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Refolding and Purification of Bothropstoxin-I, a Lys49–Phospholipase A_2 Homologue, Expressed as Inclusion Bodies in *Escherichia coli*

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Hydrolysis of phospholipids by Group II phospholipase A_2 enzymes involves a nucleophilic attack on the *sn*-2 ester bond by the His48 residue and stabilization of the reaction intermediate by a Ca^{2+} ion cofactor bound to the Asp49 residue in the protein active site region. Bothropstoxin-I (BthTX-I) is a PLA_2 variant present in the venom of the snake *Bothrops jararacussu* which shows a Asp49 to Lys substitution and which lacks hydrolytic activity yet damages artificial membranes by a noncatalytic Ca^{2+}-independent mechanism. In order to better characterize this unusual mechanism of membrane damage, we have established an expression system for BthTX-I in *Escherichia coli*. The DNA-coding sequence for BthTX-I was subcloned into the vector pET11-d, and the BthTX-I was expressed as inclusion bodies in *E. coli* BL21(DE3). The native BthTX-I contains seven disulfide bonds, and a straightforward protocol has been developed to refold the recombinant protein at high protein concentration in the presence of surfactants using a size-exclusion chromatography matrix. After refolding, recovery yields of 2.5% (corresponding to 4–5 mg of refolded recombinant BthTX-I per liter of bacterial culture) were routinely obtained. After refolding, identical fluorescent and circular dichroism spectra were obtained for the recombinant BthTX-I compared to those of the native protein. Furthermore, the native and refolded recombinant protein demonstrated identical membrane-damaging properties as evaluated by measuring the release of an entrapped fluorescent marker from liposomes.

Phospholipases A_2 (PLA_2, EC 3.1.1.4)[2] catalyze the hydrolysis the *sn*-2 acyl bonds of *sn*-3 phospholipids (1) and are currently classified into 10 groups (2). The secreted PLA_2s ($sPLA_2$) present in snake venoms are classified as either Group IA (from *Elapidae* and *Hidrophidae* venoms), Group IIA (from *Viperidae* and *Crotalidae* venoms), or Group IIB (the PLA_2 from *Bitis gabonis* venom). Groups IB and IIC include the $sPLA_2$s from mammalian pancreas and rodent testis, respectively. The catalytic mechanism of Group I and II PLA_2s involves a Ca^{2+} ion cofactor bound by Asp49 and carbonyl main chain oxygens of the neighboring calcium-binding loop, which acts in conjunction with the highly conserved active site residues His48, Tyr52, and Asp99 (3, 4). In addition to their hydrolytic activity against phospholipid substrates, many snake venom PLA_2s possess pharmacological activities, and a subfamily of myotoxic PLA_2s has been characterized in which the Asp49 is substituted by Lys (5). Crystal structures of Lys49–PLA_2s have demonstrated that the ϵ-amino group of the Lys49 is located in the position normally occupied by Ca^{2+} in the Asp49-PLA_2s (6–8). These Lys49–PLA_2s demonstrate low levels of catalytic activity, and it has been suggested that this substitution eliminates productive binding of the Ca^{2+} cofactor with the concomitant loss of hydrolytic function (9, 10). Nevertheless, the Lys49–PLA_2s retain membrane-damaging activity through a Ca^{2+}-independent mechanism (11, 12).

Bothropstoxin-I (BthTX-I) is a 13.7-kDa Lys49–phospholipase A_2 of 121 amino acids isolated from the venom

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[2] Abbreviations used: PLA_2, phospholipase A_2; $sPLA_2$, secreted PLA_2; BthTX-I, bothropstoxin-I; NTSB, 2-nitro-5-thiobenzoic acid; TFA, trifluoroacetic acid; BS^3, bis-3-sulfosiccinimidyl suberate; ITFE, intrinsic tryptophan fluorescence emission; CD, circular dichroism.

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of the viperid snake *Bothrops jararacussu* (13). In common with other Group IIA PLA_2s, the protein contains 14 cysteines which form seven disulfide bonds (14, 15). In order to study further the Ca^{2+}-independent membrane-damaging activity, we have established an expression system for BthTX-I in *Escherichia coli*. Heterologous proteins expressed in *E. coli* frequently aggregate, producing inclusion bodies which require solubilization using high concentrations of denaturating agents and subsequent dilution or dialysis under conditions which favor refolding of the recombinant protein. However, aggregation and formation of nonnative disulfide bonds during the refolding process can severely reduce yields of the final native product. We have developed a straightforward refolding protocol for the BthTX-I at high protein concentration in the presence of surfactants using a size-exclusion chromatography matrix. After refolding, recovery yields of 2.5% (corresponding to 4–5 mg of refolded recombinant BthTX-I per liter of bacterial culture) were routinely obtained.

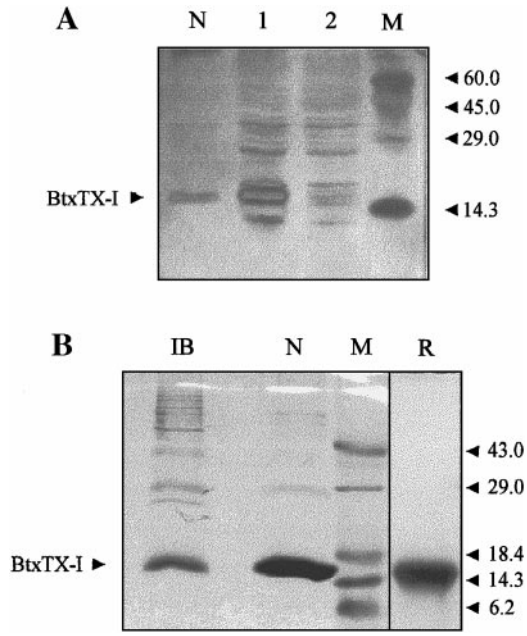

FIG. 1. (A) SDS–PAGE analysis of BthTX-I production in *E. coli* BL(21)DE3{pLysS}. Lane N, native BthTX-I purified from *B. jararacussu* venom; lane 1, extracts of whole cells transformed with pET11d + *BTXI* after 5 h of IPTG induction; lane 2, extracts of whole cells transformed with pET11d after 5 h of IPTG induction; lane M, molecular weight marker. The gel was developed by silver staining. (B) SDS–PAGE analysis of native and recombinant BthTX-I. Lane IB, inclusion bodies after sonication with lysis buffer; lane N, native BthTX-I purified from *B. jararacussu* venom; lane M, molecular weight marker; lane R, recombinant BthTX-I purified from *E. coli* inclusion bodies. The gel was developed by silver staining.

MATERIALS AND METHODS

Plasmid Construction

A complete cDNA encoding BthTX-I was isolated from *B. jararacussu* venom gland cDNA by RT-PCR, cloned into the vector pT7T3, and sequenced (GenBank/EMBL Accession No. X78599 (16)). For all subsequent plasmid manipulations, *E. coli* DH5α [*supE44* Δ*lacU169* (*ϕ80 lacZ*Δ*M15) hsdR17 relA1 gyrA96 thi-1 recA1*] was used as the cloning host. To express the protein, restriction sites for *Nhe*I and *Hin*dIII at the 5′ and 3′ extremities, respectively, together with a stop codon at the 3′ end, were introduced by site-directed mutagenesis using the unique site-elimination protocol (17). Subcloning of the *Nhe*I/*Hin*dIII fragment into the equivalent restriction sites of the expression vector pET11-d introduced an N-terminal Met-Ala extension of the translated protein sequence; therefore, this Ala codon was deleted by an additional round of site-directed mutagenesis. Nucleotide sequencing confirmed the desired final construct, denoted pET11d + *BTXI*, in which Ser1 of the BthTX-I is preceded by a Met, and a stop codon immediately follows Cys121.

Expression of BthTX-I

One-hundred fifty milliliters of growth medium (2.5 g yeast extract; 10 mM $MgSO_4$, pH 7.5; 15 μg ml^{-1} chloramphenicol; 150 μg ml^{-1} ampicillin) was inoculated with *E. coli* strain BL21(DE3){pLysS} transformed with pET11d + *BTXI* and grown at 37°C to an A_{600} of 0.6. Recombinant protein expression was induced by addition of 0.6 mM IPTG, and the culture was grown for an additional 5 h. Inclusion bodies were isolated from bacterial pellets by repeated rounds of sonication in 20 ml of lysis buffer (50 mM Tris–HCl, pH 8.0; 1 mM EDTA; 1% Triton X-100) followed by centrifugation at 12,000*g*. Expression of the desired product was confirmed by 21 cycles of N-terminal amino acid sequencing using automated Edman degradation (Procise 491 protein sequencer, Applied Biosystems) of the induced 14-kDa protein isolated by electrotransfer from SDS–PAGE gels to PVDF membranes.

Solubilization of Inclusion Bodies and Refolding

Inclusion bodies were solubilized in 0.6 ml of denaturation buffer containing sodium sulfite and the cysteine-sulfonating compound 2-nitro-5-thiobenzoic acid (NTSB) (18) (4 M GdnSCN, 2 mM EDTA, 300 mM Na_2SO_3, 50 mM NTSB). The solubilized inclusion bodies were incubated for 30 min at 45°C to ensure complete reduction and sulfonation of cysteine residues. The mixture was subsequently passed through a BioGel P6 column (2 × 3 cm, Bio-Rad) previously equilibrated in buffer A (2 M guanidinium hydrochloride; 1 mM EDTA; 50 mM Tris–HCl, pH 8.0) to separate the modified protein from excess NTSB and to exchange the buffer.

The protein peak, eluted in a total volume of ~0.8

TABLE 1

Purification Yields of BthTX-I from *E. coli* BL(21)DE3{pLysS} Cell Lysates

Purification step	Total protein (mg)	Protein concentration (mg ml^{-1})	BthTX-I (mg)	Purity (%)[a]	Recovery yield (%)
Total cell lysate[b]	151	1.0	28.0[c]	18.4	100
Sonicated inclusion bodies	20.5	34.2	9.3[c]	45.3	33.2
BioGel P6 buffer exchange column	7.4	9.3	4.0[c]	54.1	14.3
BioGel P6 refolding column	2.9	0.19	1.6[c]	55.1	5.7
Cation-exchange chromatography	1.1	0.92	0.96	86.7	2.5
Reverse-phase chromatography	0.68	0.85	0.68	>99	2.4

[a] The purity of the native BthTX-I was estimated from protein band intensity in SDS–PAGE or by the peak area during chromatography.
[b] The total cell lysate of 151 mg of protein was obtained from 150 ml of cell culture.
[c] The amount of BthTX-I was estimated from the protein band intensity in SDS–PAGE gels after scanning and image analysis.

ml, served as the starting material for the refolding process. Columns (1 × 8 cm) packed with the BioGel P6 resin were equilibrated with 3 column vol of buffer B (50 mM Tris–HCl, pH 8.0; 1 mM EDTA), followed by 1 column vol of buffer C (buffer B containing 2 mM oxidized glutathione; 4 mM reduced glutathione; 4 mM *n*-dodecyl-β-D-maltoside). The protein was eluted at a concentration of ~0.9 mg ml^{-1}, and 0.8 ml of this solution was applied by gravity flow at 150–200 μl h^{-1}. Typically sample application was complete in 4 h, after which an additional 0.7 ml of buffer C was applied at the same rate. After incubation for 12 h at 25°C and the protein was eluted with 2 column vol of buffer B. The eluate (~12 ml) was collected in a 15-ml stoppered polypropylene tubes, sealed, and incubated for an additional 36 h at 25°C with mild agitation. Since a slight flocculent precipitate was observed, the incubation product was passed through a 0.45-μm cellulose acetate/cellulose nitrate filter.

Purification of Refolded BthTX-I

The reoxidation product was adjusted to pH 7.5 with HCl and applied to a Shim-pack PA-CM column (8 × 100 mm Shimadzu Co., Kyoto, Japan) previously equilibrated with 5 column vol of buffer B at the same pH. After extensive washing with buffer B, the rBthTX-I was eluted with a step gradient of 1 M NaCl in the same buffer. This eluate was applied to a 214TP54 C_4 reverse-phase column (4.6 × 250 mm, Vydac, Hesperia, CA) previously equilibrated with a 10% acetonitrile/0.1% trifluoroacetic acid (TFA) mobile phase. The column was eluted with a gradient of a mobile phase containing 80% acetonitrile/0.09% TFA. The eluate was continuously monitored at 280 nm, and the eluted protein peaks were lyophilized and stored at −20°C. Native BthTX-I was purified from *B. jararacussu* venom by cation-exchange chromatography as previously described (13) and subsequently repurified using reverse-phase chromatography under identical conditions as the recombinant protein.

Quantification of BthTX-I

The concentration of BthTX-I was determined by scanning dried SDS–PAGE gels using a Hewlett–Packard ScanJet 3C and estimating the intensity of the protein band using standard integration software. Total protein concentration was measured using the bicinchoninic acid method (19).

Chemical Cross-Linking

Cross-linking reactions of 300 ng of purified native or recombinant BthTX-I were performed by incubating the protein at 25°C with 300 μM bis-3-sulfosuccinimidyl suberate (BS^3) in 150 mM NaCl, 25 mM Hepes, pH 7.0. In both cases incubations under the same conditions were performed in the absence of cross-linker. After 15 min, the reactions were stopped with 50 mM Tris and heated at 95°C for 3 min in sample buffer containing 5 mM β-mercaptoethanol and electrophoresed at 100 mA for 1.5 h on a 17% SDS–polyacrylamide gel (20).

Spectroscopic Characterization

Intrinsic tryptophan fluorescence emission (ITFE) spectra were measured between 300 and 450 nm with λ_{ex} = 295 nm using a Spectronic 8100 and a protein concentration of 30 μg ml^{-1}. Excitation and emission slitwidths were 4 nm and the photomultiplier tube voltage was 600 V. Spectra were corrected by subtraction of a buffer blank. Far UV circular dichroism spectra (200–250 nm) were measured with a JASCO 710 using 1-mm-pathlength cuvettes and protein concentrations of 150 μg ml^{-1} (native) and 70 μg ml^{-1} (recombinant) BthTX-I. A total of nine spectra were collected, averaged, and corrected by subtraction of a buffer blank.

Release of Entrapped Markers from Liposomes

A loss of membrane integrity results in dilution of the liposome entrapped self-quenching fluorescent dye

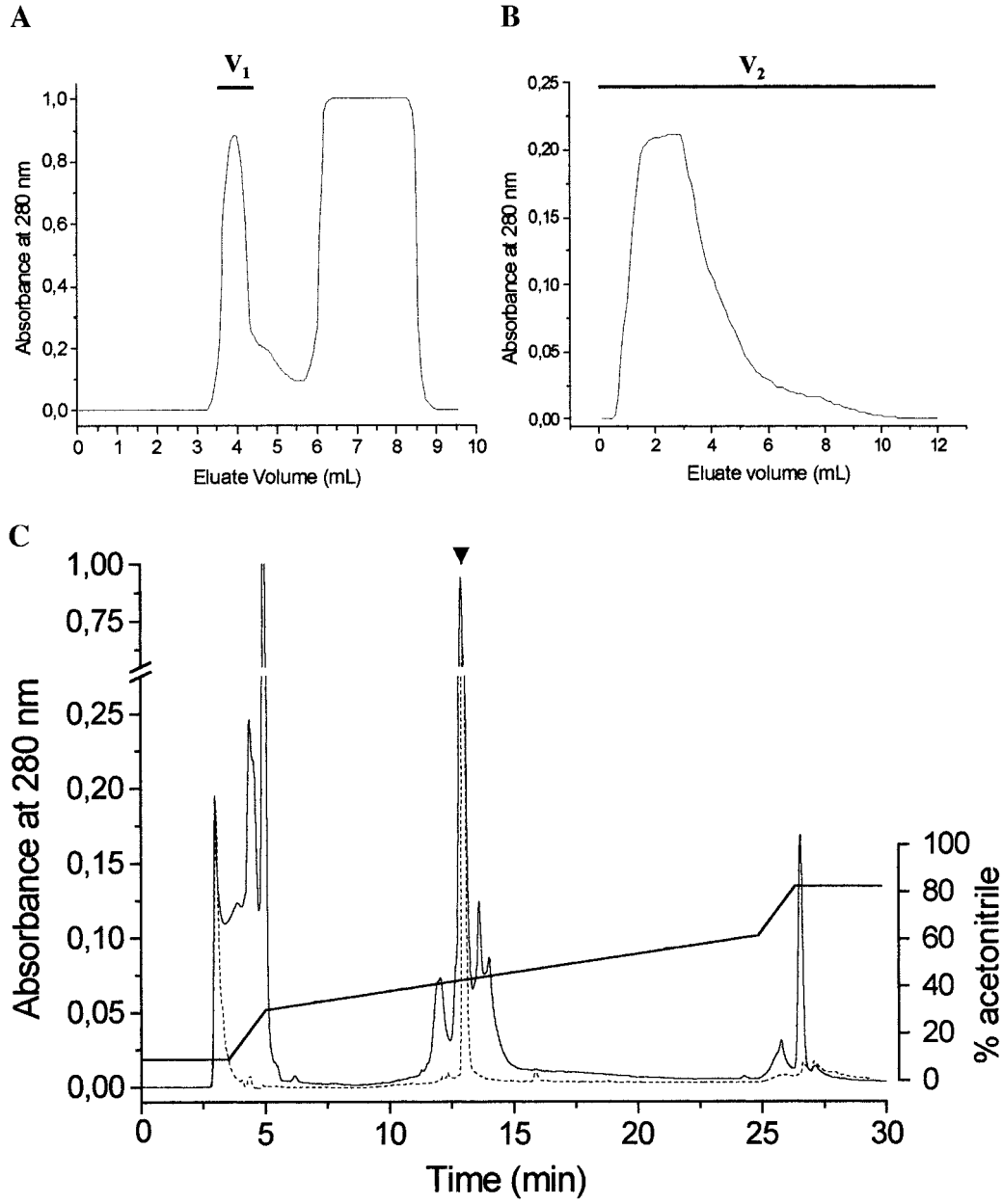

FIG. 2. (A) Elution profile of the BioGel P6 buffer exchange column. The first major peak contained the recombinant BthTX-I and was collected in the eluate volume V_1 (shown above the profile). The second major peak (eluate volumes 6–9 ml) contained excess unreacted NTSB. (B) Elution profile of the BioGel P6 refolding column. The collected eluate volume V_2 was incubated for 36 h at 25°C with mild agitation prior to the final purification by reverse-phase cromatography. (C) Elution profile of the C_4 reverse-phase column (4.6 × 250 mm, Vydac, Hesperia, CA) of native (dashed line) and refolded recombinant (solid line) BthTX-I. The column was previously equilibrated with a 10% acetonitrile/0.1% trifluoroacetic acid (TFA) mobile phase and eluted with a gradient of a mobile phase containing 80% acetonitrile/0.09% TFA (indicated by the thick solid line). The arrow indicates the elution time of the native protein.

with a consequent increase in the fluorescence signal (12). Proteins were injected to a final protein:lipid molar ratio of 1:200 to liposomes composed of a 9:1 molar ratio of egg yolk phosphatidylcholine:dimyristoyl phosphatidic acid loaded with 25 mM calceine (Sigma) in 150 mM NaCl, 25 mM Hepes, pH 7.0. The fluorescence increase was monitored at $\lambda_{em} = 520$ nm with $\lambda_{ex} = 480$ nm, and the signal was expressed as a percentage of total dye liberation on addition of 5 mM Triton X-100.

RESULTS AND DISCUSSION

As shown in Fig. 1A, the construct pET11d + *BTXI* exhibited a high level of recombinant BthTX-I overex-

pression, which after 5 h of induction with IPTG accounts for approximately 18% of total cellular protein (see Table 1). The BthTX-I is expressed in the form of inclusion bodies, and the isolation of these inclusion bodies is the first step in the purification process, as shown in Fig. 1B. Based on the amino acid sequence, the calculated molecular mass of the BthTX-I is 13675 Da (15); however, both the native and the recombinant BthTX-I demonstrate a reduced mobility on SDS–PAGE (Fig. 1B).

Figure 2A presents the chromatography profile of the BioGel P6 buffer exchange column after modification of the solubilized inclusion bodies with NTSB. Two major peaks eluted from the column, the first of which contained the sulfonated recombinant BthTX-I. The second major peak, which is well separated from the collected volume (denoted by V_1 in Fig. 1A), contained the unreacted NTSB together with the other components of the denaturation buffer. The eluate volume from the buffer exchange column was applied to the refolding column, and Fig. 2B shows the elution profile of this column after 12 h incubation at 25°C. After 36 h of incubation at 25°C, Fig. 2C shows that during reverse-phase chromatography the major peak obtained after refolding eluted at the same position as the native BthTX-I isolated from *B. jararacussu* venom. This final purification step produced refolded protein with a purity greater than 99% with a recovery yield of 2.4%, and N-terminal amino acid sequencing revealed that the first 21 amino acids of this eluted protein were identical to the native BthTX-I. Details of the overall results of the refolding and purification protocols are provided in Table 1.

Native BthTX-I forms dimers which can be covalently cross-linking with BS^3, and Fig. 3 presents a silver-stained SDS–PAGE gel of the cross-linking reaction demonstrating that both the native and the recombinant protein form dimers. A single tryptophan residue contributes to the intermolecular contacts which stabilize the dimer (15), and the ITFE (Fig. 4A) and circular dichroism (CD) (Fig. 4B) spectra of the native and recombinant BthTX-I are essentially identical, demonstrating that the microenvironment of the single Trp residue in the molecule and the secondary structure of both proteins are conserved. Functionally, the BthTX-I

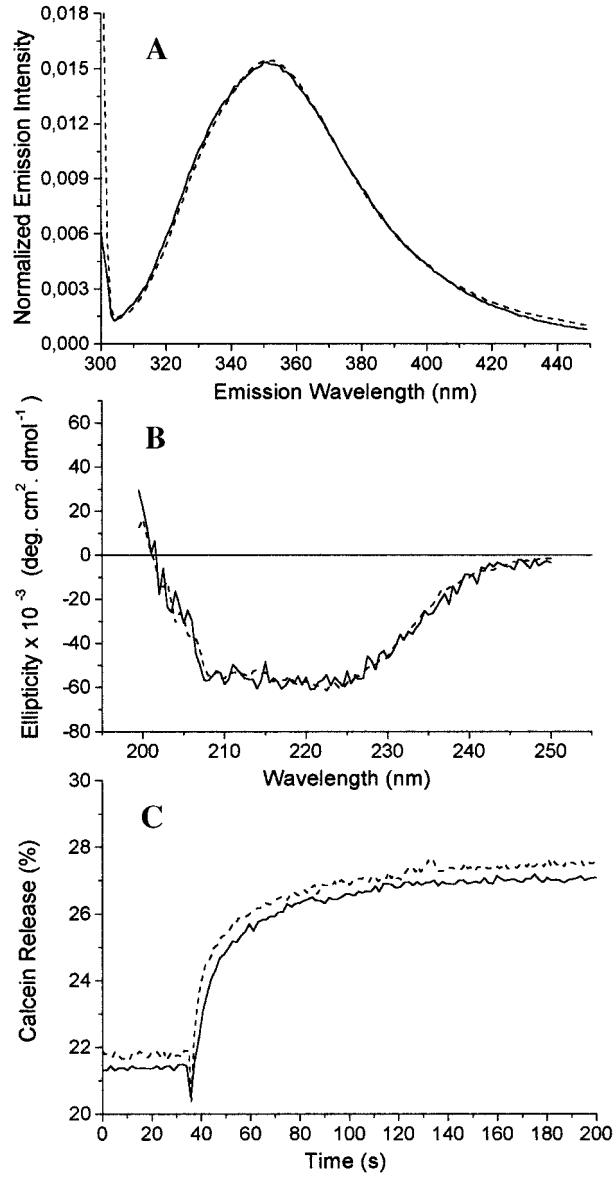

FIG. 4. Spectroscopic and functional properties of native (dashed lines) and recombinant (solid lines) BthTX-I. (A) Intrinsic tryptophan emission spectra. (B) Average of nine far UV circular dichrosim spectra. (C) Kinetics of fluorescent dye liberation from liposomes. Native or recombinant protein was added at 35 s and the signal was expressed as a percentage of total dye liberation on addition of 5 mM Triton X-100 (not shown).

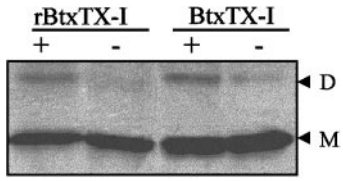

FIG. 3. Silver-stained 17% SDS–PAGE gel showing chemical cross-linking of purified native and recombinant BthTX-I after incubation in the presence (+) and absence (−) of BS^3. Monomer (M) and dimer (D) bands are indicated.

damages artificial membranes through a Ca^{2+}-independent mechanism, which in contrast to other Group II PLA_2s does not involve phospholipid hydrolysis (13). Kinetic measurements of dye release from liposomes for both native and recombinant BthTX-I are presented in Fig. 4C, which demonstrates that both samples have comparable membrane-damaging activity. No phospholipid hydrolysis was observed for either protein as measured by the phenol red colorimetric

TABLE 2

Effect of Various Surfactants on the Final Purified BthTX-I Recovery Yield

Surfactant[a]	Relative recovery yield (%)
No surfactant	0.8
n-Dodecyl-β-D-maltoside	100
n-Dodecanoylsucrose	46.8
n-hexyl-β-D-glucopyranoside	17.8
n-heptyl-β-D-glucopyranoside	14.7
n-octyl-β-D-glucopyranoside	7.8
n-nonyl-β-D-glucopyranoside	7.6
n-decyl-β-D-glucopyranoside	6.4

Note. The refolding protocol and purification procedures were as described under Materials and Methods.

[a] The refolding protocol in these experiments was as described under Materials and Methods, with the substitution of different surfactants at a final concentration of 4 mM.

assay for free fatty acid release (21) using egg yolk lecithin as a substrate (data not shown).

Initial attempts to refold recombinant BthTX-I using dilution (22) or dialysis (23) based techniques established for mammalian Group IB PLA_2s resulted in heavy precipitation and yields of <1% of product. Furthermore, dilution techniques limit the number of samples that can be processed simultaneously and dialysis techniques require large volumes of expensive buffers. Although the processing time using the hydrophobic resin refolding protocol is comparable to the other methods, a final native protein yield (4–5 mg L^{-1} of bacterial culture) offers over a 100-fold improvement in efficiency. Several columns may be manipulated simultaneously providing significant time savings for refolding of multiple samples. Furthermore, as shown in Table 1 the protocol permits refolding at high protein concentrations, which reduces buffer volumes leading to appreciable cost benefits.

During the development of the protocol described here, diverse refolding conditions were evaluated and the reverse-phase chromatogram profile provided valuable information concerning the yield of native protein. In addition to the major protein peak, the chromatogram of the refolded protein (Fig. 2) shows a series of minor flanking peaks eluting between 12 and 15 min, which we attribute to incorrectly folded BthTX-I. Although having the same mobility on SDS–PAGE, these fractions were not membrane active and gave significantly altered ITFE and CD spectra with respect to the native BthTX-I (data not shown).

Table 2 gives details of the recovery yields after refolding the protein in the presence of different surfactants at a concentration of 4 mM. It was observed that addition of surfactants with an aliphatic carbon chain of C_8 or less significantly improved the recovery yield of the final product; however, surfactants with a disaccharide head group were most effective for optimizing yields of native protein. Final yields were also influenced by the structure of the refolding matrix, and Table 3 shows that the dextran-based resins (Sephadex) gave lower yields than the polyacrylamide-based BioGel resins.

Lysozyme and carbonic anhydrase have been refolded in the presence of the dextran-based Sephacryl gel filtration resin (24, 25). The authors proposed that during application and migration of the protein through the column the chaotrope concentration decreases, and the protein adopts a compact nonnative conformation. The partially refolded protein enters the resin pores whereas the unfolded protein remains in the mobile phase volume, and this partition effect favors protein refolding over aggregation (24, 26). It might be expected that changing the resin mesh size would alter the protein partition coefficient thereby influencing the refolding process; however, we have found no clear correlation between recovery yield and the resin molecular weight cutoff. Table 3 shows that the BioGel resins P2, P6, and P100 with molecular weight cutoff values of 2, 6, and 100 kDa give comparable recovery yields, and we suggest that in the case of BthTX-I immobilization of unfolded and partially folded protein by the resin matrix favors protein refolding.

In summary, we have developed a simple and economical method for the refolding recombinant BthTX-I expressed as inclusion bodies in *E. coli*. The protocol described here uses an inexpensive polyacrylamide-based size-exclusion chromatography resin, which facilitates the simultaneous processing of multiple recombinant protein samples with final native protein yields of 4–5 mg per liter of bacterial culture. The method has been used in our laboratory to refold both native and mutant recombinant BthTX-I with excellent reproducibility and may be readily adaptable to the refolding of other disulfide-rich proteins expressed as inclusion bodies in *E. coli*.

TABLE 3

The Effect of Varying the Matrix of the Refolding Column on the Final Purified BthTX-I Recovery Yield

Refolding matrix	Relative recovery yield (%)
BioGel P6	100
BioGel P100	94.9
BioGel P2	82.8
Sephadex G25	20.6
Sephadex G100	16.0

Note. The refolding protocol and purification procedures were as described under Material and Methods.

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