Characterization of a prolinase gene and its product and an adjacent ABC transporter gene from *Lactobacillus helveticus*

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A prolinase (*pepR*) gene was cloned from an industrial *Lactobacillus helveticus* strain (53/7). Three clones, hybridizing with a gene probe specific for a peptidase shown to have activity against di- and tripeptides, were detected from a *L. helveticus* genomic library constructed in *Escherichia coli*. None of the three clones, however, showed enzyme activity against the di- or tripeptide substrates tested. One of the clones, carrying a vector with a 5.5 kb insert, was further characterized by DNA sequencing. The sequence analysis revealed the presence of two ORFs, ORF1 and ORF2 of 912 and 1602 bp, respectively. ORF2, located upstream of and in the opposite orientation to ORF1, had a promoter region overlapping that of ORF1. ORF1 had the capacity to encode a 35083 Da protein. When amplified by PCR, ORF1 with its control regions specified a 35 kDa protein in *E. coli* that was able to hydrolyse dipeptides, with highest activity against Pro-Leu, whereas from the tripeptides tested, only Leu-Leu-Leu was slowly degraded. By the substrate-specificity profile and protein homologies, the 35 kDa protein was identified as a prolinase. The activity of the cloned prolinase was inhibited by *p*-hydroxymercuribenzoate. Northern and primer-extension analyses of ORF1 revealed a 1.25 kb transcript and two adjacent transcription start sites, respectively, thus confirming the DNA sequence data. ORF2 had encoding capacity for a 59.5 kDa protein that showed significant homology to several members of the family of ABC transporters. Determination of the mRNA levels at different growth phases revealed that the *pepR* gene and ORF2 are transcribed in *L. helveticus* at the exponential and stationary phases of growth, respectively. Furthermore, two ORF2 deletion constructs, carrying the intact *pepR* gene, showed that this upstream operon adversely affected *PepR* activity in *E. coli*, which explains the enzymic inactivity of the original clones.

Keywords: *Lactobacillus helveticus*, prolinase, peptidases, ABC transporter

INTRODUCTION

Lactic acid bacteria (LAB) play an important role in the production of fermented food. Strains of *Lactobacillus* in particular are widely utilized as starters in dairy, bakery, beverage, vegetable, meat, fish and silage fermentations

Abbreviations: DIG, digoxigenin-dUTP; LAB, lactic acid bacteria; pHMB, *p*-hydroxymercuribenzoate; Pro- β NA, L-proline- β -naphthylamide; Pro-pNA, L-proline-*p*-nitroanilide.

The EMBL accession number for the sequence of the 2845 bp DNA fragment including the *pepR* and ORF2 genes reported in this paper is Z30709.

(McKay & Baldwin, 1990). LAB used in dairy fermentations are unable to synthesize many amino acids and are therefore highly dependent on proteolytic systems that have evolved to degrade milk proteins (Thomas & Pritchard, 1987). The proteolytic properties of LAB also directly affect the composition and quality of food end-products. In cheese manufacturing, the proteolytic activity of LAB affects the ripening process and is currently thought to be essential for the formation of flavour and texture (Fox, 1989; El Soda, 1993).

In recent years, the biochemical and genetic characterization of the proteolytic systems of LAB have received

considerable attention (for recent reviews, see Kok, 1990; Pritchard & Coolbear, 1993; Tan *et al.*, 1993; Visser, 1993; Kok & de Vos, 1994). In the proteolytic cascade of lactococci, and most likely also of lactobacilli, the cell-wall-associated proteinase degrades casein into peptides of different sizes and to free amino acids. Peptides formed are further hydrolysed by several different exo- and endopeptidases into smaller peptides and amino acids. The milk proteins contain a high amount of proline (Fox, 1989), resulting in the generation of proline-rich peptides during the proteinase action (Kok & de Vos, 1994). Since peptide bonds involving a prolyl residue are usually not hydrolysed by general purpose amino-, di- or tripeptidases, LAB possess several proline specific peptidases with different specificities. Activities like prolinase, prolidase, proline iminopeptidase, aminopeptidase P and X-prolyl dipeptidyl aminopeptidase have been detected in various species of LAB (Kok & de Vos, 1994; Baankreis & Exterkate, 1991; Mars & Monnet, 1995). An X-prolyl-dipeptidyl aminopeptidase gene has been cloned from lactococci (Mayo *et al.*, 1991; Nardi *et al.*, 1991) and *Lactobacillus delbrueckii* subsp. *lactis* (Meyer-Barton *et al.*, 1993). Recently, a proline iminopeptidase gene from *L. delbrueckii* subsp. *lactis* (Klein *et al.*, 1994) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Atlan *et al.*, 1994) and a prolinase gene from *Lactobacillus helveticus* CNRZ 32 (Dudley & Steele, 1994) have been described.

We are characterizing peptidases from an industrially utilized *L. helveticus* strain with highly favourable peptidolytic characteristics, in order to study gene expression, regulation and interactions of these peptidolytic enzymes. In this paper, we describe the cloning, DNA sequencing, mRNA analyses and expression of a prolinase gene (*pepR*) from *L. helveticus* 53/7 and show data of an ORF structure which was found upstream of and having overlapping promoter regions with the prolinase gene and the effect of this ORF on the activity of the cloned prolinase in *Escherichia coli*.

METHODS

Bacterial strains, plasmids and culture conditions. *L. helveticus* strain 53/7 is an industrial starter from the collection of Valio Ltd (Helsinki). When cultivated in small scale, *L. helveticus* was inoculated from a skimmed milk culture into MRS broth and grown at 42 °C without shaking. For a larger scale cultivation, a Chemap FZ-3000 bioreactor with pH control was used as described by Vesanto *et al.* (1994). *E. coli* strains DH5 α F' (Woodcock *et al.*, 1989) and CM89 (Miller & Schwartz, 1978) were grown in Luria broth and in Luria broth supplemented with 0.05 mM thiamin and 0.3 mM thymine, respectively. Ampicillin (25–50 μ g ml⁻¹) and erythromycin (0.3 mg ml⁻¹) were added when the pUC19 or pTZ18R (Pharmacia) and pJDC9 vectors (Chen & Morrison, 1987), respectively, were used in *E. coli*.

DNA techniques and screening of a *L. helveticus* genomic library. A *L. helveticus* 53/7 genomic library was constructed in *E. coli* using the pTZ18R vector. The library contained 4000 clones with an average insert size of 7 kb. The library was screened by colony hybridization (Grunstein & Hogness, 1975) using as a probe a 580 bp *EcoRI*–*EcoRV* fragment, carrying part of a *L. helveticus* CNRZ 32 gene expressing peptidase activity

against di- and tripeptides (Nowakowski *et al.*, 1993). Labelling of the probe for hybrid detection was carried out with [α -³²P]dCTP (> 3000 Ci mmol⁻¹/111 TBq mmol⁻¹, Amersham) according to the protocol of a random-primed DNA labelling kit (Amersham). Alkaline lysis (Sambrook *et al.*, 1989) was used for plasmid isolations from *E. coli* with the DNA purification systems Magic (Promega) and Flexi-Prep (Pharmacia). Other recombinant DNA techniques were performed essentially as described by Sambrook *et al.* (1989).

Peptidase activity assays. Peptidase activities in liquid cultures of *L. helveticus* and *E. coli* were determined as follows: cells were harvested by centrifugation at 7000 g for 15 min, frozen in liquid nitrogen, thawed, washed once with 50 mM HEPES (pH 7.0) or 25 mM imidazole (pH 7.0), resuspended in one-third of the original volume of the same buffer followed by sonication on ice using Ultrasonic 2000 sonicator (B. Braun) with intervals of 15 s until the cells were disrupted. After removal of the cell debris, peptidase activities were determined by two alternative methods. The iminopeptidase activity (proline-liberating) was determined using the modified method of Troll & Lindsley (1955) as described by Baankreis & Exterkate (1991). The hydrolysis of peptides not containing proline as the amino-terminal amino acid was assayed by measuring the amount of liberated α -amino termini from hydrolysis of peptide substrates with a ninhydrin reagent essentially as described earlier by Khalid *et al.* (1991), except that 50 mM HEPES pH 7.0 was used. The ability of the cloned peptidase to hydrolyse L-proline-*p*-nitroanilide (Pro-*p*NA) and L-proline- β -naphthylamide (Pro- β NA) was tested by the method of El Soda & Desmazeaud (1982) and Nagatsu *et al.* (1970), respectively. The temperature used for the enzyme assays was 37 °C.

The effect of chelating agents (EDTA and 1,10-phenanthroline), a serine protease inhibitor (PMSF) and a thiol-acting reagent (*p*-hydroxymercuribenzoate, pHMB) were measured with Pro-Leu as the substrate. Cell-free extracts were pre-incubated for 30 min with 5 mM EDTA, 5 mM 1,10-phenanthroline, 1 mM PMSF or 1 mM pHMB before the addition of the substrate. The reactivation of the enzyme after EDTA treatment was performed by adding various divalent cations to the EDTA treated cell-free extracts followed by incubation for 10 min and addition of the substrate (Pro-Leu).

Amplification of DNA by PCR and oligonucleotide synthesis.

DNA fragments were amplified by PCR as follows. The PCR mixtures consisted of 100 ng pKTH2076; 50 pmol primers; 0.2 mM each dATP, dCTP, dGTP and dTTP; 3.0 mM MgCl₂; 0.5 U Perfect Match Enhancer (Stratagene) and 1 U Dynazyme (Finnzymes) in 50 μ l reaction buffer recommended by the manufacturer. The denaturation and extension temperatures were 95 and 72 °C, respectively, and the annealing temperatures were chosen according to the *T_m* of the primers used. The inserts of the pKTH2082, pKTH2087 and pKTH2088 (Fig. 1) were synthesized using the same *pepR*-specific minus strand primer 5'-TCTTGTCGACTCTCATATTGCCATCCCCCT-3' (p0) and as the plus strand primer 5'-CAATGGATCCTATACCCGTATCTTGCCT-3' (p1), 5'-TACCGGATCCCATGA GAACCAGACGAAGC-3' (p2) or 5'-TATAGGATCCAAT TTGCACAACCTGCTGGTG-3' (p3), respectively. The hybridization probe for the detection of ORF2 transcripts was synthesized using 5'-AGCTCATCAAGTCCTGCCTC-3' and 5'-TGCTTCGCTCTGGTTCTCATG-3' as the primers.

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

DNA sequencing and sequence analysis. Sequencing was performed with an ALF DNA sequencer (Pharmacia Biotech) using the dideoxy chain-termination method (Sanger *et al.*, 1977) according to the manual of the AutoRead sequencing kit (Pharmacia). Both DNA strands of the templates were sequenced using the pUC19-specific primers for subclones in combination with sequence-specific oligonucleotides for primer walking. DNA sequences were assembled and analysed with the PC/GENE set of programs (release 6.8, IntelliGenetics). The PROSITE program of PC/GENE was used to detect specific sites and signatures in protein sequences. Hydropathy analyses were performed by the method of Kyte & Doolittle (1982) with the SOAP program of PC/GENE. For homology searches, the databases of EMBL and Swiss-Prot were used both as CD-ROM versions (release 13.0, October 1994) and directly by e-mail with the EMBL BLITZ server.

RNA methods. Total RNA was isolated from *L. helveticus* cells essentially as described (Palva *et al.*, 1988; Vesanto *et al.*, 1994). Total RNA isolation from *E. coli* cells was as described for *B. subtilis* (Palva *et al.*, 1988) without the addition of mutanolysin. RNA agarose gel electrophoresis and Northern blot were performed as described previously (Hames & Higgins, 1985). Hybridization probes were labelled with [α - 32 P]dCTP (> 3000 Ci mmol $^{-1}$ /111 TBq mmol $^{-1}$, Amersham) by using the protocol of the random-primed DNA labelling kit (Amersham) or with non-radioactive digoxigenin-dUTP (DIG, Boehringer Mannheim). DIG luminescent kit (Boehringer) was used for hybrid detection with DIG-labelled probe. The primer extension was performed essentially as described by Vesanto *et al.* (1994) using 10 pmol of the oligonucleotide 5'-TTTCGCTTGAGTGTAGTCC-3' labelled at the 5' end with [γ - 32 P]ATP (> 3000 Ci mmol $^{-1}$ /> 111 TBq mmol $^{-1}$, Amersham). The primer-extension product was analysed by gel electrophoresis using a 6% sequencing gel. The sequencing reaction of pKTH2076, performed with the same primer, was used as the marker for the 5' end determination.

RESULTS

Cloning and sequencing of the prolinase gene from *L. helveticus* 53/7

A 580 bp *EcoRI*–*EcoRV* fragment probe, carrying part of the *L. helveticus* CNRZ 32 *pepPN* gene encoding prolinase PepPN (Nowakowski *et al.*, 1993; Dudley & Steele, 1994), was used in a Southern hybridization to identify the homologous counterpart in the chromosomal DNA of *L. helveticus* 53/7 (data not shown). To clone the corresponding gene, a pTZ18R-vector-based genomic library of *L. helveticus* 53/7 in *E. coli* was screened with the same probe by colony hybridization. Three clones that gave the strongest hybridization signal were chosen for further characterization. The plasmids in these clones were designated pKTH2076, pKTH2077 and pKTH2078, respectively. All clones were, however, shown to be devoid of enzyme activity. Plasmid DNAs of the clones were isolated and digested with restriction enzymes and further analysed by Southern hybridization. Plasmids pKTH2076 and pKTH2077 both released a 1.6 kb *EcoRI* fragment and 1.1 and 0.29 kb *HindIII* fragments which were hybridization positive. These fragments were subcloned from pKTH2076 into pUC19 and designated pKTH2079, pKTH2080 and pKTH2081, respectively. A partial restriction enzyme map of the 5.5 kb *Sau3A* insert of pKTH2076 is shown in Fig. 1. Sequencing revealed an

incomplete 600 bp ORF (designated *pepR*) at the end of the 1.6 kb *EcoRI* insert of pKTH2079 which lacked a translational stop codon. Sequencing of the insert in pKTH2076 revealed that the *pepR* continued for a further 312 bp.

The *pepR* starts with ATG and is preceded, nine nucleotides upstream, by a putative RBS (AGGA) with a window of 8 bp (Fig. 2). A putative promoter region was identified 78 nucleotides upstream of the start codon with –35 (TTGCCT) and –10 (AATAAT) regions which resembled the conserved prokaryotic –35 and –10 consensus sequences. The entire DNA sequence of *pepR* with its flanking regions have been assigned to the EMBL database and only the part relevant to this paper is shown in Fig. 2.

PepR protein was 99.6% identical with the recently sequenced prolinase (PepPN) from *L. helveticus* CNRZ 32 (Dudley & Steele, 1994). The prolinase gene was renamed *pepR* to comply with the nomenclature of peptidase genes.

Analysis of the upstream region of *pepR*

Examination of the nucleotide sequence upstream of *pepR* revealed a start of another ORF (ORF2) in the opposite orientation (Figs 1 and 2). The start codon of ORF2 was located 120 nucleotides upstream of *pepR*. It is preceded by a putative promoter region comprising –35 (TTGAGA) and –10 (TATAAT) sequences which initiate 57 nucleotides upstream of the start codon (Fig. 2). ORF2 is 1602 bp long and could encode a protein of 534 amino acids. ORF2 is followed by an inverted repeat structure 36 bp downstream of the stop codon. The resultant transcript would have a $\Delta G = -109$ kJ mol $^{-1}$, and consist of a stem of 17 bp and a loop of 4 bp, and is therefore a putative transcription terminator. The putative promoters of *pepR* and ORF2 partially overlap (Fig. 2).

Analysis of the predicted amino acid sequence encoded by ORF2 revealed the presence of an ATP/GTP-binding site (residues 354–361, Fig. 2) and a signature typical of the ABC family of transporters (residues 454–465, Fig. 2) in the C-terminal half of the protein. Hydropathy analysis revealed that the N-terminal half of the protein contains stretches of hydrophobic amino acids corresponding to six transmembrane helices (data not shown). According to the homology searches, the ORF2 protein showed significant homology to several members of the ABC family of transporters, including multidrug-resistance proteins from different species and the lactococcin A transport protein. The highest overall homology was found with a transport protein CydC (39%) and a probable ATP-binding transport protein MsbA (40%) from *E. coli*.

The influence of ORF2 on expression of *pepR* in *E. coli*

Since *E. coli* carrying pKTH2076, pKTH2079 and pKTH2080 did not produce activity against di- and tripeptide substrates, a PCR fragment, carrying *pepR* with

Fig. 1. Partial restriction and deletion map of pKTH2076 carrying the cloned *L. helveticus* 53/7 *pepR* region. ORF1 (*pepR*) and ORF2 are indicated by broken arrows. pKTH2079 and pKTH2080 refer to two subclones. The inserts of pKTH2082, pKTH2087 and pKTH2088 were generated by amplification by PCR. The mRNAs, marked with the dotted arrows, refer to the sizes of the transcripts revealed by Northern blots with the *pepR*- and ORF2-specific probes. Enzyme activity was determined from cell-free extracts as free proline liberated from 2 mM substrate (Pro-Leu). Abbreviations for restriction enzymes: E, *EcoRI*; H, *HindIII*; K, *KpnI*; S, *Sau3A*.

its putative promoter and transcription terminator regions, was generated with the primers p0 and p1 (see Methods and Fig. 2) using pKTH2076 as the template. The amplified PCR product was ligated as a 1.1 kb *Bam*HI–*Sal*I fragment with pJDC9 and transferred into a peptidase-deficient *E. coli* CM 89 resulting in plasmid pKTH2082 (Fig. 1). Determination of peptidase activity of the *E. coli* (pKTH2082) revealed hydrolysis of several peptide substrates confirming the expected activity. A DNA fragment amplified by PCR with the *pepR*-specific primers from the *L. helveticus* chromosomal DNA encoded peptidase activity was indistinguishable from that of pKTH2082 (data not shown).

To test the effect of the upstream ABC transporter gene or its promoter on PepR activity, two constructs, carrying the intact *pepR* gene on a 1.1 kb fragment and 150 or 500 bp from ORF2, were generated by PCR. The primer pairs p0/p2 and p0/p3 (see Methods and Fig. 2) were used to amplify the 1.3 and 1.65 kb fragments, respectively, which were then inserted into pJDC9 and electroporated into *E. coli* CM89. The resulting plasmids were designated pKTH2087 and pKTH2088 (Fig. 1), respectively. The clones carrying pKTH2082, pKTH2087 and pKTH2088 were grown to the mid-exponential phase and PepR activity was analysed. The relative PepR activities against Pro-Leu were 100, 55 and 32% in the three clones with pKTH2082, pKTH2087 and pKTH2088, respectively. To study whether the differences in enzyme activities were due to possible copy-number variations in the three constructs, total DNAs were isolated and analysed by DNA dot-blot and Southern hybridizations. No differences were observed in the amounts of pKTH2082, pKTH2087 and pKTH2088 detected by a *pepR*-specific probe (Fig. 3a). However, the mobility of the intact pKTH2088 in agarose gels was retarded although the unit size of its linear form was unchanged (data not shown). To compare the amounts of the *pepR*-specific transcripts synthesized by these three *E. coli* clones, total RNA was isolated from exponentially growing cells and analysed by Northern blotting. The hybridization signal corresponding to the *pepR*-specific transcripts was clearly weaker from the clone carrying the largest insert (pKTH2088) than that of the other two clones, pKTH2082 and pKTH2087, respectively (Fig. 3b). Constructs carrying

larger segments of ORF2 did not show any prolinase activity and were often structurally unstable in *E. coli* (data not shown).

Expression of *pepR* and ORF2 in *L. helveticus*

The size of mRNA transcribed from the *pepR* gene was analysed by Northern blot using the 1.1 kb PCR fragment as the hybridization probe. The probe detected a 1.25 kb transcript (Fig. 4) which is in good agreement with the DNA sequence data and implies that the *pepR* gene is a monocistronic transcriptional unit. The primer-extension mapping of 5' ends of the *pepR* mRNA from exponentially growing cells revealed two start sites, separated by two nucleotides. The start sites (G and A) of the *pepR* transcripts locate immediately downstream of the putative promoter region (Fig. 2). Thus, the promoter region of the *L. helveticus* CNRZ 32 *pepR* gene (Dudley & Steele, 1994), deduced without the 5' end mapping of mRNA, was incorrectly assigned.

The transcription level of *pepR* in different growth phases was analysed by Northern hybridization (Fig. 4a). The amount of the *pepR* transcripts was highest at the end of the exponential phase, whereas most of the *pepR* mRNA detected at the stationary phase was degraded.

To study the expression of the *pepR* gene in *L. helveticus*, enzyme activity against the Pro-Leu substrate was measured as a function of growth (Fig. 5). The amount of enzyme activity increased up to the early-stationary phase, reaching the highest level 8 h after inoculation. Thereafter, the Pro-Leu-degrading activity remained relatively constant (Fig. 5).

With an ORF2-specific probe, a single 1.8 kb RNA band was detected (Fig. 4b). According to the Northern data (Fig. 4), in *L. helveticus*, the ORF2 is transcribed only at the stationary phase, whereas the transcription of *pepR* is essentially restricted to the exponential phase.

Biochemical characterization of PepR

In lysates of *E. coli*(pKTH2082), activity against the dipeptides Pro-Leu, Met-Ala and Leu-Leu was detected. Activity was highest against Pro-Leu, whereas Leu-Pro

CTTGAGTGTAGTCCCAAGTGATAACCGTGTCTAAAGTAATGATTTTAGTACCAGTTT 60
GAACTCAAAATCAGGTGTTCTACTATTGGCAACAGATTTCATTACTAAAAATCATGGTCAA

←PepR RBS (pepR) -10 (pepR)
TCATTAATAAATCCTTTCTTTTATATGTTTAAAAATAATTTTAAAGATCTTTAATTTATTG 120
AGTAATTTTTTAGGAAAGAAATATACAAATTTTTTATTAATAATCTTATGAAATTAATAAC
↑ ↑

-35 -35 (pepR) -10 p1 RBS
TATCAGATTGAGAAAAAGGCAAGATAGCGGGTATAATGTTTAAATAAAAAAGAAAGGGGT 180
ATAGCTAACTCTTTTTCGGTT

ORF2→
CTATATGAGTCTTAAATACGCTAAGAAAAGCCAACTGATTACTTACACCATTTTAGCTGC 240
M S L K Y A K K S Q L I T Y T I L A A 19

AATTAAGCCTGCAACATTGTCTTTGTGCTATATGATTGAAATTTAGTTGAACGTTGC 300
I K A C N I V F V A Y M I E I M L N V A 39

TTGCTGCTGTTCTCATGATTACATGCAATTAGTTAGATTAGCTTTATTGACTGCTCTGGG 360
S S G S H D Y M H L V R L A L L T A L G 59

ACAAATGTTGTTTATGCGCAGCAACTTTGTGTATGAAACGGTTAAGATGGGAATCATTGG 420
Q M C F M A S N F V Y E T V K M G I I R 79

TGATGTGAACATGACTTTGAAGAGAGATAATCTTCGATATCTGGTTGATCAGGGGACCCC 480
D V N M T L K R V N L R Y L V D Q G T P 99

AGATATTAAGTGGTTTGTCACTGATGACGAATGATTGAAGCAGATTGAACTAACCG 540
D I K S G L S L M T N D L K Q I E T N R 119

TGTTACTGCTCAATTAGACATGATTTTCAAGGTTTGTTCATTGTTGGTGCGATTGGTTT 600
V T A Q L D M I F Q G L S F I G A I G F 139

TGCCTTTTACAGTTTCATGCGAGATGACATTAGTATTGTTGTAGCAACGATTGACCCAGC 660
A F Y S S W Q M T L V F V V A T I A P A 159

AGTTGTGCAAAATTTACTAGCCCAATCATTACGAAAGGTCTAAGATCTGGGCCAGAC 720
V V Q I I T I P C P I I T K K S K I W A Q T 179

TAATGCTAACTATACTCAGCATGTTTCTGATTCTTAAATGGTGGCAAGCACTAAACT 780
N A N Y T Q H V S D S L N G A Q A T K L 199

GTATAACGTGCGCAAAATATCATTACGCGTGCGCAAAATGCGGCTCAACAAATGAAAA 840
Y N V R T N I I T R A A N A A Q Q M E N 219

CGCTTTGAGAAACATGACTTTAAACCAAGCTTGGGCATTGGAATTGATCTTCTGCGAGC 900
A L R N M T L T Q A W A L E L I Y S A A 239

AGAGCTTTTCTGTTTCTTATTCATTCATGATGATGATGATGATGATGATGATGATGATGAT 960
E L F C F I I P C T I G I L M M Q K G N 259

TTTAAAGTTGGTACCTTGGTCTGATGATGATGATGATGATGATGATGATGATGATGATGAT 1020
L K V G T L V M M V D L A M N F I T P V 279

TGTTACGCTTTTAAACGAATTCAACCAAGTTAAGTCGACCGTTCCAATGTTGGGAAAAGAC 1080
T L F N E P T I G V K S T V P M W E K T 299

ACAAGTTGCTTGAAGCATGTCATGAAGAATGACAAGCAGAAGATCGATCATTTTGAAG 1140
Q V A L K H V M K N D K Q K I D H F E G 319

CATGGAAGTTAATGACCTGTCTTATGTAACCTGGCAGTGATCAATAACAGATTTTGTATGG 1200
M E V N D L S I V T G S D H K Q I F D G 339

GGTAAGTCTGAGATTAAAGCCTGGTGAAGAAAGTTTGTCTTATGGCTCCAAGTGGCTGGG 1260
V S L Q I K P G E K V L L M A P S G W G 359

TAAGACGACTTTGCTTAGATTGCTCTTAGGATTGAAGCAGCCGAAGAGGGTAAGATTTT 1320
K T T L L R L L L G L K Q P K K G K I L 379

ACTGAATGGCAAAAACGTAACGGGTAATGGGACGCGCCATAACTACTATTTCATACGT 1380
L N G K N V T G N W D A A H N Y Y S Y V 399

TAACCAAAAGCGGTTTATGTTGATGATACGCTTCGTTTAAATATCAGCTTAGGAAGACA 1440
N Q K P F M F D D T L R F N I T L G R Q 419

AGTTAGTGATGAGCAATTGAAGCAAGTATTGATGAGGAGGACTGATGAGCTGGTTAA 1500
V S D E Q L K Q V I H E A G L D E L V K 439

AGAACAGGGCTTAGCAAGCAAGTTGGTGAAGAAAGCGGTAAGCAACTTTCTGGTGGTCAGAT 1560
E Q G L D K Q G S E L S G G G I A 459

TCAGAGAGTAGAAATTCGCGGTGCGCTTTGTCTGGGACACCAATTTTGTAGCAGATGA 1620
Q R V E I A R A L L S G R P I L L A D E 479

AGCAACGAGTGGCTGATCCTAAATGTGATGATGATGATGATGATGATGATGATGATGATGAT 1680
A T S A L D P K L S L D I H E T L L K N 499

TCCGAAGGTAGCGGTGCTAAGATGAGCCCAAGATTTTCCAGAAGAAAGGCTATGTT 1740
P K V A V L E V A H K I S P E E K A M F 519

TGATGTGATTATTCATTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1800
D V I I H L D K H T V E T K M 534

CCCTTATTTTATGATGATTTAATATATGAACACACCGGCACTGAAGTGCCGGTGTAGTT 1860
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CATCAGTGGTTACATCATTTAGTAGATGATCTTCTACTGTAGTTTCTTGTCTGTCTGTAT 1920

Fig. 2. Nucleotide and the deduced amino acid sequence of the ORF2 and the nucleotide sequence of the *pepR* promoter region. The predicted -10 and -35 regions of the promoter of ORF2 are underlined with a dotted line and written in bold. The predicted -10 and -35 regions of the *pepR* promoter are in bold. The 5' ends of *pepR* transcripts, found by primer

Fig. 3. Gene copy number (a) and mRNA (b) analyses of the *E. coli* CM89 clones carrying different *pepR*-ORF2 constructs. Total DNA and RNA were isolated from clones grown to same cell densities (mid-exponential phase). (a) For the dot-blot, the samples were alkali-denatured and blotted onto positively charged nylon membrane and hybridized with a DIG-labelled *pepR*-specific probe. (b) For the Northern blot, total RNA, denatured with glyoxal and DMSO, was run in a 1.0% agarose gel with 10 mM phosphate buffer, pH 6.5, followed by transfer to a positively charged nylon membrane and hybridization with a DIG-labelled *pepR*-specific probe. The dots (a) and lanes (b) from 1 to 4 show the hybridization results with the samples from *E. coli* CM89 cells carrying no plasmid, pKTH2082, pKTH2087 and pKTH2088, respectively.

and Gly-Tyr were not degraded. From six tripeptides tested, PepR only hydrolysed Leu-Leu-Leu substrate. The enzyme was unable to liberate proline from Pro-Gly-Gly, Pro-Phe-Gly-Lys or from Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys. However, prolinase did show some peptidase activity using Pro-Phe-Gly-Lys or Pro- β NA and Pro- β NA as substrates.

The effect of inhibitors on the pKTH2082-encoded prolinase in *E. coli* CM89 are summarized in Table 1. pHMB at 1 mM inhibited PepR activity by 72% whereas 1 mM PMSF and 5 mM EDTA caused only a slight reduction in PepR activity.

The prolinase from *L. helveticus* CNRZ 32 has been reported to be inactive against C-terminally modified substrate (Dudley & Steele, 1994). For comparison of the substrate specificities of the two prolinases from *L. helveticus* 53/7 and CNRZ 32, the *pepR* gene was also

extension, are marked with vertical arrows. RBS refers to the predicted ribosome-binding site. The deduced transcription terminator of ORF2 is shown with dotted arrows. Binding sites of the primers p1, p2 and p3 are overlined. Amino acids comprising the ATP/GTP-binding site and the ABC transporters family signature are in bold.

Fig. 4. Northern blot analysis of the *L. helveticus* 53/7 *pepR* (a) and ORF2 (b) transcripts at different growth phases. Strain 53/7 was grown in 2.5 l MRS broth at 42 °C using a Chemap FZ-3000 bioreactor. pH was maintained at 5.85 by the addition of 1 M NaOH. Glyoxal and DMSO were used to denature the RNA samples before loading onto agarose gel. (a) Samples run in a 0.8% agarose gel with 10 mM phosphate buffer, pH 6.5, followed by transfer to Zetaprobe membrane and hybridization with the ³²P-labelled 1.1 kb PCR fragment of pKTH2082 (Fig. 1). Lanes 1–4 show the hybridization result with the RNA samples taken 4, 6, 12 and 18 h after inoculation, respectively. (b) Samples run in a 1.5% gel followed by transfer to a positively charged nylon membrane (Boehringer Mannheim) and hybridization with a DIG-labelled 1.2 kb ORF2 PCR-fragment amplified from pKTH2076 (Fig. 1). Lanes 1–4, RNA samples taken 4, 6, 12 and 18 h after inoculation.

amplified by PCR from the chromosomal DNA of strain CNRZ 32 utilizing the same primers used for the construction of pKTH2082. The 1.1 kb PCR product was ligated as a *Bam*HI–*Sal*I fragment into pJDC9 and transferred into *E. coli* CM89. In contrast to the earlier observation (Dudley & Steele, 1994), *PepR* of CNRZ 32 was able to hydrolyse chromogenic substrates. At the same cell densities, the activities of the cell lysate from an *E. coli* clone carrying the CNRZ 32 *pepR* gene were 141, 50 and 60% of those obtained with the 53/7 *pepR* clone when using the substrates Pro-Leu, Pro-pNA and Pro-βNA, respectively, from several independent experiments with parallel measurements. No qualitative differences in

Fig. 5. Proline-specific peptidase activity in *L. helveticus* as a function of growth. For the growth conditions, see Fig. 4. The circle and triangle refer to cell density and peptidase activity against Pro-Leu (2 mM), respectively. One unit is defined as amount of enzyme activity producing a variation of 0.01 unit of absorbance at 480 nm per min.

Table 1. Inhibition of prolinase activity encoded by the *L. helveticus pepR* gene cloned in *E. coli* CM89.

The liberated proline from 2 mM substrate (Pro-Leu) in 25 mM imidazole buffer (pH 7.0) was measured.

Inhibitor	Concentration (mM)	Relative activity (%)
None		100
PMSF	1	77
pHMB	1	28
EDTA	5	76
1,10-Phenanthroline	5	55

the substrate-specificity profiles between the two prolinases were observed (data not shown).

DISCUSSION

In this work, we have cloned a proline-specific peptidase gene from an industrially used *L. helveticus* strain 53/7 with DNA hybridization, using as the probe a fragment of the *L. helveticus* CNRZ 32 prolinase *pepPN* gene (Nowakowski *et al.*, 1993; Dudley & Steele, 1994). The preliminary substrate-specificity testing of these two *L. helveticus* peptidases identifies them as prolinases (*PepR*).

The suggestion that the CNRZ 32 prolinase is able to hydrolyse tripeptides with the proline residue at the first position has been shown to be incorrect, and according to present knowledge derived from the purified enzyme it has strong preference for dipeptides over tripeptides (Dudley & Steele, 1994; W. Shao, K. L. Parkin & J. L. Steele, personal communication). Determinations of the enzyme activities with the cell-free extracts of the *E. coli* PepR clones indicate, in contrast to an earlier observation (Dudley & Steele, 1994), that neither 53/7 nor CNRZ 32 prolinase needs a free C-terminus in the substrate for peptidase activity. Both enzymes are able to hydrolyse Pro- β NA and Pro- β NA, even though the strain 53/7 prolinase is more active against these substrates. However, the rate of hydrolysis with the above chromogenic substrates was over 100-fold lower than that with the Pro-Leu substrate. The consistent activity against Pro-Phe-Gly-Lys, revealed only by the ninhydrin method, suggests that PepR might also have peptidase activity apart from that of aminopeptidases. By resequencing the inserts of pKTH2082 and of the corresponding CNRZ 32 *pepR* construct, it was confirmed that the only amino acid difference between these two enzymes is the substitution of the aspartic acid residue of strain CNRZ 32 for asparagine in strain 53/7 at position 202. This one amino acid change has to be the cause for the difference in the rate of hydrolysis with chromogenic substrates.

The prolinase of strain CNRZ 32 has been reported to share similarities of 35 and 25 % with the *Bacillus coagulans* and *L. delbrueckii* subsp. *bulgaricus* iminopeptidases, respectively (Dudley & Steele, 1994). However, using the PALING program of PC/GENE the PepRs had the overall similarities of 37, 40 and 40 % to these proline iminopeptidases from *B. coagulans* (Kitazono *et al.*, 1992), *L. delbrueckii* subsp. *bulgaricus* (Atlan *et al.*, 1994) and *L. delbrueckii* subsp. *lactis* (Klein *et al.*, 1994), respectively.

The two *L. delbrueckii* PepI enzymes are nearly identical and have a putative serine catalytic site homologous to that of hydrolases involved in degradation of cyclic compounds (Klein *et al.*, 1994; Atlan *et al.*, 1994). From the *L. helveticus* prolinase, an identical catalytic site (GQSWGG) to that of the two *L. delbrueckii* PepI enzymes can also be found. PMSF, however, inhibited PepR only slightly. This was also found with the *L. delbrueckii* subsp. *bulgaricus* PepI by Gilbert *et al.* (1994). However, a strong inhibition of PepIs with dichloroisocoumarin implies that they indeed are serine proteases (Gilbert *et al.*, 1994; Klein *et al.*, 1994). PepR was strongly inhibited by pHMB indicating that the only thiol residue is either at or near the active site of PepR. Alternatively, the inhibition may be due to the steric hindrance of pHMB. Activity of PepR was slightly inhibited by EDTA and 1,10-phenanthroline. Unexpectedly, the EDTA inhibition was observed to be strongly pH-dependent, varying from 20 % at pH 6.5 to 94 % at pH 7.7. The activity inhibited by EDTA was fully recovered by the addition of Zn^{2+} . In spite of protein homology and the identical region of the serine catalytic site with the *L. delbrueckii* PepIs, the *L. helveticus* prolinase appears to be a separate proline-specific enzyme with distinctive substrate specificity. This is supported by our

recent finding that *L. helveticus* also possesses an imino-peptidase with overall similarities of 39 and 75 % to the *L. helveticus* PepR and to the *L. delbrueckii* PepIs, respectively.

ORF2 had a deleterious effect on the activity of the cloned prolinase in *E. coli*. The loss of prolinase activity in the presence of ORF2 may be due to the competition of the overlapping promoters, since the putative ORF 2 promoter is nearly identical to the *E. coli* consensus promoter. A reduction of 45 % in the prolinase activity was observed in an *E. coli* clone carrying the promoter region and only 132 bp of ORF2. This reduction was not caused by a decrease in the plasmid copy number or by structural instability. The construct carrying 489 bp of the ORF2, encoding four transmembrane helices of the ORF2 gene product, caused the reduction of 68 % in the prolinase activity and a marked decrease in the level of *pepR* mRNA. This was accompanied by an altered plasmid mobility in agarose gels, suggesting changes in the plasmid conformation. It seems that both the promoter competition and expression of ORF2 may affect the synthesis of the *pepR* gene product in *E. coli*.

The predicted amino acid sequence of the ORF2-encoded protein showed significant overall homology to the CydC (Poole *et al.*, 1993) and MsbA (Karow & Georgopoulos, 1993) proteins suggested to be involved in the export of as-yet-unidentified molecules. The putative protein encoded by ORF2 belongs to the subfamily of bacterial ABC exporters (Fath & Kolter, 1993), and it is, therefore, tempting to assume that the ORF2 protein is involved in export. The mRNA analysis revealed that ORF2 is expressed only at the stationary phase of growth in *L. helveticus*, whereas the *pepR* gene is transcribed at the exponential growth phase. The function and possible interaction of ORF2 with prolinase expression in *L. helveticus* is currently under investigation.

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