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Bacterial Production and Refolding from Inclusion Bodies of a "Weak" Toxin, a Disulfide Rich Protein

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Abstract—The gene for the "weak" toxin of *Naja kaouthia* venom was expressed in *Escherichia coli*. "Weak" toxin is a specific inhibitor of nicotine acetylcholine receptor, but mechanisms of interaction of similar neurotoxins with receptors are still unknown. Systems previously elaborated for neurotoxin II from venom of the cobra *Naja oxiana* were tested for bacterial production of "weak" toxin from *N. kaouthia* venom. Constructs were designed for cytoplasmic production of *N. kaouthia* "weak" toxin in the form of a fused polypeptide chain with thioredoxin and for secretion with the leader peptide STII. However, it became possible to obtain "weak" toxin in milligram amounts only within cytoplasmic inclusion bodies. Different approaches for refolding of the toxin were tested, and conditions for optimization of the yield of the target protein during refolding were investigated. The resulting protein was characterized by mass spectrometry and CD and NMR spectroscopy. Experiments on competitive inhibition of ^{125}I-labeled α-bungarotoxin binding to the *Torpedo californica* electric organ membranes containing the muscle-type nicotine acetylcholine receptor ($\alpha1_2\beta1\gamma\delta$) showed the presence of biological activity of the recombinant "weak" toxin close to the activity of the natural toxin ($IC_{50} = 4.3 \pm 0.3$ and 3.0 ± 0.5 μM, respectively). The interaction of the recombinant toxin with α7 type human neuronal acetylcholine receptor transfected in the GH_4C_1 cell line also showed the presence of activity close to that of the natural toxin (IC_{50} 31 ± 5.0 and 14.8 ± 1.3 μM, respectively). The developed bacterial system for production of *N. kaouthia* venom "weak" toxin was used to obtain ^{15}N-labeled analog of the neurotoxin.

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Key words: neurotoxins, nicotine acetylcholine receptor, bacterial expression, refolding

α-Neurotoxins from snake venoms are highly specific competitive inhibitors of nicotine acetylcholine receptor (nAChR), which is a ligand-gated ion channel [1, 2]. Depending on their localization, receptors of the nAChR family are divided to two large classes—muscular and neuronal. Numerous diseases of nerve and muscle systems like epilepsy, Alzheimer's disease, Parkinson's disease, nicotine addiction, and muscular dystrophy are related to dysfunctions of nAChR [3, 4]. Thus, knowledge of neurotoxin spatial structure as well as understanding the mechanisms of neurotoxin interactions with nAChR on the molecular level is an important fundamental problem of molecular biology.

The snake venom α-neurotoxins are β-structural proteins with prevalent "three-loop" structure stabilized by a system of disulfide bonds (Fig. 1). Structurally, α-neurotoxins can be conventionally divided to three large classes: short-chain α-neurotoxins (60-62 amino acid residues (aa), four disulfide bonds), long-chain α-neurotoxins (66-75 aa, five disulfide bonds, the additional disulfide bond is located in the central loop), and "unusual" neurotoxins (62-68 aa, five disulfide bonds, the additional disulfide bond is located in the first, N-terminal, loop) (Fig. 1) [1, 5]. Short-chain and long-chain α-neurotoxins interact with equal efficiency with muscle nAChR, but only long-chain neurotoxins with the fifth disulfide bond

Abbreviations: DTT, dithiothreitol; IC_{50}, inhibition constant indicating that 50% receptor binding sites are blocked; nAChR, nicotine acetylcholine receptor; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; WTX, "weak" toxin.

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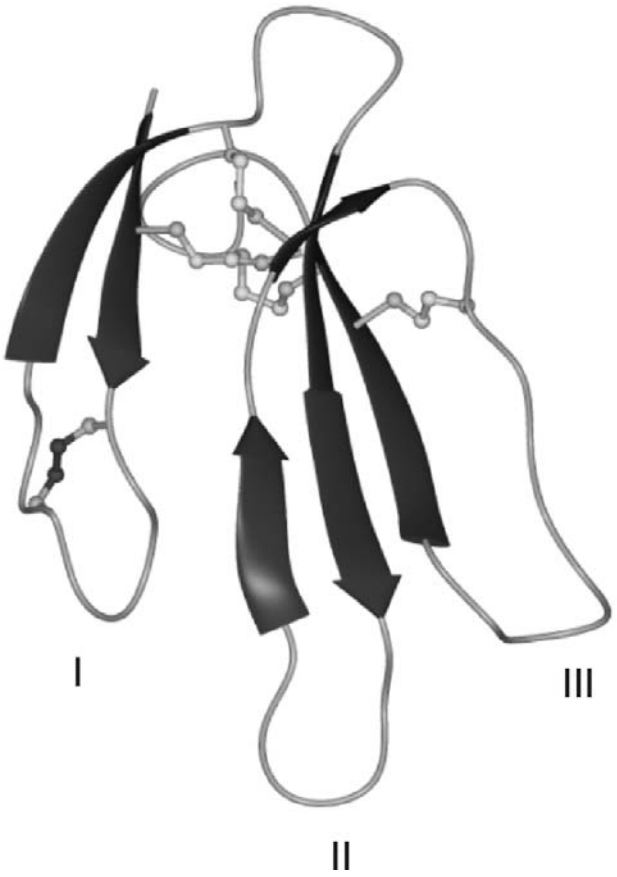

Fig. 1. WTX molecule in ribbon representation. Loops formed by β-pleated sheets are designated by Roman numerals. Zig-zag lines point to disulfide bonds. The disulfide bond in the N-terminal region of the molecule is shown in dark gray [26].

in the central loop are able to interact efficiently with α7 type neuronal nAChR [6, 7].

Both muscle nAChR and neuronal α7 type nAChR serve as targets for "unusual" toxins [8]. "Weak" toxin (WTX) from *Naja kaouthia* venom, a member of "unusual" toxin family, was found rather recently [8, 9]. It has been noted that this toxin exhibits unique biological properties. For example, compared to long-chain α-neurotoxins, WTX has lower toxicity and affinity to nAChR but finally causes practically irreversible blocking of both muscle receptors and neuronal α7 type nAChR [8]. Results of investigations *in vivo* of WTX activity show that in addition to nAChR, WTX has also other targets in the nervous system, in particular muscarinic choline receptors [10]. There are also data indicating that WTX retards proliferation of lymphocyte cell lines and increases secretion of antibodies [11]. However, unlike short-chain and long-chain neurotoxins, there is practically no information concerning the mechanisms of interaction of "unusual" toxins with nAChR. Development of efficient bacterial production systems making it possible to obtain these proteins as well as their mutant and isotope-labeled variants in milligram amounts will facilitate studying in detail functional and structural properties of "unusual" toxins.

The presence of disulfide bonds in neurotoxin molecules makes it very difficult to obtain these proteins *in vitro*. There are now only several examples of successful bacterial production of functionally active snake α-neurotoxins in amounts sufficient for structural-biological investigations [7, 12-14]. Earlier, the authors developed two systems for bacterial production of neurotoxin II from *Naja oxiana* venom and its chimeric variants with an additional disulfide bond [7, 12]. In one of these systems toxin was produced within a fused polypeptide chain with thioredoxin [12], while in another system the toxin and its chimeric variants were obtained by secretion into the periplasmic space [7]. However, in the case of WTX from *N. Kaouthia*, the necessary level of production was not achieved in any of these systems.

Therefore, the aim of this work was to develop an alternative method for WTX production in the form of *E. coli* cytoplasmic inclusion bodies and subsequent refolding. Optimal refolding conditions were investigated (concentration of oxidized and reduced glutathione forms, pH, buffer composition, presence of urea). The final yield of renatured toxin was about 5 mg/liter bacterial culture. Correct spatial structure of the resulting protein was confirmed by CD and NMR spectroscopy. Investigation of the interaction of the recombinant toxin with membrane preparations from *Torpedo californica* electric organ containing the muscle type nAChR and with GH_4C_1 cells transfected with α7 type human nAChR has shown biological activity close to that of the natural toxin. The developed bacterial system for WTX production was used to obtain for the first time the ^{15}N-labeled analog of "weak" toxin.

MATERIALS AND METHODS

Reagents of following companies were used throughout this work: ICN Biomedicals (USA), Serva Feinbiochemica (Germany), FisherBiotech (USA), Merck (USA), Difco Laboratories (USA), QIAGEN (Germany), Promega (USA), Fermentas (Lithuania), GE Healthcare (USA), and Sigma-Aldrich (USA). Membranes of *Torpedo californica* electric organ were provided through the courtesy of Prof. F. Hucho (Free University, Berlin, Germany); GH_4C_1 cells transfected by human α7 type nAChR were obtained from Eli Lilly (Great Britain). Natural "weak" toxin and α-cobratoxin from the cobra *N. kaouthia* venom were isolated as described previously [15, 16].

Cloning of the "weak" toxin gene. The WTX gene was obtained earlier [8]. Rare codons of arginine residues in positions 32, 37, and 40 were optimized using the polymerase chain reaction. Choosing codons used for

Oligonucleotide sequences used in this work

Designation	5′-3′-oligonucleotide structure
Forward primer for replacement of rare arginine codons	GCTTTAAAAAGTTACACCAACGT**CGC**CCATTGTCGTGG**CGC**TACA-TA**CGC**GGATGTGCTGATACTTGCCC
Reverse primer for replacement of rare arginine codons	GGGCAAGTATCAGCACATCC**GCG**TATGTA**GCG**CCACGACAATGG-**GCG**ACGTTGGTGTAACTTTTTAAAGC
Forward primer for WTX gene cloning into vector *pET-32a(+)*	CTGGGTACCGACGACGACGACAAGTTGACATGTCTCAATTGCCC *Kpn*I
Forward primer for WTX gene cloning into vector *pET-22b(+)/STII*	ACAAATGCGTACGCATTGACATGTCTCAATTGCCC *Bsi*WI
Forward primer for WTX gene cloning into vector *pET-22b(+)*	ACAAATGCATATGCATTGACATGTCTCAATTGCCC *Nde*I
Reverse primer for WTX gene cloning into vectors *pET-22b(+)* and *pET-32a(+)*	GGCCCGGATCCCCCGATCCTTACTAGCGGTTGCATTTGTCTG *Bam*HI

Note: Arginine codons are distinguished in bold. Restriction sites for following cloning in commercial vectors are underlined.

oligonucleotide sequences was based on their frequencies in well-expressed *E. coli* genes. The WTX gene was cloned into commercial vectors *pET-32a(+)* (Novagen, USA) at restriction sites *Kpn*I and *Bam*HI, into *pET-22b(+)* (Novagen) at restriction sites *Nde*I and *Bam*HI, and into the earlier developed vector *pET-22b(+)/STII* [7] at restriction sites *Bsi*WI and *Bam*HI. Oligonucleotide sequences of the used primers are given in the table.

Expression of WTX gene in *E. coli*. Cells of *E. coli* strain BL21(DE3) (Novagen) were transformed with vector *pET-22b/WTX* and plated on Petri dishes with LB agar and ampicillin. Colonies from a dish were inoculated into 30 ml nutrient TB medium containing ampicillin (100 μg/ml). The cells were cultured at 37°C with moderate mixing (250 rpm) to achievement of cell density in the culture corresponding to absorption level 0.6 at 600 nm. Then the cell culture was pelleted under mild conditions (1000*g*), the pellet was resuspended in sterile conditions in 1 liter of TB, and growing was continued in a Bioflow 3000 fermenter (New Brunswick Scientific, USA). Cultivation was continued at 37°C under automatic maintenance of relative oxygen content in the system at the level of 30% of the maximal possible. Rates of mixer revolution and air supply were regulated. The WTX gene expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to final concentration 0.025 mM at the cell density in culture corresponding to absorption level 1.0 at 600 nm. After induction, cell growing continued for 18 h.

To produce ^{15}N-labeled "weak" toxin, 1 liter of cell culture grown in TB medium to cell density 1.0 at 600 nm was centrifuged under mild conditions (1000*g*). The cell pellet was resuspended in sterile conditions in 1 liter of minimal medium M9 containing ^{15}N-labeled ammonium chloride (CIL, USA) as a source of nitrogen. Induction and further cultivation were carried out similarly to those in TB medium.

Washing of inclusion bodies. Cells were collected by centrifugation (10,000*g*, 20 min, 4°C) and resuspended in cold buffer containing 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0 (buffer A) at ratio 10 ml buffer per 1 g pellet. Then the cell suspension was disintegrated by ultrasound (Branson Digital Sonifier, USA) at output power 50 W and 4°C for 5 min. The resulting suspension was centrifuged at 36,000*g* for 20 min. The pellet was resuspended in buffer A with addition of 2 M urea. The suspension was transferred into a cooled glass, sonicated for 10 sec, and centrifuged under the same conditions. The procedure was repeated twice. After washing with 2 M urea, the pellet was washed twice with buffer A containing 1% Triton X-100 and then twice with Milli-Q deionized water (Millipore, USA). Washed inclusion bodies were stored at −20°C.

"Weak" toxin recovery and purification from inclusion bodies. Preliminarily washed inclusion bodies were resuspended in cooled 50 mM NaP_i, pH 7.4 (buffer B), containing 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), at a rate of 200 ml per 0.5 g of inclusion bodies. Suspension was disintegrated at the output power 50 W and at 4°C for 1 min. Then 5 mM dithiothreitol (DTT) and dry urea (Carl Roth, Germany) till 8 M were added to the mixture. In this mixture, inclusion bodies were kept for 1 h under mild mixing. The mixture was centrifuged at 36,000*g* and 4°C for 20 min to remove cell debris. The supernatant pH was adjusted by HCl addition to 5.0 and it was centrifuged again under the same conditions. Then pH of solution was adjusted to the former value 7.4. To remove contaminants of lipid origin interfering with refolding process [17, 18], the obtained protein solution was filtered through Hγ-gel preliminarily

equilibrated in buffer B which is the macroporous polymethyl methacrylate sorbent with pore permeability up to ~500-1000 kDa (joint development of State Scientific and Research Institute of Especially Pure Biopreparations, St. Petersburg, and the Institute of Bioorganic Chemistry, Russian Academy of Sciences; manufactured by Yarsintez Company, Russia). The "weak" toxin was purified using the ion-exchange resin SP-Sepharose (Amersham, USA) equilibrated in advance with buffer B containing 8 M urea and 5 mM DTT. After protein application, the column was washed with three volumes of buffer B containing 5 mM DTT, and WTX was eluted in a NaCl gradient. Immediately after chromatography, the pH in all toxin-containing fractions was adjusted to 3.0.

Refolding of reduced WTX. "Weak" toxin collected after chromatographic purification was treated by tenfold molar excess of TCEP, concentrated twofold on a YM-1000 ultrafiltration cell (Millipore), and buffer was replaced by aqueous solution containing 0.3 M NaCl, pH 3.0, by gel filtration on Sephadex G-25 (GE Healthcare, Sweden). The protein fraction after gel filtration was diluted with 50 mM Tris-HCl, pH 8.5, 1.5 M urea, 3 mM GSH, and 0.3 mM GSSG to final toxin concentration 0.1 mg/ml. The protein was renatured during 3 days at 4°C.

HPLC of neurotoxins. Neurotoxins after refolding were analyzed and purified on a 4.6 × 250-mm Jupiter A300 C4 chromatographic column (Phenomenex, USA) on a Knauer Smartline device (Germany). Proteins were eluted in 10-45% acetonitrile gradient for 50 min in the presence of 0.1% trifluoroacetic acid. The purified toxin preparation was lyophilized.

CD and NMR spectroscopy. CD spectra were obtained at room temperature on a Jasco J-810 spectrometer (Japan). The studied proteins were dissolved in 50 mM Tris-HCl, pH 8.5, to final concentration 0.05 mM. Toxin preparation with reduced disulfide bonds was obtained after addition of 5 mM TCEP.

NMR spectra were measured in aqueous solution (pH 4.5, 5% D_2O) at 32°C on an AVANCE-700 spectrometer (Bruker, Germany) with operating frequency for protons of 700 MHz and equipped with a sensor with cryogenically cooled ^{1}H-coil. The concentration of the toxins was 0.1 mM.

Mass spectrometry. Spectra were obtained on an Ultraflex TOF/TOF time-of-flight mass spectrometer (Bruker Daltonics, Germany) equipped with a MALDI ion source. Positively charged ions were detected in linear mode.

Recombinant WTX interaction with membranes of *T. californica* electric organ and GH_4C_1 cells transfected by human α7 nAChR was analyzed by its ability to compete with ^{125}I-labeled α-bungarotoxin (2000 Ci/mol) (GE Healthcare, GB) in accordance with a published method [19] with some modifications. Buffers for binding (20 mM Tris-HCl, 1 mg/ml BSA, pH 8.0) and quick washing on filters (20 mM Tris-HCl, 0.1 mg/ml BSA, pH 8.0) used in the case of membranes were also used for work with cells. Time of incubation of the preparation with WTX was increased to 2 h. The range of tested concentrations of recombinant and natural WTX was from 1 to 50 μM.

Experimental data were analyzed in the Origin 6.1 software environment (MicroCal Software Inc).

RESULTS AND DISCUSSION

Bacterial production of WTX. Previously we developed and successfully used two systems for bacterial production of short-chain neurotoxin II from *N. oxiana* venom, its isotope-labeled variants, and chimeric analogs with an additional disulfide bond in the central loop [7, 12, 20]. In one of these systems neurotoxin was produced in the form of a fused polypeptide chain with thioredoxin [12]. A special linker containing site for specific hydrolysis by enteropeptidase was placed between the neurotoxin and thioredoxin sequences. The other system, more successful concerning the final yield of target protein, was intended for bacterial secretion of the neurotoxin [7, 20].

In the course of this work, both systems were tested for bacterial production of WTX (Fig. 2, a and b). In the first system the hybrid protein WTX–thioredoxin was accumulated in soluble form in the cytoplasm. A protocol for hybrid protein purification using metal-affinity chromatography was selected. However, we failed in finding conditions for specific hydrolysis of the fused protein by enterokinase. This may be due to the presence in the "weak" toxin molecule of a disulfide bond between third and twenty-fourth cysteine residues, which, evidently,

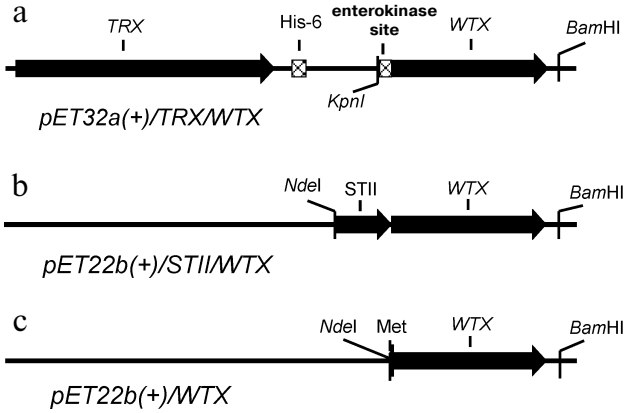

Fig. 2. Schematic representation of the WTX gene within different constructs for "weak" toxin bacterial production. a) Construct for WTX production in the form of fused polypeptide chain with thioredoxin. b) Construct for WTX secretion with STII peptide as the leader peptide [7]. c) Construct for "direct" expression of the WTX gene.

creates steric difficulties for enterokinase. The other system developed for bacterial secretion appeared to be even less successful because in this case the yield of target protein was very low (~50 μg from 1 liter of bacterial culture). Owing to this, the system on the basis of commercial vector *pET-22b(+)* was developed for "direct" expression of the WTX gene (Fig. 2c), which resulted in recombinant toxin accumulation in the bacterial cell cytoplasm in the form of inclusion bodies. To select optimal conditions for WTX production, different IPTG concentrations were tested within the range of 0.01-1 mM along with different time of cell growing after induction (3, 9, 18, 24, and 36 h). IPTG concentration 0.025 mM and 18 h incubation after IPTG addition were chosen as optimal.

WTX refolding from inclusion bodies. Previously a method was developed allowing refolding of recombinant proteins from inclusion bodies using an intermediate S-sulfitized protein derivative [17, 18, 21]. This method was probed for WTX refolding. WTX was dissolved from inclusion bodies using sodium sulfite in the presence of sodium tetrathionate, and then the preparation was purified in two steps by chromatography on DEAP-Spheronite-OH (joint work with State Scientific and Research Institute of Especially Pure Biopreparations, St. Petersburg and Institute of Bioorganic Chemistry, Russian Academy of Sciences) and on SP-Toyopearl (Tosoh, Japan). Final yield of the sulfitized toxin dissolved from inclusion bodies was about 60 mg from 1 liter of bacterial culture. However, despite high yield of the sulfitized product, its further refolding in the presence of GSH/GSSG mixture resulted in practically complete and irreversible precipitation of the recombinant toxin.

An alternative method for WTX recovery from inclusion bodies was reduction of the toxin in the presence of TCEP. Advantages of this reducing agent compared to others like β-mercaptoethanol and DTT are mentioned in a number of works [22]. In this case, the yield of reduced toxin after purification on SP-Sepharose was about 35 mg from 1 liter of bacterial culture.

In 2002, a group of French scientists described the production of "weak" toxin Wntx-5 of *Naja sputatrix* venom by chemical solid-phase synthesis [23]. In this case the disulfide bonds of the synthetic protein were closed at room temperature in acetate buffer, pH 7.8, in the presence of 1 M guanidine hydrochloride, GSH, and GSSG (molar ratio 10 : 1, respectively). We used HPLC for comparative analysis of WTX refolding in conditions described in [23] and in phosphate buffer, pH 7.4, containing GSH and GSSG in molar ratios 2 : 1, 4 : 1, and 10 : 1 at 4°C (Fig. 3a, *1-4*, respectively). As seen in Fig. 3a, the highest yield of renatured protein was observed after refolding in phosphate buffer with addition of the glutathiones at molar ration 10 : 1. Thus, summarizing our results and those of other authors [23], we can conclude that the GSH/GSSG molar ratio 10 : 1 is most effective for refolding of "weak" toxins.

Efficiency of refolding of disulfide bond containing proteins can also depend on other factors such as pH of the refolding medium [21]. It is known that efficient closure of disulfide bonds happens at pH above 7.0 [24], but the theoretically calculated isoelectric point of WTX (pH value at which total charge of the toxin molecule is zero and therefore the probability of protein precipitation increases) is 8.9. This means that the range of possible pH

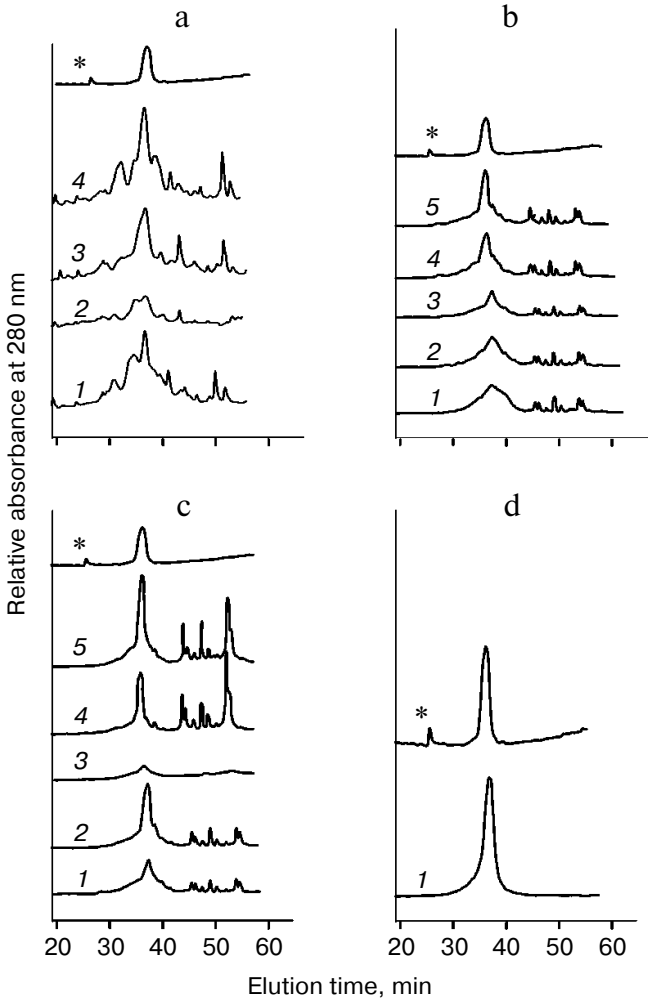

Fig. 3. WTX refolding under different conditions with monitoring by HPLC. The chromatographic profile of the natural toxin is designated everywhere by an asterisk. a) Analysis of influence of the GSH/GSSG concentration on efficiency of WTX refolding in conditions described in [23] and in phosphate buffer containing GSH and GSSG in molar ratios 2 : 1, 4 : 1, and 10 : 1, pH 7.4 at 4°C (*1-4*, respectively). b) Analysis of influence of different pH values on efficiency of WTX refolding (*1-5* correspond to pH 6.5, 7.0, 7.5, 8.0, and 8.5). c) Analysis of influence of the buffer composition on WTX refolding: *1*, *2*) buffer systems on the basis of NaP_i and Tris-HCl, respectively; *4*, *5*) Tris-HCl buffer system without and with addition of 1.5 M urea, respectively; *3*) efficiency of sulfitized WTX refolding in the determined optimal conditions). d) Chromatographic profiles of natural (*) and recombinant (*1*) toxin after refolding and final re-chromatography using HPLC.

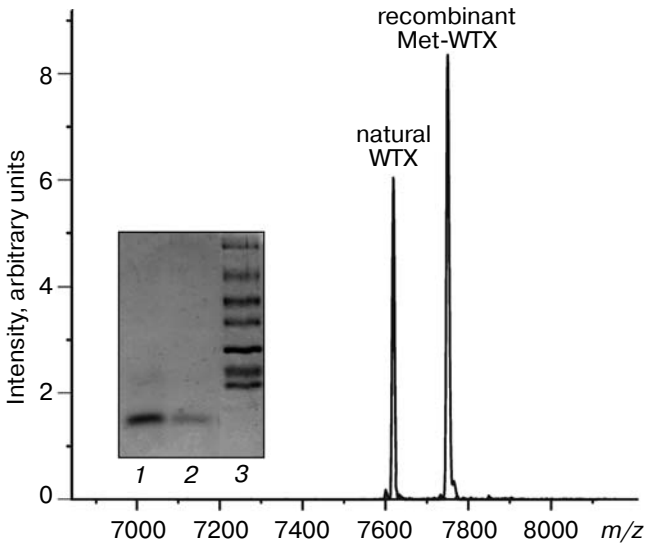

Fig. 4. Mass spectrometry of natural and recombinant "weak" toxin mixture. The insert shows electrophoretic analysis of natural (*1*) and recombinant (*2*) WTX; protein molecular mass markers (14, 18, 25, 35, 45, 66, and 116 kDa; Fermentas) (*3*).

values for WTX refolding is restricted by the interval from approximately 6.5 to 8.5. Analysis of the "weak" toxin refolding products in phosphate buffer in the presence of glutathiones at different pH (6.5, 7.0, 7.5, 8.0, and 8.5) showed the highest yield of renatured protein at pH 8.5 (Fig. 3b, *1-5*, respectively).

For further optimizing of the refolding process, the dependence of WTX refolding efficiency on the nature of buffer composition was studied. As seen in Fig. 3c (*1* and *2*), buffer on the basis of Tris-HCl is most effective for "weak" toxin refolding. The effect of urea on the recombinant protein refolding was studied under the determined optimal conditions: glutathione ratio 10 : 1, pH 8.5, and Tris-HCl buffer. This was due to the fact that even under the chosen conditions of refolding, some part of the recombinant toxin precipitated, although not so pronouncedly as in the case of refolding using the sulfitized form (Fig. 3c, *3* and *4*). Addition of 1.5 M urea to the refolding medium resulted in practically complete prevention of precipitation and increased the refolding efficiency (Fig. 3c, *5*). Thus, the following buffer was found as optimal for refolding: 50 mM Tris-HCl, pH 8.5, 1.5 M urea, and GSH/GSSG mixture (3 : 0.3 (mM)).

The dependence of toxin refolding efficiency on incubation time was studied using HPLC. The highest yield of renatured toxin was observed in the case of refolding during three days. Further lengthening of the refolding time did not increase the yield of renatured protein, while reduction in refolding time decreased the yield of final product. Under optimal conditions the final yield of renatured toxin was ~5 mg from 1 liter of bacterial culture.

The described methods for WTX production and refolding were used to obtain ^{15}N-labeled WTX. In this case the final yield of renatured toxin was ~3 mg from 1 liter of bacterial culture.

Analysis of recombinant WTX. Renatured recombinant "weak" toxin was analyzed by mass spectrometry. The experimental mass of the recombinant toxin practically coincides with the calculated mass (7750 and 7745 Da, respectively). In this case, the experimental mass of the natural toxin, which served as a control, differed from the theoretically calculated mass by the same value (7619 and 7614 Da, respectively) (Fig. 4). The difference between mass values of recombinant and natural toxins is explained by the presence of an additional methionine residue at the N-terminus of the recombinant toxin molecule. Purity of the recombinant toxin (no less that 95%) was estimated by HPLC, SDS-PAGE, and mass spectrometry (Figs. 3d and 4), as well as by NMR spectroscopy.

Full closure of all disulfide bonds in the WTX molecule upon refolding was confirmed using Ellman's reagent (5,5′-dithio-bis(2-nitrobenzoic acid)).

Investigation of recombinant WTX spatial structure. The secondary structure of the renatured toxin was studied by CD spectroscopy. The recombinant renatured toxin spectrum was identical within the limits of experimental error to the spectrum of natural toxin (Fig. 5, *1* and *2*) and pointed to dominantly β-structural arrangement of the toxin molecule. The estimates of different secondary structure elements in the recombinant toxin (50% β-structure, 40% random coil) approximately corresponded to data concerning the natural toxin (45% β-structure, 50% random coil). The analysis of the toxin secondary structure with reduced disulfide bonds revealed only the presence of disordered structure (Fig. 5, *3*).

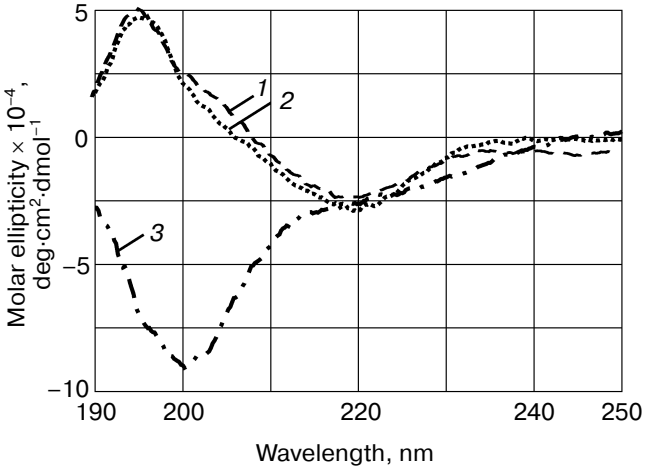

Fig. 5. Analysis by CD spectroscopy of secondary structure of natural WTX (*1*), renatured recombinant WTX (*2*), and recombinant WTX with reduced disulfide bonds (*3*).

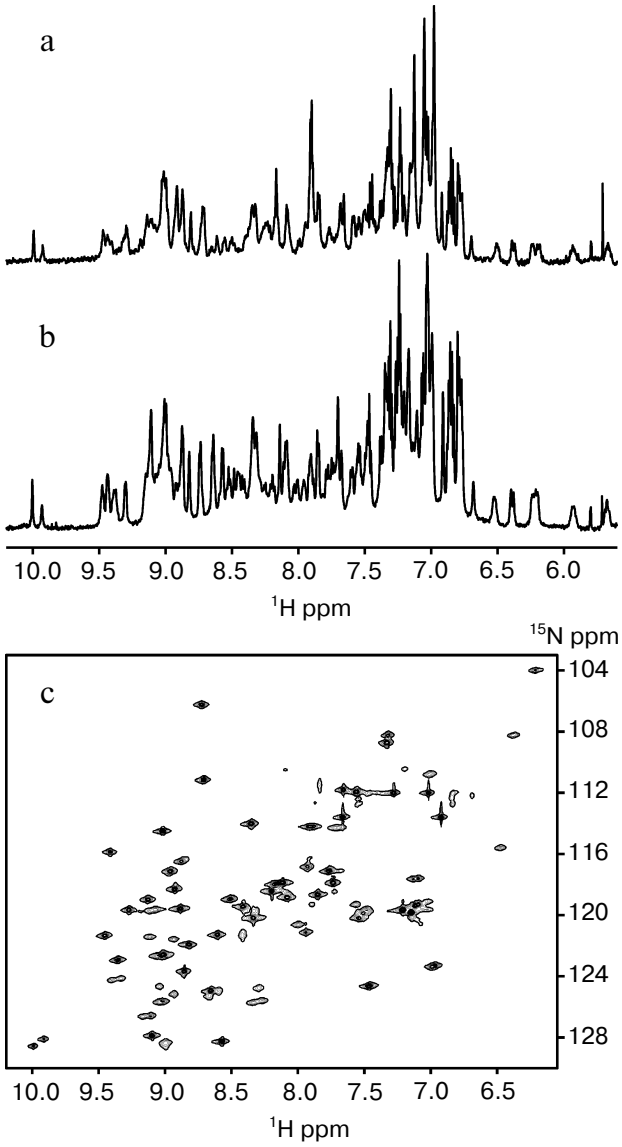

Fig. 6. ^{1}H-NMR spectra of natural (a) and recombinant WTX (b) and ^{1}H-^{15}N-HSQC spectrum of ^{15}N-labeled WTX (c).

Formation of the "weak" toxin secondary structure probably takes place simultaneously with closure of the disulfide bonds. Just this property of neurotoxins makes difficult bacterial production and subsequent refolding of these proteins.

The recombinant toxin spatial structure was also qualitatively analyzed by ^{1}H-NMR-spectroscopy. The spectrum of the recombinant toxin (Fig. 6b) was practically identical to that of the natural toxin (Fig. 6a). The observed minor differences may be due to different protocols of purification. Significant dispersion of HN-proton chemical shifts and shift of many of these signals towards weak field confirm mainly β-structural organization of the toxin molecule. At the same time, splitting of the HN signal of the unique amino acid residue tryptophan side chain in position 36, observed in the 10 ppm region, to two lines with intensity ratio 2 : 1 suggests the existence of conformational exchange in the toxin molecule. This exchange, also observed in the natural toxin [25], proceeds slowly along the NMR time scale and is evidently due to *cis-trans* isomerism of the peptide bond before amino acid residue P33.

NMR analysis of ^{15}N-labeled WTX analog showed that the extent of label incorporation is no less than 97%. The two-dimensional ^{1}H-^{15}N-HSQC spectrum of this preparation (Fig. 6c) also confirmed the presence of several conformational states of the WTX molecule.

The data indicate that the recombinant toxin not only has spatial arrangement analogous to that of the natural WTX, but also exhibits the molecular-dynamic properties of the natural toxin.

Biological activity of the recombinant WTX. The ability of the recombinant toxin to specifically interact with muscle and neuronal α7 nAChR was estimated by its competition with ^{125}I-labeled α-bungarotoxin for binding to *T. californica* electric organ membranes and to GH_4C_1 cells transfected by human α7 type nAChR, respectively.

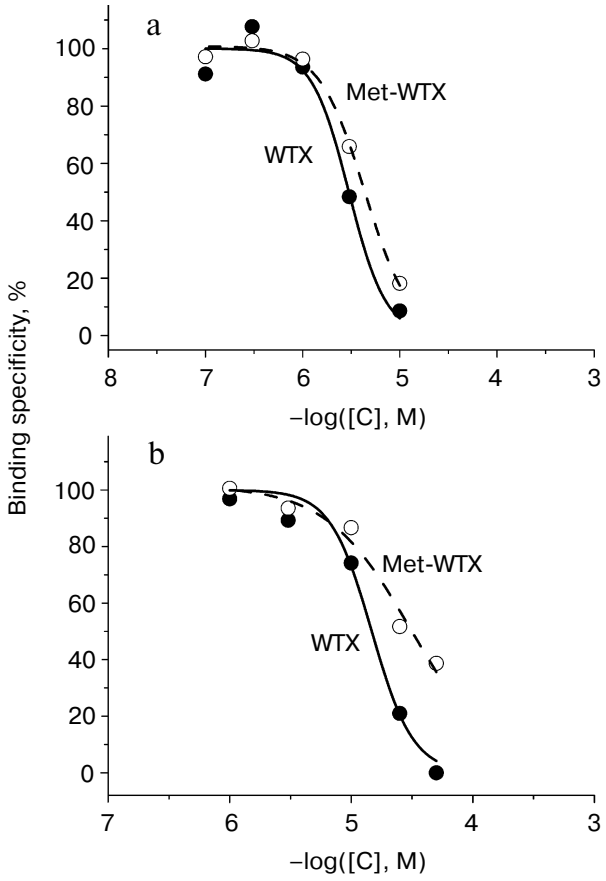

Fig. 7. Curves of competitive inhibition of ^{125}I-labeled α-bungarotoxin binding to membranes from *T. californica* containing muscle nAChR (a) and to GH_4C_1 cells transfected by human α7 type nAChR (b) plotted for recombinant and natural WTX.

The earlier obtained natural "weak" toxin isolated directly from *N. kaouthia* venom was used for comparison. Analysis of inhibition curves allowed us to determine IC_{50} values for the recombinant and natural WTX on muscle receptor (Fig. 7a; IC_{50} 4.3 ± 0.3 and 3.0 ± 0.5 μM, respectively) and neuronal α7 type nAChR (Fig. 7b; IC_{50} 31 ± 5 and 14.8 ± 1.3 μM, respectively). The activity of the recombinant product was somewhat lower than that of the natural toxin, which might be caused by the presence of an additional methionine residue at the N-terminus of the recombinant toxin molecule.

Thus, we have developed a new system for bacterial production and refolding of "unusual" snake venom toxins. According to our data, this work is the first successful example of heterogeneous production of this class of toxins. The development of this system opens new perspectives for investigation of poorly studied "unusual" neurotoxins and their interaction with nAChR by site-directed mutagenesis. Besides, the obtained isotope-labeled WTX analog makes possible studying in detail the spatial structure and intramolecular dynamics of the toxin molecule by NMR spectroscopy.

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