**Fed-batch production of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)
in soluble form in *Escherichia coli* and its purification and characterization**

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent. The aim of this study is to produce large quantities of highly pure and bioactive recombinant human TRAIL. Here, TRAIL was expressed in soluble form by pH-stat fed-batch cultivation and purified using a rapid and simple two-step chromatographic procedure. To improve the soluble yield, expression of TRAIL in *E. coli* was induced with low IPTG concentration (0.1 mM) at low temperature (28°C) supplemented with ZnSO₄ (0.5 mM), using glycerol as carbon source. Under the optimized conditions, 4.14 ± 0.19 g/L of TRAIL in soluble form was achieved at 19 h without pure oxygen. To purify the recombinant TRAIL, we developed an efficient two-step chromatographic procedure including affinity chromatography and cation-exchange chromatography, especially improved the cation-exchange chromatography using a combination of pH and NaCl gradients strategy. Consequently, 4313.5 mg of target protein with high purity (98.1%) was obtained from 2.3 liters of cell broth. Our results also showed that the purified TRAIL was with ordered secondary and tertiary structures, in homogeneous form and with strong cytotoxicity.

Keywords: TRAIL; Soluble expression; Fed-batch cultivation; Purification; Anticancer activity

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also termed Apo-2 ligand, was discovered by Wiley *et al.* in 1995 based on its sequence homology to TNF and FasL [1]. As a typical member of the TNF superfamily, TRAIL is primarily expressed as a type II membrane protein with 281 amino acids. The extracellular domain (C-terminus, amino acids 114-281) of TRAIL can be cleaved by cysteine proteases resulting in a soluble monomer. Three soluble TRAIL monomers form a homotrimer, which subsequently assembles with three receptor molecules and induces apoptosis of cancer cells [2,3]. TRAIL carries a zinc ion at the trimer interface, coordinated by the single unpaired cysteine residue of each monomer. The zinc ion is required for maintaining the native structure and stability and, hence, the biological activity of TRAIL [4].

TRAIL has received considerable attention for its selective cytotoxicity toward tumor cell lines [2]. Preclinical and clinical safety studies exhibited no toxicity of TRAIL treatment [5], in contrast, TNF or CD95L led to severe systemic toxicity [6]. Dulanermin, containing the 114-281 amino acids of TRAIL, is the only recombinant TRAIL developed for clinical application. Unfortunately, no significant anticancer activities were observed in phase II b clinical trials (randomized control trials, RCTs) when performed in non-small cell lung cancer (NSCLC) and non-Hodgkin's lymphomas [7,8], which thus lead to the termination of its clinical studies 4 years ago. However, researches never give up to bring TRAIL back on track for cancer therapy [8]. They found the limited clinical efficacies shown in previous trials are caused by many factors and some of them can be overcome. Strategies, such as identification of potent and cancer-selective TRAIL sensitizers as novel combination partners (sunitinib, lapatinib, Smac mimetics and BH3 mimetics, *etc.*, which inhibit anti-apoptotic molecules or target alternative resistance pathways), development of effective delivery systems and novel predictive biomarkers, have been considered to improve the sensitivity of TRAIL-based therapies [3,8]. Bao *et al.* [9] reported that administration of sunitinib followed by TRAIL, as well as a simultaneous administration of both agents, could serve as favorable treatment regimens for NSCLC-derived cells, which were resistant to TRAIL in the previous studies [7]. TRAIL is brought up as a potential anti-tumor agent again. Therefore, it appears very important to prepare large quantity of TRAIL with high quality and efficiency [10].

E. coli is the most commonly used prokaryotic host for the overexpression of recombinant proteins. Nevertheless, high-level expression of TRAIL in *E. coli* often leads to the formation of biologically inactive aggregates known as inclusion bodies, and thus makes a protein renaturation procedure necessary [11-15]. The renaturation procedure is usually laborious, time-consuming, costly and inefficient. Therefore, maximized expression of TRAIL in soluble form is considered good strategy for obtaining large quantities of this interesting anticancer protein.

The aim of the present study was to prepare large quantities of highly pure and bioactive TRAIL. Here, TRAIL was expressed in soluble form using pH-stat fed-batch cultivation, which is

the most widely used technique to produce various recombinant proteins by increasing cell density [16,17]. The fermentation conditions, such as carbon sources, temperature and inducer concentration were optimized. Zinc ion was added to improve the soluble yield as described by Sun *et al.* [14]. An efficient two-step chromatographic procedure was employed to obtain pure TRAIL and the characterization of this interesting anticancer protein was also described.

2. Materials and methods

2.1. Bacterial strains and plasmids

A widely used prokaryotic vector for high level expression of recombinant proteins in *E. coli*, pET-28a, was selected to construct recombinant expression vector pET-28a/TRAIL (Supplemental Fig. 1). The synthesis of cDNA of TRAIL was as described previously [1]. TRAIL (114-281) gene was amplified from the cDNA mixture with two primers: 5'- TCG ACC ATG GT GAG AAA GAG GT - 3', containing a *NcoI* site (underlined) and the start codon "ATG" (bold), and 5'- CGA CGA ATT CTT AGC CAA CTA AAA A - 3', containing an *EcoR I* site (underlined) and the stop codon "TAA" (bold). The *NcoI*-TRAIL-*EcoR I* amplified product was cloned into a pET-28a vector (Novagen) to construct pET-28a/TRAIL, then the recombinant plasmid was transformed into competent *E. coli* BL21(DE3) and transforming clones were selected on Luria-Bertani (LB) medium with 50 µg/ml kanamycin.

2.2. Shake flasks cultivation

LB medium with 50 µg/mL kanamycin was used for shake flask cultures. Expression of TRAIL (without extra amino acids or tags) was induced by IPTG. ZnSO₄ was added into the medium to improve the soluble yield [14]. The carbon source, ZnSO₄ concentration, IPTG concentration and the induction temperature were optimized.

2.3. Fed-batch cultivation and feeding strategies

Fed-batch cultivation was carried out in a 3.7-liter bioreactor (Bioengineering, Switzerland) containing 1.6 liters of batch medium. The composition of the batch medium is described in Table 1. The pH was maintained at a set-point of 7.0 by feeding 10% sodium hydroxide and 2% potassium phosphate, the temperature was set to 37°C, and the dissolved oxygen level was maintained above 25% of air saturation by automatic control of the agitation speed (up to 1,500 rpm). The pH-stat feeding strategy, which was based on the sharp pH rise upon carbon source depletion, was employed in fed-batch cultivation. When the pH rose to a value greater than its set point (pH 7.0) by 0.2 U, appropriate volume of the feeding solution (Table 1) was automatically added.

The pH-stat fed-batch cultivation process of *E. coli* BL21(DE3) with pET-28a/TRAIL continued a period of 19 hours without pure oxygen (Fig. 2). Expression of TRAIL was induced at the steady state (12 h) by IPTG with ZnSO₄ under the optimized conditions as in the shaking

109 flasks.

111 2.4. Two-step chromatographic purification of TRAIL

112 Cells were harvested, resuspended in ice-cold buffer A (20mM PBS, 50 mM NaCl, 10 μ M
113 ZnSO₄, 5 mM dithiothreitol, pH 6.4) supplemented with protease inhibitor cocktail (Roche,
114 Germany), with 1/10 volume of cell culture medium. Supernatant, collected by centrifugation after
115 sonication and filtered through a 0.22 μ m membrane, was loaded onto a Ni Sepharose 6 FF
116 column (Pharmacia) pre-equilibrated with buffer B (20 mM PBS, 50 mM NaCl, pH 6.4). TRAIL
117 was eluted with a 0 to 500 mM imidazole gradient. For further purification, the protein solution
118 was diluted with ddH₂O (1:1) (~ pH 6.4) and loaded onto a SP Sepharose FF column (Pharmacia)
119 pre-equilibrated with buffer C (10 mM PBS, 25 mM NaCl, pH 6.4). Buffer D (10 mM PBS, 80
120 mM NaCl, pH 7.4) was used to wash the contamination. TRAIL was eluted from the column using
121 Buffer E (10 mM PBS, 150 mM NaCl, pH 8.4). The progress of the purification was monitored by
122 10% SDS-PAGE and quantitatively analyzed using a BioRad scanning densitometer. The purity of
123 the final purified TRAIL was determined by reversed-phase HPLC (RP-HPLC). RP-HPLC was
124 carried out on Agilent system with a ZORBAX 300SB-C18 column (250 mm $\times$ 4.6 mm, 5 μ m)
125 with the same procedure as reported previously [18], but recorded at 280 nm. Western blot
126 analysis was performed as described, using the mouse anti-human TRAIL IgG (1:1000, ProSci
127 incorporated, USA) as primary antibody and goat anti-mouse IgG conjugated with horseradish
128 peroxidase (HRP) (Santa Cruz Biotechnology, USA) as secondary antibody [11].

130 2.5. ESI-MS analysis

131 ESI-MS was determined on an LCQ Deca XP Max mass spectrometer using a positive ion
132 mode with a scan range of m/z 150–2000 amu (Thermo-Finnigan). The samples (collected from
133 RP-HPLC) were introduced into the ESI ion source at a flow rate of 5 μ L/min. The following
134 parameters were used: nitrogen as sheath gas, 15 arb; capillary temperature, 275°C; spray voltage,
135 4.5 kV. Data acquisition and analysis were done on an XcaliburTM 1.4 SR1 workstation.

137 2.6. Circular dichroism (CD) spectroscopic analysis

138 CD measurements, used for detecting the secondary and tertiary structures of TRAIL, were
139 performed on a Jasco J-715 spectrophotometer (Jasco, Japan). TRAIL was dissolved in 20 mM
140 sodium phosphate containing 50 mM NaCl (pH 7.4) with the concentration of 0.15 mg/mL for
141 far-UV (200–250 nm) CD measurements (detecting the secondary structure) and 1 mg/mL for
142 near-UV (250–300 nm) CD measurements (detecting the tertiary structure). The following
143 parameters were used: a cuvette, path length of 1 cm; scanning speed, 100 nm/min; bandwidth, 1
144 nm; response time, 0.25 s; temperature, 25°C. Scans were collected at 0.2-nm intervals and were
145 performed three times.

2.7. Size exclusion chromatography-HPLC (SEC-HPLC) analysis

SEC-HPLC analysis, used for detecting the oligomerization state of TRAIL, was performed on a Agilent HPLC system using a Protein-PakTM 125 (7.8 mm × 300 mm) column (Waters, USA) with a mobile phase of 0.1 M phosphate buffer (PB, pH 7.0, 25°C) at a flow rate of 1 mL/min. Samples were prepared at concentrations of 1 mg/mL by diluting with mobile phase and the injection volume was 20 µL. Lysozyme (14.4 kDa), carbonic anhydrase (31.0 kDa), albumin (66.2 kDa) and γ-globulin (155 kDa) were used as molecular weight (MW) standards. Their retention times (t_R) were recorded by absorbance at 280 nm. Protein masses were calculated from a calibration curve and formula ($\lg MW = a + bt_R$).

2.8. Zinc content analysis

Purified protein with a concentration of 3 mg/mL was analyzed for zinc content using a Hitachi 180-50 atomic absorption spectrophotometer equipped with a graphite furnace. The hollow cathode lamp of zinc (10-mA lamp current), 213.8 nm wave length, air-acetylene flame, and 1.3 nm slit width were selected in this experiment.

2.9. Cytotoxicity assay

Cytotoxicity of TRAIL was determined with a sulforhodamine B assay [19]. BEL-7402 human hepatocellular carcinoma cells (from Shanghai Institute of Material Medical, Chinese Academy of Sciences) were seeded in 96-well plates (1×10^4 /well) for 24 h, then removed the supernatant and added serially diluted TRAIL. After incubation for another 72 h, cell growth was assayed using sulforhodamine B.

3. Results and discussion

3.1. Optimization of culture conditions in shake flasks

The production of TRAIL from the recombinant *E. coli* was 300.2 mg/mL when induced by 0.5 mM IPTG at 37°C for 12h in LB medium, with 128.8 mg/L in soluble form. Culture conditions were optimized in this study. Carbon sources critically influence the productivity of the metabolic by-product, and thus influence the production of recombinant proteins. Glycerol was reported to be a better carbon source rather than glucose for growth and TRAIL production because of its lower acetate productivity [15,20]. As shown in Fig. 1A, the modified LB medium with 2% of glycerol led to increase of both cell density and total TRAIL production (522.5 mg/mL), but did not for the soluble production (133.6 mg/L). ZnSO₄ was added to improve the solubility level. Fig.1B demonstrated that the cell growth was inhibited by ZnSO₄ in a dose-dependent manner and the maximum yield of soluble TRAIL was obtained when ZnSO₄ concentration was 0.5 mM. Therefore, 0.5 mM ZnSO₄ was added in induction phase but not in pre-induction phase. The soluble production was further improved by optimization of the induction temperature and it reached the peak at 28°C (Fig.1C). Moreover, 0.1mM IPTG

enhanced soluble TRAIL yields, with a production of 476.0 mg/L (Fig.1D). The modified LB medium was then used as batch medium, and the optimized ZnSO₄ concentration, inducer concentration and induction temperature conditions were utilized in fed-batch cultivation.

3.2. Fed-batch cultivation and feeding strategies

There are at least two general approaches to improve soluble protein yield in a specific organism: increasing cell density and developing solubility level. Fed-batch cultivation is the most widely used technique to produce various recombinant proteins by increasing cell density [16,17]. However, secretion of metabolic by-product acetate is one of the major drawbacks for growth and maximizing the production of recombinant proteins by fed-batch cultivation. Larger amount of acetate would secret when use glucose as carbon source than glycerol [15,20]. Therefore, glycerol was employed as carbon source in this work. Nutrient-feeding strategy also influences the production of heterogenous proteins, and the pH-stat strategy was used here. It has been reported that pH-stat feeding strategy met the cell demand well and promoted the cell capacity for expression of heterogenous proteins. Keeping pH steady state could be advantageous to avoid the accumulation of organic acid and achieve high level expression of target proteins [21]. Even though this strategy may not be optimal, it commonly led to satisfactory production of recombinant proteins [16].

It was also thought that the high induction temperature (42°C) resulted in the incorrect folding of recombinant protein and influenced the growth of the *E. coli* strain, and thereby decreased the soluble yield [22]. Therefore, optimization of induction temperature may be a good strategy to develop the solubility level. Other culture conditions like the inducer concentration also have intensive impact on the soluble yield. Turner *et al.* reported that soluble production of cyclomaltodextrinase was increased by adjusting of inducer concentration [23]. To our knowledge, only one fed-batch case was performed to improve the soluble yield of TRAIL through zinc ion feeding strategy. The zinc ion feeding strategy led to great increase of the soluble production of TRAIL (1.6 g/L), but it is only 43.2% of the total TRAIL (3.75 g/L) [14]. In the present study, we would further improve the soluble yield of TRAIL using optimized conditions.

The pH-stat fed-batch cultivation process of *E. coli* BL21(DE3) with pET-28a/TRAIL continued a period of 19 hours without pure oxygen (Fig. 2). Expression of TRAIL was induced at the steady state (12 h) by 0.1 mM IPTG with 0.5 mM ZnSO₄ at 28 °C. At the end of cultivation, a total of 2.3 liters of cell broth was harvested. The final OD₆₀₀ and soluble production of TRAIL were 83.1±3.7 and 4.14 ± 0.19 g/L, respectively. The total expressed TRAIL was 5.02 ± 0.22 g/L, which suggested that 82.5% of the target protein was expressed in soluble form. The soluble production of TRAIL reported by Sun *et al.* was 1.6 g/L at 30 h with pure oxygen, while it reached 4.14 ± 0.19 g/L at 19 h without pure oxygen in our study. Pure oxygen is one of the most expensive components for the economical production of TRAIL, and brings difficulties to scale-up fermentation. Thus, our strategy is more efficient, economical and applicable for scale-up

industrial process.

3.3. Two-step chromatographic purification of recombinant human TRAIL

Addition of Zn^{2+} often leads to increase of TRAIL trimer and decrease of inactive TRAIL forms [14], therefore 10 μM ZnSO_4 was added in buffer A. Recombinant TRAIL was purified from 2.3 L of culture obtained from fed-batch cultivation using a two-step chromatographic strategy. TRAIL was separated from contamination by affinity chromatography, resulting in a purity of 83.5%. Then, a cation-exchange chromatography using a combination of pH and NaCl gradients was followed. The pH gradient is with high resolution but low elution efficiency, while the salt gradient is with high elution efficiency but low resolution [24]. As shown in Fig. 3 and Table 2, the combination of these two strategies led to not only high resolution but also high elution efficiency. The final purified production appeared as a single ~20 kDa band on SDS-PAGE (Fig. 3A), and showed specific reaction with mouse anti-human TRAIL monoclonal antibody (Fig. 3B). The final amount and yield of purified TRAIL were 4313.5 mg and 45.3%, respectively. The purity of the TRAIL, determined with RP-HPLC, was 98.1% (Supplemental Fig. 2). Although Lin *et al.* [11] and Wang *et al.* [12] also obtained TRAIL with high purity (>96%), their purification processes were complicated (four or five steps), costly (using expensive reagents, such as enterokinase, GSSG and GSH) and with low final yield (no more than 33%). By comparison, our two-step purification process is rapid, simple, efficient and easy to be scaled up.

3.4. Characterization of recombinant human TRAIL

3.4.1. Molecular mass analysis of recombinant human TRAIL

We analyzed the molecular mass of recombinant TRAIL by ESI-MS analysis. According to the positive ESI-MS spectrum (Fig. 4), the calculated molecular mass of TRAIL was 19492.5 ± 1.8 Da, which was consistent with the size of TRAIL (19493.9 Da). This result indicates that the bacterially produced TRAIL is expressed without any unwanted amino acids and may be with no immunogenicity as that expressed in eukaryotic system.

3.4.2. Structural analysis of recombinant human TRAIL by CD spectroscopy

The structure of TRAIL was determined by CD spectroscopy. The far-UV CD spectrum of TRAIL showed a typical pattern reflecting the high content of β -sheet structures with a single negative maximum ellipticity at around 220 nm and a single positive maximum ellipticity at around 205 nm, as has been previously observed for TNF family proteins (Fig. 5A) [4,25]. Near-UV CD demonstrated strongly negative ellipticities at 275 and 285 nm as reported (Fig. 5B) [25]. These results suggested that the recombinant human TRAIL was with tight structure and ordered conformation.

3.4.3. Oligomeric state of recombinant human TRAIL

Crystallographic studies showed that, like TNF and CD40L, TRAIL was a homotrimeric jelly roll protein and transferred the apoptosis signals in its homotrimeric form [4]. Accordingly, the oligomeric state of TRAIL was analyzed by SEC-HPLC. Fig. 6 showed the elution profile of TRAIL as a single symmetrical peak at 7.330 min, which corresponded to a molecular weight of 58.7 kDa when calculated from the calibration curve and formula ($\lg MW = 2.8559 - 0.1483t_R$, $r^2 = 0.9818$). According to the monomeric polypeptide molecular mass of this molecule (19493.9 Da), it is concluded that TRAIL is indeed a trimer.

Trimers of the purified TRAIL might in heterogeneous or homogeneous forms. Lawrence *et al.* reported that the His-tagged recombinant TRAIL was heterogeneous (56% of trimers containing intersubunit disulfide) and was less potent than native TRAIL at inducing death in cancer cell lines [26]. Non-reduction SDS-PAGE was performed and demonstrated that only a small proportion of dimers, which accounted for about 5% of the total protein, were detected under non-reducing conditions, indicating TRAIL we obtained here was homogeneous (Fig. 7). It was reported that homotrimeric TRAIL carries an internal zinc atom, which means that one TRAIL trimer containing one zinc atom [27]. Therefore, the zinc content in recombinant TRAIL was tested by atomic absorption. 0.91 ± 0.04 mol zinc/mol trimer for TRAIL was measured. It revealed that the recombinant TRAIL contained a stoichiometric ratio of approximately one zinc metal ion per trimer, which strongly indicated that the purified TRAIL is in homotrimeric form.

3.4.4. Cytotoxicity analysis of purified recombinant human TRAIL

The cytotoxicity of TRAIL on cancer cells was determined with a sulforhodamine B assay. As shown in Fig. 8, TRAIL exerted a specific cytotoxic effect in a dose-dependent manner on human hepatocellular carcinoma cells BEL-7402 and the cell growth inhibition rate were 50.2%, 55.3% and 66.5% when treated with 10 ng/mL, 100 ng/mL and 1000 ng/mL of TRAIL respectively. Wang *et al.* reported the cell growth inhibition rate of BEL-7402 cells treated with TRAIL at 500 ng/mL was 51.54% [28], indicating a bit stronger cytotoxicity of TRAIL purified in our study which may benefit from its ordered conformation and homotrimeric form.

4. Conclusions

For producing large quantities of highly pure and bioactive recombinant human TRAIL in *E. coli*, TRAIL was expressed in soluble form and purified by an efficient two-step chromatographic procedure. By adding $ZnSO_4$, lowering induction temperature, optimizing IPTG concentration, using pH-stat for feeding and glycerol as the carbon source, the fed-batch strategy led to a satisfactory production of TRAIL in soluble form which was almost 2.6-fold higher than that had been reported [14]. Recently, purification of recombinant TRAIL was mainly from inclusion bodies [11-13]. Although highly pure TRAIL was obtained when purified from inclusion bodies, their purification processes were complicated, costly, time-consuming and with low recovery yield. In this study, we developed a simple and rapid two-step chromatographic procedure

including affinity chromatography and cation-exchange chromatography, especially improved the cation-exchange chromatography using a combination of pH and NaCl gradients strategy. Large quantity of target protein with high purity (~98.1%) was obtained. The purified TRAIL was characterized by CD, SEC-HPLC, atomic absorption, non-reduction SDS-PAGE, cytotoxicity and ESI-MS assays. Our results demonstrate that the purified TRAIL, with ordered secondary and tertiary structures, in homogeneous form and with strong cytotoxicity. In conclusion, our expression and purification strategies are simple, efficient and economical, thus make it possible to prepare large quantities of highly pure TRAIL protein.

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References

- [1] S.R. Wiley, K. Schooley, P.J. Smolak, W.S. Din, C.P. Huang, J.K. Nicholl, G.R. Sutherland, T.D. Smith, C. Rauch, C.A. Smith, et al., Identification and characterization of a new member of the TNF family that induces apoptosis, *Immunity*. 3 (1995) 673-682.
- [2] F.A. Kruyt, TRAIL and cancer therapy, *Cancer Lett* 263 (2008) 14-25.
- [3] B. Lim, J.E. Allen, V.V. Prabhu, M.K. Talekar, N.K. Finnberg, W.S. El-Deiry, Targeting TRAIL in the treatment of cancer: new developments, *Expert. Opin. Ther. Targets*. 19 (2015) 1171-1185.
- [4] S.G. Hymowitz, M.P. O'Connell, M.H. Ultsch, A. Hurst, K. Totpal, A. Ashkenazi, A.M. de Vos, R.F. Kelley, A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL, *Biochemistry*. 39 (2000) 633-640.
- [5] A. Ashkenazi, R.C. Pai, S. Fong, S. Leung, D.A. Lawrence, S.A. Marsters, C. Blackie, L. Chang, A.E. McMurtrey, A. Hebert, L. DeForge, I.L. Koumenis, D. Lewis, L. Harris, J. Bussiere, H. Koeppen, Z. Shahrokh, R.H. Schwall, Safety and antitumor activity of recombinant soluble Apo2 ligand, *J. Clin. Invest.* 104 (1999) 155-162.
- [6] P.T. Daniel, T. Wieder, I. Sturm, K. Schulze-Osthoff, The kiss of death: promises and failures of death receptors and ligands in cancer therapy, *Leukemia*. 15 (2001) 1022-1032.
- [7] J.C. Soria, Z. Mark, P. Zatloukal, B. Szima, I. Albert, E. Juhasz, J.L. Pujol, J. Kozielski, N. Baker, D. Smethurst, Y.J. Hei, A. Ashkenazi, H. Stern, L. Amler, Y. Pan, F. Blackhall, Randomized phase II study of dulanermin in combination with paclitaxel, carboplatin, and bevacizumab in advanced non-small-cell lung cancer, *J. Clin. Oncol.* 29 (2011) 4442-4451.
- [8] J. Lemke, S. von Karstedt, J. Zinngrebe, H. Walczak, Getting TRAIL back on track for cancer therapy, *Cell Death. Differ.* 21 (2014) 1350-1364.
- [9] Y.X. Bao, X.D. Zhao, H.B. Deng, C.L. Lu, Y. Guo, X. Lu, L.L. Deng, Schedule-dependent cytotoxicity of sunitinib and TRAIL in human non-small cell lung cancer cells with or without EGFR and KRAS mutations, *Cell Oncol. (Dordr)* (2016).
- [10] X. Dai, J. Zhang, F. Arfuso, A. Chinnathambi, M.E. Zayed, S.A. Alharbi, A.P. Kumar, K.S. Ahn, G. Sethi, Targeting TNF-related apoptosis-inducing ligand (TRAIL) receptor by natural products as a potential therapeutic approach for cancer therapy, *Exp. Biol. Med* (Maywood). 240 (2015) 760-773.
- [11] Z. Lin, H. Lei, P. Cao, Expression, purification, and in vitro refolding of soluble tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), *Protein Expr. Purif.* 51 (2007) 276-282.
- [12] D.M. Wang, L.M. Shi, High-Level Expression, Purification, and In Vitro Refolding of Soluble Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL), *Appl. Biochem. Biotechnol.* (2008).
- [13] H. Kang, A.Y. Sun, Y.L. Shen, D.Z. Wei, Refolding and structural characteristic of

355 TRAIL/Apo2L inclusion bodies from different specific growth rates of recombinant
356 *Escherichia coli*, *Biotechnol. Prog.* 23 (2007) 286-292.

357 [14] A.Y. Sun, Y.L. Shen, J.C. Yin, H. Zhang, Y.N. Tang, D.Z. Wei, Improvement of expression
358 level and bioactivity of soluble tumor necrosis factor-related apoptosis-inducing ligand
359 (Apo2L/TRAIL) by a novel zinc ion feeding strategy, *Biotechnol. Lett.* 28 (2006)
360 1215-1219.

361 [15] Q. Luo, Y.L. Shen, D.Z. Wei, W. Cao, Optimization of culture on the overproduction of
362 TRAIL in high-cell-density culture by recombinant *Escherichia coli*, *Appl Microbiol*
363 *Biotechnol.* 71 (2006) 184-191.

364 [16] K.J. Jeong, S.Y. Lee, High-level production of human leptin by fed-batch cultivation of
365 recombinant *Escherichia coli* and its purification, *Appl. Environ. Microbiol.* 65 (1999)
366 3027-3032.

367 [17] V.Q.C.H.T.H. Nguyen., L.T. Tran., Studying on high density cell cultivation of *E. coli*
368 BL21(DE3)/pET43Ins by fed-batch, *J. Biotechnol.* 136 (2008) S516-S517.

369 [18] T. Cui, Y. Gao, O.W. Liew, C.M. Puah, B. Gutte, Efficient preparation of an acyclic
370 permutant of kalata B1 from a recombinant fusion protein with thioredoxin, *J. Biotechnol.*
371 130 (2007) 378-384.

372 [19] H.M. Kim, S.B. Han, M.S. Kim, J.S. Kang, G.T. Oh, D.H. Hong, Efficient fixation procedure
373 of human leukemia cells in sulforhodamine B cytotoxicity assay, *J. Pharmacol. Toxicol.*
374 *Methods.* 36 (1996) 163-169.

375 [20] G.W. Luli, W.R. Strohl, Comparison of growth, acetate production, and acetate inhibition of
376 *Escherichia coli* strains in batch and fed-batch fermentations, *Appl. Environ. Microbiol.*
377 56 (1990) 1004-1011.

378 [21] Y.L. Shen, Y. Zhang, A.Y. Sun, X.X. Xia, D.Z. Wei, S.L. Yang, High-level production of
379 soluble tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) in
380 high-density cultivation of recombinant *Escherichia coli* using a combined feeding
381 strategy, *Biotechnol. Lett.* 26 (2004) 981-984.

382 [22] S. Paul, T.K. Chaudhuri, Chaperone mediated solubilization of 69-kDa recombinant
383 maltodextrin glucosidase in *Escherichia coli*, *J Appl Microbiol* 104 (2008) 35-41.

384 [23] P. Turner, O. Holst, E.N. Karlsson, Optimized expression of soluble cyclomaltodextrinase of
385 thermophilic origin in *Escherichia coli* by using a soluble fusion-tag and by tuning of
386 inducer concentration, *Protein Expr. Purif.* 39 (2005) 54-60.

387 [24] R. Mhatre, W. Nashabeh, D. Schmalzing, X. Yao, M. Fuchs, D. Whitney, F. Regnier,
388 Purification of antibody Fab fragments by cation-exchange chromatography and pH
389 gradient elution., *J. Chromatogr. A.* 707 (1995) 225-231.

390 [25] G.H. Nam, K.Y. Choi, Association of human tumor necrosis factor-related apoptosis inducing
391 ligand with membrane upon acidification, *Eur. J. Biochem.* 269 (2002) 5280-5287.

392 [26] D. Lawrence, Z. Shahrokh, S. Marsters, K. Achilles, D. Shih, B. Mounho, K. Hillan, K.

393 Totpal, L. DeForge, P. Schow, J. Hooley, S. Sherwood, R. Pai, S. Leung, L. Khan, B.
394 Gliniak, J. Bussiere, C.A. Smith, S.S. Strom, S. Kelley, J.A. Fox, D. Thomas, A.
395 Ashkenazi, Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions, Nat.
396 Med. 7 (2001) 383-385.

397 [27] S.S. Cha, H.C. Shin, K.Y. Choi, B.H. Oh, Expression, purification and crystallization of
398 recombinant human TRAIL, Acta Crystallogr. D. 55 (1999) 1101-1104.

399 [28] M.J. Wang, Y.X. Liu, X.L. Li, J. Shi, S.L. Liu, D.X. Zheng, Chemotherapeutic drugs
400 enhanced rsTRAIL tumoricidal activity, Zhongguo Yi Xue Ke Xue Yuan Xue Bao 26
401 (2004) 524-528.

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Figure captions

Fig. 1. Optimization of culture conditioned to improve the soluble production of TRAIL in shake flasks. (A) Comparison of cell growth and TRAIL production in two medium: LB and modified LB medium with 2% glycerol. (B) Effect of ZnSO₄ on the cell density and soluble production when induced by 0.5 mM IPTG at 37°C in mLB medium. (C) Effect of temperature on the cell density and soluble production when induced by 0.5 mM IPTG with 0.5 mM ZnSO₄ in mLB medium. (D) Cell density and soluble production of TRAIL when induced by different concentration of IPTG with 0.5 mM ZnSO₄ at 28°C in mLB medium. After induction, cells were collected, sonicated, centrifugated and analysed in a 10% SDS-PAGE. Concentration of soluble TRAIL (g/L) = concentration of total supernatant protein (g/L) × the percentage of TRAIL from the total supernatant protein (%). Concentration of total supernatant protein was determined by the method of BCA using bovine serum albumin (Pierce, USA) as the standard. The percentage of TRAIL from the total supernatant protein was measured by densitometry using the Quantity One™ v 4.5 software (BioRad).

Fig. 2. Time profiles of cell concentration OD₆₀₀ (squares) and soluble production of TRAIL (diamonds) during the pH-stat fed-batch cultivation of recombinant *E. coli*. Expression of TRAIL was induced by 0.1mM IPTG with 0.5 mM ZnSO₄ at 28 °C. The arrow indicated the point of induction. Soluble production of TRAIL was determined as described in Fig. 1.

Fig. 3. Purification of recombinant human TRAIL. (A) SDS-PAGE analysis of samples from each purification step. Lane M, protein molecular weight markers; lane 1, total supernatant protein; lane 2, sample after Ni-NTA Sepharose; lane 3, sample after SP Sepharose Fast Flow. (B) Western blot analysis of purified TRAIL.

Fig. 4. ESI-MS analysis of purified recombinant human TRAIL. The peaks at $m/z = 780.62, 813.22, 848.60 \dots 1625.41, 1773.00$ and 1950.16 corresponded to the ions $[M/25 + H]^+, [M/24 + H]^+, [M/23 + H]^+ \dots [M/12 + H]^+, [M/11 + H]^+ \text{ and } [M/10 + H]^+$, respectively (M, protein and H, proton).

Fig. 5. Secondary structure (A) and tertiary structure (B) analyses of purified recombinant human TRAIL by CD spectroscopy. The protein concentrations were 0.15 mg/mL for the far-UV CD measurements (detecting the secondary structure) and 1 mg/mL for the near-UV CD measurements (detecting the tertiary structure).

Fig. 6. SEC-HPLC chromatogram analysis of purified recombinant human TRAIL. The absorbance at 280 nm was measured, and the elution time of molecular weight standards was indicated. TRAIL, Lysozyme (14.4 kDa), carbonic anhydrase (31.0 kDa), albumin (66.2 kDa) and γ -globulin (155 kDa) were eluted at 7.330 min, 11.678 min, 8.607 min, 7.246 min and 4.512 min, respectively.

Fig. 7. SDS-PAGE analysis of purified recombinant human TRAIL under reducing (with DTT, +) and non-reducing (without DTT, -) conditions. DTT, dithiothreitol.

Fig. 8. Cytotoxicity analysis of purified recombinant human TRAIL on cancer cells *in vitro*.

441 BEL-7402 human hepatocellular carcinoma cells were incubated with serial dilutions of TRAIL
442 for 72 h, and cell growth was determined with a sulforhodamine B assay.
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444

Table 1
Medium composition

Components	Batch medium	Feeding solution
glycerol (mL/L)	2.0	300
tryptone (g/L)	10.0	150
yeast extract (gL)	5.0	75
NaCl (g/L)	10.0	0
K ₂ HPO ₄ (g/L)	4.0	0
KH ₂ PO ₄ (g/L)	2.0	0
Na ₂ HPO ₄ (g/L)	7.0	0
MgSO ₄ .7H ₂ O (g/L)	1.0	7
antifoam (mL/L)	0	10

Table 2

Purification of TRAIL from 2.3 L culture after Fed-batch cultivation.

Purification step	Vol (mL)	Concn of total protein (mg/mL) ^b	Concn of TRAIL (mg/mL) ^c	Amount of TRAIL (mg)	Yield rate (%)	Purity (%)
Supernatant	230 ^a	111.29	41.40	9522.0	100	37.2 ^d
Ni Sep 6 FF	570	13.50	11.28	6427.4	67.5	83.5 ^d
SP Sep FF	295	14.91	14.62	4313.5	45.3	98.1 ^e

^a Cells were collected from 2.3 L culture and resuspended in ice-cold buffer A with 1/10 volume of cell culture medium. Supernatant were collected by centrifugation after sonication and filtered through a 0.22 µm membrane.

^b Determined by BCA protein assay kit.

^c The product of concentration of total protein and purity.

^d Determined by densitometric scanning of the SDS-PAGE gels.

^e Determined by RP-HPLC.

Figure1

Figure2

Figure3

Figure4

Figure5

Figure6

Figure7

Figure8