Purification and Characterization of an Inhibitor of the Phospholipase A₁ in Bacillus subtilis*

(Received for publication, July 3, 1974)

SHARON S. KRAG and WILLIAM J. LENNARZ‡§
From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

SUMMARY

The protoplasts of a mutant of Bacillus subtilis 168 (B. subtilis CMKaa) are osmotically fragile when compared to protoplasts of the parent organism and contain an active, membrane-associated phospholipase A₁. A protein found in the parent organism specifically inhibits the phospholipase A₁ (KENT, C., AND LENNARZ, W. J. (1972) Proc. Nat. Acad. Sci. U.S.A. 69, 2793-2797). The inhibitor exists in both a soluble and particulate form. The soluble inhibitor is not found in the cytoplasm, but rather in a "periplasmic" fraction released from the cell during incubation with lysozyme. The soluble inhibitor has been purified to homogeneity by diethylaminoethyl-cellulose and hydroxylapatite chromatography. Its molecular weight is 28,000 to 32,000 as determined by gel filtration chromatography and 36,000 to 37,000 as determined by sodium dodecyl sulfate-urea gel electrophoresis. The inhibitor appears to enzymatically inactivate the membrane-bound phospholipase A₁ by an enzymatic process that is dependent on time and protein concentration. Binding of the inhibitor to the membrane-associated phospholipase cannot be detected.

When purified inhibitor is added to cells of B. subtilis CMKaa during treatment with lysozyme, the osmotic stability of the resultant protoplasts is similar to that of protoplasts of the wild type organism.

Mutants of Bacillus subtilis 168 which have defective cytoplasmic membranes have been isolated in our laboratory (1). The screening procedure used in isolating these mutants was based on the fact that their protoplasts were osmotically fragile when compared to protoplasts of the parent organism. The protoplasts of such a mutant, B. subtilis CMKaa, contained lower amounts of membrane lipid because of the sequential hydrolysis of the lipid by a membrane-bound phospholipase A₁ and a soluble lysophospholipase (2). When the phospholipase A₁, which is dependent on Ca²⁺ for activity, was inhibited by the addition of EDTA during the treatment of cells of the mutant with lysozyme, the osmotic stability of the protoplasts was similar to that of protoplasts of the parent organism (1).

The phospholipase A₁ was found to be inactivated by an inhibitory protein associated with both the supernatant and membranes prepared from protoplasts of the parent organism. The inhibition, which was specific for the phospholipase A₁ of CMKaa, was dependent on time and protein concentration and appeared to be irreversible (2). It was postulated that the parent organism also contained a phospholipase A₁, but that this enzyme was not detectable in the protoplasts because of the presence of the inhibitor. Protoplasts prepared from CMKaa, having no detectable phospholipase inhibitor, contained an active phospholipase A₁.

Recently, a soluble phospholipase A activity, which is Ca²⁺-dependent and inhibitor-sensitive, has been detected in the broth from cultures of both parent and mutant organisms. The relationships among the phospholipase inhibitor and the membrane-associated and extracellular phospholipases A are currently under investigation. A preliminary report of some of these studies has been presented (3).

This paper reports studies on the phospholipase inhibitor, which has been localized in both membranous and supernatant fractions prepared from the parent organism. The soluble activity is found, not in the cytoplasm, but rather in a fraction released from cells by treatment with lysozyme ("periplasmic fraction"). The inhibitor, purified to homogeneity from the supernatant fraction, appears to enzymatically inactivate the membrane-bound phospholipase A₁ of the mutant. It has been shown that the addition of purified inhibitor to cells of CMKaa during incubation with lysozyme results in the stabilization of the protoplasts of the mutant, thus correcting the defect originally observed.

MATERIALS AND METHODS

Bacterial Strains

The strains used in this study were Bacillus subtilis 168, B. subtilis Mu8465, a derivative of strain 168 auxotrophic for threonine, leucine, and methionine (4), and B. subtilis CMKaa, obtained by mutagenesis of B. subtilis Mu8465 (1).

1 S. S. Krag and M. B. Kennedy, unpublished observations.

* This work was supported by United States Public Health Service Grant 5 R01 AI06888-00 from the National Institute of Allergy and Infectious Diseases.

† Supported by Public Health Service Grant 5 T01 GM00184-15 from the National Institute of General Medical Sciences. A portion of this work is from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The Johns Hopkins University. Present address, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

‡ To whom reprint requests should be sent.
N-Ethylmaleimide and iodoacetamide were obtained from Calbiochem. Iodoacetic acid was purchased from ICN Nutritional Biochemicals Corp. Trichloroacetic acid was purchased from Eastman Kodak Co. ATP and GTP were purchased from Sigma Chemical Co. Deoxyribonuclease was obtained from ICN Nutritional Biochemicals Corp. Soybean trypsin inhibitor and ribonuclease were purchased from Worthington Biochemical Corp. Trypsin and lysozyme (three times crystallized) were purchased from Sigma Chemical Co. The sources of the other materials are noted below.

DEAE-cellulose (standard type, 0.60 mg/g) was purchased from Schleicher and Schuell. The dry powder was treated for a maximum of 2 hours alternately with 15 volumes (g/ml) of 0.5 n HCl and 15 volumes of 0.5 n NaOH. After each treatment, the cellulose was washed with distilled water until the pH of distilled water was reached. The cellulose was then equilibrated with 0.05 M Tris-Cl, pH 8.0, and stored at 4°C. Bio-Gel P-60 (100 to 200 mesh, exclusion limit 60,000 daltons as determined for globular proteins) and Bio-Gel HT (hydroxylapatite) were purchased from Bio-Rad Laboratories and were prepared for use according to their instruction manual. Blue dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) and sodium [1-14C]acetate (New England Nuclear) were purchased from ICN Nutritional Biochemicals Corp. Soybean trypsin inhibitor and ribonuclease were purchased from Worthington Biochemical Corp. Trypsin and lysozyme (three times crystallized) were purchased from Sigma Chemical Co. and myoglobin (Mann Research Laboratories, Inc.) were used as standards.

**Analytical Methods**

Protein was routinely determined by the method of Lowry et al. (5) with the use of bovine serum albumin as the standard. For the monitoring of protein in column fractions, the absorbance (Gilford 240 spectrophotometer, Gilford Instrument Laboratories, Inc.) at 260 nm or 410 nm (bene proteins) was used. With very dilute protein samples (2 to 50 µg/ml) the Waddell spectrophotometric method (6, 7) was used. Phosphate was determined in lipid samples according to the procedure of Bartlett (8). Measured conductance in micromhos was determined by a conductivity bridge (model RC16B2 with a CEL-K1X2 conductivity cell, Industrial Instruments Inc.). All readings were performed at room temperature.

**Polyacrylamide Gel Electrophoresis**

Gel electrophoresis was performed as described by Weber and Osborn (9) (with the following modifications). Samples (60 to 300 µg of protein) were dialyzed for at least 2 hours twice against a minimum of 40 volumes of distilled water at 4°C. Following dialysis, the samples were lyophilized to dryness and reconstituted with 0.05 ml of 0.5 M urea (J. T. Baker Chemical Co., purified as described (10)), 0.1 M sodium phosphate, pH 7.0, 0.1% β-mercaptoethanol (Eastman Kodak Co.) containing sufficient sodium dodecyl sulfate (Fisher Scientific Co.) so that the SDS to protein ratio (w/w) was greater than 2. A drop of glycerol and 5 µl of 0.05% bromphenol blue were added to each sample. Samples to be analyzed by electrophoresis were denatured by heating at 100°C for 2 min.

Electrophoresis was performed vertically in gels (9 × 0.5 cm) containing 0.5 M urea, 0.1% SDS, 0.1 M sodium phosphate, pH 7.0, 0.0033 M ammonium persulfate (J. T. Baker Chemical Co.), 0.15% (v/v) of N,N,N',N'-tetramethylethyleneediamine (Eastman Kodak Co.), 7.5% acrylamide (Eastman Kodak Co.), and 0.15% N,N'-methylenebisacrylamide (Eastman Kodak Co.). The gels (prerun at 2 to 3 ma/tube for 4 to 10 hours) and the electrophoresis buffer (0.5 M urea, 0.1 M sodium phosphate, pH 7.0, 0.1% SDS) were cooled to 13°C, which was the temperature at which the electrophoresis was performed (8 a tube, 8 to 10 hours) was performed.

Gels analyzed for protein were fixed for 2 to 3 hours in 10% trichloroacetic acid and stained 16 hours in a freshly prepared solution of 0.25% Coomassie brilliant blue (0.05% in 10% trichloroacetic acid) at room temperature. The gels were destained by incubation in several changes of a solution of 7.5% acetic acid, 5% methanol at 37°C. Proteins used for standards were bovine serum albumin (Miles Laboratories, Inc.), ovalbumin, cytochrome c (Sigma Chemical Co.), and myoglobin (Mann Research Laboratories, Inc.).

Gels analyzed for inhibitor activity were immediately manually cut into 0.5-cm (top 4.5 cm) or 0.2-cm slices. The slices were minced and incubated twice with 0.3-ml volumes of 0.1% SDS, 0.01 M sodium phosphate, pH 7.0 (30°C, 30 min). The solutions were combined and treated with Dowex AG 1-X2 (chloride form, 200 to 400 mesh) obtained from Bio-Rad Laboratories (11). The ratio (mg/ng) of Dowex to SDS was 20 to 50:1. The treated solutions, now free of SDS, were assayed for inhibitor activity.

**Gels and Harvesting of Cells**

Cells of B. subtilis Mu8u503 and B. subtilis CMK12 were grown in 500 ml of Medium C (1) in 2-liter flasks without baffles on a rotary shaker (340 rpm) at 30°C. The cells were harvested at an optical density of 3.0 (660 nm, mid- to late-log phase) by centrifugation (4°C) at 8000 × g for 10 min and washed with 100 ml of 0.05 M sodium phosphate, pH 7.0, containing 0.005 M MgCl2.

Cells of B. subtilis 168 were purchased in 1-pound batches from Grain Processing Corporation, Muscatine, Iowa. The cells were grown to 34-log phase in an enriched media, washed with a physiologic RC1-NaCl solution, and quick-frozen. No difference was observed between the phospholipase inhibitor obtained from B. subtilis 168 or B. subtilis Mu8u503.

Membranes from B. subtilis CMK12 were prepared as described previously (2).

**Lysis Curves**

Experiments to determine the osmotic stability of protoplasts were performed as described previously (1) with the following modifications. First, the temperature shift was not used. Second, the incubation (30°C, 2 hours) of the cells with lysozyme was performed in either 0.7 M sucrose, 0.01 M MgCl2, 0.05 M sodium phosphate, pH 7.0 (Buffer A), or Buffer A containing 0.02 M EDTA. In the latter case samples of the protoplast suspension were diluted into 0.05 M sodium phosphate, 0.01 M EDTA, containing varying concentrations of sucrose.

**Preparation of Labeled Phosphatidylethanolamine**

1-[[H]Acyl phosphatidylethanolamine (specific activity of 200 to 300 cpm/nmol) was prepared as described previously (2) from B. subtilis grown in a medium supplemented with [H]leucine (New England Nuclear) and [H]isoleucine (Schwarz-Mann or Amer- sham-Searle Corp.). Techniques for the extraction and purification of the lipid have also been described (12, 13).

1-[[H]Oleoyl phosphatidylethanolamine (3.2 × 106 cpm/nmol) was prepared as previously described (14) with [H]oleate (New England Nuclear) and bacterial phosphatidylethanolamine (Applied Science Laboratories, Inc.). The specific activity was adjusted by the addition of unlabeled bacterial phosphatidylethanolamine.

**Inhibitor Assay**

The assay measured the effect of the inhibitor on the activity of the membrane-bound phospholipase A of mutant CMK12. Samples (5 to 20 µl) of protein to be assayed for inhibitor activity were added to 5 µl of 0.01 M CaCl2 and 0 to 15 µl of distilled water (total volume of 25 µl) at 4°C. 1-[H]Acyl phosphatidylethanolamine (300 to 400 cpm/nmol) was dispersed in 0.167 M Tris-maleate, pH 8.0, and 2.0% Cetyltrimethylammonium bromide (Fisher Scientific Co.) by three 20-s periods of sonication (Sonic Bionik sonic oscillator, small probe, 20% maximum intensity). Aliquots of 10 µl (20 µg) of membranes from CMK12 and 15 µl (40 nmol) of [H]acyl phosphatidylethanolamine were added to initiate the assay. The initial activity in the incubation buffer (0.5 M NaCl, 30°C; 30 min). The final concentrations in the assay mixture were 0.05 M Tris-maleate, pH 8.0, 0.6% Cetyltrimethylammonium bromide, 0.0008 M phosphatidylethanolamine, and 0.001 M CaCl2. A zero time control and duplicate assays containing no inhibitor were routinely performed. The incubations were terminated by the addition of 3 ml of isopropanol alcohol-heptane-1 N H2SO4 (40:10:1). After the addition of 1 µl of 0.1 M Na2EDTA, the mixture was thoroughly mixed, and an incubation for 10 min at room temperature, the [H]-fatty acids, released by the action of the phospholipase, were extracted in 85% yield as previously described (15).

One inhibitor unit is defined as the amount of inhibitor needed to reduce the activity of the phospholipase of CMK12 (50 µg) by 30%. Only observed values of 15% to 60% inhibition were used to calculate specific activity (units/mg) because of the nonlinearity of the dependence of inhibition on protein concentration.
Purification of Phospholipase Inhibitor

Step A: Preparation of Supernatant Fraction—The phospholipase inhibitor was purified from the supernatant fraction of wild type B. subtilis 168. All steps were performed at 4°. Cells (approximately 2.2 kg, wet weight) were resuspended in 900 ml of 0.05 M sodium phosphate, pH 7.0, containing 0.005 M MgCl₂ and 5 mg of deoxyribonuclease. The cells were broken by passage of the suspension through either a Manton-Gaulin homogenizer (Manton-Gaulin Manufacturing Co., Inc.) or a French pressure cell (Aminco, 40 ml, American Instrument Co.) when smaller quantities of cells were processed. Unbroken cells were separated by centrifugation at 800 x g for 10 min and discarded. The supernatant was diluted 2-fold with 0.05 M sodium phosphate, pH 7.0, and was subjected to centrifugation for at least 10 hours at 80,000 x g. The supernatant fraction, which contained approximately 40 to 60% of the total cellular protein and total inhibitor units of the extract, was dialyzed against 20 liters of 0.05 M Tris-Cl, pH 8.0, for 16 hours followed by a second dialysis of 6 hours against 20 liters of the same buffer. The low speed supernatant fraction (800 x g, 10 min) was stored at 4° before high speed centrifugation (80,000 x g, 10 hours), because freezing and thawing caused the protein and inhibitor activity of the fraction to pellet during the second centrifugation.

Step B: DEAE-cellulose Chromatography (Starting Buffer Elution)—The high speed supernatant fraction (80,000 x g, 10 hours) in 0.05 M Tris-Cl, pH 8.0 (4.3 g of protein, 23.5 mg/ml) was applied to a DEAE-cellulose column (18 x 14 cm) equilibrated with the same buffer. The column was eluted with 10 liters of 0.05 M Tris-Cl, pH 8.0, and fractions of 300 ml were collected. The activity began eluting from the column in Fraction 9. The active fractions (4.6 liters, 1.14 g of protein) were dialyzed for 6 hours against 20 liters of distilled water followed by two dialyses of 16 hours each against 20 liters of 0.005 M Tris-Cl, pH 8.0. In this first chromatographic step, the ratio of column height (cm) to column diameter (cm) was varied from 1.25 to 2.5. The capacity of the column was exceeded when the ratio of protein (mg) to column volume (cm³) was greater than 18.

Step C: DEAE-cellulose Chromatography (Gradient Elution)—The inhibitor activity (4.6 liters, 1.14 g of protein in 0.005 M Tris-Cl, pH 8.0) from the previous step was applied to a second DEAE-cellulose column (35 x 2.5 cm) equilibrated with the same buffer. The activity was eluted with a linear gradient formed with 3.6 liters of 0.005 M Tris-Cl, pH 8.0, in the mixing chamber and 1.8 liters of 0.1 M Tris-Cl, pH 9.0, in the reservoir. Fractions of 0.5 ml were collected. The activity profile from this step is shown in Fig. 1. Fractions from the column were combined as follows: Peak I, Fractions 175 to 215 (260 ml); Peak II, Fractions 290 to 369 (526 ml); Peak III, Fractions 370 to 440 (452 ml); and Peak IV, Fractions 441 to 520 (512 ml). Aliquots of these combined fractions were assayed to determine the percentage of inhibitor in the various peaks. Peaks II and IV were dialyzed for 16 hours against 20 liters of distilled water and lyophilized to dryness. Peak I (36.5 mg of protein) was redissolved in 18 ml of 0.02 M sodium phosphate, pH 7.0, Peak IV (28 mg of protein) was redissolved in 9 ml of the same buffer. Both fractions were dialyzed for 16 hours against 4 liters of 0.02 M sodium phosphate, pH 7.0, and stored at -20°. In this second chromatographic step, the ratio of protein (mg) to column volume (cm³) was varied from 4 to 6.5. The ratio of column height (cm) to column diameter (cm) was 12.5 to 15. The ratio of the volume of the elution buffer to the volume of the column was 30.

Step D: Hydroxylapatite Chromatography—Peak II from the previous purification step (35 mg of protein, 2 mg/ml) was dialyzed for 16 hours against 4 liters of 0.02 M potassium phosphate, pH 7.0. The sample was applied to a hydroxylapatite column (13.5 x 9.1 cm) which had been equilibrated with 0.02 M potassium phosphate, pH 7.0. A gradient formed from 300 ml of 0.02 M potassium phosphate, pH 7.0, in the mixing chamber and 300 ml of 0.2 M potassium phosphate, pH 7.0, in the reservoir was immediately applied to the column. Fractions of 3 ml were collected; the flow rate was 0.4 ml per min. Two distinct peaks of inhibitor activity were observed (Fig. 2A). Fractions 101 to 121 (Peak 1, 76.5 ml) and Fra-
tions 123 to 150 (Peak 2, 101 ml) were pooled separately, dialyzed 6 hours against 10 liters of distilled water, and lyophilized to dryness. Peak 1 (0.56 mg of protein) was redissolved in 1.6 ml of 0.01 m sodium phosphate, pH 7.0. Peak 2 (1.5 mg of protein) was redissolved in 2.1 ml of the same buffer. Both fractions were dialyzed 16 hours against 2 liters of 0.01 m sodium phosphate, pH 7.0, and stored at -20°. In this step, the ratio of column volume (cm3) to protein (mg) was 1.5. The ratio of the volume of the elution buffer to the volume of the column was 15.

RESULTS

Localization of Phospholipase Inhibitor

Earlier studies (2) established that inhibitor activity was associated with both the supernatant and membranous fractions of extracts prepared from wild type cells. Further localization studies were performed by means of two techniques of cell disruption. In all of these studies, the total inhibitor activity did not vary significantly, being approximately 11,000 units/g, wet weight, of cells. In the first procedure, cells were broken in a French pressure cell, and the resultant extract was separated into supernatant and membranous fractions. As shown in Table I, the percentage of inhibitor found in soluble form varied from 30 to 60%, depending on the composition of the buffer used during the fractionation procedure. The only significant change in the distribution occurred when the Mg2+ concentration was increased from 0.005 m to 0.02 m. Under these conditions, the percentage of soluble inhibitor was decreased to 10%. However, the distribution of inhibitor was not simply dependent on Mg2+ concentration because the percentage of soluble inhibitor was similar in buffer containing 0.005 m MgCl2 or 0.01 m EDTA. The addition of protease inhibitors to the fractionation buffer did not affect the general pattern of the distribution.

In the second procedure, wild type cells, resuspended in isotonic buffer containing 0.015 m MgCl2, were incubated with lysozyme to convert them to protoplasts (Table II). The protoplasts were separated by centrifugation from degraded cell wall components as well as from any molecules localized between the wall and the cytoplasmic membrane ("periplasmic" fraction, S). The periplasmic fraction was then separated by centrifugation (100,000 x g, 120 min) into soluble (S2) and particulate (P1) fractions. The protoplasts were lysed by dilution into a hypotonic solution. The lysate was then separated into cytoplasmic supernatant (S3) and membranes (P2). When fractionated in this way, 10 to 30% of the inhibitor was found to be soluble. As shown in Table II, the soluble inhibitor was not in the cytoplasmic supernatant (S3), but rather in the so-called periplasmic fraction, S2, and membranes (P2), which is released from the cells by treatment with lysozyme. As shown in

Table I

Localization of inhibitor activity after disruption of cells by French pressure cell

Bacillus subtilis Mu8u5u5 cells (about 2 g wet weight) were grown, harvested, and washed with buffer as described under "Materials and Methods." Cells were resuspended in 5 ml of the specified buffer containing 1 mg of deoxyribonuclease and passed twice through a French pressure cell at 16,000 p.s.i. The suspension was subjected to a low speed centrifugation (800 x g, 10 min) to remove unbroken cells. The supernatant fraction was then subjected to a high speed centrifugation (100,000 x g, 120 min), and the resulting supernatant and pellet were analyzed for protein and inhibitor activity as described under "Materials and Methods." All procedures were performed at 4°.

<table>
<thead>
<tr>
<th>Additions to buffer</th>
<th>Recovered protein in supernatant</th>
<th>Recovered protein in pellet</th>
<th>Total recovery</th>
<th>Protein</th>
<th>Inhibitor</th>
<th>Protein</th>
<th>Inhibitor</th>
<th>Protein</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005 m MgCl2</td>
<td>61-72%</td>
<td>36-59%</td>
<td>28-39%</td>
<td>41-64%</td>
<td>89-93%</td>
<td>88-110%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02 m MgCl2</td>
<td>48%</td>
<td>8%</td>
<td>62%</td>
<td>92%</td>
<td>61%</td>
<td>74%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 m EDTA</td>
<td>76%</td>
<td>34%</td>
<td>24%</td>
<td>66%</td>
<td>98%</td>
<td>61%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005 m EDTA, 0.001 m a-TSF, 90% ethanol</td>
<td>78%</td>
<td>32%</td>
<td>22%</td>
<td>68%</td>
<td>92%</td>
<td>68%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005 m EDTA, 0.005 m a-TSF, 90% ethanol</td>
<td>54%</td>
<td>28%</td>
<td>46%</td>
<td>72%</td>
<td>100%</td>
<td>91%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Range of values from three experiments.

* a-Toluenesulfonyl fluoride.

Table II

Localization of inhibitor activity after treatment of cells with lysozyme

Bacillus subtilis Mu8u5u5 cells (about 2 g wet weight) were grown, harvested, and washed with buffer as described under "Materials and Methods." The buffer used was 0.06 m sodium phosphate, pH 7.0, containing 0.6 m sucrose and 0.015 m MgCl2. The cells were resuspended in 5 ml of buffer containing 25 mg of lysozyme and incubated without shaking at 30° for 30 min to convert the cells to protoplasts. The steps outlined below were then performed. Unless specified, all procedures were performed at 4°. The resultant fractions were analyzed for protein and inhibitor activity as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Recovered protein</th>
<th>Recovered inhibitor</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein%</td>
<td>Inhibitor%</td>
<td>Protein%</td>
</tr>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>27</td>
<td>54</td>
<td>86%</td>
</tr>
<tr>
<td>S2</td>
<td>52</td>
<td>&lt;1</td>
<td>79%</td>
</tr>
<tr>
<td>P1</td>
<td>21</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>2</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>19</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Experiment III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>88</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>21</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>10</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>38</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

* nd, not determined.
Experiments II and III, when the periplasmic fraction was subjected to centrifugation, a variable amount of activity was found in the supernatant.

In the third procedure, protoplasts, prepared with lysozyme in isotonic buffer containing 0.015 M MgCl₂, were broken by a French pressure cell instead of lysed by osmotic shock. After fractionation, 20% of the inhibitor activity was found to be soluble.

Because attempts to solubilize and purify the membrane-associated inhibitor activity were unsuccessful, all subsequent purification efforts were performed on the inhibitor localized in the supernatant fraction.

**Purification**

**DEAE-cellulose Chromatography (Starting Buffer Elution)**—The purification procedure described under “Materials and Methods” is summarized in Table III. In the first step, the high speed supernatant (80,000 × g, 10 hours, Step A under “Materials and Methods”) was applied to a DEAE-cellulose column in 0.05 M Tris-Cl, pH 8.0 (Step B). The inhibitor activity was not retained by the column whereas most of the supernatant protein was retained. In this experiment, the inhibitor was purified 13-fold with a 33% yield.

The range of purification obtained by this step in 16 separate experiments was 13- to 90-fold. The recovery of activity was variable, the maximum being 70%. No additional activity was recovered when the column was eluted with high salt (1 M KCl), a nonionic detergent (0.2% Cutsom), or a combination of both. The activity eluted from the column was not potentiated by the addition of aliquots from inactive fractions from the column. Reuse of the column also did not increase the yield of inhibitor units.

In contrast to the results obtained with the high speed supernatant (80,000 × g, 10 hours), chromatography of supernatant prepared by a low speed centrifugation (35,000 × g, 3 hours) resulted in only a 3-fold purification of the inhibitor with a 20% yield. Also, when the supernatant, separated from intact protoplasts by lysozyme treatment (“periplasmic” fraction, S₁, Table I) was subjected to high speed centrifugation (100,000 × g, 2 hours, S₂) and then applied to the DEAE-cellulose column, all the inhibitor activity was retained by the column.

The reason for the difference in behavior during chromatography on DEAE-cellulose between these inhibitor fractions and the inhibitor prepared by high speed centrifugation of cell extracts prepared by a French pressure cell is unclear.

**DEAE-cellulose Chromatography (Gradient Elution)**—When the pooled fractions containing inhibitor activity from the first DEAE-cellulose column were applied to a second DEAE-cellulose column in 0.005 M Tris-Cl, pH 8.0, all of the inhibitor activity was retained by the column. The activity was eluted from the column by a gradient of Tris-Cl, pH 8.0, with a 60% yield. As shown in Fig. 1, three peaks of activity were recovered. A small peak (Peak I, 4% of the recovered activity) preceded the major activity peak, which contained 63% of the recovered inhibitor units (Peak II). A third peak of activity (Peak IV) contained 18% of the recovered units. The inhibitor activity never reached background levels in the fractions between Peaks II and IV, and a small, fourth activity peak (Peak III, 9% of units) may have been present. The inhibitor recovered in Peak II was purified 16-fold with a 51% yield. Peak II contained 3.2% of the input protein. The inhibitor of Peak IV (2.4% of the input protein, 9.3% of the input inhibitor units) was purified 4-fold.

**Hydroxylapatite Chromatography**—The final purification step was chromatography on a hydroxylapatite column. Chromatography of Peak II from the DEAE-cellulose column described above (Fig. 1) resulted in two peaks of activity, one of which was complex (Fig. 2A). Peak 1 contained 1.6% of the input protein and 18% of the input inhibitor units. The specific activity of the inhibitor was enhanced 11-fold. The specific activity in the complex second peak was increased 10-fold; Peak 2 contained 4.3% of the input protein and 42% of the input inhibitor units. The total yield of inhibitor units from this step was 60%.

In order to rule out the possibility that the multiple peaks were simply due to a chromatographic artifact, a portion of Peak 2 was rerun on the same column. In this experiment, virtually all of the recovered inhibitor units (93%) eluted in a single major peak (Fig. 2B) which contained 46% of the input protein. Despite the difference in the steepness of the eluting gradients, this single major peak and Peak 2 of the first column have similar profiles and elution positions. Only 4% of the activity recovered from this column corresponded to Peak 1 of the first column; that amount could easily be attributed to incomplete separation on the first column. Therefore, it is clear that two distinct peaks of activity are separable upon hydroxylapatite chromatography.

**Determination of Purity**—Various fractions from the purification scheme were analyzed by polyacrylamide gel electrophoresis in the presence of urea and sodium dodecyl sulfate (Fig. 3). The efficiency of the two DEAE-cellulose columns is dramatically illustrated in a comparison of the gel of the crude supernatant fraction and the gel of Peak II from the second DEAE-cellulose column (Fig. 1). Only one protein band was discerned on the gel of Peak 1 from the hydroxylapatite column (Fig. 2A). The gel of Peak 2 from the hydroxylapatite column had two bands, one being predominant.

In order to prove that the single protein band from Peak 1 of the hydroxylapatite column corresponded to the inhibitor protein, Bio-Gel P-60 chromatography was performed. As shown in Fig. 4, the recovered protein (56%) and the recovered activity (44%) were coincident, with the specific activity in four tubes at the peak being constant. Therefore, the purification scheme outlined in Table III yielded a homogeneous inhibitor protein in the final step.

**Molecular Weight of Inhibitor**—Gel filtration chromatography on Bio-Gel P-60, illustrated in Fig. 4, was used to determine the molecular weight of the inhibitor under conditions where the protein was in its native conformation (Fig. 5A). A molecular weight in the range of 28,000 to 32,000 was determined for the following inhibitor preparations: homogeneous inhibitor from Peak 1 of the hydroxylapatite column, inhibitor from Peak 2 of
RELATIVE MOBILITY

FIG. 3. Acrylamide gel electrophoresis in SDS and urea. Samples were prepared and subjected to electrophoresis as described under "Materials and Methods" (direction of migration is downward). The samples on the gels (left to right) were: dialyzed supernatant fraction; Peak II, DEAE-cellulose chromatography, linear gradient (Fig. 1); Peak I, hydroxylapatite chromatography (Fig. 2A); and Peak 2, hydroxylapatite chromatography (Fig. 2A).

FIG. 4. Gel filtration of inhibitor after hydroxylapatite chromatography. Inhibitor protein from Peak 1 of the hydroxylapatite column in 0.5 ml of 0.02 M sodium phosphate, pH 7.0, with 10% glycerol was layered on a column of Bio-Gel P-60 (46 X 1.8 cm) equilibrated with the same buffer. The chromatography was performed at 4°C. Fractions of 1 ml were collected. Protein (O-O) and inhibitor activity (X-X) were determined as described under "Materials and Methods." The arrows indicate the elution positions of blue dextran 2000 and sodium [14C]acetate.

FIG. 5. Molecular weight determinations. A, various preparations of inhibitor (1000 inhibitor units) were combined with molecular weight standards consisting of 3 mg of ovalbumin (S1), and 3 mg of myoglobin, containing dimer (S2) and monomer (S3), in 0.5 ml of 0.02 M sodium phosphate, pH 7.0, and 10% glycerol. Samples were layered onto a Bio-Gel P-60 column (46 X 1.8 cm) and collected with 0.02 M sodium phosphate, pH 7.0. The proteins were eluted with an operating pressure of 28 cm of water at 4°C. Fractions of 1 ml were collected and analyzed for protein and inhibitor activity as described under "Materials and Methods." The relative mobility was calculated by dividing the void volume (as determined by blue dextran 2000) by the elution volume of the protein. Three preparations of inhibitor were analyzed: Peak 1, hydroxylapatite column; Peak 2, hydroxylapatite column; and Peak IV, DEAE-cellulose column, gradient elution. B, samples of protein were prepared and subjected to acrylamide gel electrophoresis in SDS and urea as described under "Materials and Methods." The mobility of the protein relative to the tracking dye was determined by an activity assay or protein stain. The proteins used as molecular weight standards were as follows: bovine serum albumin (S1), ovalbumin (S2), Bacillus PB fibrous protein (S1), myoglobin (S2), and cytochrome c (S3). The mobility of each standard was identical in two separate experiments. The preparations used were inhibitor purified 112-fold (two separate runs, activity determination) and inhibitor from Peak 1 of a hydroxylapatite column (two separate runs, protein determination).

Mechanism of Inhibition

Previous experiments utilizing crude supernatant fractions from wild type B. subtillis as the source of inhibitor and the cytoplasmic membrane of mutant CMK11 as the source
The extent of inhibition, also shown in Fig. 6, never reached 100%. After an initial preincubation of inhibitor and phospholipase to give 80% inhibition, the addition of a second portion of inhibitor resulted in little further inactivation. This result rules out the possibility that total inhibition is not achieved because the inhibitor is labile under the preincubation conditions (Fig. 6). Inhibition above 90% has never been observed.

Experiments done with both crude and purified preparations of phospholipase A1 indicated that the inhibition was dependent on both inhibitor concentration and time. In addition, the inhibitor was shown to be highly specific for the phospholipid substrate. Thus, after the inhibition is completed during the preincubation, phosphatidylethanolamine does not reverse the inhibition (17).

as depicted in the legend to Fig. 6, the phospholipase activity was then assayed for 1 min with 0.0022 M [3H]acylphosphatidylethanolamine (1190 cpm/nmol) as substrate. The homogeneous inhibitor was used to study the inactivation of the membrane-bound phospholipase. The dependence of the inhibition upon the amount of inhibitor and time of preincubation is shown in Fig. 6. There was no difference in the amount of inhibition; however, if the phospholipase, after the preincubation with varying amounts of inhibitor, was assayed in the absence of saturating or half-saturating levels of the substrate, phosphatidylethanolamine. Thus, after the inhibition is completed during the preincubation, phosphatidylethanolamine does not reverse the inhibition. However, it was shown earlier (17) that if the inhibitor, phospholipase, and phosphatidylethanolamine are added simultaneously, the phospholipid serves to decrease the rate of inactivation of the lipase by the inhibitor.

The homogeneous inhibitor was used to study the inactivation of the membrane-bound phospholipase. The dependence of the inhibition upon the amount of inhibitor and time of preincubation is shown in Fig. 6. There was no difference in the amount of inhibition; however, if the phospholipase, after the preincubation with varying amounts of inhibitor, was assayed in the absence of saturating or half-saturating levels of the substrate, phosphatidylethanolamine. Thus, after the inhibition is completed during the preincubation, phosphatidylethanolamine does not reverse the inhibition. However, it was shown earlier (17) that if the inhibitor, phospholipase, and phosphatidylethanolamine are added simultaneously, the phospholipid serves to decrease the rate of inactivation of the lipase by the inhibitor.

The homogeneous inhibitor was used to study the inactivation of the membrane-bound phospholipase. The dependence of the inhibition upon the amount of inhibitor and time of preincubation is shown in Fig. 6. There was no difference in the amount of inhibition; however, if the phospholipase, after the preincubation with varying amounts of inhibitor, was assayed in the absence of saturating or half-saturating levels of the substrate, phosphatidylethanolamine. Thus, after the inhibition is completed during the preincubation, phosphatidylethanolamine does not reverse the inhibition. However, it was shown earlier (17) that if the inhibitor, phospholipase, and phosphatidylethanolamine are added simultaneously, the phospholipid serves to decrease the rate of inactivation of the lipase by the inhibitor.
Inhibitor enzymatically inactivates the membrane-bound phospholipase.

In an attempt to delineate the molecular mechanism of the inhibitor activity, the homogeneous inhibitor was treated with various reagents which would be expected to inhibit an essential sulfhydryl group. No effect was seen on the inhibitor activity following an incubation (4°C, 40 min) with a 500-fold molar excess of N-ethylmaleimide (18), iodoacetamide, or iodoacetic acid (19). Other experiments indicated that the inhibitor activity of a crude supernatant fraction (0.96 μg, specific activity of 96 units/mg) was not destroyed by soybean trypsin inhibitor (20 or 40 μg, 30°C, 5 min) (20, 21). No loss of activity resulted when a partially purified inhibitor preparation (specific activity of 700 units/mg) was incubated (23°C, 6 hours) with an approximate 5000-fold molar excess of α-toluenesulfonyl fluoride, a general inhibitor of serine proteases (22). Nucleotides (1 to 2 × 10⁻⁴ M cyclic adenosine 3':5'-monophosphate or N6,O6'-dibutyryl cyclic adenosine 3':5'-monophosphate; 2 × 10⁻⁴ M ATP or GTP with 2 × 10⁻⁴ M MgCl₂) did not modulate the activity of the inhibitor. The inhibition does not depend on metal ions and proceeds slowly at 4°C, although the kinetics of inhibition at this temperature have not been quantitated.

**Effect of Phospholipase Inhibitor on Osmotic Stability of Protoplasts**

*B. subtilis* CMK₃₅ was originally selected because its protoplasts lyse under conditions where the protoplasts formed from the wild type are stable. This fragility could be correlated with the activity of a membrane-bound phospholipase A₁ in the protoplasts of the mutant. During the incubation with lysozyme, the action of the phospholipase, in conjunction with a soluble lysophospholipase, resulted in the production of lipid-poor protoplasts. Addition of EDTA to the lysozyme-containing incubation mixture resulted in the inhibition of the Ca²⁺-dependent, membrane-bound phospholipase and in the stabilization of the protoplasts of the mutant organism to osmotic stress (1).

If the activity of the phospholipase A₁ was responsible for the fragility of the protoplasts of the mutant, addition of the purified inhibitor, which is specific for the phospholipase A₁, should stabilize the protoplasts of the mutant. The results shown in Fig. 7 indicate that purified inhibitor added exogenously during the conversion of cells to protoplasts prevented the lysis of the protoplasts of the mutant. The amount of inhibitor added to the mutant cells was only 10 times greater than the amount of inhibitor that would have been present in an equivalent number of wild type cells.

**DISCUSSION**

During a screening procedure which compared the osmotic stability of protoplasts of mutants with that of the protoplasts of the parent, *B. subtilis* CMK₃₅ was detected because its protoplasts, being osmotically fragile, lysed while protoplasts of the parent remained intact. This fragility was thought to be due to extensive membrane lipid hydrolysis caused by the action of a membrane-bound phospholipase A₁ and a soluble lysophospholipase. A specific protein inhibitor of the phospholipase was found in the parent organism, *B. subtilis* Mu8u5u5 (1, 2).

A number of the properties of this inhibitor has now been established. The stability of the crude phospholipase inhibitor is distinctive. It retains at least 80% of its activity after treatment with heat (100°C, 5 min), urea (4 M), SDS (1%), Cutsicum (1%), or low pH (4.0). This stability is in contrast to heat-labile protein inhibitors such as the inhibitors of the DPN pyrophosphatase and 5'-nucleotidase (23, 24) but is similar to many inhibitors, most notably proteinase inhibitors (25). However, the activity of the phospholipase inhibitor is sensitive to trypsin, whereas proteinase inhibitors are quite resistant to proteolytic digestion.

The phospholipase inhibitor has been purified 2400-fold from the supernate of the wild type by chromatography on DEAE-cellulose and hydroxylapatite. It has been shown to be homogeneous by acrylamide gel electrophoresis in SDS and urea and gel filtration chromatography. The native inhibitor has a molecular weight of 28,000 to 32,000 based on gel filtration chromatography. The molecular weight under the denaturing conditions of gel electrophoresis in SDS and urea is 36,000 to 37,000. The significance of the slight difference in the values for molecular weight obtained by these two procedures is not known.

During the purification of the inhibitor at least three active forms are detected on the basis of ion exchange and adsorption chromatography. The relationship between these various molecules is unknown, although it is clear that they do not differ significantly in molecular weight. Although chromatographic artifacts were ruled out in the case of hydroxylapatite, this possibility was not excluded for DEAE-cellulose chromatography. It is also possible that multiple peaks of inhibitor activity result from limited proteolytic digestion during the purification procedure.

Addition of the purified inhibitor to the mutant CMK₃₅ corrects the defect that was used to detect it during the screening procedure. That is, if inhibitor is present during treatment of cells of CMK₃₅ with lysozyme, the resulting protoplasts, like those of the parent strain, are osmotically stable. Thus, the phenotype of the mutant under the screening conditions cannot be distinguished from that of the wild type if inhibitor purified from the wild type organism is present. This result indicates that

**Fig. 7.** Effect of exogenous inhibitor on the stability of protoplasts of the wild type (WT) and mutant. The procedure for measuring lysis of the protoplasts is given under "Materials and Methods." The incubations with lysozyme were done in either 1 ml of 0.7 M sucrose, 0.01 M MgCl₂, 0.05 M sodium phosphate, pH 7.0 (Buffer A), 1 ml of Buffer A containing 543 μg of phospholipase inhibitor (12,440 units/mg), or 1 ml of Buffer A containing 0.02 M EDTA.
the activity of the membrane-associated phospholipase A₁ does cause the osmotic fragility of the protoplasts of the mutant.

Relatively little is known about the genetic relationship between CMK₁, and the parent strain. In an attempt to show that the mutant had a single point mutation, several spontaneous revertants were isolated (data not shown). These revertants regained osmotic stability but lacked both intracellular phospholipase activity and inhibitor activity. Therefore it appears that these revertants have suppressor-like mutations.

Studies with the homogeneous protein indicate that the inactivation of the phospholipase is dependent on time and protein concentration. Moreover, under conditions where inhibition is irreversible, binding of the inhibitor to the membrane-associated phospholipase is not detectable. It appears, therefore, that the inhibitor functions as an inactivating enzyme. This mode of inhibition is uncommon; most protein inhibitors, such as proteinase inhibitors (25-27), colicin inhibitors (28, 29), and inhibitors of exoenzymes (30, 31) act by binding stoichiometrically to the enzyme.

Studies on the properties of the inhibitor indicate that a sulfhydryl or serine residue is not involved in the catalysis. Cofactors are not required for inactivation, which suggests that the inhibitor may be a hydrolytic enzyme. One possibility is that the inactivator is a phosphatase (32), although no significant effects of added nucleotides such as ATP or cyclic adenosine 3'5'-monophosphate were seen on either the phospholipase or inhibitor activity. Another possibility is that the phospholipase inactivator is a specific protease. Proteases that specifically inactivate apopyridoxal enzymes (33-35), tryptophan synthase (36), or fructose 1,6-bisphosphatase (37) have been described.

It is not possible to detect more than 90% inhibition of the phospholipase activity with the use of homogeneous inhibitor. Although the residual activity may be due to contaminating phospholipase activities, this finding suggests the possibility that the inhibitor functions to decrease the phospholipase activity rather than to completely inhibit it. This phenomenon is common with enzymes which are regulated by chemical modification (38), such as the glutamine synthetase from Escherichia coli (39). However, the fact that no phospholipase A₁ activity is detectable in extracts of the parent cell argues against this possibility.

The phospholipase inhibitor is present in cellular extracts in two forms, soluble and particulate. The amount of soluble inhibitor depends on the Mg²⁺ concentration present during the isolation procedure. Mg²⁺, which does not appear to be essential for binding, increases the amount of inhibitor bound to the membrane. This effect of Mg²⁺ in potentiating binding is similar to that seen when the solubilized membrane ATPase of Streptococcus faecalis is added back to either ATPase-depleted membranes (40) or to liposomes of phosphatidylcholine (41).

It is possible that in vivo all of the phospholipase inhibitor is bound to the cytoplasmic membrane and that the disruption of the cell by shear pressure or osmotic shock mechanically solubilizes a portion of the activity. If this were true, one might expect the soluble inhibitor to show an affinity for membranes. In experiments in which protein, Mg²⁺, Ca²⁺, or buffer concentration was varied, binding of partially purified or homogeneous inhibitor to membranes is not detected. The possibility remains however that the soluble inhibitor has lost a hydrophobic component which functions as its attachment to the membrane (42-44). Experiments performed in the presence of protease inhibitors (EDTA and α-toluenesulfonyl fluoride, Refs. 45-47) suggest that the soluble form is not produced during the isolation procedure by proteolytic digestion of the membrane-bound inhibitor.

In the hope of gaining an understanding of the function of the inhibitor, its subcellular distribution has been investigated. The majority of the phospholipase inhibitor is found associated with the cytoplasmic membrane of the cell. Some soluble inhibitor is present, not in the cytoplasm, but in the periplasmic fraction released from the cell during incubation with lysozyme. These results suggest that the inhibitor may be concentrated on the outer surface of the cytoplasmic membrane and in the compartment between the cytoplasmic membrane and the wall. The presence of such a cellular compartment is well documented in gram-negative organisms (48), but little is known about its existence in gram-positive organisms (49-52).

Recent studies have revealed that in addition to the membrane-associated phospholipase seen in the mutants, a Ca²⁺-dependent, inhibitor-sensitive phospholipase A is present in the broth of cultures of both mutant and parent organisms. There have been previous reports of extracellular phospholipases (53). For example, in another bacillus, Bacillus cereus, culture filtrates have at least two phospholipases (54). The finding of the extracellular phospholipase A, in conjunction with the subcellular localization studies of the inhibitor described above, indicates it is possible that the function of the inhibitor in the wild type is to protect the cytoplasmic membrane lipids of the organism from hydrolysis by the phospholipase during and following its secretion.

There are cases known of compartmentalization of an enzyme from its protein inhibitor. In E. coli, for example, the nucleotide diphosphate hexose pyrophosphatases are separated from their inhibitors; the hydrolases are released into the medium during the preparation of spheroplasts while the inhibitors are retained in the spheroplast (55, 56). In B. subtilis, ribonuclease is excreted into the medium (57) while its specific inhibitor is located in the cytoplasm, thus protecting the intracellular substrates (30).

Finally, it should be pointed out that all the components necessary for the regulation of the phospholipase inhibitor may not yet be known. For example, the particulate chinin synthase of yeast is specifically activated by a protease found in the vacuole fraction. This activating factor is inhibited by a soluble protein which acts by binding stoichiometrically to the activating factor (58-62). Similar inhibitors are known for the previously mentioned inactivating factors of apopyridoxal enzymes (33), tryptophan synthase (63), and fructose 1,6-bisphosphatase (37). Therefore, the phospholipase inhibitor, which appears to function as an inactivating enzyme, may itself be regulated by another enzyme or effector.

Acknowledgments—We wish to acknowledge Betty J. Earle for her expert technical assistance and Ann Fuhr for her help in the preparation of the manuscript.

REFERENCES


* S. S. Krag and M. B. Kennedy, unpublished observations.
Purification and characterization of an inhibitor of phospholipase A1 in Bacillus subtilis.
S S Krag and W J Lennarz


Access the most updated version of this article at http://www.jbc.org/content/250/8/2813

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/8/2813.full.html#ref-list-1