***Lactobacillus delbrueckii* subsp. *lactis* DSM7290 *pepG* gene encodes a novel cysteine aminopeptidase**

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A number of *Escherichia coli* clones were isolated from a *Lactobacillus delbrueckii* subsp. *lactis* gene library capable of hydrolysing the chromogenic substrate Gly-Ala- β -naphthylamide (Gly-Ala- β NA). Some of the recombinant plasmids carried by these clones have been shown to encode the cysteine aminopeptidase gene *pepC*. Nucleotide sequence analyses of the plasmid inserts of the remaining clones resulted in the identification of two adjacent ORFs encoding proteins exhibiting a high degree of similarity between themselves (72.6%) and with *PepC*. One gene, designated *pepG*, was overexpressed in *E. coli* and the crude extracts obtained were shown to be peptidolytically active both against chromogenic substrates and peptides, and in a *Salmonella typhimurium* growth test. *PepC* and *PepG* activities were compared using chromogenic β NA and *p*-nitroanilide substrates and leucine or proline-containing peptides were applied in growth experiments of recombinant *Sal. typhimurium*. The results indicate that the enzymes, although structurally related, have different substrate preferences. No enzyme activity could be ascribed to the second ORF (*orfW*), despite the production of a visible protein using a T7 RNA polymerase system. Primer extension analysis, using mRNA isolated from *Lb. delbrueckii* subsp. *lactis* DSM7290 did establish that *orfW* was transcribed.

Keywords: *Lactobacillus delbrueckii* subsp. *lactis*, cysteine aminopeptidases, gene duplication

INTRODUCTION

Lactic acid bacteria are used as starter strains for the fermentation of a large range of food products. The thermophilic lactobacilli are among the main representatives used in industrial fermentations of hard cheese and yoghurt. They are extremely fastidious for a number of nutrients and milk is not an optimal growth medium for them. The growth rate of lactobacilli in milk depends on the production of proteolytic and peptidolytic enzymes, which together with respective peptide and amino acid transport systems co-ordinately act to use casein as a source for essential amino acids. Breakdown of casein and casein-derived peptides during ripening produces the typical texture and flavour of cheese. The proteolytic activity determines the rate and extent of the

fermentation process. The manufacture as well as the quality of dairy products crucially depends on the properties of the starter organisms. A desire to understand better the proteolytic systems involved has led several research groups to study lactic acid bacteria (Pritchard & Coolbear, 1993), particularly *Lactococcus lactis* (Poolman *et al.*, 1995; Kok & De Vos, 1994). Members of the genus *Lactobacillus* have also been investigated and genes involved in proteolysis isolated and characterized (Christensen *et al.*, 1995; Dudley *et al.*, 1996; Gilbert *et al.*, 1996; Klein *et al.*, 1996; Stucky *et al.*, 1995a, b; Vesanto *et al.*, 1995; and references therein).

We have previously characterized several genes of the proteolytic system of *Lactobacillus delbrueckii* subsp. *lactis* DSM7290, a strain originally isolated from Emmentaler cheese (Klein *et al.*, 1996). In our recent screening for new peptidase genes in the multiple peptidase-deficient *Escherichia coli* strain CM89 we used the chromogenic substrate Gly-Ala- β -naphthyl-

Abbreviations: *p*NA, *p*-nitroanilide; β NA, β -naphthylamide.

The EMBL accession number for the nucleotide sequence reported in this paper is Z71782.

amide (Gly-Ala- β NA), which is not cleaved by any of the previously cloned peptidases of DSM7290. This allowed the isolation of clones with the respective hydrolytic activity. Two of the recombinant plasmids obtained were found to carry the cysteine aminopeptidase gene (*pepC*), which was subsequently sequenced (Klein *et al.*, 1994). This study describes the identification of two further genes, one of which, *pepG*, encodes a functionally active cysteine protease.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *E. coli* was grown at 37 °C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989), strain CM89 in LB supplemented with 100 μ g thymidine ml⁻¹ and *Lb. delbrueckii* subsp. *lactis* in MRS (De Man *et al.*, 1960) at 37 °C. The ability of *Salmonella typhimurium* TN1547 transformed with plasmids pJKG8 and pJKC4, to utilize Leu- and Pro-containing peptides as amino acid sources was tested by placing a few peptide crystals (Bachem) on the surface of a minimal glucose plate inoculated with the strain, as described by Carter & Miller (1984). Ampicillin or kanamycin were added when appropriate to give final concentrations of 200 and 40 μ g ml⁻¹, respectively.

Transformations. *E. coli* and *Sal. typhimurium* were transformed by electroporation using a Bio-Rad Gene Pulser as described by Dower *et al.* (1988) and O'Callaghan & Charbit (1990).

Recombinant DNA techniques. Restriction enzymes and other nucleic-acid-modifying enzymes were used as recommended by the manufacturers and molecular cloning techniques were performed essentially as described by Sambrook *et al.* (1989). Isolation of chromosomal DNA from *Lb. delbrueckii* subsp. *lactis* DSM7290 and preparation of a plasmid library in the low-copy-number vector pLG339 (Stoker *et al.*, 1982) was

performed as described previously (Klein *et al.*, 1993). Primer extension of mRNA was performed as described previously (Matern *et al.*, 1994) using oligonucleotides complementary to coding regions of *orfW* (5' GAGCCCGCTGCGTCTGGCGGCCCCGAG 3'; nt 550–575) and *pepG* (5' CATTGTA-GGAAGCTTCCAGAACACCGC 3'; nt 1934–1960).

Cloning of the *Lb. delbrueckii* subsp. *lactis* *orfW* and *pepG* genes, plasmid constructions and DNA sequencing. The genomic library of *Lb. delbrueckii* subsp. *lactis* DSM7290 (Klein *et al.*, 1993) was used to transform *E. coli* strain CM89. Transformants with Gly-Ala- β NA-cleaving activity were identified by a colorimetric plate assay as described previously (Klein *et al.*, 1993). Isolated plasmids and plasmid constructions are illustrated in Fig. 1. DNA sequence determination of the chromosomal inserts of plasmid pJKG4 was initiated with synthetic oligonucleotide primers derived from the known sequence of the vector used. Further sequencing of pJKG4 and sequencing of the inserts of pJKG6 and pJKG2 was done by a primer-walking strategy with oligonucleotides synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer. The nucleotide sequence was established for both strands using the T7 DNA polymerase sequencing kit (Sequenase 2.0; USB), which is based on the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) in the presence of [³⁵S]dATP α S (ICN) with double-stranded plasmid DNA purified by NUCLEOBOND AX100 columns (Macherey-Nagel) as template. For computer-assisted DNA and amino acid sequence analyses, MICROGENIE (Beckman), PCGENE (IntelliGenetics), and HUSAR (GENIUSnet) software was used.

Expression of *orfW* in vitro and under control of the T7 promoter. *In vitro* expression of plasmid DNA (pJKW1) was performed with a cell-free coupled transcription/translation system derived from *E. coli* (Amersham), according to the manufacturer's protocol. *In vivo* expression was carried out using *E. coli* PS1 as described by Sankar *et al.* (1993) with the modifications that vector pWSK129 (Wang & Kushner, 1991) was applied and labelling with ³⁵S-Met was omitted. LB

Table 1. Bacterial strains and plasmids

Strain	Genotype, relevant features	Reference	
<i>Lb. delbrueckii</i> subsp. <i>lactis</i> DSM7290	Source of plasmid library	Klein <i>et al.</i> (1993)	
<i>E. coli</i> K-12 CM89	F ⁻ <i>leu-9</i> Δ(<i>proAB pepD</i>) <i>met thyA pepN102 pepA11 pepB1 pepQ10</i>	Miller & Schwartz (1978)	
<i>E. coli</i> B PS1	F ⁻ <i>hsdS lacUV5::T7 gene 1 gyrA srl-300::Tn 10 recA1</i>	Sankar <i>et al.</i> (1993)	
<i>Sal. typhimurium</i> TN1547	<i>leuBCD485 pepN90 pepA16 pepB11 supQ302</i> Δ(<i>proAB pepD</i>) <i>pepP1 pepQ1 pepT1 pepE1</i>	Carter & Miller (1984)	
Plasmid*	Relevant features†	Vector	Reference
pLG339	Tc ^r Km ^r		Stoker <i>et al.</i> (1982)
pWSK129	Km ^r pT7		Wang & Kushner (1991)
pJKG2	Km ^r <i>orfW pepG</i>	pLG339	This study
pJKG4, pJKG6, pJKG8	Km ^r Δ <i>orfW pepG</i>	pLG339	This study
pJKW1	Km ^r <i>orfW</i>	pLG339	This study
pJKW2	Km ^r <i>orfW</i>	pWSK129	This study
pJKC4	Km ^r <i>pepC</i>	pLG339	Klein <i>et al.</i> (1994)

* All listed plasmids are derivatives containing the low-copy-number replicon pSC105.

† Km, kanamycin; Tc, tetracycline.

Fig. 1. Insert DNA of gene bank plasmids and subcloning of *orfW*. The initially isolated gene bank plasmids (pJKG2, pJKG4, pJKG6 and pJKG8) were isolated by their ability to provide *E. coli* CM89 with Gly-Ala-βNA-cleaving activity. pJKG2, with the largest insert, contains 5.3 kb of chromosomal DNA, including *orfW* and *pepG*. pJKW1, exclusively encoding *orfW*, was constructed by re-ligating pJKG2 cut with *PstI* and *SalI* after generation of blunt ends. pJKW2, with *orfW* under control of the T7 promoter, resulted from cloning of the *EcoNI*-*PstI* fragment containing *orfW* into pWSK129 *EcoRV*-*PstI* restriction sites. The 5' end of the tetracycline resistance determinant of the vector with its promoter (*tet*) is indicated by an open box.

(300 ml) was inoculated with 1/100 vol. of a PS1(pJKW2) overnight culture and incubated at 37 °C until an OD₆₀₀ of 0.5 was reached. T7 RNA polymerase was induced with IPTG (0.5 mM final concentration) and over a time course (0, 35, 90, 165, 210 and 420 min) 30 ml aliquots were centrifuged and cell extracts prepared by ultrasonication of the bacteria, essentially as described previously (Klein *et al.*, 1993). Inhibition of *E. coli* RNA polymerase by rifampicin was omitted.

Preparation of cell-free extracts and gel electrophoresis. Cell-free extracts from overnight cultures of *E. coli* were prepared as described previously (Klein *et al.*, 1993). Preparations contained approximately 50 mg protein ml⁻¹ as determined by the Lowry method. Crude extracts were analysed by SDS-PAGE (12.5% acrylamide) using the discontinuous buffer system of Laemmli (1970) and staining with Coomassie Blue, according to Weber & Osborn (1969). The content of PepG protein in extracts was determined by video densitometry (Cybertech CS1, Image Documentation System) with Coomassie-stained SDS gels calibrated with bovine serum albumin.

Assay of peptidase activity, measurements of pH and temperature influences and effects of various chemical reagents. Peptidase G was assayed in 0.4 ml reaction vols by incubating crude extracts of *E. coli* CM89(pJKG8) at 37 °C in the presence of 10 mM Bis-Tris, pH 7.0, and 1 mM Ala-Ala-p-nitroanilide (Ala-Ala-pNA) (Bachem) as substrate. After 30 min, samples were heated at 90 °C for 5 min. The release of p-nitroaniline was determined at 405 nm in a LKB Ultrospec plus spectrophotometer. PepC activity was determined using CM89(pJKC4) extracts and Ala-pNA as substrate.

The effect of temperature on aminopeptidase activity was determined using Ala-pNA (for PepC) or Ala-Ala-pNA (for PepG) as a substrate in the range 5–70 °C. The reaction mixture was pre-incubated for 10 min at different temperatures before the addition of the substrate.

The effect of pH on aminopeptidase activities was determined in the range pH 4–10 using a polybuffer composed of 200 mM each of malic acid, MES, HEPES and boric acid adjusted to the appropriate pH with KOH. Prior to the addition of the substrate, the extracts were equilibrated in the respective buffer for 10 min.

To study the mechanism of enzyme action, the inhibitors 3,4-dichloroisocoumarin at a concentration of 100 μM, Pefa Block at 1 μM, pepstatin A at 1 μg ml⁻¹, *trans*-epoxysuccinyl-L-

leucylamido-(4-guanidino)butane at 10 μM and 1,10-phenanthroline or EDTA at 1 mM were added to the extracts. The mixtures were incubated for 30 min at 37 °C, then the substrates (Ala-pNA for PepC, Ala-Ala-pNA for PepG) were added. After further incubation for 30 min, enzyme activity was measured spectrophotometrically as described above.

RESULTS AND DISCUSSION

Identification of *pepG* and *orfW*

Of the nine recombinant *E. coli* clones able to hydrolyse Ala-βNA, two were found to possess a plasmid carrying *pepC* (Klein *et al.*, 1994). The plasmids of the remaining clones were characterized by restriction analysis (Fig. 1) and the nucleotide sequence of one (pJKG4) was determined. A complete ORF was present which, on the basis of subsequent experimental data, was designated *pepG*. Further sequencing of the *pepG* upstream region on plasmid pJKG6 identified an adjacent 5'-terminally truncated ORF, designated '*orfW*', which showed marked similarity to *pepG*. Since the 5' terminus of *orfW* was missing on all the other plasmids, we re-screened the gene bank for clones possessing Gly-Ala-βNA-cleaving activity. Restriction analysis identified a plasmid, designated pJKG2, which contained a chromosomal insert encoding the complete coding regions of *orfW* and *pepG*. For comparative studies we separated *orfW* by deleting the *pepG*-containing *PstI*-*SalI* fragment from pJKG2. We had already isolated a plasmid, pJKG8, which exclusively contained *pepG* in its insert (Fig. 1).

Sequence analysis

Through analysis of the inserts of plasmids pJKG2, pJKG4 and pJKG6, and pJKG8 (Fig. 1), the nucleotide sequence of a 3375 bp region of the *Lb. delbrueckii* subsp. *lactis* DSM7290 genome was determined (Fig. 2). In addition to *orfW* and *pepG*, this included a region 470 nt upstream of *orfW*, encompassing a partial ORF ('*orfX*'), and extending 225 nt downstream of *pepG*.

The *orfW* coding region (55.6% G + C) initiates with an AUG start codon at position 471, preceded by a potential

Fig. 2. For legend see facing page.

ribosome binding site (RBS) (GGAG, -7 bp; Shine & Dalgarno, 1974) and terminates with a translational UAA stop codon at position 1782. This gene is followed by the *pepG* coding region (53.7% G + C) which is also preceded by a typical RBS (AGGAG, -8 bp), starts with AUG at nt 1837 and terminates with UAA at nt 3150.

The transcriptional starting points of *orfW* and *pepG* were determined by primer extension experiments (Matern *et al.*, 1994) and the upstream regions were searched for promoter motifs. The predicted -35 and -10 regions of *pepG* are separated by 17 nt, which is in agreement with the consensus spacing, with two nucleo-

Fig. 2. Nucleotide and deduced amino acid sequences of the *Lb. delbrueckii* subsp. *lactis* DSM7290 *orfW* and *pepG* genes and part of the preceding *orfX*. Relevant *Sau3A* restriction sites indicate the start points of the chromosomal inserts in recombinant plasmids. The restriction sites used for subcloning of *orfW* are indicated. The predicted -35 and -10 regions of the putative promoters are underlined. The starts of transcription, determined by primer extension, as well as the ribosome binding sites (RBS) are indicated. The inverted repeats of the potential ρ -independent terminators are denoted by facing arrows and the run of T residues is marked by dots.

tides matching the -35 consensus hexamer and four matching the -10 hexamer. The putative *orfW* promoter matches the -35 motif in four of the six

nucleotides, while the -10 region is identical to the consensus. However, these putative -35 and -10 regions are separated by 20 nt, which might result in

Fig. 3. Progressive alignment (TREE analysis) of the cysteine peptidases from *Lb. delbrueckii* subsp. *lactis* DSM7290 (LdPepC = PepC [LDPEPC]; LdOrfW = OrfW; LdPepG = PepG) with the most similar cysteine aminopeptidases from *Lb. helveticus* (LhPepC = PepC [LHPEPC]), *Lc. lactis* (LcPepC = PepC [LLAPEPC]), *S. thermophilus* (StPepC = PepC [STPEPCGE]) and with the bleomycin hydrolase from *Saccharomyces cerevisiae* (ScBlh1 = BLH1 [BLH1_YEAST]). Database entry names are given in square brackets. Identical amino acids are marked by asterisks and the most conserved regions (which can be aligned with numerous cysteine aminopeptidases) are boxed. The amino acids glutamine (Q), cysteine (C), histidine (H), asparagine (N) and tryptophan (W), which are predicted to act in substrate binding and catalysis (Kamphuis *et al.*, 1985), are indicated by arrows.

Fig. 4. Coomassie-stained SDS-polyacrylamide gels (12.5% acrylamide) showing PepG (a) and OrfW (b) expression. (a) Overexpression of PepG. Separation of crude extracts prepared from CM89(pJG8) (lane 1; lane 2, 1/10 of the same extract as in lane 1) and CM89 (lane 3). PepG, identified by its predicted molecular mass of 49711 Da is indicated (G). (b) Overproduction of OrfW from plasmid pJKW2 in the T7 expression system. Extracts of PS1(pJKW2) were prepared 0 min (lane 6), 35 min (lane 7) and 420 min (lane 8) after induction of the T7 RNA polymerase with IPTG. The position of OrfW (50 kDa) is indicated (W). Lanes 4 (a) and 5 (b), molecular mass markers. (c) Autoradiograph of a SDS-polyacrylamide gel (12% acrylamide) showing plasmid-directed protein synthesis in a coupled *in vitro* transcription-translation system. ³⁵S-Met-labelled proteins synthesized in the presence of plasmids pLG339 (lane 10) or pJKW1 (lane 11), and ¹⁴C-methylated protein standards (lane 9) were separated. The main translation product of pJKW1, predicted to be OrfW (50 kDa), is indicated (W).

inefficient gene expression. When searching for terminator structures, *p*-independent terminators ending in a run of T residues could be predicted for *orfX* ($\Delta G = -41.0 \text{ kJ mol}^{-1}$) and for *pepG* ($\Delta G = -112.2 \text{ kJ mol}^{-1}$). For *orfW* no such structure could be detected.

Sequence similarities

Comparative alignment of the *orfW* and *pepG* coding regions demonstrated them to be highly conserved, exhibiting 72.6% nucleotide sequence identity. Screening of appropriate databases (EMBL, GenBank) revealed additional similarities. The highest degree of nucleotide sequence identity, as determined by FASTA analysis (Pearson & Lipman, 1988), was with the prokaryotic cysteine aminopeptidase genes (*pepC*) from *Lb. delbrueckii* subsp. *lactis* DSM7290, *Lb. helveticus* (Fernández *et al.*, 1994; Vesanto *et al.*, 1994), *Streptococcus thermophilus* (Chapot-Chartier *et al.*, 1994), *Lc. lactis*

(Chapot-Chartier *et al.*, 1993). Identities ranged from 59.5% to 53% throughout the whole coding regions.

When the deduced protein sequences were employed for database searches (SWISS-PROT, PIR, GenPep), sequence similarities with eukaryotic cysteine aminopeptidases were also noted. The yeast bleomycin hydrolase (Magdolen *et al.*, 1993), a ubiquitous thiol proteinase with an as yet unknown function, displayed at least 33% identity with both the PepC and PepG thiol proteinases and OrfW. Minor, but significant similarities were also evident with several other thiol aminopeptidases. The latter homologies were mainly restricted to the two conserved sequence motifs of the cysteine protease family, one located at the N terminus and the second at the C terminus. The multiple sequence alignment shown in Fig. 3 summarizes these similarities. The calculated phylogenetic tree (not shown) indicates that OrfW and PepG are closely related. They build a

separate branch besides the prokaryotic PepC cysteine aminopeptidases and it might be speculated that they are the result of a gene duplication.

Gene expression in *E. coli*

As had been observed for *pepC* (Klein *et al.*, 1994), *pepG* was readily overexpressed in *E. coli*. In cell extracts of CM89(pJKG8), PepG constituted approximately 40% of the total soluble proteins of the cells (Fig. 4a). This efficient expression most probably results from *Lactobacillus* promoter structures preceding *pepG*, since in the recombinant plasmid pJKG8 no vector promoters are located upstream of the structural gene. Overexpression of *orfW*, from recombinant *E. coli* CM89(pJKW1), did not result in a detectable protein product.

In vitro transcription/translation of *orfW*

To demonstrate that *orfW* is preceded by a promoter which functions in *E. coli*, we performed a plasmid-directed *in vitro* transcription/translation with a coupled *E. coli* system. The detection of a ³⁵S-Met-labelled 50 kDa protein (Fig. 4c) indicates expression of *orfW* in *E. coli*. The presence of a promoter element, functional in *Lb. delbrueckii* subsp. *lactis*, was also evident from the primer extension experiment.

Overexpression of *orfW* in *E. coli*

In contrast to other cloned peptidase genes (i. e. *pepC*, *pepG*, *pepI*, *pepN*, *pepV* or *pepX*) (Klein *et al.*, 1996) from *Lb. delbrueckii* subsp. *lactis*, *orfW* was not expressed with sufficient efficiency for its product to be visualized in Coomassie-stained polyacrylamide gels. Therefore, we placed *orfW* under the control of the T7 promoter by subcloning it as an *Eco*NI-*Pst*I fragment into pWSK129 (Fig. 1). The resulting hybrid plasmid, pJKW2, was used to overexpress the gene in *E. coli* strain PS1. After induction of the system with IPTG, OrfW production could be monitored by the accumulation of a protein visible after staining of SDS-polyacrylamide gels with Coomassie Blue (Fig. 4b). The size of 50 kDa for OrfW was in agreement with the value deduced from nucleotide sequence analysis (50079 Da).

Partial characterization of enzyme activities

For a preliminary characterization of the two thiol aminopeptidases, PepC and PepG, we tested cleavage of β NA and *p*NA substrates by recombinant *E. coli* strains or cell-free extracts of such strains. In a second approach, the ability of the multiple-peptidase-deficient *Sal. typhimurium* TN1547, harbouring either *pepC* or *pepG*, to utilize Leu- and Pro-containing peptides as amino acid sources was determined.

PepC and PepG activities could be easily distinguished by an enzymic plate assay using β NA substrates, as several of these are exclusively cleaved by one of the two peptidases (Table 2). A further distinction could be made when the relative peptidase activities of cell

Table 2. Substrate specificities of *Lb. delbrueckii* subsp. *lactis* DSM7290 cysteine aminopeptidases PepC and PepG

Cleavage of chromogenic β NA substrates was monitored in a plate assay using transformants of *E. coli* CM89 harbouring recombinant plasmids with inserted peptidase genes of strain DSM7290. The designations of the respective plasmids are given in parentheses. The relative cleavage rates for individual substrates are indicated by: +, fast; +/–, slow; –, not detectable).

Peptidase	PepC (pJKC4)	PepG (pJKG8)
Ala- β NA	+	+
Ala-Ala- β NA	+	–
Ala-Ala-Phe- β NA	+	–
Arg- β NA	+	–
Asp- β NA	+	–
Cys(Bzl)- β NA	+	–
Gly- β NA	+	–
Gly-Ala- β NA	+	+
Gly-Phe- β NA	+	+
Gly-Pro- β NA	–	–
Gly-Trp- β NA	+	–
His- β NA	+ / –	–
Leu- β NA	+	–
Leu-Gly- β NA	+	+
Lys- β NA	+	–
DL-Met- β NA	+	–
Orn- β NA	+	–
Phe- β NA	+	–
Pro- β NA	–	–
Ser- β NA	+	–
Thr- β NA	+	–

extracts for different *p*NA-substrates (Pro-, Phe-, Tyr-, Leu-, Lys-, Gly-, Glu-, Ala-, Ala-Ala-*p*NA) were compared. The results (data not shown) confirm the broad specificity of PepC, which could degrade all tested *p*NA substrates, with the exception of Pro-*p*NA, and exhibited a moderate preference for the substrate Ala-*p*NA. In contrast, PepG could only degrade Ala-*p*NA (55% relative activity compared to PepC) and Ala-Ala-*p*NA (100% relative activity).

The *in vivo* peptidolytic activities of PepC, PepG and OrfW on Leu- or Pro-containing peptides were further examined in growth experiments with *Sal. typhimurium* TN1547. The results (Table 3) indicate that PepC and PepG, although structurally related, have different substrate specificities. Whereas PepC has a broad substrate range for β NA substrates, PepG was able to cleave more and different unmodified peptides. Further differences were observed when pH and temperature optima were determined (Fig. 5). The effect of temperature on aminopeptidase activity revealed the optimal temperature for PepC to be 37 °C, whereas PepG exhibited maximal activity between 50 and 55 °C.

The effect of pH on aminopeptidase activities was determined in the range pH 4–10, and the optimum pH

Table 3. Comparison of PepC and PepG activities

The utilization of Leu and Pro peptides by *Sal. typhimurium* TN1547(pJKG8) expressing *pepG*, and TN1547(pJKC4) expressing *pepC* was determined by crystal tests. TN1547(pLG339) was used as a negative control. The ability, conferred by cloned peptidases, to grow on peptides as a Leu or Pro source is indicated by: + + +, good growth; + +, growth; +, faint growth; -, no growth). Most of the peptides tested containing Pro (Ala-Ala-Pro-Ala, Ala-Ala-Pro-Ala-Ala, Ala-Pro, Ala-Pro-Ala, Ala-Pro-Gly, Ala-Pro-Phe, Arg-Pro, Gln-Pro, Glu-Pro, Gly-Gly-Pro, Gly-Pro, Gly-Pro-Ala, Gly-Pro-Gly-Gly, Gly-Pro-Gly-Pro, Gly-Pro-Ile, Gly-Pro-Phe, Gly-Pro-Trp, His-Pro, His-Pro-Leu, His-Pro-Val, Ile-Pro, Ile-Pro-Ile, Leu-Ala-Pro, Leu-Gly-Pro, Leu-Pro, Lys-Pro, Lys-Pro-Arg, Lys-Pro-Val, Met-Pro, Met-Pro-Gly, Phe-Pro, Pro-Ala, Pro-Arg, Pro-Gln, Pro-Gly, Pro-Gly-Gly, Pro-His-Ala, Pro-His-Asp, Pro-His-Glu, Pro-His-Gly, Pro-His-Leu, Pro-His-Phe, Pro-His-Val, Pro-Ile, Pro-Leu, Pro-Lys, Pro-Met, Pro-Phe, Pro-Pro, Pro-Pro-Gly, Pro-Pro-Pro, Pro- β -Ala, Pro-Trp, Pro-Tyr, Pro-Val, Pro-Val-Asp, Pro-Val-Gly, Ser-Pro, β -Ala-Pro, Thr-Pro, Trp-Pro, Tyr-Pro, Val-Pro, Val-Pro-Leu) could not be cleaved by either peptidase. Phg, α -aminophenylacetic acid.

Peptide	TN1547 (pJKG8)	TN1547 (pJKC4)	Peptide	TN1547 (pJKG8)	TN1547 (pJKC4)
Leu-Arg	-	-	Glu-Leu	-	-
Leu-Gly	-	-	Gly-Leu	+ + +	+ +
Leu-His	-	-	His-Leu	-	-
Leu-Ile	-	-	Ile-Leu	-	-
Leu-Leu	+ +	-	D-Ala-Leu	-	-
Leu-Met	+ +	-	D-Leu-Leu	-	-
Leu-Phe	-	-	D-Phe-Leu	-	-
Leu-Pro	-	-	D-Phg-Leu	-	-
Leu-Ser	-	-	Leu-Ala-Pro	+ + +	-
Leu-Trp	-	-	Leu-Gly-Gly	-	-
Leu-Tyr	-	-	Leu-Gly-Pro	+ +	-
Leu-Val	-	+ +	Leu-Leu-Gly	+ +	-
Leu-Phg	-	-	Leu-Leu-Leu	+ + +	-
Leu-D-Ala	-	-	Leu-Trp-Met	-	-
Leu-D-Phg	-	-	Leu-Trp-Leu	-	-
Leu- β -Ala	-	-	Leu-Tyr-Leu	-	-
β -Ala-Leu	-	-	Gly-Leu-Tyr	+	-
Lys-Leu	-	-	Gly-Leu-Gly	-	-
Met-Leu	+ +	+ +	Val-Leu-Ser	-	-
Phe-Leu	-	+ +	Ala-Val-Leu	+ + +	-
Pro-Leu	-	-	Gly-Gly-Leu	+ + +	-
Ser-Leu	+ + +	-	His-Pro-Leu	-	-
Thr-Leu	-	-	Pro-His-Leu	-	-
Trp-Leu	-	-	Val-Pro-Leu	-	-
Tyr-Leu	-	-	Leu-Trp-Met-Arg	-	-
Val-Leu	-	-	Gly-Leu-Leu-Gly	+ +	-
Ala-Leu	+ + +	-	Met-Pro-Gly	-	+
Arg-Leu	-	-	Gly-Gly-Pro	-	+ +
Asp-Leu	-	-			

was determined to be 6.5 for PepC and 7.0 for PepG. PepG has a more narrow activity range between pH 6 and 8, whereas PepC displays a broader activity range between pH 4.5 and 7.5.

To study the mechanism of enzyme action, we exposed CM89(pJKC4) or CM89(pJKG8) cell extracts (containing overproduced PepC and PepG, respectively) to a series of protease inhibitors. PepC and PepG were specifically inhibited by the cysteine protease inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane to a final relative activity of 18%

(PepC) and 20% (PepG), whereas the inhibitors 3,4-dichloroisocoumarin, pepstatin A, Pefa block, 1,10-phenanthroline or EDTA had no significant effect on enzyme activity. These results suggest that in accordance with the amino acid homologies observed, PepC and PepG can be classified as thiol proteases.

Even though OrfW sequences indicate a peptidase, no hydrolytic activity could be determined either in plate assays or crude extracts with chromogenic β NA or *p*NA substrates, nor in *Sal. typhimurium* growth tests. The genomic sequence which is so far available for DSM7290

Fig. 5. Effect of pH (a) and temperature (b) on PepC (●) and PepG (○) activities.

indicates that the chromosome is very economically covered with genes, leaving only very small non-coding regions. This observation, together with the fact that *orfW*-specific mRNA could be isolated, indicates that *orfW* does indeed encode a product. The elucidation of the function and physiological importance of this protein awaits further experimentation.

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