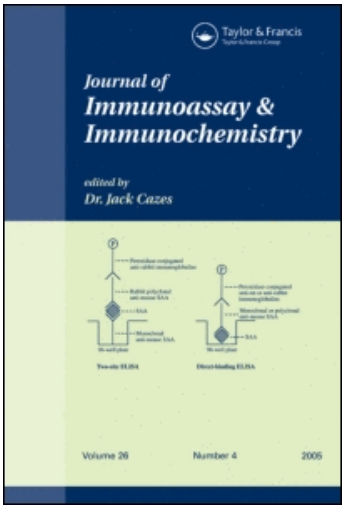

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ABSTRACT

A system was constructed for the production of alkaline phosphatase (aP)-labeled antibody single-chain F_v (scFv) fragments in *Escherichia coli*. The expression vector pASK75 was modified by sequentially inserting the *E. coli* aP coding region and the scFv cloning cassette. Engineering the cloning sites *Sfi*I and *Not*I located at the 5′ and 3′ end of the scFv gene provides an easy means to insert scFv fragments. These cloning sites are widely used in recombinant antibody technology and, thus, enable the one-step cloning of scFv fragments derived from corresponding antibody phage libraries into the expression vector. An expressed herbicide-specific scFv–aP fusion protein retained both, analyte binding and enzymatic activity, as determined by ELISA. Therefore, this

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system permits the production of scFv–aP conjugates in *E. coli*, which can replace conventionally prepared aP-labeled antibodies in immunoassays. These fusion proteins are designed to accelerate the immunochemical detection of analytes, since the assay duration is essentially reduced by omitting the use of enzyme labeled secondary antibodies.

Abbreviations: aP, Alkaline phosphatase; F_v, Fragment variable; HRP, Horseradish peroxidase; OD, Optical density; OVA, Ovalbumin; PCR, Polymerase chain reaction; scFv, Single-chain F_v; TBS, *Tris*-buffered saline

INTRODUCTION

Immunoassays are widely used as diagnostic tools for the detection of trace amounts of analytes. They are based on the specific association between antigen and antibody. Standardized immunochemical methods could be established by the supply of monoclonal and polyclonal antibodies. The development of recombinant antibody technologies and the use of bacteria to produce different antibody fragments (1,2) provide an alternative to monoclonal and polyclonal antibody production. The cultivation and expression in *E. coli* is simpler and less time consuming than the production of monoclonal antibodies in cell culture or polyclonal antisera in animals. In addition, molecular cloning techniques have facilitated the modification of antibody genes in order to improve antibody affinity or specificity. The polymerase chain reaction (PCR) offers the possibility to isolate the light and heavy chain encoding DNA sequences of antibody clones (3,4) and to construct small antibody fragments,(5,6) which evoke less background reactions in the absence of the constant regions of the antibody polypeptide chain.

Analyte detection in competitive immunoassays is carried out with primary or secondary antibodies that are labeled with sensitive reporter molecules, like fluorescent dyes or enzymes. Horseradish peroxidase (HRP) and alkaline phosphatase (aP) are commonly used enzymes for labelling antibodies by means of established coupling procedures.(7,8) However, these methods may lead to randomly cross-linked molecules, and require several steps to obtain the final antibody–enzyme conjugates. In the case of single-chain F_v fragments (scFv), analyte binding is usually visualized by the detection of a peptide-tag fused to the scFv (e.g., c-myc tag,(9) E-tag (10)). This is achieved by an anti-tag antibody chemically or covalently linked to a reporter enzyme (e.g., anti-E-tag IgG-HRP, Pharmacia). An attractive

alternative to the chemical coupling of these proteins is the construction of genetically engineered fusion proteins consisting of an enzyme and an analyte-specific antibody moiety.(11,12)

The development of a recombinant hapten detection system for scFv antibodies is reported here. To illustrate the principle, a fusion protein consisting of a herbicide-specific scFv antibody and the *E. coli* enzyme aP (Schmidt-Dannert, unpublished) was constructed. The gene of the atrazine-specific scFv antibody K47H (13) was taken for the construction of the antibody-cloning cassette. In order to produce and express the fusion product, the scFv gene flanked with the restriction sites *Sfi*I and *Not*I was inserted into the expression vector pASK75.(14) This vector contains the *Strep*-tag for the subsequent purification of proteins by streptavidin affinity chromatography due to the high affinity of the *Strep*-tag to streptavidin. This system is particularly suited for the purification of fusion proteins, which are expressed at low quantities. ScFv fragments derived from corresponding antibody libraries can easily be exchanged by restriction digest at the inserted restriction sites *Sfi*I and *Not*I, which are commonly used in the recombinant antibody production (e.g., pCANTAB 5E system by Pharmacia).

This system reduces the time expenditure needed for performing immunoassays by omitting the application of enzyme-labeled secondary antibodies. It is suitable for the preparation of aP-labeled scFv antibodies and demonstrates that recombinant scFv–aP conjugates can replace conventionally generated, enzyme-labeled antibodies in immunoassays.

EXPERIMENTAL

Recombinant DNA techniques were based on standard protocols.(15) The *E. coli* strain JM83 (16) was used for cloning and DNA preparation procedures.

Oligonucleotides were synthesized by Eurogentec, Belgium. All amplifications were carried out with the PCR kit and the T4 DNA polymerase obtained from Applied Biosystems (Weiterstad, Germany). PCR was performed in the DNA Thermal Cycler PCR Express (Hybaid, UK). The restriction endonucleases and the T4 DNA ligase were obtained from MBI Fermentas (St. Leon-Rot, Germany). Cell densities and alkaline phosphatase activity were measured using the photometer LP 300 (Lange, Germany) and the ELISA reader Spectra Fluor Plus (Tecan, Grödig, Austria), respectively. DNA sequences were determined by SequiServe (Vaterstetten, Germany).

The chemicals and other reagents, unless indicated otherwise, were obtained from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany).

Construction of the Expression Vector pscFvaP

The *E. coli* aP gene was isolated from the cloning vector pPHOA4 (Schmidt-Dannert, unpublished) by PCR using the two oligonucleotides APBACK (5′-CGATAGTCGACCGGACACCAG AAATGCCTGTTC-3′) and APFOR (5′-AGCATAGCGCTCATGGTGTAGAAGAGATCGGTC-3′). The amplification product (1.3 kb) was purified on agarose gel and digested with the restriction endonucleases *Eco*47III and *Sal*I. The restriction sites (underlined) were added to the aP gene by the PCR oligonucleotides. The digested aP fragment was inserted into the *Eco*47III/*Sal*I digested pASK75 vector. The restriction sites are located in the multiple cloning site (MCS) of pASK75. The gene of scFv K47H was amplified by PCR using the two oligonucleotides SCFVBACK (5′-GTTAGGCCTT**GGCCCAGCCGGC-C**ATGGC-3′) and SCFVFOR (5′-CTAGTCGACT**GCGGCC GC**CGTTT-GATTTC -3′). These oligonucleotides contained the recognition sites for the restriction endonucleases *Sfi*I and *Not*I (in bold letters), commonly used in recombinant antibody technology. The recognition sites for *Stu*I and *Sal*I (underlined) were inserted for cloning the scFv fragment flanked with the *Sfi*I and *Not*I-sites into the pASK75/aP vector. The amplified scFv fragment was purified on agarose gel and digested with the restriction endonucleases *Stu*I and *Sal*I. The digested scFv fragment was inserted into the *Stu*I/*Sal*I digested pASK75/aP vector, forming the plasmid pscFv(K47H)aP (cf. Figure 1).

Bacterial Expression

PscFvaP plasmids encoding the fusion protein scFv(K47H)-aP were used to transform competent *E. coli* JM83 cells by electroporation. Individual colonies were picked after overnight incubation on LB agar plates containing 0.1 mg/mL ampicillin. The colonies were incubated overnight at 30°C on a shaker at 200 rpm in LB medium containing 0.1 mg/mL ampicillin. The 1 : 50 dilution of the overnight culture in LB/Amp medium was grown at 22°C and 200 rpm until an OD_{550} of 0.5 was obtained. After the addition of 0.4 μM anhydrotetracycline (Acros, USA), the scFv(K47H)-aP fusion protein was expressed at 22°C and 200 rpm for 3 h. The bacterial cells were collected by centrifugation at 1000 × *g* for 12 min at 4°C. The cell pellet

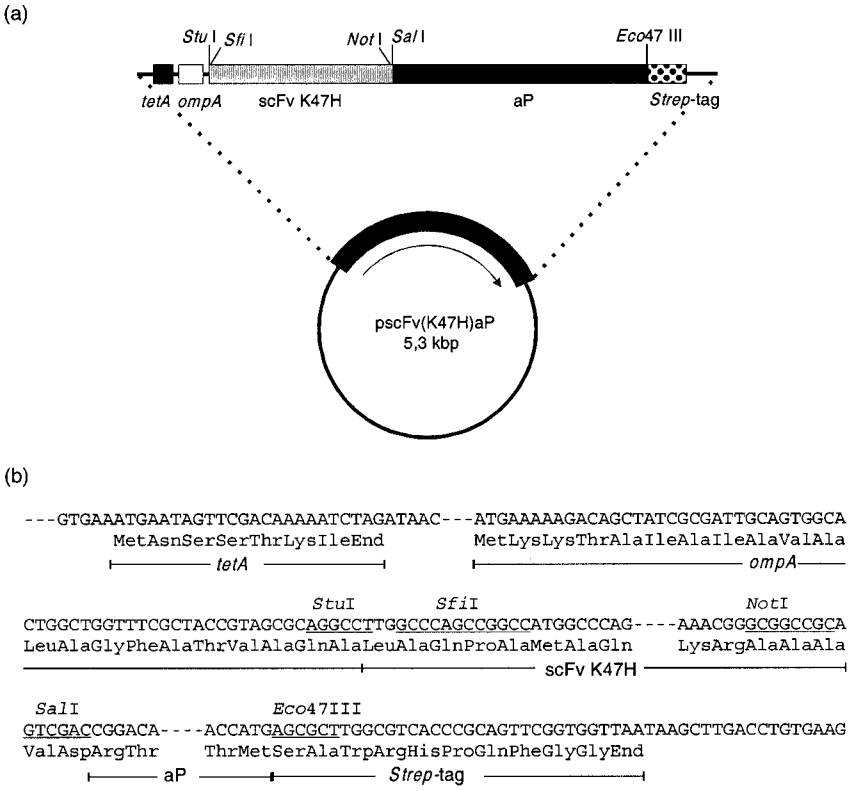

Figure 1. pscFv(K47H)aP expression plasmid. (a) Schematic representation of the vector pscFv(K47H)aP derived from the vector pASK75. The expression vector was designed to produce a recombinant scFv antibody-aP fusion protein in the periplasm of *E. coli*. The fusion protein is under the transcriptional control of the *tetA* promoter. *OmpA* and strep tag indicate the *ompA* leader and the *Strep*-tag sequences, respectively. ScFv(K47H) and aP represent the DNA sequences encoding the atrazine-specific scFv antibody fragment and the aP. The restriction enzymes *Sal*I, *Eco*47III, and *Stu*I were used for inserting the aP and the scFv genes. The system is compatible for other scFv fragments flanked with the restriction sites *Sfi*I and *Not*I. The restriction sites were incorporated in the vector via the scFv gene. (b) Nucleotide and amino acid sequences of the scFv(K47H)-aP fusion gene in pscFv(K47H)aP. The aP gene was cloned to the 5′ end of the *Strep*-tag with the restriction enzyme sites *Sal*I and *Eco*47III. The scFv fragment, including the restriction enzyme sites *Sfi*I and *Not*I, was cloned at the N-terminus of aP with the restriction sites *Stu*I and *Sal*I.

was resuspended in 100 mM *Tris*/HCl pH 8.0, 0.5 M Saccharose, 1 mM EDTA and centrifuged at 1000 × *g* for 10 min at 4°C. The obtained periplasmic cell fraction containing the soluble scFv(K47H)-aP fusion protein was dialysed (Spectra Por CE, 8.000, Spectrum, USA) overnight against 100 mM sodium chloride, 100 mM *Tris*/HCl (pH 8.0), 1 mM EDTA.

Protein and Western Blot Analysis

SDS-PAGE was carried out with a 10.5% polyacrylamide gel. For the detection of streptavidin binding activity of the fusion protein, the bands of the electrophoresed periplasmic cell fraction were transferred to a nitrocellulose membrane, which was blocked with 3% (w/v) BSA and 0.5% Tween 20 in 50 mM *Tris* buffered saline (TBS), pH 7.8, for 1 h. Avidin (2 μg/mL 50 mM TBS) was added and incubated for 10 min to suppress the background signal, which was due to the biotin carboxyl carrier protein from the cytoplasm.(17) The membrane was then washed with 0.05% Tween 20/TBS, followed by an incubation with streptavidin conjugated horseradish peroxidase for 1 h. The binding of the labeled streptavidin to the fusion protein was visualised by staining with 1% (w/v) dextran sulfate in 200 mM KH_2-citrate, 1 mM sorbic acid, pH 3.8, adding 1.67% (v/v) 1.2 mM 3,3′,5,5′-tetramethylbenzidine (TMB), 20% (v/v) dimethylsulfoxide in methanol, and 0.33% (v/v) H_2O_2 Blue precipitates developed and, after a 3 minute incubation, the colour reaction was stopped by washing with bidistilled water.

ELISA with scFv(K47H)-aP Fusion Protein

The functionality of the expressed fusion protein scFv(K47H)-aP was determined by an indirect, competitive ELISA. Ninety six-well microtiter plates (Greiner, Germany) were coated with 200 μL/well of 5 μg/mL atrazine derivative 4-chloro-6-(isopropylamino)-1,2,5 triazine-2-(6-aminohexane)-carboxylic acid (IPR) conjugated to ovalbumin (OVA) (in 50 mM sodium carbonate buffer, pH 9.6) overnight at 4°C. The washing steps were performed three times with 200 μL/well of 5 mM TBS/Tween 20, pH 7.8. One hundred μL/well of the periplasmic extract (diluted 1 : 100 in PBS) containing the fusion protein were incubated together with 100 μL/well of atrazine standards for 1 h at room temperature. After washing the plates three times with 200 μL/well of TBS/Tween 20, alkaline phosphatase activity was assayed after adding 200 μL/well aP-substrate containing 0.5 mg *p*-nitrophenyl

phosphate per mL 70 mM diethylamino buffer, pH 9.8. The absorption was measured at 405 nm with the reader Spectra Flour Plus, Tecan.

RESULTS

Synthesis of the scFv-Alkaline Phosphatase Fusion Gene Vector

A system for the production of fusion proteins, consisting of scFv antibodies and alkaline phosphatase, was constructed. For this purpose, a fusion gene encoding both an atrazine-binding scFv protein and an active alkaline phosphatase enzyme was inserted into the expression vector pASK75.(14)

The *E. coli* alkaline phosphatase gene, derived from pPHOA4 (Schmidt-Dannert, unpublished), was cloned into pASK75 using the two restriction sites *Eco*47III and *Sal*I. The restriction sites are conserved in the MCS of the expression vector. They were added at the 5′ and 3′ end of the aP gene by means of the PCR primers APBACK and APFOR. The aP amplification product was digested with the analogous restriction enzymes and inserted into the linearized pASK75.

Isolation and PCR amplification of the gene encoding the scFv(K47H) (13) and the antibody cloning sites *Sfi*I and *Not*I were performed using the scFv harbouring pCANTAB 5 E phagemid DNA as template. By inserting the scFv gene, the restriction sites, *Sfi*I and *Not*I, were engineered. Since the two restriction enzymes are commonly used in the recombinant antibody technology, they enable the one-step cloning of scFv fragments derived from compatible antibody libraries into the vector. In order to clone the scFv fragment into the aP containing pASK75 vector (pASK75/aP), the restriction sites *Sal*I and *Stu*I were added to the antibody gene by the PCR primers SCFVBACK and SCFVFOR. The restriction site *Stu*I is also located in the MCS of pASK75. Both the amplified scFv fragment and the recombinant plasmid pASK75/aP derived from transformants were digested with *Sal*I and *Stu*I. Subsequently, the digested scFv fragment was inserted upstream to the aP coding region into the linearized pASK75/aP. Figure 1a presents the modified pASK75 vector with the inserted fusion gene (pscFvaP). The restriction sites *Stu*I, *Sal*I, and *Eco*47III used for the vector construction and *Sfi*I and *Not*I for cloning of corresponding scFv fragments are also shown.

DNA sequencing of the scFv(K47H)–aP construct proved the exact cloning in frame and the identity to the corresponding scFv(K47H) and aP sequences. The sequence of the *tet*A promoter controlled fusion gene, including the peptide sequence of the *Strep*-tag, is shown in Figure 1b.

Expression of Functional Fusion Protein

Individual colonies of pscFvaP transformed *E. coli* JM83 cells were picked and incubated overnight at 30°C. The 1 : 50 dilution of the overnight culture was grown at 22°C until an OD_{550} of 0.5 was obtained. By adding anhydrotetracycline, the scFv(K47H)–aP fusion protein was expressed. After centrifugation, the bacterial cells were resolved with saccharose buffer. The obtained periplasmic cell fraction, containing the soluble scFv(K47H)–aP fusion protein, was analysed by SDS-PAGE under reducing conditions (Figure 2a). The protein bands of the electrophoresed periplasmic extract were blotted onto a nitrocellulose membrane. The streptavidin binding fusion protein was visualised with the expected size of 75 kDa by incubating the nitrocellulose membrane with streptavidin-HRP conjugate and, subsequently, with HRP-substrate (Figure 2b). The blot result proved the retained binding ability of the strep tag to streptavidin, even under reducing conditions. The affinity to streptavidin can additionally be used for protein purification via affinity chromatography, if a more refined reagent is required.

The functional characterization of the fusion protein, i.e., the binding of the scFv(K47H) to atrazine and the functionality of the aP, was carried

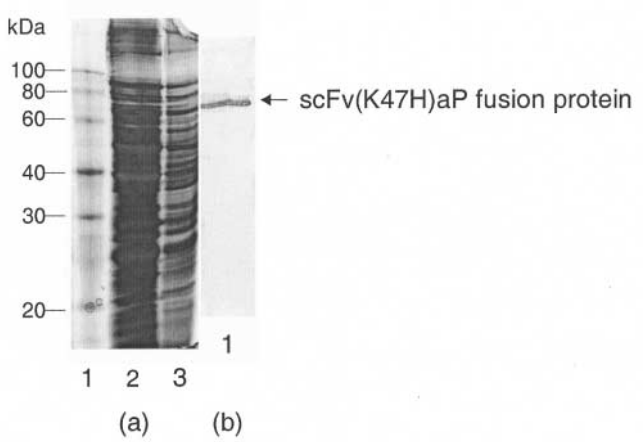

Figure 2. Characterisation of fusion protein. (a) SDS-PAGE analysis of periplasmic extract stained with silver nitrate. Molecular mass marker is shown in lane 1. Lane 2, periplasmic extract. (b) Western blot analysis of the streptavidin binding activity. Samples were electrophoresed in a SDS-gel (same conditions as a), transferred to nitrocellulose membrane, and reacted with HRP-labeled streptavidin. Lane 1, periplasmic extract.

out by indirect, competitive ELISA. The periplasmic extract (diluted 1 : 100) was applied in the ELISA. An atrazine derivative was coupled to OVA and used as coating conjugate. The enzyme reaction was recorded after various time periods at 405 nm. The atrazine calibration curves, determined at hourly intervals from 2–7 h, are shown in Figure 3. The mean value of the detection limit, based on the different calibration curves, was calculated at 30 μg/L, the IC_{50} at 56 μg/L atrazine. The OD of the aP increased with increasing incubation times. However, the detection limit and the IC_{50} of the different calibration curves only changed within a very small range, 29–31 μg/L and 54–60 μg/L atrazine, respectively. The corresponding values for the unfused scFv(K47H) were determined at 2.5 μg/L atrazine for the detection limit and 29 μg/L atrazine for the IC_{50}.(13) These differences in sensitivity may be explained by the application of the unpurified periplasmic extract containing the soluble fusion proteins to ELISA.

Cross-reactivities were determined for 8 *s*-triazines (Figure 4). They are based on the ratio of IC_{50} values obtained by ELISA. The cross-reactivity

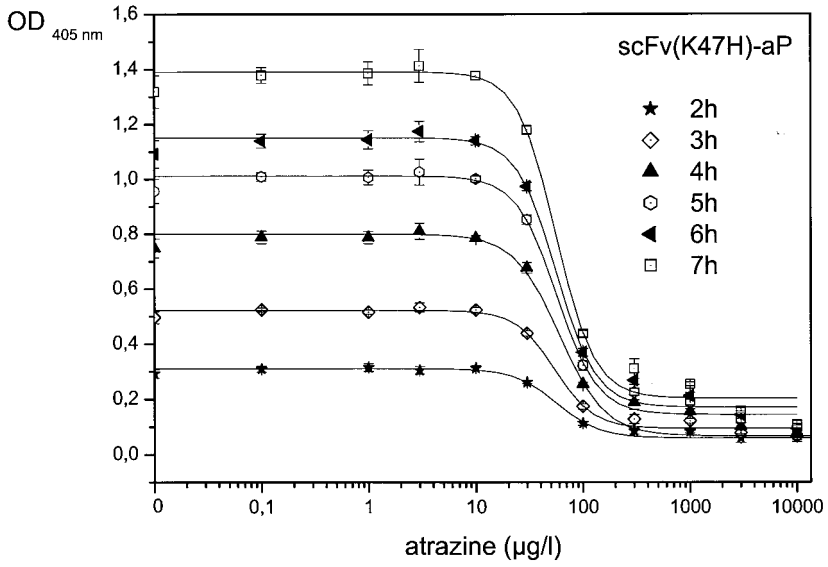

Figure 3. scFv(K47H)–aP calibration curves for the detection of atrazine based on an indirect, competitive ELISA. An atrazine derivative coupled to OVA was used as coating conjugate. One hundred μL periplasmic extract (diluted 1 : 100) containing the soluble fusion protein were incubated for 1 h together with 100 μL atrazine standards per well. Following subsequent chromogenic aP reaction, the absorbance was measured after different incubation times at 405 nm at hourly intervals from 2–7 h.

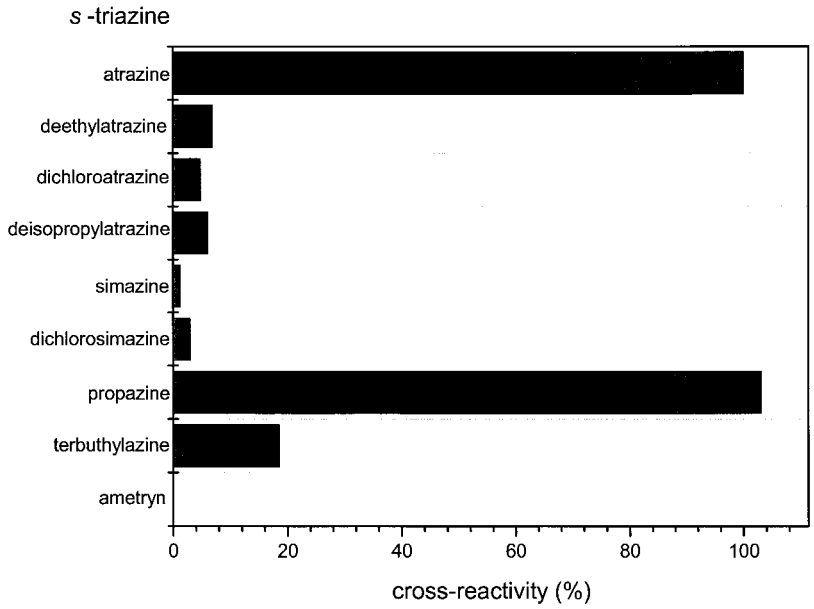

Figure 4. Cross-reactivity of the scFv(K47H)–aP fusion protein calculated by the ratio of IC_{50} values obtained by competitive ELISA. Cross-reactivities are based on atrazine (=100%).

values of the scFv–aP fusion protein to the chosen targets did not show a significant deviation from the values of the unfused K47H (cf. ref. 13).

The ELISA results indicated that the binding characteristics of the scFv(K47H) and the functionality of the enzyme aP were basically retained for the constructed fusion protein.

In conclusion, the reported vector system, pscFvaP, is suitable for generating and expressing scFv–aP fusion proteins with both the scFv-binding properties and the aP catalytic activity. The inserted restriction sites for the restriction enzymes *Sfi*I and *Not*I enable the one-step cloning of compatible scFv fragments into pscFvaP. The expressed fusion proteins can be applied in immunoassays without requirement of enzyme labeled antibodies.

DISCUSSION

A system for the simple and rapid production of scFv–aP recombinant fusion proteins was designed. The expression vector pASK75 (14) was used

for cloning and expression of the fusion proteins. This vector incorporates the signal peptide coding region of the *ompA* gene to direct translocation of the recombinant antibody fusion protein into the periplasm. The export to the *E. coli* periplasm facilitates correct folding and disulphide bond formation of recombinant antibodies, resulting in soluble, functional antibodies.(18) The extraction of functional fusion proteins from the periplasmic compartment can easily be carried out by cell lysis. Expression of the fusion protein under the control of the *tetA* promoter offers an economic alternative to IPTG-inducible promoter constructs, such as the lac promoter and its derivatives.(19) Thus, the *tetA* promoter system enables the economic large-scale production of recombinant proteins.

In addition, the incorporated *Strep*-tag facilitates the detection and purification of bacterially expressed protein.(14) In this study, the atrazine specific scFv antibody K47H (13) inserted into the phagemid pCANTAB 5E served as scFv template for the vector construction. The scFv fragment was attached to the 5′ end of the previously inserted *E. coli* aP. Simultaneously, the scFv restriction sites *Sfi*I and *Not*I incorporated into the vector. Thus, the generated expression system, pscFvaP, is compatible to phage display vectors containing the *Sfi*I/*Not*I cloning sites, e.g., the pCANTAB series. The system allows simple subcloning of any scFv fragments selected from *Sfi*I- and *Not*I-using phage display libraries to get further scFv–aP fusion proteins.

Antibody-enzyme conjugates have drawn much attention because of their potential application as immunological tools. However, it may not always be the case that both moieties of the fused protein retain their biological functions. As revealed by ELISA (cf. Figure 3), the bifunctional recombinant protein consisting of the scFv antibody K47H and the *E. coli* aP retains both the atrazine binding capacity and the aP enzymatic activity.

Similar recombinant fusion proteins with aP enzymatic activity and antigen binding capacity have been reported. Ducancel et al.(20) and Weiss et al.(21) reported the construction of recombinant F_{ab}–aP fusion proteins, while Wang et al.(22) prepared the fusion of a single Fc binding domain with aP. Also, scFv fragments have been fused to the enzyme aP using other expression systems, as in the reported study. Suzuki et al.(10) inserted a scFv–aP fusion gene into an expression vector incorporating the T7 promoter for expression, the *pelB* leader for translocation, and the *His*-tag for purification. Kerschbaumer et al.(23) reported the construction of a vector pDAP2 for producing scFv–aP fusion proteins, expressed under control of the *lac* promoter. The sequences of the *pelB* leader and the *His*-tag were also components of this vector. In contrast, Gandecha et al.(24) used a vector containing the T7 Q10 promoter and the *ompA* leader genes for expression

of a scFv–aP fusion protein, but no peptide tag coding region for the purification of the expression products.

The expression vector, pscFv(K47H)aP, described in this paper differs essentially from the expression sytems mentioned above. PscFv(K47H)aP has implemented the *tetA* promoter/operator as well as the *tet* repressor gene inserted uncoupled from the *tet* control region. The *tetR* gene is placed downstream from the constitutively expressed *bla* gene, resulting in transcriptional fusion.(14) This arrangement establishes the tight repression of the *tetA* promoter and prevents the cell death prior to induction effect, which is frequently caused by insufficient repression. In contrast to the *lac* promoter and its derivatives, the *tet* promoter system was found to be largely independent of the *E. coli* strain employed for recombinant protein production. This offers the possibility to choose the host strain according to optimised growth conditions, for instance in high cell density fermentation. In addition, the *tetA* promoter system is also inducible when cells are grown in minimal medium.(14) In conjunction with the *Strep*-tag purification technology, it facilitates the expression and isolation of heterologous gene products in *E. coli*.(25) Compared to the *His*-tag purification strategy, the immobilized metal affinity chromatography (IMAC), the *Strep*-tag system allows the purification of Ig fragments under more physiological buffer conditions.(26) The *Strep*-tag peptide displays specific binding towards streptavidin, a biochemical reagent that is commercially available in a variety of conjugates as well as immobilized to agarose. The binding is competitive with natural ligands of streptavidin, e.g., biotin or related compounds. Apart from its use for purification, the *Strep*-tag offers the additional advantage of detecting the recombinant protein in ELISA (25) or on Western blot (cf. Figure 2b) by use of streptavidin–enzyme conjugates.

In this study, we have constructed a bacterial expression system for the production of functional antibody–enzyme conjugates. The incorporation of the restriction sites, *Sfi*I and *Not*I, into the system enables the insertion of scFv fragments derived from corresponding recombinant antibody libraries by one-step cloning. The application of the scFv(K47H)–aP fusion protein in ELISA demonstrated the facilitating of immunoassays by replacing enzyme labeled secondary antibodies.

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This paper is dedicated to Professor George Guilbault on the occasion of his 65th birthday.

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