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Hydrolysis of Soluble, Linear, Un-Cross-Linked Peptidoglycans by Endogenous Bacterial *N*-Acetylmuramoylhydrolases

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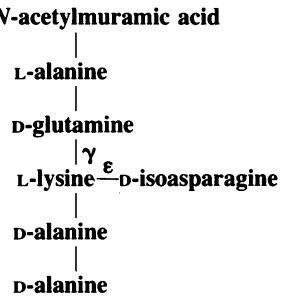
Soluble, linear, uncross-linked peptidoglycans, prepared from two autolysis-defective mutants of *Streptococcus faecium* ATCC 9790 and from *Micrococcus leuteus*, were used as substrates for studies of hydrolysis by an *N*-acetylmuramoylhydrolase (muramidase). The kinetics of hydrolysis of these substrates and the ability of the muramidases isolated from *S. faecium* ATCC 9790 and from two autolysis-defective mutants, Lyt-14 and Aut-3, to carry out transglycosylation reactions were compared with the action of hen egg white lysozyme (EC 3.2.1.17). Hydrolysis of these substrates by the endogenous streptococcal muramidases resulted in the production of disaccharide-peptide monomers with the structure



as nearly the sole product. As estimated from increases in reducing groups, hydrolysis proceeded at a linear rate for extended intervals, with consumption of up to 75% of the substrate, even at substrate concentrations well below the K_m value. Apparent K_m and relative V_{max} values for the three streptococcal enzymes were indistinguishable from each other or from those for hen egg white lysozyme. These results indicate that the autolysis-defective phenotype of these mutants cannot be attributed to differences in their muramidases. In contrast to the action of hen egg white lysozyme, the streptococcal muramidase failed to catalyze transglycosylations. The extended periods of hydrolysis at constant rates are consistent with the occurrence of multiple catalytic events after the formation of the enzyme-substrate complex.

In the preceding paper (1) we described the production and chemical characterization of soluble, linear, uncross-linked peptidoglycan (s-peptidoglycan) produced by autolysis-defective mutants of *Streptococcus faecium* ATCC 9790. Here we describe the use of these polymers and the previously described (12) s-peptidoglycan produced by *Micrococcus luteus* for studies of the mechanism of action of the endogenous, autolytic *N*-acetylmuramoylhydrolases (muramidases; EC 3.2.1.17) produced by strains of *S. faecium* (SF muramidase[s]). Use of these polymers as substrates for muramidases permitted kinetic analyses that heretofore have not been possible due to the insoluble nature of the intact cell or cell wall substrates that are commonly used. The kinetic and other studies of hydrolysis of the polymers presented here have revealed some unusual characteristics of the action of these bacterial enzymes. For example, the firm binding of these enzymes to s-peptidoglycans is consistent with previously reported (14, 16, 17) studies of the binding and action of these enzyme activities in insoluble cell walls and intact cells.

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MATERIALS AND METHODS

Bacterial cultures. The parental strain, *S. faecium* ATCC 9790, and its two autolysis-defective mutants, Lyt-14 (4) and Aut-3 (18), were stored and grown as described elsewhere (1). *M. luteus* (generously provided by D. Mirelman) was grown at 32°C with aeration in a complex medium containing 0.5% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 0.2% yeast extract (Difco), 0.5% NaCl, and 2% glucose (11). Growth was monitored turbidimetrically as described in the preceding paper (1).

Preparation of cell walls. Cell walls were prepared from exponential-phase cultures after cell disruption in a Ribi cell fractionator (Ivan Sorvall, Inc., Norwalk, Conn.) or with plastic beads in a Braun MSK homogenizer (17). Walls (25 mg) were treated at room temperature with 25 ml of 2% sodium dodecyl sulfate to inactivate endogenous autolytic activity and washed thoroughly with water as described previously (17).

Purification of the SF muramidases. The latent (proteinase-activatable) form of the SF muramidase was extracted from 180 mg of native cell walls (20 mg/ml in water) of *S. faecium* by the addition of 0.35 ml of 0.2 N NaOH to 9 ml of the aqueous wall suspension (0.008 N NaOH, final concentration). Extraction was carried out at 0°C in the presence of 0.3

mg of bovine serum albumin per ml as a stabilizing agent. The addition of NaOH was immediately followed by centrifugation (130,000 × *g*, 10 min, 4°C), removal of the supernatant, and rapid neutralization of the supernatant by the addition of 0.35 ml of 0.2 N HCl in 0.25 M sodium phosphate, pH 7.0 (3, 10). The soluble SF muramidase in 10 mM sodium phosphate, pH 7.0, was bound to a concanavalin A-Sepharose 4B affinity column (5 ml) at 4°C. The column was thoroughly washed with 10 mM sodium phosphate, pH 7.0, and the enzyme activity was eluted with 50 mM α-methylmannoside in 10 mM sodium phosphate and 50 mM NaCl, ph 7.0 (10; J. B. Cornett and G. D. Shockman, unpublished data). After dialysis against 10 mM sodium phosphate, pH 7.0, at 4°C (four times against 2 liters; 12,000-molecular-weight retention dialysis tubing) to remove the α-methylmannoside, the latent (zymogen) form of the enzyme was lyophilized and stored at −70°C. The same procedure was followed for extraction and purification of the muramidase from the parental strain and from the two autolysis-defective strains. Isolated cell walls of *S. faecium* contained 6 to 7 U of total autolysin activity per mg of dry weight (ca. 60 U/mg of wall protein). Alkaline extracts usually contained ca. 18 to 20 U of activity per mg of extracted protein, and enzyme purified by affinity chromatography had a specific activity of ca. 5.2 U/μg of protein (see below for definition of U).

Assay for SF muramidase activity. Activity of the SF muramidase was quantified by its ability to dissolve sodium dodecyl sulfate-inactivated cell walls of *S. faecium* (0.2 mg/ml) in 1.5 ml of 10 mM sodium phosphate, pH 7.0 (17). The zymogen form of the muramidase was activated by preincubation (3 min) at 0°C with trypsin (0.2 μg/ml) or bovine plasma albumin (75 μg/ml), with which contains a proteinase activity (19) that activates the zymogen. Decreases in turbidity of wall suspensions were measured at 450 nm with a Gilford 300 spectrophotometer. One unit of enzyme activity is defined as the amount which reduces the turbidity of the sodium dodecyl sulfate walls by 0.001 optical density per min at 37°C. The specific activity of the partially purified SF muramidase was 5.2 U/μg of protein, unless otherwise indicated. Muramidase preparations have been reported to have a maximum specific activity of ca. 11.5 U/μg (10), indicating that the preparations used here are ca. 50% catalytically active enzyme.

Production of s-peptidoglycan. By using the procedure of Mirelman et al. (12), incubation of *M. luteus* cells in a chemically defined medium with benzylpenicillin (a generous gift of Wyeth Laboratories, Radnor, Pa.) resulted in the secretion of s-peptidoglycan into the culture medium. This polymer has been characterized (12) as a linear, uncross-linked peptidoglycan of ca. 40 disaccharide units with ca. 50% peptide substitution of the *N*-acetylmuramic acid residues, presumably due to the action of a penicillin-insensitive amidase (8, 12). To prevent degradation of the s-peptidoglycan by an endogenous autolysin (presumably an amidase) (8), the culture medium was exposed to 95 to 100°C for 7 min before dialysis. The s-peptidoglycan was separated from other substances by gel filtration on Sephadex G-100 as described in the preceding paper (1) and detected by radioactivity or absorption at 225 nm. Fractions ($K_d \cong 0$ to 0.25) containing the *M. luteus* s-peptidoglycan were pooled, lyophilized, and stored at −70°C. Analysis of the s-peptidoglycan for reducing groups before and after acid hydrolysis indicated an average chain length of 40 disaccharide-peptide (DSP_1) units (DSP_{40}). The s-peptidoglycan polymers from *S. faecium* (strains Lyt-14 and Aut-3) had chain lengths of 42 to 48 DSP_1 units (1).

Assays for hydrolysis of s-peptidoglycans. Several different assays were used to determine the kinetics and the products of hydrolysis of s-peptidoglycans. Aqueous samples of s-peptidoglycan were lyophilized in tubes (12 by 75 mm). At 0°C, enzyme and, when appropriate, trypsin (0.2 μg/ml) or bovine plasma albumin (75 μg/ml) were added to activate the SF muramidase. Reactions were incubated in 10 mM sodium phosphate, pH 6.8, at 37°C. Estimates of reducing groups (13) from known amounts of unlabeled s-peptidoglycan as substrate proved to be the most useful assay to analyze initial rates of polymer hydrolysis. To determine the products of hydrolysis, radiolabeled polymers (synthesized in wall medium containing [^{14}C]glucose or [^{3}H]glucose, [^{14}C]lysine or [^{3}H]lysine, or [^{14}C]alanine [or a combination of these] as described previously [1]) were incubated in 10mM sodium phosphate, pH 6.8, with either SF muramidase or hen egg white lysozyme (HEWL). The products of hydrolysis were separated by paper chromatography. Paper chromatograms were run at room temperature for 72 h in solvent 1 (butanol-acetic acid-water [4:1:5, vol/vol/vol; upper phase]). In this solvent system, R_{DSP} values for the glycan oligomer(s) larger than three DSPs (DSP_n) and the glycan dimer (DSP_2) of the DSP_1 were 0 and 0.43, respectively. Radiolabeled standards (DSP_1, DSP_2, and DSP_n) were obtained from HEWL hydrolysis of radiolabeled strain Lyt-14 s-peptidoglycan, separated in the same solvent system, and identified against known standards. Although this method proved useful for analysis of hydrolysis products, it was difficult to use for kinetic analyses.

Release of DSP_1 from labeled polymers and production of reducing groups were simultaneously monitored over longer time intervals of hydrolysis to establish the coincidence of these assay methods. ^{14}C-labeled Lyt-14 s-peptidoglycan (1.17 μM) in 1.9 ml of 10 mM sodium phosphate, pH 7.0, was incubated at 37°C with 5 U of SF muramidase, which had been preactivated with trypsin, per ml. Samples (125 μl) were taken at intervals for detection of reducing groups (13) and release of radiolabeled products (DSP_1 and DSP_2), and

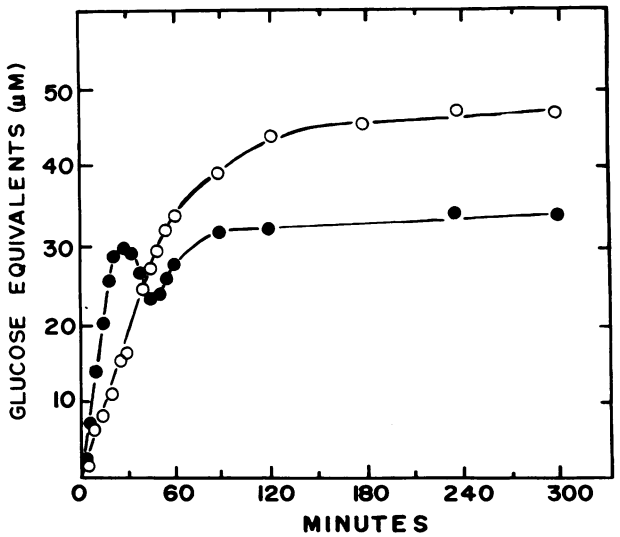

FIG. 1. Comparison of the time course of hydrolysis of *M. luteus* s-peptidoglycan by SF muramidase and by HEWL. The reaction mixtures contained 52 μM (○) or 60 μM (●) *M. luteus* s-peptidoglycan (as DSP_1) and either 10 U of trypsin-activated SF muramidase per ml (○) or 10 U of HEWL per ml (●) in 1.8 ml of 10 mM sodium phosphate, pH 6.8.

the samples were fractionated by descending paper chromatography on Whatman 1 filter paper for 48 h in solvent 1.

The assay for transglycosylase activity was similar to that used to detect this activity of HEWL (15). In general, the ability of radiolabeled low-molecular-weight compounds to be transferred to and appear in higher-molecular-weight compounds upon transglycosylation was examined by the differential mobility of these compounds on paper chromatography in solvent 1. In all experiments, HEWL was used as a control to ensure that transglycosylation could be detected. Reactions were carried out as described in the legend for Fig. 4. Reaction mixtures containing different enzyme-to-substrate ratios (with radiolabeled DSP_1 as substrate) were incubated with unlabeled s-peptidoglycan oligomers, and samples were taken at 20 and 40 min. Products were identified by paper chromatography in solvent 1 with DSP_1 and DSP_2 clearly separable from higher oligomers (DSP_n). Decreased radioactive material migrating with the mobility of DSP_1 and the appearance of radioactivity near the origin (DSP_n) were taken as an indication of transglycosylation.

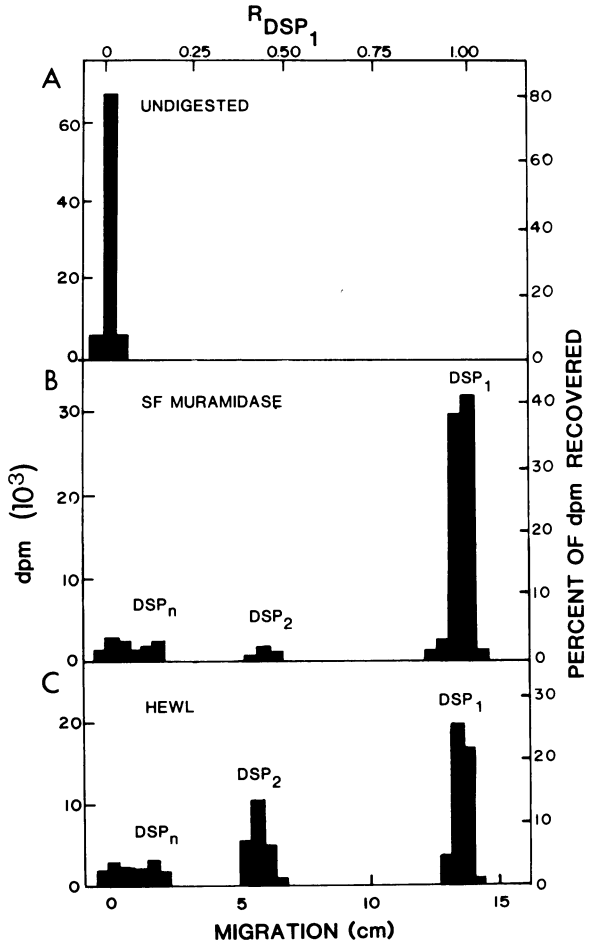

FIG. 2. Paper chromatographic analysis of the products of hydrolysis of *S. faecium* s-peptidoglycan by two muramidases. ^{14}C-s-peptidoglycan, produced by the Lyt-14 mutant (2μM, ca. 80,000 dpm), incubated at 37°C for 1 h with: (A) no enzyme; (B) SF muramidase (5 U), preactivated with 0.2 μg of trypsin per ml; or (C) HEWL (5 U) in a total volume of 200 μl of 10 mM sodium phosphate, pH 6.8. After the reactions were stopped (100°C, 10 min), samples were analyzed by descending paper chromatography (72 h) in solvent 1 as described in the text.

RESULTS

Hydrolysis of s-peptidoglycan by SF muramidase and HEWL. Several differences were noted between the actions of SF muramidase and HEWL on the s-peptidoglycan of *M. luteus* (Fig. 1). First, HEWL released only 60% of total available reducing ends over the 5-h incubation. After 30 to 40 min of hydrolysis by HEWL, a consistent decrease in the number of reducing groups was observed. Based on published data concerning the hydrolysis of various substrates by HEWL (6, 15), this decrease is consistent with the known glycosylation activity of HEWL, which can lead to net chain elongation. In contrast, hydrolysis by SF muramidase resulted in the liberation of greater than 90% of the available reducing ends (47 of the 52 μM DSP_1 present in the substrate). In addition, DSP_1 was the predominant product of hydrolysis of the substrate by SF muramidase (Fig. 2 and 3). In contrast, almost equal amounts of DSP_1 and DSP_2 were seen as products of HEWL action for 1 h (Fig. 2). The action of the SF muramidase did not result in a transient decrease in the reducing ends as seen with HEWL. Exposure of [^3H]ly-

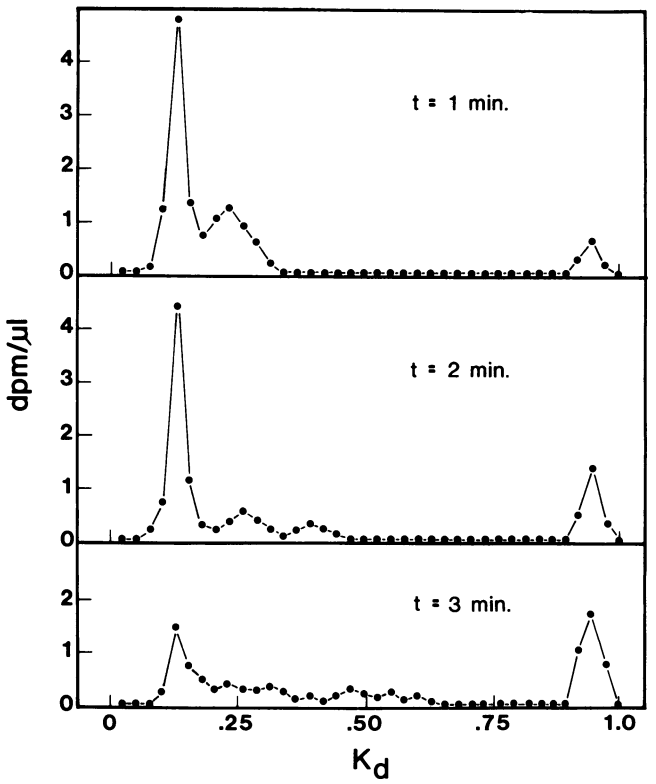

FIG. 3. Sephadex G-100 profiles of samples from a reaction mixture containing preactivated SF muramidase (17 U), 0.3 μM (chains) [^3H]lysine-s-peptidoglycan (containing 12,000 dpm), and 10 mM sodium phosphate (pH 6.8) in a total volume of 920 μl. At 0, 1, 2, and 3 min, samples (230 μl) were taken and the reaction was stopped by the addition of 0.5 ml of boiling water and heating at 100°C for 5 min. Samples were applied to the Sephadex G-100 column (1) and eluted with water (3.6-ml fractions). Counts are expressed in disintegrations per minute per microliter for each column fraction. Peak fractions of K_d greater than 0.9 were pooled, lyophilized, and chromatographed to identify the product(s) (data not shown).

sine s-peptidoglycan to SF muramidase for short intervals resulted in the production of low-molecular-weight material with a K_d greater than 0.9 (molecular size of less than ca. 5,000) upon filtration on a Sephadex G-100 column (Fig. 3). The low-molecular-weight peak (K_d over 0.9) from each time point was separately pooled, concentrated, and analyzed by paper chromatography in solvent 1. In the 1- and 2-min samples, only DSP_1 was detected. The 3-min sample contained 95% DSP_1 and 5% DSP_2. Also, no evidence of glycosylation or transglycosylation activity for the SF muramidase was observed (Fig. 4). With the SF muramidase, linear rates of hydrolysis for over 50 min and release of over 70% of the potential reducing groups at the initial reaction rate were commonly observed (Fig. 1). This prolonged, initial rate period, even at lower substrate concentrations (Fig. 5), appeared to be an anomaly in terms of predicted behavior according to the Michaelis-Menten theory.

Optimum pH and ionic strength for the action of the SF muramidase. The initial velocity of hydrolysis of *M. luteus* s-peptidoglycan by the SF muramidase exhibited optimum activity at pH 7.0 at 150 μM substrate (as DSP_1). The pH profile shows greater than 50% of the maximum rate over the rather broad range of pH 6.0 to 8.0 and at an ionic strength of 0.02 μ (Fig. 6A). When the ionic strength was varied and the pH fixed at 7.0 (Fig. 6B), the maximum activity was at 0.015 μ. Apart from a small increase in rate near this optimum value, the muramidase is relatively insensitive to ionic strength over the range of 0.005 to 0.15 μ.

Kinetic parameters of s-peptidoglycan hydrolysis. At a constant concentration of SF muramidase, increased concentrations of strain Lyt-14 s-peptidoglycan resulted in increased initial rates of hydrolysis (Fig. 5, inset). Similarly, increased enzyme concentrations increased the rates of hydrolysis of a constant substrate concentration (data not shown). The initial rate data of Fig. 5 (inset) and similar data from additional experiments were analyzed to obtain V_{max} and apparent K_m values. Figure 7 shows representative Michaelis-Menten and Lineweaver-Burk (inset) plots of hydrolysis of strain Aut-3 s-peptidoglycan by the SF muramidase. Analysis of the initial rates gave a good fit to the equation for a rectangular hyperbola, consistent with Michaelis-Menten kinetics under the conditions of the assay (Fig. 7). The Lineweaver-Burk plot indicates an apparent K_m of 73 μM in terms of DSP_1.

Comparative K_m and V_{max} values for the muramidases. The apparent K_m values for the muramidases from the parental strain and the two autolysis-defective strains of *S. faecium* were not significantly different (Table 1). However, there were some differences in relative V_{max} values, presumably due to the fact that the enzyme preparations were not of uniform purity. Based on the specific activity of the highly purified SF muramidase (10), all preparations were about 50% pure.

Time course of s-peptidoglycan hydrolysis. As described above, extended periods of initial rates of hydrolysis were observed even when the substrate concentration was well below the apparent K_m value (Fig. 1 and 5). This observation suggested that the initial combination of substrate and enzyme was the most important factor for determining the initial reaction rate. Once hydrolysis was initiated at a particular rate, it continued at this rate even when the substrate concentration (concentration of intact polymer) was reduced to a level that would have resulted in a lower initial rate. An example of these observations is seen in Fig. 5. Even at relatively high ratios of enzyme to substrate, an extended period of hydrolysis at the initial rate was observed. Together with previous data, which indicated that the SF muramidase has a high affinity for binding to intact

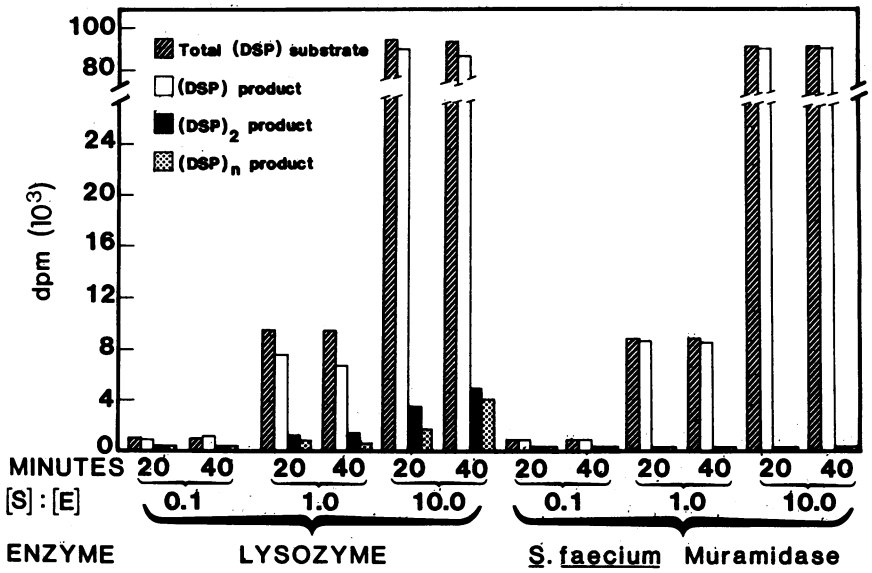

FIG. 4. Transglycosylase activity of HEWL and SF muramidase. The reaction mixtures contained 0.048, 0.48, or 4.8 μM ^{14}C-DSP_1, 0.48 μM unlabeled DSP_n, 5 U of HEWL or 5 U of preactivated SF muramidase, and 10 mM sodium phosphate (pH 6.8) in 20 μl. After 20 and 40 min at 37°C, 10-μl samples were removed, and the reactions were stopped (100°C, 7 min). Products were separated by paper chromatography (descending, 48 h) in solvent 1 and identified and quantified by detection of radioactivity. Incorporation of ^{14}C-DSP_1 into unlabeled DSP_n at or near the origin of the paper chromatogram was considered to be evidence for transglycosylation in this system. DSP_1 and DSP_2 were clearly separated and quantitatively recovered; the glycan trimer of the DSP and longer chains remained at or near the origin and are considered to be DSP_n.

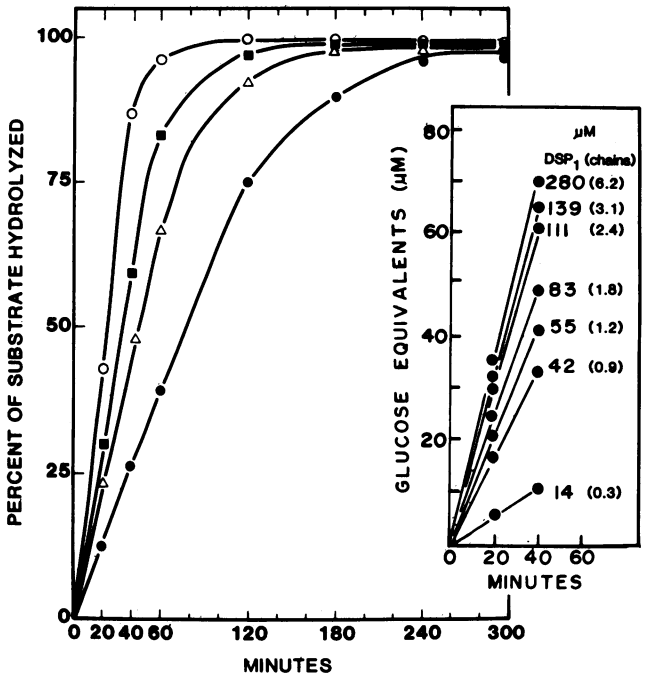

FIG. 5. Kinetics of hydrolysis of *M. luteus* s-peptidoglycan by the SF muramidase. Reaction mixtures contained 14 to 280 μM s-peptidoglycan as DSP_1 or 0.3 to 6.2 μM as glycan chains, 10 mM sodium phosphate (pH 6.8), and 10 U of SF muramidase per ml (preactivated at noncatalytic conditions [0°C] with 0.2 μg of trypsin per ml) in 1.8 ml. During incubation at 37°C, samples (200 μl) were removed at intervals, and reactions were stopped (100°C, 7 min). Products were quantified by the reducing group assay (13). Shown at right is the series of reactions containing different substrate concentrations for the first 40 min of incubation. Shown at left are four of these reactions carried to completion (0.3 [○], 1.8 [■], 3 [△] or 6.2 [●] μM s-peptidoglycan chains) and plotted as percentage of substrate hydrolyzed against time of incubation.

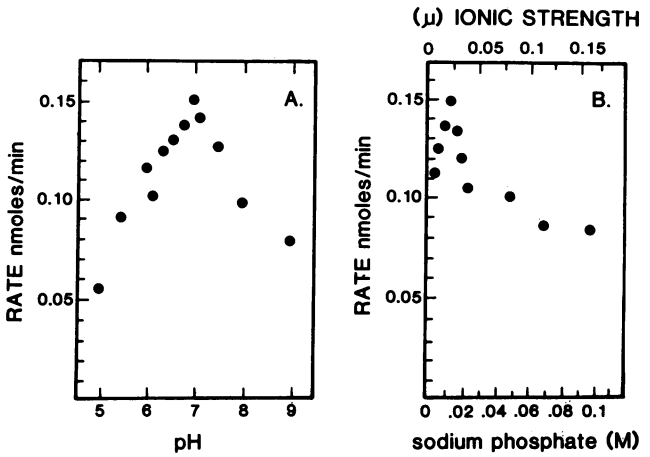

FIG. 6. Determination of the optimum pH and ionic strength for hydrolysis of *M. luteus* s-peptidoglycan by the SF muramidase. The reaction mixture contained 150 μM *M. luteus* s-peptidoglycan (in terms of DSP_1), 10 mM sodium phosphate (pH 6.8), and 85 U of trypsin-activated SF muramidase per ml in 0.2 ml. During incubation at 37°C, samples were taken at intervals for quantification of reducing groups. To determine optimum pH for hydrolysis, a constant ionic strength of ca. 0.02 μ was maintained while varying the range of pH from 5.0 to 8.0 (by varying the ratio of monobasic to dibasic 10 mM sodium phosphate). To obtain a pH of 9.0, 5 mM NaOH was added to dibasic sodium phosphate, which did not change the ionic strength significantly. Ionic strength was varied while maintaining pH 7.0 by varying the concentration of sodium phosphate.

cell walls (14, 16), these data suggested that enzyme dissociation from the s-peptidoglycan is slow and that multiple hydrolytic events may occur during the life of a single enzyme-substrate complex.

Products of s-peptidoglycan hydrolysis. The products of s-peptidoglycan hydrolysis by the SF muramidase were detected by paper chromatography and reducing group analysis and by distribution of radiolabels during hydrolysis of [^{14}C]glucose-labeled Lyt-14 s-peptidoglycan (Fig. 8). Increases of one product, DSP_1, closely paralleled the generation of new reducing ends and accounted for essentially all of the material that migrated from the origin of the chromatograms. These data are consistent with the production of one DSP_1 per glycosidic bond hydrolyzed. There was no evidence that the SF muramidase could carry out transglycosylation under incubation conditions that permitted HEWL to catalyze transglycosylation (Fig. 4).

A 10-fold molar excess of DSP_1 (2.0 μM) incubated with ^{14}C-s-peptidoglycan chains (0.2 μM) failed to affect the initial rate of product formation or the endpoint of hydrolysis of the radiolabeled substrate (data not shown).

DISCUSSION

Hydrolysis of s-peptidoglycan by HEWL (Fig. 1) resulted in a period of rapid increase in reducing groups that was followed by a decrease, an increase, and then a plateau in reducing groups. Only ca. 60% (ca. 35 of 60 μM) of the

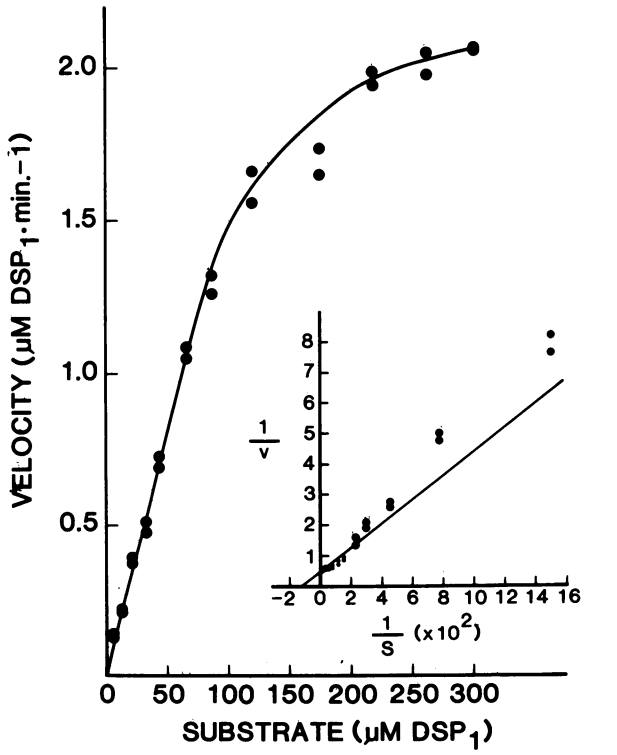

FIG. 7. Michaelis-Menten and Lineweaver-Burk (inset) plots of duplicate samples of the hydrolysis of strain Aut-3 s-peptidoglycan. Reaction mixtures contained unlabeled s-peptidoglycan (5.6 to 300 μM DSP_1), 10 U of trypsin-activated SF muramidase per ml, and 10 mM sodium phosphate (pH 6.8) in 1.8 ml. The points represent the equation for a rectangular hyperbola (2). V, Velocity; S, substrate concentration.

TABLE 1. Kinetic constants for the action of the SF muramidases[a] and HEWL[b] on s-peptidoglycans[c]

Enzyme	Apparent K_m (μM DSP_1)[d]			Apparent V_{max} (nmol of DSP_1 released · min^{-1} · mg of $enzyme^{-1}$)		
	M. luteus	Lyt-14	Aut-3	*M. luteus*	Lyt-14	Aut-3
S. faecium ATCC 9790 muramidase	87 ± 8	83 ± 12	74 ± 6	260 ± 10	210 ± 10	230 ± 10
Lyt-14 muramidase	64 ± 10	62 ± 8	60 ± 11	200 ± 10	170 ± 10	160 ± 10
Aut-3 muramidase	72 ± 8	65 ± 12	68 ± 11	190 ± 10	170 ± 10	160 ± 10
HEWL	84 ± 4	81 ± 8	77 ± 6	450 ± 20	330 ± 20	370 ± 20

[a] Molecular weight, 87,000 (10); 5.2 U/μg of protein.
[b] Molecular weight, 14,500; 22 U/μg of protein.
[c] Seventeen units of enzyme with various concentrations of substrate, with assay conditions as described in the legend to Fig. 7, were used.
[d] μM chains = μM DSP_1/chain length in terms of DSP_1.

potential reducing groups in the s-peptidoglycan substrate were produced. Analyses of hydrolysis products by paper chromatography were consistent with the production of multiple products including oligomers (Fig. 2). The interval of decreasing reducing groups observed during hydrolysis by HEWL was apparently due to a period of increased consumption of reducing groups by glycosylation (6, 15) along with hydrolysis.

In contrast, the action of the SF muramidase resulted in a continuous increase in reducing groups, with a yield of ca. 90% (47 of 52 μM) of the potential reducing groups in the s-peptidoglycan substrate. Only low-molecular-weight products were produced (Fig. 2 and 3). Also, there was an unusually long period of constant rate of hydrolysis (Fig. 5), suggesting that the molar concentration of the initial enzyme-substrate complex does not change even after a substantial fraction of substrate has been hydrolyzed.

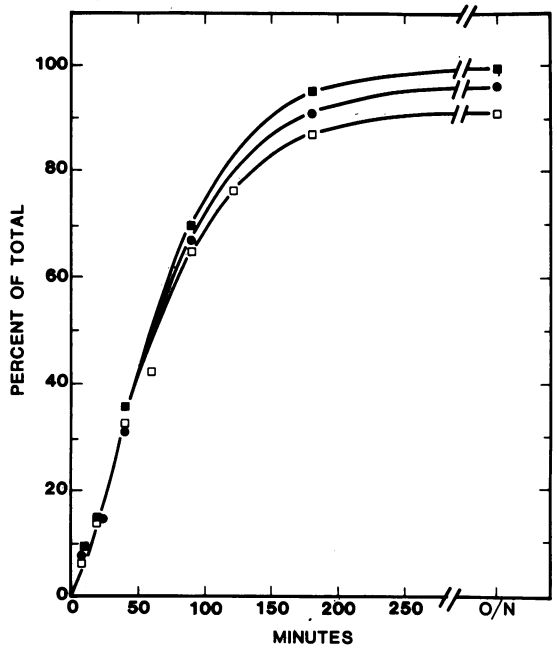

FIG. 8. Analyses of products of hydrolysis of strain Lyt-14 s-peptidoglycan by the SF muramidase. Parameters are expressed in percentage of total available glycosidic bonds between DSP_1. The reaction mixture contained 1.17 μM [^{14}C]glucose-labeled strain Lyt-14 s-peptidoglycan (as DSP_1), 5 U of SF muramidase (preactivated with trypsin at 0°C) per ml in 1.9 ml of 10 mM sodium phosphate, pH 6.8. The parameters measured and plotted are: percentage of total available reducing groups generated (□); percentage of total available DSP_1 produced (●); and percentage of ^{14}C that migrated away from the origin (■) after paper chromatography in solvent 1 for 48 h.

There was no evidence for glycosylation or transglycosylation activity by the SF muramidase as compared with observed activity for HEWL (Fig. 1 and 4). Also, the major product of SF muramidase action, DSP_1, failed to inhibit hydrolysis at inhibitor-to-substrate ratios as high as 10:1. The initial rate of hydrolysis of *M. luteus* s-peptidoglycan by the SF muramidase exhibited broad optima for both pH and ionic strength (Fig. 6). These values are essentially the same as those previously reported for the hydrolysis of sodium dodecyl sulfate-inactivated walls of *S. faecium* by the SF muramidase (17). Similar results were reported (5) for HEWL on *M. luteus* cells (broad pH optimum of 6.2 to 9.2; broad ionic strength optimum, with the peak at μ = 0.07).

When calculated in terms of potentially susceptible bonds (DSP_1), the apparent K_m values for hydrolysis of the three different s-peptidoglycans (*M. luteus* and *S. faecium* strains Lyt-14 and Aut-3) by the muramidases isolated from the three strains of *S. faecium* and HEWL (Table 1) were indistinguishable (73 ± 8 μM DSP_1). These data suggest that changes in the kinetic properties of this enzyme activity do not contribute to the autolysis-defective phenotype of the two mutants. The apparent K_m values shown in Table 1 are in terms of concentrations of total DSP_1. In terms of concentration of s-peptidoglycan chains, the apparent K_m values would be ca. 1.5 μM. Interpretation of these values depends partly on whether enzyme can bind to numerous sites on the peptidoglycan chain (e.g., at each DSP_1) or whether each polymer contains one or more unique binding sites for enzyme (e.g., at the reducing or nonreducing end of the polymer).

M. luteus s-peptidoglycan is known to differ from that of *S. faecium* in two respects: (i) not all muramic acid residues are substituted with peptide chains, and (ii) the amino acid composition of the peptide side chains differs from that of *S. faecium* (7, 12). The similarity of kinetic parameters (Table 1) for each of the enzymes on the two different substrates indicates that the above two factors do not substantially influence K_m or V_{max} values in vitro. In the case of the muramidase from strain Lyt-14, these results were somewhat unexpected, since previous studies (4) showed that although crude (alkaline) extracts of walls of the parent strain dissolved walls of both *M. luteus* and *S. faecium*, similar preparations from strain Lyt-14 failed to dissolve walls of *M. luteus* but were active in dissolving walls of *S. faecium*. This as yet unresolved discrepancy could be due to differences in nonpeptidoglycan wall polymers, peptide cross-linking of glycan chains in intact walls, or the difference in purity of enzyme preparations used. As described elsewhere (9), it seems highly likely that the ability of crude extracts of the parental strain to dissolve walls of *M. luteus*

is due to the action of a second peptidoglycan hydrolase and not to that of the SF muramidase used in these experiments.

The data presented here indicate that the mechanism of hydrolysis of peptidoglycan by the SF muramidase differs substantially from the endoglycosidase action of HEWL. Our data suggest that firm binding of the SF muramidase to the substrate greatly influences kinetic parameters and the production of a single product, DSP_1. More recent studies of the exact mechanism of SF muramidase hydrolysis have resulted in data indicating that after binding to an s-peptidoglycan chain, the enzyme processively hydrolyzes each susceptible bond, starting at or very near the nonreducing end (J. B. Barrett, D. L. Dolinger, V. L. Schramm, and G. D. Shockman, manuscript in preparation).

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