Original Article

Iranian Journal of Immunology

September 2025; 22(3):238-250.

Bioinformatic Analysis and Recombinant Expression of the Stonustoxin β-Subunit for Polyclonal Antibody Development

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ABSTRACT

Background: Stonefish (*Synanceia* spp.) are among the most venomous marine organisms. Their venom contains stonustoxin (SNTX), a heterodimeric toxin that induces severe hemolytic and myotoxic effects primarily mediated by its β-subunit.
Objective: To produce a recombinant SNTX β-subunit and develop neutralizing polyclonal antibodies against SNTX.
Methods: A DNA cassette encoding immunogenic regions of the β-*sntx* was designed using bioinformatics analysis, and codon-optimized for expression in *E. coli*. The construct was cloned into pET17b vector, and expressed in *E. coli* BL21 (DE3). The recombinant protein was purified via Ni-NTA affinity chromatography. For antibody production, rabbits were immunized subcutaneously with the recombinant protein emulsified in Freund's complete adjuvant, followed by booster doses at 2-week intervals. Antiserum was purified using protein G chromatography, and antibody titers were assessed by indirect Enzyme-Linked Immunosorbent Assay (ELISA).
Results: Epitope mapping revealed key immunogenic regions within residues 124–654 of the β-SNTX subunit. Following codon optimization, the codon adaptation index (CAI) increased to 0.93. Recombinant protein production was confirmed by SDS-PAGE and Western blot demonstrating successful purification of a 73 kDa recombinant protein (including TRX/His-tags), with a yield of 40 mg/L. Immunization of rabbits elicited a strong polyclonal IgG response, with antibody titers reaching 1:25,600 following the third booster. Purified IgG (1.8 mg/mL) exhibited high sensitivity in ELISA, detecting the recombinant β-SNTX at concentrations as low as 31.25 ng.
Conclusion: The recombinant β-SNTX subunit demonstrated strong immunogenicity, inducing high-affinity antibodies with specific binding activity against the native toxin. The resulting antiserum demonstrated significant neutralization potential, highlighting its promise for antivenom development.

Keywords: Fish Venoms, Stonustoxin, Recombinant protein, Polyclonal Antiserum, Antivenins

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Cite this article as:
Rahmani MR, Nasarian S, Samiei-Abianeh H, Aghaie SM, Sadeghi D. Bioinformatic Analysis and Recombinant Expression of the Stonustoxin β-Subunit for Polyclonal Antibody Development. *Iran J Immunol.* 2025; 22(3): 238-250,
doi: 10.22034/iji.2025.106646.3017.

Received: 2025-04-12
Revised: 2025-05-24
Accepted: 2025-08-05

INTRODUCTION

Fish represent one of the most diverse group of vertebrates, encompassing over 22,000 species— accounting for nearly half of all known vertebrates. Within this remarkable diversity, approximately 1,200 marine species are evolved venomous capabilities (1). Based on their venom apparatus and toxicological profiles, venomous fish are classified into three primary groups: *Pterois* (lionfish), *Scorpaena* (scorpionfish), and *Synanceia* (stonefish). All three groups possess specialized venom apparatus capable of causing severe local and systemic effects. Among them, stonefish are particularly noteworthy as the most venomous marine fish,, responsible for numerous cases of severe envenomation and human fatalities (2).

Stonefish can deliver venom with remarkable speed, completing envenomation in approximately 0.015 seconds during a successful strike (3). One of the striking physical features of the stonefish is its grooved dorsal spines. Typically, the venom apparatus of the stonefish consists of 12 to 13 needle-like dorsal spines, each with an average length of 15 cm, two pelvic blades, and three anal blades all covered by a main sheath (4). Apart from decorative breast thorns, all other spines are venomous. Each dorsal spine is linked to a pair of venom glands that are enclosed by a loose and thick sheath (5). Venom is secreted from the paired glands situated at the base of each spine and flows along the grooves on both sides of its anterior surface. When vertical pressure is applied to the dorsal spines, the spine tip punctures tissue, causing compression of the surrounding tissue around the venom glands. This mechanical pressure involuntarily forces venom through the spinal venom duct (6-8). Each pair of dorsal venom sacs contains approximately 5-10 mg of dry venom. The penetration depth of the spines is about 8 cm, allowing efficient delivery of venom into the victim's body. Following envenomation, the venom glands require several weeks to replenish their stores (9).

The venom is a complex mixture of proteins, including Stonustoxin (SNTX) from *Synanceia horrida* (10), verrucotoxin (VTX) and neo-VTX from *Synanceia verrucosa* (11), and trachynilysin (TLY) from *Synanceia trachynis* (10). These proteins exhibit both enzymatic and non-enzymatic activities (11). Enzymatic components include hyaluronidase, phosphatases, esterases, and acetylcholinesterase (12-14). Stonustoxin is a heterodimeric protein composed of α (79 kDa) and β (71 kDa) subunits, with a combined molecular mass of 148 kDa. It accounts for up to 9% of total venom protein content It is estimated to be 22 times more toxic than crude venom, with an LD_{50} of only 17 ng in mice (15).

SNTX exhibits potent hemolytic activity through the formation of hydrophilic pores, approximately 3.2 nm in diameter, and its activity is highly sensitive to changes in temperature and pH. In experimental models, SNTX administration at doses of 5–20 µg/kg induces a significant decrease in arterial pressure in anesthetized rats (16). Clinical manifestations of stonefish envenomation include respiratory distress, hemoptysis, tachypnea (up to 60 breaths per minute), tachycardia (heart rate ~140 beats per minute), and intense localized pain (17). SNTX also exerts neuromuscular toxicity, resulting in both local symptoms– erythema, edema, blistering, and tissue necrosis– and systemic complications such as delirium, convulsions, hypotension or hypertension, arrhythmia, and, in severe cases, cardiac failure (18).

Because antivenom remains the only effective treatment for stonefish envenomation, the development of safer and more efficient production methods is a critical priority. The use of recombinant proteins to elicit specific antibodies offers a rational and advantageous alternative to conventional antivenom derived from whole venom. This strategy offers several benefits, including enhanced safety, batch-to-batch consistency, and scalable production. This study aims to investigate the toxicological properties of recombinant β-Stonustoxin and evaluate its potential for neutralization. In the long term, this research may contribute to the development of therapeutic agents or antivenoms.

Bioinformatic Design of Recombinant Protein

The nucleotide and amino acid sequences of the β-sntx gene (accession numbers U32516.1 and Q91453) were retrieved in FASTA format from NCBI (https://www.ncbi.nlm.nih.gov/) and UniProt (https://www.uniprot.org/). Given the large size of the full-length protein and potential challenges in recombinant expression, B-cell epitope prediction was performed using IEDB tools (https://www.iedb.org/) to identify shorter, immunogenic fragments suitable for expression.

For expression and purification, a TRX fusion tag was added to the N-terminus and a His-tag to the C-terminus (before the stop codon). Restriction enzyme sites (*EcoRI* and *XhoI*) were incorporated using GeneScript's rare codon analysis tool. Codon optimization was carried out, with evaluation of GC content, codon usage bias, and codon adaptation index. RNA secondary structure was predicted and parameters such as minimum free energy and entropy were assessed using the ViennaRNA WebSuite (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) and mfold (https://www.unafold.org/mfold/applications/rna-folding-form.php) (19).

Physicochemical properties were estimated with Expasy ProtParam (https://web.expasy.org/protparam/) (20). Secondary and tertiary protein structures were predicted with the GOR IV method (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html), and I-TASSER (https://zhanggroup.org/I-TASSER/) (21). The modeled 3D structure was validated through ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) (22), PROCHECK (Ramachandran plot), ERRAT, and Verify3D all accessible via the SAVES v6.1 server (https://saves.mbi.ucla.edu/). Protein solubility and antigenicity were assessed using Protein-Sol (https://protein-sol.manchester.ac.uk/) (23), AntigenPro (https://scratch.proteomics.ics.uci.edu/), and VaxiJen (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). To predict host immune responses, an *in silico* immune simulation was conducted with the C-ImmSim server (https://wwwold.iac.rm.cnr.it/~filippo/projects/c-immsim-online.html). The protocol simulated four virtual injections administered at approximately 14-day intervals over a 60-day period, mirroring the experimental immunization schedule. Based on these integrated bioinformatics analyses, the optimized β-sntx gene fragment was synthesized by Shingene (China).

Transformation and screening of recombinant clones *E. coli* BL21 (DE3) bacteria were transformed using the calcium chloride (50 mM; Sigma-Aldrich, Cat. No. C4901) heat shock method. Transformed bacteria were plated on LB agar medium (Merck, Cat. No. 1.10285) supplemented with ampicillin for selection. To verify successful transformation, a single colony was picked and subjected to plasmid extraction by alkaline lysis. The presence of the target insert in the recombinant plasmid was confirmed by restriction enzyme digestion. The plasmid pET17b (Shingene, China) containing the gene of interest was digested with of EcoR1 (Thermo Scientific, Cat. No. ER0271) and Xho1 enzymes (Thermo Scientific, Cat. No. ER0691), corresponding to the engineered restriction sites. For digestion, 1 µL of plasmid, 5 µL of Tango buffer, and 1 µL of each restriction enzyme were combined in a 50 µL reaction volume and incubated at 37 °C for 1 hour. Digested products were analyzed by electrophoresis on a 1% agarose gel (Sinnaclon, Cat. No. EP5051). For protein expression, positive clones were cultured overnight, and 1 ml of the overnight culture was inoculated into 50 ml of LB broth containing ampicillin. When the bacterial culture reached an optical density of approximately OD_{600}=0.6, expression of the recombinant protein was induced with IPTG (Sinnaclon, Cat. No. CL5812) at a final concentration of 1 mM.

Cultures were incubated for 5 hours at 37 °C with shaking at 120 rpm. Cells were harvested by centrifugation at 6000 rpm for 5 min at 4 °C, and the bacterial pellet was collected for subsequent protein analysis.

Recombinant Protein Purification by Affinity Chromatography

The bacterial pellet obtained from the induced culture was resuspended in phosphate-buffered saline (PBS) containing 8 M urea to solubilize inclusion bodies. Cell lysis was performed by sonication at 70% amplitude for 30-seconds pulses, followed by incubation at room temperature with shaking at 140 rpm for 1 hour. The lysate was then clarified by centrifugation at 13,000 rpm for 15 minutes. The resulting supernatant was applied onto a pre-equilibrated Ni-NTA gravity-flow column packed with Ni-NTA resin (ARG Biotech, Iran Cat. No. Ni-S60). The column was washed extensively with wash buffer (100 mM NaH2PO4, 10 mM Tris-Cl, pH 8.0) to remove non-specifically bound proteins. Stepwise elution was then performed using wash buffer containing increasing imidazole concentrations (10 mM, 20 mM, and 50 mM) to remove weakly bound contaminants. The target protein was finally eluted using buffer supplemented with 250 mM imidazole, with an on-column refolding strategy to promote correct protein folding. After elution, the imidazole was removed by dialysis against phosphate-buffered saline (PBS) at 4 °C to obtain the refolded protein in a native-like state suitable for downstream applications. Purification efficiency was assessed by analyzing 20 µL aliquots collected before and after purification via 12% SDS-PAGE. Protein concentration was determined using the Bradford method, with bovine serum albumin (BSA) as the standard. A standard curve was generated using serial dilutions of BSA (0.2-1.0 mg/mL) for accurate quantification.

Confirmation of Recombinant Protein

Western blotting was conducted using an anti-His antibody to confirm expression of the recombinant protein. After SDS-PAGE, proteins were transferred electrophoretically onto a nitrocellulose membrane using transfer buffer containing 39 mM glycine, 48 mM Tris-base, 0.037% SDS, and 20% methanol. To block non-specific binding, the membrane was incubated overnight at 4 °C in PBST containing 5% skimmed milk. The next day, it was washed three times with PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 0.05% Tween-20) and incubated for 1 hour at 37 °C with an HRP-conjugated anti-6xHis antibody (dilution 1:3000; Sigma, USA). Following an additional three washes with PBST, protein detection was carried out using an HRP staining solution (3, 3'-diaminobenzidine + H2O2). Colorimetric development confirmed the presence of the His-tagged recombinant protein and the reaction was terminated by rinsing with distilled water. Bovine Serum Albumin (BSA) served as a negative control to assess antibody specificity.

Immunization and Antibody Detection

For the immunization experiments, two female New Zealand White rabbits (~1800 g) were obtained from the Razi Institute (Tehran, Iran) and housed under standardized, pathogen-free conditions at the Animal Care Facility of Imam Hossein University. All procedures were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals. Each rabbit was immunized subcutaneously with 100 µg of purified recombinant protein of purified recombinant protein emulsified in 200 µL of Freund's adjuvant (Pasteur Institute, Iran) (24). A total of four injections were administered at 14-day intervals, following a standard immunization protocol (24), with complete Freund's adjuvant used for the primary dose and incomplete Freund's adjuvant for the booster doses. Blood samples were collected from the marginal ear vein seven days after each injection. To separate the serum, samples were incubated at 37 °C for 1 hour, centrifuged at 3000 rpm for 10 minutes at 4 °C, and stored at –20 °C until further analysis.

Antibody titers were evaluated using indirect ELISA. Microplates were coated overnight at 4 °C with 5 µg of antigen in coating buffer. After washing with PBST, wells were blocked with 5% non-fat dry milk for 1 hour at 37 °C. Serial dilutions (1:100 to 1:256,000) of immune and control sera were added and incubated for 1 hour at 37 °C. Plates were washed, followed by the addition of HRP-conjugated anti-rabbit IgG (incubated for 1.5 hours at 37 °C), and color development using OPD substrate. The reaction was stopped with H_2SO_4, and absorbance was measured at 495 nm.

Statistical Analysis

Data normality was assessed using the Kolmogorov–Smirnov test. Comparisons of antibody responses across the second, third, and fourth injection time points were performed using Student's t-test. All analyses were conducted using SPSS version 29 (SPSS, IBM Corp., Armonk, NY, USA).

RESULTS

Bioinformatics Design and Confirmation of Genetic Structure

The full-length β-sntx protein sequence (700 amino acids) was obtained from the UniProt database and analyzed for linear B-cell epitopes using the BepiPred server. Its three-dimensional structure, available in the Protein Data Bank (PDB ID: 4WVM), was used to identify discontinuous B-cell epitopes via the ElliPro server. Based on the overlap of linear and conformational epitopes, the region spanning amino acids 124–654 was selected for further study (Fig. 1).

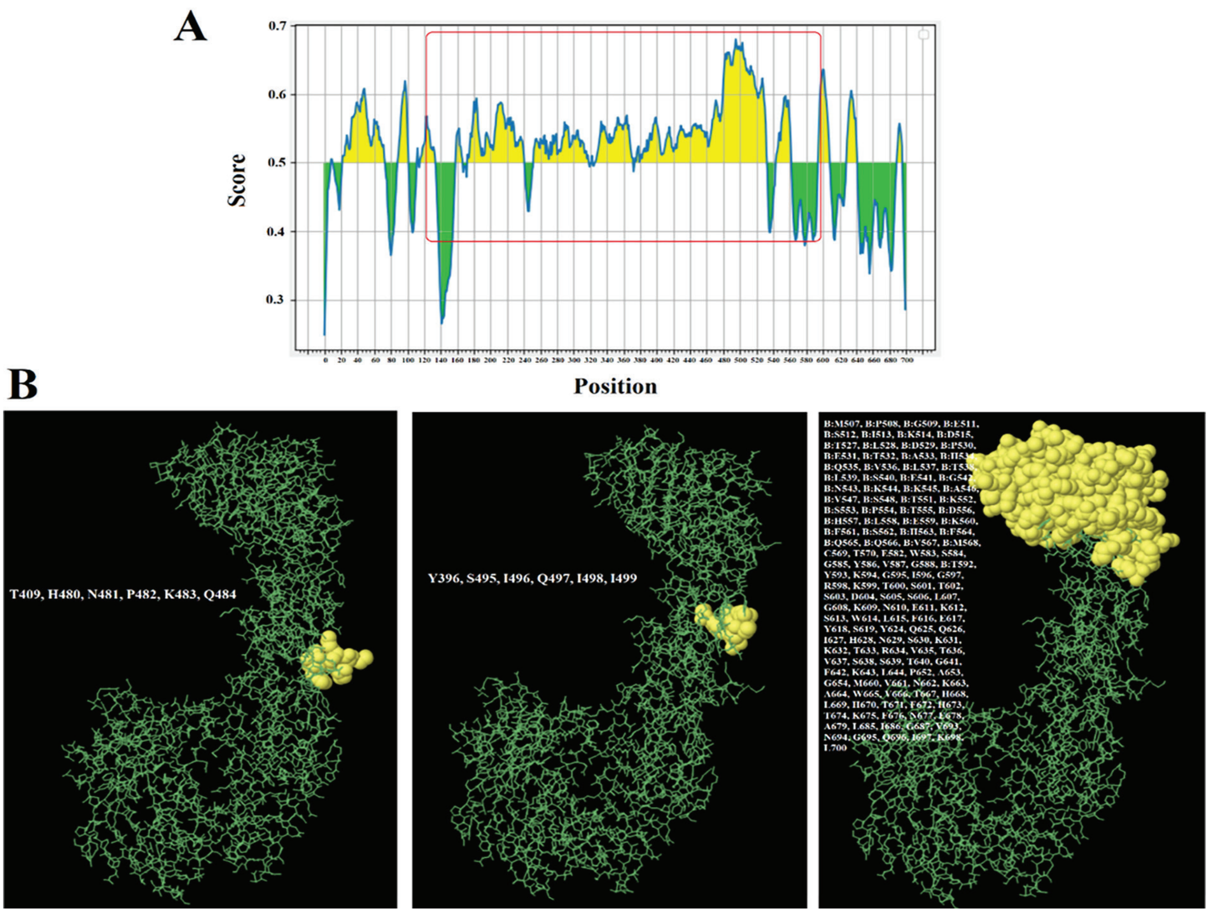

Fig. 1. Computational prediction of linear and conformational B-cell epitopes in the β-SNTx protein. (A) Linear B-cell epitope predicted using the BepiPred algorithm. The red rectangle indicates the antigenic spanning residues (amino acids 124–654) selected for experimental validation. (B) Discontinuous (conformational) epitopes predicted with ElliPro, highlighting the top- surface-exposed regions (colored patches). In both panels, the full-length β-SNTx protein is shown as green cartoon representation.

Following the selection of a suitable region from the β-stonustoxin subunit (531 amino acids in length), a codon-optimized nucleotide sequence was designed. To enhance expression and solubility, a 6×His tag was added to the C-terminus, and the *TRXA* fusion tag (108 amino acids) was fused at the N-terminus. Codon optimization increased the GC content to 44.16% and the codon adaptation index (CAI) to 0.93. mRNA secondary structure was predicted using the mfold server, indicating a favorable minimum free energy (ΔG) of –479.16 kcal/mol (Fig. 2). Physicochemical properties and solubility in *E. coli* were predicted using ProtParam and Protein-sol, respectively, with antigenicity scores summarized in Table 1. The predicted 3D structure of the recombinant β-sntx protein (rST) is shown in Fig. 3, with a ProSA Z-score of -11.5, indicating good structural quality and similarity to native proteins. The overall quality factor predicted by ERRAT was 92.93, indicating a high level of structural reliability. VERIFY3D analysis showed that 75.66% of the residues had an average 3D-1D score ≥ 0.1. *In silico* immune simulation using C-ImmSim revealed a robust and increasing antibody response over the 60-day immunization period, with four antigen injections administered at ~14-day intervals.

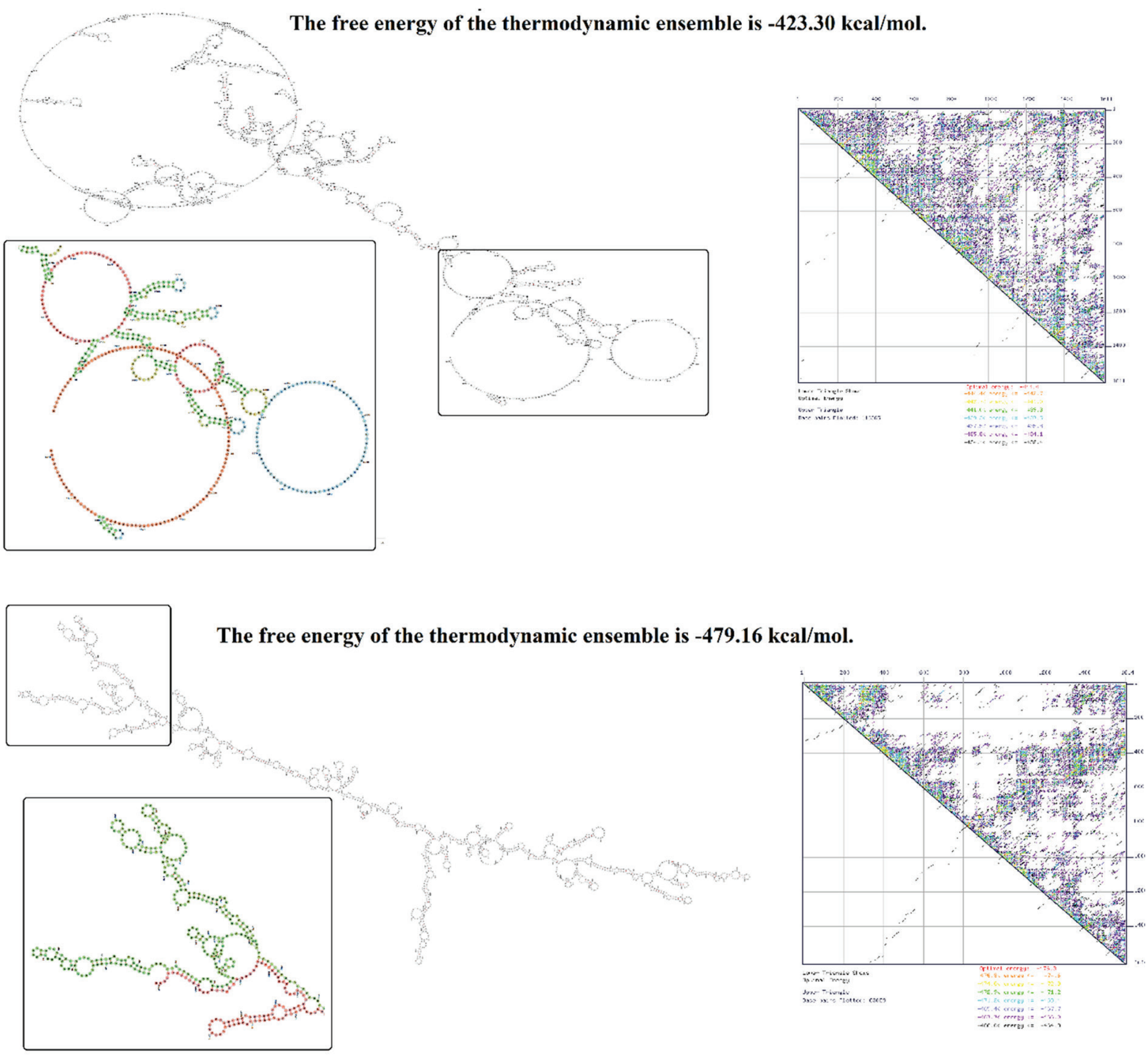

Fig. 2. Predicted mRNA secondary structures before and after codon optimization. The top panel shows the non-optimized mRNA structure with a minimum free energy (ΔG) of –423.30 kcal/mol. The bottom panel depicts the codon-optimized mRNA, demonstrating improved thermodynamic stability with a lower ΔG of –479.16 kcal/mol. In both panels, the 5′ initiation region is highlighted and enlarged for emphasis.

Table 1. Analysis of physicochemical properties, solubility, and antigenicity of TRX, β-sntx, and recombinant β-sntx (rST).

Parameter	TRX	β-sntx	rST
Number of amino acids	108	531	645
Molecular weight (Da)	11675.43	60680.90	73161.16
Theoretical pI	4.67	5.88	5.83
Negatively charged residues	16	68	84
Positively charged residues	11	59	70
Estimated half-life	>10 h (in vivo)* 1.9 h (in vitro)*	>10 h (in vivo) 7.2 h (in vitro)	>10 h (in vivo) 1.9 h (in vitro)
Instability index	4.07 (stable)	42.79 (unstable)	36.01 (stable)
Aliphatic index	103.98	76.14	80.09
Grand average of hydropathicity (GRAVY) (Hydropathicity index)	0.007 (hydrophilic)	-0.433 (hydrophilic)	-0.385 (hydrophilic)
Solubility score (Protein-sol)	0.762 (high)	0.413 (moderate)	0.439 (moderate)
Antigenicity score (VaxiJen)	0.3771 (non-antigenic)	0.4477 (antigenic)	0.4403 (antigenic)

*In vivo: In Escherichia coli, in vitro: in mammalian reticulocytes

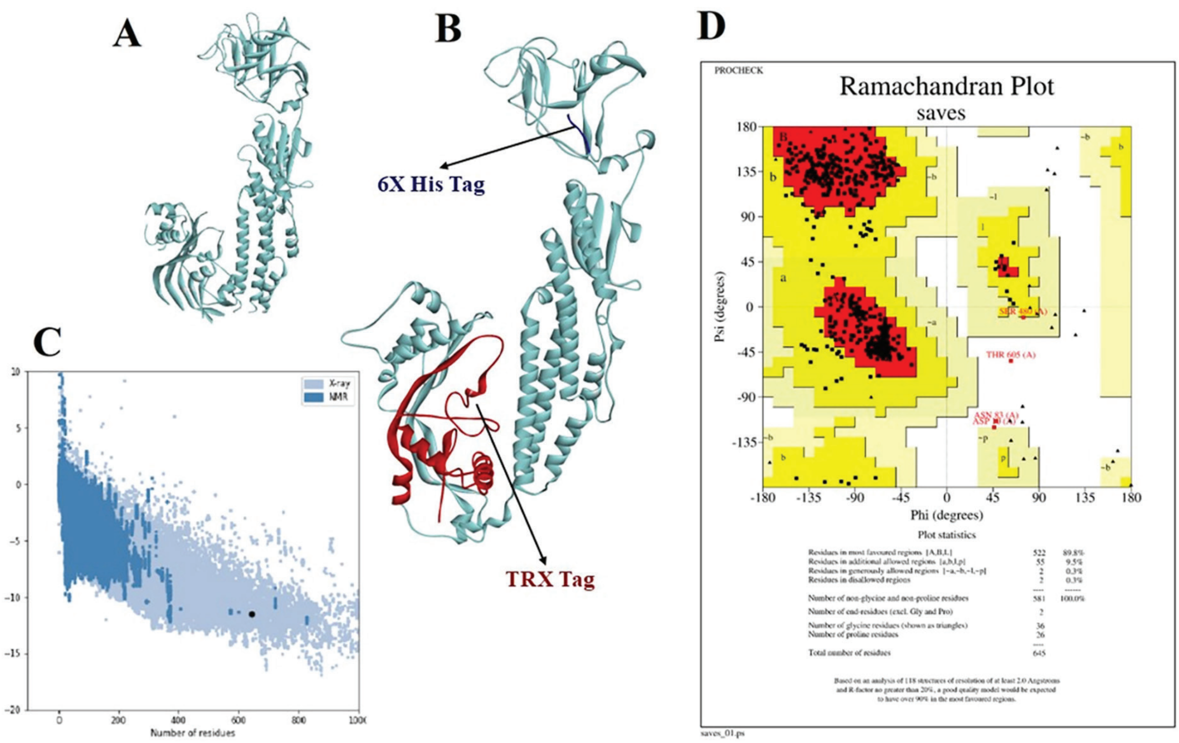

Fig. 3. Prediction and validation of the 3D structure of the recombinant rST protein. (A) Crystal structure of native Stonustoxin (PDB ID: 4WVM) determined by X-ray diffraction. (B) Predicted 3D model of rST with the TRX tag highlighted in red and the 6×His tag in blue. (C) ProSA Z-score plot of the rST model, showing a ProSA Z-score of -11.5, indicative of good structural quality and similarity to native proteins. (D) Ramachandran plot analysis of the predicted rST model, with 89.8% of residues in favored regions and only 0.3% in disallowed regions, supporting overall model reliability.

As shown in Fig. 4, total antibody titers (IgM + IgG) increased progressively with each dose.

Following synthesis, the gene encoding rST was cloned into the pET17b expression vector containing a TRX tag for enhanced solubility. The correct insertion of the 1614 bp construct—encoding the 531-amino-acid β-sntx sequence and a C-terminal 6×His tag—was confirmed by restriction digestion with *EcoRI* and *XhoI*, targeting the 5′ and 3′ ends, respectively (Fig. 5).

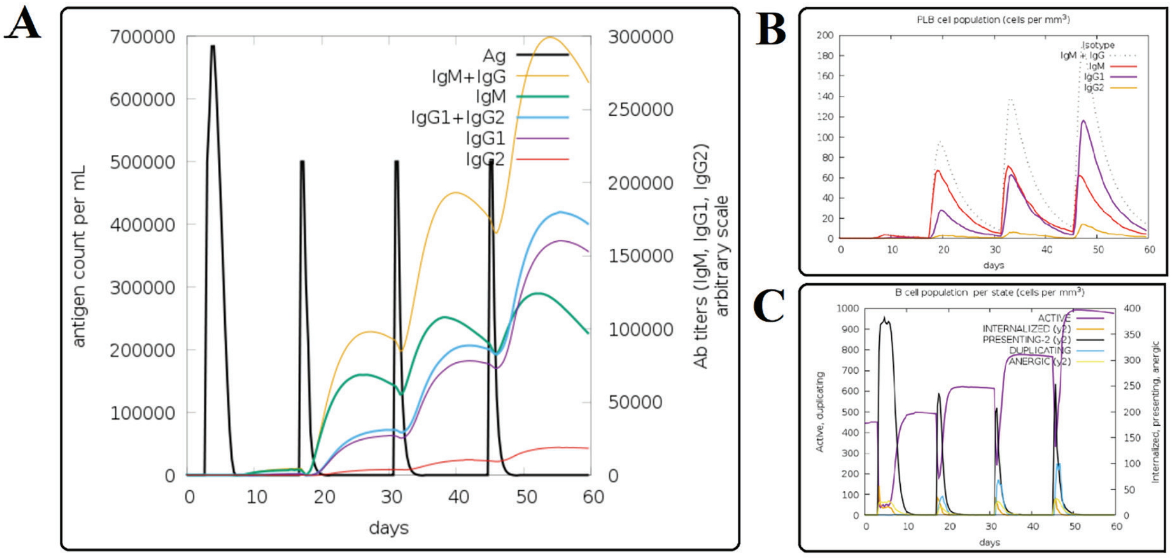

Fig. 4. *In silico* immune response simulation of rST using the C-ImmSim server. A) Antigen concentration and antibody titers over time. B) Plasma B lymphocyte population dynamics, sub-divided by isotype (IgM, IgG1, IgG2). C) B cell population according to functional state, including active, duplicating, anergic, internalized-antigen, and MHC class II-presenting cells.

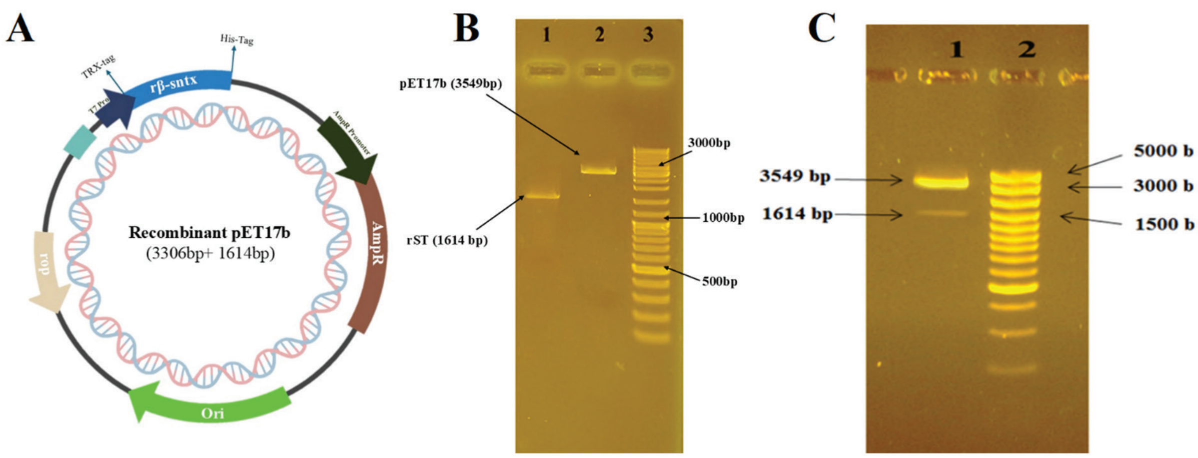

Fig. 5. Confirmation of recombinant β-sntx (rβ-sntx) gene cloning into pET17b vector. (A) Schematic representation of the recombinant pET17b plasmid containing the rβ-sntx gene, with an N-terminal TRX tag and a C-terminal 6×His tag. (B) Restriction digestion showing the rST insert (lane 1, 1614 bp) and the pET17b plasmid (lane 2, 3549 bp); lane 3: DNA ladder. (C) Double digestion of the recombinant pET17b–rST plasmid displaying the expected bands for vector and insert (lane 1); lane 2: DNA ladder.

Expression, Purification, and Confirmation of Recombinant Antigen

Recombinant protein expression was induced in *E. coli* BL21 (DE3) using 1 mM IPTG for 5 hours at 37°C. SDS-PAGE analysis (10%) revealed a protein band at ~73 kDa, consistent with the expected size (Fig. 6A). The His-tagged protein was purified using Ni-NTA affinity chromatography with 250 mM imidazole for elution (Fig. 6B). The concentration of the purified recombinant protein was determined to be approximately 350 µg/mL, with an overall expression yield of ~28 mg/L of culture. Protein quantification was performed using the Bradford assay, with bovine serum albumin (BSA) as the standard. About 20 µL of the purified protein was loaded for SDS-PAGE and Western blot analysis to confirm expression and purification efficiency. Western blot analysis confirmed the identity of the recombinant protein, with a distinct band detected at the expected position. No band was observed in the BSA control, validating the specificity of detection (Fig. 6C).

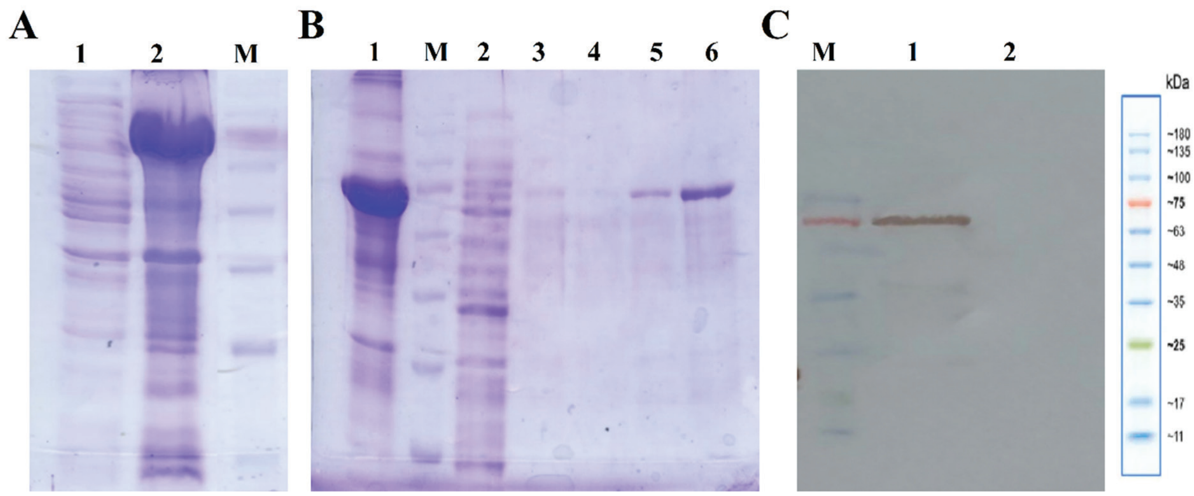

Fig. 6. Expression, purification, and immunodetection of recombinant rST protein. (A) SDS-PAGE analysis of rST protein expression in *E. coli*. Lane 1: Non-induced bacterial lysate (negative control); Lane 2: Lysate after induction with 1 mM IPTG, showing overexpression of a ~73 kDa band corresponding to rST. (B) Ni-NTA affinity chromatography purification. Lane 1: Crude bacterial lysate; Lane 2: Flow-through (unbound proteins); Lanes 3–5: Washes with 10, 20, and 50 mM imidazole, respectively; Lane 6: Eluted rtST protein with 250 mM imidazole. (C) Western blot confirming rST identity. Lane 1: Purified rST detected with anti-His tag antibody; Lane 2: Bovine serum albumin (BSA, negative control); Lane M: Protein molecular weight marker (SinaClone).BSA, bovine serum albumin,Ni-NTA, nickel-nitrilotriacetic acid, IPTG, isopropyl β-D-1-thiogalactopyranoside.

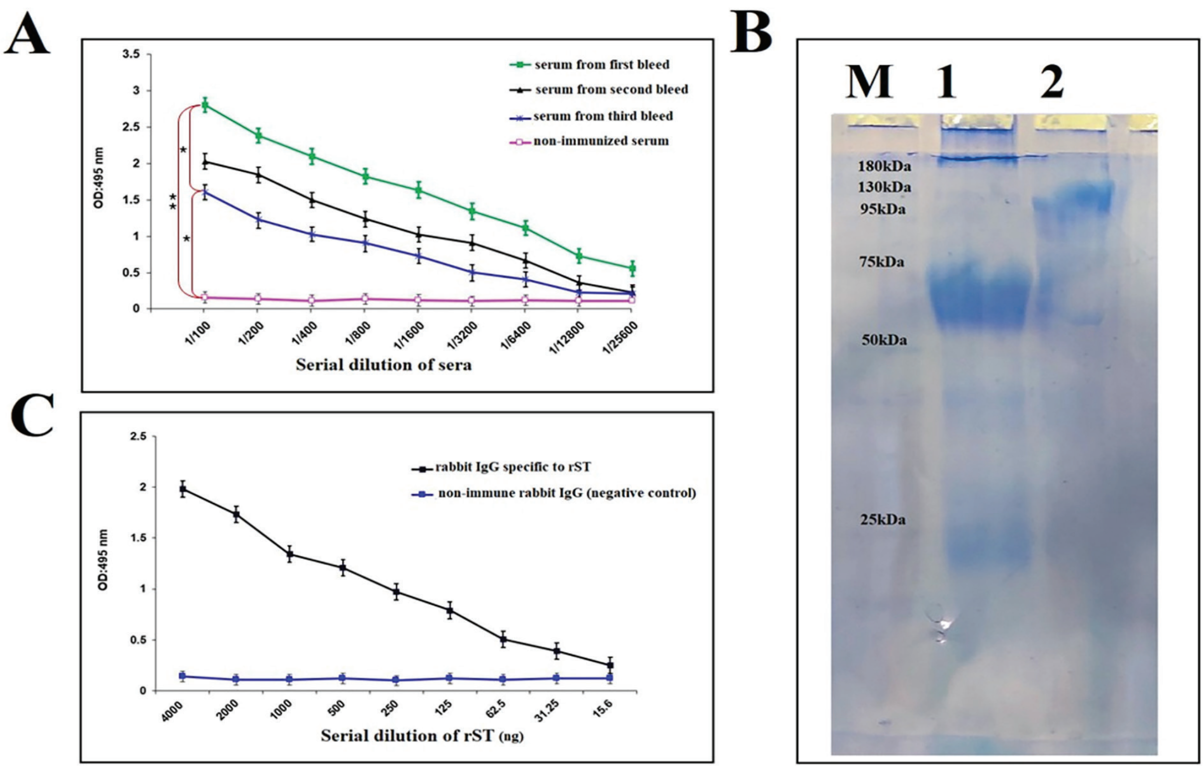

Fig. 7. Evaluation and purification of rabbit anti-rST antibodies. (A) ELISA analysis of IgG titers following antigen injections at 14-day intervals; sera were collected 7 days post-injection. Antibody levels progressively increased after each injection, rising from 1.6 (first bleed) to 2.8 (third bleed) at a 1:1000 dilution. * P <0.05, ** P <0.01 (B) SDS-PAGE analysis of purified IgG. Under reducing conditions (Lane 1), heavy (~50 kDa) and light (~25 kDa) chains were observed. Under non-reducing conditions (Lane 2), intact IgG appeared at ~150 kDa. Lane M: Protein molecular weight marker. (C) Specificity of anti-rST IgG was confirmed by ELISA, with a minimum detectable amount of 31.25 ng of recombinant β-SNTx. No signal was observed with non-immune IgG, confirming antibody specificity

Evaluation of IgG Antibody in Rabbit Groups

Antibody production was assessed using indirect ELISA after each antigen injection. Blood samples were collected weekly from test and control rabbits, and serum was analyzed for anti-SNTX β antibody titers. ELISA results showed a progressive increase in antibody levels following each subcutaneous injection, with titers rising from OD value 1.6 (first bleed) to 2.8 (third bleed) at a 1:1000 dilution (Fig. 7A). Antibody levels in immunized rabbits were significantly higher than those in the control group ($p< 0.01$). Additionally, antibody levels measured after the third injection were significantly higher than those observed after the first injection ($p< 0.05$). However, no significant difference was found between antibody levels after the third and fourth injections ($p>0.05$) (data not shown).

Antibody purification was carried out using Protein G resin. The second fraction (2 mL) contained the highest concentration of IgG (1.7 mg/mL), corresponding to a total yield of 3.4 mg. Purity was confirmed by 9% SDS-PAGE (Fig. 7B). The purified IgG yielded an OD value of 2.2 at 1,250 ng in an indirect ELISA, with a minimum detectable concentration of 9.75 ng (data not shown). The lowest detectable amount of recombinant β-sntx protein using the purified antibody was 31.25 ng (Fig. 7C).

DISCUSSION

The family *Scorpaenidae*, which includes some of the world's most venomous fish, is widely distributed in shallow tropical waters. Among its members, stonefish (*Synanceia* spp.) are considered the most toxic. According to Nabipour et al. (2016), they represent the most common cause of marine envenomation in the Persian Gulf (2). Taxonomically, this family falls under the phylum Chordata, class Actinopterygii (ray-finned fishes), order Perciformes, and suborder Cottoidei. It comprises approximately 215 species across three genera: *Pterois* (lionfish), *Scorpaena* (scorpionfish), and *Synanceia* (stonefish), with *Synanceia* being the most lethal.

According to Khoo et al.'s studies, the bioactive part of Stone toxin venom is a heterodimeric protein of ~148 kDa, consisting of α (~71 kDa) and β (~79 kDa) subunits (15). Kreger's research demonstrated potent hemolytic activity of *S. horrida* venom, especially against rabbit red blood cells, and showed its anticoagulant effects on both rabbit and human fibrinogen (10). Previous studies have reported the successful expression of antigenic regions from various fish venoms. For instance, Jafary et al. expressed the recombinant CfTX1 protein—approximately 28 kDa in size—from the venom of *Chironex fleckeri* (box jellyfish) in *E. coli*, although the expression yield was relatively low (25). In a related study, recombinant SNTXα expressed at a molecular weight of approximately 73.5 kDa was able to generate antibodies that successfully protected mice against a 2×LD50 challenge, demonstrating neutralizing potential (26). Despite such advancements, to date, there have been no published reports on the recombinant expression of the β-subunit of stonustoxin (SNTXβ) from *Synanceia horrida*. The present study is the first to report recombinant expression of the SNTXβ subunit, thereby providing a foundation for the development of targeted diagnostic tools and therapeutic agents against stonefish envenomation.

A conventional approach to neutralizing stonefish venom involves the use of antivenoms, with a commercial antivenom currently available in Australia. However, traditional antivenom production relies on venom extraction from live specimens—a process that poses significant challenges, including animal welfare concerns, batch-to-batch variability, and potential risks associated with venom handling and collection. In contrast, our recombinant strategy offers a safer, more scalable, and standardized alternative. By expressing the immunogenic β-subunit of stonustoxin (SNTXβ, residues

124–654) in *E. coli*, we eliminate the need for hazardous venom extraction (thereby enhancing laboratory safety), ensure consistent antigen quality (improving reproducibility and standardization), and focus the immune response on a defined, relevant epitope while avoiding potentially harmful components of whole venom (thereby increasing specificity and reducing off-target effects). Moreover, the use of a bacterial expression system supports cost-effective, large-scale production while minimizing ecological impact and contributing to sustainability.

To generate a specific antibody response against SNTX, it was essential to identify B-cell epitopes within the protein. Bioinformatic analysis predicted the amino acid region spanning residues 124–654 to have strong antigenic potential (Fig. 1), making it a suitable candidate for antibody production. This approach is widely applied in the design of recombinant vaccines (27, 28).

Initial sequence evaluation indicated that native SNTXβ had suboptimal stability and low predicted expression efficiency in *E. coli*. To address this, a thioredoxin (TRX) tag was fused to the N-terminus, reducing the instability index from ~42 to 36 (Table 1) and enhancing protein solubility. Codon optimization was conducted to match *E. coli* codon usage, enhancing the Codon Adaptation Index (CAI) to 0.93. Additionally, mRNA secondary structure prediction showed a reduction in minimum free energy ($\Delta G=-479.16$ kcal/mol), with fewer structural loops at the 5′ end (Fig. 2), suggesting improved translation efficiency.

The optimized SNTXβ construct was cloned into the pET17b vector and expressed in *E. coli* BL21 (DE3), with a 6×His tag facilitating efficient purification by affinity chromatography. To evaluate the immunogenicity of the recombinant SNTXβ, an indirect ELISA was conducted. Results demonstrated a progressive and significant increase in antibody titer following each booster injection (Fig. 7). Statistical analysis confirmed that antibody levels in immunized rabbits were significantly higher than in controls. These findings indicate that the SNTXβ fragment retains its immunogenic properties and is capable of eliciting a strong humoral immune response, supporting its potential use in antivenom development.

While our study successfully expressed and purified a recombinant fragment of the SNTXβ and confirmed its immunogenicity in rabbits, it presents specific limitations. Most notably, functional assays such as *in vivo* neutralization tests were not performed thereby limiting conclusions regarding the protective efficacy of the generated antibodies.

CONCLUSION

The recombinant β-subunit of stonustoxin demonstrated strong immunogenicity, inducing high-affinity antibody production in rabbits. The generated antibodies were capable of detecting as low as 9.75 ng of antigen in ELISA assays, indicating high sensitivity. While these findings suggest the potential utility of the produced antiserum, further studies—particularly *in vivo* neutralization assays—are needed to evaluate its efficacy against native venom and validate its potential for antivenom development.

ETHICAL STATEMENT

This article does not include any studies involving human participants. All relevant international, national, and institutional guidelines for the care and use of animals were strictly adhered to throughout the research. This study was approved by the Ethics Committee of Imam Hossein Comprehensive University (approval code: 1401.376).

AUTHORS' CONTRIBUTION

Mohammadreza Rahmani: research, methodology, writing: original draft. Shahram Nasarian: data management, project management, validation,. Hossein Samiei-Abianeh: research, methodology, writing: editing and review. Seyed Mojtaba Aghaie: data management. Davoud Sadeghi: data management, methodology

ACKNOWLEDGEMENT

The authors wish to thank Imam Hossein University for supporting this research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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