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Degradation of α_{s1}-CN f1-23 by aminopeptidase N and endopeptidases E, O, O2, and O3 of *Lactobacillus helveticus* WSU19 under cheese ripening conditions

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Abstract

This study determined specificities of aminopeptidase N (PepN), endopeptidase E (PepE), endopeptidase O (PepO), endopeptidase O2 (PepO2), and endopeptidase O3 (PepO3), from *Lactobacillus helveticus* WSU19 on the α_{s1}-CN f1-23 peptide, formed by residual chymosin during cheese ripening. Cell-free extracts (CFEs) were prepared from *Escherichia coli* DH5α derivatives expressing peptidase genes of *Lb. helveticus* WSU19. The α_{s1}-CN f1-23 peptide was digested by CFEs under cheese ripening conditions. Degradation pattern was analyzed qualitatively using MALDI-TOF mass spectrometry. PepN exhibited activity on α_{s1}-CN f1-23 only in the presence of an endopeptidase, particularly PepO-like endopeptidases. PepO, PepO2, and PepO3 cleaved α_{s1}-CN f1-23 predominantly at Glu_{14}–Val_{15}, forming the bitter peptide α_{s1}-CN f1-14. PepE cleaved α_{s1}-CN f1-23 primarily at Lys_3–His_4, suggesting a role for PepE in degrading bitter peptides from the N-terminus of α_{s1}-CN f1-23. Combinations of PepE/PepO and PepE/PepO2 were determined to have the potential to decrease the accumulation of α_{s1}-CN f1-14.

Keywords: Aminopeptidase; Endopeptidase; *Lactobacillus helveticus*; α_{s1}-CN f1-23; Bitter peptide; MALDI-TOF

1. Introduction

Residual chymosin in curd is important for the initial proteolysis of caseins during cheese ripening (Fox, O'Connor, McSweeney, Guinee, & O'Brien, 1996). The primary target of proteolysis by residual chymosin is α_{s1}-casein (CN), forming α_{s1}-CN f1-23 and f24-199 (Exterkate, 1987). Degradation of α_{s1}-CN f1-23 plays a role in the production of amino acid nitrogen in cheese and for the development of cheese flavor. The α_{s1}-CN f1-23 peptide in cheese is further degraded, primarily by starter proteinases (Exterkate & Alting, 1993, 1995). Degradation of α_{s1}-CN f1-23 also occurs by the action of starter peptidases. Some peptides derived from α_{s1}-CN f1-23 have been determined to be bitter, including α_{s1}-CN f1-7, α_{s1}-CN f1-9, α_{s1}-CN f1-13, α_{s1}-CN f11-14, α_{s1}-CN f14-17, α_{s1}-CN f17-21, and α_{s1}-CN f21-23 (Broadbent, Strickland, Weimer, Johnson, & Steele, 1998; Lee, Lo, & Warthesen, 1996; Lemieux & Simard, 1992). Christensson et al. (2002) hypothesized that α_{s1}-CN f1-14 is bitter because the peptide has a structure and hydrophobicity similar to α_{s1}-CN f1-13. Christensson et al. (2002) also classified α_{s1}-CN f1-17 as a bitter peptide. Accumulation of bitter peptides during ripening may result in bitter cheese, a major defect in Gouda and Cheddar cheeses (Lemieux & Simard, 1991; Shinoda, Tada, Otagiri, & Okai, 1985).

Lactobacillus helveticus WSU19 is the adjunct culture used in the manufacturing of Cougar Gold cheese, an aged Cheddar-type cheese produced using a mixed culture of *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*. Despite a long ripening time, Cougar Gold develops little

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bitterness. Studies have been conducted by our group to elucidate the debittering activity of peptidases of *Lb. helveticus* WSU19 (Fajarrini, 1999; Olson, 1998; Soeryapranata, Powers, Fajarrini et al., 2002; Soeryapranata, Powers, Hill et al., 2002; Soeryapranata, Powers, Weller, Hill, & Siems, 2004). Recently, the actions of aminopeptidase N (PepN), endopeptidase E (PepE), endopeptidase O (PepO), endopeptidase O2 (PepO2), and endopeptidase O3 (PepO3) of *Lb. helveticus* WSU19 in degrading a bitter peptide, β-CN f193-209, have been reported (Soeryapranata, Powers, & Yüksel, 2007). Sridhar, Hughes, Welker, Broadbent, and Steele (2005) reported activities of endopeptidases of *Lb. helveticus* CNRZ32 on a bitter peptide, α_{s1}-CN f1-9. However, activities of these peptidases on α_{s1}-CN f1-23 have not been reported. The objective of the current study was to characterize the specificities of PepN, PepE, PepO, PepO2, and PepO3 of *Lb. helveticus* WSU19 on α_{s1}-CN f1-23.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Recombinant *Escherichia coli* DH5α strains were grown in Luria-Bertani (LB) Broth (Invitrogen, Carlsbad, CA, USA) with vigorous shaking at 37 °C in the presence of 1 mg mL^{-1} of erythromycin (Em), purchased from Fisher Scientific (Fairlawn, NJ, USA). Table 1 describes the plasmids carried by the recombinant *E. coli* DH5α cells used in this study. Stock cultures were kept at −86 °C and working cultures were obtained from stock cultures through two transfers in appropriate media. Cloning of the peptidase genes of *Lb. helveticus* WSU19 in *E. coli* DH5α was conducted as described by Soeryapranata et al. (2007).

2.2. Preparation of cell free extracts (CFEs) from peptidase-positive clones

Cultures (150 mL) were grown to an optical density (OD) of 4.0 at 600 nm. Cells were harvested by centrifuging for 15 min (6000 × *g*, 4 °C) using an Avanti™ J-25 centrifuge (Beckman Coulter, Fullerton, CA, USA). The cells were washed twice with 75 mL of ice-cold 15.12 g L^{-1} PIPES buffer pH 7.0 (Sigma-Aldrich Co., St. Louis, MO, USA) and centrifuged for 15 min (7500 × *g*, 4 °C). The supernatants were discarded. The pellet was resuspended in 7.5 mL of ice-cold 15.12 g L^{-1} PIPES buffer at pH 7.0. The cells were ruptured by homogenizing the suspension twice with 7.5 g of 106-μm glass beads (Sigma-Aldrich Co.) for 5 min using a Red Devil Paint Mixer (Red Devil Equipment Co., Plymouth, MN, USA). The suspension was incubated on ice for 15 min before each homogenization step. The resulting suspension was centrifuged for 30 min (20,000 × *g*, 4 °C). The supernatant was removed carefully and stored at −86 °C. Protein content of the CFE was estimated by the method of Bradford (1976) using Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin (Sigma-Aldrich Co.) as standard protein.

Table 1
Designation and characteristics of plasmids used in this study

Plasmid	Relevant characteristics	References
pJDC9	Em^r, *lacZ'*, 6.95 kb	Chen and Morrison (1987)
pES1 (pJDC9::*pepE*)	Em^r, 2.7-kb *Pst*I insert containing *pepE*	Soeryapranata et al. (2007)
pES2 (pJDC9::*pepO2*)	Em^r, 5.8-kb *Sph*I-*Xba*I insert containing *pepO2*	Soeryapranata et al. (2007)
pES4 (pJDC9::*pepN*)	Em^r, 5-kb *Sac*I-*Sph*I insert containing *pepN*	Soeryapranata et al. (2007)
pES5 (pJDC9::*pepO*)	Em^r, 4.3-kb *Hin*dIII-*Sal*I insert containing *pepO*	Soeryapranata et al. (2007)
pES6 (pJDC9::*pepO3*)	Em^r, 3.4-kb *Kpn*I insert containing *pepO3*	Soeryapranata et al. (2007)

2.3. Degradation of α_{s1}-CN f1-23 by CFE(s) from peptidase-positive clone(s)

The α_{s1}-CN f1-23 peptide, synthesized by Laboratory for Biotechnology and Bioanalysis I (LBB I) at Washington State University (Pullman, WA, USA), was dissolved to a final concentration of 4 mg mL^{-1} in 9.55 g L^{-1} citrate buffer (pH 5.2) containing 40 g L^{-1} sodium chloride (JT Baker Chemical Co., Phillipsburg, NJ, USA). Reaction mixtures consisted of 400 μL of the peptide solution with CFE(s) added individually or combined to obtain approximately 50 μg of total protein per CFE. The mixtures were incubated at 37 °C. Aliquots were withdrawn at 0, 2, 4, 8, 12, 24, 48, and 72 h, followed by heat treatment at 70 °C for 20 min to terminate the reaction. Aliquots were immediately cooled in an ice-bath and stored at −86 °C. Digestion of α_{s1}-CN f1-23 by CFE from *E. coli* DH5α (pJDC9) served as the control. Digestion experiments were duplicated.

2.4. MALDI-TOF mass spectrometry analysis

Aliquots from the CFE peptide digest were desalted using ZipTip C18 (Millipore, Bedford, MA, USA) as recommended by the manufacturer. The bound analytes were eluted with 10 μL of matrix solution, a saturated solution of α-cyano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.1% trifluoroacetic acid (Sigma-Aldrich Co.). Eluate (1 μL) was deposited on the MALDI spot and air-dried. MALDI analysis was performed using a PerSeptive Biosystems (Framingham, MA, USA) DE-RP time-of-flight mass spectrometer. Sample was ionized by a 337-nm nitrogen laser pulse and accelerated under 25,000 V before entering the TOF mass analyzer. The instrument was set in the positive reflector mode with delayed extraction. The mass-to-charge ratio (m/z) was acquired between 500 and 4000 Da. Each spectrum reported is the result of 32 laser shots. A total of eight spectra (a sum of