Purification and Partial Characterization of an Aminopeptidase from *Lactobacillus lactis*

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A surface-bound aminopeptidase of *Lactobacillus lactis* cells was solubilized with lysozyme, and the extract was subjected to streptomycin sulfate precipitation, ammonium sulfate fractionation, chromatography on Sephadex G-100 and diethylaminoethyl-Sephadex A-50, and preparative polyacrylamide gel electrophoresis. The purified enzyme was homogeneous in disc electrophoretic analysis and consisted of a single polypeptide chain with a molecular weight of 78,000 to 81,000. The optimal pH and optimal temperature for enzyme activity were 6.2 to 7.2 and 47.5°C, respectively, for L-lysine-4-nitroanilide as the substrate. The enzyme was activated by Co^{2+} and Zn^{2+} ions and inhibited by Cu^{2+} , Hg^{2+} , and Fe^{3+} ions and by the metal-complexing reagents ethylenediaminetetraacetic acid, 1,10-phenanthroline, and α, α' -dipyridyl. Higher concentrations of substrate and hydrolysis products also inhibited the activity of the enzyme. The aminopeptidase had broad substrate specificity and hydrolyzed many amino acid arylamides and many peptides with unsubstituted NH_2 -terminal amino acids.

The coagulating enzymes (rennet or rennet substitutes) and the proteolytic enzymes of starter cultures play a major role in protein breakdown during the manufacturing and ripening process of cheeses (4, 16). The cheesemakers in Switzerland previously used heat-tolerant starter cultures containing *Streptococcus thermophilus* and *Lactobacillus helveticus* in the traditional Swiss manufacturing procedure for Emmentaler cheese (Swiss cheese) (18). During recent years, starter cultures containing *S. thermophilus* and *L. lactis* have been used increasingly. Proteolytic activities of many lactic acid bacteria have been studied extensively, and several proteolytic enzymes of some strains have been isolated and characterized (2, 3, 5, 6-9, 11, 14). However, there is little information available concerning the specific characteristics of proteolytic enzymes produced by *L. lactis*. During a general investigation, it became evident that *L. lactis* produces at least three distinct surface-bound proteolytic enzymes: a proteinase, an aminopeptidase, and a carboxypeptidase (hydrolyzing the substrates *N*-benzoylglycyl-L-arginine and *N*-benzoylglycyl-L-phenylalanine). Some characteristics of the three unpurified enzymes have been described elsewhere (B. Eggimann, Ph.D. thesis, Swiss Federal Institute of Technology, Zurich, Switzerland, 1980). The present

paper is a report on the purification and partial characterization of the aminopeptidase.

MATERIALS AND METHODS

Organism. Lyophilized cells of *L. lactis* (strain 1183) were a kind gift from the Swiss Federal Dairy Research Station, Liebefeld-Bern.

Media. The media used for cultivation of cells were MRS medium (12) and, for bulk cell preparations, a medium containing (per liter of the final medium): (substances A) CH_3COONa , 5 g; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 1 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.05 g; ammonium citrate, 2 g; casein Hammarsten, 10 g; peptone from casein, trypsin digested (E. Merck AG), 20 g; Tween 80, 1 g; glucose, 20 g; (substances B) yeast nitrogen base without amino acids (Difco Laboratories), 6.7 g; vitamin B12, 0.01 mg; vitamin C, 0.25 mg; guanine, adenine, uracil, and xanthine, 10 mg each. The final medium was prepared by mixing substances A (autoclaved at 115°C, 15 min) and substances B (sterilized by filtration) before inoculation and by adjusting the pH to 5.5 with 1 M HCl.

Cultivation and harvesting of the organism. The cells were subcultured twice in 10 ml of MRS medium (32 and 21 h, 42°C) and once in 200 ml of the second medium (A+B) described above (19 h, 42°C). For bulk cell preparations, 2.5 liters of the same medium (A+B) held in a glass fermentor vessel was inoculated with 1% (vol/vol) of the reactivated culture. During cultivation, a pH-stat (Metrohm) maintained the pH of the medium at 5.5 ± 0.1 by addition of 5 M NaOH. The medium was gently stirred, and sterile nitrogen, flowing continuously through the medium, maintained a low oxygen tension. During a preliminary cultivation (24 h), growth curves were established by

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enumerating viable cells by the plate count method on MRS agar and by measuring the turbidity of the medium at 600 nm. During bulk cell preparations, cell growth was controlled only by measuring the turbidity. The cultivation was stopped after 9 h at the end of the log phase (cell number, approximately 2×10^8 cells/ml), the pH was adjusted to 7.0 with 5 M NaOH, and the cells were centrifuged at $5,000 \times g$ for 10 min at 4°C. The pellet was washed twice with 0.05 M potassium phosphate buffer, pH 7.0, and resuspended in 125 ml of the same buffer.

Purification of the aminopeptidase. (i) Crude extract. As shown earlier (Eggmann, Ph.D. thesis, 1980), the aminopeptidase of *L. lactis* is surface bound and can be solubilized by the action of lysozyme. Therefore, the suspension of washed cells in phosphate buffer was incubated with lysozyme (Sigma Chemical Co.; 1 mg/ml) at 45°C for 1 h. The suspension was then centrifuged at $40,000 \times g$ and 4°C for 20 min, the pellet was resuspended in the same buffer, and centrifugation was repeated. The pooled supernatant fluids of both centrifugations gave the crude extract.

(ii) Streptomycin sulfate precipitation. All of the following purification steps were done at 4°C. Streptomycin sulfate was added to the crude extract to a final concentration of 2 mg/ml. After 2 h, the precipitated nucleic acids were removed by centrifugation at $20,000 \times g$ for 30 min.

(iii) Ammonium sulfate fractionation. Ammonium sulfate fractionation resulted in the loss of about 50% of the activity. In a preliminary attempt, we found that the greatest part of the remaining activity precipitated between 55 and 85% saturation. Solid ammonium sulfate was added to the supernatant fluid of the previous step, and the fraction precipitating between 55 and 85% saturation was dissolved in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.0.

(iv) Fractionation on Sephadex G-100. The redissolved precipitate was applied to a column (2.6 by 118 cm) of Sephadex G-100, and the proteins were eluted with the same buffer. The eluant was collected in fractions (5.4 ml), and those fractions containing aminopeptidase activity were combined.

(v) Fractionation on DEAE-Sephadex A-50. For ion-exchange chromatography, a column (1.6 by 22 cm) of diethylaminoethyl (DEAE)-Sephadex A-50 equilibrated with 0.05 M Tris buffer, pH 7.0, containing 0.1 M NaCl was used. Then 0.1 M NaCl was added to the combined fractions of the previous step, and the fractions were applied to the column. The enzyme was eluted with an NaCl gradient (0.1 to 0.3 M NaCl), and the eluant was collected in fractions (5.2 ml). For the diafiltration of the combined active fractions against Tris buffer without NaCl and for concentration, an ultrafiltration cell (membrane PM10; Amicon Corp.) was used.

(vi) Preparative gel electrophoresis. The concentrated enzyme solution of the previous step was subjected to preparative gel electrophoresis according to the method of Ornstein and Davies (17). A vertical-column apparatus for preparative electrophoresis (Shandon) was used with a separation gel (length, 15 cm; 7.5% acrylamide) and a spacer gel (2.5% acrylamide).

The upper electrode chamber contained 0.04 M Tris plus 0.04 M glycine buffer, pH 8.9, and the lower electrode chamber contained 0.1 M Tris buffer, pH 8.0. The lower electrode buffer was also used as elution buffer. During electrophoresis, the gels were cooled to 3°C and the current was kept at 35 mA. The eluant was collected in fractions (2.75 ml), and the active fractions were combined.

Analytical gel electrophoresis. After each fractionation step, a part of the active fraction was concentrated in a Minicon B-15 concentrator (Amicon), and the concentrated enzyme solutions were subjected to polyacrylamide gel disc electrophoresis as described by Maurer (13; gel system 1). After electrophoresis, the protein bands were stained with Coomassie brilliant blue G-250 in perchloric acid solution (10).

Protein determination. The protein concentrations were estimated according to the method of Ohnishi and Barr (15), using the biuret and phenol reagents with bovine serum albumin as the standard.

Enzyme assay. For determination of aminopeptidase activity a substrate solution containing 1 mM lysine-4-nitroanilide (Lys-4-NA) and 1 mM CoCl_2 in 0.05 M potassium phosphate buffer, pH 0.7, was used. To 200 μl of this substrate solution was added 20 μl of the enzyme solution and the mixture was incubated at 45°C for 2, 5, or 10 min. The reaction was stopped by the addition of 200 μl of 30% (vol/vol) acetic acid, and the color intensity of the 4-nitro-aniline produced was measured at 405 nm against a blank that had been prepared by adding the acetic acid to the substrate solution before addition of the enzyme solution. Activity was expressed in katal units. One katal equals the amount of activity that converts 1 mol of substrate/s.

Molecular weight determination. (i) Gel filtration. Molecular weight was determined by the method of Andrews (1) on a column (2.6 by 56.5 cm) of Sephadex G-100 equilibrated with 0.05 M Tris buffer, pH 7.0. For determination of the void volume, blue dextran was used; as protein markers, bovine serum albumin (67,000), egg albumin (45,000), chymotrypsinogen A (25,000), cytochrome c (12,000), and trypsin inhibitor (8,000) were used, all provided from Serva. As a control, the column of Sephadex G-100 used to purify the enzyme (step iv) was also calibrated with the above proteins, and molecular weights were determined with the same method.

(ii) SDS-polyacrylamide gel electrophoresis. In addition to gel filtration, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used for molecular weight determination. Gels (10% acrylamide) and samples were prepared according to the method of Weber et al. (19). The marker proteins were the same as for determinations by gel filtration. In addition, a set of molecular weight markers provided by BDH was also used. After electrophoresis, the gels were stained in a staining solution <0.25% (wt/vol) of Kenacid blue R (BDH) in water-acetic acid-methanol (5:1:5) for 2 h. For destaining, the gels were put into a solution of 7.5% (vol/vol) acetic acid plus 5% (vol/vol) methanol in water.

Chromatography. Substrate specificity was evaluated by thin-layer chromatography on aluminum sheets of Silica Gel 60 (Merck). For detection of hy-

drolisis products of the various assayed peptides, 1 μ l of each assay mixture was applied to the sheets and the chromatograms were developed for 4 to 5 h with an *n*-butanol-acetic acid-water solvent (4:1:1). The sheets were dried, sprayed with ninhydrin reagent (0.4 g of ninhydrin in 190 ml of ethanol plus 10 ml of 2,4,6-trimethylpyridine), and dried at 100°C for 10 min.

RESULTS

Purification of the aminopeptidase. Typical enzyme purification results are summarized in Table 1. The results of the fractionation of the aminopeptidase by gel filtration on Sephadex G-100 and by ion-exchange chromatography on DEAE-Sephadex A-50 are shown in Fig. 1 and 2. After preparative gel electrophoresis, the enzyme had only weak activity. Dialysis of the active fractions against 0.05 M Tris buffer, pH 7.0, plus 1 mM CoCl₂ did not reactivate the enzyme, nor did pre-electrophoresis of the gel, to remove persulfate, prevent partial inactivation of the enzyme during gel electrophoresis. Since the activity of the purified enzyme was weak, we did not gain enough material to do all of the investigations with the purified enzyme. For this reason, the concentrated active fractions after ion-exchange chromatography were used for some experiments. These fractions contained only a few protein bands as revealed by analytical polyacrylamide gel electrophoresis. The final product migrated as a single component in analytical polyacrylamide gel electrophoresis (Fig. 3) and in SDS-polyacrylamide gel electrophoresis.

Molecular weight. Molecular weight determination by gel filtration with the enzyme preparation after ion-exchange chromatography (step v) yielded a value of 81,000, and molecular weight determination by SDS-polyacrylamide gel electrophoresis yielded a value of 78,000. The values correspond fairly well and were confirmed by comparison of the position of the active fractions during purification on the Sephadex G-100 column (step iv) with the positions of the marker proteins on the same column. SDS-polyacrylamide gel electrophoresis showed only one band with the same molecular weight as was deter-

mined by gel filtration, indicating that the enzyme consisted of a single polypeptide chain.

Effect of pH and temperature on the activity of the enzyme. The pH optimum for the aminopeptidase, determined with partially purified enzyme, was 6.2 to 7.2, and the temperature optimum was at 47.5 °C (Fig. 4). The rate of thermal inactivation proceeded more rapidly at pH 7.0 than at pH 5.5 (Fig. 5).

Effect of substrate and end product concentration on enzyme activity. The Lineweaver-Burk plot (Fig. 6) for hydrolysis of L-lysine-4-nitroanilide (Lys-4-NA) shows that the aminopeptidase activity was inhibited by higher substrate concentrations. Addition of L-lysine to the assay mixture to a final concentration of 50 mM resulted in a total inhibition of the enzyme activity.

Effect of metal ions and enzyme inhibitors on enzyme activity. The aminopeptidase was activated only by the addition of Zn²⁺ and Co²⁺ ions to the assay mixture (Fig. 7). Of the enzyme inhibitors tested, only metal-complexing reagents gave marked inhibition. To achieve total inhibition of activity, the three metal-complexing reagents ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline, and α,α' -dipyridyl had to be added in concentrations of 10⁻⁵, 2.5 $\times$ 10⁻⁴, and 5 $\times$ 10⁻³ M, respectively.

FIG. 1. Elution pattern of the aminopeptidase from a Sephadex G-100 column. Symbols: (—) protein curve (280 nm); (O) activity curve (405 nm).

TABLE 1. Purification of *L. lactis* aminopeptidase

Purification step	Vol (ml)	Protein (mg/ml)	Act (nkat ^a /ml)	Sp act (nkat/mg)	Total recovery (%)
Crude extract	260	2.9	12.0	4.1	100
Streptomycin sulfate fractionation	258	2.9	9.5	3.3	79
Ammonium sulfate fractionation	7.3	12.4	155.4	12.5	37
Sephadex G-100	78	0.9	10.3	11.4	26
DEAE-Sephadex + ultrafiltration	26	0.4	19.0	47.5	16
Preparative gel electrophoresis	41	— ^b	0.4	—	—

^a One katal (kat) = amount of activity that converts 1 mol of substrate/s.

^b —, Amount of protein was too small to be determined by the applied method.

FIG. 2. Elution pattern of the aminopeptidase from a DEAE-Sephadex A-50 column. Symbols: (—) protein curve (280 nm); (○) activity curve (405 nm); (---) linear NaCl gradient (0.1 to 0.3 M).

FIG. 3. Polyacrylamide gel disc electrophoresis of three fractions during purification of the aminopeptidase. Conditions: Flat gel (1.5 by 220 by 110 mm), 7.5%, pH 8.9, 30 mA, 90 min, 13°C. Gel stained with Coomassie brilliant blue G-250. (1) After fractionation on Sephadex G-100; (2) after fractionation on DEAE-Sephadex A-50; (3) after preparative gel electrophoresis.

The following enzyme inhibitors did not cause any inhibition of enzyme activity: sodium iodoacetate, phenylmethylsulfonyl fluoride, sodium thioglycolate, and 2-mercaptoethanol. The simultaneous addition of either Zn^{2+} or Co^{2+} ions to the metal-complexing reagents in the assay mixture prevented inhibition of enzyme activity. When activity was inhibited by 10^{-3} M EDTA, the addition of 10^{-5} M Co^{2+} or Zn^{2+} resulted in total reactivation. For total reactivation of the

inhibition caused by 10^{-3} M 1,10-phenanthroline, the concentrations of Co^{2+} and Zn^{2+} needed were 2×10^{-3} and 10^{-3} M, respectively.

Substrate specificity. The aminopeptidase had broad substrate specificity (Table 2). The enzyme hydrolyzed preferentially peptides with basic or aromatic NH_2 -terminal amino acids. Peptides with NH_2 -terminal leucine or serine

FIG. 4. Effect of pH and temperature on the activity of the aminopeptidase. Symbols: (○) pH; (●) temperature. The substrate for determination of enzyme activities as a function of pH had been prepared in universal buffers (0.01 M sodium acetate plus 0.01 M monosodium phosphate plus 0.01 M boric acid).

FIG. 5. Rate of thermal inactivation of the aminopeptidase. Symbols: (○) pH 5.5; (●) pH 7.0. The enzyme solution was dialyzed against universal buffers (0.01 M sodium acetate plus 0.01 M monosodium phosphate plus 0.01 M boric acid, pH 5.5 or 7.0, plus 1 mM CoCl_2) and incubated at various temperatures before the enzyme assay.

FIG. 6. Inhibition of the aminopeptidase activity by the substrate Lys-4-NA. v , velocity of hydrolysis; $[S]$, substrate concentration.

FIG. 7. Effect of various metal ions on the activity of the aminopeptidase. Added metal salts (chlorides or sulfates) and substrate were in 0.05 M phthalate buffer, pH 5.5.

also were good substrates. Important for hydrolysis was also the second amino acid residue of the peptide. When the second position was occupied by a basic or aromatic amino acid, even peptides with NH_2 -terminal amino acids, other-

wise preventing hydrolysis, were hydrolyzed. Peptides having a protected amino acid or an amino acid with D configuration at the NH_2 terminus were not hydrolyzed. Relative rates of hydrolysis of L-leucine-4-nitroanilide (Leu-4-NA) and leucine- β -naphthylamide in comparison with Lys-4-NA (100%) were 13 and 1%, respectively.

DISCUSSION

An aminopeptidase of *L. lactis* was purified by several purification steps. The enzyme was strongly inhibited during preparative gel electrophoresis. Since there was only a limited amount of cell mass at our disposal after each bulk cell cultivation in the relatively small fermentor vessel, we did not further investigate the reasons for the low activity of the enzyme preparation after preparative gel electrophoresis. Analytical polyacrylamide gel electrophoresis of the pooled active fractions of the last purification step yielded a single band. Though we have no direct evidence that the band was the purified aminopeptidase, we believe that it was for the following reasons. The active fractions after preparative gel electrophoresis were concentrated to about 1/50 of the original volume before analytical and SDS-polyacrylamide gel electrophoresis. Analytical as well as SDS-polyacrylamide gel electrophoresis yielded only one band. Furthermore, the molecular weight of this protein as judged by SDS-polyacrylamide gel electrophoresis corresponded well with the molecular weight of the aminopeptidase as determined by gel filtration on Sephadex G-100.

Only a few aminopeptidases of lactic acid bacteria have been purified and characterized up to now. The aminopeptidase of *S. thermophilus* (5) has a molecular weight of 62,000, compared with a molecular weight of 78,000 to 81,000 for the aminopeptidase of *L. lactis*. The aminopeptidase of *L. lactis* was active in a broad pH range between pH 5 and 8, with a broad optimum at pH 6.2 to 7.2. The temperature optimum was at 47.5°C, and the enzyme was stable up to 60°C at pH 5.5. Similar aminopeptidases of lactic acid bacteria are those of *S. thermophilus* (5) and *L. casei* (6). Both are active within a broad pH range and have broad pH optima between pH 6 and 7. On the other hand, both enzymes are unstable at temperatures over 40°C.

The aminopeptidase of *L. lactis* showed substrate and end product inhibition. Like most of the aminopeptidases characterized up to now, the aminopeptidase of *L. lactis* seems to be a metal enzyme. The enzyme activity was inhibited by metal-complexing reagents and reactivated by the addition of Co^{2+} and Zn^{2+} ions

TABLE 2. Substrate specificity^a

Peptide class	Hydrolyzed	Not hydrolyzed
Dipeptides	Gly-Met, Gly-Tyr, Gly-Leu Gly-His, Gly-Trp, Ala-His Ala-Tyr, Ala-Trp, Leu-Leu Leu-Trp, Leu-Tyr, Trp-Trp Trp-Leu, His-Ala, His-Leu Lys-Lys, Tyr-Leu, Glu-Tyr Ser-Leu, Arg-Gly, Val-Tyr Phe-Tyr, Thr-Leu	Gly-Gly, Gly-Ile, Gly-Asp Gly-Pro, Ala-Gly, Ala-Pro Ala-Ala, Asp-Leu, Pro-Leu D-Leu-Tyr
Tripeptides	Leu-Gly-Leu, Leu-Gly-Gly Met-Ala-Ser	Gly-Gly-Gly
Tetra- and pentapeptides	Leu-Trp-Met-Arg-Phe	Gly-Gly-Gly-Gly Gly-Gly-Gly-Gly-Gly
Others	Lys-4-NA, Leu-4-NA Leucine- β -naphthylamide	<i>N</i> -acetyl-leucine-4-nitroanilide <i>N</i> ^a -benzoyl-DL-lysine-4-nitroanilide Glu-phenylalanine-4-nitroanilide <i>N</i> -acetyltyrosine-4-nitroanilide <i>N</i> ^a -benzoylglycine-Arg <i>N</i> -benzoylglycine-Phe

^a Substrates were dissolved to a final concentration of 10 mM in 50 mM Tris buffer, pH 7.0, plus 1 mM CoCl₂. Then 20 μ l of the enzyme solution was added to 100 μ l of substrate solution, and the assay mixture was incubated at 45°C for 4 h. Hydrolysis products were separated by thin-layer chromatography.

after inhibition by metal-complexing reagents. Addition of Zn²⁺ ions did not reactivate the activity of the aminopeptidase of *L. casei* and addition of Zn²⁺ ions to the aminopeptidase of *S. thermophilus* even resulted in inhibition of activity.

The aminopeptidase of *L. lactis* hydrolyzed many peptides with unsubstituted NH₂-terminal amino acids of the L configuration as well as aminoacylnaphthylamides. The broad substrate specificity may be a reason for our finding only one enzyme with aminopeptidase activity in cells of *L. lactis* (Eggimann, Ph.D. thesis, 1980).

Characteristic of the ripening process of Swiss cheese are the different periods during which the cheese is stored at different temperatures between 10 and 24°C. After production of the cheese, the pH is approximately 5.2 and reaches 5.6 to 5.8 during maturation. Comparison of these temperature and pH conditions with data in Fig. 4, shows that the conditions during maturation of the cheese are not optimal for the aminopeptidase. But during all ripening periods, especially at a temperature of 20 to 24°C, the temperature and pH values are still within those zones of the activity curves possessing distinct activity. Since the ripening process of a Swiss cheese lasts 4 to 6 months, there will still be considerable total activity of the aminopeptidase.

It was the aim of our investigation to gain more knowledge about the properties of an enzyme produced by *L. lactis*. Especially with regard to the future application of mixed starter cultures consisting of well-defined single strains

for the manufacture of Swiss cheese, it will be important to know the detailed properties of individual strains. The enzymes of the coagulant are mainly responsible for the hydrolysis of the casein in the cheese to smaller peptides (16). From the results of our investigations, we conclude that the activity of the aminopeptidase of *L. lactis* certainly is an important factor in the further degradation of the smaller peptides formed by the enzymes of the coagulant to the amino acids, resulting in changes in texture and flavor of the cheese.

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