

# Construction and Structural Modeling of a Single-Chain Fv–Asparaginase Fusion Protein Resistant to Proteolysis

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Received 14 February 2000; accepted 11 June 2000

**Abstract:** In this study, we construct a fusion protein composed of L-asparaginase (ASNase; from *Escherichia coli* AS 1.357) and a protective single-chain Fv (scFv), which was selected from a phage-display scFv library from our previous studies. The antibody moiety of the fusion protein was fused to the N-terminus of the enzyme moiety via a linker peptide, (Gly<sub>4</sub>Ser)<sub>6</sub>. Recombinant plasmid pET-SLA was constructed to express scFv–ASNase fusion to high levels in *E. coli* and the expressed product was found to form inclusion bodies. We obtained a soluble fusion protein by refolding and purification. The soluble fusion protein exhibited about 82% of the enzymatic activity of the native ASNase at the same molar concentration, and had a  $K_m$  value similar to that of the native enzyme for the substrate L-asparagine. Importantly, the fusion protein was more stable than native ASNase. In addition: (1) following treatment with trypsin,  $\alpha$ -chymotrypsin, and rennet, at 37°C for 30 min, scFv–ASNase fusion retained 94.0%, 88.8%, and 84.5% of its original activity, respectively, whereas native ASNase became inactive; and (2) ScFv–ASNase fusion had a much longer in vitro half-life (9 h) in serum than the native enzyme (2 h). The three-dimensional structure of the fusion protein was obtained by modeling with the Homology and Discover modules of the INSIGHT II software package. On the basis of the structural evidence and biochemical properties, we propose that the scFv moiety of the fusion protein may confer ASNase moiety resistance to proteolysis as a result of both steric hindrance and a change in the electrostatic surface of the enzyme. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* 70: 456–463, 2000.

**Keywords:** L-asparaginase; single-chain Fv (scFv); recombinant DNA technology; fusion protein; bacterial expression; resistance to proteolysis; three-dimensional structure modeling

## INTRODUCTION

For many medicinal proteins, immunogenicity and short serum half-lives often limit their applications. The short

half-life of therapeutic proteins is mainly caused by enzymatic proteolysis in vivo. In a previous study, using L-asparaginase (ASNase, a therapeutic agent for lymphoma and leukemia) as a model, we isolated a protective single-chain Fv (scFv) from a phage-display scFv antibody library, which enhanced the resistance of ASNase to trypsin (Guo et al., 2000). That study provided a new approach for stabilizing biologically active proteins using specific antibodies; however, it is necessary to prepare ASNase and protective scFv separately and to mix them at an optimum molar ratio to obtain a complex comprising the two. Thus, the procedure is too complex to be feasible in industrial production and medicinal application. Newsted et al. (1995) reported that, when fused to a target protein, a protective scFv could retain its protective action against trypsin proteolysis. This suggests that a fusion protein comprised of scFv and ASNase may be more suitable for use. In their report, the protective scFv derived from a protective monoclonal antibody (MAb) and the possible mechanism for protection were not discussed. However, preparation of MAb by hybridoma technology is slow and costly and phage-antibody library technology may be the better alternative. Therefore, in this study, the protective scFv used to construct the scFv–ASNase fusion was prepared by phage-display scFv library technology, and the protection mechanism of the scFv conferring ASNase resistance to proteolysis is proposed according to the results of biochemical experiments, modeling, and electrostatics analysis.

## MATERIALS AND METHODS

DNA manipulations, growth media, and buffers (unless individually specified) were as described by Sambrook et al. (1989).

### Strains and Plasmids

*E. coli* DH5 $\alpha$  [sup E44  $\Delta$ lac U169 ( $\phi$ 80 lacZ  $\Delta$ M15) hsd R17 recA end A1 gyrA96 thi-1 relA1] (TaKaRa) was used

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Contract grant sponsor: Academia Sinica, China

as the host strain for cloning of target DNA into pET-21a vector. *E. coli* BL21 (DE3) [ $F^-$  ompT hsd S ( $t8^-$  m8 $^-$ ) gal dcm (DE3)] (Novagen) was used as the host strain for expressing the fusion proteins. The vector pET-21a (Novagen) was used to construct the recombinant plasmid. Recombinant plasmid pBV-ASN was provided by Dr. Yingda Wang (Institute of Microbiology, Chinese Academy of Sciences).

### Design and Synthesis of Linker DNA

Construction of a scFv-ASNase fusion protein requires that both scFv and ASNase moieties have correct three-dimensional structures when the fusion protein is expressed. Therefore, the linker peptide between scFv and ASNase should be long and flexible. The most frequently used linker is 15 residues (Gly<sub>4</sub>Ser)<sub>3</sub> (Huston et al., 1988). The serine residues enhance the hydrophilicity of the peptide backbone to allow hydrogen bonding to solvent molecules, and the glycyl residues provide the linker with flexibility to adopt a range of conformations (Argos, 1990). These properties also prevent interaction of the linker peptide with hydrophobic interface of the individual domains. We chose to use a linker peptide of 30 residues (Gly<sub>4</sub>Ser)<sub>6</sub> to reduce tension force when the specific scFv bound to ASNase, a protein of large size (14.4 kDa). The DNA fragment encoding the linker peptide was prepared as follows.

The sense strand of the DNA comprised oligonucleotides A and B, and the antisense strand contained oligonucleotides C and D. The sequences of oligonucleotides A, B, C, and D were: A, 5'-AATTCGGTGGCGGTGGCTCGGGC-GGTGGTGGTTCGGGTGGCGGCGG-3'; B, 5'-ATCTG-GTGGCGGTGGCTCGGGCGGTGGTGGGTTCGGGTG-GCGGCGGATCTG-3'; C, 5'-TCGACAGATCCGCCGC-CACCCGACCCACCCGCCCGAGCCACC-3'; D, 5'-GCCACCAGATCCGCCGCCACCCGACCCACCAC-CGCCGAGGCCACCGCCACCG-3'. DNA was flanked by *EcoRI* and *SalI* sites (underlined). The four oligonucleotides were prepared in an automated DNA synthesizer (ABI 392). Oligonucleotides B and D were phosphorylated with T4 polynucleotide kinase. Oligonucleotides A and D were annealed as were oligonucleotides B and C. The two double-stranded fragments were ligated using standard procedures. The linker DNA was cloned into pET-21a between *EcoRI* and *SalI* sites to form a pET-link.

### Amplification of ASNase Gene (asnB) and Protective scFv Gene by Poolmerase Chain Reaction

The *asnB* gene from *E. coli* (Bonthron et al., 1990) was amplified by polymerase chain reaction (PCR) from pBV-ASN with the following primers: 5'-GCGTCGACTTAC-CCAATATCACC-3' (5' *SalI*) and 5'-CCAAGCTTT-TATTAGTACTGATTGAAG-3' (3' *HindIII*). Amplification comprised 30 cycles of denaturation for 1 min at 94°C, followed by annealing for 1 min at 55°C and extension for 1 min at 72°C. The protective scFv gene (780 basepairs)

was amplified by PCR from the p5E-scFv46 gene (Guo et al., 2000) with the following primers: 5'-AGATATACAT-ATGGCTCAGGTTTCAGCTGGTTCAGTCTGGT-3' (5' *NdeI*) and 5'-GCGAATTCACGCGGTTCCAGCGG-3' (3' *EcoRI*). Amplification comprised 30 cycles of denaturation for 1 min at 94°C, followed by annealing for 1 min at 45°C and extension for 2 min at 72°C.

### Assembly of Fusion Gene and Construction of Expression Vector

The PCR product for the *asnB* gene (Bonthron et al., 1990) was digested with *SalI* and *HindIII* and cloned into pET-link to form pET-LA. Colonies containing potential clones of pET-LA were grown in *E. coli* DH5 $\alpha$  and plasmid DNA was isolated. Clones that contained pET-LA were identified by sequence analysis of the plasmid DNA using T7 universal primers (Model 377, ABI Prism DNA Sequencer). The PCR product for the scFv gene was digested with *NdeI* and *EcoRI*, and the digested product was inserted into pET-LA between *NdeI* and *EcoRI* to yield expression vector pET-SLA.

### Expression of Fusion Protein in *E. coli* BL21 (DE3)

The expression vector pET-SLA was transformed into *E. coli* BL21 (DE3). An overnight culture (10 mL) of *E. coli* BL21 (DE3) harboring pET-SLA was inoculated into 1 L of LB medium containing 100  $\mu$ g/mL ampicillin. After incubation at 37°C with shaking to an OD<sub>600</sub> of about 0.5, the culture was induced with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The cells were grown for an additional 5 h at 37°C, harvested by centrifugation, resuspended in 40 mL of buffer A (50 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> [pH 8.0]/2 mM ethylene-diamine tetraacetic acid [EDTA]/50 mM NaCl), and stored at -20°C.

### Isolation of Inclusion Bodies and Refolding of Fusion Protein

Bacterial cells were lysed by sonication in 10-mL aliquots at 4°C. After centrifugation at 10,000g for 20 min, the pellet was washed with 10 mL of buffer A containing 1 M, 2 M, and 3 M urea, successively. The pellet was finally dissolved in 10 mL of buffer A containing 8 M urea and 100 mM  $\beta$ -mercaptoethanol, and incubated at 4°C for 2 h. The denatured fusion protein was directly diluted into 100 mL of a refolding buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> [pH 8.5]/50 mM NaCl/2 mM EDTA) and incubated at 4°C for 6 h. The refolding solution was dialyzed at 4°C against 1 L of buffer A containing 0.1 M urea for 12 h. The sample was subsequently dialyzed for 36 h at 4°C against 4 L of buffer A. After the final dialysis, the aggregated protein was removed by centrifugation at 10,000g for 20 min. The sample was further dialyzed for 36 h against 4 L of 20 mM NH<sub>4</sub>HCO<sub>3</sub>

(pH 8.0) and then concentrated by lyophilization. The sample could be stored at  $-20^{\circ}\text{C}$ .

### Further Purification of Refolded Fusion Protein

All purification steps were conducted at  $4^{\circ}\text{C}$ . About 5 mg of the refolded fusion protein was dissolved in buffer A (1 mL). A sample (1 mL) of the soluble fusion protein was applied to a  $1.0 \times 60$  cm column of high-resolution Sephacryl S-100 (Pharmacia), which had been equilibrated with 20 mM  $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$  (pH 8.0). Gel filtration was carried out at a flow rate of 20 mL/h. The fusion protein was eluted just beyond one column volume and fractions containing the fusion protein were pooled (4 mL). Low-molecular-mass ( $<30$  kDa) substances were removed by using a microconcentrator (Pall-Gelman) and the fusion protein was concentrated.

### Western Blotting

After separation with 12% (w/v) sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli et al., 1970), proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad). The primary antibody against ASNase was raised in rabbits immunized four times at intervals of 7 to 10 days with a total of 2 mg of purified ASNase. The antiserum was collected from the immunized rabbits and treated with HB101 lysate to remove anti-*E. coli* antibodies, as described by Sambrook et al. (1989). The secondary antibody was goat anti-rabbit IgG (Sigma) conjugated with HRP. The scFv was identified by mouse anti-E-tag antibody (Pharmacia) for an E-tag peptide present at the C-terminus of the protective scFv, and followed by goat anti-mouse IgG (Sigma) conjugated with HRP. ASNase and scFv were detected by a colorimetric method using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as substrate.

### ASNase Activity Assay and Resistance to Proteolysis

Enzyme activity was assayed according to Peterson and Ciegler (1969). To assess the stability of the scFv-ASNase fusion protein against proteolysis, we employed serine proteases such as trypsin and  $\alpha$ -chymotrypsin, because it was reported that the serum trypsin levels increased significantly 10 and 20 days following the start of ASNase therapy (Shimizu et al., 1998). The proteases, including trypsin type XI (from bovine pancreas),  $\alpha$ -chymotrypsin type II (from bovine pancreas), and rennet type II (from *Mucor meihie*), were purchased from Sigma. The assay was carried out as follows: scFv-ASNase fusion (20  $\mu\text{mol}$ ) and native ASNase (20  $\mu\text{mol}$ ) were treated with 25 BAEE (*N*- $\alpha$ -benzoyl-L-arginine ethyl ester) units of trypsin, 40 BTEE (*N*-benzoyl-L-tyrosine ethylester) units of  $\alpha$ -chymotrypsin, and 20  $\mu\text{g/mL}$  rennet in 20 mM  $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$  buffer (pH 7.2) in a final volume of 1 mL at  $37^{\circ}\text{C}$  for 10 to 30 min, respectively. Aliquots (200  $\mu\text{L}$ ) were taken from each of the

six aforementioned reaction mixtures at intervals and diluted to 500  $\mu\text{L}$  with 10 mM borate buffer (pH 8.4). The digestions of trypsin,  $\alpha$ -chymotrypsin, and rennet were stopped by the addition of 5  $\mu\text{L}$  of 2 mg/mL trypsin inhibitor type I-S from soybean (Sigma), 4  $\mu\text{L}$  of 500 mM PMSF (Sigma), and 2  $\mu\text{L}$  of 1 mg/mL pepstatin (Boehringer Mannheim), respectively. The enzyme activity was assayed immediately.

### Stability of ASNase and scFv-ASNase Fusion in Serum

Native ASNase and scFv-ASNase fusion (1 mg each) were hydrated with 1 mL of 20 mM  $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$  buffer (pH 7.2), mixed with an equal volume of fresh human serum, and incubated at  $37^{\circ}\text{C}$  for 1 to 11 h, respectively. Aliquots (10  $\mu\text{L}$  each) taken at intervals were diluted with 10 mM borate buffer (pH 8.4) and immediately assayed for ASNase activity.

### Molecular Modeling

An initial three-dimensional structure model for the fusion protein was built as follows. The three-dimensional structures of the Fab fragment of A human IgG 1 $\kappa$  (1VGEH), Fab fragment Ctm01 H chain (1AD9H), and Fab fragment Ctm01 B chain (1AD9B) were extracted from the protein data bank (PDB) and used as templates to simulate the VH domain of scFv. Similarly, the three-dimensional structures of  $\lambda$ III Bence Jones Protein Cle A chain (1LILA),  $\lambda$ III Bence Jones Protein Cle B chain (1LILB), and Rf-An IgM/ $\lambda$  L chain (1ADQL) were employed as templates for modeling the VL domain of scFv. The linker between VH and VL domains was generated with the loop generation function of the Homology module of the MOLECULAR SIMULATION software program (v98.0, MSI, Inc.). The structure of ASNase (Swain et al., 1993) was extracted from the PDB using code 3ECA. Assuming that the site of the protective scFv involved in binding to ASNase is at or near Lys-29 (Guo et al., 2000), the initial relative location of the scFv and ASNase molecules was determined. The long linker ( $\text{Gly}_4\text{Ser}$ )<sub>6</sub> connecting the N-terminus of the scFv molecule and the C-terminus of ASNase molecule was also generated by the INSIGHT II package.

The model was refined with the Homology and Discover modules of INSIGHT II (Flanagan et al., 1998). The refinement was performed only on the preferred structure, so any side chains involved in unfavorable interactions were adjusted manually, and any unacceptable steric overlaps caused by one to three interactions or short bond lengths were removed. Furthermore, because generation of the loop region provides extended side-chain conformations only for the residues in the loop, a side-chain conformation search with the manual rotamer was carried out.

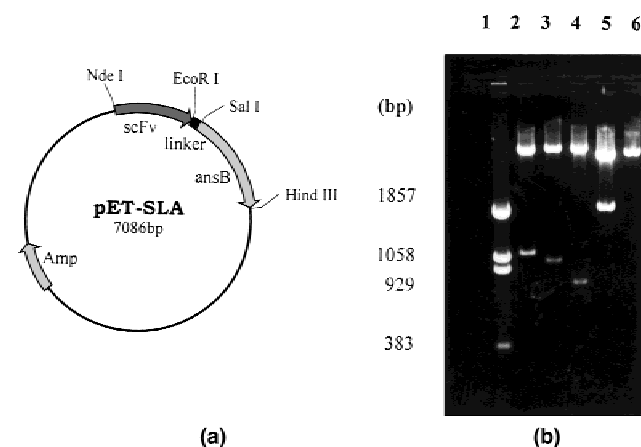
Initially, energy minimization was applied to all hydrogen atoms using the steepest descent method until convergence at 0.3 cal/ $\text{\AA}$  was achieved. One subunit of the scFv-

ASNase fusion was fixed, and a molecular dynamics run with an 8000-iteration equilibration step was employed with the temperature kept at 300 K. This procedure was repeated on each subset of the target model. Finally, energy minimization was again performed using conjugate gradients until convergence at 0.3 cal/Å. Subsequently, assembly was performed with the aforementioned results to simulate the tetrameric scFv-ASNase fusion protein. A molecular dynamics run with a 3000-iteration equilibration step was applied and followed by an energy minimization using conjugate gradients.

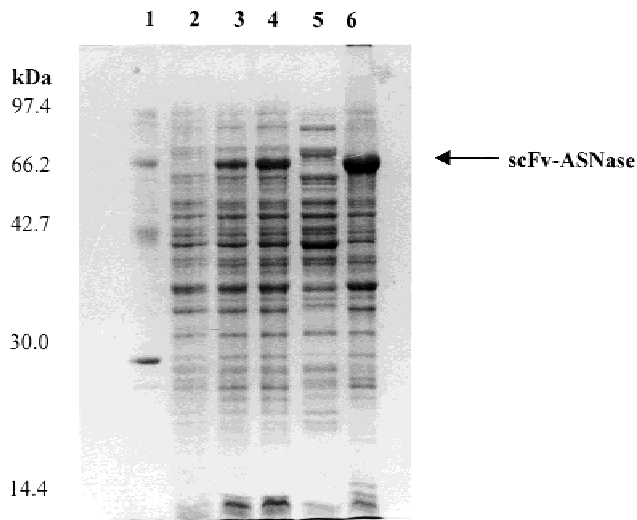
## RESULTS

### Expression, Refolding, and Purification of Fusion Proteins

The expression vector pET-SLA (Fig. 1A) was analyzed by restriction enzyme digestion (Fig. 1B) and identified subsequently by DNA sequence analysis before it was transformed into BL21 (DE3). As shown in Figure 2, the fusion protein was expressed at high levels in *E. coli* BL21 (DE3). The amount of the expressed fusion protein accounted for 15.7% of the total protein as estimated by gel scanning on a Shimadzu CS-930 densitometer. *E. coli* sonicates contained fusion proteins as inclusion bodies, which precipitated with the outer membrane fraction by centrifugation at 10,000g for 25 min. The fusion protein was partially purified by extraction with 3 M urea in buffer A as it remained insoluble in 3 M urea, but was dissolved readily in 8 M urea. Refolding of the fusion protein was performed and appeared complete after 6 h. The effect of protein concentration on folding was investigated, and the optimum protein concen-



**Figure 1.** (A) Diagram of the pET-SLA expression vector containing the scFv gene, ansB gene, and linker DNA. (B) Agarose gel electrophoresis of the fusion gene cloned into the expression vector pET-21a. Lane 1: DNA size standard pBR322/BstNI. Lanes 2-5: the vector pET-SLA after cleavage with *EcoRI* and *HindIII*, *SalI*, and *HindIII*, *NdeI* and *EcoRI*, and *NdeI* and *HindIII*, respectively, and the inserted DNA fragments of ansB-linker (1068 bp), ansB gene (978 bp), scFv gene (780 bp), and fusion gene (1860 bp) were obtained in order. Lane 6: vector pET21a digested with *EcoRI*.

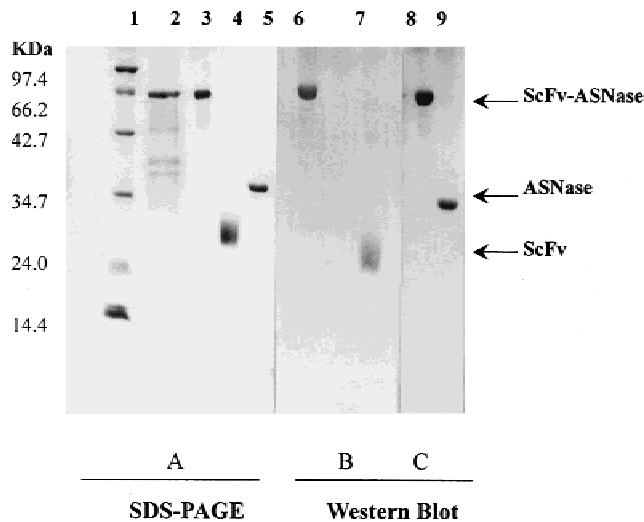


**Figure 2.** SDS-PAGE analysis of the scFv-ASNase fusion protein expressed as insoluble inclusion bodies. Lane 1: protein molecular-mass markers. *E. coli* BL21 (DE3) cells were transformed with pET-SLA, and expression of the gene was induced with 1 mM IPTG (lanes 3 and 4), no IPTG (lane 2). The cells induced with IPTG were sonicated and centrifuged, and the supernatant (lane 5) and pellet (lane 6) were analyzed on SDS-PAGE.

tration ranged from 60 to 80  $\mu\text{g}/\text{mL}$ . In addition, adding 10% glycerol to the refolding buffer increased renaturation by about 30%. The refolded fusion protein was purified to homogeneity by gel filtration and the purified protein was subjected to electrophoretic analysis on a 12% SDS-PAGE gel (Fig. 3, lane 3). The protein band was stained with Coomassie blue, and the purity was close to 95.3% as estimated by scanning on a Shimadzu CS-930 densitometer. Western blotting (Fig. 3, lanes 6-9) was performed to verify the presence of the ASNase moiety and protective scFv moiety within the scFv-ASNase fusion molecule. The apparent molecular mass of scFv-ASNase subunit was estimated to be 65.2 kDa, in agreement with the calculated molecular mass (64.697 kDa). When assayed by gel filtration of Sephadex G-75, the molecular mass of the soluble scFv-ASNase fusion was estimated to be 260 kDa, suggesting that the soluble scFv-ASNase fusion protein exists as a tetramer.

### ASNase Activity and Resistance to Proteolysis

The specific activity of the soluble fusion protein toward L-asparagine is 102 U/mg. One unit of the enzyme is defined as the amount that will catalyze the production of 1  $\mu\text{mol}$   $\text{NH}_3$  per minute with L-asparagine as substrate at 37°C. By comparing the enzymatic activity of scFv-ASNase with that of native ASNase at the same molar concentration, we found that scFv-ASNase fusion exhibited about 82% of the activity of the native enzyme. This indicates that scFv does not interfere with the active site of ASNase when fused to the enzyme. Table I shows that scFv-ASNase fusion retained 94%, 88.8%, and 84.5% of its



**Figure 3.** (A) SDS-PAGE analysis of the purified fusion protein. Coomassie blue-stained bands are shown in lanes 1–5. Lane 1: protein molecular-mass markers; lane 2: the proteins from the concentrated refolding solution; lane 3: the purified fusion protein; lane 4: the protective scFv; lane 5: ASNase. (B) Western blotting of the purified fusion protein and scFv using anti-E-tag antibody as primary antibodies and goat anti-mouse IgG as second antibodies (lanes 6–7). (C) Western blotting of the purified fusion protein and native ASNase using anti-ASNase polyclonal antiserum as primary antibodies and HRP-conjugated goat anti-rabbit IgG as secondary antibody (lanes 8–9).

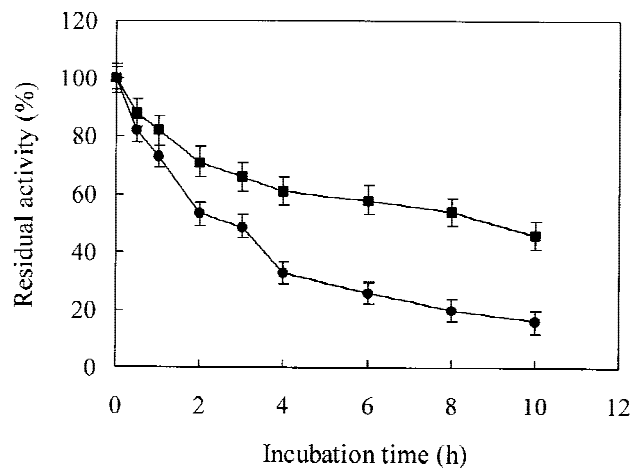
activity, respectively, after the fusion protein was treated with trypsin,  $\alpha$ -chymotrypsin and rennet at 37°C for 30 min. In contrast, the residual activity of native ASNase after the same treatment was about 0%, 15.0%, and 12.7% of the untreated enzyme, respectively.

### Stability of ASNase and scFv-ASNase Fusion in Serum

Figure 4 shows that the *in vitro* half-life of native ASNase was about 2 h, whereas the *in vitro* half-life of scFv-ASNase fusion was approximately 9 h. It is conceivable that scFv contributes to the inaccessibility of plasma proteases to the enzyme.

### Enzyme Kinetics

The apparent Michaelis constant ( $K_m$ ) was estimated by the Lineweaver-Burk method (Chaplin and Bucke, 1990). The



**Figure 4.** Retention of enzymatic activity in the presence of human serum. Native ASNase (●) and scFv-ASNase fusion (■) were incubated in fresh human serum at 37°C for different times. Values denote mean  $\pm$  SD (three different experiments).

effect of substrate concentration on the activity of native ASNase and scFv-ASNase fusion is shown in Figure 5.  $K_m$  values were  $5.08 \times 10^{-5}$  M (native ASNase) and  $6.34 \times 10^{-5}$  M (scFv-ASNase fusion). It was found that the difference in  $K_m$  for the native ASNase and fusion protein is not statistically significant. This demonstrates that the fusion of scFv to ASNase did not affect significantly the enzyme's affinity for the substrate of L-asparagine.

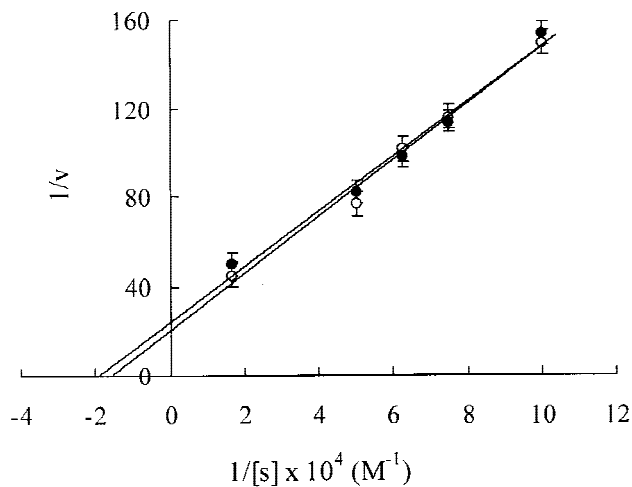
### Modeling of Three-Dimensional Structure of scFv-ASNase Fusion and Electrostatics Calculation

The three-dimensional structure of the fusion protein was modeled and refined as shown in Figure 6. The structure of the native ASNase was superimposed on that of the new scFv-ASNase assembly, and it was found that the conformation of the ASNase moiety of the fusion protein was similar to that of the ASNase molecule. The root mean square (RMS) deviation of the backbone atoms for native ASNase and ASNase fused with scFv was less than 3.0 Å. Moreover, the electrostatic potential surfaces of native ASNase and ASNase fused with the scFvs were calculated by employing the Delphi module (Nicholls and Honig,

**Table I.** Resistance of the scFv-ASNase fusion protein to proteolysis.

|                                      | Residual activity (% of the untreated enzyme <sup>a</sup> ) |        |        |        |        |
|--------------------------------------|---|--------|--------|--------|--------|
|                                      | 10 min  | 15 min | 20 min | 25 min | 30 min |
| ASNase + trypsin                     | 45.2  | 32.3   | 17.9   | 7.4    | 0      |
| ASNase + $\alpha$ -chymotrypsin      | 31.5  | 26.3   | 20.8   | 17.3   | 15.0   |
| ASNase + rennet                      | 33.0  | 27.9   | 21.3   | 15.2   | 12.7   |
| ScFv-ASNase + trypsin                | 98.6  | 98.3   | 96.2   | 95.7   | 94.0   |
| ScFv-ASNase + $\alpha$ -chymotrypsin | 97.7  | 97.1   | 95.3   | 91.8   | 88.8   |
| ScFv-ASNase + rennet                 | 98.1  | 96.9   | 93.7   | 87.4   | 84.5   |

<sup>a</sup>The residual activities of the first three samples were compared to the untreated native ASNase, and those of the last three samples were compared to the untreated scFv-ASNase fusion.

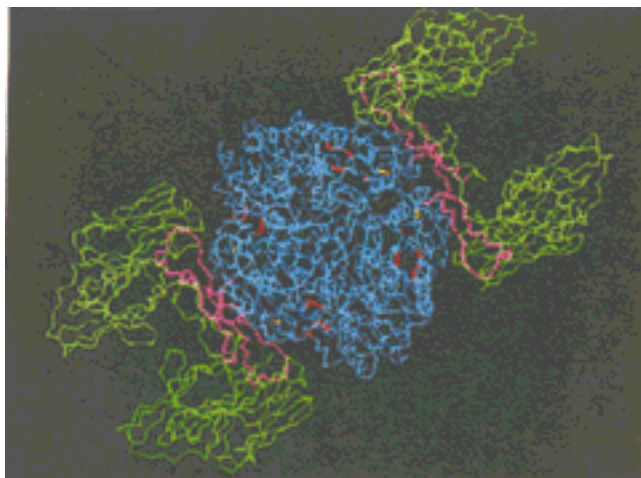


**Figure 5.** Lineweaver-Burk (double-reciprocal) plot of  $1/v$  against  $1/[S]$  giving intercepts at  $1/V_{\max}$  and  $-1/K_m$ . The apparent  $K_m$  of ASNase (●) and scFv-ASNase fusion (○). Substrate L-asparagine concentrations varied from 10 to 62  $\mu\text{M}$ . Values denote mean  $\pm$  SD (three different experiments).

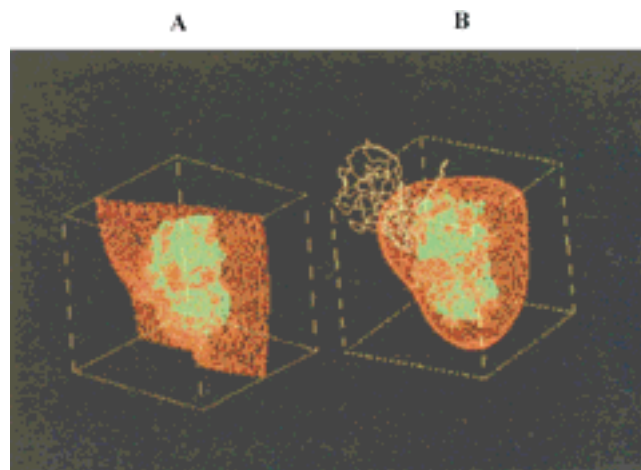
1991). Because ASNase consists of four identical subunits compacted symmetrically (Meng et al., 1985), one of its subunits was selected for analysis. As shown in Figure 7, there was an apparent curvature in the negatively charged surface of the ASNase subunit when the protein was fused with scFv.

## DISCUSSION

Improved resistance of ASNase to proteolysis has been previously reported for enzyme coupled to albumin (Poznansky



**Figure 6.** Three-dimensional structure model of scFv-ASNase fusion protein modeled by the Homology and Discover modules of the INSIGHT II software package. The enzyme and scFv moieties of the fusion protein are shown in blue and green, respectively. The linker peptide joining the ASNase and scFv moiety is shown in pink. ASNase is composed of four identical subunits compacted symmetrically; residues involved in the catalysis (Peterson et al., 1977) are shown in red. Lys-22 (yellow) is one of the cleavage sites of trypsin.



**Figure 7.** Electrostatic potential surfaces. The electrostatic potential surfaces are generated separately with the Delphi module for one subunit of native ASNase (A) and the enzyme moiety from one subunit of scFv-ASNase fusion (B). The scFv moiety is shown in yellow. The surface potential is shown in red for  $-2kT$  and green for  $2kT$  (where  $k$  is the Boltzmann constant and  $T$  is temperature), and is plotted in a linear manner. The molecule is located in the center of the grid.

et al., 1982), colominic acid (Fernandes and Gregoriadis, 1997), and polyethylene glycol (Kodera et al., 1992). The approach used to extend the half-life of ASNase in clinical application has been through covalent coupling of the enzyme to monomethoxypolyethylene glycol (mPEG). However, ASNase modified with mPEG loses much of its ASNase activity (Cao et al., 1990), and the long-term effect of accumulation in tissues of nonbiodegradable mPEG is currently unknown. Therefore, use of albumin, antibodies (Shami et al., 1991), and polysaccharide as alternatives to mPEG have been considered. We became interested in using functional antibody fragments to protect ASNase for three reasons: First, specific functional antibody fragments, such as Fab, Fv, and scFv can be selected from antibody libraries and expressed in bacteria at high levels. Using this approach complex hybridoma technology is avoided. Second, antibody fragments can be fused to their target protein to construct the fusion protein, and thus the target protein and protective scFv can be produced by a single organism. Third, antibody fragments can be finally degraded and will not accumulate in vivo.

This study has reported on the construction, expression, and stability of the scFv-ASNase fusion protein. The results have shown that the scFv moiety of the fusion protein is capable of efficiently protecting the enzyme moiety against proteolysis. For example, 94.0% of the initial activity of ScFv-ASNase fusion remained after treatment with trypsin at 37°C for 30 min. Our previous study (Guo et al., 2000) showed that 78% of the initial activity of the ASNase-scFv complex remained after treatment with trypsin at 37°C for 30 min. By comparison, the protection of ASNase by scFv was enhanced when the two proteins were fused.

The three-dimensional structure of the fusion protein was

modeled and analyzed. Computer simulation of the three-dimensional structure of the fusion protein was used to address the following two questions: (1) Is there any drastic change in the conformation of the ASNase molecule caused by its fusion with scFv? The RMS deviation of the backbone atoms of native ASNase and ASNase fused with scFv was small ( $<3.0 \text{ \AA}$ ), indicating that scFv does not interfere with correct folding of the ASNase molecule. These data are consistent with the results of the enzyme activity assay and estimated  $K_m$  value. (2) What is the possible mechanism of the protection of ASNase by scFv? The cleavage sites of the proteases (such as trypsin and  $\alpha$ -chymotrypsin) are dispersed throughout the surface of the ASNase molecule (Tetsuo et al., 1974). Thus, steric hindrance caused by the scFv moiety might not be the single factor responsible for the protection. We analyzed the electrostatic potential surfaces of ASNase (Fig. 7A), and found that there are two levels of the potential surface on each of the ASNase subunits: a positive level (in green) and a negative level (in red). We also calculated the potential surface of the ASNase subunit fused with the scFv fragment (Fig 7B). It appears that the negative surface of the ASNase subunit is curved after fusion with scFv. In the fusion protein, the negative potential surface covers a larger area than the positive surface as compared with the situation found in native ASNase. This reduces the attack of ASNase by the proteases, because the catalytic mechanisms of serine protease (trypsin and  $\alpha$ -chymotrypsin) and aspartic protease (rennet) involve a nucleophilic attack on the substrate carbonyl by the negatively charged active site (Antonov et al., 1980; Fastrez and Fersht, 1973; Kostka, 1985). Based on the three-dimensional structural model of the fusion protein, the electrostatic analysis, and the biochemical features, we propose that the protective effect of scFv on ASNase results from a combination of steric hindrance and a change in the electrostatic surface of the enzyme.

Analysis of the structure of ASNase has revealed that the N-terminus of ASNase is exposed on the surface of the molecule. In this study, scFv was fused to the N-terminus of the enzyme. In the scFv-ASNase fusion protein, the original signal peptide at the N-terminus of ASNase was deleted. This may be one of the reasons why the fusion protein was found in the form of inclusion bodies. Work is now in progress to construct a fusion protein in which scFv is fused to the C-terminus of ASNase with the original signal peptide retained.

The authors thank Dr. Yingda Wang for providing the expression vector pBV-ASN and Prof. Li Huang for performing an extensive revision of the manuscript.

## NOMENCLATURE

|      |  |
|------|--|
| bp   | base pairs                                 |
| BAEE | N $\alpha$ -benzoyl-L-arginine ethyl ester |
| BTEE | N-benzoyl-L-tyrosine ethyl ester           |
| DAB  | 3,3'-diaminobenzidine tetrahydrochloride   |
| HRP  | horseradish peroxidase                     |

|      |  |
|------|--|
| IPTG | isopropyl $\beta$ -D-thiogalactopyranoside |
| mPEG | monomethoxypolyethylene glycol             |
| PCR  | polymerase chain reaction                  |
| PDB  | Protein Data Bank                          |
| RMS  | root mean square                           |

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