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Characterization of L-Asparaginase Fused with a Protective ScFv and the Protection Mechanism

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A fusion protein of the protective scFv linked to the C-terminus of ASNase via $(Gly_4Ser)_6$ peptide was constructed. The ASNase-scFv fusion protein expressed in *Escherichia coli* exists mainly in the form of inclusion bodies, and a small amount of it was soluble. The soluble form was purified by four-step purification and it has been demonstrated that ASNase-scFv fusion exists as a dimer. By assay of the stability against proteolysis, the ASNase-scFv fusion was found to be more stable than native ASNase but less stable than scFv-ASNase fusion. The results of immunological assay indicated that the immunogenicity of the fusion proteins increased while their binding capacity with the anti-ASNase serum decreased by comparison to the native ASNase. Moreover, here the comparison of the basic physical and chemical properties of the ASNase-scFv fusion, scFv-ASNase fusion, and native ASNase is presented. Based on the structural evidence and the biochemical analysis described in this paper, the protection mechanism proposed in our previous study was further supported. The scFv moiety of the fusion protein may confer the ASNase moiety resistance to proteolysis as a result of both steric hindrance such as blocking the cleavage sites of trypsin and a change in the electrostatic potential surface of the enzyme.

Key Words: **L-asparaginase; single-chain Fv; fusion protein, protein stability, proteolysis, structural modeling.**

L-Asparaginase (EC 3.5.1.1., ASNase) is a tetrameric enzyme that catalyses the hydrolysis of L-asparagine to L-aspartic acid and ammonia (1). The enzyme is in clinical use for the treatment of acute lymphoblastic leukemia (2). However, as with other therapeutic proteins, the clinical use of L-ASNase can be limited by its short half-life, which is caused by inactivation *in vivo.* The most common form of inactivation *in vivo* is proteolysis.

In our previous study, a protective single-chain Fv (scFv) enhancing the resistance of ASNase to trypsin was selected from a phage-display scFv antibody library (3) and fused to the N-terminus of ASNase by DNA recombinant technology. The scFv-ASNase fusion exhibited ASNase activity (102 iu/mg) and increased resistance to proteolysis (4). These results demonstrate scFv does not disrupt the structural and functional integrity of active ASNase. To further elucidate the protection mechanism of scFv, the fusion of the protective scFv to the C-terminus of ASNase was investigated and the comparison of the structural models and properties of the scFv-ASNase fusion and ASNase-scFv fusion was presented in this paper.

MATERIALS AND METHODS

DNA manipulations, growth media, and buffers (unless individually specified) were as described by Sambrook *et al.* (5).

Strains and plasmids. E. coli DH5α [sup E44 Δlac U169 (ϕ80 lacZ ΔM15) hsd R17 recA end A1 gyrA96 thi-1 relA1] (TaKaRa) was used 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 host strain for expressing the fusion proteins. The vector pET21a (Novagen) was used to construct the recombinant plasmid. The recombinant plasmid pBV-ASN was provided by Dr. Yingda Wang of Institute of Microbiology, Chinese Academy of Sciences.

The proteases including trypsin Type XI (from bovine pancreas), α-chymotrypsin Type II (from bovine pancreas) and rennet Type II (from *Mucor meihie*) were purchased from Sigma.

Amplification of the ASNase gene (asnB) and protective ScFv gene by PCR. The asnB gene from *E. coli* (6) was amplified by PCR from pBV-ASN with the following primers: 5′-GGATTACCATATGGAGTTTTTCAAGAA-3′ (*Nde*I) and 5′-CGGAATTCGTACTGATTGAA-3′ (*Eco*RI). 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 was amplified by PCR from p5E-scFv46 gene (Guo *et al.*, 2000) with the following primers: 5′-GCGTCGACATGGCCCAGGTG C-3′ (*Sal*I) and 5′-

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GCAAGCTTTTATTAACGCGG TTCCAG-3′ (*Hin*dIII). 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 the fusion gene and construction of the expression vector. The linker DNA fragment encoding the peptide of $(Gly_4Ser)_6$ was inserted into pET 21a between the *Eco*RI and *Sal*I to get the recombinant pET-link. The PCR product for the scFv gene was digested with *Sal*I and *Hin*dIII and cloned into pET-link to form pET-LS. Colonies containing potential clones of pET-LS were grown in *E. coli* DH5α and plasmid DNA was isolated. Clones that contained pET-LS were identified by sequence analysis of the plasmid DNA using T7 universal primers (ABI PRISM 377 DNA Sequencer). The PCR product for the ansB gene was digested with *Nde*I and *Eco*RI, and the digested product was inserted into pET-LS between *Nde*I and *Eco*RI to yield recombinant vector pET-ALS.

Expression of the fusion protein in E. coli BL21 (DE3). The expression vector pET-ALS was transformed into *E. coli* BL21 (DE3). An overnight culture (10 mL) of *E. coli* B21 (DE3) harboring pET-ALS was inoculated into 1 L of LB medium containing 100 μg/mL ampicillin. After incubation at 30°C with shaking to an OD_{600} of about 0.5, the culture was induced with IPTG at a final concentration of 1 mM. The cells were grown for an addition period of 5 h at 30°C, and harvested by centrifugation, resuspended in 40 mL of buffer A (50 mM Na_2HPO_4–NaH_2PO_4, pH 8.0/2 mM EDTA/50 mM NaCl) and stored at −20°C.

Western blotting. After separation on a 12% (w/v) SDS–PAGE (7), proteins were electroblotted to a nitrocellulose membrane (Bio-Rad). The primary antibody against ASNase was raised in rabbits immunized four times at intervals of 7–10 days with a total of 2 mg purified ASNase. The antiserum was collected from the immunized rabbits and was treated with HB101 lysate to remove anti-*E. coli* antibodies as described by Sambrook *et al.* (5). The secondary antibody was goat anti-rabbit IgG (Sigma) conjugated with HRP. 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′-diaminobenzdine tetrahydrochloride (DAB) as substrate.

Purification of the ASNase-scFv fusion. Bacterial cells were suspended in 0.02 M Na_2HPO_4–NaH_2PO_4 (pH 8.0) buffer by 1:8 (w/v) and lysed by sonication at 4°C. After centrifugation at 10,000*g* for 20 min, the supernatant was collected. $MnCl_2$ solution was added to the above supernatant with the final concentration of 0.05 M and a white precipitate had formed and was removed by centrifugation. Subsequently, the supernatant was incubated at 50°C for 30 min and the precipitated bacterial proteins were removed by centrifugation. The supernatant was dialyzed against 0.02 M Na_2HPO_4–NaH_2PO_4 (pH 8.0) buffer at 4°C for 12 h and then concentrated by solid PEG-10000. The sample containing ASNase-scFv was absorbed on a 2.5 × 20 cm of DEAE ion-exchange column (Sigma) that had been equilibrated with 0.02 M Na_2HPO_4–NaH_2PO_4 (pH 8.0) buffer and eluted with a linear NaCl gradient (0.05–0.2 M). Peak fractions of ASNase activity were pooled. The collected sample was applied to a 1.0 × 6.0 cm column of Sephadex G-150 (Pharmacia), equilibrated with 0.02 M Na_2HPO_4–NaH_2PO_4 (pH 8.0) buffer and eluted with the same buffer. Peak fractions of ASNase activity were collected, concentrated by lyophilization and stored at −20°C.

Enzyme activity assay. ASNase, ASNase-scFv fusion and scFv-ASNase fusion preparations were assayed for enzyme activity according to Peterson and Ciegler (8). One unit of the enzyme is defined as that amount which will catalyze the production of 1 μmol NH_3 per minute with L-asparagine as substrate at 37°C.

Resistance to proteolysis. The native ASNase, scFv-ASNase fusion, and ASNase-scFv fusion (50 μmol of each) were treated with 40 BAEE units of trypsin, 80 BTEE units of α-chymotrypsin and 20 μg/mL rennet in 20 mM $NaHPO_4$–NaH_2PO_4 buffer (pH 7.2) in a final volume of 1 mL at 37°C for 10–30 min, respectively. Aliquots (200 μL of each) were taken from each of the above nine reaction mixtures at intervals and diluted to 500 μL with 10 mM borate buffer (pH 8.4). The digestions of trypsin, α-chymotrypsin and rennet were stopped by the addition of 20 μg/mL trypsin inhibitor Type I-S from Soybean (Sigma), 8 μL of 500 mM PMSF (Sigma) and 4 μg/mL pepstatin (Boehringer-Mannheim), respectively. The enzyme activity was assayed immediately.

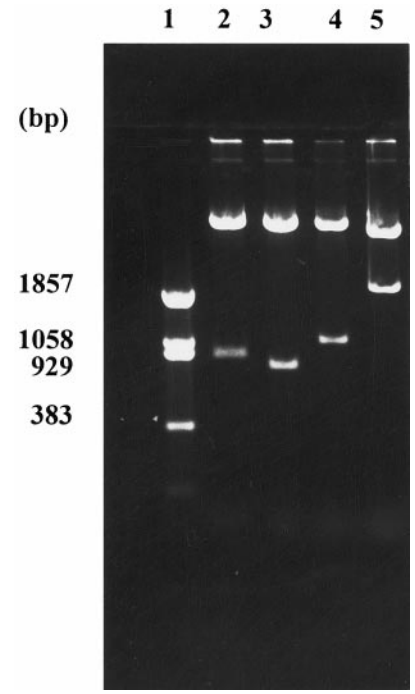

FIG. 1. Agarose gel electrophoresis analysis of pET-ALS. Lane 1, DNA size standard pBR322/*Bst*NI. Lanes 2–5, the vector pET-ALS after cleavage with *Nde*I and *Eco*RI, *Sal*I and *Hin*dIII, *Nde*I and *Sal*I and *Nde*I and *Hin*dIII, respectively, and the inserted DNA fragments of asnB gene (1044 bp), scFv gene (780 bp), asnB-Linker (1134 bp), and the fusion gene (1926 bp) were obtained in order.

Stability in human serum in vitro. The native ASNase, scFv-ASNase fusion, and ASNase-scFv fusion (1 mg of each) were hydrated with 1 mL of 0.02 M Na_2HPO_4–NaH_2PO_4 buffer (pH 7.2), mixed with an equal volume of fresh human serum and incubated at 37°C for 1~11 h respectively. Aliquots (50 μL of each) taken at intervals were diluted with 10 mM borate buffer (pH 8.4) and immediately assayed for ASNase activity.

Immunogenicity. The native ASNase, scFv-ASNase fusion, and ASNase-scFv fusion immunized the rabbits to prepare their antiserums, respectively. Subsequently, the titers of the antiserums were assayed by ELISA method.

The binding capacity with anti-ASNase serum. The native ASNase (1 mg), scFv-ASNase (1.8 mg) and ASNase-scFv fusion (1.8 mg) were hydrated in 1 mL of 0.05 M Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) respectively. 3, 6, 9, 12, 18, 24, 30, and 40 μL of samples were taken from each of the above solutions, diluted to 500 μL with 0.05 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.6) and coated on the ELISA plate at 4°C overnight. The antigen-antibody binding capacity of all the samples and anti-ASNase serum were determined by ELISA.

Enzyme kinetic. Enzyme kinetics are best described by the well-known Michaelis–Menten relationship, which takes into account the formation of an enzyme-substrate complex. The enzyme reaction was initiated by the addition of 0.5 mL of the native ASNase, scFv-

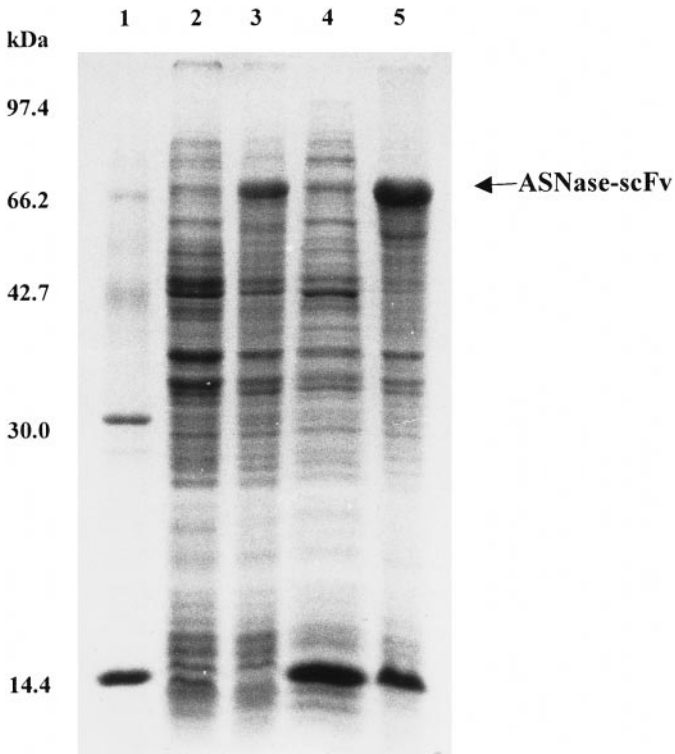

FIG. 2. SDS–PAGE analysis of the expression of ASNase-scFv fusion protein. Lane 1, protein molecular-mass markers; Lane 2, culture without IPTG induction; Lane 3, culture induced with 1 mM IPTG; Lane 4, the supernatant from the cell sonicates; Lane 5, the pellet from the cell sonicates.

ASNase fusion and ASNase-scFv fusion solution to 0.5 mL of prewarmed (37°C) substrate solution (1–12 × 10^{-5}) and the mixtures incubated for 15 min at the same temperature, respectively. The reaction was stopped by the addition of trichloroacetic acid (TCA) solution. The apparent Michaelis constant (K_m) was estimated by Lineweaver–Burk method (9).

Molecular modeling. An initial three-dimensional structure model for the fusion protein was built as follows. The three-dimensional structures of Fab fragment of A Human Igg 1κ (1VGEH), Fab fragment Ctmo1 H chain (1AD9H) and Fab fragment Ctmo1 B chain (1AD9B) were extracted from PDB and used as templates to simulate VH domain of scFv. Similarly, the three-dimensional structures of λ III Bence Jones Protein Cle A chain (1LILA), λ III Bence Jones Protein Cle B chain (1LILB) and Rf-An Igm/λ 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 Molecular Simulation Program (MSI Inc., Version 98.0). The structure of ASNase (10) was extracted from PDB with the code 3ECA. Assuming that the site of the protective scFv involved in binding to ASNase is at or near Lys-29 (3), the initial relative location of the scFv and ASNase molecules was determined. The long linker $(Gly_4Ser)_6$ connecting the N-terminus of the scFv molecule and the C-terminus of ASNase molecule was also generated by Insight II package.

The model was refined with the Homology and Discover modules of Insight II package (11). 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, since generation of the loop region only provides extended side chain conformations 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/A 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/A. Subsequently, assembly was performed with the above 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 and Purification of ASNase-scFv Fusion

The recombinant pET-ALS was analyzed by digestion (Fig. 1) and transformed to *E. coli* BL21(DE3). As shown in Fig. 2, the ASNase-scFv fusion was expressed in *E. coli* BL21 (DE3) and the express level of the fusion protein was estimated by gel scanning on a Shimadzu CS-930 densitometer. The soluble ASNase-scFv fusion accounted for 2.3% of the proteins from the supernatant of the sonicated cells (Fig. 1, Lane 4), and the ASNase-scFv fusion expressed as inclusion bodies, which accounted for 10.4% of the pellet of the sonicated cells. It was found that the refolded ASNase-scFv fusion from inclusion bodies was inactive, therefore the ASNase-scFv fusion in the supernatant was collected

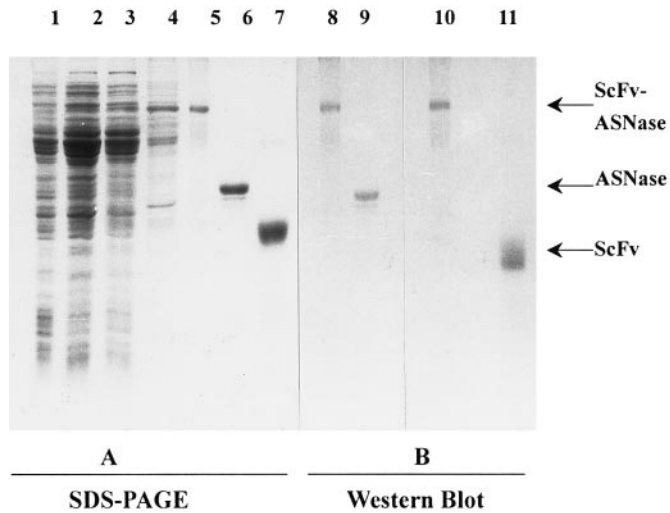

FIG. 3. (A) SDS–PAGE analysis of the purified fusion protein ASNase-scFv. Lane 1, the supernatant of the broken cells; Lane 2, purified by $MnCl_2$; Lane 3, purified by incubation at 50°C; Lane 4, the proteins from ion-exchange chromatography; Lane 5, elution fraction of Sephadex G-150; Lane 6, native ASNase; Lane 7, the protective scFv. (B) Lanes 8–9, 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 10–11, Western blotting of the purified fusion protein and native ASNase using anti-ASNase serum as primary antibodies and goat anti-rabbit IgG as secondary antibody.

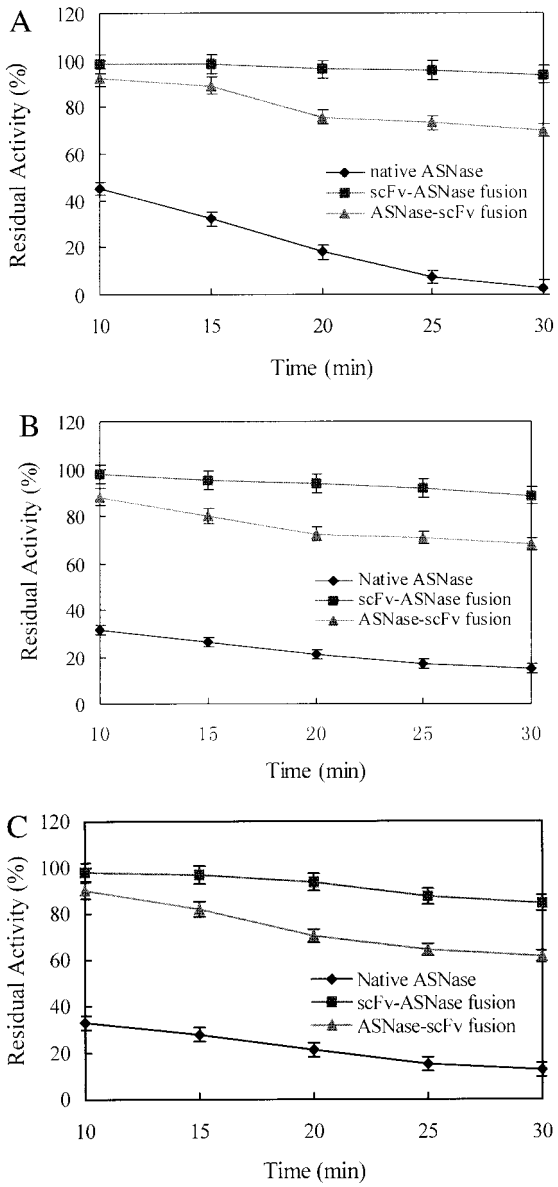

FIG. 4. The resistant ability of the fusion proteins to trypsin (A), α-chymotrypsin (B), and rennet (C) proteolysis.

and purified by 4 steps purification including DEAE ion-exchange chromatography and gel filtration. The purified protein was subjected to electrophoretic analysis on a 12% SDS–PAGE gel (Fig. 3A). Western blotting (Fig. 3B) was performed to verify the presence of the ASNase moiety and protective scFv moiety in the ASNase-scFv fusion molecule. The apparent molecular mass of ASNase-scFv subunit was estimated to 66.0 kDa, in agreement with the calculated molecular mass (64.7 kDa). Since each subunit of ASNase consisting of four identical subunits was fused with a scFv, the calculated molecular mass of the ASNase-scFv is 258.4 kDa. However, when assayed by gel filtration of Sephadex G-75, the molecular mass of the ASNase-scFv was

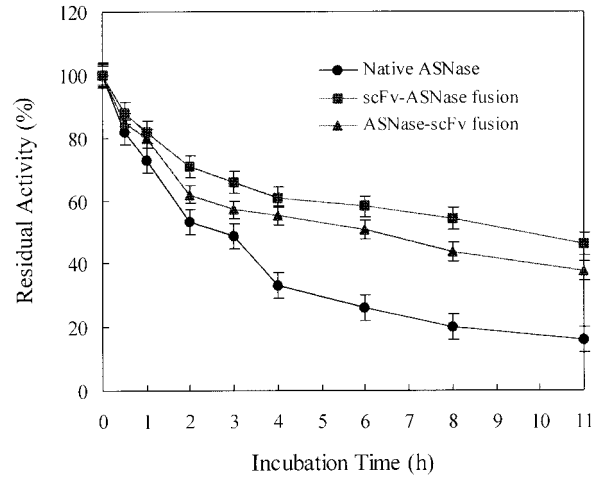

FIG. 5. Retention of enzymatic activity in the presence of human serum.

estimated 540 kDa, which is about 2-fold of the calculated molecular mass. This suggests that the ASNase-scFv fusion mainly exist as a dimer.

Resistance to Proteolysis

The results in Figs. 4A–4C show that the fusion protein scFv-ASNase and ASNase-scFv are more stable against proteolysis than the native ASNase. For example, the scFv-ASNase fusion and ASNase-scFv fusion retained 94 and 70.3% of their initial activity, respectively, after they were treated with trypsin, α-chymotrypsin and rennet at 37°C for 30 min. In contrast, the activity of the native ASNase was completely removed after the same treatment.

Stability in Human Serum in Vitro

Figure 5 shows that the half-life of native ASNase *in vitro* was about 2 h, whereas those of the scFv-ASNase

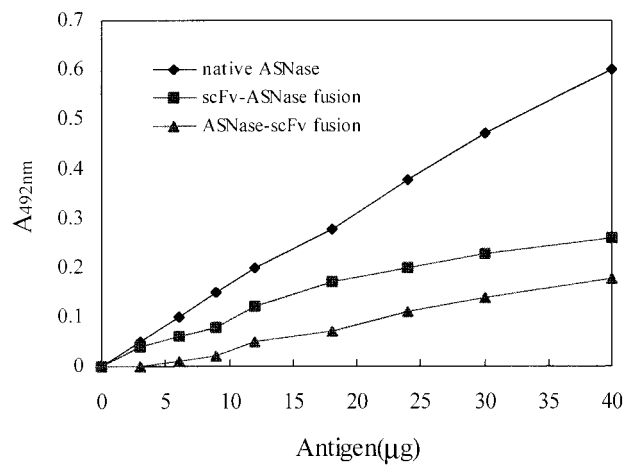

FIG. 6. The binding capacity of the fusion proteins with anti-ASNase serum.

TABLE I

Comparison of the Basic Physical and Chemical Properties

	ASNase	ScFv-ASNase	ASNase-scFv
Molecular weight	~144.5 kDa ~37.0 kDa (subunit)	~260 kDa ~65.0 kDa (subunit)	~540 kDa ~65.0 kDa (subunit)
Optimal pH[a]	7.0	8.0	8.0
Optimal temperature[b]	55°C	65°C	50°C
p*I*	4.6	5.8	5.8
K_m	5.08×10^{-5} M	6.34×10^{-5} M	1.59×10^{-4} M

[a] The native ASNase, ASNase-scFv fusion, and scFv-ASNase fusion were incubated at 37°C in 0.02 M Na_2HPO_4-Citric acid buffer (pH 3.0–8.0) and 0.02 M $Na_2B_4O_7$-NaOH buffer (pH 9.0–11.0) for 15 min and assayed for enzyme activity, respectively.

[b] The temperature optimum of the native, ASNase-scFv fusion and scFv-ASNase fusion were measured with substrate L-Asn at different temperatures ranging from 30 to 80°C.

fusion and ASNase-scFv fusion are prolonged to 9 and 6 h, respectively.

Immunogenicity and the Binding Capacity to Anti-ASNase Antibodies

The titer of each antiserum against the native ASNase, scFv-ASNase fusion and ASNase-scFv was determined by ELISA. By comparison of the titer of the antiserum against the native ASNase (0.23×10^2), the titers of the antiserums against the scFv-ASNase and ASNase-scFv fusion increase 2.1- and 2.9-fold, respectively. However, Fig. 6 shows that the binding capacity of the fusion proteins to the anti-ASNase serum was lower than that of the native ASNase.

Basic Biochemical and Physical Properties

As shown in Table 1, K_m values were 5.08×10^{-5} M (native ASNase), 6.34×10^{-5} M (scFv-ASNase fusion) and 1.59×10^{-4} M (ASNase-scFv fusion), respectively. The data indicate that the fusion of scFv to the N-terminus of ASNase did not effect the enzyme's affinity for the substrate of L-asparagine (L-Asn), but the fusion of scFv to C-terminus of ASNase decreased the enzyme's affinity for the substrate of L-Asn. In addition, the optimal pH and temperature for the activity of the fusion proteins were different from that of the native ASNase.

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

The three-dimensional structures of ASNase-scFv fusion and scFv-ASNase fusion were modeled and refined as shown in Figs. 7A and 7B. The structure of native ASNase was superimposed on the ASNase moieties of the new scFv-ASNase and ASNase-scFv assemblies, respectively. It was found that the conformation of the ASNase moiety of the scFv-ASNase fusion was almost identical with that of ASNase molecule. The RMS deviation of the backbone atoms for native ASNase and ASNase fused with scFv at its N-terminus was less than 3.0 Å. However, the RMS deviation of the backbone atoms for native ASNase and ASNase fused with scFv at its C-terminus was more than 4.5 Å. Moreover, the electrostatic potential surfaces of native ASNase and ASNase fused with scFvs were calculated by employing the program of Delphi (12). Since ASNase consists of four identical subunits compacted symmetrically (13); one of its subunits was selected for analysis of electrostatic potential surfaces. As shown in Figs. 8B and 8C, there was apparently curvature in the negative electrostatic potential surface of the ASNase subunit when the enzyme was fused with scFv.

DISCUSSION

The enzyme specific activity of the ASNase-scFv fusion (20.5 iu/mg) is significantly lower than that of the scFv-ASNase fusion (102 iu/mg). This result may be explained by their different K_m values. The K_m for the scFv-ASNase fusion is not statistically different from that of the native ASNase, whereas the K_m value of the ASNase-scFv fusion has a dramatically increase in comparison with the native ASNase. These data may suggest that the fusion of scFv to the C-terminus of ASNase affect the enzyme's affinity for the substrate L-Asn. In addition, forming dimer for ASNase-scFv can decrease the enzyme activity to some extent.

It was found that the three-dimensional structure of each subunit of ASNase molecule has not any change after fusion with a scFv. Meanwhile, it was observed that the C-terminus of ASNase involves in the interactions between the subunits of ASNase while the N-terminus of ASNase does not. When scFv was fused at the N-terminus of ASNase, the fusion did not effect the interaction between the subunits of ASNase. On the other hand, fusion at C-terminus caused the change of the interaction between the subunits of ASNase. Since the conformation of the active site of ASNase is closely relative to the interaction between the subunits of ASNase (10). The above change may affect the enzyme activity, which further demonstrate the lower enzyme activity of the ASNase-scFv fusion.

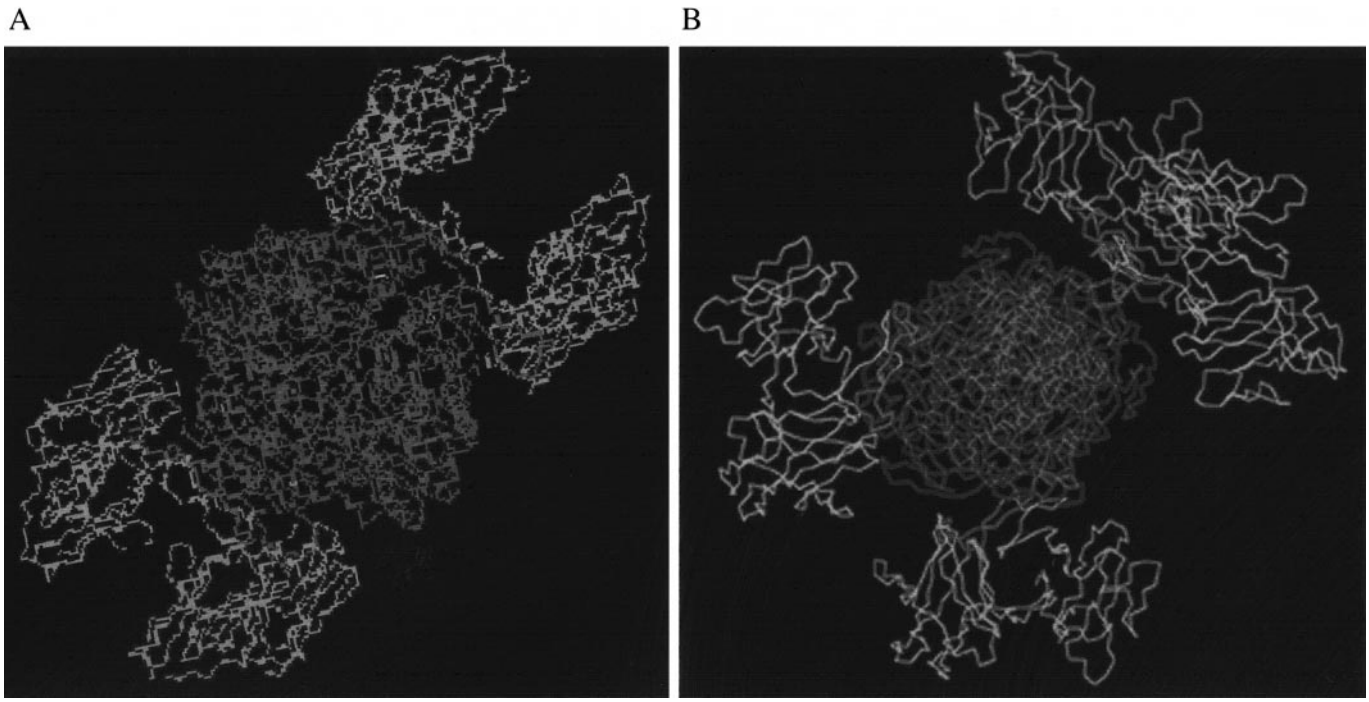

FIG. 7. Three-dimensional structure model of the fusion protein scFv-ASNase (A) and ASNase-scFv (B). The ASNase and scFv moieties of the fusion protein are the central region and four parts around the central region, respectively. ASNase is composed of four identical subunits compacted symmetrically.

The results in this paper demonstrate that the scFv moiety of the fusion protein is capable of efficiently protecting the enzyme moiety against proteolysis. In our previous study (3), the scFv can enhance the resistance of ASNase to trypsin proteolysis, which may be due to block the cleavage site of trypsin (Lys-29 of ASNase). We also proposed that the steric hindrance caused by the scFv moiety might not be the single factor responsible for the protection and the drastic change in electrostatic potential surface of ASNase molecule contributed to confer ASNase the resistance to proteolysis (4). The above protection mechanism is further supported by this study. It appears that the negative surface of ASNase subunit is curved after fusion with scFv at the C-terminus of ASNase, which is similar to the situation of the scFv-ASNase fusion.

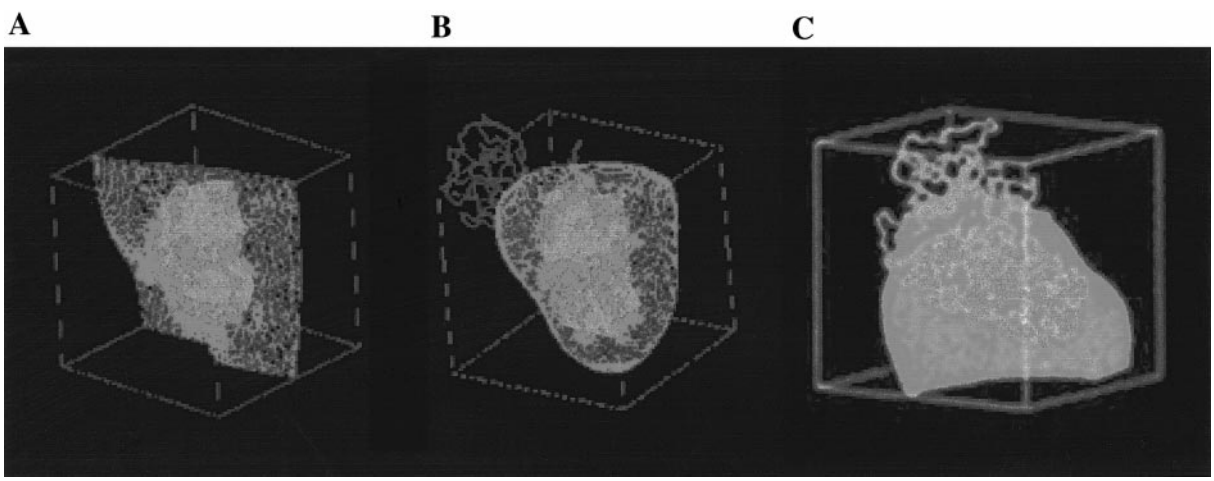

FIG. 8. Electrostatic potential surfaces. The electrostatic potential surfaces are generated separately for one subunit of native ASNase (A), the enzyme moiety from one subunit of scFv-ASNase fusion (B), and ASNase-scFv (C). The surface potential is plotted with line and the molecule located in the center of the grid.

Following the curvature, the negative potential can weaken more positive potential compared to the situation found in native ASNase. Consequently, this will reduce the attack of ASNase by the proteases because the catalytic mechanisms of serine protease (trypsin and α-chymotrypsin) and aspartic protease (rennet) involve a nucleophilic attack on the substrate carbonyl by the negatively charged active site (14–16)

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