SUBCLONING, EXPRESSION AND CHARACTERISATION OF A RECOMBINANT ANTIBODY FAB-FRAGMENT SPECIFIC TOWARDS 2,4-D

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ABSTRACT

A generic strategy was established for subcloning the VH and VL gene of antibody variable domains into the plasmid pASK85 for the expression of Fab antibody fragments. pASK85 bears coding sequences for murine constant domains including a His6-tag at the carboxy-terminal end of the constant heavy-chain domain. The VH and VL gene derived from the monoclonal antibody E2/B5 specific towards 2,4-dichlorophenoxyacetic acid (2,4-D) were used in this study. *Eschericia coli* was used as host cells for the biosynthesis of the Fab-fragment. The Fab-fragment was subsequently purified from the periplasmic extract in a single step by immobilised metal-ion affinity chromatography (IMAC). The production levels obtained were 0.5-0.8 mg purified Fab-fragments per liter *E. coli* culture. The sensitivity and cross-reactivity of the Fab-fragment determined by direct competitive ELISA were similar to those of the parental monoclonal antibody E2/B5.

Keywords: 2,4-D, antibody, competitive ELISA, Fab-fragment, IMAC

INTRODUCTION

Recombinant antibody fragments gain in comparison to immunoglobulin antibody molecule increasing importance as biological recognition elements in immunoassays and immunosensors design, since recombinant antibody fragments are amenable to refinement by means of genetic engineering. For instance, the antigen binding-site can be modified to improve the affinity towards the antigen (Chowdary and Pastan, 1999; Kusharyoto *et al.*, 2002) or to change the specificity to the analytes (Iba *et al.*, 1998; Miyazaki *et al.*, 1999).

The bacterial production of antigen-binding immunoglobulin fragments in a functional state was achieved by co-secretion of the two chains of an Fv or Fab-fragment either into the cytoplasm or into the periplasmic space of *E. coli* (Breitling & Dübel, 1997). Since then a variety of different Fv and Fab-fragments has been produced according to the strategies.

For the purification of recombinant antibody fragments from bacterial host, antigen affinity chromatography could be used in some cases. However, its application is restricted when the antigen is scarce, instable or unavailable, and it is also possible that the antibody fragments may loose their original antigen affinities. Several purification tags, such as E-tag (Malone, 1994), FLAG-tag (Knappik & Plückthun, 1994), Strep-tag (Schmidt *et al.*, 1996) or His_6-tag (Skerra *et al.*, 1991) enable simple purification of single-chain Fv antibody fragments without changing their binding properties. However, single-chain Fv fragments show practical disadvantages, since they often possess lowered affinity towards their antigen and were reported to form oligomeric structures (Griffiths *et al.*, 1993). On the other hand, non-linked Fv fragments show a tendency to dissociate into the variable domains (Breitling & Dübel, 1997). Thus, for the study of native antigen-binding properties, Fab-fragments are the molecules of choice due to their higher stability (Skerra, 1994). They are composed of two polypeptide chains, which are covalently connected by a disulfide bond, and behave as stable globular proteins.

2,4-Dichlorophenoxyacetic acid (2,4-D), which is extensively used as herbicide for controlling weeds, exhibits several toxicological

effects. Due to its mobility and widespread application, 2,4-D causes contamination of soil, ground and surface waters. Conventional methods for the detection of 2,4-D include GC or HPLC, which need an extensive sample preparation to reach the required sensitivity. As an effective alternative to instrumental methods, a fast and sensitive procedure for the detection of 2,4-D is provided by immunochemical assays. In the past, immunoassays for detecting 2,4-D were described using polyclonal antisera (Hall *et al.*, 1989, Matuszczyk *et al.*, 1996) or monoclonal antibodies (Franek *et al.*, 1994, Gerdes *et al.*, 1997).

Immunochemical assay is limited not only by the specificity and affinity of the antibody but also by its availability in large quantities. In this paper, the preparation of a 2,4-D-specific antibody Fab-fragment using recombinant technology, its purification by immobilised metal-ion affinity chromatography (IMAC) and its utilisation in ELISA are described.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase (HRP), rabbit anti-mouse antibody (RAM), ampicillin, tetramethyl–benzidine, 30% hydrogen peroxide and imida–zole were obtained from Sigma-Aldrich (Deisenhofen, Germany). N-(Dimethylamino–propyl)-N′-ethyl carbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), Tween 20 and all buffer salts were purchased from Fluka (Buchs, Switzerland).2,4-Dichlorophenoxya–cetic acid and its derivatives were available from Riedel de Häen (Seelze, Germany).

Synthesis of the hapten conjugate 2,4-D-HRP

For the synthesis of the hapten conjugate 2,4-D was coupled *via* its carboxyl group to HRP by carbodiimide/N-hydroxysuccinimide (Märtl–bauer and Terplan, 1988). Briefly, 2,4-D (100 ¼mol), NHS (130 ¼mol) and DSC (40 ¼mol) were mixed in 1 ml dried dimethoxyethane. After 1 h stirring EDC (130 ¼mol) was added and stirred for 4 h to obtain the NHS-ester of 2,4-D. To a solution of HRP (0.15 ¼mol) in 0.5 ml 0f 0.1 M sodium carbonate buffer (pH 8.5) NHS-ester solution was added drop-wise within 15 min. The mixture was then stirred for 2 h at room temperature. After centrifugation the soluble 2,4-D-HRP conjugate was purified by means of gel-filtration on Sephadex G-25 in a PD-10 column(Amersham-biotech, Uppsala, Sweden).
Construction of the plasmid pASK85-24D

The antibody variable light and heavy chain genes were subcloned into pASK85 which was designed for the bacterial expression of Fab-fragments (Skerra, 1994). The V_L encoding gene of the 24-D-specific antibody was amplified using the primers 5„-GACATCGAGCTCACCCAGTCTCCATCCTCCTTAT CT GCC-3„ and 5„-CTTCAGCTCGAG CTT GGTCCCC CCTCCGAACGTGTACGG-3„, resulting in the restriction sites *Sac*I and *Xho*I at the 5„-end and the 3„-end of the V_L coding sequence, respectively. To create the restriction sites *Pst*I at the 5„-end and *Bst*EII at the 3„-end of the V_H coding sequence, the V_H gene was am–plified using the primers 5„-GAAGTTAAACTGCAGCAGTCAGGACCTGAGCTGGTGAAG-„3 and 5„-TGAGGAGACGGT GACCGTG GTCCCTTGGCCCCAGTAAGC-3„, respectively. Enzyme recognition sites for *Sac*I, *Xho*I, *Pst*I and *Bst*EII for insertion into pASK85 are underlined. The amplified genes of the variable regions were purified by electro–phoresis on a 1.5% agarose gel and subsequently extracted with QIAprep Spin Miniprep Plasmid Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The purified V_L amplification product was digested with the restriction enzymes *Sac*I and *Xho*I, whereas the purified V_H gene was cut with *Pst*I and *Bst*EII. After another electrophoresis on 1.5% agarose gel, the digested V_L and V_H genes were isolated with Miniprep Kit. The V_H and V_L genes were consecutively ligated by T4 DNA ligase with the correspondingly digested vector pASK85. The ligation product was purified by agarose-electrophoresis and subsequent plasmid isolation with Miniprep Kit. *E. coli* DH5± cells were transformed with 2 ¼l of the purified ligation product designated pASK85-24D by heat-shock at 42 °C for 45 sec. After growing the transformed cells overnight at 37 °C, colonies were picked for plasmid extraction and subsequent sequencing, which was performed by using ABI PRISM 373 sequencer and dye terminator cycle sequencing

kit with AmpliTaq-DNA polymerase (Applied Biosystems).

Expression and purification

The vector pASK85-24D was transformed into the expression host cells *E. coli* JM101 by heat-shock at 42 ºC for 45 sec. *E. coli* JM101 harboring the expression vector pASK85-24D were grown on LB plates containing 100 ¼g/ml ampicillin. Single colonies were picked and grown overnight in 10 ml LB containing 100 ¼g/ml ampicillin at 30 °C. This culture was then diluted 1:50 with LB-medium containing 100 ¼g/ml ampicillin, grown at 30 °C in 2 l shaking flask to an OD_{550} of 0.8-1.0, after which anhydrotetracycline (IBA, Göttingen, Germany) was added to a final concentration of 0.2 ¼g/ml and growth was continued for 4 h. The cells were harvested by centrifugation (5000 *g*, 15 min, 4°C) and then resuspended in 50 mM PBS containing 300 mM NaCl. After addition of lysozyme to a final concentration of 0.5 mg/ml and incubation at room temperature for 20 min, the suspension was sonicated to isolate the Fab-fragments, which were expressed in the periplasmic space of *E. coli*.

Purification of the Fab-fragments was performed by immobilized metal-ion affinity chromatography (IMAC) on TALON™ chromatography matrix (Clontech, Heidelberg, Germany). Four ml of the matrix were loaded onto a PD-10 column and equilibrated with 50 mM PBS pH 7.2 containing 300 mM NaCl. Ten ml periplasmic extract from 0.4 l culture were applied to the column. After washing with 40 ml of the same buffer the Fab- fragments were eluted from the column with 100 mM imidazole in 50 mM PBS pH 7.2 containing 300 mM NaCl. Removal of the imidazole was performed by gel-filtration on Sephadex G-25 in PD-10 column using 50 mM PBS pH 7.2 as eluent.

ELISA

The IC_{50} values (concentrations at 50% relative absorbance (B/B_0) of the calibration curves) were determined by direct competitive ELISA. In all steps a volume of 200 µl/well was employed. After each incubations the microtiter plate (MaxiSorp, NUNC, Roskilde, Denmark) was washed five times with PBS pH 7.2 supplemented with 0.1% Tween 20. The microtiter plate wells were first coated overnight at 4°C with rabbit anti-mouse antibody (RAM; 1µg/ml in 50 mM carbonate buffer pH 9.6). The Fab-fragments (0.2 ¼g/ml in 80 mM PBS pH 7.2) were then bound to the RAM by incubation for 2 h at room temperature. The enzyme tracer was mixed with a serial dilution of the hapten and incubated in the wells at room temperature for 1 h. After incubation of 200 µl of the color reagent (400 ¼l TMB-solution (6 mg TMB in 1 ml DMSO) and 100 ¼l of 1% H_2O_2 dissolved in 25 ml of 50 mM Na-acetate buffer pH 5.5) for 15 min and stopping the reaction with 100 µl 1 M H_2SO_4 the absorbance in each well was read at 450 nm, with 620 nm as reference. Cross-reactivity was calculated as the ratio of the IC_{50} value for 2,4-D to the IC_{50} value of the cross-reacting herbicides. The limit of detection was determined by substracting the threefold standard deviation (error probability = 3%) from the maximum relative absorbance (Matuszczyk *et al*., 1996).

RESULTS AND DISCUSSIONS

Subcloning of V_H and V_L encoding genes into pASK85

The plasmid vector pASK85 is a general vector for cloning and functional expression of recombinant antibody Fab-fragments in *E. coli* (Skerra, 1994). The vector bears coding sequences for the C_H1 domain of murine class IgG1 including a His_6-tag at its carboxy-terminal end of the constant heavy chain domain. The light chain domain of pASK85 ends with the sequence for the murine $C_κ$ constant domain. To obtain the vector for the expression of 2,4-D specific Fab-fragments the antibody variable light and heavy chain genes derived from the 2,4-D specific monoclonal antibody were subcloned into the expression vector pASK85. The transfer of V_H and V_L genes into pASK85 is schematically shown in Fig. 1.

PCR amplification of the genes encoding the V_H and V_L regions were performed separately. The primers were designed to anneal at both ends of the variable gene, within framework regions 1 and 4 of V_H and those of V_L. The amplification reactions created singular restriction sites *Pst*I at the 5„-terminus and *Bst*EII at the 3„-terminus of the V_H domain, and *Sac*I at

the 5„-terminus and *Xho*I at the 3„-terminus of the V_L domain, respectively. After purification and extraction, the amplification products were subsequently digested with the corresponding restriction enzymes and inserted into the vector pASK85 in two consecutive reactions; the digested V_H gene was first inserted into pASK85, followed by insertion of the digested V_L gene (Fig. 1) to accomplish the Fab-fragment expression cassette. Since the subcloning primers were designed to anneal within the flanking regions of murine variable genes, the described procedure can be adapted to transfer the V_H and V_L genes from a phagemid system such as pCANTAB 5 E (Malone, 1994), pHEN-1 (Hoogenboom, *et al*., 1991), pSEX (Dübel *et al*., 1999) or pCOCK (Engelhardt *et al*., 1994) into the expression vector pASK85. The subcloning procedure will be accompanied by a change from the scFv format to a Fab-fragment, which is enabled by the presence of murine constant domains on pASK85 adjacent to the cloning sites. In contrast to scFv fragment, which is stabilised by an artificial peptide linker, the Fab heterodimer is stabilised by a physiological disulfide bridge between the constant domains (Skerra, 1994).

After transformation of *E. coli* DH5± cells with pASK85-24D, two independent clones were analyzed by DNA sequencing of the variable regions, which revealed that the sequences were similar (data not shown). The amino acid sequence of the V_H and V_L domain of the 2,4-D-specific antibody Fab-fragment and the assignment of the complementarity determining regions (CDRs) are shown in Fig. 2. According to the amino acid sequence, the binding-site of 2,4-D is formed by CDR L1, CDR H1 and CDR H3 with short loops, consistent with the fact that hapten coupled directly to the marker enzyme during immunization will be bound in the binding-site in form of a shallow groove.

Expression and purification of the Fab-fragment specific towards 2,4-D were performed as described (Skerra, 1994; Kusharyoto *et al*., 2002). The single His_6 tail fused to the C-terminus of the heavy chain confers sufficient affinity to immobilized metal ion in order to enable affinity purification of the functional recombinant Fab-fragments to homogeneity. When the periplasmic cell fraction of *E. coli* containing Fab24D was applied to the column containing TALON™ matrix, a chelating resin composed of Sepharose activated with carboxy-methylated aspartic acid and charged with Co^{2+} ions, single protein fraction was obtained with imidazole elution (Fig. 3). Thus, the Fab-frag–ment was efficiently purified in a single step from the periplasmic extract of *E. coli* by using IMAC on TALON™ matrix.

In SDS-PAGE, a double band corres–ponding to a molecular mass of approximately 26 kDa was observed due to reducing conditions

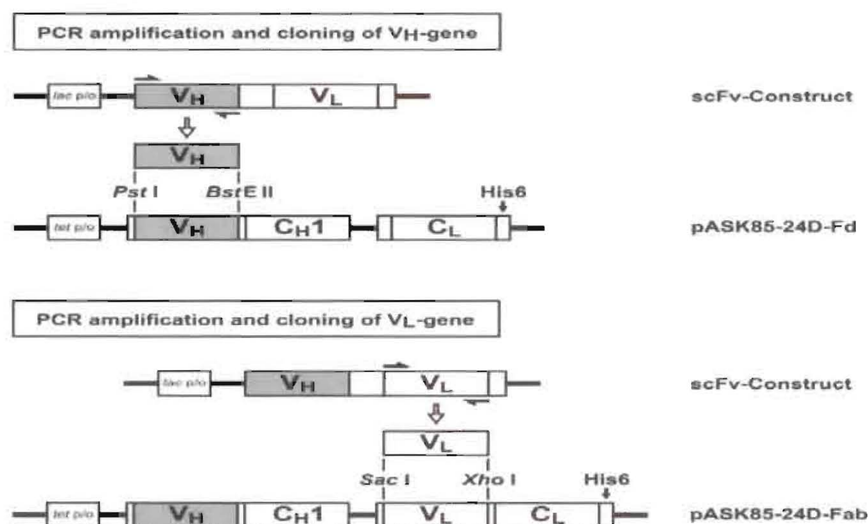

Figure 1. Steps for subcloning the V_H and V_L genes derived from the monoclonal antibody specific towards 2,4-D into pASK85

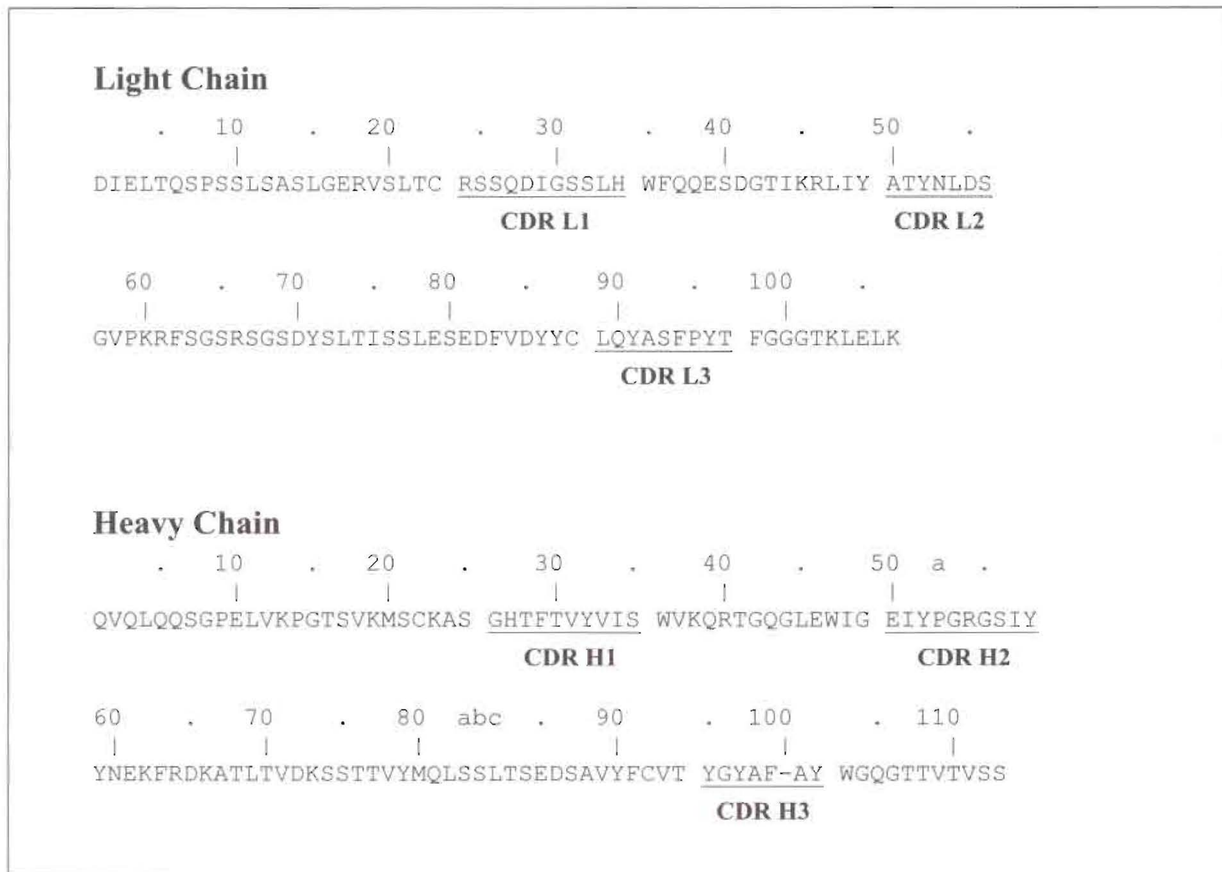

Figure 2. Amino acid sequences of the V_H and V_L domain of the 2,4-D-specific antibody Fab-fragment and the assignment of the complementarity determining regions (CDRs).

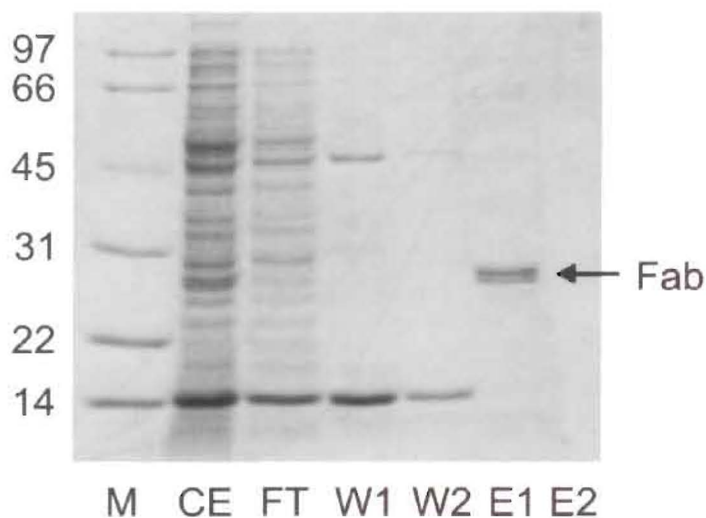

Figure 3. SDS gel electrophoresis of the purification of Fab fragment Fab24D by using IMAC on TALON™ matrix. M: marker, CE: cell extract, FT: flow-through, W1, W2: washing step 1 and 2, E1, E2: elution step 1 and 2

for SDS-PAGE, which cleaved the interchain disulfide bridge between C_L and C_H1 domains of the antibody. The production levels obtained were 0.5-0.6 mg purified Fab-fragments per liter *E. coli* culture, which were considerably lower compared to the production levels (3-4 mg/l culture) of the atrazine-specific Fab-fragment K411B (Kusharyoto *et al.*, 2002). The difference in the production levels could be solely due to the differences in the amino acid sequences of both V_H and V_L domains (Knappik & Plückthun, 1995) between the atrazine- and 2,4-D-specific antibody fragments, since the V_H and V_L genes of the atrazine-specific antibody were subcloned with the same procedure into the vector pASK85.

The functional characteristics of the Fab-fragments were determined by a direct competitive ELISA. Rabbit anti-mouse sera proved to be a suitable reagent for the immobilization of the Fab-fragments onto the surface of the MTP wells. 2,4-D was used at different concentrations and the 2,4-D-HRP tracer competed for the binding sites of the immobilised Fab-fragments. The normalised ELISA curves shown in Fig. 4 were obtained after optimising the concentration of Fab-fragment as well as the 2,4-D-HRP tracer (data not shown). The ELISA for 2,4-D showed an IC_{50} value of 13.7 nM (3.4 ¼g/l) 2,4-D, and a detection limit at 10 % inhibition of 1.1 nM (0.24 ¼g/l), which were similar to the values obtained possess similar sensitivity and selectivity as the parental monoclonal antibody. An important aspect in environmental analysis concerns the long-term stability of antibody fragments. The fragments should retain their biochemical activity upon storage at least for one year if they ought to be integrated in extensive monitoring programs or supplied on pre-coated sensor chips. As shown in Fig. 5, the recombinant antibody Fab-fragment Fab24D showed prolonged stability upon long-term storage at 4°C.

CONCLUSION

A generic strategy has been established for subcloning the VH and VL genes of antibody variable domains into the vector plasmid pASK85 for the expression of antibody Fab-fragments in *E. coli*. The ease in production of Fab-fragments in large quantities and the similarity in sensitivity and cross-reactivity between the Fab-fragments and their parental monoclonal antibody and the prolonged integrity of Fab-fragments will facilitate their availability for the development of immunosensors.

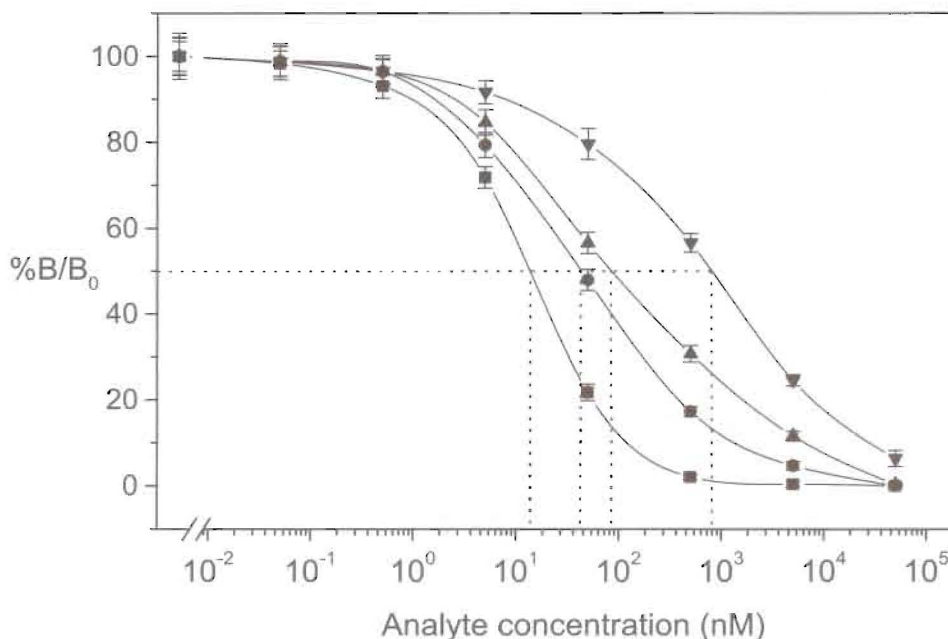

Figure 4. Normalized calibration curves of direct-competitive ELISA with the Fab-fragment Fab24D for 2,4-D (■), 2,4,5-T (●), MCPA (▲) and MCPB (▼). Dilution of the enzyme tracer: 1:5000; error bars: range of triple determination.

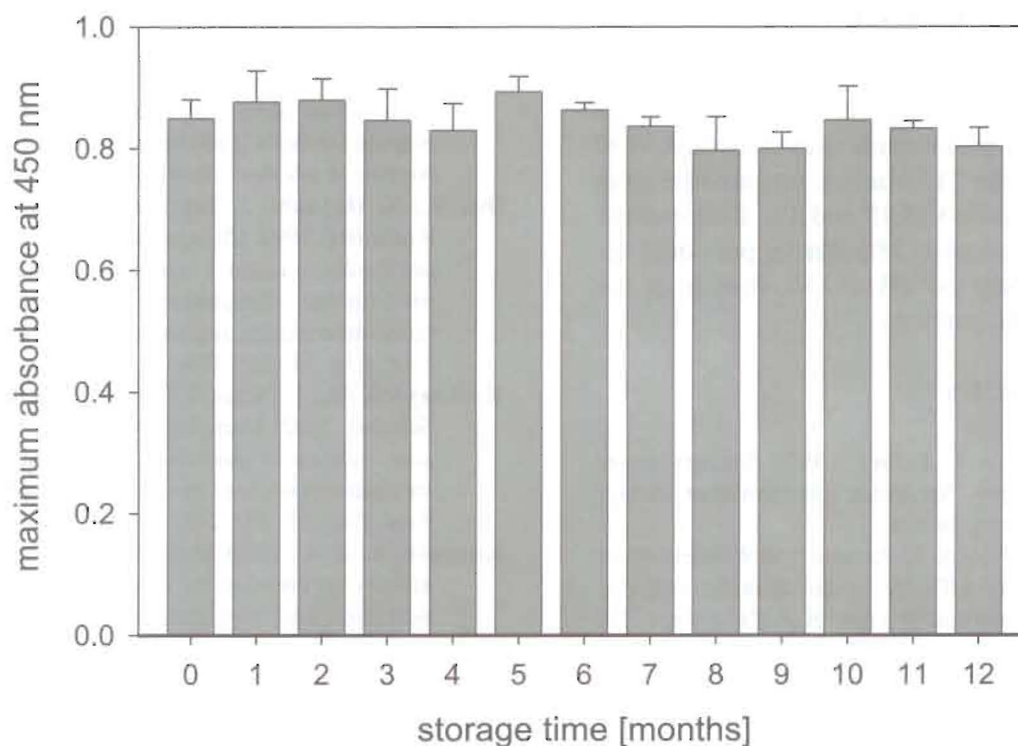

Figure 5. Stability-test of the Fab-fragment Fab24D upon long-term storage at 4°C. The concentration of the Fab-fragment was adjusted to 1 ¼g/ml in PBS buffer (80 mM, pH 7.2). The solutions of Fab-fragment were stored at 4°C. Dilution of the enzyme tracer: 1:5000; error bars: range of triple determination.

Table 1. Cross-reactivity of the Fab-fragment Fab24D towards some major derivatives of 2,4-D.

Herbicide	Structure	IC_{50} (nM)	Cross-reactivity (%)
2,4-D	Cl– –OCH_2COOH Cl	13.7	100
2,4,5-T	Cl Cl– –OCH_2COOH Cl	85.7	16
MCPA	Cl– –OCH_2COOH CH_3	44.2	31
MCPB	Cl– –$O(CH_2)_3COOH$ CH_3	806	1.7

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REFERENCES

Breitling, F. & F. Dübel. 1997. *Rekombinante Antikörper*. Spektrum Akademischer Verlag, Heidelberg-Berlin.

Chowdary, P.S. & I. Pastan. 1999. Improving antibody affinity by mimicking somatic hypermutation in vitro. *Nat. Biotechnol.* 17: 568-572.

Engelhardt, O., R. Grabherr, G. Himmler & F. Rüker. 1994. Two step cloning of antibody variable domains in a phage display vector. *BioTechniques* 17, 44-46.

Fránek, M., V. Kolár, M. Granátová & Z. Nevoránková. 1994. Monoclonal ELISA for 2,4-Dichlorophenoxyacetic acid: Characteri–zation of antibodies and assay optimization. *J. Agric. Food. Chem.* 42: 1369-1374.

Gerdes, M., M. Meusel & F. Spener. 1997. Development of a displacement immunoassay by exploiting cross-reactivity of a monoclonal antibody. *Anal. Biochem.* 252: 198-204.

Giersch, T., K. Kramer & B. Hock. 1993. Optimization of a monoclonal antibody-based enzyme immunoassay for the detection of terbuthylazine. *Sci. Total Environ.* 132: 435-448.

Griffiths, A.D., M. Malmqvist, J.D. Marks, J.M. Bye, M.J. Embleton, J. McCafferty, M. Baier, K.P. Holliger, B.D. Gorick, N.C. Hughes-Jones, H.R. Hoogenboom & G. Winter. 1993. Human anti-self antibodies with high specificity from phage display libraries. *EMBO J.* 12: 725-734.

Hall, J.C., R.J.A. Deschamps & K.K. Kreig. 1989. Immunoassays for the detection of 2,4-D and picloram in river water and urine. *J. Agric. Food. Chem.* 37: 95-108.

Hoogenboom, H., A. Griffiths, K. Johnson, D. Chisswell, D. Hudson & G. Winter. 1991. Multi-subunit proteins on the surface of filamentous phage: methodologies for dis–playing antibody (Fab) heavy and light chains. *Nucleic Acids Res.* 19: 4133-4137.

Iba, Y., N. Hayashi, J. Sawada, K. Titani, & Y. Kurosawa. 1998. Changes in the specificity of antibodies against steroid antigens by introduction of mutations into complemen–tarity-determining regions of the V_H domain. *Prot. Eng.* 11: 361-370.

Kusharyoto, W., J. Pleiss, T.T. Bachmann & R.D. Schmid. 2002. Mapping of a hapten binding site: molecular modeling and site-directed mutagenesis study of an anti-atrazine antibody. *Prot. Eng.* 15: 233-241.

Knappik, A. & A. Plückthun. 1994. An improved affinity tag based on the FLAG peptide for the detection and purification of recombinant antibody fragments. *Biotechniques* 17: 754-761.

Malone, J.A. 1994. pCANTAB 5 E: a vector designed for the expression of antibody variable regions as phage-displayed or soluble single-chain proteins (scFv). Direct Sub–mission, Pharmacia Biotech Inc.

Matuszczyk, G., D. Knopp, & R. Nießner. 1996. Development of an ELISA for 2,4-D: characterization of two polyclonal antisera. *Fresenius J. Anal. Chem.* 354: 41-47.

Miyazaki, C., Y. Ina, Y. Yamada, H. Takahashi, J. Sawada & Y. Kurosawa. 1999. Changes in the specificity of antibodies by site-specific mutagenesis followed by random mutagenesis. *Prot. Eng.* 12, 407-415.

Schiweck, W. & A. Skerra. 1995. Fermenter production of an artificial Fab-fragment, rationally designed for the antigen cystatin, and its optimized crustallization through constant domain shuffling. *Proteins: Struct. Funct. Genet.* 23: 561-565.

Schmidt, T.G., J. Koepke, R. Frank & A. Skerra. 1996. Molecular interaction between the Strep-tag affinity peptide and its cognate target, streptavidin. *J. Mol. Biol.* 255: 753-766.

Skerra, A., I. Pfitzinger & A. Plückthun. 1991. The functional expression of antibody Fv fragments in *Escherichia coli*: Improved vectors and a generally applicable purification technique. *Bio/Technology* 9: 273-278.