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Localization of Proteinase(s) near the Cell Surface of *Streptococcus lactis*

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Two criteria suggest that most of the proteinase of *Streptococcus lactis* is localized in the cell wall. (i) Intact cells possess proteinase activity when incubated with a high-molecular-weight substrate. (ii) Most of the cell-bound proteinase activity is released during spheroplast formation under conditions which result in the release of only 1% of the intracellular enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase. The solubilized cell wall, plasma membrane, and cytoplasm fractions contained 84, 0, and 16%, respectively, of the total proteinase activity with casein as substrate. The physiological role of a surface-bound proteinase in this organism is discussed.

The growth of *Streptococcus lactis* is dependent on the availability of free amino acids or small peptides, or both (14). Since these nutrients are present at low concentrations in milk, the growth of this organism will be limited unless protein is hydrolyzed (14). This suggests the presence of either surface-bound or extracellular proteinase in the organism. The present investigation (taken in part from an M.Sc. thesis submitted by N.A.S. to Massey Univ., Palmerston North, New Zealand, 1972) was undertaken to determine the localization of proteinase(s) in *S. lactis* by using the most gentle procedures available for cell fractionation, together with an improved proteinase assay. The data show that most of the proteinase activity is surface bound and released into the medium when the cell wall is removed without rupture of the spheroplast.

MATERIALS AND METHODS

Organisms and culture conditions. *S. lactis* strain C10 was obtained from W. E. Sandine, Department of Microbiology, Oregon State University, Corvallis. *S. lactis* strains H1 and ML3 and *Streptococcus cremoris* strain AM2 were obtained from the New Zealand Dairy Research Institute. Stock cultures in skim milk were stored at −75 C and subjected to minimal subculturing to avoid the accumulation of "slow acid producing variants" (4). Experimental cultures were grown at 30 C in T5 broth which consists of: lactose, 0.5%; beef extract (BBL), 0.2%; polypeptone (BBL), 0.5%; phytone peptone (BBL), 0.2%; yeast extract (BBL), 0.2%; ascorbic acid, 0.05%; $MgCl_2$, 0.01%; Na_2HPO_4, 0.85%; and KH_2PO_4, 0.2%. The lactose was autoclaved separately and the medium had a pH of 7.2. Organisms were harvested by centrifugation from cultures growing logarithmically (doubling time, 40 min) at a cell density of 0.25 mg (dry weight)/ml.

Mechanical disruption of cells. Organisms were washed three times and then suspended in 0.2 M Na_2HPO_4-KH_2PO_4 buffer (pH 6.4). A sample of this suspension (intact cells) was assayed for proteinase activity. The remaining suspension was adjusted to a bacterial density of 8 mg (dry weight)/ml, and 5-ml volumes were shaken on a Mickle disintegrator with 3 ml of Ballotini grade-12 glass beads. Complete disruption, as judged by phase-contrast microscopy, required 7 min of shaking, and the temperature was kept below 10 C. The beads were filtered off and washed. A sample of this disrupted cell suspension was assayed for proteinase activity, whereas the remainder was centrifuged (35,000 × *g*, 10 min) to give pellet 1, consisting of cell walls and membranes. The supernatant was then centrifuged (157,000 × *g*, 120 min) to give pellet 2, consisting of small particles; the supernatant was designated the cytoplasm.

Preparation of cell fractions by using phage-associated lysin. High-titer phage lysates of *S. lactis* ML3 were prepared and the phage-associated lysin was purified 30-fold by the method of Tsugita et al. (17). Phage-associated lysin had no detectable proteinase activity when assayed at 10 times the highest concentration used for cell fractionation.

Organisms in the mid-log growth phase were harvested by centrifugation, and a sample of the sedimented cells was washed twice and suspended in 0.2 M phosphate buffer (pH 6.4; intact cells). The remaining sedimented cells were resuspended in the spheroplasting medium (0.5 M sucrose, 20 mM $MgCl_2$, 0.2 M phosphate buffer, pH 6.4), and phage-associated lysin was added. The suspension was

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incubated at 30 C for 30 to 120 min (depending on the amount of lysin used) until spheroplasting was complete, as judged by phase-contrast microscopy. The suspension was then cooled to 4 C and centrifuged (35,000 × *g*, 10 min). The supernatant was centrifuged further (157,000 × *g*, 120 min), and the resulting supernatant was designated the solubilized cell wall fraction. Resuspension of the spheroplasts in 0.2 M phosphate buffer (pH 6.4) resulted in more than 99% lysis as judged by phase-contrast microscopy. This fraction is referred to as the spheroplast lysate. For the isolation of the plasma membrane fraction, organisms were suspended in 0.1 M phosphate buffer (pH 7.0) containing 20 mM $MgCl_2$ and incubated with phage-associated lysin. When lysis was complete, the lysate was centrifuged at 35,000 × *g* for 10 min. The plasma membrane pellet was washed three times with 0.02 M phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$. Cell fractions were stored at −75 C before analysis.

Release of proteinase during spheroplast formation. Organisms were harvested and resuspended in the spheroplasting medium, and the suspension was divided into two portions. Phage-associated lysin was added to one portion and the suspensions were incubated at 30 C. At intervals, samples were centrifuged (35,000 × *g*, 10 min) and the supernatant solutions were assayed for proteinase.

Proteinase assay. Casein (Hammarsten grade, British Drug Houses), labeled with ^{125}I by the method of Heuson (5), was used as the proteinase substrate. The ^{125}I-labeled casein was dialyzed extensively and had a specific activity of 10^6 counts per min per mg of casein. The assay system contained 0.5 ml of ^{125}I-labeled casein (2 mg) in 0.2 M phosphate buffer (pH 6.4) and 0.5 ml sample or buffer blank. After 6 h of incubation at 30 C, 0.4-ml samples were removed and added to 1.6 ml of unlabeled casein solution (20 mg/ml). Two samples were prepared from the blank. Trichloroacetic acid (2 ml of 24%, wt/vol) was added to each experimental sample and to one blank sample (to give the non-enzymatic release of trichloroacetic acid-soluble radioactive material), whereas distilled water was added to the other blank sample (to give total radioactivity). After mixing and standing for 20 min, samples were filtered and the filtrates (1 ml) were counted in a Packard Auto Gamma Spectrometer. Counts were corrected for both background and non-enzymatic release of radioactivity and expressed as a percentage of the total. One unit of proteinase activity was defined as the amount of enzyme which solubilized 1% of the substrate in 6 h. Proteinase activity is expressed as units per milligram of bacterial dry weight (with cell fractions these were calculated on the basis of the initial bacterial dry weight from which the fraction was derived). When less than 5% of the total radioactivity was solubilized in the assay, proteinase activity was directly proportional to enzyme concentration. Each fraction was assayed at three different concentrations where this relationship was linear. The non-enzymatic release of trichloroacetic acid-soluble radioactive material was dependent on the assay incubation time and on the trichloroacetic acid concentration used. This release, however, was very reproducible and amounted to 0.9% of the total radioactivity under the assay conditions used. The assay was found to be reproducible and quantitative when evaluated with trypsin and subtilisin.

Glycolytic enzyme assays. The assay system for aldolase contained: triethanolamine-hydrochloride buffer (pH 7.5), 0.05 M; 2-mercaptoethanol, 0.05 M; fructose-1,6-diphosphate, 1.6 mM; reduced nicotinamide adenine dinucleotide (NADH), 0.12 mM; α-glycerophosphate dehydrogenase, 1.25 U; triosephosphate isomerase, 11.5 U; and sample, in a final volume of 2.5 ml. The assay system for glyceraldehyde-3-phosphate dehydrogenase contained: $Na_4P_2O_7$ (pH 8.5), 30 mM; cysteine-hydrochloride, 4 mM; sodium arsenate, 12 mM; glyceraldehyde-3-phosphate, 2 mM; NAD, 0.6 mM; and sample, in a final volume of 2.5 ml. With both assay systems the rate of change of absorbance at 340 nm was measured. Assays were carried out at three different concentrations, and activity was proportional to concentration.

Release of intracellular enzymes during proteinase assay on intact cells and during spheroplast formation. Intact cells (2 mg [dry weight]/ml) were incubated under proteinase assay conditions. After 6 h, the suspension was centrifuged (35,000 × *g*, 10 min), and the supernatant solution was assayed for aldolase and glyceraldehyde-3-phosphate dehydrogenase activity. The organisms were resuspended in the same volume of buffer and disrupted by shaking with glass beads. The broken cell suspension was centrifuged and the cell-free extract was assayed.

Organisms (2 mg [dry weight]/ml) were incubated in the spheroplasting medium with phage-associated lysin. After 120 min, the suspension was centrifuged (35,000 × *g*, 20 min) and the supernatant was assayed. The spheroplasts were ruptured in buffer of equivalent volume to the original suspension. The lysate was centrifuged (35,000 × *g*, 30 min) and the supernatant was assayed. Activities per unit volume of the two fractions can be compared directly. The presence of sucrose did not affect the activity of either aldolase or glyceraldehyde-3-phosphate dehydrogenase.

Bacterial dry weight. Organisms were washed three times and resuspended in buffer. Samples were dried to constant weight and corrected for the weight of buffer salts present.

RESULTS

Properties of crude proteinase(s). The influence of pH, buffer strength, and temperature was evaluated on a crude proteinase preparation obtained after dialysis of phage lysin-solubilized cell walls. Controls without enzyme were included in all assays to allow for the possible effects of each condition on the stability of ^{125}I-labeled casein. The proteinase activity was markedly affected by pH, the activity falling off rapidly above pH 7 and below pH 6. It should be pointed out, however, that varying the pH or other parameters may affect the highly complex casein substrate as well as the

activity of the enzyme. Maximal activity was obtained at 30 to 32 C in 0.05 M phosphate buffer while the proteinase(s) was rapidly inactivated at 37 C. Although 0.2 M phosphate buffer depressed activity slightly, this buffer strength was used routinely to improve buffering capacity and to minimize differences in ionic composition between fractions. Addition of *p*-chloromercuribenzoic acid, cysteine, and sodium thioglycolate (all 0.2 mM) to assay systems had no effect on proteinase activity, but the addition of 1 mM ethylenediaminetetraacetic acid depressed proteinase activity by 80%.

Localization of proteinase(s). Substantial proteinase activity was exhibited by intact cells (Table 1). There was no significant leakage of two intracellular glycolytic enzymes from intact cells during proteinase assays (Table 2). In control experiments, both aldolase and glyceraldehyde-3-phosphate dehydrogenase were shown to be completely stable in 0.2 M phosphate buffer (pH 6.4) at 30 C for at least 6 h. The proteinase activity of intact cell suspensions increased by 25% on mechanical disruption (Table 1). Only 65% of the activity was recovered; of this, 75% was associated with the cell walls and membranes (pellet 1), 8% was associated with small cell fragments (pellet 2), and 17% was associated with the cytoplasm (Table 1). Neither culture supernatants nor buffers used to wash intact cells contained detectable proteinase activity. However, no attempt was made to concentrate any protein in the culture fluid, so that the possibility of an extracellular proteinase has not been excluded.

When organisms were suspended in buffered 0.5 M sucrose containing Mg^{2+} and incubated with a cell wall hydrolase (phage-associated lysin), proteinase activity was progressively released into the suspending medium (Fig. 1). Proteinase release ceased as all the spheroplasts became osmotically fragile. Under these conditions, loss of the intracellular enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase from spheroplasts was not significant (Table 2). When organisms were incubated in the same sucrose medium but without the addition of phage-associated lysin, proteinase activity was released slowly from the cells (Fig. 1). It is not clear whether this release of proteinase is due to the action of an autolysin or to some effect of sucrose on the cell. No detectable proteinase activity was released from intact cells which

TABLE 1. *Proteinase localization in fractions prepared by mechanical disintegration of S. lactis C10*

Fraction	Proteinase activity (U/mg of cell dry weight or equivalent)	Proteinase distribution (% activity of disrupted cells)
Intact cells	30.0	80
Disrupted cells	37.6	100
Pellet 1[a]	18.6	49
Pellet 2[b]	2.0	5
Cytoplasm	4.2	11

[a] 35,000 × *g*, 10 min.
[b] 157,000 × *g*, 120 min.

TABLE 2. *Release of intracellular enzymes from S. lactis during proteinase assay on intact cells and during spheroplast formation*

Determination	Enzyme	Enzyme activity[a] (μ mol/min/ml)		Percent released
		Supernatant solution	Intact cells or spheroplasts	
Proteinase assay	Aldolase	0.015	1.68	1
	GAPDH[b]	0.003	0.30	1[c]
Spheroplast formation	Aldolase	0.005	1.74	0.3
	GAPDH	0.014	1.50	1

[a] Activities in the two fractions can be compared directly. Results are the mean of two experiments.
[b] GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.
[c] Not corrected for activity lost during mechanical disintegration of cells.

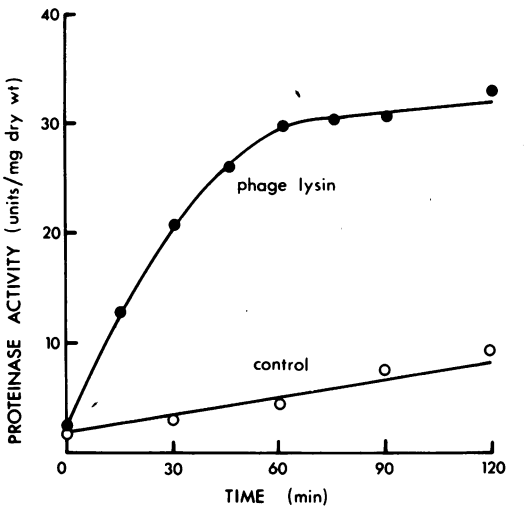

FIG. 1. *Release of proteinase from osmotically stabilized S. lactis* (○) *and during spheroplast formation with phage-associated lysin* (●).

were washed repeatedly in 0.2 M phosphate buffer (pH 6.4) at 4 C.

When organisms were converted to spheroplasts by using phage-associated lysin, 84% of the total proteinase activity was found in the solubilized cell wall fraction (Table 3). Intact cells possessed 75% of the proteinase activity found in the solubilized cell wall fraction. Of the remaining proteinase activity found in the lysed spheroplasts, none was associated with the plasma membrane (Table 3). A similar distribution of proteinase activity was found in *S. lactis* strain H1, although an even higher proportion (99%) of the activity found in the solubilized cell wall fraction was observed with intact cells.

DISCUSSION

The estimation of proteinase in cell fractions of three strains of *S. lactis* was carried out initially by using the conventional assay technique (8) based on measurement of the release of trichloroacetic acid-soluble amino nitrogen from casein. This assay proved to be too insensitive and nonspecific for the present investigation particularly where cell fractions contained appreciable amounts of non-protein nitrogen. Other difficulties which may be encountered with this method have been described (10). The casein substrate was therefore labeled with ^{125}I so that only derivatives of the added substrate were detected in the assay. The complexity of the substrate used in this investigation does not allow any distinction to be made between proteinases and peptidases. This may be particularly relevant in the interpretation of cytoplasm activities where the enzyme is likely to be a peptidase (11).

Casein prepared by acid precipitation consists of several proteins with molecular weights in the range 19,000 to 24,000. These monomers aggregate in an equilibrium reaction and, under the conditions used in this investigation, most of the protein is in the form of aggregates with molecular weights of at least 200,000, leaving little free monomer (12). It seems unlikely that even the monomeric proteins with an estimated minimal diameter of 2.0 nm (18) could penetrate the bacterial cell wall to the outside surface of the plasma membrane (16). In addition, both the casein molecules and the cell wall of *S. lactis* carry strong negative charges (7, 18) which would further restrict diffusional penetration of the casein.

TABLE 3. *Proteinase localization in fractions prepared by osmotic lysis of S. lactis C10*

Fraction	Proteinase activity (U/mg dry weight or equivalent)	Proteinase distribution
Intact cells	35.8	75[a]
Solubilized cell wall	47.8	84[b]
Spheroplast lysate	9.0	16[b]
Plasma membrane	0.0	0[b]

[a] Percentage of activity in solubilized cell wall fraction.

[b] Percentage of activity in solubilized cell wall fraction plus spheroplast lysate.

After mechanical disintegration of *S. lactis* organisms, most of the proteinase activity was recovered in the particulate fraction which consists of cell walls and plasma membranes. However, the preparation of cell fractions for studies of enzyme localization is best achieved by using the most gentle method available. This involves the enzymatic removal of the cell wall followed by osmotic lysis (6, 13). With the lactic streptococci, this is most readily achieved by the use of phage-associated lysin (T. D. Thomas, unpublished results). The hydrolysis of the cell wall of *S. lactis* with phage-associated lysin in the presence of an osmotic stabilizer was accompanied by the release of soluble proteinase activity. Under these conditions, the release of intracellular glycolytic enzymes was not significant, indicating that the spheroplasts remained intact. Of the total proteinase activity found in cell lysates, 84% was present in the solubilized cell wall fraction. The finding that intact cells of *S. lactis* strains C10 and H1 possessed 75 and 99%, respectively, of the proteinase activity found in the solubilized cell wall fractions indicates that in the intact cells most of the enzyme is accessible to the high-molecular-weight substrate, suggesting localization of the proteinase near the cell wall surface.

In the present investigation, no proteinase activity was associated with the membrane fraction which consisted of both plasma and mesosomal membranes isolated by procedures that result in the minimal damage (13). Cowman et al. (2, 3) have suggested that most of the proteinase of *S. lactis* strain 3 is membrane bound. In their experiments, cells were subjected to sonic treatment in the absence of Mg^{2+}, and the suspension was centrifuged at 27,000 $\times$ *g* for 20 min; no estimate of the degree of lysis was given. These conditions are known to disaggregate bacterial membranes so that ultracentrifugation is required to sediment the membrane components (15). The particulate fraction of Cowman et al. (3) was then treated with egg white lysozyme and centrifuged. The

resulting pellet contained proteinase activity and was assumed to represent the plasma membrane. However, the cell wall of *S. lactis* is insensitive to lysozyme and is only partly solubilized (1, 7), and it is likely that the plasma membrane fraction of Cowman et al. (3) contained much cell wall material. We consider that the criteria necessary to confirm the localization of proteinase on the membrane of *S. lactis* strain 3 have not been established.

The surface-bound proteinase(s) would appear to serve a nutritional role by hydrolyzing proteins to amino acids or peptides that are small enough to enter the cell (11). Intracellular peptidases could further hydrolyze these peptides and release their constituent amino acids, thus permitting the utilization of protein for growth. Organisms used in our investigation were grown in a broth medium containing peptides and amino acids that repress synthesis of extracellular proteinase in some bacteria (9), so that the surface-bound proteinase activity of *S. lactis* could be enhanced by growth in milk. Further investigations have shown that slow variants of *S. lactis*, which are capable of only limited growth in milk, do not possess this surface-bound proteinase when grown in a broth medium.

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