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PURIFICATION AND CHARACTERIZATION OF PROLINE-SPECIFIC PEPTIDASES FROM *LACTOCOCCUS* AND *LACTOBACILLUS*

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ABSTRACT

Purified proline-specific amino peptidases from *Lactobacillus curvatus* and from *Lactococcus lactis* were active on both X-proline dipeptidyl aminopeptidase (PepX) substrates, Gly-Pro-AMC or Gly-Pro*p*NA and on proline endopepetidase (PEP) substrates Suc-Gly-Pro-Leu-Gly-Pro, Suc-Gly-Pro-AMC, Z-Gly-Pro-AMC or Suc-Gly-Pro-*p*NA, however; activity on PEP substrates was markedly less than that on PepX substrates. The enzymes from *Lactobacillus* and *Lactococcus* hydrolyzed a number of oligopeptides containing 7-11 amino acids residues and proline at the penultimate position from N-terminus, but hydrolysis of natural PEP oligopeptide substrates containing proline residues at internal positions was negligible. The two proline-specific enzymes were strongly stimulated by NaCl and inhibited by phenylmethylsulfonyl fluoride and organic solvents.

Key words: lactococcus, lactobacillus, proline, enzymes.

INTRODUCTION

Specific enzymes are required to hydrolyze peptide bonds involving proline. Aminopeptidases and oligopeptidases, including proline-specific peptidases, degrade peptides released from casein or muscle protein by indigenous or exogenous proteinases. Many proline-containing peptides are bitter and their hydrolysis by proline-specific peptidases may remove or reduce bitterness in enzyme-modified cheese [5]. Dipeptidyl peptidases from muscle influenced a taste formation in dry-cured ham [10]. Intracellular, proline-specific iminopeptidases, prolidases and X-prolyl dipeptidyl aminopeptidases (PepX) have been isolated from different lactic acid bacteria [3] but proline endopeptidase (PEP) activity has been reported only in a crude enzyme preparation from *Lactobacillus casei* [2]. PEPs have been isolated from different animal tissues and from *Flavobacterium* and *Xanthomonas* spp. [6, 7]. Peptide bonds involving a prolyl residue are usually not hydrolyzed by general oligopeptidases and general amino-, di- or tripeptidases. Several proline-specific peptidases along with broad specificity peptidases, are needed for the efficient degradation of peptides containing internal proline residues. PEPs have also important physiological functions because they can degrade neuro-hormones [3, 9].

MATERIALS AND METHODS

Lactococcus lactis MG1363 and *Lactobacillus curvatus* 2042 were selected for isolation of proline-specific enzymes because they had high intracellular activity on proline endopoeptidase fluorogenic substrates. *Lb. curvatus* readily develops biofilms and, therefore, often contaminates cheese milk [4].

Enzymes were purified from the cytoplasmic fraction by sequential chromatography on hydroxyapatite, Sephacryl 200, Mono Q and Phenyl Superose. The prepartion of cytoplasmic fraction and purification conditions were as described for isolation of proline imino peptidase and oligopeptidases from from *Propionibacterium* [8, 9]. General, 70 kDa oligopeptidases [9] from *Lb. curvatus* and *Lc. lactis* were purified according the same procedure. Fluorogenic general endopeptidase substrate, N-succinyl-Ala-Ala-Phe-7-amido-4-methyl-coumarin (Suc-Ala-Ala-Phe-AMC), proline endopeptidase (PEP) substrates: Suc-Gly-Pro-AMC, Suc-Gly-Pro-Leu-Gly-Pro-AMC and N-carbobenzoxy-Gly-Pro-AMC (Z-Gly-Pro-AMC), X-prolyl diaminopeptidase substrates (PepX): Gly-Pro-AMC, colorgenic PEP substrate, Suc-Gly-Pro-*p*NA, colorgenic PepX substrate, Gly-Pro-*p*-nitroanilide (Gly-Pro-*p*NA), aminopeptidase substrates Leu- or Pro-*p*NA), leucine aminopeptidase and Z-prolyl-prolinal dimethyl acetal were obtained from Sigma or from Bachem. Different hormone oligopeptides were from Sigma. Casein (CN) peptides: a_{s1}-CN f1-23 and ß-CN f 60-72 were synthesized at the Biotechnology Center, University of Oslo on a peptide synthesizer (Model 431A, Applied Biosystem). All other chemicals, from different sources, were of analytical or HPLC grade.

Methods for determination of activity with fluorogenic or colorgenic substrates, pH, temperature optima and effect of chemicals were as described earlier [8, 9]. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) was used to study relative activity on oligopeptide substrates and to determine enzyme specificity. Peptides were separated on PepRPC HR 5/5 column connected to FPLC equipment (Pharmacia) operating with monitor at 214 nm [8, 9]. Activity was calculated as the percentage reduction in the area of the substrate peak. Molecular mass was determined by gel filtration chromatography on Sephacryl 200 [9] and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-

PAGE). The electrophoresis was performed using Mini-Protean II electrophoresis cell from Bio-Rad following procedure given by the manufacturer.

RESULTS AND DISCUSSION

Activity on both an X-proline dipeptidyl aminopeptidase (PepX) substrate and a proline endopeptidase (PEP) substrate was detected in purified to homogeneity enzymes from *Lactobacillus* and *Lactococcus*. However, the PepX activity was markedly higher than PEP activity (Table 1). The proline-specific enzyme from *Lactobacillus* was eluted from hydroxyapatite (Fig. 1), Mono Q (Fig. 2) and Phenyl Superose at different concentrations of elution gradients than the corresponding enzyme from *Lactococcus*. The *Lactobacillus* and *Lactococcus* PepX enzymes had molecular mass ca 95 and 90 kDa, respectively (Fig. 3). Gel filtration chromatography showed that these two enzymes are dimers. The purified enzymes did not hydrolyse aminopeptidase substrates Leu-*p*Na or Pro-*p*Na. The endopeptidase substrate Suc-Ala-Ala-Phe-AMC was not hydrolysed with or without coupled reaction with leucine aminopeptidase. This substrate is hydrolysed by chymotrypsin and with coupled reaction with leucine aminopeptidase by 70 kDa PepO from *Lactococcus* or *Lactobacillus* [9].

Figure 1. Separation on hydroxyapatite of proline specific peptidases and general oligopeptidase (PepO) from *Lb. curvatus* and *Lc. lactis*.
Arrows indicate maximum proline peptidase (PepX/PEP) activity on Gly-Pro-*p*nitranilide or Bezyloxycarbonyl-Gly-Pro-7-amido-4-methylcoumarin and PepO activity on α_{s1}-casein f-23.

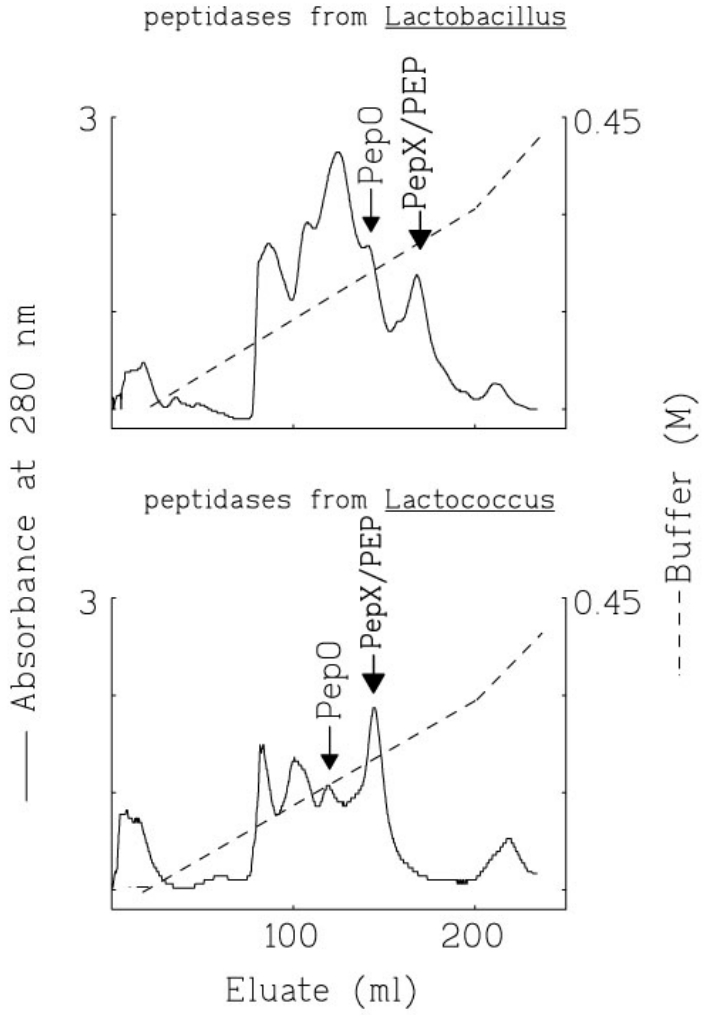


Figure 2. Purification on MonoQ PepX peptidases from *Lb. curvatus* and *Lc. lactis* Arrows indicate maximum proline peptidase (PepX/PEP) activity on Gly-Pro-*p*nitranilide or Bezyloxycarbonyl-Gly-Pro-7-amido-4-methylcoumarin

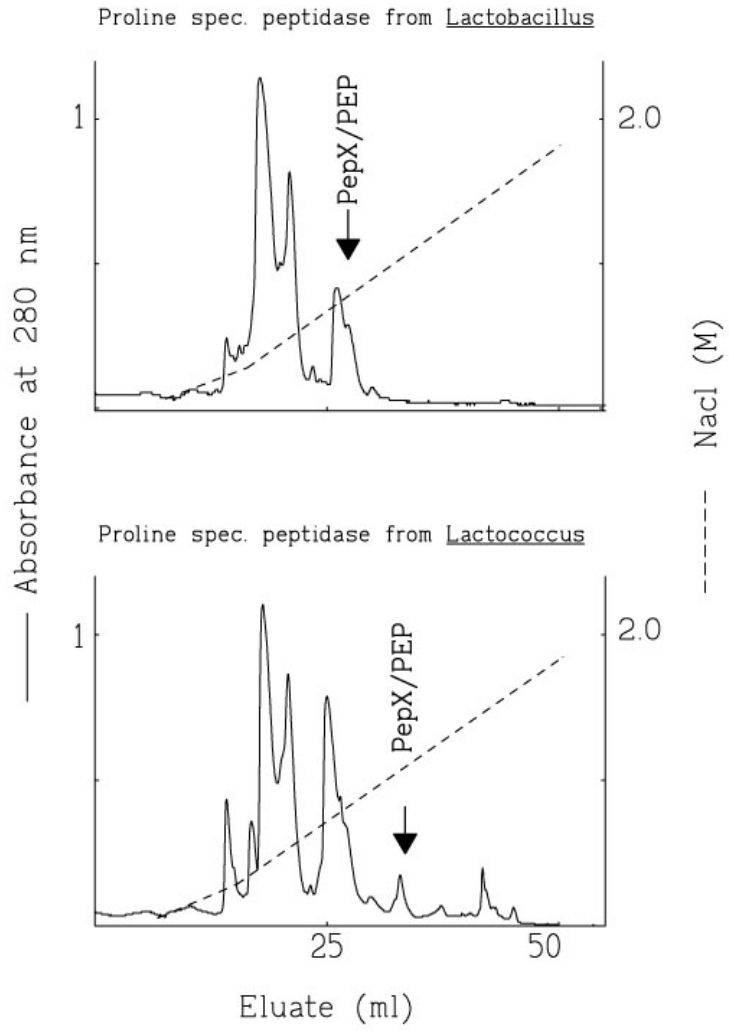
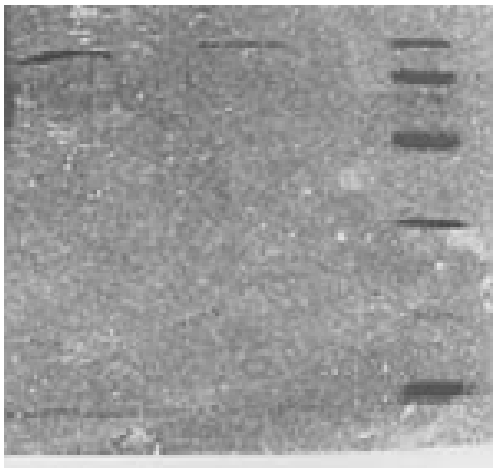


Table 1. Relative activity of proline-specific peptidases from *Lb. curvatus* and *Lc. lactis* on fluorogenic substrates

Relative actvity on:	***Lb. curvatus***	***Lb. lactis***
Substrate for X-proline dipeptidyl aminopeptidase (PepX)	%	%
Gly-Pro-AMC	100*	100
Substrates for proline endopeptidase		
Suc-Gly-Pro-Leu-Gly-Pro-AMC	28	26
Suc-Gly-Pro-AMC	10	11
Z-Gly-Pro-AMC	6	12

* Maximum activity under the incubation conditions

Figure 3. SDS-PAGE electrophoregram of purified X-prolyl dipeptide aminopeptidases (PepX) from *Lactobacillus* and *Lactococcus* Linees from left to right: Line 1 - PepX from *Lc.lactis*; Line 2 - PepX from *Lactococcus curvatus*; Line 3 - molecular mass protein markers (bands from top to bottom correspond to 97, 65, 45, 30 and 14 kDa)



Typically for PepX, the proline-specific enzymes from *Lactobacillus* and *Lactococcus* hydrolyzed a number of biologically active oligopeptides containing 7-11 amino acids residues and proline at the penultimate position from N-terminus, but hydrolysis of peptides containing proline residues in internal positions was negligible (Table 2, Fig. 4). The proline endopeptidase activity of the enzymes could, therefore be demonstrated only with synthetic peptide substrates with blocked N and C-terminus (Table 1) but not with natural oligopeptide substrates (Table 2). The proline-specific enzymes were stimulated by NaCl and inhibited by phenylmethylsulfonyl fluoride, methanol or acetonitrile (Table 3). Stimulation by NaCl or high ionic strength buffers and inhibition by is characteristic for PEP [6].

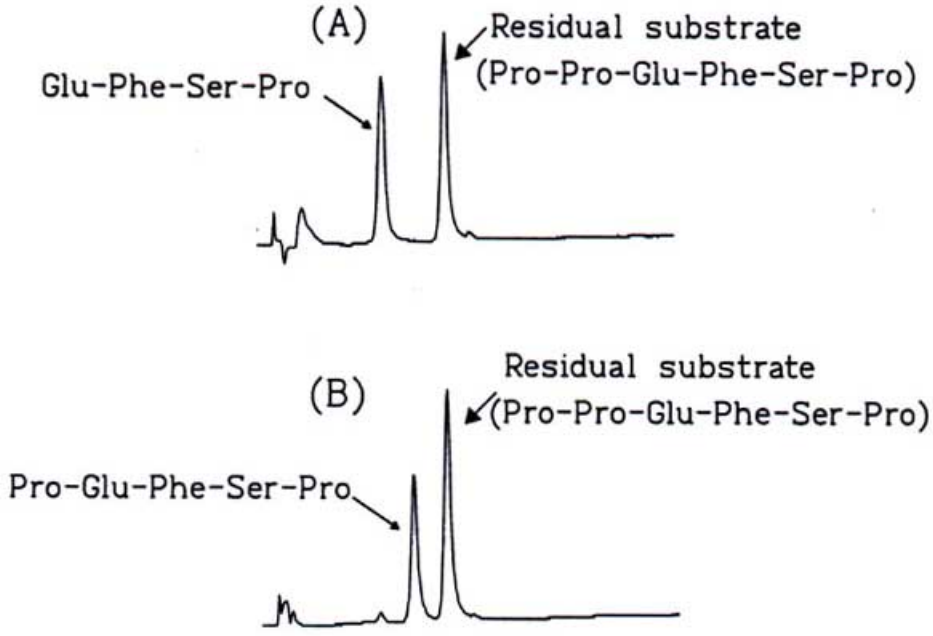
Table 2. Activity* of proline specific peptidases and general oligopeptidase PepO from *Lb. curvatus* and *Lc. lactis* on peptide substrates

Proline-specific peptidases from:	***Lactobacillus***	***Lactococcus***
Substrates for PepX		
ß-casophormin bovine (ß-CN f60-66, Tyr-Pro-Phe-Pro-Gly-Pro-Ile)	++++	++++
ß-casomorphin human (Tyr-Pro-Phe-Val-Pro-Ile)	++++	++++
ß-CN fr 60-72 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser-Leu)	++	++
Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	+++	+++
Bradykinin f2-7 (Pro-Pro-Gly-Phe-Ser-Pro)	+	+
Substrates for Proline endopeptida se		
Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	±	+
Neurotensin (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu)	–	–
Neurotensin f8-13 (Arg-Arg-Pro-Tyr-Ile-Leu)	±	–

Tyr-Bradykinin (Tyr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	±	±
Oligopeptidase PepO from		
ß-casomorphin bovine or human	–	–
Bradykinin	+++++	+++++
Bradykinin f2-7	++++	++++
Methionine enkephalin	++++	++++
Neurotensin	+++++	+++++

- Not hydrolysed; relative activity: +++++ 80-100%*; ++++ 40-60%; +++ 20-30%;
++ 5-10%; + 1-4%; ± <1%
*percent of peptide substrate peak reduction after incubation under the same conditions

Figure 4. Peptide profiles after incubation of Pro-Pro-Gly-Phe-Ser-Pro-Phe (Baradykinin f2-7) with purified proline-specific enzyme from *Lb. curvatus* (A). Chromatogram (B) of the same substrate incubated with proline iminopeptidase from *Propionibacterium* (8) is shown for comparison



The proline-specific enzymes from *Lactobacillus* and *Lactococcus* were not inhibited by Z-prolyl-prolinal dimethyl acetal (Table 3) which is highly specific inhibitor of PEP (1). PEP and PepX are distantly related members of the same family of serine proteases (6). Both PEP and PepX were inhibited by peptidyl ammonium methyl ketones, such as Z-Pro-Pro-$CH_2N^+C_5H_5$ [7].

Table 3. Effect of chemicals on activity of proline specific peptidase (PepX) and oligopeptidase PepO from *Lb. curvatus*[*]

Chemical	Residual activity %	
	PepX	PepO
Phenylmethylsulfonyl fluoride, 1 MM[**]	22	95
3,4-dichloroisocoumarin, 1 mM[**]	74	102
Z-prolyl-prolinal dimethyl acetal		
N-Tosyl-L-phenylalanine chlorometyl ketone, 1 mM[**]	95	100
o-phenathroline[***]	101	6
NaCl, 1 M	240	86
Methanol, 5%	38	ND
Acetonitrile, 5%	36	ND

[*] The effect of chemicals on PepX or PepO from *Lc lactis* was similar to that shown for PepX and PepO from *Lb curvatus*
[**]Inhibitors of serine peptidases; [***]Inhibitors of metallopeptitases; ND - not determined

The proline-specific enzymes from *Lactobacillus* and *Lactococcus* were most active at pH 6-7 and 30-35 ^{o}C, but retained ca 25% activity at pH 5 and at 7 ^{o}C.

CONCLUSIONS

The isolated enzymes from *Lactococcus* and *Lactobacillus* are likely PepX peptidases, although they also show some activity on proline endopeptidase synthetic substrates.

The enzymes may, remain quite active at the pH and temperature of cheese ripening. Activity of the two enzymes may be markedly stimulated by NaCl present in cheese.

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