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Purification and Characterization of an Aminopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2

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An aminopeptidase was purified to homogeneity from a crude cell extract of *Lactococcus lactis* subsp. *cremoris* Wg2 by a procedure that included diethyl-aminoethane-Sephacel chromatography, phenyl-Sepharose chromatography, gel filtration, and high-performance liquid chromatography over an anion-exchange column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme showed a single protein band with a molecular weight of 95,000. The aminopeptidase was capable of degrading several peptides by hydrolysis of the N-terminal amino acid. The peptidase had no endopeptidase or carboxypeptidase activity. The aminopeptidase activity was optimal at pH 7 and 40°C. The enzyme was completely inactivated by the *p*-chloromecuribenzoate mersalyl, chelating agents, and the divalent cations Cu^{2+} and Cd^{2+}. The activity that was lost by treatment with the sulfhydryl-blocking reagents was restored with dithiothreitol or β-mercaptoethanol, while Zn^{2+} or Co^{2+} restored the activity of the 1,10-phenantroline-treated enzyme. Kinetic studies indicated that the enzyme has a relatively low affinity for lysyl-*p*-nitroanilide (K_m, 0.55 mM) but that it can hydrolyze this substrate at a high rate (V_{max}, 30 μmol/min per mg of protein).

Lactococci are a very important group of bacteria in the dairy industry. They play a key role in the production of fermented milk products, such as cheese. Lactic streptococci are all nutritionally fastidious and require exogenous amino acids (23). In order to achieve growth in milk these organisms depend on their proteolytic system for the digestion of the milk protein casein into peptides and free amino acids (18). The importance of the proteolytic system for this group of bacteria has been emphasized previously (26, 27, 32).

Currently available information indicates that a cell wall-bound proteinase (5, 7, 8, 14, 22) digests the native substrate into a number of oligopeptides (21, 33). Since peptide transport in lactococci is restricted to oligopeptides of approximately 5 amino acid residues (16, 24), further degradation by extracellular peptidases into smaller peptides or amino acids is required. Several observations of amino acid and peptide transport in lactococci support this view (25; W. N. Konings, B. Poolman, and A. J. M. Driessen, Crit. Rev., in press).

Peptidase have been found in different cell fractions (4, 13, 17), but solid information about their exact locations in the cell is still lacking. A number of peptidases have been purified and characterized (9, 11, 31, 36), some of which are probably involved in extracellular peptide hydrolysis (6, 7, 37).

In this report we describe the purification and characterization of an aminopeptidase from crude cell extract of *Lactococcus lactis* subsp. *cremoris* Wg2. This enzyme appears to be distinctly different from an aminopeptidase purified previously by Geiss et al. (7) and Exterkate and de Veer (6).

MATERIALS AND METHODS

Organism and preparation of cell extract. A proteinase-negative variant of *L. lactis* subsp. *cremoris* Wg2 was obtained from the Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands. The organism was routinely maintained in 10% (wt/vol) sterile reconstituted skim milk containing 0.1% (wt/vol) tryptone (Difco Laboratories, Detroit, Mich.) and stored at −20°C. *L. lactis* subsp. *cremoris* Wg2 was grown on MRS broth (1) at a controlled pH of 6.3 in a 5-liter fermentor. Cells were harvested at an A_{660} of 0.8, washed in 200 ml of 0.05 M potassium phosphate (pH 7), and suspended in 50 ml of this buffer. Cells were disrupted by sonication (Soniprep 150; MSE Scientific Instruments, Crawley, United Kingdom) for 480 s (15 s of sonication and 45 s of rest eight times) at 10 μm at 4°C under a constant stream of nitrogen. Cell extract was obtained by centrifugation of the disrupted cells for 10 min at 20,000 × *g* and 4°C.

Enzyme assays. Lysyl-*p*-nitroanilide-hydrolyzing activities were determined by the method of Exterkate (3). An appropriate amount of enzyme was incubated for 15 min with a 2 mM solution of lysyl-*p*-nitroanilide. The reaction was stopped by the addition of acetic acid to a final concentration of 10%, and the amount of *p*-nitroanilide was measured at 410 nm (U-1100 spectrophotometer, Hitachi, Japan). All enzyme activities were measured at 30°C in 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; pH 7), unless stated otherwise.

Hydrolysis of several peptides was detected by thin-layer chromatography. Peptidase activity was assayed as follows. The reaction mixture containing 2 mM substrate in 20 mM HEPES (pH 7) and an appropriate amount of enzyme was incubated for 60 min at 30°C. The reaction mixture (10 μl) was then spotted onto a precoated silica gel 60 plate with a gel layer thickness of 0.25 cm (E. Merck, AG, Darmstadt, Federal Republic of Germany). A 75% (wt/vol) phenol-water and, on some occasions, a 4:1:1 (vol/vol/vol/) mixture of *n*-butanol-acetic acid-water were used as the mobile phase. As a control, 2 mM of each standard peptide was also spotted onto the plate. Silica gels were stained by spraying them with 0.1% (wt/vol) ninhydrin in 99% ethanol. Peptides and amino acids became visible after the silica gel was incubated in a stove for 5 min at 80°C.

DEAE-Sephacel column chromatography. A DEAE-Sephacel column (1.6 by 20 cm) was equilibrated with 10 mM K_2HPO_4-KH_2PO_4 (pH 7.0) with 0.12 M NaCl. The cell

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extract, which was obtained after sonication and centrifugation of *L. lactis* subsp. *cremoris* Wg2, was diluted with distilled water to the same ionic strength as the buffer that was used for equilibrating the DEAE-Sephacel column and was subsequently applied to the column. After the column was washed with 2 volumes of equilibration buffer, the enzyme was eluted (40 ml/h) with a linear gradient of 0.12 to 0.4 M NaCl in the same buffer. Fractions of 3 ml were collected and tested for their lysyl-*p*-nitroanilide-hydrolyzing activity. Fractions with the highest enzyme activities were combined.

Phenyl-Sepharose chromatography. A phenyl-Sepharose (CL-4B) column (1.8 by 8.5 cm) was equilibrated with 10 mM K_2HPO_4-KH_2HPO_4 (pH 7) containing 4 M NaCl. NaCl was added to the combined enzyme fractions to the same molarity as the equilibration buffer, and the enzyme solution was applied to the column. After the column was washed with 2 volumes of equilibration buffer, the enzyme was eluted (35 ml/h) with a linear gradient of 4 to 0 M NaCl in 10 mM K_2HPO_4-KH_2PO_4 (pH 7). Fractions of 2.5 ml were collected and tested for their lysyl-*p*-nitroanilide-hydrolyzing activity. Fractions with the highest enzyme activity were combined.

Gel chromatography on Sephadex G-100 super fine. The enzyme fractions obtained from the phenyl-Sepharose CL-4B column were concentrated in a filtration unit with PM-10 and YM-10 membranes (molecular weight cutoff, 10,000; Amicon Corp., Lexington, Mass.) to a final volume of about 2 ml and subsequently applied to a Sephadex G-100 super fine (SF) column (1.6 by 100 cm) which was equilibrated with 10 mM K_2HPO_4-KH_2PO_4 (pH 7) containing 0.1 M NaCl. Elution was carried out with the same buffer at a flow rate of 4 ml/h, and portions of 2 ml were collected. Fractions with the highest peptidase activity were combined.

High-performance liquid chromatography: anion-exchange chromatography. The combined fractions obtained from gel filtration were again concentrated to a volume of 2.5 ml. In each run, 400 μl of enzyme was applied to a high-resolution liquid chromatographic (HRLC) MA7P anion-exchange column (50 by 7.8 mm; Bio-Rad Laboratories, Richmond, Calif.) by injecting 100 μl four times at intervals of 2 min in a constant flow of 20 mM Tris hydrochloride (pH 7.5) containing 125 mM NaCl equilibration buffer. The column was washed with 2 ml of the same buffer, and the enzyme activity was eluted with a linear gradient of 0.125 to 0.4 M NaCl in 20 mM Tris hydrochloride (pH 7.5). The flow rate was 1 ml/min, and peak fractions were collected and tested for peptidase activity. High-performance liquid chromatography (HPLC) was performed on a multisolvent delivery system (600; Waters Associates, Inc., Milford, Mass.) with a U6K universal liquid chromatograph injector, and the A_{280} was measured with a spectrophotometer (Lambda Max model 481 LC; Millipore Corp., Bedford, Mass., and Waters Associates).

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (15). The protein samples were mixed 4:1 with sample buffer (0.05 M Tris hydrochloride [pH 6.8], 10% SDS, 22% glycerol, 10% β-mercaptoethanol, 0.18% bromphenol blue) and applied to the gels. The molecular sizes of the enzymes were estimated with the use of the following reference proteins: myosin (200 kilodaltons [kDa]), β-galactosidase (116.2 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The gels were silver stained by the method of Wray et al. (35).

Isoelectric focusing. Isoelectric focusing on slab gels was performed on a Phast system (Pharmacia, Uppsala, Sweden) with a ready-to-use 5% polyacrylamide gel containing Pharmalyte 3-9 (Phastgel; Pharmacia). The isoelectric point of the enzyme was determined by using the following references: lentil lectin, basic (pI, 8.65); lentil lectin, middle (pI, 8.45); lentil lectin, acidic (pI, 8.15); myoglobin, basic (pI, 7.35); human carbonic anhydrase B (pI, 6.55); bovine carbonic anhydrase (pI, 5.85); and β-lactoglobulin A (pI, 5.2). Gels were automatically stained by the Phast system with Coomassie brilliant blue.

Effect of divalent cations and chemical reagents on the enzyme activity. Purified enzyme (15 μg/ml) was incubated with 200 μM 1,10-phenanthroline in 20 mM HEPES (pH 7) for 10 min at 20°C. After equilibration in a cuvette for 5 min at 30°C, 1 mM lysyl-*p*-nitroanilide was added. Several concentrations of divalent cations were added, and the enzyme activity was measured by determining the release of *p*-nitroanilide, which was followed spectrophotometrically at 410 nm in a spectrophotometer (1100; Hitachi).

The effects of chemical reagents were determined by preincubating 300 μl of an appropriate enzyme solution (15 μg/ml) for 10 min at 20°C with several concentrations of different chemical reagents. The enzyme activity was determined as described above.

Reactivation of the enzyme activity by reducing reagents. Purified (15 μg/ml) enzyme was preincubated for 10 min at 20°C with 5 μM *p*-chloromercuribenzoate (pCMB) or 5 μM *O*-(3-hydroxymercuri-2-methoxypropyl) carbamylphenoxyacetate (mersalyl) in 20 mM HEPES (pH 7). A part of the treated enzyme was again incubated for 10 min at 20°C with 100 μM dithiothreitol (DTT) or 100 μM β-mercaptoethanol prior to the addition of lysyl-*p*-nitroanilide. Enzyme activity was determined (15 min, 30°C) by the release of *p*-nitroanilide and was measured spectrophotometrically at 410 nm.

Sequencing of the N terminus of the protein. The protein sequence was determined by the Edman degradation method by using a pulse-liquid sequencer (model 477A; Applied Biosystems). Samples were prepared by electroblotting the samples onto polyvinylidene difluoride membranes as described by Matsudaira (20), and after the samples were stained with Coomassie brilliant blue, they were sequenced directly. The sequencing was carried out by Eurosequence at the Department of Biochemistry, University of Groningen.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (19), with bovine serum albumin used as the standard.

Chemicals. All chemicals were reagent grade and were obtained from commercial sources.

RESULTS

In order to purify an aminopeptidase from *L. lactis* subsp. *cremoris* Wg2 to homogeneity, several methods were applied. The following steps were selected to achieve a rapid and easy procedure.

Enzyme purification. (i) Step 1. The crude cell extract was applied to a DEAE-Sephacel column. After elution with an NaCl gradient, lysyl-*p*-nitroanilide-hydrolyzing activities were obtained at several fractions at 0.18 to 0.2 M NaCl.

(ii) Step 2. The active fractions were combined and applied on a phenyl-Sepharose column. After elution the activity toward lysyl-*p*-nitroanilide was found in a peak which eluted at 50 mM NaCl.

TABLE 1. Purification of a lysyl-*p*-nitroanilide aminopeptidase from *L. lactis* subsp. *cremoris* Wg2

Purification step	Total protein (mg)	Total activity, 10^3[a]	Yield (%)	Sp act[b]	Purification (fold)
Cell extract	904	108	100	120	1
DEAE-Sephacel	81.8	65.6	61	800	6.6
Phenyl-Sepharose	27.4	35.7	33	1,300	10.8
G-100 SF	15.4	21.5	20	1,400	11.6
MA7P (HPLC)	3.3	12.7	12	3,900	31.6

[a] Total activity is expressed as nanomoles of *p*-nitroanilide released per minute.

[b] Specific activity is expressed as nanomoles of *p*-nitroanilide released per milligram of protein per minute.

(iii) Step 3. The aminopeptidase-containing fractions were combined and further purified over a G-100 SF gel filtration column.

(iv) Step 4. For the final step, the combined aminopeptidase fractions were applied on a HPLC MA7P anion-exchange column (Bio-Rad). The pure enzyme was eluted at 0.35 M NaCl in Tris hydrochloride (pH 7.5) at a retention time of 13 to 15 min (see Materials and Methods).

The enzyme purification is summarized in Table 1. By this procedure the enzyme could be purified approximately 32-fold with a yield of 12% from the crude extract.

In order to perform a clear characterization, all experiments were carried out with the most purified HPLC eluate.

Molecular weight and isoelectric point. On SDS-PAGE the purified enzyme gave only one band, even in the presence of β-mercaptoethanol. The molecular weight was estimated to be 95,000 (Fig. 1). The same result was obtained under denaturing and native conditions, which indicates that the enzyme is probably a monomer consisting of a single subunit.

The pI of the enzyme was estimated to be 6.2 by isoelectric focusing on the Phast system 5% PAA gel (Pharmacia) containing Pharmalyte 3-10 (data not shown).

Temperature dependence and effect of pH on enzyme activity. The effect of temperature on aminopeptidase activity was measured in the range of 5 to 80°C. The enzyme mixture was equilibrated for 5 min at the test temperatures before the addition of lysyl-*p*-nitroanilide. The optimum temperature for lysyl-*p*-nitroanilide-hydrolyzing activity was found to be 40°C (Fig. 2). At 55°C only 10% of the optimum activity was found.

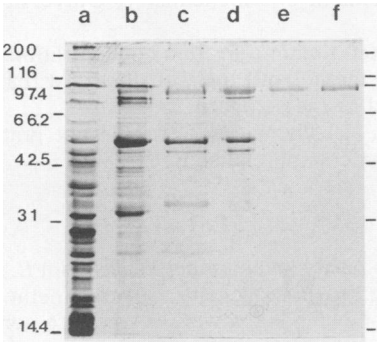

FIG. 1. SDS-PAGE analysis of the enzyme fractions during the purification of a lysyl-*p*-nitroanilide-hydrolyzing enzyme. Electrophoresis was performed on an SDS-polyacrylamide gel (10% polyacrylamide) with several reference proteins (data not shown), 30 μg of protein from crude cell extract (lane a), 15 μg of protein after the DEAE column (lane b), 10 μg of protein after the phenyl-Sepharose column (lane c), 10 μg of protein after G-100 SF gel filtration (lane d), and 5 μg (lane e) and 8 μg (lane f) of protein after MA7P column chromatography (HPLC).

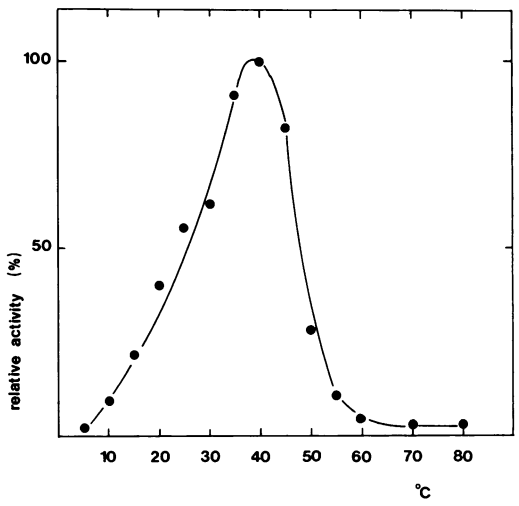

FIG. 2. Effect of temperature on the lysyl-*p*-nitroanilide-hydrolyzing activities of the purified enzyme in 20 mM HEPES (pH 7). The relative peptidase activity was measured by the release of *p*-nitroanilide, which was followed spectrophotometrically at 410 nm.

The effect of pH was determined in the range of pH 4 to 10 by using a buffer consisting of 20 mM each of malic acid, 2-(*N*-morpholino)ethanesulfonic acid (MES), HEPES, and boric acid adjusted to the appropriate pH values (Fig. 3). The optimum pH for hydrolysis of lysyl-*p*-nitroanilide appeared to be 7. At values below pH 5 and above pH 9, no hydrolyzing activity of the enzyme could be detected.

Substrate specificity. The specificity of aminopeptidase toward various peptides is shown in Table 2. The products of hydrolysis were analyzed by thin-layer chromatography. The enzyme was active toward various peptides but not with

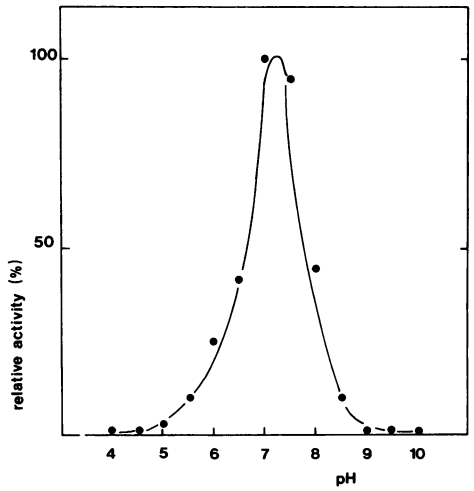

FIG. 3. Effect of pH on aminopeptidase activity measured in 20 mM malic acid–20 mM MES–20 mM HEPES–20 mM boric acid and adjusted to different pH values with KOH. The relative peptidase activity was measured by determining the release of *p*-nitroanilide, which was followed spectrophotometrically at 410 nm.

TABLE 2. Substrate specificity of an aminopeptidase from *L. lactis* subsp. *cremoris* Wg2[a]

Substrate	Activity[b]	Substrate	Activity
Leu-Leu	+	Leu-Gly-Gly	+
Leu-Gly	+	Leu-Leu-Leu	+
Ala-Glu	−	Ala-Val-Leu	+
Ala-Gly	−	Ala-Pro-Ala	+
Ala-Ala	−	Ala-Pro-Phe	+
Glu-Val	−	Ala-His-Ala	+
Glu-Ala	+	Ala-Pro-Gly	+
Gly-Lys	+	Glu-Val-Phe	−
Ile-Ala	+	Gly-Pro-Ala	+
Phe-Leu	−	Gly-Leu-Tyr	+
Phe-Val	−	Gly-Phe-Leu	+
Pro-Met	−	Gly-Ser-Ala	−
		Gly-Gly-Leu	−
Ala-2	−	Gly-Gly-Phe	−
Ala-3	+	Gly-Gly-Gly	−
Ala-4	+	Pro-Gly-Gly	−
Ala-5	+	Phe-Gly-Gly	−
Ala-6	+	Gly-Pro-Gly-Gly	−
Metenkephalin	+	Neurotensin	+
Bradykinin	+	Glucagon	+
Substance P	+		

[a] Hydrolysis of peptides was analyzed by thin-layer chromatography.
[b] +, Hydrolysis; −, no hydrolysis detectable.

dipeptides containing N-terminal proline, alanine, or phenylalanine. The hydrolytic activities of several tripeptides could also be observed, except for those of tripeptides containing N-terminal proline or glycine.

In contrast to the lack of hydrolytic action on dipeptides containing N-terminal alanine, the aminopeptidase hydrolyzed tripeptides containing N-terminal alanine, indicating that the enzyme has more affinity for oligopeptides than it does for dipeptides. This assumption is supported by the observation that the enzyme was active on several polyalanines but not on alanyl-alanine. The enzyme also acted on various large biologically active peptides like metenkephalin and bradykinin but did not hydrolyze specific carboxypeptidase substrates such as benzoyl-glycyl-lysine, carbobenzoxy-phenylalanyl-alanine, or carbobenzoxy-prolyl-alanine. Table 3 shows the relative rates of hydrolysis by the enzyme of various substrates with C-terminal *p*-nitroanilide. The highest activity was found for lysyl-*p*-nitroanilide and, to a lesser extent, for leucyl-*p*-nitroanilide. This observation indicates that this enzyme is an aminopeptidase.

The affinity of the enzyme for lysyl-*p*-nitroanilide was determined. A Lineweaver-Burk plot indicated a K_m for lysyl-*p*-nitroanilide of 0.55 mM, with a maximal rate of peptide hydrolysis of 30 μmol/min per mg (data not shown).

Effect of metal ions on enzyme activity. Treatment of the enzyme with the metal-chelating agents EDTA and 1,10-phenanthroline completely inhibited the enzyme activity (Fig. 4). The activity of the 1,10-phenanthroline-treated enzyme could be restored by 88 and 80% by the addition of 50 μM $ZnCl_2$ and 50 μM $CoCl_2$, respectively (Fig. 5). Zn^{2+} was the most effective stimulatory metal ion; but at concentrations exceeding 100 μM, Zn^{2+} became inhibitory. Other metal ions like Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+} could not restore the enzyme activity. On the other hand, no activity could be restored with any of the divalent cations when the enzyme was treated with EDTA.

The inhibitory effect of several divalent cations on the enzyme activity was also studied (Table 4). Additions of 25 μM $CuCl_2$ and 25 μM $Cd(NO_3)_2$ inhibited the activity by 92 and 65%, respectively. $ZnCl_2$ and $FeCl_2$ (both at 100 μM) also inhibited the enzyme activity, but to a lesser extent (68 and 40%, respectively). Ca^{2+}, Mg^{2+}, and Mn^{2+} had no inhibitory effect at all. All metal ions except for Cd^{2+} were added as chlorides, to prevent any influence of the anions. These results indicate that the aminopeptidase activity is dependent on the divalent cations Zn^{2+} and Co^{2+}.

Effect of various chemical reagents. The effects of various sulfhydryl group and thiol group reagents on aminopeptidase activity were studied (Fig. 4). The aminopeptidase activity was completely inhibited by the sulfhydryl-blocking reagents

TABLE 3. Relative activity of the peptidase for several *p*-nitroanilide peptides

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Lys-pNA[a]	100	Phe-pNA	4
Leu-pNA	24	Gly-pNA	<1
Met-pNA	8		
Glu-pNA	<1	Gly-Arg-pNA	<1
Ala-pNA	4	Ala-Pro-pNA	4
Pro-pNA	<1	Ala-Ala-Ala-pNA	<1

[a] pNA, *p*-Nitroanilide.

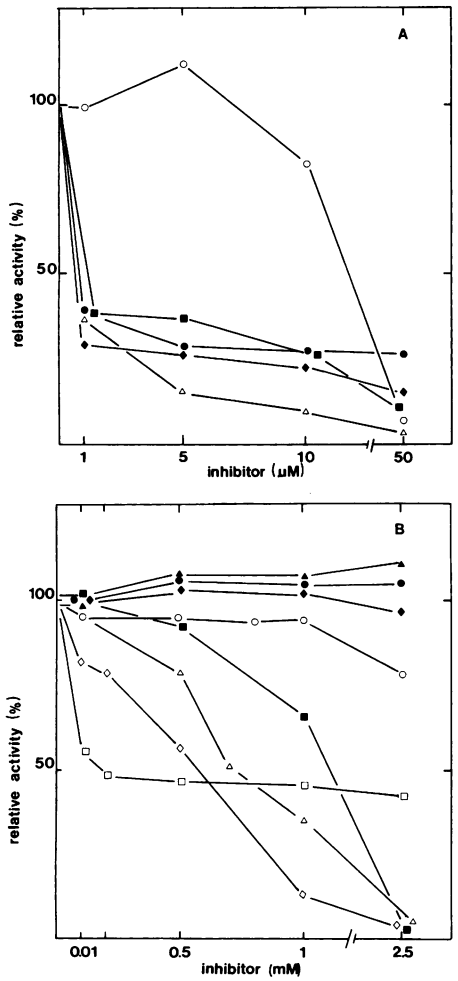

FIG. 4. Effect of various agents on aminopeptidase activity. Reaction mixtures lacking a substrate were preincubated with the agents in 20 mM HEPES (pH 7) for 10 min at 20°C prior to the addition of the substrate lysyl-*p*-nitroanilide. The relative peptidase activity was measured by the release of *p*-nitroanilide, which was followed spectrophotometrically at 410 nm. Symbols in panel A: ○, 1,10 phenanthroline; △, EDTA; ◆, pCMB; ■, *p*-chloromercuribenzene sulfonate; ●, mersalyl. Symbols in panel B: ●, DTT; ▲, β-mercaptoethanol; ◆, phenylarsine oxide; ■, L-cysteine; ○, phenylmethylsulfonyl fluoride; △, iodoacetamine; ◇, bestatin; □, *N*-ethylmaleimide.

pCMB, p-chloromercuribenzene sulfonate, and mersalyl (Fig. 4A). Sulfhydryl group reagents such as iodoacetamide and *N*-ethylmaleimide also had an inhibitory effect on the enzyme activity, but only at higher concentrations.

At 2.5 mM, phenylarsine oxide, a dithiol reagent, had no effect at all on the hydrolytic activity of the enzyme (Fig. 4B). Disulfide-reducing agents, such as DTT and β-mercaptoethanol, also had no inhibitory effect on the enzyme activity (Fig. 4B). These results suggest that the hydrolytic activity depends on one or more reactive sulfhydryl groups in the protein. This was confirmed by experiments in which the inactivating effects of pCMB and mersalyl were completely reversed by DTT or β-mercaptoethanol (Table 5).

Sequencing of the N terminus of the peptidase. The N-terminal sequence of the purified enzyme was determined. The sequencing was carried out with a pulse-liquid (gas phase) sequencer, and 32 amino acid residues were analyzed: Ala-Val-Lys-Arg-Leu-Ile-Glu-Thr-Phe-Val-Pro-Glu-Asn-Tyr-Lys-Ile-Phe-Leu-Asp-Ile-Asp-Arg-Lys-?-Thr-Lys-Ile-Lys-Gly-Gln-Val-Ala-Ile.

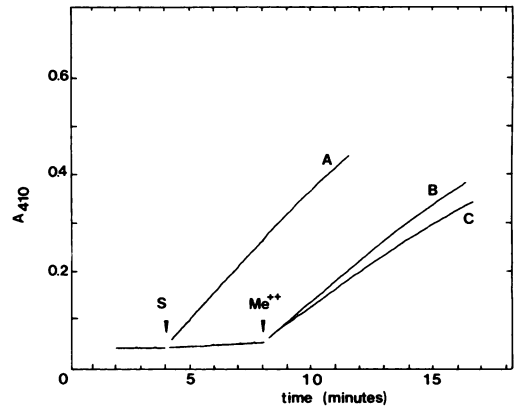

FIG. 5. Reactivation of the 1,10-phenanthroline-treated aminopeptidase by divalent cations. (A) Activity of the nontreated enzyme after addition of 2 mM lysyl-*p*-nitroanilide (S). The 1,10-phenanthroline-treated enzyme was inactive after the addition of 2 mM lysyl-*p*-nitroanilide (S to Me^{++}). The 1,10-phenanthroline-treated enzyme was reactivated after the addition (Me^{++}) of 50 μM $ZnCl_2$ (B) and 50 μM $CoCl_2$ (C). Peptidase activity was measured by the release of *p*-nitroanilide, which was followed spectrophotometrically at 410 nm.

DISCUSSION

A dipeptidase from *L. lactis* subsp. *cremoris* Wg2, which only hydrolyzed dipeptides, has already been purified by van Boven et al. (28, 31). A peptidase that was able to hydrolyze the synthetic substrate leucyl-*p*-nitroanilide was also reported for this strain.

In this report we described the purification and characterization of an aminopeptidase from crude cell extracts of *L. lactis* subsp. *cremoris* Wg2. The enzyme was purified to homogeneity from the cell extract by a four-step procedure. The purification, as estimated from the specific activity, was approximately 30-fold, with a yield of 12%. On the basis of protein, a purification of approximately 270-fold was achieved. Apparently, inactivation occurred during the purification procedure.

The purified enzyme differed from the only other described aminopeptidase purified from *L. lactis* subsp. *cre-*

TABLE 4. Effect of metal ions on aminopeptidase activity[a]

Divalent cation	% Activity at:	
	25 mM	100 mM
No addition	100	100
Zn^{2+}	90	32
Mn^{2+}	100	90
Co^{2+}	110	95
Ca^{2+}	97	97
Cu^{2+}	8	0
Fe^{2+}	75	60
Mg^{2+}	100	105
Cd^{2+}	35	4

[a] Reaction mixtures containing the intact aminopeptidase were preincubated for 10 min at 20°C with metal ions in 20 mM HEPES (pH 7) prior to the addition of lysyl-*p*-nitroanilide. The relative peptidase activity was measured by the release of *p*-nitroanilide, which was followed spectrophotometrically.

TABLE 5. Effects of pCMB-DTT and mersalyl–β-mercaptoethanol on peptidase activity[a]

Addition	% Activity
None	100
5 μM pCMB	29
5 μM pCMB + 100 μM DTT	105
5 μM mersalyl	34
5 μM mersalyl + 100 μM β-mercaptoethanol	110

[a] Purified enzyme (15 μg/ml) was preincubated for 10 min at 20°C with 5 μM pCMB or 5 μM mersalyl. A part of the treated enzyme was again incubated for 10 min at 20°C with 100 μM DTT or 100 μM β-mercaptoethanol prior to the addition of lysyl-*p*-nitroanilide. The release of *p*-nitroanilide was followed spectrophotometrically at 410 nm.

moris AC1 (7). The enzyme reported here is a monomer with a molecular weight of 95,000, whereas the aminopeptidase of *L. lactis* subsp. *cremoris* AC1 has a molecular weight of 36,000. The latter enzyme has not been characterized sufficiently to allow a profound comparison. However, certain similarities between both enzymes are obvious, such as similar pH and temperature optima and their irreversible inactivation by EDTA.

The enzyme described in this report was purified from cell extracts, but aminopeptidase activity was also found in supernatants simply by washing the whole cells in 50 mM Tris hydrochloride (pH 7). In this supernatant, no activities of intracellular enzymes could be detected (data not shown). This is an indication that the aminopeptidase is located extracellularly.

The specificity of the aminopeptidase was determined by hydrolysis of several di-, tri-, tetra-, and pentapeptides, as well as larger peptides. The results (Table 2) revealed that the enzyme has a rather broad substrate specificity. It was not active on dipeptides containing N-terminal alanine, while tri-, tetra-, penta-, and hexaalanines and tripeptides containing N-terminal alanine were hydrolyzed. These results suggest that the enzyme preferentially hydrolyzes peptides larger than dipeptides.

The enzyme was irreversibly inhibited by EDTA but not by 1,10-phenanthroline. Zn^{2+} and Co^{2+} specifically restored the enzyme activity. It is possible that one of these ions plays an essential role in the hydrolytic action. It is not clear why these metals cannot restore the activity of the EDTA-treated enzyme. Also, a cysteine residue(s) in the protein appeared to have a major role in the hydrolytic mechanism. It is possible that this enzyme is a metallo-cysteine-aminopeptidase. The kinetic studies with the substrate lysyl-*p*-nitroanilide, which appeared to be hydrolyzed the best (Table 3), revealed relatively low affinities (K_m, 0.55 mM) but high maximal rates of hydrolysis (turnover number, 45/s). The purified intracellular dipeptidase from *L. lactis* subsp. *cremoris* Wg2 (31) has maximum rates of dipeptide hydrolysis which are even 100-fold higher than those found for the aminopeptidase activity. Alanyl-alanine and phenylalanyl-leucine were not hydrolyzed at all by the aminopeptidase, while the dipeptidase activity for these peptides was very high. This suggests that the major hydrolytic activity for some dipeptides is intracellular. This is in accordance with the observed dipeptide transport found in this organism (29, 30) and recently in *L. lactis* ML3 (25).

The N-terminal amino acid sequence showed no signal peptide sequence as described by von Heijne (34). Also, no homology was found with the N terminus of the sequenced serine proteinase from *L. lactis* subsp. *cremoris* Wg2 (12). The N-terminal amino acid was an alanine, unlike the N-terminal amino acid (methionine) of most proteins. If no cleavage of an N-terminal methionine occurs, the presence of alanine as N-terminal amino acid could be an indication that the purified aminopeptidase is processed and that a signal peptide is cleaved off. This assumption is in accordance with the observation that a signal peptide is believed to be cleaved at Ala-33–Ala-34 in the serine proteinase of *L. lactis* subsp. *cremoris* Wg2 (12), leaving a proprotein with an N-terminal alanine. The same observation has been made for an extracellular peptidase of *Streptomyces* sp. strain R61, in which a 31-amino-acid signal peptide is cleaved at Ala-31–Ala-32 (2, 10).

As mentioned above, the extracellular proteinase of *L. lactis* subsp. *cremoris* Wg2 is responsible for the initial casein hydrolysis. Because of the restriction in peptide uptake, further degradation of the large polypeptides should occur. The aminopeptidase described in this report is most likely an extracellular enzyme which contributes to the further degradation of the large (casein) peptides.

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