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The presence of two proteinases associated with the cell wall of *Lactobacillus bulgaricus*

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Abstract

Whole cells of *Lactobacillus delbrueckii* subsp. *bulgaricus* ACA DC235 are able to hydrolyse casein. This proteolytic activity is greatly enhanced when cells are grown in milk rather than in a peptide-rich synthetic medium such as De Man-Sharp-Rogosa. A significant part of the caseinolytic activity can be extracted by treating the intact cells with lysozyme, which suggests that the enzyme(s) involved are associated with the cell wall. The soluble lysozyme extract has been partially fractionated by ultrafiltration using different membranes. Biphasic kinetics of irreversible thermal denaturation, partial inactivation by various agents, and selective reactivation by zinc ions indicated that the overall caseinolytic activity was due to two distinct enzymes. The first one was rapidly inactivated upon heating, inhibited by EDTA, reactivated by Zn^{2+} ions, and was probably a zinc-dependent metalloprotease. The other one was more stable towards thermal inactivation, inhibited by phenylmethanesulfonyl fluoride, insensitive to *N*-ethylmaleimide, activated by Ca^{2+} ions, and was probably a serine protease.

Keywords: *Lactobacillus bulgaricus*; Degradation of caseins; Metalloprotease; Serine protease

1. Introduction

Cell envelope proteinases are the first enzymes which act in the degradation of caseins by lactic acid bacteria. These enzymes catalyse the initial step in the hydrolysis of milk proteins by the starter proteolytic system. This process provides the cell with the peptides and amino acids essential for growth [1] and responsible for some of the organoleptic properties of the fermented products [2]. Increase of proteolytic activity results in a more rapid growth of lactic acid bacteria, which is industrially important since it ensures an appropriate rate of acid production in milk fermentation [3].

In the last few years the biochemistry and genetics of cell wall-associated proteinases have been investigated extensively in lactococcal strains. The release of the enzymes from the cell surface occurs spontaneously in a Ca^{2+}-free buffer [1,4]. Some general properties of solubilized lactococcal proteinases are high molecular masses, pH optima between 6 and 7, and a strong inhibition by phenylmethanesulfonyl

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fluoride (PMSF), suggesting they are serine-type proteinases. Fewer data are available on the proteinases present in lactobacilli. The enzyme from *L. casei* has been well characterized [6,9–11], and is very similar to the lactococcal proteinase. Cell wall proteinolytic activities have also been detected in *L. bulgaricus* [5], *L. plantarum* [6], *L. helveticus* [5,7,8] and *L. lactis* [5].

L. delbrueckii subsp. *bulgaricus*, called *L. bulgaricus* from here on, is a microorganism industrially used in yogurt production [3], in which multiple forms of proteinase, all referred to as cell-envelope bound, have been described [12–14]. This paper reports that a much larger caseinolytic activity can be released from whole cells of *L. bulgaricus* strain ACA DC235 by treating them with lysozyme than by a calcium-free buffer, and that this caseinolytic activity is due to (at least) two different enzymes, probably a serine- and a zinc-dependent proteases. This is the first time that two different proteinases associated with the cell wall are described in *L. bulgaricus*.

2. Materials and methods

2.1. Chemicals

Casein and PMSF were obtained from Sigma; *N*-ethylmaleimide (NEM) was obtained from Aldrich Chemical Co.; lysozyme and fluoresceine isothiocyanate (FITC) were purchased from Serva (Heidelberg, Germany). Skim milk was a generous gift from Union Laitiere Normande, Condé-sur-Vire, France. All other reagents used were of analytical grade and were obtained from Merck (Germany).

2.2. Bacterial strains and growth conditions

L. delbrueckii subsp. *bulgaricus* strain ACA DC235 was obtained from the dairy collection of the Agricultural University of Athens. Bacteria were grown at 40° C in 10% skim milk. Reconstituted skim milk was prepared as described by Atlan et al. [15]. The milk was inoculated from an overnight preculture of the same strain grown under the same conditions.

2.3. Preparation of the cell wall extract

After 5 h of growth, bacteria were harvested as described by Atlan et al. [15]. Cell pellets were washed twice with 50 mM Tris · HCl (pH 7.5), then resuspended in the same buffer and treated with 1 mg ml^{-1} lysozyme for 30 min at 37° C. After centrifugation the supernatant was used as soluble cell wall extract and was examined for proteolytic activity. Measurements of D-lactate dehydrogenase activity according to Le Bras and Garel [16] were used to evaluate the extent of cell lysis after lysozyme treatment.

2.4. Assay of caseinolytic activity

Caseinolytic activity was determined as described by Twinning [17], using casein labelled with fluorescein isothiocyanate as a substrate and monitoring the formation of TCA-soluble fluorescent peptides. Fluorescein thiocarbamoyl casein (FTC-casein) was prepared in the laboratory according to Twinning [17]. The standard caseinolytic activity was assayed using a mixture containing 20 μl of a 3.5 mg ml^{-1} FTC-casein solution, 10 μl of soluble cell wall extract, and 20 μl of 0.2 M Tris · HCl (pH 7.5) (assay buffer). The mixture was incubated at 45° C for the appropriate time (between 0.5 and 3 h), and the reaction was stopped by addition of 120 μl 5% TCA. After centrifugation, 150 μl of the supernatant were diluted with 850 μl of 0.5 M Tris · HCl (pH 8.5), and the fluorescence of TCA-soluble products was measured with an Amicon BS2 luminescence spectrometer, using an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Under these assay conditions, fluorescence of the TCA-soluble fraction increased linearly both with the incubation time for a fixed amount of cell wall extract, and with the amount of cell wall extract for a fixed incubation time.

2.5. Fractionation of the cell wall extract

The soluble cell wall lysozyme extract was fractionated by ultrafiltration using an Amicon cell with a YM100 membrane from. The flow-through fraction was ultrafiltered again but on a YM30 membrane using a Centricon 30 microconcentrator. All the pre-

sent results on proteolytic activity were obtained on the resulting fraction.

2.6. Inhibition by various agents

The mixture of the enzyme solution and the assay buffer was pre-incubated for 30 min at room temperature with the different reagents in concentrations as indicated in Table 1. The proteinase inhibitors used in this study were PMSF, EDTA, ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid (EGTA) and NEM. After this incubation, the residual proteolytic activity was measured by adding FTC-casein solution (3.5 mg ml^{-1}).

2.7. Effect of divalent cations

The ability of certain divalent cations to restore the proteolytic activity was studied after inhibition by EDTA or EGTA. The inhibited enzyme was incubated in presence of the indicated concentration of the divalent cation for 30 min at room temperature, and the residual caseinolytic activity was measured.

2.8. Irreversible thermal inactivation

The active fraction was diluted into assay buffer prewarmed at the inactivation temperature of 55° C. The mixture was incubated at 55° C and samples were taken after various times of incubation, chilled in cold assay buffer, and their residual proteolytic activities were measured at 45° C by the standard assay.

In some experiments the heating at 55° C was preceded by a pre-incubation of the active fraction with 1 mM PMSF or 1 mM EDTA in the presence of 1 mM Zn^{2+} ions for 30 min (see above).

3. Results and discussion

3.1. Extraction

Cells of *L. bulgaricus* strain ACA DC235 were grown in milk because this medium gave a ten-fold higher proteolytic activity (unpublished data) than the artificial broth of De Man-Rogosa-Sharpe [18]. A weak caseinolytic activity could be obtained by washing the whole cells with Ca^{2+}-free buffer, a method widely used for the proteinase release in lactococci [1,4]. However, treating the cells with lysozyme solubilized a caseinolytic activity that was at least 20 times larger, suggesting that the enzyme(s) responsible for this activity were tightly associated with the cell wall. The caseinolytic activity present in this soluble cell wall lysozyme extract decreased slowly with time because of inactivation and/or aggregation of the proteinase(s).

It was checked that the caseinolytic activity was not due to the release of an intracellular protease upon lysis of the cells. Indeed, less than 5% of the total activity of the intracellular enzyme D-lactate dehydrogenase was present in the cell wall lysozyme

Table 1
Effects of protease inhibitors and metal chelators on the caseinolytic activity from the cell wall of *L. bulgaricus* measured by the release of TCA-soluble fluorescence from FTC-casein

Addition of	Relative activity (%) [a]
Buffer	100
1 mM PMSF	55 ± 6
1 mM NEM	33 ± 4
1 mM NEM + 1 mM PMSF	≤ 10
0.5 mM EGTA	30 ± 3
0.5 mM EGTA + 0.5 mM Ca^{2+}	92 ± 7
0.5 mM EGTA + 0.5 mM Zn^{+2}	46 ± 5
1 mM EDTA	≤ 10
1 mM EDTA + 1 mM Zn^{2+}	62 ± 5
1 mM EDTA + 1 mM Co^{2+}	23 ± 3
1 mM EDTA + 1 mM Zn^{2+} + 1 mM PMSF	41 ± 5

[a] Average values from three to five independent determinations on different proteinase preparations.

extract, showing less than 5% of the cells were lysed.

3.2. Fractionation of the soluble cell wall extract by ultrafiltration

The fresh soluble lysozyme extract was ultrafiltered on an Amicon YM100 membrane, and more than 70% of total caseinolytic activity was found in the filtrate and less than 30% in the retentate. The specific activity was also higher in the filtrate. This YM100 filtrate was further ultrafiltered on a YM30 membrane, and almost no caseinolytic activity was found in the filtrate. This step did not only remove some contaminants, but also concentrated the proteinase(s) into a strongly active fraction that was used for the following studies.

3.3. Inhibition by different chemical reagents

The effects of proteinase inhibitors on the caseinolytic activity are given in Table 1. Only a partial inactivation was obtained with the serine-protease inhibitor PMSF or the thiol-reagent NEM, even when higher concentrations of inhibitor or longer incubation times were used. This indicated that the overall caseinolytic activity could be due to a mixture of a PMSF-sensitive (NEM-sensitive) and PMSF-resistant (NEM-resistant) enzymes, in roughly equal proportions. A combined treatment by both PMSF and NEM caused an almost complete inactivation, showing that the PMSF-resistant (NEM-resistant) part of the activity was NEM-sensitive (PMSF-sensitive).

3.4. Inhibition by metal chelators and re-activation by metal ions

Table 1 shows that the calcium chelator EGTA also leads to a partial inactivation, indicating that part of the caseinolytic activity is calcium-dependent. Complete recovery of the caseinolytic activity after inhibition by EGTA was obtained by the addition of Ca^{2+} ions. This re-activation did not occur when Zn^{2+} were used instead of Ca^{2+} (Table 1).

In contrast, the less specific metal chelator EDTA caused an almost complete inactivation, suggesting that the calcium-independent part of the activity also needed a metal albeit different from calcium. After inhibition by EDTA, the caseinolytic activity could be partially restored by the addition of Zn^{2+} ions (and to a lesser extent of Co^{2+} ions) (Table 1). This showed that the overall caseinolytic activity was due to two enzymes in roughly equal amounts, one that needed Ca^{2+} ions and the other that needed Zn^{2+} ions.

The fraction of the activity that needs Zn^{2+} ions was not sensitive to the presence of PMSF (Table 1), whereas we have found that the fraction that needs Ca^{2+} ions was inhibited by PMSF (this result was obtained using a slightly different fractionation and is not shown in Table 1). Therefore the overall caseinolytic activity is due to two enzymes in about equal proportions, the first one being PMSF-sensitive, NEM-resistant and Ca^{2+}-dependent, and the other being NEM-sensitive, PMSF-resistant and Zn^{2+}-dependent. It was verified that the ability to hydrolyse intact caseins into large peptides was not abolished after partial inhibition by either PMSF or NEM, which indicates that these two enzymes were genuine proteases.

NEM is a reagent rather specific for thiol groups, and NEM sensitivity of the zinc-dependent enzyme could be explained by the fact that thiol groups (together with imidazole groups) can contribute to the binding of Zn^{2+} ions in the active sites of proteins [19]. Alternatively, this enzyme could have a crucial Cys residue not involved in zinc binding [2].

3.5. Biphasic kinetics of irreversible thermal inactivation

The caseinolytic activity was irreversibly inactivated upon heating at 55° C. The kinetics of loss of activity at 55° C were biphasic, as judged from the change in slope seen in a first-order semi-logarithmic plot of the residual activity as a function of time (Fig. 1), showing the presence of two species with different heat-stabilities. By the standard method of 'peeling-back' exponentials, the loss of activity was broken down into the sum of two exponentials with roughly equal amplitudes, and largely different time constants, each corresponding to the inactivation of one species. These kinetics of inactivation confirmed that the overall caseinolytic activity was due to two

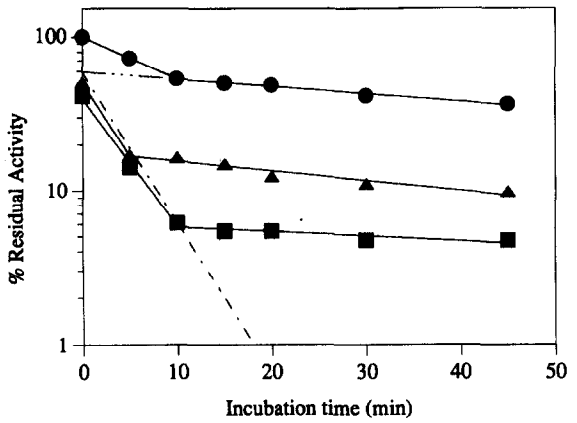

Fig. 1. Semi-logarithmic plot of the kinetics of heat inactivation. The amount of caseinolytic activity remaining after the given time of heating at 55° C was measured as described in Materials and methods without any treatment of the active fraction (●), after partial inactivation by 1 mM PMSF (▲), or after partial inactivation by 1 mM EDTA in the presence of 1 mM Zn^{2+} ions (■). The dashed lines show the decomposition of the kinetics of inactivation of the untreated active fraction (●) into a slower phase that extrapolated to zero time with an amplitude around 58% (- · · · -) and a faster phase with an amplitude of 42% (- · - · -). Note that partial inactivation prior to heating at 55° C modified only the relative proportion of these two kinetic phases, and not their rates (see text).

different enzymes in roughly equivalent amount, and showed that one was heat-labile and rapidly inactivated with a half-life of 4 min at 55° C, and the other was heat-stable and more slowly inactivated with a half-life of 65 min at 55° C.

3.6. Correspondence between heat stability and PMSF sensitivity

The kinetics of irreversible thermal inactivation were measured under the same conditions, but after previous partial inhibition by PMSF. The residual PMSF-resistant activity was lost rapidly in less than 15 min, with the same half-life as that of the heat-labile component of the overall activity (Fig. 1). This indicated that the heat-labile enzyme corresponded to the PMSF-resistant component of activity.

The inhibition by PMSF was measured after previous partial thermal inactivation by 15 min at 55° C. The residual heat-resistant caseinolytic activity was almost completely inhibited by PMSF, confirming that the activity remaining after the complete inactivation of the heat-labile component was sensitive to PMSF.

In conclusion, there was a good correspondence between the sensitivity (respectively resistance) to PMSF and the stability (respectively lability) towards temperature.

3.7. Correspondence between heat stability and zinc dependence

The kinetics of irreversible thermal inactivation were measured in the same conditions, but after previous complete inactivation by EDTA and subsequent partial reactivation by Zn^{2+} ions. The zinc-dependent component of the activity was irreversibly inactivated with a half-life of 4 min (Fig. 1), showing that the zinc-dependent enzyme corresponded indeed to the heat-labile component, in agreement with the above conclusion that the same enzyme was PMSF-resistant and zinc-dependent, and also heat-labile.

4. Conclusion

The results presented here strongly suggest that the caseinolytic activity of *L. bulgaricus* strain ACA DC235 was due to two different enzymes, probably a NEM-sensitive, heat-labile, zinc-dependent metalloprotease, and a PMSF-sensitive, heat-stable, calcium-dependent serine protease. These two enzymes contributed about equally to the total caseinolytic activity. The low amount of proteolytic activity extracted with a calcium-free buffer indicated that both enzymes were strongly attached to the cell wall. Similarly, the low amount of proteolytic activity obtained after growth in synthetic medium suggested that both enzymes were induced by growth in milk.

The presence of two different proteinases in *L. bulgaricus* and their enzymatic characteristics were not in agreement with some other reports [13,14]. This could be due to a difference in the strains used, and more work is needed to establish whether or not the same two enzymes are also present in other strains. Also, only the purification and molecular characterization of the serine protease can show whether or not it is homologous to the serine proteinase present in different strains of lactococci [1,4].

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References

[1] Kok, J. (1990) Genetics of the proteolytic system of lactic acid bacteria. FEMS Microbiol. Rev. 87, 15–42.

[2] Visser, S. (1993) Proteolytic enzymes and their relationship to cheese ripening and flavour: an overview. J. Dairy Sci. 76, 329–350.

[3] Hartley, D.L. and Denariaz, G. (1993) The role of lactic acid bacteria in yogurt fermentation. Int. J. Immunother. 9, 3–17.

[4] Pritchard, G.G. and Coolbear, T. (1993) The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. 12, 179–206.

[5] Ezzat, N., El Soda, M., Bouillane, C., Zevaco, C. and Blanchard, P. (1985) Cell wall-associated proteinases in *Lactobacillus helveticus*, *Lactobacillus bulgaricus* and *Lactobacillus lactis*. Milchwissenschaft 40, 140–143.

[6] El Soda, M., Desmazeaud, M.J., Le Bars, D. and Zevaco, C. (1986) Cell wall-associated proteinases in *Lactobacillus casei* and *Lactobacillus plantarum*. J. Food Protect. 49, 361–365.

[7] Yamamoto, N., Akino, A. and Takano, T. (1993) Purification and specificity of a cell wall-associated proteinase from *Lactobacillus helveticus* CP790. J. Biochem. 114, 740–745.

[8] Martin-Hernadez, C.M., Alting, A.C. and Exterkate, F.A. (1994) Purification and characterization of the mature, membrane-associated cell envelope proteinase of *Lactobacillus helveticus* L89. Appl. Microbiol. Biotechnol. 40, 828–834.

[9] Kojic, M., Fira, D., Banina, A. and Topisirovic, L. (1991) Characterization of the cell wall-bound proteinase of *Lactobacillus casei* HN14. Appl. Environ. Microbiol. 57, 1753–1757.

[10] Naes, H. and Nissen-Meyer, J. (1992) Purification and N-terminal amino acid sequence determination of the cell wall-bound proteinase from *Lactobacillus paracasei* subsp. *paracasei*. J. Gen. Microbiol. 138, 313–318.

[11] Holck, A. and Naes, H. (1992) Cloning, sequencing and expression of the gene encoding the cell envelope-associated proteinase from *Lactobacillus paracasei* subsp. *paracasei* NCDO 151. J. Gen. Microbiol. 138, 1353–1364.

[12] Argyle, P.J., Mathison G.E. and Chandan, R.C. (1976) Production of cell-bound proteinase by *Lactobacillus bulgaricus* and its location in the bacterial cell. J. Appl. Bacteriol. 41, 175–184.

[13] Ezzat, N., Zevaco, C., El Soda, M. and Gripon, J.C. (1987) Partial purification and characterization of a cell wall-associated proteinase from *Lactobacillus bulgaricus*. Milchwissenschaft 42, 95–97.

[14] Laloi, P., Atlan, D., Blanc, B., Gilbert, C. and Portalier, R. (1991) Cell wall-associated proteinase of *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: differential extraction, purification and properties of the enzyme. Appl. Microbiol. Biotechnol. 36, 196–204.

[15] Atlan, D., Laloi, P. and Portalier, R. (1989) Isolation and characterization of aminopeptidase-deficient *Lactobacillus bulgaricus* mutants. Appl. Environ. Microbiol. 55, 1717–1723.

[16] Le Bras, G. and Garel, J.R. (1991) Properties of D-lactate dehydrogenase from *Lactobacillus bulgaricus*: a possible different evolutionary origin for the D- and L-lactate dehydrogenases. FEMS Microbiol. Lett. 79, 89–94.

[17] Twinning, S.S. (1984) Fluoresceine isothiocyanate-labeled casein assay for proteolytic enzymes. Anal. Biochem. 143, 30–34.

[18] De Man, J.C., Rogosa, M. and Sharpe, M.P. (1960) A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23, 130–135.

[19] Glusker, J.P. (1991) Structural aspects of metal liganding to functional groups in proteins. Adv. Protein Chem. 42, 1–76.