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Single-Chain Fv with Fc Fragment of the Human IgG1 Tag: Construction, *Pichia pastoris* Expression and Antigen Binding Characterization[1]

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We report two expression vectors in *Pichia pastoris* that direct the synthesis of recombinant single chain antibody variable region (scFv), derived from anti–Z-DNA monoclonal antibody Z22. The first vector codes for a scFv fused to the Ig binding domain of staphylococcal Protein A. The second vector codes for the scFv fused to the Fc fragment of the human IgG1. The fusion partner simplified the détection and purification of the secreted protein. These constructs yielded high level expression of an scFv with specific binding activity toward a Z form of DNA, with binding activity comparable to that of the scFv molecule produced in an *Escherichia coli* expression system and the original monoclonal antibody.

Key words: antibody engineering, antibody fragment, antigen binding, *Pichia pastoris*, single-chain Fv.

The expression of recombinant antibodies and their fragments is an area of great biotechnological interest. The potential uses of recombinant antibodies in many areas of medicine drive the search for new alternative methods of large-scale production. Small antibody fragments such as single-chain Fv and Fab are promising for diagnosis and imaging due to their pharmacokinetical properties (*1*), while the whole antibody is still the choice for therapeutics. In spite of the extensive documentation on the production of antibody fragments in *Escherichia coli*, its use is being hampered by the difficulty of large-scale production of active products. Bacterial expression of antibody fragments has been widely reported but the observed levels of soluble active scFv or Fab depend greatly on the antibody structure (*2*, *3*). The use of strong promoters to drive the synthesis of insoluble cytoplasmatic proteins leads to very low yields of soluble products because refolding protocols are still empirical and hardly reproducible (*2*). To overcome these problems, eukaryotic expression vectors are preferred for the large-scale production of an antibody and its fragments. Recently, the use of lower eukaryotes such as the yeast *Pichia pastoris* has also been proposed (*4*, *5*). This methylotrophic fungus has been shown to overexpress many different recombinant proteins under control of the strong promoter AOX I. A few groups have reported the use of *P. pastoris* for expressing scFv (*6–8*). In this report we describe the modification of a *P. pastoris* expression vector that allows the single step purification of monomeric and dimeric anti–Z-DNA scFv.

The objective of this study was the overproduction of functional scFv molecules in a *P. pastoris* expression/secretion system. These vectors were derived from plasmid pPIC-9 (INVITROGEN), where the expression cassette is under the control of the strong AOX I promoter, and downstream of the α-mating type signal sequence (Fig. 1A). The multiple cloning site located downstream of the signal sequence was initially modified to introduce an *Sma*I site at the verge of the signal sequence processing site, generating the pCIP vector, to allow the cloning of the scFv fragment (Fig. 1B). To introduce these sites, two complementary oligonucleotides, TCGAGAAAAGAGAGGCTGAAGCCCGGGGTAG and AATTCTACCCCGGGCTTCAGCCTCTCTTTTC, were heat denatured and annealed by slow cooling down to room temperature in a warm 400 ml water bath. The annealed dimer possessed a *Xho*I stick end on one side and *Eco*RI on the other side. To obtain the pCIP vector, the dimer was ligated to the pPIC9 vector cut with both *Xho*I and *Eco*RI. The resulting plasmid, pCIP, retains the *Xho*I and *Eco*RI sites while a unique *Sma*I site was introduced at the signal peptide processing site (Fig. 1B).

The scFv fragment was obtained from an *E. coli* expression plasmid, pIg 16, and contains an Ig binding site of staphylococcal protein A as a C-terminal tag (scFv-SpA) (*9*). The scFv coding fragment was obtained by digestion of the pIg 16 plasmid with *Sma*I and *Eco*RI, which was introduced in the linear pCIP vector cut by means of the same double digestion. The resulting plasmid vector was named pPIg 16 (Fig. 1C).

The cDNA coding for the CH2 and CH3 domains of the human IgG 1 isotype was isolated from a human spleen cDNA library (GIBCO, BRL). Ten μg of plasmid DNA extracted from a pool of approximately 10^5 individual cDNA

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Abbreviations: AOX I, alcohol oxidase I; CH2 and CH3, constant domains 2 and 3 of IgG; ELISA, enzymatic immuno assay; Fab, antigen binding fragment; Fc, crystalline fragment; Fv, variable fragment; mAb, monoclonal antibody; scFv-SpA, single-chain Fv with staphylococcal protein A; *Z*-DNA, DNA in *Z* conformation.

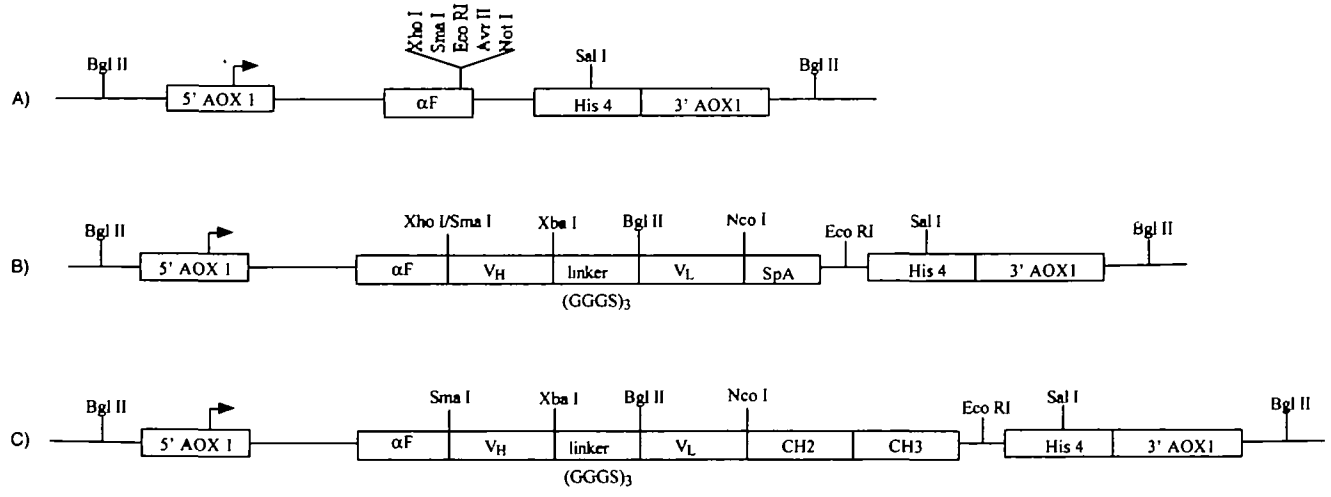

Fig. 1. **Schematic drawing of the expression cassettes used.** A: pCIP vector construction. The original vector used for cloning was pPIC-9 (INVITROGEN). This plasmid was modified so that its multiple cloning site included an *Sma*I site upstream of the original *Eco*RI site. B: pPIg 16 vector construction. Double digestion with *Sma*I/*Eco*RI allowed the introduction of the scFv of Z22 from pIg 16 (*9*). The staphylococcal protein A (SpA) fragment was used for purification purposes during the expression procedure. C: pFvFc vector construction. A further modification was the introduction of the IgG1 CH2 and CH3 domains, cloned from a human spleen cDNA library, downstream of the scFv segment. The cloning strategy for obtaining these vectors is described in the text. The expression vectors (B and C) were used to electroporate the yeast *Pichia pastoris*, strains GS115 and/or SMD1168, protease-positive and -negative, respectively (INVITROGEN), as described in the text. The symbols used in the scheme are: 5′ AOX 1: 5′ AOX 1 promoter fragment; αF: α factor secretion signal S; His 4: His 4 open reading frame; 3′ AOX 1: 3′ AOX fragment; V_H and V_L: heavy and light domains of the Z22 mAb, respectively; linker: linker peptide; CH2 and CH3: constant domains 2 and 3 of the human IgG 1 isotype, respectively; and the main restriction enzymes used for cloning.

clones was used for PCR with the primers CTCGAGATTAACCATGGCAAAACTCACACATGC and AGGGCAGCGCTGGGTGCTTTATTT. The cycling conditions were 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, repeated 34 times. The PCR product was initially cloned in the pGEM-T vector (PROMEGA). Before transferring this fragment to the expression vector, PCR mutagenesis was performed to eliminate an internal *Sma*I site and to include the appropriate restriction sites for the initial cloning in the pIg 16 vector. For this purpose, two sets of primers were used. The first set amplified the 5′ end of the Fc fragment (1-CTCGAGATTAACCATGGCAAAACTCACACATGC and 2-CATCCCGAGATGGGGGCAGGGTGT) and the second set amplified the 3′ end (3-CCATCTCGGGATGAGCTGACCA and 4-GGAATTCGGCCGTCGCACTCATTTACCC). The fragments obtained for each PCR were mixed and the complete Fc fragment was recovered by PCR with primers 1 and 4. This PCR product was cloned in the pGEM-T. The mutagenesis process leads to elimination of the internal *Sma*I site (at codon 375, following the kabat numbering, *10*) due to a silent C→T transition introduced by the complementary primers 2 and 3. Also, restriction sites *Nco*I/*Xho*I and *Eco*RI were introduced by external primers 1 and 4, respectively. The primers were designed to amplify the region between codons 232 and 478 (kabat numbering, *10*) of the IgG1 heavy chain, which includes the hinge, CH2 and CH3 domains.

The Fc fragment was cloned in the pIg 16 vector between the *Nco*I and *Eco*RI sites replacing the protein A tag. The expression cassette, including the scFv fused to the CH2-CH3 region, was then transferred to the pCIP vector as described above for the original scFv-SpA fusion. The resulting plasmid was named pFvFc (Fig. 1D). During the manipulation in the cloning strategy, all vectors were used to transform the bacterium *E. coli* (XL-1 Blue) by electroporation, followed by restriction profile analysis (data not shown).

P. pastoris GS115 and SMD1168 cells (INVITROGEN) were grown in liquid medium and made competent by resuspension in 1 M sorbitol. The cells were electroporated by means of pulse discharges (1,500 V, 25 μF, 400 Ω; Bio-Rad Gene Pulser) for 5 ms in the presence of 5–10 μg of plasmid DNA linearized with *Sal*I. This enzyme cuts within the plasmid-encoded *HIS4* gene and favors homologous recombination with the endogenous, nonfunctional *his4* gene of yeast cells. Therefore, transformants (*His*$^+$) were screened as to their capacity to grow in the absence of histidine, as described (INVITROGEN). Several *His*$^+$ transformants per construct were screened using the method described by Wung and Gascoigne (*11*) for protein secreting clones. Fifty to 100 yeast clones were plated on MM medium (yeast nitrogen base 1.34%, methanol 0.5%, biotin 0.00004%) and replica-plated in MD (yeast nitrogen base 1.34%, glucose 2%, biotin 0.00004%) with 1% agar added. The two plates were incubated for 48 h at 30°C. Recombinant scFv was induced with methanol and the secreted protein was transferred to a nitrocellulose membrane placed over the plate under many layers of towel paper to ensure culture medium diffusion throughout the membrane. After 3 h induction, the filter was removed, washed with PBST, and blocked in PBS containing 5% skim milk and 0.1% Tween 20 for 1 h at room temperature. Detection of the scFv-SpA fusion was possible due to the protein A tail of the molecule. Rabbit IgG (SIGMA) was added to a concentration of 4 μg/ml in PBS for 1 h at room temperature. After incubation with 1:2,500 alkaline phosphatase–anti-rabbit IgG conjugate (PIERCE) for 1 h, bound scFv was detected with NBT/BCIP as recommended by the man-

ufacturer (GIBCO, BRL). The FvFc fusion was tested as above except that it was detected with a 1:2,000 goat anti-human IgG1 (SIGMA), followed by 1:2,000 alkaline phosphatase–anti-goat IgG conjugate (SIGMA).

Protein expression kinetics were determined by growing clones in 25 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol) at 30°C in a shaking incubator (250 rpm) until the culture reached OD_{600} = 2.0–6.0. The cells were then centrifuged and resuspended in 100 to 200 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol) to induce protein expression. The cells were incubated for 4 days at 30°C. Aliquots of the culture supernatant were taken daily and examined by SDS-PAGE (Fig. 2A) and Western-blotting (Fig. 2B) (*12*). The recombinant scFv was detected as in the assay described above. Recombinant scFv-SpA was detected after 24 h and reached a peak around 72 h (Fig. 2). In Fig. 2B several minor bands can be observed, probably due to the activity of a protease derived from *P. pastoris*. These unspecific bands were visualized by Western-blotting, which is a highly sensitive methodology. In the other hand, when the purified recombinant protein was loaded and stained with Coomassie Brilliant Blue, only a band corresponding to the recombinant protein was observed (Fig. 2A). This indicated that a resonably pure protein with minor degradation products was obtained. Protease activity may be responsible for the decay of the recombinant protein at 96 h, albeit that the use of the protease negative (*pep*$^-$) strain, SMD1168, did not significantly improve the scFv-SpA accumulation (data not shown). Similarly, the FvFc recombinant protein exhibited the maximum accumulation at 72 h without a beneficial effect of use of the SMD1168 strain.

For large-scale expression, the clones were grown in

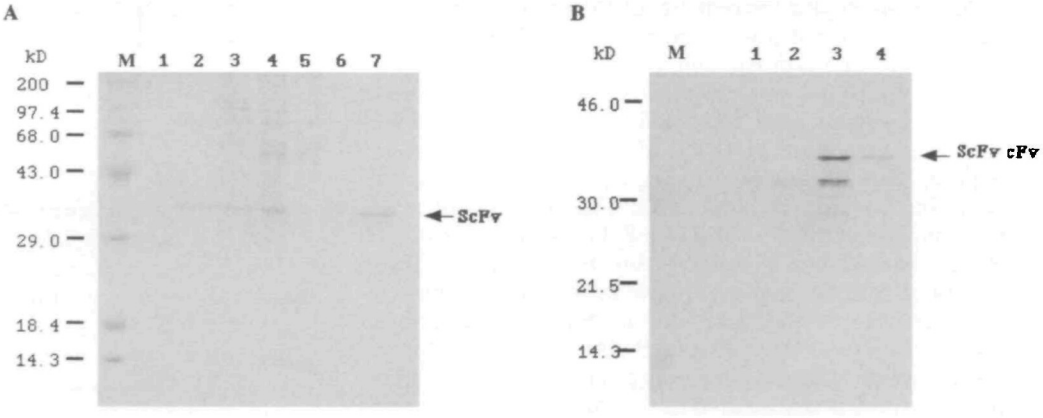

Fig. 2. **Expression and purification of the scFv-SpA fusion protein.** Recombinant clones of *P. pastoris* protease–positive strain GS115 (P+) were selected as described in the text. A: Analysis of the expressed proteins by SDS-PAGE. Aliquots (5 ml) of the culture were removed at 24 h intervals during the induction and stored at –20°C until needed. To analyze the total extract by SDS-PAGE, the samples were precipitated with 10% (final concentration) TCA (lanes 1, 2, 3, and 4). To analyze the purified recombinant proteins, the supernatant of the culture was then concentrated by filtration and run through an IgG-Sepharose column for purification of the expressed proteins (lanes 5, 6, and 7). The gel was stained with Coomassie Brilliant Blue (CBB-R250). Lanes: 1, 0 h induction; 2, 24 h induction; 3, 48 h induction; 4, 48 h induction after concentration by filtration; 5, IgG Sepharose column flowthrough; 6, IgG-Sepharose column fraction 1; 7, IgG-Sepharose column fraction 2. The positions of the molecular weight markers are indicated on the left and the scFv position is indicated by an arrow. B: Analysis of the expressed proteins by Western-blotting. Crude extracts proteins were resolved on SDS-PAGE as in A, for analysis of the total extract. The proteins were transferred to a nitrocellulose membrane and detected with 4 μg/ml rabbit IgG, followed by 1:5,000 goat anti-rabbit antibodies conjugated to alkaline phosphatase. The reaction was developed using the NBT/BCIP substrates. Lanes: 1, 0 h induction; 2, 24 h induction; 3, 48 h induction; 4, 72 h induction. The positions of the molecular weight markers are indicated.

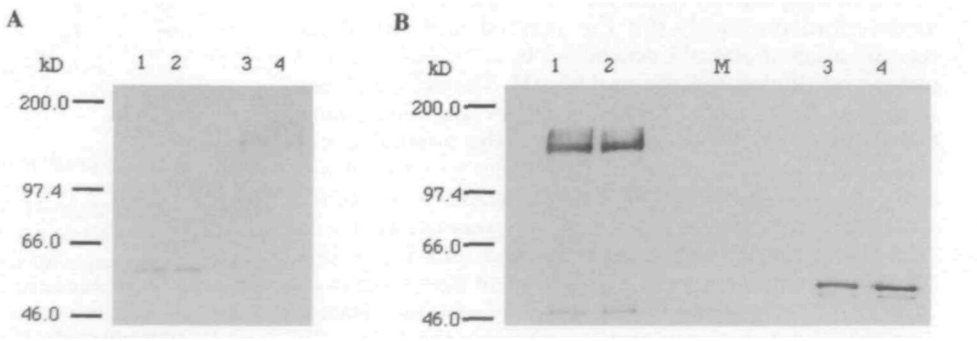

Fig. 3. **Analysis of affinity-purified soluble FvFc by SDS-PAGE (8%).** Yeast *P. pastoris* strains GS115 (P+) and SMD1168 (P–), protease-positive and -negative, respectively, were used to express the FvFc, as described in the text. The purified recombinant protein was obtained as described in the text, by running the samples through a protein A–Sepharose column. Each soluble protein [1 μg for Coomassie Blue staining (A) or 0.5 μg for Western-blotting analysis (B)] was subjected to 8% SDS-PAGE under reducing, with 2-mercaptoethanol, or non-reducing conditions. A: Coomassie Blue staining. Lanes: 1, FvFc(P+) reduced; 2, FvFc(P–) reduced; 3, FvFc(P+) not reduced; 4, FvFc(P–) not reduced. B: Western-blotting analysis. Lanes: 1, FvFc(P–) not reduced; 2, FvFc(P+) not reduced; 3, FvFc(P–) reduced; 4, FvFc(P+) reduced. Molecular mass standards are indicated (ovalbumin, 46 kDa; BSA, 67 kDa; phosphorylase *b*, 97.4 kDa; myosin, 220 kDa). In the Western-blotting analysis, the recombinant proteins were visualized by incubation with a 1:5,000 goat anti-human IgG, followed by 1:10,000 alkaline phosphatase–conjugated anti-goat IgG. The reaction was developed as in Fig. 2B.

exactly the same way as above for 72 h at 30°C under agitation. The supernatants were harvested following centrifugation and filtration through a 0.45 μm cellulose acetate filter. Protease inhibitors PMSF and pepstatin A were added to the filtrate at the concentrations of 0.07 and 0.4 μg/ml, respectively. This material was concentrated with an AMICON membrane (10000 MWCO) under pressure to a final volume of 3 ml. Media were diluted with PBS several times and then concentrated again as above to exchange the buffer. To purify the scFv, the concentrated supernatant was run through an IgG Sepharose 6B Fast Flow column (PHARMACIA) previously activated by three alternating washes with 0.5 M acetic acid, pH 3.4, and PBST (PBS and Tween 20 0.1%), and finally equilibrated with PBS. The scFv fragments were eluted with 0.5 M acetic acid, pH 3.4, and then immediately neutralized with 1.5 M Tris-HCl, pH 9.5. The purified proteins were dialyzed against PBS and then quantified using a BCA Protein Assay Kit (PIERCE) (Fig. 2). The FvFc fusion molecule was purified on protein A Agarose (SIGMA), eluted and quantified as above. The yields of the soluble recombinants Fv were 5 mg and 1.5 mg per liter of culture medium for scFv-SpA and FvFc, respectively. These production levels could be improved by the use of fermenters, reach yields comparable to those of other *P. pastoris* secreted proteins (*8*). The recombinant FvFc was visualized by SDS-PAGE (Fig. 3A) or Western-blotting (Fig. 3B), under reducing and non-reducing conditions. In these experiments it was possible to identify the monomeric and dimeric forms for FvFc, due to the presence of the disulfide bond between the hinge regions. These results were confirmed by analysing the samples by size-exclusion chromatography on a Superose-12 column (PHARMACIA) (Fig. 4, A and B). As above (Fig. 3), the results obtained for recombinant proteins on chromatography analysis demonstrated a predominant peak corresponding to monomeric and dimeric recombinant proteins for scFv-SpA (Fig. 4A) and FvFc (Fig. 4B), respectively, when compared with the molecular standard and human IgG. In addition to the predominant peaks we observed one peak corresponding to a high molecular mass and one to a lower molecular mass. These peaks probably correspond to unspecific aggregates and degradation products, respectively. It is noteworthy that the samples analyzed exhibited a retarded elution profile, probably due to unspecific interaction of the samples with the column (Fig. 4, A and B).

The binding activity of the scFv was analyzed by ELISA as described previously (*9*). The purified material at the concentration of 100 nM was added to Br-(dG-dC)$_n$–coated plates and diluted serially to 0.04 nM. The scFv-SpA isolated from *E. coli* clones harboring pIg 16 and affinity purified as above was used as a control. The original monoclonal antibody, Z22, affinity-purified from ascite fluid was also used as a control. *Z*-DNA binding activity was detected as above, except that the Z22 antibody was detected with 1:2,500 alkaline phosphatase conjugated anti-mouse IgG. All recombinant constructs were shown to bind specifically to *Z*-DNA (Fig. 5, A and B). The scFv-SpA obtained from *P. pastoris* exhibited a comparable activity to the bacterially produced scFv-Spa, although it was slightly inferior (Fig. 5A).

The CH2-CH3 tag included in this yeast expression vector to allow the production of dimeric Fv molecules was previously shown to be efficient for the production of recombinant antibody fragments in mammalian cells (*13*). The FvFc fusion protein exhibited very effective binding to *Z*-DNA comparable to that in the case of the original mAb (Fig. 5B). Considering that the FvFc molecules may be present as dimers, as suggested by the differential migration of reduced and non-reduced FvFc samples (Fig. 3, A and B) and by size-exclusion chromatography (Fig. 4B), it is expected that its avidity is comparable to that of the original mouse IgG2b Z22 mAb. The FvFc cassette was also tested in bacterial expression vector pIg 16. Although its

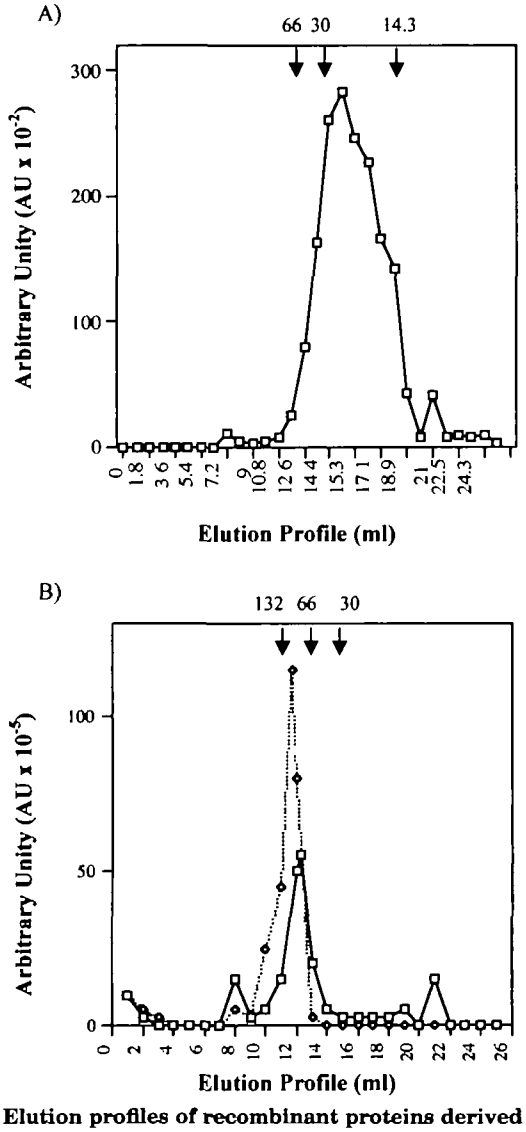

Fig. 4. **Elution profiles of recombinant proteins derived from *P. pastoris* on a Superose-12 gel filtration column.** All samples were loaded and eluted in PBS buffer, pH 7.2. The elution volumes of marker proteins are indicated by arrows and the molecular masses are given in kilodaltons (lysozyme, 14.3 kDa; carbonic anhydrase, 30 kDa; monomeric BSA, 66 kDa) in A and (carbonic anhydrase, 30 kDa; monomeric BSA, 66 kDa; dimeric BSA, 132 kDa) in B. A: Chromatography of scFv-SpA (P) derived from *P. pastoris*. For this assay approximately 10 μg (50 μl) of a sample was applied to the column, followed by 25 ml of the PBS buffer. B: Chromatography of FvFc (□). Around 5 μg (50 μl) of a sample [FvFc or human IgG (◇)] was applied to the column, followed by 25 ml of the PBS buffer. The flow rates were 0.3 ml/min in A and 0.7 ml/min in B. The elution volumes were monitored at 280 nm.

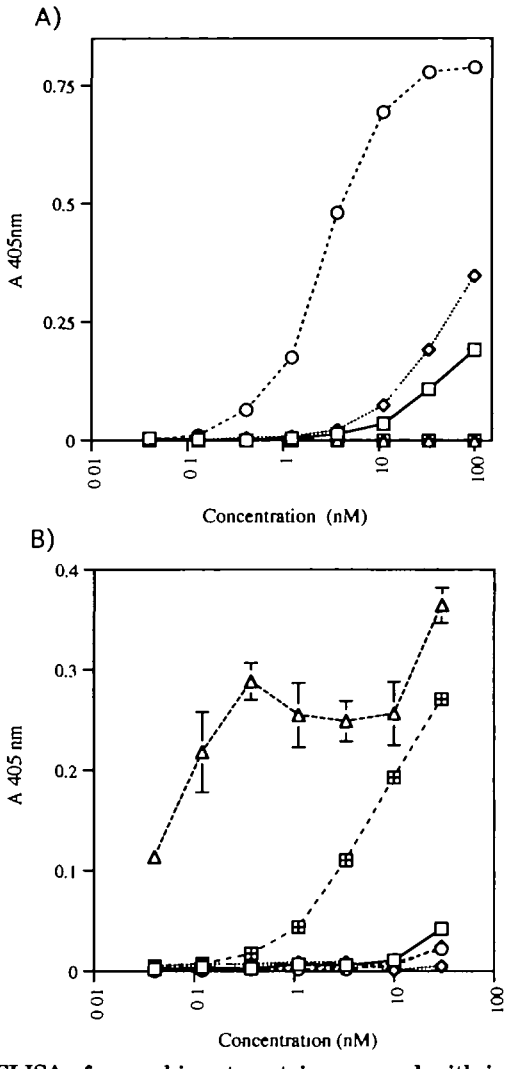

Fig. 5. **ELISA of recombinant proteins assayed with immobilized Br-(dG-dC)$_n$ (Z-DNA).** A: Direct binding of the scFv-SpA. Various concentrations of scFv-SpA (P) derived from *P. pastoris*, scFvZ22-SpA (*16*) derived from *E. coli*, and Z22 mAb were added to the wells of UV-irradiated microtiter plates (Immulon type 2; Dynatec Laboratories, Inc.) coated with 7 μg/ml of Z-DNA, followed by blocking with 3% BSA/PBST. The bound proteins were detected with 4 μg/ml rabbit-IgG, followed by 1:2,500 alkaline phosphatase-conjugated anti-rabbit IgG (for scFv construction) or 1:2,500 alkaline phosphatase-conjugated anti-mouse IgG (for Z22 mAb). Samples were added to Z-DNA containing wells (Z-DNA+) or to wells without Z-DNA (Z-DNA–). Open squares: scFv-SpA (P)/Z-DNA+; open lozenges: scFv-SpA (*16*)/Z-DNA+; open circles: Z22mAb/Z-DNA+; open triangles: scFv-SpA (P)/Z-DNA–; filled squares: scFv-SpA (*16*)/Z-DNA–; filled lozenges: Z22 mAb/Z-DNA–. B: Direct binding of the FvFc. A microwell plate (Immulon type 1; Dynatec Laboratories, Inc.) was initially sensitized with 4 μg/ml streptavidin in PBS, and blocked with 3% BSA/PBST. Immobilization of the antigen was performed by the addition of 1.5 μg/ml biotinylated Z-DNA in PBS to each well. Various concentrations of the samples were added to the plates. The bound proteins were detected with a 1:5,000 goat anti-human IgG, followed by 1:10,000 alkaline phosphatase–conjugated anti-goat IgG (for recombinant FvFc and human IgG) or 1:2,500 alkaline phosphatase–conjugated anti-mouse IgG (for Z22mAb). Open squares: FvFc/Z-DNA–; open lozenges: Z22mAb/Z-DNA–; open circles: human IgG/Z-DNA–; open triangles: FvFc/Z-DNA+; filled squares: Z22 mAb/Z-DNA+; filled lozenges: human IgG/Z-DNA+. In A and B the color reaction was determined by the addition of 1 mg/ml pNPP.

production was detectable on Western-blotting, only trace amounts of the recombinant protein could be isolated from culture media (data not shown). It is likely that the high monomer size and its tendency for dimerization could block its folding in the bacterial periplasm. The use of eukaryotic cells seems to overcome these difficulties, allowing efficient secretion of active antibody fragments. The simplicity of manipulation of this methylotrophic yeast together with its high yield of the secreted recombinant protein suggest this system as a new standard for producing recombinant antibodies, replacing the traditional bacterial expression system.

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