Purification and Characterization of an Aminopeptidase from *Lactobacillus sake*

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An aminopeptidase was purified from the cell-free extract of *Lactobacillus sake* IATA115 by ammonium sulfate fractionation and several chromatographic procedures including hydrophobic interaction, gel filtration, and anion exchange chromatography. The purified enzyme was a 35−36 kDa monomer. Activity was optimal at 37 °C and pH 7.5, and the K_m values estimated for Leu- and Met-AMC (7-amido-4-methylcoumarin) were 0.091 and 0.174 mM, respectively. The aminopeptidase exhibited maximal activity against Leu- and Ala-AMC, while Lys- and Arg-AMC were not hydrolyzed. Among peptides, highest activity was observed against Ala-Ala, Ala-Leu, and Leu-Ala, while dipeptides containing basic amino acids at the N terminus were not hydrolyzed. Serin and aspartic proteinase inhibitors had no effect on the activity. However, the enzyme was inhibited by puromycin, amastatin, bestatin, arphamenine B, and sulfhydryl group reagents but activated by reducing reagents. The presence of Hg^{2+}, Cu^{2+}, Cd^{2+}, and Ni^{2+} as well as high concentrations of chelator agents caused inhibition, while other divalent cations such as Ca^{2+}, Sn^{2+}, Mg^{2+}, Ba^{2+}, and Mn^{2+} stimulated the activity.

Keywords: *Aminopeptidase; Lactobacillus; purification; enzyme characterization*

INTRODUCTION

Lactobacilli dominate the microflora of natural fermented sausages and constitute essential agents of meat fermentation (Hammes *et al*., 1990; Samelis *et al*., 1994). Thus, the selection and application of certain lactobacilli strains as starters is of great importance for the success of the fermentation process (Hammes and Knauf, 1994), since the development of desirable changes in texture and flavor is decisively influenced by their carbohydrate and protein metabolism (Brink and Huis In't Veld, 1992). Proteolytic phenomena that occur during the dry-curing process result in a noticeable increase in small peptides and free amino acids contributing to flavor (Aristoy and Toldrá, 1995; Toldrá and Verplaetse, 1995). The exopeptidase activity involved in the release of amino acids is partly attributed to microbial enzymes (Verplaetse, 1994) and, in fact, an important aminopeptidase activity has been detected in lactobacilli species commonly found in dry fermented sausages (Montel *et al*., 1992). Particularly, *Lactobacillus sake* shows a high competitiveness accounting for the main species encountered in this kind of product, even when no inoculation has been performed (Hugas *et al*., 1992; Vogel *et al*., 1993). Additionally, a considerable aminopeptidase activity has been attributed to this species (Montel *et al*., 1992) that together with the high cell density reached in this product favors its possible contribution to flavor.

Much interest has been focused on the exoproteolytic system of lactic acid bacteria of importance in the dairy industry in relation to nutrition and flavor development, and several lactococci and lactobacilli peptidases have been purified and thoroughly characterized (Pritchard and Coolbear, 1993; Tan *et al*., 1993). However, the exoproteolytic system of meat origin lactobacilli has been scarcely studied and, in fact, only the purification of a dipeptidase from another strain of *L. sake* has been recently reported (Montel *et al*., 1995).

The objective of this work was the purification and characterization of an aminopeptidase from a *L. sake* strain IATA115, isolated from the natural flora of dry fermented sausages, to get a better knowledge of the exoproteolytic system of meat lactobacilli.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Growth Conditions. *L. sake* IATA115 was originally isolated from the indigenous flora of dry fermented sausages. The organism was routinely cultured in MRS broth (Merck, Darmstadt, Germany) at 30 °C and then maintained at either 4 or −80 °C in 15% glycerol.

Preparation of Cell-Free Extract. The microorganism 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, 4 °C), washed twice in 50 mM Tris-HCl buffer, pH 7.5, and then resuspended in the same buffer. Cells were disrupted by two passages through a French pressure cell (SLM Aminco, London, U.K.) at 100 MPa. Unbroken cells and debris were removed by centrifugation (20000*g*, 30 min, 4 °C), and the supernatant constituted the cell-free extract.

Assay of Aminopeptidase Activity. Aminopeptidase activity was determined using several amino acyl *p*-nitroanilide derivatives (Leu-pNA, Arg-pNA, and Phe-pNA), obtained from Sigma (St. Louis, MO) through the purification procedure. The reaction was carried out in multiwell plates by adding 250 μL of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM final substrate concentration, and 50 μL of enzyme. The reaction mixture was incubated at 37 °C for 15 min, and the release of each amino acid was measured by absorbency read at 405 nm in a multiwell plate reader, ELX 800 (Bio-Tek Instruments, Winooski, VT). Four replicas (samples + controls) were measured for each experimental point. One unit of enzyme activity (U) was defined as the release of 1 μmol of substrate hydrolyzed/h at 37 °C.

Characterization studies were performed using L-leucine-7-amido-4-methylcoumarin (Leu-AMC) obtained from Sigma as substrate. The reaction mixture consisted of 250 μL of 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM final substrate concentration, and 50 μL of enzyme. The release of leucine

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was determined after 10 min of incubation at 37 °C in a multiscan fluorometer (Fluoroskan II, Labsystems, Finland) at 360 and 440 nm as excitation and emission wavelengths, respectively. Four replicas (samples + controls) were measured for each experimental point.

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 45 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 45−80% ammonium sulfate saturation was applied to a 17 × 2.6 cm phenyl-Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden), previously equilibrated with 50 mM Tris-HCl, pH 7.5, containing 1.5 M $(NH_4)_2SO_4$. The column was eluted at 6 mL/min, using a linear ammonium sulfate gradient from 1.5 to 0 M (700 mL) and a final isocratic period at 0 M ammonium sulfate in the initial buffer (300 mL). Fractions of 7.4 mL were collected and assayed for aminopeptidase activity. Those fractions with aminopeptidase activity against Leu-pNA were pooled and concentrated in a Centricon 30 concentrator (Amicon, Witten, Germany).

Gel Filtration Chromatography. Concentrated fractions with Leu-pNA hydrolyzing activity were applied to a 89 × 1.6 cm Sephacryl 200 HR column (Pharmacia) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. The column was run at a flow rate of 15 mL/h, and 3 mL fractions were collected and assayed for aminopeptidase activity. Fractions containing activity against Leu-pNA were pooled and concentrated as described above.

Anion Exchange Chromatography. The active sample, obtained from the previous step, was filtered through 0.22 μm membrane filter and applied to a 6 mL Resource Q anion exchange column (Pharmacia) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. The column was eluted at 6 mL/min, applying an initial isocratic period in the equilibration buffer (18 mL), followed by a linear salt gradient from 0.1 to 0.4 M, in 50 mM Tris-HCl buffer, pH 7.5 (60 mL). The eluates were collected in 1 mL fractions and assayed for aminopeptidase activity. The active fractions against Leu-pNA were applied to the same anion exchange column but equilibrated with 50 mM Bis-Tris buffer, pH 6.0, containing 0.1 M NaCl. The column was run in the same conditions and with the same salt gradient as that described previously except for the elution buffer, since in this case 50 mM Bis-Tris pH 6.0 was used. Eluent fractions of 1 mL were collected and assayed for aminopeptidase activity.

Determination of Protein Concentration. Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. The fractions eluted from the chromatographic system were also monitored at 280 nm.

Electrophoresis. The purification was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) using 12% polyacrylamide gels (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, 0.002% bromophenol blue), boiled for 5 min, and applied into the gel. Electrophoresis was carried out at 200 V and 25 mA for about 1 h, and gels were stained with Coomassie blue R-250.

Molecular Mass Determination. The molecular mass of the native enzyme was estimated by gel filtration using a 89 × 1.6 cm Sephacryl 200 HR column (Pharmacia), as previously described. The column was calibrated using the following standard proteins (Sigma): 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 (Bio-Rad, Hercules, CA) 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 pH and Temperature on the Activity. The effect of pH was determined in the range from 4.5 to 9.0, using the following buffers: 50 mM sodium acetate, pH 4.5−5.5; 50 mM sodium phosphate pH 6.0−7.0; Tris-HCl, pH 7.5−8.5; and TAPS, pH 9.0. The activity was measured after 10 min of incubation at each pH at 37 °C. The activity was expressed as a percentage of the activity at optimum pH.

The effect of temperature on the activity was determined in the range from 5 to 55 °C. The reaction mixture, without the enzyme, was previously equilibrated in Eppendorf tubes, at each temperature. The reaction was initiated by the addition of the enzyme and, after 10 min of incubation, stopped by the addition of 100 μL of 30% (v/v) acetic acid solution. Samples were centrifuged (6000*g*, 2 min) and transferred to the multiwell plates. The fluorescence was measured through the standard method and expressed as a percentage of the activity at optimum temperature.

Determination of Kinetic Parameters. The kinetic parameters of the purified aminopeptidase were estimated for the substrates Leu-AMC and Met-AMC using concentration ranges from 0.005 to 0.1 and from 0.01 to 0.5 mM, respectively. The activity was measured continuously at 37 °C, as previously described. The K_m values were estimated from Lineweaver−Burk plots.

Effect of Chemical Agents and Metal Cations on the Activity. The effect of potential inhibitors on the aminopeptidase activity was tested by incubating the purified enzyme in the standard assay buffer and in the presence of the following chemical agents: puromycin (0.05−0.5 mM), amastatin (0.05−0.5 mM), bestatin (0.1−0.5 mM), arphamenine B (0.1−0.5 mM), dithiothreitol (DTT, 1−5 mM), 2-mercaptoethanol (1−5 mM), EDTA (0.1−20 mM), 1,10-phenanthroline (0.1−10 mM), leupeptin (0.1−0.5 mM), *p*-CMBS (0.1−1 mM), iodoacetate (0.1−5 mM), PMSF (0.1−1 mM), Pefabloc-SC (0.1−1 mM), pepstatin A (0.01−0.15 mM), ammonium sulfate (0.01−1 M), and NaCl (0.05−1 M). The effect of 1 mM metal cations was determined in the presence of the respective chloride salts ($MgCl_2$, $MnCl_2$, $CaCl_2$, $SnCl_2$, $HgCl_2$, $BaCl_2$, $ZnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, and $CdCl_2$). In all cases, the activity was assayed as previously described, after 10 min of incubation at 37 °C. Controls simultaneously run with the absence of any chemical compound were also assayed.

Substrate Specificity. The relative activity of the aminopeptidase against several aminoacyl-AMC derivatives (Leu-AMC, Ala-AMC, Met-AMC, Val-AMC, Pro-AMC, Gly-AMC, Ser-AMC, Phe-AMC, Tyr-AMC, Arg-AMC, Lys-AMC, and Pyroglu-AMC) was determined. The activity was assayed for each substrate by incubation at 37 °C for 10 min or even longer (up to 30 min) when the substrate was not hydrolyzed.

The relative activity against several peptides (Ala-Leu, Leu-Leu, Arg-Leu, Ala-Ala, Leu-Ala, Arg-Ala, Lys-Lys, Ala-Ala-Ala, Gly-Gly-Phe, Phe-Gly-Gly, Ala-Ala-Ala-Ala, and Ala-Ala-Ala-Ala-Ala) was determined by monitoring the disappearance of the substrate by capillary electrophoresis. The reaction mixture, consisting of 100 μL of respective peptide (10 mM), 250 μL of 50 mM Tris-HCl, pH 7.5, and 50 μL of enzyme was incubated at 37 °C. Samples (40 μL) were taken at different incubation times (up to 60 min), and the reaction was stopped by the addition of 30% acetic acid (10 μL). Four samples and controls were assayed for each experimental point. Samples were analyzed on a Model 270A capillary electrophoresis (Applied Biosystems, Foster City, CA). Samples were injected by vacuum (1.5 s), and running conditions were 25 kV at 35 °C on a 72 cm capillary (50 cm to detector) in 20 mM sodium citrate, pH 2.5. Detection was performed by UV absorption at 200 nm.

RESULTS

Purification of the Aminopeptidase. The results of a typical purification of an aminopeptidase from the cell-free extract of *L. sake* IATA115 are summarized in

Table 1. Purification of the Aminopeptidase from *L. sake* IATA115

purification step	total protein (mg)	total activity (U)	specific activity (U/mg)	yield (%)	purification (fold)
cell extract	573.87	1451.1	2.5	100.0	1.0
45−80% ammonium sulfate cut	393.50	1023.8	3.1	83.1	1.2
hydrophobic interaction	20.23	105.7	5.2	7.3	2.1
gel filtration	3.79	226.1	59.6	15.6	23.6
anion exchange pH 7.5	0.44	103.0	233.5	7.1	92.3
anion exchange pH 6.0	0.05	43.5	906.7	3.1	358.3

Table 2. Effect of Metal Cations on the Activity of the Purified Aminopeptidase from *L. sake* IATA115

metal salt (1 mM)	relative activity (%)	metal salt (1 mM)	relative activity (%)
control	100	$ZnCl_2$	100
$CaCl_2$	179	$CoCl_2$	92
$SnCl_2$	178	$NiCl_2$	77
$MgCl_2$	177	$CdCl_2$	63
$BaCl_2$	168	$CuCl_2$	50
$MnCl_2$	167	$HgCl_2$	0

Table 3. Hydrolysis of Various Aminoacyl-AMC Derivatives by the Purified Aminopeptidase from *L. sake* IATA115

substrate	relative activity[a] (%)	substrate	relative activity[a] (%)
Leu-AMC	100.0	Ser-AMC	0.6
Ala-AMC	93.0	Phe-AMC	0.3
Met-AMC	40.0	Tyr-AMC	0.0
Val-AMC	25.6	Arg-AMC	0.0
Pro-AMC	4.3	Lys-AMC	0.0
Gly-AMC	3.4	Pyroglu-AMC	0.0

[a] Expressed as a percentage of the activity against Leu-AMC, which was given a value of 100%.

Table 4. Hydrolysis of Peptides by the Purified Aminopeptidase from *L. sake* IATA115

substrate	relative activity[a] (%)	substrate	relative activity[a] (%)
Leu-Ala	100.0	Lys-Lys	0.0
Ala-Ala	159.8	Ala-Ala-Ala	2.5
Arg-Ala	0.0	Gly-Gly-Phe	0.0
Leu-Leu	17.4	Phe-Gly-Gly	0.0
Ala-Leu	111.7	Ala-Ala-Ala-Ala	3.5
Arg-Leu	0.0	Ala-Ala-Ala-Ala-Ala	16.4

[a] Expressed as a percentage of the activity against Leu-Ala, which was given a value of 100%.

Table 1. The first chromatographic step, on a phenyl-Sepharose column, gave three well-separated peaks showing aminopeptidase activity (see Figure 1A). Peak I, showing Leu-pNA hydrolyzing activity, eluted at 0.23 M $(NH_4)_2SO_4$, and peaks II and III eluted at 0 M $(NH_4)_2SO_4$, showing Arg-pNA (eluent volume, 710 mL) and Phe-pNA hydrolyzing activity (eluent volume 784 mL), respectively. Gel filtration chromatography of the pooled fractions containing Leu-pNA hydrolyzing activity from the previous step (peak I, Figure 1A) resulted in a single peak (see Figure 1B) with a 23.6-fold purification and 15.6% recovery. In the first anion exchange chromatography, at pH 7.5, the aminopeptidase activity eluted at 0.19 M NaCl (see Figure 1C). Finally, the partial purified enzyme was submitted to a second anion exchange step, at pH 6.0, and the purified enzyme eluted at 0.1 M NaCl (see Figure 1D). The whole purification procedure yielded 3.1% of the total activity with an increase in specificity of 358.3-fold.

Purity and Molecular Mass. The purified aminopeptidase gave a single band on SDS−PAGE corresponding to approximately 36 kDa (data not shown). The molecular mass of the native enzyme determined by gel filtration was about 35 kDa, indicating that the enzyme consists of only one subunit.

Effect of pH and Temperature on the Activity. The enzyme was active in the pH range from 5.0 to 9.0 with an optimum at pH 7.5. The activity was higher in the alkaline range, retaining 20% of the maximal activity when assayed at pH 9.0, although at pH 5.0−5.5 about 10−20% of the optimal activity was retained. The optimum temperature was found to be 37 °C. The enzyme was rapidly inactivated at 55 °C, while at 5 °C about 15% of the optimal activity remained.

Kinetic Parameters. The K_m values for Leu-AMC and Met-AMC were 0.091 and 0.174 mM, respectively.

Effect of Chemical Agents and Metal Cations. The effect of chemical agents on the aminopeptidase activity is shown in Figure 2. Puromycin, amastatin, bestatin, and arphamenine B, which are typical inhibitors of exopeptidases, caused a considerable inhibition of the enzyme (Figure 2A,B). Metal chelators, such as 1,10-phenantroline and EDTA, were strong inhibitors but only at concentrations as high as 10 and 20 mM, respectively (Figure 2C). The presence of reducing agents stimulated the aminopeptidase activity at concentrations of 1 mM of 2-mercaptoethanol and 2.5 mM of DTT (Figure 2D). The sulfhydryl group reagents, pCMBS and iodoacetate, caused about 50% (Figure 2E) and 20% (Figure 2F) enzyme inhibition, respectively. Leupeptin reduced moderately the activity (Figure 2E), while the serin proteinase inhibitors PMSF and Pefabloc-SC (F 2G) and the aspartic proteinase inhibitor pepstatin A (Figure 2H) did not seem to have a relevant effect on the activity. $(NH_4)_2SO_4$ and NaCl, which were used through the purification procedure, inhibited >50% of the enzyme activity at concentrations of 0.05 and 0.5 M, respectively (Figure 2I). The effect of metal cations was determined at 1 mM concentration of the respective metal salt (see Table 2). The presence of Ca^{2+}, Sn^{2+}, Mg^{2+}, Ba^{2+}, and Mn^{2+} notably stimulated the activity with about 70% increase. On the other hand, Hg^{2+} exerted the strongest effect, causing a complete inhibition, while Cu^{2+}, Cd^{2+}, and Ni^{2+} inhibited to a lesser extent. Finally, Zn^{2+} and Co^{2+} did not seem to have a relevant effect on the activity.

Substrate Specificity. The specificity of the major aminopeptidase for several aminoacyl-AMC derivatives is shown in Table 3. Leu- and Ala-AMC were the substrates hydrolyzed at higher rates. The substrates secondarily hydrolyzed were Met- (40%) and Val-AMC (25.6%), while the activity was clearly lower (<5%) against Pro- Gly-, Ser-, and Phe-AMC. The enzyme failed to release either basic amino acids (arginine and lysine), tyrosine, or pyroglutamic acid. Of the tested peptides, Ala-Ala, Ala-Leu, and Leu-Ala were hydrolyzed at higher rates, while dipeptides containing basic amino acids as N-terminal residues were not hydrolyzed, as shown in Table 4. The enzyme was able to hydrolyze oligopeptides of at least five residues.

DISCUSSION

Both lactobacilli and lactococci produce at least one major aminopeptidase capable of releasing a range of amino acids through its N-terminal exopeptidase activity (Arora and Lee, 1994). However, the purification of any aminopeptidase from meat origin lactobacilli has

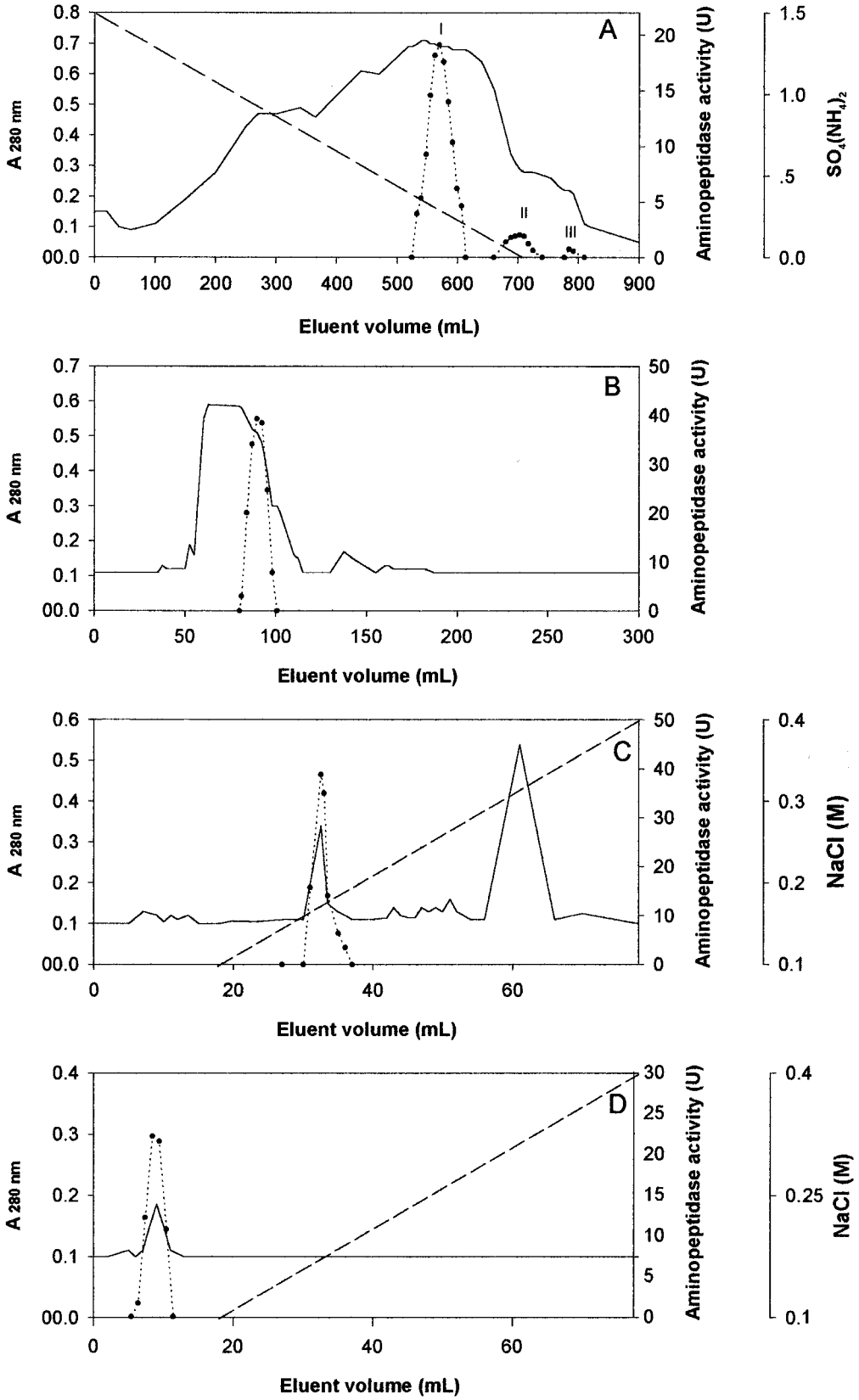

Figure 1. Purification of an aminopeptidase from *L. sake* IATA115: (· · ·) aminopeptidase activity; (—) A_{280} protein concentration; (- - -) salt gradient; (A) hydrophobic interaction chromatography (phenyl-Sepharose fast flow; peak I, fractions with Leu-pNA hydrolyzing activity; peak II, fractions with Arg-pNA hydrolyzing activity; peak III, fractions with Phe-pNA hydrolyzing activity); (B) gel filtration chromatography (Sephacryl 200 HR) of the injected fractions corresponding to peak I of the previous step; (C) anion exchange chromatography, pH 7.5 (Resource Q, 6 mL); (D) anion exchange chromatography, pH 6.0 (Resource Q, 6 mL). See Experimental Procedures for details.

not been described so far. In this paper, the purification of an aminopeptidase from *L. sake* is newly reported. Leu-pNA hydrolyzing activity constituted the peak that accounted for the strongest aminopeptidase activity after the elution from the phenyl-Sepharose column. Notably, a certain inhibition of the purified aminopep-

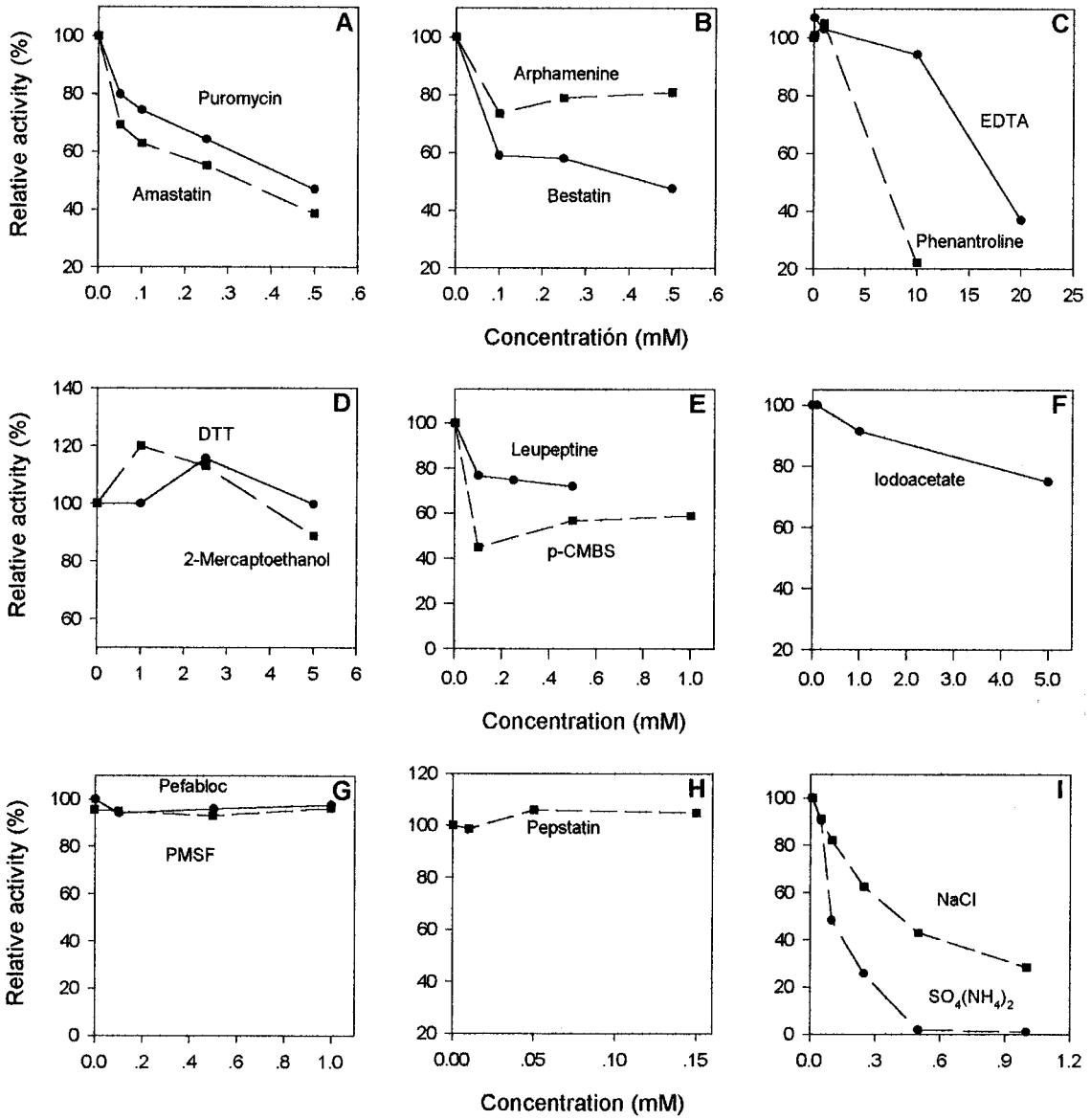

Figure 2. Effect of chemical agents on the activity of the purified aminopeptidase from *L. sake* IATA115. The activity is expressed as a percentage of that obtained in the absence of any chemical agent, which was given a value of 100%.

tidase by ammonium sulfate was deduced from the higher recovery obtained in the following purification step (gel filtration, Table 1) as observed for other peptidases (Wohlrab and Bockelmann, 1994). This was later confirmed with *in vitro* inhibition assays (Figure 2I). So, gel filtration succeeded in both desalting and removing protein. Likewise, the elution from the phenyl-Sepharose column allowed us to determine the existence of at least three aminopeptidase activities, with different specificity, in *L. sake*. In fact, the presence of an aminopeptidase that hydrolyzes Leu-pNA has already been detected by Montel *et al.* (1995) in another *L. sake* strain, although its purification has not been carried out.

Presently, the existence of two well-defined aminopeptidases with broad specificity (aminopeptidase N and C) in lactic acid bacteria has been recognized (Pritchard and Coolbear, 1993; Tan *et al.*, 1993), although the purified aminopeptidase from *L. sake* does not seem to fit with their biochemical characteristics. The molecular mass of the enzyme is 36−35 kDa, and a monomeric structure was inferred from the molecular mass estimation in natural and denaturing conditions. This contrasts with the molecular masses of the monomeric type N and the multimeric type C aminopeptidases (Pritchard and Coolbear, 1993). Exceptionally, a molecular mass of 36 kDa has been reported for an aminopeptidase similar to type N isolated from *Lactococcus lactis* subsp. *cremoris* AC1 and, even so, a possible multimeric structure was also suggested (Geis *et al.*, 1985). Thus, the low molecular mass of the purified aminopeptidase is only in agreement with the leucyl-aminopeptidase partially characterized by Klein *et al.* (1995).

The activity was optimal at 37 °C and pH 7.5 in accordance with other lactobacilli aminopeptidases. The activity at low temperature was relatively high (>15% at 5 °C), as expected according to the psychrotrophic characteristics of this species (Samelis *et al.*, 1994).

As can be deduced from the K_m values, the affinity of the enzyme for Leu-AMC is nearly twice that for Met-AMC. Accordingly, the studies of the specificity for both substrates showed that the activity against Met-AMC was near 50% of that against Leu-AMC (see Table 3). The preference for alanine- and leucine-containing peptides and the absence of hydrolysis of basic amino acids located at the N terminus of several dipeptides were in contrast to the specificity of type N and C

aminopeptidases (Khalid and Marth, 1990; Wohlrab and Bockelmann, 1993; Niven *et al.*, 1995), although the chemical nature of the amino acid groups situated at C terminus is also of significance in defining substrate specificity (Niven *et al.*, 1995) so that wider studies should be required. The activity profile of the purified aminopeptidase was only similar to that observed for the leucyl-aminopeptidase from *Lactobacillus delbrueckii* (Klein *et al.*, 1995).

The inhibition of the activity by puromycin, amastatin, bestatin, and arphamenine B is characteristic of muscle and microbial aminopeptidases (McDonald and Barrett, 1986; Harris, 1989). Likewise, amastatin, a strong inhibitor of type N aminopeptidase (Harris, 1989), also caused the highest inhibition of the purified aminopeptidase (Figure 2A). Inhibition by thiol group reagents and activation by reducing reagents were observed as commonly reported for type N aminopeptidases (Khalid and Marth, 1990; Tan and Konings, 1990; Arora and Lee, 1992, 1994; Bockelmann *et al.*, 1992; Exterkate *et al.*, 1992; Miyakawa *et al.*, 1992), indicating that sulfhydryl groups may be essential for the aminopeptidase activity. However, it is noteworthy that while type N aminopeptidases are strongly inhibited by EDTA and 1,10-phenantroline at concentrations of 0.1−1 mM (Khalid and Marth, 1990; Tan and Konings, 1990; Arora and Lee, 1992, 1994; Bockelmann *et al.*, 1992; Miyakawa *et al.*, 1992; Blanc *et al.*, 1993), considerably higher concentrations of these chelator agents were necessary to cause inhibition of the studied aminopeptidase. Therefore, at typical low concentrations effective for other aminopeptidases, this aminopeptidase would not have been considered as a metalloenzyme. On the other hand, in the case of type C aminopeptidases, the presence of EDTA caused activation (Wohlrab and Bockelmann, 1993) or no effect (Neviani *et al.*, 1989) on the activity. The inhibitory effect exerted by Cu^{2+} as well as by Hg^{2+} has been commonly reported for most aminopeptidases of broad specificity, while Co^{2+} and Zn^{2+}, which are the strongest activators of type N aminopeptidases (Pritchard and Coolbear, 1993), did not seem to have a relevant effect on the activity of the purified enzyme at the assayed concentrations. The lack of inhibition by PMSF and Pefabloc-SC was the only discrepancy found with the partially characterized aminopeptidase L (Klein *et al.*, 1995).

The presence of an additional leucyl-aminopeptidase, unaffected by chelator agents, which contributes to the release of leucine caused by the other well-defined types of aminopeptidases (N and C), has already been proposed on the basis of heat inactivation kinetics and inhibition studies in *Lactobacillus bulgaricus* (Atlan *et al.*, 1989) and *Lactobacillus helveticus* (Blanc *et al.*, 1993). Exterkate *et al.* (1992) demonstrated that in *L. lactis* subsp. *cremoris* a single intracellular aminopeptidase was responsible for both Leu- and Lys-pNA cleaving activity. More recently, a leucyl-aminopeptidase gene from *Lactobacillus delbrueckii* subsp. *lactis* has been sequenced, and the biochemical characteristics of the encoded enzyme seem to be fairly homologous to those of the major aminopeptidase purified in this study (Klein *et al.*, 1995). Due to the novelty of our purified aminopeptidase, further research on its genetic characteristics should be performed.

Therefore, this paper confirms the existence of a new aminopeptidase, with predominant specificity for leucine- and alanine-containing substrates, which appears to be forming part of the exoproteolytic system of, at least, certain lactobacilli strains. In the case of *L. sake* IATA115, this enzyme constitutes the unique aminopeptidase purified to date from a strain originally isolated from meat sources.

ABBREVIATIONS USED

AMC, 7-amido-4-methyl coumarin; pCMBS, *p*-chloromercuribenzenesulfonic acid; pNA, *p*-nitroanilide; PMSF, phenylmethanesulfonyl fluoride; TAPS, 3-[[2-hydroxy-1,1-bis (hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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