Autolytic Enzyme Associated with Cell Walls of *Bacillus subtilis*  

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**SUMMARY**

Autolysis of cell walls of the highly transformable strain 168 of *Bacillus subtilis* is accompanied by the release of N-terminal L-alanine without a concomitant release of C-terminal amino acids, reducing groups, or phosphomonoester groups. These observations show that the enzyme is an N-acylmuramyl-L-alanine amidase. Autolysis follows first order kinetics, requires an energy of activation of 9.2 kcal per mole, and results in the hydrolysis of 87% of the amide bonds between muramic acid and L-alanine. Since the enzyme is most active during logarithmic growth of the population, it is suggested that its action coupled with a transpeptidase is involved in the expansion of the cell wall.

Despite the widespread occurrence of autolytic enzymes in both gram-positive (1-6) and gram-negative organisms (7), the mechanisms of action and the physiological significance of these enzymes are poorly understood. In various species of bacteria, autolytic enzymes may occur during logarithmic growth (2), in the stationary phase of the growth cycle (5, 6), and in association with the germination of spores (4). These enzymes may therefore be involved in growth and differentiation of bacteria in addition to their function during lysis as emphasized recently by Shockman (8). The production of extracellular lytic factors might provide a selective ecological advantage either by directly initiating the lysis of other organisms or by liberating degradative enzymes such as proteases or lipases that were intracellular or were bound to the cell wall; conversely, a protease might activate the autolytic enzyme and initiate lysis (9). During studies of competence in the *Bacillus subtilis* transformation system, an autolytic enzyme was observed associated with purified cell walls of the highly transformable strain of *B. subtilis* (10). Lower enzyme activity was noted in the cell walls of numerous poorly transformable strains and in the highly competent strain grown under conditions unfavorable for the development of maximal competence. These observations suggest that the autolytic enzyme might facilitate the penetration of deoxyribonucleic acid into the cell.

The present study of the mechanism of autolysis was initiated in order to gain a further insight into the factors that influence competence. The data show that the autolytic enzyme appears during the early phase of logarithmic growth and reaches a maximum at the onset of the appearance of competence in the culture. This enzyme specifically cleaves the bond between N-acylmuramic acid and L-alanine. It therefore appears to be similar in specificity to the extracellular N-acylmuramyl-L-alanine amidase of *Streptomyces albus* (11). Preliminary reports on the mechanism of autolysis have been presented previously (12, 13).

**EXPERIMENTAL PROCEDURE**

**Preparation of Cell Walls**

*B. subtilis* 168 I-C+ was employed in these studies. The I and C refer to indole auxotrophy and competence, respectively (14). *B. subtilis* 168 I-C+ was grown for various intervals at 37°C on a rotary shaker in 2-liter flasks containing 800 ml of minimal medium (14) supplemented with 0.02% casein hydrolysate (Nutritional Biochemicals), 22 mM glucose, 24 mM L-tryptophan, and 5 mM MgSO4, which will be designated as standard minimal medium. The cells were harvested by centrifugation and disrupted by mechanical disintegration, and the cell walls were isolated as described previously (14).

**Assay of Autolytic Enzyme**—The activity of the autolytic enzyme was determined by following the decrease in optical density (600 mp) of suspensions of cell walls in Tris, NaHCO3, and (NH4)2CO3 buffers. The pH, molarity, and the temperature of the reaction mixtures are described separately with each experiment. The autolytic enzyme was inactivated by boiling the cell walls (10 mg/ml in distilled water) for 5 min. These heat-inactivated cell walls were used as substrate in the experiments with lysozyme and as the control in determinations of the C-terminal, N-terminal, and reducing groups released during autolysis. Loss in transfer of samples was minimized by performing autolysis and all of the chemical reactions for determination of N-terminal and C-terminal groups in a hydrolysate tube (18 x 150 mm) with a constriction to facilitate sealing.

**Chemical Analyses**—The release of N-terminal groups during autolysis was determined by reaction with ninhydrin (15) and 2,4-dinitro-1-fluorobenzene (16). In these experiments, 10 mg of native and 10 mg of heat-inactivated cell walls were each suspended in 2.5 ml of distilled water containing 0.12 mmoles of NaHCO3. The samples were incubated for 60 min at 40°C to effect...
autolysis of the native cell walls. There was no significant decrease in the optical density of the heat-inactivated cell walls during this incubation. A 0.1-ml aliquot of DFB 2 was added to the samples of native and heat-inactivated cell walls. As a control, samples of both native and heat-inactivated walls were handled similarly with the exception that DFB was not added. The samples were incubated for 2 hours at 40° in the dark with vigorous shaking, acidified with concentrated HCl (0.1 ml), and extracted with ether. The aqueous phase was lyophilized, suspended in 4 N HCl, and hydrolyzed for 6 hours at 105°. The samples were cooled, filtered, and extracted with ether. The ether layer was dried over (Na)2SO4 and the excess dinitrophenol was removed by sublimation. The dinitrophenol derivatives in the ether layer were identified by ascending thin layer chromatography on Silica Gel G employing two solvents (Solvent 1: butanol-1% ammonia, v/v 1:1, upper phase; Solvent 2: CHCl3, CH3OH, CH3COOH 85:14:1). The aqueous layer was analyzed on the Beckman/Spinco amino acid analyzer as described previously (14). The specific configuration of the alanine released during autolysis was determined enzymatically with l-alanine-α-ketoglutarate transaminase and d-amino acid oxidase (12).

C-Terminal Groups

Samples, 10 mg, of native and heat-inactivated cell walls were incubated in NaHCO3 for 80 min at 40° as described in the preceding paragraph. The samples were lyophilized, dried for 24 hours at 105°, and suspended in 1.0 ml of anhydrous hydrazine (Matheson, Coleman, and Bell, Inc.). The samples were sealed, hydrolyzed at 100° for 8 hours (17), cooled, allowed to react with isovaleraldehyde and ethyl acetate, and then analyzed on the Beckman/Spinco amino acid analyzer (18).

Other Chemical Analyses

The increment in reducing groups (19), Morgan-Elson-reactive material (16), and phosphorus and phosphomonoester groups (20) was determined on samples of heat-inactivated and native cell walls during autolysis.

RESULTS

Lysis of Cell Walls

Samples of cell walls of B. subtilis autolyze rapidly when suspended in 20 mM NaHCO3. Concomitant with the decrease in optical density at 600 ma there was an increase in ninhydrin-reactive material (Fig. 1), but no release of reducing groups (Fig. 1). Morgan-Elson-reactive material or phosphomonoester groups. Samples of heat-inactivated native cell walls were allowed to react with DFB before and after autolysis in 60 mM NaHCO3 buffer (pH 9.6). Under these conditions, only the native cell walls undergo autolysis. As a control, samples were treated in an identical fashion with the exception that DFB was not added. All of the samples were extracted with ether and hydrolyzed as described in “Experimental Procedure.” Chromatographic analysis of the dinitrophenol derivatives in the ether phase revealed only dinitrophenol-alanine. In order to determine the configuration of the alanine which reacted with DFB, the residual alanine in the aqueous phase was measured with α-ketoglutarate transaminase and d-amino acid oxidase. The difference between the content of l- and d-alanine in samples which were not treated with DFB and those which were treated with DFB would be a measure of the amount of L- and D-alanine liberated during autolysis. As shown in Table I this increment was solely in l-alanine. Previous studies have shown that the cell wall of B. subtilis contains 0.74 and 0.32 μmole per mg of alanine and glutamic acid, respectively (21). Therefore, 0.87 mole of N-terminal alanine per mole of glutamic acid in the mucopeptide is released during autolysis. The complete analysis of the aqueous phase on the Beckman/Spinco amino acid analyzer demonstrated that the only difference between the native autolyzed cell walls and the heat-inactivated control cell walls was in alanine. At least 80% of the e- and o- amino groups of diaminopimelic acid and greater than 85% of the amino groups of the N-acyl amino sugars are not available for reaction with DFB. The increment in N-terminal groups as measured by ninhydrin-reactive material, Fig. 1 (using alanine as the standard), is 0.52 μmole per mg which is in close agreement with the amount of
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Fig. 2. Kinetics of autolysis. The turbidometric data from three experiments similar to that described in Fig. 1 were plotted as a function of the logarithm of the ratio of the initial turbidity ($C_0$) and the turbidity at various times during incubation ($C_t$).

Fig. 3. Lysis of cell walls by lysozyme. A 1.5 mg per ml suspension of cell walls of B. subtilis was incubated with crystalline egg white lysozyme in 50 mM potassium phosphate buffer (pH 6.3) at 38°C. Samples were removed for determination of reducing groups and N-terminal groups as described in "Experimental Procedure." ○, optical density; ▲, reducing groups; ▲, N-terminal groups.

L-alanine released during autolysis as determined by the more specific enzymatic method (0.28 μmole per mg). The presence of considerable ninhydrin-reactive material at zero time is due in part to the alkaline labile amino acids which consists primarily of D-alanine, the ε-amino groups of diaminopimelic acid which are not protected (less than 20% of the total diaminopimelic acid), and the free amino groups of the amino sugars (less than 15% of the total amino sugars in the cell wall), and in part related to non-specific reaction of the ninhydrin with the cell walls which accounts for no more than 25% of the total ninhydrin-reacting material at zero time.

Alanine was the only significant C-terminal amino acid in the cell wall (Table I). There was no detectable difference in C-terminal groups between samples of heat-inactivated and autolyzed cell walls.

As shown in Fig. 2 the autolysis of cell walls follows first order kinetics. Therefore all data will be expressed as a function of the first order rate constant. Greater than 90% of the total mass of the cell wall is solubilized during this reaction.

In contrast to the 85% decrease in optical density after 90 min of autolysis, there was only a 35% decrease in optical density when cell walls of B. subtilis were incubated with lysozyme (Fig. 3). The increase in reducing groups without a concomitant increase of ninhydrin-reactive material is consistent with the proposed mechanism of action for lysozyme (22). The apparent decrease in reducing groups after 10 min of lysis was related to an alteration of the solubility of the polymers in the assay mixture.

Relationship of Growth to Activity of Autolytic Enzyme

Cell walls obtained from cells in the early exponential phase of growth autolyze more rapidly than walls isolated from stationary phase cultures (Fig. 4). In order to determine whether the rate of autolysis was a function of the activity of the autolytic enzyme or was related to the structure of the cell wall, cell walls were obtained from cells grown for various times in the standard minimal medium and were autolyzed as described in "Experimental Procedure." • — •, viable count; ■ — ■, rate of autolysis.
procedure.” The crude autolysate was then added to heat-inactivated cell walls which served as a substrate for the autolytic enzyme. Comparison of Fig. 4 and Table II reveals that the rate of lysis of cell walls is slower when heat-inactivated cell walls were used as a substrate for crude enzyme than when the native cell wall-enzyme complex was employed. Nevertheless the differences in rate of lysis of cell walls seem to be more closely related to differences in activity of crude enzyme than to intrinsic differences between preparations of cell walls from \textit{B. subtilis} grown for 5 hours and 24 hours in minimal medium. A synthetic substrate for the enzyme has not been identified. Substrates for trypsin and chymotrypsin such as benzoyl-L-arginine methyl ester and acetyl-L-tyrosine ethyl ester, and peptides such as polyalanine, Ala–Gly–Gly, and Ala–Gly were not hydrolyzed by the enzyme. Although the enzyme can be partially purified by precipitation with 60% ammonium sulfate,

### Table II

**Rate of lysis of heat-inactivated cell walls by preparations of crude autolytic enzyme**

Cell walls were prepared from \textit{B. subtilis} 168 I-C+ grown for 3, 5, 10, and 24 hours in standard minimal medium as described in “Experimental Procedure.” Samples of cell walls (0.67 mg per ml) isolated from \textit{B. subtilis} grown for 3 hours and 10 hours in standard minimal medium were suspended in 60 mM potassium phosphate buffer, pH 8.25, and incubated for 1 hour at 35° to produce autolysis. A 0.8-ml aliquot of each autolysate was added to samples of heat-inactivated walls (substrate) obtained from \textit{B. subtilis} 168 I-C+ grown for 5 hours and 24 hours in the standard minimal medium. At zero time sufficient potassium phosphate (pH 8.25) buffer was added to produce a final substrate concentration and buffer concentration of 0.67 mg per ml and 60 mM, respectively. The rate of autolysis at 35° was determined as described in “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Rate of autolysis 35°</th>
<th>5 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 °F</td>
<td>7.6 × 10³</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>10 °F</td>
<td>3.5</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

* The numbers refer to the number of hours of growth in minimal medium prior to preparation of cell walls.

### Table III

**Variation of rate of autolysis of cell walls isolated from bacteria grown in different media**

\textit{B. subtilis} 168 I-C+ was grown for 5 hours at 37° in minimal Growth Medium A (standard minimal medium as described in “Experimental Procedure”). Minimal Medium B is similar to A with the exception of a 20-fold increase in the concentration of casein hydrolysate. Penassay medium (Antibiotic Medium No. 3, Difco) was not supplemented. Cell walls were prepared from organisms grown for 5 hours at 37° in each of these media as described in “Experimental Procedure.” The sample of cell walls (3 mg) was suspended in 3.0 ml of distilled water containing 60 mM NaHCO₃ (pH 9.6) and incubated at 38°. The rate of autolysis was determined as described in “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Rate of autolysis 38°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal A</td>
<td>69.3</td>
</tr>
<tr>
<td>Minimal B</td>
<td>12.6</td>
</tr>
<tr>
<td>Penassay</td>
<td>28.9</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of temperature on autolysis. Cell walls prepared from \textit{B. subtilis} grown for 5 hours in standard minimal medium were suspended in 10 mM Tris buffer (pH 8.1) and incubated at various temperatures. The rate of autolysis was determined as described in “Experimental Procedure.” The reciprocal of absolute temperature is plotted on the abscissa and the logarithm of the rate constant on the ordinate.

Fig. 6. Effect of pH on the rate of autolysis. Samples, 3 mg, of cell walls, obtained from \textit{B. subtilis} 168 I-C+ grown for 5 hours in standard minimal medium, were added to 10 mM solutions of Tris buffer at various pH containing 20 mM KCl. Each solution was preincubated at 38° for 5 min before the addition of cell walls. The 3-ml samples were incubated at 38° and the rate of autolysis was determined as described in “Experimental Procedure.”

the failure to obtain a suitable artificial substrate has inhibited the studies with partially purified enzyme.

As shown in Table III, the chemical composition of the growth medium influences the enzymatic activity of preparations of cell...
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Or

\[ X \]

\[ 20^{-} \]

\[ C \]

\[ 10^{-2} \]

\[ 10^{-3} \]

\[ K \]

\[ CONCENTRATION (MOLAR) \]

FIG. 7. Effect of ionic strength on autolysis. Samples, 3 mg, of cell walls, obtained from B. subtilis 168 I-C+ grown for 5 hours in standard minimal medium, were added to 10 mM Tris buffer (pH 8.6) and varying concentrations of KCl. The 3-ml samples were incubated at 35°C and the rate of autolysis determined as described in "Experimental Procedure."

TABLE IV

Effect of Ions on Autolysis

Cell walls of B. subtilis 168 I-C+, 3 mg, were suspended in distilled water. At zero time sufficient Tris buffer (pH 8.6) and KCl were added to produce a final concentration of 10 mM. In other samples, sufficient volumes of divalent cations were also added at zero time to produce a final concentration of 1 mM. Total reaction volume was 3.0 ml.

<table>
<thead>
<tr>
<th>Addition</th>
<th>K ( \text{min}^{-1} \times 10^{3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.7</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>26.6</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>26.6</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>25.6</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>23.6</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>23.9</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>7.4</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>6.3</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>6.0</td>
</tr>
</tbody>
</table>

As shown in Fig. 6, the pH optimum of this enzymatic reaction is broad with a maximum at pH 9.5. The pH optimum of lysozyme and most other \( \beta \)-N-acetylhexosaminidases is below pH 7.0.

The enzyme activity was markedly influenced by ionic strength as shown in Fig. 7. Sodium ions are as efficient as potassium ions in the stimulation of autolysis. The effect of divalent ions on the rate of autolysis (Table IV) is similar in general to their effect on transformation (23). One exception is Mn⁺⁺. This divalent cation stimulates autolysis but does not influence transformation.

DISCUSSION

Enzymatic hydrolysis of cell walls may occur in two major loci: (a) cleavage of the polysaccharide, i.e., the bond between \( N \)-acylglucosamine and \( N \)-acylmuramic acid (Fig. 8, Reaction A) or (b) cleavage of the peptide chain (Fig. 8, Reactions B to E). These reactions can be readily separated by analysis of the chemical groups liberated during hydrolysis and isolation of the products of the reaction. The release of \( N \)-terminal alanine without a concomitant release of reducing groups, Morgan-Elson-reactive material, phosphomonoester groups, and \( C \)-terminal groups shows that the enzyme hydrolyzes the amide bond between \( N \)-acylmuramic acid and \( L \)-alanine (Fig. 8, Reaction B). Contamination of the preparation of cell walls by other hydrolytic enzymes must be sufficiently low so that the action of such hypothetical enzymes cannot be detected by hydrolysis of artificial substrates or release of groups other than \( N \)-terminal alanine.

The alanine in cell walls of B. subtilis occurs both in teichoic acid and in mucopeptide. The former is alkaline-labile whereas the latter is alkaline-resistant (24). Incubation of cell walls under the conditions used to produce autolysis would result in release of "alkaline-labile" \( L \)-alanine which accounts for 62% of the \( C \)-terminal alanine observed in Table I. If one assumes that the moles of \( L \)-alanine bound to the carboxyl group of \( N \)-acylmuramic acid are equal to the number of moles of glutamic acid in the remainder of the peptide, the liberation of 0.57 mole of alanine per mole of glutamic acid indicates that most of the

![Fig. 8. Model of mucopeptide subunit in the cell wall of B. subtilis 168 I-C+](http://www.jbc.org/)

The 1–4 bond between \( N \)-acyl muramic acid and \( N \)-acyl glucosamine was chosen arbitrarily. \( R, R', \) and \( R'' \) refer to the other amino acids in the mucopeptide (glutamic acid, diaminopimelic acid, and alanine, respectively).
muranyl-L-alanine residues in the mucopeptide of the wall are hydrolyzed. This mechanism predicts that at least three major types of heteropolymers should be liberated: (a) a copolymer of N-acetylgalactosamine and N-acetylmuramic acid; (b) teichoic acid; (c) cross-linked peptides. In addition there should be a small residual amount of mucopeptide. Chromatography of the autolysate on DEAE-cellulose and Sephadex G25 results in numerous fractions, some of which are chromatographically homogeneous. The isolation of polynucleotides consisting of N-acetylgalactosamine and N-acetylmuramic acid, peptides composed of alanine, diaminopimelic acid, and glutamic acid, and teicholipid which will be reported subsequently further supports the proposed mechanism of action of the autolytic enzyme.

The presence of maximal enzyme activity during logarithmic growth of the population of cells strongly suggests that the enzyme may have a physiological function related to growth. Conceivably, hydrolysis of the cross-linked peptide would permit expansion of the rigid cell wall and facilitate insertion of another small segment of cell wall that was transported through the cytoplasmic membrane on a lipid carrier. Alternately it could provide a mechanism for the insertion of teichoic acid into the basal mucopeptide. Such a model would require a transpeptidase in the cell wall to synthesize peptide bonds and form a strong three-dimensional cross-linked structure. Wise and Park (26) and Tipper and Strominger (27) have presented evidence that penicillin inhibits the biosynthesis of the cell wall in Staphylococcus by preventing transpeptidation. The concerted action of acylmuramyl-L-alanine amidase and a transpeptidase could be essential in the growth of the cell envelope. A differential retardation of biosynthesis could result in defects in the cell wall which could culminate in autolysis.

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References

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