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Journal of Chromatography A, 694 (1995) 492–497

JOURNAL OF CHROMATOGRAPHY A

Short communication

Resolution of isotoxins in the β-bungarotoxin family

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First received 27 September 1994; revised manuscript received 24 November 1994; accepted 25 November 1994

Abstract

Although only five isotoxins of the β-bungarotoxin family had been claimed, this work indicated the existence of more than sixteen isotoxins. Crude snake (*Bungarus multicinctus*) venom was divided into four main fractions by gel filtration on a Sephadex G-50 column. β-Bungarotoxin appeared in a major fraction that contained mainly the M_r 20 000 protein components. The fraction could be further resolved into eighteen peaks designated P1–P18 by HPLC on a Protein Pak SP 5PW column that was eluted with a linear gradient of 0.1–0.6 *M* CH_3COONa in 20 m*M* NaH_2PO_4–Na_2HPO_4 at pH 7.4. P3–P18 were demonstrated to be isotoxins of the β-bungarotoxin family. Results of protein sequencing for P8, P9 and P11, the three main isotoxins, confirmed that they shared a common phospholipase A_2 subunit, which was very similar to although not completely identical with the A1 chain reported previously.

1. Introduction

The β-bungarotoxin (β-BuTX) family consists of a group of isotoxins that constitute the main portion of snake (*Bungarus multicinctus*) venom. As the toxin is very specific and unique in the blockade of neuromuscular transmission [1–3], it has been exploited as an important tool in physiological, biochemical and pharamacological studies. The isotoxins of the β-BuTX family have a similar primary structure [4–6]. Each is composed of two subunits linked by a disulfide bond. One is an M_r 13 000 phospholipase A_2 (PLA_2) subunit and the other is an M_r 7000 non-PLA_2 subunit. As the isotoxins show a wide range of lethal potency, their separation to avoid contamination with one another is critical in order to correlate their structures with neurotoxicity. In this regard, the resolution of the protein components in crude venom has been attempted many times by chromatography on a CM-Sephadex column, but the reported chromatograms varied with the batch of venom [7–11]. It was often confusing to define clearly an isotoxin on the chromatogram and to compare the neurotoxicity of an isotoxin reported by

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0021-9673/95/$09.50
SSDI 0021-9673(94)01173-7

different groups. This complicates the elucidation of the structure–function relationship of β-BuTX. Here, we report the effective resolution of the isotoxins.

2. Experimental

2.1. Materials

Crude snake venom was supplied by Chen Hsin Tong Chemical (Taipei, Taiwan). CM-Sephadex C-25 and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). A Protein Pak SP 5PW column was purchased from Waters (Milford, MA, USA) and Chemcosorb-ODS-H from Chemco Scientific (Osaka, Japan). All chemicals were of analytical-reagent grade.

2.2. Purification of isotoxins in the β-BuTX family

Crude venom was fractionated by gel filtration on a Sephadex G-50 column in 0.05 *M* CH_3COONa at pH 5.0, and the fraction containing M_r 20 000 protein was further resolved by HPLC on an SP column which was washed with a linear gradient of 0.1–0.6 *M* CH_3COONa in 20 m*M* NaH_2PO_4–Na_2HPO_4 at pH 7.4. The elution was modified from our previous procedure [12].

2.3. Analysis

Protein at a concentration of 5 mg/ml in 0.1 *M* NH_4HCO_3 at pH 8.5 was digested with trypsin (protein:trypsin = 50:1, w/w) at 37°C for 24 h. The trypsin digests were reduced in 6 *M* guanidine hydrochloride–50 m*M* Tris buffer at pH 8.6 in the presence of 20 m*M* 2-mercaptoethanol at 60°C for 1 h. The reduced samples were alkylated in 20 m*M* 4-vinylpyridine in the dark at 25°C for 3 h [13]. The alkylated derivatives were resolved by HPLC on a C_{18} column (Chemcosorb-ODS-H, 7 μm, 250 × 4.6 mm I.D.).

The amino acid sequences of proteins were determined by automated Edman degradation with a gas-phase microsequencer (Model 477A protein sequencer with on-line Model 120A analyser; Applied Biosystems).

Antisera were collected from rabbits immunized with β_1-BuTX fraction which was purified by CM-Sephadex C-25 chromatography of crude venom [11,14]. Protein size was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% (w/v) gel slab (7 cm × 5 cm) in Tris–glycine buffer [15]. Electrophoresis was conducted at 60 V for 30 min and then at 120 V for 1 h. The protein bands were revealed when the gel was stained with Serva blue. Alternatively, proteins were transferred overnight from slab gels into nitrocellulose membranes by a diffusion method in phosphate-buffered saline (PBS) at pH 7.4. Blots were immunodetected by treatment with the antisera, followed by treatment with ^{125}I-labelled donkey anti-rabbit IgG antibody and fluorography.

3. Results

3.1. Resolution of isotoxins in the β-BuTX family

Crude venom was divided into four main fractions, denoted Fr. I–IV in Fig. 1, by gel filtration on a Sephadex G-50 column. Only Fr. II, which contained mainly the M_r 20 000 protein as tested by SDS-PAGE, gave a strong immunoaffinity to anti-β_1-BuTX. Taking into account the basicity of β-BuTX, we further resolved it by HPLC on an SP column into eighteen well defined peaks designated P1–P18, as shown in Fig. 2A. P1 and P2 were non-retarding fractions. P3–P18 were washed out from the column by a linear gradient of 0.1–0.6 *M* CH_3COONa at pH 7.4. Apparently, they were basic proteins: the longer the retention time of a protein, the higher may be its basicity. Under the same experimental conditions, the β_1-BuTX fraction obtained from CM-Sephadex C-25 chromatography of crude venom was resolved into three peaks (Fig. 2B), SP I–III, as defined previously [12]. Appar-

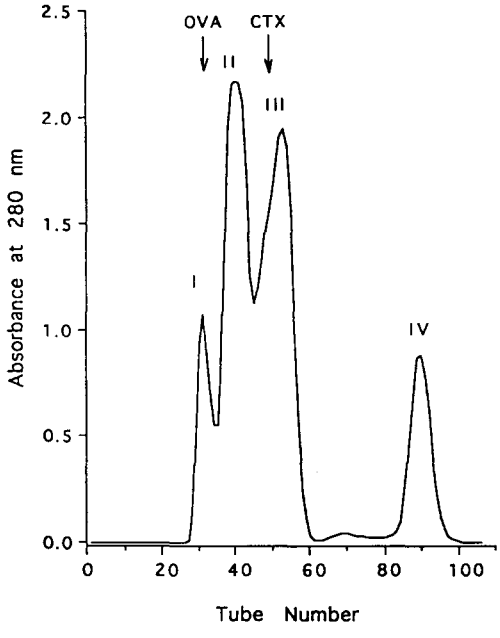

Fig. 1. Fractionation of crude venom by gel filtration. Crude venom (100 mg) in 2 ml of 0.05 *M* CH_3COONa at pH 5.0 was subjected to chromatography on a Sephadex G-50 column (110 × 1.6 cm I.D.). Fractions of 2 ml were collected and their absorbances at 280 nm recorded. The column was calibrated with ovoalbumin (OVA, M_r 45 000) and cobra cardiotoxin (CTX, M_r 7000).

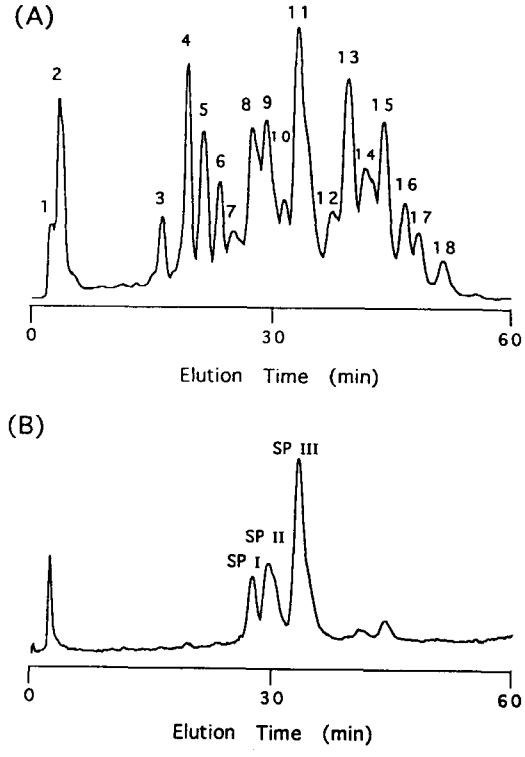

Fig. 2. Separation of isotoxins in the β-BuTX family. (A) Fr. II of Fig. 1 was resolved by HPLC on an SP column, which was washed with a linear gradient of 0.1–0.6 *M* CH_3COONa in 20 m*M* NaH_2PO_4–Na_2HPO_4 (pH 7.4) at a flow-rate of 1 ml/min. The effluent was monitored at 280 nm. Eighteen peaks are marked on the chromatogram. (B) β_1-BuTX fraction obtained from CM-Sephadex C-25 chromatography of crude venom was resolved by HPLC under the same experimental conditions as in (A).

ently, P8, P9 and P11 corresponded to SP I, SP II and SP III, respectively.

The protein samples were analysed by SDS-PAGE. Without reduction of their disulfide bonds (Fig. 3A), only one M_r 20 000 band was found in each of P7–P11 and P16–P18. There appeared one band slightly larger than M_r 20 000 in P4 and two bands around M_r 20 000 in P5, P6 and P12–P15. Traces of these two bands were detected in P1 and P2. P3 contained one M_r 20 000 band and one band smaller than M_r 20 000. Each of P3–P18 was divided into two bands, an M_r 13 000 band corresponding to a PLA_2 subunit and an M_r 7000 band corresponding to a non-PLA_2 subunit, when the protein disulfide bonds were reduced (Fig. 3B). As shown in Fig. 3C, both the M_r 20 000 band and the band slightly larger than M_r 20 000 in P3–P18 showed a strong immunoaffinity towards anti-β_1-BuTX (see Experimental), revealing that P3–P18 may share a similar antigenic determinant(s). This, together with the characteristics of SDS-PAGE shown above, supported the conclusion that P3–P18 are isotoxins of the β-BuTX family. The antisera showed a very weak affinity to the PLA_2 subunit and nearly no affinity to the non-PLA_2 subunit once they were separated by SDS-PAGE after the reduction of each isotoxin (data not shown).

3.2. *Identification of P8, P9 and P11*

Previous results from partial sequence of the N-terminal region analysis revealed that P8, P9

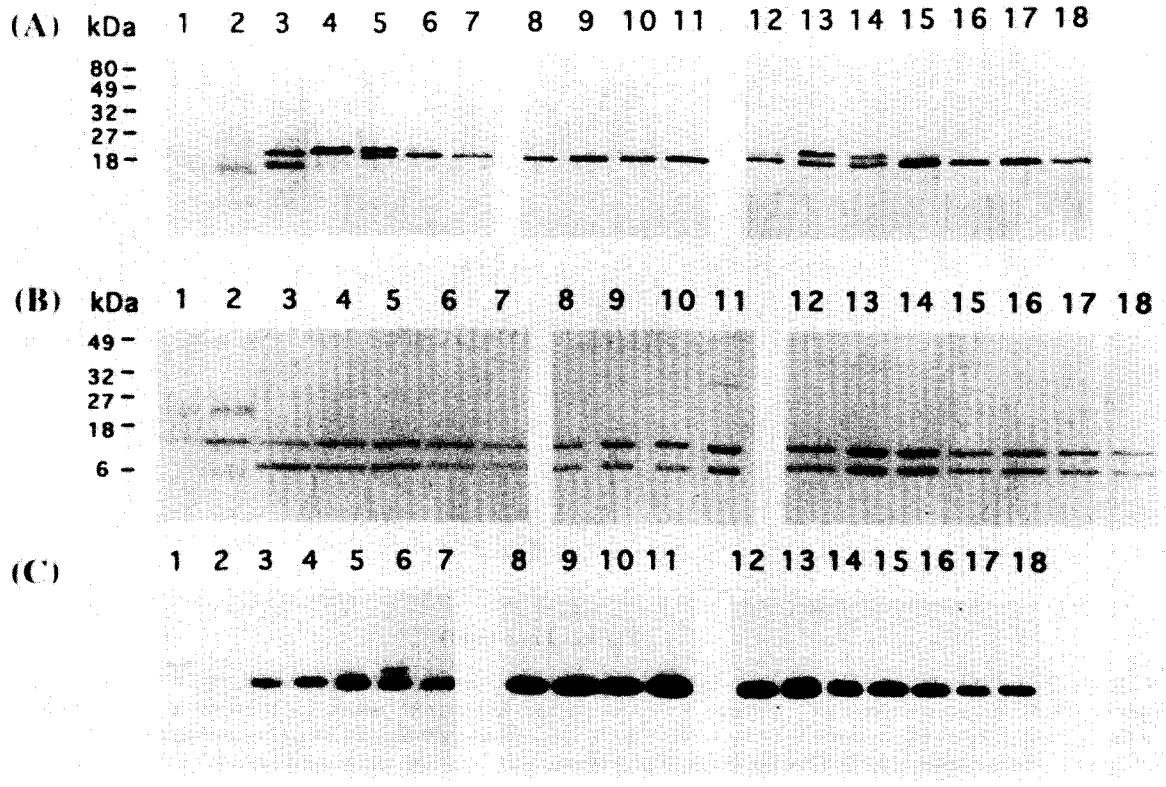

Fig. 3. The protein samples in Fig. 2A were resolved by SDS-PAGE (A) in the absence and (B) in the presence of 2-mercaptoethanol and the gels were stained with Serva blue. (C) The proteins in Fig. 2A were immunodetected by the Western blotting procedure with antisera against the β_1-BuTX fraction. The lane numbers correspond to the protein components denoted in Fig. 2A. kDa = kilodalton.

and P11 differed in their non-PLA_2 subunits and suggested that they might share a common PLA_2 subunit [12]. To corroborate the latter aspect, we determined the primary structures of their PLA_2 subunits. P8, P9 and P11 were rechromatographed on an SP column and were reductively alkylated. The two alkylated subunits of each protein could be separated by HPLC on a reversed-phase C_{18} column [12]. We found that trypsin digestion of the alkylated PLA_2 subunit of each protein remained problematic. Instead, we digested each intact protein with trypsin, followed by reductive alkylation of the trypsin digests with 4-vinylpyridine. The reaction mixture was resolved by HPLC on a reversed-phase C_{18} column (Fig. 4). The peptides of trypsin digests were randomly selected for amino acid sequence determination by automated Edman degradation. Only the peptides having partial sequences that could be aligned with the primary structure of PLA_2 subunit are summarized in Fig. 5. Peptides a–h appeared on the chromatograms of the three trypsin-digested samples. The primary structures of A1, A2, A3 and A2-like chain are also included in Fig. 5 for comparison. The partial sequences of these peptides in P11 confirmed residues 1–10, 11–17, 18–32, 65–71, 76–88, 89–95, 96–111 and 118–120 in the A1 chain reportd by Kondo and co-workers [4–6], except that $S^{66}Q$ and $Q^{103}SDY$ in the A1 chain were replaced by QS in peptide b and NSEY in peptide g, which are present in the A2-like chain deduced from a cDNA sequence [16] (Fig. 5). Nevertheless, the discrepancy between our de-

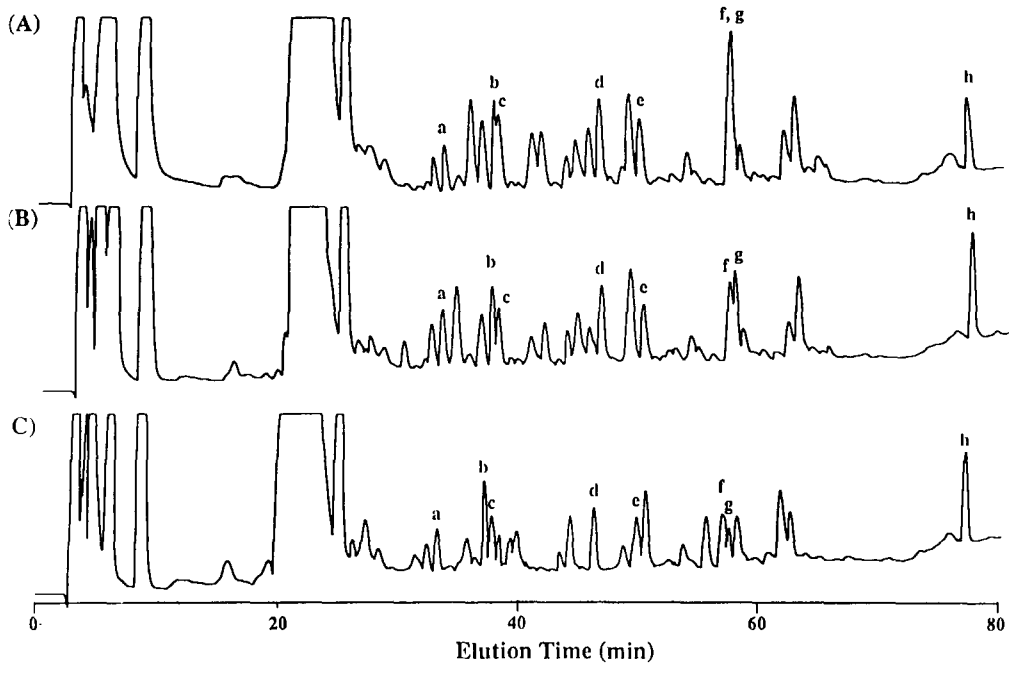

Fig. 4. Resolution of the alkylated trypsin digests of isotoxins. The alkylated derivatives of (A) P8, (B) P9 and (C) P11 were resolved by HPLC on a reversed-phase C_{18} column, which was washed consecutively with 0.1% trifluoroacetic acid (TFA) for 5 min, a linear gradient of 0–30% acetonitrile in 0.1% TFA for 60 min, a linear gradient of 30–60% acetonitrile in 0.1% TFA for 10 min and 60% acetonitrile in 0.1% TFA for 5 min. The flow-rate was 1 ml/min and the effluent was monitored at 214 nm. Peptides a–h of each protein sample were pooled for automated Edman degradation.

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          1         11        21        31        41        51
          :         :         :         :         :         :
A1        NLINFMEMIRYTIPCEKTWGEYADYGCYCGAGGSGRPIDALDRCCYVHDNCYGDAEKKHK
A2        ---------------------------------------------------------
A3        ----------------------T----------------------------------DAAIRD
A2-like   ---------------------------------------------------------

P8,P9,P11 NLINFMEMIRYTIPCEKTWGEYADYGCYCGAG
          <--- h --><- d-><------ f ------>

          61        71        81        91        101       111
          :         :         :         :         :         :
A1        CNPKTSQYSYKLTKRTIICYGAAGTCGRIVCDCDRTAALCFGQSDYIEGHKNIDTARFCQ
A2        -------------------------A-------------------R------K-H-R
A3        -------------------------A-V-----------------------------
A2-like   -----QS-------------------A---------------N-E---R------K-H-R

             **                                     * *
P8,P9,P11    TQSYSYK    TIICYGAAGTCGRIVCDCDRTAALCFGNSEYIEGHK       FCQ
             <- b ->    <------ e ------><- c -><------ g ------>    <a>
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Fig. 5. Partial sequences of peptides a–h in P8, P9 and P11. The corresponding peptides in the three protein samples showed no difference in their amino acid sequences. The partial sequences were aligned with the primary structure of the A1 chain. The primary structures of the A2, A3 and A2-like chains were also aligned with the A1 chain and the homologous residues are indicated by broken lines. The asterisks denote the sequences in peptides b and g, which disagree with the A1 chain but agree with the A2-like chain.

terminations and those of the A1 chain is minor. Peptides a–h of both P8 and P9 were proved to be the same as the corresponding peptides in P11, revealing that P8, P9 and P11 share a common PLA_2 subunit, which is most likely to be the A1 chain.

4. Discussion

This work is the first to give an effective resolution of isotoxins in the β-BuTX family. The results show two important structural features. First, there are more than sixteen isotoxins in the β-BuTX family. Previously, five types of PLA_2 subunit, A1, A2 and A3 chains reported earlier [4–6] and A2-like and A4 chains deduced recently from cDNAs [16,17], and two types of non-PLA_2 subunit, B1 and B2 chain [4–6], have been reported in the β-BuTX family. However, combination of these known subunits is insufficient to account for the formation of more than sixteen isotoxins. It turns out that more types of subunits should exist in the β-BuTX family. The gene regulation for the expression of these isotoxins and the reconstruction of subunits in the formation of isotoxins are of interest for future studies. Second, the two subunits of each isotoxin should interact to stabilize the antigenic determinant(s) of intact toxin. Apparently, the association of two subunits is essential to form an active three-dimensional structure. Therefore, separation of either subunit from the parent toxin in "native format" becomes a formidable task. Under these circumstances, correlation of the isotoxins with a common PLA_2 subunit but with different non-PLA_2 subunits, such as the situation with P8, P9 and P11 or vice versa, with their neurotoxicity may shed some light on the role of each subunit in the PLA_2 activity-dependent and the PLA_2 activity-independent neurotoxic effects, both of which are important in the blockade of neuromuscular transmission by β-BuTX [1,2].

Acknowledgement

This work was partially supported by the National Science Council, Taipei, Taiwan (Grant NSC 84-2311-B-001-069).

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