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High-Level Expression, Purification, and In Vitro Refolding of Soluble Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)

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Abstract Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a new member of the TNF superfamily. In this paper, we report the expression, purification, and preparation of a recombinant form of the extracelluar domain of the TRAIL (sTRAIL) without posttranslational modifications, which may selectively induce apoptosis of tumor cells in vitro. To obtain recombinant nonfusion sTRAIL protein, the encoding region for sTRAIL was cloned between *Kpn*I and *Bam*HI in pET32a. The Trx (thioredoxin)/sTRAIL fusion proteins were expressed in the form of inclusion bodies in *Escherichia coli* host strain BL21 (DE3). The expression level was more than 35% of total cell lysate. Inclusion bodies were disrupted, washed, and isolated at pH 9.0, and were completely dissolved in a buffer containing 2 M urea at pH 9.0. After nickel ion metal affinity chromatography, gel filtration chromatography, and renaturation, the refolded fusion proteins with a purity of >98% were obtained. Trx/sTRAIL L proteins were digested by enterokinase to both Trx and sTRAIL fragments, which then were separated by cation exchange chromatography. Cell proliferation experiments proved that the rsTRAIL (98% purity) retains its cancer-selective apoptosis-inducing properties. This result suggested that the recombinant sTRAIL may have cancer therapeutic applications.

Keywords sTRAIL · Fusion protein · Inclusion bodies · Protein expression · Refolding · Protein purification

Introduction

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

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jellyroll β-sandwich [5, 6]. The TRAIL protein undergoes posttranslational processing, including extensive glycosylation before its secretion by producing cells. However, the role of the glycosylated proteins in apoptosis induction and/or other functions of sTRAIL had not been known until now. To obtain a functional nonfusion sTRAIL and to investigate the effect of glycosylation modification on the biological activity of TRAIL, we constructed, expressed, and purified nonfusion recombinant sTRAIL from *Escherichia coli*, and identified its cancer-selective apoptosis-inducing properties. This work not only describes a new preparation strategy of sTRAIL but also introduces a highly active nonfusion protein that may have important clinical applicability.

Construction of sTRAIL Expression Vector

First strand cDNA was synthesized from the total RNA of human peripheral blood leukocytes using avian myeloblastosis virus reverse transcriptase (Promega, USA) and random primer (9 mer; Takara, Japan). The sTRAIL gene was amplified from the cDNA mixture with two primers: 5′-TAG AGG TAA CT GAG AAA GAG GT-3′, which contains a *Kpn*I site (underlined), and 5′-CGA CGG ATC CTT AGC CAA CTA AAA A-3′, which contains a *Bam*HI site (underlined). Polymerase chain reaction (PCR) was performed using pfu polymerase (Stratagene, USA) under the following conditions: first denaturation for 5 min at 95 °C; then, denaturation for 1 min at 95 °C, annealing for 30 s at 55 °C, extension for 1 min at 72 °C for 35 cycles; and last extension for 10 min at 72 °C. The amplified product was digested with *Kpn*I and *Bam*HI and then ligated to corresponding sites of the expression vector pET32a. The resulting plasmid was named pET32a/sTRAIL.

Expression of Recombinant Protein and Preparation of Inclusion Bodies

The constructed recombinant plasmid pET32a/sTRAIL was transformed into competent *E. coli* BL21(DE3). The bacteria was cultured in Luria–Bertani (LB) medium (1% bacto-tryptone, 0.5% yeast extract, 85 mM NaCl) with vigorous shaking (220 rpm) at 37 °C to a density of OD600=0.6. Then, 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to induce the expression of the recombinant protein at 37 °C for 5 h. After induction, the cultures were centrifuged at 6,000×*g* for 15 min at 4 °C. Two grams (wet weight) of pellet was resuspended in 20 ml sonic buffer (50 mM Tris–HCl, 5 mM EDTA, 0.15 mM NaCl, 1 mg/ml lysozyme, pH 8.0) for 45 min at 4 °C, and sonication was carried out on ice using an ultra-sonicator (Ningbo Instrument, China) until the cells were lysed. In the process of cell lysis, 15 mM protease inhibitor PMSF was added. The inclusion bodies were collected by centrifugation at 10,000×*g* for 10 min at 4 °C, the supernatant was discarded, and the inclusion bodies recovered.

Purification of Trx/sTRAIL

The inclusion bodies were dissolved in a buffer containing 20 mM Tris–HCl and 2 M urea (pH 9.0) to unfold the misfolded protein in the inclusion bodies completely. The fusion protein solution was adjusted to the concentration of 0.1–0.2 mg/ml (determined using a Lowry assay) and purified by affinity chromatography. A Pharmacia Chelating Sepharose Fast Flow column (XK16/10) with volume of approximately 20-ml resin was preloaded with 0.5 M Ni_2SO_4 and equilibrated with 20 mM Tris–HCl and 2 M urea (pH 9.0). The fusion protein solution was loaded onto the column at a flow rate of 0.5 ml/min; the

impurities were removed by elution with low-concentration imidazole (less than 50 mM). Finally, the fusion protein was eluted with 20 mM Tris–HCl, 2 M urea, and 0.5 M imidazole (pH 9.0) at a flow rate of 10 ml/min under the control of the AKTA purifier 100 protein purification system. After identification by SDS-PAGE, the target fraction was refined by loading on a Pharmacia Superdex 75 gel filtration column (26/60) with volume of approximately 320-ml resin which was equilibrated with 20 mM Tris–HCl, 2 M urea (pH 9.0), and then eluting with 20 mM Tris–HCl, 2 M urea (pH 9.0).

Preparation of sTRAIL

Three hundred and fifty milliliters of the purified fusion protein was slowly added to 2,000 ml refolding buffer I [20 mM Tris–HCl, 0.8 mM reduced glutathione hormone (GSH), 1.2 mM oxidized form glutathione (GSSG), pH 9.0] under constant stirring on a magnetic stirrer for 12 h at 4 °C. Then the above solution was slowly added to 3,500 ml refolding buffer II (20 mM Tris–HCl, 1 mM EDTA, pH 8.5) and stirred for 12 h at 4 °C. This solution was ultrafiltrated by Sartorius ultrafine filter (Germany) to concentrate 15- to 20-fold, the concentration of protein was adjusted to about 2 mg/ml with a buffer (20 mM Tris–HCl, pH 8.0). The refolding fusion protein was digested by EK with different enzyme concentration at various treatment times. The resulting solution of digestion was analyzed by SDS-PAGE. Then the solution was adjusted to pH 7.0 and purified by a Pharmacia HiTrap SP Fast Flow column (26/20) with volume of approximately 50-ml resin which is a cation exchange column equilibrated in 20 mM Tris–HCl buffer (pH 7.0). The protein solution was loaded onto the column at 5 ml/min, and the column was washed with 0.1 M NaCl in 20 mM Tris–HCl (pH 7.0). The purity of the eluted protein was identified by SDS-PAGE.

SDS-PAGE and Western Blotting Analysis

SDS-PAGE analysis was performed according to Laemmli [7] using 15% polyacrylamide gels. Samples of sTRAIL were mixed with equivalent 2×sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue, and 10% of 2-mercaptoethanol). Gels were stained with Coomassie brilliant blue R-250. For Western blotting, the protein was separated on a 15% polyacrylamide gel under reducing conditions and then transferred to a PVDF membrane (Millipore, USA). The membrane was washed for 60 min with TBS (50 mM Tris–HCl, 150 mM NaCl, pH 8.0), and nonspecific protein binding was blocked by incubating the membrane with 5% MTBS (5% skim milk in TBS, pH 7.4) for 1 h. After washing with TBST (TBS containing 0.05% Tween 20) for 15 min, 1:1,000 mouse anti-human TRAIL IgG (ProSci Incorporated, USA) in TBST was added and incubated for 90 min at 37 °C. The membrane was washed three times for 10 min with TBST, incubated for 60 min with a 1:500 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase (HRP; Santa Cruz Biotechnology, USA) in TBST, and subsequently washed three times for 5 min with TBST. The blot was performed using the 3,3′,5,5′-tetramethyl benzidine chemiluminescence system (Promega).

Cell Viability Assay

Human cervical cancer HeLa cells purchased from ATCC were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37 °C in a humidified

atmosphere of 5% CO_2. HeLa cells (5×10^4/100 μl) were treated for 1.5 h by challenge with recombinant human TRAIL in the concentrations indicated in Fig. 6. Cell viability was assessed by the MTT assay according to procedures previously described [8].

Estimation of Protein Concentration

Total protein concentrations of samples were determined using the Protein Coomassie Blue Assay kit, Bradford method (Pierce), according to the manufacturer's instructions, using bovine serum albumin as the reference standard.

Results

Identification of Recombinant Plasmid

pET32a plasmid is widely used to obtain high expression levels of His-Trx tagged proteins in *E. coli*. To express sTRAIL protein which can be further used in human, the restricted PCR-generated DNA fragment is subcloned using *Kpn*I and *Bam*HI cloning sites. The analysis by PCR and restriction endonuclease digestion proved that the insertion of the sTRAIL cDNA in the recombinant plasmid was correct. The ORF of sTRAIL cDNA in the recombinant plasmid was sequenced and was confirmed as the correct sequence in the recombinant plasmid. Additionally, the sTRAIL sequence followed the EK sequence to prepare sTRAIL without extra amino acid residues from the fusion protein Trx/sTRAIL. A His_6-tag in Trx/sTRAIL allows easy purification of the fusion protein by metal affinity chromatography.

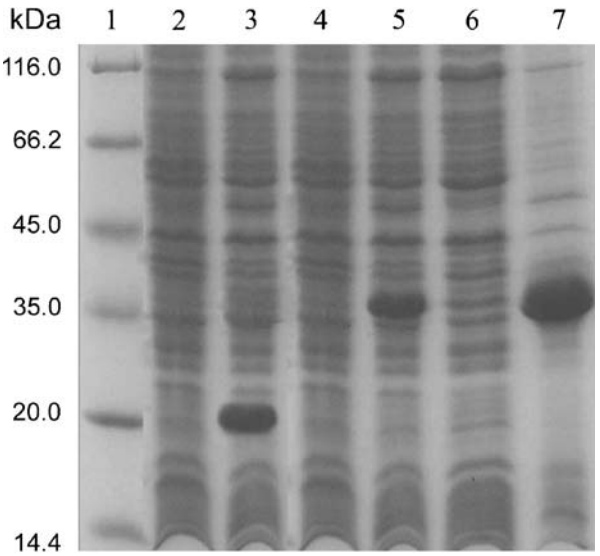

Fig. 1 SDS-PAGE analysis of Trx/sTRAIL expressed in *E. coli*. *Lane 1* Protein molecular weight markers, *lane 2* uninduced whole cell lysate of pET32a/BL21 at 37 °C, *lane 3* whole cell lysate of pET32a/BL21 induced at 37 °C, *lane 4* uninduced whole cell lysate of pET32a-sTRAIL/BL21 at 28 °C, *lane 5* whole cell lysate of pET32a-sTRAIL/BL21 induced at 28 °C, *lane 6* induced whole cell lysate of pET32a-sTRAIL/BL21 at 37 °C, *lane7* whole cell lysate of pET32a-sTRAIL/BL21 induced at 37 °C

Expression and Purification of Trx/sTRAIL

The fusion protein was highly expressed after induction at 37 °C with 0.5 mM IPTG. SDS-PAGE of cell lysates followed by scanning with the Image Master VDS image analysis system (Amersham Pharmacia) revealed a major protein band of the expected 37-kDa size (Fig. 1). The expression level of synthesized Trx/sTRAIL at 28°C was slightly less than at 37 °C. The Trx protein that had an apparent molecular weight of 20 kDa was expressed by pET32a/BL21 (Fig. 1, lane 3). The expression level of Trx/sTRAIL grown in the 5-l fed batch fermentation in synthetic medium was up to 30% of the total cellular protein. The recombinant Trx/sTRAIL protein was shown to be in the form of insoluble inclusion

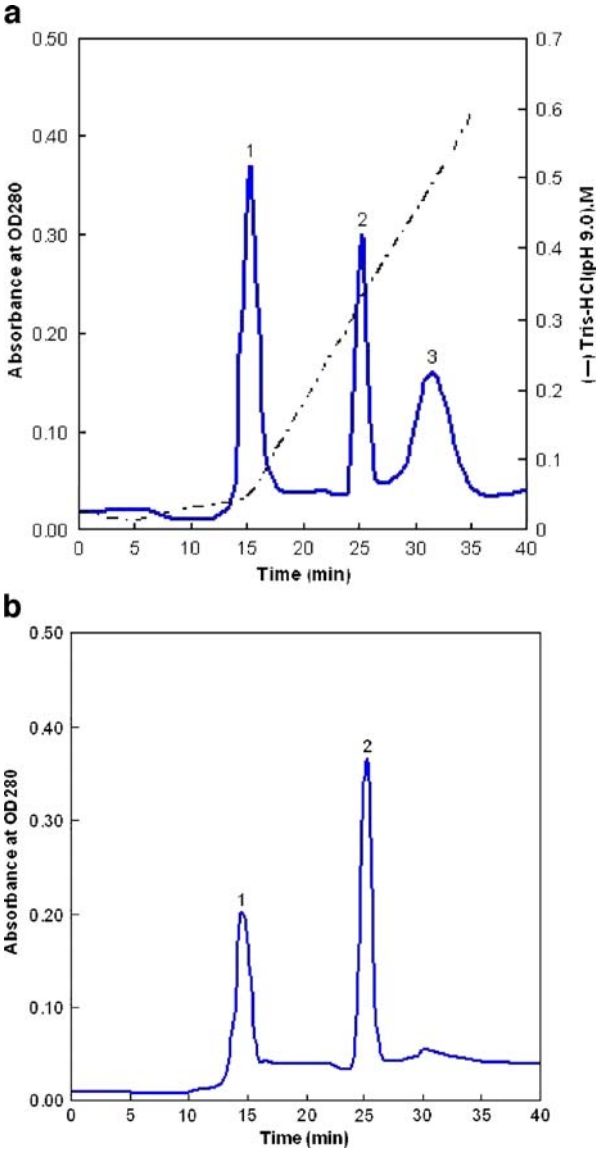

Fig. 2 The first step of purification of Trx/sTRAIL by the nickel ion metal-chelating affinity chromatography (**a**). The second step of purification of Trx/sTRAIL by Superdex 75 gel filtration chromatography (**b**)

bodies. The induced cultures were centrifuged, the cell pellets (24.6 g of wet cell paste) were disrupted with a homogenizer. Inclusion bodies were washed four times and solubilized by a 2 M urea solution. The fusion protein of inclusion body had about 50% purity. The fusion protein was loaded onto a Chelating Sepharose Fast Flow column (XK16/10). Elution revealed three dominant protein peaks. SDS-PAGE and Western blot analysis revealed that Peak 2 represented the fusion protein with a purity of about 85%, and the nontarget proteins in Peak 1 were larger-molecular-weight proteins than the fusion protein (Fig. 2a). Therefore, the fusion protein was concentrated to 10 mg/ml by PEG6000, and loaded onto Superdex 75 column. SDS-PAGE analysis revealed that Peak II represented the fusion protein (Fig. 2b). This purification step is very efficient, resulting in high purity (96%) of Trx/sTRAIL (Fig. 3, lane 4).

Preparation and Purification of sTRAIL

After two-step dilution renaturation, the fusion protein was adjusted from pH 9.0 to pH 8.0. The refolding fusion protein was digested at 25 °C with four different concentrations of EK

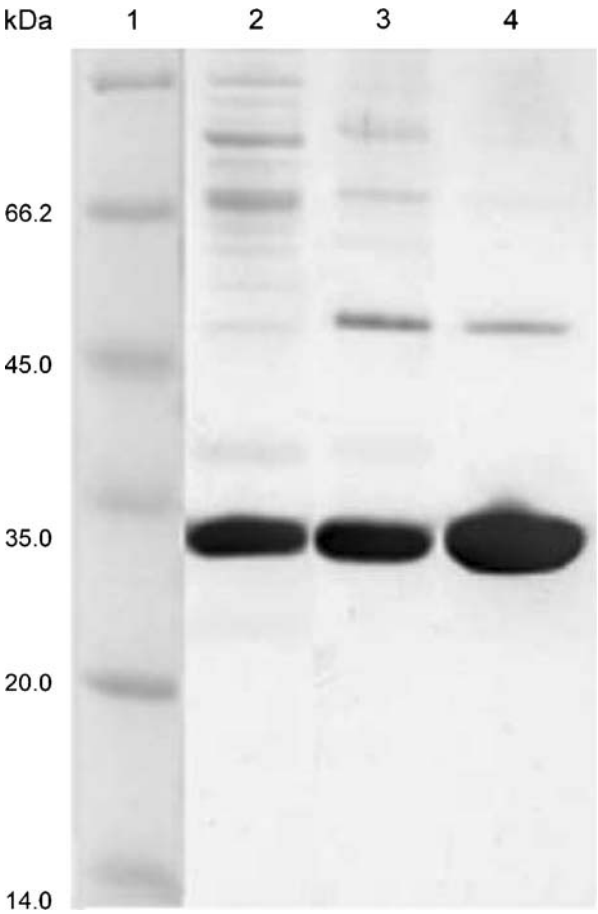

Fig. 3 The SDS-PAGE of the purified Trx/sTRAIL. *Lane 1* Protein molecular weight markers, *lane 2* inclusion bodies of Trx/sTRAIL, *lane 3* the purified Trx/sTRAIL by the nickel ion metal-chelating affinity chromatography, *lane 4* the purified Trx/sTRAIL by Superdex 75 gel filtration chromatography

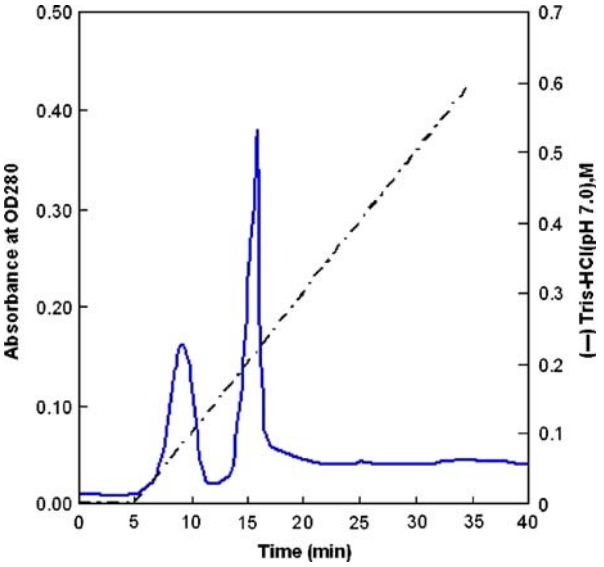

Fig. 4 Separation of Trx and sTRAIL by HiTrap SP XK26/20 chromatography

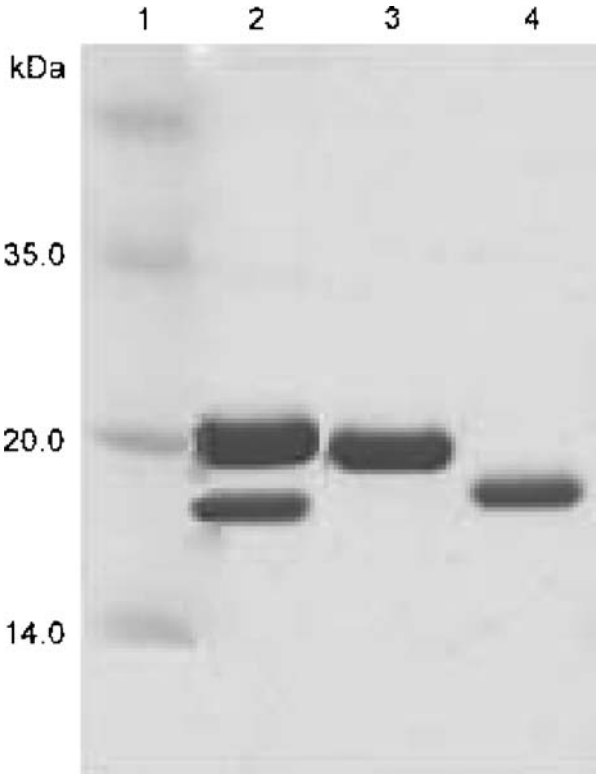

Fig. 5 SDS-PAGE of Trx and sTRAIL separated by HiTrap SP XK26/20 chromatography. *Lane 1* Protein molecular weight markers, *lane 2* digestion product of Trx/sTRAIL with EK, *lane 3* the Trx fragment, *lane 4* the sTRAIL fragment

Table 1 Purification of sTRAIL (24.6 g of wet cell paste).

Purification step	Amount of Trx/sTRAIL (amount of sTRAIL; mg)	Purity (%)	Overall yield (%)
E. coli cell lysate	635 (287)	32	100
Washed inclusion body	438 (209)	51	72
Chelating Sepharose FF	319 (188)	85	65
Superdex 75 gel filtration	204 (112)	97	39
Digested product by EK	(112)	50	39
HiTrap SP FF	(95)	98	33

[2, 1, 0.5, or 0.25%, (*w*/*w*)] for four different digestion times (16, 24, 32, or 40 h). After SDS-PAGE analysis, we concluded that the adequate digestion condition is 0.25% (*w*/*w*) EK with an incubation of 32–40 h, which caused complete digestion of the fusion protein Trx/sTRAIL to Trx and sTRAIL. The digestion product was loaded onto HiTrap SP cation exchange column (Amersham Pharmacia). Elution fraction revealed two dominant protein peaks (Fig. 4). SDS-PAGE analysis revealed that Peak 1 was the Trx fraction, and Peak 2 represented the sTRAIL protein with a purity of more than 98% (Fig. 5). The purification summary is presented in Table 1.

Finally, the identities of Trx/sTRAIL and sTRAIL were confirmed by Western blot analysis, in which the fusion protein and the sTRAIL bands were shown to be immunoreactive with a mouse mAb against human sTRAIL (R&D System, USA; data not shown).

Biological Activity of Refolded sTRAIL

To test if the expressed sTRAIL has biological activity, human cervical cancer HeLa cells were used for examining the effects of sTRAIL on cell growth by MTT assay. Renatured sTRAIL was shown to have strong cytotoxicity to human cervical cancer HeLa cells (Fig. 6). The cytotoxicity was detectable in the presence of only 100 μg/l of sTRAIL, and the ED50 was about 1.5 mg/l.

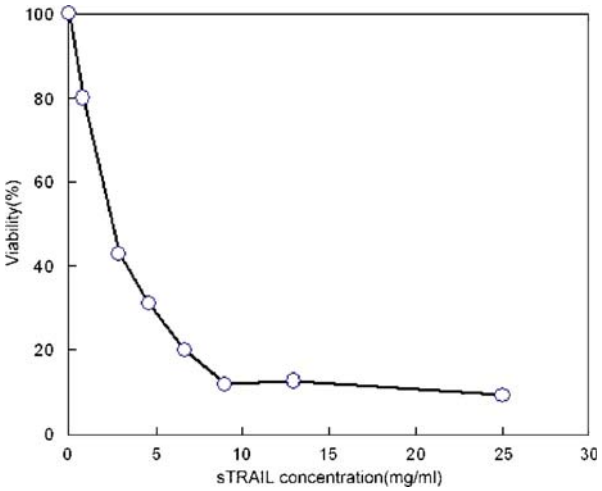

Fig. 6 Cytotoxicity of the recombinant sTRAIL to human cervical cancer HeLa cells. HeLa cells (5×10^4) were treated with sTRAIL in the different concentrations for 1.5 h at 37 °C, 5% CO_2. Cell viability was then evaluated by MTT Assay

Discussion

In this experiment, we failed to get a high expression level of sTRAIL as nonfusion protein form in some pET expression vectors: pET11b, pET22a, and pET28b. Although the sequences of the recombinant plasmids were correctly confirmed, the protein of interest was not detected on the SDS-PAGE and Western blot (data not shown). It seems that the expression of the gene sequence of sTRAIL was unfavorable in *E. coli* expression systems. Previously, there were two reports of recombinant sTRAIL expressed by *E. coli*, but they all were fusion proteins: GST/sTRAIL and sTRAIL/6×His [9, 10]. In our experiment, *E. coli* host strain BL21 (DE3) was transformed with the pET32a-sTRAIL and highly expressed the sTRAIL as fusion form (>30%). The fusion protein includes a prokaryotic cell protein thioredoxin, which could not only reduce the digestion by bacterial proteases, but could promote the expression of recombinant fusion protein like molecular chaperones [11]. It was very interesting that the inclusion bodies of Trx/sTRAIL were very difficult to dissolve in 8 M urea solution after disruption, isolation, and washing at pH range from 5 to 9. The solution worked only at pH>9. Then we found that the inclusion bodies were completely dissolved in the 2 M urea at pH 9. Therefore, we improved the washing and solution conditions which were more efficient than before, which obviously reduced the loss of target protein in the washing process, and was able to reduce the volume of refolding buffer in the renaturation process. After purification and renaturation, the fusion proteins were digested with EK which gave fragments Trx and sTRAIL. As Trx includes 6×His-tag and sTRAIL does not contain 6×His-tag, we could use nickel ion metal affinity chromatography to purify target protein. Moreover, because of their different isoelectric point (p*I* of Trx is 5.4, p*I* of sTRAIL is 8.8), we could also separate both fragments with cation exchange chromatography. Our results showed that the effect of cation exchange chromatography is significantly better than the former one.

In this paper, a method for dilution refolding of sTRAIL is presented which allows the production of large quantities of correctly refolded sTRAIL. A combination of a high yield expression system and an efficient refolding protocol made it possible to prepare sufficient quantities of sTRAIL protein.

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