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Rapid Purification of a Phospholipase-Free α-Bungarotoxin: Maintenance of Cation Barriers of Acetylcholine Receptor Membranes upon Preincubation with Purified Toxin

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The purification of highly homogeneous, phospholipase-free α-bungarotoxin (α-Bgt) from the venom of the elapid *Bungarus multicinctus* or from commercial samples of α-Bgt is described. The method combines a conventional procedure for the purification of α-Bgt [D. Mebs, K. Narita, S. Iwanaga, Y. Samejima, and C. Y. Lee (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 243-262] with high-resolution gel-filtration and cation-exchange chromatography steps to remove membrane-damaging, contaminating phospholipase activity. The procedure also removes contaminating radioactive peptides from commercial preparations of ^{125}I-α-Bgt. Apparent homogeneity of the purified α-Bgt (referred to as fraction D in the text), as well as the absence of contaminating phospholipase A_2 activity, is assessed by (i) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, (ii) gel-filtration and cation-exchange high-performance liquid chromatography, (iii) direct measurements of phospholipase A_2 activity under conditions where very low enzymatic levels should be detected, (iv) lack of interference with the passive cation permeability properties of acetylcholine receptor membranes, (v) competitive inhibition of ^{125}I-α-Bgt binding to the acetylcholine receptor membranes, and (vi) amino acid analysis and end-group (C- and N-terminus) determination. α-Bgt preparations subjected to these criteria do not exert the increase in membrane passive permeability to cations detected with other laboratory or commercial samples of α-Bgt. Availability of the new α-Bgt preparation allows for an assessment of the inertness of α-Bgt on lipid membrane properties while preventing cholinergic ligand binding to nicotinic acetylcholine receptor-rich membranes. These conditions are necessary for experiments requiring maintenance of the physical and phospholipid integrity of membranes.

The nicotinic acetylcholine receptor (AcChR)[2] is an integral membrane glycoprotein mainly located at the neuromuscular synaptic junction and in the electric organ of certain fish [see Ref. (1) for review]. Binding of the appropriate neurotransmitter (acetylcholine) to the cholinergic binding sites in the AcChR molecule elicits the formation of a short-lived cation channel which allows ion translocation through the membrane to occur, thus initiating postsynaptic membrane depolarization.

α-Neurotoxins from snake venome are able to prevent neurotransmitter binding onto the AcChR cholinergic binding sites. In particular, α-bungarotoxin (α-Bgt) is widely employed as a specific marker for

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[2] Abbreviations used: α-Bgt, α-bungarotoxin; PLA_2, phospholipase A_2; AcChR, acetylcholine receptor; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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AcChR due to its high affinity exhibited by dissociation constants for the AcChR-α-Bgt complexes in the range 10^{-10}-10^{-11} M (2, 3).

Phospholipases are also major components of snake venoms. Both α-neurotoxins and phospholipases exhibit a similar behavior through most purification procedures which makes possible cross-contamination. This problem has seemingly been solved by many authors in both fields by introducing, for instance, hydrophobic interaction chromatography (4-6) or ion-exchange chromatography (7-10) for the purification of phospholipases and α-Bgt, respectively. In any case, the relevance of possible cross-contamination depends upon the purpose of the experiments to be conducted. For instance, presence of contaminating phospholipase A_2 (PLA_2) in α-Bgt preparations does not affect the binding of the neurotoxin to AcChR (11). However, exposure of AcChR-membranes to PLA_2 activity results in a twofold effect: (i) a physical disruption of lipid-lipid and lipid-protein interactions which alter the "barrier" properties (permeability) of the membrane itself, and (ii) the prevention of the agonist-mediated AcChR cation flux, which seems to be a consequence of specific lipids released acting catalytically on the membrane-bound AcChR system (11, 12).

The procedure, first described by Mebs *et al.* (9), is widely used by researchers in the AcChR field and commercial firms for the purification of α-Bgt. However, when the purified material was analyzed by the procedures reported in this communication, we detected the presence of protein contaminants as well as associated PLA_2 activity. We describe here how the application of high-resolution chromatography steps complementing a classical purification scheme for α-Bgt (9) results in securing highly homogeneous, phospholipase-free material. This procedure may prove to be useful for studies requiring neurotoxin and preservation of the integrity of phospholipid membrane structure.

MATERIALS AND METHODS

^{125}I-α-Bgt from New England Nuclear was passed through a small DEAE-Sephadex column in 10 mM phosphate buffer, pH 7.4, for the elimination of the manufacturer's added bovine serum albumin, and was stored at 4°C.

Isolation of α-Bungarotoxin. Lyophilized venom of *Bungarus multicinctus* (Sigma Chemical Co.) was dissolved in 50 mM ammonium acetate buffer, pH 5.8, at 100 mg/ml, and was used for the purification of α-Bgt according to the procedure of Mebs *et al.* (9). This procedure includes (i) chromatography on CM-Sephadex, (ii) desalting of the α-Bgt-containing fraction on a Sephadex G-25 column and (iii) rechromatography on a second CM-Sephadex column. The main protein peak eluting from the second CM-Sephadex column (corresponding to the α-Bgt fraction) was submitted to the analytical procedures described below.

Gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out by using an exponential gradient slab gel (5-15%) with a 3% stacking gel and the discontinuous buffer system of Laemmli (13).

The amount of sample applied for SDS-PAGE analysis ranged from 10 to 50 μg protein. The protein bands were stained with Coomassie brilliant blue R-250 (Sigma Chemical Co.). Molecular weight standards were phospholipase A_2 ($M_r \sim 13{,}500$) and neurotoxin ($M_r \sim 7{,}000$) fractions from *Naja nigricollis* venom (both courtesy of Dr. H. J. Evans); and phosphorylase *b* ($M_r \sim 94{,}000$), bovine serum albumin ($M_r \sim 67{,}000$), ovalbumin ($M_r \sim 43{,}000$), carbonic anhydrase ($M_r \sim 30{,}000$), soybean trypsin inhibitor ($M_r \sim 20{,}100$) and lysozyme ($M_r \sim 14{,}300$).

Molecular weight determination of α-Bgt. Three different methods were used for the determination of the molecular weight of α-Bgt: (i) By comparison of the electrophoretic mobility of protein bands in SDS-PAGE with those of the standards; (ii) by using a HPLC sizing column (TSK-2000, Beckman) previously calibrated with the following protein standards: bovine serum albumin ($M_r \sim 67{,}100$), ovalbumin ($M_r \sim 43{,}000$), pepsin ($M_r \sim 34{,}700$), trypsinogen ($M_r \sim 24{,}000$), lactoglobulin ($M_r \sim 18{,}400$), and lysozyme ($M_r \sim 14{,}300$); and (iii) by quantitative amino acid analysis of purified α-Bgt samples. The amino acid analysis were performed as described elsewhere (14).

End-group determination. For determination of the carboxy-terminal amino acid, hydrazinolysis was performed essentially according to the method of Fraenkel-Conrat and Tsung (15).

Amino-terminal sequence was carried out by Edman degradation with a Beckman 890C automatic sequencer. The sample in 0.5 ml water was applied using the standard application technique, followed by use of the fast protein Quadrol program 972172-C (Beckman, Palo Alto, Calif.). The thiazolinone derivatives were converted to phenylthiohydantoins,

and the PTH derivatives were identified by HPLC on a Beckman C18 reverse-phase column as previously described (16).

High-performance liquid chromatography. HPLC was performed using a Beckman chromatograph Model 332. Absorbance changes were monitored at 280 or at 214 nm by using a Hewlett-Packard 3390A recorder/integrator connected to a Beckman detector Model 160.

For gel-filtration chromatography a TSK-2000 column (Altex, 7.5 mm × 30 cm) was used. The elution buffer was either 50 mM ammonium acetate, pH 6.8 (when the absorbance was monitored at 280 nm), or 10 mM phosphate buffer, pH 6.8 (for monitoring absorbance changes at 214 nm). For ion-exchange chromatography a CM-column (Altex, 6 mm × 15 cm) equilibrated in 50 mM ammonium acetate buffer, pH 5.8, was used. Experimental conditions for elution of samples are detailed in corresponding figure legends. For reverse-phase chromatography a Bondapak C-18 column (Water Associates, 3.9 × 30 cm) was used with a standard trifluoroacetic acid/acetonitrile gradient for elution of peptide samples.

Phospholipase activity measurements (PLA_2). Presence of phospholipase A_2 activity in α-Bgt samples was quantitated by using autoclaved *Escherichia coli* as substrate, whose phospholipids were labeled by *in vivo* incorporation of [1-^{14}C]oleate almost exclusively (≃95%) into the sn-2 position of the glycerol moiety (17). PLA_2 activity was expressed as the percentage hydrolysis (100% = 5 nmol phospholipid hydrolyzed). For most samples, PLA_2 activity was detected upon incubation periods of 5 min. However, for those samples with expected low levels of PLA_2 activity, the incubation period was increased up to 1 h. Assays were carried out in the presence of 5 mM $CaCl_2$ (17).

Competition-binding assays. The protein fractions obtained from HPLC were concentrated and desalted (ammonium acetate is a volatile salt) in a Savant-Speed Vacuum, and were redissolved in 50 mM ammonium acetate buffer, pH 5.8, at concentrations such that the 280-nm absorbance reading of the different protein samples were identical. These samples were then subjected to competition with ^{125}I-α-Bgt (New England Nuclear) for binding to AcChR-rich alkaline-treated membranes isolated from *Torpedo californica* electroplax (18, 19). For the competition assay, AcChR membranes were preincubated (overnight at 4°C) with increasing aliquots of the different protein fractions; then a fixed amount of ^{125}I-α-Bgt was added and the mixture was further incubated for 2 h at room temperature. Binding of ^{125}I-α-Bgt to AcChR membranes was determined by using a DEAE-cellulose filter disk assay procedure (20).

Stopped-flow kinetics of Tl^+ passive influx. The permeation of Tl^+ through AcChR membrane vesicles was monitored by a "stopped-flow/fluorescence quenching" spectroscopic procedure as described elsewhere (21). This procedure is based on the fluorescence quenching of an intravesicularly entrapped fluorophore (1,3,6,8-pyrenetetrasulfonate) by externally added cation quencher (Tl^+). As Tl^+ diffuses into the membrane vesicles, collisional quenching with the entrapped fluorophore occurs. This Tl^+ "influx" is monitored as a time-dependent decrease of the fluorescence emitted by the fluorescence probe. Spectroscopic traces are then computer-fitted to a time-dependent Stern-Volmer equation (22), which provides with apparent rate constant values for the passive cation influx process. Several 25-s spectroscopic traces were recorded for each experiment, and the values obtained for the passive Tl^+ influx rate constants were averaged. Temperature was 22°C.

RESULTS

Purification of Starting α-Bgt

The purification of the crude venom followed the same pattern and provided protein yields similar to those described by Mebs *et al.* (9). The major protein fraction from the second CM-column (referred to as starting α-Bgt) corresponded to α-Bgt as concluded from (i) Competition assays with ^{125}I-α-Bgt for binding to electroplax AcChR-membranes; (ii) SDS-PAGE, which shows a major protein band with M_r 8000; and (iii) amino acid composition analyses, which were as those reported in the literature for α-Bgt (8, 10). Nevertheless, when PLA_2 activity tests were conducted on the starting α-Bgt, the results were always positive. The extent of the PLA_2 contamination differed with the preparations. Phospholipase activity was also found in commercial purified α-Bgt from Sigma and Calbiochem and in ^{125}I-α-Bgt from New England Nuclear.

Chromatography

Because of the better resolution obtained by HPLC as compared to conventional chromatography, a CM-HPLC column was used in an attempt to fractionate the different protein components detected (Fig. 1). This resulted in the separation of the starting α-Bgt into two main components, fractions I and II.

Phospholipase activity measurements and α-Bgt competition-binding assays

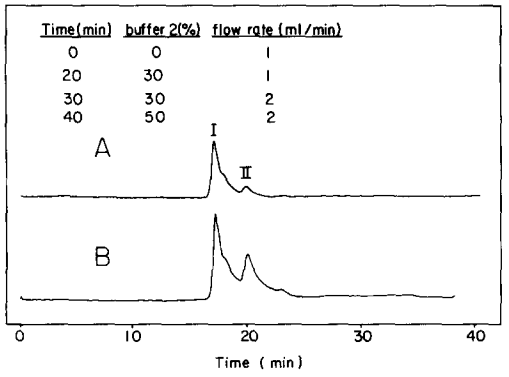

FIG. 1. Representative CM-HPLC absorbance profile at 280 nm of (A) α-Bgt as purified by the procedure indicated in Ref. (9) (starting α-Bgt in the text) and (B) commercially purified α-Bgt from Sigma Chemical Company. The column was previously equilibrated in buffer 1 (50 mM ammonium acetate, pH 5.8), and elution of protein samples was carried out by using mixtures of buffer 1 and buffer 2 (1 M ammonium acetate, pH 7.0) as indicated in the inserted elution program. Buffer 2 (%) values indicate the percentage composition of the elution buffer reached through linear gradients at indicated times.

were determined for each one of the two fractions shown in Fig. 1A. Fraction I contained the bulk of α-Bgt binding activity and low levels of PLA_2 activity, whereas fraction II accounted for most of the phospholipase activity of the original starting α-Bgt sample. Since the more purified α-Bgt fraction (fraction I) appeared to be still contaminated with, at least, PLA_2, and since α-Bgt and PLA_2 should have different molecular weights as revealed by SDS-PAGE (Fig. 2), an HPLC gel-filtration step was attempted on the starting α-Bgt.

Figure 3 shows the profile obtained for the starting α-Bgt upon the HPLC gel-filtration step. The column was previously calibrated so that the molecular weight of the different protein fractions could also be estimated (insert to Fig. 3). As shown in Table I, the two first peaks (fractions A and B) from Fig. 3 (apparent molecular weight = 24,000 and 19,000, respectively) contained most phospholipase contamination in terms of specific activity (the relative area for these peaks was considerably smaller than for the third peak); however, a significant PLA_2 activity (~26% hydrolysis) was also detected in the third peak which exhibited an apparent molecular weight of 15,800.

Fraction C from the HPLC gel-filtration step was concentrated, desalted, and then rechromatographed on the CM-HPLC column. Figure 4 shows the corresponding profile: A major peak (fraction D) containing no detectable PLA_2 activity even after long incubation time periods and able to completely inhibit the binding of ^{125}I-α-Bgt to AcChR-rich membranes, and a minor fraction containing most of or all the PLA_2-activity in fraction C applied to the column.

When ^{125}I-α-Bgt purchased from New England Nuclear was used, the elution profiles obtained under the conditions used in Fig. 1 (CM-HPLC) were further complicated by the following: (i) Although

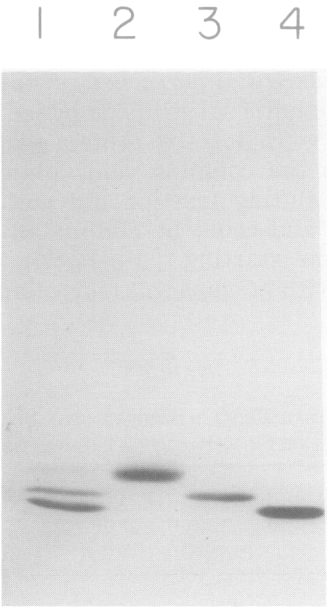

FIG. 2. Polypeptide patterns on SDS-PAGE of the following samples (see text for identification): (1) "starting" α-Bgt (major protein fraction from the second CM-column in Ref. (9) highly contaminated with PLA_2 activity; (2) fraction A from HPLC gel filtration (see Fig. 3); (3) fraction B from HPLC gel filtration (see Fig. 3); and (4) fraction D (PLA_2-free α-Bgt) from combined gel filtration and CM-HPLC (see Fig. 4). Estimates for apparent molecular weights are indicated under Results.

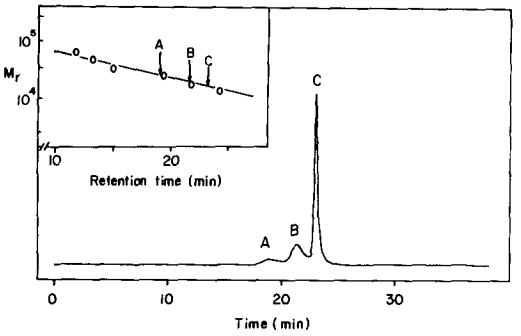

FIG. 3. Representative gel-filtration HPLC absorbance profile at 280 nm of the starting α-Bgt sample. The column was calibrated, and protein samples were eluted by using a 50 mM ammonium acetate buffer, pH 6.8. Flow rate was maintained at 0.5 ml/min. Insert: Molecular weight calibration curve of the gel-filtration column. Arrows indicate relative position for fractions A, B, and C.

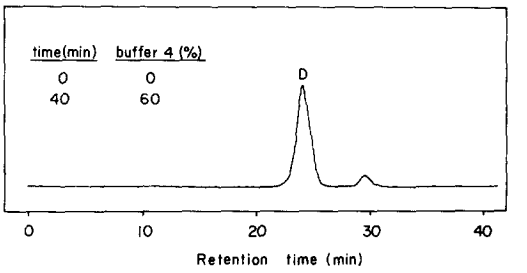

FIG. 4. Representative CM-HPLC absorbance profile at 280 nm of fraction C from Fig. 3. The column was previously equilibrated in buffer 3 (100 mM ammonium acetate, pH 5.8), and elution of protein samples was carried out by using mixtures of buffer 3 and 4 (500 mM ammonium acetate, pH 7.0), as indicated in the inserted elution program. Flow rate for elution was 0.5 ml/min. Buffer 4 (%) values indicate the percentage composition of the elution buffer reached through linear gradients at indicated times.

the shape of the elution profile in the region of the chromatogram containing fraction I and II was maintained, there was an increase for the retention times of all protein peaks which might be a consequence of a perturbed pK for Tyr-54 upon aromatic substitution by iodine, and (ii) a large radioactive protein peak eluted close to the columns void volume. This rapidly eluting fraction did not bind to AcChR, and could be eliminated by dialyzing the starting ^{125}I-α-Bgt in dialysis tubing with an apparent molecular weight cut-off of 3500. Furthermore, C-18 reverse-phase HPLC analysis of this dialyzable material revealed the presence of a heterogenous population of putative peptides, each of which had a certain amount of radioactivity associated. These peptide populations were detected in ^{125}I-α-Bgt samples regardless of prior removal of manufacturer's added bovine serum albumin by DEAE-Sephadex chromatography. The presence of a mixture of different size radioactive peptides should be expected in view of recent reports (23, 24) on the radiodecomposition of α-Bgt by the presence of bound radioactive iodine.

TABLE I

ACTIVITIES OF PROTEIN SAMPLES UPON FRACTIONATION OF STARTING α-Bgt BY GEL FILTRATION HPLC

Fraction[a]	Relative abundance (%)[b]	PLA_2 activity (%)[c]	^{125}I-α-Bgt binding inhibition
A	4.5	28.5	None[d]
B	22.5	41.0	None
C	73.0	26.0	Total

[a] In order of elution.

[b] Percentage of peak areas when monitoring the elution of protein samples at 280 nm.

[c] As indicated under Materials and Methods.

[d] ^{125}I-α-Bgt binding identical to that observed with control membranes (no preincubation with protein fractions).

Apparent Molecular Weight

Samples containing large amounts of proteins were analyzed by SDS-PAGE as represented in Fig. 2. The pattern correlated well with the data obtained by gel-filtration HPLC, three protein bands for starting α-Bgt and a single homogeneous band relative to fraction D. Molecular weight estimates for the proteins based on electrophoretic mobility were 24,000, 16,000 and 8,000 for peaks A, B, and C, respectively. Molecular weight estimates by SDS-PAGE also correlated well with those based on gel-filtration HPLC, except for fraction C ($M_r \sim$ 15,800 by gel filtration and $M_r \sim$ 8,000 by SDS-PAGE). This

apparent discrepancy may be explained if the $M_r \sim 15{,}800$ component detected by gel filtration corresponds to an aggregated form of α-Bgt, as suggested by the known tendency of α-neurotoxins to aggregate in aqueous media (10) and previous reports by other groups (7). This possibility of aggregation was strengthened by our observations upon dilution of fraction D for chromatography by HPLC gel filtration. There was a substantial increase in the retention time ($t_R \sim 35$ min) for the protein peak from this diluted sample (1 mg/ml) compared to the concentrated sample D value ($t_R \sim 23$ min, 6 mg/ml). The delayed peak from the diluted sample reversed to the $t_R \sim 23$-min position once the $t_R \sim 35$-min fraction was pooled, concentrated, and desalted, consistent with a concentration-dependent association-dissociation equilibrium.

Chemical Characterization

The homogeneous PLA_2 activity-free, α-Bgt fraction (fraction D) was subjected to amino acid analysis, yielding the expected composition according to previous papers of 74 amino acid residues (8, 10), corresponding to a molecular weight for α-Bgt near 8,000.

C- and N-terminus sequencing (followed by N-terminal sequencing of the next 8 amino acid residues) yielded Gly and Ile, respectively, in agreement with previous reports (7, 9).

Effects on Membrane Permeability

The effect of preincubation with both PLA_2-contaminated and PLA_2-free α-Bgt fractions on the passive permeability of electroplax AcChR membranes to monovalent cations was also monitored by a "stopped-flow/fluorescence quenching" technique (21). The presence of fraction D (PLA_2-free α-Bgt) preincubated with control alkaline-treated AcChR membranes did not produce significant changes in the rate of passive membrane permeation to Tl^+. Both control membranes (no toxin samples added) and membranes preincubated with PLA_2-free α-Bgt, exhibited rate constant values for passive Tl^+ influx within the 0.2 (±0.02) S^{-1} range at the operating temperature of 22°C. On the contrary, preincubation for only 5–10 min of control AcChR membranes with PLA_2-containing fractions (peak B from Fig. 3) at concentrations corresponding to 4–6% hydrolysis by the PLA_2 activity assay (i.e., slightly above the practical limit for detection of PLA_2 activity) increased the rate for passive diffusion of Tl^+ by almost one order of magnitude. Increasing either the amount of added PLA_2-containing sample or the preincubation time period further increased the passive permeability of the membranes to Tl^+. Similar phenomena were observed when preincubation of the membranes was done in the presence of PLA_2-containing, "starting" α-Bgt or with any of the α-Bgt commercial samples we tested.

DISCUSSION

Neurotoxins and phospholipases are major components of most snake venoms. Both proteins share a number of common physical properties which impair their complete separation. Nevertheless, procedures have been developed by both researchers in the neurotoxin and phospholipase fields which yield practically homogeneous preparations.

α-Bgt from *B. multicinctus* venom has been used routinely as a specific marker for the detection and quantitation of nicotinic AcChR in postsynaptic membrane preparations. Binding of α-Bgt to AcChR is extremely tight and independent of the physical integrity of the membrane. In fact, binding assays are usually done with detergent-disrupted membranes. Under these conditions the homogeneity of the α-Bgt fraction, as well as the possible presence of contaminants such as phospholipases, are of little relevance to the highly specific binding process. On the other hand, low levels of contamination with PLA_2 may become important in the monitoring of processes which require the integrity of the membrane structure when studying membrane vesicle preparations or whole cells. PLA_2 is an extremely active enzyme and, besides causing the physical

disruption of the membrane itself (25, 26), its phospholipid hydrolysis products, particularly unsaturated free fatty acids, have been shown to catalytically inhibit the ion flux event mediated by agonist binding to the membrane-bound AcChR (12). However, the presence of PLA_2 has been shown not to affect binding of α-Bgt to AcChR membranes (12).

During the course of investigating the blockade of AcChR sites and agonist-mediated cation fluxes by addition of substoichiometric amounts of α-Bgt prepared as described in the literature (9), we detected that preincubation of AcChR membranes with α-Bgt preparations greatly enhanced the rate for the passive leakage of cations from the membranes. Further analysis of the α-Bgt preparations revealed that, upon incubation of α-Bgt and radioactive phospholipids (labeled at the *sn*-2 acyl residue), a substantial PLA_2 activity was present. Also, SDS-PAGE indicated protein heterogeneity in these preparations. Presence of contaminants was confirmed in five different preparations of α-Bgt made in our laboratory following published procedures (9) as well as in commercially available α-Bgt from several manufacturers (Sigma, Calbiochem, and New England Nuclear). These findings led us to the introduction of additional resolutive separation steps in the protocol (9) that are referenced as of common usage by workers in the field.

Similarities between α-Bgt and PLA_2 that makes difficult their separation by conventional chromatography appear to include their basic and partially hydrophobic character as well as the apparent formation of α-Bgt dimers, thus exhibiting molecular weight values close to that of PLA_2 monomers.

Our best procedure combines the original method for the purification of α-Bgt (9) followed by high-resolution gel-filtration and ion-exchange chromatography steps. The resulting purified α-Bgt (referred to as fraction D) lacks detectable contaminants, its homogeneity being based on criteria which include (i) SDS-PAGE (ii) HPLC, (iii) total disappearance of PLA_2 even after long incubation time periods, while maintaining competitive inhibition of ^{125}I-α-Bgt binding activity to AcChR, and (iv) lack of interference with passive permeation of cations through the membrane while effectively blocking agonist-mediated AcChR flux responses. Other possible criteria for homogeneity such as quantitative amino acid analysis or protein end-group determination may, in our case, be considered only as circumstancial evidence for the following reasons. First, the presence on a protein sample of a low-percentage contaminant does not significantly change the results of an amino acid analysis of the sample. In fact, analysis from sample A (contaminated α-Bgt) and sample D are practically identical and undistinguishable from reported values for α-Bgt (8, 10). Second, even if the low-level contaminants could be detected from background in end-group determinations, the most common C-terminal residue reported for snake phospholipases is asparagine (25), which would not be seen by the hydrozinolysis procedure.

The complete disappearance of PLA_2 activity and the lack of effect on membrane permeability of our final α-Bgt preparation (fraction D) may be of particular interest to those working on *in vivo* systems such as cultured cells, where the disruption of the phospholipid matrix as well as the potential increase in membrane leakiness may represent undesirable processes of little relevance to α-neurotoxin utilization.

With regard to further purification of commercially available ^{125}I-α-Bgt, our procedure is equally suitable, resulting in the additional elimination of the peptide population supposedly associated to radiodecomposition of the toxin by radioactive ^{125}I (23, 24). These radioactive peptides were shown not to bind to AcChR-rich membrane preparations. Also, because of the simplicity and speed of the HPLC steps, our procedure provides a simple way for rapidly isolating labeled α-Bgt. Direct specific radioactivity determinations can be made upon removal of labeled contaminants, labeled radiodecomposition peptides, and added bovine serum albumin.

In conclusion, we have reported how the addition of high-resolution chromatographic steps to a widely accepted α-Bgt purification procedure results in homogeneous, PLA_2-free α-Bgt suitable for work requiring maintenance of the physical integrity of AcChR membranes.

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