Nucleotide sequence and distribution of the pepPN gene from Lactobacillus helveticus CNRZ32

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Abstract: The Lactobacillus helveticus CNRZ32 gene encoding a di-/tri- peptidase with prolinase activity (pepPN) was sequenced. An open reading frame of 912 base pairs was identified corresponding to a peptide with a molecular mass of 35.04 kDa. Southern hybridization indicated that the gene sequence is well conserved in strains of lactobacilli and pediococci.

Key words: Lactobacillus helveticus; Peptidase; Prolinase; Proteolysis; Proteolytic enzyme

Introduction

Lactic acid bacteria (LAB) are industrially important microorganisms that are involved in a variety of food fermentations. The proteolytic systems of LAB have received a great deal of attention over the past decade, due to the well documented role of proteolysis in cheese flavor and texture development [1]. Also, since insufficient free amino acids are present in milk to support growth to high cell density, these bacteria require a complex proteolytic system to obtain essential amino acids from casein [2].

Our laboratory is examining the proteolytic system of Lactobacillus helveticus CNRZ32 because of this organism's ability to reduce bitterness and accelerate flavor development in aged cheese [3,4]. This paper describes the nucleotide sequence and distribution of one component of this organism's proteolytic system, an enzyme that hydrolyses a variety of di- and tripeptides. The enzyme's requirement for a free carboxyl terminus and ability to efficiently hydrolyze di- and tripeptides having an N-terminal proline residue [5], suggests that it is a prolinase (Prn). Enzymes with similar substrate specificities have also been characterized from lactococci [6] and lactobacilli [7].

Materials and Methods

Bacterial strains

Escherichia coli DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, MD) and E. coli CM89 [8] were used as cloning hosts. Both strains were grown in Luria-Bertani media [9] with appropriate antibiotics at 37°C with aera-
tion, or on plates solidified with 1.5% w/v agar. Strains for Southern hybridization were obtained from our laboratory culture collection, National Collection of Food Bacteria (Reading, UK), American Type Culture Collection (Rockville, MD), L.L. McKay (University of Minnesota, Department of Food Science), Tarun Bhowmik (University of Wisconsin-Madison, Department of Food Science) and Mark E. Johnson (University of Wisconsin-Madison, Center for Dairy Research).

Molecular cloning techniques
Recombinant DNA techniques were conducted essentially as described by Sambrook et al. [9]. T4 DNA ligase and restriction endonucleases were used as recommended by the manufacturer (Gibco-BRL, Gaithersburg, MD).

DNA sequencing
Nested sets of deletions were generated using the Erase-a-Base Kit (Promega, Madison, WI). Plasmid DNA was purified by the modified mini alkaline-lysis/polystyrene glycol precipitation procedure of Applied Biosystems, and sequenced by the University of Wisconsin-Madison Biotechnology Center. Sequence analysis was done using the GCG fragment assembly program (Genetics Computer Group, Madison, WI).

Southern hybridization
Chromosomal DNA was isolated from LAB using the lysis procedure of Anderson and McKay [10], and the chromosomal isolation procedure of Marmur [11]. Probe synthesis was performed as described in the Genius kit (Boehringer Mannheim, Indianapolis, IN). Chromosomal DNA was digested with EcoRI and separated on a 0.7% agarose gel. Southern transfer was carried out as described by Southern [12] using a MagmaGraph nylon membrane (Micron Separations, Inc., Westborough, MA), except the acid hydrolysis step was omitted. Hybridization and immunological detection were carried out as described by Boehringer Mannheim. Low and high stringency was achieved by incorporating 10% or 50% formamide into the prehybridization and hybridization steps, respectively.

Results and Discussion

Subcloning of the gene
Previously, a genomic library of L. helveticus CNRZ32 was constructed in pJDC9 [13] and screened for peptidase activities [5]. One plasmid, designated pSUW9, was determined to encode a di-/tri- peptidase with an apparent specificity towards peptides with N-terminal proline residues (previously designated DTP). Tn5 mutagenesis [14] localized the Prn gene to a 2.1 kb SalI-SalI fragment (results not shown). Prn activity was observed when this fragment was subcloned in either orientation in pJDC9 (results not shown), suggesting that this fragment contains the complete pepPN gene. The restriction map of this fragment is shown in Fig. 1.

Nucleic acid sequence analysis
The nucleic acid sequence of the pepPN gene is shown in Fig. 2. A putative open reading frame encoding a 304 amino acid peptide sequence was identified. The deduced molecular mass of the peptide encoded by this sequence is 35.04 kDa. Upstream of the start codon are putative -10 (TATTAT; nucleotides -35 to -30), -35 (CTGATA; nucleotides -61 to -56) and Shine-Delgarno (AAAGGA; nucleotides -13 to -8) sequences. Located 3' to the stop codon is a putative rho-independent transcriptional terminator, with ΔG = -12.4 kcal/mol [15].

![Fig. 1. Restriction endonuclease map of the Prn+ subclone from pSUW9. Bold arrow represents limits of Prn gene, with the arrow direction indicating the direction of transcription. The location of the 0.58 kbp EcoRV-EcoRI fragment used as a probe in Southern hybridization is shown below map. Abbreviation for restriction endonucleases used: A, ApaI; E, EcoRI; B, BglII; H, HpaI; P, PstI; S, SalI; V, EcoRV.](image-url)
This gene shows significant sequence identity with proline iminopeptidase genes from *Bacillus coagulans* [16], *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ397 [7], and *Propionibacterium shermanii* ATCC 9617 (Dr. A.M. Ledeboer, personal communication). The product of these genes exhibit 35%, 25% and 19% similarity to Prn on the amino acid level, respectively. The PROSITE Dictionary of Protein Sites and Patterns [17] was used to search for structural motifs within the deduced Prn amino acid sequence. No patterns from this database were found in Prn. However, Prn does appear to contain a motif (GQSFGG; amino acids 109 to 114) that resembles the active site motif for the serine-dependent X-prolyl dipeptidyl aminopeptidase [18]. This motif site also aligned with an identical sequence in the proline iminopeptidase from *L. delbrueckii* ssp. *bulgaricus* CNRZ397, and a similar sequence in the proline iminopeptidase from *P. shermanii* (GQSFGG). A similar site was not found within the *Bacillus* enzyme, which is known to be unaffected by serine proteinase inhibitors [16]. Prn activity was found to be inhibited by 0.1 mM *p*-chloromercuribenzoate (PCMB) (data not shown). PCMB inhibition has also been demonstrable.

![Fig. 2. Nucleotide sequence of the Prn gene of *Lactobacillus helveticus* CNRZ323. The predicted amino acid sequence is shown in a single-letter code. The putative −35 (nucleotides −61 to −56) and −10 (nucleotides −36 to −31) promoter sequences, putative Shine-Delgarno sequence (nucleotides −13 to −8) and putative transcriptional termination sequence (nucleotides 922 to 932 and 938 to 948) have been underlined. The pepPN sequence was submitted to GenBank and assigned accession number U05214.](image-url)
strated with the *Bacillus* and *L. delbrueckii* ssp. *bulgaricus* enzymes. Therefore, it appears that the mechanism of catalysis may differ among members of this group of enzymes, and the mechanism of catalysis of Prn is unclear at present.

**Southern hybridization**

From the sequence data, a pepPN 0.58 kb *EcoRV*-*EcoRI* internal fragment was identified and used to synthesize a DNA probe (Fig. 1). When low stringency (10% formamide) conditions were employed, hybridization was detected with all the lactobacilli and pediococci examined (Fig. 3A, B). When high stringency (50% formamide) conditions were employed, hybridization was detected only with the *L. helveticus* strains and *L. delbrueckii* ssp. *bulgaricus* JLS160 (data not shown). Even when low stringency conditions were employed, hybridization was not detected with any of the leuconostoc (Fig. 3B), *S. thermophilus* (Fig. 3B) or lactococcal (data not shown) strains examined. While the involvement of Prn in the liberation of essential amino acids from casein is unknown, the presence of a Prn-like enzyme within a variety of LAB implies that different LAB utilize similar pathways for obtaining essential amino acids via protein hydrolysis.

Previously, an enzyme from *L. lactis* ssp. *cremoris* HP with a substrate specificity similar to that of Prn was purified and characterized [6]. The lactococcal enzyme was determined to have a subunit size of 50 kDa and was stimulated by 1mM PCMB. The difference in subunit molecular weight, sensitivity to PCMB, and lack of hybridization between the Prn probe and HP chromosomal DNA suggests that these two enzymes are unrelated.

The nucleotide sequence of the Prn gene will allow the development of strategies for its inactivation as described by Bhowmik and Steele [19]. Characterization of this *Lactobacillus helveticus* CNRZ32 derivative lacking Prn activity should give insight into the physiologic role of Prn and its role in cheese flavor development.

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*Fig. 3. Low stringency (10% formamide) Southern hybridization of *EcoRI* digested total genomic DNA samples of various lactic acid bacteria, using a probe synthesized from a 0.58 kb *EcoRV*-*EcoRI* fragment of the *Lactobacillus helveticus* CNRZ32 pepPN gene. (A) Lanes: 1, λ-*HindIII* molecular mass standards; 2, *Lactobacillus helveticus* CNRZ32; 3, *Lactobacillus helveticus* ATCC 10797; 4, *Lactobacillus delbrueckii* ssp. *bulgaricus* JLS110; 5, *Lactobacillus delbrueckii* ssp. *bulgaricus* JLS160; 6, *Lactobacillus casei* JLS20; 7, *Lactobacillus casei* ssp. *pseudoplantarum* ATCC 25598. (B) Lanes: 1, λ-*HindIII* molecular mass size standards; 2, *Lactobacillus helveticus* CNRZ32; 3, *Leuconostoc mesenteroides* ssp. *cremoris* CAFT9; 4, *Leuconostoc mesenteroides* ssp *cremoris* ATCC 19254; 5, *Pediococcus pentosaceus* NCDO 559; 6, *Pediococcus pentosaceus* ATCC 25745; 7, *Streptococcus thermophilus* S6; 8, *Streptococcus thermophilus* ST2.*
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References