Purification and Characterization of Cell Wall-associated N-Acetylmuramyl-L-alanine Amidase from Alkaliphilic Bacillus lentus C-125

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Cells of the facultative alkaliphile Bacillus sp. C-125 grown at neutral pH autolyse rapidly in alkaline buffers of pH 9-10. Alkaline autolytic activity has been found mainly in the cell wall fraction. A peptidoglycan lytic enzyme was extracted from the cell wall fraction suspended in 4 M LiCl. The enzyme was identified as N-acetylmuramyl-L-alanine amidase, with a molecular mass of 58 kDa. At low salinity, the enzyme formed an aggregate of high molecular mass. The peptidoglycan lytic reaction of this enzyme happened at pH 9.0-10.5 at 37°C. Optimum pH for the reaction was 9.7-10.0. The enzyme was most active at 60°C when assayed at pH 9.0.

Key words: alkaliphile; alkaliphilic Bacillus lentus; peptidoglycan lytic enzyme; N-acetylmuramyl-L-alanine amidase

We have analyzed the chemical composition of cell walls prepared from several alkaliphilic strains of the genus Bacillus. Several alkaliphilic strains alter their cell wall compositions when grown in an alkaline milieu. The cell walls of the alkaliphilic strains grown at alkaline pH are enriched in acidic compounds, such as acidic amino acids and uronic acids, compared with cell walls of those grown at neutral pH.

The facultative alkaliphile Bacillus lentus strain C-125 used to be called Bacillus sp. C-125, and was recently shown to be related to B. lentus. This organism also changes its cell wall composition in response to the environmental pH. The cell walls of this organism are composed mainly of A1y-type peptidoglycan and two acidic polymers, teichuronic acid and teichuronopeptide. The teichuronopeptide is composed of galacturonic acid, glucuronic acid, and N-acetylfucosamine. The teichuronopeptide is a copolymer composed of polyglucuronic acid and polyglutamic acid. The cell wall concentrations of these two acidic structural polymers increase with respect to peptidoglycan as the growth pH increases. Thus, the anion content of the cell walls is extremely great when grown at elevated pH.

The cross-linking frequencies between murein peptides of the A1y-peptidoglycan are different among the cells grown at alkaline and neutral pH. The frequency is low when the cells were grown at neutral pH. Cells of the organism grown at neutral pH and then starved in carbon sources autolyze readily at pH 9-10, although the pH is optimum for growth of the organism. In the presence of glucose, the cells are stable even in this pH region. Also, cell walls prepared from the cells grown at neutral pH autolyze rapidly at the alkaline pH. The cell lysis is due to hydrolysis of the peptidoglycan with cell-associated autolytic enzyme deregulated during carbon starvation. Cell wall-associated N-acetylmuramyl-L-alanine (MA) amidases are known in several strains of Bacillus. The major autolytic enzyme present in the cell walls of B. lentus C-125 had been assumed to be MA amidase. However, the enzyme has not been purified or characterized.

This paper describes identification of the cell wall-associated peptidoglycan lytic enzyme as an MA amidase.

Materials and Methods

Organism and cultivation medium: The facultative alkaliphile Bacillus lentus C-125 was exclusively used in this study. This organism was aerobically grown at 37°C in an alkaline medium containing (per liter of water) K$_2$HPO$_4$, 13.7 g; KH$_2$PO$_4$, 5.9 g; Na$_2$CO$_3$, 10.6 g; citric acid, 0.34 g; MgSO$_4$.7H$_2$O, 0.05 g; glucose, 5 g; peptone, 5 g; and yeast extract, 1 g. The pH of the medium was about 10. The same medium using 11.7 g of NaCl instead of Na$_2$CO$_3$ was adjusted to pH 7.2 with NaOH and used as a neutral medium.

Purification of the peptidoglycan lytic enzyme.

1. Culture of the organism. The organism was grown in 100 liters of the alkaline medium. Air was bubbled into the medium at a flow rate of 120 liter/min. The medium was aged at 400 rpm. The cells were harvested at the early stationary phase of growth and stored at −80°C until use.

2. Preparation of cell wall fraction from B. lentus C-125. The frozen cells (about 100 g, wet wt.) were suspended in 200 ml of 5 M Tris-HCl buffer (pH 8.0) with a Waring blender homogenizer. The cells were broken by sonication (20 kHz, 200 W, 10 min) in an ice water bath. Unbroken cells were removed by centrifugation (2000 x g, 15 min, 4°C). The cell walls were sedimented by centrifugation (30,000 x g, 10 min, 4°C) from the supernatant. A white upper layer on a brown precipitate was selectively scraped and collected with a micro-spatula. This preparation was stored at −80°C until use.

3. Extraction of peptidoglycan lytic enzyme from the cell wall. The cell wall fractions were thawed and combined. The cell walls prepared from 500 g (wet wt.) of the cells were repeatedly washed with 800 ml of cold 5 M Tris-HCl (pH 8.0) buffer by centrifugation (30,000 x g, 10 min, 4°C), until A$_{260}$ of the supernatant fluid became constant. Then, the cell walls were washed twice with 1 M LiCl-5 M Tris-HCl (pH 8.0). The cell walls were suspended in cold 1 M LiCl-0.02 M phenylmethylsulfonyl-fluoride (PMSF) 5 M Tris HCl (pH 8.0). To this suspension, a fourth volume of 12 M LiCl-5 M Tris-HCl (pH 8.0) was added. This mixture was stirred at 4°C for 15 min and then centrifuged (30,000 x g, 4°C, 10 min). The precipitate was again extracted with 4 M LiCl 0.02 M PMSF 5 M Tris HCl (pH 8.0). The supernatant fluids were combined and dialyzed against 5 M Tris HCl buffer (pH 8.0).

4. DEAE-cellulose column chromatography of protein extracted from the cell wall. The non-dialysable material was put on a column (2.5 x 12 cm)
of DES1 cellulose (Whatman Ltd., Maidstone, UK) equilibrated with 5 mM Tris HCl (pH 8.0) buffer. The column was eluted with a linear gradient from 0 to 0.2 M LiCl in 500 ml of the Tris HCl buffer. Fractions (5 ml) were collected. The activity was seen for B. lentus C-125 peptidoglycan lytic activity, as described below. The positive activity fractions (22 ml) were recovered, combined, and dialyzed against 50 mM KH₂PO₄-NaOH (pH 7.0) buffer.

(5) Hydroxamate chromatography of protein fractionated by DEAE-cellulose chromatography. The dialyzed sample was put on a column (1.6 x 13 cm) of hydroxamate (Nacalai Tesque, Kyoto, Japan) equilibrated with the 5 mM phosphate buffer. The column was eluted with a linear gradient from 0.005 to 0.4 M KH₂PO₄-NaOH (pH 7.0) buffer (300 ml). The active fractions (25 ml) were recovered, combined, and dialyzed against 50 mM Tris HCl (pH 8.0).

Assay of peptidoglycan lytic activity. The organism was grown in the alkaline medium. The cells in the late exponential phase of growth were harvested and immediately SDS 0.1 M NaCl. Cell walls were prepared at room temperature from the SDS-treated cells. The cell wall preparation was washed several times with 0.1 M NaCl, 0.1% NaN₃, then with deionized water. Peptidoglycan was prepared by incubating the cell walls in 5% (v/v) trichloroacetic acid at 60°C. The peptidoglycan preparation was repeatedly washed with water and used as a substrate for the lytic reaction.

The peptidoglycan was suspended in 1 mM LiCl 30 mM Na₂CO₃, NaHCO₃ (pH 9.0) to a concentration of OD₆50 0.5. The enzyme preparation was added to the suspension. The mixture was incubated at 50°C for 10 min. Each assay was routinely done twice. One unit of the peptidoglycan lytic enzyme was defined as the amount of the enzyme that decreased the OD of the suspension by 0.001 unit per minute under these conditions.

Measurement of protein. Protein was measured by the method of Lowry. Using bovine serum albumin as a standard.

Measurement of LiCl and KH₂PO₄. On the chromatographic separation of the enzyme, eluates were assayed for LiCl or KH₂PO₄ by measuring the refractive index values.

SDS PAGE of proteins. Samples were dissolved in a solution containing 2% SDS (w/v), 25% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, and 60 mM Tris HCl (pH 6.8) and heated in a boiling water bath for 5 min. The samples were run on an SDS 8% polyacrylamide gel, as described by Laemmli. The molecular mass markers used were rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), egg white ovalbumin (45.0 kDa), bovine erythrocyte carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa) obtained from Bio-Rad Laboratories, CA, U.S.A.

Measurement and identification of N-terminal amino acid residues generated by peptidoglycan hydrolysis. The cell wall sample was diaminophenylated with 20 mM dimethylfluorobenzene in 1.5% (w/v) Na₂B₄O₇ at 60°C for 30 min. To the reaction mixture, one-third volume of concentrated HCI was added. Resulting insoluble diaminophenylated (DNP) matter was partially hydrolyzed at 100°C for 15 min. The N-terminal amino acid residues were measured by A₁₂⁴ of the cleared acidic hydrolysate, using t-alanine as a reference standard.

To identify the N-terminal amino acid residue generated during the peptidoglycan hydrolysis, samples before and after the hydrolysis were also diaminophenylated. The DNP-product was washed with diethyl ether to decrease excess byproducts, such as dinitrophenol and dinitroaniline. The product was completely hydrolyzed in 4 M HCl at 100°C for 12 h. The hydrolysate was extracted twice with diethyl ether. The water phase and ether extract were separately dried up in vacuo over solid NaOH. The yellow residues were dissolved in small volumes of acetone. The DNP-compounds were developed on an Avicel thin-layer by ascending chromatography in 1.5M KH₂PO₄ KOH buffer (pH 6.0) or n-butanol pyridine-water (6:4:3, v:v:v) at room temperature in the dark together with standards of DNP-products prepared from authentic amino acids.

Materials. α-Amino acid oxidase and t-alanine dehydrogenase were purchased from Boehringer Mannheim.

Results and Discussion

Localization of the peptidoglycan lytic enzyme in the cell walls of B. lentus C-125

We first examined peptidoglycan-hydrolyzing activity in extracellular, cytoplasmic, and cell wall-associated protein fractions at pH 5.8-9.7 (Table I). Peptidoglycan prepared from the organism was used as a substrate. This peptidoglycan preparation does not contain non-peptidoglycan components, such as protein, teichuronic acid or teichuronomopeptide. The extracellular (protein, 0.08 mg/ml) or cytoplasmic (protein, 0.75 mg/ml) fractions were used without any further purification. Cell wall-associated proteins liberated with 6 M LiCl (see below; protein, 0.38 mg/ml) were assayed for the activity. Peptidoglycan-hydrolyzing activity was found mainly in the cell wall-associated protein fraction. This cell wall-associated enzyme was more active at alkaline pH than at neutral pH, and was practically inactive at pH below 7.6. The activity was almost negligible in the extracellular or cytoplasmic fraction, compared with the activity found in the cell wall fraction at alkaline pH.

Some alcaliphilic strain of Bacillus produces a murine peptide-hydrolyzing peptidase extracellularly. However, B. lentus C-125 did not produce a detectable activity of extracellular autolytic enzyme.

Liberation of the peptidoglycan lytic enzyme from the cell walls of B. lentus C-125

Autolysis of the organism was previously examined without addition of a high concentration of salts. To establish an assay system for the peptidoglycan-hydrolyzing activity, we examined the salinity at which the autolytic enzymes actively hydrolyzed the cell walls (Table II). The cell wall autolysis was the most active in the presence of 1 M LiCl. When NaCl was used instead of LiCl, the autolysis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Reaction pH 5.8</th>
<th>6.7</th>
<th>7.6</th>
<th>8.2</th>
<th>9.1</th>
<th>9.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td>NT</td>
<td>NT</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cell wall-associated</td>
<td>0.012</td>
<td>NT</td>
<td>0.034</td>
<td>0.061</td>
<td>0.101</td>
<td>NT</td>
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</table>

Table I. Localization of Peptidoglycan Lytic Enzyme
Table II. Effects of LiCl Concentration on Autolysis of Cell Walls

<table>
<thead>
<tr>
<th>Concentration of LiCl (m)</th>
<th>( \Delta \text{OD}_{660} ) (m)</th>
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</thead>
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<tr>
<td>0</td>
<td>0.085 (42%)</td>
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<tr>
<td>0.05</td>
<td>0.106 (53)</td>
</tr>
<tr>
<td>0.1</td>
<td>0.123 (61)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.142 (71)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.176 (88)</td>
</tr>
<tr>
<td>1</td>
<td>0.201 (100)</td>
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<tr>
<td>2</td>
<td>0.167 (83)</td>
</tr>
<tr>
<td>3</td>
<td>0.130 (65)</td>
</tr>
</tbody>
</table>

* The cell wall fraction was suspended (OD$_{660}$ 0.5) in 30 mM Tris HCl (pH 9.0) containing LiCl of which concentration is shown in the table.

* The samples were incubated at 37°C for 2h. Decrease in OD$_{660}$ was recorded in two experiments. Relative decrease of a mean value obtained from the experiments is shown in parentheses taking the decrease at 1 mM LiCl as 100%.

Table III. Effects of LiCl Concentration on Liberation of the Peptidoglycan Lytic Enzyme from the Cell Wall Preparation

<table>
<thead>
<tr>
<th>Concentration of LiCl (m)</th>
<th>Activity (U/mL)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>3.1</td>
</tr>
<tr>
<td>0.1</td>
<td>4.0</td>
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<tr>
<td>0.2</td>
<td>5.0</td>
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<tr>
<td>0.5</td>
<td>7.1</td>
</tr>
<tr>
<td>1</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>8.1</td>
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<tr>
<td>3</td>
<td>9.1</td>
</tr>
<tr>
<td>4</td>
<td>21.4</td>
</tr>
<tr>
<td>6</td>
<td>24.4</td>
</tr>
</tbody>
</table>

* The cell wall fraction was suspended (OD$_{660}$ 0.5) in 30 mM Tris HCl (pH 8.0) containing LiCl of which concentration is shown in the table. After incubation for 5 min at room temperature, the mixtures were centrifuged at 25,000 × g for 10 min at 4°C.

* The supernatant solution was assayed for peptidoglycan lytic activity in two experiments. A mean value is shown in the table.

was also the most active at the same concentration (results not shown). The autolytic activity found at 1 mM LiCl was 2.5 times that found in the absence of LiCl. The peptidoglycan lytic activity was evaluated in the presence of 1 mM LiCl in this study, unless otherwise described.

It is known that cell walls are tightly associated with autolytic enzymes in various microorganisms. Some of the enzymes have been liberated from the cell walls suspended in high concentrations of salt solutions. We attempted to release B. lentus peptidoglycan lytic enzyme from the cell wall preparation at high salinity (Table III). Liberation of the activity from B. lentus cell walls was significantly dependent on the LiCl concentration to which the cell walls were exposed. The peptidoglycan lytic activity was effectively released from the cell walls by exposure to 4-6 mM LiCl. SDS-PAGE of cell-wall-associated protein showed that 58-kDa protein (see below) was completely extracted with 4.5 mM LiCl (results not shown).

Purification of the peptidoglycan lytic enzyme

The peptidoglycan lytic enzyme (110 kU) was extracted from the cell walls prepared from 500 g (wet wt.) of the cells with 4 mM LiCl after washing repeatedly with the Tris buffer and twice with 1 mM LiCl (Table IV). The peptidoglycan lytic enzyme was almost completely extracted by two times extractions with 4 mM LiCl. Protein liberated from the cell walls were put on a DEAE-cellulose column. The activity eluted from the column with 0.08 M LiCl (Fig. 1). The activity-positive fractions (132 to 146) were recovered and combined. This fraction contained 71 kU of the activity. Protein fractionated by the DEAE-cellulose column chromatography was successively put on a hydroxypapatite column. The activity eluted from the column with 0.24 M KH$_2$PO$_4$ (Fig. 2) but did not elute with 0.05-1 M KCl. Recovery of the activity from hydroxypapite column was about 20% of the activity put on the column. The activity-positive fractions (67 to 71) contained three proteins with molecular masses of 40, 58, and 87 kDa, as shown in Fig. 4. Chromatographic behaviors of the lytic enzyme indicated that the enzyme was a weakly anionic protein.

These three proteins eluted at a void volume by Sephadex G150 gel chromatography, when the column was eluted with a low ionic strength buffer containing 50-100 mM NaCl (results not shown). When a portion of the sample was put on a column (7.8 X 300 mm) of TSK G-3000SW gel (Tosoh, Tokyo, Japan) with an HPLC apparatus and eluted with 3 mM LiCl-50 mM Tris-HCl (pH 7.0), the proteins were separated from one another (Fig. 3). The activity-positive fraction (retention time 53.4 min) contained a single protein with a molecular mass of 58 kDa as shown by SDS-polyacrylamide gel electrophoresis (Fig. 4). Molecular mass of the enzyme estimated on the basis of this retention time was 62 kDa, indicating that the enzyme was monomer at the high salinity. When the gel was eluted with 0.3 M LiCl in the Tris buffer, the three proteins eluted at retention time of 12.2 min. This retention time corresponded to a void volume of the column, indicating that the molecular mass of the enzyme was more than 1000 kDa. These results meant that the enzyme formed a huge aggregate at low salinity probably with the two other proteins. The purification procedures are summarized in Table IV.

MA amidase with a molecular mass of 50 kDa has been isolated from Bacillus subtilis. This amidase associates with an 80-kDa protein at low ionic strength. The latter protein is reported to be a modifier that regulates binding of the 50-kDa amidase to the cell walls. The function of 40- or 87-kDa protein found in B. lentus C-125 is still
Peptidoglycan Lytic Enzyme of Alkaliphilic Bacillus luteus

Fig. 1. DEAE-Cellulose Chromatogram of the Non-dialysable Fraction of LiCl Extracts from the Cell Walls.
The cell walls were extracted with 4 M LiCl. The non-dialysable fraction of the extracts was put on a column (2.5 \times 12 cm) of DEAE-cellulose equilibrated with 5 mM Tris HCl (pH 8.0). The column was eluted by a linear LiCl gradient as described in Materials and Methods. Fractions (5 ml) were collected and assayed for A280 (○), LiCl (△), and peptidoglycan lytic activity (●).

Fig. 2. Hydroxyapatite Chromatogram of Protein Fractionated by DEAE-Cellulose Column Chromatography.
The activity-positive fraction recovered from DEAE-cellulose chromatography was put on a column (1.6 \times 13 cm) of hydroxyapatite equilibrated with 5 mM KH2PO4, NaOH (pH 7.0). The column was eluted as described in Materials and Methods. Fractions (5 ml) were collected and assayed for A280 (○), KH2PO4 (△), and peptidoglycan lytic activity (●).

Unclear. In this study, the peptidoglycan lytic enzyme was characterized using the preparation purified partially by the hydroxyapatite column chromatography.

**Effect of reaction pH and temperature on the cell wall-lytic activity**

Lytic activity of the enzyme preparation was evaluated at various pHs. The reaction pH was adjusted to 6–11 with a variety of buffers containing 1 M LiCl (Fig. 5). The enzyme preparation degraded peptidoglycan in a range of pH between 9 and 11. The pH optimum for the hydrolytic reaction was 9.7–10.0. This optimum pH is coincident with the fact that autolysis of the cell walls is the most active at pH 10.81 Accompanying the decrease in turbidity of the suspension, the amount of N-terminal amino acids increased.80 N-Terminal amino acid-releasing activity was also active in a range of pH between 9.2 and 10.5. An amount of reducing glycosides measured using ferricya-

Fig. 3. Elution Profile of the Proteins by TSK G-3000SW Gel Chromatography.
A sample (30 μg of protein) of the activity-positive fraction from hydroxyapatite chromatography was put on a column (7.8 x 300 mm) of TSK G-3000SW gel (Tosoh, Tokyo, Japan) with an HPLC apparatus and eluted with 50 mM Tris HCl (pH 7.0) containing 3 M (solid line) or 0.1 M (dotted line) LiCl at a flow rate of 0.5 ml/min. The elution was monitored by measurement of A280. The gel chromatography was calibrated by using molecular markers (○): blue dextran, 2000 kDa; ferritin, 450 kDa; catalase, 240 kDa; aldolase, 158 kDa; bovine serum albumin, 68 kDa; hen egg albumin, 45 kDa; bovine chymotrypsinogen A, 25.5 kDa. Arrows indicate peptidoglycan lytic activity positive fractions.

nide20 did not increase at all during the hydrolysis (results not shown). Cell wall-associated MA amidases with optimum pH of 6.8–8.5 have been isolated from several Bacillus spp.9,11,12,19,21

The optimum temperature for the lytic reaction was estimated by varying the incubation temperature at pH 9.0 (Fig. 6). The optimum temperature was 60 C for both
Fig. 4. SDS-Polyacrylamide Gel Electrophoresis of Peptidoglycan Lytic Enzyme Samples during Purification Steps.

Samples were electrophoresed on an SDS 8% polyacrylamide gel. Proteins in the gel were stained with Coomassie Brilliant Blue R-250. Lanes: 1, proteins extracted from the cell wall preparation with 4 M LiCl; 2, fraction from DE-52 cellulose column; 3, fraction from hydroxypatite column; 4, fraction from 72K G-3000SW gel column eluted with 3 M LiCl.

Fig. 5. Effects of Reaction pH on the Peptidoglycan Lytic Activity.

The enzyme was added with the following buffer systems: 30 mM KH₂PO₄-NaOH (pH 6.7), 30 mM 3-N-morpholino-2-propanesulfonic acid-NaOH (pH 7 to 8), 30 mM Na₂CO₃-NaHCO₃ (pH 9 to 10), or 30 mM Na₂CO₃-NaOH (pH 10 to 12). All buffers had 1 M LiCl added. The enzyme preparation (50 U) was added to suspension (OD₆₅₀ 0.5) of the peptidoglycan preparation. Activity (decrease in turbidity at 660 nm (C)) or increase in N-terminal amino acids (●●●) was measured at 37°C for 10 min. The level of each activity at its optimum pH was defined as 100%.

Fig. 6. Effects of Reaction Temperature on the Peptidoglycan Lytic Activity.

The enzyme reaction was done using 50 U of the enzyme and peptidoglycan preparation (OD₆₅₀ 0.5) suspended in 1 M LiCl 30 mM Na₂CO₃-NaHCO₃ buffer (pH 9.0) for 10 min at 30-70°C. Decrease in turbidity at 660 nm (●) or increase in N-terminal amino acids (●●●) was measured. The level of peptidoglycan lytic activity at its optimum temperature was defined as 100%.

Mode of action of the peptidoglycan lytic enzyme on the cell walls

The N-terminal amino acid generated from the peptidoglycan with the lytic enzyme was analyzed by dimtoxyphencylation of free amino groups in the water-soluble or -insoluble products (Fig. 7). The murein peptide of the organism is composed of D- and l-alanines, L-glutamic acid, and meso-diaminopimelic acid. The chemical structure is identical to that of Bacillus megaterium. The ω-amino group of 50 to 70% diaminopimelic acid is not involved in cross-linking between the adjacent murein peptides.

The peptidoglycan lytic enzyme released water-soluble peptides composed of alanine, glutamic acid, and diamino-pimelic acid from the peptidoglycan. N-Terminal amino acids were alanine and diaminopimelic acid in the soluble products (Figs. 7A and B), and only diaminopimelic acid in the insoluble products (results not shown). When these products were diaminophenylated, mono-DNP-substituted diaminopimelic acid was recovered but not bis-DNP-substituted diaminopimelic acid. Amount of the mono-DNP-diaminopimelic acid did not increase during the enzyme treatment. Therefore, it was concluded that the mono-DNP-diaminopimelic acid was derived from the peptidoglycan, and the diaminopimelic acid was not involved in the cross-linking but not from that generated by the enzymatic hydrolysis. The enzymatic hydrolysis generated only alanine as the N-terminal residue.

The soluble peptides generated by the enzymatic hydrolysis was dimtoxyphencylated and completely hydrolyzed with HCl. This hydrolysate was developed on an Avicel thin-layer in the n-butanol-pyridine-water. In this TLC system, Rf values of free alanine and DNP-alanine
Fig. 7. Thin-layer Chromatograms of DNP-amino Acids Derived from Products Generated by the Enzymatic Hydrolysis of Peptidoglycan.

The cell wall (OD_{600}, 0.8) suspended in 2 ml of 1 mM LiCl 30 mM Na_{2}CO_{3}, NaHCO_{3} (pH 9.0) was digested with 100 U of the peptidoglycan lytic enzyme at 50°C for 4 h. After the enzyme was inactivated by heating in a boiling water bath for 10 min, the sample was centrifuged at 30,000 g for 25 min. The supernatant was dimethylsphorinated, washed with diethyl ether, and hydrolyzed with HCl. The hydrolysate was extracted with diethyl ether. Diethyl ether-soluble (A) or -insoluble (B) DNP-compounds were separately developed on Avicel cellulose thin-layers in KH_{2}PO_{4}, KOH buffer (i) or n-butanol pyridine water (ii). Lanes: A, DNP-alanine; Glu, DNP-glutamic acid; 2-DAP, bis-DNP-diaminopimelic acid; 1-DAP, mono-DNP-diaminopimelic acid; Blank, dimethylsphorin blank. 1, cell wall before incubation; 2, cell wall incubated without enzyme; 3, enzyme preparation before incubation; 4, enzyme preparation incubated without cell wall; 5, cell wall plus enzyme preparation before incubation; 6, cell wall incubated with enzyme preparation.

were 0.22 and 0.74, respectively. The free alanine was recovered from the thin-layer. This alanine was oxidized with D-amino acid oxidase, but not dehydrogenated with L-alanine dehydrogenase, indicating that L-alanine consisting of the soluble peptide was substituted with dinitrophenyl group. Thus, the peptidoglycan lytic enzyme extracted from the cell walls was MA amidas yielding N-terminal L-alanine but not carboxypeptidase yielding N-terminal D-alanine.

In recent years, two genes (cwLA and B) encoding MA amidas were cloned from B. subtilis. The molecular masses of CwLA and B are 23 and 46 kDa, respectively. The optimum pH and ionic strength for cell-wall hydrolysis of MA amidas CwLA are 8.0 and 150 mM KCl. Also, CwLB has been reported to be active at pH 8.0 and an ionic strength of 0.1 M KCl. C-125 MA amidas differs significantly from these MA amidas of B. subtilis in these properties.

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