolymph samples

Before injection of the test substance into juvenile lobsters and 2 h after injection, 50 µl of haemolymph was sampled with a Hamilton syringe, ejected into 50 µl of 0.03 N perchloric acid and immediately vortexed. The syringe was repeatedly rinsed with 10 % sodium citrate to delay clotting of the haemolymph. Haemolymph samples were centrifuged at room temperature for 8 min at 20 000 x g and the glucose concentration in the supernatant was analysed with a commercially available kit (510 A kit, Sigma). The kit consisted of the following reagents: (i) PGO enzyme capsules (each capsule, when dissolved in 100 ml distilled water, contains 500 units of glucose oxidase from *Aspergillus niger*, 100 purpurogallin units of horseradish peroxidase and buffer salts); (ii) 50 mg o-dianisidine dihydrochloride which is reconstituted in 20 ml distilled water and then used as the colour reagent; and (iii) 1mg/ml β-glucose in 0.1 % benzoic acid solution which served as the stock solution for reference amounts of glucose. An enzyme-colour reagent solution was prepared by combining 100 ml of

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enzyme solution and 1.6 ml colour reagent solution. The Sigma procedure (Procedure No. 510) is based upon the following coupled enzymatic reactions:

Glucose Oxidase

Glucose + H_20 + O_2 ⟶ Gluconic Acid + H_2O_2

Peroxidase

H_2O_2 + o-Dianisidine ⟶ Oxidised o-Dianisidine

(Colourless) (Brown)

The intensity of the brown colour measured at 425-475 nm is proportional to the glucose concentration.

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The glucose concentration in the haemolymph samples was determined as follows: 50 µl supernatant was pipetted into a glass tube with 1 ml enzyme-colour reagent solution (blank samples consisted of 50 µl 0.03 N perchloric acid with 1 ml enzyme-colour reagent). After mixing, the tubes were incubated at 37 ^{0}C for 30 min. The optical density of the samples was measured in a glass cuvette at 436 nm on a Vitatron IFP spectrophotometer and the concentration of glucose was calculated from a standard curve prepared with glucose standard solutions, viz. 8 dilutions with perchloric acid, ranging from 0-25 mg/ml. Determination of glucose concentrations were later modified for microtitre plates: 10 µl haemolymph supernatant + 200 µl enzyme colour reagent solution, in duplicate, and the optical density was then read at 450 nm with a Bio-Rad 550 microplate reader. The average difference in the glucose

concentrations after injection for each test substance was statistically compared to the difference caused after injection of sea water with the use of the Student's *t*-test.

2.4.2. Biological assay for moult-inhibiting activity

2.4.2.1. Characterisation of circulating ecdysteroids in J. lalandii and ecdysteroid quantification

2.4.2.1.1. Haemolymph sampling and extraction of ecdysteroids

Haemolymph samples were collected on 2 different occasions from the ventral sinus of adult and juvenile male lobsters (n=5 in both cases) using disposable syringes. Pure methanol was added to the haemolymph (4:1 v/v; the minimum volume of haemolymph withdrawn from an individual was 1 ml, whereas the maximum was 15 ml and the total volume collected was 51 ml). The haemolymph samples were then centrifuged at 4 ^{0}C for 10 min at 24 000 x g and the supernatants were pooled and kept on ice. One volume of methanol was added to the pellets which were extracted twice more. The pooled supernatants were dried by evaporation and then redissolved in a smaller volume of methanol for separation of the ecdysteroids.

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2.4.2.1.2. Chromatographic separation of ecdysteroids

An aliquot of the methanolic extract of haemolymph was applied to a normal phase (NP) column (Hypersil, Zorbax) attached to a Waters HPLC system with UV detection at 250 nm. The ecdysteroids were separated isocratically with dichloromethane/isopropanol/water (125:30:2; v/v/v) at a flow rate of 1 ml/min for 42 min. Fractions of 0.5 ml were collected by a fraction collector for ecdysteroid

quantification in an enzyme immunoassay (EIA, see 2.4.2.1.3. below). Both batches of methanolic extracts were separated on NP-HPLC at least 3 times. The following synthetic ecdysteroids were also applied to NP-HPLC as references: ecdysone (E), 20-hydroxyecdysone (20E), 3-dehydroecdysone (3dE), ponasterone A (PoA), 2-deoxyecdysone (2dE) and 2-deoxy-20-hydroxyecdysone (2d20E; all generously provided by Prof. R. Lafont, ENS, Paris, France).

2.4.2.1.3. Ecdysteroid quantification

An enzyme immunassay (EIA) was used to quantitate ecdysteroids in fractionated extracts of haemolymph and in the incubation medium of *in vitro* assays with Y-organs (YOs). The method, a competitive EIA, has been adapted from the one described by Porcheron *et al.* (1989) (see below) by using goat anti-rabbit IgG (Sigma) as secondary antiserum and a different enzymatic tracer: 2-succinyl-20-hydroxyecdysone coupled to peroxidase (a generous gift from Dr J.P. Delbeque, CNRS, Bordeaux, France). The principle of the EIA is, briefly, (see Fig. 7): the rabbit anti-ecdysteroid antibodies (primary antisera) are immobilised to microtitre plates by goat anti-rabbit IgG (secondary antiserum). During a 3 h incubation period the ecdysteroid sample and tracer (peroxidase-labeled ecdysteroid) compete with each other for binding sites on the immobilised primary antisera. After plate washing, the remaining peroxidase is assayed by a colour reaction using ortho-phenyldiamine as substrate and the absorption is measured at 450 nm. In greater detail, each well of the microtitre plates (96 F, Nunc/Denmark) were coated for 24 h at room temperature with 0.25 µg secondary antiserum in 200 µl 50 mM EIA buffer (0.1 M potassium phosphate buffer pH 7.4, 0.4 M NaCl, 1 mM EDTA and 0.1 % bovine serum albumin, BSA). Thereafter, 0.3 mg BSA in 100 µl distilled water was added to each well. The

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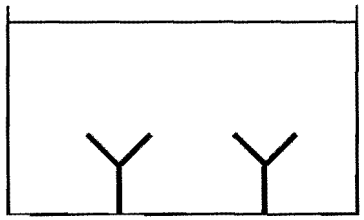
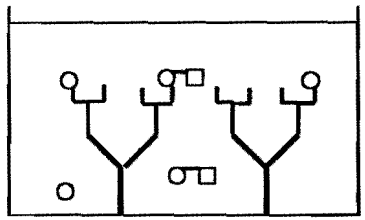
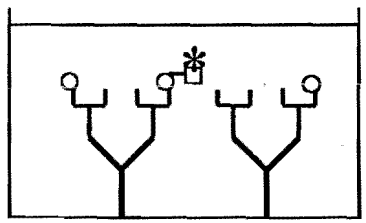


1. Coating microtitre well with goat anti-rabbit IgG



2. Immunological reaction:

- o ecdysteroids in sample
- ⊓□ 20E-peroxidase (=tracer)
- ⊤ rabbit anti-ecdysteroids



3. Revelation of enzymatic colour reaction (*)

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Fig. 7. Schematic representation of the competitive enzyme immunoassay (EIA) used to determine the titre of ecdysteroids in haemolymph samples and in tissue culture medium in which Y-organs had been incubated.

plates were stored at 4 ^{0}C and were ready for use 12 h later. Before use, the plates were extensively washed in washing buffer (0.01 M potassium phosphate buffer, pH 7.4 with 0.05 % Tween-20). In the determination of ecdysteroid quantities in YO-incubated medium, all the washing steps were manually carried out, whereas, for EIA analyses of the HPLC-separated ecdysteroids, all the washing steps were carried out by an automatic plate washer.

For the quantitation of ecdysteroids in NP-HPLC fractions, the tracer and primary antibodies were diluted in EIA buffer, and the HPLC fractions from *Jala* haemolymph were redissolved in 0.5 ml EIA buffer. Each fraction was plated in duplicate (50 µl) for EIA. Reagents were applied to the microtitre plates, final volume of 150 µl per well, in the following order (see Fig. 8): tracer, EIA buffer, ecdysteroid standards, samples and finally, the primary antibody. To determine the degree of non-specific binding, blanks were prepared in which the primary antibody and the ecdysteroid standard were omitted from the well. The degree of maximal possible binding (B_0), i.e. the enzymatic activity of the tracer bound in the absence of competitor molecules, was assessed by omission of ecdysteroid standards. All measurements were made in duplicate for blank samples, and in quadruplicate for B_0. The polyclonal antibody, AS 4919, (a gift from Prof. P. Porcheron, University of Paris XI, Paris, France) was used (1:100 000) in the EIA with haemolymph fractions because the antibody recognises a broad spectrum of ecdysteroids (Porcheron *et al.*, 1989). Calibration curves were constructed using synthetic 20E (Sigma) as standard ecdysteroid (range: 15.6-2000 fmoles in EIA buffer).

For the determination of ecdysteroid titres in the incubation medium after *in vitro* incubations of YOs from *J. lalandii*, 2-50 µl incubation medium was plated for EIA. In these EIAs, the solutions of synthetic ecdysteroids for construction of a standard

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A.

	Tracer	EIA buffer	Standard	Sample	Primary Antibody
Blank (non-specific binding)	50 µl	100 µl	-	-	-
B_0(maximal binding)	50 µl	50 µl	-	-	50 µl
Standards	50 µl	-	50 µl	-	50 µl
Samples	50 µl	-	-	50 µl	50 µl

B.

	Tracer	Medium*	EIA buffer	Standard	Sample	Primary Antibody
Blank	50 µl	50 µl	50 µl	-	-	-
B_0	50 µl	50 µl	-	-	-	50 µl
Standard	50 µl	-	-	50 µl	-	50 µl
Sample	50 µl	0-48µl	-	-	2-50 µl	50 µl

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Fig. 8. Plating scheme and volumes for EIA reagents, synthetic ecdysteroid standard solutions and, in **A**, NP-HPLC-derived fractions from extracts of haemolymph, in **B**, samples of tissue culture medium after *in vitro* incubations of YOs from *J. lalandii*.

* denotes fresh culture medium.

curve were prepared in culture medium (M199, Sigma) and the final volume of 150 µl/well was attained by the addition of culture medium (see Fig. 8). The polyclonal antibody, RUD 2 (a gift from Dr D. Sedlmeier, University of Bonn, Bonn, Germany) was used in these EIAs to obtain direct quantification of E and 3DE in samples (Von Gliscynski *et al.*, 1995). Each determination was made in duplicate. Calibration curves were constructed using either synthetic E (Sigma) or 3DE (range: 15.6–2000 fmoles; gift from Prof. R. Lafont).

In a routine EIA, the plates were incubated for 3 h at room temperature under constant but gentle agitation. Alternatively, the plates were incubated at room temperature for 2 h, incubated overnight at 4 ^{0}C and then incubated for a further hour at room temperature the following day. After incubation, the plates were washed with washing buffer. For manual washings, the reagents were flicked off from the plate, washing buffer was added at 250 µl/well with a multichannel pipette, and the plates were gently agitated for 5 min; this was repeated 3 times. The enzymatic activity of the tracer was measured using ortho-phenylenediamine (Sigma) as substrate. This was prepared by dissolving a 5 mg tablet in 12.5 ml 0.05 M phosphate citrate buffer, pH 5 with 2 µl 30 % H_2O_2 added just before use. The plate was incubated in the dark at room temperature, with constant agitation and the optical density was measured periodically at 450 nm with a microplate reader (either the Titertek Multiskan Plus, or the Bio-Rad 550 model). Standard curve fitting was done with a computer using a linear log-logit transformation (Rodbard *et al.*, 1968; software program was kindly supplied by Dr. D. Soyez) and the EIA measurements of the samples were calculated from the standard curve by computer. Results are expressed as B/B_0 x 100 as a function of the log dose, where B represents the absorbance measured on the bound fraction in the presence of competitor molecules.

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2.4.2.2. In vitro incubations of Y-organs from J. lalandii

The Y-organs (YO) of *J. lalandii* are located anteriorally near the epimeral attractor muscle, the maxillary epimera and the base of the second maxilla as described by Paterson (1968). Adult lobsters were anaesthetised by chilling in a mixture of ice and sea water. The paired YOs were dissected under sea water and placed into small glass dishes on ice, containing 1 ml of sterile incubation medium. One litre of medium consisted of 11 g M199 culture medium powder (Sigma), 20 mM Hepes, 330.2 mM NaCl, 11.7 mM $CaCl_2.2H_2O$, 17.9 mM $MgCl_2.6H_2O$, 15 mM Na_2SO_4, 6.6 mM KCl, 0.06 g penicillin and 0.13 g streptomycin. The pH was adjusted to 7.4 and the medium was sterilised by filtration through 0.22 µm filters (Millipore) and stored in sterile vials at 4 ^{0}C. After rinsing the YOs in the cold incubation medium, the medium was removed from the dish and replaced with 1 ml of fresh medium for one hour at room temperature. This medium was collected for EIA analyses and replaced with fresh medium: one organ from each pair of YOs was incubated as the control tissue in incubation medium, while the other organ was incubated in medium supplemented with a particular test substance. This substance was either a crude extract of *Jala* SGs or HPLC-purified peptide material, redissolved in M199 medium. The results were expressed as % inhibition of ecdysteroid synthesis relative to the control YO.

Crude extracts of *Jala* SGs were applied to YOs from intermoult animals during a 6 h incubation period to assess the inhibitory responses and the sensitivity of the organ assay. HPLC peaks 1, 3 and 4 (see Fig. 24 for elution profile), which correspond to truncated cHH-I, MIH and cHH-I of *J. lalandii*, were tested at doses of 1 SGE during overnight incubations. The glass dishes containing the YOs were covered with parafilm to minimise evaporative loss of media, and incubated at room temperature (21 –25 ^{0}C), under constant but gentle agitation. After incubation, the medium was

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transferred to tubes and stored at –20 ^{0}C until the ecdysteroid titre could be quantified by EIA. Pieces of YO tissues (halves and quarters) were also used in *in vitro* incubations after pilot experiments showed that the glands could be cut into representative tissue pieces of equal productivity.

2.4.2.2.1. Tissue specificity of the YO assay

Non-YO tissues of the branchiostegal region, such as muscle, epidermis, gill and hypodermis were incubated *in vitro* with M199 or crude extracts of sinus glands to show that only YO tissues produced ecdysteroids and that only these tissues were affected by extracts of sinus glands. Tissues were trimmed to approximately the same size as the YOs and were incubated overnight.

2.4.2.2.2. Production dynamics of YOs in vitro

The release of ecdysteroids from *Jala* YOs *in vitro* was assessed: YOs were incubated in 1 ml M199 for a total period of 21 h. All the media was removed and replaced with 1 ml of fresh M199 after 1, 3, 6 and 15 h of incubation. The collected samples were stored at –20 ^{0}C until EIA analyses.

2.4.2.2.3. Determination of the moult stages and the inhibitory effect of sinus glands during the moult cycle

Adult *J. lalandii* specimens were destalked; 25 µl haemolymph was withdrawn with a Hamilton syringe from the base of the 5th pair of walking legs of these animals before eyestalk ablation and at various periods thereafter. The haemolymph samples were put into tubes containing 25 µl of 0.03 N perchloric acid and vigorously vortexed. These tubes were then centrifuged at room temperature for 8 min at 20 000 x g and 15 µl of

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the supernatant was analysed, in duplicate, in a competitive EIA with AS 4919 as primary antibody. Levels of circulating ecdysteroids were correlated with the different stages of the moult cycle by observing the destalked lobsters and noting the day of ecdysis, as well as by morphological changes, such as hardness of the exoskeleton. Haemolymph samples were withdrawn up to maximally 3 weeks following ecdysis. These post-ecdysis animals were then sacrificed and their YOs were incubated *in vitro*.

YOs from lobsters undergoing different stages of the moult cycle, i.e. intermoult, premoult and postmoult, were incubated with 1 SGE of crude extracts of *Jala* SGs for 6 h and overnight incubation periods. Selection and classification of animals into these groups were made on the basis of their ecdysteroid titres in the haemolymph.

2.4.3. Biological assay to measure the inhibition of de novo protein synthesis

In contrast to the other biological assays described above, here a heterologous assay was used.

2.4.3.1. Dissection and in vitro incubations of ovaries from Penaeus semisulcatus

Female shrimps were collected from the tanks, decapitated with a pair of scissors and the dorsal carapace of the thorax and abdomen was removed. The ovaries, which extend under the dorsal carapace as a coiled, green mass were carefully removed, leaving the heart behind, and placed into ice-cold tissue culture medium. This methionine- and cysteine-free medium was similar in composition to M199 (Sigma) but was custom-made in 75 % sea water (Biological Industries Co., Beit Hamek, Israel) and the following components were added to 1 l medium before use: 3.1 mg cysteine, 1.5 mg methionine, 12 g Hepes and 150 mg (250 000 U) penicillin. The pH

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of the medium was then adjusted to 7.43. The developmental stage of each ovary was microscopically assessed by viewing the oocytes from a small tissue biopsy and measuring the average diameter of the oocytes. Suitable ovaries were those which contained oocytes larger than 350 µm and were devoid of cortical rods. The latter contains precursors for the jelly layer cover of eggs and is, therefore, indicative of the final developmental stage before spawning (Shlagman *et al.*, 1986). The selected ovaries were cut into small pieces with a sterile scalpel blade. Each piece was weighed and 45-55 mg tissue pieces were randomly placed into wells of a 24-well tissue culture plate (Corning Incorporation) containing 0.5 ml medium, on ice. Dried peptides, generated from RP-HPLC-separation of crude extracts of sinus glands from *J. lalandii* (peaks 1-6, Fig. 37), were redissolved in culture medium and added to the wells (1 SGE in 10 µl); each substance was tested in duplicate. A dose-response curve was also created by incubating ovarian tissue pieces in duplicate with 5 different concentrations of crude extracts of sinus glands from *J. lalandii* (range: 0.001-2 SGE). A positive control consisted of the incubation of ovarian tissues with crude extracts of sinus glands (0.1 SGE) from *P. semisulcatus*. Other controls were (1) non-treated ovarian tissues in which SG-derived material was omitted from the incubation, (2) short incubations of non-treated ovarian tissues, i.e. non-treated tissues were removed after 15 min in culture, and (3) incubations without any ovarian tissues.

Two µl corresponding to 30 µCi of radiolabeled amino acids (21 µCi ^{35}S-methionine + 9 µCi ^{35}S-Cysteine, Promix, Amersham) were mixed with 0.5 ml of culture medium and added to each of the culture wells. The tissue culture plates were incubated in an oxygenated chamber at 25 ^{0}C for 6 h with gentle agitation. After 15 min of incubation, the "short incubation" controls of non-treated tissues were removed from the plates and immediately frozen and stored at –70 ^{0}C, and, following the full incubation period

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of 6 h, the remaining tissue pieces were removed with forceps, put into marked tubes and also stored at –70 ^{0}C. The incubated media was left at room temperature overnight to assess the presence of contaminating fungal or bacterial growth.

2.4.3.2. Measurement of incorporated radiolabel

Tissue pieces that had been stored at –70 ^{0}C subsequent to their incubation *in vitro*, were thawed on ice. Once thawed, 0.5 ml PBS, pH 7.4, containing a protease inhibitor (2 mM Pefablock, Boehringer Mannheim) was added to each sample and the tissues were homogenised in a glass homogeniser using a battery-driven pestle at room temperature. The homogenates were centrifuged for 10 min at 4 ^{0}C, 15 000 x g. Ten µl of the resulting supernatants were spotted onto Whatman No. 1 filter paper in duplicate and air dried. The remainder of the supernatants was stored at –70 ^{0}C for protein determinations and separations (see 2.4.3.3.). Incubation medium from the wells in which no tissue pieces had been incubated was also spotted on filter paper for protein precipitation. The filter paper containing the dried supernatant and media were placed into boiling 10 % trichloroacetic acid (TCA) for 10 min; rinsed twice in water, twice in methanol and twice in acetone, then dried with hydrogen gas (H_2). Each filter was put into a numbered scintillation vial, 3ml scintillation fluid (Ultima Gold, Packard) was added and the radioactivity was measured in a scintillation counter (Kontron). Results were expressed as dpm; each sample was read twice and the average count was calculated. Background levels of radioactivity were determined as the radioactivity count in the ovarian tissues that had been incubated for only 15 min. This background count was subtracted from the average count of all the other samples to correct for "noise". Percentage inhibition of protein synthesis was calculated as follows: (non-treated – treated)/non-treated x 100.

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2.4.3.3. Determination of protein content, separation of proteins by denaturing gel electrophoresis and transfer of proteins to membranes

Supernatants of homogenised ovarian tissue pieces were thawed on ice after storage at –70 ^{0}C and the protein content was assessed in duplicate by using Bradford reagent (Bio-Rad) as follows. One µl of each sample was diluted with 799 µl distilled water and 200 µl Bradford reagent were added. Six different concentrations of 1 mg/ml BSA (range: 0-20 µg) served as a standard curve. The optical density of each sample was read on a spectrophotometer (Biochron) at 595 nm, with 800 µl distilled water + 200 µl Bradford reagent serving to zero the instrument.

Amounts of protein in the ovarian tissue pieces were then calculated by extrapolating the data obtained from the best-fit curve (i.e. y = **a**x) obtained with the protein concentrations of BSA. Thus, the concentration of the unknown sample (µg/µl) = (mean OD of unknown) divided by **a**.

After quantification of total proteins in the incubated tissue pieces, the homogenates were subjected to polyacrylamide gel electrophoresis under reducing conditions. Samples were thawed on ice, 30 µl Laemmli loading buffer (12 g/l Tris pH 6.8, 0.6 g/l EDTA, 2 % SDS, 10 % glycerol, 0.2 g/l bromophenol blue, 1 % β-mercaptoethanol) were combined with the equivalent of 30 µg of proteins from each sample and boiled for 5 min. The samples were then loaded onto 12.5 % polyacrylamide gels (15 mm thick, 10 lanes per gel), along with a prestained broad-range molecular weight marker [212 kDa (myosin) – 7.2 kDa (aprotinin), Bio-Rad]. The proteins were allowed to migrate under 150 V for 2h. The gels were then stained with Coomassie Blue solution (0.5 g Brilliant blue R250, Sigma; 20 ml acetic acid, 90 ml methanol and 200 ml distilled water; the solution was filtered through Whatman No. 1 filter paper) for 45 min at room temperature under shaking conditions. Gels were destained overnight at

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room temperature and with shaking in a solution containing 10 % acetic acid, 45 % methanol and 45 % distilled water. After recording the images by scanning, the gels were incubated for 2 h in running buffer supplemented with 0.1 % SDS and 6 M urea, this medium being changed every 30 min. Gels were then placed onto Whatman filter paper on the negatively charged transfer plate and covered with nitrocellulose membrane (0.45 µm Transblot Transfer Medium Pure Nitrocellulose Membrane, Bio-Rad) cut to the same dimensions as the gels and clearly marked. Both the filters and the membranes were first soaked in distilled water and then in transfer medium (25 mM Tris, 192 mM glycine, 15 % methanol) before use. A "sandwich" was prepared by alternately stacking filter paper, followed by a destained gel, a membrane and finally more filter paper, until all 4 gels were ready for transfer. The transfer of proteins from the gels onto the nitrocellulose membranes was carried out at 60 V for 3.5 h and migration occurred from the negative to the positive pole in transfer medium. The membranes were then exposed to autoradiography films for 1 month (X-ray film, super HR-G30, Fuji) and then automatically developed (RG II, Fuji).

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2.5. Sequence elucidation of members of the cHH/MIH/VIH peptide family in J. lalandii

2.5.1. Reduction of S-S bonds of Jala cHH-I

Peptides belonging to this peptide family are known to have 6 Cys residues with 3 connecting disulfide bonds. Four attempts were therefore made to linearise the *Jala* cHH-I molecule by reducing the S-S bonds and to prevent their reformation by alkylation (see Scheme in Fig. 9) before proceeding with sequence elucidation.

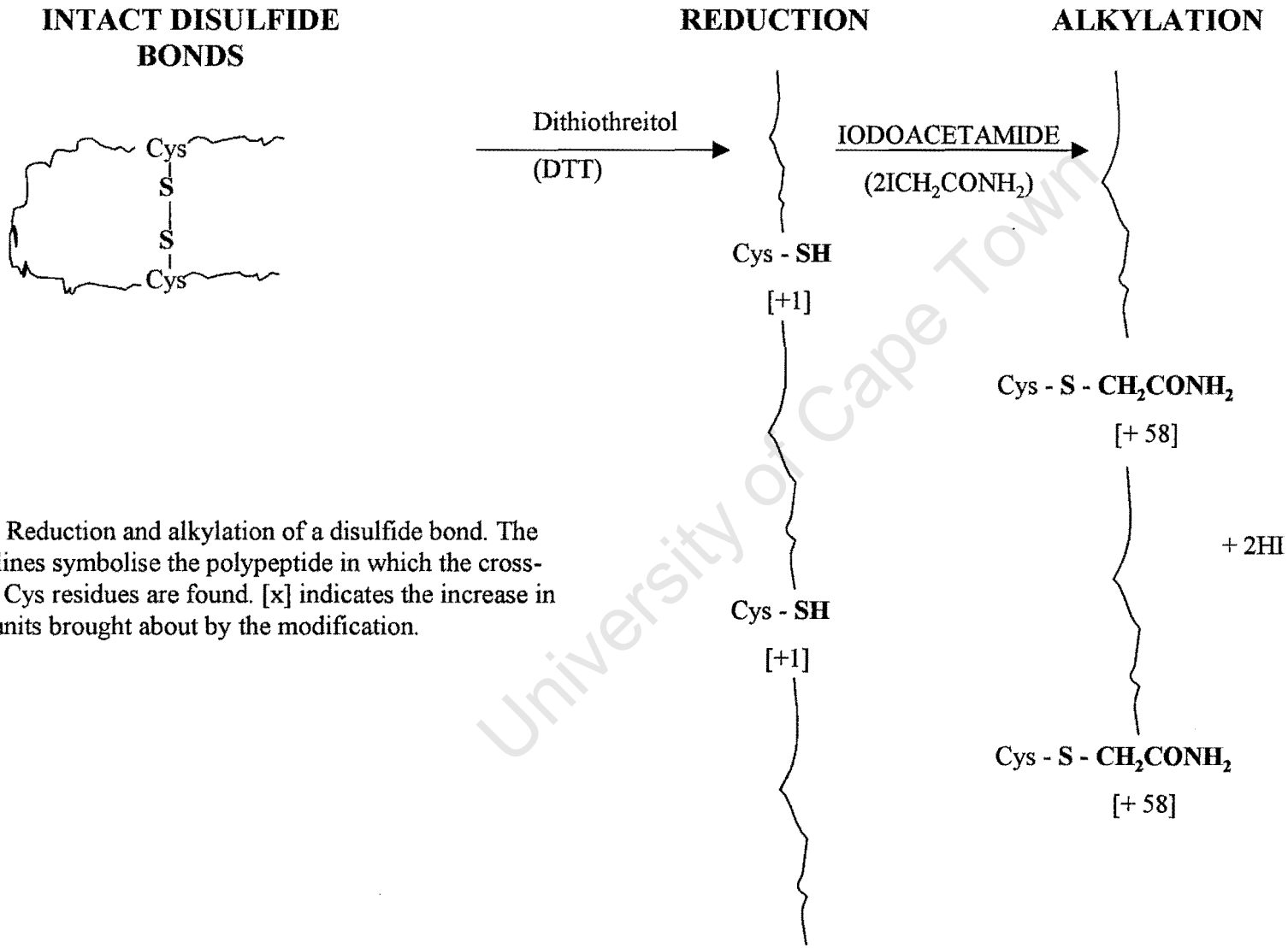

Fig. 9. Reduction and alkylation of a disulfide bond. The wavy lines symbolise the polypeptide in which the cross-linked Cys residues are found. [x] indicates the increase in mass units brought about by the modification.

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Approximately 300-600 pmol *Jala* cHH-I (corresponding to 25-50 SGs) were redissolved in 60 µl reducing buffer (0.5 M Tris-HCl buffer, pH 8.5 containing 6 M urea). Fifty µl 0.02 M dithiothreitol (DTT, Boehringer Mannheim) in reducing buffer was added. Oxygen in the sample tube was replaced by argon, an inert gas, and the mixture was incubated at 45 ^{0}C for 1 h. Thereafter, 50 µl 0.04 M iodoacetamide (Boehringer Mannheim) was added and the mixture was incubated for 20 min at 25 ^{0}C. As a control, all the reagents were incubated without peptide material. The mixtures were separated on RP-HPLC.

To determine the extent of peptide losses during this procedure, synthetic insulin was reduced, alkylated and purified on HPLC; the combined peak heights of the reduced-alkylated peptide chains were compared with the peak height of the same amount of non-reduced, non-alkylated insulin.

Reduction/alkylation using DTT and iodoacetamide was also carried out on tryptic fragment T26 (amino acid residues 1-8 and 42-50) of *Jala* cHH-I, as well as on the synthetic octapeptides AVFDQSCK (with D-Phe^3 and L-Phe^3 conformation; custom-synthesised by Dr. H. Echner, University of Tübingen, Tübingen, Germany). These mixtures were also purified on HPLC.

The success of reduction and alkylation was further monitored by removing aliquots of the peptide mixture after these different steps and analysing them by MALDI-TOF mass spectrometry (see 2.5.2.).

2.5.1.1. Reduction of disulfide bonds in peptide fragments for mass spectrometry

After enzymatic digestions of peptides, those fragments containing disulfide bonds were reduced in silanated tubes, the mixtures were then analysed by MALDI. An aliquot of each fragment (approximately 16 SGE) was dissolved in 5 µl reducing

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buffer. An equal volume of 0.02 M DTT in reduction buffer was added and the samples were incubated for 1 h at 45 ^{0}C under a layer of argon. After incubation, approximately 4 SGE of the samples were diluted 1:10 with 30 % acetonitrile and 0.01 % TFA and analysed by mass spectrometry. More recently, aliquots of the reduced samples were desalted by means of a ZipTip (Millipore). The ZipTip$_{C18}$ (C18 spherical silica: 15 µm, 200 Å pore size) is a 10 µl pipette tip with 0.5 µl resin at the tip. To desalt and remove detergents from the samples for MALDI-mass spectrometry, the following procedure was carried out as recommended by Millipore. The ZipTip was equilibrated for sample binding by aspirating 10 µl of 50 % acetonitrile in water into the tip, followed by 2 washes with 10 µl of 0.1 % TFA in water. The reduced peptide fragments were then bound to the ZipTip by aspirating and dispensing the sample 5-10 times, followed by 2 washes in 0.1 % TFA. Two to 4 µl of 50 % acetonitrile in water was pipetted into a clean sample tube using a standard pipette tip. To elute the peptides from the ZipTip, the acetonitrile solution was carefully aspirated and dispensed through the ZipTip at least 3 times. The desalted sample (0.7 µl) was spotted directly onto a gold plate with 1 µl of matrix and air-dried for mass spectrometric analyses (see 2.5.2. below).

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2.5.2. Mass spectrometry (MS)

Matrix-assisted laser desorption/ionisation (MALDI) mass spectra were first acquired on a time-of-flight (TOF) Voyager Elite Biospectrometry Workstation; this was later replaced by a MALDI-TOF Voyager-Pro DE (delayed extraction) instrument (both from PerSeptive Biosystems, Framingham, USA), equipped with a standard nitrogen laser. Data acquisition was performed by a Tektronix (TDS 520A) oscilloscope. Sinapinic acid and α-cyano-4-hydroxycinnamic acid (PerSeptive) were used as

matrices to analyse peptides in the range of 3000 - 10 000 Da and 500 - 3000 Da, respectively in the linear, positive ion, delayed extraction and reflectron modes. Dried peptides were redissolved in 30 % acetonitrile containing 0.1 % TFA. One µl of matrix (10 mg/ml in acetonitrile and 3 % TFA) was applied to 0.7 µl of sample solution on the target plate and dried. Samples containing peptide mixtures after digestions, cleavages or reduction, were not concentrated or dried in a vacuum concentrator, but were spotted directly from the reaction mixture after dilution with water or purification via ZipTip.

The software package, General Protein/Mass Analysis (GPMAW, Ver. 3.04) for Windows, was used to calculate the mass of peptides and to predict the cleavage patterns and masses of resulting fragments.

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2.5.3. N-terminal sequencing

Sequence analysis was first achieved by automated Edman degradation performed on a gas-liquid solid phase sequencer (Brandt *et al.*, 1984) linked to an on-line HPLC system, and later, on a liquid-pulsed sequencer (model 473A, Applied Biosystems). These analyses were performed by Assoc. Prof. W. Brandt (Department of Biochemistry, University of Cape Town, Rondebosch, South Africa) and Dr. S. Stoeva (University of Tübingen, Tübingen, Germany).

2.5.4. Cleavages and separation of cleavage fragments

2.5.4.1. Trypsin digestions

Batches of trypsin (25 µg sequencing grade, Boehringer Mannheim) were reconstituted in 125 µl 0.01 % TFA. Native, i.e. non-reduced peptides were redissolved in 0.1 M Tris-HCl buffer, pH 8.5: *Jala* cHH-I (125 SGE in 126 µl), *Jala*

cHH-II (285 SGE in 125 µl) and *Jala* MIH (340 SGE in 80 µl). Trypsin was added to the samples: 6 µg, 5.6 µg and 4 µg, respectively. A control reaction was always included in which no peptide was added to the incubation mixture. The digest was carried out at 37 ^{0}C for 18 h and thereafter terminated by the addition of 20 µl 0.1 N HCl and subsequent storage at 4 ^{0}C. The digest mixtures were subjected to RP-HPLC using the same column and HPLC system as described in 2.2., but with a different gradient: a 5 min isocratic step at 0 % B and then an increase to 90 % B in 90 min. The resulting peak fractions were manually collected into silanated tubes, dried *in vacuo* and used for mass analysis and N-terminal sequencing.

2.5.4.2. Endoproteinase-Asp N (endo-Asp N) digestions

Batches of endo-Asp N (2 µg sequencing grade, Boehringer Mannheim) were reconstituted in 50 µl Milli-Q water. Native, i.e. non-reduced peptides were redissolved in 50 mM sodium phosphate buffer, pH 8: *Jala* cHH-I (150 SGE in 50 µl), *Jala* cHH-II (550 SGE in 50 µl) and *Jala* MIH (350 SGE in 60 µl). The enzyme was added to the 3 samples: 1.4 µg, 1.4 µg and 1.5 µg, respectively. A control reaction was always carried out in which the peptide sample was absent. The digest was carried out at 37 ^{0}C for 18 h, terminated by the addition of 20 µl 0.1 N HCl and subsequent storage at 4 ^{0}C, and the mixtures were purified on RP-HPLC as described for trypsin (see 2.5.4.1.).

2.5.4.3. Cyanogen bromide (CNBr) cleavages

Dried, native peptide material was redissolved in 70 % formic acid: 50 SGE *Jala* cHH-I, 100 SGE *Jala* cHH-II, 125 SGE *Jala* MIH and 100 SGE truncated *Jala* cHH-I. CNBr (in formic acid) was added to a final concentration of 0.2 mg/40 µl. The

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sample mixtures were incubated in the dark under nitrogen gas for 18 h. The cleavage mixtures of *Jala* cHH-I and *Jala* cHH-II were not purified on HPLC, but dried in the vacuum concentrator and then redissolved for mass analyses and N-terminal sequencing. With the cleavage mixtures of *Jala* MIH and truncated *Jala* cHH-I, the CNBr was first evaporated from the samples and the mixtures were then applied to the HPLC for separation; the resulting peaks were collected. Only the purified C-terminal fragments (as identified by MALDI-TOF analyses) were dried and N-terminally sequenced.

Partial cleavage of cHH-I material (3 SG equivalents) was also carried out by incubating the peptide for 30 min in 10 µl 70 % TFA containing 3 mg/ml CNBr. The mixtures were dried for mass spectrometry and sequence analysis, and in the case of the partially cleaved products, also used for methylation (see 2.5.5. below).

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2.5.5. Methylation

This is a reversible modification where free carboxyl groups (COOH) are converted to $COOCH_3$ in the presence of methanol-chloride solution and is, therefore, a useful tool to investigate whether a polypeptide is C-terminally blocked. If the C-terminal carboxyl group is protected by an amide (NH_2), there can be no formation of a methyl ester (CH_2 = 14 mu). Methanol-chloride solution was prepared by bubbling HCl gas into pure methanol to a final concentration of 4-7 % gas. Peptides were redissolved in 5 µl pure methanol, and 5 µl methanol-chloride solution was added to the tube. After 25-40 min incubation at room temperature, the reaction was terminated by the addition of ice-cold Milli-Q water. The mixture was analysed by mass spectrometry, using α-hydroxy-cinnamic acid as matrix. Ten SGE of the following C-terminal fragments were methylated: MIH (D9), truncated cHH-I (CNBr 2) and cHH-I (D15).

The synthetic nonapeptide DIWRSILKA-NH_2 (custom-synthesised by Dr. H. Echner) and N-terminally deblocked RPCH (= des-pGlu-red pigment-concentrating hormone) were also subjected to methylation. A freeze-dried equivalent of 2 SGs of partial CNBr-cleaved cHH-I material (see 2.5.4.3.) was methylated at 18 °C for 20 min in 5 µl methanol-chloride solution and analysed by mass spectrometry.

As method controls, several known peptides with amidated or free C-termini were included in the methylation experiments: a hypertrehalosaemic hormone from *Carausius morosus* (code-named *Cam*-HrTH-II; Peninsula), an allatostatin from *Periplaneta americana* (*Pea*-Ast5; custom-synthesised by Dr. R. Kellner, Merck, Darmstadt, Germany) and a peptide mixture consisting of des Arg-bradykinin, angiotensin I and Glu-fibrinogen peptide B (Calibration mixture 1, PerSeptive).

2.5.6. Determination of the ratio of truncated- and non-truncated cHH peptides

Batches of five sinus glands were dissected into the following homogenisation buffers: (1) 10 % acetic acid; (2) 0.2 mM phenylmethylsulfonyl fluoride (PMSF, Boehringer Mannheim) in 10 % acetic acid; (3) 0.2 mM PMSF in distilled water; (4) 0.1 % thiodiglycol (Fluka) in 10 % acetic acid; (5) 0.1 mg/ml trypsin inhibitor (from bovine lung, Serva) in 10 % acetic acid; and (6) 0.2 mg/ml ethylenediaminetetraacetic acid, pH 8 (EDTA, Boehringer Mannheim) in water. The sinus glands were stored in solution at -20 ^{0}C and later, homogenised in a glass homogeniser for 5 min at 85 ^{0}C. The extracts were dried *in vacuo*, redissolved in 10 % acetic acid and applied to the HPLC system for peptide separation (as in 2.2.). Peaks, defined by their retention time as truncated and non-truncated cHHs, were integrated and the peak area calculated by means of Gilson's Unipoint software (ver. 1.65). This set of experiments was also repeated with batches of 10 sinus glands.

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2.6. Purification and characterisation of non-cHH-like (less-hydrophobic) peptides from J. lalandii

2.6.1. Initial purification on RP-HPLC

With every HPLC separation of crude extracts of SGs from *J. lalandii* (see 2.2.), material eluting between 0 and 20 min were continuously collected as 2 ml fractions. These fractions were immediately dried down and stored at –20 ^{0}C until further purification. The fractions were redissolved in 50 µl of 25 % acetonitrile, pooled into 1 tube for drying and again separated by HPLC in batches of 100-400 SGE of fractions (total amount repurified = 1060 SGE).

Peptides were separated on the RP-HPLC system and column, described in 2.2. Solvents were: A = 0.115 % TFA in water, B = 100 % acetonitrile with 0.1 % TFA and a gradient was applied (4 min isocratic step at 25 % B and increasing to 40 % B in 50 min). Peak fractions were collected manually and dried. Aliquots were analysed by MALDI-TOF spectrometry to assess the purity of the peak material.

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2.6.2. Subsequent purifications on RP-HPLC

Peak fractions from separate runs that corresponded to peaks 1-5 (Fig. 61), based on comparisons of the chromatograms, were pooled in batches of 350 SGE and subjected to a second purification step using the same HPLC system as described in 2.2. but with solvents: A = 0.13 % HFBA (heptafluorobutyric acid) in water, B = 100 % acetonitrile with 0.13 % HFBA. A gradient was applied from 30-60 % B in 100 min. Resulting peak fractions were manually collected; identical peaks from different runs were pooled and dried.

Those peaks that were not fully resolved, were subjected to a third purification step with different solvents: A = 0.05 % TFA in water, B = 100 % acetonitrile with 0.05 % TFA and a different gradient: 6 min isocratic step at 25 % B and increase to 40 % B in 50 min. Aliquots of the purified material were analysed by mass spectrometry.

2.6.3. Heterologous biological assays for RPCH and PDH activities

For the red pigment-concentrating hormone (RPCH) assay, shrimps (*Palaemon pacificus*) were adapted to dark conditions by being confined in a darkened aquarium in tanks lined with black plastic for at least 2 days before the assay.

For the PDH (pigment-dispersing hormone) assay, shrimps were adapted to light conditions by being kept under white light in white plastic containers in the aquarium for at least 2 days before the assay.

The assays were carried out at room temperature. The condition of the chromatophores was assessed with a Nikon dissecting microscope at the start and at regular intervals during the assay: 5 min for RPCH and 10 min for PDH. Dark-adapted shrimps were individually kept in sea water in small, black dishes for the duration of the assay, whereas light-adapted animals were isolated in white containers. Ten µl of test substances were injected through the lateral carapace and the animal was returned to its dish. For microscopic monitoring of the chromatophores, the entire dish was placed onto the microscope stage so that the shrimps were not subjected to undue stress of prolonged handling outside sea water.

Substances for injection were dissolved in sea water. These substances were: 1 SGE of HPLC fractions 1-5 (Fig. 61), synthetic RPCH (Peninsula), synthetic β-PDH (a kind gift from Prof. K. R. Rao, University of West Florida, Pensacola, Florida, United States of America) and sea water (n = 5-6); the assays were repeated at least 4 times.

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A further control included non-injected animals which were regularly monitored to see whether their chromatophoric behaviour was affected by the laboratory environment during the assay period.

2.7. Structural elucidation of non-cHH-like (less-hydrophobic) peptides

All the purified peptides were N-terminally sequenced, except the peak identified as RPCH.

2.7.1. Deblocking the N-terminus of RPCH

The suspected pGlu residue at the N-terminus of RPCH was removed by cleaving with a *Pfu* pyroglutamate amino peptidase (Takara Shuzo Co., Japan). This enzyme, purified from *Pyrococcus furiosus*, has an optimum temperature of 95-100 ^{0}C, with 90 % confirmed activity at 75 ^{0}C. Therefore, the cleavage reaction can be carried out for a shorter time compared with other pGlu peptidases.

The lyophilised enzyme was reconstituted in 50 µl buffer (supplied, and diluted in water according to the manufacturer's instructions). To establish optimum conditions for cleavage of *Jala* RPCH, 300 SGE of the pure peptide was redissolved in 45 µl buffer and 2.5 µl of the reconstituted enzyme was added. The mixture was incubated at 65 ^{0}C for a maximum period of 135 min. The equivalent of 30 SGs was withdrawn 45 and 135 min after incubation and run on HPLC. Based on these results, 900 SGE RPCH was then incubated with 5 µl enzyme at 65 ^{0}C for 3 h. Aliquots of the deblocked peptide, i.e. des-pGlu-RPCH, were subjected to mass analyses and to methylation experiments (see 2.5.5.). The remainder of the deblocked material was subjected to N-terminal sequencing.

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2.7.2. Tryptic digestion of CPRPs

The putative cHH precursor-related peptides (300 SGE dissolved in 50 µl of 0.1 M Tris-HCl buffer, pH 8.5) were digested with 1 µg trypsin in 0.01 % TFA (sequencing grade, Boehringer Mannheim) for 18 h at 37 ^{0}C and purified by HPLC. The same column, solvents and gradient system as outlined in 2.5.4.1. were used. The resulting fractions were collected manually; aliquots were retained for mass spectrometry and the rest of the fractions were dried for N-terminal sequencing.

2.7.3. Endo-Asp N digestion of CPRP-1 and -2

The dried peptide, consisting of a mixture of the two peptides (150 SGE), was redissolved in 50 µl sodium phosphate buffer, pH 8, digested with 0.3 µg endoproteinase-Asp N (sequencing grade, Boehringer Mannheim) for 18 h at 37 ^{0}C. The mixture was purified and analysed as in 2.5.4.1.

2.7.4. CNBr cleavage of CPRP-1 and -2

The dried peptide (180 SGE) was redissolved in 50 µl of 25 % acetonitrile, a small crystal of CNBr was added and brought into solution. The reaction was carried out overnight in the dark at room temperature and purified on HPLC under the same conditions as in 2.5.4.1.

2.8. Molecular biological aspects

2.8.1. RNA isolation

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Thirty XOs were dissected from freshly-ablated eyestalks of adult *J. lalandii* under sterile and RNAase-free conditions. The XOs were immediately frozen in liquid nitrogen and stored at –80 °C until the RNA could be extracted. Total RNA was isolated with a standard phenol/guanidine thiocyanate method using peqGOLD TriFast (PEQLAB) and following the manufacturer's instructions. Briefly, frozen XOs were transferred to a sterile 1.5 ml tube, 0.5 ml Trifast solution was added and the XOs were manually homogenised with a sterile, teflon-ended pestle. A further 0.5 ml Trifast was added and the sample incubated at room temperature for 5 min. Thereafter, 200 µl chloroform was added, the sample was vortexed, incubated at room temperature for 10 min and then centrifuged for 5 min at 4 °C and at 14 000 rpm on a Sorvall RMC14 (Du Pont). The top (aqueous) phase was carefully transferred into a fresh tube, and the lower layers were discarded. To the aqueous phase, 500 µl isopropanol was added to precipitate the RNA, and left at room temperature for 15 min. The sample was centrifuged at 4 °C for 10 min at 14 000 rpm. The supernatant was discarded and the pellet was rinsed (at least twice) with 1 ml 75 % ethanol, followed by a 5 min centrifugation step at 4 °C, 14 000 rpm. The pellet was redissolved in 10 µl DEPC (diethyl pyrocarbamate)-treated water and aliquots were quantified in quartz cuvettes on a UVIKON 710 spectrophotometer at wavelengths of 230, 260 and 280 nm. The amount of RNA in the sample was calculated as:

$$\frac{\text{Absorbance @ 260 nm x dilution factor of sample}}{30}$$

(where 30 is the RNA factor). The quality of the RNA was checked by electrophoresis on an ethidium bromide/agarose/formaldehyde gel: 1.5 µg and 3 µg RNA were loaded alongside a marker (RNA B) and the gel was run for several hours at 100 V. The

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RNA bands were visualised under UV light and recorded photographically. RNA samples in DEPC-treated water were stored at –70 ^{0}C.

2.8.2. Construction of a cDNA library

A cDNA library was constructed with the use of a SMART™ cDNA library construction kit from CLONTECH (User Manual PT3000-1); a flowchart of the protocol is shown in Fig. 10. The protocol utilizes the SMART III™ (**S**witching **M**echanism **A**t 5' end of **R**NA **T**ranscript) oligonucleotide in the first-strand synthesis to generate high yields of full-length double strand (ds) cDNA.

2.8.2.1. First-strand synthesis

The following reagents were combined in a sterile 0.5 ml centrifuge tube with deionised water to a final volume of 5 µl: 1 µg RNA extracted from *Jala* XOs, 1 µl SMART III™ oligonucleotide and 1 µl CDS III/3' PCR primer. The contents were mixed and incubated for 2 min at 72 ^{0}C, then cooled on ice. The following was then added to the tube to make a 10 µl reaction: 2 µl 5x First-strand buffer, 1 µl DTT (10 mM), 1 µl dNTP mix (10 µM) and 1 µl Superscript II reverse transcriptase (Life Technologies). The mixture was incubated for 1 h at 42 ^{0}C in a thermal cycler (GeneAmp 480, Techne). The tube was then cooled on ice before proceeding with cDNA amplification; the remainder of the sample was stored at –20 ^{0}C.

2.8.2.2. cDNA amplification by LD (Long Distance) PCR

The following components were added to a fresh 0.5 ml tube: 2 µl first-strand cDNA (see above), 80 µl deionised water, 10 µl 10 x cDNA PCR buffer, 2 µl dNTP mix, 2 µl 5' PCR primer, 2 µl CDS III/3' PCR primer and 2 µl 50 x Advantage® cDNA

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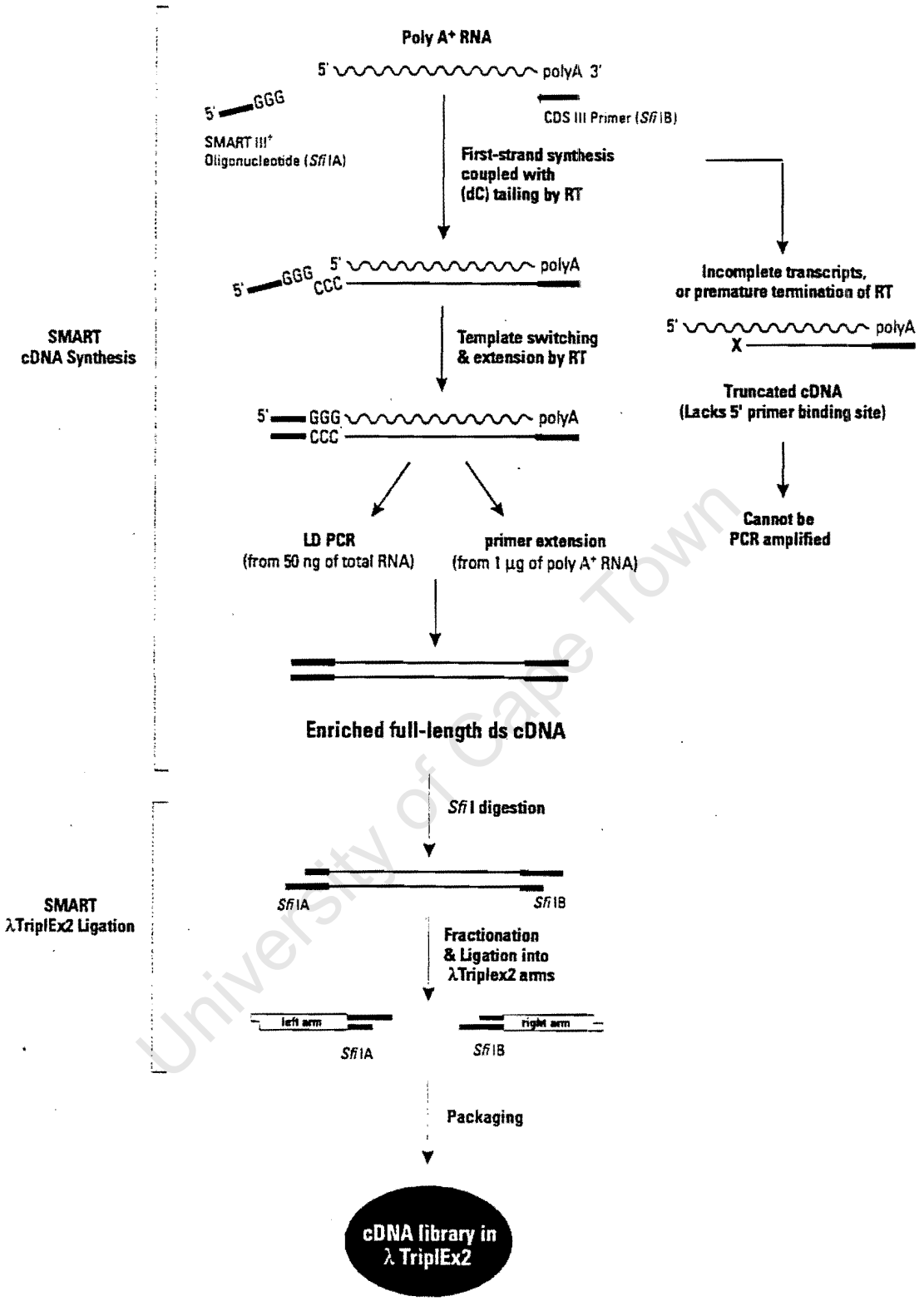

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Fig. 10. Flow diagram showing the main steps involved in the SMART cDNA library construction protocol (from CLONTECH Laboratories, Inc.).

polymerase mix. The contents were gently mixed and then placed in the preheated thermal cycler and a thermal regime selected: 95 ^{0}C for 1 min, followed by 20 cycles of a 15 s denaturing step at 95 ^{0}C and a 6 min combined annealing/extension step at 68 ^{0}C. A 5 µl sample of the PCR product was mixed with 1 µl 5x DNA Blue Run dye and analysed on a 1.1 % agarose/EtBr gel, alongside with 1 kb DNA size markers (250 ng of Lambda DNA digested with *Hind*III or a double digest with *Hind*III/*Eco* RI, MBI Furmentas). The bands were visualised under UV light. The remainder of ds cDNA was either digested with proteinase K, or stored at –20 ^{0}C.

2.8.2.3. Proteinase K digestion

Fifty µl of the amplified ds cDNA was combined with 2 µl proteinase K (20 µg/µl) in a sterile 1.5 ml tube. The contents were briefly mixed and incubated for 20 min at 45 ^{0}C. Deionised water (50 µl) was added and the sample denatured by the addition of 100 µl phenol/water/chloroform solution (nucleic acid purification grade, Applied Biosystems) and mixed continuously for 1-2 min by gentle inversion. The tube was centrifuged for 5 min at 14 000 rpm to separate the phases. The top (aqueous) layer was transferred to a clean tube, while the interface and lower layers were discarded. To the aqueous layer, 100 µl chloroform was added and the sample was gently mixed again for 1-2 min followed by a 5 min centrifugation step to separate the phases. The top (aqueous) layer was once again transferred to a clean tube and the other layers were discarded. The following was added to the aqueous layer: 10 µl 3M sodium acetate pH 5.2, 1.3 µl glycogen (20 µg/µl) and 260 µl of 100 % ethanol at room temperature. This mixture was immediately centrifuged at 14 000 rpm for 20 min at room temperature and the supernatant was carefully removed and discarded. The pellet was washed with 100 µl 80 % ethanol and centrifuged for 5 min. The pellet was

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air-dried for 15-20 min to evaporate the residual ethanol and was then resuspended in 79 µl deionised water.

*2.8.2.4. Sfi*I *digestion*

The following components were combined in a fresh 0.5 ml tube: 79 µl cDNA after proteinase K digestion, 10 µl 10x *Sfi* buffer, 10 µl *Sfi* I enzyme and 1 µl 100x BSA to give a final reaction volume of 100 µl. This was mixed well and incubated at 50 ^{0}C for 2 h.

2.8.2.5. cDNA size fractionation by CHROMA SPIN-400

Sixteen 1.5 ml tubes were labeled and arranged in a rack.The CHROMA-SPIN-400 column (CLONTECH) was prepared for the drip procedure as follows: (i) the column was warmed at room temperature for 1 h, then inverted several times to resuspend the gel matrix; (ii) air bubbles were removed from the column and the bottom cap was removed to let the column drip; (iii) the column was then attached to a ring stand and the drainage buffer could flow out under gravity; (iv) when the surface of the gel beads in the column matrix was visible and the flow rate was about 1 drop/40-60 s, the column was deemed ready. When the storage buffer stopped dripping out, 700 µl of column buffer was gently added and allowed to drip out completely (15-20 min). Only then was 100 µl of the *Sfi* I-digested cDNA carefully added to the top centre of the matrix; the sample was allowed to be fully absorbed into the surface of the matrix before 100 µl column buffer was added. This buffer was allowed to drain out of the column until there was no liquid left above the resin. The rack of 16 tubes were then placed under the column outlet and after the addition of 600 µl column buffer to the column, the single-drop fractions (30-40 µl) were sequentially collected. Three µl of

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fractions 7-16 were electrophoresed on a 1.1 % agarose/EtBr gel.The gel was run for 2 min at 150 V and the bands visualised under UV light. The fractions that contained the cDNA were pooled into a clean 1.5 ml tube. The following reagents were added to the pooled fractions: 1/10 vol. 3 M sodium acetate pH 5.2, 1.3 µl glycogen (20 mg/ml) and 2.5 vol. 100 % ethanol. The contents were mixed by gently rocking the tube back and forth and the tube was kept overnight at –20 ^{0}C. Thereafter, the tube was centrifuged at 14 000 rpm for 20 min at room temperature. The supernatant was carefully removed and discarded; the tube was recentrifuged, all the liquid was carefully removed and the pellet was air-dried for 10 min. The dried pellet of cDNA was then resuspended in 7 µl deionised water.

2.8.2.6. Ligation of cDNA to the vector

The quantity of *Jala* cDNA recovered after size fractionation was estimated by the ethidium bromide plate assay (following instructions from Stratagene). In a clean 0.5 ml tube, the following reagents were added and mixed well: 1 µl cDNA (= 50 ng), 1 µl λ TriplEx2 vector (= 500 ng/µl), 0.5 µl 10x ligation buffer, 0.5 µl ATP (10 mM), 0.5 µl T4 DNA ligase and 1.5 µl deionised water. The tube was incubated overnight at 16 ^{0}C. A λ-phage packaging reaction was carried out according to the protocol for the Gigapack III Gold Packaging kit (Stratagene). Briefly, 4 µl DNA (containing ligated *Jala* XO cDNA) was added to the Gigapack III packaging extract and mixed well with a pipette tip. The tube was then pulsed for 3-5 s in a centrifuge and incubated at room temperature for not more than 2 h. SM buffer (500 µl) was added to the tube, followed by 200 µl chloroform. After mixing gently, the tube was briefly spun to sediment the debris. The supernatant containing the phage was then ready for titre determination.

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2.8.2.7. Determining the titre of the unamplified library

E. coli XL1-Blue bacteria were grown overnight at 37 ^{0}C. The bacterial culture was centrifuged for 5 min at 5000 rpm, the supernatant was discarded and the pellet was resuspended in 7.5 ml 10 mM $MgSO_4$. The unamplified phage lysate (see above) was diluted in 1x lambda dilution buffer to 1:5 and 1:20. One µl of the diluted phage solution was added to 200 µl of the viable *E. coli* XL1-Blue overnight culture and the phage was allowed to adsorb for 10-15 min at 37 ^{0}C. Two ml of melted 50 ^{0}C LB(Lourier broth)/$MgSO_4$ top agarose was added, quickly mixed by inversion and immediately poured onto 90 mm LB agar plates prewarmed to 37 ^{0}C. The plates were cooled at room temperature for 10 min, inverted and incubated at 37 ^{0}C for 6-18 h. The resulting phage plaques, appearing as light spots in the bacterial lawn, were counted on a light box with the use of a manual counter and a marker pen. The titre of the phage was calculated as follows, where pfu refers to "plaque forming units":

$$\text{Titre of phage (pfu/ml)} = \frac{\text{no.of plaques X dilution factor X } 10^3 \text{ µl/ml}}{\text{vol. of diluted phage plated}}$$

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2.8.2.8. Library amplification

E. coli XL1-Blue bacteria, grown overnight at 37 ^{0}C, were centrifuged for 5 min at 5000 rpm, the supernatant was discarded and the pellet was resuspended in 7.5 ml 10 mM $MgSO_4$. Into 20 tubes of 4 ml volume, 500 µl of the bacterial suspension and 20 µl of *Jala* cDNA library lysate were added. The tubes were incubated at 37 ^{0}C for 15 min. Thereafter, 4.5 ml of molten 50 ^{0}C LB/$MgSO_4$ top agarose was added to each tube. The tubes were quickly inverted and the bacteria/phage/agar mixture was poured onto 15 cm agar plates that had been preheated to 37 ^{0}C. The plates were cooled at

room temperature and later inverted, stacked onto each other and incubated at 37 ^{0}C overnight. To prepare an amplified library lysate, 10 ml 1x lambda dilution buffer was added to each plate and the plates were incubated at room temperature for 8-9 h on a platform shaker. With a sterile 5 ml pipette, the lambda phage lysates were transferred into a sterile beaker. This was well mixed and poured into sterile 50 ml polypropylene screw-cap tubes; 10 ml of chloroform was added to each tube and mixed by hand. This was followed by a 10 min centrifugation step at 7000 rpm. The supernatant was collected into fresh tubes. An aliquot was used for titre determination and blue/white screening, while the remainder was dispensed into 10 ml tubes with 7 % DMSO (i.e. 7.5 ml amplified cDNA library + 525 µl DMSO, dimethyl sulfoxide). This was stored at –70 ^{0}C.

2.8.2.9. Determining the percentage of recombinant clones and titre determination of the amplified cDNA library

To determine the efficiency of the recombination and the success of the ligation, blue/white screening was carried out, i.e. *E. coli* XL1-Blue bacteria were transduced with the phage containing the *Jala* cDNA insert and the plate was screened for blue plaques on medium containing IPTG (isopropyl beta-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside). To perform blue/white screening, the same procedure was followed as described in 2.8.2.7., except that X-gal and IPTG solutions were added to the molten top agarose before plating the phage/bacteria mixture. After incubating the plates at 37 ^{0}C overnight, the ratio of the white (recombinant) to the blue (non-recombinant) plaques were calculated.

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To titre the amplified cDNA library, 10 ml of the library lysate was diluted 1/10 000 in 1x lambda dilution buffer. One µl of this was added to 200 µl bacterial cells and cultured overnight at 37 ^{0}C.

2.8.3. Primers for PCR amplification of Jala cHH cDNA

From the known amino acid sequence of *Jala* cHH-I (see Fig. 41), two non-degenerate primers were designed (using the codon usage of *Drosophila melanogaster*) for use in PCR: an upstream sense primer, *Jala* cHH pos (5' – GCCCTGTTCGAYCAGAGCTGCAAGGGCGT – 3'), encoding the N-terminal region of the cHH peptide AVFDQSCKGV, and a downstream anti-sense primer, *Jala* cHH neg (5' – CACCATCTGCACSGAGGCCACGTACTC – 3'), encoding the C-terminal region of the cHH peptide VDEYVASVQMV.

2.8.4. PCR cloning of the coding region of Jala cHH cDNA

RNA was extracted from 30 *Jala* XOs from male specimens with Trifast; first strand cDNA was synthesised and ds cDNA was amplified according to the protocol supplied with the SMART™ kit from CLONTECH (see 2.8.1. and 2.8.2. above). In the same way, RNA was also extracted from 10 XOs from female *Jala* specimens and ds cDNA was amplified. PCR reaction mixtures (total volume 50 µl) contained 5 µl ds *Jala* cDNA, 5 µl 10x cDNA PCR buffer*, 5 µl dNTP mix, 5 µl each of *Jala* cHH-pos and –neg primers (10 pmol/µl) and 0.2 µl *Taq* DNA polymerase* and sterile water. The addition of 10 µl 5x Q-Solution* was also included in some of the reaction mixes (products marked with * were obtained from the QIAGEN *Taq* PCR Kit; Q solution is an agent which consists predominantly of DMSO and Mg^{++} and which modifies the melting behaviour of DNA in a PCR reaction). As a control, reaction

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mixtures without cDNA template were included. PCR was performed with the following temperature profile: 96 ^{0}C for 1 min, followed by 30 cycles of a 45 s denaturing step at 96 ^{0}C, a 1 min annealing step at 55 ^{0}C and an elongation step at 72 ^{0}C for 1 min; a final extension step of 10 min at 72 ^{0}C concluded the cycling in a GeneAmp 480 cycler.

2.8.5. Gel extraction of specific PCR products

The PCR products from the male RNA samples were pooled and run on an agarose/EtBr gel. The same procedure was carried out with the specific PCR products from female RNA samples. The gels were briefly viewed under blue light, the DNA bands were excised from the gel with clean scalpel blades and put into preweighed Eppendorf tubes. The weight of each excised band was then determined and the DNA was extracted from the gel, following the QIAGEN QIAquick Gel Extraction protocol using the reagents supplied with the kit. Briefly, 3 volumes of QG buffer were added to each gel piece. The tubes were incubated at 50 ^{0}C for 10 min, with periodic vortexing. To each 100 mg of gel slice, 100 µl isopropanol was added. The mixture was applied in volumes of 800 µl to a QIAquick spin column that was fitted into a 2 ml collection tube. The column was then centrifuged for 1 min at room temperature, 14000 rpm in an Eppendorf 5415C centrifuge. After the centrifugation step, the buffer from the collection tube was discarded; the DNA was bound to the column. In an additional step to remove traces of agarose, 500 µl QG buffer was added to the column and centrifuged for 1 min. The column was then washed with 750 µl PE buffer and allowed to stand for 2 min before centrifuging for 1 min (this step is important for blunt-ended ligations). The centrifugation step was repeated and, finally, 30 µl elution buffer (10 mM Tris-HCl, pH 8.5) was added to the column to elute the

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bound DNA. The quantity of the recovered DNA was estimated from the intensity of the bands on an EtBr agarose gel, relative to that from known quantities of DNA in commercially available molecular weight markers.

2.8.6. Cloning of Jala-specific PCR products

The PCR products were cloned into pCR®-TOPO® vector (Invitrogen) with *Eco*R I restriction sites (see Fig. 11 for a detailed map of the TOPO® vector). The efficient 5 min, one-step cloning strategy was carried out according to the protocol supplied with the Invitrogen TOPO TA Cloning Kit. This strategy enables the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector, as illustrated in Fig. 12: the plasmid vector pCR® 2.1-TOPO®, is supplied linearised with (i) single 3'-Thymidine (T) overhangs for TA cloning, and (ii) Topoisomerase I covalently bound to the vector. *Taq* polymerase has a terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. This allows PCR inserts to ligate efficiently with the vector and occurs spontaneously within 5 min at room temperature. Thus, for the cloning reaction, 2 µl PCR product (approximately 100 ng) was mixed with 0.5 µl vector (i.e. 5 ng plasmid DNA in 50 % glycerol, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1 % Triton X-100, 100 µg/ml BSA and phenol red). The cloning reaction was carried out for 5 min at room temperature and the reaction was stopped by the addition of 0.5 µl 6x TOPO® Cloning Stop Solution (0.3 M NaCl, 0.06 M $MgCl_2$), mixed for 10 s at room temperature and placed on ice. The stop solution creates conditions that favour dissociation of topoisomerase from the DNA. The TOPO® Cloning Reaction was then transformed into chemically-competent cells in the following manner: 2 µl of the cloning reaction was added to a vial containing 50 µl of One Shot® cells (i.e. *E. coli* Top 10 strain, Invitrogen). The

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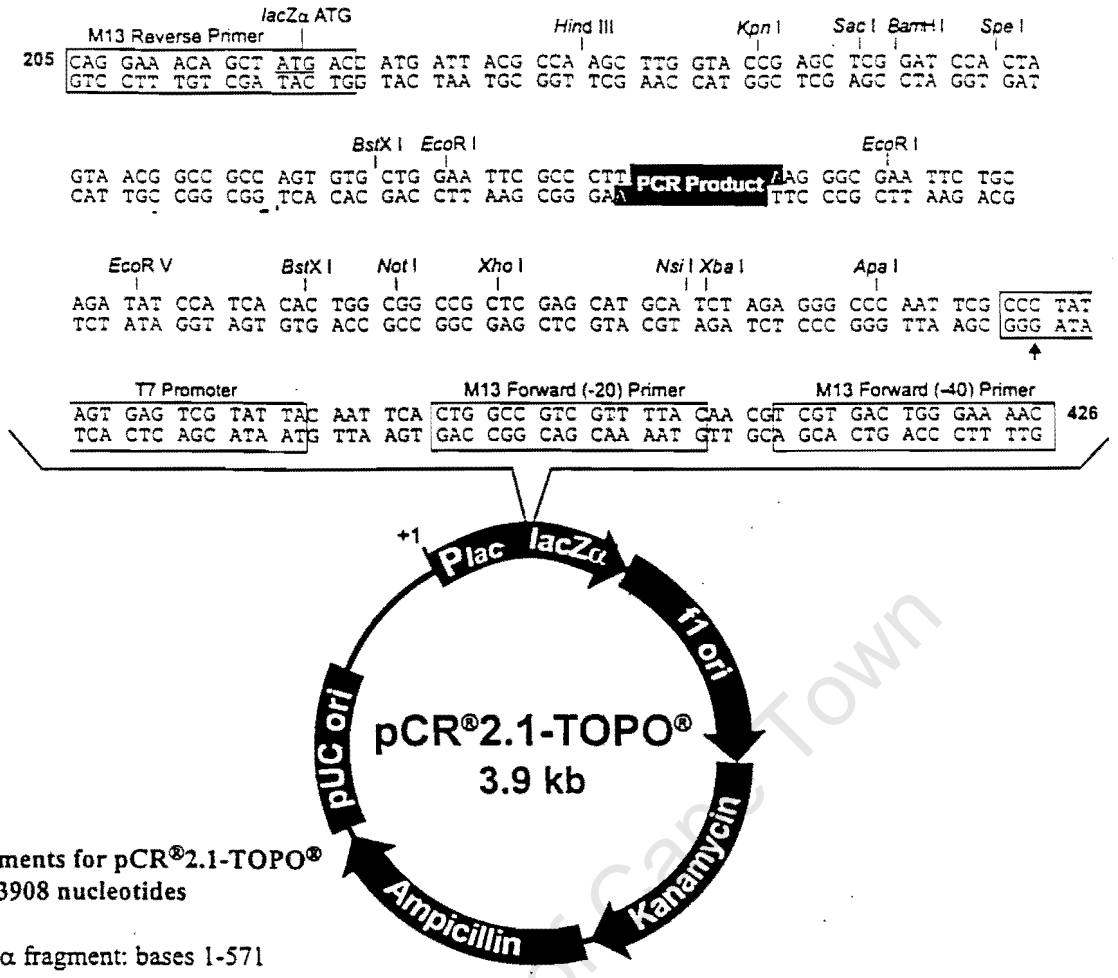

Comments for pCR®2.1-TOPO®
3908 nucleotides

LacZα fragment: bases 1-571
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
M13 Forward (-40) priming site: bases 411-426
f1 origin: bases 548-962
Kanamycin resistance ORF: bases 1296-2090
Ampicillin resistance ORF: bases 2108-2968
pUC origin: bases 3113-3786

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Fig. 11. Restriction map of the plasmid vector pCR® 2.1-TOPO® (supplied by Invitrogen) used for cloning the *Jala*-specific PCR products.

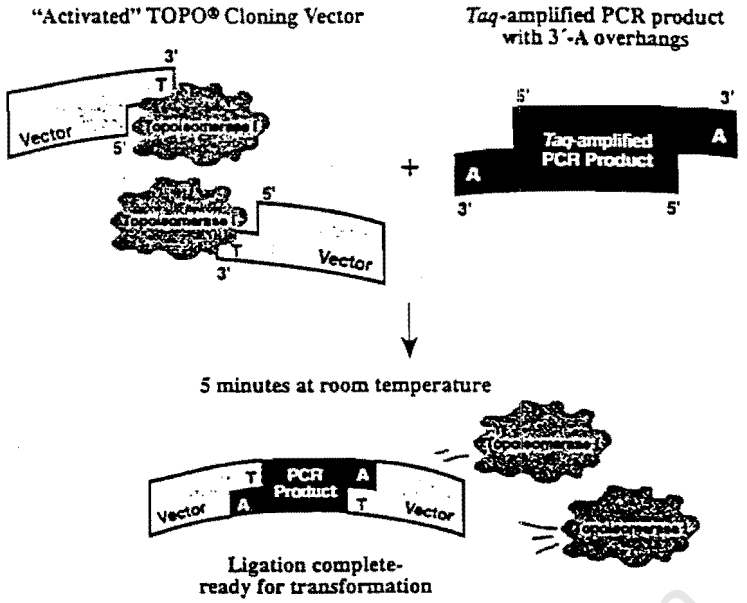

Fig. 12. A diagrammatic overview of the principle of TOPO TA Cloning (from Invitrogen). The plasmid vector is supplied with single 3' thymidine (T) overhangs for TA cloning and with covalently-bound topoisomerase I (the "activated" vector). *Taq* polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. Thus, PCR inserts ligate efficiently with the vector. The ligation activity of topoisomerase I is exploited by this system to allow efficient and spontaneous ligations within 5 min at room temperature.

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mixture was incubated on ice for 30 min, followed by a 30 s heat-shock at 42 ^{0}C. The tubes were then immediately transferred onto ice and 250 µl of room temperature SOC medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added. The tubes were then shaken horizontally at 37 ^{0}C for 30 min. From each transformation, 50 µl was spread onto ampicillin plates. The plates were incubated overnight at 37 ^{0}C and positive clones were picked for further analyses.

2.8.7. Analysis of positive clones and sequencing

Ten ampicillin-resistant, white colonies were cultured overnight in LB medium containing 50 µg/ml ampicillin. Plasmid DNA was isolated by carrying out a mini-prep (plasmid mini purification) using a kit and protocol from QIAGEN. The plasmids were then analysed for the insert by restriction analysis (i.e. digestion with *Eco*R I). Clones with inserts of the expected length were sequenced from both sides using an automated sequencer.

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2.8.8. Preparation of Jala XO cDNA library for screening

XL1-blue *E. coli* cells were resuspended in 7.5 ml 10 mM $MgSO_4$. Two µl of the unamplified *Jala* cDNA library (approximately 10.64 x 10^3 pfu) were added to 500 µl of resuspended bacterial cells and the tubes were incubated for 15 min at 37 ^{0}C. Five ml top agarose was added to the bacterial solution and rapidly poured on the surface of 15 cm $MgSO_4$ agar plates. A numbered nylon filter was deposited on the top agar for 2 min (QIABrane nylon membranes,ϕ 132 mm, QIAGEN). The filter was carefully peeled off and the plate returned to 4 ^{0}C. The filter was then placed in DNA denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 5 min, then into neutralising

solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8) for 5 min and finally, into 2x SSC solution (20x SSC stock consisted of 3 M NaCl, 3 N sodium citrate.$2H_2O$, pH 7) for 2 min. The filters were then blotted on Whatmans filter paper and the DNA was fixed to the filter by UV cross-linking. The filters were wrapped in foil, labeled and stored at – 20 ^{0}C until screening with a digoxygenin-labeled RNA probe (ROCHE Dig labeling kit).

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3. RESULTS

3.1. Comparative immunocytochemistry: localisation of cHH/MIH/VIH peptides in eyestalks of two spiny lobster and one crab species

3.1.1. Morphology of crustacean eyestalks

Longitudinal sections through the eyestalks of *J. lalandii*, *P. homarus* and *C. maenas* were stained with haematoxylin, counterstained with eosin and examined with a light microscope. Figure 13a shows the typical histological features of a decapod crustacean eyestalk: the retina, 4 optic ganglia, SG, XO and optic nerve. The retina contains the photoreceptor units, the ommatidia, each of which contains 7 retinular cells arranged in a characteristic pattern which appears flower-like when viewed in cross-section (Fig. 13c). The optic ganglia consist of the lamina ganglionaris (distal to the retina), the medulla externa, the medulla interna and the medulla terminalis. The sinus gland, a neurohaemal organ, is located on the eyestalk just beneath the exoskeleton and adjacent to the optic ganglia: the gland is comprised of axon terminals surrounding a blood sinus. The axons arise from neurosecretory cells of the medulla terminalis ganglionic X-organ (abbreviated MTGXO, or simply referred to as X-organ) and can be seen as a definitive tract leading into the sinus gland (Fig. 13b). From several morphological reports on other decapod crustaceans, it seems that the location of the sinus gland is different in the spiny lobster. Figure 13a shows that in the spiny lobster the sinus gland is situated at the transition of the medulla interna and the medulla terminalis rather than at the transition of the medulla externa and the medulla interna. The XO is located laterally to the medulla terminalis and is evident

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Fig. 13. Consecutive histological sections of the eyestalk of *P. homarus*: **a, c** stained with haematoxylin and eosin to show its morphology and **b** immunostained with anti-*Hoam* cHH to illustrate components of the neurosecretory system. **c** The retina in cross-section, showing the floral-like arrangement of the retinular cells of the ommatidia.
(*1* ommatidia, *2* lamina ganglionaris, *3* medulla externa, *4* medulla interna, *5* medulla terminalis, *6* optic nerve, *M* muscle, *S* blood sinus, *curved arrow* sinus gland, *arrow* X-organ, *open arrow* sinus gland tract
Scale bar 1 mm

as a cluster of cells with large nuclei. In Fig. 13b, only the components of the XO-SG complex are illustrated after immunocytochemical staining with an antibody raised against a cHH molecule.

3.1.2. Enzyme-linked immunocytochemistry: establishing optimal dilutions of primary antisera

The donors of the different antisera had recommended the following dilutions (pers. comm.) as optimal for the specific antigens against which the antisera had been raised: anti-*Hoam* cHH and anti-*Hoam* VIH 1:10 000 and 1:500, respectively, anti-*Capa* cHH and anti-*Capa* MIH 1: 4000, and anti-*Prbo* cHH at least 1:500 (as tested in a dot blot assay). To ascertain whether the antisera would at all recognise epitopes on eyestalk material from other crustacean species, ELISAs were carried out on crude extracts of sinus glands from *J. lalandii*, *P. homarus* and *C. maenas*. Positive immunoreactions were obtained with all the antisera on 0.25 SGE extract. Crude extracts of sinus glands from each of the 3 species were used as antigens with serial dilutions of all 5 antisera to determine an optimal dilution. Table 3 shows that anti-*Hoam* cHH strongly recognised *Jala* and *Cama* epitopes, even at antibody dilutions of 1:10 000 and 1:8000, respectively, but a more concentrated antibody solution (1:2000) was necessary to produce the same result in *Paho*. Similarly, anti-*Prbo* cHH did not recognise *Paho* epitopes as well as it did those of *Jala* and *Cama*. Both antisera raised against crab (*Capa*) antigens were most reactive with *Cama* material, followed by *Jala* antigen in the case of anti-*Capa* cHH serum. Anti-*Capa* MIH, however, only weakly recognised epitopes on *Jala* and *Paho* sinus gland material. With the anti-*Hoam* VIH serum, the most intense reaction was against sinus glands from *Cama* with an antibody solution of 1:1000 required to obtain a clear positive result.

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Table 3. Serial dilution of primary antisera (see text for abbreviations) as tested in ELISA with crude extracts of sinus glands from spiny lobster (*J. lalandii* and *P. homarus*) and crab (*C. maenas*). [SGE = sinus gland equivalents] The absorbance at 405 nm (30 min after application of the substrate) is indicated.

	Anti-*Hoam* cHH					Anti-*Prbo* cHH		Anti-*Capa* cHH			Anti-*Capa* MIH			Anti-*Hoam* VIH		
	1:10000	1:8000	1:6000	1:4000	1:2000	1:5000	1:2000	1:6000	1:4000	1:2000	1:6000	1:4000	1:2000	1:2000	1:1000	1:500
Jala 0.25 SGE	0.45	0.56	0.68	0.95	1.17	0.62	1.95	0.33	0.49	0.80	-	-	0.19	-	0.13	0.43
Paho 0.25 SGE	-	-	0.12	0.20	0.50	0.22	0.40	-	0.13	0.20	0.17	0.24	0.29	0.1	0.22	0.51
Cama 0.25 SGE	0.26	0.45	0.50	0.70	0.94	0.49	0.51	0.52	0.66	1.40	1.12	1.30	1.58	0.14	0.35	0.60

- is used where absorbance values were below 0.1.

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3.1.3. Tissue immunocytochemistry: peroxidase and immunofluorescence techniques

In all three species, an immunocytochemical reaction was observed in the sinus gland, a number of neurosecretory cells of the X-organ and, in some cases, in part of the XO-SG tract (Figs. 14-16). Only in the case of anti-*Capa* MIH was non-specific, background staining observed. This background immunoreaction was visible in striated muscle tissue and other neural tissue and was not eliminated by blocking endogenous peroxidases in the tissue sections nor by preabsorption of anti-*Capa* MIH with *Capa* MIH peptide (Fig. 17). In all instances large immunoreactive perikarya formed a distinct group at the lateroventral part of the medulla terminalis, clearly defining the shape of the X-organ. In the perikarya the immunoreaction was visible as clumps of granular material scattered around the nucleus, whereas in the sinus gland the "Herring body"-like axonal endings are stained. In all cases, the immunoreaction was more intensive in the sinus gland than in the perikarya. In the case of *P. homarus* with anti-*Hoam* cHH/VIH and anti-*Capa* MIH, parts of the axonal tract between the sinus gland and the X-organ were also immunostained (see for example, Fig.13b). The serial dilutions of primary antibodies were also carried out on tissue sections and the staining results were co-incident with those obtained from ELISA experiments (see above). However, although a visible immunoreaction was obtained in the sinus gland with the very dilute antibody solutions, the perikarya of the XOs were negative. Therefore, the antisera were used at a dilution where both components of the XO-SG complex were immunostained, viz. anti-*Hoam* cHH 1:5000, anti-*Prbo* cHH 1:5000, anti-*Capa* cHH 1:4000, anti-*Capa* MIH 1:2000 and anti-*Hoam* VIH 1:500. The immunostained axonal swellings in the sinus gland of the two spiny lobster species were indistinguishable from each other after the application of different primary

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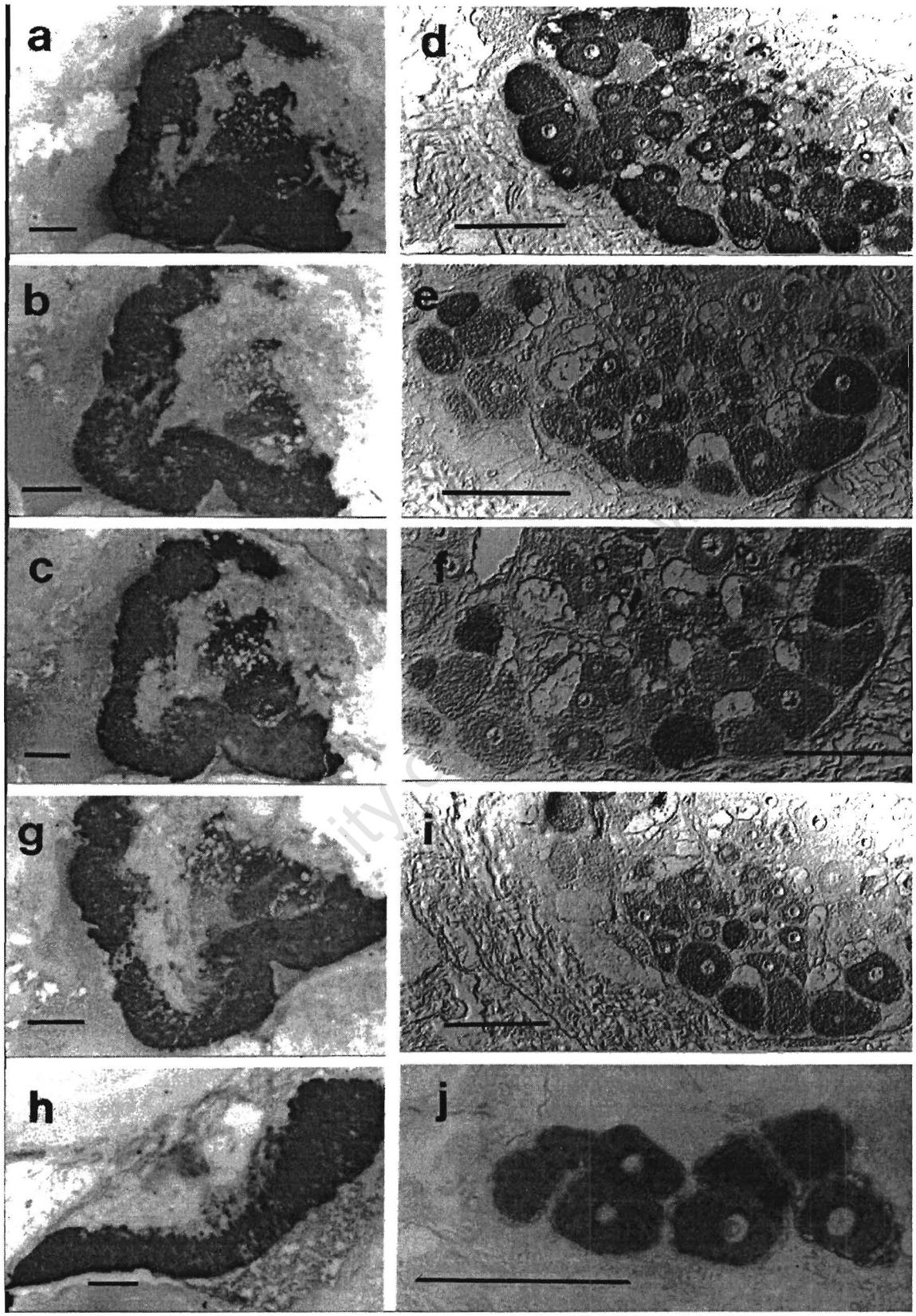

Fig. 14. Immunocytochemical staining of the neurosecretory system (sinus gland **a-c**, **g** and **h**; X-organ **d-f**, **i** and **j**) on consecutive sections (except **h**,**j**) of *J. lalandii* with the following primary antisera: anti-*Hoam* cHH (**a**,**d**), anti-*Capa* MIH (**b**,**e**), anti-*Hoam* VIH (**c**,**f**), anti-*Capa* cHH (**g**, **i**); (**h**,**j**) immunostained with anti-*Prbo* cHH.
Scale bar 50 μm

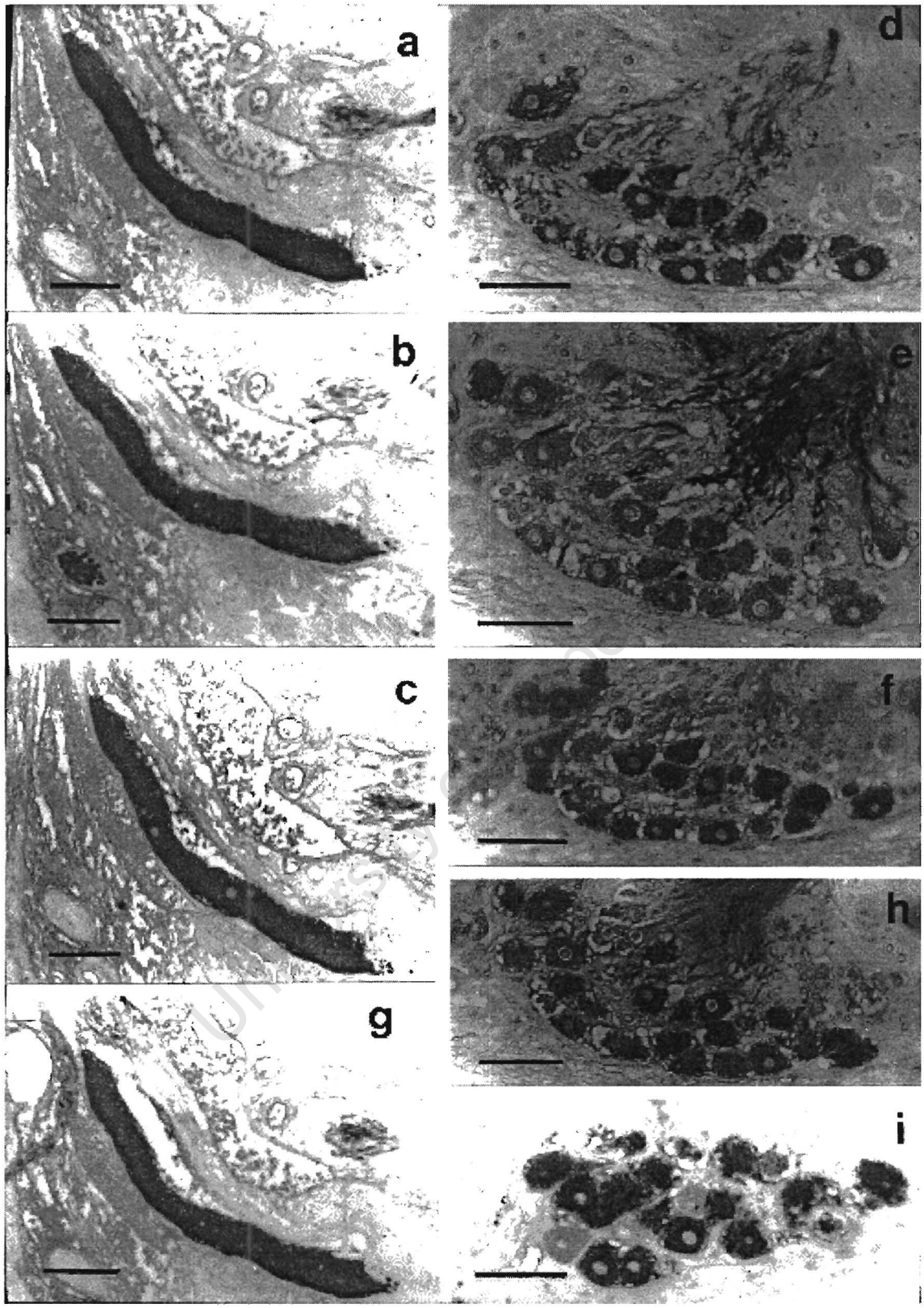

Fig. 15. Immunocytochemical staining of the neurosecretory system (sinus gland **a-c**, **g**; X-organ **d-f**, **h** and **i**) on consecutive sections of *P. homarus* with the following primary antisera: anti-*Hoam* cHH (**a**,**d**), anti-*Capa* MIH (**b**,**e**), anti-*Hoam* VIH (**c**,**f**), anti-*Capa* cHH (**g**,**h**); (**i**) immunostained with anti-*Prbo* cHH. *Scale bar* 50 μm

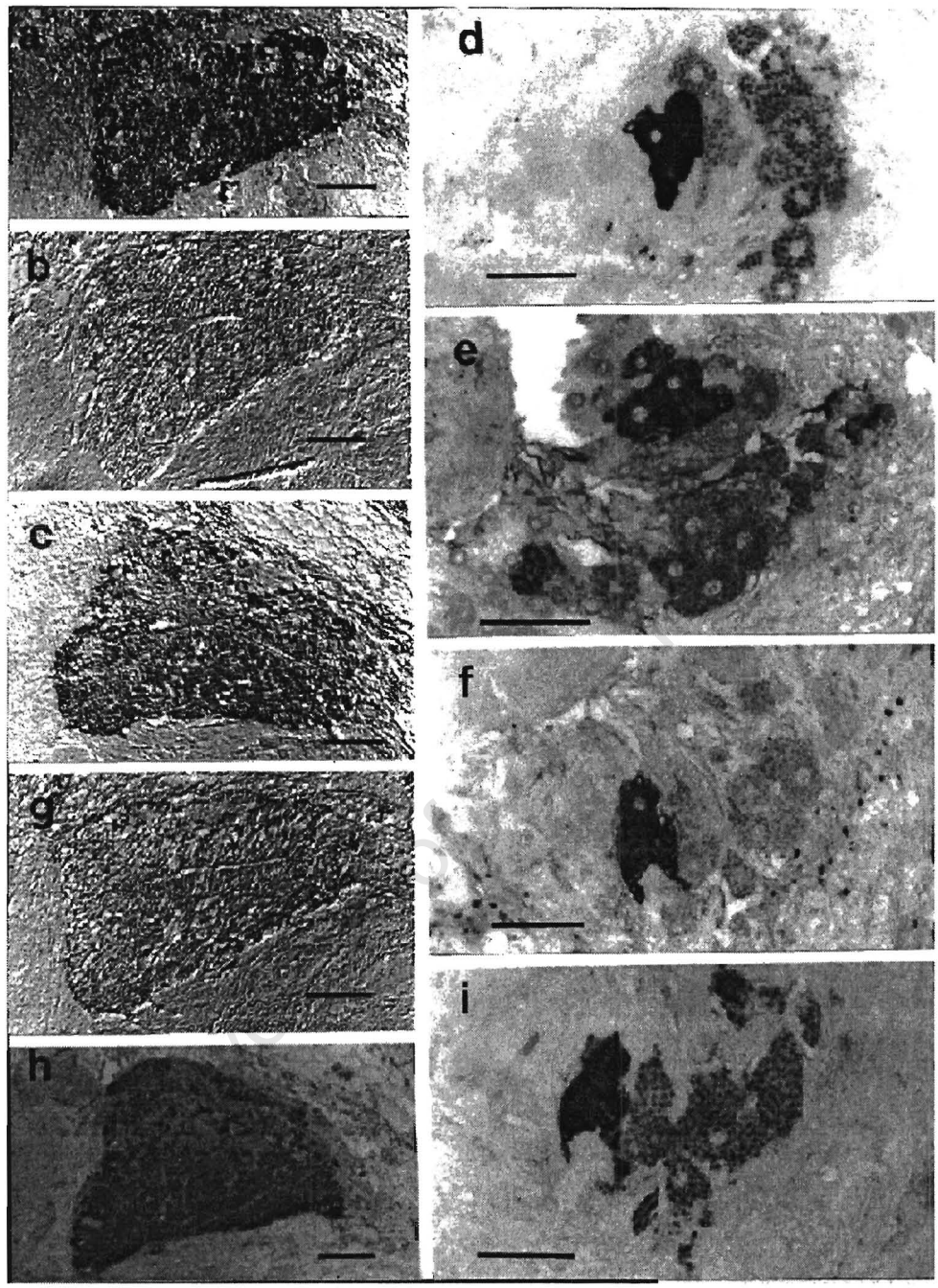

Fig. 16. Immunocytochemical staining of the neurosecretory system (sinus gland **a**-**c**, **g** and **h**; XO **d**-**f**, **i**) on consecutive sections of *C. maenas* with the following primary antisera: anti-*Hoam* cHH (**a**,**d**), anti-*Capa* MIH (**b**,**e**), anti-*Hoam* VIH (**c**,**f**), anti-*Capa* cHH (**g**, **i**); (**h**) immunostained with anti-*Prbo* cHH.
Scale bar 50 μm

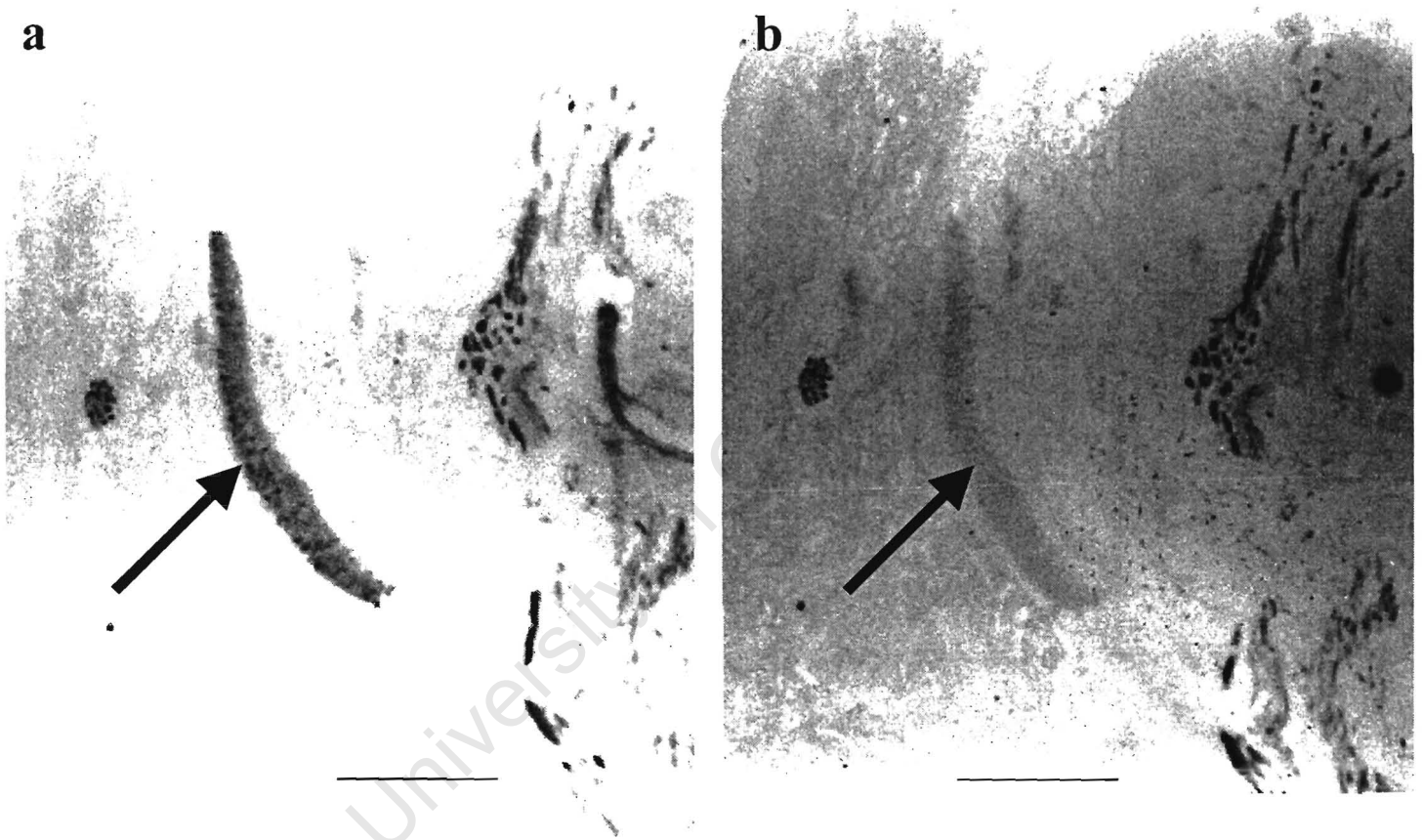

Fig. 17. Adjacent longitudinal sections through the eyestalk of *P. homarus* immunostained with **a.** anti-*Capa* MIH serum and **b.** anti-*Capa* MIH serum preabsorbed with *Capa* MIH peptide. Arrow marks position of the sinus gland. Note the presence of background staining in both **a.** and **b.** *Scale bar* 70 μm

antisera. In contrast, selective staining patterns could be observed in the axon terminals of the sinus gland of *C. maenas* after the application of different antisera (Fig. 16).

In all three investigated species, immunostaining with anti-cHH was consistently strong, whereas with anti-MIH, staining was strongest in *C. maenas* tissue; fainter staining was mostly obtained with anti-VIH in all species. Staining consecutive sections of the three crustacean species with anti-cHH, anti-MIH and anti-VIH showed immunoreactivity to all these antisera in the same perikarya, i.e. co-localisation (Figs. 14-16). However, a number of cells showed an immune response with only one antiserum, which could indicate that a subpopulation of cells produces only one neuropeptide. Alternatively, it could be speculated that the neuroendocrine cells are in different stages of secretion.

To further examine the idea of co-localisation of the cHH/MIH/VIH peptides in the same neuronal cell bodies, tissue sections were double-stained with mixtures of primary antisera and each different antiserum could be visualised by a fluorescent marker (either FITC or TRITC). In general, the staining intensity of the fluorescent-labeled secondary antisera was not satisfactory and was related to the old age of these antisera (used well after the expiry date of the product). Nevertheless, in Fig. 18a-d, co-localisation of MIH and cHH is evident in the axon terminals of the SG, as well as in one neuron of the XO in *J. lalandii* tissue. Co-localisation of MIH and VIH is shown in Fig. 18e-h.

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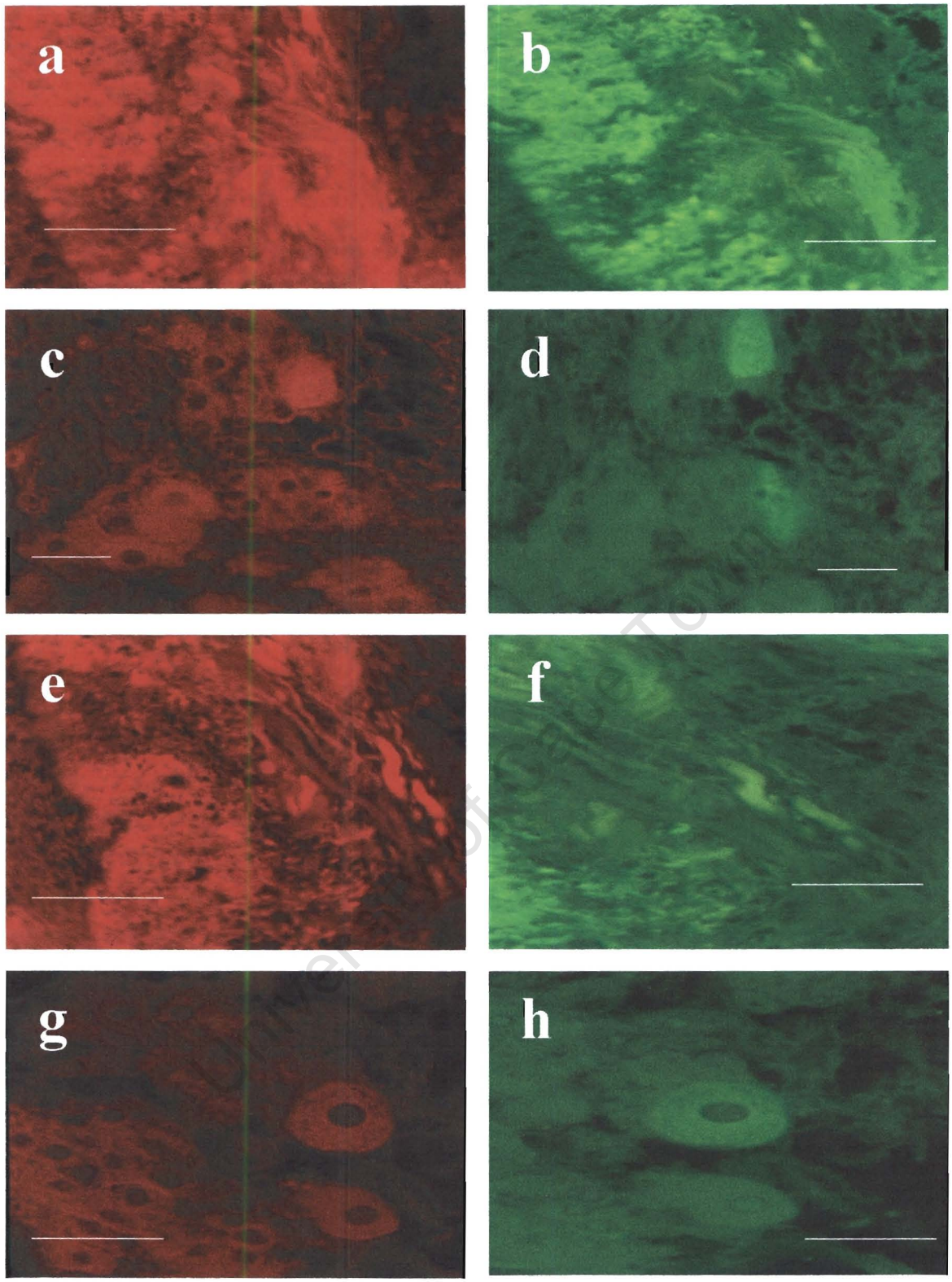

Fig. 18. A pair of longitudinal sections through the eyestalk of *J. lalandii* double-stained with (i) anti-*Capa* MIH (TRITC label: **a**,**c**) and anti-H*oam* cHH (FITC label: **b**,**d**) and (ii) anti-*Capa* MIH (TRITC label: **e**,**g**) and anti-H*oam* VIH (FITC label: **f**,**h**). **a** and **b,** and **e** and **f** show overlapping immunoreactions in the sinus gland, whereas **c** and **d**, and **g** and **h**, show co-localisation of immunoproducts in cell bodies of the X-organ. *Scale bar* 30 μm

3.1.4. Preabsorption experiments and other controls

To determine whether the apparent co-localisation of neuropeptides might be due to cross-reactivity of the antisera at the dilutions used, preabsorption experiments were carried out. The preabsorption of anti-*Capa* cHH serum with *Capa* cHH peptide, and anti-*Capa* MIH serum with *Capa* MIH peptide completely abolished the staining of cHH- and MIH-immunoreactive structures (Fig. 19). The non-specific background staining that is evident in the optic ganglia with anti-*Capa* MIH is not removed by the application of the antiserum preabsorbed with its antigen (Fig. 19i, j).

To establish whether anti-*Hoam* VIH cross-reacted with cHH or MIH, anti-*Hoam* VIH serum was preabsorbed independently with *Capa* cHH peptide and with *Capa* MIH peptide and then applied to *J. lalandii* tissue sections. These antigen-antibody complexes did not eliminate specific staining (Fig. 20). This issue was further investigated in a preliminary ELISA investigation. Putative cHH and MIH peptides were identified in HPLC peak fractions from crude sinus gland extracts of *J. lalandii* and *C. maenas* using anti-*Hoam* cHH and anti-*Capa* MIH antisera. These fractions were then preabsorbed with anti-*Hoam* VIH and tested for immunoreactivity on the putative *Jala* cHH and *Cama* MIH fractions. Figure 21 A,B shows a typical elution pattern of the fractionation of *J. lalandii* and *C. maenas* sinus gland extract on HPLC under the given chromatographic conditions. Of the *J. lalandii* peak fractions, numbered 1-12 (Fig. 21A), peaks 7-9 showed the highest cHH immunoreactivity (Fig. 21C). Fractions 7 and 9 also tested positive with anti-VIH serum; after preabsorption with these fractions, VIH immunoreactivity was reduced between 2- and 4-fold (Fig. 21E). Concerning the *C. maenas* fractions numbered 1-12 (Fig. 21B), peak 9 showed the highest MIH immunoreactivity (Fig. 21D) and was also immunopositive to the

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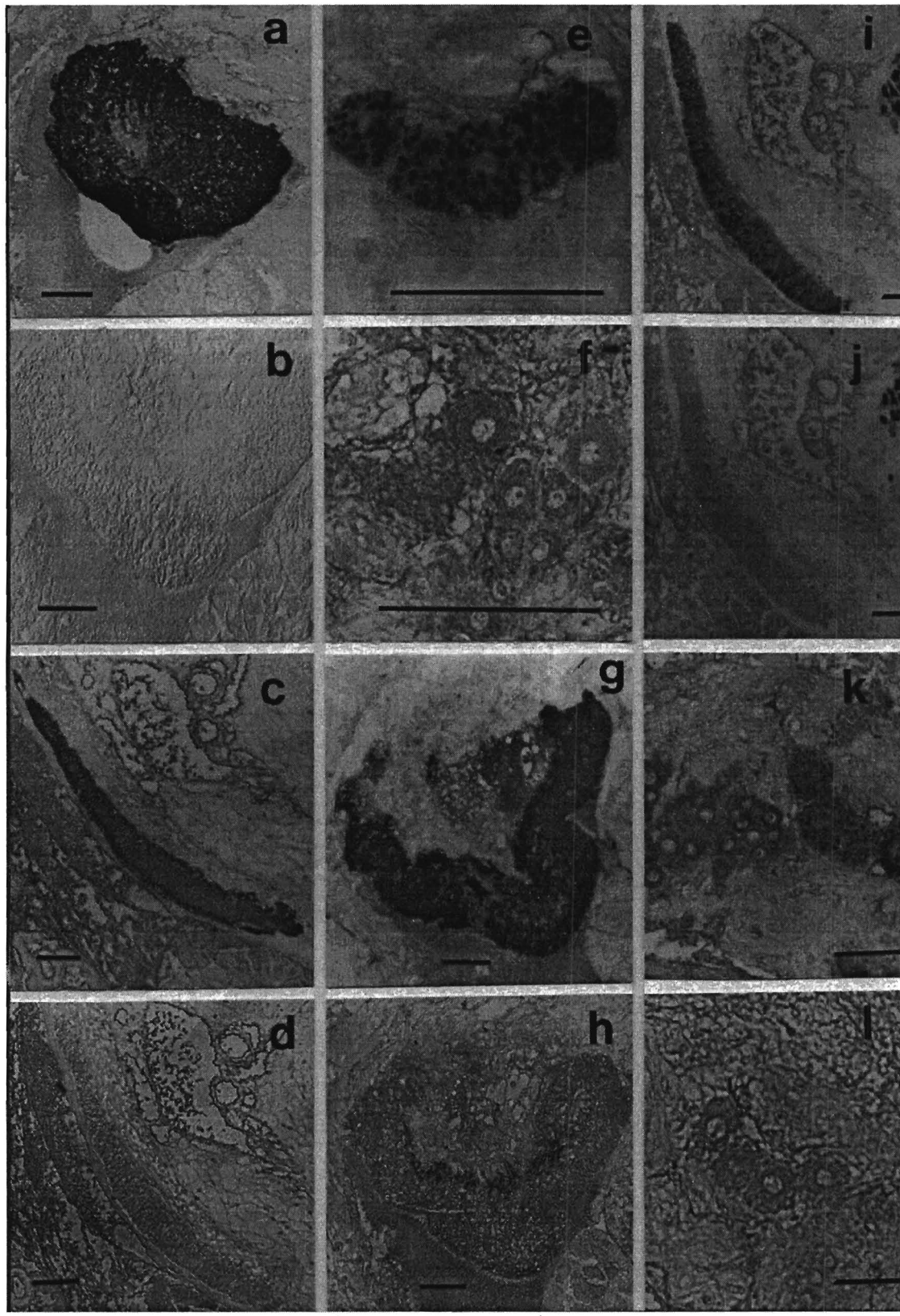

Fig. 19. Pairs of adjacent sections through the sinus glands of *J. lalandii* (**a**, **b**, **g**, **h**), *P. homarus* (**c**, **d**, **i**, **j**) and *C. maenas* (**e**, **f**, **k**, **l**) immunostained with anti-*Capa* cHH (**a**, **c**, **e**), anti-*Capa* cHH preabsorbed with *Capa* cHH (**b**, **d**, **f**), anti-*Capa* MIH (**g**, **i**, **k**) or anti-*Capa* MIH preabsorbed with *Capa* MIH (**h**, **j**, **l**).
Scale bar 50 μm

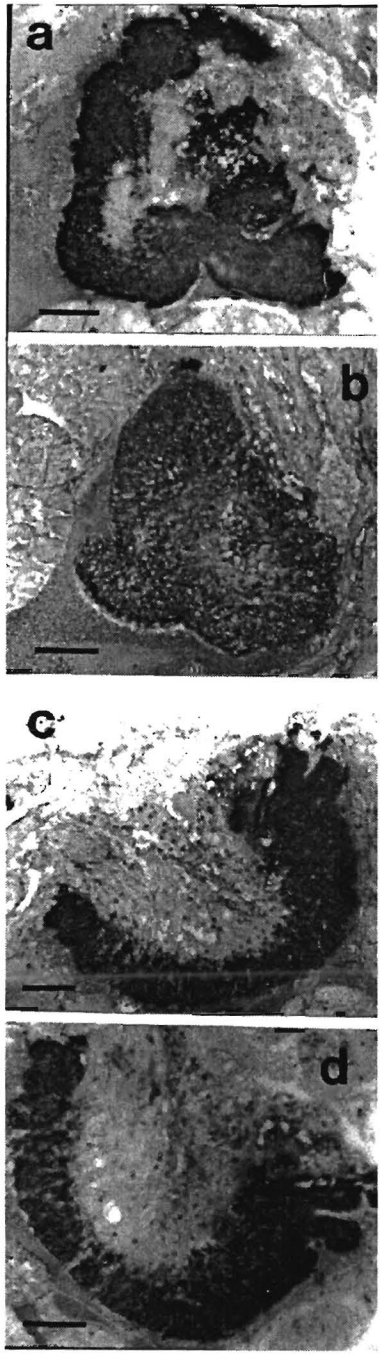

Fig. 20. Pairs of adjacent sections through the sinus gland of *J. lalandii* immunostained with (**a**, **c**) anti-*Hoam* VIH, (**b**) anti-*Hoam* VIH preabsorbed with *Capa* cHH and (**d**) anti-*Hoam* VIH preabsorbed with *Capa* MIH.

Scale bar 50 µm

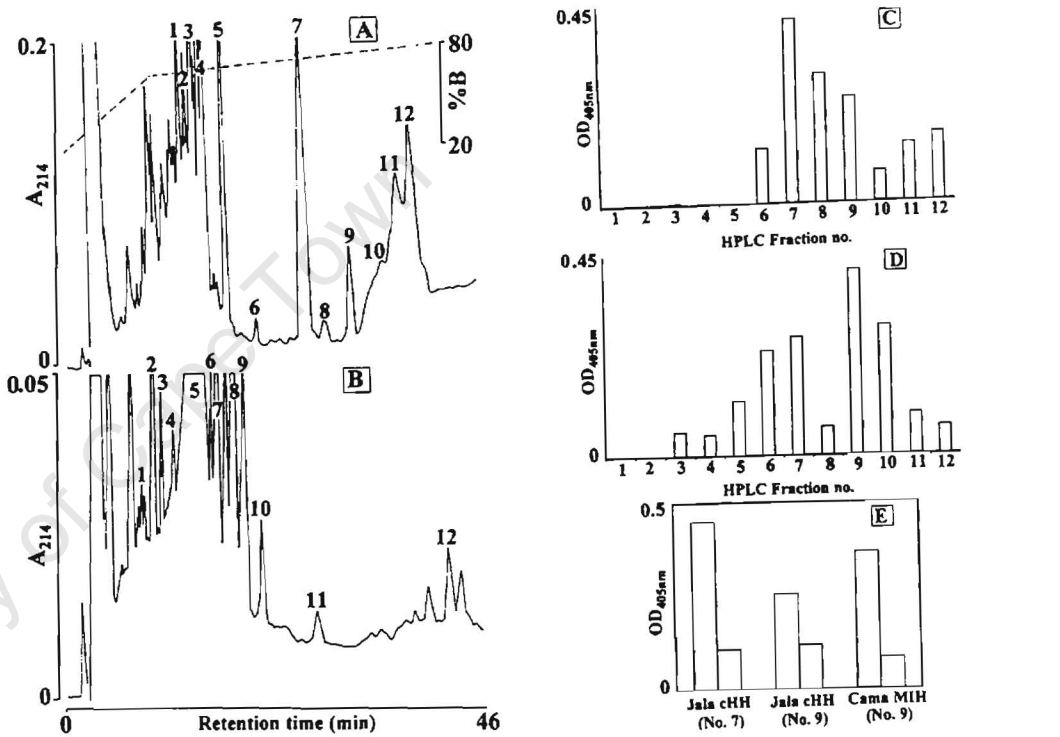

Fig. 21. HPLC profile of extracts of **A.** 12 sinus glands from *J. lalandii* and **B.** 6 sinus glands from *C. maenas*. The gradient is indicated by dotted lines; solvent A= 0.11 % trifluoracetic acid (TFA) in water, solvent B= 0.1 % TFA in 60 % acetonitrile. **C.** Identification of *Jala* cHH in HPLC fractions in **A** by ELISA with anti-*Hoam* cHH. **D.** Identification of *Cama* MIH in HPLC fractions in **B** by ELISA with anti-*Capa* MIH. **E.** Immunoreactivity of anti-*Hoam* VIH antiserum on putative *Jala* cHH and *Cama* MIH before and after preabsorption with the respective cHH or MIH peptides as shown by ELISA.

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anti-*Hoam* VIH serum. After preabsorption of anti-*Hoam* VIH serum with peak 9 material, the resultant immunoreactivity showed a 4-fold reduction. To determine whether different peptides of the spiny lobster are indeed reacting with different antisera, peak fractions obtained from HPLC separation of *J. lalandii* crude extracts of SG were tested by ELISA using anti-*Hoam* cHH, anti-*Hoam* VIH, anti-*Capa* cHH and anti-*Capa* MIH antisera. The HPLC fractionation pattern obtained (not shown) was similar to that of Fig. 21A and the eluted peaks were numbered identically as in Fig. 21A. Fig. 22 shows that anti-*Capa* cHH recognises the same two peaks as anti-*Hoam* cHH, viz. peaks 7 and 9. However, an additional major peak (peak 5) is strongly recognised by anti-*Capa* cHH. This peak 5 is, however, the sole peak recognised by anti-*Capa* MIH. In contrast, anti-*Hoam* VIH did not recognise a unique peak but weakly cross-reacted with the three major HPLC peaks, 5, 7 and 9 (Fig. 22).

In all cases of tissue immunocytochemistry or ELISA, substitution of the primary antisera with PBS or normal sera did not result in specific staining (results not shown).

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3.2. Purification and characterisation of cHH/MIH/VIH family peptides from *J. lalandii*

3.2.1. Sinus gland preparation, peptide separation and characterisation by antisera and mass spectrometry

The sinus gland of *J. lalandii* was easily visible, even with the naked eye, as a pale-blue disc on the eyestalk, lying just beneath the exoskeleton and adjacent to the optic

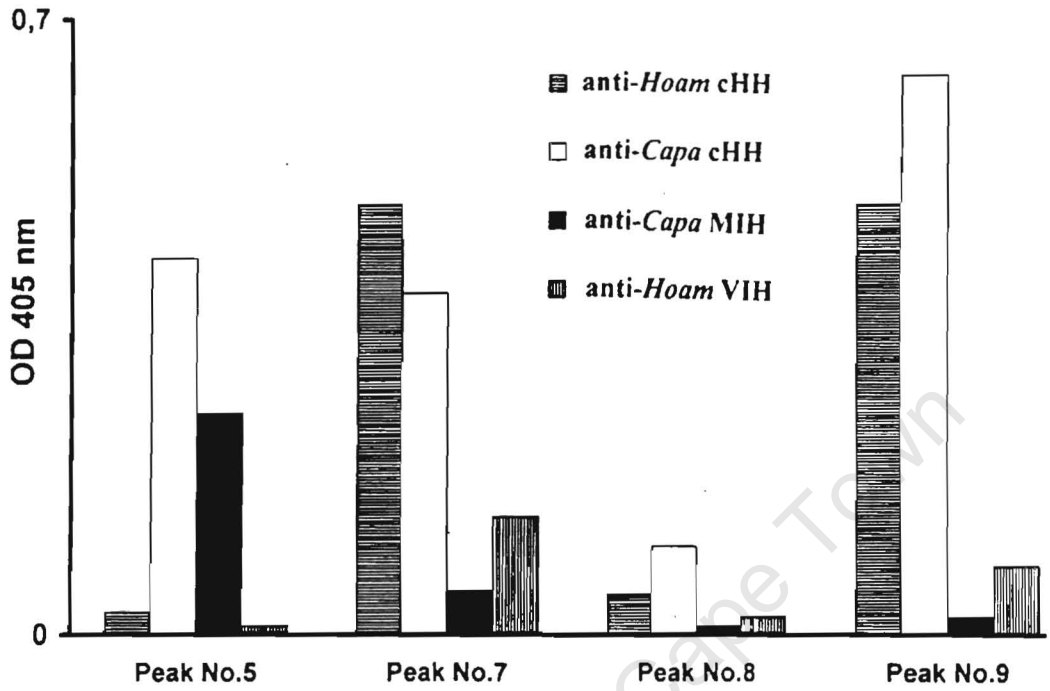

Fig. 22. Immunoreactivity, as shown by ELISA, of anti-*Hoam* cHH, anti-*Capa* cHH, anti-*Capa* MIH and anti-*Hoam* VIH on HPLC-separated peak fractions from crude extracts of sinus glands from *J. lalandii* (see Fig. 21A for a typical elution profile).

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ganglia (Fig. 23). Sinus glands could only be dissected whole from freshly-ablated eyestalks; when older material was used (stored at 4 ^{0}C or frozen) the gland was visibly disintegrated and the peptide yield was markedly reduced. Therefore, SGs were quickly dissected from fresh material and kept in ice-cold homogenisation solution. In pilot experiments, 0.1 N HCl was used with 3 different homogenisation techniques: (1) the tissue was disrupted by short bursts of sonication, heated for 5 min at 85 ^{0}C, cooled on ice and then stirred for 1h at room temperature. The homogenate was centrifuged at 14 000 rpm for 20 min at 4 ^{0}C and the supernatant collected and dried (see Soyez *et al.*, 1990). (2) The tissues were sonicated on ice, centrifuged at room temperature for 20 min; supernatant was collected and re-extracted twice more (see Soyez *et al.*, 1987). (3) The tissues were sonicated very briefly on ice and centrifuged at 4 ^{0}C for 15 min; the supernatant was collected and the pellet was re-extracted. Sinus glands were also prepared in 10 % acetic acid and homogenised in the same manner as for samples in 0.1 N HCl. The success of each method was assessed by the reproducibility of the chromatogram and the peptide yield (by comparing the peak height of identical peaks). The validity of pre-separation of crude extracts over a C18+ Sep-Pak column was also assessed.

Under the same chromatographical conditions, 10 % acetic acid was found to be the better medium for the preparation of *J. lalandii* sinus gland material and homogenisation in hot acetic acid resulted in the most reproducible HPLC chromatograms with the highest peptide yields (results not shown). The method of homogenisation described in 2.2., using a glass homogeniser without sonication, became the method of choice for preparing crude extracts of sinus glands from *J. lalandii*. Different HPLC gradients were also tested in pilot experiments to obtain the

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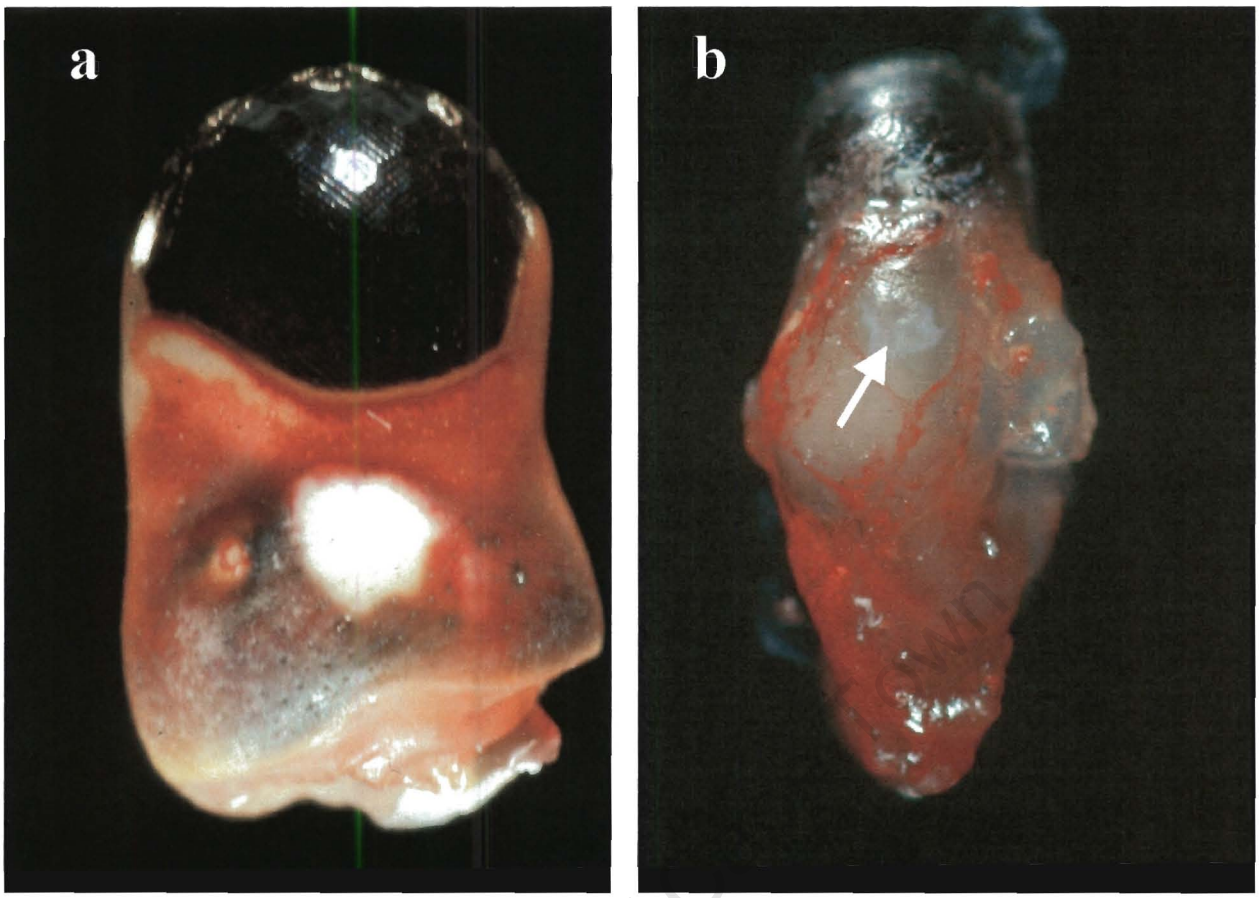

Fig. 23. The eyestalk of *J. lalandii* **a.** encapsulated in the exoskeleton and **b.** removed of its exoskeleton. The sinus gland is seen as a pale blue disc (arrow) on the white neural tissue of the optic ganglia.

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best resolution of the more-hydrophobic material in the sinus glands. A gradient from 20-62 % B in 10 min, followed by a linear increase to 80 % B in 50 min, resulted in a good separation of the late-eluting, more-hydrophobic peak material. Figure 24 shows the typical HPLC chromatogram of an extract of 100 SGs from *J. lalandii*. Fractions of one ml were collected, aliquots thereof were tested in ELISAs with antisera raised against *Hoam* cHH, *Hoam* VIH, *Capa* cHH and *Capa* MIH to identify similar peptides in *J. lalandii*. From Fig. 25A, B it is clear that all 5 peak fractions are recognised by the cHH/MIH/VIH antisera. Fractions corresponding to peaks 1, 2, 4 and 5 were strongly reactive with anti-*Hoam* cHH (Fig. 25A) and anti-*Capa* cHH (Fig. 25B). These fractions were also all recognised by anti-*Hoam* VIH, so that no unique peak was identified by this antiserum (Fig. 25A). Anti-*Capa* cHH antiserum, in addition to recognising fractions of peaks 1, 2, 4 and 5, reacted also with fractions that corresponded to peak 3 (Fig. 25B). Peak fraction 3, however, was the sole peak clearly recognised by anti-*Capa* MIH antiserum (Fig. 25B). Mass analyses of peaks 1-5 by MALDI-TOF, revealed the following $[M+H]^+$ data: 7666.41, 7613.53, 9007, 8381 and 8358, respectively.

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3.2.2. Biological activity: hyperglycaemia

A homologous assay, using live juvenile specimens of *J. lalandii*, was established to measure glucose concentration in haemolymph samples in response to injected substances. Dried material of HPLC peaks 1-5 (Fig. 24) were redissolved in sea water, injected at 1 SGE/50 µl and its effect on glycaemia was measured 2 hr after injection. No significant increase in glucose concentration was obtained after the injection of

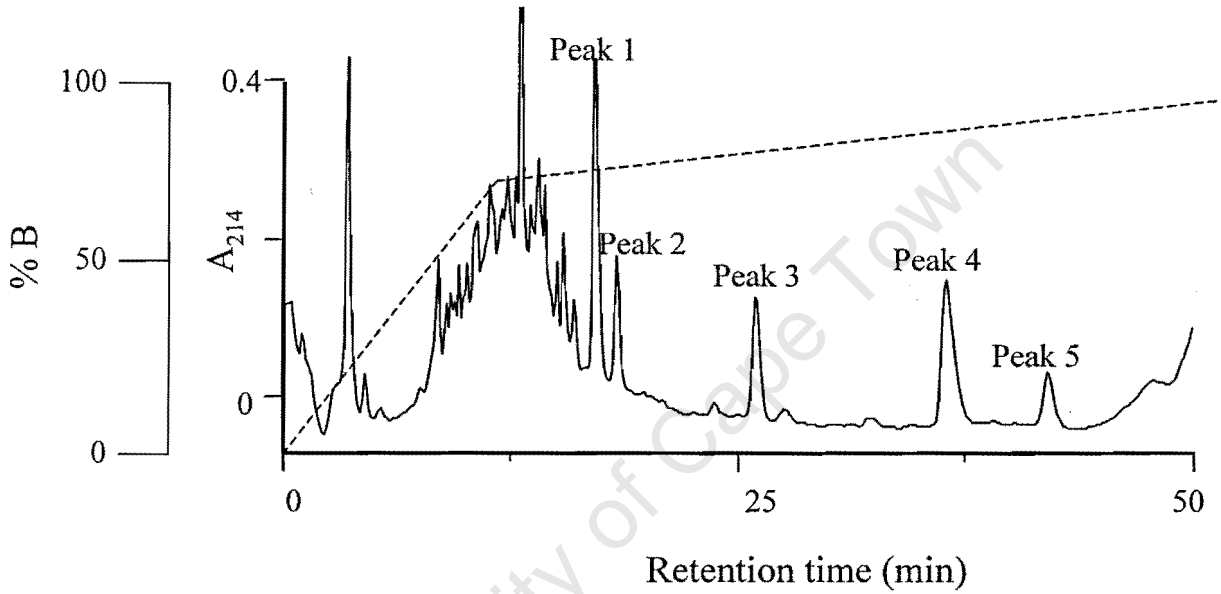

Fig. 24. A typical RP-HPLC fractionation of an acetic acid extract of 100 sinus glands from *J. lalandii* on a Nucleosil C-18 column. Gradient (indicated by dotted lines) with solvents A: 0.11 % TFA in water and B: 0.1 % TFA in 60 % acetonitrile. Peaks were labeled in order of elution.

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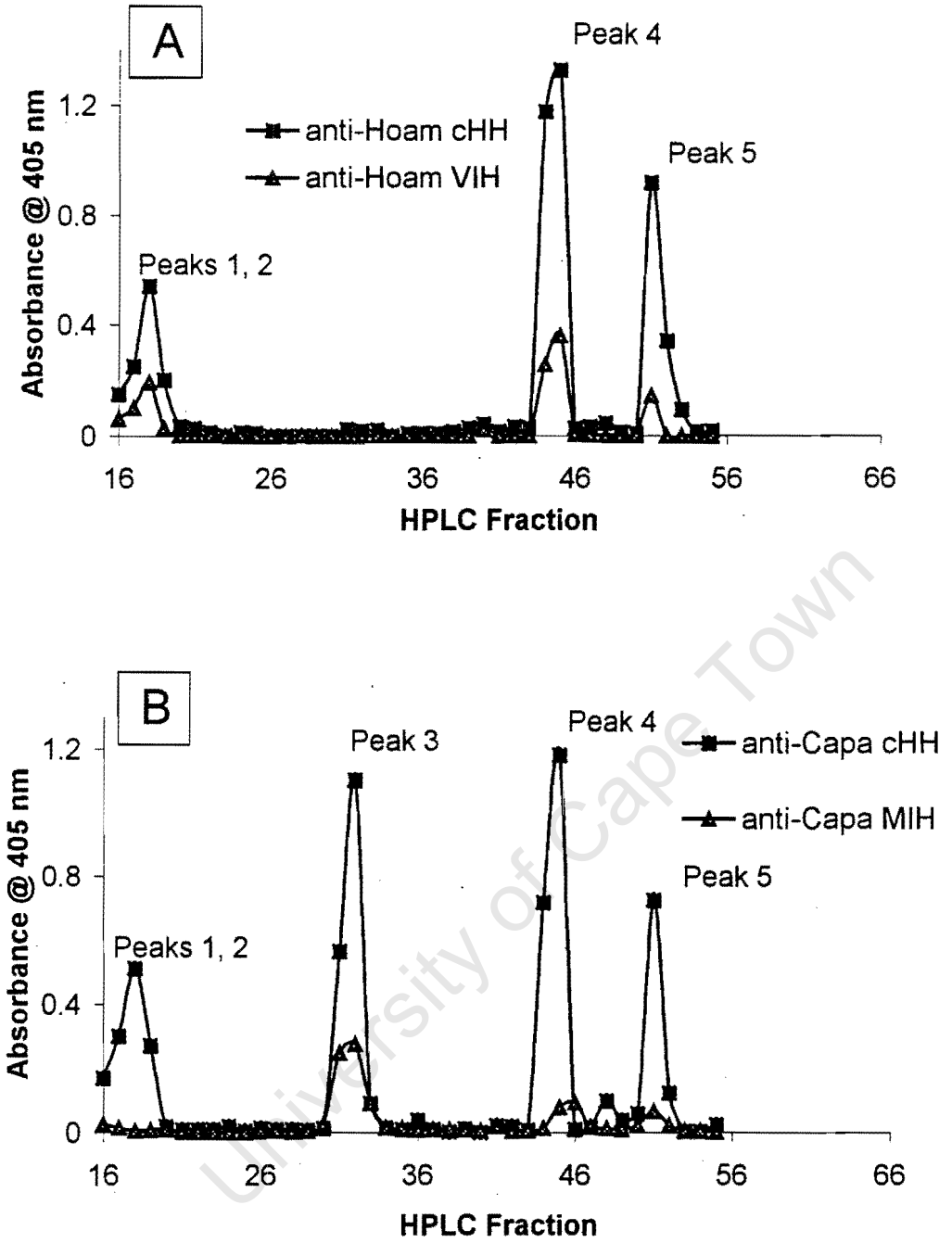

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Fig. 25. Immunoreactivity, as shown by ELISA, of **A** anti-*Hoam* cHH and anti-*Hoam* VIH, and **B** anti-*Capa* cHH and anti-*Capa* MIH sera on aliquots of HPLC-separated fractions from crude extracts of sinus glands from *J. lalandii* (see Fig. 24 for HPLC elution profile).

sea water and peaks 1-3 (Fig. 26). Significant increases in the glucose concentration were consistently obtained after the injection of crude extracts of *Jala* sinus glands and peaks 4 and 5 (Fig. 26). On the basis of their hyperglycaemic activities *in vivo*, peaks 4 and 5 are referred to as *Jala* cHH-I and *Jala* cHH-II, respectively.

3.2.3. Biological activity: effects on ecdysteroid synthesis

The biological activity of a putative moult-inhibiting peptide was deduced from the indirect evidence of ecdysteroid synthesis in the Y-organs (YOs), dissected from adult specimens of *J. lalandii* and incubated *in vitro*. The ecdysteroid titre in the incubation medium was determined by EIA.

3.2.3.1.Characterisation of circulating ecdysteroids

To establish a meaningful EIA for the quantitative assessment of the biological significance of *Jala* MIH on ecdysteroid production, the ecdysteroids present in the haemolymph of *J. lalandii* specimens were firstly identified. Ecdysteroids extracted from haemolymph were separated by normal-phase (NP) HPLC (Fig. 27A); aliquots of the collected fractions were assayed by EIA using the antiserum AS 4919. Four immunoreactive compounds were detected (Fig. 28). Their retention times on HPLC were compared with those of known synthetic ecdysteroids to establish their identity (Fig. 27B). The major immunoreactive compound had the same retention time as 20E and a minor peak had the same retention time as E. In haemolymph samples from adult specimens, the concentration of 20E was more than 5050 pg/ml, whereas samples from juvenile specimens had a much higher concentration, viz. 72 ng/ml. No compound co-migrating with ponasterone A was detected, and the 2 less-polar

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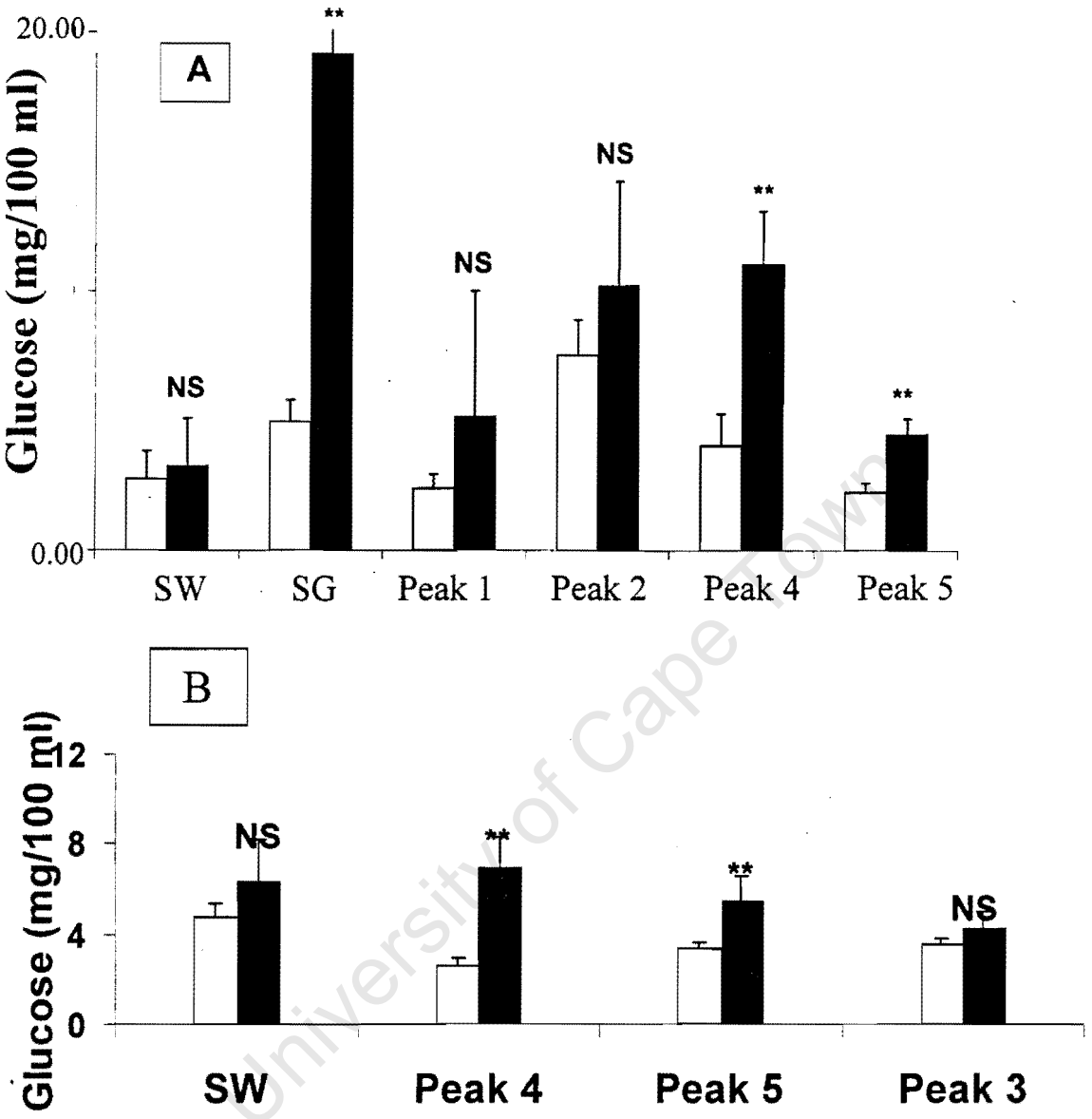

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Fig. 26. Effects of peaks 1-5 on glycaemia in juvenile *J. lalandii*. Before injection, 50 µl of haemolymph was withdrawn (blank bar), and then again 2h after injection (black bar) of sea water (SW; n=5), 0.5 SGE of crude extracts of sinus glands (SG; n=8), or 1 SGE of peak material from HPLC peak 1 (n=10), peak 2 (n=10), peak 3 (n=12), peak 4 (n=10) and peak 5 (n=11). Average values are indicated ± SD. **Significant difference ($p<0.05$) when compared with the injection of sea water, using the Student's t-test. NS, not a significant difference.

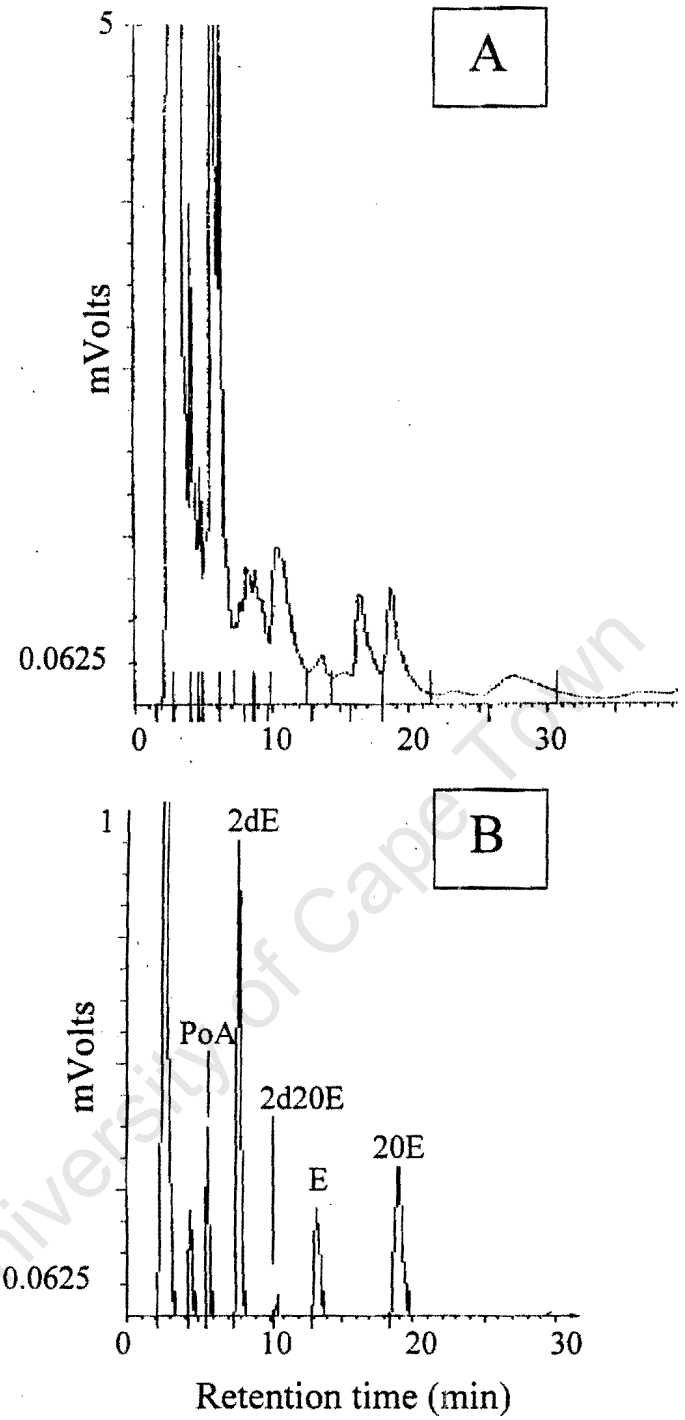

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Fig. 27. **A.** Profile of the compounds extracted from 5 ml haemolymph of adult (intermoult) *J. lalandii* and separated by NP-HPLC over a Hypersil column. A flow rate of 1 ml/min with the isocratic mixture of dichloromethane/isopropanol/water (125:30:2) was maintained. **B.** Retention times of synthetic ecdysteroids in a reference run using the same NP-HPLC system, column, solvents and isocratic conditions as in **A**. Reference compounds used: ecdysone (E), 20-hydroxyecdysone (20E), Ponasterone A (PoA), 2-deoxyecdysone (2dE) and 2-deoxy-20-hydroxyecdysone (2d20E).

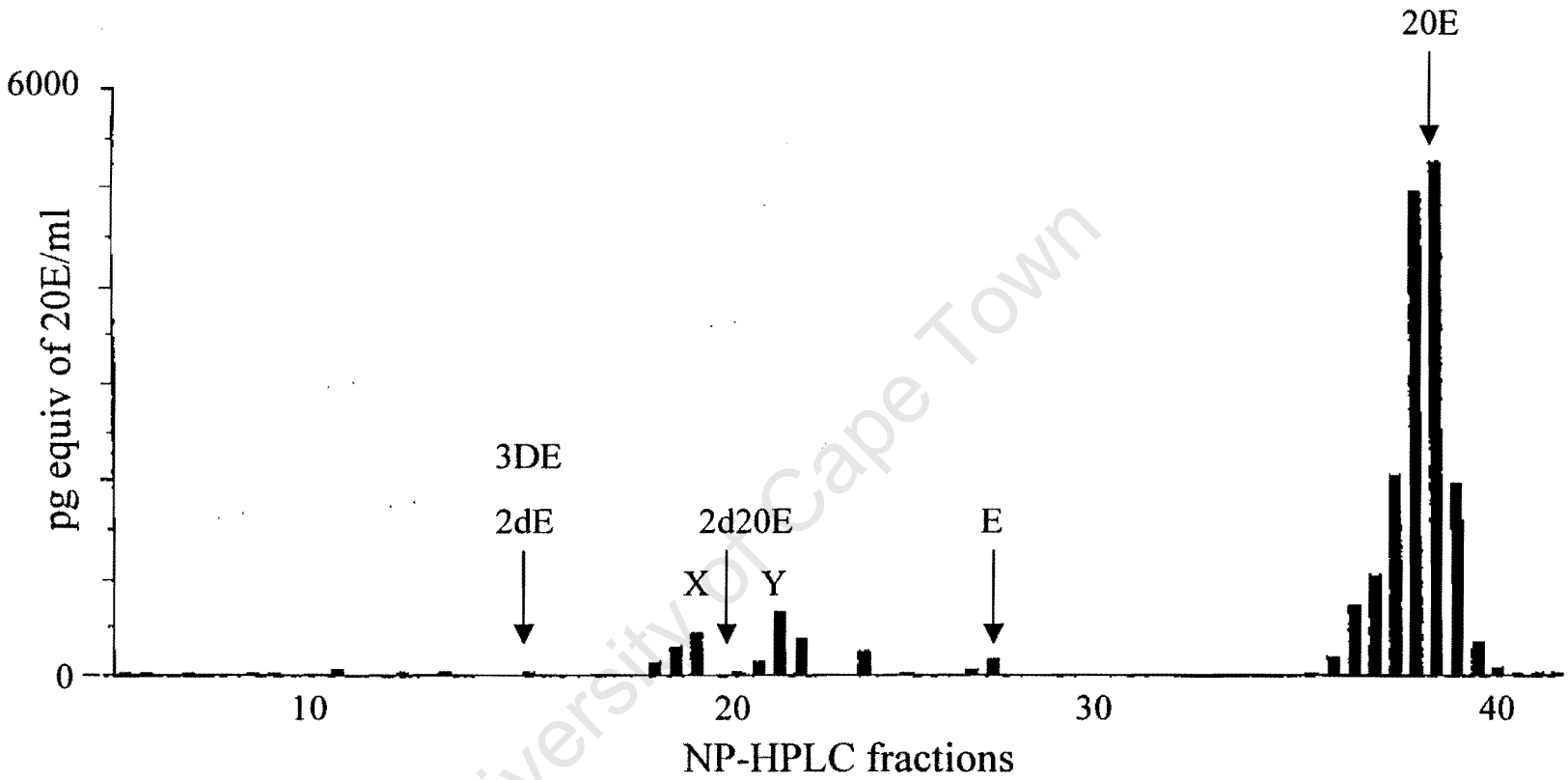

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Fig. 28. EIA profile of ecdysteroid compounds separated from haemolymph of *J. lalandii* specimens by NP-HPLC (see Fig. 27A for typical profile). NP-HPLC fractions of 0.5 ml were collected and analysed in the assay with AS 4919 as antiserum. EIA activity is expressed as pg equivalents of 20E. Arrows indicate the retention time of reference ecdysteroids: ecdysone (E), 20-hydroxyecdysone (20E), 3-dehydroecdysone (3DE), 2-deoxyecdysone (2dE) and 2-deoxy-20-hydroxyecdysone (2d20E). Unidentified, immunoreactive compounds are labeled as "X" and "Y".

immunoreactive compounds did not correspond to any of the known ecdysteroids (Fig. 28). For the purpose of designing an EIA for the *in vitro* incubation of YOs of *J. lalandii*, however, 20E was considered to be the major circulating ecdysteroid and, hence, it is assumed that either E or 3DE or both, are secreted by the YOs of the spiny lobster.

3.2.3.2 In vitro incubations of Y-organs

3.2.3.2.1. Location and dissection of YOs

The anatomy of the neuroendocrine system of the spiny lobster is not particularly well-documented and the lack of such an atlas/dissecting guide is sorely felt. Paterson (1968) describes the position of the YO in *J. lalandii* as follows: "...it is generally located near the ventral body wall and has been interpreted by Gabe as a thickening of the hypodermis." (page 204). And "It lies almost horizontally near the anterior limit of the epimeral attractor muscle and is also close to the maxillary epimera and the base of the second maxilla." (page 204). Further, "...the lateral diverticulum of the dorsal lobe of the digestive gland bulges into the anterior part of the branchiostergite." (page 205). With this information, many dissections were carried out in an attempt to locate the YOs. The successful dissection is initiated by removing the lateral carapace and the underlying tissue of the cephalothoracic region, i.e. the branchiostergite (Fig. 29A). With a blunt-bladed knife, the exoskeleton is carefully removed from the underlying tissue and discarded. With the epidermis-side down in a dissecting dish, the second maxilla can be seen with its broad basal plate (scaphognathite) onto which the hepatic (digestive) gland protrudes (Fig. 29B).

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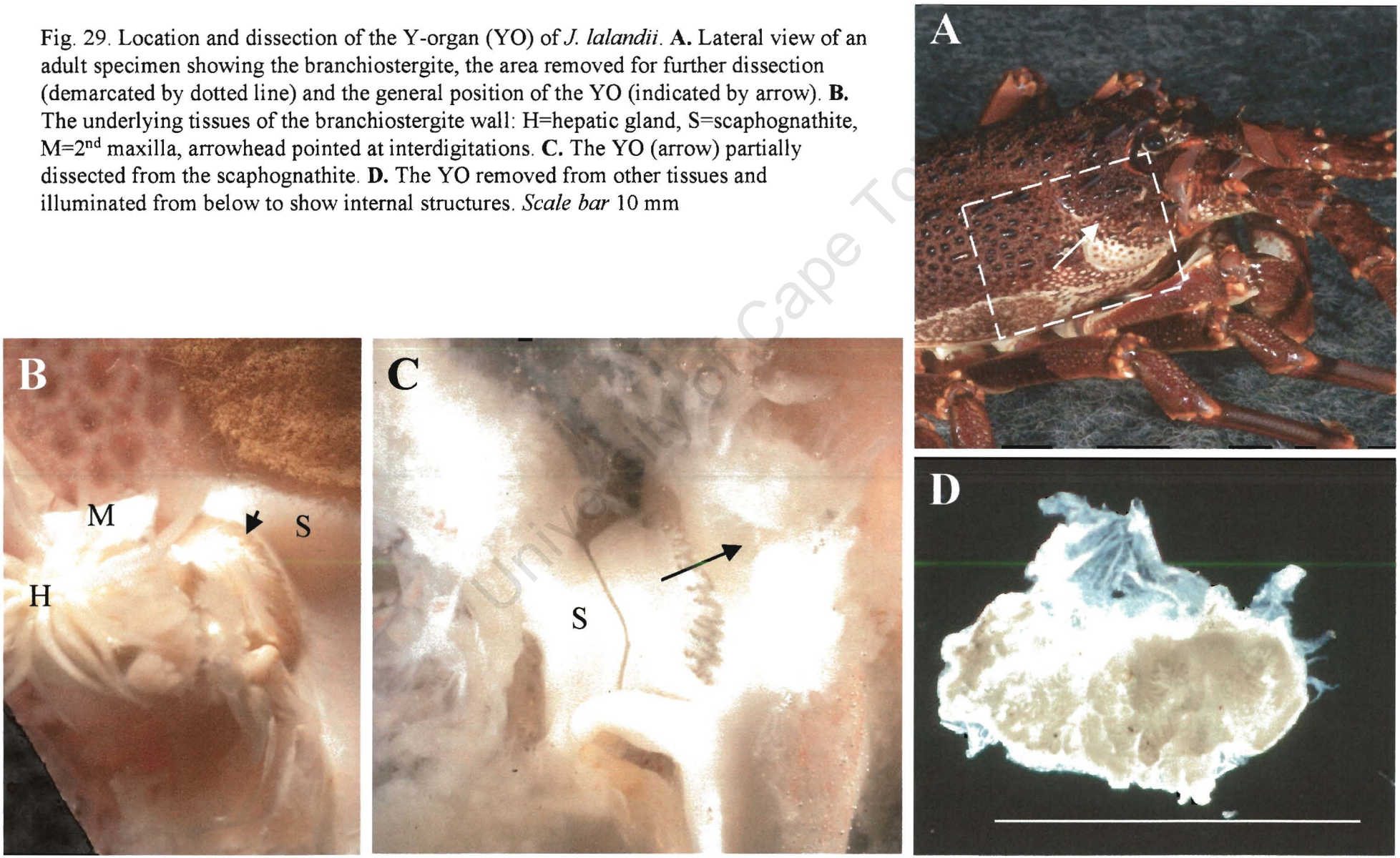

Fig. 29. Location and dissection of the Y-organ (YO) of *J. lalandii*. **A.** Lateral view of an adult specimen showing the branchiostergite, the area removed for further dissection (demarcated by dotted line) and the general position of the YO (indicated by arrow). **B.** The underlying tissues of the branchiostergite wall: H=hepatic gland, S=scaphognathite, M=2nd maxilla, arrowhead pointed at interdigitations. **C.** The YO (arrow) partially dissected from the scaphognathite. **D.** The YO removed from other tissues and illuminated from below to show internal structures. *Scale bar* 10 mm

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Behind the interdigitation on the scaphognathite lies the YO, arranged in a slight crescent to follow the contours of this ridged area. The convoluted ridges of the sac-like YO are pale-yellow in colour in freshly-killed animals and can be seen as a thickening of the inner epidermal wall (Fig. 29C). In adult specimens of *J. lalandii* (carapace length = 9.2 cm) the YO was 10 mm long and approximately 5 mm high (Fig. 29D). Because of the relatively large size of the YOs, these tissues could not be incubated in wells of a microtitre plate, but were incubated in glass block (embryo) dishes (diameter of well= 25mm with maximum volume capacity of 2 ml). For *in vitro* incubations, either the whole YO was used with the contralateral organ as the control tissue or, the YO was bisected through the vertical axis and the halves were incubated or, the halved YO was further bisected through the horizontal axis to produce quarters for incubation. In the latter case, the two upper pieces were compared with each other, and the two lower pieces were directly compared with each other, due to the crescent arrangement of the YO tissue (as shown in Fig. 30).

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3.2.3.2.2. In vitro incubations of YO and related tissues

Because the YO is such a diffuse structure in the spiny lobster, it was not always easy, at first, to cleanly dissect out only YO tissue for incubations. Thus, other surrounding tissues such as muscle, gill, epidermis and hypodermis were incubated in tissue culture medium to assess whether these tissues produced anything that would interfere with the EIA and thereby, influence the interpretation of the results. Non-YO tissues were incubated from the branchiostergal regions of 6 adult lobsters, thus n = 12 for each tissue type; no ecdysteroids were detected by EIA in the incubation medium from all these samples and the absorbance values were identical to that of

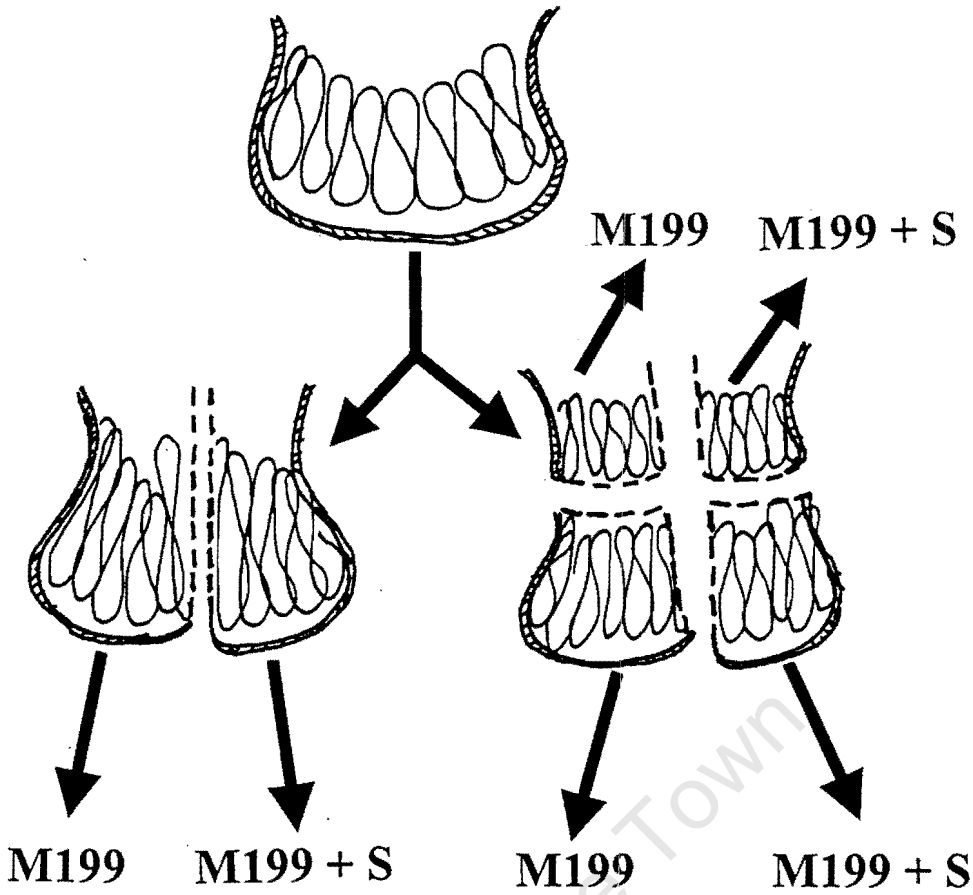

Fig. 30. Scheme showing the further dissections of the YO of *J. lalandii* for *in vitro* incubations. M199, tissue culture medium; M199 + S, tissue culture medium supplemented with a test substance, such as crude extracts of sinus glands or purified peptides.

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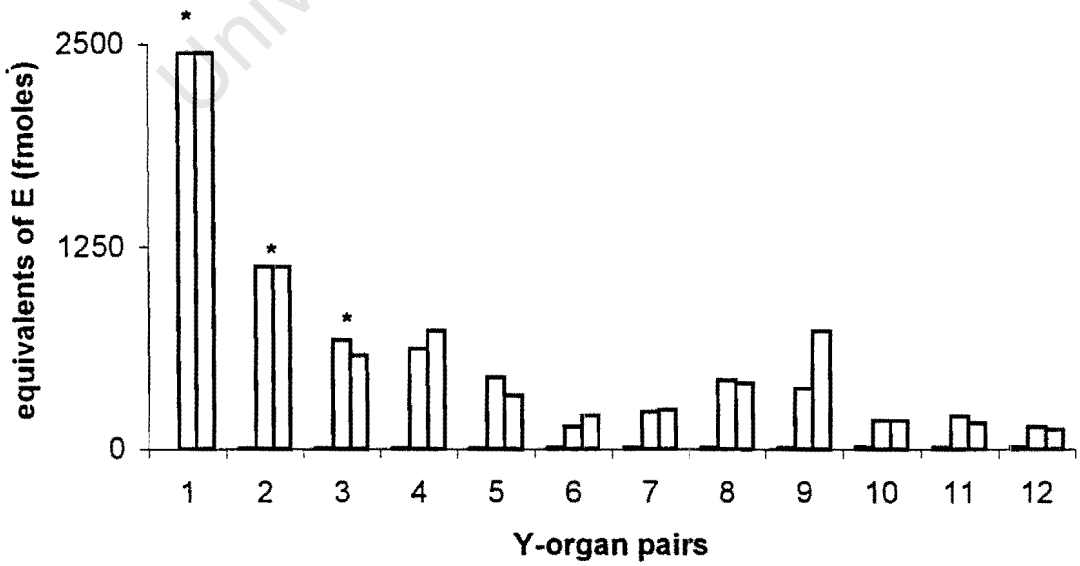

Fig. 31. Rates of ecdysteroid secretion from 3 pairs of whole YOs (marked with *) and 9 pairs of halved YOs from *J. lalandii* shown as paired bars 4-12.

medium that were incubated without any tissue pieces (results not shown). The influence of surrounding tissues was also investigated by incubating a YO with the surrounding epidermis still intact, and comparing this with its contralateral YO from which the surrounding tissue had been removed. Comparable amounts of ecdysteroids were measured in these samples (results not shown) To determine the variability between YOs, pairs of YOs were cultured in M199 overnight. Fig. 31 shows that there is mostly little variation between ecdysteroid secretions of YOs from the same pair, but much more variation in ecdysteroid titre between glands from 2 different specimens. Further, halved organs were generally also representative with regards to equal ecdysteroid secretion (Fig. 31). Quartered YOs (derived as shown in Fig. 30) similarly demonstrated equivalent production of ecdysteroids (results not shown). As a rule, all YO tissue pieces and whole YOs were incubated for 1 h at room temperature in M199, this medium was removed and replaced with fresh medium containing the substances to be tested. In this way, the initial rates of ecdysteroid secretion could be compared and, where these preincubation values varied by more than 20 %, the data was discarded.

The time-course pattern of ecdysteroid secretion *in vitro* was established by withdrawing all the culture medium after a specified time of incubation and replacing it with fresh medium. Maximum secretory rates were observed during the first 3 h and, thereafter, the rate of secretion was lowered; after 6 h of incubation there was not much further increase in ecdysteroid secretion (Fig. 32). However, for convenience, some of the YO incubations were carried out overnight, i.e. for more than 6 h.

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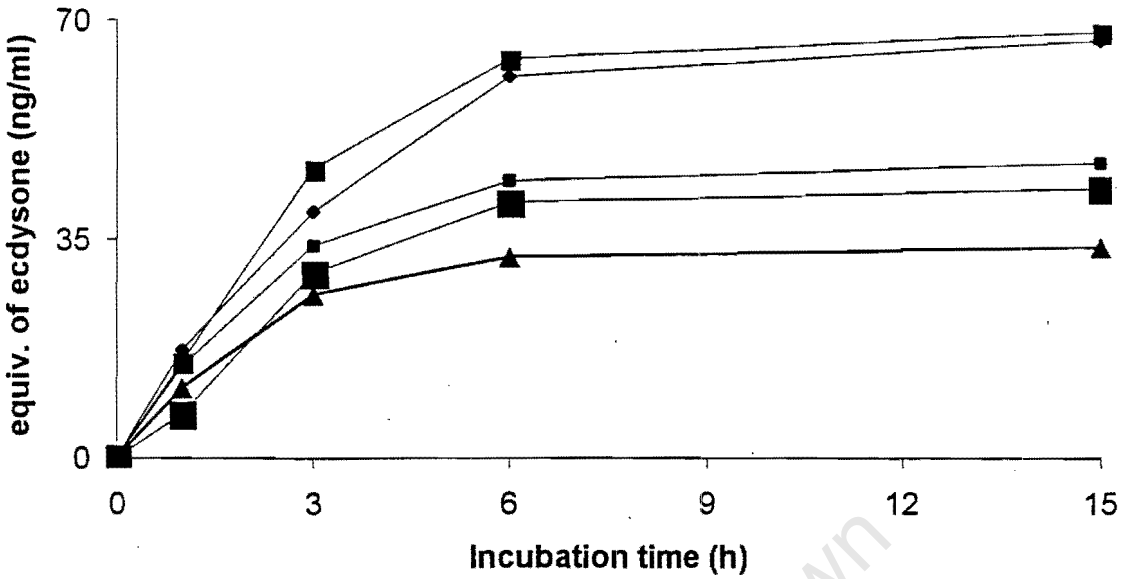

Fig. 32. Time-course profile of ecdysteroid secretion by the YOs of *J. lalandii in vitro*. Five YOs were incubated in 1 ml medium M199 for a total of 21 h. Medium was removed and replaced after 1 h, 3 h, 6 h and 15 h of incubation and subjected to EIA to determine the titre of ecdysteroids. Results are expressed as ng ecdysone per ml of incubation medium.

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Table 4. Ecdysteroid titre of individual lobsters before and after eyestalk ablation as determined by EIA analyses on haemolymph samples.

	Ecdysteroids (equiv. of 20E ng per ml)						
	Before	Post-ablation (days)					
Animal		5	23	31	36	48	80
1	10	0.2* **(d. 12)**	0.33	0.30	-	-	-
2	39.41	n.s.	1.39* **(d. 30)**	0.29	-	-	-
3	0.11	n.s.	0.74	0.89	1.10	2	4.25
4	0.09	n.s.	0.42	0.67	0.61	0.62	0.65
5	34* **(d. 12)**	n.s.	0.26	0.19	-	-	-
6	0.69	n.s.	2.35	3.26	4.42	8.74	210
7	0.07	n.s.	0.29	0.27	0.31	0.52	0.28
8	0.1	n.s.	0.41	0.42	0.27	0.57	1.41
9	188.07	118.55	2.16* **(d. 27)**	-	-	-	-

*Indicates the last sample taken before ecdysis; the exact day of ecdysis is indicated in bold letters; n.s. = no samples taken; - means animal sacrificed.

3.2.3.2.3. Ecdysteroid production and inhibition in relation to moult stage

It is not easy to distinguish the different moult stages in the spiny lobster by virtue of external parameters, except in the cases of pre- and post-ecdysis when the exoskeleton is extremely soft. The circulating ecdysteroid titre was therefore, used as an additional indicator of the moult stage, with dissection as confirmation. However, the study did not lend itself to accurately sub-divide moulting stages, for example as early-, mid-, or late-premoult, therefore, the animals were broadly classified into intermoult, premoult and postmoult groups.

Haemolymph samples were analysed in EIAs with AS 4919 as antiserum. In this EIA system, it was found that animals in intermoult exhibited on average 15.14 ± 10.34 ng 20E/10 ml haemolymph, 98 ± 174 ng 20E/ml haemolymph for premoult animals, and an average of 2 ± 1 ng 20E/10 ml haemolymph was measured in postmoult animals (values are presented as mean ± SD and n=10 in each case). In the case of premoult animals the developmental stage could be confirmed by dissection and looking at the formation of the new cuticle. Confirmation for postmoult and intermoult stages came from observations of the lobsters held for months in the aquarium (noting when the animal had previously moulted) and by the condition of the carapace (postmoult lobsters were generally blemish-free). Nine adult male specimens of *J. lalandii* were also destalked. Haemolymph samples were collected before destalking and periodically thereafter. Ecdysteroid titres were determined until the specimens were maximally 2 weeks post-ecdysis. From this data (Table 4) and its correlation with ecdysis, it is apparent that a broad classification of the moulting stage, on the basis of circulating levels of 20E, can indeed be made. What is further noticeable is that there is a dramatic and significant decline in ecdysteroid titres

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before ecdysis (Table 4); the decline is so pronounced that ecdysteroid levels which are normally measured for intermoult or postmoult animals were recorded at this time. Animals 1, 2, 5 and 9 were already in the premoult phase at the time of destalking, whereas all the other animals were in the postmoult (or post-ecdysis) phase of the moult cycle (as seen by their ecdysteroid titres in Table 4).

Lobsters from different moult stages were sacrificed and their YOs incubated with crude extracts of sinus glands of *J. lalandii* for 6 h to determine the effect of putative inhibitory peptide(s) during development. The results are depicted in Fig. 33. Animals in inter- or premoult were, thus, preferred for YO incubations with purified peptides (see below), because their tissues responded to MIH, whereas those from postmoult animals did not. With respect to postmoult YOs, no inhibition of ecdysteroid synthesis was recorded for 3 out of 5 pairs of organs after 6 hr, whereas the other 2 pairs showed 32 and 3 % inhibition, respectively, which accounts for the average value of 7 % inhibition in Fig. 33. It should be noted that the same experiments were performed, in parallel to these above, but with an overnight incubation period of approximately 18 hours. With this longer incubation, the observed inhibition increased (not significantly) in intermoult YOs to 62 ± 13 % (n=10) and decreased slightly but not significantly in premoult YOs to 49.25 ± 16 % (n=4). When post-moult YOs were incubated overnight with crude extracts of sinus glands, 3 out of 5 pairs showed no inhibition of ecdysteroid secretion, whereas 2 pairs showed 59 and 24 % inhibition, respectively, which accounts for an average value of 16.6 ± 26 % inhibition.

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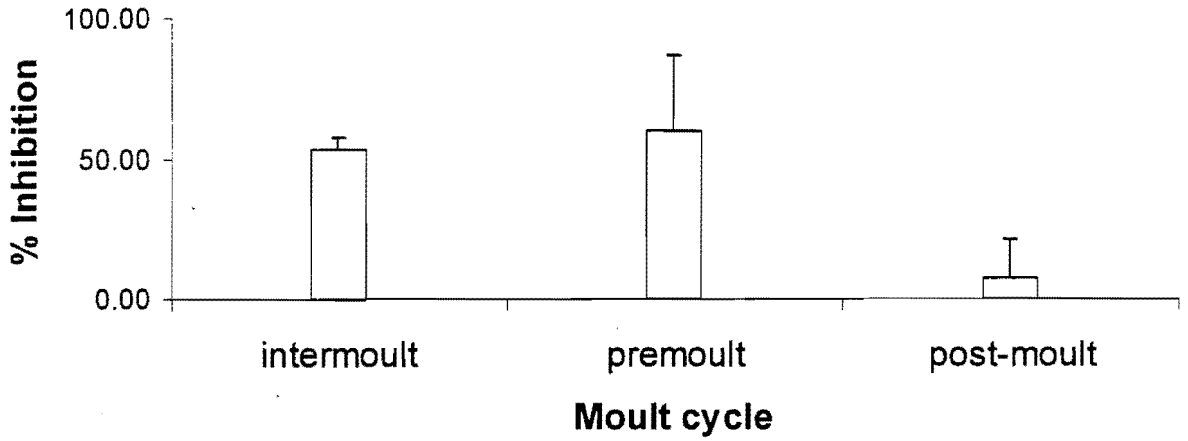

Fig. 33. Inhibition of ecdysteroid production in YOs dissected from adult *J. lalandii* specimens at different stages of the moult cycle. Pairs of halved YOs were used and crude extracts of sinus glands (1 SGE) were applied in each case. Values are expressed as a percentage of inhibition ± SD (n=8, 5 and 5, respectively for intermoult, premoult and postmoult groups).

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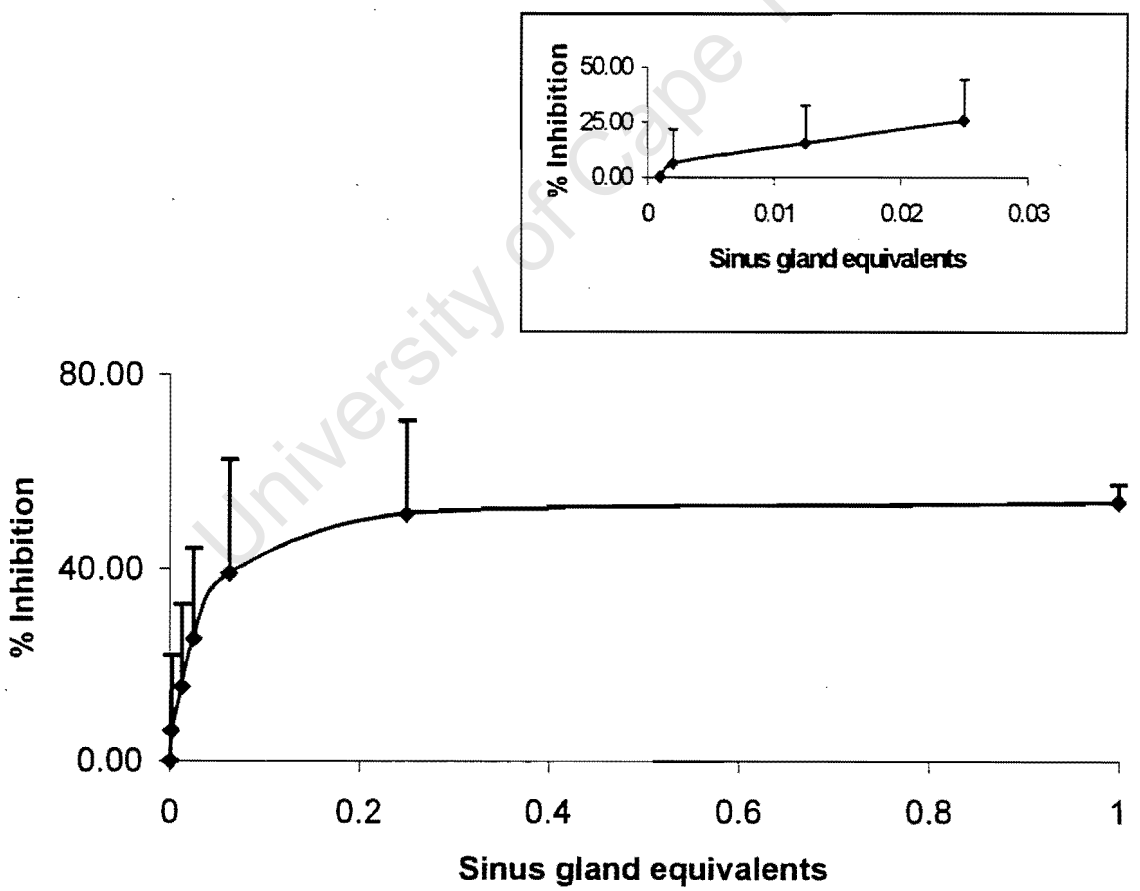

Fig. 34. Percentage of inhibition (± SD) of ecdysteroid synthesis *in vitro* by YOs from intermoult *J. lalandii* as effected by different concentrations of crude extracts of sinus glands, n= 5, 7, 7, 5, 5 and 8, for the respective doses from 0.001 to 1 SGE. The lower doses (0.001 to 0.025 SGE) are shown in the insert for clarity.

3.2.3.2.4. Dose-dependent inhibition of ecdysteroid synthesis by crude extracts of sinus glands

The inhibitory effect induced by crude extracts of sinus glands on YOs from intermoult *J. lalandii* specimens was examined at doses between 0.001 and 1 SG equivalents over a 6 h incubation period. Figure 34 shows that a typical saturation curve was obtained, with 1 SGE producing an inhibition of 53 % and the lowest dose of 0.001 SGE did not have an inhibitory effect. The first dose to give a significantly different effect to 1 SGE was 0.025 SG, which produced an inhibition of 25.4 %.

3.2.3.2.5. Inhibition of ecdysteroid synthesis in response to various Jala peptide neurohormones

RP-HPLC-derived peak material from *J. lalandii* sinus glands were examined for an inhibitory effect on the synthesis of ecdysteroids from YOs. Pairs of YOs were dissected from adult lobsters and incubated overnight with 1 SGE of peak fractions 1, 3 and 4 (see Fig. 24 for HPLC trace). Figure 35 shows that peak fractions 3 and 4 significantly inhibited the ecdysteroid titre compared with peak fraction 1. Whereas peak fraction 4 (*Jala* cHH-I) was nearly as efficient as the putative MIH (peak fraction 3) in suppressing the ecdysteroid synthesis (no significant difference between the effect of *Jala* cHH-I and *Jala* MIH), the truncated form of *Jala* cHH-I (peak fraction 1) had no effect in this assay. Based on its ability to suppress the ecdysteroid output from YOs *in vitro*, HPLC peak fraction 3 is henceforth referred to as *Jala* MIH.

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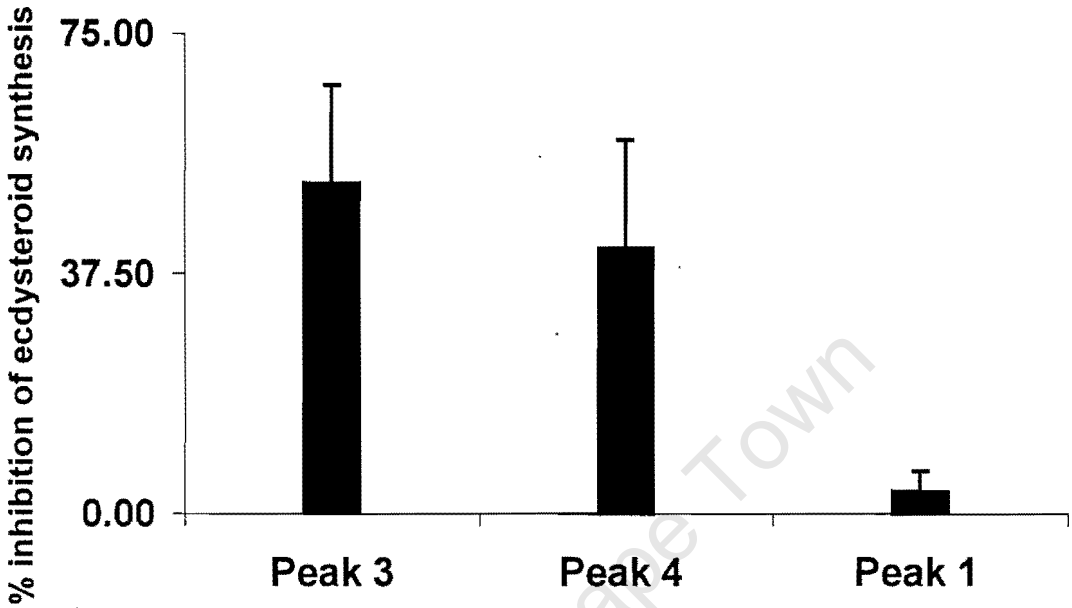

Fig. 35. Percentage inhibition (± SD) of ecdysteroid production *in vitro* by *J. lalandii* YOs when treated with peptides purified from *Jala* SGs by HPLC. The YOs were incubated overnight with 1 SGE of HPLC peak fractions (see Fig. 24 for profile): 1 (n=8), 3 (n=12) and 4 (n=10); the medium was analysed by EIA.

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Biological activity: inhibition of de novo ovarian protein synthesis

3.2.4.1. Inhibition of de novo protein synthesis in prawn ovaries by extracts of Jala SGs

Vitellogenic ovaries, dissected from *Penaeus semisulcatus* (*Pese*), were successfully used in a heterologous assay with peptide material from *J. lalandii*. A dose-response curve for inhibition of total protein synthesis was generated by incubating pieces of *Pese* ovary with increasing doses of crude extracts of *Jala* SGs. The concentration ranged from 0.001-2 SGE. As a reference, ovary tissue pieces were also incubated with homologous SG extract, viz. 0.1 SGE of crude extracts of SG from *Pese*. Background incorporation of radiolabeled amino acids was estimated from the tissues incubated for a short time only (15 min; typically 15.29 dpm/µg protein) and the normal level of synthesis was assessed from incubating pieces of ovary tissue in medium containing only radiolabeled amino acids, i.e. no treatment with SG extracts (typically 152.52 dpm/µg protein). The crude extracts of *Jala* SGs induced a dose-dependent inhibition of total protein synthesis (Fig. 36). The maximum inhibitory effect of 57 % inhibition of protein synthesis was achieved with 2 SGE crude *Jala* extracts, whereas an inhibitory effect of 52 % was achieved with only 0.1 SGE of a crude extract from *Pese* sinus glands (Fig. 36).

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3.2.4.2. Protein synthesis in ovaries incubated with peptides purified from J. lalandii sinus glands

Pieces of ovary tissues were incubated with 1 SGE of HPLC peak material obtained by fractionating extracts of sinus glands from *J. lalandii* (see Fig. 37 for HPLC trace). A peak with $[M+H]^+$ of 8400 Da was only purified from a few batches of *Jala*

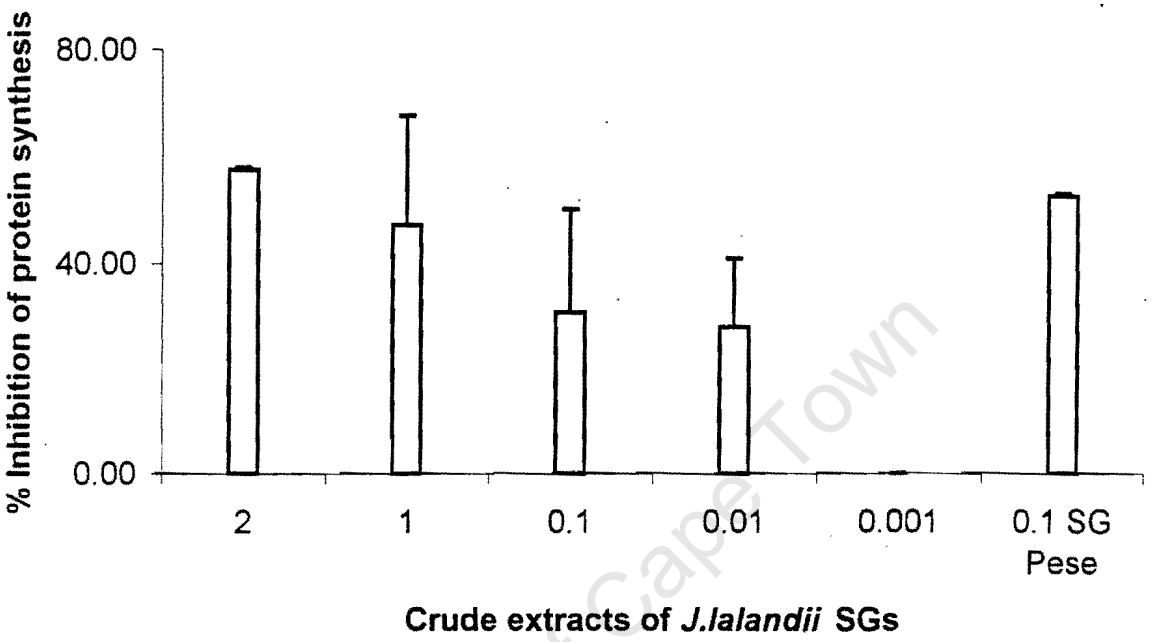

Fig. 36. Effects of increasing doses of crude extracts of *J. lalandii* (*Jala*) sinus glands on protein synthesis in ovaries from *Penaeus semisulcatus* (*Pese*); 1 dose of homologous extract, i.e. 0.1 SGE *Pese* was also administered as a positive control. Results are expressed as mean percentage inhibition ± SD (n=4 from 2 ovaries with mean oocyte diameters of 353 µm and 431 µm, respectively).

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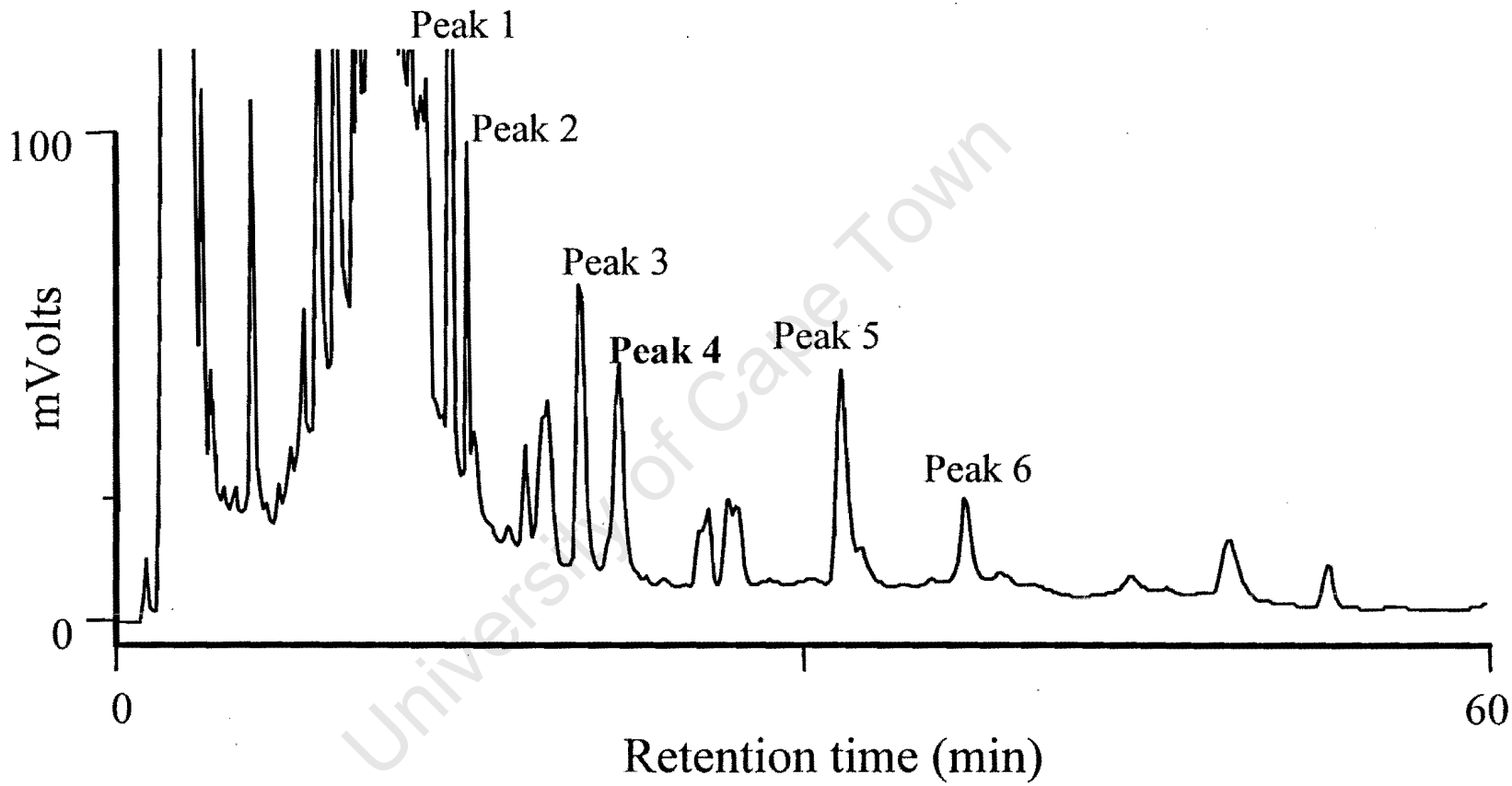

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Fig. 37. An atypical chromatogram showing the RP-HPLC fractionation of 80 sinus glands of *J. lalandii* on a Nucleosil C-18 column. The same column, HPLC and solvent system were used as in Fig. 24. Six peaks are labeled according to order of elution. The 4th peak (indicated in bold letters), with protonated mass of 8400 Da, does not typically appear with fractionation of *Jala* sinus glands. The effect of these 6 peak fractions on *de novo* protein synthesis in ovaries were tested at 1 SGE each (see Fig. 38).

SGs and this peak 4 was included in the *in vitro* assay, along with the cHHs (peaks 5 and 6, Fig. 37), MIH (peak 3, Fig. 37) and the 2 peaks with cHH immunoreactivity (peaks 1 and 2, Fig. 37). Figure 38 shows that peak fractions 1 and 2 did not inhibit protein synthesis in the ovaries, whereas peak 6 and peak 4 caused 48.59 ± 7.02 % and 33.51 ± 8.87 % inhibition, respectively. Peak material of fractions 5 and 3 produced an inhibition of 20.23 ± 14.16 % and 14.90 ± 13.26%, respectively (values are mean ± SD, n = 4).

An electrophoretic profile of the proteins occurring in the ovary of a vitellogenic prawn is depicted in Fig. 39. A number of prominent bands are visible in the Coomassie Blue-stained SDS gel of ovarian homogenates, which relates to the 3 sub-units of vitellin (VT) with a molecular weight of 200, 120 and 80 kDa, respectively; a less abundant protein with molecular weight of around 33 kDa corresponds to the shrimp ovarian peritrophin-like protein (SOP, Fig. 39A). The inhibition of *de novo* protein synthesis in the ovary, as measured by the incorporation of radiolabeled amino acids following their 6 h incubation with various substances (see above), was also reflected in the electrophoretic profile of ovarian proteins, as visualised on autoradiographed gels (Fig. 39 B, C). After the protein content of each homogenate (from the ovary pieces) had been determined, total protein loaded per lane could be standardised to facilitate direct comparisons between samples; the intensity of the bands on the autoradiograph reflects the abundance of the radiolabeled proteins. Figure 39B shows a clear increase in the intensity of the protein bands with the decreasing dose of crude extracts of *Jala* sinus glands; in fact, there is no visible difference in the amounts of proteins synthesised in lanes 5 (treated with 0.001 SGE of *Jala* extracts) and 6 (not treated with SG extracts). This agrees with the data in

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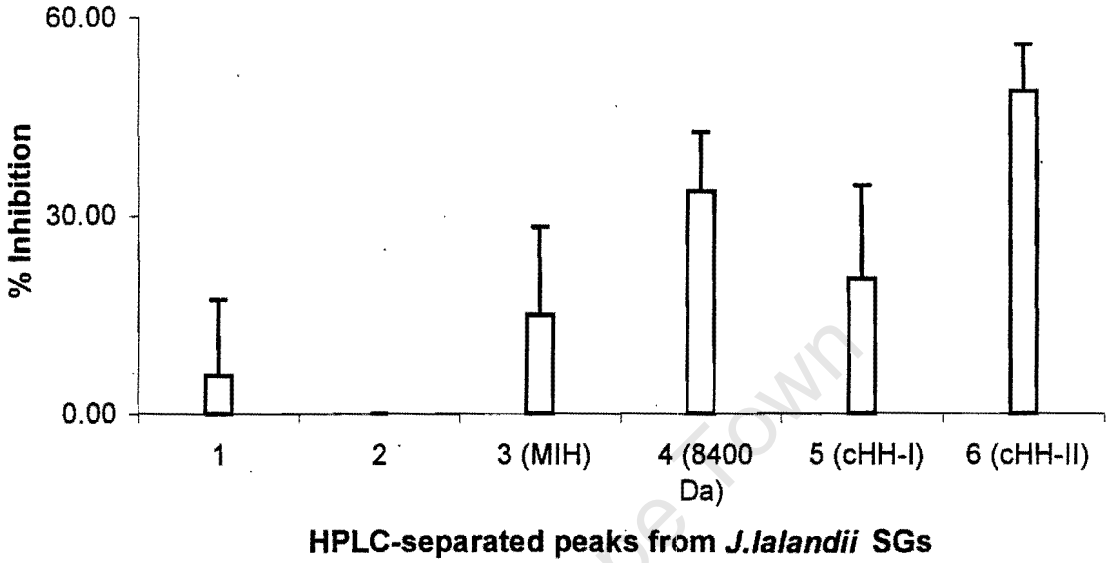

Fig. 38. The effect of various peptides, isolated from sinus gland extracts of *J. lalandii*, on protein synthesis in ovaries from *P. semisulcatus*. The values represent the percentage of inhibition in fragments removed from 2 ovaries (mean ± SD; n=4). The chromatographic profile of the sinus gland peptides is shown in Fig. 37.

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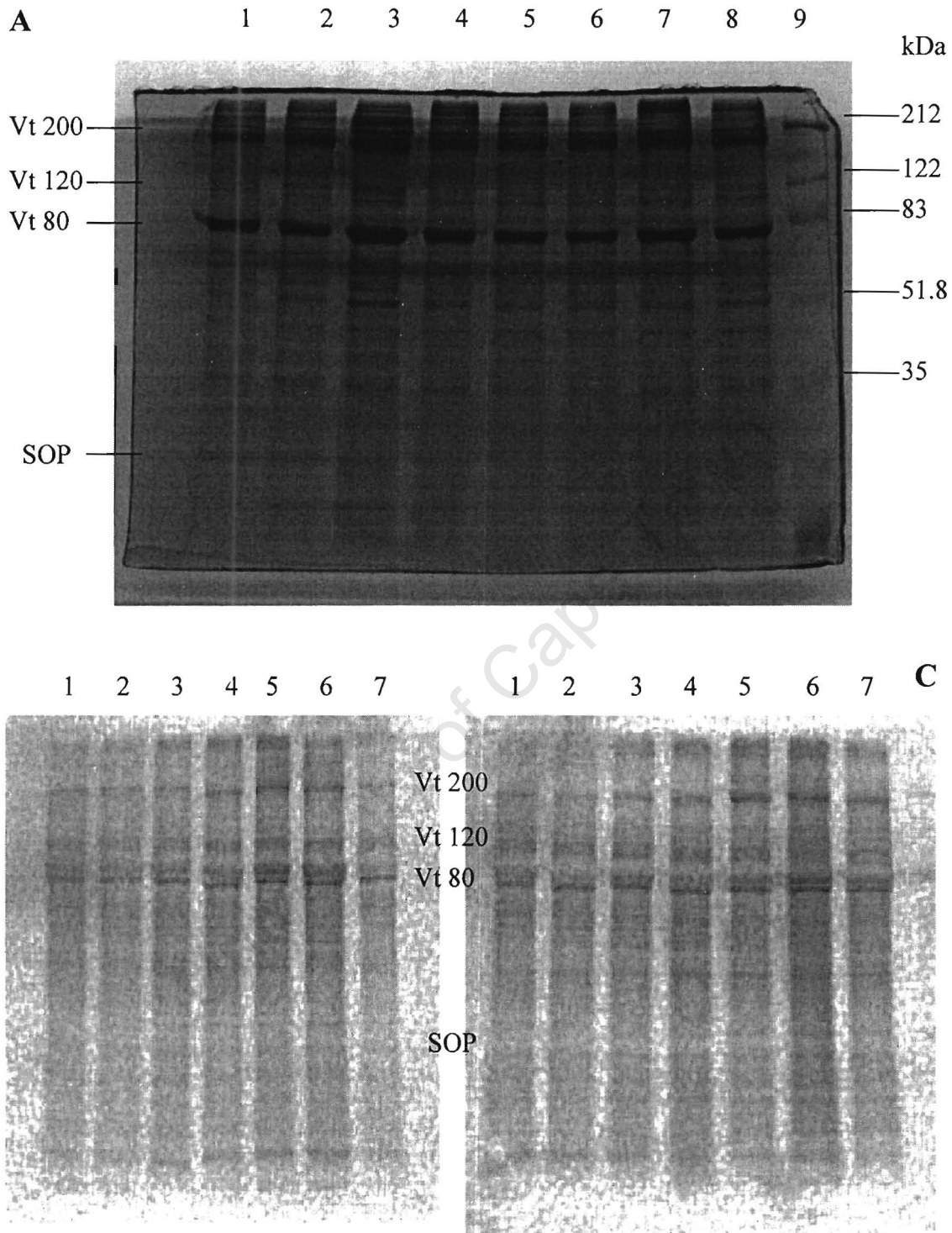

Fig. 39 **A.** 12.5 % SDS-PAGE (stained with Coomassie Blue) of ovarian homogenates of *Penaeus semisulcatus*. The equivalent of 30 µg of proteins from different homogenates were loaded onto the gel (lanes 1-8). A molecular weight marker was loaded in lane 9: weights correspond to myosin (212 kDa), β-galactosidae (122 kDa), BSA (83 kDa), ovalbumin (51.8 kDa), carbonic anhydrase (35 kDa), soybean trypsin inhibitor (28.4 kDa), lysozyme (20 kDa) and aprotinin (7.2 kDa). Proteins were transferred to nitrocellulose membranes and exposed to autoradiographic film to reveal the effect of **B.** different doses of crude extracts of *Jala* sinus glands on *de novo* protein synthesis: 2 SG (lane 1), 1 SG (lane 2), 0.1 SG (lane 3), 0.01 SG (lane 4), 0.001 SG (lane 5), untreated ovary (lane 6), 0.1 SG of *Pese* (lane 7) and **C.** peptides purified from *Jala* sinus glands: lanes 1-7 correspond to ovaries treated with peaks 5, 6,4, 3, 1 and 2 (see Fig. 37); untreated ovary was loaded into lane 7.

Fig. 36 which was derived at from counting dpm values of the samples. In Fig. 39C, the effect of purified *Jala* sinus gland peptides on *de novo* protein synthesis in the ovary can be seen. The vitellin bands, e.g. Vt 200, in lanes 5 (peak fraction 1 of Fig. 37) and 6 (peak fraction 2 of Fig. 37) have the same strong intensity as that of non-SG treated ovaries (lane 7, Fig. 39C). This Fig. 39 further shows that ovaries treated with peak fractions 6 and 4 (of Fig. 37) have the highest inhibition of protein synthesis. It is further apparent that total protein synthesis was inhibited and not specifically the egg proteins.

3.3. Sequence elucidation of cHH/MIH/VIH peptides from J. lalandii

Conventionally, disulfide linkages are reduced and alkylated to linearise the molecule before determining its primary sequence. The extent of peptide loss during this procedure was examined with the use of synthetic insulin. The process of reduction/alkylation was successful in that the A and B chains of insulin were separable on HPLC but the combined peak area of the peaks corresponding to these chains, however, did not equal that of the non-reduced insulin and represented a loss of 43 % of the starting material (results not shown). Even bigger losses in peptide yield were experienced after reduction and alkylation of *Jala* cHH-I (results not shown). It was, thus, decided not to linearise the cHH/MIH/VIH peptides from *J. lalandii* by reduction and alkylation in order to elucidate their primary structures, but rather to use non-reduced (i.e. native) sample material. Reduction and alkylation was, however, carried out as a pilot experiment on 25 SGE of *Jala* cHH-I and –II, and each step was monitored by mass spectrometry to determine its success. Fig. 40

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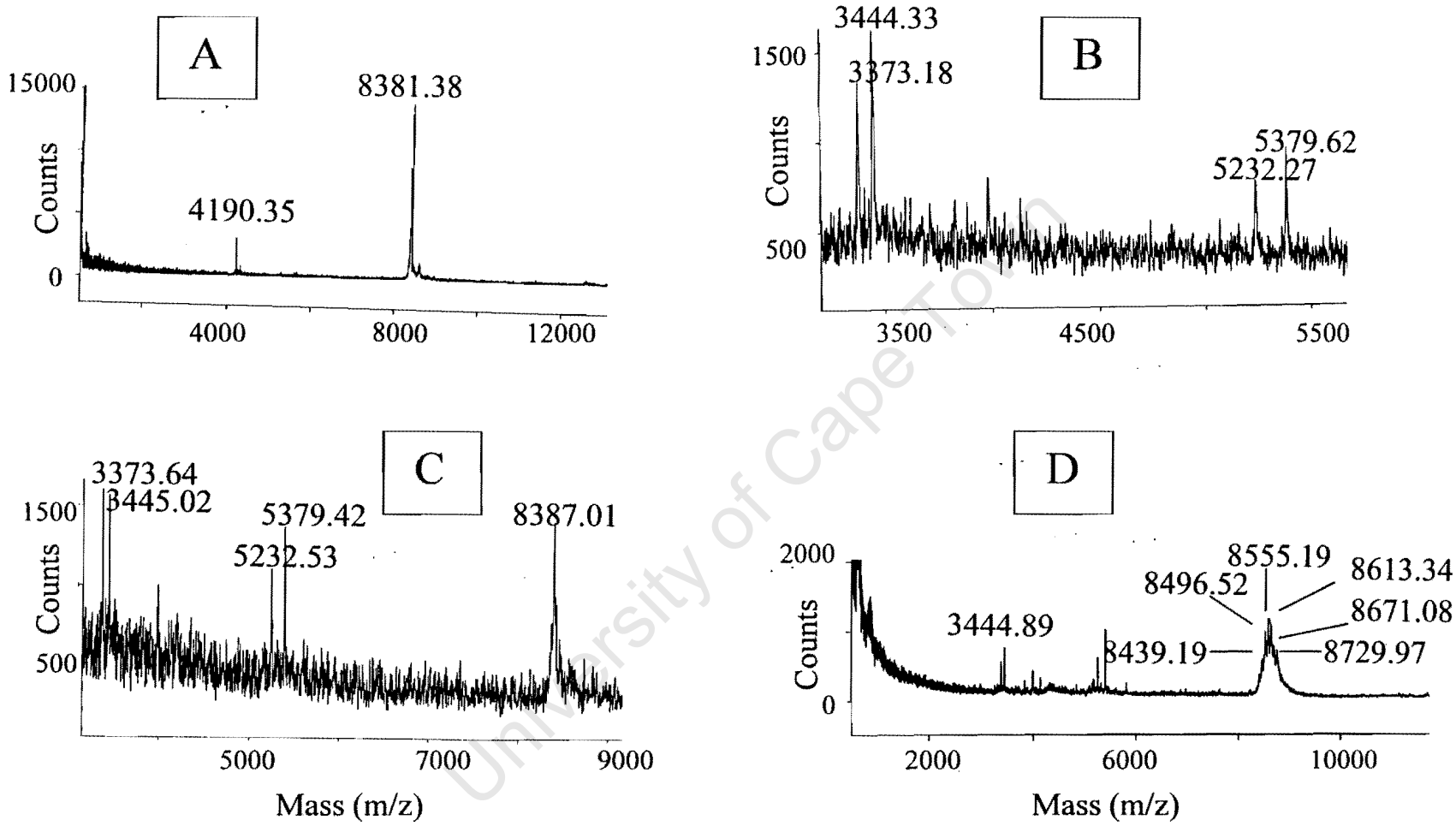

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Fig. 40. MALDI-TOF mass spectra of *Jala* cHH-I obtained with sinapinic acid as matrix: **A.** native, non-reduced peptide; **B.** reduction buffer only; **C.** *Jala* cHH-I after reduction of the disulfide bridges and, **D.** after alkylation of the reduced bridges. Aliquots of the peptide were removed at each step of the reduction/alkylation procedure, diluted with acetonitrile/TFA to lower the buffer salt content in the sample, and spotted onto the sample plate for MS.

shows that it is possible to carry out reduction of disulfide bonds on a small scale and still demonstrate the results by MALDI-TOF MS. In Fig. 40A the protonated mass peak of *Jala* cHH-I (8381) and its doubly-charged ion (4190) is visible before modification of the peptide. Fig. 40C illustrates a mass shift of +6 units (8387), which indicates that the reduction of the disulfide bonds have been successfully carried out. In Fig. 40D the "family" of 6 mass peaks in the 8000+ Da range, corresponds to the alkylation of the 6 Cys residues of the reduced *Jala* cHH-I peptide (+58 mass units for each Cys). In both Fig. 40C and D, mass peaks relating to buffer components are also present (cf. mass peaks in Fig. 40B). The strategy to elucidate the amino acid sequence of the cHH/MIH/VIH peptides present in *J. lalandii* was, thus, to cleave only non-reduced peptides (by means of enzymes and chemicals), separate and sequence the resulting peptide fragments, reduce the disulfide linkages of the fragments, and analyse the reduced mixtures on MALDI-TOF mass spectrometry.

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3.3.1. Jala cHH-I

3.3.1.1. Jala cHH-I: trypsin digest.

N-terminal sequence analysis of native cHH-I peptide (peak 4 in Fig. 24) yielded the first 30 amino acid residues (Fig. 41). This provided evidence that the amino terminus of this peptide is not blocked. Cys residues at positions 7, 23 and 26 were identified by sequence homology (conserved residues in decapod cHHs) and by the smaller increases of the PTH dehydro-alanine derivative compared with Ser in the Edman degradation cycle. From the sequencing data, a yield of 100 ng (± 12 pmol) of cHH-I peptide per SG has been estimated. Trypsin digestion of non-reduced purified cHH-I produced a tryptic map (Fig. 42A) whose peptide fragments were

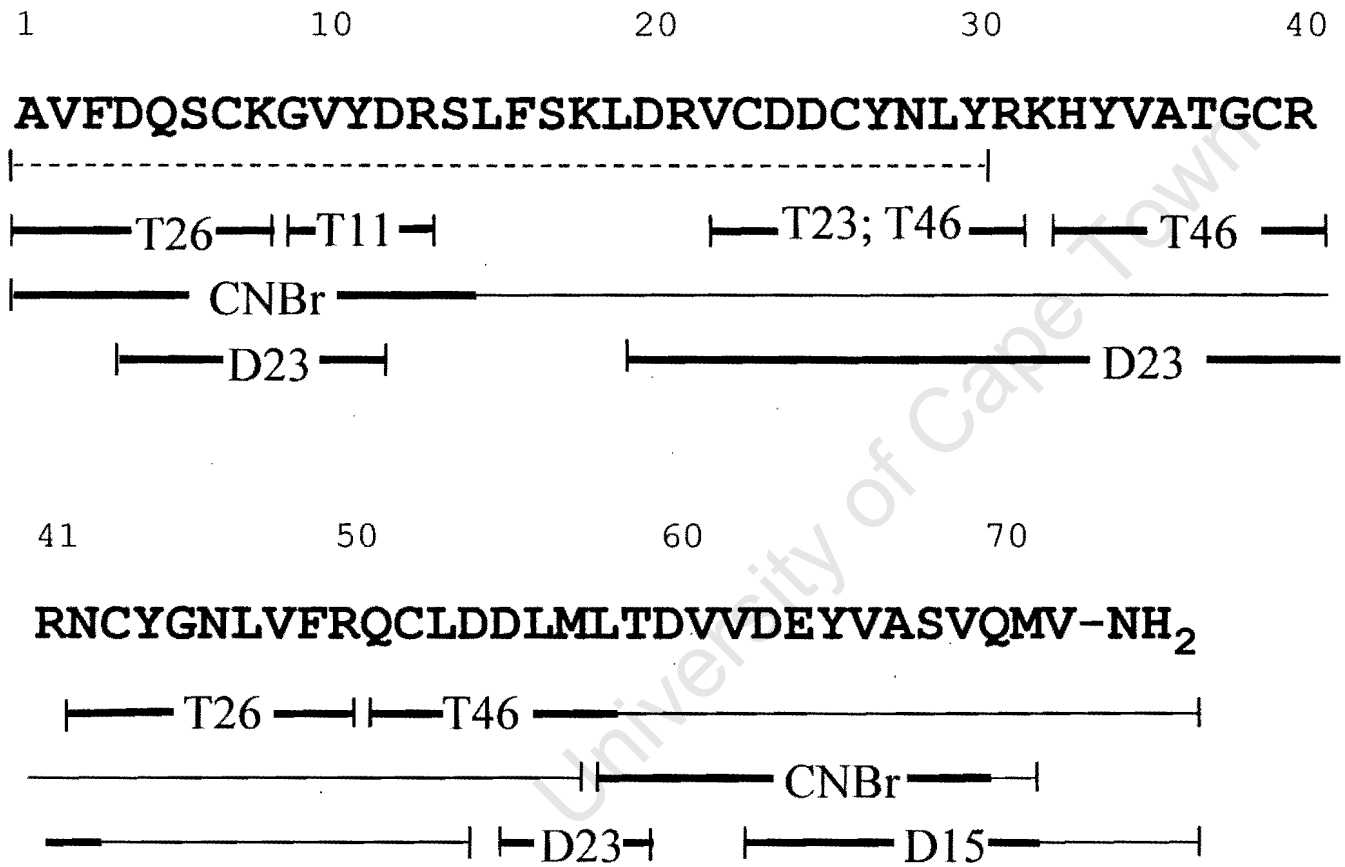

Fig. 41. Primary structure of cHH-I from *Jasus lalandii* as deduced from sequenced peptide fragments: Tx, tryptic peptides; Dx, endoproteinase Asp-N peptides; CNBr, cyanogen bromide-cleaved products. Note: The dark line indicates the sequenced residues while the faint line indicates the unsequenced residues of a particular fragment. The dotted line shows residues identified by N-terminal sequencing of native *Jala* cHH-I. Amidation was established by methylation of the C-terminal fragment (58-72) of a partial CNBr cleavage.

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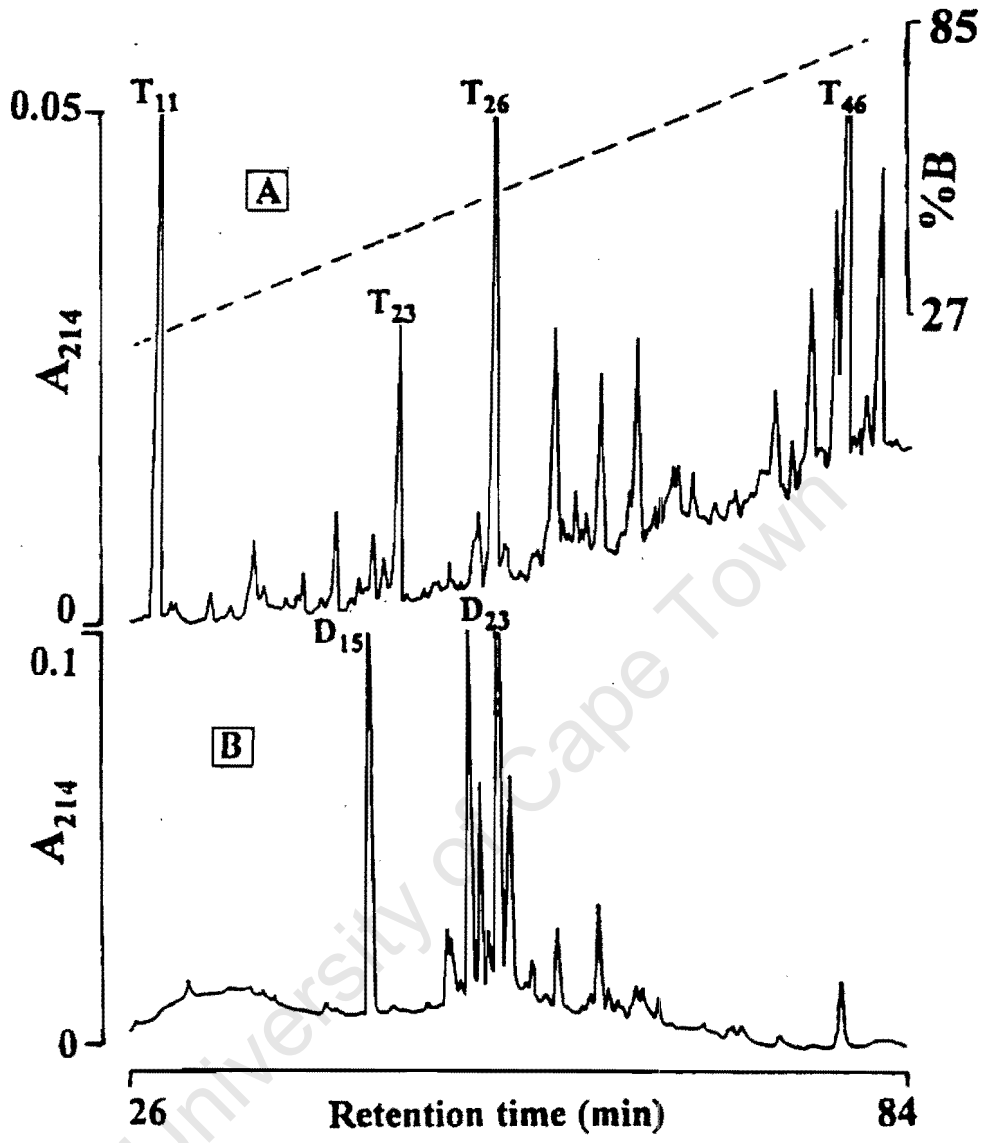

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Fig. 42. Peptide maps of native cHH-I from *J. lalandii* SGs after digestion with **A**. trypsin and **B**. endo-Asp-N. The same column, solvents and HPLC system were used as in Fig. 24. A linear gradient (dotted line) from 0 % B to 99 % B was developed in 90 min after an isocratic step at 0 % B for 5 min. Peptides that were selected for sequencing analysis are numbered in the order of elution. (Tx, tryptic peptides; Dx, endoproteinase Asp-N peptides)

subjected to mass spectrometry. The 4 major peaks (T11, T23, T26 and T46) were N-terminally sequenced. It became apparent that T11 was an individual peptide chain, whereas T26 and T46 consisted of 2 and 3 peptide chains, respectively, linked by disulfide bonds. Arrangement of these sequences was aided by (a) sequence information obtained from Edman degradation of native *Jala* cHH-I, (b) sequence similarity with *Hoam* cHH-A (Tensen *et al.*, 1991) and (c) mass spectrometric analysis of reduced T26 and T46 fragments (Table 5; Fig. 41). Cystine residues were assigned on the basis of cHH sequence homology, mass analysis and an increase in PTH dehydro-alanine derivatives in Edman degradation. Table 5 shows how well the mass data of the reduced peptide fragments correlate with that of the sequenced peptide chains. Figure 43 shows, as an example, the mass analyses of tryptic fragments T26 and T46 after reduction of the disulfide bonds. Surprisingly, although fragment T23 (residues 22-31) contained 2 Cys residues, it was sequenced as a single peptide chain, whereas it should have formed part of the linked peptides of T46.

Residues at position 32 and 41 could not be established by sequencing the tryptic fragments. However, K^{32} and R^{41} were later identified by sequencing data obtained from overlapping Endo-Asp N digest fragments (see 3.3.1.2. below). Further, one of the peptide chains of tryptic fragment T26, N^{42}-R^{50}, yielded an $[M+H]^+$ ion of 1981 following an overnight digestion with trypsin (see Table 5). With a partial tryptic digest of only a few hours duration, however, a fragment corresponding to T26 on HPLC yielded an $[M+H]^+$ ion of 2137 (results not shown). The resulting mass difference of 156 Da corresponds to the mass of Arg, and it was deduced that R^{41} was cleaved off from R^{40} and N^{42} during the extended period of digestion.

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Table 5. Relative mass of peptides obtained from peptide fragments generated by enzymatic and chemical cleavage of non-reduced cHH-I purified from *Jasus lalandii* sinus glands.

PEPTIDE	POSITION	M_r ACCORDING TO: MALDI-TOF $[M+H]^+$ NON-REDUCED	REDUCED[1]	SEQUENCE
TRYPSIN				
T_{11}	9 - 13	609		608
T_{23}	22 - 31	1262		1261
T_{26}	1 - 8; 42 - 50	1981		
	1 - 8		898	896
	42 - 50		1086	1084
T_{46}	33 - 40; 22 - 31; 51 - 72	4648		4649
	33 - 40		907	905
	22 -31		1263	1261
	51 - 72[2] (51 - 58)		2488	2484
CYANOGEN BROMIDE				
Complete CNBr digest	1 - 57[2] (1 - 14)	6684[3]		6684[3]
	58 - 71[2] (58 - 69)	1519[3]		1518[3]
Partial CNBr digest	58-71	1519[3]		1518[3]
	58-72	1666		1665
ASP-N PROTEASE				
D_{15}	63 - 72	1006		1139
D_{23}	4 - 11; 20 - 53	5006		5012
	4 - 11		900	897
	20-53[2] (20 - 42)		4117	4114
	55-59[4]			

[1]Mass of reduced fragments is measured by MALDI as $[M+H]^+ + n[H]^+$, where n = number of Cys. (Reduction by DTT adds H to each S). [2]Expected positions as deduced from the entire sequence. The fragment was only partially sequenced (sequenced positions in brackets). [3]Mass when homoserine lactone is formed. [4] Fragment co-eluted with D23, see text.

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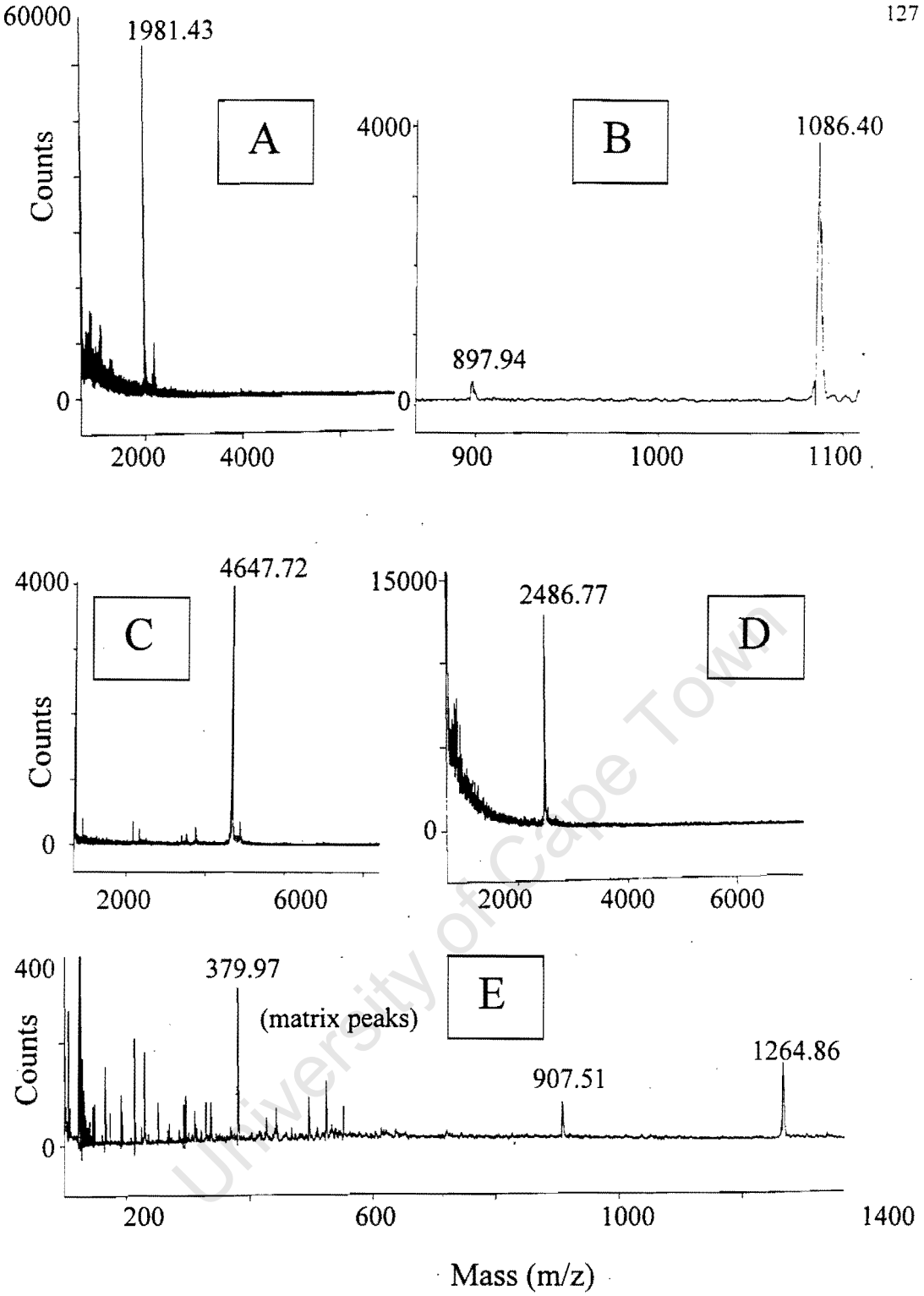

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Fig. 43. MALDI-TOF mass spectra of *Jala* cHH-I tryptic fragment T26 and T46 before reduction (sinapinic acid matrix; **A**, **C**) and after reduction of the disulfide bonds (cinnamic acid matrix; **B**, **D**, **E**). **A**, **B** = T26; **C**, **D**, **E** = T46.

From N-terminal sequencing data it also became clear that, in addition to other peptide chains (see Table 5 and Fig. 41), tryptic fragment T46 contained the C-terminal peptide chain of cHH-I; this chain was, however, only partially sequenced up to residue 58 (Fig. 41).

3.3.1.2.Jala cHH-I: endo-Asp N digest

Non-reduced cHH-I peptide was digested with endo-Asp N to generate overlapping peptide fragments. The resulting peptide map is shown in Fig. 42B. The 2 major peaks of this digest (D15 and D23) were selected for further analysis. Sequencing data showed D15 to be the carboxyl-terminal peptide fragment, and residues 63-72 were identified (Fig. 41).The mass discrepancy between the calculated D15 sequence and the actual fragment mass obtained by MALDI, i.e. 1139 versus 1005 (Table 5), may be explained by a loss of D^{63} (= 115) in the N-terminal position, as well as a H_2O molecule (= 18) from the adjacent E^{64} residue during MALDI-TOF analysis. Sequencing data of D23 showed that this fragment consisted of 2 peptide chains linked by disulfide bridges, and amino acid residues 4-11 and 20-53 were identified (Fig. 41). From the obtained sequence it is clear that endo-Asp N failed to cleave between C^{23} and D^{24}, as well as between D^{24} and D^{25}. A probable reason for this may be that the two flanking Cys residues (position 23 and 26) form disulfide bonds and so the enzyme is excluded from this region by steric hindrance. The presence of only 2 peptide chains in this fragment was confirmed by mass analyses on a mixture of the reduced products of fragment D23 (Table 5). As with D15, the mass of the non-reduced D23 fragment is also less than that calculated from its sequence (Table 5). These odd results may stem from the interactions between the N-terminal Asp and its

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adjacent residues during the MALDI procedure. Quite surprisingly, a third peptide chain, residues 55-59 without a Cys residue, was also sequenced from D23 peak material (Fig. 41), thus co-eluting with fragment D23.

3.3.1.3. Jala cHH-I: CNBr cleavage and methylation

The assigned Met at position 57 enabled the execution of a cyanogen bromide cleavage of native cHH-I. MS analysis of the completely cleaved peptide mixture revealed that homoserine lactone products were present (Table 5) and N-terminal sequencing identified residues 58 to 69 (Fig. 41). To unambiguously determine whether the C-terminal residue (V^{72}) is amidated, methylation was carried out on a C-terminal fragment of cHH-I and analysed by MALDI-TOF MS. This fragment for methylation was obtained from partial cleavage of cHH-I with CNBr which yielded $[M+H]^+$ ions of 1519 and 1666 (Fig. 44) that corresponded to the cleaved residues 58-71 with a C-terminal homoserine lactone, and to residues 58-72 of the uncleaved fragment, respectively, (see Table 5). The $[M+H]^+$ ion of the uncleaved C-terminal fragment shifted from 1666 to 1708 when the fragment was converted to its methyl ester (Fig. 44). This corresponded to an increase of 42 Da, thus revealing the incorporation of 3 methyl groups (i.e. 3 x 14 Da) and, since fragment 58-72 contains 3 free carboxyl groups, viz. D^{60}, D^{63} and E^{64}, it was concluded that (V^{72}) must be amidated.

The accuracy of the methylation method and the correct interpretation of the ensuing results were controlled by including several peptides of known sequences. Figure 45 shows that, under the current conditions for methylation, all free carboxyl groups are esterified.

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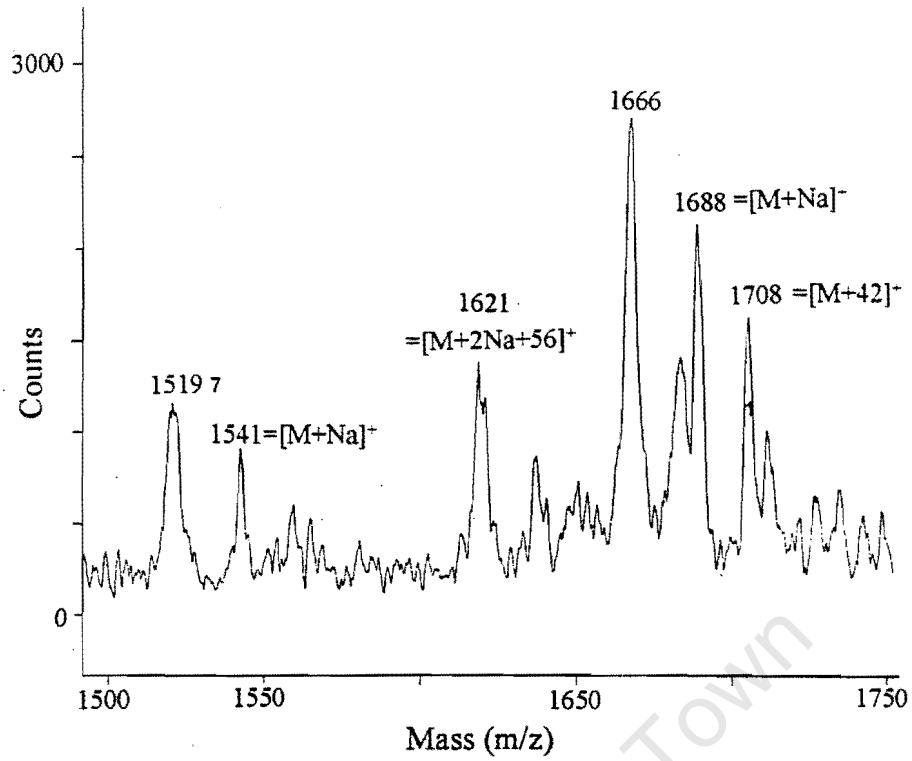

Fig. 44. MALDI-TOF analysis of the C-terminal fragments of *Jala* cHH-I following partial CNBr cleavage and methylation. The [M+H]+ ions of 1519 and 1666 correspond to the homoserine lactone product of fully cleaved residues 58-71 and the partially-cleaved residues 58-72, respectively. Interpretation of other mass peaks are indicated. The increase of 42 m.u. relates to 3x14 m.u. and 56 m.u. corresponds to 4x14 m.u.

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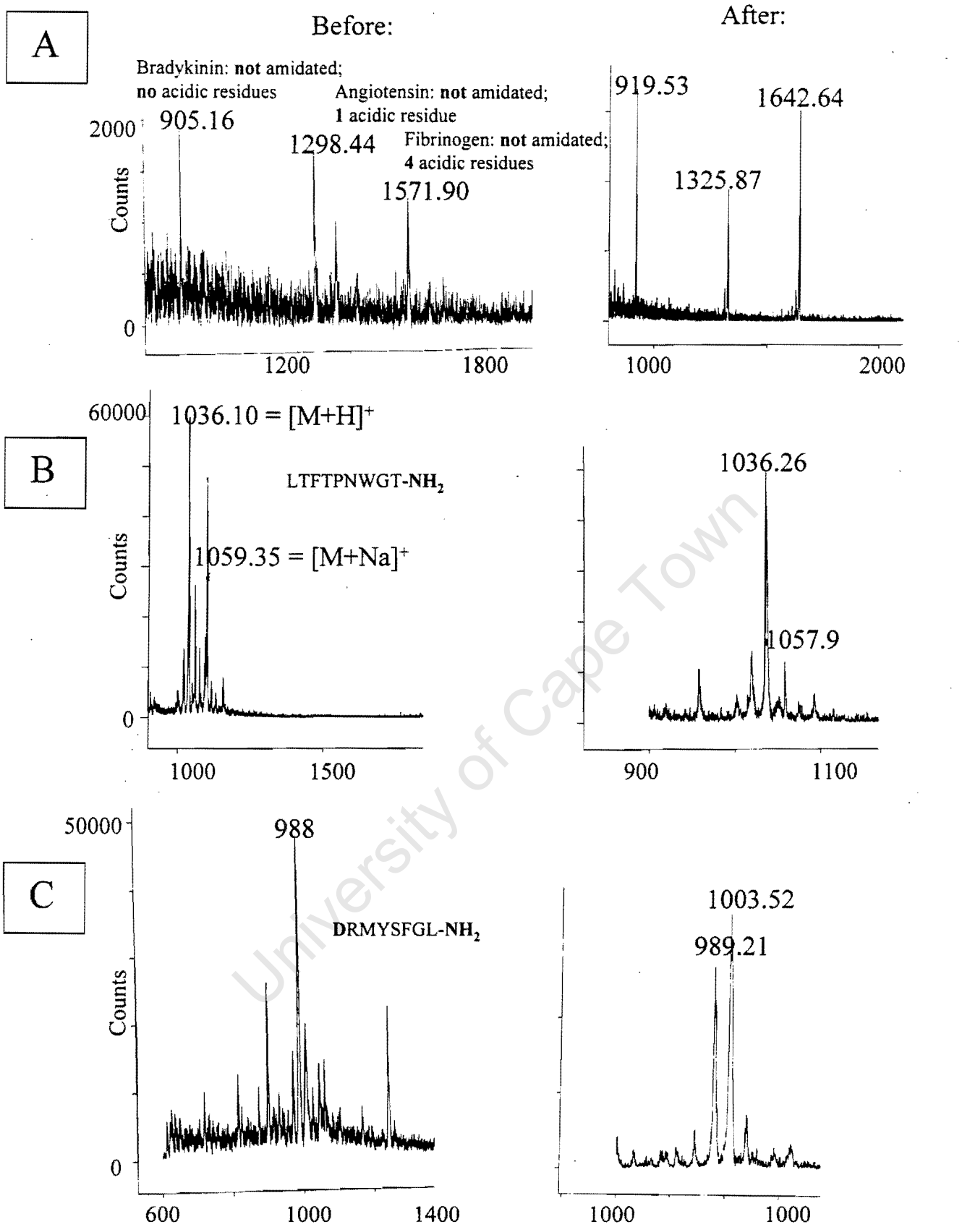

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Fig. 45. Methylation of synthetic peptides and analyses by MALDI-TOF MS: mass spectra before and after methylation. **A.** calibration 1 standard mix (PerSeptive), **B.** des pGlu-*Cam*-HrTH-II, **C.** *Pea*-Ast 5 (see 2.5.5. for explanation of abbreviations). In **B** and **C** the primary peptide structure is given as single-letter code with acidic residues and amide groups indicated in bold letters.

3.3.1.4.Investigation into chirality of Jala cHH-I

To determine whether *Jala* cHH-I was composed of a D-Phe^3 or an L-Phe^3, 2 synthetic octapeptides, AVFDQSCK, which corresponds to the N-terminal residues 1-8 of *Jala* cHH-I, were made with the different conformations of the Phe^3 residue. In order to purify the native octapeptide from *Jala* cHH-I, reduction and alkylation was performed on tryptic fragment T26 (see Fig. 42A). Aliquots, collected for MS after each step in the procedure, indicated the success of the method (Fig. 46). The reduced and alkylated mixture of fragment T26 was then purified on HPLC (Fig. 46C). To facilitate direct comparison between the reduced/alkylated native octapeptide and the synthetic octapeptides, the latter were also subjected to reduction and alkylation. The retention times on HPLC and mass of these synthetic peptides were established after these procedures (Fig. 46A,B). Figure 46C shows a clear peak, corresponding to the L-Phe^3 form of the octapeptide, from native cHH-I but no such peak relating to the D-Phe^3 form of the peptide. The second, later-eluting peak in Fig. 46C is the second peptide chain of this tryptic fragment (residues 42-50, see also Table 5).

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3.3.2. Jala cHH-II

3.3.2.1. Jala cHH-II: trypsin digest

N-terminal sequencing of *Jala* cHH-II (see peak 5 of Fig. 24) resulted in the first 20 amino acids (Fig. 47), indicating a free N-terminus. The Cys residue at position 7 was assigned by sequence homology with other cHHs and by the smaller increase of the PTH dehydro-alanine derivative compared with Ser in the Edman degradation cycle. The peptide yield of cHH-II has been estimated at 3 pmol/SG. A tryptic map

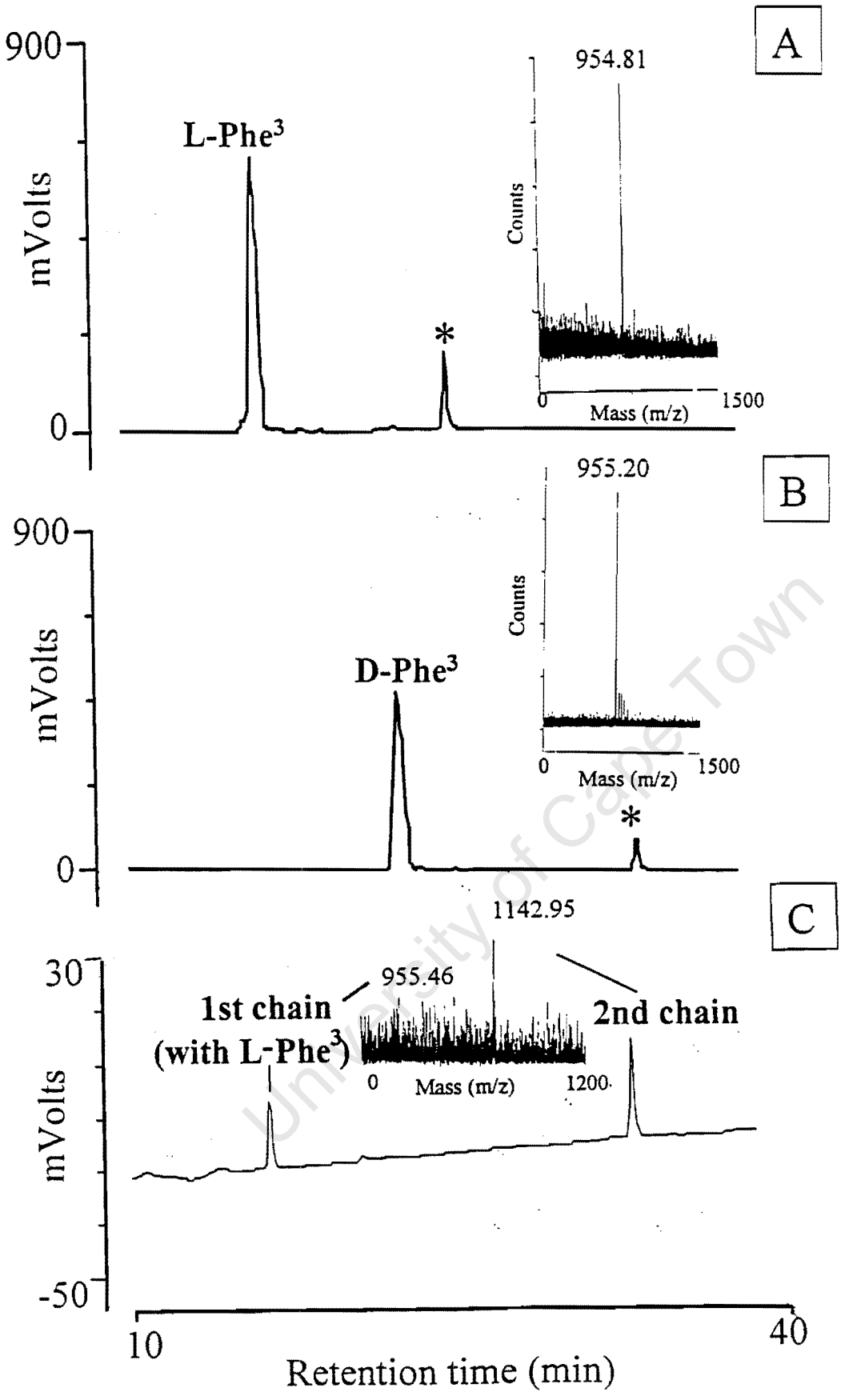

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Fig. 46. Chromatographic and mass spectrometric analyses of 2 synthetic octapeptides corresponding to the N-terminal sequence of *Jala* cHH-I, but with different conformations of the third residue, viz. **A.** L-Phe^3 and, **B.** D-Phe^3 after the procedure of reduction/alkylation. **C.** Tryptic fragment T26 of *Jala* cHH-I after reduction/alkylation.
The samples were applied to a Nucleosil C18 column under a gradient of 15 % B for 4 min, followed by a linear increase to 50 % B in 35 min, using the same HPLC and solvent system as described in 2.2. The asterisk (*) marks minor impurities in the synthetic peptides.

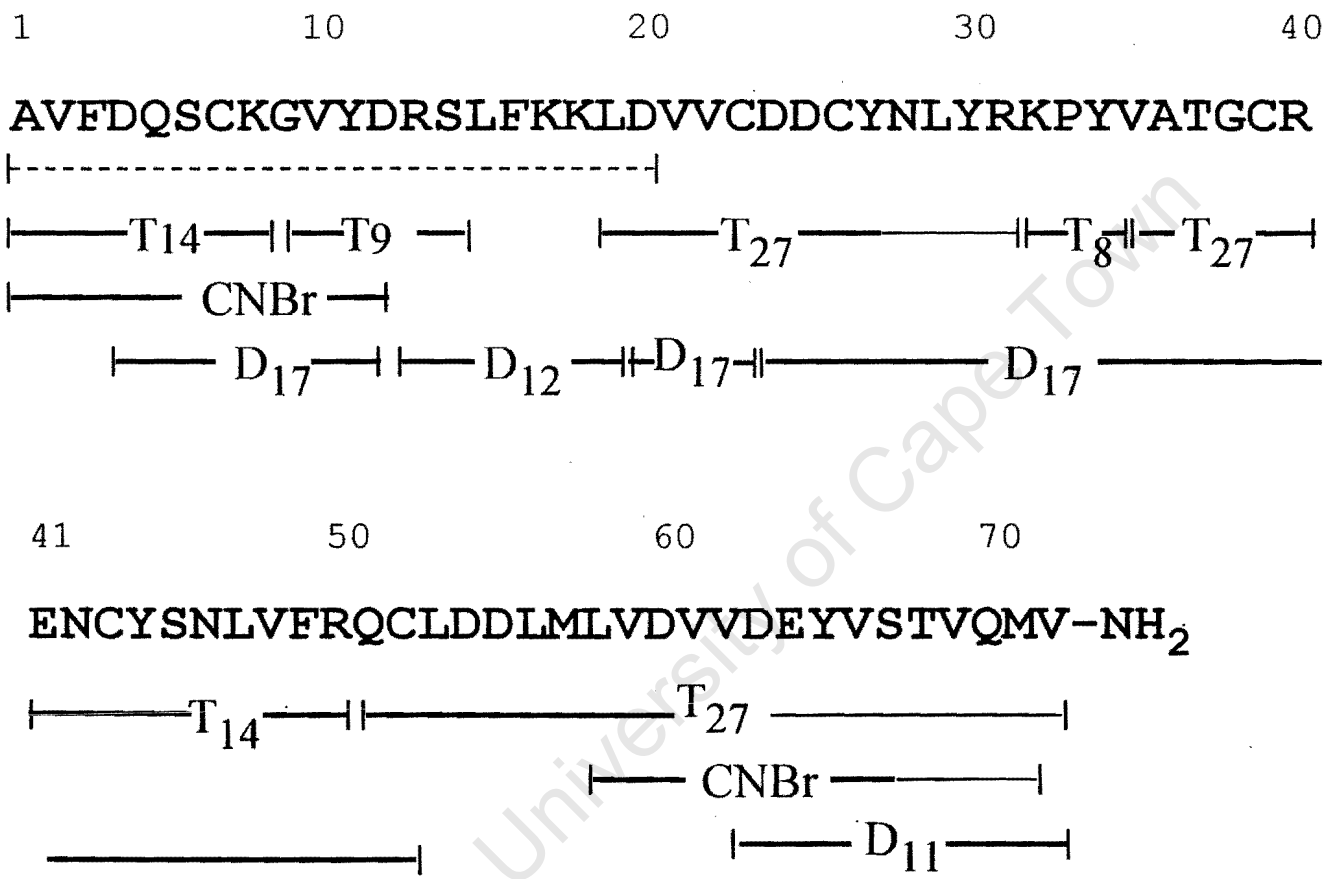

Fig. 47. Primary structure of *Jala* cHH-II as deduced from sequenced peptide fragments: Tx, tryptic peptides; Dx, endoproteinase Asp-N peptides; CNBr, cyanogen bromide-cleaved products. Note: The dark line indicates the sequenced residues, while the faint line indicates the unsequenced residues of a particular fragment. The dotted line shows residues identified by N-terminal sequencing of the native, uncleaved cHH. Amidation was inferred from sequence homologies and mass spectrometry.

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of non-reduced *Jala* cHH-II revealed 4 major peptide fragments (Fig. 48A) that were analysed by MALDI-TOF mass spectrometry and subjected to Edman degradation. Although no mass data was forthcoming from fragment T8, it became apparent from sequencing data that T8 and T9 were single peptides (Fig. 47; Table 6). Moreover, T8 was the atypical result of a tryptic digest and, more likely, resulted from a chymotrypsin contamination in the trypsin preparation. It was also evident from mass data and sequence information that tryptic fragments T14 and T27 consisted of 2 and 3 peptide chains, respectively, linked by disulfide bonds (Fig. 47; Table 6). Interpretation of the sequence information was facilitated by knowledge of the first 20 amino acid residues, sequence similarity with *Jala* cHH-I and mass spectrometric analyses of the fragments in both the non-reduced and the reduced form (Table 6). Figure 49 shows mass analyses data of tryptic fragment T14 before and after reduction of the disulfide bonds, as an example.

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3.3.2.2. Jala cHH-II: endo-Asp N digest

Further sequence information and confirmation resulted from overlapping fragments following digestion of non-reduced cHH-II material with endo-Asp N. Three major fragments were purified from this digest (Fig. 48B) for Edman degradation and mass analyses. It became apparent that fragments D11 and D12 were single peptide chains, whereas D17 consisted of 3 peptide chains linked by the intact disulfide bonds (Table 6). Clearly, also, D11 represented the C-terminus of *Jala* cHH-II (Fig. 47).

3.3.2.3. Jala cHH-II: CNBr cleavage

Native, non-reduced cHH-II was chemically cleaved with CNBr. This cleavage with CNBr took advantage of the 2 Met residues in the cHH-II sequence, viz. M^{57} and M^{71}

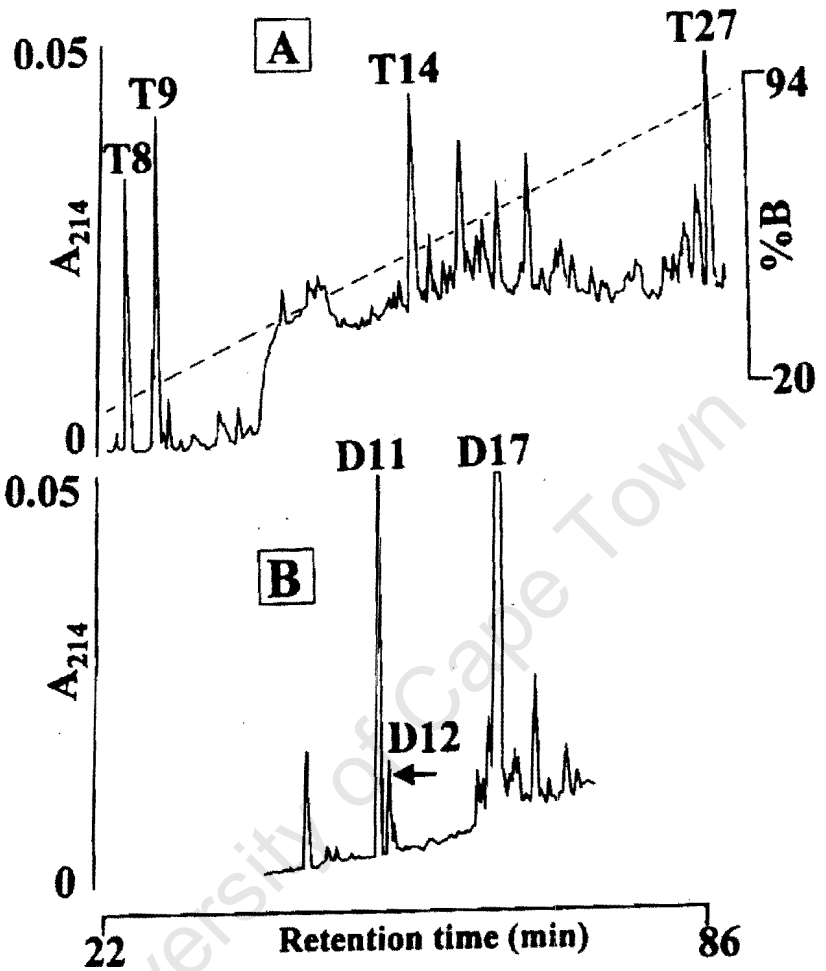

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Fig. 48. Peptide maps of native *Jala* cHH-II after digestion with **A.** trypsin and **B.** endoproteinase Asp-N. The same column, HPLC system and solvents were used as in Fig. 24. A linear gradient (dotted line) from 0 % B to 99 % B was developed in 90 min after an isocratic step at 0 % B for 5 min. Peptides that were selected for sequencing analysis are numbered in the order of elution. (Tx=tryptic peptide; Dx=endo-Asp N-derived peptide).

Table 6. Average protonated mass of peptides obtained from peptide fragments generated by enzymatic and chemical cleavages of non-reduced cHH-II purified from sinus glands of *Jasus lalandii*.

PEPTIDE		POSITION OF RESIDUES	OBSERVED MASS $[M+H]^+$: NON-REDUCED	OBSERVED MASS $[M+H]^+$: REDUCED	CALCULATED M_r: FROM SEQUENCE
Trypsin:	T8	32-34	No mass data		407
	T9	9-13	609.68		609.65
	T14	1-8; 41-50	2140.09		2140.38
		1-8		897.34	898.00[a]
		41-50		1244.52	1245.38[a]
	T27	19-31; 35-40; 51-72	4672		4671.38
		19-31		1592.64	1591.77[a]
		35-40		606.96	606.71[a]
		51-72		2516.30	2514.93[a]
Endo-Asp N:	D11	63-72	1174		1170
	D12	12-19	1006.8		1007.21
	D17	4-11; 20-23; 24-53	4939(K^+ adduct)		4900.54
		4-11		900	899.98[a]
		20-23		436.26	435.51[a]
		24-53		3604	3609[a]
Cyanogen bromide:		1-57	6636		6632.56[b]
		58-71	1571 (Na^+ adduct); 1587 (K^+ adduct)		1549. 71[b]

[a] Mass of reduced fragment is calculated as $[M+H]^+ + n(H)^+$, where n=number of Cys. (Reduction by DTT adds H to S of each Cys).
[b] Mass when homoserine lactone is formed.

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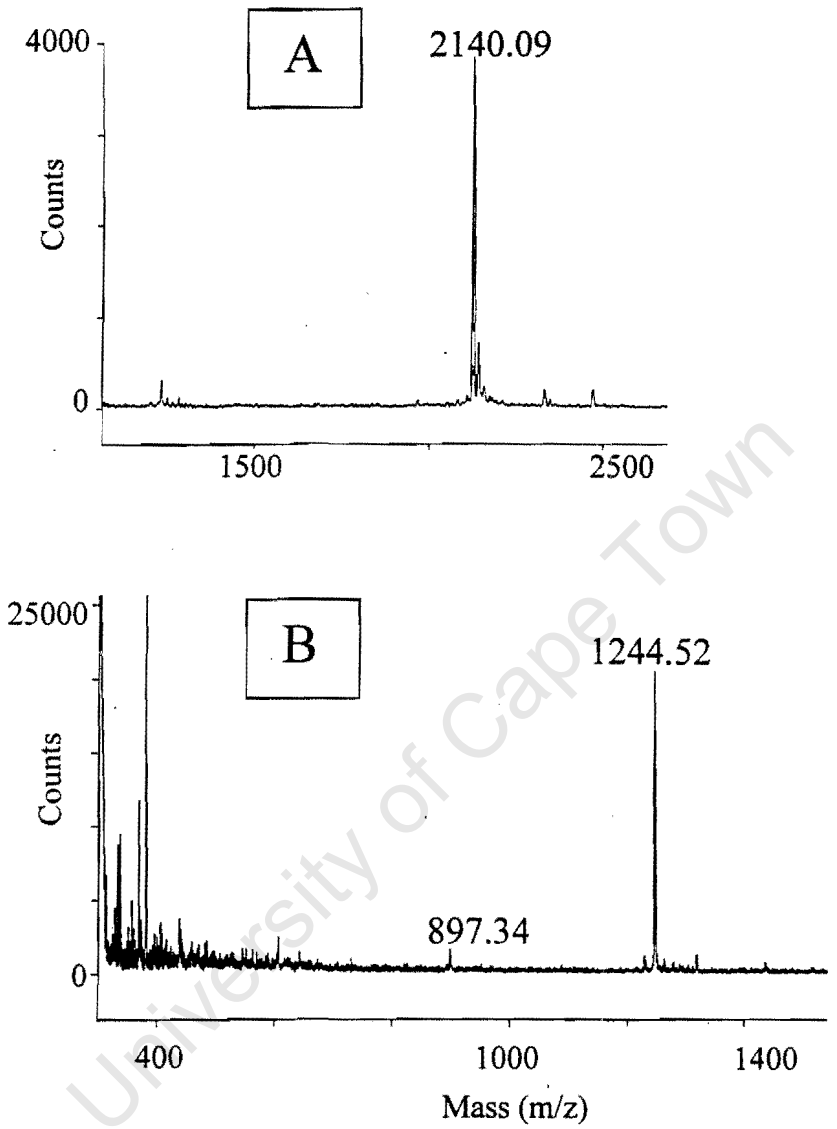

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Fig. 49. MALDI-TOF mass spectra of tryptic fragment T14 of *Jala* cHH-II **A.**before and, **B.** after reduction of the disulfide bonds with DTT.

(Fig. 47) and mass analyses of the resulting mixture indicated homoserine lactone products corresponding to chemically-modified residues 1-57 and residues 58-71 (Table 6). For the latter residues, 2 masses were obtained which corresponded to the sodiated ($[M+Na]^+$) and the potassiated ($[M+K]^+$) ions. Amidation of *Jala* cHH-II was not experimentally established, but was deduced from the average mass obtained from the whole molecule and that from the sequence information.

3.3.3. Jala MIH

3.3.3.1. Jala MIH: trypsin digest

N-terminal sequence analysis of native MIH peptide (see peak 3 of Fig. 24) isolated from 50 sinus glands, resulted in the first 40 amino acid residues, thus indicating a free N-terminus (Fig. 50). Cys residues at positions 6, 23, 26 and 39 were identified by smaller increases of the PTH dehydro-alanine derivatives compared with Ser in the Edman degradation cycle, as well as by sequence homology to other members of the cHH family of peptides. The yield of *Jala* MIH has been estimated at 6 pmol/SG. A tryptic map of non-reduced *Jala* MIH revealed 7 major peaks (Fig. 51A) that were analysed by mass spectrometry and subjected to Edman degradation. From the sequence data and mass analyses, it became apparent that fragments T1, T3, T6 and T11 were single peptides (Fig. 50; Table 7). Fragments T1 and T3 exhibited identical mass and sequence data, despite having different retention times on HPLC (Table 7; Fig. 50A). Tryptic fragments T15, T17 and T18 consisted of more than one peptide chain, but linked to other peptide chains by the intact disulfide bonds (Table 7). Furthermore, from the sequencing data, it appeared that these 3 fragments represented the same region of the MIH peptide but that T15 consisted of 4 peptide

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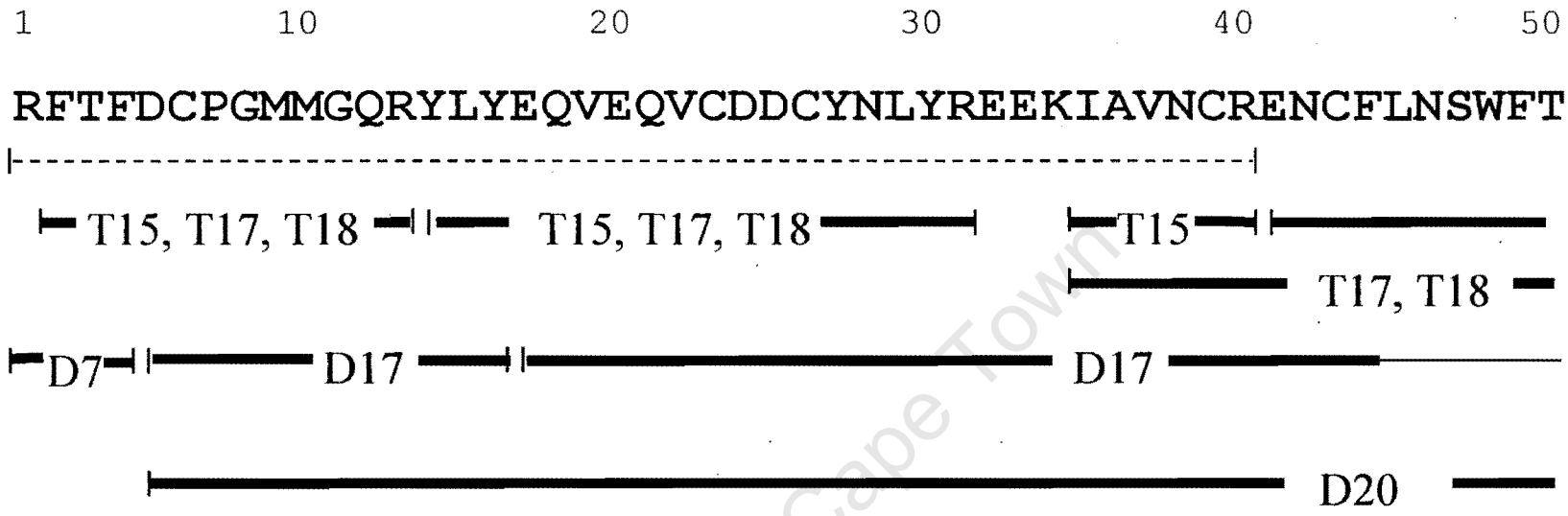

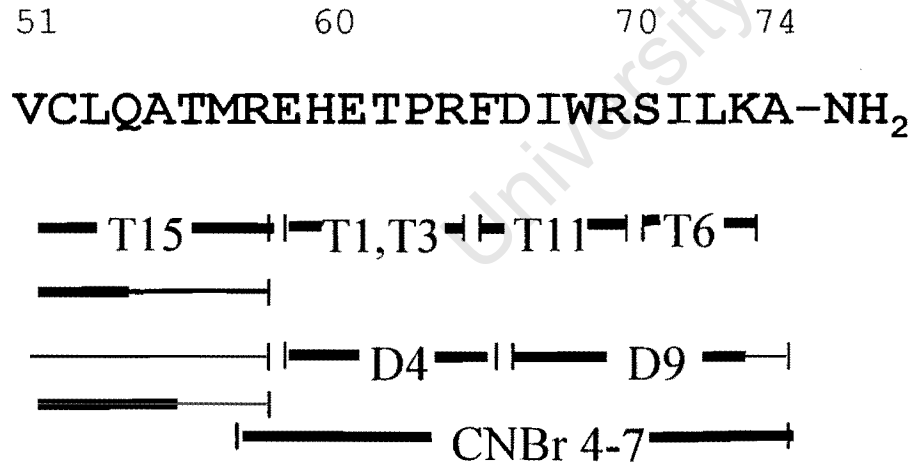

Fig. 50. Primary structure of a putative MIH from *J. lalandii* as deduced from sequenced peptide fragments: Tx, tryptic peptides; Dx, endoproteinase Asp-N peptides; CNBr, cyanogen bromide-cleaved products. Note: the dark line indicates the sequenced residues of a particular fragment, while the faint line corresponds to the unsequenced residues. The dotted line represents residues identified by N-terminal sequencing of the native, uncleaved MIH. Amidation was established by methylation of the C-terminal fragment D9 (residues 66-74).

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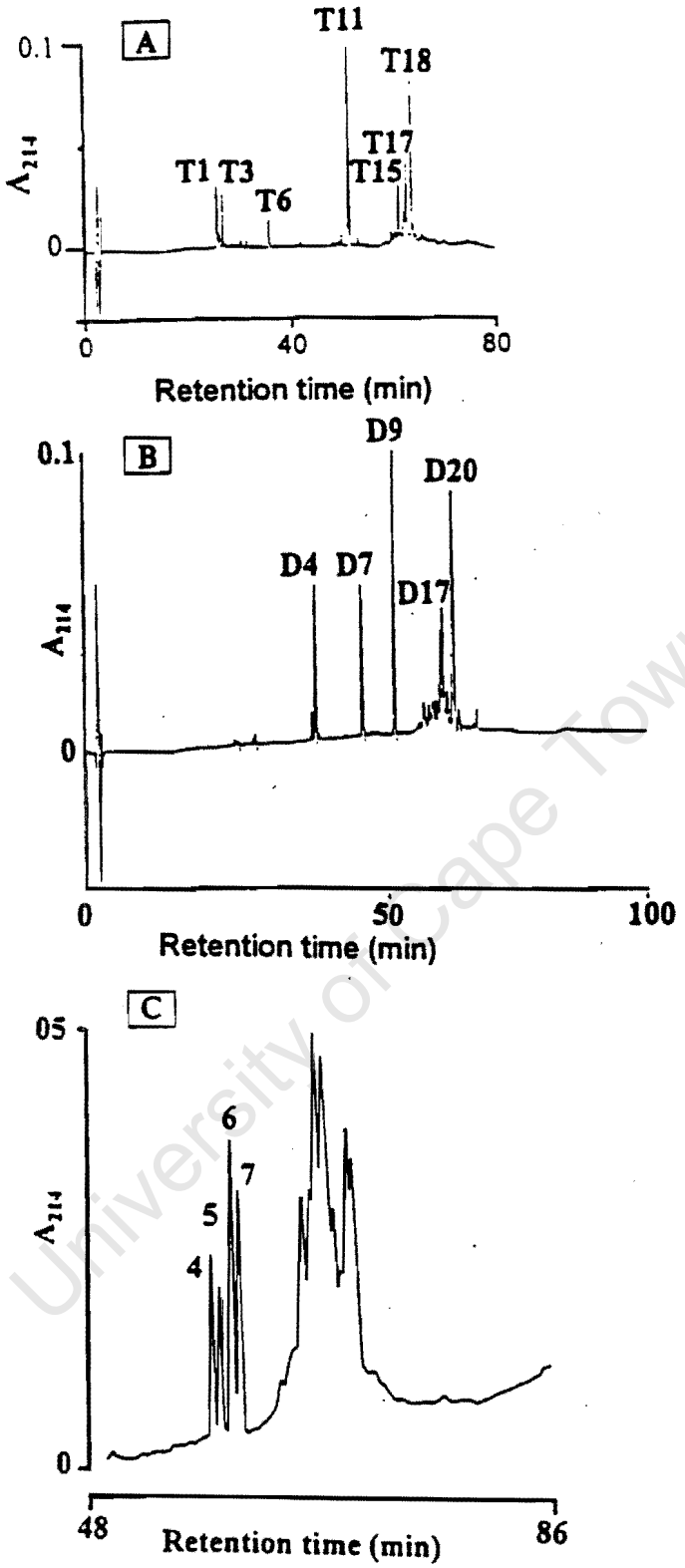

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Fig. 51. Peptide maps of native *Jala* MIH after digestion with **A.** trypsin and **B.** endoproteinase Asp-N, and **C.** after cleavage with cyanogen bromide. The HPLC system, column and solvents are identical as described in Fig. 24. After an isocratic step of 0 % B for 5 min, a linear gradient from 0 to 99 % B was developed in 90 min. Peptides that were selected for sequencing analysis are numbered in order of elution.

Table 7. Average protonated mass of peptides obtained from peptide fragments generated by enzymatic and chemical cleavages of non-reduced MIH purified from sinus glands of *Jasus lalandii*.

PEPTIDE		POSITION OF RESIDUES	OBSERVED MASS $[M+H]^+$: NON-REDUCED	REDUCED	CALCULATED $[M+H]^+$: FROM SEQUENCE
Trypsin:	T1	59-64	768.97		768.80
	T3	59-64	768.93		768.80
	T6	70-73	No mass data		460.59
	T11	65-69	736.90		736.84
	T15	2-13; 14-31; 35-40; 41-58	6540.10		6539.43
		2-13		1390.77	1390.63[a]
		14-31		2317.60	2317.54[a]
		35-40		671.94	675.81[a]
		41-58		2164.66	2164.51[a]
	T17	2-13; 14-31; 35-58	6543.89 [Na^+ adduct]		6521.41
		2-13		1391.77	1390.63[a]
		14-31		2321.01	2317.54[a]
		35-58		2825.40	2821.31[a]
	T18	2-13; 14-31; 35-58	6532.03		6539.43
		2-13		1392.66	1390.63[a]
		14-31		2319.60	2317.54[a]
		35-58		Not found	2821.31[a]
Endo-Asp N:	D4	59-65	915.81		915.97
	D7	1-4	570.38		570.66
	D9	66-74	1101.01		1101.31
			1123 (Na^+ adduct)		
	D17	5-16; 17-58	6498.4		6494.42
		5-16		1436.30	1434.69[a]
		17-58		5068.57	5066.68[a]
	D20	5-58	6476.96		6476.33
Cyanogen bromide:	CNBr4	58-74	2153.93		2154.45
	CNBr5	58-74	2139.74		2154.45
	CNBr6	58-74	2181.91		2154.45
	CNBr7	58-74	2209.96		2154.45

[a] Mass of reduced fragment is calculated as $[M+H]^+ + n(H)^+$, where n=number of Cys. (Reduction by DTT adds H to S of each Cys).

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chains, whereas T17 and T18 had only 3 chains (Table 5) – apparently, trypsin did not cleave at R^{40} in the latter 2 fragments. Interpretation of the sequence information was facilitated by knowledge of the first 40 amino acid residues and mass spectrometric analyses of fragments in both, the non-reduced and the reduced form (Table 7). Figure 52 shows the mass analyses of tryptic fragment T15 before and after reduction of the disulfide bonds.

3.3.3.2. Jala MIH: endo-Asp N digest

Further sequence information and confirmation resulted from the overlapping fragments following digestion of native MIH with endo-Asp N proteinase. Five major peaks were separated from this digest mixture (Fig. 51B) for mass analyses and Edman degradation. The resulting data indicated that fragments D4, D7 and D9 were single peptide chains, whereas D17 consisted of 2 chains (Table 7, Fig. 50). Fragment D20 was sequenced as a single peptide chain from residue 5 to 55, presumably extending up to residue R^{58}, judging from the known cleavage specificity of the proteinase, as well as the mass of this fragment (Table 7, Fig. 50).

The interpretation of the sequencing data at hand, led to the identification of D9 as the carboxyl-terminal peptide fragment of *Jala* MIH and residues D^{66}-K^{73} were sequenced. The calculated protonated mass for this sequence, viz. 1030 Da, did not match with the actual mass observed from the fragment D9, viz. 1101 Da. Thus, the sequence was deemed incomplete. The mass difference of 71 indicated that an Ala residue might have remained unsequenced.

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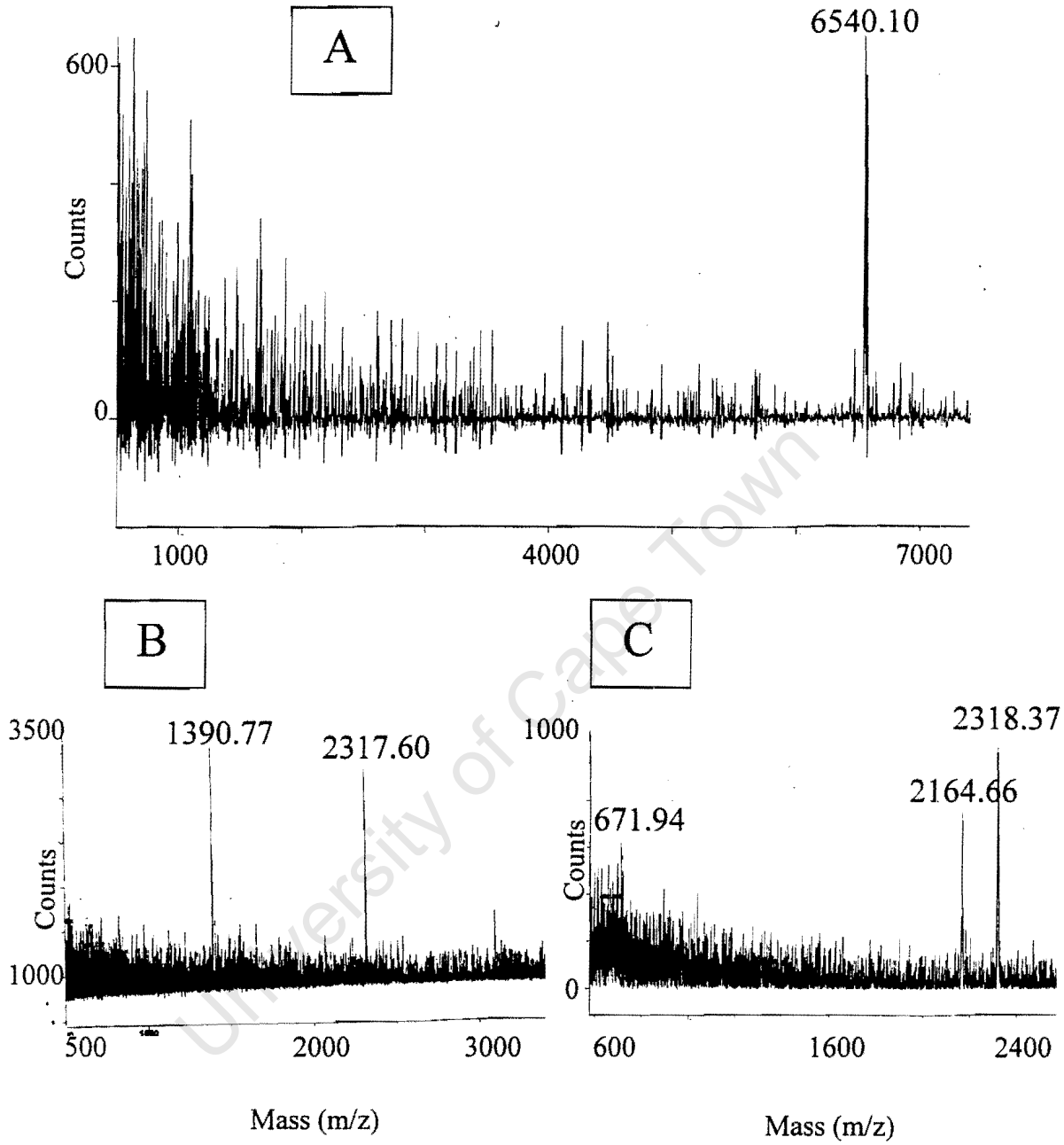

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Fig. 52. MALDI-TOF mass spectra of tryptic fragment T15 of *Jala* MIH **A.** before and, **B, C** after reduction of the disulfide bonds.

3.3.3.3. Jala MIH: CNBr cleavage and amidation

To confirm our assignment of the short fragments at the C-terminal region of MIH and to elucidate the full sequence of *Jala* MIH, native MIH material was chemically cleaved with CNBr and the resulting mixture purified on HPLC. This cleavage particularly took advantage of the M^{57}. Four peaks (Fig. 51C) were selected for N-terminal sequencing; the molecular mass of these fragments indicated that they could be the predicted C-terminal fragment starting with residue R^{58}, although not all the peaks displayed the calculated mass for such a fragment (Table 7). All 4 peaks yielded the identical amino acid sequence and thereby revealed the identity of the last amino acid residue of *Jala* MIH unequivocally as A^{74} (Fig. 50). This was confirmed by the construction of a synthetic nonapeptide: DIWRSILKA-NH_2; the characteristics of the synthetic peptide were compared with that of fragment D9 (see 3.3.3.2. above). Firstly, the native fragment D9 co-eluted with the synthetic nonapeptide when co-injected on RP-HPLC (Fig. 53); secondly, the 2 peptides had the identical $[M+H]^+$ of 1101 on MALDI-TOF (Fig. 54). The protonated mass of the whole MIH molecule ($[M+H]^+ = 9007$) agrees with the value calculated for an amidated structure and this was further substantiated by methylation experiments performed with the native C-terminal fragment D9 and the synthetic nonapeptide DIWRSILKA-NH_2. With both peptides, the $[M+H]^+$ ion of the fragment shifted from 1101 to 1115 (Fig. 54B), corresponding to the methyl ester formed by the esterification of the only acidic residue in the fragment, viz. D^{66}. It was, thus, concluded that *Jala* MIH has an amidated C-terminus.

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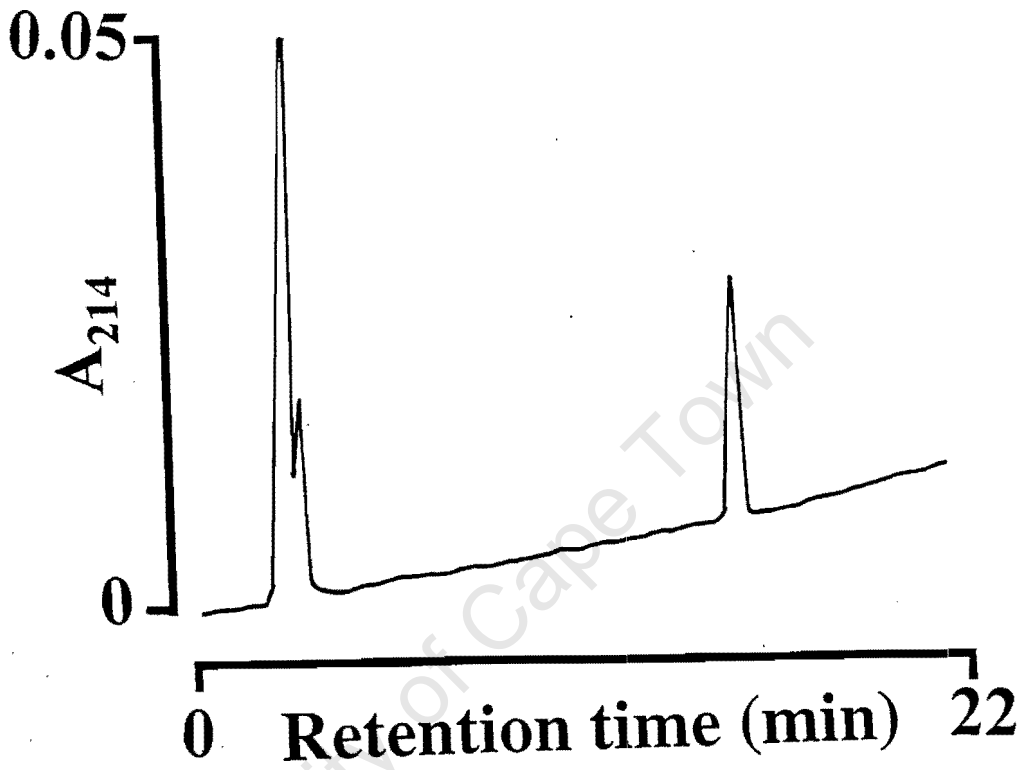

Fig. 53. Co-elution of fragment D9 of *Jala* MIH with the synthetic nonapeptide, DIWRSILKA-NH_2, on RP-HPLC. The same column, HPLC and solvent system system was used as described in 2.2. A gradient of 35-75 % B in 30 min was applied to the system.

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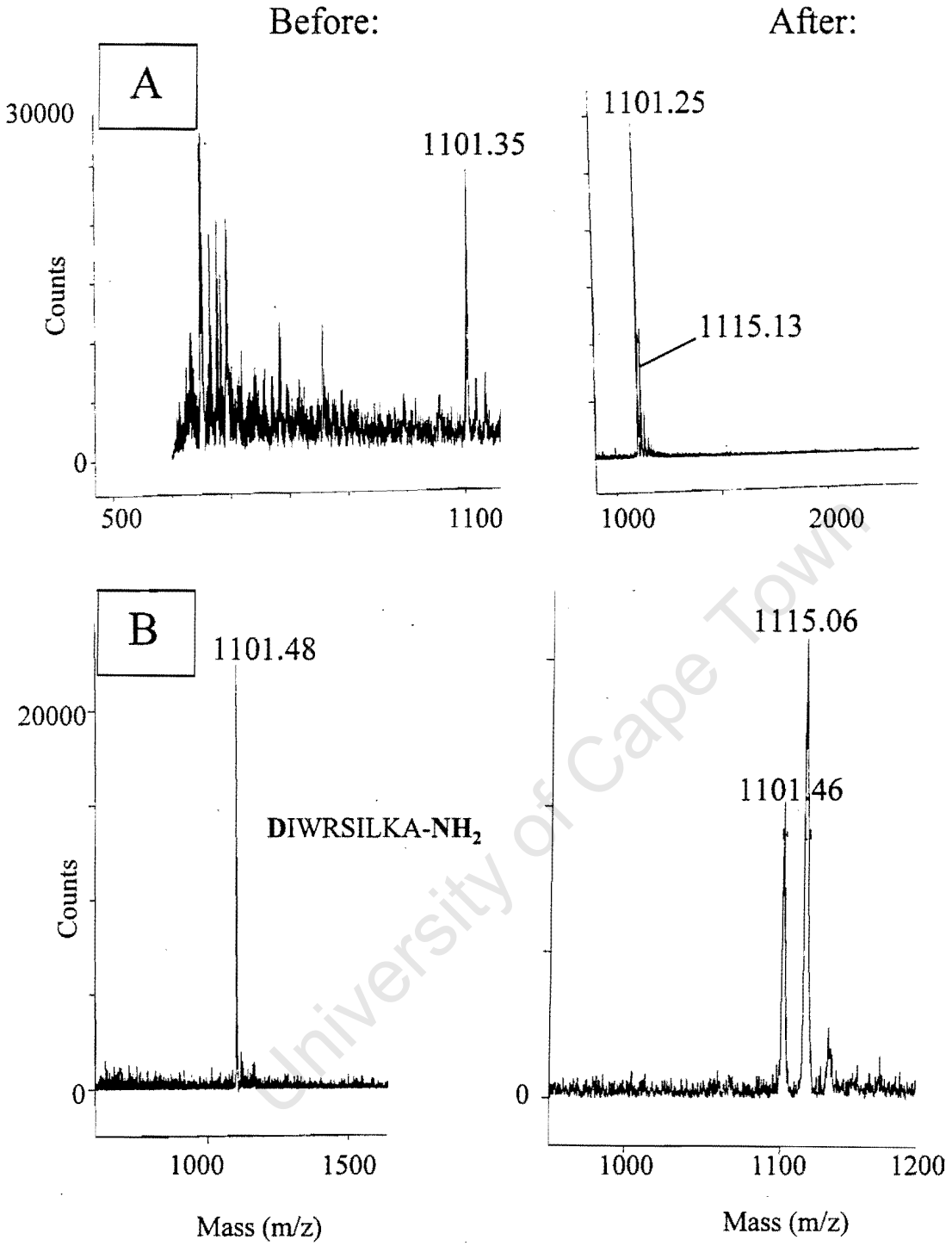

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Fig. 54. Methylation of **A.** the C-terminal fragment of *Jala* MIH, i.e. D9, and **B.** the synthetic nonapeptide, DIWRSILKA-NH_2, as monitored by MALDI-TOF MS.

3.3.4. Truncated Jala cHHs

The mass data obtained from peaks 1 and 2 (Fig. 24), did not fit into the accepted mass range of peptides belonging to the cHH/MIH/VIH family, viz. 8000-9000+ Da, yet the peaks contained cHH epitopes (as illustrated in ELISA, Fig. 25) but no hyperglycaemic activity (Fig. 26). N-terminal sequencing of peak 1 and peak 2 yielded sequence information of residues 1-62 (peak 1) and residues 1-60 (peak 2). The primary structure of peak 1 was 100 % identical to the first 62 residues of *Jala* cHH-I and, that of peak 2 was 100 % identical to the first 60 residues of *Jala* cHH-II (Fig. 55). Peak 1 and peak 2 are therefore, referred to as being truncated forms of *Jala* cHH-I and –II, respectively. Truncated cHH-I was chemically cleaved with CNBr and the mixture was purified on HPLC (Fig. 56). N-terminal sequencing of the resulting CNBr peak 1 yielded residues L^{58}-Y^{65} (Fig. 55A). The theoretical mass $[M+H]^+$ of this CNBr fragment was calculated to be 952 Da; CNBr peak 1 displayed a protonated mass of 976 Da (Fig. 56), which corresponds to 952 Da + Na^+. CNBr-derived peak 2 displayed a mass that correlates to the N-terminal fragment (Fig.56). The theoretical mass calculated for a truncated *Jala* cHH-I peptide, i.e. A^1- Y^{65}, is 7665 Da. This fits well to the $[M+H]^+$ value of 7666.41 Da obtained by MALDI-TOF (see Fig. 57). Theoretical calculations of the mass of a *Jala* cHH-II peptide that is truncated at residue Y^{65}, matched exactly the observed mass, viz. 7613 Da (Fig. 57). The question of C-terminal amidation was addressed by carrying out methylation experiments on the CNBr-derived peak 1 of truncated cHH-I (Fig. 58). Before methylation, CNBr peak 1 yielded $[M+H]^+$ = 951 Da (Fig.58A); this shifted to 1007 Da after methylation (Fig. 58B). This means an increase of 56 m.u., which corresponds to the formation of 4 methyl esters. Since this fragment contains only 3

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A

1 10 20 30 40

AVFDQSCKGVYDRSLFSKLDRVCDDCYNLYRKHYVATGCRRNCYGN

50 60 65

LVFRQCLDDLMLVDVVDEY

CNBr1

Fig. 55. Sequence eludication of **A.** truncated cHH-I and **B.** truncated cHH-II. The dotted line represents residues identified by N-terminal sequencing. CNBr, cyanogen bromide-cleaved products.

B

1 10 20 30 40

AVFDQSCKGVYDRSLFKKLDVVCDDCYNLYRKPYVATGCRENCYSN

50 60

LVFRQCLDDLMLVDVVDEY

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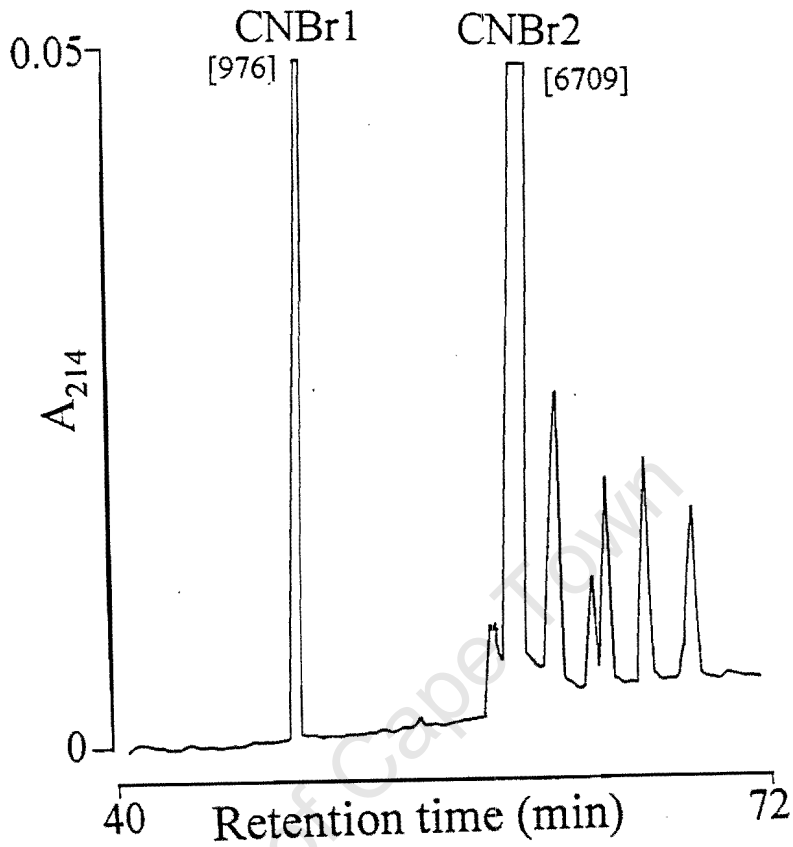

Fig. 56. Peptide map of truncated *Jala* cHH-I after chemical cleavage with CNBr. The same column, HPLC system and solvents were used as in Fig. 24. A linear gradient from 0-99 % B was developed in 90 min after an isocratic step at 0 % B for 5 min. Peptide fragment 1 was selected for sequencing analysis on the basis of its mass (mass data are indicated as $[M+H]^+$).

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free carboxyl groups on residues D^{60}, D^{63} and E^{64}, it can be concluded that Y^{65} is not amidated and provided a fourth free carboxyl group for esterification.

3.3.4.1. Origin of the truncation of Jala cHHs

To determine whether these truncated cHH peptides were induced by the procedure of tissue extraction and purification, freshly-dissected sinus glands were homogenised in different media (see 2.5.6. for details) and, after HPLC analyses of the resulting extracts, the peak area of the truncated peptides were compared with that of the intact cHHs as a means of quantitation. The results, summarised in Table 8 and with chromatographic examples in Fig. 59, show that certain homogenisation buffers can limit the ratio of the truncated cHHs and favour the "preservation" of the intact cHHs. Such buffers in which the intact cHHs predominate, appear to be those containing PMSF (an inhibitor of serine proteases) and thiodiglycol (an anti-oxidant) (Table 8). EDTA resulted in such overall low yields of peptide, that further quantification was not possible. The presence of trypsin-inhibitor also did not prevent a quantitative shift from intact cHHs to truncated cHHs (Table 8). Clearly, also, our preferred homogenisation buffer, i.e. 10 % acetic acid without additives, is not the best medium to limit the breakdown of cHHs; *Jala* cHH-II seems to be more vulnerable to truncation under these conditions than *Jala* cHH-I (Table 8).

3.3.5. "8400 Da" Jala peak

Peak 4 (Fig. 37) was not typically present in HPLC separation of *Jala* SGs, yet it appeared on several chromatographic traces. The presence of this peak cannot be attributed to any obvious deviations from the standard protocol followed in preparing

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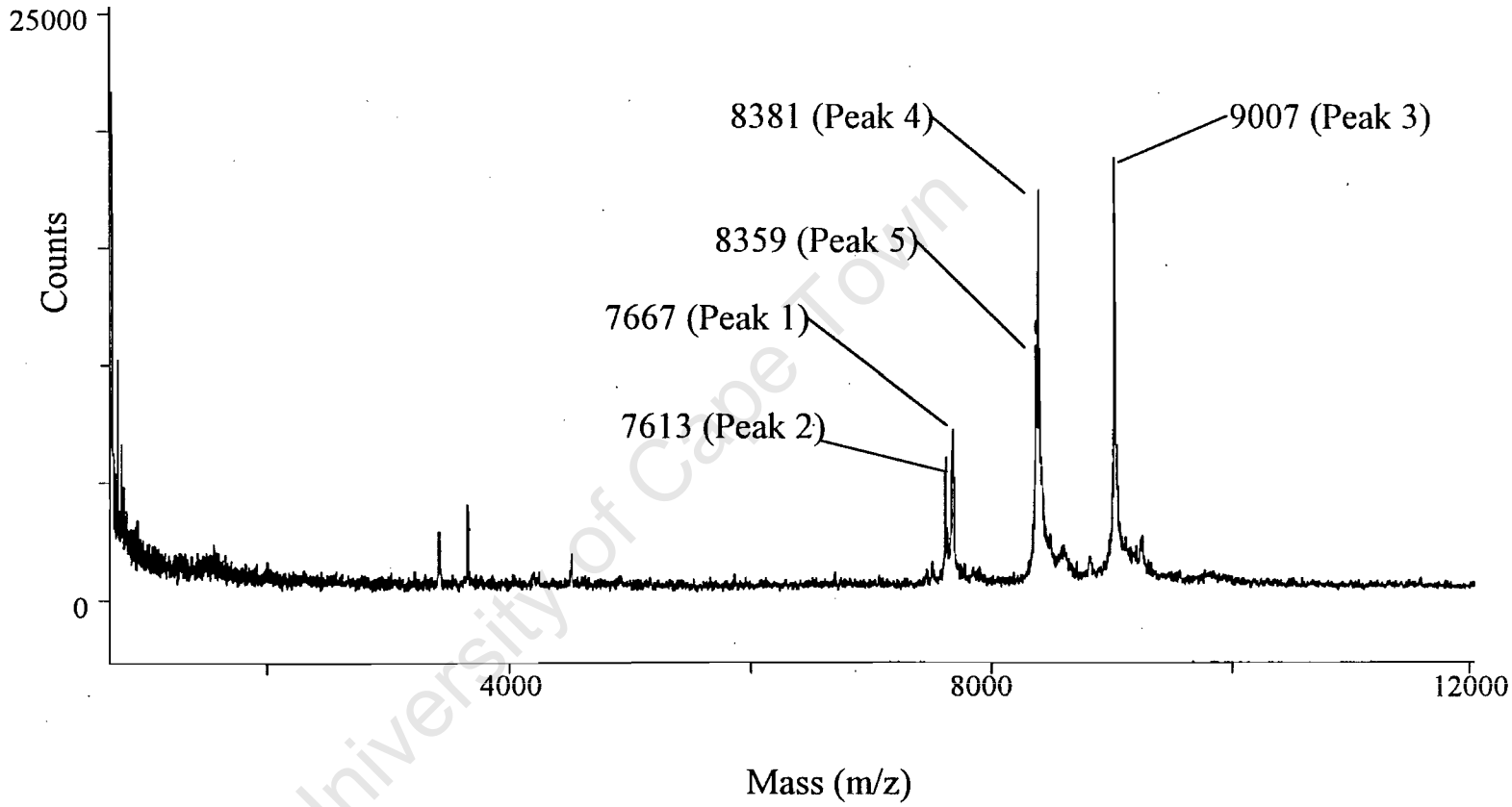

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Fig. 57. MALDI-TOF spectrum of a crude extract of sinus glands from *J. lalandii* (with sinapinic acid as matrix) before purification by RP-HPLC. The masses correspond to specific HPLC peak fractions, indicated in brackets (see Fig. 24 for elution profile).

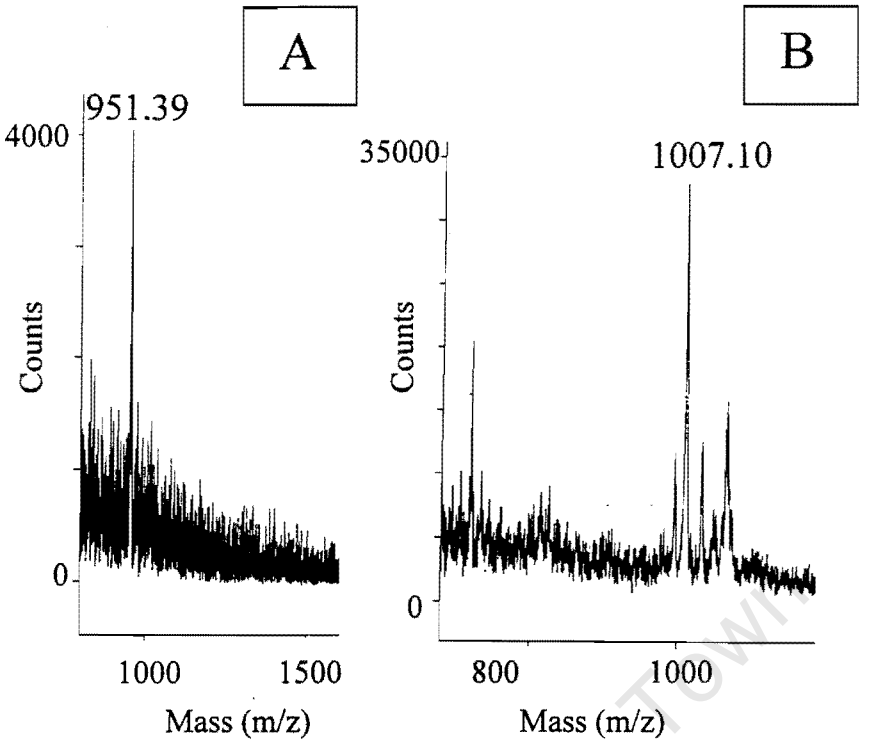

Fig. 58. Mass spectra of the C-terminal fragment of truncated *Jala* cHH-I, ie. Fragment CNBr1: **A.** before and, **B.** after methylation.

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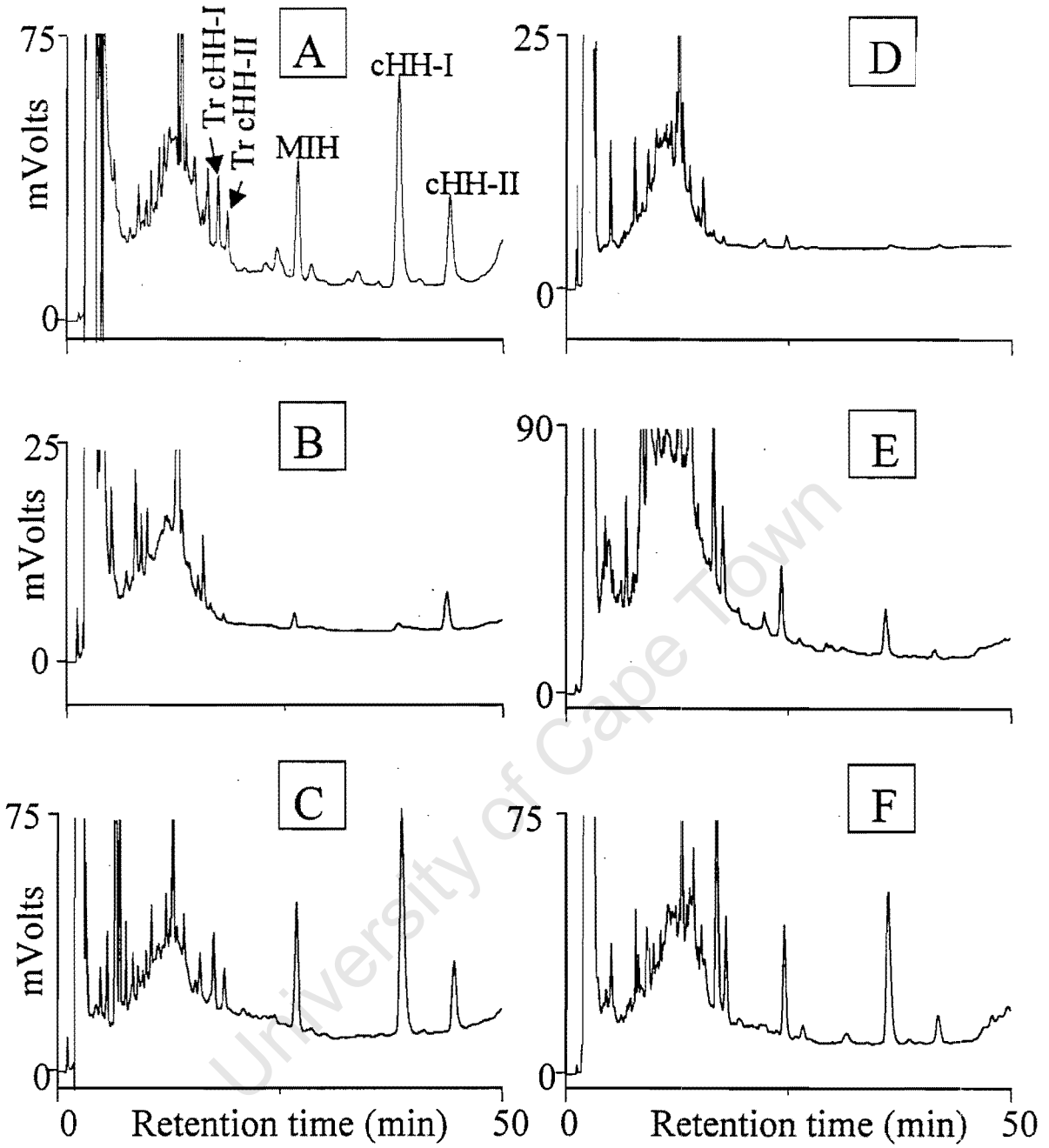

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Fig. 59. Chromatographic comparisons of peptide yields after extraction from *J. lalandii* sinus glands in different homogenisation solutions: **A.** PMSF in acetic acid, **B.** PMSF in water, **C.** thiodiglycol, **D.** EDTA, **E.** trypsin inhibitor and, **F.** acetic acid.

Table 8. Peak areas of truncated cHH peptides and non-truncated cHHs obtained from HPLC separations of crude extracts of sinus glands from *J. lalandii*

Homogenising buffer	Trunc. cHH-II:Trunc. cHH-I	cHH-I:Trunc. cHH-I	cHH-II:Trunc. cHH-II	cHH-II:cHH-I
PMSF in acid (n=4)	2.27 ± 0.58	0.23 ± 0.08	0.37 ± 0.15	3.57 ± 1.07
PMSF in water (n=2)	0.24 ± 0.02	0.13 ± 0.07	0.11 ± 0.02	0.24 ± 0.17
Thiodiglycol (n=4)	1.21 ± 0.42	0.15 ± 0.05	0.41 ± 0.04	3.18 ± 0.15
Trypsin inhibitor (n=4)	2.47 ± 0.73	2.14 ± 0.48	4.43 ± 0.85	5.05 ± 0.9
10 % acetic acid (n=4)	2.94 ± 0.39	1.72 ± 0.78	2.84 ± 0.97	4.97 ± 0.25
EDTA (n=4)	-	-	-	-

Sinus glands were prepared and extracted at 85 ° C in different buffers (see 2.5.6. for abbreviations used). The peak areas are expressed and compared as a ratio of 2 specified peaks. Values are mean ± SD, where n = 2-4 with 5 sinus glands per sample.
- Yields of peptides were too low for analyses/integration

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the SGs, nor in the extraction and purification of peptide material. Although this peak displayed biological activity in the protein synthesis assay with prawn ovaries (see Fig. 38), it cannot be termed a "vitellogenesis-inhibiting hormone" without further, more rigorous tests. In an ELISA with anti-*Hoam* cHH serum, this peak 4 displayed an immunoreaction that equaled the intensity of immunoreactions obtained with crude extracts of sinus glands and the equivalent amount of purified *Jala* cHH-I (average absorbance reading of 0.4 was recorded 20 min after substrate application). N-terminal sequencing of this peak yielded sequence information up to residue 52. This sequence (results not shown) fits exactly to the first 52 residues of *Jala* cHH-I. Peak fraction material (120 SGE) was digested with trypsin and purified on HPLC. The resulting fragments were subjected to MALDI-TOF MS analyses. Fig. 60 compares a tryptic map of *Jala* cHH-I (Fig. 60C) with that of the 8400 Da peak 4 (Fig. 60A). The tryptic fragment containing the C-terminal peptide of *Jala* cHH-I displayed a protonated mass of 4648.39 Da (Fig. 60C). On the shallow gradient employed for this separation, a tryptic peak originating from the 8400 Da peptide had approximately the same retention time but displayed a mass of 4665.77 Da (Fig. 60A); this is thought to be the equivalent C-terminal fragment of the 8400 Da peak 4.

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3.4. Characterisation and sequence elucidation of less-hydrophobic Jala peptides

3.4.1. Purification by RP-HPLC

Early-eluting material were collected as 2 ml fractions from HPLC runs in which cHH/MIH/VIH peptide peaks were purified from *Jala* SG extracts (see for example

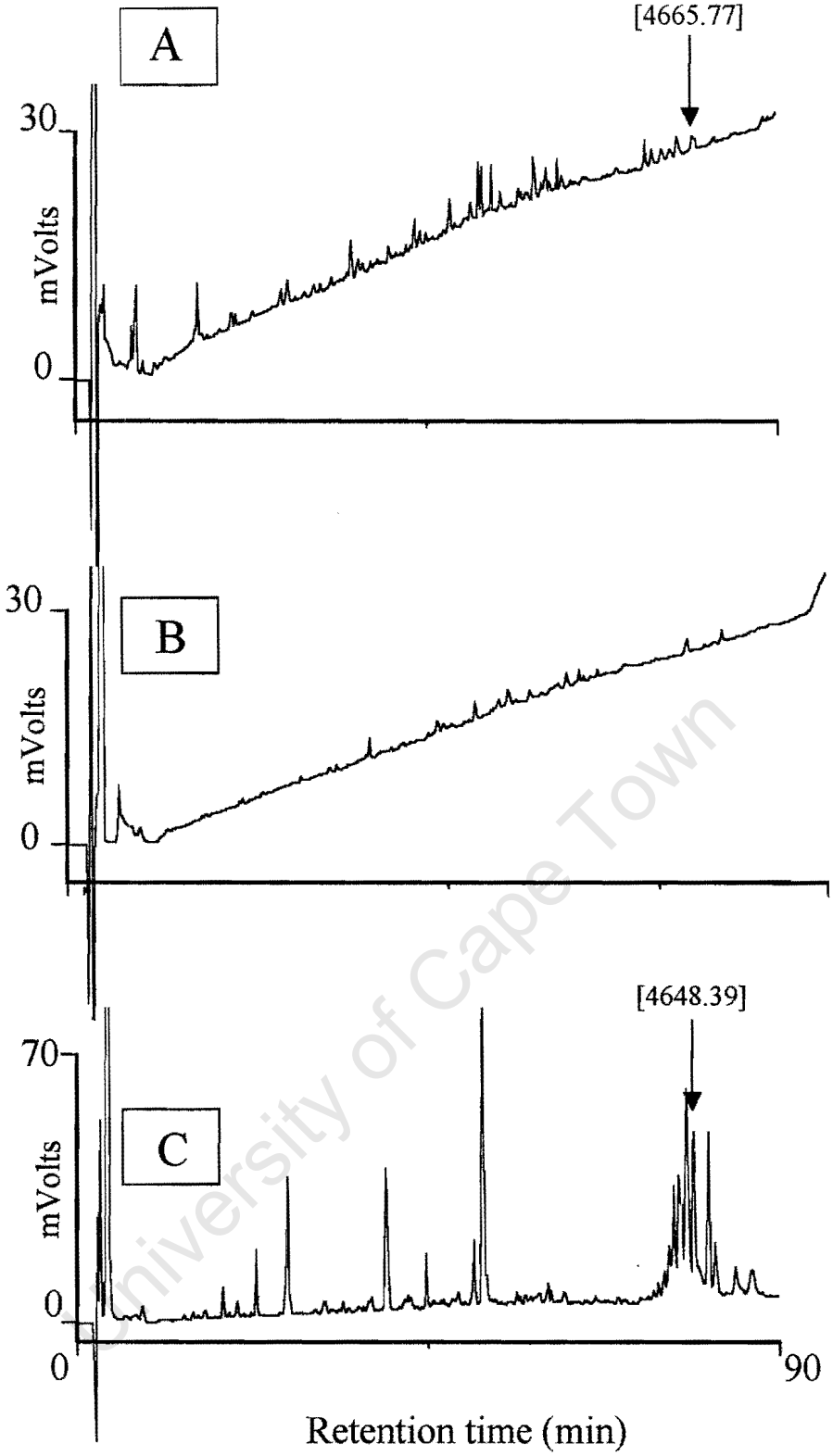

Fig. 60. Tryptic maps of **A.** Peak 4 (of chromatogram in Fig. 37) and, **C.** *Jala* cHH-I. **B.** Separation of reduction buffer alone. The same gradient was used as in Fig. 42. $[M+H]^+$ values of the fragments containing the C-terminal ends are indicated for Peak 4 (**A**) and cHH-I (**B**).

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Fig. 24). These early-eluting fractions were later pooled and separated by employing different HPLC gradients and ion-pairing reagents. Figure 61 shows the result of a typical first separation of the pooled, less-hydrophobic fractions. Mass spectrometric analyses on the resulting peaks revealed that the fractions were impure (results not shown). The fractions were roughly grouped into 5 areas on the basis of retention time (Fig. 61). Similar areas from other HPLC runs were pooled and dried for further purification (see below).

3.4.2. Purification, characterisation and sequence elucidation of RPCH

Figure 62 illustrates the further purification of peptides in area 1 of Fig. 61 by using a different system of HPLC solvents. The four peaks were analysed by MS (see Fig. 62A for data). Peak fraction 1 had a mass identical to that of synthetic RPCH ($[M+Na]^+ = 953$) and co-eluted with synthetic RPCH on RP-HPLC (Fig. 62B). This peak fraction was also shown to have biological activity as an RPCH: injection of 1 SGE into dark-adapted shrimps (*P. pacificus*) resulted in the concentrating of pigment in the integumental erythrophores (Fig. 63). The injection of sea water did not alter the state of the chromatophores (results not shown); peak fraction 1 of Fig. 62A is considered to be the red pigment-concentrating hormone in *J. lalandii* and is, therefore called *Jala* RPCH.

Since all known RPCHs are N-terminally blocked by a pyroglutamate residue (pGlu), *Jala* RPCH was first cleaved with a pyroglutamate aminopeptidase before N-terminal sequence analyses. Figure 64 shows the progression of the deblocking as monitored over time on HPLC. The deblocked peptide yielded the following amino acid sequence: LNFSPGW

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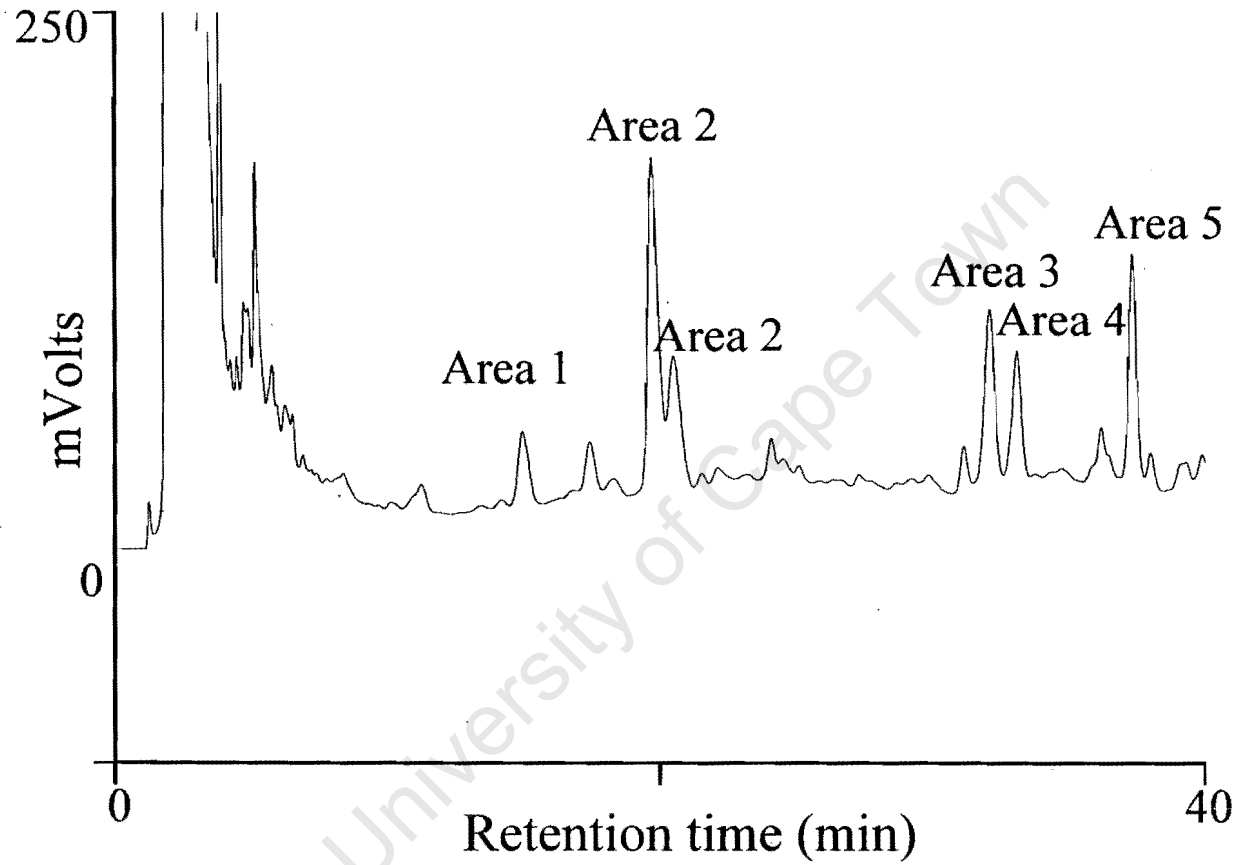

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Fig. 61. Re-purification on RP-HPLC of a batch of early-eluting material (300 SGE) from extracts of *J. lalandii* sinus glands. Less-hydrophobic fractions, originating from first HPLC separations such as that shown in Fig. 24, were pooled for re-purification, using the same column as in Fig. 24. A linear gradient from 25-40 % B was developed in 50 min after a 4 min isocratic step at 25 % B. Solvent A = 0.115 % TFA in water, solvent B = 100 % acetonitrile with 0.1 % TFA, UV detection at 214 nm and a flow rate of 1 ml/min. The peak fractions resulting from this separation were collected as distinct areas. Similar areas from the re-purification of other batches were pooled for MS analyses and further steps of purification.

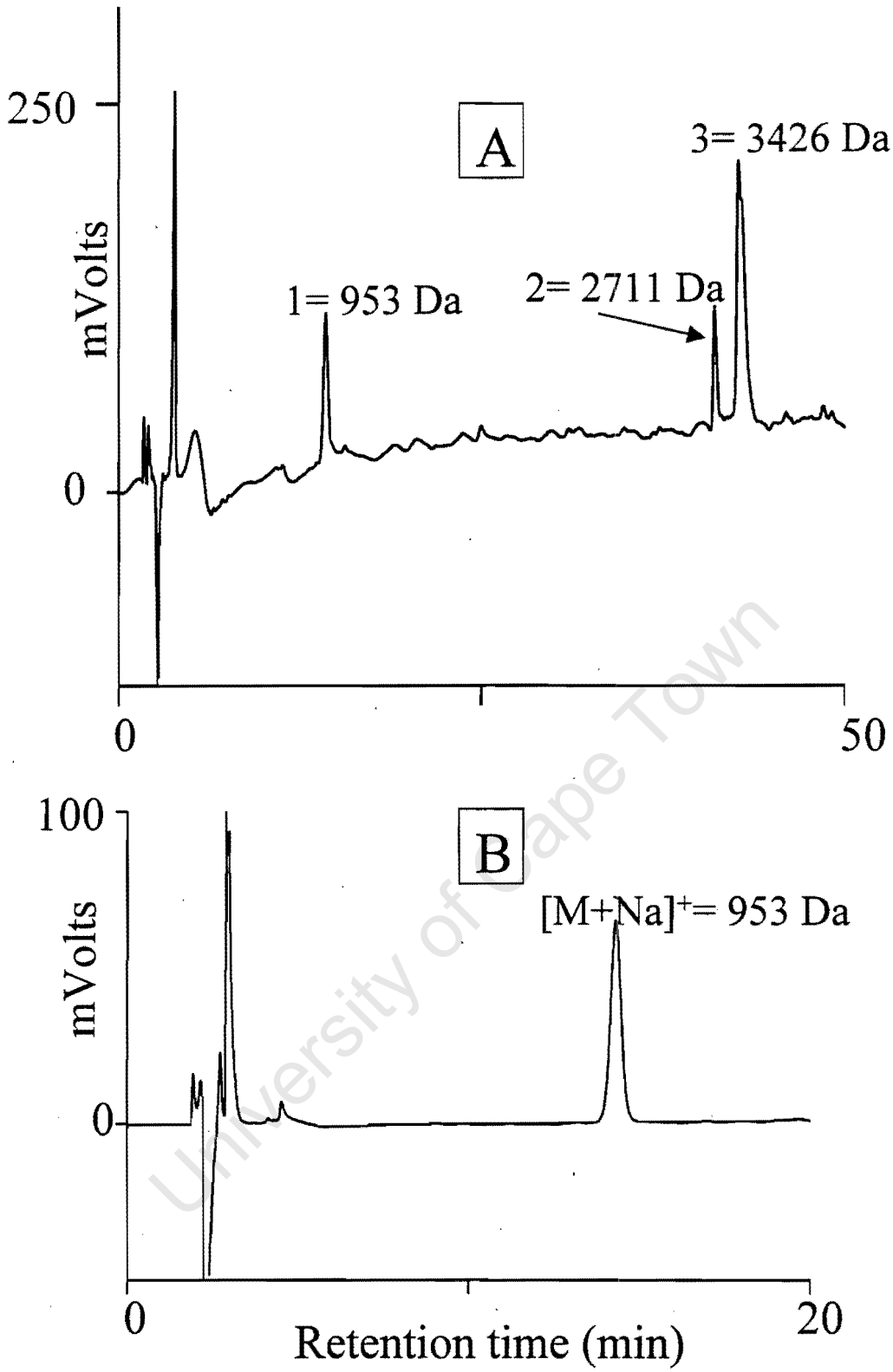

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Fig. 62. **A.** Separation of red pigment-concentrating hormone (RPCH) from other peptides of *J. lalandii*. Pooled fractions of Area 1 (see Fig. 61) were applied to a Nucleosil C18 column and subjected to a linear gradient of 30-60 % B in 100 min, where solvent A = 0.13 % HFBA in water and solvent B = 0.13 % HFBA in acetonitrile. The resulting peptides are labeled in the order of elution and aliquots were analysed by MS with cinnamic acid as matrix. Mass data are indicated. **B.** Co-elution of peak fraction 1 (see **A.** above) with synthetic RPCH. The same column, HPLC, solvents and gradient was used as in **A.** above.

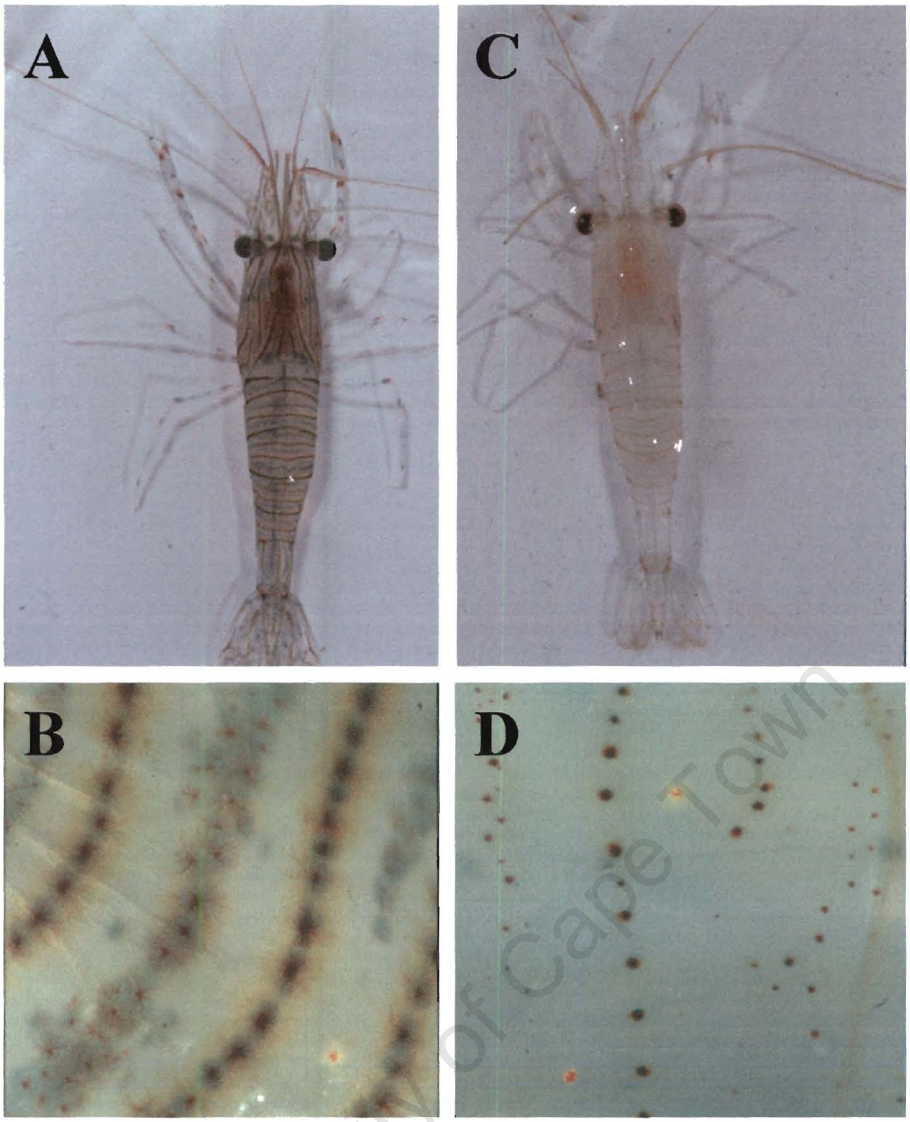

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Fig. 63. Biological identification of RPCH from sinus glands of *J. lalandii*. **A, B** Dark-adapted *Paleamon pacificus* before injection of peak fraction 1 (see Fig. 62A for chromatographic profile). **C, D** *P. pacificus* 5-10 min after injection of 1 SGE of peak fraction 1. **B** and **D** is a close-up of the shrimp showing the distribution of pigment in the dispersed and concentrated states, respectively.

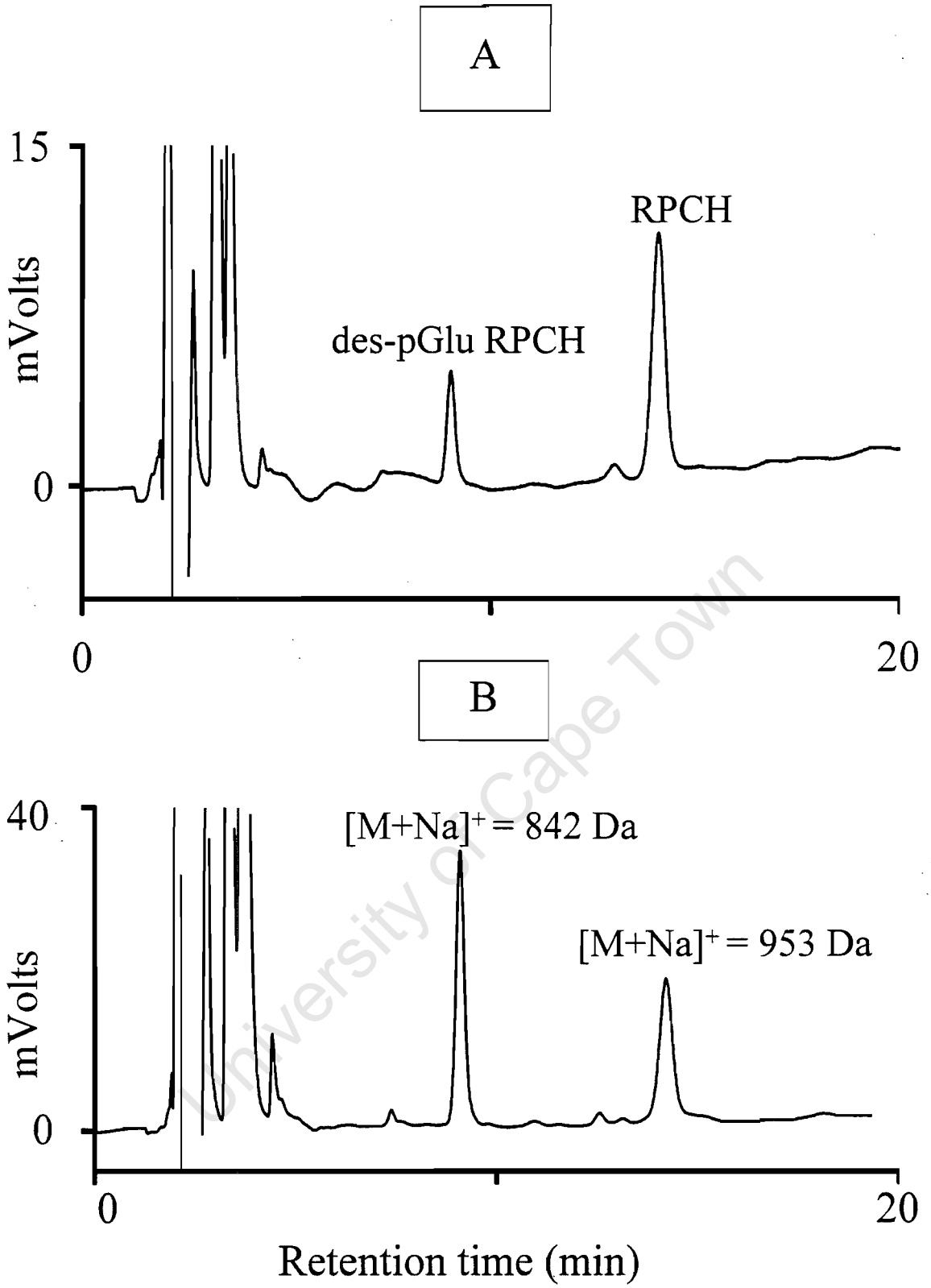

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Fig. 64. Cleavage of *Jala* RPCH with pyroglutamate (pGlu) amino peptidase. Aliquots of the mixture were removed at different time intervals of the digestion and applied to HPLC under the following gradient: 6 min isocratic step at 25 % B, followed by a linear increase to 40 % B in 50 min. Solvent A= 0.115 % TFA in water; solvent B= 0.1 % TFA in 100 % acetonitrile. **A.** 45 min and, **B.** 135 min after incubation of *Jala* RPCH with pGlu amino peptidase. Mass data are indicated in **B**.

and a mass of $[M+H]^+$ = 820.24, as well as $[M+Na]^+$ = 842.13. The difference in mass between the deblocked and the blocked peptide = 111 corresponds to the mass of a pGlu residue. To determine if *Jala* RPCH is amidated, methylation was performed. Figure 65 shows that there was no shift in the mass of the peptide, which indicates that *Jala* RPCH is C-terminally blocked.

3.4.3. Purification of CPRPs

3.4.3.1. CPRP-1 and -2

Interest area 2 consisted of a poorly-resolved doublet (see Fig. 61). Attempts were made to separate and resolve these 2 peaks by HPLC with 0.13 % HFBA instead of TFA added to the solvents. However, the 2 peaks were not separated at all and eluted as one, lumpy peak (Fig. 66A). This was then subjected to a last purification step with 0.05 % TFA in the solvents. Figure 66B shows that this was also insufficient to resolve the 2 fractions of the doublet. The doublet was N-terminally sequenced and yielded 2 sequences (25 residues long) that were identical to each other, except for a single substitution at position 18 (Fig. 67). From sequence homology to other cHH precursor-related peptides, these sequences were recognised as CPRPs and the peptides are referred to as *Jala* CPRP-1 and -2. Mass spectrometric data obtained from the CPRP doublet ([M+H]+ = 3406 and 3416, respectively) did not match the theoretical mass calculated for the sequenced 25 amino acid residues (3386.7 and 3396.8, respectively). The sequences were, therefore, deemed incomplete and, to elucidate the C-terminus, a digestion with trypsin was carried out. The resulting major peaks were subjected to MALDI-TOF MS, and peak T8 (Fig. 68A) was selected for Edman degradation. This resulted in information about residues 11-31

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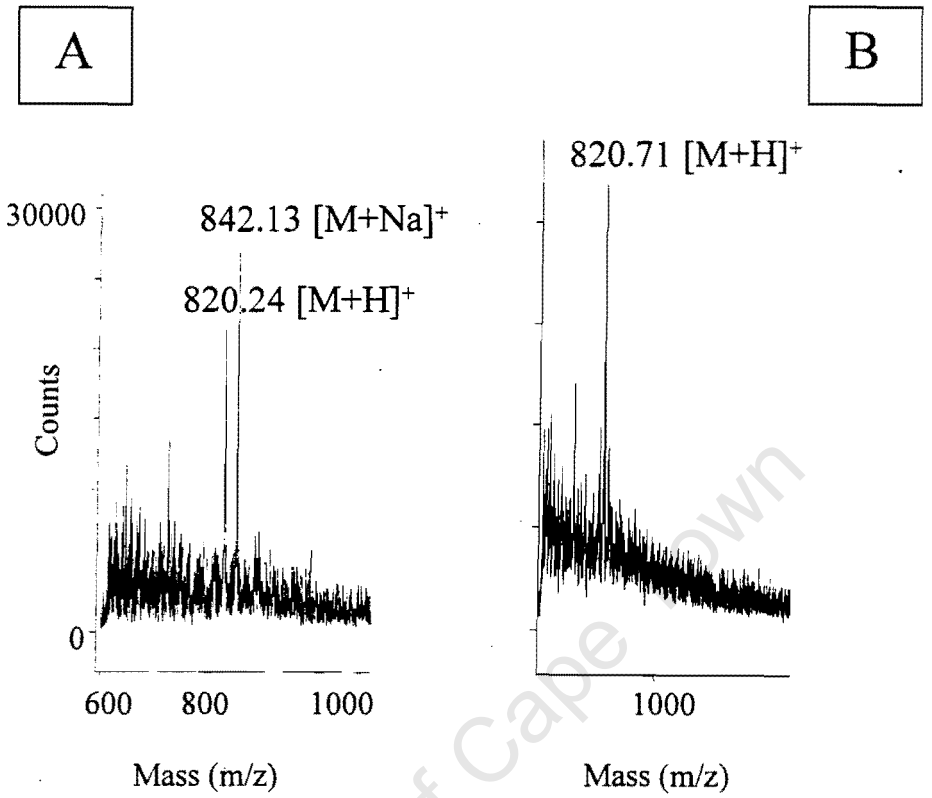

Fig. 65. Mass analysis of *Jala* RPCH: **A.** before and **B.** after methylation.

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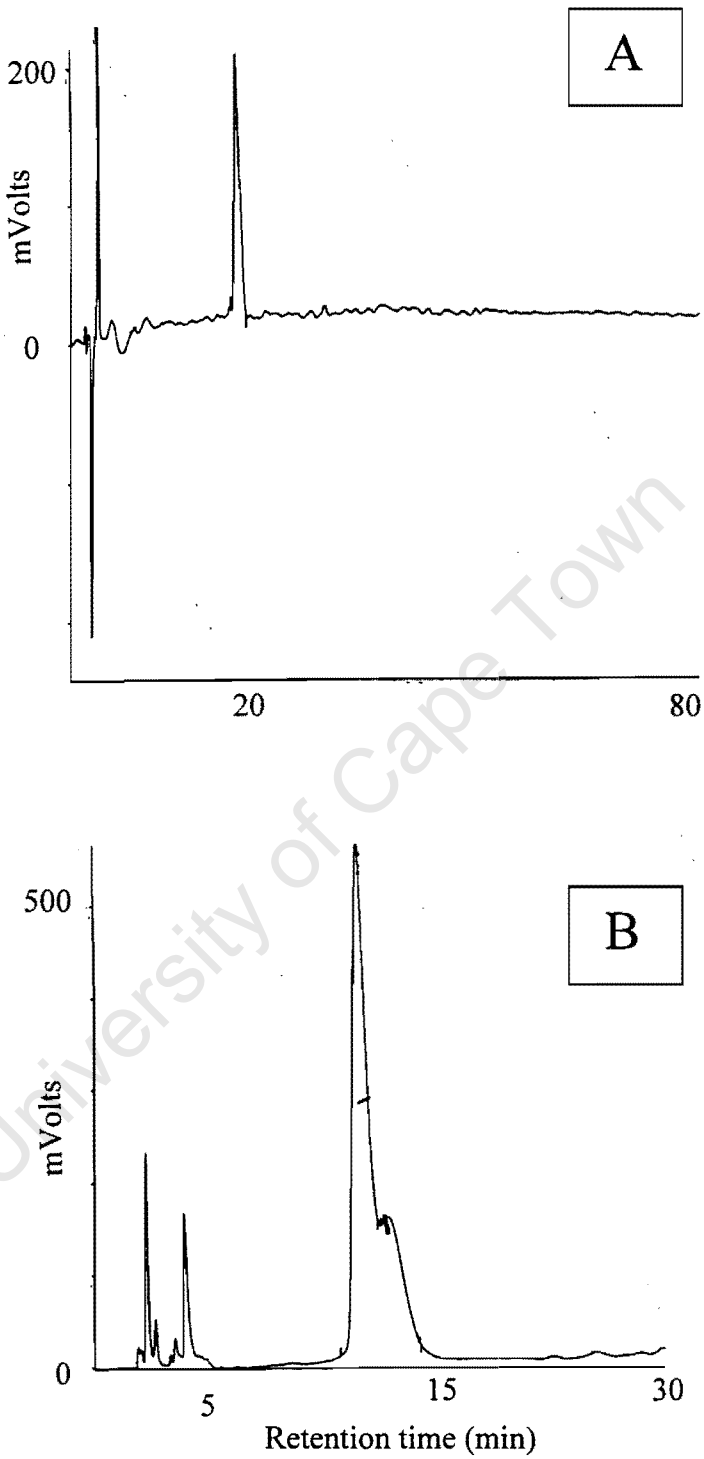

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Fig. 66. Re-purification of peak fractions from Area 2 (see Fig. 61). **A.** A second purification step with a linear gradient from 30-60 % B in 100 min was applied. Solvent A = 0.13 % HFBA in water, solvent B = 0.13 % HFBA in acetonitrile. **B.** A third purification step with a linear increase from 25-40 % B in 50 min, following a 6 min isocratic step at 25 % B. Solvent A = 0.05 % TFA in water and Solvent B = 0.05 % TFA in acetonitrile.

CPRP-1/2*:

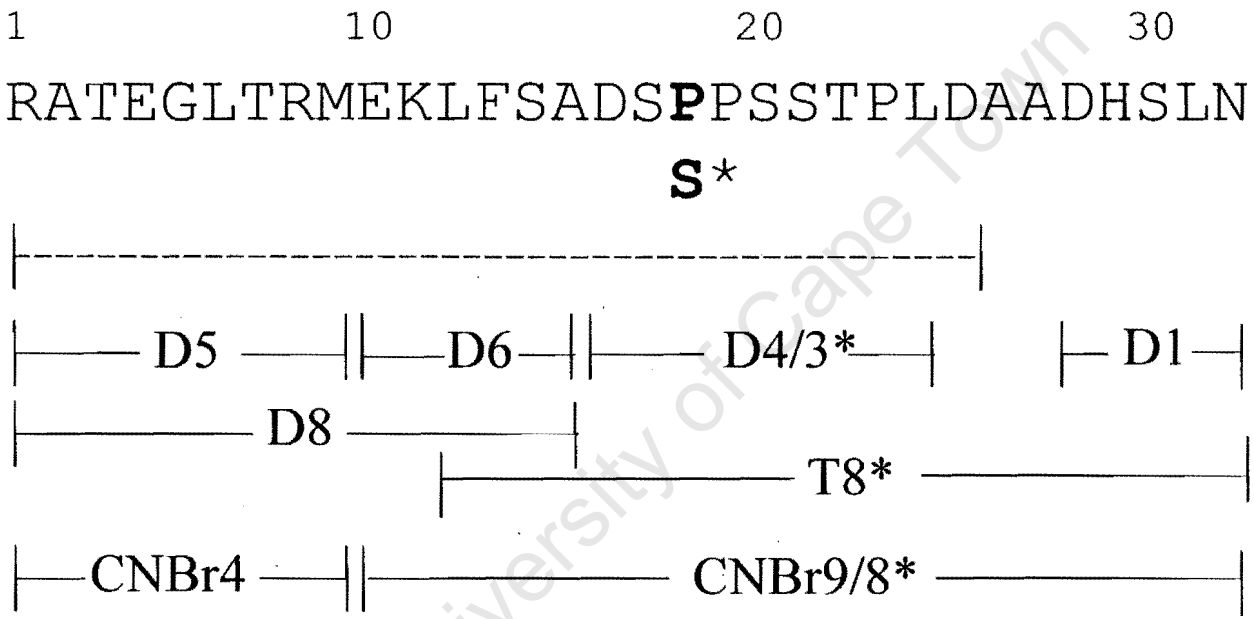

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Fig. 67. Primary structure elucidation of *Jala* CPRP-1 and CPRP-2* as deduced from sequenced peptide fragments: Tx = tryptic peptides; Dx = endoproteinase Asp-N peptides; CNBr = cyanogen bromide-cleaved products. The dotted line shows residues identified by N-terminal sequencing of the uncleaved CPRP peptides. Note that CPRP-1 and -2 differ only by 1 substitution at position 18, therefore, CPRP-2-specific fragments are indicated by *.

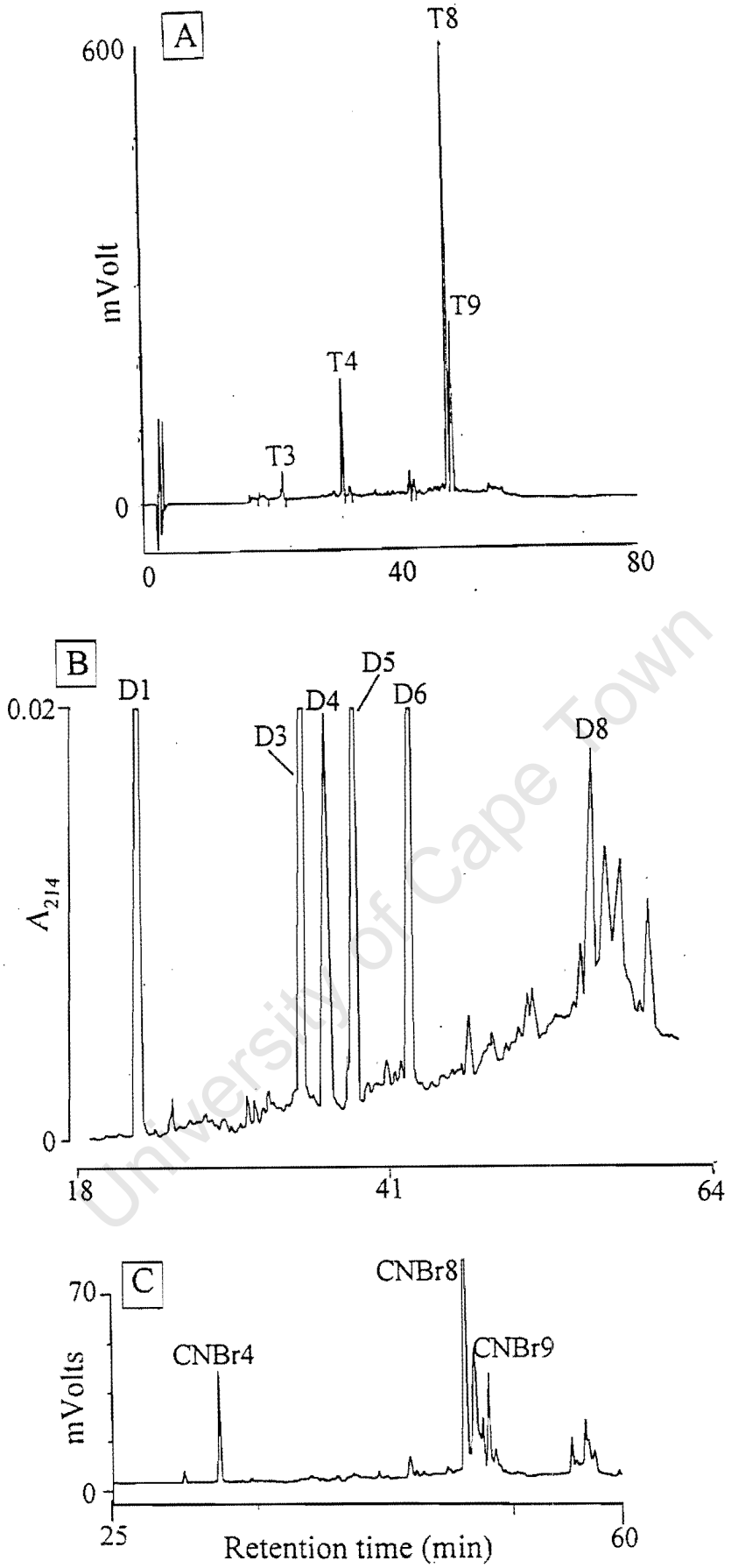

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Fig. 68. Peptide maps of a mixture of *Jala* CPRP-1 and –2 after digestion with **A.** trypsin and **B.** endo-Asp N, and cleavage with **C.** cyanogen bromide. The same column, HPLC and solvent system were used as in Fig. 24. A linear gradient from 0-99% B was developed in 90 min after an isocratic step at 0 % B for 5 min. Peptides that were selected for sequencing analysis are numbered in the order of elution. (Tx=tryptic peptide; Dx=endo-Asp N-derived peptide; CNBr=cyanogen bromide-derived product).

(Fig. 67); the calculated masses for sequences R^1-L^{31} was 116 m.u. less than the observed masses. Fraction material from the doublet was then digested with endoproteinase Asp-N and the mixture purified on HPLC (Fig. 68B). Six peak fractions were selected for N-terminal and mass spectrometric analyses (Fig. 67, Table 9). It became clear that fragment D1 was the C-terminal end of the peptides and residues 28-32 were elucidated (Fig. 67). This was confirmed by chemical cleavage with CNBr, where HPLC peak fractions 8 and 9 (Fig. 68C) proved to be the C-terminal fragments. This cleavage took advantage of residue M^9 (Fig. 67, Table 9).

3.4.3.2.CPRP-3 and -4

Interest area 5 (see Fig. 61) was subjected to RP-HPLC with HFBA as ion-pairing reagent. Four fractions were separated (Fig. 69). Peak fractions 1 and 2 were N-terminally sequenced and both yielded identical residues 1-29 (Fig. 70). From sequence homology to other cHH precursor-related peptides, these peaks were recognised as CPRPs and are referred to as *Jala* CPRP-3 and -4, since 2 other CPRPs have also been identified (see above). The calculated mass for the sequence of peak 2 (named CPRP-4) did not match the mass observed for this fraction, viz. 3646.

Peak fraction material of *Jala* CPRP-3 and -4 was then digested with trypsin and the digests were purified on RP-HPLC (Fig. 71). N-terminal sequencing of tryptic fragment T3 (Fig. 71A) confirmed that CPRP-3 consisted of residues 1-29 only (Fig. 70, Table 10). Sequence information from fragments T2 and T4 of CPRP-4 (Fig. 71B) confirmed the N-terminal residues and provided additional information about the C-terminus of CPRP-4 (Fig. 70). It appears thus, that CPRP-4 has 36 amino acid

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Table 9. Average protonated mass of peptide fragments generated by enzymatic and chemical cleavages of CPRP-1 and CPRP-2 purified from sinus glands of *J. lalandii*. Note: CPRP-1 and CPRP-2 differ only by one substitution at position 18.

Peptide		Position of residues	Observed mass $[M+H]^+$	$[M+H]^+$ calculated from sequence
Trypsin	T8	12-32 (P^{18})	2154	2141
		12-32 (S^{18})	2133.13	2130.99
Endo-Asp N	D1	28-32	not determined	585.58
	D3	16-24 (S^{18})	890.80	890.91
	D4	16-24 (P^{18})	900.20	900.95
	D5	1-9	1035.20	1035.20
	D6	10-15	not determined	694.80
	D8	1-15	1711.48	1710.99
Cyanogen bromide	4	1-9	1035.25	1035.20
	CNBr8	10-32 (S^{18})	2389.48	2389.53
	CNBr9	10-32 (P^{18})	2398.97	2399.57

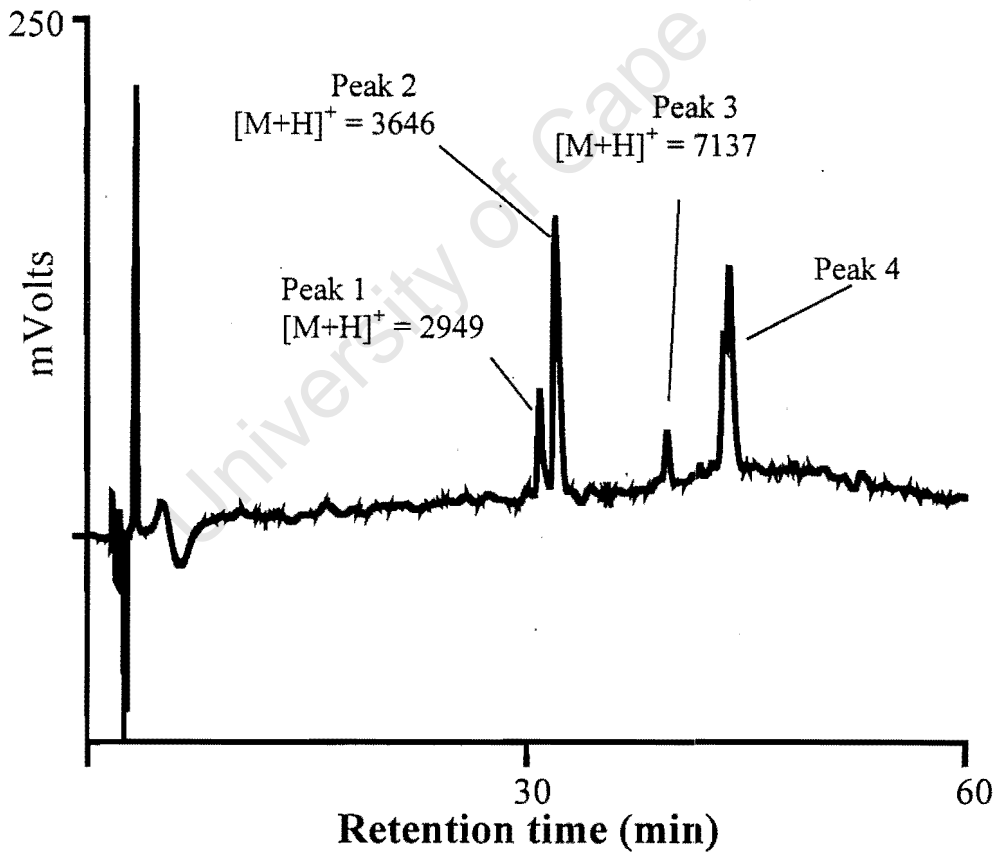

Fig. 69. RP-HPLC purification of peptides from fraction material from Interest area 5 (see Fig. 61). The resulting peaks are labeled in the order of elution and $[M+H]^+$ data are indicated in square brackets. Peak 4 contained more than one mass (not shown) and was further purified on HPLC (see Fig. 72). The HPLC solvents and gradient are the same as that in Fig. 62A.

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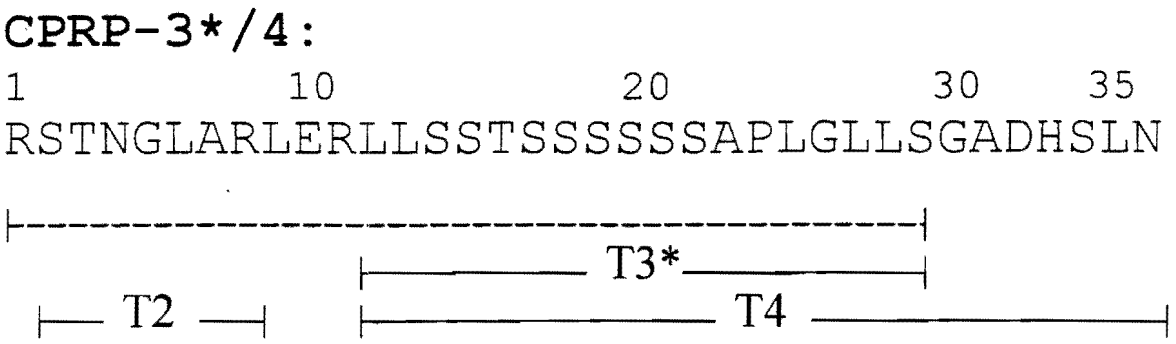

Fig. 70. Primary structure elucidation of *Jala* CPRP-3 and CPRP-4 as deduced from sequenced tryptic peptide fragments (Tx). The dotted line shows residues identified by N-terminal sequencing of the uncleaved CPRP peptides. Note: CPRP-3 appears to be a truncated version of CPRP-4 and consists only of amino acids 1-29; peptide fragments generated from CPRP-3 are marked by *.

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Table 10. Average protonated mass of peptide fragments generated by tryptic cleavages of CPRP-3 and CPRP-4 purified from sinus glands of *J. lalandii*. Note: CPRP-3 is a truncated version of CPRP-2, the former extends only to residue 29, whereas the latter ends at residue 36.

Peptide		Position of residues	Observed mass $[M+H]^+$	$[M+H]^+$ calculated from sequence
(i) CPRP-3:				
Trypsin	T3	12-29	1692	1693
(ii) CPRP-4:				
Trypsin	T2	2-8	719.57	719
	T4	12-36	2389.38	2389.57

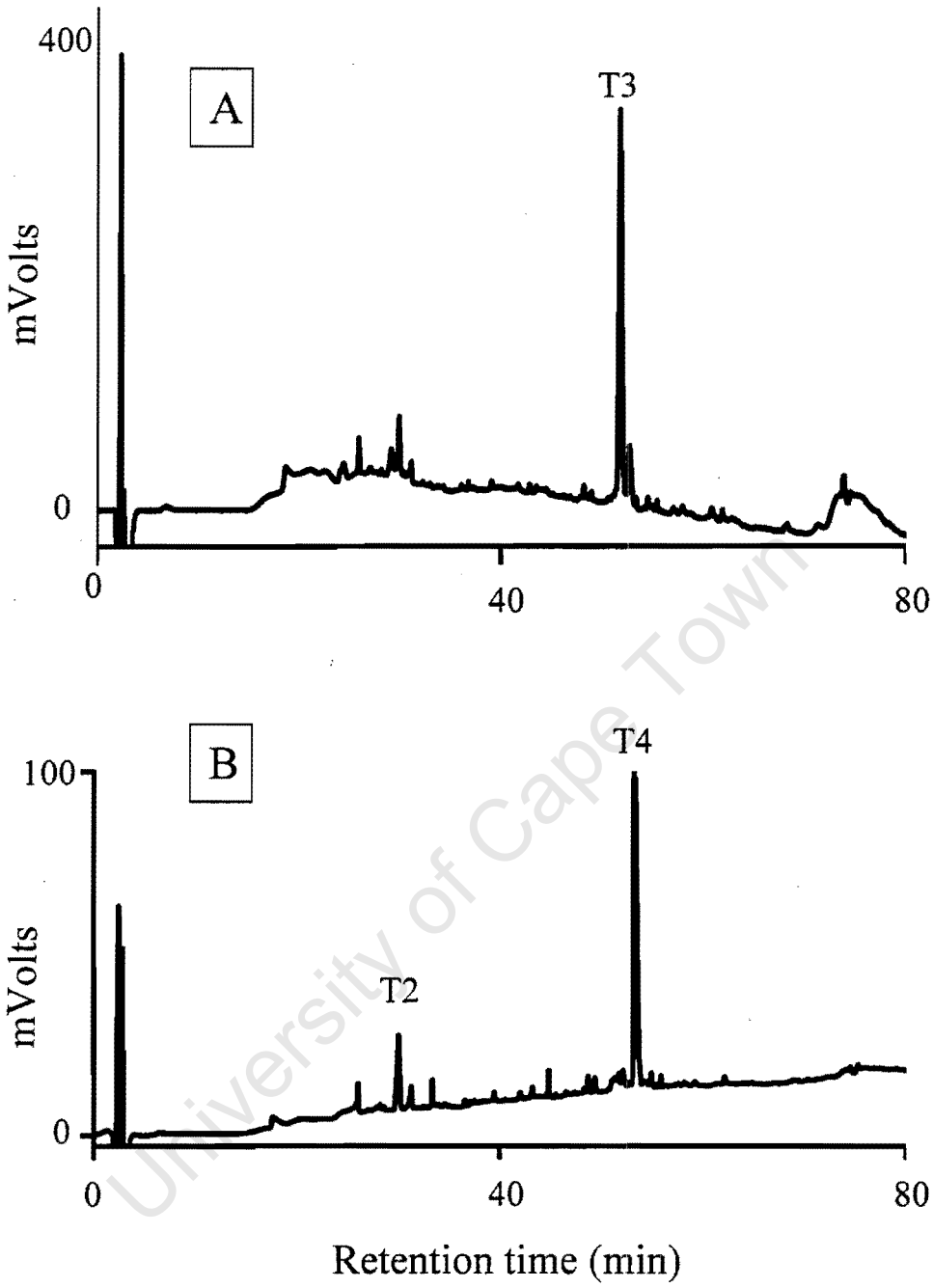

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Fig. 71. HPLC purification of **A.** *Jala* CPRP-3 and **B.** *Jala* CPRP-4, after digestion with trypsin. Peaks that were selected for further analyses are numbered in the order of elution. The solvents and gradient are the same as for Fig. 68.

residues and that CPRP-3 is a C-terminally truncated form of CPRP-4, having only 29 amino acid residues.

An attempt was made to further purify the doublet peak fraction 4 of Fig. 69 with HFBA in the HPLC solvents. Figure 72 shows that a better resolution was obtained. These peaks produced $[M+H]^+$ data of 7186 and 7484 (Fig. 72) and were not investigated further.

3.4.3.3. Attempted characterisation of the CPRPs

The purified *Jala* CPRPs were tested in ELISAs for immunoreactivity with anti-*Hoam* cHH serum. The cHH serum did not recognise any of the CPRPs (results not shown). The CPRPs were also injected into light-adapted *P. pacificus* to investigate a possible role in pigment dispersal. None of the CPRPs had a convincing PDH effect (Fig. 73).

3.4.4. Purification of other HPLC areas of interest

Interest area 3 (see Fig. 61) was further purified on HPLC with HFBA solvents. These peaks were collected; all yielded $[M+H]^+$ data of approximately 7000 Da (results not shown). These peak fractions were not investigated further.

Similarly, interest area 4 gave rise to 2 peaks after HPLC with HFBA solvents; both of these peaks yielded [M+H]+ data of approximately 7400+ Da (results not shown). These fractions were also not studied further.

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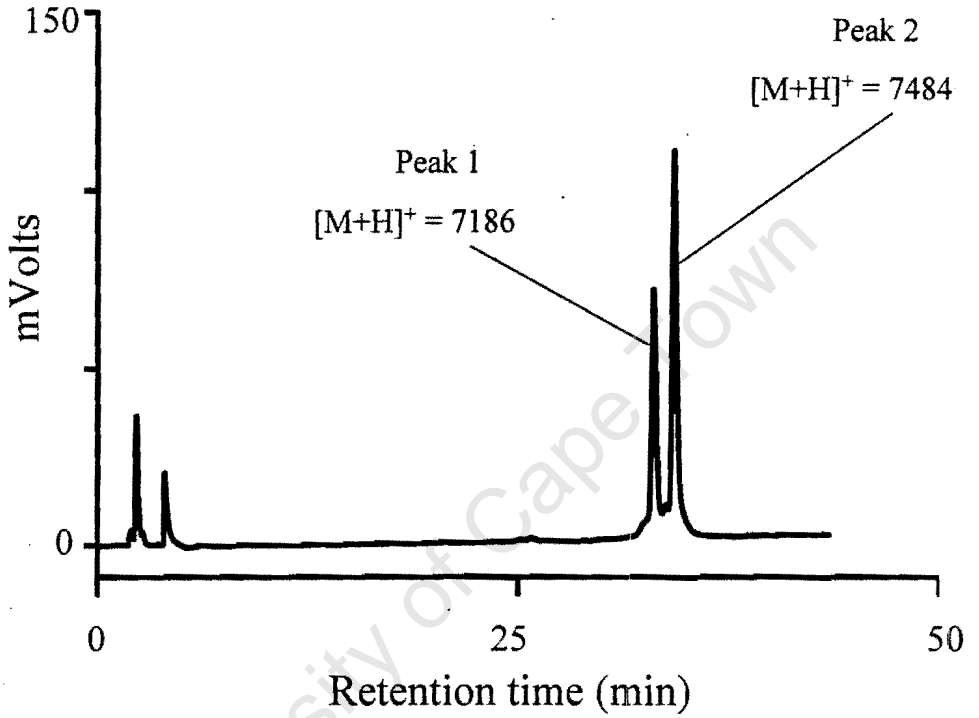

Fig. 72. Attempted HPLC separation of the doublet (peak fraction 4) in Fig. 69. Identical gradient and solvents were used as in Fig. 62A. MALDI-TOF data are indicated.

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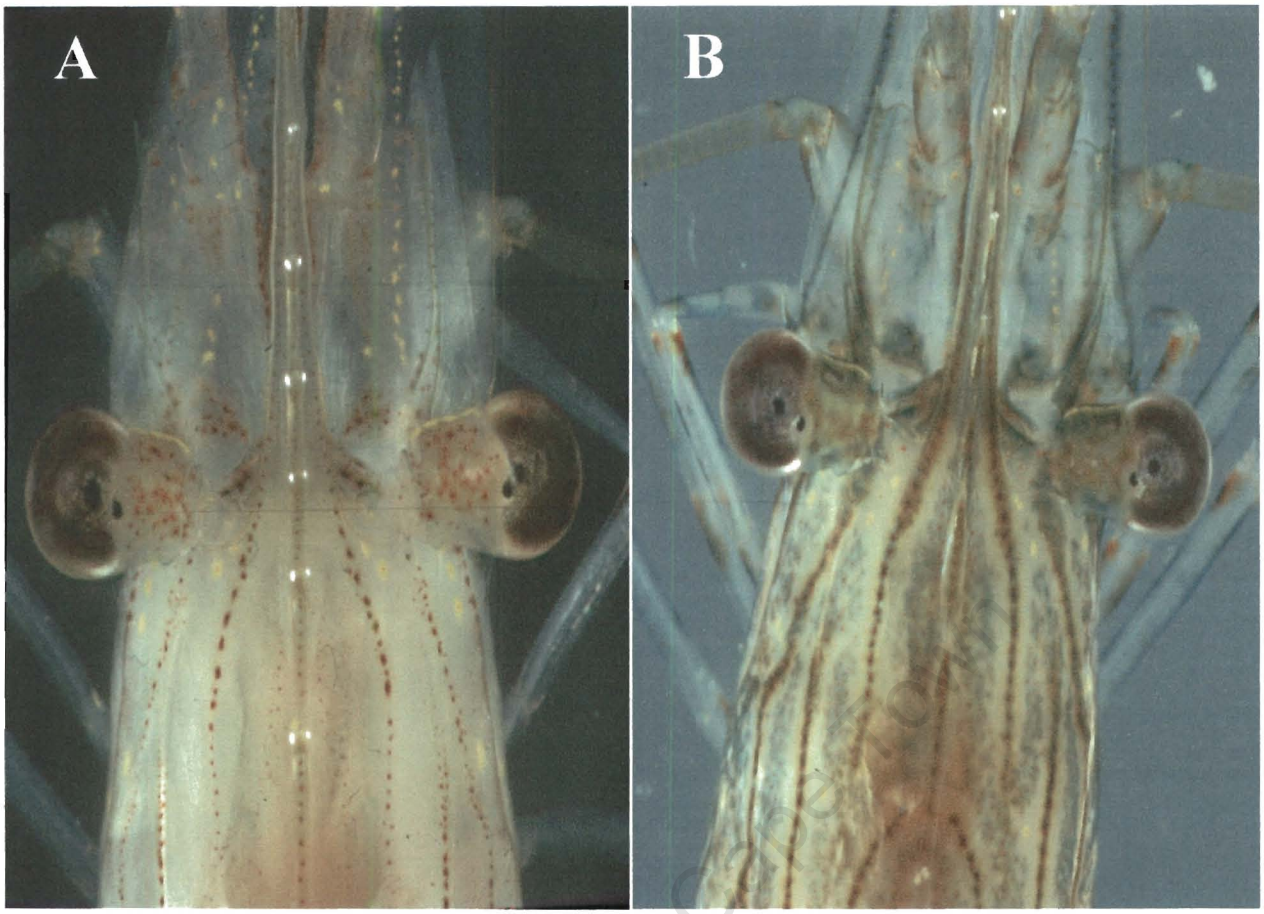

Fig. 73. Biological assay for pigment-dispersing hormone, showing the head of a light-adapted *P. pacificus* **A.** before, and **B.** after injection of synthetic β-PDH. *Jala* CPRP 1-4 were also assayed in shrimps, but failed to elicit a PDH effect.

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3.5. Preliminary investigation of cHH/MIH/VIH peptides from sinus glands of Palinurus gilchristii

Figure 74A shows a typical HPLC chromatogram of an extract of 50 sinus glands from the spiny lobster *P. gilchristii.* Aliquots of peak material were subjected to mass analyses and ELISA. Immunoreactions with anti-*Hoam* cHH serum were visible in material from 4 peaks only, the most intense reaction coincided with peak 16 (see Fig. 74B). Anti-*Capa* MIH serum was also used in the ELISA and only weakly recognised peak 14,which is the major peak of the separation profile (Fig. 74). Surprisingly, the more-hydrophobic peaks 18 and 21 were not recognised as cHH-family peptides by anti-*Hoam* cHH and anti-*Capa* MIH sera. Mass spectrometry provided $[M+H]^+$ data peaks 7,8 and 14 only, as 7578, 7686 and 8524 Da, respectively (results not shown). As the major peak, peak fraction 14 was selected for N-terminal sequencing; a partial sequence of 30 residues was elucidated which indicated an unblocked N-terminus. The partial sequence (not shown) is 100 % identical to first 30 residues of *Jala* cHH-I.

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3.6. Molecular biological aspects

3.6.1. RNA extraction

Total RNA was extracted from 30 X-organs of *J. lalandii* specimens. This amounted to a total of 14 µg RNA, as quantified spectrophotometrically. The good, non-degraded quality of the RNA is visible in Fig. 75. The RNA, redissolved in DEPC-treated water, was stored at –70 ^{0}C when not in use.

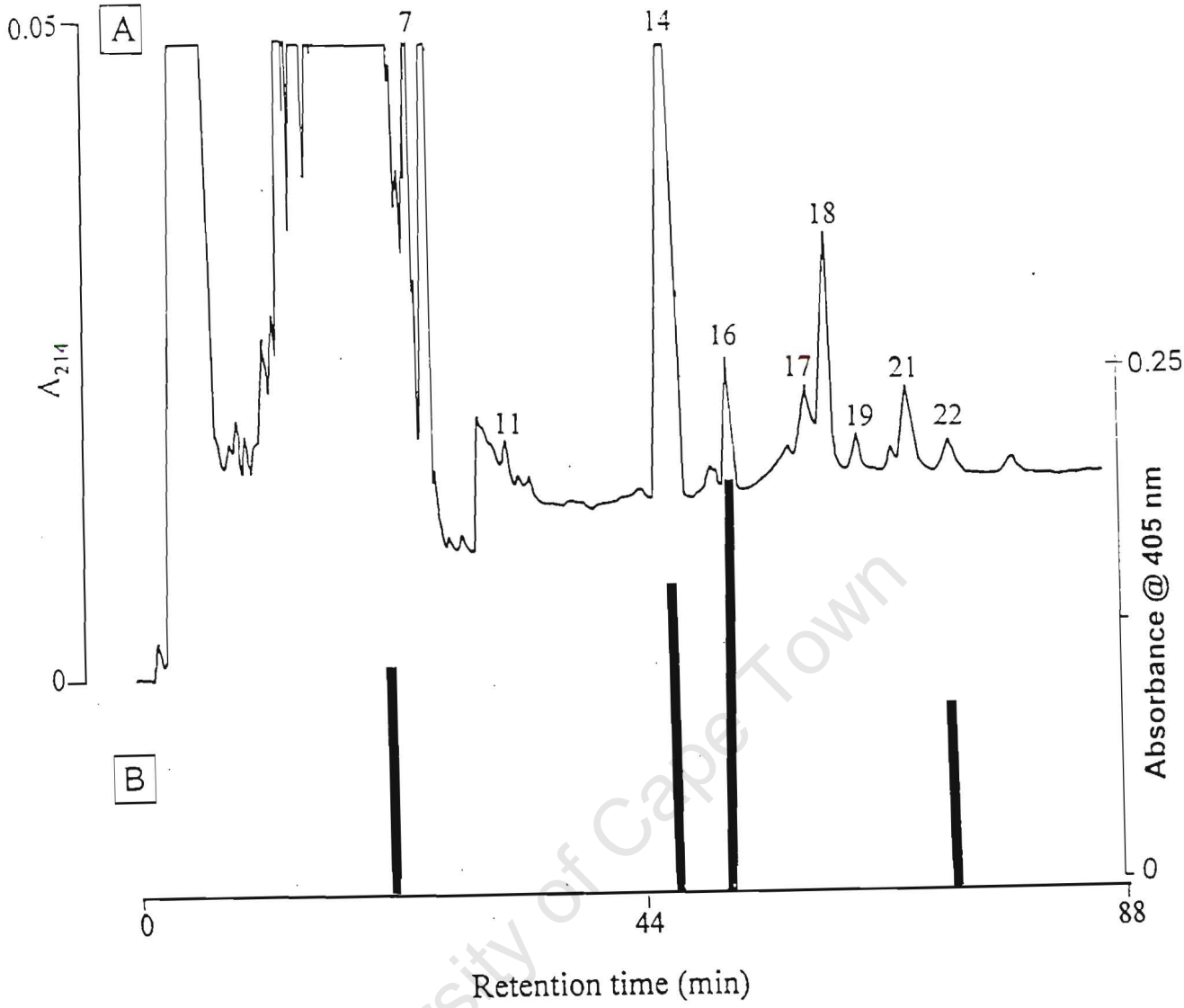

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Fig. 74. **A.** A typical RP-HPLC fractionation profile of an extract of 50 sinus glands from the spiny lobster *Palinurus gilchristii*. Peak fractions are labeled in the order of elution. Identical separation conditions, column and solvents were used as for the separation of peptides from sinus glands of *J. lalandii* (see legend to Fig. 24). HPLC peak fractions were collected and tested in ELISAs for immunoreactivity with **B.** anti-*Hoam* cHH: values above 0.1 are illustrated, and anti-*Capa* MIH sera (results not shown; only peak fraction 14 reacted weakly with the MIH serum: absorbance of 0.067).

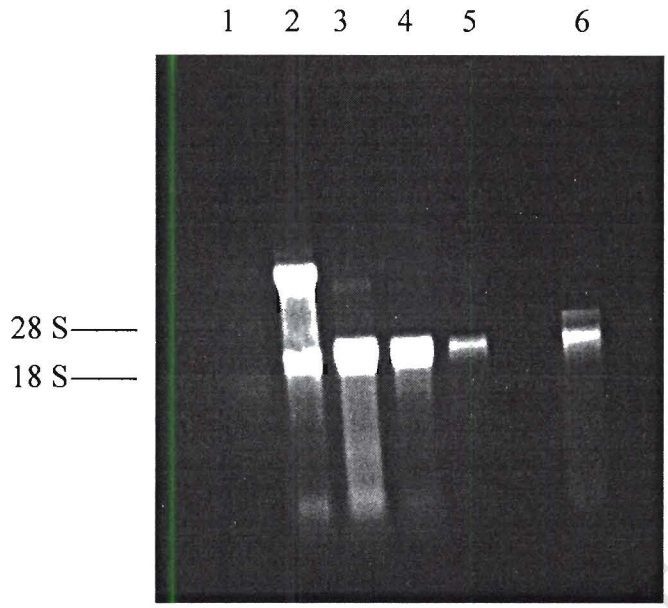

Fig. 75. Electrophoresis of 1.14 µg total RNA, extracted from X-organs of *J. lalandii* (lane 6), separated on an ethidium bromide agarose gel. An RNA marker was loaded in lane 1, while lanes 2-5 contain RNA from various insect tissues.

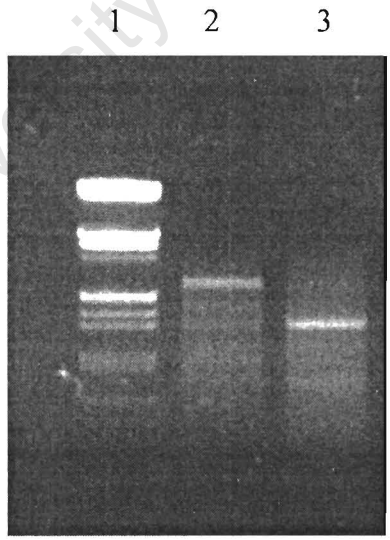

Fig. 76. Ethidium bromide 1.1 % agarose gel containing double strand cDNA of *J. lalandii* (lane 3), derived from X-organ RNA. Lane 1 contains the molecular weight marker *λ*/*Hind*III + *Eco*RI, while lane 2 contains cDNA of an insect.

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3.6.2. Construction of cDNA library

The library was constructed following the protocol and reagents supplied with the SMART™ kit from CLONTECH. One µg of total RNA from *Jala* XOs was used to synthesise a first strand of cDNA that was then amplified by PCR to produce double stranded (ds) cDNA. The success of the amplification is visualised in Fig. 76. Two to three µg of the amplified ds cDNA was digested with proteinase K to inhibit any further enzyme activities, and thereafter, was digested with *Sfi* I. This *Sfi* I-digested ds cDNA was fractionated by virtue of size on CHROMA SPIN columns. Peak fractions containing cDNA were clearly detected in fractions 7-11 under UV in an EtBr gel (Fig. 77). Thus, fractions 6-12 were pooled, the ds cDNA was precipitated, dried and redissolved in water. The quantity of ds cDNA recovered after size-fractionation was estimated by the EtBr plate assay. Figure 78 shows that the recovered *Jala* cDNA corresponds, in intensity, to 50 ng of *Lambda* DNA per 0.5 µl. Fifty ng of the recovered ds *Jala* cDNA was then mixed with 500 ng of TriplEx2 vector in a ligation reaction. The ligated cDNA (400 ng) was packaged into a λ phage.

Lysates of the unamplified *Jala* cDNA library were diluted 1:5 and 1:20 with 1x lambda dilution buffer, added to *E. coli* and titred after an overnight incubation period. The 1:5 dilution produced near-confluent plaque growth, whereas the 1:20 dilution produced well-separated plaques which amounted to 5.32×10^6 plaque forming units (pfu)/ml.

The cDNA library was amplified, yielding a titre of 6.5×10^9 pfu/ml with 92 % recombinant plaques.

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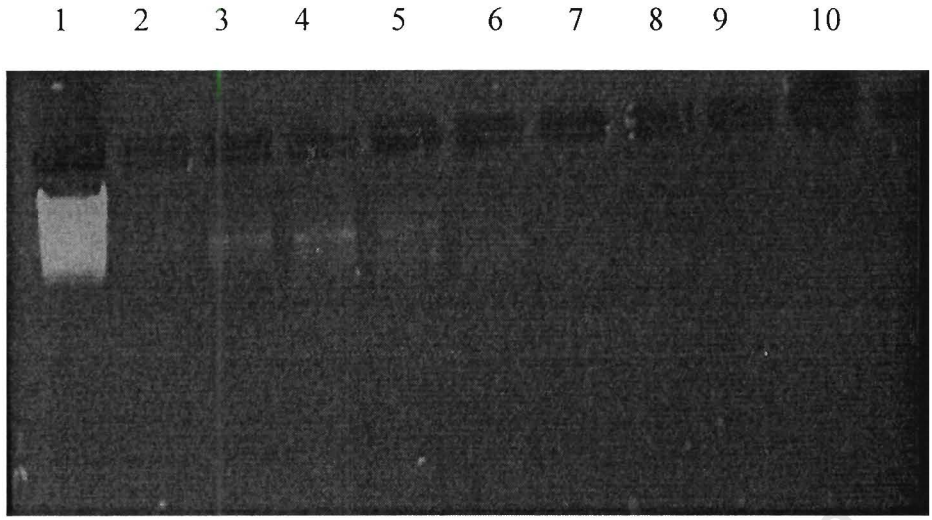

Fig. 77. Gel electrophoresis of 3 µl of cDNA fractions (derived from RNA of *J. lalandii* X-organs) after size fractionation. Lane 1 contains a molecular weight marker (*λ*/*Hind*III + *Eco*RI) and lanes 2-10 contain cDNA fractions 7-15, respectively.

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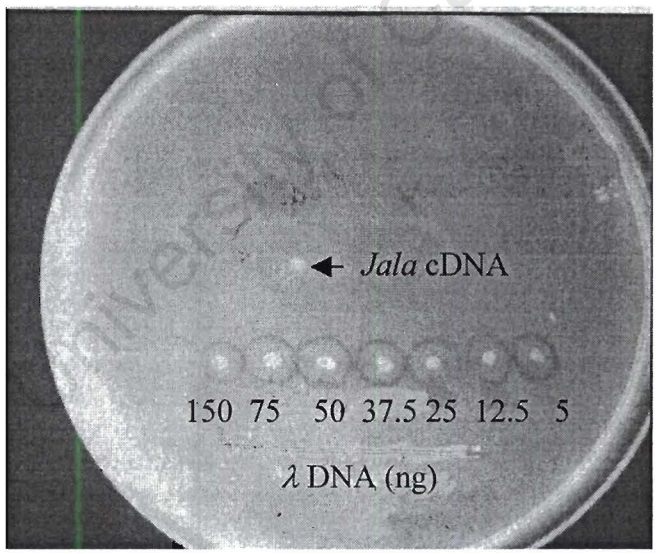

Fig. 78. Ethidium bromide plate assay to estimate the quantity of *J. lalandii* cDNA (derived from RNA of X-organs). Different concentrations of *λ* DNA were spotted onto the plate (the same volume as *Jala* cDNA, viz. 0.5 µl) and the relative intensity of the latter was compared to the DNA standard. The approximate yield of *Jala* cDNA after size fractionation (i.e. recovery of full-length transcripts) correspond to 100 ng/µl.

3.6.3. PCR cloning of the coding region of Jala cHH cDNA

Several PCR reactions were carried out with degenerate primers encoding *Jala* cHH-I. These experiments were all unsuccessful, despite designing several degenerate primers and modifying the PCR cycling protocol in various ways. It was then decided to design non-degenerate primers by using the codon usage for *Drosophila melanogaster* and by carefully selecting the part of the amino acid sequence to which the primers should correspond to. In PCR reactions with these non-degenerate primers that encoded the termini of *Jala* cHH-I, a clear DNA band of about 240 bp in size was detected in samples that contained *Jala* cDNA as template, regardless of whether the cDNA was amplified from RNA that had been extracted from male or female specimens (Fig. 79). This PCR product band was not detected in control samples in which ds *Jala* cDNA template was omitted (lane 6, Fig. 79). It also appears that the absence of Q solution did not have an obvious effect on the PCR reaction (compare lanes 2 and 3 with lanes 4 and 5 of Fig. 79). After extraction of the DNA bands from the gel, the recovered 200+ bp fragment was estimated at a quantity of 50 ng/µl, based on the relative intensity of the band compared with standard amounts of DNA fragments from *Lambda* DNA digested with *Hind* III (Fig. 80).

3.6.3.1. TOPO® cloning of 200+ bp PCR product and its DNA sequence

The 200+ bp PCR product was successfully cloned into the TOPO® vector and transformed into *E. coli* cells (One shot® cells). White plaques with ampicillin-resistance conferred by the TOPO® vector, were picked and amplified overnight in LB medium with ampicillin. The plasmid DNA was isolated by mini-prep.

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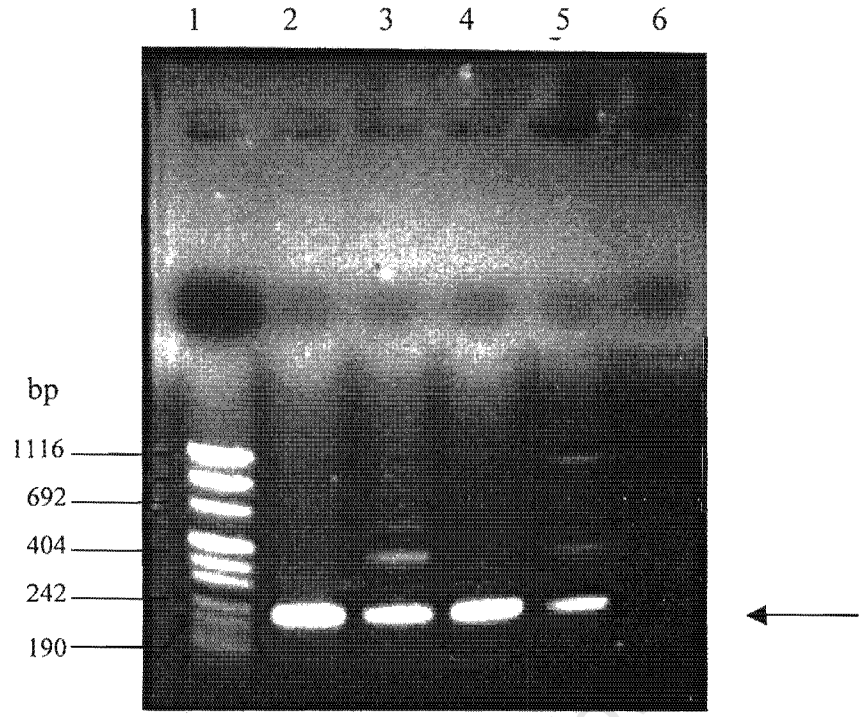

Fig. 79. DNA gel electrophoresis of a 216 bp product (arrow) from *J. lalandii* cDNA derived from RNA of X-organs from females (lanes 2 and 4) and males (lanes 3 and 5). These fragments were generated by PCR using non-degenerate *Jala* cHH-I primers and aliquots of ds *Jala* cDNA template. The molecular weight marker is pUC Mix Marker 8 (lane 1). PCR reactions with samples loaded in lanes 2 and 3 were carried out in the absence of Q solution, whereas those in lanes 4 and 5 were performed with Q solution. Lane 6 contains reaction mixture in which the cDNA template had been omitted.

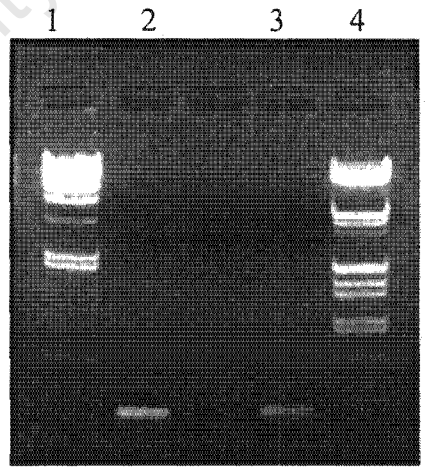

Fig. 80. Quantitation gel electrophoresis. The 216 bp PCR products (see Fig. 71) of *Jala* cDNA from females were pooled, electrophoresed, the cDNA fragment was extracted from the gel and quantified on an ethidium bromide 1 % agarose gel (lane 2). The same procedure was carried out with the PCR fragment derived from male cDNA (lane 3). Lanes 1 and 4 contain molecular weight markers (*λ*/*Hind*III and *λ*/*Hind*III + *Eco*RI, respectively). The fragment corresponded to approximately 50 ng cDNA/μl compared with DNA standards. Only the fragment from female spiny lobster specimens (lane 2) was cloned.

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On sequencing of clones, the following sequence information was obtained, some of which corresponded to the TOPO® vector itself, others corresponded to the non-degenerate primers and others to a novel sequence (Scheme 1 illustrates how these different sequences could originate from the clone):

Vector:

TTTTNNGCCCGCTCAGATTAACCCTCCTAANGGGACTAGTCCTGCAGGTTTAAAC

GAATTCGCCCTT

Primer: GCCGTGTTCGATCAGAGCTGCAAGGGCGT

Novel sequence:

GTACGACCGCTCGCTCTTCAGCAAGCTGGACCGGGTCTGCGACGACTGCT

ACAACCTCTACAGGAAGCACTACGTGGCCACCGGCTGCAGGAGAAACTG

CTACGGCAACCTGGTCTTCCGCCAGTGTCTGGACGACCTGATGCTGGTGG

ACGTGGTGGAC

Primer: GAGTACGTGGCCTCCGTGCAGATGGTG

Vector:

AAGGGCGAATTCGCGGCCGCTAAATTCAATCGCCCTATAGTGAGTCGTATTACA

ATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCA

ACTTAATCGGCTTGCAGCACATCCCCCTTTCGCAGCTGGCGTAATAGCGAAGAG

GCCCGACCGATCGCCTTCCAACAGTTGCGCAGCCTATACGTACGGAAGTTTAAG

GGTTACACCTATAAAAGAGAAGCCCGTATCGCTGTTGNGGATGACNGAGGGTAT

TATTGANCNCCGGCACGATGGGATCCCTGGCATGACGTTGTGNAANAAGCNTCC

GGAACTTACCGGGGGCTATGGGAAAAG

When the novel DNA sequence was aligned and compared using BLAST software, its deduced peptide sequence showed 100 % identity to the partial *Jala* cHH-I sequence, spanning amino acid residues Y^{11}-C^{52}. Thus, cDNA encoding a part of one

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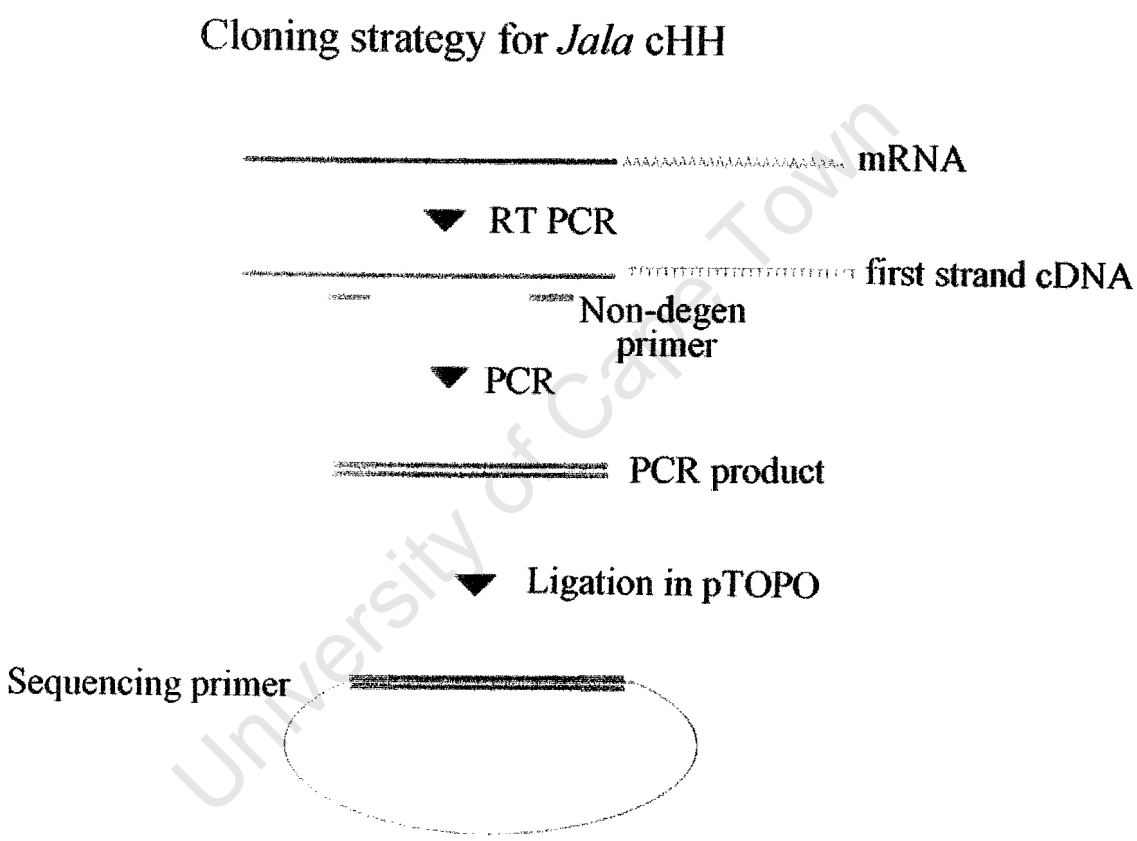
of the hyperglycaemic hormones was cloned; this partial cDNA also contains a region that is common to *Jala* MIH, viz. V^{22}CDDCYNLYR^{31}.

This 200+ bp cDNA is therefore a potentially useful fragment as a probe with which to screen the *Jala* cDNA library for the genes encoding members of the cHH/MIH/VIH peptide family.



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Scheme 1. A diagrammatic representation of the cloning strategy employed for a cHH from *J. lalandii*. The sequencing results of the amplified PCR product relates to the known sequences of the TOPO vector (indicated by black ellipsoid), the 2 non-degenerated primers (indicated by blue bars) and the novel sequence which corresponds to *Jala* cHH-I (indicated by green lines).

4. DISCUSSION

Peptide hormones appear to be the master regulators of many physiological processes in both invertebrates and vertebrates. Neuropeptides, i.e. peptides that are synthesised by neurons, also fall under this category. The best-known neuroendocrine complex is the vertebrate hypophyseal-hypothalamus system. The equivalent of this system in invertebrates is the brain-corpora cardiaca/corpora allata system in insects and the X-organ-sinus gland complex in crustaceans.

Many decades ago, neuroendocrine functions were identified by classical physiological experiments of tissue extirpation and implantation. In this way, it was ascertained that a number of processes in decapod crustaceans were under the control of factors that were produced in the eyestalks. By the same means, observations were recorded on the sudden hypertrophy of certain organs following eyestalk ablations With improved microscopy and histological techniques, neuroendocrine and neurohaemal organs could be identified, described and named.

It was during this early period of investigation into neuroendocrine tissue that the X-organ and its associated neurohaemal organ, the sinus gland, were described (Hanström, 1939; Bliss and Welsh, 1952; Passano, 1953). Specific extirpation of these tissues resulted in the same outcomes as with eyestalk ablation and the involvement of the XO-SG complex in processes of moulting, growth, sexual maturation, regulation of metabolism, colour adaptation and osmoregulation was confirmed.

In the current study, the XO-SG system in a palinuran decapod species has been investigated by means of immunocytochemistry at the light microscopical level and

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several neurohormones have been isolated from the sinus glands and have been chemically and functionally characterised.

4.1. Immunocytochemical aspects

The localisation of peptides belonging to the cHH/MIH/VIH neuropeptide family in the XO-SG complexes of 3 different decapod crustacean species was achieved in the present study with the use of heterologous antisera. In most of the immunocytochemical studies on cHH/MIH/VIH peptides, homologous antisera were used because it was thought that there would not be cross-reactivity between different crustacean species and infraorders due to substantial peptide differences. The idea of species- or systematic group-specific neurohormones was established on the basis of bioassays on interspecific hyperglycaemic activity in which a number of crustacean species were cross-injected with crude eyestalk or sinus gland extracts, or with purified cHHs (Leuven *et al.* 1982; Keller *et al.* 1985). The conclusion drawn from these studies was that cHH is specific at the infraorder level, e.g. the hormone isolated from a brachyuran would only have a hyperglycaemic effect in other members of that group, and will have no effect on astacideans and *vice versa*. This biological specificity was further borne out by immunological cross-experiments using immunodiffusion techniques with anti-*Astacus leptodactylus* cHH serum and anti-*Carcinus maenas* cHH serum in which there was also no interspecific cross-reactivity among the infraorders (Leuven *et al.* 1982). With the use of a more sensitive immunodetecting method, however, viz. tissue immunocytochemistry, Gorgels-Kallen *et al.* (1982) showed cross-reactivity of anti-*A. leptodactylus* cHH serum with other astacideans (*Orconectes limosus, Nephrops norvegicus, Homarus americanus*), as

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well as with a palinuran (*Palinurus vulgaris*) and a caridean species (*Palaemon serratus*). Thus, the immunocytochemical study indicated an immunologically detectable relationship between the cHHs of different crustacean infraorders. This is not so surprising in view of the close sequence homology of cHHs across the systematic groups as recently determined by biochemical methods. For example, the sequence homology is 81 % between *O. limosus* and *H. americanus* cHHs and 61 % between *O. limosus* and *C. maenas* cHHs (Keller 1992).

In the present study I report on comparative tissue immunocytochemical and ELISA results that confirm the conclusions drawn by Gorgels-Kallen *et al.* (1982) and extend the immunological relationship of different cHHs to include the brachyuran infraorder. This was achieved by the use of anti-cHH serum from two different astacideans (*H. americanus* and *Procambarus bouvieri*) and from a brachyuran (*Cancer pagurus*) (the primary structures of cHH from these species are shown in Fig. 81). The cHH antisera showed clear cross-reactivity on palinuran eyestalk tissue (*Jasus lalandii* and *Panulirus homarus*), as well as on brachyuran tissue (*C. maenas*). The specific immunostaining of the sinus gland and X-organ perikarya with anti-*Capa* cHH serum was completely abolished when the antiserum was preadsorbed with HPLC-purified *Capa* cHH. The specificity of the anti-*Hoam* cHH and anti-*Prbo* cHH sera could not be tested in such preadsorption experiments because of the unavailability of purified *Hoam* and *Prbo* cHH peptides. However, since the staining pattern is comparable to that obtained with anti-*Capa* cHH, it can be accepted that the immunoreactive results are true positive.

In this immunocytochemical study, I have also extended the comparative investigation to include anti-*Capa* MIH serum and anti-*Hoam* VIH serum. Thus far, VIH has only been isolated and sequenced from *H. americanus* (Soyez *et al.* 1991), *P. bouvieri*

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	10	20	30	40	50	60	70
Jala cHH-I[1]	AVFDQSCKGV	YDRSLFSKLD	RVCDDCYNLY	RKHYVATGCR	RNCYGNLVFR	QCLDDLMLVD	VVDEYVASVQ MV-NH_2
Jala cHH-II[1]	AVFDQSCKGV	YDRSLFKKLD	VVCDDCYNLY	RKPYVATGCR	ENCYSNLVFR	QCLDDLMLVD	VVDEYVSTVQ MV-NH_2
Jala cHH-II[2]	AVFDQSCKGV	YDRSLFQKLD	LVCDDCYNLY	RKPYVATGCR	ENCYGN		
Jala cHH-I[2]	AVFDQSCKGV	YDRSLFGKLD	RVCDDCYNLY	RKPYVATGCR	QNCYGN		
Hoam cHH-A[3]	pEVFDQACKGV	YDRNLFKKLD	RVCEDCYNLY	RKPFVATTCR	ENCYSNWVFR	QCLDDLLLSD	VIDEYVSNVQ MV-NH_2
Hoam cHH-B[3]	pEVFDQACKGV	YDRNLFKKLN	RVCEDCYNLY	RKPFVIVTCR	ENCYSNRVFR	QCLDDLLMID	VIDEYVSNVQ MV-NH_2
Prbo cHH**[4]	pEVFDQACKGI	YDRAIFKKLD	RVCEDCYNLY	RKPYVATTCR	QNCYANSVFR	QCLDDLLLID	VVDEYISGVQ TV-NH2
Cama cHHII[8]*	pEIYDTSCKGV	YDRALFNDLE	HVCDDCYNLY	RTSYVASACR	SNCYSNLVFR	QCMDDLLMMD	EFDQYARKVQ MV-NH_2
Capa Chhii[6*]	pEIYDTSCKGV	YDRGLFSDLE	HVCDDCYNLY	RNSYVASACR	SNCYSNVVFR	QCMEELLMMD	EFDKYARAVQ MV-NH_2
Peja cHH-VI[7]	LVFDPSCAGV	YDRVLLGKLN	RLCDDCYNVF	REPNVATECR	SNCFYNLAFV	QCLEYLLPPS	LHEEYQANVQ MV-NH_2
Peja cHH-III[7]	SLFDPACTGI	YDRQLLRKLG	RLCDDCYNVF	REPKVATGCR	SNCYHNLIFL	DCLEYLIPSH	LQEEHMAAMQ TV-NH_2
Maro cHH [8]	AILDQSCKGI	FDRELFKKLD	RVCDDCYNLY	RKPYVAIDCR	RGCYQNLVFR	QCIQDLQLMD	DLDEYANAVQ TV-NH_2
Pemo cHH[9]	SLSFRSCTGA	YDRELLVRLD	RVCEDCYNVY	RDVGVAAECR	SNCFHNEVFL	YCVDYMFRPR	QRNQYRAALQ RL-NH_2
Meen cHHB[10#]	SLFDPSCTGV	KDRELLGRLN	RVCDDCYNVF	REPKVATECR	SHCFLNPAFI	QCLEYIIFEV	LHEEYQANVQ LV-NH_2
Pesc cHH[11]	ANFDPSCTGV	YDRELLGRLS	RLCDDCYNVF	REPKVATECR	SNCFYNPVFV	QCLEYLIPAD	LHEEYQAHVG TV-NH_2

Fig. 81. Comparison of the amino acid sequences of cHHs from four different infraorders, viz. Palinuridae: [1]*Jasus lalandii* (current study) and [2] the partial cHH sequence reported from *J. lalandii* (Yasuda, 1997); Astacidae: [3]*Homarus americanus* (Tensen *et al.*, 1991) and [4]*Procambarus bouvieri* (Aguilar *et al.*, 1995); Brachyura: [5]*Carcinus maenas* (Kegel *et al.*, 1989) and [6]*Cancer pagurus* (Chung *et al.*, 1998), and Penaeidae: [7]*Penaeus japonicus* (Yang *et al.*, 1995, 1997), [8]*Machrobrachium rosenbergii* (Sithigorngul *et al.*, 1999a,b), [9]*Penaeus monodon* (Udomkit *et al.*, 2000), [10]*Metepenaeus ensis* (Gu *et al.*, 2000; # deduced from nucleotide sequence) and [11]*Penaeus schmitti* (Huberman *et al.*, 2000). Similarities between the sequences of the palinurid and astacidean species are indicated by boldface letters. Similarities between all the infraorders are underlined. * cHH-I differs from –II by having a free N-terminus; **the cHH sequence of *Procambarus bouvieri* is identical to that of *P. clarkii*, and cHH from *Orconectes limosus* differs from this by only one residue (L^{62}). The truncated *Jala* cHH sequences of the current study is identical to the first 65 residues of *Jala* cHH-I and –II presented above.

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(Aguilar *et al.*, 1992) and the isopod *Armadillidium vulgare* (Greve *et al.*, 1999) whereas MIH sequences are known from crab, prawn and crayfish species (see Fig. 82).

To date, MIH antisera have only been used in immunocytochemistry on brachyuran tissue (anti-*C. maenas* MIH serum reacted positively with eyestalk tissue sections from five different crab species; Dircksen *et al.* 1988) and prawn (an antiserum raised against a synthetic peptide which represented the C-terminal sequence of *Peja* MIH specifically differentiated between *Peja* MIH and cHH peptides and reacted positively with the XO-SG complex of *Penaeus japonicus*; Shi *et al.*, 1998). In the present study I show reactivity of anti-*Capa* MIH serum on brachyuran as well as palinuran eyestalk tissue. Anti-*Capa* MIH serum was recognised by antigens in the axon terminals of the sinus gland and by the granular contents of the X-organ perikarya. The weaker intensity of the immunoreaction on palinuran tissue, relative to the strong reaction in the crab tissue, might be explained by the degree of sequence similarity between the different MIHs. With regards to *C. maenas* MIH, MIH from other crabs, such as *Callinectes sapidus* and *C. pagurus*, are 79.5 % and 84.5 % identical, respectively (see Chung *et al.* 1996), whereas the astacidean *Procambarus clarkii* MIH is only 48 % identical (Nagasawa *et al.* 1996). Comparisons between the sequence of MIH from *J. lalandii* and *C. pagurus* show indeed only 41 % sequence identity (Fig. 82). Background staining associated with neural tissue of the optic ganglia was also observed with the use of anti-*Capa* MIH, despite attempts to block endogenous peroxidase activity. The specificity of anti-*Capa* MIH serum was, however, unequivocally shown by preabsorption with pure *Capa* MIH peptide. The application of the MIH + anti-MIH preabsorbed complex completely abolished the specific

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Fig. 82. Alignment of the peptide structure of a putative MIH from *J. lalandii* (*Jala*) with those purified or deduced from nucleotide sequences (*) from other decapod crustacean infraorders. Astacidae: [1] *Procambarus clarkii* (Nagasawa *et al.*, 1996), and [9]*P. bouvieri* (Aguilar *et al.*, 1996); Penaeidae: [7]*Penaeus japonicus* (Yang *et al.*, 1996), [8]*Metapenaeus ensis* (Gu and Chan, 1998) and [10]*P. vannamei* (Sun, 1994); and Brachyura: [2]*Callinectes sapidus* (Lee *et al.*, 1995), [3]*Carcinus maenas* (Webster, 1991), [4]*Cancer pagurus* (Chung *et al.*, 1996), [5]*Charybdis feriatus* (Chan *et al.*, 1998) and [6]*Cancer magister* (Umphrey *et al.*, 1998). Identical amino acid residues, with reference to the brachyuran MIHs, are shown in bold. The (-) indicates the gap(s) introduced into the amino acid sequence to align the Cys residues and to allow for maximal identity. Amidation is shown by: -NH_2. Overall identity of the amino acid sequence (%) is compared with *Jala* MIH and indicated to the right of the primary structures.

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immunostaining in the SG-XO system, whereas the non-specific, background staining remained unaffected.

In this immunocytochemical study, positive immunostaining of the SG-XO system with anti-*Hoam* VIH was obtained on both palinuran and brachyuran tissue, albeit weaker reactions than were observed with anti-cHH sera. This confirms an earlier ELISA report that VIH does not seem to be species- or systematic group-specific based on cross-reactivity of anti-*Hoam* VIH on brachyuran, caridean and isopod sinus gland extracts, but contradicts the finding that anti-*Hoam* VIH serum does not recognise sinus gland material of palinurans (Meusy *et al.* 1987). Apart from the negative immunocytochemical result of anti-*Hoam* VIH with palinuran tissue from *Jasus paulensis*, negative results were also reported with sinus glands from the astacideans *A. leptodactylus* and *O. limosus* (Meusy *et al.* 1987). In contradiction to this, cross-reactivity of anti-*Hoam* VIH has been illustrated for the crayfish *N. norvegicus* (Giulianini *et al.*, 1998). Due to the unavailability of pure *Hoam* VIH peptide, I could not use preabsorbed antibody-antigen complexes to demonstrate the specificity of the anti-VIH reaction. However, I investigated this issue in ELISA experiments in which putative cHH peptides from *J. lalandii* and MIH peptides from *C. maenas* were identified from HPLC-generated peaks from crude extracts of sinus glands and used for absorption of anti-*Hoam* VIH. This absorbed antiserum was then applied to the putative cHH and MIH peptides in another ELISA. The putative cHH and MIH peptides were also treated with unadsorbed anti-*Hoam* VIH to determine the degree of cross-reactivity between the antiserum and these peptides. Observations from previous ELISA experiments have indicated that similar antigenic determinants

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reside in cHH and VIH peptides of *H. americanus*, which leads to cross-reactivity of anti-*Hoam* VIH with *Hoam* cHH peptide (Meusy and Soyez 1991). This may therefore account for the observed positive immunoreaction between anti-VIH antiserum and the identified cHH fractions in the current study. The cross-reactivity of anti-*Hoam* VIH with *C. maenas* MIH, although never tested before, may also not be surprising, since it is known that MIH and VIH share greater sequence identity with each other (51% - 53 %) than with any cHH (19 %) (Chung *et al.* 1996). Preabsorbed complexes of anti-*Hoam* VIH with either pure *C. pagurus* cHH or pure *C. pagurus* MIH peptide did not eliminate specific staining when applied to *J. lalandii* tissue sections in the present study. Specific immunostaining was also not eliminated in the ELISA investigation when preabsorbed anti-*Hoam* VIH and *J. lalandii* cHH peptide, or anti-*Hoam* VIH and *C. maenas* MIH were applied, thus indicating that VIH-like antigens are being positively identified after the suppression of cross-reactivity with cHH and MIH antigens. However, anti-VIH serum did not react with a unique peptide in ELISA investigations on peptides separated by HPLC from crude extracts of sinus glands from *J. lalandii* and it may be speculated that the VIH peptide(s) has(have) not been resolved with the given HPLC gradient but may be co-eluting with the other peaks. MALDI-TOF analyses of the HPLC peaks, however, revealed only 1 mass per peak, which argues for the purity of the separated peaks and cross-reactivity of anti-VIH serum with cHH and MIH epitopes.

A further result, emanating from the use of serial sections in the present study, revealed co-localisation of cHH, MIH and VIH antigens in the X-organ perikarya. Partial co-localisation of cHH and VIH antigens have also been documented in

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immunocytochemical studies with *H. americanus* (Kallen and Meusy 1989), and has been confirmed at the mRNA level by in situ hybridisation (De Kleijn *et al.* 1992); in both studies, homologous antisera and cRNA probes were used. Co-localisation of cHH and VIH peptides and their mRNAs has also been reported for *Homarus gammarus* using antisera and cRNA probes against *H. americanus* (Rotllant *et al.*, 1993). In another study where heterologous antisera (anti-*O. limosus* cHH and anti-*Hoam* VIH) were used on *N. norvegicus* tissue sections, co-localisation of the two hormones was not apparent (Giulianini *et al.*, 1998). Thus, there is no clear trend emerging about the dual synthesis of cHH and VIH in the same perikarya and the expected co-release of the two hormones. Similarly with co-localisation of MIH and cHH immunoreactivity: in both immunocytochemical and in situ hybridisation studies with specific *C. maenas* antisera and cRNA probes, respectively, co-localisation of cHH and MIH in *C. maenas* was not observed (Dircksen *et al.* 1988; Klein *et al.* 1993a). Yet, co-localisation of MIH and cHH has been reported in *P. japonicus* with specific antisera raised against *Peva* cHH and MIH (Shih *et al.*, 1998). The fact that the preabsorption experiments with *Capa* antisera and respective peptides eliminated all positive immunoreactions in tissue immunocytochemistry in the current study, points to the possibility that the observed results must be specific for the particular antisera. A logical conclusion, therefore, follows that cHH and MIH peptides are indeed co-localised in some neurons. Co-localisation of cHH/MIH/VIH peptides in the present study was also investigated by double staining with a cocktail of two primary antisera and detection of immunoreactivity by immunofluorescence. This

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method could not be fully optimised, largely because of the age (post-expiry) of the flourescent-labeled secondary antisera. Despite this, however, evidence of co-localisation of cHH with MIH and VIH immunoreactivity in *J. lalandii* was obtained. More information regarding this co-synthesis/secretion of *Jala* hormones may be obtained from embarking on in situ hybridisation studies with specific cRNA probes; such studies must still be undertaken. In view of the sequence homologies, crab MIH is similar to lobster VIH, and since lobster cHH and VIH are co-localised, it does not seem improbable to find that crab cHH and MIH also have partial co-localisation. At present, there is no explanation for the observed discrepancy between the results of the present study and that of earlier workers, with respect to immunostaining of *C. maenas* tissues, except to point out that different antisera (anti-*C. pagurus* MIH serum in the present study and anti-*C. maenas* MIH serum in earlier studies) and different methods of study have been carried out (indirect peroxidase technique in the present study and the peroxidase-antiperoxidase technique was used by Dircksen *et al.* 1988). Although *C. pagurus* MIH is recognised by MIH receptors on *C. maenas* Y-organs, and both of these MIHs exhibit similar biological activity (Webster 1993), radioimmunoassays of *C. pagurus* MIH using *C. maenas* MIH antiserum showed limited cross-reactivity (see Chung *et al.* 1996), therefore, suggesting differences in their antigenic determinants. In the current ELISA study on HPLC-generated fractions from crude extracts of *J. lalandii* sinus glands, anti-*C. pagurus* MIH serum clearly recognised one peak only, whereas anti-*C. pagurus* cHH serum recognised this same putative MIH fraction in addition to the two cHH peaks. Thus, one would

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speculate that the *J. lalandii* MIH peptide may structurally be more similar to the crab cHH than to the lobster cHH peptide. There is, however, not a significant difference in the sequence identity between *Jala* MIH and *Hoam* cHH (27 %), and that between *Jala* MIH and *Capa* cHH (28 %), and it must be assumed that anti-*Capa* cHH serum is not as specific as anti-*Hoam* cHH and anti-*Prbo* cHH serum. It can further be postulated that anti-*Capa* cHH serum may be recognising the residues V^{22}-R^{31}, which is a highly conserved region in the *Jala* MIH, *Jala* cHH and *Capa* cHH peptides (cf. Fig. 81 and 83).

The current immunocytochemical study has shown that members of the cHH/MIH/VIH peptide family in the palinurans *J. lalandii* and *P. homarus* are sufficiently homologous to peptides from the astacideans *H. americanus* and *P. bouvieri* and to the brachyuran *C. pagurus* to achieve cross-reactivity with heterologous antisera. This cross-reactivity resulted, largely, in very specific immunoreactions that could be abolished by preadsorbing the antiserum with its specific peptide. In the case of *J. lalandii* and *C. maenas*, the immunoreactions were attributed to distinct HPLC peaks, following the separation of peptide fractions in extracts of sinus glands. These antisera could then be used as useful tools with which to identify cHH/MIH/VIH peptides after HPLC separations.

Further information from this study relates to morphology of the eyestalk of decapod crustaceans, especially the XO-SG complex. Whereas the optic ganglia and X-organ of the crab and spiny lobster species are no different than what have previously been described for other crustaceans (see Cooke and Sullivan, 19982), the position of the

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sinus gland relative to the underlying optic ganglia varies. In the current study, there is confirmation of the location of the sinus gland in *C. maenas* (at the medulla interna; cf. Jaros and Keller, 1979), and information is supplied on the position of the sinus gland in *J. lalandii* and *P. homarus* (between the medulla interna and the medulla terminalis). This relatively distal position of the sinus gland in the spiny lobster agrees with a previous report on *Panulirus interruptus* (Mancillas *et al.*, 1981) and has also been reported for the prawn *P. japonicus* (Shih *et al.*, 1998). In other crustaceans the sinus gland is situated at the transition between the medulla externa and the medulla interna (e.g. *A. leptodactylus*: Van Deijnen *et al.*, 1985) or at the medulla interna (e.g. *N. norvegicus*: Giullianini *et al.*, 1998).

Results from the present comparative immunocytochemical study indicate co-localisation of cHH/MIH/VIH neuoropeptides in the X-organ, which suggests dual synthesis and processing of the peptides in the perikarya and simultaneous release from the axon terminals. Since the inhibitory hormones (VIH and MIH) are assumed present in high titres in the circulation for most of the year to be effective in suppressing growth and reproductive development, and since cHH is also required on a daily basis, it is perhaps not so strange to have frequent co-release of these necessary hormones into the haemolymph. Further studies at the molecular biological level are needed to confirm the co-localisation and to monitor the rate of mRNA synthesis (transcription of the genes) over different periods of the day, moulting and reproductive cycles.

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4.2. Functional characterisation of cHH/MIH/VIH peptides from sinus glands of J. lalandii

Crude extracts of sinus glands, prepared in hot acetic acid to minimise the action of degradative proteases, were applied to RP-HPLC. A gradient was designed to separate and resolve the hydrophobic material from such extracts and the resulting pattern of fractionation was observed by monitoring the absorbance of peptide bonds of the eluting material at a wavelength of 214 nm. In this manner, reproducible separations were achieved and the peak material was subjected to immunoassays, mass spectrometry and biological assays to characterise and identify cHH/MIH/VIH peptides of *J. lalandii*.

4.2.1. Assessment of hyperglycaemic activity

In the current study, *in vivo* homologous assays were carried out with juvenile spiny lobsters (i.e. sexually immature animals to minimise the possible influence of reproductive cycles on glycaemia; Dean and Vernberg, 1965a) under controlled temperature conditions of the aquarium room (12–14 ^{0}C), since temperature is also known to affect glycaemia in decapod crustaceans (Dean and Vernberg, 1965b). In a number of crustacean species, the secretory rhythm of cHH over a 24 hour period has shown that glucose levels peaked during the night and then stabilised during late-morning hours (Dean and Vernberg, 1965b; Reddy *et al.*, 1981; Kallen, 1985). Therefore, to ensure a lowered and stable level of glucose in the haemolymph, assays in the current study were always carried out after 10h00. In pilot experiments, juvenile *J. lalandii* specimens of roughly the same size and weight classes were starved for a few days prior to the injection of 1 SGE of crude homologous extracts or sea water.

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Two hours post-injection, a significant increase ($p<0.05$) in glucose concentration was measured in the haemolymph of sinus gland-injected lobsters compared with sea water-injected animals. Two hours were thus selected as the time frame over which to measure a change in glycaemia following injection of a test substance. These pilot experiments further showed that there was quite a wide variation in initial glucose values of the juveniles, despite having selected similar sized animals and the fact that all the juveniles were maintained under similar aquarium conditions, including the feeding regime. Such variations have been reported before and attempts to counteract this have included eyestalk ablations and prolonged periods of starvation (see for example Aguilar *et al.*, 1995; Sithigorngul *et al.*, 1999a). To prevent unnecessary trauma to the juvenile lobsters and not to interfere with their overall hormonal balance, it was decided not to ablate their eyestalks and, instead, to group and select animals for injection based on their initial glucose levels. In this way, the standard deviations inside the group were lowered and the significance of the change in glycaemia could be unequivocally established. This non-random selection of the test animals did not bias the outcome of the experiments, since the current study has shown that all the juveniles showed a similar level of response after the injection of crude extracts of sinus glands, regardless of whether they had low or high initial glucose concentrations. Furthermore, the initial glucose levels also did not affect the response of the lobsters to sea water injections. These assay conditions were, thus, applied to discover which of the peaks, separated on HPLC from crude extracts of *J. lalandii* sinus glands, showed hyperglycaemic activity. From the immunological

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characterisation of the five peaks typically separated in the current study, four had shown a positive immunoreaction with anti-*Hoam* cHH serum (peaks 1, 2, 4 and 5 of Fig. 24), whereas all 5 peaks had reacted positively with anti-*Capa* cHH serum. Biological activity was only associated with peaks 4 and 5, eliciting a significant increase in glucose levels of 170 and 81 %, respectively. These bioactive peptides are thus code-named *Jala* cHH-I and *Jala* cHH-II in the order of elution, as well as abundance (cHH-I being the major peak).

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4.2.2. Determination of MIH activity

4.2.2.1. Characterisation of circulating ecdysteroids in J. lalandii

The moult cycle of crustaceans is under the control of two hormones: a peptide hormone that has an inhibitory effect (MIH) and a steroid hormone that has a stimulatory effect (moulting hormone = ecdysteroid). The accepted, simplified, paradigm for moult control in crustaceans is that high titres of MIH suppress the synthesis of ecdysteroids and only when this repressive action of MIH is lifted, can sufficient ecdysteroids be produced to reach a critical level in the haemolymph, which then initiates ecdysis (see Webster, 1998). Ecdysteroids are produced and secreted in various chemical forms from a pair of thoracic glands known as the Y-organ. For example, ecdysone (E) and 25-deoxyecdysone (25dE) are secreted from *C. maenas* Y-organs (Lachaise *et al.*, 1989), ecdysone and 3-dehydroecdysone (3DE) are

secreted in *Cancer antennarius* (Spaziani *et al.*, 1989), *Procambarus clarkii* (Sonobe *et al.*, 1991), *Orconectes limosus* (Böcking *et al.*, 1993) and *Penaeus vannamei* (Blais *et al.*, 1994). Research has shown that these ecdysteroids are further modified by peripheral tissues in the proximity of the Y-organs (see Lachaise *et al.*, 1993): E and 25dE are hydroxylated to give rise to 20-hydroxyecdysone (20E, which is the active form of the ecdysteroids) and ponasterone A, respectively; 3DE is reduced by 3β-reductase into ecdysone and then converted into 20E (Böcking *et al.*, 1995). Thus, it is possible to deduce which form of ecdysteroids is produced by the Y-organs from the ecdysteroids present in the haemolymph. In the present study, extracts of haemolymph samples from *J. lalandii* were subjected to normal phase (NP) HPLC; the retention times of the resulting peak fractions were compared to the retention times of known ecdysteroid compounds chromatographed under the same conditions, and the fractions were tested for immunoreactivity in an EIA with AS 4919 (an antibody that recognises a variety of different ecdysteroid forms; Porcheron *et al.*, 1976). In the current study, peak fractions that had the same retention times as E and 20E on NP-HPLC were immunoreactive, which suggests that these are the major forms of ecdysteroids occurring in the haemolymph of *J. lalandii* and further, that either E or 3DE or both, i.e. E and 3DE, are produced by the Y-organs of this spiny lobster species. Based on these conclusions, an EIA was designed to titre the ecdysteroids produced *in vitro* by *J. lalandii* Y-organs in the presence of various substances. Two other less-polar compounds also eluted as minor peaks on NP-HPLC from *J. lalandii* haemolymph in the current study. These peaks could not be identified

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by their retention times but were immunoreactive with AS 4919 antisera. It could be argued that, depending on the extent of cross-reactivity of these unknown peaks with AS 4919, the final conclusions concerning the ecdysteroids of *Jala* Y-organs could greatly differ. I have, however, presumed that these unknown peaks are well recognised by AS 4919 and hence, conclude that 20E is the major circulating ecdysteroid in *J. lalandii*. The moulting hormone, 20E, was incidentally first isolated in crustaceans from carcasses of the spiny lobster *Jasus lalandei* (Hampshire and Horn, 1966) and was later also extracted from insects, a crayfish and a pine tree (Galbraith *et al.*, 1967). Prior to this, ecdysone was first purified from insects by Butenandt and Karlson in 1954 (see Smith and Sedlmeier, 1990).

4.2.2.2. Y-organ incubations and EIAs

The biological activity of a putative moult-inhibiting hormone has mostly been deduced from the indirect evidence of ecdysteroid production from *in vitro* incubations of Y-organs – a reliable method that was first introduced by Soumoff and O'Connor (1982). Coupled with sensitive methods of immunodetection, such as radio- or enzyme-immunoassays, this biological assay has provided a means to quantitatively assess the effect of compounds on the output of ecdysteroids from the moulting glands. Instrumental to the success of such a biological assay are two considerations: (i) dissection of the Y-organ, i.e. knowledge of the location and clear identification of the moulting gland; (ii) accurate quantification of the ecdysteroids

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produced *in vitro*, i.e. selecting the appropriate antibody to best recognise the ecdysteroid and thereby to provide accurate interpretations of the ecdysteroid titre.

In the current study, successful explantation of the Y-organ was confirmed by immunoreactivity of the secreted products with antisera raised against ecdysteroids, as well as by demonstrating that the productivity of the gland can be altered by the presence of crude extracts of sinus glands, a source of the moult-inhibiting peptide. A survey of the more recent literature on the gross anatomy and location of the Y-organs highlights the inaccurate descriptions of older reports and subsequent misidentifications, especially confusions between the Y-organ and the mandibular organ (see Spaziani, 1990; Aiken, 1980; Lachaise *et al.*, 1993). The lack of adequate illustrations regarding the anatomical position of Y-organs have further compounded this problem. One of the few undisputed reports is the description of the brachyuran Y-organs as clearly discernable, discrete glands, which is accompanied by drawings of the location of these glands (see Lachaise *et al.*, 1993). Another good report gives the description of the position of the Y-organ relative to the mandibular organ in *Orconectes limosus*, and this is also supplied with a lucid sketch for orientation (Burghause, 1975). The only attempt to locate the Y-organs of a spiny lobster is documented in a very descriptive report by Paterson (1968). However, the emphasis on gross anatomical features, such as muscles and their points of insertion, without an adequate diagram to clearly mark the position of the Y-organ, has not made this report very useful. Therefore, in the current study on *J. lalandii*, careful attention was given to the location of the Y-organs and the best approach to remove the organs for

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in vitro incubations. It appears that the Y-organs of *J. lalandii* are larger and not as diffuse in structure as reported for clawed lobsters and crayfish (see for example, Lachaise *et al.*, 1993). Morphologically, the structure that I have removed and identified as the Y-organ of *J. lalandii* in the present study, matches that of Paterson (1968), except that the crescent-shaped Y-organ is yellowish, not whitish in appearance *in vivo*. To confirm my conclusions, several tissues in the cephalothorax have also been incubated and analysed for the production of ecdysteroids, but only the identified Y-organ tissues have given positive results in the EIA. It was with absolute certainty therefore, that these organs have been removed for *in vitro* biological assays.

The second consideration of the assay, viz. that of antibody selection to confidently recognise the produced ecdysteroids, was also carefully addressed in the current study. Characterisation of the circulating ecdysteroids had pointed to the likelihood of E, 3DE or both compounds being produced by the Y-organs of *J. lalandii*. To optimally bind these ecdysteroids in an EIA for their exact quantification, the RUD-2 antibody was used; this polyclonal antiserum was shown to have a high specificity towards both E and 3DE (Von Gliscynski *et al.*, 1995).

The Y-organ assays of the current study have clearly demonstrated the presence of a factor in the sinus glands of *J. lalandii*, which inhibits ecdysteroid synthesis by homologous Y-organs. The maximum inhibition of 50-60 % of the control Y-organs achieved by 1 SG, has also been reported by investigations on other species (e.g.

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Webster, 1986; Sefiani *et al.*, 1996; Terauchi *et al.*, 1996). This inhibition, however, appears to be dependant on the moulting stage of the Y-organ donor: crude extracts of sinus glands in the present study were unable to inhibit the synthesis of ecdysteroids in Y-organs that were obtained from post-ecdysis animals, i.e. postmoult, whereas significant inhibition was achieved in Y-organs from intermoult and premoult spiny lobsters (54 and 60 %, respectively). Variation in the response of Y-organs from prawns at different premoult stages to homologous sinus gland extracts have already been reported, where ecdysteroid synthesis in late premoult animals was inhibited by less than 10 % and mid-premoult animals were inhibited by 72 % (Sefiani *et al.*, 1996). A similar phenomenon was observed in the crab, *C. maenas* (Webster and Chung, 1999).

In the current study, the ecdysteroid titre in the haemolymph of *J. lalandii* was measured over different times of the moult cycles, viz. intermoult, premoult and postmoult. The results show a fluctation pattern that is very similar to that recorded in *P. vannamei* (Blais *et al.*, 1994), *O. limosus* (Willig and Keller, 1973) and *Callinectes sapidus* (Lee *et al.*, 1998) with the maximum concentration of ecdysteroids occurring during the premoult phase of the cycle and a decline in the titre evident already before ecdysis (late premoult). This change in circulating ecdysteroids in *C. sapidus* correlated to the levels of MIH mRNA in the eyestalk, thus affirming the model of moult control in crustaceans: MIH mRNA levels dropped during premoult and then increased in the postmoult stage, remaining high during intermoult (Lee *et al.*, 1998). Just after ecdysis (postmoult), the Y-organs of *J. lalandii* produce very low amounts

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of ecdysteroids when incubated *in vitro* and this is also reflected in the circulating ecdysteroid titre of the present study. The decrease in circulating ecdysteroid titres during the critical stages immediately before and after ecdysis is not brought about just by the influence of MIH, since this fluctuation in ecdysteroid titres are also observed in eyestalk-ablated lobsters in the present study and by others (Hopkins, 1983). Recent experiments have demonstrated that ecdysteroid production by the Y-organs of *O. limosus* is under negative feedback control by circulating ecdysteroids (Dell *et al.*, 1999). This might explain the observed drop in titre before ecdysis, whereas the observed low circulating titre of ecdysteroids immediately after ecdysis (a period from 3 days to about 3 weeks post-ecdysis was under observation in this study) is directly connected with the low production rates in the Y-organs. The contributing factors for this low production of ecdysteroids are not known at this stage; it does not seem to be MIH, since the postmoult Y-organs in the current study were not negatively influenced by sinus gland extracts. A possible reason why postmoult Y-organs are not responsive to crude extracts of sinus glands in the present study may be that these Y-organs are not yet expressing receptors for MIH. This was not investigated and remains speculation at this stage but which seems to be refuted by an earlier study on crabs. Specific receptors for MIH (and cHH) on the Y-organs of intermoult brachyuran crabs have been demonstrated and MIH peptides from 2 other brachyuran crab species could bind effectively to the *C. maenas* MIH receptor, which suggests that there is a high degree of conservation in the binding domains of the brachyuran MIH peptides (Webster, 1993). It was, surprisingly, reported that neither

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the density nor the affinity of the MIH and cHH receptors on the Y-organ changed significantly during the moult cycle of the crab, which may suggest that the incompetence of the Y-organ to respond to MIH/cHH during late premoult and early postmoult could be due to uncoupling of the signal transduction pathways (Webster and Chung, 1999).

In the current study, moult stage was estimated from the circulating ecdysteroid titres, coupled with observations of the condition of the exoskeleton. Previous studies have determined moult stages from various external, macroscopic changes in the exoskeleton, microscopic examinations of setal development on various appendages and measurement of the size of gastroliths (see Willig and Keller, 1973; Lyle and MacDonald, 1983; Chan *et al.*, 1988). Classically, the moult cycle of crustaceans is divided into four phases: (i) metecdysis (postmoult) = the period immediately following ecdysis; (ii) anecdysis (intermoult) = a period of tissue growth and accumulation of food reserves; (iii) proecdysis (premoult) = a period of active changes (morphological and physiological); (iv) ecdysis (moulting) = the actual act of shedding off the old exoskeleton (Drach, 1939). These phases were further subdivided by investigators, on the basis of observation of morphological changes during the moult cycle. Normal variations in this pattern arising from species differences, as well as the varied terminology used in the different studies, have left a rather confusing protocol for determining moult stage of crustaceans. It was for this reason that ecdysteroid titres in the haemolymph of *J. lalandii* were correlated with moult stage. As shown in

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the current study, the classic stages could be accurately and relatively easily be discerned on this basis but attempts were not made to further divide the moult stages. Intermoult spiny lobsters were preferred as Y-organ donors in the current study because of the clear inhibition effected by sinus glands on these organs. A typical saturation curve was achieved when crude sinus gland extracts of *J. lalandii* were applied in different doses to the Y-organs from intermoult animals. One SGE, the highest dose tested, effected a 54 % inhibition, whereas the lowest dose of 0.001 SGE did not have any effect on ecdysteroid synthesis. The lowest dose to have an inhibitory effect was 0.002 SGE with 6 % inhibition, whereas the first dose to elicit an inhibitory response that was significantly different to that caused by 1 SGE, was 0.025 SGE (25.4 % inhibition).

To identify the moult-inhibiting factor(s) from the sinus glands of *J. lalandii* in the current study, 1 SGE of HPLC-separated fractions were added to *in vitro* Y-organ incubations. Peak 3 (of chromatogram in Fig. 24) showed the most pronounced inhibitory effect (52 %), peak 4 also inhibited ecdysteroid synthesis, whereas peak 1 had a negligible effect. Peak 4 had also exhibited a significant hyperglycaemic effect and showed strong cHH immunoreactivity in the current study and was characterised as a cHH. Peak 3, on the other hand, had no effect in the hyperglycaemic bioassay and was the only peak that showed MIH immunoreactivity, although it was also recognised by one of the three different anti-cHH sera. Further, peak 3 displayed the mass characteristics of a typical MIH peptide, whereas peak 4 had a mass in the range

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of the cHH peptides. On this basis, peak 3 was identified as *Jala* MIH, the moult-inhibiting hormone of the spiny lobster. Crustacean hyperglycaemic hormone from other crustacean species have also exhibited moult-inhibiting activity, e.g. *C. maenas* (Webster and Keller, 1986), *H. americanus* (Chang *et al.*, 1990), *P. vannamei* (Sefiani *et al.*, 1996), *C. pagurus* (Chung *et al.*, 1996), *P. japonicus* (Yang *et al.*, 1996) and *P. clarkii* (Yasuda *et al.*, 1994). It was demonstrated in *C. maenas* that two different membrane-bound receptors occurred on the Y-organ: one type specifically bound MIH and the other bound cHH only (Webster, 1993). These results seem to implicate the direct involvement of cHH in the moulting cycle.

4.2.3. In vitro ovarian cultures

In crustaceans, vitellogenesis is under the control of an inhibitory peptide hormone, vitellogenesis- or gonad-inhibiting hormone (VIH/GIH), which is synthesised and released from the XO-SG complex. Vitellogenesis is a phase of the reproductive cycle that is dominated by yolk protein production and accumulation of these proteins into the oocytes. The accumulated lipoprotein, vitellin (Vt), is derived from a circulating precursor molecule, vitellogenin (Vg). By definition Vg is the extraovarian form of Vt (Fainzilber *et al.*, 1992); it has been demonstrated that both Vg and Vt of the prawn *Penaeus semisulcatus* consists of 3 polypeptide subunits (200, 120 and 80 kDa) but have a different lipid composition: by comparison with Vg, Vt carried more triacylglycerols and hardly any diacylglycerols (Lubzens *et al.*, 1997). While the hepatopancreas, haemocytes and adipose tissue are potential

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sources of Vg in other crustaceans (Kerr, 1969; Meusy and Payen, 1988), the ovary itself is a major site of Vg/Vt synthesis in prawns (Fainzilber *et al.*, 1989; Quackenbush, 1989; Browdy *et al.*, 1990; Shafir *et al.*, 1992). Whereas it is accepted that VIH mediates an effect by inhibiting Vg/Vt production, it is still not clear whether VIH acts only on the ovary or also on the extra-ovarian sources of Vg (see Thurn and Hall, 1999).

Over the years, a number of methods have been employed to identify the putative VIHs; to date, only 3 VIHs have been characterised and sequenced. The first VIH peptide was isolated from a lobster and functionally characterised in a heterologous *in vivo* assay where injections of the VIH peptide into shrimps significantly retarded the growth of their oocytes relative to control animals (Soyez *et al.*, 1987). In many of the larger decapod crustacean species, such as lobsters, spiny lobsters and crayfish, *in vivo* assays cannot be of a homologous nature because these species usually have prolonged reproductive cycles, with egg laying occurring only once per annum. Prawns and shrimps, with their much shorter reproductive cycles, are therefore used in heterologous assays. The second VIH peptide to be sequenced was isolated from a crayfish and identified by an *in vitro* heterologous assay where the incorporation of radiolabeled leucine into yolk proteins of a prawn was measured after treatment with the VIH peptide; it must be noted, however, that VIH activity was only achieved with an unphysiologically high dose of 10 SGE (Aguilar *et al.*, 1992). Both of these assays indicated that the VIH peptide was not acting in a species- or group-specific manner as has been reported for the cHH peptide (see Leuven *et al.*, 1982). Recently, a VIH was sequenced from a terrestrial isopod and biologically characterised in 2 homologous assays: (i) *in vitro* incubations of female adipose tissue with or without

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VIH and the determination of vitellogenin quantities by immunoprecipitation, and (ii) an *in vivo* assay based on the delay of the onset of vitellogenesis: in these assays female woodlice were maintained under environmental conditions that would naturally stimulate vitellogenesis; some of these females were then periodically injected with the VIH peptide while others received a Ringer solution and the time of egg-laying was monitored (Greve *et al.*, 1999). This assay took full advantage of the relatively short reproductive cycle of the woodlice.

In the current study an attempt was made to identify vitellogenesis-inhibiting peptides from the sinus gland of *J. lalandii* by utilising a heterologous *in vitro* assay in which the incorporation of radiolabeled amino acids into proteins of prawn ovaries was assessed. It has been shown that along the length of the prawn ovary, ovarian development is not uniform and that different regions of the ovary may exhibit different levels of protein synthesis (Thurn and Hall, 1999). Therefore, to remove any bias from the study, ovary tissue pieces were randomly placed into the tissue culture wells and each substance (crude extract or purified peptide) was tested in duplicate. Vt was synthesised *de novo* in the ovary pieces (as judged by the electrophoretic mobility of proteins, which was analysed using gel electrophoresis under denatured conditions, followed by autoradiography) and this synthesis was inhibited in a dose-related manner by the addition of crude extracts of sinus glands from *J. lalandii*. Although this showed that sinus gland peptides from a spiny lobster could elicit a

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biological effect on penaeid prawn ovaries at a physiological dose, the inter-species difference was evident in the different doses of heterologous (*J. lalandii*: 2 SGE) versus homologous (*P. semisulcatus*: 0.1 SGE) extracts required to achieve the same percentage of inhibition. The lowest dose of *J. lalandii* sinus gland extracts tested in these assays, viz. 0.001 SGE, did not inhibit *de novo* protein synthesis in the prawn ovaries and the lowest dose to produce a significantly different effect from the maximum dose of 2 SGE, was 0.1 SGE. These experiments further showed that the crude extracts of sinus glands, regardless of its origin (i.e. homologous or heterologous), inhibited total protein synthesis and not specifically just Vt. This general inhibition of ovarian protein synthesis was also reported from other studies (Khayat *et al.*, 1998; Thurn and Hall, 1999).

Not all the cHH-like peptide fractions separated from sinus gland extracts of *J. lalandii* could inhibit *de novo* protein synthesis in the prawn ovaries. Applied at a dose of 1 SGE, peak fractions 1 and 2, corresponding to truncated *Jala* cHH-I and -II (see chromatogram in Fig. 37), did not have any effect in the assay, whereas the peak fractions corresponding to *Jala* cHH-I, *Jala* cHH-II and *Jala* MIH resulted in inhibition of protein synthesis. *Jala* cHH-II caused the highest inhibition (49 %), which was significantly different to the effect of peak 4 (34 %) and *Jala* cHH-I (20 %). The inhibitory effect of cHH-II approximates the maximum inhibition observed with 1 SGE of crude extracts of *Jala* sinus glands, whereas *Jala* MIH inhibited protein synthesis in the prawn ovaries by about only 15 %. It seems therefore, that

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VIH activity, as defined in the present study to inhibit vitellogenin/vitellin synthesis in the ovaries, is not associated with a unique peptide of *J. lalandii* and that *Jala* cHH-II (the minor isoform of hyperglycaemic hormone) has greater VIH activity than cHH-I (the major isoform). Furthermore, the unidentified/uncharacterised peak 4 also shows significant VIH activity in this heterologous bioassay, whereas MIH has only a small effect of less than 20 % inhibition. Peptides belonging to the cHH family from *P. japonicus* were also shown to affect protein, as well as mRNA synthesis in *in vitro* incubated ovaries from *P. semisulcatus* (Khayat *et al.*, 1998). The results from the current study are in agreement with this previous study, viz. that the cHH peptides substantially inhibited protein synthesis in the ovary while the MIH peptide had only a slight effect. In the current study, none of the selected peptides elicited a stimulatory response on ovarian protein synthesis, which signifies that a gonad stimulating hormone (GSH) was not included among the studied peptides. The absence of a GSH was similarly shown among the cHH/MIH/VIH peptides of *P. japonicus* (Khayat *et al.*, 1998), whereas a GSH in the sinus glands of *P. bouvieri* was reported to enhance protein synthesis in *P. vannamei* by 300 % (Huberman *et al.*, 1995).

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4.3. Sequence elucidation of cHH/MIH/VIH peptides from J. lalandii

Peptides belonging to the cHH/MIH/VIH peptide family from *J. lalandii* were identified in an ELISA with antisera raised against cHH, MIH and VIH peptides from other decapod crustacean species. Mass spectrometry on aliquots of these

immunoreactive peaks revealed that the single HPLC purification step had yielded pure or uncontaminated peaks, since only one mass was obtained per fraction. Further, mass analyses revealed that most of the immunoreactive peptides were in the 8-9 kDa mass range, while 2 others were around 7.6 kDa. All the immunoreactive peaks were tested in biological assays (see above) and were selected for primary sequence elucidation. This was successfully achieved with non-reduced peptide material; linearisation by means of breaking the disulfide bonds was only carried out on a micro-scale with selected peptide fragments and analysed by mass spectrometry to confirm the assignment of the sequences. In this way, less starting material could be used to elucidate the full sequence of a peptide compared with studies that followed the conventional strategy of reducing the disulfide bonds prior to sequencing. This novel approach that I employed with success in the current study, relied heavily on accurate mass spectrometric determinations and reliable software for calculating and predicting peptide mass following various manipulations, such as enzymatic digestion and chemical cleavages. With the aid of a MALDI-TOF instrument and a GPMAW software package (see Materials and Methods), I was able to implement my strategy to assist in the elucidation of the primary sequence of 5 cHH-like peptides from *J. lalandii.*

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4.3.1. Crustacean hyperglycaemic hormones

From a typical separation of *J. lalandii* sinus glands in the current study, four peaks were recognised by anti-cHH sera, and only two of these displayed biological activity

in a hyperglycaemic assay.

4.3.1.1. Jala cHH-I

The most abundant peptide from *J. lalandii* sinus glands (100 ng/sinus gland = 12 pmol), peak 4 (of chromatogram in Fig. 24), had displayed strong hyperglycaemic activity, as well as MIH and some VIH activity in biological assays of the current study. By omitting the step of reducing the disulfide bonds known to be conserved in these peptide members, the complete sequence was obtained from material purified from only 387 SGs, whereas previous studies needed between 600 and 6000 SGs (Yang *et al.*, 1995; Huberman and Aguilar, 1988; Chang *et al.*, 1990; Huberman *et al.*, 1993). Mass spectrometry played an important role in assigning the sequences and was particularly efficient because accurate mass spectra could quickly be obtained with sub-picomole quantities. Figure 81 gives the complete cHH-I sequence from the spiny lobster *J. lalandii* and compares it with the major cHH structures from the lobster *H. americanus*, the crayfish *P. bouvieri*, *P. clarkii* (Yasuda *et al.*, 1994) and *O. limosus* (Kegel *et al.*, 1991), as well as the crab *C. maenas* and *C. pagurus*, the prawn *P. japonicus*, *Macrobrachium rosenbergii*, *Penaeus monodon*, *Metapenaeus ensis* and *Penaeus schmitti*. Also included in this comparison is the partial sequence of cHH-I isolated from *J. lalandii* (Yasuda, 1997), which was published while my own work was in progress. The complete *Jala* cHH-I structure is, on average, 76 %, 74 %, 65 % and 60 % identical to lobster, crayfish, crab and prawn species listed in Fig. 81. Interestingly, in one aspect *Jala* cHH resembles more the cHH of prawns and isopods,

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despite its overall lower homology to this group: it contains a free N-terminus, whereas other cHHs have an N-terminus blocked by pyroglutamate. The widely-accepted theory about an N-terminally blocked peptide concerns the increased stability and biological activity of blocked versus unblocked peptides. However, this hypothesis was recently challenged by the finding that the minor cHH isoform of *C. maenas* has a free N-terminus and displays identical biological activity and rates of release and degradation compared with the N-terminally blocked major cHH isoform of the crab (Chung and Webster, 1996).

A comparison of the complete *Jala* cHH-I structure presented here and that of the previously determined partial *Jala* cHH-I structure (residues 1-46; Yasuda, 1997) shows distinct differences (residue mass given in brackets below):

S^{17} (87) versus G^{17} (57), H^{33} (137) versus P^{33} (97), R^{41} (156) versus Q^{41} (128). The sum of the mass differences between these pairs of residues is 98 Da; this may account for the 100 Da mass difference recorded for *Jala* cHH-I in each case (8380 in the present study and 8280 reported by Yasuda, 1997). Since the mass of the partially sequenced *Jala* cHH-I can be substantiated by the 3 amino acid substitutions above, it does not seem likely that sequencing errors have occurred. On the other hand, the unequivocal evidence in the present study shows that the complete *J. lalandii* sequence is correct: (a) S^{17} was clearly identified in at least two attempts of Edman degradation of native cHH-I peptide, (b) H^{33} was confirmed by sequencing overlapping tryptic and endo-Asp N-generated peptide fragments; it is also known that trypsin will not cleave when Lys is followed by Pro (Croft, 1980; Tensen *et al.*,

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1991), and (c) R^{41} was first deduced from a mass difference of 156 between a partial- and a fully digested cHH-I sample, and was then confirmed by sequencing data from an endo-Asp N digest fragment, as well as from the sequencing information of the truncated cHH-I peptide (peak 1 in Fig. 24) which yielded residues 1-62 in Edman degradation (see 4.3.1.2. below). I therefore conclude that the partial cHH sequence (Yasuda, 1997) may not have been generated from SGs isolated from *J. lalandii* specimens but from another spiny lobster species whose cHH may be as much as 93 % homologous to that of *J. lalandii*. Since *J. lalandii* is an endogenous species of the west coast of South Africa, it seems likely that some other species may have been used in the studies by Yasuda. It can be speculated to be a Japanese spiny lobster but proper identification of the species would help to get insights into the phylogenetic relationship between the genera of spiny lobsters. It would be very interesting, indeed, to have this particular species properly identified. In the current study, 2 other spiny lobster species which occur locally along the South African coasts have been included in preliminary investigations. Tissue immunocytochemistry with eyestalk sections from *Panulirus homarus* indicates that, like in the case of *J. lalandii*, there is sufficient homology between cHH/MIH/VIH peptides of this spiny lobster and those from a brachyuran crab, and 2 astacideans. From ELISAs on crude extracts of sinus glands, however, it became clear that the extracts from *J. lalandii* gave more intense reactions with the different antisera than did extracts from *P. homarus*. This would suggest that the *J. lalandii* peptides have a greater sequence identity with the known peptides than do the peptides from *P. homarus*. The current study did not provide any comparative

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peptide sequence information from *P. homarus* to corroborate the results of the immunocytochemical study, but extracts of sinus glands from a third spiny lobster species, *Palinurus gilchristii*, were prepared and fractionated in the same manner as for *J. lalandii*. Mass analyses and immunological investigations by ELISA of the resulting hydrophobic peak fractions were carried out. In this way, a putative cHH was identified and subjected to Edman degradation. The partial sequence of the putative *Pagi* cHH peptide (residues 1-30) is 100 % identical to *Jala* cHH-I. This was a rather surprising result because the chromatographic behaviour and the mass data of *Pagi* cHH differed to that of *Jala* cHH-I: the putative *Pagi* cHH displayed greater hydrophobicity (eluting about 4 min later than *Jala* cHH-I on RP-HPLC) and is also 143 mass units heavier. It is my belief, therefore, that the primary structure of *Pagi* cHH will show some differences to *Jala* cHH-I in the remaining part of the peptide. Homologous biological assays for hyperglycaemia could not be carried out because there is no laboratory access to live specimens of *P. gilchristii*, but the relative abundance of this peptide, judging from peak areas on the HPLC chromatogram (Fig. 74), is a strong indication that this peak is indeed a cHH. In cHH isolated from crayfish species, such considerable sequence identity has been reported, for example: *P. bouvieri* and *P. clarkii* have identical sequences, while *O. limosus* differs from them by only one amino acid substitution (Kegel *et al.*, 1991; Huberman *et al.*, 1993; Yasuda *et al.*, 1994). This reflects the evolutionary relationship between the crayfish species; similarly, the identical peptide sequences of *J. lalandii* and *P. gilchristii* also reflects their close ancestry in the infraorder Palinuridae. In this context it is necessary

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again to reflect on the data of Yasuda (1997); certainly by sequence identity, the species he studied appears to be a palinurid lobster but cannot be *J. lalandii* or *P. gilchristii*. His data, and information from the current study reveals that 3 out of the 49 palinurid species listed by Phillips *et al.*, (1980) have cHH peptide sequences that are more than 90 % identical. Studies have been undertaken on the relationships of the genera within the Palinuridae, and the evolution of these spiny lobsters (see George and Main, 1967) which propose that the genus *Jasus* evolved independently from the genera *Palinurus* and *Panulirus*, and that *Jasus* had evolved first, while *Panulirus* evolved more recently. It would be interesting to study the phylogenetic relationships of the Palinuridae genera at the level of neuropeptides, specifically cHH and preprocHH to see if it supports the proposed relationships. Practically, such a study would be feasible because: (i) a total of 27 species of the genera *Jasus*, *Palinurus* and *Panulirus* are commercially-fished in the world's oceans (see Phillips *et al.*, 1980) and an adequate supply of sinus gland material could therefore be available; (ii) cHH is the most abundant neuropeptide in the sinus gland and (iii) can be recognised immunochemically by antisera raised against cHH from astacideans.

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In a number of decapod crustacean species, cHH isoforms with the same peptide sequence and molecular mass have been reported. These isoforms elute from HPLC as doublet peaks and they differ only by the configuration of the third amino acid residue, i.e. either L- or D-Phe[3] (*P. bouvieri*, Aguilar *et al.*, 1995; *O. limosus*, De Kleijn *et al.*, 1994a; *H. americanus*, Soyez *et al.*, 1994; *P. clarkii*, Yasuda *et al.*, 1994 and Yasuda,

1997). To investigate the possibility of such conformational polymorphism in *J. lalandii*, the N-terminus of *Jala* cHH-I was studied to assess whether the third amino acid was L-Phe^3 or D-Phe^3. This was done in an indirect manner: (i) two synthetic peptides were made that corresponded to the first 8 amino acid residues of *Jala* cHH-I but one with L-Phe^3 and the other with D-Phe^3. These synthetic peptides had the same mass but distinctly different retention times on RP-HPLC. (ii) The native N-terminal octapeptide of *Jala* cHH-I had been sequenced earlier in the current study as one of two peptide chains from the tryptic fragment T26 and could then be isolated from this fragment by reducing and alkylating the disulfide bonds between Cys^7 and Cys^{43}. (iii) The retention times on HPLC of the isolated native octapeptide was compared with that of the synthetic L- and D-Phe^3 octapeptides. The results indicate that the octapeptide fragment from *Jala* cHH-I is a homogenous peak consisting of peptide material with L-Phe^3 only.

4.3.1.2. Jala cHH-II

Peak 5 (see Fig. 24) was identified as a second and minor isoform of cHH (molecular mass = 8357 Da) in *J. lalandii*, which also displayed significant VIH activity. With a yield of only 3 pmol of cHH-II per sinus gland and greater hydrophobicity of this peptide, considerably more material had to be purified to obtain the full primary structure than what was needed for cHH-I. It is clear from sequence comparisons of the two *Jala* cHH peptides that the nature of the cHH heterogeneity in the spiny lobster is clearly genetic in origin and not due to post-translational modifications. This

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difference in cHH isoforms, as well as the free N-terminus of cHHs in the spiny lobster has also been observed in prawns (Sefiani *et al.*, 1996; Yang *et al.*, 1997; Gu *et al.*, 2000) and differs from the situation in true crabs, true lobsters and crayfish (Soyez *et al.*, 1994; Yasuda *et al.*, 1994; Aguilar *et al.*, 1995; Chung and Webster, 1996; Chung *et al.*, 1998).

The 7 amino acid substitutions in the primary sequence of *Jala* cHH-II (Fig. 81), perfectly accounts for the observed mass difference of 23 Da between *Jala* cHH-II and *Jala* cHH-I, and the two peptides are thus 90 % identical to each other. In the only other study on cHH peptides from a palinurid species, two isoforms were reportedly also isolated from *J. lalandii* and partially sequenced (residues 1-46; Yasuda, 1997; Fig. 81) during the time when my own work was in progress. There are, however, discrepancies between sequencing data emanating from the afore-mentioned study and the current study (see also 4.3.1.1. above). Yasuda (1997) has reported the cHH peptides to have a mass of 8280 and 8258 Da, respectively for cHH-I and –II: thus, 100 Da less for each peptide compared with the data for *Jala* cHH-I and –II of the current study. The 100 Da mass difference between *Jala* cHH-II of the current study and the partial cHH-II of Yasuda (1997) can, however, not be explained by the observed 3 amino acid substitutions (residue mass given in brackets): K^{17}(128) versus Q^{17}(128); V^{21} (99) versus L^{21}(113); S^{45}(87) versus G^{45}(57). Since the sum of the mass differences between these pairs of residues is only 16 Da, we predict that further substitutions will be discovered in the complete sequence of

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the cHH-II peptide reported by Yasuda (1997). In fact, the mass difference of 22 Da between the partial cHH-I and –II peptides (Yasuda, 1997) can also not be explained by the amino acid substitutions and substantiates my prediction of further sequence changes. With the complete elucidation of *Jala* cHH-II in the present study, it appears that the partially sequenced cHH peptides (Yasuda, 1997) were not isolated from *Jasus lalandii* but from another, perhaps closely-related species. The accuracy of the assignment of the amino acid sequence of *Jala* cHH-I and –II is confirmed by the sequencing data of the truncated cHHs (see 4.3.1.3. below), where the uninterrupted N-terminal sequencing of the native peptides up to residues 62 and 60, eliminated the opportunity for misinterpretation of the primary structural data.

A comparison of the complete *Jala* cHH-II structure with cHH structures of other decapod crustaceans, shows 76-82 %, 63-67 %, and 42-71 % sequence identity with cHHs from true lobster and crayfish, true crab and prawn species, respectively (Fig. 81). Clearly, cHH is a very conserved molecule, with many stretches of conserved residues throughout most of the molecule; this could account for the observed cross-reactivity in the immunocytochemical component of the current study.

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4.3.1.2. Truncated Jala cHH peptides

In addition to the two biologically active cHHs, two other peptides (peaks 1 and 2 in Fig. 24) were isolated from the sinus glands of *J. lalandii* in the present study. Although these peptides were recognised by the anti-*H. americanus* cHH serum, their

lack of hyperglycaemic activity, mass of 7000+ Da and their less-hydrophobic behavior in RP-HPLC indicated that they could not be true cHHs as defined earlier (see Introduction). Sequencing data shed some light on this puzzle: the two peaks had exactly the same amino acid sequence as the *Jala* cHH-I and *Jala* cHH-II peptides, except that they were truncated at residue Y^{65} and were, therefore, referred to as truncated cHH-I/-II. This sequence identity with the *Jala* cHHs explained why the anti-cHH serum could recognise epitopes on the truncated molecules, whereas the truncation itself may be the reason for the lack of biological activity of the truncated cHHs. It is not presently possible to identify the exact region of the cHH molecule that is responsible for functional activity, since there are no synthetic or genetically-modified variants of cHH available as yet. However, since the truncated cHHs in the present study could not induce hyperglycemia under the same assay conditions as the non-truncated cHHs, one could speculate that the amino acid residues from position 66-72 may be important in this regard. The potential role of the C-terminus of cHH in interacting with the cHH receptor has been alluded to before (Huberman *et al.*, 1993); this theory, based on the relative degrees of homology of cHH (especially between residues 61 and 68) from species belonging to different infraorders, seems to be supported by results of interspecies bioassays for hyperglycaemia. In such bioassays, crude extracts of eyestalks or purified peptides isolated from one brachyuran species could induce hyperglycaemia in another brachyuran species but failed to elicit such a hyperglycaemic effect in an astacidean species, and *vice versa* (Kleinholz and Keller, 1973; Keller *et al.*, 1985). Thus, the biological specificity of cHH was demonstrated at

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the infraorder level. This rule, however, cannot always be strictly applied: extracts of eyestalks from a palinuran spiny lobster, *Palinurus vulgaris*, significantly raised the glucose concentration in the astacidean crayfish, *Astacus leptodactylus* (Leuven *et al.*, 1982). Indeed, when we examine the C-terminal region (residues 61-72) of the cHH molecules in Fig. 81, there is a high degree of sequence identity between the palinuran and astacidean species, viz. 66-83 %, compared with 42-50 % identity between the astacurans and brachyurans. This could explain the observations made by Leuven *et al* (1982) from their bioassays. Biological differences of cHH isoforms are also evident within the same species. For example, dose-response relationships of the five cHH peptides of *P. japonicus* showed that, although they were equally potent in homologous bioassays, *Peja* cHH-VI had a considerably higher efficacy, thus producing a higher increase in glucose levels than did *Peja* cHH-III (Yang *et al.*, 1997). Since these two peptides of the prawn share an overall identity of 68 % but are only 50 % identical in the C-terminal region of residues 61-72, one can assume that this region may well affect the efficacy of the cHH molecule.

Also, when one considers the change of charge in the cHH molecule in this region (residues 61-72; Fig. 81), either by suppression or introduction of charged residues, one finds that there is no change between the spiny lobster and the crayfish/true lobster. On the other hand, the introduction of a positive charge is the net result of amino acid substitutions between brachyurans and astacideans/palinurans, brachyurans and penaeids, and between astacideans/palinurans and penaeids. Thus, the variation in

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the C-terminal portion of the cHH molecule from the different infraorders, may explain some of the observed functional differences through lack of receptor recognition or inability of the cHHs to bind to the receptor due to strategic structural differences (amino acid substitutions) in that region. This can, however, only be unequivocally shown when suitably modified, synthetic cHH peptides are available for cross-injection experiments across the different infraorders. Some progress towards the synthesis of cHH/MIH/VIH peptides have been reported: the ion-transporting peptide of the locust *Schistocerca gregaria* has been completely chemically synthesized, with the correct disulfide linkages and C-terminal amidation (King *et al.*, 1999); using recombinant DNA technology, prawn MIH was successfully expressed in *Eschericia coli* to produce a large quantity of biologically active hormone (Ohira *et al.*, 1999), recombinant low molecular weight protein (LMWP) from the spider, *Latrodectus mactans tredecimguttatus* has also been engineered and expressed in *E. coli* (Gasparini *et al.*, 1994) and prawn cHH was recently expressed in bacterial cells and recovered with biological activity (Gu *et al.*, 2000). Nevertheless, the importance of the C-terminus of cHHs for biological functioning is also implied by the negative results of the truncated cHHs in various biological assays in the current study. For example, the lack of MIH activity of truncated *Jala* cHH-I on Y-organs, and the failure of both truncated *Jala* cHHs to inhibit protein synthesis in the ovary.

Finally, since both cHH peptides are truncated at a Tyr residue in *J. lalandii*, chymotryptic activity is implicated in this mode of truncation. The present study has shown that the addition of PMSF, which inhibits serine proteases, such as

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chymotrypsin, and an anti-oxidant, such as thiodiglycol, could lower the incidence (ratio) of truncation. It is our opinion that the *Jala* cHHs are susceptible to protease activity, and that this degradation can be minimised during extraction procedures by the addition of protease inhibitors.

4.3.1.3. Jala MIH

In this study, I report on the characterisation and complete sequence elucidation of a neuropeptide with MIH activity, which was isolated from the sinus gland of the palinurid lobster, *Jasus lalandii*. This peptide (peak 3 in Fig. 24) was identified as a moult-inhibiting hormone by means of (i) a homologous *in vitro* biological assay, (ii) immunoreactivity with anti-MIH serum, and (iii) mass and sequence homology to previously identified MIHs. It is, therefore, referred to as *Jala* MIH in the current study. The peptide has 74 amino acid residues, a free N-terminus, is amidated at the C-terminus and did not demonstrate any hyperglycaemic activity in an *in vivo* homologous bioassay, although the previously-identified hyperglycaemic hormone *Jala* cHH-I (peak 4 in Fig. 24) was shown to have an inhibitory effect on ecdysteroid synthesis in this study. Such a functional complexity of cHH is not unknown and has been described for several species (Van Herp, 1998; Webster, 1998).

A single HPLC purification step yielded sufficiently pure material of *Jala* MIH which is a relatively less-hydrophobic peptide than the 2 cHHs found in this species (see Fig. 24). *Jala* MIH was uniquely recognised and bound in an ELISA by an antiserum raised against the MIH peptide of *C. pagurus*. The complete primary sequence of *Jala* MIH was obtained from a combination of automated sequencing and MALDI-TOF

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mass analyses of peptide fragments generated by enzymatic digests and cleavages of the native, non-reduced peptide material. Several anomalies are apparent when we consider the HPLC and sequence data of a number of these fragments. Some of the anomalies can be explained by incomplete enzymatic digestions due to inaccessible cleavage sites and possibly, steric hindrances brought about by the conformation of the molecule around the 3 disulfide bridges (consider the tryptic fragments T15, T17 and T18, as well as the endo-Asp fragments D17 and D20 in Fig 51A, B and the data in Table 7). In the case of tryptic fragments T1 and T3, the molecular mass and amino acid sequences are identical to each other, yet they exhibited different retention times on HPLC. This may be explained by H^{60} being differently protonated in the 2 fragments. The 4 distinct peak fractions observed in RP-HPLC after cleavage with CNBr (peaks 4-7, Fig. 51C) yielded identical amino acid sequences which corresponded to the C-terminus of the MIH peptide. Their differential elution can be explained by a persistent mass difference of 28 units in the case of peak fractions 4, 6 and 7 which can be accredited to the addition of a formyl group (27.995 Da) and multiples thereof due to the use of 70 % formic acid in the cleavage mixture; the difference of 14 mass units from peak fraction 5 is inexplicable.

Amidation of *Jala* MIH was definitively established by methylation and subsequent MALDI-TOF analyses of the native fragment D9, which yielded a mass shift characteristic of an amidated peptide, as was shown by the identical behaviour of the synthetic amidated counterpart of D9. The average molecular mass measured for the

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entire *Jala* MIH peptide, i.e. 9006 Da, fits well with the mass calculated from the amino acid sequence of the amidated MIH peptide.

Figure 82 shows the alignment of the amino acid sequences of the putative *Jala* MIH neuropeptide with those from other crustacean species either obtained by means of peptide sequencing or deduced from the nucleotide sequences. It must be noted that in the latter case, the peptide itself was never isolated and its biological activity was, hence, also not established for several species; function was merely inferred from sequence identity with other peptides of known MIH activity. Further, gaps in the amino acid sequence of some of the peptides have been introduced in Fig. 82 to align all the Cys residues and to allow for a maximal degree of identity. It is clear that all the putative MIH peptides thus far isolated from the brachyuran crabs conform to a distinct type, viz. 78 residues long, free N- and C-terminus, 6 Cys residues in conserved positions and sequence identity in excess of 70 %. From the other infraorders, viz. Penaeidae (represented by 3 species) and Astacidea (represented by 2 species) there is no structural conformity of the MIH peptides. In the former group, all 3 putative MIH peptides apparently have free N- and C-termini, but range in length from 72-77 residues with sequence identity of between 30 and 79 %. Whereas the Cys residues from *P. vannamei* MIH are not in the positions typical for the brachyuran MIHs, but rather in the positions typical of the cHH peptides, those from the other 2 penaeid species conform to the brachyuran type. Concerning the 2 astacidean MIH peptides, both are C-terminally blocked, whereas only 1 is N-terminally blocked. The

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latter, isolated from *P. bouvieri*, is a 72 residue peptide with 90 % sequence identity to the hyperglycaemic peptide found in that species but which is only active in the MIH bioassay (Aguilar *et al.*, 1996) while the former was isolated from *P. clarkii* and is more typical of the brachyuran MIHs with 44 % sequence identity to *C. maenas* MIH. The present study supplies the first report on the structure of a putative MIH in Palinuridae. The 74 residue amidated peptide shares 36-43 % sequence identity with other putative MIHs and is most similar to *P. clarkii* MIH (Fig. 82) and has 32-34 % identity with the 2 cHH peptides isolated from *J. lalandii* (Fig. 83). Unique to *Jala* MIH is the location of the first Cys residue at position 6 in the sequence. In both, the cHH and MIH peptides from other crustacean species, the first Cys residue occurs at position 7 and a shift in the Cys residues between the 2 types of peptides occurs with the second Cys residue (C^{23} in the case of cHHs versus C^{24} in the case of MIHs). This apparent atypical alignment of the Cys residue in *Jala* MIH may be attributed to a deletion on the gene encoding this peptide, but this cannot be verified yet. The structural data, however, presented here of a MIH from a palinurid species, supplies further information that the highest interspecific similarity of the molecule is found in discreet regions: (i) closely clustered around the Cys residues and (ii) in the C-terminal 7-10 amino acid residues (a hexapeptide with the sequence WXXILXA) which may point to the importance of these regions in maintaining the conformation of the entire molecule and may suggest that the C-terminal region is not involved in any species-specific activity of the MIH peptide. There is tentative evidence that the C-terminal region of the cHH molecule, contrary to MIH, may be important not only for

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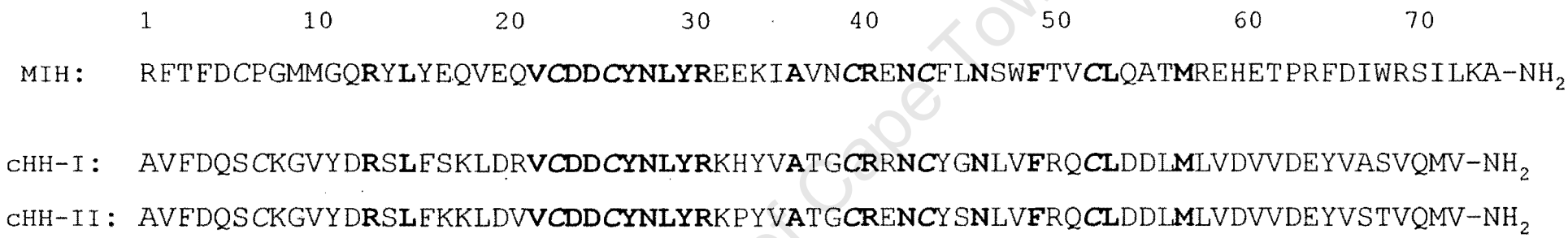

Fig. 83. Comparison of the amino acid sequence of *Jala* MIH and that of the hyperglycaemic peptides found in *J. lalandii*: *Jala* cHH-I and *Jala* cHH-II. Identical residues are indicated in bold.

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functional activity *per se* but also for functional differences between different infraorders, as discussed in 4.3.1.2. The current study has shown that C-terminally truncated cHHs (peaks 1 and 2 in Fig. 24) from *J. lalandii* do not elicit hyperglycaemia in homologous bioassays *in vivo* nor do they affect protein synthesis in a heterologous *in vitro* assay, and that truncated *Jala* cHH-I also does not inhibit ecdysteroid synthesis *in vitro*, which reaffirms the importance of the C-terminal part of cHH for functional activity. The question of how the cHH molecule can elicit multiple functions, and which exact regions of the cHH and MIH molecules are important for biological activity remain, as yet, unanswered.

4.3.1.4. The atypical 8400 Da peak

The fractionation of acetic acid extracts from *J. lalandii* sinus glands on RP-HPLC provided a typical separation profile of five clear peaks in the hydrophobic elution zone (see Fig. 24for example). However, atypical separations, resulting in 6 or more peaks in this region, were suddenly and inexplicably obtained (see Fig. 37). The only possible natural contributing factor may be the season during which these glands were collected: ordinarily, sinus glands were prepared *en masse* during the austral summer months of December to March. The sinus gland extracts that resulted in the atypical separations were collected and prepared during the austral autumn and winter period (April to August). During this time the South African spiny lobsters moult and the females enter the reproductive stage (Heydorn, 1969). It is possible, therefore, that the changed physiological status of the animals may also be reflected in the profile of

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their neuropeptide content in the sinus gland. This would then mean that the 8400 Da peak is naturally occurring only at certain times in the life-cycle of the spiny lobster. On the other hand, the different elution pattern may have resulted from degradation processes induced and perpetuated by certain unnoticed laboratory conditions over that time, e.g. old homogenisation buffers, oxidation of peptides, protease activities due to incorrect handling of the glands. It must be said, however, that dissection, extraction and HPLC conditions were kept as uniform as possible over time.

Peak 4 (Fig. 37) eluted just before MIH and was characterised to have a protonated molecular mass of 8400 Da, thus, 19 mass units heavier than *Jala* cHH-I and 607 mass units lighter than *Jala* MIH. Does this peak represent breakdown material of the MIH peptide or the cHHs? If the latter, then how does one reconcile the observed increase in mass with degradation? This would only be a feasible option if oxidation of the Met^{71} residue of *Jala* cHH-I has taken place; such a modification would increase the mass by 16 mass units and further, render the peptide less hydrophobic. N-terminal sequencing of the native 8400 Da peak revealed that the 52 amino acid residues of the partially sequenced peptide, are identical to that of *Jala* cHH-I, a clear indication that this peak is not directly linked to *Jala* MIH. A small portion of the 8400 Da peak material was subjected to digestion with trypsin and the resulting HPLC chromatogram was compared with that of *Jala* cHH-I to see if there were any unique fragments. This was coupled with mass spectrometry on the fragments. The results show that a hydrophobic tryptic fragment of peak 4, of co-incident retention time with

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the C-terminal fragment of *Jala* cHH-I, displayed a mass of approximately 18 mass units more than the corresponding fragment of cHH-I. This could be interpreted as evidence for the theory about Met-oxidation of *Jala* cHH-I, since Met^{71} is contained in these C-terminal fragments.

Functionally, the 8400 Da peak was tested in an *in vitro* assay to assess its ability to inhibit protein synthesis in prawn ovaries: the peak displayed substantial inhibitory activity, as did the full-length cHHs but not the truncated cHHs. This indicates that if this is indeed a modified *Jala* cHH-I, then the modification does not interfere with some of its biological activities. Unfortunately, there was not sufficient material to test in a hyperglycaemic assay, or to complete the sequence elucidation. It is my expectation, from the above evidence, that this spurious peak represents the Met-oxidised form of *Jala* cHH-I and is merely an artefact arising from handling of the sinus gland material. However, one should keep an open mind and not merely dismiss the idea that this peak 4 might represent a novel, seasonally-expressed peptide without first conducting further investigations into the nature, the functional potential and the full sequence of this 8400 Da peak.

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4.4. Sequence elucidation and characterisation of non-cHH/MIH/VIH peptides

Crude extracts of sinus glands prepared from *J. lalandii* were subjected to RP-HPLC in order to separate the cHH/MIH/VIH peptide family peaks in the current study. In the process, the less-hydrophobic peptide material was not well separated but were

collected, pooled and dried for further separation. The gradient conditions were changed to achieve this subsequent purification. From mass spectrometric analyses, it became clear that this purification step was not sufficient and more separation steps were carried out with different ion-pairing reagents to resolve peaks with different ionic characteristics. Peaks within defined regions of the separation chromatograms, and certain pure, individual peak material were tested in chromatophoric biological assays.

4.4.1. Red pigment-concentrating hormone (RPCH)

After the first re-purification of the less-hydrophobic sinus gland material, the chromatogram could be divided into 5 regions of interest (see Fig. 61). Material from all of these regions was redissolved in sea water and 1 SGE was injected into dark-adapted shrimps (*Palaemon pacificus*). Only peak material from the first region of interest on the chromatogram had a blanching, i.e. pigment-concentrating, effect on the shrimps. MALDI-TOF analyses revealed that this region contained more than one peptide, which was confirmed by a subsequent HPLC step. Peak fraction 1 from this purification (Fig. 62A) was identified as RPCH by mass analyses, biological activity and co-chromatography with synthetic RPCH. The primary sequence of this peak was obtained following digestion with a pyroglutamate (pGlu) aminopeptidase. The presence of the suspected pGlu residue at the N-terminus was confirmed by mass spectrometry on the peptide before and after treatment with the amino peptidase. The amino acid sequence of *Jala* RPCH is not a novel structure and is 100 % identical to

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RPCHs previously isolated from other crustaceans. Hence, structural evidence from a variety of decapod crustacean infraorders to date (caridean, penaeid, astacidean, palinuran and brachyuran) shows that RPCH is a highly conserved peptide in crustaceans and is only present in one form (see Table 1). This differs remarkably from the structurally homologous AKH peptides in insects, which can occur as an octa-, nona-, or decapeptide; most insect species have more than one isoform of AKH, arising as distinct gene products and not resulting from post-translational modification (see Gäde, 1997), although the latter has been shown as well (Gäde *et al.*, 1992)

Despite attempts in the current study, there was no success in isolating a neurohormone with pigment-dispersing activity from the sinus glands of *J. lalandii*. This antagonist to RPCH readily causes a darkening of light-adapted shrimps by stimulating dispersion of the pigments in epidermal chromatophores. In addition, PDH causes the translocation of pigment in extraretinular cells of the crustacean compound eye to provide a protective screen under light conditions (Rao and Riehm, 1993). In the current study, crude extracts of sinus glands and purified peptide peak fractions from *J. lalandii* sinus glands were assayed for PDH activity in light-adapted shrimps. Only injections of synthetic PDH resulted in pigment dispersal of the epidermal chromatophores in the present study. Furthermore, mass analyses of *Jala* sinus glands and purified peptides from these extracts did not reveal the presence of a typical PDH entity (mass approximately 1900 Da) at all. It was, thus, concluded that the hot acid extraction procedure of the present study is not conducive to maintaining the chemical

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integrity of a PDH. Evidence that the PDH molecules are susceptible to oxidation and proteolytic enzymes has been documented (see Josefsson, 1983). But is there a biological relevance for PDH in the spiny lobsters? The characteristic colours of lobsters are due to carotenoids, mainly astaxanthin, that occur in the pigmented layer just beneath the epicuticle of the exoskeleton (see Phillips *et al.*, 1980), and unlike other non-translucent crustaceans, such as crabs and crayfish, lobsters do not display pronounced colour changes. The fiddler crab, *Uca pugilator*, is frequently selected as a PDH assay animal because of its ability to respond to the background colour by lightening or darkening the melanophores, erythrophores and leucophores (see Sandeen, 1950; Rao and Riehm, 1989). In my opinion, PDH does not obviously affect integumentary pigment changes in spiny lobsters, but is surely involved in the screening effect of the pigment layers in the eyes. The compound eyes of crustaceans are categorised by their position: (i) the appostion type is typical of animals who usually live under high light intensity; the retinular cells extend from the basement membrane to the crystalline core of the ommatidium; (ii) the superposition type is typical of nocturnal or deep sea animals who are exposed only to low light intensities; in this eye type there is a clear zone between the crystalline cone and the rhabdome of the ommatidium (Phillips *et al.*, 1980). Lobsters have the superposition type of eyes. Pigment movements within retinular cells are stimulated mainly by the direct action of light, whereas the extra-retinular ommatidial pigment cells are under the control of RPCH and PDH (Rao and Riehm, 1988). Under prolonged exposure to high light intensity (e.g. 2.5 hr in sunshine for *Nephrops norvegicus*), the visual pigment of the

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eye is bleached, the structure of the rhabdomes is destroyed and blindness results (Loew, 1976). It is, however, not certain if other lobsters are very susceptible to light-induced blindness since many of the commercially important palinurid species and *Homarus* species commonly occur in much shallower water than *N. norvegicus* and are, therefore, more exposed to relatively high light intensities.

4.4.2. Crustacean hyperglycaemic hormone-precursor-related peptides (CPRPs)

The CPRP, previously called peptide C (Weidemann *et al.*, 1989), was demonstrated to be part of the preprohormone structure of cHH in several crustacean species (see Van Herp, 1998; Ohira *et al.*, 1997a, b). Comparisons of the preprohormones of cHH, MIH and VIH further illustrated that only the cHH precursor had a precursor-related peptide. This difference at the precursor level and the degree of peptide sequence identity (53 % between VIH and MIH, and only 19 % identity between cHH and VIH/MIH) has led to the postulation of a cHH subfamily versus a VIH/MIH subfamily (see Van Herp, 1998). The CPRPs characteristically have multi-serine amino acid residues (Fig. 84) and their biological functions have not been determined yet. In the present study, peptide material which corresponded to four HPLC peaks from the less-hydrophobic material of *J. lalandii* sinus glands were sequenced and found to resemble the CPRPs (see Fig. 84). The *Jala* CPRPs 1-4 have been named in the order of elution. *Jala* CPRP-1 and –2 eluted as a doublet from the second region of interest; the doublet could not be completely separated under the different chromatographic conditions employed. Mass analyses confirmed the sequencing data that these two

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                1          10          20          30          38
Cama[1]:    RSTQGYGRMDRILAALKTSPMEPSAALAVQHGTTHPLE
Capa[2]:    RSAQGMGKMERLLASYRGALEPSTPLGDLSGSLGHPVE
Caca[3]:    RSADGFGRMESLLTSLRGSAESPAALGEASAA--HPLE
HoamA[4]:   RSVEGASRMEKLLSSSN-SPSST PLGFLSQD--HSVN
HoamB[4]:   RSVEGVSRMEKLLSSI--SPSST-PLGFLSQD--HSVN
Orli[5]:    RSVEGSSRMDRLLSS--GSSSSE-PLSFLSQD--QSVS
PejaIII[6]:RSFDA-----------SPSAT----- SGN--HSLN
Pemo[7]:    RS----------------SPEAS-PVT--SL---HTSD
MeenA[8]:   ------------------SPVEAASPWVE-----HRLVRR
MeenB[8]:   ----------------- SPVEAASSPRVD----HRLVRR
Jala1:    RATEGLTRMEKLFSAD--SPPSSTPL---DAAD-HSLN
Jala2:    RATEGLTRMEKLFSADS-SP-SSTPL---DAAD-HSLN
Jala3:    RSTNGLARLERLLSSTSSSSSSA-PLGLLS
Jala4:    RSTNGLARLERLLSSTSSSSSSA-PLGLLSGA-DHSLN
```

Fig. 84. Alignment of crustacean hyperglycaemic hormone-precursor-related peptides from various decapod crustaceans: [1]*C. maenas* (Weidemann *et al.*, 1989), [2]*C. pagurus* (Chung *et al.*, 1998), [3]*Cardisoma carnifax* (Newcomb, 1987), [4]*H. americanus* (De Kleijn *et al.*, 1995), [5]*Orconectes limosus* (De Kleijn *et al.*, 1994a), [6]*Penaeus japonicus* (Ohira *et al.*, 1997a), [7]*Penaeus monodon* (Udomkit *et al.*, 2000), [8]*Metepenaeus ensis* (Gu *et al.*, 2000), *Jasus lalandii* (present study). Gaps (-) have been introduced into the shorter sequences for maximal identity; identical residues are indicated in bold.

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peptides, 32 amino acids in length, are identical, except for an amino acid substitution at position 18 (P^{18} versus S^{18}). *Jala* CPRP-3 and –4, on the other hand, are more hydrophobic (eluted from the fifth region of interest on the chromatogram in Fig. 61) and could be completely purified on HPLC. Sequence elucidation showed that *Jala* CPRP-3 (29 amino acids long) is identical to *Jala* CPRP-4 (36 amino acids long), but the former represents a C-terminally truncated form of the latter. The presence of four CPRP-like structures in *J. lalandii* is puzzling at the moment, since only two cHH peptides have been elucidated to date. It is possible that *Jala* CPRP-1 and –2 is a display of genetic microheterogeneity in the populations of *J. lalandii* found along the west coast of South Africa; the presence of only one variant, i.e. either S^{18} or P^{18}, in populations of spiny lobsters from different sampling areas can best be demonstrated by the fractionation of a pair of sinus glands from individual spiny lobsters with the aid of micro-HPLC, coupled with MALDI-TOF analyses. Alternatively, this issue can also be clarified by cDNA cloning of *Jala* CHH to determine its preprohormone structures. Similarly, it may be argued that *Jala* CPRP-4 is a full-length precursor-related peptide and that *Jala* CPRP-3 is a mere artefact of peptide degradation and that, therefore, only two CPRPs are found in *J. lalandii* sinus glands: either *Jala* CPRP-1 and –4, or *Jala* CPRP-2 and –4. At this stage no definite answers can be provided, although a previous study on *C. maenas* also reports on the finding of three CPRPs when only two cHHs have been elucidated (Chung and Webster, 1998). There is always still the possibility that a hitherto undetected cHH-like peptide occurs in the sinus gland, perhaps a very hydrophobic peptide that does not elute on the gradients

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used in the current study and which can account for the "extraneous" precursor-related peptides. CPRPs have also been isolated from other crustacean species, such as *O. limosus*, *H. americanus* and *C. maenas* (Tensen *et al.*, 1991b) and *C. pagurus* (Chung *et al.*, 1998). Alignment of the different CPRPs in Fig. 84 reveals certain regions of identical amino acids and also shows that the CPRPs may vary in length, even within one species. Strikingly different in *Jala* CPRP-1 and –2, is the R^1A^2 residues compared with the conserved R^1S^2 of other CPRPs. Other exceptions are the CPRP structures of *Metapenaeus ensis*, where the first 2 residues are SP (Fig. 84).

The CPRP of *C. pagurus* was recently studied with immunocytochemical and radioimmunoassay techniques and revealed the following: CPRP co-localises with cHH in the eyestalks and pericardial organs; CPRP was stochiometrically released with cHH, and the main target tissue for CPRP appeared to be the hypodermis (Wilcockson *et al.*, 1998). In the current study it was shown that the CPRPs are not recognised by anti-cHH sera (confirmation that cHH and CPRP do not have common epitopes). I have also established that the CPRPs do not play a role in integumental pigment migration, i.e. they could not elicit RPCH or PDH activity in biological assays.

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4.5. Molecular biology aspects

Molecular biology techniques have been successfully applied by several investigators to the field of crustacean neuroendocrinology (see De Kleijn and Van Herp, 1995; Van Herp, 1998). To initiate study on the spiny lobster neuropeptides at this level, a cDNA library was constructed from RNA of *J. lalandii* X-organs in the current study,

using commercially available kits. The amplified *Jala* cDNA library showed a high titre with more than 90 % recombination and an average insert size of about 1.5 kbp. This preliminary study has, thus, provided an available library which will later be screened with various *Jala* cDNA probes in order to obtain information about the number and primary sequences of preprocHHs, as well as other eyestalk neuropeptide precursors. In future studies, too, cRNA probes will be made for *Jala* cHHs and MIH which will be useful to study the expression of these neuropeptide mRNAs under specified environmental or developmental conditions, as well as to achieve clarity about co-expression of *Jala* cHH and MIH in the same cells. Towards this end, PCR reactions were carried out in the present study with two synthetic oligonucleotides, deduced from the amino acid sequence of *Jala* cHH-I and corresponding to the N- and C-terminus of the peptide, and cDNA from *Jala* X-organs as template. The sequence data obtained from the resulting PCR product indicates 100 % sequence identity with the partial sequence of *Jala* cHH-I, viz. residues Y^{11}-C^{52}. This partial cDNA also encodes a region that is common to both *Jala* cHHs and to *Jala* MIH and is, thus, a potentially useful candidate to serve as a probe for screening the *Jala* cDNA library for genes encoding both of these hormones. A similar strategy has recently been applied, with success, to isolate a cDNA encoding a cHH-like peptide from the silkworm *Bombyx mori* (Endo *et al.*, 2000).

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4.6. Concluding comments

A number of neuropeptide hormones have been isolated, functionally characterized and sequenced for the first time from the eyestalk of the spiny lobster *Jasus lalandii*. This species is a valued commodity in South Africa because it not only generates much-needed foreign revenue for this developing country, but it also provides employment and upliftment to mainly impoverished coastal communities. To date, the South African rock lobster industry has complied with the increasing overseas demand by increasing the fishing yields, extending the lobster collection season and reducing the legal size limit of lobsters. These changes in fisheries management policies place enormous pressure on the sustainability of the natural resource, especially when tons of lobsters are also periodically killed by deoxygenated coastal waters (as had occurred in 1999 during severe black-tide conditions). It is at a time like this when purely ecological research reaches its limit – not that such studies should be under-rated, but one should also acknowledge the worth of knowing which physiological and biochemical parameters impact most on the biology of these large, but also vulnerable crustaceans.

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The present study has laid the foundation for future non-ecological research on *J. lalandii* by providing structural information of important bioregulators. Some information can already be implemented practically by the existing lobster industry, for example, the accurate assessment of moulting stage will eliminate mortalities (and the resulting decrease in revenue) when only the hardiest animals, i.e. those in intermoult,

are selected for live export. With the methodology and information at hand from the current study, specific antisera can be raised and cRNA probes can be made to study these neuropeptide regulators during development and during an array of environmental conditions, which will produce useful basic information for budding aquaculturists of *J. lalandii*. Gene manipulation and recombinant DNA technology with regards to *J. lalandii* neuropeptide hormones, are also not far-off possibilities anymore.

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