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SNAKE VENOM PHOSPHODIESTERASE: SIMPLE PURIFICATION WITH BLUE SEPHAROSE AND ITS APPLICATION TO POLY(ADP-RIBOSE) STUDY*

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SUMMARY A rapid method of purifying snake venom phosphodiesterase has been developed using Blue Sepharose or blue dextran/Sepharose as an affinity adsorbent. A sixty-fold purification of the enzyme from commercial preparations is achieved in a single step with a yield of 60%. The purified enzyme preparation is essentially free from phosphatase activities and exhibits a major protein band on SDS-polyacrylamide gel electrophoresis. Chain length analysis of poly(ADP-ribose) exemplifies the usefulness of this technique.

Snake venom phosphodiesterase (exonuclease I, EC 3.1.4.1) has been widely used for structural analyses of nucleic acids (1) and poly(ADP-ribose) (2). Partially purified preparations are commercially available, but are considerably contaminated by nonspecific alkaline phosphatase (EC 3.1.3.1) and 5'-nucleotidase (EC 3.1.3.5), which interfere with precise quantitative analyses. The techniques so far employed for purifying phosphodiesterases involve acetone precipitation (3), acid treatment (4), ion exchange (5-10) and affinity chromatographies (11-13). However, none of these methods are satisfactory either in the removal of contaminating activities or in the yield of phosphodiesterase activity.

Recently, affinity chromatography on Blue Sepharose or blue dextran/Sepharose has been used for purification of various enzymes

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Abbreviation: SDS, sodium dodecyl sulfate.

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which interact with NAD and other nucleotides. The affinity has been attributed to the structural similarity between the blue chromophore (Cibacron Blue F3GA) and NAD (14, 15). Taking advantage of the phosphodiesterase activity to cleave NAD, a possible interaction of the enzyme with blue chromophore was examined. We found that the enzyme specifically binds to and elutes from Blue Sepharose under certain conditions, and that this chromatography is very effective in eliminating phosphatases. This report describes a rapid procedure applicable to commercial preparations of phosphodiesterase. A structural analysis of poly(ADP-ribose) is also presented to exemplify the potency of this purification.

MATERIALS AND METHODS

Phosphodiesterases (Type II and Type VI (dried venom), from Crotalus adamanteus; and Type VII, from Crotalus atrox) and thymidine 5'-monophospho-p-nitrophenyl ester were purchased from Sigma. Phosphodiesterase (VPH, from C. adamanteus) was obtained from Worthington. Bovine albumin (Fraction V, fatty acid poor or free) was from Miles. Blue Sepharose, Sepharose 4B and blue dextran 2000 were obtained from Pharmacia. Blue dextran/Sepharose 4B was prepared according to the method of Ryan and Vestling (16); immobilized blue dextran was estimated to be 12 mg/g packed Sepharose. [^{32}P]AMP was prepared from [α-^{32}P]ATP (the Radiochemical Centre, Amersham) with the aid of myokinase (17). [Ade-^{14}C]poly(ADP-ribose) (5,000 cpm/nmol of ADP-ribose residue) was prepared by a modification of the method of Sugimura et al. (18).

The assay mixture for phosphodiesterase contained 40 µmol of Tris-acetate (pH 8.8), 0.4 µmol of thymidine 5'-monophospho-p-nitrophenyl ester, 10 µmol of $MgCl_2$ and enzyme in 1.0 ml. The reaction was started by the addition of enzyme and the increase in absorbance at 400 nm was monitored at 24°. The activity of nonspecific phosphatase was determined by incubating, for 6 hours at 37°, the mixture (1.0 ml) containing 150 µmol of glycine-NaOH (pH 9.1), 0.4 µmol of p-nitrophenyl phosphate, 10 µmol of $MgCl_2$ and enzyme. The time course was linear at least for 10 hours. 5'-Nucleotidase activity was measured in the reaction mixture (0.1 ml) containing 15 µmol of glycine-NaOH (pH 9.1), 10 nmol of [^{32}P]-AMP (300,000 cpm), 1.0 µmol of $MgCl_2$ and enzyme. Reaction was carried out for 2 hours at 37° and terminated by the addition of acid-activated charcoal (Norit A), 0.4 ml, suspended in distilled water (10% w/v). The mixture was centrifuged at 1,500 X g for 10 min, and a 0.15 ml-aliquot of the supernatant was counted in a Triton-toluene scintillator. The time course was nearly linear for 2 hours. For all these enzymes, one unit was defined as the activity that liberates one µmol of p-nitrophenol (ε_{400} = 17,000 cm^2 $mole^{-1}$) or radioactive phosphate per min under the conditions employed.

SDS-polyacrylamide gel electrophoresis was performed by a modification of the method of Weber and Osborn (19) in slabs containing 7.5% polyacrylamide and 0.1% SDS. The gel was stained with Coomassie Brilliant Blue R. Protein was determined by the method of Lowry et al. (20).

RESULTS AND DISCUSSION

Purification of phosphodiesterase on Blue Sepharose C. adamanteus venom phosphodiesterase purchased from Sigma was dissolved in 5 mM potassium phosphate containing 10 mM Tris-Cl and 50 mM NaCl (pH adjusted to 7.5), and applied to a Blue Sepharose column. A bulk of protein including most of nonspecific phosphatase and 5'-nucleotidase passed through the column, while all phosphodiesterase activity was retained (Fig. 1). The column was washed with 10 mM potassium phosphate containing 10 mM Tris-Cl and 50 mM NaCl; this eluate contained another small fraction of phosphatases. Phosphodiesterase was then eluted by elevating the phosphate concentration to 30 mM. The recovery of phosphodiesterase in this fraction was 62% and the contamination of phosphatases was reduced to less than 1/200 of the original. Blue dextran/Sepharose, prepared in our laboratory, was also useful for this purification. In this case, however, due to a smaller (empirically one-third to one-fifth) capacity than Blue Sepharose, it was necessary to use a larger column or omit the washing with 10 mM potassium phosphate.

Table I shows the summary of purification. Purification from other commercial products (phosphodiesterase from C. atrox and dried venom of C. adamanteus) is also presented. The yield of phosphodiesterase from C. atrox was higher but the separation from phosphatases was poorer than those of the C. adamanteus enzyme under identical conditions. The purification worked on dried venom as effectively as on partially purified products in terms of the ratio of phosphatase activities before and after the chromatography. For reference, Table I includes our analysis on the pro-

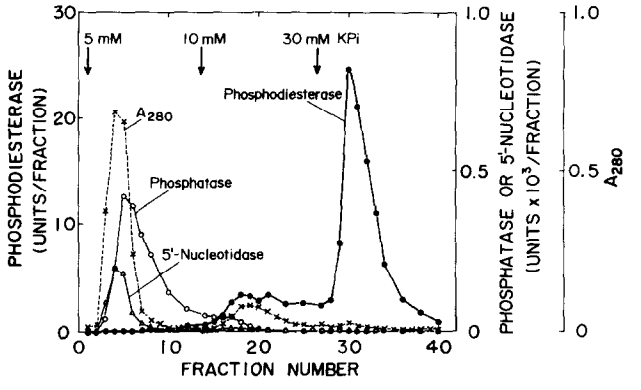

Fig. 1. Purification of phosphodiesterase on Blue Sepharose. All column operations were performed at 24°. A blue Sepharose column (0.7 X 8 cm) was equilibrated with 5 mM potassium phosphate containing 10 mM Tris-Cl and 50 mM NaCl (pH 7.5). A sample of phosphodiesterase (Sigma, Type II, 5.66 mg protein) dissolved in 1.5 ml of the equilibration buffer was applied to the column. The column was washed with 18 ml of the equilibration buffer, followed by 20 ml of 10 mM potassium phosphate in 10 mM Tris-Cl and 50 mM NaCl (pH 7.5). The concentration of phosphate was then raised to 30 mM in the same Tris-NaCl solution. Fractions (1.5 ml each) were collected at a flow rate of 12 ml/hour. After determination of A_{280}, bovine serum albumin was added (0.5 mg/ml). Enzyme activities were assayed on aliquots dialyzed separately against 50 mM Tris-Cl (pH 8.5) containing 10 mM $MgCl_2$ at 4°.

ducts of Worthington, which were reportedly treated with acid to inactivate 5'-nucleotidase (4).

Fairly specific binding of phosphodiesterases to Blue Sepharose is indicative of the affinity of the enzyme for blue chromophore, but does not necessarily exclude a possible ionic interaction between this basic protein (1) and the anionic ligand (15). Stability of purified phosphodiesterase The phosphodiesterase activity, as was eluted, was very unstable at 0° or lower temperatures, presumably due to a low concentration of protein. The enzyme was stabilized by mixing with bovine serum albumin[1/] (final

1/ Among various commercial preparations, the products of Miles (Fraction V, fatty acid poor, and Fraction V, fatty acid free) were the lowest in the contaminating 5'-nucleotidase activity (0.21 and 0.095 X 10^{-6} unit/mg protein); the activity in other commercial products ranged from 3.1 to 5.3 X 10^{-6} unit/mg.

Table I. Summary of purification of phosphodiesterases

Preparation	Protein	Phosphodiesterase		Nonspecific phosphatase	5'-Nucleotidase
		Total activity	Specific activity	Total activity	
	mg	units (%)	units/mg	units X 10^3	
C. adamanteus					
Sigma (Type II) (Lot 86C-9590)					
Commercial	5.66	169 (100)	29.9	2.78	1.12
Purified on BS[a]	0.064	107 (62)	1,672	0.011	0.0022
Purified on BDS[b]	0.30	110 (65)	367	0.053	0.014
Sigma (Type VI) (Lot 92C-1531-9)					
Commercial	6.00	39 (100)	6.5	19.0	12,300
Purified on BDS	0.47	22 (56)	46.8	0.59	225
Worthington (VPH) (Lot 36C900)					
Commercial	2.09	100	47.8	1.74	55.2
Worthington (VPH) (Lot 36H743)					
Commercial	1.23	100	81.3	0.80	0.33
C. atrox					
Sigma (Type VII) (Lot 55C-9530)					
Commercial	3.21	119 (100)	37.1	9.30	3.77
Purified on BS	0.19	112 (94)	589	0.21	0.036
Sigma (Type VII) (Lot 124C-9640)					
Commercial	2.06	256 (100)	124	6.13	1.50
Purified on BDS	0.15	181 (71)	1,207	0.18	0.030

a) Blue Sepharose.
b) Blue dextran/Sepharose.

concentration, 0.1 - 0.5 mg/ml) and kept frozen at -20°. No loss of activity was detected after one month under these conditions or after several times of freeze-thaw. Inclusion of 20% glycerol in buffers did not affect the chromatographic profiles shown above and replaced albumin in stabilizing the phosphodiesterase activity.

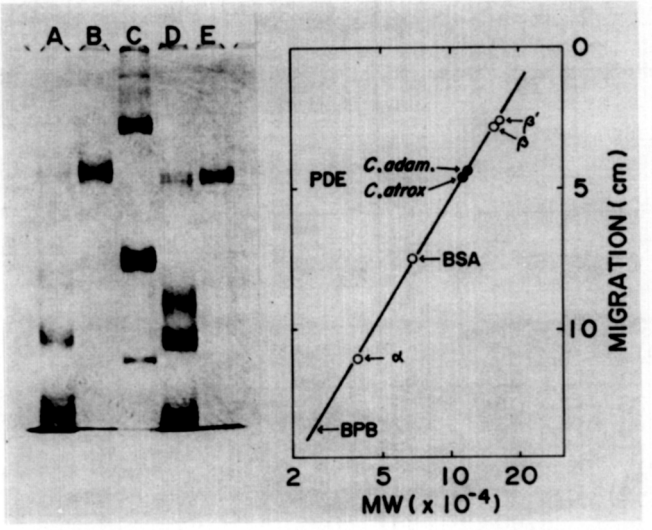

Fig. 2. SDS-polyacrylamide gel electrophoresis (left) and molecular weight determination (right). Lanes A and B, phosphodiesterase (Sigma, Type II) unpurified, 30 μg, and purified on Blue Sepharose, 10 μg, respectively; C, standard proteins with following molecular weights (E. coli RNA polymerase (subunit α 39,000, β 155,000, β' 165,000), 5 μg, and bovine serum albumin (68,000), 5 μg); D and E, phosphodiesterase (Sigma, Type VII) unpurified, 30 μg, and purified on Blue Sepharose, 10 μg, respectively.

Purity and molecular weights of purified phosphodiesterases The phosphodiesterases of C. adamanteus and C. atrox, purified as above, gave one each prominent protein band on SDS-polyacrylamide gel electrophoresis (Fig. 2 (left)). Another minor band was also detected near a tracking dye in both cases, but the phosphodiesterase activity appeared to associate with the major band since further purification on Sephadex G-100 gave a single band corresponding to this major band (results not shown). Purity of the phosphodiesterase preparations judged by the staining density was, at least, 70% for C. adamanteus and 50% for C. atrox.

From the electrophoretic mobilities, molecular weights of phosphodiesterase protomers of C. adamanteus and C. atrox were estimated at 120,000 and 112,000, respectively (Fig. 2 (right)).

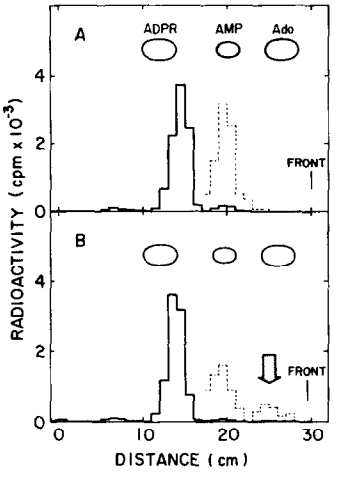

Fig. 3. Phosphodiesterase digested products of poly(ADP-ribose). [Ade-^{14}C]poly(ADP-ribose) (15,000 cpm, average chain length 24) was digested (12 hours, 37°) by one unit of phosphodiesterase (Sigma, Type II) purified on Blue Sepharose (A) or unpurified (B) in the presence of 50 m$\underline{M}$ Tris-Cl (pH 8.0) and 10 m$\underline{M}$ $MgCl_2$. The total reaction mixture was applied on paper (Whatman No. 1), and chromatographed in a solvent system, isobutyric acid : 1 $\underline{M}$ NH_4OH : 0.1 $\underline{M}$ EDTA/Na_2 (100:60:1.6, v/v/v). Dotted bars represent 20-fold scaled-up replots of a part of radioactivity (solid bars). The arrow indicates dephosphorylated product(s).

These values are close to, but not identical with, those in other reports (9-11).

Application of purified phosphodiesterase to analysis of poly(ADP-ribose) Poly(ADP-ribose) is a macromolecule synthesized from NAD in the nucleus of eukaryotes. It is composed of a linear sequence of repeating ADP-ribose units, and the average chain length is calculated from the ratio of isoADP-ribose to AMP after digestion with snake venom phosphodiesterase (2). We tested the effect of the present purification on the chain length analysis (Fig. 3). In both cases with purified (A) and unpurified (B) preparations of phosphodiesterase, the main products were isoADP-ribose, which migrated slightly ahead of marker ADP-ribose, and AMP. The unpurified enzyme, however, produced additional product(s) (marked

by an arrow) that migrated close to adenosine. The average chain lengths were calculated to be 23.8 and 40.8 ADP-ribosyl units in A and B, respectively. It is evident that degradation of AMP, and possibly of isoADP-ribose, by contaminating phosphatases led to erroneous estimation in the unpurified enzyme case.

This result clearly demonstrates the importance of the purity of phosphodiesterase preparations in structural studies on poly(ADP-ribose). This would hold true also for nucleic acids. The rapid chromatographic procedure described in this communication provides phosphodiesterase which is practically free of phosphatases, and thus will be useful for analytical purposes.

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