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Refolding of disulfide containing peptides in fusion with thioredoxin

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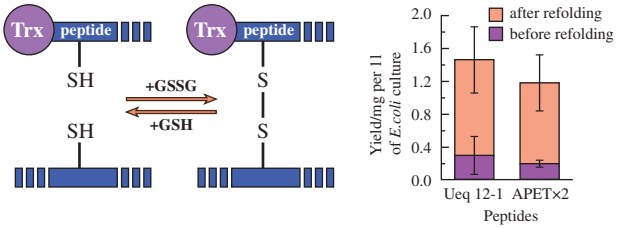
A protocol for refolding of thioredoxin-fused cysteine-rich peptides *via* addition of oxidized/reduced glutathione reagent directly to unfolded soluble fused protein has been developed. This procedure allows one to skip the steps of inclusion bodies purification, denaturation/disulfide reduction as well as lyophilization before oxidative folding, and thus to improve the yield of cysteine-rich peptides in their production using *E. coli* expression system.



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The biological activity of cysteine-rich peptides, including a variety of animal toxins and plant defense peptides, ensures their great pharmaceutical potential.[1–3] Contrary to the peptides with one or two disulfide bridges, whose production is typically an efficient process with a high yield of correctly folded chain with proper disulfide bonds, the preparation of their counterparts with three or more disulfide bridges remains a challenge because the number of possible disulfide pairings increases dramatically with every additional couple of cysteine residues.[4,5]

Since the disulfide bond formation is a post-translational process, it requires a special environment, and only the proper folding provides an activity to peptide. Available methods for the production of cysteine-rich peptides are the following: (i) chemical synthesis of unfolded linear peptide or its heterologous expression as an insoluble material, followed by *in vitro* reorganization into the active form, the last step being called refolding and (ii) production of the peptide in its native conformation using heterologous expression systems. The chemical synthesis is limited by the peptide length but can provide a large amount of cysteine-rich peptide in its linear form. Using the following *in vitro* refolding, it is possible to obtain a properly folded peptide with 30–70% efficiency.

The recombinant expression methods represent an attractive alternative especially when peptides are difficult to obtain by the chemical synthesis. Several approaches are described for heterologous expression in bacteria.[6] However, for cysteine-rich peptides their expression in *Escherichia coli* with no protein fusion typically results in the formation of an inclusion body and the need for the following *in vitro* refolding of the insoluble product, this inclusion body refolding step being quite laborious. An alternative approach consists in maximization of production of recombinant cysteine-rich proteins in their soluble form. Thus, Novagen's pET-32(a–c) vectors have been designed for cloning and high-level expression of peptide sequences as fusion proteins with 109 amino acid protein thioredoxin (Trx), which enhances solubility and stability of the recombinant proteins and catalyzes the formation of disulfide bonds in the cytoplasm of *E. coli*.[7,8]

Depending on the target peptide, the expression using pET-32 based system can result in: (i) a peptide folded completely correctly,[9–12] (ii) a peptide folded mostly correctly and in amount enough to neglect its insoluble or misfolded (incorrectly assembled) byproducts,[13–15] (iii) an array of crude products with different disulfide binding patterns[16] as well as (iv) mainly insoluble product, which requires refolding.[17,18] To maximize the correct folding of recombinant proteins, different *E. coli* strains such as Origami B[19] or SHuffle[20] have been used. Refolding can be considered as an additional approach to the preparation of active peptides, and it typically consists of denaturation, reduction of the enclosed disulfide bonds and the following folding of the linear recombinant peptide into its native form.

We have developed a simple protocol for the refolding of soluble Trx-fused proteins, which can assist in the production of peptides that are incompletely and/or incorrectly folded. Here we describe a modified method for the recombinant production of two peptides from sea anemone venoms, namely APETx2 (or Pi-AITX-Ael2b, PDB ID: P61542), a 42 amino acid toxin isolated from *Anthopleura elegantissima*, and Ueq 12-1 (or τ-AnmTx Ueq 12-1, PDB ID: C0HK26), a 45 amino acid peptide from *Urticina eques*. Several protocols for the APETx2 production were reported. First, an efficient method for its chemical synthesis was developed based on native chemical ligation and folding.[21] Then, APETx2 was obtained using the yeast *Pichia pastoris* in 2–4 mg l^{-1} yield after purification.[22] Expression in the *E. coli* periplasmic system resulted in its yield of ~1 mg dm^{-3}.[23] Considering the Ueq 12-1 peptide, the bacterial recombinant production gave highly variable yields from 0.1 to 0.7 mg dm^{-3} *E. coli* culture. APETx2 is known to be the most potent and selective inhibitor of ASIC3-containing homomeric and heteromeric channels,[24,25] whereas Ueq 12-1 represents a positive modulator of TRPA1 channels and is considered as a potential analgesic lead with

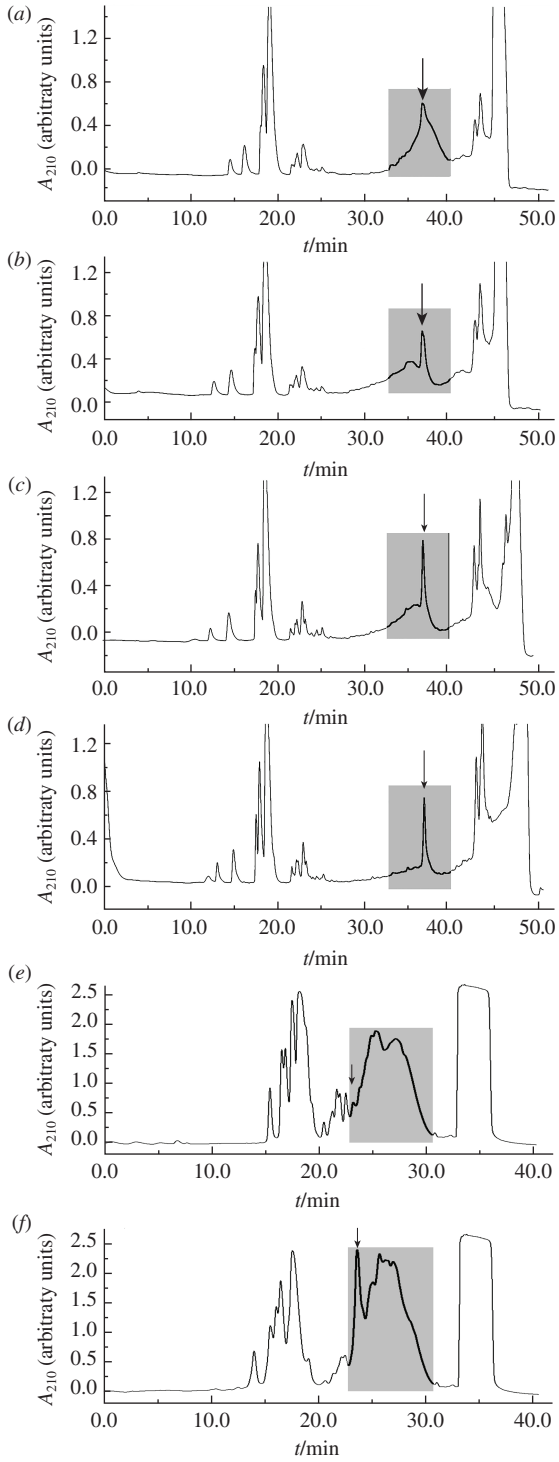

Figure 1 Separation of Trx-peptides fragments by HPLC after BrCN cleavage using a Jupiter C5 column 250×10 mm with detection at 210 nm, target peptide zones are filled in gray and correctly folded peptides are marked by arrows. Trx-APETx2 protein (~0.5 mg), 0–60% linear gradient of acetonitrile: (*a*) no refolding, (*b*) refolding for 24 h, (*c*) refolding for 48 h and (*d*) refolding for 72 h. Trx-Ueq 12-1 protein (~1 mg), 0–32% linear gradient of acetonitrile for 32 min, then increase to 70% of acetonitrile: (*e*) no refolding, (*f*) refolding for 5 days.

antibacterial properties.[26] APETx2 has six cysteine residues corresponding to three disulfide bridges and belongs to an 'inhibitor cystine knot' peptide type, while Ueq 12-1 bears 10 cysteine moieties, *i.e.* five bridges, and has a special W-shaped structure partially resembling defensin-like peptides of mammals.[26]

The synthetic genes coding for mature peptides and the Met codon for the subsequent cleavage by BrCN were constructed with the codon usage optimized for *E. coli* and then cloned into

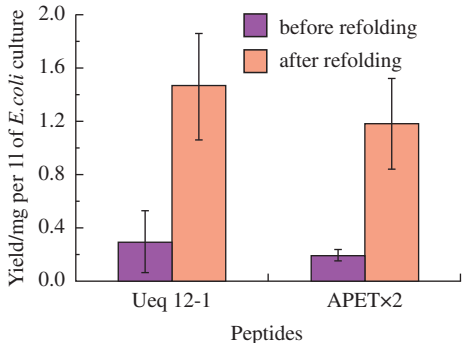

Figure 2 Recombinant polypeptide yields as means ±SD (n = 5) after cleavage by BrCN followed by HPLC purification.

the expression vector pET32b(+) for thioredoxin fusion protein.[26] These constructs were employed to transform different *E. coli* strains, after that the bacterial cells were cultivated at 25°C for 18 h. The heterologous expression of Trx-peptides in *E. coli* BL21 (DE3) strain did not produce the active peptide. For the SHuffle strain the yields were still low, namely 0.2 ± 0.04 mg dm^{-3} (mean ± SD, n = 5) for APETx2 and 0.3 ± 0.23 mg dm^{-3} (mean ± SD, n = 5) for Ueq 12-1. Moreover, an additional step was required, that involved chromatographic separation of the peaks, corresponding to the molecular weight of properly folded peptides, from the misfolded ones and other byproducts [Figure 1(*a*),(*e*)]. Therefore, to increase the final yields, the refolding procedure was developed.

Given the low yield of active peptides fused with Trx that catalyzed the cysteine bond formation, we proposed to supplement additional redox agents directly to the soluble Trx-peptides to form an oxidized environment appropriate for the disulfides reduction/oxidation as a mimic of their isomerization.[4] Reduced (GSH) and oxidized (GSSG) glutathiones represent typical redox agents, which reduce and oxidize disulfide bridges in a random way providing molecules with all possible array of disulfide-bonded folds. Since the probability of favorable folding outcome decreases by a factor of 10^3 for one extra pair of cysteines, and taking into account the Trx chaperon activity, we have designed the following preparation and refolding protocol for the soluble peptide fusions with Trx in a glutathione buffer. After cultivation the cells were harvested, resuspended in a buffer for metal-affinity chromatography (300 mM NaCl, 50 mM Tris-HCl, pH 7.5), ultrasonicated and centrifuged to remove insoluble particles. The fusion proteins were isolated from the supernatant using HisPur Ni–NTA metal affinity resin with 500 mM NaCl, 50 mM Tris–HCl and 150 mM imidazole (pH 7.5) as an elution buffer. Then Trx-peptides in the elution buffer were diluted by water up to the protein concentration of 1 mg ml^{-1}. GSH and GSSG were added directly to the fused proteins solutions up to concentrations of 4 mM and 1 mM, respectively, and the folding was carried out at 10–15 °C for 2–5 days. Then the fusion proteins were cleaved in the dark at room temperature using BrCN with an addition of HCl up to 0.2 M concentration as described.[27] Target peptides were isolated from the reaction mixture using reverse-phase HPLC. The purity of the recombinant peptides was confirmed by N-terminal sequencing and ESI mass spectrometry. The final yields of the peptides after refolding were estimated to be 1.2 ± 0.4 and 1.46 ± 0.39 mg dm^{-3} *E. coli* culture for APETx2 and Ueq 12-1, respectively (n = 5) (Figure 2).

APETx2 expressed in *E. coli* BL21 (DE3) was correctly folded within 3 days [Figure 1(*a*)–(*d*)]. The use of SHuffle strain proved to be crucial for production of the properly folded active Ueq 12-1 peptide, and five days were required for the following effective refolding [Figure 1(*e*),(*f*)]. The recombinant peptides with correct folding revealed the expected effects on their molecular targets (Figure 3), namely APETx2 significantly inhibited the peak

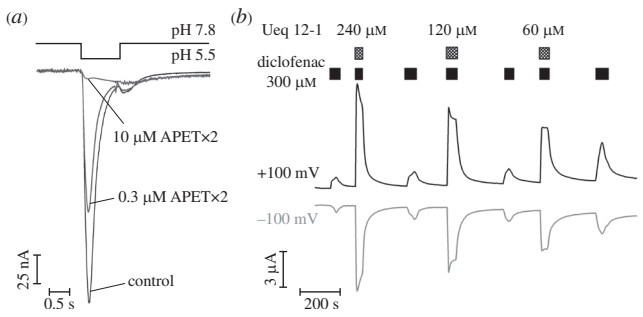

Figure 3 Biological activity of the recombinant peptides: (*a*) inhibitory effect of APETx2 on pH drop-induced activation of human ASIC3 and (*b*) TRPA1 currents evoked in response to 300 μM diclofenac in the presence of Ueq 12-1.

amplitude of ASIC3 current [Figure 3(*a*)], while Ueq 12-1 potentiated diclofenac-induced currents of TRPA1 [Figure 3(*b*)].

Using the refolding method developed, we obtained the active APETx2 peptide in the yield similar to that described for *E. coli* periplasmic expression system.[23] As for Ueq 12-1, our refolding protocol resulted in twofold increase in the yield, *viz.* 1–2 mg dm^{-3} *E. coli* culture, and enabled the robust peptide production.

Thioredoxin relevance for the refolding process was confirmed by additional tests. The fusion proteins were cleaved by BrCN, and misfolded target peptides were collected and subjected to the same refolding procedure. Under these conditions, misfolded APETx2 precipitated completely within 3 days, while the ratio of folded/misfolded Ueq 12-1 remained unchanged. Therefore, this refolding protocol is not valid for misfolded peptides themselves and is appropriate only for their fused forms.

In summary, the protocol elaborated for refolding of thioredoxin-fused cysteine-rich peptides represents the simplified process as compared with existing refolding strategies, because it allows one to skip the steps of additional purification of inclusion bodies, denaturation/disulfide reduction and lyophilization before oxidative folding.[18] As a result, the production of the peptide can be achieved in six steps only, namely expression of the fusion protein in *E. coli*, lysis, isolation of the fusion protein, direct refolding by oxidized/reduced glutathione reagent *in vitro*, cleavage and final purification.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2020.03.028.

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