Cloning, heterologous expression, and sequencing of a novel proline iminopeptidase gene, pepl, from Lactobacillus delbrueckii subsp. lactis DSM 7290

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The gene for proline iminopeptidase from Lactobacillus delbrueckii subsp. lactis DSM 7290 coding for an enzyme that hydrolyses the synthetic substrate L-prolyl-β-naphthylamide (Pro-βNA) was cloned in Escherichia coli. An enzymic plate assay was used to screen for positive clones. The gene, designated pepl, was subcloned into vector pUC18 and sequenced. The nucleotide sequence revealed an 882 bp open reading frame encoding 294 amino acids, coding for an enzyme with a calculated molecular mass of 32883 Da. By cloning under control of the lac promoter the peptidase was highly expressed. Sequence analysis showed that pepl is of a new sequence type, distinct from all peptidases so far sequenced. Amino acid homology to the active site of a Pseudomonas putida esterase and inhibitor studies of the enzyme imply involvement of a serine residue in catalysis.

Keywords: iminopeptidase, serine protease, Lactobacillus delbrueckii subsp. lactis, nucleotide sequence analysis

INTRODUCTION

Lactobacilli growing in milk need to possess an efficient enzymic system to utilize the abundant nutrients present. Using a coordinated combination of proteinases, peptidases, and amino acid and peptide transport systems, they are able to use milk protein, especially casein, as a source of amino acids essential for growth. The proteolytic system of lactobacilli used as starter cultures in the industrial dairy fermentations is an important factor influencing dairy product quality (e.g. organoleptic properties, or taste, and texture). This has resulted in increased fundamental research concerning the genes and corresponding enzymes involved, which may contribute to the improvement of industrial strains. Additionally the genes of individual proteinases or peptidases may serve as food-grade markers in plasmids suitable for genetic engineering.

The large peptides generated by proteinases during casein degradation are further hydrolysed into smaller peptides and amino acids by the action of peptidases. The catalytic activity of the proline iminopeptidase (EC 3.4.11.5) is to cleave N-terminal proline residues from peptides. This bond is less susceptible to the action of aminopeptidases of broad specificity because proline residues confer structural constraints on the peptide (Yaron & Naider, 1993). Casein has an extraordinarily high content of proline residues (16.7% for β-casein; Casey & Meyer, 1985) and the iminopeptidase is able to remove the blocking proline residues, thus making the peptides again accessible for other enzymes. Proline iminopeptidase activity has been reported for a variety of organisms, but as far as we know only a Bacillus gene has been cloned and sequenced (Kitazono et al., 1992). This paper deals with the cloning, sequencing and expression of the proline iminopeptidase from Lactobacillus delbrueckii subsp. lactis DSM 7290 in Escherichia coli.

METHODS

Bacterial strains, plasmids, and growth conditions. Escherichia coli K12 strain ER1562 (Raleigh et al., 1988) was used for molecular cloning and strain CM89 (Miller & Schwartz, 1978) for preparation of cell extracts. The low-copy-number vector pLG339 (Stoker et al., 1982) was from N. Stoker and pUC18...
(Yanisch-Perron et al., 1985) was obtained from Pharmacia. E. coli was grown at 37 °C in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37 °C. Ampicillin or kanamycin were added to concentrations of 200 µg ml⁻¹ and 40 µg ml⁻¹, respectively.

**Transformations.** E. coli was transformed by electroporation using a Bio-Rad Gene Pulser as described by Dower et al. (1988).

**Recombinant DNA techniques.** Restriction enzymes and other nucleic-acid-modifying enzymes were used as recommended by the manufacturers. Isolation of plasmid DNA from E. coli was performed as described by Sambrook et al. (1989).

Isolation of chromosomal DNA from L. delbrueckii subsp. lactis DSM 7290 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).

**Molecular cloning of the L. delbrueckii subsp. lactis pepI gene.** A gene bank of size-fractionated partial Sau3A fragments of total L. delbrueckii subsp. lactis DNA constructed with vector pLG339 (Stoker et al., 1982) was used to transform E. coli strain F1R1562 as described previously (Klein et al., 1993). Colonies with proline iminopeptidase activity were identified by a plate staining method originally described by Miller & Mackintosh (1974). If the chromogenic substrate Pro-βNA is cleaved, the reaction of the β-naphthylamine with fast garnet GBC (Sigma) can be monitored by the formation of a red, non-diffusible azo dye. During subcloning the transformants, selected on ampicillin- or kanamycin-containing LB-plates, were screened for those showing proline iminopeptidase enzyme activity with Pro-βNA as a substrate.

**DNA sequence analysis.** DNA sequencing of the chromosomal insert of pJK505 was carried out with a pair of universal sequencing primers adjacent to the multiple cloning site of pUC18. Synthetic oligonucleotide primers, deduced from the investigated sequence, were synthesized (Applied Biosystems model 392), and allowed direct sequencing of double-stranded plasmid DNA purified by NUCLEOBOND AX100 columns (Machery-Nagel). The DNA sequence of each strand was determined using the T7 DNA polymerase sequencing kit (Pharmacia), which is based on the dideoxynucleotide chain-termination method (Sanger et al., 1977), in the presence of [³²P]dATPβS (Amersham). For computer-assisted sequence analysis the Microgenie (Beckman), PC-Gene (IntelliGenetics), and HUSAR (GENIUSnet) software were used.

**Preparation of cell extracts.** Cell pellets of E. coli from a 50 ml overnight culture were washed with 50 mM Tris/HCl pH 8.0, pelleted by centrifugation and resuspended in 500 µl of the same buffer. The bacteria were sonicated on ice (Bandelin sonifier; Sonopuls HD60) until more than 90% of the cells were broken. Cell debris was removed by centrifugation at 51 000 g and 4 °C for 60 min in a Hereus Biofuge RS28.

The supernatant of the preparation contained approximately 50 mg protein ml⁻¹ as determined by the Lowry method and was used for enzyme assays and SDS-PAGE (Laemmli, 1970). The content of Pepl protein in cell extracts was determined videodensitometrically (Cybertech CSI, Image Documentation System) with Coomassie-stained SDS-gels, calibrated with bovine serum albumin standards.

**Enzyme assay and effects of various chemical reagents.** For characterization of the enzyme, p-nitroanilide (p-NA) substrates (Bachem) were dissolved in water and added to the reaction mixture [10 mM Tris/HCl, pH 8.0, and varying amounts of cell extracts (10–2000 ng)] to a concentration of 1 mM in a volume of 250 µl. Release of p-nitroaniline was measured after a 10 min incubation at 37 °C at 405 mm in a LKB Ultraspec Plus spectrophotometer.

To study the mechanism of enzyme action, the inhibitors 3,4-dichloroisocoumarin (3,4-DCI) at a concentration of 0.1 mM, pepstatin A at 1 µg ml⁻¹, L-trans-epoxyoxycinnamoylucylamide(4-guanidino)-butane (E-64) at 0.1 mM, and 1,10-phenanthroline or EDTA at 1 mM were added to the extracts and incubated for 30 min at 37 °C. The substrate Pro-pNA was added and activity was measured spectrophotometrically by the release of p-nitroaniline.

**RESULTS AND DISCUSSION**

**Cloning of the L. delbrueckii subsp. lactis DSM7290 pepI gene**

E. coli ER1562 was transformed with the plasmid library of DSM 7290 chromosomal DNA in pLG339 and the pepI gene could be isolated from colonies showing peptolytic activity on the substrate Pro-pNA. In the plate assay this substrate was not cleaved by the E. coli host strain, nor was it cleaved by E. coli harbouring the recently cloned genes for X-prolyl-dipeptidyl aminopeptidase (Meyer-Barton et al., 1993) and aminopeptidase N (Klein et al., 1993) from L. delbrueckii subsp. lactis DSM 7290. From approximately 6500 transformants screened, four Pro-pNA-cleaving colonies could be detected. Restriction

![Fig. 1. Genetic map of pJK502. The 3.2 kb insert of chromosomal DNA is indicated as a hatched segment, with the pepI gene located along the black arrow. The linear expansion of the insert illustrates the subcloning steps. The different restriction fragments used for subcloning into vector pUC18, all spanning the pepI gene, are indicated as shaded bars.](image-url)
efficiently expressed than in pSU1. We further succeeded in observing a considerable increase after cloning the promoter and the structural gene and PepI is less expressed in cloning the smallpepl-expressing 1140 bp HincII/JacII fragment from pSUl into pUCl8, resulting in pSUll9. Overexpression of Pep1 protein in E. coli. Crude cell extracts of E. coli CM89 harbouring plasmids pJK502 or pJK505, both coding for pepI, were subjected to SDS-PAGE (Fig. 3). A 34 kDa protein could be detected; this value corresponds well with the molecular mass of the enzyme predicted from nucleotide sequence analysis.

The subcloning experiments, which were performed in order to minimize the pepI coding region, are outlined in Fig. 1. Cloning of the 3.2 kb Spbl fragment from low-copy-number plasmid pJK502 into high-copy-number vector pUC18, resulting in pJK505, increased gene dosage, but this had only a minor effect on protein overexpression. The knowledge of the complete pJK505 nucleotide sequence allowed cloning of defined DNA fragments. The Psbl fragment from pJK505 was subcloned into pUC18, both possible insert orientations were obtained and both constructs expressed PepI protein, thus indicating that the pepI promoter sequence is functional in E. coli. But only the plasmid (pSU1) with the lac promoter upstream of pepI showed a drastic increase of PepI expression (Fig. 3). Plasmid pSU2, with pepI on a 1.8 kb SauI fragment, has a spacing region of 1 kb between the lac promoter and the structural gene and PepI is less efficiently expressed than in pSU1. We further succeeded in cloning the small pepI-expressing 1140 bp HincII/SacII fragment from pSU1 into pUC18, resulting in pSU119. The amount of PepI protein expressed was correlated with the copy number of the vectors used, but a considerable increase could be observed after cloning under control of the lac promoter. In E. coli CM89(pSU119) the pepI product constituted about 50% of the cytoplasmic proteins (Fig. 3). This remarkable high-level expression of the pepI product appeared not to be lethal for the E. coli host, but the high-copy-number plasmids were structurally unstable and PepI activity was lost after approximately 80 generations with antibiotic selection. In earlier experiments with pepX (encoding X-prolyl-dipeptidyl aminopeptidase; Meyer-Barton et al., 1993), we were not successful in cloning the peptidase gene into pUC18, and with pepN (aminopeptidase N; Klein et al., 1993) subcloning into high-copy-number vectors resulted in extremely unstable plasmids. A reason for this was probably the strong overexpression in E. coli under control of the original promoter even if cloned in low-copy-number plasmids.

A protein which could be associated with ORF2 gene product (198 kDa) was not detectable by SDS-PAGE.

Homologies of the L. delbrueckii subsp. lactis sequence.

Searching the EMBL database with the investigated nucleotide sequence resulted in no significant homologies; only after translation of the two proposed open reading frames were sequences with homologies detected in the protein databases.

The ORF2 gene product is similar to a repressor protein of Bacillus subtilis purl (Honjo et al., 1990) (Fig. 4). In Bacillus this repressor is involved in reduction of extracellular and cell-associated protease levels, when present in high copy number, and it is considered to function as a transcriptional regulator. The physiological function of the lactobacillal ORF2 gene product remains to be investigated.

For pepI the most significant homology is observed in a small, but functionally important segment of a 2-hydroxy-6-oxohexa-2,4-dienoate hydrolase (Menn et al., 1991), an enzyme involved in degradation of toluene, which contains the serine catalytic site (Fig. 5). Comparison of both protein sequences over their entire length results only in a non-significant homology of 23-8% (70 in 294 amino acids). This result shows that pepI is of new sequence type, distinct from prolyl-aminopeptidase, the only one having so far been cloned and sequenced being from Bacillus coagulans (Kitazono et al., 1992), also distinct from the Pro-ß-naphthylamidase from porcine liver (Matsushima et al., 1991), which turned out to be a carboxylesterase, and from all other aminopeptidases hitherto sequenced.

The absence of a signal peptide sequence and the hydrophilicity plot of the amino acid sequence according to Kyte & Doolittle (1982), which does not show transmembrane domains, indicate that PepI of L. delbrueckii subsp. lactis might be an intercellularly located enzyme. No secreted peptidase has yet been found in lactic acid bacteria. However, an extracellular location of peptidases is discussed since most peptides released from casein by the proteinase are too large to be taken up by the...
**Fig. 2.** For legend see facing page.
Fig. 2. Nucleotide sequence of the chromosomal insert of pJK505. The upper strand codes for pepI (nt 410-1291) and the lower strand for a gene (nt 1812-1294) with high homology to the pail repressor of B. subtilis. The deduced amino acid sequences are given below the nucleotide sequence. Five base pairs upstream of the putative pepI ATG start codon is a ribosome-binding site with a tetranucleotide stretch (AGGA) complementary to the E. coli 16S rRNA (Shine & Dalgarno, 1974); sequences that resemble promoter -10 and -35 elements (Van de Guchte et al., 1992) are underlined (conserved nucleotides in bold type). An inverted repeat (overlined at bp 1396 to 1422) with a AG value of approximately -18 kcal mol⁻¹ (−75.3 kJ mol⁻¹) might function as transcription terminator. Some pertinent restriction enzyme sites are also indicated.

To determine the substrate specificity of PepI a variety of p-NA substrates (Pro-, Ala-, Arg-, Asp-, Glu, Gly-, His-, Leu-, Lys-, Phe-, Ala-Pro-, Gly-Ala-, Gly-Phe-, Gly-Pro-, Gly-Trp-p-NA) were incubated with cytoplasmic extracts of CM89(pSU119), but among these only Pro-p-NA was effectively cleaved. This rapid test is only an indication of enzyme specificity, valuable if compared with the specificities of other cloned peptidases, and of course a more detailed characterization will be performed with purified enzyme. The purification and characterization of PepI will be described in the near future, as well as its subcellular location in Lactobacillus and determination of the N-terminal sequence, in order to confirm the coding region of the nucleotide sequence.
Proteins were visualized by Coomassie staining. Separation of crude cell extracts by SDS-PAGE (12%, w/v, acrylamide). Proteins were visualized by Coomassie staining. The gel contained molecular mass markers proteins (both flanking lanes) and cell extracts of E. coli CM89(pLG339) (lane 1), CM89(pJK502) (lane 2), CM89(pJK505) (lane 3), CM89(pSU1) (lanes 4 and 5; 1:10 of cell extract in lane 4), CM89(pSU2) (lane 6), CM89(pSU119) (lane 7) and CM89(pUC18) (lane 8). The position of Pep1 is marked by an arrowhead.

Fig. 3. Expression of Pep1 protein in E. coli strain CM89. Separation of crude cell extracts by SDS-PAGE (12%, w/v, acrylamide). Proteins were visualized by Coomassie staining.

Fig. 4. Alignment resulting from CLUSTAL analysis of the ORF2 gene product (173 amino acids) as predicted from pJK505 nucleotide sequence analysis, with the B. subtilis pail1 repressor protein (172 amino acids), the translation product of EMBL entry M36471. Values of 45% identity and 84% similarity were calculated. Identical (*) and similar () amino acids are marked.

Fig. 5. Homology of the pep1 gene product with 2-hydroxy-6-oxohepta-2,4-dieneoate hydrolase of P. putida (Swissprot database entry: TODF_PSEPU) resulting from BLASTX analysis. The Pep1 amino acid sequence starting at nucleotide 644 is numbered as in Fig. 2. Identities, 14/37 (37%); positives, 23/37 (62%).

ADDENDUM

After the completion of this work and acceptance of our manuscript, we became aware that cloning and sequencing, as well as purification and characterization, of a proline iminopeptidase from a closely related subspecies, Lactobacillus delbrueckii subsp. bulgaricus, was about to be published (Atlan et al., 1994; Gilbert et al., 1994). Comparison of the sequences revealed a very high homology not only of the deduced Pep1 amino acid sequence (identities: 287 of 295 amino acids) but also for the up- and downstream regions of the reported nucleotide sequence. As could be expected from this, the enzymatic properties of the enzymes are very similar (unpublished).

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