The Autoproteolysis of Lactococcus lactis Lactocepin III Affects Its Specificity towards β-Casein

BENEDICTE FLAMBARD AND VINCENT JUILLARD*
Unité de Recherches Laitières et Génétique Appliquée, Institut National de la Recherche Agronomique,
F-78350 Jouy-en-Josas, France

Received 22 May 2000/Accepted 13 September 2000

The effect of autoproteolysis of Lactococcus lactis lactocepin III on its specificity towards β-casein was investigated. β-Casein degradation was performed by using either an autolysin-defective derivative of L. lactis MG1363 carrying the protease genes of L. lactis SK11, which was unable to transport oligopeptides, or autoprotoelyzed enzyme purified from L. lactis SK11. Comparison of the peptide pools by high-performance liquid chromatography analysis revealed significant differences. To analyze these differences in more detail, the peptides released by the cell-anchored protease were identified by on-line coupling of liquid chromatography to mass spectrometry. More than 100 oligopeptides were released from β-casein by the cell-anchored proteinase. Analysis of the cleavage sites indicated that the specificity of peptide bond cleavage by the cell-anchored protease differed significantly from that of the autoprotoelyzed enzyme.

Due to their limited capacity for synthesizing amino acids (3), lactococci have to utilize exogenous nitrogen sources for optimal growth. The amino acid requirements appear to be strain specific, but most Lactococcus lactis strains need at least Leu, Ile, Val, and His for growth (15, 28). In milk, the concentrations of several essential amino acids, especially those of Ile and Leu (less than 1 mg liter−1), are very low (10, 18). In addition, milk peptides are a poor source of Leu and Met (16). Thus, for optimal growth in milk, lactococci depend on utilization of casein (18, 29). A complex proteolytic system is needed for casein hydrolysis. According to proposed models, lactocepin (EC 3.4.21.96; previously named cell envelope proteinase PrtP) (34) is involved in the first step of casein degradation. Only some of the oligopeptides released by lactocepin are taken up by the oligopeptide transport system (Opp) and subsequently cleaved into amino acids by intracellular peptides (for recent reviews, see references 17 and 22).

Two different types of lactocepins (lactocepin I and lactocepin III) have been identified in lactococci on the basis of their specificity for caseins (44). Lactocepin I cleaves β-casein preferentially and κ-casein to a lesser extent. In contrast, lactocepin III cleaves β-, κ-, and αs1-caseins. There are only 44 differences in the amino acid sequences of lactocepin I and III, and 5 of them are responsible for the differences in specificity between the two lactocepins (7, 40).

Lactocepin purification requires release of the enzyme from the cell (30), which results from autoproteolysis of the protein in a Ca2+-free buffer (24, 25). Autoproteolysis takes place in the C-terminal part of the protein, presumably in the B domain of the protein, and results in a 145-kDa enzyme, compared to the 186-kDa cell-anchored lactocepin III (1). The action of purified lactocepins towards caseins has been studied extensively (32, 33, 37, 38). In particular, most, if not all, of the peptides released from β-casein by purified lactocepin I have been identified by on-line coupling of liquid chromatography (LC) to mass spectrometry (MS) (19). Nevertheless, autoproteolysis of lactocepin may result in a conformational change in the enzyme. It is worth noting that the B domain of lactocepin III, which presumably contains the autoproteolysis site, has been reported to play an important role in the stability of the enzyme (1). Thus, the question of the specificity of the anchored form of lactocepins has to be addressed.

The use of a genetically engineered strain of L. lactis made it possible to analyze the pool of peptides released from β-casein by the anchored form of lactocepin I (21). The composition of the pool of peptides was clearly identical to that obtained with the purified enzyme, indicating that autoproteolysis of lactocepin I did not affect the specificity of the enzyme towards β-casein, as suggested elsewhere (8). In contrast, there has been no clear demonstration of such conservation (or alteration) of specificity after autoproteolysis of lactocepin III. A previous study suggested that the peptides released by the anchored form of lactocepin III might differ from those released by the purified form (8). Nevertheless, there has been no report on the composition of the peptide pool released from caseins by the native form of lactocepin III. The aim of the present study was, therefore, to analyze in great detail the peptides released from β-casein by the anchored form of lactocepin III. A comparison of the data obtained with previously published data indicated that autoproteolysis of lactocepin III affects its specificity towards β-casein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. L. lactis GFI100, an autolysin- and oligopeptidase transport-deficient derivative of L. lactis MG1363 (21), was a generous gift from B. Poolman (University of Groningen, Groningen, The Netherlands). Plasmids pNZ521 and pNZ511, which encode a wild-type lactocepin III and a C-terminally truncated lactocepin III that is completely secreted into the growth medium, respectively (4), were generous gifts from W. de Vos (Netherlands Institute for Dairy Research, Ede, The Netherlands). Plasmids were introduced into recipient strains by transformation by the procedure of Dornan and Collins (5). The presence of plasmids in transformed strains was checked by agarose gel electrophoresis. Lactococcal strains were grown at 30°C in M17 broth (41) or in chemically defined medium (34) supplemented with 1% (wt/vol) glucose or 1% (wt/vol) lactose and chloramphenicol (5 µg ml−1) when required. The pH of the culture medium was controlled at 6.5. The strains were stored at −80°C in M17 broth containing 0.5% (wt/vol) glucose, 10% (vol/vol) glycerol, and the appropriate antibiotics (each at a concentration of 5 µg ml−1).

Lactocepin isolation. The procedure used to isolate lactocepin III from L. lactis SK11 relied on the autoproteolytic properties of the enzyme (24). Cells were washed twice in 50 mM Tris-HCl (pH 8) containing 30 mM CaCl2. Lactocepin was released from the cell envelope by incubation for 30 min at 30°C in 50
mM Tris-HCl (pH 8). Cells were removed by centrifugation (10,000 × g for 10 min at 4°C), and CaCl2 (final concentration, 2 mM) was added to the lactocepin-containing supernatant. Further purification of lactocepin was achieved by anion-exchange chromatography using a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) and a linear 0 to 0.35 M NaCl gradient for 50 min. No release procedure was required for isolation of lactocepin from L. lactis GF1005 (secreted enzyme). Lactococcal cells were removed from an overnight culture in casein-containing chemically defined medium by centrifugation (10,000 × g for 10 min at 4°C). Lactocepin-containing supernatant was ultrafiltered through a 30,000-Da-cutoff membrane (YM30; Amicon Corp., Beverly, Mass.), and the residual unfiltered solution was subjected to anion-exchange chromatography.

Lactocepin activity was estimated by using fluorescein isothiocyanate-labeled casein (Sigma Chemical Co., St. Louis, Mo.) as the substrate (42). The absence of peptidase activity in lactocepin solutions was checked by using substrates specific for different peptidases (i.e., Lys-p-nitroanilide, Glu-p-nitroanilide, Gly-Pro-p-nitroanilide, and bradykinin) as previously described (19).

### β-Casein purification

The method used for purification of β-casein was adapted from the method of Guilou et al. (13). Total casein was obtained by centrifugation at 30°C. Hydrolysis was stopped by removing the cells by centrifugation (10,000 × g at 4°C), and CaCl2 (final concentration, 2 mM) was added to the lactocepin-pension (OD650, 24; corresponding to 4.8 mg of protein ml⁻¹) to prepare a 3 h digested lactocepin solution.

### RESULTS

The transport assays used were adapted from previously described assays (11, 23). Cells were grown to an OD650 of approximately 0.8 in chemically defined medium containing 17 free amino acids as the nitrogen source (34). Prior to transport assays, cells were deenergized for 30 min at 30°C with 10 mM 2-deoxy-d-glucose. For each transport assay, cells (OD650: 1; corresponding to 0.2 mg of protein ml⁻¹) were preincubated for 5 min in the presence of 25 mM glucose. Peptide uptake was monitored by determining the increase in the intracellular concentration of free amino acids after dapsyl chloride derivatization by using HPLC analysis. The dapsylated amino acids were separated at 40°C on a reverse-phase C18 column (Nucleosil; 250 by 4.6 mm; Shandon HPLC, Cheshire, United Kingdom).

### LC-MS analysis

The peptides soluble in 1% TFA were identified by on-line coupling of HPLC to MS, essentially as previously described (19, 21). Peptides were separated at 40°C on a reverse-phase C18 HPLC column (150 by 0.5 mm; Perkin-Elmer Corp., Norwalk, Conn.). Solvents A and B were 0.1% (vol/vol) formic acid–4 mM ammonium acetate and 0.1% (vol/vol) formic acid–4 mM ammonium acetate–90% (vol/vol) acetonitrile in MilliQ water, respectively. A linear 5 to 75% solvent B gradient was applied for 100 min. The flow rate was 5 μl min⁻¹. By flow splitting the eluate, about 5% of the sample was introduced into a single quadrupole MS (API 100; Perkin-Elmer Corp.). The MS was used in the positive-ion mode, and full-scan spectra were recorded at mass-to-charge ratios (m/z) between 200 and 2,000. To generate multiply charged ions without fragmentation, a low nozzle voltage (40 V) was used; to generate fragment ions by collision-induced dissociation from peptides, a higher nozzle voltage (170 V) was used.

### Transport experiment

The transport assays used were adapted from previously described assays (11, 23). Cells were grown to an OD650 of approximately 0.8 in chemically defined medium containing 17 free amino acids as the nitrogen source (34). Prior to transport assays, cells were deenergized for 30 min at 30°C with 10 mM 2-deoxy-d-glucose. For each transport assay, cells (OD650: 1; corresponding to 0.2 mg of protein ml⁻¹) were preincubated for 5 min in the presence of 25 mM glucose. Peptide uptake was monitored by determining the increase in the intracellular concentration of free amino acids after dapsyl chloride derivatization by using HPLC analysis. The dapsylated amino acids were separated at 40°C on a reverse-phase C18 column (Nucleosil; 150 by 4.6 mm; Shandon HPLC). Solvent A was 10 mM sodium citrate (pH 6.2). Solvent B was acetonitrile–25 mM sodium citrate (60:40) (pH 6.2). A linear 5 to 75% solvent B gradient was applied for 75 min. The flow rate was 5 μl min⁻¹. By flow splitting the eluate, about 5% of the sample was introduced into a single quadrupole MS (API 100; Perkin-Elmer Corp.). The MS was used in the positive-ion mode, and full-scan spectra were recorded at mass-to-charge ratios (m/z) between 200 and 2,000. To generate multiply charged ions without fragmentation, a low nozzle voltage (40 V) was used; to generate fragment ions by collision-induced dissociation from peptides, a higher nozzle voltage (170 V) was used.

### RESULTS

Hydrolysis of β-casein by cell-anchored and autoproteolyzed forms of lactocepin III. Purified β-casein was digested for 3 h by lactocepin III purified from L. lactis SK11 or by the autolysin-defective strain L. lactis GF1004 carrying the lactocepin III genes of L. lactis SK11. On the one hand, lactocepin III was purified by a two-step procedure (release from the cell envelope by autodigestion, followed by ion-exchange chroma-
tography). SDS-PAGE and immunoblotting experiments indicated that active fractions contained the purified proteinase and several autoproteolysis fragments (24). No other proteins could be detected in the lactocepin preparation. In particular, lactocepin-containing fractions were free of peptidase activity. This preparation of lactocepin is called autoproteolyzed lactocepin below. On the other hand, when \textit{L. lactis} GF1004 was used, no lysis of the cells could be detected during the incubation period, as reported previously for a related strain (\textit{L. lactis} GF200) (2, 21), and no release of lactocepin into the incubation mixture could be detected. Hydrolysis experiments were repeated at least three times and yielded similar results.

The concentration of cell-anchored lactocepin (i.e., cell density) and that of autoproteolyzed lactocepin were adjusted to the same initial proteolytic activity, as determined by measuring the rate of isothiocyanate-labeled casein hydrolysis. The activity of the cell-anchored lactocepin remained constant during the incubation period, suggesting that no significant autoproteolysis occurred. In contrast, the activity of the autoproteolyzed lactocepin decreased with time, despite the presence of calcium in the incubation mixture. To counterbalance this loss of activity, fresh autoproteolyzed lactocepin was added to the incubation mixture during the incubation period in order to keep the enzymatic activity as constant as possible.

Accumulation of \(\beta\)-casein degradation products was analyzed by HPLC, which yielded similar traces for all repetitions. No TFA-soluble peptides were detected when \(\beta\)-casein was incubated in the presence of \(\beta\)-casein and the autoproteolyzed lactocepin (Fig. 1). Several peaks were detected only when \(\beta\)-casein was hydrolyzed by the cell-anchored lactocepin (e.g., retention times of 16.5, 19.7, 22.3, 33.8, and 38.8 min). These peptides were not detected when \(\beta\)-casein was omitted from the reaction mixture, suggesting that they were effectively derived from \(\beta\)-casein. In contrast, several peaks seemed to be specifically released by the autoproteolyzed form of lactocepin, since they were not detected in the peptide pool produced by the cell suspension (e.g., peaks eluting at 19.0, 23.3, 35.4, and 36.3 min).

During the initial stages of degradation (up to 100 min), TFA-soluble peptides released by the cell suspension were detected mainly in the late region of the chromatogram (i.e., the region corresponding to hydrophobic [or large] peptides), and the hydrophilic (or short) peptides were released when longer incubation periods (more than 100 min) were used. In contrast, both hydrophilic and hydrophobic peptides were detected in the initial stages of \(\beta\)-casein degradation by the autoproteolyzed form of the lactocepin.

**Additional \(\beta\)-casein hydrolysis experiments.** Additional \(\beta\)-casein degradation experiments were performed by using \textit{L. lactis} GF100, the \textit{L. lactis} GF1004 parental Prt \(^+\) strain (Table 1). As expected, no TFA-soluble peptides could be detected when \(\beta\)-casein was incubated in the presence of only \textit{L. lactis} GF100. Addition of autoproteolyzed lactocepin III to the incubation mixture resulted in liberation of peptides. HPLC analysis of this pool of peptides revealed a chromatographic trace similar to that obtained in the presence of \(\beta\)-casein and the autoproteolyzed lactocepin (Fig. 2). In particular, the peaks eluting at 19.0, 23.3, 35.4, and 36.3 min, which were specific for the autoproteolyzed form of the enzyme, were detected, whereas the peaks specific for the cell-anchored form of lactocepin III (16.5, 19.7, 22.3, 33.8, and 38.8 min) were not detected. Similarly, the pool of peptides released from \(\beta\)-casein by the autoproteolyzed lactocepin was not modified when it was incubated in the presence of \textit{L. lactis} GF100, as indicated by HPLC analysis (data not shown).
The genetically engineered strain *L. lactis* GF1005 produces a truncated form of lactocepin III, which lacks the C-terminal LPKTG anchor (4). Consequently, the enzyme is secreted into the growth medium. This form of lactocepin was purified and is called secreted lactocepin below.

*b*-Casein was digested by the secreted lactocepin. The HPLC profile corresponded to that obtained with the anchored enzyme, and none of the peptides observed only in the presence of autoproteolyzed lactocepin (e.g., the peptides eluting at 19.0, 23.3, 35.4, and 36.3 min) were detected (data not shown).

Identification of peptides released from *b*-casein by the anchored form of lactocepin. The main peptides released from *b*-casein by autoproteolyzed lactocepin III have already been identified (38). Consequently, only the peptides released by cell-anchored lactocepin III were identified by on-line coupling of HPLC to MS by using scan nozzle voltages ranging from 70 to 170 V. At a low nozzle voltage, multiply charged ions were generated, so that a mass could be assigned to each eluted compound (even in the case of coeluting materials). Increasing the nozzle voltage resulted in collision-induced dissociation of eluted peptides. Analysis of the dissociation fragments made it possible to identify the initial peptides (19, 21). About 100 different peptides were identified in the pool, indicating that a large number of *b*-casein peptide bonds were cleaved (about 55%). The locations of the peptides from *b*-casein suggested that they did not originate from a particular region of the substrate (Fig. 3). More than 50% of the peptides released from *b*-casein contained eight or fewer amino acids. To ensure that these peptides were not excreted by the cells, *L. lactis* GF1004 was incubated for 3 h in the absence of *b*-casein, and the external medium was subjected to LC-MS analysis. Although several peaks were detected by UV after HPLC separation, only a few masses were recorded, suggesting that most of the compounds could not be ionized. Nevertheless, none of the recorded masses could be assigned to *b*-casein fragments. These results, therefore, indicated that the peptides effectively resulted from degradation of *b*-casein by the cell-anchored lactocepin, whereas the cells incubated in the absence of *b*-casein did not release significant amounts of peptides.

Several free amino acids were detected in the external medium following incubation of *b*-casein with the cell suspension. All of them were also detected when the cell suspension was incubated in the absence of *b*-casein. Since the concentrations of free amino acids were in the same range in both cases, the data strongly suggested that the presence of free amino acids in the peptide mixture resulted from passive leakage from the cells, as previously reported (11, 23).

Several masses (range, 212.8 to 389.3 amu) could be assigned to di- or tripeptides. These compounds could not be identified by LC-MS analysis, since the masses of the potential dissociation fragments are too small to be distinguished from the background. To check for the possible presence of di- or tripeptides in the pool of peptides released from *b*-casein, transport experiments were performed with *L. lactis* VS772. This strain is able to transport di- and tripeptides but not oligopeptides (43). The accumulation of free amino acids in the cells corresponded to that observed when *L. lactis* CFS62, a strain unable to translocate any peptide (9), was used. These results suggested that intracellular accumulation of amino acids by the Opp-defective strain resulted from translocation of free amino acids rather than transport of di- or tripeptides followed by internal degradation by amino-, di-, or tripeptidases. To confirm this hypothesis, the mixture of casein-derived peptides was first deprived of free amino acids by performing a transport experiment with *L. lactis* CFS62. A second transport experiment was then performed by using *L. lactis* VS772 and the residual mixture of peptides. No intracellular accumulation of amino acids was observed. Altogether, these results indicate that no di- or tripeptides were released from *b*-casein by lactocepin III.

Specificity of the cell-anchored lactocepin III towards *b*-casein. Lactocepin has a significant binding area consisting of...
eight amino acids (31). Consequently, the environment of each cleavage site has been studied. The presence of a Phe or His residue at position P4 (i.e., the fourth residue on the C-terminal side of the cleavage site), the presence of a Tyr residue at position P1, or the presence of an Asn at position P9 (i.e., the second residue on the N-terminal side of the cleavage site) prevented hydrolysis of the peptide bond by the cell-anchored lactocepin. In contrast, the presence of an Asp or Gly residue at position P6, the presence of a Thr residue at position P3, the presence of a Met residue at position P1, or the presence of a Phe, Ser, or Asn residue at position P9 resulted in cleavage of the peptide bond. According to these preferences, only two cleavages (Pro67-Asn68 and Gln167-Ser168) were expected (due to the positive action of Asn and Ser residues at position P9, respectively) and not observed, whereas only one peptide bond (Glu5-Leu6) was cleaved even though it was expected to remain intact (due to the negative action of Asn at position P9).

On the other hand, the frequencies of the different classes of residues (i.e., acidic, basic, hydrophobic, hydrophilic) at specific positions close to the cleavage site were compared to those encountered in the whole β-casein sequence. The frequencies of acidic residues at positions P4 and P9 (6% in both cases) and the frequencies of basic residues at positions P9 and P3 (6% in both cases) were slightly lower than those in β-casein (10%). Similarly, the frequencies of hydrophobic residues at positions P4, P5, and P9 (26, 27, and 28%, respectively) were lower than those in β-casein (37%). In contrast, hydrophilic residues at position P6, hydrophobic residues at position P9, and basic residues at position P1 were encountered slightly more frequently than they were in β-casein (40, 52, and 12%, compared to 37, 48, and 10%, respectively).

**DISCUSSION**

The use of an autolysin-defective derivative of *L. lactis* MG1363 carrying the protease genes of *L. lactis* SK11 made it possible to study the action of the cell-anchored lactocepin III towards β-casein. More than 100 different oligopeptides released from β-casein were identified, whereas no di- or tri-peptides could be detected. Such a high number of peptides suggests broad specificity of the enzyme, which was confirmed by the analysis of the proximity of the cleaved bonds. Only particular rules could be identified (positive influence of specific residues at positions P6, P3, P1, and P9 during cleavage), which made it possible to explain only 38% of the observed cleavage sites.

On-line coupling of LC to MS resulted in identification of a considerably larger number of peptides than previously reported (38). This is in fact not surprising, since previously identification was performed by N-terminal sequencing of peptides. Such an approach requires pure peptides, and that is the reason why the HPLC traces differed when these two forms of lactocepin III were used.
acid composition of these peptides (i.e., a large excess of Glu, Gln, and Leu).

An alternative explanation could be the presence of another surface-associated protease, since at least one other protease is located on the cell surface of L. lactis (35). Such a protease would not act on casein, since the lactocepin-negative strain did not release any peptide from β-casein. Thus, this protease should act synergistically with lactocepin, by further degrading the peptides released by the lactocepin. This hypothesis is not consistent with (i) the fact that the pool of peptides released by the autoproteolyzed lactocepin is not modified when it is subjected to the action of a lactocepin-negative strain and (ii) the fact that the secreted lactocepin produced an HPLC trace identical to that obtained with the cell-anchored enzyme. Altogether, these arguments rule out the hypothesis that another cell proteinase explains the differences observed between the cell-anchored lactocepin and the autoproteolyzed lactocepin.

The only remaining explanation for the differences is that autoproteolysis of lactocepin III affects its specificity towards β-casein. As a matter of fact, autoproteolyzed lactocepin III has been reported to release Gln19-Val209 as one of the major peptide products (38). The Tyr193-Gln194 peptide bond was not cleaved by the cell-anchored lactocepin III. Seven other peptide bonds were found to be cleaved by the autoproteolyzed form of lactocepin III, but they were not hydrolyzed by the lactocepin anchored to the cell; these bonds were the Leu16-phospho Ser17, Leu25-Val209, Tyr48-Pro59, Ile67-Pro75, Trp143-Pro144, Met184-His185-Gln186, and Ser196-Lys198 bonds (36). These observations clearly indicate that there is a difference in specificity between the two forms of the enzyme (i.e., cell-anchored lactocepin III and autoproteolyzed lactocepin III).

The model proposed for autoproteolysis of lactocepin involves a change in the conformation of the enzyme in the absence of calcium. This change results in exposure of an as-yet-unidentified autoproteolysis site, which is masked in the presence of calcium (6). According to Bruinenberg et al. (1), the autoproteolysis site is most likely located in the B domain of the protein, about 500 amino acids from the C-terminal anchor. Interestingly, the secreted form of lactocepin III, which displayed specificity towards β-casein identical to that of the cell-anchored form, lacks the last 311 C-terminal residues (45). Therefore, the data strongly suggest that (i) the specificity of the cell-anchored form of lactocepin III does not depend on an interaction of the enzyme with the cell wall and (ii) the amino acids involved in the change in specificity are located in this deleted region. It is worth noting that the C-terminal region has been reported to play an important role in the stability of the enzyme, as it contains a calcium-binding site (1, 39). On the other hand, a comparison of the data of Juillard et al. (19) and Kunji et al. (21) clearly indicates that both cell-anchored and autoproteolyzed forms of lactocepin I release similar peptides from β-casein. The last 311 C-terminal residues of lactocepin III, which are apparently involved in the change in specificity of the enzyme, exhibit some amino acid variations compared to the C-terminal part of lactocepin I, namely, the presence of a duplicate 60-residue region in lactocepin III and seven amino acid substitutions (4, 20). Lactocepin engineering should make it possible to identify which of these differences between lactocepins I and III are involved in the change in specificity of lactocepin III during autoproteolysis.

ACKNOWLEDGMENTS

We thank J.-C. Huet for LC-MS analyses, D. Le Bars for amino acid analyses, B. Poolman for providing L. lactis GF100, and W. de Vos for providing plasmids pNZ521 and pNZ511.

REFERENCES


