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Location of Peptidases Outside and Inside the Membrane of *Streptococcus cremoris*

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Peptidase activity determinations involving native cells of *Streptococcus cremoris* and completely disrupted cell preparations, as well as experiments concerned with peptidase activity distribution among cell fractions obtained by a damage-restrictive removal of the cell wall and release of intracellular material, suggest the presence of peptidases with distinguishable locations. Alanyl, leucyl, and prolyl aminopeptidase activities are most likely located in the cell wall-membrane interface, showing no detectable association with the membrane. Lysyl aminopeptidase is present not only in this location, but also as an intracellular enzyme. Endopeptidase activity and glutamate aminopeptidase activity appear to be weakly associated with the membrane. The locations of these two peptidase activities, unlike those of the former aminopeptidase activities, impose a restriction on their expression. Results of experiments concerned with permeabilization of the membrane and findings regarding an effect of the local environment of the enzymes on their pH activity profiles are evaluated and considered as being indicative of the proposed location. The possible implications of these findings with respect to protein utilization during growth of the organism in milk are discussed.

Streptococcus cremoris constitutes the main flora in mixed cultures used as starters in cheesemaking. The organism is responsible for flavor development during the ripening of cheese, among other things, by virtue of its ability to degrade large peptides liberated from casein by the action of chymosin and cell wall proteinases to small peptides and amino acids (14). Endo- and exopeptidases (2, 4, 5), rather than the additional action of the cell wall proteinases (see reference 13), are considered to be responsible for the degradation of these peptides to transportable units during the growth of the organism in milk, thus being involved in satisfying the nutritional demands of the cell. Previous results (4, 5) already strongly indicate that some of these peptidases, in one way or another, are located in association with the membrane, although direct proof of their supposed location has not been obtained due to the difficulties of preparing protoplasts and membranes without causing dissociation of the enzymes from the membrane.

In this paper, results are described which are indicative of the location of peptidases near the outside surface of and in the membrane, suggesting that peptide hydrolysis as well as transport of peptides in *S. cremoris* is a function of the membrane.

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MATERIALS AND METHODS

Organism. A strain of *S. cremoris* with the designation HP was used. This organism was originally obtained from the Commonwealth Scientific and Industrial Research Organization, Australia.

Maintenance, growth, and harvesting of the organism. The strain was maintained at −40°C in pasteurized milk (30 min, 98°C), reconstituted (10% [wt/vol]) from spray-dried, nonfat milk powder (23). Growth of the organism was started in this milk, which had been precentrifuged for 15 min at 16,000 × *g*, by inoculation with 0.1% of a 6- to 7-h culture grown in milk, and was continued for 14 h at 30°C (final pH, 4.2 to 4.3, stationary phase) or for 14 h at 20°C (final pH, 5.4, logarithmic phase). The (coagulated) milk was adjusted to pH 6.8 with 1 N NaOH. Then 1% (wt/vol) trisodium citrate · $2H_2O$ was added to clear the culture liquid (22). The cells were obtained by centrifugation (15 min at 16,000 × *g*).

Preparation of cell fractions. The cells were washed twice with an excess of 0.1 M NaH_2PO_4–NaOH buffer (pH 7.2) and resuspended in the same buffer. Ultrasonically prepared lysates of spheroplasts and soluble and particulate fractions were obtained as described previously (2).

Removal of the cell wall and release of intracellular material. The method adopted to remove the cell wall and to induce complete lysis was based on the observations made by Metcalf and Deibel (19) and Goodman et al. (7, 8) with respect to lysis of enterococcal streptococci, and by Otto et al. (21) with respect to lysis of *S. cremoris*.

The present method involves the use of logarithmic phase cells of *S. cremoris* HP and a relatively high lysozyme concentration. The cells were washed once with 0.05 M NaH_2PO_4–NaOH buffer (pH 7.0) and once with distilled water and were then suspended in distilled water to an absorbance of 0.5 at 650 nm with a 1-cm light path after a 250-fold dilution. The pH of the suspension was adjusted carefully to pH 7.0, allowing the cells to ferment until no more acid was produced. To the suspension was added an equal volume of a solution of lysozyme (Boehringer, Mannheim, West Germany) in water (6 mg/ml), and the mixture was stirred gently for 60 min at 30°C. Part of the suspension was centrifuged (15 min at 47,000 × *g*), and the supernatant and particulate fraction were tested for enzyme activities. Another part of the lysozyme-treated cell suspension was disrupted ultrasonically to obtain maximum enzyme activities.

Estimation of lysis. The release of glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) during treatment of cells with lysozyme was used to estimate the extent of lysis. G6PDH was assayed in the supernatant by using the UV test

method (Boehringer), and the activity was expressed as a percentage of the maximum G6PDH activity determined in completely disrupted, lysozyme-treated cells. Complete disruption of these cells was accomplished by ultrasonication (2), following the increase in G6PDH in the supernatant until this maximum activity was reached.

Butanol treatment of cells. Cells were treated with *n*-butanol solutions in 0.1 M NaH_2PO_4–NaOH buffer at pH 7.8 and 25°C as described previously (4). This treatment results in an impairment of membranal organization (9).

Determination of endo- and aminopeptidase activity. For the determination of the endopeptidases P_{37} and P_{50} and alanyl and prolyl aminopeptidase activity, the method involving nitroanilide derivatives of amino acids as the substrates was used (2). The activity was expressed as nanomoles of *p*-nitroaniline per minute per milliliter of cell suspension or cell preparation at 37°C by measuring the release of *p*-nitroaniline at 410 nm (E_{410} = 8,800). Both endopeptidase activities were detectable with the same substrate, *N*-glutaryl-L-phenylalanine-4-nitroanilide.

Leucyl, lysyl, and glutamate aminopeptidase activities were assayed in the same way at 37°C and under the indicated conditions, using L-leucine-4-nitroanalide, L-lysine-4-nitroanilidedihydrobromide, and L-glutamic acid-4-nitroanilide (Merck, Germany), respectively, in 0.1 M NaH_2PO_4–NaOH buffer as the substrates. Substrate concentrations were 0.5 or 1.0 mM. Under the conditions of the assay, the reactions proceeded linearly with time.

RESULTS

Peptidase activities detectable with cells. Cells of *S. cremoris* have been shown to exhibit two endopeptidase activities (P_{37} and P_{50}, demonstrating optimum activity at 37 and 50°C, respectively); and alanyl aminopeptidase, prolyl aminopeptidase, and pyrrolidone carboxylate peptidase activity (2, 3). In addition to these activities, three other specific aminopeptidase activities were detected: those of leucyl, lysyl, and glutamate aminopeptidase. Like all other peptidases detected so far, these three aminopeptidases were found entirely in the soluble fraction after ultrasonic disruption of lysozyme-treated cells.

The pH optimum for the soluble leucyl and lysyl aminopeptidase was 7.0 to 7.2, and that for the soluble glutamate aminopeptidase was 8.0 to 8.2 when determinations were performed at 37°C. The temperature optima determined at the optimum pH were 37°C for both leucyl and lysyl aminopeptidase, and 55°C for glutamate aminopeptidase.

The aminopeptidase activities, when isolated, appear to be associated with proteins which show different mobilities in gel electrophoresis at pH 7.2 (unpublished data). Also, several other results (see below) suggest that we are dealing with different enzymes. For the time being, they are therefore treated as distinct enzymes.

Comparison of peptidase activities of intact cells with total activities. A comparison of activities assayed with intact cells and with completely disrupted cells obtained after spheroplast formation and ultrasonication is shown in Table 1.

In the case of prolyl, alanyl, and leucyl aminopeptidase activity, intact cells exhibit an even higher activity than the total activity found in an equivalent amount of completely disrupted cells. The latter activity is entirely in the soluble form; no activity could be detected with the particulate fraction. The other peptidases mentioned in Table 1 showed a marked increase in activity on disruption of cells. This increase was less obvious for the endopeptidase P_{50}. Lysyl aminopeptidase activity was even increased 20-fold when determinations were performed at pH 7.2.

Location of peptidases at the outside surface of, or associated with, the membrane. To prove unequivocally that the peptidases are associated with the membrane, a method has to be used which permits the removal of the cell wall and the complete release of intracellular material without release of the peptidases that are possibly weakly associated with the membrane. All of the methods tried so far appeared to result in the detection of the peptidases in the soluble fraction. Otherwise, these peptidases appeared to remain associated with the particulate fraction of gradually leaking spheroplasts obtained by limited removal of the cell wall (2, 23). Results have been obtained which suggest an association of peptidases with the membrane (3, 4, 5).

A simple procedure, described in the preceding section, appeared to satisfy the requirements stipulated above. A low ionic environment has been shown to be essential to lysozyme binding to the cell and to efficient cell wall removal in enterococcal streptococci (19). Cellular integrity was preserved under these conditions, as almost all cellular proteins, DNA, and RNA remained with the protoplasts. Similar observations were recently made by Goodman et al. (7, 8). According to Kruse and Hurst (10), lysis of cells of lactic streptococci did not occur after treatment with lysozyme unless salt or sodium dodecyl sulfate was added. However, in their studies, the decrease of optical density was used as a criterion of lysis. No mention was made of the leakage of intracellular material as the result of bursting before salt- or sodium dodecyl sulfate-induced lysis. Addition of salt after the incubation of logarithmic phase cells at a relatively high concentration of lysozyme appeared to be an essential criterion in the formation of membrane vesicles from *S. cremoris* cells (21).

Although only a slight change in the optical density of the suspension was observed, a burst percentage of up to 99%

TABLE 1. Peptidase activities[a] in preparations of completely disrupted cells of *S. cremoris* HP

Cells from:	pH of assay	Activity of:							*n*
		Endopeptidase		Aminopeptidase (AP)					
		P_{50}	P_{37}	Glu-AP	Pro-AP	Ala-AP	Leu-AP	Lys-AP	
Stationary phase	7.8	123 (93–144)	522 (407–631)	410 (350–527)	54 (50–63)	43 (41–48)	53 (50–57)	216 (175–242)	5
	7.2	ND[b]	ND	ND	71 (61–79)	50 (47–52)	76 (71–83)	1970 (1,610–2,264)	4
Logarithmic phase	7.2	ND	ND	ND	(61–67)	(46–54)	(44–68)	(696–793)	2

[a] Activities were assayed in 0.1 M NaH_2PO_4–NaOH buffer and are expressed as a percentage of the activities detectable with whole cells. Mean values of *n* experiments, with the extremes in parentheses, are shown.

[b] ND, Not determined.

TABLE 2. Release of peptidase activities from cells of S. cremoris HP during cell wall removal[a] and lysis[b]

Peptidase	Release of activity[c]
Endopeptidase	
P_{50}	17 (10–27)
P_{37}	15 (11–23)
Aminopeptidase (AP)	
Glu-AP	10 (8–13)
Pro-AP	99 (94–100)
Ala-AP	96 (90–100)
Leu-AP	90 (80–96)
Lys-AP	89 (80–97)

[a] For details, see the text.

[b] Lysis was expressed as the percentage of G6PDH released during the lysozyme treatment. The mean value was 92; the extremes were 80 and 99.

[c] Activities in the supernatants were assayed at pH 7.2 in 0.05 M NaH_2PO_4–NaOH buffer and are expressed as a percentage of the total activity in the completely disrupted cell suspension. Mean values (n = 5), with the extremes in parentheses, are shown.

was achieved by the present method after 60 min of incubation, as measured by the release of glucose-6-phosphate dehydrogenase (Table 2). Impairments of the membrane, at least with respect to a peptidase function, now seem to be limited since 85 to 90% of the total glutamate aminopeptidase and endopeptidase activities are suggested to remain with the particulate fraction. The total endopeptidase activity at 50°C (P_{50}) was approximately 23% (extremes, 15 and 34%) of that determined at 37°C; hence, it appears to contribute in logarithmic phase cells only to a very small extent to the total activity determined at 37°C (P_{37}). The enzymes exhibit an almost unrestricted activity in the presence of lysozyme (Table 3). Relief from restriction was effected within a very short period after the addition of lysozyme, since maximum activities were already detected at time zero of the incubation (data not shown). Glutamate aminopeptidase activity could indeed be recovered almost entirely in the membrane fraction. However, only 25 and 18% of the total endopeptidase activity could be recovered in the membrane and soluble fractions, respectively, indicating a loss of approximately 60%.

If a phosphate buffer (0.1 M) was present during the treatment of cells with lysozyme, clearing of the suspension was obvious, and significantly increased levels of endopeptidase and glutamate aminopeptidase were found in the soluble fraction. Addition of salt after a cell suspension in water had been treated with lysozyme also resulted in clearing.

Unlike the endopeptidase and glutamate aminopeptidase, the other four peptidase activities, alanyl, leucyl, prolyl, and lysyl aminopeptidase activity, are released to the extent of 90 to 100% by lysozyme treatment (Table 2). However, when treatment with lysozyme was performed under conditions which are supposed to protect the protoplasts from bursting (e.g., in the presence of sucrose), these aminopeptidases remained for the most part with the stabilized protoplasts. A slight release of these aminopeptidases did not correlate in most cases with the extent of leakage of G6PDH, which nevertheless was observed at all sucrose concentrations used (from 0.25 to 0.75 M) (data not shown).

Membrane perturbation. The ability of n-alkanols to perturb lipid-protein and lipid-lipid interactions is well known (9, 15–17). Observed effects on membrane enzymes have been ascribed to reversible changes in the membrane order and to irreversible impairment of supramembranal organization, or else to a reversible association of the alkanol with the enzyme. The latter phenomenon has been observed with pyrrolidone carboxylate peptidase in S. cremoris (5) and might also be the explanation of reversible inactivation of the other peptidases in this organism in the presence of relatively low concentrations of an alkanol (4).

In the next experiment (Fig. 1), milk-grown stationary-phase cells were treated with increasing concentrations of n-butanol at pH 7.8 and 25°C. In agreement with previous results concerning cells harvested from a synthetic medium (4), a maximum increase of both endopeptidases, P_{37} and P_{50}, was observed with milk-grown cells when 3.5 and 5 to 6% (vol/vol) n-butanol, respectively, was used. However, the maximum increase was approximately four times higher with milk-grown cells, which is mainly due to a lower detectability of the enzyme in situ. Glutamate aminopeptidase also showed maximum increase in activity when 5 to 6% n-butanol was used. The endopeptidase P_{50} exhibited, unlike what can be observed upon solubilization, an increase of six times the initial activity. Taking into account the simultaneous detection of P_{37} and P_{50} at 37°C, the increase of P_{37}—like that of glutamate aminopeptidase—appears to be of the same order as those increases observed on solubilization (Table 1).

At higher concentrations than those causing optimum activity, both endopeptidases were irreversibly inactivated, as is demonstrated for P_{37} with strains exhibiting only (strain Wg2) or mainly (strain TR) this endopeptidase activity (Fig. 2). Glutamate aminopeptidase was not inactivated. The other peptidases, with the exception of lysyl aminopeptidase, showed no activation at all at pH 7.8 or at a lower pH. If lysyl aminopeptidase was measured at pH 7.0, a sharp increase in activity was detectable.

Comparison of pH profiles of peptidases in situ and of the soluble peptidases. The location of enzymes outside or inside the membrane may have consequences for the pH dependency characteristics, owing to the presence of charges in the cell wall and in the membrane, and to a possible buffering effect of the membrane (18). Negative charges, known to be present in the wall-membrane interface and on the membrane surface, can affect H^+ distribution in such a way that a lower pH in the vicinity of the enzyme than that actually measured prevails. This must result in an apparent shift of the pH activity profile of the enzyme in situ to alkaline values when compared with that of the soluble enzyme. Deviations in the pH profile, in comparison with that of the soluble enzyme, may also be expected if an enzyme is in the membrane. In that case, and apart from an effect on H^+

TABLE 3. Endopeptidase P_{37} and glutamate aminopeptidase (glu AP) activity[a] of a lysozyme-treated cell suspension and of the PLM complex[b] of S. cremoris HP

Preparation	Activity of:	
	Endopeptidase P_{37} (n = 9)	Glu-AP (n = 7)
Cells	20 (11–34)	50 (32–68)
Cells (lysozyme-treated)	97 (75–111)	93 (84–108)
PLM fraction	25 (7–39)	110 (83–135)
Soluble fraction	18 (8–30)	16 (2–27)

[a] Activities were assayed at pH 7.2 in 0.05 M NaH_2PO_4–NaOH buffer and are expressed as a percentage of the total activity in the completely disrupted cell suspension. Mean values of n experiments, with the extremes in parentheses, are shown.

[b] For details, see the text.

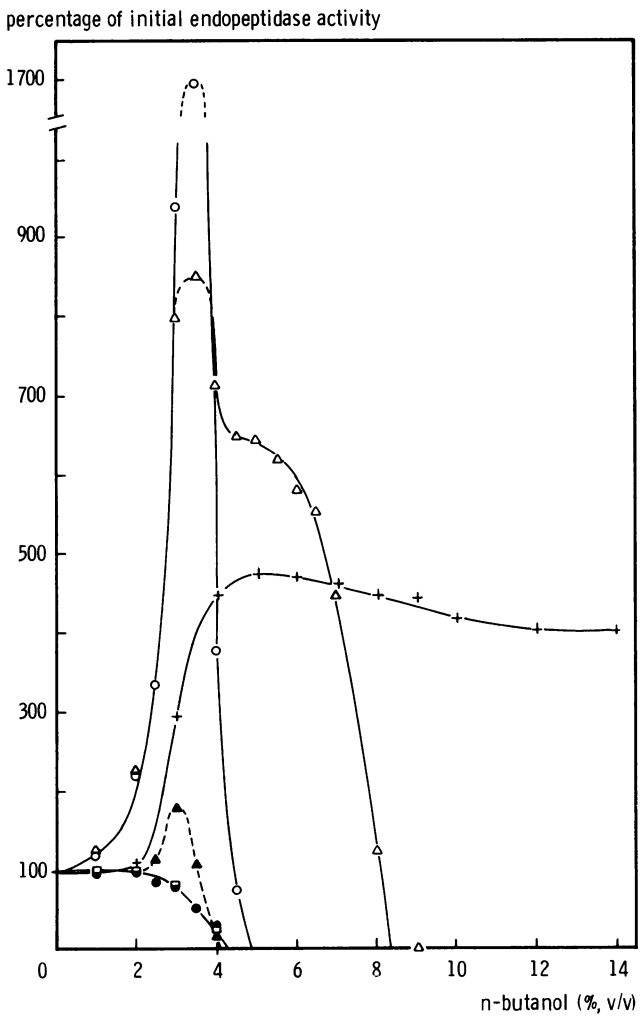

FIG. 1. Effect of treatment of stationary phase cells of *S. cremoris* HP with *n*-butanol on peptidase activities (for details, see reference 4). Residual activities determined in 0.1 M NaH_2PO_4–NaOH buffer at the indicated pH and at 37°C are expressed as percentages of controls treated in the absence of *n*-butanol. Symbols: △, endopeptidase P_{37} and P_{50}, pH 7.8; ○, lysyl aminopeptidase, pH 7.0; ▲, lysyl aminopeptidase, pH 7.8; ●, lysyl aminopeptidase, pH 8.2; □, alanyl (leucyl, prolyl) aminopeptidase, pH 7.2; +, glutamate aminopeptidase, pH 7.8.

distribution, activity should, within the same pH range, vary between much narrower limits than that of the free enzyme because of a buffering effect (18). Both effects may together strongly perturb the pH profile.

A shift was found with alanyl, leucyl, and prolyl aminopeptidase activities. For the leucyl aminopeptidase, this is shown in Fig. 3. The effect was more distinct at low buffer molarities. The relative positions of these profiles are also representative of the other two aminopeptidase activities. The shifts were not evoked by changing the buffer molarity of the incubation mixture containing the soluble enzyme. Also, no thermal inactivation of the soluble enzyme could be established under the experimental conditions.

The cell-bound lysyl aminopeptidase activity shows a pH dependency curve which diverges from that of the soluble enzyme and from that of the former aminopeptidases in situ.

When we look at the pH profiles of the endopeptidase and glutamate aminopeptidase (Fig. 4), a picture is seen that is totally different from that of the aminopeptidases mentioned above (Fig. 3). The enzymes show in situ less variation in activity within a comparable pH range than do the soluble enzymes and appear to shift to the alkaline side in the case of glutamate aminopeptidase.

Treatment of cells with butanol solution (3.5% [vol/vol]) results in a profile of the unrestricted peptidase activity which is similar to that of the corresponding soluble activity (data not shown).

DISCUSSION

The results described above suggest that there are a number of peptidases in *S. cremoris* which are located in a cell wall-membrane interface and have no detectable association with the membrane, and others which are associated with the membrane, though weakly.

The following arguments considering these results are indicative of the proposed locations. First, if the substrates used in the determination of peptidase activities associated with native cells (Table 1) had to enter or to pass through the cell membrane to meet the enzyme, one would expect that the ratio of the activity of the enzyme in situ to that of the solubilized enzyme would be less than one. This is certainly not the case for prolyl, alanyl, and leucyl aminopeptidase activities. Taking into account the fact that amino acid

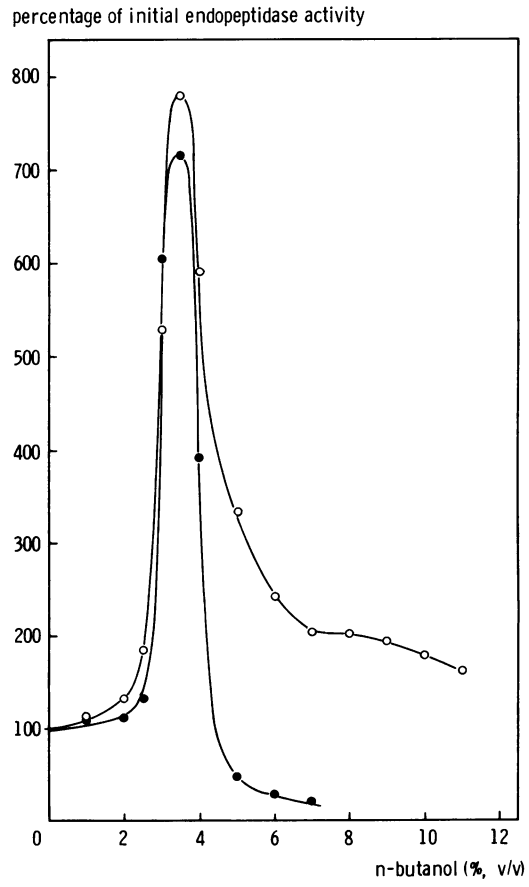

FIG. 2. Effect of treatment of stationary phase cells of *S. cremoris* Wg2 and TR with *n*-butanol on endopeptidase activity. For details, see the legend to Fig. 1. Symbols: ●, strain Wg2; ○, strain TR.

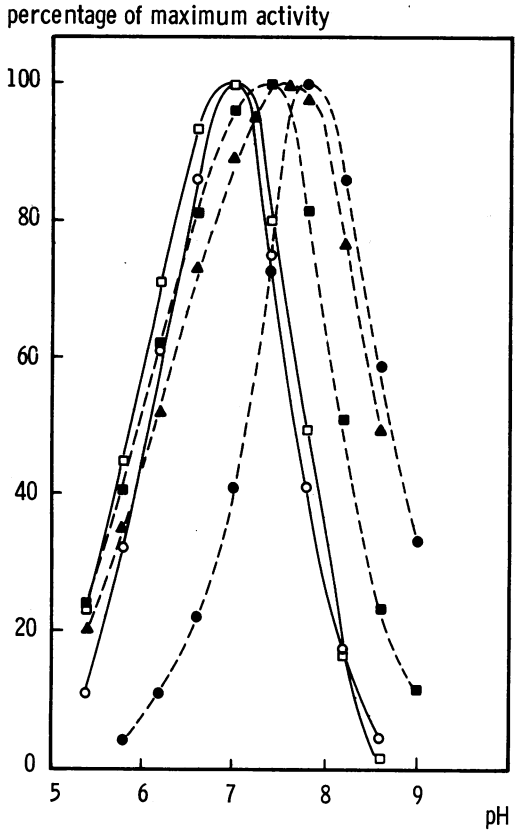

FIG. 3. pH dependency profiles of leucyl aminopeptidase and lysyl aminopeptidase in situ and in the soluble state determined at 37°C in a buffer consisting of (unless indicated otherwise) 0.05 M sodium acetate, 0.05 M sodium dihydrogen phosphate, and 0.05 M boric acid. Symbols: □, soluble leucyl aminopeptidase; ○, soluble lysyl aminopeptidase; ●, lysyl aminopeptidase in situ; ■, leucyl aminopeptidase in situ; ▲, leucyl aminopeptidase in situ; the molarity of the buffer constituents was, in this case, 0.025 M.

nitroanilides do not easily enter the cell membrane (6), it is most probable that these activities are located outside the membrane, at least without any restriction for the active center to associate with the substrate. The endopeptidase P_{37}, glutamate aminopeptidase, and lysyl aminopeptidase did show a considerably increased activity upon disruption of cells. Apparently, these enzymes are located in such a way that their activity in situ is restricted. The second argument concerns the damage-restrictive fractionation of cells. Although lysis was nearly 100%, clearing of the suspension of lysed lysozyme-treated cells did not occur. It could be induced by the addition of salt. We suggest that an enduring integrity of a complex which consists of residual cell wall polymers, lysozyme, and the membrane (PLM complex) left of the cell envelope after treatment of the cells with lysozyme in a low-ionic-strength medium. This conclusion is based on the detection of such a complex in lactic streptococci (10) and enterococcal streptococci (8) after incubation with lysozyme, and on the observation that addition of salt causes complete disintegration of the complex, resulting in clearing of the suspension. Damage of the membrane seems to be a prerequisite for lysis in the sense of clearing (1, 8). From the present experiments, it can be concluded that glutamate aminopeptidase is associated with this PLM function. Loss of endopeptidase activity during the fractionation step after the incubation with lysozyme can be attributed to separation of two components which both are essential in the release of *p*-nitroaniline from the substrate. One of these components is associated with the PLM fraction, and the second component can be found in the soluble fraction (unpublished data). Since both enzymes already showed almost completely unrestricted activity in the presence of lysozyme, a nonenzymatic effect of lysozyme seems to be responsible. This effect may be identical to the membrane permeabilizing effect exerted by lysozyme as a result of an unspecific binding of this basic protein to the membrane which affects the structure of the latter (see reference 1). Considering this effect of lysozyme, the restriction of glutamate aminopeptidase activity and endopeptidase activity is most likely due to reduced accessibility as a result of the association of the enzymes with the membrane. Apparently, the presence of buffer during the action of lysozyme or the addition of salt afterwards not only prevents the formation of the PLM complex or causes its disintegration, respectively, but also causes damage to the membrane (1, 8). This damage manifests itself in the dissociation of the peptidases and perhaps other membrane constituents.

From the results, one may also conclude that alanyl, leucyl, and prolyl aminopeptidases, because of their ability to react in situ without restriction, are entrapped in polymeric structures of the cell wall-membrane interface, and that

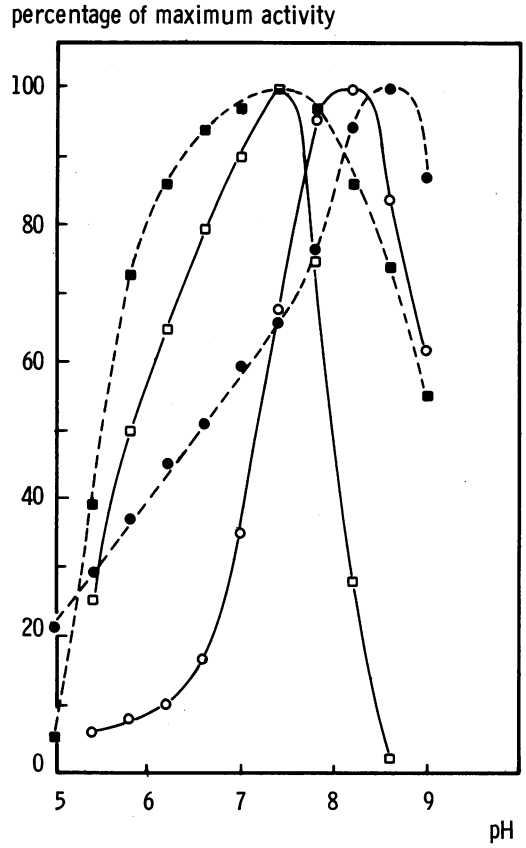

FIG. 4. pH dependency profiles of endopeptidase and glutamate aminopeptidase in situ and in the soluble state determined at 37°C in a buffer consisting of 0.05 M sodium acetate, 0.05 M sodium dihydrogen phosphate, and 0.05 M boric acid. Symbols: □, soluble endopeptidase; ○, soluble glutamate aminopeptidase; ■, endopeptidase in situ; ●, glutamate aminopeptidase in situ.

lysyl aminopeptidase, because of its restricted activity in situ, is present not only in this position but also as an intracellular enzyme. The enzymes in the interface most likely remain entrapped in polymeric structures, e.g., residual cell wall polymers (8, 10) and lipoteichoic acids (24), if cell wall removal is performed in an osmotically stabilized medium. They escape almost completely if swelling of the protoplasts occurs during cell wall removal in water.

Two further considerations sustain the proposed locations of the various peptidases. The results of the experiments involving *n*-butanol (Fig. 1), if evaluated in relation to its known action on the membrane (9, 15–17) and to the foregoing, lead to the conclusion that the effect on P_{50} and P_{37}, glutamate aminopeptidase, and lysyl aminopeptidase is not due to any direct effect on the catalytic function of the enzymes. It is merely the result of an opening of an activity-restricting location induced by structural changes which enhance the permeability of the membrane to the substrate and thus even allow detection of the intracellular lysyl aminopeptidase. Apparently, the other peptidases are associated with the membrane in such a way that their position imposes a restriction on their expression, most likely by the presence of a hydrophobic barrier.

The results concerning the observed shift of the pH activity profile of alanyl, prolyl, and leucyl aminopeptidase (Fig. 3) rule out any possible effect resulting from a different local ionic strength in the vicinity of the enzyme in situ or from thermal inactivation. The results therefore leave the possibility that a local effect on H^+ distribution (18) is exerted by negative charges at the membrane surface. They confirm in that case the assumed location of the enzymes at the outside surface of the membrane. Further, the results furnish an explanation of the decrease in activity on solubilization of these enzymes (Table 1).

The pH activity profiles of the endopeptidase and glutamate aminopeptidase in situ, compared with those of the soluble enzyme, suggest an additional effect caused by a buffering action (18), which results in activities varying between narrower limits than those, within the same pH range, of the free enzyme. The fact that treatment of cells with butanol results in abolition of the above-mentioned effects on the pH activity profiles confirmed that microenvironmental conditions responsible for these effects are found in the membrane.

With respect to the leucyl aminopeptidase activity, our results are contradictory to those obtained by Law (11). The fact that a short incubation period of logarithmic phase cells with lysozyme at 37°C (11) or incubation of stationary phase cells at a relatively low lysozyme concentration (1) did not result in the release of (leucyl) aminopeptidase activity from cells of *S. cremoris* may be attributed to a very limited solubilization of the cell wall. The observed release of cell wall proteinase activity under the same conditions, which was considered to be indicative of cell wall removal, is most probably mainly due to rapid diffusion of the proteinase into the supernatant and not to lysozyme action in the first place (20).

The peptidases mentioned in the present study are, in concerted action with cell wall-associated proteinases, assumed to be involved in protein utilization during growth of the organism in milk by making small peptides and free amino acids available for transport over the membrane (2). The location of some peptidases (e.g., the endopeptidases and glutamate aminopeptidase) requires penetration of the substrate into the membrane. Results obtained with the specific pyrrolidone carboxylate peptidase (5) with respect to reversible inhibition by homologous series of organic solvents have indicated hydrophobic subsite interactions between the substrate and the enzyme molecule which are essential to the catalytic function of this enzyme. Reversible inactivation of the other peptidases by relatively low concentrations of alkanols (4) also suggests that essential hydrophobic interactions are involved in catalysis. If this phenomenon or the observed restrictions or both are, in fact, common to the peptidases located near or in the membrane, they add to the possibility that during growth of the organism in milk, the more hydrophobic peptides have the advantage over other peptides in the competition for degradation. On the whole, there could be a demand for peptides with specific properties, and such peptides can be obtained most efficiently by the cell through the action of its own cell wall proteinase system, preferably with casein as the substrate. This view offers an alternative explanation of those observations in which *S. cremoris* fails to grow on certain peptide fractions because, according to the authors, the size limit for utilization with respect to transport (seven or eight amino acid residues) is exceeded (12; R. Otto, Ph.D. thesis, University of Groningen, The Netherlands, 1981). The ability of the organism to degrade such peptides before transport has not been taken into consideration. According to the present view, the organism should grow, since degradation of the peptides can be expected, but growth will not occur because of the lack of properties which enable the peptides to associate with the peptidases. If this view appears to be true, a re-interpretation with respect to peptide utilization by lactic streptococci is needed.

ACKNOWLEDGMENT

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