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Single-Chain Fv Fragment of Catalytic Antibody 4f4f with Glycosidase Activity: Design, Expression, and Purification

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Abstract Constructs, encoding a single-chain variable fragment of a catalytic antibody 4f4f (scFv-4f4f) with glycosidase activity, were made by combining the coding sequences for the heavy and light chain variable domains with a sequence encoding a linker (GGGGS). Using three different plasmid systems, single-chain antibodies were expressed separately in *Escherichia coli*, demonstrating significant differences in the expression level and amounts in soluble form of the recombinant protein. The protein expression from pET3a-scFv-4f4f was up to 20% of the total soluble proteins and, more importantly, the proteins were mostly found in a soluble form. An SDS-PAGE analysis of the purified single-chain proteins, yielding higher than 5 mg from a 1-l culture, showed a single band corresponding to its molecular weight of 29,100. A preliminary study shows that the expressed scFv-4f4f is catalytically active. The catalytic parameters for the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside by scFv-4f4f are being investigated.

Key words: Single-chain Fv fragment, catalytic antibody, engineering, expresssion

Antibodies with an affinity for transition states in enzyme reactions have been shown to catalyze a wide variety of reactions since the idea was first postulated by Jencks [14], ranging from efficient esterase reactions to reactions difficult to be achieved by existing enzymes [16, 23, 24]. Among them, the antibodies of selective glycosidic bond cleavages were given relatively less attention in light of recent advances in glycoconjugate chemistry and glycobiology [20]. Catalytic antibodies with glycosidase activity have been developed mostly through hapten design. Early efforts focused on the positively charged or conformationally restricted analogues of oxocarbenium ion intermediates, which yielded a rate acceleration with the order of 10^2–10^3 [22, 27]. *In vitro* immunization using the structural and electronic features of carrier-free haptens further produced antibodies which catalyze the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside with a rate increase (k_{cat}/k_{uncat}) of about 10^4 [30]. The more recent design of a chair-like transition state analog derived from a natural glycosidase inhibitor, nojirimycin, resulted in an antibody, 4f4f, that catalyzes the hydrolysis with a rate enhancement of about 10^5 [32]. The kinetic effects of these antibodies elicited in response to various transition state analog haptens can be best illustrated by the three-dimensional structural features of the variable region and complementary combining site. Nevertheless, three-dimensional structural studies have been limited by the availability of sufficient amounts of antibodies and by difficulties in expressing them in soluble forms [25]. An expression system of an artificially constructed combination of heavy and light chains was reported to provide high expression levels and improve the genetic approaches of Fab fragments [28].

Recently, single-chain variable fragments (scFv), comprising V_H and V_L domains of monoclonal antibodies connected by a flexible linker to stabilize their association, have been developed successfully, yielding the smallest antibody fragment containing a complete antigen binding site [3, 11, 15]. The first catalytic scFv construction was reported by Gibbs *et al.* [6] to show that the recombinant protein had the same catalytic parameters as the parent antibody (Ab) from which it was derived, although they were expressed as inclusion bodies. In addition, scFvs exhibited an improved cellular penetration compared with Ab or an antigen-binding fragment (Fab), making them useful for targeted cancer therapy [17, 21].

Among the glycosidase antibodies elicited against designed haptens, which revealed enhanced kinetic effects,

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the antibody 4f4f binding to a nitrobenzyl derivative of nojirimycin was the most efficient. To determine the catalytic mechanism and kinetic enhancement of 4f4f and its related antibodies, structural studies of their fragments with a complete combining site are essential. The crystallization of Fab fragments often suffers from the difficulty in obtaining homogeneous proteins after digestion with papain. The expression of soluble single-chain antibodies in a bacterial system is a prerequisite to attain a suitable homogeneity and sufficient amount for crystallization. This report describes the construction, expression, and purification of a single-chain variable fragment of 4f4f with a (GGGGS) linker, scFv-4f4f, which is catalytically active.

Isopropyl-β-D-thiogalactoside (IPTG) and ampicillin were purchased from Boehringer Mannheim (Mannheim, Germany). Restriction endonucleases and T4 DNA ligases were purchased from TaKaRa Biotechnology (Shiga, Japan). The oligonucleotide primers were synthesized using an Applied Biosystems DNA synthesizer (Foster, CA, U.S.A.) at Bioneer Co (Seoul, Korea). DEAE and phenyl Sepharose resins and a Superdex 200 HR 10/30 column were purchased from Amersham Pharmacia Biotech. (Uppsala, Sweden). *E. coli* strains DH5α and BL21 (DE3) were used for the transformation and expression of the scFv protein. pET3a, pMAL-c2, and pGEX-4T vectors were purchased from Novagen (Madison, U.S.A.), New England Biolab (Beverly, U.S.A), and Pharmacia Biotech. (Uppsala, Sweden), respectively.

The PCR amplification was carried out for 30 cycles in a Fine PCR (Robotics, U.S.A.). An initial cycle of 94°C for 2 min, 50°C for 1 min, and 72°C for 2 min was followed by 30 cycles of 72°C for 2 min, 92°C for 1 min, and 50°C for 1 min. A final single incubation at 72°C for 10 min yielded PCR products which were purified by phenol/chloroform extraction and ethanol precipitation. The variable domain sequences of 4f4f were amplified by a two-step PCR using linker primers. The first PCR was carried out using primers, V_H-R and V_H-linker-F for the V_H domain and V_L-F and V_L-linker-R for the V_L domain (Table 1). The first PCR products were used as templates for the second PCR with primers, V_L-F and V_H-R. The second products were then digested with *Bam*H1 and ligated into the expression vector pET3a which included a T7 promoter designed to express scFv in the orientation of V_L-linker-V_H (Fig. 1). The ligation mixture was used to transform *E. coli*

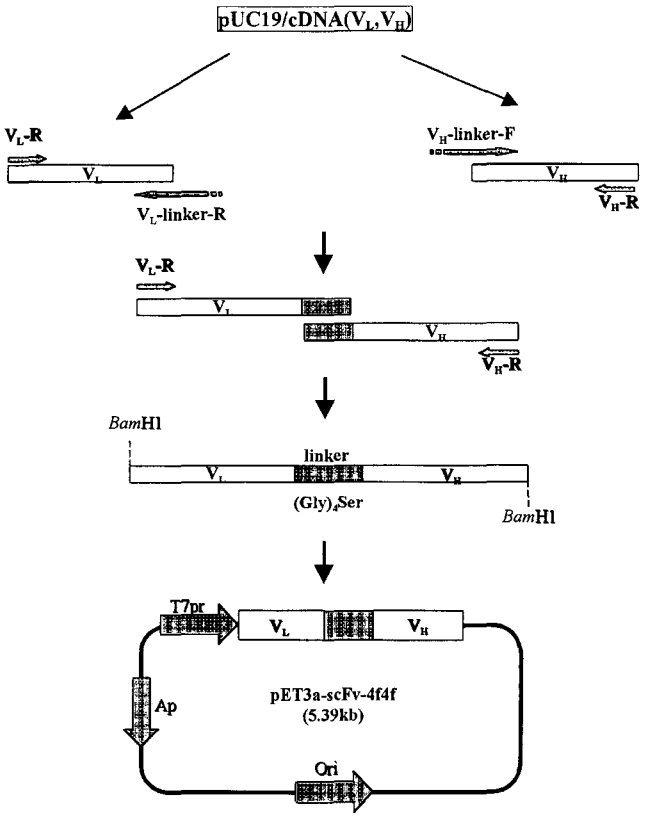

Fig. 1. Construction of pET3a-scFv-4f4f.
V_L, V_H, and linker are indicated; The primers used for the first and the second PCR are shown with shaded arrows. *Bam*H1 sites at the end of the N-terminus of V_L and C-terminus of V_H were used for ligation into pET3a. T7pr and Ap represent T7 polymerase and ampicillin resistance, respectively.

BL21(DE3) cells, which carry a copy of the T7 RNA polymerase gene under *lacUV5* control, and protein expression was induced by the addition of IPTG [26]. Additional pairs of different vectors and transformants, pMAL-c2 and pGEX-4T with DH5α and BL21(DES), respectively, were also used with the same procedure as described above, to produce different expression vector systems.

The transformed bacteria were grown at 37°C and scFv protein expression was induced in the presence of IPTG at 28°C for 4 h. Proteins from the cultured cells were purified using ammonium sulfate precipitation, DEAE Sepharose ion-exchange and phenyl Sepharose hydrophobic chromatography, and Superdex 200 gel filtration FPLC. The expression

Table 1. Oligonucleotide primer sets used for cloning of immunoglobulin genes.

V_L-F	5'-GCAGGATCCGACATTGTGCTGACC-3'
V_L-R	5'-CGCGGATCCTGATTTCCAGCTTGG-3'
V_H-F	5'-CAAGGATCCCAGGTCCAGCTGCAGG-3'
V_H-R	5'-GACGGATCCTGAGGAGACGGTGACC-3'
V_L-linker-R	5'-GGAGCCGCCGCCGCCAGAACCACCACCACCTGATTTCCAGCTTGG-3'
V_H-linker-F	5'-GGCGGCGGCGGCTCCGGTGGTGGTGGTTCTCAGGTCCAGCTGG-3'

level and purity of the recombinant proteins produced were checked by SDS-PAGE.

The kinetic parameters on *p*-nitrophenyl-β-D-glucopyranoside were measured under conditions where less than 1% of the substrate was hydrolyzed by low concentrations of scFv (about 0.1 mg/ml), as described by Yu *et al.* [31]. The assay mixture of scFv-4f4f and its substrate was incubated at 37°C and the product hydrolysis was determined by injecting onto a C_{18} column HPLC and subsequent monitoring at 315 nm.

The transformed *E.coli* cells with different plasmids as described above were grown separately at 37°C and the expression of scFv proteins was induced at 28°C by the addition of IPTG. Their respective expression levels were determined by SDS-PAGE using 12% gels. It was found that the scFv fusion proteins from pGEX-4T were expressed mostly as inclusion bodies and only a small fraction of those from pMAL-c2 was found in a soluble form. In contrast, the recombinant scFv protein from pET3a was found mainly in soluble forms and its expression level was the highest in the transformed *E. coli*. Therefore, pET3a was superior to the pGEX-4T or pMAL-c2 expression plasmid in recombinant protein expression levels and, more importantly, the amount of scFv-4f4f in soluble form (data not shown). The protein expression from pET3a-scFv-4f4f increased continuously for 4 h after IPTG induction, remained constant for an additional 2 h, and thereafter decreased. ScFv-4f4f was expressed in amounts up to 20% of the total soluble proteins depending on the cell growth conditions. The expression of scFv in the transformed cells containing the pET3a-scFv-4f4f plasmid is shown in Fig. 2 (lane 2).

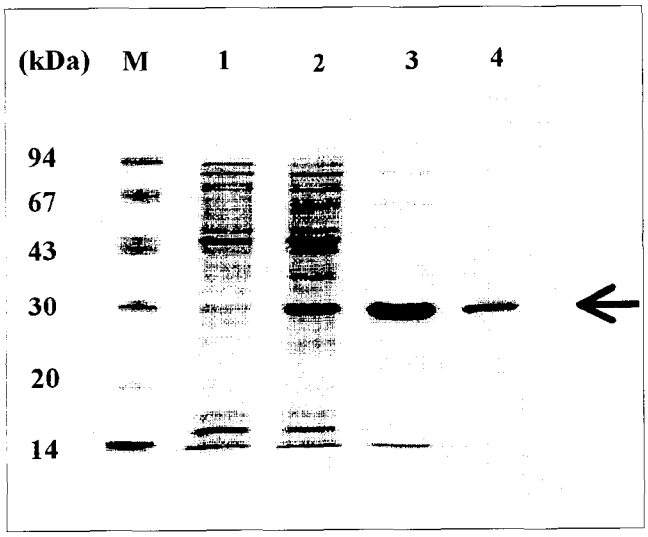

Fig. 2. Expression and purification of pET3a-scFv-4f4f recombinant proteins monitored by SDS-PAGE on 12% gels using Coomassie Blue staining.

The purified scFv-4f4f recombinant protein is indicated by an arrow. Lane M, molecular weight markers; lane 1, uninduced soluble fraction of cell lysates; lane 2, soluble fraction after IPTG induction; lane 3, fraction after DEAE-ion-exchange chromatography; lane 4, fraction after Superdex 200 FPLC.

The purification of scFv-4f4f recombinant proteins was carried out using ammonium sulfate precipitation, ion-exchange and hydrophobic chromatography, and gel filtration FPLC in sequence. DEAE-ion-exchange chromatography using a 0–0.5 M sodium chloride gradient was found to be the most effective step during purification. SDS-PAGE analyses of the purified scFv showed a single band, demonstrating its molecular weight of 29,100 (Fig. 2, lane 4). The final yield of scFv-4f4f expression was higher than 5 mg/l of culture. Preliminary examination showed the catalytic activity of scFv-4f4f to be similar to that of the parent antibody (Ab) (Fig. 3). The kinetic parameters of the parent Ab were k_{cat} of 0.168 h^{-1} and K_m of 22 μM, affording a value of specificity constant of 7.6×10^3 $M^{-1}h^{-1}$ [31]. There have been a number of reports showing that recombinant scFv or Fab fragments usually have lower affinities, presumably due to incorrect folding, and also lower catalytic activities, closely related to the extent of the conformation stabilization, than their native and intact antibody (Ab) [15, 18, 19]. Proteolytically stable and flexible linker peptides are needed to join protein domains. Inspection results of the molecular model constructed for Fv fragments were reported to show that the C-terminus of the V_H domain and the N-terminus of the V_L domain contained sequences flexible enough to serve as part of the linker of only two residues [5, 8]. The presence of very short linkers may result in a tendency to form dimers and multimers. With the ELISA experiments, however, the shorter linkers were shown to exhibit significantly higher hapten binding activity than the original fragment [1, 12]. On the other hand, the conformational rigidity of the linker rendered the peptide bond less susceptible to proteases [29]. The 5 amino acids peptide linker is rich in the small

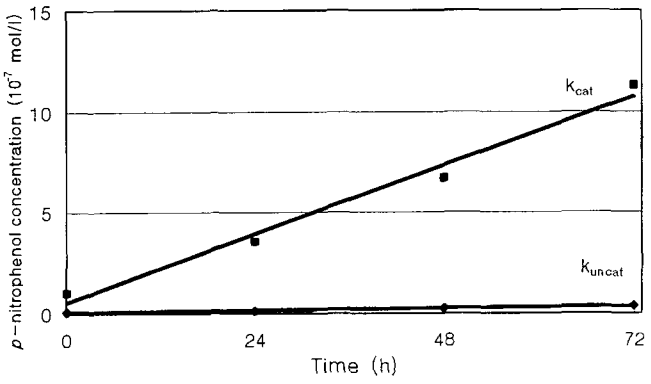

Fig. 3. Glycosidase activity of scFv-4f4f at 1 mM *p*-nitrophenyl-β-D-glucopyranoside.

The slope, k_{cat}, shows *p*-nitrophenol cleaved over time as measured on HPLC at 315 nm. The background hydrolysis represented as k_{uncat} was monitored with the substrate only.

glycine and polar serine residues, providing necessary flexibility and allowing hydrogen bonds to solvent molecules on the surface. These properties are believed to prevent the peptide linker from penetrating into the hydrophobic interface formed in an association of the protein domains [14]. The (GGGGS) interdomain linker is reported to be fully hydrated and fluctuating in configuration [2]. Accordingly, the 5 amino acids peptide linker is believed to confer the conformational flexibility of scFv-4f4f, yielding a catalytically active single-chain fragment.

Previously developed antibodies with glycosidase activity (AA series) exhibited a rate enhancement of the order of about 10^2- 10^3 [27]. One antibody, AA71.17, showed enzyme kinetics with an apparent second-order rate constant of 2.3×10^3 M^{-1} h^{-1} [30]. The refinement of hapten design improved the acceleration of the rate in the order of up to 10^5. One of these antibodies, 4f4f, demonstrated high reactivity and substrate affinity. Since the pseudo-first-order rate constant for the water-catalyzed reaction (k_{H_2O}) is 1.3×10^{-8} min^{-1}, the value of k_{cat}/k_{H_2O} for the 4f4f-catalyzed reaction is 2.2×10^5 [31]. A monoclinic crystal of the Fab fragment of AA71.17 was obtained, which could diffract to 2.6 Å resolution (unpublished results). However, the crystallization of the Fab fragment of 4f4f was not successful, partly due to the limited supply of monoclonal antibodies from *in vitro* immunization and partly due to the inhomogeneity of Fab fragments resulting from papain digestion. With the successful expression of catalytically active scFv, it was possible to obtain a sufficient amount for the crystallization of homogeneous scFv. In addition, this system should facilitate further structural studies, and consequently, the two series of glycosidase antibodies can be compared. β-glucosidases in general have catalytic constants of the order of about 10^5 h^{-1}, yet have affinity constants of the order of 10^{-3} M [7, 9, 10], approximately 100-fold weaker than those of glycosidase antibodies. Carbohydrate antigens rarely have affinity constants for antibodies that significantly exceed 10^{-6} M [13]. Recent structural studies of catalytic antibodies provide a clue as to how a hapten binding site can effect a given chemical transformation, revealing much about convergent evolution [4]. Despite significant structural differences in the combining site, the conformations of catalytic residues were found to be similar. One of the long-term goals of this research project is to find the differences in the catalytic efficiencies of glycosidase antibodies and to assess their utility as groundwork strategies for the development of antibodies with enhanced glycosidase activity. Mutational studies in hand will make mechanistic approaches possible. Furthermore, the structural role of the linker to improve hapten binding or perhaps conformational flexibility/ stabilization remains an area for further investigation.

In conclusion, the variable regions of the catalytic antibody 4f4f which catalyzes the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside were cloned and expressed in *E. coli* as Fv fragments. Among the different expression plasmids studied, it was possible to express and purify high yields of soluble recombinant scFv-4f4f which is catalytically active.

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