High-yield expression of the recombinant, atrazine-specific Fab fragment K411B by the methylotrophic yeast Pichia pastoris

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Abstract

In this report, we describe the high-yield secretory expression (~ 40 mg l^{-1}) of pure, atrazine-specific Fab fragments (K411B) from P. pastoris that was achieved by co-integration of the genes encoding the heavy and light chains (both under the control of the alcohol oxidase promoter) into the genome of the yeast cells. Antibody-expressing clones were selected by SDS-PAGE and ELISA and fed-batch fermentations were carried out in a 5 l scale. Both chains of the Fab were successfully expressed upon methanol induction and almost no other proteins were secreted into the media. Approximately 30 % of the two chains formed the active Fab fragment containing the intermolecular disulphide bond, as determined by Western blot analysis under non-reducing conditions.

Crude culture supernatant was used to study the binding properties of the Fab fragment toward different s-triazines by means of competitive ELISA: the IC_{50} value for the detection of atrazine was determined from the standard curve as 3 µg l^{-1}, which is one magnitude higher than the value obtained with the parental mAb K4E7 but equals that obtained when the same Fab fragment was expressed in E. coli cells. In addition, the cross-reactivity pattern of the Fab from Pichia is comparable to that of E. coli and the parental mAb K4E7.

Keywords:

antibody fragment, Pichia pastoris, herbicide, fermentation

Introduction

Recombinant antibody (rAb) fragments are usually produced in E. coli as expression system (reviewed in (Harrison and Keshavarz-Moore, 1996)) and purified from the cell extract. However, bacteria often are unable to fold properly or to secrete eukaryotic proteins such as antibodies, thus leading to low yields of soluble and active proteins even after denaturation and refolding of intracellularly accumulated inclusion bodies. In addition, even single mutations can dramatically influence the expression-yield of correctly folded antibody fragments in E. coli (Ito et al., 1993; Duenas et al., 1995; Knappik and Pluckthun, 1995; Ulrich et al., 1995), which may possibly account for the failure to improve the properties of rAb fragments by mutagenesis.

To overcome these problems other expression systems such as mammalian (Dorai et al., 1994; Jost et al., 1994), insect (Putlitz et al., 1990), yeast (Bowdish et al., 1991; Davis et al., 1991), plant (Firek et al., 1993), and in vitro translation systems (Nicholls et al., 1993) have been used to produce rAb fragments (compared in (Verma et al., 1998)). The main advantage of yeast is that it is eukaryotic, has well understood genetics, and can be manipulated and cultivated easily and inexpensively. Proteins produced in yeast also are subjected to typical eukaryote-specific, post-translational modification mechanisms, e.g. proteolytic processing, folding, glycosylation and formation of disulfide bridges. Due to the high yields obtained, the methylotrophic yeast Pichia pastoris has become a popular protein expression system (reviewed in (Cregg, 1999)), including several single-chain antibody fragments and a diabody (Ridder et al., 1995; Eldin et al., 1997; FitzGerald et al., 1997; Luo et al., 1997a; Luo et al., 1997b; Luo et al., 1998). However, the expression of the more stable, heterodimeric Fab fragments using this system has not been reported so far.

Only, HLA-DR2αβ (Kalandadze et al., 1996) and bovine follicle-stimulating hormone (Fidler et al., 1998) have been successfully expressed as heterodimeric, biologically active

proteins in P. pastoris. The secretory expression of a complete dioxin-specific monoclonal antibody (mAb) by P. pastoris has been described (Ogunjimi et al., 1999), but only a small percentage of the heavy and light chains formed the functional, active tetramer (H_2L_2). An even higher yield of functionally active antibody fragments could result from the formation of the smaller, dimeric Fab fragments after co-expression of the heavy and light chain DNA sequences in P. pastoris. The objective of this study was to develop high-level expression and secretion of Fab fragments in P. pastoris. We used the well characterized atrazine-specific antibody fragment K411B (Kramer and Hock, 1996), previously produced in E. coli. Its target atrazine is a herbicide which is banned in several European countries, but can still be detected in ground- and drinking water. It is a very persistent environmental contaminant with a broad spectrum of serious negative effects on health. Thus, an accurate and rapid method is needed to detect this herbicide. Immunoassays that employ polyclonal antibodies (pAb), mAb (Eremin et al., 1994), or rAb fragments (Ward et al., 1993; Kramer and Hock, 1996; Longstaff et al., 1998) have been developed to facilitate the detection of atrazine, but the conventional production of large amounts of pesticide-specific antibodies is expensive and requires appropriate conjugates consisting of a small analyte and a large molecule for the immunization of the animals. In contrast, large-scale production of rAb fragments in micro-organisms is inexpensive and their properties can be manipulated as desired by genetic engineering. Thus, in future rAb fragments, especially Fab fragments due to their higher stability, are expected to play an essential role also in food and environmental immunoanalysis (Kramer and Hock, 1995), and therefore appropriate production systems are of high industrial importance.

Materials and Methods

Micro-organisms, media, plasmids and oligonucleotides

E. coli DH5α [F^- endA1 hsdR17(rk^-, mk^+) supE44 thi-1 λ^- gyrA96 relA1 Δ(argF-laczya)U169] was used for maintenance and propagation of most of the plasmids applied or made in this study. E. coli XL 1 Blue [supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac^-] (Stratagene, Heidelberg, Germany) was used as host for the transformation of plasmids after site-directed mutagenesis. Both strains were cultivated in $LB_{lowsalt}$ (10 g l^{-1} yeast extract, 10 g l^{-1} peptone, 5 g l^{-1} NaCl), supplemented with 50 mg l^{-1} nalidixic acid and, if required, with 25 mg l^{-1} Zeocin (Invitrogen, Carlsbad, CA).

Pichia pastoris GS115 (his4 Mut^+) (Invitrogen, Groningen, Netherlands) was used as host for the expression experiments. The following media were employed in the cultivation of Pichia cells under different conditions: YPD medium (1 % yeast extract, 2 % peptone, and 2 % glucose); YPDS medium (YPD medium supplemented with 1 M sorbitol); BMGY medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, and 1 % glycerol); BMMY medium (BMGY, but using 0.5% filter-sterilized methanol instead of 1 % glycerol). The media were supplemented with 100 mg l^{-1} Zeocin. For plates, 1.5 % agar was added to the media.

The Fab fragment K411B is derived from hybridoma line K4E7 (Giersch, 1993) which secretes mAb against atrazine. Using the variable regions of K411B, scFv K411B* was produced using the pCANTAB 5E vector (Kramer and Hock, 1996). The Fab fragment K411B had been generated previously by cloning the sequences coding for the variable regions in frame with the constant domains from pASK 85 (Bandtlow et al., 1996) and expressed in E. coli K12 W3112. The resulting vector pASK-K411B was used as a template

for the amplification of genes encoding the complete heavy- and light chains of Fab fragment K411B.

The E. coli – P. pastoris shuttle vectors pPICZαA (Invitrogen) and pPICZα-ΔPme were used to subclone the genes encoding the Fab fragment K411B under control of the alcohol oxidase (AOX1) promoter. The pPICZα-ΔPme vector was derived from pPICZαA by introducing a point mutation into the AOX1 promoter by site-directed mutagenesis to destroy the Pme I restriction site.

Oligonucleotides used for PCR or sequencing (Table 1) were purchased from ARK Scientific (Darmstadt, Germany), MWG Biotech (Ebersberg, Germany), or Interactiva (Ulm, Germany).

Recombinant DNA technologies and transformations

Unless stated otherwise, standard DNA technologies (Sambrook et al., 1989) were used. The QIAprep Spin Miniprep Kit, the Plasmid Midi Kit, and the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) were used for plasmid DNA and DNA gel extractions, respectively. Restriction enzymes and other DNA modifying enzymes were purchased from different suppliers (New England BioLabs, Beverly, MA; Boehringer-Mannheim, Mannheim, Germany; GIBCO-BRL-Life-Technologies, Eggenstein, Germany; and MBI Fermentas, St. Leon-Rot, Germany) and used according to the manufacturers' instructions.

Site-directed mutagenesis was performed with the Quik-Change-mutagenesis Kit (Stratagene).

DNA-sequencing reactions were carried out on both strands of double-stranded templates using the Taq Ready Reaction Dye Deoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). The sequencing products were analyzed on a 373 DNA Sequencer (Applied Biosystems).

Standard protocols (Sambrook et al., 1989) were used for the preparation and transformation of competent E. coli cells. Transformation of P. pastoris was made by following the Invitrogen electroporation method (Invitrogen,). The integration of the linearized vectors into the genome of recombinant P. pastoris clones was confirmed by PCR with antibody gene-specific primers and genomic DNA as template. Genomic DNA from P. pastoris was prepared according to the Invitrogen manual (Invitrogen,).

Shake-flask cultivation of P. pastoris and secreted expression of Fab fragments

P. pastoris transformants were grown in YPDS medium at 30°C, 200 rpm until OD_{600} ~15. Two hundred microliters of this preculture were used to inoculate 25 ml BMGY and incubated overnight. The yeast cells were collected by centrifugation (5 min, 3000 x g, 4-10 °C) and transferred to the BMMY induction medium until an OD_{600} of 1.0 was reached. Induction was maintained by the addition of 0.5 % methanol (v/v) every 24 hours. After a 96-hour induction, the cells were harvested and the supernatants stored at 4°C. The proteins in the supernatant were concentrated either by ultrafiltration using a polyethersulfone membrane (Ω-series; Pall Filtron, Dreieich, Germany) with a nominal molecular weight limit of 10 kDa or by precipitation with trichloroacetic acid (TCA): equal amounts of supernatant and 48 % TCA were mixed and incubated on ice for 30 min. After centrifugation (20800 x g, 15 min, 4°C), the pellet was washed twice with the same volume of acetone, air-dried and then resuspended in 50 mM phosphate buffer (pH 7.0).

Fed-batch cultivation of Pichia pastoris in a bioreactor

A 5 l benchtop fermentor (Infors GmbH, Einsbach, Germany) with pH, temperature, agitation, dissolved oxygen (DO), and air flow control was used.

Fermentation inoculum was prepared by inoculating 100 ml of BMGY medium with 2 ml of an overnight culture in a 500 ml shake flask followed by incubation overnight (200 rpm,

30°C). This preculture was added (OD_{600} of ~ 1.5) to the bioreactor containing 4 l of 1 % yeast extract, 2 % peptone, and 0.5 % methanol, and grown initially as batch culture at 27°C, pH 6.0 (adjusted with 10 % ammonium hydroxide), DO > 20 %, agitation at 400 rpm and air flow rate of 7 l min^{-1}. After exhaustion of the methanol, detected by a rapid increase in DO concentration, the fed-batch phase was began by continuously feeding 40 % methanol, 1 % yeast extract and 2 % peptone. Depending on the oxygen consumption of the culture, the feeding rate (initially ~2.7 ml h^{-1}) was gradually increased (up to 22.5 ml h^{-1}) to keep the DO level between 20 % and 30 %. The cells were centrifuged (3000 – 5000 x g, 20 min, 4°C) after four days. The supernatant, as well as the samples that had been taken twice daily during the fermentation process, were analyzed by SDS-PAGE and ELISA.

SDS-PAGE and Western Blot analysis

Ten microliters of cell lysate or 10-fold concentrated supernatant were mixed with 10 µl of 2x SDS-sample buffer (Sambrook et al., 1989), supplemented with 200 mM DTT for gels run under reducing conditions, and separated on 12.5 % polyacrylamide gels with a 4 % stacking gel.

For protein estimations, Coomassie-stained SDS-gels were analyzed by densitometry using Scion Image (Rasband, 23.07.1998 revision date).

After equilibration of gel and nitrocellulose membrane in transfer buffer (25 mM Tris, 142 mM glycine, 20 % (v/v) methanol), the cells were electroblotted onto a membrane (Biorad transblot SD; 15 V, 20 min).

After blocking the dried membrane was probed with a mouse-anti-$(his)_6$-mAb (Sigma-Aldrich Chemicals, Steinheim, Germany; dilution 1:1000 in 1 % albumin / TS-buffer (10 mM Tris pH 7.5, 0.85 % (w/v) NaCl); 1h, 20 – 25°C) followed by a sheep-anti-mouse IgG coupled to horseradish peroxidase (Boehringer-Mannheim, Mannheim, Germany; dilution: 1:1000 in 1 % albumin / TS-buffer; 1 h, 20 – 25°C). Bound antibodies were detected using

2.8 mM 4-chloro-1-naphtol and 0.167 % (v/v) hydrogen peroxide in 15 mM phosphate buffer, pH 6, as substrate.

Antibody-immobilized ELISA

A competitive ELISA for the determination of the standard binding curves of the Fab fragments towards atrazine and other cross-reactive s-triazines was performed as previously described (Giersch et al., 1993), except that crude Pichia culture supernatants containing the recombinant Fab fragments were used instead of the parental mAb K4E7. The synthesis of the atrazine-C_6-POD tracer was described previously (Wittmann and Hock, 1989). To achieve low IC_{50} values and low detection limits, and absorbance values above 0.2, tracer- and antibody concentrations were optimized. The rabbit-anti-mouse-IgG-precoated microtiter plate was coated with Pichia-derived recombinant Fab fragments by diluting the fermentation supernatants 1- to 100-fold and incubating the plate for 1 hour at 20 – 25°C. For the competition reaction, atrazine standard solutions (0 - 10 mg l^{-1}) were incubated with the atrazine-C_6-POD tracer solution (1:4000 – 1:10000).

Results

Construction of the pPICZα-Fab expression vector

The pPICZα-Fab expression vector, which contains the sequences encoding both chains of the Fab K411B, each fused directly in frame with the α-factor signal sequence of S. cerevisiae under the control of the AOX1 promoter, was cloned in two steps (Fig. 1):

The two genes were individually fused directly in frame to the α-factor signal sequence using splicing-by-overlap-extension PCR (Horton et al., 1989). The sequences for the heavy chain including a $(his)_6$-tag-sequence (Hochuli et al., 1987) and for the light chain were amplified by PCR. pASK-K411B was used as template and the primer pairs Fab-5 / Fab-6

and Fab-1 / Fab-2, respectively. Both PCRs introduced an EcoRI-site at the 3'-end of the coding sequences and a region corresponding to the 3'-end of the α-factor sequence at the 5'-end of the amplified regions. In a second PCR reaction, the α-factor signal sequence was amplified and a SfuI site was introduced at the 5'-end, using pPICZαA as template and the primer pair Fab-3 / Fab-4. The resulting PCR product was combined in a third PCR reaction with the amplified light or heavy chain respectively as template (primer pairs: Fab-3 / Fab-6 and Fab-3 / Fab-2). This reaction fused sequences encoding the antibody chains with the α-factor signal sequence. Both PCR products were double-digested with EcoRI and SfuI and ligated into pPICZαA and pPICZαA-ΔPme, which were previously cut with the same enzymes, to produce the plasmids pPICZα-H, pPICZα-L, pPICZα-ΔPme-H, and pPICZα-ΔPme-L.

To combine both genes into a single vector, silent mutations were introduced into each of the heavy- and light-chain coding sequences to destroy BamHI-sites. All vectors (pPICZα-H, pPICZα-L, pPICZα-ΔPme-H, pPICZ-ΔPme-L) containing a monomer of one of the two antibody genes were double-digested with BglII and BamHI. DNA fragments encoding a heavy-chain expression cassette were ligated into linearized (BglII or BamHI) monomer vectors that held a light-chain expression cassette. DNA fragments encoding a light-chain expression cassette were ligated into linearized monomer vectors containing a heavy-chain expression cassette. Additionally it was taken into account that the resulting vector carried only one PmeI site in order to ensure proper linearization before transformation into P. pastoris.

The two genes and their orientation towards each other could result in eight different heterodimeric vectors (pPICZα-Fab1 to 8) because of the introduced restriction sites (BamHI, BglII), which recognize different sequences but generate compatible sticky ends.

All eight were transformed and propagated in E. coli DH5α, but only four (pPICZα-Fab-1, -3, -5, -7) that contained both, heavy and light chain sequences in the same orientation were transformed into P. pastoris.

The sequences encoding the heavy and light chains of the Fab fragment K411B integrated into the Pichia genome. Their presence was confirmed by PCR using genomic DNA as template and antibody gene-specific primers (Fab-7/Fab-8 for the amplification of the heavy-chain region and Fab-9/Fab-10 for the light chain region), followed by agarose gel electrophoresis. All clones tested contained both genes (Fig. 2, lanes 2 – 6, 8 and 9; fragments of ~350 bp for the heavy chain region, 300 bp for the light chain region).

Expression of the recombinant Fab fragments in shake flask cultures and in a 5 l bioreactor

Nine transformants (7 of Mut^+ and 2 of Mut^S phenotype of heterologous DNA integration were used for expression studies in shake flask experiments. Secretion of the heterologous Fab fragment K411B into the culture medium was monitored by SDS-PAGE and ELISA. We found only one protein, with a molecular mass of ~ 29 kDa, when 10-fold concentrated supernatants of methanol-induced cells were used, resolved on SDS-PAGE (denaturing conditions) and then stained with Coomassie blue (Fig. 3). No band appeared in the supernatants of uninduced cultures or in the supernatant from a Pichia clone transformed with pPICZαA. The similar molecular mass of the two chains (24.2 kDa for the light and 25.0 kDa for the heavy chain) prevented their resolution by SDS-PAGE. However, the two chains could be separated by isoelectric focusing (data not shown). We performed a Western blot (Fig. 3B) with the proteins in the supernatants of clone 4A from non-induced (lanes 1 and 3) and induced (lanes 2 and 4) recombinant Pichia cells and an anti-$(his)_6$-antibody, which binds to the $(his)_6$-tag of the heavy chain. No proteins were detected in the supernatant of non-induced cultures (Fig. 3B, lanes 1 and 3). We detected a 29 kDa protein,

corresponding in size to the band appearing after SDS-PAGE (Fig. 3A, lane 2) under reducing conditions (Fig. 3B, lane 2), thus confirming the presence of the heavy chain. We detected two bands under non-reducing conditions (Fig. 3B lane 4). The upper band (~ 45 kDa) probably represents the complete Fab, while the lower band (~ 25 kDa) probably corresponds to the heavy chain fused to the $(his)_6$-tag.

Three Mut^+ clones had the highest expression rates in the shake flask experiments and were chosen for fed-batch fermentations in a 5-l scale. The fermentation supernatants contained much higher amounts of Fab fragments and could be applied directly to SDS gels without having to be concentrated. An SDS-PAGE of culture supernatants taken at different times during the fermentation process showed that a significant amount of antibodies was already secreted after one day of induction. Densitometric analysis of the Coomassie stained SDS-PAGE determined a Fab concentration in the supernatant of ~ 40 mg l^{-1}. No influence of the order of genomic integration of the sequences for heavy and light chain on the expression level was observed by SDS-PAGE.

Binding properties of the recombinant antibodies

We assayed the P. pastoris-derived Fabs directly from the supernatant in a direct, competitive ELISA. Atrazine was used at different concentrations and the atrazine-C_6-POD tracer competed for the binding sites of immobilized Fab fragments. The supernatants of all of the induced recombinant clones led to the expected sigmoidal binding curves (Fig. 4A), whereas the negative control (supernatant of induced P. pastoris containing pPICZαA) gave no signal. The different amplitudes of the curves might be due to different expression levels of the clones. The normalized (Giersch et al., 1993) curves (Fig. 4B) revealed no significant difference in the affinity of the Fabs from clones containing the sequences for the heavy and

light chains in different orders, as measured by IC_{50}. The median IC_{50} of the P. pastoris Fab amounts to ~ 3 µg l^{-1} (~14 nmol l^{-1}).

Crossreactivity of the Fab K411B derived from P. pastoris clone 4A was determined by comparison with standard binding curves of the major cross-reactants of the mAb K4E7 among the s-triazines (propazine, terbuthylazine, simazine, cyanazine, and deethylatrazine), all of which have a chlorine atom at position 4 of the triazine ring (Fig. 4C). The resulting crossreactivity pattern (Fig. 4D) is similar to that of the parental mAb K4E7 as well as the scFv fragment expressed in E. coli (Kramer and Hock, 1996).

Discussion

We showed for the first time that recombinant P. pastoris cells can secrete functional Fab fragments after induction with methanol. Expression of heterodimeric proteins in P. pastoris usually relies on co-transformation and genomic integration of two vectors, each containing the sequence encoding one subunit of the protein (Kalandadze et al., 1996; Fidler et al., 1998). In contrast, our strategy was to construct a single expression vector with sequences encoding each antibody chain in a separate expression cassette but with the same regulatory sequences in either AOX1 promoter and termination signal. Thus, both heterologous sequences are integrated into the genome of all transformants as shown by PCR and only a single selectable marker is required for expression. In contrast, only a small portion of the transformants gained after the co-transformation of two vectors carry both genes (Kalandadze et al., 1996) as also in these cases only a single selection marker is used. The different intensities of the bands on the agose gel (Figure 2) corresponding to the amplified heavy and light chains is probably due to experimental variation as the PCR conditions for the two chain has not been optimized.

The expression level (40 mg l^{-1}) of the Fab K411B after fermentation of recombinant P. pastoris cells in a 5 l bioreactor is not high when compared to other heterologous proteins that are expressed in the range of g l^{-1} (Sreekrishna and Kropp, 1996). Nevertheless, the yield is much higher than that reported for other secreted heterodimers, e.g. 300 – 400 µg l^{-1} for HLA-DR2 (Kalandadze et al., 1996), and 17.5 – 61 µg l^{-1} for ovine-follicle stimulating hormone (oFSH). The Fab expression level in P. pastoris is similar to that observed for the parental mAb (50 – 100 µg ml^{-1}) expressed by the hybridoma clone K4E7 (Giersch, 1993).

P. pastoris secretes Fab into the culture supernatant as extremely pure protein (Fig. 3A) that can be used for practical applications without any further purification. In contrast, antibody fragments produced in E. coli often accumulate as intracellular or periplasmic inclusion bodies and usually must be refolded and purified (Better and Horwitz, 1989).

Approximately 30% of the Fab fragments produced by P. pastoris were correctly folded. Although this value is not optimal, it is not unusual: Expression of scFv mutants with a C-terminal cysteine, which allows the formation of an intermolecular disulfide bridge in bivalent dimers, resulted in a monomer:dimer ratio of 3 to 1 (Luo et al., 1997a). Ten percent and 33% of the secreted subunits of the ovine-follicle stimulating hormone oFSHα and oFSHβ, respectively, formed stable dimers (Fidler et al., 1998).

The difference in the apparent molecular weight of the heavy chain under reducing and non-reducing conditions is probably due to the intramolecular disulfide bonds, which are intact under non-reducing conditions but broken under reducing conditions. Thus, incomplete crosslinking of the two chains probably results from the failure of one of the chains to find a partner, although both chains are expressed at similar level as determined by isoelectric focusing, rather than the result of inproper formation of disulfide bonds by Pichia.

Ogunjimi et al. (Ogunjimi et al., 1999) produced a functional murine antibody to dioxin. Its secretory expression by P. pastoris resulted in total protein yields between 10 and 36 mg l^{-1}.

Only a very small portion of the two chains, however, constituted the intact antibody (H_2L_2). Western Blot analysis under non-reducing conditions revealed that in this case, heavy and light chains were secreted as monomers or as heavy chain-light chain (HL) dimers. We assume that the ratio of functional protein to total expression level decreases as the number of subunits needed to assemble an intact Ab increases. Thus, the Fab fragment with its high expression level and an appropriate thermal stability during fermentation, is an optimal antibody fragment for secreted expression in Pichia pastoris, combining the advantages of scFvs (Luo et al., 1997b) and of mAbs (Ridder et al., 1995).

The Fab K411B has binding properties (IC_{50} ~ 3 µg l^{-1} (~14 nmol l^{-1}), crossreactivity pattern) that are identical to those obtained with the Fab K411B expressed in E. coli W3112 under the control of the tetracycline promoter of pASK-K411B (data not shown). The parental mAb K4E7 has an IC_{50} of ~ 0,2 µg l^{-1} (~ 0,9 nmol l^{-1}), which is one order of magnitude lower than that obtained with the recombinant antibodies. This difference also is seen between the scFv K411B and the corresponding mAb K4E7 (Kramer and Hock, 1996) but the reason is not known.

In conclusion, we showed that Fab fragments of high purity can be produced at high levels by P. pastoris. Our findings suggest that P. pastoris is an attractive organism for the production of recombinant antibody fragments, including both homo- and hetero-multimers. In particular, dimeric Fab fragments were present at a good ratio between expression level of functional protein and total expression level at a high purity. Therefore, Pichia strongly suggests the possibility of Fab production for use in diagnostic or therapeutical applications as well.

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References

Bandtlow, C., Schiweck, W., Tai, H.-H., Schwab, M.E. and Skerra, A. (1996) The Escherichia coli-derived Fab fragment of the IgM/κ antibody IN-1 recognizes and neutralizes myelin-associated inhibitors of neurite growth. Eur. J. Biochem. 241, 468-475.

Better, M. and Horwitz, A.H. (1989) Expression of engineered antibodies and antibody fragments in microorganisms. Methods in Enzymology 178, 476-496.

Bowdish, K., Tang, Y., Hicks, J.B. and Hilver, D. (1991) Yeast expression of a catalytic antibody with chorismate mutase activity. J. Biol. Chem. 266, 11901-11908.

Cregg, J. (1999) Gene expression systems, Vol. 1. Academic Press, San Diego.

Davis, G.T., Bedzyk, W.D., Voss, E.W. and Jacobs, T.W. (1991) Single chain antibody (SCA) encoding genes: one-step construction and expression in eukaryotic cells. Biotechnology (N Y) 9, 165-169.

Dorai, H., McCartney, J.E., Hudziak, R.M., Tai, M.S., Laminet, A.A., Houston, L.L., Huston, J.S. and Oppermann, H. (1994) Mammalian cell expression of single-chain Fv (sFv) antibody proteins and their C-terminal fusions with interleukin-2 and other effector domains. Biotechnology (N Y) 12, 890-897.

Duenas, M., Ayala, M., Vazquez, J., Ohlin, M., Soderlind, E., Borrebaeck, C.A. and Gavilondo, J.V. (1995) A point mutation in a murine immunoglobulin V-region strongly influences the antibody yield in Escherichia coli. Gene 158, 61-66.

Eldin, P., Pauza, M.E., Hieda, Y., Lin, G., Murtaugh, M.P., Pentel, P.R. and Pennell, C.A. (1997) High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris*. J. Immunol. Methods 201, 67-75.

Eremin, S.A., Samsonova, Z.V. and Egorov, A.M. (1994) Immunochemical methods for the assay of herbicides of the 1,3,5-triazine group. Russ. Chem. Rev. 63, 611-622.

Fidler, A.E., Lun, S., Young, W. and McNatty, K.P. (1998) Expression and secretion of a biologically active glycoprotein hormone, ovine follicle stimulating hormone, by Pichia pastoris. J. Mol. Endocrinol. 21, 327-336.

Firek, S., Draper, J., Owen, M.R., Gandecha, A., Cockburn, B. and Whitelam, G.C. (1993) Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. Plant Mol. Biol. 23, 861-870.

FitzGerald, K., Holliger, P. and Winter, G. (1997) Improved tumour targeting by disulphide stabilized diabodies expressed in *Pichia pastoris*. Protein Eng. 10, 1221-1225.

Giersch, T. (1993) A new monoclonal antibody for the sensitive detection of atrazine with immunoassay in microtiter plate and dipstick format. J. Agric. Food Chem. 41, 1006-1011.

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

Harrison, J.S. and Keshavarz-Moore, E. (1996) Production of antibody fragments in Escherichia coli. Ann. N Y Acad. Sci. 782, 143-158.

Hochuli, E., Döbeli, H. and Schacher, A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighboring histidine residues. Journal of Chromatography 411, 177-184.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77, 61-68.

Invitrogen. Manual for Pichia expression vectors pPICZα A, B, and C - Version A. In:, San Diego, CA.

Ito, W., Iba, Y. and Kurosawa, Y. (1993) Effects of substitutions of closely related amino acids at the contact surface in an antigen-antibody complex on thermodynamic parameters. J. Biol. Chem. 268, 16639-16647.

Jost, C.R., Kurucz, I., Jacobus, C.M., Titus, J.A., George, A.J. and Segal, D.M. (1994) Mammalian expression and secretion of functional single-chain Fv molecules. J. Biol. Chem. 269, 26267-26273.

Kalandadze, A., Galleno, M., Foncerrada, L., Strominger, J.L. and Wucherpfennig, K.W. (1996) Expression of recombinant HLA-DR2 molecules. Replacement of the hydrophobic transmembrane region by a leucine zipper dimerization motif allows the assembly and secretion of soluble DR alpha beta heterodimers. J. Biol. Chem. 271, 20156-20162.

Knappik, A. and Pluckthun, A. (1995) Engineered turns of a recombinant antibody improve its in vivo folding. Protein Eng 8, 81-89.

Kramer, K. and Hock, B. (1995) Rekombinante Antikörper in der Umweltanalytik. Lebensmittel- und Biotechnologie 2, 49-56.

Kramer, K. and Hock, B. (1996) Recombinant single-chain antibodies against s-trianzines. Food Agricul. Immunol. 8, 97-109.

Longstaff, M., Newell, C.A., Boonstra, B., Strachan, G., Learmonth, D., Harris, W.J., Porter, A.J. and Hamilton, W.D. (1998) Expression and characterisation of single-chain antibody fragments produced in transgenic plants against the organic herbicides atrazine and paraquat. Biochim. Biophys. Acta 1381, 147-160.

Luo, D., Geng, M., Noujaim, A.A. and Madiyalakan, R. (1997a) An engineered bivalent single-chain antibody fragment that increases antigen binding activity. J. Biochem. (Tokyo) 121, 831-834.

Luo, D., Geng, M., Schultes, B., Ma, J., Xu, D.Z., Hamza, N., Qi, W., Noujaim, A.A. and Madiyalakan, R. (1998) Expression of a fusion protein of scFv-biotin mimetic peptide for immunoassay. J. Biotechnol. 65, 225-228.

Luo, D., Mah, N., Krantz, M., Wishart, D., Jacobs, F. and Martin, L. (1997b) High level secretion of single-chain antibody in *Pichia* expression system. Biotechnology Techniques 11, 759-761.

Nicholls, P.J., Johnson, V.G., Andrew, S.M., Hoogenboom, H.R., Raus, J.C. and Youle, R.J. (1993) Characterization of single-chain antibody (sFv)-toxin fusion proteins produced in vitro in rabbit reticulocyte lysate. J. Biol. Chem. 268, 5302-5308.

Ogunjimi, A.A., Chandler, J.M., Gooding, C.M., Recinos, A. and Choudary, P.V. (1999) High-level secretory expression of immunologically active intact antibody from the yeast *Pichia pastoris*. Biotechnol. Lett. 21, 561-567.

Putlitz, J.z., Kubasek, W.L., Duchene, M., Marget, M., Specht, B.-U.v. and Domdey, H. (1990) Antibody production in baculovirus-infected insect cells. Bio/Technology 8, 651-654.

Rasband, W. (23.07.1998 revision date) [Online] ScionImage Beta 3b, based on NIH-Image for Macintosh and modified for Windows by Scion Corporation, Frederick, Maryland, USA; http://www.scioncorp.com. In:. Scion Corporation.

Ridder, R., Schmitz, R., Legay, F. and Gram, H. (1995) Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast Pichia pastoris. Biotechnology 13, 255-260.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. Cold spring Harbor Laboratory Press, New York.

Sreekrishna, K. and Kropp, K. (1996) Pichia pastoris. In: K. Wolf (Ed) Non-Conventional Yeasts in Biotechnology. Springer, Berlin, p. 203-252.

Ulrich, H.D., Patten, P.A., Yang, P.L., Romesberg, F.E. and Schultz, P.G. (1995) Expression studies of catalytic antibodies. Proc Natl Acad Sci U S A 92, 11907-11911.

Verma, R., Boleti, E. and George, A.J. (1998) Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. J Immunol Methods 216, 165-181.

Ward, V.K., Schneider, P.G., Kreissig, S.B., Hammock, B.D. and Choudary, P.V. (1993) Cloning, sequencing and expression of the Fab fragment of a monoclonal antibody to the herbicide atrazine. Protein Eng 6, 981-988.

Wittmann, C. and Hock, B. (1989) Improved enzyme immunoassay for the analysis of s-triazines in water samples. Food and Agricultural Immunology 1, 211-224.

Table 1: Primers for PCR or sequencing reactions

Oligonucleotide	**Nucleotide sequence***	**Annotations**
Fab-1	5’-gaaaagagaggctgaagctgacatcgagctcacccagtc-3’	
Fab-2	5’-ccgccggaattctcactaacactcattcct-3’	Eco RI
Fab-3	5’-acaactaattattcgaaacgatgagatttcc-3’	Sfu I
Fab-4	5’-agcttcagcctctcttttctcgagagataccc-3’	
Fab-5	5’-gaaaagagaggctgaagctgaagttaaactgcagcagtc-3’	
Fab-6	5’-ccgccggaattcttattaatggtgatggtgg-3’	Eco RI
Fab-7	5’-gtttcaagagatgattcc-3’	
Fab-8	5’-tttggtgctagaagccgg-3’	
Fab-9	5’-aggacagacttcaccctcac-3’	
Fab-10	5’-ggtatagctgttatgtcgttc-3’	

* Restriction sites are italicised.

Figure legends

Figure 1: Two-step cloning strategy for construction of the expression vectors containing the genes encoding the light and heavy chain of the Fab fragment K411B, each under the control of the AOX1 promoter: First, both of the two genes are fused directly in frame to the α-factor signal sequence of Saccharomyces cerevisiae by splicing-by-overlap-extension PCR. In the second step, the two genes are cloned into one vector. Due to the position and orientation of the two genes towards each other 8 vectors were obtained, four of which (pPICzα-Fab-1, -3, -5, -7) were found to have both genes in the same orientation and were chosen for transformation. Restriction sites used for each cloning step are underlined.

Figure 2: Integration of both genes encoding the heavy and light chains of the Fab K411B into the P. pastoris genome was confirmed by PCR using antibody gene-specific primers (A: heavy chain specific; B: light chain specific) and genomic DNA as template. The tested clones contain both genes (lanes 2 – 6, 8, 9) in their genome. Lanes 1 and 7: molecular weight standard (1 kb-ladder); lanes 2 – 6, 8, 9: different recombinant clones; lane 10: genomic DNA from cells transformed with pPICZαA served as negative control; lane 11: plasmid DNA (pPICZα-Fab) served as positive control.

Figure 3: Analysis of the P. pastoris-secreted Fab fragment K411B by SDS-PAGE and Western blotting.

A) Coomassie-stained SDS-PAGE of 10-fold concentrated but unpurified culture supernatants under reducing conditions (+DTT). Lane 1: molecular mass standard; lane 2: sample taken before induction; lane 3: sample taken after 4 days of methanol induction.

B) Western blot analysis of supernatants from recombinant Pichia cultures before (lanes 1 and 3, negative control) and after 4 days (lanes 2 and 4) of methanol induction using an anti-$(his)_6$-antibody which binds the $(his)_6$-tag fused to the heavy chain. Culture supernatants were separated by SDS-PAGE under reducing (+DTT, lanes 1 and 2) and non-reducing (-DTT, lanes 3 and 4) conditions. Molecular mass markers are shown. Under reducing conditions (lane 2) a band of the same size as that on the SDS-gel was detected. Under non-reducing conditions two bands are visible: the upper one at ~ 45 kDa represents the complete Fab; the lower one at ~ 25 kDa corresponds to the heavy chain, which is not crosslinked to a light chain. A dimer/monomer ratio of ~ 30 % was determined by densitometry.

Figure 4: Binding properties of the Fab K411B expressed by different recombinant Pichia clones assayed using a direct, competitive ELISA and unpurified supernatants from recombinant, methanol-induced Pichia cultures.

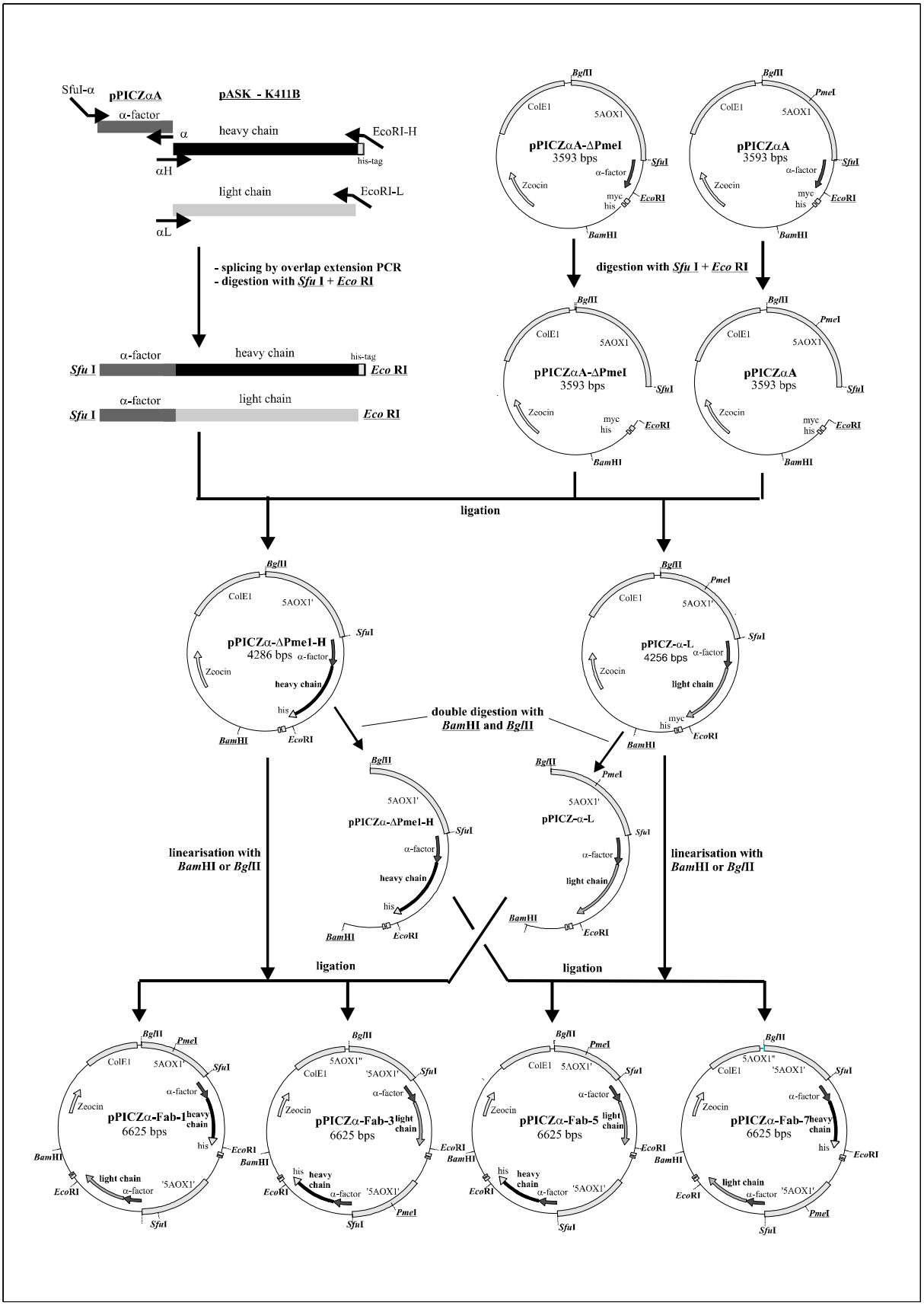
A) Sigmoidal standard binding curves were obtained with crude culture supernatants from different recombinant Pichia clones after methanol induction for 4 days. Clones containing the sequences for the two chains in different orders in their genomes are indicated by the same number. Letters indicate different clones of the same integration type. No signal was obtained from a strain transformed with pPICzαA (negative control). The different absorption values at low atrazine concentrations are due to different expression levels.

B) Normalization of the standard binding curves. The IC_{50} values obtained with the Fab fragments from all recombinant clones is between 3 and 4.5 µg l^{-1}.

C) Normalized standard binding curves towards different s-triazines, obtained with the Fab K411B secreted from clone 4A.

D) The cross-reactivity pattern of the Fab K411B from clone 4A is similar to that from the parental, monoclonal antibody of the hybridoma cell line K4E7. Abbreviations: A, atrazine; C, cyanazine; T, terbuthylazine; P, propazine; D, deethylatrazine; S, simazine.

Figure 1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α-factor
α
αH
pASK - K411B
heavy chain
EcoRI-H
his-tag
light chain
EcoRI-L
αL
- splicing by overlap extension PCR
- digestion with Sfu I + Eco RI
α-factor
heavy chain
his-tag
Sfu I
Eco RI
α-factor
light chain
Sfu I
Eco RI
BglII
ColE1
5AOX1
pPICZαA-ΔPmeI
3593 bps
α-factor
SfuI
Zeocin
myc
his
EcoRI
BamHI
PmeI
pPICZαA
3593 bps
digestion with Sfu I + Eco RI
ligation
pPICZα-ΔPme1-H
4286 bps
heavy chain
5AOX1'
pPICZ-α-L
4256 bps
light chain
double digestion with
BamHI and BglII
linearisation with
BamHI or BglII
ligation
pPICZα-Fab-1
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Figure 2

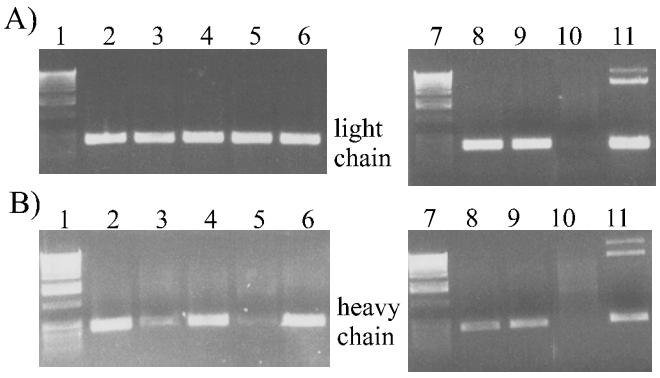


Figure 3

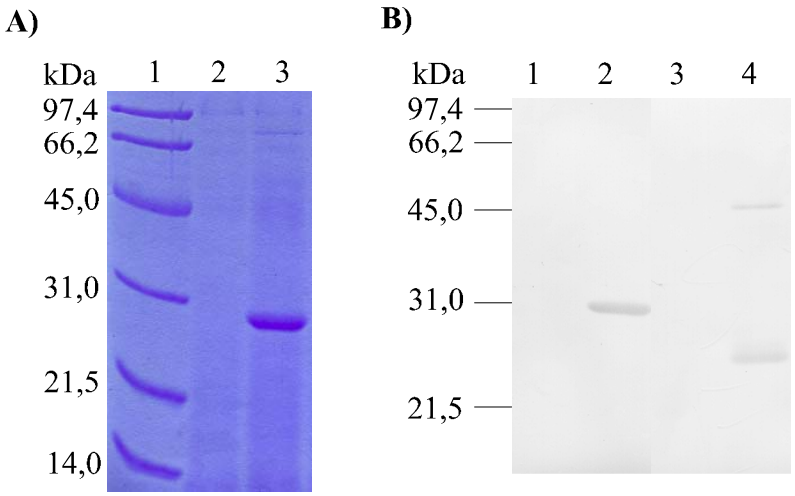
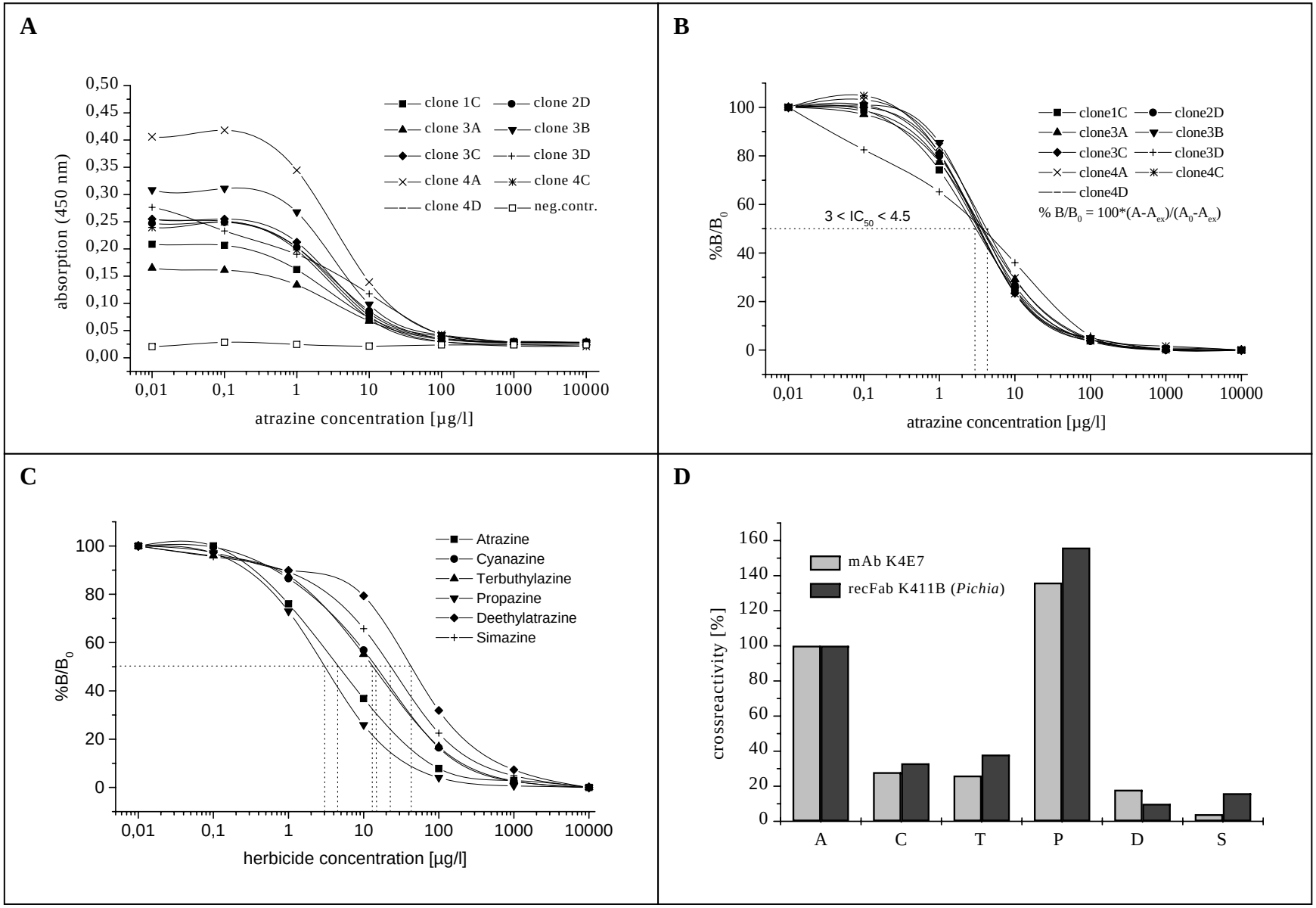


Figure 4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0,40
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0,30
0,25
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0,15
0,10
0,05
0,00
clone 1C
clone 2D
clone 3A
clone 3B
clone 3C
clone 3D
clone 4A
clone 4C
clone 4D
neg.contr.
0,01
0,1
1
10
100
1000
10000
atrazine concentration [µg/l]
B
%B/B0
100
80
60
40
20
0
clone1C
clone2D
clone3A
clone3B
clone3C
clone3D
clone4A
clone4C
clone4D
3 < IC50 < 4.5
% B/B0 = 100*(A-Aex)/(A0-Aex)
atrazine concentration [µg/l]
C
%B/B0
Atrazine
Cyanazine
Terbuthylazine
Propazine
Deethylatrazine
Simazine
herbicide concentration [µg/l]
D
crossreactivity [%]
160
140
120
100
80
60
40
20
0
mAb K4E7
recFab K411B (Pichia)
A
C
T
P
D
S