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PURIFICATION OF SYNTHETIC CARDIOTOXIN BY AFFINITY CHROMATOGRAPHY

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SUMMARY

A polypeptide containing 60 amino acids with 4 disulphide bonds, synthesized by the solid-phase method, was highly purified by anticardiotoxin–Sepharose affinity chromatography following gel filtration and CM-cellulose chromatography. The identification of the final product as cardiotoxin was confirmed by thin-layer chromatography on silica gel, polyacrylamide gel electrophoresis, amino acid analysis, circular dichroism spectra, N-terminal analysis and four biological tests.

INTRODUCTION

Solid-phase polypeptide synthesis[1] is a rapid and efficient way of preparing short and medium-length peptides. Some biologically active peptides, such as ACTH[2] and β-endorphin[3] have been synthesized by this technique. In the same range of peptide chain length, *i.e.* 10–40 amino acid residues, the method also has been applied to the studies of structure–function relationships[4]. However, the total synthesis of long polypeptides, such as cobrotoxin (62 amino acid residues)[5] or ribonuclease (124 amino acid residues)[6] by this technique still can not be achieved without modification of the method as currently used. The problems usually encountered in the solid-phase method are the existence of contaminants in the product with properties similar to those of the desired polypeptide, and the lack of suitable purification techniques to separate such closely related compounds. One of the solutions is the use of affinity chromatography to purify the synthetic product[7].

We have been engaged upon the chemical synthesis of Taiwan cobra cardiotoxin[8], which has 60 amino acid residues with 4 disulphide bonds, in order to investigate the molecular mechanism of its biological activities. Preliminary synthesis of cardiotoxin by the stepwise solid-phase method[9] gave a product with *ca.* 75% of the natural biological activity after purification by gel filtration and repeated CM-cellulose chromatography. This paper reports the re-synthesis of cardiotoxin, with the same protecting groups, in a highly purified form by using anticardiotoxin–Sepharose affinity chromatography for the final purification.

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EXPERIMENTAL

Amino acids were purchased from Kyowa Fermentation Company, Tokyo, Japan. Chloromethylated styrene–divinylbenzene copolymer (2% cross-linked, 0.95 mmol Cl/g), *tert.*-butyloxycarbonyl carbazate and 1-hydroxybenzotriazole (HOBt) were purchased from Protein Research Foundation, Osaka, Japan. Dicyclohexylcarbodiimide (DCC) and trifluoroacetic acid (TFA) were from Pierce, Rockford, Ill., U.S.A. and distilled before use. Dichloromethane was purified[10] and distilled from P_2O_5. Dioxane was distilled from sodium. Sephadex G-50 and Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. CM-cellulose (CM-11) was purchased from Whatman, Clifton N.J., U.S.A. Liquid HF, in steel cylinders, was from Matheson, East Rutherford, N.J., U.S.A. Other reagents were obtained from Wako, Osaka, Japan. All solvents were purified to meet reagent grade purity.

Thin-layer chromatography (TLC) was performed on silica gel with the solvent system: *n*-butanol–pyridine–acetic acid–water (30:20:6:24). The spot was detected by ninhydrin reagent.

Native cardiotoxin isolated from crude venom[11] was a gift of Professor T. B. Lo, of this Institute. Concentrations of native or synthetic cardiotoxin were determined from the absorbance at 280 nm using extinction coefficient, $E^{1\%}_{1\,cm} = 6.82$, as standard. A Hitachi UV–visible spectrophotometer, Model 624, was used to measure the absorbance.

Circular dichroism (CD) was measured with a Jasco J-20 spectropolarimeter under constant nitrogen gas flush. The data were expressed in terms of mean residue ellipticity, $[\theta]$, with a mean residue weight of 112.3.

Disc gel electrophoresis[12] was carried out for 30 min at room temperature in 0.35 *M* β-alanine acetate (pH 4.5) with 10% of acrylamide. A current of 6 mA per tube was applied. Protein bands were stained with Amido Black.

Amino acid analyses were carried out by amino acid analyser (Jeol JLC-6AH) according to the accelerated method[13]. Peptide hydrolysis was performed with constant-boiling HCl under vacuum at 110° for 22–24 h. Cystine was determined by analysis of cysteic acid after performic acid oxidation[14] and hydrolysis.

N-Terminal group analysis was carried out by the dansyl method[15], and two-dimensional chromatography of Dns-amino acid was performed on a polyamide layer[16].

CM-cellulose chromatography was performed in a 1.5 × 34 cm column with an initial buffer of 0.1 *M* ammonium acetate (pH 6.8). A gradient with respect to salt concentration was achieved by introducing 0.5 *M* ammonium acetate (pH 6.8) through a mixing chamber containing 200 ml of starting buffer. A fraction of 4 ml was collected and the peptide was detected by measuring the absorbance at 280 nm.

All BOC-amino acid derivatives were synthesized according to the established method[17]. The side-chain protecting groups used were as follows: Lys, Z; Arg, Tos; Asp, Bzl; Ser, Bzl; Thr, Bzl; Tyr, Bzl; Cys, Bzl.

Preparation of anticardiotoxin sera and the immunoadsorbent

The antisera were obtained from rabbits (2.5–3 kg) that had been injected at weekly intervals for five weeks with increasing amounts of cardiotoxin (from 0.3 mg/ml to 0.5 mg/ml) in an emulsion of 0.9% saline–Freund's adjuvant (1:1). The sera

obtained after bleeding were chilled in an ice bath, and an equal volume of 4.2 *M* ammonium sulphate was added slowly. The mixture was allowed to stand at 4° for at least 20 h, and immunoglobulins were collected by centrifuging (10,000 *g*) for 10 min. The precipitates were dissolved in 20 ml of 0.01 *M* phosphate buffer (pH 8.0) and dialysed against 100 volumes of the same buffer for 5 h. The protein content determined[18] was 10 mg/ml, with bovine serum albumin as standard.

Sepharose 4B was activated with CNBr[19]. 20 ml of Sepharose 4B and 4.1 g of CNBr were used. Anticardiotoxin sera in phosphate buffer (15 ml), prepared as above, was added to the activated Sepharose. The mixture was stirred for 40 min at 4°, then allowed to stand at the same temperature for at least 16 h. The mixture was filtered and washed three times with an equal volume of 3 *M* sodium chloride solution, followed by 0.01 *M* phosphate saline buffer (PBS), pH 7.2. The protein content of the filtrate was determined and *ca.* 80% of protein in the antisera was coupled to the polymer.

The prepared immunoadsorbent was packed in a column (1 × 10 cm) equilibrated with PBS buffer. Different amounts of native cardiotoxin were applied to determine the loading capacity of the affinity column. Desorption of antigen was carried out using 20 m*M* acetic acid–1 *M* sodium chloride solution (pH 2.2).

Bioassays

To determine the muscle contracture-inducing activity, the biventer cervicis muscle of baby chick was prepared[20] and suspended in 20 ml of Krebs solution. The isometric contractures were recorded with a Grass FT 03 force-displacement transducer attached to a Grass 7D polygraph[21].

Toxicity was assayed by intraperitoneal injection in mice weighing 15–20 g. Eight mice were used at each dose and the LD_{50} was calculated[22].

Ouchterlony double diffusion[23] was carried out at room temperature with 1% of agarose in 0.01 *M* phosphate buffer (pH 8.0) for 16 h. Anticardiotoxin serum and the antigen to be tested were placed in separate wells.

The haemolytic activity[24] upon guinea-pig red blood cells was expressed as a percentage of haemolysis, with native cardiotoxin as standard.

Solid-phase synthesis

Starting from 2 g of BOC-Asn resin, prepared according to the method of Marglin[25], a polypeptide resin (3.7 g) with the cardiotoxin sequence was synthesized according to the method described previously[9]. Cleavage of the peptide resin with liquid HF and isolation of the reduced polypeptide with a Sephadex G-50 column were also carried out as described before.

Oxidation and purifications

The unprotected polypeptide (120 mg) was lyophilized and dissolved in 0.05 *M* phosphate buffer (pH 6.8) in the presence of 0.2% sodium azide. The concentration of polypeptide was adjusted to 10^{-5} *M* and the solution allowed to stand at room temperature for 5 days. It was then filtered to remove some precipitated materials, and the filtrate was lyophilized and rechromatographed through a Sephadex G-50 column (2.5 × 60 cm), which was equilibrated and eluted with 0.05 *M* ammonium

acetate. The fraction corresponding to native cardiotoxin was then chromatographed on the CM-cellulose column.

Purification by anticardiotoxin sera affinity chromatography

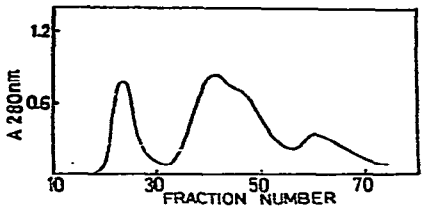
The product obtained from the second run on the CM-cellulose column was lyophilized (11 mg) and dissolved in PBS buffer (2 ml), and then further purified by passing through the immunoadsorbent prepared as described above.

RESULTS

Synthesis and purifications

Starting with 2 g of BOC-Asn resin, we obtained a final weight of uncleaved peptide resin of 3.7 g, a yield of 31%. After HF cleavage, the gel filtration chromatogram of the synthetic product in a reduced state still showed a number of side-products resulting from the synthetic process.

After oxidation, the synthetic peptide was purified on the Sephadex G-50 and CM-cellulose columns (Figs. 1 and 2). A final yield of 11 mg was obtained after the second run on the CM-cellulose column. Some impurities were still present, as indicated by TLC and disc gel electrophoresis.



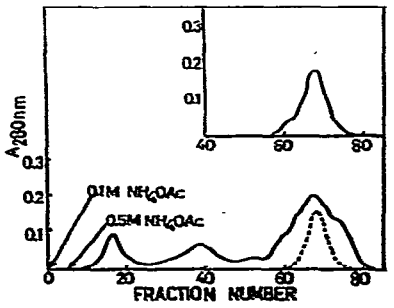


Fig. 1. Chromatography of the synthetic product on Sephadex G-50. After oxidation, the product was applied to the column (2.5 × 60 cm) and eluted with 0.05 *M* NH_4OAc. 5 ml per fraction was collected. Fraction II yielded 58 mg.

Fig. 2. CM-cellulose chromatography of the synthetic product. The conditions were as described in the Experimental section. 4 ml per fraction were collected. – – –, Elution profile of native cardiotoxin. The inset shows the curve from a second run on the CM-cellulose column under the same conditions.

Affinity purification

The immunoadsorbent as prepared can load *ca.* 5 mg of native cardiotoxin at a flow-rate of 0.2 ml/min. As shown in Fig. 3, the excess cardiotoxin was eluted in the leading fraction. When the synthetic product from the second CM-cellulose run (11 mg) was passed through the immunoadsorbent with PBS as eluent, and then desorbed by elution at a low pH and in a high salt medium, a highly purified polypeptide was obtained (4 mg) that was homogeneous on TLC (R_F, 0.45) and disc gel electrophoresis (Fig. 4). The leading fraction, with the tailing band on TLC, showed complex substances.

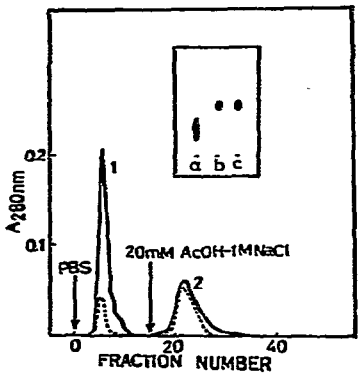


Fig. 3. Affinity chromatography of the synthetic product on the anticardiotoxin–Sepharose column. After the second run on CM-cellulose, the synthetic product (11 mg) was applied to the column (1 × 10 cm) and eluted with PBS. Desorption was performed with 20 m*M* AcOH–1 *M* NaCl (pH 2.2). A fraction of 3 ml/15 min was collected. – – –, Elution profile of native cardiotoxin (7 mg) under the same conditions. The inset shows the TLC of the eluate of the synthetic product: a, fraction 1; b, fraction 2; c, native cardiotoxin (R_F 0.45).

Further characterization

After desalting on a Sephadex G-25 column (2 × 10 cm), the amino acid composition (Table I) and the CD spectrum (Fig. 5) of the highly purified polypeptide were almost the same as those of native cardiotoxin. Small shifts and slightly low values for the ellipticities were found in the CD spectrum. End-group analysis indicated that leucine was the N-terminal residue, as it is in native cardiotoxin.

Several biological activities were compared with those of native cardiotoxin and are listed in Table II. The contracture-inducing activity (Fig. 6) was almost as

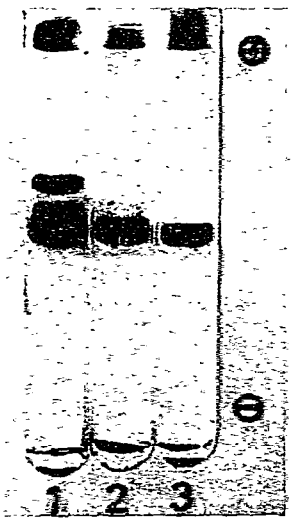


Fig. 4. Disc gel electrophoresis of synthetic cardiotoxin in 10% polyacrylamide gel at pH 4.5. 1 = Synthetic cardiotoxin after the second run on CM-cellulose (50 μg); 2 = synthetic cardiotoxin after further purification by affinity chromatography; 3 = native cardiotoxin (50 μg).

TABLE I

AMINO ACID ANALYSES OF NATURAL AND SYNTHETIC CARDIOTOXINS

0.5 mg of sample in 0.5 ml of constant-boiling HCl was sealed under vacuum and hydrolysed at 110° for 22 h.

Amino acid	*Calcd.*	*Natural cardiotoxin*	*Synthetic cardiotoxin*
Lysine	9	9.1	8.6
Arginine	2	2.1	1.4
Aspartic acid	2	6.2	6.2
Asparagine	4		
Threonine	3	2.9	2.2
Serine	2	1.6	1.4
Proline	5	4.5	4.2
Glycine	2	2.1	2.1
Alanine	2	1.9	2.0
Half-cystine	8	7.8*	7.4*
Valine	7	7.0	7.5
Methionine	2	1.6	1.2
Isoleucine	1	1.1	1.2
Leucine	6	6.1	5.2
Tyrosine	3	2.8**	2.6**
Phenylalanine	2	1.6	2.1

* Determined as cysteic acid by performic acid oxidation and hydrolysis.
** Determined in the presence of 0.2% phenol.

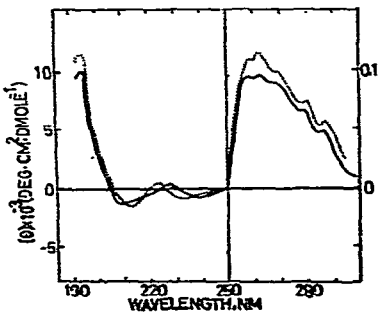


Fig. 5. CD spectra of synthetic (———) and native (— — —) cardiotoxins.

TABLE II

BIOLOGICAL ACTIVITIES OF NATURAL AND SYNTHETIC CARDIOTOXINS

Tests	*Natural cardiotoxin*	*Synthetic cardiotoxin**
Antigenicity	+**	+**
Toxicity (LD_{50}, μg/g)	2.4	2.6***
Haemolysis (%)	100	88§
Muscle contracture	+§§	+§§

* Purified from affinity chromatography.
** The precipitation lines formed are characteristic of identity.
*** As reported previously[9], repeated CM-cellulose column chromatography (three times) gave a product with $LD_{50} = 3.3$ μg/g in mice.
§ Obtained from the average of two independent assays on guinea-pig erythrocytes.
§§ The contracture patterns are identical.

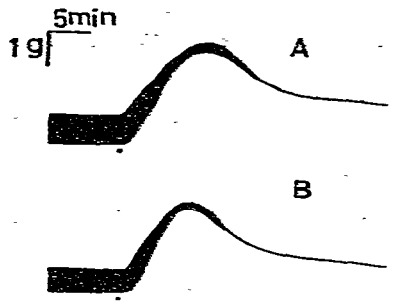


Fig. 6. Muscle contracture-inducing activity of synthetic (A) and native (B) cardiotoxins at a concentration of 1.5 μM. The dot indicates the point of application.

great as that of native cardiotoxin. Haemolysis and lethality were *ca.* 90% of the native peptide values.

DISCUSSION

From the physical, chemical and biological characteristics of the synthetic product, it is believed that a chemically pure polypeptide with the same structure and conformation as cardiotoxin has been synthesized. The biological activities of synthetic peptide are *ca.* 90% of those of native cardiotoxin.

In comparison with our first synthesis of cardiotoxin, the affinity column chromatography is a key step for the purification of this synthetic polypeptide with a high content of disulphide bonds. The classical methods of gel filtration and CM-cellulose column chromatography can hardly be applied to the purification of such a complex polypeptide synthesized by the stepwise solid-phase method and with conventional protecting groups in amino acid side-chains[9].

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