Characterization of the Cell Wall-Bound Proteinase of *Lactobacillus casei* HN14

M. KOJIC, D. FIRA, A. BANINA, AND L. TOPISIROVIC*

*Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 283,
P.O. Box 794, 11001 Belgrade, Yugoslavia*

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Lactobacillus casei HN14, which was isolated from homemade cheese, produces an extracellular, cell wall-bound proteinase. The HN14 proteinase can be removed from the cell envelope by washing the cells in a Ca^{2+} -free buffer. The activity of the crude proteinase extract is inhibited by phenylmethylsulfonyl fluoride, showing that the enzyme is a serine-type proteinase. Considering the substrate specificity, the HN14 proteinase is similar to the lactococcal PI-type enzyme, since it hydrolyzes β -casein only. *Lactobacillus casei* HN14 appeared to be plasmid free, which suggests that the proteinase gene is chromosomally located. Chromosomal DNA of this strain hybridizes with DNA probes Q1 (which contains a fragment of the *prtM* gene) and Q6 and Q92 (which contain fragments of the *prtP* gene); all three probes originated from the proteinase gene region of *Lactococcus lactis* subsp. *cremoris* Wg2. A restriction enzyme map of the proteinase region of *Lactobacillus casei* HN14 was constructed on the basis of hybridization experiments. Comparison of the restriction enzyme maps of the *Lactobacillus casei* HN14 proteinase gene region and those of lactococcal proteinase gene regions studied so far indicates that they are highly similar.

In the last few years, the biochemistry and genetics of cell wall-bound proteinases have been intensively investigated in strains of *Lactococcus lactis* subsp. *lactis* (8), *Lactococcus lactis* subsp. *cremoris* (3, 17), and *Lactococcus lactis* subsp. *lactis* biovar diacetylactis (14). Some general properties of lactococcal proteinases are high molecular weights, pH optima from 6 to 7, and isoelectric points of 4.4 to 4.55 (18, 19). Furthermore, the enzymes are stabilized or activated by Ca^{2+} ions. Release of the enzymes from the cell surface is significantly increased in the presence of a calcium-chelating agent such as EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] or EDTA. This suggests that proteinase release is the result of autocatalytic degradation, which is normally inhibited by Ca^{2+} ions (19). In addition, the proteinases are inhibited by phenylmethylsulfonyl fluoride (PMSF) or diisopropylfluorophosphate, both of which are specific inhibitors of serine-type proteinases (18, 19).

Considering their caseinolytic specificities, lactococcal cell wall-bound proteinases have been divided into two groups. The PI-type proteinases, exemplified by *Lactococcus lactis* subsp. *cremoris* HP, predominantly hydrolyze β -casein. On the other hand, the PIII-type proteinases, detected in *Lactococcus lactis* subsp. *cremoris* AM1 among others, cause degradation of α_{s1} - and κ -caseins in addition to β -casein (18, 24).

In all lactococcal strains studied to date, proteinase genes are located on plasmids of different sizes (8, 18). A proteinase gene region of *Lactococcus lactis* subsp. *cremoris* Wg2 was cloned and sequenced (16, 17). Interestingly, in close proximity to the proteinase gene (*prtP*), another gene, named *prtM*, was located. The *prtM* gene codes for a membrane-located lipoprotein which is essential for activation of the proteinase (9). Removal of the *prtM* gene resulted in the elimination of proteolytic activity, but synthesis and secretion of proteinase were not affected (10). An identical

genetic organization of the proteinase gene region of *Lactococcus lactis* subsp. *cremoris* SK11 was found (25).

Much less is known about proteinases of lactobacilli. They have been detected in *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Lactobacillus lactis* and they have only been biochemically characterized (2, 7). It was found that *Lactobacillus plantarum* and *Lactobacillus acidophilus* have proteinases which can also be removed from the cell wall by repeated washing of whole cells in a Ca^{2+} -free buffer (15). Both *Lactobacillus* enzymes are serine-type proteinases, and it was estimated that the *Lactobacillus acidophilus* proteinase had a molecular size of 145 kDa. However, to our knowledge there are no published reports of studies on the genetics of lactobacillus proteinases.

In this paper, we describe the isolation and characterization of a proteinase from *Lactobacillus casei* HN14 which belongs to the cell wall-bound proteinases. In addition, we have made a preliminary determination of the genetic structure of the proteinase gene region.

MATERIALS AND METHODS

Bacterial strains and media. *Lactobacillus casei* HN14 was originally isolated from homemade hard cheese by standard microbiological procedures for the detection of lactic acid bacteria. It was grown at 30°C in skim milk, and the final pH of the curd was 4.4. Carbohydrate fermentation was determined by using API 50CH (API System S.A.; Montelieu-Vercieu, France). *Lactococcus lactis* subsp. *lactis* 712 and its plasmid-cured derivative, *Lactococcus lactis* subsp. *lactis* MG1363, were used as proteinase-positive and proteinase-negative controls, respectively. *Lactobacillus casei* HN14 was grown in MRS broth (Difco, Detroit, Mich.). Agar plates were prepared by adding agar (1.5%, wt/vol) to MRS broth. Strains of *Lactococcus lactis* subsp. *lactis* were grown in M17 medium (23) supplemented with glucose (0.5%, wt/vol), to which agar (1.5%, wt/vol) was added when a solid medium was necessary. Induction of proteinase production was achieved by using milk-citrate agar (4.4%

* Corresponding author.

FIG. 1. Action of proteinases on α_1 -casein (A), β -casein (B), and κ -casein (C). Lanes: s, starting substrate; 1, *Lactococcus lactis* subsp. *lactis* 712; 2, *Lactococcus lactis* subsp. *lactis* MG1363; and 3, *Lactobacillus casei* HN14. Reaction mixtures were incubated at 30°C for 5 h.

reconstituted skim milk, 0.8% Na citrate, 0.1% yeast extract, 0.5% glucose, 1.5% agar [wt/vol]).

Casein hydrolysis. Proteolytic activity was detected by using a modified procedure of Hill and Gasson (12). After several subsequent inoculations on milk-citrate agar plates, cells were collected and resuspended in Na phosphate buffer (pH 7.2) to an approximate density of about 10^{10} cells per ml. The cell suspension was mixed with 5 mg of α_1 -, β -, or κ -casein (Sigma Chemie GmbH, Deisenhofen, Germany) per ml dissolved in the same buffer at a 1:1 volume ratio. The resulting mixtures were incubated at 30°C. At times indicated below, samples were taken and centrifuged and supernatants were mixed with solubilization buffer (125 mM Tris-HCl (pH 6.8), 10 mM disodium EDTA, 4% sodium dodecyl sulfate (SDS), 25% glycerol, 5% 2-mercaptoethanol, 0.07% bromophenol blue) at a 1:1 volume ratio. Samples were heated at 100°C for 3 min and analyzed by SDS-polyacrylamide (15%, wt/vol) gel electrophoresis (SDS-PAGE).

Crude extracts of proteinase were obtained by repeated washing of whole cells in a Ca^{2+} -free buffer, which is a general method for isolation of cell wall-bound proteinases (12). After proteinase induction on milk-citrate agar plates, cells were collected (100 mg, wet weight) and incubated in 100 μ l of Na phosphate buffer (pH 7.2) for 30 min at 30°C. Subsequently, the mixtures were centrifuged and the pellets were resuspended in the same buffer. After repeated incubation, the supernatants were pooled and tested for proteolytic activity towards β -casein.

Analysis of casein degradation. Analysis of casein hydrolysis was performed by using SDS-PAGE, as described previously (12). The protein samples, prepared as described above, were analyzed on acrylamide gels (15%, wt/vol) by using vertical slab electrophoresis cells (Bethesda Research Laboratories [BRL] Life Technologies, Inc., Gaithersburg, Md.). Gels were run for 20 h at 20 mA constant current and stained with Coomassie brilliant blue G250 (SERVA, Heidelberg, Germany).

Isolation of DNA, restriction analysis, and electrophoresis. Plasmid DNA was isolated as described by Anderson and McKay (1). Chromosomal DNA was isolated by following the procedure described by Hopwood et al. (13). Restriction

FIG. 2. Kinetics of β -casein degradation by proteinases from *Lactobacillus casei* HN14 (A) and *Lactococcus lactis* subsp. *lactis* 712 (B). Samples were taken at the following times: 0 min (lanes 1), 15 min (lanes 2), 30 min (lanes 3), 1 h (lanes 4), 2 h (lanes 5), 4 h (lanes 6), and 8 h (lanes 7). Lanes s, β -casein.

enzymes were purchased from BRL and used as described in the manufacturer's instructions. Agarose gel electrophoresis of DNA was performed with 1% (wt/vol) gels and TBE buffer (89 mM Tris, 89 mM boric acid, 25 mM disodium EDTA, pH 8.5).

Southern hybridization. The proteinase gene probes, Q1, Q6, and Q92 from *Lactococcus lactis* subsp. *cremoris* Wg2, were kindly provided by J. Kok. They were labeled by using the BioNick Labelling System (BRL). DNA on 1% (wt/vol) agarose gels was transferred to PhotoGene nylon membranes (BRL) as described by Southern (22). After transfer, the hybridization experiments were performed with the nonradioactive PhotoGene Nucleic Acid Detection System (BRL). Labeling of probes and hybridization were both carried out essentially as described in the BRL Instruction Manual.

RESULTS

Strain characterization. *Lactobacillus casei* HN14 was originally isolated from homemade cheese by using standard microbiological procedures for detection of lactic acid bacteria. During the screening of the plasmid content of natural isolates from our laboratory collection, *Lactobacillus casei* HN14 appeared to be plasmid free. Repeated attempts to isolate plasmid DNA by using procedures designed for the

FIG. 3. Degradation of β -casein by crude extracts of proteinases. Lanes: 1, *Lactococcus lactis* subsp. *lactis* MG1363 (negative control); 2, *Lactobacillus casei* HN14; 3, *Lactobacillus casei* HN14 with PMSF (2 mg/ml); and s, β -casein. Reaction mixtures were incubated at 30°C for 4 h.

detection of large, low-copy-number plasmids in lactic acid bacteria (1) gave the same result, i.e., they indicated that *Lactobacillus casei* HN14 does not contain plasmid DNA.

Proteinase activity. The ability of *Lactobacillus casei* HN14 to hydrolyze α_{s1} -, β -, and κ -caseins was tested after induction of proteinase on milk-citrate agar plates. As controls, proteinase-positive *Lactococcus lactis* subsp. *lactis* 712 and the proteinase-negative derivative of this strain, *Lactococcus lactis* subsp. *lactis* MG1363, were used. It was found that the proteinase from *Lactobacillus casei* HN14 gave a pattern of β -casein degradation similar to that of the proteinase from *Lactococcus lactis* subsp. *lactis* 712 when whole cells were used in the test (Fig. 1). Comparison of the kinetics of β -casein degradation caused by the HN14 and 712 proteinases obtained when the same amounts of cells were used in the experiment showed that the proteolytic activity of *Lactobacillus casei* HN14 was lower than that of *Lactococcus lactis* subsp. *lactis* (Fig. 2). This could be explained

either by the lower activity of the HN14 proteinase itself or by the smaller quantity of the enzyme synthesized by *Lactobacillus casei* HN14. On the other hand, neither strain showed significant hydrolysis of α_{s1} - or κ -casein within 5 h of incubation at 30°C (Fig. 1), suggesting that the HN14 proteinase is similar to the PI-type lactococcal proteinases. Incubation at 40°C resulted in less degradation of β -casein by the HN14 proteinase (data not shown).

A crude proteinase extract from *Lactobacillus casei* HN14 was obtained by washing the cells in a Ca^{2+} -free buffer. The proteinase extract gave a pattern of β -casein degradation almost identical to that obtained with the whole cells. The extract from the negative control, Prt^- strain *Lactococcus lactis* subsp. *lactis* MG1363, did not show any proteolytic activity (Fig. 3). Activity of the HN14 proteinase was inhibited by PMSF. It was found that a concentration of PMSF of 2 mg/ml completely inhibited the activity of the crude proteinase extract (Fig. 3).

Hybridization experiments. Total DNA from *Lactobacillus casei* HN14 was hybridized with proteinase gene probes originating from *Lactococcus lactis* subsp. *cremoris* Wg2. Probe Q1 contains a fragment of the *prtM* gene, and probes Q6 and Q92 contain cloned DNA fragments corresponding to the active site of the proteinase and the 3' terminus of the *prtP* proteinase gene, respectively (16). As controls, we used plasmid DNA from *Lactococcus lactis* subsp. *lactis* 712, which contains a proteinase gene highly similar to that from *Lactococcus lactis* subsp. *cremoris* Wg2 (18), and chromosomal DNA from *Lactococcus lactis* subsp. *lactis* MG1363. Positive hybridization signals were obtained with all three probes (Fig. 4). On the other hand, a probe containing an insertion sequence element from *Lactococcus lactis* subsp. *cremoris* Wg2 (11) did not give a positive signal in hybridization experiments with DNA from *Lactobacillus casei* HN14 (data not shown).

In order to construct a preliminary restriction enzyme map of the HN14 proteinase gene, total DNA from *Lactobacillus casei* HN14 was digested either with *EcoRI*, *HindIII*, *ClaI*, *BglII*, or *BamHI* restriction enzymes individually or with combinations of the enzymes. A restriction map was constructed on the basis of hybridization experiments and is presented in Fig. 5.

FIG. 4. (A) Digested total DNA isolated from *Lactobacillus casei* HN14; (B, C, and D) Southern hybridization of the digested DNA with proteinase probes Q1 (B), Q6 (C), and Q92 (D). Lanes: 1, λ DNA-*EcoRI* and *HindIII* standard; 2, *HindIII*; 3, *HindIII* and *BamHI*; 4, *HindIII* and *ClaI*; 5, *HindIII* and *EcoRI*; 6, *BamHI* and *BglII*; 7, *BamHI* and *ClaI*; 8, *BamHI* and *EcoRI*; 9, *HindIII* and *BglII*; 10, nondigested chromosomal DNA; 11, plasmid DNA from *Lactococcus lactis* subsp. *lactis* 712; 12, nondigested chromosomal DNA from *Lactococcus lactis* subsp. *lactis* MG1363.

FIG. 5. Restriction enzyme maps of the proteinase gene regions from *Lactococcus lactis* subsp. *lactis* 712 (A), *Lactococcus lactis* subsp. *cremoris* Wg2 (B), and *Lactobacillus casei* HN14 (C). H, *Hind*III; E, *Eco*RI; B, *Bgl*II; C, *Cla*I; BA, *Bam*HI. The thick lines represent a proteinase gene region functionally cloned into *Lactococcus lactis* (17, 18). The restriction enzyme map of the proteinase gene region of *Lactobacillus casei* HN14 was constructed from Southern hybridization experiments. Restriction enzyme maps of lactococcal proteinase genes are as described in reference 18.

DISCUSSION

The ability of lactic acid bacteria to grow in milk depends on the amount of free amino acids and peptides of low molecular weight in milk. Since the concentrations of these compounds are low, the presence of a functional proteolytic system is of great importance for strains which are used in industrial dairy fermentations. By the action of cell wall-bound proteinases on casein, which is the major component of milk proteins, peptides of different sizes are released and then hydrolyzed by peptidases located in or on the outer surface of the cell membrane. The importance of the cell wall-bound proteinase system in lactic acid bacteria has been discussed in a number of papers in the last several years (4–6, 20, 21). Because of the genetics and biochemistry of cell wall-bound proteinases, almost all experimental data are obtained with lactococcal enzymes. Data on the biochemical properties and genetics of extracellular proteolytic enzymes in lactobacilli are very limited.

Lactobacillus casei HN14 obviously has a cell wall-bound proteinase similar to the proteinase of *Lactococcus lactis* subsp. *lactis* 712. As determined by the substrate specificity, the HN14 proteinase is similar to the PI-type lactococcal proteinases, since it hydrolyzes β -casein only. Furthermore, the enzyme is more active at 30 than at 40°C. The activity of the *Lactobacillus casei* HN14 proteinase was inhibited by PMSF, suggesting that the enzyme belongs to the serine-type proteinases.

Another interesting feature of *Lactobacillus casei* HN14 is the location of its proteinase gene. It was found that the HN14 proteinase gene is probably located on the chromosome, which is in contrast to the proteinase genes of lactococci studied by others, which are all plasmid encoded (8, 18). Interestingly, the restriction enzyme map of the HN14 proteinase gene region is very similar to the restriction enzyme maps of the lactococcal proteinase genes studied to date. The similarity is especially pronounced in the regions of the genes corresponding to the active site and C terminus of the proteinases (Fig. 5). However, it seems that one of the *Cla*I sites is missing in the HN14 proteinase gene region. After digestion of the plasmid and the chromosomal DNAs isolated from *Lactococcus lactis* subsp. *lactis* 712 and *Lactobacillus casei* HN14 with the *Hind*III and *Cla*I restriction enzymes, respectively, two bands of different sizes which hybridized with probe Q1 were obtained (data not shown). This is very interesting, since it is known that the *Cla*I sites

in the lactococcal proteinase gene regions are flanking promoters which are responsible for expression of the *prtM* and *prtP* genes (25). In addition, hybridization with a probe containing an insertion sequence element from *Lactococcus lactis* subsp. *cremoris* Wg2 did not give any positive signals. It is known that this element is located in close vicinity of the *prtM* gene in the lactococcal proteinase gene region (11). All these data suggested that the genetic organization of the region containing the *prtM* gene in *Lactobacillus casei* HN14 seems to some extent to be different from that in lactococci.

Taking all obtained similarities among proteinase gene regions of lactococci and *Lactobacillus casei* HN14 into account, one can speculate that they have the same origin. Thus, it would be of great interest to clone and sequence the HN14 proteinase gene and to elucidate its genetic structure; such experiments are in progress in our laboratory.

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REFERENCES

- Anderson, D. G., and L. L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.* **46**:549–552.
- Bouillanne, C., C. Zevaco, and P. Blanchard. 1985. Cell wall associated proteinases in *Lactobacillus helveticus*, *Lactobacillus bulgaricus* and *Lactobacillus lactis*. *Milchwissenschaft* **40**: 140–143.
- de Vos, W. M., P. Vos, H. de Haard, and I. Boerrigter. 1989. Cloning and expression of the *Lactococcus lactis* subsp. *cremoris* SK11 gene encoding an extracellular serine proteinase. *Gene* **85**:169–176.
- Exterkate, F. A. 1976. Comparison of strains of *Streptococcus cremoris* for proteolytic activities associated with the cell wall. *Neth. Milk Dairy J.* **30**:95–105.
- Exterkate, F. A. 1979. Accumulation of proteinase in the cell wall of *Streptococcus cremoris* strain AM1 and regulation of its production. *Arch. Microbiol.* **120**:247–254.
- Exterkate, F. A., and G. J. C. M. de Veer. 1985. Partial isolation of and degradation of caseins by cell wall proteinase(s) of *Streptococcus cremoris* HP. *Appl. Environ. Microbiol.* **49**:328–332.
- Ezzat, N., C. Zevaco, M. El Soda, and J.-C. Gripon. 1987. Partial purification and characterization of a cell wall associated proteinase from *Lactobacillus bulgaricus*. *Milchwissenschaft* **42**: 95–97.
- Gasson, M. J. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**:1–9.
- Haandrikman, A. J. 1990. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
- Haandrikman, A. J., J. Kok, H. Laan, S. Soemitro, A. M. Ledebroer, W. N. Konings, and G. Venema. 1989. Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. *J. Bacteriol.* **171**:2789–2794.
- Haandrikman, A. J., C. van Leeuwen, J. Kok, P. Vos, W. M. de Vos, and G. Venema. 1990. Insertion elements on lactococcal proteinase plasmids. *Appl. Environ. Microbiol.* **56**:1890–1896.
- Hill, S. H. A., and M. J. Gasson. 1986. A quantitative screening procedure for the detection of casein hydrolysis by bacteria, using sodium dodecyl sulphate polyacrylamide gel electrophoresis. *J. Dairy Res.* **53**:625–629.
- Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf. 1985. Genetic manipulation of streptomycetes. John Innes Foundation, Norwich, United Kingdom.

14. Kempler, G. M., and L. L. McKay. 1979. Characterization of plasmid deoxyribonucleic acid in *Streptococcus lactis* subsp. *diacetylactis*: evidence for plasmid linked citrate utilization. *Appl. Environ. Microbiol.* **37**:316–323.
15. Kok, J. 1990. Genetics of the proteolytic system of lactic acid bacteria. *FEMS Microbiol. Rev.* **87**:15–42.
16. Kok, J., K. J. Leenhouts, A. J. Haandrikman, A. M. Ledeboer, and G. Venema. 1988. Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2. *Appl. Environ. Microbiol.* **54**:231–238.
17. Kok, J., J. M. van Dijk, J. M. B. M. van der Vossen, and G. Venema. 1985. Cloning and expression of a *Streptococcus cremoris* proteinases in *Bacillus subtilis* and *Streptococcus lactis*. *Appl. Environ. Microbiol.* **50**:94–101.
18. Kok, J., and G. Venema. 1988. Genetics of proteinase of lactic acid bacteria. *Biochimie* **70**:475–488.
19. Laan, H., and W. Konings. 1989. Mechanism of proteinase release from *Lactococcus lactis* subsp. *cremoris* Wg2. *Appl. Environ. Microbiol.* **55**:3101–3106.
20. Law, B. A. 1977. Dipeptide utilization by starter streptococci. *J. Dairy Res.* **44**:309–317.
21. Law, B. A. 1979. Extracellular peptidases in group N streptococci used as cheese starters. *J. Appl. Bacteriol.* **46**:455–463.
22. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517.
23. Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29**:807–813.
24. Visser, S., F. A. Exterkate, C. J. Slangen, and G. J. C. M. de Veer. 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine α_{s1} -, β - and κ -casein. *Appl. Environ. Microbiol.* **52**:1162–1166.
25. Vos, P., M. van Asseldonk, F. van Jeveren, R. Siezen, G. Simons, and W. M. de Vos. 1989. A maturation protein is essential for production of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. *J. Bacteriol.* **171**:2795–2802.