APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2002, p. 1980–1987 Vol. 68, No. 4
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.4.1980–1987.2002

Purification and Characterization of an Arginine Aminopeptidase from *Lactobacillus sakei*

Yolanda Sanz* and Fidel Toldrá

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), 46100 Burjasot (Valencia), Spain

Received 9 July 2001/Accepted 21 January 2002

An arginine aminopeptidase (EC 3.4.11.6) that exclusively hydrolyzes basic amino acids from the amino (N) termini of peptide substrates has been purified from *Lactobacillus sakei*. The purification procedure consisted of ammonium sulfate fractionation and three chromatographic steps, which included hydrophobic interaction, gel filtration, and anion-exchange chromatography. This procedure resulted in a recovery rate of 4.2% and a 500-fold increase in specific activity. The aminopeptidase appeared to be a trimeric enzyme with a molecular mass of 180 kDa. The activity was optimal at pH 5.0 and 37°C. The enzyme was inhibited by sulfhydryl group reagents and several divalent cations (Cu^{2+}, Hg^{2+}, and Zn^{2+}) but was activated by reducing agents, metal-chelating agents, and sodium chloride. The enzyme showed a preference for arginine at the N termini of aminoacyl derivatives and peptides. The K_m values for Arg-7-amido-4-methylcoumarin (AMC) and Lys-AMC were 15.9 and 26.0 μM, respectively. The nature of the amino acid residue at the C terminus of dipeptides has an effect on hydrolysis rates. The activity was maximal toward dipeptides with Arg, Lys, or Ala as the C-terminal residue. The properties of the purified enzyme, its potential function in the release of arginine, and its further metabolism are discussed because, as a whole, it could constitute a survival mechanism for *L. sakei* in the meat environment.

In bacteria, a number of cellular processes involve proteolytic degradation; these include protein maturation, protein turnover, signal peptide processing, regulation of gene expression, and nutrition (5, 18). The role of the proteolytic system of dairy lactic acid bacteria in major physiological processes such as the utilization of exogenous proteins as nutrients and gene regulation is well established (16). This system is responsible for the supply of free amino acids essential for optimal growth in milk (14). Besides, milk protein-derived peptides may function as regulators of proteolytic enzymes (8, 12, 27). In addition, proteolytic activity plays a technological role, contributing to the development of texture and flavor in dairy products through the generation of peptides and free amino acids, which may be precursors of aromatic compounds (11). The biochemistry and genetics of the proteolytic system of dairy lactic acid bacteria have been the subjects of exhaustive research. This system is essentially integrated by a cell wall-associated proteinase, peptide transport systems, and a pool of intracellular peptidases. Two general aminopeptidases (PepC and PepN) are commonly found in both lactococci and lactobacilli. These enzymes have wide specificity and can hydrolyze basic, aromatic, and hydrophobic amino acid residues from oligopeptides (16).

Lactobacillus sakei is often the predominant species in meat and meat products; it is also used as a starter culture for sausage production. Therefore, the metabolic traits that allow this species to compete effectively in the meat ecosystem are under investigation (1, 4, 30). The proteolytic events that occur during meat processing lead to the generation of small peptides and free amino acids, which are considered to be flavor compounds (2, 31). The proteolytic products also have a positive physiological effect on lactic acid bacteria, since addition of proteolytic enzymes promotes the growth of these bacteria during meat fermentation (3). Several meat-derived *Lactobacillus* spp. have shown proteolytic activity on sarcoplasmic and myofibrillar proteins, and *L. sakei* in particular has been demonstrated to play an important potential role in amino acid generation (6, 7, 26). Thus, fundamental biochemical research has been focused on intracellular peptidases of *L. sakei*, and a number of peptidases have been purified (20, 22, 24, 27). Unlike the general aminopeptidases previously identified in dairy lactic acid bacteria, the only aminopeptidase purified from *L. sakei* so far does not hydrolyze N-terminal arginine (22). However, arginine is an essential amino acid for *L. sakei* and specifically promotes its growth in meat (19, 21). The concentration of free arginine in raw meat is low, although it is relatively abundant in muscle myofibrillar proteins. Thus, arginine-specific aminopeptidase activity could be important for the release of the free amino acid, as it could be further channeled into the arginine deiminase pathway and used as an energy source, constituting a survival mechanism for *L. sakei* (4). The only work reported on the purification of such a bacterial enzyme, named arginine aminopeptidase (EC 3.4.11.6), in a closely related genus was carried out on the oral bacterium *Streptococcus mitis*, which has two forms of this enzyme (13).

This work reports the purification and characterization of an arginine aminopeptidase from *L. sakei* which has not been previously described for dairy lactobacilli or lactococci. The properties of the purified enzyme and its possible relevance in the generation of arginine in the meat environment are discussed.

* Corresponding author. Mailing address: Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Apartado 73, 46100 Burjasot (Valencia), Spain. Phone: 34 96 3900022. Fax: 34 96 3636301. E-mail: yolsanz@iata.csic.es.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *L. sakei* CECE (Colección Española de Cultivos Tipo) 4808, originally isolated from sausages (25), was routinely grown in MRS broth or agar (Oxoid, Basingstoke, Hampshire, United Kingdom)

at 30°C. For purification purposes, the organism was grown in 1.5-liter batch cultures of MRS broth (Oxoid) at 30°C without agitation. The medium was inoculated with an overnight culture (0.5%) and incubated until a final optical density of 3.5 at 660 nm was reached.

Enzyme assay. Arginine aminopeptidase activity was determined throughout the purification and characterization work by using L-arginine-7-amido-4-methylcoumarin (AMC; Sigma, St. Louis, Mo.) as a substrate. The reaction mixture consisted of 250 μl of 50 mM citric acid–NaOH (pH 5.0) containing 0.1 mM substrate and 50 μl of enzyme. During the purification work, aminopeptidase activity was also determined against Leu-AMC (Sigma) as described elsewhere (22). Unless otherwise stated, the release of fluorescence was determined after 10 min of incubation at 37°C in a multiscan fluorimeter (Fluoroscan II; Labsystems, Oy, Finland) at excitation and emission wavelengths of 360 and 440 nm, respectively. Three replicas were measured for each experimental point. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per h at 37°C.

Purification. The cell extract used for purification was obtained by lysozyme and ultrasonic treatments as previously described (6, 26). The aminopeptidase was purified as described below.

(i) Ammonium sulfate fractionation. The cell extract was fractionated with ammonium sulfate in two steps. The first step involved addition of ammonium sulfate to the cell extract to 50% saturation. After incubation for 20 min at 4°C, the cell extract was centrifuged at 15,000 × *g* for 20 min at 4°C. The pellet obtained was discarded, and the supernatant was subjected to the second step, which involved addition of ammonium sulfate to 70% saturation, as described above. The final precipitate was collected by centrifugation (at 15,000 × *g* for 20 min at 4°C) and dissolved in approximately 10 ml of 50 mM Tris-HCl (pH 7.5).

(ii) Hydrophobic interaction chromatography. The protein fraction obtained by ammonium sulfate precipitation was applied to a phenyl-Sepharose Fast Flow column (23 by 1.3 cm; Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.5 M $(NH_4)_2SO_4$. The proteins retained were eluted at 2 ml/min, by using a linear gradient from 0.5 to 0 M $(NH_4)_2SO_4$, in 50 mM Tris-HCl (pH 7.5) (500 ml). The eluent was collected in 6-ml fractions. The active fractions were pooled and concentrated by ammonium sulfate precipitation (70% saturation) as described above.

(iii) Gel filtration chromatography. The precipitated sample was dissolved in 1.0 ml of 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and was applied to a Sephacryl 200 HR column (100 by 1.5 cm; Pharmacia) equilibrated with the same buffer. Proteins were eluted at 10 ml/h, and fractions of 3 ml were collected.

(iv) Anion-exchange chromatography. The active sample obtained from the previous chromatographic step was applied to a Resource Q anion-exchange column (6 ml; Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.2 M NaCl. Proteins were eluted at 6 ml/min by applying an initial isocratic step in the equilibration buffer (18 ml), followed by a linear gradient from 0.2 to 0.3 M NaCl in the same buffer (60 ml). The eluent was collected in 1-ml fractions.

Every chromatographic separation was carried out in a fast protein liquid chromatography (FPLC) system (Pharmacia) except for the gel filtration step, which was performed by classical chromatography.

Determination of protein concentration. The protein concentration was determined by the bicinchoninic acid (BCA) method with the BCA protein assay reagent (Pierce, Rockford, Ill.).

Determination of molecular mass. Purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4% acrylamide stacking gels and 10% acrylamide resolving gels (17). The molecular mass of the denatured enzyme was estimated by using a broad-range molecular weight protein standard (Bio-Rad, Richmond, Calif.). Proteins were visualized after Coomassie brilliant blue R-250 staining. The relative molecular mass of the native enzyme was determined by gel filtration on a Sephacryl 200 HR column as described above. The column was calibrated with the following standard proteins (Sigma): β-amylase (200 kDa), aldolase (158 kDa), albumin (68 kDa), chymotrypsinogen A (25 kDa), and cytochrome *c* (12.4 kDa).

Effects of pH and temperature on aminopeptidase activity. The effect of pH on aminopeptidase activity was determined in the range from pH 3.5 to 8.5 by using the following buffers: 50 mM citric acid–NaOH, pH 3.5 to 5.5; 50 mM sodium acetate, pH 4.0 to 5.5; 50 mM sodium phosphate, pH 6.0 to 7.0; and Tris-HCl, pH 7.5 to 8.5. The effect of temperature was determined at the optimum pH in the range from 6 to 40°C, by adapting the incubation time to the stability of the enzyme at each temperature, as previously described (22). In every case, activity was expressed as a percentage of the activity obtained at either the optimum pH or the optimum temperature.

Effects of chemical agents and metal cations on aminopeptidase activity. The effects of potential inhibitors or activators on arginine aminopeptidase activity were determined by addition of several chemical agents and metal salts, at 0.1 or 1 mM, to the reaction buffer. Activity was assayed as described above and expressed as a percentage of the activity obtained in the absence of the added compound.

Substrate specificity. The relative activities of the arginine aminopeptidase against several fluorescent substrates were determined by the standard activity assay. The relative rates of hydrolysis of several aminoacyl-*p*-nitroanilide (pNA) substrates were also determined. The reaction mixture consisted of 250 μl of 50 mM citric acid–NaOH (pH 5.0) containing 0.5 mM substrate and 50 μl of enzyme. Absorbance at 415 nm was determined in a multiplate reader (Ultramark Model 550; Bio-Rad) after 30 min of incubation. Relative activities against various dipeptides were assayed by monitoring their disappearance by high-performance liquid chromatography (HPLC) using a 1050 Hewlett-Packard HPLC system (Waters Corporation, Milford, Mass.). The reaction mixture consisted of 100 μl of 50 mM citric acid–NaOH (pH 5.0), 25 μl of 10 mM peptide, and 25 μl of enzyme. The mixture was incubated at 37°C for as long as 60 min, and the reaction was stopped by addition of 25 μl of 30% acetic acid. Samples (20 μl) were loaded onto a Spherisorb SCX column (25 by 0.46 cm; Teknokroma, Barcelona, Spain). Separation was carried out by using a sodium chloride gradient between two solvents: 20% (vol/vol) acetonitrile in 6 mM HCl (solvent A) and 20% (vol/vol) acetonitrile in 6 mM HCl containing 1 M NaCl (solvent B). Peptides were eluted in a linear gradient from 0 to 55% solvent B for 8 min at a flow rate of 1.2 ml · min^{-1} at 40°C. Detection was carried out at 214 nm by using a variable-wavelength UV detector. All dipeptides, fluorogenic substrates, and chromogenic substrates were obtained from Sigma.

Determination of kinetic parameters. Kinetic parameters of the purified enzyme were estimated for Arg-AMC and Lys-AMC by using concentrations ranging from 0.005 to 0.1 mM. Activity was measured continuously at 37°C as described above. Kinetic parameters were calculated from Lineweaver-Burk plots.

TABLE 1. Purification of arginine aminopeptidase from *L. sakei*

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	1,295.02	182.1	0.14	100.0	1.0
50–70% ammonium sulfate cut	743.40	112.1	0.15	61.6	1.1
Phenyl-Sepharose	13.34	20.4	1.53	11.2	10.9
Sephacryl 200 HR	0.55	18.1	32.91	9.9	235.1
Resource Q	0.11	7.7	70.0	4.2	500.0

RESULTS

Purification of the enzyme. An arginine aminopeptidase of *L. sakei* was purified by selective fractionation with ammonium sulfate and three chromatographic steps. The results of each purification step are shown in Table 1 and Fig. 1. The Arg-AMC hydrolyzing activity eluted from the phenyl-Sepharose column as a unique peak at 0.16 to 0.14 M $(NH_4)_2SO_4$. This activity partially coeluted with Leu-AMC hydrolyzing activity (Fig. 1A). The chromatographic step on the gel filtration column allowed the complete separation of the contaminant aminopeptidase activity (eluent volume, 84 ml) from the Arg-AMC hydrolyzing activity (eluent volume, 60 ml) (Fig. 1B). The Arg-AMC hydrolyzing activity eluted from the Resource Q column as two separate peaks: a minor fraction at 0.22 M NaCl and a major fraction at 0.29 M NaCl (Fig. 1C). The fractions corresponding to the second peak showed a unique protein band when analyzed by SDS-PAGE (Fig. 2). The whole procedure resulted in a recovery yield of 4.2% and a 500-fold increase in specificity.

Molecular mass. The molecular mass of the purified enzyme estimated by SDS-PAGE analysis was approximately 60 kDa (Fig. 2, lane 6). The relative molecular mass of the native enzyme estimated by gel filtration on a Sephacryl 200 HR column was around 180 kDa. These results suggested that the purified enzyme is a trimer.

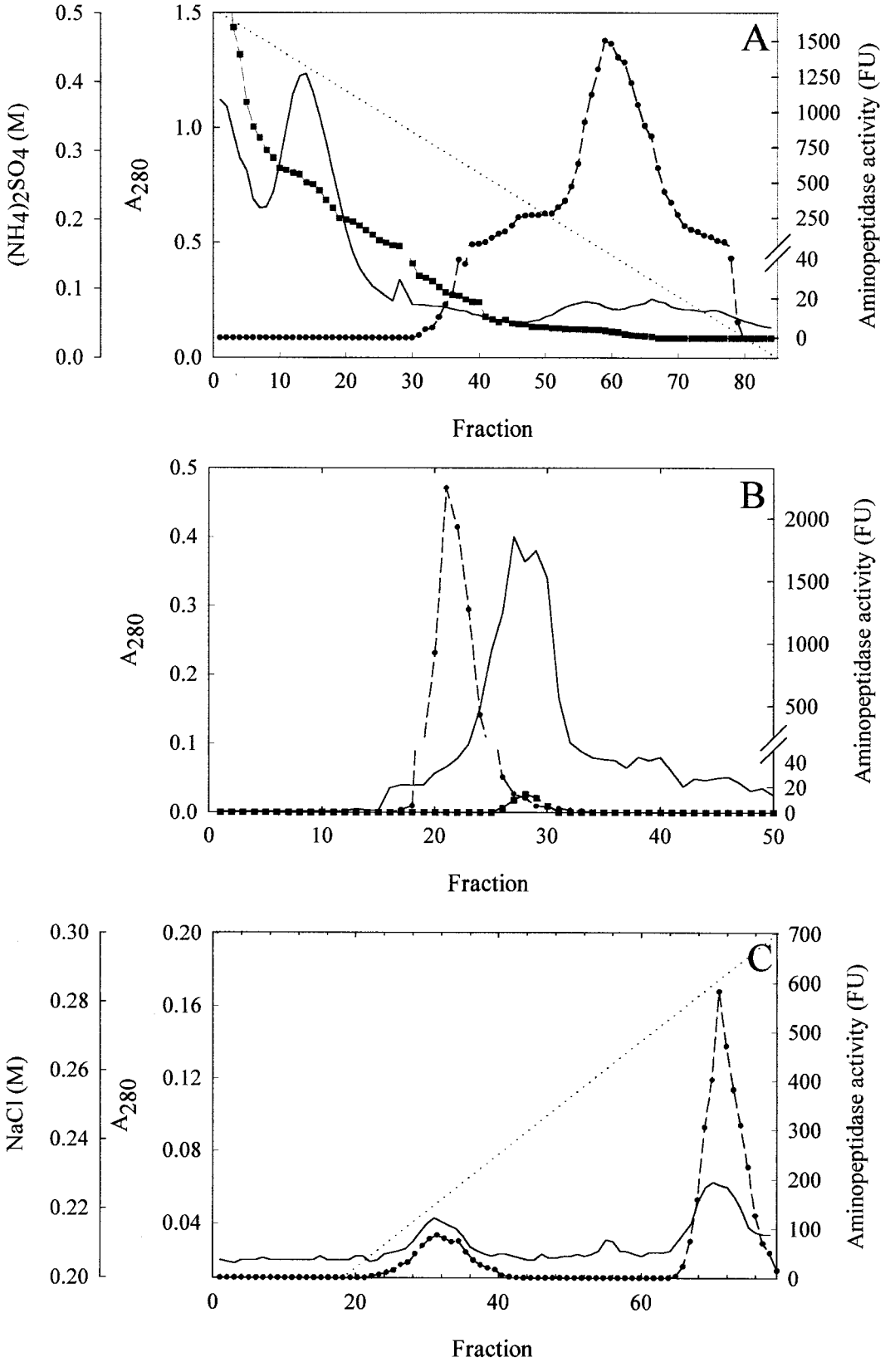

FIG. 1. Chromatograms from different steps in the purification of *L. sakei* arginine aminopeptidase. Proteins were detected by measuring the absorbance at 280 nm (solid line); aminopeptidase activity was assayed against Arg-AMC, at pH 5.0 (solid circles), and Leu-AMC, at pH 7.5 (solid squares) (see Materials and Methods for details) and expressed in fluorescence units (FU). (A) Phenyl-Sepharose chromatography with $(NH_4)_2SO_4$ gradient (light dotted line); (B) Sephacryl 200 HR chromatography; (C) Resource Q chromatography with NaCl gradient (light dotted line).

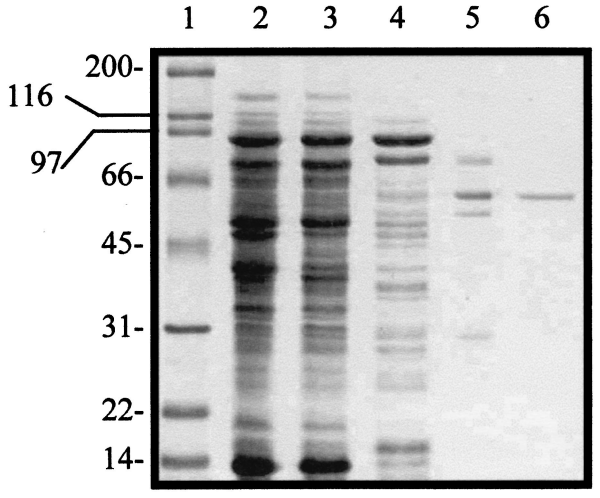

FIG. 2. Electrophoretic analysis (SDS-PAGE) of arginine aminopeptidase active fractions obtained in different purification steps. Lane 1, molecular weight markers (in thousands); lane 2, cell extract; lane 3, 50 to 70% ammonium sulfate cut; lane 4, phenyl-Sepharose chromatography; lane 5, Sephacryl 200 HR chromatography; lane 6, Resource Q chromatography.

Effects of pH and temperature. The purified enzyme showed activity in a narrow pH range (4.0 to 6.0), with an optimum at pH 5.0, in both 50 mM sodium acetate–acetic acid and 50 mM citric acid–NaOH (Fig. 3A). The activity was negligible at pH values beyond 4.0 and 6.0. The activity of the purified enzyme was optimal at 37°C but was sharply reduced at 40 to 45°C (Fig. 3A). The reduction in enzyme activity at high temperatures was not due to a decay in stability during the incubation time (5 min) (Fig. 3B). At low temperatures such as 6 to 15°C, the enzyme retained 20 to 30% of its optimal activity (Fig. 3A).

Effects of chemical agents and metal cations. The effects observed in the presence of potential inhibitors or activators of the enzyme are shown in Tables 2 and 3. The sulfhydryl group reagent iodoacetate completely inhibited aminopeptidase activity, while reducing agents, particularly dithiothreitol, activated the enzyme. These results suggested that sulfhydryl groups are involved in the catalytic activity. However, the other cysteine protease inhibitor, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), had no effect on the activity.

The chelating agent EDTA had a stimulating effect (28 to 46%) on arginine aminopeptidase activity (Table 2). The serine protease inhibitors phenylmethylsulfonyl fluoride and 3,4-dichloroisocoumarin, the cysteine and serine protease inhibitor leupeptin, and typical inhibitors of exopeptidases, such as puromycin and bestatin, did not have any remarkable effect on the activity (Table 2).

The presence of Cu^{2+}, Hg^{2+}, and Zn^{2+} caused a complete inhibition at 1.0 mM, while less pronounced effects (10 to 30% inhibition) were observed in the presence of the remaining divalent cations (Table 3). The enzyme was activated by increasing concentrations of NaCl, with a maximum at 0.6 M (Fig. 4). The presence of high concentrations (0.5 to 3 M) of ammonium sulfate, used in the purification procedure, provoked 40 to 75% inhibition of the aminopeptidase activity (Fig. 4).

Substrate specificity. The purified enzyme was active only against the AMC and pNA derivatives of basic amino acids (Table 4). The hydrolysis rates obtained with lysine-derived substrates were only about 4% of those obtained with arginine-derived substrates. The enzyme did not show endoproteinase activity, as it did not hydrolyze *N*-α-benzoyl-Arg-AMC (Table 4). Among all dipeptides tested, only those containing basic residues at the N-terminal position were hydrolyzed. The hydrolysis rates of peptides with the sequence Lys-X (where X stands for Ala or Lys) were approximately 30% of those of peptides with the sequence Arg-X. The nature of the amino acid residue at the C terminus of dipeptides also had an effect on hydrolysis rates. Activity was maximal toward peptides containing either Arg, Lys, or Ala as the C-terminal residue of Arg-X dipeptides, while the presence of Phe, Ile, Leu, or Asp at this position reduced the hydrolysis rates to values similar to those obtained with dipeptides containing lysine at the N terminus (Table 5).

Kinetic parameters. K_m and V_{max} were determined for Arg-AMC and Lys-AMC. The K_m values were 15.9 and 26.0 μM, respectively, and the V_{max} values were 211.4 and 11.1 $\mu mol \cdot h^{-1} \cdot mg^{-1}$, respectively.

DISCUSSION

L. sakei arginine aminopeptidase (EC 3.4.11.6), identified in this study, is the first exopeptidase found in lactobacilli or lactococci that exclusively releases N-terminal basic residues. The enzyme, also known as aminopeptidase B or arginyl aminopeptidase, was first purified from rat liver and at present is known to be widely distributed in a number of animal tissues (10). In contrast, the presence of this exopeptidase in microorganisms is not well documented. In bacteria, various peptidases with overlapping specificities can hydrolyze basic N-terminal residues from oligopeptides, but only two forms of an arginine aminopeptidase have been described, in the oral bacterium *S. mitis* (13).

Purification of *L. sakei* arginine aminopeptidase was achieved by ammonium sulfate fractionation and three consecutive chromatographic steps. Ammonium sulfate fractionation allowed the concentration of the initial cell extract but apparently did not result in a significant increase in specific activity. However, it should be considered that the measurements of the activity at this stage must have been negatively affected by the presence of ammonium sulfate in the sample. In fact, it was demonstrated later that this salt is a strong reversible inhibitor of the purified enzyme (Fig. 4). Gel filtration chromatography on the Sephacryl 200 HR column was a critical step for separating the contaminant Leu-AMC hydrolyzing activity from the purified enzyme and increasing the specificity. Unexpectedly, the Arg-AMC hydrolyzing activity was split into two separate peaks in the last chromatographic step on the strong anion-exchange column. The fractions eluting at low sodium chloride concentrations were also endowed with dipeptidase activity on peptides which did not contain arginine or lysine as the N-terminal residue (data not shown). The major fraction of activity containing the purified enzyme eluted at high sodium chloride concentrations, as is characteristic of arginine aminopeptidases from *S. mitis* (13). Thus, the enzyme was completely separated from other peptidases, and its specific activity was substantially increased. Moreover, the recovery yield obtained was very low, which could be due to the facts that (i)

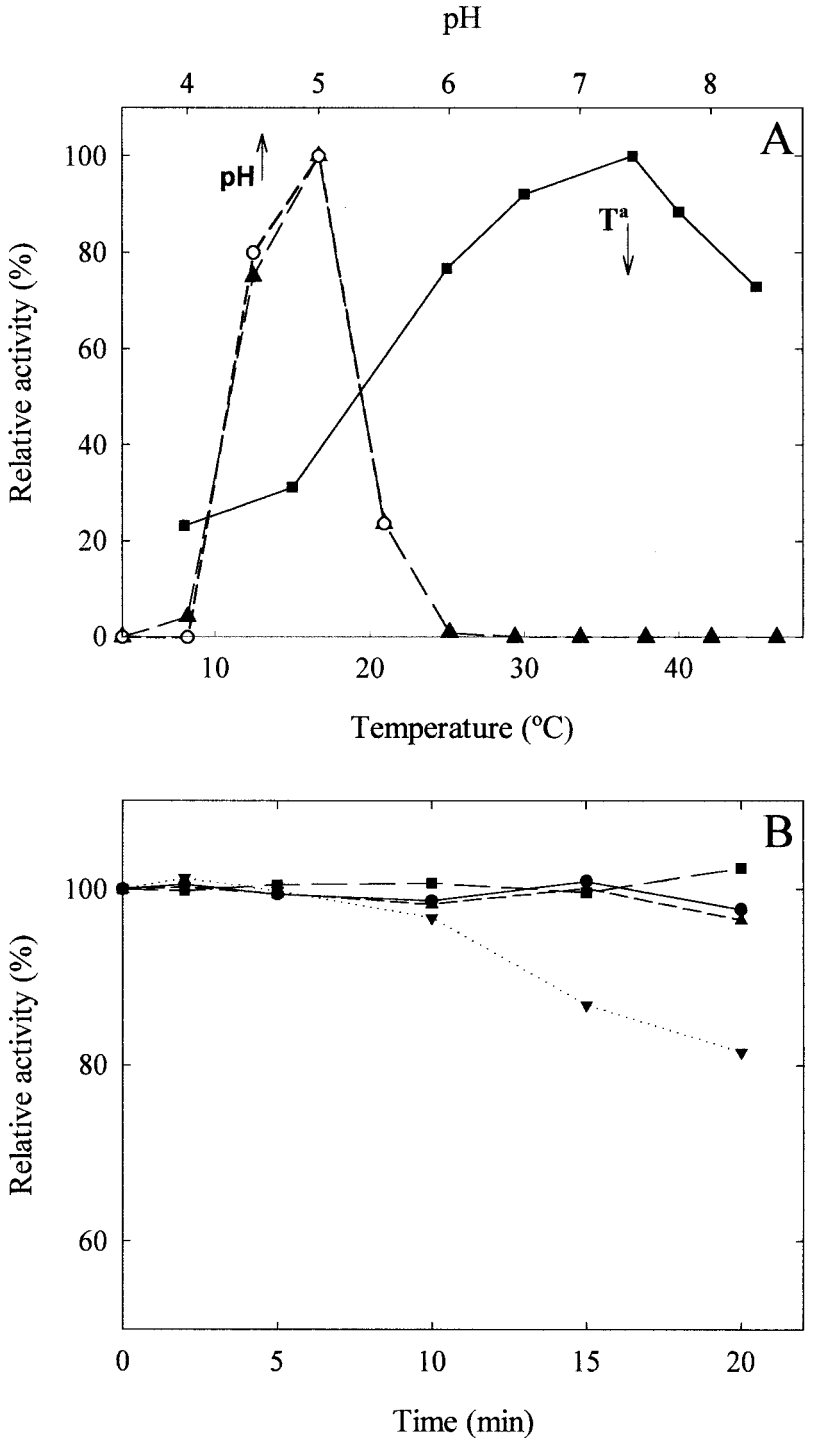

FIG. 3. (A) Effects of pH and temperature on arginine aminopeptidase activity from *L. sakei* using Arg-AMC as a substrate. Symbols: ▲, activities in 50 mM citric acid–NaOH at varying pHs; ○, activities in 50 mM sodium acetate–acetic acid at varying pHs; ■, activities at varying temperatures. Activities at optimal pH and temperature were assigned a value of 100%. (B) Stability of arginine aminopeptidase at different temperatures: 30°C (●), 37°C (■), 40°C (▲), and 45°C (▼). Initial activities were assigned a value of 100%.

other peptidases, previously characterized, also eluted from the phenyl-Sepharose column at low concentrations of ammonium sulfate, so that it was necessary to establish a narrow salt gradient (0.5 to 0 M) for a long time (250 min; 500 ml of elution volume) to partially separate all these activities (22, 24), resulting in a relatively broad peak of arginine aminopeptidase activity from which only four fractions with maximal activity were pooled to avoid contaminants; (ii) at least one other peptidase which also hydrolyzes Arg-AMC was previously detected in a cell extract of *L. sakei* (23); and (iii) the

TABLE 2. Effects of chemical agents on the activity of purified arginine aminopeptidase

Chemical agent	Relative activity[a] at:	
	0.1 mM	1.0 mM
EDTA	128	146
o-Phenanthroline	117	95
Dithiothreitol	119	129
2-Mercaptoethanol	103	116
Iodoacetate	0	0
Leupeptin	110	106
E-64	109	101
Bestatin	97	101
Puromycin	94	81.5
Phenylmethylsulfonyl fluoride	100	90
3,4-Dichloroisocoumarin	88	84

[a] Expressed as a percentage of the activity obtained in the absence of any added chemical agent, which was assigned a value of 100%.

TABLE 4. Relative activities of purified arginine aminopeptidase on various fluorescent (AMC-derived) and colorimetric (pNA-derived) substrates

Substrate	Relative activity[a]
Ala-AMC	0.0
Arg-AMC	100.0
Gly-AMC	0.0
Leu-AMC	0.0
Lys-AMC	4.3
Met-AMC	0.0
Pro-AMC	0.0
Ser-AMC	0.0
Tyr-AMC	0.0
Val-AMC	0.0
N-α-Benzoyl-Arg-AMC	0.0
Arg-pNA	100.0
Lys-pNA	3.6

[a] Expressed as a percentage of the rate of hydrolysis of Arg-AMC or Arg-pNA, each of which was assigned a value of 100%.

enzyme could have lost stability through the purification process.

The molecular mass of the purified enzyme was 180 kDa, and it likely consists of three equal subunits of 60 kDa. The apparent molecular masses of forms I and II of the enzyme found in *S. mitis* were 62 and 362 kDa, respectively; only the latter is a multimeric enzyme. The enzymes characterized from mammalian tissues are monomers with molecular masses that vary (52 to 105 kDa) depending on origin (9, 10, 15, 29).

The acidic and narrow optimal pH of *L. sakei* arginine aminopeptidase differs from those of the other arginine aminopeptidases previously characterized. This enzyme is usually functional over a broad pH range, with an optimum close to 7.0 (10) or even higher in the case of the two forms of the enzyme identified in *S. mitis*. The general aminopeptidases (PepN and PepC) from dairy lactic acid bacteria, which hydrolyze arginine at the N termini of oligopeptides and would partly fulfill the function of an arginine aminopeptidase, have optimal activity around pH 7.0 (16). On the other hand, the dipeptidase and tripeptidase characterized in *L. sakei* possess wide specificity, hydrolyzing arginine but only from di- and tripeptides, respectively, and with optimal activity at neutral or slightly basic pHs (20, 24). Therefore, the presence of the purified enzyme in *L. sakei* could confer on this organism the advantage of obtaining free arginine and thereby surviving under acidic stress conditions. In *L. sakei*, free arginine resulting from proteolysis could be further channeled to the arginine deiminase pathway, allowing the generation of ATP and ammonia, which may compensate for reductions in pH (4).

TABLE 3. Effects of metal cations on the activity of purified arginine aminopeptidase

Metal salt	Relative activity[a] at:	
	0.1 mM	1.0 mM
$CaCl_2$	81	89
$CoCl_2$	77	72
$CuCl_2$	0	0
$HgCl_2$	0	0
$MgCl_2$	78	80
$ZnCl_2$	18	0

[a] Expressed as a percentage of the activity obtained in the absence of any added metal salt, which was assigned value of 100%.

The purified enzyme seemed to be a cysteine protease according to the inhibition observed in the presence of sulfhydryl blocking reagents and the activation by reducing agents, as was the case for the two forms of the *S. mitis* enzyme (13). In contrast, the mammalian enzyme is a Zn-dependent metalloprotease, although in some cases, cysteine or disulfide bonds also seem to be necessary for the enzyme activity (9, 10). The lack of inhibition of both the *L. sakei* and *S. mitis* enzymes by bestatin was also in contrast with the strong inhibitory effect generally observed for mammalian arginine aminopeptidases (9, 10). Metal chelating agents promoted the activity of the bacterial arginine aminopeptidases (*L. sakei* and *S. mitis* enzymes), while they inhibited the mammalian enzymes. The cations Cu^{2+}, Hg^{2+}, and Zn^{2+} were potent inhibitors of the enzymes from both *L. sakei* and *S. mitis.* Remarkably, *L. sakei* aminopeptidase was activated by sodium chloride, which may favor its activity during meat-curing processes. The equivalent mammalian enzyme is also characterized by its chloride ion activation, while that effect was not observed in either of the forms of the *S. mitis* enzyme (13).

L. sakei arginine aminopeptidase exclusively releases basic residues, while the enzymes from other sources showed slight hydrolysis of N-terminal residues other than arginine or lysine (9, 13). The preference of the bacterial enzymes for arginine versus lysine at the N terminus of different substrates was remarkably higher than that of the mammalian enzymes. Thus, the ratios of hydrolysis rates obtained with Arg-derived substrates to those obtained with Lys-derived substrates were 25 for the *L. sakei* enzyme and 83.3 and 13.9 for the two forms of the *S. mitis* enzyme. In contrast, the ratios of hydrolysis rates obtained with Arg-derived substrates to those obtained with Lys-derived substrates were much lower for mammalian enzymes, e.g., 2.5 and 2.0 for rat liver and porcine liver enzymes, respectively (13). Among dipeptides, the maximal hydrolysis rate was detected against Arg-Arg, which also indicated that the catalytic activity is highly specific in order to release free arginine in *L. sakei*, which could promote the growth of this organism in meat (19, 21). The kinetics of the hydrolysis of both Arg-AMC and Lys-AMC also indicated that the *L. sakei*

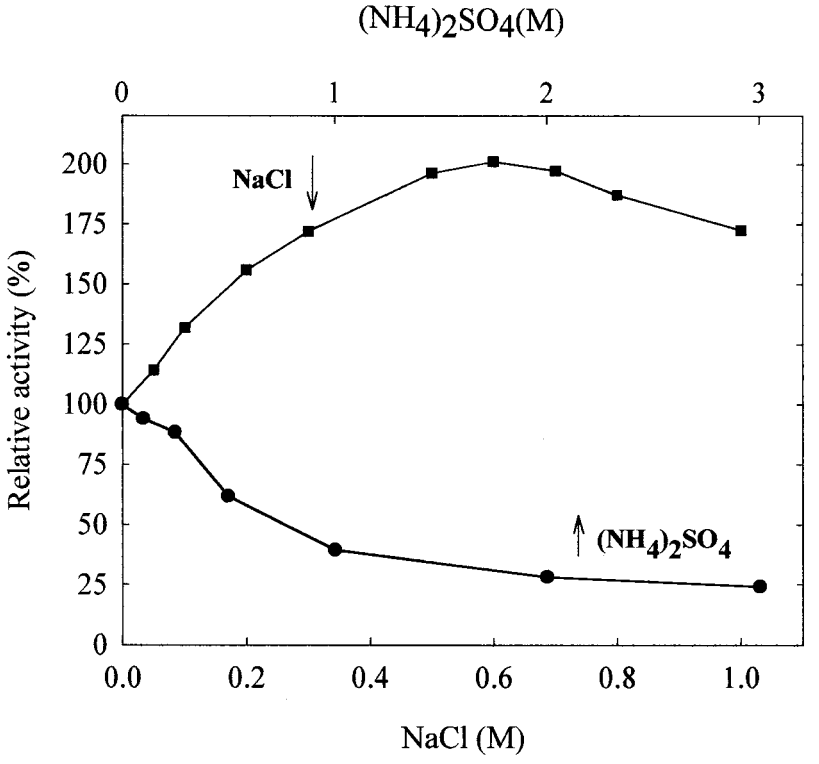

FIG. 4. Effects of NaCl (■) and $(NH_4)_2SO_4$ (●) on arginine aminopeptidase activity from *L. sakei* using Arg-AMC as a substrate. Activity in the absence of any salt was assigned a value of 100%.

enzyme has higher affinity and maximal catalytic activity on Arg-AMC.

Overall, a novel arginine aminopeptidase has been described with particular biochemical properties such as its acidic optimum pH and the activating effect of sodium chloride, which may be of the utmost importance for physiological and technological roles in the meat preservation and fermentation processes. Therefore, further research is needed in order to establish the involvement of the purified enzyme in the release of arginine, energy generation, and neutralization of the low pH values due to fermentation processes.

TABLE 5. Relative activities of purified arginine aminopeptidase on various peptides

Peptide	Relative activity[a]
Arg-Arg	100
Arg-Lys	88
Arg-Ala	82
Arg-Phe	35
Arg-Ile	32
Arg-Leu	25
Arg-Asp	25
Lys-Ala	33
Lys-Lys	30
Ala-Ala	0
Ala-Lys	0
Asp-Ala	0
Gly-Ala	0
Leu-Ala	0

[a] Expressed as a percentage of the rate of hydrolysis of Arg-Arg, which was assigned a value of 100%.

ACKNOWLEDGMENTS

This work was supported by grant AGL2000-2007 from CICYT (Madrid, Spain). The postdoctoral contract to Y. Sanz from MEC (Madrid, Spain) is acknowledged.

REFERENCES

1. **Amanatidou, A., M. H. J. Bennik, L. G. M. Gorris, and E. J. Smid.** 2001. Superoxide dismutase plays an important role in the survival of *Lactobacillus sake* upon exposure to elevated oxygen. Arch. Microbiol. **176:**79–88.
2. **Aristoy, M.-C., and F. Toldrá.** 1995. Isolation of flavour peptides from raw pork meat and dry-cured ham, p. 1323–1344. *In* G. Charalambous (ed.), Food flavours: generation, analysis and process influence. Elsevier Science B.V., Amsterdam, The Netherlands.
3. **Blom, H., B. F. Hagen, B. O. Pedersen, A. L. Holck, L. Axelsson, and H. Naes.** 1996. Accelerated production of dry fermented sausage. Meat Sci. **43:**S229-S242.
4. **Champomier-Verges, M.-C., M. Zúñiga, F. Morel-Deville, G. Pérez-Martínez, M. Zagorec, and S. D. Ehrlich.** 1999. Relationships between arginine degradation, pH and survival in *Lactobacillus sakei*. FEMS Microbiol. Lett. **180:**297–304.
5. **Charlier, D., A. Kholti, N. Huysveld, D. Gigot, D. Maes, T.-L. Thia-Toong, and N. Glansdorff.** 2000. Mutational analysis of *Escherichia coli* PepA, a multifunctional DNA-binding aminopeptidase. J. Mol. Biol. **302:**411–426.
6. **Fadda, S., Y. Sanz, G. Vignolo, M.-C. Aristoy, G. Oliver, and F. Toldrá.** 1999. Hydrolysis of pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sakei.* Appl. Environ. Microbiol. **65:**578–584.
7. **Fadda, S., Y. Sanz, G. Vignolo, M.-C. Aristoy, G. Oliver, and F. Toldrá.** 1999. Characterization of pork muscle protein hydrolysis caused by *Lactobacillus plantarum.* Appl. Environ. Microbiol. **65:**3540–3546.
8. **Flambard, B., J. Richard, and V. Juillard.** 1997. Interaction between proteolytic strains of *Lactococcus lactis* influenced by different types of proteinase during growth in milk. Appl. Environ. Microbiol. **63:**2131–2135.
9. **Flores, M., M.-C. Aristoy, and F. Toldrá.** 1993. HPLC purification and characterization of porcine muscle aminopeptidase B. Biochimie **75:**861–867.
10. **Foulon, T., S. Cadel, and P. Cohen.** 1999. Aminopeptidase B (EC 3.4.11.6). Int. J. Biochem. Cell Biol. **31:**747–750.
11. **Fox, P. F., and J. M. Wallace.** 1997. Formation of flavor compounds in cheese. Adv. Appl. Microbiol. **45:**17–85.
12. **Guedon, E., P. Renault, and S. D. Ehrlich.** 2001. Transcriptional pattern of

genes coding for the proteolytic system of *Lactococcus lactis* and evidence for coordinated regulation of key enzymes by peptide supply. J. Bacteriol. **183:** 3614–3622.
13. **Hiraoka, B. Y., K. Fukasawa, and M. Harada.** 1983. Purification and characterization of two novel arginine aminopeptidases from *Streptococcus mitis* ATCC 9811. J. Biochem. **94:**1201–1208.
14. **Juillard, V., D. Le Bars, E. R. S. Kunji, W. N. Konings, J.-C. Gripon, and J. Richard.** 1995. Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk. Appl. Environ. Microbiol. **61:**3024–3030.
15. **Kawata, S., S. Takayama, K. Ninimiya, and S. Makisumi.** 1980. Purification and some properties of porcine liver aminopeptidase B. J. Biochem. **88:** 1025–1032.
16. **Kunji, E. R. S., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings.** 1996. The proteolytic systems of lactic acid bacteria. Antonie Leeuwenhoek **70:** 187–221.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
18. **Lazdunski, A. M.** 1989. Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. FEMS Microbiol. Rev. **5:**265–276.
19. **Montel, M.-C., and J. Labadie.** 1986. Specific nutritional requirements of Lactobacilli spp. from meat. Zentbl. Bakteriol. Mikrobiol. Hyg. B **183:**23–27.
20. **Montel, M.-C., M.-P. Seronine, R. Talon, and M. Hébraud.** 1995. Purification and characterization of a dipeptidase from *Lactobacillus sakei*. Appl. Environ. Microbiol. **61:**837–839.
21. **Newton, K. G., and C. O. Gill.** 1978. The development of the anaerobic spoilage flora of meat stored at chill temperatures. J. Appl. Bacteriol. **44:** 91–95.
22. **Sanz, Y., and F. Toldrá.** 1997. Purification and characterization of an aminopeptidase from *Lactobacillus sakei*. J. Agric. Food Chem. **45:**1552–1558.
23. **Sanz, Y., and F. Toldrá.** 1997. Aminopeptidase activities from *Lactobacillus sakei* in models of curing ingredients and processing conditions for dry sausage. J. Food Sci. **62:**1211–1213, 1234.
24. **Sanz, Y., F. Mulholland, and F. Toldrá.** 1998. Purification and characterization of a tripeptidase from *Lactobacillus sakei*. J. Agric. Food Chem. **46:**349–353.
25. **Sanz, Y., M. Hernandez, M. A. Ferrus, and J. Hernández.** 1998. Characterization of *Lactobacillus sake* isolates from dry-cured sausages by restriction fragment length polymorphism analysis of the 16S rRNA gene. J. Appl. Microbiol. **84:**600–606.
26. **Sanz, Y., S. Fadda, G. Vignolo, M.-C. Aristoy, G. Oliver, and F. Toldrá.** 1999. Hydrolysis of muscle myofibrillar proteins by *Lactobacillus curvatus* and *Lactobacillus sakei*. Int. J. Food Microbiol. **53:**115–125.
27. **Sanz, Y., and F. Toldrá.** 2001. Purification and characterization of an X-prolyl-dipeptidyl peptidase from *Lactobacillus sakei.* Appl. Environ. Microbiol. **67:**1815–1820.
28. **Sanz, Y., F. C. Lanfermeijer, P. Renault, A. Bolotin, W. N. Konings, and B. Poolman.** 2001. Genetic and functional characterization of *dpp* genes encoding a dipeptide transport system in *Lactococcus lactis.* Arch. Microbiol. **175:**334–343.
29. **Söderling, E.** 1982. Effect of Cl^- on the function of the Cl-activated arginine aminopeptidase purified from human erythrocytes. Arch. Biochem. Biophys. **216:**105–115.
30. **Stentz, R., and M. Zagorec.** 1999. Ribose utilization in *Lactobacillus sakei*: analysis of the regulation of the *rbs* operon and putative involvement of a new transporter. J. Mol. Microbiol. Biotechnol. **1:**165–173.
31. **Verplaetse, A.** 1994. Influence of raw meat properties and processing technology on aroma quality of raw fermented meat products, p. 45–65. *In* Proceedings of the 40th International Congress on Meat Science and Technology. ICoMST Committee, The Hague, The Netherlands.