Purification of Phosphodiesterase from Bothrops atrox
Venom, with Special Consideration of the
Elimination of Monophosphatases*

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In fragments of nucleic acids bearing 3'-monophosphoryl
groups, both terminals may be determined by degrading the
fragment with a massive dose of a single enzyme, venom phos-
phodiesterase (1-5). To be reliable, this method requires a
preparation of phosphodiesterase which is both highly potent
and free from contaminating interfering enzymes. The most
dangerous contaminant is venom endonuclease, which was re-
cently purified (3). Fortunately, the optimal pH of the en-
donuclease is 5, and the enzyme has almost no activity at pH 8.9,
the optimum for phosphodiesterase. Equally fortunate are the
differences in charges between the two enzymes that permit their
rather effective separation on either carboxymethyl- or diethyl-
aminoethyl cellulose.

The preceding paper (5) describes the purification and prop-
erties of the two monophosphatases which contaminate venom
phosphodiesterase. In the earlier methods of preparation of
phosphodiesterase (4, 6-8), the major effort was directed toward
the removal of 5'-nucleotidase, but the nonspecific phosphatase
was totally neglected. This paper describes a modified pro-
cEDURE for the preparation of phosphodiesterase. The pro-
cEDURE was aimed at obtaining phosphodiesterase free from
both contaminating monophosphatases. This objective was
achieved in respect to 5'-nucleotidase but only partly at-
tained in respect to the nonspecific phosphatase.

EXPERIMENTAL PROCEDURE

Dried venom of Bothrops atrox was obtained from suppliers in
Brazil through the courtesy of Dr. K. H. Slotta. The ethano-
yzed cellulose powder was purchased from the Chromatography
Corporation of America, Morton Grove, Illinois. A sample of a
polyacrylamide gel with a water regain of approximately 15 was
kindly provided by Pharmacia, Uppsala, Sweden. Polyacrylam-
ide gels of different compositions were prepared and treated ac-
Cording to Hjerten and Mosbach (9).

Column Electrophoresis—The column was packed with ethano-
yzed cellulose, and the experiments were performed according
to Porath (10).

Analytical Methods—Phosphodiesterase was determined as
follows. The reaction mixture contained 1.0 ml of 0.1 M Tris-
HCl buffer, pH 8.9, 1.2 ml of 0.001 M calcium di-p-nitrophenyl
phosphate, 0.2 ml of enzyme solution, and water up to 3 ml.
The reaction mixture was incubated at 37° and stopped by the addi-
tion of 3 ml of 0.05 N NaOH. The absorbancy was determined at
400 mp against a blank which contained all components but
the enzyme solution. A unit of phosphodiesterase was defined
as the amount of enzyme liberating 1 µmole of p-nitrophenol per
minute, at 25°. The measurements were made at 37° and recal-
culated with the coefficient of 0.44. The new unit = 32.5 of the
units previously used in this laboratory (4, 7).

The nonspecific phosphatase was determined in the identical
manner except that the reaction mixture was composed of 1.0
ml of 0.1 m glycine-NaOH buffer, 1.2 ml of 0.001 M p-nitrophenyl
phosphate, 0.3 ml of 0.1 M MgCl₂, 0.1 ml of enzyme solution,
and water up to 3 ml. A unit was defined as the amount of
phosphatase liberating 1 µmole of p-nitrophenol per minute, at
25°. A factor of 0.70 was established to recalculate values ob-
tained at 37°.

To determine 5'-nucleotidase, the reaction mixture contained
0.1 ml of 1 x glycine buffer, pH 9.0, 0.1 ml of 0.1 M MgCl₂, 0.3
ml of 0.01 M AMP, 0.1 ml of enzyme solution, and 0.4 ml of water.
The liberated phosphate was determined by the method of Fiske
and SubbaRow (11). A unit was defined as the amount of en-
zyme liberating 1 µmole of inorganic phosphate per minute, at
25°. A coefficient of 0.25 was used to recalculate the results ob-
tained at 37°.

RESULTS

Purification Procedure

Step 1—This was a further modification of the acetone pre-
cipitation procedure previously used (6, 7). Venom, 10 g, was
dissolved in 600 ml of cold water, stirred for 30 minutes in an
ice bath, and filtered on a Buchner funnel. To the filtered solu-
tion, placed in the ice bath, 400 ml of 0.5 M acetate buffer, pH
3.8, were added with stirring. Immediately thereafter, 725 ml
of acetone (cooled to -20°) were rapidly added to attain 42% acetone concentration (the volumes are assumed to be additive).
Step 4—Gel filtration of the second precipitate from Step 3 on Sephadex G-100 was performed in 0.02 M Tris-HCl, pH 8.9 (Fig. 1). All three enzymes came in the first peak without any separation among them (nonspecific phosphatase and 5'-nucleotidase not shown in the figure). In regard to overall purification, however, this step was the most favored in the procedure, as the potency was increased approximately 10-fold with an 85% recovery.

Gel filtration on Sephadex G-200 was also tried. The three enzymes were all retarded to nearly the same degree. A slight separation within the peak was observed, the maximal activity for both monophosphatases appearing one or two tubes before the phosphodiesterase maximum, but this could not be used for further increasing the acetic acid concentration was not helpful. When the second precipitation was made with 46.5% acetic acid instead of 45% (2, 6), only 97% of phosphodiesterase was recovered in the third precipitate, and the ratio of the nonspecific phosphatase to phosphodiesterase was only slightly decreased.

Step 2—Ethanol fractionation at pH 8.9 between 33 and 66%: This was identical with Step 3 in the previous procedure (7). The ethanol precipitation at pH 6.0 (Step 2 in the previous procedure) was omitted, as it was found that gel filtration removed the same components as the pH 6.0 precipitation. The 66% ethanol precipitate was dissolved in a small volume of water.

Step 3—The repeated acetone precipitation of pH 4.0 was performed as described previously (4), except that the acetic acid concentration in the first precipitation was raised from 44 to 45%. This precipitate was discarded and the second precipitate, obtained with 50% acetic acid, was dissolved in 35 ml of 0.1 M Tris-HCl, pH 8.9.

Step 4—Gel filtration of the second precipitate from Step 3 on Sephadex G-100 was performed in 0.02 M Tris-HCl, pH 8.9 (Fig. 1). All three enzymes came in the first peak without any separation among them (nonspecific phosphatase and 5'-nucleotidase not shown in the figure). In regard to overall purification, however, this step was the most effective in the procedure, as the potency was increased approximately 10-fold with an 85% recovery.

Gel filtration on Sephadex G-200 was also tried. The three enzymes were all retarded to nearly the same degree. A slight separation within the peak was observed, the maximal activity for both monophosphatases appearing one or two tubes before the phosphodiesterase maximum, but this could not be used for further increasing the acetic acid concentration was not helpful. When the second precipitation was made with 46.5% acetic acid instead of 45% (2, 6), only 97% of phosphodiesterase was recovered in the third precipitate, and the ratio of the nonspecific phosphatase to phosphodiesterase was only slightly decreased.

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Fig. 1. Step 4 of the purification procedure. Gel filtration of the precipitate from Step 3 on a Sephadex G-100 column (4.4 X 50 cm; 760 ml, total volume). The precipitate had been dissolved in 35 ml of 0.1 M Tris-HCl, pH 8.9. Equilibrating and eluting buffer, 0.02 M Tris-HCl, pH 3.9. Fraction volume, 6.0 ml. The first 150 ml of eluate were collected separately before starting the fraction collector. O---O, Protein concentration expressed as A280 per ml; @---@, phosphodiesterase activity, units per 0.2 ml.

Fig. 2. DEAE-cellulose chromatography of the phosphodiesterase peak from Step 4 (tubes 28 to 36 in Fig. 1). Column dimensions, 1.9 X 34 cm; total volume, 96 ml. Equilibrating buffer, 0.02 M Tris-HCl, pH 8.9. Stepwise elution with Tris-HCl buffers of pH 8.9 and indicated molarity. Fraction volume, 6.0 ml. O---O, Protein concentration expressed as A280 per ml; @---@, phosphodiesterase activity, units per 0.2 ml; @---@, 5'-nucleotidase activity, units per 0.1 ml X 104.

After 30 minutes of stirring, the precipitate which formed was centrifuged off at 0° (20 minutes, 10,000 X g). This precipitate served as starting material for the purification of 5'-nucleotidase and the nonspecific phosphatase (5). The supernatant solution was transferred to a bath at -17° and 95 ml of -20° acetone were added with stirring (45% acetone concentration). After 2 hours of stirring, the precipitate was centrifuged off at -17° and discarded. The supernatant solution was again placed in the -17° bath, and 180 ml of -20° acetone were added (50% acetone concentration). The mixture was stirred for 1 hour and then centrifuged at -17°. The precipitate was dissolved in 0.1 M Tris-HCl, pH 8.9, and the pH was adjusted to 8.9 by the addition of 1 N NaOH.

The first two precipitations in this step leave less than 1% of the nonspecific phosphatase in the third precipitate, whereas in the older procedure (7), this precipitate contained 8% of the phosphatase. The change of pH from 4.0 to 3.8 also results in a substantially increased recovery (70%) of phosphodiesterase in the third precipitate.

Further increasing the acetic acid concentration was not helpful. When the second precipitation was made with 46.5% acetic acid instead of 45% (2, 6), only 97% of phosphodiesterase was recovered in the third precipitate, and the ratio of the nonspecific phosphatase to phosphodiesterase was only slightly decreased.

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Step 4—Gel filtration of the second precipitate from Step 3 on Sephadex G-100 was performed in 0.02 M Tris-HCl, pH 8.9 (Fig. 1). All three enzymes came in the first peak without any separation among them (nonspecific phosphatase and 5'-nucleotidase not shown in the figure). In regard to overall purification, however, this step was the most effective in the procedure, as the potency was increased approximately 10-fold with an 85% recovery.

Gel filtration on Sephadex G-200 was also tried. The three enzymes were all retarded to nearly the same degree. A slight separation within the peak was observed, the maximal activity for both monophosphatases appearing one or two tubes before the phosphodiesterase maximum, but this could not be used for

Fig. 3. Effect of temperature on stability of phosphodiesterase. Phosphodiesterase purified through Steps 1 to 5 was diluted with 0.1 M Tris-acetate of different pH values to a concentration convenient for the assay and kept in a water bath for 30 minutes at specified temperatures. After heating, the solutions were cooled rapidly in an ice bath and the activity was measured as described in "Experimental Procedure."
summary of purification procedure

<table>
<thead>
<tr>
<th>Step</th>
<th>pH</th>
<th>Total Ass</th>
<th>Phosphodiesterase</th>
<th>5'-Nucleotidase</th>
<th>Non-specific phosphatase</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td>Total activity</td>
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<td>total activity</td>
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<tr>
<td>Crude venom (10 g)</td>
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<td>124.6</td>
<td>0.0093</td>
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<td>1. Acetone precipitation</td>
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<td>4. Sephadex G-100</td>
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<td>0.0066</td>
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<td>5. DEAE-cellulose</td>
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<td>25.9</td>
<td>2.39</td>
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<td>6. Dialysis and heating to 60°*</td>
<td>6.0</td>
<td>10.3</td>
<td>11.7</td>
<td>1.14</td>
<td>0.006</td>
</tr>
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</table>

* The ratio phosphatase/phosphodiesterase = 0.006/11.7 = 1/1950 represents not only the relative activities, but also the relative number of monophosphate and diphosphate bond cleavages, because the units for both enzymes are defined on the same basis.

purification purposes without sacrificing 70 to 80% of the phosphodiesterase. Even then the improvement would not be substantial. As the potency as well as the recovery of phosphodiesterase was approximately 10% lower than after G-100, gel filtration on G-200 was not included in the final purification.

The difference in retardation on G-200 suggested that a gel with a water regain between 10 and 20% (these are the water regain values for Sephadex G-100 and G-200, respectively) might be useful. No such Sephadex gel is commercially available, but Pharmacia kindly provided us with a sample of a polyacrylamide gel with a water regain of approximately 15%. The protein separation pattern on this gel was more favorable than on G-100, but the recovery and potency of the phosphodiesterase were lower; no purification with regard to the monophosphatase was achieved.

Gel filtration on different polyacrylamide gels (9) gave patterns resembling those obtained from Sephadex gels. Potency and recovery of phosphodiesterase were nearly the same as in the case of Sephadex G-200. The monophosphatases were not separated from phosphodiesterase.

Step 6—The phosphodiesterase peak from Sephadex G-100 (tubes 25 to 36 in Fig. 1) was applied to a column of DEAE-cellulose, previously equilibrated with 0.02 M Tris-HCl of pH 8.9. Elution was performed first with the equilibrating buffer and then with 0.05 M and 0.4 M Tris-HCl of the same pH. The main phosphodiesterase peak together with the nonspecific phosphatase and some 5'-nucleotidase activity was eluted with 0.05 M buffer (Fig. 2, nonspecific phosphatase not shown in the figure), whereas most of the 5'-nucleotidase was eluted with 0.4 M buffer in a protein peak which did not contain any nonspecific phosphatase. When the main phosphodiesterase peak was tested for activity towards 5'-AMP and 3'-AMP with 0.5 unit of phosphodiesterase and 48 hours of incubation, the ratio of the two mononucleotidase activities was found to be the same as reported for the purified nonspecific phosphatase (5). This indicates that no specific 5'-nucleotidase remained.

The thermostability of phosphodiesterase from Step 5 was tested at different pH values (Fig. 3). It was found that at pH 6.0 the loss of activity during 30 minutes at 60° was only 22%, much less than at alkaline pH values. With a concentrated enzyme solution the stability of the nonspecific phosphatase was

<table>
<thead>
<tr>
<th>Outer solution</th>
<th>pH</th>
<th>Total phosphodiesterase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before dialysis</td>
<td>After dialysis</td>
</tr>
<tr>
<td></td>
<td>units</td>
<td>units</td>
</tr>
<tr>
<td>Deionized H₂O</td>
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<td>0.0201</td>
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<tr>
<td>Sodium acetate</td>
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<tr>
<td>Sodium acetate</td>
<td>5.0</td>
<td>0.0440</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>6.0</td>
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</tr>
<tr>
<td>Tris-HCl</td>
<td>7.0</td>
<td>0.0302</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>8.0</td>
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</tr>
<tr>
<td>Tris-HCl</td>
<td>9.0</td>
<td>0.0412</td>
</tr>
<tr>
<td>Glycine-NaOH</td>
<td>10.0</td>
<td>0.0412</td>
</tr>
<tr>
<td>Glycine-NaOH</td>
<td>12.0</td>
<td>3.01</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of pH on stability of phosphodiesterase. Phosphodiesterase purified through Steps 1 to 5 was diluted with 0.1 M buffers of different pH values to a concentration convenient for the assay. Sodium acetate was used for pH 5.0 and 6.0, Tris-HCl for pH 8.0 and 9.0, and glycine-NaOH for pH 10.3. The solutions were kept in a water bath at 37°C, and after different times, aliquots were withdrawn and tested for activity as described in "Experimental Procedure."
phosphodiesterase was then tested under the same conditions; the loss of activity was 42%. On the basis of these observations, Step 6 of the purification procedure was devised.

Step 6—Heating to 60° at pH 6.0: Phosphodiesterase from Step 5 was dialyzed against 0.1 M sodium acetate buffer, pH 6.0, and heated in a water bath to 60° for 80 minutes. The temperature was not allowed to exceed 60°. After heating, the sample was cooled rapidly in an ice bath and then frozen.

Table I summarizes the purification procedure. The figures for Step 6 are averages for five different samples, recalculated to a starting \(A_{280}\) value of 10.8.

Attempts to separate the nonspecific phosphatase from phosphodiesterase by column electrophoresis (10) at pH 4.2, 8.0, and 9.5 were unsuccessful.

Stability of Phosphodiesterase—When stored frozen at \(-20^\circ\), the enzyme appears to be stable for at least 2 months. The effect of pH and incubation time on the stability of the purified enzyme at 37° was tested (Fig. 4). An initial decrease in activity was found at all pH values, but in the range 6.0 to 9.0, the curves leveled off after approximately 25% of the activity had been lost. At pH 5.0, 50% of the activity was lost after the first 30 minutes, but 5% was still present after 32 hours.

In earlier publications on venom phosphodiesterase, it has been noted that activity is lost during dialysis and lyophilization, but no quantitative data have been presented. Table II shows that significant losses occur at all pH values. In the pH range 7.0 to 9.0, the difference between concentrated and diluted solutions is small, but at lower as well as at higher pH values concentrated solutions are much more stable than diluted solutions. The same is true for dialysis against deionized water.

Lyophilization of the dialyzed samples gave widely varying results, the losses of activity being 25 to 60% for concentrated solutions and 40 to 65% for diluted solutions. The concentrated solutions were most stable at pH 9 to 10, whereas the values for diluted solutions did not seem to be correlated with the pH values.

The effects of pH and dialysis on the nonspecific phosphatase closely parallel the values found for phosphodiesterase. These effects thus could not be used for selective denaturation of the phosphatase.

Discussion

The method described is the first conscientious attempt to eliminate the nonspecific phosphatase (5) from the preparation of venom phosphodiesterase. The acetone precipitation previously used removed the bulk of the nonspecific phosphatase, but the modification described in the present paper (Step 1) resulted in a further 10-fold decrease in the amount of contaminating phosphatase.

The remaining phosphatase, however, was so tenaciously held that the ratio of phosphatase to phosphodiesterase remained constant in a number of precipitates obtained by gradual increase of acetone concentration. This ratio remained constant through chromatography on either DEAE- or CM-cellulose, gel filtration, and electrophoresis. The only method found to improve this ratio was thermal denaturation at \(pH\) 6.0. This step is costly in terms of phosphodiesterase, but not prohibitive. The over-all yield of phosphodiesterase is approximately 10%, comparable to that reported by Sung et al. (7).

The degree of purification is slightly lower than that reported previously (7, 8) owing to the fact that heating (Step 6) destroys more than 50% of phosphodiesterase without removing the denatured protein from solution. After Step 5, however, the potency of the pooled peak of phosphodiesterase is almost twice as high as that obtained by other methods. Potencies higher than 2.8S (95 in units previously used in this laboratory) have been found in some chromatographic fractions, suggesting that the phosphodiesterase after Step 5 still contains an appreciable amount of inert proteins.

The ratio of the nonspecific phosphatase to phosphodiesterase after Step 6 of the purification procedure is approximately 1:2000 (Table I). This ratio was obtained with \(p\)-nitrophenyl phosphate to determine the nonspecific phosphatase and calcium di-p-nitrophenyl phosphate to determine phosphodiesterase. From the point of view of usefulness of venom phosphodiesterase in the study of nucleotide sequence, this ratio serves only as a crude approximation. It has been previously shown that during the process of purification of phosphatase (5), the relative ability to hydrolyze \(p\)-nitrophenyl phosphate and 3'-AMP changes from 37:1 to 2:1. In the preparations of phosphodiesterase, this ratio for the contaminating phosphatase approaches 10:1. However, even this figure does not allow estimation of the functional ratio of phosphatase to phosphodiesterase, because the rate of hydrolysis by the latter enzyme is influenced by the chain length of the substrate.

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References

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