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Purification and characterization of an aminopeptidase from *Lactobacillus helveticus* JCM 1004

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Abstract

An aminopeptidase was purified to homogeneity from a cell-free extract of *Lactobacillus helveticus* JCM 1004 by ammonium sulfate precipitation and chromatography on DEAE–Sepharose, Sephacryl S-300 HR, HiLoad 26/60 Superdex 200pg and Mono-Q 10/10. The purified aminopeptidase had a trimeric structure and a molecular mass of ~129 kDa. The enzyme was optimally active at pH 7.0 and 40 °C. The enzyme was a metallopeptidase, strongly activated by Co^{2+} and inhibited by Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} and EDTA. The enzyme showed high activity toward *p*-nitroanilide derivatives (pNA) of amino acids and a peptide, dipeptides and tripeptides that had hydrophobic amino acids (Leu, Ala and Phe) or diaminocarboxylic acids (Lys and Arg) at the N-termini but not *p*-nitroanilide derivatives or peptides with proline at their N-termini or C-termini, such as Pro–pNA, Gly–Pro–pNA, Pro–Leu and Ala–Pro.

Keywords: *Lactobacillus helveticus*; Aminopeptidase; Purification; Characterization

1. Introduction

Lactobacillus helveticus is a common constituent of mesophilic lactic starters used in the cheese industry. However, these nutritionally fastidious bacteria require a greater concentration of free amino acids or peptides than is present in milk (Prichard & Coobear, 1993; Thomas & Pritchard, 1987). Proteolytic activity is thus very important for growth in milk. Proteolysis during lactic fermentation is carried out mainly by proteolytic enzymes of lactic acid bacteria and it is well known that lactic acid bacteria produce several endopeptidases and exopeptidases that hydrolyze milk proteins and release the free amino acids required for cell growth. These proteolytic enzymes are also particularly important in the formation of flavours during cheese ripening (Fox, 1989; Visser, 1993).

Some researchers have been interested in screening combinations of lactic acid bacteria to improve the organoleptic and rheological properties of cheese. In related studies, a few peptidases have been isolated from *Lactobacillus acidophilus*, *Streptococcus diacetylactis* (Desmazeaud & Zevaco, 1979) and *Lactobacillus lactis* (Eggimann & Bachmann, 1980) and their properties have been characterized. However, there have been fewer studies of proteolytic enzymes from *L. helveticus* than studies of enzymes from other lactic acid bacteria used in fermented milk products. The present work reports the purification and some characterization of an aminopeptidase from *L. helveticus* JCM 1004.

2. Materials and methods

2.1. Reagents

Diethylaminoethyl (DEAE)–Sepharose, Sephacryl-S-300 HR, HiLoad 26/60 Superdex 200pg and Mono-Q

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10/10 were purchased from Amersham Biosciences AB, Uppsala, Sweden. *p*-Nitroanilide (pNA) derivatives of amino acids and a peptide, dipeptides and tripeptides were obtained from Sigma Chemical Co., St. Louis, Missouri, USA, Wako Pure Chemical Industries Ltd., Tokyo, Japan and Peptide Institute Inc., Osaka, Japan. Protein assay calibration kits for gel filtration and SDS electrophoresis were purchased from Amersham Bioscience UK Ltd., Little Chalfont, Buckinghamshire, UK. High-performance centrifugal concentrators were purchased from Orbital Biosciences, LLC, Winsor Lane, Tosfield, USA. Other chemicals and reagents used were also of analytical grade and were obtained from Wako Pure Chemical Industries Ltd., Tokyo, Japan.

2.2. Microorganisms, growth conditions and preparation of cell-free extract

L. helveticus JCM 1004 was obtained from the Institute of Physical and Chemical Research (RIKEN), Wako, Saitama, Japan. The organisms were grown in 4 l of MRS (0.5% yeast extract, 0.5% polypeptone, 0.2% lactose, 1.0% glucose and 0.1% $MgSO_4 \cdot 7H_2O$) media at 37 °C for 20 h, with shaking at 120 rpm.

Cells were harvested by centrifugation at 5,000*g* for 10 min at 10 °C and washed three times with distilled water. The cells were suspended in 50 mM Tris–HCl buffer (pH 7.0) and sonicated for 25 min at 10 °C. The cell debris was removed by centrifugation at 15,000*g* for 15 min at 10 °C. Clear supernatant was lyophilized and used as cell-free extract.

2.3. Measurement of aminopeptidase activity

Aminopeptidase activity was measured during purification by the procedure of Soda and Desmazeaud (1982) with L-lysine-*p*-nitroanilide (16.4 mM) as the substrate. The incubation mixture contained 0.05 ml of substrates, 2.85 ml of 50 mM Tris–HCl 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 by absorbance at 410 nm. One unit of aminopeptidase activity was defined as the amount of enzyme that produced an increase of 0.1 unit of A_{410} per min at 37 °C.

2.4. Determination of proteins

Protein concentrations were determined throughout the purification procedure by the method of Lowry, Rosebrough, Farr, and Randall (1951) with bovine serum albumin as the standard.

2.5. Purification of the enzyme

2.5.1. Ammonium sulfate precipitation

The cell-free extract was dissolved in 50 mM Tris–HCl buffer (pH 7.0) and proteins in the cell-free extract were concentrated by salting out with solid ammonium sulfate at about 90% of saturation. The precipitate was dissolved in 50 mM Tris–HCl buffer (pH 7.0) and dialysed against the same buffer.

2.5.2. DEAE–Sepharose chromatography

Six millilitres of crude enzyme preparation were applied to a DEAE–Sepharose column (2.6×60 cm^2) equilibrated with 50 mM Tris–HCl buffer (pH 7.0). Proteins were eluted at a flow rate of 2.0 ml/min with a linear NaCl gradient (250 ml buffer – 250 ml 200 mM NaCl buffer). The fractions containing the aminopeptidase activity were pooled, dialyzed against 50 mM Tris–HCl buffer (pH 7.0) and lyophilized.

2.5.3. Sephacryl S-300 HR chromatography

The lyophilized fraction from the previous step was dissolved in 6.0 ml of 50 mM Tris–HCl buffer (pH 7.0) and applied to a Sephacryl S-300 column (2.6×60 cm^2) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.0) containing 200 mM NaCl. Proteins were eluted with the same solution at a flow rate of 2.0 ml/min. The fractions containing the aminopeptidase activity were pooled and then concentrated using high-performance centrifugal concentrators equipped with membrane MW 5000.

2.5.4. HiLoad 26/60 Superdex 200pg chromatography

Eight millilitres of sample obtained from the previous step were applied to a HiLoad 26/60 Superdex 200pg column (2.6×60 cm^2) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.0) containing 200 mM sodium chloride. Proteins were eluted at a flow rate of 2.0 ml/min with the same solution. The fractions containing the aminopeptidase activity were pooled and concentrated using a microultrafiltration system equipped with membrane MW 10000.

2.5.5. Mono-Q 10/10 chromatography

One millilitre of the concentrated enzyme solution was applied to a Mono-Q 10/10 column (1.0×10 cm^2) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.0). Proteins were eluted with a linear NaCl gradient (50 ml buffer – 50 ml 200 mM NaCl buffer) at a flow rate of 2.0 ml/min; the gradient went from 0 to 90 ml over 45 min. The fractions containing the aminopeptidase activity were pooled, dialyzed against 50 mM Tris–HCl buffer (pH 7.0) and lyophilized.

2.6. Effects of pH on the enzyme activity

The effects of pH from 4.5 to 9.0 on the enzyme activity were measured in 50 mM Tris–HCl buffer at 37 °C with L-lysine-*p*-nitroanilide as the substrate.

To assess the effect of pH on the enzyme stability, the enzyme was dissolved in 50 mM Tris–HCl buffers within the pH range 4.0–9.5 and incubated for 30 min at 37 °C. The residual activity was subsequently measured at 37 °C with L-lysine-*p*-nitroanilide as substrate, as described above.

2.7. Effects of temperature on the enzyme activity and stability

The effects of temperature, from 19 to 46 °C, on the enzyme activity were measured in 50 mM Tris–HCl buffer (pH 7.0) with L-lysine-*p*-nitroanilide as the substrate.

To assess the thermal stability of the enzyme, the enzyme solutions were incubated for 30 min at temperatures ranging from 30 to 65 °C. The residual activity was subsequently measured at 37 °C with L-lysine-*p*-nitroanilide as the substrate as described above.

2.8. Molecular mass determination

The relative molecular mass of the purified enzyme was determined by gel filtration HPLC using a column of HiLoad 26/60 Superdex 200pg (2.6×60 cm^2) equilibrated with 50 mM Tris–HCl buffer, pH 7.0, containing 200 mM NaCl. Aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used as M_r standards.

The molecular mass and purity of the enzyme were also determined by SDS–PAGE according to the method of Laemmli Laemmli (1970) with gels containing 12% acrylamide. Phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as molecular size markers.

2.9. Effects of metal ions and EDTA on enzyme activity

The enzyme was pre-incubated in the presence of various metal ions (Ca^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}) and EDTA for 30 min at 37 °C at a final concentration of 1.0 or 2.0 mM in 50 mM Tris–HCl buffer, pH 7.0. The enzyme activity was measured after incubation for 30 min at 37 °C with L-lysine-*p*-nitroanilide as the substrate.

2.10. Substrate specificity

Substrate specificity, of the purified aminopeptidase on a number of peptides, was determined using the Cd-ninhydrin assay (Folkertsma & Fox, 1992). The reaction mixture consisted of 0.05 ml enzyme preparation, 0.05 ml substrate (10 mM in methanol or deionized water) and 0.4 ml 50 mM Tris–HCl buffer, pH 7.0. The mixture was incubated for 30 min at 37 °C and the reaction was terminated by the addition of 1 ml Cd-ninhydrin reagent. The contents were thoroughly mixed and heated at 84 °C for 5 min and then cooled immediately on ice; the absorbance at 507 nm was measured. One unit (U) of enzyme activity was defined as the amount of enzyme required to give an increase of one absorbance unit at 507 nm per min under the assay conditions. The hydrolytic activity of the purified aminopeptidase against several *p*-nitroanilide derivatives of amino acids and a peptide was determined as described above. Rates of Lys–pNA and Leu–Arg hydrolysis were taken as 100%.

3. Results

3.1. Purification of aminopeptidase from L. helveticus JCM 1004

The specific aminopeptidase activity observed with Lys–pNA as the substrate at each purification step is summarized in Table 1. The enzyme was purified about 116-fold from the cell-free extract by ammonium sulfate fractionation and four steps of column chromatography and the recovery activity was about 9.5%. The enzyme

Table 1
Purification of aminopeptidase from *Lactobacillus helveticus* JCM 1004

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Cell-free extract	1216	612	0.5	100	1.0
Ammonium sulfate fractionation	463	516	1.1	84.3	2.2
DEAE–Sepharose	58	309	5.3	50.5	10.6
Sephacryl S-300	15	167	11.1	27.3	22.2
Superdex G-200	3	82	27.3	13.4	54.6
Mono-Q 10/10	1	58	58	9.5	116

obtained from the final Mono-Q 10/10 chromatography step showed a single protein band upon SDS–PAGE examination (Fig. 5).

3.2. Effects of pH and temperature on activity

The enzyme showed high activity in the pH range 6.0–7.0, with optimum activity at pH 7.0 (Fig. 1). Very little activity was found at pH 9.0, but about 25% of the maximum activity was found at pH 5.0. The enzyme was stable for 30 min over the pH range 4.5–8.0 (Fig. 2).

The effects of temperature on the enzyme activity and stability are shown in Figs. 3 and 4, respectively. At pH 7.0, the enzyme showed high activity at temperatures from 34 to 43 °C, with maximum activity at 40 °C. The enzyme was stable at temperatures up to 40 °C but rapidly lost activity at temperatures above 45 °C.

3.3. Molecular mass determination

The relative molecular mass of the enzyme was estimated to be 129 kDa by gel filtration on a HiLoad 26/60 Superdex 200pg column. Electrophoresis on SDS-Polyacrylamide gel showed one band for the enzyme with a molecular mass of ~43 kDa under denaturing conditions (Fig. 5). These results suggest that the enzyme is composed of three subunits of equal size.

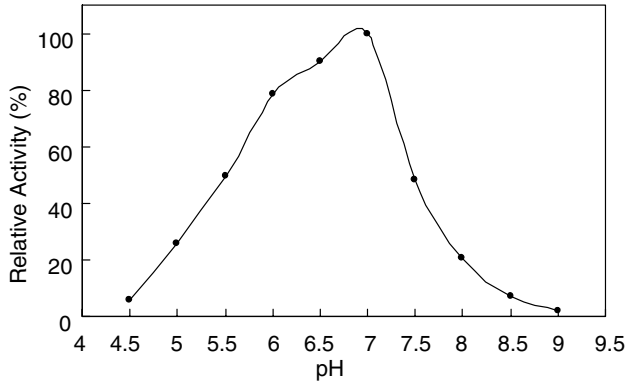

Fig. 1. pH dependence of enzyme activity with L-lysine-*p*-nitroanilide as the substrate.

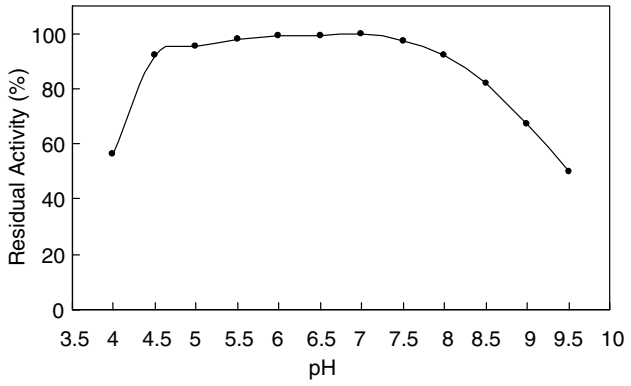

Fig. 2. Residual activity of aminopeptidase after 30 min incubation at pH ranging from 4.0 to 9.5. The enzyme activity was measured as described in Section 2.

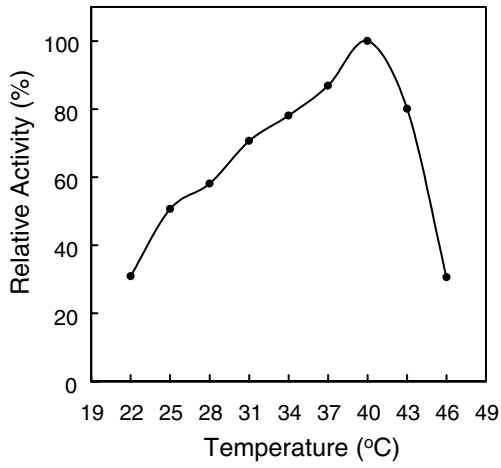

Fig. 3. Effects of temperature on the activity of aminopeptidase.

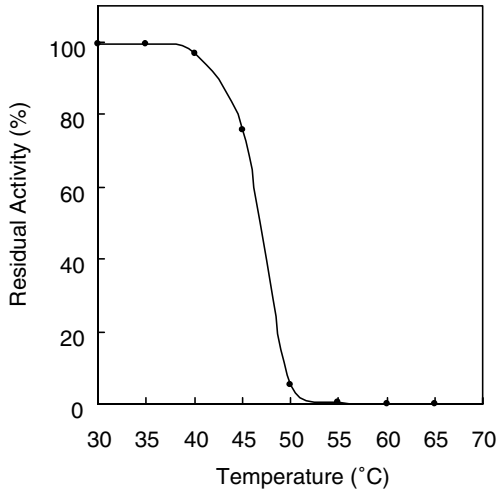

Fig. 4. Residual activity of aminopeptidase after 30 min incubation at temperatures ranging from 30 to 65 °C. The enzyme activity was measured as described in Section 2.

3.4. Effects of metal ions and EDTA on enzyme activity

The effects of various divalent metal ions and EDTA on the enzyme activity are summarized in Table 2. The enzyme was strongly activated by Co^{2+} and inhibited by Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} and EDTA. The enzyme was not significantly affected by Mg^{2+}, Ca^{2+} or Mn^{2+}.

3.5. Substrate specificity

The substrate specificity of the purified enzyme was tested with several pNA derivatives of amino acids and a peptide, dipeptides and tripeptides. The relative rates of

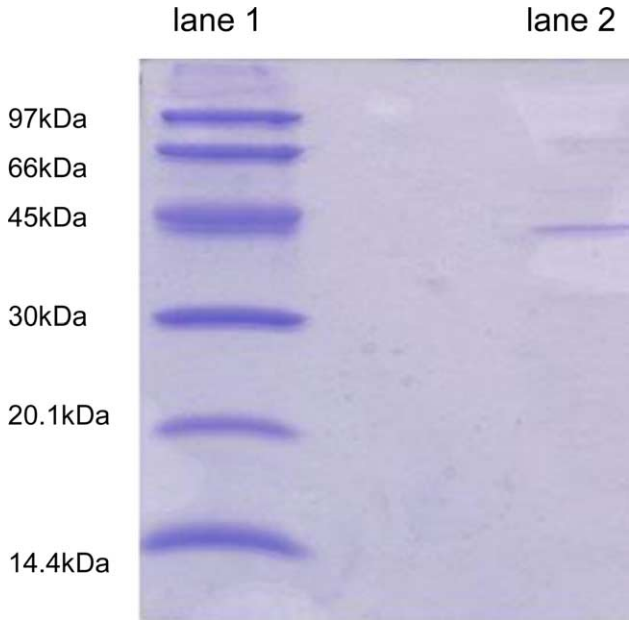

Fig. 5. SDS–PAGE of fractions containing aminopeptidase obtained after chromatography on Mono-Q (lane 2) and molecular mass standards (lane 1).

Table 2
Effect of metal ions and EDTA on enzyme activity

Compound	Relative activity (%)	
	Compound concentration (mM)	
	1.0	2.0
None	100	100
$CoCl_2$	151	168
$MgCl_2$	99.6	96.8
$CaCl_2$	102	97.1
$ZnCl_2$	80.2	71.1
$MnCl_2$	101	95.2
$CuCl_2$	71.7	60.0
$NiCl_2$	73.9	70.1
$FeCl_2$	45.7	41.8
EDTA	61.4	58.3

hydrolysis for various substrates are summarized in Table 3. The rate of Lys–pNA hydrolysis is taken as 100%. The enzyme was active towards a number of dipeptides with relatively hydrophobic amino acids at their N-termini but did not hydrolyze dipeptides containing proline at their N-termini nor C-termini.

4. Discussion

In this study, an aminopeptidase from the cell-free extract of *L. helveticus* JCM 1004 was purified to homogeneity about 116-fold over the cell-free extract with a recovery activity of 9.5%.

The molecular mass of the purified enzyme was ~129 kDa by gel permeation chromatography and ~ 43 kDa by SDS–PAGE, suggesting a trimeric structure for the native enzyme. Similar molecular masses have been reported for the trimer aminopetidase from *Streptococcus cremoris* (Exterkate & De Veer, 1987) and similar subunit molecular mass has also been reported for the aminopeptidases from *Staphylococcus chromogenes* (Yoshpe-Besancon et al., 1993), *Streptococcus faecium* and *Brevibacterium* (Mousesyan et al., 1992; Sullivan, Muchnicky, Davidson, & Jago, 1977). However, the enzyme purified in this study is different (in molecular masses) from aminopeptidases of *L. helveticus* LHE-511 (monomer, ~92 kDa) (Miyakawa, Kobayashi, Shimamura, & Tomita, 1992), *L. lactis* subsp. *cremoris* AM2 (hexamer, ~0300 kDa) (Neviani, Boquien, Monnet, Thanh, & Gripon, 1989), *L. acidophilus* R-26 (trimer, ~156 kDa) (Edward & David, 1984) and *Lactobacillus curvatus* DPC2024 (dimer, ~64 kDa) (Magboul & McSweeney, 1999).

Table 3
Substrate specificity of the enzyme

Substrate	Relative activity (%)
Lys–pNA	100
Leu–pNA	55
Ala–pNA	46
Arg–pNA	87
Phe–pNA	18
Gly–Pro–pNA	0
Phe–Ala	17
His–Leu	7
Pro–Leu	0
Lys–Gly	6
Lys–Asp	2
Arg–Gly	5
Arg–Glu	2
Leu–Arg	100
Leu–Met	72
Leu–Tyr	29
Leu–Phe	35
Leu–Gly	2
Ala–Gly	3
Ala–Ala	7
Ala–Pro	0
Ala–Val	9
Ala–Met	17
Lys–Tyr–Lys	39
Lys–Trp–Lys	35
Arg–Ser–Arg	46
Leu–Leu–Tyr	30
Leu–Gly–Gly	7

The enzyme showed maximal activity at pH 7.0 and 40 °C, which are a similar pH and temperature to the optimum conditions of aminopeptidases from strains of *Lactococcus* and *Lactobacillus* (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). Most aminopeptidases from lactic acid bacteria are rapidly inactivated at acidic pH (Desmazeaud & Zevaco, 1979; Morsi, Desmazeaud, & Bergere, 1978; Neviani et al., 1989). In contrast, the aminopeptidase from *L. helveticus* JCM 1004 is more stable over a wide pH range (4.5–8.0). Enzyme stability

under acidic conditions (pH 4.5–5.0) probably plays an important role in cheese ripening.

Like some of the aminopeptidases from lactic acid bacteria, the enzyme isolated in this study was activated by Co^{2+} and inhibited by Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} and EDTA (Edward & David, 1984; Kaminogawa, Ninomiya, & Yamauchi, 1984; Magboul & McSweeney, 1999; Miyakawa et al., 1992). These results suggest that the aminopeptidase from *L. helveticus* JCM 1004 is a metalloenzyme.

The aminopeptidase from *L. helveticus* JCM 1004 hydrolyzed dipeptide-type *p*-nitroanilide derivatives containing hydrophobic amino acids (Leu, Ala and Phe) and diaminocarboxylic acids (Lys and Arg) at the N-termini but not *p*-nitroanilide derivatives or peptides with proline at their N-termini nor C-termini, such as Pro–pNA, Gly–Pro–pNA, Pro–Leu and Ala–Pro. The enzyme showed broad substrate specificity with leucyl and alanyl dipeptides. The enzyme activities observed with lysyl or arginyl tripeptides such as Lys–Tyr–Lys, Lys–Trp and Arg–Ser–Arg, were considerably higher than those observed with the dipeptides (Table 3). Similar specificities were observed for aminopeptidases isolated from *L. helveticus* ITG LH1 and LHE 511 (Degraeve & Martial-Gros, 2003; Miyakawa et al., 1992).

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