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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 α_{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

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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.

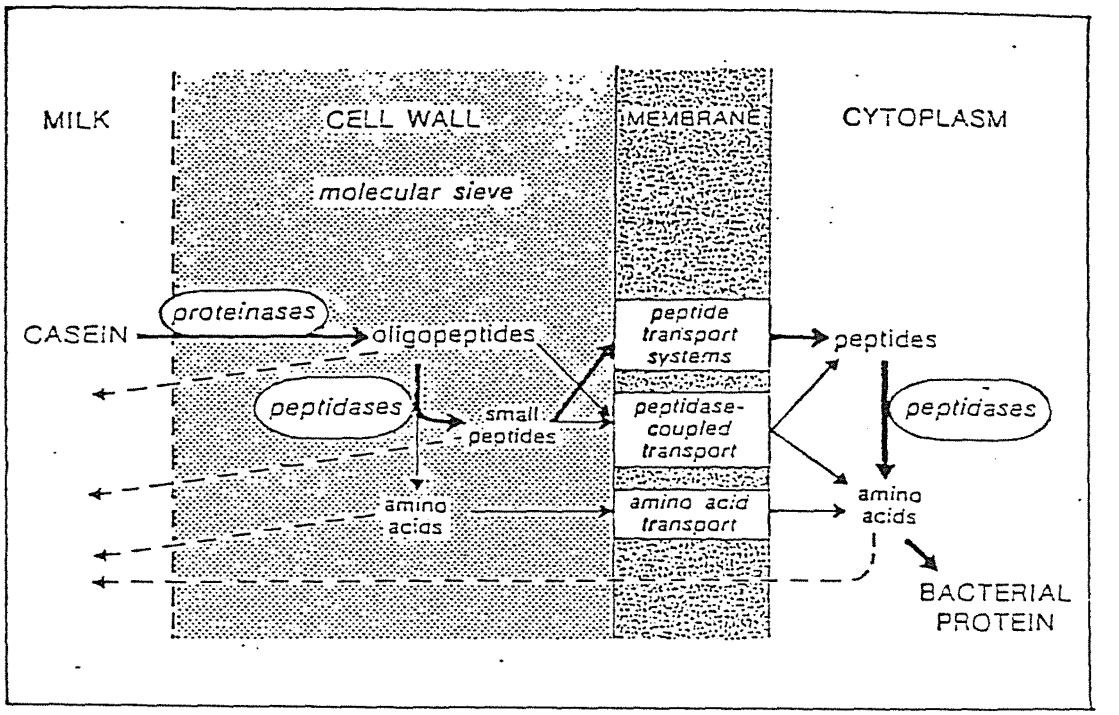


FIGURE 1.1 UTILISATION OF CASEIN FOR GROWTH OF LACTIC STREPTOCOCCI IN MILK.
(Reproduced from Thomas & Pritchard, 1987)

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.

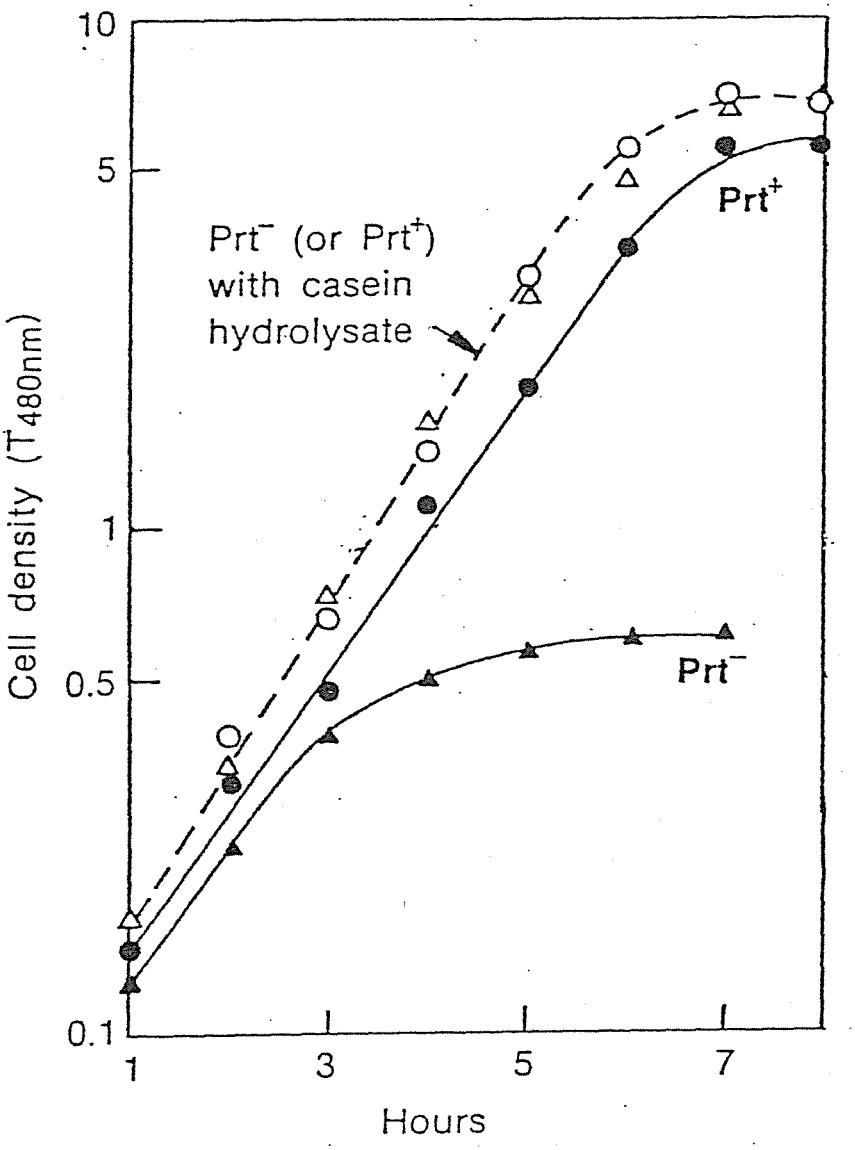


FIGURE 1.1.1

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 (α_{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 α_{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 α_{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.

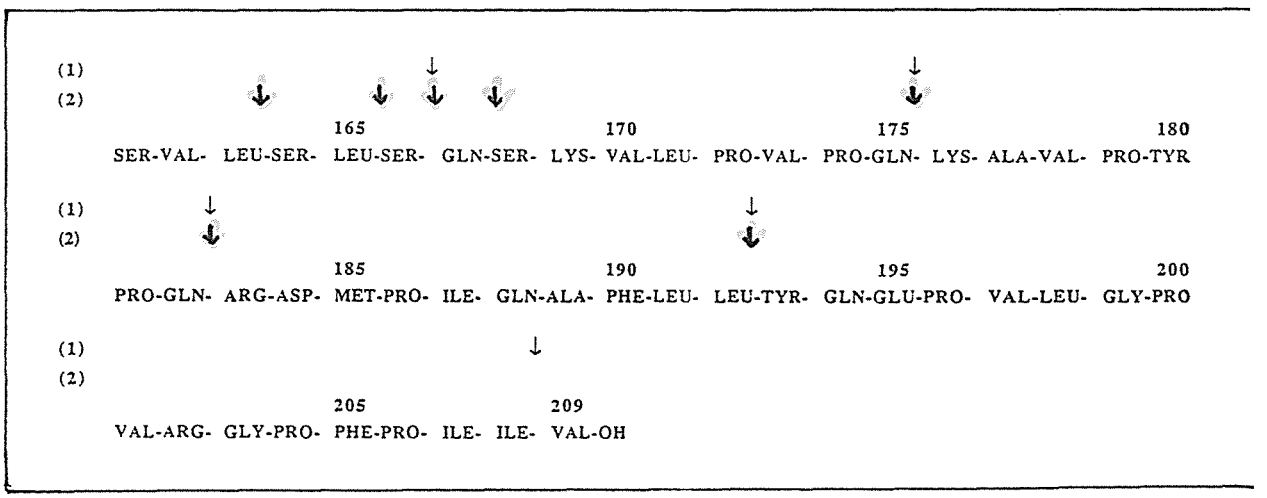


FIGURE 1.4.1
BONDS HYDROLYSED IN β- CASEIN BY CELL WALL PROTEINASE FROM *S. LACTIS* 763 AND *S. CREMORIS* HP

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.

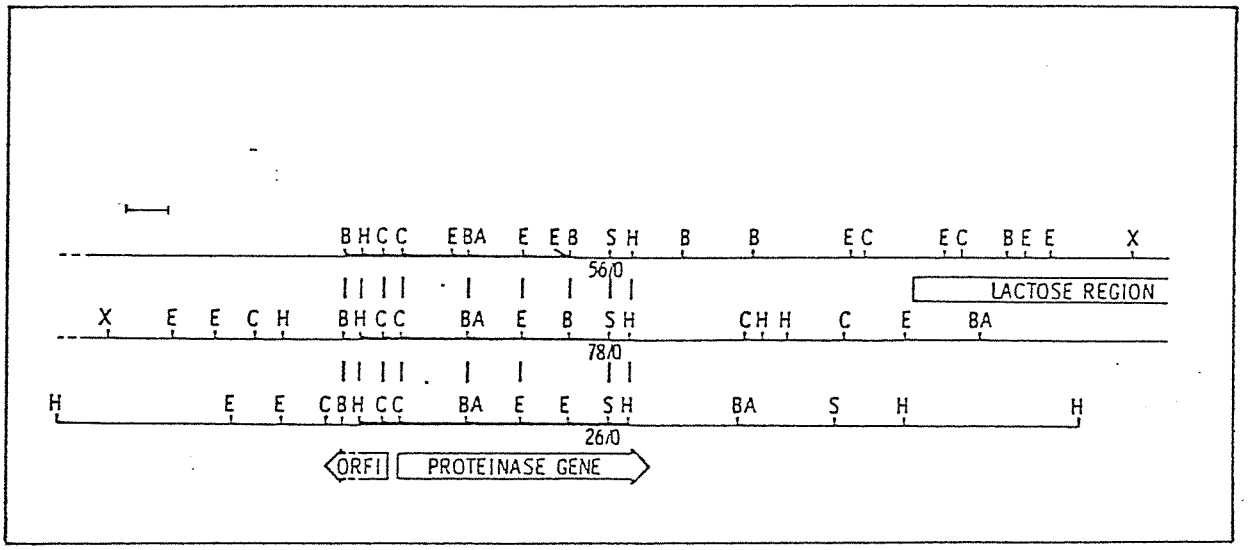


FIGURE 1.5.1 ALIGNMENT OF THE RESTRICTION ENZYME MAPS OF THE PROTEINASE PLASMIDS FROM *S. LACTIS* 712, *S. CREMORIS* SK11 AND *S. CREMORIS* Wg2.

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).

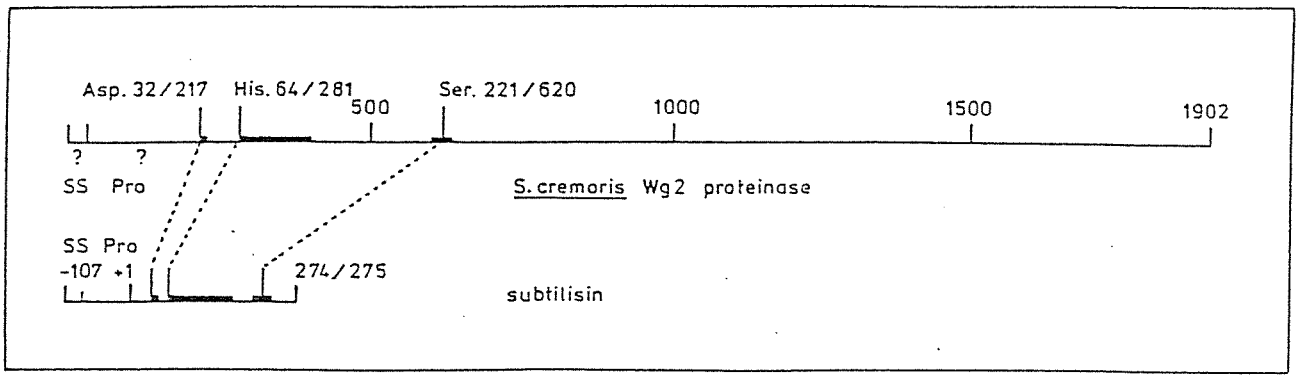


FIGURE 1.5.2 HOMOLOGY COMPARISON OF THE PROTEINASE GENE FROM *S. CREMORIS* Wg2 AND SUBTILISIN

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.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1.GROWTH MEDIA

Low heat reconstituted skim milk was obtained from the New Zealand Dairy Research Institute. Polypeptone and Soy- peptone used in the broth medium were from Gibco Lab., USA. Lactose was from BDH, England, beef extract from Merck, W. Germany, yeast extract from BBL, USA, ascorbate from Scientific Supplies, Auckland and sodium β-glycerol phosphate from Sigma Chemical Co.,USA.

2.1.2 SUBSTRATES FOR ENZYME ASSAY

α_{s1}- casein, β-casein, κ-casein and α-lactalbumin were from Sigma. β-lactoglobulin purified from bovine whey was kindly provided by Dr. J. W. Tweedie, Massey University. Fluorescein isothiocyanate Isomer I was obtained from Sigma. L- lysine 7- amido-4-methylcoumarin (lys-AMC) and L- glycylproline 7- amido-4-methylcoumarin (gly-pro-AMC) were prepared as the hydrobromide salts by Mr. R. Lloyd, Massey University. Sodium pyruvate (Type II), NADH and D- fructose 1,6 bisphosphate were also from Sigma.

2.1.3 CHROMATOGRAPHIC RESINS

Diethylaminoethane (DEAE) cellulose- 23 resin for anion exchange chromatography was from Whatman Biochemicals Ltd., England. Sephacryl S- 300 resin for gel permeation chromatography (GPC) was obtained from Pharmacia Fine Chemicals, Sweden. The Monobead C- 18 column for reverse phase- high performance liquid chromatography (RP-HPLC) was from Waters Associates, USA.

2.1.4 BUFFERS AND SOLVENTS

All buffers were analytical grade whenever possible.Tris buffer was obtained from United States Biochemical Corporation , USA and MES buffer was from Boehringer Mannheim GmbH, W. Germany. Glycine was from May and Baker, England. Redistilled hydrochloric acid containing phenol was used for the hydrolysis of peptides for amino acid analysis. Glass- distilled trifluoroacetic acid used for RP-HPLC was from Halocarbon, USA. HPLC- grade acetonitrile was obtained either

from May and Baker, England or British Drug Houses (BDH), England.

2.2 METHODS

2.2.1 BACTERIAL STRAINS

The following strains of lactic streptococci were obtained from the New Zealand Dairy Research Institute, Palmerston North:

S.lactis H1 strain 4122
S. lactis H1 strain 4125
S. lactis H1 strain 4760
S. cremoris H2
S. cremoris SK11

The *S. lactis* strains 4122, 4125 and 4760 were derived from *S. lactis* H1 which in the wild type strain contains four plasmids (Crow *et al.*, 1983). *S. lactis* strain 4122 was derived from partially plasmid- cured *S. lactis* H1, retaining only a resident Lac^+Prt^+ plasmid (Lac^+= lactose metabolising, Prt^+= containing proteinase activity). *S. lactis* strain 4125 was totally plasmid- cured and hence, phenotypically, Lac^-Prt^-. *S. lactis* strain 4760 was derived by transferring the Lac^+Prt^+ plasmid from *S. cremoris* strain H2 into *S. lactis* strain 4125.

2.2.2 GROWTH MEDIA

Reconstituted skim milk medium (RSM)

RSM was prepared by thoroughly blending 10g of low heat reconstituted skim milk powder in distilled water using a homogeniser, then making up to 100 ml volume of medium (Thomas and Turner, 1977). Sterilised glucose and casitone, sufficient to give final concentrations of 0.05% and 0.02% (w/v) respectively were added to this medium to maintain the Lac^- Prt^- *S. lactis* strain 4125.

Experimental reconstituted skim milk medium (ERSM)

In order to grow the bacteria to the high cell density desirable for enzyme extraction, RSM was buffered by adding 3 ml of 2.5M solution of sodium β-glycerophosphate

per 100 ml of medium. This avoids the coagulation of the milk medium by the lactic acid produced during the growth of the bacteria.

M17-lactose broth medium

Broth medium was prepared according to Terzaghi and Sandine (1975). Each litre of medium contained :

Polypeptone	5 g
Soy peptone	5 g
Yeast Extract	2.5 g
Ascorbate	0.5 g
Na ß-glycerophosphate.$5H_2O$	10 g
Beef extract	5 g
1M $MgCl_2$	1.0 ml
Lactose	5 g

All growth media was autoclaved at 15 psi for 15 minutes, except when larger volumes of ERSM were used when the autoclaving time was extended.

2.2.3 MAINTENANCE OF BACTERIAL CULTURES

Stock bacterial cultures were stored in RSM, frozen at -70°C. For routine maintenance, freshly prepared RSM was inoculated with thawed stock (1% inoculum) and incubated until the medium was almost fully coagulated (about 7 hours at 30° C). The coagulated culture was stored at 4°C and subcultured immediately prior to being grown in large volumes of ERSM for enzyme extraction.

2.2.4 GROWTH OF BACTERIA FOR ENZYME EXTRACTION

Freshly prepared ERSM medium was inoculated with a new subculture of bacteria (using a 1% inoculum) and the cultures grown at 22° C for 14 hours or until the A_{480} of the cells in alkaline EDTA was in the range 0.1- 0.15 units (see Section 2.2.5).

2.2.5 ESTIMATION OF BACTERIAL CELL DENSITY

The bacterial cell density of milk cultures was estimated following the method of Kanasaki *et al.* (1985). Aliquots of the culture grown in ERSM were diluted three fold with ice cold, 0.2% (w/v) EDTA solution. Several drops of 1M NaOH was added to dissolve the casein micelles in milk, thus minimizing its opacity. The suspension was further diluted three fold and the A_{480} measured. Milk medium without bacteria treated similarly was used as a blank.

2.2.6 HARVESTING BACTERIA FROM ERSM CULTURES

The culture was neutralised to pH 7.0 with 1 M NaOH and 6 ml of 25% (w/v) trisodium citrate was added per 100 ml of culture to chelate calcium and other cations. To minimize the release of the cell wall- associated proteinase enzyme during chelation the culture was maintained on ice (refer Section 2.2.8). The citrate- treated culture was centrifuged at 12 000 g for five minutes at 4°C. Pelleted cells were rapidly washed twice by resuspending in ice- cold, 50 mM sodium acetate/ phosphate buffer, pH 6.4 and recentrifuged. The washed cells harvested from 100 ml of ERSM culture were finally resuspended in 5 ml of 50 mM phosphate buffer, pH 6.4, or in the appropriate buffer for the study of the pH dependence of proteinase released (refer Section 3.3.3).

2.2.7 DRY WEIGHT DETERMINATION

To estimate rapidly the quantity of bacteria from each harvest, a standard curve relating the dry weight of cells to A_{480} of the washed cell suspension was prepared. Replicate aliquots of the cells were dessicated overnight at 60°C on preweighed, unused milk bottle caps and then reweighed. The same volume of buffer without bacteria was also dried to account for the weight due to buffer salts. The A_{480} of a series of dilutions of the cell suspension was measured and the absorbance values plotted against cell dry weight. A linear relationship of cell dry weight to A_{480} over the range 0- 0.4 mg cell dry weight was obtained. Thus, the cell dry weight in subsequent harvests could be determined from the standard curve by measuring A_{480} of the washed cell suspension.

2.2.8 RELEASE OF CELL WALL ASSOCIATED PROTEINASE

The procedure used for the release of cell wall- associated proteinase was based on the studies of Mills and Thomas (1978). They established that most of the caseinolytic activity of the lactic streptococci could be released by incubation of the cells in buffer free of calcium. Washed cells were resuspended in an appropriate buffer to a cell density of around 20 mg cell dry weight per ml. Phosphate buffer (50 mM, pH 6.4) was routinely used for this except where otherwise specified. The cell suspension was incubated at 30° C for varying lengths of time and then centrifuged at 12 000 g for 5 minutes at 4° C. The supernatant containing the crude cell wall- associated proteinase was retained for further study.

Pelleted cells following the extraction of the proteinase were stored in sealed tubes at -20°C. The cell pellets were subsequently disrupted in a French Press (Wabash hydraulic Press, USA) and then centrifuged at 12 000 g for 5 minutes (4° C). The amount of protein, proteinase activity and lactate dehydrogenase activity in the supernatant from both whole cell suspension and disrupted cells were determined as described below.

2.2.9 PROTEINASE ASSAY

The method of Twining (1984) based on the rate of production of TCA soluble fluorescent peptides from fluorescein isothiocyanate (FITC) conjugated casein was used routinely for proteinase assay in this study. Preliminary studies (Pritchard, unpublished) had shown that this is a convenient, reliable and sensitive assay for proteinase activity in lactic streptococci. Moreover, it is less expensive than assays based on ^{14}C- labelled caseins used extensively in previous studies. Since the proteinases from lactic streptococci were known to be invariably active towards β- casein, whilst only some strains produce proteinases active towards α_{s1}- casein (Visser *et al.*, 1986), FITC- β casein was used as the substrate in the present study.

Preparation of FITC β-casein.

200 mg of β casein was dissolved in 20 ml of 50 mM carbonate buffer, pH 9.5, containing 150 mM NaCl 5 mg of FITC (Isomer I) powder was dissolved in a minimal volume of buffer and slowly added to the protein solution while stirring gently.The conjugation reaction was carried out in a brown glass bottle, in the dark, at 25° C for one

hour. To remove uncoupled dye, the conjugation mixture was dialysed at 4°C in the following solutions (overnight dialysis in each)

2 l 0.5% charcoal suspension in distilled water (twice)
2 l 50 mM Tris/ HCl buffer at pH 8.5
2 l 50 mM Tris/ HCl buffer at pH 7.2.

The dialysed conjugate was diluted with an equal volume of the final dialysis buffer and stored at -20°C as 4 ml aliquots in dark brown bottles.

The molar ratio of conjugated FITC to β casein was estimated by measuring the A_{490} of the conjugate and the protein concentration by the Lowry method, using β casein as a standard. A value of 0.9 moles of FITC per mole of β casein was obtained using E_{490} for FITC = 61 000 l mol^{-1} cm^{-1} and M_r β casein ~ 29 000.

Assay of proteinase activity

Proteinase activity towards FITC- β casein was measured using the following reaction mixture:

50 mM NaOAc/ H_2PO_4	100 µl
FITC- β casein (0.5% protein)	100 µl
Enzyme extract	100 µl

The reaction mixture was incubated at 22° C and 60 µl aliquots were removed at various time intervals for up to two hours and then added to 150 µl of 5% (w/v) trichloroacetic acid in Eppendorf tubes. The TCA precipitation was maintained at room temperature for precisely 30 minutes and then spun for two minutes at full speed in an Eppendorf Microcentrifuge. 100 µl of the supernatant was added to 3 ml of 500 mM Tris / HCl buffer, pH 8.5 and the relative fluorescence ratio (RF) of the solution measured in an Amicon SP500 Ratio Spectrofluorometer using an emission wavelength of 525 nm and an excitation wavelengths of 490 nm.

Non- enzymatic release of TCA soluble fluorescent material was determined as a control by substituting the enzyme extract with an equal volume of 50 mM phosphate buffer, pH 6.4. The initial RF of the reaction mixture (time= 0 min) was obtained by adding TCA to the enzyme extract before adding the FITC-β casein. A standard solution of 0.07 $mgml^{-1}$ of FITC dye in 500 mM Tris/ HCl buffer, pH 8.5 was used to standardise the

Spectrofluorometer. One unit of proteinase activity was defined as one RF unit / hour.

Assay of peptidase activity

The chromogenic substrate glycylprolyl-7-amido, 4-methylcoumarin (gly-pro- AMC) was used to assay for the dipeptidyl peptidase activity in eluted fractions during the purification of the proteinase. Activity towards lysine- AMC was also assayed.Both peptidase activities were assayed using the following protocol :

Fraction	50 µl
gly-pro- AMC or lys- AMC	50 µl
100 mM MES buffer pH 6.8	100 µl

The reaction mixture was incubated at 22° C for 30 minutes (gly-pro- AMC) or 1 hour (lys- AMC) and then diluted to a total of 3 ml with distilled water. The relative fluorescence ratio of the diluted mixture was measured at Excitation λ=385nm and Emission λ=460nm.

2.2.10 LACTATE DEHYDROGENASE ASSAY

To estimate the extent of cell lysis during the release of cell wall- associated proteinase from whole cell suspension, the activity of the intracellular enzyme, lactate dehydrogenase, was assayed in the supernatant of both whole and disrupted cells. The assay method used was adapted from Crow and Pritchard (1977). 0.1 ml of cell extract was incubated for two minutes with :

0.1 M MES buffer, pH 6.9	2.6 ml
5 mM NADH	0.1ml
15 mM D-Fructose 1,6 bisphosphate	0.1ml

0.1 ml of 150 mM sodium pyruvate was added to start the reaction and the decrease in absorbance at 340 nm followed in a Gilford Spectrophotometer Model 260 at 25°C. One enzyme unit of activity was defined as one µmole of NADH oxidised per minute calculated using an E_{340} for NADH of 62 00 $l\ mol^{-1}\ cm^{-1}$.

2.2.11 PROTEIN DETERMINATION

The Lowry (or Folin) method (Lowry et al., 1951) was used to estimate the amount of protein in all samples. Absorbance of the protein solution was measured at 580 nm instead of above 600 nm. Bovine serum albumin was used as the protein standard.

2.2.12 DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS

The method of Laemmli (1970) was adopted for the discontinuous electrophoresis of proteins in polyacrylamide gels. The running gel was prepared as 8% acrylamide (w/v), and 0.26% (w/v) bisacrylamide in 75 ml of 0.39 M Tris/ HCl buffer pH 8.9. A stacking gel of 5% acrylamide, 0.26% bisacrylamide (w/v) was made in 30 ml of 60 mM Tris/ HCl buffer pH 6.7. SDS was added to 0.1% for denaturing gels. The gels were polymerised by adding 0.1% (v/v) of TEMED and 0.1% (v/v) of a 50% (w/v) solution of ammonium persulphate.

The gels were electrophoresed in a Pharmacia GE/ 4 Vertical Slab Gel Apparatus temperature controlled at 16°C, at 10 mA/ gel until the bromophenol blue dye- front reaches the stacking gel/ running gel boundary and thereafter at 20 mA/ gel until the dye- front is about 2 cm from the end of the gel.

0.125% Coomassie Brilliant Blue-R dissolved in methanol: acetic acid: water (9:1:10) was used to stain for protein bands either at 60°C for one hour or overnight at room temperature.The gels were destained with the methanol: acetic acid: water solution without Coomassie Blue.

Discontinuous electrophoresis in pre- prepared 8- 25% (SDS) Polyacrylamide gels were also run in mini Phast™ Gel System (Pharmacia) under programmed instructions for both electrophoresis and Coomassie- staining

2.2.13 REVERSE PHASE- HIGH PERFORMANCE LIQUID-CHROMATOGRAPHY (RP-HPLC)

1% trifluoroacetic acid- soluble peptides resulting from proteinase action (refer Section 3) were filtered through Acro LC-13 membranes (0.2 μm pore size, Gelman Sciences Ltd., USA). The filtered solution was fractionated using a monobead C-18 reverse phase-

HPLC column (10 μm bead diameter, 10 Å pore diameter, Waters Associates, USA). The peptides were eluted with a gradient of acetonitrile in 0.1% TFA and the A_{220} of the eluate monitored. Selected peaks were collected and vacuum dried for analysis of size and purity by Fast Atom Bombardment Mass Spectrometry (FAB- MS), amino acid composition and amino acid sequence.

2.2.14 AMINO ACID COMPOSITION AND SEQUENCE ANALYSIS

Vacuum dried peptides from RP-HPLC were hydrolysed in 0.5 ml of 0.5 M phenolated-HCl in vacuum sealed test tubes for 24 hours at 100°C. 16 hour- hydrolyses were done when necessary to confirm the quantities of serine and threonine residues. The hydrolysates were vacuum dried and analysed in an automated amino-acid analyser (Beckman, USA). Amino acid sequence was obtained by the automated Edman- sequencer (Applied Biosystems, USA).

3 RESULTS

3.1 EXTRACTION OF CELL WALL- BOUND PROTEINASE FROM *S. LACTIS* H 1

The aim of the work described in this section was to examine the effects of various factors on the release of the cell wall bound- proteinase from cells of *S. lactis* H1. As reviewed in the Introduction (Section 1.2), extensive work done previously has established the cell wall location of the proteinase " released " by incubating cells in Ca^{++}- free buffer. The primary aim of the following experiments was not to reevaluate previous work but to optimise the conditions for release of the cell wall enzyme and also to assess the extent to which leakage of intracellular enzymes might be occurring during the incubation of the bacteria in Ca^{++}- free buffer.

The transconjugant *S. lactis* strain 4760, obtained by transferring the Lac^+Prt^+ plasmid from *S. cremoris* H2 into a plasmid- cured strain of *S. lactis* H1, was used for this study. Thus the strain used for much of the work to be described is an *S. lactis* strain but the proteinase being studied is, genetically, that from the *S. cremoris* H2 since the Prt^+ plasmid is known to encode the cell wall proteinase (Kok & Venema, 1988). This strain was constructed for a previous study in this laboratory to characterise the Lac^+Prt^+ plasmid (Appleby, 1986) and had been used for preliminary studies on proteinase release and characterization (Pritchard, unpublished). These preliminary studies had suggested that the transconjugant strain produces higher levels of cell wall- bound proteinase than either the *S. lactis* H1 4122 with its own resident Prt^+ plasmid or the *S. cremoris* H2 strain from which the Prt^+ plasmid in strain 4760 had been obtained. In the present study, a comparison of the proteinase activity released from *S. lactis* strain 4760, *S. lactis* strain 4122 and *S. cremoris* H2 will be reported in Section 3.1.7.

3.1.1 RELIABILITY OF THE PROTEINASE ASSAY BASED ON FITC-β CASEIN AS A SUBSTRATE

Most studies on proteinase activity have been reliant on the use of radiolabelled substrates. $^{14}CH_3$- labelled milk caseins have generally been used as the substrate to assay for the cell wall- bound proteinase of the lactic streptococci. Until recently, fluorescent labelled protein derivatives were found to lack the sensitivity desirable for the assay of proteinase activity.

Twining (1984) reported the use of fluorescein isothiocyanate (FITC)- labelled casein for the assay of various proteases. Cleavage yielding TCA- soluble fluorescent peptides occurred in a linear time dependent manner. In addition, the assay was sensitive enough to detect sub-nanogram quantities of the enzymes. In comparison with radiolabelled substrates, FITC is inexpensive, more stable, less hazardous and easily disposable. For the above reasons, FITC- labelled casein was used as the substrate to assay for the activity of the cell wall- bound proteinase in this study.

The method of Twining (1984) used Hammarsten casein which is a mixture of the different bovine caseins. Since the proteinase studied in this work was found to be specific for β- casein (see Section 3.3), FITC- labelled β casein has been used for all but a few of the experiments described in this thesis. The molar ratio of FITC to casein used in the conjugation reaction mixture as specified by Twining is 2.0. The molar ratio of FITC to β-casein in the main batch of the conjugate prepared in the present study was 0.89. In another preparation using a molar ratio of FITC to β- casein of 3.0 in the conjugation mixture, a value of 1.0 was obtained for the conjugate. These values compare reasonably well with the ratio of 0.65 reported by Twining.

Since the FITC casein was used in the present study mainly for assaying the relative levels of proteolytic activity released under different conditions and for monitoring activity during purification, a systematic study of the FITC conjugation process was not undertaken. However, in the light of the subsequent findings on the sites of β- casein cleavage by the enzyme, the low ratio of FITC to casein raises some interesting questions which will be discussed later (Section 4.1).

In the experiments to be described in the following sections the FITC- casein hydrolysing activity will be referred to simply as ' proteinase '. Proteolytic enzymes not capable of hydrolysing this substrate will not be measured by this method.

The FITC-β casein conjugate used for the assay of proteinase activity in this study was shown to give a linear rate of hydrolysis over two hours of incubation at 22° C with crude preparations of the enzyme (Figure 3.1.1).

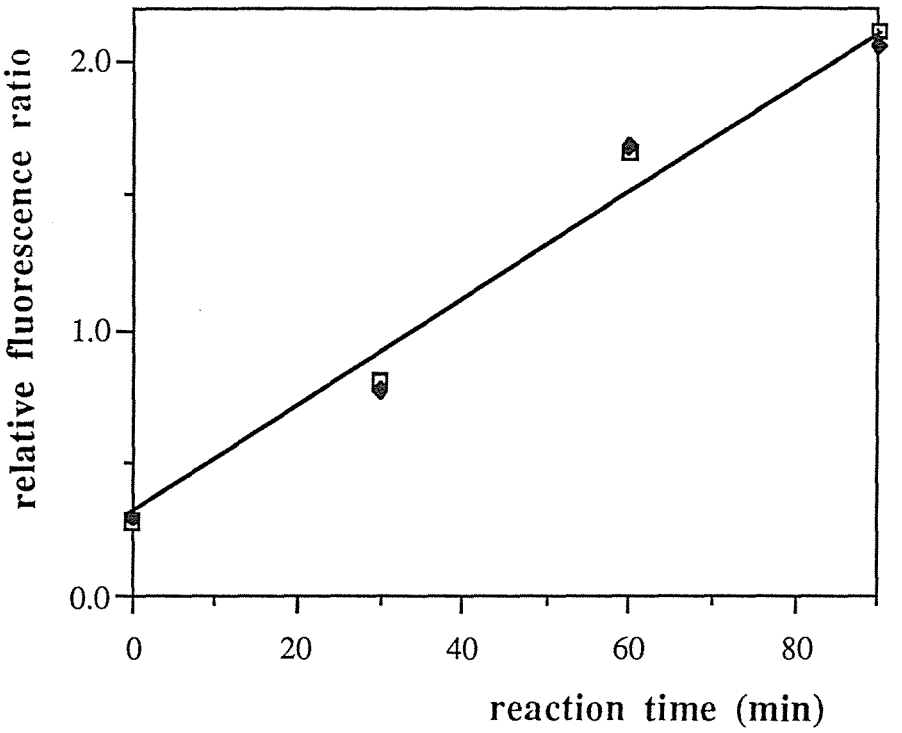


FIGURE 3.1.1 LINEARITY OF PROTEINASE HYDROLYSIS OF FITC-β CASEIN

Duplicate samples of 100 µl crude enzyme released from cell suspensions of *S.lactis* 4760 previously grown in milk medium was incubated at 22° C with 100 µl FITC-β casein and 100 µl 50 mM NaOAc / H_2PO_4 buffer at pH 6.4 for up to two hours. 60 µl aliquots were removed at 30 minute- intervals and 150 µl 5% (w/v) TCA added to stop the reaction. A blank (reaction time=0 min) was prepared by adding the TCA to the enzyme and buffer before the addition of the substrate. The precipitation of TCA insoluble material was left for precisely 30 minutes at room temperature and then centrifuged at full speed in an Eppendorf Microcentrifuge for 2 minutes. 100 µl of the supernatant containing TCA soluble material was added to 3 ml of 500 mM Tris / HCl buffer pH 8.5 and the relative fluorescence (RF) of the solution measured in an Aminco Ratio Spectrofluorometer using Excitation λ=490 nm and Emission λ=525nm. A plot of RF value against reaction time is shown here.

3.1.2 CELL WALL- BOUND PROTEINASE ACTIVITY IN RELATION TO THE GROWTH OF *S. LACTIS* 4760

In order to ascertain whether the level of proteinase activity released by *S. lactis* 4760 was dependent on the age of the culture, the bacteria were grown in ERSM medium for 23 hours at 22° C. The growth of the milk culture was monitored by determining the A_{480} in alkaline EDTA of samples removed at regular time intervals, as well as by the acidity, determined by titrating the samples to pH 7 with 50 mM NaOH. The log A_{480} and titratable acidity are plotted against the age of the culture in Figure 3.1.2.

Bacteria harvested at early- log, mid- log and early stationary phase of growth were incubated in Ca^{++}-free buffer for one hour to release cell wall proteinase as described in the legend to Figure 3.1.3. The amount of leakage of intracellular enzymes during the incubation of the cells in buffer was assessed by measuring the activity of the intracellular enzyme lactate dehydrogenase in the supernatant after sedimenting the cells.

As shown in Figure 3.1.3, the proteinase activity per ml of supernatant decreased with increasing age of the culture, being highest in samples from the early- log phase while the total protein released increases with increasing age. Unfortunately, in this early experiment the bacterial density in the cell suspensions prepared from the three different samples was not determined, so activity released cannot be expressed on a cell dry weight basis. However, as estimated from the A_{480} of the samples removed from the culture, the density of the cell suspension containing the early- log phase cells would have been only about half of that containing the early stationary phase cells. Thus it is clear that the proteinase activity released from cells harvested from the early- log phase is considerably greater than when harvested from the mid- log or early stationary phase. No LDH activity was released in any of the samples. It might have been anticipated that stationary phase cells would have been more ' leaky ' than early- log phase cells but this is not the case at least for the short incubation time (one hour) used in this experiment.

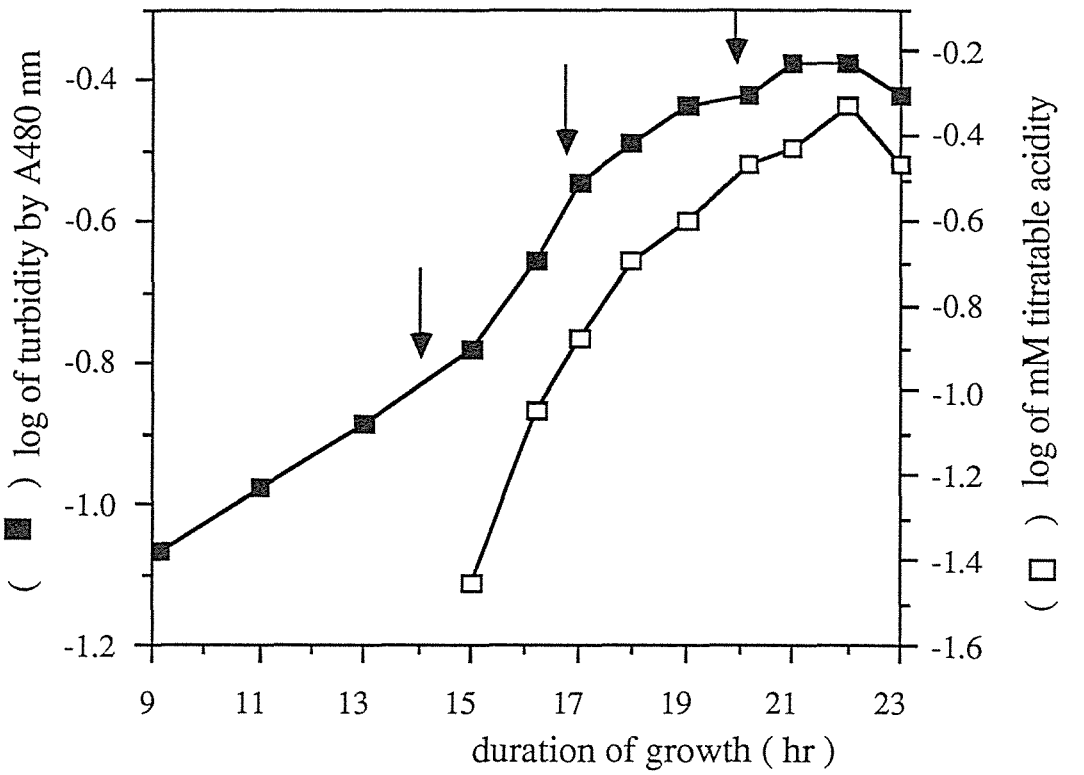
From the results of the above experiments, a routine method in which bacteria were harvested from cultures grown to early- log phase (A_{480} ~ 0.1-0.12 or approximately 14 hours after inoculation) at 22° C was used for the release of cell wall- bound proteinase.

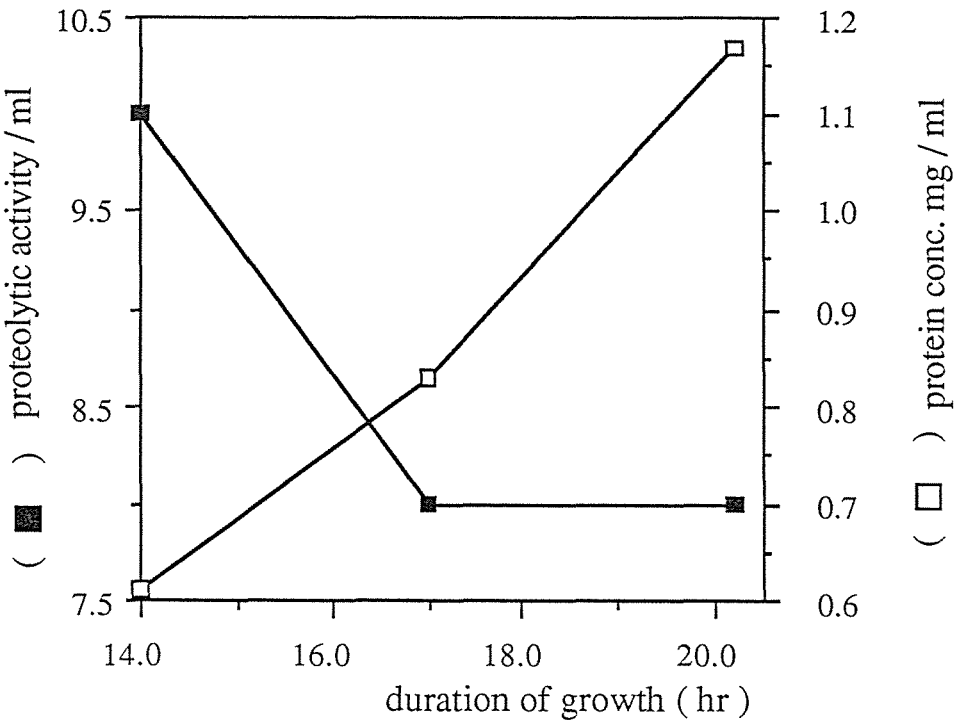
FIGURE 3.1.2 GROWTH OF *S. LACTIS* 4760 AT 22° C IN MILK MEDIUM

1.5 ml of a fully coagulated culture of *S. lactis* 4760 previously grown at 30° C in reconstituted skim milk medium (RSM) was inoculated into 150 ml RSM buffered with 4.5 ml of 2.5 M Na β–glycerophosphate (ERSM). The culture was grown for 23 hours at 22° C. Duplicate 5 ml aliquots were removed at the time intervals shown in the graph. One sample was chilled on ice immediately and then 10 ml of ice cold 0.2% EDTA and 3 drops of 10 M NaOH added. A 1 ml aliquot of the EDTA treated sample was diluted with an additional 2 ml of EDTA and A_{480} measurements taken (i.e. turbidity). To the other 5 ml sample, pH measurements were taken and the volume of 50 mM NaOH required to titrate the sample to pH 7.0 was recorded. The $\log_{10}$ of the turbidity by A_{480} and titratable acidity are plotted against the time at which the samples were removed. Arrows indicate cells harvested for release of proteinase from separate milk cultures grown for 14, 17 and 20 hours respectively in the same conditions as that described above.

FIGURE 3.1.3 RELEASE OF PROTEINASE ACTIVITY IN RELATION TO GROWTH OF *S. LACTIS* 4760 IN MILK.

Washed cells previously grown in ERSM at 22° C for 14, 17 and 20 hours as described in Figure 3.1.2, were incubated in 50 mM NaOAc / H_2PO_4 buffer pH 6.4 at 30° C for one hour. The cell suspensions were centrifuged and the supernatant assayed for FITC β casein- hydrolysing activity, as described in Figure 3.1.1, and protein concentration by the Folin method. One unit of proteolytic activity = one RF unit / hour. The proteolytic activity and protein concentration per ml of the supernatant are plotted against the time at which the growth of the cells in ERSM was stopped and the cells harvested.


(■) log of turbidity by A480 nm
-0.4
-0.6
-0.8
-1.0
-1.2
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
(□) log of mM titratable acidity
9
11
13
15
17
19
21
23
duration of growth (hr)


(■) proteolytic activity / ml
10.5
9.5
8.5
7.5
1.2
1.1
1.0
0.9
0.8
0.7
0.6
(□) protein conc. mg / ml
14.0
16.0
18.0
20.0
duration of growth (hr)

3.1.3 COMPARISON OF THE AMOUNT OF PROTEINASE RELEASED FROM *S. LACTIS* 4760 HARVESTED FROM M-17 BROTH- AND MILK GROWN CULTURES

It has been reported previously that the amount of proteinase activity which can be released from lactic streptococci is dependent on the growth medium (Exterkate, 1976 and1985). In general, less proteinase activity could be obtained from bacteria grown in media rich in protein hydrolysates and free amino acids than from cells grown in milk. The aim of the following experiment was to confirm the value of using milk as the growth medium in order to obtain a high yield of cell wall- bound proteinase.

Cultures of *S. lactis* 4760 were grown in M-17 broth medium (refer section 2.2.2) and in ERSM to comparable cell density, harvested and finally resuspended in 50 mM phosphate buffer pH 6.5 to give the same final concentration of cell dry weight per ml. The cells were incubated in Ca^{++}- free buffer at 30° C. Samples taken at 0, 0.5, 1 and 2 hours were centrifuged and the supernatant assayed for proteinase activity.

The results summarised in Figure 3.1.4 confirm that more proteinase activity was released from milk grown cells than from cells grown in M-17 medium. Proteinase activity continued to be released up to two hours of incubation from milk- grown cells while no further increase in proteinase release occurred after one hour of incubation from broth grown cells.

3.1.4 EFFECTS OF INCUBATION TIME ON ENZYME RELEASE AND CELL LEAKAGE

In the previous experiment it was found that proteinase release from milk- grown cells incubated in buffer continued up to two hours. A more detailed study of the effect of incubation time on the release of proteinase was therefore undertaken.

Washed cells harvested from ERSM- grown culture were incubated at 30° C in Ca^{++}- free buffer at pH 6.4. Aliquots were removed before incubation and after 0.5, 1, 2 and 3 hours of incubation. Proteinase and lactate dehydrogenase activities were measured both in the supernatant after centrifuging to pellet the cells and in cell- free extracts prepared by disrupting the pelleted cells in a French Press.

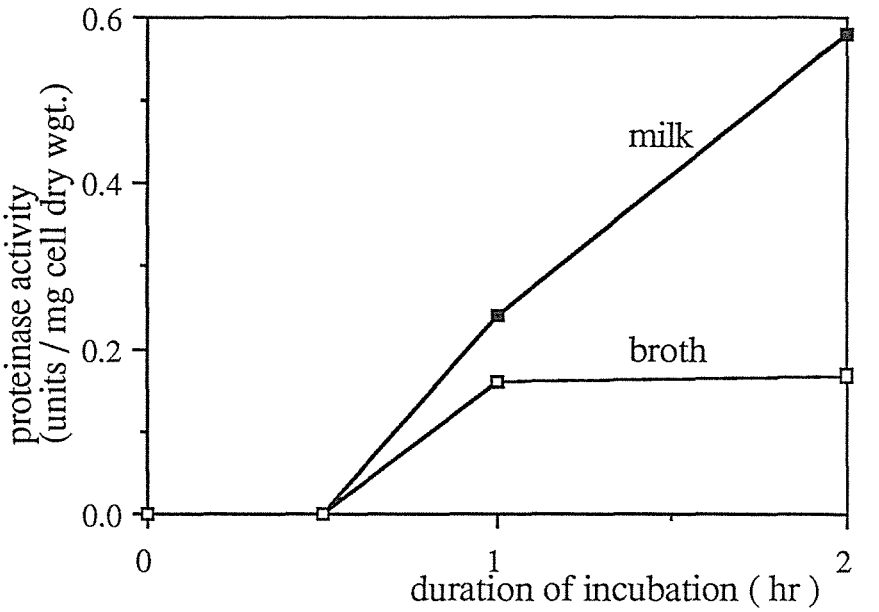
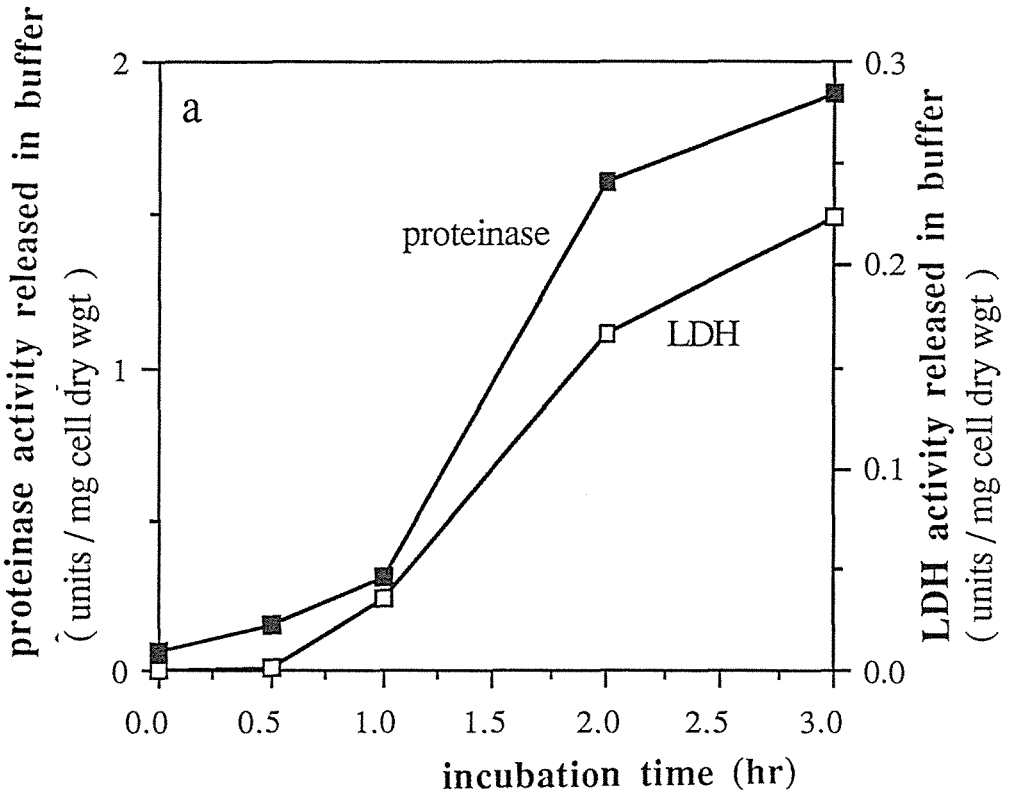
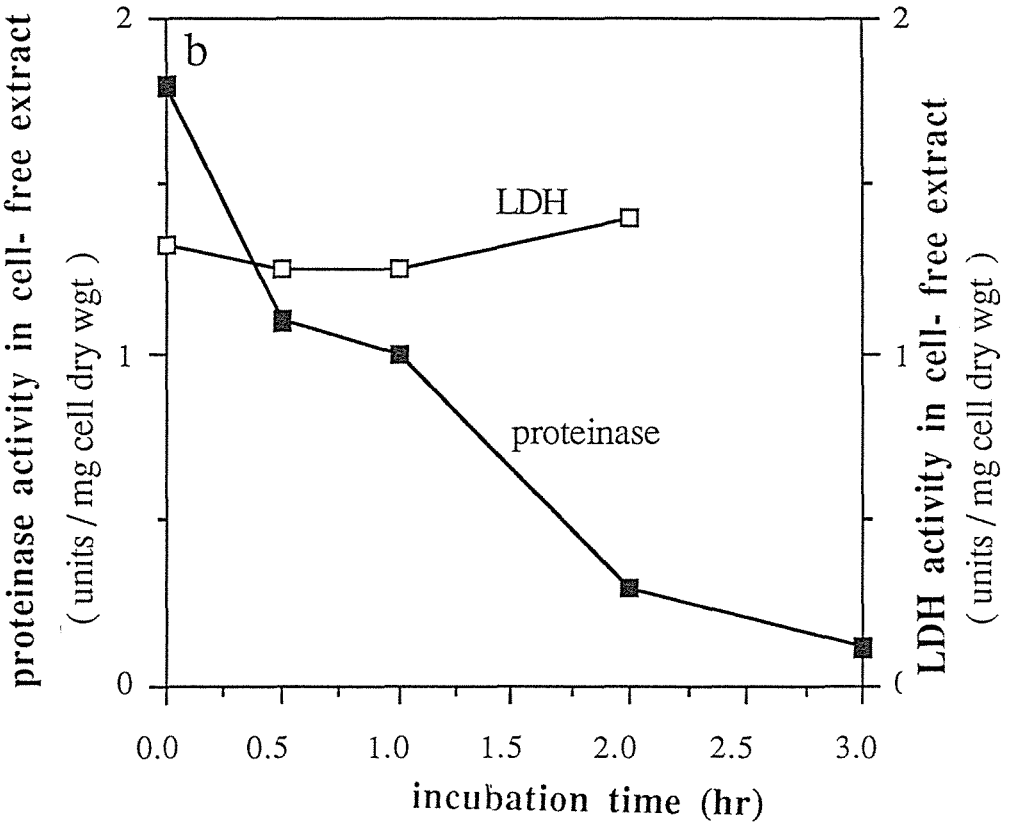


FIGURE 3.1.4 COMPARISON OF PROTEINASE RELEASE FROM MILK- AND BROTH- GROWN CELLS.

2 ml of previouly coagulated milk cultures of *S. lactis* 4760 was inoculated into 200 ml each of milk medium (ERSM) and M-17 broth medium. The cells were grown at 22° C for 14 hours and then harvested. The washed cells were finally resuspended in 10 ml 50 mM HPO_4 / H_2PO_4 buffer pH 6.4 and incubated at 30° C.

2 ml aliquots removed after 0, 0.5, 1 and 2 hours of incubation were centrifuged and the supernatant assayed for proteinase activity with FITC-β casein. The A_{480} of the washed cell supensions measured prior to incubation indicated that the cell dry weight per ml from the milk- grown and broth- grown cells were similar (26.8 and 28 mg / ml cell suspension respectively).The amount of proteinase activity per mg cell dry weight, released from the cells is plotted against the duration of incubation in buffer.


a
proteinase
LDH
proteinase activity released in buffer
(units / mg cell dry wgt)
LDH activity released in buffer
(units / mg cell dry wgt)
incubation time (hr)
0
1
2
0.0
0.1
0.2
0.3
0.0
0.5
1.0
1.5
2.0
2.5
3.0


b
LDH
proteinase
proteinase activity in cell- free extract
(units / mg cell dry wgt)
LDH activity in cell- free extract
(units / mg cell dry wgt)
incubation time (hr)
0
1
2
0.0
0.5
1.0
1.5
2.0
2.5
3.0

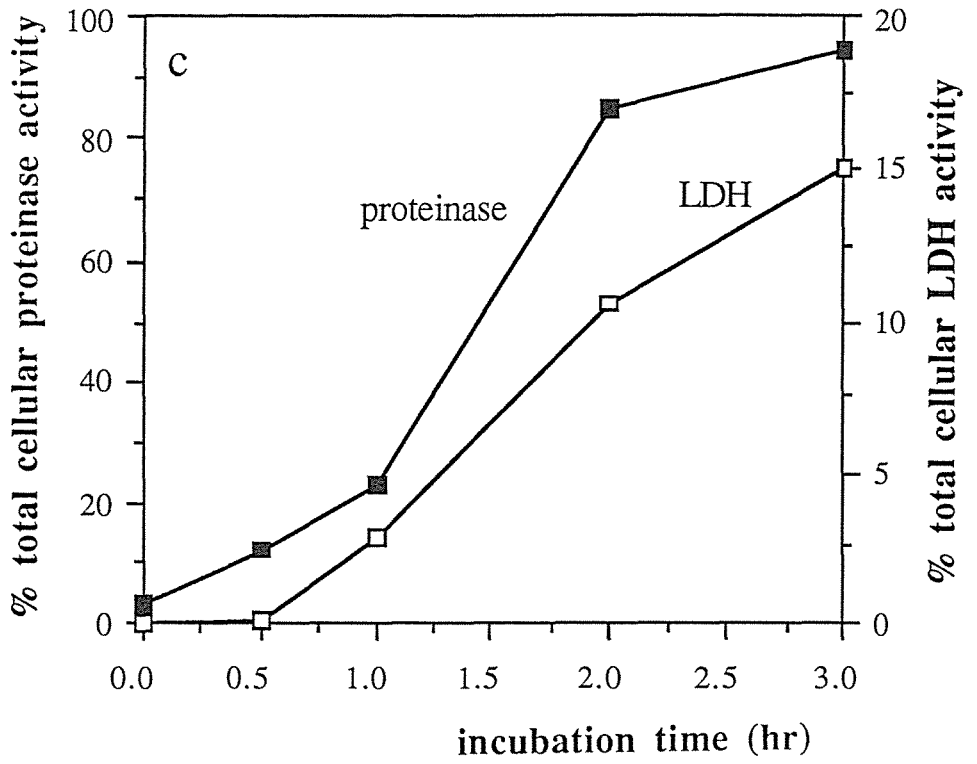


FIGURE 3.1.5 TIME DEPENDENT RELEASE OF PROTEINASE AND LDH ACTIVITY FROM *S. LACTIS* 4760

3 ml of fully coagulated culture of *S. lactis* 4760 was inoculated into 300 ml of ERSM and grown at 22° C for 14 hours. The cells were then harvested and finally resuspended in 15 ml of 50 mM NaOAc / H_2PO_4 buffer at pH 6.4. The dry weight of cells in the cell suspension estimated by A_{480} was ~ 27 mg ml^{-1}. 2.5 ml was removed and maintained on ice (i.e. O hour of incubation) while the remaining 12 ml of cell suspension was incubated at 30° C. 3 ml aliquots were removed after 0.5, 1, 2 and 3 hours of incubation, centrifuged and the supernatant assayed for proteinase and LDH activity.

A plot of proteinase and LDH activity released in buffer per mg cell dry weight against the duration of incubation is shown in (**a**). The pelleted cells were resuspended in buffer, the cell dry weight estimated and then disrupted in a French Press. The disrupted cells were centrifuged and the supernatant containing soluble cell free- extract was assayed for proteinase and LDH activity.as shown in (**b**). The LDH activity of the cell free- extract from the 3 hour incubation was not assayed.A plot of the amount of proteinase and LDH activity as a percentage of the total cell activity (i.e. activity from whole cell + cell free-extract) is shown in (**c**). The percentage of total cellular LDH activity for the 3 hour incubation was estimated using the average value of LDH activity from the 0, 0.5, 1 and 2 hour incubations.

Proteinase activity released from the cells suspended in buffer increased linearly up to one hour and then more rapidly between one and two hours of incubation as shown in Figure 3.1.5a.There was a corresponding fall in the proteinase activity of the cell free- extract prepared by disrupting the cells after the various incubation time (Figure 3.1.5b). As shown in Figure 3.1.5c, when proteinase activity is expressed as a percentage of total FITC- casein hydrolysing activity (i.e. supernatant plus cell- free extract activity), by three hours about 95% of the total activity was released into the buffer.

Lactate dehydrogenase activity, used as an indicator of cell leakage, was barely detectable after half an hour of incubation but thereafter increased almost linearly with the incubation time such that after three hours the released lactate dehydrogenase activity accounted for almost 15% of the total activity.

These results suggest that, while most of the proteinase activity released into the buffer during the first half hour of incubation was not from the interior of the cells, it is possible that the proteolytic activity released during longer times of incubation may be partly due to leakage of intracellular activity (using LDH as the criterion for leakage).

In subsequent sections the proteinase activity present in the buffer following incubation of the cell suspensions will be referred to as ' released enzyme '. The extent to which the release process represents true release of the cell wall bound- enzyme rather than leakage from lysed or leaky cells will be discussed later.

3.1.5 EFFECTS OF pH ON ENZYME RELEASE

The release of proteinase from cells incubated in Ca^{++} free- buffer for one hour at 30^{o} C was investigated in various buffers, covering the pH range of 5.2-7.6. Washed cells harvested from ERSM- grown cultures were incubated at 30^{o} C for one hour in different buffers containing 50 mM acetate, acetate / phosphate or phosphate ions, at pH 5.2, 5.8, 6.4, 7.0 and 7.6 as described in the legend to Figure 3.1.7. After the incubation, the cell suspensions were centrifuged and the supernatant assayed for proteinase and LDH activity.

A preliminary experiment investigating the effects of buffers covering a range of pH values from pH 4.6 - 7.6 on the proteinase assay showed that the activity varied with the pH of the buffer. Since the volume of the supernatant used in the proteinase assay

constituted a third of the total volume of the assay mixture, the pH of the reaction mixtures containing the different buffers would vary such that it would not be possible to separate the effects of pH on release of proteinase activity from those due to pH dependent activity changes.

To overcome the effect of varying pH on the proteinase activity, the concentration of the assay buffer was increased from 50 mM to 500 mM. Due to the small volume of the total assay mixture (300 µl) the pH of the reaction could not be measured directly except by simulating the conditions of the reaction mixture in proportionately larger volumes. By this method, the higher concentration of assay buffer was found to maintain the pH of the reaction mixture at a constant value irrespective of the range of pH values of the supernatant used in the present experiment. In addition, as shown in Figure 3.1.6, the use of the higher concentration of buffer in the proteinase assay stimulated the proteinase activity only slightly without causing any spontaneous (non- enzymatic) release of FITC from the FITC- casein conjugate.

The effects of pH on the release of proteinase are summarised in Figure 3.1.7. An increase in the release of proteinase activity was observed when the pH of the release buffer was increased from pH 5.2 to pH 7.0. Above pH 7.0 however, the release of proteinase declined. It was also observed that the different buffer ions used to obtain the different pH values also affected the amount of proteinase released. At pH 5.8 and 6.4, buffers containing acetate as the buffering ions were compared with those containing only phosphate ions. The presence of acetate in the buffer lead to a decrease in the release of proteinase suggesting that acetate ions may be inhibiting the release.

The level of LDH activity released into the buffer was also dependent on pH in a manner similar to the release of proteinase activity. Since the release of LDH indicates the extent of leakage of intracellular enzymes these results suggest that the effect of pH on proteinase release may be, at least in part, a consequence of pH dependent changes in cell leakage. The result is somewhat surprising since it might have been expected that cell leakiness would not have varied greatly over the pH range studied.

From the results of the above experiments, it was concluded that the optimum conditions for the release of proteinase from the lactic streptococci involved incubating the cells at 30° C in Ca^{++}-free phosphate buffer at pH 6.4 for not more than 3 hours. A pH of 6.4 was used rather than 7.0 since the buffer used for the proteinase assay was at pH 6.4. It was thus convenient to use the same pH for release as that used for the assay since the enzyme could then be assayed in the release buffer without adjustment of pH. These conditions

FIGURE 3.1.6 INFLUENCE OF ASSAY BUFFER CONCENTRATION ON PROTEINASE ACTIVITY.

80 µl of crude proteinase from *S. lactis* 4760 released into Ca^{++}free- buffer was incubated at 22° C with 80 µl of FITC-β casein and 80 µl of either 50 mM or 500 mM NaOAc / H_2PO_4 buffer pH 6.4. 60 µl aliquots were removed after 30, 50 and 100 minutes and the RF of TCA soluble- hydrolysis products measured. Incubation in 500 mM buffer in the absence of proteinase was also carried out. To obtain blank RF values (i.e at 0 minute), TCA was added to 20 µl of enzyme and 20 µl of buffer before the addition of 20 µl of FITC-β casein. All reactions were carried out in duplicates.

FIGURE 3.1.7 pH DEPENDENT RELEASE OF PROTEINASE AND LDH FROM *S. LACTIS* 4760

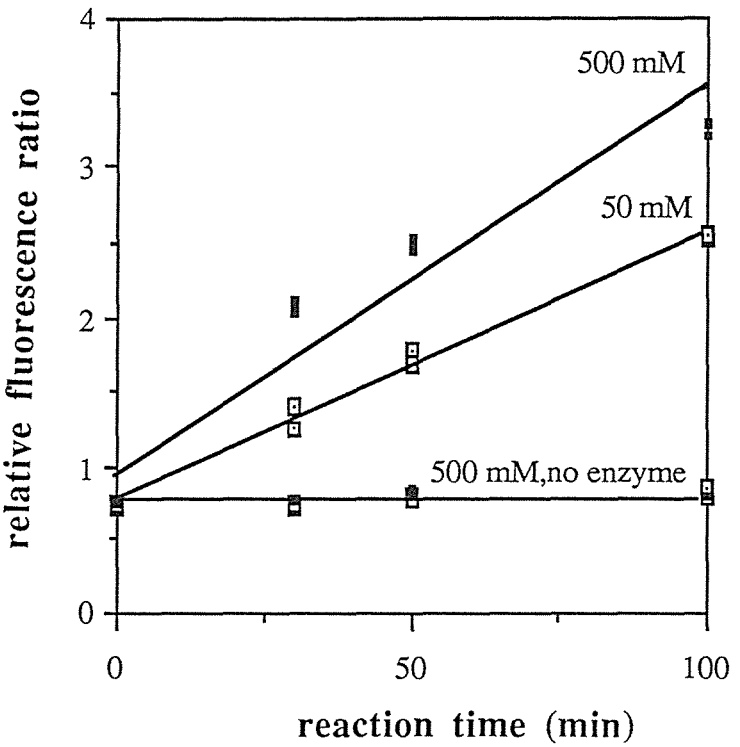
4 ml of fully coagulated culture was inoculated into 400 ml of ERSM and grown at 22° C for 14 hours. The cells were harvested, washed extensively with 50 mM NaOAc/ H_2PO_4 buffer pH 6.4 and divided into 8 equivalent samples. The cell suspensions were centrifuged and the pelleted cells resuspended in 3 ml of each of the following buffers :

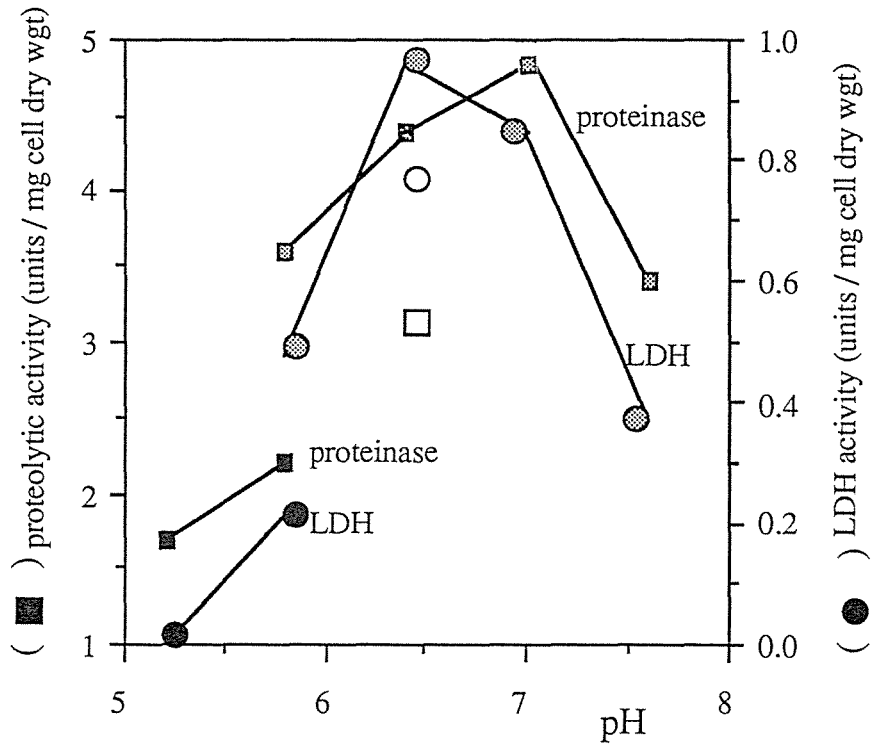
50 mM NaOAc / AcOH buffer pH 5.2 and pH 5.8 (● , ■);

50 mM HPO_4 / H_2PO_4 buffer pH 5.8, 6.4, 7.0 and 7.6 (◍ , ▣);

50 mM NaOAc / H_2PO_4 buffer pH 6.4 (○ , □).

0.5 ml of each of the cell suspensions was removed to determine cell dry weight and the remaining 2.5 ml incubated at 30° C for one hour and then centrifuged to pellet the cells. The supernatant was assayed for proteinase and LDH activity in duplicates. Proteinase activity was carried in 500 mM assay buffer instead of the usual 50 mM buffer so that the pH of all the reaction mixtures was maintained regardless of the varying pH values of the supernatant. A plot of proteinase activity (squares) and LDH activity (circles) against the pH of the release buffer is shown.


relative fluorescence ratio
reaction time (min)
500 mM
50 mM
500 mM,no enzyme
0
1
2
3
4
0
50
100


(■) proteolytic activity (units / mg cell dry wgt)
(●) LDH activity (units / mg cell dry wgt)
pH
proteinase
LDH
proteinase
LDH
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
5
6
7
8

were used to release proteinase for the purification and study described in section 3.2.

3.1.6 EFFECTS OF Ca^{++} ION CONCENTRATION ON CELL LEAKAGE DURING ENZYME RELEASE

In the previous two sections, the release of LDH was shown to be dependent on the duration of incubation and pH of the cell suspensions in a manner similar to the release of proteinase activity. In general, any increase in the the amount of proteinase released was accompanied by an increase in the release of LDH activity, suggesting that part of the proteinase activity released into the buffer may be due to leakage of intracellular enzymes.

Since the presence of Ca^{++} ions in the buffer have been shown to inhibit the release of cell wall bound- proteinase (Mills & Thomas, 1978), it was of interest to determine if the addition of Ca^{++} ions to the buffer would also inhibit the release of LDH activity and whether the release of the proteinase could be only partially inhibited by Ca^{++}. To investigate the effect of Ca^{++} on the release of the proteinase into the buffer, the possible effects of Ca^{++} ions on the activity of the enzyme had to be taken into account. It was shown that the presence of up to 2.66 mM $CaCl_2$ added to the proteinase assay mixture did not alter the activity of the enzyme significantly (Figure 3.1.8). Since in the following experiment the concentration of $CaCl_2$ which would inevitably be transferred from the release buffer into the assay mixture did not exceed 2 mM, any changes observed in the release of proteinase activity will be due to the effect of Ca^{++} on the release process and not on the enzyme activity in the assay.

Washed cells harvested from ERSM- grown culture were incubated for one hour at 30° C in buffer containing 0, 0.5, 1, 2 and 4 mM $CaCl_2$ respectively. The cell suspensions were centrifuged and the supernatant containing released enzymes assayed for proteinase and LDH activity. The cell pellets were disrupted in a French Press and the supernatant retained after centrifugation was also assayed.

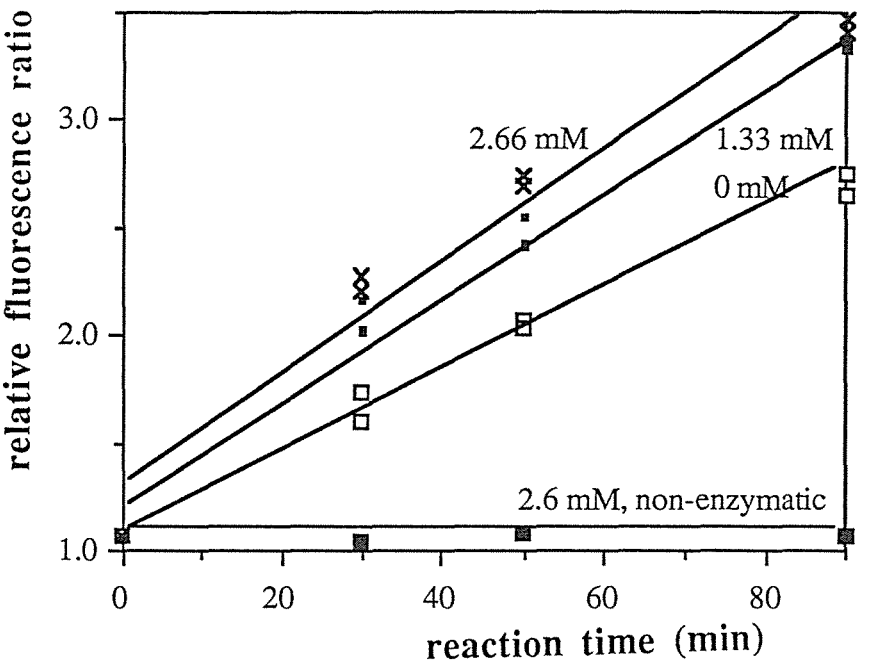
A plot of the percentage of total cellular proteinase activity released into the buffer against the $CaCl_2$ concentration showed that while the release of proteinase activity declined from 34% in Ca^{++}- free buffer to about 9% in buffer containing 2 mM $CaCl_2$, the release of LDH activity was not altered (Figure 3.1.9). Increasing the concentration of $CaCl_2$ from 2 mM to 4 mM did not result in any further significant decline in either the release of proteinase activity or LDH activity. These results are consistent with the possibility that part of the proteinase activity released in buffer containing more than 2 mM $CaCl_2$ may be due to leakage of intracellular enzymes during the incubation of the cells for one hour.

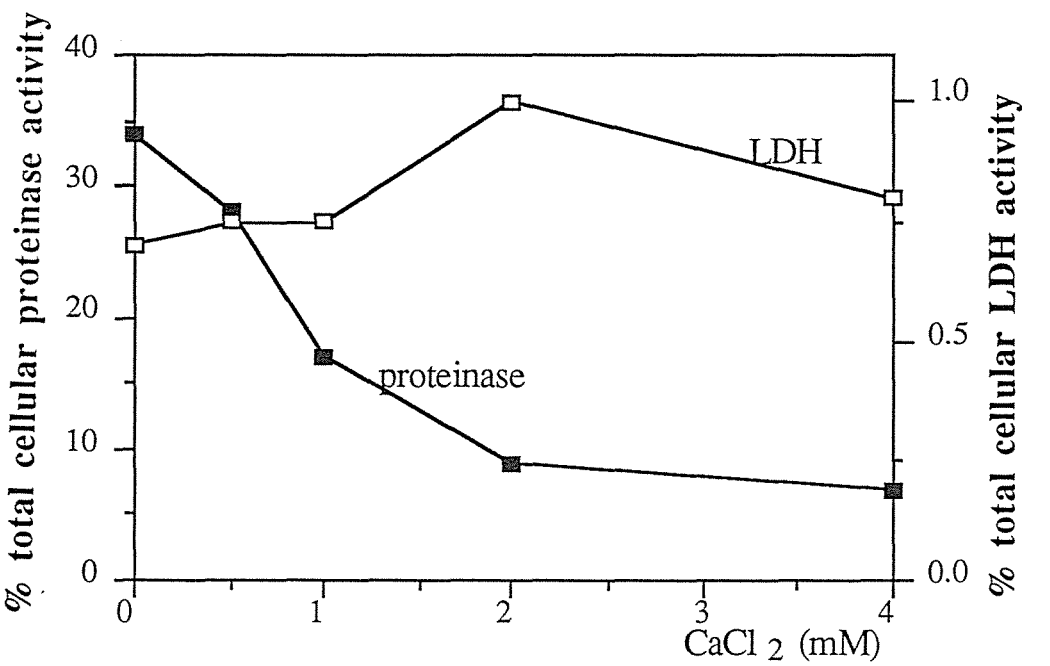
FIGURE 3.1.8 INFLUENCE OF THE PRESENCE OF Ca^{++} IONS IN THE ASSAY BUFFER ON PROTEINASE ACTIVITY

80 µl of crude proteinase released from *S. lactis* 4760 was incubated at 22° C with 80 µl of FITC-β casein and 80 µl of 50 mM NaOAc / H_2PO_4 buffer pH 6.4 containing 0 (□), 1.33 (▪) and 2.66 mM $CaCl_2$ (×) respectively. 60 µl aliquots were removed after 30, 50 and 90 minutes and the RF of TCA soluble hydrolysis product measured. Incubation of FITC-β casein in buffer containing 2.66 mM $CaCl_2$ in the absence of proteinase was also carried out (■). Blank RF values (0 minute) was obtained from incubations of proteinase and buffer in the presence of TCA before FITC-β casein was added. All reactions were carried out in duplicates.

FIGURE 3.1.9 EFFECTS OF Ca^{++} ON THE RELEASE OF PROTEINASE FROM *S. LACTIS* 4760

Washed cells harvested from 200 ml ERSM- grown culture were resuspended in 50 mM HPO_4/H_2PO_4 buffer , pH 6.4 containing 0, 0.5, 1, 2 and 4 mM $CaCl_2$ to a cell dry weight of around 9 $mgml^{-1}$. The suspensions were incubated for one hour at 30° C. The cells were pelleted by centrifugation and the supernatant assayed for proteinase and LDH activity. The pelleted cells were disrupted in a French Press, centrifuged and the supernatant containing soluble cell- free extract subsequently assayed for proteinase and LDH activity. The proteinase (▬) and LDH (□) activity released from intact cells expressed as a percentage of the total amount of activity present in the cell (i.e released plus cell- free extract) are plotted against the concentration of $CaCl_2$ in the release buffer.


relative fluorescence ratio
3.0
2.0
1.0
2.66 mM
1.33 mM
0 mM
2.6 mM, non-enzymatic
0
20
40
60
80
reaction time (min)


% total cellular proteinase activity
40
30
20
10
0
LDH
proteinase
1.0
0.5
0.0
% total cellular LDH activity
0
1
2
3
4
CaCl 2 (mM)

However, since only 0.7% of the total cellular LDH was released into the buffer, it is unlikely that the residual 9% of total cellular caseinolytic activity could have originated from an intracellular source.

3.1.7 COMPARISON OF THE RELEASE OF PROTEINASE FROM THE *S. LACTIS* H1 STRAINS 4760, 4122 AND 4125 AND FROM *S. CREMORIS* H2

The inter-relationship between *S. lactis* strain 4760, S. *lactis* H1 strain 4122 and *S. cremoris* H2 was defined in the introduction to section 3.1. To summarise, *S. lactis* H1 strain 4122 was cured of its single Lac^+ Prt^+ plasmid and the resulting Lac^-Prt^- *S. lactis* H1 strain 4125 was transformed with an equivalent plasmid from *S. cremoris* H2 to generate the Lac^+Prt^+ *S. lactis* 4760. Therefore, although *S. lactis* 4760 is a daughter strain of *S. lactis* H1 strain 4122, the proteinase released from the bacteria is that encoded by an *S. cremoris* H2 plasmid.

The aim of the following experiments was to compare the release of proteinase from *S. lactis* 4760 with that from its parent strain, *S. lactis* H1 strain 4122 and from its Prt^+ plasmid donor, *S. cremoris* H2.

3.1.7.1 Comparison of the release of proteinase from *S. lactis* H1 strains 4760, 4122.and 4125.

In the present study, the release of the proteinase encoded by the *S. cremoris* H2 Prt^+ plasmid present in *S. lactis* strain 4760 was compared to the release of the enzyme encoded by the Prt^+ plasmid present in *S. lactis* H1 strain 4122. Any release of proteinase not encoded by the plasmids was accounted for by using the plasmid cured Lac^- Prt^- *S. lactis* H1 strain 4125 as a control.

In order to grow the Lac^- Prt^- *S. lactis* H1 strain 4125 the milk medium had to be suppplemented with 0.05% (w/v) glucose and 0.02% (w/v) casitone. These supplements were also added to the milk medium used for growing the Prt^+ strains 4122 and 4760 since it is possible that the addition of casitone to the milk may affect the synthesis or release of proteinase. Any differences between the release of proteinase from *S. lactis* strain 4760 grown in the casitone- supplemented medium and that from cells grown in medium without added casitone (only 0.05% glucose supplementation) was also investigated.

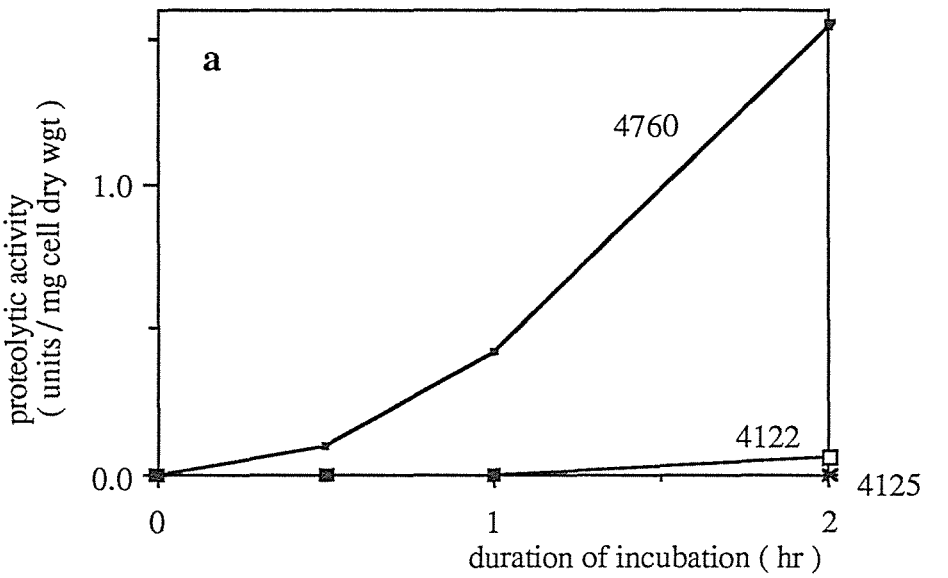
Washed cells harvested from the milk grown cultures were incubated in Ca^{++}-free buffer at pH 6.4, at 30° C. The cell suspensions were centrifuged after 0, 0.5, 1 and 2 hours of incubation and the supernatant assayed for proteinase and LDH activities. The amount of activity released into the buffer from the various strains grown in supplemented ERSM is plotted against duration of incubation in Figure 3.1.10a and 10b. No proteinase activity was released from *S. lactis* H1 strain 4125 during the 2 hours of incubation.This suggests that the proteinase released from the Prt^+ strains 4122 and 4760 must be plasmid encoded. Very little activity was released from *S. lactis* H1 strain 4122 incubated for more than one hour in comparison to *S. lactis* strain 4760 which released a significantly greater amount of proteinase activity. This finding is consistent with previous observations (L. Pearce, personal communication) that the strain 4122 coagulates milk much more slowly than the transconjugant strain 4760.

From Figure 3.1.10b it can be seen that the release of LDH during the 2 hour incubation in buffer is very different for the three strains. The increased rate of LDH leakage occurring after 1 hour in strain 4760 did not occur in the plasmid-free strain 4125. On the other hand the leakage of LDH from strain 4122 carrying its 'native' Lac^+Prt^+ plasmid was much greater than that from strain 4760 especially in the first hour of incubation and contrasts with the much lower release of proteinase activity from strain 4122 compared to strain 4760. These differences in LDH leakage between the strains differing only in their plasmid complements are unexpected but have not been further investigated.

The results comparing the release of proteinase and LDH from *S. lactis* strain 4760 previously grown in glucose and casitone supplemented milk medium to that from cells grown only in glucose supplemented medium are summarised in Figure 3.1.11a and 11b.The amount of proteinase released from the cells grown in unsupplemented medium was about twice the activity released from cells grown in casitone-supplemented medium suggesting that the presence of casitone may have partly repressed proteinase synthesis. The level of LDH released or cell lysis was markedly greater (~ 10 fold) from the cells grown in unsupplemented medium.This observation suggests that the addition of casitone to the milk medium may significantly reduce the amount of cell lysis.

FIGURE 3.1.10 COMPARISON OF THE RELEASE OF PROTEINASE FROM *S. LACTIS* strains 4122, 4125 AND 4760

Culture of *S. lactis* strains 4122, 4125 and 4760 were grown in 150 ml of ERSM supplemented with 0.05% (w/v) glucose and 0.02% (w/v) casitone for 14 hours at 22° C. The cells were harvested, resuspended in 8 ml 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4, and incubated at 30° C for 2 hours. The dry weight of cells in the suspensions was 28 mg/ml for strains 4122 and 4760 and 22 mg/ml for strain 4125. Aliquots of the cell suspension were removed after 0, 0.5, 1 and 2 hours of incubation, centrifuged and the supernatant assayed for proteinase and LDH activity according to the methods outlined in section 2. A plot of proteinase activity released against the duration of the incubation is shown in **(a)** and that for LDH in **(b)**.


a
4760
4122
4125
1.0
0.0
0
1
2
proteolytic activity
(units / mg cell dry wgt)
duration of incubation (hr)

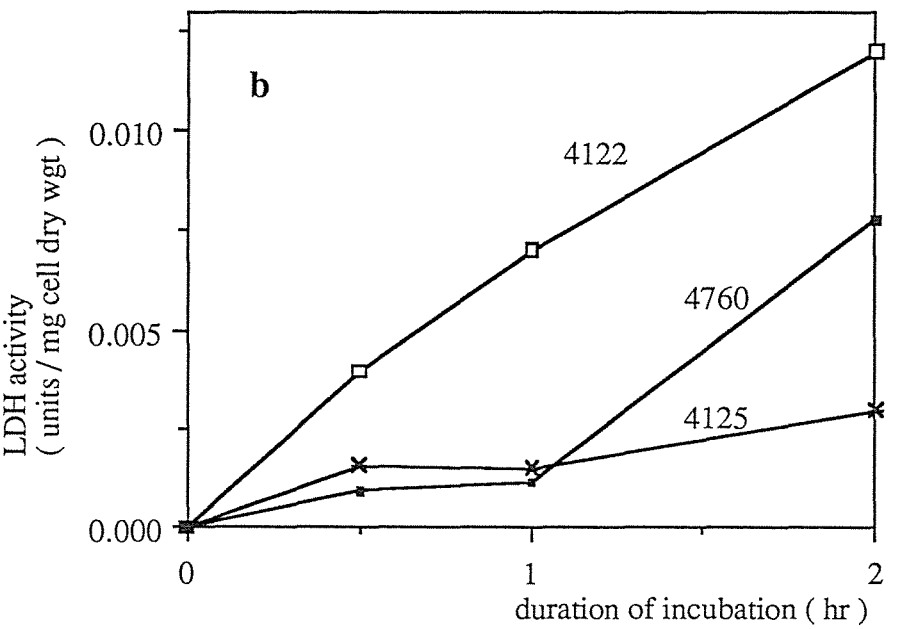
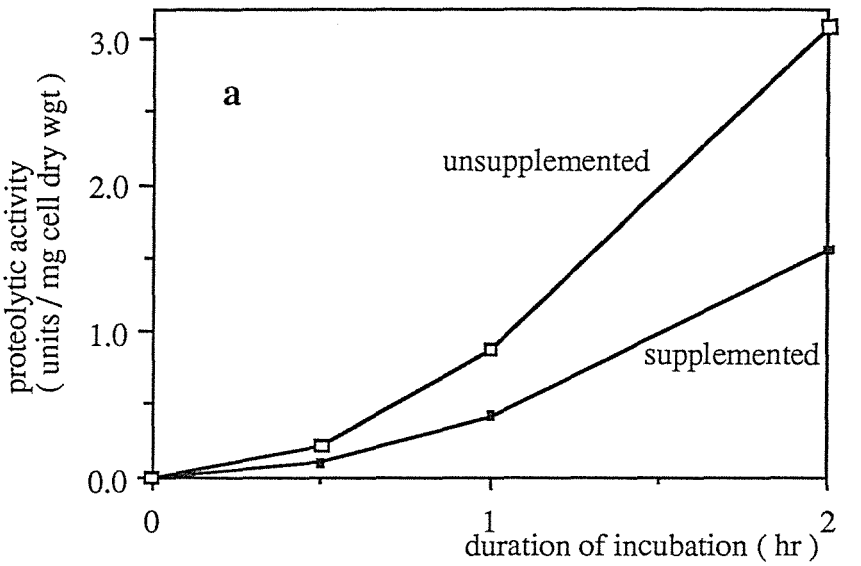

b
4122
4760
4125
0.010
0.005
0.000
0
1
2
LDH activity
(units / mg cell dry wgt)
duration of incubation (hr)

FIGURE 3.1.11 COMPARISON OF THE RELEASE OF PROTEINASE FROM *S. LACTIS* 4760 GROWN IN CASITONE-SUPPLEMENTED AND UNSUPPLEMENTED MILK.

S. lactis 4760 was grown for 14 hours at 22° C in 150 ml of ERSM supplemented with 0.05% (w/v) glucose either with or without casitone added to 0.02% (w/v). The cells were harvested and then incubated in 8 ml of 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4, for 2 hours. The dry weight of cells in the suspension of cells harvested from casitone- supplemented medium was 28 mg/ml compared to 20 mg/ml in the cells harvested from unsupplemented growth medium. Samples removed after 0, 0.5, 1 and 2 hours of incubation were centrifuged and the supernatant assayed for proteinase (**a**) and LDH (**b**) activity.


a
unsupplemented
supplemented
proteolytic activity
(units / mg cell dry wgt)
3.0
2.0
1.0
0.0
0
1
2
duration of incubation (hr)

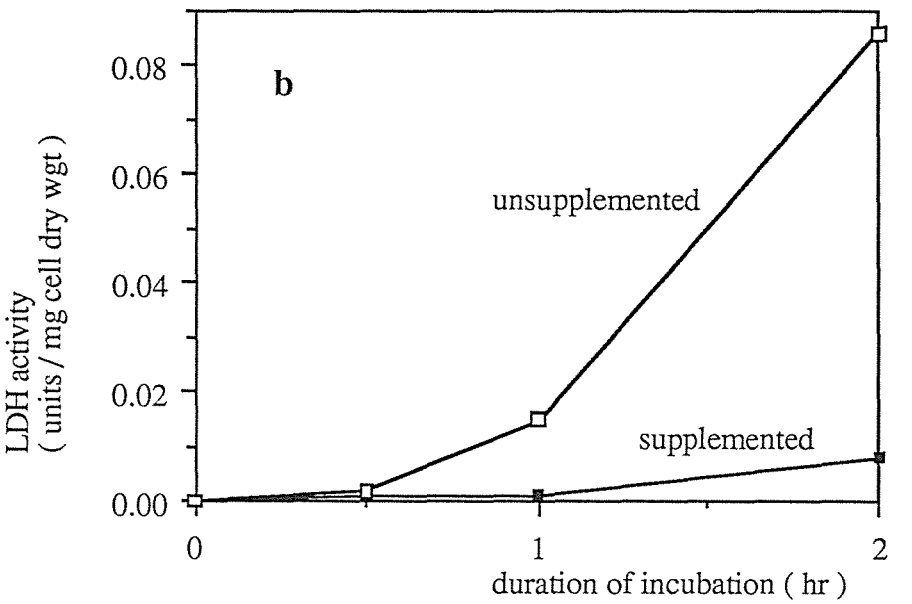
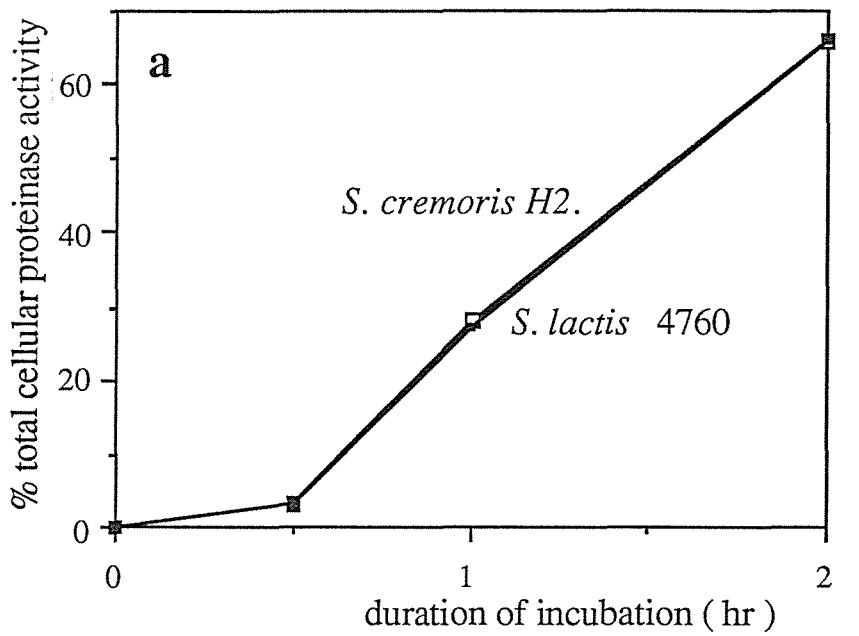
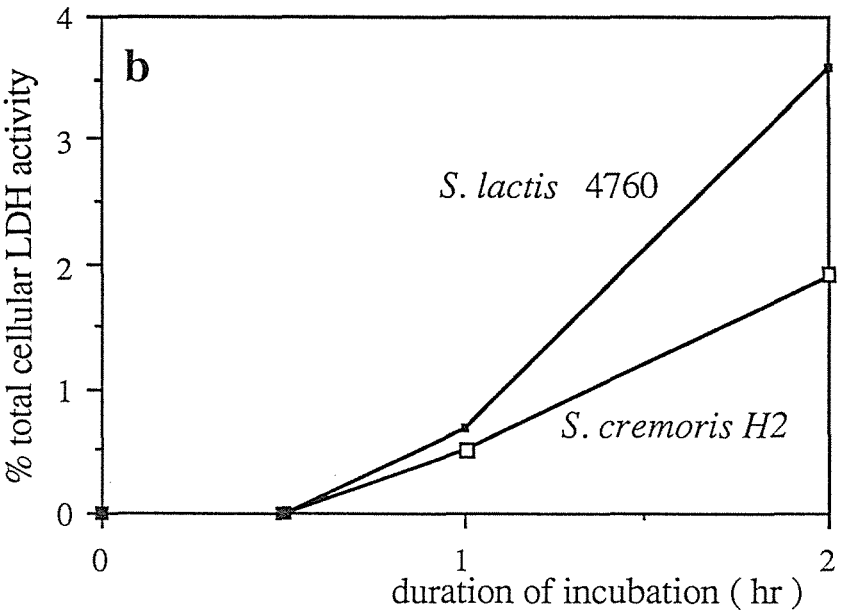

b
unsupplemented
supplemented
LDH activity
(units / mg cell dry wgt)
0.08
0.06
0.04
0.02
0.00
0
1
2
duration of incubation (hr)

FIGURE 3.1.12 COMPARISON OF THE RELEASE OF PROTEINASE FROM *S. CREMORIS* H2 AND *S. LACTIS* 4760

150 ml ERSM cultures (1% inoculum) of *S. lactis* 4760 and of *S. cremoris* H2 were grown for 14 hours at 22° C. The cells were harvested and then resuspended in 8 ml 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4. The cell dry weight in the washed cell suspension of *S. lactis* 4760 was 26.5 mg/ml while that for *S. cremoris* H2 was 29 mg/ml. The cells were incubated at 30° C and samples removed after 0, 0.5, 1 and 2 hours of incubation were centrifuged and the supernatant assayed for proteinase and LDH activity. The cells pelleted from the centrifugation were disrupted in a French Press and then centrifuged and the supernatant assayed for proteinase and LDH activity. The activity released into buffer over the duration of incubation is shown as a percentage of the total cellular proteinase activity in **(a)** and total cellular LDH activity in **(b)** where total cellular activity is taken as the sum of the activity released into buffer and that in the disrupted cell supernatant.


a
% total cellular proteinase activity
60
40
20
0
S. cremoris H2.
S. lactis 4760
0
1
2
duration of incubation (hr)


b
% total cellular LDH activity
4
3
2
1
0
S. lactis 4760
S. cremoris H2
0
1
2
duration of incubation (hr)

3.1.7.2 Comparison of the release of proteinase from *S. lactis* 4760 and *S. cremoris* H2.

S. cremoris H2 and *S. lactis* 4760 were grown to equivalent cell density at 22° C in ERSM, harvested and finally incubated in Ca^{++}-free buffer at 30° C for 0, 0.5, 1 and 2 hours. The cell suspensions were centrifuged and the supernatant assayed for proteinase and LDH activity.

The results summarised in Figure 3.1.12a and 12b showed that both strains released very similar amounts of proteinase activity into the buffer. However, *S. lactis* 4760 released more LDH activity than *S. cremoris* H2 when the cells were incubated for two hours. This suggests that the release of the proteinase encoded by the same plasmid from the two different cells may be accompanied by more extensive cell lysis in the transconjugant *S. lactis* 4760 than the natural host of the plasmid, *S. cremoris* H2. (Note : This comparison between strains 4760 and H2 was not carried out until the enzyme purification studies reported in the next section were completed. In the light of the above results it would possibly have been better to have used *S. cremoris* H2 as the source of the cell wall proteinase.)

3.2 PARTIAL PURIFICATION OF PROTEINASE RELEASED FROM *S. LACTIS* 4760.

This section describes the partial purification of the proteinase activity released by incubation of milk grown cells of *S. lactis* 4760 in Ca^{++}-free buffer using conditions based on the work described in the previous section. The aims of the work described in this section were :

(a) to partially purify the cell wall- bound proteinase. It has been found by previous workers attempting to purify the proteinase that the enzyme undergoes autoproteolysis during purification yielding two or more molecular weight forms of the enzyme which, however, retain proteolytic activity (Exterkate *et al.*, 1987). The extent of autoproteolysis may well increase with the degree of purification so that it is necessary to balance the desirability of obtaining a very high degree of purification against the disadvantages of extensive autoproteolysis with prolonged purification procedures.

(b) to investigate further the possibility that the proteinase released during incubation in Ca^{++}-free buffer may be partially due to the presence of intracellular proteinase

Table III STABILITY OF CRUDE CELL WALL PROTEINASE

Crude proteinase was released from *S. lactis* 4760 cells harvested from ERSM grown culture by incubating the washed cells in 50 mM HPO_4/H_2PO_4 buffer at 30° C for three hours. The proteinase was concentrated in an Amicon Diaflo Cell over PM 30 membrane and then 1 ml aliquots diluted with an equal volume of each of the appropriate buffers. 0.5 ml samples were subsequently removed and treated with either Ca^{++} or glycerol. All samples were assayed for proteinase activity with FITC-β casein after storage at 4° C for 5 and 120 hours. The values for the relative fluorescence ratio of TCA soluble material from the samples after two hours of incubation at 24° C are tabulated. The RF value for incubation of FITC-β casein without enzyme was 0.9.

TABLE III

conditions	untreated		1 mM $CaCl_2$		4 mM $CaCl_2$		10% glycerol		30% glycerol	
hours of storage	5	120	5	120	5	120	5	120	5	120
Buffer										
50 mM $NaOAc/H_2PO_4$ pH 6.4	3.9	3.8	3.7	3.4	4.0	3.5	3.6	3.1	3.5	3.4
100 mM Tris/HCl pH 7.5	3.6	3.3	not done		not done		not done		3.4	3.3
100 mM Tris/HCl pH 8.0	3.5	3.0	3.4	3.2	3.5	3.1	not done		3.0	3.0
100 mM HPO_4/H_2PO_4 pH 8.0	3.4	3.0	3.4	3.0	not done		not done		3.1	3.0

which has been released by leakage or lysis of cells. In the following procedure a 3 hour incubation was used to obtain high yields of released enzyme. As indicated by the results described in the previous section there is considerable ' leakage ' of the intracellular enzyme LDH after this duration of incubation so if intracellular proteolytic activity is contributing to released proteinase it should be readily detected after 3 hours of incubation.

Crude proteinase released during a 3 hour incubation of bacteria harvested from one to four litres of ERSM- grown culture was purified by anion exchange chromatography on DE-23 cellulose followed by gel permeation chromatography on Sephacryl S- 300 column. The extent of the purification was analysed by discontinuous electrophoresis on 8% polyacrylamide gel in non- denaturing conditions.

To obtain suitable conditions for purification and storage of the purified enzyme with minimal loss of activity or stability, a preliminary experiment was carried out to investigate the stability of the crude enzyme in different conditions (Table III). Four aliquots of crude enzyme released by incubating cells for 3 hours at 30° C in 50 mM phosphate buffer at pH 6.4 were diluted with an equivalent volume of each of the following buffers : 50 mM NaOAc / H_2PO_4 pH 6.4 ; 100 mM Tris / HCl pH 7.5 ; 100 mM Tris / HCl pH 8.0 and 100 mM HPO_4 / H_2PO_4 pH 8.0. To five equivalent aliquots from each of these four different buffer systems, $CaCl_2$ or glycerol was added to give the following final concentration : untreated; 1 mM $CaCl_2$; 4 mM $CaCl_2$; 10% glycerol ; 30% glycerol.The addition of Ca^{++} ions was done to find out if the enzyme could be more stable in the presence of the Ca^{++} as has been reported for previous purifications of the proteinase (Geis *et al.*, 1985).

The samples were stored at 4° C for 5 and 120 hours and subsequently assayed for proteinase activity towards FITC- β casein by measuring the relative fluorescence of the TCA- soluble hydrolysate after two hours of incubation at 22° C. From the results summarised in Table III it was found that the enzyme in the crude supernatant was more or less equally stable under all conditions used. There was a slight loss of activity after storage of the enzyme for 120 hours at pH 7.5 and pH 8.0 suggesting that the enzyme was possibly more stable at pH 6.4 than at the higher pH values. Addition of up to 4 mM $CaCl_2$ did not alter the stability of the enzyme.

From the results summarised above, it was decided that the released proteinase could be purified by DE- 23 anion exchange chromatography in 10 mM Tris / HCl buffer at pH 8.0 without any significant loss of activity. Subsequent gel permeation chromatography of the partially purified enzyme was carried out in 50 mM NaOAc / H_2PO_4 buffer at pH 6.4 so

that the enzyme could be stored in the same buffer. Glycerol was added to 30% v/v if the enzyme was to be stored for longer periods at -20° C.

3.2.1 ANION EXCHANGE PURIFICATION OF CRUDE CELL WALL PROTEINASE

Crude cell wall proteinase was released by incubating milk- grown cells of *S. lactis* 4760 in Ca^{++}-free buffer (50 mM phosphate buffer, pH 6.4) at 30° C for 3 hours.The supernatant was equilibrated with 10 mM Tris / HCl buffer pH 8.0 by extensive washing over PM30 ultrafiltration membranes in an Amicon Diaflo concentrator at 6° C until the pH was about 8.0. (This procedure was used in perference to dialysis since there was greater loss of activity on dialysis.) The diafiltered enzyme was applied to a 3.3 cm x 12 cm column of DEAE- 23 cellulose and subsequently eluted with a linear gradient of 0 - 1 M NaCl at 6° C. The fractions were assayed for proteinase activity and the presence of protein monitored by A_{280}. The elution profile of the enzyme purification is shown in Figure 3.2.1. Only one peak of proteinase activity was obtained which was eluted at 0.2 M NaCl.

In a subsequent purification of another batch of proteinase the column was eluted with a step wise gradient of 0.1 M, 0.2 M and 0.3 M NaCl followed by a linear gradient of 0.6-1 M NaCl (Figure 3.2.2). The fractions were assayed for proteinase activity as well as for two different aminopeptidase activities known to be present in the bacteria ; glycyl-prolyl dipeptidyl peptidase activity and lysine aminopeptidase activity. These two amino peptidases were assayed using gly-pro- AMC and lys- AMC as described in Section 2.2.9.

Only a single peak of proteinase activity was detected (Fig.3.2.2a). The gly- pro- AMCase activity was eluted in about the same fractions as the proteinase activity (Fig. 3.2.2b) so that it was not possible to separate the two different enzyme activities at this stage of the purification. Low activity towards lys-AMC was detected (Fig. 3.2.2c), which eluted at a higher concentration of NaCl than the proteinase and gly-pro- AMCase activities. The fractions containing the proteinase activity were pooled and subjected to further purification by gel permeation chromatography on Sephacryl S-300.

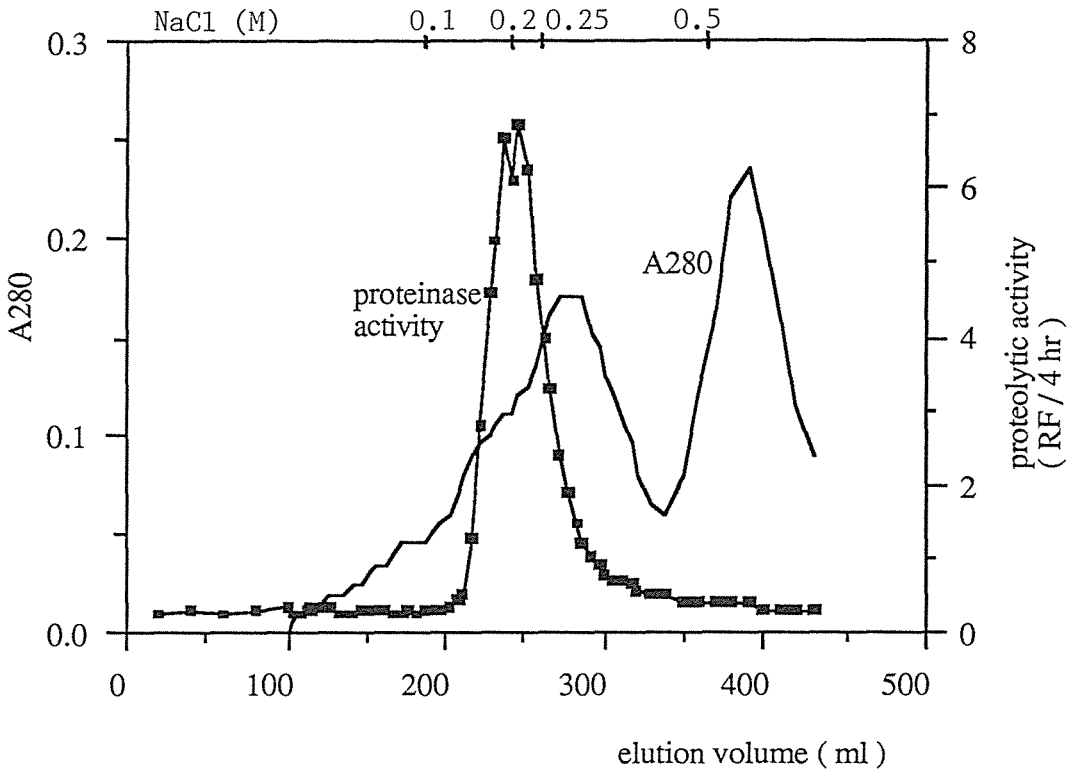


FIGURE 3.2.1 DEAE- 23 CELLULOSE ELUTION PROFILE OF CRUDE PROTEINASE RELEASED FROM *S. LACTIS* 4760.

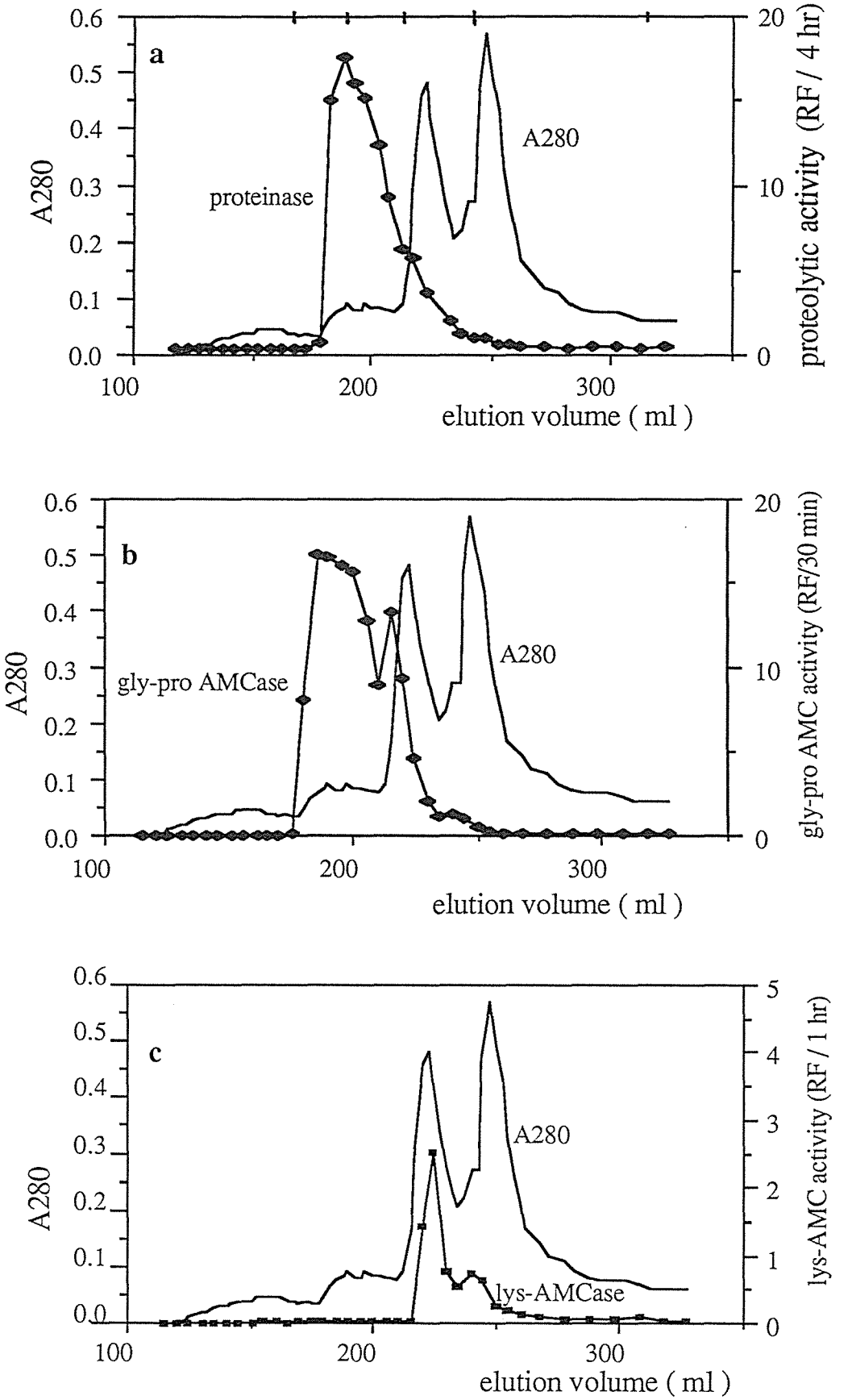
S. lactis 4760 cells harvested from one litre of ERSM culture previously grown for 14 hours at 22°C were incubated in 100 ml of 50 mM HPO_4 / H_2PO_4 buffer, pH 6.4, for 3 hours at 30° C. The cell suspension (24 mg/ml cell dry weight) was centrifuged and the supernatant equilibrated with 10 mM Tris/HCl pH 8.0 buffer by diafiltration as described in the text. The diafiltered sample was purified on the DEAE-cellulose column (12 cm X 3.3 cm) at 6° C, eluting with a linear gradient of 0- 0.6M NaCl in 10 mM Tris/HCl buffer, pH 8.0.and a flow rate of 1 $mlmin^{-1}$.

The A_{280} and conductivity of the fractions were measured. Proteinase activity was detected by incubating 100 µl of eluted fractions with 30 µl of FITC-β casein and 30 µl of 500 mM NaOAc / H_2PO_4 buffer, pH 6.4 for 4 hours. 150 µl of 5% (w/v) TCA was added and after 30 minutes, the RF value of 150 µl of TCA soluble products was determined.

FIGURE 3.2.2 ANION EXCHANGE CHROMATOGRAPHY OF CRUDE PROTEINASE RELEASED FROM *S. LACTIS* 4760

Washed cells harvested from 2.5 litres of ERSM- grown culture were incubated in 50 mM HPO_4/ H_2PO_4 buffer at 30° C for three hours. The cell suspension was centrifuged and the supernatant containing crude proteinase re-equilibrated in 10 mM Tris/HCl buffer, pH 8.0 by filtering over a PM-30 membrane in an Amicon Diaflo Cell. The diafiltered proteinase was purified by ion- exchange on DEAE-23 cellulose at 6° C, eluting at a flow rate of 1 ml min^{-1} with stepwise elution using 0.1 M, 0.2 M, 0.3 M NaCl followed by a linear gradient of 0.6 M to 1.0 M NaCl in 10 mM Tris/HCl buffer, pH 8.0.

The presence of protein in the eluted fractions was monitored by measuring A_{280}. Proteinase activity (**a**) was detected by assaying with FITC- β casein as described in the legend to Figure 3.2.1. The fractions were also assayed for the presence of dipeptidyl- peptidase activity with gly- pro-AMC (**b**) and amino- peptidase activity with lys-AMC (**c**) as outlined in section 2.2.9.


a
A280
proteinase
A280
proteolytic activity (RF / 4 hr)
elution volume (ml)
b
A280
gly-pro AMCase
A280
gly-pro AMC activity (RF/30 min)
elution volume (ml)
c
A280
A280
lys-AMCase
lys-AMC activity (RF / 1 hr)
elution volume (ml)

3.2.2 GEL PERMEATION CHROMATOGRAPHY ON SEPHACRYL S-300

Pooled fractions containing proteinase and dipeptidyl- peptidase activity eluted from the ion-exchange column (Figure 3.2.2) were concentrated by ultrafiltration and loaded onto a 3 cm x 75 cm column of Sephacryl S-300 equilibrated with 50 mM NaOAc / H_2PO_4 buffer at pH 6.4. The protein content of the fractions was too dilute to be detected either by A_{280} or by the Folin assay. The fractions were assayed for both proteinase activity and gly-pro-AMC dipeptidyl peptidase activity (Figure 3.2.3).

As shown in Figure 3.2.3, proteinase activity was eluted at about 200 ml elution volume. This elution volume was obtained consistently in several other purifications. The gly-pro-AMCase activity was eluted in a slightly larger elution volume but before all the proteinase activity had been eluted. Fractions from the first half of the elution peak were pooled separately (Peak A) from those containing most of the dipeptidyl peptidase activity (Peak B).

The percentage yield of the proteinase and the relative increase in specific activity towards FITC- β casein of the released enzyme was calculated at different stages of the purification (Table IV). In general, the purification method used above gave a low yield of proteinase activity. Only ~ 20% of the activity present in the crude cell wall extract was recovered in the proteinase peak after gel filtration (i.e. the sum of the recoveries in the two partial peaks). The increase in specific activity of the enzyme after gel filtration was difficult to calculate because the protein concentration of the partially purified enzyme was too low to be accurately determined by the Folin method even after concentrating the sample 10 fold. The 100 fold increase in specific activity shown in Table IV is subject to considerable error because of the difficulty in measuring the protein concentration accurately.

Samples from each step of the purification were examined by discontinuous electrophoresis under non- denaturing (native) conditions in 8% polyacrylamide slab gels (Figure 3.2.4). As the protein concentration of the samples was very low it was necessary to concentrate an aliquot from each of the samples by freeze-drying and then reconstitute to a volume of 50 µl with 10 mM Tris / HCl containing 2 mM EDTA so that the amount of protein was sufficient to be visualised by staining with Coomassie Blue-R. Since there was no convenient or rapid method available to detect proteinase activity on polyacrylamide gels by

FIGURE 3.2.3 GEL PERMEATION CHROMATOGRAPHY OF ION-EXCHANGE PURIFIED PROTEINASE

Pooled fractions containing proteinase activity from the ion- exchange chromatography of crude proteinase (Figure 3.2.2) were fractionated by gel filtration through an S- 300 column (75 x 3 cm) eluting with 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4 at 6° C. The column was eluted at a flow rate of 0.5 ml min^{-1} and 2.5 ml fractions were collected. The protein content of the fractions was too dilute to be detected either by A_{280} or the Folin method of protein determination. The fractions were assayed for the presence of proteinase as well as dipeptidyl- peptidase activity (gly- pro- AMC peptidase). Fractions containing proteinase activity were pooled into two separate subpools, Peaks A and B (as indicated by the vertical line) so that Peak B contained most of the dipeptidyl-peptidase activity.

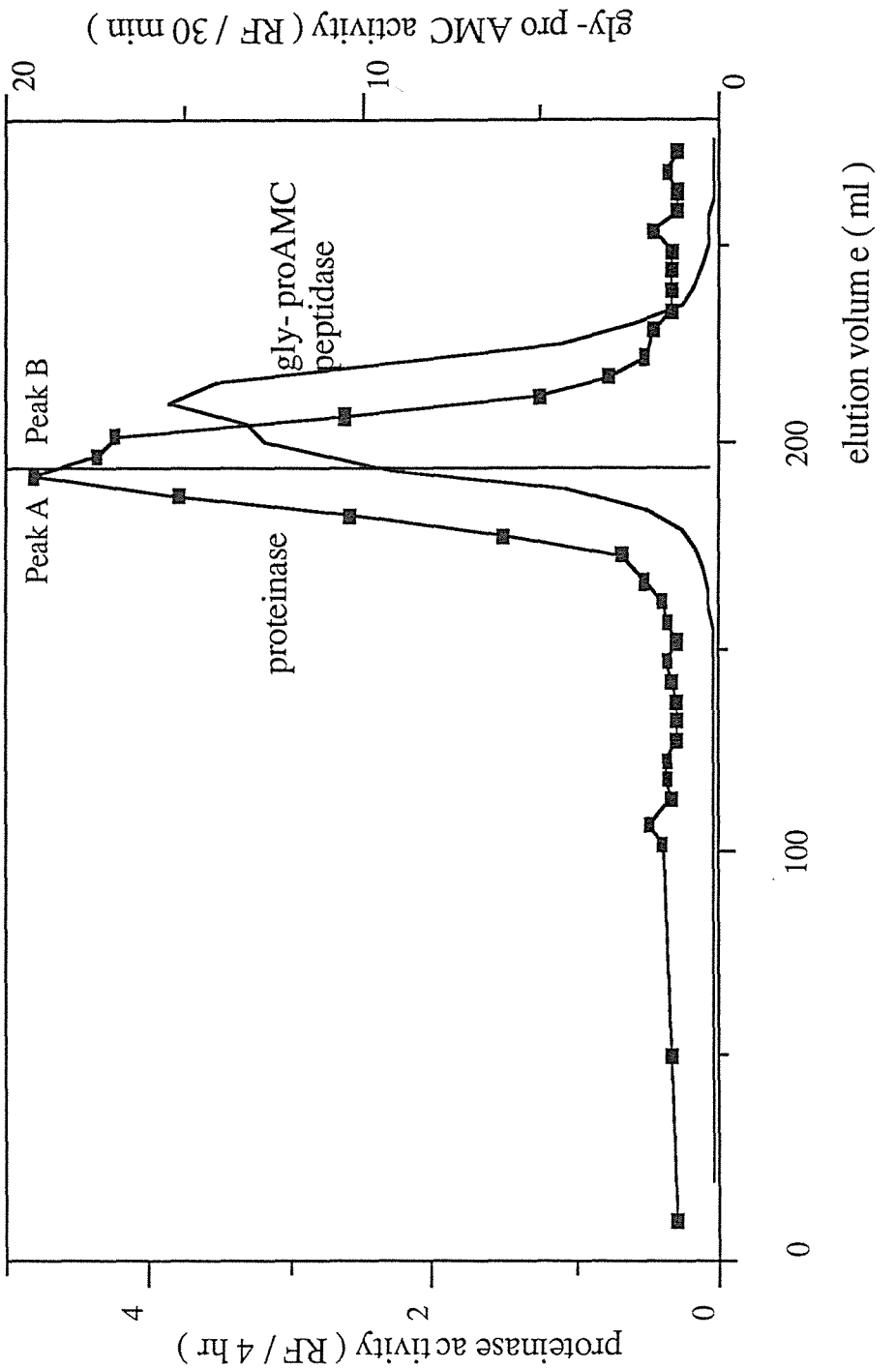

proteinase activity (RF / 4 hr)
gly- pro AMC activity (RF / 30 min)
elution volume (ml)
Peak A
Peak B
proteinase
gly- proAMC
peptidase
0
2
4
0
10
20
0
100
200

TABLE IV RECOVERY OF ACTIVITY DURING PURIFICATION OF PROTEINASE FROM MILK- GROWN *S. LACTIS* 4760

Samples retained during each step of the purification described in Figure 3.2.2 and 3.2.3 were assayed for proteinase activity with FITC-β casein and protein concentration determined with the Folin method. "Fold Purification" refers to the increase in the specific activity of the purified samples relative to unpurified enzyme. "% Yield" refers to the recovery of total proteinase activity in the purified samples relative to the unpurified enzyme. The protein concentration of the enzyme after S- 300 gel filtration was too low to be determined accurately by the Folin method even after a ten fold concentration of the sample and therefore the specific activity and fold purification could not be calculated.

TABLE IV

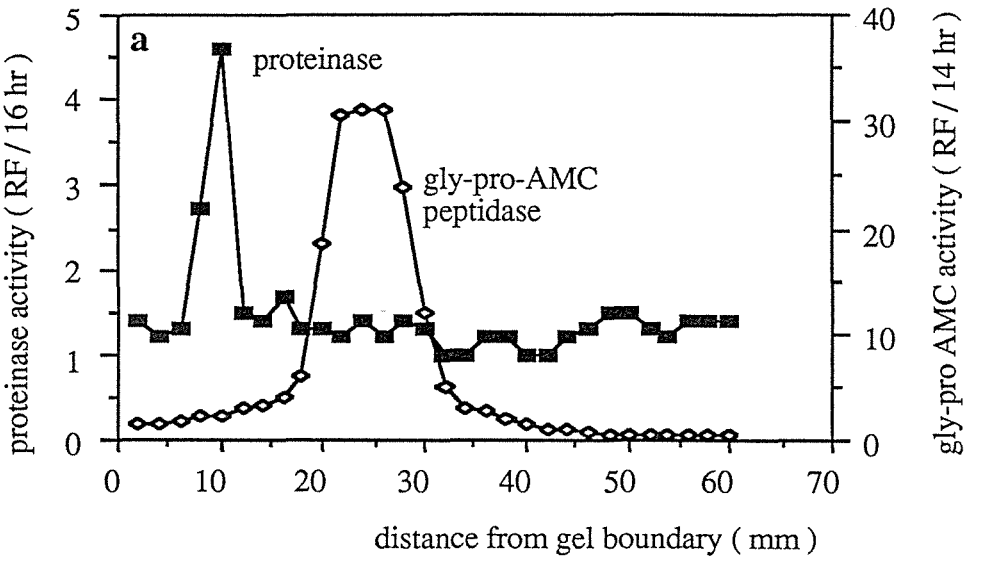
SAMPLE	TOTAL VOLUME ml	PROTEIN mg / ml	TOTAL PROTEIN mg	ACTIVITY ΔRF / hr / ml	SPECIFIC ACTIVITY ΔRF / hr / mg	TOTAL ACTIVITY ΔRF / hr	FOLD PURIFI-CATION	%YIELD
CRUDE PROTEINASE	125	0.6	75	11.2	18.6	1398.6	1	100
DIAFILTERED	55	0.6	33	11.2	18.6	615	1	46
DEAE-23 PURIFIED	25	0.09	2.15	12.4	164	309	7.7	25
S-300 G.P.C. PEAK A	17.5	0.003 ?	0.05 ?	7.1	2474 ?	123.7	132.6 ?	13
S-300 G.P.C. PEAK B	20	0.01 ?	0.192 ?	5.5	572.4 ?	110	31 ?	12

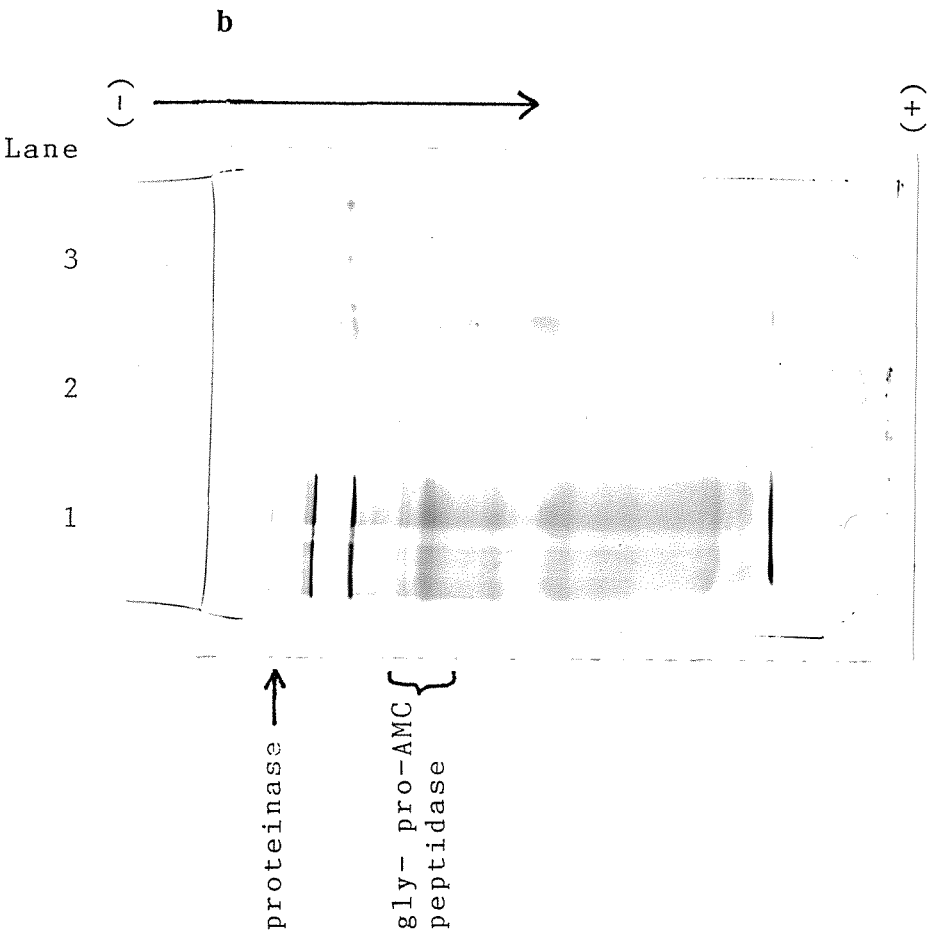
FIGURE 3.2.4 DISCONTINUOUS ELECTROPHORESIS OF PARTIALLY PURIFIED PROTEINASE FROM *S. LACTIS* 4760 ON 8% POLYACRYLAMIDE GEL

Duplicate samples of crude and partially purified proteinase were freeze dried and resuspended in 50 µl of 10 mM Tris/HCl buffer, pH 8.0 containing 2 mM EDTA. 1 µl of bromophenol blue solution containing 50% (v/v) glycerol was added to each of the samples which were subsequently electrophoresed according to the methods described in section 2.2.12. The gels were run at 10 mA per gel until the dye- front was below the stacking/ running gel boundary and then 20 mA until the dye- front was about 2 cm from the bottom of the running gel.

One gel was fractionated into 2 mm slices and then assayed for proteinase and dipeptidyl- peptidase activity. The proteinase and dipeptidyl-peptidase activities in an electrophoresed sample of crude cell wall extract is shown in **(a)**. Proteinase assay involved incubating each gel slice for 16 hours at 22° C with 50 µl of FITC-β casein and 50 µl of 500 mM NaOAc / H_2PO_4 buffer, pH 6.4. The reaction was stopped by adding 200 µl of 5% (w/v) TCA (after removal of the gel slices) and then, after 30 minutes, the precipitate was pelleted by centrifugation in a microcentrifuge. 200 µl of TCA- soluble material was transferred into 3 ml of 500 mM Tris/HCl buffer, pH 8.5 and RF values measured. Peptidase activity was assayed by incubating the gel slices for 14 hours at 22° C with 100 µl of gly- pro-AMC and 400 µl of 100 mM MES buffer, pH 6.8. RF values (Excitation λ= 385 and Emission λ= 460) were measured after diluting the reaction mixture with 2.5 ml of distilled water. The proteinase activities of the purified samples were too low for detection.

The other gel was stained for protein with Coomassie Blue **(b)**. Lane 1, crude cell wall proteinase (752 µg protein); Lane 2, DEAE-23 ion- exchange purified proteinase (197 µg) and Lane 3, S- 300 purified proteinase Peak A and B (approximately 17 µg, see Table IV).


a
proteinase
gly-pro-AMC
peptidase
proteinase activity (RF / 16 hr)
gly-pro AMC activity (RF / 14 hr)
distance from gel boundary (mm)
0
10
20
30
40
50
60
70
1
2
3
4
5


b
(-)
(+)
Lane
3
2
1
proteinase
gly- pro-AMC
peptidase

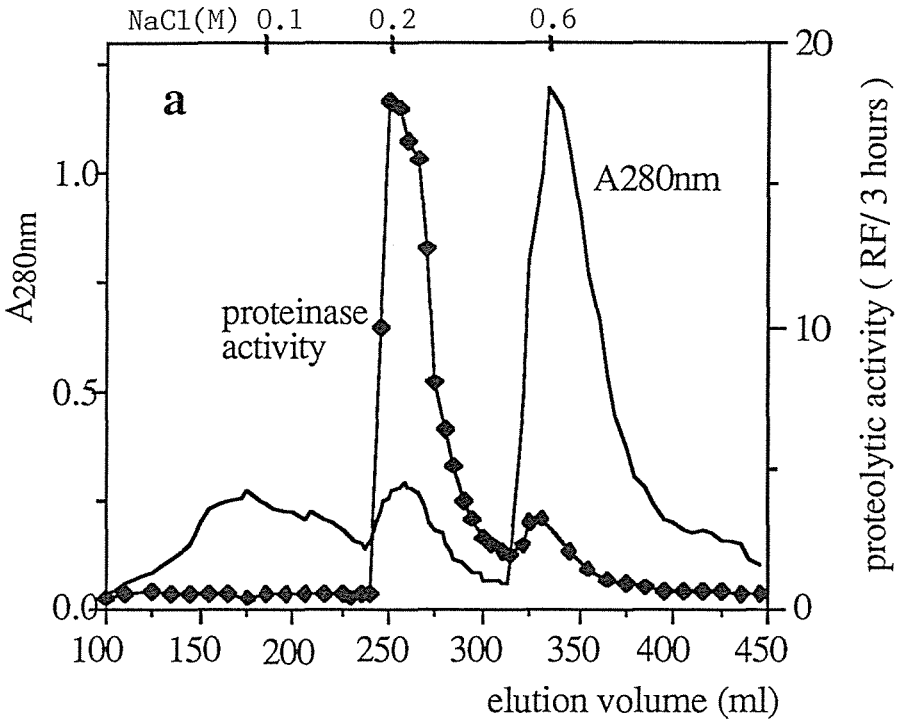
activity staining, proteinase activity was located by slicing 2 mm sections along the length of the gel from the boundary between the stacking gel and running gel and then assaying each section individually for proteinase activity using FITC β-casein. Equivalent slices from a duplicate gel were also assayed for gly-pro-AMC dipeptidyl peptidase activity. The level of activity present in the purified samples of the proteinase even after concentration was insufficient for detection following polyacrylamide gel electrophoresis. Only in the crude cell wall extract was activity detectable.

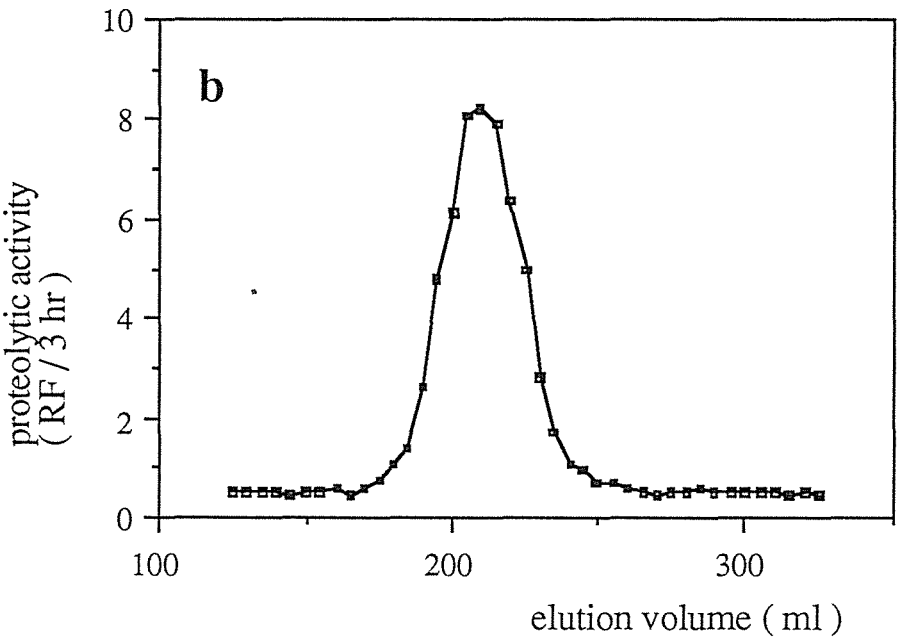
A plot of the proteinase and peptidase activities against the distance of the gel slices from the stacking gel / running gel boundary is shown in Figure 3.2.4a. The relative distance of the peak of proteinase or peptidase activity from the stacking / running gel boundary to the distance of the bromophenol blue dye- front was measured and the equivalent position of Coomassie Blue-R stained protein band (Figure 3.2.4b) was taken as an indication of the position of the enzyme. From Figure 3.2.4a and 4b, a single zone of activity running near the origin was found and this was well separated from the dipeptidyl peptidase band which ran further from the origin.

The purification method and subsequent polyacrylamide gel analysis described above was repeated and very similar results were obtained. The activity profiles on both ion-exchange and gel permeation chromatography were very reproducible (Figure 3.2.5a and 5b) and again there was insufficient activity in the partially purified enzyme for detection following PAGE analysis.

FIGURE 3.2.5 PURIFICATION OF PROTEINASE RELEASED FROM 4 LITRE- MILK CULTURE OF *S. LACTIS* 4760

Washed cells of *S. lactis* harvested from 4 litres of ERSM- grown culture were incubated for 3 hours at 30° C and the released proteinase purified by ion- exchange on DEAE- 23 cellulose (**a**) followed by gel filtration (**b**) as previously described in Figure 3.2.2 and 3.2.3 above except that the enzyme was eluted from the ion- exchange column in a step- wise sequence using 0.1M, 0.2M, 0.6M and 1M NaCl. A_{280} and proteinase activity of the eluted fractions are shown. The A_{280} elution profile for the gel filtration is not shown because the protein concentration of the fractions was too dilute.


NaCl(M) 0.1
0.2
0.6
a
A280nm
proteinase activity
A280nm
1.0
0.5
0.0
20
10
0
100 150 200 250 300 350 400 450
elution volume (ml)
proteolytic activity (RF/ 3 hours)


b
proteolytic activity (RF / 3 hr)
10
8
6
4
2
0
100
200
300
elution volume (ml)

3.3 ACTIVITY OF PARTIALLY PURIFIED PROTEINASE FROM *S. LACTIS* 4760 TOWARDS BOVINE MILK PROTEINS.

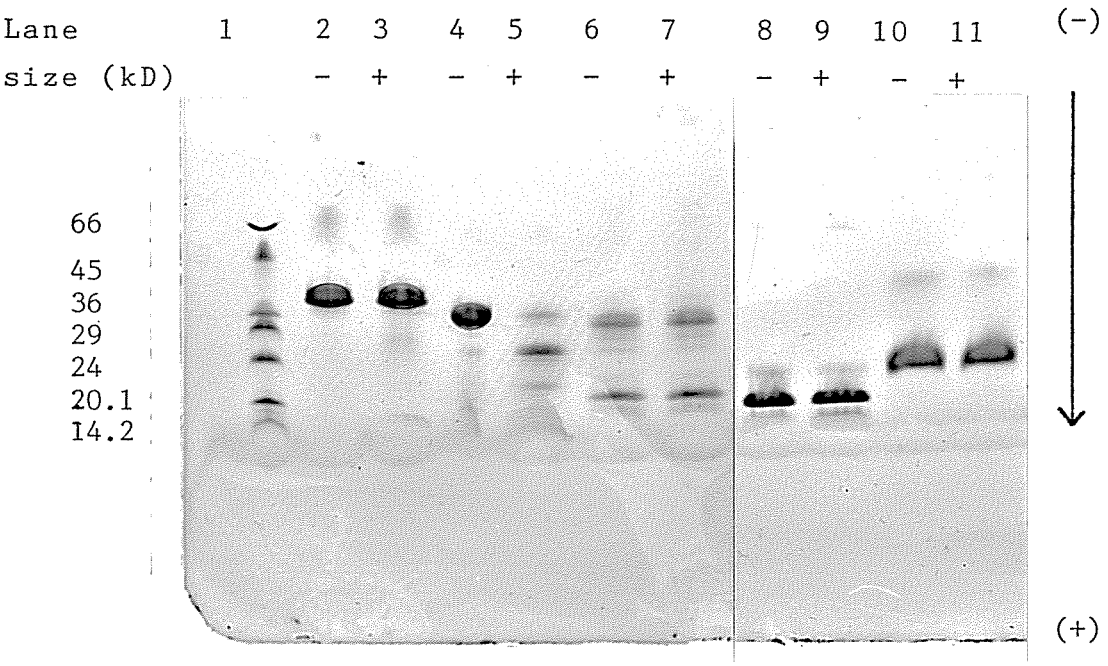
As described in the Introduction (section 1.3), the cell wall proteinases from various strains of *S. lactis* and *S. cremoris* have been classified into two types, namely the HP- and the AM1- types, distinguished by their different specificities towards α_{s1}-, β- and κ- casein. Proteinases belonging to the HP- type were active only towards β-casein whereas the AM1- type hydrolyses both α_{s1}- and β- casein. Both types of proteinase are also able to hydrolyse κ- casein slowly (Visser *et al.*, 1986).

The aim of the experiments described in this section was to identify the type of proteinase which has been partially purified from *S. lactis* 4760 by studying its ability to cleave the different milk caseins, α_{s1}-, β- and κ- caseins as well as the whey proteins, α- lactalbumin and β- lactoglobulin. Partially purified enzyme was incubated for twenty four hours at 24° C with each of the substrates as described in the legend to Figure 3.3.1. A control was prepared for each of the substrates by incubating the protein with 50 mM NaOAc / H_2PO_4 buffer pH 6.4 without proteinase. At the end of the incubation period, the samples were electrophoresed in pre- prepared 8- 25% gradient polyacrylamide gel in a Phast ™ electrophoresis system and the gel subsequently stained with Coomassie blue.

The electrophoretic patterns generated by the action of the enzyme activity on the various milk proteins are shown in Figure 3.3.1. These patterns have been consistently obtained on several occasions. The results showed that of the various milk caseins tested, only β-casein was hydrolysed by the partially purified enzyme of *S. lactis* H1 strain 4760. The inability of the enzyme to hydrolyse α_{s1}- casein is characteristic of the HP type of proteinase suggesting that the 4760 enzyme may be of that type. The heterogeneity of the κ- casein in the undigested control is probably due to variations in the carbohydrate and / or phosphate content (Vreeman *et al.*, 1977). No hydrolysis products were evident when the electrophoretic pattern of κ- casein incubated with the proteinase was compared to that of protein incubated without enzyme. The proteinase was unable to hydrolyse the whey proteins α- lactalbumin and β- lactoglobulin. (The faster migrating of the two protein bands from the β- lactoglobulin sample corresponds to the β- lactoglobulin monomer.)

FIGURE 3.3.1 SDS- PAGE PATTERN OF CLEAVAGE OF BOVINE MILK PROTEINS INCUBATED WITH *S. LACTIS* 4760 PROTEINASE

20 µl of partially purified proteinase was incubated with 20 µl of each of the various milk proteins (10 mg ml^{-1}) at 24° C for 24 hours. Incubations of the milk proteins in 50 mM NaOAc/ H_2PO_4 buffer, pH 6.4 without enzyme were also prepared. The reaction was stopped by adding 20 µl 10% SDS and 5 µl β- mercaptoethanol. 40 µl of 10 mM Tris/ HCl buffer containing 2 mM EDTA was added and the samples heated at 100° C for five minutes. 0.5 µl of bromophenol- blue was added and 1 µl aliquots of the samples were electrophoresed in mini 8- 25% SDS- Phast™ polyacrylamide gels in an automated Phast™ Electrophoresis System. Coomassie Blue- stained protein bands are shown. (+) and (-) refers to incubations with and without enzyme respectively. Lanes 1, standard molecular weight markers; Lanes 2 and 3, α- casein; Lanes 4 and 5, β- casein; Lanes 6 and 7, κ- casein; Lanes 8 and 9,α- lactalbumin; Lanes 10 and 11, β- lactoglobulin.


Lane 1 2 3 4 5 6 7 8 9 10 11
(-)
size (kD) - + - + - + - + - +
66
45
36
29
24
20.1
14.2
(+)

3.4 SPECIFICITY OF β CASEIN CLEAVAGE BY THE PARTIALLY PURIFIED PROTEINASE

At the time of commencement of the present study, a recently published paper (Monnet *et al.*,1986) reported that the proteinase from *S. lactis* 763 catalysed cleavage of the β-casein molecule at five specific sites all located in the C- terminal 42- residue region of the 209 residue β- casein molecule. There was no obvious specificity of the enzyme for bonds adjacent to any particular amino acid or consensus sequence of amino acids (see section 1.4). A major aim of the present study was to investigate the specificity of cleavage of casein by the proteinase partially purified from *S. lactis* 4760 and to compare the findings with those obtained by Monnet *et al.* (1986).

The proteolytic products of the β- casein generated by the proteinase were separated by Reverse Phase- High Performance Liquid Chromatography (RP- HPLC) on a C-18 column and subsequently analysed for purity, amino acid composition and sequence.

3.4.1 RP- HPLC SEPARATION OF THE HYDROLYSIS PRODUCTS OF β-CASEIN

The partially purified proteinase stored in 50 mM NaOAc / H_2PO_4 buffer pH 6.5 was incubated at 22° C for 4, 16 and 24 hours with β- casein. A control was prepared by incubating the β- casein in buffer without enzyme. The reaction was stopped by adding TFA and the mixture left to stand for 3 to 16 hours. It was then centrifuged for 3 minutes at full speed in an Eppendorf Microcentrifuge to sediment the 1% TFA- insoluble material.

The 1% TFA- soluble peptides in the supernatant were separated by RP- HPLC as described in the Methods (section 2.2.13) and the legends to Figure 3.4.1. A linear gradient of 5%- 60% acetonitrile in 0.1% TFA applied over 60 minutes was used to elute the peptides at a flow rate of 1 ml min^{-1}. Typical elution profiles are shown in Figure 3.4.1a to 1e. Elution of the peptides was monitored by A_{220}. At least six peaks can be distinguished. The size of all 6 peaks was observed to increase with the duration of the hydrolysis.

The peptide profiles as shown in Figure 3.4.1a to 1e were obtained using the partially purified proteinase eluted from the Sephacryl S-300 column (see elution profile in Figure 3.2.3b). The proteolytically active fractions from this column were collected as two sub-pools since the later fractions contained a high level of the glycyl-prolyl dipeptidyl

peptidase activity (gly- pro AMCase). Separate incubations of β- casein with the two sub-pools from the S-300 column peak were carried out. When the proteinase contaminated with the gly-pro AMCase activity was incubated with β- casein for 16 hours the HPLC elution profile was identical with that obtained using the proteinase preparation with low gly-pro AMCase (compare Figure 3.4.1c and 1e). Thus the dipeptidyl peptidase does not appear to be degrading the peptide products of the proteinase action.

Peaks 5 and 6 in the elution profile in Figure 3.4.1 were not separated well enough for further analysis of the amino acid composition or sequence. The peaks were further separated when the linear gradient of 5%- 60% acetonitrile used to elute the column was applied in 90 minutes at 2 ml min^{-1} as described in the legend to Figure3.4.1f. (Previously the gradient was applied in 60 minutes at 1 ml min^{-1}).

3.4.2 ANALYSES OF β- CASEIN PEPTIDES ELUTED FROM RP- HPLC

The peaks labelled 1, 2, 3, 4 from Figure 3.4.1a- 1d and peaks 5 and 6 from Figure 3.4.1f were dried under vacuum and analysed for amino acid composition and sequence. Peaks 1, 2, 5 and 6 were also analysed by FAB- MS. The results of the analyses are summarised in Table V.

Peak 1 was a pure oligopeptide yielding a single molecular ion of 802.8 by FAB-MS and a single amino acid sequence. The amino acid composition of the peptide also confirmed the homogeneity of the peptide. By searching for the position of the peptide sequence on the known sequence of β- casein, it was shown that the peptide in Peak 1 corresponded to the sequence of residues from position 176- 182 of the β- casein molecule (Figure 3.4.3). The calculated molecular weight of the peptide in this sequence agreed exactly with that obtained by the FAB-MS spectrum shown in Figure 3.4.2.

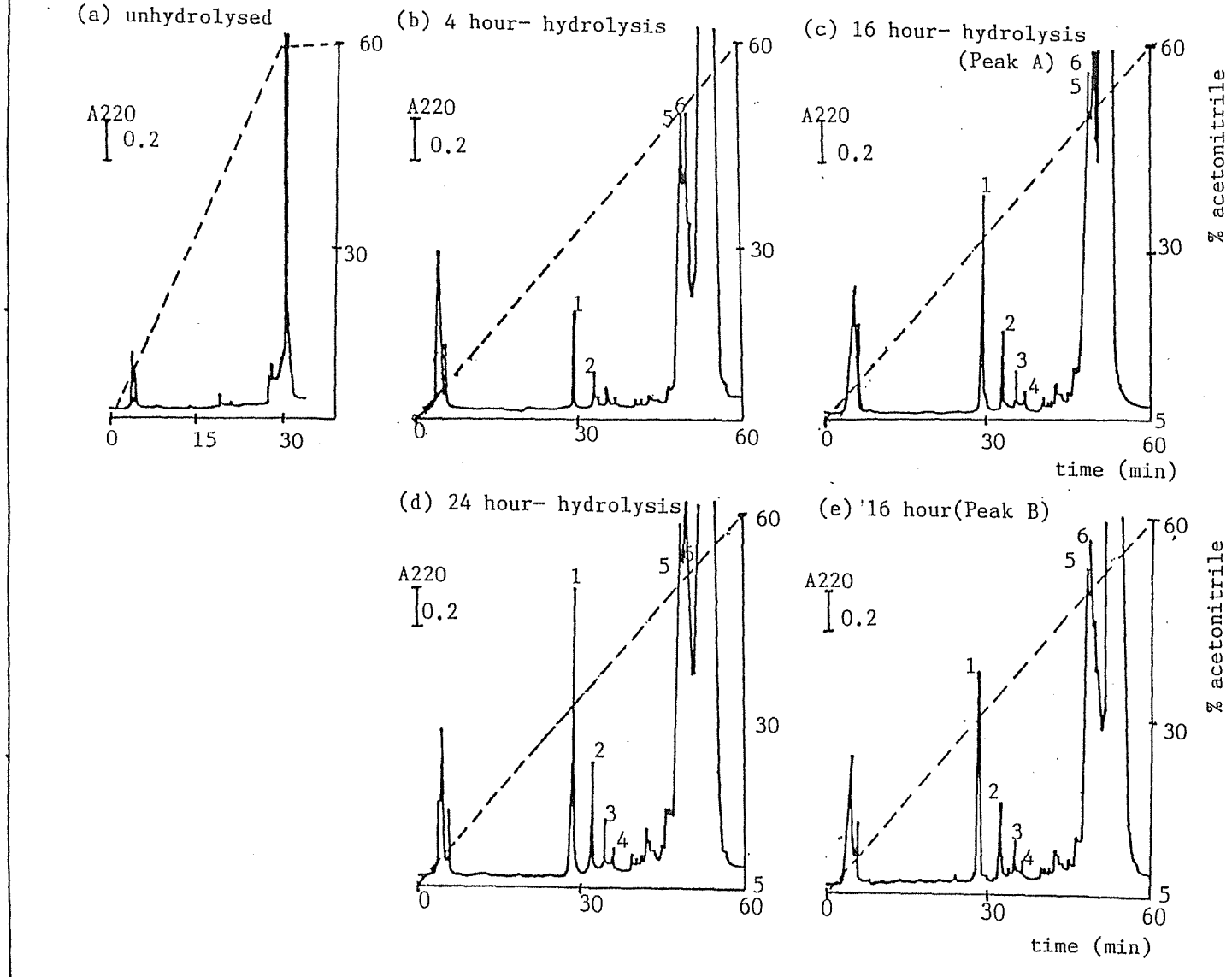
The remaining five peaks each gave a mixture of two peptide sequences. The sequence of the peptides in these peaks (shown in Table V) were deduced by comparing the relative sizes of the amino acid peaks identified from the automated sequencer. The assumption was made that the smaller of the two amino acid peaks in each cycle originated from the peptide present in smaller amounts. This assumption seemed valid since the sequence so obtained could be identified in the known sequence of the β- casein molecule. [However, it may not always correctly reflect the actual relative proportions of the peptides in the sample. For example the relative proportions of the amino acid unique to each of the two fragments in peak 5 (compare glycine with aspartic acid) deduced from the analysis of the amino acid composition of the sample suggests the predominance of the fragment 194- 209 while the

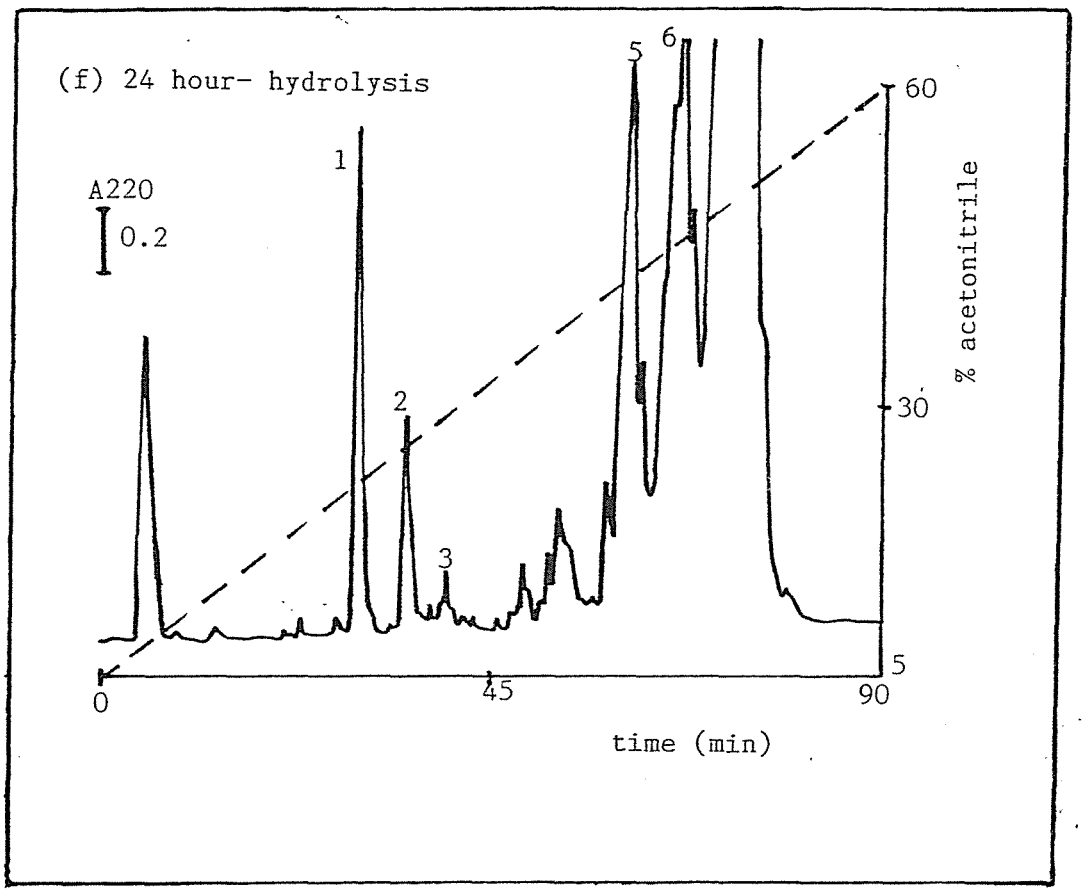
FIGURE 3.4.1 SEPARATION BY RP- HPLC OF TFA- SOLUBLE HYDROLYSATES OF β- CASEIN INCUBATED WITH PARTIALLY PURIFIED PROTEINASE FROM *S. LACTIS* 4760

400 µl of β- casein (10 mg ml^{-1}) was incubated with 200 µl of the partially purified proteinase, Peak A, from the purification described in Figure 3.2.3, at 24° C for 4 hours (**b**), 16 hours (**c**) and 24 hours (**d**). Incubation of β- casein with 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4 without enzyme was also carried out as a control (**a**). β- casein incubated with the partially purified proteinase containing the majority of the dipeptidyl- peptidase activity (gly- pro-AMCase) in Peak B from the same purification was also carried out for 16 hours (**e**). 300 µl of 3% TFA was added and after 3- 16 hours at 6° C the samples were subsequently microcentrifuged.

Samples were filtered through Acro-LC-13 membranes (0.2 µm pore diameter) and then separated on a C- 18 reverse- phase HPLC column. The column was eluted at a flow rate of 1 ml min^{-1} with a linear gradient of 5- 60% acetonitrile in 0.1% TFA solution over 30 minutes (**a**) or 60 minutes [(**b**) to (**e**)]. In (**f**), 600 µl of another batch of partially purified proteinase (Figure 3.2.5) was incubated with 600 µl of β- casein for 24 hours at 24° C and then the reaction stopped by adding 600 µl of 3% TFA. 1.5 ml of the 1% TFA soluble hydrolysate was separated on the C-18 RP- HPLC column, eluting at 2 ml min^{-1} with a linear gradient of 5- 60% acetonitrile over 90 minutes.The A_{220} elution profile of the separations are shown.

The peaks labelled 1 to 4 from figures (**d**) and (**e**), 5 and 6 from figure (**f**) were vacuum dried and then analysed by FAB- MS, amino acid composition and amino acid sequence as described in section 3.4.


(a) unhydrolysed
A220
0.2
60
30
0
15
30
(b) 4 hour- hydrolysis
A220
0.2
1
2
5
6
60
30
0
30
60
(c) 16 hour- hydrolysis
(Peak A)
A220
0.2
1
2
3
4
5
6
60
30
5
0
30
60
time (min)
% acetonitrile
(d) 24 hour- hydrolysis
A220
0.2
1
2
3
4
5
6
60
30
5
0
30
60
(e) 16 hour(Peak B)
A220
0.2
1
2
3
4
5
6
60
30
5
0
30
60
time (min)
% acetonitrile


(f) 24 hour- hydrolysis
A220
0.2
1
2
3
5
6
60
30
5
% acetonitrile
0
45
90
time (min)

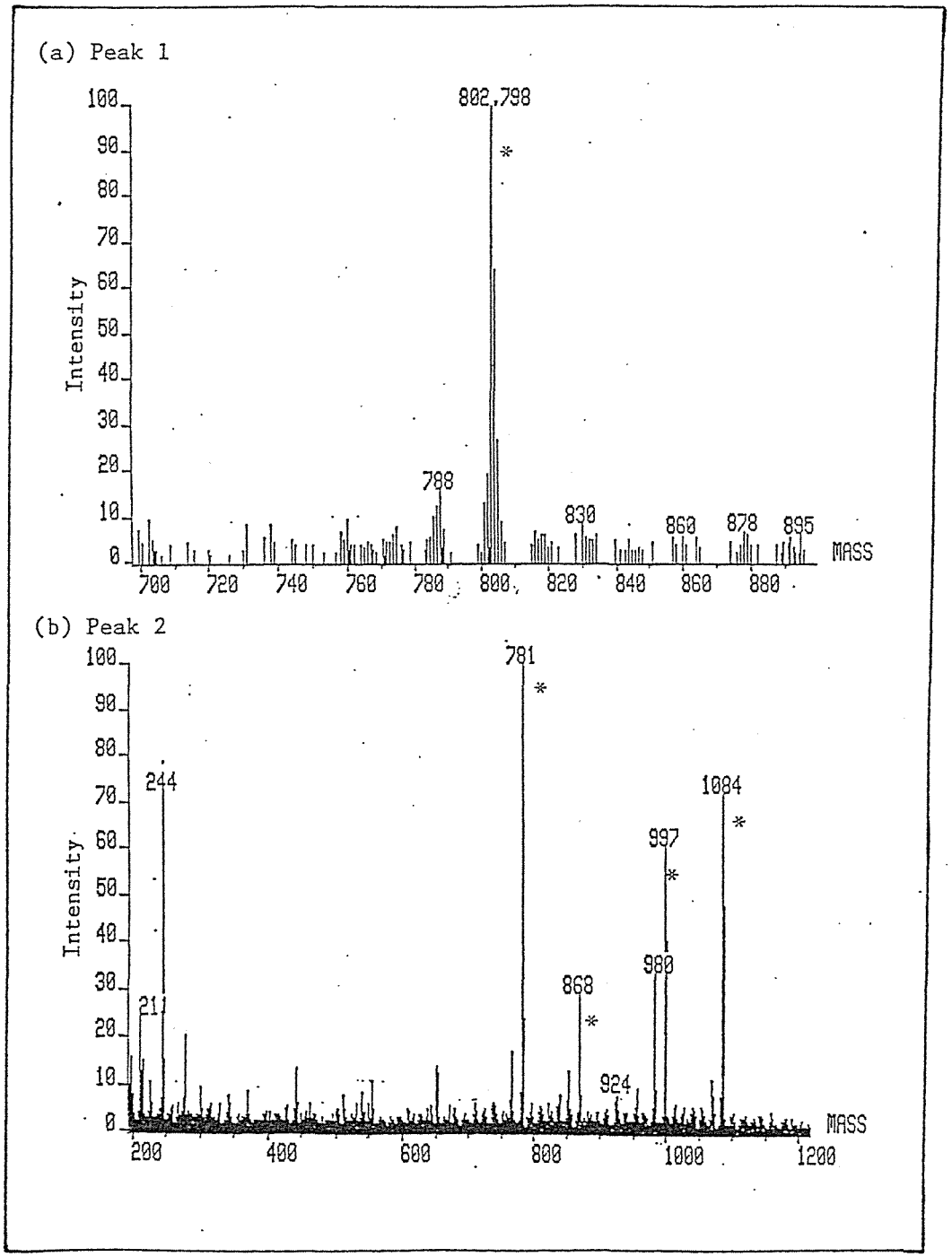


FIGURE 3.4.2 FAB- MASS SPECTRUM OF β- CASEIN PEPTIDES ELUTED FROM RP- HPLC

The FAB- mass spectrum of the peptide(s) in Peaks 1 and 2 from Figure 3.4.1 are shown in (a) and (b) respectively. The molecular ion species marked with an asterisk correspond to the calculated mass of the peptides containing the sequence of residues in the β- casein molecule shown in Table V.

TABLE V RESULTS OF THE ANALYSIS OF β- CASEIN PEPTIDES SEPARATED BY RP- HPLC

Peaks 1- 6 from Figure 3.4.1 were analysed for amino acid composition, sequence and where appropriate, FAB- MS, as outlined in section 2.2.13 and 2.2.14. The sequence of amino acids marked with an asterisk in Peaks 2, 3 and 4 represented the major peaks eluted from the automated Edman- sequencer. The FAB- MS of Peaks 3 and 4 was not carried out due to the small amount of sample collected from the RP- HPLC. No molecular ion- species was detected in the FAB- Mass spectrum of Peak 6. The amino acid sequence of this peak indicated that it came from the N- terminus of the β- casein molecule and since the analysis of its amino acid composition suggested that it may be a large N- terminal fragment of the β- casein molecule, the sequence analysis was stopped after the first six residues were identified.

TABLE V

PEAK #	AMINO ACID COMPOSITION	AMINO ACID SEQUENCE	FAB-MS MH^+ ION	POSITION IN β CASEIN
1	$Glx_1Pro_2Ala_1Val_1Tyr_1Lys_1$	$_2$HN-Lys-Ala-Val-Pro-Tyr-Pro-Gln-OH	802.8	176 - 182
2	$Ser_1Glx_{1.6}Pro_{2.3}Val_2Leu_1Lys_1$	$_2$HN-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-OH	997	167 - 175?
		$_2$HN-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-OH*	1084	166 - 175
3	$Ser_{2.5}Glx_2Val_2Leu_{1.7}Lys_1$	$_2$HN-Ser-Val-Leu-Ser-Leu-Ser-OH	not done	161 - 166
		$_2$HN-Ser-Leu-Ser-Gln-Ser-Lys-OH*		164 - 169
4	$Ser_{2.6}Glx_1Pro_{1.4}Val_2Leu_2$	$_2$HN-Ser-Val-Leu-Ser-Leu-Ser-OH*	not done	161 - 166
		$_2$HN-Ser-Leu-Ser-Gln-Ser-Lys-OH		164 - 169
5	$Asx_1Glx_4Pro_6Gly_3Ala_1Val_4Met_1Ile_3Leu_4Phe_2Arg_2$	$_2$HN-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu--Leu-Tyr-OH*	1367	183 - 193
		$_2$HN-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg--Gly-Pro-Phe-Pro-Ile-Ile-Val-OH	1717	194 - 209
6	$Asx_1Thr_1Ser_1Glx_3Pro_2Val_1Ile_1Leu_1Lys_1$	$_2$HN-Arg-Glu-Leu-Glu-Glu-Leu- stopped	not detected	N- terminal fragment?

contrary was suggested from the relative size of the peaks in the elution profile of the amino acid from the automated sequencer.]

The two peptides in Peak 5 corresponded to the sequences between residues 183- 193 and 194- 209 of the β- casein molecule. The calculated mass of each of these fragments was confirmed by the results of the mass spectrum.

Peak 2 was found to contain two overlapping sequences corresponding to the sequences between residues 167- 175 and 166- 175 of the β- casein molecule. The predicted masses of the molecular ion- species of these fragments (i.e. 997 and 1084 respectively) were detected in the mass spectrum. However, two other molecular ion- species with mass values of 781 and 868 were also present in the mass spectrum but they were not detected by the automated sequencer. These ions correspond exactly to the calculated masses of the sequence between residues 169- 175 and 168- 175 respectively of the β- casein molecule.

The two peptides present in Peaks 3 and 4 were probably derived from the same overlapping regions of the β- casein molecule corresponding to the sequences between residues 161- 166 and 164- 169. The relative proportions of these two peptides in Peaks 3 and 4 appeared to be different. The mass spectra of the two peaks were not obtained because of the small quantities of the sample. The fragment containing residues 164- 169 present in these peaks overlaps with the fragments from Peak 2 lying between residues 166- 175.

Peak 6 was not sequenced entirely. The observation that the first 6 residues sequenced correspond to the N- terminus of the β- casein molecule and the inability to detect any molecular ion from the FAB-MS (mass number beyond 2000 is not detected) suggest that it may be a large N- terminal fragment of the β- casein molecule possibly remaining after the enzyme catalysed hydrolysis. It would have been interesting to have determined the C- terminal amino- acid(s) of this sample to find out which part of the β- casein molecule the peak represents. This was not carried out however, due to time constraints.

Summarising the results obtained, it can be seen that the partially purified proteinase from *S. lactis* 4760 cleaves the β- casein molecule at sites largely consistent with those reported for the proteinase from *S. lactis* 763 (Monnet *et al.*, 1986). The proteinase cleaves at sites lying within a limited region at the C- terminal end of the β- casein. The major peptide products arise from cleavage at the previously reported sites between residues 166- 167, 175- 176, 182- 183 and 193- 194.

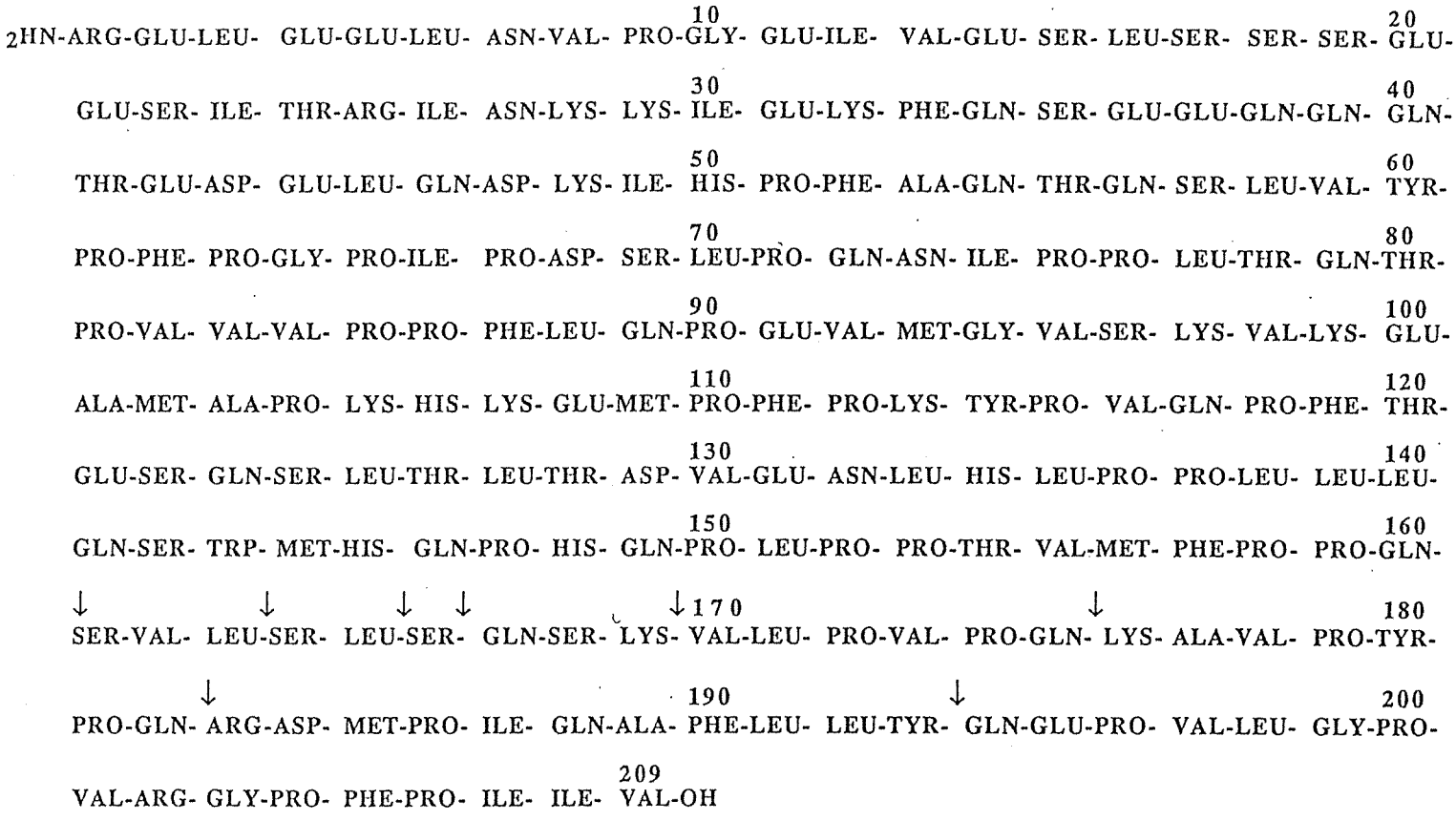


FIGURE 3.4.3 SITES IN β- CASEIN CLEAVED BY THE PARTIALLY PURIFIED PROTEINASE FROM *S. LACTIS* 4760

The sequence of peptides identified in Table V were matched with the known sequence of β- casein to define the cleavage sites indicated by the vertical arrows. Comparison of these cleavage sites with those previously reported is shown in section 4.4.

However 3 additional sites identified in the present study at which limited cleavage may occur are those between residues 160- 161, 164- 165, 165- 166 and 169- 170. The cleavage sites between residues 164- 165 and 165- 166 have also been reported in a recent poster abstract by Visser *et al.* (1987) for a partially purified proteinase from *S. cremoris* HP.

The proteinase from the *S. cremoris* HP also cleaves β- casein at the specific positions reported for *S. lactis* 763 (Monnet *et al.*, 1986). Since the proteinase from *S. cremoris* HP has been shown to exhibit the HP- type of proteinase activity (Visser *et al.*, 1986), on the basis of its inability to hydrolyse α_{s1}- casein and the similarity of its β- casein cleavage pattern on gel electrophoresis, it was suggested that the proteinase from *S. lactis* 763 studied by Monnet *et al.* (1986) could also be an HP- type. Therefore it is likely that the proteinase from the transconjugant *S. lactis* H1 strain 4760 presently studied is also an HP type proteinase based on the similar pattern of β- casein cleavage to that reported for the *S. cremoris* HP described above as well as its inactivity towards α_{s1}-casein as described in Section 3.3 above.

3.5 COMPARATIVE STUDIES ON THE PROTEINASES RELEASED FROM *S. LACTIS* 4760, *S. CREMORIS* H2 AND *S. CREMORIS* SK11

The results presented in section 3.4 suggest tht the proteinase released from the transconjugant *S. lactis* 4760 has the characteristic activities of the HP- type proteinase since it cleaves β- casein but not α_{s1}- casein. The aims of the experiments in this section were to compare the specificity of the proteinase from *S. lactis* 4760 to :(a) that of the proteinase released from *S. cremoris* , the strain from which the Prt plasmid in *S. lactis* 4760 was derived and, (b) that of the proteinase released from *S. cremoris* SK11 which has been reported to be an AM1- type of proteinase. To recapitulate, this type of proteinase is able to hydrolyse α_{s1}- and β- casein in contrast to the HP- type which is generally only active towards β- casein (Visser *et al.*, 1986). The specific sites at which the AM1- type proteinase cleaves β- casein have not been reported. Therefore, a preliminary study to define the AM1- specific β- casein cleavage sites was also carried out.

A comparison of the time dependent release and partial purification of the proteinase from both *S. cremoris* H2 and SK11 with that of the *S. lactis* 4760 enzyme, studied in the previous sections, will be described. The activity of the partially purified enzymes on α_{s1}- , β- and κ- caseins and the whey proteins α- lactalbumin and β- lactoglobulin was

investigated by discontinuous electrophoresis on SDS- and urea polyacrylamide gels. The TFA soluble- peptide products of the enzymic hydrolysis of β- casein were fractionated by RP- HPLC and then analysed by FAB- MS. Amino acid composition and amino acid sequence analysis was carried out so that the enzyme cleavage sites on the β- casein molecule could be determined.

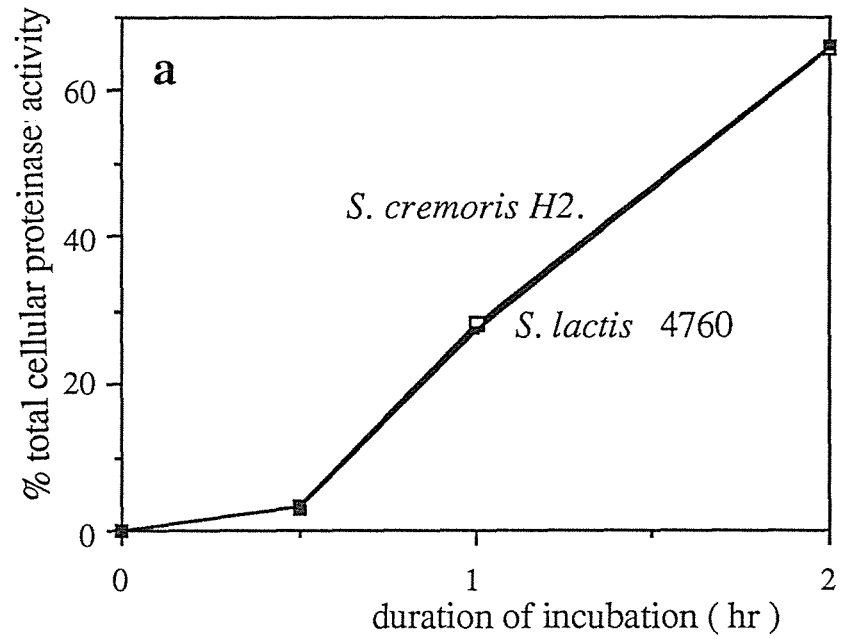
3.5.1 COMPARISON OF THE RELEASE OF PROTEINASE ACTIVITY FROM *S. LACTIS* 4760 WITH THAT FROM *S. CREMORIS* H2 AND SK11.

In a previous section (section 3.1.7) the release of proteinase from *S. cremoris* H2 was compared to the release from *S. lactis* 4760. Results from that experiment are summarised again in Figure 3.5.1a and 1b. Both cells released very similar amounts of proteinase activity into the Ca^{++} - free buffer. The *S. lactis* 4760 however, were more leaky since the cells released more LDH activity than did *S. cremoris* H2 when incubated for more than 2 hours.

Using the same procedure, the release of proteinase from *S. cremoris* SK11 and *S. lactis* 4760 was compared. The cells were harvested after growth in ERSM for 14 hours at 22° C. The cell density of the *S. lactis* 4760 culture was approximately twice that of the *S. cremoris* SK11 culture at harvest. The harvested cells were incubated at 30° C in Ca^{++} - free phosphate buffer, pH 6.4, for 0, 0.5, 1 and 2 hours. The cell suspensions were centrifuged and the supernatant assayed for proteinase and LDH activity. From Figure 3.5.2a, it can be observed that *S. cremoris* SK11 and *S. lactis* 4760 released very similar amounts of proteinase activity when the cells were incubated for up to one hour. However, when the cells were incubated for 2 hours the 4760 cells released rather more proteinase activity than did the SK11 cells (0.54 compared to 0.42 units / mg cell dry weight respectively). Likewise, the release of LDH activity of the two strains incubated for up to one hour was similar (Figure 3.5.2b), but the *S. lactis* 4760 cells released significantly more LDH activity than did the *S. cremoris* SK11 when the cells were incubated for 2 hours (cf. 0.022 and 0.0071 units/ mg cell dry weight, which represent 1.4% and 0.31% of the total cellular LDH activity for *S. lactis* 4760 and *S. cremoris* SK11 respectively). These results indicate that the proteinase released from both *S. cremoris* H2 and SK11 may be less contaminated with intracellular enzymes than the proteinase released from *S. lactis* 4760.

FIGURE 3.5.1 COMPARISON OF THE RELEASE OF PROTEINASE FROM *S. CREMORIS* H2 ANDS. *LACTIS* 4760

150 ml ERSM cultures (1% inoculum) of *S. lactis* 4760 and of *S. cremoris* H2 were grown for 14 hours at 22° C. The cells were harvested and then resuspended in 8 ml 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4. The cell dry weight in the washed cell suspension of *S. lactis* 4760 was 26.5 mg/ml while that for *S. cremoris* H2 was 29 mg/ml. The cells were incubated at 30° C and samples removed after 0, 0.5, 1 and 2 hours of incubation were centrifuged and the supernatant assayed for proteinase and LDH activity. The cells pelleted from the centrifugation were disrupted in a French Press and then centrifuged and the supernatant assayed for proteinase and LDH activity. The activity released into buffer over the duration of incubation is shown as a percentage of the total cellular proteinase activity in (a) and total cellular LDH activity in (b) where total cellular activity is taken as the sum of the activity released into buffer and that in the disrupted cell supernatant.


a
% total cellular proteinase activity
60
40
20
0
S. cremoris H2.
S. lactis 4760
0
1
2
duration of incubation (hr)

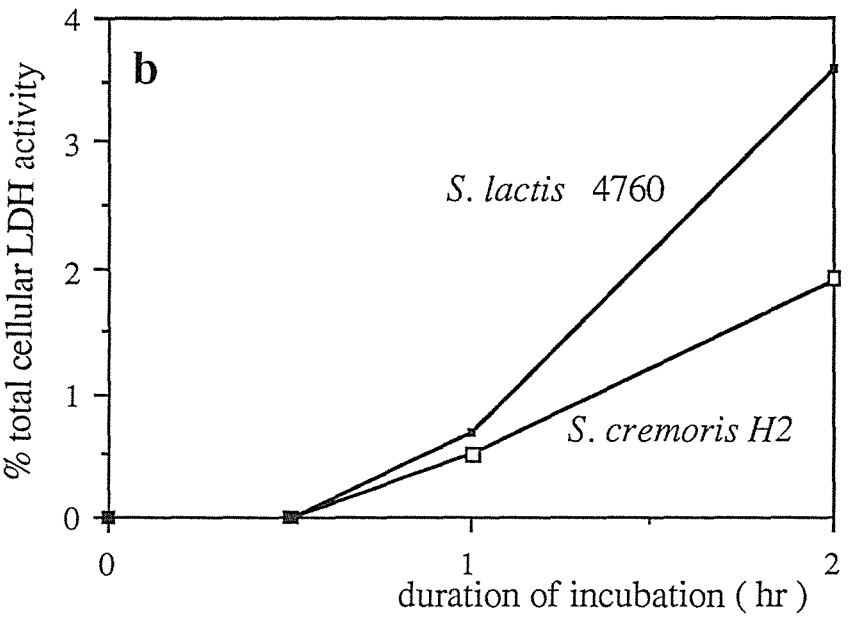
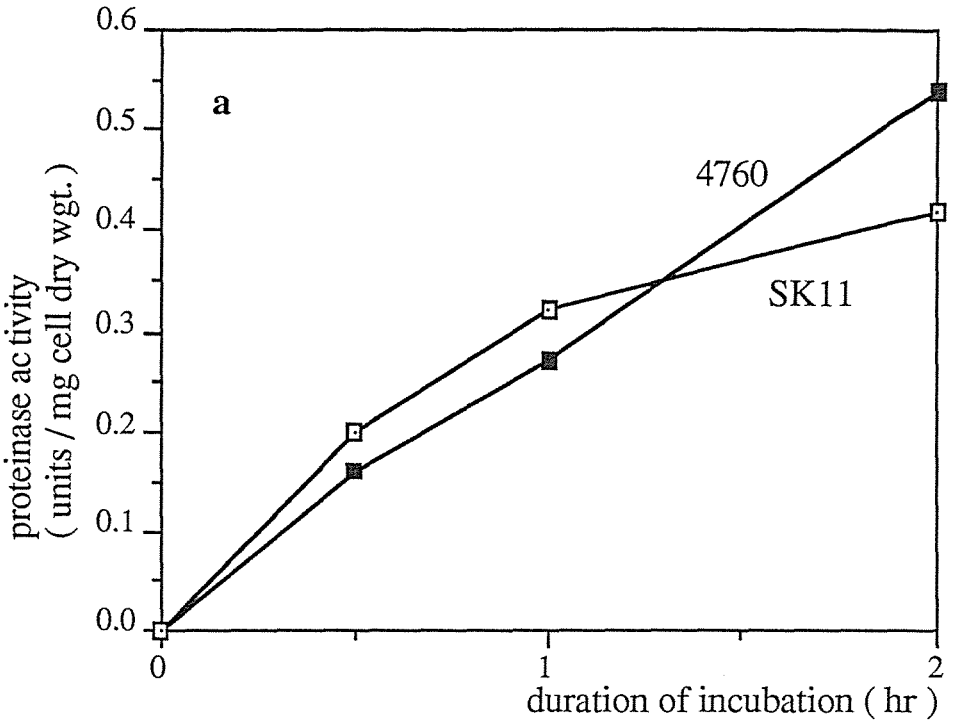
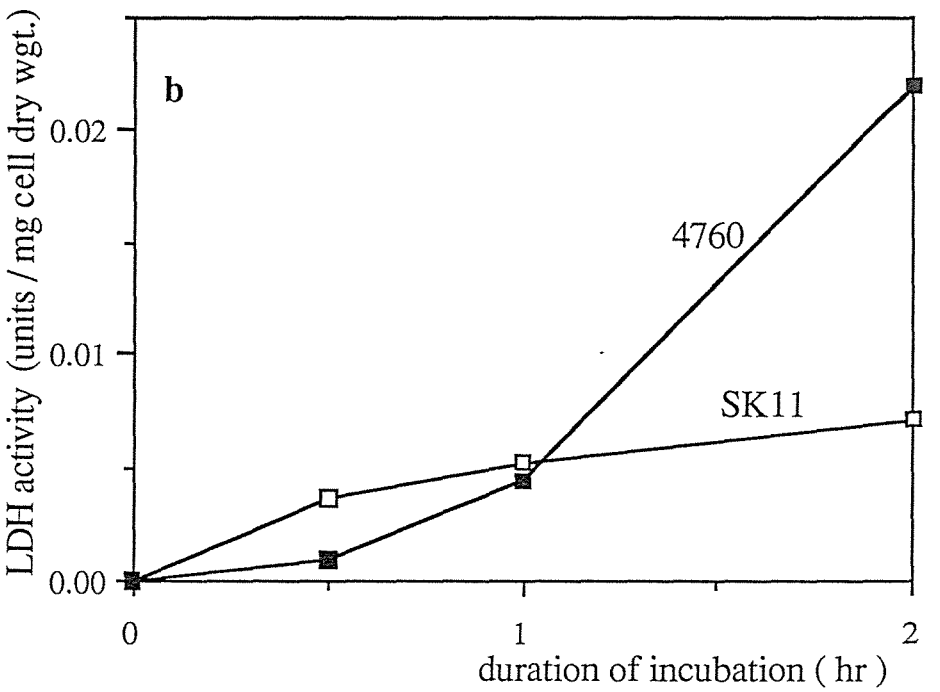

b
% total cellular LDH activity
4
3
2
1
0
S. lactis 4760
S. cremoris H2
0
1
2
duration of incubation (hr)

FIGURE 3.5.2 COMPARISON OF THE PROTEINASE RELEASE FROM *S. CREMORIS* SK11 AND *S. LACTIS* 4760.

150 ml cultures of *S. cremoris* SK11 and *S. lactis* 4760 were grown in ERSM for 14 hours at 22° C. The cells were harvested and then incubated in 8 ml of 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4. The SK11 cell suspension contained about 13.5 mg/ml by cell dry weight while that containing the 4760 cells was 25 $mgml^{-1}$ cell dry weight. Samples removed after 0, 0.5, 1 and 2 hours of incubation were centrifuged and the supernatant assayed for proteinase (**a**) and LDH activity (**b**).


a
4760
SK11
proteinase activity
(units / mg cell dry wgt.)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
duration of incubation (hr)


b
4760
SK11
LDH activity (units / mg cell dry wgt.)
0.02
0.01
0.00
0
1
2
duration of incubation (hr)

3.5.2 PARTIAL PURIFICATION OF PROTEINASE RELEASED FROM *S. CREMORIS* H2.

Proteinase released into Ca^{++} free- buffer after a 3 hour incubation of *S. cremoris* H2 cells, harvested from a 14 hour culture grown in 2 l of ERSM, was purified by the methods of ion- exchange and gel filtration described section 3.2.

The proteinase was purified by anion- exchange on a DE- 23 cellulose column, eluting with a linear gradient of 0- 0.4 M NaCl followed by a steeper gradient of 0.4- 1.0 M NaCl in 10 mM Tris/ HCl buffer, pH 8.0. The A_{280} and proteinase activity profiles of eluted fractions are shown in Figure 3.5.3a. A single peak of proteinase activity was eluted at 0.2 M NaCl. The proteinase from *S. lactis* 4760 was also eluted from the same column at 0.2 M NaCl (section 3.2.1).

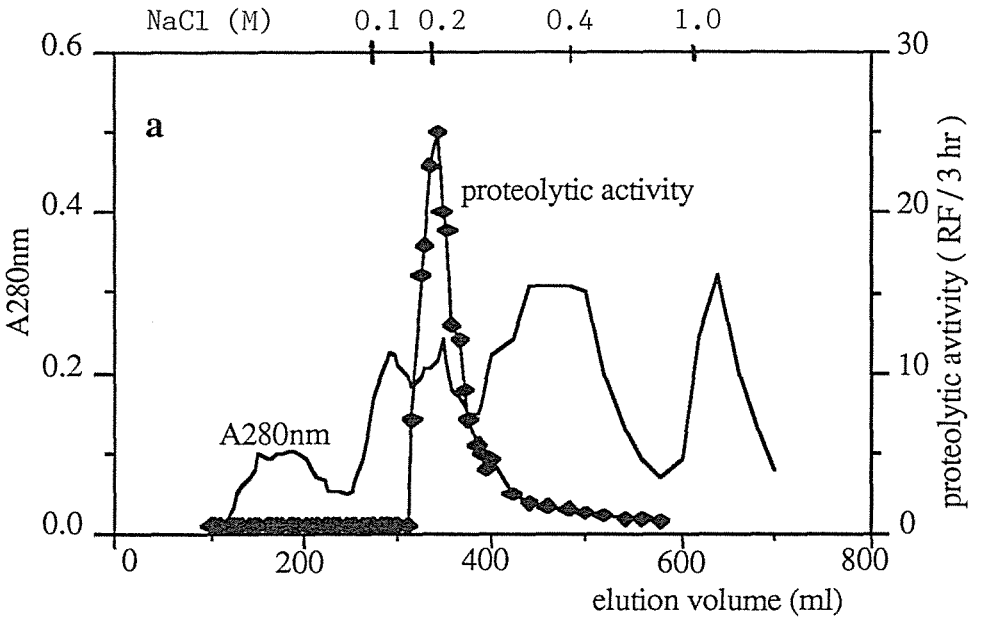
When the pooled fractions containing the proteinase activity were fractionated on Sephacryl S- 300 gel filtration column, only a single peak of proteinase activity was eluted (Figure 3.5.3b). The protein concentration of the fractions was too dilute to be detected by A_{280}. The volume of buffer required for elution of the proteinase from the S- 300 column (225 ml) was somewhat greater than that required for elution of the proteinase from *S. lactis* 4760 (200 ml). During the gel filtration of the *S. cremoris* H2 proteinase, the temperature of the cold room (normally 6° C) rose to 25° C due to plant failure. It is possible that the higher temperature of the purification may have led to a limited autoproteolysis of the proteinase yielding a lower molecular weight form which still retained proteolytic activity. The purification of the proteinase was not repeated, however, due to the shortage of time.

The results of the purification are summarised in Table VI. The 30% yield and 7 fold increase in specific activity of the partially purified enzyme after ion- exchange is very similar to the values obtained after the ion- exchange purification of the enzyme from *S. lactis* 4760 (25% and 7.7 fold respectively, see section 3.2). Since the protein concentration of the S- 300 purified enzyme was too dilute to be accurately determined by the Folin method it was not possible to estimate the increase in specific activity of the partially purified enzyme.

FIGURE 3.5.3a and 3b PARTIAL PURIFICATION OF PROTEINASE RELEASED FROM *S. CREMORIS* H2

Proteinase released from a 3- hour incubation of cells of *S. cremoris* H2 harvested from 2 litres of ERSM- culture grown at 22° C for 15 hours was purified by the method of ion- exchange chromatography on DEAE-23 cellulose (**a**) as described for the *S. lactis* 4760 enzyme in section 3.2. The proteinase was eluted from the ion- exchange column with a linear gradient of 0- 0.4 M NaCl followed by a steeper gradient of 0.4 M- 1.0 M NaCl in 10 mM Tris/HCl buffer, pH 8.0. A_{280} and proteinase activity of eluted fractions are shown.

The pooled fractions containing proteinase activity eluted from the ion- exchange column were purified by gel filtration on Sephacryl S- 300 (**b**), eluting with 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4. Only the proteinase activity of the fractions are shown as the protein concentration was too dilute to be detected by A_{280}.


NaCl (M)
0.1 0.2
0.4
1.0
a
proteolytic activity
A280nm
A280nm
proteolytic avtivity (RF / 3 hr)
elution volume (ml)
0.6
0.4
0.2
0.0
30
20
10
0
0
200
400
600
800

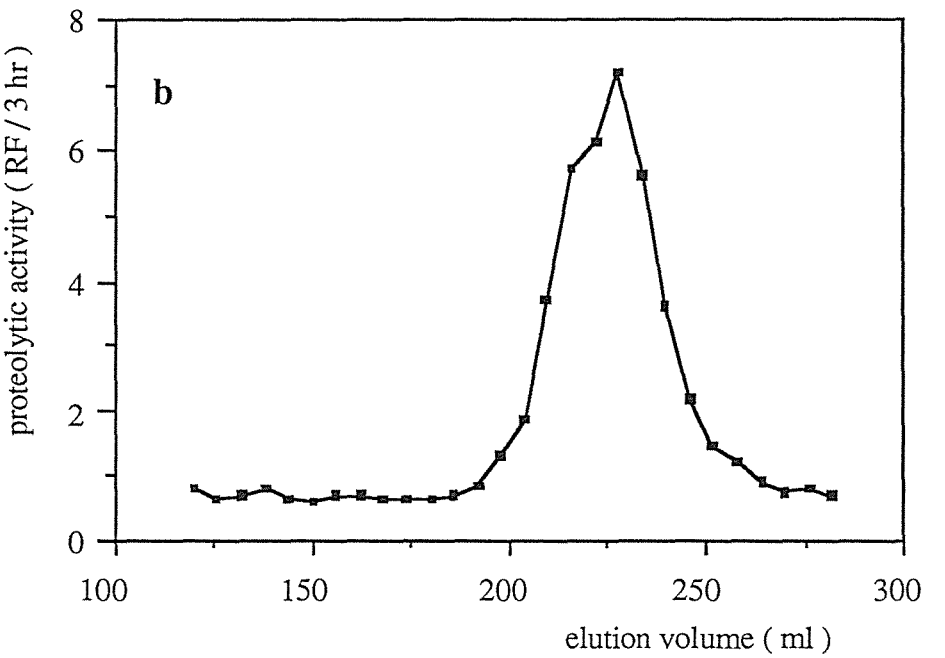

b
proteolytic activity (RF / 3 hr)
elution volume (ml)
8
6
4
2
0
100
150
200
250
300

TABLE VI RECOVERY OF ACTIVITY DURING PURIFICATION OF PROTEINASE FROM *S. CREMORIS* H2

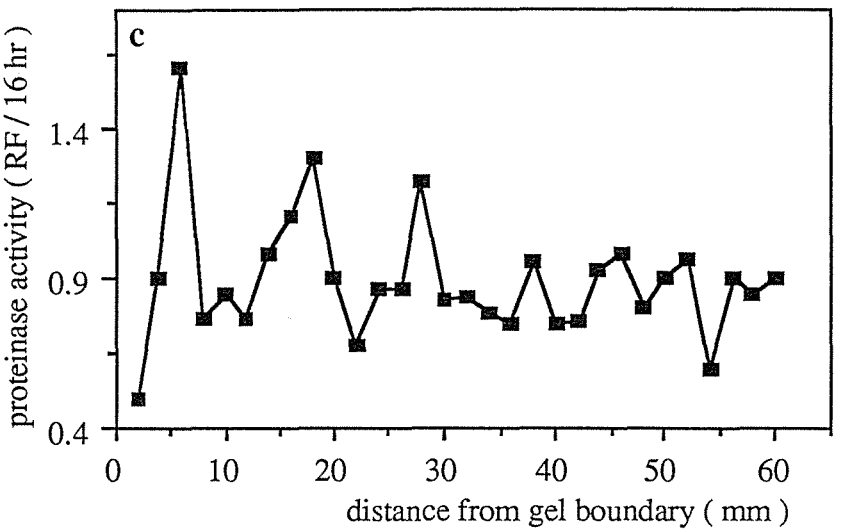
Fold purification refers to the increase in specific activity of the purified samples relative to crude enzyme. % Yield refers to the percentage ratio of total activity recovered in the purified samples to that initially present in the crude extract. The protein concentration of the S- 300 purified sample was too dilute to be determined by the Folin method even after a ten fold concentration of the sample. Therefore the specific activity of the sample could not be estimated.

TABLE VI

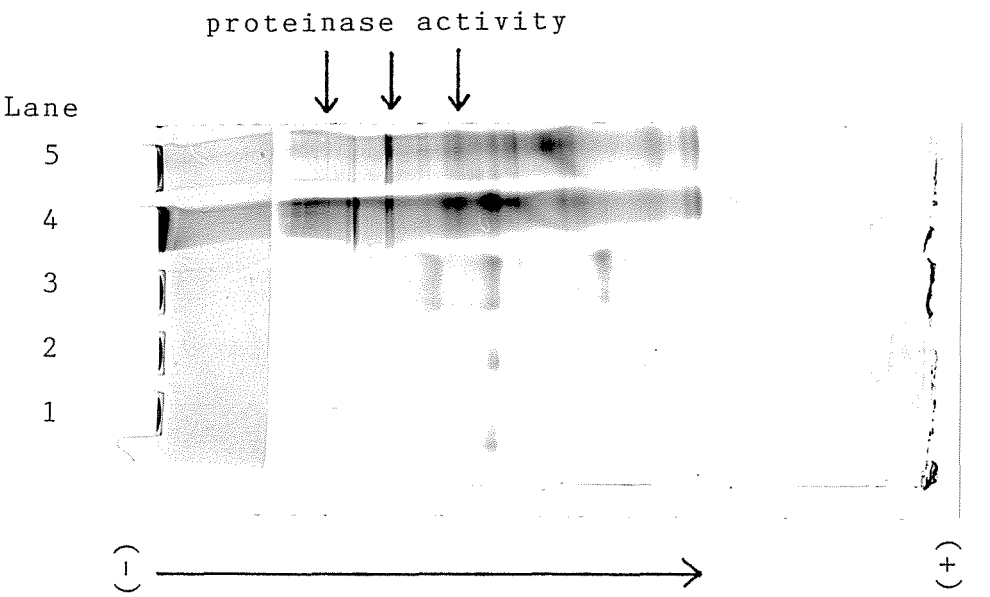
SAMPLE	TOTAL VOLUME ml	PROTEIN mg / ml	TOTAL PROTEIN mg	ACTIVITY ΔRF / hr / ml	SPECIFIC ACTIVITY ΔRF / hr / mg	TOTAL ACTIVITY ΔRF / hr	FOLD PURIFI-CATION	% YIELD
CRUDE PROTEINASE	100	0.63	63	50.5	80.16	5050	1	100
DIAFILTERED	90	0.45	40.5	52	115.5	4680	1.4	93
DEAE-23 PURIFIED	30	0.08	2.4	44.5	556.3	1335	7	30
S-300 PURIFIED	20	?	?	18.5	?	370	?	7

FIGURE 3.5.3c AND 3d DISCONTINUOUS ELECTROPHORESIS OF PARTIALLY PURIFIED PROTEINASE FROM *S. CREMORIS* H2 ON 8% POLYACRYLAMIDE GEL

Duplicate samples of crude proteinase and partially purified proteinase from *S. cremoris* H2 were separated on an 8% polyacrylamide gel. The gel sample of crude proteinase was assayed for proteinase activity (**c**) as described in the legend to Figure 3.2.4. The distance of the dye- front from the stacking gel / running gel boundary was 76 mm. Coomassie Blue- stained protein bands are shown in (**d**). Lane 1 and 2, S- 300 purified sample (1 ml); Lane 3, DE-23 ion- exchange sample (72 µg protein); Lane 4, diafiltered sample (126 µg) and Lane 5, crude proteinase (324 µg).


c
proteinase activity (RF / 16 hr)
1.4
0.9
0.4
0
10
20
30
40
50
60
distance from gel boundary (mm)

(d)


proteinase activity
Lane
5
4
3
2
1
(-)
(+)

An analysis of the purified enzyme by discontinuous electrophoresis on 8% polyacrylamide gel under native conditions of was carried out. The gels containing crude proteinase sample were fractionated into 2 mm slices and assayed for proteinase activity. A profile of the proteinase activity along the length of the gel is shown in Figure 3.5.3c and the protein stained gel in Figure 3.5.3d. The amount of protein in the electrophoresed samples of the partially purified enzyme is too low for locating the Coomassie stained bands corresponding to the proteinase activity peaks in Figure 3.5.3c. At least three peaks of proteinase activity were detected in the gel fractions. This is in contrast to the presence of a single peak of proteinase activity in the electrophoresed samples of the proteinase released from *S. lactis* 4760. However, the highest activity peak was located at an equivalent position (relative to the migration of the dye front) to the single proteinase peak obtained from *S. lactis* 4760 (see section 3.2).

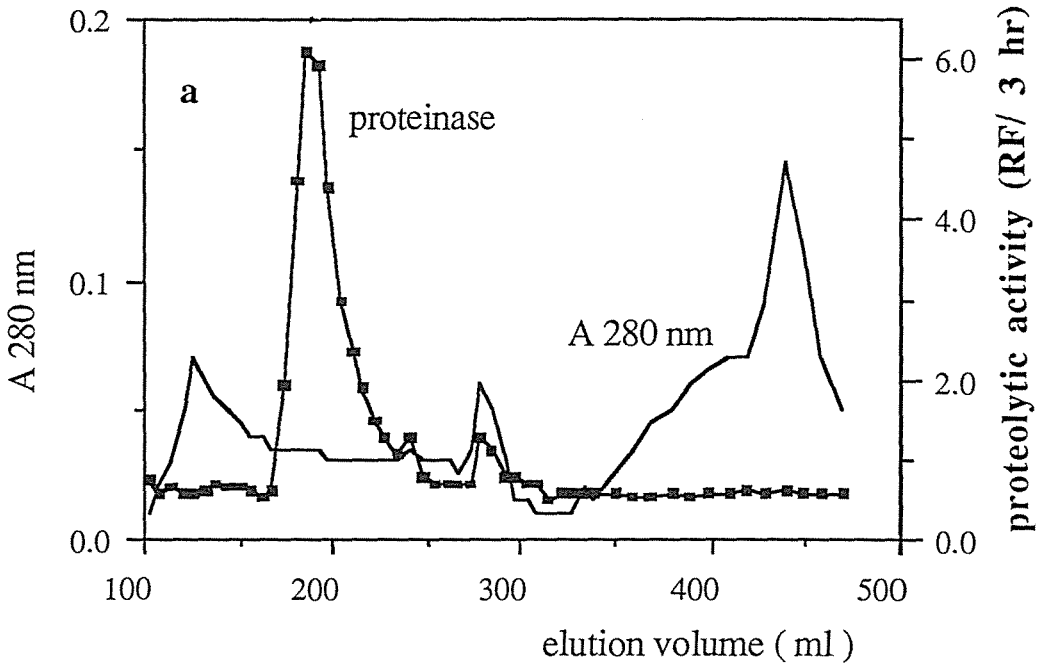
3.5.3 PARTIAL PURIFICATION OF RELEASED PROTEINASE FROM *S. CREMORIS* SK11

A 14 hour- culture of *S. cremoris* SK11 grown in 2 litres of ERSM was harvested and the cells incubated at 30° C in Ca^{++}free- phosphate buffer, pH 6.4 for 3 hours. The crude proteinase released from the cell suspension was purified using the same procedures as for *S. lactis* 4760 and *S. cremoris* H2 proteinases.

The total protein released from the SK11 cells during incubation in buffer was much less than for *S. lactis* 4760 and *S. cremoris* H2. This is evident in the elution profile from the DEAE cellulose ion- exchange chromatography (Figure 3.5.4a : note the difference in A_{280} scale compared to that in Figure 3.5.3a). Only a single peak of proteinase activity was eluted from the ion- exchange purification. The recovery of the proteinase activity (about 22%, Table VII) was slightly lower compared to the values obtained for the purified proteinase from *S. cremoris* H2 (30%) and *S. lactis* 4760 (25%). The lower recovery was partly due to a greater loss of activity after filtration of the crude proteinase in the Diaflo cell. It is possible that more enzyme could have adhered irreversibly to the Diaflo PM- 30 membrane because the concentration of total protein in the crude extract was low. The 2 fold increase in specific activity of the proteinase after the ion- exchange step was low compared to the 7 to 8 fold increase of the *S. lactis* 4760 and *S. cremoris* H2 enzymes. Note however that the specific activity of the proteinase in the crude supernatant was about 3 fold greater for SK11 preparation compared to either the H2 or the 4760 cells.

FIGURE 3.5.4a and 4b PARTIAL PURIFICATION OF PROTEINASE FROM *S. CREMORIS* SK11

Proteinase was released during a 3 hour- incubation at 30° C of washed cells of *S. cremoris* SK11 harvested from ERSM culture previously grown for 16 hours at 22°C. The crude enzyme was purified according to the methods described for the *S.lactis* 4760 enzyme in section 3.2. The enzyme was eluted from the ion- exchange column with a linear gradient of 0- 0.6 M NaCl followed by a steeper gradient of 0.6- 1.0 M NaCl in 10 mM Tris/HCl buffer, pH 8.0 (**a**).Fractions containing proteinase activity were subsequently purified by gel filtration on an S- 300 column, eluting with 50 mM $NaOAc/H_2PO_4$ buffer, pH 6.4 (**b**). A_{280} of the fractions from the gel filtration was not detected because the protein content of the fractions was too dilute.


a
proteinase
A 280 nm
A 280 nm
0.2
0.1
0.0
6.0
4.0
2.0
0.0
100
200
300
400
500
elution volume (ml)
proteolytic activity (RF/ 3 hr)

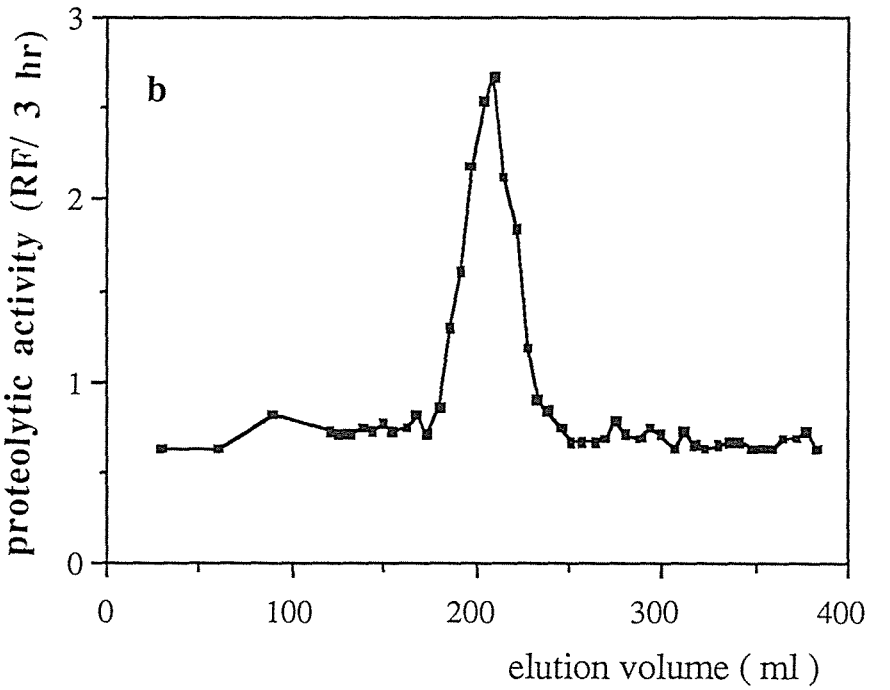

b
proteolytic activity (RF/ 3 hr)
3
2
1
0
0
100
200
300
400
elution volume (ml)

TABLE VII RECOVERY OF ACTIVITY DURING PURIFICATION OF PROTEINASE FROM *S. CREMORIS* SK11

Fold purification refers to the increase in specific activity of the purified samples relative to crude enzyme. % Yield refers to the percentage ratio of total activity recovered in the purified samples to that initially present in the crude extract. The protein concentration of the S- 300 purified sample was too dilute to be determined by the Folin method even after a ten fold concentration of the sample. Therefore the specific activity of the sample could not be estimated.

TABLE VII

SAMPLE	TOTAL VOLUME ml	PROTEIN mg / ml	TOTAL PROTEIN mg	ACTIVITY ΔRF / hr / ml	SPECIFIC ACTIVITY ΔRF / hr / mg	TOTAL ACTIVITY ΔRF / hr	FOLD PURIFI-CATION	% YIELD
CRUDE PROTEINASE	100	0.06	6.0	15	250	1500	1	100
DIAFILTERED	36	0.08	2.88	24.8	310	893	1.24	60
DEAE-23 PURIFIED	35	0.0185	0.65	9.27	498	324	2	22
S-300 PURIFIED	30	?	?	10.1	?	303	?	20

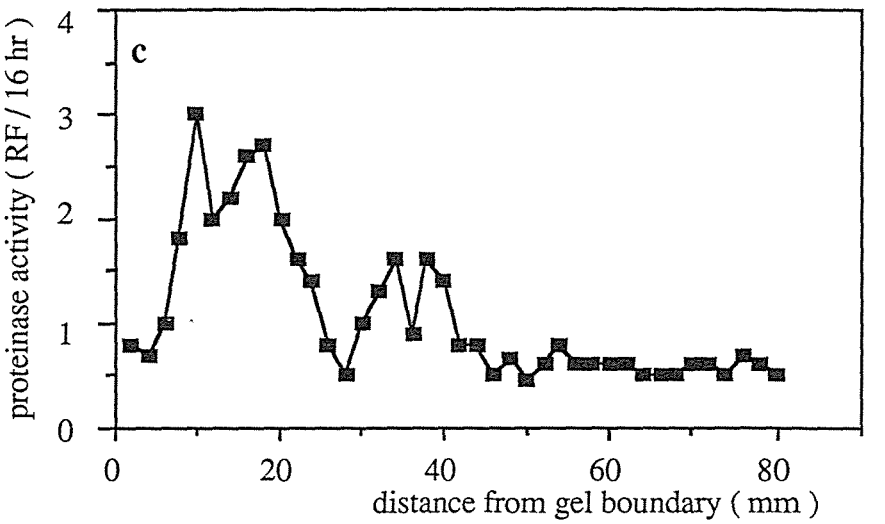


FIGURE 3.5.4c PROTEINASE ACTIVITY OF 2 mm NATIVE 8% POLYACRYLAMIDE GEL SLICES OF CRUDE PROTEINASE RELEASED FROM *S. CREMORIS* SK11

Duplicate samples of diafiltered proteinase (80 µg), DE-23 purified (37 µg) and S-300 purified proteinase (3 ml) were freeze dried and reconstituted in 50 µl 10 mM Tris/ HCl buffer, pH 8.0 containing 2 mM EDTA. 1 µl bromophenol containing 50% glycerol was added to the samples and then electrophoresed in 8% polyacrylamide as previously described. The tract containing one of the duplicate samples of diafiltered proteinase was cut from the gel and fractionated into 2 mm slices to assay for proteinase activity. The remaining gel was stained for protein with Coomassie Blue.Although proteinase activity could be detected in the gel slices, no Coomassie stained protein bands could be detected. The proteinase activity of the gel slices were plotted against the gel distance from the stacking/ running gel boundary as shown above. The distance of the bromophenol blue dye- front from the gel boundary was 90 mm.

Following further purification of the pooled DEAE ion- exchange fractions on a S-300 gel filtration column, the protein concentration of the eluted fractions were too low to be detected by A_{280}. The profile of proteinase activity in the eluted fractions is shown in Figure 3.5.4b. A single proteinase activity peak was eluted in about 200 ml elution buffer, similar to that obtained for the elution of the *S. lactis* 4760 proteinase. The protein concentration of even a 10 fold concentrated sample of the purified enzyme was too low to estimate by the Folin method to enable a quantitative estimate of the increase in specific activity of the enzyme (Table VII).

Analysis of the samples of the *S. cremoris* SK11 proteinase purified by the above methods by discontinuous electrophoresis on 8% polyacrylamide gels under native conditions was carried out as described for the enzyme from *S. cremoris* H2 and *S. lactis* 4760. Coomasie blue-stained protein bands even from the unpurified enzyme could not be detected on the gel due to the low concentration of protein and the limited volume of the samples which could be concentrated. However, proteinase activity was detected in the gel slices of the unpurified enzyme, as shown in Figure 3.5.4c. At least two bands of proteinase activity were detected. The slower migrating band was located at a position similar to the electrophoresed enzyme from *S. cremoris* H2 and *S. lactis* 4760. The observed electrophoretic pattern of the SK11 proteinase was confirmed by repeating the gel fractionation of a further sample of the enzyme.

To summarise, the purification of the proteinase released from *S. cremoris* H2, SK11 and *S. lactis* 4760 by the same methods gave very similar results. For all three strains, only one peak of proteinase activity was obtained. The electrophoretic patterns of the proteinase obtained from the crude supernatant indicate that, in contrast to that released from *S. lactis* 4760, the proteinase activity released from the *S. cremoris* strains may consist of lower molecular weight forms in addition to the higher molecular weight form observed for all three strains. The proteinase activity of the enzymes purified by ion- exchange and gel filtration was too low for detection on the gels. Therefore it is not possible to conclude that the lower molecular weight forms, possibly derived by autoproteolysis of the higher molecular weight form detected in the crude enzyme, are present in the purified enzyme preparation. Other workers (Exterkate & de Veer, 1987) have, however, shown that the multiple forms obtained by gel electrophoresis of purified samples of the proteinase are due to autoproteolytic degradation of a single higher molecular weight form of the enzyme.

3.5.4 ACTIVITY OF PARTIALLY PURIFIED PROTEINASES FROM *S. CREMORIS* H2 AND SK11 TOWARDS VARIOUS MILK PROTEINS

The partially purified proteinase from *S. cremoris* H2 and SK11 described in the previous sections were incubated with α_{s1}-, β- and κ- caseins and the whey proteins α- lactalbumin and β- lactoglobulin according to the methods described in section 3.3. The samples were subsequently electrophoresed on 8- 25% gradient polyacrylamide gels in a Phast ™ system.

The Coomassie blue stained electrophoretic pattern of the gels are shown in Figure 3.5.5a and 5b. Among the various milk proteins, only β- casein was hydrolysed by the enzyme from both *S. cremoris* H2 and SK11. This preferential hydrolysis of β- casein is similar to the specificity of the partially purified proteinase from *S. lactis* 4760 and is characteristic of the HP- type of proteinase (Visser *et al.*, 1986). The inability of the SK11 proteinase to catalyse the hydrolysis of α_{s1}- casein is in marked contrast to the findings of Visser *et al.* (1986).

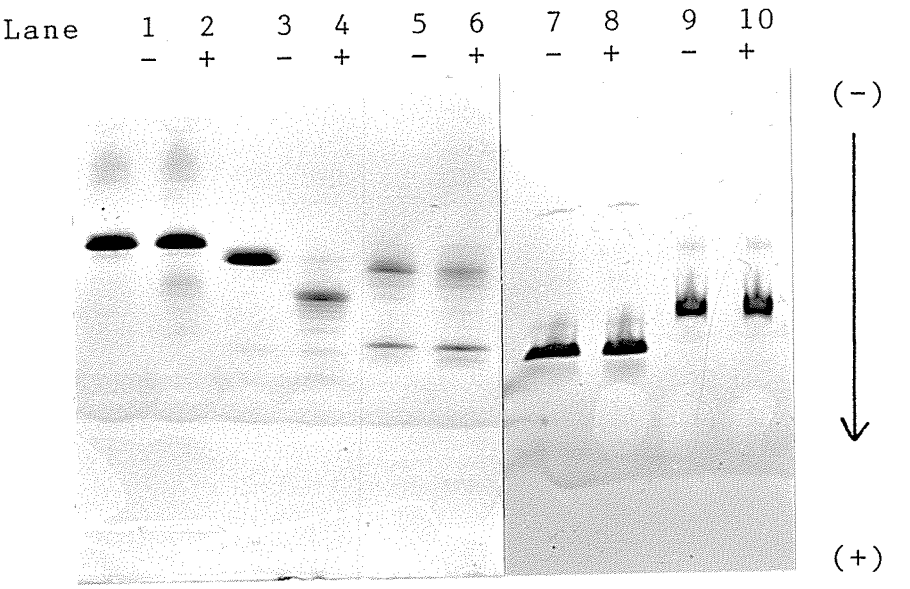
A further comparison of the activity of the enzymes from the three strains towards α_{s1}- and β- casein was carried out by discontinuous electrophoresis on 8% urea polyacrylamide gels using larger gels and higher protein loadings.The electrophoretic pattern of the α_{s1}- and β- casein samples incubated with the proteinase are shown in Figure 3.5.6a and 6b respectively. Virtually no hydrolysis of α_{s1}- casein by the proteinase from *S. lactis* 4760, *S. cremoris* H2 or SK11 could be detected as suggested by the presence of the same pattern of faint bands for all the three enzymes. Therefore the inability of the SK11 proteinase to cleave α_{s1}- casein was confirmed.

It can be observed that the electrophoretic pattern of β- casein cleavage by the *S. cremoris* H2 proteinase is very similar to that obtained with the *S. lactis* 4760 enzyme. This observation is not surprising since the proteinase enzyme from both strains is encoded by the same plasmid (i.e.from *S. cremoris* H2). However, the cleavage pattern for *S. cremoris* SK11was different from those generated by the *S. lactis* 4760 or the *S. cremoris* H2 enzymes. It is possible that the observed difference in the gel pattern may be due not to a difference in cleavage specificity but to a low level of proteolytic activity of the *S. cremoris* SK11 proteinase. Ideally, a series of samples taken at various stages of digestion should be examined to test this possibility.

FIGURE 3.5.5a and 5b SDS- PAGE OF THE CLEAVAGE OF VARIOUS BOVINE MILK PROTEINS BY THE PROTEINASE FROM *S. CREMORIS* H2 AND SK11.

The partially purified proteinases from *S. cremoris* H2 (**a**) and SK11 (**b**) were incubated with α_{s1}-, κ- and β- casein, and the whey proteins, α- lactalbumin and β- lactoglobulin as described in the legend to Figure 3.3.1 and section 3.4.4. (+) and (-) refers to incubation with and without enzyme respectively. Lanes 1 and 2, α_{s1}- casein; Lanes 3 and 4, β- casein; Lanes 5 and 6, κ- casein; Lanes 7 and 8, α- lactalbumin; Lanes 9 and 10, β- lactoglobulin.

(a) H2



(b) SK11

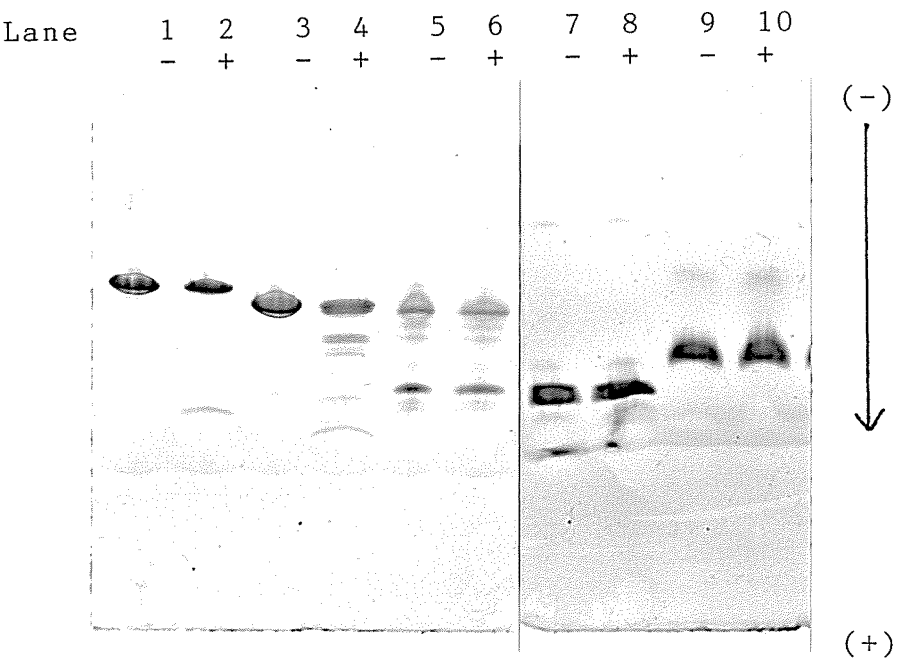
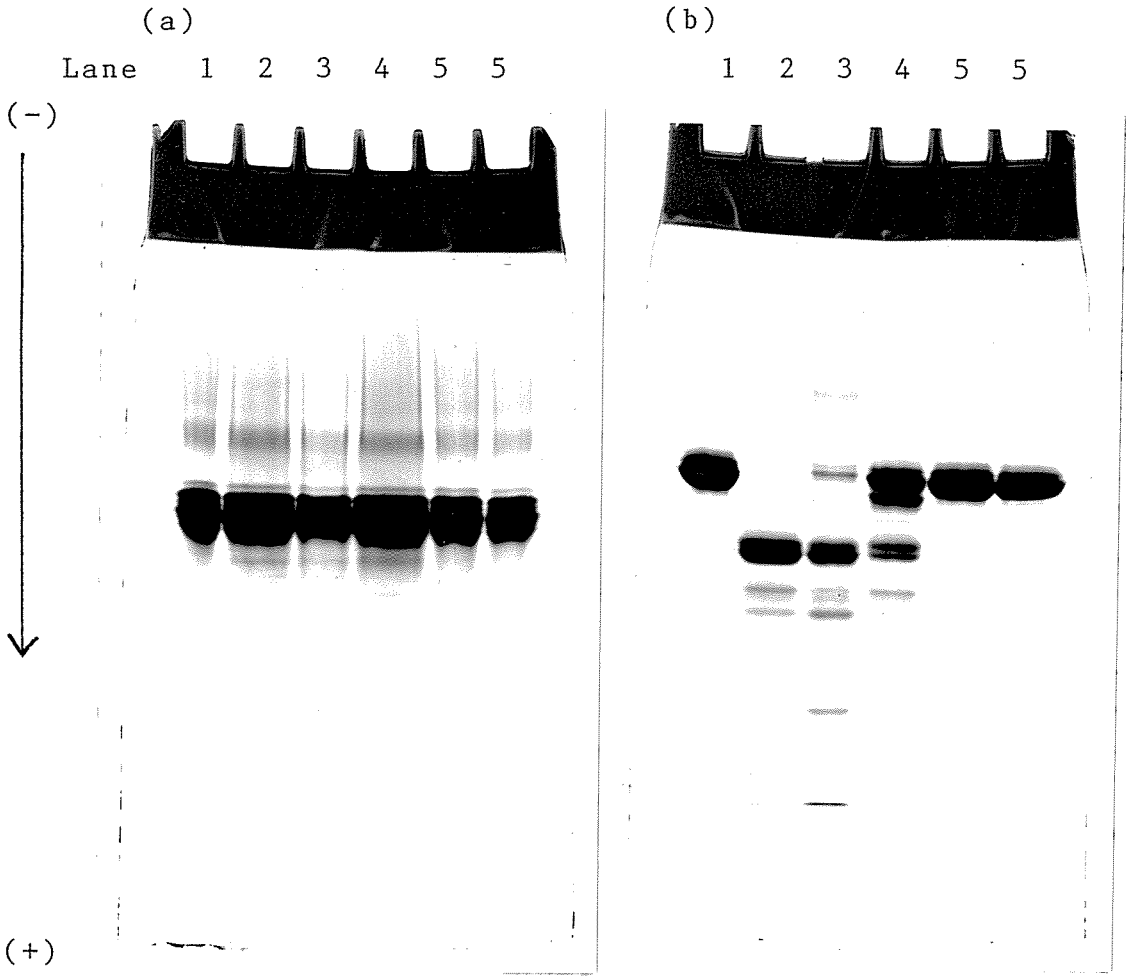


FIGURE 3.5.6a and 6b DISCONTINUOUS ELECTROPHORESIS OF α_{s1}- AND β- CASEIN HYDROLYSATES ON 8% POLYACRYLAMIDE GEL IN 4 M UREA

20 µl of α_{s1}-casein (**a**) and β– casein (**b**) at 10 $mgml^{-1}$ were incubated at 24° C with 20 µl of partially purified proteinase from *S. lactis* 4760 and *S. cremoris* SK11 for 50 hours and *S. cremoris* H2 for 24 hours respectively. It was necessary to add up to 30 µl of glycerol to the incubation mixture prior to loading onto the gel due to the density of the running gel buffer prepared in 4 M urea. The gels were electrophoresed according to the methods outlined in section 2.2.12. Lanes 1 and 5, incubation without enzyme; Lane 2, incubated with proteinase from *S. lactis* 4760; Lane 3, proteinase from *S. cremoris* H2 and Lane 4, proteinase from *S. cremoris* SK11.


(a)
(b)
Lane 1 2 3 4 5 5
1 2 3 4 5 5
(-)
(+)

However, the HPLC profiles obtained from a time course digestion of β- casein by the proteinase from *S. lactis* 4760 (Figure 3.4.1, section 3.4) do not show major qualitative changes in the degradation pattern at the different sample times.

3.5.5 ANALYSIS OF THE β- CASEIN CLEAVAGE SITES OF THE PROTEINASE FROM *S. CREMORIS* H2 AND SK11.

The partially purified proteinase from *S. cremoris* H2 and SK11 was incubated with β- casein for 24 hours and 50 hours respectively according to the methods specified in section 3.4. The incubation of the SK11 enzyme was carried out for a longer period due to the relatively low rate of activity of the enzyme.

TFA soluble products of the reaction mixtures were separated by RP- HPLC on a C- 18 column with a linear gradient of 10- 40% acetonitrile in one hour followed by a 42- 90% gradient of acetonitrile in 20 minutes. The elution of the peptides monitored by A_{220} is shown in Figure 3.5.7b and c. For comparative purposes the β- casein hydrolysates obtained from a 50 hour digestion with the partially purified proteinase from *S. lactis* 4760 (stored in 30% glycerol) and fractionated using the same gradient was carried out (Figure 3.5.7a).

The RP- HPLC profile of the β- casein peptides from *S. cremoris* H2 proteinase digests is very similar to that from *S. lactis* 4760. FAB- MS and amino acid sequence analysis of Peaks 1 and 15 (Table VIII) confirmed that they correspond to the peptides present in the *S. lactis* 4760 enzyme digests which are eluted at the same concentration of acetonitrile, i.e Peaks 1 and 5 respectively (see Table V, section 3.4). These peptides are derived from the sequences 176- 182 and 183- 193 respectively in the β- casein molecule. FAB- MS analysis of Peak 18 and 19 suggest that these peptides are more than 2 000 in molecular weight and may represent the N- terminal fragments of the hydrolysed β- casein molecule.

While not constituting a complete analysis of the peptides produced, these data together with the similar electrophoretic pattern of β- casein hydrolysis suggest that the proteinase from *S. cremoris H2* probably cleaves β- casein at the same sites as the *S. lactis* 4760 enzyme. This is not surprising since *S. cremoris* H2 is the donor of the proteinase plasmid in *S. lactis* 4760.

FIGURE 3.5.7 RP- HPLC SEPARATION OF β- CASEIN PEPTIDES GENERATED BY THE PARTIALLY PURIFIED PROTEINASE FROM *S. CREMORIS* H2 AND SK11.

400 µl of the partially purified proteinase from *S. cremoris* H2 (**b**) and SK11 (**c**) were incubated at 24° C with 200 µl of β- casein (10 $mgml^{-1}$) for 24 and 50 hours respectively. For comparative purposes, incubation of the casein for 50 hours with the partially purified proteinase from *S. lactis* 4760 previously stored at -20° C in 30% glycerol was also carried out (**a**). The 1% TFA soluble fractions from the incubations were separated on a C- 18 reverse phase HPLC column as described in section 3.5.3. The peaks labelled 1- 6 in the elution profile for the peptides generated by the 4760 enzyme were shown to correspond to the peaks of the same number shown in Figure 3.4.2 by amino acid sequence analysis. The results of the FAB- MS analysis, amino acid sequence and composition of the peaks labelled 1 and 15 in (**b**) and those labelled 1- 4 in (**c**) are summarised in Table VIII and IX respectively.

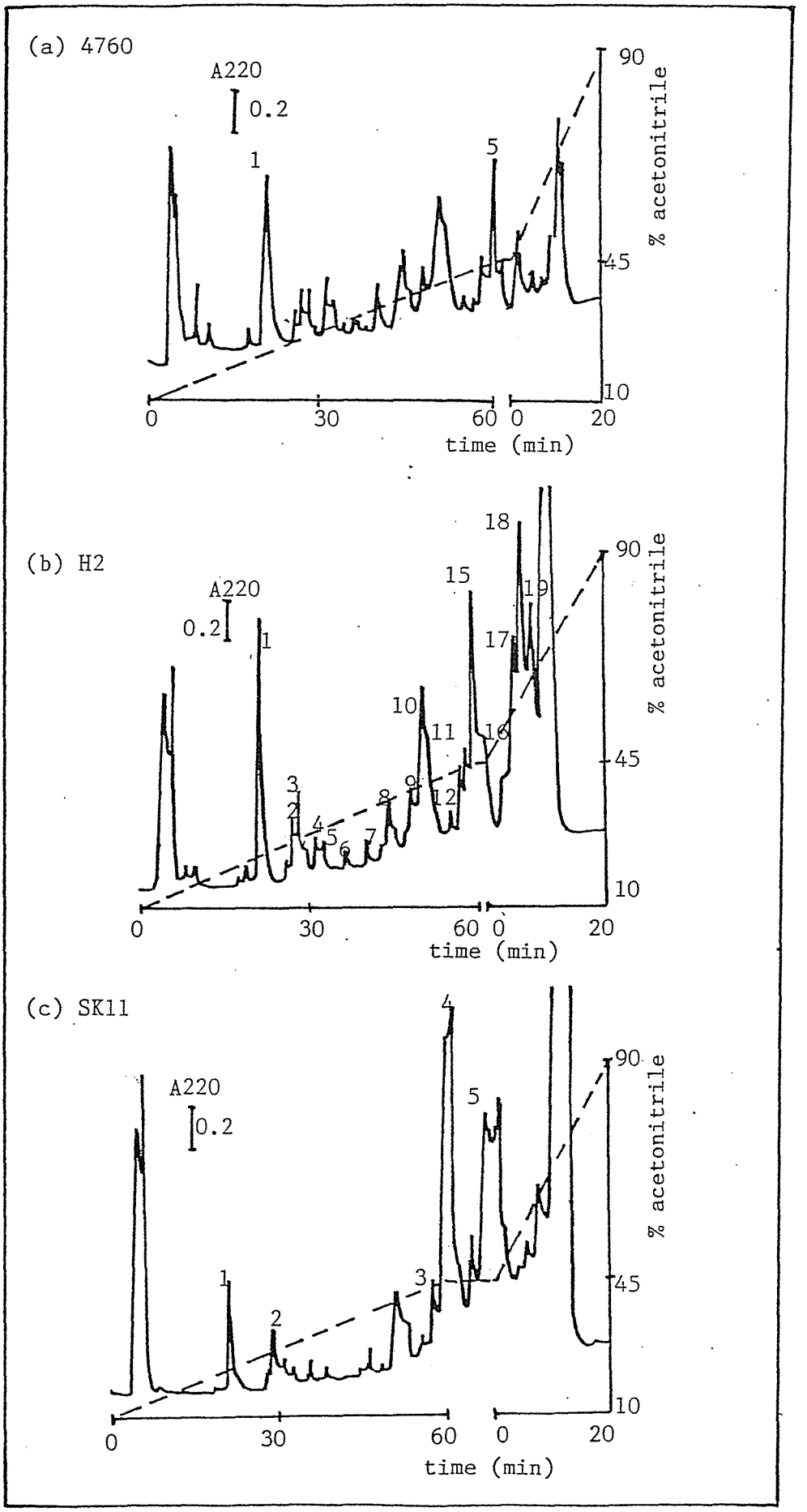

(a) 4760
A220
0.2
1
5
90
45
10
% acetonitrile
0
30
60
0
20
time (min)
(b) H2
A220
0.2
1
2
3
4
5
6
7
8
9
10
11
12
15
16
17
18
19
90
45
10
% acetonitrile
0
30
60
0
20
time (min)
(c) SK11
A220
0.2
1
2
3
4
5
90
45
10
% acetonitrile
0
30
60
0
20
time (min)

TABLE VIII AND IX ANALYSIS OF RP- HPLC SEPARATED β-CASEIN PEPTIDES PRODUCED BY THE ACTION OF PARTIALLY PURIFIED PROTEINASES FROM *S. CREMORIS* H2 AND SK11

The labelled peaks eluted from the RP- HPLC of the β- casein hydrolysates after incubation with the proteinases from the *S. cremoris* H2 and SK11 shown in Figure 3.5.7b and 3.5.7c respectively were analysed for amino acid composition, sequence and by FAB- MS wherever specified in Table VIII (H2) and Table IX (SK11).

TABLE VIII

PEAK #	AMINO ACID COMPOSITION	AMINO ACID SEQUENCE	FAB-MS MH+ ION	POSITION IN β CASEIN
1	not determined	$_2$HN-Lys-Ala-Val-Pro-Tyr-Pro-Gln-OH	802	176 - 182
15	not determined	$_2$HN-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe -Leu-Leu-Tyr-OH	1367	183 - 193

TABLE IX

PEAK #	AMINO ACID COMPOSITION	AMINO ACID SEQUENCE	FAB-MS MH+ ION	POSITION IN β CASEIN
1	$Glx_1Pro_2Ala_1Val_1Tyr_1Lys_1$	$_2$HN-Lys-Ala-Val-Pro-Tyr-Pro-Gln-OH	802	176 - 182
2	$Ser_1Glx_2Pro_{2.3}Val_2Leu_{1.4}Lys_{1.3}$	not determined	not done	166 - 175 ?
3	$Asx_1Glx_5Pro_8Gly_{2.5}Ala_1Val_5Met_{0.7}Ile_{3.3}$ $Leu_5Tyr_1Phe_2Arg_{2.3}$	$_2$HN-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe -Leu-Leu-Tyr-OH	1367 1716	183 - 193 194 - 209 ?
4	$Asx_1Thr_{0.8}Ser_1Glx_{3.3}Pro_{2.2}Val_{1.6}Ile_1Leu_{1.7}$	not determined	not detected	N- terminal fragment ?

The profile of the β- casein peptides generated from *S. cremoris* SK11 enzyme appears to be similar to the profile obtained for *S. cremoris* H2 and *S. lactis* 4760 enzymes over that part of the gradient eluted by 10- 42% acetonitrile but differs over the range from 42- 90% acetonitrile. The peaks numbered 1 to 4 were analysed for amino acid composition and sequence (Table IX). Peak 1 was identical in sequence and amino acid composition to the Peak 1 from *S. lactis* 4760 proteinase digests corresponding to the sequence 176- 182 of the β- casein molecule (Table V, section 3.4). FAB- MS gave a single molecular ion species corresponding to the calculated molecular weight of the sequence. The amount of peptide from Peak 2 was not sufficient for FAB-MS or sequence analysis but the amino acid composition of the peptide was analysed. The composition is very similar to that obtained for the Peak 2 from *S. lactis* 4760 digests which corresponds to sequence 166-175 of the β- casein (Table V).

The sequence of Peak 3 is identical to the smaller of the two peptides present in Peak 5 of the *S. lactis* 4760 digests. This peptide corresponds to the sequence 183- 193 of the β-casein molecule. The molecular ion species for the sequence was also detected in the FAB-MS trace. However, the presence of another molecular ion in the FAB- MS of mass 1716 was not detected from the sequencer. This fragment has been shown to correspond to the sequence 194- 209 of the β- casein molecule present in Peak 5 of the *S. lactis* 4760 digest. The amino acid sequence of Peak 4 is very similar to that of Peak 6 from the *S. lactis* 4760 digest. As with the *S. lactis* peptide, no molecular ion species could be detected from this peak suggesting that the *S. cremoris* SK11 peptide(s) in this peak may be large N-terminal fragment(s).

From the partial analysis of the β- casein peptides produced by the action of the *S. cremoris* SK11 proteinase, no novel sequence differing from those generated by the *S. lactis* 4760 enzyme could be deduced. This suggests that the proteinase from *S. cremoris* SK11 may cleave the β- casein molecule at very similar sites to the enzyme from *S. cremoris* H2 and *S. lactis* 4760, which has been shown to be an HP- type proteinase (section 3.3). These observations are in contrast to the different pattern of the β- casein cleavage in polyacrylamide gels described in section 3.5.2. It can also be recalled from section 3.5.2 that the *S. cremoris* SK11 enzyme was unable to hydrolyse α_{s1}- casein to any great extent unlike the activity of the AM1- type activity shown to be present in this strain (Visser *et al.*, 1986). Further discussion concerning the apparent conflict of the results described in this section, which suggest the presence of an HP- type of proteinase in *S. cremoris* SK11, to previously published findings that the enzyme is of the AM1- type will be presented in section 4.

4 DISCUSSION

The progress in research on the proteolytic system of the lactic streptococci has advanced rapidly in the past decade. The central role of the proteolytic system both for starter growth and for the production of cheese with desirable flavour and texture has provided impetus for further work in this area. While the present study was in progress, several reports on work of direct relevance to the aims of this investigation have been published or appeared in abstract form, particularly from the major research group in Holland.

In general, the main results obtained in the present study are very similar to those recently reported apart from a few but important differences. The following sections discuss the results described in section 3, in the light of previously reported findings.

4.1 PROTEINASE ASSAY USING FITC- β CASEIN

The fluorescent isothiocyanate (FITC) conjugated β- casein prepared according to Twining (1984) proved to be a suitable substrate for proteinase assay in the above studies. The hydrolysis of the FITC-β casein by the proteinase is linear with time (up to 2 hours studied).

Although no systematic study was carried out to investigate the conjugation process during the preparation of the substrate, the low molar ratio of FITC coupled to β- casein raises some interesting questions in light of the cleavage specificity of the proteinase. The starting molar ratio of FITC to β- casein in the conjugation mixture was 2- 3. The ratio was kept low to minimize non- specific binding of the dye to protein (Nairn, 1976). Since the ratio obtained in the conjugated protein is 0.8- 1.0, not all of the dye molecules could have coupled to the protein and when the ratio is less than 1.0, not all the protein molecules could have been labelled. [The ratio obtained may be a slight underestimate since the E_{490} for FITC used in the calculation is that for the free dye which may be 1.1 to 1.2 fold greater than that for conjugated dye (Nairn, 1976).]

FITC is known to react with the side- chain amine (NH_2) group of lysine residues at the pH of the conjugation mixture (pH 9.0), (Nairn, 1976). Since there are eleven lysine residues in the β- casein molecule, it is possible that those residues more exposed in the structure of the protein (at pH 9.0) would react.preferentially. From the analysis of the β- casein cleavage sites (section 3.4), the proteinase appears to cleave only within the 49 residues at the C-terminus of the molecule. This region contains only two lysine residues. If the

enzyme cleaves the conjugated β- casein in a similar manner to its hydrolysis of the unconjugated molecule, then the fluorescence in the TCA soluble products must include those from the C- terminal region.

Since no apparent bond- specificity or consensus sequence is evident in the proteinase-catalysed cleavage of β- casein, it is possible that the observed cleavage at the C- terminus may be due to the region being structurally more accessible to cleavage and therefore also more exposed to dye- coupling.

However, it is also possible that the conjugated protein is more open or denatured in structure than the unconjugated protein so that the molecules may be randomly labelled with dye. The proteinase may also hydrolyse the conjugated molecule more extensively. A systematic study of the conjugation process involving making tryptic digests of the conjugated protein followed by analysis of the labelling of specific peptides would reveal whether the dye is coupled randomly. It would also be of interest to investigate the nature of the peptide products of hydrolysis of FITC- labelled β- casein by the lactic streptococcal proteinase using the same approach as those used in the present study for unconjugated casein.

Since the FITC- β casein assay was used mainly for comparative studies, a systematic study to compare the sensitivity of the assay with those using radiolabelled substrates (eg. $^{14}CH_3$- casein) was not carried out. It is clear, however, that the assay using the fluorescent conjugate is both simple and sensitive: 200 mg of β- casein coupled with 5 mg of FITC was sufficient for all the assays described above and only microlitre quantities of both substrate and enzyme were required. Added advantages include the relatively inexpensive cost of the dye compared to radioactive labels and no special disposal measures are required since the dye is not a radiation hazard.

4.2 RELEASE OF PROTEINASE INTO Ca^{++}- FREE BUFFER

The results of the studies aimed at optimising the conditions for proteinase release in Ca^{++}-free buffer (described in section 3.1) have largely confirmed earlier findings on the release process by Mills & Thomas (1978) but also show some differences. The main difference found in the present study was the pH dependence of the release of proteinase. The optimum pH for the release of proteinase during incubation reported by Mills & Thomas (1978) was between pH 7.8 to 8.0 with significantly lower rates of release at pH values less than 7.0. Subsequently, other workers have also isolated proteinase by incubation in

buffers at pH values above 7.0 [pH 7.3 and pH 7.5 buffers respectively by Hugenholtz *et al.* (1984) and Geis *et al.* (1985).]

In contrast, the optimum pH for release of proteinase was found in the present study to be between pH 6.4 to 7.0 while the amount of proteinase released at pH values between 7.0-8.0 was markedly lower (Figure 3.1.7). Exterkate & De Veer (1985) and Monnet *et al.* (1987) have used buffers at pH values of 6.5 and 7.0 respectively to release the proteinase for purification.

It is quite possible that the pH dependence of proteinase release may vary according to the strain of lactic streptococci. In addition, the nature of the buffer ions may affect the pH dependence of proteinase release. In this study, less proteinase was released in acetate buffer than in phosphate buffer at the same pH. In contrast to the Tris buffer used by Mills & Thomas (1978) to cover the higher pH values, phosphate buffer was used in this study.

Another feature of the release process found in the present study is the apparent lysis or leakage of cells which often seems to increase with the increasing release of proteinase. For example, when the release of cellular proteinase activity increased from 23% at one hour to 94% at three hours of incubation, there was also a marked increase of leakage of intracellular lactate dehydrogenase from 3% to 15%. Likewise, the pH dependence of proteinase release was similar to the pH dependence of cell leakage. These observations are in contrast to the findings of Mills & Thomas (1978) on the release of proteinase from *S. cremoris* AMI which released up to 81% of its proteinase activity with leakage of less than 1% total cellular LDH activity.

The extent of lysis or leakage may, however, vary according to the strain or growth conditions. Thus in the present study (section 3.1.7) the *S. cremoris* strains H2 and SK11 were found to be much less leaky than *S. lactis* 4760 while all three strains released comparable amounts of proteinase. In comparison of the release of proteinase from *S. lactis* 4760 harvested from casitone- supplemented milk medium with cells from unsupplemented medium, it was found that while the amount of proteinase released from the cells grown in unsupplemented media was only two- fold greater than that from cells grown in casitone-supplemented medium, there was a ten- fold greater leakage of intracellular LDH from the former cells.

In a study of proteinase released from several different species of *Lactobacillus* , Ezzat *et al.* (1985) showed that proteinase released by incubating cells in Ca^{++}- free buffer was accompanied by less leakage of intracellular LDH than when proteinase was released by

lysozyme treatment to solubilise the cell wall. However, it is interesting to note that while the proteinase released by lysozyme treatment was contaminated with more LDH, the amount of proteinase so released was, on average, higher than that released by incubating the cells in Ca^{++}- free buffer. Also, the amount of LDH released was greater than 1% of the total cellular activity for most strains (eg. up to 55% when 91% of proteinase was released from *Lactobacillus lactis* NRZ 252).

The increase leakage of LDH which has frequently been found to accompany an increased yield of proteinase in the studies described above may indicate an intracellular source for part of the proteinase so released. However, it could also mean that complete release of all the cell wall proteinase requires conditions which may cause the cells to become more leaky or susceptible to lysis. Other evidences from the present study suggest that it is unlikely that a large proportion of the proteinase activity released into Ca^{++}- free buffer is intracellular in origin. Since the release of cell wall proteinase has been shown to be specifically inhibited by Ca^{++}(Mills & Thomas, 1978), addition of a sufficient concentration of Ca^{++} to inhibit the release of the cell wall proteinase should leave a residual level of proteinase activity if some of this activity originates from cell lysis. This amount of residual proteinase should correlate with the degree of lysis measured by LDH release. However, while a residual level amounting to 9% of total cell casein- hydrolysing activity was released at concentrations of Ca^{++} ions which apparently saturate inhibition (Figure 3.1.10) this was accompanied by leakage of less than 0.8% of total cellular LDH.

It is certainly unlikely that the partially purified proteinase used for the above studies could be wholly of intracellular origin because incubation of cells for 3 hours released almost 100% of the total proteinase activity while only up to 15% total cellular LDH was detected in the crude proteinase preparation. It should be emphasised at this point that the term "proteinase" refers, throughout this discussion, to enzyme activity capable of hydrolysing casein. If there is any intracellular activity present in the proteinase released by the 4760 cells, it must be very similar in properties to the cell wall proteinase since only one peak of proteinase activity was eluted from the ion- exchange and gel filtration column during purification of the crude enzyme. Although it is still possible that there may be some leakage of proteinase enzymes form the cell cytoplasm which are involved in the turnover of bacterial protein and which may be able to hydrolyse β- casein, the amount of such enzymes in the crude cell wall proteinase preparation is probably negligible.

It has been proposed that the release of cell wall proteinase from the lactic streptococci involves events which are specifically inhibited by Ca^{++} ions. Mills & Thomas (1978) suggested that the proteinase may be loosely entrapped in the cell wall and therefore

conditions which affect the permeability of the cell wall would also affect the release of the proteinase. For example, changes in the electrostatic interactions between charged polymers of the cell wall due to changes in pH or ionic composition which induce swelling of the walls may allow the loosely entrapped proteinase to be released.

The specific inhibition of release by Ca^{++} ions, however, may also indicate that this ion is directly involved in the binding of the enzymes to the cell wall. Exterkate & de Veer (1987) proposed that the proteinase of the lactic streptococci may exist as aggregates held together at or near the cell wall by Ca^{++} bridges. Since *S. cremoris* ML1 has been found to be the only strain of lactic streptococci which secretes the proteinase into the growth medium (Exterkate, 1976), a comparison of the sequence of this enzyme or the gene encoding it with that from the other strains of lactic streptococci may help identify the structural features responsible for binding Ca^{++} if they are present, and the relationship between the Ca^{++}-binding regions and cell wall aggregation sites of the enzymes.

For example, if the absence of a particular Ca^{++}- binding region in the proteinase from *S. cremoris* ML1 could be correlated to the absence of cell wall binding region, then the theory of Exterkate & de Veer (1987) that Ca^{++} is directly involved in the binding of the enzyme to the cell wall would be supported. Interestingly, deletion of a large portion of the C- terminus in the *S. cremoris* Wg2 proteinase has been found to result in the secretion of the normally cell wall- bound proteinase into the growth medium (De Vos, 1987). This finding suggests that the proteinase may be bound to the cell wall via a region near the C-terminus but whether or not the binding to the cell wall is mediated by Ca^{++} bridges has not been investigated.

A more recent theory on the release of proteinase from lactic streptococci suggests that it is due to autoproteolytic cleavage of a form of the enzyme covalently linked to the cell wall (Kok & Venema, 1988). This idea was based largely on the observation that several proteolytically active forms of the enzyme could be isolated which differ in size and immunological identity (Exterkate & de Veer, 1987; Hugenholtz *et al.*, 1984, 1987; Kok *et al.*, 1988). In addition, when the serine proteinase inhibitor, PMSF, was included in the release buffer, no proteinase could be detected by immunoprecipitation with anti- cell wall proteinase- antisera, suggesting that a proteolytic step is involved in the release of the proteinase (Laan, H., quoted in Kok & Venema, 1988).Although the site(s) of autoproteolysis in the proteinase has not been shown, a stretch of 4 amino acid residues near the C- terminus of the enzyme from *S. cremoris* HP has been shown to be identical to one of the cleavage sites of *S. lactis 763* proteinase in β- casein (Kok *et al.*, 1988; Monnet *et al.*, 1986). To find out if this site in the enzyme is actually used for the release of the

proteinase from the cell wall through autoproteolysis, purification and determination of the N- terminal sequences of the proteinase could be done. Alternatively, site directed mutagenesis to alter the gene sequence encoding the proposed autoproteolytic site could be carried out.

4.3 PARTIAL PURIFICATION OF PROTEINASE

A question that has been much discussed in the literature on the proteinase activity of lactic streptococci is whether there are multiple forms of the enzyme (Thomas & Pritchard, 1988). The partial purification of released proteinase from *S. lactis* 4760 described in section 3.2 revealed the presence of only one peak of proteinase activity by both ion-exchange and gel filtration chromatography.

Analysis of the proteinase in the crude cell wall extract by electrophoresis on native polyacrylamide gels revealed only one band of proteinase activity which appears to comprise only a very small proportion of the total protein released from the cells (Figure 3.2.4). [Smaller protein bands not demonstrating proteinase activity were present in the electrophoretic separation of the partially purified enzyme. However, further purification of the enzyme was not attempted, firstly due to the already low yield of enzyme and secondly, due to the likelyhood of autoproteolysis of the enzyme when further purified.] Monnet *et al.* (1987) reported the purification of a single 80 kD proteinase from *S. lactis* 763, which also comprised a very small proportion of the total proteinase from the cells (less than 0.2%). Smaller protein bands without proteinase activity were also present in the partially purified proteinase from this strain.

The proteinase released from the *S. lactis* 4760 is encoded from the Prt^+- plasmid transferred from *S. cremoris* H2. In contrast to the 4760 cells however, the crude proteinase released from the H2 cells showed at least two bands of proteinase activity on gel electrophoresis. It is not clear why the H2 proteinase should be more heterogeneous than that from the 4760 cells. Multiple forms of proteinase have been separated by Cliffe & Law (1985), Hugenholtz *et al.* (1984, 1987) and Exterkate & de Veer (1987). Exterkate & de Veer (1987) have provided evidence for the view that the presence of multiple forms of the enzyme are due to autoproteolysis of a single high molecular weight form of the enzyme. They observed the appearance of several proteinase active bands with sizes ranging from 60- 118 kD in the electrophoretic separation of the purified proteinase from *S. cremoris* HP which, before purification, only consisted of two proteinase bands 126 and 133 kD in size respectively.

Likewise, the presence of more than one proteinase activity in the crude proteinase released from *S. cremoris* H2 described in this study may be an artifact of autoproteolysis, since the larger proteinase present in the crude enzyme preparation corresponded in size to the single enzyme released from *S. lactis* 4760. The cleavage pattern of β- casein by the partially purified proteinase from the H2 and 4760 cells described in section 3 is similar if not identical. This suggests that if the smaller proteinase released from the H2 cells are different from the larger enzyme, they must exhibit the same activity towards β- casein. Alternatively, if the smaller enzyme arose by autoproteolysis of the larger form, then the autoproteoysis did not alter the specificity of the enzyme activity.

Addition of Ca^{++} ions has been used to stabilise the enzyme from autoproteolysis during purification (Geis *et al.*, 1985; Exterkate & de Veer, 1987). Although most serine proteinases have been shown to be stabilised by Ca^{++}, the stabilising effect of Ca^{++} on the proteinase from the lactic streptococci has not been proven conclusively. Studies with crude enzyme (section 3.2) showed that addition of up to 4 mM Ca^{++} did not alter the stability of the enzyme. Although Exterkate & de Veer (1987) found that loss of proteinase activity was reduced by adding Ca^{++}, they also found a similar effect with Mn^{++} ions. Moreover, neither Ca^{++} nor Mn^{++} ions could fully restore the partial loss of proteinase activity effected by adding EDTA or citrate, suggesting that other factors and/or bivalent cations removed by the chelating agents may be required to stabilise the enzyme.

The specific inhibition of proteinase release from the cell wall by Ca^{++} has also led to the suggestion that the release of the enzyme may involve an autoproteolytic process and that addition of Ca^{++} stabilises the cell wall enzyme from the autoproteolysis and hence from being released (Kok & Venema, 1988, see section 1.5). The influence of Ca^{++} on the release and stability of the proteinase from the lactic streptococci is an interesting subject to pursue because the milk environment in which the bacteria are grown as starters, is rich in Ca^{++}. If the abundance of Ca^{++} in milk prevents the release of the cell wall proteinase into the milk medium, this would be an advantage for the survival of the bacteria since the proteolytic products of the enzyme would be close to the cell membrane and could be taken up by the bacteria before being able to diffuse and be used up by neighbouring cells.

4.4 ACTIVITY OF THE CELL WALL PROTEINASE

The partially purified proteinase form *S. lactis* 4760 was shown to be active towards β-casein only, with no apparent cleavage of α_{s1}- and κ- casein or the whey proteins, α-lactalbumin and β- lactoglobulin. Since the inability of the proteinase to cleave α_{s1}- casein is characteristic of the HP- type proteinase described by Visser *et al.* (1986), the enzyme from the 4760 cells is probably of that type, although this criterion, by itself, is insufficient to establish its identity with the HP- type proteinase.

The preferential hydrolysis of β- casein has been reported for the proteinase from *S. lactis* 763 (Monnet *et al.*, 1986) and from a wide variety of *S. cremoris* strains (Exterkate & de Veer, 1985; Visser *et al.*, 1986; Geis *et al.*, 1985). This observation raises the question as to whether the proteolytic activity of starter bacteria in milk is also largely limited to the degradation of β- casein. Since β- casein is present in milk as micellar aggregates with α_{s1}- and κ- caseins, it might be expected to be less exposed to hydrolysis than when it is present in the soluble form used to characterise the activity of the proteinase. Exterkate & de Veer (1985) have found that β- casein was preferentially hydrolysed by the cell wall proteinase of *S. cremoris* HP whether present as soluble or micellar form. However, they proposed that in the case of the hydrolysis observed with the micelles, the β- casein was probably hydrolysed after extrusion as a soluble form. Analysis of the actual distribution of the β-casein (as well as α_{s1}- and κ- caseins) between the soluble and micellar forms would be useful to substantiate these proposals.

The sites at which β- casein is cleaved by the proteinase from *S. lactis* 4760 (section 3.4 and Figure 4.1) are very similar to those formed for the *S. lactis* 763 enzyme (Monnet *et al.*, 1986) as well as those recently reported for the enzyme from *S. cremoris* HP (Visser *et al.*, 1987). Like the 763 and HP cells, the proteinase from *S. lactis* 4760 cleaves at sites located near the C- terminal end of the β- casein molecule between residues 166-167, 175 - 176, 182- 183 and 193- 194. The sequence of amino acids Pro-X-Pro-Gln could be identified as preceeding the cleavage sites between residues 175-176 and 182- 183. However, apart from this consensus sequence, no other hallmark of the enzyme cleavage specificity could be found. The cleavage sites between residues 207 and 209 by the *S. lactis* 763 enzyme (Monnet *et al.*, 1986) was not observed for either the 4760 enzyme of this study or the *S. cremoris* HP enzyme studied by Visser *et al.* (1987).

The cleavage sites found for the *S. cremoris* HP enzyme (Visser *et al.*, 1987) between residues 163- 164 and between residues 165- 166 were also found in the present study for the 4760 enzyme but were not reported by Monnet *et al.* (1986). Visser *et al.* (1987) also

reported a cleavage site between residues 167 and 168. While a peptide starting from serine 168 was not identified in peak 2 of the HPLC profile (Figure 3.4.1), a peptide of mass 868 corresponding to that of the sequence 168- 175 was identified as a minor constituent in this peak.

Thus this study has confirmed all of the sites reported by Visser *et al.* (1987) for the *S. cremoris* HP proteinase including three additional sites not found for the *S. lactis* 763 enzyme (Monnet *et al.*, 1986). In addition a minor cleavage site between residues 160- 161 has been identified in the present study (Figure 4.1). This cluster of additional sites, all of which lie on the N- terminal of a serine residue, produced only minor components of the range of peptides resolved by HPLC and may have been overlooked in the study by Monnet *et al.* (1986). The fact that one of the peptides present in the minor peak 3 terminated at lysine 169 (Table V) suggests that there may be yet another minor cleavage site between residue 169 and 170 but the predicted peptide 170- 175 has not been detected in the present study.

Apart from relatively small differences, the proteinase from *S. lactis* 4760 described in the present study and the enzymes from *S. lactis* 763 (Monnet *et al.*, 1986) and *S. cremoris* HP (Visser *et al.*, 1987) all cleave within the last 49 residues at the C- terminal end of the β- casein molecule. It is uncertain whether the observed cleavage pattern may be imposed by the structure of the β- casein molecule where this C- terminal region could be more exposed to hydrolysis. It is of interest to note here that the relative amounts of the various peptides eluted from the RP- HPLC is correlated to their position in the β- casein molecule, i.e. the quantities of peptides decreased as their distance from the C- terminus increased. This may suggest that the proteinase cleaves the β- casein progressively from the C- terminal end of the molecule, possibly from regions that are more structurally exposed to those that are less exposed to cleavage.

The limitation of cleavage of β- casein by the cell wall proteinase to the C- terminal region of the molecule described above raises some interesting questions in relation to the growth of the lactic streptococci in milk. Whether or not the bacteria cleave β- casein in milk in a pattern similar to that observed with soluble β- casein, the fragment between residues 193 to 209 separated from the solution studies has been shown to be identical to a peptide isolated from a Gouda- type cheese made with *S. cremoris* HP as the starter organism (Visser *et al.*, 1983b). The presence of this peptide in the final cheese product suggests that it may not be used by the bacteria as a source of amino acids for protein synthesis.

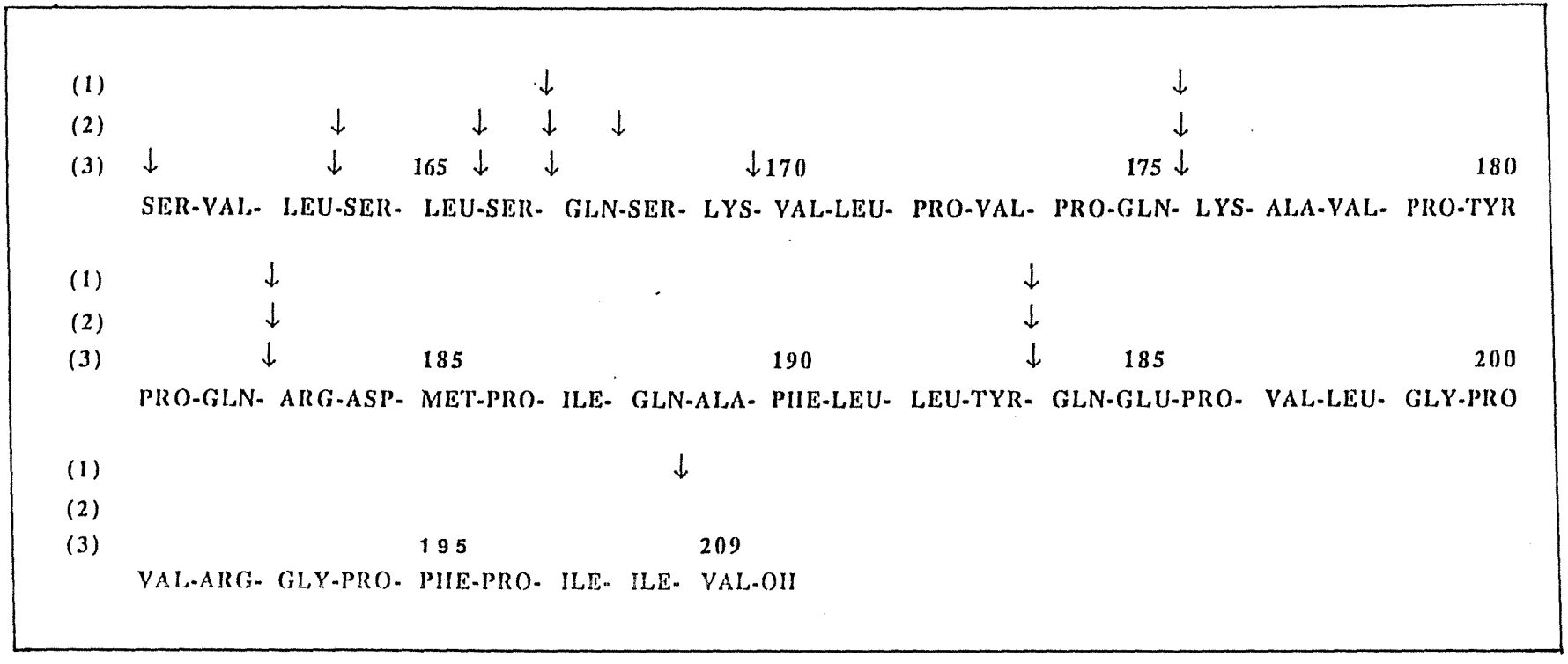


FIGURE 4.1. COMPARISON OF THE βCASEIN CLEAVAGE SITES BY THE PROTEINASES FROM *S. LACTIS* 763, *S. LACTIS* 4760 AND *S. CREMORIS* HP (WITHIN THE C- TERMINAL 49 RESIDUES).

(1) *S. lactis* 763 (Monnet *et al.* (1986).

(2) *S. cremoris* HP (Visser *et al.* (1987).

(3) *S. lactis* 4760.

It would appear unlikely that the peptides from residues 161 to 209 of the β- casein molecule could be the only part of the milk proteins used as a nitrogen source for bacterial growth since this region does not include all the amino acids essential for the bacterial protein synthesis (eg. histidine is absent in this region). Experiments to study the incorporation of labelled amino acids into bacterial protein during growth in media supplemented with defined- and labelled- protein substrates may help in clarifying the relationship between the observed proteinase activity from the studies described above and the actual process of protein hydrolysis in milk cultures.

Comparison of the β- casein cleavage specificity of the proteinase released from *S. cremoris* H2 to that from *S. lactis* 4760 (section 3.5.4) indicates that the transconjugant 4760 strain does indeed produce a similar proteinase to that produced by its plasmid donor, strain H2. This is hardly surprising but it does confirm that the specificity of the partially purified proteinase is determined by the plasmid- encoded enzyme since the DNA homology studies of the Prt gene in *S. lactis* H1, the plasmid recipient component of 4760, indicate that it may be quite different from the *S. cremoris* H2 Prt gene (L. Pearce, personal communication).

When the specificity of β- casein cleavage by the proteinase form *S. cremoris* SK11 was compared to that for the *S. lactis* 4760 enzyme, no apparent difference in the peptide products could be found although a complete analysis of all the HPLC peaks was not carried out. Although previous workers (Exterkate *et al.*, 1985;Visser *et al.*, 1986) have shown that the SK11 enzyme is an AM1- type, characterised by its ability to hydrolyse α_{s1}- casein as well as β- casein, the α_{s1}- casein- hydrolysing activity was not observed in this study. Visser *et al.* (1986) also showed that the pattern of β- casein cleavage by the SK11 proteinase, as visualised by polyacrylamide gel fractionation of digests, differed from that obtained for the HP- type proteinase. The electrophoretic pattern of SK11 proteinase- β- casein digests, obtained in the present study, was also different from that obtained using the 4760 enzyme but the differences were not evident in the HPLC profile of the peptide products.

The SK11 strain used in this study originated from the Dairy Research Institute (DRI, Palmerston North). It was sent to the United Kingdom and the Dutch workers, who have used this strain in specificity studies, presumably obtained it from the U.K. source. An isolate of the U.K. SK11 strain was subsequently sent back to the DRI. This isolate was found to have some alterations in phage- sensitivity, suggesting that the strain may have changed slightly. It is possible that some change in the plasmid- encoded proteinase may have also occurred. Further work with this SK11 isolate and with other AM1- type strains

would be required to verify the observed proteinase activity of the SK11 strain revealed by the preliminary studies described above.

To summarise, the sudies described above have largely confirmed the findings of previous work on the cell wall proteinase of the lactic streptococci. The observations that the enzymes preferentially hydrolyse β- casein with no obvious bond specificity and that the cleavage sites are limited to the C- terminal 49 residues of the β- casein molecule, raise some questions if these observations represent the actual situation in milk. First of all, how is the β- casein brought into contact with the proteinase when it is mainly present as micellar aggregates with α_{s1}- and κ- caseins? Secondly, could the limited hydrolysis of β- casein provide a sufficient nitrogen source for bacterial growth to high cell density?

Another important consequence of the preferential hydrolysis of β- casein by the lactic streptococcal proteinase is the influence it may have on the production of bitter flavour in cheese (Visser *et al.*, 1983b). The balance of rapid fermentation and reduced bitterness in cheese is a subject of economic importance in the cheese manufacturing industry. As described in the Introduction (section 1.6), the bacterial strains known to cause bitter flavour in cheese possess the HP- type of proteinase activity. These strains also coagulate milk rapidly. On the other hand, strains known to produce non- bitter flavours have the AM1- type proteinase and coagulate milk only slowly. Since these two types of proteinase have been shown to generate different patterns of β- casein cleavage as revealed by polyacrylamide gel electrophoresis of hydrolysis products (Visser *et al.*, 1986), it was suggested that the peptides generated by the HP- type proteinase may contribute to bitter cheese flavour.

While the biochemical and physiological basis for the relationship between particular strains and the production of bitter flavour is complex and controversial (Thomas & Pritchard, 1987), including considerations of growth rate, relative proportions of Prt^- and Prt^+ variants in the starter culture as well as the relative activities of proteinases and peptidases, it is nevertheless important to extend the work done in the present study on the nature of the peptide products of proteinase action on specific milk proteins. In particular the apparent conflict between the findings in the present study, that the low molecular weight peptides produced by the action of the SK11 proteinase in β- casein are the same as those produced by the HP- type proteinase from *S. cremoris* H2 (and *S. lactis* 4760), and the assignment of the SK11 proteinase to a " non- bitter " category (Visser *et al.*, 1986) in contrast to the H2 strain which is known to be a "bitter " strain needs to be resolved.

Since the genes for the HP- and AM1- type proteinase have been isolated (Kok & Venema, 1988), cloning of the HP- type proteinase into the AM1- type strains and vice- versa could be carried out and the growth rate as well as the effect of the growth rate of the "recombinant" bacteria in milk on cheese flavour examined. Studies on the peptidase enzymes are equally important to contribute to this area of starter growth in cheese production.

REFERENCES

Appleby, R. D. (1986)
A restriction endonuclease map of the *Streptococcus cremoris* lactose plasmid pD121 and location of the gene for tagatose 1, 6- bisphosphate aldolase.
Honours Dissertation, Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand.

Cliffe, A. J. and Law, B. A. (1985)
Discontinuous polyacrylamide gel electrophoresis of cell wall proteinase from variants of *Streptococcus lactis*.
J. Appl. Bacteriol. **58**, 245- 250.

Crow, V. L. and Pritchard, G. G. (1977)
Fructose 1,6 diphosphate- activated L- lactate dehydrogenase from *Streptococcus Lactis* : kinetic properties and factors affecting activation.
J. Bacteriol. **131**, 82- 91.

Crow, V. L., Davey, G. P., Pearce, L. E. and Thomas, T. D. (1983)
Plasmid linkage of the D- tagatose 6- phosphate in *Streptococcus lactis* : Effect on lactose and galactose metabolism.
J. Bacteriol. **153**, 76- 83.

De Vos, W. M. (1986)
Gene cloning in lactic streptococci.
Neth. Milk Dairy J. **40**, 141- 154.

De Vos, W. M. (1987)
Gene cloning and expression in lactic streptococci.
FEMS Microbiol. Revs. **46**, 281- 295.

Exterkate, F. A. (1975)
An introductory study of the proteolytic system of *Streptococcus cremoris* strain HP.
Neth. Milk Dairy J. **29**, 303- 318.

Exterkate, F. A. (1976)
Comparison of strains of *Streptococcus cremoris* for proteolytic activitites associated with the cell wall.
Neth. Milk Dairy J. **30**, 95- 105.

Exterkate, F. A. (1979)
Accumulation of proteinase in the cell wall of *Streptococcus cremoris* strain AM1 and regulation of its production.
Archives of Microbiol. **120**, 247- 254.

Exterkate, F. A. (1983)
A dual- directed control of the cell wall proteinase production in *Streptococcus cremoris* AM1: a possible mechanism of regulation during growth in milk.
J. Dairy Sci. **68**, 562- 571.

Exterkate, F. A. and de Veer, G. J. C. M. (1985)
Partial isolation and degradation of caseins by cell wall proteinase(s) of *Streptococcus cremoris* HP
Appl. Env. Microbiol. **49**, 328- 332.

Exterkate, F. A. and de Veer, G. J. C. M. (1987)
Complexity of the native cell wall proteinase of *Lactococcus lactis subspecies cremoris* HP and purification of the enzyme.
Syst. Appl. Microbiol. **9**, 183- 191.

Ezzat, N., El Soda, M., Bouillanne, C., Zevaco, C. and Blanchard, P. (1985)
Cell wall- associated proteinase in *Lactobacillus helveticus*, *Lactobacillus bulgaricus* and *Lactobacillus lactis*.
Michwissenschaff **40** , 140- 143.

Gasson, M. J. (1986)
In: Biomolecular Engineering in the European Community (Magnien E., ed.), Final report, Martinus Nijhoff Publishers, Dordrecht, pp. 465- 472

Geis, A., Bockelmann, W. and Teuber, M. (1985)
Simultaneous extraction and purification of a cell wall associated peptidase and β– casein specific protease from *Streptococcus cremoris* AC1.
Appl. Microbiol. Biotechnol. **23**, 79- 84.

Geis, A., Kiefer, B. and Teuber, M. (1986)
Proteolytic activities of lactic acid streptococci isolated from dairy starter cultures.
Chem. Mikrobiol. Technol. Lebensm. **10**, 93- 95.

Hugenholtz, J., Exterkate, F. A. and Konings, W. N. (1984)
The proteolytic system of *Streptococcus cremoris* - An immunological analysis.
Appl. Env. Microbiol. **48**, 1105- 1110.

Hugenholtz, J. van Sinderen, D., Kok, J. and Konings, W. N. (1987)
The cell wall- associated proteases of *Streptococcus cremoris* Wg2
Appl. Env. Microbiol. **53**, 853- 859.

Kanasaki, M., Breheny, S., Hillier, A. J. and Jago, G. R. (1975)
Effect of temperature on the growth and acid production of lactic acid bacteria, 1. A rapid method for the estimation of bacterial populations in milk.
Aust. J. Dairy Technol. **30**, 142- 144.

Kok, J., van Dijl, J. M., van der Vossen, J. M. B. M., and Venema, G. (1985)
Cloning and expression of a *Streptococcus cremoris* proteinase in *Bacillus subtilis* and *Streptococcus lactis*..
Appl. Env. Microbiol. **50**, 94- 101.

Kok, J. and Venema, G. (1988)
Genetics of proteinases of lactic acid bacteria.
Biochimie **70**, 475- 488.

Kok, J., Leenhouts, K. J., Haandrikman, A. J., Ledeboer, A. M. and Venema, G. (1988)
Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2.
Appl. Env. Microbiol. **54**, 231- 238.

Laemmli, U. K. (1970)
Cleavage of structural proteins during the assembly of the head of bacteriophage T4.
Nature **227**, 680- 685.

Law, B. A., Sezgin, E. and Sharpe, M. E. (1976)
Amino acid nutrition of some commercial cheese starters in relation to their growth in peptone-supplemented whey media.
J. Dairy Res. **43**, 291- 300.

Law, B. A. and Kolstad, J. (1983)
Proteolytic systems in lactic acid bacteria.
Antonie van Leeuwenhoek **49**, 225- 245.

Law, B. A. (1984)
Flavour development in cheeses. In: Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk (Davies, F. L. and Law, B. A., Eds.), Elsevier Applied Science Publishers, London, pp. 187- 208 and 209- 228.

Lawrence, R. C. and Pearce, L. E. (1968)
The case against the use of unpaired single starter strains.
N.Z. J. Dairy Sci. Technol. **3**, 137- 139.

Lawrence, R. C., Creamer, L. K. and Gilles, J. (1987)
Texture development during cheese ripening.
J. Dairy Sci. **70**, 1748- 1760.

Lowrie, R. J., Lawrence, R. C., Pearce, L. E. and Richards, E. L. (1972)
Cheddar cheese flavour, III. The growth of lactic streptococci during cheesemaking and the effect on bitterness development.
N.Z. J. Dairy Sci. Technol. **7**, 44- 50.

Lowry, O. H., Rosebrough, N. J., Fair, A. L. and Randall, R. J. (1951)
Protein measurement with the Folin phenol reagent.
J. Biol. Chem. **193**, 265- 275.

McKay, L. L., Baldwin, K. A.(1975)
Plasmid distribution and evidence for a proteinase plasmid in *Streptococcus lactis* C2.
Appl. Microbiol. **29**, 546- 548.

Marshall, V. M. E. and Law, B. A. (1984)
The physiology and growth of dairy lactic- acid bacteria. In: Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk. (Davies, F. L. and Law, B. A., Eds.), Elsevier Applied Science Publishers, London, pp. 67- 98.

Martley, F. G. and Lawrence, R. C. (1972)
Cheddar cheese flavour, II. Characteristics of single strain starters associated with good or poor flavour development.
N.Z. J. Dairy Sci. Technol. **7**, 38- 44.

Mills, O. E. and Thomas, T. D. (1978)
Release of cell wall- associated proteinase(s) from lactic streptococci.
N.Z. J. Dairy Sci. Technol. **13**, 209- 215.

Mills, O. E. and Thomas, T. D. (1981)
Nitrogen sources for growth of lactic streptococci in milk.
N.Z. J. Dairy Sci. Technol. **16**, 43- 55.

Monnet, V., LeBars, D. and Gripon, J. (1986)
Specificity of a cell wall proteinase from *Streptococcus lactis*. NCDO 763 towards bovine β-casein.
FEMS Microbiol. Lett. **36**, 127- 131.

Monnet, V., Le Bars, D. and Gripon, J. (1987)
Purification and characterisation of a cell wall proteinase from *Streptococcus lactis* NCDO 763.
J. Dairy Res. **54**, 247- 255.

Monnet, V., LeBars, D., Neviani, E. and Gripon, J. C. (1987)
Partial characterisation and comparison of cell wall proteinase from 5 strains of *Streptococcus lactis*.
Lait **67**, 51- 61.

Nairn, R. C. (1976)
Fluorescent Protein Tracing, Churchill Livingston, Longman Group United, England 4th. ed., pp 30 and 61.

Ohmiya, K. and Sato, Y. (1975)
Purification and properties of an intracellular proteinase from *Streptococcus cremoris*..
Appl. Microbiol. **30** , 738- 745.

Pearce, L. E., Skipper, N. A. and Jarvis, B. D. W. (1974)
Proteinase activity in slow lactic acid- producing variants of *Streptococcus lactis*.
Appl. Microbiol. **27**, 933- 937.

Reiter, B. and Oram, J. D. (1962)
Nutritional studies on cheese starters,1. Vitamin and amino acid requirements of single strain starters.
J. Dairy Res. **29**, 63- 77.

Rice, G. H., Stewart, F. H. C., Hillier, A. J. and Jago, G. R. (1978)
The uptake of amino acids and peptides by *Streptococcus lactis*.
J. Dairy Res. **45**, 93- 107.

Scheiffer, K. H., Kraus, J., Dvorak, C., Kilper-Bulz, R., Collins, M. D., and Fischer, W.(1985)
Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus ge. nov.*
Syst. Appl. Microbiol. **6**, 183- 195.

Stadhouders, J., Hup, G., Exterkate, F. A. and Visser, S. (1983)
Bitter flavour in cheese. 1. Mechanism of the formation of the bitter flavour defect in cheese.
Neth. Milk Dairy J. **37**, 157- 167.

Sullivan, J. J. and Jago, G. R. (1972)
The structure of bitter peptides and their formation from casein.
Aust. J. Dairy Technol. **27**, 98- 104.

Terzaghi, B. E. and Sandine, W. E. (1975)
Improved medium for lactic streptococci and their bacteriophages.
Appl. Microbiol. **29**, 807- 813.

Thomas, T. D., Jarvis, B. D. W. and Skipper, N. A. (1974)
Localisation of proteinase(s) near the cell surface of *Streptococcus lactis*.
J. Bacteriol. **118** , 329- 333.

Thomas, T. D. and Turner, K. W. (1977)
Preparation of skim milk to allow harvesting of starter cells from milk cultures.
N.Z. J. Dairy Sci. Technol. **12**, 15- 21.

Thomas, T. D. and Mills, O. E. (1981)
Proteolytic enzymes of starter bacteria.
Neth. Milk Dairy J. **35**, 255- 273.

Thomas, T. D. and Pritchard, G. G. (1987)
Proteolytic enzymes of dairy starter cultures.
FEMS Microbiol. Revs. **46**, 245- 268.

Twining, S. S. (1984)
Fluorescein isothiocyanate- labelled casein assay for proteolytic enzymes.
Anal. Biochem. **143**, 30- 34.

Visser, S. (1981)
Proteolytic enzymes and their action on milk proteins. A review.
Neth. Milk Dairy J. **35**, 65- 88.

Visser, S., Hup, G., Exterkate, F. A. and Stadhouders, J. (1983a)
Bitter flavour in cheese, 2. Model studies on the formation and degradation of bitter peptides by proteolytic enzymes from calf rennet, starter cells and starter cell fractions.
Neth. Milk Dairy J. **37**, 169- 180.

Visser, S., Slangen, K. J., Hup, G. and Stadhouders, J. (1983b)
Bitter flavour in cheese.3. Comparative gel- chromatographic analysis of hydrophobic peptide fractions from twelve Gouda- type cheeses made with *Streptococcus cremoris* strain HP.
Neth. Milk Dairy J. **37**, 181- 192.

Visser, S., Exterkate, F. A., Slangen, C. J. and de Veer, G. J. C. M. (1986)
Comparative studies of the action of cell wall proteinases from various strains of *Streptococcus cremoris* in bovine α_{s1}-, β- and κ- casein.
Appl. Environ. Microbiol. **52**, 1162- 1166.

Visser, S., Exterkate, F. A., Slangen, C. J. and de Veer, G. J. C. M. (1987)
Specificity of a cell wall proteinase from *Streptococcus cremoris* HP in its action on bovine β-casein. poster abstract from the 2nd. Symposium on Lactic Acid Bacteria, Wageningen, The Netherlands.

Vreeman, H. J., Both, P., Brinkhuis, J. A. and van der Spek, C. (1977)
Purification and some physicochemical properties of bovine κ– casein.
Biochim. Biophys. Acta **491**, 93- 103.

Yu, P. L., Appleby, R. D., Pritchard, G. G. and Limsowtin, G. K. Y. (1989)
Restriction mapping and localisation of the lactose- metabolising genes of plasmid pD1- 21 from *Streptococcus cremoris*.
Appl. Microbiol. Biotechnol. (in press).