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Autoproteolysis of the Extracellular Serine Proteinase of *Lactococcus lactis* subsp. *cremoris* Wg2

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The molecular masses of purified extracellular serine proteinase of a number of *Lactococcus lactis* strains vary significantly, and these molecular mass values do not correspond to the values estimated on the basis of genetic data. The discrepancies can only partially be explained by N-terminal processing during maturation of the precursor enzyme and by C-terminal cleaving during the release from the cell envelope. With a monoclonal antibody that binds in the active site region of the *L. lactis* proteinase, the processing of the released proteinase was followed. At 30°C the proteinase was degraded with a concomitant loss of β-casein hydrolytic activity. In the presence of $CaCl_2$, proteinase degradation was inhibited, and new degradation products were detected. The specific serine proteinase inhibitors phenylmethylsulfonyl fluoride and diisopropylfluorophosphate also inhibited proteinase degradation. Two major high-molecular-mass proteinase fragments (165 and 90 kDa) were found to have the same N-terminal amino acid sequence as the mature proteinase, i.e., [Asp-1–Ala-2–Lys-3–Ala-4–Asn-5–Ser-6, indicating that both fragments were formed by cleavage at the C terminus. The N terminus of a proteinase fragment with low molecular mass (58 kDa) started with Gln-215. In this fragment part of the active site region was eliminated, suggesting that it is proteolytically inactive. Unlike larger fragments, this 58-kDa fragment remained intact after prolonged incubations. These results indicate that autoproteolysis of the *L. lactis* subsp. *cremoris* Wg2 proteinase ultimately leads to inactivation of the proteinase by deletion of the active site region.

Lactococci are important organisms in starter cultures used in cheese manufacture. A dominant species in Dutch cheese starters is *Lactococcus lactis*. Growth of *L. lactis* depends on the availability of essential amino acids, which can be taken up by the cells as small peptides or as free amino acids (for reviews, see references 11 and 18). In milk these nutrients are present at low concentrations, and growth of *L. lactis* depends on a proteolytic system to achieve hydrolysis of casein. The proteolytic system of *L. lactis* consists of a cell envelope-associated extracellular serine proteinase, a number of (extracellular and intracellular) peptidases, and transport proteins which facilitate the uptake of peptides and free amino acids.

The initiation of the breakdown of casein occurs via the extracellular serine proteinase. Biochemical and genetic data show that the proteinases of different *L. lactis* strains are very similar and that the genes are highly homologous (8, 17). Furthermore, the lactococcal proteinases show remarkable homology to the serine proteinases of the subtilisin family (7, 10, 22).

The molecular weight of the purified proteinase of a number of *L. lactis* strains was shown to vary between 80,000 and 140,000, while the gene encodes for a protein of 200 kDa, with the exception of strain SK11, which encodes for a 206-kDa proteinase (8, 17). The decrease in molecular weight can partially be explained by N-terminal processing of the precursor enzyme molecule. Cleavage of the signal sequence during export and cleavage of the prosequence in the presence of the *prtM* gene product results in a 20-kDa reduction of the molecular mass of the proteinase (4, 10, 23). These degradation steps have been visualized on immunoblots by the use of monoclonal antibodies directed against the proteinase of *L. lactis* subsp. *cremoris* Wg2 (16). It was shown that the proteinase when attached to the cells has a molecular weight of 180,000, which is compatible with the molecular mass calculated for the mature proteinase. Further reduction of the molecular weight occurs during the isolation and purification of the proteinase. During the isolation of the *L. lactis* proteinase, the enzyme is released by (repeated) incubation of the cells in a Ca^{2+}-free (or EDTA-containing) buffer (15). The proteinase release from the cells involves an autoproteolytic cleavage of the C-terminal membrane anchor region. The largest proteinase that could be detected after release was 165 kDa, which was still larger than the usually purified enzyme. Similar discrepancies were found for the proteinase of *L. lactis* subsp. *lactis* NCDO763 and *L. lactis* subsp. *cremoris* SK11 (7, 20, 22).

To study the proteinase in more detail, purification is required in order to rule out effects from other cell components. During purification the composition of the released proteinase changed, and proteinase derivatives were formed with other proteolytic characteristics than the native enzyme. This paper describes the changes which take place during the purification of the released proteinase fraction of *L. lactis* subsp. *cremoris* Wg2. These changes result in a decline of the caseinolytic activity. The effect of inhibiting compounds on the degradation of proteinases was also studied. The proteinase degradation was followed with monoclonal antibody Wg2-1 (16). Because of their homogeneity and unique specificity, these monoclonal antibodies can provide information about the presence of their epitopes. Monoclonal antibody Wg2-1 binds to a region near the Ser-433 residue of the active site of the *L. lactis* proteinase and this antibody has been used to provide information about fragments which include (part of) the active site region (14). With the monoclonal antibodies (16) the proteinase derivatives can be identified specifically in crude preparations.

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MATERIALS AND METHODS

Bacterial strains and media. *L. lactis* subsp. *cremoris* Wg2 was routinely maintained in 10% (wt/vol) sterile reconstituted skim milk and stored at −20°C. *L. lactis* was grown at 29°C in MRS broth (1) supplemented with 15 mM $CaCl_2$, 1% lactose (wt/vol), and with other modifications as previously described (6). The pH of the culture was controlled at 5.9. The organisms were harvested when the culture reached the stationary phase of growth.

Proteinase isolation. The proteinase of *L. lactis* subsp. *cremoris* Wg2 was isolated as described previously (15). First, the cells were washed twice in 50 mM Tris hydrochloride (pH 7.8) containing 15 mM $CaCl_2$, and subsequently suspended in 50 mM Tris hydrochloride (pH 7.8)–15 mM EDTA. The volume of this incubation buffer was 1/10 of the culture volume. After 25 min of incubation at 29°C, the proteinase-containing supernatant was separated from the cells by centrifugation. These samples were used to monitor proteinase degradation. To stop this process and for the analysis on immunoblots, the supernatant was precipitated and concentrated 10-fold by perchloric acid precipitation. Perchloric acid was added to a final concentration of 10% (vol/vol) and the mixture was stored on ice for 20 min. After 10 min of centrifugation (12,000 × *g*), precipitated material was dissolved in 100 mM Tris hydrochloride (pH 7.8). Samples of 5 μl were applied to the gel.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western (RNA) blotting were performed as described previously (12, 15, 18). After SDS-PAGE, protein was transferred to Immobilon-P polyvinylidene difluoride transfer membranes (Millipore Corp., Bedford, Mass.). Proteinase on the blot was detected with biotinylated monoclonal antibody Wg2-1 (16). The monoclonal antibodies Wg2-1 were biotinylated by using *N*-hydroxysuccinimidobiotin (Pierce, Rockford, Ill.) as described by the manufacturer. Bound monoclonal antibodies were visualized by incubation with alkaline phosphatase coupled to streptavidin (Boehringer GmbH, Mannheim, Germany), and reaction with 150 μg of 5-bromo-4-chloro-3-indolylphosphate per ml and 300 μg of nitroblue tetrazolium (Sigma) per ml in 50 mM carbonate (pH 9.8)–1 mM $MgCl_2$.

Proteolytic activity measurements. Proteinase activity was measured as described before with fluorescamine-labeled β-casein (0.1% [wt/vol] β-casein) as the substrate in 30 mM sodium 2-(*N*-morpholine)ethanesulfonic acid (MES; pH 6.5) (16, 21). Standard assays were performed at 30°C for 60 min.

Amino acid sequence determinations. Proteins blotted to polyvinylidene difluoride membranes from SDS-slab gels were visualized by staining with Coomassie blue R250; the appropriate bands were cut out and used for sequence determination (19). Automated amino-terminal sequence determinations were performed in a gas-phase sequencer (470 A; Applied Biosystems, Foster City, Calif.) connected online with a 120 A PTH analyzer.

RESULTS

Immunodetection and degradation of the released proteinase. The cell envelope-associated proteinase was released by incubating the cells in an EDTA-containing buffer (15). After Western blotting and immunodetection with monoclonal antibody Wg2-1, seven proteinase fragments could be identified in the released proteinase preparation (Fig. 1, lane 1). The molecular masses of these fragments as judged by SDS-PAGE were 165, 130, 110, 90, 73, 68, and 58 kDa. During incubation the composition of the released proteinase fraction changed, and the proteolytic activity towards β-casein decreased. To study the changes in the composition of the released proteinase fraction, samples were analyzed by immunodetection after 0, 6, 24, 48, and 96 h of incubation at 30°C (Fig. 1, lanes 1 to 5), and by determination of the caseinolytic activity (Fig. 2).

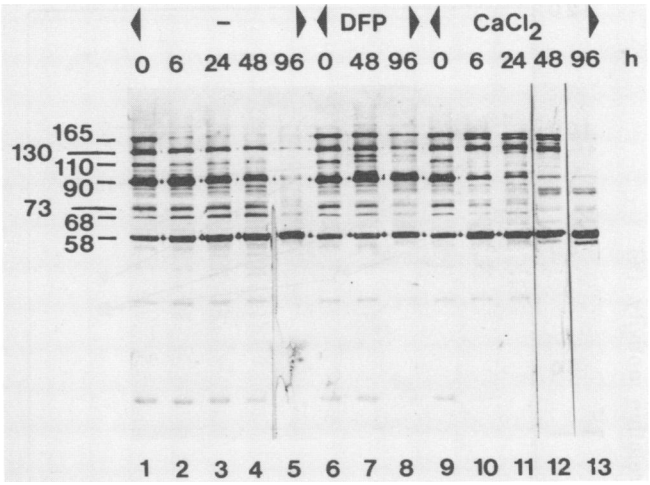

FIG. 1. Western blot of the released proteinase fraction of *L. lactis* subsp. *cremoris* Wg2 after immunodetection with monoclonal antibody Wg2-1. The released proteinase fraction (lane 1) was incubated at 30°C (lanes 1 to 5), in the presence of 5 mM DFP (lanes 6 to 8), or in the presence of 30 mM $CaCl_2$ (lanes 9 to 13). The incubation times of the different samples are indicated in the margin at the top. The respective molecular masses of the proteinase fragments are indicated (in kilodaltons) in the margin at the left.

The intensity of a number of fragments on Western immunoblots decreased or disappeared during incubation, indicating that these fragments were degraded. The relative intensities of the proteinase fragments p165 and p110 decreased significantly within 6 h and were hardly detectable after 48 h. (The different proteinase products are indicated by p plus the number which corresponds to the apparent molecular mass in kilodaltons.) The intensities of p130 and p90 also decreased, but more slowly than p165 and p110. After 96 h, the fragments were still visible, although very faint. On the other hand, the relative intensities of p73 and p68 increased in the first 24 h, but decreased after more than 48 h of incubation. This indicates that after prolonged incubations the breakdown of these proteinase fragments was faster than the formation. During the whole incubation time the intensity of proteinase fragment p58 became more and more dominant. After 96 h it was the strongest band detectable in the sample.

The hydrolytic activity on β-casein of the proteinase fraction was monitored during the incubation. No casein hydrolysis could be detected after 6 h of incubation of the released proteinase fraction (Fig. 1, lane 2). Actually, after 25 min, the fraction was inactivated by 50% (Fig. 2). This suggests that the proteinase fragments p130 and p90 and the bands below 90 kDa (Fig. 1, lane 2) are not any more proteolytically active towards casein.

Effects of proteinase inhibitors. The effects of the specific serine proteinase inhibitors phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) on the degradation of the *L. lactis* subsp. *cremoris* Wg2 proteinase was

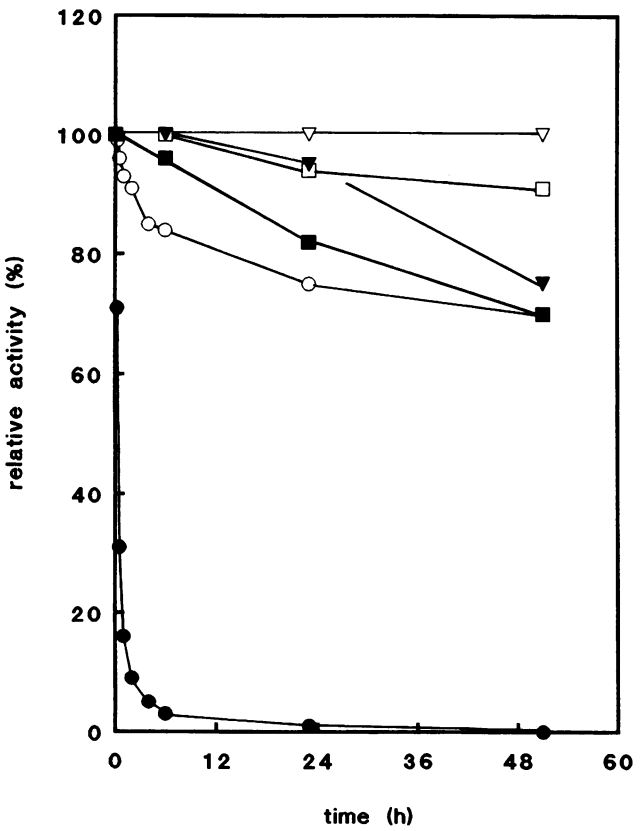

FIG. 2. Changes in the relative caseinolytic activities of the released proteinase fraction of *L. lactis* subsp. *cremoris* Wg2. The fractions were incubated in the presence (open symbols) or in the absence (closed symbols) of 30 mM $CaCl_2$ at 4 (▽, ▼), 15 (□, ■), and 30°C (○, ●).

studied. The proteinase fraction was incubated with 5 mM of either inhibitor. In the presence of DFP the degradation pattern as visualized on the immunoblots did not change significantly within 48 h of incubation at 30°C, except for the appearance of a 120-kDa proteinase fragment which was not observed in the control (Fig. 1, lanes 6 to 8). A slight decrease of all the intensities of all bands except for p58 was detected after 96 h of incubation. These observations indicated that the degradation of proteinase was strongly inhibited by DFP. Similar observations were made with 5 mM PMSF (data not shown).

Effects of $CaCl_2$ and temperature on proteinase degradation. The *L. lactis* proteinase was incubated in the presence or absence of 30 mM $CaCl_2$ at 4, 15, and 30°C, respectively. Incubation at 30°C in the presence of $CaCl_2$ resulted in the formation of the following new proteinase fragments: within 6 h, p160; 24 h, p125 and p55; and 48 h, p80 (Fig. 1, lanes 9 to 13). Another remarkable difference observed in the presence of $CaCl_2$ was a delayed decline of p165 and p130. Both were still detectable after 48 h of incubation. On the other hand, the intensities of the fragments p110, p90, p73, and p68 remained very low even after 6 h of incubation (Fig. 1, lane 10). Interestingly, a strong increase of p58 and p55 was observed. After 96 h of incubation, all bands of the proteinase fragments were very faint except for those of p80, p58, and p55. The effects of incubation temperature on the proteinase degradation were determined in the presence and absence of $CaCl_2$. At lower temperatures the changes in the proteinase patterns on immunoblots were the same as detected at 30°C, but the changes occurred after longer incubation times (data not shown), indicating that the degradation rate was decreased.

In the presence of 30 mM $CaCl_2$ the caseinolytic activity of the proteinase decreased much more slowly than in the control (Fig. 2). A further increase of the $CaCl_2$ concentration had no further effect (data not shown). The caseinolytic activities decreased more slowly at lower temperatures (Fig. 2). At 15°C the activity decreased only by 29%, and at 4°C if decreased by 25% after 48 h. These data indicate that $CaCl_2$ and lower temperatures prevent the loss of caseinolytic activity.

N-terminal amino acid determination. The N-terminal amino acid residues of the proteinase fragments p165, p90, and p58 were determined. The first six N-terminal amino acid residues of the 165- and 90-kDa fragments were Asp-Ala-Lys-Ala-Asn-Ser, which is the same N terminus as in the mature protein. The N terminus of p58 was found to be Gln-Asp-Asn-Glu-Met-Val, which indicates that the proteinase was cleaved between residues Leu-214 and Gln-215. This cleavage site is located between the His-94 and Ser-433 residues of the active site of the lactococcal proteinase (Fig. 3).

DISCUSSION

In this paper it is demonstrated that the *L. lactis* proteinase is (further) degraded after release from the cell envelope, which leads to significant changes in the enzyme preparation. During the purification of the proteinase these changes result in the isolation of a proteinase derivative with a

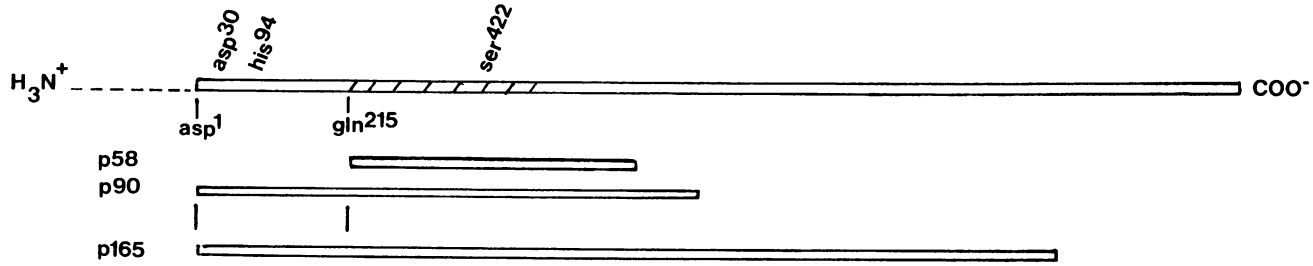

FIG. 3. Schematic representation of the mature *L. lactis* proteinase and the locations of three identified degradation fragments, viz., p165, p90, and p58. The antigen-binding region of monoclonal antibody Wg2-1 (▨) is indicated. Asp-217, His-281, and Ser-620 are the amino acid residues of the active site. The numbering of the amino acid residues is changed compared with the numbering of Kok et al. (10); in this paper the N-terminal amino acid of the mature proteinase molecule is amino acid residue 1 (Asp-1).

significantly lower molecular weight than the mature (released) enzyme. Controversial data for the molecular weight of the *L. lactis* proteinase revealed by genetic and biochemical studies can be explained by proteolysis.

The observation that serine proteinase inhibitors DFP and PMSF both inhibited the degradation of the *L. lactis* proteinase strongly implies that the proteinase breakdown is an autoproteolytic event. Moreover, after inactivating the proteinase by substitution of the active site residue Asp-30 into Asn, no proteinase fragments below 165 kDa could be detected (5). DFP (and PMSF) did not inhibit autoproteolytic activity totally, nor did these inhibitors completely inhibit caseinolytic activity (2). Probably the remaining very low activities are sufficient for slow autoproteolysis. These inhibitors are not suitable for application in a purification protocol to isolate active and intact proteinase, since the inhibition of the proteinase is irreversible.

An autoproteolytic mechanism was previously reported for the release of the proteinase from the cell envelope, and this activity continues after the release (15). Autoproteolysis of the *L. lactis* proteinase apparently occurs at a number of specific cleavage sites in the molecule, resulting in a specific degradation pattern as visualized by immunodetection with monoclonal antibody Wg2-1 (Fig. 1). The decrease of proteinase fragments with a relatively high molecular weight was accompanied by a decrease in the caseinolytic activity. In the absence of $CaCl_2$, p165 and p110 were degraded rapidly (Fig. 1) with a concomitant decrease of the caseinolytic activity (Fig. 2). In the presence of $CaCl_2$ this degradation of p165 and the decline of the caseinolytic activity was much slower, suggesting a significant role of p165 in the overall activity for casein hydrolysis. The N terminus of this fragment was the same as that of the mature *L. lactis* proteinase, indicating that degradation occurred at the C terminus (15). The results also indicate that caseinolytic activities of the other detected fragments are very low. This could be due to either the lack of the active site or the lack of a substrate binding site. For p90 the N terminus was found to be intact, suggesting that in this fragment a large part of the C terminus was deleted.

The increase of the low-molecular-mass fragments p73 and p68, together with the decrease of the fragments above 90 kDa, suggests that they are derived from high(er)-molecular-mass degradation fragments (Fig. 1). In the end, autoproteolysis leads to the inactivation of the proteinase. An example of such an end-product is p58. It is formed after hydrolysis of the peptide bond between Leu-214 and Gln-215, resulting in a deleted active site region (Fig. 3). In the absence of other proteolytic activity the component is stable for prolonged periods of incubation (Fig. 1, lanes 5 and 13). Other fragments—not detectable with this antibody—can also be formed as end-products of the degradation process.

High concentrations of $CaCl_2$ and lower temperature dramatically inhibited the decline of the caseinolytic activity (Fig. 2). The caseinolytic activity of the proteinase could also be stabilized by $MgCl_2$ (13). This stabilizing effect of Ca^{2+} has also been reported for the proteinases of *L. lactis* subsp. *cremoris* HP and AC1. Ca^{2+} concentrations of less than 5 mM have been found to stimulate the caseinolytic activity of the proteinases of *L. lactis* subsp. *cremoris* HP, AC1, Wg2, and subsp. *lactis* NCDO 763 (2, 3, 15, 20). Higher Ca^{2+} concentrations partially inhibit the activity. Ca^{2+} (and other divalent cations) can play a significant role in the conformation of the *L. lactis* proteinase. The role of Ca^{2+} in relation to the conformation of the *L. lactis* proteinase has been discussed previously (9, 15). It has been suggested that Ca^{2+} can affect the conformation in such a way that autoproteolytic release from the cell envelope is prevented. In the presence of Ca^{2+} the conformation is possibly more rigid than in its absence. This can result in a decreased interaction with β-casein and to a higher extent with itself. Furthermore, the more rigid and possibly altered conformation can unmask other cleaving sites, which can change the degradation pattern. The proteinase degradation products p160, p125, p80, and p55 appeared only in the presence of Ca^{2+}, which indicates that the autoproteolytic proteinase specificity is modified by these ions. The inhibited breakdown of p165 and p130 and the accelerated degradation of p110 and p95 in the presence of Ca^{2+} can also be the results of an altered specificity.

Autoproteolytic breakdown of the *L. lactis* proteinase occurs after release from the cells. During purification of the *L. lactis* proteinase, autodegradation is the main reason for loss of enzyme activity. The proteinase degradation can be inhibited by the irreversible serine proteinase inhibitors DFP and PMSF and is partially inhibited by Ca^{2+} and low temperatures.

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