Letter to the Editor

Efficient Expression and Purification of Fc-fragment-binding Domain and Its Application to Immunoglobulin G Purification*

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The number of therapeutic monoclonal antibodies used in clinical trials has recently increased dramatically, leading to the development of optimized downstream purification processes[1]. Staphylococcal protein A (SPA), a cell-wall protein of *Staphylococcus aureus*, has been developed as a universal ligand for immunoglobulin G (IgG) purification because it binds specifically to the Fc portion of the IgG molecule of many mammals[2]. However, certain characteristics of SPA severely restrict the advancement of the antibody industry. This ligand is expensive because it must be highly purified from *S. aureus*[3]; it may cause immunogenic responses in patients should it contaminate the product because SPA itself has proven pathogenic[4]; and it tends to lose activity under harsh elution, washing, and sterilization conditions. Therefore, a novel purification tool is quite urgently required for the large cell cultures used in antibody production[1].

The IgG-Fc-fragment-binding domain, also designated the 'Z domain', is derived from the B domain of SPA. Previous research has shown that two Z domains joined end to end (designated the 'ZZ domain') have a similar molar binding capacity for IgG as native SPA[5]. Many studies have reported the use of the ZZ domain as a fusion partner[6-7] or immunoassay agent[8-9], but very little research has focused on its application in the affinity purification of IgG. In this study, the ZZ domain was produced and immobilized on a matrix to generate a ZZ-sepharose affinity resin, and the performance of this resin in IgG purification was examined.

Firstly, an expression vector for the ZZ domain was constructed. A single copy of the Z sequence was generated with a recombinant PCR method using four primers (P1, P2, P3, and P4; Table 1), which amplified the whole Z domain (GenBank accession no. M74186). The Z fragment was then amplified from the recombinant PCR product using the primer pair P9 and P11 (Table 1). The resulting fragment was digested with *Hin*dIII and *Xho*I and inserted into the corresponding sites on the pET32a vector (Novagen, Merck, Germany) to produce pET32a-Z. An additional Z fragment amplified from pET32a-Z with the primer pair P7 and P8 (Table 1) was inserted into the *Kpn*I and *Hin*dIII sites of pET32a-Z to produce pET32a-ZZ (Figure 1A). The ZZ fragment was thus expressed under the control of the phage T7 promoter as a thioredoxin carrier fusion protein with a hexahistidine tag (His tag) to ensure its high-level soluble expression and to simplify the purification process (Figure 1A).

Table 1. Primers Used to Construct the pET32a-ZZ Expression Vector. Restriction Enzyme Recognition Sites Are Underlined and the Termination Codon Is Boxed

Primers	Nucleotide Sequence	Use
P1	5′-AATTCTGCATCTTCCGAACCTGAACGAAGAACAGC GTAACGCATTCATTCAGAGCCTG-3′ (42-99)	P1, P2, P3, and P4 cover the full-length sequence of the Z gene (the figures in parentheses show the locations the primers in the Z sequence)
P2	5′-AGCCAGAAGGTTTGCAGACTGGGACGGGTCGTCT TTCAGGCTCTGAATGAATGC-3′ (82-135)	
P3	5′-GACAACAAATTTAACAAAGAACAGCAGAACGCAT TCTACGAAATTCTGCATCTTCCGA-3′ (1-58)	
P4	5′-AACTTTCGGTGCCTGAGCGTCGTTTAGCTTTTTCG CTTCAGCCAGAAGGTTTGCAG-3′ (119-174)	
P9	5′-CCCAAGCTTGACAACAAATTTAACA-3′ (*Hin*dIII)	Construction of pET32a-Z
P11	5′-AAACTCGAGTTAAACTTTCGGTGCC-3′ (*Xho*I)	
P7	5′-CCCGGTACCGACAACAAATTTAACA-3′ (*Kpn*I)	Construction of pET32a-ZZ
P8	5′-AAAAAGCTTAACTTTCGGTGCCTGA-3′ (*Hin*dIII)	

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Secondly, the ZZ domain was expressed in and purified from *Escherichia coli* cells. Competent *E. coli* BL21 (DE3) cells produced with $CaCl_2$ treatment were transformed with the expression vector pET32a-ZZ. Single colonies were selected from the plates and the expression of the exogenous fragment was induced with lactose (5 mmol/L). The cells were harvested by centrifugation and the cell pellet was resuspended to a concentration of 0.067 g/mL in buffer A [50 mmol/L Tris-HCl (pH 7.9), 50 mmol/L NaCl, 0.5 mmol/L EDTA, 5% glycerol] and then disrupted by sonication. Following centrifugation at 12 470 × g for 10 min at 4 °C, the supernatant was loaded onto a nickel chelating column (GE Healthcare, Shanghai, China) previously equilibrated with 20 mmol/L phosphate-buffered saline (PBS, pH 7.4) containing 30 mmol/L imidazole. The column was washed with 10 volumes of equilibration buffer and the adsorbed protein was eluted with 100 mmol/L imidazole in 20 mmol/L PBS (pH 7.4). The fractions containing the ZZ protein, as ascertained with SDS-PAGE, were pooled and concentrated with an ultrafiltration device (UFC900396 Amicon® Ultra-15 Centifugal Filter Concentrator, Millipore, Shanghai, China). The concentrated solution was then loaded onto a PD-10 desalting column (GE Healthcare) to buffer exchange the ZZ protein with a 10 mmol/L $(NH_4)_2CO_3$ solution. The fractions containing the pure ZZ protein were pooled and lyophilized for long-term storage at -70 °C. The protein concentration was determined with the Bradford method using a Braford kit (KeyGen Biotech Co., Ltd, Nanjing, China). The protein purity was analyzed with SDS-PAGE under reducing conditions using a 15% separation gel and a 5% stacking gel (Figure 1B).

According to the SDS-PAGE analysis (Figure 1B), abundant recombinant ZZ protein was expressed in *E. coli* cells, with an apparent molecular weight of 28 kDa. The observed molecular weight was consistent with the theoretical value. The recombinant ZZ protein accounted for approximately 47.4% of the total bacterial protein, determined with Bandscan 5.0, and almost all was present in the supernatant after the cells were disrupted by sonication (Figure 1B). The recombinant ZZ protein was successfully purified with Ni-Sepharose resin. The final purity was estimated to be 98.3% (Figure 1B), and the yield was 125 mg of recombinant ZZ protein from 1 L of cultured cells.

Thirdly, the binding activity of the ZZ peptide was confirmed with an immunoblotting analysis and enzyme-linked immunosorbent assay (ELISA). After SDS-PAGE, the protein was transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membrane (0.45 μm) at 60 mA for 2 h. After the membrane was blocked with 3% bovine serum albumin (BSA; Bio Basic Inc. Shanghai, China) in 20 mmol/L PBS (pH 7.4) for 2 h, it was incubated for 1 h with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Jackson Immuno Research, Shanghai, China) diluted 1:5 000. The membrane was then washed five times with PBS and the HRP signal was visualized with diaminobenzidine (Bio Basic Inc.). The recombinant ZZ protein was thus detected as a distinct band of color (Figure 1C). These results demonstrated that the thioredoxin and His tag did not affect the binding activity of the ZZ domain.

An ELISA was performed to confirm the IgG-binding capacity of the recombinant ZZ protein. Serial dilutions of the ZZ fusion protein were coated onto a 96-well microplate and incubated overnight at 4 °C. After the microplate was washed three times with PBS (pH 7.4), any unoccupied sites on it were blocked with 200 μL of 3% BSA in PBS for 2 h. After three washes, the plate was incubated with 100 μL of 1:5 000-diluted HRP-conjugated rabbit anti-goat IgG (Jackson Immuno Research, Shanghai, China) for 1 h at 37 °C. After three washes, the HRP was reacted with TMB substrate (100 μL/well) for 10 min, after which the reaction was stopped with 2 mol/L H_2SO_4 (50 μL/well) and the absorbance was read at 450 nm. As shown in Figure 2A, the recombinant ZZ protein bound specifically to rabbit anti-goat IgG.

Fourthly, the ZZ domain was immobilized on a Sepharose matrix. The recombinant ZZ domain was coupled to the *N*-hydroxysuccinimide (NHS)-activated Sepharose Fast Flow matrix (GE Healthcare), according to the manufacturer's protocol. The purified recombinant ZZ (10 mg) was dissolved in 1 mL of coupling buffer solution [0.2 mol/L $NaHCO_3$, 0.5 mol/L NaCl (pH 8.3)]. The ZZ ligand solution was then added to Sepharose beads and the suspension was gently agitated overnight at 4 °C. The suspension was then filtered and mixed with 1 mL of 0.1 mol/L Tris-HCl (pH 8.5) for 2 h at 4 °C to block any unreacted NHS groups. This was followed by several washing cycles, each comprising 3 mL of acetate buffer (pH 4.0) followed by 3 mL of Tris-HCl buffer (pH 8.5). The concentrations of the recombinant ZZ protein before and after the coupling reaction were measured. The ligand density was calculated to be 2.08 μmol/mL of drained medium.

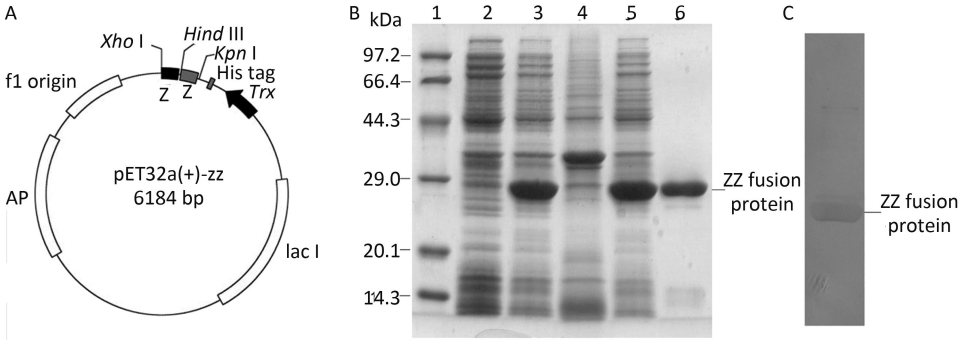

Figure 1. (A) Plasmid map of the pET32a-ZZ expression vector. Z, Z gene; Trx, thioredoxin; His tag, hexahistidine tag. (B) SDS-PAGE analysis of the expression and purification of the recombinant ZZ protein. Lane 1, molecular weight standards; lane 2, total protein of cells containing pET32a-ZZ before lactose induction; lane 3, total protein of cells containing pET32a-ZZ after lactose induction (fivefold dilution); lane 4, insoluble fraction after sonication (fivefold dilution); lane 5, supernatant after sonication (fivefold dilution); lane 6, recombinant ZZ protein purified by Ni^{2+} affinity chromatography. (C) Immunoblotting analysis of the recombinant ZZ protein. Total protein of cells containing pET32a-ZZ after lactose induction (fivefold dilution), resolved with SDS-PAGE and transferred to a PVDF membrane, then detected with an HRP-conjugated rabbit anti-goat antibody. The position of the ZZ fusion protein is indicated.

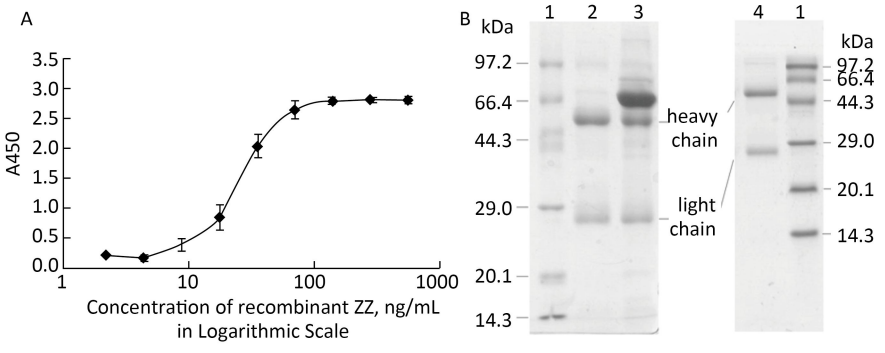

Figure 2. (A) ELISA of the recombinant ZZ protein. Serial dilutions of the recombinant ZZ protein were coated onto a 96-well microplate and then detected with an HRP-conjugated rabbit anti-goat antibody (n=3). A graph of the ELISA results for the recombinant ZZ protein; the concentration of the recombinant ZZ protein solution is shown on a logarithmic scale on the x axis and the corresponding absorbance is shown on the y axis. The concentrations of the recombinant ZZ protein solutions were as follows: 2.18, 4.36, 8.71, 17.42, 34.84, 69.69, 139.38, 278.75, and 557.50 ng/mL. (B) SDS-PAGE analysis of the proteins eluted from the ZZ-Sepharose column and a protein A-Sepharose column. Lane 1, molecular weight standards; lane 2, proteins eluted from the ZZ-Sepharose column; lane 3, IgG sample mixed with human serum; lane 4, proteins eluted from a commercial protein A-Sepharose column. The positions of the immunoglobulin heavy and light chain are indicated.

Finally, the ZZ-Sepharose resin was used to purify IgG from human serum. To prepare an affinity chromatography column, the ZZ-Sepharose resin was packed into a 100×6.6 mm i.d. glass column. The IgG sample was prepared by dissolving human IgG (Solarbio, Beijing, China) in PBS (pH 7.4) to a concentration of 2.0 mg/mL and mixing it with the same volume of human serum diluted 1:20. This mixture was then applied to the ZZ-Sepharose column. After the unbound material was eluted from the column with 10 column volumes of PBS (pH 7.4), the bound material was eluted with 0.1 mol/L

glycine (pH 2.7) and was immediately neutralized with a few drops of 1 mol/L Tris-HCl (pH 9.0) to prevent the denaturation of the immunoglobulin. As a comparison, a HiTrap Protein A HP column (GE Healthcare) was also used for IgG purification. Reduced SDS-PAGE of the proteins eluted from the ZZ-Sepharose column showed only two clear bands (Figure 2B), which were consistent with the IgG heavy chain (MW 50 kDa) and IgG light chain (MW 25 kDa). These results demonstrate that the ZZ-Sepharose column specifically binds IgG and can purify it from human serum. The IgG-binding capacity of ZZ-Sepharose was measured as 14.6 mg human IgG/mL of drained resin, which was similar to the capacity of the protein A-Sepharose column (-20 mg human IgG/mL medium).

In this study, the relatively low-antigenicity IgG-Fc-binding ZZ domain was used instead of protein A to make an affinity resin for IgG purification. This method provides a safe and useful strategy for IgG purification.

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AUTHOR CONTRIBUTIONS

LAO Xing Zhen, ZHOU Ya Li, and ZHENG Heng conceived and designed the experiments. LAO Xing Zhen and ZHOU Ya Li performed the experiments and analyzed the data. LAO Xing Zhen and ZHOU Ya Li wrote the paper.

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