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Classification of *Lactococcus lactis* cell envelope proteinase based on gene sequencing, peptides formed after hydrolysis of milk, and computer modeling

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ABSTRACT

Lactococcus lactis strains depend on a proteolytic system for growth in milk to release essential AA from casein. The cleavage specificities of the cell envelope proteinase (CEP) can vary between strains and environments and whether the enzyme is released or bound to the cell wall. Thirty-eight *Lc. lactis* strains were grouped according to their CEP AA sequences and according to identified peptides after hydrolysis of milk. Finally, AA positions in the substrate binding region were suggested by the use of a new CEP template based on *Streptococcus* C5a CEP. Aligning the CEP AA sequences of 38 strains of *Lc. lactis* showed that 21 strains, which were previously classified as group d, could be subdivided into 3 groups. Independently, similar subgroupings were found based on comparison of the *Lc. lactis* CEP AA sequences and based on normalized quantity of identified peptides released from α_{S1}-casein and β-casein. A model structure of *Lc. lactis* CEP based on the crystal structure of *Streptococcus* C5a CEP was used to investigate the AA positions in the substrate-binding region. New AA positions were suggested, which could be relevant for the cleavage specificity of CEP; however, these could only explain 2 out of 3 found subgroups. The third subgroup could be explained by 1 to 5 AA positions located opposite the substrate binding region.
Key words: cell envelope proteinase (CEP), *Lactococcus lactis* ssp. classification, computer modeling, substrate binding

INTRODUCTION

Milk contains a relatively small amount of free AA and small peptides; however, lactic acid bacteria (**LAB**) need 4 to 16 essential AA to grow (Chopin, 1993). This makes LAB dependent on a proteolytic system that can break down caseins in milk to AA and peptides. The proteolytic system in *Lactococcus lactis* consists of a cell envelope proteinase (**CEP**), also called lactocepin or PrtP, which is located outside the cell and initiates the breakdown of casein into medium-sized peptides. These peptides are transported across the cell membrane by at least 3 different types of specialized transport systems. Inside the cells, intracellular peptidases hydrolyze medium-size peptides into smaller peptides and AA (Kunji et al., 1996; Savijoki et al., 2006).

One of the first classification systems divided the CEP into 3 groups based on their ability to degrade different parts of casein: CEP type PI preferentially hydrolyzes β-CN, whereas CEP type PIII also hydrolyzes α_{S1}-CN and κ-CN; an intermediate group PI/PIII was also defined (Visser et al., 1986). About 98% homology of CEP sequences was found between *Lc. lactis* ssp. *cremoris* SK11 (CEP type PIII) and *Lc. lactis* ssp. *cremoris* Wg2 (CEP type I; Vos et al., 1989), which made it possible, using recombinant DNA technology, to construct hybrid proteins by swapping regions of the 2 CEP with new proteolytic properties. The AA sequences and proteolytic properties were compared by using a computer model of SK11 CEP with a template based on the crystal structure of subtilisin (Vos et al., 1991). The homology between subtilisin and *Lc. lactis* CEP is seen especially in the catalytic domain around the residues of the active site aspartic acid (Asp30), histidine (His94), asparagine (Asn196), and serine (Ser433). Comparison between the cleavage sites and the CEP model made it possible to suggest 5 AA positions (131, 138, 142, 144, and 166) to be part of the substrate binding pocket and 2 other AA positions (747 and 748), which were thought to be important in electrostatic interactions with caseins (Vos et al., 1991).

Exterkate et al. (1993) provided the foundation of a new classification system of CEP. They compared the selected AA positions found by Vos et al. (1991) plus 2 extra residues (177 and 763) with the ability to hydrolyse the peptide α_{S1}-CN (f1–23) from 16 CEP purified from various *Lc. lactis* strains. This resulted in 7 classification groups (named a to g) that were later extended to 8 groups (a to h; Broadbent et al., 1998). This classification system is hereafter referred to as

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Exterkate's classification system. The specificity of *Lc. lactis* CEP cleavage of intact casein was reviewed in Kunji et al. (1996). Specificity and activity depend on whether purified or native-bound CEP is used (Exterkate and Alting, 1993; Flambard and Juillard, 2000). Environmental factors such as salt, pH, and water activity also influence the specificity (Reid and Coolbear, 1998, 1999). Finally, expression of CEP depends on the growth medium (Exterkate, 1979, 1985).

Cheddar cheese contains residual chymosin and plasmin activity that initiates the primary casein breakdown. The CEP from LAB hydrolyses the medium-sized peptides to smaller peptides, which are transported into the cell by the specialized transport systems. Intracellular peptidases continue the breakdown of peptides when *Lc. lactis* are grown in milk. Proteolysis in cheese is a major contributor to flavor formation and structure development of Cheddar cheese (Upadhyay et al., 2004).

More knowledge and new technology have been gained since the last update of the *Lc. lactis* CEP classification system. Faster and cheaper sequencing of LAB genomes indicates that the a to h classification system is not consistent and does not fully describe the variation of CEP.

The aim of this study was to consider a subdivision of Exterkate's classification system group d, strains of which are the most commonly used in Cheddar cheese production. This was based on comparison of the *Lc. lactis* CEP AA sequences and normalized quantity of identified peptides after hydrolysis in milk. Furthermore, new AA positions important in the substrate binding region were suggested based on a computer model of *Lc. lactis* ssp. *cremoris* SK11 CEP with *Streptococcus* C5a CEP as template.

MATERIAL AND METHODS

Bacteria Preparation

Thirty-four different *Lc. lactis* ssp. *cremoris* and *Lc. lactis* ssp. *lactis* strains (Chr. Hansen A/S, Hørsholm, Denmark) were genome sequenced in this study. Four *Lc. lactis* ssp. *cremoris* strains (University of Copenhagen, Frederiksberg, Denmark) were not genome sequenced. However, published CEP sequences of strains (www.uniprot.com) with the same strain names (Wg2, HP, NCDO763, and SK11) were used in the analysis. Stocks were prepared by growing strains in reconstituted laboratory milk (9.5% total solids, autoclaved 15 min, 121°C; hereafter, **A-milk**) and incubated overnight at 30°C, diluted 1:10 with A-milk, and transferred to ampules, which were stored at −80°C until use.

Gene Sequences

Genome sequencing was performed at Geneservice (Cambridge, UK) or Beijing Genome Institute (BGI, Hong Kong) on purified DNA originating from single-strain samples using the next-generation sequencing (NGS) platform of Illumina Inc. (San Diego, CA). Sequencing strategies used were 36-bp single reads or 36-, 90-, or 100-bp paired-end reads with 500-bp spacer size. De novo assemblies of the genome sequences were performed using the algorithm in CLC Genomics Workbench (CLC Bio, Århus, Denmark), resulting in a variable number of contigs. The CEP sequences were identified by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi; CLC Genomics Workbench) of the contigs resulting from de novo assembly against the nucleotide sequence of the CEP from *Lactococcus lactis* ssp. *cremoris* HP (GenBank accession no. AF247159). Only strains without sequencing ambiguities as revealed by the de novo conservation profile were included in the study. The gene sequences were aligned using the software Jalview (www.jalview.org).

Modeling of CEP

In the absence of an experimentally determined 3-dimensional structure for any *Lc. lactis* CEP, a homology model for *Lc. lactis* ssp. *cremoris* SK11 lactocepin (P15292) was constructed. First profile-profile alignment (HHPRED) at the Max Planck Institute for Developmental Biology website (http://toolkit.tuebingen.mpg.de/hhpred) was used to find the most suitable template available, the alignment of which was then passed to the MODELER (Sali and Blundell, 1993) server at the same website for determination of the predicted 3-dimensional model.

Identification of Milk-Derived Peptides

The A-milk was inoculated with 1% (vol/vol) of each strain from stock ampules for 20 h at 30°C, followed by reinoculation with 1% (vol/vol) in A-milk and incubation under the same conditions. The hydrolysis experiment was carried out in fresh skim milk, which was pasteurized (30 min at 64°C) before use. Then, 50 mL of skim milk was inoculated with 1% of each strain and incubated for 24 h at 30°C. The acidified milk was centrifuged (3,400 × g, 10 min, at 5°C), the supernatant was heated (10 min at 85°C) to stop potential enzyme reactions, and the samples were stored at −20°C until analysis. The experimental work was carried out in duplicate on 2 different days.

Fifteen microliters of each supernatants was analyzed by liquid chromatography-mass spectrometry (LC-MS)

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