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# A STUDY OF THE CELL WALL- ASSOCIATED PROTEINASE OF LACTIC STREPTOCOCCI

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry at Massey University.

*KEE HUAT NG*

1988

# ABSTRACT

The cell wall proteinase of *Streptococcus lactis* 4760 was released by incubation of milk-grown cells in $Ca^{++}$- free buffer. The effects of duration of incubation, pH and presence of $Ca^{++}$ ions on the release of proteinase activity was investigated. The extent of leakage of intracellular enzymes during incubation was monitored by the appearance of lactate dehydrogenase activity in the incubation buffer. The proteinase released from the cells was partially purified by ion- exchange and gel permeation chromatography and then analysed for activity towards various milk- proteins. Only a single proteinase was evident from the purification. This enzyme was active towards β- casein but showed no apparent cleavage of $\alpha_{s1}$- and κ- caseins nor the whey proteins, α- lactalbumin and β- lactoglobulin. The enzyme cleaved the β- casein molecule within the C- terminal 49 residues, generating four main peptides containing residues 167- 175, 176- 182, 183- 193 and 194- 209, and smaller amounts of peptides corresponding to the overlapping sequences 161- 166, 164- 169 and 166- 175. The four main peptides are identical to those generated by an *S. lactis* 763 proteinase described by Monnet *et al.* (1986) and by an *S. cremoris* HP proteinase recently described by Visser *et al.* (1987). No apparent specificity of enzyme action was evident. A preliminary study of the cell wall proteinase from *S. cremoris* SK11, a strain reported to produce a proteinase with a different specificity, suggested that the enzyme may hydrolyse the β- casein molecule at the same sites as those cleaved by the *S. lactis* 4760 enzyme.

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Kee H. Ng

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **AcOH** | acetic acid |
| **CIE** | cross- immunoelectrophoresis |
| **DEAE** | diethylaminoethyl |
| **EDTA** | ethylenediaminetetra- acetic acid |
| **ERSM** | experimental reconstituted skim milk medium |
| **FITC** | fluorescein isothiocyanate |
| **$HPO_4/H_2PO_4$** | phosphate buffer |
| **LDH** | lactate dehydrogenase |
| **MES** | 2-(N-morpholino-) ethanesulfonic acid |
| **NADH** | nicotinamideadeninedinucleotide (reduced form) |
| **NaOAc** | sodium acetate |
| **PAGE** | polyacrylamide gel- electrophoresis |
| **PMSF** | phenylmethylsulfonylfluoride |
| **RF** | relative fluorescence |
| **RP-HPLC** | reverse phase- high performance liquid chromatography |
| **RSM** | reconstituted low heat skim milk medium |
| **SDS** | sodium dodecyl sulfate |
| **TRIS** | tris-(hydroxymethyl-) aminomethane |
| **TCA** | trichloroacetic acid |
| **TFA** | trifluoroacetic acid |

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# 1 INTRODUCTION

The Group N or lactic streptococci are aerotolerant anaerobes unable to synthesize most of the amino acids and vitamins essential for bacterial growth. These Gram positive bacteria propagate well, however, in 'rich' media such as milk. *Streptococcus lactis* * subspecies *lactis* and *cremoris* (*S. lactis* and *S. cremoris* in short ) are important 'starter bacteria' selectively added to milk to initiate the production of cheese. Two aspects of the bacterial growth physiology relevant to this process are :

(i) the ability to ferment rapidly, the milk sugar lactose, to lactic acid at moderate temperatures (30-37° C). This process provides virtually all the ATP required for bacterial metabolism. The lactic acid produced lowers the pH of milk to about 4- 5 units. This not only inhibits the growth of competing and/ or undesirable bacteria but also causes the precipitation of the milk protein, casein, i.e., the initial step in cheese production.

(ii) the proteolysis of milk proteins to support bacterial growth. This process is necessary to provide the nitrogen sources required to support bacterial growth especially to the high cell densities required for rapid fermentation of milk. Proteolysis continues during the ripening and maturation of cheese and affects both the development of flavour and texture of the final cheese product. Two groups of enzymes participate in the proteolytic system of the bacteria : (a) proteinases which are defined as the enzymes which make the initial cleavage of the native proteins in milk, probably to large oligopeptides and (b) peptidases, including the exo- and endo- peptidases which are capable of hydrolysing the oligopeptides to amino acids which can then be used for bacterial protein synthesis.

Current evidence indicates that the proteinases initiating bacterial proteolytic activities are associated with the cell wall of the lactic streptococci, while the peptidases are found in the cell wall, cell membrane as well as the cytoplasm (Figure 1.1). In the following sections, the literature on proteinase enzymes of the lactic streptococci will be reviewed. Available information on both the proteinase and peptidases can be found in several recent reviews (Thomas & Mills, 1981; Law & Kolstad, 1983; Marshall & Law, 1984; Thomas & Pritchard, 1987).

* Although *Streptococcus lactis* has been re-classified as *Lactococcus lactis subspecies lactis* (Schleifer *et al.*, 1985), the former name is still widely used in the literature and will be used in this thesis.

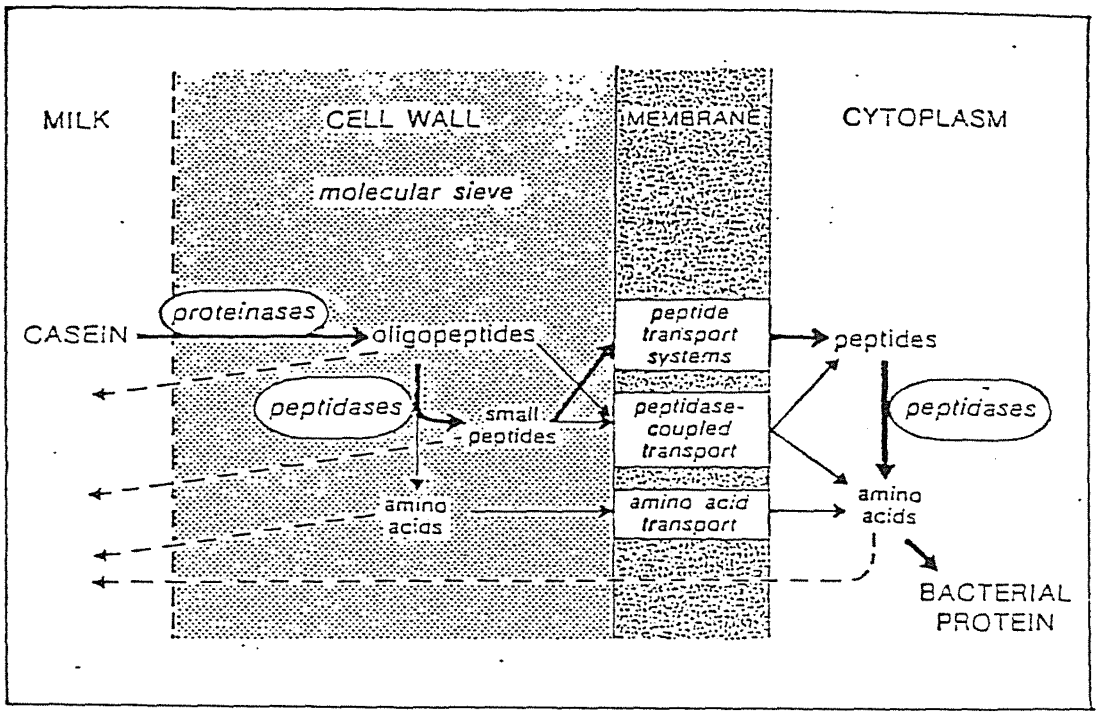



FIGURE 1.1 UTILISATION OF CASEIN FOR GROWTH OF LACTIC STREPTOCOCCI IN MILK.
(Reproduced from Thomas & Pritchard, 1987)

The importance of the proteolytic systems for the growth of the lactic streptococci in milk is treated in section 1.1. Evidence leading to the conclusion that the proteinase activity is cell wall- bound is covered in section 1.2. Although no extensive purification and characterization of the proteinases have been carried out, the available literature, especially the recent work on the proteinase complex of *S. cremoris* is reviewed in sections 1.3 and 1.4. Recent genetic analysis particularly on the proteinase gene of *S. cremoris* is summarised in section 1.5. A brief summary of the role of the enzymes in relation to starter growth for the development of cheese flavour is treated in section 1.6. In section 1.7 the aims of the present study are outlined.

## 1.1 IMPORTANCE OF PROTEOLYSIS FOR THE GROWTH OF LACTIC STREPTOCOCCI IN MILK.

Lactic streptococci are dependent on extracellular sources of amino acids to support the protein synthesis required for bacterial growth (Reiter & Oram, 1962). A comparison of the amount of amino acids present as free amino acids, or released from hydrolysis of the non-protein nitrogen fraction, in milk to the amino acid composition of the cell protein from bacteria isolated from fully coagulated milk cultures is shown in Table I. This indicates that the non- protein nitrogen fraction in milk is not a sufficient source of nitrogen to support the synthesis of bacterial protein in the coagulated milk cultures. Hence milk protein must provide a further source of nitrogen for bacterial growth to high cell densities. An extracellular proteolytic system would be required to hydrolyse the milk proteins to amino acids or peptides small enough to be transported through the bacterial cell membrane (Law *et al.*, 1976: Rice *et al.*, 1978).

The first indication of extracellular proteinase activity in lactic streptococci was shown for *S. lactis* C10 by Thomas *et al.* (1974). They demonstrated casein- hydrolysing activity by cell wall and cell membrane components separated after disintegration of the intact cells by sonication (see section 1.2). Spontaneously occurring mutants of the streptococci which lacked the proteolytic activity ($Prt^-$ strain) were found to be able to grow to only about 25% of the high cell densities reached by wild- type ($Prt^+$) parental strains (Pearce *et al.*, 1974; Mills & Thomas, 1981). The growth rate of the mutants was increased to that comparable to the wild-type when casein hydrolysates were added to the growth medium (Figure 1.1.1). These observations suggested that the casein- hydrolysing activity of the $Prt^+$ strain was located extracellularly and that the absence of the proteolytic activity in the mutant strain limited the growth of the bacteria once the non- protein nitrogen sources of the milk

media became depleted.

A systematic study (Mills & Thomas, 1981) to define the actual contributions of the different potential nitrogen sources to the synthesis of bacterial protein during starter growth in milk provided further evidence for the role of the bacterial proteolytic system in using the nitrogen sources for growth. $^{14}C$- labelled amino acids, peptides and milk proteins were added separately to the growth media and the incorporation of radioactivity in the bacterial protein after several generations of growth were measured. The incorporation of radioactivity into the bacterial protein did not continue after two generations of growth when $^{14}C$- labelled amino acids or peptides were added to the growth media but continued to increase up to six generations of growth when $^{14}C$-labelled milk protein was provided as the nitrogen source. This observation suggested that the milk protein must be hydrolysed to support the bacterial protein synthesis at the high cell densities.

## TABLE I

Amino acid requirements of lactic streptococci and the minimum concentrations required for bacterial protein synthesis compared with the levels of amino acids present in milk in low molecular weight form. (Reproduced from Thomas & Pritchard, 1981.)

| Amino acid | Requirement for growth [a] | | Minimum concentration required for *S. cremoris* AM2 cell protein synthesis [b] (μg/ml) | Concentration in milk (μg/ml) | |
|---|---|---|---|---|---|
| | *S. cremoris* | *S. lactis* | | free [c] | NPN [d] |
| Asp | − | − | 29.4 | 5.0 | 25.7 |
| Thr | − | − | 14.9 | 1.3 | 10.8 |
| Ser | ± | − | 12.0 | 3.7 | 18.8 |
| Glu | + | + | 40.1 | 35.9 | 78.1 |
| Pro | + | − | 8.8 | 0.8 | 5.6 |
| Gly | ± | − | 11.9 | 5.3 | 19.6 |
| Ala | ± | − | 19.2 | 3.5 | 9.6 |
| Cys | − | − | ND [e] | nd [f] | nd |
| Val | + | + | 14.7 | 2.6 | 11.0 |
| Met | + | + | 6.5 | nd | 3.7 |
| Ile | + | + | 12.5 | 0.8 | 6.4 |
| Leu | + | + | 21.5 | 1.2 | 7.3 |
| Tyr | ± | − | 10.4 | ND | ND |
| Phe | + | ± | 15.8 | ND | ND |
| Lys | ± | − | 23.1 | 4.1 | 18.4 |
| His | + | + | 5.9 | 2.8 | 4.0 |
| Trp | ± | − | ND | ND | ND |
| Arg | ± | ± | 13.1 | 1.6 | 3.5 |

[a] + = required by all strains tested: ± = required by some of the strains tested; − = not required [6].
[b] Based on the amino acid composition of *S. cremoris* AM2 protein [7], given that the final cell density in coagulated milk is ~ 500 μg (dry weight) bacteria/ml [8], 52% fo bacterial dry matter is protein [7] and assuming the cell does not synthesize amino acids. Similar data were obtained for *S. cremoris* E8 [7].
[c] Values from [7]. Similar levels of free amino acids have been found in aseptically drawn milk [9]. Aston [9] reported Met, Tyr and Phe levels of 0.0, 1.3 and 0.8 μg/ml, respectively.
[d] Amino acids resulting from acid hydrolysis of the non-protein nitrogen (NPN) fraction (soluble in 12% TCA) [7].
[e] ND = not determined.
[f] nd = not detectable.

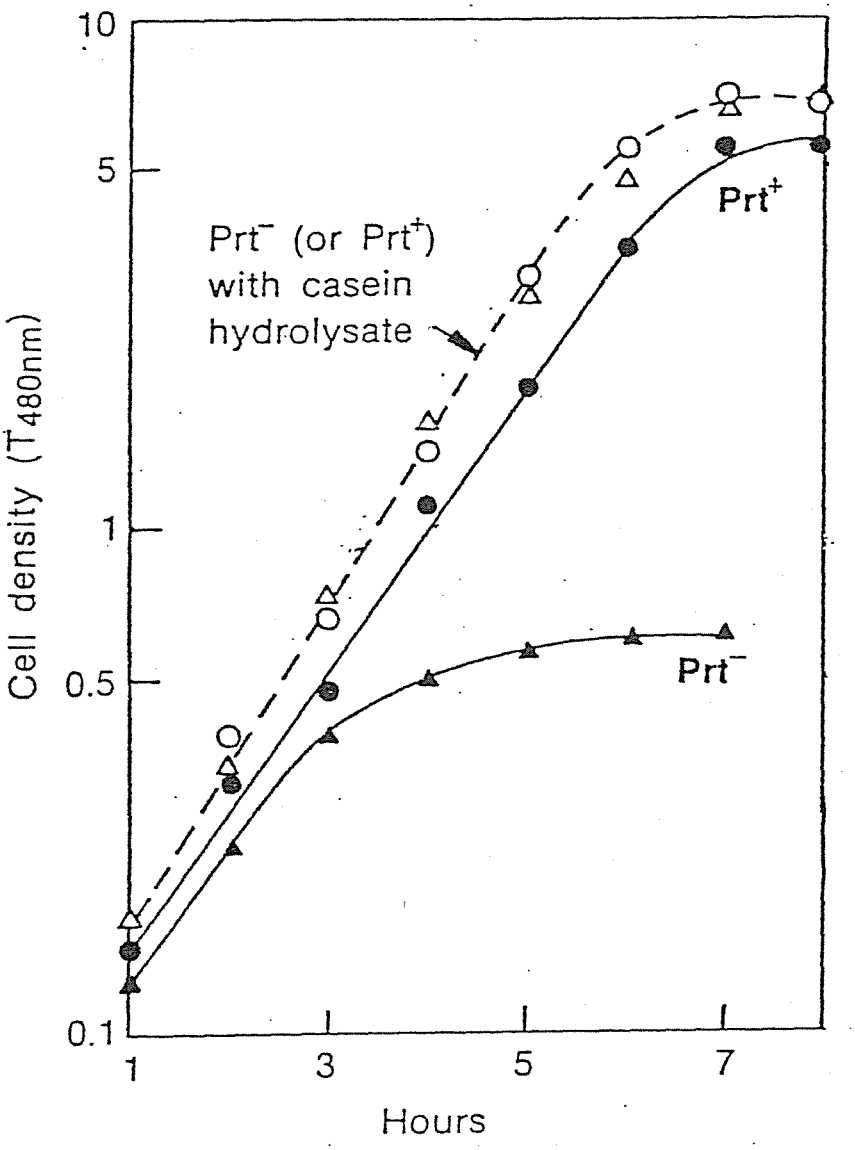



FIGURE 1.1.1

COMPARISON OF THE GROWTH OF PRT⁻ MUTANTS AND WILD TYPE OR PRT⁺ STRAINS OF *S. CREMORIS* 266 IN LOW HEAT- SKIM MILK.

Growth of starter bacteria in unsupplemented low heat- skim milk (solid lines) and supplemented with enzyme- hydrolysed casein (broken lines, 10 mg Trypticase/ml) are shown (adapted from Mills & Thomas, 1981).

## 1.2 LOCATION OF PROTEINASE ACTIVITY IN LACTIC STREPTOCOCCI

The requirement for hydrolysis of milk protein to support bacterial growth to maximum cell densities indicates that the proteinase required to hydrolyse the protein must be located extracellularly. Unlike most Gram positive bacteria such as *Bacillus spp* . there is little evidence to show that the proteinase enzyme(s) of the lactic streptococci is / are secreted into the growth medium. Only *S. cremoris* ML1 was shown to secrete the enzyme to the cell exterior (Exterkate, 1976). For all other strains examined, the proteinase enzymes appear to be located in the cell wall.

The first demonstration that the proteinase activity of the lactic streptococci is located at or near the cell wall was reported by Thomas *et al.* (1974). Intact cells were shown to have casein- hydrolysing activity which could be separated with the particulate fraction of sonicated cells consisting mainly of cell membrane and cell wall material. When the cell wall components of the bacteria were solubilised with phage lysin under osmotically stable conditions to prevent lysis of the cell membrane, 84% of the proteolytic activity of the intact cells was detected in the cell wall component with less than 1% leakage of intracellular marker enzymes (eg. aldolase, glyceraldehyde 3-P dehydrogenase and lactate dehydrogenase). Cells of *S. cremoris* HP treated with lysozyme which disintegrates the cell wall also resulted in the recovery of the proteolytic activity of intact cells with the cell wall fraction (Exterkate, 1975).

A method for detecting proteinase activity in samples separated in discontinuous polyacrylamide gel electrophoresis under non- denaturing conditions was developed by Cliffe & Law (1985). The gels containing electrophoresed samples were soaked with casein prior to staining for protein with Coomassie Blue and the presence of a clear band against a protein- stained background was taken as indication of casein- hydrolysing activity. When cell wall and intracellular fractions prepared from lysozyme treated cells of *S lactis* NCDO 712 were electrophoresed, four clear bands were detected only from the cell wall samples indicating that the casein- hydrolysing activity was cell wall- associated. The absence of corresponding bands when the gels were soaked with casein in the presence of proteinase inhibitors and also when the cell wall samples were prepared from proteinase-deficient mutants confirmed that the bands were due to the extracellular proteolytic activity.

During their attempts to isolate the cell wall- associated proteinase from *S. lactis* C10 by solubilising the cell wall with phage lysin, Mills & Thomas (1978) discovered that cells simply suspended in buffer released more proteinase activity than did cells suspended in the

presence of the phage lysin (with less than 1% leakage of intracellular enzymes). This observation provided further support for the proposal that the proteinase activity of the cells was located extracellularly. The release of proteinase activity into the buffer was dependent on the duration of the incubation of cells as well as the pH of the buffer and the temperature. The optimum conditions for the release were 31° C and pH 7.8 and the release was shown to be specifically and markedly inhibited when $Ca^{++}$ ions were added to the buffer. Preparation of cell wall- associated proteinase from *Lactobacillus spp* using both the lysozyme treatment method and the incubation of cells in $Ca^{++}$ free- buffer showed that the latter preparation was less contaminated with intracellular enzyme resultant from cell leakage (Ezzat *et al.*, 1985). In recent studies incubation of lactic streptococci in $Ca^{++}$ free-buffer has been widely used as the preferred method to prepare the cell wall proteinase.

Recently, an immunogold labelling technique was used to locate the proteinase from cells of *S. cremoris* Wg2 (Hugenholtz *et al.*, 1987). Polyclonal antibodies were raised against the proteinase enzyme released into $Ca^{++}$ free- buffer and purified by Cross Immuno-Electrophoresis (CIE, see section 1.3). The antibody was labelled with protein A- gold particles and incubated with whole cells. Sections of cells examined under the electron microscope (EM sections) were reported to have the gold- labelled antibodies deposited only outside the cell wall region. However, closer examination of the EM sections showed the presence of some gold particles in the cell cytoplasm as well. Thus the authors' claim that the proteinase was clearly located outside the cell wall was not wholely convincing. Considering the possibility that the polyclonal antibodies may bind to a variety of bacterial cell wall antigens, the inclusion of proteinase deficient mutants ($Prt^-$) as controls probed with the same labelled antibodies would have improved this method for direct localisation of the proteinase.

No conclusive evidence for the presence of an intracellular casein- hydrolysing activity has been reported. Although an intracellular proteinase was purified from disrupted cells of *S. cremoris* by Ohmiya & Sato (1975) no rigorous criteria to eliminate the possibility of contamination with cell wall material was applied to the preparation of the proteinase from the disrupted cells. The remarkable similarity of the properties of this enzyme with the enzyme released in $Ca^{++}$ free- buffer (see section 1.4) together with the evidence presented in this section all point to the conclusion that the casein- hydrolysing proteinase activity of the lactic streptococci is located near or at the surface of the cell wall.

## 1.3 CLASSIFICATION OF LACTIC STREPTOCOCCI ACCORDING TO THE TYPE OF THE CELL WALL- ASSOCIATED PROTEINASE

Successive studies aimed at clarifying the complexity of the proteinase enzymes from various strains of *S. cremoris* have resulted in the classification of the strains based on the differences in the activity and immunological cross-reactivity of their proteinase enzymes (Table II). Initial studies involved the investigation of the pH- and temperature- dependence of the proteinase activities of whole cells of various strains of *S. cremoris*. (Exterkate, 1976).Three types of proteinase activities were distinguished ; two acid proteinases designated PI and PIII, which were optimally active at 40° C and 30° C respectively, and a neutral proteinase, PII, maximally active at 30° C.

The PII activity however, has recently been shown to be an artifact of the PI activity at 30° C (Visser *et al.*, 1986). Therefore, the *S. cremoris* strains studied were classified into three groups, based on the presence of either only the PI- or PIII- type of proteinase activity or both as shown in Table II. However, the distinction of the PI- and PIII- activity based on a difference of 10° C in optimum temperature requirements is questionable since the proteinase activity partially purified from cells of *S. cremoris* AC1 was shown to exhibit a PI type of activity but the activity assayed with whole cells was characteristic of the PIII type (Geis *et al.*, 1985).

TABLE II
CLASSIFICATION OF *S. CREMORIS* STRAINS
(adapted from Kok & Venema, 1988).

| Strain | Classification based on proteinase- | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | activity | | | specificity | | immunology | | | |
| Wg2, HP, C13 | PI | PII | | HP | | A | | B | |
| E8 | PI | | (PIII) | HP | AM1 | A | | | C |
| TR | PI | PII | PIII | HP | (AM1) | A | | B | C |
| FD27 | PI | PII | PIII | HP | AM1 | A | | B | C |
| AM1, SK11 | | | PIII | | AM1 | A | A′ | (B) | C |

The *S. cremoris* strains have also been classified by Cross-Immuno-Electrophoretic analysis (CIE), of their proteinase enzymes (Hugenholtz *et al.*, 1984). Proteinase from the different strains released into $Ca^{++}$- free buffer was partially purified and used to raise polyclonal antibodies. The partially purified proteinase from each strain was first separated on the basis of size by gel electrophoresis in one direction and then isoelectric focusing was performed in a perpendicular direction in the presence of the antibodies raised against the partially purified proteinase from the different strains. Initially, four immunologically distinct proteins, A, A', B and C were found which were very similar in size (Hugenholtz *et al.*, 1984).

The A and B proteins were very similar if not identical in size and were only separated during the isoelectric focusing step in the presence of antibodies. The A' protein is smaller in size than the A protein but the two proteins have recently been shown to share the same antigenic determinants, and protein C which is the smallest of the proteins, has been shown to be immunologically identical to protein B (Hugenholtz *et al.*, 1987). Therefore all the strains shown in Table II would be classified as one group, all having two immunologically distinct proteins A/A' and B/C distinguished by the CIE technique. Antibodies specific for protein A and B in partially purified proteinase from *S. cremoris* Wg2 have been raised after separation by CIE and used to determine if both the proteins were responsible for proteolytic activity. When the antibodies specific for either protein A or B were added to the partially purified proteinase solution, about half the proteinase activity was removed, suggesting that both proteins contributed almost equally to the proteolytic activity (Hugenholtz *et al.*, 1987).

A more recent attempt to probe the complexity of the proteinase enzymes of *S. cremoris* based on differences in specificity was reported by Visser *et al.* (1986). Using gel electrophoretic techniques to look for differences in the action of partially purified enzymes on the different milk caseins ($\alpha_{s1}$-,β- and κ- caseins), two types of activity were distinguished (Table II). The HP- type was capable of hydrolysing only β- casein in contrast to the AM1- type which was also capable of hydrolysing $\alpha_{s1}$- casein. The gel electrophoretic pattern of β- casein cleavage by the two types of enzyme was also different. By this method, the *S. cremoris* strains were classified into three groups, possessing either the HP- type or AM1- type of proteinase or both.

The classification of the *S. cremoris* strains into three groups based on the presence of a PI and/or PIII type of activity and that based on the presence of an HP- and/or AM1- type of casein- hydrolysing activity shown in Table II are essentially the same, suggesting that the

PI activity may be related to the HP- type while the PIII enzyme may correspond to the AM1- type of activity. In contrast, the separation of the proteinases of the bacteria based on the CIE technique suggests that all the *S. cremoris* strains have proteinases with the same immunological reactivity, i.e., A/A' and B/C and therefore should be classified as one group.

From Table II, it can be seen that whether or not the strains showed an HP- or AM1- or both type of activity, they all show the A and B type of immunological response. It is possible that the A and B antigenic determinants may involve small differences in those regions of the enzyme which do not affect the specificity of the enzyme activity so that enzymes having the HP- and AM1- type of specificity could not be distinguished by the CIE- method. Recent data on the proteinase gene has shown that both the A and B proteins are encoded on the same gene and that the proteinase genes from *S. cremoris* strains having either the AM1- or the HP- type proteinase activity are highly homologous (Kok & Venema, 1988). These genetic results will be reviewed in section 1.5.

## 1.4 CHARACTERISATION OF PARTIALLY PURIFIED CELL WALL-ASSOCIATED PROTEINASE

Extensive purification of the proteinase from lactic streptococci has been hindered by the difficulties in preventing autoproteolysis of the purified proteinase. However, detailed characterisation of the partially purified proteinase from various strains of *S. cremoris* (Geis *et al.*, 1985 and 1986; Hugenholtz *et al.*, 1987; Exterkate & de Veer, 1987; Visser *et al.*, 1986) and *S. lactis* (Monnet *et al.*, 1987) has been reported. The results from these studies suggest that the proteinase enzymes of the lactic streptococci are generally high molecular weight serine proteases (145- 60 kD) and are activated or stabilised by calcium. Unlike most serine proteases, the lactic streptococci are optimally active in weakly acidic instead of alkaline conditions.

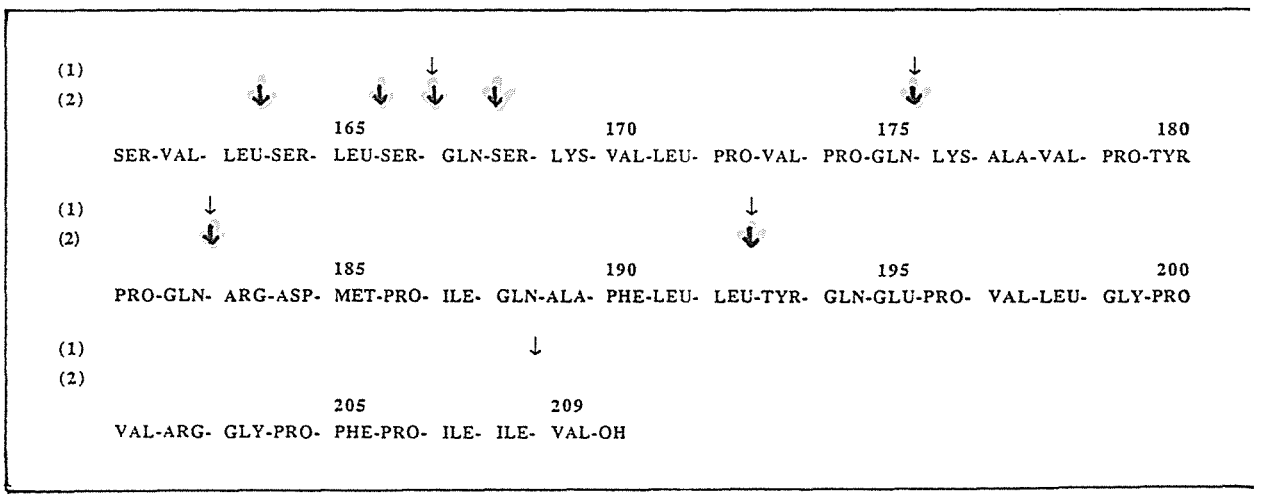
Proteinase enzymes ranging from 145 kD to 60 kD were isolated during attempts to purify the enzyme. A single proteinase was purified from *S. cremoris* Wg2 (Hugenholtz *et al.*, 1987) and from *S. cremoris* AC1 (Geis *et al.*, 1985) and these were very similar in size (140 and 145 kD respectively). Monnet *et al.* (1987) also purified only a single proteinase from *S. lactis* NCDO 763 which was about 80 kD. In contrast, Exterkate & de Veer (1987) isolated several proteinase enzymes of about 60, 84, 118, 126 and 133 kD in size from *S. cremoris* HP.

Despite the size differences, all the proteinases from the different bacterial strains have an isoelectric point at pH 4.5- 4.6 and are optimally active in weak acid conditions (pH 5.5- 6.5). In addition, the activity of the enzymes was inhibited by serine proteinase inhibitors, particularly phenylmethylsulfonylfluoride (PMSF) and partially inhibited by EDTA. A slight activation of enzyme activity by $Ca^{++}$ was reported in all cases as was shown originally in crude proteinase preparation by Mills & Thomas (1978) although the presence of more than 1 mM $Ca^{++}$ was found to markedly inhibit the enzyme activity (Geis *et al.*, 1985). The partial inhibition of enzyme activity by EDTA was not restored by addition of $Ca^{++}$ (Exterkate & de Veer, 1987) thus suggesting that metal ions other than $Ca^{++}$ may be required for the enzyme activity. At this point, it is interesting to note that the intracellular proteinase purified from disrupted cells of *S. cremoris* H61 by Ohmiya & Sato (1975) has properties very similar to those of the cell wall- bound enzymes described above. This enzyme was reported to be about 140 kD in size, optimally active around pH 6.5- 7.0 at 30° C and was activated by $Ca^{++}$. It is likely that this 'intracellular' proteinase was from the cell wall as the authors did not ascertain that the disrupted cell supernatant was not contaminated by cell wall components.

During the purification of the proteinases from *S. cremoris* HP (Exterkate & de Veer, 1987) and *S. cremoris* AC1 (Geis *et al.*, 1985), $Ca^{++}$ was added to stabilise the enzyme against autoproteolysis. Proteolytically active enzymes of lower molecular weight (60- 118 kD) were obtained by Exterkate and de Veer (1987) after the partially purified enzyme, originally consisting of only the 126 and 133 kD proteins, was dialysed and diafiltered in distilled water. Therefore, it was suggested that these lower molecular weight enzymes may have originated from a larger enzyme by autoproteolysis due to the absence or diluted amounts of $Ca^{++}$ in the distilled water. However, the stabilising effect of $Ca^{++}$ against autoproteolysis remains questionable since the single 140 kD proteinase purified from *S. cremoris* Wg2 by Hugenholtz *et al.* (1987) was purified in the absence of any added $Ca^{++}$.

Another common feature of the proteinases purified from the various strains of *S. cremoris* and *S. lactis* is the preferred cleavage of β- casein among the various milk proteins (Exterkate & de Veer, 1985; Visser *et al.*, 1986; Geis *et al.*, 1986; Monnet *et al.*,1987). The classification of *S. cremoris* strains based on the differential caseinolytic activity of the proteinase was mentioned in section 1.3. Briefly, only two types of activity, the HP- and the AM1- type were distinguished. Both types cleave β- casein, possibly with different specificities as suggested from the difference in the gel electrophoretic pattern of the hydrolysate but the AM1- type is also capable of hydrolysing $\alpha_{s1}$- casein albeit at a slower rate than that of β- casein (Visser *et al.*, 1986).

Detailed studies on the β- casein cleavage specificity of the partially purified proteinase from *S. lactis* 763 has been published recently (Monnet *et al.*, 1986). Trifluoroacetic acid-soluble hydrolysates of β- casein from incubations with the enzyme were separated by reverse phase HPLC. Five distinct peptides were eluted, which were present in increasing amounts in the samples of β- casein incubated with enzyme for longer periods of time. Analysis of the amino acid composition and identification of the N- and C- terminal residues of the peptides indicated that they originated from a limited 43- residue region of the C- terminal end of the β- casein molecule (Figure 1.4.1). When the amino acid sequences around the cleavage sites were examined for any specificity of the enzyme action, no specific residue or consensus sequence of residues could be identified. An almost identical cleavage pattern of β- casein by the *S. cremoris* HP proteinase was recently reported (Visser *et al.*, 1987). The β- casein cleavage sites of the AM1- type proteinase have not yet been published.




## FIGURE 1.4.1
## BONDS HYDROLYSED IN β- CASEIN BY CELL WALL PROTEINASE FROM *S. LACTIS* 763 AND *S. CREMORIS* HP

Black arrows indicate the cleavage sites specific to the *S. lactis* 763 (Monnet *et al.*, 1986) and red arrows those sites specific to the *S. cremoris* HP enzyme (Visser *et al.*, 1987). Only the C- terminal 49 residues are shown.

Whether or not the β- casein cleavage by the lactic streptococci shown by the studies just described above is similar to that in milk awaits further investigations. Interest in this area has mainly been concerned with the possible role that the proteinase may play in the development of cheese flavour since one of the peptides generated by the enzyme action mentioned above has been identified with a 'bitter- tasting' peptide previously found in cheese made using *S. cremoris* HP as the starter organism (Visser *et al.*, 1983b). A brief review of the possible role of the proteinase activity of the lactic streptococci in the production of bitter flavour in cheese is treated in section 1.6.

## 1.5 GENETICS OF THE PROTEINASE FROM LACTIC STREPTOCOCCI

Populations of lactic streptococci show a high frequency of mutation to produce slow growing variants which coagulate milk very slowly. These mutants were shown to be deficient in proteinase activity (Pearce *et al.*, 1974). The first direct demonstration that the proteinase gene was plasmid encoded was shown by the simultaneous loss of proteinase activity from *S. lactis* C2 with the loss of the plasmids (McKay & Baldwin, 1975). Gasson *et al.* (1983) have confirmed this observation by showing that the proteinase gene of *S. lactis* NCDO 712 was linked to a 33 MDal plasmid. Using the casein- staining technique of Cliffe & Law (1985) to detect proteinase activity on polyacrylamide gels (see section 1.2) two proteinase active bands were detected from variants of the streptococci carrying only the 33 MDal plasmid which disappeared when the plasmid was cured from the bacteria. Conjugation experiments on various *S. cremoris* strains have also shown that the proteinase genes are plasmid encoded (Kok & Venema, 1988).

The proteinase gene from *S. cremoris* Wg2 has been cloned (Kok *et al.*, 1985) and detailed restriction mapping and nucleotide sequencing of the gene were carried out (Kok *et al.*, 1988). The proteinase genes from *S. cremoris* SK11 (De Vos, 1986) and *S. lactis* 712 (Gasson, 1986) have also been isolated and extensively characterised. A comprehensive review on the recent progress in the genetics of the proteinase of the lactic streptococci has been published (Kok & Venema, 1988).

Comparison of the restriction maps and nucleotide sequence of the proteinase genes from the three strains of lactic streptococci (Figure 1.5.1) showed highly extensive homology (98% homology between the nucleotide sequences of the *S. cremoris* strains and even more between *S. cremoris* Wg2 and *S. lactis* 763) and suggests that there may be one common or slightly modified gene among the lactic streptococci (De Vos, 1987). This

observation is in apparent conflict with the biochemical characterisation of the enzymes as described in section 1.3 (Table II), where the proteinase from the *S. cremoris* strains Wg2 and SK11 were shown to have different types of activity based on the specificity of casein cleavage and temperature- and pH- dependence of activity. Since the genes from the two strains are very similar, the changes involved in specifying the type of enzyme activity are likely to be restricted to a highly specific region of the gene. Restriction mapping and hybridization studies on the Prt- plasmid from *S. cremoris H2*, the source of the proteinase described in this thesis have also shown a high degree of homology to that from *S. cremoris* Wg2 (Yu *et al.*, in press).

The proteinase gene from *S. cremoris* Wg2 was successfully cloned into *B. subtilis* and a proteinase- deficient strain of *S. lactis* (Kok *et al.*, 1988). The cloned gene in the Prt$^-$ mutant of *S. lactis* was able to restore proteinase activity to the streptococci thus indicating that the gene was expressed.

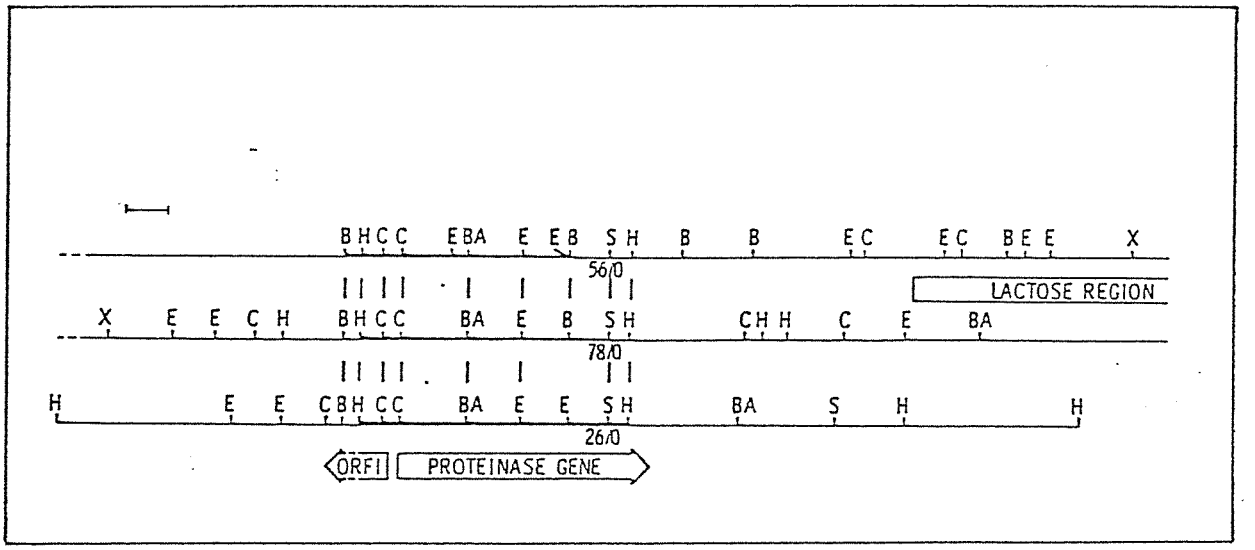
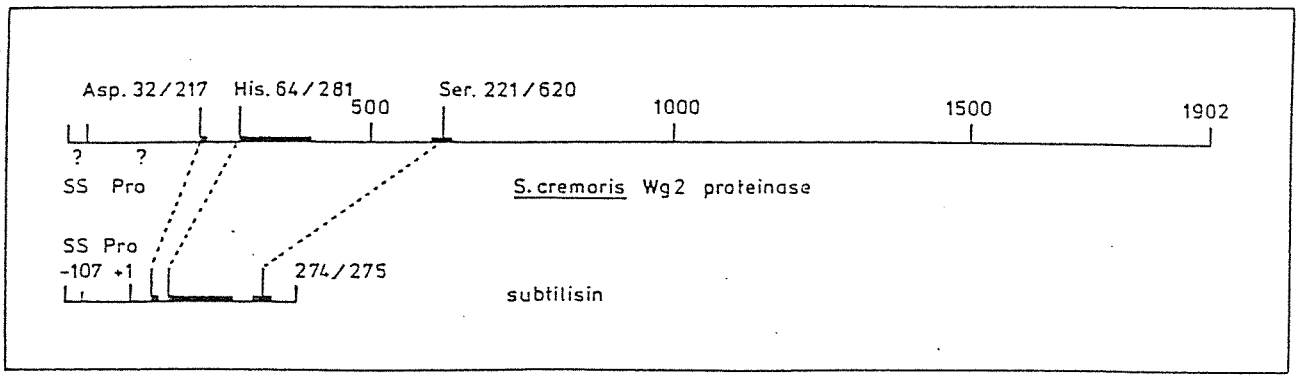



FIGURE 1.5.1 ALIGNMENT OF THE RESTRICTION ENZYME MAPS OF THE PROTEINASE PLASMIDS FROM *S. LACTIS* 712, *S. CREMORIS* SK11 AND *S. CREMORIS* Wg2.

The physical maps of the proteinase plasmids of *S. lactis* 712 (top, 56 kb), *S. cremoris* SK11 (middle, 78 kb) and *S. cremoris* Wg2 (bottom, 26 kb) are aligned such that maximal overlap is obtained in the region where the proteinase genes are located. The bar represents 1 kb of DNA. B: *Bgl* II; BA: *Bam* HI; C: *Cla* I; E: *Eco* RI; H: *Hind* III; X: *Xho* I. Modified from Kok & Venema (1988).




## FIGURE 1.5.2 HOMOLOGY COMPARISON OF THE PROTEINASE GENE FROM *S. CREMORIS* Wg2 AND SUBTILISIN

Homologous regions are shown as thick lines, drawn to scale on a linear map of the whole proteinase and compared with a linear map of subtilisin. SS refers to signal sequence. Numbers refer to amino acid residues (taken from Kok & Venema,1988).

Immunological analysis (CIE) of the proteinase expressed from the *S. cremoris* Wg2 gene cloned in the *S. lactis* showed the presence of both the A and the B type of immunological reactivity, suggesting that both the A and B proteins must be encoded on the same gene. At this point it is interesting to note that the gene sequence of the *S. cremoris* proteinase was shown to predict a protein 200 kD in size (Kok *et al.*, 1988) yet the isolated protein was only about 140 kD. Thus, in comparing the genetic and biochemical data described above, one is left with a picture of a gene encoding one protein which when isolated from the cell has a smaller size than predicted and possesses one type of proteinase activity (HP) but two types of immunological reactivities (A and B).

Several proteolytically active proteins of various sizes were isolated during the purification of the *S. cremoris* HP enzyme by Exterkate & de Veer, (1987). It was suggested that the smaller proteins may have originated from a larger enzyme by the occurrence of autoproteolysis during the purification. It has also been suggested that autoproteolysis of the enzyme at different sites may be responsible for generating enzymes with different structural conformations and which may thus exhibit differences in immunological reactivity (Kok & Venema, 1988). Processing of the enzyme by proteolysis has also been proposed to explain the difference in size of the isolated enzyme and that predicted from the gene sequence of the *S. cremoris* Wg2 proteinase.

This proposal was based mainly on two observations :

(i) Homology with subtilisin. The nucleotide sequence of the gene revealed extensive homology between parts of the *S. cremoris* Wg2 proteinase and the subtilisin family of serine proteinases (Kok *et al.*, 1988) which are only about 30 kD in size (Figure 1.5.2). In addition the presence of a 33 amino acid long signal peptide- like sequence at the N-terminus coding region of the gene suggests that like subtilisin, the streptococcal proteinase may also be synthesised as a pre-pro- molecule. Subsequent removal of this sequence prior to maturation of the proteinase would account for part of the size difference between the predicted and the known molecular weight of the proteinase. Although the presence of a pro- sequence similar to that present in subtilisin which may be responsible for proteolytic activation of the proteinase has not been conclusively shown, it is conceivable that its removal would contribute to further size reduction of the nascent proteinase.

(ii) Proteolytically active enzyme with deleted C- terminus. The gene fragment (from *S. cremoris* Wg2) originally cloned lacked the coding region for the C- terminal 130 amino acids of the proteinase but could still complement proteinase deficiency when transferred to a Prt $^-$ strain of *S. lactis* (Kok *et al.*, 1985). *In vitro* deletion of a large region at the 3' end of the complete gene also showed that this region was not essential for the activity of the proteinase (Kok *et al.*, 1988). However, the truncated proteinase expressed from a similarly deleted gene was found to be secreted into the growth medium (De Vos, 1987).

Since the proteinase has been shown to be located near the cell wall and was released only in $Ca^{++}$- free buffer (see section 1.2), it has been proposed that the release of the proteinase might also involve a deletion of the C- terminal region by self digestion of the enzyme in the absence of $Ca^{++}$ and consequent reduction in size (Kok & Venema, 1988). Interestingly, a stretch of 4 amino acids present near the C- terminal region of the proteinase from *S. cremoris* HP has been shown to be identical to one of the digestion sites of the *S. lactis* 763 proteinase in β- casein (Kok *et al.*, 1988; Monnet *et al.*, 1986). It remains to be established, however, whether this site is actually used for the release of the proteinase from the cell wall. Further studies to define the nucleotide sequence of the active site of the proteinase may help explain the consistently observed presence of only the HP- and AM1- type of activity (or PI and PIII respectively) among the various strains of lactic streptococci.

Work on the regulation of the synthesis of lactic streptococcal proteinase has been initiated in a series of studies with *S. cremoris* AM1 by Exterkate (1976, 1979, 1983) largely based on the observation that cells grown in broth media showed less proteinase activity than those grown in milk. Since there is a higher content of non- protein nitrogen sources in the broth media to support bacterial protein synthesis, it is conceivable that the availability of the substrates for protein synthesis will affect the rate of protein metabolism of the bacteria and may involve the regulation of proteinase gene expression. With the presence of cloned proteinase genes now available, rapid progress in this study may be anticipated.

## 1.6 ROLE OF PROTEINASE ACTIVITY IN STARTER BACTERIA ON THE DEVELOPMENT OF BITTER FLAVOUR IN CHEESE

The proteolytic activity of the lactic streptococci is important not only to support the fermentative growth of the bacteria in milk but also in the development of cheese flavour and texture in the process of cheese maturation and ripening. Extensive reviews on the various aspects of the process are available (Law, 1984; Law & Kolstad, 1983; Visser, 1981; Stadhouders *et al.*, 1983; Lawrence *et al.*, 1987).The present section includes a brief review of the role of the starter proteinase in the development of bitter flavour in cheese.

Starter bacteria were first shown to contribute directly to the development of bitter flavour in cheese when variants of *S. cremoris* AM1 and AM2 that coagulate the milk very slowly produced cheese that were non- bitter (Lawrence & Pearce, 1968).The possibility of the involvement of starter proteinase activity was raised when the variants were subsequently shown to be deficient in proteinase activity (Martley & Lawrence, 1972; Pearce *et al.*, 1974, see section 1.2). When the 'non- bitter' strains were grown to high cell densities they produced bitter flavour (Lowrie *et al.*, 1972).

More direct evidence for the involvement of the proteolytic activity of starter bacteria on bitter flavour in cheese was demonstrated by the occurence in the milk culture of low molecular weight peptides suspected of being derived from the C-terminus of β-casein due to their hydrophobic properties (Sullivan & Jago, 1972; Visser *et al.*, 1983). It can be recalled from section 1.3 that the proteinase enzymes isolated from various strains of *S. cremoris* were shown to preferentially cleave β- casein among the various casein molecules. One of the fragments generated by the enzyme cleavage (see Figure 1.4.1, section 1.4) has been found to be identical to one of the 'bitter' peptides found in cheese made with *S. cremoris* HP as the starter bacteria (Visser *et al.*,1983; Monnet *et al.*, 1986).

It is of interest that the classification of the strains of *S. cremoris* based on the studies of the proteinase activity, described in section 1.3, is also correlated to the classification of the strains on the basis of whether or not they produce cheese with bitter flavour. For example the AM1 and SK11 strains known to produce 'non- bitter cheese' were shown to exhibit the AM1 type- (or PIII) proteinase activity while the strains shown to have the HP- type of activity are all known to produce 'bitter cheese'.

## 1.7 AIMS OF THE THESIS

From the above review of the literature on the present progress in the research on the proteinase enzymes of the lactic streptococci, it is apparent that there are still inconsistencies between genetic and biochemical data concerning the complexity of the proteinase system. The present study is intended to contribute to the biochemical characterisation of the enzyme.

The principle aims of this study are:

(1) To study the process of proteinase release from lactic streptococci in order to define conditions which give maximum yields of enzyme for subsequent purification.
(2) To partially purify the released proteinase to a degree that permits its action on milk proteins to be characterised.
(3) To define the sites at which the partially purified proteinase hydrolyse the β- casein molecule.

Most of the work described was carried out on a single strain of *S. lactis*. A limited amount of comparative work on the proteinase from other strains of lactic streptococci was also carried out.