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Original article

Partial purification and characterization of intracellular proteinases from *Lactococcus lactis* subsp *lactis* MG1363

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Summary – Three caseinolytic proteinases (P1, P2, P3) were isolated from the cytoplasm of plasmid-free *Lactococcus lactis* subsp *lactis* MG1363 by sequential chromatography on DEAE-cellulose, Sephacryl 200, CM-cellulose, Phenyl Sepharose or hydroxyapatite and MonoQ ion exchanger. Minor caseinolytic fractions were separated during successive purification steps and the final preparations, although free from aminopeptidase activities, showed impurities by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), indicating autolysis or a very complex caseinolytic system. Proteinase P1 was a dimer with molecular mass (M_s) of ca 66 kDa by SDS-PAGE and 124 kDa by gel filtration and it was most sensitive to EDTA. Proteinase P2 (M_s ca 68 kDa by SDS-PAGE and 64 kDa by gel filtration) and P3 (M_s ca 52 kDa SDS-PAGE and 47 kDa by gel filtration) were strongly inhibited by phenylmethylsulfonyl fluoride. Proteinase P1 was most active at pH 7.0 and 35 °C, while proteinases P2 and P3 had optima at pH 7.5 and 45 °C. Urea-PAGE of digests of α- or β-casein showed differences in the peptide pattern released by the three proteinases after 6 or 20 h incubation. The peptides released by P1, P2 and P3 were also different from those produced by a crude preparation of the cell envelope-associated proteinase from *L lactis* subsp *lactis* 712, the parental strain of *L lactis* subsp *lactis* MG1363. Reverse-phase fast protein liquid chromatography also showed differences between peptides released from α_{s1}-casein by P1, P2 and P3.

intracellular proteinase / *Lactococcus lactis*

Résumé – Purification partielle et caractérisation de protéinases intracellulaires de *Lactococcus lactis* ssp *lactis* MG 1363. *Trois protéinases caséolytiques (P1, P2, P3) ont été isolées du cytoplasme sans plasmide de* Lactococcus lactis *ssp* lactis *MG1363 par chromatographie séquentielle sur cellulose DEAE, Sephacryl 200, CM-cellulose, Phenyl Sepharose ou hydroxyapatite et échange d'ions MonoQ. Les fractions caséinolytiques mineures ont été séparées lors de ces étapes de purifi-*

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cation successives, puis les préparations finales, bien que sans activité aminopeptidasique, ont montré des impuretés par SDS-PAGE, indiquant une autolyse ou un système caséolytique très complexe. La protéinase P1 était un dimère (masse moléculaire d'environ 66 kDa par SDS PAGE et 124 kDa par gel filtration) et elle était la plus sensible à l'EDTA. Les protéinases P2 (masse moléculaire d'environ 68 kDa par SDS PAGE et 64 kDa par gel filtration) et P3 (masse moléculaire d'environ 52 kDa par SDS PAGE et 47 kDa par gel filtration) étaient fortement inhibées par du phényl méthyl sulfonyl fluorure. La protéinase P1 était plus active à pH 7,0 et 35 °C, tandis que les protéinases P2 et P3 avaient un optimum à pH 7,5 et 45 °C. L'électrophorèse urée PAGE d'hydrolysats des caséines α *et* β *a montré des différences dans le profil peptidique libéré par les trois protéinases après 6 ou 20 heures d'incubation. Les peptides relargués par P1, P2 et P3 étaient également différents de ceux produits par une préparation brute de protéinases associées à l'enveloppe cellulaire de* L lactis *ssp* lactis *MG 712, issu de* L lactis *ssp* lactis *MG1363. La chromatographie RP-FPLC a également montré des différences entre les peptides libérés à partir de la caséine* α_{s1} *par P1, P2 et P3.*

***Lactococcus lactis* / protéinase intracellulaire**

INTRODUCTION

To date, the most extensively studied proteolytic enzymes of lactic acid bacteria (LAB) are cell envelope-associated (CEP) proteinases and intracellular aminopeptidases. Oligopeptidases (PepO, PepF) which do not hydrolyze casein are also quite well characterized (see Monnet et al, 1994; Fox et al, 1995; Law and Haandrikman, 1996).

Intracellular caseinolytic activity comprises a marked portion of the total proteolytic activity of prt$^+$ and prt$^-$ lactococci (Muset et al, 1989; Crow et al 1993a; Stepaniak and Fox, 1995). Westhoff et al (1971) separated four caseinolytic fractions from the intracellular material of slow acid-producing, presumably prt$^-$ variant, of *Lactococcus lactis* subsp *lactis*. Ohmiya and Sato (1975) found only one proteinase in the intracellular fraction from *L cremoris* subsp *cremoris*. Likewise, Muset et al (1989) found one principal intracellular proteinase in the prt$^-$ variant of *L lactis* subsp *lactis* NCDO 763. However, Akuzawa et al (1990) identified several intracellular endopeptidases, with or without caseinolytic activity, from *L lactis* subsp *lactis* IAM 1198. Work by Desmazeaud and Zevaco (1976) and Muset et al (1989) indicates that the proteolytic system of lactococci may, in addition to the caseinolytic proteinases and typical oligopeptidases (which hydrolyze various oligopeptides but not proteins), contains endopeptidases with relatively high activity on oligopeptides and low activity on casein.

Generally, intracellular turnover of proteins and peptides is an indispensable function of all living cells (Orlowski, 1990; Hilt and Wolf, 1996). Orlowski (1990) proposed that degradation of intracellular proteins is initiated by a ca 700 kDa multicatalytic proteinase complex (MPC), while the function of intracellular endopeptidases, including prolyendopeptidases and oligopeptidases, is a further hydrolysis of released by MPC oligopeptides. MPC (termed also *proteasomes*) were well studied in eukaryotic cells but simpler MPC were also found in archaebacteria (Orlowski, 1990; Hilt and Wolf, 1996).

CEP and lactococcal oligopeptidase probably contribute to secondary proteolysis during cheese ripening by degrading peptides released by chymosin (Fox et al, 1995). Intracellular lactococcal proteinase was detected by an immunological technique in Gouda cheese ripened for 1 month (Akuzawa and Yokoyama, 1993). However, the significance of intracellular caseinolytic proteinases from LAB for cheese ripening remains unknown.

Some of the principal intracellular lactococcal proteinases which have been char-

acterized to various degrees (Westhoff et al, 1971; Ohmiya and Sato, 1975; Muset et al, 1989; Akuzawa et al, 1994; Akuzawa and Okitani, 1995) showed differences in general properties (table I), indicating a complex intracellular proteolytic system in lactococci. We reported that cytoplasm of *L lactis* subsp *lactis* MG1363 contains caseinolytic fractions separable by anion-exchange chromatography (Stepaniak and Fox, 1995). The objective of this study was to characterize the major intracellular proteinases from this microorganism.

MATERIALS AND METHODS

Microorganism

Plasmid-free *L lactis* subsp *lactis* MG1363 and its parental strain, prt$^+$ *L lactis* subsp *lactis* 712, were from the culture collection of the Department of Microbiology, University College, Cork (Ireland). The microorganisms were cultivated for 14 h at 30 °C (1% inoculum; final pH, ca 5.0) in M17 broth (Merck, Darmstadt, Germany) supplemented with 0.5% glucose. The harvested cells were washed briefly in acetate-phosphate buffer, pH 6.4, and then incubated for 30 min at 30 °C, with shaking, in 50 mmol/L Tris-HCl buffer, pH 7.5, to release cell envelope-associated crude proteinase (Crow et al, 1993b). The procedure of Crow et al (1993b) was used to prepare intracellular fractions from lysozyme/mutanolysin-treated cells of *L lactis* subsp *lactis* MG1363.

Determination of cell yield

A cell suspension (2 mL) was deposited by filtering on a 0.22 μm pre-weighted filter, washed with water, freeze-dried and the weight of dried cells determined.

Casein and peptide substrates

Fluorescent casein was prepared by the method of Twinning (1984) and freeze-dried. α_{s1} - and β-Caseins were prepared by urea fractionation and purified on DEAE-cellulose (Fox and Guiney, 1972) using a NaCl gradient (0–0.5 mol/L) in phosphate buffer containing 4.5 mol/L urea and 10 mmol/L mercaptoethanol.

α_{s1}-Casein f157-164 was synthesized at the Biotechnology Centre, University of Oslo (Norway), using a Model 431A peptide synthesizer (Applied Biosystems Inc, San Jose, CA, USA).

Isolation of intracellular proteinases

The yield of *L lactis* subsp *lactis* MG1363 cells was 1.35 g of dry matter per litre. Proteolytic enzymes were isolated from the cytoplasmic fraction using a five-step purification procedure.

Step 1: Anion-exchange chromatography. The cytoplasmic fraction obtained from cells harvested from 20 L of culture was first applied to a column (2.1 x 75 cm) of DEAE-cellulose equilibrated with 20 mmol/L phosphate buffer, pH 6.5. The proteins were eluted at a flow rate of 18 mL/h with a linear NaCl gradient from 0.1 to 0.6 mol/L. The fractions with strong caseinolytic activity were pooled, dialyzed for 24 h against 5 mmol/L phosphate buffer, pH 7.0, and concentrated by freeze drying.

Step 2: Gel filtration chromatography. Caseinolytic fractions from chromatography on DEAE-cellulose were redissolved in a small volume of 50 mmol/L phosphate buffer, pH 7.0, and further purified by gel filtration chromatography on Sephacryl 200 (column size 2.1 x 73 cm) equilibrated with 50 mmol/L phosphate buffer, pH 7.0, containing 0.15 mol/L of NaCl. Elution with the same buffer was at the flow rate of 12 mL/h; 3 mL fractions were collected.

Step 3: Cation-exchange chromatography. The pooled fractions from gel filtration were further purified by chromatography on CM-cellulose (column size 1.5 x 35 cm). The column was washed with 20 mmol/L acetate buffer, pH 5.25, and a linear salt gradient (0.1 to 0.4 mol/L of NaCl) was used to elute adsorbed protein. The flow rate was 10 mL/h and 2 mL fractions were collected, dialyzed and concentrated by freeze-drying.

Step 4: The fourth purification step involved chromatography either on hydroxyapatite or Phenyl Sepharose; the pooled proteolytic fractions from the first three purification steps were divided into two portions. One portion was chromatographed on hydroxyapatite (column size 0.21 x 10 cm) at a flow rate of 12 mL/h using a linear potassium phosphate buffer gradient, 0.01 to 0.4 mol/L, pH 6.0; 1 mL fractions were collected. The second portion was purified on Phenyl Sepharose (column size 1.6 x 10 cm) using a linear ammonium sulphate gradient (1.7 to 0

mol/L) in 50 mmol/L phosphate buffer, pH 7.0, at a flow rate of 0.5 mL/min; fractions (1 mL) with high proteolytic activity were collected. Better separation of nonenzymatic and caseinolytic proteins was obtained by chromatography on Phenyl Sepharose.

Step 5: Secondary ion-exchange chromatography. Active fractions from Phenyl Sepharose were finally purified on a ready-packed MonoQ column HR 5/5 (Pharmacia Biotech, Uppsala, Sweden). The proteins were eluted using a NaCl gradient in 20 mmol/L Bis-Tris propane buffer, pH 6.5, at a flow rate of 0.75 mL/min. The centers of the protein peaks were collected manually. The first three purification steps were performed at 4 °C, but chromatography on hydroxyapatite, Phenyl Sepharose and MonoQ was at room temperature on columns connected to a fast protein liquid chromatography system (FPLC; Pharmacia-Biotech).

Determination of enzymatic activities

Caseinolytic activity was measured routinely in 50 mmol/L phosphate buffer, pH 6.0, by the method of Twinning (1984) using fluorescent casein (0.5%) as substrate. Activity in chromatographic fractions was measured after incubation at 30 °C for 20 h or after 6 h during characterization of the enzymes. The incubation mixture contained 0.02% NaN_3. The purified enzymes were diluted to approximately the same activity at pH 6.0 and 30 °C. The fluorescence of the control sample (to which purified enzyme was added after incubation of substrate) was less than 5% of that measured in the enzyme-containing sample after 6 h of incubation. Activity expressed as an increase in arbitrary fluorescence units was measured using a fluorescence HPLC monitor (Shimadzu corporation, Kyoto, Japan).

PepN and PepX aminopeptidase activities on Leu-pNA and Arg-Pro-pNA, respectively, were measured as described by Stepaniak and Fox (1995).

Protein content

Protein content was measured by the method of Bradford (1976).

Electrophoresis

Urea-polyacrylamide gel electrophoresis (Urea-PAGE) was used to separate peptides released from α_{s1}- or β-caseins by purified proteinases. Electrophoresis was performed by the method of Andrews (1983); gels were stained as described by Blakesley and Boezi (1977). SDS-PAGE was performed in Mini-Protean II electrophoresis cell (Bio-Rad, Herculers, CA, USA) using the procedure recommended by the manufacturer.

Molecular mass determination

The molecular mass (M_s) was determined by SDS-PAGE and gel filtration on a TSK 3000SW column (60 cm x 7.5 mm, TosoHaas, Stuttgart, Germany).

Electroblotting and N-terminal analysis

Electroblotting and staining of peptides released from β-casein by intracellular proteinases and separated by urea-PAGE was performed as described by Singh et al (1995). Some peptides extracted from the membrane used for electroblotting were partially sequenced at National Food Biotechnology Centre, University College, Cork (Ireland) by Edman degradation on an automated pulsed liquid-phase protein-peptide sequencer (model 477A; Applied Biosystems Inc, Foster City, CA, USA). Liberated amino acids were detected as their phenylthiohydantoin derivatives using a model 120 A analyzer (Applied Biosystems).

Reverse-phase chromatography

Reverse-phase chromatography with PepRC HR5/5 column (Pharmacia) was used to separate 1% trifluoroacetic acid (TFA)-soluble peptides released from α_{s1}-casein by the purified proteinases and to determine degradation of α_{s1}-casein fragment 157-164 (α_{s1}-CN f157-164). Chromatographic conditions were as described by Stepaniak and Fox (1995).

Effect of pH, temperature and inhibitors

Effect of pH at 30 °C was measured using universal buffer (Dawson et al, 1969). The effect of temperature and inhibitors was measured using phosphate buffer, pH 6.0. Proteinases were incubated with inhibitors at pH 6.0 at room temperature. After 10 min, the inhibitor-enzyme buffer mixture was diluted ten-fold with buffered fluorescein-labelled casein so that the final con-

centration of substrate was 0.5% and the final concentration of phosphate buffer was 50 mmol/L.

RESULTS AND DISCUSSION

Purification and molecular mass (M_s)

Chromatography of the cytoplasm from *L lactis* subsp *lactis* MG1363 on DEAE-cellulose resolved two major and two minor protein peaks with caseinolytic activity (fig 1). Gel filtration on Sephacryl 200 resolved the first proteolytic fraction into two major and one minor peak (fig 2A), while the second caseinolytic fraction was only partially resolved (fig 2B). As shown in figures 1 and 2, the main proteolytic fractions were denoted as proteinases P1, P2 and P3. A substantial amount of noncaseolytic proteins were removed from each of the three proteinases by chromatography on CM-cellulose, Phenyl-Sepharose or hydroxyapatite. One minor peak was separated from P1, P2 and P3 by chromatography on CM-cellulose. Traces of proteolytic activity were separated from P1, P2 and P3 by chromatography on Phenyl-Sepharose (data not shown) and in the case of P1, a minor proteolytic peak was identified in the eluate from MonoQ (fig 3). The three proteinases were eluted from MonoQ column at different NaCl concentrations (fig 3).

Following purification by the five-step procedure, proteinases P1, P2 and P3 were not homogeneous by SDS-PAGE, although the specific activity of P1 and P2 increased markedly (table I) and the three enzymes were free from PepN and PepX activities. The purification procedure was repeated with similar results. SDS-PAGE showed one major and three minor protein bands in P1, one major and one minor band in P2 and one major and two minor bands

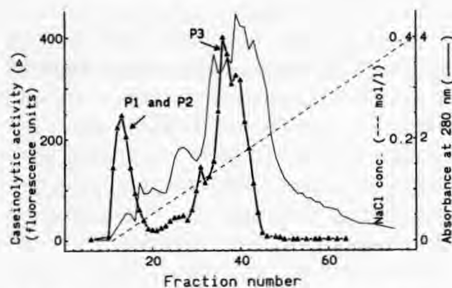

Fig 1. Separation of caseinolytic activity in the cytoplasm of *L lactis* subsp *lactis* MG1363 on DEAE-cellulose. Activity was measured on fluorescent casein. P1, P2 and P3: proteinases 1, 2 and 3, respectively.
Fractionnement de l'activité caséolytique dans le cytoplasme de L lactis *ssp* lactis *MG1363 sur cellulose DEAE. L'activité a été mesurée sur une caséine fluorescente. P1, P2 et P3 : protéinases 1, 2 et 3 respectivement.*

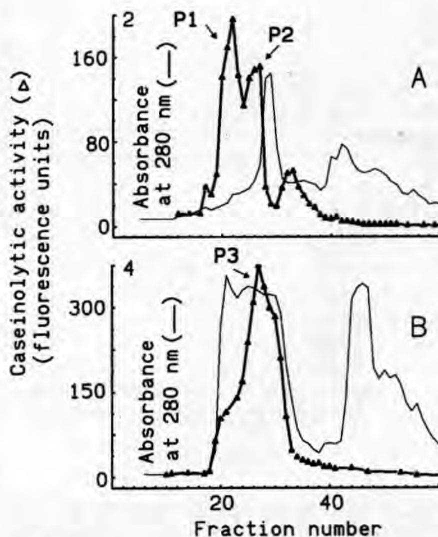

Fig 2. Separation of proteinases P1 from P2 (A) and further purification of proteinase P3 (B) from the cytoplasm of *L lactis* subsp *lactis* MG1363 by chromatography of the proteolytic fraction from DEAE-cellulose on Sephacryl 200.
Séparation des protéinases P1 de P2 (A) et purification ultérieure de la protéinase P3 (B) du cytoplasme de L lactis *ssp* lactis *MG1363 par chromatographie sur Sephacryl 200 des fractions protéolytiques obtenues sur cellulose DEAE (fig 1).*

in P3. No protein bands of M_s > 67 kDa were present in the electrophoregrams of P1, P2 and P3. Comparison of the M_s of the major protein band on SDS-PAGE electrophoregram of proteinase P1 with the M_s of the highest proteolytic fraction collected from the gel filtration column indicated that the enzyme was a dimer. The molecular masses established in the same way for P2 and P3 indicated that these enzymes were monomers (table I).

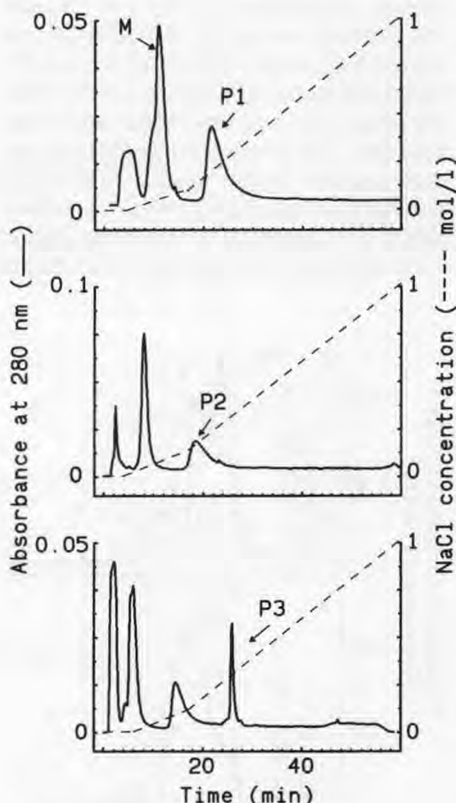

Fig 3. Final (fifth step) purification of proteinases P1, P2 and P3 from *L lactis* subsp *lactis* MG1363 by secondary ion-exchange chromatography on MonoQ. Approximately 30% of each enzyme recovered from purification on Phenyl Sepharose was loaded on the column. M: minor proteolytic fraction.

Purification finale (5e étape) des protéinases P1, P2 et P3 de L lactis *ssp* lactis *MG1363 par chromatographie d'échange d'anions secondaire sur monoQ. Environ 30 % de chaque enzyme récupérée sur la phényl sépharose ont été chargés sur la colonne. M : fraction protéolytique mineure.*

In agreement with other reports (Westhoff et al, 1971; Muset et al, 1989; Akuzawa et al, 1990; Stepaniak and Fox, 1995), the cytoplasm of *Lactococcus* contains several proteinases which are eluted from anion exchangers between 0 and 0.4 mol/L of NaCl. Akuzawa et al (1990) separated three major and two minor caseinolytic fractions from the cytoplasm of *L lactis* subsp *lactis* IAM 1198 by chromatography on DEAE-cellulose. The caseinolytic fractions were further resolved by gel filtration, yielding proteinases which differed markedly in molecular mass and belonged to different biochemical classes. Serine and cysteine proteinases in the fraction eluted from DEAE-cellulose at ca 0.05 mol/L of NaCl were isolated and characterized by Akuzawa et al (1994) and Akuzawa and Okitani (1995) (table I). Muset et al (1989) found two major proteinases in prt^+ *L lactis* subsp *lactis* NCDO 763, but in case of the prt^- variant, proteinase activity eluted from a MonoQ column before the NaCl gradient was markedly reduced. According to Westhoff et al (1971), Ohmiya and Sato (1975) and Akuzawa and Okitani (1995), anion exchange and gel filtration chromatography (see table I) were sufficient to purify the proteinases to homogeneity. The data from chromatography (figs 1–3) and SDS-PAGE indicate that the cytoplasm from *L lactis* subsp *lactis* MG1363 contained several major and minor proteinases or that the intracellular proteinases were susceptible to autolysis. Together with proteinase P1, P2 and P3 from this study, intracellular lactococcal proteinases represent a group of enzymes of markedly diverse molecular masses (table I). As established by gel filtration M_s, of proteinase P1, proteinase LLP-IIC_2 and the proteinase reported by

Table I. General characteristics of lactococcal intracellular proteinases.
Caractéristiques générales des protéinases intracellulaires de Lactococcus.

	P1 (this study)	*P2 (this study)*	*P3 (this study)*	*LLP-IIC$_2$ from* L lactis *subsp* lactis *IAM 1198 (Akuzawa & Okitani, 1995)*	*LLP-IIC$_1$ from* L lactis *subsp* lactis *IAM 1198 (Akuzawa et al, 1994)*	*From* L lactis *subsp* cremoris *H61 (Ohmiya & Sato, 1975)*	*From* L lactis *subsp* lactis *NCDO 763 (Muset et al, 1989)*	*From* L lactis *subsp* lactis *No 3 (Westhoff et al, 1971)*
Type	Metallo	Serine	Serine	Cysteine	Serine	Metallo	Metallo	*
M_s (kDa) by gel filtration	124	62	47	12	140	140	93	
by SDS-PAGE	66	68	52	12	67	ND	ND	3.3
sedimentation velocity								
pH optimum	7.0	7.5	7.5	5.5–6.0	6.0–6.5	7.0	7.5	7.6
Temp optimum (°C)	35	45	45	30	30–35	30	45	ND
Purification by successive chromatography on:	DEAE-cellulose, Sephacryl 200, CM-cellulose, Phenyl Sepharose, MonoQ			DEAE-cellulose, Sephadex G-100	DEAE-cellulose, Sephadex G-100 P-cellulose, DEAE-cellulose, TSK-G 3000 SW	DEAE-cellulose Sephadex G-200	MonoQ, Superose 12	DEAE-Sephadex Bio-Gel P-30 Bio-Gel P10
Fold purification	291	266	57	105	111	150	12	160

* Not sensitive to p-hydroxymercuribenzoate (p-HMB); M_s: molecular mass; ND: not determined.

Ohmiya and Sato (1975) were similar, but proteinase LLP-IIC_2 was classified as serine, while the other two enzymes were metalloproteinases (table I). Occurrence of the major low molecular mass proteinases reported by Westhoff et al (1971) and Akuzawa and Okitani (1995) were not confirmed by this study. Cultivation conditions may influence the concentration of intracellular lactococcal proteinases (Ohmiya and Sato, 1975) and, therefore, at the time of isolation different proteinases may occur as principal or minor enzymes. Proteasomes also hydrolyze caseins and these enzyme-complexes may be degraded to active subunits (Orlowski, 1990). It is warranted to determine if: 1) proteasomes occur in LAB; 2) some intracellular proteinases may be products of their degradation; and 3) the function of intracellular proteinase corresponds to that of proteasomes in eukaryotic or mammalian cells.

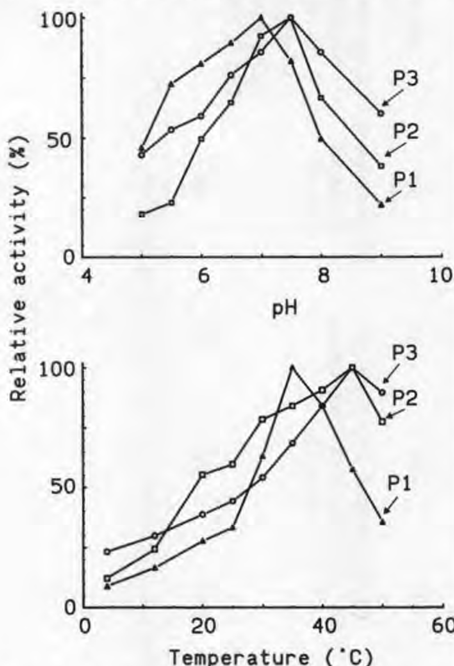

Fig 4. Effect of temperature and pH on the activity of cytoplasmic proteinases P1, P2 and P3 from *L lactis* subsp *lactis* MG1363.
Effet de la température et du pH sur l'activité des protéinases cytoplasmiques P1, P2 et P3 de L lactis *ssp* lactis *MG1363.*

Effect of pH and temperature

P1 differed from both P2 and P3 with respect to pH and temperature optima (table I, fig 4). However, P1, P2 and P3 and the proteinases studied earlier had, with the exception of proteinase LLP-IIC_2, pH optima characteristic for neutral enzymes and temperature optima between 30 and 45 °C (table I). P2 was relatively somewhat more active at pH 5–5.5 than P1 and P3, but all three enzymes showed marked activity at 12 and 50 °C. A low activation energy was characteristic of proteinase LLP-IIC_2 (Akuzawa and Okitani, 1995).

Effect of inhibitors

Results in table II indicate that proteinase P1 is a metalloenzyme while P2 and P3 are serine proteinases. However, both P2 and P3 were partially inhibited by metal chelators and *p*-hydroxymercuribenzoate at a concentration of 5 mmol/L. Treatment of the proteinases with inhibitors at a concentration of 1 mmol/L gave less conclusive results. Metalo-, serine and cysteine proteinases were found in cytoplasm from lactococci (table I). Noncaseinolytic lactococcal endopeptidases (PepO, PepF) are metalloenzymes (Monnet et al, 1994; Stepaniak and Fox, 1995; Law and Haandrikman, 1996).

Specificity of P1, P2 and P3 proteinases

Urea-PAGE of β-casein hydrolysates show marked differences in the peptide patterns produced by the three intracellular proteinases from *L lactis* subsp *lactis* MG1363 and the CEP from *Lactococcus lactis* subsp *lactis* 712, but the differences between electrophoregrams of peptides released by the four enzymes from α_{s1}-casein are less

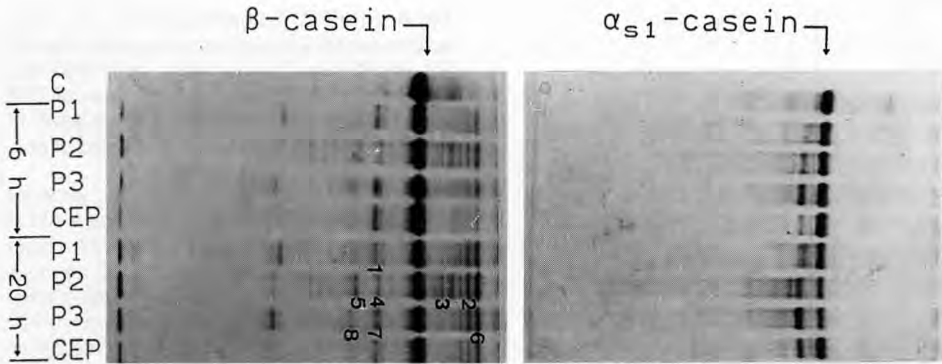

Fig 5. Urea-PAGE electrophoregrams of α_{s1}- and β-casein incubated with proteinases P1, P2 and P3 from *L lactis* subsp *lactis* MG1363 and crude cell envelope-associated proteinase (CEP) from *L lactis* subsp *lactis* 712 for 6 or 20 h. C: control incubated for 20 h without proteinases.

Band	N-terminal sequence	Position on β-casein
1 :	Arg-Glu-Leu-Glu-Glu-Leu-Asn-X..	1-7-X...
2 :	Ala-Gln-Thr-Gln-Ser-X...	53-57-X...
3 :	Contained 2 peptides:	
	Lys-Ile-Glu-Lys-Phe-X...	29-33-X...*
	Ala-Gln-Thr-Gln-X...	53-56-X...
4 :	Arg-Glu-Leu-Glu-X...	1-4-X...
5 :	Arg-Glu-Leu-Glu-X...	1-4-X...
6 :	Contained 2 peptides:	
	Asp-Lys-Ile-His-Pro-Phe-X...	47-52-X...
	Ala-Gln-Thr-Gln-Ser-X...	53-57-X...
7 :	Arg-Glu-Leu-Glu-Glu-Leu-Asn-X...	1-7-X...
8 :	Arg-Glu-Leu-Glu-Glu-X...	1-5-X...

*N-terminal sequence of γ1-casein

Électrophorégramme urée PAGE des caséines α et β incubées pendant 6 ou 20 heures avec les protéinases P1, P2 et P3 de L lactis *ssp* lactis *MG1363 ou les protéinases brutes associées à l'enveloppe cellulaire de* L lactis *ssp* lactis *712 (CEP). C; témoin incubé 20 heures sans protéinase.*

distinct (fig 5). Amino acid sequencing of some peptides released from β-casein showed that most of them were N-terminal fragments of β-casein (fig 5). Reverse-phase (RP) FPLC chromatograms of TFA-soluble peptides released from α_{s1}-casein also indicated differences in the specificity of P1, P2 and P3 (fig 6).

Proteinases P1, P2, P3 and CEP hydrolysed (RP-FPLC chromatograms are not shown) α_{s1}-CN f157-164, a minor peptide in the chymosin hydrolysate of α_{s1}-casein (McSweeney et al, 1993), which was not degradable by the lactococcal PepO (Stepaniak and Fox, 1995).

The intracellular metalloproteinase (M_s 49.5 kDa by gel filtration) from *L lactis* subsp *lactis* biovar *diacetylactis* characterized by Desmazeaud and Zevaco (1976) and Zevaco and Desmazeaud (1980) cleaved β-casein slowly at Pro_{186}-Ile_{187} and Ala_{189}-Phe_{190}. The enzyme was identified in chromatographic fractions using oxidized insulin. It also slowly hydrolyzed α_{s1}-casein, but it rapidly degraded two β-casein-derived peptides and bradykinin. Its specificity was similar to that of thermolysin

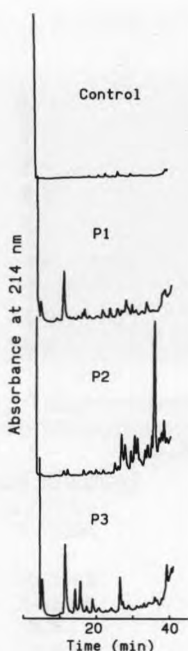

Fig 6. RP-FPLC chromatograms of 1% TFA-soluble peptides released from α_{s1}-casein by P1, P2 and P3 from *L lactis* subsp *lactis* MG1363. C: control as in figure 5. A large peak of TFA eluted at the beginning and a large peak of undigested α_{s1}-casein eluted at the end of acetonitrile gradient are not shown.
Chromatogrammes RP-FPLC de peptides solubles dans l'acide tri-fluoroacétique à 1 % et libérés de la caséine α_{s1} par P1, P2 ou P3 à partir de L lactis *ssp* lactis *MG1363. C: témoin, comme sur la figure 5. Un large pic d'acide tri-fluoroacétique élué au début ainsi qu'un large pic de caséine α_{s1} non hydrolysée éluée à la fin du gradient d'acétonitrile ne sont pas montrés.*

Table II. Effect of specific inhibitors on the activity of intracellular proteinases P1, P2 and P3 from *L lactis* subsp *lactis* MG1363.
Effet des inhibiteurs spécifiques sur l'activité des protéinases intracellulaires P1, P2 et P3 de L lactis *ssp* lactis *MG 1363.*

Inhibitor (5 mmol/L)	*P1*	*P2*	*P3*
	Relative	*activity*	*(%)*
Control	100	100	100
PMSF*	78	8	14
EDTA	21	90	67
o-Phenanthroline	35	38	30
p-HMB**	72	41	30
Iodoacetic acid	73	10	19

or 70 kDa lactococcal PepO. The proteinase characterized by Muset et al (1989) (table I) also slowly hydrolyzed β-casein. The specificity of proteinases studied by Westhoff et al (1971) and Muset et al (1989) on oxidized insulin chain B was similar to that of thermolysin.

CONCLUSION

The cytoplasm of prt⁻, plasmid-free *L lactis* subsp *lactis* MG1363 contains a complex caseinolytic system comprising proteinases of apparently different specificity. The three major proteinases (P1, P2 and

P3) are quite active at the pH and temperature of ripening cheese. Assessment of the ability of P1, P2 and P3 to hydrolyze β-CN f193-209 is warranted because this peptide is resistant to degradation by chymosin, lactococcal CEP (Exterkate et al, 1995) and lactococcal, 70 kDa PepO (Stepaniak et al, 1996). Immunological studies may reveal if the intracellular proteinases P1, P2 and P3 are related to the minor proteolytic fractions detected during the purification process or with intracellular lactococcal proteinases studied by other authors.

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REFERENCES

Akuzawa R, Yokoyama K (1993) Behavior of a proteinase, indicated low activation energy from *Lactococcus lactis* ssp *lactis* during cheese ripening. *Jpn J Dairy Food Sci* 42, 145-151

Akuzawa R, Okitani A (1995) Purification and characterization of a cysteine proteinase with low activation energy from *Lactococcus lactis* spp *lactis* IAM 1198. *J Dairy Sci* 78, 2609-2616

Akuzawa R, Yokoyama K, Matsuishi M, Okitani A (1990) Isolation and partial characterization of intracellular proteinases in *Lactococcus lactis* ssp *lactis* IAM 1198. *J Dairy Sci* 73, 3385-3392

Akuzawa R, Yagi N, Kimura M, Okitani A (1994) Purification and characterization of a serine proteinase from *Lactococcus lactis* spp *lactis* IAM 1198. *Anim Sci Technol [Jpn]* 65, 22-32

Andrews AT (1983) Proteinases in normal bovine milk and their action on caseins. *J Dairy Res* 50, 45-55

Blakesley RW, Boezi JA (1977) A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Anal Biochem* 82, 580-582

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254

Crow VL, Coolbear T, Holland R, Pritchard GG, Martley F (1993a) Starters as finishers: starter properties relevant to cheese ripening. *Int Dairy J* 3, 423-460

Crow VL, Holland R, Coolbear T (1993b) Comparison of subcellular fractionation methods for *Lactococcus lactis* ssp *lactis* and *Lactococcus lactis* ssp *cremoris*. *Int Dairy J* 3, 599-611

Dawson RMC, Elliott D, Elliott WH, Jones KM (1969) *Data for Biochemical Research*. Clarendon Press, Oxford, UK

Desmazeaud MJ, Zevaco C (1976) General properties and substrate specificity of an intracellular neutral protease from *Streptococcus diacetylactis*. *Ann Biol Biochim Biophys* 16, 851-868

Exterkate FE, Alting AC, Slangen CJ (1995) Conversion of α_{s1}-casein- (24-199)-fragment and β-casein under cheese conditions by chymosin and starter peptidases. *System Appl Microbiol* 18, 7-12

Fox PF, Guiney J (1972) A procedure for partial fractionation of the α_{s1}-casein complex. *J Dairy Res* 39, 49-53

Fox PF, Singh TK, McSweeney PLH (1995) Biogenesis of flavour compounds in cheese. *In: Chemistry of Structure-Function Relationships in Cheese* (EL Malin, MH Tunick, eds) Plenum Press, New York, USA

Hilt W, Wolf D (1996) Proteasomes: destruction as a programme. *Trends Biochem Sci* 21, 96-102

Law J, Haandrikman A (1996) Proteolytic enzymes of lactic acid bacteria. *Int Dairy J* (in press)

McSweeney PLH, Olsen NF, Fox PF, Healy A, Hojrup P (1993) Proteolytic specificity of chymosin on bovine α_{s1}-casein. *J Dairy Res* 60, 401-412

Monnet V, Nardi M, Chopin A, Chopin MC, Gripon JC (1994) Biochemical and genetic characterization of PepF, an oligopeptidase from *Lactococcus lactis*. *J Biol Chem* 269, 32070-32076

Muset G, Monnet V, Gripon JC (1989) Intracellular proteinase of *Lactococcus lactis* ssp *lactis* NCDO 763. *J Dairy Res* 56, 765-778

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

Orlowski M (1990) The multicatalytic proteinase complex, a major extralysosomal proteolytic system. *Biochemistry* 29, 10289-10297

Singh TK, Fox PF, Healy A (1995) Water-soluble peptides in Cheddar cheese. Isolation and identification of peptides in the diafiltration retentate of the water-soluble fraction. *J Dairy Res* 62, 629-640

Stepaniak L, Fox PF (1995) Characterization of the principal intracellular endopeptidase from *Lactococcus lactis* subsp *lactis*. *Int Dairy J* 5, 699-713

Stepaniak L, Gobbetti M, Søhaug T, Fox PF, Højrup P (1996) Peptides inhibitory to endopeptidase and aminopeptidase from *Lactococcus lactis* ssp *lactis* MG1363, released by chymosin, trypsin and chymotrypsin from bovine β-casein. *Z Lebens Unters Forsch* 202, 329-333

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

Westhoff DC, Cowman RA (1971) Substrate specificity of the intracellular proteinase from a slow acid producing mutant of *Streptococcus lactis*. *J Dairy Sci* 54, 1265-1269

Westhoff DC, Cowman RA, Swaisgood HE (1971) Characterization of an intracellular proteinase of a slow acid producing mutant of *Streptococcus lactis*. *J Dairy Sci* 54, 1259-1264

Zevaco C, Desmazeaud MJ (1980) Hydrolysis of β-casein and peptides by intracellular neutral protease of *Streptococcus diacetylactis*. *J Dairy Sci* 63, 15-24