Purification and Characterization of Intracellular Aminopeptidase from *Pseudomonas fluorescens* ATCC 948

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ABSTRACT

Pseudomonas fluorescens ATCC 948 expressed cell-associated peptidase activity, which was shown by subcellular fractionation to be primarily in the cytoplasmic fraction. An aminopeptidase of broad specificity was purified 129-fold over the crude extract with an 11% recovery by ion-exchange chromatography, biogel filtration, and affinity chromatography. The enzyme had a monomeric structure and a molecular weight of about 50,000. The optimal activity occurred at pH 7.5 and 45°C. A heat treatment of 75°C for 1 min was necessary to inactivate the enzyme irreversibly. The aminopeptidase was strongly activated by Co^{2+}, completely inhibited by EDTA and 1,10-phenanthroline, and weakly inhibited by sulfhydryl-blocking agent, suggesting that the aminopeptidase likely is a metalloenzyme with a thiol group at its active site. The enzyme showed high activity with β-naphthylamide derivatives that had a hydrophobic AA (Leu, Ala, or Phe) or diaminomonocarboxylic acid (Lys or Arg) at the N terminus. The occurrence of only one aminopeptidase active on Leu, Lys, or Arg aminoacyl bonds was demonstrated by the competitive inhibition of Lys-β-naphthylamide and Arg-β-naphthylamide on the activity against Leu-*p*-nitroanilide. Kinetic studies conducted on Leu-β-naphthylamide showed a Michaelis-Menton constant of .3 m*M*, a maximum velocity of 10 nkat, and an energy of activation of 6200 cal/mol.
(**Key words**: *Pseudomonas fluorescens*, aminopeptidase, characterization, bitter peptides)

Abbreviation key: K_m = Michaelis-Menton constant, **KPB** = potassium-phosphate buffer, ***p*-NA** = *p*-nitroanilide, **β-NA** = β-naphthylamide.

INTRODUCTION

Extracellular proteinases of *Pseudomonas fluorescens* have been studied extensively because of their ability to form bitter peptides in dairy products (3, 21, 24). To date, only a few studies exist on the properties of cell-associated peptidases of *P. fluorescens*. Levy and Goldman (22) reported some characteristics of carboxypeptidases from certain pseudomonas, McKellar (25) studied the Leu aminopeptidase activity in whole cells, and Shamsuzzaman and McKellar (30) partially purified and characterized an endopeptidase and an aminopeptidase. Keogh and Pettingell (20) determined a correlation between cell-associated peptidases in raw milk and age gelation of milk after UHT treatment. In previous articles (14, 15), we established the peptidase profiles of *P. fluorescens* strains by the API ZYM enzyme (Analytab Products, Plainview, NY) system and further proposed a debittering action of cell-associated peptidase on bitter milk protein hydrolyzates.

In recent years, the peptide hydrolase systems of lactic acid bacteria used as starters (5, 26), cheese-related bacteria (11), and other microorganisms (27, 29) have been extensively characterized. Intracellular aminopeptidases, also known as α-aminoacylpeptide hydrolases

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(E.C.3.4.1.1), were purified and characterized from *salivarius* ssp. *thermophilus* (28), *Lactobacillus casei* ssp. *casei* (2), *Lactococcus lactis* ssp. *cremoris* (12), *Lactobacillus delbrueckii* ssp. *bulgaricus* (5), and *Lactobacillus helveticus* (26). The principal difference between bitter and nonbitter strains of lactic acid bacteria used as starters for cheese making seems to be the amount of intracellular aminopeptidase (8). In addition, aminopeptidase activity was fundamental for flavor (32) and absorbable peptide formation in yogurt production (1).

On the basis of these considerations, a better understanding of the aminopeptidase system in *P. fluorescens* is important physiologically and, especially, to elucidate its possible debittering role for use as an additive enzyme in dairy products. In the present study, the cellular localization and purification of the aminopeptidase system of *P. fluorescens* ATCC 948 were attempted to characterize the activity as a prerequisite for the future assays as a crude debittering enzyme preparation for use in dairy products.

MATERIALS AND METHODS

Reagents

Biogel P-300 was purchased from Bio-Rad Laboratories (Richmond, CA). The DEAE-Sephadex A-50 and AH-Sepharose 4B were from Pharmacia Fine Chemicals (Uppsala, Sweden); H-Leu-, H-Lys-, H-Pro-, H-Arg-, H-Phe-, H-Hys-, H-Ala, H-Met-, H-Gly-, H-Tyr-, H-Phe-, H-Asp-, H-Iso-, H-Trp-, Bz-DL-Arg-(BANA), H-Gly-Pro-, H-Phe-Pro-, H-Leu-Gly-, H-Ser-Tyr-, H-Gly-Gly-, H-Gly-Phe- and H-Ala-Pro-β-naphthylamides (β-NA) and H-Leu-, H-Lys-, and H-Arg-*p*-nitroanilides (*p*-NA) were from Bachem Feinchemikalien AG Products (Budendorf, Switzerland). The DNase used to digest nucleic acids in the crude extract was from Amersham Ltd. (Oakville, ON, Canada).

Culture Conditions and Preparation of Crude Enzyme Extract

Pseudomonas fluorescens ATCC 948 was maintained and propagated in nutrient broth. Cultures (100 ml in 500-ml Erlenmeyer flasks) were shaken at 160 rpm at 28°C until absorbance at 620 nm was 1.1 to 1.4 (stationary phase). Cells were harvested at 6000 × *g* for 15 min, washed three times with potassium-phosphate buffer (KPB; pH = 7.0, .05 *M*), and then were resuspended at 10% (vol/vol, wet weight) in the same buffer. The cell suspension was treated by an Ultrasonic Ltd. A 180 G Instrument (PBI International, Milan, Italy) at 20 kcps for 30 min. The crude enzyme extract was centrifuged (15 min, 10,000 × *g*) and filtered prior to use (pore size: .22 μm, Syrfil Filter; Nucleopore, Costar Corporation, Cambridge, MA).

Subcellular Fractionation

The subcellular distribution of aminopeptidase activity was determined by the modified procedure of Shamsuzzaman and McKellar (30). The cells from a 100-ml culture were resuspended in 25 ml of .05 *M* Tris buffer, pH = 8.0, containing 20% sucrose, 3 m*M* EDTA, and 10 mg/ml of lysozyme. After incubation at 37°C for 60 min and cooling on ice, the periplasmic fraction was obtained by centrifugation at 14,500 × *g* for 10 min. The spheroplasts, after being washed with Tris buffer containing 20% sucrose, were lysed by resuspension in 5 ml of Tris buffer containing 500 μg of DNase, .1 *M* NaCl (6), and 50 μ*M* $MgCl_2$. The mixture was incubated at 37°C for 30 min and then made up to 25 ml with Tris buffer. The cytoplasmic fraction was obtained by centrifugation at 27,000 × *g* for 10 min. The pellet was resuspended in 25 ml of buffer and centrifuged at 755 × *g* for 15 min to remove unbroken cells and to obtain the cloudy supernatant that represented the membrane fraction.

Enzyme Assays

Aminopeptidase activity at each purification step was measured by the method of Goldberg and Rutenburg (16). The microassay mixture contained 60 μl of KPB (.05 *M*, pH 7.0), 20 μl of substrate (1.66 m*M* in .05 *M* KPB), and 20 μl of enzyme solution. After incubation for 90 min at 37°C, the reaction was stopped with .1 *M* acetic acid. Using the molar coefficient of extinction 10.1 m*M*/cm for naphthylamine, one unit (nanokatal) of the peptidase activity was defined as the quantity of enzyme that hydrolyzed 1 nmol/s of substrate. Specific activity was defined as the units of the peptidase ac-

tivity per milligram of protein used in the assays. Aminopeptidase activity in the presence of *p*-NA substrates was determined by the method of El Soda and Desmazeaud (10).

The enzyme activities of the column eluates were determined by reading the absorbance at 560 nm in the method just described (16).

Protein Assay

The protein concentration in enzyme solutions was determined spectrophotometrically by the method of Lowry et al. (23) using BSA as standard. Protein concentrations of column eluates were estimated by reading absorbance at 280 nm.

Purification of Enzyme

Step 1: Ion-Exchange Chromatography. The crude enzyme extract was first applied to a DEAE-Sephadex column (1.6 × 40 cm), equilibrated with .05 *M* KPB, pH 7.0, containing .1 *M* NaCl. After washing the column with the same buffer (30 ml), proteins were eluted at a flow rate of 12 ml/h with a linear NaCl gradient, .1 and .5 *M*. The fractions with aminopeptidase activities were pooled separately, dialyzed 24 h at 4°C against .05 *M* KPB, pH 7.0, concentrated 10-fold by freeze-drying (Edwards MOD E1PTB; Edwards, Milan, Italy), and resuspended in the same buffer.

Step 2: Gel Filtration. The dialyzed and concentrated fractions from the previous step were applied to a Biogel P-300 column (2.6 × 70 cm), equilibrated with .1 *M* KPB, pH 7.0. Elution with the same buffer was at a flow rate of 6 ml/h, and 3-ml fractions were collected. Fractions with aminopeptidase activities were pooled and lyophilized to a 5-fold concentration.

Preparation of L-Leu-Gly and AH-Sepharose 4B. The AH-Sepharose 4B was washed with water (100 ml/ml), and 5 ml of gel was transferred to 10 ml of aqueous solution of L-Leu-Gly (200 mg/10 ml). The pH was adjusted to 4.5 with 1 *M* HCl, 5 ml of a solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (20 mg/ml) were added (4), and the pH was adjusted to 4.5 over 1 h. The reaction mixture was then mixed at room temperature (20°C) for 20 h in a shaker. The gel was washed with water (150 ml/ml of gel), suspended in .01 *M* KPB (pH = 7.0), and packed in a column (1 × 12 cm), which was then equilibrated with the same buffer.

Step 3: Affinity Chromatography. The pooled fraction from the Biogel P-300 column was applied to the affinity column. The column was washed with 50 ml of .01 *M* KPB, pH 7.0, followed by a linear NaCl gradient from .05 to 1.0 *M* to elute adsorbed protein. The flow rate was 8 ml/h, and 2.5-ml fractions were collected and pooled after measurement of activity. The purified aminopeptidase was dialyzed and concentrated 10-fold by freeze-drying.

The overall purification factor was calculated for each step by dividing the specific activity at that step by the initial specific activity. The overall percentage of activity recovered was calculated as the activity of the material recovered at that purification step divided by the total activity of the initial material.

Properties of Purified Enzyme

Molecular Weight. The molecular weight of the purified enzyme was estimated by gel filtration (mean of three determinations) on a Biogel P-300 column (100 × 1.6 cm). Catalase (240,000), phosphorylase B (94,000), BSA (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000) were used as molecular weight markers. The elution buffer was .05 *M* KPB, pH 7.0, containing .5 *M* NaCl.

The method of Weber and Osborn (34) was also used. The marker proteins used for the 12.5% SDS-PAGE were phosphorylase B, BSA, ovalbumin, and soybean trypsin inhibitor (20,000). The gels were stained with silver nitrate by the method of Henkeshoven and Dernick (18).

pH Optimum. The optimal pH range for activity of the purified enzyme was determined in .1 *M* K-phthalate (pH 4.5 to 5.5), .1 *M* KPB (pH 5.5 to 8.0), and .1 *M* Na-tetraborate (pH 8.0 to 9.5) buffers at 37°C. The appropriate buffer (70 μl) was mixed with the enzyme solution (25 μl), and the reaction was initiated by addition of 15 μl of 3.3 m*M* Leu-β-NA. The reaction was allowed to proceed for 90 min, and the released naphthylamine was determined as described previously (16).

Temperature Optimum. The effect of temperature from 5 to 55°C on the activity of the purified enzyme was determined in .05 *M*

KPB, pH 7.0, after 90 min of incubation. By means of the logarithm of the percentage of activity and by the reciprocal of temperature, an Arrhenius plot was used to determine the activation energy in the presence of the Leu- and Arg-β-NA substrates.

Heat Stability. To estimate the thermal stability of the purified enzyme, portions (300 μl) were treated at pH 7.0 in .05 *M* KPB at different temperatures (60 to 75°C) for 5 to 30 min in 3-ml glass tubes. At intervals, 20-μl samples were cooled, and the remaining activity was measured at 37°C in the same buffer with Leu-β-NA, Arg-β-NA, or Pro-β-NA (only for the 60 and 65°C temperatures), as previously described. The decimal reduction times (heating time for 90% inactivation) were calculated from plots of the logarithm of residual activity against heating time.

To ascertain the influence of Ca^{2+} on the thermal stability of the purified enzyme, 1.0 m*M* (final concentration) Ca^{2+} was added to the reaction mixture prior to the heat treatment.

Substrate Specificity. Substrate specificity of the purified enzyme was determined as described previously using H-Leu-, H-Lys-, H-Pro-, H-Arg-, H-Gly-, H-Tyr-, H-Phe-, H-Hys-, H-Ala-, H-Asp-, H-Iso-, H-Trp-, H-Met-, H-Gly-Pro-, H-Phe-Pro-, H-Leu-Gly-, H-Ser-Tyr-, H-Gly-Phe-, H-Ala-Pro, and H-Gly-Gly-β-NA. The activities on Leu- and Gly-Phe-β-NA were taken as a maximum of activity for aminoacyl and dipeptidyl substrates, respectively.

The competitive inhibition of Leu substrate-cleaving activity of the purified enzyme was determined at 37°C for 90 min in a reaction mixture containing 20 μl of Leu-*p*-NA (1.66 m*M*), 20 μl of enzyme solution, 60 μl of KPB (.05 *M*, pH 7.0), and .0825 to .99 m*M* concentrations of Arg- or Lys-β-NA.

Effect of Divalent Cations, Metal Chelators, and Inhibitors. A mixture containing 20 μl of the purified enzyme solution and 60 μl of divalent cations or inhibitors (final concentration 1.0 m*M*) in .05 *M* KPB, pH 7.0, was incubated for 30 min at 37°C. Reaction was initiated by addition of 20 μl of Leu-β-NA (1.66 m*M*), and the enzyme activity was assayed under standard conditions. Inhibition was expressed as a percentage of the activity without modifiers.

Reactivation of Enzyme Activity. The purified enzyme (50 μl), preincubated (30 min at 37°C) with EDTA or 1,10-phenantroline, was incubated again for 30 min at 25°C with 1.0 m*M* (final concentration) of various divalent cations prior to addition of Leu-β-NA, and the enzyme activity was determined.

Enzyme Kinetics. Enzyme solution was incubated with various concentrations of substrate (Leu-β-NA), yielding a range of final substrate concentration from .11 to 1.6 m*M*. The plot representation of Hanes (17) was used to calculate the Michaelis-Menton constant (K_m) and the maximum apparent velocity.

RESULTS

Subcellular Fractionation

The data in Table 1 show that the overwhelming majority of Leu-, Lys-, and Arg-aminopeptidase activities were located in the cytoplasmic fraction; activity was relatively

TABLE 1. Subcellular distribution of peptidase activities of *Pseudomonas fluorescens* ATCC 948.

	Activity[1]				
Cell fraction	BANA[2]	Leu-β-NA[3]	Lys-β-NA	Arg-β-NA	Pro-β-NA
Intact cells	100	100	100	100	100
Extracellular fluid	. . .	. . .	. . .	. . .	. . .
Periplasm	68.0	22.8	31.5	25.3	48.1
Membrane	. . .	.3	. . .	.5	. . .
Cytoplasm	282.3	82.5	77.5	87.4	21.1

[1]Expressed relative to intact cells as 100%.

[2]Bz-DL-Arg-β-naphthylamide.

[3]β-NA = β-Naphthylamide.

low in the periplasmic fraction. The Pro-aminopeptidase activity was highest in the periplasmic fraction. Aminopeptidase activities were not present in the membrane cell of *P. fluorescens* ATCC 948. As shown by Shamsuzzaman and McKellar (30), lysis of the cells noticeably increased endopeptidase activity, probably because of difficult entry of Bz-DL-Arg-β-NA into the intact cell.

Purification of the Enzyme

The specific peptidase activity observed with Leu-β-NA at each purification step is shown in Table 2. The enzyme was purified approximately 129-fold over the cell-free extract, and the activity yield was about 11%. The elution profile of the aminopeptidase by ion-exchange chromatography on DEAE Sephadex is shown in Figure 1A. After elution with the NaCl gradient, Leu-β-NA-hydrolyzing activities were highest from .35 to .38 *M* NaCl, but a lower NaCl concentration was necessary to elute the endopeptidase activity. The most active fractions, after concentration, were applied to a Biogel P-300 column (Figure 1B). The peak with aminopeptidase activity was located between two major protein peaks. Gel filtration increased the specific activity 44-fold compared with that of the cell-free extract and 1-fold with respect to the ion-exchange column. The elution profile of the pooled and concentrated material from the Biogel P-300 column onto a L-Leu-Gly and AH-Sepharose 4B is shown in Figure 1C. Using a NaCl gradient from .62 to .65 *M*, affinity chromatography gave a partially increased specific activity almost 3-fold compared with that for the previous step.

Properties of Purified Enzyme

Molecular Weight. The molecular weight of the aminopeptidase from *P. fluorescens* ATCC 948 was 49,000, as estimated from its elution volume from Biogel P-300. The SDS-PAGE showed a single protein band, which corresponded to a monomer of a molecular weight of 51,000.

pH Optimum. The optimal pH for aminopeptidase activity was determined with various buffers at pH 4.5 to 9.0 (Figure 2). The enzyme had an optimal pH at 7.5, and activity was relatively high between pH 5.0 and 8.5.

Temperature Optimum. At pH 7.0, with Leu-β-NA as substrate, the optimal temperature for the purified aminopeptidase was 45°C (Figure 3). The relative activity at 5°C was 23%, and about 75% of the optimal activity remained at 55°C. An Arrhenius plot was used to calculate an energy of activation of 6200 and 5400 cal/mol in the presence of Leu- and Arg-β-NA, respectively.

Optimal pH and temperature were the same when Arg-β-NA was used as substrate (data not shown).

Heat Stability. The purified aminopeptidase was relatively stable at 65°C for 30 min (Figure 4). At 70°C, the decimal reduction time of the enzyme activity was calculated to be 23 min. Activity was undetectable in samples heated at 75°C for 1 min. The presence of 1.0 m*M* Ca^{2+} in the reaction mixture did not modify the heat stability of the purified enzyme. The heat stability in the presence of Arg-β-NA did not differ from the results in Figure 4 (data not shown).

Substrate Specificity. Hydrolytic activity of the purified enzyme toward various aminoacyl

TABLE 2. Purification of aminopeptidase from *Pseudomonas fluorescens* ATCC 948.

Purification step	Total protein	Total activity	Specific activity	Yield of activity	Purification
	(mg)	(unit)[1]	(unit/mg)	(%)	(-fold)
Cell-free extract	1324	102.5	.077	100	1
DEAE Sephadex[2]	27	68	2.518	66	33
Biogel P-300[3]	12.2	41.3	3.385	40	44
L-Leucyl-glycine and AH-sepharose 4B[1]	1.2	11.9	9.916	11	129

[1]Quantity of enzyme that hydrolyzed 1 mmol/s of Leu-β-naphthylamide.

[2]Pharmacia Fine Chemicals (Uppsala, Sweden).

[3]Bio-Rad Laboratories (Richmond, CA).

and dipeptidyl derivatives is summarized in Table 3. The rate of Leu-β-NA hydrolysis was taken as 100. Among the aminoacyl-β-NA, those containing hydrophobic AA (Leu, Ala, or Phe) or diaminomonocarboxylic acids (Lys or Arg) were significantly hydrolyzed. The relative activity for Pro-β-NA was 9%. The dipeptidyl derivatives were poor substrates for the aminopeptidase; activities were less than 10% of the maximum found with aminoacyl substrates. Nevertheless, hydrolysis was higher in the presence of Phe at the N terminus.

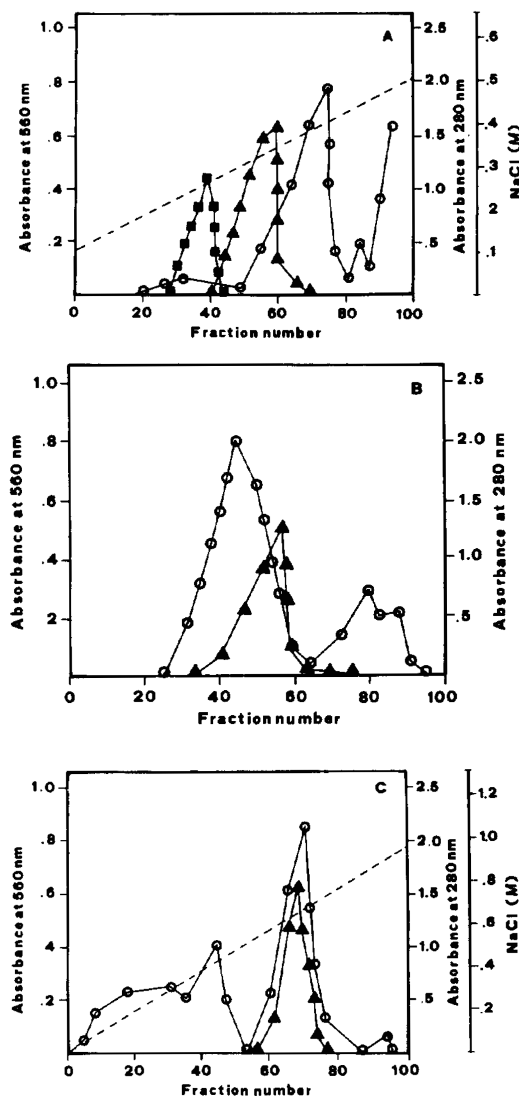

Figure 1. Purification of the aminopeptidase by ion-exchange chromatography on DEAE Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) (A), gel filtration on Biogel P-300 (Bio-Rad, Richmond, CA) (B), and affinity chromatography on leucyl-glycine-AH and Sepharose 4B (Pharmacia) (C). Endopeptidase activity (■), aminopeptidase activity (▲), protein absorbance at 280 nm (O), and NaCl concentration in the buffer (---).

Competitive inhibition of the cleaving activity of Leu-aminoacyl was demonstrated by β-NA substrate in the presence of *p*-NA compounds (Figure 5). Inhibition of the activity on Leu-*p*-NA was nearly complete in the presence of Lys- or Arg-β-NA at concentrations of .3 and .88 m*M*, respectively.

Effect of Divalent Cations, Metal Chelators, and Inhibitors. The effect of AA modifying agents on enzyme activity was used to identify the active site. The influence of various divalent ions is shown in Table 4. The enzyme was strongly activated by Co^{2+}, but other metal ions caused slight to marked inhibition of the activity. Although Hg^{2+}, Cd^{2+}, Cu^{2+}, and Ag^{2+} strongly inhibited the enzyme, its activity was not significantly affected by Ca^{2+}, Zn^{2+}, Mg^{2+}, or Ni^{2+}. The effects of various other reagents on enzyme activity are shown in Figure 6. The enzyme was almost completely inhibited by EDTA and 1,10-phenantroline at 1.0 m*M*; slightly activated by a sulfhydryl group protective reagent such as Cys, mercaptoethanol, and dithiothreitol; and slightly inhibited by a sulfhydryl-modifying compound (*p*-chloromercuribenzoic acid). Both EDTA and 1,10-phenantroline irreversibly inhibited the aminopeptidase because none of the cations restored any enzymatic activity.

Enzyme Kinetics. The relationships between hydrolytic velocity and substrate concentration were calculated for Leu- and Arg-β-NA (Figure 7). Using a reciprocal plot of Hanes, the affinity was expressed by a K_m of .3 and .42 m*M* and by a maximum velocity of 10 and 8.4 nkat for Leu- and Arg-β-NA, respectively. With both substrates, inhibition occurred at concentrations of 1.2 m*M*.

DISCUSSION

Proteinases of *Pseudomonas* spp. play an important role in the specific formation of bitter peptides in dairy products (3, 21, 24). However, only Shamsuzzaman and McKellar (30) have partially purified and characterized cell-associated peptidase in *P. fluorescens*. We previously analyzed the cell-associated pepti-

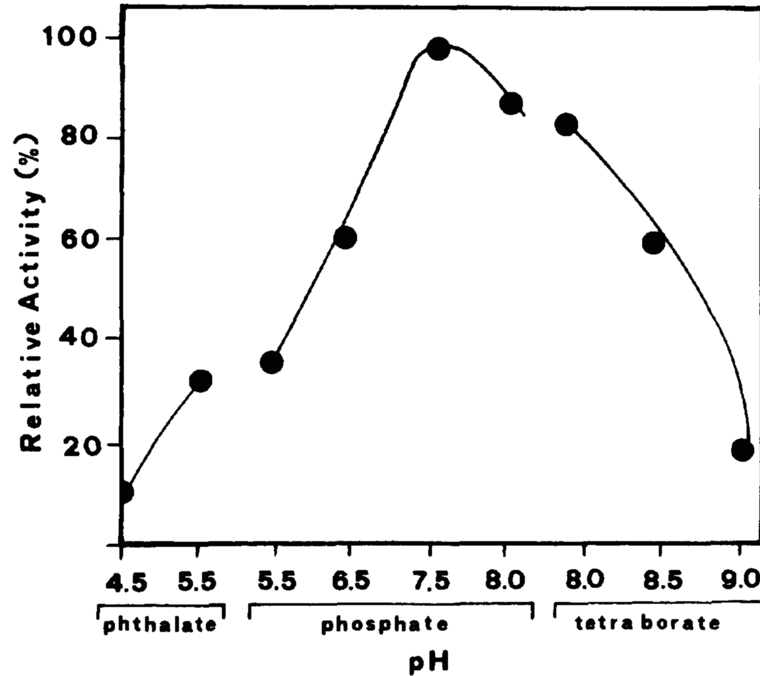

Figure 2. Effect of pH on the activity of purified aminopeptidase.

dase profile of *P. fluorescens* ATCC 948 (15) and investigated the peptidase activity of whole cells on hydrolyzed milk proteins (14), suggesting a debittering activity. In this investigation, we purified and characterized the cell-associated aminopeptidase from *P. fluorescens* ATCC 948.

Subcellular fractionation of *P. fluorescens* ATCC 948 spheroplasts revealed the presence of the highest endopeptidase, Leu-, Lys-, and Arg-β-NA activities in the cytoplasmic fraction; the Pro-β-NA activity was located predominantly in the periplasmic fraction. Thus, these activities appear to be intracellular.

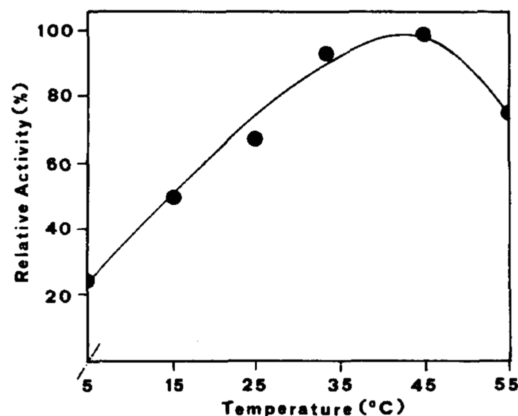

Figure 3. Effect of temperature on the activity of purified aminopeptidase.

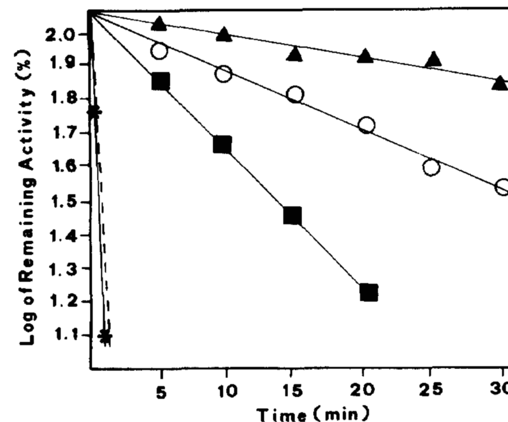

Figure 4. Heat stability of purified aminopeptidase at 60°C (▲), 65°C (○), 70°C (■), and 75°C (∗); heat treatment at 75°C in presence of 1 m*M* Ca^{2+} (—).

TABLE 3. Relative activities of the purified aminopeptidase on various substrates.

Substrate	Relative activity
	(%)
Aminopeptidases	
Leu-β-NA[1]	100
Arg-β-NA	115
Pro-β-NA	9
Lys-β-NA	85
Gly-β-NA	20
Tyr-β-NA	53
Phe-β-NA	68
Hys-β-NA	5
Ala-β-NA	62
Asp-β-NA	1
Iso-β-NA	1
Trp-β-NA	1
Met-β-NA	8
Dipeptidyl aminopeptidases	
Gly-Phe-β-NA	100
Gly-Pro-β-NA	85
Phe-Pro-β-NA	90
Ser-Tyr-β-NA	9
Leu-Gly-β-NA	15
Ala-Pro-β-NA	70
Gly-Gly-β-NA	11

[1]β-NA = β-Naphthylamide.

TABLE 4. Effect of divalent cations on aminopeptidase activity.

Ion	Relative activity
	(%)
Blank[1]	100
Mg^{2+}	92
Ca^{2+}	87
Fe^{2+}	54
Zn^{2+}	77
Cu^{2+}	8
Ag^{2+}	15
Cd^{2+}	28
Ni^{2+}	88
Hg^{2+}	2
Co^{2+}	164

[1]Blank value denotes enzyme activity without effector.

By ion-exchange chromatography, gel filtration, and affinity chromatography, an aminopeptidase active on Leu-β-NA was purified 129-fold. Enzymes with molecular weights of 117,000 and 130,000 (trimer) in *Streptococcus cremoris* (now named *Lactococcus lactis* ssp. *cremoris*) (6, 13), 78,000 to 81.000 (monomer) in *Lactobacillus lactis* (7), 62,000 in *Streptococcus salivarius* ssp. *thermophilus* (28) and 95,000 in *Lb. delbrueckii* ssp. *bulgaricus* (5) have been reported. *Pseudomonas fluorescens* 32A aminopeptidase had a molecular weight of 44,000 (30). The aminopeptidase of this study was a monomer of molecular weight about 50,000.

The *P. fluorescens* ATCC 948 aminopeptidase was active over a broad pH range, from pH 5.5 to 8.5, maintaining about 30% of maxi-

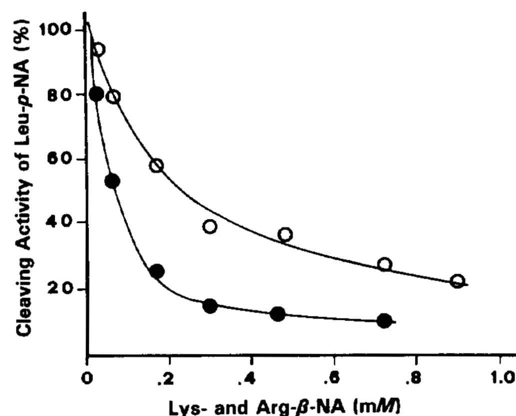

Figure 5. Competitive inhibition of initial cleaving activity of L-Leu-*p*-nitroanilide (%) of purified aminopeptidase by various concentrations of Lys- (●) and Arg-β-NA (○). NA = Nitroanilide.

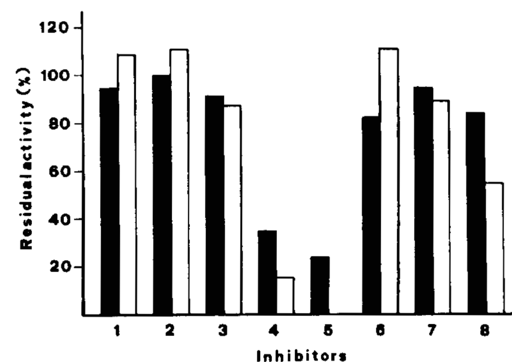

Figure 6. Effect of inhibitors at 1.0 (□) and .1 (■) m*M* on aminopeptidase activity: 1) mercaptoethanol, 2) cysteine, 3) hydroxyquinoline, 4) EDTA, 5) 1,10-phenantroline, 6) dithiothreitol, 7) iodoacetate, and 8) *p*-chloromercuribenzoate.

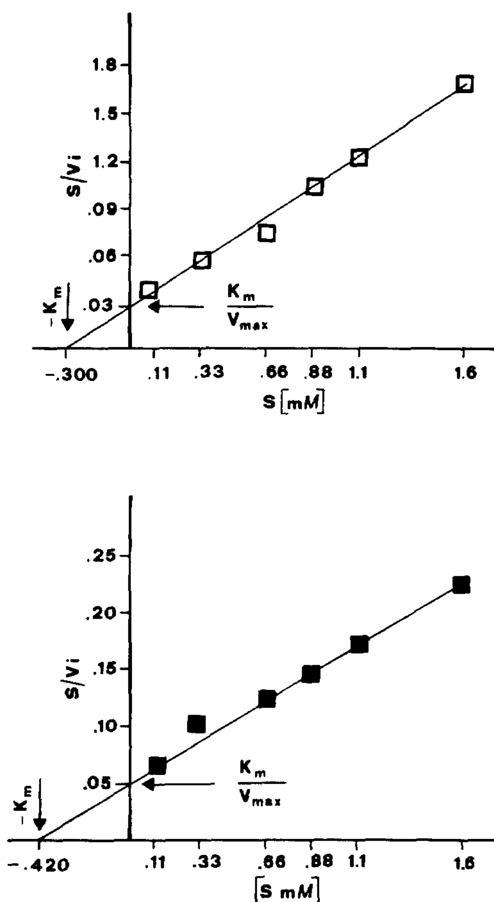

Figure 7. Hanes plot of activity of purified aminopeptidase with Leu-β-naphthylamide (□) and Arg-β-naphthylamide (■), as substrates. K_m = Michaelis-Menton constant, V_{max} = maximum velocity, S/Vi = substrate/initial velocity, and S = substrate.

mum activity at pH 5.0. This 7.5 optimum was similar to that of the aminopeptidase of *Lb. helveticus* LEE-511 (26) and *Lb. casei* ssp. *casei* LGG (2) and is characteristic for lactic acid bacteria with debittering properties (8).

The optimal temperature for activity of our aminopeptidase on Leu-β-NA was 45°C, and 70% of the initial activity remained after a heat treatment of 70°C for 10 min. Because the logarithm of Leu-β-NA activity decreased linearly with inactivation time, without a change in the slope (monophasic kinetics) for all the temperatures assessed, a single enzyme was probably responsible for the hydrolysis of substrate (12). The same inactivation kinetics was determined for the 60 and 65°C temperatures in presence of Pro-β-NA.

The activity of the purified aminopeptidase was strongly activated by Co^{2+} and irreversibly inhibited by chelating reagents such as EDTA and 1,10-phenantroline. These properties indicate that this aminopeptidase is a metalloenzyme that is comparable with those isolated from several lactic acid bacteria (2, 5, 12, 26) and different from the aminopeptidase of *P. fluorescens* 32A characterized by Shamsuzzaman and McKellar (30). Stimulation of the enzyme activity by Co^{2+} ions suggested that some heavy metal ions may have a certain affinity for the structure of the active center of the enzyme. The presence of these ions in dairy products (19) may contribute to the stability of this enzyme (2). The purified aminopeptidase was negatively affected by 1.0 *M* concentration of sulfhydryl-modifying reagent and positively stimulated by the presence of Cys. These findings suggest a possible involvement of a functional sulfhydryl group near or at its active site.

Because the purified enzyme hydrolyzed a wide range of the aminoacyl derivatives tested and exhibited an aminopeptidase specificity profile similar to that detected with whole cells (15), it could account for almost all of the aminopeptidase activity detected with whole cells. This enzyme, according to the definition used for similar activities in the lactic acid bacteria (2, 8), could be defined as a general aminopeptidase. In particular, activity was highest on Leu-, Lys-, and Arg-β-NA substrates. The low activity on Pro-β-NA excluded the presence of iminopeptidase activity in the purified enzyme (2, 31). However, because hyrolysis of Pro-β-NA was detected by using the periplasmic fraction or the whole cells (14), *P. fluorescens* ATCC 948 may also possess a distinct iminopeptidase enzyme (EC 3.4.11.5). The poor activity on dipeptidyl substrate and the high molecular weight determined for the dipeptidyl aminopeptidase (EC 3.4.14) (6) excluded the possibility that the purified aminopeptidase might have contained this enzyme. The occurrence of only a single intracellular aminopeptidase exhibiting cleaving activities was indicated by the competitive action of β-NA derivatives of Lys and Arg against Leu-*p*-NA and substantiated by the

results for heat stability. Because the activity of the aminopeptidase of *P. fluorescens* ATCC 948 was highest when hydrophobic AA (Leu, Ala, or Phe) or diaminomonocarboxylic acid (Lys or Arg) occupied the N terminus of the peptides, the previous results obtained on hydrolyzed milk protein (14) can be confirmed, and the properties of this aminopeptidase can be shown to be similar to those of several aminopeptidases of debittering or nonbitter lactic acid bacteria used as starters (8, 9, 11). A general aminopeptidase with a strong affinity for the same two AA (Arg and Leu) with quite different characteristics was also present in *Mycoplasma salivarium* (31).

The K_m values presented in this paper cannot be directly compared with values reported by other authors because various aminoacyl substrates were used. However, the affinity of *P. fluorescens* ATCC 948 aminopeptidase for Leu- and Arg-β-NA was in the range of values found in other experiments: K_m of .3 m*M* for Leu-β-NA and of .42 m*M* for Arg-β-NA were estimated in the present study, but K_m of .22 and .12 m*M* for Leu-*p*-NA (2, 26), .55 and .03 m*M* for Lys-*p*-NA (5, 33), and .18 m*M* for Ala-β-NA (35) were reported for the lactic acid bacteria and *Escherichia coli* strains, respectively.

CONCLUSIONS

The results obtained with synthetic peptides as substrate showed several properties of the purified aminopeptidase. The broad specificity, the high activity on peptides with hydrophobic AA at the N-terminus, and the relative stability to heating and to acidic conditions suggest that the general aminopeptidase from *P. fluorescens* ATCC 948 may be an important debittering enzyme for using as additive in various dairy products, as previously supposed (14). Because of the ability of *Pseudomonas* spp. proteinases to generate bitter peptides and because of the ability of the peptidases of the same microorganisms to cleave the peptides produced by its own proteinases, the aminopeptidase of *P. fluorescens* ATCC 948 may have a particular aptitude to remove the bitter flavor in milk products with a high level of contamination by *Pseudomonas* ssp.

More work on the specificity of the *P. fluorescens* ATCC 948 aminopeptidase and on its action on bitter peptides isolated from casein is needed to confirm this hypothesis.

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