PURIFICATION OF EXONUCLEASE (PHOSPHODIESTERASE) FROM THE VENOM OF CROTALUS ADAMANTEUS.*

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Received September 19, 1974

Summary. A four step procedure, including chromatography on Concanavalin-A Sepharose, and leading to a 350 fold purification of exonuclease from venom of Crotalus adamanteus is described. The yield is about 35%. The enzyme is essentially free of 5'-nucleotidase, the nonspecific monophosphatase, and endonuclease. The preparation is homogeneous on disc electrophoresis without and with sodium dodecyl sulfate.

Venom exonuclease (phosphodiesterase) had been studied in this laboratory since 1955 (see the reviews 1,2). Lately, it was found that venom exonuclease is a glycoprotein. It is strongly held on Concanavalin-A Sepharose column, while endonuclease and the nonspecific phosphatase are eluted, but not 5'-nucleotidase (3). Recently, a step was devised to elute exonuclease from Con-A Sepharose. In combination with three other steps, the new method leading to a homogeneous preparation of exonuclease is described.

Experimental Procedures

Materials. Lyophilized venom of Crotalus adamanteus was obtained from the Miami Serpentarium (Miami, Florida). Three different batches were used. Concanavalin-A Sepharose 4B,

* Supported by Contract AT(11-1)3225 from the Atomic Energy Commission and Grant GB36811X from the National Science Foundation.

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QAE-Sephadex A-50 and Ficoll 400 were purchased from Pharmacia Fine Chemicals (Piscataway, New Jersey). Bis-p-nitrophenyl phosphate, sodium salt was obtained from Sigma (St. Louis, Missouri) and p-nitrophenyl thymidine 5'-phosphate from P-L Biochemicals (Milwaukee, Wisconsin). Bio-Gel P-200 was purchased from Bio-Rad Laboratories (Rockville Centre, New York), and α-methyl-D-mannopyranoside from Pfanstiehl Laboratories (Waukegan, Illinois). All other chemicals were reagent grade; water was triple distilled.

Methods. The assay mixture contained in 1 ml: 5 μmoles bis-p-nitrophenyl phosphate, 10 μmoles $MgCl_2$, 100 μmoles Tris HCl, pH 9.0. Preincubation at 37° lasted 5 min, enzyme was added and incubated for the next 5 min. The reaction was stopped by 2 ml 0.1 N NaOH; the absorbancy was read at 400 nm, using 17,600 as molar extinction coefficient (4); activity was expressed in μmoles x min^{-1} x ml^{-1}. One present unit is equal to 2.28 units previously used in this laboratory. Determinations of 5'-nucleotidase and of the nonspecific phosphatase were described previously (4). Endonuclease was determined by the method of Kunitz (5).

Results and Discussion

Preliminary Inactivation of 5'-Nucleotidase. Five grams of venom were suspended in 225 ml of water and stirred for 1 hr at room temperature. A small amount of insoluble material was removed by centrifugation at 5,000 x g for 15 min. To the solution of venom 225 ml of 0.2 M acetic acid was added with stirring. The mixture was incubated at 37° for 3 hr to

inactivate 5'-nucleotidase (Fig. 1); the mixture was then cooled down to 2°.*

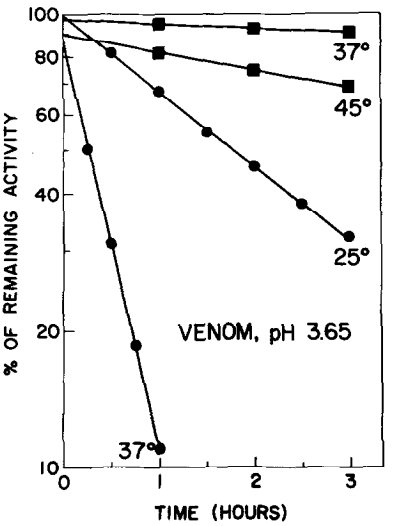

Fig. 1 Inactivation of 5'-nucleotidase. To a sample of the clarified water extract of venom (200 mg/9 ml) 0.2 M acetic acid was added to attain pH 3.65. The mixture was divided into 4 portions and each portion was incubated at a temperature indicated on the graph. Aliquots were withdrawn at indicated time intervals and were assayed for phosphodiesterase ■——■, and 5'-nucleotidase ●——●.

Step 1. Acetone (362.5 ml) precooled to -17° was added slowly to keep the temperature below 5°. The mixture was stirred for 30 min in an ice bath and then centrifuged at 0° for 30 min (13,000 x g). The supernatant was transferred to an alcohol bath (-17°), 6.5 ml of 1 M sodium acetate was added to attain pH 4.0, it was followed by 43.5 ml of 1 M sodium acetate, pH 4.0, and stirred for 15 min. Acetone (22.5 ml) was added and the mixture was stirred at -17° for 60 min. The

*This treatment decreased specific activity of 5'-nucleotidase from 14 x 10^{-1} to 1.4 x 10^{-2} u/A_{280}. It also decreased an apparent molecular weight of the nonspecific phosphomonoesterase. If the pretreated venom was directly passed through Bio-Gel P-200, the nonspecific phosphatase appeared in the second peak rather than the first.

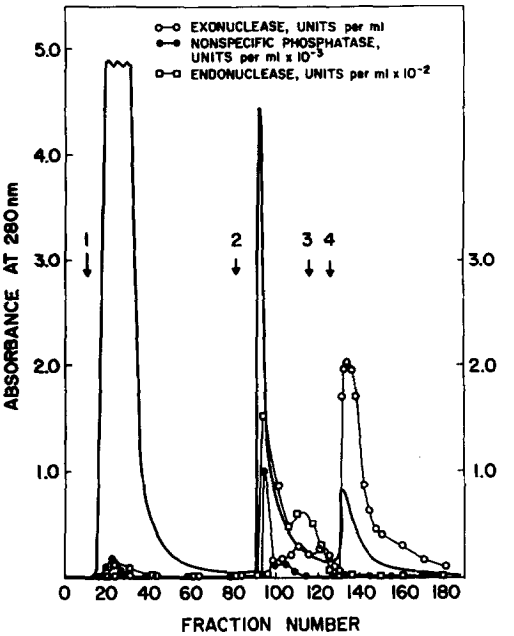

Fig. 2 Chromatography on Con-A Sepharose 4B. The column (35 x 1.5 cm) was equilibrated with 0.2 M sodium acetate buffer, pH 6.0 at room temperature (~22°). The dialyzed enzyme preparation from step 1 (~25 to 30 ml) was charged on the column at a rate of 20 ml/hr and was followed (the first arrow) by the original buffer until the absorbance of the effluent at 280 nm fell to 0.05 (the second arrow). At that time 0.05 M α-methyl-D-mannopyranoside in equilibrating buffer was started. This eluted endonuclease and the non-specific phosphatase with about 10-12% of exonuclease. When absorbance at 280 nm fell below 0.1, the buffer was changed to 0.05 M sodium phosphate, pH 7.2 containing 1 M NaCl and 0.3 M α-methyl-D-mannopyranoside. After 30 ml of this solution were passed through (1 1/2 hr), arrow 3, the flow was stopped for 6 hr and was resumed again, arrow 4, with a rate reduced to 10 ml/hr. Volume of each fraction was 3 ml. The right ordinate refers to activities of the three enzymes. Coefficients are given on the figure.

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mixture was then centrifuged at 13,000 x g for 30 min at -17°. The supernatant was returned to the alcohol bath (-17°) and 115 ml of acetone (-17°) was added. Stirring was continued for 30 min and the precipitate containing exonuclease was collected by centrifugation (13,000 x g, -17°, 30 min). It was dissolved in 20 ml of 0.2 M sodium acetate, pH 6.0, dialyzed against 1

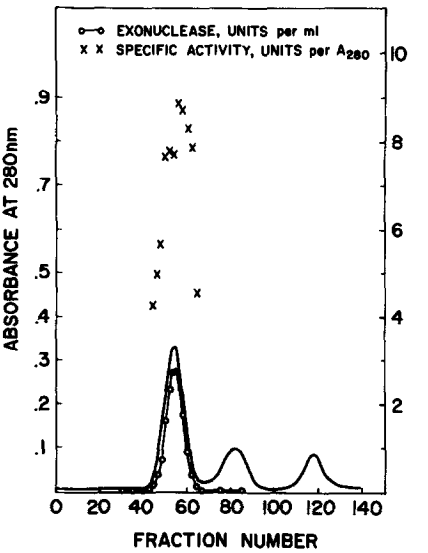

Fig. 3 Molecular sieving on Bio-Gel P-200. The column (80 x 2.5 cm) was equilibrated with 0.2 M sodium acetate, pH 6.0, at room temperature. The enzyme from step 2, after dialysis and concentration with Ficoll was charged on the column in upward direction, 15 ml per hr, 3 ml per fraction. Numbers of right ordinate apply to both activity and specific activity.

liter of 0.2 M sodium acetate, pH 6.0, overnight at 4° and clarified by brief centrifugation at 4°.

As a result of this step, the contamination by 5'-nucleotidase was reduced to 2.7×10^{-4}, endonuclease to 0.29×10^{-2} and the nonspecific phosphatase to 6.2×10^{-5}.

Step 2. The dialyzed solution was absorbed on Con-A Sepharose and contaminating endonuclease with the nonspecific phosphatase were removed (3). In order to elute exonuclease (Fig. 2, tubes 130-180), a significant increase in the ionic strength, pH and concentration of mannoside were required. Furthermore, a 6 hr equilibration was allowed. As a result, more than 50% exonuclease was recovered. Contaminating phosphatases and endonuclease could not be detected using 0.1 unit of exonuclease.

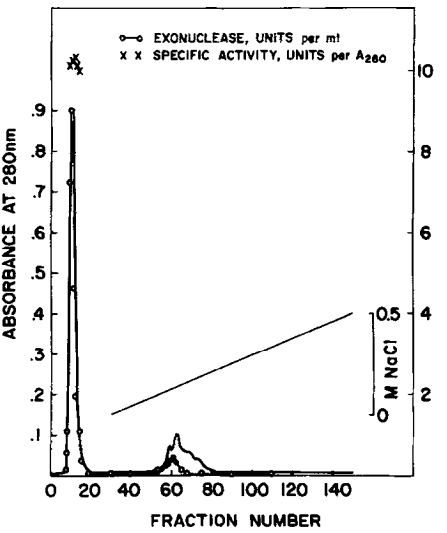

Fig. 4 Chromatography on QAE-Sephadex A-50. Column (30 x 1.1 cm) was equilibrated with 0.01 Tris HCl, pH 8.5 at 4°. The material (peak 1 from step 3) after concentration and dialysis was charged at a rate 20 ml/hr and was followed by the same buffer. With tube 30, a gradient 0 to 0.5 M NaCl in the same buffer (2 x 150 ml) was started. This eluted small amount of exonuclease with a low specific activity. Molarity of NaCl is marked on the right hand side of the graph, numbers of right ordinate refer to activity and specific activity.

Table I

Summary of Purification Procedure

Step of Purification	Protein A_{280}	Total Activity Units	Specific Activity w/A_{280}	Yield %
Pretreated venom	5900	157	0.027	100
Acetone	586	150	0.26	96
Con-A Sepharose	26.5	75	2.8	48
Bio-Gel P-200	9.8	69	7.0	44
QAE Sephadex A-50	5.7	58	10.2	37

Table II

Parallelism of Phosphodiesterase and Pyrophosphatase Activities of the Venom Exonuclease During the Purification Steps.

Step of Purification	Phosphodiesterase Activity	Pyrophosphatase Activity	Ratio
	μ equivalent x min^{-1} x ml^{-1}*		
Venom	2.05	14.21	0.142
Acetone	7.22	51.30	0.140
Con-A Sepharose	1.14	7.98	0.142
Bio-Gel P-200	2.28	16.34	0.139
QAE-Sephadex	10.45	78.85	0.138

*Aliquots of the enzyme at different steps were checked for both activities toward bis-p-nitrophenyl phosphate and ATP using the pH-stat method as previously described (6).

Step 3. The exonuclease eluted in step 2 was dialyzed against Ficoll (at 4°) almost to dryness. The dialysis tubing was washed with a total of 10 ml of 0.2 M sodium acetate, pH 6.0, and clarified by centrifugation at 4,000 rpm, 10 min at 4°. The solution was charged on a column of Bio-Gel P-200 (Fig. 3). The enzyme appears in tubes 40 to 65, the contents of which were pooled.

Step 4. The collected material from the previous step was concentrated to dryness on Ficoll at 4°, dissolved in a minimal amount of 0.01 M Tris HCl, pH 8.5, dialyzed against the same buffer for 6 to 8 hrs at 4°. It was chromatographed on QAE-Sephadex, Fig. 4. The enzyme appears in the first fraction, showing a constant specific activity across the peak.

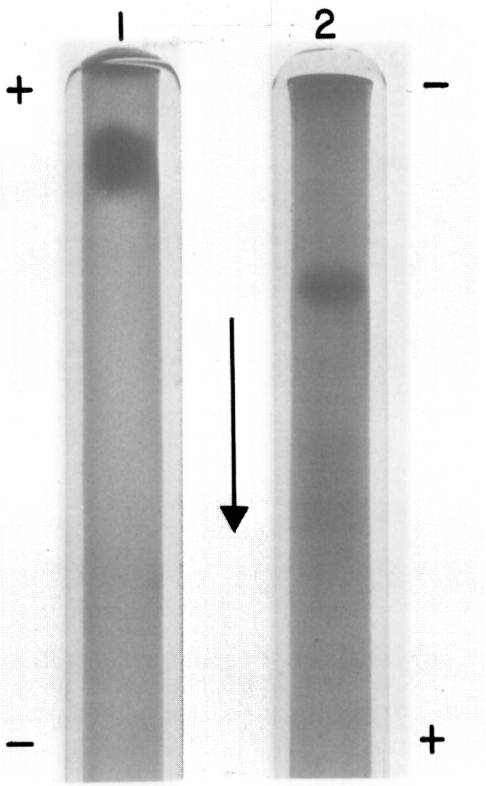

Fig. 5 Polyacrylamide gel electrophoresis of the material collected from QAE-Sephadex (step 4). 1- at pH 4, without SDS; 2- at pH 7, with 7.5% SDS.

The enzyme was pooled, and for convenience, divided into samples of 1 unit each, and lyophilized. Lyophilization results in a loss of about 10% of activity; little loss occurs on further storage at -17°.

Table I summarizes the purification procedure. An overall purification of exonuclease is about 350 fold, with a yield of about 35%. The nonspecific phosphatase and endonuclease are eluted prior to exonuclease from Con-A Sepharose (Fig. 2). The inactivation of 5'-nucleotidase is accomplished prior to chromatography on Con-A Sepharose, which retains the last traces of this contaminant.

The enzyme thus purified is homogeneous on disc electrophoresis at pH 4 without SDS, and at pH 7 with SDS (Fig. 4). The parallelism between the phosphodiesterase and pyrophosphatase activity throughout all steps of purification (Table II) leads to a conclusion that both activities are the intrinsic property of the same enzyme molecule.

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