Purification and Characterization of a Tripeptidase from *Lactobacillus sake*

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A tripeptidase was purified to homogeneity from the cell extract of *Lactobacillus sake* by ammonium sulfate precipitation, hydrophobic interaction chromatography, gel filtration chromatography, and two steps of anion exchange chromatography. After SDS−PAGE a single band of protein was detected of approximately 55 kDa. A similar molecular mass was estimated by gel filtration. The tripeptidase activity was optimal at pH 7.0 and at 40 °C. The enzyme was strongly inhibited by metal chelators, reducing agents, and bestatin while thiol group reagents, serine proteinase inhibitors, and aspartic proteinase inhibitors had no effect on the activity. The enzyme was activated by Mn^{2+} and almost totally inhibited by Zn^{2+} and to a lesser extent by Sn^{2+}. The enzyme only exhibited activity against tripeptides, and those hydrolyzed at higher rates were Ala-Ala-Ala, Ser-Ser-Ser, and Leu-Gly-Gly.

Keywords: *Tripeptidase; lactobacilli; purification; enzyme characterization; metallo-enzyme*

INTRODUCTION

Dry-fermented sausages constitute the main fermented meat products in which the metabolic activities evolved by lactic acid bacteria (LAB) are of vital importance not only for preservation but also for texture and flavor development (Hammes et al., 1990; Montel et al., 1992). Proteolytic events during dry-curing processes result in an increase in free amino acids and peptides (Toldrá and Verplaetse, 1995). The concentration and composition of these compounds have been correlated to flavor (Kato et al., 1989; Verplaetse, 1994), and their generation is partly attributed to microbial peptidases either from the inoculated organisms or from the indigenous flora (Verplaetse, 1994; Toldrá and Verplaetse, 1995). Accordingly, peptidase activity has been detected in the main LAB species which dominate the flora of dry-fermented sausages (Montel et al., 1992).

In comparison to the position in meat fermentations, a significant amount of information on the proteolytic system of dairy starter cultures is available. Dairy LAB have a complex proteolytic system which consists of a cell wall-associated proteinase and at least 13 different intracellular peptidases (Pritchard and Coolbear, 1993; Tan et al., 1993). These enzymes have been thoroughly studied due to their essential role in both bacterial nutrition and flavor development (Mulholland, 1991; Visser, 1993). Special attention has been focused on lactococci (Pritchard and Coolbear, 1993) and, more recently, on lactobacilli peptidases (Klein et al., 1994), resulting in many of these peptidases being purified and characterized, biochemically and genetically.

These studies have largely been confined to the dairy starters, and there is scarce information on peptidases from bacteria more associated with meat fermentations. *Lactobacillus sake* constitutes one of the main species isolated from naturally fermented sausages, and it is also widely used as a starter (Hammes et al., 1990; Montel et al., 1993). This lactobacilli species has been assessed to have substantial peptidase activity, and as a consequence, it has been proposed to have a potential role in flavor development (Montel et al., 1992). The purification and characterization of a dipeptidase from *L. sake* was recently described (Montel et al., 1995), constituting the first characterization of a peptidase from this species. Besides, an aminopeptidase has just been also purified and characterized (Sanz and Toldrá, 1997). However, further studies are required to improve our knowledge about peptidases from meat-originating lactobacilli and to allow a systematic investigation of their possible role in fermented meat processing.

The objective of this work was the purification and characterization of a tripeptidase from the cell extract of *L. sake*, isolated from the natural flora of dry-fermented sausages, in order to advance the knowledge of the proteolytic system of lactobacilli associated with meat fermentations.

EXPERIMENTAL PROCEDURES

Organism and Growth Conditions. *L. sake* IATA115 (CECT4808) was originally isolated from the indigenous flora of dry fermented sausages. The organism was routinely cultured in MRS broth (Oxoid, Hampshire, U.K.) at 30 °C and then maintained either at 4 or at −80 °C in 15% glycerol.

Preparation of Cell Extract. The organism was grown in 1.5 L batch cultures of MRS broth. Following two subculturings it was inoculated at 5% and incubated at 30 °C for 16 h. Cells were harvested by centrifugation (10000*g*, 30 min, at 4 °C), washed twice in 50 mM Tris-HCl buffer, pH 7.5, and resuspended in the same buffer. The cells were disrupted by two passages through a French Pressure Cell (SLM Amicon, U.K.) at 100 MPa. Unbroken cells and debris were removed by centrifugation (30 min, 20000*g*, 4 °C), the supernatant constituting the cell-free extract.

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Determination of Tripeptidase Activity. Peptidase activity was determined as described by Mulholland et al. (1993). The reaction mixture, containing 10 mM Gly-Gly-Phe (100 mL), 50 mM phosphate buffer, pH 7.0 (250 mL), and enzyme sample (50 mL), was incubated at 40 °C for 15 min. The reaction was stopped by the addition of 30% acetic acid (100 mL), and the mixture was centrifuged at 5000*g* for 5 min. Four samples and one control were assayed for each experimental point. Samples were assayed on a model 270A capillary electrophoresis apparatus (Applied Biosystems, Warrington, U.K.). Following sample injection (1.5 s by vacuum), running conditions were 30 kV at 45 °C for 15 min on an uncoated 72 cm capillary (Applied Biosystems, Warrington, U.K.) in 20 mM sodium citrate buffer, pH 2.5. Electrophoretic mobility was monitored by measuring absorption at 200 nm. The data was collected using a Roseate chromatography data analysis package with a 100 mV input (Drew Scientific). One unit of activity was defined as the amount of enzyme that hydrolyzed 1 mmol of substrate per hour at 40 °C.

Enzyme Purification. *Ammonium Sulfate Fractionation.* The cell-free extract was fractionated with ammonium sulfate in two steps by addition of the reagent at 4 °C. The precipitate formed between 40 and 80% saturation was collected by centrifugation at 10000*g* for 30 min and dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.5.

Hydrophobic Interaction Chromatography. The sample obtained at 40−80% ammonium sulfate saturation was applied to a 17 × 2.6 cm phenyl-sepharose fast flow column (Pharmacia Biotech, Uppsala, Sweden) kept at 4 °C, previously equilibrated with 1.5 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl buffer, pH 7.5. The bound proteins were eluted using a 1.5−0 M ammonium sulfate in 50 mM Tris-HCl buffer, pH 7.5 gradient, at a flow rate of 6 mL/min. Fractions of 7.4 mL were collected, and those containing tripeptidase activity were pooled and concentrated in a Centricon-30 concentrator.

Gel Filtration Chromatography. Concentrated fractions with Gly-Gly-Phe hydrolyzing activity were applied to a 89 × 1.6 cm sephacryl 200 HR column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. Gel filtration chromatography was run under refrigeration (4 °C) at a flow rate of 15 mL/h, and 3 mL fractions were collected and assayed for tripeptidase activity. Eluent fractions containing the activity were pooled and concentrated to 1 mL as described above.

Anion Exchange Chromatography. Tripeptidase activity eluted from the gel filtration column was filtered through 0.22 mm membrane filter and injected onto a 6 mL Resource Q anion exchange column (Pharmacia Biotech, Uppsala, Sweden) kept at 4 °C and equilibrated with starting buffer, 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. The bound proteins were eluted at a flow rate of 6 mL/min, applying an initial isocratic period in the starting buffer (18 mL) followed by a linear salt gradient from 0.1 to 0.4 M NaCl in 50 mM Tris-HCl, pH 7.5 (60 mL). Then 1 mL fractions were collected and assayed for tripeptidase activity. Fractions with activity were applied to the same anion exchange column equilibrated in 50 mM Tris-HCl buffer pH 8.0, containing 0.2 M NaCl. The second anion exchange chromatography was run in the same conditions except that the gradient was established between 0.2 and 0.4 M NaCl in 50 mM Tris-HCl buffer pH 8.0. Fractions of 1 mL were collected and assayed for tripeptidase activity.

Determination of Protein Concentration. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Gel Electrophoresis. The purification of the enzyme was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) according to Laemmli (1970). Samples were mixed 1:1 with the sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% sorbitol, 5% 2-mercaptoethanol and 0.002% bromophenol blue), boiled for 5 min and applied to 12% polyacrylamide gels. Electrophoresis was carried out at 200 V and 25 mA, and gels were stained with Coomassie blue R-250 (Laemmli, 1970) and silver (Nielsen and Brown, 1984).

Molecular Mass Determination. The molecular mass of the native purified enzyme was estimated by gel filtration using a Sephacryl 200 HR column (Pharmacia Biotech, 89 × 1.6 cm) as described previously. The column was calibrated using the following standard proteins: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa), and finally, blue dextran was used to estimate the void volume.

The molecular mass of the enzyme in denaturing conditions was also determined by SDS−PAGE as described above. The following reference proteins were run simultaneously with the samples: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Effect of Temperature and pH on the Activity. The effect of pH on the activity was determined in the pH range from 4.0 to 9.0. The buffers used were 50 mM sodium acetate, pH 4.0−5.5; 50 mM sodium phosphate pH 6.0−7.0; Tris-HCl pH 7.5−8.5; and 10 mM TAPS pH 9.0. The enzyme was previously equilibrated with each buffer at 4 °C for 20 min prior to addition of the substrate. Peptidase activity was measured according to the method previously described.

The effect of temperature on the activity was determined in the range from 10 to 60 °C using Gly-Gly-Phe as substrate. The reaction mixture, without the enzyme, was previously incubated at each temperature. The reaction was initiated by the addition of the enzyme and the peptidase activity was measured after 15 min of incubation at each temperature.

Determination of Kinetic Parameters. The enzyme was incubated in the optimum reaction buffer containing various substrate concentrations ranging from 0.1 to 2.0 mM for Phe-Gly-Gly and from 1 to 14 mM for Gly-Gly-Phe, and the activity was determined as normal. K_m was estimated from Lineweaver−Burk plots for both substrates.

Effect of Chemical Agents and Metal Cations on the Activity. The chemical agents and metal cations (listed in Table 2) were previously incubated with the enzyme, in the reaction buffer, at 4 °C for 20 min. The reaction was initiated by adding the substrate (Gly-Gly-Phe), and the activity was measured after 15 min of incubation at 40 °C, as described previously. The relative activities were determined by comparing against an untreated sample.

Substrate Specificity Determination. The relative activity of the tripeptidase against different dipeptides, tripeptides and longer peptides (listed in Table 3) was determined by monitoring the disappearance of the initial substrate concentration at 40 °C, after incubation for 15 min, in the conditions described previously. When substrates were not hydrolyzed in 15 min, the incubation was prolonged for 30 min.

Assaying with activity against Leu-, Phe-, and Arg-*p*-nitroanilide substrates was determined spectrophotometrically on a Thermomax microplate reader (Molecular Devices, Crawley, U.K.). The reaction mixture consisted of 50 mM sodium phosphate buffer, pH 7.0 (250 mL), containing the substrate at a final concentration of 1 mM, and enzyme (50 mL). Multiwell plates were incubated at 37 °C for 30 min, and the absorbance was measured at 405 nm.

RESULTS

Purification of Tripeptidase. A tripeptidase was purified to homogeneity from the cell-extract of *L. sake* through the purification procedure described above. The results obtained in each purification step are shown in Table 1. The enzyme eluted from the phenyl-sepharose column at 0.26 M ammonium sulfate and 59.0% of the total activity was recovered. Specific Gly-Gly-Phe hydrolyzing activity was enriched 18.2-fold. The CE electropherogram for the hydrolysis of Gly-Gly-Phe at this stage of the purification also shows the further breakdown of the dipeptide product, Gly-Phe, as the

Table 1. Purification of the Tripeptidase from *L. sake*

purification step[a]	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	purification (fold)
cell extract	560.00	3664.2	6.5	100.0	1.0
40−80% ammonium sulfate cut	404.60	3274.5	8.1	89.4	1.2
hydrophobic interaction	18.32	2162.9	118.1	59.0	18.2
gel filtration	4.70	1924.4	410.3	52.5	63.1
first anion exchange	0.03	226.4	6698.2	6.2	1030.5
second anion exchange	nd[b]	55.5	nd	1.5	nd

[a] See Experimental Procedures for details. [b] Value not detected by Bradford assay.

formation of the free amino acid, phenylalanine, indicating the presence of other peptidase activity. It was also observed that the tripeptidase activity partially coeluted from this column with a Leu-pNA hydrolyzing activity suggesting other aminopeptidase activities were possibly present, too (data not shown).

Gel filtration resulted in a 63.1-fold purification with 52.5% of the initial activity recovery. The tripeptidase fraction, however, still contained activity against Leu-pNA and Gly-Phe. The initial anion exchange chromatography effectively separated the tripeptidase with a 1030.5-fold purification from the contaminating Leu-pNA activity which eluted at 0.19 NaCl, compared to the Gly-Gly-Phe hydrolyzing activity, which eluted at 0.24 M NaCl. Some Gly-Phe hydrolysis was still detectable in the fraction corresponding to the maximum Gly-Gly-Phe hydrolyzing activity. This was separated from the tripeptidase in the second anion exchange chromatography step, and the resulting fraction gave a single band on SDS−PAGE. The whole purification procedure yielded 1.5% of the total activity. However, the final purification factor was not determined since the protein content was not detectable by Bradford assay in the final step.

Purity and Molecular Mass. The purity of the enzyme was confirmed by SDS−PAGE after silver staining. A single protein band was detected on denaturing gels corresponding to approximately 55 kDa (Figure 1). The molecular mass of the native enzyme determined by gel filtration was about 54 kDa, indicating that the enzyme is monomeric.

Effect of pH and Temperature on the Activity. The relative activity was studied in the pH range from 4.0 to 9.0. The optimum pH for the tripeptidase activity was 7.0. The enzymatic activity sharply decreased out of its optimum pH, but still retained 15% of the optimum activity at pH 5.0 and 8.5. The activity at pH beyond the mentioned values was negligible. On the other hand, the optimal temperature for tripeptidase activity was 40 °C. At temperatures higher than 50 °C, or lower than 10 °C, less than 20% of the activity was detected.

Effect of Chemical Agents and Metal Cations on the Activity. The effect of several chemical agents at 1 mM concentration are shown in Table 2. The activity was strongly inhibited by metalloenzyme inhibitors (1,10-phenanthroline and EDTA), thiol-reducing reagents (dithiothreitol and 2-mercaptoethanol) and bestatin. The serine proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF), sulfhydryl reagents such as iodoacetic acid and *p*-chloromercuribenzenesulfonic acid, and the aspartyl-proteinase inhibitor pepstatin A did not have any significant effect on the activity. The effect of cations at 1 mM on tripeptidase activity is also shown in Table 2. The enzyme was completely inhibited by

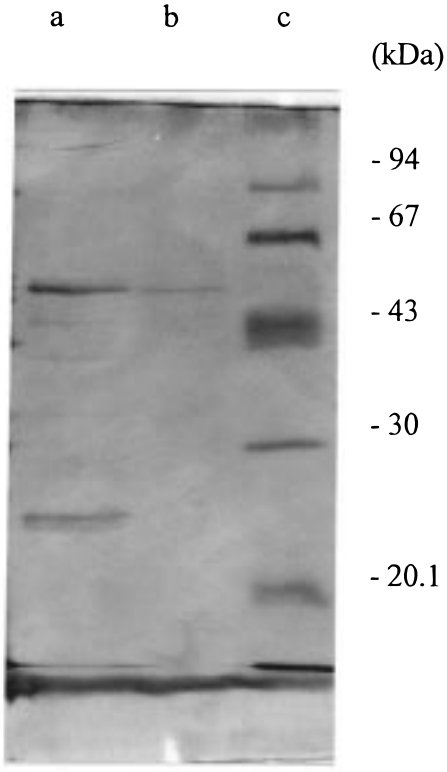

Figure 1. SDS−PAGE analysis of the active fractions of the last purification steps of the tripeptidase from *L. sake*. Lanes: (a) active fractions after the first anion exchange chromatography; (b) purified enzyme after the second anion exchange chromatography; (c) molecular mass standard proteins (kilodaltons). Protein bands were visualized by silver staining. See Experimental Procedures for details.

Table 2. Effect of Chemical Agents (1 mM) and Metal Cations (1 mM, except NaCl 0.5 mM) on the Purified Tripeptidase from *L. sake*

chemical agents and metal cations	rel activity (%)	chemical agents and metal cations	rel activity (%)
control	100.0	iodoacetic acid	98.7
EDTA	7.0	bestatin	0.0
1,10-phenanthroline	0.0	NaCl	115.2
dithiothreitol	2.3	$CaCl_2$	118.0
2-mercaptoethanol	1.0	$MgCl_2$	105.6
pepstatin A	99.0	$ZnCl_2$	3.2
p-chloromercuribenzene-sulfonic acid	107.0	$MnCl_2$	216.0
		$SnCl_2$	75.6
PMSF	100.1		

Zn^{2+} at this concentration and to a lesser extent by Sn^{2+}, while Mn^{2+} appears to be a potent activator of the enzyme.

Substrate Specificity. The specificity for substrates was determined against several dipeptides, tripeptides and oligopeptides, listed in Table 3. The enzyme has broad specificity against a range of tripeptides. The

Table 3. Substrate Specificity of the Purified Tripeptidase from *L. sake*

substrate	relative activity (%)	substrate	relative activity (%)
Ala-Ala-Ala	100.0	Phe-Gly	0.0
Ser-Ser-Ser	82.6	Gly-Phe	0.0
Leu-Gly-Gly	78.5	Met-Phe	0.0
Phe-Gly-Gly	73.3	Ala-Ala-Ala-Ala	0.0
Tyr-Gly-Gly	73.1	Phe-Gly-Gly-Phe	0.0
Arg-Gly-Asp	62.5	Gly-Pro-Gly-Gly	0.0
Lys-Lys-Lys	62.4	Ala-Ala-Ala-Ala-Ala	0.0
Val-Gly-Gly	46.4	Val-Tyr-Ile-His-Pro-Phe	0.0
Met-Ala-Ser	12.1	Phe-pNA	0.0
Gly-Gly-Phe	1.3	Leu-pNA	0.0
Glu-Gly-Phe	0.0	Arg-pNA	0.0
Ala-Pro-Gly	0.0.		

substrates hydrolyzed at higher rates were Ala-Ala-Ala, Ser-Ser-Ser, and Leu-Gly-Gly. The hydrolyzing activity was also high against aromatic and basic amino acids at N-terminal position. N-terminal Gly was hydrolyzed at lower rates (Gly-Gly-Phe), and no activity was detected against amino acids (Glu-Gly-Phe) and tripeptides containing Pro at second position (Ala-Pro-Gly). Neither dipeptides, peptides containing more than 3 residues, nor aminoacyl-*p*-nitroanilide derivatives were hydrolyzed.

Kinetic Parameters. The K_m of the tripeptidase using Phe-Gly-Gly and Gly-Gly-Phe as substrates were 1.1 and 13.0 mM, respectively, indicating low affinity for the last substrate.

DISCUSSION

The existence of tripeptidase activity in LAB is well documented (Mou et al., 1975; Desmazeaud and Zevaco, 1979; Law, 1979; Kaminogawa et al., 1984; Abo-Elnaga and Plapp, 1987). However, until now, only two lactococci (Bosman et al., 1990; Bacon et al., 1993) and two lactobacilli (Bockelmann et al., 1995; Bockelmann, 1995) tripeptidases have been purified to homogeneity and thoroughly characterized. Likewise, the purification of a tripeptidase from meat origin lactobacilli has not been previously described. In this report, a tripeptidase from the cell extract of *L. sake* was purified to homogeneity with an specific activity enrichment of more than 1030-fold.

In LAB, a wide range of peptidases have been reported that can hydrolyze tripeptides, but they are also capable of cleaving other sized peptides. Although Table 3 clearly shows that Gly-Gly-Phe is not the optimum substrate for the tripeptidase and has high K_m at 13.0 mM indicating a low affinity, Gly-Gly-Phe was used to follow the purification as it was believed to be the most specific substrate available for assaying the tripeptidase, particularly in early steps of purification. The CE analysis method was then able to monitor the formation of the dipeptide, Gly-Phe, specifically due to the tripeptidase activity. The CE method was also able to monitor any further breakdown of the Gly-Phe dipeptide by monitoring for Phe formation in the same assay. This was important as a strong dipeptidase activity was present, which was not separated from the tripeptidase until the last purification step. The presence of dipeptidase activity in another *L. sake* strain (starter L110 from Texel, France) has already been reported and characterized (Montel et al., 1995). Therefore, at least three exopeptidases form part of the proteolytic system of *L. sake*, the tripeptidase reported in this paper and the dipeptidase (Montel et al., 1995) and aminopeptidase hydrolyzing Leu-pNA (Sanz and Toldrá, 1997) previously purified. It is likely, as is the case with other LAB species, that other peptidase activities are also present.

The SDS−PAGE and gel filtration analysis indicates that the purified tripeptidase is a monomer with a molecular mass of 54−55 kDa. This contrasts with the molecular mass reported for other LAB tripeptidases from *Lactococcus* (Bosman et al., 1990; Bacon et al., 1993) and *Lactobacillus* (Bockelmann et al., 1995) which seem to be multimeric of two and three subunits, respectively. However, the tripeptidase from *L. sake* has a molecular mass similar to that reported for another tripeptidase from mammalian origin (Doumeng and Maroux, 1978). The tripeptidase studied has optimum activity at pH 7.0, in agreement with the optimum pH of *Lactococcus lactis* subsp. *diacetylactis* CNRZ 267 (Desmazeaud and Zevaco, 1979) and the lactococci strains studied by Kaminogawa et al. (1984) and close to pH 7.5 reported for *L. lactis* subsp. *cremoris* Wg2 (Bosman et al., 1990). The temperature found to be optimal is 40 °C as observed by Bockelmann et al. (1995). Nevertheless, maximum tripeptidase activity at more acid (Bockelmann et al., 1995) and basic pH (Bacon et al., 1993) and in a different temperature range has also been found (Desmazeaud and Zevaco, 1979; Law, 1979; Bosman et al., 1990; Bockelmann, 1995).

The substrate specificity study indicates that the purified peptidase is a true aminotripeptidase. It hydrolyzed only tripeptides yielding the N-terminal residue and the intact dipeptide. No C-terminal hydrolysis was observed. The hydrolysis of Ala-Ala-Ala, Leu-Gly-Gly, Phe-Gly-Gly, Tyr-Gly-Gly, Val-Gly-Gly, and Gly-Gly-Phe has also been reported for other LAB tripeptidases (Bosman et al., 1990; Bacon et al., 1993; Bockelmann et al., 1995). In common with these other tripeptidases it appears likely that prolyl-containing peptides are not hydrolyzed for the tripeptidases characterized to date.

As commonly found for other LAB peptidases, the studied tripeptidase is a metalloenzyme, indicated by the strong inhibitory effect of the chelating agents, EDTA and 1,10-phenanthroline. The most stimulatory divalent cation resulted to be Mn^{2+} as also reported by Bockelmann et al. (1995). However, other metals such as Zn^{2+} and Co^{2+} at lower concentrations have often been reported as strong activators (Kaminogawa et al., 1984; Bosman et al., 1990). Tripeptidase inhibition by thiol-reducing reagents is also confirmed in this study in agreement with other LAB tripeptidases (Kaminogawa et al., 1984; Bosman et al., 1990; Bockelmann et al., 1995) although activation by reducing agents or inhibition by sulfhydryl-blocking reagents has been noted by other authors (Desmazeaud and Zevaco, 1979; Bockelmann et al. 1995).

In summary, the peptidase purified from *L. sake* is the first tripeptidase described in meat lactobacilli. Its possible role in the breakdown of hydrophobic tripeptides and the release of amino acids of interest in flavor development during meat curing still needs to be determined, taking into consideration the conditions of the manufacturing process.

ABBREVIATIONS USED

CE, capillary electrophoresis; LAB, lactic acid bacteria; pNA, *p*-nitroanilide; PMSF, phenylmethanesulfonyl

fluoride; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid, [[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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