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ISOLATION OF HIGH-SPECIFIC-ACTIVITY SUBUNITS OF CHOLERA TOXIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Facile, rapid procedures for the separation of active cholera toxin subunits were developed, based on high-performance liquid chromatography (HPLC) with a Nucleosil C_8 reversed-phase column. These procedures were capable of completely resolving subunits A and B as well as S-carboxymethylated or reduced α-, γ-, and β-chains. The binding of HPLC-purified B subunit to GM_1 ganglioside was essentially identical to that of cholera toxin when compared on a molar basis. The adenosine 5'-diphosphate-ribosyltransferase activity of HPLC-purified A subunit, reduced α-chain, or carboxymethyl α-chain was also determined to be reasonably high compared to that of cholera toxin or commercially prepared A subunit.

INTRODUCTION

The primary causal agent in cholera is a multimeric molecular weight (M_r) 84 000 protein, produced by the bacterium *Vibrio cholerae*[1]. The protein is composed of two non-covalently associated subunits or protomers, A (M_r 29 000) and B (M_r 58 000). Subunit A is an adenosine 5'-diphosphate (ADP)-ribosyltransferase and is responsible for the stimulation of adenylate cyclase by catalyzing the ADP-ribosylation of a guanosine 5'-triphosphate (GTP)-binding regulatory protein of the cyclase multimer[2]. The function of the subunit B is to bind the toxin specifically to GM_1 ganglioside on the cell surface[3].

The B subunit component of the toxin is an aggregate of five β-chains (β_5), each of M_r 11 600, that strongly associate non-covalently. The A subunit is composed of two chains, α (M_r 23 000) and γ (M_r 5400), held together by a single disulfide bond. The A subunit initially occurs as a single chain that is subsequently nicked enzymically to give the final two-chain arrangement[4,5].

The purification of cholera toxin was initially developed by Finkelstein and LoSpalluto[6]. Isolation of the component chains of cholera toxin is usually achieved by gel permeation chromatography under rigorous dissociating conditions on Sephadex G-75 or Bio-Gel P-60[7,8]. Phosphocellulose chromatography has also been

employed to isolate specific toxin subunits[5]. Although it is generally held that the various activities of the toxin are able to withstand denaturing conditions, little attention, in fact, has been directed to toxin subunit specific activity. One exception is the report of Lai *et al.*[9] which demonstrated renaturation of the α-chain after 8 M urea treatment.

In view of the relative expense of cholera toxin subunits and the widespread research application of these proteins to the study of adenosine 3′,5′-monophosphate (cAMP)-related phenomena, a facile and rapid method of isolating highly purified toxin subunits possessing high specific activity would be of considerable value.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography (HPLC) was performed with a Beckman/Altex liquid chromatography system comprised of a Model 421 programmer and two Model 110A pumps (Beckman Instruments, Houston, TX, U.S.A.). Column effluents were monitored with a Hitachi 155-40 variable-wavelength UV–VIS detector (Hitachi Scientific, Mountainview, CA, U.S.A.) and a 3390A Hewlett-Packard integrator (Hewlett-Packard, Houston, TX, U.S.A.) and were collected with a LKB 2111 Multirac fraction collector (LKB Instruments, Houston, TX, U.S.A.). Amino acid analysis was carried out on a Beckman 121M analyzer (Beckman Instruments). Acrylamide gel electrophoresis utilized a Bio-Rad Model 360 mini vertical slab-gel apparatus (Bio-Rad Labs., Richmond, CA, U.S.A.).

Materials

Nucleosil C_8 reversed-phase columns, 5 μm and 10 μm, 25 cm × 4.6 mm I.D., were obtained from Alltech Assoc. (Houston, TX, U.S.A.). Guard columns were from Waters Assoc. (Milford, MA, U.S.A.) and were packed with Synchropak RSC from Synchrom (Linden, NJ, U.S.A.). Cholera toxin and cholera toxin A subunit were purchased from List Biological Labs. (Campbell, CA, U.S.A.). Solvents for HPLC were from Burdick & Jackson (Muskegon, MI, U.S.A.). Reagents for amino acid analysis were obtained from Beckman Instruments. Most other protein modification reagents were bought from either Pierce (Rockford, IL, U.S.A.) or from Sigma (St. Louis, MI, U.S.A.). Polystyrene microtiter plates were purchased from Dynatech Labs. (Alexandria, VA, U.S.A.). Nicotinamide adenine dinucleotide (NAD^+), polyarginine (M_r 40 000), and alkaline phosphatase substrate were from Sigma. Ethylene glycol was a Fisher Certified Reagent (Fisher Scientific, Houston, TX, U.S.A.) and [^{14}C]NAD^+ (400 mCi/mmol) was a product of New England Nuclear (Boston, MA, U.S.A.). Alkaline phosphatase-conjugated anti-rabbit IgG, produced in goat, was obtained from Miles-Yeda, Israel.

HPLC fractionation of cholera toxin subunits

Cholera toxin was fractionated with the use of two separate gradient programs with two solvents: (A) 0.1% trifluoroacetic acid (TFA); and (B) 0.1% TFA in acetonitrile. A 90-min gradient program started with 0% solvent B and continued for 12 min, at which time a 0–25% solvent B gradient was started. At 25% solvent B (18 min), a second gradient to 60% solvent B (80 min) was begun. At 80 min, the program

was completed, and solvent B was decreased to 0% by 90 min. A second gradient program of 45-min duration began at 35% solvent B, which was increased to 48% in 35 min. At 35 min, the gradient of solvent B was programmed to return to 35% by 40 min and was held there for an additional 5 min. Immediately after collection, fractions were dialyzed against 1% acetic acid. More recently, we have been collecting HPLC fractions into an equal volume of 0.1% TFA. Toxin samples were usually injected in the buffer in which it was sold.

ADP-ribosyltransferase assay

Measurement of ADP-ribosyltransferase activity was carried out essentially according to Lai et al.[9] with the following modifications. Ethylene glycol (10%) was included to reduce α-chain aggregation[10]. Assay mixtures of toxin or subunits in 100 μl 0.1 M 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) buffer (pH 7.0) contained 3.18 mg/ml dithioerythritol (DTE), and 0.103 mg/ml bovine serum albumin. Toxin mixtures were pre-activated at 37°C for 1 h prior to reaction; however, mixtures of A subunit and α-chain were not pre-activated.

Ganglioside-binding assay

The ganglioside-binding assay employed was similar to that reported by Sack et al.[11]. Reversed-phase HPLC-prepared subunit B and cholera toxin were dialyzed against phosphate buffered sodium chloride (PBS) [0.05 M sodium phosphate buffer (pH 7.0), containing 0.15 M sodium chloride] and titered on polystyrene enzyme-linked immunosorbent assay (ELISA) plates, coated with GM_1 ganglioside. Aliquots of up to 10 μl of each protein were diluted to a total volume of 200 μl with PBS, containing 0.05% Tween-20. Serial dilutions were made, and then the mixtures were incubated for 2 h at 24°C on a shaker platform. The wells were washed with 0.15 M sodium chloride, containing 0.05% Tween-20 (Tween-saline), and affinity chromatography-purified rabbit anti-B subunit polyclonal antibody was added in the presence of PBS-Tween. The plates were incubated as above, washed, and affinity chromatography-purified, alkaline phosphatase-conjugated goat anti-rabbit IgG was added. After incubation and washing, the relative amounts of specifically bound cholera toxin and B subunit were determined colorimetrically with p-nitrophenyl phosphate at 405 nm.

Reduction and alkylation

Reduction and carboxymethylation reactions were carried out essentially according to Hirs[12]. Reaction mixtures were non-dissociating and contained 10% ethylene glycol. Both reduction and alkylation were carried out at 24°C for 45 min each.

Amino acid analysis

Samples were hydrolyzed with 5.7 M hydrochloric acid in vacuo at 107°C for 24 h. Amino acid compositional analysis was carried out on a Beckman 121M analyzer, using the single column methodology on Beckman AA20 resin, as specified by the manufacturer.

Polyacrylamide gel electrophoresis

Homogeneity of the HPLC-prepared cholera toxin subunits was evaluated by

12% sodium dodecyl sulfate-polyacrylamide gel electropohoresis (SDS-PAGE) with a mini vertical slab-gel apparatus, essentially according to the method of Laemmli[13].

RESULTS

The fractionation of cholera toxin by Nucleosil C_8 reversed-phase HPLC with a 90-min gradient elution program is shown in Fig. 1A. Re-injection chromatograms of subunits A and B are given in Fig. 1B and C, respectively. No cross-contamination of the two subunits was evident. This conclusion was supported by the ADP-ribosyltransferase assay, in which no activity was detected in the B subunit fraction (Table I). Fractionation of the toxin into its component chains by C_8 HPLC, following reduction and carboxymethylation under non-dissociating conditions, is shown in Fig. 2A. Similarly, A subunit was also resolved into carboxymethyl-γ (Cm-γ) and carboxymethyl-α (Cm-α), as demonstrated in Fig. 2B. These isolations were achieved immediately following the reduction and alkylation reactions without prior removal of the reagents. Dithioerythritol-reduced cholera toxin without alkylation gave an elution profile virtually identical with that of the carboxymethylated chains in Fig. 2A. We found that the Nucleosil C_8 column was capable of resolving reasonably large amounts of toxin, as shown in Fig. 3. As much as 2 mg per single injection was
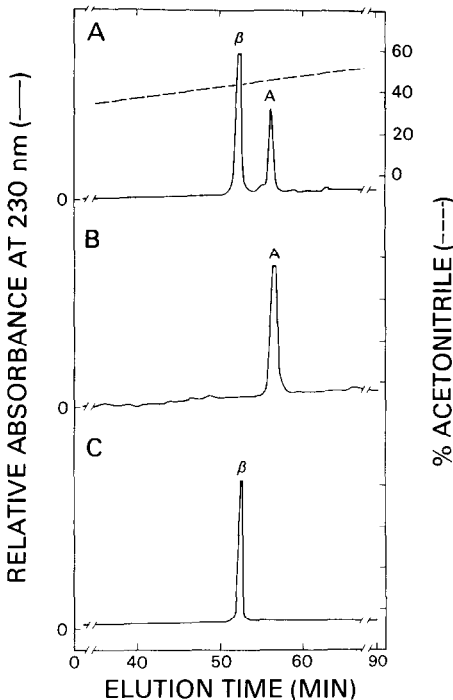

Fig. 1. Fractionation of cholera toxin and component subunits by HPLC on a Nucleosil C_8 reversed-phase 10-μm column (25 cm × 4.6 mm I.D.) with a gradient elution system of 0.1% (TFA) and 0.1% TFA in acetonitrile. Total elution program time was 90 min. (A) 100 μg cholera toxin; (B) 100 μg of A subunit, prepared by HPLC; (C) 50 μg of B subunit, prepared by HPLC.

TABLE I

ADP-RIBOSYL TRANSFERASE ACTIVITY OF CHOLERA TOXIN AND COMPONENT POLY-PEPTIDES

ADP-ribosylation of polyarginine for each protein measured is expressed relative to cholera toxin on a molar basis of α chain. Results are the average of duplicate determinations.

Protein	Relative specific activity (%)
Cholera toxin (+ DTE)	100
Cholera toxin (+ DTE, − polyarginine)	0
Cholera toxin (− DTE)	14
Cholera toxin (+ DTE and TFA)*	57
Cholera toxin (+ DTE, TFA and acetonitrile)**	43
Cholera toxin (+ DTE and guanidine hydrochloride)***	48
Cholera toxin (+ DTE and urea)§	28
A subunit (+ DTE, TFA and acetonitrile)§§	58
A subunit (− DTE, + TFA and acetonitrile)§§	18
A subunit (+ DTE, commercial)	60
A subunit (− DTE, commercial)	8
α chain (+ DTE, TFA and acetonitrile)§§	52
Cm-α chain (TFA and acetonitrile)§§	43
B subunit (+ DTE, TFA and acetonitrile)§§	0
No protein control	0

* Pre-exposed to 0.1% TFA for 1 h at 24°C.

** Pre-exposed to 0.1% TFA and 48% acetonitrile for 1 h at 24°C.

*** Subjected to 0.05 M Tris–HCl buffer, pH 7.0, containing 6 M guanidine–hydrochloride for 9 h at 24°C.

§ Subjected to 0.05 M Tris–HCl buffer, pH 7.0, containing 8 M urea for 9 h at 24°C.

§§ Nucleosil HPLC-purified.

applied. For separating larger quantities of A and B subunits, the 45-min gradient elution program shown in Fig. 3 was used, since it gave greater resolution than the 90-min program. However, the 90-min gradient elution program (Figs. 1 and 2) was required to separate all three chains simultaneously, or to separate the γ-chain from the reagent peaks after reduction and alkylation of the A subunit. Identification of the fractions and evidence of the homogeneity of the isolated components of the toxin were obtained by PAGE (Fig. 4) and amino acid compositional analysis (data not shown).

The relative biological activity of HPLC-isolated toxin A subunit and its α-chain was evaluated by measurement of ADP-ribosyltransferase activity as shown in Table I. The activity of the B subunit was determined by measurement of binding to GM_1 ganglioside (Fig. 5).

DISCUSSION

A rapid and complete fractionation of the A and B subunits of cholera toxin can readily be achieved by C_8 reversed-phase HPLC, as shown in Figs. 1 and 2. In 40 min, amounts of up to 2 mg of toxin can be fractionated. The maximum load capacity for this size of column was not established, however. Protein recoveries from
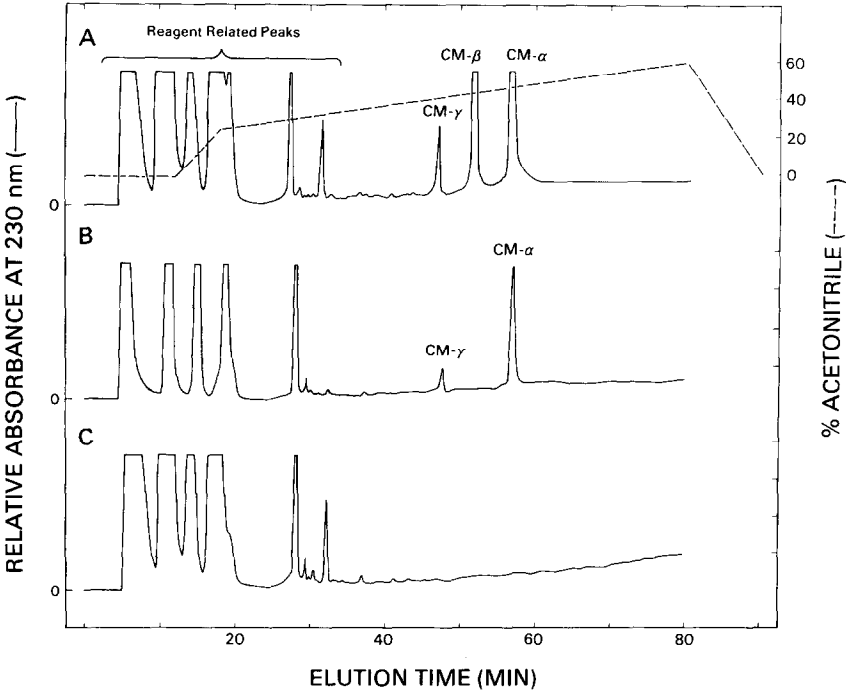
Fig. 2. Fractionation of carboxymethylated cholera toxin chains on Nucleosil C_8, as in Fig. 1. (A) Reduced and carboxymethylated cholera toxin (100 μg); (B) reduced and carboxymethylated A subunit (25 μg); (C) reagent blank from the reduction and alkylation reaction.
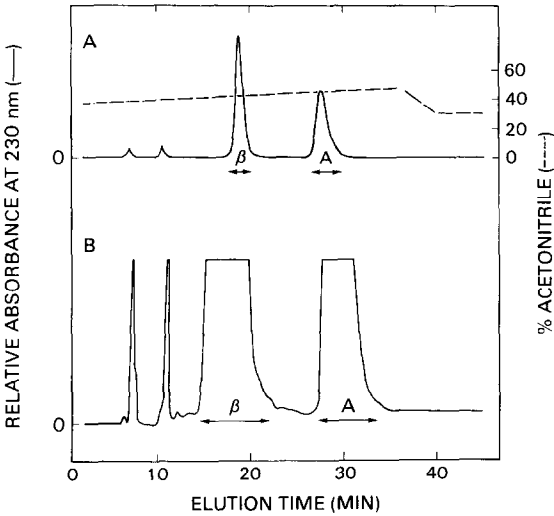

Fig. 3. Fractionation of cholera toxin by Nucleosil C_8, as in Fig. 1, but with a gradient elution time of 45 min. (A) 100 μg of cholera toxin; (B) 2 mg of cholera toxin.

(−)

A −

α −

β/γ −

−43 K
−25.7 K
−18.4 K
−14.3 K
−12.3 K
−6.2 K
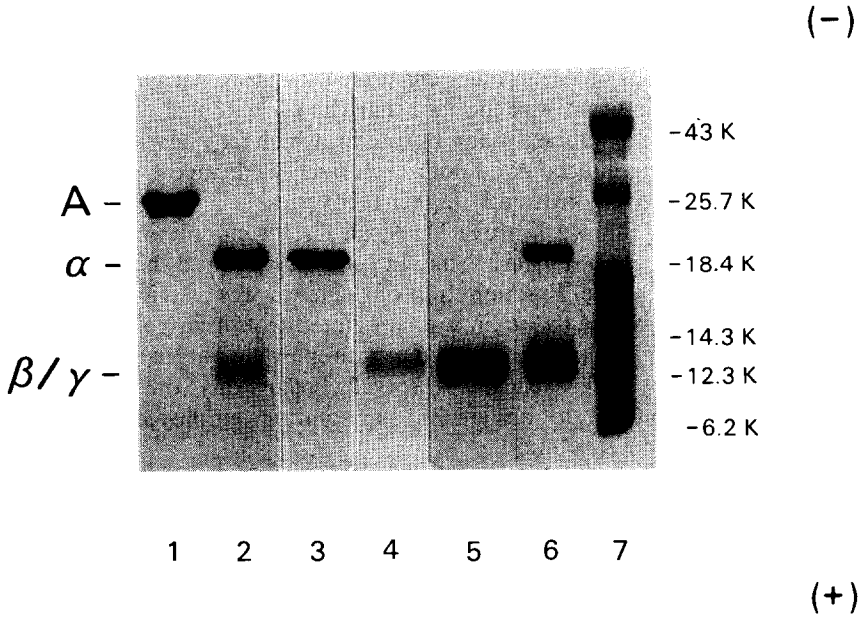
1   2   3   4   5   6   7

(+)

Fig. 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of HPLC-purified subunits of cholera toxin: lane 1, unreduced subunit A; lane 2, reduced subunit A; lane 3, reduced α-chain; lane 4, reduced γ-chain; lane 5, reduced β-chain; lane 6, reduced cholera toxin; lane 7, molecular weight standards.
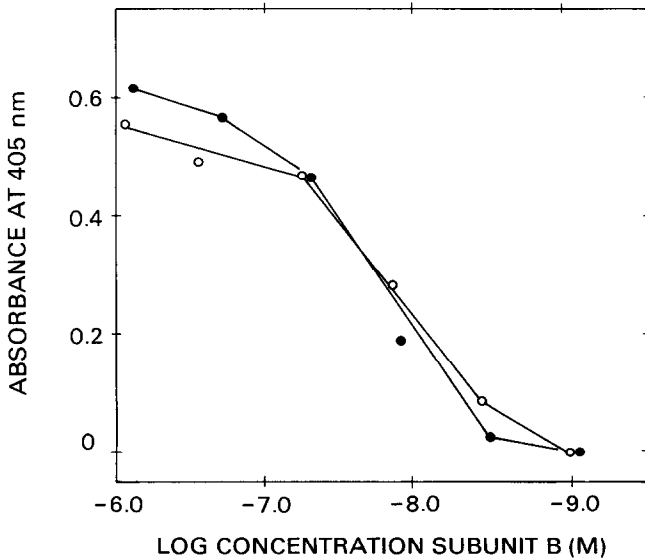

Fig. 5. Binding of cholera toxin and HPLC-purified B subunit to Gm_1 ganglioside-coated microtiter plates and measurement by ELISA with immunoaffinity chromatography-purified rabbit anti-B subunit and alkaline phosphatase-labeled goat anti-rabbit IgG. ●—●, Cholera toxin; ○—○, B subunit.

the C_8 column were typically about 80% under optimal conditions and varied between 60% and 80%, depending on the condition and age of the column and the guard column. The recoveries of A subunit were somewhat more variable than that of B. An additional consideration was the fact that the C_8 columns we employed were also used to fractionate a number of other proteins, which may have contributed to some non-specific binding effects. A column exclusively dedicated to toxin fractionation would probably give consistently higher recoveries.

During the course of this work, three Nucleosil C_8 10-μm columns were employed. They all performed essentially the same. In general, we found this column to be very durable and highly stable. Fractionation of the toxin was also attempted on a Nucleosil C_8 5-μm column, but the toxin was not eluted from that column. Some variation in subunit elution times was evident, depending on the nature and concentration of the buffer in which the toxin was dissolved. Presumably, this was due to some counter ion effect; however, all buffers and salt solutions employed gave complete resolution of the A and B subunits.

A major problem encountered during this study was the insolubility of the A subunit after exposure to mild acid. Some improvement in solubility could be achieved by the use of ethylene glycol, as reported by Tomasi *et al.*[10]. However, we found that the problem of insolubility could be largely overcome by quick adjustment of pH from acidic to alkaline buffers. Exposure of the A subunit to pH values near its isoelectric point ($pI \cong 6$)[14] during dialysis resulted in considerable precipitation.

It is important to emphasize that the HPLC procedure employed did not involve dissociating conditions other than the intrinsic effect of the 0.1% TFA and acetonitrile in the gradient eluents. These conditions not only dissociated A and B subunits but also disrupted the β_5 subunit interaction. Evidence for this was obtained by heating the B subunit for 3 min at 100°C in 1% acetic acid, containing 6 M guanidine hydrochloride, and immediately injecting the mixture into the Nucleosil column. No change in elution time was observed for the B subunit, suggesting that the eluted peak was, in fact, β-chain rather than β_5. Reduced and carboxymethylated B subunit also gave an elution time almost identical to that of untreated B subunit. It is known that dissociation of β_5 under acidic urea-denaturing conditions can be reversed after dialysis[15]. Gel permeation HPLC confirmed that the Nucleosil-prepared β-chain had reaggregated to the β_5-form. Conceivably, the Nucleosil column could be employed to establish the association constant of the β-chain.

HPLC-prepared B subunit showed almost the same binding to GM_1 ganglioside as intact cholera toxin, compared on a molar basis (Fig. 5). Similarly, the A subunit, prepared by HPLC, demonstrated reasonably high ADP-ribosyltransferase activity, as compared with conventionally prepared commercial A subunit and cholera toxin (Table I). In general, the A subunit and the α-chain preparations were found to have transferase activities about 40–60% of that of intact DTE-treated toxin. Treatment of intact toxin with TFA, TFA and acetonitrile, urea, or guanidine also resulted in a *ca.* 50% loss of transferase activity. The urea-treated toxin had somewhat lower activity, perhaps due to carbamylation by cyanate. The observed loss of activity with the A subunit and α-chain preparations, as well as with chemically treated intact toxin, may be due to α-chain aggregation in the course of the assay after reduction with DTE, as reported by Tomasi *et al.*[10]. Aggregation of α-chain may not occur as readily in the case of reduced, untreated toxin if the α-chain is still

attached to $\gamma\beta_5$. Evidence for a reduced but undissociated form of cholera toxin was obtained by means of ultracentrifugation studies by Tomasi *et al.*[16]. Alternatively, reduced cholera toxin that has not yet dissociated into free α-chain may be a transition form having intrinsically greater specific activity than reduced α-chain, when compared on a molar basis.

ACKNOWLEDGEMENTS

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