Purification and characterization of the free form of the lactococcal extracellular proteinase and its autoproteolytic cleavage products

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(Received 17 October 1990; revised 1 March 1991; accepted 5 March 1991)

In addition to the cell wall proteinase, *Lactococcus lactis* subsp. *cremoris* produced significant amounts of a free extracellular proteinase. The free proteinase activity was highest in the late exponential and early stationary phase of growth, whereas the cell wall activity was highest in the last half of the exponential phase. Both proteinase forms had a pH optimum between 4.6 and 5.8, and they behaved similarly upon anion exchange and hydrophobic interaction chromatography, chromatofocusing and gel filtration, indicating that they were related. Purification to homogeneity, as judged by SDS-PAGE, resulted in a 50 000–80 000-fold increase in the specific activity of the free proteinase. It contained two major protein species (termed pro150 and pro115) with proteinase activity. As judged by SDS-PAGE, the M_r values of pro150 and pro115 were 150 000 and 115 000, respectively, and by chromatofocusing the isoelectric points were 4.3 and 4.1, respectively. Upon gel filtration, pro150 and pro115 had M_r values of 300 000 and 125 000, respectively, indicating that pro150 was a dimer and pro115 a monomer. Pro115 was an autodegradation product of pro150. Other distinct autodegradation products had M_r values of 90 000 (p90), 53 000 (p53), 37 000 (p37) and 30 000 (p30). These had little if any proteinase activity. Pro115, p90 and p53 had a common N-terminal sequence with that reported for the cell wall proteinase. Judging from its N-terminal sequence and M_r , p30 was derived from the C-terminal half of p53. Cleavage of pro150 to pro115 generated p37.

Introduction

The proteolytic enzymes of lactococci are of importance in the use of these bacteria in the production of fermented milk products. Degradation of milk proteins is essential for growth of these bacteria on milk products as well as for the development of the products' texture and flavour (Fox, 1989; Law & Kolstad, 1983; Thomas & Mills, 1981). Thus, knowledge as to the types of proteinases that appear extracellularly during growth of lactococci – the substrate specificity and biochemical characteristics of the proteinases, and how the enzymes vary in different strains – is of importance in the use of lactococci in food production. Consequently, a proteinase(s) associated with the cell wall has been the subject of much research and the genetics of this proteinase have recently been well characterized (Haandrikman *et al.*, 1989; Kok *et al.*, 1988a, b; Kok & Venema, 1988; Vos *et al.*, 1989a, b).

In the present study we found that many strains of lactococci released significant amounts of proteinase activity into the growth medium. We report here the purification to homogeneity of the free extracellular

proteinase of one strain. The enzyme was characterized and directly compared to the cell wall proteinase to determine whether the free proteinase was novel or was related to the cell wall proteinase. In addition, characteristic autoproteolytic degradation products as well as areas of the proteinase which are prone to autoproteolytic cleavage have been identified.

Methods

Bacterial strains and media. *Lactococcus lactis* subsp. *cremoris* NCDO 1201, obtained from the National Collection of Food Bacteria, AFRC Institute of Food Research, Reading, UK, was grown at 30 °C in M17 broth (Oxoid) without lactose, but supplemented with 0.5% (w/v) glucose.

Assay for proteinase activity. Proteinase activity was assayed by measuring production of trichloroacetic acid (TCA)-soluble radioactive peptides from an [³H]acetylcasein substrate, essentially as described by Hatcher *et al.* (1977). The assay was carried out in 200 µl reaction mixtures containing 200 mM-sodium phosphate (pH 5.3), radioactive casein (5×10^4 c.p.m.; sp act: 50 c.p.m. ng⁻¹) and various amounts of proteinase-containing fractions. The reaction mixtures were incubated for 2 h at 37 °C. One hundred microlitres of a 2.5% (w/v) solution of unlabelled casein in 20 mM-sodium phosphate (pH 7.1) and 1 M-KCl were then added to each reaction mixture, followed by

200 μ l ice-cold 7.5% (w/v) TCA. The reaction mixtures were then centrifuged at 14000 g for 10 min, and the radioactivity in the supernatants was measured in a liquid scintillation counter. One enzyme unit (EU) was defined as the amount of enzyme that degrades 1% of the ^3H -labelled casein into TCA-soluble products under standard assay conditions.

Ammonium sulphate precipitation and binding to Phenyl Sepharose. For purification of the free extracellular proteinase, an overnight culture in the early stationary phase was rendered cell-free by centrifugation at 4000 g for 15 min. Three hundred grams $(\text{NH}_4)_2\text{SO}_4$ per litre of cell-free culture medium (10–4 °C) were then added. The precipitated proteins were pelleted by centrifugation at 7000 g for 20 min and solubilized in 20 mM-Bistris, pH 5.8 (50 ml per litre culture medium); this resulted in a 200–300-fold increase in the specific activity of the proteinase and a 20-fold concentration (see Table 1). Four grams of $(\text{NH}_4)_2\text{SO}_4$ were then added per 50 ml of the solubilized protein preparation, and it was applied to a 10 ml Phenyl Sepharose CL-4B (Pharmacia) column which had been equilibrated with 20 mM-Bistris, pH 5.8, and 0.6 M- $(\text{NH}_4)_2\text{SO}_4$. The proteinase was eluted with 15–20 ml 20 mM-Bistris, pH 5.8. This resulted in a 1000–5000-fold increase in the specific activity of the proteinase and a yield of 200–300% (see Table 1).

For purification of the cell wall-associated proteinase, bacteria were harvested in the late exponential phase of growth by centrifugation at 4000 g for 15 min. The bacteria were washed once in 20 mM-Tris/HCl, pH 7.2, and 15 mM- CaCl_2 (200 ml per litre of culture), and then suspended with stirring for 4 h at 20 °C in 20 mM-Tris/HCl, pH 7.2, and 15 mM-EDTA (40 ml per litre of culture). The CaCl_2 was included in the former buffer in order to minimize release of the cell wall proteinase when washing the cells, whereas the presence of EDTA in the latter buffer was to optimize its release (Laan & Konings, 1989). The Ca^{2+} -containing washing buffer did not have significant proteolytic activity, and thus the free proteinase did not contribute to the activity in the cell-wall-derived proteinase preparation. The bacteria were then removed by centrifugation, and $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of 1 M to the supernatant, which contained the cell-wall-derived proteinase, after which the supernatant was applied to a 5 ml Phenyl Sepharose CL-4B column. The cell-wall-derived proteinase was eluted from the column with 10 ml 20 mM-Tris/HCl, pH 7.2. This resulted in a nearly 10-fold purification and a recovery of 80–90% compared to that obtained after release of the proteinase (see Table 1). When starting with equal volumes of cell cultures, about five times more of the free than the cell wall proteinase was obtained at this stage (Table 1).

Ion exchange and hydrophobic interaction chromatography. The proteinase which eluted from the Phenyl Sepharose column was transferred to 20 mM-Bistris, pH 5.8, by gel filtration using Sephadex G-25 PD-10 columns (Pharmacia), and subsequently applied to a Mono Q HR 5/5 anion exchange column (Pharmacia) equilibrated with 20 mM-Bistris, pH 5.8. The proteins were eluted with a gradient of NaCl in the Bistris buffer. Ammonium sulphate to a final concentration of 1 M was added to the proteinase-containing fractions, which were then applied to a Phenyl Superose HR 5/5 column (Pharmacia) equilibrated with 20 mM-Bistris, pH 5.8, and 1 M- $(\text{NH}_4)_2\text{SO}_4$. The proteins were eluted from the column by decreasing the concentration of $(\text{NH}_4)_2\text{SO}_4$.

Chromatofocusing and gel filtration. The proteinase obtained after hydrophobic interaction chromatography was transferred to 25 mM-methylpiperazine, pH 5.7, by gel filtration using the PD-10 columns as described above, and then applied to a Mono P HR 5/20 column (Pharmacia) equilibrated with the methylpiperazine buffer. The proteins were eluted from the column with polybuffer 74, pH 3.5 (Pharmacia). The proteinase was then applied to a Sephacryl S-200 (Pharmacia) column (90 $\times$ 2.6 cm) and eluted with 0.1 M-NaCl in

20 mM-sodium phosphate, pH 7.0. The column was calibrated with blue dextran, ferritin (M_r 440000), catalase (232000), aldolase (158000), bovine serum albumin (67000), ovalbumin (43000), chymotrypsinogen (25000) and RNAase A (13700). All chromatography was performed at 20 °C, with flow rates of 1 ml min $^{-1}$ and with the use of the FPLC system (Pharmacia).

SDS polyacrylamide gel electrophoresis (SDS-PAGE). Protein fractions were analysed by SDS-PAGE using the Phast-System (Pharmacia), and the 8–25 % gradient polyacrylamide gels were stained using the PhastGel Silver Kit (Pharmacia). The high- M_r standards (Pharmacia) were myosin (M_r 212000), α -macroglobulin (170000), β -galactosidase (116000), transferrin (76000) and glutamic dehydrogenase (53000). The low- M_r standards (Bio-Rad) were phosphorylase b (M_r 97400), bovine serum albumin (66200), ovalbumin (43000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400).

Amino acid sequence analysis. Proteins to be sequenced were concentrated using Centricon Microconcentrators (Amicon). N-terminal amino acid sequence analysis was performed on these samples using an Applied Biosystems 477A protein sequencer. The phenylthiohydantoin-derivatized amino acid residues were determined on line by an Applied Biosystems 120 phenylthiohydantoin analyser. The proteins needed for N-terminal sequencing were purified from 6–9 litres of culture.

Results and Discussion

Proteinase activity in various growth phases, and pH optimum

In addition to the well-characterized cell-wall-associated proteinase, *Lactococcus lactis* subsp. *cremoris* produced a free extracellular proteinase(s). Out of 28 arbitrarily chosen strains tested in the early stationary phase of growth, 22 released significant amounts of proteinase activity into the growth medium, the amount of activity released often being comparable to the amount associated with the cell wall (data not shown). This prompted us to purify the free proteinase and directly compare it to the cell wall-associated proteinase. The strain NCDO 1201 produced the largest amount of free extracellular activity, and the proteinase from this strain was consequently chosen for further study.

The amount of proteolytic activity present during the various growth phases was determined, in order to obtain high yields for purification. The free activity was highest at the very end of the exponential phase and in the early stationary phase, whereas the cell-wall-associated activity was greatest during the last half of the exponential phase, and fell in the early stationary phase (Fig. 1). Thus, the kinetics of accumulation of the proteinases suggested that they might be related, as did the fact that the two enzymes had the same pH optimum – between pH 4.6 and 5.8 (data not shown). This pH optimum was similar to the value of 5.5–6.0 reported by Geis *et al.* (1985), but significantly less than the 6.2–6.6 reported by

Fig. 1. Amount of free (○—○) and cell-wall-associated (●—●) proteinase activity during growth of a culture of *L. lactis* subsp. *cremoris* NCDO 1201. Samples were taken at the indicated times and the OD_{610} (●—●) was measured. The bacteria in the samples were pelleted and resuspended in an equal volume of 20 mM-sodium phosphate, pH 5.5, and the proteinase activity in the supernatant (free proteinase) and bacterial suspension (cell-wall-associated proteinase) was measured.

Exterkate & de Veer (1987) for the cell wall proteinases from other strains.

Anion exchange and hydrophobic interaction chromatography

Upon chromatography of the partially purified proteinase fractions (see Methods) on a Mono Q anion exchange column, the free and cell wall proteinases eluted from the column at similar salt concentrations. However, the free proteinase had a broader elution profile and was thus more heterogeneous than the cell wall proteinase (Fig. 2). The cell wall proteinase was also somewhat heterogeneous, in that two partly overlapping activity peaks were obtained (Fig. 2*b*). The heterogeneity was in part due to the fact that the proteinase activity derived from two major proteins, one of which was a cleavage product of the other (results presented below). Moreover, immunological studies suggest that the cell wall proteinase may exist in two or more distinct conformations (Hugenholtz *et al.*, 1984, 1987; Kok *et al.*, 1988*a*; Laan *et al.*, 1988), and this conformational heterogeneity might also explain the heterogeneous chromatographic behaviour.

Upon further purification by hydrophobic interaction chromatography, the free proteinase eluted at a slightly higher salt concentration than the cell wall proteinase (Fig. 3), and thus was overall somewhat less hydrophobic. At this stage the specific activity had increased by 70000–80000-fold, with a 200–300% yield, for the free proteinase, and by 500–600-fold, with a 40–50% yield, for the cell wall proteinase (Table 1). For both proteinases a specific activity of about 40000 was

Fig. 2. Chromatography on a Mono Q anion exchange column of the (a) free and (b) cell wall proteinases which eluted from the Phenyl Sepharose column. The proteinases were from a 3 litre culture. No activity was detected in the flow-through fraction.

attained (Table 1), and this appears to be the specific activity of the pure enzymes. In some experiments, however, this specific activity was not attained for the free proteinase until after chromatofocusing and gel filtration, and these two subsequent steps were consequently included in the purification scheme. A significant increase in the purity of the cell wall proteinase was not attained upon further chromatography, but it was nevertheless carried out in order to compare this enzyme's chromatographic characteristics with those of the free proteinase.

Chromatofocusing and gel filtration

The elution profiles obtained upon chromatofocusing the free and cell wall proteinases were nearly identical (Fig. 4). In both cases there were two activity peaks with coinciding A_{280} peaks which eluted with apparent isoelectric points of 4.3 and 4.1. The two elution profiles differed in that there was relatively more of the 4.3 isoelectric point proteinase component in the free than in the cell-wall-derived preparation. There was also an A_{280} peak eluting with an isoelectric point of 4.2 (Fig. 4), and this peak was due to an inactive degradation product of the proteinases (see results below). The isoelectric points are similar to the values of 4.40–4.55 obtained upon

Table 1. Purification of the free extracellular and cell wall proteinases

Fraction	Free extracellular proteinase						Cell wall proteinase					
	Vol. (ml)	Total A_{280} *	Enzyme units	Sp. act.†	Purif. (-fold)‡	Yield (%)	Vol. (ml)	Total A_{280} *	Enzyme units	Sp. act.†	Purif. (-fold)‡	Yield (%)
Culture medium	3000	87000	46000	0.5	1	100	—	—	—	—	—	—
Extract derived from cell wall	—	—	—	—	—	—	120	420	30000	70	1	100
(NH ₄) ₂ SO ₄ precipitation	150	650	82000	125	250	175	—	—	—	—	—	—
Binding to Phenyl Sepharose	50	60	134000	2200	4400	290	30	45	25000	550	8	80
Anion exchange chromatography	8	6	135000	22500	45000	290	4	0.75	27000	36000	500	88
Hydrophobic interaction chromatography	10	3	117000	39000	78000	250	8	0.3	13000	43000	610	43
Chromatofocusing	5	1.5	42000	28000	56000	90	4	0.2	8000	40000	550	26
Gel filtration	30	0.35	13000	37000	74000	28	30	0.04	1400	35000	500	5

* Total A_{280} is the absorbance at 280 nm multiplied by the volume.

† Specific activity is the enzyme activity (in EU) divided by the absorbance at 280 nm.

‡ Purification (-fold) is the increase in specific activity.

Fig. 3. Hydrophobic interaction chromatography on a Phenyl Superose column of the (a) free and (b) cell wall proteinases which eluted from the Mono Q column. The proteinases were from a 3 litre culture. No activity was detected in the flow-through fraction.

isoelectric focusing the cell wall proteinase from other strains (Exterkate & de Veer, 1987; Hugenholtz *et al.*, 1987).

Upon gel filtration of the free 4.3 isoelectric point proteinase component, a major activity and A_{280} peak eluted at an M_r of about 300000, with a marked shoulder at an M_r of 175000 (Fig. 5a). The free 4.1 isoelectric point component eluted with an M_r of about 125000 (Fig. 5b). Similar results were obtained with the cell wall proteinase (data not shown). The material absorbing at 280 nm which eluted after the proteinase activity peaks (Fig. 5) was due to auto-degradation products, since the A_{280} in this part of the elution profile was minimal when the proteinase was inactivated by exposure to HCl (pH 2.5) prior to gel filtration (data not shown).

Gel electrophoretic analysis of the proteinase

The proteinase fractions obtained after gel filtration were concentrated by chromatography on a Mono Q anion exchange column at pH 7.5 and subsequently analysed by SDS-PAGE. Two major proteins (termed pro150 and pro115) with proteinase activity were identified in the free proteinase preparation (Fig. 6, lanes 2 and 3; similar data were obtained for the cell wall proteinase, but are not presented). Pro150 eluted with an M_r of 300000 upon gel filtration (Fig. 5a); it was released from the Mono Q column at about 0.22 M-NaCl (data not shown), and its M_r as judged by SDS-PAGE was 150000 (Fig. 6, lane 2). It thus appears to be a dimer under non-denaturing conditions. The M_r of pro150 as determined by SDS-PAGE is similar to the values of 130000–150000 previously reported for the mature cell wall proteinase of

Fig. 4. Chromatofocusing on a Mono P column of the (a) free and (b) cell wall proteinases which eluted from the Phenyl Superose column. The proteinases were from a 3 litre culture. No activity was detected in the flow-through fraction.

other strains (Bockelmann *et al.*, 1989; Exterkate & de Veer, 1987; Geis *et al.*, 1985; Hugenholtz *et al.*, 1987). Pro115 eluted with an M_r of about 125 000 upon gel filtration (Fig. 5b); it was released from the Mono Q column at about 0.25 M-NaCl (data not shown), and its M_r as judged by SDS-PAGE was 115 000 (Fig. 6, lane 3). Thus, pro115 is a monomer under non-denaturing conditions. Pro115 is a degradation product of pro150, as judged by the cleavage products obtained upon incubating pro150 at 37 °C (see results below).

Upon concentrating pro115 on the Mono Q column prior to SDS-PAGE, a major A_{280} peak also eluted at 0.23 M-NaCl. This peak contained a protein of M_r 90 000 (termed p90; Fig. 6, lane 4; similar data were obtained for the cell wall proteinase, but are not presented). As judged by the cleavage products obtained upon incubating pro115 at 37 °C, and the fact that pro115 and p90 had the same N-terminus (see results below), p90 is a degradation product of pro115. The active site of the proteinase is in the N-terminal half of the enzyme (Kok *et al.*, 1988b; Vos *et al.*, 1989a), and p90 should consequently contain the active site. Despite this,

Fig. 5. Gel filtration on a Sephacryl S-200 column of the free proteinase which eluted with an isoelectric point of (a) 4.3 and (b) 4.1 upon chromatofocusing. The proteinase was from a 3 litre culture.

p90 had little if any proteinase activity, since no activity peak coincided with the A_{280} peak of p90 when it was chromatographed on the Mono Q column (data not shown).

Analysis by SDS-PAGE of fractions obtained upon chromatofocusing (Fig. 4) revealed that the peaks eluting with isoelectric points of 4.3 and 4.1 were due to pro150 and pro115, respectively, whereas the A_{280} peak eluting with an isoelectric point of 4.2 was due to p90 (data not shown). Similar analysis of fractions obtained upon anion exchange chromatography (Fig. 2) revealed that pro150 was in fractions 9–12 and pro115 in fractions 13–15 (data not shown). Upon hydrophobic interaction chromatography (Fig. 3), pro150 was similarly shown to elute slightly ahead of pro115 (data not shown).

The relative amount of pro150 as compared to pro115 was higher in the free than in the cell-wall-derived preparation. The two proteinase preparations were otherwise similar, indicating that the free and cell wall proteinases were related. The differences observed in their chromatographic behaviour may be explained by the relatively greater amount of pro150 in the free

Fig. 6. Analysis by SDS-PAGE of the free proteinase and its autoproteolytic cleavage products. Lanes 1 and 14 show the high- and low- M_r standards, respectively. Lanes 2, 3, 4 and 13 show the purified prol50, prol15, p90, and p53 and p30, respectively. The preparations analysed in lanes 3, 4 and 13 were used for N-terminal sequencing. Lanes 5–8 and 9–12 show the autoproteolytic cleavage products obtained upon incubating, respectively, prol50 and prol15 at 37 °C for the times indicated. The final chromatofocusing and gel filtration steps of the purification procedure were not included when isolating prol50 and prol15 for the degradation experiment shown in lanes 5–12. Lanes 1–4 are from the same gel, and similarly for lanes 5–12 and 13–14.

preparation combined with the fact that prol50 is less hydrophobic, has a slightly higher isoelectric point, and binds less tightly to anion exchangers than prol15.

Autoproteolytic cleavage and N-terminal amino acid sequencing

To identify autodegradation products, prol50 and prol15 were incubated at 37 °C and subsequently analysed by SDS-PAGE (Fig. 6, lanes 5–12). Incubating prol50 for 2 and 4 h resulted in a relative increase in prol15 and p90 as well as proteins of M_r less than 60000 (Fig. 6, lanes 5–7). Similarly, incubating prol15 for 2 and 4 h resulted in a relative increase in p90 (Fig. 6, lanes 9–11). Incubating prol50 for 30 h resulted in the degradation of prol50, prol15 and p90, and the accumulation of three distinct proteins of M_r about 53000, 37000 and 30000 – termed, respectively, p53, p37 and p30 (Fig. 6, lane 8). Only p53 and p30 were obtained upon incubating prol15 for 30 h (Fig. 6, lane 12), indicating that p37 was produced upon cleavage of prol50 to prol15. Since prol50 was a dimer and prol15 a monomer, dimerization of prol50 depended on a sequence in p37.

An attempt was made to purify these degradation products from each other. However, p53 and p30 co-eluted upon both anion exchange chromatography and chromatofocusing, whereas p37 was not recovered. The fraction obtained after chromatofocusing contained on a molar basis roughly two times more of p53 than p30, as judged by the relative intensity of the two bands detected

upon SDS-PAGE analysis of the chromatofocusing fraction (Fig. 6, lane 13).

The N-terminal sequence was determined for prol15, p90, p53 and p30 in order to: (i) definitely show that the free and cell wall proteinases were related and that the degradation products in fact derived from the proteinase, (ii) identify autoproteolytic cleavage sites and thereby gain insight into the amino acid specificity of the proteinase, and (iii) obtain information as to the parts of the mature proteinase from which the cleavage products stemmed. Two sequences were obtained for the fraction containing both p53 and p30 (Fig. 6, lane 13). The first nine amino acids of the two sequences were Asp-Ala-Lys-Ala-Asn-Ser-Met-Ala-Asn and Gln-Asp-Asn-Glu-Met-Val-Gly-Thr-Pro, respectively. There was 1.5–2.0 times more of the former sequence than the latter, indicating that the former sequence was that of p53 and the latter that of p30. Moreover, this is consistent with the determined M_r of 30000 for p30 (see Fig. 7). Both prol15 and p90 had the same N-terminal sequence as p53.

The nucleotide sequence was recently determined for the gene encoding the cell wall proteinase of *L. lactis* subsp. *cremoris* strains Wg2 and SK11 (Kok *et al.*, 1988b; Vos *et al.*, 1989a). The proteinase gene from these two strains codes for a protein with, respectively, 1902 and 1962 amino acids, and there is an overall homology between the genes of 98% (44 amino acid substitutions). The cell wall proteinase from strain SK11 is synthesized as a pre-pro-protein (Vos *et al.*, 1989a, b). The pre-pro-sequence is removed by cleavage between Thr-187 and

Fig. 7. A model illustrating the autoproteolytic cleavage sites and products, and the parts of the mature proteinase from which the cleavage products derived. Although not determined, it is proposed that the N-terminal sequence of pro150 also starts with amino acid residue number 188, as is the case for the mature cell-wall-derived proteinase from *L. lactis* subsp. *cremoris* strain SK11 (Vos *et al.*, 1989*a, b*). The amino acid numbers 186, 187, 400 and 401 (Pro, Thr, Gly and Leu), the total length of 1900–2000 amino acids for the initial translation product, and the amino acid numbering were all derived from the nucleotide sequence of the proteinase gene of strains Wg2 (Kok *et al.*, 1988*b*) and SK11 (Vos *et al.*, 1989*a*).

Asp-188, generating the mature cell wall proteinase with Asp-188 as the N-terminal amino acid. Judging from the N-terminal sequencing, the pro150 degradation products pro115, p90 and p53 all contained Asp-188 at the N-terminus, whereas p30 contained Gln-402. These sequences are identical to the corresponding sequences of the cell wall proteinases from strains Wg2 and SK11 (Kok *et al.*, 1988*b*, Vos *et al.*, 1989*a*). It thus appears that the extracellular proteinase activity produced by *L. lactis* subsp. *cremoris* is due to basically one type of proteinase which may be present both in a cell-wall-associated and a free form, although small structural differences may exist in the proteinase obtained from different strains (Kok *et al.*, 1988*b*, Kok & Venema, 1988; Vos *et al.*, 1989*a*).

A summary of the results on the autoproteolytic cleavage and N-terminal sequencing is shown in Fig. 7. The figure also illustrates from what parts of the mature proteinase the cleavage products derived, as judged from their N-terminal sequence and their M_r values. The active site of the proteinase involves Asp-217, His-281 and Ser-620 (Kok *et al.*, 1988*b*; Vos *et al.*, 1989*b*), and this part is most resistant to autoproteolytic cleavage as judged by the degradation products obtained (Fig. 7). It is interesting to note that the cleavage site between amino acid numbers 401 and 402 was adjacent to Gln (Fig. 7), in view of the fact that of seven identified sites in casein at which the cell wall proteinase cleaves, five were adjacent to Gln (Visser *et al.*, 1988). Cleavage at the autoproteolytic cleavage site between amino acid residues 401 and 402, and at a cleavage site between residues 640 and 740, and between residues 975 and 1075,

inactivates the proteinase. Altering the amino acid sequence in these regions may be a means by which proteinases less prone to autoproteolytic inactivation may be generated.

We are grateful to Dr Ingolf Nes for his continuing interest and helpful discussions.

This work was supported by the Agricultural Research Council of Norway.

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