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IDENTIFICATION AND CHARACTERISATION OF SPECIFIC ALLERGENS OF ABALONE (*HALIOTIS MIDAE*; MOLLUSCA) AND OTHER SEAFOOD SPECIES

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DECLARATION

I, Andreas L. Lopata, 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 or any part of is has been, is being, or is to submitted for another degree in this or any other University.

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Andreas L. Lopata

September 1999

AN

MEINEN PARTNER KASTURI

UND MEINE FAMILIE IN SÜD AFRICA

UND IN DEUTSCHLAND

And God said, "Let the waters bring forth swarms of living creatures."

God created the great sea monsters and all the living creatures of every kind that creep, which the waters brought forth in swarms.

1st Chapter of Genesis
Verses 20 and 21

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List of Abbreviations

ABTS	2,2'azin-di (3-ethyl-benxthiazoline) sulfonic acid
BSA	bovine serum albumin
DBPCFC	double blind-controlled food challenge
DDT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
HDM	house dust mite
HUCS	human umbilical cord serum
MoAb	monoclonal antibody
MW	molecular weight
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PVDF	Immobilon-P polyvinylidene difluoride
RAST	Radioallergosorbent test
RPMI	Roswell Park Memorial Institute 1640 medium
RT	room temperature
SDS	sodium dodecyl sulfate
SPT	skin prick test
TEMED	N,N,N,N'-tetramethylene diamine

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ABSTRACT

Shellfish and fish are sources of potent allergens in sensitised individuals. In view of the limited information available from published studies, especially on mollusc allergens, more detailed characterisation of the major allergens from different shellfish and fish is necessary to accurately predict cross-allergenicity. The need for a detailed characterisation of the immune response and the allergens in an indigenous mollusc species, followed a recent increase in patients presenting in the Cape Town area, with Type I allergic reactions after ingestion of abalone (*Haliotis midae*).

The first objective of this study was to determine the frequency and spectrum of reported hypersensitivity to abalone and other related seafood species in the Western Cape of South Africa. 105 volunteer subjects with suspected seafood allergy were recruited by means of a detailed seafood allergy questionnaire, advertised in the local press. The analysis of the questionnaire demonstrated clearly the importance of abalone, which was the third most frequently reported species (35%), after rock lobster and shrimp, of the 26 seafood species implicated in allergic reactions. Allergy to seafood was confirmed by the presence of specific IgE using in-house and commercial radioimmunoassays (RASTs).

A novel Abalone-RAST identified specific IgE to abalone in 17/38 subjects who reported adverse reaction following the ingestion of abalone. The novel Abalone-RAST correlated positively not only with the phylogenetic closely related Snail-RAST ($p<0.01$) but also with squid and several indigenous crustacean species. Among subjects with multiple seafood allergies, the immune responses to molluscs species were found to be diverse. The presence of species specific allergens in the mollusc group was supported by the very low frequency of concurrent sensitivity to all mollusc species (13%) compared to concurrent sensitivity between mollusc and among the crustacea and fish group (42% and 56% respectively). The persistence of specific IgE, following a period of more than three years of seafood avoidance, was demonstrated in the mollusc species studied.

The second objective was to investigate the specific immune responses to local seafood in more detail using RAST-inhibition experiments, skin prick tests (SPTs) and Western blots to demonstrate the presence of species-specific allergens. Sensitivity for the detection of abalone allergy was improved using an additional in-house SPT in six RAST negative subjects (23/38, 61%). RAST-inhibition experiments with abalone extract demonstrated the highest degree of cross-reactivity with the Snail-RAST. In addition, a strong inhibition was achieved using commercial crustacean RASTs, confirming the presence of cross-reacting allergens in species of the same and other seafood groups. However, the low inhibitions achieved with the indigenous black mussel and squid extracts indicated that their allergen compositions differ from the species utilised in the commercial RASTs. Unique species-specific protein bands could be detected by SDS-gel electrophoresis, which clearly distinguished related mollusc species. These have not been demonstrated previously. Western blot analysis of different mollusc species identified several prominent allergens. An unexpected finding was the appearance of novel specific IgE binding reactivity after cooking the abalone. Several IgE binding proteins with similar molecular weights could be detected in immuno blots of indigenous crustacean and fish species.

The next objective was to characterise the hypersensitive reactions to the local abalone species (*H. midae*) in detail and identify the allergens found in this mollusc species. The questionnaire on abalone sensitive subjects revealed that asthma-like symptoms and the delayed onset of symptoms were frequent in sensitised individuals (42% and 34% respectively). :This has previously only been reported for snail and in one case report on abalone. Surprisingly, the five abalone sensitive subjects who were studied in more detail had concurrent sensitivity to HDM and, analysed by RAST-inhibition, demonstrated two distinct types of responses. One set of subjects demonstrated a strong inhibition by HDM, indicating clearly for the first time that cross-reacting allergens or epitopes must exist between the food allergens from abalone and the air borne allergens found in HDM.
SDS-gel electrophoresis demonstrated common but in addition species-specific protein bands even between very closely related abalone species from South Africa, Australia and Japan. Western blot analysis revealed two major allergens with molecular weights of about 38 and 45 kDa. Their remarkable thermal stability was demonstrated by various *in-vitro* and *in-vivo* assays. These two allergens were also present in extracts of other indigenous mollusc species, and surprisingly in some local crustacean and fish species. The individual immune responses to mollusc species were very heterogeneous for each analysed individual. The 38 kDa allergen is believed to belong to the protein family of tropomyosins, as was supported by specific IgE binding to recombinant tropomyosin of shrimp. This novel 45 kDa allergen of the South African abalone (*H. midae*), was registered with the WHO International Union of Immunological Societies (IUIS) as Hal m 1. It is only the second allergen recognised for a mollusc species after Tod p 1 from squid.

A further aim of these studies was to generate monoclonal antibodies (MoAbs) to the mollusc allergens using the hybridoma technology. The MoAbs were used, due to their consistent specific binding, to identify cross-reacting allergens among species of different seafood groups. In addition, I attempted to develop an immunologic test to distinguish between abalone species from different parts of the world. This test is of importance for the South African police to identify for forensic purposes unequivocally, fresh or processed abalone tissue exported illegally from South Africa to the Far East. Three ELISA- and Western blot assay positive clones were analysed. They demonstrated highly individual binding profiles when binding to fresh and cooked mollusc species was analysed. MoAb clones 2.11 and 2.12 (generated to the same protein of abalone) lacked complete binding to four and two of the ten abalone species respectively, allowing for very distinct species identification. Furthermore, antibody binding to cross-reacting proteins in crustacea and fish was also detected. Western blot results demonstrated clearly that these two antibodies bind to different epitopes on the same protein, making them very useful as tool for allergen and species identification. Finally, an amino acid analysis of the 3 purified antibody binding fractions of abalone was conducted. These proteins were rich in glutamine and asparagine, like tropomyosin, but differed significantly from tropomyosin with respect to serine content.

The immunological findings using different patient specific sera and the monoclonal antibodies generated, provide important new information and insights into the concordant and multiple positive sensitivity to molluscs, crustacea and fish, and new information about the complexity and stability of immune responses to seafood and mollusc allergens observed in allergic subjects.

Chapter I:

A Literature Review on Adverse Reactions to Seafood, their Clinical Characteristics, Available *in-vivo* and *in-vitro* tests and known allergens in molluscs and related species

INTRODUCTION

Seafood constitutes an important food for humans, particularly as a source of animal protein. Global fish production now exceeds 100 million tonnes per year, and about 70% is available for direct human consumption (Moody *et al.*, 1993). The current global seafood consumption is 14 kg/capita/year (Leung and Chu, 1998). Increased consumption due to the high nutritive value of seafood and the promotion of a healthy diet, has also lead to the more frequent reporting of adverse reactions, including immunologically-mediated reactions.

The South African Fishing industry has a long history. Long before the establishment of the Dutch colony at the Cape in 1652, fishing had already been taking place in South African waters, at first only by indigenous people, but later also by merchantmen on passing ships. From what little is known about the inhabitants of South Africa before the arrival of the San (Bushman), Khoi (Hottentot) and Black people, it seems that marine fauna was one of their principal food sources. The finding of large mounds of the shells of edible molluscs such as mussels, oysters and abalone led to these people being called Shellmound Man [Stuttaford, 1997 #249].

Allergy to fish is common among fish-eating populations and in fish processing communities. Many of the published studies assessing the prevalence of fish allergy have been performed in Scandinavian countries and Spain (Aas, 1966; Bernhisel-Broadbent *et al.*, 1992a; Pascual *et al.*, 1992). Crustaceans are also known as an important and common cause of allergic reactions to food (Daul *et al.*, 1993). Hypersensitivity to members of the mollusc group however has only been reported in few studies (Castillo *et al.*, 1994; Lehrer and McCants, 1987; Miyazawa *et al.*, 1996). Although there have been studies on the molecular characterisation of various inhalant and contact allergens, as reviewed by Lehrer et al. (Lehrer *et al.*, 1996), until the past decade there have been few studies defining food allergens at the molecular level.

It is clear that food allergy is increasing in prevalence e.g. oral allergy syndrome or peanut allergy (Yunginger, 1990), as has been observed for inhalant allergy. However, life-threatening immediate reactions appear to be increasing and the pattern of reactions to different foods is changing with the change in diet. In addition, biotechnology makes it possible to develop (or produce) recombinant food proteins with unforeseen allergenicity (Bindslev-Jensen, 1998; Lehrer, et al., 1996).

In the next chapter I will define adverse reactions to seafood and their clinical symptoms and describe diagnostic methods currently available. Furthermore, I will review the known chemical characteristics of known food allergens focusing particularly on the allergens identified in seafood.

1. Description and Classification of Important Seafood

Patients with allergy to seafood may fail to identify the offending seafood species, often as a result of confusion regarding the different common names used to describe various seafood (see below). The two invertebrate phyla of arthropoda and mollusca are generally referred to as "shellfish". All major phyla are represented in the waters of the world. Most seafood species are edible and most are consumed even in small amounts around the world. The three most important seafood groupings most frequently consumed in the Western world include the mollusca, arthropoda and pisces (Chordates). More exotic "creatures" such as seacucumber, jellyfishes, sea urchins and abalone are widely consumed seafood's in other parts of the world, particularly in the East. In Southern Africa there are over 10 000 species of marine plants and animals, representing about 15% of all coastal marine species known world-wide [Branch, 1994 #251.

During the last decade, as concerns about dietary fat and cholesterol have increased in the Europe and United States seafood has become a more prominent component of the diet. In 1990, consumption of seafood reached 7 kg per capita in the USA, while world consumption averaged 14 kg per capita (Leung and Chu, 1998). Total USA commercial landings and imports equalled over 4 million tons for 1990 (Moody, et al., 1993), of which over 600,000 tons were classified as shellfish, including shrimp, crab, lobster and crawfish.

In South Africa the total commercial catch on seafood in 1995 was approximately 6 million kg of which the bulk of the production is consumed domestically [Stuttaford, 1997 #249]. The economic impact is immense, as the wholesale value of about 1.7 billion Rand had increased by over 40% since 1993. The main fish species caught are anchovy, followed by hake and pilchard (herring) with over 100,000 tons each. Among the crustacea, rock lobsters and prawns gained 3,000 and 500 tons in 1995 respectively and among the molluscs squid and abalone 7,000 and 600 tons respectively. In addition, South Africa is one of the worlds leading exporter of abalone and abalone products to the Far East.

The major edible seafoods that induce allergic reactions belong to three phyla (Table 1.I). The first phylum is the Mollusca which includes the classes Gastropoda, consisting of abalone, conches, limpets, snails and whelks; Bivalvia include mussels, clams, cockles, oysters and scallops and Cephalopoda; octopuses and squids (Moody, et al., 1993). The other two large groups are the class of Crustacea (Shrimp, Lobster, Crab) and Osteichthyes (bony fish).

Table 1.I: Classification of seafood groups causing allergic reactions.

PHYLUM	CLASS	COMMON NAME
MOLLUSCA	Gastropoda	Abalone, Snail, Limpet, Alikreukel
	Bivalvia	Clam, Mussel, Oyster, Scallop
	Cephalopoda	Octopus, Squid
ARTHROPODA	Crustacea	Lobster, crayfish, Shrimp, Crab, Rock Lobster
CHORDATA	Condrichthyes	Sharks, Rays
	Osteichthyes	Cod, Hake, Salmon, Sardine, Mackerel, Yellowtail, Tuna

One has to bear in mind that South African common English or Afrikaans names are used to describe many species that have no close phylogenetic relationships. Furthermore, classifications of species on taxonomic bases are subjected to continuous changes in taxonomic classifications over the years. Common names used in this thesis are based on those utilised by the South African seafood industry [Stuttaford, 1997 #249]. They differ in many instances from the European or North American names (Pharmacia, 1993).

Table 1.II: English, South African and scientific names for various seafood species

Group	Common Names		Scientific Names
	English	South African	
MOLLUSCS	- Abalone	- Perlemoen	- *Haliotis midae*
	- Giant periwinkle	– Alikreukel	- *Turbo sarmaticus*
	- Squid	– Tjokka	- *Loligo vulgaris reynaudii*
CRUSTACEAN	- Rock lobster	- Crayfish	- *Jasus lalandii*
FISH	- Hake	- Haddock	- *Merluccius capensis* or *Merluccius paradoxus*
	- Hake	– Stockfish	
	- Cape Salmon	– Geelbek	- *Atractoscion aequidens*
	- Kabeljou	– Kob	- *Argyrosomus hololepidotus*
	- Herring	- Pilchard	- *Sardinops sagax*

When patients identify the offending seafood species, usually by common name, there is often confusion regarding common names or deceptive marketing practices. For example in South Africa herring is also called pilchard, "marsbanker" is actually a mackerel, Haddock is hake, kob is kabeljou, and Cape salmon is not related to the fresh water salmon (see Table 1.II). Among the crustacean and molluscs, it is important to note that the South African rock lobster (*Jasus lalandii*) belongs to a different class as the European and North American lobster (*Homarus gammarus* or *H. americanus* respectively), abalone is locally also known as "perlemoen" and squid is also called "tjokka".

1.1 Definitions of Adverse Reactions to Food

Adverse reactions to foods are of considerable importance in today's society, especially given the recent introduction of new allergens as eating habits change, and the use of new industrial technologies which result in food ingredients appearing in unexpected forms. The diagnosis of an allergic reaction to foods is often difficult, since non-allergic food intolerance is also very common.

Adverse reactions to foods may be classified on the basis of the mechanism of the reaction (Bindslev-Jensen, 1998; Bindslev-Jensen *et al.*, 1994), which will be described below:

Food Allergy; mostly IgE mediated, but also non-IgE reactions observed (see 1.2)

- **Toxic Reaction** (see 1.3)
- **Food Intolerance**; non-immune mediated (1.4)

1.2 Mechanisms of Food Allergy

Clinical and experimental observations both support a role for mast cells, basophils and IgE antibody in immediate reactions to foods. Allergen specific IgE on mast cell surfaces which reacts with food extracts has been demonstrated by immediate wheal and erythema reactions in the dermis after local injection of seafood extracts. These reactions were first been observed in 1921 by Prausnitz-Kustner (Prausnitz and Kustner, 1921) by transferring passively IgE by intracutaneous injection of allergic sera into the skin of a normal recipient. Subsequent local injection of the fish extract to which the donor was sensitive, resulted in a wheal and flare reaction.

The nature of adverse reactions resulting from ingestion, dermal contact or inhalation of various seafood cooking vapours and their temporal pattern to exposure of onset support the view that these are Type I hypersensitivity reactions (classification of allergic reactions see below). The induction of allergic sensitisation and the effector mechanisms in Type I allergic reactions are summarised in Figure 1.1.

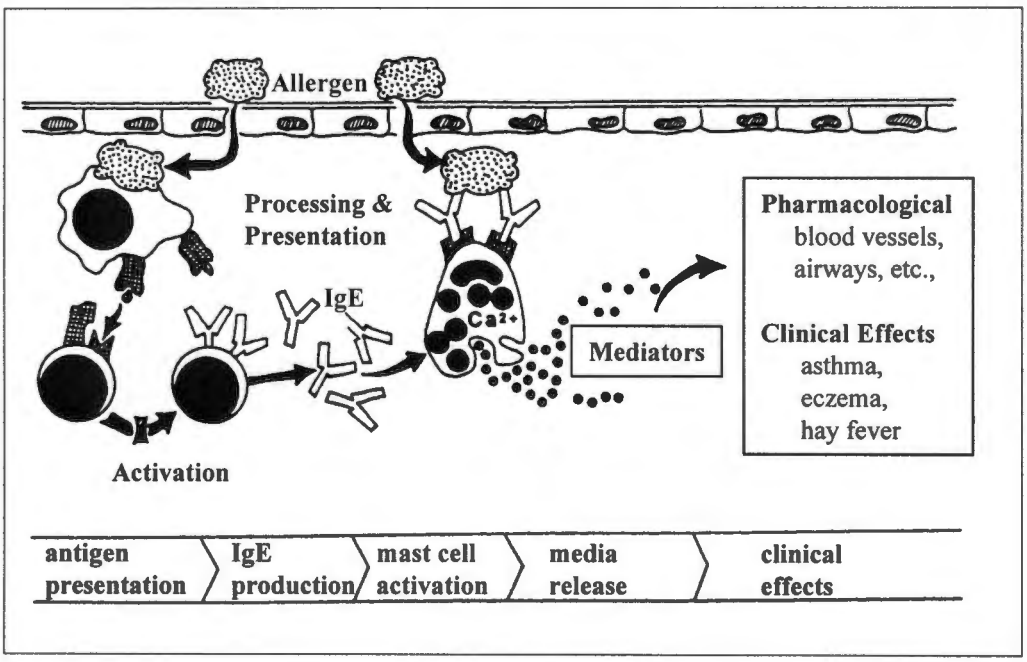


Figure 1.1: Induction and effector mechanisms in IgE-mediated hypersensitivity. (Source: Lehrer SB et al.; Critical Reviews in Food Science and Nutrition, 1996).

The induction of an allergic response is initiated when an allergen (antigen) enters the body via a mucosal surface. The specific IgE response is mediated through the presentation of the processed allergen by antigen presenting cells that present peptide fragments of the allergen to T lymphocytes. Allergens tend to favour a Th2 rather than a Th1 lymphocyte response; recognition between the Th2 and antigen specific T-cells results in the release of soluble factors such as interleukins 4, 5 and 13. This in turn leads to B-cell proliferation and differentiation and the production of allergen specific IgE antibodies. Upon re-exposure to the allergen, the sensitised subject will develop an allergic reaction through interaction with IgE bound on IgE receptors on the surface of mast cells and basophils. The recognition of IgE epitopes of these allergens by the antibodies will trigger the release of preformed and newly synthesised mediators which in turn elicit the clinical signs an symptoms of allergic diseases including hayfever, asthma and anaphylaxis.

However, not all immunologic adverse reactions are mediated by IgE antibodies. For example, studies on cow's milk allergic (Keller *et al.*, 1996) and shrimp allergic patients (Morgan *et al.*, 1990) indicated an elevated level of IgG4, but their detection appears to have little diagnostic value.

In general immunological reactions are divided into Type I, II, III and IV reactions after Cooms and Gell (Table 1.III).

Table 1.III: Classification of Hypersensitivity Reactions

Type	Mediator	Onset of Symptoms	Example
I	IgE	Acute Phase: 10-20 min Late Phase: 4-6 hours	Anaphylaxis
II	IgG, IgM	Minutes to hours	Transfusion Reaction
III	IgG, Complement	6-18 hours	Arthus Reaction
IV	Sensitised T-lymphocytes	1-2 days	Tuberculin Reaction

Each type may be involved in food allergy, but laboratory confirmation of such reactions is difficult, and overlap may occur with a given reaction. In some delayed reactions sensitised T-cells can be identified but the mechanisms of delayed food reactions are currently poorly understood.

1.3 Toxic Food Reactions

1.3.1 Scombrotoxism

Scombroid Fish Poisoning occurs from the ingestion of spoiled fish (Brown, 1993; Merson *et al.*, 1974; Morrow *et al.*, 1991; Smart, 1992). The fish belong usually to the major family of the *Scombridae* i.e. tuna, mackerel and bonito. However, non-scombroid fish, such as mahi-mahi, yellowtail, herring, sardines and anchovy have been implicated as causes of scombrotoxism. A recent report of Scombroid fish poisoning in the Western Cape has been linked to spoiled yellowtail (Muller *et al.*, 1992). Fish with a high content of red meat which turns brown upon cooking contain large amounts of free histidine in their muscle tissue. When the fish is improperly refrigerated or when refrigeration is delayed, histidine is converted to histamine by bacteria containing the enzyme, histidine decarboxylase. Enterobacteriaceae, such as *Proteus morganii* and *Klebsiella pneumonia*, contain this enzyme and are often implicated in Scombroid poisoning.

Clinically, Scombroid poisoning resembles an IgE-mediated allergic reaction. Symptoms usually occur within 1-2 hours after the ingestion of spoiled fish and symptoms include flushing, sweating, headache, nausea, vomiting, diarrhoea, hives and oral burning sensation. These symptoms may be confused with a Type I allergy reaction, resulting in an incorrect diagnosis.

1.3.2 Shellfish Poisoning by Marine Algae

A major cause of adverse seafood reactions is the presence of numerous toxins in fish and shellfish. The phenomenon of "Red Tide" is a significant contributor. Red tides occur when a warm surface layer is created above colder waters by heating, or by fresh water run off. As a result, blooms of toxic organisms can rapidly appear in these upper layers. This proliferation may be so profuse that the colour of the seawater changes to red, brown or even green (Anderson, 1994; Luckas, 1992). The toxic algae are taken up by filterfeeders such as mussels and oysters, accumulated in the tissue of this invertebrates and in turn consumed by humans.

Blooms of microalgae that cause seafood poisonings have been reported in South Africa as far back as in 1948 when people became ill after eating toxic mussel (Grindley, 1969). Since then, toxic blooms have been regularly recorded along the South African coast causing immense marine mass mortality and human illnesses (Horstman, 1981; Horstman *et al.*, 1991; Pitcher *et al.*, 1993).

Common algae toxins are:

1.3.2.1 PSP Toxins:

Among the phycotoxins, PSP has been recorded most frequently and accounted for the majority of human fatalities. Toxins which cause Paralytic Shellfish Poisoning are produced by dinoflagellates, most of which belong to the genus *Alexandrium*. The PSP toxins include the well characterised saxitoxin and over 20 related compounds (Luckas, 1992; Oshima, 1995) which are accumulated by shellfish during filter feeding. Toxication results primarily from the blockage of muscular and neuronal sodium channels. Symptoms after ingestion of toxic shellfish range from tingling sensations around lips and face to headaches, nausea and diarrhoea. In severe cases death through respiratory failure has been reported within 2-24 hours after ingestion (Sakamoto *et al.*, 1987; Sims, 1987).

1.3.2.2 DSP Toxins:

Diarrhetic Shellfish Poisoning is produced by algae of the *Dinophysis* or *Prorocentrum* genera. This toxins contain several components including Okadaic acid which is a potent inhibitor of protein phosphatase enzymes (Luu *et al.*, 1993). The symptoms of DSP include nausea, abdominal pain, diarrhoea and vomiting which can be confused with symptoms of an Type I allergic reaction. Chronic exposure to DSP toxins may also promote tumour formation in the digestive tract (Suganuma *et al.*, 1989) (Amzil *et al.*, 1992).

1.3.2.3 Ciguatera Fish Poisoning:

This fish poisoning causes more human illnesses than any other seafood toxicity (Sakamoto, et al., 1987). Occurring tropically and subtropical, it affects up to 50 000 individuals annually. Ciguatoxin is the major component, which concentrates via the food chain in certain reef fishes. The toxin binds to voltage-dependent sodium channels producing symptoms of tingling of the hands and feet, low heart rate and blood pressure and even death through respiratory failure (Gillespie *et al.*, 1986; Hokama *et al.*, 1993).

In most of the poisonings illustrated above, the symptoms encountered are almost identical to those experienced in Type I allergic reactions and only accurate diagnostic procedures will exclude a genuine seafood allergy and consequently allow seafood into the diet.

1.3.3 Other types of adverse reactions to seafood

1.3.3.1 Red Soft Coral

A marine coelenterate (*Dendronephthytia nipponica*), commonly associated with spiny lobster harvesting, is responsible for occupational asthma (Onizuka *et al.*, 1990). Fisherman complained about inflammation and itching of the skin and conjunctivitis.

1.3.3.2 Parasite Contamination

1.3.3.2.1 Sea-squirt

Hoja (sea-squirt) allergy was first reported in 1950 among oyster farmworkers in Japan (Jyo *et al.*, 1989). Sea squirt is a parasite belonging to the protochordates (*Styella plicata, S. clava, Halocynthia roretzi*), commonly attached to oyster shells, expelling fluid and contaminating the oyster shells when it is removed from the water. Asthma, rhinitis and conjunctivitis were associated with this occupational allergy. An allergenic carbohydrate chain of a sea squirt antigen, termed Gi-rep, was finally characterised by Oka and colleagues (Oka *et al.*, 1987).

1.3.3.2.2 Anisakis

World-wide increasing allergies to a foodborne parasite, *Anisakis simplex,* have been linked to the ingestion of this nematode which causes human Anisakiasis ('herring worm disease') and induces immune reactions. *Anisakis simplex* is a nematode belonging to the Anisakidae family, which includes *Anisakis*, *Pseudoterranova*, and *Contracaecum*, found as parasites in the gut of fish, cephalopods (squid) , crustaceans, and marine mammals. Studies on *Anisakis* in South Africa (Webb, 1998) identified an very high infection rate of close to 100% in commonly consumed fish such as: yellowtail, snoek, kinglip, hake and even squid (calamari).

Hypersensitivity and allergic reactions have been observed including symptoms such as urticaria, angiodema and severe anaphylactic reactions (Fernandez de Corres *et al.*, 1996; Fraj Lazaro *et al.*, 1998; Kasuya *et al.*, 1990). The reactions may be mistaken for a seafood allergy, as revealed by a study from Japan, where mackerel-induced urticaria was more often caused by *Anisakis* than by the fish (Kasuya, et al., 1990). *Anisakis* antigens have been demonstrated to be heatstable and the allergic reaction to be mediated by IgE (Audicana *et al.*, 1997; Garcia *et al.*, 1997).

1.4 Food Intolerance

The definition "food intolerance" is used when the history and/or the provocative tests clearly prove a food as the cause but there is no evidence that the immune system is involved (Bindslev-Jensen, et al., 1994). Symptoms of food intolerance can be the same as food allergic symptoms and also can be related to the gastrointestinal and respiratory tract and/or the skin. Nevertheless, some reactions after ingesting food or food additives belong to the group of psychological or psychosomatic reactions.

In general adverse reactions caused by food intolerance are divided into the following groups:

- **Enzymatic**
  Most well known is lactase deficiency which means that the person is unable to digest the milksugar lactose.

- **Pharmacological**
  Caused by food additives or may depend on the direct effect of vasoactive amines naturally found in foods such as histamine, tyramine, phenylethylamine and serotonin. Large amounts of histamine and tyramine are found in canned fish, fish autolysates and tuna.

- **Undefined**
  Food additives which cause intolerance are for example: azo dyes (tartrazine), flavours (monosodium glutamate, MSG; 'Chinese restaurant syndrome') and preservatives (sulphites etc).

1.5 Identification of Seafood Species

One of the problems in identifying clinical reactions to seafood is (as explained above) that patients may not in fact be ingesting what they think, because of the widespread problem of commercial substitution and the incorrect identification of the offending seafood species by common names (see Table 1.II).

Certain fish products and species have a much higher commercial value than closely related congeners and typically, many of the identifying morphological features of these species are lost during processing and packaging (Bartlett and Davidson, 1991; Ram *et al.*, 1996; Sotelo *et al.*, 1995). This provides an opportunity for fraudulent substitution of high value products with lower value products and/or the mislabelling of produce. For example, cheaper smoked trout (*Oncorhynchus mykiss*) is difficult to distinguish by taste and appearance alone from more expensive smoked salmon (*Salmo salar*) for which it has been substituted (Carrera *et al.*, 1998; Carrera *et al.*, 1996). Similar practices occur with flatfish, where European plaice (*Pleuronectus platessa*) and flounder (*Platichthys flesus*) are substituted for the more valuable sole (*Solea solea*) (Cespedes *et al.*, 1998). Huang et al. (Huang *et al.*, 1995) describe how sought-after fillets

of the red snapper (*Lutjanus campechanus*) were substituted in 70% of samples from across Florida with congeneric species. Other processed products such as canned tuna are also subject to fraudulent mislabelling since prices of several species vary on different markets (Quintero *et al.*, 1998). These problems are not confined to fish species alone. For example An et al. (An *et al.*, 1990) developed a technique for identifying and quantifying the degree of adulteration of rock shrimp (*Sicyonia brevirostris*) in seafood products.

The accurate identification of seafood species has recently become very important in a legal case involving canned abalone species, which are usually indistinguishable from each other after processing.

The abalone *H. midae* is the only commercially exploited abalone of the six species that occur on the South African coast. The commercial quota stands at 550 metric tons and the recreational diver exploit about the same volume. In addition, poaching of abalone stocks has an serious impact on the South African abalone industry. It is estimated, that in just one area in the Western Cape province at least 40 tons of *H. midae*, valued at approximately US $1 million to local producers, is poached annually. Abalone poaching has increased in recent years following the establishment of sophisticated syndicates who illicitly export processed abalone to the Far East. However, abalone poaching and illegal trade are international problems for most major abalone fisheries. In California, these activities have caused a sharp decline in stocks and the possible extinction of at least one species, *H. soreness*.

Several alleged poachers have been acquitted in South African trials where the state has been unable to prove that the confiscated tissue is of the local abalone. Poachers claimed that the abalone in question are not *H. midae*, but instead *H. spadicea* (a congener for which less stringent regulations exist) or that the product in question is not abalone at all. For this reason, a robust method was required to identify fresh or processed abalone to species level, and would conform to the legal requirements for forensic purposes. Nevertheless, abalone poaching is in South Africa a complex socio-political issue, and will only be solved by the development of a dynamic multidisciplinary holistic approach. It is here that the development of an immunological identification method of different abalone species could make a significant contribution.

In general, there are two types of methods available for the differentiation of species:

1.5.1 DNA based methods for species identification

In the legal case mentioned above, the South African abalone (*Haliotis midae*) were allegedly marketed in cans falsely labelled as "Product of Australia" (apparently containing another *Haliotis* species). The contents were proved to be of South African origin using a DNA based species identity test (Sweijd *et al.*, 1998). As a result of this case, the South African police now have a molecular tool to help curb the precipitous decline of this abalone species, due to over-exploitation.

1.5.2 Protein based methods for species identification

While protein based methods are generally regarded as less robust than DNA based methods, they nevertheless have several benefits, such as simplicity and speediness, and are still widely applied (Hsieh *et al.*, 1997; Huang, et al., 1995; Sotelo *et al.*, 1993). Since the value of seafood is linked to its freshness, many of the cytosolic proteins are usually intact when they reach the market. However, in highly processed products, such as smoked, canned and dried seafood, most of the higher molecular weight proteins are denatured and rendered useless for analysis, although this is not always the case (Hsieh, et al., 1997). At the species level, a relatively high degree of interspecific polymorphism is apparent and thus, where the tissue is in a suitable condition, useful distinguishing characters are readily available from proteins (Powell *et al.*, 1995). With proteins, the principle is that different species will possess unique proteins or be differentiated on the basis of structural polymorphism's of a protein. Three types of protein based techniques have been used to assay these polymorphism's in marine species, namely total protein analyses, locus specific allozyme markers and serological methods (Cunniff, 1998).

Isoelectric focusing of total proteins of nine species of sunfish separated putative parvalbumin proteins (Whitmore, 1990). The migration of solubilised seafood proteins to their respective positions on the gel (SDS-PAGE) separates them into a species-specific banding pattern which can be regarded as phenotypic expressions of the genetic information and which consistently differentiate the species concerned (Sotelo, et al., 1993) and (Whitmore, 1990; Yman, 1993).

Serological methods have been employed for species identification purposes in several of the examples cited above. A number of analytical techniques can be employed to detect the reaction between the antigen and antibody in order to test for the presence of the species-specific proteins in question (An, et al., 1990; Carrera, et al., 1996; Sotelo, et al., 1993). While serological methods generally require a high degree of expertise to execute properly, they do lend

themselves to the development of portable non-laboratory based kits which would be of advantage to compliance officials in the field. Shepard & Hartmann (Shepard and Hartmann, 1996) have developed such a technique which uses monoclonal antibody-coated glass beads that allow a technician to process up to 18 samples an hour for diagnosing the identity of sailfish species (*Istiophorus albicans*) in a non-laboratory environment. Similarly, Carrera et al. (Carrera *et al.*, 1997) have developed a colorimetric ELISA method using species-specific rendered polyclonal antibodies bound to the paddles of immunostick tubes for identifying smoked salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*) and bream (*Brama raii*).

1.6 Clinical Manifestation of Seafood Allergy

Allergy to seafood can elicit almost any allergic symptom and sign, but some are more widely demonstrated than others. Patients may have a single symptom, but often there is a multi-organ involvement.

Symptoms can be divided into four main groups (Bindslev-Jensen, et al., 1994; Daul, et al., 1993; Lehrer and Salvaggio, 1990):

- **Generalised Reactions**
  Anaphylaxis and exercise-induced anaphylaxis eventually causing death
- **Respiratory Reactions**
  Asthma and rhinitis; rarely occur without other organ involvement
- **Cutaneous Reactions**
  Skin reactions, including acute urticaria and/or angiodema are common.
  Atopic dermatitis is more common in children
- **Gastrointestinal Reactions**
  Abdominal pain, nausea, vomiting and diarrhoea
- **Other Reactions**
  Conjunctivitis, oral allergy syndrome, food aversion

The pattern of immediate allergic reactions following the ingestion of seafood is similar to that reported to other foods (Daul, et al., 1993). Shellfish were the second most commonly implicated food in a recent study of subjects with food-induced rhinitis or asthma (Oehling *et al.*, 1992). The appearance of adverse symptoms resulted not only from ingestion, but also from inhalation of cooking vapours (Pascual *et al.*, 1996) or handling seafood.

Dermatological manifestations include flushing, urticaria, angiodema and less common, atopic dermatitis. Gastrointestinal symptoms include nausea, diarrhoea and/or abdominal cramping. Respiratory reactions such as wheezing and/or rhinitis are frequently reported and even fatal anaphylaxis in several cases (Maulitz *et al.*, 1979; Yunginger, 1992; Yunginger *et al.*, 1988)

Some studies indicate (Daul *et al.*, 1987) that atopic individuals are at greater risk of anaphylaxis than non-atopic. In addition, it is interesting to note that respiratory symptoms following ingestion and/or inhalation of vapours of fish or shellfish are very common (Dannaeus and Inganas, 1981; Lehrer *et al.*, 1990). Reactions are reported to be almost always occurring within 2 h of exposure. However, late phase reactions occurring 3-6 hours after ingestion have been reported to snow crab (Cartier *et al.*, 1984) cuttlefish (Shibasaki *et al.*, 1989) and limpets (Morikawa *et al.*, 1990).

Another IgE mediated reaction, the "oral allergy syndrome" (OAS) is frequently recorded in patients with crustacean allergy. The OAS appears generally commonly as "cluster of hypersensitivity" where patients are simultaneously sensitive to pollen and fruits and/or vegetables. Symptoms occur while or within minutes of ingesting the offending food. Local oral symptoms, include itching of the lips, mouth or pharynx and angiodema of the lips, tongue, palate and throat. Systemic manifestations such as urticaria, rhinitis, asthma or anaphylaxis have been recorded (Ortolani *et al.*, 1989; Pastorello *et al.*, 1995).

1.7 Seafood Allergy Diagnosis

1.7.1 History

Many people, because of personal reasons or health concerns, wish to continue to eat seafood. It is therefore important to document that any adverse reaction was indeed IgE-mediated and to identify the specific seafood species without any doubt. A precise and detailed history is, as with other allergies, very important. Information should be obtained concerning the putative foods, nature of the symptoms and whether exercise exacerbates the reaction. Furthermore, the atopic status of a patient should be determined by history and by SPT or RAST with common inhalant allergens (Bousquet and Davies, 1998).

However, patient identification of the offending seafood species, usually by common name, may fail to provide adequate information. This is often the result of confusion regarding common

names or deceptive marketing practices. Thus, it is beneficial to perform diagnostic procedures to confirm or refute the seafood allergy and to identify the implicated seafood species without any doubt.

1.7.2 Skin testing

Skin tests should be the second step in the diagnosis. They have been a primary diagnostic tool in the field of allergy since their introduction by Charles Blackley in 1865 [Korenblat, 1992 #220]. Skin testing is a very sensitive method of evaluating hypersensitivity to an offending allergen by demonstrating an IgE-mediated allergic reaction of the skin. Methods include scratch tests, intradermal injection and prick tests. The skin prick test (SPT) involves the superficial penetration of the skin, preferably on the forearm, with a disposable lancet. Extracts of the offending food are applied and a positive test is indicated by the development of a wheal within 10-30 minutes that is 3 mm or larger in diameter than a diluent control. Skin testing should not be performed if a subject experienced previously an anaphylactic reaction to an allergen.

Diverse results with the SPT are not only influenced by a given population but also by the use of commercial and/or in-house extracts. It has been implicated that the freshness of the offending food is a possible factor in lack of sensitivity and specificity. Despite the drawbacks of false positive/negative results obtained, skin prick testing is, if performed properly and with the appropriate food extracts, a quick, sensitive and reproducible technique.

1.7.3 Radioallergosorbent test (RAST)

The RAST is an *in-vitro* diagnostic test which is used to demonstrate food-allergen specific IgE antibodies. In this procedure, allergens are covalently bound to a solid-phase support and reacted with specific IgE antibodies in patients serum. The CAP-RAST from Pharmacia is used exclusively throughout all South African pathology laboratories. There is a range of over 200 different food allergens that can be tested for with the CAP-RAST test. Among these food allergens there are 30 different seafood species. However, most of those species are of European origin and not indigenous to South Africa and therefore the reliability of this serum test is in question. The amount of specific IgE antibodies to a given allergen can be exactly quantified. The results are reported semi-quantitavely (i.e. in classes 0 to 6) and fully quantitatively in units of IgE. By definition, 1 U equals 2.4 ng of IgE.

1.7.4 Enzyme-linked immunosorbent assay (ELISA)

The ELISA is also an *in-vitro* diagnostic test where the allergen extract is covalently bound directly to a solid-phase or indirect via a first antibody, generally serum IgE or specific IgG generated in rabbit or mice. The binding of specific IgE antibodies to the allergen is visualised by adding a colourimetric substrate to the antibody-enzyme conjugate. The results are compared with the optical density (OD) of a standard curve and are generally quantitative.

1.7.5 Double-Blind Placebo-Controlled Food Challenge (DBPCFC)

The "gold standard" for diagnosis of food allergy and for identification of the offending agent is the DBPCFC. Food challenges are highly sensitive, specific and performed in order to reproduce the spectrum of symptoms reported in the patient's history. However, due to similar clinical symptoms of seafood allergy and seafood intolerance, additional laboratory tests have to be included (Bindslev-Jensen, et al., 1994; Zinn *et al.*, 1997). Food challenges should be conducted in a hospital or clinic setting to ensure that appropriate action is taken if an unsuspected anaphylactic reaction occurs (Sampson and Metcalfe, 1992). Up to 8 grams of raw fish may be given to confirm a negative DBPCFC. Foods may be lyophilised, purified or used in the natural form, delivered in capsules or other foods possibly masked by concentrated black current juice. However, the process of freeze-drying may destroy or alter food antigens. Using lyophilised fish for DBPCFC, 21% of challenges produced false negative results (Bernhisel-Broadbent, et al., 1992a), which was confirmed in a follow-up study investigating various preparation methods (Bernhisel-Broadbent *et al.*, 1992b). Furthermore, the fish challenge should come into direct contact with the oral mucosa, to induce symptoms such as the oral allergy syndrome (OAS), frequently observed in patients with crustacean sensitivity (Daul, et al., 1993).

1.8 Food Allergens

In recent years, our knowledge of the structure of many of the important allergens has increased greatly due to the application of sophisticated molecular biological and protein chemistry techniques. The complete amino acid sequences of more than 110 recorded allergens is known. Data obtained from several studies have been extensively reviewed (Marsh and Freidhoff, 1992; Stewart and Thompson, 1996) and they clearly indicate that, from a structural point of view, allergens are indistinguishable from conventional non-allergenic antigens. Factors known to contribute to antigenicity such as dose of antigen and mucosal permeability will also apply to allergens entering the gastrointestinal and the respiratory tract.

The general properties of allergens comprising several groups are discussed in the following chapters and the recommended allergen nomenclatures by WHO/IUIS (King *et al.*, 1994) applied. Characterised allergens are assigned specific names according to the international standards set by the WHO/IUIS. The first three letters of the genus and the first letter of the species are used to indicate the source. An Arabic number is assigned according to the order of their identification. For example, the first allergen described in codfish, *Gadus callarias,* is designated Gad c 1. The current inclusion criteria on to the official list of allergens are:

1) A prevalence of IgE reactivity above 5% of all identified allergens and
2) A minimum of 5 patients with IgE reactivity (regardless the number of patients included).

1.8.1 General Characteristics

Foods contain a wide variety of proteins, yet only a few are known allergens. It is tempting to conclude that the likelihood of allergenicity correlates with the degree of exposure to a particular protein and the dominance of this protein in a food. In plants, many of the allergens are storage proteins presenting up to 80% of the total protein in the offending foods (Yunginger, 1990). However, proteins that occur only in minor amounts in foods can also be major food allergens. This has been shown for Gad c 1 from codfish where this major allergen is not a predominant protein. On the other hand, major components of many foods, such as actin, myosin and tropomyosin from chicken, beef and pork have not been identified as major allergens. Why certain proteins are more allergenic than others is not yet fully understood (Lehrer, et al., 1996).

Most food allergens are very stable molecules and resist the effects of cooking, processing or digestive processes (Astwood *et al.*, 1996) with the exception of labile allergens in fresh fruits (Dreborg and Foucard, 1983; Hannuksela and Lahti, 1977). Generally, food allergens are glycoproteins with an acidic isoelectric point. Most known food allergens have a molecular weight between 10 and 70 kDa. The upper size limit of food allergens is probably imposed by limitations of mucosal absorption.

A prerequisite for any potential allergen is the ability to stimulate the immune system. One common property is the polyvalence to enable the molecule to trigger an immune response by bridging IgE antibodies on the surface of mast cells. Polyvalence means that the proteins have several epitopes. The portions of the allergen/antigen molecule that interacts with antibodies are defined as epitopes. Depending on their reactivity one differentiates between T-cell and B-cell epitopes. Whether they are different from each other and/or vary from allergen to allergen is still controversial. It is generally thought that T-cell epitopes are linear and B-cell epitopes can be

either linear or conformational. T-cell epitopes are generally small peptide fragments with 10-18 amino acid residues and recognised by special receptors (TCR) after processing by antigen presenting cells (APC). Linear epitopes are composed of only the amino acid sequence itself and have a minimum of 6 residues. However, our knowledge of the structure of food allergens is limited compared to the wealth of information available on inhalant allergens (Taylor and Lehrer, 1996).

1.8.2 Multiple Allergens

Most allergenic foods have more than one allergen. In an attempt to organise significant allergens according to their patient reactivity an classification system has been developed. Allergens to which more than 50% of sensitive patients react are described as "major allergens", in contrast to "minor allergens" where a minority of patients react (Lehrer and Salvaggio, 1990). However, one cannot ignore minor allergens that can cause serious reactions in sensitised subjects.

In addition, some allergens occur as a group of proteins having very similar physical, chemical and immunochemical structures. They differ only slightly in their isoelectric points (Lehrer and Salvaggio, 1990) (Larsen, 1995; Lindstrom *et al.*, 1996) and are termed "isoallergens". At least 67% of the amino acid residues in these proteins must be identical. The slight differences observed are due to differences in the carbohydrate moiety, degree of protein amidation or genetic variation.

1.8.3 Allergen Groups

It has become clear that the majority of allergens can be divided into several broad groupings, based either on demonstrable biological activity or significant homology with proteins of known function.

A: Hydrolytic enzymes

Many of the allergens from plants, bacteria, fungi and house dust mites are hydrolytic enzymes. These proteins are usually involved in cleaving substrates by the addition of water. They include the following groups:

Proteases

Serine proteases; often occupational allergens such as trypsin, chymotrypsin and subtilisins. Cysteine proteases; mainly in mites but also occupational allergens as papain (from papaya). Aspartate proteases; major allergens are pepsin and rennin. Metalloproteinases; collagenase from *Clostridium*

Carbohydrases

Including amylase, cellulase, xylanase, lysozyme (hen egg white and latex) and polygalacturonase

Ribonucleases

Grass pollen and fungal allergens

Non-hydrolytic enzymes

Several allergens from pollen pectins and other enzymes such as enolases, aldolase and soybean lipoxygenases

B: Enzyme inhibitors

Inhibit proteases and/or amylase activity

C: Transport proteins

Proteins for substances such as lipids, pheromones, electrons, oxygen, iron

D: Regulatory proteins

Ca-binding calmodulins (Gad c 1 from codfish); heat shock proteins (house dust mite)
Tropomyosins (from shrimp species and house dust mites)

Several allergens have been shown to possess regulatory properties, encountered as antigens in food allergy, parasite infection and autoimmunity. They include tree, weed and grass pollen profilins (Fuchs *et al.*, 1997; Valenta *et al.*, 1991), fungal and mite heat shock proteins (Aki *et al.*, 1994; Kumar *et al.*, 1993) and tropymyosins. The regulatory properties of these proteins are varied, although several appear to be associated with actin. For example, plant profilin and tropomyosins are involved in actin binding and muscle contraction respectively (Elsayed and Apold, 1983; Konstadoulakis *et al.*, 1993; Reese *et al.*, 1995; Valenta, et al., 1991) Tropomyosins have been shown to be important shellfish allergens. Calmodulins also play a regulatory role via calcium modulation and are found for example in parvalbumins from codfish, carp and salmon (Elsayed and Apold, 1983; Lindstrom, et al., 1996). The heat shock proteins are thought to perform a number of regulatory functions, including binding to actin and various receptors.

We now know the complete amino acid sequences of more than 110 recorded allergens, with an additional 130 allergen entries in the GeneBank database. Among the thousands of seafood species consumed by mankind only very few allergens have been identified (Table 1.IV).

Table 1.IV: Summary of characterised seafood allergens and their references.
Note: **C** = complete; **P** = partial and **AA** = aminoacid analysis.

Allergen source	Allergens	MW (kDa)	A.A. sequence data	Reference
FISH				
Gadus Callarias (Cod)	Gad c 1; allergen M	12	**C**	(Elsayed *et al.*, 1973)
Salmo salar (Atlantic salmon)	Sal s 1; parvalbumin	12	**C**	(Lindstrom, et al., 1996)
CRUSTACEA				
	Antigens I	21	--	(Hoffman *et al.*, 1981)
(Shrimp)	Antigen II	38	**AA**	
Penaeus indicus	SA-I;	8.2	--	(Naqpal *et al.*, 1989)
(Indian shrimp)	SA-II; tropomyosin	34	**AA**	
Parapenaeus fissurus (Taiwan shrimp)	Par f 1; tropomyosin	39	**P**	(Lin *et al.*, 1993)
Penaeus indicus (Indian shrimp)	Pen i 1; tropomyosin	34	**P**	(Shanti *et al.*, 1993)
Penaeus aztecus (Brown shrimp)	Pen a 1; tropomyosin	36	**P**	(Daul *et al.*, 1994)
Metapenaeus ensis (Greasyback shrimp)	Met e 1; tropomyosin	34	**C**	(Leung *et al.*, 1994)
MOLLUSC				
Todarodes pacificus (Pacific squid)	Tod p 1	38	**P**	(Miyazawa, et al., 1996)
Haliotis midae (Abalone)				This study

1.9 Seafood Allergies

A heterogeneous group of proteins is ingested in a normal diet. The diet itself of course varies, in part according to age, but also depending on the regional eating habits. Theoretically, any food can elicit an allergic reaction, however, only about 100 different foods have been documented as causing allergic reactions. Of these only a few foods or food groups account for more than 90% of allergic reactions; they include peanuts, tree nuts, eggs, fish and crustacea (Sampson and Metcalfe, 1992) (Taylor and Lehrer, 1996). The consumption of fish/shellfish or inhalation of cooking vapours (Elsayed and Aas, 1970; Pascual, et al., 1996) are frequent causes of IgE-mediated reactions.

1.9.1 Prevalence

The actual prevalence of seafood allergy is difficult to estimate accurately. In general, the estimates for allergy to food is some 2-4% of children under the age of 6 and up to 2% of adults have reproducible allergic reactions. However, at least one in four atopic adults believe that they have experienced an allergic reaction to some kind of food. Most of the published studies assessing the prevalence of fish allergy have been performed in Scandinavian countries and Spain. The prevalence of immediate-type fish allergy is higher when the intake of fish plays a greater part in the diet of the observed community. It is estimated that 3% of 3-year old Finish children are allergic to fish (Bernhisel-Broadbent, et al., 1992b; Pascual, et al., 1992). In Norway, a large percentage of the population works in fishing and related industries, and fish forms a large part of their diet. The prevalence of fish allergy approaches 1/1 000 in the general population of Norway (Aas, 1966). However, the prevalence to a particular species of fish is difficult to establish as most studies refer only to cod or to 'fish' in general. The prevalence of seafood allergy in South Africa is as yet unknown.

Shellfish are a frequent cause of adverse food reactions. Exact data on the prevalence are unknown but Daul et al (Daul, et al., 1993) estimated that more than 250,000 individuals in the United States alone have developed allergic reactions to shellfish. Like other seafood, a higher incidence of allergy to shellfish would be expect in geographical areas where the consumption is high. Allergic reactions to molluscs, and consequently mollusc allergens, have not been as well studied as those of fish or crustacean. However, it has recently become apparent that molluscs are significant food allergens in exposed populations (Carrillo *et al.*, 1992; Carrillo *et al.*, 1994; Lehrer and McCants, 1987; Morikawa, et al., 1990). In a study conducted in Spain, 50% (24/48) of subjects with allergic reactions to shellfish reported sensitivity to squid.

1.9.2 Allergens

The most comprehensive study on an seafood allergen has been the analyses of the allergen from codfish, Gad c 1 (Elsayed and Aas, 1970). This Ca-binding allergen from cod shares about 34% homology with similar proteins from hake, carp and pike which may explain cross-reactivity in fish-allergic patients.

Allergens from different shrimp species, crab, lobster and crayfish have been demonstrated to be cross-reactive to one another by skin test and immunoassay (Daul, et al., 1993; Leung *et al.*, 1998b). They are all heat-stable glycoproteins with a MW between 34 and 38 kDa and an acidic isoelectric point. These antigens belong all to the protein family of tropomyosins and seem to be the major antigens in the crustacean analysed.

Adverse or anaphylactic reactions after ingestion of mollusc have been frequently reported including various groups and species such as abalone, snails, limpets, squid and mussels. However, the first allergen of a mollusc species has only very recently been identified by Miyazawa et al. (Miyazawa, et al., 1996) in squid (*Todarodes pacificus*), using column chromatography and immunoblotting. The isolated head-stable protein (Tod p 1) has a molecular weight of 38 kDa and reveals a high homology with tropomyosins of a snail (*Biomphalaria glabrata*) and the allergen of a shrimp (Met e 1). It is suggested that this major allergen from squid is also a muscle tropomyosin.

Summary, objectives for the thesis and the problem of abalone poaching

Fish and shellfish play an important role in human nutrition and are particularly popular in certain parts of the world. The recent trend to more healthy eating habits and the substitution of meat with seafood in the diet has resulted in even greater demands for fish and shellfish consumption world wide.

Fish and shellfish are potent allergens in sensitised individuals. With the increase in seafood consumption, an increasing number of patients exhibiting allergic or toxic reactions to seafood are being identified. The type of sensitisation to specific seafood is very much related to the regional diet.

The major edible seafoods that induce allergic reactions belong to three phyla: the chordata (fish), the arthropoda (crustacea) and the mollusca (gastropoda, bivalvia, cephalopoda). To date very few of the allergens in seafood have been characterised. The best known allergen in seafood are the Allergen M (Gad c 1) from cod fish, a parvalbumin, and the major allergens from different shrimp species, all belonging to the protein family of tropomyosins. Very little information exists for the mollusc group, in which recently an allergen has been identified in squid (*Todarodes pacificus*).

In view of the limited information available from published studies, more detailed characterisation of the major allergens from different shellfish and fish is necessary to accurately predict cross allergenicity. A better knowledge of the major allergens in seafood will also assist particularly with the development of more sensitive and specific *in vitro* an *in vivo* diagnostic tests and provide further information and explanation for commonly observed multiple sensitivities to different seafoods observed in some patients.

This thesis focused on the identification and characterisation of the immune response to abalone in a cohort of 38 subjects with reported mollusc allergy in the Western Cape of South Africa, and analysed in detail the major allergens of *Haliotis midae* (abalone; gastropoda), an abalone species indigenous to the Southern African Coast. The detailed characterisation of the allergens in *Haliotis midae* followed from a recent increase in patients presenting in the Cape Town area (Western Cape) to the UCT Allergology Clinic with Type I allergic reactions after having ingested "perlemoen" (the local name for abalone). Since there were no commercial tests available for testing for specific sensitivity to *Haliotis midae*, I set out to develop reliable

specific *in vitro* and *in vivo* tests. In order to develop an understanding of the specific immune responses to perlemoen I have addressed the following questions:

1) What are the immunological characteristics of the major allergens in the local abalone species (*Haliotis midae*; "perlemoen") ?
2) Are the allergens in abalone species-specific, or related to similar allergens in local mollusc, crustacean and fish species?
3) Do abalone sensitive patients demonstrate concordant sensitivity to other seafood species or groups?
4) Does crossreactivity exist between patients allergic to abalone with patients allergic to other seafood species?

In addition I have attempted to develop an immunological diagnostic test to distinguish between abalone species from different parts of the world. The need for such a test arose from the increasing incidence of abalone poaching in South Africa and illegal export to the Far East, where highest prices are commanded for this delicacy. Identification of the source of canned abalone is difficult by simply inspecting the food. A reliable immunologic test to confirm the origin of a species in canned abalone (which is often falsely labelled) has practical forensic importance for legal prosecution of poachers.

CHAPTER II:

An Analysis of *In Vitro* Specific IgE Responses to local Seafood in a Patient Cohort from the Western Cape of South Africa as Detected by Commercial- and In-house RASTs

1. Introduction

World-wide, seafood represents one of the most important groups of allergens in the induction of food allergy. Most of the published studies assessing the prevalence of seafood allergy have been performed in the Scandinavian countries and Spain (Castillo, et al., 1994; Neil'O *et al.*, 1993). In general the type of sensitisation to seafood is related to the regional diet. Shrimp allergy is common in the Southern USA (Daul, et al., 1987), while fish allergy is common in the Scandinavian countries and Spain (Crespo *et al.*, 1995c). Very little information exists for the mollusc group regarding symptoms, frequency of involved species and the relevant allergens.

With the increased consumption of seafood, the rate of adverse reactions is believed to be rising. In recent years, subjects with adverse reactions to abalone (*Haliotis midae*; also locally known as 'perlemoen') have been reported to the Allergology Unit of Groote Schuur Hospital with increasing frequency. We therefore embarked upon a study of patients with a history of adverse reactions to abalone and other seafood species in a cohort of volunteers to determine the reported clinical symptoms and the species implicated to be responsible. Sensitisation to the seafood was confirmed by CAP-RAST where these were available. For abalone and yellowtail reactions (a fish species frequently reported locally in adverse reactions) I developed an in-house RASTs (radioallergosorbent assay) to detect the presence of specific IgE.

In vitro studies were conducted on samples obtained from this cohort of patients to asses the co-reactivity of positive IgE responses to seafood allergens and the correlation between reported sensitivity and confirmation using laboratory tests.

2. Material and Methods

2.1 Patients

Patients were recruited for this evaluation using advertisements in the daily newspapers, the hospital newsletter and local medical journals. A detailed questionnaire (see appendix) was distributed to 148 individuals who volunteered to participate in the study. The production, collection and analysis of the questionnaires were conducted with the assistance of Ms C Zinn (Department of Dietetics, UCT). The questionnaire sought details of the specific seafood species implicated, the symptoms produced upon ingestion of the suspected foods, the time between food ingestion and symptom onset and the ingredients used for the preparation of the seafood

dish. Other details such as family history of food allergy and atopy status were also obtained. Individuals who were willing to undergo a series of diagnostic tests (*in vitro* and *in vivo*) signed a consent form and had a blood sample taken. Those who reported previous anaphylaxis were not subjected to any *in vivo* (Skin prick) tests.

2.2 Specific IgE RAST

RASTs were performed using the Pharmacia UniCAP System (Pharmacia, Uppsala, Sweden) for 12 seafood species: blue mussel (f37), oyster (Rf290), squid (Rf258), snail (Rf314), lobster (f80), shrimp (f24), crayfish (Rf320), crab (f23), salmon (f41), tuna (f40), mackerel (Rf206) and hake (Rf307). The results were regarded as positive when binding values exceed 0.35 ku/l. By definition, 1U of IgE equals 2.4 ng.

For some of the implicated seafood species was no commercial RAST available. In-house RASTs were therefore developed and performed for abalone (*Haliotis midae*) and yellowtail (*Seriola lalandi*). The in-house RAST for abalone were prepared using CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Raw and cooked abalone extract were coupled to the Sepharose using 4 mg/ml and 2 mg/ml of protein respectively. Sepharose 4B was activated according to the manufacturer's instructions, apart from the inactivation step, which was performed with glycine rather than with ethanolamine. A volume of 100 µl of serum was incubated with 100 µl of the allergen-coupled Sepharose suspension in duplicate, while shaking, at room temperature overnight. The Sepharose was then washed three times with PBS-T, 20 µl of iodine 125-labelled anti-IgE (Pharmacia) containing approximately 20 000 cpm added and incubated overnight. Excess ^{125}I-labelled anti-IgE was eliminated by washing and centrifugation, as above, and bound radioactivity was counted using a Beckman gamma counter. Results were expressed as the percentage bound of the total counts added per minute. A positive reaction was regarded as percentage of binding exceeding three times the binding obtained with the negative control.

2.3 Ethics approval

Ethical approval for the study was obtained from the Ethics and Research Committee of the University of Cape Town, and written consent was obtained from each of the patients studied.

3. Results

3.1 Patient demographics

Out of the 148 questionnaires distributed, 105 were returned. 89 of these individuals (85%) were from Cape Town, 16 were from other areas in the Western Cape of South Africa. The ages of the patients ranged from 7-74 years, the mean age being 41.2 years. The majority of the subjects were females. Sera from 80 subjects were obtained for RAST and Western-blot studies. A flowdiagram of the studies of the samples and their selection for further studies reported in chapters II to V is presented in Figure 2.1.

3.2 Seafood species implicated

The questionnaires reported reactions to 10 of the most common Southern African seafood species known to be implicated in adverse reactions (see Table 1.I for the classification of seafood species causing allergies). These included:

Molluscs: abalone, oyster, black mussel and snail;

Crustacea: rock lobster (locally called "crayfish") and prawns;

Fish: yellowtail, hake, salmon and mackerel.

Among the mollusc group the most frequently reported species to cause adverse reactions was abalone in 35% (Fig.2.2 A). The other species reported were black mussel (33%), oyster (24%), snail (16%) and squid (11%). Four mollusc species were each reported by single individuals; limpet, alikreukel (periwinkle), white mussel and scallop. In the crustacea group the most frequently reported species was prawn (47%) (Fig. 2.2 B) followed by rock lobster (44%), shrimp (13%) and crab (12%). Most subjects referred to rock lobster by the common local name as "crayfish".

The most frequent fish species incriminated were hake (25%) (Fig. 2.2 C), followed by yellowtail (22%), salmon and mackerel (15% each), kingklip (13%), snoek (11%) and tuna (3%). Two subjects each reported haddock, cob and sole, and carp, trout and one subject reported an adverse reaction to pilchard.

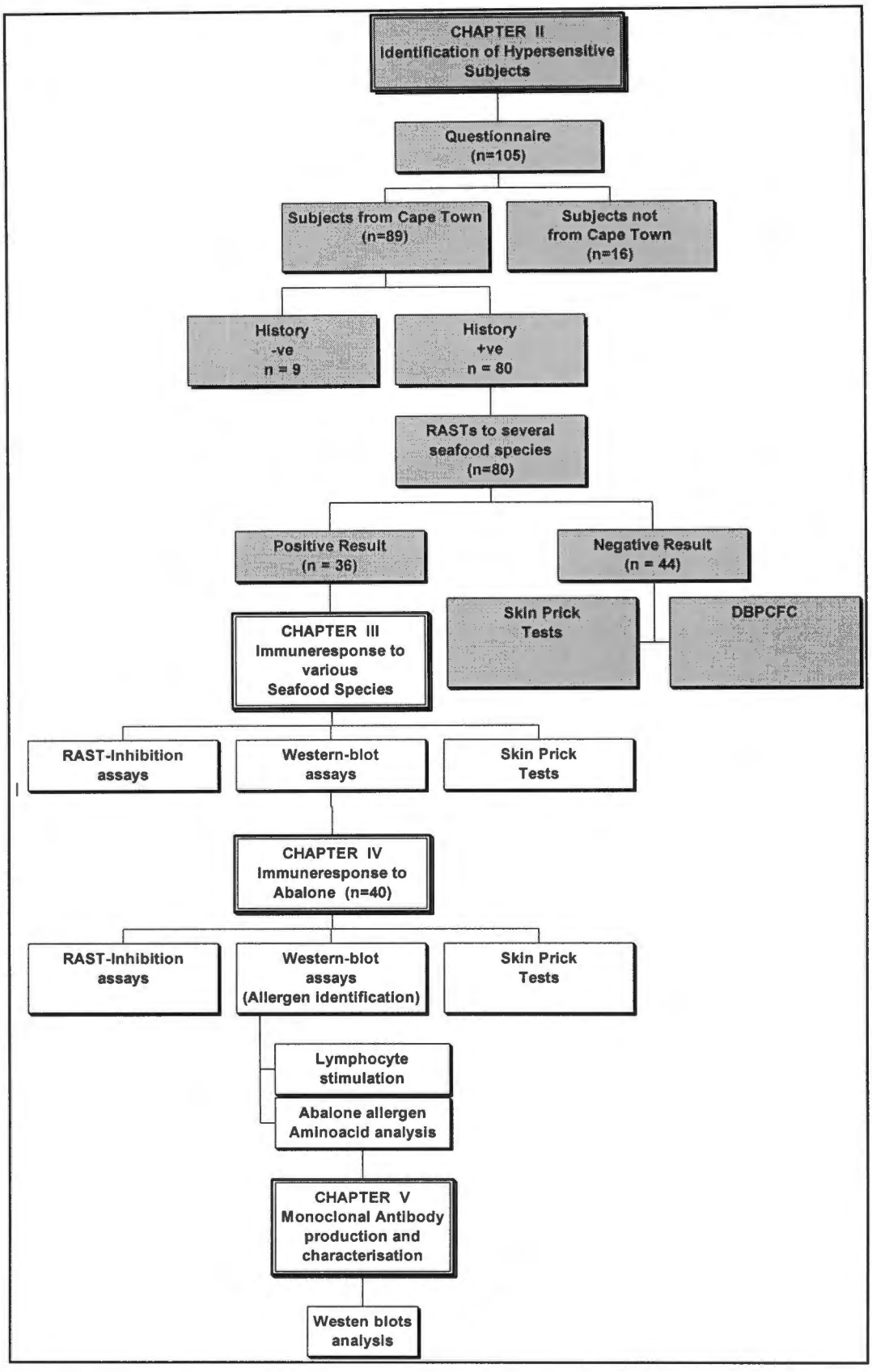
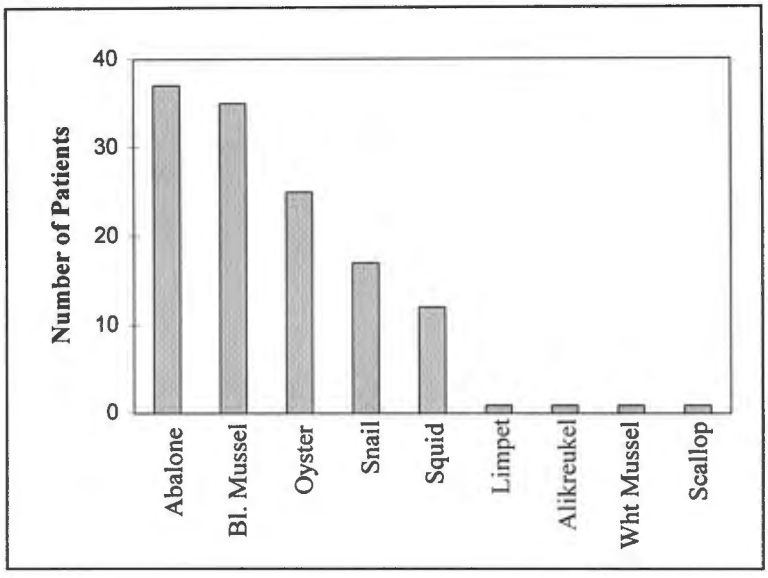
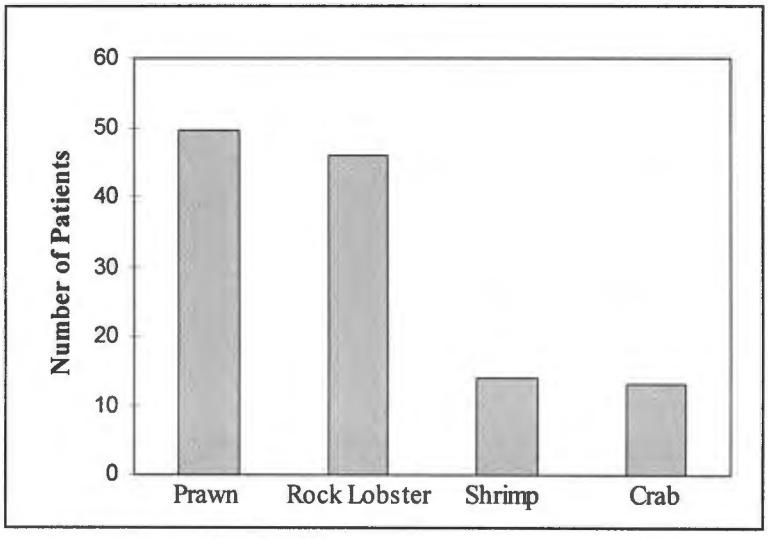


Figure 2.1: Flowdiagram of sample selection, diagnosis and analysis of allergy to seafood by different *in vitro* and *in vivo* methods.

A) Mollusc Species



B) Crustacean Species



C) Fish Species

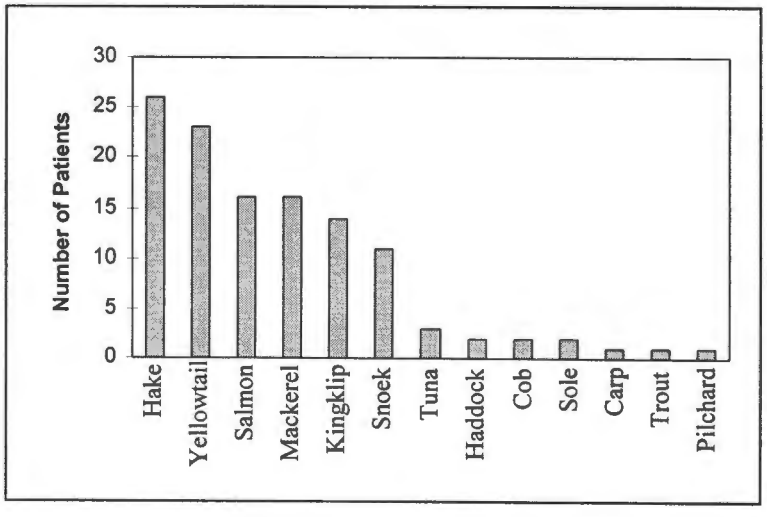


Figure 2.2: Frequency of seafood species implicated in allergic reactions to seafood reported in the questionnaire by 105 subjects with perceived seafood allergy. Note: Bl. Mussel = Black Mussel, Wht. Mussel = White Mussel.

3.3 Symptoms

The symptoms experienced by the subjects were divided into four categories: cutaneous, gastro-intestinal, respiratory and miscellaneous (Table 2.I). The total number of symptoms reported by the 105 subjects was 517, of which 33% were in the cutaneous category, 25% in the gastrointestinal tract (GIT), 17% in the respiratory system and 25% in the miscellaneous category. Table 2.I displays the most frequent symptoms in these categories.

In the cutaneous category itching and swelling of the throat were very common, followed by itching of the lips and/or tongue. The most common symptoms in the GIT group were nausea and vomiting and in the respiratory system wheezing. In the remaining category, anxiety was the most common symptom experienced followed by flushing.

Within two hours of consumption of seafood 68 subjects (65%) had adverse reactions and 24 subjects (23%) stated symptoms only after two to seven hours. The remaining 13 subjects reported varying intervals on several occasions. Not only the ingestion but also handling of seafood and inhaling the odours of raw or cooked seafood caused also adverse reactions in 27 subjects of the population sample (26%).

Table 2.I: Frequency of reported symptoms in subjects with perceived seafood sensitivity. **A)** in 61 subjects to the mollusc group, **B)** in 40 subjects to the crustacea group and **C)** in 38 subjects to the fish group.

A) Mollusc

SYMPTOMS	PERCENTAGE (%)
Nausea/vomiting/abdominal pain	61
Oropharyngeal itching/swelling	53
Anxiety	49
Diarrhoea	43
Asthma/wheezing	41
Urticaria/eczema	39
Flushing	34
Dizziness	26
Headache	23

B) Crustacea

SYMPTOMS	PERCENTAGE (%)
Oropharyngeal itching/swelling	100
Nausea/vomiting/abdominal pain	78
Asthma/wheezing	61
Anxiety	48
Urticaria/eczema	44
Flushing	22
Diarrhoea	17
Dizziness	13
Headache	9

C) Fish

SYMPTOMS	PERCENTAGE (%)
Oropharyngeal itching/swelling	66
Nausea/vomiting/abdominal pain	53
Urticaria/eczema	53
Asthma/wheezing	42
Flushing	40
Headache	29
Anxiety	26
Diarrhoea	18
Dizziness	13

3.4 Specific IgE assessment by RAST

Frequency of IgE responses

The frequency of specific IgE responses to 12 seafood species as determined by CAP-RAST in 80 subjects is presented in the three different species groupings as demonstrated in Figure 2.3. In-house RASTs determined specific IgE to abalone and yellowtail.

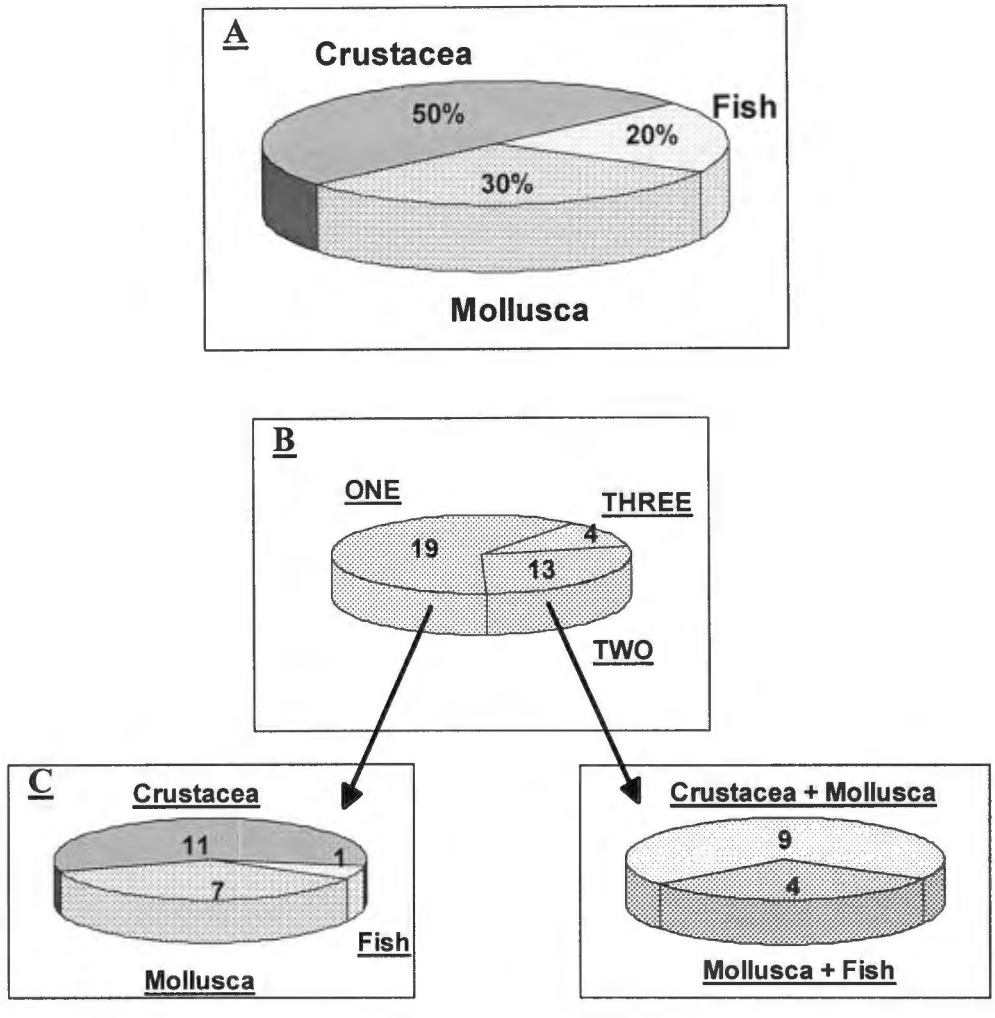


Figure 2.3: Overall distribution of positive CAP-RAST results in the different seafood groups. **A)** 36 subjects had positive results to one or more seafood species in the different groups (n=131). **B)** Positive RAST results of subjects to species in only one, two or all three seafood groups and their distribution among the different groups (n= number of subjects). **C)** Distribution of subjects with monosensitivity in one seafood group or with concurrent results in two groups.

Among the 80 subjects tested 36 had a total of 131 +ve RASTs. 65 (50%) of this +ve RASTs were in the crustacean group, 40 (30%) among the mollusc and 26 (20%) among fish (Fig. 2.3 A). The majority of subjects (n=19) had +ve RAST response to one or several species in only one of the three seafood groups (Fig. 2.3 B) followed by reactions in two different groups (n=13) and to all three groups (n=4). The majority of reactions to only one seafood group was to crustacea (n=11) (Fig. 2.3 C), followed by mollusc (=7) and fish with only one subject. Co-reactivity, as observed by concordant positive RAST results, among the three groups was the highest between mollusc and crustacea with 9 individuals, whereas no subject was +ve to crustaceans and fish only. Among the 9 fish allergic individuals, 4 were positive to all three groups and 4 to fish and molluscs whereas one was sensitive to fish only. None of the subjects had positive RAST results to crustacea and fish simultaneously without being also sensitised to mollusc.

I identified in each of the major three seafood groups subjects who had a positive IgE response to all four species tested in each group. However, in the mollusc group only 3/24 RAST positive subjects were positive to all four species, whereas in the group of crustacean and fish, 10/24 and 5/9 subjects respectively, had simultaneous responses to all four species in their group. These results indicated that the immune responses to mollusc are more diverse than to the other compared seafood groups.

Quantitative assessment of IgE response and co-reactivity

Figure 2.4 demonstrated the specific commercial IgE RAST results and the in-house abalone RAST results of all 24 mollusc sensitive subjects. The strongest IgE response was to oyster with a mean of 4.5 ku/l, followed by mussel, snail and squid with mean values of 2.5, 2.5 and 1.6 ku/l respectively. The specific IgE response to abalone was expressed as %-binding with a mean of 12.1%. Simultaneous reactivity to all four mollusc CAP-RASTs was found in 3 subjects with a strong decreasing immune response from oyster- to squid RAST (Fig. 2.4 B).

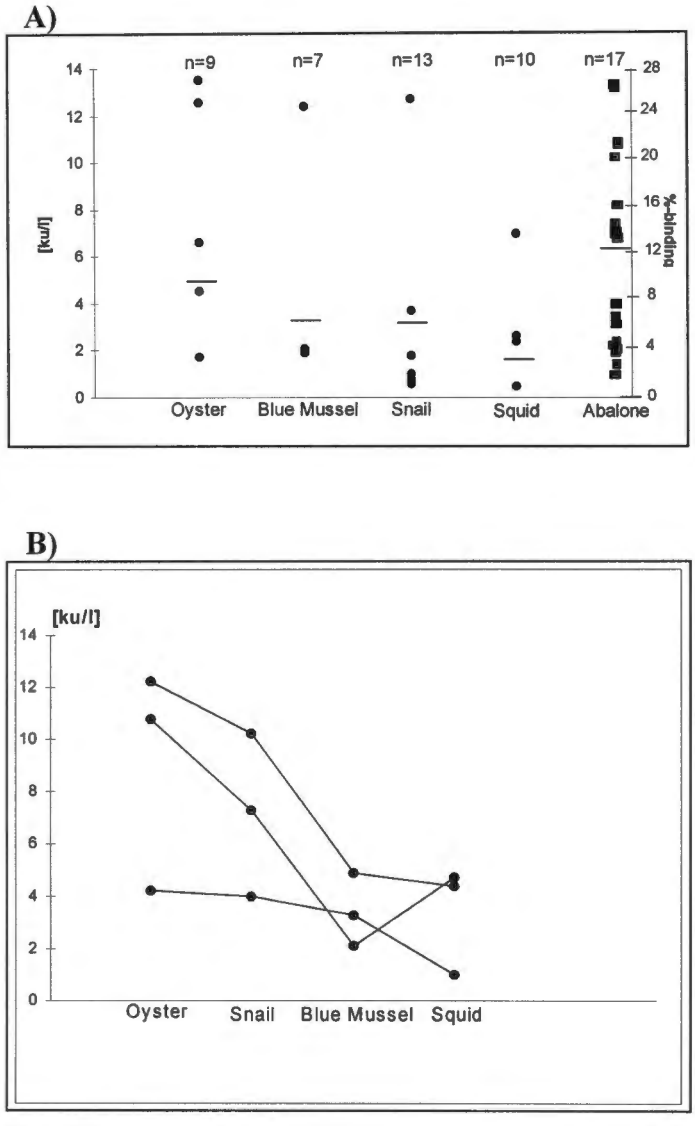


Figure 2.4: **A)** Distribution of specific IgE responses to different mollusc species in twenty-four subjects with seafood allergy and their mean values for each species in ku/l and the in-house Abalone-RAST in %-binding. The following CAP-RASTs were used: Oyster (Rf290), Snail (Rf314), Blue Mussel (f37), Squid (Rf258). **B)** Comparison of concurrent IgE response of three subjects to four different mollusc species.

The IgE response of 24 crustacean sensitive subjects to langoustine, crayfish, lobster, shrimp and crab was illustrated in Figure 2.5 with a mean of 13.6, 6.4, 6.0, 5.0 and 5.0 ku/l respectively. The mean value for the Langoustine-RAST was far higher (but not significantly) than the means for the other four crustacean species. However, there were only 8 sera available for Langoustine-RAST testing as compared to more than 13 available sera for the other crustacea RASTs. Therefore, the results for the Langoustine-RAST might not be representative for the whole group. Co-reactivity among the five different crustacean species was found in 6 subjects (Fig. 2.5 B). The immune responses observed to the different crustacean species are not very different from each other.

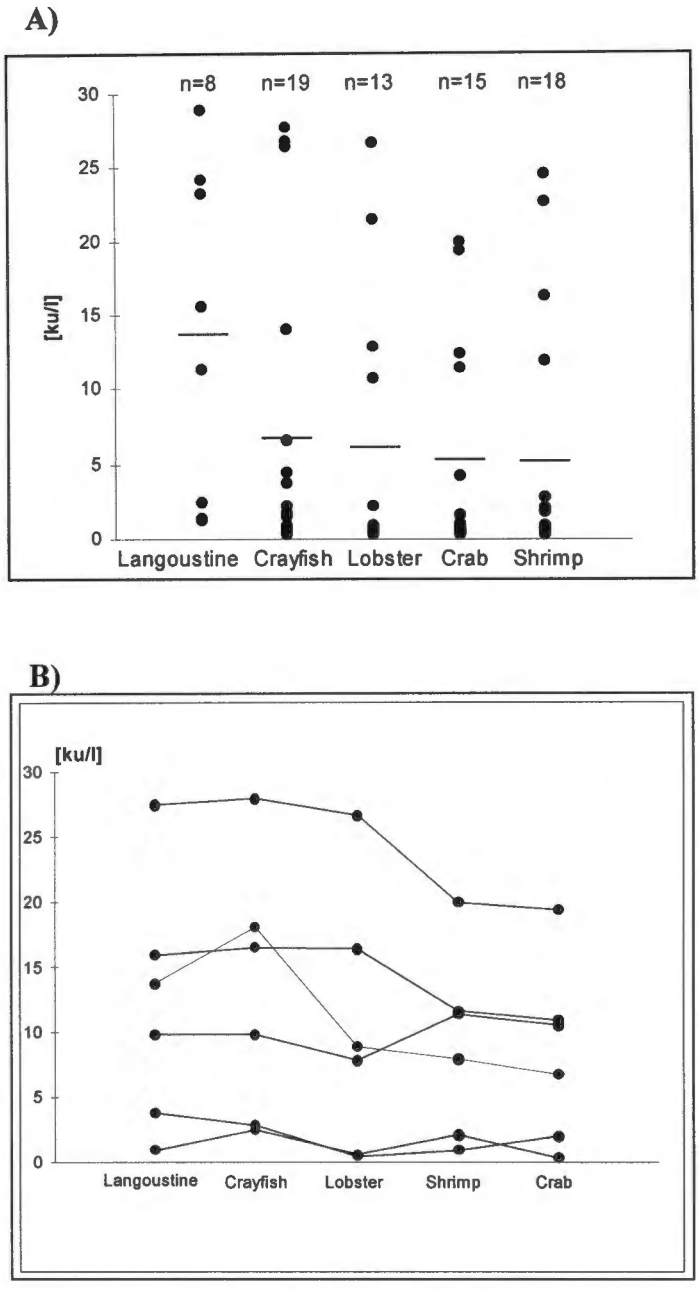


Figure 2.5: **A)** Distribution of specific IgE responses to different crustacean species in twenty-four subjects with seafood allergy and their mean values for each species in ku/l. The following CAP-RASTs were used: Langoustine (Rf304), Crayfish (Rf320), Lobster (f80), Shrimp (f24), Crab (f23). **B)** Comparison of concurrent IgE response of six subjects to five different crustacean species.

The immune response of the 9 fish sensitive subjects to hake, mackerel, tuna and salmon are presented in Figure 2.6, with mean values ranging from 6.6 ku/l to 2.4, 2.0 and 1.4 respectively. Co-reactivity among the four different fish species was found in five subjects (Fig. 2.6 C) with the strongest immune response to hake and decreasing to tuna or salmon.

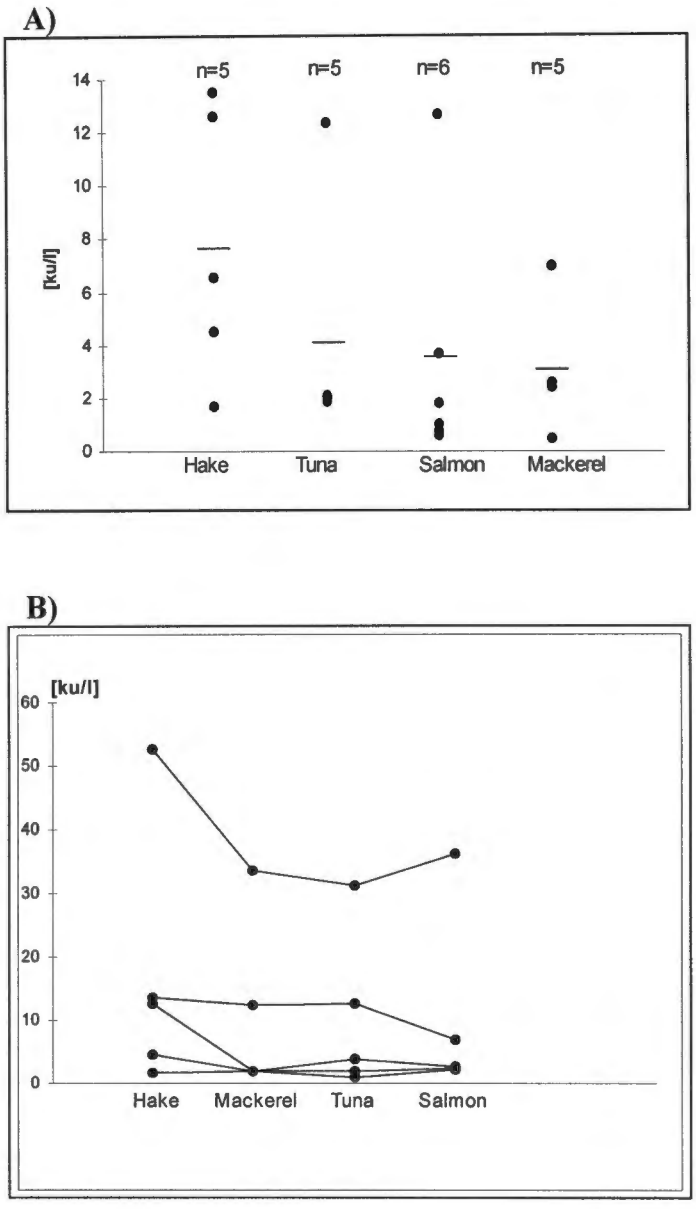


Figure 2.6: **A)** Distribution of specific IgE responses to different fish species in nine subjects with seafood allergy and their mean values for each species in ku/l. The following CAP-RASTs were used: Hake (Rf307), Mackerel (Rf206), Tuna (f40), Salmon (f41). **B)** Comparison of concurrent IgE response of five subjects to four different fish species.

Correlation of Co-reactivity

Several subjects demonstrated co-reactivity to some or all mollusc species and also to members of the crustacea and fish group. Comparing the RAST ratios and analysing them by linear regression and student t-test showed a positive correlation among the different mollusc only for five of the ten possible pairs (Table 2.II). The specific IgE response to abalone correlated among the different molluscs only with snail (r=0.79) and squid (r=0.97) whereas there was no significant correlation with blue mussel and oyster.

Interestingly, the two closely related bivalve species (oyster and blue mussel) are not positively correlated. In contrast, the two representatives, snail and squid of the other two mollusc classes, bivalvia and cephalopoda, are positively correlated.

The two representative species of the class gastropoda, the abalone and snail RAST both demonstrate positive correlation to some crustacean RASTs; abalone to crayfish, shrimp and crab and snail to lobster, shrimp and crab. There was no positive correlation of any mollusc or crustacea species with any of the four fish species.

In contrast to the very heterogeneous group of mollusc, among the group of crustacea and fish all species showed a very high correlation ($p<0.01$) to each other.

Frequency of reported allergy and RAST result

Further analysis of the RAST results compared the positive RAST results to a particular seafood species with the reported species in the initial questionnaire replied. In the mollusc group, abalone specific IgE antibodies were identified in 17 of 27 subjects with a history of abalone sensitivity (Fig. 2.7 A) followed by 7/26 for mussel, 9/16 for oyster, 12/12 for snail and 10/10 for squid. In this seafood group the lowest number of positive RAST results compared to the reported adverse reactions was to blue mussel with only 27%. The in-house RAST identified in 63% of the reported abalone sensitive subjects a specific IgE response.

In the fish group, the four fish species analysed by RAST had positive results ranging from 28% for hake to 83 % for tuna (Fig. 2.7 C).

In contrast to these two seafood group were the results in the crustacea group. All subjects with a history of allergic reactions demonstrated specific IgE (Fig. 2.7 B) resulting in a 100% confirmation of their reactions.

Table 2.II: Correlation matrix of linear regression of CAP-RASTs results to different seafood species with each other by student t-test.
Note: * significant P< 0.05; ** significant P<0.01; n= number of subjects with concurrent sensitivity.

CAP-RASTs	Abalone	Snail	Squid	Blue Mussel	Oyster	Lobster	Crayfish	Shrimp	Crab	Hake	Mackerel	Salmon
SNAIL	0.79 ** n=9											
SQUID	0.97 * n=4	0.62 n=6										
BLUE MUSSEL	0.67 n=5	0.95 ** n=7	-0.15 n=4									
OYSTER	0.09 n=4	0.93 ** n=7	0.94 ** n=6	0.42 n=5								
LOBSTER	0.64 n=8	0.70 * n=8	0.16 n=5	0.58 n=5	-0.10 n=5							
CRAYFISH	0.77 * n=9	0.45 n=9	0.71 n=4	-0.93 n=4	0.28 n=6	0.90 ** n=10						
SHRIMP	0.75 * n=7	0.76 * n=8	0.27 n=5	0.65 n=5	0.15 n=6	0.97 ** n=10	0.97 ** n=10					
CRAB	0.78 * n=7	0.35 n=7	0.83 n=4	-0.90 n=4	0.09 n=5	0.97 ** n=10	0.93 ** n=10	0.98 ** n=9				
HAKE			0.35 n=5									
MACKE-REL			-0.24 n=5			-0.64 n=3	-0.68 n=3	-0.72 n=3	-0.66 n=3	0.97 ** n=5		
SALMON			0.34 n=5			-0.46 n=3	-0.47 n=3	-0.51 n=3	-0.48 n=3	0.94 ** n=5	0.97 ** n=5	
TUNA			0.37 n=5							0.96 ** n=6	0.97 ** n=5	0.99 ** n=6

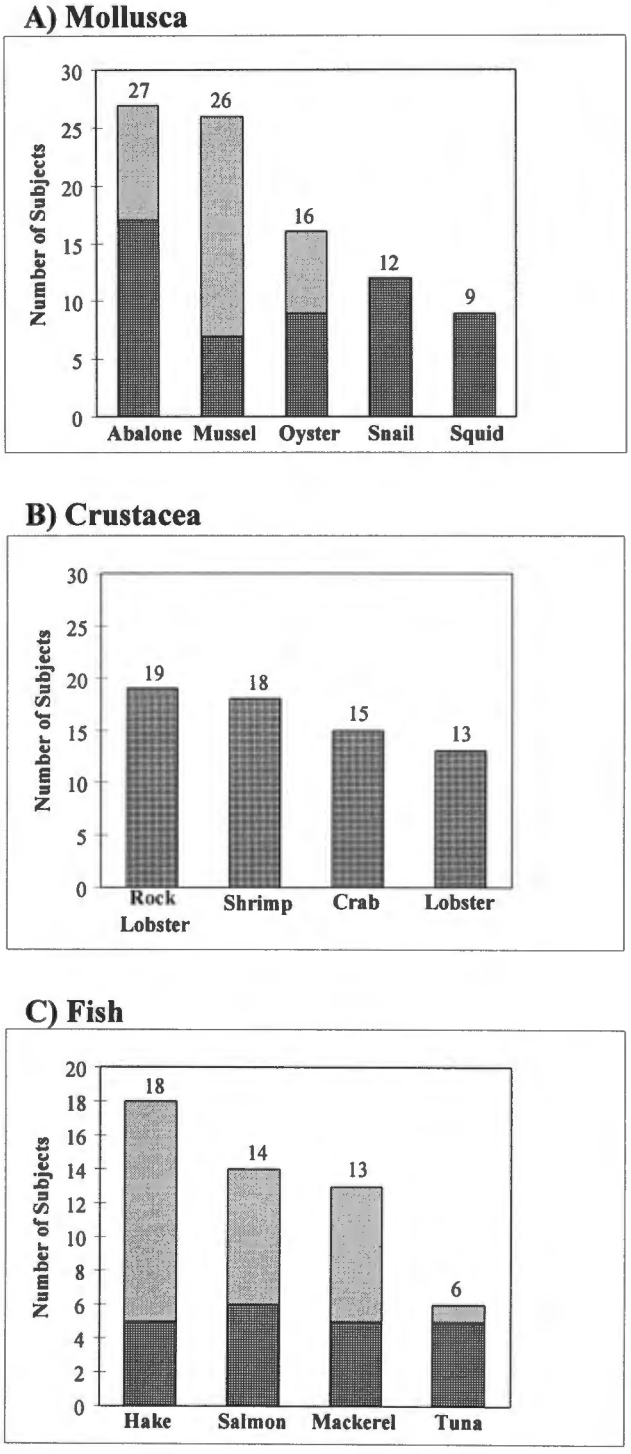


Figure 2.7: Frequency of species implicated in perceived seafood allergy as reported by 80 subjects to mollusca **A)**, crustacea **B)** and fish **C)**. Number of subjects which implicated a particular species is indicated by the black bar and positive RAST results by a hatched bar (≡).

However, screening all seafood sensitive subjects for the different species I identified several individuals with positive RAST results to species, which have not been reported as inducing an adverse reaction. Analysing the seafood sensitive subjects for the offending species in the three groupings one becomes aware of the fact that the utilised seafood species for the commercial CAP-RASTs are mostly of European origin. The limitation of the availability of RASTs especially to local mollusc species is highlighted in Table 2.III.

Table 2.III: Classification of mollusc species causing allergies, local representative species, available commercial CAP-RASTs and their scientific names. N.A. = not available

Class	Orders	Some Local Species	Species used for commercial CAP-RAST
GASTROPODA	ABALONE	- Abalone: *Haliotis midae*; *H. spadicea*	- N.A.
	SNAIL	- Snail (brown garden): *Helix aspersa*	- Snail: *Helix aspersa* Rf314
	WHELK	- 'Restaurant' snail: *Achatina fulgens*	- N.A.
		- Giant Periwinkle: *Turbo sarmaticus*	- N.A.
BIVALVIA	MUSSEL	- Black Mussel: *Choromytilus meridionalis*	- N.A.
		- White Mussel: *Donax serra*	- N.A.
			- Blue Mussel: *Mytilus edulis* f37
	OYSTER	- Cape Rock Oyster: *Strimostrea margaritacea*	- N.A.
		- Red Oyster: *Ostrea atherstonei*	- Oyster: *Ostrea edulis* f290
	CLAM		- Clam: *Ruditapes sp.* f207
	SCALLOP		- Scallop: *Pecten sp.* Rf338
CEPHALOPODA	SQUID	- White Squid: *Loligo vulgaris reynaudii*	- Squid: *Loligo sp.* Rf258
		- Red Squid: *Todaropsis eblanae*	- N.A.
			- Pacific Squid: *Todarodes pacificus* f58
	OCTOPUS	- Octopus: *Octopus sp.*	- Octopus: *Octopus vulgaris* f59

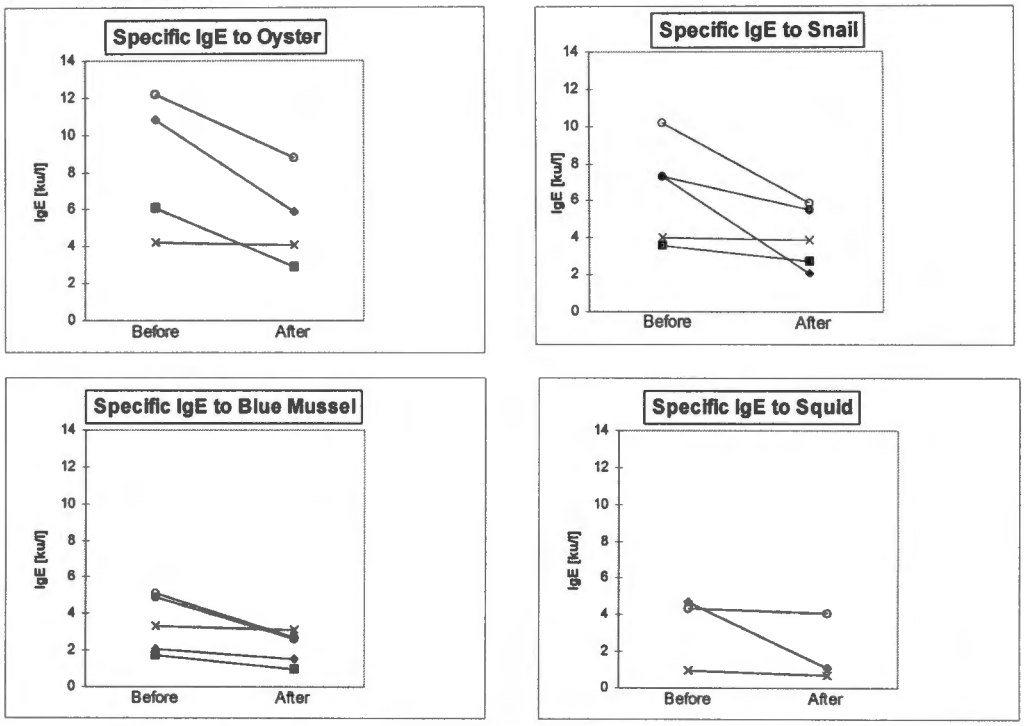
In the group Gastropoda no RAST is available for abalone, limpets or whelks. The only species offered as a commercial RAST is for snail (*Helix aspersa*). This delicacy, often consumed as starter in restaurants, originates from France where the above species is found in vineyards. Unfortunately, because of financial reasons, local restaurants use predominantly a different snail species (*Achatina fulgens*) coming from Asia.

In the group of Bivalvia no RAST is available for the commonly consumed black and white mussel species (*Choromytilis meridionalis* and *Donax serra* respectively). Instead the blue mussel species (*Mytilus edulis*) is offered which does not occur in the Southern Hemisphere. Moreover, the Cape rock oyster (*Strimostrea margaritacea*) is not offered as RAST, but the European oyster (*Ostrea edulis*) which is probably the nearest related species to our Red oyster (*Ostrea atherstonei*). However, there are other RASTs available in this group, for clam and scallop species, which are both very rarely consumed in South Africa. The commercial RASTs available for the Chephalopoda group seem to have the best association to our local squid and octopi species with members of the genus *Loligo*, *Todarodes* and *Octopus*.

3.5 Persistence of the immune responses to seafood over a longer period

Six subjects, with concurrent allergy to mollusc and/or crustacea, were followed up 3-4 years after their first consultation in our Allergology Clinic and the immune response to different mollusc and crustacean species compared by RAST reactivity (Fig. 2.8). All subjects were recommended to avoid any seafood and to wear a "Medic-Alert" symbol. In all subjects but one, the concentration of specific IgE antibodies to all tested seafood species decreased on average by about 50%. The one subject which did not demonstrate a decreased immune response over three years admitted to occasionally ingesting seafood, in particular rock lobster and prawns, and taking oral antihistamines before the meals to contain the symptoms.

A) Mollusca



B) Crustacea

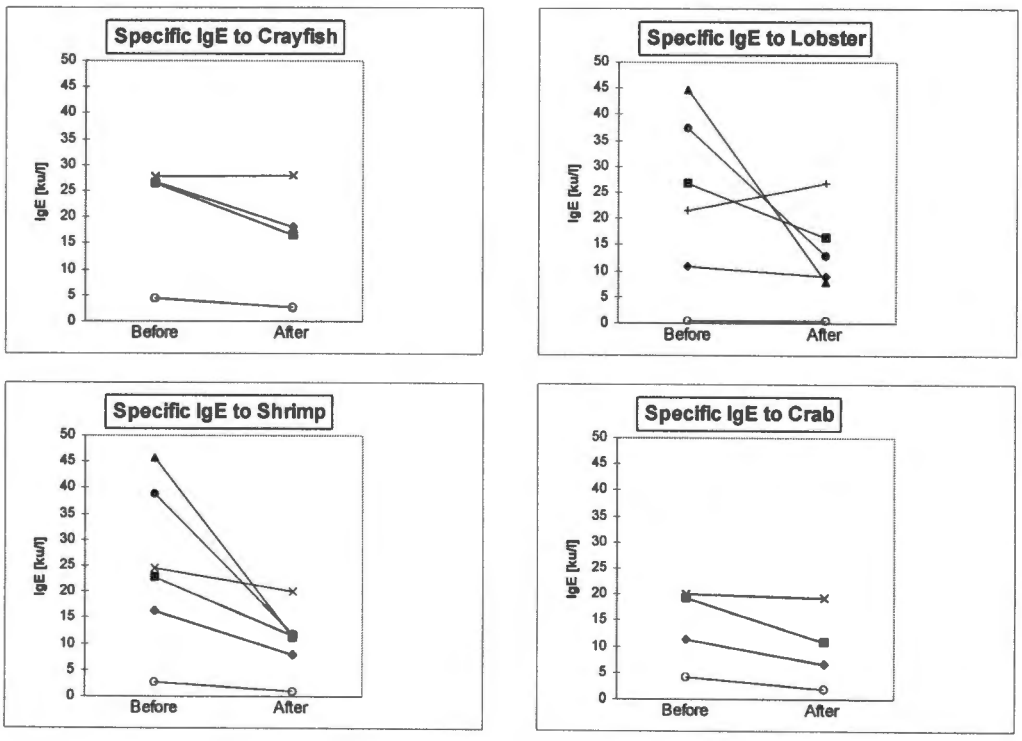


Figure 2.8: Specific IgE reactivity of six seafood sensitive subjects before consultation in our clinic and after 3-4 years as measured by CAP-RAST in ku/l. All subjects, except -+- , demonstrated a decrease in IgE reactivity to different species among the mollusc **A)**, and crustacea group **B)**.

4. Discussion

4.1 Questionnaire

Symptoms following exposure to seafood

The majority of subjects reported symptoms of adverse reactions such as swelling of the throat and nausea/vomiting (51% and 54% respectively). Urticaria, wheezing, anxiety and flushing were each reported by over one third of all 105 subjects. These distribution of symptoms is not representative of classical food allergy (Sampson, 1999). One reason could be the relative high mean age of 41.2 year caused by some quite old people. Because allergy is not common in older people some symptoms might be distorted. Furthermore, because of studying a selected patient cohort, the results from these data cannot be extrapolated for the entire geographical population. However, analysing the questionnaire according to adverse reactions in the three different seafood groupings certain symptoms are more frequent than others.

Itching and swelling of the throat seems to be a particularly common symptom reported by all subjects with reported sensitivity to crustaceans. In the other two groups only two third of the subjects were affected by this symptom. In the mollusc sensitive group diarrhoea and dizziness were a far more frequent problem than in the other groups. Anxiety was not as common in the fish group as it was in the other groups, but urticaria and flushing was more frequent.

A study on seven squid sensitive subjects demonstrated that respiratory and cutaneous symptoms varied between 43%-57% (Carrillo, et al., 1992). This was supported in a study of five subjects with sensitivity to limpet and abalone (Morikawa, et al., 1990). These findings are similar to the reported symptoms by mollusc sensitive subjects in my study.

Symptoms experienced while or within minutes of ingesting the offending food is called the "oral allergy syndrome" (OAS) and is frequently observed (70%) in crustacean sensitive subjects (Daul *et al.*, 1988; Daul, et al., 1993). However, urticaria was more frequent (83%) followed by gastrointestinal symptoms (35%).

Fish was reported to induce mainly urticaria/eczema (80%) in a study conducted on 21 fish sensitive subjects from Spain (Crespo *et al.*, 1995b). Skin problems were followed by gastrointestinal symptoms in 19%. However, the OAS was reported to a much lower extend with about 10% and inhalation of fish odours caused respiratory symptoms in more than 50%. A different study on 39 subjects with a self-administered questionnaire also reported mainly cutaneous symptoms (70%), followed by wheezing (54%) and gastrointestinal symptoms (26%) (Helbling *et al.*, 1996). The later study reported very similar results found in my study group.

The comparison of my data with results of other studies demonstrates that a self-administered questionnaire to subjects with have their hypersensitivity not yet confirmed by laboratory tests can differ in the frequencies of reported symptoms. However, the general trend of specific frequent symptoms in a particular seafood group correlates well with most other studies.

Nevertheless, it is appeared that not all reported adverse reactions were due to allergic responses. The unexpected high incidence of diarrhoea in the mollusc group correlates well with the symptoms experienced by subjects ingesting local black mussels contaminated by Red Tide algae. This phenomenon is observed every year on our coastline, causing extensive damage among the marine life (Horstman, et al., 1991; Pitcher, et al., 1993). The toxins generated by this algae cause Diarrhoetic Shellfish Poisoning (DSP) among filterfeeders such as black mussels and oysters and in turn produce Type I allergic reactions in humans consuming this contaminated shellfish (Luckas, 1992; Sakamoto, et al., 1987).

Flushing and urticaria was very frequent among fish sensitive subjects. These are both typical symptoms of Type I allergic reactions. Nevertheless, these symptoms are also very often experienced after ingestion of spoiled fish, particularly certain species with dark meat such as tuna, mackerel and yellowtail. The fish species involved belong mainly to the family scombroidae and the toxication is therefore called Scombroid Fish Poisoning (Brown, 1993; Merson, et al., 1974; Morrow, et al., 1991; Smart, 1992). A recent report of Scombroid fish poisoning in the Western Cape has been linked to spoiled yellowtail (Muller, et al., 1992). Because of the high incidence of symptoms such as flushing etc. and the fact that no fish specific IgE were found I would like to suggest that scombroid fish poisoning was, at least for some of the studied subjects, the cause of their reactions.

The time of onset of Type I hypersensitivity symptoms after ingestion of the offending food was usually between a few minutes and up to two hours. In addition, handling of seafood caused symptoms in over 27% of this sample population. This has also been observed by other research groups were contact with seafood by handling or inhaling cooking vapours generated allergic symptoms (Carrillo, et al., 1992; Cartier *et al.*, 1986; Glass *et al.*, 1998; Lemiere *et al.*, 1996).

However, a large number of subjects among the mollusc sensitive group (34%) demonstrated delayed reactions, occurring between 2 and 7 hours after ingestion especially of abalone. The delay of onset of reactions has also been reported in previous studies on seafood allergy. These studies reported late phase reactions occurring 3.5 hours after ingestion of squid in a young girl

(Shibasaki, et al., 1989) and 3 hours after ingestion of limpets and abalone in five subjects (Morikawa, et al., 1990). In addition also a crustacean species, the snow crab, were reported to induces late reactions in 23 of 46 subjects (Cartier, et al., 1984). Most studies involved different mollusc species indicating that delayed reactions may be a common feature for this seafood group. The one study on snow crab and delayed reactions (Cartier, et al., 1984) involved subjects with occupational asthma and the route of sensitisation was via the respiratory tract.

One has to bear in mind that the immediate reaction (less than 2 hours after ingestion) underlies a different mechanism compared to the late phase reaction. Immediate-type (Type I) reactions involve soluble antigen interacting with IgE bound to high affinity receptors (FcεRI) mainly on mast cells or basophils. Antigens (allergens) induce the release of mediators (such as histamine) and several cytokines including IL-5 and IL-3. Reactions delayed-in-time are called late-phase responses (LPR) and are initiated when antigens are presented to sensitised CD4+ T lymphocytes. T lymphocytes of the T helper (Th) type 1 release cytokines such as IL-2 and IFN-γ, opposed to Th2 lymphocytes which release typically IL-4 and IL-5 cytokines. The classical delayed-type hypersensitivity (Th1 lymphocytes) are the tuberculin reaction and contact dermatitis, whereas the Th2 response is frequently found in chronic allergic inflammation such as chronic asthma and atopic eczema. However, present evidence suggest that the LPR may have both an immediate type response as well as an delayed component.

The pattern of immediate allergic reactions following the ingestion of seafood is in general similar to that reported to other seafoods (Daul, et al., 1993). However, asthma like symptoms were a common feature in mollusc and crustacean sensitive individuals. In a recent study shellfish were the second most commonly implicated food in subjects with food-induced rhinitis or asthma (Oehling, et al., 1992). This could be confirmed in my study, where between 41% and 61% of the subjects in the different seafood groups (see Table 2.I) experienced asthma and wheezing after ingestion of seafood.

Summarising my data and the findings of other studies it appears that the allergens from mollusc, including the South African abalone, induced a late phase reaction in several sensitised subjects. Further studies have to be conducted to identify the nature of these allergens and why the reaction is often delayed compared to the immediate reaction observed to crustaceans and fish. One explanation could be the rubbery consistency of mussel tissue especially of abalone and limpets that could during the longer digestion period result in a slow release of the allergens. In addition, the stability to digestion of particular allergens could be a significant parameter as was

demonstrated in a gastric model by Astwood et al (Astwood, et al., 1996). However, these hypotheses need to be investigated in a separate study *in vivo*.

Seafood species implicated

The two most frequent seafood species reported to cause allergic reactions belonged to the group of crustacea; prawn and rock lobster accounted for 47% and 44% of the reactions respectively. However, I observed that in the Western Cape, three mollusc species were among the five most frequent reported seafood species overall to cause adverse reactions. The particular species included abalone, black mussel and oyster representing 35%, 33% and 24% of all reactions respectively. Reported reactions to fish were mainly from hake and yellowtail representing 25% and 22% of all reactions. A study conducted in Spain on 355 children demonstrated that fish was the second most frequent implicated food group (30%), wheras hypsersensitivity to crustacean and molluscs were found in only 6% and 3% respectively (Crespo *et al.*, 1995a). These findings confirmed the overall importance of crustacea sensitivity among seafood allergic subjects. However, it is clear that in the Cape, molluscs play an equally important role in inducing allergic reactions in local subjects.

4.2 Specific IgE response as determined by RAST

Looking at the overall distribution of specific IgE responses among the three main seafood groups analysed by CAP-RAST it is again clear that over 80% of the reactions occurred among the mollusc and crustacean species (Fig.2.3 A). 90% of all subjects reacted to one or two seafood groups but not to all three groups simultaneously. Most subjects had reactions to crustacean or mollusc species only. However, 25% reacted to both groups and another 10% to mollusc and fish concurrently. Some of the subjects had in addition positive RASTs to house dust mite (HDM) as described in detail in chapter III.

It has been reported previously that cross-reactivity exists between related seafood species but also between arthropods including species such as caddis fly, cockroaches and mites (De Maat-Bleeker *et al.*, 1995). Lehrer observed similar cross-reactivities in subjects with crustacean and oyster allergy (Lehrer and McCants, 1987). Leung and colleagues (Leung *et al.*, 1996) identified cross-reacting allergens not only among several crustacea and mollusc species but also in addition to cockroach, grasshopper and fruit fly. Inhibition studies with recombinant tropomyosin supported the view that this cross-reacting allergens share immuno dominant epitopes. RAST-inhibition studies in chapter III will analyse the observed co-reactivities in more detail and demonstrate possible cross-reactivities among the different seafood species.

IgE responses to different seafood species: Monosensitivity versus concordant sensitivity

Comparing the level of the specific IgE responses to the different mollusc species by RAST (Fig. 2.4 A), it is clear that the IgE levels to oyster was more than four times higher than to squid. This observation was confirmed by analysing the concurrent immune responses of three patients to all four mollusc RASTs which showed also decreasing levels of IgE from oyster to squid (Fig. 2.4 B).

Monosensitivity to molluscs was encountered in 9/24 (38%) of the patients sensitive to molluscs by CAP-RAST. In contrast to the mollusc group only 29% in the crustacea (7/24) and 33% in the fish (3/9) group demonstrated monosensitivity. Interestingly, of the nine subjects with mono allergy, the majority responded to snail or squid (four subjects each) and only one to oyster whereas none responded to blue mussel only. These findings suggested that snail and squid have very specific allergens and that the bivalvia (oyster and blue mussel) have mainly common allergens shared with the other two investigated mollusc species.

In addition, the mollusc group had not only the highest number of mono allergies but also the lowest number of subjects with concurrent sensitivity to all four molluscs (3/24=13%). This was in strong contrast to the crustacean and fish group where 42% (10/24) and 56% (5/9) respectively displayed specific IgE to all investigated species in their group. These results supported the view that the different crustacea and fish species in contrast to the mollusc group share common allergens demonstrated by concurrent sensitivity to several species of the same group.

Subjects with concordant RAST reactivity to all analysed crustacea species (Fig. 2.5) demonstrated decreasing levels of reactivity from langoustine to crab, but the levels were not significantly different from each other. These results are in strong contrast to reactivities in the fish group (Fig. 2.6). The strongest response was to hake, with a much lower IgE reactivity to the other fish species, which were not much different from each other.

The observation that the immune responses to the different mollusc species are so diverse could be partially explained by a much higher allergen concentration in the utilised seafood extracts for the preparation of the commercial CAP-RASTs. This could explain the stronger IgE response to e.g. the utilised oyster extract compared with the squid. In addition, it is also possible that the specific oyster allergen/s are more potent or more stable to degradation thereby loading more active allergens onto the CAP-RASTs solid phases during manufacturing. However, the lower response or complete lack of binding to particular mollusc species could also be explained by the

utilisation of species which are not found in South Africa and therefore lack the relevant species specific allergen.

Furthermore, in the mollusc group the immune response of subjects with concurrent specific IgE only to mollusc but not to any other species from the crustacea group demonstrated lower RAST reactivity as compared to subjects with reactivity to both seafood groups (Table 2.IV). In the group of crustacea was the same trend observed (significant with $p<0.05$; t-test) with much higher specific IgE values for subjects with concurrent mollusc/crustacea allergy than for subjects with an immune response to crustacean species only. Concurrent allergies to species in the mollusc and crustacea group were observed to induce a strong immune response. The cause for this observation could be the presence of common allergens in some species, which are prevalent in both groups and provoked therefore a cross-reacting immune response.

The correlation study on all subjects with concurrent specific immune response to seafood (see Table 2.II) supported this hypothesis. Only five of the ten possible associated mollusc RASTs demonstrated a significant correlation. Abalone IgE as expected, correlated well with IgE to the other gastropod, snail ($p<0.01$) but also to the cephalopod squid ($p<0.05$). Oyster demonstrated a very strong correlation with snail specific IgE and squid ($p<0.01$), however, there was no correlation with specific IgE to the other bivalvia member, the blue mussel. Comparing the mollusc group with the crustacea, only the two gastropod species, the abalone and the snail demonstrated a significant correlation with the four crustacean species (all $p<0.05$). Interestingly, all the tested crustacean and fish RASTs correlated highly significantly among each group (all $p<0.001$). This affirmed that the major allergen/s in the crustacean or fish group must be highly conserved and reflected in a high degree of cross-reactivity.

Similar comparisons of mollusc reactivity with co-reactivity to fish or crustacean in a patient cohort have not been previously published to my knowledge yet. However, correlations between IgE responses among oyster and squid sensitive subjects and crustacean allergens have been implied in several publications. Lehrer et al. (Lehrer and McCants, 1987) pointed out that their is a significant correlation between oyster and crustacean RAST-reactivity suggesting common antigenic structures. In contrast to Lehrer's finding, I could not demonstrate such correlation's with oyster IgE but to the local frequently eaten abalone and snail. This could imply that the frequency of consumption of a particular seafood species in a region is a very important factor in determining and analysing cross-reactivities among seafood groups.

These findings indicated that the concurrent immune reactivity to different seafood species varies among the mollusc group far more than among the crustacean or fish and that for some or even all mollusc species a common allergen with crustacean may possibly be present. The possible role of species specific or cross-reacting allergens could only be investigated further by RAST-inhibition studies and the use of Western blotting techniques to identify the molecular characteristics of the allergens in question.

The cross-reactivity among the different seafood species and the characterisation of the allergens are analysed and compared in chapter III.

Table 2.IV: Specific IgE responses of all RAST+ve subjects to different mollusc and crustacea species and concurrent reactivity. The mean values and standard deviations are presented and the correlation analysed between MC-IgE and M-IgE and C-IgE respectively. Note: * = $p<0.05$ (t-test).

MC = Mollusca + Crustacea specific IgE; M = Mollusc specific IgE only;
C = Crustacea specific IgE only.

RASTs	Specific IgE (mean in ku/l)	MC-IgE	M-IgE	C-IgE
MOLLUSC				
ABALONE (%-Binding)	12.1% (n=17)	10.6% ±8.2 (n=12)	6.2% ±1.9 (n=5)	
OYSTER	4.5 ±4.7 (n=9)	5.7 ±4.9 (n=7)	0.8 (n=2)	
MUSSEL	2.5 ±1.5 (n=7)	2.9 ±1.2 (n= 6)	0.4 (n=1)	
SNAIL	2.5 ±3.1 (n=13)	3.7 ±3.4 (n= 10)	1.3 ±1.0 (n=3)	
SQUID	1.6 ±1.6 (n=10)	2.3 ±2.0 (n=6)	0.7 ±0.3 (n=4)	
CRUSTACEA				
CRAYFISH	6.4 ±9.7 (n=19)	8.6 ±10.6 (n=10)		1.3 ±1.1* (n=9)
LANGOUS-TINE	13.5 ±9.7 (n=8)	13.5 ±9.7 (n=8)		
SHRIMP	4.9 ±8.0 (n=18)	7.6 ±9.4 (n=11)		0.7 ±0.5* (n=7)
LOBSTER	6.0 ±9.0 (n=15)	6.5 ±9.3 (n=12)		0.7 ±0.2 (n=3)
CRAB	5.0 ±7.1 (n=15)	7.2 ±7.9 (n=10)		0.6 ±0.2* (n=5)

4.3 Change of specific IgE with time

The persistence of a specific IgE response to seafood allergens could be demonstrated by measuring the concentration of specific IgE over a period of several years (Fig. 2.8) in a small cohort of patients who I was able to follow up. 3-4 years after their first consultation at the Allergology Clinic, all six subjects illustrated a decrease of specific IgE antibodies to all tested seafood species, on average by about 50%. None of the subjects received medical treatment for their allergy to seafood but were informed about their condition and asked to avoid ingestion of any seafood. All subjects but one had avoided seafood since they were informed about their allergy to seafood. One subject could not resist the temptation of eating seafood and did consume occasional small pieces (especially of prawns and rock lobster) after taking oral antihistamines. His specific IgE values did not change significantly after four years from the first serum samples, and even increased as was observed for the Lobster-RAST. However, looking at all the RAST results after the first serum analysis only one of the six subjects acquired a negative specific IgE immune response as measured by CAP-RAST to one of the 8 different seafood species (squid).

These results confirmed that allergy to seafood persisted even after avoiding the offending allergen for several years and that food avoidance may not protect a subject of an allergic response by lowering the amount of specific IgE. This was supported by the fact that in 27 subjects (26%) of my sample population, allergic reactions were not only triggered by ingestion but also experienced after inhaling cooking odours or even handling seafood. Another reason for the persistence of specific IgE could be the occurrence of cross-reacting allergens in other food (other than seafood) or in inhalant substances.

In a similar study conducted by Daul et al. (Daul *et al.*, 1990), the levels of shrimp-specific IgE in 11 sensitised subjects were relatively constant during a 24 months period and not affected by a DBPCFC with shrimp. The possibility of incidental stimulation of IgE- mediated hypersensitivity was discussed in previous studies where the inhalation of seafood odours or fumes played an important role. Carillo could demonstrate this for seven patients with sensitivity to squid (Carrillo, et al., 1992), Cartier et al. (Cartier, et al., 1984) from inhaling vapours of cooked crab and several other groups for fish (Crespo, et al., 1995b; Dominguez *et al.*, 1996). In one study occupational allergy to clam was demonstrated by specific inhaling challenge (Desjardins *et al.*, 1995). Accidental exposure to seafood could not only elicit clinical symptoms and but also have some effect in delaying the development of tolerance in sensitised subjects.

4.4 Correlation with history

Analysed individually, the highest frequency of specific RASTs among all positive in-house RASTs and CAP-RASTs was observed for abalone and rock lobster with about 50% each (Fig. 2.7). The lowest frequency was observed for blue mussel, tuna and hake with 17% each. This high frequency of hypersensitivity to abalone reflected the commonness of allergy to an mollusc species in the Western Cape of South Africa.

It is interesting to note that the low confirmation of specific IgE following an adverse reaction to black mussel (6 of 26 reported adverse reactions) was associated with a high incidence of symptoms not often correlated with Type I hypersensitivity reactions such as anxiety, flushing, headache and dizziness. This observation may be explained with the high incidence of Red Tides on the West Coast of South Africa and their association with Diarrhetic (DSP) and Paralytic (PSP) shellfish poisoning (Horstman, et al., 1991; Pitcher, et al., 1993).

The low frequency of +ve-RASTs to tuna and hake in this study was also observed by other groups where tuna was considered to be more hypoallergenic (Bernhisel-Broadbent, et al., 1992b; James *et al.*, 1997) than other fish species. Analysis of local fish species extracts by SDS-PAGE and Western blot analysis indicated that hake and tuna have a very low concentration of Gad c 1 like allergens in the molecular range of 13 kDa (see chapter III). In addition, contamination with possible toxins such as scombroid toxins can cause symptoms similar to a Type I hypersensitivity reaction. Scombroid toxication is known world-wide (Gellert *et al.*, 1992; Smart, 1992) and also has been reported recently in South Africa (Muller, et al., 1992) where the fish species implicated was yellowtail.

The low frequency of fish allergy in my study is supported by data from our diagnostic service at Groote Schuur Hospital for the Red Cross Children's Hospital. Over a period of four years between 1994 and 1998 460 children were tested for suspected allergy to fish. Only 18% had specific IgE antibodies as detected using the CAP-RAST for cod fish. Nevertheless, whether cod fish is the correct species to use in this locality will be analysed in the following chapters.

Summary and Conclusion

The analysis of the questionnaire demonstrated that abalone was the third most reported species acounting for over one third of all reactions. Thus, in the Western Cape of South Africa, molluscs, and in particular abalone (*Haliotis midae*), play an important role in inducing allergic reactions to local seafood species. Further analysis of the questionnaires showed that delayed reactions were more frequently reported among mollusc sensitive subjects, and asthma like symptoms were not uncommon.

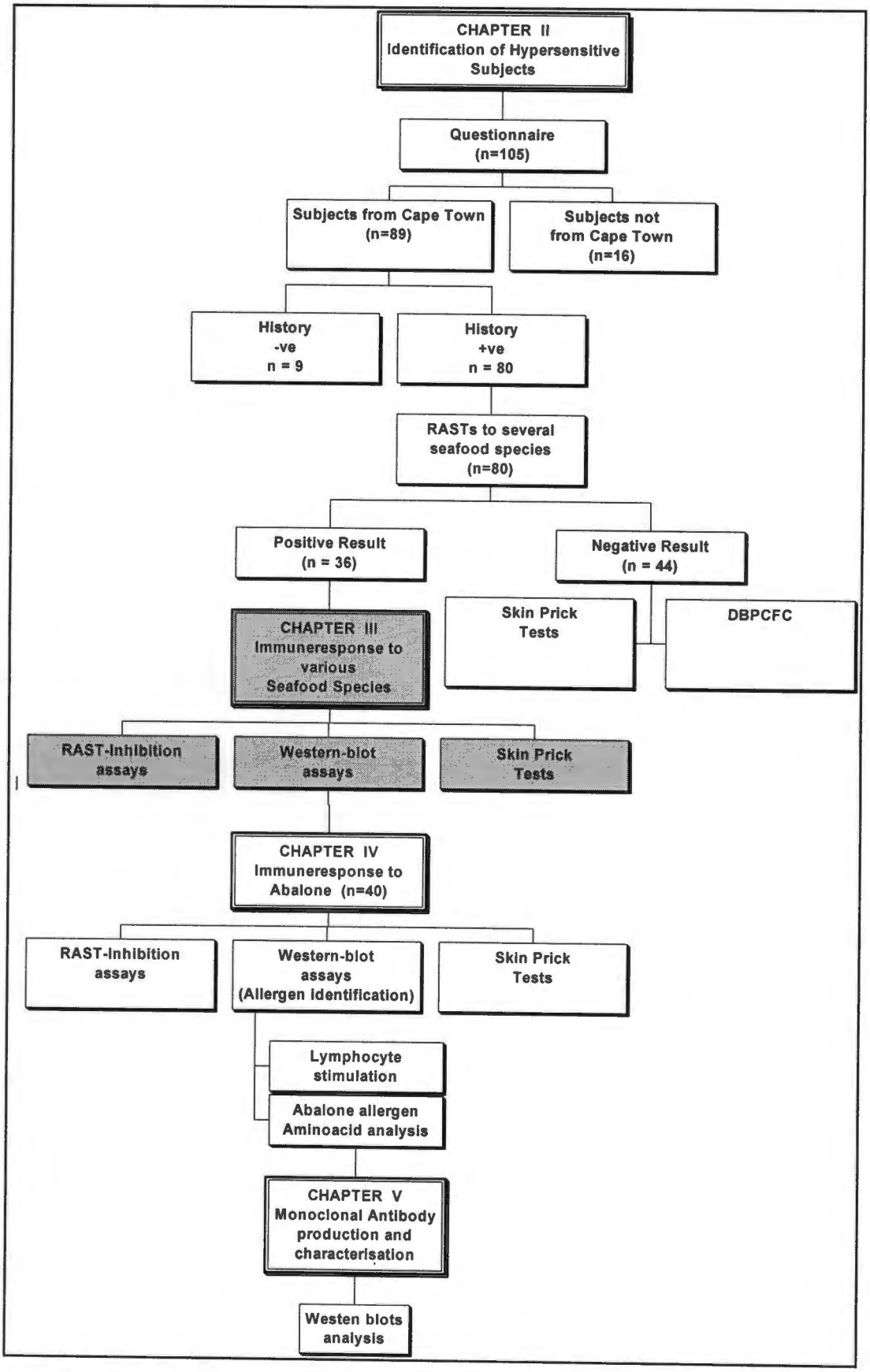
The high frequency of positive RAST results correlating with a history of an adverse reaction observed for in abalone sensitive subjects is a partial indicator of the sensitivity of the developed in-house RAST since double blind placebo controlled challenge studies were not performed. The specific IgE responses to the different seafood species by RAST illustrated that the immune responses to the mollusc species were considerably more diverse than the responses to crustacea or fish. Monosensitivity to a particular mollusc species was found in more than two thirds of the subjects with sensitivity to molluscs by RAST. Among the mollusc group, the majority of subjects with monosensitivity, reacted to snail or squid, suggesting that these species have specific allergens that are not cross-reacting. These findings were supported by the observation that concurrent sensitivity to other species in the mollusc group was infrequent, when compared to the crustacea and fish group.

Further evidence for mollusc specific allergens came from a series of correlation studies, which demonstrated a low number of significant correlations among the different mollusc RASTs (only 50%) as compared to the results among the crustacea and fish species. These results suggested that major allergen/s in the crustacean or fish had a high degree of cross-reactivity or concordance when compared with the members of the mollusc group. However, the presence of common allergens, particular between the mollusc and crustacean group, was suggested by the fact that concurrent allergy between this two groups often induced a very strong immune response. The persistence of elevated specific IgEs to seafoods, demonstrated on several subjects, could result from additional immune stimulation generated by cross-reacting allergens in other food or in inhalant substances.

To explore and investigate the possibility of cross-reactivity among the different seafood species and to characterise the offending allergens, I have performed RAST-inhibition studies and Western blot analysis on several species, the results of which are presented in chapter III. Furthermore, *in-vitro* tests with in-house prepared skin prick solutions determined the clinical immuno reactivity to local seafood species.

CHAPTER III

An Investigation of the Specific Immune Responses to Indigenous South African Mollusc, Crustacean and Fish species using RAST-Inhibition, Western Blotting and Skin Prick Tests



Flowdiagram of samp`le selection, diganosis and analysis of allergy to seafood by different *in-vitro* and *in-vivo* methods.

1. Introduction

In chapter II I have analysed the frequency of hypersensitivity to different species of ingested mollusc and other seafood species including the group of crustacea and fish in the Western Cape of South Africa. It is apparent from this study that food allergy to mollusc is very frequent in South African subjects with hypersensitivity to seafood.

The purpose of this study was to identify the antigenic and allergenic components in different mollusc species occurring at the South African shores and compare them with the allergens in other seafood species. Furthermore I have compared the specific immune response of allergic subjects to different molluscs and other seafood by RAST-inhibition studies to identify cross-reacting species. Western blot techniques were employed to identify the particular allergens. Furthermore skin prick tests (SPTs) with in-house produced seafood extracts should give additional information on the immune response of subjects with positive or negative RAST results.

2. Material and Methods

2.1 Study population

36 of 80 patients had specific IgE to seafood confirmed by CAP-RAST (see chapter II). RAST+ve subjects were studied further using skin prick tests, RAST-inhibition and Western blots studies. RAST-ve subjects were included using skin prick tests.

2.2 Preparation of extracts

For the *in vivo* and *in vitro* experiments protein extracts were prepared from various common seafood species caught on the South African Coast and fresh frozen prior the extraction (see Table 3.I). Some of the more common species were in addition cooked for 10 minutes and extracts generated with these tissues. These extracts are referred as raw (fresh) versus to cooked extracts. Most specimens were provided by the Department of Zoology (University Cape Town) and by the "Two Oceans Aquarium" situated at the Waterfront (Cape Town). In brief: edible portions were removed, cut into small pieces and the extracts prepared. The protein concentration was determined using a dye-binding method (BCA-protein assay, Pierce). A house dust mite (HDM) extract of *Dermatophagoides pteronyssinus* was provided by Dr B Nurse (Department of Immunology) who tested the potency of this extract in previous proliferation studies on HDM allergic patients.

To obtain solutions for skin prick tests (SPT), extracts were diluted in 50% glycerol (v/v), sterile filtered and stored at -80^0 C. The extracts were retested after 3 months on SDS-PAGE and demonstrated no degradation whatsoever (see 3.1). All the extracts for SPT were tested for pyrogens using the Chromogenic Limulus Amebocyte Lysate Test (LAL; Bio-Whittaker; Walkersville; USA).

2.3 Skin prick test (SPT)

To confirm allergy to seafood in the RAST negative subjects with a firm history of allergic reactions after consumption of seafood (44/80), *in vivo* tests such as Skin Prick Tests and Double Blind Placebo Controlled Food Challenge (DBPCFC) gave additional information about the immune response of this subjects.

DBPCFC on seven fish sensitive subjects (but RAST -ve) in our study cohort were performed in a separate study by Prof. Paul Potter assisted by C Zinn (Zinn, et al., 1997). One of the seven subjects with RAST- and SPT- negative results responded positive to the oral challenge. Other researchers in several other studies have performed food challenges with fish and their protocols are readily available. However, a DBPCFC with molluscs on sensitised subjects have not been reported in previous studies. I therefore declined this part of the study as the health risk to the individuals appeared to high and we did not have in patient facilities to observe delayed reactions. It may however be possible for the clinicians to study the mollusc sensitive cohort using DBPCFC in the future at our academic hospital.

Prof. Paul Potter (with informed consent) performed skin prick tests with extracts of raw seafood, on the forearm of 36 of the 80 subjects tested by RAST. These subjects were selected on the base of availability and consent as many individuals where not able to visit our clinic since they lived up the coast. This group included RAST-positive and -negative subjects in the different seafood groups. The panel of 19 different seafood species consisted of 12 in-house extracts (see 2.2 for production) to common South African seafood species such as: abalone, oyster, black mussel, white mussel, squid, rock lobster, prawn, hake, yellowtail, Cape salmon, snoek and kingklip. The seven commercial glycerinated extracts (Soluprick, ALK Laboratories, Horsholm, Denmark) consisted of blue mussel, crab, shrimp, cod, herring, plaice and mackerel. Histamine dihydrochloride 10 mg/ml was used as positive control and a diluent of glycerol/saline as negative control. Subjects were instructed not to take any antihistamines 48 hours prior to and on the day of the skin prick tests. The SPT were read after 15 minutes and were considered to be positive if the diameter of the wheal was at least 3 mm greater than the negative control. Blood

pressure, pulse and PEFR (peak expiratory flow rate) were monitored prior to and after the SPT. Skin tests to the panel of in-house allergens were also performed on 10 non-allergic individuals who served as negative controls.

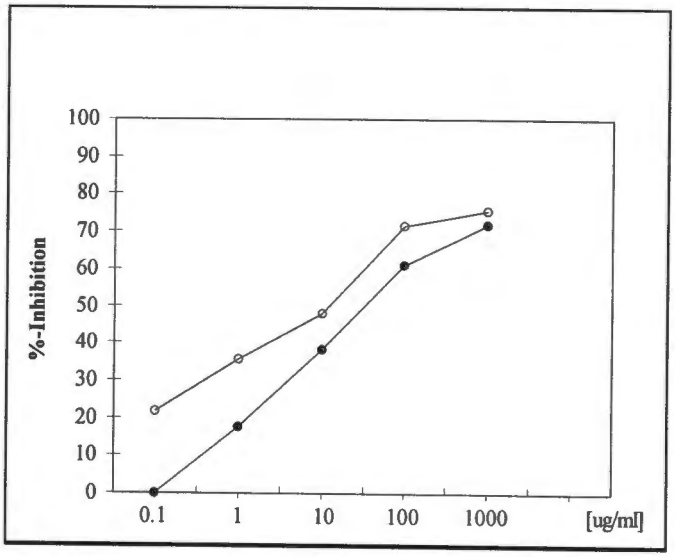
2.4 RAST and RAST-Inhibition Studies

Immunochemical analyses of cross-reacting allergens in various shellfish extracts were performed by RAST-inhibition assay using UniCAP-RASTs (Pharmacia, Uppsala, Sweden). Inhibition curves were performed with selected CAP-RASTs (Blue Mussel and Lobster) to identify a sufficient high antigen concentration to block all specific IgE antibodies. Pooled sera from 4 subjects was pre-incubated over night with tenfold serial dilutions of raw and cooked black mussel and rock lobster extracts (80 µl of serum + 20µl of inhibitor) to achieve a final concentration ranging from 0.1 µg/ml to 1000 µg/ml (Fig. 3.1). Inhibition was measured as percent binding as compared to the un-inhibited CAP-RAST. The value for 0%-inhibition was obtained by using PBS instead of inhibitor in the assay. From this pilot experiment I decided to use a final concentration of 500 µg/ml for all utilised seafood extracts for further inhibition studies (concentration range similar to other studies).

$$\%\text{-Inhibition} = 100 - \frac{\text{RAST value with inhibitor}}{\text{RAST value with PBS}} \times 100$$

The following CAP-RASTs were used for the assays with serum samples of 4-5 different sensitised subjects; Snail (Rf314), Squid (Rf258), Blue Mussel (f37), Oyster (Rf290) with extracts of raw indigenous abalone, snail, squid, black mussel, oyster and rock lobster. To determine the cross-reactivity between mollusc and crustacea I also inhibited some crustacean RASTs with seafood extracts as follows; Langoustine (Rf304), Lobster (f80) and Crayfish (Rf320) with extracts of two different indigenous rock lobster and one langoustine species as well as abalone, black mussel and hake. Since cross-reactivity of seafood with arthropod species has been observed in previous studies I also inhibited house dust mite (HDM) CAP-RAST with some seafood extracts and vice versa all the above mentioned seafood RASTs with extract of HDM.

A) Blue Mussel CAP-RAST



B) Lobster CAP-RAST

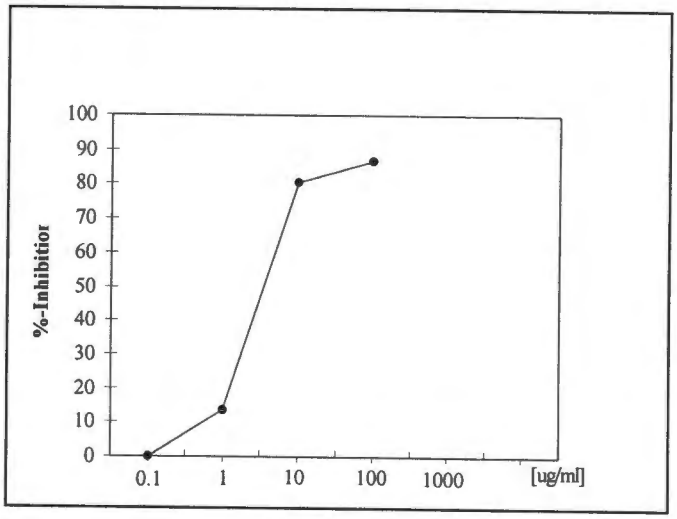


Figure 3.1: CAP-RAST-inhibition with increasing protein concentrations of different seafood extracts; **A)** Blue Mussel-RAST (*Mytilus edulis*) with extracts of raw (•) and cooked (0) South African black mussel (*Choromytilus meridionalis*), and **B)** Lobster-RAST (*Homarus gamarus*) with extracts of raw South African rock lobster (*Jasus lalandii*) (•). A serum pool of four seafood sensitive subjects was used in both experiments representing one set of data.

2.5 SDS-gel electrophoresis and Western blotting

Seafood extracts were separated on 11% SDS-PAGE (Laemmli, 1970) or gradient gels (4%-16%) under reducing or non-reducing conditions were referred respectively. Molecular weight markers (BDH, Electran low molecular weight marker; and at later stage of study, Amersham, Rainbow Marker) were included to estimate the molecular weights of the separated proteins. After heating the samples for 5 minutes in boiling water, 20 µl of sample (containing 20 µg of protein for staining and about 2 µg for Western blotting) were loaded into each pocket of the gel. Electrophoresis was carried out for about 3 hours at 20 mA, on a Vokan SAE (2761) power pack. After electrophoresis gels were stained with Coomassie Brilliant Blue (Sigma) to

display the protein banding patterns. For Western blotting proteins were electroblotted onto Polyvinyldifluor membrane (Hybond-PVDF, Amersham) employing the semidry method (Coligan *et al.*, 1994). Two graphite blocks were used with a sandwich of a shammy and filter paper on each side of the blocks pre-soaked in transfer buffer (Towin buffer). The allergen bound membranes were blocked for 1 hours in 1% blocking reagent (BM, Chemiluminescence assay, Boehringer Mannheim), washed twice in phosphate-buffered-saline containing Tween-20 (PBS-T) followed by incubation overnight with serum samples (1/30). The blots were washed again in PBS-T and incubated for 2 hours with a mouse anti-human IgE antibody (DAKO; 1/5,000), washed, incubated with biotinylated rabbit anti-mouse antibody (DAKO; 1/5,000) and finally incubated in streptavidin-peroxidase (1/30,000) for another 30 minutes. After an overnight wash, the blots were developed using the chemiluminescence detection system (Boehringer Mannheim) and exposed to Kodak x-ray film. After exposure, to visualise the protein bands, the membranes were stained with Coomassie Brilliant Blue for 10 minutes and rinsed in water.

3. Results

3.1 Seafood extracts and SDS-gel electrophoresis

The extracts of indigenous seafood species were prepared and the protein concentrations determined as displayed in Table 3.I. The extraction of cooked tissue of the different seafood species generated in general a lower protein concentration. For SDS-gel electrophoresis, the protein contents of 'raw' and 'cooked' extracts were adjusted to equal concentrations.

Table 3.I: Seafood species utilised for the production of extracts and protein concentration for the extracts of fresh (raw) and cooked tissues in mg/ml in the three seafood groups, **A)** mollusc, **B)** crustacea **C)** fish. Note: ND = not done.

A) MOLLUSC

No	Common name	Scientific name	Fresh protein concentration mg/ml	Cooked Protein concentration mg/ml
1	Abalone (Perlemoen)	*Haliotis midae*	8.0	2.0
2	Abalone	*Haliotis spadicea*	4.3	N.D.
3	Limpet	*Patella granularis*	4.0	N.D.
4	Periwinkle	*Oxystele spp.*	2.5	1.3
5	Snail	*Helix aspersa*	6.3	N.D.
6	Black Mussel	*Choromytilus meridionalis*	5.0	0.7
7	Mediteranian Mussel	*Mytilus galloprovincialis*	6.5	1.0
8	Ribbed Mussel	*Aulacomya ater*	4.0	1.1
9	White Mussel	*Donax serra*	11.0	2.1
10	Oyster	*Striostrea margaritacea*	4.5	N.D.
11	Squid	*Loligo vulgaris reynaudii*	2.0	0.5
12	Octopus	*Octopus vulgaris*	8.0	1.0

B) CRUSTACEA

No	Common name	Scientific name	Fresh protein concentration mg/ml	Cooked protein concentration mg/ml
1	Shrimp	*Metapenaeus monoceros*	3.5	0.8
2	Tiger Prawn	*Panaeus monodon*	5.0	2.0
3	Zebra Prawn	*Panaeus semisulcatus*	16.0	N.D.
4	Crab	*Cancer spp*	4.5	1.3
5	Rock Lobster, East coast	*Panulirus homarus*	17.0	1.2
6	Rock Lobster, South coast	*Palinurus gilchristii*	16.0	1.3
7	Rock Lobster West coast	*Jasus lalandii*	6.0	1.5
8	Langoustine	*Palinurus spp*	16.0	3.1

C) FISH

No.	Common name	Scientific name	Fresh protein concentration mg/ml	Cooked protein concentration mg/ml
1	Pilchard/Sardine	*Sardinops sagax*	15.5	N.D.
2	Hake	*Merluccius paradoxus*	9.0	4.0
3	Cape Salmon	*Atractoscion aequidens*	14.0	0.9
4	Cob/Kabeljou	*Argyrosomus hololepidotus*	11.0	N.D.
5	Kingklip	*Genypterus capensis*	4.5	2.5
6	Monk fish	*Lophius vomerinus*	5.0	N.D.
7	Angelfish (Pomfret)	*Brama brama*	7.0	N.D.
8	Yellowtail	*Seriola lalandi*	11.0	3.5
9	Marsbanker (Cape Horse Mackerel)	*Trachurus trachurus capensis*	6.0	N.D.
10	Chub Mackerel	*Scomber japonicus*	5.0	3.4
11	Tuna	*Thunnus albacares*	16.0	0.5
12	Snoek	*Thyrsites atun*	10.0	4.0
13	Sole	*Austroglossus pectoralis*	5.5	3.8
14	Rainbow Trout	*Oncorhynchus mykiss*	5.6	N.D.
15	Canadian Salmon	*Salmo salar*	15.0	N.D.
16	Jacopever	*Helicolenus dactylopterus*	8.0	N.D.
17	John Dore	*Zeus faber*	16.0	N.D.

All the prepared extracts were separated by reducing SDS-gel electrophoresis (Laemmli, 1970) to verify the extraction of soluble proteins from the different seafood tissues and to identify species-specific protein patterns. The electrophoretic separation of the mollusc extracts demonstrated that most of the proteins were in the molecular range of 25-50 kDa (Fig. 3.2 A). However, very closely related mollusc species such as the two different abalone species as well as the four different mussels and the two different cephalopod species demonstrated distinct banding pattern. This banding pattern allowed the differentiation of some of the species by SDS-gel electrophoresis only. Cooking of the mollusc species prior the extraction showed that some species (except for abalone and three mussels) exhibited strong bands in the 15 kDa range which were not as dominant in the raw extracts (Fig. 3.2 B). In general, the banding patterns were similar to the raw extracts but did demonstrate the loss of some bands and the expression of new ones for some of the species.

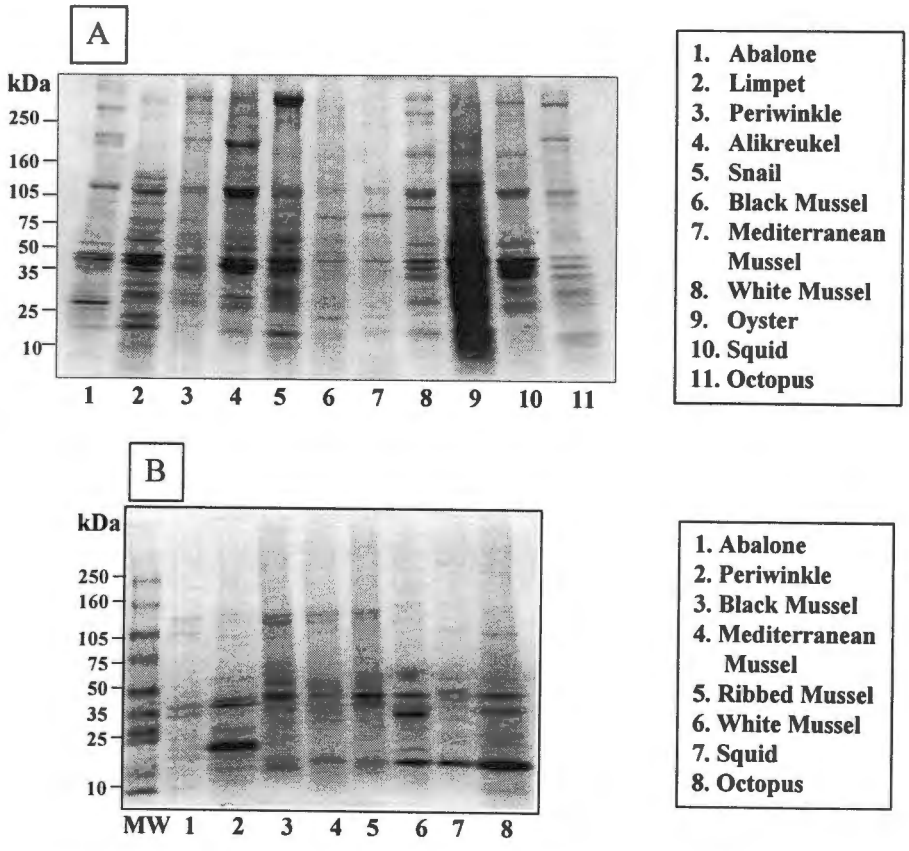
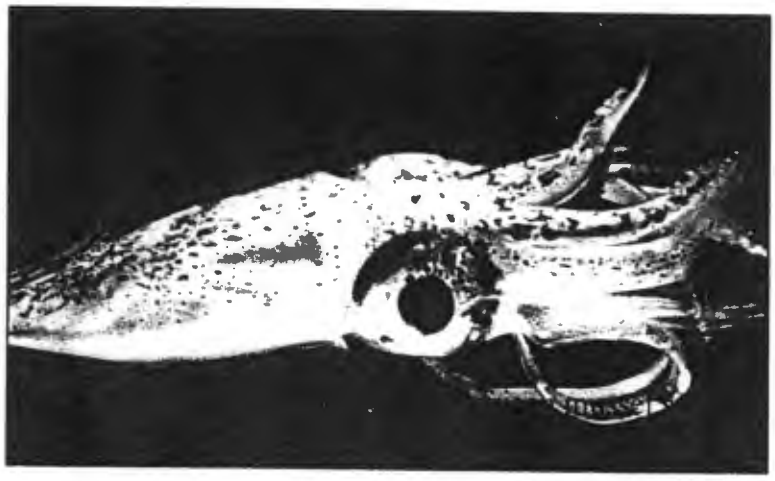


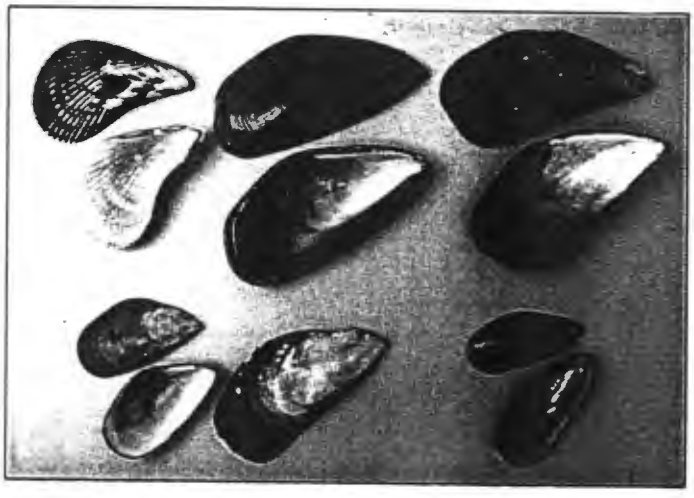
Figure 3.2: SDS-gel electrophoresis of extracts of raw (**A**) and cooked (**B**) local mollusc species. The species names are labelled from 1 to 8. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

For the crustacean extracts the separated proteins had higher molecular weights of up to 80 kDa but with a common strong banding pattern in the 35-40 kDa range (Fig. 3.3). Unlike the mollusc extracts, the crustacean extracts of cooked species showed a striking loss of protein bands in the molecular range of 50-100 kDa but new band appeared for most species at about 110 kDa. However, the proteins between 20-35 kDa seemed to be very heat stable. All six cooked crustacean species had two major proteins with almost identical molecular weights of about 20 kDa and 35 kDa respectively which were more dominant in the cooked extracts.

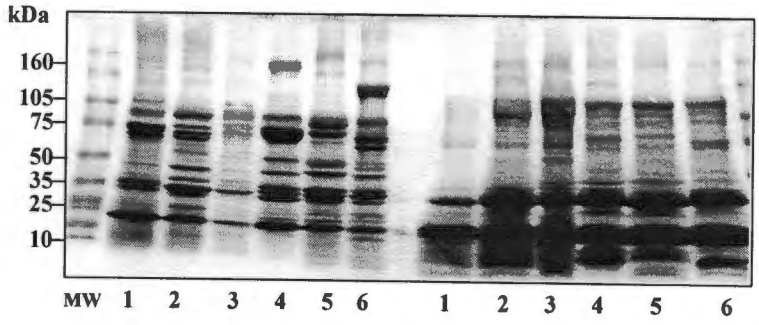
Picture of South African octopus (*Octopus vulgaris*)



Picture of South African Squid (*Loligo vulgaris reynaudii*)



Picture of five different South African mussel species, with the Black Mussel in the middle of the top row.



1. Shrimp
2. Prawn
3. Langoustine
4. Rock Lobster West Coast
5. Rock Lobster South Coast
6. Rock Lobster East Coast

Figure 3.3: SDS-gel electrophoresis of extracts of South African crustacean species. The species names are labelled from 1 to 6, the left side is the raw extracts and on the right side the cooked extracts. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

Also the fish extracts illustrated a major protein range from about 30-80 kDa (Fig. 3.4 A). In addition there were strong bands in the molecular range of 10-14 kDa for some of the species observed (e.g. cape salmon, yellowtail, hake) which were in comparison not present in extracts of different crustaceans (see Fig. 3.4 A). Cooking had also a weakening effect on the protein bands in the upper molecular weight range (Fig. 3.4 B). Two dominant protein bands remained in all seven fish species tested; with about 10-15 kDa and 35 kDa. However, two species (mackerel and hake) showed much weaker bands than the other five species.

The skin prick test solutions with glycerol were stored at -80^{0}C until further use. Testing for pyrogens established that the produced extracts were free of endotoxins (results not shown). Only the Cape salmon extract demonstrated a raised endotoxin concentration, indicating bacterial contamination. A second extract produced from a fresh fish proved to be pyrogen free. The SPT solutions of all three seafood groups separated by SDS-gel electrophoresis after 3 month storage at -80^{0}C demonstrated no degradation products whatsoever (Fig. 3.5, for mollusc and crustacean species; Fig. 3.6, for fish and crustacean species).

Picture of West Coast Rock Lobster (*Jasus lalandii*)

Picture of South Coast Rock Lobster (*Palinurus gilchristi*)

Picture of East Coast Rock Lobster (*Panulirs homarus*)

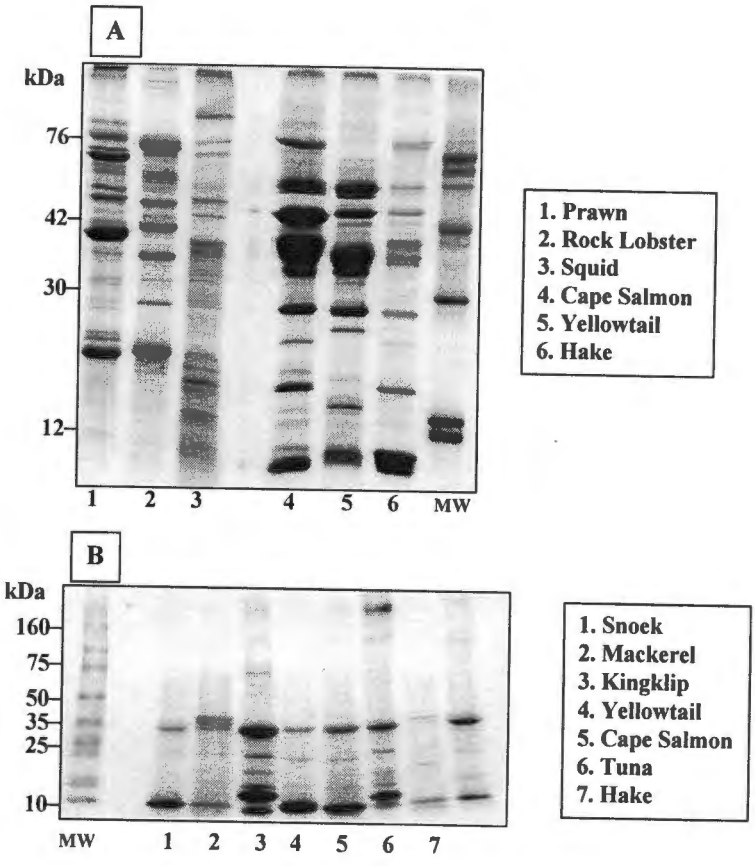


Figure 3.4: SDS-gel electrophoresis of extracts of South African fish species and crustacean species (A; lanes 1-3). The species names are labelled from 1 to 7; **A)** raw extracts and **B)** cooked extracts. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

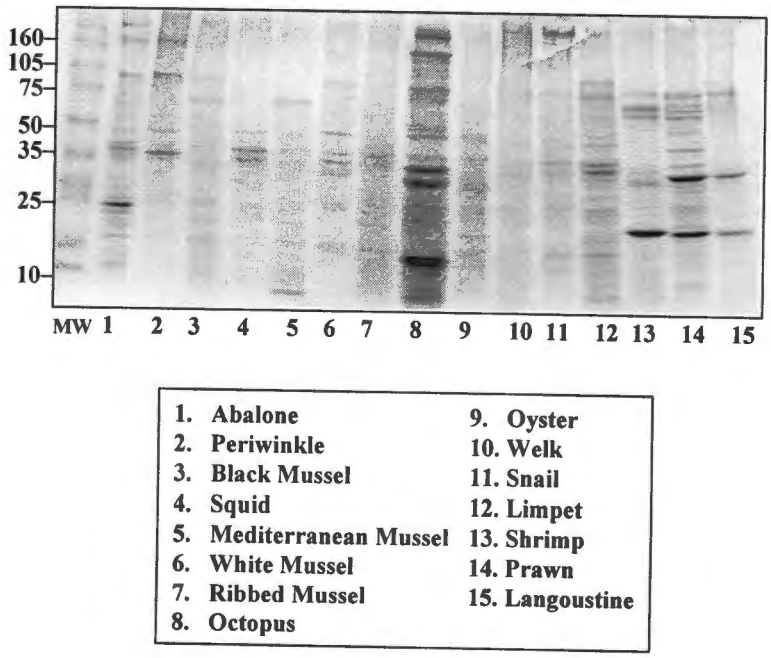


Figure 3.5: SDS-gel electrophoresis of glycerinated solutions used for skin prick tests (SPT) after five month of storage at -80^{0}C. The species names of different South African mollusc and crustacea extracts are labelled from 1 to 15. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

3.2 Skin prick test

When tested for immuno reactivity by skin prick tests, all examined mollusc RAST +ve subjects reacted positively (6/6) to five different in-house prepared mollusc extracts (Table 3.II A). In addition, SPTs on RAST-ve subjects revealed fifty positive results out of 138 performed tests (50/138 = 36%) using in-house extracts of local species of abalone, oyster, squid, black- and white mussel. The commercial extract of blue mussel was also positive in 9/33 (27%) of RAST-ve subjects. In the comparative group, 1 of 10 subjects had a positive response each to oyster, squid, black mussel, blue- and white mussel. However, no positive reaction was obtained with the abalone extract in this control group.

3.3 RAST-Inhibition

To asses the specificity of IgE binding and possible cross-reactions and determine species specific immune responses, RAST-inhibition studies were performed on five subjects by using different seafood extracts (see section 2.4). In addition, I included house dust mite (HDM) extract for these inhibition studies to analyse possible cross-reactions with mollusc allergens as has been observed by other research groups.

Both inhibition curves for Blue Mussel-RAST (f37) and Lobster (f80) verified that a concentration of 500 µg protein per ml of seafood extract were sufficient to obtain a maximal inhibition of specific serum IgE after preincubation (Fig. 3.1). The amount of residual IgE binding in the serum gave an indication about the specificity of the antibodies developed against a specific seafood allergen or allergens. This indicated that a strong inhibition of binding (maximum is 100%) reflected a high degree of cross-reactivity. Similar or even identical IgE binding epitopes must exist on the allergens in the observed (preincubated) seafood extract and the analysed seafood species utilised in the commercial RAST. Consequently a weak inhibition characterised a low degree of cross-reacting allergens.

RAST-inhibition studies were conducted on four mollusc sensitive subjects (Car, Fre, Ver, Koc) with different seafood extracts. These four subjects had concurrent specific IgE to all four mollusc RASTs. For the inhibition experiment of the Snail-RAST and HDM-RAST I included a fifth sensitised subject (Ket). The RAST results of these five subjects to the different seafood and HDM are presented in Table 3.III.

Table 3.II: Specific IgE and Skin Prick reactivity (SPT) in seafood sensitive subjects with in-house prepared extracts of local species and commercial extracts for **A)** mollusc, **B)** crustacea and **C)** fish. The relevant seafood RASTs are in bold. N.A. = Not available, N.D. = Not done.

A) Mollusc

Species	RAST +ve subjects	In-house SPT +ve and RAST +ve	In-house SPT +ve and RAST -ve	Commercial SPT +ve and RAST –ve
Snail	13	N.D.	N.D.	N.D.
Squid	10	3/3	8/28	N.D.
Blue Mussel	7	1/1	N.D.	9/33
Oyster	9	2/2	11/22	N.D.
Abalone	17	8/8	6/20	N.A.
Black Mussel	N.A.	N.D.	10/33	N.A.
White Mussel	N.A.	1/1	13/33	N.A.

B) Crustacean

Species	RAST +ve subjects	In-house SPT +ve and RAST +ve	In-house SPT +ve and RAST –ve	Commercial SPT +ve and RAST -ve
Crayfish	19	N.D.	N.D.	N.D.
Langoustine	8	N.D.	N.D.	N.D.
Lobster	15	N.D.	N.D.	N.D.
Shrimp	18	5/5	N.D.	6/20
Crab	13	3/3	N.D.	5/16
Cape Rock Lobster	N.A.	3/3	8/30	N.A.
Prawn	N.A.	4/4	11/38	N.A..

C) Fish

Species	RAST +ve subjects	In-house SPT +ve and RAST +ve	In-house SPT +ve and RAST -ve	Commercial SPT +ve and RAST –ve
Hake	5	4/4	3/15	N.D.
Mackerel	5	2/2	N.D.	4/18
Tuna	5	N.D.	N.D.	N.D.
Salmon	6	N.D.	N.D.	N.D.
Yellowtail	7	6/7	0/10	N.A.

The tested in-house extracts of local crustacean and fish species were also positive on all RAST +ve subjects (15/15 and 6/6 respectively; Table 3.II B and 3.II C). In addition, with these extracts a positive reaction was observed in 28% and 20% respectively of RAST -ve subjects (19/68 and 3/15 respectively). The commercial extracts for crustacea and fish produced also positive results in 31% and 22% respectively (11/36 and 4/18 respectively).

The positive SPT-results indicated that a negative RAST response does not rule out an immunologic sensitivity to abalone or other seafood species.

Table 3.III: RAST reactivity of five subjects to different seafood species in ku/l or percent (%) for abalone. Note: '--' indicates negative RAST results.

	SUBJECTS				
CAP-RASTs (ku/l)	**Car**	**Fre**	**Koc**	**Ver**	**Ket**
OYSTER	4.1	2.9	5.9	8.8	3.5
BLUE MUSSEL	3.1	1.0	1.5	2.7	2.6
SNAIL	3.9	2.7	2.1	5.9	5.5
SQUID	0.7	0.4	1.1	4.1	--
ABALONE (%-binding)	40.6	13.1	22.5	15.8	19.8
CRAYFISH	28.0	16.6	18.1	2.8	14.1
LANGOUSTINE	27.5	15.9	13.8	3.8	10.4
SHRIMP	20.0	11.7	8.0	0.9	11.9
CRAB	19.4	11.0	6.8	2.0	12.4
LOBSTER	26.7	16.4	8.9	0.5	12.9
HAKE	--	--	--	--	--
TUNA	--	--	--	--	--
SALMON	0.6	--	--	--	--
MACKEREL	--	--	0.5	--	--
HDM	20.4	4.4	15.2	12.1	46.5

To achieve maximum inhibition I used seafood extracts of indigenous species with the closest relationship in the different seafood group (see Table 1.I in chapter I) to the species utilised in the commercial CAP-RASTs. Table 3.IV displayed the scientific and common names of the seafood species used for the commercial CAP-RASTs and the inhibiting indigenous species extract with the closest association. For example in the case of the Snail-RAST I used the common garden snail (*Helix aspersa*), which is identical to the species used for the commercial RAST.

Table 3.IV: Species- and common names of utilised CAP-RASTs for inhibition assays and the indigenous species with closest homology used for maximum inhibition.

CAP-RASTs	**Scientific Names of commercial species**	**indigenous species**	**Common Names**
SNAIL	Helix aspersa	Helix aspersa	Garden Snail
SQUID	*Loligo spp.*	*Loligo vulgaris reynaudii*	White Squid
BLUE MUSSEL	*Mytilus edulis*	*Choromytilus meridionalis*	Black Mussel
OYSTER	*Ostrea edulis*	*Crassostrea margaritacea*	Cape Rock Oyster
LOBSTER	*Homarus gammarus*	*Jasus lalandii*	Rock Lobster West Coast
LANGOUS-TINE	*Palinurus spp.*	*Panulirus homarus*	Rock Lobster East Coast
CRAYFISH	*Astacus astacus*	No species available; freshwater!	
HOUSE DUST MITE	*Dermatophagoides pteronyssinus*	*Dermatophagoides pteronyssinus*	

Comparison of the mean inhibition values of the different extracts demonstrated which seafood possesses the strongest allergen homology to the utilised CAP-RASTs. For the Snail- and Oyster-RAST the indigenous species extracts caused the strongest inhibition compared to the other extracts with 83% and 89% respectively (Table 3.V). Compared to these results the Blue Mussel- and Squid-RAST were only inhibited by 70% and 53% respectively by their indigenous species. These low inhibition values could reflect the distant species used for the inhibition experiment which are locally available. In contrast to the mollusc RASTs, the three crustacean RASTs were all inhibited by three related local available crustacean species between 72% and 85% (Table 3.VI). This indicated that the crustacean species do have very closely related allergens causing an strong inhibition in all tested extracts.

Table 3.V: Inhibition of **Mollusc** and House Dust Mite (HDM) CAP-RASTs with different indigenous seafood extracts. Inhibition is displayed in %-inhibition for each individual patient and each seafood extract. Inhibitions with "homologues" in-house extracts are boxed in grey. The mean values and standard deviation (S.D.) are given for each inhibition experiment, not including the negative reactions. Note: N.D. = not done.

CAP-RASTs	Patients	Snail	Squid	Black Mussel	Oyster	Rock Lobster East Coast	HDM
SNAIL	Car	59.7	79.3	41.5	91.4	92.7	0.0
	Fre	95.4	90.9	57.0	93.3	95.4	0.0
	Koc	93.4	29.0	65.6	86.7	65.6	41.8
	Ver	82.5	56.1	61.0	80.6	64.7	56.9
	Ket	84.1	82.5	N.D.	N.D.	82.3	0.0
	Mean ± S.D.	**83.0 ± 14.2**	**67.5 ± 25.1**	**56.2 ± 10.4**	**88.0 ± 5.6**	**80.2 ± 14.5**	**49.3**
SQUID	Car	82.4	84.9	75.0	83.5	56.3	0.0
	Fre	78.7	50.0	46.6	74.7	50.6	0.0
	Koc	96.5	55.7	84.6	92.2	62.2	71.6
	Ver	78.3	23.0	38.8	71.3	28.3	22.7
	Mean ± S.D.	**83.0 ± 8.5**	**53.4 ± 25.6**	**61.2 ± 22.0**	**80.4 ± 9.4**	**49.3 ± 14.8**	**47.1**
BLUE MUSSEL	Car	81.4	92.7	79.0	90.2	76.6	0.0
	Fre	78.2	62.9	83.2	80.6	91.4	4.2
	Koc	83.9	49.8	84.0	87.3	72.2	50.3
	Ver	56.4	0.0	34.9	67.9	8.1	0.0
	Mean ± S.D.	**74.9 ± 12.6**	**68.4 ±26.9**	**70.2 ±23.7**	**81.5 ±9.9**	**80.0 ± 10.0**	**27.2**
OYSTER	Car	92.5	96.0	74.2	95.5	88.9	0.0
	Fre	89.1	88.4	52.6	93.7	93.4	0.0
	Koc	94.5	27.0	58.3	87.3	59.2	25.9
	Ver	85.5	20.7	44.8	79.0	35.9	27.7
	Mean ± S.D.	**90.4 ± 3.9**	**58.0 ± 39.6**	**57.4 ± 12.4**	**88.8 ± 7.5**	**69.3 ± 26.9**	**26.8**
HOUSE DUST MITE	Car	22.1	13.8	N.D.	N.D.	47.0	22.1
	Fre	68.2	70.6	N.D.	N.D.	80.8	9.8
	Koc	0.0	0.0	N.D.	N.D.	4.5	91.2
	Ver	1.8	10.8	N.D.	N.D.	26.9	73.9
	Ket	0.0	10.0	N.D.	N.D.	0.0	58.7
	Mean ± S.D.	**30.7 ± 34.0**	**26.3 ± 29.5**			**39.8 ± 32.4**	**51.234.4**

Table 3.VI: Inhibition of **Crustacean** CAP-RASTs with different in-house seafood extracts and extract of House Dust Mite (HDM). Inhibition is displayed in %-inhibition for each individual patient and each seafood extract. The mean values and standard deviation (S.D.) are given for each inhibition experiment, not including the negative reactions. Note: N.D. = not done

CAP-RASTs	Patients	Rock Lobster East Coast	Rock Lobster South Coast	Langoustine	Black Mussel	Hake	HDM
LOBSTER	Car	86.7	78.7	79.9	9.4	0.0	0.0
	Fre	88.5	84.1	84.0	12.5	3.5	0.0
	Ver	83.5	81.7	0.0	0.0	0.0	0.0
	Ket	81.8	78.5	80.8	16.5	12.6	0.0
	Mean ± S.D.	**85.1 ± 3.0**	**80.7 ± 2.6**	**81.5 ± 2.1**	**12.8 ± 3.5**	**8.0**	**0.0**
LANGOUS-TINE	Car	85.8	79.8	80.1	39.6	12.6	0.0
	Fre	84.9	80.4	78.1	15.2	9.2	N.D.
	Ket	78.6	74.6	77.4	18.9	0.0	N.D.
	Mean ± S.D.	**83.1 ± 3.9**	**78.2 □ 3.1**	**78.5 ± 1.4**	**24.5 ± 13.1**	**10.9**	**0.0**
CRAYFISH	Car	88.2	N.D.	76.4	12.6	N.D.	0.0
	Fre	73.5	N.D.	82.3	11.6	N.D.	26.9
	Koc	74.7	N.D.	59.0	60.5	N.D.	57.6
	Mean ± S.D.	**78.8 ± 8.1**		**72.5 ± 12.1**	**28.2 ± 27.9**		**42.2**

Comparing overall the potency of the different extracts one notes that the snail- and oyster extracts achieve very strong inhibitions on all four seafood RASTs with values ranging from 75%-90% demonstrating common allergens or IgE binding epitopes in these extracts. On the other hand rock lobster extract inhibits the Snail-and Blue Mussel-RASTs very strongly (80%), but less effective than the Oyster- and Squid-RAST (69% and 49% respectively). The squid and black mussel extracts also demonstrated some degree of inhibition for some of the RASTs, however the standard deviations (S.D.) were very high (e.g. Blue Mussel-RAST with squid extract; 68.4% ± 26.9). The inhibition by abalone extract will be analysed in chapter IV (3.3).

The HDM extract had also a medium to strong inhibition effect on most of the analysed seafood RASTs. However, some of the subjects demonstrated no inhibition with the HDM extract but showed on the contrary a strong inhibition of HDM-RAST with seafood extracts. It was interesting to note that all five subjects with sensitivity to mollusc had also specific IgE to HDM (4-46 ku/l; Table 3.III). The antigenic relationship between this arthropod and snail (a gastropod) has been demonstrated previously by several research groups and will be investigated in more detail in Chapter IV.

3.4 Immunoblots of patients to molluscs, crustacea and fish

Protein extracts of abalone and other seafood extracts were separated by SDS-PAGE and the IgE-binding components analysed by immuno printing with sera of sensitised subjects. The two Western blots in Fig. 3.6 demonstrated the IgE binding pattern of two subjects to six different mollusc species. The immune response of both subjects was very heterogeneous with between 1 to 3 apparent major allergens for each species. The allergens of abalone, snail, limpet and squid seemed to be similar with some additional allergens.

However, the IgE binding pattern demonstrated the existence of distinct different allergens among most species. The major allergens for abalone extracts have a molecular weight (MW) of about 38-45 kDa, which seemed also to be present in snail and limpet (both gastropoda) and in both mussel species (except for subject B). Additional allergens were present with higher MWs of about 70-80 kDa for the mussel species and limpet. Squid demonstrated only one allergen for both subjects with a molecular weight of about 35 kDa.

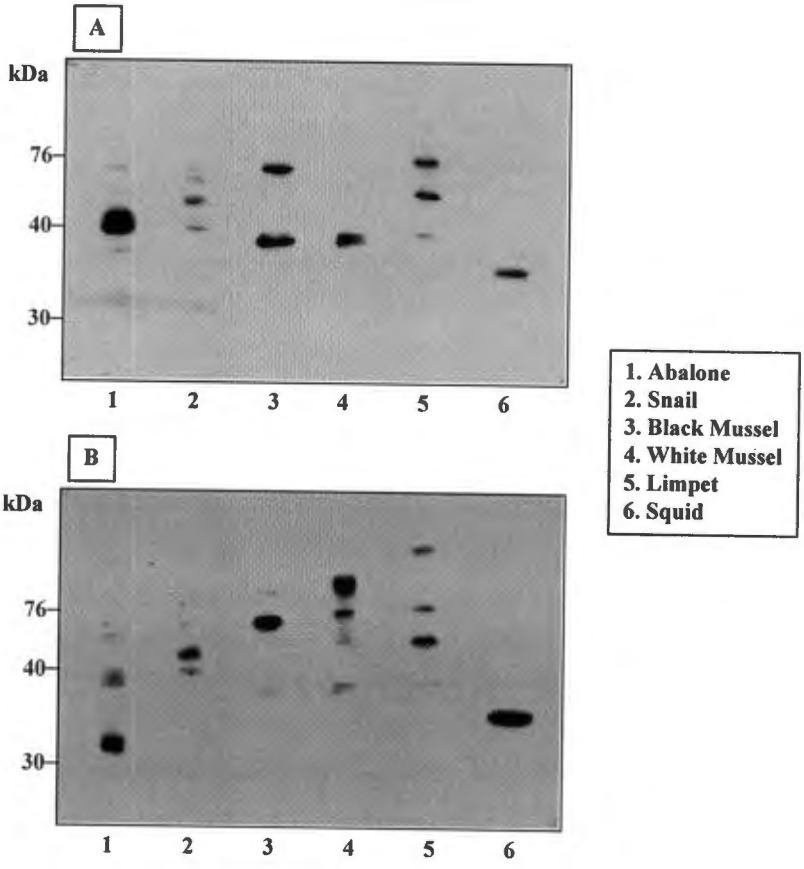


Figure 3.6: Western blot of IgE antibody reactivity of two sensitised subjects (**A** and **B**) to South African mollusc species. The species names are labelled from 1 to 6 and the molecular weights (MW) are indicated on the left side in kilodalton (kDa).

To demonstrate the resistance of the allergens to heat and therefore denaturation of proteins the extracts of cooked mollusc species were immuno blotted. The immunoblots of some representative subjects illustrated that some of the allergens were heat stable (Fig. 3.7 and 3.8) whereas other allergens seem to be sensitive to heat. Subject Car and Fre demonstrated clearly the increase of IgE binding activity of several proteins after cooking of abalone and periwinkle. In fact, subject Fre showed no IgE binding on any raw mollusc extract but to four of the seven cooked extracts.

Subject Ver demonstrated a mixed immune response to three raw species, which were lost after cooking. Vice versa, three species gained IgE reactivity only after cooking. However, for both gastropods, the abalone and periwinkle, the immune response was similar for the raw and cooked extract. No binding whatsoever was observed for squid.

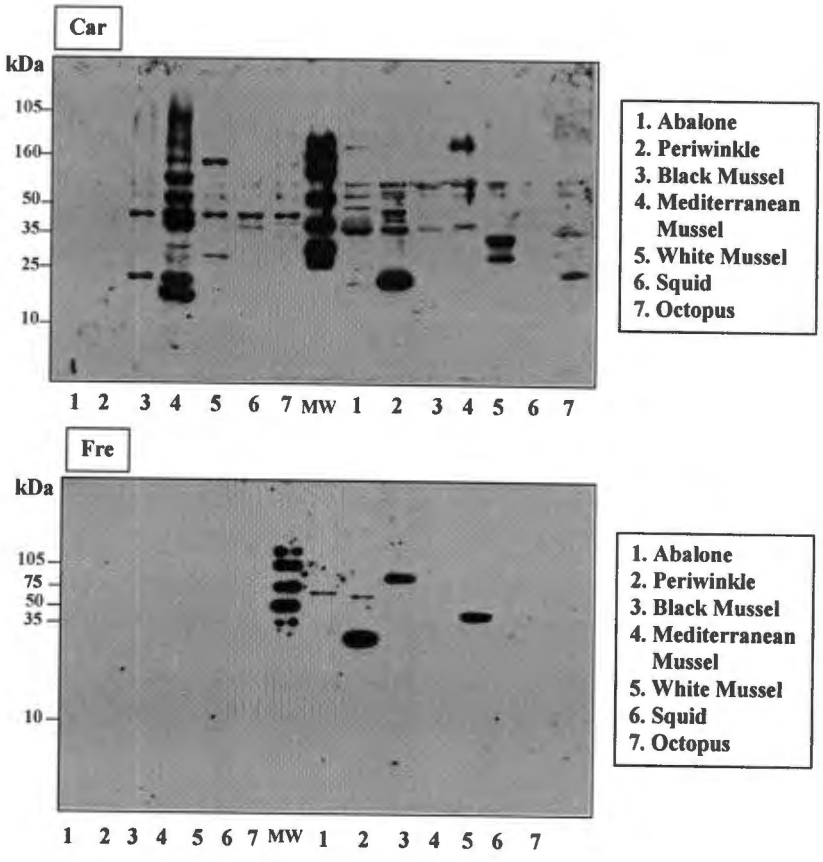


Figure 3.7: Western blots of subjects Car and Fre to raw and cooked mollusc species. The species names are labelled from 1 to 7, the left side is the raw extracts and on the right side the cooked extracts. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

Immunoblots of several crustacean extracts with sera of two sensitised subjects (Fig. 3.9) demonstrated for A) allergens in the molecular range of about 22 kDa for three of the six species investigated. In addition, all six crustacean species showed binding at about 70 kDa for subject A and B. Both subjects also demonstrated binding to allergens between 36 to 40 kDa in all analysed species. However, the immune response of subject A showed distinct different allergens in rock lobster from the East Coast compared to the other very close related rock lobsters from the South-and West Coast.

For one additional subject the IgE reactivity to only rock lobster (West Coast) but not to two other indigenous crustacean species could be demonstrated by Western blotting (Fig. 3.10).

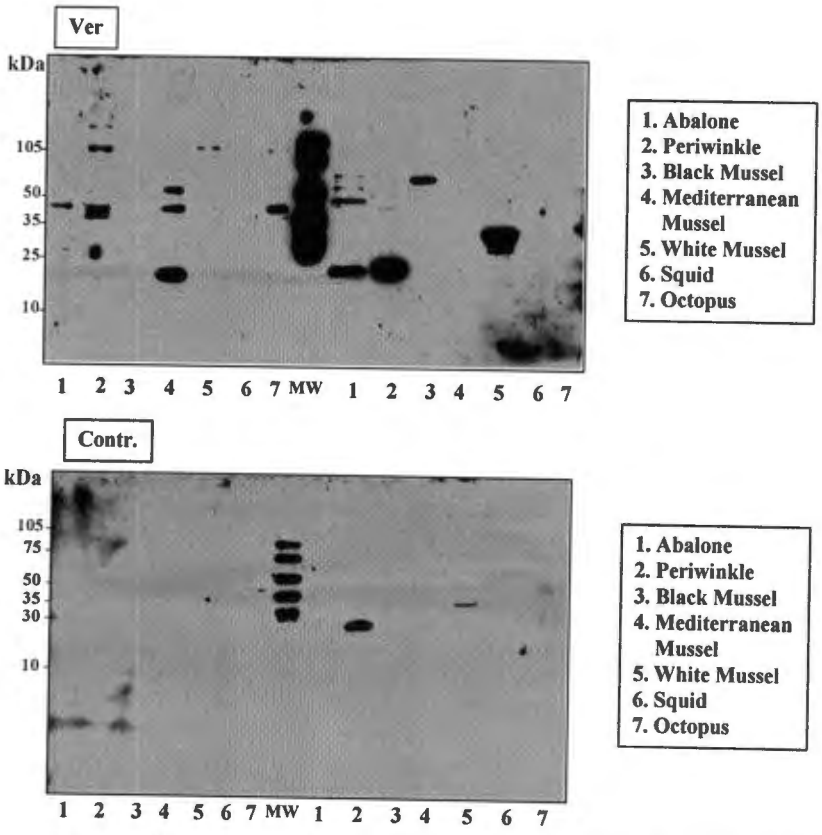


Figure 3.8: Western blots of subject Ver and a control subject to raw and cooked mollusc species. The species names are labelled from 1 to 7, the left side is the raw extracts and on the right side the cooked extracts. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

Cooking of crustacean species was generally indicated by a decrease of allergenicity (Fig. 3.11 and 3.12). However, some of the allergens were remarkably resistance to heat treatment. The different crustacean species analysed lost all (except one species) of their higher MW allergens but the 20 kDa proteins seemed to be stable for most of the six species tested for. Also most of the allergens in the 35-50 kDa range lost their activity. One subject (Ver) demonstrated after cooking an increase in IgE reactivity for an 35 kDa allergen and another subject (Fre) demonstrated activity to almost all 20 kDa allergens after cooking but not to the raw extracts.

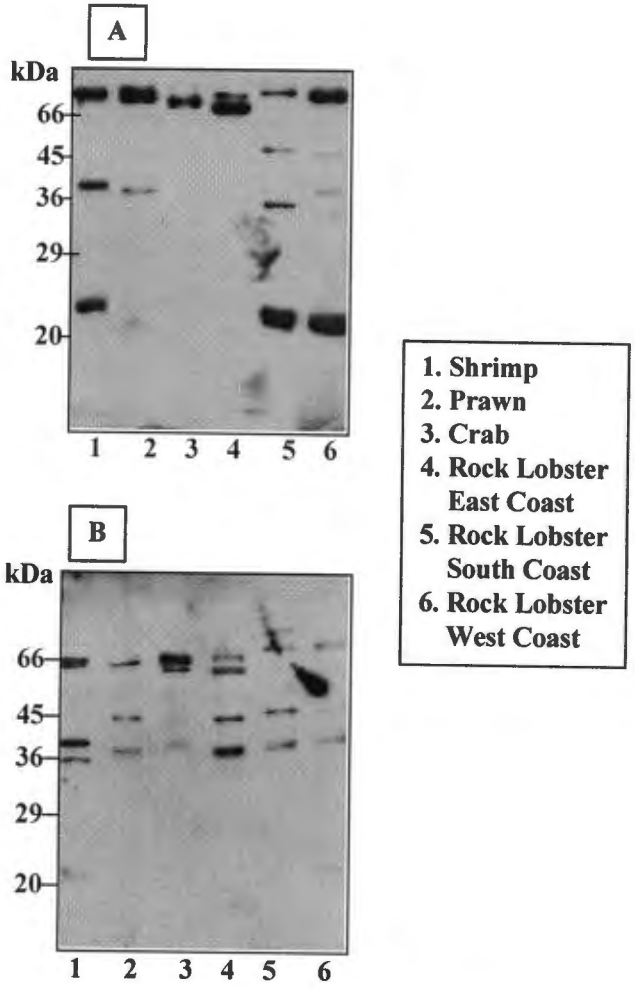


Figure 3.9: Western blot of IgE reactivity of two sensitised subjects (**A** and **B**) to South African crustacean species. The species names are labelled from 1 to 6 and the molecular weights are indicated on the left side in kilodalton (kDa).

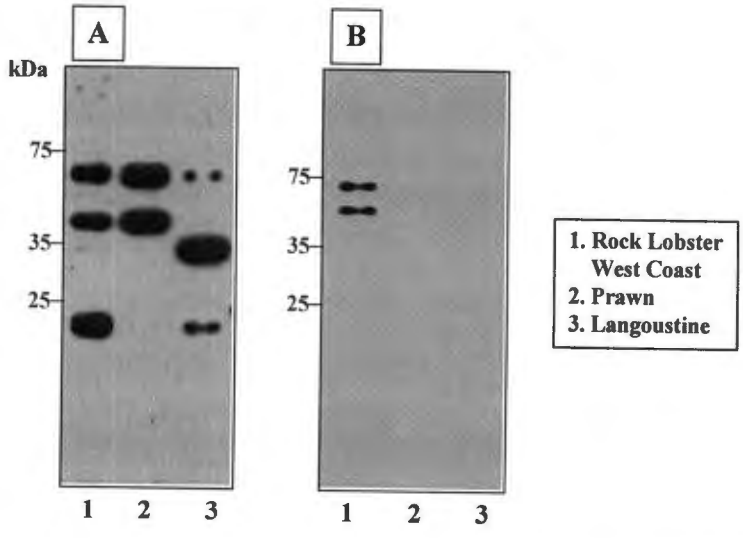


Figure 3.10: Western blot of IgE reactivity of two hypersensitive subjects with multiple **A)** and monosensitivity **B)** to different local crustacean species. The molecular weights are indicated on the left side in kilodalton (kDa).

Western blots of two fish sensitive subjects to seven different raw and cooked indigenous fish species demonstrated a clear loss of reactive bands after cooking (Fig. 3.13). Most of the 75 and 35-50 kDa bands disappeared. However, for both subjects it appeared that the activity to bands between 10-20 kDa increased after cooking for snoek, kingklip, yellowtail and Cape salmon.

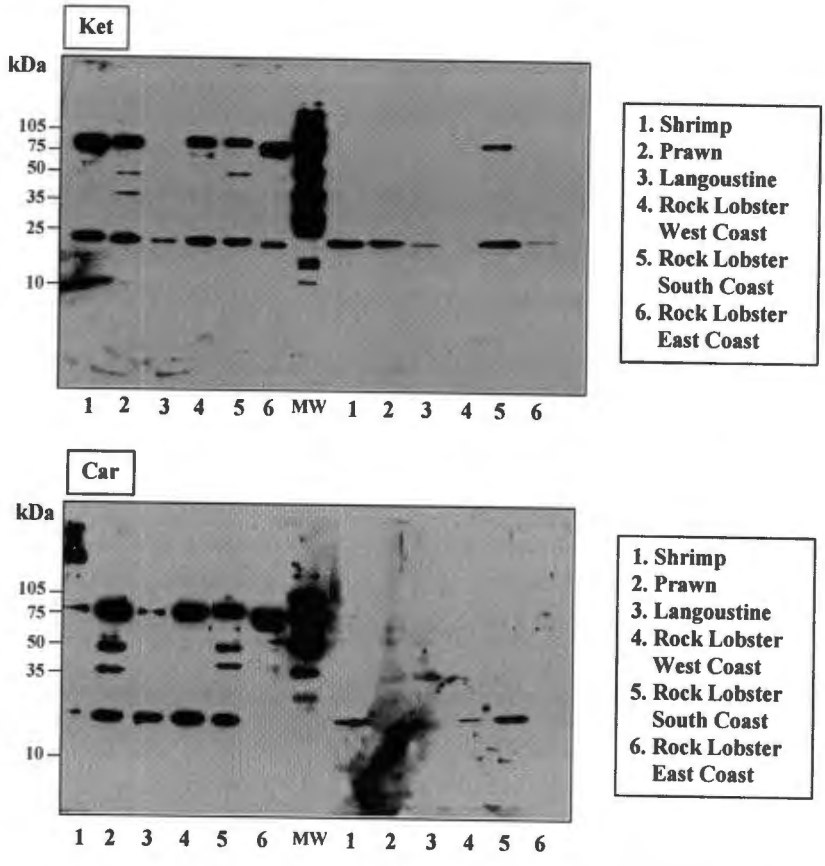


Figure 3.11: Western blots of subjects Ket and Car to raw and cooked crustacean species. The species names are labelled from 1 to 6, the left side is the raw extracts and on the right side the cooked extracts. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

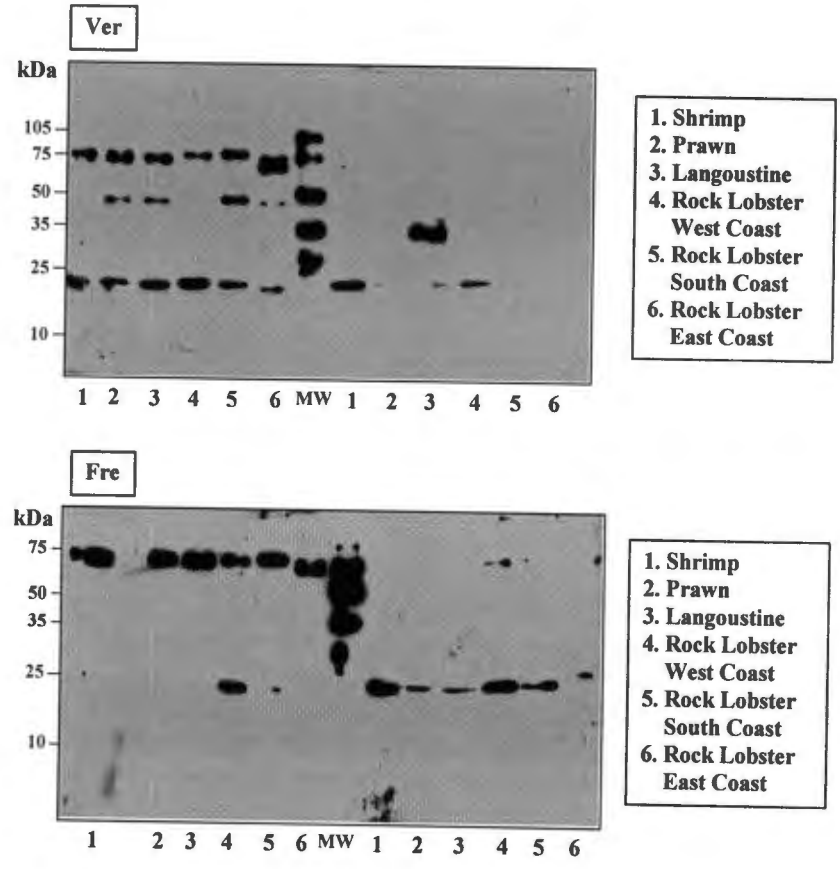


Figure 3.12: Western blots of subjects Ver and Fre to raw and cooked crustacean species. The species names are labelled from 1 to 6, the left side is the raw extracts and on the right side the cooked extracts. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

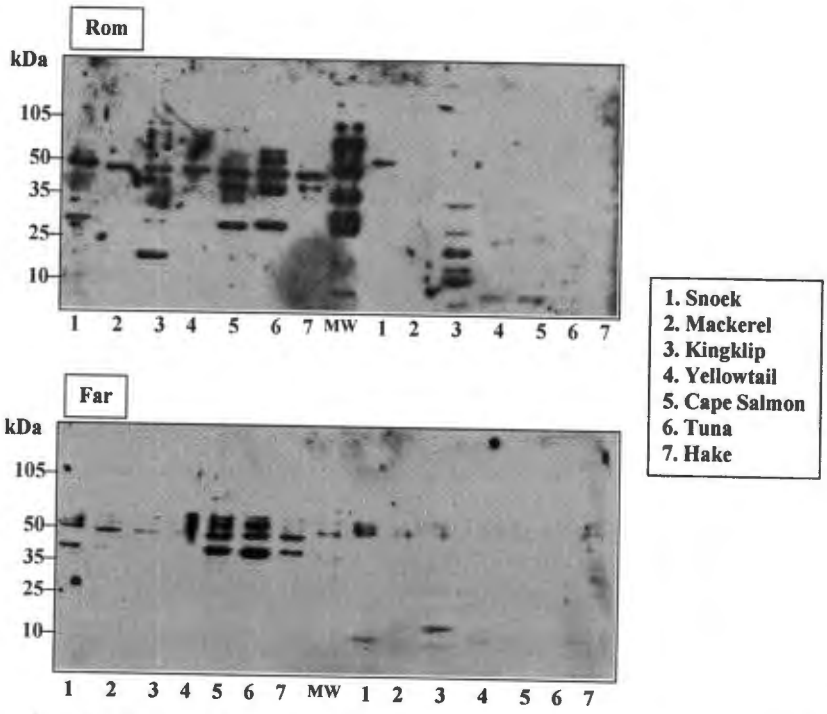


Figure 3.13: Western blot of IgE antibody reactivity of two subjects to raw and cooked fish species respectively. The species names are labelled from 1 to 7 and the molecular weights (MW) are indicated on the left side in kilodalton (kDa).

4. DISCUSSION

4.1 Skin Prick reactivity (SPT)

A distinction between food allergy and food intolerance (both present with very similar symptoms) depends on whether the involvement of the immune system can be verified. In addition, subjects with a convincing history of hypersensitive reaction to ingested seafood were frequently tested RAST negative. We also tested subjects with RAST +ve and -ve results to determine their *in vivo* immune response by SPT by using in-house prepared extracts of indigenous seafood species.

When tested for immuno reactivity by skin prick tests, all examined mollusc, crustacea and fish RAST +ve subjects reacted positively to the different in-house prepared seafood extracts. In addition, positive reactions were also observed in RAST -ve subjects from the same cohort of subjects. Among the mollusc group SPTs with in-house extracts revealed the highest frequency of 36% positive reactions of 138 skin tests (50/138, Table 3.II). This was followed by crustacean and fish sensitive subjects with 27% and 20% respectively. This supported clearly the hypothesis of species specific immune responses among mollusc sensitive subjects, as already indicated in chapter II by the very heterogeneous and diverse RAST and immunoblot results among the different mollusc species (Table 2.II and Fig. 3.8; 3.9). The few commercial SPTs available demonstrated similar responses among the different groups. However, one can not overlook the fact that the strong overall immune response among the mollusc sensitive subjects is mainly produced by the strong response to oyster extract. The potency of this extract was confirmed by the RAST-inhibition studies on different mollusc species (see Table 3.V). Still, it is possible that the oyster extract had a higher concentration of cross-reacting allergens or that these allergens are more stable than other seafood extracts. The high frequency of positive SPT-results by in-house extracts among the different seafood sensitive subjects indicated clearly that a negative RAST-response does not rule out an immunologic response to abalone or other seafood species.

A large study conducted by Ortolani et al. (Ortolani, et al., 1989) in the late 80's assessed the reliability of RAST tests compared to SPT tests with commercial extracts and fresh foods. The specificity varied among the different fruits and vegetables between 40%-93%. However, all tests were negative in the control group and it was therefore concluded that false positive results may result from cross-reactivity with pollen allergens. In addition, the SPT with fresh foods proved more sensitive than commercial SPT or RAST in confirming a history of oral allergy syndrome (OAS) to certain alimentary allergens, such as apple, orange and tomato. The oral

allergy syndrome is also a very common symptom among subjects with sensitivity to seafood but especially to crustacean (Table 2.I) where 100% of the subjects experienced this symptom. The SPT could in this group therefore be of greater importance.

Similar comparative studies on SPT for seafood allergens have not been conducted. However, a few studies showed results on seafood sensitivity involving SPT for some species in the mollusc, crustacea and fish group. Nevertheless, it is expected that the sensitivity and specificity will be improved by complementing the RAST test in subjects with a positive history of seafood hypersensitivity. This was confirmed in my study by a positive SPT in almost one third of all RAST-negative subjects among the three different seafood groups in whom we were able to conduct skin tests.

A study conducted on fish sensitive subjects by Bernhisel-Broadbent (Bernhisel-Broadbent, et al., 1992a) determined whether patients allergic to one fish species can safely eat other fish species. Skin prick tests were positive to all investigated 10 fish species in eight of the 11 patients, and the remaining three patients had at least two positive fish SPTs. However, *in vitro* tests such as inhibition experiments, gel electrophoresis and oral challenges gave evidence of IgE-specific cross-reactivity which not necessarily correlated with symptomatic fish allergy. In addition, these fish-hypersensitive patients were able to consume one or more other fish species without adverse allergic reactions.
I observed in my study also subjects with positive RAST- or SPT-reactivity to seafood species which were not implicated in an adverse reaction. These reactions could have been generated by cross reacting allergens or epitopes. In addition, it could mean that the subjects are already sensitised to some seafood species without being sensitive, yet. However the clinical implication of this has to be determined.

In the mollusc seafood group several studies involved food allergies to snail which support the findings on fish sensitive individuals. A study on the correlation of respiratory problems after eating snails (Amoroso *et al.*, 1988) tested extensively an aqueous extract of a common Mediterranean land snail (*Euparipha pisana*). SPTs on 70 subjects with allergies to the more common allergens of the Mediterranean area gave a positive reaction in 61% of the subjects of which 19% gave also a positive RAST response to snail. No SPT-snail-positive reactions were obtained by using the same extract on 30 non allergic subjects. This confirms that skin prick tests are in some cases not only more specific but also more sensitive than RAST tests. However, as

this study was conducted in the 80's, the possibility of cross-reaction between snail- and HDM sensitivity was not considered.

In a very recent investigation the development of snail specific IgE antibodies was studied during immunotherapy for HDM. It was demonstrated in 17 patients that the immune response to snail showed a significant increase after 1-2 years of treatment (van Ree *et al.*, 1996a). RAST reactivity to snail and shrimp was observed in 3 subjects. However, a clear IgE response to snail was confirmed by a positive SPT test for 6/10 patients. The authors concluded that the induction of IgE during mite immunotherapy might occasionally cause allergy to foods of invertebrate animal origin and demonstrated that the cross-reacting allergen tropomyosin from snail, shrimp and mite was involved.

This data supports the previous study by Amoroso et al. (Amoroso, et al., 1988) that a specific IgE response to a seafood such as snail can be frequently found in subjects with allergies to common allergens and aeroallergens. Moreover, it was demonstrated in particular for aeroallergens, which are found in HDM, that these reactivities are not con-current allergies but characteristic cross-reactivities. Furthermore, because of the close phylogenetic relationship of snails and abalone (both are gastropoda in the mollusc seafood group) the same correlation can be expected also for the indigenous abalone species.

RAST-inhibition experiments on different seafood RASTs with different indigenous seafood species from different seafood groups should give more information on the allergenic cross-reactivities of the various species. In addition I tested also the inhibitory capacity of extract of house dust mite (HDM) to analyse the suggested cross-reactivity between mollusc allergens and a common aeroallergen.

4.2 RAST-inhibition

For maximum inhibition I utilised extracts of local seafood species with the closest morphological association (here specified as being "homologous" extracts). The inhibition experiments in my study indicated clearly that the indigenous seafood species with the closest homology to the commercial RASTs were the extracts of snail and oyster. Inhibition values of 83% and 89% were achieved for the Snail- and Oyster-RAST respectively. This was not surprising because for the in-house prepared snail extract the identical local snail species (*Helix aspersa*) was used (Table 3.IV). However, the indigenous oyster species is similar but not identical to the *Ostrea edulis* used for the commercial RAST. Also for the Mussel- and Squid-

RAST inhibition it was not possible to use identical species since they are not consumed locally. The inhibition values for the available "homologous" extracts were therefore not only lower but produced also much higher standard deviations (70% ±24% and 53% ±26% respectively). These data indicated that the allergen pattern of indigenous mussel and squid species differ from the species used in the commercial RASTs. However, it is also possible that the concentrations of the specific allergens differ in the particular indigenous species meaning that the major allergens could be different.

This can possibly be supported by the fact that the local oyster extract not only had a strong inhibitory effect on the homologous Oyster-RAST but also on the other mollusc RASTs with values between 82-88% inhibition. Nevertheless, these results could also be a reflection of similar antigenic epitopes that are shared among the different mollusc species causing a strong inhibition of the four commercial RASTs.

However, not only the oyster extracts but also the snail extract demonstrated a very high inhibitory potency, with values ranging from 75% (for Blue Mussel-RAST) to 90% (for Oyster-RAST). However, the inhibition results were quite similar across the members of the different mollusc groups indicating, as for the oyster extract, the existence of cross-reacting epitopes in the abalone. The least inhibitory potency on the different mollusc RASTs demonstrated the extract of the local black mussel. Surprisingly, a very low inhibition was even attained on the Oyster-RAST (57%), since both mollusc species belong to the same group of bivalvia and should be very close related. The inhibitory effect of abalone extract on different seafood RASTs will be analysed in chapter IV.

In contrast to the mollusc RASTs, the three crustacean RASTs were all strongly inhibited by three local available crustacean species (Table 3.VI) which are phylogenetically not very closely related. Unexpectedly, also abalone extract demonstrated some degree of inhibition on the crustacean RASTs (26%-34%), which was much stronger than the values produced by the black mussel extract (also member of the mollusc family) with 13%-28%. In comparison extracts of local hake, which is not related whatsoever to crustacean, caused as expected almost no inhibition (0%-12%). Interestingly, the crustacean extract (of local rock lobster) demonstrated on the contrary inhibition on mollusc RASTs at various degrees, from 8% to 95% for the four different individuals.

These results indicated that the crustacean species do have very close related allergens causing a

strong inhibition in all tested crustacean RASTs and that these allergens could be close related to some mollusc species. This could be demonstrated in particular for the two members of the gastropod group, snail and abalone.

However, one reason for the inconsistent data for homologues inhibitions might be that the concentration of 500 µg used for the assays was to low to reach a maximum inhibition. From the initial inhibition curves in figure 3.1 I decided to use this maximum concentration while at both ends of the sigmoid curve the inhibition capacity drops substantial. This means that a tenfold increase in allergen concentration does not change the inhibition very much. However, titration curves produced in my study for Mussel- and Lobster-RASTs (see Fig. 3.1) demonstrate very similar EC50 values with about 10 µg compared to other studies. For crossreacting crustacean species the values ranged from 1-80 µg (Lehrer, 1986; Lehrer and McCants, 1987) and 40 and 150 µg for limpet and abalone respectively (Morikawa, et al., 1990). It would have been better to produce a dose respond curve for each subject and extract and to calculate from these assays the concentration giving 50% inhibition (EC50). The comparison of these values would probably have reduced the standard deviations measured. Furthermore, some of the specific IgE level, in particular to squid, are very low. In fact of the five subjects investigated only one had a strong response with 4.1 ku/l whereas the other three positive values were at or below 1 ku/l (see Table 3.III). Consequently, the inhibition values for the squid RAST have to be interpreted cautiously. However, RAST inhibition studies conducted by other research groups also utilised single maximum protein concentrations to compare allergenicity. The protein concentration in these studies ranged from 1000 µg (cockroach, house dust mite and shrimp) (Witteman *et al.*, 1995) to 10,000 µg for crutacean and oyster (Lehrer and McCants, 1987) or extract were used with no exact protein concentrations (Carrillo *et al.*, 1991).

Some subjects (e.g. Ver in Table 3.V) demonstrated distinct species specific immune responses to some mollusc and crustacean RASTs. The Blue Mussel-, Oyster- and Squid-RASTs demonstrated no or only very weak inhibition by abalone, squid or rock lobster extracts whereas the other subjects were very strongly inhibited. Among the crustacean extracts, also the Lobster-RAST was not inhibited by langoustine or the two mollusc extracts. However, the actual un-inhibited RAST value for Lobster was very weak, in fact the weakest of all values for seafood in this experiment. Therefore, the low inhibition values to crustacean for subject Ver could be an artefact caused by the very low values measured. Nevertheless, the distinct inhibition results of subject Ver on the molluscs demonstrated clearly the existence of species specific reactions among mollusc species of different groups. The IgE antibodies produced by this subject seemed

to bind to particular allergens or epitopes on the RASTs that are not found in the seafood extracts utilised for the inhibition experiments.

Subjects with monosensitivity were found in my study in all three seafood groups. Moreover, the group of mollusc-RAST positive subjects included more than one third of individuals (9/24=38%) with sensitivity to one species only. Nevertheless, as discussed already above, the RAST reactivities of these subjects were much lower than the RAST values of subjects with concurrent sensitivity to crustacea and mollusc. In addition, subjects with single sensitivity to only one species were also found among crustacean (7/24=29%) and fish (3/9=33%).

To investigate the possibility of cross-reactivity between seafood and arthropod allergens, I analysed also the inhibitory capacity of HDM extract, which contain common inhalant allergens. Very surprisingly, some subjects demonstrated a very strong inhibition by the HDM extract whereas other subjects IgE binding to the seafood RASTs were not reduced. Vice versa, the HDM-RAST was inhibited by the seafood extracts for some subjects but not all. It is important to note that the five subjects with concurrent sensitivity to seafood had also raised specific IgE to HDM (see Table 3.III). This concurrent sensitivity could be responsible for some degree of cross-reactivity between the seafood and HDM allergens. Nevertheless, this possible cross-reactivity was analysed in more detail in chapter IV including Western blot analysis of the possible common allergens.

Various studies have demonstrated cross-reactivity among different species in the same or other seafood group. For example the cross-allergenicity between grand keyhole limpet, abalone and keyhole limpet hemocyanin was demonstrated by RAST-inhibition on one patient (Morikawa, et al., 1990). In a study conducted by Lehrer et al. (Lehrer and McCants, 1987) it was demonstrated that a significant inhibition of the Oyster-RAST was obtained with oyster or crustacea extracts. This was also confirmed in my inhibition experiments with different seafood species. However, the Oyster-RAST results of the 19 subjects in Lehrers study with sensitivity to oyster only and concurrent oyster- and crustacea sensitivity, showed a good correlation to different crustacean RASTs which could not be demonstrated in my subjects (see chapter II; Table 2.II). Instead, the Abalone- and Snail-RAST were positively correlated in my study to almost all of the crustacean-RASTs indicating a allergenic correlation between crustacean and gastropods rather than to oyster. For the RAST-inhibition study by Lehrer in-house produced seafood RASTs were utilized to detect specific IgE antibodies. Therefore, the good correlation between the Oyster- and the Crustacean-RASTs could be based on the similarity of consumed and tested species. In

my study I used extract of the indigenous oyster species *Crassostrea margaritacea* for the inhibition assay, but the commercial Oyster-RAST with the species *Ostrea edulis* for the detection of specific IgE (see Table 3.IV).

Furthermore, Lehrer's study demonstrated that subjects with sensitivity to oyster only had minimal RAST reactivity. I could confirm this not only for oyster, but also in addition for snail, squid and mussel (see chapter II; Table 2.IV). In fact, all subjects with concurrent mollusc and crustacea allergy illustrated a much higher RAST reactivity then subjects with sensitivity to oyster, mussel, squid or snail only. These results support the possibility that subjects can demonstrate sensitisation to a particular seafood species, which is frequently consumed in their region, and that a sensitive and specific detection of a specific immune response depends on the correct species used for *in-vivo* and *in-vitro* test.

Other groups also found a correlation between mollusc and crustacean sensitivity. Cross reactivity between squid and shrimp and other crustaceans was demonstrated by reverse immunoassay inhibition studies (Carrillo, et al., 1992). In addition, cross reactivity could not be demonstrated between squid and octopus, which are both cephalopods, nor between squid and other molluscs. Correlation of RAST results of my study subjects with concurrent sensitivity could not confirm this but demonstrated a different relationship. Squid sensitivity demonstrated a very strong correlation to Oyster-RAST. However, no correlation to any other mollusc was observed nor to any crusatacean RAST. The RAST inhibition experiment supported this with very strong inhibition values for Squid-RAST with oyster extract. In addition also extracts of other mollusc and a crustacean species (rock lobster) showed a significant inhibition on the Squid-RAST. Cross-reactivity between squid and octopus was not investigated by RAST-inhibition in my study. Nevertheless, I demonstrated by Western blotting distinct different allergen patterns for both cephalopods (see 3.9).

The hypothesis of species-specific seafood allergens was supported also among crustacean by a study by Morgan et al (Morgan *et al.*, 1989), where three subjects (of 16 Shrimp-RAST positive) with monosensitive RAST reactivity to only one particular shrimp species were found. In addition RAST inhibition experiments on two subjects demonstrated qualitatively different allergens in brown and white shrimp extracts, supporting the hypothesis that there are species-specific allergens in very close related shrimp species.

Sensitivity to indigenous fish species was not analyzed by RAST-inhibition experiments. However, studies by various groups (Hansen *et al.*, 1997) on up to 17 different fish species (de Martino *et al.*, 1990) suggested the presence of cross-reacting antigen(s) in cod, bass, dentex, eel, sole, and tuna. Results of this study demonstrated that cod allergy might be, on the whole, a reliable index of fish allergy, but cod-positive children may perhaps tolerate some other species, which will have to be tested for possible inclusion in their diet. It is to note here that fish cod is not found in South Africa and frequently mistaken with kob, a common name for local kabeljou.

4.3 SDS-PAGE and Western-blot analysis of raw and cooked mollusc and related seafood species

The electrophoretic separation of protein extracts of different seafood species has been used in the past to distinguish closely related species (Hsieh, et al., 1997; Rehbein, 1995). At the species level, a relatively high degree of interspecific polymorphism is apparent and thus, where the tissue is in a suitable condition, useful distinguishing characters are readily available from proteins (Powell, et al., 1995). With proteins, the principle is that different species will possess unique proteins or be differentiated on the basis of structural polymorphisms of a protein.

Molluscs and the generation of new allergens during processing

Indeed, the separation of the local mollusc extracts by SDS-PAGE and staining with Coomassie Blue demonstrated that even the very close related mussel species have species specific proteins. However, most of the proteins were in the molecular weight range of 25-50 kDa and were very difficult to distinguish. The Western blot analysis of different indigenous mollusc species with sera of six sensitised subjects clearly demonstrated species specific allergen (Fig. 3.6 to Fig. 3.8). In particular a common 45 kDa allergen is prominent in most of the species and was found to be heat resistant for most of them. In addition other common allergens with molecular weights of about 38 kDa and 20 kDa were heat stable. Most subjects demonstrated a very specific banding pattern to each individual mollusc species. For abalone an common allergen of about 45 kDa was found which was also present in all the other species identified by one or the other subject. Cooking often had a reducing effect on the allergen binding. However, in the case of this 45 kDa protein, for subjects Car and Fre it was found that cooking increased the IgE reactivity. In chapter IV this particular allergen was analysed in more detail for abalone and other related species.

The three indigenous mussel species demonstrated, for each particular subject, a very distinct banding pattern indicating the existence of species specific allergens. In addition, IgE binding sites for the black and white mussel species were only generated after cooking for subjects Fre and Ver (Fig. 3.7 and 3.8) and were not present in the raw extracts. Cooking seemed to have little effect in the generation of new allergens or their epitopes on the two cephalopod species analysed. Squid and octopus had two very distinct allergens of about 35-45 kDa. However, subject Ver demonstrated binding only to octopus but not to squid. This lack of binding supported the previous data of the RAST inhibition study. Here, the extract of indigenous squid had very little inhibitory effect on the Squid-RAST. This confirms that the two squid species, *Loligo spp.* for the RAST and our indigenous species *Loligo vulgaris reynaudii* (see Table 3.IV) have different allergens which could be demonstrated quantitative (by RAST inhibition) as well as qualitative (Western blot).

Cooking had a very strong increasing effect of IgE binding on four of the seven molllusc species for subject Fre. This demonstrated clearly that heat and therefore denaturing of proteins must not necessarily mean a decrease of reactivity. Denaturing of proteins involves also the change of the secondary and tertiary structure, which in turn exposes segments of the allergen which are not found in intact proteins. This new or additional potential IgE binding sites or epitopes can, as demonstrated for subjects Fre, increase the binding of specific antibodies. This was especially demonstrated for abalone and periwinkle, both members of the gastropod group.

Others have also reported hypersensitive reactions to other members of the gastropod group. Anaphylactic reactions were reported by two subjects after ingestion of limpet's (Carrillo, et al., 1991). Sensitivity was confirmed by SPT, RAST and basophil histamine release assay. However, the patients responded only to cooked limpet but not to raw extract, possibly by exposure of specific IgE binding epitopes during the heating process. The specific increase of IgE binding could also be demonstrated by Western blotting for abalone and periwinkle, both gastropods such as the limpet.

Grand keyhole limpet, a limpet species from Asia, was the offending food in five cases where subjects where hospitalised with respiratory problems (Morikawa, et al., 1990). SPT and RAST demonstrated sensitivity to limpet and abalone. By immunoblotting, the major IgE binding proteins of grand keyhole limpet appeared to have molecular weights of 38 and 80 kDa, but further characterisation has not been done (Maeda *et al.*, 1991). These immunoblots on a mollusc species closely related to abalone, have obviously different allergens from the ones observed in

abalone. However, the immunoblots to two subjects in my study on raw limpet demonstrated allergens in the 40 to 76 kDa range which is similar to the finding by Maeda et al.

Another closely related mollusc species is snail, which has been implicated in several studies on food allergy. Skin prick tests with boiled snail extract (*Euparipha pisana*) on 70 atopic subjects from Italy gave a positive response in 61% of the subjects (Amoroso, et al., 1988). RAST reactivity was demonstrated in 19%, and 15% of these subjects reported asthma symptoms after ingestion of snails. Immunoblotting of the boiled extract resulted in several IgE reactive protein bands ranging from 12-66 kDa. In my study I utilised the indigenous snail species *Helix aspersa* which is not only served in restaurants as so called 'escargot', but also used for the commercial Snail-RAST. Western blotting demonstrated mainly bands between 40 to 50 kDa and appeared to be very similar to the abalone allergens. The relationship of this closely related gastropod species but also to house dust mite is analysed and discussed in more detail in chapter IV.

Very recently the first major allergen of a mollusc species, squid (*Todarodes pacificus*), was identified by Miyazawa et al. (Miyazawa, et al., 1996) using column chromatography and immunoblotting. The isolated heat-stable protein had a molecular weight of 38 kDa and was named Tod p 1. Analyses of amino acid sequences of peptides derived from the squid allergen revealed a high homology with tropomyosin of a snail (*Biomphalaria glabrata*) and the allergenic tropomyosin (Met e 1) of a shrimp. This strong homology, ranging from 40-100% for the snail protein and up to 85% for Met e 1, suggested that Tod p 1 is the squid muscle protein tropomyosin. The indigenous squid species *Loligo spe.* used in my Western blot studies demonstrated IgE binding to a similar molecular weight allergen of about 35 kDa which could also be tropomyosin.

Tropomyosin has been implicated in several other studies to be the major allergen of different crustacean species. Leung and colleagues (Leung, et al., 1996) have recently compared the immuno blot reactivity of 9 shrimp sensitive subjects to different crustacean and mollusc species. All nine sera demonstrated IgE reactivity to the 38 kDa protein when tested against ten common molluscs. In addition, other allergens, in particular a 49 kDa antigen was detected in most molluscs and also abalone. Preabsorption of the sera with recombinant shrimp tropomyosin resulted in complete loss of IgE reactivity to the 38 kDa protein band in all mollusc extracts but also to the other minor allergens. However, preabsorption or inhibition studies of the sera with the extracts of the analysed mollusc or crustacean species were not presented. The immunoblots produced in my study demonstrated higher molecular weight allergens in most analysed mollusc

species. In addition, the RAST inhibition studies conducted in my study demonstrated clearly that immuno dominant epitopes are shared among mollusc but also between crustacean species.

Relationship with Crustacean allergens

The separation of the crustacean proteins by SDS-gel electrophoresis showed proteins in the molecular range from 20 to 80 kDa which, made it difficult to detect a species specific profile. In the molecular range between 30-40 kDa all crustacean species had similar protein bands.

Western blot analysis of raw indigenous crustacean species demonstrated IgE reactivity mainly in the range of 20 kDa and 75 kDa (Fig. 3.9). However, for most species in addition allergens of 36 and 50 kDa were present. Interestingly, the three very close related rock lobster species showed binding patterns which made it possible to distinguish them from each other. One subject showed binding only to a rock lobster but not to prawn or langoustine (Fig. 3.10). Cooking resulted in the loss of most of the high molecular allergens whereas the 20 kDa IgE activity was mostly preserved. Furthermore, the reactivity of these allergens were, for subject Fre, only activated after cooking. Interestingly, this subject demonstrated the same heat activation of IgE binding in addition for mollusc species.

Recent studies have identified tropomyosin with 38 kDa as the major allergen in shrimp (Daul, et al., 1994; Leung, et al., 1994). However, allergens in other crustacean species have only been characterised very recently in a spiny lobster *Panulirus stimpsoni* and from the American lobster *Homarus americanus* (Leung, et al., 1998b).The allergens of both crustacean were characterised by molecular cloning and coded for a 34 kDa protein for the spiny lobster (named Pan s I) and for the American lobster (Hom a I). However, both new allergens show a significant homology to shrimp tropomyosin, which was supported by immunoblot inhibition studies. These findings could not be fully supported by the results of my study. Some of the subjects do demonstrate binding to allergens in this molecular range. However, most of the IgE reactivity seemed to be located in the 75 kDa range for the raw extracts and 20 kDa for he cooked species. To characterise these new allergens, which have not been described in the literature before, inhibition studies with recombinant tropomyosin would have to be conducted to identify e.g. common epitopes. Nevertheless, sequence analysis of protein fragments would give more information about the homology to known allergens.

It has been shown that the tropomyosin proteins are conserved among invertebrates including not only shellfish but also arthropods. This latter observation encouraged Leung and colleagues to suggest that persons sensitive to shellfish should undergo further study for potential cross-reactive inhalant sensitivity to arthropods. (Leung, et al., 1996). The allergenic relationship between indigenous shellfish species and arthropods is analysed and discussed in more detail in chapter IV.

Relationship with Fish allergens

The separation of local fish extracts by SDS- electrophoresis (Fig. 3.4) demonstrated protein bands in the molecular range of 30 to 70 kDa. In addition strong bands are present at about 12 kDa. The banding pattern clearly distinguished the different fish species from each other. It is well documented in the literature that the major allergen of cod fish has a MW of 14 kDa. This band is also after cooking the different fish species still clearly visible, yet the molecular weight seems to be slightly lower.

However, some of the fish species clearly indicated a weaker 12 kDa protein band including tuna, whereas fish like kingklip, yellowtail and Cape salmon illustrated a very strong band (see Fig.3.4). This is supported by the fact that in this patient cohort studied, tuna sensitivity is only indicated by 3 subjects of causing an adverse reaction compared to kingklip and yellowtail which have the strong 12-14 kDa band and are implicated by 14 and 23 subjects respectively.

Other studies on fish sensitivity also observed that tuna is often indicated as hypoallergenic fish (Bernhisel-Broadbent, et al., 1992b; James, et al., 1997). However, one has to note here that particular tuna is more commonly consumed canned instead of raw, especially here in South Africa. For canning purposes the cooked fish is heated over 100^0C for several hours, which denatures the proteins extensively. This by itself generates a loss of allergenicity and therefore generated a hypoallergenic form of fish. Nevertheless, my own observations of immuno blots of various canned tuna products indicated that, depending on the brand name, some extracts demonstrated only a minor loss of allergenicity (data not shown). This decrease of allergenicity in canned tuna and salmon products was also observed by Bernhisel-Broadbent and colleagues (Bernhisel-Broadbent, et al., 1992b) on Western blots and confirmed by DBPCFC. However, there are no published data on the altered allergenicity of different brands of canned tuna products to prove that all this products are actually hypoallergenic.

Other allergens have been detected by Western blot in cod extracts (Dory *et al.*, 1998). Several IgE-reactive protein bands have been identified over a wide molecular weight range (12-130 kDa). It was shown that storage conditions may influence the relative distribution of IgE-reactive protein bands. This could have clinical and analytical implications as the indigenous fish species demonstrated IgE reactive bands mainly between 10 and 60 kDa (Fig. 3.13). However, cooking the fish species resulted in a drastic loss of almost all higher weight allergens whereas for some fish species the IgE reactivity of the 10-15 kDa band rather increased. This may account for the fact that that concurrently clinical sensitivity to fish and molluscs was not found in my patient cohort.

Summary and Conclusion

The hypothesis of species specific immune responses among mollusc sensitive subjects was clearly supported by the data obtained in my analyses of sera from this patient cohort.

Analysing the RAST results among the different mollusc species showed a very heterogeneous and diverse response of sensitised subjects to the different mollusc species. When tested for immuno reactivity by skin prick tests with in-house extracts, positive reactions were also observed in RAST-ve subjects, with the highest frequency found in the mollusc sensitive group.

RAST-inhibition experiments demonstrated that abalone extract showed the strongest inhibition values for the closely related gastropod species, snail. Additional inhibition of the other mollusc RASTs supported the existence of some cross-reacting allergens in the abalone, *Haliotis midae*. However, the very low RAST-inhibition with the indigenous mussel and squid species indicated that their allergen compositions differ strongly from the species used in the commercial RASTs. In addition, the RAST-inhibition of crustacean RASTs with extract of abalone revealed a strong inhibitory effect on the three utilised crustacean RASTs. This finding supported the existence of cross-reacting allergens not only among mollusc species, but also among different seafood groups in this case, the crustaceans.

Western-blot studies on the very closely related indigenous mussel and cephalopod species as well as in local crustaceans demonstrated species-specific allergens. RAST analysis demonstrated monosensitivity to only one species in a particular seafood group especially among the group of molluscs (more than one third of the RAST+ve individuals).

SDS-gel electrophoresis demonstrated that even the very closely related mussel species have species-specific proteins, which allow a clear distinction. Western blot analysis of different indigenous mollusc species identified two prominent allergens particular in abalone with molecular weights of 38 kDa and 45 kDa. These and other mollusc allergens appeared to be very resistant to heat and denaturation. Increased immuno activity following heating and the generation of "new" allergens could be demonstrated in two subjects.

Western blotting of raw and cooked indigenous crustacean species could not confirm the heat resistance reported by several research groups for the major 38 kDa allergen, identified as tropomyosin. Instead a 20 kDa protein was demonstrated to be immuno reactive after cooking which has not been described before as well as allergen binding patterns for raw crustacean in the molecular range of 20-75 kDa.

To summarise, Western-blot techniques identified subjects with a defined immune response to a specific seafood species. This study suggests that in the confirmation of the diagnosis in subjects with single sensitivity to species within the group of molluscs, the RAST may not be useful as an single diagnostic instrument. It is suggested that the percentage of seafood sensitive subjects testing positive by skin test and RAST will be increased by use of extracts from more than one species of a particular seafood. In addition species specificity is important because it may explain the intermittent symptoms of some study subjects after consuming certain seafood. I propose therefore the following flowdiagram (Figure 3.14) which demonstrates the selection of analytical techniques for the establishment of an immune reaction to ingested seafood. Cross-reactivity among seafood sensitive subjects to mite allergens has been established by an RAST-inhibition assay. It might, therefore, be interesting to screen mite allergic patients for allergy to abalone, also a member of the gastropod group in areas where abalone is harvested and widely consumed.

Only detailed characterisation of the specific allergens will answer the questions raised about possible cross-reacting allergens. In the following chapter IV I have analysed the immune responses to abalone in more detail, and characterised the novel allergens.

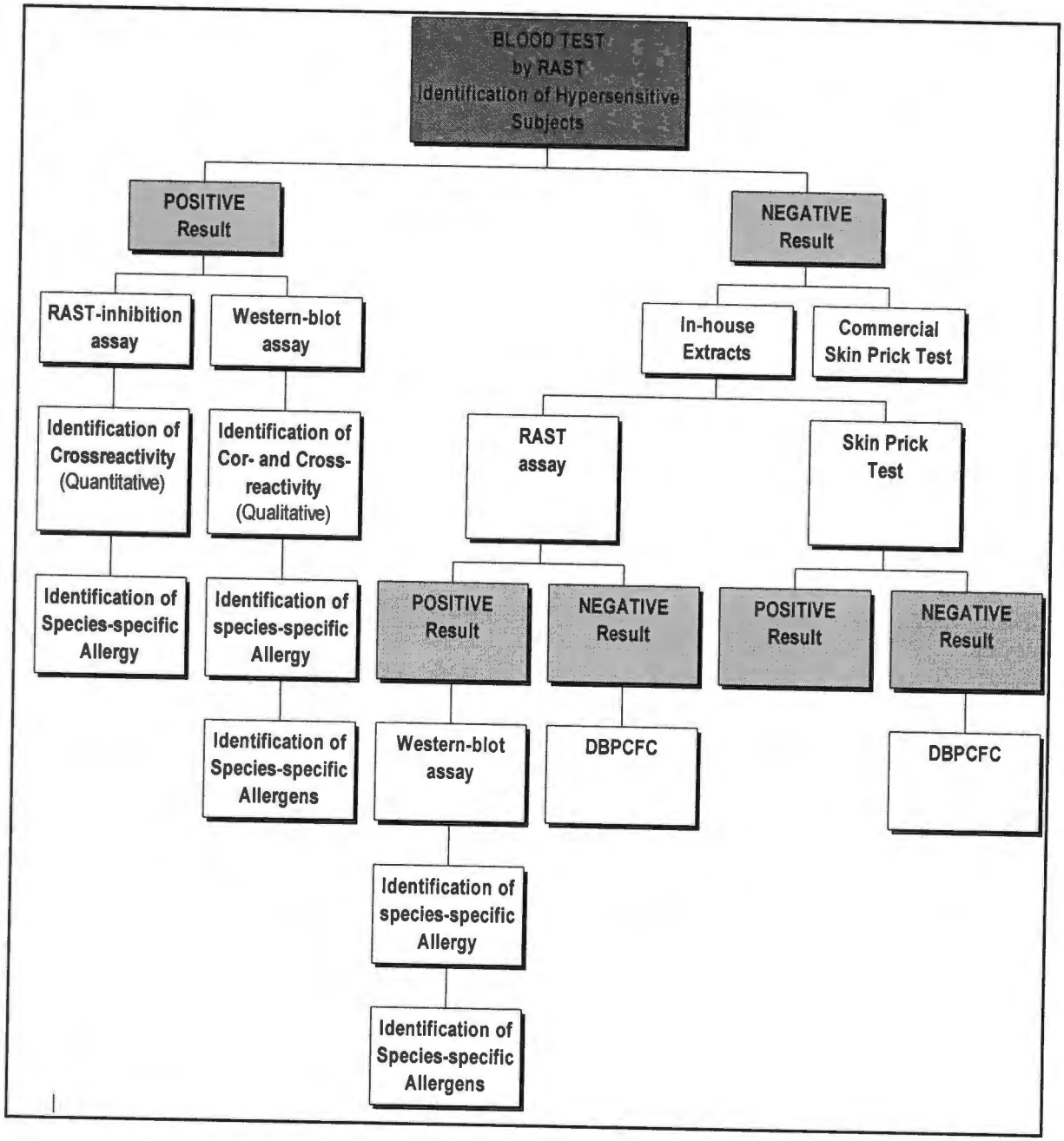
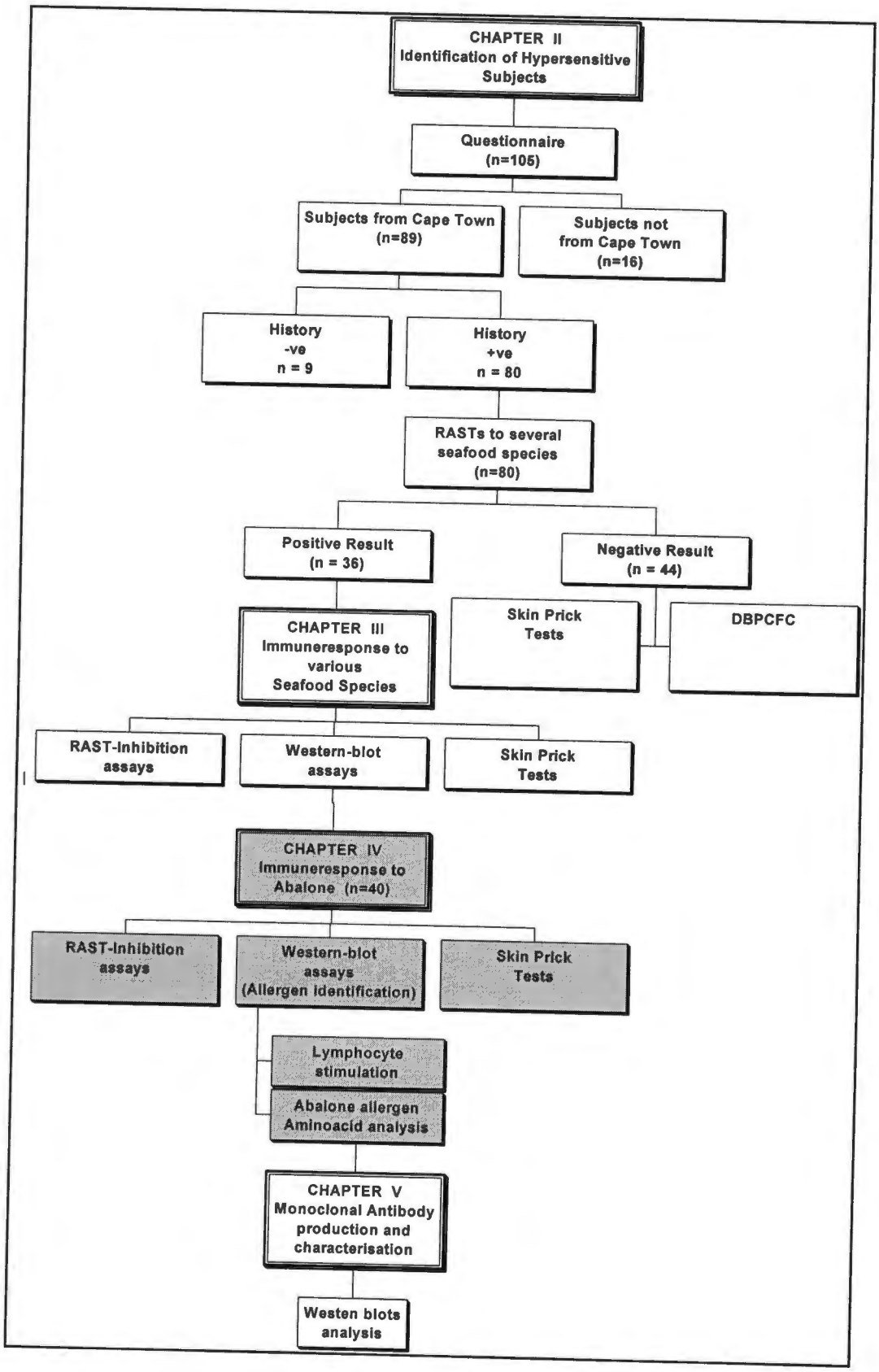


Figure 3.14: Flowdiagram of identification of subjects sensitised to different seafood species by different *in-vivo* and *in-vitro* methods.

CHAPTER IV:

Immunological characteristics of Hypersensitive Reactions and Identification of a Unique 45 kDa IgE Binding Protein in South African Abalone (*Haliotis midae*)



Flowdiagram of sample selection, diagnosis and analysis of allergy to seafood by different *in-vitro* and *in-vivo* methods.

1. Introduction

In chapter III I identified 38 subjects who reported hypersensitivity to abalone and other molluscs. Since little is known about the clinical features of abalone hypersensitivity specifically and the allergens involved, I have investigated these 38 subjects and their immune responses in more detail, using an in-house Abalone-RAST and skin prick tests (SPT) as already described in chapter II (3.4) and chapter III (3.2) respectively. In addition, Western blotting and lymphocyte proliferation assays where performed to characterise the abalone allergens in more detail and determine their stability to denaturation. Furthermore, the abalone extract was characterised in more detail using RAST-inhibition studies as mentioned already in chapter III (3.3). The allergenic components were isolated using SDS-gel electrophoresis, the allergens isolated and the amino acid composition analysed.

2. Material and Methods

2.1 Patients

As already analysed in chapter II (3.3), the questionnaire administered to individuals with perceived seafood allergy was analysed specifically for subjects with perceived allergy to abalone. 38 subjects with symptoms reported after ingestion of abalone were recruited from this survey, for this more in-depth study. A comparative group consisted of 10 subjects from the survey who reported sensitivity to fish (Teleosti) but not to molluscs or crustaceans. The comparative group was utilised as controls for the *in-vitro* and *in-vivo* tests.

2.2 Preparation of extracts

Seafood species used in the present study were caught on the South African Coast and freshly frozen prior to preparation of the extracts (see chapter III for species names and extraction). Abalone tissues from Australia and Japan were generous gifts from Dr S.M. Degnan (University of Queensland, Australia) and Dr T. Naganuma (Hiroshima University, Japan) respectively. The house dust mite (HDM) extract was obtained from Dr. B. Nurse (Department of Immunology, UCT), the snail (*Helix aspersa*) caught in local gardens in the Cape Town area and the locusts (*Locusta migratoria*) supplied by the Department of Zoology (UCT). For skin prick tests (SPT) the extracts were diluted in 50% glycerol (v/v) and stored at -80^{0} C until used.

Picture of five different South African abalone species, with *Haliotis midae* as the largest species on the right side.

2.3 Skin prick tests

Skin prick tests were performed, with informed consent, on the forearm of 24/38 subjects. SPTs were not performed on the 10 subjects whose sera displayed abalone RAST results exceeding 10% binding in view of the unknown risk of inducing a generalised reaction. Four of the 38 subjects did not come to the clinic for the skin tests because they had to travel a long distance to the hospital. Five in-house prepared extracts were used for the skin testing: abalone, oyster, black mussel, white mussel, squid and one commercial (Soluprick, ALK Laboratories, Horsholm, Denmark) blue mussel (*Mytilus edulis*) extract. The skin prick test was performed as already described in chapter III (2.3).

2.4 Specific IgE RAST

Abalone specific IgE levels were determined using duplicate serum samples and a in-house Abalone-RAST (see Chapter II (2.2) for RAST production). A positive reaction was regarded as percentage of binding exceeding 3 times the binding obtained with the negative control. Commercial RASTs were performed using the Pharmacia CAP System RIA (Pharmacia, Uppsala, Sweden). The RAST results of the in-house Abalone-RAST were correlated with the commercial seafood RASTs by linear regression.

2.5 RAST-inhibition assay

To validate the in-house Abalone-RAST I developed and to analyse possible cross-reactivities to other seafood species, I pre-incubated sera of abalone sensitive subjects with increasing protein concentrations of fresh (raw) and cooked abalone and extracts of black mussel, rock lobster and house dust mite (0.01-1000 µg/ml).). The method for the RAST inhibition has been described in detail in chapter III (2.4).

To determine the cross-reactivity between abalone allergens and allergens of other molluscs, crustaceans and fish species I inhibited the following commercial CAP-RASTs with extract of South African abalone; Snail (Rf314), Squid (Rf258), Blue Mussel (f37), Oyster (Rf290), Lobster (f80), Langoustine (Rf304), Crayfish (Rf320). As mentioned in chapter III, I also investigated the cross-reactivity between snail and the close related abalone species with house dust mite (HDM).

To measure the antigenic activity of eluted proteins from SDS-gels (see below), I pre-incubated 20 µl of the eluted fractions with 80 µl of pooled serum from three subjects with concurrent abalone and snail sensitivity and measured the inhibition of the Snail-RAST.

2.6 IgE ELISA-inhibition assay

The binding of proteins to different surfaces can change the antibody recognition and therefore the detection limits of the particular antigen. The binding of hydrophilic or hydrophobic proteins will be greatly facilitated depending on the type of ELISA plates utilised. During Western blotting proteins bind to the apolar membrane via strong hydrophobic forces whereas the antigens for the RAST assays are bound covalently to the matrix. I investigated the influence of binding abalone allergens onto a different matrix by comparing the RAST results with similar inhibition experiments using enzyme-linked immunosorbent assay (ELISA). The cross-reactivity of various seafood extracts was measured by pre-incubation of serum of abalone sensitive subjects with different extracts and measuring the residual specific IgE. A decrease in IgE binding demonstrates the existence of cross-reacting proteins. For the IgE-ELISA inhibition assay protein extract of raw (fresh) abalone was used as the solid phase antigen (on Polysorp plates, NUNC). 1:5 dilutions of patient sera were preincubated with 10-fold serial dilution's of the various extracts with concentrations ranging from 0.5 mg/ml to 5 ng/ml. IgE binding was detected by using an in-house produced monoclonal mouse antihuman IgE antibody and biotinylated rabbit anti-mouse IgG antibody (DAKO).

2.7 *In-vitro* proliferation assay of PBML's

Peripheral blood mononuclear cells (PBMLs) were isolated from heparinized blood (Ficoll-Hypaque gradient) and cultured at 10^5 cells/well for 6 days with 100 µg/ml protein extract of raw and cooked abalone according to Coligan (Coligan and et.al., 1994). The cells were pulsed on day 6 with 0.5 µCi(^{3}H) thymidine per well 18.5 h before harvesting on filter paper and counting for 1 min in a Beta-counter (Beckman, LS 3800). The results are expressed as stimulation index (SI).

$$SI = \frac{\text{Stimulation induced, dpm}}{\text{Control, dpm}}$$

2.8 SDS-gel electrophoresis and Western blotting

SDS-gel electrophoresis was performed as described in chapter III (2.5) (Laemmli, 1970). Western blotting for IgG reactivity was modified from the method used to identify IgE reactivity. A mouse monoclonal antibody anti-human IgG (DAKO, 1/5000) was incubated for 2 hours and after a washing step detected by a rabbit anti-mouse antibody (DAKO, 1/10000). All the other steps were identical to chapter III (2.5).

2.9 Allergen purification

To purify allergens from abalone extract I separated the protein extract by SDS-gel electrophoresis (see 2.8) and stained the proteins using 0.1% Coomassie Blue in 50% methanol. The desired protein bands were excised using a scalpel, crushed to very small pieces with a spatula and transferred into an eppendorf tube. Western blotting studies (see chapter III) identified two major allergens with approximate molecular weights 38 kDa and 45 kDa. However, two protein bands with approximate 42 kDa and 49 kDa were very close to the identified allergen bands. To ensure that I identified the major allergens of abalone all four proteins were purified and analysed for antibody binding.

The proteins were extracted using three different extraction methods (Ausubel *et al.*, 1995).

A) Acid extraction

300 µl of 80% trifluoroacetic acid (TFA, MERCK) were added to the gelpieces and incubated overnight at 4^0C. After centrifugation the eluted protein solutions were dialysed overnight into 0.1 M PBS, the volume reduced to 100µl using a vacuum centrifuge (Speed Vac, Savant SC 110) and stored frozen at -20^0C until further use.

B) Electroelution

300 µl of Tris-buffer (0.1 M, 0.4% SDS, pH 8.3) were added to the gelpieces and electroeluted using the 'SIXPAC GEL ELUTER (GE 200, Hoefer, San Francisco, USA) at 50 Volt over a duration of 2 hours. The electroeluted protein solutions were dialysed overnight into 0.1 M PBS, the volume reduced to 100µl using a vacuum centrifuge and stored frozen at -20^0C until further use.

C) SDS extraction

300 µl of 0.4 M NH_4HCO_3 with 3% SDS were added to the gelpieces and incubated overnight at 4^0C. After centrifugation the eluted protein solutions were dialysed overnight into 50 mM NH_4CO_3, the volume reduced to 100µl using a vacuum centrifuge and stored frozen at -20^0C until further use.

The eluted protein fractions were all separated by SDS-gel electrophoresis and transferred onto PVDF membrane for Western blotting with a serum pool of three abalone sensitive subjects. For some of the eluted protein fractions the IgE binding activity was analysed using Snail-RAST inhibition (see 2.5).

3. RESULTS:

3.1 Study group and Symptoms

The ages of the 38 subjects who reported reactions to abalone and other molluscs and participated in this study ranged from 17 years to 74 years with a mean age of 44 years. Twenty four subjects (63%) were female and fourteen (38%) had a family history of food allergy to other foods.

The comparative group consisted of 10 subjects who reported sensitivity to fish only. These subjects had a mean age of 36 years and 6 were female. As part of the questionnaire, the subjects were asked to describe, in detail, the symptoms they experienced during an adverse reaction. Symptoms were divided into 4 categories: cutaneous, gastrointestinal, respiratory and others (Table 4.I).

The symptoms for subjects with reported reactions to all mollusc species were already analysed in chapter II (3.3).

Table 4 I: Frequency of reported symptoms of 38 subjects with perceived allergy to abalone.

Symptoms	PERCENTAGE (%)
Nausea/vomiting/abdominal pain	**61**
Diarrhoea	**48**
Asthma/wheezing	**42**
Oropharyngeal itching/swelling	**39**
Anxiety	**35**
Urticaria/eczema	**32**
Flushing	**32**
Dizziness	**26**
Headache	**23**

The total number of symptoms reported by the 38 subjects was 105, of which the gastrointestinal symptoms were most frequent, with 61% for nausea/vomiting and 48% for diarrhoea. The next most frequent symptoms were asthma and wheezing with 42% followed by cutaneous symptoms such as oropharyngeal itching (39%) and urticaria/eczema (32%). The least frequent symptoms were headaches (23%). Delayed onset of symptoms of up to 7 hours, were reported in 13 (34%) of our subjects

3.2 Specific IgE assessment by RAST and Correlation of Co-reactivity

The RAST reactivity to 5 different molluscs and other seafood species in subjects with reported abalone-sensitivity is demonstrated in Table 4.II. The RAST reactivity of all seafood sensitive subjects was already analysed in chapter II (3.4). Elevated RAST %-binding to abalone was detected in 45% of all sera tested (17/38). However, specific IgE was also detected to mollusc species that were not reported by some subjects as having induced clinical symptoms. None of the 10 comparative subjects who reported hypersensitivity to fish displayed specific IgE binding to any of the 5 molluscs. For the comparative subjects the mean RAST value for abalone was 0.6% binding (SD ±0.3%, n=10).

Table 4.II: RAST results of seventeen subjects with positive Abalone-RAST (in %-binding) to different seafood species in ku/l. The mean values and standard deviation (S.D.) are given.
Note: '--' indicates negative RAST results; N.A. = not available

RASTs	(ku/l) ± S.D.	In-house RAST %-binding ± S.D.	Number of subjects
OYSTER	8.2	N.D.	2
SNAIL	3.4 ±4.0	N.D.	5
BLUE MUSSEL	2.8 ±2.3	N.D.	3
SQUID	2.0 ±2.0	N.D.	3
ABALONE (%-binding)		< 10% = 4.8 ±1.1 > 10% = 24.3 ±9.9	10 7
CRAYFISH	11.6 ±12.2	N.D.	9
SHRIMP	11.4 ±10.3	N.D.	7
CRAB	9.8 ±8.2	N.D.	7
LOBSTER	9.2 ±10.5	N.D.	8
HAKE	14.4	N.D.	2
TUNA	2.1	N.D.	1
SALMON	0.7	N.D.	2
MACKEREL	0.5	N.D.	1

60% (10/17) of Abalone-RAST +ve patients also had elevated specific IgE to the phylogenetic closely related snail. The RAST ratios of the Abalone- and Snail-RAST, compared and analysed by linear regression, showed a positive correlation ($r = 0.79$, $p \leq 0.01$). However, seven subjects demonstrated specific IgE for abalone, but not for snail. Figure 4.1 shows this correlation excluding the highest value each for two subjects, with and without concurrent IgE to snail. These two values are with 41% and 35% respectively, exceptionally higher than the majority of

the RAST results which have an mean value of 10.3% (±S.D. 7.0%). These values are possibly artefacts, caused by insufficient washing during the last step of the RAST (see chapter II; 2.1).

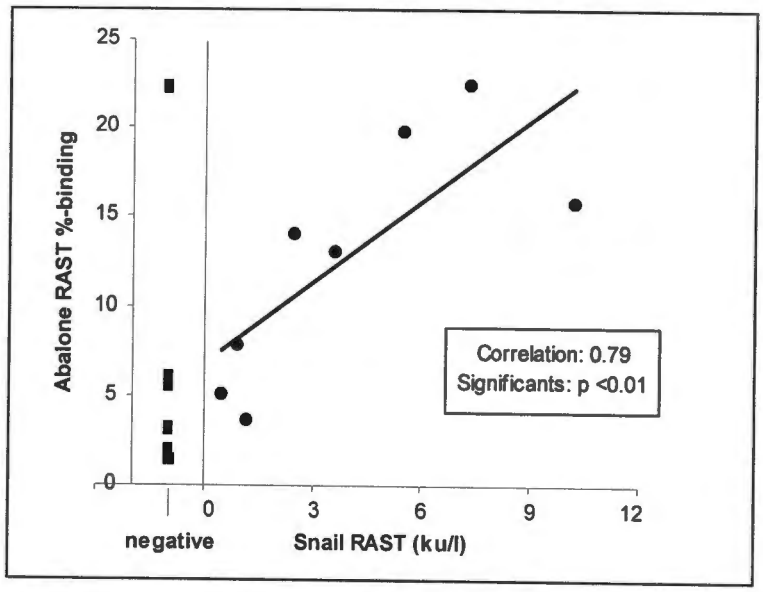


Figure 4.1: Correlation of positive abalone in-house RAST reactivity with commercial Snail-RAST reactivity (•). Subjects demonstrated specific IgE to both abalone and snail (n=9). Seven subjects presented with positive in-house RAST to abalone but negative commercial Snail-RAST (■).

The positive correlated co-reactivity was not only observed between Abalone-RAST and the Snail-RAST, but also to a second mollusc species, the Squid-RAST ($r = 0.97$, $p \leq 0.05$; see chapter II, Table 2.II). There was no significant correlation to the Blue Mussel- and Oyster-RAST. However, surprisingly abalone was also positive correlated to three of the four crustacean RASTs evaluated (crayfish, shrimp, crab; $p<0.05$).

3.3 RAST inhibition studies

To asses the specificity of IgE binding to the Abalone-RAST and possible crossreactions to other mollusc and seafood species, RAST inhibition studies were carried out on five abalone sensitive subjects. Because of the observed cross-reactivity between snail and house dust mite (HDM) and the close phylogenetic relationship between abalone and snail (both gastropoda), I included HDM extract for the inhibition study.

RAST-inhibition results of Abalone-RAST with raw (fresh) and cooked abalone demonstrated clearly that cooking did not diminish the IgE binding activity (Fig. 4.2). Comparing the dose of an extract required to inhibit 50% of the abalone IgE binding gives an index of the antigenic relationship. Cooked abalone has the highest inhibitory effect with about 0.5 µg/ml followed by raw abalone with about 5 µg/ml. Black mussel and HDM demonstrated some degree of cross-

reactivity at a much higher antigen concentrations of over 500 µg/ml. However, two inhibition points for the inhibition curve with raw and cooked abalone each did not fit the curve very well and made the calculation of the values for 50% inhibition not very accurate.

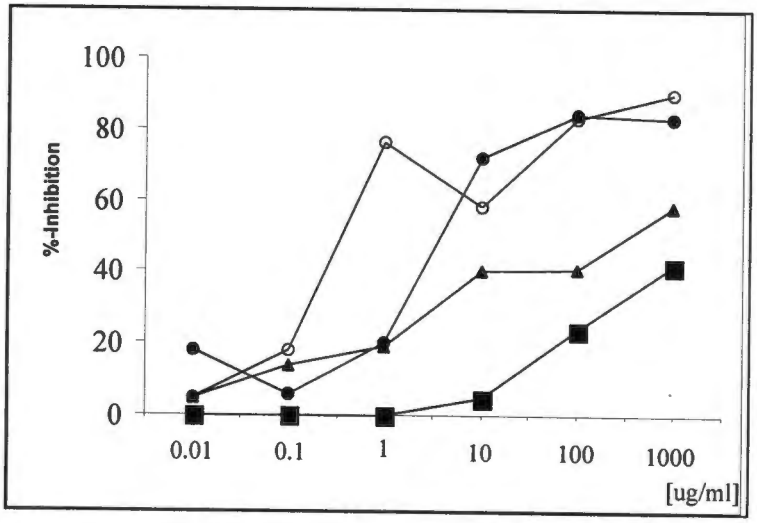


Figure 4.2: Abalone-RAST inhibition with increasing protein concentrations of different seafood extracts. Raw (•) and cooked (o) abalone (*Haliotis midae*), black mussel (*Choromytilus meridionalis*, ▲) and house dust mite (■).

The highest inhibition of the commercial mollusc RASTs with abalone extract (Table 4.III) was achieved for the Snail- and Blue Mussel-RAST with up to 95%. The Oyster- and Squid-RAST followed with 92% and 85% respectively. However, for some subjects only a marginal inhibition was achieved and for subjects Ver no inhibition whatsoever of the Blue Mussel-RAST.

Table 4. III: Inhibition of seafood and House Dust Mite (HDM) CAP-RASTs with Abalone and HDM extracts. Inhibition is displayed in %-inhibition for each individual patient and each extract. The mean values and standard deviation (S.D.) are given for each inhibition experiment, not including the negative reactions.

CAP-RASTs	Patients	Abalone	Snail	HDM
SNAIL	Car	86.3	59.7	0.0
	Fre	94.8	95.4	0.0
	Koc	49.4	93.4	41.8
	Ver	49.8	82.5	56.9
	Ket	81.1	84.1	0.0
	Mean ± S.D.	**72.2 ± 21.2**	**83.0 ± 14.2**	**49.3**
SQUID	Car	85.4	82.4	0.0
	Fre	65.7	78.7	0.0
	Koc	73.1	96.5	71.6
	Ver	13.8	78.3	22.7
	Mean ± S.D.	**59.5 ± 31.5**	**83.0 ± 8.5**	**47.1**
BLUE MUSSEL	Car	94.7	81.4	0.0
	Fre	78.4	78.2	4.2
	Koc	70.8	83.9	50.3
	Ver	0.0	56.4	0.0
	Mean ± S.D.	**81.3 ± 12.2**	**74.9 ± 12.6**	**27.2**
OYSTER	Car	90.7	92.5	0.0
	Fre	91.6	89.1	0.0
	Koc	44.5	94.5	25.9
	Ver	16.0	85.5	27.7
	Mean ± S.D.	**60.7 ± 37.0**	**90.4 ± 3.9**	**26.8**
HOUSE DUST MITE	Car	14.5	22.1	22.1
	Fre	68.0	68.2	9.8
	Koc	0.0	0.0	91.2
	Ver	6.3	1.8	73.9
	Ket	0.0	0.0	58.7
	Mean ± S.D.	**29.6 ± 33.5**	**30.7 ± 34.0**	**51.2 ± 34.4**
LOBSTER	Car	24.4	N.D.	0.0
	Fre	32.0	N.D.	0.0
	Ver	0.0	N.D.	0.0
	Ket	21.0	N.D.	0.0
	Mean ± S.D.	**25.8 ± 5.6**		**0.0**
LANGOUS-TINE	Car	36.7	N.D.	0.0
	Fre	42.2	N.D.	N.D.
	Ket	22.9	N.D.	N.D.
	Mean ± S.D.	**33.9 ± 9.9**		**0.0**
CRAYFISH	Car	24.4	N.D.	0.0
	Fre	17.1	N.D.	26.9
	Koc	45.1	N.D.	57.6
	Mean ± S.D.	**28.8 ± 14.5**		**42.2**

Figure 4.3 summarises these results for the four different mollusc RASTs. It appeared that the analysed subjects demonstrated two sets of responses.

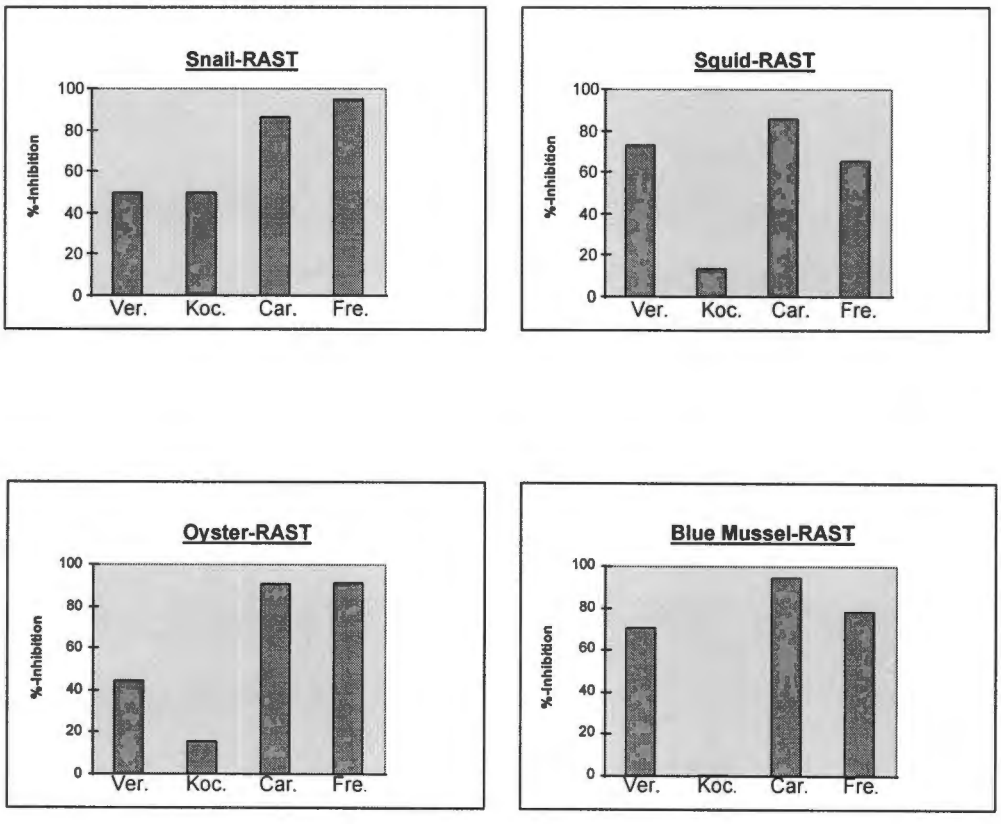


Figure 4.3: RAST-inhibition results of four subjects with in-house prepared abalone extract. The four CAP-RAST used for this assay were Snail (Rf314), Squid (Rf258), Oyster (f290) and Blue Mussel (f37).

The sera of subjects Car and Fre are very strongly inhibited with abalone extract (65-95%), whereas Ver and Koc show a much lower inhibition (0-73%). However, the snail-RAST is very strongly inhibited by abalone in both groups with 87% and 50% respectively indicating a strong antigenic relationship between abalone and snail, both members of the class of Gastropoda. The crustacean RASTs were also inhibited by the abalone extract, between 14-42% for subjects Car and Fre and to a lower degree (0-45%) for subjects Ver and Ket.

Table 4.IV showed these two distinct groups with their mean values of two subjects (or three for group A on Snail-RAST) for different seafood extracts used as inhibitor. The inhibition of the different seafood and HDM-RAST with extracts of squid and rock lobster also demonstrated a similar response with subjects Car and Fre (and Ket for the Snail-RAST) showing a much higher inhibition as Ver and Koc.

Table 4. IV: RAST-inhibition results of seafood and HDM CAP-RASTs with extracts of different local seafood extracts and HDM. The mean %-inhibition values of two subject groups (A and B) are presented. The shadowed boxes indicate the inhibition values for "homologous" extracts whereas the values in bold indicate the strongest inhibition for a specific CAP-RAST.

A: Subjects Car and Fre (and Ket for SNAIL-RAST)

CAP-RAST	Abalone	Snail	Squid	Oyster	Black Mussel	Rock Lobster	Hake	HDM
SNAIL	87	80	85	**92**	48	90	N.D.	0
SQUID	75	**80**	70	78	60	53	N.D.	0
OYSTER	90	90	90	**94**	62	90	N.D.	0
BLUE MUSSEL	**85**	80	75	**85**	81	84	N.D.	2
LOBSTER	28	N.D.	N.D.	N.D.	11	**84**	6	0
HDM	40	45	40	N.D.	N.D.	**60**	N.D.	16

B: Subjects Ver and Koc

CAP-RAST	Abalone	Snail	Squid	Oyster	Black Mussel	Rock Lobster	Hake	HDM
SNAIL	50	**85**	40	83	62	65	N.D.	57
SQUID	45	**90**	40	82	60	45	N.D.	45
OYSTER	35	**85**	25	83	50	48	N.D.	25
BLUE MUSSEL	30	70	25	**77**	60	40	N.D.	25
LOBSTER	10	N.D.	N.D.	N.D.	7	**85**	4	0
HDM	5	3	5	N.D.	N.D.	15	N.D.	**30**

The inhibition of HDM-RAST with abalone and snail also showed a much stronger inhibition of Car, Fre and Ket serum IgE (14%-81%) than for Ver and Koc (0%-27%). At the contrary, inhibition with homologous extract of HDM achieved with Car and Fre serum only 16% whereas Ver and Koc reached about 83%. Figure 4.4 illustrated these findings which clearly showed the two distinct responses.

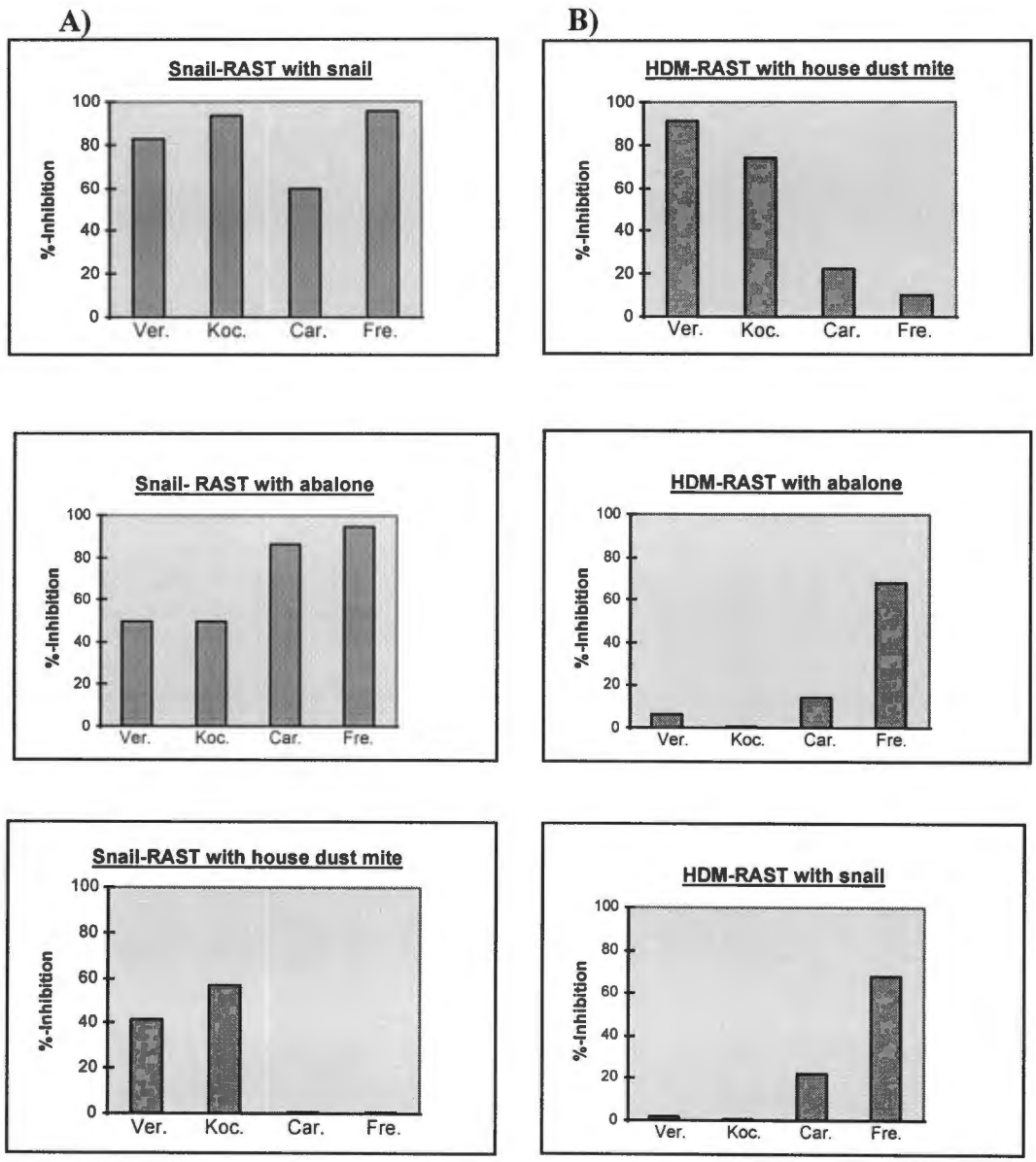


Figure 4.4: RAST-inhibition results of four subjects with in-house extracts of abalone, snail and house dust mite (HDM). **A)** Snail-RAST and **B)** HDM-RAST. Inhibition is expressed as residual percent (%) binding.

The finding of high inhibition of Snail-RAST with HDM extract (57%) and low inhibition of HDM-RAST with snail extract (3%) suggested that for some subjects (here Koc, Ver) the IgE antibodies may primarily be raised to the aeroallergens. However, for other subjects with concurrent allergy to HDM this seems not to be the case. For two out of the five studied subjects (Car, Fre) I observed just the opposite effect, with no inhibition of the Snail-RAST with HDM and a high inhibition of the HDM-RAST with snail or abalone extract (up to 68%).

3.4 ELISA-inhibition assay

An ELISA-inhibition assay was performed on two subjects using 7 different extracts at increasing protein concentrations ranging from 0.005-500 µg/ml. These 2 subjects (Ver and Ket) were representatives of the group of the 9 subjects with concurrent RAST reactivity to abalone and snail. Inhibition data for one of these subjects is presented in Figure 4.5. Comparing the inhibition of 50% of the abalone IgE binding gives an index of the antigenic relationship. The most potent inhibitors are, as expected, raw and cooked abalone at a concentration of less than 1 µg/ml followed by snail with about 9 µg/ml. The required concentration for rock lobster and black mussel is much higher with about 25 µg/ml. Sea urchin and house dust mite however, inhibit by only 10% with a 500-times higher concentration (500 µg/ml). I observed that some of the sera with a high %-binding in the Abalone-RAST did not bind to antigens bound to the ELISA plate or the PVDF membrane (immunoblotting), possibly as a result of changes in the conformational epitopes of the allergens on the solid phase.

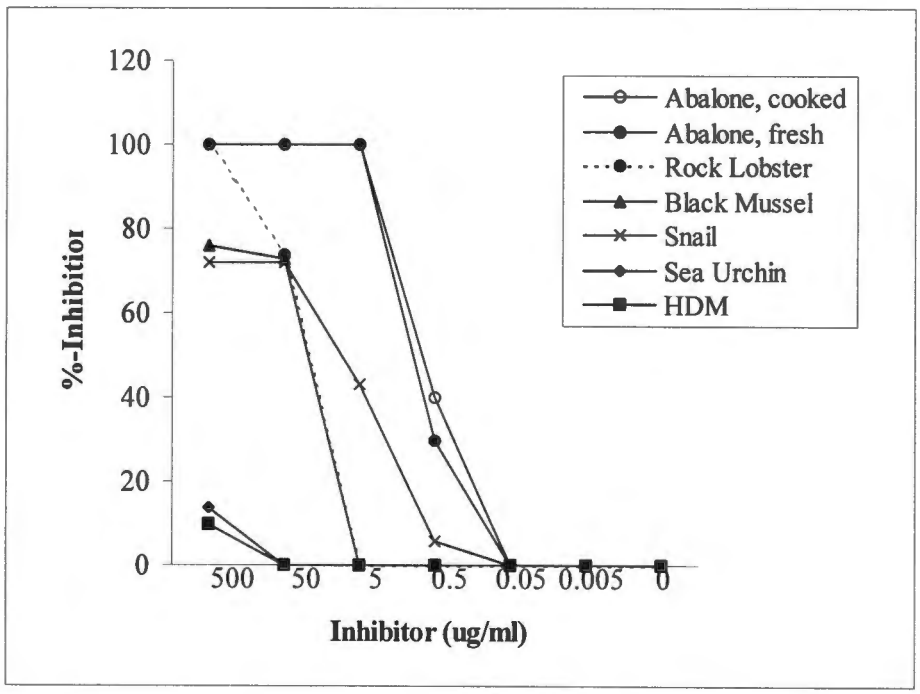


Figure 4.5: Allergenic activity of different extracts as compared by inhibition ELISA. Increasing concentrations of abalone (fresh and cooked), snail, rock lobster, black mussel, sea urchin, and house dust mite (HDM) extracts were tested for their ability to inhibit the abalone-ELISA.

3.5 Skin prick test

The skin prick test reactivity in abalone sensitive subjects to in-house mollusc extracts is presented in Table 4.V. Skin prick tests were only performed on subjects with less than 10%-binding in the Abalone-RAST in view of a possible risk of inducing a generalised reaction. All

of the eight abalone RAST +ve subjects tested were skin prick positive. In addition, six subjects with RAST-ve results responded positive to the abalone extract. Skin reactivity of subjects with general seafood sensitivity was tested for in chapter III (Table 3.II).

Table 4.V: Positive Skin Prick Test (SPT) reactivity extracts in abalone sensitive subjects to different in-house mollusc extracts and one commercial extract.

Extracts	Abalone sensitive subjects n=24	Comparative subjects n=10
Abalone	14	0
Oyster	9	1
Black Mussel	10	1
Blue Mussel (commercial)	6	1
White Mussel	5	1
Squid	7	1

The correlation of SPT results and RAST %-binding for abalone is illustrated in Figure 4.6. The mean Abalone-RAST value for SPT+ve subjects was 3.7% (SD ±3.7%) and for SPT-ve subjects was 0.5% (SD ±0.2%). These results clearly show that skin test negative individuals, in general, had little or no reactivity to the Abalone-RAST. In addition it was demonstrated that a negative RAST result did not exclude an immunological sensitivity to abalone as demonstrated on six SPT +ve but RAST -ve individuals.

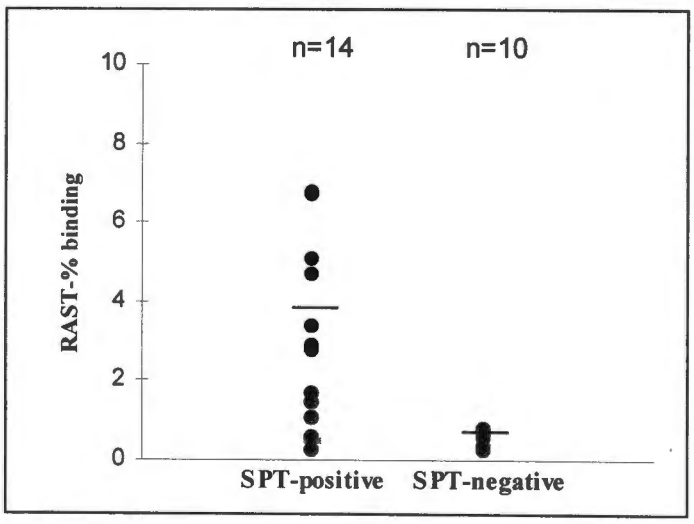


Figure 4.6: Correlation of IgE reactivity of abalone sensitive subjects as measured by in-house RAST in %-binding with Skin Prick Test (SPT) reactivity. The mean values and standard deviation (±S.D.) for the SPT-positive group are 3.7 ±3.7% and for the negative group 0.5 ±0.2%.

In the comparative group 1/10 subjects had a positive SPT to oyster, squid, black mussel, blue mussel and white mussel (Table 4.V). No positive reaction was obtained with the abalone extract in this control group.

In seven subjects I found that a protein concentration as low as 100 µg/ml abalone extract generated a wheal of about 50% the size induced by the extract used in our study (3000 µg/ml). Most of the RAST positive subjects (11/17=62%) had avoided contact with abalone for between 2-10 years but still demonstrated significant skin and RAST reactivity. Comparing the SPT and/or RAST activity with reported symptoms I found a strong association of respiratory symptoms with positive SPT or RASTs to abalone (92%) compared to subjects with negative results (60%).

3.6 *In-vitro* proliferation assay of PBML's

To compare the immunological response of lymphocytes of RAST positive with RAST negative subjects both with a convincing history of hypersensitivity to abalone, I exposed peripheral blood mononuclear cells (PBMLs) to extracts of abalone. The PBMLs of subject "Si" show a significant proliferative response to raw (fresh) and cooked abalone extract, as well as subject "Vi" who had negative RAST results for abalone (Fig. 4.7). The two abalone extracts did not induce a prominent response in the control subjects.

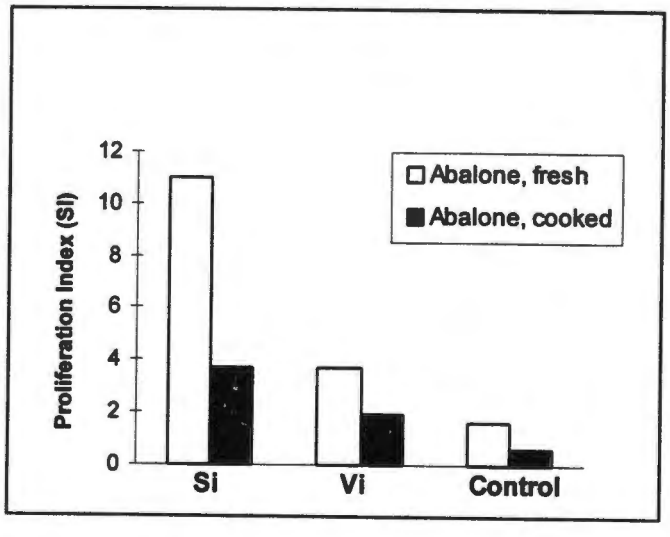


Figure 4.7: Proliferative response of PBMLs of subjects Si and Vi to fresh (raw) and cooked abalone. Subjects Si had a strong positive reaction to the abalone in-house RAST, whereas subject Vi demonstrated a negative IgE reactivity. The control subject demonstrated a very weak response. Results are given as ratios of stimulation index (SI): allergen-induced proliferation (dpm) per spontaneous proliferation of control (dpm).

3.7 SDS-gel electrophoresis and Western-blots

Protein extracts of different abalone species from South Africa, America and Asia were separated by SDS gel electrophoresis according to their molecular weights and the IgE binding components analysed by immunoblotting with different sera. The SDS gel electrophoresis of the different abalone species illustrated proteins with molecular weights ranging from 17 to over 200 kDa (Fig. 4.8). Also the abalone species are phylogenetically very close related, the protein banding patterns varied from species to species and made therefore a clear species identification possible.

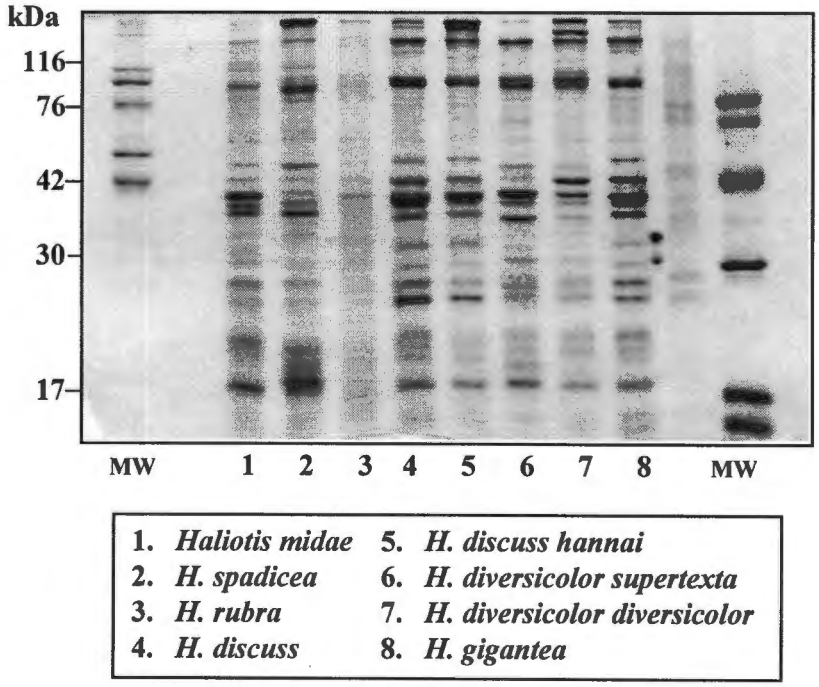
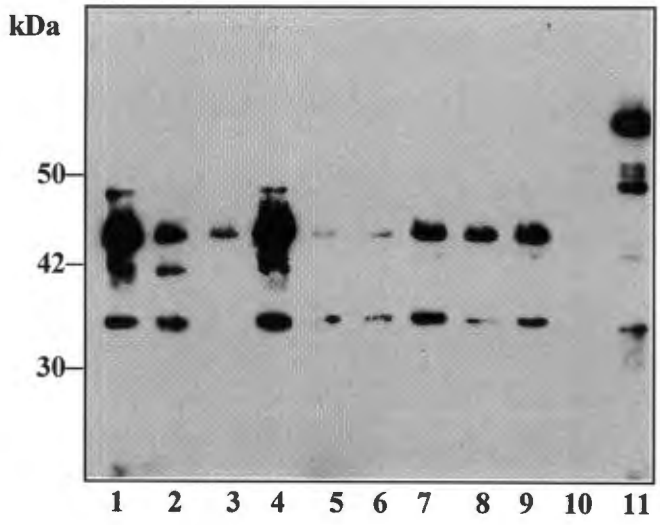


Figure 4. 8: SDS-gel electrophoresis of extracts of eight different abalone species from South Africa (*H. midae and H. spadicea*), Australia and Japan. The species names are labelled from 1 to 8. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

Western blotting of these abalone species and analysing the IgE binding reactivity with sera of abalone sensitive subjects demonstrated two major allergens in most of these molluscs (Fig. 4.9, Western-blot of one representative subject). The IgE binding pattern of the two South African abalone species (*H. midae* and *H. spadicea*) were almost identical to the Japanese species *H. discus discus*. However, these three species differed from the other six species which were again very similar among each other. There was a similar IgE binding pattern to snail (*Helix aspersa*) with additional bands in the higher molecular weight range but there was no binding to limpet (also a gastropod).

I investigated the stability of the identified allergens and boiled abalone tissue for 15 minutes at 100^0C. The proteins were, after extraction, separated by SDS-gel electrophoresis and demonstrated a striking loss of most of the protein bands except in the range of 35-45 kDa (Fig. 4.10 A). I also extracted tissue from dried abalone which was confiscated by the South African police force from local poachers which dry the abalone before illegal trade to the Far East.

These extracts demonstrated protein bands mainly in the range of 40-76 kDa. Two proteins with a molecular weight of about 45 kDa and 38 kDa were identified by Western blot to be the major allergens (Fig. 4.10 B). After cooking and drying the abalone, additional IgE binding proteins were detected with higher and lower molecular weights respectively. Western blots analysis illustrated the exceptional heat resistance and stability of the identified allergens in fresh, cooked and dried abalone.



1. *Haliotis midae*
2. *H. spadicea*
3. *H. rubra*
4. *H. discus discus*
5. *H. discus hannai*
6. *H. sieboldii*
7. *H. diversicolor supertexta*
8. *H. diversicolor diversicolor*
9. *H. gigantea*
10. Limpet
11. Snail

Figure 4.9: Western blot of IgE antibody reactivity of an abalone sensitive subject to different abalone and mollusc species. The species names are labelled from 1 to 11. The molecular weights are indicated on the left side in kilodalton (kDa).

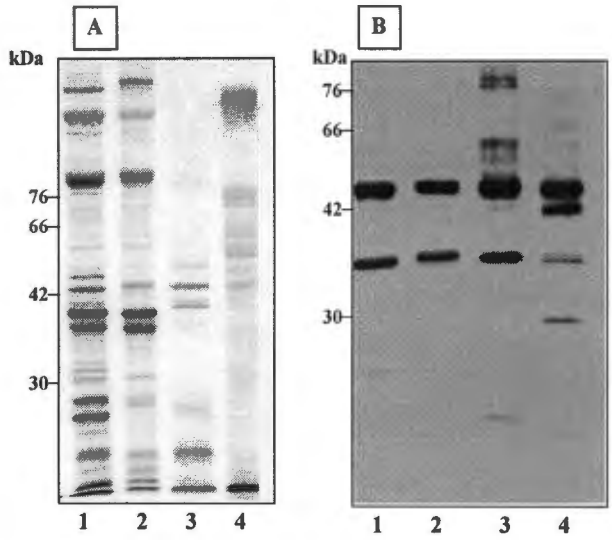


Figure 4.10: **A)** SDS-gel electrophoresis of four different abalone extracts: South African abalone *Haliotis midae* (1), Japanese abalone *Haliotis rubra* (2), cooked *H. midae* (3) and dried *H. midae*. **B)** Western blot of serum IgE reactivity of abalone sensitive subject to the identical extracts. The molecular weights are indicated on the left side in kilodalton (kDa).

SDS-gel electrophoresis of the abalone proteins under non-reducing conditions produced a complete loss of binding to the 38 kDa protein (Fig. 4.11) but not to the 45 kDa band.

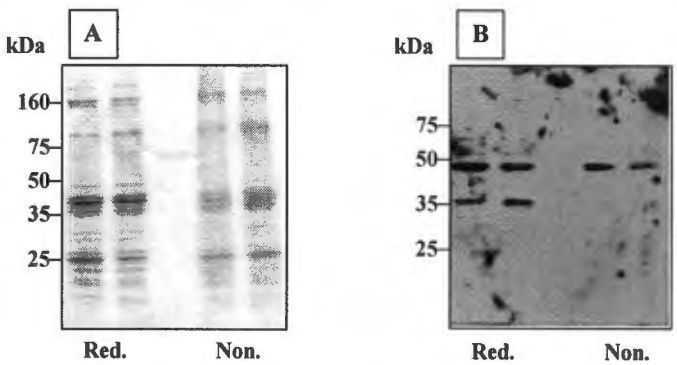


Figure 4.11: SDS-gel electrophoresis of reduced (Red.) and non-reduced (Non.) extracts of *H. midae* (abalone) (**A**; blotting membrane stained with Coomassie Blue). Western blot of IgE serum reactivity of a abalone sensitive subject to reduced and non-reduced extract of abalone (**B**). The molecular weights are indicated on the left side in kilodalton (kDa).

Sera from Abalone-RAST positive subjects (identified in chapter II) were screened for IgE binding to the South African abalone species *H. midae* (Fig. 4.12). I found that not all of these sera demonstrated IgE binding reactivity to the abalone allergens bound to the surface of the PVDF membrane. Electroblotting of the allergens onto the membrane possibly affected conformational changes of the antibody binding epitopes. Five subjects displayed IgE binding consistently to a distinct 45 kDa allergen, fulfilling all the required criteria for registration with the WHO International Union of Immunological Societies (IUIS):

1) A prevalence of IgE reactivity above 5% of all identified allergens 2) A minimum of 5 patients with IgE reactivity (regardless the number of patients included). The novel allergen from abalone was named Hal m 1 according to the recommended procedure of the IUIS.

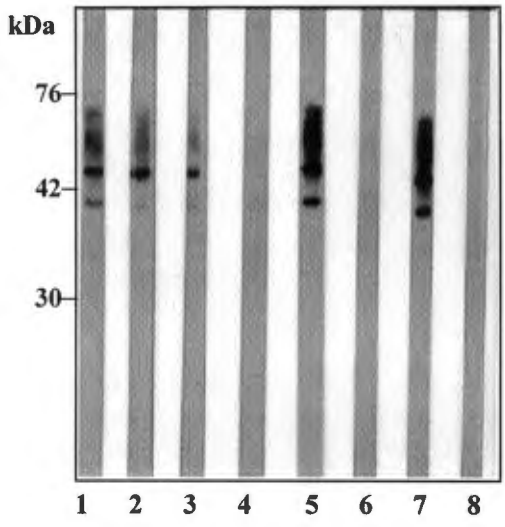


Figure 4.12: Western blot of serum IgE reactivity of abalone-sensitive subjects to extract of abalone (*H. midae*). Lanes 1 to 7 represent sera of Abalone-RAST+ve subjects. Lane 8 represence a negative control of normal sera. Molecular weights are indicated on the left in kilodalton (kDa).

Since I employed different molecular markers during the different stages of my thesis, I have obtained different molecular weights for the particular allergens of abalone. The molecular weight for the lower allergen varied from 36-38 kDa and the higher allergen between 42 and 49 kDa. Thus, in chapter III the allergen Hal m 1 was measured as 45 kDa, but for the registration with the IUIS it was reported as 49 kDa allergen. During the purification stages for the aminoacid analysis the same allergen was analysed with the new molecular markers as 42 kDa protein. Difficulties such as these arise because of the inconsistency observed with commercial molecular weight markers and have been observed by others.

I also investigated IgG binding of sensitised subjects to abalone proteins and demonstrated antibody binding only to few proteins with molecular weights of mainly 42 kDa and below 30 kDa in raw as well as cooked extracts (Fig. 4. 13). However, also control sera of non-sensitised subjects demonstrated identical binding patterns to the sensitised subjects. Thus IgG binding does not appear to have any diagnostic value, or correlation with clinical symptoms.

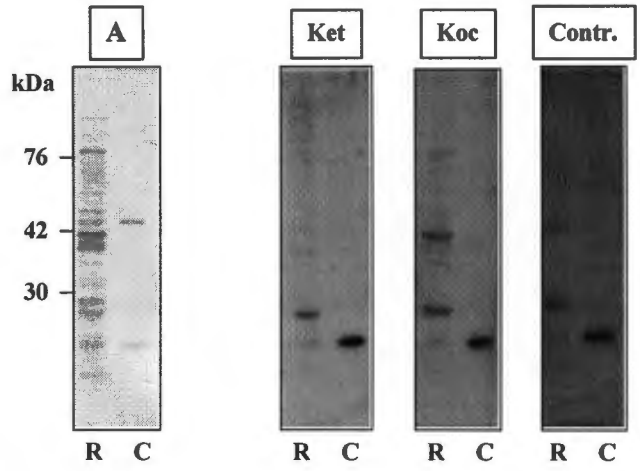


Figure 4.13: SDS-gel electrophoresis of extract of raw (R) and cooked (C) abalone extract (A; blotting membrane stained with Coomassie Blue) and IgG binding of two abalone sensitive subjects (Ket and Koc) and a control subject (Contr.).

Cross-reactivities

The identified 45 kDa allergen in abalone was also detected in other mollusc species. For some subjects (Ver) the 45 kDa allergen was found only in particular species such as periwinkle, Mediterranean Mussel (but not black - or white mussel) and in octopus (but not in squid) (chapter III; Fig. 3.9). In other subjects, such as Car (chapter III; Fig. 3.8), the 45 and 38 kDa allergens in abalone and periwinkle were only activated after cooking.

These two allergens were also found in some local crustacean species (chapter III; Fig. 3.12 and 3.13) such as prawn, langoustine and rock lobster, but not in shrimp. In addition each of the seven local fish species I have studied seemed to have one or more of the allergens with the same molecular weights (chapter III; Fig. 3.14).

Because of the concurrent RAST reactivity of some subjects to abalone, snail and house dust mite (HDM) and the reported cross-reactivity of allergens between snail and HDM I analysed the IgE binding profiles of five abalone sensitive subjects to these three extracts. Figure 4.14 showed that the major IgE binding protein bands of the HDM extract had an molecular weight of about 25 kDa. This particular band was found also in four of the five abalone extracts but not in the snail extract. In addition, the IgE binding 45 kDa protein band was also identified in the HDM extract by the serum of subject Fre.

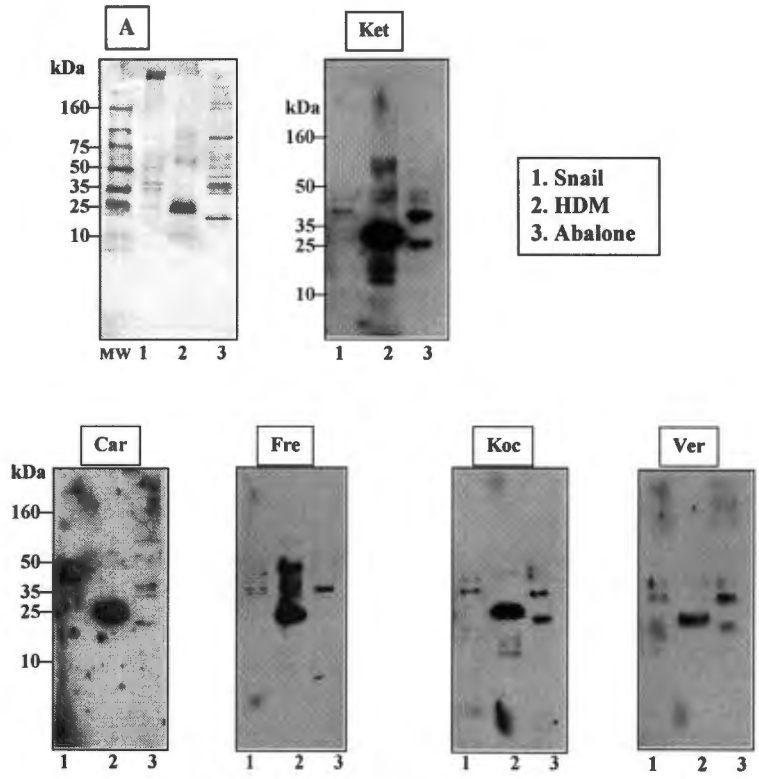


Figure 4.14: Western blot of serum IgE reactivity of abalone sensitive subjects to extracts of snail, house dust mite (HDM) and abalone. One representative blotting membrane stained with Coomassie Blue is displayed in **A**. The species names are indicated from 1 to 3. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

House dust mites belong to the group of arachnida and are phylogenetic close related to insects. Insects are also a common cause of inhalant allergies as was reported for cockroaches, chironomide larvae but also for grasshoppers (locusts). In South Africa cockroaches and locusts are frequently found and as for the cockroaches are also reported to be involved in inhalant allergies. A recent report of allergic symptoms by animal handlers in a local locust breeding facility at the University of Cape Town encouraged me to also analyse possible cross-reactivities with the allergens of this insect. One of the subjects (Ket) appeared to demonstrate binding to the 45 kDa allergen in abalone but also in *Locusta*. However, several additional allergens were identified, which will be followed up in a separate study.

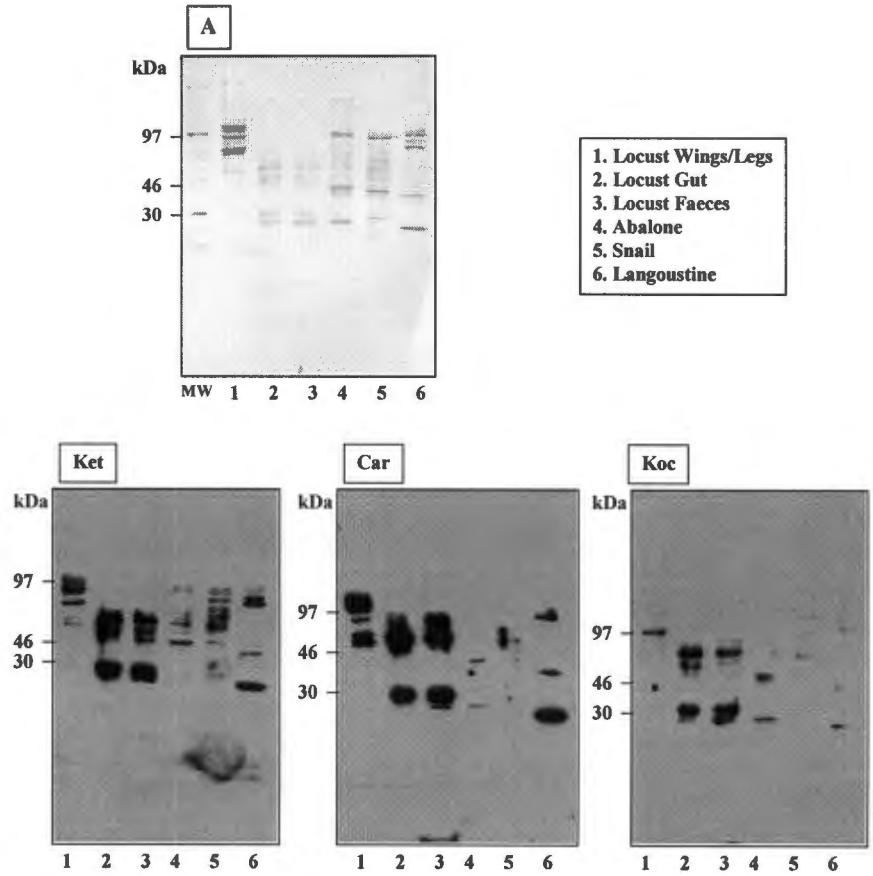


Figure 4.15: SDS-gel electrophoresis of different extracts of *Locusta migratoria* and seafood species (A). The extracts are labelled from 1 to 6. Serum IgE reactivity of three abalone sensitive subjects (Ket, Car, Koc) as demonstrated by Western blot. The molecular weights (MW) are indicated on the left side in kilodalton (kDa).

The 45 kDa abalone allergen seemed not to be present in any of the three locust extracts but a 25 kDa allergen, similar to the one found in the HDM extract, was also present in four of the five subjects. Very similar IgE binding patterns were attained with sera of three subjects with occupational allergy to locusts. Also all three abalone sensitive subjects demonstrate a strong IgE binding to the locust extracts, yet none of them reported allergic reactions to any insects.

I suspected that the abalone allergen with the molecular weight of about 38 kDa could be similar to allergens already described in various crustacean species. This allergen belongs to the protein family of tropomyosins and was recently generated as recombinant protein by Professor Leung and colleagues (Leung, et al., 1994). I received as a generous gift a sample of this recombinant protein from Prof. Leung and tested three subjects for IgE binding by Western blot (Fig. 4.16). All three subjects, but not the control subject, demonstrated strong binding to an about 60 kDa band and weaker band at about 116 kDa protein. It is to note that this allergen has a much higher molecular weight as recombinant fusion protein compared to the natural allergen Met e I which has 34 kDa and is found in a shrimp (*Metapenaeus ensis*).

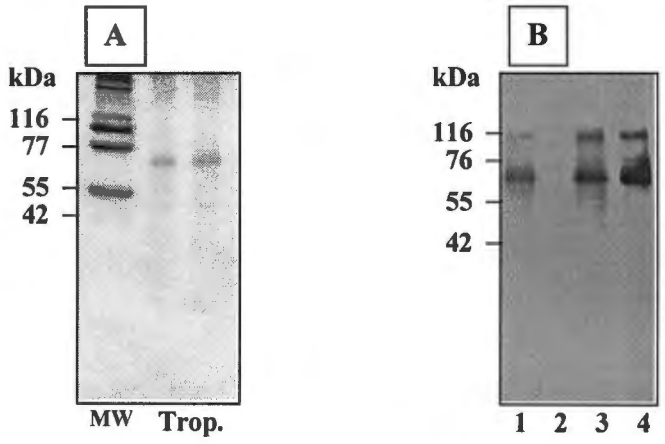


Figure 4.16: SDS-gel electrophoresis of recombinant tropomyosin from shrimp (*Metapenaeus ensis*) (**A**) and serum reactivity by Western blot of four abalone sensitive subjects labelled 1 to 4 (**B**). Subject number two is not sensitive to abalone. The molecular weights are indicated in kilodalton (kDa) on the left.

3.8 Allergen purification

To isolate and purify the two identified allergens of abalone I applied three different extraction methods (see 2.9) and analysed the proteins by Western blotting with a serum pool of five abalone sensitive subjects. The acid extraction of four isolated gel bands with the molecular weights of 38, 42, 45 and 49 kDa resulted surprisingly in a double band for all five fractions. The molecular weights of this two proteins were about 65 kDa (Fig. 4.17 A) and demonstrated IgE reactivity. The electroelution of the same proteins resulted also in four immunoreactive double bands with identical molecular weights to the acid extraction (Fig. 4.17 B). Both extraction methods must have had some denaturing or aggregation effect on the proteins, unfolding hidden epitopes, which in turn made specific IgE binding possible to all four extracted proteins. Because of lack of specific binding after extraction of the two previously identified allergens, I employed a third extraction method. High concentration of SDS in ammonium buffer seemed to elute all four protein fractions much better. The molecular weights of the extracted proteins were identical to the protein bands isolated from the whole abalone extract (Fig. 4.17 C). However, some additional bands with lower intensity were observed. Western blots of these proteins resulted in IgE reactivity to fractions 2 and 3 with molecular of 42 and 45 kDa respectively. It appeared that the proteins are well preserved by this extraction method. However, additional IgE binding epitopes were exposed on these proteins as demonstrated by binding to the protein fractions #2 and #3, whereas in the whole extract only binding to the 38 and 45 kDa allergens was observed.

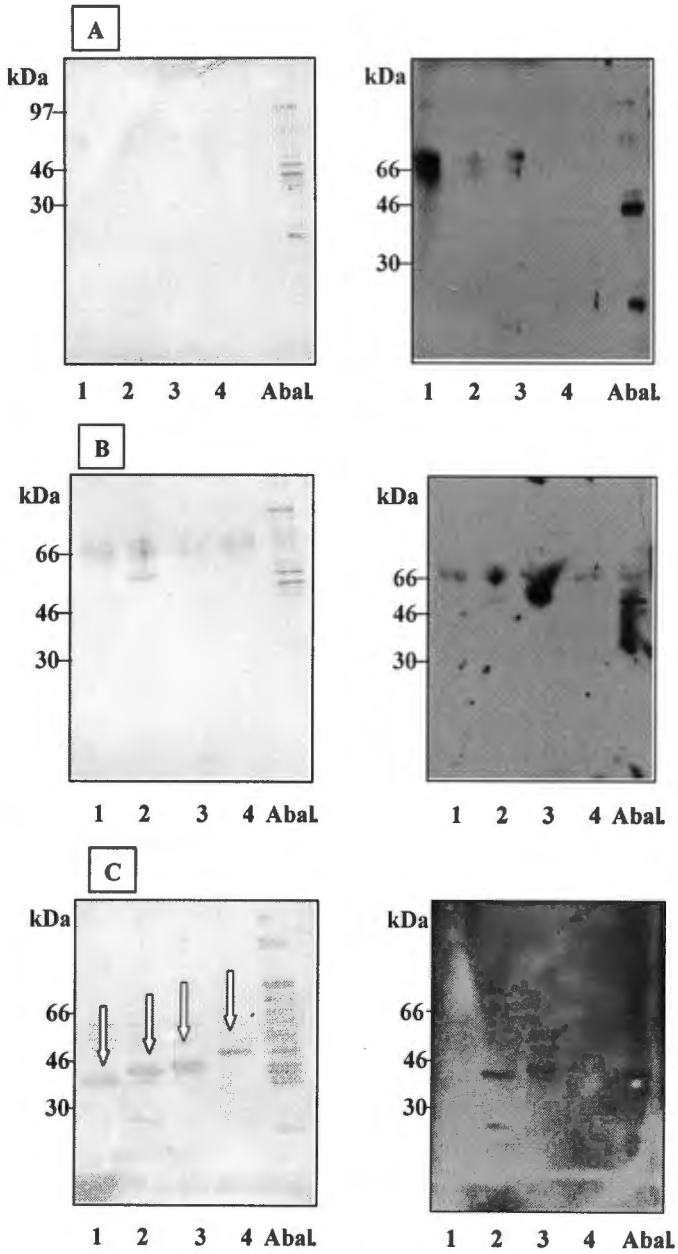


Figure 4.17: Purification of abalone allergens with trifluoroacetic acid (TFA) (**A**) and by using electroelution (**B**) and SDS extraction (**C**). Blotting membranes stained with Coomassie Blue are displayed on the left side and the Western blot IgE reactivity of abalone sensitive subjects on the right side. The protein fractions are labelled 1 to 5 and the abalone extract as Abal. The molecular weights are indicated on the left side in kilodalton (kDa).

I wanted to demonstrate not only the IgE binding activity but also the binding capacity of the allergens in the fluid phase, which does not have the risk of interference of potential IgE binding site with the solid phase. I therefore performed an RAST-inhibition assay with the eluted protein fractions on the Snail-RAST which demonstrated (as already analysed in chapter III) strong inhibition by whole abalone extracts. The same inhibition experiment with the four protein fractions after the SDS-extraction showed the highest inhibition for fraction #1 (64%) with decreasing inhibition to 24% for fraction #3.

Both inhibition experiments established that the 49 kDa protein produced the weakest reactivity and the 38 kDa protein the strongest and that all the extracted proteins must have some IgE binding epitopes exposed which are not present in proteins #2 and #4 in the untreated abalone extract.

3.9 Amino acid analysis

The amino acid compositions of the three antibody binding fractions were analysed using the OPA (orthophthalaldehyde) method (Klapper, 1982). All three proteins, including the 38 kDa and 45 kDa abalone allergens, were rich in glutamine, asparagine, alanine, leucine, lysine and serine. Table 4.VI compares the mole-% ratios of the three abalone proteins with two tropomyosin allergens of shrimps. The abalone proteins were similar to tropomyosin allergens from shrimp and chicken in respect to glutamine and asparagine content but had a higher content of serine residues.

Table 4.VI: Amino acid composition (in Mole-%) of abalone protein fractions (#1-#3) in comparison with tropomyosins from two shrimp species (Par f 1 and Met e 1 respectively) and chicken tropomyosin. Note: the data for Met e 1 and chicken were determined from deduced amino acid sequences from cDNA clones.

Amino acid	#1[1]	#2[1]	#3[1]	Par f 1[2]	Met e 1[3]	Chicken[4]
Alanine (A)	9	10	10	7	11	12
Cysteine (C)	0	0	0	0	0	0
Phenylalanine (F)	4	4	3	6	1	1
Glycine (G)	0	0	0	9	2	1
Histidine (H)	0	0	0	3	0	0
Isoleucine (I)	6	4	6	5	1	3
Lysine (K)	7	8	7	8	9	14
Leucine (L)	10	10	9	9	12	13
Methionine (M)	2	3	4	2	2	2
Asparagine (N)	13	12	12	11	13	12
Proline (P)	0	0	0	4	0	0
Glutamine (Q)	14	15	15	13	26	23
Arginine (R)	4	5	5	4	8	5
Serine (S)	15	13	13	5	6	6
Threonine (T)	6	6	7	5	3	3
Valine (V)	6	6	6	6	5	3
Tyrosine (Y)	4	4	3	3	1	2
Total (%)	100	100	100	100	100	100

[1] This study; [2] (Lin *et al.*, 1993); [3] (Leung *et al.*, 1994); [4] (Goodling *et al.*, 1987)

4. Discussion

4.1 Study group and Symptoms

Subjects who reported specifically allergic reactions after eating abalone were recruited from the previous study on immune responses to South African seafood species. These 38 subjects were analysed in more detail for their immune response to abalone and other seafood species.

In the abalone sensitive patients used for this analysis, next to gastrointestinal symptoms, (61%), asthma and wheezing were very frequent symptoms (42%). A recent study on 48 crustacea and mollusc sensitive subjects demonstrated asthma like symptoms in 38% of the study population and rhinitis in 29% (Castillo, et al., 1994). A different study on limpets, a closely related gastropod species to abalone, described six patients with severe bronchospasm 30 to 120 minutes after eating this mollusc species (Carrillo, et al., 1994).

The association of consumption of snails, also very closely related to abalone, and the development of asthma like symptoms has been reported in the eighties (Amoroso, et al., 1988). The data obtained in this study suggested that eating snail (*Euparipha pisana*), a common food in Mediterranean countries, could give serious allergic reaction such as asthma. Most of the subjects in this study were also sensitized to common allergens in the Mediterranean area including house dust mite (HDM). A similar association was found in a study from Spain on 10 subjects with food allergy to the snail (*Helix terrestre*) (de la Cuesta *et al.*, 1989). Eight of the ten subjects had only respiratory problems and six had no gastrointestinal or skin symptoms, which are usually seen in cases of food allergy.

In a recent study conducted by van Ree and colleagues (van Ree *et al.*, 1996b) it was demonstrated by RAST analysis that in a group of 28 patients with asthma after consumption of snail, that all had combined sensitisation to snail and mite.

A survey in Spain on general food allergy and reported symptoms (Oehling, et al., 1992) observed that 18.5% of the patients presented exclusively with respiratory symptoms. In addition this study found that foods such as snails and eggs have a strong predilection for the bronchial tree as the shock organ.

A uninvestigated aspect of abalone sensitivity is the relatively frequent occurrence of delayed onset of symptoms (up to 7 hours), reported in more than one third of the subjects. Furthermore, type I sensitivity could only be confirmed in one third of these subjects by positive SPT and/or RAST. Delayed reactions have been reported to cuttlefish and limpets but not with crustacean or fish (Morikawa, et al., 1990; Shibasaki, et al., 1989). Delayed symptoms are a frequent and

characteristic feature of abalone and mollusc hypersensitivity in my experience and may account for the lack of apparent sensitivity of SPT and RAST in these patients.

4.2 Specific IgE assessment and RAST-correlation

Testing the abalone sensitive subjects against 4 different mollusc species using commercial RASTs reflected both the specificity and co-reactivity of the studied abalone sensitive subjects. The closest related mollusc species available commercially is the Snail-RAST (*Helix aspersa*) which is also a gastropod. 'Abalone sensitivity' could have been detected in just over half of the abalone RAST+ve subjects by screening with the Snail-RAST only. Furthermore, almost 40% of the abalone sensitive subjects had neither a positive skin test- nor RAST reactivity in spite of a convincing Type I reaction on history. Mechanisms of their food hypersensitivity is thus not clear. The lack of RAST reactivity in some subjects with histories of abalone sensitivity may be partially related to the long timespan which had lapsed since they had last eaten abalone. However, two third of the RAST+ve subjects had avoided contact with abalone for between 2-10 years but still demonstrated significant skin and RAST reactivity.

Other reasons for the lack of immunologic responses in some of the patients included the fact that some individuals may have falsely indicated abalone as offending seafood or have reacted to other allergens or irritants ingested together with abalone. These includes food additives (Sampson and Metcalfe, 1992) or possibly contamination by algae toxins that may elicit adverse reactions by non-immunologic mechanism (Luckas, 1992; Sakamoto, et al., 1987) (Pitcher, et al., 1993). Recently toxicated abalone (*H. midae*) were found along the West Coast of South Africa, but also in abalone farms (Prof Pete Cook; personal communication), where the Red Tide algae *Alexandrium minutum* caused poisoning by PSP toxins (Paralytic Shellfish Poisoning).

Concurrent- and Cross-reactivities

Several subjects demonstrated co-reactivity to some or all mollusc species and also to species from the crustacea and fish group. Comparing the RAST ratios and analysing them by linear regression demonstrated a positive correlation between abalone and two of the four mollusc species (chapter II; table 2.II). As I expected the two closely related gastropd species, snail and abalone, demonstrated significant correlation, and in addition to squid (a cephalopod). Surprisingly, abalone was also correlated to three crustacean RASTs (crayfish, shrimp and crab), suggesting the presence of cross-reacting allergens in abalone and crustacea. However, it was unexpected to find not positive correlation between blue mussel and oyster, both belonging to the group of bivalves and are very closely related.

To assess possible crossreactions of abalone allergens with other mollusc and seafood species RAST inhibition assays were conducted on the different commercial seafood RASTs. As predicted from the RAST correlation analysis the abalone extract produced the strongest inhibition with Snail-RAST. Moreover, also the Squid-, Oyster- and Blue Mussel-RAST were inhibited by up to 90% (Fig. 4.3). However, these results varied for each individual: for example, one subject (Koc), did not demonstrate any inhibition of the Blue Mussel-RAST and very weak inhibition of the Oyster- and Squid-RAST.

Very surprisingly, the five abalone sensitive subjects with concurrent sensitivity to HDM (see chapter III; Table 3.III) demonstrated two distinct responses. The HDM extract demonstrated a strong inhibitory effect on most seafood RASTs for two subjects (Ver and Koc), wheras no inhibition was achieved for the other two subjects (Car and Fre). Interestingly, the inverse inhibition of HDM-RAST with the four seafood extracts showed the opposite results with the highest inhibition for Car and Fre and almost no inhibition for Ver and Koc. The apparent explanation for the latter two subjects could be the primary sensitisation to the aeroallergens from HDM and hence the existence of cross-reacting epitopes in seafood. For the two subjects, Car and Fre, it is reasonable to assume that they were primarily sensitised by seafood. The developed IgE antibodies cross-reacted to epitopes on the HDM-RAST, which can be inhibited, by epitopes in the different seafood extracts. Even so, the two distinct different responses of these four subjects indicated clearly that cross-reacting allergens or epitopes must exist between local seafood species such as abalone and HDM

A different approach in evaluating shared allergenic determinants among different species was illustrated using the ELISA-inhibition assay. However, these experiments demonstrated qualitative very similar inhibition results as the RAST-inhibition assays. This confirmed that there was no apparent influence of IgE binding fixing the antigens to a different matrix.
There is very little information in the literature on mollusc sensitivity and cross-reactivity to other species among the mollusc group. A case report demonstrated by RAST inhibition the existence of cross-reacting allergens in a Mediterranean abalone and limpet species (Morikawa, et al., 1990). Cross-reactivity between mollusc and other seafood species such as oyster have been analysed. Lehrer et al (Lehrer and McCants, 1987) showed a strong inhibition of the Oyster-RAST by different crustacean species. Also the RAST results of subjects with con-current sensitivity to oyster and crustaceans showed a strong correlation. However, the association between oyster sensitivity and other mollusc species was not analysed. A correlation

between oyster and crustacean RAST results was not found in my study, but rather between abalone and snail and several crustacean species.

In a different seafood group, the crustacea, species specificity could be demonstrated by RAST inhibition. In a group of 31 shrimp sensitive subjects, three subjects reacted only to one ore the other of two analysed shrimp species (Morgan, et al., 1989). These results support the hypothesis of species specific crustacean allergens specifically among some shrimps.

For fish sensitive individuals it was shown that among different fish species more allergens must exist than the major allergen Gad c 1 mostly described in the literature. RAST inhibition testing of 39 fish sensitive subjects with 17 different fish species demonstrated significant cross-reactivity among pollack, salmon, trout, and tuna; and between mackerel and anchovy (Helbling, et al., 1996). A recent study on eight fish sensitive subjects demonstrated inhibition of an in-house Cod-RAST with extracts of mackerel, herring, and plaice but not by shrimp (Hansen, et al., 1997). These results demonstrated that cross-reactivities among different fish species are very species specific. Yet, the clinical relevance of these in vitro results needs to be investigated further.

Clinical reports have suggested an unusual frequency in the number of patients with food allergy to snails who are also allergic to the house- dust mite (HDM). In a recent study conducted by van Ree and colleagues (van Ree, et al., 1996b) it was demonstrated by RAST analysis that in a group of 28 patients who developed asthma after consumption of snail, that all had combined sensitization to snail and mite. A few sera also had, in addition, specific IgE antibodies to mussels and shrimp. RAST inhibition studies showed that most IgE antibodies against snail were cross-reactive with house-dust mite. In contrast, the mite RAST was not significantly inhibited by snail, indicating that house dust mite was the sensitizing agent.

Already in the late 80's (Koshte *et al.*, 1989) a study conducted on cross-reactivity of arthropod and seafood allergens investigated the possibility that subjects with IgE antibodies to an inhalant insect allergen, such as caddis fly, cross-react to allergens in mussels, oysters, shrimps and crabs. RAST inhibition and immunoblot studies suggested that a cross-reacting caddis fly allergen could possibly trigger an allergic reaction during the first exposure to shellfish.
In my RAST inhibition study I also included house dust mite (HDM) extract to investigate the proposed correlation to this arthropod. The five subjects with concurrent immune response to all four mollusc species demonstrated two distinct responses. One group reacted exactly as van Ree

described in their study. However, three of the five subjects demonstrated no inhibition of Snail-RAST with HDM extract but a strong inhibition of HDM-RAST with snail and very weak with homologues mite extract. My study only involved five sensitised subjects compared with van Ree's study with 28 snail sensitive subjects (van Ree, et al., 1996b). Nevertheless, none of the subjects in van Ree's study showed such a distinct response as the three subjects in my study. In addition, in van Ree's study an about six times higher extract concentration was used for the inhibition experiment indicating that my approach was not only more sensitive but probably also more specific. However, the contrasting RAST-inhibition results indicate also that cross-reacting epitopes must exist in the snail and mite extract and that the primary sensitization must not necessarily be caused by the inhalant allergen of mites.

These results clearly demonstrated that the heterogeneous immune responses of sensitive subjects to the different analysed mollusc species is most probably produced by the various composition of allergens among the mollusc species. To answer these questions Western blot analyses of the different seafood extracts were conducted (see 4.6).

4.3 Skin prick test

The Skin Prick Tests (SPT) on abalone sensitive subjects demonstrated reactivity in all Abalone-RAST+v subjects. In addition six RAST-ve subjects had skin reactivity indicating that the SPT is more sensitive and also very specific since none of the subjects of the control group reacted to this in-house SPT. A comparison of SPT results with RAST reactivity demonstrated 3.7% binding for the SPT+ve group and only 0.5% binding in the SPT-ve group.

A similar study on subjects from the Mediterranean area with snail (*Euparipha pisana*) extract demonstrated positive skin reactivity in 61% of the study population (Amoroso, et al., 1988) while none of the control group reacted to the same extract. However, only 19% of the tested sera where RAST+ve using an in-house RAST with a mean value of 4.8% compared to the control group with 0.5%. These RAST values are very similar to my findings on abalone sensitive subjects verifying the specificity and sensitivity of the in-house SPT and RAST.
Furthermore, I found in my study a strong association of positive SPT or RAST results to abalone with respiratory symptoms as compared to subjects with negative results. Amoroso and colleagues (Amoroso, et al., 1988) found in their study also that eating snails could give serious allergic reactions such as asthma. A different study from Spain on subjects who developed allergic symptoms following ingestion of a different snail species (*Helix terrestre*) reported that 8/10 patients had pulmonary symptoms (de la Cuesta, et al., 1989). All subjects reacted skin

positive and in addition two subjects also to extract of limpet, a very close related mollusc species.

However, a very recent study on subjects with snail specific IgE did not demonstrate a positive skin response in all snail positive subjects (van Ree, et al., 1996a). Only 6/10 subjects responded positively. However, the investigated patients underwent a house-dust mite immunotherapy and this was accompanied by the induction of specific IgE against snail. It seems from this data that the cross-reacting allergens between house dust mite and snail do not necessarily provoke a positive skin response if the primary sensitisation is induced by inhalant allergens. This is in contrast to my study where I found a positive skin response in all Abalone-RAST+ve subjects where the route of sensitisation was via ingestion.

Other closely related mollusc species also produced bronchospasm after ingestion and were tested for skin reactivity. A study of six subjects with respiratory problems after ingestion of a Mediterranean limpet species were tested by SPT for reactivity of raw and cooked limpet (Carrillo, et al., 1994). The cooked limpet demonstrated a much stronger skin reactivity compared with the raw extract. In my study I did not include the cooked abalone for skin testing but the strong IgE binding activity of cooked abalone was demonstrated by RAST- and ELISA inhibition assays.

Comparing the SPT results with other seafood species in the literature illustrated that there are many studies conducted on skin sensitivity to various fish species. In 20 children with positive RAST to cod-fish SPT were performed with 17 different species (de Martino, et al., 1990). It could be shown that the fish eel extract caused the highest frequency of positive SPT (85%) and dogfish the least (10%). However, it was also demonstrated that skin reactivity was also present for fish species which where clinically tolerated by some of the subjects.

4.4 *In-vitro* proliferation assay of PBML's

I have demonstrated for one subject each with positive- or negative-RAST and skin results and convincing history to abalone that the lymphocytes display strong proliferative responses toward abalone antigens (Fig. 4.7). Interestingly also lymphocyts of subject Vi, which had negative RAST and SPT results, demonstrated proliferative response to abalone allergens. Subject Vi experienced delayed reactions after ingestion of abalone and the presentation of antigens to sensitised CD4+ T lymphocytes, producing a typical Type IV reaction is possible.

In addition, cooked abalone also stimulated the proliferation though the response was markedly reduced. Further studies need to be performed to assess whether proliferative assays are a reliable alternative to confirm food-hypersensitivity in RAST-ve subjects who report delayed symptoms to abalone.

4.5 SDS-gel electrophoresis, Western blots and Cross-reactivities

Protein extracts of abalone were separated by SDS-gel electrophoresis and the IgE binding proteins analysed using Western blotting techniques. The separation of the total protein extract of eight different abalone species demonstrated after staining species specific protein banding patterns even between very closely related subspecies. A similar approach of species differentiation by gel electrophoresis has been conducted successfully for some European fish species and some crayfish species (Powell, et al., 1995; Rehbein, 1995; Yman, 1993). However, to my knowledge there is no comparative analysis of any mollusc species published, as demonstrated for abalone in my thesis. Species identifications may also be conducted by analysing the specific DNA using PCR techniques as was recently accomplished for the two local abalone species (Sweijd, et al., 1998).

Western blotting of extracts of South African abalone (*Haliotis midae*) revealed two major thermo stabile allergens with an molecular weight of 38 and 45 kDa. Furthermore, these allergens were found in eight different *Haliotis* species from South Africa, Australia and Japan. In addition, these two allergens seemed to be present also in other mollusc species such as the different mussel and cephalopod species. However, additional IgE binding proteins were identified, also after cooking the tissue, which varied from subject to subject.

A study on a limpet from the Mediterranean (also a gastropod such as abalone), demonstrated activity only in the cooked extract (Carrillo, et al., 1991) (Carrillo, et al., 1994). RAST and Histamine assays were used to show activity, but no Western blotting technique to demonstrate IgE binding proteins. For the South African abalone I demonstrated by Western blot, RAST inhibition and Lymphocyte stimulation assay that both, the raw and cooked abalone contain active allergens.

The stability of the abalone allergens is comparable with a similar property of an allergen found in shrimp (Hoffman, et al., 1981) (Lehrer, et al., 1990). The shrimp allergens are released into the cooking water and contain their activity as was demonstrated by RAST inhibition assays. The major allergens of crustaceans, identified in different shrimp, lobster and crab species (Leung *et al.*, 1998a; Leung, et al., 1998b; Reese, et al., 1995; Shanti, et al., 1993), all belong to the family of tropomyosin proteins with molecular weights between 34 to 38 kDa. The tropomyosins are muscle proteins which function as actin-binding proteins in muscle and non-muscle tissues and are highly conserved during the process of evolution.

Leung and colleagues have recently described the cross-reaction between mollusc and crustaceans. It was demonstrated by Western blot that a 38 kDa protein cross-reacted in different crustacean and mollusc species including a *Haliotis* species from the USA (*H. diversicolor*) (Leung, et al., 1996). Unlike to my study, there was no additional IgE binding demonstrated to any other allergens in abalone by the nine subjects investigated. For the comparative study by Leung subjects with reported allergy to shrimp only were analysed. It may be that the primary sensitisation to a mollusc, as was demonstrated in my study, generates an immune response different from sensitisation to a crustacean. Therefore the IgE reactivity analysed by Western blot will demonstrate a different allergen pattern.

It is to expect that closely related seafood species may share similar allergenic epitopes, accounting for a degree of concurrent allergenicity. I tested therefore recombinant tropomyosin from shrimp (Leung, et al., 1994) for IgE binding of abalone sensitive subjects. IgE binding was demonstrated, however, this recombinant protein was expressed as fusion protein with about 60 kDa and was therefore not directly comparable with the natural allergen.

Only very recently the first allergen from a mollusc species has been identified and sequenced (Miyazawa, et al., 1996). This allergen has a molecular weight of 38 kDa and was identified as squid tropomyosin with a high homology to a snail species. Whether or not the 38 kDa allergen of abalone also belongs to the protein family of tropomyosins has to be confirmed using amino acid analysis, or better amino acid sequencing.

I have clearly shown in five abalone sensitive subjects that the 45 kDa abalone protein binds specific IgE is a major allergen. Furthermore, other IgE binding proteins were identified which varied individually. The identified 45 kDa allergen of *Haliotis midae* was identified as a novel mollusc allergen and designated as Hal m 1 according to the nomenclature of the IUSI. However,

as explained in 3.7, the new allergen was previously incorrectly reported as having a molecular weight of 49 kDa but this will be corrected as soon as information on the amino acid sequence is available.

Cross-reactivities to arthropods

The presence of cross-reacting allergens between snail and arthropods (mites, insects) has been demonstrated in several studies (De Maat-Bleeker, et al., 1995; Guilloux *et al.*, 1998). To investigate the existence of cross-reacting allergens between abalone and arthropod allergens, which contain potent aeroallergen, I compared the IgE binding profiles of abalone and snail with house dust mite (HDM) and locust (insect).

The major IgE binding protein of the HDM extract had a molecular weight of 25 kDa. Very surprisingly an IgE binding protein with about the same molecular weight was also found in four of the five analysed subjects and in addition also the 45 kDa protein in both extracts for subject Fre. Interestingly the same 25 kDa of HDM, in addition to several higher molecular allergens, seemed also to be present in extract of an indigenous grasshopper species (*Locusta migratoria*). This insect was reported in several studies to be the source of potent inhalant allergens which cause respiratory symptoms (Burge *et al.*, 1980; Tee *et al.*, 1988). Cross-reacting allergens in crustacean sensitised subjects and grasshoppers and cockroaches were also demonstrated by Leung et al (Leung, et al., 1996). However, only a 38 kDa allergen was found on Western blots whereas I detected the major allergens with 25, 30 and 62 kDa but not with 38 kDa. However, the clinical relevance of these findings is unknown, as none of these abalone sensitive subjects recalled any allergies to insects.
Similar allergen analysis by Vuitton et al (Vuitton *et al.*, 1998) demonstrated by Western blotting several cross-reacting allergens between snail and HDM.

However, allergen analysis cannot answer he question about the primary route of sensitisation. The RAST inhibition assays I conducted on Snail- and HDM-RASTs clearly demonstrated that, even so the IgE binding profiles are identical, the primary sensitisation of one group of subjects was probably HDM. This was already suggested by several research groups (van Ree, et al., 1996b) but the opposite, primary sensitisation as in this study by a seafood has not been demonstrated yet to my knowledge. These results indicated that both food- and aeroallergens of very different source may have the same or similar allergens or epitopes.

4.6 Allergen purification

The aminoacid analysis of the two identified abalone allergens, and protein # 2, demonstrated that all three proteins are rich in acidic acids such as glutamine and asparagine. In addition leucine and lysine were quite common. However, such an amino acid composition pattern is not remarkable, compared with those of serum albumins from different animals (ginazza, 80) or various tropomyosins from shrimps or chicken (Leung, et al., 1994; Lin, et al., 1993); Goodling, 1987 #312]. The complete amino acid sequences of more than 110 recorded allergens are known and extensively reviewed (Marsh and Freidhoff, 1992; Stewart and Thompson, 1996). They clearly indicate that, from a structural point of view, allergens are indistinguishable from conventional antigens. Furthermore, comparisons of primary amino-acid sequences of allergenic proteins or tertiary protein structure has not yielded any unique or typical pattern (Lehrer, et al., 1996). However, amino-acid similarities occur with a number of proteins in our environment. Most known food allergens have molecular weights between 10 and 70 kDa (Taylor and Lehrer, 1996) and are glycoproteins with acidic isoelectric points. A pre-request for any potential allergen is the ability to stimulate the immune system by containing at least two IgE antibody-reactive sites in order to trigger mediator release. Resistance of many food allergens to heat, such as the abalone allergens, suggests that conformational epitopes are not critical for IgE binding (Astwood, et al., 1996). However, our knowledge of the structure of food allergens is limited compared to the wealth of information available on inhalant allergens (Taylor and Lehrer, 1996). A comprehensive analysis and comparison of the abalone allergens with known allergens will only be possible by direct sequencing of peptides generated by chemical or enzymatic cleavage and detailed epitope mapping.

Further studies of the biochemical and immunological characterisation of the identified abalone allergens with in-house produced monoclonal antibodies and cross-reactivities to other mollusc and seafood species are presented in chapter V.

Summary and Conclusion

The questionnaire administered to abalone sensitive subjects revealed that asthma like symptoms and the delayed onset of symptoms were not uncommon in sensitised individuals. Delayed symptoms have previously only been reported for snail and a case report on abalone.

The results of my study indicated that hypersensitivity to abalone (*Haliotis midae*) can be confirmed by an in-house RAST and the sensitivity of testing improved, using an additional skin prick test with in-house extracts in RAST negative subjects. The in-house Abalone-RAST was positive correlated with the phylogenetic closely related Snail-RAST. Conversely, the commercial Snail-RAST did not identify 6 subjects with specific abalone IgE which were only detected using the novel in-house RAST for abalone confirming unique species-specific allergens. However, the presence of cross-reacting allergens was demonstrated by inhibition experiments of in-house and commercial RASTs with extracts of abalone and other indigenous seafood species.

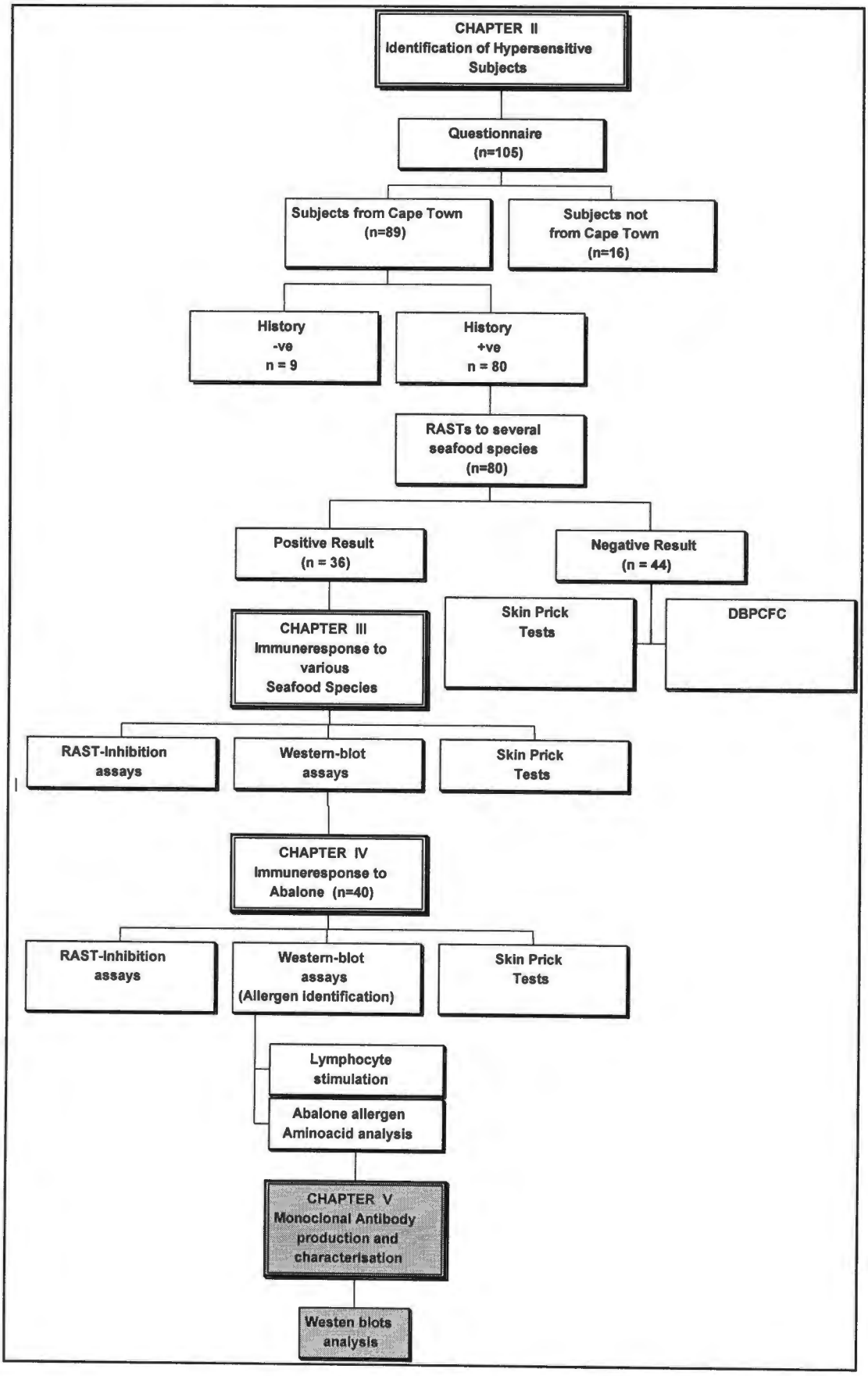
Very surprisingly, RAST-inhibition experiments of the five abalone sensitive subjects with concurrent sensitivity to HDM demonstrated two distinct types of responses. These results indicated clearly for the first time that cross-reacting allergens or epitopes must exist between abalone and the house dust mite (HDM) which is a common cause of inhalant allergies in South Africa. Western blot studies supported these findings with cross-reacting 45 kDa and 25 kDa allergens between abalone and two arthropod species, the HDM and an insect species (*Locusta migratoria*).

Western blotting of extracts of South African abalone (*Haliotis midae*) revealed two major allergens with an molecular weight of 38 and 45 kDa and remarkable thermal stability. These two allergens where also present in extracts of other indigenous mollusc species as well as in some local crustacean and fish. However, the immune response of sensitised individuals was very heterogeneous for each analysed individual, demonstrated by different binding patterns. The existence of different species specific proteins in even closely related mollusc species was demonstrated by SDS-gel electrophoresis.

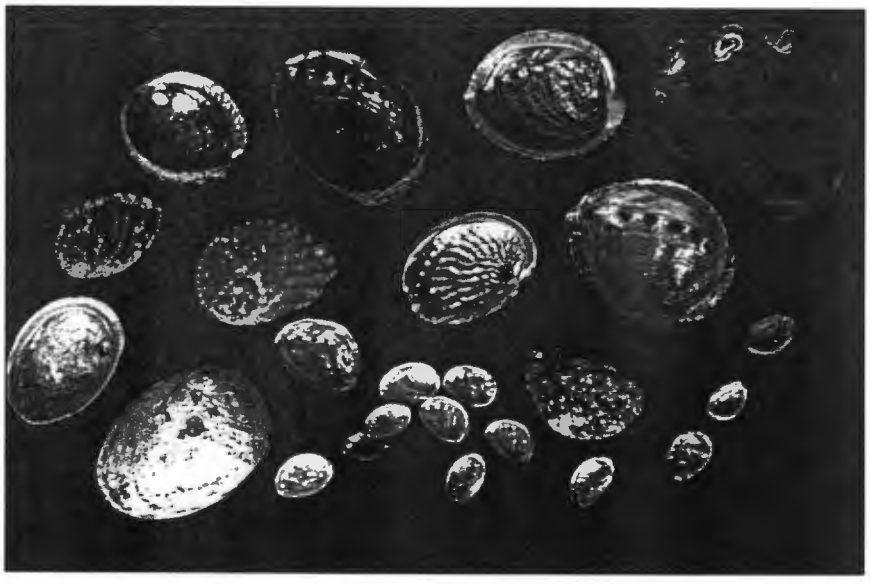
The 38 kDa allergen is believed to be abalone tropomyosin as was supported by specific IgE binding to recombinant tropomyosin of shrimp. The novel 45 kDa allergen of abalone (*H. midae*) was registered with the International Union of Immunological Societies (IUIS) and named Hal m1 according to the recommended procedure. Hal m1 is only the second identified allergen of a mollusc species after Tod p1 in 1996. Amino acid analysis of the two abalone allergens revealed a high contents of acidic residues. However, the amino acid composition pattern is not remarkably different from other non-allergenic proteins.

CHAPTER V

Generation and Characterisation of Monoclonal Antibodies generated to South African Abalone (*Haliotis midae*) allergens



Flowdiagram of sample selection, diagnosis and analysis of allergy to seafood by different *in vitro* and *in vivo* methods.



Picture of different abalone species from various parts of the world

1. Introduction

Several IgE binding sites have been identified in the extract of abalone using SDS-gel electrophoresis and Western blotting. Five of the abalone RAST positive subjects demonstrated binding to two major allergens with a molecular weight of about 38 and 45 kDa. The later unique 45 kDa heat-stable protein has been identified as a new seafood allergen and been designated as Hal m 1 according to the International Union of Immunological Societies allergen nomenclature regulation (Lopata *et al.*, 1997). Hal m 1 like bands were also detected in other mollusc species such as mussels, squid and octopus but also in some crustacean species such as shrimp, prawn and rock lobsters.

To be able to detect the same or similar abalone allergens specifically in other molluscs species and possibly also in other related seafood species, I generated monoclonal antibodies (MoAbs) using hybridoma technology. For this purpose three abalone proteins with molecular weights between 38 and 49 kDa were extracted after SDS-gel electrophoresis and used for the immunisation of mice. Monoclonal antibodies have the advantage that they are homogenous in immunoglobulin subclass, specificity and affinity and bind to the same epitope. The specificity of the produced antibodies was tested by ELISA and Western blot assay.

The generated monoclonal antibodies were used to distinguish *Haliotis midae* from different abalone species. The abalone *H. midae* is the only commercially exploited abalone of the six species that occur on the South African coast. However, the incidence of abalone poaching in South Africa has increased in recent years following the establishment of sophisticated syndicates who procure, process (dry and canned abalone) and illicitly export the abalone to the Far East. Because of forensic difficulties in identifying tissue of *Haliotis midae* and differentiating it from other abalone species I was asked by the South African Police Force if I could develop a robust analytical diagnostic method, to support identification by the Department of Zoology at the University of Cape Town.

The aim of this study was to develop monoclonal antibodies to abalone allergens and to study their specificity by immunoblot analysis of different mollusc species, and compared with representative species of the crustacea and fish group. Polyclonal antibodies were generated in rabbits to the same purified antigens from *H. midae* and to extract of *H. spadicea*. I also attempted to develop an immunological diagnostic test to distinguish abalone species from

different parts of the world, which could be used to identify the source of canned abalone which has been incorrectly labelled.

2. Material and Methods

2.1 Patients sera

Sera were collected from subjects with history of respiratory, dermatological or gastrointestinal symptoms occurring after ingestion of abalone (*Haliotis midae*) (see chapter IV for details). 17 of 38 subjects demonstrated elevated specific IgE antibodies to abalone extract (in-house RAST binding > 3%). Five of these subjects also illustrated IgE binding to several proteins in the abalone extract by Western blotting.

2.2 Purification of abalone allergens for immunisation

Four proteins (including the two identified allergens) of abalone extract were purified by preparative SDS-gel electrophoresis. The gels were soaked in KCl solution (0.02 M) for 15 minutes followed by a 5 minute rinse with distilled water which produced white bands. Because two proteins were closely located to the 38 and 45 kDa allergens, four protein band were cut out, squeezed through a syringe and left in extraction buffer (50 mM NH_4HCO_3; 0.1% SDS) overnight (o/n) at room temperature (RT). Subsequently the extracted proteins were analysed for purity by immunoblotting with patients serum or staining with Coomassie Blue. The proteins were subsequently numbered as fraction 1 to 4 with the approximate molecular weights of; #1= 38 kDa, #2= 42 kDa, #3= 45 kDa and #4= 49 kDa (see Fig. 5.9). Only fractions #1, #2 and #3 were used for monoclonal antibody production.

2.3 Production of polyclonal antibodies in rabbits

For the production of polyclonal antibody 50 μg of each of the four protein fraction (antigens) were used to immunise one New Zealand rabbit. In addition, as control sera polyclonal antibodies were produced to another South African abalone species (*H. spadicea*) by using 450 μg of whole protein extract. The two New Zealand rabbits received the extracted proteins in Complete Freund's Adjuvant (Difco) subcutaneously injected at several sites. Subsequent immunisations were performed two, four and eight weeks after the first immunisation with the same protein concentration in Incomplete Freund's Adjuvant.

2.4 Production of monoclonal antibodies (MoAbs)

BALB/c mice were immunised twice at day 0 and 17 with 50µg each with purified proteins #1, #2 and #3. Two weeks after the second immunisation, the sera were tested for antibody production using a direct ELISA. A final booster was given intraperitoneally three days before the fusion. The spleen cells of the immunised mice were fused with SP2 mouse myeloma cells using the PEG fusion technique (Harlow and Lane, 1988). The produced hybridomas were screened by ELISA and Western blot and cloned further by limiting dilution (see 2.5).

2.5 Screening for antibody reactivity

The fusion products were screened for ELISA reactivity against abalone extract. Hybridomas producing abalone specific antibodies were subcloned, by diluting the antibody producing clones to only one cell per well (limiting dilution), and further screened by ELISA to the purified protein fractions. During the coating of the antigens on the ELISA plates, possible preferential orientation of the antigen on the plastic surface might hide some epitopes of the antigens. For this reason the produced antibodies were also tested for Western blot reactivity to total abalone extract.

ELISA

Immunoplates (Polysorp, Nunc) were coated with extracted antigens at a concentration of 3 µg/ml o/n at 4°C. Plates were blocked with blocking-buffer (0.1% human albumin, Fraction V) for 30 minutes and incubated further with 100 µl supernatant of the different wells for two hours and tested for immuno reactivity. All steps were followed with five washes of PBS-Tween20 (PBS-T) and all incubation steps were for 30 minutes at RT. Rabbit anti mouse-biotin antibody (Dako; 1:5000) was added and after incubation followed by Horse radish peroxidase (HRP)-streptavidin (DAKO; 1:10000). Mouse anti-beta galactosidase MoAb and normal mouse serum were included as negative controls. Antibody binding was detected using 2,2-Azino-di (3-ethyl-benzthiazoline) sulphonic acid (ABTS) as substrate and the absorbance (OD) read at 405 nm after 30 minutes incubation.

Western blot

For screening of Western blots with in-house generated monoclonal antibodies to abalone allergens, the allergen bound membranes were blocked for 1 hours in 1% blocking reagent (Boehringer Mannheim), and incubated for two hours with condition medium (1/300 in PBS), followed by two washes with PBS-T (10 min each). Rabbit anti mouse-biotin antibody (Dako; 1:5000) was added for 30 min followed by incubation with HRP-streptavidin (DAKO; 1:30000).

After an overnight wash, the blots were developed using the chemiluminescence detection system (Boehringer Mannheim) according to the manufacturers instruction and exposed to Kodak X-ray film.

2.6 Cross-reactivity to proteins in other related seafood species

To verify immunogenic cross-reactivity of abalone derived MoAbs to other abalone and mollusc species, extracts were separated by SDS-gel electrophoresis and analysed using the Western blotting technique. Furthermore, other seafood species from the crustacea and fish group were tested for the presence of cross-reacting proteins or epitopes.

2.7 Isotyping of antibodies

Hybridoma culture medium containing monoclonal antibodies was used to determine the isotype of the antibodies by a standard ELISA procedure (Coligan, et al., 1994).

2.8 Purification of MoAb

The purification of the produced monoclonal antibodies from cell culture fluid was achieved by affinity chromatography. The separation was performed using protein G Sepharose 4 Fast Flow (Pharmacia LKB) according to the manufacturers manual. In brief: the pre-swollen Sepharose was re-suspended in starting buffer (20 mM phosphate buffer; pH 7.0) in a sintered glass filter and transferred to a plastic syringe (10ml) used as a column. After application of the culture medium and rinsing the column, the bound IgG antibodies were eluted with the elution buffer by lowering the pH (0.1 M glycine-HCl; pH 2.7). To avoid damage of the eluted antibodies, about 50 µl of 1 M Tris-HCL (pH 9.0) were added before collection. The antibody fraction was analysed for purity by SDS-gel electrophoresis under reducing and non-reducing (no DTT added) conditions.

2.9 Purification of abalone allergens

To identify MoAb binding to proteins from abalone, the isolated MoAbs were utilised for affinity chromatography. The purified MoAb 1.4 was coated onto paramagnetic polystyrene beads, tosylactivated Dynabeads M-450 (Dynal, Oslo, Norway). The coating of the beads with antibodies and the elution of the antigen were performed according to the manufacturers manual. The concentration of the isolated proteins were however to low for amino acid analysis.

3. Results

3.1 MoAb directed to protein #1

ELISA with total abalone extract indicated that the first fusion resulted in 12 positive wells out of a total of 72 wells (3x 24 well plates). A well was considered positive with an OD reading of more than 1.0. Furthermore, the positive wells were tested by ELISA for specificity to the different purified protein fractions #1, #2, #3 and #4 respectively. All the supernatants of this 12 positive wells demonstrated also binding on Western blots of total abalone extract. However, all possessed reactivity to several proteins over a broad molecular weight range from about 20-80 kDa.

After expanding the positive wells in 60 mm dishes and re-testing on ELISA for the different protein fractions it was noticed that 3 clones were unstable and lost their reactivity (MoAbs 1.2, 1.7 and 1.8). I decided to expand clone 1.4 on a 96-well plate, which resulted in 35 wells containing one or more cell clones. Of this 35 wells only one was positive in ELISA (OD reading of 1.097). This well contained a one cell clone and could therefor be expanded in 2x 60 mm dishes (1A, 1B). This single cell clone was tested again after 6 days for cross-reactivity on ELISA (see Table I).

Table 5.I: Reactivity of expanded single cell clone 1.4 against protein #1, #2, #3 and #4 as determined by ELISA (OD at 405 nm).

Sample	1A	1B
Protein #1	2.225	2.099
Protein #2	0.151	0.174
Protein #3	0.078	0.078
Protein #4	0.092	0.086

This test for cross-reactivity of clone 1.4 by ELISA demonstrated the specificity of this antibody to protein #1. Furthermore, this antibody was tested for immuno reactivity by Western blots on different abalone and mollusc species as well as other seafood species (see section 3.5). Establishing the isotype of this produced MoAb demonstrated that this antibody was of the IgG1 type.

3.2 MoAb directed to protein #2

A second fusion resulted in 9 positive wells of 72 with an OD reading of over 1.0 and three weaker wells with OD readings below 1.0. After further expansion of these 9 positive wells, ELISA established cross-reactivity to the other abalone proteins #1, #3 and #4.

All the supernatants of this 12 positive wells demonstrated also binding on Western blots of total abalone extract. However, all possessed reactivity to several proteins with molecular weights close to the 42 kDa protein #2. Sample wells 2.5 and 2.12 demonstrated binding only to protein #2. I decided to expanded clones 2.12 and also 2.11, because of cross-reaction to #1 of the later, on a 96-well plate, which resulted in 32 wells containing one or more cell clones. Of these 35 wells only one each were positive in the ELISA assay. These wells contained a one cell clone and could therefore be expanded in 2x 60 mm dishes. These single well clones were growing very slowly and the supernatants were frozen at -80^{0}C for further characterisation. The isotypes of this MoAb anti-protein #2 was determined to be also of the type IgG1.

3.3 MoAb directed to protein #3

The third fusion was not as successful as the first two, since there was only one well that had an OD higher than 1.0. There were 4 other wells with OD reading between 0.4-0.8. After further expansion of these 9 wells cross-reactivity was established to the other abalone proteins #1, #2 and #4 by ELISA. All the supernatants of this 5 positive wells demonstrated also binding on Western blots of total abalone extract. However, samples 3.1, 3.3 and 3.4 demonstrated in addition cross-reactivity to other proteins.

I decided to clone the well 3.2 but the cloning to a single clone resulted in no positive clones. A repeat of this experiment with two 96-well plates was also not successful. The original clone 3.2 was grown in a 60 mm dish and tested on ELISA for reactivity. Unfortunately the negative results confirmed that this clone was unstable and did not produce antibodies.

3.4 Polyclonal antibodies produced to abalone

For future ELISA studies on abalone allergens, polyclonal antibodies were produced to a mixture of the purified proteins #1, #2 and #3 from *H. midae*. In addition, one rabbit was immunised with whole protein extract of another South African abalone species, *H. spadicea*. The serum antibody titer was determined by ELISA to whole protein extract of the respective abalone species.

A serum dilution of 1:5,000 was sufficient to give an OD reading of about 1.5.

Testing the sera for antibody binding by Western blot demonstrated a qualitative very different binding profile for the two sera (Fig. 5.1, B). The rabbit sera to the three extracted *H. midae* proteins demonstrated as expected binding in the 40 kDa range. However, in the two South African species binding to several proteins was observed, whereas in the other seven abalone species analysed only one major band of about 42 kDa appeared. The limpet extract showed no binding whatsoever and the snail only to a very high molecular weight protein.

The binding profile of the antisera developed to a whole extract of *H. spadicea* (Fig. 5.1, A) was quite different. There were two major proteins recognised with molecular weights of about 90 kDa and 120 kDa. However, this serum did not demonstrate binding to the limpet extract but appeared to bind to two proteins with similar molecular weights as the rabbit anti *H. midae* serum.

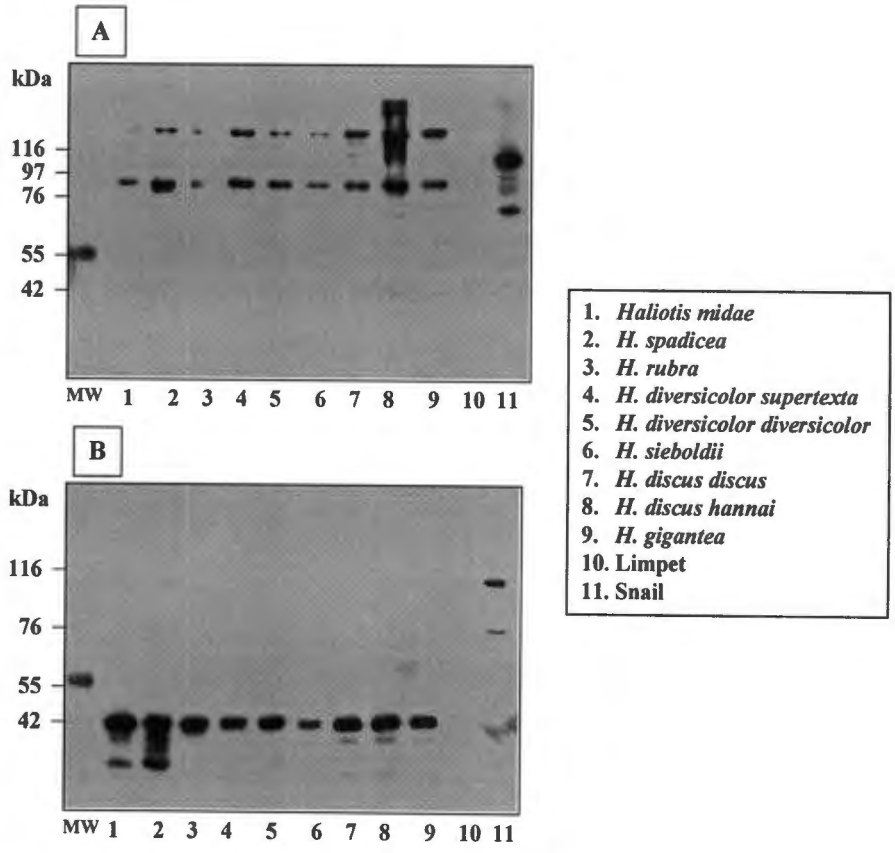


Figure 5.1: Western blot of rabbit IgG antibody binding to different abalone and other mollusc species. **A)** Rabbit serum raised against total protein extract of *Haliotis spadicea* and **B)** serum raised against three purified proteins from *H. midae* (both South African abalone species).

3.5 Cross-reactivity of selected monoclonal antibodies to proteins in abalone and other related seafood species

To assess possible cross-reactions of the generated monoclonal antibodies to proteins (or their epitopes) in other abalone species and related seafood, antibody binding was analysed by Western blot studies. Figure 5.2 shows that the three analysed MoAbs bound to proteins with similar molecular weights in the ten different abalone species from South Africa, Australia and Japan. MoAb 1.4 recognised predominately the same protein of about 42 kDa in all analysed species. In addition binding to a 35 kDa protein was observed in three species (*H. spadicea*, *H. rubra* and *H. diversicolor diversicolor*). MoAb 2.11 demonstrated binding to a protein with a MW of about 42 kDa and MoAb 2.12 to a 45 kDa protein. MoAb 2.11 showed additional binding to a 35 kDa protein in some species. However, MoAb 2.11 did not show any binding whatsoever to four of the ten species analysed (*H. discus discus, H. discus hannai*, *H. silboldii* and *H. gigantea*). A similar lack of protein recognition was observed for MoAb 2.12 for abalone species *H. spadicea* and *H. diversicolor supertexta.*

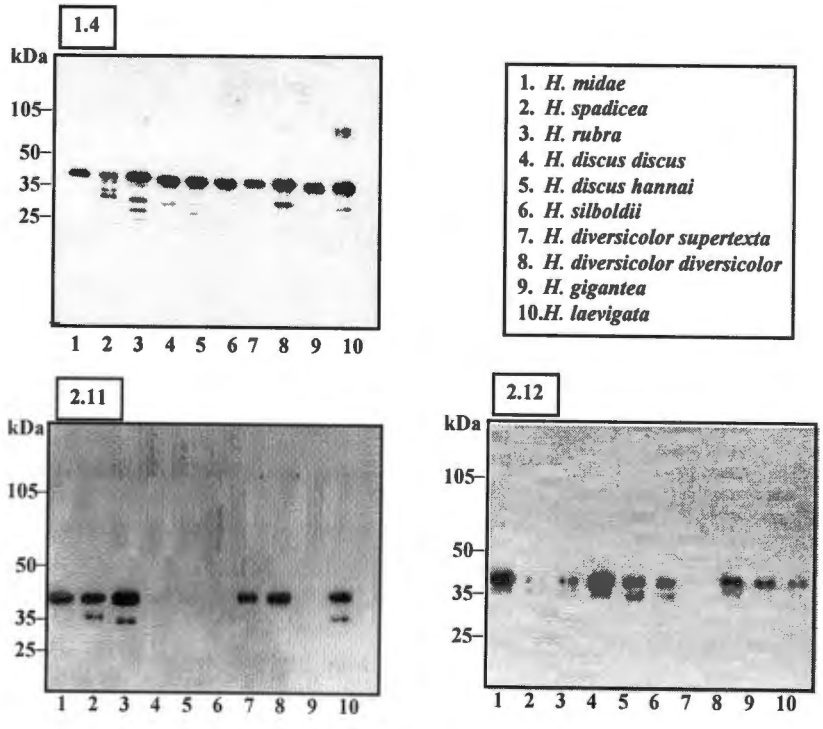


Figure 5.2: Western blot of antibody reactivity of monoclonal antibodies 1.4, 2.11 and 2.12 to different abalone species. The species names are labelled from 1 to 10, while the species 1 and 2 are from South Africa. The molecular weights are indicated on the left side in kilodalton (kDa).

The produced antibodies demonstrated also a very different binding profile from each other when binding to closely related mollusc species was observed (Fig. 5.3). MoAb 2.11 showed binding only to proteins in abalone and black- and white mussel and a very weak signal to periwinkle but no binding to snail, limpet, and squid. In contrast MoAb 2.12 recognised the same

protein of about 45 kDa in all analysed mollusc species with additional bands in abalone, black- and white mussel.

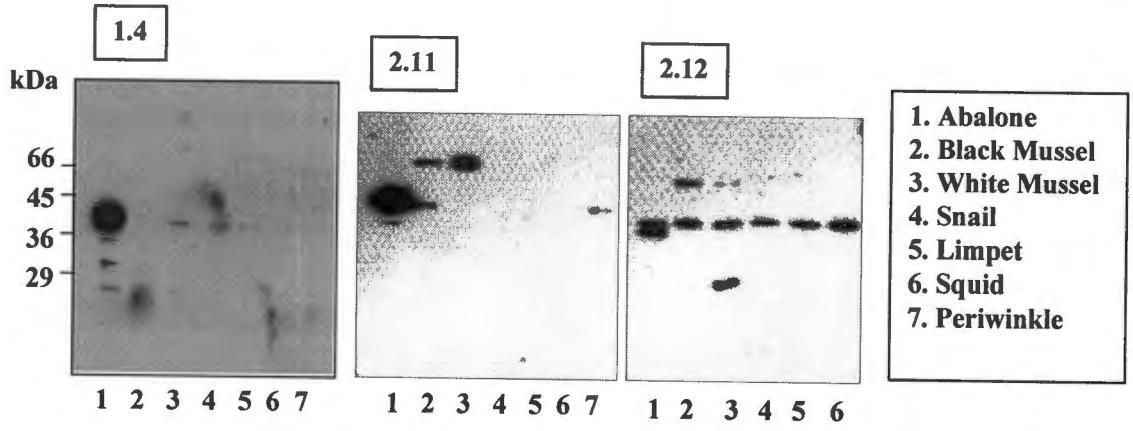


Figure 5.3: Western blot of antibody reactivity of monoclonal antibodies 1.4, 2.11 and 2.12 to different mollusc species. The species names are labelled from 1 to 7. The molecular weights are indicated on the left side in kilodalton (kDa).

To investigate the stability of the detected cross-reacting proteins and/or their epitopes, tissue of the different seafood species was cooked before protein extraction (see chapter III, 2.2 for details). Figure 5.4 demonstrated that MoAb 1.4 binds only to the heat-treated alikreukel extract and the raw abalone (lane 9). MoAb 2.11 and 2.12 recognised a major protein in all mollusc species, which was corresponding to the 45 kDa protein. However, MoAb 1.4 and 2.11 seemed to lose antibody reactivity to heat treated abalone (*H. midae*).

Binding to seafood species from the crustacea and fish group was also analysed. All of the MoAbs seemed to demonstrate no binding to cooked crustacea except to East Coast rock lobster for MoAb 2.11 (Fig. 5.5; lane 6). Analysing binding to different raw crustacean species showed that only the indigenous South Coast rock lobster (but not from the East- or West Coast) demonstrated a 35 kDa band which bound MoAbs 1.4 and 2.11.

The produced MoAbs 1.4 and 2.12 demonstrated also moderate binding to indigenous cooked and raw fish species respectively (Fig. 5.6). The molecular weights of these low MW proteins were about 25 kDa. However, MoAb 2.12 lost the antibody reactivity to cooked fish, quite in contrast to its reactivity to cooked mollusc. MoAb 2.11 did not demonstrate any binding to fish proteins.

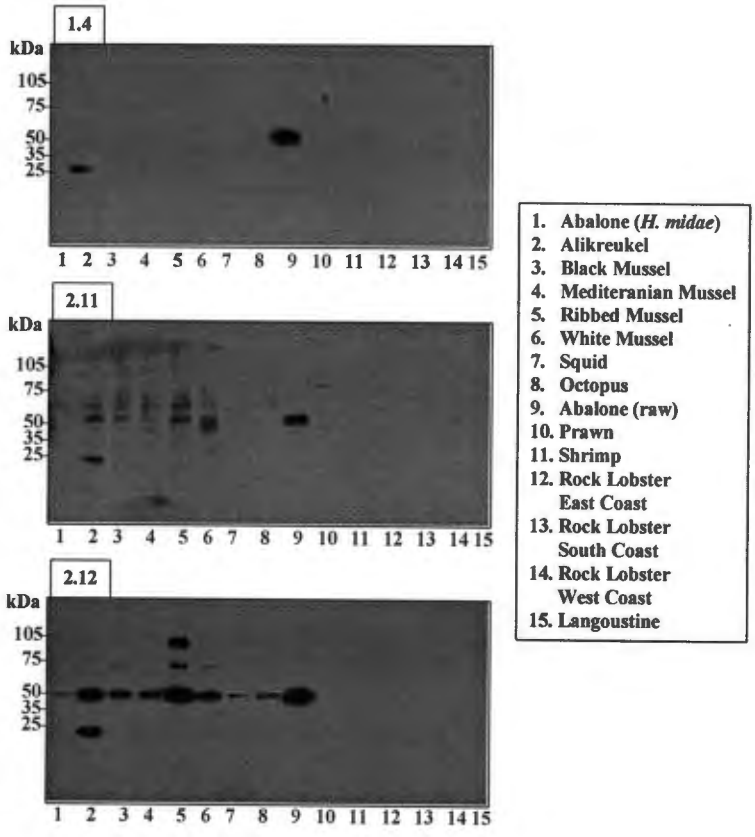


Figure 5.4: Western blot of antibody reactivity of monoclonal antibodies 1.4, 2.11 and 2.12 to cooked mollusc and crustacean species. The species names of the cooked specimens are labelled from 1 to 15, except lane number nine demonstrates raw abalone. The molecular weights are indicated on the left side in kilodalton (kDa).

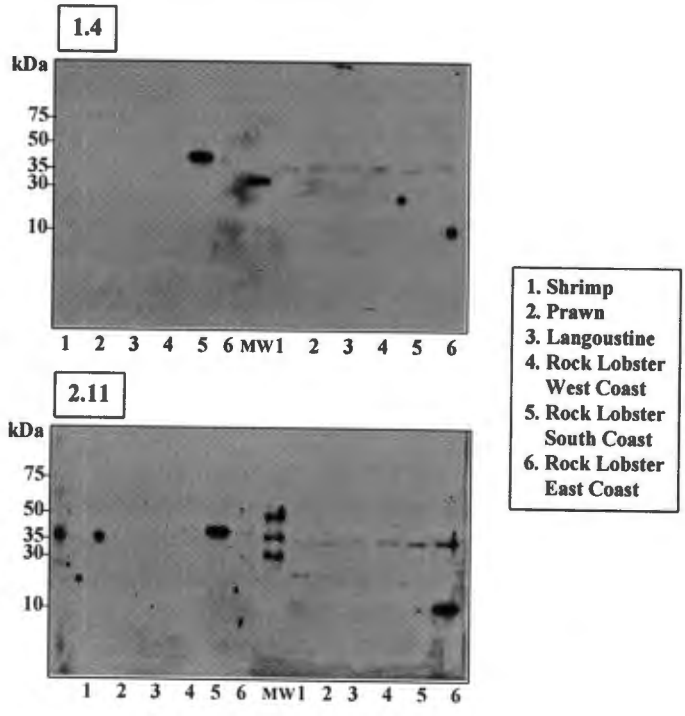


Figure 5.5: Western blot of antibody reactivity of monoclonal antibodies 1.4 and 2.11 to different crustacean species. The species names are labelled from 1 to 6. The left side of the blots is raw (fresh) extracts and the right side represents cooked specimens. The molecular weights are indicated on the left side in kilodalton (kDa).

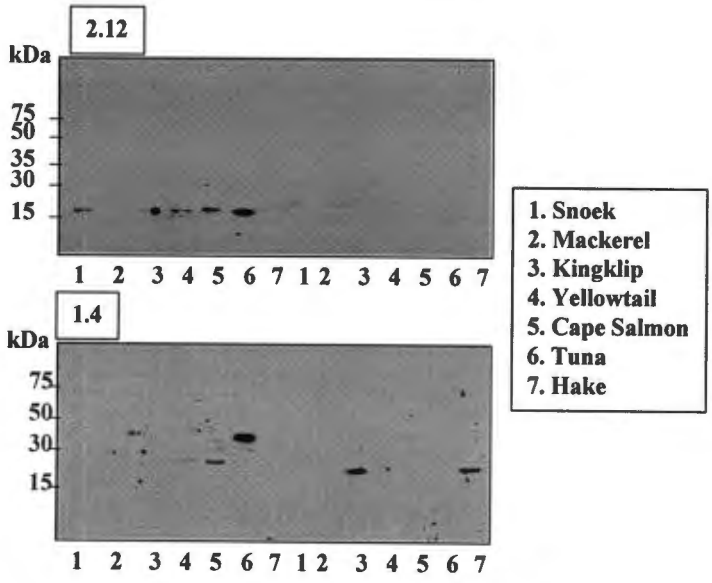


Figure 5.6: Western blot of antibody reactivity of monoclonal antibodies 2.12 and 1.4 to different fish species. The species names are labelled from 1 to 7. The extracts on the left side are raw (fresh) extracts and the right side represents cooked fish specimens. The molecular weights are indicated on the left side in kilodalton (kDa).

The antibody binding profiles of the different MoAbs to different mollusc, crustacean and fish species are summarised in Table 5.II.

In addition to show cross-reactive binding to other arthropod species I analysed the binding ability to extracts of house dust mites. As explained and demonstrated in chapter IV (3.7), the 45 kDa allergen was recognised by abalone sensitive subjects in abalone and mite extracts. However, Figure 5.7 demonstrated that the MoAbs did not bind to any proteins in the house dust mite extract.

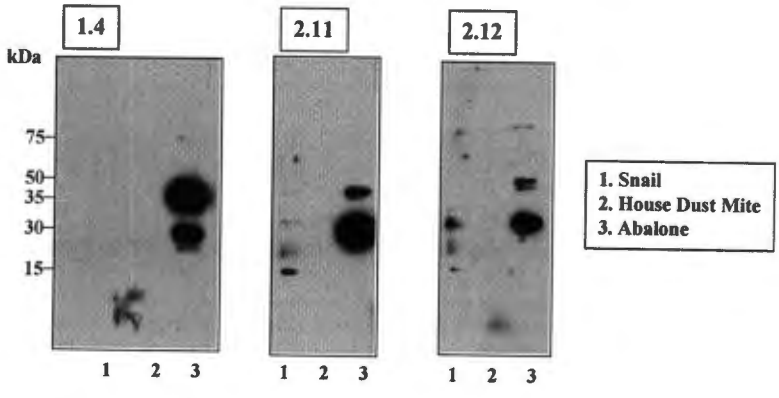


Figure 5.7: Western blot of antibody reactivity of monoclonal antibodies 1.4, 2.11 and 2.12 to extracts of snail, house dust mite and abalone. The species names are labelled from 1 to 3 and the molecular weights are indicated on the left side in kilodalton (kDa).

Table 5.II: Monoclonal antibody reactivity to different molluscs and also to crustacea and fish species on Western blot.
Note: + = binding to raw extracts; - = no binding to raw extracts; ( ) = binding to cooked species.

MoAb	Iso-type	MW (kDa)	Abalone (*H. midae*)	Peri-winkle	Black Mussel	White Mussel	Snail	Limpet	Squid	Rock Lobster	Tuna	Hake
1.4		42	+ (-)									
	IgG1	38	+ (+)									
		35								+ (-)	+ (-)	
		25	- (-)	- (-)	- (-)	+ (-)	- (-)	- (-)	- (-)		- (-)	- (+)
2.11		66			+ (-)	+ (-)						
	IgG1	42	+ (-)		+ (+)	- (+)						
		35								+ (+)		
2.12		66			+ (-)	+ (+)			- (+)			
		45	+ (+)	+ (+)	+ (+)	+ (+)	+	+	+ (+)			
	IgG1	42	+ (-)									
		35				+						
		25									+ (-)	- (-)

3.6 MoAb purification and allergen extraction

The specificity of the produced MoAb and antigen binding was analysed by affinity chromatography. MoAb 1.4 was purified using protein G Sepharose and analysed by SDS-gel electrophoresis under reducing conditions. The molecular weights of the generated antibody fragments had as expected molecular weights (MWs) of 50 kDa and 25 kDa (results not shown) and confirmed the purity of the isolated antibody. The antibody was bound onto paramagnetic Dynabeads (according to manufacturer instructions) and extracts of raw or cooked abalone were added. The proteins of raw and cooked abalone which where specifically bound by MoAb 1.4 were eluted, separated by electrophoresis and analysed for IgE binding activity on Western blot (Fig. 5. 8). The isolated antigen of raw abalone had a MW of about 42 kDa (lane 1) whereas the cooked extract demonstrated additional bands with MWs of 39, 38 and 35 kDa. The same proteins separated under non-reducing (no DTT added) conditions showed additional band with MWs of lower that 10 and higher than 100 kDa for the cooked extract. However, the protein of the raw extract demonstrated IgE binding only to the over 100 kDa band.

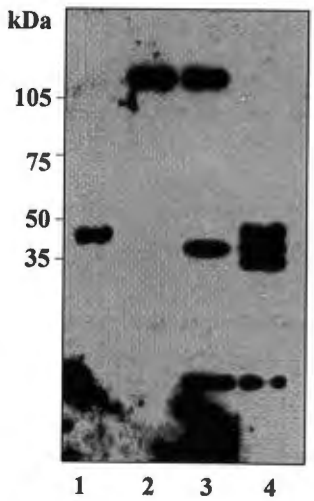


Fig. 5.8: Western blot reactivity of monoclonal 1.4 to purified abalone proteins by affinity chromatography. Lanes 2 and 3 were run under non-reducing conditions (without DTT). Proteins in lane 1 and 2 are from raw abalone and lanes 3 and 4 from cooked abalone.

Furthermore, MoAb binding was analysed to the four extracted abalone proteins by the SDS-extraction method (see chapter IV; 3.8). MoAb 1.4 bound as expected to protein #1 (38 kDa) but in addition to #2 with 42 kDa and several lower molecular weight proteins (Fig. 5.9). MoAb 2.11 demonstrated binding to the 42 kDa protein and in addition to several lower molecular weight proteins similar to MoAb 1.4. MoAb 2.12 bound only to the 45 kDa protein which was already observed for the extracts of other mollusc species (see also Fig. 5.4).

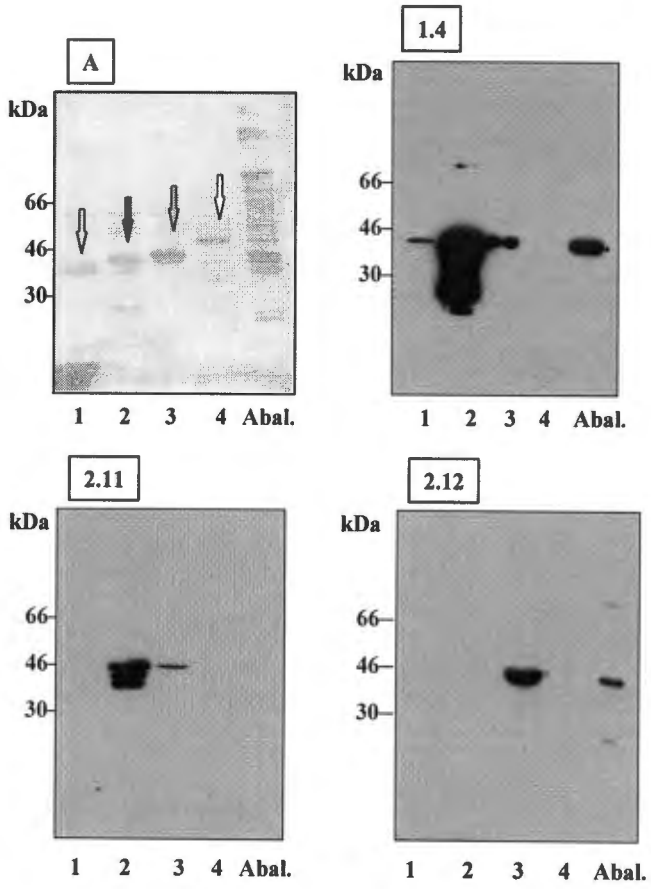


Figure 5.9: Western blot reactivity of monoclonal antibodies 1.4, 2.11 and 2.12 to purified abalone proteins by the SDS-extraction method (see chapter IV; 3.8). The blotting membrane is stained with Coomassie Blue (**A**). The protein fractions are labelled from 1 to 4 and the molecular weights are indicated on the left side in kilodalton (kDa).

4. Discussion

I demonstrated in chapter IV that five of the abalone RAST positive subjects demonstrated binding to two major allergens with a molecular weight of about 38 and 45 kDa. However, twelve Abalone-RAST+ve subjects did not demonstrate IgE binding to abalone allergens on the immunoblot, possibly because of conformational changes of the proteins during transfer onto the membrane. In order to identify cross-reacting allergenic proteins or identical epitopes on proteins from other seafood species, I have generated specific antibodies. To ensure not only homogeneity in immunoglobulin subclass, but also a high degree of specificity, monoclonal antibodies were produced in mice.

Furthermore, the generated monoclonal antibodies were also used to distinguish *Haliotis midae* from other abalone species. The need for this technique has resulted from increased abalone poaching, the effects of which are beginning to affect the legitimate abalone industry in South Africa, which employs several hundred people and also provides a significant source of foreign currency. Several alleged poachers have been acquitted in cases where the state has been unable to prove that the confiscated shellfish is in fact the local abalone, *Haliotis midae.* For this reason a method was required that would unequivocally identify fresh or processed abalone to species level, and which would conform to the legal requirements for forensic purposes.

As a result of the problem of abalone poaching from the shores of the Cape Coast, South Africa, I was approached by the Department of Zoology (UCT) and the South African Police to develop a fast and accurate immunological technique to distinguish different abalone species from each other. In this study I used Western blot analysis with monoclonal antibodies which I generated to abalone allergens, to identify and differentiate the different abalone species. In addition, the correct identification of seafood species may also be important from the clinical point of view. Because of the incorrect identification of the offending seafood species by common names and the widespread problem of commercial substitution, patients may in fact not ingesting what they think. These was demonstrated for several fish species such as salmon (Carrera, et al., 1998), sole (Cespedes, et al., 1998) and red snapper (Quintero, et al., 1998) but also for shrimp species (An, et al., 1990).

The antibody reactivity of MoAb 2.11 and 2.12 demonstrated that even very closely related subspecies can be clearly differentiated such as *H. discus discus* from *H. discus hannai and H. diversicolor supertexta* from *H. diversicolor diversicolor*. These results demonstrated explicitly that the two monoclonal antibodies, developed against the same purified protein from *H. midae*, recognised different epitopes on the same protein. This showed that at the species level, a relatively high degree of interspecific polymorphism is apparent and therefore useful distinguishing characters are readily available from proteins (Powell, et al., 1995). Recently a PCR technique has been developed for the forensic identification of two South African abalone species, *Haliotis midae* and *H. spadicea* (Sweijd, et al., 1998). However, while protein based detection and differentiation methods are generally regarded as less robust than DNA based methods, they nevertheless have several benefits. They are inexpensive, give rapid results and lend themselves to the development of portable non-laboratory based kits, which can be used for field work in unsophisticated areas (Hsieh, et al., 1997; Huang, et al., 1995; Sotelo, et al., 1993). Adulteration of seafood products using immunological techniques have been developed for sailfish species (Shepard and Hartmann, 1996) as well as for the identification of salmon from related species (Carrera, et al., 1997).

The fact that the three MoAbs bound to several proteins in the analysed abalone and mollusc species suggested that several proteins had similar or identical epitopes. These epitopes were very heat stable for MoAb 2.12 but reduced binding was observed for MoAbs 2.11 and 1.4. Different epitopes on the same protein seemed to have diverse stability to denaturation. Furthermore, purification of abalone allergens by affinity chromatography with MoAb 1.4 demonstrated several protein bands in the cooked extract below 42 kDa, whereas from the raw extract only the 42 kDa protein demonstrated antibody binding. In addition, MoAb bound to the purified proteins after SDS-extraction demonstrated binding not only to the 38 kDa protein, but additional binding to a protein with 42 kDa.

As described above, the cooking process as well as the SDS treatment could have exposed similar or identical epitopes on other proteins. This was also demonstrated for IgE binding of abalone sensitive subjects to cooked abalone (see chapter III; 3.4). Similar results were reported by Reese et al. (Reese, et al., 1995) after chemical cleavage and enzymatic digestion of Pen a 1, the major shrimp allergen from the brown shrimp. Most of the cleavage products, with MWs ranging from 1.5 to 20 kDa, yielded IgE and MoAb binding peptides which were similar but not identical. It appeared form this study that this allergen Pen a 1 (a tropomyosin protein) from shrimp, may have several similar epitopes. The same research group demonstrated on peptide

digests that the IgE binding epitopes are restricted to certain parts of the allergen as the reactivity pattern varied by subject and were not reproduced by the MoAbs (Reese *et al.*, 1996). Furthermore, comparison with mammalian tropomyosin suggested that these epitopes appear not to be located in the highly homologous parts of the tropomyosin molecule.

In addition, one can not exclude the existence of isoallergens in abalone, which have been extensively studied for several aeroallergens (Larsen, 1995). Isoallergens are identical with respect to molecular size, but when analysed more closely, they may differ at up to 25 % of the amino acid residues. This means that the difference between isoallergens may be comparable to the difference between allergens from different species. In a study with MoAbs generated to a 39 kDa allergen from the Taiwan shrimp (Par f 1) it was demonstrated that one antibody cross-reacted to a similar protein in crab (Lin, et al., 1993). This confirmed that the same epitopes can be found on some proteins causing cross-reactivities of antibodies. In addition it was shown that six isoforms existed for Par f 1 which had no marked difference in their amino acid composition.

In addition to the MoAbs, I generated polyclonal antibodies in rabbits to a mixture of the three *H. midae* antigens as well as whole protein extract of *H. spadicea*. The immure response of the rabbits to the antigens was compared. The produced antisera can in addition be used for further development of ELISA assays to detect and measure the concentrations of abalone antigens. The polyclonal antisera to whole protein extract of *H. spadicea* were produced as control sera to the anti *H. midae* sera. Interestingly, the antisera produced to the whole protein extract of the other abalone species, *H. spadicea*, demonstrated binding to mainly two proteins with molecular weights of about 100 kDa. This is very surprising as electrophoretic separation of whole abalone extracts demonstrated similar protein patterns for both species with MW ranging from 15 kDa to over 200 kDa (Fig. 4.8) and one would expect to generate antibodies to a broader protein range. The IgE response of hypersensitive subjects (see Fig. 4.9 and 4.12) to abalone is directed mainly to proteins in the MW range of 35-50 kDa. The dissimilar immune response of rabbits could be explained by the different route of immunisation; ingestion opposed to subcutaneous injection. The particular higher MW proteins could be more resistant to breakdown after injection into the rabbits causing them to be more available for an immune response. However, the proteins could also have more allergenic epitopes, which stimulated a stronger immune response than the extracted proteins used for antibody production.

The rabbit injected with the mixture of the three purified proteins from *H. midae* responded mainly to a 42 kDa antigen. I expected to observe a similar response of the immune system to all

three proteins, however the 38 kDa and 45 kDa proteins did not generate any IgE antibodies. One explanation could be the extraction with high concentrations of SDS which might have altered or destroyed antigenic epitopes. The strong immune response to the 42 kDa protein was probably enhanced by the bound SDS during the extraction procedure. SDS bound to proteins gives not only a net negative charge to the molecule but in addition unfolds coiled or globular protein structures exposing more potential epitopes. Nevertheless, an individual different response of the rabbits can not be excluded.

Western blot analysis of the MoAbs to different fish species demonstrated significant binding only by MoAb 2.12. Five of seven analysed fish species had antibody reactivity to a protein of about 25 kDa. However, the epitope seemed also to be heat sensitive, demonstrated by the loss of binding after cooking. The sensitivity of allergenic epitopes was also demonstrated for allergens in codfish. MoAb studies with anti-parvalbumin, which is the major fish allergen (Gad c 1), demonstrated binding to several high molecular weight proteins (Dory, et al., 1998). It was assumed that these proteins were aggregates of the lower molecular weight allergen, greatly influenced by storage conditions of the fish.

Western blot analysis of different indigenous crustacean species demonstrated that MoAb 1.4 and 2.11 bound to one protein only with a MW of about 35 kDa. This protein was only found in the South Coast rock lobster but not in the other rock lobster or crustacean species. It seemed that this particular antibody binding epitope could only be found in one of the six analysed crustacean species. Whether or not this protein is a representative member of the tropomyosin family has to be investigated further by amino acid analysis. A positive confirmation would propose that the same epitope could be found on a abalone and a crustacean protein.

In my study I analysed the MoAbs also for antibody reactivity to other arthropod antigens, in addition to the various crustacean species. In the literature, a monoclonal antibody to the house dust mite *Dermatophagoides pteronyssinus* (HDM) was described, that cross-reacted with an IgE-binding antigen present in insects, crustacea (e.g. shrimp) and other invertebrates (Witteman *et al.*, 1994). It was shown that this MoAb recognised tropomyosin as the cross-reactive allergen in shrimp allergic patients. However, the fact that my MoAbs to abalone did not recognise epitopes on HDM allergens does not exclude the existence of cross-reacting proteins. IgE binding studies in chapter IV demonstrated that for some subjects (Fig. 4.15; Ket) a cross-reacting 45 kDa protein existed and other proteins of lower MW.

A comparative study of mollusc with crustacean allergens revealed cross-reactivity between major squid and shrimp allergens (Miyazawa, et al., 1996). Specific monoclonal antibodies to shrimp tropomyosin identified a 38 kDa protein which was the major allergen of the squid, *Todarodes pacificus*, and is believed to be squid muscle protein tropomyosin. Furthermore, a comparative studies with recombinant crustacean allergens gave immunological evidence that tropomyosin is a cross-reactive allergen among crustacean and mollusc (Leung, et al., 1996). A 38 kd protein was identified as tropomyosin and was shown to share immunodominant epitopes among all species of crustaceans and molluscs tested by specific absorption studies. However, additional IgE binding proteins were identified in several analysed mollusc species. Nevertheless, to my knowledge, no study on a monoclonal antibody generated to mollusc allergens has been published yet and the cross-reactivity with other seafood species has not previously been analysed.

Summary and Conclusion

Three monoclonal antibodies (MoAbs) were generated to two abalone (*H. midae*) allergens with 38 kDa and 45 kDa. Two proteins, with 42 kDa and 45 kDa, were recognised by the MoAbs in most of the ten abalone species analysed. Lack of binding to some of the close related species suggested that the MoAbs bind to different epitopes on the same protein. Species identification by Western blotting of close related subspecies is possible using these monoclonal antibodies. Hybridomas generated to the 45 kDa allergen Hal m 1 (see chapter IV) appeared to be unstable. Nevertheless, the MoAbs produced to other abalone proteins cross-reacted to this allergen.

Western blot analysis of different mollusc species revealed cross-reacting epitopes for MoAb 2.12 on 45 kDa proteins which were found to be very heat stable. IgE binding studies in chapter III and IV to different mollusc extracts demonstrated that a 45 kDa protein is a major allergen in abalone. Furthermore, a 35 kDa protein was identified in several abalone species and in addition to one crustacean species. Serum IgE of abalone sensitive subjects demonstrated (chapter IV) binding to a 38 kDa protein. Known allergens in these molecular weight ranges between 34 kDa and 39 kDa have been described in many crustacean species and in squid (also a gstropod) and belong most probably to the protein family of tropomyosins. Recently, a 38 kDa allergen of a squid species has been identified as tropomyosin suggesting that the allergen from abalone (also a gastropod) could also belong to the same family allergen.

The question if the MoAbs generated to abalone in this study recognise the same proteins and/or epitopes on crustacean and fish allergens can only be answered with studies on protein digests of the abalone allergens. Antibody binding studies on protein digests and subsequent peptide sequencing will enable one to compare these proteins with known sequences in a protein database. From the Western blot studies on the abalone allergens it can be concluded that MoAb 1.4 can be used to characterise the 38 kDa allergen of abalone and compare this protein with the tropomyosin allergens identified in different shrimp species. Furthermore, MoAb 2.12 demonstrated strong binding to the 45 kDa allergen not only in abalone but in addition in other mollusc species and can therefore be used for epitope mapping.

SUMMARY AND FUTURE CONSIDERATION

World-wide, seafood represents one of the most important groups of allergens in the induction of food allergy. While some information on the prevalence of seafood allergy comes from Scandinavian countries and Spain, there is no information available on the frequency to different seafood species in South Africa. In addition, very little information exists world-wide for the mollusc group regarding symptoms, frequency of involved species and the relevant allergens.

The objectives of my study were first to determine the frequency and spectrum of reported hypersensitivity to abalone and other seafood species in South Africa. Furthermore, the specific immune responses to local seafood species were analysed by various *in-vitro* and *in-vivo* tests. Subsequently the hypersensitive reactions to the local abalone species, *Haliotis midae*, were characterised in more detail and the allergens identified. Monoclonal antibodies generated to abalone allergens were used to identify cross-reacting allergens among different seafood species and to differentiate between closely related abalone species for forensic identification.

The analysis of the questionnaires demonstrated that of the 26 species implicated in adverse reactions to seafood, three mollusc species were among the five most frequently reported species. Abalone, *H. midae*, was the most reported mollusc species with more than one third of all reactions and thus, this seafood species plays an important role in inducing allergic reactions to local seafood species. Furthermore, delayed onset of hypersensitive reactions were very frequently reported among abalone sensitive subjects. Delayed reactions may be the reason for the negative SPT and RAST results for subjects with positive history of allergic reaction to ingested abalone. Whether or not the consistency of the rubbery abalone tissue, or the stability of the mollusc allergens to digestion are significant parameter has to be investigated in further studies. It may be possible to study mollusc sensitive subjects in the future, using DBPCFC at our academic hospital.

IgE binding studies identified two major abalone allergens with molecular weights of 38 kDa and 45 kDa. These two allergens where also present in extracts of other indigenous mollusc species as well as in some crustacean and fish. Cross-reacting allergens between abalone and arthropods such as crustacean, house dust mites and locusts were clearly demonstrated by RAST inhibition and Western blot studies. It appeared that the allergens in the analysed species of the crustacean and fish group are highly conserved and shared a high degree of cross-reactivity. In

addition, I observed that concurrent IgE reactivity to molluscs and crustaceans induced a stronger immune response possibly produced by cross-reacting allergens.

In the mollusc group very diverse and heterogeneous species-specific allergens were demonstrated by Western-blot, RAST-correlation and -inhibition studies. Additional RAST and SPT assays with extracts of indigenous seafood species will increase the number of seafood sensitive subjects testing positive in South Africa.

The abalone and other mollusc allergens were found to be very resistant to heat as was demonstrated by ELISA, RAST and Lymphocyte proliferation assays. Even generated "new" allergens were demonstrated by Western blotting. The 38 kDa allergen is believed to be the abalone tropomyosin, and the novel 45 kDa allergen was registered with the WHO/IUIS and named Hal m 1 according to the recommended procedure. Amino acid analysis of these allergens identified high contents of acidic residues and in particular serine. However, such an amino acid composition pattern is not remarkably different from other non-allergenic proteins. Future enzymatic digestion of the purified allergens and sequencing of the resulting peptides will allow a detailed comparison with known proteins and allergens. Furthermore, IgE and monoclonal antibody binding regions can be identified by epitope mapping on the generated allergen fragments.

Monoclonal antibodies generated to abalone allergens identified cross-reacting proteins or antibody binding epitopes in various mollusc species but also to a lesser extent in different crustacean and fish species. However, analysis of ten different abalone species demonstrated that species specific epitopes exist in very close related mollusc species.

Monoclonal antibody studies on mollusc antigens and the cross-reactivity with other seafood species have not previously been published. As a result of these studies, a diagnostic test was developed to distinguish between closely related abalone species from different parts of the world. The development of a user-friendly immunological field kit is in progress to identify the origin of abalone tissue samples, in unsophisticated areas of South Africa and in areas where poaching of abalone is a problem.

APPENDIX

SOUTHERN AFRICAN FISH ALLERGY STUDY QUESTIONNAIRE

Allergology Unit, UCT Medical School, Observatory, 7925

Fax No: 021-448 6116
Tel No: 021-406 6147

Contact Persons: Prof P C Potter - 406 6320
Dr G Marais (Registrar) - 406 6373
Mr A Lopata (Natural Scientist) - 406 6151
Miss C Zinn (Dietician) - 797 8840
Mrs J Higgins (Snr Secr) - 406 6147

1(a) **PATIENT'S SURNAME:** .....

(b) **PATIENT'S NAME:** .....

(c) **PATIENT'S DOB:** .....

(d) **ADDRESS:** .....

.....

(e) **TEL NO** HOME:..... WORK:.....

		Y	N
2. Do you suffer from	(i) allergic rhinitis (hayfever) and conjunctivitis (itchy eyes)	☐	☐
	(ii) asthma	☐	☐
	(iii) eczema	☐	☐
	(iv) swellings of face/mouth/skin (hives or wheals)	☐	☐

		Y	N
3(a)	Is there any history of food allergies in your family?	☐	☐

(b) If yes state: (i) Your relationship to the allergic patient/s

.....

(ii) The nature of the allergy:

.....

(iii) The severity of the allergy/symptoms:

.....

4. Which food/s do you suspect are causing the symptoms (specify precisely the fish or seafood that you are suspicious of):

Yellowtail	☐	Black Mussel	☐
Hake	☐	Crayfish	☐
Salmon	☐	Prawns	☐
Mackerel	☐	Perlemoen	☐
Oyster	☐	Snails	☐
Others (Please specify)	☐		

.....

.....

5(a) Do your symptoms, following ingestion of fish or seafood, include the following?

		Y	N
(i)	urticaria (hives/itchy wheals)	☐	☐
(ii)	eczema	☐	☐
(iii)	abdomimal pain	☐	☐
(iv)	nausea/vomiting	☐	☐
(v)	diarrhoea	☐	☐
(vi)	wheezing/tight chest/difficulty breathing	☐	☐
(vii)	itching of tongue or lips	☐	☐
(viii)	swelling/itching of throat	☐	☐
(ix)	dizziness or collapse	☐	☐
(x)	headache	☐	☐
(xi)	flushing	☐	☐
(xii)	feeling of anxiety	☐	☐

6. List any other symptoms experienced: .....

.....

7. State the length of time between ingestion of the food and the onset of the specific symptoms.

.....

Y N

8(a) Have you taken any medications to help relieve your symptoms? ☐ ☐

(b) If yes, what do you take? .....

and does it help? .....

9(a) State your age at which the symptoms first started .....

(b) Approximately how many times has the reaction occurred? .....

(c) When was the most recent occurrence of the reaction? .....

(d) Have you eaten the food since? .....

(e) Does it happen <u>everytime</u> you eat the specified food? .....

(f) Does touching the food ever cause any reaction? .....

10. What is the minimum quantity of food which produces the symptoms? (State approximate size and weight)

.....

11. How soon was the food/s eaten after being prepared?

.....

12. In what form/state is the food/s eaten when it causes the symptoms?

Cooked ☐ Raw ☐ Tinned ☐ In a mixture (paella, casserole) ☐

Other (specify) ☐

.....

13(a) In what environment is the suspected food eaten?

	Y	N
Home	☐	☐
Restaurant	☐	☐
Cafe	☐	☐
Other		

(b) If at home, how was the food prepared? (State in detail spices, sauces, etc.)

.....

.....

14(a) Do you suffer from any other diseases/medical problems? (including allergies) Y ☐ N ☐

(b) If yes, list them .....

(c) Do you react to drugs? Y ☐ N ☐

(d) Do you react to food additives/preservatives/colourants (e.g. sulphites, MSG, benzoates, tartrazine, sulphur dioxide? Y ☐ N ☐

15(a) Are you on any medication/vitamins? Y ☐ N ☐

(b) If yes, list them .....

.....

16. Describe, in detail, the events which occur after the suspected seafoo allergen has been ingested. (ie. sequence, nature, other food/s eaten simultaneously/afterwards). Describe a typical attack.

.....

.....

.....

.....

17(a) Has this allergy problem been investigated before? Y ☐ N ☐

(b) If yes, what were the procedures and results? .....

.....

.....

(c) Which doctor was consulted? (Name & address)

.....

.....

.....

18. Would you donate a small amount of blood (20mls - 5 spoons) in order that we could perform laboratory tests to precisely identify your allergy?

Y ☐ N ☐

In some cases the only way to be sure that you can safely eat a seafood where there has previously been doubt is to actually ingest a small amount under controlled conditions in hospital with a doctor standing by to treat any possible reactions.

This is a well established procedure at the other Food Allergy Units in Europe. We would never conduct such challenges if the cause of your allergy was quite obvious and confirmed by the blood tests.

19. Would you consent to participating in a careful food challenge at the hospital under controlled supervision as a morning procedure?

Y ☐ N ☐

I ..... hereby consent to participate in the fish allergy study. I understand that this will involve blood tests. I understand that there is a small risk of a clinical reaction should I have a food allergy challenge. If there is a need for a food allergy challenge or a skin prick test, this will be fully explained to me and I will have the opportunity to take a decision about this at a later date at which I will sign formal consent if I agree to the performance of the careful food challenge at Groote Schuur Hospital.

SIGNATURE: .....

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