Stonustoxin Is a Novel Lethal Factor from Stonefish
(Synanceja horrida) Venom

cDNA CLONING AND CHARACTERIZATION*

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Stonustoxin (SNTX) is a multifunctional lethal protein isolated from venom elaborated by the stonefish, Synanceja horrida. It comprises two subunits, termed α and β, which have respective molecular masses of 71 and 79 kDa. SNTX elicits an array of biological responses both in vitro and in vivo, particularly a potent hypotension that appears to be mediated by the nitric oxide pathway. As a prelude to structure-function studies, we have isolated and sequenced cDNA clones encoding the α- and β-subunits of SNTX from a venom gland cDNA library. The deduced amino acid sequence of neither subunit shows significant homology with any known protein. Protein sequence alignment does, however, show the subunits to be 50% homologous to each other and implies that they may have arisen from a common ancestor. The subunits of this novel toxin lack typical N-terminal signal sequences commonly found in proteins that are secreted via the endoplasmic reticulum. Golgi apparatus pathway, indicating the possibility of its being secreted by a non-classical pathway, which is not clearly understood. The SNTX subunits have been expressed in Escherichia coli as clevable fusion proteins that cross-react with antibodies raised against the native toxin. To the best of our knowledge, this is the first complete sequence of a fish-derived protein toxin to be reported.

Stonefish are regarded as the most dangerous venomous fish in the world (1). Envenomation mediated by their venom apparatus elicits an array of symptoms, including an instantaneous and sharp pain at the site of the puncture wound, edema, hypotension, respiratory distress, convulsions, and death within 6 h (2–4). As with venoms produced by other organisms, stonefish venom is a mixture of enzymes and non-enzymatic proteins. Enzymatic activities detected in stonefish venom include hyaluronidase, alkaline phosphomonoesterase, 5'-nucleotidase, arginine amidase, arginine esterase, and proteinase activities (5). The hyaluronidase component from the venom of the stonefish Synanceja horrida has been purified (6). A proteinaceous lethal factor with a molecular weight of around 150,000 was first partially purified from the venom of S. horrida by Austin et al. (7). Subsequent to this, Deakin and Saunders (8) obtained a partially purified (10-fold) lethal factor from the same species. No further studies on this lethal protein were reported for the next 2 decades.

Recently, we purified a lethal toxin from the venom of S. horrida, indigenous to the shallow waters of the Indo-Pacific oceans and designated it stonustoxin (SNTX)1 (9). This toxin has a molecular weight of 148,000 and comprises two subunits, termed α (71 kDa) and β (79 kDa). It was found to be devoid of phospholipase A2, proteinase, and hyaluronidase activities, and had an LD50 (intravenous) of 17 ng/g (9). Lethal factors have also been isolated from the venoms of other species of stonefish that display comparable biological activities. These include a 158-kDa cytolytin with lethal properties purified from the venom of Synanceja trachynis (10), a 90-kDa monomeric lethal protein with hemolytic activity isolated from the venom of Synanceja verrucosa (11) and verrucotoxin, a 322-kDa tetrameric protein with lethal, cytolytic, and hypotensive activities, also isolated from the venom of S. verrucosa (12). All display LD50 values similar to that of SNTX.

Many of the symptoms associated with envenomation by S. horrida have been accredited to SNTX. It possesses edema-inducing, vascular permeability-increasing, and hemolytic (in vitro) activities (9, 13). SNTX is also a potent hypotensive agent in vivo, with doses of 20 ng/g causing a rapid and very marked fall in blood pressure from which rats fail to recover (13). Extremely low concentrations (0.1–1 nM) were also shown to induce endothelium-dependent vasorelaxation of isolated rat aorta. These in vitro vasorelaxant effects were abolished by the NO synthase inhibitor Nω-nitro-L-arginine methyl ester and the guanylyl cyclase inhibitor methylene blue. This suggests that SNTX's cogent vasorelaxant activity is mediated via the release of an endothelium-dependent relaxant factor, most probably NO or a NO-yielding substance (13). Similar mechanisms involving endothelium-derived relaxant factor also appear to mediate the endothelium dependent in vitro relaxant effects of melittin (14, 15), a 26-amino acid hemolytic peptide that is the main component of honey bee venom (16). At higher concentrations (50–330 nM), SNTX is myotoxic and irreversibly interferes with neuromuscular function. Based on these results, we concluded that marked hypotension is the primary cause of the toxin's lethality (17). Despite the growing pharmacological data, the toxin's detailed mechanism(s) of action at the molecular level remains to be elucidated.

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Cloning and Characterization of Stonustoxin

As a first step toward establishing structure-function relationships of this potent hypotensive toxin, we initiated molecular cloning studies at both the genomic and cDNA levels. In a recent paper, we described the characterization of a genomic region partially encoding the β-subunit of SNTX (18). The partial amino acid sequence of this subunit showed no homology to other known protein sequences, intimating that SNTX represents a novel class of protein toxins. In this paper we report the complete amino acid sequences of the α- and β-subunits of SNTX as determined through cloning and sequencing of the cDNAs encoding them.

**EXPERIMENTAL PROCEDURES**

cDNA Library Construction—Live stonefish (S. horrida) were purchased from local fishermen, and total RNA was isolated from excised venom glands using the guanidinium isothiocyanate extraction procedure described by Chirgwin et al. (19). Poly(A)+ RNA was isolated by oligo(dT)-cellulose chromatography (20). One µg of mRNA was transcribed into blunt-ended cDNA using a cDNA synthesis kit (Amerham) following the manufacturer’s instructions. The RibonClone EcoRI adaptor ligation system (Promega) was used to ligate EcoRI adaptors onto the 5′ and 3′ ends of the cDNA. This ligated cDNA was then cloned into the phage vector λgt10 (Promega). Recombinant phage DNA was packaged into viable phage particles using packaging extracts (Amerham) and plated on E. coli to generate the primary library (~5 × 10⁶ plaque-forming units/ml).

Screening for SNTX Subunit cDNAs—A previously described 1899-bp β-subunit genomic PCR fragment (18) was labeled with 32P by random priming (Amersham) and used to screen approximately 10,000 plaques for β-subunit cDNAs. Seven independent clones were identified and their DNA isolated using the DEAE-cellulose method of Helms et al. (21). The cDNA inserts were excised from phage DNA by EcoRI digestion, visualized by gel electrophoresis, and submitted to hybridization analysis with the genomic PCR fragment using the method of Southern (22). Two of the clones harboring the largest inserts (approximately 4.4 kb) yielded identical EcoRI restriction products, which hybridized analogously to the genomic PCR fragment. One of these was therefore chosen for further characterization by DNA sequencing.

PCR was used to amplify α-subunit related cDNA directly from the bacteriophage library using one sequence-specific primer and the λgt10 reverse primer. The sequence-specific primer was based on the fragment MPINQGY, generated via isolation and sequencing of tryptic peptides of the purified α-subunit. It was antisense so as to allow for amplification of coding regions 5′ to the tryptic fragment. It was degenerate and contained inosine at positions of high variance in the genetic code. Its sequence was: 5′-AA/GTA ICC (CTTTG/GAATT IAT IGG CAT-3′. PCR was carried out using 1 µl of the recombinant phage stock that had been heated at 96°–100 °C for 5 min as the template. The cycling parameters were 95 °C, 1 min; 50 °C, 1 min; 72 °C, 2 min. Reaction yielded several products, and one of these (approximately 500 bp) was purified for gel electrophoresis using the Geneclean procedure (Bio101) and subcloned into the TA vector pT7 Blue (Novagen). Sequencing of this fragment revealed it to encode a 148-amino acid polypeptide that showed 40% sequence homology to the β-subunit (amino acids 364–512 of the mature β-subunit protein). This PCR product was radiolabeled and used to screen approximately 10,000 plaques of the cDNA library. This yielded five independent clones. The DNA from these clones was purified as described above, and cDNA inserts were excised by EcoRI digestion and visualized by gel electrophoresis. Of the five clones isolated, two gave identical EcoRI restriction patterns. These clones also contained the largest inserts of approximately 3.2 kb. One of these was therefore chosen for further characterization by DNA sequencing.

Sequence Determination of cDNA Clones—Sequencing was carried out using the dideoxy chain termination method of Sanger et al. (23). All sequencing subclones were constructed in the vector pUC 19 using standard procedures (24) and sequenced on both strands with the forward and reverse M13 primers. Sequence-specific oligonucleotide primers (well DNA Service, Edinburgh, United Kingdom) were employed where necessary to ensure adequate overlap of sequences generated. DNA sequencing was carried out on an automated sequencer (Applied Biosystems Inc. (ABI), model 373A) using the manufacturer’s own protocol and reagents. Analysis of sequencing data was carried out using the Seqed program (ABI).

Reconstruction of Recombinant α- and β-Subunit Genes and Their Expression as Fusion Proteins—The pMAL protein fusion and expression system (New England Biolabs) was used for the expression of the toxin subunits as soluble fusion proteins. PCR was used to amplify the α- and β-subunit structural genes from the corresponding bacteriophage clones. The 5′ primers used were designed so as to incorporate the thrombin recognition sequence, whereas the 3′ primers provided unique XbaI sites and two stop codons. The primer sequences were: αF, 5′-CTG GTC CCG GGT GAA GCC CTT CCT TCA GAT GTA ATC-3′; αR, 5′-TAA TTC TAG ATT ATC AAA GTA TTC GAG TCC C-3′; βF, 5′-CTG GTC CCG GGT GAA GCC CTT CCT TCA GAT GTA ATC-3′; βR, 5′-GTG CCT ACT ATT ACA ATT TAA TGG CAT GAC C-3′. The PCR reactions were carried out as described above, using 1 µl of phage stock as template. The PCR products were extracted with phenol/chloroform and concentrated by ethanol precipitation. The reconstructed genes were blunt-ended by treatment with T4 DNA polymerase and then cloned with the restriction endonuclease XbaI after heat inactivation of the T4 DNA polymerase. They were then gel-purified and ligated to pMALc vector DNA restricted with XmnI and XhoI. This ligated the generated plasmids pFRo and pFRβ. After transformation into competent E. coli PR700 cells (New England Biolabs), individual clones were grown at 37 °C in liquid broth containing 100 µg/ml ampicillin until A600 reached ~0.6. Cell induction was initiated by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. Induced cells were left to express at room temperature for 1.5 h prior to harvest by centrifugation at 4 °C for 20 min at 5,000 g. The cell pellet was resuspended in column buffer (0.2 mM NaCl, 1 mM EDTA, 20 mM TrisCl, pH 7.4) and left overnight at −20 °C.

Purification of Fusion Proteins—After thawing of the frozen cell pellet, lysozyme was added to a final concentration of 0.25 mg/ml and the mixture was left on ice for 20 min. The protease inhibitor phenylmethanesulfonyl fluoride and benzamidine were added to final concentrations of 1 mM and the cells lysed by sonication. After centrifugation for 20 min at 10,000 g, crude extracts were applied to an amyllose affinity resin column (3 mg of fusion protein/ml of amyllose). Unbound proteins were eluted from the amyllose resin by washing with 10 volumes of column buffer. The bound α- and β-subunit fusion proteins were eluted by the addition of 1 M D-thiogalactoside. Prior to cleavage fusion proteins, CaCl2 was added to a final concentration of 2.5 mM. Fusion proteins were cleaved at 26 °C for 2–6 h by using thrombin (1% w/v, Sigma).

Isolation and Sequencing of Tryptic Fragments of the α-Subunit—The α- and β-subunits of SNTX were first separated by SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride-type membranes (ABI). Following staining and destaining, the bands were excised and subjected to in situ tryptic digestion (25). Typically, the digestion was performed in a volume of 50–100 µl at 37 °C for 24 h, and the enzyme: substrate ratio used was 1:20. The peptide extract was then fractionated by reverse phase HPLC using a RP-300 column (2.1 × 100 mm, Applied Biosystems) using 0.1% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid/0.5% acetonitrile (solvent B) eluents. Peptides were detected at 214 nm, and fractions were collected manually. Selected peptides were subjected to N-terminal sequencing using an Applied Biosystems model 477A sequencer.

Generation of Anti-SNTX Antibodies—Polyclonal anti-SNTX antibodies were obtained from rabbits by the subcutaneous injection of native SNTX (120 µg) emulsified in complete Freund’s adjuvant. Subsequent boosts used similar amounts of toxin in incomplete Freund’s adjuvant. Antibodies in the sera were capable of detecting 10–50 ng of SNTX as determined by Western analysis.

**Gel Electrophoresis, Immunoblotting, and Glycoestimation—**Proteins were analyzed by SDS-polyacrylamide gel electrophoresis on 8% gels and Coomassie Brilliant Blue staining. Immunoblot analysis was done by electroblotting proteins from the SDS gel onto a polyvinylidene difluoride membrane using established procedures (24). This was treated with total rabbit antiserum against native SNTX. The carbohydrate-specific periodic acid Schiff (PAS) staining method (26) was used to stain venom glycoproteins and purified SNTX after fractionation by SDS-PAGE. Ovalbumin (1 µg) and mucin (5 µg) were used as positive controls. After staining with PAS, the presence of other, non-glycosylated proteins was established by staining with Coomassie Brilliant Blue.

**Determination of SNTX Free Thiol Groups—**Thiol moieties present in the SNTX subunits were titrated with 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) as described by Habeeb (27). SNTX was purified from stonefish venom (9), and 450 µg was dissolved in 3 ml of denaturing solution (6 M guanidine hydrochloride, 1 mM EDTA, 0.1 M sodium phosphate buffer, pH 8.0). DTNB (10 nm, 0.1 ml) in 0.1 M sodium
phosphate buffer (pH 7.0) was added and the reaction incubated for 20 min at room temperature. The absorbance was read at 412 nm using a reagent blank. An extinction coefficient of 13,600 was used to calculate the number of free thiol groups/molecule of SNTX.

**Protein Estimation and N-terminal Sequencing**—Protein concentrations were calculated based on determined amino acid analysis data obtained using an Applied Biosystems model 420A derivatizer/aminoc acid analyzer. Protein sequencing blots were performed according to Matsudaira (28). N-terminal sequencing of proteins was carried out by Edman degradation on an Applied Biosystems model 470A automated protein sequencer coupled with a model 120A phenylthiohydantoin analyzer.

**RESULTS AND DISCUSSION**

SNTX is a novel protein toxin in that its primary means of lethality is a potent hypotension, which is endothelium-dependent and appears to be mediated by the NO pathway (17). As a first step toward gaining further information concerning structure-function relationships in SNTX, we have cloned α- and β-subunit cDNAs and have expressed these in E. coli.

**Isolation and Sequencing of cDNAs Encoding SNTX α- and β-Subunits**—Recently, we obtained a partial-length genomic clone, which enabled the determination of approximately 70% of the β-subunit’s amino acid sequence (18). This was used to screen approximately 10,000 plaques of the stonefish venom-gland cDNA library. Seven independent clones were isolated, and the sequence of the first 3040 bp of one of these harboring the largest insert size (approximately 4.4 kb) is shown in Fig. 1B.

In order to clone the α-subunit, a 500-bp cDNA fragment was first amplified by PCR from the venom-gland cDNA library. This was carried out using a primer based on the known amino acid sequence of an α-subunit tryptic digest peptide fragment and the Agt10 reverse primer. Sequencing of this fragment showed it to encode a 148-amino acid polypeptide that exhibited 40% homology with amino acids 364–512 of the deduced β-subunit sequence. Furthermore, the first deduced amino acid 5' to the peptide fragment on which the sequence-specific reverse primer was designed was Arg. This was to be expected, as the α-subunit peptide fragment was generated via a tryptic digest. Screening of the cDNA library with this PCR product yielded five independent clones, two of which gave identical EcoRI restriction products and contained the largest inserts (approximately 3.2 kb). The sequence of the first 2377 bp of one of these clones is shown in Fig. 1A.

**Analysis of cDNA Sequences**—The 5'-untranslated region of the β-subunit cDNA contains a stop codon in the same reading frame as the first initiation codon at position 61, following which 2100 bp encode a 700-amino acid protein before the first translation termination. The 3' region is about 1 kb long. The identity of the clone and the correct reading frame is established by the presence of four α-subunit tryptic peptides (underlined in Fig. 1A) present in the deduced amino acid sequence. The predicted α-subunit initiating methionine shared the nucleotide sequence features identified with the assumed β-subunit initiating methionine (Fig. 1, A and B). In addition, sequence alignment of the deduced α- and β-subunit sequences (Fig. 2) aligns this methionine with the one predicted to initiate translation in the β-subunit. The predicted initiating Met is followed by a serine, which as with alanine or glycine in the second position, favors removal of the initiator methionine and N-acetylation of the N-terminal amino acid (31, 32). Such acetylation would therefore account for the observed blocked N terminus of the α-subunit after removal of the initiator methionine (9).

The 3' regions of both α- and β-subunit cDNAs contain AT-rich regions (boxed in Fig. 1, A and B). Similar regions have been identified in the mRNAs of several cytokines and oncogenes that impart instability (33–35). It remains to be seen whether or not these sequences serve to destabilize the SNTX subunit mRNAs.

**Analysis of Deduced Amino Acid Sequences**—The deduced α- and β-subunit amino acid sequences (Fig. 1), respectively, contain 699 and 702 amino acids after removal of the putative initiating methionines. The β-subunit has a calculated molecular weight of 79,290. This value is in accordance with the value of 79,000 determined by SDS-PAGE and HPLC (9). The α-subunit has a predicted molecular weight of 79,388. This value differs somewhat from the 71-kDa value derived by SDS-PAGE and HPLC (9). The results of our expression studies (see below) suggest that the observed discrepancies is due to anomalous behavior of the protein in the methods thus far used to establish its molecular weight.

Comparison of the deduced subunit amino acid sequences with those of other proteins using the BLAST algorithm (36) revealed no significant homology and was indicative of SNTX being a novel protein. This further supports our earlier suggestion (18) that SNTX is a member of a new class of marine toxins. Sequence alignment of the α- and β-subunits (Fig. 2) shows them to be considerably homologous, displaying an overall identity of 50% and a similarity of 70%. Several insertions/deletions of 1 or 2 amino acids at various positions are also apparent in both subunits, and the nature of these infer that these two genes have probably evolved separately from a common ancestor after gene duplication and not one from the other.

**Glycosylation**—Potential Asn glycosylation sites are present in the deduced amino acid sequences of both the α-(6 sites) and β-subunits (2 sites). In order to see if these were actually glycosylated, both purified SNTX and crude venom were electrophoresed using SDS-PAGE and stained by the PAS staining method. Purified SNTX did not stain at all by PAS, and only a single band of approximately 60 kDa was stained in the crude venom (data not shown). This band corresponds to the venom hyaluronidase, which has been purified and shown to be a glycoprotein (6).

**Free Sulfhydryl Groups**—The deduced amino acid sequences of the SNTX subunits contain 15 cysteine residues (7 in α-subunit and 8 in β-subunit). The number of free thiol groups per molecule of SNTX were determined by titration with DTNB. This revealed the presence of 5 free cysteines/molecule of SNTX. The remaining 10 cysteines are therefore possibly involved in the formation of five disulfide bridges. SDS-PAGE analysis of SNTX under both reducing and non-reducing con-
ditions show that these are intrachain linkages (data not shown), indicating that the two subunits are associated via some non-covalent interaction. Alignment of the determined subunit amino acid sequences shows that 5 cysteine residues (shaded in Fig. 2) are conserved between them. This makes it possible for the retention of two identically placed disulfide bridges in each subunit, which may contribute to the formation of a conserved essential framework around which evolutionary modifications can be made.

Is SNTX Secreted by a Non-classical Pathway?—Several observations lead us to suggest that the secretion of SNTX differs from the classical model of eukaryotic protein secretion, which is typically mediated by N-terminal leader sequences (37, 38). Neither of the deduced subunit N-terminal sequences has characteristics of a typical signal peptide (39), lacking positively charged amino acid residues and a stretch of between 7 and 16 apolar residues. The deduced β-subunit sequence immediately following the predicted initiating Met matches that determined by direct sequencing of secreted protein collected from the venom gland's lumen. SNTX lacks modifications associated with proteins that are secreted via the ER-Golgi pathway. These include the absence of Asn-linked glycosylation at potential sites and the presence of free cysteines. In this respect SNTX is similar to several other proteins...
including factor XIIIa, interleukin 1-β, and thioredoxin (reviewed in Ref. 40). It has been postulated that these exit the cell through a novel secretory pathway that does not include the ER-Golgi apparatus. (d) Gopalakrishnakone and Gwee (41) carried out a detailed morphological study on the S. horrida venom gland. They reported that the venom-secreting cells were unique in that they did not possess Golgi apparatus and rough endoplasmic reticulum, features typically associated with protein-secreting cells. A novel, holocrinetype of secretion was suggested that differs from the classical model seen in snakes, scorpions, and spiders. Such a non-classical mode of secretion could therefore account for SNTX's observed lack of typical leader sequences and post-translational modifications associated with the ER-Golgi apparatus.

Predicted Cytolytic Site and Mechanism—The membrane-permeating ability of many peptides and proteins can be attributed to the presence of membrane-spanning hydrophobic segments or amphipathic α-helices and β-sheets (42, 43). The hydropathy profiles of the SNTX subunits (44) show them to be predominantly hydrophilic in nature with no potential membrane-spanning regions (data not shown). The cytolytic activity of SNTX might therefore be mediated via a "barrel-stave" mechanism, whereby membrane-spanning amphipathic α-helices present in toxin monomers aggregate to form pores (42). Since acationicsite flanked by a hydrophobic surface has been implicated in the cytolytic activity of several proteins and polypeptides (43), we searched the subunit sequences for the presence of amphipathic helices and cationic sites. The secondary structure prediction method of Garnier et al. (45) predicts the presence of four α-helices in the SNTX subunits that are at least 20 residues long (one in α-subunit, three in β-subunit). One of these is predicted between Val-293 and Leu-325 of the β-subunit, and construction of an "Edmunson wheel" (46) reveals this to be amphipathic, particularly for the first 20 amino acids (Val-293 to Asp-312) as shown in Fig. 3. An amphipathic α-helix is also predicted between Thr-274 and Leu-293 of the α-subunit (Fig. 3). The N terminus of this helix is flanked by a region that contains many basic residues (Lys-267, Arg-269, Arg-270, and His-272), which might represent a cationic site that is thought to be important in mediating the cytolytic activity of many toxins (43).

Expression of the SNTX Subunits as Fusion Proteins in E. coli—Our observation that the SNTX subunits lacked certain post-translational modifications associated with the ER-Golgi apparatus enforced the viability of their expression in a bacterial system. We therefore chose to individually express the subunits in E. coli as fusions with maltose-binding protein (MBP). PCR was used to reconstruct the cDNAs encoding the mature sequences of the SNTX subunits, thus allowing for their subcloning into the XmnI and XbaI sites of the expression vector pMALc. This placed each subunit in-frame, downstream of the MBP and factor Xa recognition sequences contained within the vector. As initial experiments resulted in the production of fusion proteins that were resistant to factor Xa cleavage (data not shown), the forward PCR primers were modified so as to incorporate sequences encoding the thrombin restriction site downstream from the factor Xa cleavage site. Bacterial lysates before and after induction were analyzed by SDS-PAGE and Coomassie Blue staining. Proteins with molecular weights of approximately 120,000 were expressed upon

Fig. 2. Sequence alignment for homology between SNTX α- and β-subunits. Sequences were aligned using the Seqed program (ABI). Single-letter amino acid notation is used. α and β represent the sequences of the SNTX α- and β-subunits. Amino acids are numbered beginning at the assumed N-terminal Pro for the β-subunit and the assumed N-terminal Ser residue for the α-subunit. Sets of identical amino acids are boxed, and conservative substitutions (49) are indicated by colons. Conserved cysteine residues are shaded.
predicted amphiphilic a-subunits. These are depicted by Edmunson helical wheels (46). A, Coomassie-stained gel of Laemmli 8% polyacrylamide gel of protein standards (lane 1). Lane 2, uninduced extract from E. coli harboring pJFα; lane 3, induced extract of E. coli harboring pJFβ; lane 4, uninduced extract from E. coli harboring pJFB; lane 5, induced extract from E. coli harboring pJFB; lane 6, affinity-purified a-subunit-MBP fusion protein (5 μg); lane 7, a-subunit-MBP fusion protein (5 μg) cleaved with thrombin; lane 8, affinity-purified β-subunit-MBP fusion protein (4 μg); lane 9, β-subunit-MBP fusion protein (4 μg) cleaved with thrombin; lane 10, native SNTX (4 μg). Arrows represent in order of decreasing molecular mass: 1) SNTX a- and β-subunit-MBP fusion proteins (~120 kDa, lanes 3, 5, 6, and 8); 2) recombinant and native SNTX β-subunit (~79 kDa, lanes 9 and 10); 3) recombinant and native SNTX a-subunit (~71 kDa, lanes 7 and 10); 4) MBP (~42 kDa, lanes 7 and 9). B, immunoblot of identical gel using rabbit anti-SNTX polyclonal antibodies. Arrows depict hybridization to the following proteins in order of decreasing molecular mass: 1) SNTX a- and β-subunit-MBP fusion proteins (~120 kDa, lanes 3, 5, 6, and 8); 2) recombinant and native SNTX β-subunit (~79 kDa, lanes 9 and 10); 3) recombinant and native SNTX a-subunit (~71 kDa, lanes 7 and 10); 4) product formed from nonspecific thrombin hydrolysis of β-subunit-MBP fusion protein (~50 kDa, lane 9).

**Fig. 3.** Predicted amphiphilic a-helices in SNTX a- and β-subunits. Two potential amphiphilic a-helices were predicted in the SNTX subunits. These are depicted by Edmunson helical wheels (46). A, predicted amphiphilic a-helix from Thr-274 to Leu-293 in SNTX a-subunit. B, predicted amphiphilic a-helix from Val-293 to Asp-312 in SNTX β-subunit.

induction (Fig. 4A, lanes 3 and 5), which were of the expected size (MBP, ~42 kDa; SNTX a- and β-subunits, ~79 kDa). Lysates from 1-liter cultures expressing each subunit were prepared by sonication after lysozyme treatment. After centrifugation, the supernatants containing the soluble fusion proteins were purified by affinity chromatography on amylose resin columns (Fig. 4A, lanes 6 and 8). This typically yielded between 3 and 5 mg of both a- and β-subunit fusion proteins/liter of culture. Cleavage of the fusion proteins was carried out by addition of thrombin (1% w/v). This resulted in liberation of the recombinant subunits from the MBP carriers (Fig. 4A, lanes 7 and 9). The migrations of the recombinant a- and β-subunits were similar to those of the native toxin (Fig. 4A, compare lanes 7 and 9 with lane 10). While having a predicted molecular weight of 79,388, the recombinant a-subunit migrated as a protein with an apparent molecular weight of approximately 71,000. This makes it likely that the molecular weight of the native a-subunit is in the order of 79,000. The recombinant SNTX subunits were further characterized by N-terminal sequencing. This showed the first 2 amino acids of the recombinant β-subunit to be the expected residual Gly and Ser from the thrombin cleavage site. The following 8 amino acids characterized were identical to those of the native β-subunit N-terminal sequence (9). The N-terminal sequence of the recombinant a-subunit agreed with that deduced from the cDNA. It also commenced with the expected Gly and Ser residues left over from the thrombin cleavage site. Western blotting of the recombinant proteins (Fig. 4B) using rabbit antibodies raised against native SNTX further confirmed the identity of the expressed proteins. Both a- and β-subunit fusion proteins (Fig. 4B, lanes 3, 5, 6, and 8) as well as the cleaved a- and β-subunits (Fig. 4B, lanes 7 and 9) showed hybridization to the anti-SNTX antibodies. This was more pronounced for the re-
combinant β-subunit, both as a fusion and when cleaved. Strong hybridization was also observed to an approximately 50-kDa fragment generated during cleavage of the β-subunit fusion protein (Fig. 4B, lane 9). This fragment may have arisen from non-specific thrombin cleavage (47) after Arg-460 in the potential site YLFRNL, along with that expected at the polylinker region. Its calculated molecular weight is 52,057. Further characterization of this band is currently being undertaken.

Ducancel et al. (48) reported that the fused sea-snake neurotoxin, erabutoxin-a, was more immunogenic than the native toxin. The SNTX fusion proteins might therefore have a possible use in the development of new antisera against stonefish envenomation. We have yet to establish whether or not they can elicit an immunological response. Their ability to do so would provide important immunological tools for further delineation of the biological roles of the individual SNTX subunits. We are currently purifying the recombinant subunits with the view to testing them for biological activity.

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