Selecting and expressing protective single-chain Fv fragment to stabilize L-asparaginase against inactivation by trypsin

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Four non-inhibitory specific single-chain Fv (sc Fv) fragments directed against L-asparaginase (ASNase) of *Escherichia coli* were selected from a synthetic phage-display scFv library. The scFv46 fragment could enhance the resistance of ASNase to trypsin proteolysis, with 70% of the initial ASNase activity present after the ASNase–scFv46 complex had been treated with trypsin for 30 min at 37 °C, whereas little residual activity was detected without the scFv46 fragment. The *scFv46* gene was cloned to an expression vector pET-21a and expressed at high levels (about 45% of total cell protein) in *E. coli* BL21 (DE3) as inclusion bodies. The refolded and purified scFv46 fragment was proved to protect ASNase, and the protective effect was further confirmed by SDS/PAGE. It was found that under optimum conditions of molar ratio of scFv to ASNase, incubation time and temperature, the residual activity of the ASNase–scFv46 complex could reach about 78% after treatment with trypsin for 30 min at 37 °C. The results demonstrated that scFv fragments prepared by phage-antibody library technology could be used to protect target proteins.

Introduction

One limitation to the clinical use of most therapeutic proteins is their short half-lives as a result of inactivation *in vivo*. The most common form of inactivation *in vivo* is enzymic modification, particularly enzymic proteolysis [1]. Protecting medically important proteins from proteolytic inactivation may be achieved by use of poly(ethene glycol) [2], albumin [3] and antibodies [4–6].

L-Asparaginase (ASNase) [7], a therapeutic agent for leukaemia and lymphoma, could be protected by both polyclonal antibodies and monoclonal antibodies against proteolytic inactivation. In 1973, Naomi [4] reported that polyclonal antibodies could increase the resistance of ASNase to trypsin and pronase digestion, and that the protection didn't depend on the Fc antibody fragment. In 1984, Jemmerson and Stigbrand [5] used a monoclonal antibody to block the single trypsin cleavage site of placental alkaline phosphatase. In 1992, Shami et al. [6] proposed that the specific monoclonal antibody could protect ASNase by blocking the cleavage site and the residual enzyme activity of the complex was about 70% after it was treated with trypsin for 25 min at 37 °C.

However, preparation of monoclonal antibodies by hybridoma technology is slow and costly and phage-antibody library technology [8,9] may be a better alternative. Antibodies have been fused to the N-terminus of the minor coat protein pIII of the phage or to the C-terminal domain of pIII and displayed as Fab fragments or as single-chain Fv (scFv) fragments. The scFv fragment consists of VH and VL domains (the variable regions of the heavy and light chains, respectively) linked by a flexible polypeptide [10,11]. Moreover, functional recombinant antibody fragments such as Fv, Fab and scFv fragments have been expressed successfully in *E. coli* [12–14].

We now report the screening of the protective scFv fragments for ASNase from a phage-display library, and analysis of the stability of the ASNase–scFv complex.

Materials and methods

The synthetic phage-display antibody library [15] was in scFv format and obtained from the MRC Center, University of Cambridge, Cambridge, U.K. In this library, the genes of the scFv fragments were cloned into *Sfi*I and *Not*I sites of the phagemid pHEN1. ASNase was isolated and purified from *E. coli* AS 1.357 according to Meng et al. [16].

Selection of phage-display scFv fragments against ASNase

Coating and blocking A Nunc immunotube (Maxisorp, Gibco Life Technologies) was coated overnight with 100 μg/ml

Abbreviations used: ASNase, L-asparaginase; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactoside; scFv, single-chain Fv.

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ASNase in 50 mM $NaHCO_3$ buffer (pH 9.6) at 4 °C. The tube is rinsed with PBS (7.305 g of NaCl, 2.306 g of anhydrous Na_2HPO_4, and 1.314 g of $NaH_2PO_4 \cdot 2H_2O$ per litre, pH 7.2), and the uncoated sites on the Nunc immunotube were blocked with PBS containing 2% skimmed milk for 2 h at 37 °C.

Panning and propagating The phage antibodies were added into the Nunc immunotube to bind to ASNase. The Nunc immunotube was rinsed 10 times with PBS/0.1% (v/v) Tween 20 and 10 times with PBS, and then eluted with 100 mM triethylamine (pH 11.6). The eluate was neutralized immediately with 1.0 M Tris/HCl (pH 7.4), and was taken to infect *E. coli* TG1. Infected *E. coli* TG1 were grown until an A_{600} value of 0.5 was reached. Then the protocol "Preparing phage antibodies" [17] was followed to rescue the phagemid for the next round of selection. Each round of selection was carried out as described above, until specific phage-display scFv fragments were selected by ELISA detection.

ELISA

Plates (Costar) were coated with 10 μg/ml ASNase in 50 mM $NaHCO_3$ buffer (pH 9.6) at 4 °C overnight. After three washes in PBS, 100 μl of phage scFv fragments or soluble scFv fragments were added to the plates, and incubated for 2 h at 37 °C. Phage-display scFv fragments were detected by horseradish peroxidase (HRP)-conjugated anti-M13 antibodies (Pharmacia Biotech). Soluble scFv fragments were detected by primary mouse 9E10 antibodies and secondary HRP-conjugated anti-mouse antibodies (Sigma). 9E10 antibodies could recognize the c-Myc peptide at the C-terminus of the scFv fragment. Detection was performed in substrate buffer [0.8 mg/ml *o*-phenylene diamine in 0.1M citrate buffer, pH 5.0 and 0.03% (v/v) H_2O_2] for 20 min at room temperature. The reaction was stopped with 25 μl of 1 M H_2SO_4 per well and the absorbance was determined at 490 nm using a plate reader.

Expression and purification of soluble scFv fragments

The phagemid pHEN1 can be used directly for expression of scFv as soluble fragments, when an amber stop codon is encoded at the junction of the antibody gene and phage minor-coat-protein pIII gene. When expressed in suppressor strains (such as *E. coli* TG1) the pIII fusion protein is produced and packaged into phage, whereas when it is expressed in non-suppressor strains (such as *E. coli* HB2151) soluble antibody fragments are secreted into the bacterial periplasm, and thence into culture broth.

E. coli HB2151 were infected with each selected pHEN1 phagemid for 30 min at 37 °C. Single colonies were inoculated in 2 × TY medium (16 g of tryptone, 10 g of yeast extract and 5 g of NaCl in 1 litre) containing 100 μg/ml ampicillin and 0.1% glucose, and scFv-fragment expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) when cells were grown to $A_{600} = 0.9$. Colonies were incubated overnight at 30 °C and the scFv fragments that had secreted into the culture supernatant were harvested by centrifugation at 10800 ***g*** for 15 min. For small-scale preparation (original culture volume < 100 ml), the supernatant can be concentrated about 10-fold by reversed dialysis against dry granular poly(ethene glycol)-10000 and purified by gel filtration on Sephadex G-75 (Pharmacia Biotech).

Sequencing and cloning of the *scFv* gene

The *scFv* gene was cut with the *Sfi*I and *Not*I restriction enzymes from the phagemid pHEN1 and ligated into pCANTAB 5E (Pharmacia Biotech) that had been cut previously with the same two restriction enzymes. The DNA sequence of the *scFv* fragment gene was analysed using an auto-sequencer (ABI Prism™ 377 DNA sequencer). A DNA fragment next to the *Not*I site of pCANTAB 5E encodes an E-tag epitope (GAPVPYPDPLEPR; Pharmacia Biotech), which allows confirmation by ELISA. The E-tag epitope was introduced at the C-terminus of scFv by PCR and the primers were designed as followed: the forward primer, 5′-AGATATACATATGGCTCAGGTTCAGCTG-GTTCAGTCTGGT-3′; and the reverse primer, 5′-AGCA-AGCTTTTATTATGCGGCACGCGG-3′. The PCR product was digested with *Nde*I and *Hin*dIII, and ligated into pET21a (Novagene) digested with *Nde*I and *Hin*dIII. The restriction-endonuclease digestion, ligation and transformation were performed according to Sambrook et al. [18].

Isolation, denaturation and renaturation of the inclusion bodies

The cells were collected from the culture broth by centrifugation at 5000 ***g*** for 15 min. The cells (wet weight of 0.5 g) were resuspended in 3 ml of 25 mM Tris/HCl (pH 8.0) buffer containing 0.3 mM sucrose and 25 mM EDTA, and disintegrated by sonication. Inclusion bodies were precipitated from the crude homogenate by centrifugation at 12000 ***g*** for 10 min. To purify inclusion bodies from adherent impurities, the pellet was washed twice with 10 mM Tris/HCl (pH 8.0) containing 0.1 M NaCl, 1 mM EDTA and 1% Triton X-100, and then once with 2 M urea. Then, inclusion bodies were dissolved in the denaturing buffer (0.1 M Tris/HCl, pH 8.0/6 M guanidine hydrochloride/0.5% 2-mercaptoethanol) with a protein concentration of less than 10 mg/ml, and incubated at 4 °C for 4 h. The solubilized polypeptide (1 ml) was diluted directly into 100 ml of refolding buffer (0.1 M Tris/HCl, pH 8.0/0.3 M L-arginine/2 mM EDTA/2 M guanidine hydrochloride) and incubated at

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4 °C for 24 h. The solution was dialysed against buffer (0.1 M urea/20 mM Tris/HCl, pH 8.0/5 mM NaCl) with stirring at 4 °C for 12 h, and then against PBS/0.1% (v/v) Tween 20 at 4 °C for 24 h. The refolded antibodies were concentrated by lyophilization, then dissolved in 1.5 ml of PBS and applied to a Sephadex G-75 column (10×60 mm). The protein was eluted with PBS at 26 ml/h.

Results

Selection of phage-display scFv fragments against ASNase

Specific phage-display scFv fragments to ASNase were selected from the phage scFv fragment library, which contains at least 10^8 different clones. The low-stringency condition (such as antigen at a high concentration of 100 μg/ml) was performed in the initial rounds so as not to lose rare binders, and more stringent conditions (10 μg/ml antigen) was employed in the later rounds. After five rounds of selection, four single colonies (numbers 10, 24, 46 and 97) were selected to produce the specific scFv fragments with high binding activity examined by ELISA (Figure 1).

Expression and purifcation of soluble scFv fragments

The selected scFv fragments were expressed as a soluble fraction after the phagemid pHEN1 was transformed into *E. coli* HB2151. By the induction of IPTG, soluble scFv fragments were expressed, and the purified scFv fragments recovered from culture supernatant were applied to SDS/PAGE (Figure 2). However, it was found that the expression level of soluble scFv fragments was so low that it was difficult to collect and purify the antibodies on a large scale.

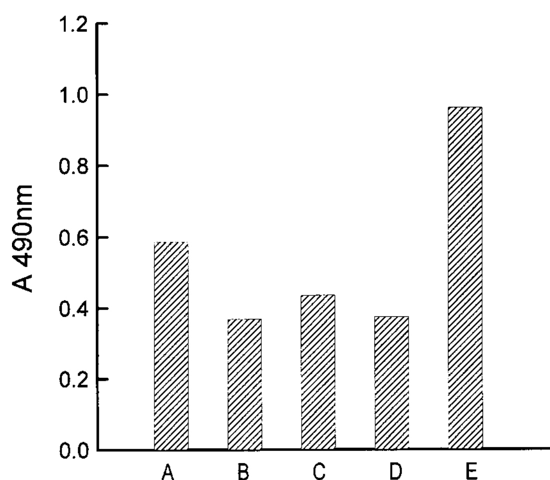

Figure 1 ELISA detection of the binding activity of phage scFv fragments with ASNase

The binding of phage scFv fragments prepared from colonies 10 (A), 24 (B), 46 (C) and 97 (D) to ASNase was detected by ELISA. HRP-conjugated anti-M13 antibodies were used to recognize the phage scFv fragments. E, binding activity of HRP-conjugated anti-M13 antibodies to M13 phage (positive control).

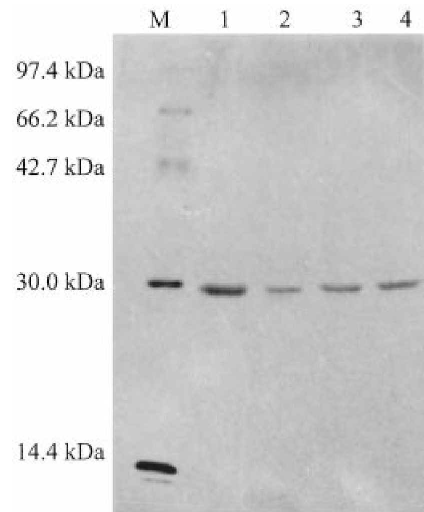

Figure 2 SDS/PAGE (12% gel) analysis of the expression of soluble scFv fragments

Lane 1, protein molecular-mass marker; lanes 2–5, purified soluble scFv fragments nos. 10, 24, 46 and 97 respectively.

Table 1 Stability of ASNase–scFv complexes

ASNase (5 units, free or bound with scFv) was incubated at 4 °C in 2 ml of 10 mM borate buffer (pH 8.3) overnight. The molar ratio of scFv to ASNase was 10:1. The following day, all the samples were incubated at 37 °C and some were treated with 1 unit of trypsin. Enzyme activity was assayed according to Peterson [22].

	Fraction of initial activity (%)				
Time …	10 min	15 min	20 min	25 min	30 min
ASNase	98	95	94	92	92
ASNase–scFv 10	98	96	96	94	94
ASNase–scFv 24	100	98	98	96	95
ASNase–scFv 46	98	98	98	95	95
ASNase–scFv 97	100	98	96	94	94
ASNase+trypsin	45	32	18	7	0
ASNase–scFv 10+trypsin	48	36	20	11	5
ASNase–scFv 24+trypsin	52	40	29	19	12
ASNase–scFv 46+trypsin	90	86	78	73	70
ASNase–scFv 97+trypsin	51	39	27	13	8

Selecting scFv fragments to protect ASNase against trypsin

The protective effect of specific scFv fragments on ASNase was assessed by determining the residual enzyme activity of different ASNase–scFv complexes after treatment with trypsin. As shown in Table 1, fragment scFv46 could protect the enzyme against trypsin efficiently and the residual enzyme activity was about 70%, whereas the other three scFv

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  1 ATGGCCCAGG TGCAGCTGGT GCAGTCTGGG GCTGAGGTGA AGAAGCCTGG GGCCTCAGTG
 61 AAGGTTTCCT GCAAGGCTTC TGGATACACC TTCACTAGCT ATGCTATGCA TTGGGTGCGC
121 CAGGCCCCCG GACAAAGGCT TGAGTGGATG GGATGGATCA ACGCTGGCAA TGGTAACACA
181 AAATATTCAC AGAAGTTCCA GGGCAGAGTC ACCATTACCA GGGACACATC CGCGAGCACA
241 GCCTACATGG AGCTGAGCAG CCTGAGATCT GAAGACACGG CCGTGTATTA CTGTGCAAGG
301 TTGACGCGTA ATAAGTTTAA GTCGCGTGGT CATTGGGGCC AAGGTACCCT GGTCACCGTG
          ↓                                                   ↓
361 TCGAGAGGTG GAGGCGGTTC AGGCGGAGGT GGCTCTGGCG GTGGCGGATC GTCTGAGCTG
421 ACTCAGGACC CTGCTGTGTC TGTGGCCTTG GGACAGACAG TCAGGATCAC ATGCCAAGGA
481 GACAGCCTCA GAAGCTATTA TGCAAGCTGG TACCAGCAGA AGCCAGGACA GGCCCCTGTC
541 ATTGTCATCT ATGGTAAAAA CAACCGGCCC TCAGGGATCC CAGACCGATT CTCTGGCTCC
601 AGCTCAGGAA ACACAGCTTC CTTGACCATC ACTGGGGCTC AGGCGGAAGA TGAGGCTGAC
661 TATTACTGTA ACTCCCGGGA CAGCAGTGGT AACCATGTGG TATTCGGCGG AGGGACCAAG
                   ↓
721 CTGACCGTCC TAGGTGCGGC CGCAGGTGCG CCGGTGCCGT ATCCGGATCC GCTGGAACCG
                                            E -tag
781 CGTGCCGCAT AG
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Figure 3 DNA sequence of the *scFv46* gene

The DNA fragments of bp 1–366, 367–411 and 412–735 encoded heavy-chain, linker and light-chain domains of the scFv46 fragment respectively (shown by arrows). The DNA sequence encoding the E-tag peptide from vector pCANTAB 5E is underlined.

fragments had little protective effect on ASNase against inactivation by trypsin. This indicated that the scFv46 fragment could block the trypsin cleavage site on the surface of the ASNase molecule.

The four specific scFv fragments did not directly influence the enzyme activity of ASNase. This demonstrated that the four scFv fragments were non-inhibitory antibodies to ASNase. This may be explained by a combination of factors: (i) the binding sites for the antibody are located on the surface of the enzyme molecule, whereas the active sites of the enzyme are located in the inner part of the enzyme molecule [19]; (ii) the low-molecular-mass substrate (L-asparagine) is unsusceptible to steric interference caused by antibodies.

Sequencing, cloning and high-level expression of the *scFv46* gene

The DNA sequence of the *scFv46* gene was analysed as shown in Figure 3, which consists of 735 bp encoding 245 amino acid residues (heavy chain, 122 amino acids; linker, 15 amino acids; and light chain, 108 amino acids). Since the expression level of the soluble scFv fragment was low, the *scFv* gene needed to be amplified by PCR and cloned to a high-level expression vector for further research. The *scFv46* gene was cloned into *Nde*I and *Hin*dIII sites of the expression vector pET21a and the resulting plasmid, designated pET-scFv46, was identified by double restriction-endonuclease digestion (Figure 4). *E. coli* BL21 (DE3) was transformed with the recombinant plasmid pET-scFv46 and protein expression was induced in Luria–Bertani medium by addition of IPTG (1 mM) at a cell density with $A_{600} = 0.5$. A significant amount of foreign protein was observed 4 h after IPTG induction (Figure 5, lane 4). The mobility pattern of the protein was as expected (scFv46, ≈ 28 kDa). Furthermore, the highly expressed protein was confirmed to be the scFv46 fragment by Western blotting (Figure 5, lane 5). The yield of the scFv46 fragment accounted for about 45% of the total bacterial protein, estimated by TLC scanning (dual-wavelength TLC scanner CS-930). However, the scFv46 fragment was found in inclusion bodies. After isolation, renaturation and purification (Figure 6, lanes 4–6), about 25 mg of the scFv46 fragment was obtained from 500 ml of culture, with an overall recovery of about 22%.

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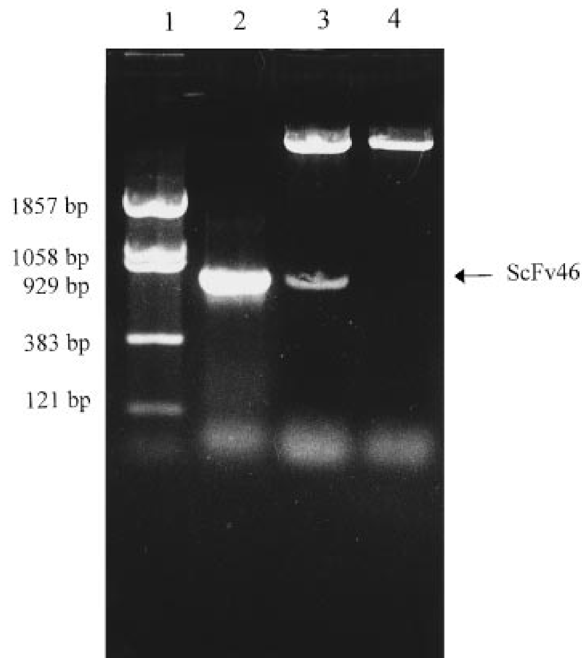

Figure 4 Agarose-gel electrophoresis of the recombinant plasmid pET-scFv46

Lane 1, DNA molecular-mass marker pBR322/*Bst*NI. Lane 2, the PCR product of *scFv46* (804 bp). Lane 3, the plasmid pET-scFv46 digested by *Nde*I and *Hin*dIII. Lane 4, the vector pET-21a digested by *Hin*dIII.

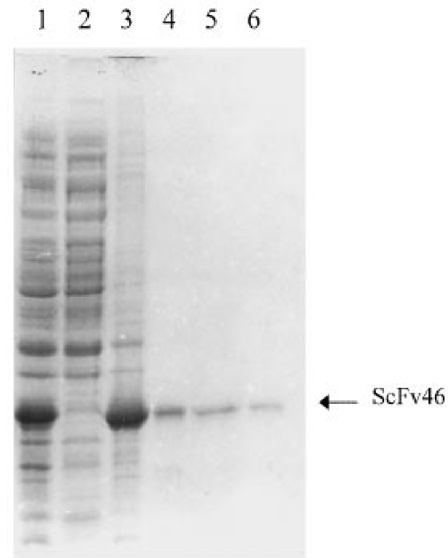

Figure 6 Purification of scFv46

Aliquots from the purification of scFv46 were analysed on SDS/PAGE (12% gel). The recombinant bacteria BL21 (DE3)/pET-scFv46 were harvested and broken by sonication. The crude homogenate (lane 1) was centrifuged at 12000 *g* for 10 min to obtain the supernatant (lane 2) and sediment (lane 3). Lane 4, inclusion bodies of scFv46 isolated from adherent impurities by 10 mM Tris/HCl (pH 8.0) buffer (containing 0.1 M NaCl, 1 mM EDTA and 1% Triton X-100) and urea wash. Lane 5, denatured and renatured scFv fragment. Lane 6, elution fraction from Sephadex G-75.

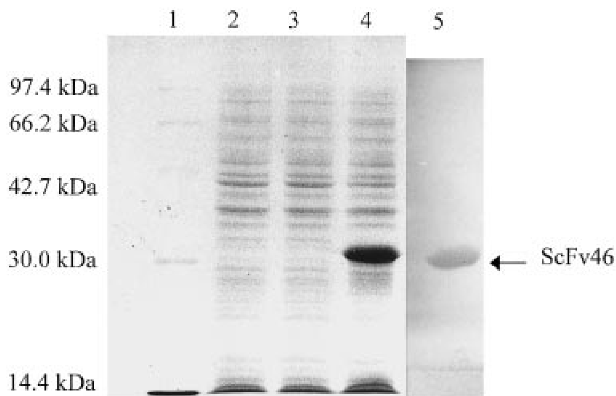

Figure 5 Expression of scFv46 in *E. coli*

Lane 1, protein molecular-mass marker. Lane 2, proteins from host *E. coli* BL21 (DE3). Lane 3, proteins from un-induced transformant. Lane 4, proteins from transformant induced by IPTG. The cells were resuspended in PBS, mixed with an equal volume of 2 × protein sample buffer and heated at 95 °C for 5 min. The samples were analysed on SDS/PAGE (12% gel) [23] and detected by Coomassie Brilliant Blue R-250 staining. Lane 5, proteins from lane 4 were transferred on to nitrocellulose membrane and probed with anti-E-tag antibody (8 μg/ml) [24].

Confirmation of the protective effect of the scFv46 fragment by SDS/PAGE

The scFv46 fragment recovered from inclusion bodies was used to protect the ASNase. As shown in Figure 7, trypsin

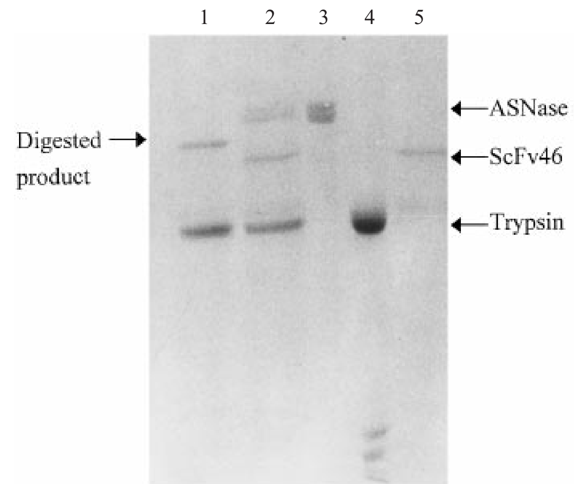

Figure 7 SDS/PAGE evidence for the protective effect of scFv46 on ASNase

Lane 1, 4 μg of free ASNase treated with trypsin. Lane 2, 4 μg of ASNase ($\approx$ 14.4 kDa) was bound with 9 μg of scFv46 fragment ($\approx$ 2.8 kDa; molar ratio of antibody to enzyme, 10:1) and treated with trypsin. Lane 3, free ASNase (4 μg). Lane 4, trypsin (2 μg). Lane 5, scFv46 fragments (9 μg). Trypsin-treated samples were prepared as followed: 4 μg of ASNase (free or mixed with 9 μg of scFv46) was incubated in 10 mM sodium borate buffer, pH 8.3, overnight at 4 °C. Then the samples (lanes 1 and 2) were treated with 5 μg of trypsin (40 units) for 30 min at 37 °C. All the samples were analysed on SDS/PAGE (13.5% gel) and detected by Coomassie Brilliant Blue staining.

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reduced the apparent molecular mass of ASNase monomers by 3–4 kDa to ≈ 31 kDa (Figure 7, lane 1), which was consistent with the report in [6] that cleavage of trypsin at lysine-29 results in loss of an N-terminal peptide with a calculated molecular mass of 2.647 kDa. It was also shown that scFv46 fragments could protect ASNase against trypsin cleavage (Figure 7, lane 2). This indicates that the scFv46 fragment blocks the trypsin cleavage site on the surface of ASNase molecule.

The effect of molar ratio, incubation temperature and time on protection

The molar ratio of scFv to enzyme, the temperature and the time of incubation for the binding of scFv and enzyme influenced the protective action of scFv46 on ASNase. After the treatment with trypsin, the residual activity of the complex (antibody–enzyme, molar ratio of 6:1) was about 66%, which was close to the residual activity of about 70% when the molar ratio was 10:1. Therefore, the molar ratio of 6:1 was employed. Incubation required 4 h, and the optimum incubation temperature was 37 °C. Under these optimum conditions, the residual activity of the ASNase–scFv46 complex was up to about 78% after the complex was treated with trypsin for 30 min at 37 °C.

Discussion

In this study, a scFv46 fragment enhancing ASNase resistance to trypsin proteolysis was selected from a phage-display scFv fragment library. After the treatment with trypsin, the residual activity of the ASNase–scFv46 complex was about 78% under optimum conditions. We found that the scFv fragment selected from the phage antibody library had a similar protective effect on ASNase as the monoclonal antibodies [6] prepared by hybridoma technology.

The new approach detailed in this paper applied phage-display technology and DNA recombinant technology, which avoided hybridoma technology [20] and simplified the process of purification. The approach can be summarized as follows. First, with a target protein as antigen, specific phage scFv fragments should be selected from a phage antibody library. Secondly, the selected scFv fragments should be expressed as a soluble fraction. Thirdly, the protective scFv fragments of the target protein should be selected. Fourthly, the gene of the protective scFv fragment may be cloned and highly expressed using DNA recombinant technology.

Successful selection of a protective scFv fragment for a target protein from a phage antibody library is dependent on library size and diversity, so a large library is necessary to select a protective antibody for a target protein. The phage scFv fragment library used in this study consists of about 10^8 members, which is sufficient to select specific antibodies for different antigens. Furthermore, phage-display antibody libraries containing 6.7×10^9 members have recently been constructed using a new strategy [21].

scFv fragments are of special interest as protecting agents. First, scFv fragments could be expressed in prokaryotic and eukaryotic systems since the single-gene design of scFv facilitates intracellular expression. Secondly, scFv fragments can be fused with their particular antigen via a flexible peptide linker to form a fusion protein. Thus target protein and protective scFv can be produced by a single organism. We are now working on the construction of two forms of fusion genes for the scFv46 and ASNase genes and the expression of the fusion proteins to study their stability and activity.

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