VENOM EXONUCLEASE (PHOSPHODIESTERASE) IMMOBILIZED ON CONCANAVALIN-A-SEPHAROSE.*

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Summary. Highly purified, immobilized venom exonuclease was prepared by a two step procedure. No significant contamination with monophosphatases or endonuclease was detected. The immobilized exonuclease hydrolyzed to completion Poly A and ApAp, and was stable for at least a month.

The immobilization of an enzyme offers advantages of increased stability and of using the same preparation many times. The starting material for purification of venom exonuclease is expensive and the procedure is laborious. In the process of purification of venom exonuclease (to be published), it became apparent that it is a glycoprotein. Exonuclease appears on disc electrophoresis in the region containing glycoproteins; it is fairly stable in acid, and shows limited solubility in acetone. Furthermore, L. Dolapchiev and W. Ostrowski (personal communication) observed that treatment with neuraminidase changed the electrophoretic behavior of a commercial preparation of venom exonuclease. It was, therefore, decided to investigate the immobilization of exonuclease on a commercially available Con-A-Sepharose.

Experimental Procedures

Materials. Lyophilized venom of Crotalus adamanteus was

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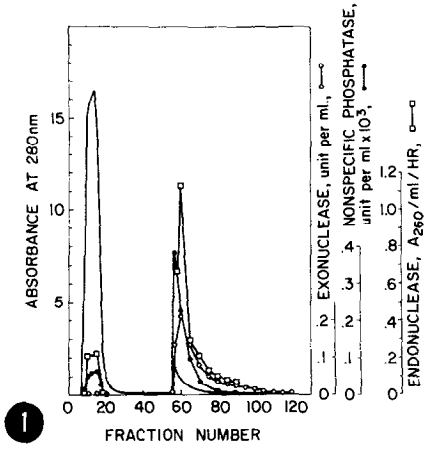
obtained from the Miami Serpentarium (Miami, Florida). Concanavalin-A-Sepharose was purchased from Calbiochem (Los Angeles, California). Both p-nitro phenyl phosphate and bis-p-nitro phenyl phosphate, sodium salt, were purchased from Sigma (St. Louis, Missouri). E. coli alkaline phosphatase was from Worthington. Poly A was from Miles (Elkart, Indiana), dinucleotide ApAp was prepared according to Sulkowski et al. (1); adenosine 5'-phosphate was from P. L. Biochemicals (Milwaukee, Wisconsin). Methyl α-D-Mannopyranoside was obtained from Pfanstiehl Laboratories (Waukegan, Illinois). All other chemicals were reagent grade; water was triple distilled.

Methods. The activities of exonuclease and of 5'-nucleotidase were determined according to Sulkowski and Laskowski (2). Nonspecific phosphatase was determined by the method of Sulkowski et al. (3). Endonuclease was assayed by the acid soluble method essentially as described by Georgatsos and Laskowski (4), except that perchloric acid was used rather than trichloroacetic.

Results and Discussion

Five grams of venom were dissolved in 225 ml of water, clarified by centrifugation, and acidified to pH 3.6 by the addition of 225 ml of 0.2 M acetic acid. After 3 hours incubation at 37°, 50 ml of 1 M sodium acetate, pH 4.0, were added and the mixture was fractionated with acetone according to Williams et al. (5). The fraction rich in exonuclease (54 units) was passed through a column of Con-A-Sepharose, as shown in Fig. 1. Approximately 95% of the protein passed through. No exonuclease could be detected in the eluant. However, endonuclease and nonspecific phosphatase were detectable. The column was then washed with 0.1 M α-methyl-D-mannoside. About 20% of the exonuclease passed through, whereas over 90% of the non-



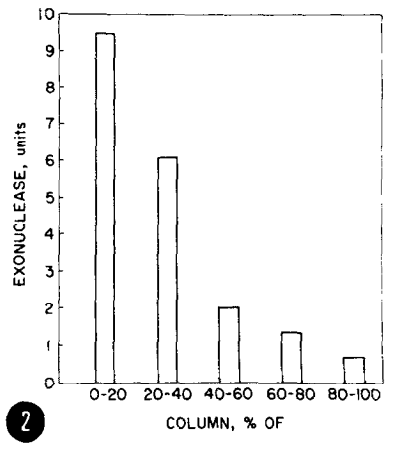
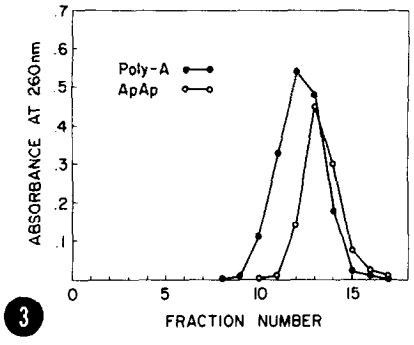


Fig. 1 Immobilization of venom exonuclease on Con-A-Sepharose. The column (27 x 1.2 cm) was equilibrated with 0.5 L of 0.2 M sodium acetate, pH 6.0, at 4°. The material obtained after acetone precipitation was taken up in 40 ml of 0.2 M sodium acetate, pH 6.0, and dialyzed against 1 L of the same buffer for 15 hours at 4°. It was clarified by a brief centrifugation. The material (40 ml, 18 A_{280} unit per ml) was applied onto the column by means of a peristaltic pump. The column was washed with equilibrating buffer (0.25 L) and then with 0.1 M α-methyl-D-mannoside in equilibrating buffer (0.35 L). Fractions (5 ml) were collected at a flow rate of 0.4 ml per min.

Fig. 2 Distribution of immobilized venom exonuclease on Con-A-Sepharose column. The content of the column described in Fig. 1 was extruded and divided into five segments from top (0-20%) to bottom (80-100%). Material from each segment was washed with 20 ml of 0.05 M Tris-HCl, pH 8.5, containing 0.1 M sodium chloride. The washing was repeated four times. To estimate the activity of exonuclease, 50 µl of suspension (20 ml) was added to incubation mixture (3 ml) and stirred for 10 min. The reaction was stopped with 0.05 M sodium hydroxide (3 ml) and the beads were allowed to settle. Absorbancy at 400 nm was read and units of activity corresponding to each segment of the column were calculated.

specific phosphatase and endonuclease were effectively removed. The Con-A-Sepharose was extruded from the column and cut into 5 segments. The results (Fig. 2) show that Con-A-Sepharose has a high binding capacity for exonuclease. The 3 lower segments contained only about 20% of the immobilized enzyme. The first two segments were mixed and a new column was poured (14 x 1 cm).



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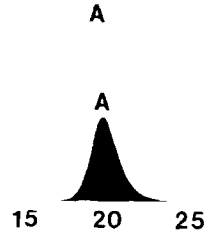

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Fig. 3 Procedure for use of venom exonuclease immobilized on Con-A-Sepharose. All operations were performed at room temperature. A column (14 x 1 cm) was packed with the material obtained from the portion (0-40%) of the column described in Fig. 2. The column was equilibrated at pH 8.5 with 0.05 M Tris-HCl containing 0.1 M NaCl and 1 mM $MgCl_2$ (50 ml). The solution (1 ml) of Poly A (1 mg/ml) in equilibrating buffer was allowed to flow into the bed of the column and was washed in with an additional 1 ml of buffer. The flow was interrupted for 15 min. and then resumed (0.3 ml per min.). Fractions (1 ml) were collected; 0.2 ml portions were withdrawn, diluted to 4 ml volume and the absorbance at 260 nm was read. The hydrolysis of ApAp (20 A_{260} units) was performed in 0.1 M Na-acetate, pH 6, containing 1 mM $MgCl_2$, for 1 hour. The fractions (10-14) from each experiment were pooled and chromatographed on paper (6) for the identification of products.

Fig. 4 Demonstration of the extent of phosphatase activity immobilized on Con-A-Sepharose. A sample (10 µl) of Poly A digest, eluted from the column, Fig. 3, was chromatographed on the Varian Aerograph (7), and the elution of adenosine was recorded (top). A sample (10 µl) of the digest after treatment with alkaline phosphatase was chromatographed (bottom) for comparison (100% dephosphorylation).
Abscissa: elution time (minutes).

The performance of this column was tested with Poly A as substrate (Fig. 3). The products were chromatographed on paper (6), and only one spot with the R_f of a mononucleotide was observed. Lack of products with smaller R_f excluded pAp which would have been present if the enzyme preparation were contaminated with venom endonuclease. It also showed that the reaction with exonuclease was complete. No nucleoside could

be detected on paper (6); however, when a sample of the eluant was chromatographed on the Varian Aerograph (7), a trace amount of A was detected (Fig. 4). Fig. 4 also shows the amount of nucleoside obtained after dephosphorylation of an identical aliquot of products with alkaline phosphatase. Dephosphorylation was calculated to be 1%.

The immobilized enzyme was then used to hydrolyze ApAp. Oligonucleotides bearing 3' monophosphate are 1000 times more resistant to exonuclease at pH 9 than the corresponding 5'-oligonucleotides; but only 200 times when the pH is lowered to 6.0 (8), at which pH, the residual activity of monophosphatases is depressed. The column was therefore equilibrated with 0.1 M sodium acetate, pH 6.0, containing 1 mM $MgCl_2$, charged with 20 A_{260} units of dinucleotide, which was allowed to remain on the column for 1 hour at room temperature. After that time elution was started. The elution profile is shown in Fig. 3. The eluant was chromatographed on paper (6), and only A and pAp were detected and found to be in equimolar quantities.

After allowing the column to stand for a month at room temperature in 0.05 M Tris-HCl buffer, pH 8.5, containing 0.1 M sodium chloride, the poly A was charged on the column, and as before the hydrolysis was complete; the experiment with ApAp was also successfully repeated suggesting that immobilization of exonuclease results in stabilization of the enzyme.

Finally, an experiment was performed to test the binding of 5'-nucleotidase on Con-A-Sepharose. To this end the content of 3 vials of exonuclease (phosphodiesterase, Worthington VPH) were dissolved in 1.5 ml of 0.2 M sodium acetate pH 6.0 and passed through a 5 ml column of Con-A-Sepharose at room temp-

erature. The sample contained 1.2 unit of exonuclease and 0.005 unit of 5'-nucleotidase prior to passing through column. The eluant (25 ml), contained neither exonuclease nor 5'-nucleotidase. When the column was washed with 0.1 M α-methyl-D-mannoside (25 ml), about 15% of the exonuclease was washed out, but no 5'-nucleotidase was detected. Additional washing with 0.5 M α-methyl-D-mannoside in the same buffer eluted neither exonuclease nor 5'-nucleotidase. The amount of exonuclease absorbed to the column was sufficient to hydrolyze 1 mg of Poly A in 6 hours. After 16% of the products was recovered as adenosine. Should the commercial preparation of venom phosphodiesterase (exonuclease) be used for immobilization, the 5'-nucleotidase must be first inactivated at low pH (3.6) as previously described (2).

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