A new, broad-substrate-specificity aminopeptidase from the dairy organism *Lactobacillus helveticus* SBT 2171

Masahiro Sasaki, Boukje W. Bosman and Paris S. T. Tan

Author for correspondence: Paris S. T. Tan. Tel: +31 50 574 57 42. Fax: +31 50 574 57 66.

Snow Brand European
Research Laboratories
B. V., Zernikepark 6, 9747
AN, Groningen, The
Netherlands

An aminopeptidase with a very broad substrate specificity was purified to homogeneity from *Lactobacillus helveticus* SBT 2171 by FPLC. The enzyme was purified 144-fold from a cell-free extract with a yield of 16%. The purified enzyme appeared as a single band on an SDS-PAGE gel. It had a molecular mass of 95 kDa and an isoelectric point of 4.9. The enzyme hydrolysed a large range of naphthylamide- and nitroanilide-substituted amino acids, as well as several di-, tri- and oligopeptides. It also exhibited significant proline-iminopeptidase-like activity, since it hydrolysed several proline-containing peptides. Prolyl-*p*-nitroanilide was hydrolysed with a low affinity (Michaelis-Menten constant 0.6 mM) and a $V_{\max}$ of $2.5 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ while lysyl-*p*-nitroanilide was hydrolysed with a high affinity [K_m 0.003 mM; $V_{\max}$ $37.5 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]. The aminopeptidase activity, which was optimal between pH 6.0 and 8.0 and at 50 °C, was very stable at 30 °C for more than 7 d. The activity lost by treatment with the thiol-blocking reagents could be restored with β -mercaptoethanol, while Co^{2+} and Mn^{2+} restored the activity of the EDTA-treated enzyme. Immunological experiments with antibodies raised against the aminopeptidases from *Lactococcus lactis* and *Lb. helveticus* clearly showed that both aminopeptidases are at least immunologically different from each other.

Keywords: lactic acid bacteria, *Lactobacillus helveticus*, proteolytic system, aminopeptidase

INTRODUCTION

The importance of the proteolytic system of lactic acid bacteria for the modern dairy industry is well established and widely recognized. Compounds derived from casein degradation play a key role in the flavour development during the ripening of cheese. The subject has been reviewed extensively (Kok & de Vos, 1994; Pritchard & Coolbear, 1993; Tan *et al.*, 1993). In lactococci, the best studied lactic acid bacteria, the proteolytic cleavage of casein is initiated by cell-wall-bound proteinases that are well characterized both genetically and biochemically. Peptides, released from casein by the proteinase, are taken up via di-, tri-peptide (DtpT) and oligopeptide (Opp) transport systems, of which the latter is essential in the process of casein utilization during growth on milk (Kunji *et al.*, 1995). Intracellularly located peptidases subsequently degrade the peptides further to amino acids. Several peptidases have been purified from lactococci

(endopeptidases: PepO, PepF, LEP; aminopeptidases: PepN, PepC; glutamylaminopeptidase: PepA; X-prolyl dipeptidyl aminopeptidase: PepX; tripeptidase: PepT; dipeptidase: DIP) and the structural genes of most of the enzymes have been cloned and sequenced.

Although interest in the proteolytic systems of lactobacilli has increased over the last few years, they have been studied to a much lesser extent. For instance, from *Lactobacillus helveticus*, which is isolated from sour milk, cheese starter cultures and Emmenthal and Gruyère cheese (Law, 1984), only a few proteolytic enzymes have been purified or cloned (Miyakawa *et al.*, 1992; Nowakowski *et al.*, 1993; Blanc *et al.*, 1993; Dudley & Steele, 1994). *Lb. helveticus* is one of the most nutritionally fastidious lactic acid bacteria, and requires more exogenous amino acids than any other lactic acid bacterial species (Morishita *et al.*, 1981; Chopin, 1993). This aspect makes the study of its proteolytic system of great interest.

Previously we reported that *Lb. helveticus* SBT 2171 is the most powerful proteolytic strain out of 178 other *Lacto*-

Abbreviation: *p*-CMBS, *p*-chloromercuribenzenesulfonate.

bacillus and *Lactococcus* strains. Further studies showed that several individual enzymes were responsible for this superior activity (Sasaki *et al.*, 1995a, b; Tan *et al.*, 1995). This report describes the purification and characterization of a new general aminopeptidase from a crude cell-free extract from *Lb. helveticus* SBT 2171. This enzyme has a broad substrate specificity and appears to be distinctly different from aminopeptidases purified previously from other lactic acid bacteria.

METHODS

Organisms and preparation of cell-free extract. *Lb. helveticus* SBT 2171 was obtained from the Snow Brand Type Culture Collection, Tokyo, Japan. *Lactococcus lactis* subsp. *cremoris* Wg2 was obtained from the Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands. The organisms were routinely subcultured (3 ×) in MRS broth (Merck) at 30 or 37 °C and maintained as 'stock-culture' in MRS containing 10% (v/v) glycerol at -55 °C.

Lb. helveticus SBT 2171 was grown in 6 l MRX broth to an OD₆₆₀ of 1.0 (Sasaki *et al.*, 1995a, b), harvested by centrifugation at 7500 g for 10 min at 4 °C, washed twice in 250 ml 50 mM HEPES (pH 7.0), and suspended in 60 ml of this buffer to an OD₆₆₀ of 100. Cells were disrupted by sonication (Vibra Cell, Sonics & Materials) for four periods of 10 min at 0 °C. Cell-free extracts were obtained by centrifugation of the disrupted cells for 10 min at 30000 g and 4 °C.

Enzyme assays. Acyl-*p*-nitroanilide-hydrolysing activities were determined by the method of Exterkate (1976). Enzyme was incubated for 15 min with a 2 mM solution of acyl-*p*-nitroanilide. The reaction was stopped by the addition of acetic acid to a final concentration of 10% (v/v), and the *p*-nitroaniline formed was measured at 410 nm (Hitachi U-2000 spectrophotometer). All enzyme activities were measured at 30 °C in 50 mM HEPES (pH 7.0).

Hydrolysis of several peptides was detected by TLC. Peptidase activity was assayed as follows. A reaction mixture consisting of a peptide (5 mM) in 50 mM HEPES (pH 7.0) and purified enzyme was incubated at 30 °C. After 2.5 h, the reaction was stopped by the addition of acetic acid to a final concentration of 10% (v/v). The reaction mixture (2–5 µl) was then spotted onto a HPTLC precoated silica gel 60 plate (E. Merck). A mixture of *n*-butanol/acetic acid/water (3:1:1, by vol.) or in some cases 75% (w/v) phenol/water was used as mobile phase. As a control, 2 mM of each peptide substrate was also spotted on the plate. The plates were stained by spraying with 0.1% (w/v) ninhydrin in 99% (v/v) ethanol. Peptides and amino acids became visible after incubation for 5 min at 80 °C.

The LRA ZYM AP kits were obtained from Laboratoire de Recherche API. To each cupule, containing dehydrated chromogenic substrates, 75 µl 50 mM HEPES (pH 7.0) containing 0.55 µg purified enzyme was added after which the strips were incubated at 37 °C for 3.5 h. Hydrolysing activities were determined as described in the manual provided by the manufacturer. The activities were measured by comparing the colour developed to the colour chart provided by the manufacturer.

HPLC analysis. The HPLC study was performed with a 2157 automatic sampler, a low pressure mixer, a 2248 pump, a VWM 2141 detector (all Pharmacia LKB) and a Epson LQ-570⁺ recorder. The HPLC system was controlled and the data

acquisition was performed with the HPLC manager software (Pharmacia LKB). The reaction mixture contained 100 µl 2 mM peptide substrate in deionized water, 10 µl enzyme solution and 100 µl 100 mM HEPES buffer (pH 7.0). After incubation for 5 h at 30 °C, 10 µl of the mixture was injected on a Capcell Pak SG120 C18 column (4.6 × 250 mm; Shiseido). The mobile phase was 0.1% (v/v) trifluoroacetic acid in acetonitrile/H₂O, which was increased linearly from 0–60% acetonitrile in 15 min at a flow rate of 1.0 ml min⁻¹. The effluent was monitored at 214 nm.

Q-Sepharose ion-exchange column chromatography. A HiLoad Q-Sepharose 26/10 (Pharmacia LKB) column of the FPLC (Pharmacia LKB) apparatus was equilibrated with 20 mM Tris/HCl (pH 8.0). The cell-free extract, which was obtained after sonication and centrifugation of *Lb. helveticus* SBT 2171, was filtered through a 0.22 µm membrane filter and was subsequently applied to the column. The enzyme was eluted with a flow rate of 2 ml min⁻¹ with a linear gradient of 0.1–0.4 M NaCl in the same buffer. Fractions of 4 ml were collected and tested for lysyl-*p*-nitroanilide-hydrolysing activity. The highest activity was found in several fractions which were eluted at 0.28–0.32 M NaCl.

Phenyl-Superose chromatography. A Phenyl-Superose HR 5/5 column (Pharmacia LKB) of the FPLC apparatus was equilibrated with 20 mM Tris/HCl (pH 7.5) containing 1.7 M (NH₄)₂SO₄. Before the enzyme solution was applied to the column, (NH₄)₂SO₄ was added to the pooled enzyme fractions to the same concentration as in the equilibration buffer. The enzyme was eluted with a linear gradient of 1.36–0.6 M (NH₄)₂SO₄ in 20 mM Tris/HCl (pH 7.5) with a flow rate of 0.5 ml min⁻¹. Fractions of 1 ml were collected and tested for their lysyl-*p*-nitroanilide-hydrolysing activity. The highest activity was found in the peak fraction which eluted at 1.25–1.15 M (NH₄)₂SO₄.

Gel-filtration chromatography on Sephacryl S-100 HR. The enzyme solution was concentrated in a filtration unit with Amicon YM-10 membranes (molecular mass cutoff 10 kDa) to a final volume of about 3 ml and subsequently applied to a HiLoad Sephacryl S-100 HR column 26/60 which was equilibrated with 20 mM Tris/HCl (pH 7.5) containing 0.2 M NaCl. Elution was carried out with the same buffer at a flow rate of 1.5 ml min⁻¹ and fractions of 3 ml were collected.

Mono Q column chromatography. A Mono Q HR 5/5 column (Pharmacia LKB) of the FPLC apparatus was equilibrated with 20 mM Tris/HCl (pH 7.0). The enzyme solution obtained by the gel filtration was concentrated as described above and subsequently diluted with 20 mM Tris/HCl (pH 7.0). The enzyme was eluted from the column with a linear gradient of 0.1–0.35 M NaCl in the same buffer with a flow rate of 1 ml min⁻¹. Fractions of 0.5 ml were collected and tested for lysyl-*p*-nitroanilide-hydrolysing activity. The purified aminopeptidase was eluted at 0.21–0.23 M NaCl in 20 mM Tris/HCl (pH 7.5).

This purification procedure was repeated and all characterization experiments were carried out with the purified peptidase, in duplicate and in some cases in triplicate. Most of the characterization experiments were repeated and all results were reproducible.

SDS-PAGE. SDS-PAGE was performed on a Mini-Protein II (Bio-Rad) apparatus with a 12% (w/v) polyacrylamide gel. Conditions and sample preparations were as described by the manufacturer. The low molecular mass SDS-PAGE protein standards, supplied by the same company, were used as reference proteins. The gels were stained with Coomassie Brilliant Blue R250 and with Silver Stain Plus (Bio-Rad).

Isoelectric focusing. Isoelectric focusing on slab gels was performed on a Phast system with ready-to-use PhastGel IEF 3–9 and PhastGel IEF 4–6.5 (Pharmacia LKB). The isoelectric point of the enzyme was determined by using the pI references supplied by the same company. Gels were automatically stained with silver by the Phast system.

Preparation of the antisera and immunoblotting. The purified aminopeptidase of *Lb. helveticus* SBT 2171 was separated by SDS-PAGE (10% gel) and subsequently stained by CuCl_2 as described by Lee *et al.* (1987). After the protein band was excised from the gel and destained, it was electroeluted from the gel strip by using a Bio-Rad 422 Electroeluter. The conditions and sample preparation were as described by the manufacturer. The antiserum to the enzyme was raised in rabbits after immunization by using the following protocol. A 50 μg sample of electroeluted aminopeptidase was injected with complete Freund's adjuvant into the rabbit. After 12 and 22 d, a first and a second booster injection of a further 50 μg was given. The rabbit was bled 11 d after the second booster injection. After the serum was prepared from blood, polyclonal antibodies were purified with a protein A column (Pharmacia LKB). The antibodies from *Lc. lactis* subsp. *cremoris* Wg2 were obtained from the Department of Microbiology, University of Groningen, The Netherlands. Immunoblotting experiments were carried out as described previously by Sasaki *et al.* (1995b).

Effect of divalent cations and chemical reagents on the enzyme activity. Purified enzyme (1 μg protein ml^{-1}) was preincubated with 20 μM EDTA in 50 mM HEPES (pH 7.0) for 10 min at 20 °C, and divalent cations were then added at different concentrations and left for another 10 min at 20 °C. After equilibration in a cuvette for 5 min at 30 °C, 1 mM lysyl-*p*-nitroanilide was added. The reaction was stopped after 5 min by addition of 10% (v/v) acetic acid. The enzyme activity was determined by measuring the *p*-nitroaniline formed at 410 nm in a Hitachi U-2000 spectrophotometer.

The effects of chemical reagents were determined by preincubating purified enzyme (1 μg protein ml^{-1}) 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 agents. Purified enzyme (1 μg protein ml^{-1}) was preincubated with 200 μM *p*-chloromercuribenzenesulfonate (*p*-CMBS) in 50 mM HEPES (pH 7.0) for 10 min at 20 °C. Some of the treated enzyme was again incubated with different concentrations of β -mercaptoethanol for 10 min at 20 °C prior to the addition of 1 mM lysyl-*p*-nitroanilide. The enzyme activity was determined as described above.

Amino acid analysis. Amino acid analysis of the purified enzyme was performed with an Aminoquant (Hewlett-Packard) by Eurosequence.

Protein determination. Protein concentrations were determined by using the Bio-Rad Bradford Microassay procedure, with BSA as the standard.

RESULTS

Enzyme purification

The enzyme purification is summarized in Table 1. By this procedure, the enzyme could be purified from the crude extract approximately 144-fold with a yield of 16%. SDS-PAGE analysis of the enzyme fractions during the

purification of the lysyl-*p*-nitroanilide-hydrolysing enzyme is shown in Fig. 1.

Molecular mass and isoelectric point

On SDS-PAGE gels, the purified enzyme gave only one band (also in the presence of β -mercaptoethanol). The molecular mass was estimated to be 95 kDa on an SDS-PAGE gel which was stained with Coomassie Brilliant Blue R250 (Fig. 1, lanes 1–7) and with silver (Fig. 1, lane 8). The molecular mass was confirmed by gel filtration (results not shown), which indicates that the enzyme is probably a monomer consisting of a single subunit.

The pI of the enzyme was estimated to be 4.9 by isoelectric focusing on a 5% (w/v) polyacrylamide gel containing Pharmalyte 4–6.5 in the Phast system (data not shown).

Immunoblotting of the purified enzyme

Purified aminopeptidase from *Lb. helveticus* SBT 2171 (Fig. 2, lanes 3 and 4) and cell-free extract from *Lc. lactis* subsp. *cremoris* Wg2 (Fig. 2, lane 2) were tested with monoclonal (a) and polyclonal (b) antibodies raised against the purified aminopeptidase from *Lc. lactis* subsp. *cremoris* Wg2. The results clearly show that a 95 kDa band became visible in the cell-free extract from *Lc. lactis* subsp. *cremoris* Wg2, indicating the presence of PepN. No immunoresponse was observed with the purified aminopeptidase from *Lb. helveticus* SBT 2171.

In addition, immunoblotting experiments were done with polyclonal antibodies which were raised against the purified aminopeptidase from *Lb. helveticus* SBT 2171 (Fig. 2c). The results with the cell-free extracts from strain SBT 2171 (Fig. 2c, lane 5) show that only one clear band was revealed, with an electrophoretic mobility identical to that of the aminopeptidase, which indicates that the antiserum obtained was monospecific. No immunoresponse whatsoever was observed with the cell-free extracts from *Lc. lactis* subsp. *cremoris* (Fig. 2c, lane 2). These results strongly indicate that the 95 kDa aminopeptidases from *Lb. helveticus* SBT 2171 and *Lc. lactis* subsp. *cremoris* Wg2 are immunologically distinct from each other.

Effect of temperature and pH on enzyme activity

The effect of temperature on the aminopeptidase activity was measured in the range 5–80 °C. The enzyme mixture was equilibrated for 5 min at the test temperatures before the addition of the substrates. The optimum temperature for lysyl-*p*-nitroanilide and for prolyl-*p*-nitroanilide hydrolysis was found to be 50 °C (data not shown). The enzyme activity was stable when preincubated for 30 min up to 50 °C. When preincubated aseptically by passing through a 0.22 μm pore-size filter at 30 °C, the enzyme activity remained stable for more than 1 week, but decreased rapidly when preincubated at 55 °C for more than 30 min (data not shown).

Table 1. Purification of a lysyl-*p*-nitroanilide-hydrolysing aminopeptidase from *Lb. helveticus* SBT 2171

Purification step	Total protein (mg)	Total activity ($\mu\text{mol min}^{-1}$)	Yield (% activity)	Specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]	Purification (-fold)
Cell-free extract	1246.00	286.6	100.0	0.23	1.0
HiLoad Q-Sepharose HP (26/10)	80.75	204.3	71.3	2.53	11.0
Phenyl-Superose HR (5/5)	10.68	153.9	53.7	14.41	62.7
HiLoad Sephacryl S-100 HR (26/60)	3.11	86.5	30.2	27.81	120.9
Mono Q HR (5/5)	1.38	45.6	15.9	33.04	143.7

Fig. 1. SDS-PAGE analysis of the enzyme fractions during the purification of the aminopeptidase from *Lb. helveticus* SBT 2171. Electrophoresis was performed on an SDS-PAGE gel (12%, w/v, polyacrylamide) with several reference proteins (lanes 1 and 7), 6 μg protein from crude cell-free extract (lane 2), 3.3 μg protein after the Q-Sepharose HP (lane 3), 1.8 μg protein after the Phenyl-Superose (lane 4), 0.8 μg protein after the Sephacryl S-100 HR (lane 5) and 0.5 μg and 1.3 μg protein after Mono Q column chromatography (lanes 6 and 8). Lanes 1–7 were stained with Coomassie Brilliant Blue R250 and lane 8 was stained with silver. By this procedure, the enzyme was purified three times (lane 8, third purification) and the results were reproducible. **Fig. 2.** Western blots of analysis of cell-free extract from *Lc. lactis* subsp. *cremoris* Wg2 and *Lb. helveticus* SBT 2171. The blots were incubated with monoclonal antibodies (a) and polyclonal antibodies (b) raised against the 95 kDa aminopeptidase from *Lc. lactis* subsp. *cremoris* Wg2 and polyclonal antibodies (c) raised against the 95 kDa aminopeptidase from *Lb. helveticus* SBT 2171. Lanes: 1, reference proteins; 2, 50 μg cell-free extract from *Lc. lactis* subsp. *cremoris* Wg2; 3 and 4, 16.5 μg and 5.5 μg purified aminopeptidase from *Lb. helveticus* SBT 2171; 5, 50 μg cell-free extract from *Lb. helveticus* SBT 2171. The results were reproducible.

The effect of pH was determined in the pH range 4.0–10.0 by using a buffer consisting of 20 mM each of malic acid, MES, HEPES and boric acid, adjusted to the appropriate pH values (data not shown). The optimum pH for

hydrolysis of lysyl-*p*-nitroanilide and for prolyl-*p*-nitroanilide appeared to be in the range 6–8. At values below pH 5.0 and above pH 9.0, no hydrolysing activity of the enzyme could be detected.

Table 2. Substrate specificity of the aminopeptidase from *Lb. helveticus* SBT 2171 towards various chromogenic peptides

Dehydrated chromogenic β -naphthylamide (β NA) substrates in the LRA ZYM AP cupules and the *p*-nitroanilide (pNA) substrates (2 mM) were incubated with purified enzyme (0.55 μ g in 50 mM HEPES, pH 7.0) from *Lb. helveticus* SBT 2171 at 30 °C for 3.5 h and 15 min, respectively. +, Hydrolysed; –, not hydrolysed. The experiments were carried out with the purified peptidase, in duplicate. All results were reproducible.

Substrate	Hydrolysis	Substrate	Hydrolysis
Ala- β NA	+	Ala-Arg- β NA	+
β -Ala- β NA	+	Arg-Arg- β NA	+
Arg- β NA	+	Asp-Ala- β NA	+
Asp- β NA	+	Asp-Arg- β NA	+
Gln- β NA	+	Glu-Glu- β NA	+
Glu- β NA	+	Glu-His- β NA	+
γ -Glu- β NA	–	Gly-Ala- β NA	+
Gly- β NA	+	Gly-Arg- β NA	+
His- β NA	+	Gly-Gly- β NA	+
Ile- β NA	+	Gly-Phe- β NA	+
Lys- β NA	+	Gly-Pro- β NA	–
Met- β NA	+	Gly-Trp- β NA	+
Orn- β NA	+	His-Phe- β NA	+
Phe- β NA	+	His-Ser- β NA	+
Pro- β NA	+	Leu-Ala- β NA	+
Hyp- β NA	+	Leu-Gly- β NA	+
Pyr- β NA	–	Lys-Ala- β NA	+
Ser- β NA	+	Lys-Lys- β NA	+
Thr- β NA	+	Phe-Arg- β NA	+
Trp- β NA	+	Phe-Pro- β NA	–
Tyr- β NA	+	Pro-Arg- β NA	+
		Ser-Met- β NA	+
Lys-pNA	+	Ser-Tyr- β NA	+
Pro-pNA	+		
		Ala-Pro-pNA	–
Ala-Phe-Pro- β NA	–	Gly-Pro-pNA	–
His-Leu-His- β NA	+		
Phe-Pro-Ala- β NA	–	Lys-Ser-MeO- β NA	+
Val-Tyr-Ser- β NA	+	Ac-Gly-Lys- β NA	–
		B-Cys- β NA	+
Ala-Phe-Pro-Ala- β NA	–	Bz-Ala-MeO- β NA	–
Leu-Leu-Val-Tyr-Ser- β NA	+	Bz-Leu- β NA	–
		Cbz-Arg-MeO- β NA	–
		Cbz-Gly-Gly-Arg- β NA	–

Substrate specificity

The specificity of the aminopeptidase towards various chromogenic peptides is shown in Table 2. The substrate specificity was characterized by a rapid LRA ZYM AP system. The hydrolysis of the *p*-nitroanilides was measured spectrophotometrically (see Methods). Out of 61 chromogenic peptides, 47 were rapidly hydrolysed by the purified aminopeptidase, which indicates a broad substrate specificity. Only a few additional peptides were hydrolysed by the enzymes in the cell-free extract from *Lb. helveticus* SBT 2171 (data not shown). These obser-

vations strongly indicate that the aminopeptidase plays a major role in the proteolytic system of *Lb. helveticus* SBT 2171. Proline-containing peptides, such as Pro-*p*-nitroanilide, Pro- β -naphthylamide and Pro-Arg-*p*-nitroanilide, were also hydrolysed by the purified aminopeptidase. However, no hydrolysis was detected for peptides containing a proline at the second or third position. Except for B-Cys- β -naphthylamide, none of the peptides blocked at the N- and C-termini were hydrolysed, which indicates that the enzyme has no endopeptidase-like activity. γ -Glu- β -naphthylamide and Pyr- β -naphthylamide were not hydrolysed by the purified

Table 3. Relative activity of the aminopeptidase with various substrates

The *p*-nitroanilide (pNA) substrates (2 mM) were incubated with purified enzyme (in 50 mM HEPES, pH 7.0) from *Lb. belveticus* SBT 2171 at 30 °C for 5 min. The amount of pNA was measured at 410 nm. An absolute A_{214} value of 0.87 corresponds to 100%. The experiments were carried out with the purified peptidase, in duplicate. All results were reproducible.

Substrate (2mM)	Relative activity (%)
Lys-pNA	100
Arg-pNA	74
Ala-pNA	47
Leu-pNA	29
Pro-pNA	7
Glu-pNA	3
Gly-pNA	3
Val-pNA	<1
Asp-pNA	<1

aminopeptidase. In addition, the relative activity towards various acyl-*p*-nitroanilides has been determined (Table 3).

The specificity of the purified enzyme towards various di-, tri- and oligopeptides was also determined qualitatively (Table 4). The products of hydrolysis were analysed by TLC. The enzyme was active towards various peptides but not with dipeptides containing glutamic acid (negatively charged residue) or C-terminal proline. The purified aminopeptidase had less affinity for some glycine-containing peptides or dipeptides containing N-terminal proline. However, Pro-Phe and Pro-Tyr were hydrolysed by the enzyme. Most of the tripeptides were hydrolysed, including some tripeptides containing N-terminal proline. In contrast to the lack of hydrolysis of some di- and tripeptides containing N-terminal proline (Pro-Leu, Pro-Gly-Gly), the purified aminopeptidase hydrolysed tetra- and pentapeptides which contained the same residues at the N-terminus (Pro-Leu-Gly-Gly and Pro-Gly-Lys-Ala-Arg). This suggests that the enzyme has more affinity for oligopeptides than for di- or tripeptides containing a proline at the N-terminus.

The biologically active peptides neurotensin, substance P and β -casomorphin were not hydrolysed by the aminopeptidase. Clearly, the enzyme had no affinity towards peptides containing a negatively charged amino acid (Glu) at the N-terminus or proline as the second N-terminal amino acid. Also the insulin β -chain, starting with Phe-Val-, was not hydrolysed, probably because it is too large. The enzyme was further incubated with various peptide fragments of dynorphin A: f(1–6), f(1–8), f(1–10), f(1–13), f(1–17). HPLC analysis of the peptide products revealed that, except for dynorphin A f(1–13) and f(1–17), all peptides were hydrolysed, suggesting that the enzyme preferentially hydrolyses peptides not bigger than 10 residues (data not shown).

The kinetics of the enzyme for prolyl- and lysyl-*p*-nitroanilide hydrolysis were determined. A Lineweaver–Burk plot indicated a very low apparent K_m for lysyl-*p*-nitroanilide of 3 μ M, with a maximal rate of peptide hydrolysis of 37.5 μ mol min^{–1} (mg protein)^{–1}. The apparent K_m and V_{max} values of the aminopeptidase towards prolyl-*p*-nitroanilide were estimated to be 0.6 mM and 2.5 μ mol min^{–1} (mg protein)^{–1}, respectively (data not shown).

Effect of metal ions on enzyme activity

Treatment of the enzyme with the metal-chelating agent 1,10-phenanthroline (0.1 mM) inhibited the enzyme activity only partially (65 %), while EDTA, even at lower concentrations, inhibited the activity almost completely (data not shown). The activity of the EDTA-treated enzyme could be restored to more than 168 % and 93 % of the native enzyme by the addition of 100 μ M CoCl₂ and 100 μ M MnCl₂, respectively. Zn²⁺ and Fe²⁺ at concentrations of 25 μ M could also activate the enzyme (94 % and 78 %, respectively). However, at higher concentrations, both metal ions became inhibitory. Mg²⁺, Ca²⁺ and Cu²⁺ could not restore the enzyme activity.

The stimulatory and inhibitory effect of several divalent cations on the native enzyme activity was also studied. CoCl₂ activated the enzyme activity, while additions of CuCl₂, FeCl₂ and ZnCl₂ were inhibitory. MgCl₂, CaCl₂ and MnCl₂ hardly affected the enzyme activity. These results indicate a metallo-enzyme character for the aminopeptidase.

Effect of various chemical reagents

The effect of various thiol-blocking reagents on aminopeptidase activity was studied (data not shown). The aminopeptidase activity was inhibited by the thiol-blocking reagents *p*-CMB and *p*-CMBS (both at 0.2 mM) by 32 and 68 %, respectively. The inactivating effect of *p*-CMBS was completely reversed by 0.5 mM β -mercaptoethanol. The other thiol-blocking reagents iodoacetic acid and *N*-ethylmaleimide had no significant effect on the activity, even at higher (2 mM) concentrations. Disulfide-reducing agents, such as DTT and β -mercaptoethanol (at 0.25 mM and 0.5 mM, respectively), had a slight stimulatory effect on the enzyme activity, while a slight inhibition (21 %) was observed with phenylarsine oxide (2 mM).

PMSF, a serine-proteinase inhibitor, inhibited the aminopeptidase only slightly, even at higher concentrations (2 mM). Bestatin, a leucyl-leucine analogue and therefore a potential competitor for binding at the active pocket of the enzyme, inhibited the lysyl-*p*-nitroanilide-hydrolysing (1 mM lysyl-*p*-nitroanilide) activity by 44 and 75 % at 1 and 2 mM, respectively. L-Cystine and glutathione (both reduced and oxidized form) had no inhibitory effect. These results suggest that the hydrolytic action depends on one or more reactive thiol groups in the protein.

In addition, the prolyl-*p*-nitroanilide-hydrolysing activity of the aminopeptidase was determined in the presence of

Table 4. Substrate specificity of the aminopeptidase from *Lb. helveticus* SBT 2171 towards various di-, tri- and oligopeptides

Peptides (2–5 mM) were incubated with purified enzyme (0.55 µg in 50 mM HEPES, pH 7.0) at 30 °C for 2.5 h. Hydrolysis of the di-, tri and tetrapeptides was determined by TLC, whereas that of the tetra- and larger peptides was determined by HPLC. +, Hydrolysed; –, not hydrolysed. The experiments were carried out with the purified peptidase, in duplicate and in some cases in triplicate. All results were reproducible.

Substrate	Hydrolysis	Substrate	Hydrolysis
Ala-Ala	+	Pro-Tyr	+
Ala-Glu	–	Pro-Val	–
Ala-Leu	+	Ser-Leu	+
Ala-Met	+	Tyr-Ala	+
Ala-Phe	+	Tyr-Leu	+
Ala-Pro	–	Tyr-Pro	–
Ala-Ser	–	Val-Ala	–
Arg-Glu	–	Val-Glu	–
Arg-Leu	+	Val-Leu	+
Glu-Ala	–		
Glu-Glu	–	Ala-Ala-Ala	+
Glu-Val	–	Glu-Val-Phe	+
Gly-Gly	–	Leu-Gly-Gly	+
Gly-His	+	Leu-Leu-Leu	+
Gly-Leu	–	Met-Gly-Gly	+
Gly-Phe	+	Pro-Gly-Gly	–
Gly-Pro	–	Pro-Phe-Lys	+
Gly-Tyr	+	Pro-Pro-Pro	–
His-Ala	+	Pro-Tyr-Ala	+
His-Leu	+	Pro-Val-Gly	–
His-Lys	+	Tyr-Gly-Gly	+
Leu-Ala	+		
Leu-Gly	+	Gly-Pro-Arg-Pro	–
Leu-Leu	+	Gly-Pro-Gly-Gly	–
Leu-Met	+	Pro-Leu-Gly-Gly	+
Leu-Phe	+	Pro-Phe-Gly-Lys	+
Leu-Pro	–		
Leu-Ser	+	Leu-Trp-Met-Arg-Phe	+
Leu-Tyr	+	Pro-Gly-Lys-Ala-Arg	+
Leu-Val	+		
Lys-Leu	+	Methionine Enkephalin	+
Met-Ala	+	Neurotensin	–
Met-Leu	+	Insulin β -chain	–
Phe-Ala	+	Substance P	–
Phe-Leu	+	β -Casomorphin	–
Phe-Met	+		
Phe-Pro	–	(Ala) ₂	+
Phe-Val	+	(Ala) ₃	+
Pro-Ala	–	(Ala) ₄	+
Pro-Leu	–	(Ala) ₅	+
Pro-Phe	+	(Ala) ₆	+
Pro-Pro	–		

EDTA and 3,4-dichloroisocoumarin. The results (data not shown) showed that the prolyl-*p*-nitroanilide-hydrolysing activity of the purified enzyme was almost completely inhibited (>97 %) by 50 µM EDTA, while 500 µM 3,4-dichloroisocoumarin had hardly any effect on the enzyme activity (<10 % inhibition).

The effect of NaCl and KCl on the aminopeptidase activity was also determined. Both Na⁺ and K⁺, at concentrations of 300 mM, stimulated the lysyl-*p*-nitroanilide-hydrolysing activity by 118 and 136 %, respectively, at pH 7.0, while under the same conditions the hydrolysis of prolyl-*p*-nitroanilide was strongly inhibited

Table 5. Amino acid composition of an aminopeptidase from *Lb. helveticus* SBT 2171

Amino acid	Percentage of amino acid residues in enzyme	Amount of amino acid residues in enzyme (total molecular mass 93 086 Da)
Aspartate/asparagine	14.92	127
Glutamate/glutamine	18.57	158
Arginine	3.53	30
Histidine	0.24	≤2*
Lysine	1.06	9
Serine	5.40	46
Threonine	5.29	45
Cysteine	0.47	≤4*
Proline	2.23	19
Glycine	5.64	48
Alanine	11.28	96
Valine	6.11	52
Methionine	1.18	10
Isoleucine	5.29	45
Leucine	11.52	98
Phenylalanine	4.58	39
Tyrosine	2.70	23
Tryptophan	ND	ND

ND, Not detectable.

* The estimation of small amounts of amino acids is less reliable.

(data not shown). At the pH present during the ripening of cheese (pH 5.4), the activity was lower than at pH 7.0 but the stimulatory effect of NaCl on the hydrolysis of lysyl-*p*-nitroanilide was higher. Also at pH 5.4 the hydrolysis of prolyl-*p*-nitroanilide was inhibited by NaCl. These results suggest that the activity depends on the NaCl concentration.

Amino acid analysis

The amino acid composition of the purified enzyme was determined (Table 5). The number of negatively charged amino acids is higher than the positively charged residues, which is in accordance with the low isoelectric point of 4.9 of the enzyme (see above). On the basis of the amino acid composition, the protein has about 850 amino acids.

DISCUSSION

Although general aminopeptidases from lactic acid bacteria exhibit several similarities, the purified enzyme from *Lb. helveticus* clearly differs from the previously described lactococcal aminopeptidases (Pritchard & Coolbear, 1993). For instance, different molecular mass values have been reported for all other described lactococcal aminopeptidases (Geis *et al.*, 1985; Exterkate & de Veer, 1987; Neviani *et al.*, 1989; Niven, 1991; Sahlström *et al.*, 1993). The 95 kDa aminopeptidase purified from *Lc. lactis* subsp. *cremoris* Wg2 (Tan & Konings, 1990; Tan *et al.*, 1993) is an exception; however, immunoblotting experiments

with polyclonal and monoclonal antibodies raised against the aminopeptidase from *Lc. lactis* subsp. *cremoris* Wg2 showed no immunoresponse with the purified aminopeptidase from *Lb. helveticus* SBT 2171. Moreover, the polyclonal antibodies raised against the purified aminopeptidase from SBT 2171 revealed no immunoreaction with any of the proteins from *Lc. lactis* subsp. *cremoris* Wg2. These results confirm earlier experiments with cell-free extracts from *Lb. helveticus* SBT 2171 (Sasaki *et al.*, 1995b) and strongly indicate that the 95 kDa aminopeptidase from *Lb. helveticus* SBT 2171 is at least immunologically different from that of *Lc. lactis* subsp. *cremoris* Wg2. The aminopeptidase from SBT 2171 is most likely also different from the 95 kDa aminopeptidase from other lactococcal strains, since cell-free extracts of various *Lc. lactis* strains revealed an immunoresponse of 95 kDa with the above-mentioned antibodies (Tan *et al.*, 1992a).

The aminopeptidase from *Lb. helveticus* SBT 2171 also differs with respect to other characteristics from the aminopeptidase from *Lc. lactis* subsp. *cremoris* Wg2. The enzyme reported here has an isoelectric point of 4.9, a temperature optimum of 50 °C and an affinity (K_m) for lysyl-*p*-nitroanilide of 3 µM, whereas the corresponding characteristics of the aminopeptidase from *Lc. lactis* subsp. *cremoris* Wg2 are 6.2, 40 °C and 0.55 mM, respectively.

An important characteristic of the aminopeptidase from *Lb. helveticus* SBT 2171 is its broad substrate specificity and in particular its ability to hydrolyse proline-containing peptides. The proline-iminopeptidase-like activity of the

enzyme has a K_m of 0.6 mM on prolyl-*p*-nitroanilide and a V_{max} of 2.5 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$, which is comparable with the kinetics of the 50 kDa proline iminopeptidase from *Lc. lactis* subsp. *cremoris* HP [K_m 2.6–125 mM and V_{max} 0.3–10 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$; Baankreis & Exterkate, 1991], the 46 kDa proline iminopeptidase from *Lactobacillus casei* subsp. *casei* LGG [K_m 0.6 mM and V_{max} 1.7 nmol min $^{-1}$ (mg protein) $^{-1}$; Habibi-Najafi & Lee, 1995] and the 70 kDa prolyl aminopeptidase from *Lb. helveticus* LHE-511 [about 5 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$; Miyakawa *et al.*, 1994]. The proline iminopeptidases from *Lc. lactis* subsp. *cremoris* HP and *Lb. helveticus* LHE-511 exclusively hydrolyse proline-containing peptides, while that of *Lb. casei* subsp. *casei* LGG also hydrolyses a few other peptides (however at lower rates).

Experiments with specific inhibitors clearly showed that the prolyl-*p*-nitroanilide- and the lysyl-*p*-nitroanilide-hydrolysing activities of the purified aminopeptidase from SBT 2171 are almost completely (>97%) inhibited by 50 μM EDTA. In contrast, the peptidases from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 (Gilbert *et al.*, 1994), *Lb. helveticus* LHE-511 (Miyakawa *et al.*, 1994) and *Lb. casei* subsp. *casei* LGG (Habibi-Najafi *et al.*, 1995) were not inhibited by EDTA (1 mM). Furthermore, the prolyl-*p*-nitroanilide-hydrolysing activity of the enzyme in this study was hardly inhibited by 500 μM 3,4-dichloroisocoumarin, whereas the proline iminopeptidase from *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 397 (Gilbert *et al.*, 1994) was clearly inhibited (80%) by 100 μM of the serine type inhibitor. These results confirmed that the aminopeptidase from SBT 2171 was not contaminated with traces of proline iminopeptidase type of enzymes.

The ability to hydrolyse proline-containing peptides distinguishes this enzyme from previously described general aminopeptidases from other lactobacilli. For instance, the purified and characterized aminopeptidases from *Lb. helveticus* LHE-511 (Miyakawa *et al.*, 1992), *Lb. helveticus* CNRZ32 (partially purified; Khalid & Marth, 1990), *Lb. helveticus* ITGL1 (Blanc *et al.*, 1993), *Lactobacillus acidophilus* R-26 (Machuga & Ives, 1984), *Lactobacillus lactis* (Eggimann & Bachmann, 1980), *Lb. casei* subsp. *casei* LLG (Arora & Lee, 1992) and *Lb. delbrueckii* subsp. *bulgaricus* B14 (Bockelmann *et al.*, 1992; Wohlrab & Bockelmann, 1993) exhibited no hydrolytic action on peptides containing proline.

Recently, immunological experiments were performed with antibodies against various proteolytic enzymes from *Lb. helveticus* SBT 2171 and with various *Lactobacillus* type strains (results not shown). The results clearly indicated that the aminopeptidase reported here is immunologically different from other lactobacilli such as *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *delbrueckii*, *Lb. delbrueckii* subsp. *lactis* and *Lactobacillus fermentum*.

The amino acid requirements of various lactic acid bacteria have been investigated by Chopin (1993). From these results, it appears that the *Lb. helveticus* strains, examined by Morishita *et al.* (1981), have a greater need

for amino acids than other lactic acid bacteria strains. *Lb. helveticus* is nutritionally fastidious on all amino acids except glycine, alanine, cysteine and serine. This implies that in order to grow in milk, *Lb. helveticus* has to obtain all these amino acids from the available casein. A powerful aminopeptidase which is active towards a broad range of substrates, including proline-containing peptides, would play a crucial role in the release of these amino acids.

The aminopeptidase could hydrolyse fragments up to 10 residues. In lactococci it has been shown that most peptidases appear to be cytoplasmic, while some of them are also located in the cell envelope (Tan *et al.*, 1992b, 1993). This may also be the situation in *Lb. helveticus*. As a logical consequence, endopeptidases should be present and active towards larger peptides prior to the action of the aminopeptidase, while other proteolytic enzymes as well as peptide and amino acid transport systems have to fulfil the amino acid requirements of *Lb. helveticus*.

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