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Comparative Study of Action of Cell Wall Proteinases from Various Strains of *Streptococcus cremoris* on Bovine α_{s1}-, β-, and κ-Casein

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Partially purified cell wall proteinases of eight strains of *Streptococcus cremoris* were compared in their action on bovine α_{s1}-, β-, and κ-casein, as visualized by starch gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and thin-layer chromatography. Characteristic degradation profiles could be distinguished, from which the occurrence of two proteinases, represented by strain HP and strain AM_1, was concluded. The action of the HP-type proteinase P_I (also detectable in strains Wg_2, C_{13}, and TR) was established by electrophoretic methods to be directed preferentially towards β-casein. The AM_1-type proteinase P_{III} (also detectable in strain SK_{11}) was also able to degrade β-casein, but at the same time split α_{s1}- and κ-casein more extensively than did P_I. Strain FD_{27} exhibited mainly P_I activity but also detectable P_{III} degradation characteristics. The cell wall proteinase preparation of strain E_8 showed low P_I as well as low P_{III} activity. All proteinase preparations produced from κ-casein positively charged degradation products with electrophoretic mobilities similar to those of degradation products released by the action of the milk-clotting enzyme chymosin. The differences between P_I and P_{III} in mode of action, as detected by gel electrophoresis and thin-layer chromatography, were reflected by the courses of the initial degradation of *methyl*-^{14}C-labeled β-casein and by the effect of α_{s1}- plus κ-casein on these degradations. The results are discussed in the light of previous comparative studies of cell wall proteinases in strains of *S. cremoris* and with respect to the growth of this organism in milk.

The cell wall proteolytic system of *Streptococcus cremoris* plays an important role in the initial breakdown of the substrate casein during growth in milk and also during the ripening of cheese, for the manufacture of which the organism is widely used as a starter. By this action on casein, peptides are released which can penetrate the cell wall and reach peptidases situated at the outside surface of or in the cell membrane (1–5, 15). By the action of these peptidases transportable products are formed which serve as a source of nitrogen and (essential) amino acids (11, 15).

Knowledge about the rate and the pathway of casein degradation by *S. cremoris* may answer the question which casein component(s) is, in the absence of free amino acids and peptides, utilized as a nitrogen source for the initiation and continuation of growth in milk.

Previous results concerning casein degradation by the action of the cell wall proteinase of strain HP, as detected by thin-layer chromatography (TLC), indicated a preferential splitting of β-casein. This could be confirmed following the degradation of *methyl*-^{14}C-labeled β-casein in the presence and absence of α_{s1}- and κ-casein (5).

In the present paper we describe experiments in which partially purified cell wall proteinases of eight strains of *S. cremoris*, including strain HP, were compared in their action on α_{s1}-, β-, and κ-casein, as visualized by starch gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and TLC, and also in their action on *methyl*-^{14}C-labeled β-casein.

MATERIALS AND METHODS

Origin of organisms. The strains of *S. cremoris* designated C_{13}, E_8, and HP were originally obtained from the Commonwealth Scientific and Industrial Research Organisation, Australia; AM_1, SK_{11}, and TR originated from the New Zealand Dairy Research Institute; FD_{27} and Wg_2 were from the collection of the Netherlands Institute for Dairy Research.

Growth and harvesting of organisms. Organisms were grown in and harvested from pasteurized milk under conditions reported by Exterkate (4).

Isolation and purification of cell wall proteinases. Purified cell wall proteinases were obtained by repeated extraction of intact cells in the absence of Ca^{2+} and fractionation of the crude enzyme material on a Sephacryl-S300 column as described by Exterkate and De Veer (5). The extent of lysis during treatment of the cells was estimated by measuring the release of the intracellular marker enzyme glucose-6-phosphate dehydrogenase (4). In all cases cell lysis appeared to be absent.

Estimation of proteinase activity. Proteinase activity was estimated by measuring the initial release over a 20-min period of trichloroacetic acid-soluble radioactive products from *methyl*-^{14}C-labeled whole casein (specific activity, 34,000 cpm mg^{-1}) or *methyl*-^{14}C-labeled β-casein (specific activity, 37,000 cpm mg^{-1}) (1). Activity is expresssed as cpm per milliliter per minute.

Preparation of caseins. Whole casein was prepared by isoelectric precipitation from bovine skim milk at pH 4.6. From this, α_{s1}-casein (genetic variant B) was isolated as described by Schmidt and Payens (14). β-Casein (genetic variant A^1/A^2) was obtained essentially by the method of Payens and Heremans (12) from the fraction insoluble in 1.7 M urea, which was solubilized at pH 7, dialyzed against water at 4°C, and then freeze-dried. κ-Casein (genetic variant B) was prepared by the method of McKenzie and Wake (9).

Incubation of caseins with cell wall proteinases. To 800 μl of a 1.5% (wt/vol) solution of the casein in 0.02 M ammonium acetate buffer (pH 6.2), equilibrated at 15°C, 80 μl of the enzyme solution in the same buffer was added (each enzyme

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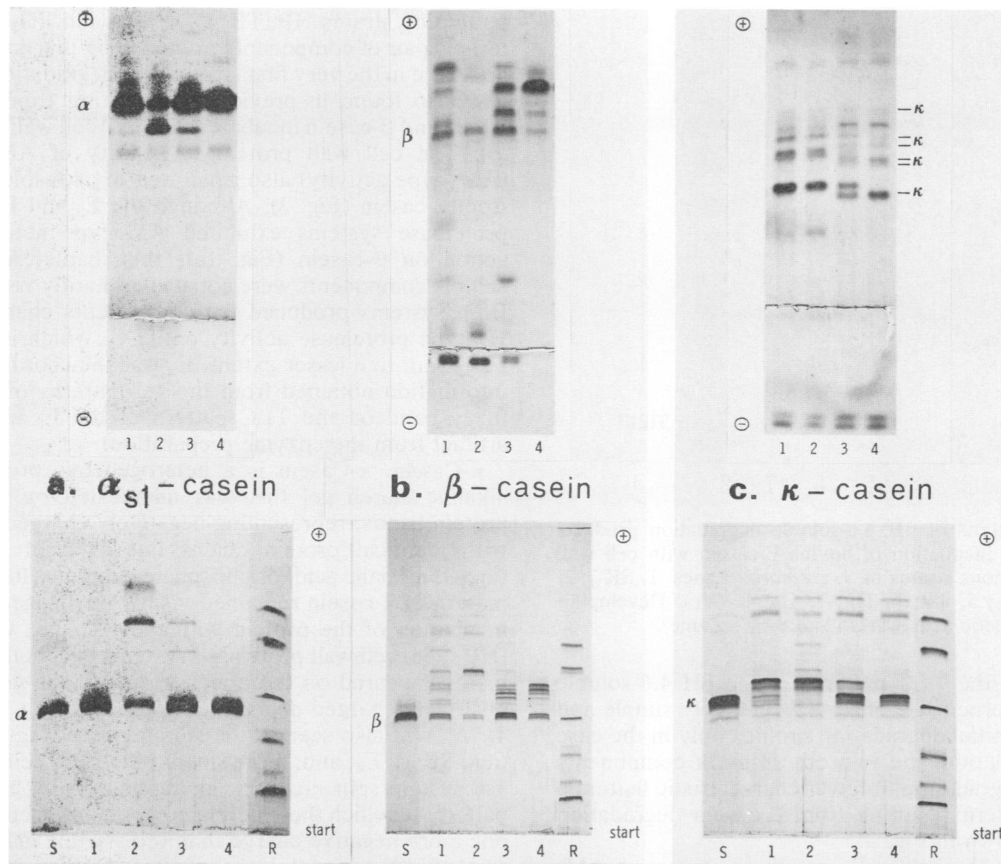

FIG. 1. Gel electrophoretic patterns showing the action of cell wall proteinases from various strains of *S. cremoris* on bovine α_{s1}-casein (a), β-casein (b), and κ-casein (c). Top panels: Alkaline starch gels after 24 h of incubation. Bottom panels: SDS-PAGE gels after 8 h of incubation. The positions of intact casein components are indicated. Lanes: 1, E_8; 2, AM_1; 3, FD_{27}; 4, HP; S, starting substrate; R, mixture of reference proteins consisting of phosphorylase B (M_r 94,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 29,000), soybean trypsin inhibitor (M_r 21,000), and lysozyme (M_r 14,300). In all cases the patterns for degradation by SK_{11} proteinase (not shown) were similar to those shown for AM_1; the patterns for degradation by TR, Wg_2, and C_{13} (not shown) were indistinguishable from those for HP.

stock solution had been standardized to yield an activity of approximately 575 cpm ml^{-1} min^{-1} with *methyl*-^{14}C-labeled whole casein as the substrate). The reaction mixture was incubated at 15°C, and several samples were taken immediately (ca. 1 min) after the start of the incubation and again after 3, 8, and 24 h. Each time, one sample (50 μl) was freeze-dried and subjected to alkaline starch gel electrophoresis; a second sample (10 μl) was freeze-dried and subjected to SDS-PAGE; a third sample (200 μl) was acidified to pH 4.6 with 10 μl of 1 M acetic acid and centrifuged in an Eppendorf centrifuge for 3 min, and the pH 4.6-soluble degradation products after freeze-drying were dissolved in 25 μl of distilled water and subjected to TLC.

Gel electrophoresis and TLC. Urea-starch gel electrophoresis in the presence of 2-mercaptoethanol was performed at pH 8.6 by the method of Schmidt (13). Gels were loaded with 75 μl of a solution containing 0.3% (0- and 3-h incubations), 0.6% (8-h incubation), or 1% (24-h incubation) of the freeze-dried protein material (wt/vol). Protein bands were stained with Amido black 10-B.

SDS-PAGE by the method of Laemmli (8) was carried out on gels (1.5 mm thick) containing 12% acrylamide; a Bio-Rad vertical slab gel electrophoresis cell (model 220) was used. Gels were loaded with 10 μl of a 0.15% (wt/vol) solution of the freeze-dried protein material. After electrophoresis the gels were stained with Coomassie brilliant blue G-250.

TLC of pH 4.6-soluble hydrolysis products was done on ready-to-use silica gel plates (10 by 10 cm; Kieselgel 40 F_{254}; Merck), which were developed either in *n*-butanol–acetic acid–water (24:4:8 by volume) or in *n*-butanol–acetic acid–acetone–water (14:4:10:8 by volume). Bands were made visible by spraying with a ninhydrin reagent (5).

RESULTS

Analysis of casein degradation by gel electrophoresis and TLC. Typical starch and SDS gel electrophoretic patterns of three casein components incubated with the cell wall proteinase fraction of various *S. cremoris* strains are shown in Fig. 1. For space-saving reasons we have deleted the lanes representing degradations by SK_{11} proteinase (identical to those shown for AM_1, lane 2) and by TR, C_{13}, and Wg_2 proteinases (identical to the patterns shown for HP, lane 4). As will be discussed below, E_8 (lane 1) and FD_{27} (lane 3) showed mixed types of proteinase activity. From the results of separate blank experiments (not shown) it was concluded that the degradation patterns could indeed be ascribed to the action of the added enzyme preparations on the various

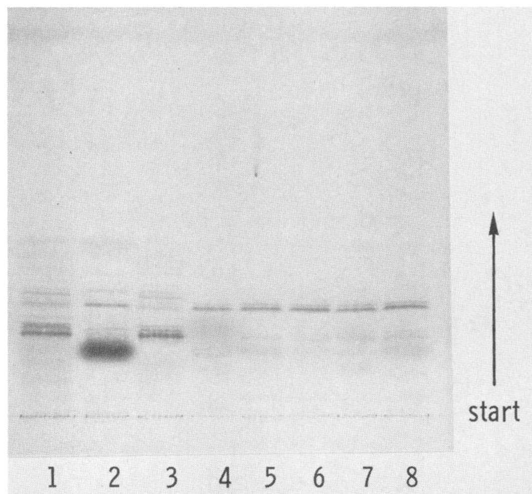

FIG. 2. TLC patterns of pH 4.6-soluble degradation products obtained after a 3-h incubation of bovine β-casein with cell wall proteinases from various strains of *S. cremoris*. Lanes: 1, SK_{11}; 2, E_8; 3, AM_1; 4, FD_{27}; 5, TR; 6, HP; 7, C_{13}; 8, Wg_2. Developing system: *n*-butanol–acetic acid–water (24:4:8 by volume).

caseins. As far as the TLC patterns of the pH 4.6-soluble products are concerned, we observed relatively simple and easily detectable initial degradation profiles only in the case of β-casein degradation, the two other casein components showing slower degradation or fewer characteristic features. The 3-h TLC pattern resulting from β-casein degradation experiments is shown in Fig. 2.

α_{s1}-Casein. With the cell wall proteinase system of AM_1 and SK_{11} an extensive, mutually identical degradation of α_{s1}-casein was evident from the patterns appearing on the starch gels (Fig. 1a, top, lane 2) and on the SDS-PAGE gel (Fig. 1a, bottom, lane 2). The same patterns developed at a lower and much lower rate, respectively, for the action of the cell wall proteinase systems of strains FD_{27} and E_8 (Fig. 1a, lanes 3 and 1, respectively). The identical splitting of α_{s1}-casein by these four strains may be ascribed to a common type of proteinase activity (hereafter called AM_1-type activity) located in the cell wall. The proteinases from strains HP, TR, C_{13}, and Wg_2 did not form breakdown products from α_{s1}-casein, with the exception of a faint (double) band of higher mobility on the starch gel (Fig. 1a, top, lane 4). The weak band of lowest relative mobility of all the samples in the starch gels originated from an artifact in the starting α_{s1}-casein.

β-Casein. The cell wall proteinase systems of AM_1 and SK_{11} (AM_1-type proteinase activity) produced a typical and identical breakdown of β-casein, which on the starch gel (Fig. 1b, top, lane 2) was characterized by a prominent, slightly positively charged component; the proteinase system of E_8 (lane 1) showed a similar (AM_1-type) degradation pattern for β-casein, but at the same time produced some additional components, as was also evident by SDS-PAGE (Fig. 1b, bottom) patterns. In contrast to α_{s1}-casein, β-casein was clearly degraded by the cell wall proteolytic systems of strains HP, TR, C_{13}, and Wg_2 (Fig. 1b, lane 4); this group of strains showed identical specificity of action on β-casein (designated HP-type activity). Strain FD_{27} (Fig. 1b, lane 3) mainly showed HP-type activity, but also exhibited a (relatively weak) AM_1-type proteinase activity. Figure 2 shows the TLC patterns of pH 4.6-soluble degradation products of β-casein. As expected, typical HP-type activity was demonstrated by strains HP, TR, C_{13}, Wg_2, and FD_{27}; the characteristic pair of components produced by this group of strains appeared in the very first stage of the degradation process, as was also found in previous degradation experiments with whole and β-casein incubated with HP cell wall proteinase(s) (5). The cell wall proteinase activity of AM_1 and SK_{11} (AM_1-type activity) also generated pH 4.6-soluble products from β-casein (Fig. 2). Although the E_8 and FD_{27} cell wall proteinase systems exhibited AM_1-type activity in their action on β-casein (Fig. 1b), the characteristic pH 4.6-soluble components were not at all or hardly visible in Fig. 2. Both systems produced the components characteristic for HP-type proteinase activity on TLC, which in the case of FD_{27} and, to a lesser extent, E_8 was in accordance with the information obtained from the gel patterns of Fig. 1b (the fuzzy band on the TLC pattern of the E_8 incubate is an artifact from the enzyme preparation).

κ-Casein. κ-Casein is a heterogeneous protein. On the alkaline starch gel this was manifested by a number of protein bands, representing negatively charged components with identical protein chains but different carbohydrate (including sialic acid) or phosphate contents (10, 17). This gel pattern of κ-casein remained visibly unchanged after a 24-h incubation of the protein with the HP, TR, C_{13}, and Wg_2 (HP-type) cell wall proteinase systems except that additional bands appeared on the opposite side of the gel slot due to positively charged degradation products (Fig. 1c, top, lane 4). As was also seen in the starch gels of Fig. 1c, the AM_1 (and SK_{11}), E_8, and, more slowly, the FD_{27} cell wall proteinase system split κ-casein, giving an equally heterogeneous pattern, of which the different components seem to carry just one more negative charge than the starting material. At the same time these proteinases produced the positively charged components also generated by the other (HP-type) cell wall systems. We found the electrophoretic mobility of these positive components, which on the SDS-PAGE gel (Fig. 1c, bottom) corresponded to the components of highest mobility, to be similar to those observed in the degradation pattern obtained during the splitting of κ-casein by the milk-clotting enzyme chymosin (result not shown). Therefore, apart from the two positively charged components found by incubation with all of the proteinase systems studied, one could also in the case of the κ-casein substrate distinguish between gel patterns obtained with AM_1-type (including AM_1 SK_{11}, E_8, and FD_{27} proteinases) and HP-type (including HP, TR, C_{13}, and Wg_2 proteinases) proteinase activities.

Degradation of *methyl*-^{14}C-labeled β-casein. The foregoing results suggested the presence of a single but common (HP-type) proteinase activity in the cell wall of strains HP, C_{13}, W_{g2}, and TR. Likewise, AM_1 and SK_{11} can be grouped by the fact that these strains exhibited only one (AM_1-type) proteinase activity. Strains E_8 and FD_{27} seem to contain mixed proteinase activity. β-Casein was degraded by both AM_1- and HP-type proteinase activities, but in characteristically different ways. It has already been established that the degradation of *methyl*-^{14}C-labeled β-casein by the cell wall proteinase of strain HP proceeds without the interference of α_{s1}-casein and with only a slight inhibition exerted by κ-casein; a biphasic progress curve typical of *methyl*-^{14}C-labeled β-casein degradation was obtained (5). In the present study similar progress curves were obtained for the action of the cell wall proteinase system of C_{13}, Wg_2, and TR, but also for that of strain FD_{27}. In all cases only a slight effect of α_{s1}- and κ-casein was observed when both were added simultaneously to the incubation mixture in concentrations reflecting the stoichiometry in whole casein. This is illustrated

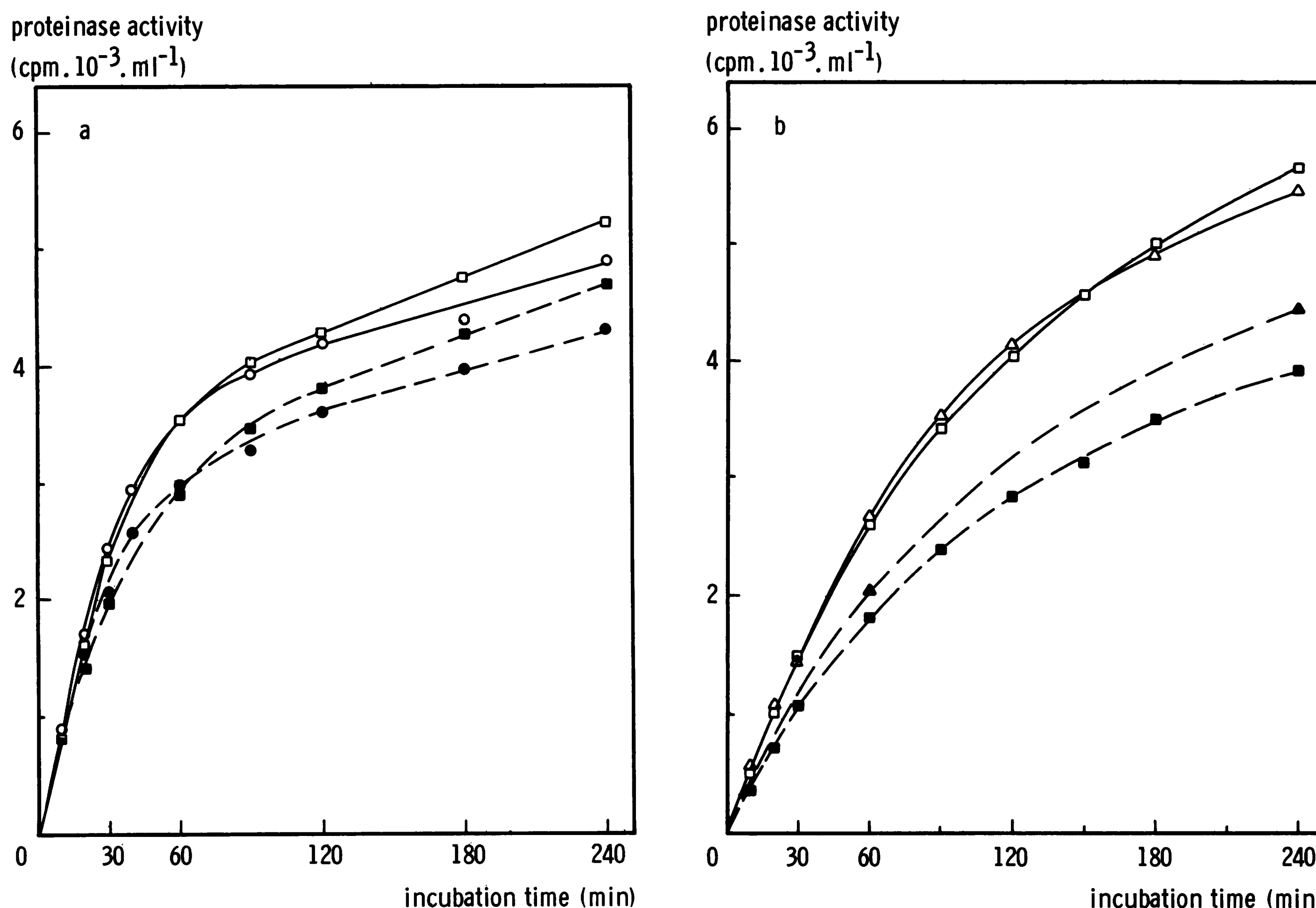

FIG. 3. Appearance of trichloroacetic acid-soluble *methyl*-^{14}C-labeled hydrolysis products during the incubation of *methyl*-^{14}C-labeled β-casein (0.1%, wt/vol) at 30°C and pH 6.2 with cell wall proteinase(s) of different strains in the absence (open symbols) or presence (solid symbols) of α_{s1}-casein (0.1%, wt/vol) and κ-casein (0.025%, wt/vol). (a) Strains Wg_2 (○, ●) and FD_{27} (□, ■); (b) strains AM_1 (□, ■) and E_8 (△, ▲). The initial rates of degradation by the two proteinase systems compared in each figure were the same. The buffer was 0.05 M sodium acetate–0.05 M sodium dihydrogen phosphate.

in Fig. 3a for strains Wg_2 and FD_{27}. The second phase showed a somewhat higher rate of product formation in case of the FD_{27} proteinase system.

methyl-^{14}C-labeled β-casein degradation by the AM_1-type proteinase of strains AM_1 and SK_{11} proceeded in a different way and was significantly inhibited in the presence of α_{s1}- plus κ-casein. Similar results were obtained with the cell wall proteinase system of E_8, although less effect of α_{s1}- plus κ-casein was observed (Fig. 3b).

DISCUSSION

Cell wall proteinases of strains of *S. cremoris*. Highly purified cell wall proteinases appeared to lose their activity rather quickly (5). For this reason we used the present, partially purified but relatively stable proteinase preparations in degradation studies with the various casein components. The results described in this paper allow the differentiation of strains of *S. cremoris* by the specificity of the cell wall-associated proteinase(s) in its action on α_{s1}-, β-, and κ-casein. This differentiation can be brought into agreement with the classification in five different types proposed previously (2) and endorsed later by immunological analysis of the cell wall proteinase systems (6). At least four immunologically different cell wall-associated protein components were detected by crossed-immunoelectrophoresis; each type of strain seems to produce a characteristic combination of these components. The original basis for the classification (2) needs to be adapted to the present results. These results indicate that strains HP, C_{13}, Wg_2 (type 3 in reference 2) and TR (type 4 in reference 2) seem to possess one (HP-type) kind of proteinase activity (= P_I), which is characterized by a preferential action on β-casein as shown by gel electrophoresis (Fig. 1b), resulting in the rapid initial release of relatively small products from this substrate, as judged by TLC (Fig. 2) (5). It has been established that α_{s1}- and κ-casein are less susceptible to this type of proteinase. The apparent neutral proteinase activity (P_{II}) detectable in these strains (1, 2) could be carried back to a different stability of P_I under the different conditions of pH and temperature applied earlier to discriminate between P_I and P_{II}; evidence for this was obtained from experiments with the purified enzyme (Exterkate and De Veer, unpublished data). In strains AM_1 and SK_{11} (type 5 in reference 2), a different (AM_1-type) proteinase activity (= P_{III}) could be detected. This proteinase, unlike P_I, is able to degrade not only β-casein but also α_{s1}- and κ-casein equally well. The difference between P_I and P_{III} in their mode of action as detected by gel electrophoresis and TLC was confirmed by a difference in the courses of the initial degradation of *methyl*-^{14}C-labeled β-casein and the

effect of α_{s1}- plus κ-casein on these degradations. The possibility of the presence in the strains mentioned so far of another proteinase(s) which either remains associated with the cell wall during the isolation procedure or is not present at a detectable level in the final preparation cannot be excluded. In this respect it is possible that strain TR, previously shown to contain P_{III} (2), does not exhibit enough P_{III} activity to be detectable in the present experiments. Unlike TR, the cell wall proteinase preparation of strain FD_{27} (type 4 in reference 2) did show detectable P_{III} activity but exhibited mainly P_I activity, as may be concluded from the α_{s1}-, β-, and κ-casein degradation patterns. P_{III} activity in this strain may be responsible for the somewhat increased rate of the second phase of *methyl*-^{14}C-labeled β-casein degradation compared with that by the action of P_I alone (Fig. 3a). Strain E_8 (type 2 in reference 2) exhibited relatively low P_{III} but also very weak P_I activity. This weak P_I activity may be responsible for the slight deviation of the progress curve of *methyl*-^{14}C-labeled β-casein degradation from that obtained by the action of P_{III} alone (Fig. 3b). It should be mentioned here that the diffusion of proteinase activity from the E_8 cell wall is not efficient and does not result in cells with a significantly decreased cell wall-bound proteinase activity. This might indicate a firmly bound proteinase in addition to easily removable but relatively low P_I and P_{III} activity.

A remarkable feature of the κ-casein degradation by the proteinase system of all strains studied is the appearance of a component which in electrophoresis behaves like para-κ-casein released by chymosin action. This product might result either from a nonspecific action, to be attributed to P_I as well as to P_{III}, or from a specific action of a different enzyme. The second possibility could be interesting to the dairy industry, since it would mean that lactic streptococci possess a milk-clotting enzyme which might be useful for cheesemaking purposes.

Proteolysis and growth in milk. Cell wall proteinases are essential for optimal growth of lactic streptococci in milk. The rate of proteolysis may determine the maximal specific growth rate (μ_{max}) of these organisms in milk. Mutual differences in the μ_{max} values for different strains in milk appear to be the same as those established in a medium containing peptides and amino acids as the N source, although μ_{max} for each strain in milk is about 35 to 40% lower; this reduction was imputed to a limitation imposed by the rate of proteolysis (7). Such a conclusion does not, however, take into account that differences in the growth rates of these strains in milk may also be determined by the specificity of the proteinase(s) involved. It would suggest that in milk the provision of the cell with peptide substrates is equally efficient in each strain in terms of suitability of these peptides as a source of nitrogen and essential amino acids. However, in our opinion it seems more likely that the μ_{max} for growth in milk (7) is mainly determined by the pool of free amino acids and peptides present in milk and responsible for the initial production of up to 25% of the total cell mass found in coagulated milk cultures (15). Milk proteins become important in a later stage of the growth, when relatively high cell densities have been reached. They may be responsible for an even lower μ_{max} (16; F. A. Exterkate, unpublished result), which will then be determined by both the specificity of the proteinase(s) and the rate of proteolysis. The latter limits the growth rate in milk, possibly because of the restricted accessibility of micellar casein and limitations with respect to the concentration of soluble casein (5).

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