An X-prolyl dipeptidyl aminopeptidase (*pepX*) gene from *Lactobacillus helveticus*

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The X-prolyl dipeptidyl aminopeptidase gene (*pepX*) of an industrially used *Lactobacillus helveticus* strain has been detected by nucleic acid hybridization, cloned, characterized and sequenced. One ORF of 2379 bp with coding capacity for a 90·6 kDa protein (PepX) was found. The ORF was preceded by a typical prokaryotic promoter region. An inverted repeat structure with ΔG of $-84\cdot1$ kJ mol⁻¹ was found downstream of the coding region. The deduced amino acid sequence of the 90·6 kDa protein showed 49·3, 49·4 and 77·7% homology with the PepX proteins from *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *lactis*, respectively. Northern blotting revealed a 2·6 kb transcript and one transcription start site was identified via primer extension analysis using an A.L.F. sequencer. In a bioreactor study, the expression of *pepX* in *Lb. helveticus* was studied as a function of growth. Transcription of *pepX* was typical of exponential growth phase expression. The *pepX* gene has been cloned into pKK223-3 and expressed at a high level in *Escherichia coli* JM105. PepX was purified to homogeneity by ion-exchange and hydrophobic interaction chromatography. Optimum PepX activity was observed at pH 6·5 and 45 °C. According to gel filtration analysis, PepX is a dimer of 165 kDa. The enzyme was inactivated by heavy metal ions such as Cu²⁺, Cd²⁺ and Zn²⁺. EDTA and 1,10-phenanthroline did not decrease PepX activity significantly. It was completely inhibited by *p*-hydroxymercuribenzoate and reactivated by adding DTT, and strongly inhibited by PMSF. PepX is thus a metal-independent serine peptidase having functional sulfhydryl groups at or near the active site.

Keywords: *Lactobacillus helveticus*, X-prolyl dipeptidyl aminopeptidase, peptidases

INTRODUCTION

Lactic acid bacteria play an important role in a variety of food fermentations. Strains of lactococci and lactobacilli used in dairy fermentations are fastidious organisms which require a variety of amino acids for growth. Since the concentrations of small peptides and amino acids in milk are insufficient to support their growth to high density, these bacteria are dependent on a complex proteolytic system for the liberation of amino acids from milk proteins. The proteolytic system is composed of a cell-envelope-associated proteinase, membrane-bound

transport systems, and numerous cytoplasmic peptidases. Recently, extensive biochemical and genetic analyses of individual components of the proteolytic system of lactic acid bacteria and their role in cheese ripening and flavour formation has been conducted (Kok, 1990; Pritchard & Coolbear, 1993; Tan *et al.*, 1993; Visser, 1993; Kok & De Vos, 1994). The major milk proteins, caseins, are rich in the imino acid proline (Fox, 1989) and specialized enzymes are required to hydrolyse peptide bonds involving proline residues; therefore, proline-specific peptidases such as X-prolyl dipeptidyl aminopeptidase (PepX), aminopeptidase P, proline endopeptidase, proline iminopeptidase, prolinase and prolidase are thought to be important components of these proteolytic systems (Kok & De Vos, 1994; Baankreis & Exterkate, 1991). PepX cleaves dipeptidyl residues from peptides by hydrolysing the peptide bond at the carboxyl side of the proline residue

Abbreviations: gly-pro-pNA, L-glycyl-L-prolyl-*p*-nitroanilide; PepX, X-prolyl dipeptidyl aminopeptidase; pHMB, *p*-hydroxymercuribenzoate.

The EMBL, GenBank and DDBJ accession number for the nucleotide sequence reported in this paper is Z48236.

when this imino acid is the penultimate N-terminal residue. In addition to peptidase activity, PepX also has amidase and esterase activities (Yoshpe-Besancon *et al.*, 1994).

Casey & Meyer (1985) first reported the presence of PepX in lactic acid bacteria. PepX has now been purified from a wide range of lactic acid bacteria including *Streptococcus thermophilus* (Meyer & Jordi, 1987), *Lactococcus lactis* subsp. *cremoris* (Kiefer-Partsch *et al.*, 1989; Booth *et al.*, 1990), *Lc. lactis* subsp. *lactis* (Zevaco *et al.*, 1990), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Atlan *et al.*, 1990; Miyakawa *et al.*, 1991; Bockelmann *et al.*, 1991), *Lb. delbrueckii* subsp. *lactis* (Meyer & Jordi, 1987), *Lb. acidophilus* (Bockelmann *et al.*, 1991), *Lb. casei* subsp. *casei* (Habibi-Najafi & Lee, 1994) and *Lb. helveticus* (Khalid & Marth, 1990; Miyakawa *et al.*, 1994). However, *pepX* genes have only been cloned and sequenced from *Lc. lactis* subsp. *cremoris* (Mayo *et al.*, 1991), *Lc. lactis* subsp. *lactis* (Nardi *et al.*, 1991) and *Lb. delbrueckii* subsp. *lactis* (Meyer-Barton *et al.*, 1993).

In this paper we describe the cloning, DNA sequencing, mRNA analysis and expression of the *pepX* gene of *Lb. helveticus* 53/7. Also overexpression, purification and characterization of the cloned *Lb. helveticus* PepX from *Escherichia coli* is described.

METHODS

Bacterial strains, plasmids and growth conditions. *Lb. helveticus* strain 53/7 is an industrial starter from the collection of Valio Ltd (Helsinki, Finland). *Lb. helveticus* was cultivated in MRS broth (Difco) at 42 °C without shaking. Bioreactor cultivation was as described previously (Vesanto *et al.*, 1994). *Escherichia coli* strains DH5 α F' (Woodcock *et al.*, 1989) and JM105 (Pharmacia) were grown in Luria broth. Erythromycin (500 μ g ml⁻¹) or ampicillin (25–50 μ g ml⁻¹) was added to the growth medium when the pJDC9 and pKK223-3 (Pharmacia) vectors, respectively, were used in *E. coli*.

Construction and screening of a *Lb. helveticus* genomic library. A *Lb. helveticus* genomic library was constructed in λ gt10 using λ -DNA *in vitro* packaging and cDNA rapid cloning modules (λ gt10; Amersham). Instead of the Amersham cDNA rapid adaptor ligation module, a RiboClone *Eco*RI Linker System (Promega) was employed.

Oligonucleotide synthesis. Oligonucleotides were synthesized with an Applied Biosystems DNA/RNA synthesizer model 392 and purified by ethanol precipitation or with NAP-10 columns (Pharmacia).

Amplification of DNA by PCR. DNA was amplified by PCR in reaction conditions recommended by the manufacturer of Dynazyme DNA polymerase (Finnzymes). The specific primer pair used was 5' CAACGGATCCGCATGCTCAAGCAGCT-TAAG 3' and 5' CTGAGGATCCAGTCGACAAAGGCGGC 3', giving rise to a 2.8 kb DNA fragment carrying the *pepX* gene. For construction of an overexpression system for PepX in *E. coli* two synthetic primers were designed: 5' GGTACCC-GGGCTGACAAAGGAGAAAAATATG 3' and 5' TGATC-CCGGGGATCAAACGGGTTATGGC 3', giving rise to a 2.5 kb DNA fragment carrying the *pepX* gene.

DNA sequencing. Sequencing was performed on an A.L.F. DNA Sequencer (Pharmacia). The dideoxy-sequencing

reactions (Sanger *et al.*, 1977) were performed according to the AutoRead Sequencing Kit manual (Pharmacia). Both DNA strands were sequenced using λ gt10- and pUC19-specific primers and different sequence-specific oligonucleotides for primer walking.

Other DNA methods. Plasmid DNAs from *E. coli* clones were isolated by using Wizard Minipreps (Promega) or FlexiPrep (Pharmacia) kits. Restriction enzymes were used as recommended by the manufacturers. Ligations and other standard DNA methods were according to Sambrook *et al.* (1989). *E. coli* strains were transformed by electroporation using a Bio-Rad Gene Pulser.

RNA isolation, Northern (RNA) blotting and primer extension.

Total RNA was isolated from *Lb. helveticus* cells as described by Vesanto *et al.* (1994). RNA gel electrophoresis and Northern blotting were performed as described by Hames & Higgins (1985). Total *Lb. helveticus* 53/7 RNA, denatured with glyoxal and dimethyl sulfoxide, was run on a 1.0% (w/v) agarose gel with 10 mM sodium phosphate buffer, pH 6.5, followed by transfer to a positively charged nylon membrane (Boehringer Mannheim). Hybridization probes were labelled with digoxigenin-dUTP (DIG, Boehringer-Mannheim). A DIG luminescent detection kit (Boehringer) was used for hybrid detection. Primer extension was performed with total RNA isolated from exponentially growing cells essentially as described by Myöhänen & Wahlfors (1993) using an A.L.F. DNA Sequencer (Pharmacia) with the fluorescein-labelled oligonucleotide 5' CTGCTGAAGTCCCAGACTTG 3'. Briefly, 40 μ g of DNA-free total RNA was ethanol-precipitated with the labelled primer (10 pmol). For the reaction, 46 U of avian myeloblastosis virus (AMV) reverse transcriptase was used. The reaction mixture was extracted with phenol/chloroform (1:1, v/v) and chloroform, ethanol-precipitated and dissolved in 4 μ l water. Stop buffer (4 μ l) from the AutoRead Sequencing Kit (Pharmacia) was added, and the whole mixture was loaded with sequencing reactions into a 6% (w/v) sequencing gel. The sequencing reaction of pKTH2097, performed with the same primer, was used as the marker for the 5' end determination. The transcription start site was determined by comparing the retention time of the primer extension product with that of the products from the sequencing reaction.

Peptidase activity assays. Bacterial colonies and λ plaques were screened by a plate-staining procedure described by Nardi *et al.* (1991) using L-glycyl-L-prolyl- β -naphthylamide (Sigma) as the substrate in 50 mM HEPES (pH 7.0) buffer with Fast Garnet GBS sulfate salt (Sigma) (2 mg ml⁻¹). From liquid cultures, the enzyme activity was determined according to El Soda & Desmazeaud (1982) using 2 mM L-glycyl-L-prolyl-*p*-nitroanilide (gly-pro-*p*NA; Sigma) in 50 mM HEPES (pH 7.0) buffer. *Lb. helveticus* cells were disrupted with an Ultrasonic 2000 sonicator (Braun) or permeabilized by chloroform. During protein purification PepX activity was determined at 37 °C using 1.2 mM gly-pro-*p*NA in 50 mM Tris/HCl, pH 7.5. The total volume of the reaction was 430 μ l and the reaction was stopped by the addition of 1 ml 30% (v/v) acetic acid. The release of *p*-nitroaniline was followed at 410 nm. Hydrolysis of *p*NA was linear in the range 0–1.2 absorbance units, where one unit corresponds to the release of 1 μ mol *p*-nitroaniline min⁻¹. A molar extinction coefficient of 8800 M⁻¹ cm⁻¹ (Bockelmann *et al.*, 1991) was used to calculate the specific enzyme activity as units (mg protein)⁻¹. The protein content was determined by the Bio-Rad protein assay using BSA (Sigma) as a standard. Diluted enzyme samples of 1 ml were mixed with 250 μ l Coomassie reagent, and the absorbance was measured at 595 nm.

Computer analysis. The DNA sequences obtained from A.L.F. were assembled and analysed with the PC/GENE set of programs (Release 6.8, IntelliGenetics). The databases of EMBL and Swiss-Prot were used for searching for homologous protein and nucleic acid sequences.

Construction of an overexpression system for the *Lb. helveticus* PepX in *E. coli*. The *pepX* gene was isolated by PCR. For this purpose two PCR primers were designed containing *Xma*I sites at their 5' ends for the cloning (see above). The *Xma*I fragment, carrying the structural gene of *pepX* with its own ribosome binding site, was ligated with the pKK223-3 vector (Pharmacia), downstream of the inducible *tac* promoter and transferred into the *E. coli* JM105 host (Pharmacia). The expression of PepX was characterized by cultivating the cells to a density of 50 Klett units (filter 66), followed by 1 mM IPTG induction and measurement of product accumulation during 5 h by SDS-PAGE and measurement of PepX activity against the gly-pro-pNA substrate.

Purification of PepX. The crude extract of *E. coli* JM105 carrying the *pepX* construct in pKK223-3 was prepared as follows. Luria broth (1.8 l) supplemented with ampicillin (50 µg ml⁻¹), was subcultured with 36 ml of an overnight culture. Bacteria were grown to the cell density of 50 Klett units (filter 66), followed by induction of *pepX* expression with 1 mM IPTG. Two hours after induction the cells were harvested by centrifugation (8000 g, 20 min) and washed with 200 mM Tris/HCl, pH 8.0. The pellet was resuspended in 54 ml lysis buffer (20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.5 mg lysozyme ml⁻¹) and gently stirred for 45 min at 4 °C. The volume of the crude extract was brought up to 100 ml and then treated with bovine pancreatic RNase (Sigma) and DNase I (Boehringer) for 30 min at room temperature. The cell debris was removed by centrifugation (4 °C, 10000 g, 40 min). The cell-free extract was fractionated by 50 and 80% (w/v) (NH₄)₂SO₄ precipitations in two steps. The precipitates were collected by centrifugation (4 °C, 6000 g, 30 min), solubilized in 50 mM Tris/HCl, pH 7.5 (20 ml), and dialysed first against deionized water for 60 min and then overnight against 50 mM Tris/HCl, pH 7.5.

Prior to Q-Sepharose chromatography the dialysed sample (24 ml) was filtered through a 0.45 µm membrane filter (Millex HA, Millipore). A Q-Sepharose column (gel bed 100 ml, diam. 5 cm, Pharmacia) was equilibrated with the starting buffer (50 mM Tris/HCl, pH 7.5). After loading the sample, the column was washed until the absorbance at 280 nm was near zero. Proteins were eluted in a linear gradient of 0–0.3 M NaCl in starting buffer (flow rate 5 ml min⁻¹, gradient volume 1 l, fraction size 10 ml), and the fractions were tested for gly-pro-pNA-hydrolysing activity. The active PepX fractions (110 ml) were concentrated 110-fold by ultrafiltration through a 30 kDa cut-off membrane (Amicon). For the last purification step, NaCl was added to a final concentration of 4 M to the concentrated fraction. Half of the concentrated fraction was centrifuged (4 °C, 10000 g, 10 min) and purified by hydrophobic interaction chromatography (Phenyl-Superose HR 5/5, Pharmacia). Proteins were eluted in a linear gradient of 4–0 M NaCl in 50 mM Tris/HCl buffer, pH 7.5 (flow rate 0.5 ml min⁻¹, gradient volume 15 ml, fraction size 1 ml), and assayed for gly-pro-pNA-hydrolysing activity.

Determination of pH and temperature optima. For determination of the pH optimum in the range of pH 4–10, a buffer system containing either MES, HEPES, malic acid or boric acid (50 mM each) was employed. Reactions were incubated at 37 °C for 8 min. The reactions were stopped with 1 ml 30% acetic

acid and the absorbance was measured at 410 nm. The optimum temperature was estimated by the same method in the temperature range 0–70 °C with 50 mM MES, pH 6.5.

Inhibition studies. Potential inhibitors were added to final concentrations of 0.1 and 1 mM. Reaction mixtures were preincubated at room temperature for 30 min and contained 50 mM MES buffer, pH 6.5 (1.2 mM gly-pro-pNA was used as a substrate). Reactivation was performed by adding reactivating agent (final concentration 1 mM) to the reaction mixture and incubating for an additional 10 min at room temperature. Reactions were incubated at 40 °C for 8 min and stopped with 30% acetic acid. The activities were compared with the untreated control, which was taken as 100%.

Molecular mass determination. The molecular mass of the native PepX was determined by gel filtration chromatography (Superdex 75 and 200 HR 10/30, Pharmacia) equilibrated with 50 mM Tris/HCl pH 7.5, containing 0.15 M NaCl. The columns were calibrated with catalase (232 kDa), aldolase (158 kDa), albumin (68 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa). SDS-PAGE was performed with a 10% (w/v) polyacrylamide gel (Laemmli, 1970). The gel was stained with Coomassie Brilliant Blue R250. MWH-SDS proteins (Pharmacia) were used as marker standards.

RESULTS AND DISCUSSION

Cloning and sequence analysis of the *pepX* gene from *Lb. helveticus* 53/7

Southern hybridization was used to examine homology between a 1.5 kb *Eco*RI–*Kpn*I fragment probe derived from the *Lb. helveticus* CNRZ32 *PepX* gene (Nowakowski *et al.*, 1993) and chromosomal DNA of *Lb. helveticus* 53/7. The Southern analysis gave positive results but the *pepX* gene could not be isolated from a *Lb. helveticus* gene library established in the pTZ18R vector carrying large chromosomal inserts (approx. 7 kb) (Varmanen *et al.*, 1994), suggesting that the flanking regions of *pepX* may be harmful in *E. coli*. Instead, the *pepX* gene was finally isolated from a *Lb. helveticus* 53/7 genomic library established in λ gt10. Screening of 26 000 plaques resulted in the detection of one positive clone. This clone was determined to contain an insert of 3.6 kb.

The DNA sequence analysis of the 3.6 kb insert revealed one ORF of 2379 bp with the capacity to encode a protein of 793 amino acids with a calculated molecular mass of 90 571 Da (Fig. 1). Using primers designed up- and downstream of the ORF, the putative *pepX* gene was amplified by PCR, cloned into pJDC9 and designated pKTH2097. The *E. coli* clones carrying pKTH2097 were confirmed to encode PepX activity by demonstrating activity of permeabilized cells and in the *in situ* enzymic plate assay on PepX-specific substrates. The *pepX* starts with ATG at position 235 and a termination codon TAA is located at position 2614 (Fig. 1). A typical prokaryotic ribosome binding site, AGGAGA (Shine & Dalgarno, 1974; Stormo *et al.*, 1982) was identified 11 nucleotides upstream of the initiation codon. A putative promoter with –35 (TTGGTT) and –10 (TAAAT) regions found 61 and 38 nucleotides upstream of the initiation codon, respectively, was identified. A putative ρ -independent type transcriptional terminator was found 13 nucleotides

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GCATGCTCAAGCAGCTTAAGCGTTCAATTTATTATTGCAATTTACGTCCTTTTGTGTTTTTACTAAATAAATCAAAAGTCCCATAAAAATATCCTTAATAAATAAATCTAATTACTTATA - 120
TTATGGACTATTTTATCTTACTTAAAAATTTCTTTATTGATTTTCATTTTATTTGGTTCTCTCTTTAAACGGGTAAATATAAGGTAATAATACTGACAAAGGAGAAAAATATGAAA - 240
TATAACCAATATGCTTACGTTGAAACCGACTTTCAACAACAAGTTAAAGAAATTAATCGATATCAACTTCTTACCTAAAAATTAATCAAGTCTGGGACTTCAGCAGTCTTTTAGCTAAATTA - 360
Y N Q Y A Y V E T D F Q Q Q V K E L I D I N F L P K N Y Q V W D F S S L L A K L - 42
GTTAAAAATGCAATCGCTGAGGCTAAAACAGATGCGCTAAAAATGCCAAACTTGGCGAATTTGCGAGTTTCTGATCACCAGACCTTGGCTGATTTTCTTAAGGAAAAAGCCAAACAGAAAT - 480
V K N A I A E A K T D A A K N A K L A E F A V S D H Q T L A D F L K E K P T E I - 82
GGTACCGAACCAATTTCTACAACGTAGCTCTGCAACTTCTTGGCTACCACTGCTCACTACGACTATGATTTTGTGATGCCAACCGGTTTCATGCAAGAAATGCTTTGCCATTTGTGCAAGAC - 600
G T E Q F Y N V A L Q L L G Y H V H Y D Y D F A D P T G F M Q R N A L P F V Q D - 122
ATCAGCGACAACTCAAAATTAATCTCTGCCTTTTACCGCTTGTCTCAATACCGTGTCTAAAAACGGACAAATCTTGTCTGATGTGATGGCAGGCAAGGCTACTTTACTCAATCTCTGGGT - 720
I S D N Q K L I S A F Y R L L N T R A K N G Q I L L D V M A G K G Y F T Q F W G - 162
CAAAACAAGTTCAAGTTCTCAATGGTAAATCTATCCCTGTTTTGACACAAATAAGGTCATTCGTGAAGTCGTTTATGTGCAAACTGATCTTGATACTGACCATGATGGCAAGAGTGAT - 840
Q N K F K F F N G K S I P V F D T N K V I R E V V Y V E T D L' D T D H D G K S D - 202
TTAATTCAAGTTACTGTTTTTCCGTCCTGAAGAACTAATAAGGGCTTAAAGTACCTGCACTCTACACTGCATCCCCACTTTTGGCGGAATCATCGCCAAACGAAACGCAACCTAATAC - 960
L I Q V T V F R P E E T N K G L K V P A L Y T A S P Y F G G I I A N E K R N H N - 242
GTTGACGAAAACTCTCTGACTCTACCGAATGGAATGATCCACAATACATTCACTCTCCAATCGTTAAGGCTGAAAAGCCAGATGGTTCAAGCCGTCCAGCTACAGAGGAGGCAGTGAC - 1080
V D E N L S D S T E W N D P Q Y I H S P I V K A E K P D G S S R P A T E E A V H - 282
AAGTCTCTTACCCATTAAACGAATACATGCTAGCTCGCGGTTTTGTAGCGCTTTTGTGCTGCAATTTGGTACTCGCGGCGAGGATGGTGTCCGCATCACTGGTGTCCAGAGAAACT - 1200
K S S Y P L N E Y M L A R G F A S V F A G A I G T R G S D G V R I T G A P E E T - 322
GAATCAGCTGCTGCAGTTATCGAATGGCTGATGGTGATCGTTGCTCTACCTGACAGAACCGGTTCAAACTACAGCTGATTTGGTGCAACGGCAACATCGGTATGACCGGTCGT - 1320
E S A A A V I E W L H G D R V A Y T D R T R T V Q T T A D W C N G I G M T G R - 362
TCATATCTTGGTACTTTTGCATTCGCCATTCGCCAATCTGGCGTTTAAAGGCTTAAAGCTTGTCTGTTGAAGCCGCAATTTTCATCATGGTACGACTACTACCGTGAACATGGTTTGGTT - 1440
S Y L G T L Q I A I A T T G V K G L K T V A E A A I S S W Y D Y Y R E H G L V - 402
ATTGCTCTGAAGCTTGGCAAGGTGAAGACCTTGATTTGCTCGCTGAAACCTGTCAATCAAACTATGGGATCGCGGTTTCACTTAAGATCAAGCTGAATACGACAAATGCAAAAG - 1560
I A P E A C Q G E D L D L L A E T C Q S N L W D A G S Y L K I K P E Y D K M Q K - 442
CAGTTGCGTGAAAAAGAACCGGTAATCTGCTCAATCTGACTTCTGGGAAGCTAGAACTACCGTCACCACTGATGGTATCAAAATGCTCTCGGATTTCCGTTTCAATGGTTTAAAT - 1680
Q L R E K E D R N T G Q Y S D F W E A R N Y R H H A D G I K C S W I S V H G L N - 482
GACTGGAATGTTAAGCCAAAGAACGTTTACAAAATCTGGCAACTAGTTAAAAAGATGCCAATGAAGCATCACTCTTCTTGACCAAGGTCGCACTACAATATGAACAACTTCGTTTCC - 1800
D W N V K P K N V Y K I W Q L V K K M P M K H H L F L H Q G P H Y N M N N F V S - 522
ATCGACTTTACAGACTTCATGAACCTCTGGTTTGTTCATGAACCTTTAGGCATTGAAAAATATGCTTACAATCAATGGCCAAAGGTTATGATTCAAGATAAATCTGCAAGCTGCAAGTGG - 1920
I D F T D F M N L W F V H E L L G I E N N A Y N Q W P T V M I Q D N L Q A D K W - 562
CATGAAGAACCTGACTGGAGCAATGATTTAGGCCAAGAAAGATCTACTACCTACTGATGAAGGCGCAACTTTTCAAGATGGCAACGGCAAGACACAAAGTCTTTACCGATGTTGGC - 2040
H E B P D W S N D L G Q E K I Y Y P T D E G E L F Q D G N G K A Q K S F T D V G - 602
GGTATTGAATTCAGAAAGCTGGTATTTTCAGAAAGCGATGGCAATACAAGTTCAATTTGCGGTGATGAAAAATGGGCTAAGCCAAAGTTTACGTTTGAACCTGATGAATTTATCCACCT - 2160
G I E F K K A G I S E S D W Q Y K F I C G D E K W A K P S L R F E T D E F I H P - 642
ACTACCATCGTGGGTCGTCAGAAAGTTAAAGTTAGAGTTTCTGCTAGTCTGCAAAAGGTTGAGATTTCGTTGCTTGTGAACTGGGCGAAAGACAAAGCTTACCGCTACTCTCTAAA - 2280
T T I V G R P E V K V R V S A S L P K G E I S V A L V E L G E R Q R L T A T P K - 682
TTCTTAATGCGTGGCGGCAAGAATTAGGCTACCGTTTGGCACTGACACTTTACAAGAATTTGTTCCAGATAAAAGACTAAAGCCAAAGTTGATTACTAAGGCTCACATGAATTTGCAA - 2400
F L M R G G Q E L G Y R F G T D T L Q E F V P D K K T K A K L I T K A H M N L Q - 722
AACTTCAAGGATATGAAGAAGCCTGAAGCAATCGACGCGGATAAGTTCTACGACTTGGACTTCTGCTTCAACCTACTTATTACACCATCCCATCTGGCAGCAAGCTTGCTTTGATCATT - 2520
N F K D M K K P E A I D A D K F Y D L D F L L Q P T Y Y T I P S G S K L A L I I - 762
TATTCACCTGACCAAGCATGACTAAGCGTCCGCTTGAAGATGAACTTACACAATGATTTAGCTAATACTGAAATCAAGTTTATGAAAAATAATTAATCTTAACGAAAGACAGGCA - 2640
Y S T D Q G M T K R P L E D E T Y T I D L A N T E I K F Y E K - 793
TTCAATCGAATGCCTGTCTTTTGTGCTATTCAATAACTTTTATACTCGCCATAACCCGTTGATCAAGCTTATCATATGGAATAAAATTTCAAAAGCAGCCGAATTAATGCAATAACG - 2760
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Fig. 1. The nucleotide and deduced amino acid sequences of the *Lb. helveticus* 53/7 *pepX* gene. The predicted -35 and -10 regions of the putative promoter are underlined. The transcription start site, determined by primer extension, is indicated by a vertical arrow. RBS refers to the putative ribosome binding site which is indicated by a dashed line. Conserved amino acid residues involved in the active site are indicated by bold type and the active site serine with an asterisk. The stop codon is written in bold type. The putative transcription terminator is shown with dashed arrows.

downstream from the stop codon with a ΔG of $-84.1 \text{ kJ mol}^{-1}$ (Fig. 1).

The alignment (PALIGN program of PC/GENE) of the deduced amino acid sequence of *pepX* (793 amino acid residues) with those of the *pepX* genes from *Lc. lactis* subsp. *lactis* (Mayo *et al.*, 1991), *Lc. lactis* subsp. *cremoris* (Nardi *et al.*, 1991; 763 amino acids) and *Lb. delbrueckii*

subsp. *lactis* (Meyer-Barton *et al.*, 1993; 792 amino acids) revealed overall homologies of 49.3, 49.4 and 77.7%, respectively. A search for other homologous proteins in the Swiss-Prot data bank revealed no additional homologies.

The active site of *PepX* from *Lc. lactis* has been identified (Chich *et al.*, 1992) and a sequence resembling that was

also found from the deduced protein sequence of PepX of *Lb. helveticus* 53/7 (Fig. 1). The consensus sequences surrounding the active site serine in *Lc. lactis* and *Lb. helveticus* are G-K-S-Y-L-G and G-R-S-Y-L-G, respectively.

With PC/GENE programs, neither membrane-associated helices nor any putative signal peptide were found in the deduced amino acid sequence of PepX, suggesting intracellular location. In lactococci, both extracellular (Kiefer-Partsch *et al.*, 1989) and intracellular (Meyer *et al.*, 1989; Booth *et al.*, 1990; Zevaco *et al.*, 1990) locations for PepX have been suggested. Tan *et al.* (1992) found, by using immunoblotting and immunogold labelling, that PepX in *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* was intracellular, although some peripheral labelling was detected. The N-terminal amino acid sequences of the purified PepX proteins from *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* were identical to those deduced from the *pepX* genes, indicating that the enzymes were not subjected to processing at the N terminus (Nardi *et al.*, 1991; Mayo *et al.*, 1991). Furthermore, the sequenced *pepX* genes of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lb. delbrueckii* subsp. *lactis* did not reveal any putative signal sequences. These results strongly support an intracellular location for PepX (Nardi *et al.*, 1991; Mayo *et al.*, 1991; Meyer-Barton *et al.*, 1993).

mRNA analysis

The size of mRNA transcribed from the *pepX* gene was analysed from exponentially growing *Lb. helveticus* cells by Northern blotting with a DIG-labelled *pepX*-specific hybridization probe. The probe detected a 2.6 kb transcript (Fig. 2), which is in good agreement with the size of the *pepX* gene, thus confirming that the *pepX* gene is a monocistronic transcriptional unit. The primer extension mapping of the 5' end of *pepX* mRNA, performed with total RNA from exponentially growing *Lb. helveticus* cells, revealed one transcription start site located 24 nucleotides upstream of the start codon (data not shown). The direct localization of the transcription start site was determined with an automated A.L.F. sequencer by comparing the retention time of the primer extension product with those of the products of a sequencing reaction performed with the same primer. The signal in the sequencing chromatogram with the same retention time as the primer extension signal represents the first nucleotide of the mRNA. The reliability of this method has been tested by localizing the 5' end of a *Lb. helveticus* dipeptidase gene by this and the conventional primer extension methods (unpublished data). The mapping of the 5' end of the *pepX* transcript confirmed the location of the promoter region predicted from the DNA sequence (Fig. 1).

Expression of *pepX*

Expression of the *pepX* gene in *Lb. helveticus* 53/7 was studied in a pH-controlled culture in MRS broth by measuring activity against the gly-pro-pNA substrate and the amount of *pepX* transcripts was measured as a function

Fig. 2. Expression of the *Lb. helveticus* 53/7 *pepX* as a function of growth. *Lb. helveticus* 53/7 was grown in 2.5 l MRS broth using a Chemap FZ-3000 bioreactor. The pH was maintained at 5.85 by the addition of 1 M NaOH. (a) Change in cell density (●) and peptidase activity (■) against a gly-pro-pNA substrate with time. One unit (U) is defined as the amount of enzyme activity producing a variation of 0.01 A_{410} unit min^{-1} at 37 °C. (b) Northern blot analysis of the *Lb. helveticus* 53/7 *pepX* transcripts. Total *Lb. helveticus* 53/7 RNA, denatured with glyoxal and dimethyl sulfoxide, was run in a 1.0% agarose gel with 10 mM sodium phosphate buffer, pH 6.5, followed by transfer to a positively charged nylon membrane (Boehringer Mannheim) and hybridization with the DIG-labelled 2.8 kb *pepX*-specific PCR fragment. RNA molecular mass markers (BRL) were used to determine the size of transcripts. Lanes 1–4 refer to RNA samples taken 4, 6, 12 and 18 h after inoculation, respectively.

of growth (Fig. 2). The amount of enzyme activity increased up to the early stationary phase of growth reaching the highest level 8 h after inoculation. During the rest of the growth period analysed, the activity against the gly-pro-pNA substrate remained relatively constant (Fig. 2a). To measure the steady-state level of *pepX* transcripts, total RNAs were isolated from *Lb. helveticus* cell samples taken 4, 6, 12 and 18 h after inoculation and analysed by Northern blotting (Fig. 2b). According to the Northern blot data, the steady-state amount of *pepX* transcripts was highest during exponential growth, whereas most of the mRNA detected at the stationary phase, was degraded. Like *pepX*, the level of transcripts of three other *Lb. helveticus* peptidase genes (*pepC*, *pepD* and *pepR*) followed a typical exponential-phase expression (Vesanto *et al.*, 1994 and unpublished results), whereas the level of the *Lb. helveticus* *pepN* transcripts remained relatively stable in the same conditions throughout the entire growth period analysed (Varmanen *et al.*, 1994).

Overexpression and purification of PepX

The expression vector was constructed by cloning a 2.5 kb PCR fragment carrying the *pepX* gene into the pKK223-3 vector and designated pKTH2098. The amount of PepX after IPTG-induction increased throughout the entire growth phase of 5 h as judged by SDS-PAGE, whereas the highest level of PepX activity

Fig. 3. SDS-PAGE analysis of PepX expression and purification in *E. coli*. Lanes: 1, molecular mass markers; 2 and 3, crude extracts isolated 2 h after IPTG induction from *E. coli* JM105 with the pKK223-3 vector and from *E. coli* JM105 with pKK223-3 carrying the *pepX* gene, respectively; 4, purified PepX (0.7 µg). Gel electrophoresis was performed in a 10% polyacrylamide gel followed by staining with Coomassie Brilliant Blue R-250.

was obtained 2 h after induction (Fig. 3), suggesting the formation of inactive aggregates at the later state of growth. The yield of PepX was up to 10–20% of the total cellular proteins. Using two chromatographic steps, PepX was purified to homogeneity. The results are summarized in Table 1. The cell-free extract (180 mg protein) was first fractionated by ammonium sulphate precipitation in two saturation steps (50 and 80%). The precipitate formed at 50% saturation contained only a minority of gly-pro-*p*NA hydrolysing activity, whereas the second precipitate at 80% saturation contained the majority of the gly-pro-*p*NA hydrolysing activity and was used for further purification. In the first chromatographic step, the dialysed and filtered sample was applied onto a Q-Sepharose anion exchange column and eluted in 0–0.3 M NaCl gradient. Gly-pro-*p*NA hydrolysing activity eluted at 0.29 M NaCl. During the second step, the active fractions were pooled, concentrated and further purified by hydrophobic interaction chromatography (Phenyl-Superose). The major absorbance peak with the maximum PepX activity was detected at 2 M NaCl and was determined to be an electrophoretically pure enzyme (Fig.

Fig. 4. Effect of temperature (●) and pH (○) on PepX activity.

3). The specific activity was enriched about 36-fold and the yield of total activity was 4.1%.

Characterization of the purified PepX protein

The *Lb. helveticus* PepX had a sharp pH optimum at pH 6.5 and a broad temperature optimum between 37 and 45 °C (Fig. 4). At temperatures above 45 °C, the activity of PepX decreased sharply. These results are in good agreement with the pH and temperature optima of PepXs from *Lb. acidophilus* and *Lb. delbrueckii* subsp. *bulgaricus* (Bockelmann *et al.*, 1991), *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus* and *Lb. helveticus* CNRZ32 (Khalid & Marth, 1990). PepX from *Lb. helveticus* LHE-511 had the same pH optimum, but the temperature optimum was 50 °C (Miyakawa *et al.*, 1994). Also higher temperature optima, near 50 °C, have been reported for the PepX enzymes of *Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. casei* subsp. *casei* by Meyer & Jordi (1987), Miyakawa *et al.* (1991) and Habibi-Najafi & Lee (1994). A higher pH optimum, pH 8.5, was reported for the *Lc. lactis* subsp. *lactis* PepX (Zevaco *et al.*, 1990).

Table 1. Purification of the cloned *Lb. helveticus* 53/7 PepX from *E. coli* JM105

Purification step	Total protein (mg)	Total activity (units)*	Specific activity [units (mg protein) ⁻¹ †	Purification factor	Yield activity (%)
Cell-free extract	180.0	442.8	2.5	1.0	100
(NH ₄) ₂ SO ₄ (80% saturation)	38.2	107.3	2.8	1.1	24
Q-Sepharose	5.0	43.7	8.7	3.5	10
Phenyl-Superose	0.2	18.0	90.0	36.0	4.1

* One unit corresponds to the release of 1 µmol *p*-nitroaniline min⁻¹.

† A molar extinction coefficient of 8800 M⁻¹ cm⁻¹ (Bockelmann *et al.*, 1991) was used for *p*-nitroaniline.

Table 2. Effect of metal ions on PepX activity

Control samples not treated by metal ions are taken as 100 %.

Divalent cation	PepX activity (%) at ion concentration of:	
	0.1 mM	1 mM
Control	100	100
Mg ²⁺	69	95
Ca ²⁺	71	108
Co ²⁺	86	81
Mn ²⁺	80	102
Zn ²⁺	30	10
Cu ²⁺	10	14
Cd ²⁺	17	12

Table 3. Effect of chemical reagents on PepX activity

Control samples not treated by chemical reagents are taken as 100 %.

Reagent	PepX activity (%) at reagent concentration of:	
	0.1 mM	1 mM
Control	100	100
2-Mercaptoethanol	83	101
DTT	107	144
1,10-Phenanthroline	87	69
EDTA	71	108
PMSF	53	24
pHMB	0	0
pHMB + DTT	ND	162
pHMB + 2-mercaptoethanol	ND	68

ND, not determined.

Habibi-Najafi & Lee (1994) reported two optima at pH 7.0 and 9.5 for *Lb. casei* subsp. *casei* PepX.

The effect of divalent cations and various inhibitors on the *Lb. helveticus* 53/7 PepX are summarized in Tables 2 and 3, respectively. PepX activity was strongly inhibited by Cu²⁺ and Cd²⁺ at concentrations of 0.1 and 1 mM (Table 2), which is in good agreement with other PepXs (see references below), except that Cu²⁺ inhibited only slightly the PepX activity of *Lb. casei* subsp. *casei* (Habibi-Najafi & Lee, 1994). Strain 53/7 PepX was also strongly inhibited by Zn²⁺. PepX from *Lb. helveticus* CNRZ32 was also inhibited by 0.1–2.5 mM Zn²⁺ (Khalid & Marth, 1990), in contrast to PepX from *Lb. helveticus* LHE-511, which was slightly activated by 1 mM Zn²⁺ (Miyakawa *et al.*, 1994). PepX activity was not inhibited by 1 mM Ca²⁺ and Mn²⁺. Other divalent cations such as 1 mM Mg²⁺ and Co²⁺ had only a minor effect on the enzyme activity.

The PepX activity was strongly inhibited by 0.1 and 1 mM *p*-hydroxymercuribenzoate (pHMB), partially restored by reducing agents like 2-mercaptoethanol and

completely restored by DTT (Table 3). This result is similar to PepXs from *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* (Meyer & Jordi, 1987; Miyakawa *et al.*, 1991, 1994; Khalid & Marth, 1990; Miyakawa *et al.*, 1994) and suggests the involvement of a sulfhydryl group in catalysis. Alternatively, the inhibition might be due to the steric hindrance by the thiol modifying agent rather than the direct role of cysteine in catalysis. DTT and 2-mercaptoethanol alone had no effect on enzyme activity, indicating that intact disulfide groups were not essential for enzyme activity. PepXs from *Lb. casei* subsp. *casei* and *Lc. lactis* subsp. *lactis* were, however, not inhibited by sulfhydryl inhibitors such as iodoacetate or pHMB, indicating that no functional sulfhydryl group was involved in catalysis in these enzymes (Habibi-Najafi & Lee, 1994; Zevaco *et al.*, 1990; Lloyd & Pritchard, 1991). EDTA at concentration of 1 mM had no effect on PepX activity, while 1,10-phenanthroline, another potential metallo-protease inhibitor reduced the activity slightly (Table 3). This result is similar to PepXs from *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. acidophilus* (Khalid & Marth, 1990; Miyakawa *et al.*, 1994; Bockel-

mann *et al.*, 1991). The addition of the serine enzyme inhibitor, PMSF, reduced activity to about 24–53%, indicating that the active site includes a serine residue. This result is consistent with results obtained with PepXs from *Lb. helveticus* CNRZ32, *Lb. helveticus* LHE-511 and *Lb. casei* subsp. *casei* (Khalid & Marth, 1990; Miyakawa *et al.*, 1994; Habibi-Najafi & Lee, 1994).

The affinity of *Lb. helveticus* 53/7 PepX for the gly-pro-pNA substrate (10–1000 µM final concentration) was studied by measuring the initial rate of the reaction with a continuous assay in thermostat-controlled cuvettes at 40 °C, pH 6.5. The K_m value of 280 µM was calculated by non-linear regression analysis. For the PepX from several lactic acid bacteria, K_m values of 284, 289, 451 and 71 µM have been reported (Bockelmann *et al.*, 1991).

The molecular mass for the native enzyme was calculated to be around 165 kDa by gel filtration, indicating that the enzyme probably consists of two subunits. The discrepancy between the observed dimer molecular mass and the molecular mass expected from the monomeric size might be due to the shape of the PepX dimer when compared to the globular marker proteins. A dimer form has also been reported for PepXs from *S. thermophilus*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* with molecular sizes of 150–200 kDa (Meyer & Jordi, 1987; Kiefer-Partsch *et al.*, 1989; Zevaco *et al.*, 1990, Lloyd & Pritchard, 1991; Bockelmann *et al.*, 1991). Booth *et al.* (1990) calculated a molecular mass of 117 kDa for the active form of the PepX from *Lc. lactis* subsp. *cremoris*. In contrast to our results, monomer forms have been described for *Lb. helveticus* CNRZ32 and LHE-511, where the molecular mass of CNRZ32 PepX was estimated only by native PAGE (Khalid & Marth, 1990; Miyakawa *et al.*, 1994). Moreover, a monomer form has been described for *Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. casei* subsp. *casei* (Atlan *et al.*, 1990; Meyer-Barton *et al.*, 1993; Habibi-Najafi & Lee, 1994) and also a trimer form with a molecular mass of 270 kDa for *Lb. delbrueckii* subsp. *bulgaricus* (Miyakawa *et al.*, 1991). Some of the differences in molecular masses for the native PepXs might be due to the slight variations in pH and NaCl concentrations in gel filtration.

In conclusion, we have characterized the X-prolyl dipeptidyl aminopeptidase (PepX) gene and protein of an industrial *Lb. helveticus* strain. The *pepX* gene was shown to be a monocistronic transcriptional unit having a typical exponential-phase expression. The PepX protein was overexpressed in *E. coli* and purified to homogeneity by only two steps. The purified PepX protein was shown to be a dimeric intracellular serine peptidase possessing characteristics closely resembling those of other X-prolyl dipeptidyl aminopeptidases.

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