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# CHARACTERIZATION AND REGULATION OF SERINE EXOPROTEASES AND COLLAGENASE IN VIBRIO ALGINOLYTICUS

by

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## ABSTRACT

The production of an extracellular collagenase and serine proteases by Vibrio alginolyticus during stationary phase was inhibited by a temperature shift from 30 to 37°C and by lack of oxygen. V. alginolyticus had identical growth rates at 30 and 37°C. Aeration did not affect the growth rate of stationary phase cells when the exoproteases were being produced. Macromolecular synthesis in stationary phase cells was not affected by temperature. The regulation of exoprotease production by temperature and oxygen is specific and has implications regarding the ecology of V. alginolyticus. The synthesis of a 100 000 molecular weight protein was induced in V. alginolyticus by either raising the temperature from 30 to 37°C, a lack of oxygen or $(NH_4)_2SO_4$. Histidine stimulated synthesis of a 52 000 molecular weight protein. The possibility that these proteins have a regulatory role in exoenzyme synthesis is discussed.

Cerulenin inhibited the production of extracellular collagenase and serine proteases. Although quinacrine markedly inhibited collagenase production, only transient inhibition of serine protease production was observed. O-phenanthroline, however, which also inhibits the penicillinase-releasing protease, severely inhibited serine protease production. The anaesthetic lidocaine inhibited

both collagenase and serine protease production. Lidocaine and quinacrine added simultaneously had a synergistic effect on inhibition of collagenase production. Addition of quinacrine and lidocaine altered synthesis of four different proteins.

A low molecular weight peptone fraction in the molecular weight range from 350 to 1 500 induces collagenase production. Digestion of this peptone inducer with purified V. alginolyticus or Clostridium histolyticum collagenase markedly reduced their inducing ability, whereas digestion with trypsin, pepsin or pronase had no effect. The results suggest that a high molecular weight product with the triple helix structure is not required for induction of collagenase.

The release of $[^{3}H]$-proline from collagen matrices produced by smooth muscle cells was shown to be a sensitive assay for bacterial collagenase and was utilized to show that V. alginolyticus produces a basal constitutive level of collagenase. The constitutive levels of collagenase were affected by aeration but not by temperature.

The number and molecular weights of extracellular alkaline proteases produced by V. alginolyticus were determined by gelatin-PAGE. Three major bands and two minor bands of protease activity with molecular weights of 28 000, 22 500, 19 500, 15 500 and 14 500 (proteases 1,2,3,4 and 5) were obtained after gelatin-PAGE. The activities of the five

proteases were inhibited by serine protease inhibitors but not by inhibitors of trypsin-like enzymes. Histidine which inhibited collagenase activity did not inhibit the alkaline serine protease activity. The production of protease 1 was enhanced by histidine and inhibited by growth at 37°C.

Gelatin-PAGE of a commercial V. alginolyticus collagenase preparation revealed four bands of activity which were identified as collagenases with molecular weights of 45 000, 38 500, 33 500 and 31 000. The collagenase preparation was contaminated with two serine proteases.

# CHAPTER 1



# INTRODUCTION

## 1.1 EXOENZYMES

Exoenzymes are produced by both Gram-negative and Gram-positive bacteria. An exoenzyme is one which exists free in the medium surrounding the cells or which may be released from the cells by protoplasting. The primary secretion event may thus be regarded as the transmembrane passage of the protein (Glenn, 1976). Bacterial exoenzymes fall in the molecular weight range from 12 000 to 500 000, the majority being within the range 20 000 to 40 000. Amino acid analysis showed that cysteine is either absent or present in very low quantities. No other difference in overall amino acid composition distinguishes these proteins as a group (Glenn, 1976).

The synthesis of exoenzymes may occur at any stage throughout the growth cycle. This is reflected by the various functions of these enzymes. The main function of many exoenzymes is the degradation of polymers to supply the cells with an assimilable source of nutrients. Some bacteria release antibiotics or lytic factors which may be a method of protection or a supply of nutrients. Other proteases are involved in sporulation, cell wall metabolism and DNA-mediated transformation (Priest, 1977).

A substantial proportion of extracellular enzymes appear to

be inducible by their substrate or closely related compounds (Pollock, 1962; Priest, 1977). Inducers are frequently large molecules that are not likely to enter the cell. It is generally accepted that a low basal level of constitutive exoenzyme degrades its substrate and the resultant low molecular weight products enter the cell and induce further exoenzyme synthesis. Regulation of exoenzyme synthesis shows marked differences among organisms. Many exoproteins, however, are subject to end product inhibition particularly by amino acids, and to catabolite repression (Glenn, 1976).

Secretion of exoproteases is dealt with in Section 1.7.

## 1.2 COLLAGENASES AND COLLAGEN

Unless otherwise stated the following information is taken from the works of Seifter & Harper (1970), Mandl (1972), Keil (1979) and De Crombrugghe & Pastan (1982).

Mandl (1972) defined collagenases as enzymes capable of solubilizing insoluble fibrous collagen by peptide bond cleavage under physiological conditions of pH and temperature. In general vertebrate collagenases can only cleave collagen whereas bacterial and low eucaryote collagenases can degrade proteins other than native collagen. Vibrio alginolyticus and Clostridium histolyticum collagenases will both cleave gelatin and Entomophtora collagenase will degrade the B

chain of insulin (Keil, 1979).

One of the main functions of bacterial collagenases is to aid invasion in the connective tissues of the host by rapid degradation of collagen. Breakdown of tissue proteins has been observed in many bacterial infections and has stimulated the study of proteolytic enzymes of a variety of micro organisms. Among bacteria, collagenolytic activity has been demonstrated with three anaerobic genera (Clostridium, Bacteroides and Staphylococcus) and with five aerobic genera (Streptomyces, Vibrio, Pseudomonas, Bacillus and Aeromonas).

Animal and human collagenases are involved in normal tissues (skin and bone), where remodelling goes on continuously and in pathological conditions including wound healing in the skin and cornea, rheumatoid arthritis and other inflammatory and degenerative joint diseases.

Collagen, a most abundant and versatile protein comprises approximately 33% of the total protein in mammalian organisms. There are at least six genetically distinct types of collagen molecule. The different types predominate in different tissues and the predominant type is tissue dependent. Initially the molecule is in a form termed procollagen which is about one and a half times as long as the final molecule. This is cleaved by proteolysis and post-translational modification such as hydroxylation of selected

proline and lysine residues and glycosylation of selected residues, takes place. The resulting collagen molecule, molecular weight 300 000, is a triple helix of three $\alpha$ peptide chains. Each peptide chain contains approximately 1 055 amino acids, the smallest of which, glycine, occurs as every third amino acid throughout the molecule except for the two short telopeptides of 16 and 25 amino acids at the N- and C-termini of the molecule, respectively. The strands wind around one another so that the glycine residues are always on the inside, allowing the strands to coil closer together. The non-glycine amino acids on the outside of the molecule determine the intermolecular interactions. The prevalence of the apolar regions containing the sequences, glycine-proline-hydroxyproline and glycine proline-alanine presumably account for the reason that collagen is stable against common proteolytic enzymes with specificities directed toward polar and aromatic residues (Mandl, 1972). Of the three $\alpha$-chains, two are identical (except in cod fish collagen), the third one differs slightly in amino acid composition. Each $\alpha$-chain is arranged in the form of a left-hand helix and these three minor helices are then arranged in a right-hand spiral about a common axis to form a super helix, the collagen molecule, which is stabilized by interpolypeptide hydrogen bonds and hydrophobic interactions. The collagen molecules are staggered to produce a fibril, the fibrils aggregate to yield visible, functional tissue. The telopeptides comprise 1% of the collagen molecule and interact during

polymerization of tropocollagen to form fibrils. Removal of the telopeptides leaves the triple helices intact but greatly reduces their ability to aggregate and form fibrils.

## 1.3 V. ALGINOLYTICUS COLLAGENASE

Welton & Woods (1973, 1975) described the isolation from cured hides of an aerobic, halotolerant, collagenolytic, Gram-negative bacterium which was originally classified as an Achromobacter iophagus strain. This strain has subsequently been reclassified as a V. alginolyticus strain (NCIB 11038) by M. Hendrie of the National Collection of Industrial Bacteria, Aberdeen, Scotland. V. alginolyticus produces an extracellular collagenase (EC 3.4.24.3) as the cells enter the stationary phase of growth. Keil-Dlouha et al. (1976) showed that no zymogen or cell-accumulated enzyme is present in the first stage of exponential growth and that collagenase synthesis is in direct correlation with a particular state of the bacterial growth cycle. Collagenase is induced by collagen and its high molecular weight fragments, peptone and gelatin. The enzyme cleaves the X-Gly bond in the sequence Pro-X-Gly-Y where Y is generally proline or alanine and X is a neutral amino acid. Vibrio collagenase splits this bond in the helical regions of collagen and in a number of synthetic peptide substrates. Analogous bonds in $\beta$-casein can also be cleaved (Gilles & Keil, 1976).

The autodigestion of V. alginolyticus collagenase results in the formation of at least three fractions active against the synthetic substrate (PZ-Pro-Leu-Gly-Pro-D-Arg, Fluka) as well as native collagen (Keil-Dlouha, 1976). The simplest form of active collagenase is a dimer composed of two subunits of molecular weight 35 000 each (Keil-Dlouha & Keil, 1978). Keil-Dlouha & Keil (1978) were able to isolate another homogeneous form of V. alginolyticus collagenase of molecular weight 80 000. This form was subsequently found to consist of two subunits of molecular weight 35 000, each of which binds non-covalently a peptide of molecular weight 5 000. The amino acid composition of subunits of both 70 000 and 80 000 collagenases is the same.

V. alginolyticus collagenase resembles in its amino acid composition, molecular weight of the subunit and metal dependence, two bacterial metallo-proteases thermolysin and the neutral protease from Bacillus subtilis. Vibrio collagenase (subunit) and thermolysin have practically identical molecular weights (35 000 and 34 300), and a very similar content of basic amino acids, 9 and 10 arginines, 10 and 11 lysines and both contain 8 histidines. In thermolysin, B. subtilis protease and Vibrio collagenase a single histidine residue is involved in the catalytic activity of the enzymes (Herry & Keil-Dlouha, 1978). $Ca^{2+}$ ions are required for both the binding of the enzyme to the collagen substrate and for full catalytic activity (Seifter & Harper, 1970).

## 1.4 NITROGEN METABOLISM AND THE REGULATION OF V. ALGINOLYTICUS EXOENZYMES BY THE HUT SYSTEM

Unless otherwise stated, the information in this section is taken from the works of Tyler (1978) and Magasanik (1978).

In all biological systems the assimilation of nitrogen into macromolecules is essential for growth. The metabolic pathways for nitrogen metabolism can be divided into two classes: the assimilatory pathways for utilization of nitrogen and the biosynthetic pathways for production of the nitrogen-containing compounds in the cell. In virtually all cells glutamate and glutamine serve as nitrogen donors for biosynthetic reactions. A general control element, glutamine synthetase, is thought to mediate the formation of enzymes involved in the assimilation of nitrogen into glutamate and glutamine. Glutamine can only be synthesized by the addition of ammonia to glutamate in a reaction catalyzed by glutamine synthetase. However, in the enteric bacteria glutamate can be produced by a variety of reactions, (i) from ammonia and 2-oxoglutarate by a reaction catalyzed by glutamate dehydrogenase or by a coupled reaction catalyzed by glutamine synthetase and glutamate synthase, (ii) as a direct product of degradation of an amino acid, or (iii) from amino groups of another amino acid and 2-oxoglutarate by a transamination reaction.

Deadenylylated glutamine synthetase has a molecular weight of 600 000 and contains twelve identical subunits. The

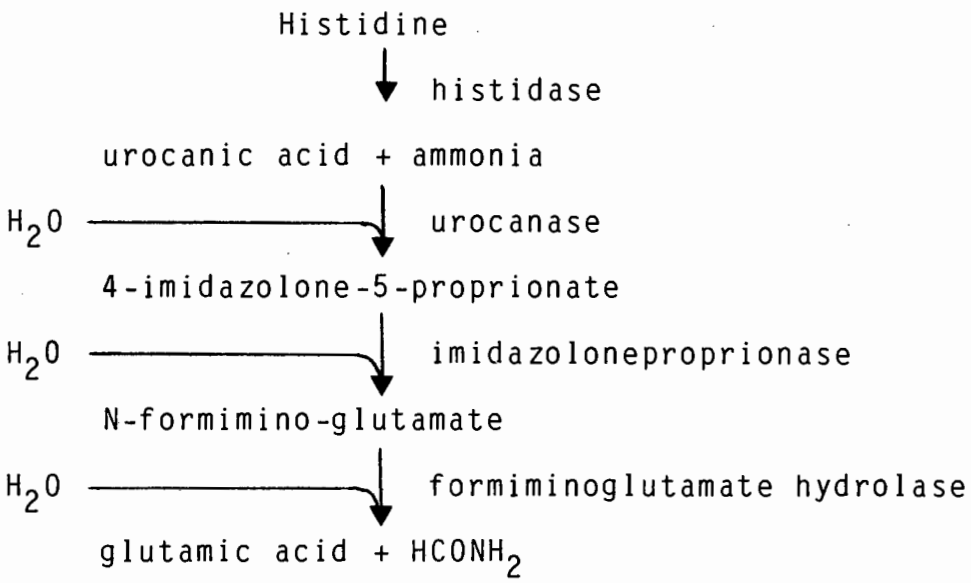
divalent cation $Mg^{2+}$ or $Mn^{2+}$ is required for stability. The enzymatic activity of glutamine synthetase is regulated by the following mechanisms: (i) by the interconversion of a relaxed (inactive) and taut (active) form in response to variations in concentrations of divalent cations, (ii) by cumulative feedback inhibition by various endproducts of glutamine metabolism, and (iii) by a cascade system consisting of several metabolite regulated enzymes and a small regulatory protein, which together modulate adenylylation of a specific tyrosyl residue on each subunit of glutamine synthetase (Ginsburg & Stadtman, 1973). Maximum biosynthetic activity is obtained when the enzyme is completely unadenylylated and decreases over a wide range as the degree of adenylylation increases.

The hut operon is the model system for glutamine synthetase regulation. Although the ability to degrade L-histidine is widely distributed among bacteria, Escherichia coli does not have this ability. However, the hut genes of Klebsiella aerogenes and Salmonella typhimurium have been introduced into E. coli where they function in the same way as in the strains from which they were derived (Tyler & Goldberg, 1976).

Studies of the hut system in S. typhimurium strains LT-2 and 15-59 and in K. aerogenes revealed clustering of the histidine utilization genes into two adjacent operons: hut MIGC and hut PUH (Smith & Magasanik, 1971; Goldberg

& Magasanik, 1975). The structural genes for the four enzymes responsible for histidine degradation are hut H (histidase), hut U (urocanase), hut I (imidazoloneproprionase) and hut G (formiminoglutamate hydrolase). Mutations in any of these genes leads to the loss of the ability to use histidine as a source of nitrogen and to a deficiency in the corresponding enzyme. The product of the hut C gene is a repressor (Hagan & Magasanik, 1973; Smith & Magasanik, 1971). The pathway of histidine degradation is shown in Figure 1.

FIGURE 1:




Regulation of the hut system is affected by both positive and negative controls. Negative control is exerted by the product of the hut C gene, the repressor, which binds to the operator regions of the two operons and can be released by the inducer, urocanate (Hagan & Magasanik, 1973, 1976). In S. typhimurium the hut repressor has

greater affinity for the right-hand operator than the left-hand operator (Hagan & Magasanik, 1976). As a result the basal level components of the left-hand operon are considerably higher and a lower level of inducer is required for induction. The regulation of the two operons by the repressor is similar for S. typhimurium and K. aerogenes, though in the latter organism the left-hand operon is more tightly controlled. The fact that urocanate and not histidine is the inducer of the hut enzymes casts histidase in the role of an inducer-producing enzyme and urocanase as an inducer-destroying enzyme. To allow the cell to regulate the activities of these two enzymes with regard to each other, the hut U and hut H are found in the same operon (Brill & Magasanik, 1969; Meiss et al., 1969; Smith & Magasanik, 1971 and Hagan et al., 1974).

Positive control of the hut system is mediated by catabolite-activating protein (CAP) charged with cAMP (Prival & Magasanik, 1971), and by non-adenylylated glutamine synthetase (Tyler et al., 1974). Much of the evidence indicating that glutamine synthetase can regulate transcription came from studies on the hut genes of K. aerogenes (Prival & Magasanik, 1971; Prival et al., 1973). Prival & Magasanik, (1971) showed that histidase and proline oxidase of K. aerogenes can escape from catabolite repression exerted by glucose provided the source of nitrogen is growth-rate limiting. In 1973 they were able to correlate this "escape" to the cellular level of glutamine synthetase.

In cells growing with an excess of ammonia, the level of glutamine synthetase is low, whereas in cells growing on a growth rate-limiting nitrogen source, the level of glutamine synthetase is high. Furthermore in glutamine-requiring mutants histidase does not escape from catabolite repression, even when the nitrogen source is growth rate-limiting. In S. typhimurium no escape from catabolite repression is observed. However, in hybrid strains of E. coli or K. aerogenes, the glutamine synthetase of S. typhimurium activates synthesis of histidase from the hut S15-59 genes (Bloom et al., 1977). Bloom et al. (1977) therefore proposed that a specific transcription factor may be necessary for glutamine synthetase to activate transcription of the hut genes. A candidate for such a factor would be RNA polymerase. This model would suggest that the interaction of the glutamine synthetase of S. typhimurium with the hut DNA causes only a partial melting of the promotor region, not sufficient for the S. typhimurium RNA polymerase to move into the open complex with the DNA, but sufficient for the K. aerogenes or E. coli enzyme. Alternatively, this factor may facilitate the interaction of glutamine synthetase with DNA (Bloom et al., 1977).

Regulation by glutamine synthetase CAP-cAMP and the repressor would thus ensure operation of the hut system according to the needs of the cell. Cells growing in a medium containing glucose, ammonia and histidine require neither CAP-cAMP nor nonadenylylated glutamine synthetase activation.

However, glucose limitation would result in an increase in CAP charged with cAMP which would stimulate transcription of hut operons. Similarly, during ammonia limitation non-adenylylated glutamine synthetase levels would increase causing transcription of hut operons. Finally, if histidine was used up, urocanate could no longer inactivate the repressor, but useless transcription of the hut operon would not take place (Magasanik, 1978). Long et al. (1981) first implicated the involvement of the hut system in the regulation of the extracellular alkaline proteases produced by V. alginolyticus (Lecroisey et al., 1975; Long et al., 1981).

Alkaline protease activity is sensitive to catabolite repression by a number of carbon sources including glucose, and by amino acids and ammonium ions. Cyclic AMP, dibutyryl cAMP and cGMP did not relieve catabolite repression. Histidine, however, was able to relieve repression by glucose and ammonia but where concentrations of glucose and ammonia are high enough, histidine has no effect.

The inducer of alkaline protease activity is urocanic acid, and histidine induces by virtue of its conversion to urocanic acid. Studies with the hut H2 and hut H1 mutants by Bowden et al. (1982) confirmed these results. The hut H1 and hut U1 mutants lack histidase and urocanase activity respectively. Alkaline protease activity was stimulated by urocanic acid and not histidine in the hut H1

mutant. Whereas in the *hut U1* mutant which lacked urocanase activity, histidine and urocanic acid were both able to induce protease synthesis. Bowden *et al*. (1982) also studied induction of the *hut* enzymes. Although urocanic acid and not histidine induced alkaline protease activity, the *hut* enzymes were only induced by histidine. In the *hut H1* mutant which cannot convert histidine to urocanic acid, histidine was shown to induce the *hut* enzymes. This suggests a similarity with the *B. subtilis* system in which histidine also induces the *hut* enzymes, unlike *E. coli* and *S. typhimurium* in which urocanic acid is the inducer. A separate control mechanism is suggested for the extracellular collagenase produced by *V. alginolyticus* by the inhibition of collagenase synthesis by histidine in the wild type strain and in the *hut* mutants.

## 1.5 DETECTION OF PROTEASE ACTIVITY BY PAGE

In view of the many important processes that are catalyzed and regulated by specific proteases, a study of extracellular proteolytic reactions is of increasing interest. The characterization of these reactions may present difficulties which vary depending on the reaction conditions and the degree of purity of the system. To solve this problem Granelli-Piperno & Reich (1978) explored the potential of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as a general method for separating extracellular proteases. Their technique exploited the fact that the

inactivation by SDS of at least one specific protease-plasminogen activator is reversible, since the activity can be restored by removal of the SDS with nonionic detergent. Samples treated with SDS were electrophoresed on SDS-polyacrylamide gels. The SDS was removed by washing the gels in nonionic detergent solutions. The slab gel was then layered onto a second indicator gel consisting of fibrin and agar. Zones of fibrin degradation, corresponding to the position of proteases in the overlay, can be seen as clear areas in an opaque background on dark field illumination, or as clear areas in a dark blue background after fixation and staining with Coomassie Blue or amido black.

Granelli-Piperno & Reich (1978) established the usefulness of this method by the number of different proteases able to retain their activity on these gels. Results were reproducible and sensitive, allowing detection of proteases in less than 1 $\mu\ell$ of fresh plasma. Catalytic activity of proteases bound to inhibitors could be unmasked by this method, suggesting a use for identifying antibody complexes.

Finally, by substituting different protease substrates for fibrin, the technique could become widely applicable. Heussen & Dowdle (1980) reported a further modification of this technique. It was based on the observation that if the protease substrate (gelatin & plasminogen) is copolymerized into the matrix of the SDS-PAGE at the time of casting, it is

retained during electrophoresis, providing *in situ* substrates for separated bands. This modification has the advantages of ease of manipulation, secondly, the relatively compact structure and smaller pore size of the matrix limits lateral diffusion of proteolytic activity. Thirdly, the use of gelatin provides a substrate susceptible to a wider variety of proteases. Finally, molecular weight markers are visible in the same slab gel. This technique, however, is not suitable for crude enzyme preparations where non-proteolytic protein bands may interfere with the proteolytic bands. The emergence of proteolytic bands at different rates may also be observed in the Granelli-Piperno & Reich (1978) technique but not in the Heussen and Dowdle (1980) technique where bands are only visible after the reaction has been terminatedby staining.

## 1.6 MICROBIAL PROTEASES AND THEIR INHIBITORS

Hartley (1960) classified proteases on the basis of mechanism of action rather than origin, specificity or physiological action due to the lack of knowledge regarding the latter characteristics. The four groups thus classified were: serine proteases, thiol proteases, acid proteases and metal proteases. Most microbial proteases can be classified according to these groups. Morihara (1974) further subdivided the four groups according to side-chain specificity (i.e. primary specificity).

Serine proteases can thus be divided into four groups: trypsinlike proteases, alkaline proteases, myxobacter α-lytic protease, and staphylococcal protease. Thiol proteases further consist of two groups: clostripain, a protease from Cl. histolyticum and streptococcal protease. Metal chelator-sensitive proteases are classified into the groups: neutral and alkaline proteases and myxobacter AL-1 proteases I and II. The acid proteases are not further subdivided.

Various types of specificity are exhibited: (i) against basic, acidic, aromatic or hydrophobic, and rather small aliphatic amino acid residues at the carboxyl side of the splitting point in a peptide substrate (serine and thiol proteases); (ii) against basic and bulky or hydrophobic amino acid residues at the amino side (metal proteases); and (iii) against aromatic or hydrophobic amino acid residues at both sides (acid proteases).

Proteases usually split internal peptide bonds when they hydrolyse large molecular peptides and protein substrates; the enzymes are therefore called endopeptidases. Table 1 overleaf shows the groups of enzymes, their specificity and inhibitors.

Protease inhibitors are widely distributed in plants, animals and microorganisms. Their gross physiological function is the prevention of unwanted proteolysis. With the exception of macroglobulins, which "inhibit" proteases

TABLE 1

| Group | Inhibitor | Specificity |
|---|---|---|
| Serine proteases | | |
| Trypsinlike | Diisopropylfluorophosphate (DFP), tosyl-L-lysine chloromethyl ketone (TLCK), soybean trypsin inhibitor. | Basic amino acid residues at the carboxyl side of the splitting point. |
| Alkaline | DFP | Aromatic or hydrophobic amino acid residues at the carboxyl side of the splitting point. |
| Myxobacter α-lytic protease | DFP | Small aliphatic amino acid residues at the carboxyl side of the splitting point. |
| Staphylococcal | DFP | Aspartic or glutamic acid residues at the carboxyl side of the splitting point. |
| Thiol proteases | | |
| Clostripain | p-Chloromercuribenzoate (TLCK) | Basic amino acid residues at the carboxyl side of the splitting point. |
| Streptococcal | pCMB | Broad. |
| Metal-chelator sensitive proteases | | |
| neutral | Ethylenediamine tetraacetic acid (EDTA), O-phenanthroline | Hydrophobic or bulky amino acid residues at the amino side of the splitting point. |
| Alkaline | EDTA, O-phenanthroline | Broad |

Table 1 continued overleaf.

Table 1 continued:

| Group | Inhibitor | Specificity |
|---|---|---|
| Myxobacter protease I | EDTA | Small molecular amino acid residue at either or both side(s) of the splitting point. |
| Myxobacter protease II | EDTA | Lysine residue at the amino side of the splitting point. |
| Acid proteases | Diazoacetyl-DL-norleucine methyl ester. | Aromatic or hydrophobic amino acid residues at both sides of the splitting point. |

of all classes, individual protein inhibitors inhibit only proteases belonging to a single mechanistic class. The inhibitors are exceptional among proteins since they tend to retain their inhibitory activity upon replacement of their reactive site residue by another residue. In some cases such a substitution leads to a change in inhibitory activity: e.g. $Arg^{63} \rightarrow Trp^{63}$ in soybean trypsin inhibitor (Kunitz) leads to the conversion of a trypsin inhibitor to a chymotrypsin inhibitor.

## 1.7 SECRETION OF EXOENZYMES

Biological membranes present a diffusion barrier for macromolecules such as proteins, but transfer of a large number of specific proteins across membranes is an important physiological activity of virtually all cells. In 1971 Blobel and Sabatini proposed the "signal hypothesis" for secretion of proteins in eucaryotes. In the last 10 years the mechanism of secretion of proteins in procaryotes has been greatly studied, and a model has emerged based on the original signal hypothesis.

The essential feature of the signal hypothesis is the occurrence of a unique sequence of codons, located immediately to the right of the initiation codon, which is present only in those mRNAs whose translation products are to be transferred across a membrane. Translation of the signal codons results in a unique sequence of amino acid residues (15-30 residues) on the amino terminus of the nascent chain. Emergence of this signal sequence of the nascent chain from within a space in the large ribosomal subunit triggers attachment of the ribosome to the membrane, thus providing the topological conditions for the transfer of the nascent chain across the membrane (Blobel & Dobberstein, 1975). Upon emergence of the signal peptide from the membrane, it is cleaved from the protein by a specific protease (Lampen, 1978). The protein chain is now free to be released into the external medium or to assume its specific conformation in the outer membrane. Folding of the protein

into its three-dimensional conformation takes place either during or after secretion. This model does not allow for accumulation of an intracellular pool of protein for secretion. The signal sequence of exported proteins is not always cleaved nor is it always situated at the amino terminus. Chicken ovalbumin has been shown to contain an internal signal sequence which is not cleaved from the exported protein (Lingappa et al., 1978, Lingappa et al., 1979).

Sucrose fermenting yeast strains carrying one or more SUC genes synthesize two forms of the enzyme invertase, a secreted glycoprotein and a cytoplasmic form containing no carbohydrate. The polypeptide portions of these two forms are encoded by a single structural gene but are synthesized from distinct mRNAs. Perlman et al. (1982) showed that the cytoplasmic and secreted polypeptides are identical in amino acid sequence carboxy terminal to the cytoplasmic polypeptide initiator methionine. The secreted polypeptide however, contains an amino terminal signal sequence of 19 amino acid residues that is cleaved during secretion to generate the glycopeptide. There is no precursor-product relationship between these two forms.

The secretion of penicillinase by B. licheniformis is unusual in involving a membrane-bound form of molecular weight 33 000, as well as an extracellular form of molecular weight 29 000 (Lampen, 1978). Though the membrane penicillinase can be converted to the exoenzyme, this is not an obligatory

step. Conversion is effected by cleavage by the penicillin-releasing protease (PR-protease). The stable binding of the membrane-bound form of penicillinase was accounted for by the presence of an $NH_2$-terminal phosphatide, (Yamamoto & Lampen, 1976 a,b), however, the presence of lipid could not be confirmed.

Smith et al. (1981) were able to isolate yet a larger precursor of molecular weight 36 000 along with the other two forms of penicillinase. The extra sequence on the 36 000 molecular weight form was found to be a hydrophobic signal sequence. Some 33 000 molecular weight molecules were found to be even more hydrophobic indicating the possibility of a lipoprotein form. This was supported by the fact that the 33 000 molecular weight fraction had incorporated glycerol. Nielsen et al. (1981) confirmed these results showing that the polypeptide chain of the 33 000 molecular weight form contains a cysteinyl residue at or near the $NH_2$-terminus as well as glycerol and fatty acid residues.

Membrane penicillinase from B. licheniformis bears a striking resemblance to the major outer membrane lipoprotein of E. coli. The polypeptides of both contain a hydrophobic sequence at the amino terminus consisting of a cysteinyl residue, glycerol and fatty acid. Moreover, when the B. licheniformis gene was introduced by a λ lysogen into E. coli the membrane penicillinase produced contained an identical hydrophobic sequence (Nielsen et al., 1981;

Lai et al., 1981). Inouye et al. (1977) determined the complete amino acid sequence for the peptide extension of the prolipoprotein of the E. coli outer membrane. This sequence includes the tetrapeptide, Leu-Ala-Gly-Cys. The same tetrapeptide has been found in prepenicillinase of B. licheniformis as deduced from DNA sequence of the cloned gene (Neugebauer et al., 1981; Nielsen et al., personal communication, quoted by Lai et al., 1981). The amino acid sequence at the amino terminus could thus be a common recognition site for posttranslational modification enzymes.

The determination of the amino acid sequence of the peptide extension of prolipoprotein in E. coli and subsequent analysis enabled Inouye et al. (1977) to propose a mechanism for the translocation of prolipoprotein across the membrane. The extended region can be divided into four regions: two hydrophobic regions and two hydrophilic regions alternating with each other, each playing a role in translocation. The first section (S-1) is hydrophilic and may be involved in leading the initial attachment of the prolipoprotein to the membrane by forming ionic interactions between the positively charged section S-1 and the negatively charged surface of the membrane. Following S-1 is a long hydrophobic section I-1 which is most likely inserted into the membrane. The second hydrophilic section (S-2) follows, keeping the carboxyl terminal end of section I-1 on the surface of the membrane, or may be inserted

into the membrane together with the following hydrophobic section I-2.

Cotranslational secretion has now been directly demonstrated in bacteria. Smith et al. (1977) radioactively labelled E. coli spheroplasts with [$^{35}$S]-labelled acetyl-methionyl methylphosphate (AMMP). When the spheroplasts were disrupted and fractionated, the polysomes recovered contained radioactivity, and the radioactivity was shown to be attached as peptidyl-tRNA. Moreover, when the labelled polysomes were allowed to complete and release their chains, a periplasmic protein, alkaline phosphatase could be identified from amongst them. Using this procedure Smith et al. (1977) demonstrated cotranslational secretion of $\alpha$-amylase in B. subtilis, the toxin of Corynebacterium diptheriae and of the penicillinase of B. licheniformis.

Smith et al. (1977) further demonstrated virtually exclusive synthesis of alkaline phosphatase, $\alpha$-amylase, penicillinase and diptheria toxin on membrane-bound polysomes of various organisms, while a cytoplasmic protein (elongation factor G of E. coli) was made exclusively on free polysomes. In support of this work Randall & Hardy (1977) showed that membrane-bound polysomes synthesize proteins of the outer membrane and a secreted periplasmic protein, the maltose-binding protein in E. coli, while the elongation factor Tu, a soluble cytoplasmic protein, is synthesized by free polysomes. However, not all procaryotes follow this

rule. Precursor synthesis of subunit A of cholera toxin has been shown to be cytoplasmic (Nichols et al., 1979 as quoted by Davis & Tai, 1980).

There is no universal temporal processing pattern to which all proteins conform (Josefsson & Randall, 1981). The time at which the signal sequence is cleaved is independent of the mode of secretion, i.e. posttranslational or co-translational secretion. One protein, ampC $\beta$-lactamase, is processed entirely cotranslationally while another, TEM $\beta$-lactamase, is processed entirely posttranslationally (Josefsson & Randall, 1981). Other proteins show a mixture of both modes of processing. Cotranslational processing was shown to be a late event, being initiated only after the polypeptides had elongated to 80% of their full length. This requirement for a critical size to initiate processing may be necessary for folding of the polypeptide chain into a structure recognised by the processing enzyme, for access to the enzyme or for translation of a sequence required to activate processing.

Several models may account for the energy needed to transport proteins across membranes. Davis & Tai (1980) favour the model in which an organised membrane structure surrounds the secreted chain and transports it, unidirectionally, by transducing metabolic energy. Such an active carrier would differ from the passive tunnel postulated by Blobel & Dobberstein (1975), but shares the possibility that the "channel" is induced, by the signal sequence, to assemble

in the fluid membrane from mobile components.

Recent work shows increasing evidence for the involvement of membrane potential in the secretion process. A role for membrane potential has been implicated for the proper insertion and processing of the M13 precoat protein within the cytoplasmic membrane (Date et al., 1980; Date et al., 1980). Daniels et al. (1981) found that the addition of a proton ionophore, carbonylcyanide m-chlorophenylhydrazone (CCCP), at concentrations that dissipate the membrane potential can prevent the processing of several periplasmic proteins in E. coli, including the leucine-specific binding protein and β-lactamase. Valinomycin, a potassium ionophore, also inhibited processing of the leucine-specific binding protein. They suggest that the membrane potential plays a role in orientating the signal sequence within the membrane such that it is accessible to the leader peptidase. Enequist et al. (1981) at the same time, also proposed that the export of most proteins requires an energized membrane. They not only used uncouplers such as CCCP but also examined export in an uncA mutant. The uncA mutant cannot maintain a proton-motive force in the absence of respiration whereas the wild type can hydrolyse ATP to generate an electrochemical potential across the membrane. Under anaerobic conditions the uncA mutant showed inhibition of processing of the maltose binding protein. Uncouplers blocked maturation of three outer membrane proteins, OmpF, Omp A and LamB and two periplasmic binding proteins.

The genetic manipulation of bacteria to yield mutants or recombinants altered in various functions, provides a powerful tool for the study of the secretory process. Bassford & Beckwith (1979) isolated mutants of E. coli in which the maltose binding protein (MBP), which is normally secreted into the periplasmic space, accumulates in the cytoplasm in precursor form, due to mutations in the signal sequence. They also isolated fusion strains in which the lac Z gene encoding the normally cytoplasmic enzyme $\beta$-galactosidase is fused to the mal E gene. The product is a hybrid protein which has the MBP amino-terminal sequence and $\beta$-galactosidase enzyme activity. Such a strain containing the entire signal sequence of MBP was isolated but was unable to export the hybrid protein into the periplasm. Bassford & Beckwith (1979) suggested that the amino-terminal signal sequence is altered such that it is not recognised by the cell's export apparatus. Such experiments demonstrate the importance of the signal sequence. Moreno et al. (1980) constructed a Lam B-lac Z fusion strain whose product contains the entire signal sequence of lam B protein linked to the $\beta$-galactosidase enzyme. Although no alterations of the signal sequence had occurred the hybrid protein was not exported. Either the signal sequence is not sufficient for export or restrictions exist as to which amino acid sequences can be extruded across a membrane. To determine whether the mature portion of the exported protein is required for export across the cytoplasmic membrane, Ito & Beckwith (1981)

studied secretion of amber fragments of E. coli MBP. A fragment lacking 25-35 amino acid residues at the C-terminus was secreted at normal levels, suggesting that this sequence is not required. Further, results suggested that the C-terminal two-thirds of the polypeptide sequence, is not involved in secretion.

Ito et al. (1981) utilized the gene fusion technique to demonstrate that there is a common step in the secretion of periplasmic and outer-membrane proteins. They studied a hybrid protein containing a substantial portion of the amino terminal sequence of the MBP attached to β-galactosidase. This protein becomes stuck to the cytoplasmic membrane inhibiting the processing and localization of a number of E. coli outer-membrane and periplasmic proteins. These results suggest that outer-membrane and periplasmic proteins share a common step in localization before the polypeptide becomes accessible to the processing enzyme.

Numerous proteins are thus transported across or into the membrane, several at the same site on the membrane. Blobel (1980) proposes that the polypeptide chains contain discrete "topogenic" sequences which determine the locality of the polypeptide. Four types of topogenic sequences would be necessary: signal sequences to initiate translocation; stop-transfer sequences to interrupt translocation; sorting sequences for subpopulations of proteins with an identical travel object; and insertion sequences which

would initiate unilateral integration of proteins into the lipid bilayer.

## 1.8 AIM OF THIS STUDY

Many important processes in bacterial cells are catalyzed by specific proteases. The study of extracellular proteolytic reactions is therefore of interest. In recent years the knowledge of exoprotease production and regulation has greatly increased. Although there are many reports of true exoprotease production and secretion by Gram-positive bacteria and of periplasmic space proteins by Gram-negative bacteria, information on true exoprotease production and secretion by Gram-negative bacteria is very limited. Secretion of proteins across the cytoplasmic membrane and integration into the outer membrane has been reported in the Gram-negative bacteria, *E. coli* (Ito *et al.*, 1977) and *S. typhimurium* (Osborn *et al.*, 1972). However, apart from exoenzyme secretion in *V. alginolyticus* (Welton & Woods, 1973, 1975; Long *et al.*, 1981) and *S. marcescens* (Bromke & Hammel, 1979) there appear to be few extensive reports on protein secretion across both inner and outer cell membranes. *V. alginolyticus* produces an extracellular collagenase with a high specific activity (Lecroisey *et al.*, 1975; Keil-Dlouha, 1976) as well as other extracellular serine proteases. The study of regulation and secretion of these extracellular proteases will provide valuable information with regard to the processes carried out in

bacterial cells. The classical model for regulation of genes is that proposed by Jacob & Monod (1961) for induction and repression. The production of many exoenzymes is influenced by environmental factors such as individual nutrients, temperature and oxygen. These regulatory mechanisms will be further discussed in the following chapters of this thesis.

# REGULATION OF COLLAGENASE AND ALKALINE PROTEASE PRODUCTION

**Summary:** The production of an extracellular collagenase and serine proteases by *V. alginolyticus* during stationary phase was inhibited by a temperature shift from 30 to 37°C and by a lack of oxygen. There was no difference in the growth rate at 30 and 37°C. Aeration did not affect the growth rate of stationary phase cells when the exoproteases were being produced. Macromolecular synthesis in stationary phase cells was not affected by temperature. There was no rapid release of the exoproteases after temperature shift down, and chloramphenicol inhibited the production of collagenase when added at the time of temperature shift down from 37 to 30°C. The regulation of exoprotease production by temperature and oxygen is specific and has implications regarding the ecology of *V. alginolyticus*.

The synthesis of a 100 000 molecular weight protein was induced in *V. alginolyticus* by either raising the temperature from 30 to 37°C, lack of oxygen or the addition of $(NH_4)_2SO_4$. These conditions all inhibited the synthesis of collagenase and the serine proteases. Methionine sulfone which reversed the repression of collagenase synthesis by $(NH_4)_2SO_4$, decreased the synthesis of the

100 000 molecular weight protein. Histidine stimulated the production of the extracellular serine protease activity. Histidine also induced the synthesis of a 52 000 molecular weight protein. The synthesis of the 52 000 and 100 000 molecular weight proteins showed opposite responses to histidine and the levels of the 100 000 molecular weight protein were always low in the presence of histidine. Repression of serine protease production at 37°C was reversed by histidine.

## 2.1 INTRODUCTION

Little is known about the mechanisms which regulate the synthesis and secretion of extracellular enzyme production by microorganisms. Studies have been most frequently performed on the spore-formers, since in these microorganisms enzyme production appears to be related to the sporulating process inside the cell (Mandelstam & Waites, 1968). However, these studies have not elucidated the mechanism of control of these enzymes.

Many reports of exoenzyme regulation are concerned with the effect of the levels of individual nutrients in the extracellular environment. This regulation is divided into end product inhibition and catabolite repression. A widely reported example of end product inhibition is the repression of exoprotease synthesis by amino acids; this

has been reported in a number of bacterial genera, including Bacillus spp. (Daatselaar & Harder, 1974; Haavik, 1981). Sarcina sp. (Bissell et al., 1971), Arthrobacter (Hofsten & Tjeder, 1965) and Vibrio (Reid et al., 1978; Long et al., 1981). In some organisms, for example, Aeromonas proteolytica (Litchfield & Prescott, 1970a,b) and a marine organism strain SA1 (Daatselaar & Harder, 1974) an efficient regulatory mechanism has developed. Endopeptidase and aminopeptidase production is repressed by high concentrations of amino acids, and induced by low concentrations, indicating an efficient mechanism for utilization of carbon and energy sources for growth and the production of extracellular enzymes.

Extracellular collagenase and serine protease production by V. alginolyticus are subject to end product inhibition by various amino acids and ammonium ions (Reid et al., 1978; Long et al., 1981). Collagenase production is inhibited by isoleucine and proline, which have been identified as the most common repressive amino acids in other bacteria (Glenn, 1976). Glycine and glutamine also repress collagenase production. The severe repression by glutamine suggested to Reid et al. (1978) that the enzymes involved in glutamine metabolism may be implicated in collagenase regulation. This hypothesis was supported by derepression of collagenase production by methionine sulfone, in the presence of ammonium ions. Glutamine, leucine and isoleucine were the most repressive amino acids

of alkaline protease production. Histidine was found to inhibit collagenase production but greatly increased alkaline protease synthesis. This suggests that there are separate control mechanisms for these two exoproteases.

In addition to end-product repression, some exoenzymes are subject to catabolite repression. Catabolite repression is the permanent repression of inducible or constitutive enzyme synthesis that occurs in the presence of glucose or some other rapidly metabolised carbon source (Priest, 1977).

Exoenzyme synthesis in V. parahaemolyticus (Tanaka & Iuchi, 1971), P. maltophilia (Boethling, 1975), S. marcescens (Bromke & Hammel, 1979), and in Vibrio (Reid et al., 1978; Long et al., 1981) is sensitive to catabolite repression. Cyclic adenosine 3',5'-monophosphate (cyclic AMP) overcomes catabolite repression of many inducible enzymes in E. coli (De Crombrugghe et al., 1969), however, it does not overcome catabolite repression in all cases. Extracellular collagenase and alkaline protease produced by V. alginolyticus are subject to catabolite repression by a number of different substrates but this repression is not relieved by cyclic AMP or cyclic guanosine 3',5'-monophosphate (cyclic GMP) (Reid et al., 1978; Long et al., 1981).

Environmental factors other than individual nutrients in the extracellular environment such as temperature and

oxygen also play a role in exoenzyme regulation but have not been widely investigated. The expression of the nitrogen fixation (nif) operon in the facultative anaerobe K. pneumoniae is sensitive to repression by $NH_4^+$ (Eady et al., 1978), oxygen (St. John et al., 1974; Eady et al., 1978) and temperature (Hennecke & Shanmugam, 1979). The hut operon in this organism is regulated by $NH_4^+$ and cyclic AMP (Prival et al., 1973), and by oxygen (Goldberg & Hanau, 1980). Studies on the regulation of nitrogenase synthesis by temperature (Hennecke & Shanmugam, 1979) and oxygen (Hill et al., 1981) have implicated the synthesis of specific proteins which appear to have a regulatory role.

Another approach to investigating exoenzyme regulation has been to look for regulatory mutations. Regulatory mutations at loci designated pap (Yoneda & Maruo, 1975), HPr (Higerd et al., 1972), sac $U^h$ and sac $Q^h$ (Kunst et al., 1974) have been reported to increase extracellular enzyme synthesis. However, the mechanism for enhanced secretion is not known.

Reid et al. (1978) and Long et al. (1981) investigated end product inhibition and catabolite repression of exoenzyme synthesis by V. alginolyticus. This study undertook the investigation of temperature and oxygen regulation and the involvement of possible regulatory proteins in extracellular collagenase and serine protease production. Since

histidine inhibits collagenase production (Reid et al., 1978) but enhances protease production (Long et al., 1981), the effect of histidine on intracellular protein synthesis was also examined.

## 2.2 MATERIALS AND METHODS

All % values are w/v unless otherwise stated.

### 2.2.1 Maintenance of V. alginolyticus

Media are listed in Appendix A. Specialized chemicals and their sources are listed in Appendix B.

For long term maintenance of V. alginolyticus, the strain was kept in the Tris-HCl buffer pH 7,6 containing $\underline{c}$ 5 mg $m\ell^{-1}$ of bovine Achilles' tendon collagen. For routine use the culture was maintained on the complex medium of Welton & Woods (1973).

### 2.2.2 Growth Conditions and Exoenzyme Production

To facilitate aeration loosely fitting aluminium foil caps were used and culture volumes were 5 to 10% of the flask volume (Reid, 1981).

Samples (10 mℓ) of overnight V. alginolyticus cultures in 2,5% casamino acids medium or minimal medium were inoculated into 100 mℓ 2,5% peptone or minimal media. Cultures were aerated on a Gallenkamp orbital incubator for 4 h at 30°C. The cells were harvested by centrifugation, washed and resuspended in 10 mℓ of 0,25% peptone, 0,5% tryptone or low SNP media at an $E_{600}$nm of 8,0 (concentrated stationary phase cultures). Cultures were incubated with or without aeration (standing cultures and cultures shaking at 140 rev. $min^{-1}$ on an orbital shaker) at 30 or 37°C. Samples were removed at time intervals and assayed for collagenase and protease activity. Growth was followed turbidimetrically at 600 nm using an MSE Spectro-plus spectrophotometer.

### 2.2.3 Collagenase Assay

Collagenase activity was assayed using the synthetic substrate phenylazobenzyloxycarbonyl-L-propyl-L-leucyl-glycyl-L-propyl-D-arginine (PZ-Pro-Leu-Gly-Pro-Arg) (Fluka, Buchs, Switzerland). The method used was adapted from that of Wünsch & Heidrich (1963). PZ-Pro-Leu-Gly-Pro-Arg (100 mg) was dispersed in 1 mℓ methanol and made up to 100 mℓ with Tris-HCl buffer pH 7,6. Substrate solution (200 µℓ) was added to 50 µℓ culture sample at 37°C and incubated for 15 min. The reaction was stopped by the addition of 0,5 mℓ of 0,5% citric acid. The total reaction volume was then extracted with 2,5 mℓ of ethyl acetate by

vigorously mixing on a Fisons Whirlimix for 15 secs. When the inorganic phase had settled, 2 mℓ of the overlying organic phase was withdrawn, briefly dried with ≤ 0,15 g anhydrous sodium sulphate, and the absorbance read at 320 nm. Each sample was assayed in duplicate and each experiment repeated at least twice (Reid, 1981). Collagenase activity was expressed as nkat $m\ell^{-1}$ where one katal is the amount of activity that converts one mole of substrate per second (Florkin & Stotz, 1973); (Reid, 1981).

### 2.2.4 Azocasein Protease Assay

Proteolytic activity was assayed using the substrate, azocasein. The method used was adapted from that of Millet (1970) and was as follows: 1 mℓ of a 0,5% solution of the substrate in 0,2 M Tris buffer, pH 7,2, was incubated with 0,5 mℓ of the test culture for 10 or 30 min at 37°C. The reaction was stopped by adding 2 mℓ of a 10% trichloroacetic acid (TCA) solution. After standing for 30 min at 5°C the mixture was filtered through Whatman paper No. 4, and 1,5 mℓ of 0,5 N NaOH was added to each 1,5 mℓ of filtered solution. The absorbance was determined at 440 nm.

This method was adapted to give maximum extracellular proteolytic activity produced by *V. alginolyticus*: 1 mℓ of a 2% solution of azocasein in 0,1 M Tris-HCl buffer,

pH 9,0, was incubated with 1 mℓ culture supernatant for 30 min at 37°C. The reaction was stopped by adding 2 mℓ of a 10% TCA solution and the assay procedure of Millet (1970) followed from here.

One unit of alkaline protease activity is defined as the amount of enzyme that gives an increase in absorbance of 0,1 at 440 nm in 30 min at 37°C. Each sample was assayed in duplicate and experiments repeated at least twice.

### 2.2.5 Determination of RNA, Protein and Lipid Synthesis

The effect of temperature and aeration on growth was determined on exponential and concentrated stationary phase cultures. Exponential phase cultures were obtained by inoculating overnight cultures (10 mℓ) into 90 mℓ fresh medium. The concentrated stationary phase cell suspensions were obtained as described above.

RNA and protein synthesis were determined on aerated stationary phase cell suspensions used for exoprotease production by the incorporation of $[^{3}H]$-uracil (2 $\mu$g m$\ell^{-1}$, 1 $\mu$Ci m$\ell^{-1}$) and $[^{14}C]$-protein hydrolysate (2 $\mu$g m$\ell^{-1}$, 0,5 $\mu$Ci m$\ell^{-1}$) respectively into TCA-precipitable material by the method of Eichenlaub & Winkler (1974). Samples (0,5 mℓ) were mixed with 0,5 mℓ cold 10% TCA containing 2 $\mu$g m$\ell^{-1}$ uracil or protein hydrolysate and placed on ice

for 30 min. The precipitate was collected on glass fibre filters using a millipore filter and washed with 5 mℓ cold 5% TCA and 5 mℓ cold 1% acetic acid. The filters were then dried at 50°C for 60 min, added to 10 mℓ scintillation fluid and counted in a Beckman scintillation counter.

Lipid synthesis was determined by the incorporation of $[^{14}C]$-acetate (2 μg $m\ell^{-1}$, 0,2 μCi $m\ell^{-1}$) into lipid fractions. Lipid was extracted using chloroform and methanol by the method of Bligh & Dyer (1959). Gently shake 1 mℓ sample with 1 mℓ chloroform and 2 mℓ methanol for 1 min. Add another 1 mℓ chloroform and shake for further 1 min. Add 1 mℓ distilled water and shake for 1 min and then filter on a millipore filter apparatus. A few minutes are allowed for complete separation and clarification and then the alcoholic layer is removed by aspiration. Samples are then placed at 50°C overnight to evaporate, 10 mℓ scintillation fluid is added to each sample vial and counted in a Beckman scintillation counter.

### 2.2.6 Toluene Treatment of Cells

Cells were treated with toluene to determine whether they would release preformed collagenase or become permeable to the substrate and indicate the presence of preformed collagenase. Toluene (0,2 mℓ) was mixed with 0,5 mℓ samples of culture medium and cell-free supernatant and

the collagenase activity was determined in the supernatant and in uncentrifuged culture samples.

### 2.2.7 Radioactive Labelling, Electrophoresis and Autoradiography

Concentrated stationary phase *V. alginolyticus* cultures were prepared in 2 mℓ low molecular weight peptone (refer to Chapter 4 for preparation) or low SNP media in a 25 mℓ flask and incubated under various conditions. The cells were pulse-labelled for 10 or 30 min by the addition of [$^{35}$S] -methionine (20 $\mu$Ci m$\ell^{-1}$). The cells were collected by centrifugation in a microfuge, washed twice with 0,4 M NaCl and resuspended at a 100-fold concentration of the original cell volume, in electrophoresis buffer containing 2,3% sodium dodecyl sulphate (SDS), 10% glycerol, 5% (v/v) mercaptoethanol and 0.0625 M Tris, pH 6,8. The samples were then boiled for 2 min and 2 $\mu\ell$ were added to 5 mℓ Scintillator 299 scintillation fluid and radioactivity measured in a Packard Tri-carb scintillation counter. Sample volumes containing $\leq$ 20 000 cpm were resolved by discontinuous linear and gradient SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE), according to the methods described by Laemmli (1970) and O'Farrell (1975). The samples were stacked at 100 V and then resolved on 0,5 x 140 x 170 mm slab gels at 200 V at 20°C. The gels were stained with Coomassie Brilliant Blue (0,05%), destained, washed and dried. Immediately prior to exposure to X-ray film, the dried gels were sprayed with En$^3$hance™ surface

autoradiography enhancer. Labelled protein bands were visualised by exposure of the dried gels to Kodak X-Omat MA X-ray film at -20°C for 14 d (Laskey & Mills 1975).

Growth hormone, ovalbumin, human albumin and transferrin with molecular weights 22 000, 43 000, 70 000 and 90 000 respectively were used as molecular weight markers in the linear gels. Cytochrome C, $\beta$-lactalbumin $\alpha$-chymotrypsinogen, ovalbumin and bovine serum albumin with molecular weights 12 300, 18 400, 25 700, 43 000 and 68 000 were used as molecular weight markers in the gradient gels.

The autoradiograph strips were scanned with a Beckman Du-8 spectrophotometer with a gel scanner attachment.

## 2.3 RESULTS

### 2.3.1 Effect of Temperature and Oxygen on Growth and Metabolism

Although the collagenase and alkaline protease were only produced in stationary phase, the effect of temperature and oxygen on growth of exponential and concentrated stationary phase cultures was investigated. No significant difference was found in growth rates of V. alginolyticus cultures over a temperature range from 25 to 44°C (Table 2.1 and Figure 2.1). The growth curves of concentrated stationary phase cultures at 30 and 37°C were very similar

TABLE 2.1: Effect of temperature on the exponential growth rate of V. alginolyticus in casamino acid medium.

| Time (h) | Growth rate expressed as a percentage of the growth at 30°C. | | | | |
|---|---|---|---|---|---|
| | 27°C | 34°C | 37°C | 39°C | 44°C |
| 2 | 79 | 116 | 99 | 114 | 115 |
| 4 | 96 | 102 | 104 | 122 | 103 |
| 6 | 103 | 107 | 100 | 107 | 110 |
| 7 | 107 | 105 | 100 | 107 | 109 |

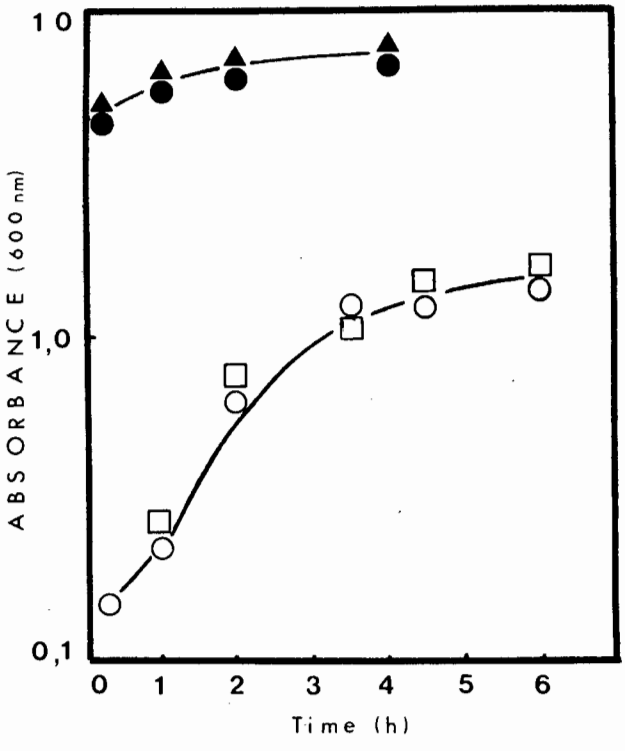



FIGURE 2.1: Effect of temperature on the growth of V. alginolyticus in casamino acid medium. Exponential aerated cultures at 30(0), 34(□) and 37°C (△). Concentrated stationary phase cultures aerated at 30°(●) and 37°C(▲). Each point is calculated as the average of four independent determinations.

(Figure 2.1) and shifting these cultures between 30 and 34°C, and between 30 and 37°C did not affect the growth curves.

The effect of temperature on RNA and protein synthesis was determined at the time of exoprotease production in aerated stationary phase V. alginolyticus cultures (Table 2.2). The levels of total RNA and protein synthesis during stationary phase were virtually unaffected by incubation at temperatures between 30 and 37°C. The addition of rifampicin (100 $\mu g\ m\ell^{-1}$) and chloramphenicol (100 $\mu g\ m\ell^{-1}$) caused 86 and 100% inhibition in RNA and protein synthesis respectively.

The growth rate of exponential phase cultures of V. alginolyticus was affected by aeration (Figure 2.2). Aerated cultures grew faster than standing cultures. However, aeration of concentrated stationary phase cultures at the time of exoprotease production did not enhance growth (Figure 2.2).

### 2.3.2 Effect of Temperature and Oxygen on Collagenase and Alkaline Protease Production

The stability of the collagenase and the alkaline protease in cell-free supernatant samples was determined in standing and shaking flasks at 30 and 30°C. Both enzymes were stable under standing and shaking conditions at 30 and 37°C and no loss in activity was observed over 6 h (Table 2.3). The highest yields of collagenase and serine

TABLE 2.2: Effect of temperature on RNA, protein and lipid synthesis in aerated stationary phase *V. alginolyticus* cells. RNA and protein synthesis were determined by the incorporation of $[^{3}H]$-uracil and $[^{14}C]$-protein hydrolysate respectively into TCA-precipitable material. Lipid synthesis was determined by incorporation of $[^{14}C]$-acetate into lipid fractions. Incorporation expressed as cpm $min^{-1}$ determined over 10 min.

| Culture Conditions | RNA synthesis$_1$ (cpm $min^{-1}$) | Protein synthesis$_1$ (cpm x $10^1$ $min^{-1}$) | Lipid synthesis$_1$ (cpm x $10^1$ $min^{-1}$) |
|---|---|---|---|
| 30°C | 430 | 250 | 370 |
| 37°C | 400 | 230 | 360 |
| 30°C + rifampicin (100 $\mu g$ $m\ell^{-1}$) | 60 | - | - |
| 30°C + chloramphenicol (100 $\mu g$ $m\ell^{-1}$) | - | 0 | - |

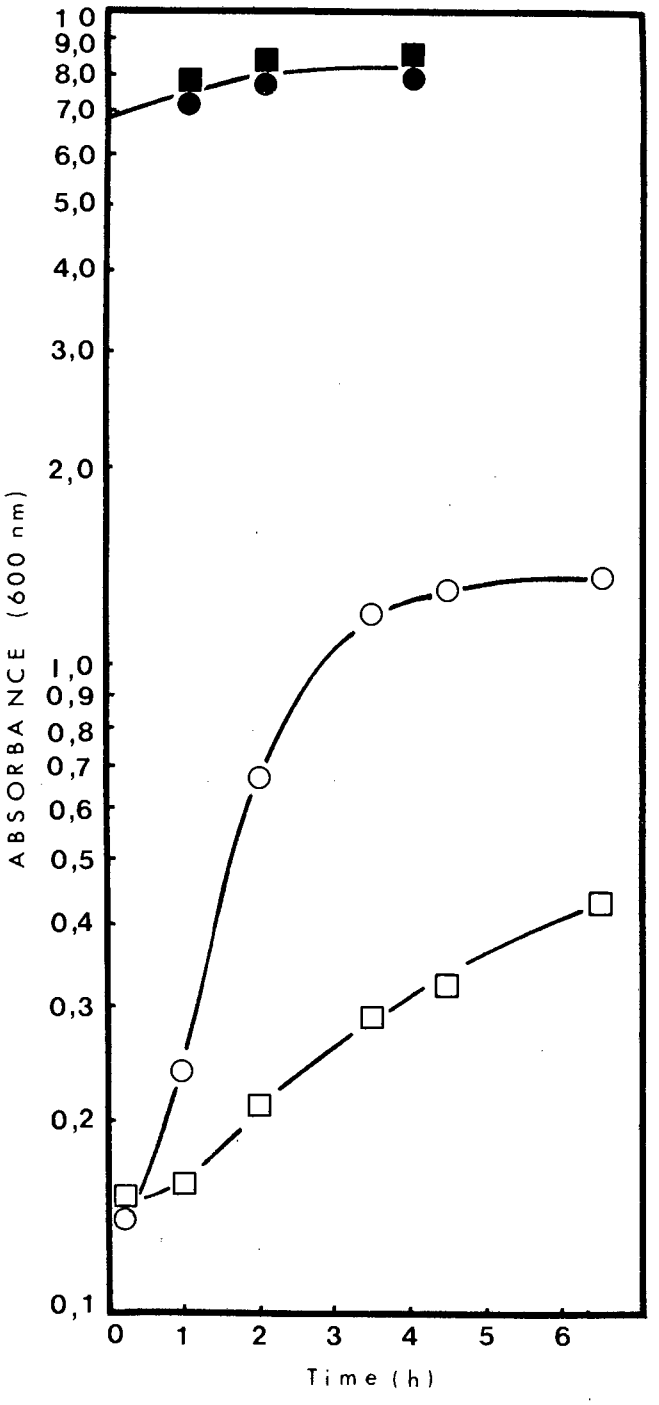



FIGURE 2.2: Effect of oxygen on the growth of V. alginolyticus in casamino acid medium. Exponential cultures at 30°C with (○) and without aeration (□). Concentrated stationary phase cultures at 30°C with (●) and without aeration (■). Each point is calculated as the average of four independent determinations.

TABLE 2.3: Stability of the collagenase and alkaline protease. Cell-free supernatant samples were assayed at time 0 h and then again after 6 h standing and shaking at 30 and 37°C.

| Enzyme Sample | Collagenase activity (nkat $m\ell^{-1}$) | | Protease activity (units $m\ell^{-1}$) | |
|---|---|---|---|---|
| | 0 h | 6 h | 0 h | 6 h |
| Standing 30°C | 18 | 18 | 0,6 | 0,6 |
| Shaking 30°C | 18 | 18 | 0,6 | 0,6 |
| Standing 37°C | 18 | 18 | 0,6 | 0,6 |
| Shaking 37°C | 18 | 18 | 0,6 | 0,6 |

protease were produced by V. alginolyticus at 30°C (Figure 2.3). No collagenase and reduced amounts of alkaline protease were produced at 37°C. Synthesis of the exoproteases was reinitiated after a temperature shift from 37 to 30°C (Figure 2.3). A temperature shift from 30 to 37°C had the opposite effect and enzyme production was inhibited (Figure 2.4). There was no difference in the production of the enzymes between 25 and 30°C but at temperatures above 30°C lower levels of enzyme were produced (incubation at 34 and 37°C resulted in a 50 and 100% reduction in collagenase activity respectively).

Aeration also affected collagenase and alkaline protease production. In shaking aerated cultures at 30°C normal levels of both enzymes were produced but in standing cultures at 30°C markedly lower levels of the collagenase and the alkaline protease were synthesized (Table 2.4). Shifting the cultures from shaking to standing conditions inhibited enzyme production and vice versa.

### 2.3.3 Effect of Temperature on Enzyme Secretion

If temperature inhibited a process involved in the secretion of the exoenzymes, an intracellular accumulation of collagenase and protease should occur at 37°C. This was investigated by treatment of a culture with chloramphenicol (100 $\mu g\ m\ell^{-1}$) at the time of shift down from 37 to 30°C. No release of collagenase was detected in the presence of

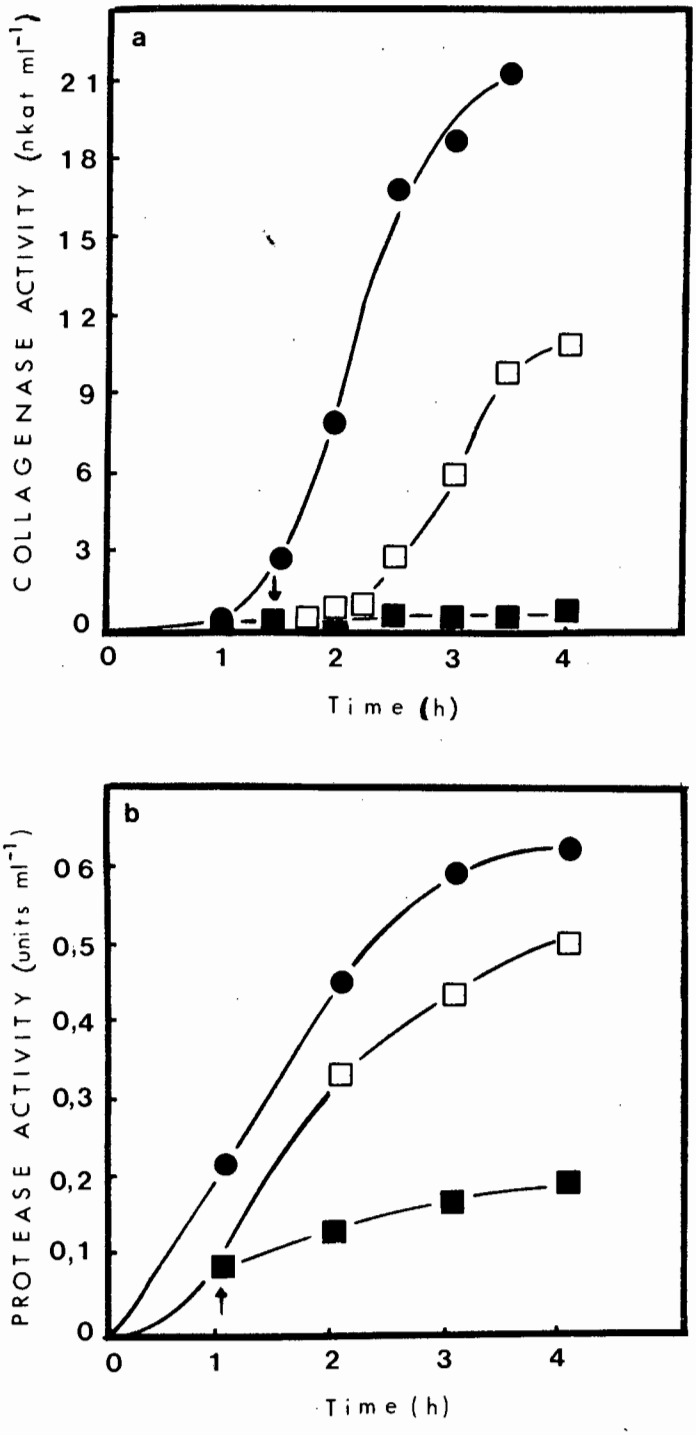



FIGURE 2.3: Effect of temperature on collagenase (a) and serine protease (b) production by V. alginolyticus. Cultures incubated at 30°C (●); 37°C (■) and initially at 37°C but shifted down to 30°C at 1 or 1,5 h (□). The arrow indicates the time of temperature shift.

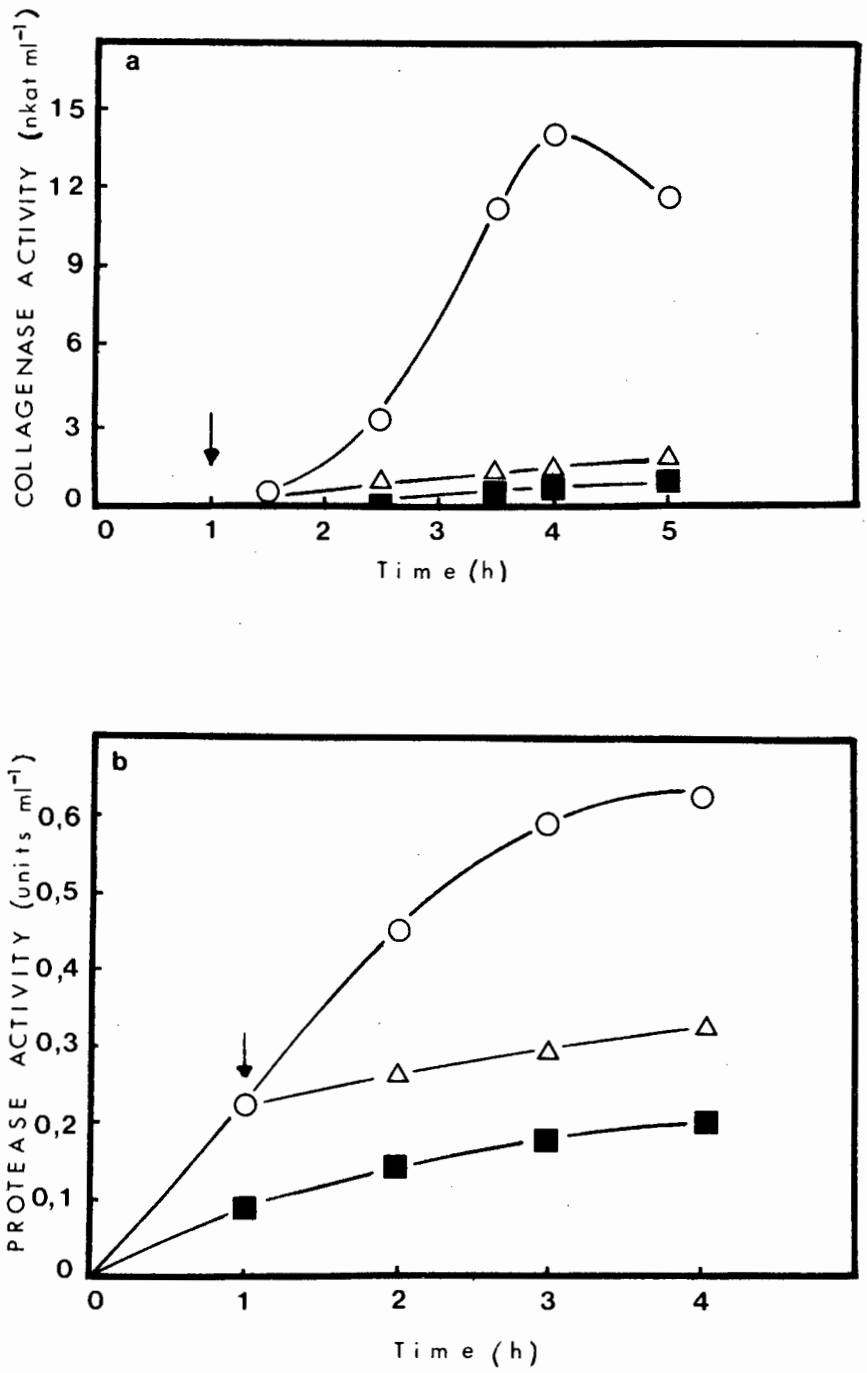



FIGURE 2.4: Effect of temperature on collagenase (a) and serine protease (b) production by V. alginolyticus. Cultures incubated at 30°C (○); 37°C (■) and initially at 30°C but shifted up to 37°C at 1 h (△). The arrow indicates the time of temperature shift.

TABLE 2.4: Effect of aeration on collagenase and serine protease production at 30°C. Exoproteases were determined after 90, 120 and 150 min in concentrated stationary phase cultures which were aerated by shaking or allowed to stand. Enzyme production was also determined in cultures which were shifted at 120 min from shaking to standing conditions and *vice versa*.

| Culture Conditions | Collagenase activity (nkat $m\ell^{-1}$) | | | Protease activity (units $m\ell^{-1}$) | | |
|---|---|---|---|---|---|---|
| | 90 | 120 | 150 min | 90 | 120 | 150 min |
| Shaking for 150 min | 2,9 | 6,6 | 8,9 | 0,30 | 0,35 | 0,38 |
| Standing for 150 min | 0,2 | 0,5 | 0,8 | 0,19 | 0,23 | 0,25 |
| Shaking 120 min Standing 30 min | 2,9 | 6,6 | 7,2 | 0,30 | 0,32 | 0,34 |
| Standing for 120 min Shaking 30 min | 0,2 | 0,5 | 2,2 | 0,19 | 0,23 | 0,30 |

chloramphenicol after shift down (Figure 2.5). In addition toluene treatment of cells incubated at 30 and 37°C revealed no intracellular accumulation of either exoproteases (Table 2.5).

A lipid intermediate is required for the secretion of the collagenase and alkaline protease (refer to Chapter 3). Incubation at 30 and 37°C did not affect the rate of total lipid synthesis (Table 2.2).

### 2.3.4 Effect of Temperature and Oxygen on the Synthesis of Intracellular Proteins

The effect of temperature (30 and 37°C) and aeration on the synthesis of proteins in *V. alginolyticus* cells was determined by pulse-labelling with [$^{35}$S]-methionine for 10 and 30 min in the low molecular weight peptone medium. Densitometric comparison of the labelled intracellular protein bands after SDS-PAGE of extracts from aerated 30 and 37°C cultures indicated that the synthesis of a 100 000 molecular weight protein (referred to as 100K protein throughout this thesis) was greatly increased at 37°C (Figures 2.6 and 2.7). No consistent variations in the other protein bands were observed at 30 and 37°C. The increased synthesis of the 100K protein was also observed at 37°C in pulse-labelled cells in low SNP medium (Figure 2.13). The effect of temperature shifts from 30 to 37°C and *vice versa*, on the synthesis of the 100K protein was also

of shift down from 37 to 30°C. Temperature shift from 37 to 30°C with (▽) and without chloramphenicol (□) at time 1,5 h. The arrow indicates the time of the temperature shift.

TABLE 2.5: Effect of toluene treatment on collagenase activity at 30 and 37°C.

| Age of culture (min) | Collagenase activity (nkat $m\ell^{-1}$) | | | |
|---|---|---|---|---|
| | Supernatant samples | | Uncentrifuged culture samples | |
| | 30 | 37 | 30 | 37 |
| 60 | 0,86 | 0,72 | 0,86 | 0,72 |
| 90 | 2,59 | 1,29 | 2,16 | 0,78 |
| 120 | 13,42 | 4,89 | 10,37 | 3,45 |
| 150 | 17,29 | 5,18 | 15,16 | 6,05 |

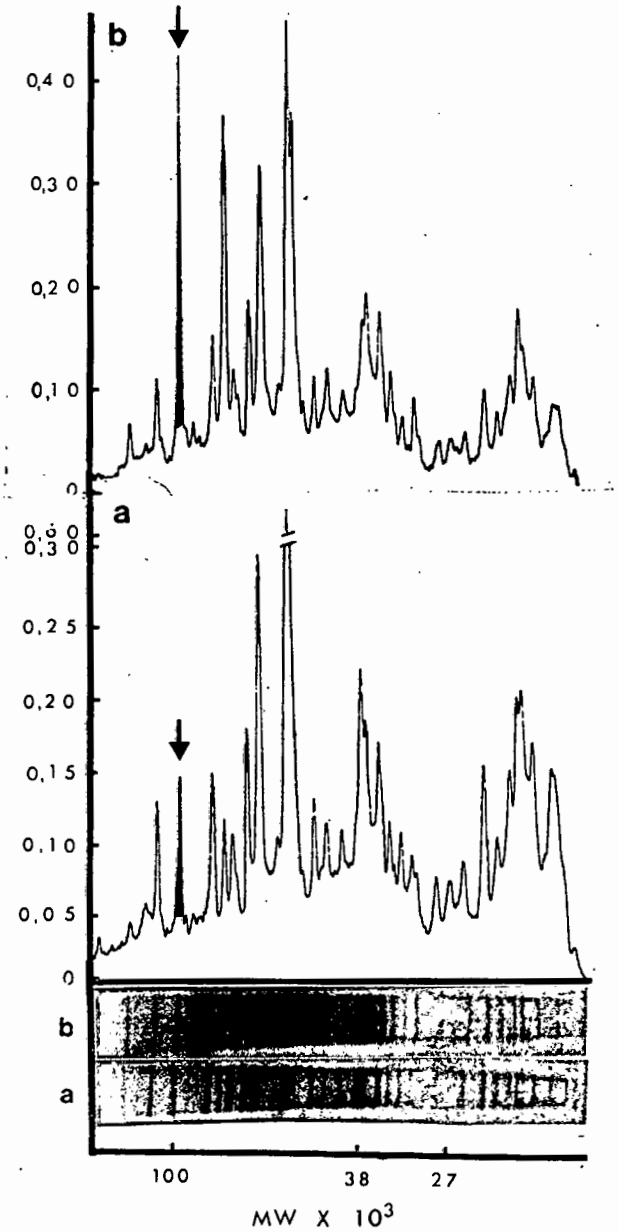



FIGURE 2.6: Densitometric comparison of $[^{35}S]$ - methionine labelled protein bands after 10% linear SDS-PAGE of extracts of aerated peptone cultures at 30(a) and 37°C(b). The cells were pulse-labelled for 10 min. The 100K protein is indicated by the arrow.

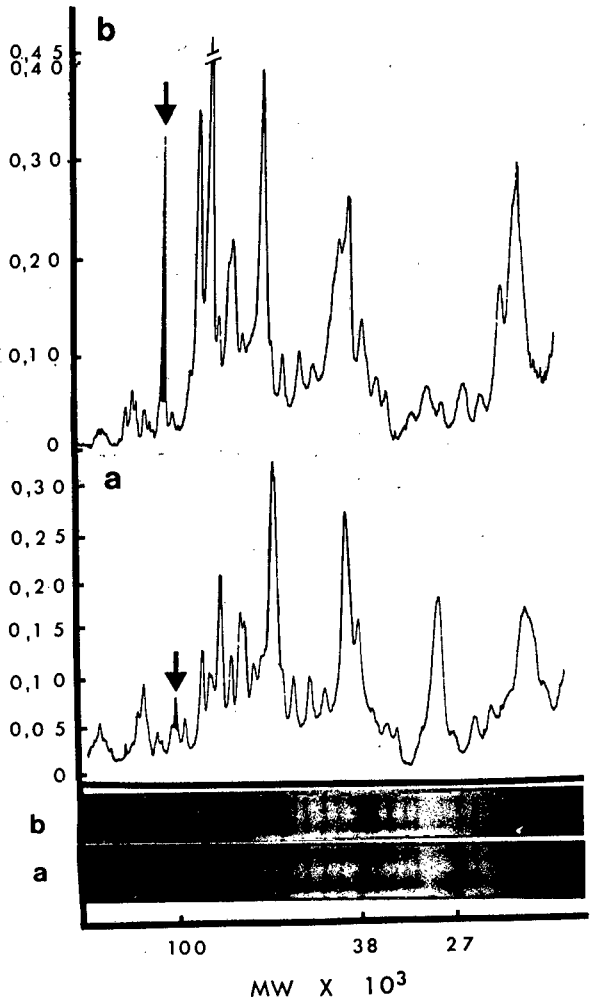



FIGURE 2.7: Densitometric comparison of $[^{35}S]$ - methionine labelled protein bands after 10% linear SDS-PAGE of extracts of aerated peptone cultures at 30 (a) and 37°C (b). The cells were pulse-labelled for 30 min. The 100K protein is indicated by the arrow.

determined. Cells shifted from 30 to 37°C showed an increased synthesis of the 100K protein within 10 min (Figure 2.8) and 30 min (Figure 2.9) after the shift. There was a lag of 30 min before basal levels of the 100K protein were observed in cells which were shifted from 37 to 30°C.

Densitometric comparisons of labelled protein bands after SDS-PAGE of extracts of aerated and non-aerated low-molecular-weight peptone cultures at 30°C indicated that the synthesis of the 100K protein was increased in non-aerated cultures pulse-labelled for 10 and 30 min which did not produce collagenase or serine protease (Figures 2.10 and 2.11). No other significant or consistent differences in the pulse-labelled protein profiles were observed in aerated and non-aerated cultures.

### 2.3.5 Effect of Histidine on the Synthesis of Intracellular Proteins

The addition of histidine (0,5%) to minimal media enhanced the production of serine alkaline protease activity at 30°C (Long et al., 1981), but inhibited collagenase activity (Reid et al., 1978). The effect of histidine on the synthesis of intracellular proteins was investigated by pulse-labelling aerated cells with $[^{35}S]$-methionine for 30 min in low SNP medium at 30 and 37°C. The extracellular serine protease activities of the cultures were also determined. Densitometric comparison of the labelled

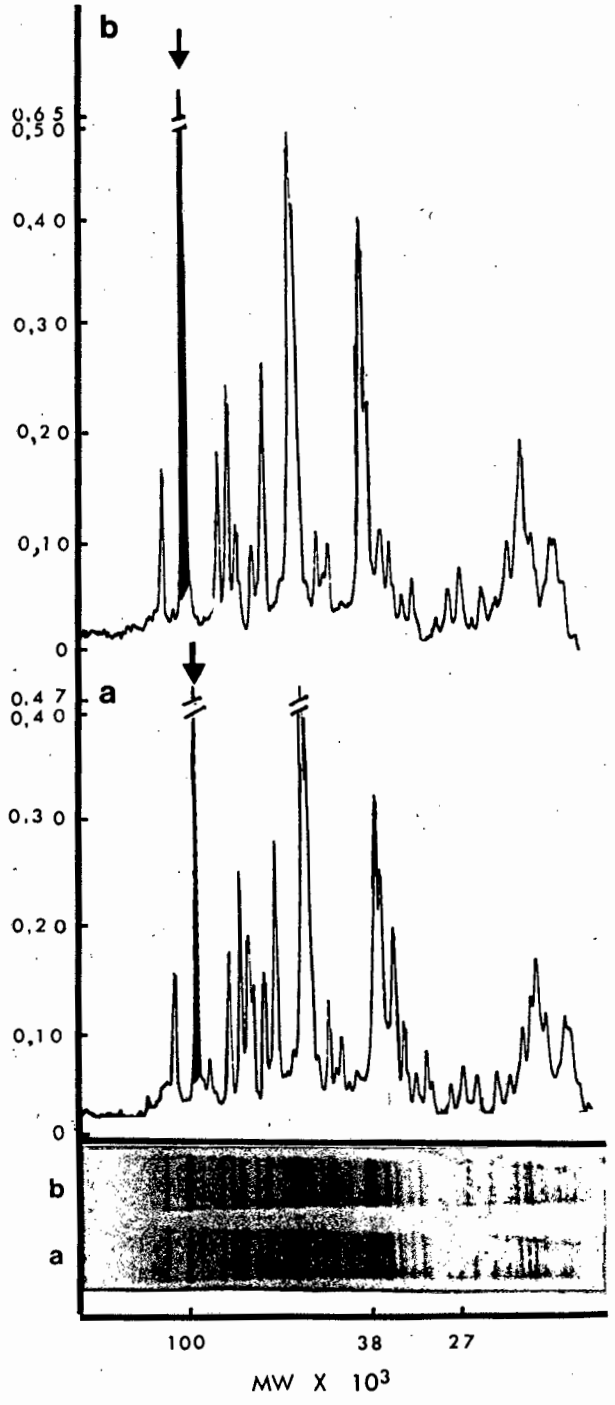



FIGURE 2.8: Densitometric comparison of $[^{35}S]$- methionine labelled protein bands after 10% linear SDS-PAGE of extracts of aerated peptone cultures shifted from 30 to 37°C (a) and from 37 to 30°C (b). The cells were pulse-labelled for 10 min after the shift. The 100K protein is indicated by the arrow.

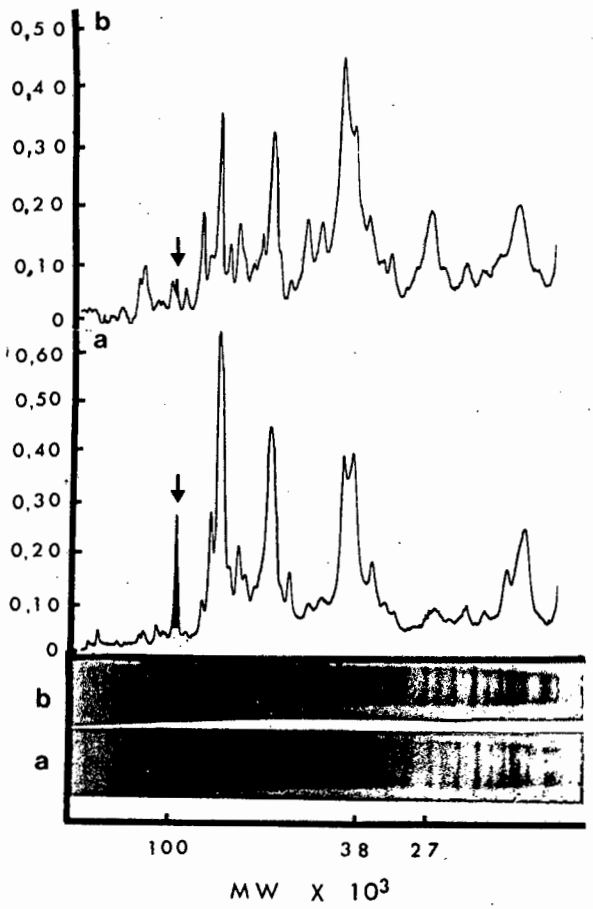



FIGURE 2.9: Densitometric comparison of $[^{35}S]$- methionine labelled protein bands after 10% linear SDS-PAGE of extracts of aerated peptone cultures shifted from 30 to 37°C (a) and from 37 to 30°C (b). The cells were pulse-labelled for 30 min after the shift. The 100K protein is indicated by the arrow.

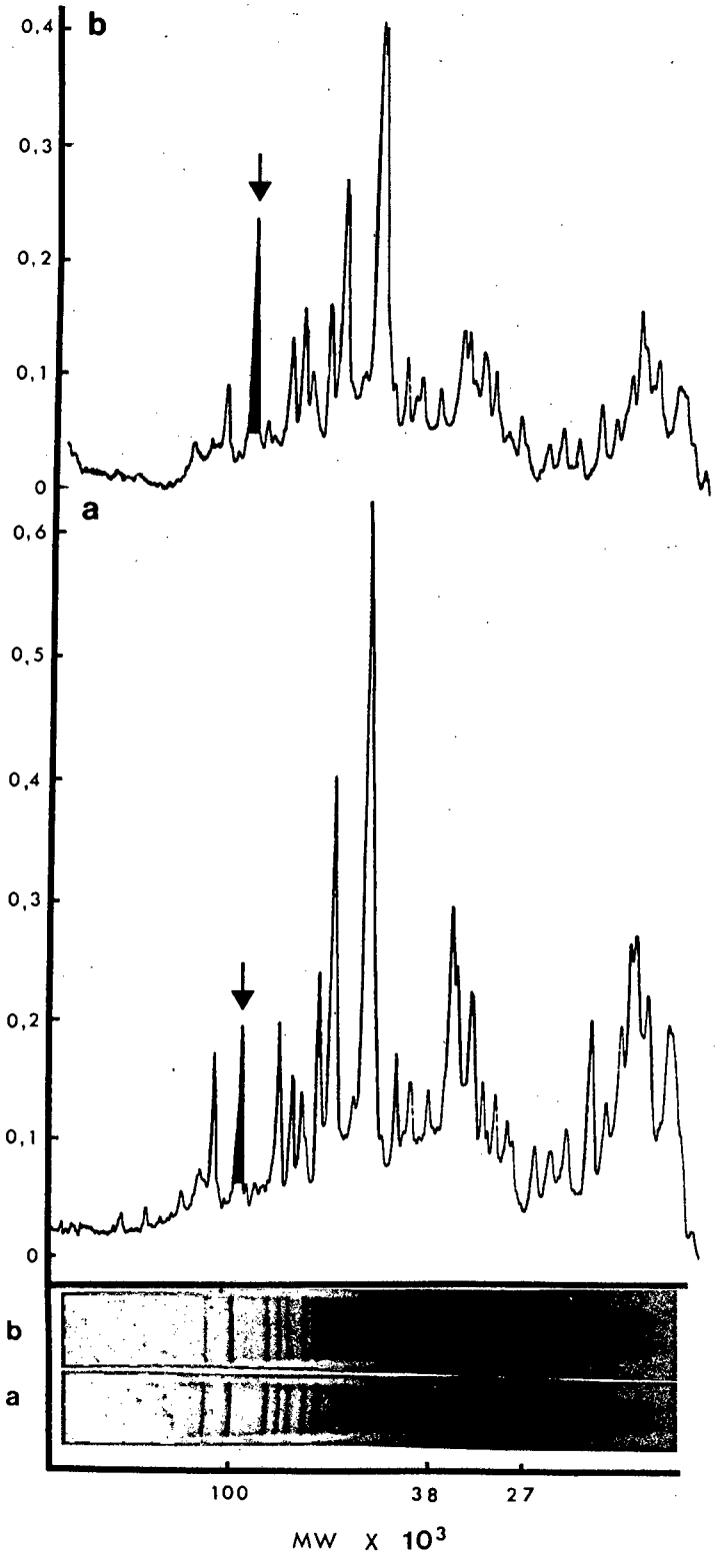



FIGURE 2.10: Densitometric comparison of $[^{35}S]$-methionine labelled protein bands after 10% linear SDS-PAGE of extracts of peptone cultures incubated at 30°C with (a) and without (b) aeration. The cells were pulse-labelled for 10 min. The 100K protein is indicated by the arrow.

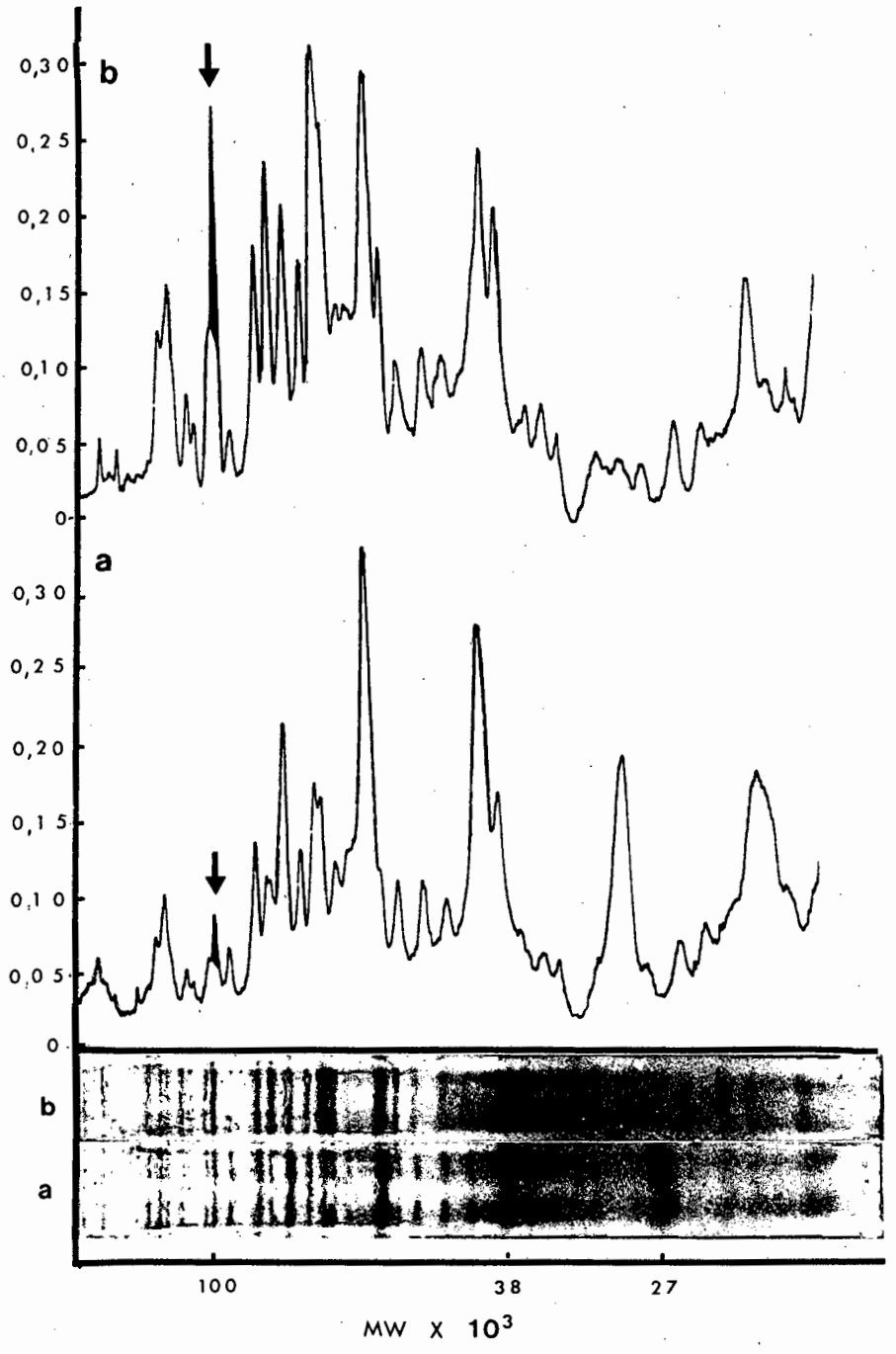



FIGURE 2.11: Densitometric comparison of $[^{35}S]$-methionine labelled protein bands after 10% linear SDS-PAGE of extracts of peptone cultures incubated at 30°C with (a) and without (b) aeration. The cells were pulse-labelled for 30 min. The 100K protein is indicated by the arrow.

protein bands after SDS-PAGE of extracts from 30°C cultures in the presence and absence of histidine indicated that a 52 000 molecular weight protein (referred to as 52 K protein throughout this thesis) was induced by histidine (Figure 2.12). Histidine did not affect the synthesis of the 100K protein at 30°C and low levels of this protein were observed (Figure 2.12). At 37°C in the absence of histidine there was very little synthesis of the 52K protein (Figure 2.13). However, the production of the 100K protein was stimulated (Figure 2.13). The addition of histidine to cultures incubated at 37°C induced the synthesis of the 52K protein but the synthesis of the 100K protein was not enhanced (Figure 2.13). Determination of extracellular serine protease activity indicated that histidine overcame the repression of protease production by temperature (37°C). No extracellular serine protease activity was detected at 37°C in the absence of histidine but in the presence of histidine (0,5%) 2,0; 2,5 and 2,7 units $m\ell^{-1}$ of serine protease activity were obtained after 1, 2 and 3 h incubation respectively. At 30°C, in the presence of histidine, 2,2; 3,0 and 3,2 units $m\ell^{-1}$ of serine protease activity were obtained after 1, 2 and 3 h respectively.

### 2.3.6 Effect of $(NH_4)_2SO_4$ on the Synthesis of Intracellular Proteins

The production of collagenase and the serine proteases was subject to end product repression by $(NH_4)_2SO_4$ (200 mM-N) (Reid et al., 1978; Long et al., 1981). Methionine sulfone

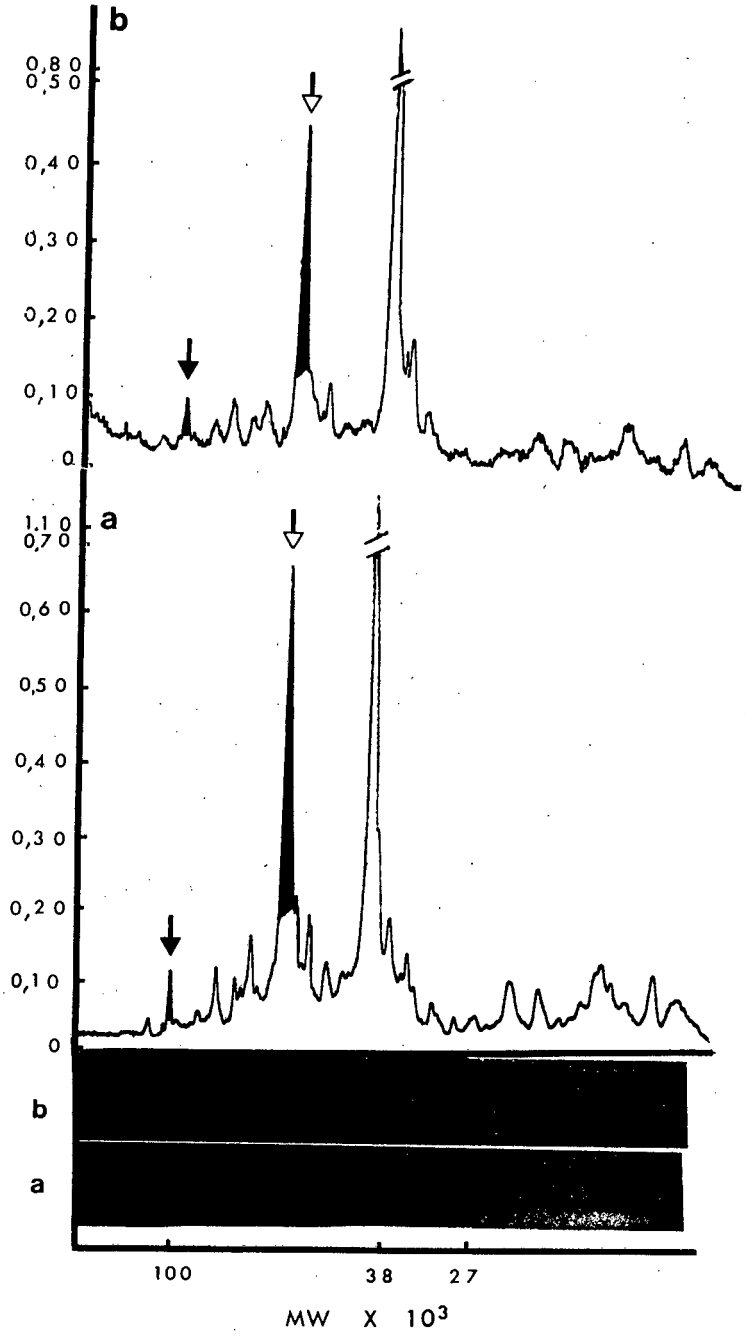



FIGURE 2.12: Densitometric comparison of $[^{35}S]$- methionine labelled protein bands after (6-15%) gradient SDS-PAGE of extracts of aerated minimal medium cultures with (a) and without (b) histidine (0,5% w/v). The cells were pulse-labelled for 30 min in cultures incubated at 30°C. The positions of the 52K and 100K proteins are indicated by open and closed arrows respectively.

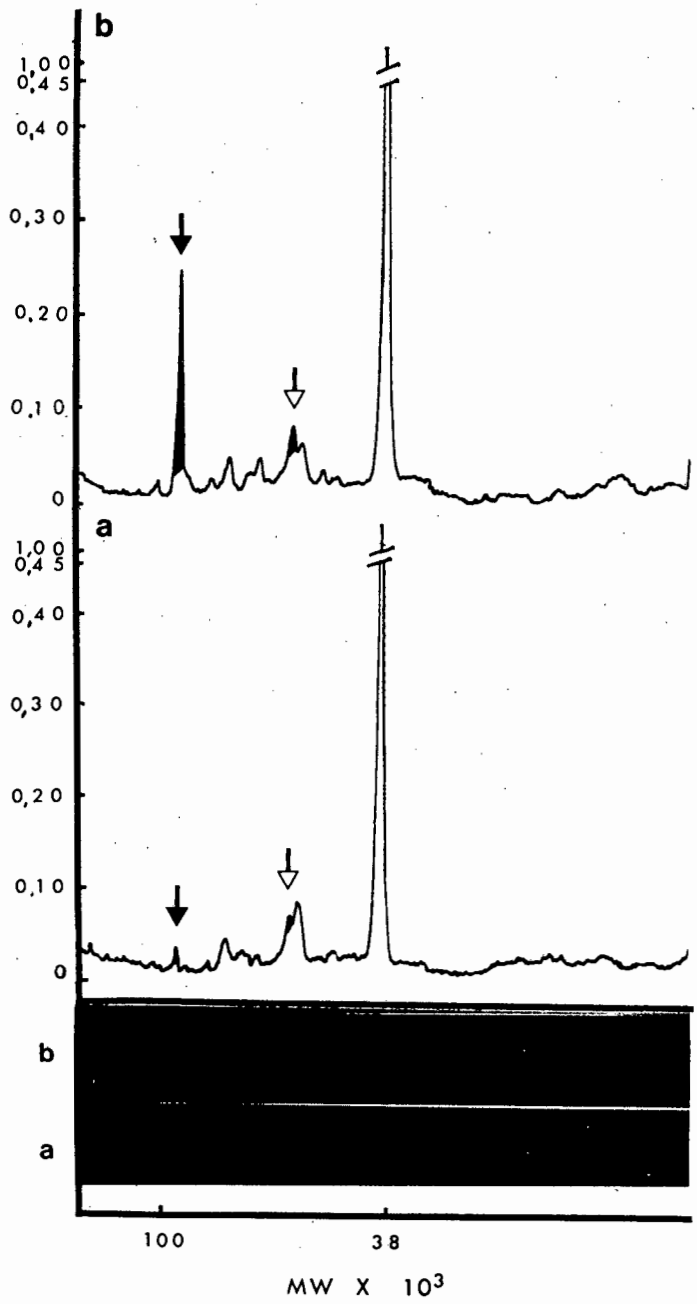



FIGURE 2.13: Densitometric comparison of $[^{35}S]$ - methionine labelled protein bands after (6-15%) gradient SDS-PAGE of extracts of aerated minimal medium cultures with (a) and without (b) histidine (0,5% w/v). The cells were pulse-labelled for 30 min in cultures incubated at 37°C. The positions of the 52K and 100K proteins are indicated by open and closed arrows respectively.

has been shown to reverse the repression of nitrogenase synthesis in K. aerogenes (Brenchley, 1973).

The effect of $(NH_4)_2SO_4$ and methionine sulfone on the synthesis of intracellular proteins was investigated in low molecular weight peptone medium at 30°C. Densitometric comparison of the labelled intracellular protein bands after SDS-PAGE indicated that the level of the 100K protein was increased when collagenase production was repressed by $(NH_4)_2SO_4$ (Figure 2.14). The addition of methionine sulfone to $(NH_4)_2SO_4$ repressed cultures, caused a reduction in the level of the 100K protein (Figure 2.14). Inconsistent variations in other protein bands were also observed. A 38 000 molecular weight protein appeared to be enhanced by $(NH_4)_2SO_4$ and methionine sulfone (Figure 2.14).

## 2.4 DISCUSSION

The collagenolytic V. alginolyticus strain was originally isolated from damaged hides and shown to cause leather decay (Welton & Woods, 1973; Woods et al., 1973). Although the source of infection is the animal itself and the bacteria are on the hides of living animals, no sign of hide decay was observed on either live animals or hides immediately after slaughtering (Rawlings et al., 1974). Since the bacteria are in the hair follicles and sweat glands and as there is a difference in temperature between the internal

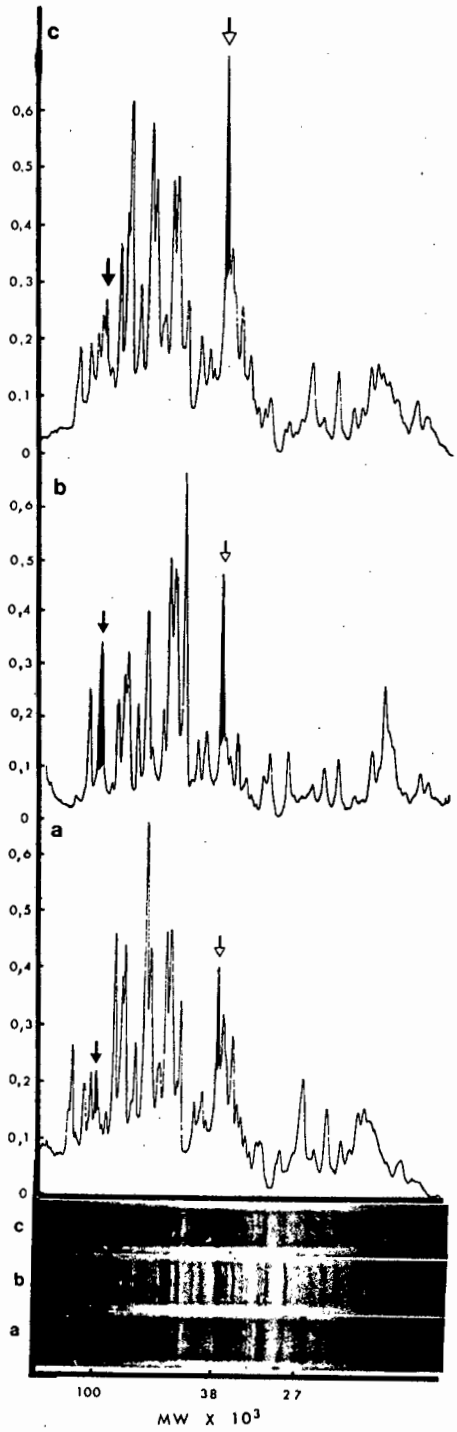



FIGURE 2.14: Densitometric comparison of $[^{35}S]$ - methionine labelled protein bands after 10% linear SDS-PAGE of extracts of an aerated peptone culture(a) and cultures containing $(NH_4)_2SO_4$ (200 mM-N)(b) and $(NH_4)_2SO_4$ (200 mM-N) + methionine sulfone (25 $\mu g/m\ell^{-1}$) (c). Cultures were incubated at 30°C and pulse-labelled for 30 min. The positions of the 38 000 and 100 000 molecular weight proteins are indicated by open and closed arrows respectively.

layers of a hide of a live animal and the same region once it is removed from the animal, the effect of temperature on collagenase and alkaline protease synthesis was investigated.

Collagenase and alkaline protease production were found to be regulated by temperature and oxygen. A shift up from the optimum temperature (30°C) to 34 or 37°C markedly inhibited collagenase production and reduced alkaline protease production. Temperature shifts from 37 to 30°C reinitiated extracellular enzyme synthesis, whereas shifts from 30 to 37°C inhibited enzyme synthesis.

Aeration also affected collagenase and alkaline protease production. Non-aerated cultures showed markedly reduced levels of exoenzyme synthesis.

The growth rates of V. alginolyticus cells were remarkedly constant over a broad range of temperatures from 25 to 44°C. The range of temperatures over which growth is possible may be as much as 50 degrees for some bacteria (Stanier et al., 1976). However, the growth rate of bacteria over a temperature range varies greatly. E. coli has a near constant growth rate over a small range of temperatures (37 to 42°C) (Herendeen et al., 1979). It is surprising, therefore, that a constant growth rate is exhibited by V. alginolyticus over such a wide temperature range.

Since growth and macromolecular synthesis are not affected by temperature at the time of exoprotease production, the control of exoprotease production by temperature is likely to be specific. It is unlikely that temperature inhibited the secretion of the enzymes as there is no rapid release of collagenase and serine proteases after temperature shift down. Furthermore, chloramphenicol inhibited the production of the enzymes when added at the time of temperature shift down. This suggests that temperature affects the synthesis of specific proteins. The involvement of specific proteins in the regulation of nitrogenase synthesis in K. pneumoniae by temperature and oxygen has been suggested by Hennecke & Shanmugam (1979) and Hill et al. (1981). Several other enzyme systems in the enterobacteriaceae are apparently regulated by $O_2$, fumarate reductase in E. coli (Spencer & Guest, 1973), respiration rate in bacteria (Harrison, 1976) expression of ChlC gene, the structural gene for nitrate reductase, in E. coli (Fimmel & Haddock, 1979), the hut operons in K. pneumoniae (Goldberg & Hanau, 1980) and hydrogenase and hydrogenlyase in Aerobacter aerogenes, E.coli Proteus vulgaris and S. oranienburg (Pichinoty, 1962).

Temperature has also been shown to affect the formation of bacterial flagella (Fuerst & Hayward, 1980) fatty acid and phospholipid composition of low obligately psychrophilic Vibrio spp (Bhakoo & Herbert, 1979), and regulation of peptidoglycan biosynthesis in E. coli during growth temperature up-shift (Vanderwel & Ishiguro, 1981).

To determine whether temperature and oxygen affect the synthesis of specific proteins, V. alginolyticus cells were labelled under various conditions, and the intracellular protein profiles were examined. Synthesis of a 100K protein was induced by either raising the temperature to 37°C, lack of oxygen or the addition of $(NH_4)_2SO_4$. These conditions inhibited the synthesis of collagenase and serine proteases. This suggests that the 100K protein is not one of the exoproteases and molecular weight determinations of the collagenase and serine proteases confirm this suggestion. Collagenase consists of two subunits of molecular weight 35 000 each (Keil-Dhoula & Keil, 1978) and the serine proteases all have molecular weights $< 30\ 000$ (refer to Chapter 5).

It is unlikely that the 100K protein represents the accumulation of a precursor protein which is then processed as the 100K protein did not accumulate in the presence of quinacrine and lidocaine (refer to Chapter 3). The 100K protein may have a role as a repressor of collagenase and serine protease synthesis. Studies with methionine sulfone which reversed the repression of collagenase synthesis by $(NH_4)_2SO_4$ and decreased the synthesis of the 100K protein support the suggestion that the 100K protein has a regulatory role.

Temperature regulation of nitrogenase synthesis in K. pneumoniae (Hennecke & Shanmugam, 1979) has been shown to involve the

synthesis of a 60 000 molecular weight protein at increased levels at 37°C but is repressed at 30°C when nif-encoded proteins are synthesized. Hill et al. (1981) reported that the nif L product is involved as a negative effector in the $O_2$-regulatory mechanism specific to nitrogen fixation.

Investigations on the control of the stimulation of serine protease production by histidine suggest that a 52K protein may also have a regulatory role. The synthesis of the 52K protein and the 100K protein showed opposite responses to histidine and the levels of the 100K protein were always very low in the presence of histidine. Furthermore, histidine reversed the repression of serine protease synthesis at 37°C. A possible explanation of these results is that the 52K protein acts as a negative effector of the 100K protein which results in the synthesis of the serine proteases at the non-permissive temperature.

Further work involving the isolation of mutants is necessary. Mutants which have overcome inhibition by temperature and lack of oxygen and which do not show serine protease enhancement with histidine would be valuable in determining the exact nature of these regulatory mechanisms. As regards the 100K and 52K proteins which appear to have a role in regulation by temperature, oxygen and histidine, it would be necessary to isolate and purify these proteins.

# THE EFFECT OF CERULENIN, QUINACRINE AND LIDOCAINE ON EXOPROTEASE SECRETION

**Summary:** Cerulenin inhibited the production of extracellular collagenase and serine proteases by 75 and 50% respectively. Although quinacrine markedly inhibited collagenase production, only transient inhibition of serine protease production was observed. O-phenanthroline which also inhibits the penicillinase-releasing protease, severely inhibited serine protease production. The anaesthetic lidocaine inhibited both collagenase and serine protease production. Lidocaine and quinacrine added simultaneously to cultures, showed a synergistic effect on inhibition of collagenase production. Treatment with lidocaine and quinacrine showed altered synthesis of three different intracellular proteins.

## 3.1 INTRODUCTION

The production of exoenzymes depends on the formation of a proper functional link between the ribosomes and the membrane (Smith et al., 1977) and hence is expected to be vulnerable to subtle alterations in membrane organisation (Fishman et al., 1980). Alterations in the physical state of bacterial membranes can be achieved by treating cells with antibiotics such as cerulenin (which inhibits

lipid synthesis and causes changes in membrane composition, Ōmura, 1976), and with a variety of alcohols and anaesthetics. Cerulenin was originally isolated as an antifungal antibiotic (Hata et al., 1960). It inhibits fatty acid synthesis by specific inactivation of -ketoacyl-acyl carrier protein synthetase (D'Agnolo et al., 1973). Cerulenin has been shown to affect the production of extracellular proteins in B. amyloliquefaciens (Paton et al., 1980), S. aureus enterotoxin A production (Berkeley et al., 1978), levansucrase production by B. licheniformis (Caulfield et al., 1979) and B. subtilis (Petit-Glatron & Chambert, 1981), and assembly of chromatophore membranes of Rhodopseudomonas sphaeroides (Broglie & Niederman, 1979). In B. licheniformis a protease removes the phospholipopeptide sequence from membrane-bound penicillinase, allowing it to be secreted. This protease is inhibited by quinacrine and O-phenanthroline (Traficante & Lampen, 1977).

Studies on the effects of cerulenin and quinacrine on the synthesis of S. aureus enterotoxin A (Berkeley et al., 1978) and on levansucrase production by B. licheniformis (Caulfield et al., 1979) suggested that a lipid intermediate/exoenzyme releasing protease mechanism was involved in the secretion of these enzymes. This work was supported by Paton et al. (1980) who studied the effect of cerulenin on extracellular and membrane proteins in B. amyloliquefaciens. Their results suggested that cerulenin affected the availability of lipid directly concerned with the secretion process.

Membrane proteins were unaffected by cerulenin. However, Petit-Glatron & Chambert (1981) found that in B. subtilis export of levansucrase is not related to fatty acid synthesis. In the presence of cerulenin concentrations which completely inhibited de novo fatty acid synthesis, the half-life of levansucrase mRNA was unmodified, as was the lag time of induction of levansucrase. They suggested that cerulenin inhibition of exoenzyme synthesis is a result of its physicochemical interaction with the membrane. This was supported by inhibition of levansucrase synthesis by ethanol and dodecanoic acid. These two compounds modify the membrane and inhibit exoenzyme synthesis but are not metabolized by the cells.

Recently, more workers have been studying the effects of alcohols and anaesthetics on membranes and related functions. The interaction of local anaesthetics with membranes has been studied using natural eucaryotic membranes (Feinstein et al., 1975), artificial membranes (Papahadjopoulos, 1972) and bacterial cells (Silva et al., 1979). Silva et al. (1979) studied the membrane effects of chlorpromazin, nupercaine, tetracaine and procaine using B. cereus, B. megaterium, B. subtilis and S. faecalis, protoplasts from S. faecalis and isolated membranes from B. subtilis. The effects of the anaesthetics included growth inhibition, reduction in the number of viable cells, lysis of protoplasts, permeability changes, characteristic ultrastructural alterations and inhibition of membrane-bound enzymatic activities.

The membrane activity of local anaesthetics is dependent on their interaction with the phospholipid components of biomembranes (Papahadjopoulos, 1972). This interaction is fundamentally of a hydrophobic character. Several anaesthetics have been shown to cause increased fluidity of membranes (Papahadjopoulos et al., 1975; Feinstein et al., 1975; Silva et al., 1979), and by adding their own bulk to the lipoid molecules they can increase molecular packing in the membrane (Shanes, 1960).

Silva et al. (1979) suggest that the disturbances in membrane phospholipids by anaesthetics may account for their inhibition of membrane-bound enzymatic activities. Tribhuwan et al. (1970) and Tribhuwan & Pradhan (1977) reported that whereas phenethyl alcohol and procaine suppressed the formation of a secreted enzyme, alkaline phosphatase, in E. coli the formation of $\beta$-galactosidase, a cytoplasmic enzyme, was not preferentially inhibited. Both phenethyl alcohol and procaine inhibited the conversion of inactive monomer subunits to active dimer alkaline phosphatase enzyme. Studies by Lazdunski et al. (1979) and Pagès & Lazdunski (1981) with phenethyl alcohol and procaine also showed inhibition of processing of periplasmic proteins in E. coli. Procaine also inhibits carbohydrate transport (Granett & Villarejo, 1981) and the production of the major outer membrane protein of E. coli (Pugsley et al., 1980).

Since synthesis of extracellular proteases in bacteria is closely linked to their secretion, it is conceivable that

both processes may be selectively inhibited by changes in the cytoplasmic membrane, and not just post-translational events. The local anaesthetics procaine and piperocaine block induction of plasmid-determined enzymatic activities involved in the metabolism of n-alkanes in P.patida (Benson, 1979). Benson suggests that these compounds prevent induction by altering the membrane such that inducer recognition cannot take place. The exact mechanisms of induction and secretion of exoenzymes in V. alginolyticus are not understood. The following work was carried out in an attempt to clarify the situation.

## 3.2 MATERIALS AND METHODS

The details of the materials and methods utilized in the following experiments have been described in Chapter 2.

### 3.2.1 Growth Conditions, Enzyme Production and Enzyme Assays

Samples (10 mℓ) of overnight V. alginolyticus cultures in 2,5% casamino acids or minimal media were inoculated into 100 mℓ 2,5% peptone or minimal media. Cultures were incubated for 4 h at 30°C in an orbital shaker. The cells were harvested by centrifugation, washed and resuspended in 0,25% small molecular weight peptone fraction (prepared as in Chapter 4), 0,25% whole peptone or 0,5% tryptone. Samples were removed at time intervals and assayed for

collagenase by means of the Wünsch & Heidrich (1963) method, or for serine protease activity by means of the azocasein assay. All inhibitors were added at 0 h after resuspension of cells into fresh medium unless otherwise stated.

Protein synthesis was determined by the incorporation of $[^{14}C]$-protein hydrolysate (2 $\mu g\ m\ell^{-1}$, 0,5 $\mu Ci\ m\ell^{-1}$) into TCA-precipitable material by the method of Eichenlaub & Winkler (1974). Lipid synthesis was determined by incorporation of $[^{14}C]$-acetate (2 $\mu g\ m\ell^{-1}$, 0,2 $\mu Ci\ m\ell^{-1}$) into lipid fractions by the method of Bligh & Dyer (1959).

### 3.2.2 Radioactive Labelling, Electrophoresis and Autoradiography

Concentrated stationary phase V. alginolyticus cultures were pulse-labelled for 30 min by the addition of $[^{35}S]$ methionine (20 $\mu Ci\ m\ell^{-1}$). The cells were collected by centrifugation in a microfuge, washed twice with 0,4 M NaCl and resuspended at a 100 fold concentration of the original cell volume in electrophoresis buffer. Sample volumes containing $\leq$ 20 000 cpm were resolved by discontinuous linear and gradient SDS-PAGE according to the methods described by Laemmli (1970) and O'Farrell (1975). Dried gels were sprayed with En$^3$hance$^{TM}$ spray, surface autoradiography enhancer prior to exposure to Kodak X-Omat X-ray film at -20°C for Du-8 (Laskey & Mills, 1975). The autoradiograph strips were scanned with a Beckman Du-8 spectrophotometer with a gel scanner attachment.

## 3.3 RESULTS

### 3.3.1 Effect of Cerulenin, Quinacrine and O-phenanthroline on Collagenase and Serine Protease Activity

Cerulenin inhibited the production of collagenase and serine protease by 75 and 50% respectively (Figures 3.1 and 3.2). Cerulenin did not inhibit the cell-free exoprotease activities (Table 3.1). Although cerulenin (5 $\mu g\ m\ell^{-1}$) did not affect the growth of V. alginolyticus cultures, it caused a 50% reduction in lipid synthesis (Figure 3.3).

Quinacrine, an inhibitor of the penicillinase-releasing protease (Traficante & Lampen, 1977), markedly inhibited collagenase production but caused a transient inhibition of serine protease production (Figures 3.1 and 3.2). O-phenanthroline, however, severely inhibited serine protease production (Figure 3.2) but not cell-free enzymatic activity (Table 3.1). Quinacrine (25 $\mu g\ m\ell^{-1}$) had no effect on growth or total protein synthesis (Figure 3.4). Cell-free collagenase and serine protease activity were not affected by quinacrine (25 $\mu g\ m\ell^{-1}$) (Table 3.1).

### 3.3.2 Effect of Lidocaine on Collagenase and Serine Protease Activity

Lidocaine (5 mM) inhibited collagenase and serine protease production by 46 and 43% respectively (Figures 3.5 and 3.6).

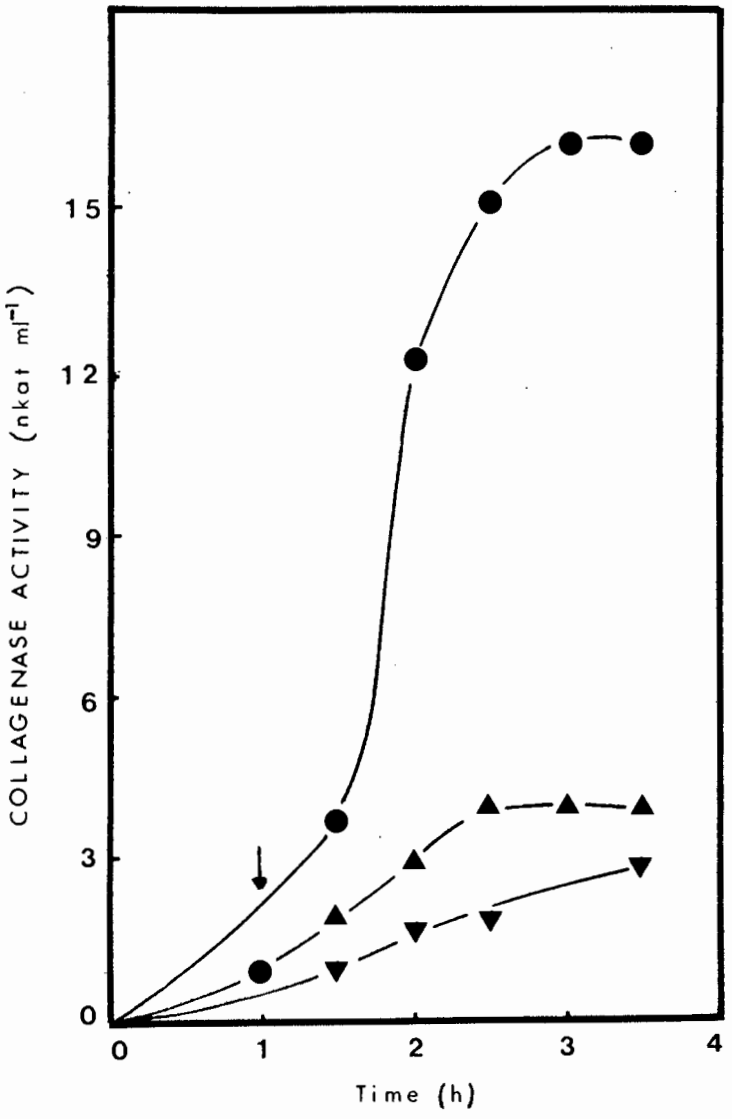



FIGURE 3.1: Effect of cerulenin and quinacrine on the production of collagenase in V. alginolyticus. Control (●); cerulenin (5µg mℓ$^{-1}$) (▲) and quinacrine (5µg mℓ$^{-1}$) (▼) added at 1 h (↓).

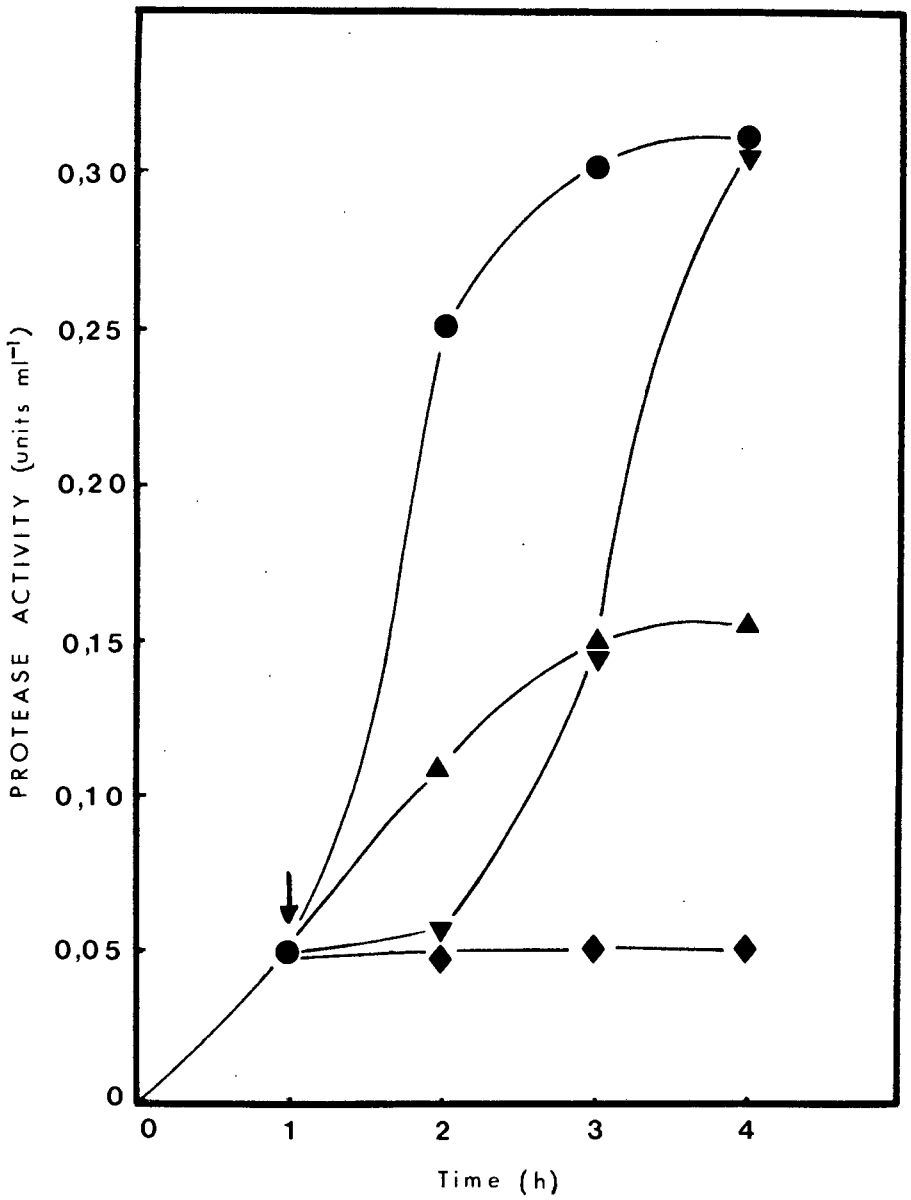



FIGURE 3.2: Effect of cerulenin, quinacrine and O-phenanthroline on serine protease production in V. alginolyticus. Control (●); cerulenin (0,5µg mℓ$^{-1}$) (▲); quinacrine (25µg mℓ$^{-1}$) (▼) and O-phenanthroline (50µg mℓ$^{-1}$) (◆).

TABLE 3.1: Effect of various inhibitors on cell-free collagenase and serine protease activity.

| Inhibitor (conc.) | % control cell-free collagenase activity after incubation for 4 h. | % control cell-free serine protease activity after incubation for 4 h. |
|---|---|---|
| Cerulenin (5 $\mu g\ m\ell^{-1}$) | 102 | |
| (0,5 $\mu g\ m\ell^{-1}$) | | 100 |
| Quinacrine (5 $\mu g\ m\ell^{-1}$) | 105 | |
| (25 $\mu g\ m\ell^{-1}$) | | 98 |
| O-phenanthroline (100 $\mu g\ m\ell^{-1}$) | 38 | |
| (50 $\mu g\ m\ell^{-1}$) | | 95 |
| Lidocaine (5 mM) | 97 | 100 |

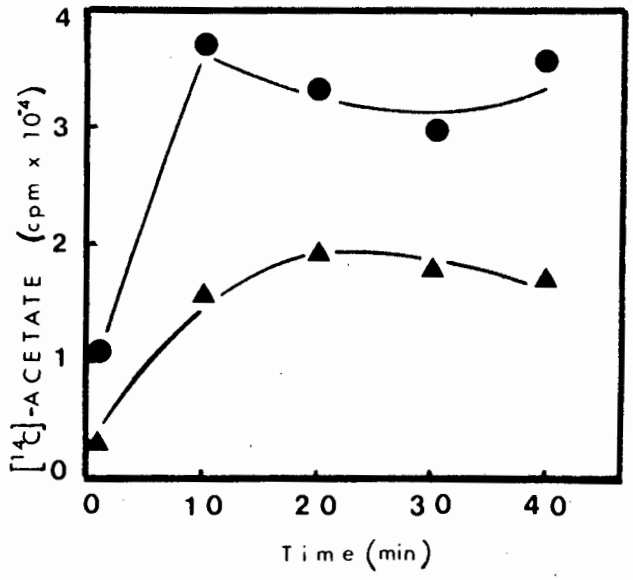



FIGURE 3.3: Effect of cerulenin on Lipid synthesis in aerated stationary phase V. alginolyticus cells. Lipid synthesis was determined by incorporation of $[^{14}C]$-acetate into Lipid fractions. Control (●) and cerulenin (5μg $m\ell^{-1}$) (▲) added at 0 h.

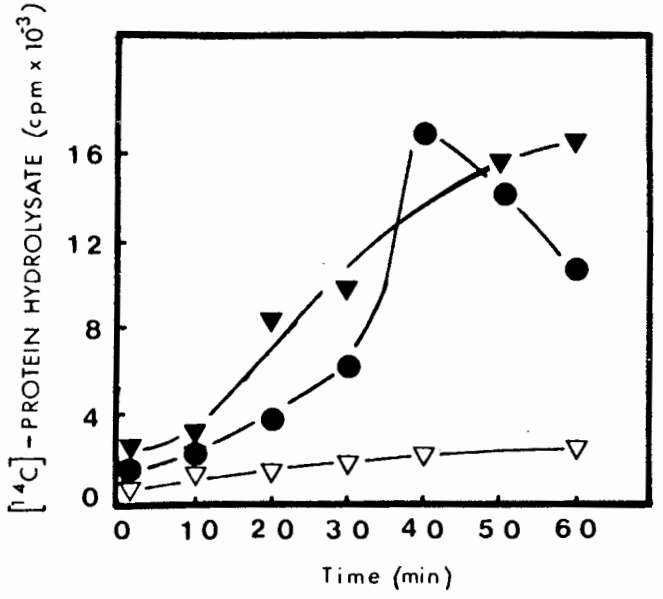



FIGURE 3.4: Effect of quinacrine on protein synthesis in aerated stationary phase V. alginolyticus cells. Protein synthesis was determined by incorporation of $[^{14}C]$-protein hydrolysate into TCA-precipitable material. Control (●); quinacrine (5,0μg $m\ell^{-1}$) (▼); and chloramphenicol (100μg $m\ell^{-1}$) (▽) added at 0 h.

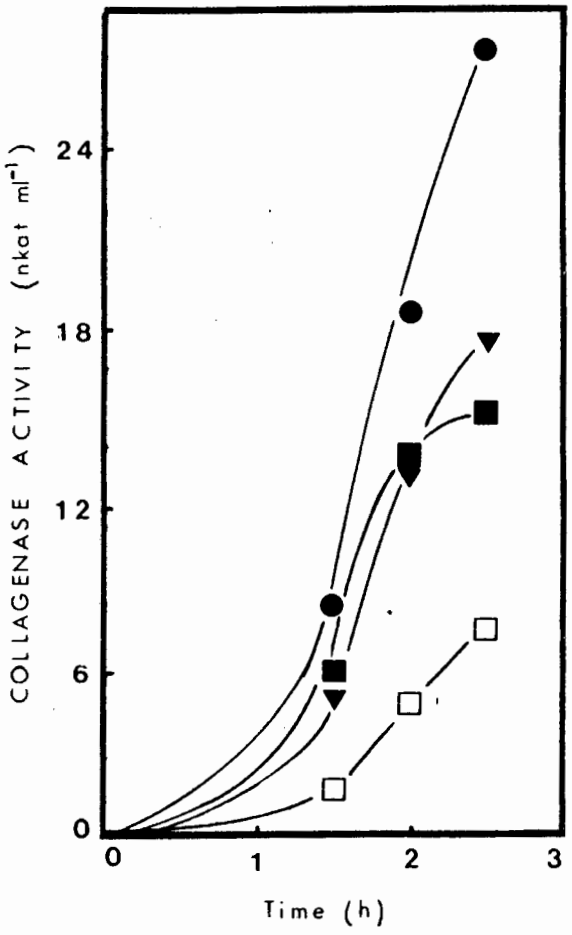



FIGURE 3.5: Effect of lidocaine and quinacrine on the production of collagenase in V. alginolyticus. Control (●); quinacrine (0,5µg mℓ$^{-1}$) (▼); lidocaine (5 mM) (■) and quinacrine (0,5µg mℓ$^{-1}$) and lidocaine (5 mM) added simultaneously (□) at 0 h.

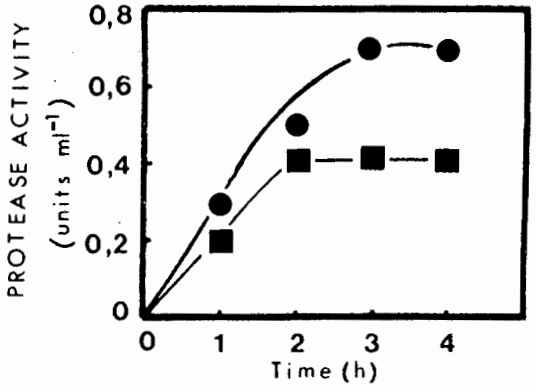



FIGURE 3.6: Effect of lidocaine on serine protease production in V. alginolyticus cells. Control (●) and lidocaine (5 mM) (■) added at 0 h.

Lidocaine (5 mM) had no effect on growth or cell-free collagenase or serine protease activity (Table 3.1).

Lidocaine (5 mM) and quinacrine (0,5 $\mu g\ m\ell^{-1}$) added simultaneously to V. alginolyticus cultures had a synergistic effect on the inhibition of collagenase activity (Figure 3.5).

### 3.3.3 Effect of Lidocaine and Quinacrine on Intracellular Protein Synthesis

Densitometer tracings of $[^{35}S]$-methionine labelled protein bands after SDS-PAGE of extracts of aerated peptone cultures at 30°C with and without quinacrine and/or lidocaine were compared after 30 min (Figure 3.7). The synthesis of three proteins was affected. Synthesis of a 62 000 molecular weight protein was slightly inhibited by lidocaine or quinacrine alone, and markedly inhibited when both substances were added simultaneously. A 52 000 molecular weight protein showed increased synthesis in the presence of lidocaine alone, but decreased synthesis in the presence of both lidocaine and quinacrine. This protein migrates at the same position as the protein which was stimulated by histidine in low SNP medium. The third protein, molecular weight 38 000 showed increased synthesis in the presence of quinacrine or quinacrine plus lidocaine.

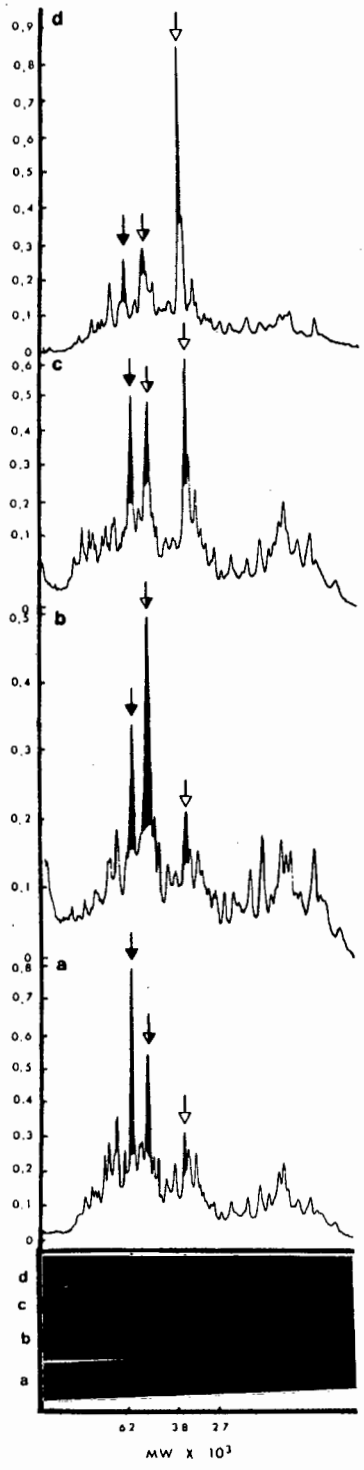



FIGURE 3.7: Densitometric comparison of $[^{35}S]$-methionine labelled protein bands after 10% linear SDS-PAGE of extracts of an aerated peptone culture (a) and cultures containing lidocaine (5 mM) (b), quinacrine (0,5 μg $m\ell^{-1}$) (c) and lidocaine (5 mM) + quinacrine (0,5 μg $m\ell^{-1}$) (d). Cultures were incubated at 30°C and pulse-labelled for 30 min. The positions of the 38 000, 52 000 and 62 000 molecular weight proteins are indicated by open, half-open and closed arrows respectively.

## 3.4 DISCUSSION

The effects of cerulenin and quinacrine on the production of collagenase and serine protease in *V. alginolyticus* are similar to those reported for the production of exoproteins in *Bacillus* spp. (Caulfield *et al.*, 1979, Fishman *et al.*, 1978, 1980), and *S. aureus* enterotoxin A production (Berkeley *et al.*, 1978). Inhibition of exoenzyme activity by cerulenin linked exoenzyme synthesis and fatty acid synthesis, suggesting the presence of a phospholipoprotein. Fishman *et al.* (1978) and Paton *et al.* (1980) were able to show that the inhibitory effect of cerulenin on exoprotease synthesis could be partially reversed by addition of fatty acids prepared from the lipids extracted from *B. licheniformis* and *B. amyloliquefaciens* respectively.

Petit-Glatron & Chambert (1981) found that in *B. subtilis* fatty acid synthesis and extracellular protein production are not inhibited by the same cerulenin concentration and therefore questioned the current interpretation of results obtained with cerulenin. In *V. alginolyticus* cells cerulenin at a concentration (5 $\mu g\ m\ell^{-1}$) which caused a 50% reduction in lipid synthesis inhibited collagenase by 75%. This suggests that *de novo* fatty acid synthesis is required for the formation of extracellular collagenase.

Intracellular protein profiles of *V. alginolyticus* cultures treated with cerulenin suggest that cerulenin does not act at the level of translation (discussed below).

Furthermore, experiments by Reid et al. (1981) suggest that any non-specific effect on transcription is not significant. Reid et al. (1981) found that although RNA synthesis is inhibited immediately with rifampin, collagenase synthesis continues for 30 to 60 min after addition of rifampin.

Serine protease synthesis, on the other hand, was inhibited by 0,5 $\mu g\ m\ell^{-1}$ cerulenin, whereas fatty acid synthesis was inhibited by 5 $\mu g\ m\ell^{-1}$ cerulenin. Fishman et al. (1978) showed definite alterations in membrane composition by treatment with cerulenin. It is possible that alterations in membrane composition of V. alginolyticus cells caused by cerulenin may be responsible for inhibition of serine protease activity. It is unlikely that de novo lipid synthesis is required for secretion of the serine proteases. These results suggest a complex situation involving these extracellular enzymes and further work is necessary to provide substantial evidence of the exact nature of secretion.

Inhibition of collagenase and serine protease synthesis by quinacrine and O-phenanthroline suggests that both of these exoenzymes are produced in a precursor form which must be cleaved before export can take place.

The anaesthetic, lidocaine, inhibits the conversion of a 42 000 molecular weight precursor to mature outer membrane protein a in E. coli K12 (Gayda et al., 1979). It has also been shown to inhibit lactose transport in E. coli (Villarejo et al., 1980). Lidocaine inhibited collagenase

and serine protease production by 46 and 43% respectively. When lidocaine and quinacrine were added to V. alginolyticus cultures simultaneously, a synergistic effect on inhibition of collagenase production was observed, suggesting that these two compounds inhibit collagenase production at different steps.

Densitometric comparison of $[^{35}S]$-methionine labelled protein bands after SDS-PAGE of extracts of aerated peptone cultures at 30°C with and without quinacrine and/or lidocaine revealed that synthesis of three proteins of molecular weight 62 000, 52 000 and 38 000 had been altered after 30 min labelling.

Synthesis of the 100K protein found to be involved in temperature and oxygen regulation of extracellular enzymes in V. alginolyticus (refer to Chapter 2), was not affected by quinacrine or lidocaine. This confirms the suggestion that the latter compounds affect processing and secretion events rather than transcription.

Increased synthesis of the 38 000 molecular weight protein in the presence of quinacrine may represent a block in processing of collagenase subunits into an active dimer enzyme. Collagenase consists of two subunits of molecular weight 35 000 each (Keil-Dlouha & Keil, 1978). Increased synthesis of a 38 000 molecular weight protein was also observed under conditions of $(NH_4)_2SO_4$ repression (refer

to Chapter 2). Specific immunoprecipitation (Josefsson & Randall, 1981) is necessary to identify these proteins.

# INDUCTION OF COLLAGENASE

**Summary:** A low molecular weight peptone fraction in the molecular weight range from 350 to 1500 induces collagenase production. Digestion of these peptone inducer molecules with purified V. alginolyticus or Cl. histolylicum collagenase markedly reduced their inducing ability, whereas digestion with trypsin, pepsin or pronase had no effect. Autoclaving the peptone fraction had no effect on its inducing ability. The results suggest that a high molecular weight product with the triple helix structure is not required for induction of collagenase.

The release of [$^3$H]-proline from collagen matrices produced by smooth muscle cells was shown to be a sensitive assay for bacterial collagenases and was utilized to show that V. alginolyticus cells produce a basal constitutive level of collagenase. The constitutive levels of collagenase were affected by aeration but not by temperature.

## 4.1 INTRODUCTION

The production of bacterial exoenzymes may be inducible, partially constitutive or completely constitutive. These terms, however, are relative since it is unlikely that there

are any inducible enzymes whose basal rate of formation is zero. Enzyme induction is therefore a qualitative increase in the rate of production of a specific enzymically active protein and involves de novo synthesis of that particular protein from its constituent amino acids (Pollock, 1959).

Although inducing activity is restricted to substances closely related to the substrate, inducers need not be substrates, or substrates inducers. One of the problems involved in studying substrate induction is that inducers are frequently large molecules unable to enter the cell (e.g. hyaluronic acid, starch, chitin and collagen). In several cases, however, it has been found that low molecular weight products are as effective as the high molecular weight substrates in inducing exoenzyme synthesis. Pollock (1962) found that purified tetrasaccharide and trisaccharide could induce streptococcal hyaluronidase as efficiently as purified undegraded hyaluronic acid, molecular weight $\leq 10^6$. In both B. stearothermophilus (Welker & Campbell, 1963) and B. licheniformis (Saito & Yamamoto, 1975) maltotetraose was found to be the most effective inducer of $\alpha$-amylase. Loriya et al. (1977, quoted by Braun & Schmitz, 1980) traced induction of the extracellular protease of Serralia marcescens by albumin to leucine. Large inducer molecules would, therefore, induce enzyme synthesis directly by means of their breakdown products.

Just as there may be a number of different (albeit closely related) compounds which can induce a particular enzyme in one strain of micro organism, a single inducer may often specifically stimulate the formation of more than one enzyme (not necessarily very closely related). Hockenhull & Herbert (1945) found that if Cl. acetobutylicum was grown on maltose only maltase was formed, however, if grown with starch, both amylase and maltase were produced. Induction of a specific enzyme may not only lead to sequential induction (where the product of the first reaction induces a second enzyme) but may also affect formation of other proteins in the cell.

Coleman (1981) studied regulation of extracellular protein formation in Staphylococcus aureus (Wood 46) and a low α-toxin-producing variant of this strain. Although five extracellular proteins were produced at a low level by the variant, the differential rates of total extracellular protein formation by the wild type organism and the variant were identical. This suggests that the reduced ability to produce the five proteins is compensated for by an increased ability to produce all other extracellular proteins.

The induction of collagenase has been studied in Vibrio B30 (Dreisbach & Merkel, 1978) and in V. alginolyticus (Welton & Woods, 1975; Keil-Dlouha et al. 1976; Reid et al., 1980). Collagenase could be induced by collagen, hydrolyzed collagen or gelatin. OIn V. alginolyticus, peptone was also shown to be an inducer (Welton & Woods,

1975; Reid et al., 1980). Reid et al. (1980) showed that inducer molecules in peptone are in the molecular weight range from 1 000 to 60 000. The present study investigated the possibility of a low molecular weight inducer below 1 000. Keil-Dlouha et al. (1976) reported that the synthetic peptide substrate, PZ-Pro-Leu-Gly-Pro-D-Arg, the collagenase inhibitor, Pro-Leu-Sar-Pro, and the low molecular weight fragments from the peptic hydrolysate of collagen all failed to induce collagenase production.

In the previously reported experiments on the induction of collagenase the enzyme was assayed by the method of Wünsch & Heidrich (1963) which utilizes a chromogenic synthetic substrate. Since this method lacks the sensitivity of a radioactive assay the production of collagenase was investigated by using a sensitive radioactive assay procedure which involves the release of [$^{3}$H]-proline from a labelled collagen matrix (Jones & Scott-Burden, 1979).

## 4.2 MATERIALS AND METHODS

### 4.2.1 Fractionation of Peptone

Peptone (1g) was dissolved in 10 mℓ distilled water and dialyzed (membrane retention ~ 1 000 to 8 000 MW) against distilled water for 24 h at 4°C. The dialysate and the medium surrouding the dialysis bag (low molecular weight

peptone fraction) were lyophilized and tested for collagenase inducing activity. The cells were grown up as described in Chapter 2 and collagenase activity assayed by the method of Wünsch & Heidrich (1963).

The low molecular weight peptone fraction (50 mg in 0,25 mℓ 0,01 M Tris-HCl buffer pH 7,6) was layered onto a Sephadex G25 superfine column (600 mm by 9 mm). Samples (2 mℓ) were eluted with 0,01 M Tris-HCl buffer pH 7,6 at a flow rate of 1 mℓ per 3,8 min. Fractions 13 to 16; 18 to 20; and 21 to 24 were pooled, inoculated with V. alginolyticus cells and tested for inducing activity. The column was calibrated with cytochrome C (molecular weight 12 500); insulin A chain (molecular weight 5 733), vitamin B 12 (molecular weight 1 355) and phenol red (molecular weight 354, 38).

### 4.2.2 Enzymatic Digestion of Low Molecular Weight Peptone Fraction

The low molecular weight peptone fraction was subjected to digestion with purified V. alginolyticus collagenase, Cl. histolyticum collagenase, trypsin, pepsin and pronase at an enzyme to substrate ratio of 1:125 for 18 h at 37°C. After digestion the enzymes were inactivated by autoclaving. These flasks were then inoculated with washed minimal medium-grown cells to give an absorbance at 600 nm of 8. The control consisted of heat inactivated enzyme added to

undigested low molecular weight peptone fraction. No effect on the low molecular weight peptone fraction by heat inactivated collagenase, trypsin, pepsin or pronase was observed.

### 4.2.3 Production of Dishes Containing Labelled Collagen Matrices

Smooth muscle cells were used to produce a [$^3$H]-proline labelled matrix in 35 mm culture dishes by the method of Jones & Scott-Burden (1979). The cells cultured in the presence of [$^3$H]-proline synthesized an extensive radioactively labelled matrix containing glycoprotein(s), elastin and collagen, which remained on the bottom of the culture dishes following the removal of the producer cells by mild alkaline treatment. The matrix was treated with trypsin and elastase and sequential enzyme analysis demonstrated that 34% of the proline radioactivity was released by trypsin, a further 43% by elastase (Jones & Scott-Burden, 1979). After treatment with trypsin and elastase the remaining radioactivity could only be released enzymatically with collagenase. All the experiments were carried out with collagen matrices which had been treated with trypsin and elastase.

### 4.2.4 Growth Conditions for Collagen Induced Enzyme Studies

Overnight *V. alginolyticus* cultures (10 mℓ) in 2,5% casamino

acids medium were inoculated into 2,5% peptone medium (100 mℓ). After 4 h incubation with aeration at 30°C, the cells were harvested, washed twice by centrifugation and resuspended in 20 mℓ low SNP medium. Samples (2 mℓ) were added to the [$^3$H]-proline labelled collagen dishes which were incubated at 30°C in an orbital shaking incubator (130 rpm). Samples (0,1 mℓ) were removed at different time intervals, added to 5 mℓ scintillation fluid and the radioactivity determined in a Packard Liquid Scintillation Spectrometer. Digestion of the collagen matrix was also carried out with either 1 or 0,75 mg $m\ell^{-1}$ solutions of V. alginolyticus and Cl. histolyticum collagenase preparations. Total digestion of collagen matrices was determined by digestion with 2N NaOH for 12 h at 30°C followed by neutralization with concentrated HCl.

## 4.3 RESULTS

### 4.3.1 Fractionation of Peptone

The peptone dialysate and low molecular weight peptone fraction were tested for their inducing ability. The dialysate when added to low SNP medium and the small molecular weight peptone fraction in 0,1 M Tris-HCl buffer pH 7,6 both showed inducing activity (Figure 4.1).

Fractionation of the low molecular weight peptone fraction

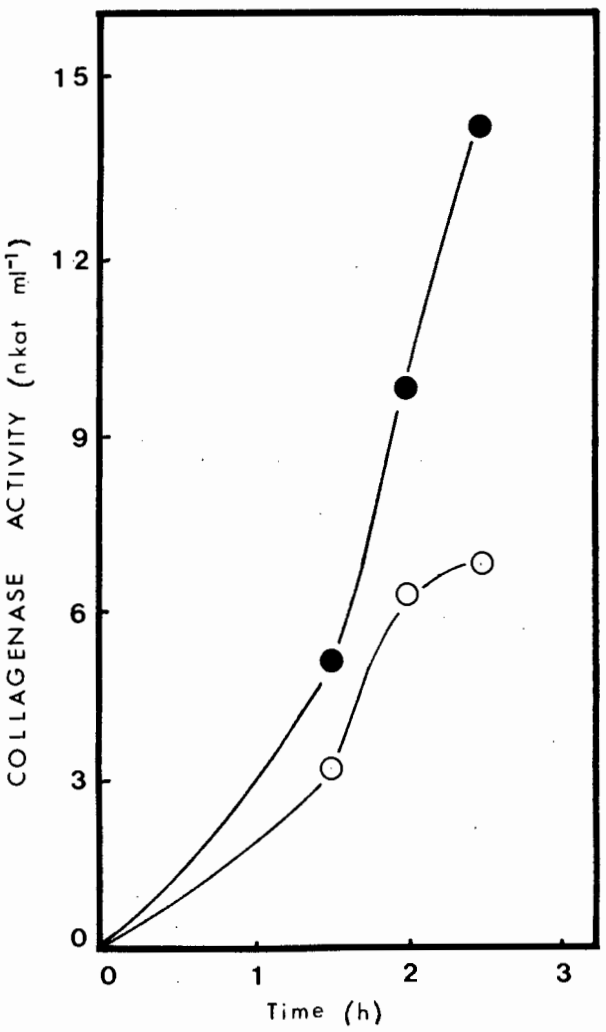



FIGURE 4.1: Induction of collagenase by peptone dialysate (0,25% w/v)(●) and the low molecular weight peptone fraction (0,25% w/v) (○).

on a Sephadex G25 superfine column gave one major peak and two minor peaks (Figure 4.2). The protein concentration of fractions pooled under peaks 1, 2 and 3 were 0,05, 0,04 and 0,03% respectively. These protein concentrations were too low to perform reliable induction experiments and the fractions under all three peaks were pooled for all further experiments. The three peaks fall into the molecular weight range from 350 to 1 500.

Autoclaving (121°C for 20 min) the low molecular weight peptone fraction before or after dialysis did not alter its ability to induce collagenase (Figure 4.3). It is, therefore, heat-stable.

Different concentrations of the low molecular weight peptone fraction were tested for maximum induction of collagenase. A concentration of 0,25% gave maximum induction, with decreasing inducing ability of greater and lower concentrations (Figure 4.4).

Digestion of the low molecular weight peptone fraction by purified V. alginolyticus and Cl. histolyticum collagenase ($1 mg/ml^{-1}$) caused a marked decrease in inducing ability of this peptone fraction (Table 4.1). Digestion by V. alginolyticus collagenase for 18 h completely destroyed inducing ability of the small molecular weight peptone fraction. Digestion with trypsin, pepsin and pronase ($1 mg\ m\ell^{-1}$) had little or no effect on the inducing activity

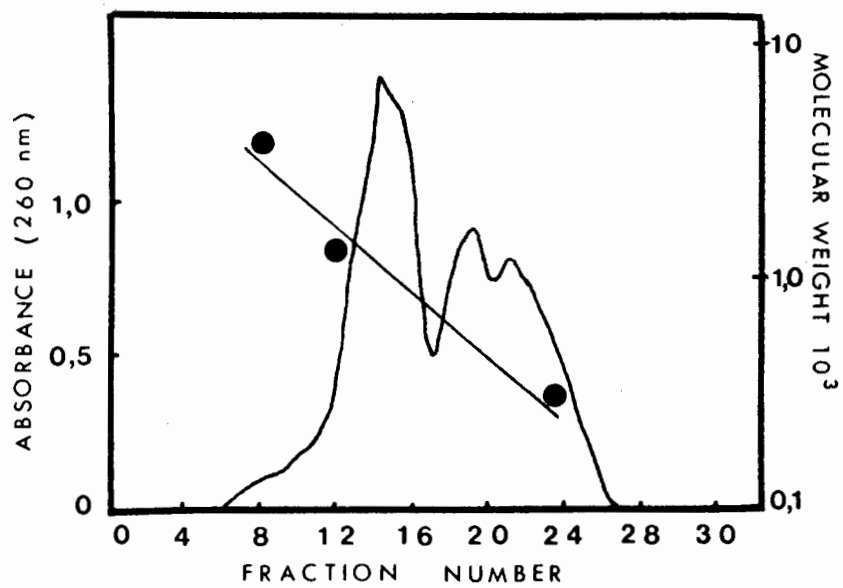



FIGURE 4.2: Gel filtration of low molecular weight peptone fraction on Sephadex G-25 superfine. The protein concentration of fractions under each peak was too low for reliable induction experiments, so fractions under all three peaks were pooled.

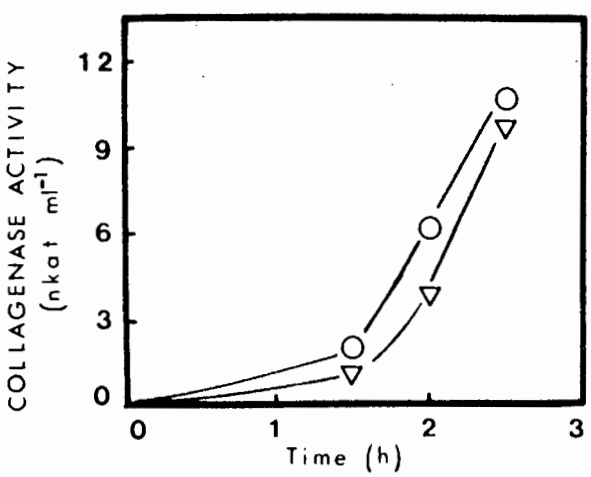



FIGURE 4.3: Effect of heat on the ability of the low molecular weight peptone fraction to induce collagenase. Low molecular weight peptone fraction autoclaved before dialysis (0,25% w/v) (▽) and peptone fraction autoclaved after dialysis (0,25% w/v) (○).

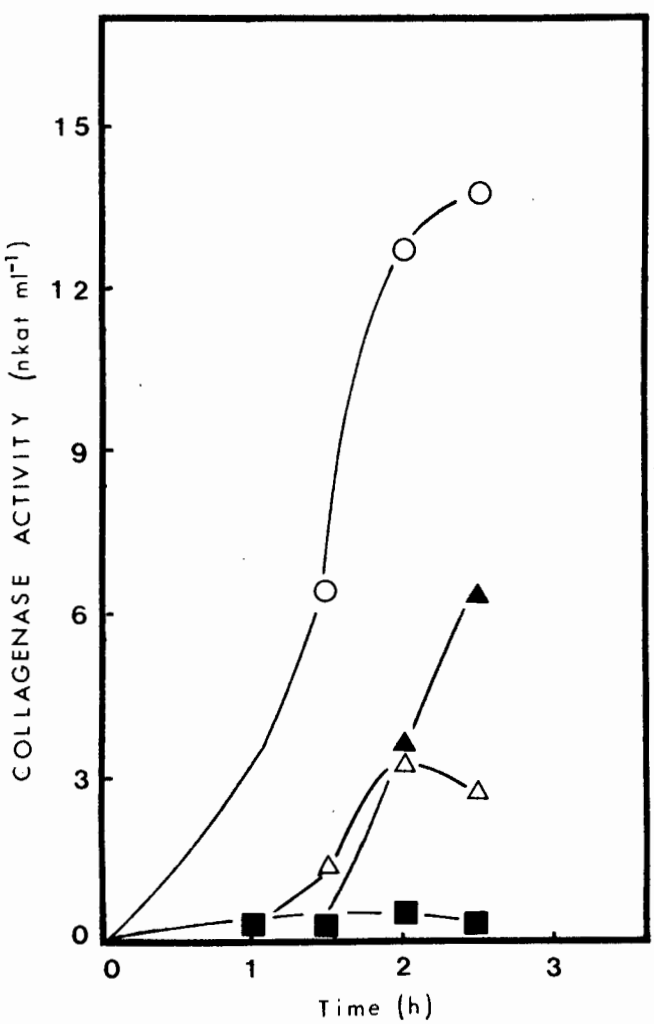



FIGURE 4.4: Effect of different concentrations of low molecular weight peptone fraction on its ability to induce collagenase production. Maximum induction occurs at a concentration of 0,25% (w/v) with decreasing inducing ability of greater and lower concentrations. 1% (w/v)(■); 0,75% (w/v)(▲); 0,25 % (w/v) (○) and 0,125% (w/v) (△).

TABLE 4.1: Effect of enzymatic digestion of low molecular weight peptone fraction (0,25% w/v) for 18 h on its ability to induce collagenase.

| Enzyme | % Control collagenase activity after digestion of substrate |
|---|---|
| Cl.histolyticum collagenase | 29 |
| V. alginolyticus collagenase | 0 |
| Trypsin | 95 |
| Pepsin | 108 |
| Pronase | 113 |

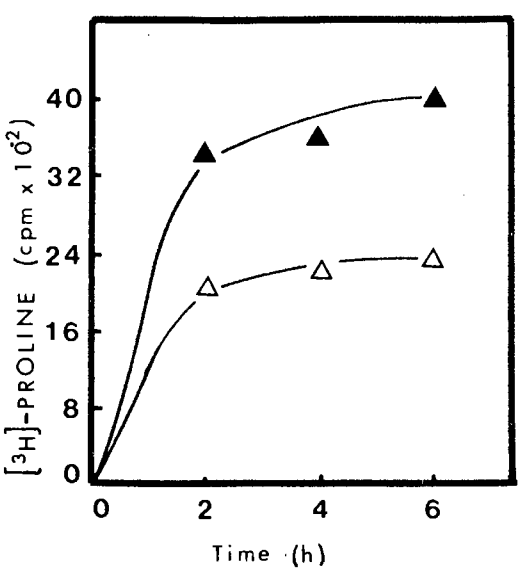



FIGURE 4.5: The release of $[^3H]$-proline from the collagen matrix by Cl. histolyticum and V. alginolyticus collagenases (1 mg $m\ell^{-1}$) incubated at 30°C for 6 h. Release by Cl. histolyticum collagenase (▲) and by V. alginolyticus collagenase (△).

of the peptone fraction over the same time period. Collagenase activity remains stable over a period of 22 h. (Reid, 1981).

### 4.3.2 Radioactive Collagen Matrix Assay

The suitability and specificity of the release of $[^3H]$-proline from the collagen matrices had been previously established for mammalian collagenases by Jones & Scott-Burden (1979). Further experiments showed that the assay system was a sensitive assay procedure for bacterial collagenases. The release of $[^3H]$-proline from the collagen matrix by Cl. histolyticum and V. alginolyticus collagenases (1 mg $m\ell^{-1}$) incubated at 30°C for 6 h was 39 751 and 22 960 cpm $m\ell^{-1}$ respectively (Figure 4.5) and after 12 h was 40 387 and 25 257 cpm $m\ell^{-1}$ respectively. The percentage of total radioactivity solubilized by Cl. histolyticum and V. alginolyticus collagenases, calculated after 12 h incubation at 30°C was 90,6% and 56,7% respectively.

The addition of $5 \times 10^8$, $5 \times 10^9$ and $5 \times 10^{10}$ cells $m\ell^{-1}$ caused the release of 200, 1 100 and 2 250 cpm $m\ell^{-1}$ $[^3H]$-proline respectively after 6 h incubation at 30°C (Figure 4.6). All experimental data has been corrected for the low release of radioactivity obtained in the controls. Collagenase activity in the inoculated samples was not detected by the assay of Wünsch & Heidrich (1963).

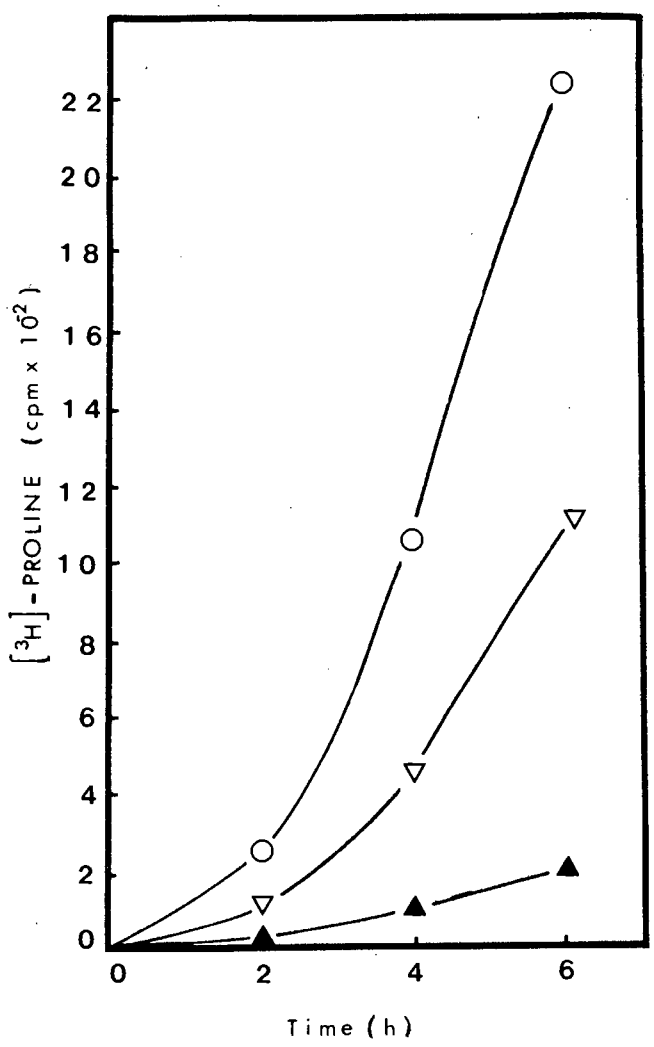



FIGURE 4.6: The effect of cell concentration on the release of $[^3H]$-proline from the collagen matrix by V. alginolyticus cells. Cell concentration $5 \times 10^8$ (▲); $5 \times 10^9$ (▽); and $5 \times 10^{10}$ (○) cells $m\ell^{-1}$.

### 4.3.3 Effect of Temperature, Oxygen and Histidine on the Release of [$^3$H]-Proline by V. alginolyticus Cultures

Release of [$^3$H]-proline from collagen matrices by V. alginolyticus cells was not affected by a shift up in temperature to 37°C (Figure 4.7). When a cell-free collagenase preparation from V. alginolyticus was incubated with labelled collagen matrices at 30° and 37°C, no difference in the release of radioactivity was observed, indicating that collagenase activity was not affected by temperature changes between 30 and 37°C (Figure 4.8).

The addition of histidine (0,5%) to the culture dishes resulted in a 75% decrease in the release of [$^3$H]-proline from the collagen matrices.

The release of [$^3$H]-proline from the collagen matrices by V. alginolyticus cells after 6 h incubation at 30°C was affected by aeration. Shaking the dishes on a rotary shaker at 0, 70, 100 and 130 rpm resulted in the release of 2 000, 4 400, 6 500 and 9 100 cpm $m\ell^{-1}$ [$^3$H]-proline respectively (Figure 4.9).

### 4.3.4 Constitutive Basal Levels of Collagenase

The sensitive radioactive assay method was utilized to determine whether V. alginolyticus cells produced a constitutive basal level of collagenase and whether the cells

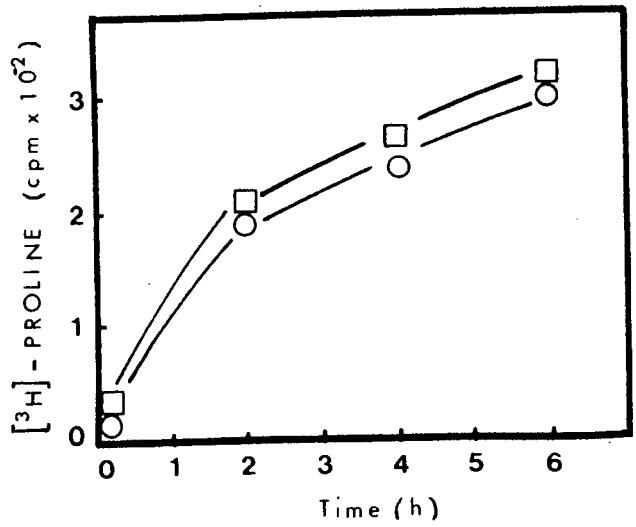



FIGURE 4.7: The effect of temperature on the release of $[^3H]$-proline from the collagen matrix by V. alginolyticus cells. Aerated cultures at 30 (O) and 37°C (□).

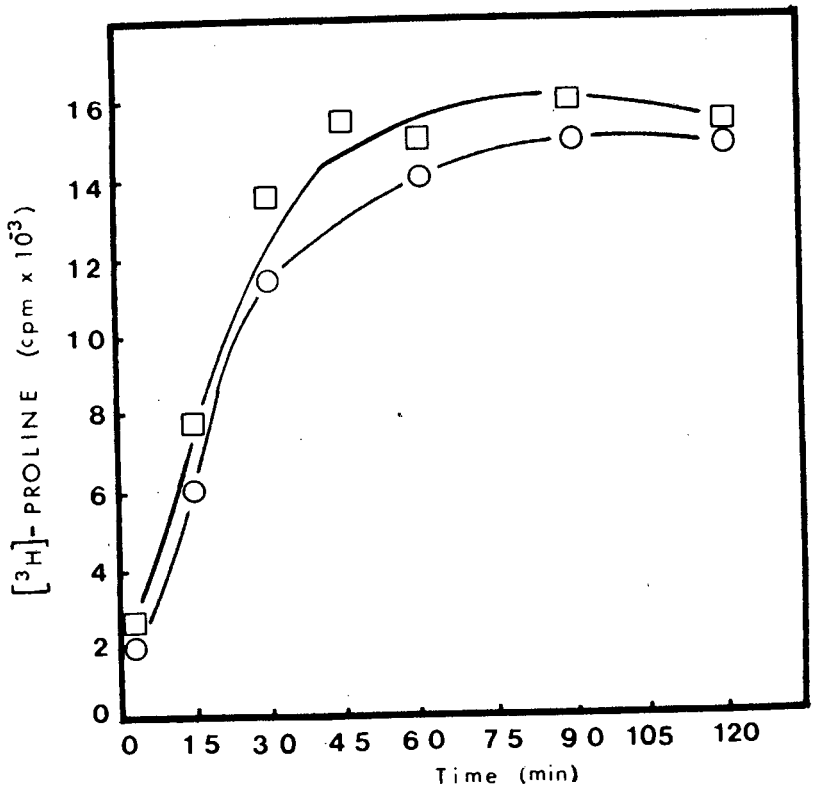



FIGURE 4.8: Effect of temperature on $[^3H]$-proline release from collagen matrices by cell-free V. alginolyticus cultures. Culture dishes incubated at 30 (O) and 37°C (□).

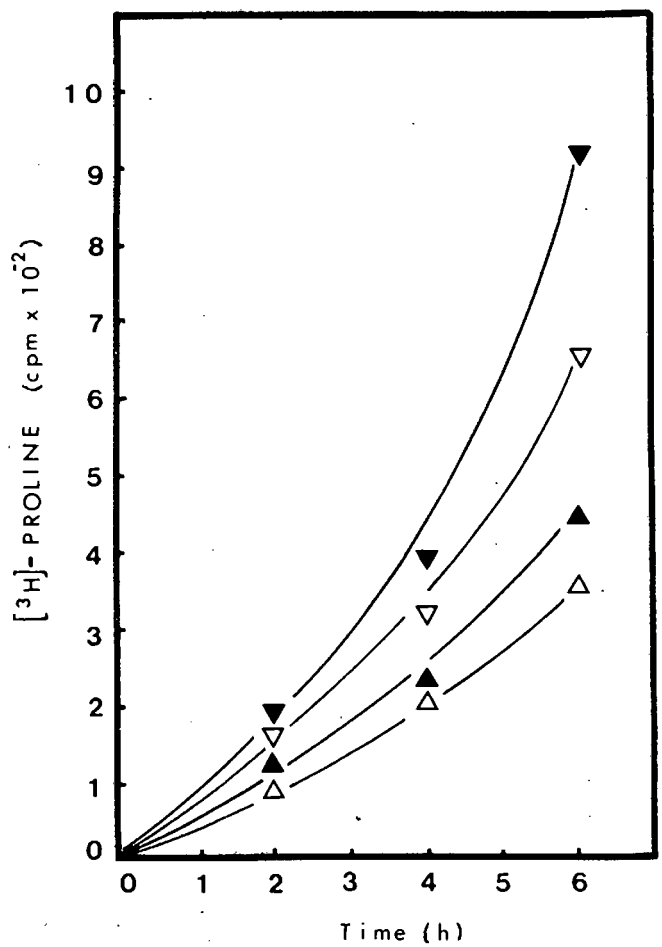



FIGURE 4.9: Effect of aeration on the release of $[^3H]$-proline from the collagen matrix by *V. alginolyticus* cells. Culture dishes were aerated on a rotary shaker at 0 (△); 70 (▲); 100 (▽) and 130 (▼) rpm.

could be presensitized to produce collagenase. Low SNP cultures which did not contain a collagenase inducer were resuspended and incubated for 6 h at 30°C in culture dishes with and without collagen matrices. The cultures containing collagen matrices showed release of [$^3$H]-proline (Figure 4.10). After 6 h incubation the supernatants from the culture dishes with and without collagen matrices were added to fresh culture dishes containing collagen matrices. The release of [$^3$H]-proline by uninduced cell-free culture supernatants was more rapid than with induced cell-free culture supernatants (Figure 4.10). The lower activity of the supernatants from dishes containing collagen matrices was presumably due to the affinity and binding of collagenase by the anchored substrate in the first culture dish. The washed cell suspensions from both the induced and uninduced cultures were added to fresh collagen matrices and exhibited similar levels of collagenase activity (Figure 4.10).

## 4.4 DISCUSSION

Peptide substrates such as Z-Gly-Pro-Leu-Gly-Pro (Nagai et al., 1960); Z-Gly-Pro-Gly-Gly-Pro-Ala (Grassmann & Nordwig, 1960) and PZ-Pro-Leu-Gly-Pro-D-Arg (Wünsch & Heidrich, 1963) have been frequently used in collagenase assays, since they have many advantages over soluble, and especially, insoluble collagen (Nordwig, 1971). However, enzymes digesting these peptide substrates but not native

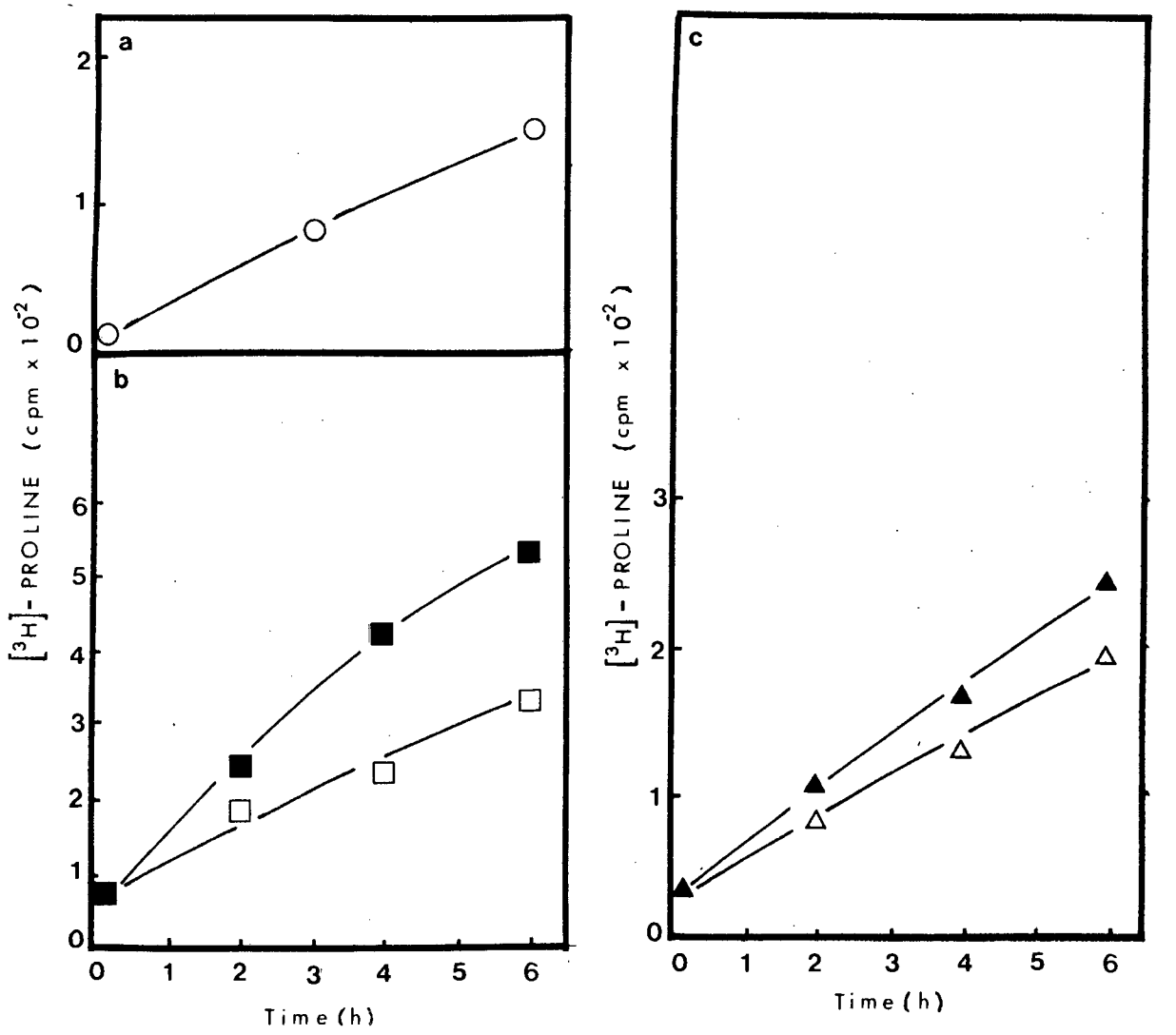



FIGURE 4.10: Constitutive basal levels of collagenase produced by V. alginolyticus cells. Production of collagenase by cells added to a collagen matrix (a). Collagenase activity of supernatant low SNP culture solutions from V. alginolyticus cells pregrown in the presence (□) and absence (■) of collagen (b). The release of $[^3H]$-proline in the solution pregrown in the presence of collagen has been corrected for the amount of radioactivity carried over from the initial collagen matrix. Production of collagenase by the washed cell suspensions from the V. alginolyticus cells pregrown in the presence (△) and absence (▲) of collagen (c).

collagen have been found. Mitchell (1968) found a non-collagenolytic protease in Cl. histolyticum culture filtrates capable of cleaving the Wünsch & Heidrich (1963) peptide, 2-Pro-Leu-Gly-Pro-D-Arg.

In 1969, Waldvogel & Swartz found that an extracellular fraction of P. aeruginosa able to hydrolyse a synthetic hexapeptide, Cbz-glycyl-L-Propyl-glycyl-glycyl-L.Propyl-L-alanine, with an amino acid sequence very similar to collagen (Schoellman & Fisher, 1966), had no detectable effect on reconstituted collagen. Thus the only suitable substrate for screening for collagenolytic enzymes is collagen itself. However, peptide substrates remain valuable for investigating properties, reaction mechanisms, etc., of pure collagenases.

Dreisbach & Merkel (1978) studied induction of collagenase in a marine bacterium, Vibrio B30. They found that the lowest molecular weight fraction of collagen hydrolysate capable of induction was between 1 000 and 10 000. Studies on the induction of collagenase in V. alginolyticus showed a similar molecular weight range for inducer molecules. Keil-Dlouha et al. (1976) showed that a macromolecular fraction of collagen (molecular weight 8 000 to 30 000) was necessary for induction, and Reid et al. (1980) reported collagenase inducer molecules in peptone with a molecular weight range from 1 000 to 60 000.

There have been no reports, however, of a peptide capable of inducing collagenase synthesis in V. alginolyticus. Experiments in our laboratory with specific peptides failed to induce collagenase (unpublished results).

Keil-Dlouha et al. (1976) reported that low molecular weight fragments from the peptic hydrolysate of collagen did not possess inducing ability. Similarly, the synthetic peptide substrate, PZ-Pro-Leu-Gly-Pro-D-Arg, or the inhibitor, Pro-Leu-Sar-Pro, of collagenase did not induce enzyme activity.

The low molecular weight peptone fraction isolated from whole peptone in this study has a molecular weight range from 350 to 1 500.

Digestion of this low molecular weight peptone fraction by V. alginolyticus and Cl. histolyticum collagenase for 18 h reduced its inducing ability by 100 and 71% respectively. The enzymes trypsin, pepsin and pronase had little or no effect on the inducing ability of the peptone fraction. Keil-Dhoula et al. (1976) and Reid et al. (1980) reported similar results when high molecular weight collagen or peptone inducers were enzymatically digested. They suggested that a collagenase sensitive bond in collagen is required for induction. However, since no low molecular weight inducer could be found, Keil-Dlouha et al. (1976) proposed that although the collagenase-sensitive bond is

important in induction, it is not sufficient and that the tertiary structure of the collagen molecule must also play a role in induction. The present study suggests that the tertiary structure of collagen is not important. Dreisbach & Merkel (1978) reported that autoclaved collagen and gelatin can act as inducers of collagenase produced by Vibrio B30, therefore the native state of the molecule is not essential for induction.

Reid et al. (1980) found that the peptone inducers of collagenase (molecular weight range 1 000 to 60 000) acted as inhibitors of collagenase activity. This could explain the reduced enzyme activity observed when high (0,75 and 1,00%) concentrations of the low molecular weight peptone fraction were tested for inducing ability.

The release of [$^3$H]-proline from collagen matrices attached to culture dishes is a very sensitive and specific assay for bacterial collagenases. Further examples of the use of a radioactive assay procedure for detecting collagenase activity are: use of labelled collagen to detect collagenolytic activity in amphibian tissues (Gross & Lapiere, 1962), in mammalian tumor tissue (Labrosse et al., 1976), an assay for vertebrate collagenase (Terato et al., 1976), and an assay for bacterial collagenolytic activity (Hu et al., 1978).

Previous studies utilizing the Wünsch & Heidrich (1963) assay

procedure were unable to detect a basal level of collagenase production. However, the radioactive assay procedure described here has indicated that uninduced V. alginolyticus cells produce a basal constitutive level of collagenase. This suggests that the basal levels of collagenase, while attached to the cell envelope, could act as the recognition site for the inducer molecules and trigger the induction mechanism. V. alginolyticus cells could not be presensitized to produce collagenase by growth on two consecutive collagen matrices in fresh medium.

The production of low levels of collagenase in the culture dishes is enhanced by aeration. This effect of oxygen is similar to that shown previously in peptone induced cultures described in Chapter 2, using the synthetic peptide assay. This suggests that oxygen affects both the regulation of the basal constitutive collagenase production and the induced production of high levels of collagenase.

Although previous studies on peptone induction of collagenase suggested that temperature plays a regulatory role in collagenase synthesis, no effect on the collagenase synthesis by V. alginolyticus cells in labelled collagen cultures was detected when cells were incubated at 37°C.

In addition temperature had no effect on the activity of cell-free collagenase incubated at 30 and 37°C. This would suggest that basal constitutive collagenase is not regulated by temperature.

# CHARACTERIZATION OF EXTRACELLULAR ALKALINE PROTEASES

**Summary:** The number and molecular weights of extracellular alkaline proteases produced by *V. alginolyticus* were determined by gelatin-PAGE. Three major bands of protease activity with molecular weights of 28 000, 22 500 and 19 500 (proteases 1, 2 and 3) and two minor bands of protease activity with molecular weights of 15 500 and 14 500 (proteases 4 and 5) were obtained after gelatin-PAGE. The activities of the five proteases were inhibited by serine protease inhibitors but their activities were not affected by inhibitors of trypsin-like enzymes. Histidine which inhibited *V. alginolyticus* collagenase did not inhibit the activities of the alkaline serine proteases. The production of protease 1, however, was enhanced by histidine. Protease 1 production was also inhibited by growth at 37°C.

Gelatin-PAGE of a commercial *V. alginolyticus* collagenase preparation revealed four bands of activity which were identified as collagenases with molecular weights of 45 000, 38 500 33 500 and 31 000. The collagenase preparation was contaminated with two serine proteases.

## 5.1 INTRODUCTION

The most widely distributed group of proteolytic enzymes of both microbial and animal origin are the serine proteases (Hartley, 1960). They are characterized by the presence of a serine residue at their active site. A common test for these enzymes is the inhibition of their hydrolase activity by the reaction of this serine residue with DFP. They are generally neither inhibited by metal chelating reagents and thiol poisons nor activated by metal ions or reducing agents (Matsubara & Feder, 1971).

Serine proteases are generally optimally active at alkaline pH, but they exhibit a fairly broad pH profile for hydrolysis of proteins, peptides and synthetic substrates covering the pH range from neutrality to pH 11 (Matsubara & Feder, 1971). Some enzymes, however, do have a sharp pH profile such as the Streptomyces alkaline proteases pH 9 to 10 (Morihara et al., 1967), pH 10.6 to 10.8 (Mizusawa et al., 1964 quoted by Mizusawa et al., 1969) and pH 11 to 11.5 (Morihara et al., 1967). The serine proteases also exhibit a broad range of substrate specificities (Matsubara & Feder, 1971).

The gross physiological function of protein inhibitors is the prevention of unwanted proteolysis. With the exception of macroglobulins, which "inhibit" proteases of all classes, individual protein inhibitors only affect proteases belonging to a single mechanistic class. Of these the

most studied are inhibitors of serine proteases. In each inhibitor molecule there exists on the surface one peptide bond, the reactive site which combines with the enzyme in a substrate-like manner and serves as a substrate for the enzyme. Contact occurs over a small portion of the enzyme and the inhibitor, but numerous van der Waals interactions, hydrogen bonds and salt bridges are formed. In each case complex formation occurs with relatively small conformational change; it is predominantly a lock and key interaction (Laskowski & Kato, 1980).

The characterization of extracellular V. alginolyticus proteases by polyacrylamide gel electrophoresis and various protease inhibitors was investigated. In order to define the proteolytic activity of a biological system, it is important to establish the number of proteases present in the system. This is necessary to characterize the system, as a guide for purification and as a basis for investigating the relationships that exist between different proteases.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Production of Dishes Containing Labelled Collagen Matrices

Smooth muscle cells were used to produce a $[^3H]$-proline labelled matrix in 35 mm culture dishes by the method of Jones & Scott-Burden (1979). The cells cultured in the presence of $[^3H]$-proline synthesized an extensive radio-

actively labelled matrix containing glycoprotein(s), elastin and collagen. After treatment with trypsin and elastase the remaining radioactivity could only be released enzymatically by collagenase. All the experiments were carried out with collagen matrices which had been treated with trypsin and elastase.

### 5.2.2 Growth Conditions for Enzyme Studies

Overnight V. alginolyticus cultures (10 mℓ) in 2,5% casamino acid medium were used to inoculate 100 mℓ 2,5% peptone medium cultures which were incubated with aeration for 4 h at 30°C. The cells were harvested, washed twice and re-suspended in 20 mℓ low SNP medium.

### 5.2.3 Enzyme Assays

Collagenase was assayed by the method of Wünsch & Heidrich (1963) utilizing the synthetic collagenase substrate, PZ-Pro-Leu-Gly-Pro-D-Arg.

Collagenase activity was expressed as nkat $m\ell^{-1}$ where one katal is the amount of activity that converts one mole of substrate per second (Florkin & Stotz, 1973). Alkaline protease activity was assayed using the synthetic substrate azocasein. One unit of alkaline protease activity is defined as the amount of enzyme that gives an increase

in absorbance of 0,1 at 440 nm in 30 min at 40°C.

### 5.2.4 Polyacrylamide Gel Electrophoresis (PAGE)

Extracellular proteases and collagenase produced by V. alginolyticus were characterized by linear PAGE in gels containing SDS and gelatin as a copolymerized substrate by the method of Heussen & Dowdle (1980). Cultures were sedimented by centrifugation in a microfuge and 1,0 mℓ supernatant samples were mixed with 0,1 mℓ SDS (25%) and 0,1 mℓ glycerol and incubated at 37°C for 30 min. PAGE was carried out at 4°C in 10% linear SDS-gelatin-polyacrylamide gels at pH 7,6 and 8,8 at constant currents of 20 and 8 mAmp respectively.

After electrophoresis the gels were washed in Triton X-100 for 1 h at 4°C to remove the SDS. The gels were incubated in 0,1 M glycine (pH 7,6 or 8,8) for 3,5 h at 37°C and then stained with 2% amido black.

Phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor with the molecular weights 94 000, 67 000, 43 000, 30 000 and 20 100 respectively were used as molecular weight markers. Effects of the following protease inhibitors on the activities of the proteases were determined by adding the inhibitors to the gelatin gels during incubation in 0,1 M glycine: DFP (10 mM), phenylmethyl-sulfonyl fluoride (10 mM), EDTA (10 mM), O-phenanthroline (1 mM), TLCK (1 mM), tosyl-phenylalanine-chloromethylketone (1 mM). nitrophenylguanidinobenzoate

(NPGB) (1 mM), benzamidine (10 mM), pCMB (1 mM), iodoacetamide (10 mM), soybean trypsin inhibitor (1 mM), lima bean inhibitor (1 mM), bovine pancreatic trypsin inhibitor (trasylol) (1 mM), and ovomucoid (1 mM). Inhibition of the proteases by histidine (0,5%) and the divalent cations, $CaCl_2$, $MgCl_2$, $MgSO_4$, LiCl and $ZnCl_2$ (1mM) was also determined.

## 5.3 RESULTS

### 5.3.1 Characterization of Proteases by Gelatin-PAGE

The number and molecular weights of the extracellular proteases produced by V. alginolyticus cells in low SNP medium containing the collagen matrices (described in Chapter 4) were determined by gelatin-PAGE at pH 7,6 and 8,8. Three major bands of protease activity with molecular weights of 28 000, 22 500 and 19 500 (proteases 1, 2 and 3 respectively) and two minor bands of protease activity with molecular weights of 15 500 and 14 500 (proteases 4 and 5 respectively)were obtained at pH 7,6 (Figure 5.1). The two minor bands of activity were absent at pH 8,8 (Figure 5.1). The production of the two minor proteases varied but they were always produced in small amounts and were often not detected after gelatin-PAGE at pH 7,6. Total alkaline protease activity in samples taken from the collagen matrix cultures could be measured by the azocasein assay

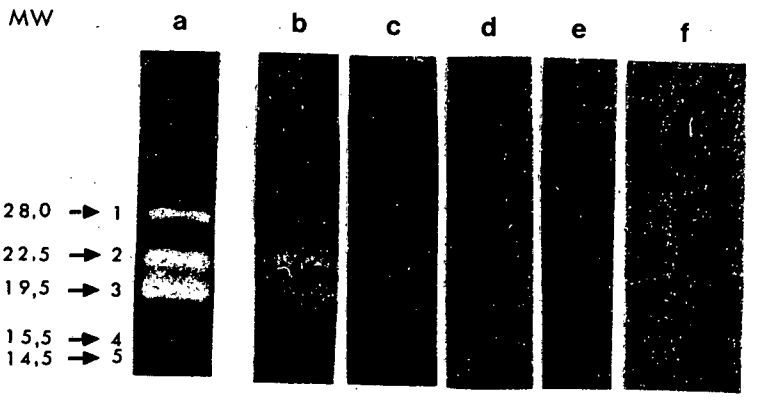



FIGURE 5.1: Effect of DFP and PMSF on extracellular protease activity of V. alginolyticus cultures after gelatin-PAGE at pH 7,6 and 8,8. The lanes represent: culture supernatants without inhibitors at pH 7,6 (a) and 8,8 (b), with DFP (10 mM) at pH 7,6 (c) and 8,8 (d), and with PMSF (10 mM) at pH 7,6 (e) and 8,8 (f). MW, molecular weight ($10^2$). The arrows indicate the positions of proteases 1,2,3,4 and 5.

method (6,8 units $m\ell^{-1}$ after 10 h incubation at 30°C). No collagenase activity as measured by the Wünsch & Heidrich (1963) assay utilizing the synthetic collagenase substrate (PZ-Pro-Leu-Gly-Pro-Arg) could be detected.

### 5.3.2 Effect of Inhibitors

The effect of various inhibitors (Table 5.1) on the activities of the proteases at pH 7,6 and 8,8 was investigated after gelatin-PAGE by adding the inhibitors to the gelatin gels during incubation in 0,1 M glycine. All five proteases were inhibited at pH 7,6 and 8,8 by the serine protease inhibitors DFP and PMSF (Figure 5.1, Table 5.1), suggesting that they are all serine proteases. EDTA inhibited all the proteases but O-phenanthroline only inhibited protease 1 at pH 7,6 and 8,8 and proteases 4 and 5 only at pH 7,6. (Figure 5.2, Table 5.1). The five serine proteases were not inhibited by sulphydryl protease inhibitors (pCMB and iodoacetamide) or by inhibitors of trypsin-like enzymes (TLCK, TPCK, NPGB, benzamidine, soybean trypsin inhibitor, lima bean inhibitor, trasylol and ovomucoid). The divalent cations $CaCl_2$, $MgCl_2$ and $MgSO_4$ had no effect on any of the five proteases. LiCl inhibited proteases 4 and 5 at pH 7,6 and $ZnCl_2$ inhibited proteases 1 and 3 at pH 8,8 and proteases 4 and 5 at pH 7,6 (Figure 5.2).

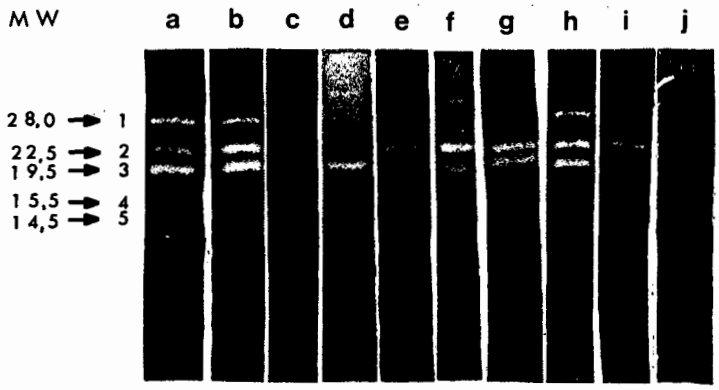



FIGURE 5.2: Gelatin-PAGE of extracellular protease activity of V. alginolyticus cultures at pH 7,6 and 8,8. The lanes represent: culture supernatants without inhibitors at pH 7,6 (a) and 8,8 (b), with EDTA (10 mM) at pH 7,6 (c) and 8,8 (d), with O-phenanthroline (1 mM) at pH 7,6 (e) and 8,8 (f), with LiCl (1 mM) at pH 7,6 (g) and 8,8 (h), and with $ZnCl_2$ (1 mM) at pH 7,6 (i) and 8,8 (j). The arrows indicate the positions of proteases 1,2,3,4 and 5.

TABLE 5.1: Effect of protease enzyme inhibitors and divalent cations on the activity at pH 7,6 and 8,8 of extracellular proteases produced by V. alginolyticus cells. After gelatin-PAGE the inhibitors were added to the gelatin gels during incubation in 0,1 M glycine for 3,5 h. The gels were then stained with amido black.

| Inhibitor | Protease and molecular weight | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 28 000 | | 2 22 500 | | 3 19 500 | | 4 15 500 | 5 14 500 |
| | pH 7,6 | pH 8,8 | pH 7,6 | pH 8,8 | pH 7,6 | pH 8,8 | pH 7,6 | pH 7,6 |
| DFP, PMSF, EDTA | +* | + | + | + | + | + | + | + |
| O-phenanthroline | + | + | -** | - | - | - | + | + |
| TLCK, TPCK, NPGB, benzamidine, pCMB, lodoacetamide, soybean trypsin inhibitor, lima bean inhibitor, trasylol, ovomucoid | - | - | - | - | - | - | - | - |
| $CaCl_2$, $MgCl_2$, $MgSO_4$ | - | - | - | - | - | - | - | - |
| LiCl | - | + | - | - | - | - | + | + |
| $ZnCl_2$ | - | + | - | - | - | + | + | + |
| Histidine | - | - | - | - | - | - | - | - |

* Total inhibition of protease activity after gelatin-PAGE.

** No inhibition of protease activity after gelatin-PAGE.

### 5.3.3 Effect of Histidine and Temperature on the Production of the Serine Proteases

The addition of histidine (0,5%) to the gelatin gels during incubation in 0,1 M glycine did not affect the activities of the five serine proteases (Table 5.1). However, the addition of histidine to the low SNP medium during the growth of the cells in the collagen culture dishes (described in Chapter 4) caused the increased production of protease 1, did not affect production of protease 2 and inhibited production of protease 3 at pH 7,6 and 8,8 (Figure 5.3). The effect of histidine on the production of the two minor proteases 4 and 5 was difficult to determine and variable results were obtained. The production of alkaline extracellular protease activity is specifically inhibited by temperature (37°C) (refer to Chapter 2). Gelatin-PAGE analysis of supernatants from stationary phase peptone cultures incubated at 30 and 37°C indicated that the synthesis of protease 1 was markedly inhibited at 37°C (Figure 5.3). The production of protease 3 at 37°C was also inhibited but to a lesser extent than protease 1. Production of protease 2 was not affected by changes in temperature between 30 and 37°C. The effect of temperature on the production of the minor proteases 4 and 5 could not be determined due to the small and variable amounts of these enzymes which were produced.

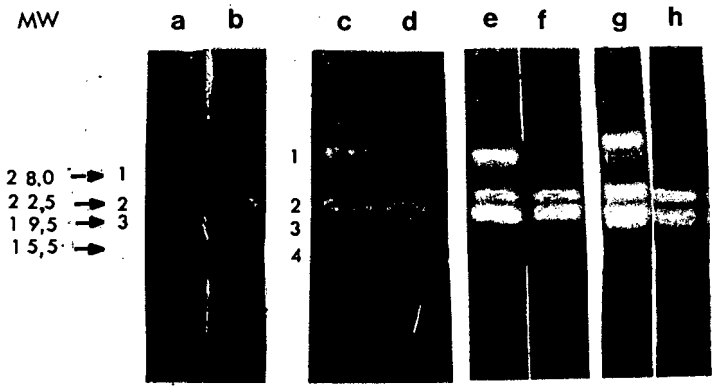



FIGURE 5.3: Effect of histidine and temperature on the production of extracellular serine proteases by V. alginolyticus at pH 7,6 and 8,8. The lanes represent: culture supernatants with (a and c) and without (b and d) histidine (0,5% w/v) at pH 7,6 (a and b) and 8,8 (c and d); culture supernatants at 30 (e and g) and 37°C (f and h) at pH 7,6 (e and f) and 8,8 (g and h). The arrows indicate the positions of proteases 1,2,3 and 4.

### 5.3.4 Gelatin-PAGE of a Concentrated Collagenase Preparation

Gelatin-PAGE of a concentrated commercial preparation of *V. alginolyticus* collagenase revealed six bands of activity at pH 8,8 and 7,6 with molecular weights of 45 000, 38 500, 33 500, 31 000, 19 000 and 16 000 (proteases 1, 2, 3, 4, 5 and 6 respectively) (Table 5.2, Figure 5.4). The 19 000 and 16 000 molecular weight bands were identified as serine proteases as they were inhibited by DFP and PMSF. The other four bands of activity (proteases 1, 2, 3 and 4) were not affected by these inhibitors at pH 8,8 (Table 5.2, Figure 5.4). Proteases 1, 2, 3 and 4 were inhibited by PMSF at pH 7,6 when added to the gels during incubation with 0,1 M glycine. However, when the concentrated commercial collagenase sample was incubated with PMSF (1 mM) for 15 min at 30°C and then assayed for collagenase activity using the synthetic collagenase substrate, PZ-Pro-Leu-Gly-Pro-Arg (Wünsch & Heidrich, 1963) no difference in the activity of the PMSF-treated collagenase (0,55 nkat $m\ell^{-1}$) and the control collagenase sample (0,52 nkat $m\ell^{-1}$) was observed.

Benzamidine, pCMB, O-phenanthroline and the divalent cations, $MgCl_2$ and LiCl inhibited proteases 1, 2, 3 and 4 at pH 7,6 only (Figure 5.5). $ZnCl_2$ inhibited proteases 1, 2, 3 and 4 at both pH 7,6 (Figure 5.5) and 8,8 (Figure 5.4) and EDTA inhibited all six proteases at these pH values (Figures 5.4 and 5.5). The inhibitors of trypsin-like enzymes (TLCK, NPGB, soybean trypsin inhibitor, lima bean inhibitor,

TABLE 5.2: Effect of protease enzyme inhibitors and divalent cations on the activity at pH 7,6 and 8,8 of concentrated commercial V. alginolyticus collagenase. After gelatin-PAGE the inhibitors were added to the gelatin gels during incubation in 0,1 M glycine for 3,5 h. The gels were then stained with amido black.

| Inhibitor | Protease and molecular weight | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 45 000 | | 2 38 500 | | 3 33 500 | | 4 31 000 | | 5 19 000 | | 6 16 000 | |
| | pH 7,6 | pH 8,8 | pH 7,6 | pH 8,8 | pH 7,6 | pH 8,8 | pH 7,6 | pH 8,8 | pH 7,6 | pH 8,8 | pH 7,6 | pH 8,8 |
| DFP, PMSF | *+ | **- | + | - | + | - | + | - | + | + | + | + |
| Benzamidine, pCMB, O-phenanthroline | + | - | + | - | + | - | + | - | - | - | - | - |
| EDTA | + | + | + | + | + | + | + | + | + | + | + | + |
| TPCK | - | - | - | - | - | - | - | - | - | - | - | + |
| TLCK, NPGB, soybean trypsin inhibitor, lima bean inhibitor, trasylol, ovomucoid, iodoacetamide | - | - | - | - | - | - | - | - | - | - | - | - |
| $CaCl_2$ | - | - | - | - | - | - | - | - | - | - | - | - |
| $MgCl_2$ | + | - | + | - | + | - | + | - | - | - | - | - |
| $MgSO_4$ | - | - | - | - | - | - | + | - | - | - | - | - |
| LiCl | + | - | + | - | + | - | + | - | - | - | - | - |
| $ZnCl_2$ | + | + | + | + | + | + | + | + | - | - | - | + |
| Histidine | + | + | + | + | + | + | + | + | - | - | - | - |

* Total inhibition of protease activity after gelatin-PAGE.
** No inhibition of protease activity after gelatin-PAGE.

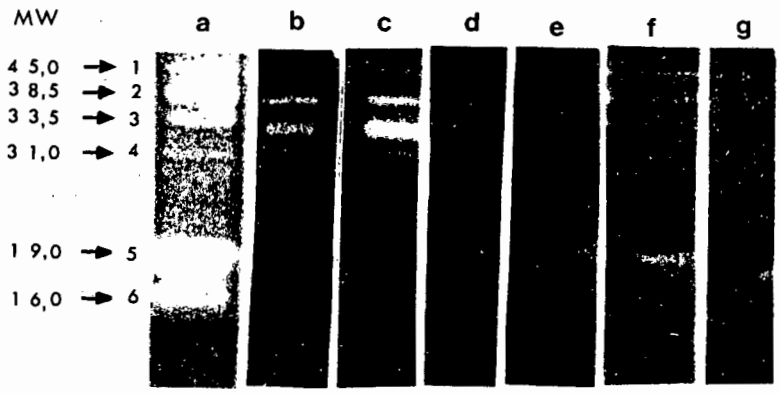



FIGURE 5.4: Gelatin-PAGE of a concentrated V. alginolyticus collagenase preparation at pH 8,8. The lanes represent: control collagenase preparation (a); collagenase preparation treated with DFP (10 mM)(b); PMSF (10 mM)(c); histidine (0,5% w/v)(d); O-phenanthroline (1 mM)(e); $ZnCl_2$ (1 mM)(f) and EDTA (10 mM)(g). MW, molecular weight ($10^2$). The arrows indicate the positions of collagenase bands 1,2,3,4 and serine proteases 5 and 6.

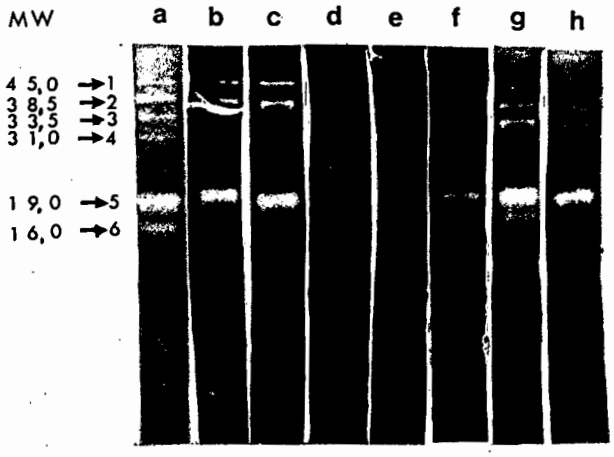



FIGURE 5.5: Gelatin-PAGE of a concentrated V. alginolyticus collagenase preparation at pH 7,6. The lanes represent: control collagenase preparation (a); collagenase preparation treated with benzamidine (10 mM)(b); pCMB (1 mM)(c); O-phenanthroline (1 mM)(d); EDTA (10 mM)(e); $MgCl_2$ (1 mM)(f); LiCl (1 mM)(g) and $ZnCl_2$ (1 mM)(h). The arrows indicate the positions of collagenase bands 1,2,3,4 and serine proteases 5 and 6.

trasylol and ovomucoid) and the divalent cation $CaCl_2$, did not affect the activity of any of the proteases. In some cases a slight enhancement of proteases 1, 2, 3 and 4 was obtained when treated with $CaCl_2$.

## 5.4 DISCUSSION

V. alginolyticus produces three major and two minor serine proteases as identified by the serine protease inhibitors, DFP and PMSF. The minor proteases are produced in small amounts and it is concluded that the extracellular serine protease activity is largely due to proteases 1, 2 and 3. These five proteases are not collagenases as they are inhibited by serine protease inhibitors which do not inhibit V. alginolyticus collagenase activity (Lecroisey et al., 1975). Furthermore, the activities of the proteases are not inhibited by histidine which inhibits V. alginolyticus collagenase activity (Lecroisey et al., 1975). Studies on the effects of inhibitors and pH on activity, and histidine and temperature on production suggest that the three major serine proteases are different enzymes and not autodigestion fragments of one or two enzymes. The two minor proteases (4 and 5) have similar molecular weights and are inhibited by the same inhibitors. Protease 5 could be a degradation product of protease 4.

Although the five serine proteases could be detected in culture supernatants by gelatin-PAGE, no collagenase enzymes

could be detected in the same supernatants by this technique. The supernatants possessed collagenase activity as they caused the specific release of [$^3$H]-proline from collagen matrices (refer to Chapter 4). Collagenase enzymes were only detected by gelatin-PAGE in concentrated and partially purified enzyme preparations from V. alginolyticus.

Long et al. (1981) showed that serine protease production in V. alginolyticus cultures is stimulated by histidine. This stimulation of the total serine protease activity is due to the production of a single serine protease (protease 1). The production of the other four proteases is either inhibited or unaffected by histidine. Histidine affects the production of the enzymes since the serine protease enzyme activities of cell-free samples were not affected by histidine. Studies on the effect of temperature indicate that the production of serine protease 1 is also specifically regulated by temperature and that this enzyme is responsible for the majority of the serine protease activity in V. alginolyticus cultures assayed by the azocasein method.

The four bands of collagenase activity in the commercial V. alginolyticus collagenase preparation are likely to be partial digests of the same enzyme. Keil-Dlouha (1976) and Reid et al. (1980) reported that autodegradation of V. alginolyticus collagenase results in the production of at least three active fragments. The degradation

of this commercial collagenase preparation may also be due to the two contaminating serine proteases which were detected.

The gelatin-PAGE technique utilized here for visualising enzymatic activity is an extremely useful one. However, it does have limitations, the nature of the substrate being one such limitation. This problem can be partially overcome by utilizing the technique of Granelli-Piperno & Reich (1978) in which the electrophoretic slab gel is layered onto a second indicator gel which contains the substrate. The position of the proteases can be seen as clear areas in an opaque background on dark field illumination. This method allows a greater variety of substrates to be used, enabling visualisation of a greater number of enzymes.

# CONCLUSION

The aim of this work was to characterize and study the regulation of the extracellular proteases produced by V. alginolyticus.

V. alginolyticus was found to produce three major and two minor serine exoproteases which were identified by gelatin-PAGE, as well as an extracellular collagenase. Most of the proteolytic activity measured by means of the azocasein assay is protease 1 activity. Further work should include the isolation of these proteases by a method such as ion exchange chromatography to obtain further information with regard to the relationships between these proteases.

Regulation of collagenase and the serine proteases by temperature, oxygen and histidine acts at the level of transcription, affecting the synthesis of specific proteins. There are few reports on temperature control of exoproteases, however, this represents an important mechanism of environmental regulation. Temperature control of nitrogenase synthesis in K. pneumoniae (Hennecke & Shanmugam, 1979), is also exerted at the transcriptional level. Further biochemical and genetic research on the temperature control mechanism should include the isolation of mutants able to

synthesize the exoproteases at 37°C and mutants in the synthesis of the 100K protein. Histidine, by virtue of its conversion to urocanic acid, induces alkaline serine protease production (Long *et al.*, 1981), whereas histidine inhibits collagenase production. Histidine is also involved in the regulation of the *hut* enzymes in *V. alginolyticus* (Bowden *et al.*, 1982).

This study demonstrated that the synthesis of a specific protein (52K protein) increased and decreased in a reciprocal manner to that shown by the proteases under histidine control. Isolation of mutants in which the serine proteases do not respond to histidine and mutants which do not synthesize the 52K protein should be included in future research.

There are no detailed reports on secretion across both cell membranes in Gram-negative eubacteria. *V. alginolyticus* is thus a model system for studying the mechanism of secretion across both membranes. Quinacrine and lidocaine were found to affect secretion and processing of the exo-enzymes rather than transcription. Accumulation of a 38 000 molecular weight protein, possibly the collagenase subunit (molecular weight 35 000, Keil-Dlouha & Keil, 1978), occurred in the presence of quinacrine and lidocaine. Positive identification of this protein could be achieved by a method such as specific immunoprecipitation followed by two-dimensional PAGE of extracted proteins from *V. alginolyticus* cultures.

The present study has shown that there is a low basal constitutive level of collagenase in V. alginolyticus. This collagenase may act as a receptor site on the cell wall for binding of the collagen inducer molecule. There have been no reports of a peptide capable of inducing collagenase. This study suggests that such a peptide may be isolated from peptone. A peptone fraction of molecular weight range 350 to 1 500 is capable of inducing collagenase. The inducer molecule must have a collagenase sensitive bond for induction but it is suggested that for induction by this peptide, the tertiary structure is not vital.

The value of bacteria as model cells in biological research has been well illustrated. The experiments described here illustrate the value of a Gram-negative bacterium such as V. alginolyticus in research on regulation and secretion of exoproteases.

# APPENDIX A

## MEDIA

### Casamino Acids Medium

| | |
|---|---|
| vitamin free Casamino Acids | 2,5 g |
| Tris-HCl buffer, pH 7,6 (as below) | 100 mℓ |

### Complex Medium Agar

| | |
|---|---|
| casein hydrolysate | 17,0 g |
| NaCl | 23,4 g |
| glycerol | 10,0 g |
| $Na_2SO_3$ | 0,1 g |
| nutrient broth | 8,0 g |
| soytone | 3,0 g |
| tryptone | 0,5 g |
| vitamin free Casamino Acids | 0,5 g |
| yeast extract | 2,0 g |
| glucose | 5,0 g |
| Difco agar | 15,0 g |
| distilled water | 1 000 mℓ |

Adjust the pH to 7,6 with NaOH.

## GELATIN-PAGE BUFFERS AND SOLUTIONS

### Acrylamide-bis-acrylamide (30:1) Stock (A-bis A)

| | |
|---|---|
| acrylamide | 30 g |
| N,N'-methylene-bis-acrylamide | 1 g |

Make up volume to 100 mℓ with distilled water.
Add 5 g active charcoal and stir for 8 h, filter through Whatman's paper No. 1.

Catalysts

N N N'N' tetramethylene-ethylene diamine (TEMED)
Use undiluted.

Ammonium persulphate (10% w/v) in distilled water.

Gelatin

Dissolve 1 g of gelatin in ≤ 80 mℓ distilled water by boiling, allow to cool and adjust volume to 100 mℓ.

Incubation buffer

| | |
|---|---|
| Glycine (0,1 M) | 15,01 g |
| distilled water | 1 000 mℓ |

Adjust the pH to 9,0 with NaOH.

Running Gel Buffer (RGB)

| | |
|---|---|
| Tris (1,5 M) | 18,17 g |
| SDS (10% w/v) in distilled water | 4,0 mℓ |
| distilled water | 96,0 mℓ |

Adjust the pH to 8,8 with concentrated HCl.

Reservoir buffer

| | |
|---|---|
| Tris (0,025 M) | 6,06 g |
| Glycine (0,192 M) | 28,82 g |
| SDS (10% w/v) in distilled water | 20 mℓ |
| distilled water | 1 980 mℓ |

Adjust the pH to 8,5.

Stacking Gel Buffer (SGB)

| | |
|---|---|
| Tris (0,5 M) | 6,057 g |
| SDS (10% w/v) in distilled water | 4,0 mℓ |
| distilled water | 96,0 mℓ |

Adjust the pH to 6,8.

Stain and Destaining Solutions

Stain:

| | |
|---|---|
| Amido black (1% w/v) in distilled water | 10 mℓ |
| destain solution | 100 mℓ |

Destain:

| | |
|---|---|
| acetic acid | 10 mℓ |
| methanol | 30 mℓ |
| distilled water | 60 mℓ |

Triton X-100

| | |
|---|---|
| Triton X-100 | 2,5 mℓ |
| distilled water | 97,5 mℓ |

Preparation of 10% Gelatin-gel

| Solutions | Running Gel | Stacking gel |
|---|---|---|
| A-bis A | 30 mℓ | 0,40 mℓ |
| RGB | 22,5 mℓ | |
| SGB | | 0,50 mℓ |
| Gelatin | 9,0 mℓ | |
| distilled water | 28,5 mℓ | 3,10 mℓ |
| ammonium persulphate | 0,2 mℓ | 0,2 mℓ |
| TEMED | 0,1 mℓ | 0,1 mℓ |

MINIMAL MEDIUM

| | | |
|---|---|---|
| Salts solution: | $K_2HPO_4$ | 10,5 g |
| | $K\ H_2PO_4$ | 4,5 g |
| | sodium citrate | 0,47 g |
| | $(NH_4)_2SO_4$ | 1,00 g |
| | distilled water | 100 mℓ |
| | $MgSO_4.7H_2O$ | 0,1 g |
| | (added cold in solution) | |
| glucose: | 20% (w/v) in distilled water | |
| Water-NaCl solution: | NaCl | 23,4 g |
| | distilled water | 1 000 mℓ |
| To make up: | salts solution | 8 mℓ |
| | glucose | 1 mℓ |
| | water-NaCl solution | 70 mℓ |

## PAGE BUFFERS AND SOLUTIONS

### Acrylamide-bis-acrylamide stock

| | |
|---|---|
| acrylamide | 29,2 g |
| N,N'-methylene-bis-acrylamide | 0,8 g |

Make up volume to 100 mℓ with distilled water.

### Catalysts

Refer to catalysts for gelatin-PAGE.

### RGB

| | |
|---|---|
| Tris (1,5 M) | 18,17 g |
| SDS | 0,4 g |
| distilled water | 100 mℓ |

Adjust the pH to 8,8 with concentrated HCl.

### Reservoir Buffer

| | |
|---|---|
| Tris (0,067 M) | 8,13 g |
| Glycine (0,238 M) | 35,75 g |
| SDS | 2,5 g |
| distilled water | 2 000 mℓ |

### SGB

| | |
|---|---|
| Tris (0,5 M) | 6,057 g |
| SDS | 0,4 g |
| distilled water | 100 mℓ |

Adjust the pH to 6,8.

## Stain and Destain Solutions

| | |
|---|---|
| Stain: | |
| Coomassie blue | 0,05 g |
| destain solution | 100 mℓ |
| Destain: | |
| acetic acid | 270 mℓ |
| distilled water | 2 430 mℓ |
| propan-2-01 | 900 mℓ |

## Preparation of 10% Acrylamide Gels

| Solutions | Running Gel | Stacking Gel |
|---|---|---|
| A-bis A | 12 mℓ | 2 mℓ |
| RGB | 8,2 mℓ | |
| SGB | | 3 mℓ |
| distilled water | 13,65 mℓ | 7 mℓ |
| ammonium persulphate | 160 µℓ | 64 µℓ |
| TEMED | 18 µℓ | 13 µℓ |

## Peptone Medium

| | |
|---|---|
| Peptone/low molecular weight peptone fraction | 0,25 or 2,5 g |
| Tris-HCl buffer, pH 7,6 (as below) | 100 mℓ |

## SNP Medium (low)

| | |
|---|---|
| disodium succinate (hexahydrate)(2 mM) | 0,54 g |
| $(NH_4)_2SO_4$ (1 mM) | 0,132 g |

| | |
|---|---|
| $KH_2PO_4$ (1 mM) | 0,136 g |
| Tris-HCl buffer, pH 7,6 (as below) | 1 000 mℓ |

Tris-HCl Buffer

| | |
|---|---|
| NaCl | 23,4 g |
| $CaCl_2\ 2H_2O$ | 0,29 g |
| Tris (0.1 M) | 12.10 g |
| distilled water | 1 000 mℓ |

Adjust the pH to 7,6 with concentrated HCl.

Tryptone

| | |
|---|---|
| Tryptone | 0,5 g |
| Tris-HCl buffer, pH 7,6 (as above) | 100 mℓ |

# APPENDIX B

## CHEMICALS

Benzamidine - Sigma, Saint Louis, Missouri.

Cerulenin - Makor Chemicals Ltd., Jerusalem, Israel.

Collagenase, Cl. histolyticum - Koch-Light Laboratories, Colnbrook, Bucks, England.

Collagenase, V. alginolyticus - Boehringer Mannheim (SA) (Pty) Ltd.

Cytochrome C - Miles Laboratories, Goodwood, South Africa.

DFP - Sigma, Saint Louis, Missouri.

EDTA - Merck, Darmstadt, Federal Republic of Germany.

En$^3$hance$^{TM}$ surface spray - New England Nuclear, Boston, Mass.

Growth hormone - State Vaccine Institute, Cape Town, SA.

Histidine hydrochloride - Merck, Darmstadt, Federal Republic of Germany.

Human albumin - Western Province Blood Transfusion Service, Cape Town, SA.

Insulin A chain - Sigma, Saint Louis, Missouri.

Iodoacetamide - BDH Chemicals Ltd., Poole, England.

Lidocaine hydrochloride - a gift from Dr. K. Ivanetich, Department of Medical Biochemistry, University of Cape Town, Cape Town, SA.

Lima bean inhibitor - Sigma, Saint Louis, Missouri.

Molecular weight markers for gelatin-PAGE - Pharmacia Fine Chemicals, Uppsala, Sweden.

Molecular weight markers for gradient PAGE - BRL Inc., Neu-Isenberg, Federal Republic of Germany.

NPGB - Sigma, Saint Louis, Missouri.

O-phenanthroline - Sigma, Saint Louis, Missouri.

Ovalbumin - State Vaccine Institute, Cape Town, SA.

Ovomucoid - Worthington Biochemical Corporation, Freehold, New Jersey.

pCMB - Sigma, Saint Louis, Missouri.

Peptone - Difco Laboratories, Detroit, Michigan.

Phenol red - Merck, Darmstadt, Federal Republic of Germany.

PMSF - Merck, Darmstadt, Federal Republic of Germany.

Quinacrine dihydrochloride - Sigma, Saint Louis, Missouri.

Soybean trypsin inhibitor - Sigma, Saint Louis, Missouri.

TLCK - Sigma, Saint Louis, Missouri.

TPCK - Sigma, Saint Louis, Missouri.

Transferrin - Sigma, Saint Louis, Missouri.

Trasylol - Bayer SA (Pty) Ltd., Cape Town, South Africa.

Vitamin $B_{12}$ - Merck, Darmstadt, Federal Republic of Germany.

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