Reinvestigation of the Purification and Characterization of Castor Bean Hemagglutinin†

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Hemagglutinin was extracted from castor beans and purified by gel filtration on Sephadex G-75 followed by DEAE-cellulose column chromatography. The molecular weight of castor bean hemagglutinin (CBH) was estimated to be 130,000 by SDS-polyacrylamide gel electrophoresis and its isoelectric point to be 7.8 by isoelectric focusing electrophoresis. CBH possessed the identical N-terminal amino acids (Ile and Ala) and a very similar amino acid composition to those of ricin D, whereas its toxicity for mice was about 1% of that of ricin D.

We first separated hemagglutinin from ricin, a toxic protein in castor bean (*Ricinus communis*), by adsorption chromatography on a hydroxyapatite column, purified it by ion exchange cellulose chromatography and shown that the toxicity and hemagglutinating activity found are the properties of two different species of protein possessing similar structures.^{1,2)}

Recently castor bean hemagglutinin (CBH) has been isolated in several laboratories and characterized. It has been shown that CBH consists of four polypeptide chains of similar size and possesses similar sugar specificity to that of ricin.^{3~5)}

In the present paper we have purified CBH from the seed produced in Thailand by an improved method using gel filtration on a Sephadex G-75 column followed by DEAE-cellulose column chromatography, reinvestigated its physicochemical properties by recent techniques and compared them with those of ricin D.

MATERIALS AND METHODS

Material. The crude CBH was prepared from castor beans produced in Thailand without selection of color according to the preparation procedure of crude ricin as described previously.⁶⁾

Gel filtration. Gel filtration was performed through a Sephadex G-75 (Pharmacia) column with 0.05 M borate-HCl buffer of pH 8.0.

DEAE-cellulose column chromatography. A column of DEAE-cellulose (Brown) was prepared and equilibrated with 5 mM Tris-HCl buffer, pH 8.5. After dialysis against the same buffer, the protein was applied to the column and eluted stepwise with increasing concentrations of NaCl in the same buffer.

Affinity chromatography. Affinity chromatography on Sepharose 4B was carried out according to Nicolson and Blaustein.⁷⁾ The protein was applied to a Sepharose 4B (Pharmacia) column previously washed with 0.2 M NaCl-5 mM phosphate buffer, pH 7.1. After washing the column with the same buffer, the adsorbed protein was eluted by a linear gradient from 0 to 0.1 M lactose in the same buffer.

Disc electrophoresis. Polyacrylamide gel electrophoresis was performed at pH 4.0 using 5% gel by the method of Orstein.⁸⁾ The protein in the gel was detected by staining with amide black 10B.

Ampholine electrophoresis. Homogeneity and the isoelectric points were determined by the electrofocusing technique⁹⁾ with a LKB 8101 electrofocusing column in 0.8% carrier ampholyte (pH 3.5~10) at 5°C for 48 hr. After electrophoresis, the solution was taken out in 1.6 ml fractions at a flow rate of 1 ml/min, and the pH and protein concentration of each fraction were measured.

Molecular weight estimation. Molecular weight was determined by polyacrylamide gel electrophoresis in sodium dodecylsulfate (SDS) according to Weber and Osborn.¹⁰⁾ The protein was treated with SDS with or without β -mercaptoethanol at 50°C for 3 hr

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prior to electrophoresis. The marker proteins (cytochrome c, aldolase, hen egg albumin and catalase) were purchased from Boeringer Mannheim GmbH (Germany).

Ultraviolet absorption. The ultraviolet absorption spectra of CBH was determined in an aqueous solution with a Cary Model 14 Recording Spectrophotometer and the amount of CBH in the solution was weighed after drying at 100°C in vacuum.

Amino acid and N-terminal amino acid analyses. The amino acid composition and the N-terminal amino acid of CBH were analyzed as described in the previous paper.¹¹⁾ The lyophilized protein was hydrolyzed with 6 N HCl in an evacuated sealed tube at 108°C for 24 hr and was analyzed with a JEOL amino acid auto-analyzer (Type JLC-6AH). N-Terminal amino acids of CBH were identified on a silica gel plate using Solvent VM after Edman degradation modified by Iwanaga *et al.*¹²⁾

Cytoagglutination and toxicity assay. Cytoagglutinating activity and toxicity were determined as previously described.¹³⁾

RESULTS

Purification of CBH

The crude CBH, obtained by ammonium sulfate precipitation, was applied to the Sephadex G-75 column and eluted with 0.05 M borate buffer, pH 8.0. As shown in Fig. 1, the crude CBH was separated into three fractions—F-1, F-2 and F-3—and the highest hemagglutinating activity was found in fraction F-1 (Table I). The protein in fraction F-1 was

FIG. 1. Gel Filtration of Crude Castor Bean Hemagglutinin through Sephadex G-75.

The crude CBH was applied to the column prewashed with 0.05 M borate buffer (pH 8.0) and developed with the same buffer.

precipitated with ammonium sulfate, dissolved in water, dialyzed against water and finally against 5 mM Tris-HCl buffer, pH 8.5. After removal of the insoluble material produced during dialysis, the supernatant was loaded on DEAE-cellulose previously equilibrated with 5 mM Tris-HCl buffer, pH 8.5, and the protein was eluted by a linear gradient of NaCl from 0 to 0.2 M. As shown in Fig. 2, the protein was eluted in a single but broad peak, indicating the presence of more than one component. When the elution was carried out stepwise by

TABLE I. HEMAGGLUTINATING ACTIVITY AND TOXICITY OF DIFFERENT FRACTIONS FROM CASTOR BEAN

Purification steps	Fractions	Hemagglutinating activity		Toxicity	
		Minimum conc. (OD at 280 nm)	%	MLD ₅₀ (OD at 280 nm)	%
Gel filtration on Sephadex G-75 (Fig. 1)	F-1	0.0030	63		
	F-2	0.037	5.1		
	F-3	0.20	0.95		
DEAE-cellulose column chromatography (Fig. 3)	A	0.0019	100	0.213	1.08
	B	0.0022	86	0.275	0.84
	C	0.0026	73		
	D	0.0047	40		
Isoelectric focusing electrophoresis	I	0.0047	40	0.285	0.81
	II	0.0047	40		
Ricin D		0.120	1.58	0.0023	100

FIG. 2. DEAE-cellulose Column Chromatography of Fraction F-1 Obtained by Gel Filtration on Sephadex G-75 (Fig. 1).

Approximately 80 mg of fraction F-1 in Fig. 1 was applied to the column (1.5 × 16 cm) of DEAE-cellulose equilibrated with 5 mM Tris-HCl buffer (pH 8.5) and eluted by a linear gradient from 0 to 0.2 M NaCl in the same buffer (each 150 ml).

FIG. 3. DEAE-cellulose Column Chromatography of Fraction F-1 Obtained by Gel Filtration on Sephadex G-75 (Fig. 1) and Polyacrylamide Gel Electrophoresis of the Fractions Obtained in This Fig.

Approximately 30 mg of fraction F-1 obtained in Fig. 1 were applied to the column (1.5 × 14 cm), equilibrated with 5 mM Tris-HCl buffer (pH 8.5), and eluted by stepwise increases of NaCl concentration. Polyacrylamide gel electrophoresis was carried out at pH 4.0 using 7.5% gel concentration. (a) ricin D, (b) fraction A, (c) fraction B, (d) fraction C and (e) fraction D.

increasing NaCl concentration, as shown in Fig. 3, the protein was separated into four fractions—A, B, C and D. Hemagglutinating

activity was detected in all four fractions, but major fraction A possessed the highest activity (Table I). On polyacrylamide gel electrophoresis, as shown in Fig. 3, these four fractions gave a single band with almost identical mobilities at pH 4. On the other hand, the mobilities of these fractions on 5% polyacrylamide gel in the presence of SDS were almost the same and their molecular weights were near 130,000. After treatment with β -mercaptoethanol, fractions B, C and D were broken down into two bands corresponding to mol. wt. 34,000 and 31,000, whereas fraction A gave one minor third band near mol. wt. 32,000 in addition to these two main bands.

The purity of the fraction A was examined by isoelectric focusing electrophoresis. As shown in Fig. 4, the main component of fraction A moved to pH 7.8, but small amounts of the proteins were found both at the acidic side of the main peak as a shoulder (II) and at pH 4.9 as a peak (III). The main fraction (I) gave only two bands on SDS-polyacrylamide gel electrophoresis after treatment with β -mercaptoethanol, and still had strong hemagglutinating activity and about 1% of the

FIG. 4. Ampholine Electrophoresis of CBH-A.

Isoelectric focusing electrophoresis was carried out using 0.8% carrier ampholytes at 5°C for 48 hr. After electrophoresis, the solution was taken out at a flow rate of 1 ml/min in 1.6 ml fractions, and the pH of each was measured at 17°C.

FIG. 5. Affinity Chromatography of CBH-A on a Sepharose 4B Column.

Approximately 8 mg of CBH-A were applied to the column (1.5×14 cm) prewashed with 0.2 M NaCl-5 mM phosphate buffer (pH 7.1). After washing with 70 ml of the buffer, the adsorbed CBH-A was eluted by a linear gradient from 0 to 0.1 M lactose in the same buffer (150 ml eah). The experiment was performed at 4°C.

FIG. 6. Ultraviolet Absorption Spectrum of CBH-A. The ultraviolet absorption spectrum of CBH-A was determined in an aqueous solution (0.448 mg/ml) with a Cary 14 Recording Spectrophotometer.

toxicity of ricin D (Table I). These fractions A, B, C and D were referred to as CBH-A, -B, -C and -D, respectively.

Properties of CBH-A

Isoelectric point of CBH-A was estimated to be 7.8 by isoelectric focusing electrophoresis.

TABLE II. AMINO ACID COMPOSITIONS OF CASTOR BEAN HEMAGGLUTININ A AND RICIN D

The values were expressed by mole % after HCl-hydrolysis for 24 hr.

Amino acids	CBH-A	Ricin D
Asp	12.77	12.46
Thr	8.04	7.34
Ser	7.67	7.76
Glu	10.10	10.68
Pro	6.24	5.45
Gly	7.34	7.54
Ala	7.13	7.75
Val	5.68	6.07
Met	1.23	1.25
Ile	6.76	7.33
Leu	8.99	8.80
Tyr	3.58	4.19
Phe	3.80	3.77
Lys	2.67	1.88
His	1.36	1.25
Arg	6.64	6.70

The binding affinity of CBH-A to Sepharose 4B was examined by chromatography. As shown in Fig. 5, CBH-A bound to Sepharose 4B in 0.2 M NaCl-5 mM phosphate buffer, pH 7.1, at 4°C and eluted as a single peak by a linear gradient of lactose from 0 to 0.1 M in the same buffer.

A typical absorption spectrum of CBH-A is shown in Fig. 6. The maximum and minimum absorptions of an aqueous CBH-A solution were found at 280 nm and 250 nm, respectively. The ratio of a 1% aqueous solution of CBH-A at 280 nm was estimated to be 13.62.

Amino acid composition

The amino acid composition of CBH-A after a 24 hr hydrolysis is shown in Table II in comparison with that of ricin D obtained with the same hydrolysis conditions. Both compositions are very similar, but distinctly differ especially in Thr, Glu, Pro, Ala, Ile, Tyr and Lys. The N-terminal amino acids of CBH-A were identified to be alanine and isoleucine by the Edman degradation.

DISCUSSION

Our present data showed that hemagglutinin

in the seed of castor beans produced in Thailand is heterogeneous and can be separated into several fractions by stepwise elution in DEAE-cellulose column chromatography. The minor third band, which has always been found after reduction and electrophoresis on SDS-polyacrylamide gel by other authors,^{3~5)} was found only in the first main fraction (CBH-A) of these, but isoelectric focusing electrophoresis revealed that this minor third polypeptide chain is due to a contaminating protein. Since SDS-polyacrylamide gel electrophoresis after reduction of a mixture of the purified CBH-A and ricin D gave the band of this third polypeptide chain, there is one possibility that this derives from a contaminating ricin dimer. The present result that purified CBH-A and CBH-B, which do not contain the third band, possess still 1% of the toxicity of ricin D suggests that this toxicity is not due to the contaminant, ricin, but is the proper character of CBH.

The molecular weight of CBH-A determined by SDS-polyacrylamide gel electrophoresis is 130,000 which is larger than 120,000 obtained by other authors.^{3~5)} This value is twice of a sum of the molecular weights of two constituent chains (mol. wt. 31,000 and 34,000).

Affinity of CBH-A to Sepharose 4B is stronger than that of ricin D and CBH-A is adsorbed on Sepharose even at room temperature where ricin D is eluted without lactose.

The isoelectric point of CBH-A is slightly higher than that of ricin D, whereas the optical density at 280 nm is slightly lower than that of ricin D, probably due to slight differences in their amino acid compositions.

REFERENCES

- 1) G. Funatsu, *Nippon Nôgeikagaku Kaishi*, **34**, 139 (1960).
- 2) T. Takahashi, G. Funatsu and M. Funatsu, *J. Biochem.*, **51**, 288 (1962).
- 3) L. G. Gürtler and H. J. Horstmann, *Biochim. Biophys. Acta*, **295**, 582 (1973).
- 4) G. L. Nicolson, J. Blaustein and M. Etzler, *Biochemistry*, **13**, 196 (1974).
- 5) S. Olsnes, E. Saltvedt and A. Pihl, *J. Biol. Chem.*, **249**, 803 (1974).
- 6) M. Ishiguro, G. Funatsu and M. Funatsu, *Agric. Biol. Chem.*, **35**, 724 (1971).
- 7) G. L. Nicolson and J. Blaustein, *Biochim. Biophys. Acta*, **266**, 543 (1972).
- 8) L. Orstein, *Ann. New York Acad. Sci.*, **121**, 321 (1964).
- 9) O. Verterberg and H. Sevensson, *Acta Chem. Scand.*, **20**, 1820 (1966).
- 10) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
- 11) G. Funatsu, S. Miyauchi, N. Yoshizuka and M. Funatsu, *Agric. Biol. Chem.*, in press.
- 12) S. Iwanaga, P. Wallen, N. J. Gröndahl, A. Henschen and B. Blombäck, *Eur. J. Biochem.*, **8**, 189 (1969).
- 13) G. Funatsu, S. Miyauchi and M. Funatsu, *Agric. Biol. Chem.*, **40**, 639 (1976).