Autolysis in Strains of Viridans Streptococci

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SUMMARY

Seven strains of viridans streptococci of the species *Streptococcus sanguis*, *S. mutans* and *S. mitis* were investigated for autolysis. The effect of pH, salt concentration and temperature on the autolytic process was studied in Na,HPO₄/NaH₂PO₄ buffer. Whole cells and walls of all strains autolysed most rapidly at pH values above 7. Autolysis of whole cells of *S. sanguis* and one strain of *S. mitis* (ATCC15909) was maximal in 0.05 to 0.2 M buffer, while the two *S. mutans* strains and *S. mitis* ATCC15912 showed maximal autolysis in 0.5 and 1.0 M buffers. Cultures harvested in the stationary phase of growth possessed only slightly decreased autolytic activity compared with those from the exponential phase. Whole cells autolysed more rapidly at 37 °C than at 45 °C and 10 °C. Autolysis of isolated walls of three strains of *S. mitis* (ATCC903, ATCC15909 and ATCC15912) was maximal at pH 7.0 and 7.5 and in 1.0 M buffers. *Streptococcus mitis* ATCC15909 also showed maximal lysis in 0.01 M and 0.5 M buffers. An endopeptidase action of the autolytic system of *S. mitis* ATCC15912 was indicated by the progressive release of soluble amino groups during autolysis of the walls. No release of reducing groups was observed. Several free amino acids were released during autolysis of these walls, alanine, lysine and glutamic acid being in greatest quantity.

INTRODUCTION

Autolysins, that is, bacterial enzymes or enzyme systems which can cause bacterial lysis by the hydrolysis of various bonds in the peptidoglycan of the wall, have an important physiological role in, for example, division, cell separation and remodelling of shape (Ghuysen & Shockman, 1973; Rogers, 1970; Fan, 1970b). In addition, autolytic enzymes may be involved in the final stages of peptidoglycan synthesis in at least one species (Pooley et al., 1972; Pooley & Shockman, 1969). Linder (1974a) showed that autolysis of some of the cells in a population may release cell-bound enzymes specific for substrates unable to penetrate the cell.

Autolytic enzymes are suitable for preparing spheroplasts and protoplasts (Mohan et al., 1965; Joseph & Shockman, 1974) and for isolating bacterial membranes (Salton & Freer, 1965). Autolysis under controlled conditions is also an effective and gentle method for disintegration of cells to extract cell products (Linder, 1974b; Davis & Salton, 1975).

The viridans streptococcus, *S. mitis* ATCC903, has been investigated for autolysis (Linder, 1974b, c). Whole cells of this strain revealed unusual lysis behaviour compared with other strains of the Lactobacillaceae (Coyette & Shockman, 1973; Coyette & Ghuysen, 1970; Shockman et al., 1961; Neujahr & Logardt, 1973): (i) the rate of autolysis was maximal in buffers of high molarity; (ii) stationary-phase cells autolysed as readily as exponential-phase cells; and (iii) lysis was maximal at pH values above 7.

The aim of the present investigation was to determine optimum conditions for autolysis.
of whole cells and walls of *S. sanguis*, *S. mutans* and *S. mitis*. The mode of action of the autolytic system in the strain with the highest autolytic activity, *S. mitis* ATCC15912, was investigated.

**METHODS**

**Bacterial strains.** *Streptococcus sanguis* strains 804 (Carlsson, 1965) and OPC1 (Carlsson, 1968), *S. mutans* strains KPSK2 (Carlsson, 1968) and IB (Krasse, 1966), and *S. mitis* strains ATCC15909, ATCC15912 and ATCC903 were used.

**Cultivation technique and nutrient medium.** Bacteria were grown anaerobically at 37 °C and pH 6.5 in a stirred fermenter (FG 500, Biotec, Stockholm, Sweden) with automatic pH control. The medium, PPI, contained proteose peptone and 2% (w/v) glucose (Linder, Holme & Frostell, 1974).

**Lysis of whole cells.** Bacteria were harvested in the late-exponential growth phase (exp. cells) and in the stationary phase about 30 min after glucose exhaustion (stat. cells). They were washed twice in cold (5 to 7 °C) 0.01 M-sodium phosphate buffer pH 6.8, resuspended in appropriate incubation buffers, homogenized with a tissue grinder (A. H. Thomas, Philadelphia, U.S.A.), and incubated at 37 °C for 20 h.

**Preparation of walls.** Bacteria harvested in the late-exponential growth phase were washed twice in cold 0.01 M-sodium phosphate buffer pH 6.8, resuspended in 20 ml of the same buffer at a concentration equivalent to about 80 mg dry wt ml\(^{-1}\), and disintegrated in the X-press by the method of Edebo (1960). To the resulting suspension, deoxyribonuclease (20 μg ml\(^{-1}\)) and ribonuclease (2 μg ml\(^{-1}\)) were added. After 10 min at 20 °C, the suspension was centrifuged (3000 g; 20 min) to remove the remaining whole cells. The supernatant fraction, carefully decanted, was centrifuged (10000 g; 20 min) to sediment the walls, which were then washed four times in cold 0.01 M-sodium phosphate buffer pH 6.8. All experiments were made with freshly prepared walls.

The degree of contamination of the wall preparations by whole cells was estimated by viable count. Wall preparations of strains of *S. mitis* suspended in 20 ml 0.01 M-sodium phosphate buffer pH 6.8 yielded less than 10\(^2\) viable cells per ml.

**Preparation of walls free from autolytic activity.** Wall-bound autolytic enzymes can be inactivated to yield autolysin-free walls that are suitable as substrate(s) for isolated autolysins (Fan, 1970a; Coyette & Shockman, 1973) Walls of *S. mitis* ATCC15912, free from autolytic activity, were prepared by heating to 100 °C for 15 min. Those of *S. mitis* ATCC903, however, could not be completely inactivated by heating to 100 °C for 15 to 30 min or by treatment with sodium dodecylsulphate (SDS) 1% (w/v; final concentration) at 37 °C for 3 h, although lysis was decreased by 70 to 80% compared with controls. For these walls, exposure to low pH gave autolysin-free walls. Walls incubated at pH 4.9 in 0.05 M-sodium phosphate buffer at 20 °C for 15 min showed no lysis during this period, and no lysis during subsequent incubation for 20 h in 0.01 M-sodium phosphate buffer pH 7.5.

Autolysin-free walls were used as controls in experiments where autolysis was followed by thin-layer chromatography of dinitrophenylated products of wall autolysates.

**Measurement of autolysis.** Lysis was determined by the decrease in extinction at 550 nm, using a Zeiss PMQ II spectrophotometer, and is expressed as the percentage decrease in the initial extinction. All readings were made in the range 0.100 to 0.800 by diluting the sample in the same buffer or in distilled water.

**Analytical procedures.** The autolysis of walls was followed chemically by measuring the solubilization of N-terminal amino groups and reducing groups. Free amino groups were determined by the reaction with 2,4-dinitrofluorobenzene (DNFB) as described by Ghuysen,
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Tipper & Strominger (1966). The liberation of reducing groups was assayed by the sensitive method of Park & Johnson (1949). Samples of walls were incubated for autolysis for various periods of time and then centrifuged at 20,000 g for 15 min. The supernatants were stored frozen until assayed for free amino groups and reducing groups. The dinitrophenylated amino acids were also estimated by thin-layer chromatography on silica-gel plates (Merck, Darmstadt, Germany) according to Ghysen et al. (1966).

Chemicals. DNase I, RNase A type I-A, DNFB and DNFB-amino acids were all obtained from Sigma.

RESULTS

Autolysis of whole cells

Whole cells of S. mitis ATCC903 were not studied as their autolysis has already been reported (Linder, 1974b). With the other strains, samples were harvested from the exponential and stationary growth phases, washed twice and suspended in Na₂HPO₄/NaH₂PO₄ buffer to give an extinction of about 12, equivalent to approximately 4 mg dry wt ml⁻¹. The effect of pH and salt concentration was studied. The results (Figs 1 and 2) show that all strains autolyzed in phosphate buffer, the extent of lysis being affected by pH and salt concentration. The pH optimum for autolysis of these six strains, as for the previously-studied S. mitis ATCC903 (Linder, 1974b), was above pH 7. One strain, S. sanguis 804, also had a high autolytic activity at pH 4.5 (Fig. 1a). Streptococcus mitis ATCC15909 autolysed to a high degree over a broad range of pH values above 5.5 (Fig. 1e). Exp. cells exhibited higher autolytic activity than stat. cells except for two strains, S. mutans IB and S. mutans KPSK2 (Figs 1d, e), which showed maximal lysis in stat. cells. The pH curves for exp. cells and stat. cells were almost parallel in the other strains, with differences in lysis of 5 to 20 %.

Salt concentration had a more varied affect on lysis than pH. Some strains (Figs 2b, e) showed maximal lysis in 0.05 to 0.2 M buffers, while S. mitis ATCC15912 and exp. cells of S. mutans IB showed increasing autolysis with increasing salt concentration (Figs 2f, d). As can be seen from Figs 1 and 2, S. mitis ATCC15912 and S. mitis ATCC15909 exhibited the highest autolytic activity, being at maximum more than 80 and 60 % respectively.

Whole cells (both exp. and stat.) were also tested for autolysis at 45 °C and 10 °C at various pH values and at various salt concentrations, as in the experiments at 37 °C. Optimal autolysis at 45 °C occurred at the same values of pH and salt molarity as in the experiments at 37 °C. Maximum autolysis was 0 to 5 % less at 45 °C than at 37 °C for both exp. and stat. cells. Autolysis also occurred at 10 °C but at a reduced rate: the maximum autolytic activity was 40 to 50 % of that at 37 °C.

Autolysis of walls

To study autolysis of walls, three strains of S. mitis, ATCC15912, ATCC15909 and ATCC903, were chosen because of their high autolytic activity as demonstrated here and previously (Linder, 1974b). Walls were incubated for 20 h in Na₂HPO₄/NaH₂PO₄ buffer under the same conditions of pH, salt molarity and temperature as for whole cells. The pH optimum for strain ATCC15912 was pH 7.0, and for strains ATCC15909 and ATCC903, pH 7.5 (Fig. 3a). Salt concentration affected lysis of the walls differently in the three strains (Fig. 3b). Walls of strain ATCC15912 exhibited maximum lysis at high salt concentration (1.0 M), while those of strains ATCC903 and ATCC15909 showed two maxima, one at 0.01 M and the other at 0.5 to 1.0 M.

In experiments performed at 45 °C and at 10 °C (Figs 4a, b, c), wall lysis was reduced by
10 to 20% at 45 °C and by 30 to 40% at 10 °C compared with lysis at 37 °C. In strain ATCC903, lysis at 10 °C was reduced by about 80% and remained at a practically constant level over the pH range studied.

Mode of action of the autolytic enzyme system of S. mitis ATCC15912

Figures 1 to 3 show clearly that, of all the strains tested, S. mitis ATCC15912 had the highest autolytic activity for both whole cells and walls. Autolysis of this strain was characterized by a decrease in extinction and by release of soluble N-terminal amino groups. From an initial extinction of the wall suspensions of about 21 (corresponding to 9 mg dry wt ml⁻¹), autolysis started without lag (Fig. 5) and was accompanied by a progressive release of soluble amino groups that was proportional to the decrease in extinction. No release of reducing groups was detected.

Dinitrophenylated amino acids were identified by comparison with known standards of DNFB-derivatives of amino acids on thin-layer plates of silica gel (Ghuysen et al., 1966). Walls were autolysed at 37 °C for 1, 3 and 20 h in 0.2 M-NaH₂PO₄/Na₂HPO₄ buffer, pH 7.5. Two controls were used: a zero-time autolysis supernatant, and the supernatant of autolysin-free walls incubated in the same buffer as the test samples for 20 h at 37 °C. Dinitro-
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Fig. 2. Autolysis of whole cells of six strains of viridans streptococci in sodium phosphate buffer pH 7.5 of different molarities. Strains and symbols as in Fig. 1.

Fig. 3. Autolysis of walls of three *S. mitis* strains in sodium phosphate buffer at different (a) pH values, (b) molarities. ■, ATCC903; ●, ATCC15909; ▲, ATCC15912.
Fig. 4. Effect of temperature on autolysis of walls from three *S. mitis* strains in 0.2 M-sodium phosphate buffer of different pH values. (a) ATCC903; (b) ATCC15909; (c) ATCC15912. △, 10 °C; ○, 37 °C; □, 45 °C.

Fig. 5. Kinetics of autolysis of *S. mitis* ATCC15912 walls, incubated in 0.2 M-sodium phosphate buffer pH 7.5 at 37 °C. Extinction (●) was measured at 550 nm, and free amino groups (■) were determined with 1-fluoro-2,4-dinitrobenzene. Alanine was used as the standard.

Table 1. *Amino acids released into the soluble fraction during autolysis of walls of S. mitis* ATCC15912

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration (μmol ml⁻¹)</th>
<th>Amino acid</th>
<th>Concentration (μmol ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.868</td>
<td>Alanine</td>
<td>1.134</td>
</tr>
<tr>
<td>Histidine</td>
<td>trace</td>
<td>Cystine</td>
<td>trace</td>
</tr>
<tr>
<td>Arginine</td>
<td>ND</td>
<td>Valine</td>
<td>0.726</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.297</td>
<td>Methionine</td>
<td>0.135</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.379</td>
<td>Isoleucine</td>
<td>0.476</td>
</tr>
<tr>
<td>Serine</td>
<td>0.658</td>
<td>Leucine</td>
<td>0.700</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.812</td>
<td>Tyrosine</td>
<td>0.185</td>
</tr>
<tr>
<td>Proline</td>
<td>0.305</td>
<td>Phenylalanine</td>
<td>0.285</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.684</td>
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</tr>
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ND, Not detected.
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phenylated amino groups of alanine, glutamic acid, lysine, valine, serine, glycine and leucine occurred in all three test supernatants (1, 3 and 20 h). All spots which were visible in the 20 h tests were also seen at 1 and 3 h, although they were fainter. No dinitrophenylated products were obtained from the two controls.

The release of free amino acids during autolysis of walls at 37 °C for 5 h was investigated using an amino-acid analyser. Autolysin-free walls were incubated in parallel as a control. Between 14 and 16 amino acids were released into the soluble fraction during autolysis (Table 1), alanine, lysine and glutamic acid being released in greatest quantity.

DISCUSSION

It was suggested in a previous paper that autolysis in *S. mitis* is a physiologically useful mechanism for release of enzymes (Linder, 1974a). In this strain (ATCC903), lysis in sodium phosphate buffer is regulated by the salt concentration. A striking characteristic of autolysis of this strain is that a constant proportion of the cells is resistant to autolysis regardless of whether they are harvested in the exponential growth phase or in the stationary (autolytic) phase. The potentiating effect of high salt concentration on lysis in *S. mitis* ATCC15912 and *S. mutans* in is similar to that in *S. mitis* ATCC903 but has not been reported with other bacteria. However, in *S. faecalis* ATCC9790, autolysis of exp. cells is most rapid at phosphate buffer concentrations of 0.01 M and 0.3 M (Shockman et al., 1961). The present investigation has shown that all strains of viridans streptococci tested autolyse in phosphate buffer and that cells harvested in the stationary phase possess almost the same autolytic activity as exponential cells. This contrasts with the autolytic behaviour of *S. faecalis* ATCC9790 (Shockman et al., 1961) and strains of Lactobacillus (Coyette & Ghuysen, 1970; Neujahr & Logardt, 1973).

Many of the amino acids released during autolysis of the walls of *S. mitis* ATCC15912 were probably not part of the peptidoglycan portion of the wall and may represent a poly-peptide or protein. The observation that autolysin-free walls did not release free amino groups or amino acids indicates that the peptide or protein may have been a wall protein rather than an intracellular or membrane contamination of the wall preparation. It appears that the autolytic system of *S. mitis* ATCC15912 contains endopeptidases which hydrolyse bonds both within the tetrapeptide of the peptidoglycan and within the wall protein. Bacteriolytic enzymes splitting bonds within the tetrapeptide are extremely rare; only two reports on such enzymes have appeared (Neujahr & Logardt, 1973; Welker, 1971). These enzymes are of considerable physiological importance and of experimental and taxonomic interest. We have attempted to isolate the autolytic enzymes of *S. mitis* ATCC15912 but without success. Soluble enzyme activity could not be recovered from the supernatant fluid after wall lysis, perhaps because the autolytic enzymes are firmly bound to the walls, as in *S. faecalis* (Shockman, Pooley & Thompson, 1967), and are released only at unusually high concentrations of salt (Pooley, Porres-Juan & Shockman, 1970). It is also possible that the enzymes are rapidly inactivated after release from the wall. Thus Coyette & Shockman (1973) found that only 20 % of the original wall lytic activity could be recovered in the supernatant after lysis of walls of *Lactobacillus acidophilus* for 16 h at 37 °C.

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REFERENCES


