A Phospholipase Specific for Sphingomyelin from Clostridium perfringens

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SUMMARY

An enzyme that hydrolyzes sphingomyelin to ceramide and phosphorylcholine has been purified from the growth medium of Clostridium perfringens. The activity of the enzyme is stimulated about 2-fold by magnesium chloride and by diethyl ether. The activity of the enzyme is completely inhibited by 10^{-2} M ethylenediaminetetraacetic acid or 10^{-3} M calcium chloride. Lysolecithin and dipalmitoyl lecithin are hydrolyzed at about 10% of the rate of sphingomyelin. No hydrolysis of phosphatidylserine, phosphatidylethanolamine, or phosphatidylinositol is detected. The enzyme does not catalyze the exchange of phosphorylcholine-14C into sphingomyelin.

Clostridium perfringens produces a hemolytic factor, α-toxin, which Macfarlane and Knight (1) found to hydrolyze lecithin to phosphorylcholine and diglyceride. Using partially purified preparations of this enzyme, phospholipase C (phosphatidylcholine: choline phosphohydrolase, EC 3.1.4.3)) various workers (2) have also observed hydrolysis of sphingomyelin. However, the hydrolysis of lecithin and sphingomyelin has been generally considered to be caused by a single enzyme (3, 4). More recently, Slein and Logan have found that crude extracts of Bacillus cereus also hydrolyze both lecithin and sphingomyelin (5), but that when this enzyme preparation was purified, the enzymes hydrolyzing lecithin and sphingomyelin were partially separated (6). A phospholipase that hydrolyzes sphingomyelin but not lecithin has been detected in extracts of rat liver and brain (7, 8), and an enzyme apparently capable of specifically hydrolyzing sphingomyelin has also been detected in certain strains of Staphylococcus pyogenes (9). This paper reports that the phospholipase C of C. perfringens also can be resolved into two enzymes. One enzyme preferentially hydrolyzes sphingomyelin, and the other preferentially hydrolyzes lecithin.

MATERIALS AND METHODS

Bovine brain sphingomyelin was purchased from Mann or Pierce Chemical Company (Rockford, Illinois) and was over 95% pure on thin layer chromatography. DL-α-Dipalmitoyl lecithin, DL-α-dipalmitoyl cephalin, phosphatidyl-l-serine, lyssolecithin (crystalline), cholesteryl palmitate, and phosphorylcholine calcium salt from Sigma, phosphorylcholine-14C (9.4 mCi per mmole) from Tracerlab (Waltham, Massachusetts), boron trifluoride from Applied Science Laboratories (State College, Pennsylvania), and thin layer plates (Silica Gel H) from Brinkmann Instruments, Inc. Antiserum to the toxins of C. perfringens was purchased from Eli Lilly and Company (Indianapolis). N-Palmitoyl-DL-erythro sphingomyelin-14C (labeled in the methyl groups of choline), N-palmitoyl-DL-erythro sphingomyelin, and N-palmitoyl-DL-erythro-ceramide were gifts of Dr. J. Kanfer, National Institutes of Health.
lecithin, and other phospholipids was also measured at pH 7.8 (37) in a Radiometer TTTT titrator equipped with an O.Dich recorder as previously described (12). The phospholipids were suspended by sonic disruption in 0.154 M NaCl just prior to their use. Each reaction mixture contained 1.5 mg of phospholipid and 308 μmoles of sodium chloride in a total volume of 2.0 ml.

Protein was measured by the method of Lowry et al. (13) with an insulin standard or by a spectrophotometric method (14). For removal of calcium which might have been present in some sphingomyelin preparations, the sphingomyelin was dissolved in chloroform-methanol (2:1, v/v), and the solution was treated with 0.1 HCl. Other methods have been described previously (12).

**RESULTS AND DISCUSSION**

Enzyme Purification—C. perfringens (ATCC 10543) was grown as described by Cassidy, Jourdian, and Roseman (15). The bacteria were removed by centrifugation and discarded, and the material precipitating from the growth medium between 50 and 85% saturation with ammonium sulfate was collected, dissolved in distilled water, and lyophilized. This lyophilized preparation (Lot. NEU 6602, supplied by Worthington Biochemical Corporation, Freehold, New Jersey) was used for further purification. In a typical preparation, 12.0 g (dry weight) of NEU 6602 containing 3.3 g of protein was dissolved in 120 ml of 0.154 M NaCl and applied to a column (8 × 110 cm) of Sephadex G-100 equilibrated in 0.01 M Tris-HCl, pH 6.0. The flow rate of the column was 15 ml per hour. The peak of θ-toxin activity emerged at 2280 ml. Sphingomyelin-hydrolyzing activity was detected in two partially resolved peaks between 2660 and 3080 ml (Fig. 1). The first peak was the sphingomyelin-hydrolyzing enzyme. The second peak corresponded to the position of lecithinase C which also hydrolyzes sphingomyelin but at a much lower rate than lecithin.

The material emerging between 2660 and 3080 ml was pooled, the pH was adjusted to 7.5 with sodium hydroxide, the protein was measured, and the enzyme was applied to a DEAE-cellulose column equilibrated with 0.01 M Tris-HCl, pH 7.5. A column size was chosen so that there was 1 ml of DEAE-cellulose per mg of protein. After the enzyme was applied, the column was washed with 4 volumes of 0.01 M Tris-HCl (pH 7.5). A linear gradient of NaCl in 0.01 M Tris-HCl (pH 7.5) was then applied. Sphingomyelin-hydrolyzing activity emerged at conductivity of 2.0 mohms⁻¹. The individual fractions were rapidly frozen in acetone and Dry Ice and stored at −20°. No loss of enzyme activity was detected through 2 months of storage. The purification is summarized in Table I. The apparent increase in enzyme yield is probably caused by the removal of inhibitors during purification.

Sephadex G-100 fractions containing lecithin-hydrolyzing activity were pooled and purified on DEAE-cellulose (0.01 M Tris-HCl, pH 7.5) in a similar manner. This enzyme was also eluted at a conductivity in a 1-cm cell of about 2 to 4 mohms⁻¹. The individual fractions were rapidly frozen in acetone and Dry Ice and stored at −20°. No loss of enzyme activity was detected through 2 months of storage. The purification is summarized in Table I. The apparent increase in enzyme yield is probably caused by the removal of inhibitors during purification.

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Preparation NEU 6602 was purified on shorter Sephadex G-100 columns and at somewhat higher flow rates, less complete separation of the sphingomyelin-specific and lecithin-specific enzymes was achieved. However, the enzymes could then be separated on a DEAE-cellulose column (0.01 M Tris-HCl, pH 6.0) provided that about 1 mg of protein was used for each cubic centimeter of DEAE-cellulose. The lecithin-specific enzyme was unsadolized and the sphingomyelin-specific enzyme was absorbed and eluted with sodium chloride. When the lecithin-specific enzyme was dialyzed extensively, a large amount of brown pigment was removed, after which the enzyme could be adsorbed on DEAE-cellulose, as described above in the large scale preparations.

**Sphingomyelin-hydrolyzing Enzyme**

Properties of Purified Enzyme—The optimal activity of the sphingomyelin-cleaving enzyme was at pH 7.8 to pH 8.8 (Fig. 2). The reaction was linear with time for up to 40 min (Fig. 3). The reaction rate declined when about 100 μmoles of phosphorylcholine were produced. Sphingomyelin hydrolysis is proportional to the amount of enzyme protein up to the formation of 100 μmoles of phosphorylcholine. The apparent Kₘ for sphingomyelin was approximately 8 × 10⁻⁴ M (Fig. 4).

**Inhibitors**—The enzyme was completely inactivated by a 30-min preincubation with 10⁻² M EDTA and 60% inhibited by 10⁻² M 2-mercaptoethanol. Sphingomyelin-cleaving activity was also diminished by an antiserum prepared against the toxins

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**TABLE I**

**Enzyme purification**

Information about details of purification is contained in the text.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume</th>
<th>Total protein</th>
<th>Total enzyme activity</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate, 50 to 80%</td>
<td>120</td>
<td>3.3</td>
<td>53.6</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>410</td>
<td>0.3</td>
<td>61.0</td>
<td>173</td>
<td>11</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>396</td>
<td>0.021</td>
<td>64.0</td>
<td>3080</td>
<td>190</td>
</tr>
</tbody>
</table>

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1 The studies reported here were performed with a 50 to 80% ammonium sulfate fraction which is relatively low in phospholipase C activity but which contains another substance of interest to us (16). However, the enzymes might be best prepared from the 0 to 80% ammonium sulfate fraction of the growth medium.
Sphingomyelin Phospholipase

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Fig. 2. The pH dependence of sphingomyelin hydrolysis. Each 0.4-ml reaction mixture contained 2.9 mg of bovine brain sphingomyelin, 5.2 μmoles of buffer, and 4.7 μg of enzyme protein (2500 units per g). Incubation was for 40 min at 37°. The buffers used were: O, sodium glycylglycinate; ●, Tris-HCl; △, sodium glycinate.

Fig. 3. Time course of sphingomyelin hydrolysis. Each incubation mixture of 0.4 ml contained 0.58 mg of sphingomyelin, 5.2 μmoles of Tris-maleate buffer at pH 7.8, and 6.3 μg of enzyme protein (3500 units per g). A larger amount of enzyme was used here than in other experiments, because the Tris-maleate buffer was inhibitory.

Fig. 4. Dependence of the rate of hydrolysis on substrate concentration. Each incubation mixture of 0.4 ml contained 5.7 μg of enzyme protein (3000 units per g) and 5.2 μmoles of Tris-Cl buffer at pH 8.55. The incubation period was 40 min.

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of C. perfringens. However, exposure to iodoacetate, iodoacetamide, N-ethylmaleimide, or diisopropylfluorophosphate at 10^{-2} M did not diminish sphingomyelin-hydrolyzing activity. Also, ceramide at 1 mg per ml and the sodium salt of phosphorylcholine at 10^{-1} M were not inhibitory. The phosphorylcholine inhibition studies were performed in the pH-stat. Enzymatic activity was also decreased about 60% when the assay was done in Tris-maleate buffer instead of sodium glycinate.

Activators—Sphingomyelin-hydrolyzing activity was increased about 2-fold when 0.2 volume of diethyl ether was layered over the reaction mixtures. The ether evaporated during the reaction. Stimulation of lecithinase C activity by ether has been observed previously (17). Enzymatic activity was also increased about 2-fold when the sphingomyelin was sonically disrupted in a mixture of 0.15 M sodium chloride and 0.077 M sodium glycinate buffer rather than directly in 0.15 M sodium chloride, and the buffer was subsequently added. These increased rates are probably caused by a change in the dispersion of water-insoluble sphingomyelin.

Ions—Sphingomyelin-hydrolyzing activity was increased by the addition of MgCl₂ and completely inhibited by 10^{-2} M CaCl₂ (Table II). Enzyme activity was reduced 80% by 10^{-3} M ZnCl₂, 64% by 10^{-4} M CuCl₂, 47% by 10^{-3} M FeSO₄, 25% by 10^{-3} M MnCl₂ or BaCl₂, and 10% by 10^{-3} M Ni(C₂H₃O₂)₂.

Substrate Specificity—Lysolecithin and lecithin were hydrolyzed by the enzyme but at a much lower rate than sphingomyelin (Table III). However, no hydrolysis of phosphatidylserine or dipalmitoyl cephalin was detected (Table IV). No hydrolysis of an impure preparation of phosphatidylinositol was detected when 15 μg of enzyme were incubated with 6 mg of phosphatidylinositol in a total volume of 2.0 ml in the pH-stat at pH 7.8. Also, no hydrolysis of cholesteryl palmitate at 3 mg per ml could be detected in the pH-stat.

Exchange Reaction—Exchange of phosphorylcholine-¹⁴C into sphingomyelin was tested by incubating 506,000 cpm (40 μmole) of phosphorylcholine-¹⁴C with 1.6 μg of enzyme for 40 min in a standard reaction mixture. Although 155 μmole of phosphorylcholine were produced in the forward reaction, incorporation of phosphorylcholine-¹⁴C into sphingomyelin was not detected.

Products of Enzyme Reaction—One product was identified as phosphorylcholine and ceramide.
phosphorylcholine as follows. (a) A water-soluble compound containing phosphorylcholine in an organic form was found to accumulate in reaction mixtures. This material had the same RF as phosphorylcholine when chromatographed on Whatman No. 1 paper in phenol saturated with water (RF 0.84) and in the upper phase of butanol-acetic acid-water (4:1:5) (RF 0.11). The phosphate-containing spots were located by the method of Runeckles and Krotkov (18). (b) A 1.8-ml reaction mixture (pH 7.8) containing 1.0 mg of N-palmitoyl sphingomyelin, 13,000 cpm of N-palmitoyl sphingomyelin-32P, 8 μg of enzyme, 21 μmoles of Tris-maleate buffer, and 20% ether (v/v) was incubated for 4 hours at 37°. The reaction mixture was extracted with 9 ml of chloroform-methanol (2:1, v/v), and the aqueous phase was washed with 2 ml of synthetic upper phase (19). When aliquots were chromatographed in phenol saturated with water or butanol-acetic acid-water (4:1:5), only one radioactive compound was present. The RF of that compound was identical with that of phosphorylcholine. The aqueous phase was then evaporated to dryness, 100.2 mg of unlabeled phosphorylcholine calcium salt were added, and the combination of labeled and unlabeled compounds was dissolved in 0.45 ml of warm water. The calcium salt was then crystallized as described by Wittenberg and Kornberg (20). The specific activity of the product on successive crystallizations was 13.7, 14.1, and 15.6 dpm per pmole. These values agreed closely with the expected 90% (NH4)2SO4 fraction.

The other product was identified as ceramide as follows. (a) In all reaction mixtures, a compound accumulated with the same RF as authentic ceramide in thin layer chromatography on silica gel plates. The RF in chloroform-methanol-acetic acid (92:2:4) was 0.44, and in chloroform-methanol-H2O (65:25:4), the RF was 0.92. (b) A 6-ml reaction mixture containing 9 mg of bovine brain sphingomyelin and 8 μg of enzyme was incubated for 18 hours at 37° in a pH-stat. A control without enzyme was also run. The reaction mixture was mixed with 19 volumes of chloroform-methanol (2:1, v/v), and 2 volumes of water were added to give two phases. The organic phase was washed with 4 volumes of theoretical upper phase and evaporated to dryness. The residue was dissolved in chloroform and applied to a silica gel column. The column was eluted sequentially with chloroform-methanol-water-soluble organic phosphate released during the reaction. Each reaction mixture in a total volume of 2.0 ml contained 15 mg of phospholipid, 308 μmoles of sodium chloride, and, where indicated, 2 μmoles of MgCl2, 2 μmoles of calcium chloride, or 2 μmoles of both ions. Reaction rates were measured in the pH-stat for 20 min at 37° (pH 7.8). The rate of the fastest reaction was set equal to 100. Each reaction mixture contained either 2 μg of sphingomyelin-hydrolyzing enzyme (2500 units per g), 5 μg of the lecinith-hydrolyzing enzyme (1000 units per g), or 20 μg of the 0 to 80% (NH4)2SO4 fraction.

**TABLE II**

**Effect of MgCl2 and CaCl2 on sphingomyelin hydrolysis**

Each reaction mixture contained 0.41 mg of sphingomyelin and 5.2 μmoles of sodium glycinate buffer at pH 8.6. The incubation was 40 min. The specific activity of the enzyme was 3500 units per g.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration</th>
<th>Enzyme</th>
<th>Phosphorylcholine produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg</td>
<td>mmoles</td>
</tr>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.48</td>
<td>34</td>
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<tr>
<td>MgCl2</td>
<td>10^-4</td>
<td>0.48</td>
<td>87</td>
</tr>
<tr>
<td>MgCl2</td>
<td>10^-4</td>
<td>0.48</td>
<td>86</td>
</tr>
<tr>
<td>MgCl2</td>
<td>10^-4</td>
<td>0.48</td>
<td>60</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>1.06</td>
<td>102</td>
</tr>
<tr>
<td>CaCl2</td>
<td>10^-3</td>
<td>1.06</td>
<td>5</td>
</tr>
<tr>
<td>CaCl2</td>
<td>10^-4</td>
<td>1.06</td>
<td>50</td>
</tr>
<tr>
<td>CaCl2</td>
<td>10^-5</td>
<td>1.06</td>
<td>96</td>
</tr>
</tbody>
</table>

**TABLE III**

**Comparison of relative rates of hydrolysis of sphingomyelin, egg lecithin, and dipalmitoyl lecithin by crude and purified phospholipase C**

Each reaction mixture in a total volume of 2.0 ml contained 1.5 mg of phospholipid, 308 μmoles of sodium chloride, and, where indicated, 2 μmoles of MgCl2, 2 μmoles of calcium chloride, or 2 μmoles of both ions. Reaction rates were measured in the pH-stat for 20 min at 37° (pH 7.8). The rate of the fastest reaction was set equal to 100. Each reaction mixture contained either 2 μg of sphingomyelin-hydrolyzing enzyme (2500 units per g), 5 μg of the lecinith-hydrolyzing enzyme (1000 units per g), or 20 μg of the 0 to 80% (NH4)2SO4 fraction.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Phosphorylcholine produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>2.0</td>
<td>117</td>
</tr>
<tr>
<td>Dipalmitoyl lecithin</td>
<td>2.0</td>
<td>12</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Dipalmitoyl cephalin</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>

**TABLE IV**

*Substrate specificity of enzyme*

Each reaction mixture of 0.4 ml contained 0.58 mg of phospholipid and 5.2 μmoles of sodium glycinate buffer at pH 8.8. Incubation was for 40 min, and the enzyme had a specific activity of 2500 units per g.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Phosphorylcholine produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>0.81</td>
<td>92</td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>0.81</td>
<td>9</td>
</tr>
</tbody>
</table>

3, 10, 50, and 80% methanol-chloroform (v/v) and finally with methanol. The fractions were assayed for fatty acid amides as described by Bradley (21). Fatty acid amide-containing material, 3.9 μmoles, was eluted with 3% methanol-chloroform as was authentic ceramide. The yield of 3.9 μmoles of fatty acid amide agreed well with the recovery of 4.7 μmoles of phosphorylcholine in the aqueous phase. The material eluted with 3% methanol-chloroform also had the same RF as standard ceramide in thin layer chromatography. (c) Another reaction mixture containing 1.5 μmoles of N-palmitoyl sphingomyelin, 7.8 μg of enzyme, and 14 μmoles of glycine buffer was incubated at pH 8.8 for 4 hours at 37°. The reaction mixture was extracted as described above and chromatographed on silica gel. The material eluted with 3% methanol-chloroform (v/v) was evaporated to dryness and analyzed by infrared spectroscopy (Fig. 5). The unknown compound had an infrared spectrum essentially identical with that of ceramide published by Rouser et al., as shown in Fig. 13 of Reference 22.
Comparison of Properties of Sphingomyelin- and Lecithin-
specific Enzymes—Hydrolysis of phospholipids by a phos-
pholipase C produced by C. perfringens has been previously
considered to be attributable to a single enzyme. In most
studies, egg lecithin has been the substrate used, and it has been
found that hydrolysis of lecithin is dependent on the presence of
calcium (3). When the hydrolysis of sphingomyelin was previ-
ously investigated, calcium was routinely added to the reaction
mixtures and much more lecithin than sphingomyelin was
found to be hydrolyzed during incubations lasting 1 to 3 hours
(3). With enzyme at the ammonium sulfate stage of purifica-
tion, we found in the presence of calcium that the rate of lecithin
hydrolysis was linear for 1 to 2 hours, whereas the rate of sphin-
gomyelin hydrolysis was linear for 10 to 15 min and then declined
in agreement with the findings of Matsumoto (2). The reason
for the decrease in the rate of sphingomyelin hydrolysis is unclear,
but may be caused by activation of a protease by calcium.

The rate of sphingomyelin hydrolysis, however, was greatly
increased when calcium was omitted from reaction mixtures
(Table III). After the crude enzyme preparation was purified
on Sephadex G-100, it became clear that there were two phos-
pholipases present (Fig. 1). The first to emerge was an enzyme
with high specificity for sphingomyelin. Its activity on both
sphingomyelin and lecithin was completely inhibited by calcium
ions at 10^{-4} M. Thus, the activity of the enzyme would not have
been detected in crude extracts when calcium was added. The
inhibition by calcium was not caused by destruction of the sphin-
gomyelin-hydrolyzing enzyme by a calcium-activated protease,
since MgCl_2 elevated the rate of sphingomyelin hydrolysis when
added to reaction mixtures which had undergone a prior incuba-
tion with CaCl_2.

The second enzyme to emerge has the characteristics of the
well known phospholipase C of C. perfringens. It had its
highest activity on the hydrolysis of egg lecithin when calcium
chloride at 10^{-4} M was present, and was 10% as active when
calcium was omitted (Table III). It had much less activity on
sphingomyelin, and this activity was further diminished when
calcium was absent, suggesting that the ability to hydrolyze
sphingomyelin is an intrinsic property of the lecithin-specific
denzyme. Synthetic dipalmitoyl lecithin was also hydrolyzed,
and calcium chloride doubled its rate of hydrolysis. Whether
calcium was present or absent, dipalmitoyl cephalin, animal
cephalin, and phosphatidylserine were hydrolyzed at less than
10% of the rate of dipalmitoyl lecithin.

Since calcium ions completely inhibited the hydrolysis of
sphingomyelin by the sphingomyelin-specific enzyme (Table III)
and calcium ions stimulated the hydrolysis of sphingomyelin by
lecithinase C, one could test to see if both activities were present
in crude extracts by measuring the rate of sphingomyelin hy-
drolysis with or without calcium chloride. In addition to
preparation NEU-6602, the presence of large amounts of a
sphingomyelin-specific enzyme was detected in one out of four
other preparations tested. However, when the one other active
preparation was passed over a Sephadex G-100 column, no
separation of the sphingomyelin- and lecithin-specific enzymes
was achieved. Furthermore, the two activities were inseparable
on chromatography on DEAE-cellulose and carboxymethyl
cellulose. No explanation for the inability to resolve the two
enzymes is yet apparent. Preliminary studies have indicated
that the activity of sphingomyelinase is increased when C.
perfringens is grown on medium enriched with sphingomyelin.
Such preparations may be useful in preparing the sphingomyelin-
specific enzyme.

Acknowledgments—We thank Dr. Julius Kanfer for his many
suggestions during the course of this work as well as for his gift
of various substrates. We also thank Dr. Andrew Gal for
performing the infrared spectra.

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