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RAPID METHOD FOR SEPARATION AND PURIFICATION OF FOUR ISOENZYMES OF PHOSPHODIESTERASE FROM *TRIMERESURUS FLAVOVIRIDIS* (HABU SNAKE) VENOM

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SUMMARY

Four isoenzymes of phosphodiesterase were identified in *Trimeresurus flavoviridis* venom. A rapid and highly reproducible column chromatographic procedure on CM-Sephadex C-25, QAE-Sephadex A-25 and Sephadex G-100 was developed for the purification of these isoenzymes with a yield of 83%. All four isoenzymes are metalloglycoproteins having negligible amounts of phosphomonoesterase activity.

INTRODUCTION

Snake venom is a complex mixture of proteins and polypeptides. The individual components of venom are potentially useful in both therapy and research, especially in molecular biology[1,2]. Snake venom phosphodiesterases (phosphodiesterase I, 5′-exonuclease; oligonucleate 5′-nucleotidohydrolase; E.C. 3.1.4.1) are helpful in the elucidation of the structure and nucleotide sequence of nucleic acids[3–6].

Snake venom phosphodiesterase (PDE) provides an excellent model for kinetic investigations of an enzyme interacting with a macromolecule[7–9]. Several attempts have been made to isolate this enzyme from snake venoms and two types of PDE preparations were obtained, (i) homogeneous preparations useful for mechanistic studies and (ii) phosphatase-free preparations helpful in nucleic acid chemistry. Iwanaga and Susuki[10] have reviewed the various methods for phosphodiesterase purification from snake venoms. Dolapchiev *et al.*[11] employed a four-step procedure, including acetone precipitation, affinity chromatography, gel filtration and ion-exchange chromatography, to purify a PDE from *Crotalus adamanteus* venom with 35% yield. Oka *et al.*[12] were successful in improving the recovery of the enzyme from the same venom source to 60% by a single-step procedure on a pseudoaffinity column of Blue Sepharose–Sephadex. However, this phosphatase-free preparation was heterogeneous. Another homogeneous preparation was obtained from *Vipera aspis* venom in a yield of about 40% by DEAE-cellulose chromatography and preparative electrophoresis[13]. Only small amounts could be handled in this method, and efforts to purify this enzyme employing gel filters and ion exchangers were unsuccessful[13].

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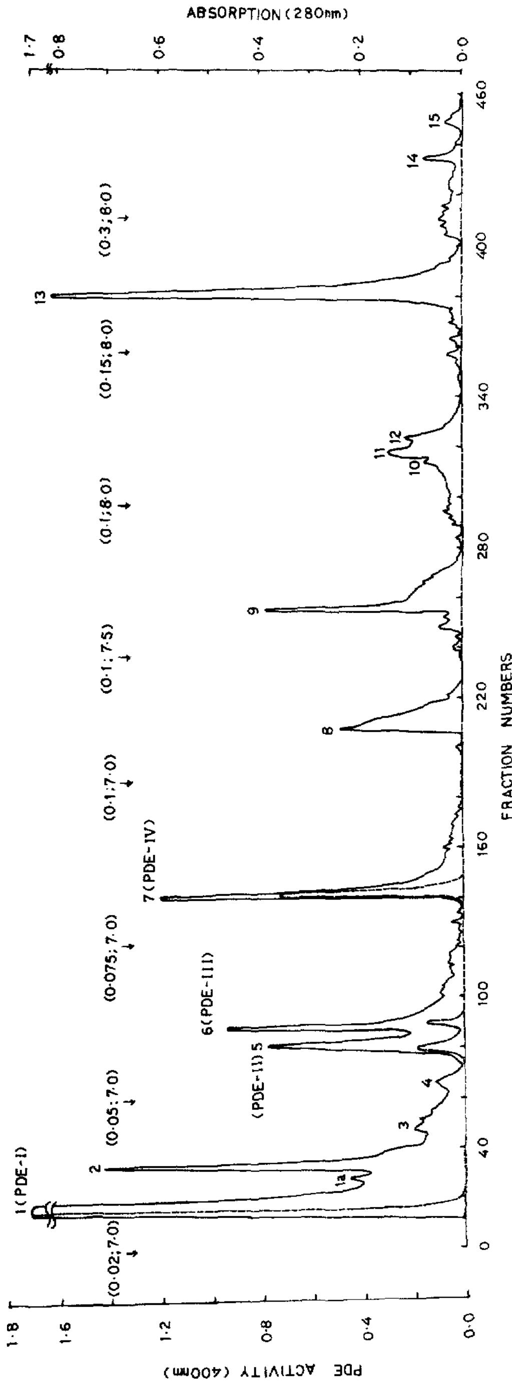

Fig. 1. CM-Sephadex C-25 column chromatography of *Trimeresurus flavoviridis* (Habu snake) venom. Loading, 450 mg in 2.0 ml of phosphate buffer (0.02 *M*, pH 7.0); column, 123 × 1.4 cm I.D.; flow-rate, 35 ml/h; fraction volume, 5 ml; room temperature. Elution was carried out stepwise with phosphate buffers of molarities and pH values as indicated. Recovery of PDE activity: 83%. Solid line, amount of protein; broken line, PDE activity.

Thus in all these methods either the enzyme could not be isolated in pure form or poor yields were obtained.

Earlier, we reported the fractionation of *V. russelli*[14] and *Naja naja*[15,16] venoms and a single-step purification of cobra neurotoxin to homogeneity[17,18] on CM-Sephadex C-25 columns. In this paper, we report a rapid highly reproducible procedure for the isolation and purification of four isoenzymes of PDE from *Trimeresurus flavoviridis* (Habu snake) venom. All four PDEs are metalloglycoproteins free from phosphomonoesterase (PME) activity. This provides a method for purifying large amounts of PDE with high yields.

EXPERIMENTAL

Trimeresurus flavoviridis (Habu snake) venom was a generous gift from Dr. Yoko Aniya (Naha City, Japan). CM-Sephadex C-25 (4.5 mequiv./g), QAE-Sephadex A-25 (3.4 mequiv./g) and bis-*p*-nitrophenyl phosphate, sodium salt, were purchased from Sigma (St. Louis, MO, U.S.A.) and *p*-nitrophenyl phosphate from Centron Research Lab. (Bombay, India). The colorimetric measurements were made in a Bausch and Lomb Spectronic-2000 instrument. All chemicals and reagents used were of analytical-reagent grade.

Assay of enzyme activities

The PDE assay mixtures contained bis-*p*-nitrophenyl phosphate (5 m*M*) in Tris–HCl buffer (0.02 *M*, pH 8.7) in a total volume of 1 ml. The reaction was carried out at 37°C for 20 min and terminated by adding 2 ml of 0.1 *M* sodium hydroxide solution. The PME assay mixture contained *p*-nitrophenyl phosphate (100 m*M*) in Tris–HCl (0.1 *M*, pH 8.0) in a total volume of 1 ml. The reaction was carried out at 37°C for 30 h. The liberated *p*-nitrophenol was measured at 400 nm.

EDTA inhibition of the PDE activity was studied by preincubating the enzyme for 10 min at 37°C with 5 m*M* EDTA in Tris–HCl buffer (0.02 *M*, pH 8.7), then assaying the PDE activity as described.

Protein was determined by Miller's modification of Lowry's method[19]. The carbohydrate content was determined by the phenol–sulphuric acid method of Dubois *et al.*[20] using glucose as a standard. The carbohydrate content is expressed as per cent glucose units.

Electrophoretic studies were carried out on 5% polyacrylamide gel at pH 8.3 according to Davis[21]. The protein bands were stained using Amido Black 10B.

Purification of PDE isoenzymes

Trimeresurus flavoviridis venom was fractionated on a CM-Sephadex C-25 column (123 × 1.4 cm I.D.) equilibrated in phosphate buffer (0.02 *M*, pH 7.0). Venom protein (450 mg) was dissolved in 2.0 ml of starting buffer and applied to the column. The column was eluted by stepwise gradients of phosphate buffers of various molarities and pH values (indicated in Fig. 1). Fractionation was carried out at room temperature. Elution of proteins from the column was monitored at 280 nm and the elution of enzymes was studied by PDE assay of 0.1-ml aliquots from each of the tubes. The fractions were pooled (Fig. 1), desalted on Sephadex G-25 and lyophilized. The specific activity and the recovery of PDE isoenzymes from the CM-Sephadex column were calculated.

The non-retained fraction (first fraction eluted in the starting buffer) from the CM-Sephadex C-25 column was sub-fractionated with Tris buffers on a QAE-Sephadex column. Protein (30 mg) was dissolved in 1.0 ml of starting buffer and applied to the column. The column was eluted using stepwise gradients of Tris–phosphoric acid buffers of various molarities and pH values (indicated in Fig. 2). The tubes containing PDE activity were pooled, desalted and lyophilized.

PDE-II and PDE-III were separated from the contaminants by gel filtration on a Sephadex G-100 column (69 × 1.2 cm I.D.). The purity of all the isoenzymes was calculated.

RESULTS

T. flavoviridis venom contains PDE activity (0.9 units/mg). It was fractionated on a CM-Sephadex C-25 column into 15 fractions (Fig. 1). The elution of PDE from the column indicated the presence of four isoenzymes, which we named PDE-I, -II, -III and -IV. The overall PDE activity recovered from the column was 83%. PDE-I was the major isoenzyme, contributing 77% of the total activity, and was eluted in the starting buffer (Fig. 1). PDE-II, -III and -IV contribute comparatively little to the total activity (Table I).

The non-retained fraction was further fractionated on QAE-Sephadex A-25 into nine fractions (Fig. 2). The PDE elution profile showed the presence of only one isoenzyme, eluted in the starting buffer. The recovery from the QAE-Sephadex column was almost quantitative.

PDE-II and -III from CM-Sephadex C-25 contained a minor contamination of phospholipase A_2 (unpublished observations). However, both of the preparations were free from phosphatase activity and the contaminants accounted for less than 5%. Comparatively high-molecular-weight PDE was separated from contaminants by gel filtration on Sephadex G-100. The recovery of the enzymes or proteins was observed in PDE-IV and no further steps were required for its further purification. The purity achieved after the second step of purification is shown in Table II. PDE-I was purified more than 44-fold.

The electropherogram of the isoenzymes of PDE on 5% polyacrylamide gel

TABLE I

RECOVERY OF PHOSPHODIESTERASE ISOENZYME FROM A CM-SEPHADEX COLUMN

Proteins were determined by Miller's modification[19] taking a bovine serum albumin standard. PDE activity was determined as described under Experimental.

Preparation	*Specific activity (units*/mg)*	*Purification factor*	*Total recovery (units*)*	*Contribution to total PDE (%)*
Crude	0.9	1.0	405.0	100
PDE-I	2.0	2.2	311.7	77.0
PDE-II	2.9	3.2	13.0	3.2
PDE-III	1.5	1.7	6.9	1.6
PDE-IV	0.6	0.7	5.0	1.2

* 1 unit of PDE activity is defined as the amount of enzyme required to liberate *p*-nitrophenol equivalent to 1.0 absorbance at 400 nm in 20 min under the defined experimental conditions.

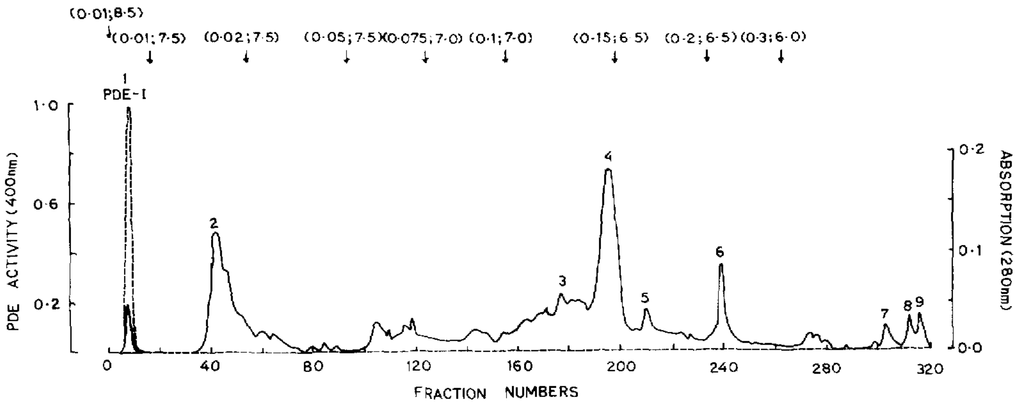

Fig. 2. QAE-Sephadex A-25 column chromatography of non-retained fraction from CM-Sephadex column. Loading, 30 mg in 1.0 ml of Tris phosphate buffer (0.01 *M*, pH 8.5); column, 57 × 1.4 cm I.D.; flow-rate, 40 ml/h; fraction volume, 5 ml; room temperature. Elution was carried out stepwise with Tris–phosphate buffers of molarities and pH values indicated. The pH values of all the buffers were adjusted to the required values using 4% phosphoric acid. Recovery of PDE activity: 100%. Solid line, amount of protein; broken line, PDE activity.

at pH 8.3 is shown in Fig. 3. All four isoenzymes showed a single Amido Black-positive band. In addition, we observed that in the first step on the CM-Sephadex column a phospholipase A_2 (fraction 13) was purified to homogeneity. Details on the characterization of this enzyme will be published elsewhere.

Isoenzymes of PDE are glycoproteins. The carbohydrate content of these isoenzymes is shown in Table II. PDE-I had the highest carbohydrate content. The effect of EDTA on the PDE activity of isoenzyme was recorded (Table II). All four isoenzymes were inhibited by EDTA.

The PME activity of all the PDE preparations for up to 30 h is shown in Fig. 4. Among the PDEs, PDE-III showed some PME activity. The amount of PME activity decreased in the order PDE-III > PDE-II > PDE-IV > PDE-I ≈ 0. However, the PDE activity predominated over the very low PME activity in all the preparations.

TABLE II

SOME PROPERTIES OF PURIFIED PHOSPHODIESTERASE ISOENZYMES FROM *TRIMERESURUS FLAVOVIRIDIS* VENOM

Carbohydrate was determined by the phenol–sulphuric acid method[20]. Protein was determined by Miller's modification[19] and EDTA inhibition was studied as described under Experimental.

Isoenzyme	*Purification factor*	*Carbohydrate content (% glucose units)*	*Inhibition by EDTA (%)*
PDE-I	44.4	23.8	100
PDE-II	6.9	3.8	100
PDE-III	4.2	ND*	100
PDE-IV	0.6	10.5	98.5

* ND = not determined.

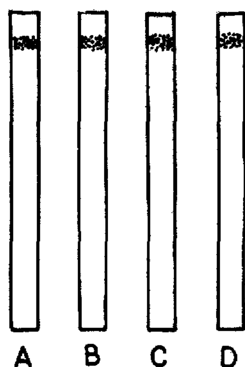

Fig. 3. Electropherograms of phosphodiesterase isoenzymes on polyacrylamide gel (5%) at pH 8.3. A, PDE-I; B, PDE-II; C, PDE-III; D, PDE-IV.

DISCUSSION

Snake venom is a rich source for several enzymes, which contribute to the effects of venom poisoning[2]. However, the exact role of the venom enzymes is not very clear. Snake venom PDE preparation had an LD_{50} value of 4 mg/kg (i.v.) in mice, which is fairly toxic[22]. These actions may be due to the enzyme or they may be due to the presence of some other protein in the preparation, as several bands were observed on disc gel electrophoresis[22]. The pathological and pharmacological roles of PDE in snake venom poisoning are not understood, possibly because of the non-availability of homogeneous preparations.

The PDE enzyme has been purified to homogeneity from *C. adamanteus*[11] and *V. aspis*[13] venoms. However, the yield of PDE in both the procedures is very low. We have developed a rapid and highly reproducible chromatographic procedure for the separation and purification of four isoenzymes of PDE from *T. flavoviridis* venom. Habu venom was fractionated on CM-Sephadex C-25 into 15 fractions and all four isoenzymes were separated in a single step (Fig. 1). The recovery of PDE activity from the CM-Sephadex column was 83% (Table I). Isoenzymes of PDE were further purified on QAE-Sephadex A-25 (Fig. 2) and Sephadex G-100 columns with quan-

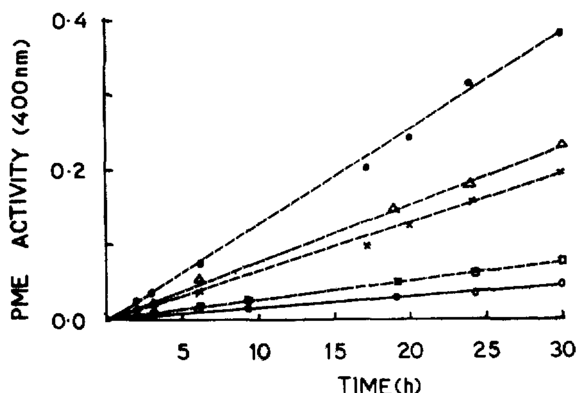

Fig. 4. Phosphomonoesterase activity of phosphodiesterase isoenzyme of Habu snake venom. ○, Auto hydrolysis; □, PDE-I; △, PDE-II; ●, PDE-III; ×, PDE-IV.

titative recovery of the enzymes. Hence the yield of PDEs in this procedure is very high compared with the earlier methods[11–13].

Large amounts of venom proteins can be handled in this procedure, a distinct advantage over the method of Ballario *et al.*[13]. Up to 600 mg of protein can be applied to a CM-Sephadex column of the same dimensions without affecting the resolution. The same procedure could possibly be scaled up for larger loads with columns of greater dimensions.

All four isoenzymes are glycoproteins with varying amounts of carbohydrates (Table II). The inhibition of these PDE preparations by EDTA indicates that a metal is required for their activity. However, no metal ion was added to the assay mixture. The metal ion essential for the PDE activity may be found to the enzyme itself. Hence, all four isoenzymes from *T. flavoviridis* venom seem to be metalloglycoproteins. This is in accordance with earlier observations on *C. adamanteus* venom PDE[9,11].

The four PDE isoenzymes purified by the present procedure were shown to have negligible amounts of PME activity (Fig. 4). Venom PDEs free from phosphatase are important in nucleic acid research and all four preparations may be used as tools in this area.

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