ISSN 0582-9879 Acta Biochimica et Biophysica Sinica 2004, **36** (2): 118–122 **CN** 31-1300/Q

Purification and Characterization of Recombinant sTRAIL Expressed in *Escherichia coli*

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Abstract As a potential anti-tumor protein, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has drawn considerable attention. This report presented the purification and characterization of soluble TRAIL, expressed as inclusion bodies in *E. coli*. sTRAIL inclusion bodies were solubilized and refolded at a high concentration up to 0.9 g/L by a simple dilution method. Refolded protein was purified to electrophoretic homogeneity by a single-step immobilized metal affinity chromatography. The purified sTRAIL had a strong cytotoxic activity against human pancreatic tumor cell line 1990, with ED_{50} about 1.5 mg/L. Circular dichroism and fluorescence spectrum analysis showed that the refolded sTRAIL had a structure similar to that of native protein with β-sheet secondary structure. This efficient procedure of sTRAIL renaturation may be useful for the mass production of this therapeutically important protein.

Key words sTRAIL; inclusion bodies; refolding; immobilized metal affinity chromatography; characterization

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a recently characterized member of the tumor necrosis factor (TNF) superfamily. The soluble form of TRAIL exhibited strong apoptotic activity against various tumor cell lines with minimal cytotoxicity toward normal tissues both *in vitro* and *in vivo* [1–4]. The crystal structures of human soluble TRAIL (sTRAIL) and its complex with death receptor-5 revealed that the individual sTRAIL subunit mostly consisted of antiparallel β-sheets organized to form a jellyroll β-sandwich [5,6]. As a potential anti-tumor agent, sTRAIL has drawn a great deal of attention. Many researchers have cloned human sTRAIL gene into *Escherichia coli* expression vector to get sTRAIL [7,8]. However, high expression levels of recombinant proteins in *E. coli* often lead to the formation of insoluble inclusion bodies, resulting in a significant loss of native sTRAIL [9]. Therefore, an efficient and convenient refolding system would benefit the preparation and purification of sTRAIL.

Renaturation involves the removal or dilution of denaturant to produce a chemical environment that favors folding. The removal or dilution of denaturants can be achieved by a variety of methods including dilution [10], dialysis [11], gel filtration [12], diafiltration [13], immobilization on a solid support [14], and by the use of reversed micelles [15]. The simplest method is dilution refolding. Its main disadvantage is that the yield is usually lower.

In this work, a stepwise addition strategy was applied to the sTRAIL renaturation process. The renaturation volume by stepwise addition of denatured protein into the refolding solution was reduced. By stepwise increasing the denatured protein concentration, high renaturation yield was obtained.

Materials and Methods

Chemicals

Tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), guanidinium chloride (GdnHCl), urea, Triton X-100 were purchased from Bebco.

Received: October 15, 2003 Accepted: November 27, 2003

This work was supported by a grant from the National High Technology Research and Development Program of China (No. 2002AA2Z345A) and the Key Disciplinary Foundation of Shanghai

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HPLC-grade acetonitrile and trifluoroacetic acid were purchased from Merck. Electrophoresis reagents were purchased from Sigma. All buffers were prepared with deionized water purified by the Milli-Q SP/UF system (Millipore). All other chemicals were of analytical grade.

Expression of sTRAIL and recovery of its inclusion bodies

Recombinant *E. coli* C600/pBV-*sTRAIL* (encoding 114–281 aa of TRAIL, constructed by the Second Military Medical University), was cultured as described previously [16]. Fermentation was carried out at 30 ℃ and pH 7.2. Recombinant protein expression was induced at A_{600} of 90 by a temperature shift to 42 ℃. After 4 h induction, cells were harvested by centrifugation. The cell pellets were suspended at 4 ℃ in 0.05 M sodium phosphate buffer (PBS, pH 7.4) containing 0.02 M EDTA and 3% Triton X-100. Cell disruption was carried out by high-pressure homogenization and the inclusion bodies were collected by centrifugation at 15,000 *g* for 30 min. The inclusion body pellets were washed with 0.05 M PBS (pH 7.4) containing 1 M NaCl, 6 M urea and 0.5% Triton X-100, and then stored at –70 ℃.

Solubilization of inclusion bodies

The inclusion body pellets were suspended in 0.05 M pH 8.5 Tris-HCl buffer containing 5 M GdnHCl and 0.03 M DTT. After stirring at 4 ℃ for 2 h, insoluble material was removed by centrifugation (25,000 *g*, 20 min). The inclusion body preparation was diluted with the same buffer to a final protein concentration of 7.2 g/L.

Refolding of recombinant sTRAIL

sTRAIL was renatured at 4 ℃ in 0.4 L 0.05 M Tris-HCl buffer (pH 7.4) containing 0.4 M *L*-arginine, 2 mM DTT, 0.5 M urea, and 0.5 M NaCl. Totally 60 ml inclusion body solution was added to the renaturing buffer in 6 steps with 1 h interval after each step. The reaction mixture was dialyzed overnight against 0.05 M pH 7.4 Tris-HCl buffer containing 0.3 M NaCl, 1 mM DTT, 0.1 M urea at 4 ℃. The dialyzed samples were centrifuged at 25,000 *g* for 30 min at 4 ℃, and the supernatant was used for further purification.

Single step purification of refolded sTRAIL on Ni-chelating column

20 ml samples were loaded at 1 ml/min onto Ni-chelating Sepharose column FF (Amersham Pharmacia) equilibrated with pH 7.5 buffer containing 0.05 M NaH_2PO_4, 0.3 M NaCl and 10 mM imidazole. The column was washed with five volumes of the same buffer (flow rate 2 ml/min). The bound proteins were eluted with linear gradient of 10–80 mM imidazole using AKTA explorer 100 system (Amersham Biosciences) at a flow rate of 1 ml/min. Protein fractions were analyzed by SDS-PAGE. The relevant fractions were pooled and used for spectroscopic analysis.

Spectroscopic analysis

The fluorescence spectra of the native, denatured and refolded sTRAIL were recorded with a Cary eclipse Fluorescence spectrophotometer (Varian, USA). The emission spectrum excited at 295 nm was monitored in the range of 300 nm to 400 nm. The bandwidths were 2 nm for both the excitation and the emission wavelengths. The step resolution and the integration time were 1 nm and 1 s, respectively. The CD spectra of the native, denatured and refolded proteins were obtained by Jasco 715 spectropolarimeter. A cuvette with 0.2-cm path length was used for all CD spectral measurements. The CD spectra were obtained with a scan speed of 20 nm/min and band width of 2 nm. Scans collected at 0.2 nm intervals with a response time of 0.25 s were accumulated 3 times. All CD spectra were corrected by subtracting the spectrum of the buffer solution.

Protein assay

The bioactivity of sTRAIL was monitored by a cell lytic assay [17]. The protein concentration was analyzed by Bradford method [18]. Protein assay was carried out by SDS-PAGE as described by Laemmli [19].

Results

Solubilization and refolding of sTRAIL

After extensive washing, most cellular proteins were separated from the inclusion bodies. The purity of the inclusion bodies reached 80%. The inclusion bodies were solubilized with 5 M GdnHCl plus 0.03 M DTT, approximately 95% inclusion bodies were dissolved. The application of DTT effectively enhanced the solubilizing ability of denaturants. The possible reason may be the existence of large amount of disulfide bonds in the inclusion bodies.

The addition of 0.4 M *L*-arginine, 0.5 M urea and 0.5 M NaCl in the refolding buffer increased the refolding efficiency of sTRAIL from about 13% to 51%. These additives helped the inclusion bodies gradually dissolved

into the refolding buffer, with a final protein concentration of 0.9 g/L. This renaturation method allowed the efficient use of refolding buffer by significantly reducing the reaction volume.

Purification of renatured sTRAIL

The refolded sTRAIL was loaded onto an immobilized metal affinity column and eluted with a linear imidazole gradient. Fig. 1 showed the elution profile of the refolded sTRAIL. A major protein peak appeared at about 40 mM imidazole. A_{280}/A_{260} values of the fractions of this peak were higher than 1.5. Inset is Coomassie-stained SDS-PAGE result of this peak, showing a single band at 19.6 kD. This peak was found to have strong cytotoxic

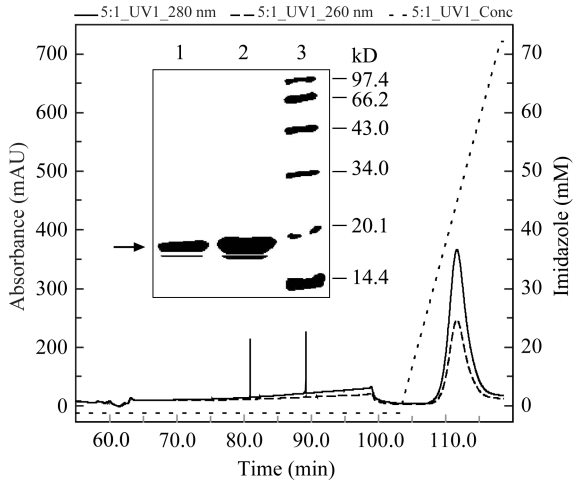

Fig. 1 Elution profile of sTRAIL on Ni-chelating column

Samples were loaded at 1 ml/min onto Ni-chelating Sepharose column FF. The bound proteins were eluted with linear gradient of 10–80 mM imidazole using AKTA explorer 100 system. sTRAIL was eluted in the gradient of 40–60 mM imidazole. Inset shows the Coomassie-stained SDS-PAGE of the major peak. 1, purified sTRAIL (12 μg); 2, purified sTRAIL (24 μg); 3, protein marker. The arrow indicates the sTRAIL protein.

activity against human pancreatic tumor cell line 1990 (generous gift from the Second Military Medical University). Although the protein was not expressed with a histidine tag, sTRAIL by itself had a weak affinity for immobilized metal and could be eluted with low concentrations of imidazole. Analytical reverse-phase HPLC was further used to examine the purity of sTRAIL. As shown in Fig. 2, renatured sTRAIL was purified to homogeneity. The recovery of sTRAIL from the affinity column was around 75%. The overall yield of the purified refolded sTRAIL from the inclusion bodies was about 35%.

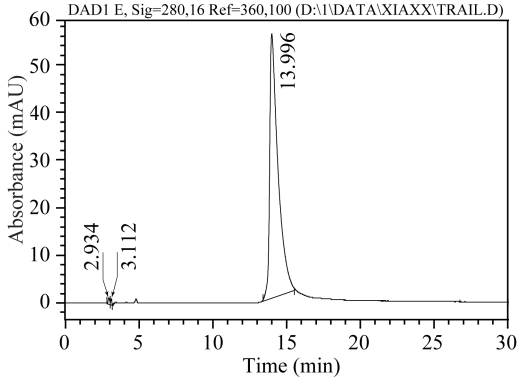

Fig. 2 HPLC analysis of the purified recombinant sTRAIL

The experiment was performed using a Sephasil Protein C4 column (12 μm 300 Å ST 4.6/250, Pharmacia). The column was equilibrated with 38% acetonitrile and 0.1% trifluoroacetic acid, and the proteins were eluted with a linear gradient of 38% to 62% acetonitrile and 0.1% trifluoroacetic acid over 40 min at a flow rate of 1 ml/min at 40 ℃.

Characterization of renatured sTRAIL

The UV spectrum of purified refolded sTRAIL showed an absorbance maximum at 276 nm and a shoulder at 283 nm, which was typical for a protein containing tyrosine and tryptophan residues. To study the secondary and tertiary structure of sTRAIL, we compared the far-UV CD and fluorescence spectra of native, refolded and chemically denatured proteins [Fig. 3(A, B)]. The unfolded protein exhibited a maximum emission band at 350 nm almost matching that of free tryptophan. The fluorescence intensity of the refolded protein decreased in the range of 330–400 nm, increased in the range of 305–330 nm, and the maximum wavelength was blue-shifted. The fluorescence spectrum of the refolded protein was almost identical to that of the native protein with maximum

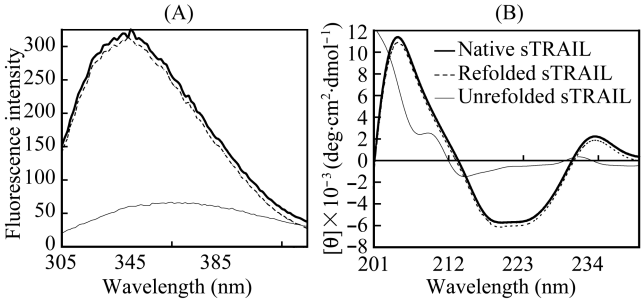

Fig. 3 Fluorescence (A) and CD (B) spectra of native, refolded, and unfolded sTRAIL

Prior to spectroscopic analysis, sTRAIL was dialyzed overnight against 20 mM PBS (pH 7.5) containing 1 mM DTT at 4 ℃. To obtain unfolded sTRAIL, it was dissolved in 6 M GdnHCl. The protein concentration was 0.2 g/L.

emission at 330 nm, indicating that the overall tertiary structure was recovered after refolding. The CD spectrum of sTRAIL showed a high content of β-sheet structure with a single negative maximum ellipticity around 220 nm as observed in TNF family proteins. CD spectra of the refolded and native proteins were also identical in the range of 200–250 nm with very similar intensities at 208 and 220 nm.

Renatured sTRAIL was shown to have strong cytotoxicity to human pancreatic tumor cell line 1990 (Fig. 4). The cytotoxicity was detectable in the presence of only 100 μg/L of sTRAIL and the ED_{50} was about 1.5 mg/L, comparable to the activity of sTRAIL expressed in the same recombinant *E. coli*.

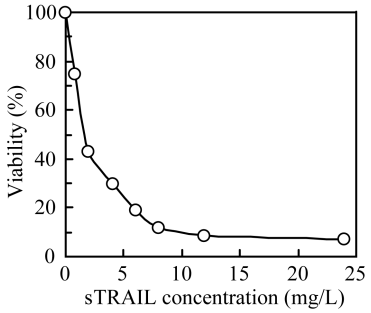

Fig. 4 Cytotoxicity of renatured sTRAIL to human ancreatic tumor cell line 1990

Discussion

We presented the preparation of *E. coli*-expressed sTRAIL with a high-yield refolding process. The overall yield of the protein using conventional dilution refolding method was less than 10% (data not shown). However, the renaturation procedure in this work obtained an overall yield over 35%, indicating that the stepwise addition method was beneficial for improving the yield of bioactive protein.

Refolding yield was mainly limited by aggregation since denatured proteins were generally prone to form aggregates in the refolding process. In conventional dilution method, the denatured protein solution was added into the refolding buffer in one batch, and renaturation at this relatively high denatured protein concentration was prone to form aggregates. In the stepwise method, the denatured protein solution was added in several steps to decrease the denatured protein concentration to a lower level. The addition of *L*-arginine together with urea and NaCl could dramatically decrease the formation of aggregates.

The purity of inclusion body was another important factor to influence the yield of refolding. According to Thatcher [20], peptidoglycans, lipids, nucleic acids, lipopolysaccharides, and membrane proteins were the major impurities of inclusion body preparations, and the concentration of protein impurities ranged from 5%–65% depending on the cultured condition. In our experiment, the initial purity of sTRAIL inclusion bodies shown by SDS-PAGE was about 32.5%, and the molecular weights of the major contaminant bands were about 31 kD, 37 kD and 40 kD. Addition of 0.5% Triton X-100 in washing buffer could effectively remove the 31 kD band, and 6 M urea could remove the 37 kD and 40 kD bands. After washing, the purity of the inclusion bodies reached 80%, facilitating the following refolding and purification.

TNF family proteins have a high sequence homology and their crystal structures have similar jellyroll β-sheet [21,22]. In particular, the number and location of tryptophan residues in sTRAIL, TNF-α and lymphotoxin (LT) are conserved. There are two tryptophans, Trp^{154} and Trp^{231}, per monomer. The red shift of λ_{max} and the decrease of tryptophan fluorescence intensity when sTRAIL was denatured indicated that the environment surrounding the tryptophan residues of denatured sTRAIL became more polar than that of the native form. Circular dichroism and fluorescence spectrum analysis suggested that the structure of renatured sTRAIL in this work was consistent with the previous prediction that sTRAIL had β-sheet secondary structure [23,24].

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Edited by
You-Shang ZHANG