Production of scFv recombinant fragments against 2,4-dichlorophenoxyacetic acid hapten using naïve phage library

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ABSTRACT: Three single chain variable fragment (scFv) antibodies against 2,4-dichlophenoxyacetic acid (2,4-D) herbicide were produced by the Griffin1.library. The selection of the scFv from the phage library was carried out by 2,4-D-protein coated tubes with different levels of hapten substitution in the conjugate. The scFv phage clones were isolated within the five round library panning and the antibodies were expressed in *Escherichia coli* **HB2151**. The recombinant products were purified by metal affinity chromatography yielding 200 μg of pure scFv per 1 liter of bacterial culture. The antibody fragments provided steep curves in conventional indirect ELISA having the IC_{50} values from 10.2 to 14.5 ng/ml established for 2,4-D standard. Interestingly enough, the recombinant ScFv E1 antibody exhibited 68% cross-reactivity with 2,4-dichlorphenol (2,4-D = 100%), and 38.0% with methylchlorophenoxyacetic acid (MCPA) whereas reaction with other phenoxyacetic compounds was low. Similar characteristics were obtained for other two recombinant products. Low stability for the isolated scFv antibodies was found in storage buffer even in the presence of stabilizers and protease inhibitors. Factors influencing stability of the recombinant antibodies are discussed.

Keywords: naïve phage library; selection; phage antibody; 2,4-D hapten; antibody stability

Recombinant antibodies represent the next generation of immunochemical reagents extending options of poly- and monoclonal antibodies for application to clinical, environmental and food analysis (Spinks, 2000; Souriau and Hudson, 2001; Hock *et al*., 2002). The molecular biology tools such as vectors (Horn *et al*., 1996) and phage display libraries (McGregor, 1996; Knappik *et al*., 2000) have been developed and used for the preparation of the recombinant antibodies against both protein antigens and small hapten structures. Human and animal lymphocytes have been used as a source of genetic material for the construction of phage libraries. Li *et al*. (2000) used lymphocytic genetic material of a rabbit immunized simultaneously with 4 different herbicide-protein immunogens. The method employing immunized phage library enabled the authors to produce high affinity scFv antibodies specific for mecoprop, atrazine, simazine, and isoproturon herbicides within one procedure. Recently, the same kind of the immunized phage library was used for production of several antibody fragments against haptens such as atrazine (Charlton *et al*., 2001), ampicilin (Burmester *et al*., 2001) and picloram (Tout *et al*., 2001). Additionally, the robust potential enabling production of recombinant antibodies against wide spectra of antigens was demonstrated using large naïve phage libraries containing up to 2×10^9 different phage particles (Griffiths *et al*., 1994; Nissim *et al*., 1994; Hanes *et al*., 2000; Knappik *et al*., 2000). The libraries were successfully utilized for isolation of phage clones producing functional fragments against progesterone by Gram *et al*. (1992), copper complex of 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (Cu-TETA) and yttrium complex of 1,4,7,10-tetraaza-4,7,10-tris(carboxymethyl)-1-cyclododecylacetyl (Y-DOTA) prepared by DeNardo *et al*. (1999), aflatoxin-B1 by

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Moghaddam *et al*. (2001) or microcystin by Strachan *et al*. (2002).

Selection of high-affinity binding between antigenic (hapten) determinant and phage particles is a key step of the successful panning process in the phage library technology. However, phage particles having on their surface various numbers of antibody fragments may be a cause of low-affinity to hapten determinant or non-specific interactions. Thus, selection of functional antibodies can be suppressed due to the undesirable avidity binding whereas inapplicable antibodies can be predominantly selected. To overcome this problem, separation by biotin-streptavidin system was employed by Hawkins *et al*. (1992). In the system, high-affinity interaction of biotin-labeled hapten in solution and the capture on streptavidin coated surface was achieved. The final step, elution of specific phage particles, was performed in this method by non-labeled hapten. Charlton *et al*. (2001) employed for the affinity selection hapten-conjugate coated tubes in combination with elution by triethylamin or hapten molecules. Stringency of selection process was achieved by reduced concentration of the hapten conjugate on the coating surface.

In this work, the separation system based on the hapten coated tubes was chosen to explore potential of the naïve Griffin1.library to produce antibody fragments against 2,4-D herbicide. The strategy, tested within 5 panning cycles, was based on the premise that low density of hapten in the coating conjugate can improve selection efficiency. The final aim of the work was to produce recombinant antibodies and to characterize their properties by immunoassay parameters, DNA analysis and product stability.

MATERIAL AND METHODS

Library, enzymes, reagents and standards

The Griffin1.library, established by Griffiths *et al.* (1994), was kindly provided by the Centre for Protein Engineering, MRC Technology, 20 Park Crescent, London W1B 1AL, England. Restriction enzymes, Thermus aquaticus DNA polymerase were purchased from Promega (Madison, USA); Sephadex G-25 was from Sigma, (St. Louis, USA) and HPLC purified PCR primers were product of custom synthesis (Laboratory of Molecular Plant Physiology, Masaryk University, Brno, Czech Republic). 2,4-D conjugates with bovine serum albumin (BSA) and porcine thyroglobulin (TG) possessing hapten density 2.3 and 26.5 for 2,4-D-BSA and 44.1 for 2,4-D-TG (mol/mol) were prepared in previous work of Franek *et al*. (1994). Deionized water (Millipore apparatus) was used in all experiments. Hybridoma clone producing monoclonal antibody 9E10 against anti c-myc epitope tag present on C-termini of scFv molecules was obtained from The European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). The cells were grown on Dubelcco's modification of Eagle's medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum. Peroxidase labeled swine-anti-mouse antibody was from SevaPharma a.s. (Prague, Czech Republic).

Buffers, solutions

The following solutions and buffers were used: as a coating buffer was used 50 mM carbonate buffer, pH 9.6; phosphate-buffered saline (PBS), pH 7.2 was composed of 10 mM phosphate buffer, and 145 mM NaCl; PBS-T assay buffer was prepared by mixing 100 ml of PBS with 0.1 ml Tween 20; tetramethylbenzidine solution (TMB) was composed of 10 mg per ml TMB in dimethylsulfoxide; acetate buffer was 100 mM sodium acetate/citric acid buffer, pH 5.5; substrate solution (TMB+H_2O_2) was prepared by adding 200 μl of 6% H_2O_2 to 21 ml of acetate buffer; osmotic shock buffer, pH 8.0 was prepared from 30 mM Tris, 30% sucrose and 1 mM EDTA.

Bacterial strains

Escherichia coli **TG1** suppressor strain (K12, *del(lac-pro), supE, thi, hsdD5*/F'*traD36, proA+B+, lacIq, lacZdelM15*) *E. coli* **HB2151** non-suppressor strain (K12, *ara, del(lac-pro), thi*/F'*proA+B+, lacIq, lacZdelM15*).

Phage library selection and screening

2,4-D coating conjugates (4 ml) at the concentration of 50 μg/ml in PBS were incubated in Nunc maxisorp tubes overnight at room temperature, washed with PBS and blocked with 1% BSA in PBS at 37°C for 2 hours. Suspension of phage particles (approximately 10^{12} particles) in 1% BSA in PBS was added to the coated tubes and incubated at 37°C on a rotating turntable at room temperature

for 30 min and without rotating for further 60 min. Non-specifically absorbed phage particles were extensively washed out using PBS-T solution. Bound phage particles were then eluted by 1 ml of 0.1 M triethylamine. The first selection round was based on 10 min and subsequent 20 min triethylamine mediated particles desorption from the coated conjugates. For quick neutralization, 0.5 ml 1 M Tris, pH 7.4 was added to 1 ml of the eluted phage. Half of the eluted phage suspension (0.75 ml) was infected into 10.25 ml exponentially growing TG1 cells in 2×TY broth (Sambrook *et al*., 1989) and remaining bound phage particles were recovered by adding 4 ml TG1 cells into the selection tube. The cells were pooled and plated on TYE agar (Sambrook *et al*., 1989) containing 1% glucose and 100 μg/ml ampicilin. After an overnight incubation at 30°C, colonies were scratched into 2 ml of 2× TY-Amp-15% glycerol. Aliquots were stored at –80°C. Phages were rescued using 500 ml of exponentially growing TG1 culture prepared from 100 μl aliquot of the scratched glycerol stock. M13KO7 helper phage (Pharmacia Biotech, Uppsala, Sweden) was added at 20× multiplicity (phage/bacterial cell) to 50 ml of the culture which was incubated at 37°C without shaking for 30 min. Infected cells were pelleted, resuspended in 500 ml 2×TY with 100 μg/ml of ampicilin (Amp), 25 μg/ml of kanamycin (Kan) and 1% glucose and incubated with shaking at 30°C overnight. Phage particles were isolated from the supernatant by two successive precipitations with 1/5 volume PEG (20% polyethylene glycol 8 000 w/v, 2.5 M NaCl) as described by Griffiths *et al*. (1994). Titres of phage particles were determined as plaque forming units on Petri dishes with LB media containing 50 μg/ml of ampicilin (Sambrook *et al*., 1989). The selection and amplification cycles were repeated in 4 subsequent panning rounds with the following modifications: The only phage particles obtained from the second (20 min) desorption process were further used. Two panning approaches were employed using phage rescued from the first panning cycle as the start point for additional four cycles of selection. The first panning approach used 4 subsequent selection rounds of phage particles in tubes coated with 50 μg/ml 2,4-D-BSA conjugate (hapten density 2.3 mol/mol). The two conjugates were used for the panning in the fifth cycle of selection (2.3 mol 2,4-D/mol BSA and 44.1 mol 2,4-D/mol TG). The second selection approach was based on the same procedure, however high hapten density 2,4-D-BSA conjugate (26.5 mol/mol) was employed for coating. The obtained clones were screened for scFv production by ELISA after 3rd–5th cycle of the selection procedure.

Expression and purification of scFv

ScFv exhibiting competition between protein conjugates and free hapten in ELISA were produced from *E. coli* **HB2151** harboring pHEN2 vector. The cells were used for the production of the recombinant species as follows: 10 ml of the overnight culture were used to inoculate LB media (0.5 l) in the 2 l Erlen-Mayer flasks. Bacterial pellets were resuspended in 15 ml of osmotic shock buffer. The suspension was stirred on ice bath for 10 min supernatant was collected after centrifugation at 3 000 g for 10 min. The cells were treated with 5 mM-$MgSO_4$ and the same procedure was repeated as described above. The supernatants collected were pooled and used for metal affinity chromatography purification of hexahistidine tagged scFv. Crude *E. coli* periplasmic fraction was mixed with the Ni.NTA-agarose resin (Qiagen, Hilden, Germany), stirred at 4°C for 30 min and packed to a chromatography column. Subsequent elution was performed by 2 ml of 250 mM imidazole in PBS buffer. Eluted scFv fractions were dialyzed against 3 l of PBS for 12 hours. The amount of the dialyzed product was determined by $UV_{(280\ nm)}$ absorbance and stabilized in storage buffer containing 15% glycerol, 0.5% ovalbumin and 1 mM-protease inhibitor PMSF at –80°C. The activity of the native scFv product was determined by indirect ELISA.

Indirect ELISA procedure

2,4-D protein conjugates in coating buffer of concentration 0.2 μg/ml were pipetted into wells of microtitre plates and the plates were incubated at room temperature overnight. Each well was washed three times with 300 μl of the PBST buffer and 50 μl of the 2,4-D standard in PBS solution and 50 μl soluted scFv in PBST buffer was added into the wells. Unbound compounds were removed by washing after one hour incubation at room temperature. Then 100 μl of the mouse antibody 9E10 of concentration 2 μg/ml and HRP labeled anti-mouse antibody (1 : 2 000 stock dilution) were added into the wells. The plates were incubated at room temperature for 1 hour. After washing, 100 μl

of the TMB/H_2O_2 substrate solution was added into each well. The enzymatic reaction was stopped after 15 min incubation at room temperature by adding 100 µl of 1 M H_2SO_4. The absorbance of the developed colour was measured at 450 nm.

Stability of scFv clones

Frozen aliquots of scFv E1 were thawed subsequently in week intervals and incubated at 4°C and 37°C within the range of 7 weeks. The scFv aliquots were incubated in storage buffer, or storage buffer with added ovalbumin (0.5 mg/ml) and protease inhibitor (1 mM PMSF). At the end of the 7th week, antibody binding of respective aliquots towards hapten-coated conjugate were quantified by ELISA. Binding of scFv antibodies was compared to freshly thawed scFv and relative amount (%) of scFv active fraction determined as proportion of relative absorbance as determined by ELISA.

ScFv sequence determination

Plasmid DNA was isolated from 10 ml overnight culture of *E. coli* harboring 2,4-D phagemide clones by Wizard Plus SV minipreps kit (Promega, Madison, USA). ScFv coding segments on the plasmids were amplified by PCR using specific primers Forlink seq 5′-GCC ACC TCC GCC TGA ACC-3′ and pHEN seq 5′CTA TGC GGC CCC ATT CA-3′. The DNA sequence was determined via ABI PRISM® BigDye™ terminator cycle sequencing kit. The procedure was carried out in a 20 µl Ready Reaction Mixture containing 20 ng of polymerase chain reaction (PCR) product and 10 pmol of sequencing primer. The mixture was subjected to 25 cycles PCR consisting of 96°C for 15 s, 47°C for 15 s and 60°C for 4 min. The analysis of the sequencing reaction products was done by ABI prism 310 capillary electrophoresis analyzer (Applied Biosystems, Foster City, USA). The two pHEN2 plasmid inserts per each scFv clone were sequenced from both 5′ and 3′ end directions. Nucleic acid sequence data were analyzed and converted to amino acid sequence using BioEdit sequence alignment software. Canonical class assignment of particular CDR was done by automated method of Martin and Thornton (1996) using WWW interface at http://www.rubic.rdg.ac.uk/abs/chothia.html.

RESULTS

Selection and production of anti 2,4-D binders

The employed selection procedure, based on two panning strategies, is depicted in Figure 1. About 1×10^{12} phage particles were added into hapten coated tubes in each round of selection. Bound

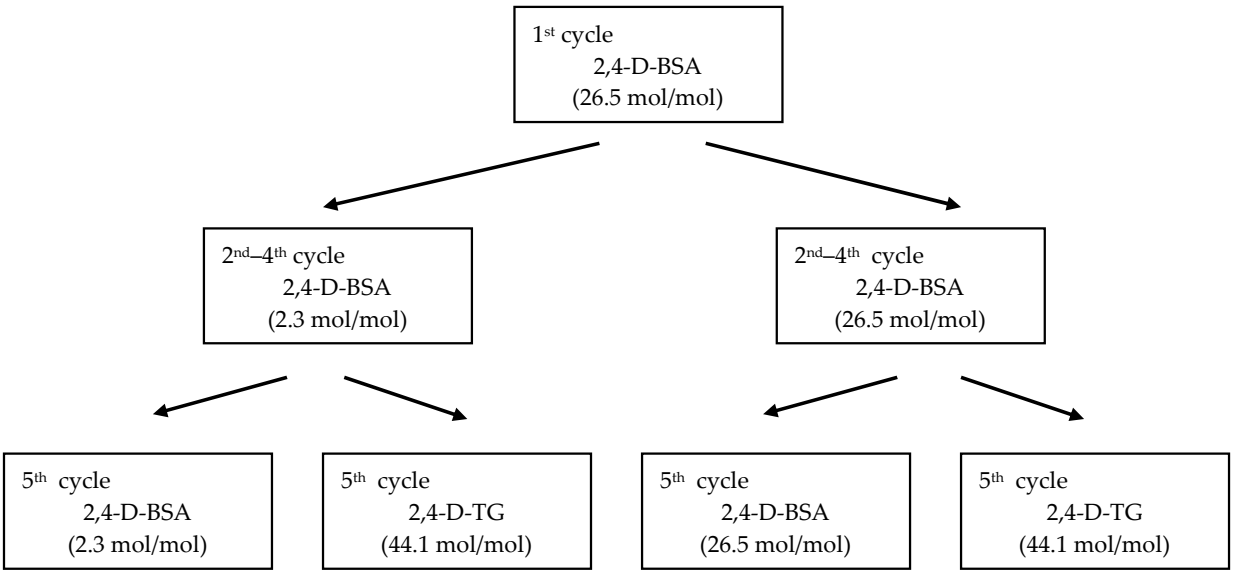


Figure 1. Selection strategy for phage antibodies against 2,4-D

phage particles were double eluted with TEA (triethylamine), initially for 10 min and subsequently for further 20 min. In the first selection cycle, eluted particles were used to infect *E. coli* **TG1** cells from both eluates, while in the subsequent cycles only the second eluate was used to infect the cells. The 95 phage clones picked from Petri dish in each of 3rd–5th selection cycles were tested for scFv production by indirect ELISA. No positive clones were indicated in the 3rd cycle. When the low hapten density strategy was used, 10 and 7 of the 95 clones exhibited good antibody binding in the 4th and 5th round, respectively. On the contrary, the second selection approach based on the high hapten density in the coating conjugate did not yield any functional phage antibodies within the whole panning procedure. The positive clones obtained in the first procedure were expressed in *E. coli* **HB2151** from pHEN2 plasmid. The effect of the cultivation time on the expression yield is presented in Figure 2. The 1 mM IPTG was used for scFv induction in exponentially growing cells ($OD_{600\ mn}$ = 0.5) in the course of 4–24 hours. Binding activity expressed here in percentage is proportional to the amount of the scFv antibody produced by the cells in media. It is apparent from the results that 6 hour induction was optimal for formation of the maximum scFv E1 yield, while longer incubations resulted in lower antibody production. The scFv fragment obtained was purified from crude *E. coli* periplasmic fractions by metal affinity chromatography. The product showed 95% purity as single band with size approx. 40-kDa on the densitogram of SDS-PAGE. The production yielded 200 μg of the pure scFv E1 fragment per one liter of culture media. The yields found for the scFv-E2 and H7 antibodies were comparable to scFv-E1 in the same induction interval.

Immunochemical characterization of scFv

Using recombinant scFv E1, E2 and H7 antibodies, ELISA was optimized in terms of coating conjugate dilution, hapten density conjugate and antibody dilution. The results are shown in Figure 3. The obtained calibration curves are steep and therefore quantitative in the range of 5–50 ng/ml. The IC_{50} values (50% binding inhibition) for the scFv E1, E2 and H7 were 10.2, 12.8 and 14.5 ng/ml, respectively. The results of cross-reactivity are presented in Table 1. Surprisingly, low specificity was found for 2,4-dichlorphenol. The 68.8–80.2% of the cross-reactivity of the phenol in all three recombinant antibodies demonstrates the fact that the antibodies are not able to recognize chemical change at the site of the phenoxy moiety (-O- versus -OH-) in the 2,4-D hapten determinant. Relatively high cross-reactivity was indicated also for MCPA (24.9–38.0%) whereas reaction with other tested compounds was low.

The analysis of scFv coding sequences

The amplified DNA fragments coding for the scFv-E1, E2 and H7 were characterized by sequenc-

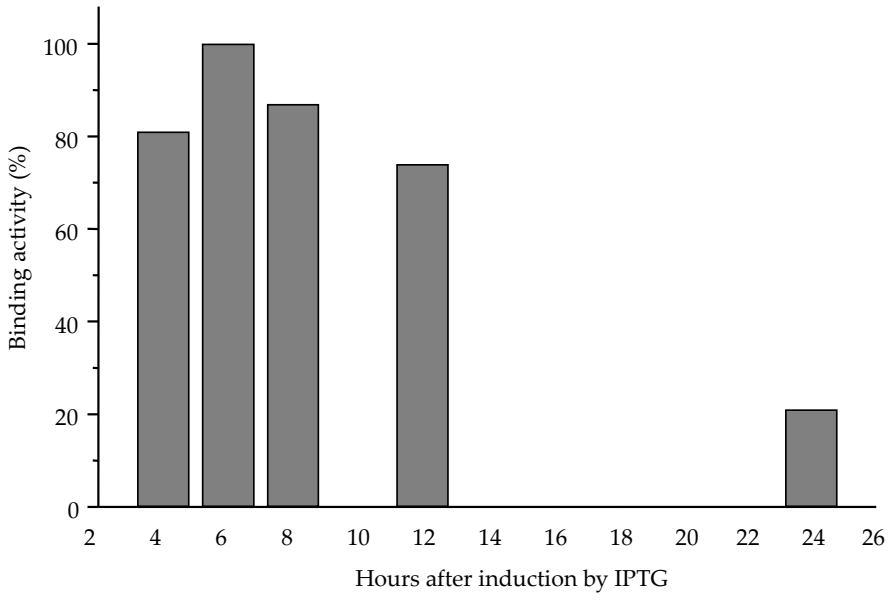


Figure 2. Production characteristics of scFv-E1 expressed from pHEN2 vector in *Escherichia coli* HB2151

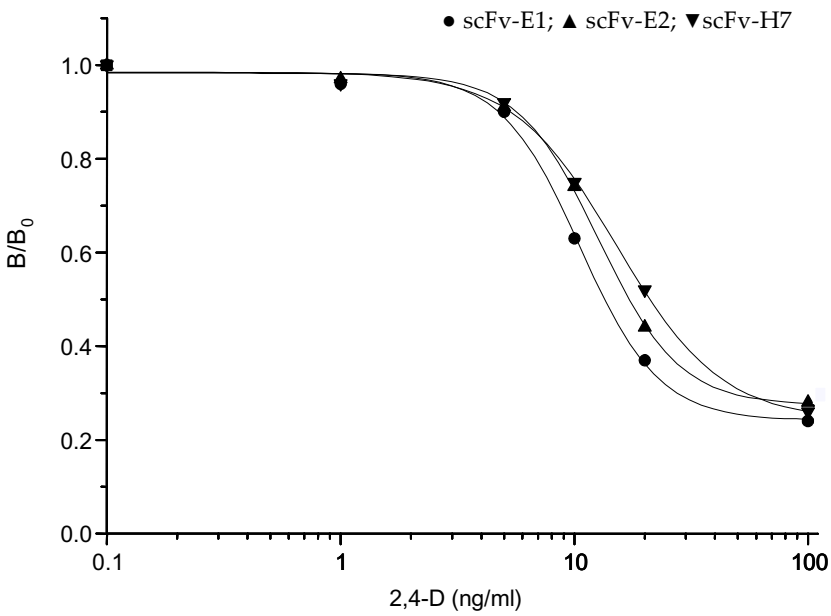
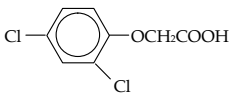
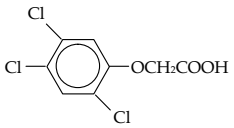
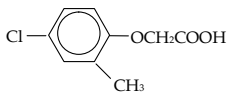
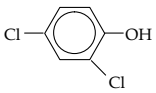
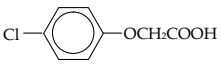
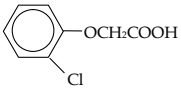


Figure 3. Indirect ELISA calibration curves for 2,4-D using scFv antibodies

Table 1. Cross-reactivity among 2,4-D and related compounds for scFv antibodies

	Cross-reactivity (%)*		
	scFv-E1	scFv-E2	scFv-H7
2,4/D 	100	100	100
2,4,5-T 	2.3	6.9	8.1
MCPA 	38.0	31.2	24.9
2,4-dichlorphenol 	68.8	80.2	69.2
p-chlorophenoxyacetic acid 	3.1	2.4	1.8
o-chlorophenoxyacetic acid 	2.8	1.5	0.7

*Cross-reactivity (%) is the IC_{50} of 2,4-D divided by the IC_{50} of the respective cross-reactant (times 100). IC_{50} value represents the concentration (ng/ml) of 2,4-D or cross-reactant lowering the absorbance to 50% compared to calibration zero

ing analysis. Amino acid sequences, presented in Table 2, were deduced from the obtained nucleotide data. As shown in Table 2, the amino acid sequences shared relatively low sequence homology, especially in the CDR area. Comparison of the scFv homology was based on the number of amino acid differences among the 3 examined clones. Apparently, the degree of homology for the scFv-E1 and E2 pair was about two times higher than the homology of scFv-E1 and scFv-H7 antibody species. The hypervariable regions were further characterized by canonical classification according to Chothia *et al*. (1992). This approach allows approximate shape of CDR loops within the particular binding site and evaluates the degree of structural similarity of the CDR loops among different binding sites. The length of the CDR and the key amino acid residues are the relevant parameters determining the type of the canonical class. It is apparent from Table 3, that the same canonical classes can be ascribed to all CDR for scFv-E1, E2 and scFv-H7 fragment.

The stability of scFv

Since stability of the recombinant product is an important factor for assay performance, the effect

Table 2. Amino acid sequence alignment of scFv-E1, E2 and H7 antibody fragments*

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            Fw-1                          CDR-H1       Fw-2
scFv E1  QVQLVQSGAEVKKPGASVKVSCKAS GYTFTSYGIS WVRQAPGKGLEWMG
scFv E2  QVQLVQSGAEVKKPGESLKISCKAS GYSFISYWIG WVRQMPGKGLEWMG
scFv H7  QVQLVQSGAGVVQPGRSLRLSCAAS GFTFSSYSMH WVRQAPGKGLEWVG

            CDR-H2                    Fw-3                          CDR-H3
scFv E1  WISAYNGNTN YAQKLQKRVTITADTSASTAYMELSSLRASDTAVYYCAR HRRN
scFv E2  WIYAGNSNTR YSQSLQKQVTISADKSISTAYLQWSSLKASDTAMYYCAR HRRN
scFv H7  WVMWFDGTEK YSQESVKGRTISADNSKSTLFLQMNSLRADDTAVYYCAR EPDW

          Fw-4         linker           Fw-1                    CDR-L1
scFv E1 WGQGTLVTVSR GGGGSGGGGSGGGGS SELTQDPAVSVALGQTVRITC TDSSVRSYYVH
scFv E2 WGQGTLVTVSS GGGGSGGGGSGGGGS QALTQPSSVSGAPGQRVTISC TDSSSRNYYVH
scFv H7 WGDRGALDVSS GGGGSGGGGSGGGGS QSLTQDTLVAGAPGQRVTITC RASQGISYYVH

             Fw-2              CDR-L2     Fw-3
scFv E1  WYQQKPGQAPVLVIY GKNNRPS GIPDRFSGSSSGNTASLTITGAQAEDEADYYC
scFv E2  WYQQLPGTAPKLLIY GNSNRPS GVPDRFSGSKSGTSASLAITGLQAEDEADYYC
scFv H7  WYQQKPGKAPKLLMY EASSLES GVPSRFSGSGSGTEFTLTISSLQPDDFAAYYC

          CDR-L3       Fw-4
scFv E1 NSRDSSGNHVV FGGGTKLTVL
scFv E2 QSYDSSLSGSV FGGGTKLTVL
scFv H7 QHYNTYPYTSV FGQGTKLTVL
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* Positions of respective complementarity determining regions (CDR H1–3) variable domain of heavy chain and (CDR-L1–3) for variable domain of light chain are indicated by bold symbols. The (Fw 1–4) are framework parts of antibody domains

Table 3. Cannonical assignment of CDR sequencies of recombinant antibody fragments determined by Chothia *et al*. (1992) classification

Recombinant antibody	CDR classification				
	CDR 1 light	CDR2 light	CDR3 light	CDR1 heavy	CDR2 heavy
ScFv-E1	2	1	5	1	2
ScFv-E2	2	1	5	1	2
ScFv-H7	2	1	5	1	2

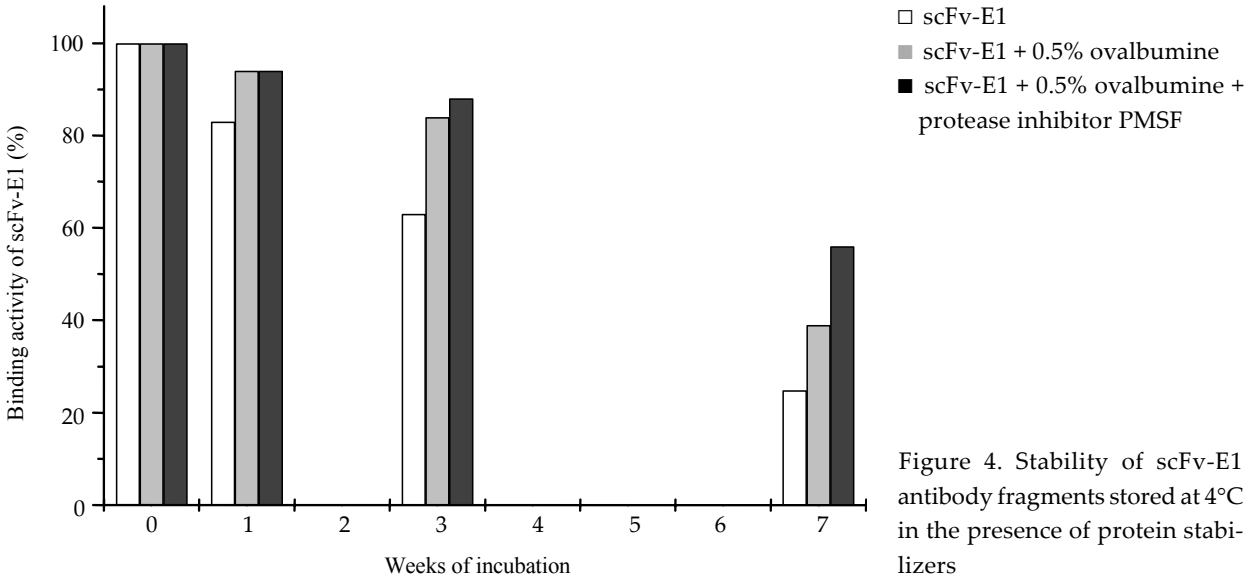

Figure 4. Stability of scFv-E1 antibody fragments stored at 4°C in the presence of protein stabilizers

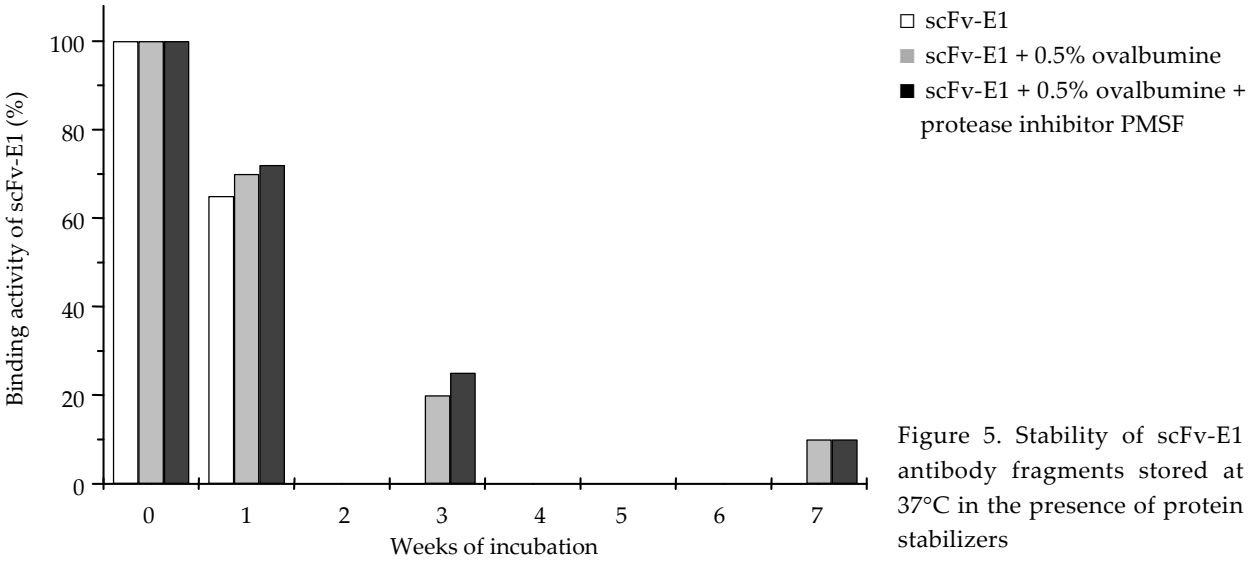

Figure 5. Stability of scFv-E1 antibody fragments stored at 37°C in the presence of protein stabilizers

of two additives in storage buffer solution on the product stability was investigated. The measurement was carried out under conditions described in the section Methods. Figures 4 and 5 illustrate the decline of scFv-E1 binding activity in the presence of protein stabilizers at 4°C and 37°C in the course of a 7-week period. As shown in Figure 4, under the presence of the protein stabilizers the binding

of scFv declined about 14% after 3 weeks and 45% after 7 weeks when compared to the initial values. On the contrary, binding decline of 38% and 77% was observed in buffer without stabilizers during 3 and 7 weeks, respectively. As expected, incubation at 37°C resulted in much stronger decrease of binding activity when compared to 4°C incubation (Figure 5). This decline reached 74% after 3 weeks of storage and approached to zero after 7 weeks of incubation. Presence of stabilizers in storage buffer attained 10% of binding activity at the end of the tested period.

DISCUSSION

Although the Griffin1.library has been broadly used for production of recombinant antibodies to high-molecular antigens, its application to hapten compounds has been rare so far. To our knowledge production of recombinant fragments against 2,4-D herbicide in this laboratory is only the third case of hapten antibodies formation using this library (Griffiths *et al*., 1994; Strachan *et al*., 2002). We believe that strategy based in this work on the use of low hapten density in the coated conjugate, together with optimized elution steps, improved selection ability of the library. This approach can be an alternative to the established selection methods of the selection such as affinity separation based on phage particles capture by biotin labeled hapten on magnetic particles and subsequent elution with free hapten (Hawkins *et al*., 1992) or decreasing concentrations of hapten conjugate in coated tubes used by Charlton *et al*. (2001). The approach suggested in this work allows modulation of the hapten density on the selection surface in order to reduce potential avidity binding formation between phage molecules and hapten protein conjugates.

In this work, the established limit of detection was 4 ng/ml of 2,4-D for the respective scFv antibodies which is about twenty-fold lower sensitivity in comparison to the monoclonal antibody E2/G2 raised against 2,4-D in the previous work (Franek *et al*., 1994). The assay sensitivity for the scFv antibodies is similar to those scFv obtained by Tout *et al*. (2001) and Kramer (1998) for picloram and atrazine. The authors produced the scFv fragments using direct cloning of variable parts of antibody genes from immunized mice or employed recombinant phage antibody system from RPAS, Pharmacia. The supersensitive antibody against atrazine was produced by Charlton *et al*. (2001) using phage library. The limit of detection (1–2 ppt) established in ELISA is about three orders of magnitude higher compared to the above reported antibody reagents. Additionally, recombinant fragments derived from parent monoclonal species against parathion and coplanar PCBs exhibited similar sensitivity compared to the monoclonal immunoassays (Garrett *et al*., 1997; Chiu *et al*., 2000).

The phage antibodies, produced in this work, exhibited strong reactivity between 2,4-D and 2,4-dichlorinated phenol. This means that the antibodies were not able to recognize chemical changes at the site of the phenoxy moiety (-O- versus -OH) in the 2,4-D hapten determinant and thus represent rather model products with a strong non-specific response to the above phenol. Reactions with other cross-reactants were not surprising.

About 45% binding decline at 4°C in the course of 7 weeks in our scFv antibodies shows only short term stability of the scFv products in solution. The cause of this phenomenon can be ascribed to factors such as intrinsic thermodynamical instability of particular scFv molecule (Worn and Plueckthun, 2001) or to the presence of proteases in storage buffer. As reported by Kramer *et al*. (2002), purification process based on metal affinity chromatography was not effective enough to remove the traces of enzymes from the culture matrix. It can be noted that the presence of protease inhibitors as well as ovalbumin stabilizer in the storage buffer improved stability of the recombinant products prepared in this work. As regards the albumin, it is unclear whether effect of this stabilizer was achieved by decreasing denaturation rate of scFv or by decreased protease activity. Surprisingly, stability of our recombinant product is low when compared to the product obtained by Kramer *et al*. (2002). Whereas scFv produced in this laboratory showed significant binding decrease in the course of 7 weeks, the same decline was indicated for the Kramer's recombinant product within 7 months. Unfortunately, many scFv fragments are not stable for longer periods. Even when provided with superb antigen-binding properties, they often fail to show convincing effects during practical applications, because they tend to denature and aggregate under the conditions faced in practice (Reiter *et al*., 1994; Willuda *et al*., 1999). It can be shown, however, that this is not an intrinsic drawback of the scFv format, but rather a property of particular scFv coding sequences, which can be corrected by engineering, reviewed by Worn and Plueckthun (2001).

The amino acid sequences of clones, particularly in the CDR areas, demonstrate pretty clear homology (motifs) considering that T is similar to S, K to R, V to I, F to Y (Table 2). Additionally, canonical classification of the CDR was found to be completely identical as shown in Table 3. It appears from the data that immunochemical characteristics of the produced antibody fragments (Table 1, Figure 3) are compatible with these results.

CONCLUSIONS

Further development in agri-food analytical immunochemistry depends almost exclusively on the ability to develop novel antibodies in a more cost-effective manner (Spinks, 2000). Phage antibodies produced by naïve recombinant phage libraries represent an alternative to the conventional and monoclonal antibodies. In this work, the 2,4-D herbicide was chosen as a model hapten to explore potential of the naïve Griffin1.library to produce functional antibodies for immunoassay techniques, especially for ELISA. The selection of the scFv from the phage library was carried out by 2,4-D-protein coated tubes with different levels of hapten substitution in the conjugate. This strategy allowed production of three recombinant fragments with binding affinity to the herbicide hapten. Immunoassays incorporating these antibodies provided steep calibration curves for 2,4-D in the range of 4–100 ng/ml and high cross-reactivity with 2,4-dichlorphenol. Although naïve Griffin1.library has achieved wide popularity in high-molecular weight antigens, to our knowledge, recombinant fragments prepared in our laboratory is only the third case of hapten binding scFv production by using this library.

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