



Article Primary Structure and Conformation of a Tetrodotoxin-Binding Protein in the Hemolymph of Non-Toxic Shore Crab Hemigrapsus sanguineus

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Abstract: Tetrodotoxin (TTX)-binding proteins are present in toxic TTX-bearing animals, such as pufferfish and gastropods. These may prevent autotoxicity. However, TTX-binding proteins are also found in the nontoxic marine shore crab, *Hemigrapsus sanguineus*. Here, we isolated the TTX-binding protein, HSTBP (*Hemigrapsus sanguineus* TTX-binding protein), from the hemolymph of *H. sanguineus* and elucidated its primary structure using cDNA cloning. HSTBP, a 400 kDa acidic glycoprotein by gel filtration high-performance liquid chromatography, comprises 3 subunits, 88 kDa (subunit-1), 65 kDa (subunit-2), and 26 kDa (subunit-3) via sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reduced conditions. The open reading frame of the cDNA comprises 5049 base pairs encoding 1683 amino acid residues, and the mature protein contains 1650 amino acid residues from Arg³⁴ to Ser¹⁶⁸³. The three subunits are arranged in tandem in the following order: subunit-3 (Arg³⁴-Gln²⁶¹), subunit-1 (Asp²⁶²-Phe¹¹³⁸), and subunit-2 (Val¹¹³⁹-Ser¹⁶⁸³). A BLAST homology search showed weak similarity of HSTBP to clotting proteins of crustaceans (29–40%). SMART analysis revealed a von Willebrand factor (vWF)-type (⇒delete hyphen) D domain at Phe¹³⁸⁷-Gly¹⁵⁴⁴. We confirmed that the recombinant protein of HSTBP subunit-2 containing the vWF-type (⇒delete hyphen) D domain bound to TTX at a molecular ratio of 1:1.

Keywords: tetrodotoxin; tetrodotoxin-binding protein; hemolymph; shore crab *Hemigrapsus sanguineus*; cDNA cloning; primary structure; characterization; clotting proteins; vitellogenin; von Willebrand factor-type D domain

1. Introduction

Tetrodotoxin (TTX), a potent neurotoxin, is a selective sodium channel blocker that prevents the influx of sodium ions through voltage-gated sodium (Na_v) channels in most neurons and muscle cells. It causes paralysis with rapid and progressive muscular weakness [1,2]. TTX was initially discovered in pufferfish from the family Tetraodontidae and is widely found in various taxa, including marine and terrestrial eukaryotes, as well as bacteria [3,4]. Some TTX-bearing animals possess large amounts of TTX, exceeding their lethal doses. The minimum lethal dose of the toxic pufferfish *Takifugu niphobles* (presently *Takifugu alboplumbeus*) by intraperitoneal injection is 35 mouse unit (MU)/g body weight (equivalent to 7.7 μ g TTX/g body weight) [5], while the toxicity level of



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Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). *T. niphobles* (*T. alboplumbeus*) is over 1000 MU/g in the liver and ovary [6]. Here, one mouse unit (MU) is defined as the amount of TTX required to kill a 20 g mouse in 30 min via intraperitoneal injection and is equivalent to 0.22 μ g TTX [7]. The minimum lethal dose of the xanthid crab, *Atergatis floridus*, injected at the chela is 50–100 MU/g body weight (equivalent to 11–22 μ g TTX/g body weight) [8]. The toxicity of the crab was more than 100 MU/g with the highest score at 9000 MU/g, which coexisted with TTX and paralytic shellfish toxins (PSTs) [9–11]. Although how TTX-bearing organisms acquire, accumulate, and retain high concentrations of TTX in their bodies is poorly understood, it likely relates to unique mechanisms that prevent autotoxicity, such as the resistance of Na_v channels in TTX-bearing animals are mutated in the P-loop regions of Na_v1.4, reducing the binding affinity for TTX [12,13]. The latter is that TTX-binding proteins bind to TTX and neutralize it. TTX-binding proteins have been identified in pufferfish, gastropods, and shore crabs.

Matsui et al. [14] purified a TTX-binding protein from the plasma *T. niphobles* (*T. alboplumbeus*) and analyzed a partial amino acid sequence of the purified protein. Then, Yotsu-Yamashita et al. [15] elucidated the primary structure and characteristics of the TTX-binding protein from the plasma *Takifugu pardalis* and identified it as pufferfish saxitoxin (STX) and tetrodotoxin-binding protein (PSTBP). PSTBP is a glycoprotein with a molecular mass of 200 kDa consisting of 104 kDa subunits that binds to not only TTX but also STX, the representative principle of paralytic shellfish toxins (PSTs) (\Rightarrow Change to "PSTs"), preferring STX to TTX. PSTBP is widely distributed in the genus *Takifugu* of pufferfish and among most organisms (\Rightarrow Change to "tissues of *T. pardalis*"); therefore, it may function as a carrier protein for TTX [16]. Recently, other TTX-binding proteins were found in the ovaries of the pufferfish *T. pardalis* and *Takifugu flavidus*. They were identified as the vitellogenin subfragment, von Willebrand factor (vWF) type D domain, implicating TTX transport from the liver to the ovary because vitellogenin is synthesized in the liver and moves to the ovary via circulating blood [17,18]. Additionally, peroxiredoxin-1, a class of mammalian antioxidants from the pufferfish *Takifugu bimaculatus* binds to TTX [19].

The occurrence of TTX-binding high molecular weight substances (HMWS) has been reported in the muscles from five species of Taiwanese gastropods, with TTX-binding capacities of 0.12–0.65 MU/mg protein (equivalent to 0.026–0.14 μ g TTX/mg protein) [20]. HMWS with a molecular mass of 434 kDa, including two subunits, 272 and 47 kDas, was purified from the muscle of the toxic gastropod *Nitica line* (\Rightarrow Change to "*lineata*"). The purified HMWS showed an increased TTX binding capacity of 4.2 MU/mg protein (equivalent to 0.92 μ g TTX/mg protein), although the primary structure and the features of HMWS are unclear.

The shore crab, *Hemigrapsus sanguineus*, has a TTX-binding protein in the hemolymph, despite being non-toxic [21]. Yamamori et al. [22] investigated the resistance of non-toxic marine crabs to TTX and revealed that *H. sanguineus* was much more resistant to TTX than other crab species. However, the nerves of *H. sanguineus* were significantly sensitive to TTX, as those of other species. In contrast, intraperitoneal or intravenous injection of the hemolymph into mice prior to TTX injection effectively protected them from the lethal activity of TTX. Furthermore, Shiomi et al. [23] demonstrated the neutralizing effect of hemolymph against TTX in mice in three different ways: simultaneous injection of hemolymph and TTX, pre-injection of hemolymph, and post-injection of hemolymph. Shiomi et al. [23] also obtained TTX-binding HMWS with a molecular mass over 2000 kDa by gel filtration on a Sepharose 6B column. Thereafter, we purified and characterized the TTX-binding protein by successive ultrafiltration, lectin affinity column chromatography, and gel filtration high performance liquid chromatography (HPLC) [21]. The TTX-binding protein is an acidic glycoprotein with a molecular mass of 400 kDa and isoelectric point (pl) of 3.5. This selectively binds to TTX with a neutralizing ability of 6.7 MU/mg protein (equivalent to 1.5 μ g TTX/mg protein) but not to PSTs.

In this study, we evaluated the TTX-binding activity of *H. sanguineus* hemolymph using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Next, we isolated

the TTX-binding protein, elucidated its primary protein structure by cDNA cloning, and characterized it. The mature protein contains 1650 amino acid residues, comprising three subunits: 98.8, 60.8, and 25.5 kDas. We then produced a recombinant protein of subunit-2 (60 kDa) and confirmed the binding of the protein with TTX. This study provides a reference for applying the protein to possible antidotes for TTX intoxication because of its selective binding to TTX and its effective neutralizing ability against TTX.

2. Materials and Methods

2.1. Materials

Hemigrapsus sanguineus were collected from Tokyo Bay, Tokyo Metropolis, Japan. The hemolymph was extracted from the hemocoel with a syringe, the inside of which was wetted with 0.1 M sodium oxalate to prevent coagulation and centrifuged at 18,800× (Add "×") g for 15 min at 4 °C. The supernatant was used as the hemolymph. Hemolymph samples were pooled to purify the TTX-binding protein. To determine the TTX-binding activity of each crab, hemolymph samples were individually prepared from each specimen of five female and five male crabs. The details of the specimens used are listed in Table 1. Viscera obtained from the specimens were used for extracting total RNA.

Sample No.	Sex	Carapace Width (cm)	TTX-Binding Ability (μg TTX/mL)
1	Female	2.7	1.20
2	Female	2.5	1.25
3	Female	3.3	1.31
4	Female	2.5	1.40
5	Female	3.1	1.40
6	Male	3.8	1.20
7	Male	3.8	1.28
8	Male	3.9	1.29
9	Male	3.5	1.49
10	Male	3.2	1.55
Mean \pm S.D.			1.34 ± 0.12

Table 1. TTX-binding activity of the shore crab hemolymph sample.

TTX was partially purified from the ovary of *T. pardalis* by the method of Nagashima et al. [24], with a specific toxicity of 2800 MU/mg. Crystalline TTX (Fuji Film Wako Pure Chemical Industries, Osaka, Japan) was used as the standard for LC-MS/MS analysis. All other reagents used were of analytical grade.

2.2. Isolation of TTX-Binding Protein

TTX-binding proteins were isolated according to a previous method [21]. Six hundredµg TTX was added to the pooled hemolymph sample (95 mL, 5300 mg protein). The mixture was allowed to stand for 30 min at 4 °C and ultrafiltered through a 5k molecular weight cut-off filter (Ultracel 5 kDa, Millipore, MA, USA). After the concentrate was diluted with 0.5 M NaCl-0.01 M sodium phosphate buffer (pH 7.4), it was salted out with 50% saturation ammonium sulfate and centrifuged at 18,800× *g* for 15 min to precipitate. The precipitate (3970 mg protein) was dissolved in 0.5 M NaCl-0.01 M sodium phosphate buffer (pH 7.4) and subjected to a Con A-Sepharose column (1.5×30 cm, GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with the saline buffer containing 1 mM CaCl₂ and 1 mM MgCl₂. The column was washed with the buffer and then eluted with 0.15 M methyl- α -D-mannopyranoside-0.5 M NaCl-0.01 M sodium phosphate buffer (pH 7.4) at a flow rate of 20 mL/h. The eluate was collected in 4 mL portions.

Active fractions containing TTX-binding proteins were collected and concentrated by ultrafiltration using a 5k filter. The TTX-binding protein was run through a MonoQ 5/50 GL anion exchange column (0.5×5.0 cm, GE Healthcare Bio-Sciences) and eluted with

a linear gradient of 0.2–1.0 M NaCl in 0.01 M sodium phosphate buffer (pH 7.4) at a flow rate of 0.5 mL/min. The TTX-binding protein was purified by preparative electrophoresis (AE-6750, Atto, Tokyo, Japan) and re-chromatographed with a MonoQ 5/50 GL column (0.5×5.0 cm, GE Healthcare Bio-Sciences) with the same conditions as described above. The TTX-binding protein was monitored by measuring the absorbance at 280 nm and TTX-binding activity, as described below. Hereafter, the isolated TTX-binding protein is referred to as *Hemigrapsus sanguineus* TTX-binding protein (HSTBP).

2.3. N-Terminal Amino Acid Sequencing of TTX-Binding Protein, HSTBP

The isolated TTX-binding protein was subjected to SDS-PAGE on a polyacrylamide gel (E-D310L, Atto) and electrically transferred onto a polyvinylidene difluoride membrane (Clearblot P, Atto) according to the manufacturer's instructions. Amino acid sequence analysis of the TTX-binding protein was performed using an automatic gas-phase sequencer (492HT; Applied Biosystems, Foster City, CA, USA).

2.4. Molecular Cloning of TTX-Binding Protein, HSTBP

Total RNA was extracted from 2 g of the shore crab viscera with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). mRNA was purified from total RNA (1.5 mg) using an mRNA purification kit (GE Healthcare Bio-Sciences) and converted to cDNA, followed by ligation of a Marathon cDNA adaptor using a Marathon cDNA Amplification kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions.

Degenerate primers were designed based on the amino acid sequence of the subfragments of HSTBP and are shown in Table 2. The forward primer, HSTBP-S3F, and reverse primer, a Marathon adaptor primer (AP1), were used for the 3' rapid amplification of cDNA ends [RACE] (Thermo Fisher Scientific, Waltham, MA, USA). Amplification was carried out using TaKaRa Taq HS DNA polymerase (Takara Bio Inc., Shiga, Japan) under the following conditions: 35 cycles of 98 °C for 10 s, X °C for 30 s, and 72 °C for 2.5 min, in which the annealing temperature, X was adapted between 50 and 65 °C. The subsequent nested PCR used the degenerate primers HSTBP-S3N (forward primer) and AP2-2 (reverse primer) for 3'RACE. 5'RACE fragment of subunit 3 of HSTBP (HSTPB-sub3) was amplified with HSTBP-S3R and AP1, using a Mighty Amp DNA polymerase (Takara Bio Inc.) under the following conditions: 98 °C for 2 min and 35 cycles of 98 °C for 10 s, 59 °C for 15 s, and 68 °C for 2 min. The downstream sequences were determined by 3'RACE using forward primers (HSTBP-S3F5, HSTBP-S3F6, and HSTBP-S1Fk2) and reverse primer, AP1, respectively. The PCR products were subcloned into the pT7Blue T-vector (Novagen, Madison, WI, USA) and cloned into Escherichia coli JM109 competent cells (Nippon Gene Co., Ltd., Tokyo, Japan). The cloned plasmids were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and 3130 Genetic Analyzer (Thermo Fisher Scientific). The strategy used for HSTBP cDNA cloning is illustrated in Figure S1.

2.5. Computational Analyses

The amino acid sequences were analyzed for similarity using the standard protein BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 14 December 2022). Signal peptides in the N-terminal segments of HSTBP and other clotting proteins were predicted using the SignalP 6.0 server (https://services.healthtech.dtu.dk/service.php? SignalP, accessed on 14 December 2022) [25]. Multiple amino acid sequence alignments of HSTBP and other clotting proteins were performed with the Clustal W algorithm [26] using the MEGA XI software (https://www.megasoftware.net/, accessed on 14 December 2022) [27]. Molecular Evolution Genetics Analyses Version XI) software [27]. The theoretical molecular mass and electric point (p*I*) were estimated using DNASIS pro (Hitachi Software Engineering, Tokyo, Japan). The SMART program (http://smart.embl-heidelberg.de/, accessed on 14 December 2022) [28] was used to determine the conserved domains of HSTBP.

	Sequence	Base	Tm (°C)
Forward primer			
HSTBP-S3F	5'-CARGCIGAYATIWSITAYCARTAYAA-3'	26	53
Nested forward primer			
HSTBP-S3N	5'-TAYCARTAYAARTAYACIGGIACIGTIGC-3'	29	57
5'-RACE reverse primer			
HSTBP-S3R	5'-TGTCCAATCATGGTGTCCTCTGTGAG-3'	26	61
3'-RACE forward primer			
HSTBP-S3F5	5'-CTAGCAGCAAGACCAGCAGCAAG-3'	23	62
HSTBP-S3F6	5'-AGCAAGACTGGCACCAAGACTGG-3'	23	62
HSTBP-S1Fk2	5'-CATCCTGGTACCACTCCTGAATAACC-3'	26	62
Marathon adaptor primer			
AP1	5'-CCATCCTAATACGACTCACTATAGGGC-3'	27	62
AP2-2	5'-ACTATAGGGCTCGAGCGGCCGCC-3'	23	67
Sequence primer			
T7 promoter	5'-TAATACGACTCACTATAGGG-3'	20	52
SP6 promoter	5'-TATTTAGGTGACACTATAG-3'	19	47
HSTBP-S1IF	5'-CAGATCACAGCGAGAACACG-3'	20	57
HSTBP-S1IR	5'-CAGGAAGGAGTCAAGACTTGC-3'	21	58
HSTBP-S2IF	5'-CTGCAGGTGACCACTGTCTTAG-3'	22	60
HSTBP-S2IR	5'-CGTGACTGCTATAGTGAGTAGG-3'	22	58
HSTBP-S2IF2	5'-CTAACCAGCACAAGCTTCATCC-3'	22	58
HSTBP-S2IR2	5'-CATGATACTCCCACGCTGTG-3'	20	57
HSTBP-S2IF3	5'-GAGTACAGGCTTGTGTTTGATCC-3'	23	58
HSTBP-S2IR3	5'-AACATCCACCACAGTCAAGTCC-3'	22	58

Table 2. Primers used in this study. \Rightarrow Need space between "Base" and "Tm (C)".

The three-dimensional structures of HSTBP sub-2 (Val¹¹³⁹-Ser¹⁶⁸³) and vWF type D domain TfVWF from *T. flavidus* were predicted using Colabfold (version 1.4.0) [29]. Structural models were viewed using PyMOL.

2.6. Recombinant Protein of TTX-Binding Protein Subunit 2 (rHSTBP-sub2) and Its Binding with TTX

A partial gene fragment corresponding to HSTBP subunit 2 (HSTBP sub-2) was synthesized based on the cDNA sequence (accession number LC733238) using an artificial gene synthesis system (Eurofins Genomics K.K., Tokyo, Japan). Codon usage of the fragment sequence was optimized for *E. coli*. To insert the restriction enzyme sites, the fragment was amplified by PCR using primers containing NdeI (forward primer, 5'-GTCGTCATATGGTCGAACAGGAAGAAAC-3') and BamH1 (reverse primer, 5'-CAGCCG GATCCTTAACTGCGGATTTGGCGAAAAC-3') recognition sequences. The amplicon was cloned into the pGEM[®]-T vector (Promega, Madison, WI, USA) and transformed into DH5 α competent *E. coli* (Nippon gene Co., Ltd.) following the manufacturer's instructions. The plasmid and empty pET16b vector (Merck Millipore, Burlington, MA, USA) were digested with NdeI and BamHI (Nippon Gene Co., Ltd.). Both the fragment and linearized pET16b vector were ligated and transformed into DH5 α competent *E. coli* (Nippon gene Co., Ltd.) to produce the expression construct.

The expression construct was transformed into Rosetta-Gami B (DE3) pLysS *E. coli* (Merck Millipore). rHSTBP sub-2 was overexpressed by incubation at 37 °C for 4 h in a shaking incubator (150 rpm) with 100 μ g/mL of ampicillin, 34 μ g/mL of chloramphenicol, and 0.3 mM isopropyl β -D-thiogalactopyranoside (IPTG) after 2 h-preculture in the same conditions, except for IPTG. The cells were harvested by centrifugation (3000× *g*, 4 °C, 20 min) and the inclusion body was collected by centrifugation (10,000× *g*, 4 °C, 15 min) after treatment with 0.15 M NaCl-0.05 M Tris-HCl buffer (pH 8.0) containing 1% Triton-X100, 0.001% RNase, and 0.001% DNase I (Nippon gene). The inclusion body was solubilized with 6 M guanidine hydrochloride-0.15 M NaCl-0.05 M Tris-HCl buffer (pH 8.0) and applied to a Ni-IMAC resin (Bio-Rad Laboratories, Hercules, CA, USA). The recombinant protein

was eluted with 0.5 M imidazole-0.15 M NaCl-0.05 M Tris-HCl buffer (pH 8.0) after washing with 0.025 M imidazole-0.15 M NaCl-0.05 M Tris-HCl buffer (pH 8.0).

The rHSTBP sub-2 fraction was reduced with β -mercaptoethanol and refolded by a dilution and dialysis as follows: the protein fraction was added dropwise into 25 volumes of the saline buffer containing 0.5 M L-arginine, 1 mM glutathione, and 0.1 mM glutathione disulfide with stirring. The diluted fraction was dialyzed twice against 0.5 M L-arginine-0.15 M NaCl-0.05 M Tris-HCl buffer (pH 8.0) and ultrafiltered with an Ultracel 5 kDa ultrafiltration disc (Merck Millipore) to replace the buffer by 0.02 M Tris-acetate buffer (pH 7.4). The rHSTBP sub-2 was mixed with TTX and allowed to stand for 3 h at 4 °C. The mixture was applied to a Sephacryl S-300 column (2 × 29 cm, GE Healthcare Bio-Sciences) and eluted with 0.02 M Tris-acetate buffer (pH 7.4) at a flow rate of 0.5 mL/min. The eluate was fractionated in a 4-mL portion. Each 1 mL aliquot of the fractions was mixed with 10 µL of acetic acid and heated in a boiling water bath for 10 min to liberate TTX bound to rHSTBP sub-2. TTX was analyzed by LC-MS/MS, as described below.

2.7. Measurement of TTX Binding Activity

For the hemolymph, $500-\mu$ L aliquots of the samples were mixed with 20 µg TTX and allowed to stand for 60 min at 4 °C. The mixture was ultrafiltered with an Amicon Ultra-0.5 mL centrifugal filter Ultracel-3K (nominal molecular weight cut-off 3000, Merck Millipore) to remove free TTX. Milli-Q water was added to the centrifugal filter, and ultrafiltration was repeated twice to concentrate to 100 µL. TTX bound to the TTX-binding protein was separated from the protein by adding 100 µL of 0.1% (v/v) acetic acid and heating in a boiling water bath for 10 min.

For the active fractions from the purification steps, $100-\mu$ L aliquots of the fractions were mixed with an equal volume of 0.1% (v/v) acetic acid and heated in a boiling water bath for 10 min.

TTX was determined using a Waters Acquity UPLC and TDQ triple-quadrupole tandem mass spectrometry (Waters, Midford, MA, USA) [30]. A TSKgel Amide-80 column ($2.0 \times 150 \text{ mm}$, 3-µm particle size; Tosoh, Tokyo, Japan) was maintained at 25 °C and eluted with 0.016 M ammonium formate (pH 5.5): acetonitrile (40:60, v/v) at a flow rate of 0.2 mL/min. The eluate was introduced into the ion source of electrospray ionization-mass spectrometry, ionized by the positive mode, and detected in multiple reaction monitoring mode, *m*/*z* 320 > 162, with a collision energy of 45 eV.

Student's *t*-test was used to test for significant differences, with a significance level of 5%.

2.8. Analytical Methods

The molecular mass of the isolated TTX-binding protein was determined by gel filtration HPLC on a TSKgel G300SW_{XL} column (0.78 × 30 cm, Tosoh) with 0.5 M NaCl-0.01 M sodium phosphate buffer (pH 7.4) at a flow rate of 0.5 mL/min. Four reference proteins (Bio-Rad Laboratories), thyroglobulin (670 k), bovine gamma globulin (158 k), chicken ovalbumin (44 k), and equine myoglobin (17 k) were used to calibrate the column. TTX-binding protein was estimated by SDS-PAGE on a C520L gel (Atto). Prior, the protein was dissolved in $2\times$ buffer containing 0.1 M dithiothreitol and 2% SDS and heated in a boiling water bath for 5 min. The proteins were stained with Rapid CBB KANTO (Kanto Chemical Co., Inc., Tokyo, Japan). Precision Plus Protein Standards (Bio-Rad Laboratories) were used as the reference. Native-PAGE was performed using a C7.5 gel (Atto).

Protein levels were measured using bovine serum albumin as a standard protein using the Lowry method [31]. The protein concentration of rHSTBP sub-2 eluted by Sephacryl S-300 column chromatography was measured with a protein assay kit using a Qubit 4 fluorometer (Thermo Fisher Scientific).

3. Results

3.1. TTX-Binding Activity of the H. sanguineus Hemolymph

As shown in Table 1, all the hemolymph samples used were bound to TTX. TTXbinding activity ranged from 1.20 to 1.55 μ g TTX/mL. There was no difference in TTXbinding activity among individuals or between female and male samples (p > 0.05). The former ranged from 1.20 to 1.40 μ g TTX/mL (mean \pm S.D. 1.32 \pm 0.09 μ g TTX/mL) and the latter from 1.20 to 1.55 μ g TTX/mL (mean \pm S.D. 1.36 \pm 0.15 μ g TTX/mL). No relationship was observed between TTX-binding activity and crab size (carapace width).

3.2. Isolation of TTX-Binding Protein

TTX-binding protein was isolated using five purification steps. In the first step of salting out, TTX-binding protein was recovered from the 30 to 50% saturation ammonium sulfate fraction. The TTX-binding protein was subjected to lectin affinity chromatography on a Con A-Sepharose column and eluted in the methyl- α -D-mannopyranoside eluate fraction (data not shown). Next, the active fraction containing the TTX-binding protein was chromatographed by anion-exchange HPLC on a MonoQ 5/50 GL column and eluted with 0.6–0.8 M NaCl in 0.01 M sodium phosphate buffer (pH 7.4) (data not shown). Finally, the TTX-binding protein was re-chromatographed using a MonoQ 5/50 HPLC after preparative electrophoresis. TTX-binding protein was detected at retention times of 44–46 min (Fraction I) and 48–52 min (Fraction II) (Figure 1). Native PAGE analysis revealed that both fractions produced only one band, confirming their homogeneity (Figure 1). Fraction I showed the TTX-binding activity of 0.22 µg TTX/mg protein and Fraction II of 0.33 µg TTX/mg protein. Because Fraction II had higher TTX-binding activity and more TTX-binding protein than Fraction I, Fraction II was identified as *H. sanguineus* TTX-binding protein (HSTBP) and used in further experiments.



Figure 1. MonoQ anion-exchange HPLC of active fraction obtained from preparative electrophoresis. Sample: active fraction obtained from preparative electrophoresis; column: MonoQ5/50 GL (0.5×5 cm); mobile phase: 0.2–2.0 M NaCl-0.01 M sodium phosphate buffer (pH 7.4); flow rate: 0.5 mL/min; detection: absorbance at 280 nm; inlet: Native-PAGE; I; peak I obtained from Mono Q anion-exchange HPLC, II; peak II obtained from MonoQ anion-exchange HPLC.

3.3. Characterization of TTX-Binding Protein, HSTBP

The molecular mass of the isolated TTX-binding protein, HSTBP, was measured at 400 kDa using gel filtration HPLC on a TSKgel G3000SW_{XL} column (Figure 2A,B). In contrast, the molecular masses estimated by SDS-PAGE were approximately 88, 65, and 26 kDa (Figure 2C). These results suggested that HSTPB has a dimeric conformation consisting of three subunits with molecular masses of 88 (HSTBP sub-1), 65 (HSTPB sub-2), and 26 kDa (HSTBP sub-3).



Figure 2. Molecular mass of TTX-binding protein: (**A**) gel filtration HPLC on a TSKgel G3000SW_{XL} column (0.78 × 30 cm); (**B**) calibration curve for the determination of TTX-binding proteins by gel filtration HPLC; •: TTX-binding protein, \diamond : standard proteins, a: thyroglobulin (670 kDa), b: bovine γ -globulin (158 kDa), c: chicken ovalbumin (44 kDa), d: equine myoglobin (17 kDa); (**C**) SDS-PAGE. I; peak I obtained from MonoQ anion-exchange HPLC, II; peak II obtained from MonoQ anion-exchange HPLC; ①, ②, and ③ indicate subunits-1, -2, and -3, respectively; (**D**) SDS-PAGE before (-) and after (+) deglycosylation treatment with PNGase F; ①, ②, and ③ indicate subunits-1, -2, and -3, respectively. Transferrin was used as a control of N-glycosylated protein.

Based on the chromatographic results of the purification procedures, HSTBP is most likely an acidic glycoprotein. HSTBP was adsorbed onto an anion exchange MonoQ 5/50

GL column. It was also retained on the lectin affinity Con A-Sepharose column and eluted with an eluent containing methyl- α -D-mannopyranoside. The molecular mass of HSTBP sub-2 (65 kDa) was decreased to 63 kDa after digestion with the glycopeptidase, PNGase F (Figure 2D), indicating the presence of an N-linked carbohydrate side chain. However, that of the other two subunits, HSTBP sub-1 (88 kDa) and HSTBP sub-3 (26 kDa), did not change.

3.4. cDNA Cloning and Molecular Properties of HSTBP

The N-terminal amino acid sequences of HSTBP sub-1, sub-2, and sub-3 were determined as ¹DPDPARLIQPINDTLWVLTR²⁰, ¹VEQEETQTQTVITHMQRVEELAINF²⁵, and ¹RGGLQADISYQYKYTGTVATWVSED²⁵, respectively (Table 3).

Subunit No.	Molecular Mass (kDa)	Amino Acid Sequences
1	88	DPDPARLIQPINDTLWVLTR
2	65	VEQEETQTQTVITHMQRVEELAINF
3	26	RGGLQADISYQYKYTGTVATWVSED

Table 3. N-terminal amino acid sequences of TTX binding protein subunits.

A cDNA fragment was amplified by 3'RACE using the degenerate primers HSTBP-S3F and HSTBP-S3N. These were designated from the amino acid sequence of HSTBP sub-3 as forward primers and AP1 and AP2 as reverse primers (Figure S1). Based on the nucleotide sequence of the PCR product (682 bp), the remaining 5' region sequence was determined by 5'RACE using AP1 and HSTBP-S3R. The PCR product (632 bp) contained a 5'-untranslated region (Figure 3). Next, a cDNA fragment was amplified by 3'RACE using two primer sets: HSTBP-S3F5/AP1 and HSTPB-S3F6/AP1. A PCR product of 1289 bp was obtained, which included the nucleotides encoding 20-amino acids of the N-terminus of HSTBP sub-1. The remaining 3'region was identified by 3'RACE using HSTBP-S1Fk2 and AP1 primers. The 3364 bp PCR product was obtained, which contained 23-amino acid residues of the N-terminus of HSTBP sub-2 and 3'-untranslated region. Here, the nucleotide sequence of the full-length HSTBP cDNA (5391 bp) was determined (accession number LC733238). The accuracy of this sequence was verified using re-cloning experiments. The initial ATG codon is present at nucleotides 91–93. In the 3'-untranslated region, a polyadenylation signal AAT AAA was identified in nucleotides 5341–5346, and a poly(A) tail at nucleotide 5368. An open reading frame (ORF) comprised of 5049 bp, encoding a precursor protein of 1683 amino acid residues from the putative initiating Met¹ to the putative last Ser¹⁶⁸³ was also identified. The amino acid sequences of the N-terminal proteins of HSTBP sub-1, HSTBP sub-2, and HSTBP sub-3, determined by protein sequencing, were all found at positions 34-58 (HSTBP sub-3), 262-281 (HSTBP sub-1), and 1139-1163 (HSTBP sub-2) of the deduced molecule (Figure 3).

In Figure 4, the deduced amino acid sequences of the TTX-binding protein were aligned with the clotting proteins of shrimp and crab from the suborder Pleocyemata. SignalP-6.0 Server predicted that HSTBP comprised a signal peptide (Met¹ to Gly¹⁷) and a mature protein starting with Leu¹⁸. However, HSTBP sub-3 began with Arg³⁴, suggesting the absence of a peptide from Leu¹⁸ to Ala³³ by translational modification. The mature protein of HSTBP likely consisted of 1650 amino acid residues from Arg³⁴ to Ser¹⁶⁸³. The theoretical molecular masses of HSTBP and its subunits (HSTBP sub-1, HSTBP sub-2, and HSTBP sub-3) were calculated at 185,080.6 Da, 98,822.3 Da, 60,784.6 Da, and 25,509.7 Da, respectively, using the DNASIS Pro (Hitachi Software Engineering). The isoelectric point (p1) of HSTBP was estimated to be 5.26 using DNASIS pro (Hitachi Software Engineering). A BLAST homology search showed weak identity of the deduced amino acid sequence of HSTBP to clotting proteins of crustaceans, *Portunus trituberculatus* (accession number XP_045118682.1) (40.2%), *Scylla paramamosain* (accession number AYK27566.1) (39.9%), *Pacifastacus leniusculus* (accession number AAD16454.1) (29.9%), *Homarus americanus* (accession number KAG7156252.1) (29.1%), and *Procambarus clarkii* (accession number AYD41596.1) (29.1%).

GCC ACC ACC ACC GCC GCC GCC ACG AGA GGG CTT GGA TCG TCT AGA AAG GTT TCT CAT CTT GAG TGG GCC CGC CGC CGC CGC TGG GCC AC 106 GCC ACC CTC ACT CTC TTG CTG GCC GGG TGT CAG GGC CTT TCC ATT GCT CAG CAG GAG CAT GAA GCC CCG CGC TAC ACT GTA GCA CGA GGG GGG CTG CAG GGG GAG 210 40 TAC CAG TAT AAG TAC ACG GGC ACG GTG GCC ACA TGG GTA TGG GAG GAC CCC ACA CAG TTT TCG GGG TGG GCC TTG AGG GCG GAC GTG GAG CTG GAG AGG 315 75 316 ACT CAA GCT GGC AAC CTC CGC GTG AAA GTG AGC AAC GTG GAG GTG GCC GAG ACC GAG AAC GAG GAT TCA TGC GAC CCC CAG AAG AAC ATC GAG TTT GAC TAC CAC 420 110 41. CÓC MÃ BÝC CÓI BỐC BÝC BỘC GÓC CÚC CÍB BHB CHO VÚC TÚC CHU BHC BÝC GÓP GCH BÓC BÚC BHE THÍ CÍC VHB BÚC CÓC BHP BHB CÓP CIL THE BÚC 41. CÚC MÃ BHC CÓI BÓC BÝC BÚC CÚC CIL CÍB BHB CÁP VÍC TÚC CÚC NH GUB BHC BÁC BÓP GÓP GÓP BHB THÍ CÍC VHB BÚC CÓC BHP BHB CÓP CIL THE BÚC 525 145 ACC AAC GTA OGG CGA GGC ATC ATC TAC TOG CTC CCT CTC GCT GTC CTC ACG GGT CAT AAT CCT TCA OGC CTC ACG CTC ACG GAG GAC ACC ATG ATT GGA 630 180 THE STREAM OF THE STREAM OF THE CALL AT A AND CTC TCC AND TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAA GAC GAG AAG ATC GAA GAA GAC GCA GCA AGA ATA ANG CTC TCC AND TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAA GAC GAG AAG ATC GAA GAC GCC CAA AGA ATA ANG CTC TCC AND TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAA GAC GGA AGA TC GAA GAC GCC CAA AGA ATA ANG CTC TCC AND TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAA GAC GGA AGA TC GAA GAC GCC CAA AGA ATA ANG CTC TCC AND TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAA GAC GAG AAG ATC GAA GAC GCC CAA AGA ATA ANG CTC TCC AND TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAA GAC GAG AAG ATC GAA GAC GCC CAA AGA ATA ANG CTC TCC ANG TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAA GAC GAG AAG ATC GAA GAC GCC CAA AGA ATA ANG CTC TCC ANG TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAA GAC GAG AAG ATC GAA GAC GCC CAA AGA ATA ANG CTC TCC ANG TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAG AAG ATC GAA GAC GAC GAC ATA ATA TCC TCA AGA ATA ANG CTC TCC ANG TAC CAR TCA GCA CTC GAA GAC GGG AAA CAG GAG AAG ATC GAA GAC GAC GAC ATA TAC 735 215 840 250 214 945 285 GAG GGC TGC GAG GAG AAC CAC TTT ACT GTG AAG ATT AAC GGC AAT GAC GAG TCA GGG GAC AAG ACA CAG CCC AGC TCA CAA GGC ACC TTC CTC ATT AGG GGC GAG 1050 320 946 E G C E E N H F I V K I R G A D D D C ... I C GAT GC CAC GAC AAA TTC ATC AGG ACA AAG ACA AAG CAG AGG GTG 1155 CTA GGG GGC ATG ACA CGC CTG GAG AGG GCC GAA GTG AATC GTC GAC GTG GTC TAC ACT ACT GAT GC CAC GAC AAA TTC ATC AGG ACA AAG ACA AAG CAG GTG 1155 1051 321 CTG GAG CTG AGA GCA GTG AGA CAT GCG GAG GTG CAT GTG GAA AGT GCA CAG GAG TTG GAG AGA AGG ACA CTC AGC TTT GAA ACT AAG GAA GGG CCG CTT ATT TTA L E L R A V R H A E V H V E S A O E L E R R T L S F E T K E G P L I L ACT GIC GAG GAG CAG CTG ACA GGC ATT TAC CCC ACA GAG CTA CAA GCC CAA GCC ATT ATA GCA AAG ATC AAA GAA GAC ATG GAG TTG CTT AAT GAA CCA GAT CAC 1366 AGC GAG AAC ACG CCA CCC TTC CTG GAC ACT TTC CGG CAT GTG GTG GAG CGC ATC GCC GTG CTG GCC AAG TCT AAG ATT GAG TCC CTC ATT TCC CAG GTG CCT GAC 1470 ACT CCG AGA GTC AAA AAA ACT CTG TAC CAA GCA CTT TTG ACC TCC GGG ACC AAG GAG GCA CTG GCC TAC CTC TTG GAG AAG CTC AGT GAT GAG GCT TTC CAG CAA 1575 T P R V K K T L Y Q A L L T S G T K E A L A Y L L E K L S D E A F Q Q 498 1471 CAG AAC CCC AGC CTT GTA TTC CAG GTC TTC CAG GCC TCT ACA ATG ACA GTC ACG GAC CCG TCA CTC ATC CTT ACC ATTG GTG GAC AGT ATG GAT GAC O N P S L V F O V F O A S T M T V T D P S L I P T L L O L V D S M D D AGA OGT GGG GAC COA CTC AAG ACC ACA GCC CTT CTC AGC ACC GCC GAC GCC GAG AGC TAG TAC TGC CTT GAC CCC CAG GTG GCT GAG AGC TGT GAC CAG CGA TGC GAC AAT GGC GCA AGE CITI GAC TOC TITC CIE COC ATT CITC ACT CIE GGT CITC AAT GAT CAL TIG AAG TOG AAG CAA TIG GITI CAC CIE CAA GOC ATG TGC AIG CIE 1890 D N G A S L D S F L P I L T R G L N D P L K W K Q L V H L Q A M C M L 600 1786 566 GGA ACA CCG CTC AOC CTG CCA TAC CTG GAG CCC ATC ATT GAA GGG AAG AAG GAT GAT AAT GCG GAC CTG AGG ATC AGC GCC ATC CAC TGT CTC AAA GCA GAG CTT G T P L S L P Y L E P I I E G K N D D N A D L R I S A I H C L K A E L 1891 601 1995 635 CTG CCC AAA ACA GCT CAC TCA AGG GTG CTG GAA CAC CCTG GTA CCAC CCT CTG AAT AAC CCG TCA GAG TTC CCC GAG GAG AGA AGC ATG GCC TTC CTC GTC ATC AGC 1996 634 ACA TGG GAG COG AGG AAC CTG TCT TGG TGG GAG CAC ATC GCA CTC TCC ACG TGG AGG GAA AGC TCA CAT CAA GTG GCC GCC TTT ATC TAC TCT GCC CTC ACC GCC T W E P T N L S W W E H I A L S T W R E S S H O V A & F T V S & T T N 2101 CTC AGT GAA GGA AAC CAT CCC CGC TCA CTT AGT GCC AAG AAA GTT GTT CAT CTT GCT AAG CAT CCT GGC AAG ATA ACC CTC GCC CAA TCT GCC TAT 2310 2206 ATA TAC TTG AAA GAA GAT GTG GAA AAC TGT AAA GAA GGA GGA GAT TTG AATA GAC GCC TGG GCC GCC TCC ACA CTG ACC GTC TTC CCA GCA GCA GTT TAT TAC 2411 2311 CTG CAG GTG ACC ACT GTC TTA GGG ATG CGT CAG AGA GAG ATG GCT GTC TTC TTT AGT AAT TAC CTC CAG CGT GTT TAT AAG AGG GGC AAT CCG TTC CAG TGG GAC ATA AAG GAA GAT TCA GAA GAC TCC TCC TTC GAA ATA TTT GAG GAA TTA CAG GAA CAA CTG ATG CTA ACC AGC ACA AGC TTC ATC CAT AAG GAA ACT TTT ACT 2622 TIT GCT TCA ATC TTA GAA CCT TGG AGT TAC CTT TCT TCA TCA TCA TCA GAA GGG GAA CAT CGT CAA CCA GGG GCA TTT GGA CCT TGG AGT TAC CTG GAT TAC AAT CGA 2730 2731 CCA ATC ATC TTC CGC TTC ACA GAT TCA ACA CTA GCA TTT CCA TTG GAC TTG GGT CTC CCT GTC ATT GTT AAG CTT GAA GAA GAC TTT GGC TTC TAT CTT AGA GGA 2835 881 P I I F R F T D S T L A F P L D L G L P V I V K L E E D F G F Y L R G 915 2941 CAG ATC AGA TTG CCT CTC CGG AGT AGA GGA GAA TGG CGC CCA GTA AGA CTG ACA CTG CG ACT TGA AAA GTT AATC CTT ATA GAA ATG CGG CGC TGC AGA 3151 GAG AGC AGC TCA AAG TCT COG TTG CTA GAC AAG TTA GOG ATC CAC TTG AGC TGT GAG TGG ACA GGT GAC TCC CAG CAG GCA GGA GAC CCC AAG ACT GTC ATC AGA E S S S K S R L L D K L G I H L T C E W T G D F Q Q P G D P K T V I R GRE THE GRICCT PART GRE GRIT ATC CREAR ARC THT GRE TRAGE GRIT GRE GTH GRIT GCE AGG GTC TOCC GRIT ATC GRIT ATC GRIT ATT GRIA GRIT ATC GRIT 1054 NAA NAA GAG ATT NAG ATC AGC AGG GGG CCA TTT GAG GAG GGA GGG AGC GAT GGA GGA GGG AGC GGA TGAT GAT TAT GAG GGA CAA AGTT TCT GGC GGC TCT TCA GAC TTT 3455 GAG ATC TTC AGC CAG ACT TCC AGT GGT GGC GGA GAT TTT GTT GAG CAA GAA GAG ACG CAG ACC CAG ACT GTC ATC ACT CAC ATG CAG AGG GTG GAG GAG GTT GCT E I F S Q T S S G G G D F V E Q E E T Q T Q T V I T H M Q R V E E L A 3466 1126 TTT ACA TAC GET GTG ATA AAC AGC GTA AGC TTG AGA ATT GAG CCA GAT GCT GGC CCT CAA AAG TAT GAG TTT GCC ATC TCC CTG ACT ACC AGC AAA CAG 3675 3571 ATA A 1161 3676 1196 GAG AAG CTT GGT GTG AAG GCT CCA ACG AAT TTC CAC TTG ACG TTT GCC AAT AGC TCG GTG ACG GAA ATC TTC GGC GAC AGC TAT TGG ACA TGT ATT GAT GCA AGT 3786 E K L G V K A P T N F H L T F A N S S V T E I F G D S Y W T C I D A S 1236 3781 12?" CTA TAT TCT CCT TAC CTG ACG CCT TTG ATG CCC AAG GAG GTG ATC CTT GAG ACA GAT CTG TCT GAC GAC TTC TTG CTG ACA GAG GCT TAT TGT GAG 3865 GAC CCA ACT ATA CTG TAC CTC AAG GCT CGC CAG GAC GTG AGC ACC ACA GCC TTG GAG TAC CTT CAA GCT GAG GCA GAG AGG GAG ACG TGT GAC GGT TCC CTC CCC 3990 3886 CAG CGC CTA GAC CAG GAC ATC ATC GAC AAT GTT GAA GCC TTC CGA GCC TGG TCG ATT CAT AAC TTG GAC TTG ACT GTG GTG GAT GTT GTC AGG GAC AGA GTT TCC 4095 3991 CAA TCA GAC AAT TIG ATC TCT GTT GAT GCT TTG CTA AAT CTG AAG GTG AAT GAG TGG AAG TTG AGG ACG ACT CCT CAT GGA ACA TTT GTG TAT TCT GTG CCG 4200 GCT GAG TCC CTC GAT GTG ATA ACC AGT AGC TAT GTC CAA AAC AAT GTG TTT GAT GTC ATC AGT AGC AGC CTC GAC AAA GTG TGC TAC CTG ACA ACG CAT TCG ATT 4305 4201 AGG ACT TTT GAT GGG CTT GTC CAT GAG TTT GAA CCC TCC TCT TGC TGG ACC ACT GCA GCC ATC CAC ATT CCT CCA CCT ACT TCC CTT GCA ACG AAG GCC 4410 4306 CTA CAA GTG GCT GTA CAG GGC CGC TAC ATT GAC GAC CAG TOG GAA GTT CGC ATC AGC TGG CCT TTA TCT GGG CTG ATC CTC GAC GTA ACA AAA TCG GAG ATA CTG 4315 4411 1441 GTC AAC GAG GAA CGA CTC AGT GGT CAA AGC GAC AAA TIT GTG GTG CAC CGT CTA CAG GAC AGC CAG CTG GTG GAG AGA GTG CTG GTG GTG GTG GGC AAT 4516 1476 4621 1511 của tức yếu đặc cầu đác các vớc yếu vậu đất từa vậc vắc các đác cát đặc vậu củi đác tác vấu củi đác tác vấu vậu 4726 GAT ACT TGT GAC AAA GAA GTT TTC COG ACC ACT GGC TTG TCA CAG CAA AAT GTA ATG GAA AAT TGC ACA GG3 TG3 GAG TAT CAT GAG CGC ACC TCC CCA GCA GGA 4995 D T C D K E V F R T T G L S Q Q N V M E N C T A W E Y H E R T S P A G 1615 4831 1581 TTA CTC TGC CGC TCC AGA GTT CCT GTA CCT GTG TGT CTG CCA GGC TGT GAG GTC GGC ATA GCA TTC ACT AGT AAT CTA AAG TAT GAC TGC TCG CAG CCG TCC TCC 5000 4936 TCC GCA GAG CAA CAA CAA CAA TAT GAC GGC AGC AGC AGG CAG TGC CTC AGC AAA GTC CTG CCA GCC ACC ATT GCC GCT GGG TGC TTC CGC CAG ATC AGA AGT TAG GGT 5145 5041 CTT CTG TTT GGA ATT AGG GTC TCA AGA AAT TAA GCA ATG CTT CTG GTC TCA GTA GCT AGA ATT ACC CAT CAA GCA GTG GTG GTG CTT ACA CTG CCT ACT CAC TAT AGC 5256 5251 AGT CAC GCC GCC GCC GCC GCC TCT GCC ATA TITT GAC AGT AAT ACA AAA CTA AAC ACT CCA CAG TITT GCA ATA ATA AAG TGC AGG TITG ACT TCT GAA AAAT AAA GAA AAT TAT 5355

Figure 3. Nucleotide and deduced amino acid sequence of cDNA. N-terminal amino acid sequences of TTX-binding protein subunits are shown in gray. Underlines indicate primers. Protein N-glycosylation site is boxed. Amino acids marked in magenta, blue, green, and red represent signal peptides and cleaved peptides, subunit 3, subunit 1, and subunit 2, respectively.

HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus leniusculus Procambarus clarkii	1 1 1 1 1	NSLFAATLTLLLAGCOOLSIAQQEHEA PRYTVARGGLQAD ISY QYK YT GTVÄTWYSED PTOFSGSÄLRADVELERTQAGNLRVKVSNVEVAETNEKASOD 	100 82 82 42 80 80
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus leniusculus Procambarus clarkii	101 83 83 43 81 81	POKNIE FDYHPMD PADAALLEOTFLIOVDAATVEYLKVPEEPLWVTNVRRGIIYSLPLAVLTGHNPSRLTFRLTEDTMIGG YNWYSQIKLSSY GFEOGGFPYREMD PEDAALLQIPFLVHVNESOUSYMEVPSEPAWVSNIRRGIINTIPLVVLTAOHPSRLSFGLKEETMIGG FNWYSMIKLTGH FRROEGFPYREMD PEDAALLQIPFLVHVNSOUSYMEVPSEPAWVSNIRRGIINTIPLVVLTGOHPSRLSFGLKEETMIGS FNWYSMIKLTGH TRSPLPIOYHPLEOGKDLEMPFEVSFTOPEGTEFLNAPEEPVNITNIRKSINTITIVSKGOAFKKTTFSHNGTHAGGNGACKSMISLICIPOE TRAPLPIPHVLYSKELLEKFPOLPEGREGEHLVGRAEAPNITNIKKAJVARFSIPPILANIGRRINSAKKTTFFENELGMAGROKVSMIHLNKE TOAPLPIPLVEGKELLEKFPOLPEGRGEEHLVGRAEAPNITNIKKAJVARFSIPPILANIGRRINSFKLPAVSVNETLIHGETTWVSVIRLNE	194 176 176 142 180 180
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus leniusculus Procambarus clarkii	195 177 177 143 181 181	GSÄLEDGKOEEK I EVDVORY I TSER I I GOPGAK GR PK TKR PSK TRK TSSK TSK TGTKT GTKR PGPQD PDPARL I OP IND LWVL TRT D NEG DAKV ESKKI EEVI EVDVOR FOR GAKST TAPKOKNKK TGRKKS PGK TRP FOR PTAL TVA PIOTLFIVT RSL DOQ DAV ESKKI EEVI EVDVOR FOR GAKST TAPKOKNKK TGRKKS PGK TRP STATE GTANGEHOR EAVI EGO I ORHYM FOR GAKST SKARK SV SKOSK KGAKSSKR I G EAR ERNOR EAAMEED I OREEOD FEBRA FSSKV PSK TSK TOTSK TTK TTGTK TTOTKT TGTKT TGTK TO APAKL TPP I MT LWSMSR TV DFDS EAR RENOR EAAMEED I OREEOD FEBRA FSSKV PSK TSK TOTSK TTK TTGTKT TGTKT TGTK TGTK TGTK TGTK	287 249 249 213 274 264
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	288 250 250 214 275 265	CE-ENHFTVK INGNDESADK TORSSOCTFLIRGELGGMTRLERAEVNON IVVYTTD CK-DKHFTMK INGNKESVENTORSSOCTFLIRGELGAVVRLERAEVEGNIMITODODGFIVTSKOTLOLTSVOEVETISIS	376 337 337 311 371 359
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus leniusculus Procambarus clarkii	377 338 338 312 372 360	RTLSFETKEGPLILTVEEOLTGIYPTELOAOAIIAK TKEDMELLNE - PDHSENTPPFLDTFRHVVERIAVLPKSKIESLISOVPDT PRVKKTLYOA RALSFSVADGRDJ TIKEKLIGLSPAAEOVEEMMAGILEDLSIIRR - NMAGGEAPEFLKVFORLAEMLILSWTEIEDIVSRIATS TROKYIIYOA RALSFSVADGRDST TIKEKLIGLSPAAEOVEDMAGVEDLSIIRR - NMAGGDAPEFLKVFORLVERNILSWTEIEDIVSRIATS TROKYIIYOA RLTDLRERGDETHPNLEOAVTGVPITSOLITE KEMIVSGLKTVASRMSTTPHSTOPLVREISIVEAVATITROEIDSVYSMITODELVDKKTRIFFEI FOVSGEDEPGHDIHPSLEOLVTGIRITGOVTE KYLIIKLLKMVLKMTTHPYSTOPLVKDMSTLVDAASIISRPE EALCSGIEHEELMAKKLEIFFEL FSVSEGEPGHDVHPSLEOLVTGIRITGOVTE KYLIIKLLKMVLKMTTHPYSTOPLVKDLSILVEAVSILSKPEIGALTSGIEREELMAKKLEIFFEL	471 431 431 411 471 459
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	472 432 432 412 472 460	LL TSGTKEA LAYLLEKLSDEAFOQONPSLVFQVFQASTMT VTDPSLIPTLLQLVDSMDDRRGDP-LKTTALLSIAKLAROYGLDPQVAESCDOR ALASGRDEVLIPMLELKNONDFWFHYOILOSPLTPVRNALLPAMLEVISTGEDESGAKALSLUNIAKLANNYGLNSETONLOPG VITSGRDEVLIPILEGMKNDOSSEVLUGTLLOSPLTPVRNALLPAMLEVIKIGDNPNAALSLINIAKLANNYGLNSETONLOPG LMTAGTEPTINVLIEANDOGTIROFYSVFITTEEKIGTIMKSSFSIPKLMEVVNKLTWONEERVIKSIALLNFAKLAHOYGLSPDKOMMEFGDDSCVPOH VITGGRDEVIKINDPERKTINVSVKTILGHTTSKNPLLIPDIDIDEGTITWOEENOGFKMILLUNASLAKOLGISDKWHEFGDDSCVPOH LVTAGTEPAIRYLLDKIKDPERKTINSVKTLUTTVENFHLSVKSPLVIPDIIVLEGALSWTEDDQEFKISVLINLASLAKOLGISSEKWETFGYNTCDPEY	564 520 519 511 571 559
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus leniusculus Procambarus clarkii	565 521 520 512 572 560	- CONGASLOSFLPILTRGLNDPLK - WKQLVHLQANGWLGTPLSLPYLEPIIGGKNDDN - ADLRISAIHG LKAELLPKTAHSRVLDILVPLLNNPSEFP - COTDFILTNFLPLLSRGLYDGEK - WKRIVTVOAVGALGSPYSIPYLOPIIGGTNRED - IDVRINAISCITNDHLPKTAINVVFDSLVPLUKKPTEFP - COTDFILTFELPMLSRNIVDHEK - WKRIVTQAALGSPYSIPYLOPIIGGTNRED - IDERINAISCITNDHLPKTAINVVFDSLVPLUKRPTEFP - SSPDLILVDFLPILVGGLODTDSPTWKRLVYLOALANLGTPGIIDVLKHILGDTESH - LALRWNAGFSLSSTNLPETARSTVFDLUMPVFENIGENV - TOTPGIISHFLPTILGGVODDESSLWIRLVYLOALANLGTPGIIDVLKHFLGKPSTNIHVLRTNAILGISGVIPTAOSSYGLUMPVFENTGENN - TPDGIIENFLPTILGGVODDESSLWIRLVYLOALANLGTPGIDVLKHFLUKRFSTNIHVLRTNAIGSLSSTNLPETARSTVFDLUMPVFENTGENN - TPDGIIENFLPTILGGVODDESSLWIRLVYLOALANLGTPGIDVLKHFLUKRFSTNIHVLRTNAIFGISGIYLPKTAKSTVFSLUMPVFENTGENN - TPDGIIENFLPSLVGGIQDDESSLWVKLVYLOALANLGLPGTIDVLKHFLUKRFVKRSTNIHVLRTNAIFGISGIYLPKTAKSTVFSLUPVFENTGENN	659 615 614 609 671 659
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	660 616 615 610 672 660	E ERSMAFLV ISTWEPTALSWWEH IALSTWRESSHQVAAF IYSAL TALSEGNHPRSLSAKKVVHLAKHPGKMETNTLAQGAY IYLKEDVEN CKEGENLNMA E IRSMAFLV LTTWEPS - LSWWEEVAFSTWE EKSPQVESVISST LTTLSE ONDSRSKVAKVVHLAKHPSDDDGET ITRSIVLIMEEVVRNCOMELKTKMA E IRSMAFLV LTTWEPS - LSWWEEVAFSTWE EKSPQVSVISST LITLSEOSDSRSKSAKVVHLAKHPDDBGEGPINKSVUL IMDE ENTRM OVRPKMKLA E VRNIAFL TMVMKOPS - VNWERMAVSTWE EPSOVANFISTI ITSLANGKHPLSMSARVVHLAKPTTLS THSSMTYLSE YLHNGETNSRISFG E VRAVAFLAMMTFKPS - LAWWER IA ISTWRDPSOVANFISTI ITSLANGKHPLSMSARVVHLAKPTTLS THSSMTYUSE YLHNGETNSRISFG E VRAVAFLAMMTFKPS - LAWWER IA ISTWRDPSSOVASFVSSTFTSLANSNYPLSK I A SRVYHLVK PAPHVS THSAMVYLNDYLYNC SQUYFNFG E VRAVAFLAMMTYEPS - LAWWER IA ISTWRDPSSOVASFVSSTFTSLANSNYPLSK I A SRVYHLVK PAPHVS THSAMVYLNDYLYNC SQUFFISG	759 714 713 705 767 755
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	760 715 714 706 768 756	WVASTLTVFPAAAFYSLQVTTVLGMRQREMAVFFSNYLGRVYK-RGNPEQWDIKESEDSETFFGIFEELQEOLMLTSTSFIHKETFTFASILEPL WLVSTKSIFPSSIYWQLQVVSTMGGTQEKGFIFTNVLGDYKNMYAAIHAREKDYEDGELFSELVAELIEELELIFENNEDEFPTFGMLEPL WLISTKSIFPSSIYWQLOUVSTMGGTGEKGLIFTNILGDYKNHTEIHSRKENYEDGELFNETVAELTEELEVLDNNEEGHFIFGLLEPL WLANTQGFPNKILYLOMNTFFGFTSEVKATLDQONLDEWRKFYNYFYIEHHSRGOYRGGAFUVQOLVIELKEDLGVETSLPSSINLWFRIGEAFY WFASNQGLPSKILYQLGHTFIGFTSEFVKATLDQONLDEWRKFYNYFYIEKHHSQOYRGGAFUVQOLVIELKEDLGVETSLPSSINLWFRIGEAFY WFASNQGLPSKILYQLGHTFIGFTSEFVKATLDQONLDEWRKFYNYFYIEKHHSGOYRGGAFUVQOLVIELKEDLGVETSLPSSINLWFRIGEAFY WFASNQGLPSKILYQLGHTFIGFTSEFVKATLDQONLDEWRKFYNYFYIEKHSKDAETVQEMFDEUVDULGIHTEKENDMSFYIKI.TDFI	853 809 808 805 864 852
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	854 810 809 806 865 853	H ILFS YEGEHROPGAF GPWSFLD-YNRPIIFRFTDSTLAFPLDLGLPVIVKLEED FGFYLRGDTGDFEUGHKHSVNVKTLVPWT PLLAVPGSRHASSMNIVFLWTLMS-ERIPAHFRMPEMILAFPSDLGLPVVKYEEDVAFMLGVTMGTGVTVETN	937 898 897 902 963 950
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	938 899 898 903 964 951	EG - RAAGAGLESNMQIRLPLRSRGEWR PVKLTLTLPTS · KVNLI EMSGCMYTSLTPLFPFTGAALSPEEP-ATSLGRKVVWKGEVLESSKSRLLDKL NG - ITVGVGIENSFDVNLMLSADVERROGKDVVVLSVSPEQKAKI I YLSSVMYSSMTKLYHVVDSLLTSEESDESTGLGRRVIWNRRFKEGT DLL LPDT MG - MAVGYGFESFDVNLMUTVNLNIRGGTKVVLTISPEQKAKI I SLSNVMYTSMTKLYHVVDSLTSEESDESTGLGRRVIWNRFKEGT DLL LPDT OMNFAVGAGLHMNLSVTVPIVLSGILDMRKGEIOLTVEPLNNKVQLVDSHNP FTVLGGFPTTVYTOGHOYMTIKNTDPEVFSRTTGALPSVV KK. F GVGAGLHNVSLVL PFSVHTNLDMRHSELKVTIEPODSSKVQLFNIHNV P FTVVTGPFPTAVHTQGAEYKK I SAFPLETLVHKHQVL PAIA	1032 995 994 997 1057 1044
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	1033 996 995 998 1058 1045	G IHL TCEWIGD FOOPGD PK TV IR-ELH PNOLHYON FEYRLYFD PTSSOSKVIVF IGH YYKKEIKISTOGPFEOVSOGAT-DYD Y POVSGGSSD FE GLHLLKWSCDLEMPSDF TNVIE-KLLISOLYVON FEWNLIVDLENSRMKIVT FTSFDKKEROEFPEOETDYDOG FTOLS. DLDLFSOP POSPSOTS GLHKLRWTCDLEMSSYFTXVIK-KVELWOTIYON FEWNLIVDLENSRMKIVT FTSFDKKEROEFPEOTDYDOG ITOLS. DLDLFSOP POSPSOTS GLSVESSWSADF EFPINFASIH ROPFDLFTPAYKLWEYSLELN PETSTRKITTTFOYTMORRENLWEOVDEGEFGGESO-GSDYSOMS GLSVEASWAKD FPSOFD FLHFSDDFDFINPAYKSWDYSIVLD PAASTTKST FTFTAYVILEKAED IVOORFEGFGGETQGAADY VDFS-OTHVGG- GLSVEASWAKD FPSOFD FLIFFSDDFDFLIPAYKAWDYTILYD PAASTTKSTFTFTTYVILEKLED FORGOT-SEOFHOETQ-SDYFDRSADYFDFSVGG	1126 1093 1092 1086 1153 1141
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	1127 1094 1093 1087 1154 1142	I FSOTSSGGGDFVE-OEETO TOTVITHMORVEELA INFTYGVINSVSLRIEPDA GPOKYEFA ISLITSKOEKLGVKA PINFHLTFANSSVTEIFGDS YW GFOGF-EVPNEVIFDROODHTSSLDNYXGRMOEMIM-PSGTLYTA IMTVEPDNSTOKYDVAVTLASIDG-NVNEKOSIKVHVVFMSPALGFFGDTHMT GFDDFGEGANEAVFDROODHSSSFNYVGRMOEMIM-PSGTLYTA IMTVEPENSTKYDVAVTLASIDG-NVNEKOSIKVHVVFMSPITGSLGDTHMT 	1225 1189 1189 1176 1252 1240
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	1226 1190 1190 1177 1253 1241	G ID AS LYS PYL TPLMPK EV I LETDLSDDFLLTYKKAAY GEDPPILYLKARODVS TTALEYLGAEAERE TODGSFPORRDTODG GLDIKWETFFVLPILPKELLS TDLATNFLMNLYSGGH EGPPOMKMOGMFGVSPAAVQNIGST. EETGVGDLGKRRPTYDLIIGATWTOD GLDVKVETFFVLPIVPKELSSTDLATNFLVSLYGGH OGRAVMKOMGOMFGVSPAAVQNIGST. EETGVGDLGKRRPTYDLIIGATWTOD GVNLTYVKLFFLPMATTEEVLSSNFHTTVDAEYVGGH OGRAVMKOMGOMFGVSPAAVQNIGST. EETGVGDLGKR GNVQLKFFLPMATTEEVLSNFHTTVDAEYVGGS SSPVLEVGGTLDVSEKKLEHIREELA-KE GPEITHLLPIDIITSPLYDHAN KAYWEED GNVQLVK PAFLPMPTLEEVLSDNFHTTIAEYVGGS SSPVLEVQGTLDVSOEVVRRIREELA-KE QDEITHLLPIDIITSPLYDHAHFTARWTED GNVQLVK PAFLPMPTLEEVLSVNFHTTIEATYFDGESGSSAPVLEVQGTLDVSOEVVRRIREEVA-KEQDLADSULPIDVITSPLYDHAHFTARWTED	1306 1280 1282 1276 1351 1339
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	1307 1281 1283 1277 1352 1340	I I DNVEAFRAWS I HNLDLTVVDVVRDRVSQSDNL ISVDALLNLKVNEWKLRT I GPHGT FVYSVPAESLDV I TSSYVQNNVFDV I SSSLDKV <mark>C</mark> YL FRHLYRAVED I TLNQLOSLSTANVKIEGDRPONELI I RGVLNE EEWNVTVTRPELTSIFLVP OMLFLSPYDTFVS-FEKVCHV FRHLFRAVEALTINQLOSLSTANVKSEGORPONELI I RGVLNE EEWNVTVTRPEVTSVFLVP OVLYLS	1400 1362 1364 1376 1451 1439
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	1401 1363 1365 1377 1452 1440	TTHSIRTFDGLVHEFEPSSWITTAAIHIPP-PPLSSLÄTKALOVAVQARYIDDQWEVRISWPLSALILDVTKSEILVNEEPLSGQSDKFVVHRLOD TSDSVQTYDGLTYAFQGPSWITATIFSDSKTPLLWQEVYNDLQMAVQYRFT-DQWEVRLTAPSYAVVIDUTRSDIFVNDEPLIGQNEKFIVHRLON TSDSVQTYDGLTYAFQGPSWITATIFSDSKLDMYSNEAYNDLQMAVQYRFT-DQWEVRLTAPSYAVVIDUTRSDIFVNDEPLIGQNEKFIVHRLON EDTOYRTLDGTWFFSHOPDACWTTASIFHADPE-DPEAMEGALWYRHT-DQWEVRITAPSYAVVIDUTRSDILVNGPYEER-DKTYEFIRLSDAG EKTKVXTHDGVFFPHPDOSWTTLGGHOLGOGKGPLWYINMARFT-DEWEVRIVAFFGVIVFTKSQLUVNGPYEER-DKTYEFIRLSDAG ETTKVRTHDGVKFPYHPDNGWTTLGSFHADPE-EEAPLNAVVSARFT-EEWEVRIVGNFGVIVEFTKSQLUVNGEPZEBOEIVRFSVED	1495 1458 1460 1468 1544 1532
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus leniusculus Procambarus clarkii	1496 1459 1461 1469 1545 1533	SQLV EVGRVLVWVGNTVEIRISDVYYA EVTGLGGNYDGEPSNDMKGPLGGLYDDALHTTAWSSFALGGSRLALRAKKRKLEEGDDTGDKEVFRTTGLSQ RMMIKIGMVLIRVGDTYDMVSDUYYGAVKGIGCNYDGEPINDMMGFTGCLSX.VTLAIHPR LLIFADGCF-IKVSDKVEILDFOULRGKIEGLGKMDGERSDLVGFKGIVTNLTIFALSMTTBSODGDVFALOSKKDEVELFGKTGSRESVIPTGVSH LTTKKMMAFAIKVSDKVEILDHOULRGKIEGLGGKMDGEKSDLVGFKGIVTNLTIFALSMTTKEGGAOFSLRSKKDEVELFGKTGPRETHIPTGVSH YFMLKSATLSIKVSDKJEULHINIAGUGGGGGGGGGGGUGGDGGKTDDLVGFGGIVGDPALFALMTTKGDGGAOFSLRSKKAVENGGEVPRETHIPTGVSH	1595 1519 1522 1567 1644 1632
HSTBP Portunus trituberculatus Scylla paramamosain Hormarus americanus Pacifastacus Ieniusculus Procambarus clarkii	1596 1520 1523 1568 1645 1633	QNV MEN G TAWEY HER TSPAGLLGRSRV FV PVG LPG E VG IAFTSNLKYD GSQPSSS - A EQQOQYDGSRQCLSKV LPA TIAAGCFRQIRS LEY GSYRLGKVY PS LCKVY PS DVVYD GTEWLY QEK IFG - NYKGKSMV FTPFG KIEGEATEN ITKPIAYD GYWSEERVU QLGSTDKACY PHTYV TNY PASCV PH PNV LYD GTEWQY VER FFG - DYRGKSMV FTPFG KIEGEATEN ITKPIAYD GYWSEERVU QLGSTDKACY PHTYV TNY PASCV PH PNV LYD GTEWQY VER FFG - DYRGKSMV FTPFG KIEGEATEN ITKPIAYD GYWSEERVU GLGSTDKACY PHTYV TNY PASCV PH PNV LYD GTEWGY VER FFG - DYRGKTIDATPVG KDTGKATRY TLPIAYD GQV FGG PNV LYD GTEWGY VER FFG - DYRGRTIDATPVG KDTGKATRY TLPIAYD GQV FGH RNNTTHGECHPHSIITTF PASCV P	1683 1534 1537 1649 1722 1710

Figure 4. Deduced amino acid sequence alignment of TTX binding protein, HSTBP, with clotting proteins of shrimp and crab from the suborder Pleocyemata. Accession numbers for clotting proteins

(DDBJ/EMBL/GenBank databases): XP_045118682.1 for *Portunus trituberculatus*; AYK27566.1 for *Scylla paramamosain*; KAG7156252.1 for *Hormarus americanus*; AAD16454.1 for *Pacifastacus leniusculus*; AYD41596.1 for *Procambarus clarkii*. The residues identical with those of HSTBP are shown by gray and gaps by dashes. Signal peptides are boxed in straight lines. Cysteine residues are highlighted in white on a black background. Putative N-glycosylation sites are indicated by closed circles. Amino acid numbers are shown at the left and right.

Analysis using the SMART program showed a lipoprotein N-terminal domain at Leu³⁷-Thr⁷⁰⁴ and vWF type D domain at Phe¹³⁸⁷-Gly¹⁵⁴⁴. A three-dimensional structure model of HSTBP sub-2 containing the vWF type D domain was constructed using Colabfold (Figure 5A). It comprised three independent domains (N-domain, M-domain, and C-domain), and the M-domain corresponded to the vWF type D domain (Figure 5A). Figure 5B compares the 3D structural models of the vWF type D domains between HSTBP sub-2 and TfVWF from the pufferfish *Takifugu flavidus* liver [18]. The key amino acids Asp¹¹⁵, Val¹¹⁷, and Lys¹²² in TfVWF were predicted to mediate binding to TTX using in silico structural and docking analyses [18]. However, they were not conservative in the HSTBP sub-2.

(B)



Figure 5. (**A**) Three-dimensional structure of HSTBP sub-2; (**B**) three-dimensional structures of vWF type D domain of HSTBP sub-2 (left) and TfVWF (right).

(A)

3.5. Binding of rHSTBP-sub2 with TTX

The rHSTBP-sub2 was successfully produced by the *E. coli* expression system and solubilized using the L-arginine-aided refolding procedure. It produced a single band on SDS-PAGE with a molecular mass of 60 kDa, indicating its homogeneity (Figure 6A). The rHSTBP sub-2 was combined with TTX and subjected to gel filtration chromatography on a Sephacryl S-300 column. The elution profile is shown in Figure 6B. TTX was detected in two fractions, Fr. 11 and Frs. 24–45. The former fraction contained TTX bound to a high molecular weight substance, rHSTBP-sub2, and the latter fraction contained TTX in free form. Based on the concentrations of proteins and TTX in Fr. 11 and the molecular masses of rHSTBP sub-2 (60 kDa) and TTX (319 Da), TTX-binding activity of rHSTBP sub-2 was measured at 5.51 µg TTX/mg, equivalent to 1:1 stoichiometry TTX/protein.



Figure 6. Recombinant protein of TTX-binding protein subunit-2, rHSTBP sub-2: (**A**) SDS-PAGE, lane 1: marker; lane 2: sample; white arrow indicates recombinant protein of TTX-binding protein subunit-2, rHSTBP sub-2; (**B**) Sephacryl S-300 column chromatography; Sample: recombinant protein of TTX-binding protein subunit-2, rHSTBP sub-2, mixed with TTX; column: Sephacryl S-300 (2×29 cm); mobile phase: 0.02 M Tris-acetic acid (\Rightarrow Change to "acetate") buffer (pH 7.4); flow rate: 0.5 mL/min. Fraction: 4 mL/Fr.

4. Discussion

We isolated the TTX-binding protein HSTBP from the hemolymph of *H. sanguineus* by salting out with ammonium sulfate, lectin affinity chromatography on a Con A-Sepharose column, ion-exchange HPLC on a MonoQ 5/50 GL column, and preparative electrophoresis. HSTBP is an acidic glycoprotein with a molecular mass of 400 kDa by gel filtration HPLC on a TSKgel G3000SW_{XL}. This protein comprised three subunits of 88 (subunit-1), 65 (subunit-2), and 26 kDa (subunit-3) by SDS-PAGE under reduced conditions, suggesting that HSTBP is a homodimer. We elucidated the primary structure of the protein using cDNA cloning. The ORF of the cDNA consisted of 5049 bp encoding 1683 amino acid residues, and the mature protein contained 1650 amino acid residues from Arg³⁴ to Ser¹⁶⁸³; cDNA cloning showed that the three subunits were arranged in tandem in the following order: subunit-3 (Arg³⁴-Gln²⁶¹ with a calculated molecular mass of 25,509.7), subunit-1 (Asp²⁶²-Phe¹¹³⁸ with the calculated molecular mass of 98,822.3), and subunit-2 (Val¹¹³⁹-Ser¹⁶⁸³ with the calculated molecular mass of 60,784.6). Subunit-1 may be cleaved by post-translational modification because the molecular mass estimated by SDS-PAGE was approximately 10 kDa smaller than that calculated. Subunit-2 may have an N-type sugar chain because the molecular mass estimated by SDS-PAGE was higher than that predicted and decreased

in molecular mass by deglycosylation with PNGase F. Furthermore, the subunits may have been bound by disulfide bonds. On SDS-PAGE, HSTBP segregated into three subunits under reduced conditions, while it did not enter the polyacrylamide gel under non-reduced conditions due to aggregation. These results imply the formation process of HSTBP as follows: after translation, the TTX-binding protein (Met¹-Ser¹⁶⁸³) with a molecular mass of 188,565.4 is cleaved off the signal peptide (Met¹-Gly¹⁷) and the subsequent peptide (Leu¹⁸-Ala³³), glycosylated with the subunit-2 region, separated into three subunits, and cleaved off the C-terminal region of subunit-1. These modified subunits may then rearrange via disulfide bonds to form dimers.

A previous study determined the molecular mass of the TTX-binding protein to be approximately 400 kDa by gel filtration on a TSKgel G3000SW and approximately 72 and 82 kDa by SDS-PAGE, indicating that the TTX-binding protein comprised at least two kinds of subunits with molecular masses of approximately 72 and 82 kDa [21]. In contrast, this study showed three subunits with molecular masses of 88, 65, and 26 kDa on SDS-PAGE; cDNA cloning verified that the TTX-binding protein comprised three subunits. The previous study might have overlooked the third subunit (subunit-3 with a molecular mass of 26 kDa in the present study) because a high-molecular-weight calibration kit containing myosin (212 kDa), macroglobulin (170 kDa), β -galactosidase (116 kDa), transferrin (96 kDa), and glutamic dehydrogenase (53 kDa) was used as a marker. The shore crab hemolymph has a TTX-binding protein other than HSTBP, as determined by ion-exchange HPLC on a MonoQ 5/50 GL column (Figure 1). This protein could be an isoform of HSTBP because it exhibited the same pattern as HSTBP on SDS-PAGE (Figure 2). Further studies are necessary to elucidate the sequences of other TTX-binding proteins.

SMART program analysis identified a vWF type D domain in HSTBP subunit 2. It has been reported that the vWF type D domain may be involved in the toxification of pufferfish. Yin et al. [17] demonstrated the presence of a pufferfish toxin-binding protein in the ovaries of *T. pardalis* (*Takifugu pardalis* ovary toxin-binding protein with a molecular mass of 10 kDa, TPOBP-10) and identified it as a vitellogenin-1-like protein [*Takifugu rubripes*] subdomain, a vWF type D domain. Furthermore, Qiao et al. [18] confirmed that the vWF type D domain bound TTX using a recombinant protein of TfVWF from *T. flavidus* liver. Surface plasma resonance analysis evaluated the weak affinity of the protein to TTX with an equilibrium dissociation constant of 2.92×10^{-3} M. Although kinetic analysis has not been performed in this study, it is evident that HSTBP sub-2 binds TTX at a 1:1 molar ratio. The binding mechanism of the TTX-binding protein HSTBP remains to be elucidated in the context of TfVWF in the pufferfish liver.

Barber et al. [32] observed that the small shore crab *Hemigrapsus oregonesis* along the coasts of British Columbia, Canada, developed resistance to STX when exposed to the red tide bloom of *Gonyaulax catenella* (presently *Alexandrium pacificum* [Group V]). In contrast, crabs did not resist TTX administration. Notably, the shore crabs belonging to the genus *Hemigrasus* showed differing resistance activities to the toxins between the two species of *H. sanguineus* and *H. oregonesis*: the former exclusively resisted TTX, whereas the latter resisted only STX. *H. oregonesis* induced the production of a STX-resistant protein complex with a molecular mass of 145 kDa in the viscera by exposure to the wild toxic dinoflagellate bloom and by artificial treatment with STX in a dose-dependent manner, suggesting the involvement of the STX-resistant protein in detoxifying activity of crabs [33]. However, the TTX-binding protein of *H. sanguineus* hemolymph is not likely associated with TTX toxification, but by binding the toxin. Further investigation is in progress on the disposition of the TTX-binding protein in shore crab tissues and the distribution among shore crab species to clarify the physiological role of the protein.

Otherwise, TTX-binding protein could be a promising target for developing an antidote and applied to diagnostics for TTX intoxication because of its unique properties of specific binding to TTX and neutralization against TTX toxicity. Furthermore, it would be applicable to biochemical research to purify, identify, and analyze TTX in biological samples because we previously developed affinity chromatography using partially purified TTX-binding protein as a ligand [34].

5. Conclusions

This study revealed a novel TTX-binding protein, HSTBP, from the hemolymph of non-toxic shore crab *H. sanguineus*. The protein comprises three subunits, Arg³⁴-Gln²⁶¹ (subunit-3), Asp²⁶²-Phe¹¹³⁸ (subunit-1), and Val¹¹³⁹-Ser¹⁶⁸³ (subunit-2). HSTBP showed a weak similarity (29–40%) to clotting proteins of crustaceans and conserved vWF type D domain at Phe¹³⁸⁷-Gly¹⁵⁴⁴ in subunit-2. The recombinant protein of subunit-2 bound TTX at a molecular ratio of 1:1. Therefore, HSTBP may neutralize TTX and prevent the lethal toxicity of TTX. It could be applicable to possible antidotes for TTX intoxication.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse11010181/s1, Figure S1: Strategy of 5' RACE and 3' RACE.

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