Recombinant expression, different downstream processing of the disulfide-rich anti-tumor peptide Ranpirnase and its effect on the growth of human glioma cell line SHG-44

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Abstract. Ranpirnase (Onconase) is a frogspawn-derived disulfide-rich peptide with ribonuclease activity that may be used for tumor treatment. In the present study, we established an efficient approach for preparing mature ranpirnase which may be used for research and therapeutic purposes. The designed ranpirnase precursors carried a 6xHis-tag and were recombinantly expressed in *Escherichia coli*. After S-sulfonation, the precursors were purified by immobilized metal-ion affinity chromatography. Following removal of the tag by aminopeptidase cleavage, cyclization and *in vitro* oxidative refolding, the mature ranpirnase was obtained with considerable yield, and the yield of mature ranpirnase was ~50-60 mg per liter cultures. In addition, ranpirnase inhibited the growth of human glioma cells SHG-44 in a dose-dependent manner. Thus the present study has provided an efficient approach for the preparation of active ranpirnase and its analogues for future studies.

Introduction

Ranpirnase (Onconase) was isolated from oocytes or early embryos of the northern leopard frog (*Rana pipiens*) and is a polypeptide with 104 amino acid residues and four disulfide bonds (Fig. 1). Ranpirnase is the smallest member of the ribonuclease (RNase A) superfamily, and appears to be a promising drug with broad clinical application in tumor treatment due to its moderate cytotoxicity, unique synergy, low immunogenicity and few side effects (1-10). Between 1996 and 2004, Tamir Biotechnology, Inc. (formerly Alfacell Corporation) successively conducted clinical investigations regarding the effects of ranpirnase on breast cancer, pancreatic cancer, renal cell carcinoma, non-small cell lung cancer and malignant mesothelioma, for which the therapeutic effect was the most significant with few side effects. Ranpirnase is currently used as a drug for malignant mesothelioma in a phase IIIb clinical trial and for non-small cell lung cancer in a phase II clinical trial.

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Materials and methods

Materials. The oligonucleotide primers were chemically synthesized at BioSune (Shanghai, China). Aminopeptidase and Papain were purchased from Sigma-Aldrich (St. Louis, MO, USA). Agilent reverse-phase columns (analytical column, Zorbax 300SB-C18, 4.6x250 mm; semi-preparative column, Zorbax 300SB-C18, 9.4x250 mm) were used in the experiments. The peptide was eluted from the columns with an acetonitrile gradient composed of solvent A and B. Solvent A was 0.1% aqueous TFA, and solvent B was acetonitrile containing 0.1% TFA. The elution gradient was as follows: 0 min, 10% solvent B; 3 min, 10% solvent B; 53 min, 60% solvent B; 55 min, 100% solvent B; 56 min, 100% solvent B, and 57 min, 10% solvent B. The flow rate for the analytical column was 0.5 ml/min, and that for the semi-preparative column was 1.0 ml/min. The eluted peptide was detected by UV absorbance at 280 and 214 nm.

Gene construction, recombinant expression, and purification of 6xHis-Ranpirnase. The gene of 6xHis-Ranpirnase was constructed from two chemically synthesized DNA primers according to a previous study (11). Briefly, we designed two primers with the following sequences: P1 5'-CCATCACCATC ATATGCAGGATTGGCTGACCTTTCAAAAAAAACATA TTACGAACACTCGTGAT-3'; and P2 5'-TGAATTCTTAACA AGAGCCAACGCCCACGAAGTGGACCGGTGCCTGGTT TTCGCACGTAACACAAAATTTATTGGTGCT-3'; and four DNA fragments with the following sequences: F1 5'-CTGT TTCACTGCAAAGATAAAAATACCTTCATCTATTCTCG CCCGGAACCGGTTAAAGCGATTTGCAAAGGCATT ATC-3'; F2 5'-TTTACCTGAGCGACTGTAACGTAACCTCG CGCCCGTGCAAATATAAACTGAAAAAGAGCACCAAT AAATTTTGTGTT-3'; F3 5'-GGTTACGTTACAGTCGCT CAGGTAAAACTCGCTCGTGGTCAGGACATTTTTTGG AGGCGATAATGCCTTTGCAAATCGC-3'; and F4 5'-GGT GTTTTTATCTTTGCAGTGAAACAGGTTAGTGCTCATG ATGTTATCACAGTCCACATCACGAGTGTTCGTAAT ATG-3'. These DNA fragments were linked by T4 DNA polymerase and dNTP, and constructed the Onconase gene. After

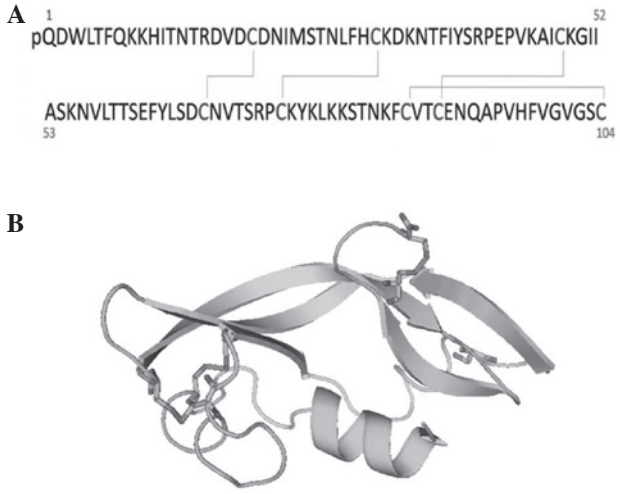

Figure 1. (A) The amino acid sequence and disulfide linkages of mature ranpirnase. (B) The previously reported Crystal structure of ranpirnase (Protein Data Bank ID: 3PHN).

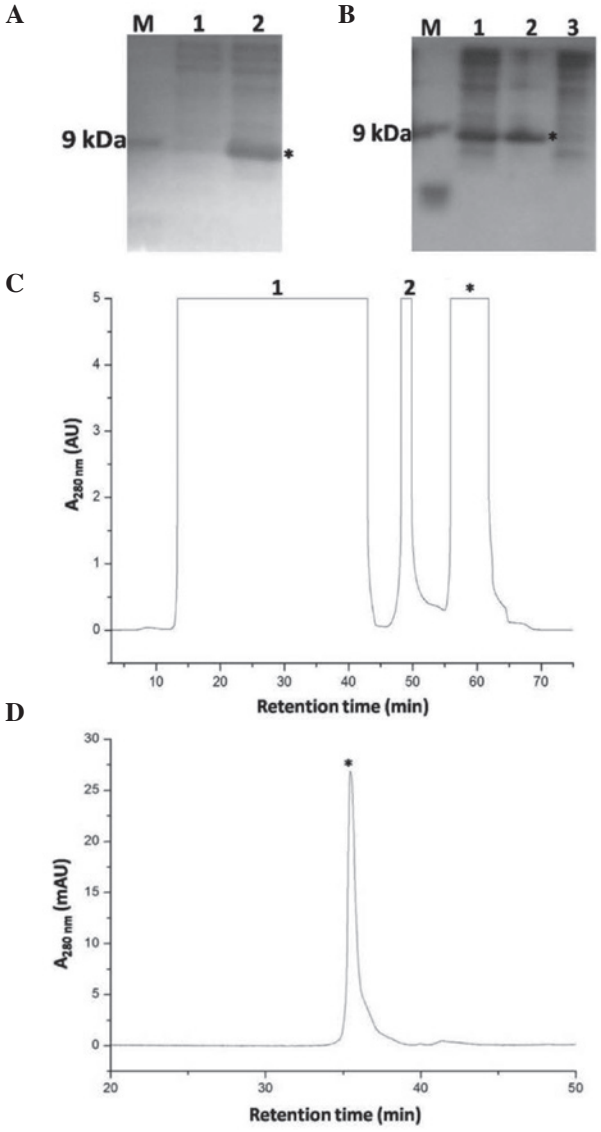

Figure 2. (A) SDS-PAGE analysis of 6xHis-ranpirnase expression. M, Marker; lane 1, before IPTG induction; lane 2, after IPTG induction. The band of 6xHis-ranpirnase was indicated in lane 2 after induction by IPTG (indicated by a star). (B) SDS-PAGE analyses of the 6xHis-ranpirnase precursor at the primary purification stage. M, Marker; lane 1, total lysate; lane 2, P, pellet; lane 3, supernatant. The band of 6xHis-ranpirnase was indicated by a star. (C) FPLC profile of the S-sulfonated 6xHis-ranpirnase purified with an immobilized metal-ion affinity column (Ni^{2+} column). Lane 1, flow-through; lane 2, eluted fraction by 30 mM imidazole; eluted S-sulfonated 6xHis-ranpirnase precursor by 250 mM imidazole was indicated by a star. (D) HPLC profile of the S-sulfonated 6xHis-ranpirnase precursor eluted from the Ni^{2+} column. The peak of S-sulfonated 6xHis-ranpirnase precursor was indicated by a star.

annealing, elongation by T4 DNA polymerase, cleavage by restriction enzyme *Nde*I and *Eco*RI, the DNA fragment was ligated into a pET28a vector pretreated with the same restriction enzymes. Its sequence was confirmed by DNA sequencing. Thereafter, the expression construct pET28a/6xHis-Ranpirnase was transformed into *Escherichia coli* (E. *coli)* strain BL21 (DE3) star, and the transformed cells were cultured in liquid TB medium (with 100 μg/ml ampicillin) to OD600 nm=2.5 at 37˚C with vigorous shaking (7 x g). After being induced by 0.8 mM of isopropyl thio-β-D-galactoside (IPTG) at 37˚C for 6-8 h, the *E. coli* cells were harvested by centrifugation (5,000 x g, 10 min), re-suspended in lysis buffer (50 mM Tris-HCl, pH 8.5, 0.5 M NaCl), and lysed by sonication. After centrifugation (10,000 x g, 15 min), the inclusion body pellet was re-suspended in solubilizing buffer (50 mM Tris-HCl, 6 M guanidine chloride, pH 8.5) and S-sulfonated by the addition of solid sodium sulfite and sodium tetrathionate to a final concentration of 200 and 150 mM, respectively. The S-sulfonation reaction was carried out at 4˚C with gentle agitation for 2-3 h. After centrifugation (10,000 x g, 15 min), the supernatant was loaded onto a Ni^{2+} column that was pre-equilibrated with the washing buffer (50 mM Tris-HCl, 3 M guanidine chloride, pH 8.5). The S-sulfonated precursor was eluted from the column by step-wise increase of imidazole concentration in the washing buffer. The eluted S-sulfonated 6xHis-Ranpirnase was subjected to dialysis in water at 4˚C overnight in order to remove imidazole. After centrifugation (6,000 x g, 10 min), the pellet was re-suspended in solubilization buffer (50 mM Tris-HCl, 2.4 M guanidine chloride, pH 8.5).

Aminopeptidase cleavage of the S-sulfonated ranpirnase precursors, cyclization and in vitro refolding. The S-sulfonated ranpirnase precursors (6xHis-Ranpirnase) were digested by aminopeptidase (peptide enzyme molar ratio 2,000:1) in the digestion buffer (2.4 M guanidine chloride, 50 mM Tris-HCl, 0.1 mM $Zncl_2$, pH 8.5) at 37˚C overnight, and the N-terminal of the digestion products was directly cyclized at 30˚C overnight by cyclotransferase purified from Papain (crude powder from Papaya Latex, Sigma) according to the procedures mentioned in Zerhouni *et al* (12). The cyclization mixture was initially treated with 50 mM dithiothreitol (DTT) at room temperature for 15 min. Subsequently, the treated mixture was 50-fold diluted into the pre-incubated refolding buffer (0.5 M L-arginine, 1.0 mM EDTA, 3.0 mM oxidized glutathione, pH 8.5). The refolding reaction was carried out at 4˚C for 6-8 h. The refolding mixture was then acidified to pH 3.0 by trifluoroacetic acid and subjected to C18 reverse-phase high-performance liquid chromatography (HPLC). The eluted refolded ranpirnase fraction was manually collected, lyophilized and analyzed by mass spectrometry.

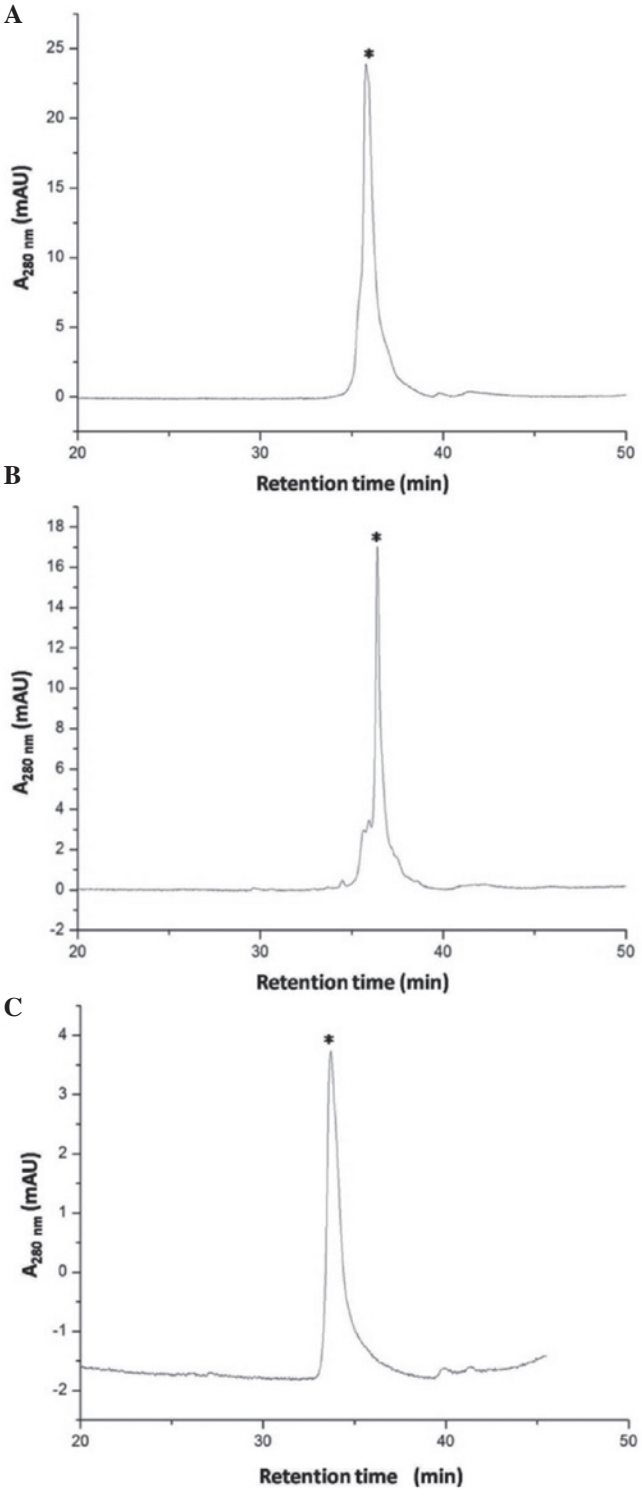

Figure 3. Aminopeptidase cleavage of S-sulfonated 6xHis-ranpirnase precursors, cyclization and *in vitro* refolding of the S-sulfonated ranpirnase. (A) HPLC profile of the digestive products of the S-sulfonated 6xHis-ranpirnase precursors with aminopeptidase. The peak of the S-sulfonated ranpirnase was indicated by a star. (B) HPLC analysis of the cyclization of the digestive products. The peak of the S-sulfonated ranpirnase cyclized by cyclotransferase was indicated by a star. (C) HPLC analysis of ranpirnase refolding mixture derived from the S-sulfonated ranpirnase. The peak of the mature ranpirnase was indicated by a star.

The effect of the folded ranpirnase on human glioma cell line SHG-44. Human glioma cell line SHG-44 was obtained from the cell library of the Shanghai Institute and cultured in 96-well plates at a density of $1x10^4$/ml Dulbecco's modified Eagle's medium, supplemented with glutamine, penicillin, streptomycin and 10% fetal calf serum. The folded ranpirnase was added to the cells after plating overnight. The cells were then examined using MTT assay following treatment with the folded ranpirnase in CO_2 incubator for 36 h, allowing ≥3-4 rounds of application at different concentrations of the folded ranpirnase.

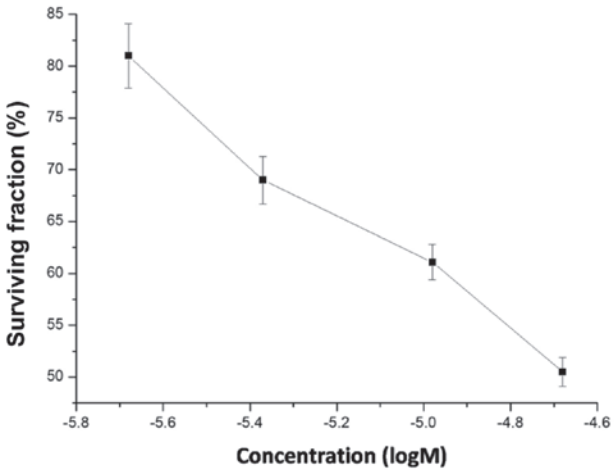

Figure 4. The cytotoxic effect of ranpirnase on SHG-44 cell lines. The MTT assay was performed after 36 h of incubation with different concentrations of ranpirnase.

Results

Expression, and purification of ranpirnase precursors. The recombinant plasmid pET-Ranpirnase was constructed, and 6xHis-Ranpirnase was recombinantly expressed in the *E. coli* strain BL21(DE3)star under IPTG induction. As analyzed by tricine SDS-PAGE, a band with a molecular weight of ~12 kDa was significantly increased following IPTG induction (Fig. 2A). After the *E. coli* cells were lysed by sonication, the precursor was present in the pellet (Fig. 2B), suggesting that 6xHis-Ranpirnase formed inclusion bodies. The inclusion bodies were solubilized by 6 M guanidine chloride and then treated with sodium sulfite and sodium tetrathionate to obtain an S-sulfonated precursor. The S-sulfonated precursor was then subjected to immobilized metal-ion affinity chromatography (Ni^{2+} column) (Fig. 2C). The S-sulfonated precursor (indicated by a star) was eluted by 250 mM imidazole from the Ni^{2+} column, and the eluted S-sulfonated precursor was subjected to dialysis to remove imidazole. Following centrifugation, the pellet was re-suspended in the solubilization buffer.

Aminopeptidase cleavage of ranpirnase precursors, cyclization and in vitro refolding of S-sulfonated ranpirnase. The dialysised S-sulfonated 6xHis-Ranpirnase was analyzed by C18 reverse-phase HPLC (Fig. 2D). The measured molecular mass of the eluted peak (indicated by a star) was 13,566, which was similar to the expected value (13,561.8) of the S-sulfonated precursor, and it was confirmed in later studies that the peak was the expected S-sulfonated 6xHis-Ranpirnase. The dialysised S-sulfonated precursors were digested by aminopeptidase, and the N-terminal of the digestion mixture was directly cyclized

by cyclotransferase. The digested and cyclized S-sulfonated ranpirnase was analyzed by C18 reverse-phase HPLC, as shown in Fig. 3A and B. The measured molecular mass of the eluted peak (indicated by a star) was 12,485 and 12,466, respectively, similar to the expected values (12,484 and 12,467) of the digested and cyclized S-sulfonated ranpirnase, and it was confirmed in later studies that the peaks were the expected digested and cyclized S-sulfonated ranpirnase. As shown in Fig. 3C, in *in vitro* refolding the measured molecular mass of the eluted peak (indicated by a star) was 11,819, consistent with the expected value 11,819.5 of the refolded ranpirnase.

The effect of the folded ranpirnase on human glioma cell line SHG-44. To determine the cytotoxic effect of ranpirnase on the SHG-44 cell line, MTT assay was performed after incubation with ranpirnase. The ranpirnase concentration required for 50% cell survival (the surviving fraction is 50%, SF0.5) was determined as shown in Fig. 4. Following co-incubation with the SHG-44 cell line for 36 h, the ranpirnase concentration for SF0.5 was ~15 μM.

Discussion

Ranpirnase is a disulfide-rich peptide with 104 amino acids and four disulfide bonds. Although the recombinant expression procedure of ranpirnase has been previously reported by Notomista *et al* (2), we found that ranpirnase was autocatalytically cyclized at 30˚C overnight with secondary reaction products after digestion with aminopeptidase. Thus, we utilized cyclotransferase to catalyze ranpirnase cyclization after digestion with the aminopeptidase to reduce the secondary reaction products. Additionally, the plant cyclotransferase, which is resistant to chemical denaturation, simplified the purification procedure following digestion with the aminopeptidase because it could be used in the buffer containing guanidine chloride at a high concentration level, which made the ranpirnase precursors soluble at a high concentration for the high digestive efficiency with aminopeptidase.

The folded 6xHis-Ranpirnase could not be efficiently digested by aminopeptidase probably due to steric hindrances. Thus, we employed an S-sulfonation approach, by which the eight cysteine residues of the ranpirnase precursors were reversibly modified by sulfonate moieties, in order to improve digestion. This also reduced the crosslink between two ranpirnase molecules, rendering ranpirnase precursors highly soluble in the enzyme digestion buffer for efficient cleavage with aminopeptidase. After removal of the N-terminal 6xHis tag, the S-sulfonated ranpirnase was efficiently refolded *in vitro* with ~70% yield under optimized condition, and the final yield of mature ranpirnase was ~50-60 mg per liter cultures. In addition, ranpirnase inhibited the growth of human glioma cells SHG-44 in a dose-dependent manner. Thus, the present study has provided an efficient approach for the preparation of active ranpirnase and its analogues for future studies.

Acknowledgements

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