Partial Isolation and Degradation of Caseins by Cell Wall Proteinase(s) of Streptococcus cremoris HP

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The cell wall proteinase fraction of Streptococcus cremoris HP has been isolated. This preparation did not exhibit any activity due to either specific peptidases known to be located near the outside surface of and in the membrane or intracellular proteolytic enzymes. By using thin-layer chromatography for the detection of relatively small hydrolysis products which remain soluble at pH 4.6, it was shown that β-casein is preferentially attacked by the cell wall proteinase. This was also the case when whole casein or micelles were used as the substrate. κ-casein hydrolysis is a relatively slow process, and α₁-casein degradation appeared to proceed at an extremely low rate. These results could be confirmed by using ¹⁴CH₃-labeled caseins. A relatively fast and linear initial process of ¹⁴CH₃-labeled β-casein degradation is not inhibited by α₁-casein and only slightly by κ-casein at concentrations of these components which reflect their stoichiometry in the micelles. Possible implications of β-casein degradation for growth of the organism in milk are discussed.

MATERIALS AND METHODS

Growth and harvesting of the organism. Stationary-phase cells of S. cremoris HP were obtained from milk-grown cultures as described previously (8).

Preparation of a crude cell wall proteinase fraction. Cells from 12.5 liters of milk culture were washed twice with 0.05 M sodium acetate–0.05 M sodium dihydrogen phosphate buffer (pH 6.5). Care was taken to keep the temperature during these washings between 0 and 4°C to prevent the release of proteinase activity (13). After these washings the cells were resuspended in the same buffer at 25°C, stirred gently for 30 min, and then centrifuged (10 min, 16,000 × g). Resuspension of the cells was repeated several times. The supernatants were pooled, adjusted to pH 5.4, and dialyzed overnight against an excess of distilled water. The powder obtained after freeze-drying was extracted with distilled water, and the precipitate was discarded. The extract was freeze-dried again (crude cell wall proteinase fraction, 56 mg of protein) and dissolved in 10 ml of 0.05 M ammonium acetate buffer (pH 5.4).

Sephacryl S300 column fractionation. Portions of 2.5 ml of crude cell wall proteinase were applied to a Sephacryl S300 column (30 by 2.5 cm) equilibrated with 0.05 M ammonium acetate (pH 5.4), and elution was performed with the same buffer at a rate of 0.65 ml·min⁻¹. Fractions (5 ml) were collected. Those fractions indicated as proteolytic active were pooled, dialyzed overnight, and freeze-dried, and the powder was again extracted with 5 ml of water. The final degradation of partially purified cell wall proteinase in water contained 0.5 mg of protein · ml⁻¹.

Degradation of ¹⁴CH₃ casein. The purified cell wall proteinase fraction was used to study the breakdown of ¹⁴CH₃-labeled whole casein (specific activity, ca. 34,000 cpm · mg⁻¹) or ¹⁴CH₃-labeled β-casein (specific activity, 37,000 cpm · mg⁻¹) by following the release of trichloroacetic acid (TCA) (6% [wt/vol])-soluble products, essentially according to the method described earlier (5).

Degradation of micelles, whole casein, and the casein components. Solutions of whole casein (0.2%, [wt/vol]) or α₁-, β-, and κ-casein (0.1%, [wt/vol]) in 0.05 M ammonium acetate (5 ml) or a suspension of micelles in the same buffer (5 ml) supplemented with 10 mM Ca²⁺ were incubated with 100 μl

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spray consisting of 0.4% (wt/vol) ninhydrin in n-butanol-acetic acid (96:4 [vol/vol]).

Isolation of micelles and caseins. Caseins were isolated according to generally accepted methods (see reference 12). For the isolation of micelles, skim milk was centrifuged for 90 min at 100,000 x g and 15°C. The micelles were suspended in 0.05 M ammonium acetate buffer (pH 6.2) supplemented with 10 mM CaCl₂ by means of a Potter-Elvehjem tube. After a second centrifugation, the micelles were resuspended in the same buffer at a concentration equal to the micelle concentration in milk.

Gel electrophoresis and isoelectrofocusing. Sodium dodecyl sulfate–8.5% polyacrylamide gel electrophoresis (imidazole buffer, pH 7.0) and analytical isoelectrofocusing (pH 4 to 6) were performed essentially according to the instructions of LKB-Produkter AB, Bromma, Sweden (application notes 306 and 250, respectively) by using the LKB 2117 multiphor.

Protein quantification. Proteins were estimated according to the method of Bradford (2) by using crystalline bovine serum albumin (fraction V; BDH, Poole, England) as the standard.

RESULTS

Partial purification of the cell wall proteases. During the incubation of cells of S. cremoris HP at pH 6.5 and 25°C, proteinase activity (P₁ + P₂) (5, 6) appeared in the supernatant mainly during the first 30 min (Fig. 1a). The reduction in activity measured with the collected cells was much smaller than the activity appearing in the supernatant. On the one hand this may be due to the variety of the peptide bonds that are split in whole casein, which gives rise to nonlinear

FIG. 1. Release of proteinase activity from the cell wall of S. cremoris HP (a) and TR (b). Cold (0 to 4°C) collected cells were suspended in 0.05 M sodium acetate–0.05 M sodium dihydrogen phosphate buffer (pH 6.5) at 25°C. After the indicated time intervals, a sample (0.5 ml) of the cell suspension was centrifuged (10 min: 12,000 x g), and the supernatant (C) and cells (O) were resuspended in cold (0 to 4°C) buffer at pH 5.4 (0.5 ml) and tested for proteinase activity at pH 5.4 and 30°C (5). After 10 and 60 min of incubation, collected cells were resuspended in buffer at pH 6.5 and 25°C, and the proteinase activity in the supernatant (o, △) and that associated with the cells (■, ▼) was determined after 10 min of incubation. Proteinase activities are expressed as cpm·ml⁻¹ detectable after 20 min of incubation with the substrate ¹⁴CH₃-labeled whole casein.

(50 μg of protein) of the partially purified cell wall proteinase preparation at pH 6.2 and 30°C. Samples (0.5 ml) were withdrawn from the incubation mixture at the indicated time intervals and adjusted to pH 4.6 with 10 μl of an acetic acid solution to precipitate casein and the larger hydrolysis products. The supernatant was freeze-dried and dissolved in 25 or 50 μl of distilled water. Equal amounts of each of the samples were applied to thin-layer plates (Kieselgel 40 F254; E. Merck AG, Darmstadt, Federal Republic of Germany) and developed with n-butanol-acetic acid–water (24:4:8 [vol/vol]). The products were made visible with a ninhydrin

FIG. 2. Gel filtration of crude cell wall proteinase (±15 μg of protein) of Sephacryl S300. The A₂₈₀ was recorded. For details see the text. Proteinase activity (O) was assayed as described (5) and expressed as cpm·ml⁻¹ after 20 min of incubation with the substrate ¹⁴CH₃-labeled whole casein. The arrow marks the void volume.
progress curves (see below), and on the other hand it may be due to accessibility of the proteinase in situ. Continued incubation resulted in a further decrease of activity detectable with the cells, without any increase of proteinase activity in the supernatant being detected. No reduction in proteinase activity of these cells upon continued incubation was detected when they were first resuspended in fresh buffer at pH 5.4. At this pH the permeability of the cell wall for the proteinase is assumed to be reduced almost completely (13). Since all activity determinations were also performed at a pH of 5.4, the activity measured with cells is not due to additional released enzyme.

Cells collected after 10 or 60 min and resuspended in fresh buffer at pH 6.5 and 25°C showed a rapid additional release of activity. This procedure can be repeated with similar results until the cell wall is exhausted. Such a cascade-like release of proteinase activity was observed with all strains studied so far, including those belonging to different types with respect to their cell wall proteolytic system (6). No lysis of cells, as measured by the release of glucose-6-phosphate dehydrogenase (8), could be detected during the whole wash procedure. Some strains showed only a poor release of proteolytic activity. Cells of such strains showed no or only slight additional reduction of proteolytic activity after maximum values in the supernatant had been reached (Fig. 1b).

A typical elution profile obtained with the crude cell wall proteinase fraction loaded on a Sephacryl S300 column is shown in Fig. 2. Both proteinase activities P_1 and P_2, detectable in this strain HP, were found in the same fractions. None of the peptidase activities known to be located near the outside surface or in the membrane, and which contaminated the crude cell wall fractions at detectable levels, could be detected in the pooled column fractions covered by the indicated bar. The final preparation showed six bands after sodium dodecyl sulfate-polyacrylamide electrophoresis and analytical isoelectrofocusing. It was used to perform the experiments described in the following paragraphs. Under the conditions of these experiments no decrease of proteinase activity could be detected. Further purified preparations of the proteinase appeared to show remarkable increased instability.

Degradation of caseins. α-, β-, and κ-casein, whole casein, and micelles were used as the substrates for cell wall proteinase activity. β-Casein was degraded relatively quickly when compared with α- and κ-casein (Fig. 3a through c and 4b). In fact, the degradation of α-casein proceeded very slowly under the present conditions. The degradation pattern obtained with β-casein within the first 10 to 15 min (and already detectable within a few seconds [Fig. 4a]) changed only gradually over the following period of hours (Fig. 3b). With κ-casein and α-casein a gradual increase of the intensity of the different bands was seen over the whole incubation period. The degradation of whole casein (Fig. 3d and 4b) and micelles (data not shown) is mainly that of β-casein, since initially the degradation products typical of β-casein degradation are produced.

FIG. 3. Appearance of hydrolysis products soluble at pH 4.6 followed over a 4-h period of degradation of α- (a), β- (b), κ- (c), and whole (d) casein by the action of the cell wall proteinase system of S. cremoris HP. Samples were taken after 15 s (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 30 min (lane 5), 60 min (lane 6), 180 min (lane 7), and 240 min (lane 8) of incubation. For details see the text.
Degradation of 14CH3-labeled caseins. A typical relationship between the release of TCA-soluble products from 14CH3-labeled β-casein by the action of the purified cell wall protease and time is shown in Fig. 5. An initially linear progress curve at a relatively high rate was followed by a lower rate of product formation. The initial phase appeared to approximate, independent of the enzyme concentration, a maximum value (viz., ca. 4,000 cpm · ml−1) with a rate higher than that expected for the second phase. In the presence of both αs-casein (0.1% [wt/vol]) and κ-casein (0.025% [wt/vol]), the initial-phase degradation of 14CH3-labeled β-casein was inhibited by only 17%. This was due to the presence of κ-casein, since αs-casein alone had no inhibitory effect, whereas κ-casein at the same concentration (0.1% [wt/vol]) showed a significant inhibition of 14CH3-labeled β-casein degradation of ca. 40%.

Degradation of 14CH3-labeled whole casein also showed an initially rapid, but nonlinear phase of product formation followed by a relatively slow phase (Fig. 6).

DISCUSSION

Studies with respect to proteinases of S. cremoris have been limited until now. More data have been obtained concerning proteinases from S. lactis (for a review see reference 10a).
Umemoto and Itoh (19) described some characteristics of the proteolytic activity of *S. cremoris* H-61, which shows an optimum activity at 60°C. With respect to its identity and its location in situ, the isolation procedure does not warrant the assumption of the authors that the proteolytic activity under consideration is a "cell-surface proteinase," since isolation was started with a cell lysate obtained by salt-induced lysis of lysozyme-treated cells (8). Likewise, the procedure followed by Ohmiya and Sato (15) for the isolation of intracellular proteinase from the same organism may have resulted in the release of cell wall proteinase activity (1, 13).

Our results with respect to the release of proteinase from cell walls of lactic streptococci, as originally described by Mills and Thomas (13), may give some additional information. First, the enzymes apparently diffuse through the cell wall into the medium until an equilibrium has been reached. This explains the cascade-like release. Second, in the case of cells showing a high rate of diffusion of the proteinase(s) through the cell wall (strain HP), a peripheral accumulation of proteinase may occur initially, followed by a redistribution in the cell wall at the moment that the equilibrium has been established. This explains the continuing decrease in detectable activity with such cells (and no decrease or a smaller one with cells of strains like TR). Substrate diffusional limitations, which reduce the effective substrate concentration for an increasing amount of proteinase molecules, will be introduced. At pH 5.4, however, the initial situation will be maintained due to reduced cell wall permeability (13).

β-Casein appears to be the most suitable substrate among the caseins for the cell wall proteinase(s) of *S. cremoris* HP. It is preferentially attacked, apparently also when micelles are offered, although in that case it may well be that degradation of extruded, soluble β-casein has been detected (see reference 17). The same order of preference with respect to caseins is suggested by the results obtained with a proteinase present in an extract of *S. cremoris* H-61 (15). However, the kinetic data presented do not give any substantial information due to the possibility of significant differences in progress curves, especially the initial velocities.

The results obtained with 14CH3-labeled caseins reflect the visualized initial degradation process remarkably well. A rapid initial degradation of β-casein is the most likely main process involved in the occurrence of the initial rapid phase of TCA-soluble product formation observed during the degradation of 14CH3-labeled whole casein. In the presence of the other caseins, first-phase degradation of β-casein is inhibited, but the effect is small at the relative concentrations present in whole casein.

Products of β-casein released during the initial phase may be essential both with respect to initiation and continuation of growth of *S. cremoris* HP in milk. This should imply that β-casein alone or in combination with other caseins is a more stimulatory and efficient substrate for growth than any other casein or combination without β-casein. In milk, the possibility of preferential β-casein utilization for optimal growth without limitation due to substrate accessibility and without severe competitive interference of other caseins exists. Growth may be mainly supported by degradation of extruded soluble β-casein (3, 16), leaving micellar casein unattacked. Since soluble β-casein is in equilibrium with micellar β-casein and additional β-casein is released from the micelles so as to reestablish equilibrium upon removal of soluble β-casein (3), it may well be that during growth of the organism the cells are continuously provided with subsequently delivered soluble β-casein. Preliminary results from growth experiments in synthetic media supplemented with different caseins or with milk fractions strongly support an essential role for soluble (β-)casein (unpublished data).

**LITERATURE CITED**