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Purification of an X-prolyl-dipeptidyl aminopeptidase from the cell wall proteolytic system of *Lactococcus lactis* subsp. *cremoris*

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Summary. The β-casein specific cell wall proteolytic system of *Lactococcus lactis* subsp. *cremoris* P8-2-47 contains a metal-independent X-prolyl-dipeptidyl-aminopeptidase. Suitable substrates for its assay are Gly-Pro-nitroanilide and Ala-Pro-nitroanilide. It is suggested that the function of the enzyme is to cleave the proline-rich sequences of β-casein, as shown by the degradation of β-casomorphin. It is a serine proteinase with a monomer molecular mass of about 90000 daltons, a temperature optimum of 45°-50° C, and a pH optimum of about 7.

Introduction

Lactococcus lactis subsp. *cremoris* P8-2-47 is able to grow in milk due to a β-casein-degrading proteolytic system located in the cell wall (Thomas and Pritchard 1987). The first enzyme in the necessary cascade of reactions from the initial breakdown of β-casein to the transport of amino acids and peptides into the cytoplasm is a highly β-casein-specific protease in most strains, called *Lactococcus* β-caseinase in this communication. This enzyme, which is usually coded for on plasmids (Kok et al. 1985), has an apparent molecular mass of 145000 daltons (Geis et al. 1985). The nucleotide sequence of its gene has recently been established (Kok et al. 1988). The β-casein peptides generated by the purified β-caseinase have been isolated and characterized. The acid soluble peptides of β-casein contain between two and 24 amino acids (Monnet et al. 1986; Bockelmann 1987). The peptide bonds specifically split by the β-caseinase are located between phenylalanine 52 and glycine 94, and glutamic acid 160 and glutamic acid 194 of the β-casein peptide chain. All these peptides disappear when incubated with intact cells of *L. lactis* subsp. *cremoris* P8-2-47. Since large peptides cannot be transported through the bacterial cell membrane, other enzymes found in the cell wall fractions, including a dipeptidase (van Boven et al. 1988), an aminopeptidase (Geis et al. 1985) and a membrane bound peptidase (Exterkate and de Veer 1987) must complement the β-caseinase. Using peptides isolated from a *Lactococcus* β-caseinase digest of β-casein and synthetic bovine β-casomorphin as substrates, we detected an additional metal-independent peptidase activity in the β-casein specific proteolytic system. Due to the discovered specificity for X-prolyl moieties, Gly-Pro-4-nitroanilide turned out to be an excellent model substrate for the characterization of this new enzyme.

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Materials and methods

Bacteria and culture conditions. Lactococcus lactis subsp. *cremoris* P8-2-47 was isolated from a mesophilic dairy starter culture (Andresen et al. 1984). The organism was routinely maintained frozen in litmus milk at −74° C. For the preparation of cells, M17 broth (Terzaghi and Sandine 1975) was inoculated with 1% bacteria, pregrown for several cycles in litmus milk and passaged once in M17 broth. Incubation was at 30° C for 16 h.

Isolation and purification procedure. Step 1: peptidase activity was solubilized from the cells by repeated washings (2-3 times) of intact cells with 50 m*M* Tris-HCl buffer, pH 7.5 (Geis et al. 1985); 20 g cells (wet weight) were suspended in 200 ml buffer, stirred for 30 min at 20° C and collected by centrifugation. Step 2: anion exchange chromatography (*Q*-Sepharose Fast Flow, Pharmacia, Uppsala, Sweden); a linear gradient of 0-0.3 *M* NaCl in 50 m*M* TRIS-HCl, pH 7.5 was used. Step 3: chromatofocusing (Mono-P HR 5/20 column, Pharmacia); the

start buffer was 0.025 *M* Bis-TRIS-HCl, pH 6.3; proteins were eluted with 50 ml polybuffer 74 (Pharmacia) diluted 1:10 with distilled water and adjusted to pH 4.0 with HCl. Step 4: anion exchange chromatography (Mono-*Q* HR 5/5 column, Pharmacia); proteins were eluted in a gradient of 0–0.3 *M* NaCl in 50 m*M* TRIS-HCl, pH 7.5. Gel filtration was performed on an HPLC column (G2000SW 7.5 mm × 600 mm, Pharmacia-LKB) in 50 m*M* TRIS-HCl, pH 7.5, including 0.2 *M* NaCl.

Electrophoresis. The molecular weight and the purification status of the enzyme were estimated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). For activity staining, gels according to Laemmli were used without SDS. Activity staining was performed in 10% PAGE by the method of Miller and Mackinnon (1974). As substrates, Tyr-Pro-Phe or β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) were used. Liberated Phe and Ile were detected in a coupled enzyme reaction in which *o*-dianisidine was oxidized in the presence of L-amino acid oxidase (Boehringer Mannheim, FRG).

Peptidase assay with gels under non-denaturating conditions. To distinguish several peptidase activities present in the wash fluid, enzyme extracts were run in native gels. The gels were cut into strips in the direction of the slots, which were cut again into 1–1.5 mm parallel pieces and incubated in parallel with different substrates (Lys-4-NA for the aminopeptidase, Tyr-Pro-Phe for the prolylpeptidase and Leu-Gly for the dipeptidase). By this procedure, it was possible to associate enzyme activities with the defined protein bands seen on the gels.

Standard prolylpeptidase assay. Prolylpeptidase activity was determined at 37°C using 1 m*M* Gly-Pro-4-nitroanilide (Bachem, Bubendorf, Switzerland) in 50 m*M* TRIS-HCl, pH 7.5, in a total volume of 0.5 ml. The reaction was stopped by the addition of 1 ml of 30% acetic acid. The release of nitroaniline was spectrophotometrically followed at 410 nm. Activities are expressed as micromoles nitroaniline released per minute per milligram of protein (= 1 Unit); protein was determined by the Coomassie Brilliant Blue method (Sedmak and Grossberg 1974) with bovine serum albumin as a standard.

Thin-layer chromatography (TLC). The identification of amino acids was achieved by TLC on HPTLC-silica gel 60 (Merck, Darmstadt, FRG) DC-plates (10 × 10 cm). As solvents, butanol/acetic acid/water (4:2:2) and chloroform/methanol/ammonia (4:4:2) were used; detection was with 0.1% ninhydrin in acetone and incubation was for 10–15 min at 80°C.

Results and discussion

Washing of intact cells of mesophilic lactococci with TRIS-HCl buffer induces the release of cell surface proteins including a β-caseinase (Exterkate 1975, 1976). Analysis of this supernatant by PAGE followed by activity staining yielded three distinct zones of active peptidases: (1) a dipeptidase (relative electrophoretic mobility compared to bromophenol blue: rem, 0.44) splitting the dipeptide Leu-Gly, which seems to be identical with the enzyme described recently from Koning's laboratory (van Boven et al. 1988); (2) an aminopeptidase (rem, 0.19) splitting the artificial substrate L-Lys-4-nitroanilide (Geis et al. 1985); (3) a peptidase (rem, 0.1) splitting β-casomorphin and Gly-Pro-4-nitroanilide. The latter enzyme is of special interest since it appears to be the first demonstration of a peptidase in the cell wall proteolytic system of *L. lactis* subsp. *cremoris* which is able to degrade the proline rich peptides that are abundant in β-casein. Similar enzymes from *Lactobacillus lactis* and *Streptococcus thermophilus* were described by Meyer and Jordi (1987). Since these enzymes were purified from crude cell free extracts, a cell wall location was not established.

The peptidase from *L. lactis* subsp. *cremoris* P8-2-47 was enriched 300-fold by three chromatography steps (Fig. 1). The SDS-PAGE of peptidase-containing fractions is shown in Fig. 2, revealing a protein with a molecular mass of about 90000 daltons. By gel filtration on an HPLC G2000SW column a molecular mass of 160000–180000 daltons was found, suggesting a dimer configuration of the native enzyme, as also de-

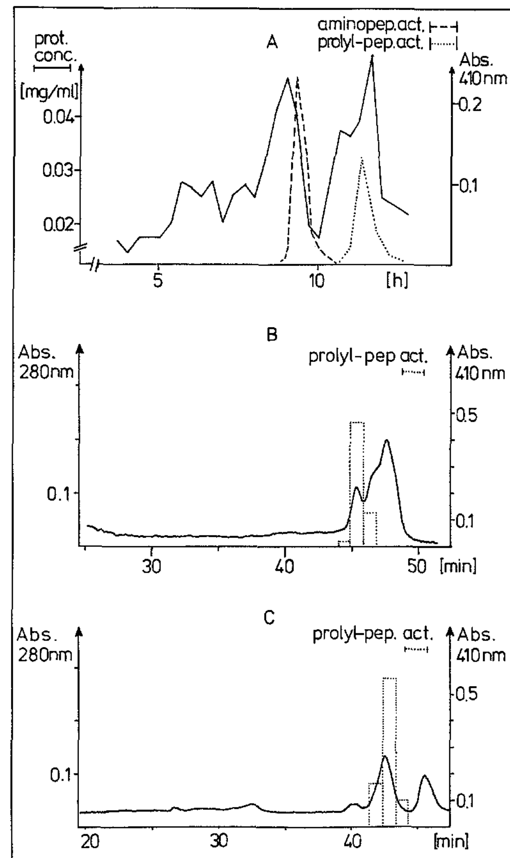

Fig. 1. Purification of the X-prolyl-dipeptidyl-aminopeptidase by chromatography on Q-Sepharose Fast Flow **(A)**, Mono-P HR 5/20 **(B)**, and Mono-Q HR 5/5 **(C)**

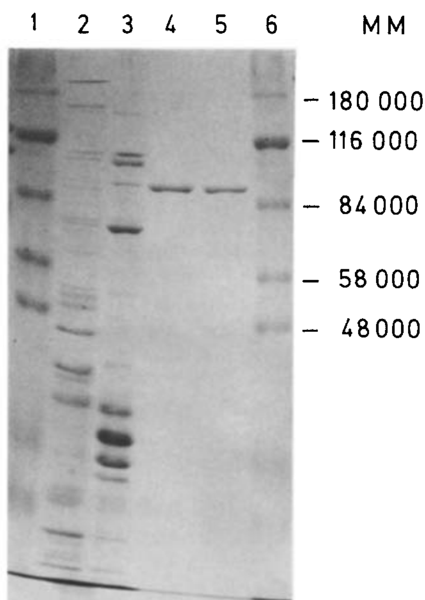

Fig. 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the X-prolyl-dipeptidyl-aminopeptidase from the cell wall of *Lactococcus lactis* subsp. *cremoris* P8-2-47: lanes *1* and *6*, molecular mass marker proteins (SDS-Blue, Sigma, Deisenhofen, Fed Rep Germany); lane *2*, cell wash supernatant; lane *3*, most active Q-Sepharose fraction (step 1); lanes *4* and *5*, prolylpeptidase after three steps of purification (maximum specific activity, lane *5*)

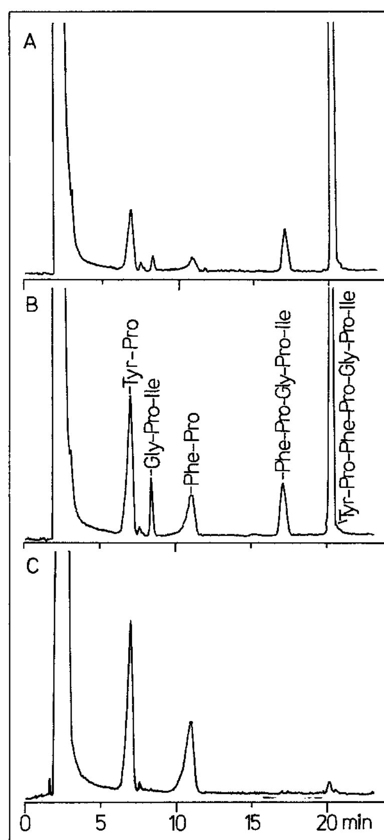

Fig. 3A–C. Reversed phase HPLC (RP-18 column) of hydrolysis products of β-casomorphin generated by *L. lactis* subsp. *cremoris* P8-2-47 prolyl-peptidase. Recording of the chromatograms was performed at 220 nm. Buffer system: (a) 10 m*M* K phosphate buffer, pH 7.2; (b) acetonitrile + buffer a (60:40). Gradient: 0% b–80% b in 40 min. The reaction mixture (2.5 nmol β-casomorphin in 50 µl 50 m*M* TRIS-HCl buffer, pH 7.5) was incubated with different amounts of enzyme for 2.5 h at 37° C. The peaks were identified with the help of thin-layer chromatography on the basis of the reference compounds Tyr-Pro, Phe-Pro, Gly-Pro, β-casomorphin and the free amino acids. **A.** Enzyme activity adequate to cleave 0.45 nmol of the added β-casomorphin. **B.** Enzyme activity adequate to cleave 0.95 nmol of β-casomorphin. **C.** Enzyme activity adequate to cleave β-casomorphin completely (>2.5 nmol)

scribed for *Lb. lactis* and *S. thermophilus* (Meyer and Jordi 1987).

The quantitative enrichment data are summarized in Table 1. Evidence for specificity of the enzyme towards β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) is given in Fig. 3. The main split products detected by HPLC were identified as Tyr-Pro and Phe-Pro (Fig. 3C). The missing peptide Gly-Pro was eluted in the salt peak which also contained the free amino acid Ile. This was confirmed by TLC with appropriate standards. The kinetics of β-casomorphin degradation is indicated in Fig. 3. At a constant incubation time of 2.5 h and a concentration of β-casomorphin of 40 µg/ml, low enzyme concentrations produced the dipeptide Tyr-Pro and the pentapeptide Phe-Pro-Gly-Pro-Ile as the main products (Fig. 3A). At medium enzyme concentrations, the dipeptide Phe-Pro and the tripeptide Gly-Pro-Ile appeared in addition (Fig. 3B). When high enzyme concentrations were applied, only Tyr-Pro, Phe-Pro, Gly-Pro, and Ile were detected by HPLC (Fig. 3C) or TLC. This indicates that the attack of the enzyme on β-casomorphin starts from the aminoterminal end leading to X-proline dipeptides and free isoleucine. The enzyme is therefore considered to be

Table 1. Purification and yield of the X-prolyl-dipeptidyl-aminopeptidase from the cell wall of *Lactococcus lactis* subsp. *cremoris* P8-2-47. Substrate used in the assays was Gly-L-Pro-4-nitroanilide

Purification step	Total protein (mg)	Specific activity (Units)[a]	Purification factor	Yield (%)
1. Raw extract	172.0	0.3	1	100
2. Q-Sepharose	9.4	1.5	5	27
3. Mono-P	0.35	18	60	12
4. Mono-Q	0.04	90	300	7

[a] Units of specific activity are micromoles of nitroaniline released per minute per milligram of protein

an X-prolyl-dipeptidyl-aminopeptidase. Gly-Pro-4-nitroanilide turned out to be a suitable substrate for further characterization of the enzyme; Ala-Pro-nitroanilide was cleaved at a 15% slower rate.

The pH optimum has a broad peak between pH 6.5 and 8 and the temperature optimum is between 45° C and 50° C. Above 50° C the enzyme is rapidly inactivated. The enzyme is not inactivated by 1 m*M* EDTA, in contrast to the amino- and dipeptidase (Geis et al. 1985; van Boven et al. 1988). Since the prolylpeptidase is severely inhibited by 0.1 m*M* phenylmethylsulphonylfluoride and diisopropyl fluorophosphate, we propose that it belongs to the class of proteases which have a serine residue in the active centre.

Since the wash supernatants of lactococci contain peptidase activities able to degrade peptides generated by the well-characterized, plasmid-coded β-caseinase (Bockelmann 1987), the possible role of the detected prolylpeptidase seems to match the high proline content of β-casein (35 residues out of 209 amino acids). In our laboratory, the enzyme has been detected in all 30 lactococcal strains investigated, including β-caseinase negative and plasmid free strains. This finding suggests that the enzyme might be coded for on the *Lactococcus* chromosome. Further genetic and biochemical studies of the new prolylpeptidase are in progress in our laboratory. These studies will help to develop a more precise model of how β-casein is actually split and used as a nitrogen source by lactococci growing in milk. The possible role of the enzyme in cheese ripening including degradation of bitter peptides remains to be established.

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References

Andresen A, Geis A, Krusch U, Teuber M (1984) Plasmidmuster milchwirtschaftlich genutzter Starterkulturen. Milchwissenschaft 39:140–143

Bockelmann W (1987) Das proteolytische System von Milchsäurebakterien. PhD. Thesis, University of Kiel

Boven A van, Tan PST, Konings WN (1988) Purification and characterization of a dipeptidase from *Streptococcus cremoris* WG2. Appl Environ Microbiol 54:43–49

Exterkate FA (1975) An introductory study of the proteolytic system of *Streptococcus cremoris* strain HP. Neth Milk Dairy J 29:303–318

Exterkate FA (1976) The proteolytic system of a slow lactic acid producing variant of *Streptococcus cremoris* HP. Neth Milk Dairy J 30:3–8

Exterkate FA, de Veer GJCM (1987) Partial isolation and degradation of caseins by cell wall proteinase(s) of *Streptococcus cremoris* HP. Appl Environ Microbiol 53:1482–1486

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

Kok J, van Dijl JM, van der Vossen JMBM, Venema G (1985) Cloning and expression of a *Streptococcus cremoris* proteinase in *Bacillus subtilis* and *Streptococcus lactis*. Appl Environ Microbiol 50:94–101

Kok J, Leenhouts KJ, Haandrikman AJ, Ledebeer AM, Venema G (1988) Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* WG2. Appl Environ Microbiol 54:231–238

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Meyer J, Jordi R (1987) Purification and characterization of X-prolyl-dipeptidyl-aminopeptidase from *Lactobacillus lactis* and from *Streptococcus thermophilus*. J Dairy Sci 70:738–745

Miller CG, Mackinnon K (1974) Peptidase mutants of *Salmonella typhimurium*. J Bact 120:355–363

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

Sedmak JJ, Grossberg SE (1977) A rapid, sensitive, and versatile assay for protein using Coomassie Brilliant Blue G250. Anal Biochem 79:544–552

Terzaghi BE, Sandine WE (1975) An improved medium for lactic streptococci and their bacteriophages. Appl Microbiol 29:807–813

Thomas TD, Pritchard GG (1987) Proteolytic enzymes of dairy starter cultures. FEMS Microbiol Rev 46:245–268

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