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Purification and Molecular Characterization of a Tripeptidase (PepT) from *Lactobacillus helveticus*

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A tripeptidase (PepT) from a thermophilic dairy starter strain of *Lactobacillus helveticus* was purified by four chromatographic steps. PepT appeared to be a trimeric metallopeptidase with a molecular mass of 150 kDa. PepT exhibited maximum activity against hydrophobic tripeptides, with the highest activity for Met-Gly-Gly (K_m, 2.6 mM; V_{max}, 80.2 μmol · min^{-1} · $μg^{-1}$). Some of the hydrophobic dipeptides were slowly hydrolyzed, distinguishing the *Lactobacillus* PepT from its counterpart in mesophilic *Lactococcus lactis*. No activity against tetrapeptides or amino acid *p*-nitroanilide derivatives was observed. The *pepT* gene and its flanking regions were isolated by PCR and sequenced by cyclic sequencing. The sequence analyses revealed open reading frames (ORFs) 816 bp (ORF1) and 1,239 bp (ORF2) long. ORF2 encoded a 47-kDa PepT protein which exhibited 53% identity with the PepT from *L. lactis*. The mRNA analyses indicated that *pepT* conforms a novel operon structure with an ORF1 located upstream. Several putative −35/−10 regions preceded the operon, but only one transcription start site located downstream of the first putative −10 region was identified. An inverted repeat structure with Δ*G* of −64.8 kJ/mol was found downstream of the PepT-encoding region.

Lactobacillus helveticus strains are frequently used as starter lactic acid bacteria (LAB) in manufacturing of Swiss- and Italian-type cheeses (17). They possess a comprehensive proteolytic system that supplies from casein the amino acids essential for growth in milk. One of the favorable characteristics of the thermophilic *L. helveticus* strains is their high proteolytic activity compared to those of other LAB (47, 50, 60). The proteolytic system of *L. helveticus* has also been demonstrated to contribute to the acceleration of cheese-ripening process, to reduction of bitterness, and to improvement of flavor development in manufacturing of Cheddar- and Gouda-type cheeses (1, 3, 4, 16, 18, 50). Furthermore, the milk fermented by an *L. helveticus* strain has been reported to have antihypertensive properties (30, 60, 61), which may be of particular interest for designing functional foods.

The components of the proteolytic system of the mesophilic LAB *Lactococcus lactis* have been studied at genetic and biochemical levels to the extent that there is a good understanding of the function of each component involved in the proteolytic pathway (12, 27, 28, 36, 52). The proteolytic system consists of cell wall-associated proteinase, peptide and amino acid transport systems, and numerous intracellular peptidases. Although an increasing amount of attention has been paid to unravel the proteolytic system in lactobacilli and other thermophilic LAB, much of the available data are still largely based on reports obtained from enzyme purifications and characterizations. The first study concerning the peptide transport systems in lactobacilli has recently been reported (37). The genetic data concerning lactobacillar cell wall proteinases are also relatively limited; so far, proteinase genes have been cloned and characterized from two thermophilic (21, 39) and one mesophilic (24) *Lactobacillus* species. Most of the genetic characterization of the lactobacillar proteolytic system has focused on *L. delbrueckii* and *L. helveticus* strains from which several of the peptidase genes have been cloned and sequenced, including genes encoding aminopeptidases (PepC, PepN, and PepL), proline-specific peptidases (PepI, PepQ, PepR, and PepX), endopeptidases (PepE, PepG, and PepO), and dipeptidases (PepD and PepV) (11). However, the PepT enzymes, required at later stages of the proteolytic pathway, remain uncharacterized (11). In *Lactococcus*, *pepT* (35) encodes a metal-dependent peptidase showing activity only for tripeptides (7). Unclassified metal-dependent tripeptidases have been enzymatically characterized from *L. delbrueckii* (5, 6), *Lactobacillus sake* (43), and *Pediococcus acidilactici* (46). These enzymes were shown to prefer hydrophobic tripeptides (5, 6, 43), and particularly tripeptides with NH_2-terminal methionine were efficiently hydrolyzed by the *L. delbrueckii* enzymes (5, 6). Tripeptidases could, thus, play an important role in cheese manufacture, since methionine is believed to be a precursor of volatile aroma compounds essential for flavor development during cheese ripening (9, 19, 20, 62). Furthermore, the concentration of methionine, one of the essential amino acids required by LAB (25, 47), is very low in milk; therefore, these particular peptidases of the proteolytic system may have a significant role also in the liberation of methionine from milk casein.

This work is part of a larger project that focuses on characterization of the proteolytic system from the industrial *L. helveticus* strain 53/7. In this work, we report the purification and biochemical characterization of an intracellular PepT from *L. helveticus*. The cloning, DNA sequencing, and mRNA analysis of the corresponding gene are also described. To our knowledge, this is the first report of characterization of *pepT* from *Lactobacillus* or any other thermophilic LAB.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. For PepT purification *L. helveticus* 53/7 was subcultured twice in skim milk and once in lactobacillus MRS (de Man, Rogosa, and Sharpe) broth (Difco). A final cultivation of 10 liters of MRS containing 0.5% Casitone and lactose was subsequently inoculated with a 4% overnight culture. Cells were grown for 8 h under slight agitation (100 rpm) at

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42°C and harvested by centrifugation (7,000 × *g*, 15 min, 4°C) at the end of the exponential phase of growth. *Escherichia coli* DH5αF′ (59) was grown in Luria broth. Erythromycin (300 μg ml^{-1}) was added to the growth medium when the pJDC9 vector (10) was used in *E. coli*.

Preparation of cell extract. Harvested cells (approximately 55 g) were mixed with glass beads (diameter, 0.1 mm), and the pH of the cell paste was adjusted to 7.0 by 1 M Trizma base. Cells were disrupted in a homogenizer (Vibrogen VI4) for 10 min at 4°C. The crude extract was separated by washing the glass beads several times by 50 mM sodium phosphate buffer (pH 7.0) and centrifuged (7,000 × *g*, 30 min, 4°C) to remove cell fragments. The crude extract (1,300 ml) was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) at 8°C for 16 h and then centrifuged (7,000 × *g*, 30 min, 4°C) prior to chromatography.

Determination of protein concentration. The protein concentrations were estimated with the Bio-Rad protein assay reagent for the Bradford dye-binding method (8), with bovine serum albumin (Sigma) as the protein standard.

Peptidase assays. Enzyme activities during purification steps were determined by the coupled L-amino acid oxidase–peroxidase–*o*-dianisidine system essentially as described by Wohlrab and Bockelmann (58) or by the method of El-Soda and Desmazeaud (15). Enzyme activity determined by the coupled enzyme reaction was calculated by using a molar extinction coefficient of 8,100 M^{-1} cm^{-1}. Based on the previous purification reports of the lactococcal and lactobacillar tripeptidases (5, 6, 7, 43), Leu-Gly-Gly was chosen for monitoring the PepT purification from *L. helveticus*.

Purification methods. (i) Ion-exchange chromatography on DEAE-Sepharose. The dialyzed and centrifuged crude extract (1,230 ml) was loaded onto a DEAE-Sepharose Fast Flow (FF) column (gel bed, diameter of 5.0 cm and height of 14 cm) equilibrated with 3 column volumes of starting buffer (50 mM sodium phosphate [pH 7.0]). Proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in starting buffer (gradient volume, 2,500 liters; flow rate, 8 ml/min; fraction size, 8 ml).

(ii) Hydrophobic interaction on phenyl-Sepharose. The fraction containing highest PepT activity (8 ml) was brought up to 4 M with NaCl, centrifuged (10,000 × *g*, 10 min, 4°C) to remove minor impurities, and subsequently purified by hydrophobic interaction chromatography (HiTrap FF test kit, low levels of phenyl substitution). Samples of 2 ml were applied to a HiTrap HIC column equilibrated with 50 mM sodium phosphate (pH 7.0) containing 4 M NaCl. Proteins were eluted in a decreasing step gradient of 4 to 0 M NaCl in the same buffer (bed volume, 1 ml; flow rate, 2 ml/min). The fractions containing majority of PepT activity (27 ml) were pooled and concentrated 50-fold by ultrafiltration through a 30-kDa-cutoff membrane (Amicon).

(iii) Gel filtration on Superdex 200. The concentrate (650 μl) obtained by ultrafiltration was further purified by gel filtration (Superdex 200 HR10/30) in 100-μl aliquots. The column was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl buffer (flow rate, 0.2 ml/min; fraction size, 0.2 ml).

(iv) Ion-exchange on MonoQ. Two fractions showing the highest PepT specific activity from each gel filtration run were pooled (2.4 ml) and applied to a MonoQ (HR5/5) column equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. Proteins were first eluted in a sharp gradient of 0.15 to 0.3 M NaCl in the running buffer (flow rate, 0.5 ml/min; gradient volume, 8.4 ml; fraction size, 0.25 ml) and then in a slow NaCl gradient of 0.3 to 0.4 M in the same buffer (gradient volume, 5.2 ml; fraction size, 0.15 ml).

Determination of molecular mass. After each purification step, the purity of PepT active fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (29), with gels containing 12% acrylamide. Gels were stained with Coomassie blue R 250 (Sigma), and the molecular mass of the denaturated PepT was estimated by using a low-molecular-weight protein standard (Pharmacia Biotech). The relative molecular mass of the purified PepT (10 μg) in native form was determined by gel filtration on a Superdex 200 HR10/30 column, using the running conditions described above for gel filtration on Superdex 200. The column was calibrated with catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), and ovalbumin (43 kDa).

Temperature and pH dependence of PepT activity. The pH optimum was determined with morpholinoethanesulfonic acid, HEPES, and Trizma buffers (Sigma). Briefly, 80 μl of 100 mM buffer (pH 5.5 to 9) was mixed with 10 μl (75 ng) of enzyme preparation and 10 μl of 20 mM Leu-Gly-Gly substrate. The reactions were incubated for 15 min at 37°C. To estimate the thermostability, the enzyme preparation was preincubated for 15 min in 80 μl of 100 mM HEPES buffer (pH 7.5) at various temperatures between 25 and 60°C. The reaction mixtures were combined with 10 μl of 20 mM Leu-Gly-Gly and then incubated for another 15 min at 37°C. The extent of Leu-Gly-Gly hydrolysis in each experiment was assayed by a modification of the Cd-ninhydrin method (14).

Effects of divalent cations and chemical reagents on PepT activity. The effects of metal ions and chemical reagents were determined by preincubating the enzyme preparation in 90 μl of 100 mM HEPES buffer (pH 7.5) with 0.1 and 1.0 mM divalent cation or chemical reagent for 10 min at room temperature. The reactions were initiated by adding 10 μl of 20 mM Leu-Gly-Gly and incubated for 15 min at 37°C. The residual PepT activity was determined by the Cd-ninhydrin method (14). Activities were compared to that of the untreated control, which was taken as 100%.

Substrate specificity assays with PepT. PepT activities toward different substrates were determined under the reaction conditions described above. The extent of hydrolysis with 2 mM tripeptide, dipeptide, and tetrapeptide and with 1 mM Lys-*p*-nitroanalide (*p*NA), Leu-*p*NA, Pro-*p*NA, Gly-Pro-*p*NA substrates after 15 min was measured by the Cd-ninhydrin method (14) and the method of El-Soda and Desmazeaud (15), respectively. The substrate hydrolyzed at highest rate was taken as 100%.

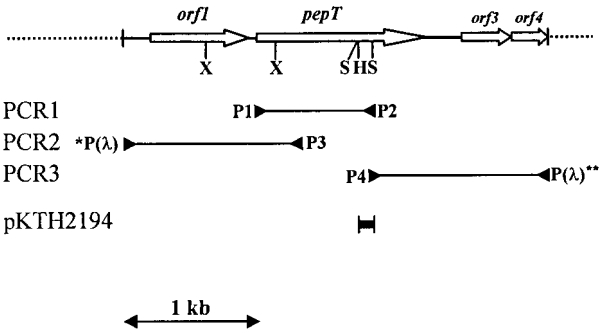

FIG. 1. Partial restriction map of the *L. helveticus* 53/7 *pepT* gene and its flanking regions. The positions and orientations of ORF1, *pepT*, ORF3, and ORF4 are indicated by arrows. PCR1 refers to a PCR fragment obtained with a degenerate primer pair P1-P2. pKTH2194 refers to the *E. coli* subclone carrying an insert from a *Sau*3AI-digested PCR1. PCR2 and PCR3 are the PCR products amplified from the *L. helveticus* 53/7 genomic library by *pepT* (P3 and P4)- and λgt10 [*P(λ) forward; P(λ)**, reverse]-specific primers. Abbreviations for restriction enzymes: X, *Xba*I; S, *Sau*3AI (only 2 of the 14 *Sau*3AI recognition sites are shown); H, *Hin*dIII.

Determination of kinetic parameters. Kinetic parameters for Met-Gly-Gly and Leu-Gly-Gly were determined by incubating 50 to 75 ng of the enzyme preparation under the reaction conditions described above by using substrate concentrations ranging from 0.5 to 10 mM. The extent of hydrolysis was determined by the Cd-ninhydrin method (14). The experimental data were evaluated by nonlinear and linear regression analyses with the program GRAFIT (Sigma). Specific activity was expressed as micromoles of substrate hydrolyzed per microgram of protein per minute under the reaction conditions used.

Determination of NH_2-terminal amino acid sequence for PepT. The purified PepT preparation was concentrated by Ultrafree-0.5 centrifugal filter device (Millipore) and then dried in a Speed-Vac. PepT (3.0 μg) was separated on an SDS–12% polyacrylamide gel and transferred electrophoretically onto a polyvinylidene difluoride membrane (Immobilon P; Millipore) as described by Matsudaira (32). The amino acid sequence of the NH_2-terminal region of the intact protein was determined by degrading the protein in an Applied Biosystems Procise sequencing system.

DNA syntheses, molecular cloning, and sequencing of *L. helveticus pepT*. Oligonucleotides were synthesized with an Applied Biosystems model 392 DNA/RNA synthesizer and purified by ethanol precipitation or with NAP-10 columns (Pharmacia Biotech). PCR was used to synthesize DNA fragments by using reaction conditions recommended by the manufacturer (Finnzymes). Total DNA from *L. helveticus* was isolated as described previously (57) without guanidine hydrochloride treatment. Plasmid DNAs from *E. coli* clones were isolated by using the Wizard Minipreps (Promega). Other molecular cloning techniques were performed essentially as described in reference 42. The sequencing reactions were carried out either on PCR fragments or on plasmid DNAs according to the Thermo Sequenase fluorescent labeled primer Cycle sequencing kit manual by using an A.L.F. DNA sequencer (Pharmacia Biotech). Both DNA strands were sequenced by using fluorescein-labeled pUC19, λgt10, or different sequence-specific primers.

A 1.1-kbp fragment of *L. helveticus pepT* was amplified by PCR from *L. helveticus* chromosomal DNA using degenerate oligonucleotides designed according to the NH_2-terminal region of *L. helveticus* PepT (P1; 5′-TACTGGATCCC[A/T]CG[T/C]TT[T/C][C/T]T[A/T/G]AA[A/G]TA[T/C]GT[T/C]AA[A/G]G-3′ [Fig. 1]) and to the conserved regions of known PepT proteins from *L. lactis* (35) and *Salmonella enterica* serovar Typhimurium (34) (P2; 5′-TACTGTCGAC[G/A]CC[A/G]TC[A/G]GT[G/A]CC[G/A]CC[A/G]CG[A/G]AT[A/T]GG-3′ [Fig. 1]). The PCR fragment of 1.1 kb was digested with *Sau*3AI and cloned into *Bam*HI site of pJDC9 in *E. coli*. One plasmid clone carrying a 102-bp fragment of the *pepT* gene was designated pKTH2194 (Fig. 1). *pepT*-specific oligonucleotides were designed for isolating the complete *pepT* gene and its flanking regions from the *L. helveticus* genomic library established in λgt10 (55). The genomic library was screened by PCR amplification with *pepT*- and λgt10-specific primers, resulting in fragments of 1.3 and 1.1 kb (Fig. 1), which subsequently were analyzed by PCR sequencing.

Computer analyses of the DNA and the deduced amino acid sequences were performed with the PC/GENE set of programs (version 6.70; IntelliGenetics) and with programs on the ExPaSy server. Sequence comparisons were performed with the EMBL/GenBank and SWISS-PROT/PIR databases.

Transcription analyses. Total RNA was isolated with an RNeasy Mini kit according to the instructions provided by Qiagen. Cell lysis was performed as follows. *L. helveticus* cells were disrupted with glass beads (1:3; diameter, 0.1 mm) for 45 s in a cell homogenizer (Vibrogen V14). The crude extract was obtained by washing the glass beads gently with 100 mM Tris-Cl (pH 7.0) containing 1 mM EDTA at 4°C. The glass beads and cell debris were removed by centrifugation (10,000 × *g*, 2 min, 4°C), and total RNA was subsequently isolated with an RNeasy Mini kit. A total RNA sample of 15 μg was subjected to RNA gel electrophoresis and Northern blotting, performed as described by Hamens and Higgins (22). For Northern blot analysis, a 1.1-kb PCR fragment (PCR1 [Fig. 1]) was labeled with digoxigenin (DIG)-dUTP (Boehringer). A DIG luminescence detection kit (Boehringer) was used for hybrid detection. The primer extensions were performed with an A.L.F. sequencer (Pharmacia Biotech) as described earlier (55) with total RNA (30 μg) isolated from exponentially growing cells (6 h after inoculation). The primer extension reactions were purified with a QIAquick PCR purification kit (Qiagen) prior to DNA analysis with an A.L.F. sequencer. The antisense fluorescein-labeled oligonucleotides used for primer extensions were P3 (5′-AACGTTAGAAGGAATTTCAGC-3′) and P4 (5′-CACCACAAATAAAGCCAAGAG-3′). Reverse transcription (RT) PCR was carried out as follows. Total RNA (5 μg) isolated from cells withdrawn at the exponential phase of growth and the antisense oligonucleotide P3 were used for cDNA synthesis as described earlier for the primer extension. PCR was performed with 1/10 of the cDNA reaction as template and with primers P3 and P5 (5′-CGCTATGGGAAGAAAAGGTAG-3′). To confirm that no contaminating DNA material was present in the RT-PCR, the RNA sample (1 μg) without RT reaction was PCR amplified with the same primer pair.

Nucleotide sequence accession number. The nucleotide sequences of ORF1 and ORF2 (Fig. 1) and their deduced amino acid sequences are available from the EMBL sequence database under accession no. AJ243321.

RESULTS

PepT purification. PepT from *L. helveticus* 53/7 was purified by four chromatographic steps. The elution profiles of PepT during DEAE-Sepharose, phenyl-Sepharose, Superdex 200, and MonoQ chromatography are shown in Fig. 2, and the final results of the purification procedure are summarized in Table 1. After DEAE-Sepharose chromatography, the majority of Leu-Gly-Gly-hydrolyzing activity was found in two separate activity peaks which eluted at 0.36 and 0.39 M NaCl (Fig. 2). The Lys-*p*NA-hydrolyzing activity peak eluted early in the NaCl gradient (data not shown); according to the enzyme characterizations performed with other *L. helveticus* peptidases, this activity most likely originated from PepC and PepN enzymes (53, 54). Leu-Gly-Gly proved to be a specific substrate for separating the PepT activity from the PepN and PepC activity in *L. helveticus*, as the Lys-*p*NA-hydrolyzing fractions showed only slight activity for this substrate (data not shown). One-third of the total PepT activity after the DEAE-Sepharose chromatography was found in the first PepT activity peak (0.36 M NaCl) (data not shown). In addition to Leu-Gly-Gly, these fractions were active also on Leu-Gly and Gly-Pro-*p*NA. The second activity peak (0.39 M NaCl) contained ca. two-thirds of the total of Leu-Gly-Gly-hydrolyzing activity, and the fraction with the highest specific activity (1.32 U/mg [Table 1]) was used for further purifications. The MonoQ purification step resulted in a single protein band on a Coomassie blue-stained SDS-polyacrylamide gel (Fig. 3). The specific activity of the purified PepT was enriched 354-fold, with a yield of 0.2%.

Characterization of PepT. The molecular mass of the pure PepT was estimated to be around 47 kDa by SDS-PAGE analysis (Fig. 3) and around 150 kDa by gel filtration (data not shown). These results indicate that *L. helveticus* PepT in its native form is composed of three subunits of an approximately equal size.

PepT was found to have a broad pH optimum between pH 6 and 8, the maximal activity being at pH 7.5 (data not shown). The heat stability studies indicated that PepT was relatively heat labile, with approximately 45% of the PepT activity remaining after incubation for 15 min at 45°C (data not shown). No activity loss was observed in the temperature range of 25 to 37°C (data not shown).

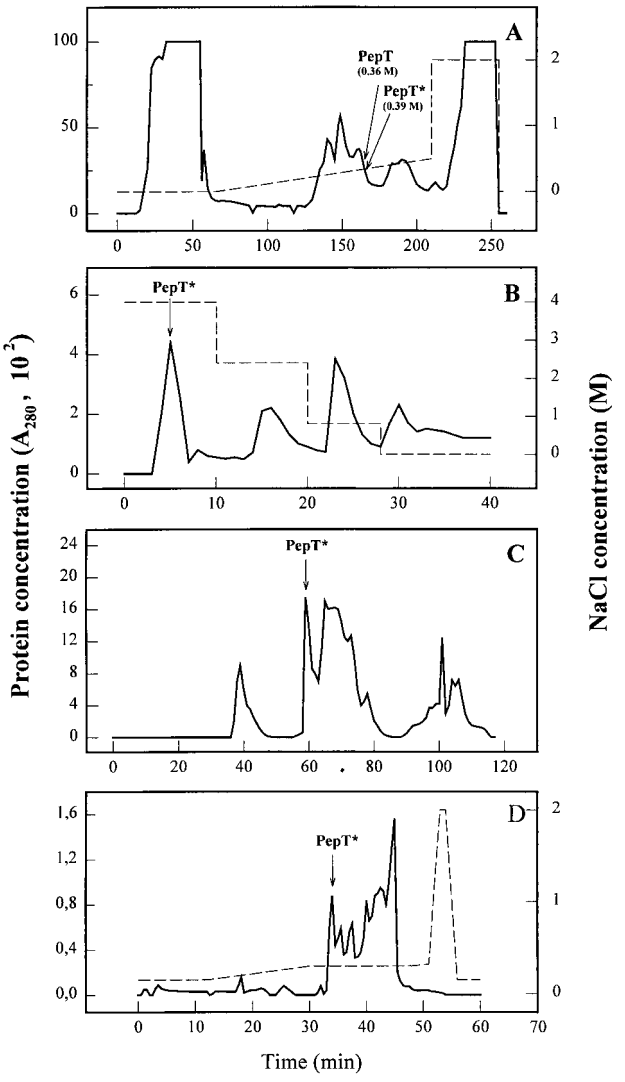

FIG. 2. Purification of *L. helveticus* 53/7 PepT. (A) DEAE-Sepharose FF anion-exchange chromatography; (B) phenyl-Sepharose HiTrap FF (low sub) chromatography; (C) Superdex 200 HR10/30 gel filtration chromatography; (D) MonoQ anion-exchange chromatography. Protein concentration (——) and NaCl gradient (– – –) are indicated. Fractions containing Leu-Gly-Gly-hydrolyzing activity are marked with arrows; fractions used for further purification are marked with asterisks.

The effects of various metal ions and chemical reagents on PepT activity at concentrations of 0.1 and 1.0 mM are shown in Table 2. Cd^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+} were strongly inhibitory at concentrations of 0.1 and 1.0 mM. Although PepT was not

TABLE 1. Purification of *L. helveticus* PepT

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg^{-1})	Purification (fold)	Activity yield (%)
Cell extract	1,300.0	4,420.00	693.0	0.16	1	100
DEAE-Sepharose FF	8.0	9.60	12.7	1.32	8	1.8
Phenyl-Sepharose FF	27.0	0.80	4.6	5.75	36	0.7
Superdex 200	2.4	0.09	2.0	22.22	139	0.3
MonoQ HR5/5	0.6	0.03	1.7	56.67	354	0.2

[a] One unit corresponds to the hydrolysis of 1 μmol of Leu-Gly-Gly per μg of protein per min.

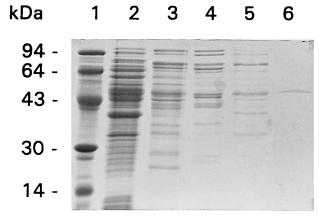

FIG. 3. SDS-PAGE analysis of PepT purification from *L. helveticus* 53/7. Lane 1, low-molecular-weight markers; lane 2, crude extract after dialysis and centrifugation; lanes 3 to 6, PepT fractions after anion-exchange chromatography (DEAE-Sepharose), hydrophobic interaction chromatography (phenyl-Sepharose HiTrap), gel filtration (Superdex 200), and anion-exchange chromatography (MonoQ), respectively.

affected by 0.1 mM Zn^{2+}, 1.0 mM Zn^{2+} completely abolished the enzyme activity. Both 0.1 and 1.0 mM Mg^{2+} had a stimulatory effect on PepT. PepT was totally inhibited by metalloenzyme inhibitors such as EDTA, EGTA and 1,10-phenanthroline. After inhibition with 0.1 mM EDTA and EGTA, PepT activity could not be restored with 1.0 mM Mg^{2+} or Ca^{2+}. The serine protease inhibitors phenylmethylsulfonyl fluoride and 3,4-dichloroisocoumarin had no significant influence on enzyme activity. PepT was partially inhibited by the reducing agents 2-mercaptoethanol and dithiothreitol. Iodoacetic acid did not significantly affect PepT activity, whereas another sulfur-reactive agent, *p*-hydroxymercuribenzoic acid, almost totally inactivated PepT.

The substrate specificities of PepT for various di-, tri-, and tetrapeptides and amino acid *p*NA derivatives are summarized in Table 3. PepT was capable of hydrolyzing all tripeptides tested, Met-Gly-Gly being the most suitable substrate for this enzyme. Tripeptides containing phenylalanine at the NH_2-terminal position were not hydrolyzed, whereas tripeptides containing proline at the NH_2 terminus and in the second position were hydrolyzed, but to an extent only 2 to 3% of that of Met-Gly-Gly. Also, the hydrophobic dipeptides Leu-Gly and Leu-Leu were slowly hydrolyzed. PepT showed no activity for tetrapeptides and amino acid *p*NA derivatives.

TABLE 2. Effects of chemical reagents and divalent cations on the PepT activity

Reagent and/or cation	Relative activity (%)	
	0.1 mM	1.0 mM
None	100	100
EDTA	0	0
EGTA	0	0
1,10-Phenanthroline	22	0
Dithiothreitol	88	28
β-Mercaptoethanol	90	58
Phenylmethylsulfonyl fluoride	100	60
3,4-Dichloroisocoumarin	100	100
Iodoacetic acid	93	88
p-Hydroxymercuribenzoic acid	75	11
Ca^{2+}	106	95
Co^{2+}	32	38
Cd^{2+}	2	0
Cu^{2+}	0	0
Mg^{2+}	120	168
Mn^{2+}	47	35
Zn^{2+}	112	9
Reagent + cation[a]		
EDTA + Mg^{2+}	ND[b]	0
EGTA + Mg^{2+}	ND	0
EDTA + Ca^{2+}	ND	0
EGTA + Ca^{2+}	ND	7

[a] 0.1 mM EDTA or EGTA + 1 mM cation.
[b] ND, not determined.

TABLE 3. Substrate specificity of purified PepT

Substrate (1 mM)	Activity (%)
Met-Gly-Gly	100
Leu-Gly-Gly	34
Leu-Leu-Ala	33
Ala-Ala-Ala	26
Leu-Leu-Leu	15
Gly-Leu-Tyr	15
Lys-Gly-Gly	13
Tyr-Gly-Gly	11
Ala-Leu-Ala	7
Val-Gly-Gly	6
Pro-Gly-Gly	3
Ala-Pro-Leu	2
Phe-Gly-Gly	0
Leu-Leu	9
Leu-Gly	4
Met-Gly	0
Leu-Pro	0
Pro-Leu	0
Lys-Lys	0
Gly-Leu-Leu-Gly	0
Leu-Trp-Met-Arg	0
Leu-*p*NA	0
Lys-*p*NA	0
Pro-*p*NA	0
Gly-Pro-*p*NA	0

The K_m and V_{max} values were determined with Met-Gly-Gly and Leu-Gly-Gly as the substrates. K_m values were 2.6 and 0.6 mM for Met-Gly-Gly and Leu-Gly-Gly, respectively; V_{max} values were 80.2 and 6.8 $\mu mol \cdot min^{-1}$ per μg of protein, respectively.

NH_2-terminal sequencing of PepT. The 47-kDa protein band was separated by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and analyzed in a gas-pulsed liquid sequencer. The NH_2-terminal sequence of the purified PepT was NH_2-M-E-Y-P-N-L-L-P-K-F-L-K-Y-V-K-V-N. Protein homology searches revealed a high identity with the NH_2-terminal sequences of the purified tripeptidases from *Pediococcus pentosaceus* (75%) (46) and *L. lactis* (70%) (35), indicating that the purified enzyme possessed tripeptidase-like activity.

Cloning and sequencing of the gene encoding PepT. A degenerate primer pair was designed according to the NH_2-terminal amino acid sequence of the purified *L. helveticus* PepT and according to the conserved amino acid region deduced from the *L. lactis* (35) and *Salmonella* serovar Typhimurium (34) *pepT* genes. PCR amplification resulted in a 1.1-kb DNA (Fig. 1, PCR1) fragment which was digested with *Sau*3AI, ligated with pJDC9, and subsequently transformed into *E. coli* DH5α. All transformants were shown to carry identical inserts of 102 bp, and one of these plasmid clones was designated pKTH2194 (Fig. 1). Sequence analysis revealed a significant match with the *L. lactis pepT* (35), confirming that the amplified PCR fragment contained part of the *L. helveticus pepT* structural gene. The *pepT* gene and its flanking regions from the *L. helveticus* genomic λgt10 library were isolated by PCR amplification, and the resulting PCR fragments of 1.1 and 1.3 kb (Fig. 1) were sequenced with λgt10 and several sequence-specific primers. The assembled sequence data (3,106 bp) revealed four open reading frames (ORFs), ORF1 (816 bp),

ORF2 (1,239 bp), ORF3 (364 bp), and an incomplete ORF4 (283 bp) (Fig. 1).

ORF2 (1,239 bp) was found to encode a PepT protein of 413 amino acids with a calculated molecular mass of 46.8 Da (data not shown), in good accordance with the molecular mass of purified PepT (Fig. 3). PepT starts at position 991 with the ATG start codon and ends at position 2229 with tandem TAG TGA stop codons. A consensus ribosome-binding site (45), AG GAG, is located 10 nucleotides upstream of the start codon. However, no obvious promoter-like sequences were found. Immediately downstream of the translation stop codons, a 20-bp palindromic sequence (positions 2257 to 2277) is located. Furthermore, an apparent rho-independent transcription terminator was identified 121 nucleotides downstream of the stop codon, with a ΔG of -64.8 kJ/mol (48). The NH_2-terminal amino acid sequence deduced from the *pepT* sequence is identical to that for the intact protein (data not shown), which showed that PepT is not subjected to maturation at its NH_2-terminal part. Further computer analyses did not reveal any membrane-spanning domains or hydrophobic segments encoding a putative signal peptide, confirming the intracellular location of PepT. Comparison of the deduced amino acid sequence of PepT against the protein databases showed a high degree of identity with PepT proteins from *L. lactis* (53%) (35), *Bacillus subtilis* (45%) (44), *Salmonella* serovar Typhimurium (43%) (34), and *E. coli* (42%) (BAA35949). Three highly conserved regions were also identified in approximately the same position in these PepTs (data not shown). One of these regions (Thr-139 to Ala-185), suggested to represent a metal-binding region (34, 35), is also found in *L. helveticus* PepT (data not shown). Multiple alignment of this subsequence with the corresponding sequences from *L. lactis*, *B. subtilis*, and *Salmonella* serovar Typhimurium revealed that over 50% of the amino acids were identical (data not shown).

mRNA analyses. The size of *pepT*-specific mRNA in exponentially growing *L. helveticus* cells was determined by Northern blotting. DIG-labeled PCR1 used as the hybridization probe detected a 2.3-kb transcript (Fig. 4A), suggesting that *pepT* is expressed as part of an operon. According to DNA sequence analyses, the size of the 2.3-kb transcript most likely is consistent with the size of a dicistronic operon containing the *pepT* gene and the upstream ORF1. RT-PCR was used to confirm that *pepT* and ORF1 are expressed through the same mRNA. The RT-PCR resulted in a 0.63-kb PCR fragment (Fig. 4B and C), which corresponds to the size predicted on the basis of DNA sequence analysis. An equal amount of total RNA sample without RT reaction was amplified with the same primers to confirm that no contaminating chromosomal DNA material was present. The 5′ end of the ORF1-*pepT* dicistronic transcript was determined by primer extension mappings from exponentially growing cells with two primers, P3 and P4, designed downstream of possible transcription initiation sites of ORF1 and the *pepT* gene (see Materials and Methods). Only one transcription initiation site in the ORF1-*pepT* region was found, 78 nucleotides upstream of the ORF1 initiation codon (data not shown). Mapping of the 5′ end of the ORF1-*pepT* transcript also confirmed the location of the putative promoter region suggested on the basis of DNA sequence analyses (data not shown).

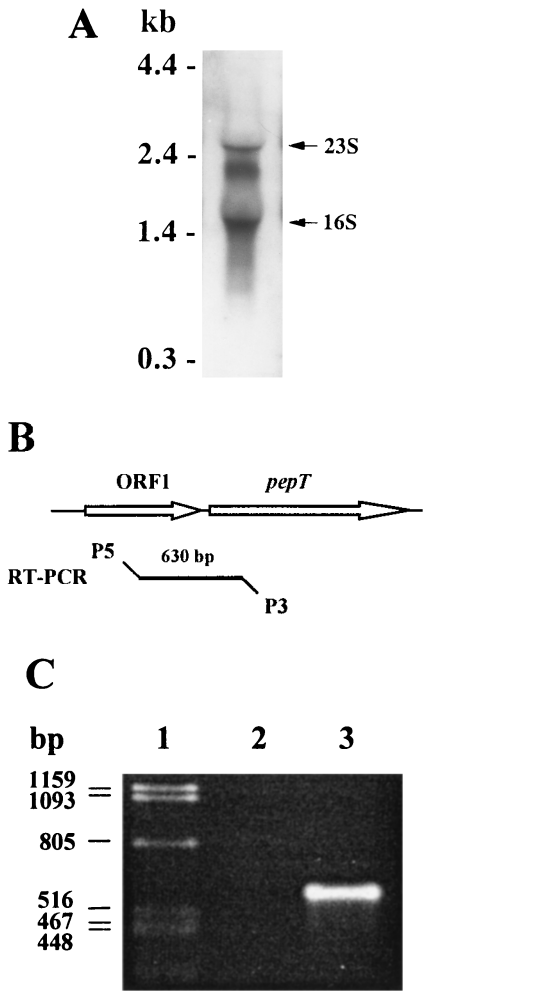

FIG. 4. Northern (A) and RT-PCR (B and C) analyses of *L. helveticus* 53/7 *pepT* expression. (A) Northern blot analysis was performed with total RNA isolated from exponentially (6 h) growing *L. helveticus* cells. Numbers on the left denote positions of RNA molecular size markers (Gibco BRL). (B) Schematic representation of RT-PCR analysis of ORF1 and *pepT* mRNAs. (C) Agarose gel electrophoresis of RT-PCR and control PCR samples. Lane 1, part of the *Pst*I-digested λ DNA; lanes 2 and 3, PCR amplification products of the control and cDNA preparations with primer pair P5-P3, respectively. The two amplification reactions were performed with the same amount of total RNA isolated from *L. helveticus* 53/7.

DISCUSSION

In this study, we describe the purification and molecular characterization of a PepT from *L. helveticus*. From other *Lactobacillus* species, three enzymes with tripeptidase-like activity have recently been purified and characterized (5, 6, 43). As reported in several studies, PepC and PepN are capable of cleaving wide range of various tripeptides (27). However, in this case they were found to hydrolyze Leu-Gly-Gly only at a very low rate or if not at all. The first purification step resulted in two PepT activity-containing peaks; first was active also on other peptide substrates, while the second showed activity only for Leu-Gly-Gly. The PepT activity in the first peak most likely resulted from the combined activity of X-prolyl dipeptidyl aminopeptidase (PepX) and dipeptidase (PepD) enzymes, which was also supported by the properties determined for recombinant *L. helveticus* PepX (K. Savijoki, unpublished data) and PepD (56) enzymes.

Molecular weights of native LAB tripeptidases reported in literature vary between 55 and 105 kDa (2, 5, 6, 7, 41, 43, 46). The number of subunits also varied in these enzymes, for which two (2, 6, 7, 46) and three (5, 41) subunits were reported. The *L. helveticus* PepT has optimum activity at pH 7.5, comparable

to that of *Lactococcus* PepT (7). The proposed cell wall-located tripeptidases purified from *L. lactis* subsp. *cremoris* IMN-C12 (41) and *L. delbrueckii* (5) were found to have slightly acidic pH optima. However, no genetic data to confirm the extracellular location of these enzymes are yet available. These tripeptidases were also found to be more stable during heat treatment than the *L. helveticus* PepT.

Both the *L. lactis* PepT (7) and the PepT characterized here were found to belong to the group of metallopeptidases. The *L. helveticus* PepT was stimulated by Mg^{2+}, as has been reported for tripeptidases from *L. delbrueckii* (6) and *P. pentosaceus* (46). However, Mg^{2+} did not significantly affect the activities of other LAB tripeptidases (5, 7, 41, 43). The cations Zn^{2+} and Co^{2+} have been reported to be strong activators for some tripeptidases at low concentrations (5, 7, 26), whereas only slight stimulation of the *L. helveticus* PepT was obtained with 0.1 mM Zn^{2+}. Partial inhibition of the *L. helveticus* PepT by both disulfide- and sulfhydryl-modifying reagents suggest a requirement of disulfide and/or sulfhydryl groups for retaining maximal enzyme activity. Conversely, the partial inhibition due to steric hindrances caused by these reagents could not be excluded. The *L. helveticus* PepT is a trimer containing two cysteine residues (data not shown) in each of its subunits: Cys-147, in the putative metal-binding region, and Cys-372, close to the other conserved region thought to play an important role in PepT activity (34, 35). Both sequence analyses and inhibition assays suggest that this region in the COOH-terminal part of the PepT protein is involved in substrate binding. Most of the LAB tripeptidases were also inhibited by disulfide-modifying reagents, whereas the sulfhydryl-modifying reagents had either stimulatory or inhibitory effects on these enzymes (2, 5, 6, 7, 41).

In common with other lactobacillar tripeptidases (5, 6, 41), the *L. helveticus* PepT preferred hydrophobic tripeptides. Particularly high activity observed with Met-Gly-Gly suggests that this enzyme may play an important role in flavor formation during cheese ripening. As Phe-Gly-Gly appeared to be a suitable substrate for the other enzymes (5, 6, 41), the *L. helveticus* PepT showed no activity for this substrate. The affinities of different substrates for PepT were clearly affected by the nature of the NH_2-terminal amino acid of each of the substrates tested. Although Met-Gly-Gly appeared to be hydrolyzed at the highest rate, it was shown to have three-times-lower affinity for the enzyme than Leu-Gly-Gly. Similar results were obtained from the lactococcal PepT, which showed lower K_m values with Leu-Gly-Gly than with Met-Gly-Gly (2). In contrast to the *Lactococcus* PepT (7) and the tripeptidases from *Lactobacillus* (5, 6, 41), the *L. helveticus* PepT was also able cleave Leu-Leu and Leu-Gly. The *L. helveticus* PepT was able to liberate the NH_2-terminal amino acid from tripeptides with proline in either the first or the central position. This kind of unique and broad substrate specificity may suggest that *L. helveticus* PepT supplements the activities of other peptidases.

The attempts to clone the *pepT* gene in *E. coli* and in *L. lactis* were unsuccessful. Therefore, the *pepT* gene was isolated by PCR and analyzed by PCR sequencing. DNA sequence analyses of the *pepT* gene revealed several inverted repeat structures (data not shown), which probably caused some of the instability problems during cloning on plasmids. Sequence and mRNA analyses revealed that *pepT* is expressed through a 2.3-kb transcript containing ORF1 and the *pepT* gene. ORF1 encodes a protein homologous to an unknown protein from *Streptococcus mutans*, where the corresponding gene is located downstream of a gene locus involved in the dTDP-L-rhamnose synthesis pathway (51). The operon structure was also suggested for *L. lactis pepT*, but no transcriptional analysis to confirm this is available (35). Identities of the other proteins encoded by the ORFs adjacent to the *L. lactis pepT* also are still unknown. The ORF1-*pepT* operon was expressed at a moderately high level (data not shown), which is an unexpected result due to the lack of the well-conserved −10 region. Whether the inverted repeat structure and/or the A+T-rich element (13, 31, 33, 40) located upstream of ORF1 is involved in the ORF1-*pepT* operon expression remains to be studied. Also, the identity and effect of the protein encoded by ORF1 require further examination.

Sequence analysis of the *pepT* downstream region revealed an additional ORF, ORF3 (Fig. 1), showing significant identity to members of the GntR family DNA-binding proteins (data not shown) (23). The highest degree of identity was found with a putative transcription regulator from *B. subtilis* (34%) (38), where the corresponding gene partially overlaps with a gene encoding a putative copper ABC transporter protein. In *L. helveticus*, ORF3 is also followed by a putative ABC transporter (Fig. 1, ORF4); however, no inverted or direct repeat that may function as a probable DNA-binding site was found in close vicinity to ORF3 or ORF4. It is not yet known whether the 20-bp palindromic sequence preceding the ORF1-*pepT* transcription terminator is involved in transcription regulation of the downstream genes and/or in transcription termination of the ORF1-*pepT* operon. As no obvious GntR operator-like sequences (23, 49) could be identified in the palindrome region or in the upstream regions close to ORF3 and ORF4, further examination is needed to locate the accurate DNA-binding site and to determine the specific role of this putative regulator protein in *L. helveticus*.

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