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NEUROTOXINS OF BUNGARUS MULTICINCTUS VENOM
PURIFICATION AND PARTIAL CHARACTERIZATION[†]

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Running Title: Bungarus multicinctus Neurotoxins

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Abbreviations used: AChE, acetylcholinesterase; α -Bgt, alpha-bungarotoxin; Asx, Aspartic Acid/Asparagine; β -Bgt, beta-bungarotoxin; β -NAD, nicotinamide-adenine dinucleotide; CM, carboxymethyl; CD, circular dichroism; DMS, dimethyl sulfoxide; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; MCD, magnetic circular dichroism; NBS, N-bromosuccinimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TEMED, N,N,N',N'-tetramethylethylene-diamine.

ABSTRACT

The purification to homogeneity of nine neurotoxic components of the venom of Bungarus multicinctus is described. The purified components include α -bungarotoxin and two other post-synaptic toxins and β -bungarotoxin and five other pre-synaptic toxins. The purified toxins have been characterized by electrophoresis, isoelectric focusing, amino acid analysis, N-terminal amino acid determination, and immunodiffusion. The post-synaptic neurotoxins constitute a discrete antigenic class with molecular weights of 7000-8500 daltons, isoelectric points (pI) of 9.0-9.2, and N-terminal isoleucine or methionine. The pre-synaptic neurotoxins constitute a second antigenic group with molecular weights of 20-22,000 daltons and pI 8.8-9.7. Fractions 10 thru 13 exhibit a chain structure consisting of a 6-7000 dalton light chain and a 11-15,000 dalton heavy chain apparently covalently stabilized by inter-chain disulfides. Fractions 9A and 14 were single chains of 11-14,000 daltons which resemble the sequenced pre-synaptic neurotoxin notexin (Halpert and Eaker, 1975, J. Biol. Chem. 250, 6990). All of the pre-synaptic toxins have a single tryptophan and N-terminal aspartic acid or asparagine.

INTRODUCTION

The protein neurotoxins of snake venoms have received increasing attention both as systems for the study of structure-function relationships (Tu, 1973, Karlsson, 1974) and as tools in the study of synapses (Changeux et al, 1970, von Hahn and Honegger, 1974). The majority of purified toxins from the Elapidae and Hydrophidae families are highly specific post-synaptic neurotoxins targeted to the nicotinic acetylcholine receptor of the neuromuscular junction (Lee, 1972). In addition, in some Elapid venoms there occur pre-synaptic neurotoxins directed to the nerve terminal of the neuromuscular junction (Lee, 1972). Both toxin varieties are found in the venom of the Formosan banded krait, Bungarus multicinctus (Lee et al, 1972). The two predominant components of the venom, α -bungarotoxin (post-synaptic) and β -bungarotoxin (pre-synaptic), have been studied in some detail (Mebs et al, 1972, Kelly and Brown, 1974). However, there are other functional homologues of these neurotoxins in the same venom which have not been structurally characterized. Initial pharmacological studies (Lee et al, 1972, Dryden et al, 1974) have suggested that several components should be examined more closely for their possible neurochemical application. In our initial report (Eterović et al, 1975), we presented a fractionation scheme and some toxicological and spectral properties of the bungarotoxins. In this paper, we present details for further purification and several aspects of structural analysis which will facilitate the reproducible identification of the same toxins in different laboratories. In addition, we can now begin to identify generic features of the two pharmacological classes of bungarotoxins. In the case of post-synaptic neurotoxins, a picture of striking structural conservation has emerged (Yang, 1974). For pre-synaptic toxins, less is known. However, they appear to share phospholipase a_2 enzymatic activity and some sequence homology (Halper and Eaker, 1975, Strong et al, 1976).

MATERIALS

Bungarus multicinctus crude venom was purchased from Miami Serpentarium. Guanidine hydrochloride (ultrapure) was from Heico. N-bromosuccinimide was 99%-pure reagent from Aldrich and was recrystallized twice from water before use. Iodoacetic acid was from K and K Laboratories and was recrystallized from petroleum ether (30-60°) and diethyl ether. Bio-Rex 70, Bio-Gel P30, acrylamide, and bis-acrylamide were from Bio-Rad. Sephadex G50 and CM-Sephadex C50 were from Pharmacia. Bungarus multicinctus horse serum anti-venom, TEMED, trypsin, alpha-chymotrypsin, leucine aminopeptidase, carboxypeptidases A and B were from Sigma. Dansyl chloride, dimethyl suberimide, 4N methanesulfonic acid ampules, and constant boiling HCl ampules were from Pierce Chemical. Ampholines were purchased from LKB-Produkter AB. Polyamide sheets (mikropolyamid F1700) were from Schleicher and Schull. Diaflo ultrafiltration membranes were from Amicon. CM-cellulose was from Whatman.

METHODS

Fractionation of Crude Venom

Thirteen components were obtained by ion exchange chromatography on CM-Sephadex as described previously (Eterović et al, 1975).

Purification of CM-Sephadex Fractions

For the first experiments, pure α -bungarotoxin was obtained from fraction 3 by rechromatography on CM-cellulose as previously described (Eterović, et al, 1975). For later experiments, fractions were concentrated by pressure dialysis to 1-2 ml and applied to a 2.6 x 40 cm Sephadex G50 (fine) column. The column was run at 4°C with upward elution by 0.1 M ammonium acetate (pH 6.8) at 20 ml/h. Peaks of absorbance at 280 nm detected by a Chromatronix Model 220 UV monitor were pooled, and then desalted and concentrated by ultrafiltration (UM-2 Diaflo). These fractions were applied to a 2.5 x 20 cm column of Bio-Rex 70 (sodium form, 200-400 mesh). The column was rinsed with 150 ml of 50 mM sodium acetate (pH 6.0) and the proteins were eluted with a linear gradient of 0-1 M NaCl (1.5 l) in the same buffer. Lethality was determined after each chromatographic step (Eterović, et al, 1975). Final fractions were desalted, concentrated and either lyophilized, or stored frozen in dilute neutral buffer. Lyophilized components were stored dessicated at -20°C.

Assay of Enzymatic Activities

Acetylcholinesterase (EC: 3.1.1.7) was determined by the method of Ellman et al, (1961). The substrate was acetylthiocholine at a final concentration of 6×10^{-4} M. As a blank for each determination, identical assays were done in the presence of 10^{-5} M eserine (an anticholinesterase). Hyaluronidase (EC: 4.2.99.1) was measured by the turbidimetric method of DiFerrante (1956) with hyaluronic acid at several concentrations. Phosphomonoesterase (EC: 3.1.3.1) activity was measured with p-nitrophenyl

-7-

phosphate as substrate following the method of Lowry (1957). Nucleotide pyrophosphatase (NADase, EC: 3.6.1.9) was determined by the method of Kornberg and Pricer (1950) with 8 or 27 mM β -NAD. Phospholipase A (EC: 3.1.1.4) was measured by the titrimetric method of Saito and Hanahan (1962). Proteolytic activities were screened using azocasein (Fritz et al, 1974). Arginine esterase was measured by the hydrolysis of BAPNA (N-benzoyl-dl-arginine-p-nitroanilide HCl) which produces a product absorbing at 405 nm. Proteinase inhibitor assays employed BAPNA as a synthetic substrate for purified trypsin (Sigma Type XI) to which assay aliquots were added (Fritz et al, 1974).

Electrophoresis

Phenol:acetic acid:urea gels were prepared by an adaptation of the procedure of Takayama et al, (1966). In brief, solid acrylamide, bis-acrylamide, 12 g urea, 200 μ l TEMED, and 2 mg sodium dithionite were stirred until dissolved in 15 ml distilled water. The solution was then clarified through HAWP Millipore filters. Glacial acetic acid (14 ml) was added, and the mixture was stirred for 15 min. Ammonium persulfate (150 mg) was then added and the solution briefly stirred. Gels were prepared in 7 mm x 8 cm glass tubes that had been cleaned and subsequently treated with Kodak Photo-Flo. Gels were overlaid by degassed glacial acetic acid and incubated for 15 min at 50°C. The glacial acetic acid was drawn off, replaced, and the gels reincubated for another 10 min. The acetic acid was drawn off again and replaced with 75% acetic acid. The gels were pre-electrophoresed for an hour with 10% acetic acid as electrode solution. Samples were applied in 10-15 μ l of phenol: glacial acetic acid: 8M urea (2v/1v/1v) and run at 4 ma/gel until the marker pyronin Y has reached the gel end.

Sodium dodecyl sulfate-urea gels were prepared by the method of Eipper (1974). Sodium dodecyl sulfate gels were prepared according to Laemmli (1970).

For cross-linking 200 µg of lyophilised protein were dissolved in 20 µl of 0.2M triethanolamine (pH 8.5) containing 100 µg dimethyl suberimide and incubated for 3 hours at 25°C (Davies and Stark, 1970). The cross-linked products and standards were then solubilized and fractionated by the Laemmli SDS-gel procedure. For reduction and alkylation of the cross-linked proteins, 100 µg were taken and incubated for 17 h in 0.2M Na borate buffer (pH 9.0) with 1% mercaptoethanol. Iodoacetic acid (1 mg) was added and the proteins were incubated for an additional hour in the dark at 25°C.

Polyacrylamide gradient slab gels, incorporating the buffer system of Laemmli (1970) were prepared by increasing the concentration of acrylamide in the direction of migration. A linear gradient of acrylamide (10-20% or 17.5-20%), with the ratio of acrylamide to bis-acrylamide (20: 1 w/w), was cast as the separating gel and overlaid by a 5% stacking gel. Each gel accommodated up to 10 samples (10-50 µg protein) which were boiled with and without mercaptoethanol (5%) for 2 min and analyzed by electrophoresis.

Gels were stained in all cases by the procedure of Fairbanks et al, (1971).

Isoelectric-Focusing

Isoelectric-focusing in a pH 3-10 gradient was performed according to the procedures of Vesterberg (1972), Fawcett (1968), and Trump and Singer (1970) using 7.5% and 10% polyacrylamide gels and both chemical and photopolymerization. The pH profile was determined by slicing a control gel containing no protein into 2 mm sections which were incubated in 250 µl deionized water overnight and then the pH of each was measured. Proteins were fixed and rinsed with repeated washes of 10% TCA over 4 days and then stained by either Coomassie Blue, Naphtol blue black, or the Schiff's -PAS procedure. Where elution of the focused material was desired, fixation was omitted and the gel was sliced and the sections were incubated as above in water.

N-Terminal Amino Acid Determination

Protein (30 nmoles) was dansylated and hydrolysed as delineated by Gros and Labouesse (1969) with the modifications of Zanetti et al, (1970). Dansyl amino acids were chromatographed on 3 x 3 cm Schleicher and Schuell polyamide sheets with two-dimensional development in 3% formic acid followed by benzene:acetic acid (9:1 v/v). To resolve dansylaspartic and dansylglutamic acid, a subsequent development in the second direction with ethyl acetate: methanol:acetic acid (20:1:1 v/v) (Briel and Neuhoﬀ, 1972) was used. Unknowns were identified by comparison to standard dansyl-amino acids.

Immunodiffusion

Purified materials were tested by the double diffusion Ouchterlony microtechnique for antigenic similarities. Agarose layers (1%) were prepared on degreased glass slides as described (Clausen, 1969). Commercial horse antisera (dissolved in 10 ml sterile 0.9% saline) (5 μ l) was placed in the center wells at 1 cm distance and allowed to diffuse for 40 h at 4°C. Precipitin bands were stained after saline rinsing with 1% amido black in 10% acetic acid for 30 min. Agarose layers were destained by repeated rinsing in 10% acetic acid.

Amino Acid Analysis

Toxins (1 to 1.5 mg) were hydrolysed with 1 ml constant boiling HCl in sealed tubes maintained at $110^{\circ} \pm 1^{\circ}$ in an oil bath (Dow Corning 550). Prior to sealing, the tubes were flushed twice with dry nitrogen and evacuated to less than 20 microns Hg. Hydrolyses were run for 20 to 72 h. The hydrolysates were analysed in a Beckman Model 120C using the two-column system of Spackman et al, (1960). Values for serine, threonine, cystine, tyrosine, and histidine were corrected to zero time and values

for valine, leucine, isoleucine, alanine, glycine, and phenylalanine were extrapolated to plateau levels at long hydrolysis times. In cases where an appreciable decomposition of cystine occurred, the values for proline were also corrected to zero time since cysteine is co-eluted with proline in the system used. (Its presence can be detected by the ratio of absorbances at 570 and 440 nm which is 7.56 for proline and 1.46 for cysteine).

The best integral fit of amino acid residues to the experimental data was obtained by a computer analysis (Katz, 1968). The residue mole fractions are multiplied by an integer representing the total number of amino acids. The result is compared to the nearest integer and the difference squared. A minimum in the sum of differences squared will occur at the point of best fit of integral residues to the experimental data. Results were weighted to give different reliabilities to different amino acids and to account for either absolute or percentage error following the recommendations of Katz.

Independent composition analyses, including tryptophan, were obtained by hydrolysis in 4N methanesulfonic acid (115°, 22h) after the procedure of Simpson et al, (1976). Tryptophan values were confirmed by oxidation with N-bromosuccinimide in both the presence and absence of urea (Spande and Witkop, 1967), and by magnetic circular dichroism in 6M guanidine hydrochloride (Barth et al, 1972). Total sulfur was obtained from x-ray fluorescence (see below) and compared with the values for cystine plus methionine. Titration with DTNB was used for the detection of free sulfhydryl groups (Robyt et al, 1971).

Carboxymethylated reduced toxins were prepared by reducing 3-5 mg protein dissolved in 1 ml of 0.1M Tris base (pH 8.5) with a hundredfold molar excess over protein of solid dithiothreitol. Samples were maintained at 25°C overnight under a nitrogen barrier and solid iodoacetic acid was then added.

The samples were incubated for an hour in the dark and the reaction quenched by acidification. The carboxymethylated derivatives were isolated by gel filtration on a 1.6 x 50 cm Sephadex G10 (superfine) column equilibrated with 10% acetic acid. The CM-toxins were lyophilized and stored. Cystine content was obtained as carboxymethylcysteine after acid hydrolysis.

Sulfur Determination by X-ray Fluorescence

Total sulfur content of the protein samples was determined with a recently described non-dispersive vacuum soft X-ray fluorescence spectrometer (Hebert and Street, 1974). The spectrometer features 6 anodes which provide characteristic X-rays for sample excitation and determinations of the elements from oxygen to iron. The present experiments were primarily aimed at a sulfur determination and only a cursory examination was made to determine the presence of Na, Mg, Al, Si, P, Cl, K, and Ca at $\mu\text{g/ml}$ level in some samples. The analysis required 50 μl of aqueous solution or homogeneous suspension containing approximately 1 mg of sample per ml. The present group of unknowns contained from 14 to 53 μg of protein per sample.

An important consideration in performing vacuum soft X-ray analyses is the amount of incident radiation which may be converted to heat in the sample. This is especially true of biological samples which may contain easily decomposed or volatile components. The present spectrometer operates at anode power levels of 2 watts or less and the amount of exciting radiation reaching the sample after filtration is estimated to be less than 1 mwatt per cm^2 . Repeated analyses with several of the protein sample disks over periods of hours, and then again after several weeks revealed no observable sample deterioration or decline in sulfur content.

RESULTS

Purification

The initial fractionation on CM-Sephadex separated the crude venom into 13 protein peaks as indicated by positive Lowry reaction and UV absorbance (Eterović et al., 1975). Eleven of the protein fractions were lethal to mice (neurotoxic). Fraction 3 contained the toxin designated α -Bgt and fraction 10 contained the toxin previously designated β -Bgt (Eterović et al., 1975). Toxicological traits classified fractions 3 and 6 thru 8 as post-synaptic neurotoxins (" α -type") and fractions 9 thru 14 as pre-synaptic (" β -type"). Purity was determined by assay of enzymatic activities detectable in the venom (Lee et al., 1972) and by sodium dodecyl sulfate disc gel electrophoresis (Laemmli, 1970).

Our patterns for 4 enzyme activities (Table I) resembled that reported by Lee et al., (1974). One exception was acetylcholinesterase which had earlier been found to elute slightly before the α -Bgt peak but which we found to elute immediately after fraction 3(α -Bgt). (Clark et al., (1972) found AChE in both elution positions). No proteolytic or proteolytic inhibitor activities were found in the crude venom or in chromatographic fractions. A thrombin-like arginine esterase was detected in fraction 4+5 (so designated because of variable chromatographic resolution). After the CM-Sephadex separation, fractions 2 thru 7 were the most heavily contaminated by enzymes whereas fractions 9 thru 11 contained only traces of nucleotide pyrophosphatase and AChE. Further purification of fraction 3 from CM-Cellulose yielded pure α -Bgt which showed no activity for the enzymes tested. When 10-40 μ g of each fraction was subjected to SDS-electrophoresis on 7.5 or 10% gels, they displayed a high degree of homogeneity, with the exception of fractions 2 and 4+5. These latter fractions manifested multiple high molecular weight bands probably due to the enzymes

which had been detected by their activities (Table I). All other fractions were 90-100% low molecular weight material (Figure 1). High loading of the gels permitted detection of high molecular weight contaminants in some fractions, in agreement with the enzyme assays.

To eliminate high molecular weight contaminants, proteins were fractionated by gel filtration on Sephadex G50 (Figure 2). Fraction 3 contained a contaminant eluting at the void volume which was identified as AChE. Fractions 6, 7 and 8 also contained several components, including non-toxic material excluded from the gel at the void volume. A small tryptophan-containing peptide whose properties are consistent with an identified snake venom tripeptide (Kato et al, 1966); was present in fraction 6. The toxic components of 3, and 6 thru 8 all elute at identical volumes. Fraction 9 was separated into two components, one toxic (9A) and one non-toxic (9B). Fractions 12 thru 14 had varying degrees of contamination with proteins eluting both before and after the toxic components. In all cases, there was quantitative recovery of the applied protein. The toxins of fractions 10 thru 14 eluted in an identical position indicative of a uniformly higher molecular weight than for fractions 3, ^{and} 6 thru 8. Calibration of the preparative column with standard proteins indicated approximate molecular weights (Table II). The α -toxins eluted at a position calibrated at 15-16,000 daltons. To investigate whether this was attributable to dimerization under the mild conditions employed, 0.2M NaCl was incorporated into the column eluant. The elution positions were not altered. Indeed, identical calibrated weights were obtained by preparative scale chromatography on a 2.6 x 40 cm Bio-Gel P30 column. There was adsorption to Bio-Gel P30 since yields were reduced to 70-80% with pronounced trailing of peaks.

After removal of non-toxic high molecular weight materials, isoelectric focusing in polyacrylamide gel (Figure 3) revealed that several fractions were significantly cross-contaminated. A final fractionation step and gradient

elution was introduced using the cation exchanger Bio-Rex 70. Final fractions gave single bands on phenol: acetic acid: urea gels, SDS - urea gels, and upon isoelectric focusing.

Stability and Storage of Purified Toxins

Lethality was stable to extremes such as acid pH, high temperature, non-reducing denaturants and brief exposure to organic solvents. The native toxins are relatively resistant to proteolytic damage by chymotrypsin, trypsin, pepsin, carboxypeptidase, and aminopeptidases. They can be stored frozen or lyophilized. There is no tendency for the toxins to aggregate on freeze-drying (Karlsson et al, 1972) but the toxins do aggregate to less active forms with repeated freezing and thawing. They are very soluble at all pH's.

The toxic activities are susceptible to cystine cleavage by either reducing agents or basic pH. The β -type toxins are much more sensitive to alkaline damage and will denature above pH 10. The α -toxins, on the other hand, can be stored at up to pH 12 for short periods without damage. Alkaline denaturation is immediately detectable by an increase in turbidity indicative of reduced solubility. Control experiments showed that the toxins are resistant to cleavage by bacterial contaminants, but are readily deamidated with a concomitant change in the isoelectric point.

Isoelectric Focusing

The purified toxins were all highly basic proteins ($pI > 9$) (Table III). This was corroborated by eluting the unfixed basic proteins and testing for toxicity. Using calibrated time-to-death curves as a rough gauge of concentration, 75-90% of the applied protein could be recovered. In the presence of urea, multiple bands were observed after isoelectric focusing (Table III). This may reflect either natural variation in the "isotoxins" of each purified venom component or a carbamylation artifact. Storage of the β -type toxins in urea at pH 9 resulted in a rapid reduction in activity paralleled by a loss of free amino

groups as determined by ninhydrin. Consequently, it appears that the toxins are readily carbamylated (Stark et al, 1960). Although fresh recrystallized urea was used in all experiments, it is possible that the minor bands with lower pI's may be carbamylated derivatives.

Selective staining indicated that no purified component was a glyco- or lipoprotein.

Purity and Molecular Weight by SDS-PAGE Technique

In SDS-gel systems in the absence of a reducing agent, single bands were observed in all fractions, with the exception of fraction 14, which gave evidence of heterogeneity. With reduction, however, fractions 10 thru 13 split into two electrophoretic bands. Since fractions 10 thru 13 gave single electrophoretic bands in the strongly dissociative but non-reducing systems described above, the constituent chains may be maintained by inter-chain disulfides in the native molecule.

The initial results with SDS-gels (Figure 1) and gel electrofocusing (Fig. 3) suggested that these proteins might prove difficult to accurately analyze by conventional SDS-gel procedures since, like the anomalous histones (Panyim and Chalkley, 1971), they are small and highly basic. Thus, several more elaborate approaches were used. Estimates of the unreduced weight were obtained by calibration of a linear gradient SDS-slab gel. Although this procedure has limitations, we nevertheless obtained molecular weights consistent with other techniques. Fraction 7 was very similar to α -Bgt. On the other hand, fraction 8 appeared inordinately small by this procedure, approximately 5,000 daltons, which may be due to unreliability from inadequate standards. All of the β -types appeared to be 20-21,000 daltons in agreement with gel filtration. Running the toxins on identical gradients under reducing conditions

did not alter the size of fractions 7 and 8, indicating that they are clearly single polypeptide chains. Smaller chains were obtained in all the pre-synaptic toxin fractions. Fractions 10 thru 13 had chains of very similar weights; 7-8,000 daltons for the lighter and 11,500-12,500 daltons for the heavier. Fraction 9A and 14 appeared to be homodimers. Fraction 14 was notably more heterogeneous, giving three separate heavier bands with reduction.

The cross-linking procedure of Davies and Stark (1970) was employed to give a separate measure of oligomeric structure and it gave very similar results to those obtained using SDS-gels with the exception that the chain values were uniformly larger. This, however, is frequently observed with cross-linked proteins, even when calibrated against cross-linked standards (Davies and Stark, 1970). To reduce the overall charge on the cross-linked molecules (unchanged by DMS cross-linkages), they were reduced and carboxymethylated and again electrophoresed on disc gels with comparably treated standards. Values for the heavy and light chains in the β -type and the single chain of the α -type were all lower. These results are summarized in Table IV.

Amino Acid Composition

Table V summarizes the data of amino acid analyses. The method used to calculate results assumes good recoveries from acid hydrolysis, homogeneity, and a single tryptophan per mole of protein. Results obtained from 22 h methane sulfonic acid hydrolyses of the fractions agreed very closely with those obtained by HCl hydrolyses and gave independent evidence for the existence of a single tryptophan. The existence of a single tryptophan was originally indicated from the magnetic circular dichroism spectra (Hanley, Vickery, and Bennett, manuscript in preparation). Subsequent quantitative studies using NBS oxidation (Fig. 4), indicated a single tryptophan whose modification eradicated its

contribution to the fluorescence spectra (Fig. 4). The residual fluorescence was characteristic of tyrosine.

Except for fractions 8 and 13, molecular weights obtained from computer minima of the squared differences from integer values of the experimental amino acid composition agree well with the values from other techniques. From their basic isoelectric point, we predict that the bulk of the glutamic and aspartic acid residues are amidated. Ammonia was observed in all analyses; but even when corrected to zero time, these values were not considered reliable since the toxins were customarily purified in ammonium acetate buffers and residual ammonium after desalting could introduce a significant contribution.

Notable destruction of threonine, serine, cystine, and tyrosine was observed. Values were extrapolated to zero time and the tyrosine content was compared to independent values obtained from magnetic circular dichroism. We have given the MCD values preference in this determination. Even with correction, the serine and threonine values may still be low owing to their non-linear destruction kinetics in these proteins.

Alanine and methionine are absent from many Elapid toxins (Karlsson, 1973). Alanine was found in all the bungarotoxins representing 4-6 mole fraction percent. This alanine was present in higher concentrations than histidine, serine, valine, methionine, leucine, or phenylalanine. Methionine concentrations were the lowest of all the amino acids. There were consistently higher tyrosine-to-tryptophan ratios in the β -type than in the α -type toxins. Except for the high proportion of basic residues and the high cysteine content, the compositions of the toxins were very similar to those found for soluble, globular proteins (Jukes, et al, 1975).

Complete reduction and alkylation were difficult to achieve in the β -type toxins 9A thru 14. Long incubation times (24-36 h) with strong denaturants

(6M guanidine hydrochloride or 2M guanidine perchlorate) and a large molar excess of reducing agent were necessary. Carboxymethyl cysteine values after acid hydrolysis were low in several instances which may reflect the known acid hydrolytic instability of CM-amino acids. No evidence was obtained for reaction with lysines or histidines under these alkylating conditions. α -Type toxins could be reduced and alkylated satisfactorily under normal conditions.

N-terminal amino acid analyses gave the expected isoleucine for α -Bgt (Mebs et al., 1972; Clark et al., 1972), which was recovered as a mixture of isoleucine and its allo- isomer. Fractions 7 and 8 gave methionine as the N-terminal amino acid and all of fractions 9A thru 14 gave Asx as the N-terminal amino acid. No attempt was made to quantitate the N-terminal amino acid liberation and dansylated arginine and ϵ -lysine were observed at low levels in all samples.

Sulfur Determination by X-Ray Fluorescence

Examples of some typical spectra are given in Fig. 5. The silicon peak (Si) is caused by an impurity in the Lexan. The observed sensitivity for sulfur was of the order of 50 ng in a typical 4 min run. Each sample was run for a total of 12 min or more. A summary of the results is given in Table VI.

Absorption corrections were applied to the observed sulfur intensities to correct for differences between the standard Li_2SO_4 solution and the various estimated sample weights and compositions. A variation in weight of protein in the sample of a factor of 3 (from 15 to 50 μg), results in a correction of about 3%, which in most cases is less than the estimated uncertainty in the analyses. The corrections were estimated using the coefficients tabulated by McMaster et al., (1969). A more complete description of absorption corrections and sample preparations as applied to solutions when analyzing for the elements

from sodium to iron has been presented (Hebert and Bowman, 1975, Hebert and Street, 1974).

The X-ray fluorescence determinations served two purposes: 1) the determination of total protein sulfur, assumed to represent the cystine plus methionine content, and 2) the analysis of ionic contamination. The latter was of interest to judge the success of the desalting and to screen for the presence of any tightly bound ionic co-factors. There were no appreciable amounts of Na, Mg, Al, Si, P, Cl, K, or Ca in the samples.

The cysteine content was very high in these proteins, as with other snake venom neurotoxins (Tu, 1973). The high proportion of disulfides accounts for the resistance of these toxins to non-reducing denaturation and susceptibility to reduction (Eterović et al., 1975). Free sulfhydryl groups were not found in any fraction. The number of half-cystines plus methionine agrees within 10% of the amino acid analyses values for fractions 7 and 8. The values for fractions 9A and 14 agree within a single sulfur per protein. We found 16 half-cystines per 20,500 daltons for fraction 10 (β -Bgt) from both the X-ray and amino acid analyses. This value contrasts with the 20 half-cystines reported by Lee et al., (1972) and Kelly and Brown (1974). The larger number is very likely caused by contamination with fraction 11, which has 22 half-cystines. This is a fraction whose complete separation from β -Bgt is difficult and migrates identically in standard SDS-gel systems. Sulfur values from X-ray fluorescence for fractions 12 and 13 were considerably higher than the total sulfur from amino acid composition. We have chosen the X-ray values as correct, attributing the low cystine obtained after hydrolysis to incomplete compensation by the extrapolation to zero time. Corroboration for the higher cystine content in these fractions has come from the magnitude of a near UV circular dichroism disulfide signal which we have demonstrated to be linearly dependent on the cystine content (Hanley, Vickery, and Bennett, manuscript in preparation).

Extinction Coefficients

For the convenience of subsequent workers, we have determined the extinction coefficients of the various purified fractions using a magnetic circular dichroism technique (Hanley, Vickery, and Bennett, manuscript in preparation) and confirmed (within 3%) the results by quantitative amino acid analysis of samples with known absorption. These values are indicated in Table VII.

Immunodiffusion

Using antiserum to crude venom, purified components were screened for antigenic similarities by spur formation. Fraction 3 gave the reaction of partial identity with fractions 7 and 8. Fractions 7 and 8 gave the reaction of complete identity with each other. Fractions 3, 7, and 8 did not share determinants with fractions 9A thru 14. Fractions 9A and 14 gave the reaction of partial identity with fraction 10 thru 13. Fractions 10 thru 13 were immunologically identical to the reacting commercial antiserum.

DISCUSSION

The natural heterogeneity of the neurotoxins in Bungarus multicinctus venom makes it an ideal source for the purification of either post-synaptic (α -type) or pre-synaptic (β -type) toxins. α -Type bungarotoxin can be obtained in high yield and purity after only two chromatographic steps (Eterović et al., 1975). However, our experience with the other toxins has established that obtaining them in a homogeneous condition requires more extensive repurification. β -Bungarotoxin is a good example. Previous groups have reported amino acid compositions which differ in the amount of cystine and serine from that we reported here (Lee et al., 1972; Kelly and Brown, 1974). We attribute this to the cross-contamination of fraction 10 (β -Bgt) with fraction 11 since, in both cases, fraction 11 is not separated in the fractionation schemes. In the latter instance (Kelly and Brown, 1974), β -Bgt was declared "pure" after a single

chromatographic resolution of crude venom into only six components, which makes it rather likely that the properties described pertain to the family of our fraction 10 thru 13. More recently, Wernicke et al., (1975) reported a persistent contaminant of their β -Bgt preparation detectable on cellulose acetate electrophoresis. Donlon et al., (1975) have reported a " β -Bgt" that is the most basic fraction in the crude venom and has a molecular weight near 11,000 daltons. In both of these cases, the degree of contamination or identity of their purified component cannot be gauged without an amino acid analysis. The properties of the toxin studied by the latter group are very close to those of our fraction 14.

With improperly purified or identified toxins subsequent pharmacological study may be quantitatively, or indeed, qualitatively, compromised.

The purified toxins fall into two antigenic classes in correspondence with their pharmacological categories. The post-synaptic toxins 3 (α -Bgt), 7 and 8 have identical preparative Sephadex G50 elution volumes, calibrated to dimeric molecular weights. The incorporation of 0.2M NaCl did not alter this behavior; but it should be noted that Mebs et al., (1972) used 0.5M NaCl at pH 8.5 to determine the gel filtration weight of α -Bgt. Dimerization of toxins has been noted before, (Tu, 1973) and may be a consequence of the high concentration. Alternatively, the toxins may be sufficiently asymmetric so as to behave like a larger molecule owing to rapid rotation in solution. Some evidence for this asymmetry has been observed by X-ray diffraction studies of erabutoxin (Low et al., 1976).

Variations in the mobility of fractions 7 and 8 make it difficult to gauge their weights relative to that of α -Bgt or to judge whether any of these are behaving anomalously. For example, on SDS-gradient gels, fraction 8 has a

higher mobility than α -Bgt, but on phenol: acetic acid: urea gels and on SDS-urea gels it has lower mobilities. In view of the sensitivity of SDS-techniques to charge effects (Panyim and Chalkley, 1971), it is possible that this is the source of the variation. Consequently, the lower mobility of α -Bgt may be due to its higher pI than fraction 8. By all techniques, fraction 7 appears very near α -Bgt in weight (7800-8700 daltons by SDS-PAGE and 8300 daltons based upon amino acid composition). Fraction 8 appears smaller than α -Bgt by SDS-PAGE, but larger by amino acid composition. Fractions 7 and 8 are more like each other than like α -Bgt in composition. Sequencing studies of post-synaptic neurotoxins have established that they fall into two categories: 1) Type I or "short" neurotoxins (60-62 residues, 4 disulfides, 6800-7000 daltons), and 2) Type II or "long" neurotoxins (71-74 residues, 5 disulfides, 7800-8100 daltons) (Tü, 1973, Yang, 1974). Fractions 7 and 8 may be long neurotoxins by these criteria, but have the unusual N-terminal methionine. Their higher content of arginine and aspartic acid than in α -Bgt is more typical of the class of long neurotoxins than is the content in α -Bgt (Yang, 1974). It is possible that the final word on their similarities and differences to α -Bgt must await sequencing.

The second antigenic group consists of the pre-synaptic neurotoxins fractions 9A thru 14. Fractions 10 thru 13 resemble each other closely. They each have 1 tryptophan, the same N-terminal amino acid, similar isoelectric points, molecular weights, and comparable amino acid compositions (with the exception of increasing cystine content from fractions 10 thru 13). They can be separated into smaller chains, but only under reducing conditions. We have pursued the original suggestion of Kelly and Brown (1974) that the chain structure was maintained by inter-chain disulfides by trying a variety of dissociating conditions in both electrophoresis and chromatography (Hanley and Bennett, manuscript in preparation). Chains have been obtained only with reduction and

with the complete loss of biological activities. For this reason, we prefer to term these "chains" rather than sub-units, since they cannot, so far, be isolated with a retention of any activity. We now have evidence that the chain structure is maintained by a single disulfide between the light and heavy chains (Hanley and Bennett, manuscript in preparation). When fractions 10 thru 13 are reduced and alkylated, the light chain is not detectable in SDS-gel systems unless first cross-linked. Subsequent isolation of the light chain has established that it in turn may be composed of smaller chains, similar to crotoxin A (Horst, et al., 1972). It is refractory to N-terminal amino acid analysis, which explains the origin of the single N-terminal Asx in β -type fractions. We would predict that the molecular weight values obtained with reduced/alkylated/cross-linked chains would most nearly approximate the true weights and indeed, the amino acid composition of the isolated chains of β -Bgt has given weights of 15,000 for the heavy chain and 5500 for the light chain (Hanley and Bennett, manuscript in preparation). The N-terminal Asx arises from the heavy chain which we have established by tryptic mapping to be homologous to phospholipase a_2 enzymes (Eaker, 1975).

The amino acid compositions presented by Lee et al., (1972) and Kelly and Brown (1974) for β -Bgt differ from ours in the cystine and serine content, as noted earlier, and in the tryptophan content. We have established by methane-sulfonic acid hydrolysis, MCD, and NBS oxidation that there is a single tryptophan in each of the fractions 9 thru 14. This tryptophan is localized to the heavy chain (Hanley and Bennett, manuscript in preparation) and is partially buried, as indicated by its shift in fluorescence maximum (Eterović et al., 1975) towards 330 nm and in its relative inaccessibility to NBS (consuming 8 moles per tryptophan oxidized).

The molecular weight reported by Lee et al., (1972) for β -Bgt has been re-

calculated by us to be about 22,000 based on the amino acid composition. The "sub-unit" weights reported by Kelly and Brown for β -Bgt, 8800 and 12,400 are higher and lower respectively than our best values for these chains. On Sephadex G50, we have noted a clear separation of the chains, unlike the co-migration noted by Kelly and Brown on Sephadex G75. Kelly and Brown do not give an isoelectric point for their purified component, but their description suggests it is a weakly basic protein, which is clearly not the case. We have observed that applying the samples directly to the gels even under an ampholyte spacer, gives artifactual results in isoelectric focusing. For this reason, we have polymerized our samples into the gel. We have obtained identical results by either photo- or chemical polymerization or by using glycerol density-gradients to stabilize the focusing. Consequently, all of the β -type toxins are highly basic.

Fractions 9A and 14 are unusual in their toxicology (Eterović et al., 1975), structure, and antigenic properties. They resemble notexin, the sequenced pre-acid synaptic neurotoxin (Halpert and Eaker, 1975). The amino/compositions are compared in Table VIII. They are both single chains homologous to the heavy chains of the fractions 10 thru 13 (Hanley and Bennett, manuscript in preparation). The ambiguity in their pharmacological action (Dryden et al., 1974) may be attributable in the case of 9A to a failure to purify completely from small amounts of the post-synaptic toxin. Since fraction 9 marks the transition in the chromatographic profile from post- to pre-synaptic toxins, we have observed that as much as 30% of the material in fraction 9 can be post-synaptic contaminants after the initial fractionation step. Fraction 14, on the other hand, has an unusual pharmacology that is currently the subject of more extensive research. Both of these fractions exhibit the myotoxic effects of notexin (Hanley, unpublished observations).

We have omitted in our tabulation of the enzyme activities in the venom

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the endogenous phospholipase activities recently discovered in pre-synaptic neurotoxins (Halpert and Eaker, 1975; Howard, 1975; Strong et al., 1976). We have confined our attention to the non-neurotoxic phospholipase as an index of contamination. The occurrence and characteristics of this activity in the pre-synaptic fractions will be the subject of a future report. We have, however, indicated that the phospholipase structure is found on the heavy chain. The light chain may serve to confer preference for the nerve terminal membrane and in this way, act as an "affinity label". It may, on the other hand, not act as a recognition site, but rather as a structural modifier which alters the already toxic, notexin-like heavy chain secondary structure in a manner so as to recognize and attack nerve terminal phospholipids with greater selectivity.

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TABLE I: Enzymic Activities in Fractions of the Venom of Bungarus multicinctus

Fraction	Acetylcholin- esterase	Phospho- lipase A	Nucleotide pyrophospha- tase	Phosphomono- esterase	Hyaluronidase ^a	Arginine Esterase
	$\mu\text{moles hydrolyzed/ mg protein/ min}$			$\frac{\text{nmoles}}{\text{mg} \cdot \text{min}}$	$\frac{\mu\text{g}}{\text{mg} \cdot \text{min}}$	$\frac{\mu\text{moles/min}}{\text{mg protein}}$
2	12	12.3	0.1	6.7		
3	37	24.0	4.8	4.3		
α -Bgt ^b						
4+5	739	3.2	10.5	7.3		8.4
6	80		0.1	19.9		.5
7	2		*	17.3		
8	*		*	6.5		
9	*		*			
10	*		*			
11	**					
12	*				3.2	
13	*				6.0	
14	*				25.0	

^aHyaluronidase was inhibited by excess substrate. Optimum substrate concentrations varied with the concentration of enzyme and so did the ratio of enzyme to optimum substrate concentration and the specific activities. The numbers shown were obtained with 150 μg of protein per ml, and the substrate concentrations of 43, 144 and 192 $\mu\text{g/ml}$ respectively.

^b α -Bgt was purified from fraction 3 by gradient elution from CM-Cellulose.

* Trace activity. Absence of number indicates no detectable activity.

TABLE II: Summary of Gel Filtration Data of Bungarotoxins

Fraction	Neurotoxic ¹ Activity	Earlier Names ²		K _{av} ³		Apparent ⁴ Molecular Weight
		Lee	Dryden	Sephadex G50	Bio-Gel P30	
3 (α-Bgt)	Post	II ₂	5c	.28	.21	15-16,000
7	Post	III ₁	7	.28	.21	15-16,000
8	Post	III ₂	8	.28	.21	15-16,000
9A	Pre & Myotoxin	IV ₂	9	.28	.17	15-17,500
10 (β-Bgt)	Pre	V	10	.12	.14	20,21,000
11	Pre			.11	.13	20,500-21,500
12	Pre	VI	11	.10	.12	21-22,000
13	Pre	VII	13	.11	.13	20,500-21,500
14	Pre & Post	VIII	12	.08	.10	22-23,000

¹Activities defined by toxicology (Eterović et al., 1975) and pharmacological studies (Lee et al., 1972; Dryden et al., 1974). Pre: pre-synaptic neurotoxin, Post: post-synaptic neurotoxin

²From Lee et al., 1972; Dryden et al., 1974

³K_{av} is defined as $\frac{V_e - V_o}{V_t - V_o}$ where V_e is the elution volume of the peak concentration of a solute; V is the void volume (indicated by blue dextran); V_t is the total column bed volume (indicated by potassium dichromate) (Ackers, 1972)

⁴From calibration curve of K_{av} vs. (molecular weight) using chymotrypsinogen, myoglobin, lysozyme, ribonuclease, cytochrome c, pancreatic trypsin inhibitor, and insulin as standards.

TABLE III: Isoelectric Points of Bungarotoxins

<u>Fraction</u>	<u>No Urea</u>	<u>4M Urea</u>	
		<u>Major</u>	<u>Minor</u>
3	9.2	9.2	9.0
7	8.8	8.8	9.5, 9.3
8	9.0	9.0	9.2, 8.9, 8.7, 8.5, 7.8
9A	8.8	8.8	9.0
10	9.4	9.4	9.2
11	9.4	9.4	
12	9.3	9.3	9.5
13	9.2	9.2	9.7, 8.9
14	9.7	9.7	9.5, 9.3, 9.2

TABLE IV: Molecular Weights of Bungarotoxins and Their Constituent Polypeptide Chains

<u>Fraction</u>	<u>SDS Gradient¹ Slab Gel</u>	<u>Reduced/SDS¹ Gradient Slab Gel</u>	<u>DMS/SDS² Cylindrical Gels</u>	<u>Reduced/Alkylated² DMS/SDS Cylindrical Gels</u>
3	8000	8000	8400	8100
7	8000	8000	8700	7800
8	5000	5000	7000	6800
9A	21,000	11,500	16,000	14,500
10	21,500	8500 11,500	8500 18,000	6000 15,000
11	20,500	7000 11,500	9000 18,500	6800 15,000
12	21,000	7000 11,500	8400 18,000	7000 15,000
13	21,500	7000 12,500	7600 13,500	7200 14,000
14	21,500	11,300 12,000 12,500	11,500	11,000

¹Measured with gradients of acrylamide from 17.5-20% (2 experiments); 10-20% (1 experiment)

²Measured on 12% and 15% Laemmli (1970) SDS-gels

TABLE V: Amino Acid Composition of Bungarotoxins. Fractions 7 through 14.

	FRACTION 7		FRACTION 8		FRACTION 9A		FRACTION 10		FRACTION 11		FRACTION 12		FRACTION 13		FRACTION 14	
	Moles	Integer	Moles	Integer	Moles	Integer	Moles	Integer	Moles	Integer	Moles	Integer	Moles	Integer	Moles	Integer
	Mole		Mole		Mole		Mole		Mole		Mole		Mole		Mole	
LYS	5.23	5	6.42	6	7.90	8	13.18	13	14.32	14	15.77	16	18.88	19	12.92	13
HIS	1.15	1	1.34	1	2.77	3	4.82	5	5.79	6	6.06	5	6.25	6	3.21	3
ARG	4.77	5	5.76	6	10.82	11	14.05	14	14.11	14	15.12	15	17.26	17	8.71	9
ASP	8.50	9	8.91	9	18.13	18	23.01	23	22.27	22	22.77	23	23.86	24	12.08	12
THR	7.11	7	5.62	6	9.27	9	10.28	10	11.30	11	11.19	11	10.17	10	6.94	7
SER	3.44	4	3.79	4	4.40	4	3.42	4	6.93	7	6.83	7	5.97	6	5.32	5
GLU	5.10	5	5.98	6	8.39	8	12.29	12	11.82	12	12.09	12	13.21	13	7.85	8
PRO	5.00	5	1.89	2	5.28	5	6.94	7	8.18	8	7.84	8	9.82	10	6.07	6
GLY	4.55	5	2.97	3	12.63	13	17.72	18	15.97	16	16.09	16	16.29	16	8.20	8
ALA	3.26	3	2.15	2	9.24	9	11.38	11	10.87	11	10.64	11	11.35	11	5.92	6
Half-CYS	9.78	<u>10</u>	9.94	<u>10</u>	14.04	<u>14</u>	16.29	<u>16</u>	20.18	<u>22</u>	22.13	<u>30</u>	20.78	<u>26</u>	12.57	<u>12</u>
VAL	2.70	3	1.82	2	2.29	2	5.00	5	4.54	5	3.69	4	5.58	6	3.93	4
MET	1.00	1	.94	1	1.61	2	1.95	2	1.97	2	2.04	2	1.61	2	0.61	1
ILE	2.61	3	2.81	3	4.92	5	8.48	9	8.47	9	8.21	8	8.53	9	5.67	6
LEU	3.19	3	3.10	3	4.70	5	7.38	7 (8)	6.32	6	5.90	6	7.61	8	4.90	5
TYR	1.84	<u>2</u>	4.26	<u>4</u>	9.93	<u>10</u>	13.28	<u>14</u>	13.82	<u>14-15</u>	16.10	<u>16</u>	13.18	<u>14-15</u>	7.06	<u>9</u>
PHE	2.26	2	3.18	3	4.15	4	6.02	6	5.99	6	6.13	6	6.14	6	2.72	3
TRP	.95	1	.84	1	.98	1	.86	1	.92	1	.89	1	.92	1	.54	1
Total number of amino acids/mole	74		72		131		177		186		198		204		118	
Molecular Weight	8300		8500		15,100		20,400		21,500		23,000		23,700		13,800	
N-terminal	Met		Met		Asx		Asx		Asx		Asx		Asx		Asx	

Moles of amino acid per mole of protein were calculated from the μ moles of amino obtained from the analyses and the μ moles of protein hydrolyzed. This last value was calculated from A_{280} of the solution to be hydrolyzed and the ϵ_{280} of the respective protein. Values for half-cystine in frame were obtained by subtracting the values for methionine from total sulphur content obtained from X-ray fluorescence spectrum (Table VI). Values for tyrosine in frame were obtained from MCD spectra (Table VII). Values are extrapolated from at least one analysis at each time point; 20-22 hrs, 44 hrs and 68-72 hrs. Every fraction has more than one determination at the 20-22 hrs time point, including a methanesulfonic acid hydrolysis for tryptophan.

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TABLE VI: Determination of Sulfur Content of Bungarotoxins by
X-ray Fluorescence

Fraction	$\mu\text{g S} / \text{ml}^b$	moles S / mole protein ^a
α -Bgt	32.2 $\pm$ 1.1	10.4
7	35.0 $\pm$ 1.5	11.8
8	27.8 $\pm$ 1.0	10.2
9A	24.5 $\pm$ 1.6	16.3
10 (β -Bgt)	19.7 $\pm$ 0.5	17.8
11	27.0 $\pm$ 1.0	24.1
12	26.0 $\pm$ 0.9	31.6
13	26.8 $\pm$ 1.3	27.2
14	21.0 $\pm$ 0.9	13.1

^a Protein molar concentration was calculated from A_{280} and the extinction coefficient

^b For a solution with $A_{280} = 1.0$

TABLE VII: Tyrosine-tryptophan Ratios and Extinction Coefficients
of Bungarotoxins

Fraction	Tyrosine ^a Tryptophan	Integral Value	$\epsilon_{\max}^b$	0.1% A_{280}
			(mM-cm) ⁻¹	(cm ⁻¹)
α -Bgt	2.2	2	10.5	1.32
7	2.2	2	10.9	1.31
8	4.0	4	13.5	1.59
9A	10.2	10	21.3	1.41
10 (β -Bgt)	13.7	14	29.6	1.45
11	14.5	14-15	29.8	1.39
12	16.1	16	35.4	1.54
13	14.5	14-15	34.2	1.44
14	8.9	9	20.1	1.46

^a Method of calculation based on Barth et al., (1972)

^b Maximum was found to be 278-279 nm for the α -type fractions and 277-278 nm for the β -type fractions.

^c These values were calculated from the values for ϵ_{280} and molecular weight estimates from Table V

TABLE VIII: Comparisons with Bungarotoxin Amino Acid Compositions

	<u>α-Bgt</u>	<u>Fr.7</u>	<u>Fr.8</u>	<u>Fr.10</u> <u>β-Bgt</u>	<u>β-Bgt¹</u> <u>(Kelly)</u>	<u>β-Bgt²</u> <u>(Lee)</u>	<u>Notexin³</u>	<u>Fr.9A</u>	<u>Fr.14</u>
LYS	6	5	6	13	13	13	11	8	13
HIS	2	1	1	5	5	5	3	3	3
ARG	3	5	6	14	15	14	5	11	9
ASP	4	9	9	23	24	22	18	18	12
THR	7	7	6	10	12	12	3	9	7
SER	6	4	4	4	6	6	3	4	5
GLU	5	5	6	12	13	12	7	8	8
PRO	8	5	2	7	8	8	5	5	6
GLY	4	5	3	18	18	16	10	13	8
ALA	5	3	2	11	12	11	9	9	6
Half-CYS	10	10	10	16	20	20	14	14	12
VAL	5	3	2	5	4	4	4	2	4
MET	1	1	1	2	2	2	2	2	1
ILE	2	3	3	9	9	8	4	5	6
LEU	2	3	3	7	7	7	4	5	5
TYR	2	2	4	14	13	13	10	10	9
PHE	1	2	3	6	6	6	5	4	3
TRP	1	1	1	1	4	3	2	1	1
Total	74	74	72	177	191	182	119	131	118
Molecular Weight	7983	8350	8540	20,440	22,130	21,110	13,580	14,850	13,770

¹ Kelly and Brown (1974)

² Lee et al. (1972)

³ Halpert and Eaker (1975)

FIGURE LEGENDS

- Figure 1: Sodium dodecyl sulfate electrophoresis of protein fractions after first chromatographic step. Protein (20 μ g) was applied in 20 μ l of Laemmli solution (1970) for fractions 3 and 6. In all other fractions, 40 μ g was applied in 40 μ l Laemmli solution. These are 12% gels run at 4 ma/gel until the marker bromophenol blue had reached the gel end. Gels were stained and destained as in Methods.
- Figure 2: Gel filtration of fractions from first chromatographic step on Sephadex G50. Hatched peaks indicate the toxic components.
- Figure 3: Polyacrylamide gel isoelectric focusing of fractions after second chromatographic step, 40 μ g of each fraction was focused in 10% gels at 100v for 1 hour and then 200v for 2 hours. Gels were exhaustively fixed and rinsed in 10% TCA and stained as in Methods.
- Figure 4: Oxidation of α -Bgt (fraction 3) and β -Bgt (fraction 10) by N-bromosuccinimide and fluorescent spectra of purified products of reaction. After oxidation, proteins were desalted on Sephadex G25 and lyophilized. One hundred μ g of protein was used in all cases to obtain the spectra on a Hitachi-Perkin-Elmer MPF 2A with excitation at 286 nm and 6 nm slit widths. Oxidized samples were excited at 280 nm.

Figure 5: Some typical 4-min X-ray fluorescence spectra for a) LiSO_4 standard solution (58.3 μg sulfur/ml), b) protein fraction 10 (β -Bgt), H_2O rinse solution. The largest peak is due to scattered exciting radiation at an energy of 3 KeV from silver L X-rays. The vertical scale corresponds to 10^4 X-ray pulses per channel at full scale.

SODIUM DODECYL SULFATE ELECTROPHORESIS OF
BUNGAROTOXINS AFTER CM-SEPHADEX
CHROMATOGRAPHY

Fraction No.

XBB 759-6652

Fig. 1

XBB 763-2142-A

Fig. 3

(a)

(b)

(c)

XBB 756-4737

Fig. 5

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