Applied and Environmental Microbiology, Feb. 1990, p. 381–388
0099-2240/90/020381-08$02.00/0
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Vol. 56, No. 2

Purification and Partial Characterization of a Prolyl-Dipeptidyl Aminopeptidase from *Lactobacillus helveticus* CNRZ 32

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Received 12 July 1989/Accepted 3 November 1989

X-prolyl-dipeptidyl aminopeptidase, which hydrolyzed Gly-Pro-*p*-nitroanilide (relative activity [RA] = 100%) and Arg-Pro-*p*-nitroanilide (RA, 130%), was purified to homogeneity from the cell extract of *Lactobacillus helveticus* CNRZ 32. The enzyme also hydrolyzed Ala-Pro-Gly (RA, 11%) and Ala-Ala-*p*-nitroanilide (RA, 2%) but was not active on Ala-Leu-Ala, dipeptides, and endopeptidase and carboxypeptidase substrates. The enzyme was purified 145-fold by streptomycin sulfate precipitation, ammonium sulfate fractionation, and a series of column chromatographies on DEAE-cellulose, arginine-Sepharose 4B, and glycyl-prolyl-AH-Sepharose 4B. The purified enzyme appeared as a single band on native polyacrylamide gel and sodium dodecyl sulfate-polyacrylamide gel electrophoreses and had a molecular weight of 72,000. Optima for activity by the purified enzyme were pH 7.0 and 40°C. The enzyme was incubated at 40°C for 15 min with various metal ions. It was activated by Mg^{2+} (2.5 mM), Ca^{2+} (0.1 to 2.5 mM), Na^{+} (10 to 50 mM), and K^{+} (10 to 50 mM) and was inhibited by Hg^{2+} (0.1 to 2.5 mM), Cu^{2+} (0.1 to 2.5 mM), and Zn^{2+} (0.1 to 2.5 mM). Enzyme activity was partially inhibited by EDTA (1.0 mM, 20 h at 40°C), 1,10-phenanthroline (1.0 mM, 15 min at 40°C), phenylmethylsulfonyl fluoride (1.0 mM), *N*-ethylmaleimide (1.0 mM), and iodoacetate (1.0 mM). It was completely inhibited by diisopropyl fluorophosphate (1.0 mM, 2 h at 40°C) and *p*-chloromercuribenzoate (1.0 mM, 15 min at 40°C). The enzyme was not affected by dithioerythritol (1.0 to 10 mM).

X-prolyl-dipeptidyl aminopeptidase, also known as post-proline dipeptidyl aminopeptidase IV, dipeptidyl aminopeptidase IV, dipeptidyl peptidase IV, or glycylprolyl-β-naphthylamidase, EC 3.4.14.5 (26), from mammalian sources has been purified and extensively studied (11, 17, 18, 33, 39) but less so when obtained from microorganisms such as *Flavobacterium meningosepticum* (40), *Streptococcus thermophilus* (28), *Streptococcus mitis* (12), *Lactobacillus lactis* (28), and *Saccharomyces cerevisiae* (1). The enzyme releases N-terminal dipeptides sequentially from polypeptides with unsubstituted N termini of the types X-Pro-Y- or X-Ala-Y- provided that the penultimate residue is proline or alanine (X and Y can be any amino acid provided Y is not Pro or Hyp). The enzyme hydrolyzes the peptide bond at the carboxyl side of the proline or alanine residue, producing X-Pro or X-Ala and Y-. The rate of hydrolysis is greater when proline is the penultimate residue, resulting in the X-Pro-Y- peptide having higher susceptibility to the enzyme than the X-Ala-Y- peptide (4, 16, 17).

The physiological purpose of X-prolyl-dipeptidyl aminopeptidase in lactobacilli may be to participate with aminopeptidase, dipeptidase, and proteinase as mediators in releasing essential amino acids from milk proteins which are necessary for maximum cell growth. The proteolytic enzyme systems complement each other in splitting most, if not all, types of peptide bonds. *Lactobacillus helveticus* is a fastidious organism which requires preformed amino acids in its growth medium (32). Concentrations of free amino acids in milk are not sufficient to support bacterial growth to high cell densities or rapid acid production, both of which are necessary to manufacture fermented milk products such as cheese (37, 38).

Bovine milk protein consists of about 80% caseins composed of α_{s1}, α_{s2}, β-, and κ-caseins in a ratio of 4:1:3:1, respectively (36). A high proportion of caseins consists of proline residues, with α_{s1}, α_{s2}, β-, and κ-caseins containing up to 17, 10, 35, and 20% proline, respectively. β-Lactoglobulin and α-lactalbumin, however, contain 8 and 2% proline, respectively. Prolyl-dipeptidyl aminopeptidase may facilitate digestion of proline-rich protein such as casein, thus liberating essential amino acids from peptide chains. Heymann and Mentlein (15) showed that release of proline from bovine β-casein increased in the presence of dipeptidyl peptidase IV, resulting in an increase in the overall yield of free amino acids. In the absence of dipeptidyl peptidase IV, degradation of β-casein by aminopeptidase would stop at peptide bonds involving the amino group of proline, thus preventing further hydrolysis of β-casein by aminopeptidase.

Ripening of cheese, a casein-rich substrate, is a complex process which depends on enzymes of bacteria functioning in the proper sequence. Lactobacilli occurring in cheese as adventitious bacteria contribute to the ripening process. Controlled and accelerated ripening of cheese remains a sought-after goal. An understanding of the enzymes produced by lactobacilli will provide valuable information and may bring us closer to this goal. *L. helveticus* CNRZ 32 was chosen for this study because it has been used successfully to hasten the ripening of cheese (2, 3). This communication reports the presence of prolyl-dipeptidyl aminopeptidase and describes the purification and partial characterization of the enzyme from the cell extract of *L. helveticus* CNRZ 32.

MATERIALS AND METHODS

Chemicals. DEAE-cellulose (DE52) was obtained from Whatman, Inc. (Hillsboro, Oreg.). Arginine-Sepharose 4B and AH-Sepharose 4B were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, N.J.). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). All peptide derivatives and molecular weight markers were obtained from Sigma Chemical Co. (St. Louis, Mo.). Aquacide I

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(sodium salt of carboxymethyl cellulose) was obtained from Calbiochem-Behring (La Jolla, Calif.). All other chemicals used were of analytical grade.

Bacterial strains and growth conditions. *L. helveticus* CNRZ 32 was supplied by the Center for Dairy Research, University of Wisconsin, Madison. *L. helveticus* ATCC 10797 and *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 12278 were obtained from the American Type Culture Collection (Rockville, Md.). The organisms were grown in 3 liters of MRS broth (Difco Laboratories, Detroit, Mich.) (8) in a fermentor (Norman D. Erway Glassblowing Laboratory, Oregon, Wis.) at 37°C for 10 h (*L. helveticus* CNRZ 32 and ATCC 10797) or 16 h (*L. delbrueckii* subsp. *bulgaricus* ATCC 12278). The pH was allowed to decrease by lactic acid fermentation to 5.85 where it was maintained by the addition of a neutralizer containing 20% Na_2CO_3 in 20% NH_4OH (30) with a pH controller (type 45 AR; Chemtrix Inc., Hillsboro, Oreg.). The culture was stirred with a T-line stirrer (G. K. Hellar, Floral Park, N.Y.) at 150 rpm (constant speed controller; Cole Parmer Instrument Co., Chicago, Ill.).

Preparation of cell extracts. Cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and washed three times with cold saline (0.85%) solution. The cells were disrupted by grinding with alumina (Sigma) at 4°C for 20 min and suspended in 0.01 M Tris hydrochloride buffer (pH 7.5) in 1/10th the original volume. The cell debris and alumina were removed by centrifugation twice at 10,000 × *g* for 20 min.

Enzyme assays. Prolyl-dipeptidyl aminopeptidase was assayed by the method of El Soda and Desmazeaud (9) with arginine-proline-*p*-nitroanilide and glycylproline-*p*-nitroanilide (6.4 mM) as the substrates. The incubation mixture contained 0.05 ml of substrate, 2.85 ml of 0.01 M phosphate buffer (pH 7.0), and 0.1 ml of an appropriately diluted enzyme. Incubation was at 37°C for 20 min. The reaction was stopped by the addition of 0.5 ml of 30% acetic acid, and the extent of hydrolysis was measured at A_{410}. Amino/iminopeptidase activity was measured by the procedure of El Soda and Desmazeaud (9) with L-arginine-*p*-nitroanilide and L-proline-*p*-nitroanilide (16.4 mM) as the substrates, respectively. One unit of aminopeptidase activity was defined as the amount of enzyme which produced a variation of 0.1 unit of A_{410} per min at 37°C (10). Prolyl-dipeptidyl aminopeptidase activity was calculated in the same manner as aminopeptidase activity.

Substrate specificity. Hydrolyses of *p*-nitroanilide derivatives of amino acids and peptides were observed by the standard enzyme assay procedure described above. Enzyme activities toward dipeptides (Leu-Pro, Met-Gly, Tyr-Gly, and Gly-Phe) or peptides with blocked α-amino acids such as *N*-benzyloxycarbonyl-glycyl-L-proline (N-CBZ-Gly-Pro), N-CBZ-Ala-Pro-Tyr, and N-CBZ-Ala-Pro-Leu were assayed in a reaction mixture containing 1.8 ml of substrate (0.5 mM in 0.01 M phosphate buffer, pH 7.0) and 0.2 ml of purified enzyme incubated at 40°C, and the amount of α-amino acids or peptides released was determined by the ninhydrin method as described by Moore and Stein (31).

Protein determination. Protein contents were determined by the method of Lowry et al. (25) with bovine serum albumin as the standard. Elution profiles of the proteins separated by DEAE-cellulose chromatography and arginine-Sepharose 4B and glycyl-prolyl-AH-Sepharose 4B affinity chromatographies were measured at A_{280}.

Gel electrophoresis and histochemical staining to detect prolyl-dipeptidyl aminopeptidase and aminopeptidase activities. Polyacrylamide gel electrophoresis (PAGE) was done by the procedure of Davis (7) with 7% gel and 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.5. Prolyl-dipeptidyl aminopeptidase and aminopeptidase activities were detected on the gels after electrophoresis by the method described by Miller and MacKinnon (29) with slight modifications. The gel was incubated in 10 ml of 0.05 M HEPES buffer (pH 7.0) containing 20 mg of fast garnet GBC salt, 10 μl of cobalt chloride (0.05 mM), and 100 μl of substrate dissolved in *N*,*N*-dimethylformamide (10 mg/ml). The substrates for prolyl-dipeptidyl aminopeptidase were lysylproline 4-methoxy-β-naphthylamide and glycylproline 4-methoxy-β-naphthylamide, whereas the substrates for amino/iminopeptidase were L-leucine-β-naphthylamide and L-proline-β-naphthylamide, respectively. Both enzyme activities were detected on the gels as orange bands.

Purification of prolyl-dipeptidyl aminopeptidase from *L. helveticus* CNRZ 32. All steps were done at 4°C. The cell extract was prepared as described above.

(i) Streptomycin sulfate precipitation. Streptomycin sulfate was added to the cell extract to a final concentration of 0.22 mg/ml. This was calculated based on 1 ml of a 10% solution of streptomycin sulfate per A_{260} of 1,500 for the crude extract (34). The resulting suspension was stirred for 10 min. After 2 h, the precipitated nucleic acids were removed by centrifugation at 10,000 × *g* for 20 min.

(ii) Ammonium sulfate fractionation. The streptomycin supernatant fraction from the previous step was fractionated by salting out with solid ammonium sulfate to 40 to 75% saturation. The precipitate formed was collected by centrifugation at 10,000 × *g* for 20 min, dissolved in a minimum amount of 0.01 M Tris hydrochloride buffer (pH 7.5), and then dialyzed for 24 h against 0.01 M Tris hydrochloride buffer containing 10 mM NaCl (pH 7.5) at 4°C. The dialyzed fraction was analyzed for protein concentration and for prolyl-dipeptidyl aminopeptidase activity by measuring the release of *p*-nitroaniline at A_{410} from glycylproline-*p*-nitroanilide.

(iii) DEAE-cellulose chromatography. The DEAE-cellulose column (1.0 by 20 cm) was equilibrated with 0.01 M Tris hydrochloride buffer containing 10 mM NaCl, pH 7.5. The dialyzed fraction containing about 960 mg of protein from the ammonium sulfate fraction was applied to the column at a flow rate of 16 ml/h. The column was washed with the equilibration buffer, and the enzyme was eluted with a linear gradient of 0.01 to 0.5 M NaCl in the same buffer. Fractions of 4.2 ml each were collected and tested for protein concentration by measuring the A_{280} and for prolyl-dipeptidyl aminopeptidase activity by measuring the A_{410}. Fractions with the highest enzyme activities (numbers 58 to 60) were pooled and dialyzed overnight against 0.01 M Tris hydrochloride buffer (pH 7.5) at 4°C and then concentrated to about 6 ml by immersing the dialysis tubing in Aquacide I for 45 min.

(iv) Arginine-Sepharose 4B affinity chromatography. The concentrated enzyme fraction obtained from the previous step was applied to an arginine-Sepharose 4B column (1.6 by 8 cm) previously equilibrated with 0.01 M Tris hydrochloride buffer, pH 7.5. The column was washed with the same buffer, the enzyme was eluted with a linear gradient of NaCl (0 to 0.5 M) in the same buffer at a flow rate of 12 ml/h, and 5-ml fractions were collected. The enzyme-active fractions (62 to 67) were pooled and dialyzed against 0.01 M Tris hydrochloride (pH 7.5) at 4°C and then concentrated in Aquacide I.

(v) Preparation of glycyl-prolyl-AH-Sepharose 4B affinity column. Carbodiimide coupling of glycyl-proline to AH-

TABLE 1. Specific activity and relative mobility of prolyl-dipeptidyl aminopeptidase (DAP), aminopeptidase, and proline iminopeptidase of *L. helveticus* CNRZ 32, *L. helveticus* ATCC 10797, and *L. delbrueckii* subsp. *bulgaricus* ATCC 12278

Substrate	*L. helveticus* CNRZ 32				*L. helveticus* ATCC 10797			*L. delbrueckii* subsp. *bulgaricus* ATCC 12278		
	Sp act[a]	R_f[b]			Sp act[a]	R_f[b]		Sp act[a]	R_f[b]	
		DAP	AP1	AP2		DAP	AP1		DAP	AP1
Arg-pro-*p*-NA[c]	1.24				0.67			1.38		
Gly-pro-*p*-NA	0.76				0.46			0.81		
Leu-*p*-NA	7.00				5.50			3.00		
Pro-*p*-NA	0.17				0.20			0.17		
Lys-pro-β-NA[d]		0.17				0.17			0.17	
Gly-pro-β-NA		0.17				0.17			0.17	
Leu-β-NA			0.55	0.84			0.44			0.34
Pro-β-NA			0.55				0.44			0.34

[a] Units of activity per minute per milligram of protein at 37°C.
[b] Ratio of the distance traveled by the band of activity to that of the tracking dye.
[c] *p*-NA, *p*-Nitroanilide.
[d] β-NA, β-Naphthylamide.

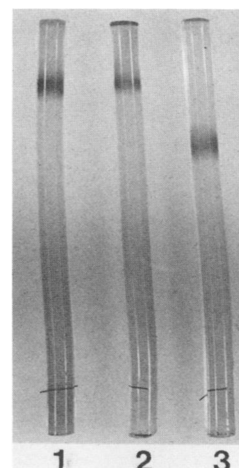

FIG. 1. Peptidase-active bands detected on polyacrylamide gels after electrophoresis of cell extract of *L. helveticus* CNRZ 32. Lanes: 1, prolyl-dipeptidyl aminopeptidase detected with lys-pro-β-naphthylamide; 2, prolyl-dipeptidyl aminopeptidase detected with gly-pro-β-naphthylamide; 3, aminopeptidase (AP1) detected with pro-β-naphthylamide.

Sepharose 4B was based on procedures previously described (13, 28; Pharmacia LKB Biotechnology, *Affinity Chromatography—Principles and Methods*). AH-Sepharose 4B (4 g) was allowed to swell in 0.5 M NaCl at room temperature for 20 min. The gel was then washed with 800 ml of 0.5 M NaCl on a sintered glass filter to remove additives followed by washing with double-distilled water (adjusted to pH 4.5) until the chloride ion was no longer detected in washings. The gel was then suspended in 20 ml of water (pH 4.5). The ligand solution was prepared by dissolving 87 mg of glycyl-proline in 1 ml of double-distilled water (pH 4.5) and then added to the gel. The mixture was stirred gently, and the pH was immediately adjusted to between 4.5 and 6.0. Solid 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.77 g) was added to the gel-ligand mixture to a final concentration of 0.1 M. The pH was maintained between 4.5 and 6.0 for 1 h by the addition of dilute HCl. The volume was adjusted to 40 ml, and the suspension was gently mixed in a shaker for 24 h at room temperature. The gel was thoroughly washed six times alternately with an acidic buffer (0.1 M sodium acetate, pH 4.0) containing 0.5 M NaCl and a basic buffer (0.1 M carbonate-bicarbonate, pH 8.3) containing 0.5 M NaCl. This was followed by washing with distilled water until the chloride ion was absent from the eluent. The gel was then packed into a column (1.6 by 8 cm) and equilibrated with 0.01 M Tris hydrochloride buffer, pH 7.5.

(vi) Glycyl-prolyl-AH-Sepharose 4B affinity chromatography. The concentrated enzyme from the previous step was further purified on a glycyl-prolyl-AH-Sepharose 4B column (1.6 by 8 cm) equilibrated with 0.01 M Tris hydrochloride buffer, pH 7.5. The column was first washed with the same buffer and then eluted with a linear gradient of NaCl (0 to 0.5 M) in the same buffer at a flow rate of 10 ml/h, and 5-ml fractions were collected. Active fractions (55 to 57) were used to characterize the enzyme.

(vii) PAGE. The purity of the enzyme preparation at each purification step was examined by sodium dodecyl sulfate (SDS)-PAGE with the Laemmli buffer system (22, 23), a 4% stacking gel, and a 7.5% running gel. The protein samples were mixed 1:1 with sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue), boiled for 4 min, and applied to the gels. Proteins in the gels were stained with Coomassie brilliant blue R.

Native PAGE was done by the procedure of Davis (7) with a 7% running gel and 0.1 M HEPES buffer, pH 7.5. Matched pairs of gels were prepared. One of the gels was stained for protein with Coomassie brilliant blue R. The other was used to detect prolyl-dipeptidyl aminopeptidase activity with lysylproline 4-methoxy-β-naphthylamide as a substrate by the procedure of Miller and MacKinnon (29) as described previously.

Determination of molecular weight. The molecular weight of the purified enzyme was estimated by SDS-PAGE by the method of Laemmli (22, 23) with a 4% stacking gel and a 7.5% running gel. Carbonic anhydrase (29.0 kilodaltons [kDa]), ovalbumin (45.0 kDa), bovine serum albumin (66.0 kDa), phosphorylase *b* (97.4 kDa), β-galactosidase (116.0 kDa), and myosin (205.0 kDa) were used as molecular size markers.

Effect of metal ions on enzyme activity. The effect of metal ions on prolyl-dipeptidyl aminopeptidase activity was determined by using purified enzyme which had been dialyzed against 0.01 M Tris hydrochloride buffer (pH 7.5) for 24 h at 4°C. The dialyzed enzyme was incubated in the presence or absence of various metal ions ($CaCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$,

TABLE 2. Purification of prolyl-dipeptidyl aminopeptidase from *L. helveticus* CNRZ 32

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	285	3,172	2,530	0.8	100.0	1.0
Ammonium sulfate (40–75%)	83	960	1,243	1.3	49.0	1.6
DEAE-cellulose	12	77	807	10.5	31.9	13.1
Arginine-Sepharose 4B	30	7.3	249	34.1	9.8	42.6
Gly-Pro-AH-Sepharose 4B	15	1.2	140	116.0	5.5	145.0

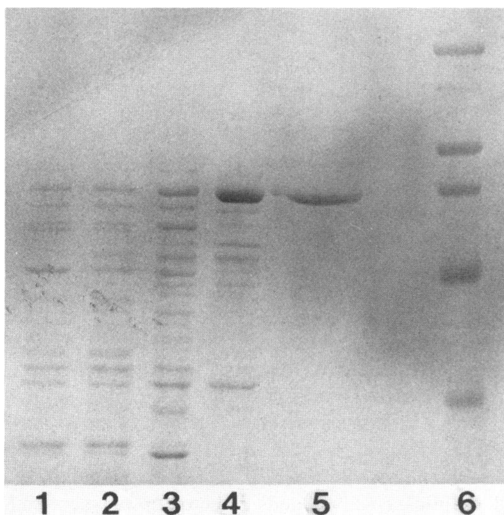

FIG. 2. SDS-PAGE of cell extract and of prolyl-dipeptidyl aminopeptidase active fractions obtained from different purification steps. Lanes: 1, crude cell extract; 2, after ammonium sulfate fractionation, 40 to 75% saturation; 3, after DEAE-cellulose chromatography; 4, after arginine-Sepharose 4B chromatography; 5, purified prolyl-dipeptidyl aminopeptidase (10 μg of protein) after glycyl-prolyl-AH-Sepharose 4B chromatography; 6, molecular size markers in kilodaltons (top to bottom): myosine (205.0), β-galactosidase (116.0), phosphorylase *b* (97.4), bovine serum albumin (66.0), ovalbumin (45.0), and carbonic anhydrase (29.0). Each sample except for the purified enzyme was loaded on the basis of 25 μg of protein.

$CuCl_2$, $HgCl_2$, KCl, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, NaCl, $ZnCl_2$) for 15 min at 40°C to a final concentration each of 0.1, 1.0, or 2.5 mM in 0.01 M Tris hydrochloride buffer, pH 7.0. The enzyme activity was determined with glycylproline-*p*-nitroanilide as a substrate.

Effects of inhibitors, reducing agents, and metal chelators on enzyme activity. Effects of inhibitors, reducing agents, and metal chelators on enzyme activity were examined with 0.01 M phosphate buffer, pH 7.0. The purified enzyme was preincubated with the various agents for 15 min at 40°C. EDTA and diisopropyl fluorophosphate (DFP) were also preincubated with the enzyme for longer periods of 20 and 2 h, respectively. Glycylproline-*p*-nitroanilide (6.4 mM) was then added as a substrate, and enzyme activity was measured after incubation at 40°C for 15 min.

RESULTS

Enzymatic activities of prolyl-dipeptidyl aminopeptidase, proline iminopeptidase, and aminopeptidase. Intracellular prolyl-dipeptidyl aminopeptidase was found in the three strains of lactobacilli used in this study together with aminopeptidase and proline iminopeptidase (Table 1). Aminopeptidase activities were highest in all three strains, whereas proline iminopeptidase activities were quite low and prolyl-dipeptidyl aminopeptidase activities were relatively high. The presence of two separate enzymes, an amino/iminopeptidase and a prolyl-dipeptidyl aminopeptidase, was confirmed by histochemical staining of polyacrylamide gels after electrophoresis (Fig. 1). This was further substantiated by the different relative mobility (R_f) values of prolyl-dipeptidyl aminopeptidase and amino/iminopeptidase in the three strains.

Purification of dipeptidyl aminopeptidase. Results of the purification of prolyl-dipeptidyl aminopeptidase are summarized in Table 2. Elution profiles of the enzyme from columns of DEAE-cellulose, arginine-Sepharose 4B, and glycyl-prolyl-AH-Sepharose 4B were prepared but are not shown in this report. In the final fractionation step of the enzyme on the glycyl-prolyl-AH-Sepharose 4B affinity column, the enzyme was eluted as a sharp, single peak. The enzyme was purified about 145-fold from the cell extract with a yield of 5.5%, and the preparation exhibited a single band by both SDS-PAGE (Fig. 2) and native PAGE (data not shown). Purification was best by affinity chromatography on the glycyl-prolyl-AH-Sepharose 4B column.

Purity of prolyl-dipeptidyl aminopeptidase. The SDS-PAGE pattern of enzyme obtained from different purification steps showed that only a single, sharp band was detected after the final purification step on the glycyl-prolyl-AH-Sepharose 4B affinity column (Fig. 2). The electrophoretic pattern of the purified enzyme on native PAGE after staining with Coomassie brilliant blue R also showed one protein band. Using lysylproline 4-methoxy-β-naphthylamide as a substrate, we found that the enzyme activity coincided with the protein band (data not shown) using the staining method described by Miller and MacKinnon (29).

Molecular weight. The molecular weight of the enzyme was estimated to be about 72,000 by SDS-PAGE (Fig. 2).

Effect of pH on enzyme activity. The effect of pH from 5.0 to 8.5 on prolyl-dipeptidyl aminopeptidase activity was determined at 40°C with 0.01 M sodium phosphate buffer. The optimum for hydrolysis of glycylproline-*p*-nitroanilide by the enzyme was at pH 7.0 (Fig. 3).

Effect of temperature on enzyme activity. The effect of temperature from 5 to 65°C on enzyme activity was determined with 0.01 M phosphate buffer, pH 7.0. The optimum temperature for prolyl-dipeptidyl aminopeptidase activity was 40°C (Fig. 3). At 65°C, the enzyme still had 34% of the activity noted at 40°C.

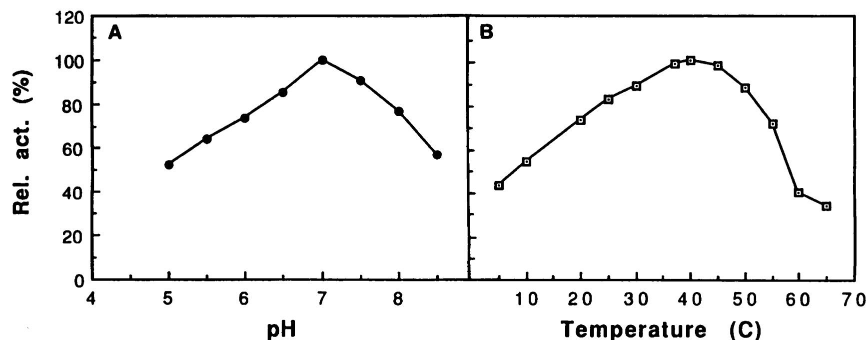

FIG. 3. Effect of pH (A) and temperature (B) on prolyl-dipeptidyl aminopeptidase activity.

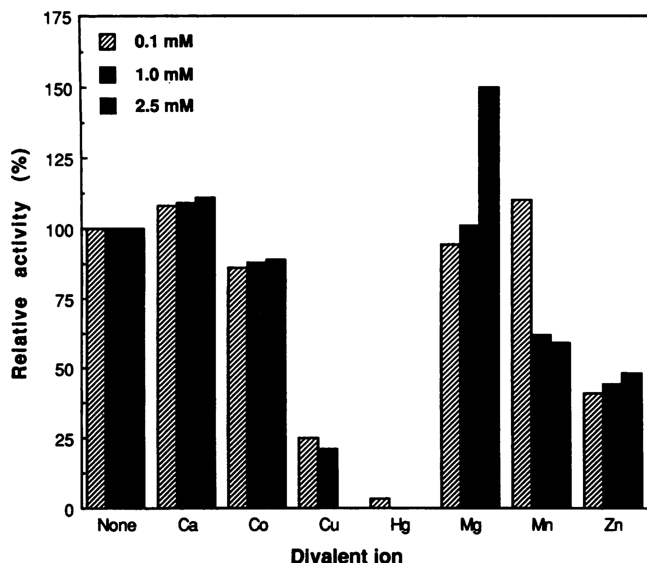

FIG. 4. Effect of divalent ions on prolyl-dipeptidyl aminopeptidase activity.

Effect of metal ions on enzyme activity. The enzyme was inhibited by several divalent metal ions (Fig. 4). Among the sources of divalent ions studied, $HgCl_2$, $CuCl_2$, and $ZnCl_2$ caused marked inhibition, with complete inhibition of enzyme activity by 1.0 mM $HgCl_2$ and 2.5 mM $CuCl_2$. The enzyme was activated 1.5-fold by 2.5 mM $MgCl_2$, whereas $CaCl_2$ enhanced enzyme activity slightly. Addition of 0.1 mM $MnCl_2$ also stimulated enzyme activity, but at concentrations exceeding 1.0 mM, $MnCl_2$ became inhibitory. The sources of monovalent ions, NaCl and KCl, increased the enzyme activity at concentrations greater than 10 mM (Fig. 5). All metal ions were added as chlorides to prevent any influence of anions.

Effects of inhibitors, reducing agents, and metal chelators on enzyme activity. The influences of several agents on the activity of prolyl-dipeptidyl aminopeptidase are summarized in Table 3. The rate of hydrolysis of glycylproline-*p*-nitroanilide in the absence of any inhibitor, reducing agent, or metal chelator was taken as 100%. The enzyme activity was reduced to 46% when incubated in 1.0 mM phenylmethylsulfonyl fluoride (PMSF), whereas it was completely inhibited by 1.0 mM DFP, a specific inhibitor for serine protease, after incubation for 2 h. Total inhibition of enzyme activity also was observed by incubation with *p*-chloromercuribenzoate (PCMB). The metal-complexing agents EDTA and 1,10-phenanthroline also decreased enzyme activity, indicating that there was a metal ion requirement. *N*-Ethylmaleimide and sulfhydryl inhibitors such as iodoacetate had some inhibitory effect on activity of prolyl-dipeptidyl aminopeptidase, whereas dithioerythritol apparently had no effect on enzyme activity.

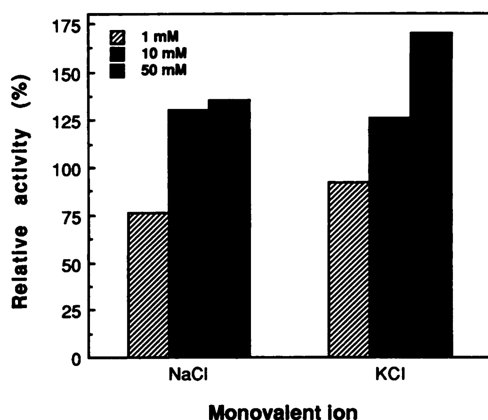

FIG. 5. Effect of monovalent ions on prolyl-dipeptidyl aminopeptidase activity.

TABLE 3. Inhibition of prolyl-dipeptidyl aminopeptidase activity by several inhibitors, reducing agents, and metal chelators

Reagent	Concn (mM)	Relative activity (%)[a]
None	0.0	100
EDTA		
15 min	0.1	98
15 min	1.0	96
20 h	1.0	38
Dithioerythritol	0.1	88
	1.0	96
	10.0	100
DFP	0.1	77
15 min	1.0	13
2 h	1.0	0
N-Ethylmaleimide	0.1	85
	1.0	30
PCMB	0.1	8
	1.0	0
PMSF	0.1	80
	1.0	46
Iodoacetic acid	0.1	83
	1.0	68
1,10-Phenanthroline	0.1	84
	1.0	37

[a] Rate of hydrolysis of glycylproline-*p*-nitroanilide in the absence of any inhibitor, reducing agent, or metal chelator was taken as 100%.

Substrate specificity of prolyl-dipeptidyl aminopeptidase. The purified enzyme was incubated with *p*-nitroanilide derivatives of amino acids or dipeptides and with peptides of free and blocked α-amino acids. Arg-Pro-*p*-nitroanilide had the highest activity among the substrates, followed by Gly-Pro-*p*-nitroanilide (Table 4). The purified enzyme also hydrolyzed Ala-Pro-Gly but not Ala-Leu-Ala. When the purified enzyme was incubated with dipeptides, these substrates were not hydrolyzed. Neither simple amino acid *p*-nitroanilides nor CBZ peptides were hydrolyzed by the enzyme.

DISCUSSION

X-prolyl-dipeptidyl aminopeptidase was detected in the three strains of lactobacilli we studied. This was distinct from the aminopeptidase, as evidenced by the different R_f values of the enzymes after PAGE of the cell extracts (Fig. 1). The enzyme specifically hydrolyzed glycylproline-*p*-nitroanilide and arginine-proline-*p*-nitroanilide. *L. helveticus* CNRZ 32 had two aminopeptidases, AP1 and AP2, with R_f values corresponding to 0.55 and 0.84, respectively (Table

TABLE 4. Relative activity of dipeptidyl aminopeptidase with various substrates.

Substrate	Relative activity (%)[a]
Gly-Pro-*p*-nitroanilide	100
Arg-Pro-*p*-nitroanilide	130
Ala-Ala-*p*-nitroanilide	2
Ala-Pro-Gly	11
Ala-Leu-Ala	0
Pro-*p*-nitroanilide	0
Leu-Pro	0
Met-Gly	0
Tyr-Gly	0
Gly-Phe	0
N-CBZ-Gly-Pro	0
N-CBZ-Ala-Pro-Tyr	0
N-CBZ-Ala-Pro-Leu	0
N-Suc-Phe-*p*-nitroanilide	0
N-Benzoyl-DL-Arg-*p*-nitroanilide	0
N-CBZ-Gly-Pro-Arg-*p*-nitroanilide	0

[a] The rate of hydrolysis of glycylproline-*p*-nitroanilide was taken as 100%.

1). AP1 reacted with both leucine and proline, whereas AP2 was specific for leucine only (the peptide hydrolase systems of the three strains will be described in another report).

The other lactobacilli, *L. helveticus* ATCC 10797 and *L. delbrueckii* subsp. *bulgaricus* ATCC 12278, each had one aminopeptidase (AP1) with R_f values similar to that of proline iminopeptidase, indicating that the aminopeptidase could also react with proline. The presence of two iminopeptidases in *L. helveticus* FAM 176/102, *Lactobacillus leichmanii* ATCC 4797, *S. thermophilus* FAM 101/15, and *S. thermophilus* 176/4 was reported by Casey and Meyer (6). They also found that the R_f values of an iminopeptidase and X-prolyl-dipeptidyl aminopeptidase of *S. thermophilus* were similar. This could be caused by presence of two enzymes with the same R_f value or an X-prolyl-dipeptidyl aminopeptidase able to react with proline. It was later shown that the X-prolyl-dipeptidyl aminopeptidase of *S. thermophilus* had no iminopeptidase activity since the purified enzyme did not react with proline (28). The three strains of lactobacilli in our study also had only one prolyl-dipeptidyl aminopeptidase, as demonstrated by histochemical staining of gels after electrophoresis. Most lactobacilli and streptococci possess only one active prolyl-dipeptidyl aminopeptidase, with the exception of *Lactobacillus brevis* ATCC 8287, which had two bands (6).

In the present study, X-prolyl-dipeptidyl aminopeptidase was purified from the cell extract of *L. helveticus* CNRZ 32 by several steps. After the final purification step on the glycyl-prolyl-AH-Sepharose 4B column, a purified X-prolyl-dipeptidyl aminopeptidase was obtained. Upon exposure to native PAGE and SDS-PAGE, the enzyme was detected as a single, sharp band after staining with Coomassie brilliant blue R. The enzyme from the native gel also was stained with a substrate solution to detect activity on the gel. An orange band was detected in the same region as the protein band, indicating prolyl-dipeptidyl aminopeptidase activity (data not shown). The selective isolation and purification of prolyl-dipeptidyl aminopeptidase by affinity chromatography on glycyl-prolyl-AH-Sepharose 4B was highly effective and resulted in an enzyme purified to homogeneity. Kojima et al. (21) obtained dipeptidyl peptidase IV from the human submaxillary gland and used a similar affinity column to purify the enzyme so it was free from any contaminating aminopeptidase activity.

The purified enzyme catalyzed hydrolyses of peptides and peptide derivatives containing proline residues in the position penultimate to the free amino termini. This was shown by hydrolyses of Arg-Pro-*p*-nitroanilide, Gly-Pro-*p*-nitroanilide, and Ala-Pro-Gly by the purified enzyme at rates of 130, 100, and 11% of the rate toward Gly-Pro-*p*-nitroanilide, respectively, thus indicating that the purified enzyme hydrolyzed X-Pro-Y- peptide almost specifically. The N-terminal position may be occupied by any one of several residues, including Pro, Leu, Arg, and Phe (27). The purified enzyme was classified as a prolyl-dipeptidyl aminopeptidase because of its strong preference for substrates having a penultimate prolyl residue. It also can hydrolyze the X-Ala-Y- peptide when alanine occupies the penultimate position to the free amino terminal. This was shown by hydrolysis of Ala-Ala-*p*-nitroanilide. The rate of hydrolysis with alanine, however, was lower than with proline. Similar results were obtained by Fukasawa and Harada (12), Meyer and Jordi (28), and Yoshimoto and Tsuru (40) with dipeptidyl aminopeptidases of other bacteria. However, this was not true for the yeast enzyme. Substitution of the proline residue in Ala-Pro-*p*-nitroanilide with an alanine residue resulted in a complete loss of hydrolysis of Ala-Ala-*p*-nitroanilide by dipeptidyl aminopeptidase yscV from *S. cerevisiae* (1). The X-Pro-Y- peptide of the type Gly-Pro-Pro or Gly-Pro-Hyp is resistant to enzymatic attack since the third residue from the N-terminal is occupied by Pro or Hyp (14, 20, 33). However, we did not test the substrate specificity of our enzyme against peptides of the type X-Pro-Pro or X-Pro-Hyp.

The enzyme purified in our study clearly differed from the other bacterial dipeptidyl aminopeptidases in its molecular weight. The molecular weight of our purified enzyme was estimated to be about 72,000 by SDS-PAGE. The dipeptidyl aminopeptidase of *S. mitis* had a molecular weight of 120,000 by gel filtration and 53,000 by SDS-PAGE (12). This enzyme from *S. thermophilus* had a molecular weight of 165,000 ± 15,000 by gel filtration and 80,000 ± 5,000 by SDS-PAGE (28), whereas that of *L. lactis* had a molecular weight of 165,000 ± 15,000 by gel filtration and 90,000 ± 5,000 by SDS-PAGE (28). Similarly, the dipeptidyl aminopeptidase of *F. meningosepticum* had a molecular weight of 160,000 by gel filtration and 75,000 by SDS-PAGE (40). Both native PAGE and SDS-PAGE revealed our purified enzyme as a single band with a subunit molecular weight of 72,000, whereas the other bacterial dipeptidyl aminopeptidases were dimers with two subunits (12, 28, 40).

The prolyl-dipeptidyl aminopeptidase purified from *L. helveticus* CNRZ 32 was most active at pH 7.0. This optimum pH was similar to that of the prolyl-dipeptidyl aminopeptidase isolated from *L. lactis* (28). The enzymes from *S. thermophilus* (28), *F. meningosepticum* (40), *S. cerevisiae* (1), and *S. mitis* (12), however, had broad pH optima with the highest activities between pH 6.0 and 8.7, 7.4 and 7.8, 6.5 and 8.2, and 7.0 and 7.5, respectively. The optimum pH values for bacterial and yeast dipeptidyl aminopeptidases were higher than that for mammalian dipeptidyl aminopeptidase, which was reported as 5.5 to 6.0 (18).

The optimum temperature for activity by our enzyme was 40°C. This was 5°C lower than for the dipeptidyl aminopeptidases of *S. thermophilus* (28) and *F. meningosepticum* (40) and 10 to 15°C lower than for the dipeptidyl aminopeptidase of *L. lactis* (28). In contrast, the mammalian enzyme had a

higher optimum temperature of 60°C (39). The stability of prolyl-dipeptidyl aminopeptidase in this study was greater than that of the other bacterial dipeptidyl aminopeptidases. At 50°C, 88% of the initial activity of our enzyme remained after 15 min of incubation, whereas only 65% remained for the *F. meningosepticum* enzyme (40). The enzymes from *L. lactis* and *S. thermophilus* also were less stable, with 50% of the activity of *L. lactis* remaining after 20 min of incubation at 50°C and only 20% remaining for *S. thermophilus* (28). The enzyme from *S. mitis* (12) was relatively stable below 50°C, but activity was almost lost at 55°C after 10 min. In contrast, the enzyme isolated in this study still had 34% of its activity remaining after 15 min at 65°C. All the bacterial enzymes, however, were less thermostable than the mammalian enzyme, which retained about 50% of its activity when incubated at 72°C for 15 min (39).

Various compounds which might affect the activity of the purified enzyme were examined. Incubation of the enzyme with *N*-ethylmaleimide and sulfhydryl inhibitors such as iodoacetate and PCMB decreased the prolyl-dipeptidyl aminopeptidase activity, and complete inhibition of enzyme activity was achieved with 1.0 mM PCMB. The results indicate the possible involvement of a functional sulfhydryl group(s) at or near the active site. These compounds, however, did not inhibit the activity of the enzymes of *F. meningosepticum* (40) and *S. mitis* (12) but slightly decreased the activity of the mammalian enzyme at 1.0 mM concentration (39). *N*-Ethylmaleimide also had no inhibitory effect on the enzymes of *S. thermophilus* and *L. lactis*, although both were sensitive to PCMB and iodoacetate, with the enzyme from *L. lactis* being more sensitive to the sulfhydryl-blocking agents than the enzyme from *S. thermophilus* (28).

Incubation of the enzyme from this study with dithioerythritol had no effect on enzyme activity, suggesting that an intact disulfide group(s) was not essential for the mechanism of action by this enzyme. The enzyme was totally inhibited by DFP, a specific inhibitor of serine protease. The high sensitivity of the prolyl-dipeptidyl aminopeptidase to DFP suggested involvement of a serine residue in catalysis. Bacterial dipeptidyl aminopeptidases of *S. mitis* (12), *F. meningosepticum* (40), *L. lactis*, and *S. thermophilus* (28), mammalian dipeptidyl aminopeptidase IV from rat brain (18), and yeast dipeptidyl aminopeptidase yscV of *S. cerevisiae* (1) were all found to be serine peptidases. PMSF at 1.0 mM concentration reduced the activity of our enzyme to 50% and that of the *L. lactis* enzyme to 65% (28). In contrast, the enzyme of *F. meningosepticum* (40) was hardly affected by PMSF, whereas the enzymes from *S. thermophilus* (28) and *S. cerevisiae* (1) were markedly inhibited by PMSF.

The purified prolyl-dipeptidyl aminopeptidase was activated by $MgCl_2$ and $CaCl_2$ and inhibited by metal-chelating agents such as EDTA, $HgCl_2$, and 1,10-phenanthroline, thus indicating a requirement for metal ion(s). The same inhibition pattern was observed with the enzymes from *S. mitis* (12) and *S. thermophilus* (28), whereas the enzyme from *F. meningosepticum* (40) was not affected by EDTA and 1,10-phenanthroline. A decrease in activity was also observed by prolonged incubation of the enzyme of *L. lactis* in EDTA, but the enzyme was less sensitive to 1,10-phenanthroline (28). This was consistent with our finding that the purified enzyme was inhibited by EDTA only after prolonged incubation.

This study on purification and partial characterization of prolyl-dipeptidyl aminopeptidase from *L. helveticus* CNRZ 32 provides information on the likely role of this enzyme in the overall complex cheese-ripening process which depends on bacterial enzymes functioning in the proper sequence. This enzyme may be important in the ripening of cheese since milk protein is rich in proline. The physiological function of prolyl-dipeptidyl aminopeptidase probably is to participate in release of proline from proline-rich proteins. Peptides containing proline give rise to bitter flavor (19, 35), and this flavor defect may occur during accelerated ripening of cheese (24). Degradation of casein which results in accumulation of a high proportion of hydrophobic amino acid such as proline, leucine, and phenylalanine can result in development of bitter flavor (38). Bitterness will not develop if sufficient peptidases are present to ensure that bitter peptides are degraded as they are formed (5). The peptide hydrolase systems of *L. helveticus* CNRZ 32 also include aminopeptidases with broad substrate specificities (N. M. Khalid, M. El Soda, and E. H. Marth, unpublished data). They cleaved amino acid residues sequentially from the N terminus, and hydrolysis stopped when a residue followed by proline was reached.

The role of prolyl-dipeptidyl aminopeptidase is to further hydrolyze the N-terminal X-Pro-Y- bond which is formed to release other essential amino acids from the peptide chains (27). After the action of prolyl-dipeptidyl aminopeptidase, the newly formed N-terminal X-Pro may be hydrolyzed by a dipeptidase. The presence of a dipeptidase which hydrolyzed dipeptides including the X-Pro type has been detected in *L. helveticus* CNRZ 32 (Khalid et al., unpublished data).

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences at the University of Wisconsin–Madison, the Malaysian Agricultural Research and Development Institute, Serdang, Malaysia, and the Center for Dairy Research at the University of Wisconsin.

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