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Short communication

The peptide hydrolase system of *Lactobacillus reuteri*

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Abstract

Peptide hydrolase system of *Lactobacillus reuteri* CRL 1098, a lactic acid bacteria of sourdough origin, was investigated. This microorganism has a broad range of peptidases consisting of an active aminopeptidase, X-Prolyl-dipeptidylamino-peptidase, dipeptidase and tripeptidase. Aminopeptidase, iminopeptidase and endopeptidase are most likely located in the cytoplasmic fraction showing no detectable association with the cell membrane, while dipeptidase and tripeptidase are mainly associated with the latter fraction. The peptidases are metalloenzymes activated by Co^{2+} and inhibited by Cu^{2+}, Hg^{2+}, Cd^{2+} and by metal-complexing reagents. The aminopeptidase activity inhibited by EDTA can be restored by Mn^{2+} while that of di- and tripeptidase treated with 1,10-phenantroline can be restored by Zn^{2+} and Co^{2+}, respectively. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Nitrogen is a growth-limiting factor for lactic acid bacteria (LAB) in several food ecosystems (Laan et al., 1989). Only by means of their proteolytic system, consisting of proteinases and peptidases, LAB are able to hydrolize proteins into small peptides and amino acids necessary for growth, which can be transported through the cell membrane (Smid et al., 1991). Proteolytic enzymes also play a key role in ripened food products and in different ecosystems including wheat and bran sourdoughs (Spicher, 1987). The production of flavour precursors such as free amino acids or sugars is important for aroma development in sourdough; thus, strains with high metabolic activity are preferentially selected (Spicher, 1983; Gobbetti et al., 1994). The characterization of the proteolytic system of non-dairy LAB could extend the knowledge on the significance of this bacterial group in various food ecosystems.

The present study was undertaken to characterize the peptide hydrolase system of *Lactobacillus reuteri* in order to better know the role it plays in sourdough fermentations.

2. Materials and methods

2.1. Organism and preparation of cell fractions

Lb. reuteri CRL 1098 used in this study was isolated from sourdough and belongs to the culture collection of Centro de Referencia para Lactobacilos

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(CERELA), Tucumán-Argentina. The organism was grown in MRS broth (De Man et al., 1960) at 37 °C. Bacterial cells were harvested by centrifugation at $7000 \times g$ for 15 min at 4 °C at the end of the exponential phase, washed twice with 10 mM potassium phosphate buffer, pH 7.0, and resuspended in the same buffer. Cells were disrupted with a French press. Cell extracts were obtained by centrifugation (International Equipment, model B-22M centrifuge) of disrupted cells ($9000 \times g$, 15 min, 4 °C) and the supernatants obtained were referred to as crude cell-free extracts (CE). The cell walls and membrane fractions were pelleted by a second centrifugation ($145,000 \times g$, 60 min, 4 °C) and resuspended in 10 mM potassium phosphate buffer.

2.2. Enzyme assays

2.2.1. Aminopeptidase (AP) activity

Aminopeptidase (AP) activity was measured on chromogenic substrates *p*-nitroaniline (*p*-NA) derivatives of L-anomers of leucine, lysine, alanine, valine, proline, methionine, phenylalanine, glutamic acid and glycine, by the method of El Soda and Desmazeaud (1982). The assay mixture contained 50 μl of the substrate (20 mM in methanol), 2.6 ml of potassium phosphate buffer (10 mM, pH 6.0) and 25 μl of enzyme solution. After incubation at 37 °C for 15 min, the reaction was stopped with 30% (v/v) acetic acid, and the color intensity of the 4-nitroaniline produced was measured at 410 nm in a Cecil Model 2021 series 2000 spectrophotometer. The concentration of *p*-nitroaniline was calculated from the derived value of the molar absorption coefficient ($E_{410} = 8800\ M^{-1}\ cm^{-1}$). One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of *p*-nitroaniline per minute under the assay conditions stated above.

2.2.2. X-Prolyl-dipeptidyl aminopeptidase (PDA) and endopeptidase (EP) activities

X-Prolyl-dipeptidyl aminopeptidase was measured on Gly-Pro-*p*-NA and endopeptidase activity on *N*-succinyl L-phenyl-alanine-*p*-NA (Suphepa), and *N*-glutaryl L-phenyl-alanine-*p*-NA (Gluphepa) as substrates. The hydrolysis of synthetic substrates was measured by the method of El Soda and Desmazeaud (1982). The enzyme units were defined as before.

2.2.3. Dipeptidase (DP) and tripeptidase (TP) activities

DP and TP activities were measured on Leu-Leu, Gly-Gly, Leu-Pro, Gly-Tyr and Leu-Leu-Leu, Gly-Gly-Gly, DL-Leu-Gly-DL-Phe, Leu-Gly-Gly as substrates, respectively. The hydrolysis of synthetic substrates was carried out at 37 °C and the activities were determined by the estimation of α-amino groups by the Cd–ninhydrin method (Doi et al., 1981). The assay mixture contains potassium phosphate buffer 10 mM pH 7.0 (400 μl), substrate (20 mM; 50 μl) and enzyme solution (50 μl). After incubation at 37 °C for 15 min, the reaction was stopped by addition of Cd–ninhydrin reagent and further incubated at 85 °C for 5 min; the reaction mixture was cooled and the enzyme activity was determined by reading the A_{505}. An enzyme unit was defined as the amount of enzyme required to release 1 μmol amino acid per minute.The specific activity was defined as units of enzyme per milligram protein used in the assay.

2.2.4. Protein determination

The protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

2.3. Effect of pH and temperature

The effect of pH on the enzymes activities was examined at 37 °C in the pH range 3.5–9.0. The buffers used were: citrate buffer (pH 3.5–6.0), potassium phosphate buffer (pH 5.6–8.0) and Tris–HCl buffer (pH 7.0–9.0). The effect of temperature was determined at pH 7.0 in the temperature range of 20–50 °C.

2.4. Effect of chemical reagents and divalent cations

Various cations were added as chloride or sulphate salts to the cell-free extract to assess their effect on enzyme activities. Different chemical reagents were also used: dithiothreitol (DTT), β-mercaptoethanol, L-cysteine and ethylenediaminetetraacetic acid (EDTA) (dissolved in distilled water), phenylmethylsulphonylfluoride (PMSF) (dissolved in propanol); 1,10-phenanthroline (dissolved in ethanol) and *p*-chloromercuribenzoate (*p*-CMB) (dissolved in 1 N NaOH). A mixture containing cell-free extract

and 0.1 or 1.0 mM (final concentration) chemical reagents or divalent cations in 10 mM potassium phosphate buffer, pH 6.0 for AP and pH 7.0 for and DP and TP, was incubated at 37 °C for 30 min. Reactions were initiated by adding the specific substrates.

2.4.1. Reactivation of the enzyme activities inhibited by chelating reagents

The reactivation of the inhibited enzymes by EDTA or 1,10 phenantroline was investigated preincubating the cell-free extract at 37 °C for 30 min with the chelating agents in 10 mM potassium phosphate buffer (pH 6.0 for AP and pH 7.0 for DP and TP). Subsequently, divalent cations (Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+} or Zn^{2+}) were added separately to the enzyme mixture to give a final concentration of 1.0 mM before adding the specific substrates to start the reaction.

2.4.2. Chemicals

All the synthetic chromogenic substrates: L-Leu-*p*-NA, L-Ala-*p*-NA, L-Phe-*p*-NA, L-Pro-*p*-NA, L-Glu-*p*-NA, L-Lys-*p*-NA, Gly-*p*-NA, L-Met-*p*-NA, L-Val-*p*-NA, Gly-Pro-*p*-NA, *N*-succinyl L-phenylalanine-*p*-NA (Suphepa), *N*-glutaryl L-phenylalanine-*p*-NA (Gluphepa) and di- and tripeptides: Leu-Leu, Gly-Gly, Leu-Pro, Gly-Tyr; Leu-Leu-Leu, Gly-Gly-Gly, DL-Leu-Gly-DL-Phe and Leu-Gly-Gly were obtained from Sigma (St. Louis, MO, USA).

3. Results and discussion

Lb. reuteri CRL 1098 has a broad spectrum of aminopeptidase (AP) that are active against the substrates assayed. The highest enzyme activity with *p*-NA derivates was obtained when the diaminomonocarboxylic acid (Lys) was present at the N-terminal position (Table 1). The activity with Lys-*p*-NA was 2.4 times higher than that obtained with Leu-*p*-NA, which was the standard *p*-NA derivative used in the assay. Low enzymatic activity was detected on Gly-*p*-NA, Val-*p*-NA and Glu-*p*-NA. Pro-*p*-NA was also hydrolyzed by the microorganism indicating the presence of proline iminopeptidase (PIP) activity. Since gluten (the wheat storage protein) contains a high amount of proline residues, the presence of PIP enzyme would be an important criteria for selecting sourdough starter cultures (Gobbetti et al., 1996b). The enzymes of *Lb. reuteri* CRL 1098 are comparable to other AP enzymes isolated from *Lb. lactis* subsp. *cremoris* Wg2 (Tan and Konings, 1990), *Lb. helveticus* (Miyakawa et al., 1992), *Lb. casei* (El Abboudi et al., 1992) and *Lb. sanfrancisco*, a key sourdough lactobacillus (Gobbetti et al., 1996a).

Table 1
Hydrolysis of chromogenic substrates by cell-free extract of *Lb. reuteri* CRL 1098

Substrate	Relative activity (100%)[a]
Leu-*p*-NA	100 ± 0.0[b]
Ala-*p*-NA	45 ± 3.5
Phe-*p*-NA	50 ± 4.0
Pro-*p*-NA	14 ± 1.7
Glu-*p*-NA	14 ± 2.0
Lys-*p*-NA	240 ± 5.0
Gly-*p*-NA	15 ± 2.3
Met-*p*-NA	70 ± 3.0
Val-*p*-NA	11 ± 3.3
Gly-Pro-*p*-NA	136 ± 6.4

[a] Expressed as percentage of the activity against L-Leu-*p*-NA, which was given a value of 100%.
[b] Mean ± standard deviation of three independent measurement.

Among the various substrates assayed, maximum DP and TP activities were observed against Leu-Leu and Leu-Leu-Leu, respectively (Table 2). This fact and the low enzyme activity on some Leu-Gly would indicate similarity among the DP enzymes of *Lb.reuteri* CRL 1098 and those isolated from other LAB strains (Wohlrab and Bockelmann, 1992; Gobbetti et al., 1996a).

The enzymes AP, PIP and EP were detected only in the cell-free extract fraction while DP and TP enzymes were mainly found associated to the cell membrane-wall fraction (results not shown). In this fraction, the enzymatic activities were 2.74 (DP) and 2.2 (TP) times higher than those obtained in crude cell-free extracts. These results are in agreement with those reported for *Lb. sanfrancisco* (Gobbetti and Corsetti, 1997).

The optimum pH for cell-free extract AP, DP and TP activities was about 6.0, 8.0 and 6.5, respectively, and the optimal temperature was about 37 °C

Table 2
Hydrolysis of di- and tripeptides by cell-free extract of *Lb. reuteri* CRL 1098

Substrate	Relative activity (100%)[a]
Leu-Leu	100 ± 0.0[b]
Gly-Gly	70 ± 1.8
Leu-Pro	7 ± 0.0
Gly-Tyr	3 ± 0.05
Leu-Leu-Leu	100 ± 4.0
Gly-Gly-Gly	46 ± 2.2
DL-Leu-Gly-DL-Phe	0 ± 0.03
Leu-Gly-Gly	64 ± 2.5

[a] Expressed as a percentage of the activity against Leu-Leu and Leu-Leu-Leu, which were given a value of 100% for dipeptidase and tripeptidase activities, respectively.

[b] Mean ± standard deviation of three independent measurements.

for AP and DP and about 45 °C for TP (results not shown).

Table 3 shows the effect of chelating agents and group-specific inhibitors on the cell-free extract enzyme activities. The enzymes were inhibited by ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline (metal-complexing reagents) as well as by *p*-chloromercuribenzoate (*p*-CMB), a sulfhydryl-blocking reagent. Dithiothreitol (DTT), cysteine and β-mercaptoethanol (thiol groups reducers) and phenylmethylsulphonylfluoride (PMSF) (potent inhibitor of serine-proteases) had no apparent effect on the enzyme activities.

The influence of various divalent metal ions on the enzymes activities is shown in Table 4. The AP activity appeared to be stimulated by Co^{2+}, Ca^{2+} and Mn^{2+}. It was inhibited by Zn^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+}. *p*-CMB (1 mM; Table 3) and the heavy metal Hg^{2+} (1 mM; Table 4) inhibited the AP activity by 65% and 100%, respectively, indicating that the AP is a metalloenzyme and at least one sulfhydryl group is essential for activity. The stimulation of AP by Co^{2+} has been observed previously in *Lb. delbrueckii* subsp. *bulgaricus* (Bockelmann and Schulz, 1992), *Streptococcus mitis* ATCC 903 (Andersson et al., 1992), *Lb. casei* (El Abboudi et al., 1992), *Bacillus* sp. N_2 (Lee et al., 1998) indicat-

Table 3
Effect of chemical reagents on cell-free extract enzyme activities

Chemical reagents[a]	Concentration (mM)	Relative activity (%)[b]		
		AP	DP	TP
No addition		100 ± 0.0[c]	100 ± 0.0	100 ± 0.0
DTT	0.1	96 ± 1.6	96 ± 2.4	110 ± 2.0
	1.0	88 ± 1.9	84 ± 2.1	80 ± 0.9
β-mercapto-ethanol	0.1	93 ± 2.1	99 ± 3.0	103 ± 3.0
	1.0	109 ± 2.9	91 ± 1.4	82 ± 2.1
L-cysteine	0.1	117 ± 3.1	93 ± 0.9	104 ± 2.6
	1.0	90 ± 3.0	83 ± 1.1	98 ± 2.0
EDTA	0.1	15 ± 0.7	50 ± 2.8	45 ± 1.7
	1.0	12 ± 0.6	35 ± 1.1	22 ± 0.3
1,10-phenantroline	0.1	47 ± 3.7	21 ± 1.3	6 ± 0.2
	1.0	16 ± 2.0	16 ± 1.7	5 ± 0.4
PCMB	0.1	45 ± 1.1	58 ± 3.5	30 ± 1.3
	1.0	35 ± 2.9	24 ± 1.6	13 ± 1.1
PMSF	0.1	110 ± 3.5	110 ± 3.9	92 ± 3.7
	1.0	89 ± 2.0	99 ± 2.9	87 ± 2.7

[a] DTT: dithiothreitol, EDTA: ethylenediaminetetraacetic acid, PCMB: *p*-chloromercuribenzoate, PMSF: phenylmethylsulfonyl fluoride.

[b] Aminopeptidase (AP), dipeptidase (DP) and tripeptidase (TP) activities were determined against Leu-*p*-NA, Leu-Leu, and Leu-Leu-Leu as substrates, respectively, as described in Section 2.

[c] Mean ± standard deviation of three independent measurements.

Table 4
Effect of metal ions on the cell-free extract enzyme activities

Divalent cation	Concentration (mM)	Relative activity (%)		
		AP	DP	TP
No addition		100 ± 0.0[a]	100 ± 0.0	100 ± 0.0
Co^{2+}	0.1	124 ± 3.8	120 ± 2.2	117 ± 1.3
	1.0	141 ± 3.5	141 ± 2.7	130 ± 1.4
Mn^{2+}	0.1	160 ± 2.2	128 ± 1.8	116 ± 3.7
	1.0	112 ± 3.3	95 ± 2.3	121 ± 5.5
Fe^{2+}	0.1	115 ± 3.9	66 ± 2.3	141 ± 2.5
	1.0	67 ± 3.5	55 ± 3.8	157 ± 1.9
Cu^{2+}	0.1	75 ± 2.7	54 ± 2.9	41 ± 3.0
	1.0	17 ± 2.5	8 ± 0.8	0 ± 0.0
Ca^{2+}	0.1	125 ± 5.0	125 ± 3.6	106 ± 1.6
	1.0	137 ± 2.6	157 ± 2.2	108 ± 1.3
Hg^{2+}	0.1	50 ± 1.7	70 ± 3.6	75 ± 4.7
	1.0	0 ± 0.0	2 ± 0.5	63 ± 1.6
Mg^{2+}	0.1	93 ± 2.0	103 ± 1.5	128 ± 2.8
	1.0	89 ± 2.7	178 ± 4.2	142 ± 2.5
Zn^{2+}	0.1	73 ± 4.1	193 ± 2.9	148 ± 2.3
	1.0	13 ± 1.5	234 ± 2.4	155 ± 2.8
Cd^{2+}	0.1	41 ± 3.6	80 ± 2.4	85 ± 2.5
	1.0	10 ± 0.4	54 ± 2.7	48 ± 2.6

[a] Mean ± standard deviation of three independent measurements.

ing that Co^{2+} is playing a role in the activity or stability of aminopeptidase.

Ions like Zn^{2+}, Ca^{2+}, Mn^{2+} (0.1 mM), Mg^{2+} and Co^{2+} seem to stimulate DP while Fe^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} have a stimulatory effect on the TP (Table 4).

After inhibition by 1.0 mM EDTA, the AP activity could almost completely be restored by the addition of 1.0 mM Mn^{2+} and in a less extent by Mg^{2+}, Zn^{2+} and Ca^{2+} (data not shown). These results support the hypothesis that Mn^{2+} plays a role in the activity or stability of *Lb. reuteri* AP. The AP activity could not be restored by any divalent cation when treated with 1,10-phenanthroline (results not shown) in contrast to the DP and TP activities which could be completely restored by 1 mM Zn^{2+} and Co^{2+}, respectively (results not shown). Mn^{2+} partially restored both enzymatic activities.

Results presented here provide evidences of an active peptide hydrolase system in *Lb. reuteri* CRL 1098, which has a broad range of peptidase enzymes. Since wheat flour contains considerable amounts of low molecular weight peptides, the presence of di- and tripeptidases in starter cultures might be more important than other proteolytic enzymes for the predominance of LAB in sourdoughs (Gobbetti et al., 1996b).

The peptidase enzymes of *Lb. reuteri* CRL 1098 would be involved in the further degradation of small peptides formed during sourdough fermentation. The amino acids released by this way could stimulate the interaction between LAB and yeasts and contribute to or act as precursors for flavour development during the wheat sourdough fermentation and baking.

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References

Andersson, C., Linder, L.E., Sund, M.L., Lonnies, H., 1992. Purification and characterization of an aminopeptidase from *Streptococcus mitis* ATTC 903. Curr. Microbiol. 25, 261–267.

Bockelmann, W., Schulz, Y., 1992. Purification and characterization of an aminopeptidase from *Lactobacillus delbrueckii* subsp. bulgaricus. Int. Dairy J. 2, 95–107.

Bradford, M.M., 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.

De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23, 130–135.

Doi, E., Shibata, D., Matoba, T., 1981. Modified colorimetric ninhydrin methods for peptide assay. Anal. Biochem. 118, 173–182.

El Abboudi, M., El Soda, M., Pandian, S., Simard, R.E., Olson, N., 1992. Partial purification and characterization of aminopeptidase from debittering and non-debittering strains of *Lactobacillus casei*. Milchwissenschaft 47, 366–370.

El Soda, M., Desmazeaud, 1982. Les peptides des lactobaciles du group thermobacterium. I Mise en évidence de ces activitiés chez *L. helveticus*, *L. .acidophilus*, *L. lactis* et *L. bulgaricus*. Can. J. Microbiol. 28, 1181–1187.

Gobbetti, M., Corsetti, A., 1997. *Lactobacillus sanfrancisco* a key sourdough lactic acid bacterium: a review. Food Microbiol. 14, 175–187.

Gobbetti, M., Simonetti, M.S., Rossi, J., Cossigani, L., Corsetti, A., Damiani, P., 1994. Free D- and L-amino acid evolution during sourdough fermentation and baking. J. Food Sci. 59, 881–884.

Gobbetti, M., Smacchi, E., Corsetti, A., 1996a. The proteolytic system of Lactobacillus sanfrancisco CB1: purification and characterization of a proteinase, a dipeptidase, and an aminopeptidase. Appl. Environ. Microbiol. 62, 3220–3226.

Gobbetti, M., Smacchi, E., Fox, P., Stepaniak, L., Corsetti, A., 1996b. The sourdough microflora. Cellular localization and characterization of proteolytic enzymes in lactic acid bacteria. Lebensm.-Wiss. Technol. 29, 561–569.

Laan, H., Smid, E.J., Tan, P.S.T., Konings, W.N., 1989. Enzymes involved in the degradation and utilization of casein in *Lactococcus lactis*. Neth. Milk Dairy J. 43, 327–345.

Lee, G.D., Chun, S.S., Kho, Y.H., Chun, H.K., 1998. Purification and properties of an extracellular leucine aminopeptidase from the *Bacillus* sp. N2. J. Appl. Microbiol. 84, 561–566.

Miyakawa, H., Kobayashi, S., Shimamura, S., Tomita, M., 1992. Purification and characterization of an aminopeptidase from *Lactobacillus helveticus* LHE-511. J. Dairy Sci. 75, 27–35.

Smid, E.J., Pooleman, B., Koninsgs, W.N., 1991. Casein utilization by lactococci. Appl. Environ. Microbiol. 57, 2447–2452.

Spicher, G., 1983. Baked goods. In: Rehm, H.J., Reed, G. (Eds.), Biotechnology, vol. 5. Verlag Chemie, Weinheim, pp. 1–80.

Spicher, G., 1987. The microflora of sourdough: XXII. Comunications: the *Lactobacillus* species of wheat sourdough. Z. Lebensm.-Unters. Forsch. 184, 300–303.

Tan, P.S.T., Konings, W.N., 1990. Purification and characterization of an aminopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. 56, 526–532.

Wohlrab, Y., Bockelmann, W., 1992. Purification and characterization of a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Int. Dairy J. 2, 354–361.