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Partial Purification and Characterization of a Cell Wall Bound Proteinase from *Lactobacillus casei*

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ABSTRACT

The cell wall-bound proteinase from Lactobacillus casei *NCDO 151 was partially purified and characterized. Its properties appeared to be different from the two proteolytic components of the same strain described by Ezzat* et al. *(1988).*

The proteinase has pH optima at 4·8 using haemoglobin and at 5·6 with casein as substrate, temperature optimum at 35–37°C and is inactivated after 20 min at 50°C. The molecular weight, as estimated from molecular sieve chromatography, is approximately 150 000. Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) indicated a tetrameric composition. The isoelectric point is 4·8. Serine proteinase inhibitors strongly inhibit this enzyme, but also metal- chelating compounds affect its activity. The proteinase is thus to be regarded as a serine proteinase. The ions Ca^{2+} *and* Co^{2+} *enchance proteinase activity, while* Zn^{2+} *and* Cu^{2+} *suppress it. The proteinase does not exhibit peptidase activity and it poorly degrades synthetic substrates of other serine proteinases.*

INTRODUCTION

Much attention has been paid to proteinases of lactic acid bacteria due to their role as starter bacteria in fermented milk products. Growth of lactic acid bacteria depends on the supply of amino acids. Thomas and Mills

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(1981) demonstrated that the level of some essential amino acids and peptides in milk is too low to support optimal growth of lactic acid bacteria. Thus the presence of extracellular and cell wall-bound proteinases is of importance for degradation of proteins, in order to create transportable peptides for further degradation by intracellular peptidases.

Partial purification and characterization of cell wall-bound proteinases from mesophilic streptococci strains have recently been reported by Geis *et al.* (1985), Exterkate and de Veer (1985, 1987), Visser *et al.* (1986), Monnet *et al.* (1986, 1987*a,b*) and Hugenholtz *et al.* (1984, 1987).

Data concerning cell wall-bound proteinases from lactobacilli are more limited. Properties of partly purified cell wall proteinases have been reported for *L. bulgaricus* (Argyle *et al.*, 1976; Chandan *et al.*, 1982; Ezzat *et al.*, 1987), *L. lactis* (Ezzat *et al.*, 1985), *L. helveticus* (Vescovo & Bottazzi, 1979; Zevaco & Gripon, 1988) and *L. plantarum* (El Soda *et al.*, 1986).

Extensive studies were also carried out on the proteolytic system of *L. casei*, especially on strain NCDO 151 (El-Soda *et al.*, 1978*a,b*; El-Soda & Desmazeaud, 1981; Abo-Elnaza & Plapp, 1987). Subcellular localization of different peptide hydrolases, their general properties and the influence of some factors on enzyme production were determined. El-Soda *et al.* (1986), demonstrated the presence of cell wall associated proteinases in this strain. Ezzat *et al.* (1988) revealed three cell wall-bound proteinase activities after chromatography of the crude cell wall extract on DEAE-Sephacel. Two of them were further investigated. One had an optimum activity at pH 7·0 and 40°C and was strongly inhibited by the serine proteinase inhibitor phenylmethylsulphonyl fluoride (PMSF). The second one, with optimum activity at pH 7·5 and 45°C, was completely inactivated by *p*-chloromercuribenzoic acid (PCMB). None of them were affected by EDTA. This paper describes partial purification and physiochemical characterization of a cell wall-bound proteinase from *Lactobacillus casei* NCDO 151, different from the proteolytic active components from the same strain described by Ezzat *et al.* (1988).

MATERIALS AND METHODS

Organism and culture conditions

Lactobacillus casei NCDO 151 was kindly supplied from the collection of the INRA Centre, Jouy-en-Josas, France. The culture was grown overnight in MRS broth (Difco, MI, USA) at 30°C. The medium contained 20 mM $CaCl_2$ and β-glycerophosphate (pH 7·4) at a final concentration of 50 mM.

Extraction of cell wall-bound proteinase

The cells were harvested by centrifugation at 10000 g for 3 min. The cell pellet was washed twice with 50 mM β-glycerophosphate buffer, pH 7·0 containing 20 mM $CaCl_2$. Extraction of cell wall-bound proteinase was performed by resuspending the cells five successive times in 1/30 of the culture volume of 50 mM Tris-HCl buffer, pH 7·5 at room temperature for 1 h. The supernatants from the last four extractions were combined and filtered through 0·45 μm Millipore filters. This extract was designated as the crude cell wall proteinase extract.

The leakage of intracellular enzymes during extraction was estimated by assay of aldolase (Test kit, Catalog No. 234848, Boehringer Mannheim, GmbH Diagnostica) and lactate dehydrogenase (Reeves & Fimognari, 1966). Results were expressed as % of activity of these enzymes in the total extract obtained after disintegration of the pellet in a Braun Melsungen homogenizer (Stuttgart, West Germany) with ballotini beads (0·15 mm).

Determination of proteolytic activity

The determination of proteolytic activity with ^{14}C-methylated casein was performed as described by Exterkate (1975). Radioactive substrate was prepared according to Donnelly *et al.* (1980), and its specific activity was $4{\cdot}5 \times 10^4$ cpm/mg.

Standard enzyme activity conditions

All enzyme activity studies were performed in 50 mM Na-phosphate buffer pH 6·0 at 35°C unless otherwise indicated.

One unit of proteolytic activity was defined as the amount of enzyme releasing 1% of the initial radioactivity of C^{14}-labelled casein after 10 min incubation.

Purification

The crude cell wall proteinase extracts were applied to Fast Protein Liquid Chromatography (FPLC) (Pharmacia Fine Chemicals, Uppsala, Sweden) equipped with an anion-exchange column (Mono Q HR 5/5) (Pharmacia) and equilibrated with 20 mM Tris-HCl buffer, pH 8·4. The enzyme was eluted with a linear gradient of sodium chloride (0–0·5M) at a flow rate of 1 ml/min. Proteolytic fractions eluted from Mono Q were pooled and applied to a chromatofocusing column (Mono P, HR 5/20, Pharmacia). The pH gradient from 6 to 4 was formed by equilibrating the column with 25 mM bis-Tris

buffer, pH 6·3 and eluting with Polybuffer 74 (Pharmacia) adjusted to pH 3·8 with HCl.

Molecular weight determination

Molecular weight determinations were performed using gel chromatography and SDS–PAGE employing silver stain for visualization. For gel chromatography, FPLC equipped with a Superose 12 HR 10/30 column (Pharmacia) was used with 50 mM Tris-HCl pH 7·1 containing 0·1 M NaCl at a flow rate of 1 ml/min as the eluting buffer.

After radioactive labelling of the proteinase with the specific serine proteinase inhibitor, ^{3}H-diisopropylfluorophosphate (^{3}H-DFP) (Amersham, UK), SDS–PAGE was performed. The cells harvested from 800 ml of culture were suspended in 7 ml β-glycerophosphate buffer, pH 7·0 and 40 μl (40 μCi) of ^{3}H-DFP added. After incubation at 37°C for 40 min the reaction mixture was centrifuged and cell wall proteinase was extracted from the cell pellet as described earlier. The supernatants were pooled and concentrated by ammonium sulphate precipitation at 70% saturation. The precipitate was dissolved in 0·8 ml 50 mM Tris-HCl buffer, pH 7·5 and 40 μl loaded on to the gel. The SDS–PAGE was carried out according to the slab gel technique of Laemmli and Favre (1973). After electrophoresis the gel was fixed in 25% v/v isopropanol and 10% v/v acetic acid in water, dried at 60°C for 3 h and exposed to X-ray film (Hyperfilm MP, Amersham, UK).

Determination of isoelectric point

Preparative flat bed isoelectric focusing was carried out in a layer of granulated Sephadex G-200 Superfine according to the method of Frey and Radola (1982) employing 2% ampholines, Pharmalyte 4–6·5 and LKB 2·5–4 (1:4) (Pharmacia LKB, Sweden). The proteolytic fractions obtained from Mono Q chromatography were dialyzed against 1% glycine overnight at 4°C before applying to the gel layer.

Protein determination

Protein concentrations were determined according to Bradford (1976), employing the Bio-Rad protein assay-kit (Bio-Rad, Ca, USA).

Effect of pH on activity

The effect of pH on proteolytic activity was determined with two substrates, ^{14}C-casein and ^{14}C-haemoglobin (Sigma, Mo. USA). The proteolytic

activity on casein was measured in 50 mM Na–phosphate buffer (pH 5·6–8·0) and 50 mM citrate–phosphate buffer (pH 5·4–6·2) and on haemoglobin in 50 mM citrate–phosphate buffer (pH 3·8–6·0). In order to estimate pH stability, the enzyme was incubated for 24 h at 4°C in 50 mM Na–phosphate buffer at pH 5·6 or 8·4.

Effect of temperature on activity

Proteinase activity was assayed at different temperatures from 30°C to 55°C. To estimate thermostability, the enzyme was preincubated at various temperatures from 35°C to 55°C for 20 min. The solutions were then cooled, and the remaining activity was measured at 35°C.

Effect of bivalent cations

The effects of cations on proteolytic activity were determined by preincubating the enzyme in a 1 mM solution of a salt of the cation at room temperature for 30 min before addition of substrate.

Effect of inhibitors

The effect of inhibitors on proteolytic activity was detected by preincubation with enzyme solution for 30 min at room temperature before addition of substrate.

Detection of peptidase activity

Table 1 lists the synthetic substrates used to test for peptidase activity. Concentrations of substrates in the reaction mixtures for di, tri, carboxy and aminopeptidase activity determinations were 0·5 mM and, for endopeptidase activity, 1 mM. Final concentrations in amidase and esterase activity tests were 0·5 and 0·3 mM, respectively. Enzyme was incubated with each substrate for 1 h.

RESULTS AND DISCUSSION

Leakage of intracellular enzymes as judged from aldolase and lactate dehydrogenase activity was less than 2% (results not shown here). Proteolytic activity of *L. casei* extract thus seems to be solely due to the action of cell wall-bound proteinase.

With the number of extractions the proteolytic activity increased, while

TABLE 1
Synthetic Substrates for Detection of Peptidase Activity

Enzyme activity	*Substrate*	*Obtained*	*Reference from*
Dipeptidase	Glu-Tyr Pro-Phe	Sigma	El-Soda & Desmazeaud (1982)
Tripeptidase	D-Ala-Gly-Gly Leu-Leu-Leu	Serva	El-Soda & Desmazeaud (1982)
Carboxypeptidase	Z-Gly-Tyr	Serva	El-Soda & Desmazeaud (1982)
	Z-Pro-Ala	Serva	El-Soda & Desmazeaud (1982)
Aminopeptidase	L-Ala-*p*-NA	Serva	El-Soda &
	L-Leu-*p*-NA	Fluka	Desmazeaud (1982)
	L-Leu-*z*-naphthyl-amide	Fluka	Nagatsu *et al.* (1970)
Endopeptidase	Glutaryl-L-Phe-*p*-NA	Serva	El-Soda & Desmazeaud (1982)
	Z-Phe-Val-Arg-*p*-NA	Sigma	El-Soda & Desmazeaud (1982)
	N-tosyl-Gly-Arg-*p*-NA	Sigma	El-Soda & Desmazeaud (1982)
	N-tosyl-Gly-Pro-Lys-*p*-NA	Sigma	El-Soda (1982) Desmazeaud (1982)
	N-succinyl-Ala-Ala-Ala-*p*-NA	Sigma	El-Soda & Desmazeaud (1982)
	Succinyl-L-Ala-Ala-Pro-Phe-*p*-NA	Boehringer Mannheim GmbH	El-Soda & Desmazeaud (1982)
Amidase	*N*-benzoyl-DL-Arg-*p*-NA HCl	Fluka	Arnon (1970)
Esterase	*N*-benzoyl-L-Tyr-ethylester	Fluka	Rick (1965)

the amount of protein decreased, leading to an improved specific activity for each consecutive extraction. This is in agreement with the cascade like release observed during the successive extractions of proteinases from *S. lactis* (Monnet *et al.*, 1987*a*), *S. cremoris* (Exterkate & deVeer, 1985) and *L. bulgaricus* (Ezzat *et al.*, 1987). The lower activity observed in the first extract may be due to the presence of residual Ca^{2+} from the washing buffer, since Ca^{2+} has been shown to inhibit the release of cell wall bound proteinase (Mills & Thomas, 1978). Also, Ezzat *et al.* (1988) working with the same strain, reported the lowest activity in the first extract. However, after the second extraction they observed decreasing activity, which is contrary to our results.

Anion-exchange chromatography at pH 8·4 of the cell wall bound crude

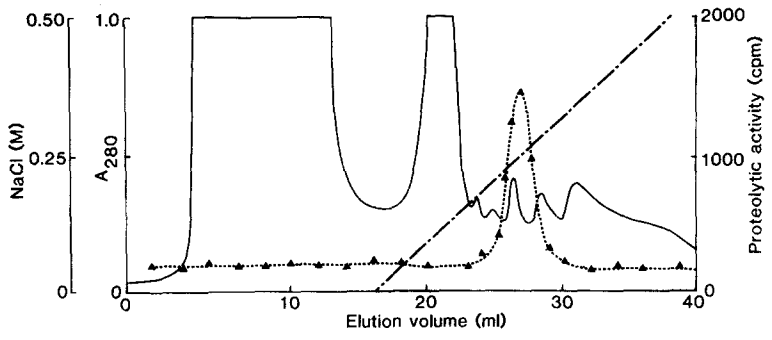

Fig. 1. Anion-exchange chromatography of the crude cell wall extract from *L. casei*. ▲, proteinase activity; ——, OD_{280} and — · —, (NaCl).

extract gave a single peak with proteolytic activity eluting at 0·25 mM NaCl (Fig. 1). At this stage the average activity yield was 130% of the original activity in the crude cell wall preparation and a 6-fold increase in specific activity was observed (Table 2). The increase in activity may be due to the removal of inhibitory proteins from the sample preparations. The proteolytic fractions eluted from Mono Q were further purified by chromatofocusing. The activity was eluted at pH 4·3–4·15 (Fig. 2). The activity profile showed two peaks not completely separated. At this step the proteinase was purified 17-fold over the initial extract and the protein recovery was 5%. This purification procedure did not result in a homogeneous proteinase as judged from electrophoresis (data not shown). Ezzat *et al.* (1988) using ion-exchange chromatography on a DEAE-Sephacel column revealed three active components.

The conditions under which we cultivated the organism differed from those used by Ezzat *et al.* (1988) with respect to time and pH. At the time of harvesting the pH of the culture medium was 4·5 whereas they maintained the pH during growth at 6·0.

TABLE 2

Levels of Activity Observed during Purification of Cell Wall Bound Proteinase from *Lactobacillus casei*

	Total protein (mg)	*Total activity (units)*[a]	*Specific activity (units/mg)*	*Purification index*	*Protein yield (%)*
Crude cell wall proteinase extract	20	940	47	1·0	100
Post Mono Q	4·32	1 226	283	6·0	22
Post Mono P	1·02	800	784	16·7	5

[a] For definition, see 'Materials and Methods' section.

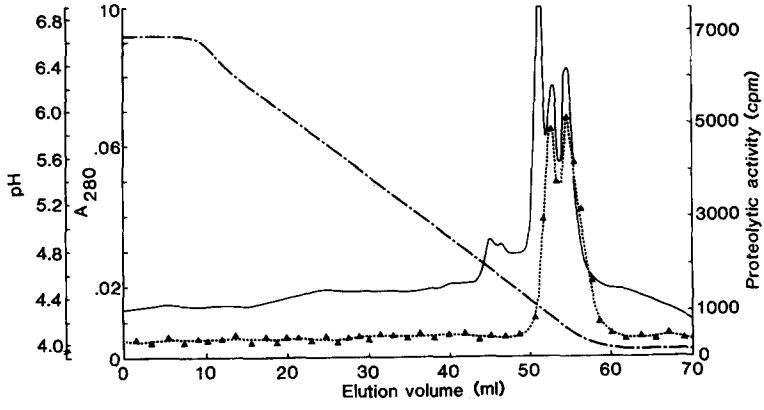

Fig. 2. Elution profiles after chromatofocusing of the proteolytically active fraction from anion-exchange chromatography. ▲, proteinase activity; ——, OD_{280} and —·—, pH.

Cliffe and Law (1985) observed for *S. lactis* that the number of proteinases produced was dependent on the composition of culture medium. Thus it seems that the different medium pH could influence the appearance of different proteolytic components. A DEAE-Sephacel column revealed three active components.

After anion-exchange chromatography the proteinase was subjected to molecular weight determination. By molecular sieve chromatography, the proteinase showed a similar elution volume to that of aldolase, thus indicating a molecular weight of approximately 150 000. This value was also obtained after chromatography of crude cell wall extract. Additionally, fractions with maximum radioactivity were found at the same elution volume, after chromatography of the ^{3}H-labelled proteinase, indicating that we are dealing with an enzyme similar to other proteinases from lactic acid bacteria (Thomas & Pitchard, 1987). However, SDS-PAGE of radioactive labelled (^{3}H-DFP)–enzyme complex indicated a molecular weight of 36 000 (Fig. 3). The same molecular weight was observed in samples not treated with mercaptoethanol before electrophoresis. These results indicate that the enzyme is tetrameric. Two less distinct proteins bands visualized in the gel represent molecular weights of 95 000 and 140 000 and may indicate the existence of two monomer proteinases or one monomer and its self digestion product. However, these bands are of a much lower protein content than the band representing the 36 kD enzyme–inhibitor complex. The heterogeneity of all cell wall proteolytic systems is not fully understood and is further complicated by self digestion. Exterkate and de Veer (1987), demonstrated two active components in the cell wall of *S. cremoris* H.P. both originating from the same proteinase. Also Zevaco and Gripon (1988) showed that two separated proteolytic fractions from *L. helveticus* of different molecular

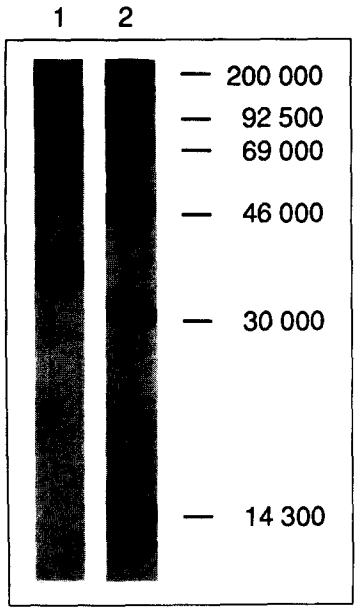

Fig. 3. 1. SDS–PAGE of ^{3}H-DFP-labelled proteinase(s) from *L. casei*. 2. Radioactive MW standard.

weights, but similar properties and specificity represented the same enzyme.

Hugenholtz *et al.* (1987) and Kok *et al.* (1988) have discussed the apparent existence of multiple proteinases, due to enzyme processing and autoproteolytic activity. Exterkate (1975, 1976) and Cliffe and Law (1985) suggest the existence of several individual proteinases in one bacterium, while Monnet *et al.* (1987*b*) only discovered one proteinase in five strains of *S. lactis*. Whether the proteolytic fraction described here is composed of one or more components remains unclear.

The partly purified enzyme after anion-exchange chromatography (Mono Q) was used for further characterization. Preparative electrofocusing indicated an isoelectric point for the *L. casei* proteinase at pH 4·8.

The effect of pH on the activity of this proteinase against haemoglobin and casein is shown in Fig. 4A. The optimum pH was 4·8 when ^{14}C-methylated haemoglobin was used as substrate and is similar to that observed by Monnet *et al.* (1987*a*) for *S. lactis* NCDO 763 and only slightly higher (0·3 units) than the results obtained for other strains of *S. lactis* (Monnet *et al.*, 1987*b*). With ^{14}C-methylated casein the optimal proteolytic activity was at pH 5·6. This corresponds with the pH optima of proteinases from *S. cremoris* (Geis *et al.*, 1985; Exterkate, 1976) and *L. bulgaricus* (Argyle *et al.*, 1976), but is different from those found by Ezzat *et al.* (1988) for the two proteolytic fractions from the same strain as used in the present study. They reported pH optima of 7–7·5.

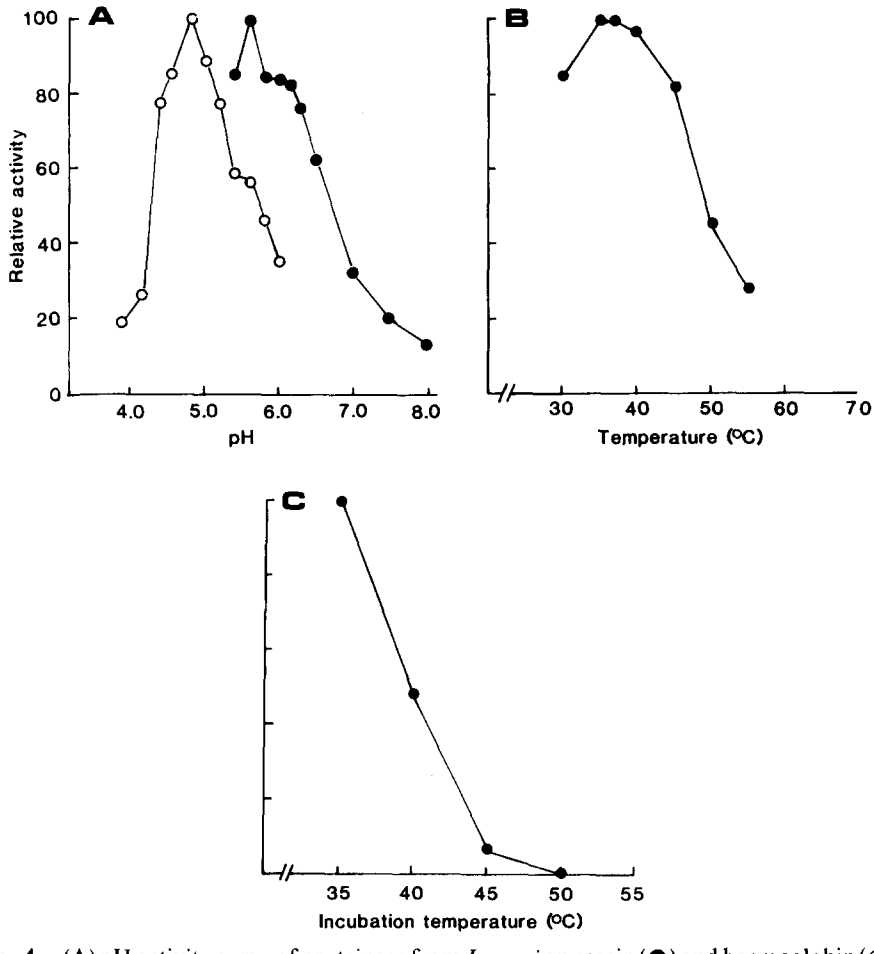

Fig. 4. (A) pH activity curve of proteinase from *L. casei* on casein (●) and haemoglobin (○). (B) Temperature optimum. (C) Thermostability of the purified cell wall proteinase.

Enzyme stability was examined at pH 8·4 and 5·6 at 4°C over 24 h. No decrease in activity was observed at pH 8·4. At pH 5·6 there was only 6% reduction of activity. The effect of temperature on proteolytic activity is shown in Fig. 4B and C. Hydrolysis of ^{14}C-methylated casein was optimal at 35–37°C and declined sharply above 40°C contrary to the findings for the two proteinases studied by Ezzat *et al.* (1988) derived from the same strain as we used in the present investigation. They reported temperature optima of 42 and 47°C.

The enzyme was completely inactivated after 20 min preincubation at 50°C. The instability of the *L. casei* proteinase at elevated temperatures is in agreement with Exterkate and de Veer (1987) who reported high instability of *S. cremoris* proteinase at temperatures above 30°C. Proteinase from *S.*

TABLE 3
Effect of Bivalent Cations at 1mM Concentration on the Partly Purified Cell Wall Proteinase from *L. casei*

Cation	*Relative activity* (%)
Control	100
Co^{2+}	128
Ca^{2+}	120
Mg^{2+}	104
Mn^{2+}	105
Zn^{2+}	76
Cu^{2+}	72

lactis is thermosensitive and quickly inactivated at 40°C (Monnet *et al.*, 1987*a*).

Table 3 shows the effect of various cations on enzyme activity of *L. casei* proteinase. Addition of Ca^{2+}- and Co^{2+}-ions gives a stimulatory effect of about 20%. An activating effect of Ca^{2+} was also reported for proteinase from *S. lactis* NCDO 763 (Monnet *et al.*, 1987*a*) and *S. cremoris* AC1 (Geis *et al.*, 1985). According to Exterkate and de Veer (1987) some cations have a structural function and stabilize the enzyme molecules in an active configuration.

Zn^{2+}- and Cu^{2+}-ions reduced the initial activity of the *L. casei* proteinase by 24 and 28%, respectively. This may be due to precipitation of the substrate by these ions, as indicated by Exterkate and de Veer (1987). Mg^{2+}- and Mn^{2+}-ions did not influence the activity.

The effect of different inhibitors on enzyme activity is shown in Table 4. It should be noted that the reduction in enzyme activity due to the presence of EDTA and EGTA was fully recovered by addition of Ca^{2+}- or Co^{2+}-ions.

Proteinase activity was inhibited to 54 and 41% of the initial value by addition of 1 mM phenylmethylsulphonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP), respectively (Table 4). Increasing the concentration of DFP to 5 mM almost completely inhibited the enzyme. The thiol proteinase inhibitor, *p*-chloromercuribenzoic acid (PCMB), showed an inhibitory effect of 35%. However, cysteine which stimulates thiol proteinase activity also showed an inhibitory effect (13%), thus indicating that the enzyme is not a real thiol proteinase. Ezzat *et al.* (1988) found that one of the proteolytic fractions originating from the same strain as our proteinase was strongly inhibited by PMSF while the other was inactivated by PCMB, but neither was affected by EDTA. Altogether our results support the hypothesis of the isolation of a different enzyme and indicate

TABLE 4
Effect of Inhibitors on the Partly Purified Cell Wall Proteinase from *L. casei*

Inhibitor	*Concentration (mM)*	*Obtained from*	*Relative activity* (%)
Control	—		100
Leupeptin	0·1	Boehringer Mannheim GmbH	98
Leupeptin	1·0	Boehringer Mannheim GmbH	94
BPTI	0·1	POCH	91
BPTI	1·0	POCH	75
Turkey ovomucoid	0·001	Sigma	100
Chicken ovomucoid	0·001	Sigma	100
PMSF	1·0	Boehringer Mannheim GmbH	54
DFP	1·0	Aldrich	41
DFP	2·0	Aldrich	19
DFP	5·0	Aldrich	6
DFP	10·0	Aldrich	0
TLCK	1·0	Boehringer Mannheim GmbH	98
TPCK	1·0	Boehringer Mannheim GmbH	100
EDTA	1·0	Sigma	57
EDTA	5·0	Sigma	34
EGTA	1·0	Sigma	65
EGTA	5·0	Sigma	53
Pepstatin	0·1	Boehringer Mannheim GmbH	98
PCMB	1·0	Boehringer Mannheim GmbH	65
Cysteine	1·0	Sigma	87
DTT	1·0	Sigma	100
Iodoacetamide	1·0	Sigma	100
Iodoacetic acid	1·0	Sigma	93

Abbreviations: BPTI: Bovine pancreas trypsin inhibitor; PMSF: phenylmethylsulfonyl fluoride; DFP: diisopropylfluorophosphate; TLCK: tosyl-L-lysine chloromethylketone; TPCK: tosylaminophenylethyl chloromethylketone; EDTA: ethylenediaminotetraacetic acid; EGTA: ethyleneglycol-aminoethylether tetraacetic acid; PCMB: parachloromercurybenzoic acid; DTT: dithiotreitol.

that the proteinase described here is a serine proteinase which is affected by EDTA in a similar way to cell wall-bound proteinases from other lactic acid bacteria (Geis *et al.*, 1985; Monnet *et al.*, 1987*a*). This enzyme was not affected by TLCK and TPCK typical inhibitors for trypsin or chymotrypsin-like proteinases. The ability of our proteinase to hydrolyze the range of synthetic substrates listed in Table 1 was investigated. None were hydrolyzed and the enzyme had neither carboxypeptidase nor aminopeptidase activity. *L. casei* proteinase poorly degrades synthetic substrates of other serine proteinases. Only one of the chymotrypsin substrates, succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, appeared to be slightly

degraded by the enzyme. The results confirm those of El-Soda *et al.* (1986), who did not demonstrate any of these activities in the cell wall fractions of *L. casei* and *L. plantarum*. No peptidase activity could be detected in cell wall extracts of *S. lactis* NCDO 763, either (Monnet *et al.*, 1987*a*).

In conclusion the cell wall proteinase isolated from *L. casei* is closely related to proteinases from the mesophilic streptococci *S. lactis* (Monnet *et al.*, 1987*a*) and *S. cremoris* (Geis *et al.*, 1985) and that of *L. bulgaricus* (Argyle *et al.*, 1976). The apparent discrepancies between the results described in this paper and the data of Ezzat *et al.* (1988) concerning pH and temperature optima, effect of EDTA and number of proteolytic components detected, indicates that growth conditions may influence the type of proteinases formed.

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