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# Cloning and Characterization of a Single Chain Antibody to Glucose Oxidase from a Murine Hybridoma

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**Glucose oxidase (GOD) is an oxidoreductase catalyzing the reaction of glucose and oxygen to peroxide and gluconolacton (EC 1.1.3.4.). GOD is a widely used enzyme in biotechnology. Therefore the production of monoclonal antibodies and antibody fragments to GOD are of interest in bioanalytics and even tumor therapy. We describe here the generation of a panel of monoclonal antibodies to native and heat inactivated GOD. One of the hybridomas, E13BC8, was used for cloning of a single chain antibody (*scFv*). This *scFv* was expressed in *Escherichia coli XL1-blue* with the help of the vector system *pOPE101*. The *scFv* was isolated from the periplasmic fraction and detected by western blotting. It reacts specifically with soluble active GOD but does not recognize denatured GOD adsorbed to the solid phase. The same binding properties were also found for the monoclonal antibody E13BC8.**

**Keywords:** Bacterial expression, Glucose oxidase, Monoclonal antibody, Single chain antibody

## Introduction

Immunoglobulins are stable molecules that can bind a vast number of antigens. The benefit of high stability is combined with the drawback of complex folding mechanisms which can only be performed in higher eukaryotes. A promising way to achieve easy modification and cheap large- scale production is the recombinant expression of antibody fragments in *Escherichia coli,* first described in 1988 for Fv fragments (Skerra and Plückthun, 1988) and single chain Fv fragments (Bird *et al.*, 1988; Huston *et al.*, 1988). As the expression of complete antibodies is not possible in the prokaryotic system, these single chain Fv fragments (*scFv*) consist only of the variable regions of light and heavy chain. Both are fused via a peptide linker. *ScFv* have additional advantages if used for example in tumour therapy. Because of their smaller size they are easily able to penetrate tissue and are less immunogenic (Löffler *et al.*, 2000). The efficient expression of active antibody fragments in *E. coli* is, therefore, of great technological importance.

In the last decade the production of *scFv* against an enormous variety of antigens was performed. There are two general strategies to select *scFv,* either from naive or preimmunized libraries via phage display (Hoogenboom, 2002; Hust and Dübel, 2004) or by cloning from recently generated hybridoma cell lines (Toleikis *et al.*, 2004). Whereas phage display allows the selection of a large number of new clones from several species, e.g. human, rabbit or chicken, and antibodies against toxic and non-immunogenic antigens, the cloning of *scFv* from established hybridoma cells usually leads to antibody fragments with similar binding characteristics compared to the known parental monoclonal antibody.

A rewarding target is the production of *scFv* against enzymes. In bioanalytics and diagnostics an interesting enzyme in addition to horseradish peroxidase (HRP) and alkaline phosphatase (AP) is glucose oxidase (GOD). The enzyme, first described in 1942 under the name notatin (Coulthard *et al.*, 1945), is a 160 kD homodimer (Wohlfahrt *et al.*, 1999). It is found among others in *Aspergillus* and *Penicillium* species. There is also a noteworthy content of GOD in honey which may lead to the antimicrobial wound healing effect of bee honey (Bang *et al.*, 2003). GOD belongs to oxidoreductases and catalyses the oxidation of glucose. Glucose and oxygen react to form β-D-glucono-δ-lacton and hydrogen peroxide. The enzyme is commercially used in the synthesis of glucuronic acid (Ray & Banik, 1999) and as marker enzyme in several enzyme immunoassays (Suffin *et al.*, 1979). As the enzymatic reaction of GOD leads to the release of peroxide, the coupling of GOD to tumor targeting antibodies

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might be a promising strategy in the treatment of malignant diseases (Ng *et al*., 2002). Another approach would be the use of bispecific antibodies against a tumor antigen and glucose oxidase. Such bispecific molecules can also recombinantly be generated. In 2004, Ascione *et al*. (2004), described the selection of a human anti-GOD *scFv* from a naive antibody library using phage display.

We describe the generation of a panel of monoclonal antibodies to GOD and the successful cloning of a *scFv* specifically binding GOD from one of the generated murine hybridoma cell lines. Future aims of this work will be its application in bispecific molecules targeting GOD and tumor associated antigens and also in assays employing the channeling of substrate between two enzymes closely linked via recombinant antibodies. Furthermore, monoclonal or single chain antibodies could be useful in the detection and quantification of glucose oxidase in several microbial strains.

## Materials and Methods

Generation of monoclonal antibodies. The hybridoma technology (Köhler and Milstein, 1975) was applied for the generation of murine monoclonal GOD-specific antibodies. Female C57/bl6 mice were immunized with 70 µg heat denatured GOD (Sigma) for fusions LU2 and E9 or 70 µg GOD + 250 µg catalase (Sigma) for fusion E13 with 100 µl complete Freund's adjuvant, respectively. Mice were boosted with 70 µg GOD + 250 µg catalase in phosphate buffered saline (PBS) (for fusions E9 and E13) or 70 µg heat denatured GOD (fusion LU2). Four days later, the spleens of the mice were fused with X63-Ag8.653 myeloma cells (ATCC: CRL-1580) using standard fusion with 25% or 42% PEG 1550 (Micheel *et al*., 1994) or electrofusion technique (Schenk *et al*., 2004). Briefly, the spleen/myeloma cell ratio was about 2 : 1 in 10% PEG 8000 and the voltage ranged from 3000-3500 V/cm. Following fusion, the cells were plated into ten 96-well plates (Nunc, Wiesbaden, Germany) per mouse and cultured in RPMI 1640 medium containing 10% fetal calf serum.

Selected hybrids were cultivated, cloned, subloned and stored in liquid nitrogen according to standard methods.

Monoclonal antibodies were tested for specificity using either a solid-phase enzyme immunoassay with GOD at the solid phase (5 µg/ml in PBS) followed by the monoclonal antibody and peroxidase conjugated secondary goat anti-mouse Ig antibody (Sigma) or with goat anti-mouse Ig at the solid phase followed by the monoclonal antibody, GOD and in the last step a mixture of peroxidase, glucose and o-phenylene diamin (OPD). Classes and subclasses of monoclonal antibodies were determined with GOD at the solid phase (for LU2) or with the subclass specific antibodies at the solid phase (for E9 and E13).

To obtain large quantities of monoclonal antibodies, the hybridoma cells were propagated in large culture flasks and the monoclonal antibody was purified from the supernatant using a protein A column (BioTeZ, Berlin, Germany). Elution was performed at acidic pH (3.5) using 100 mM citric acid and NaOH buffer. The fractions were neutralized using 1/10 end volume of 3.5 M Tris-HCl, pH 9.0 immediately.

Cloning of scFv. Total RNA was extracted from about $10^6$ cells of the mouse hybridoma cell line E13BC8 with the Invisorb RNA Kit II purchased from InViTek, Berlin, Germany. Synthesis of cDNA was carried out using MuMLV (MBI Fermentas, St. Leon-Rot, Germany) and oligo-$(dT)_{14}$ primer (BioTeZ, Berlin, Germany) following the protocol recommended by the supplier. Amplification of the genes for variable fragments of heavy and light chain was performed via polymerase chain reaction (PCR) with primers described in Table 1 and CombiPol (InViTek GmbH, Berlin, Germany) according to standard conditions (Schenk *et al*., 1995). PCR products were resolved by electrophoresis in an 1.5% agarose gel and the bands corresponding to about 350 bp were recovered with Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany). To generate cohesive ends, the purified fragments were digested overnight with MluI, BamHI and NcoI, HindIII, for $V_L$ and $V_H$ respectively. After purification via agarose gel electrophoresis the fragments were ligated into pOPE 101 (Schmiedel *et al*., 2000) using T4-DNA-Ligase (MBI Fermentas). Ligation was performed in two steps: at first $V_H$ was inserted into the vector, the plasmids were amplified in *E. coli* and after that $V_L$ was inserted into the $V_H$ containing vector. For amplification of the plasmids and later expression *E. coli* XL-1 blue cells were transformed via electroporation following standard protocol (Potter 1988). Positively selected clones on 2YT medium containing 1% glucose and 100 µg/ml carbenicillin (2YTGC) were tested for correct inserts using restriction analysis. Plasmids were also sequenced with a sequencing device from Applied Biosystems, Langen, Germany.

**Protein expression.** Positive clones were grown overnight in 2YTGC at 37°C. One ml of this culture was inoculated in 100 ml fresh 2YTGC and grown for additional 3 h at 37°C and 225 rpm. Then the cells were harvested by centrifugation and grown overnight at 30°C and 225 rpm in fresh 2YTC containing 100 µM isopropyl-D-thiogalactoside (IPTG). *ScFv* was isolated from culture supernatant, periplasmic and osmotic shock fraction. Periplasmic fraction was obtained by incubating the *E. coli* cell pellet with periplasmic buffer (25% glucose; 20 mM Tris-HCl pH 7.5; 1 mM EDTA) for 20 min on ice. The osmotic shock fraction was extracted from the remaining cell pellet for 20 min on ice using 5 mM $MgSO_4$ (Dübel *et al*., 1995). The different fractions were separated in a gradient (7.5-15%) SDS-PAGE according to Laemmli (1970) and stained with Coomassie blue or blotted on a nitrocellulose membrane membranes (PROTRAN NC BA3, Schleicher & Schuell, Dassel, Germany) with a semi-dry blotting apparatus. The membrane was blocked overnight with PBS/5%NCS (neonatal calf serum) and incubated with monoclonal anti-myc-*tag* antibody 9E10 (1 µg/ml in PBS/NCS) (Chan *et al*., 1987) for one hour at room temperature. After washing twice with PBST (PBS + 0.5% Tween20), the membrane was incubated with peroxidase conjugated goat anti-mouse IgG (1 : 10.000 in PBS/NCS, Dianova, Hamburg, Germany) for one hour at room temperature. The membrane was washed three times with PBST and stained with diaminobenzidine (0.8 mg/ml DAB; 0.4 mg/ml $NiCl_2$; 0.01% $H_2O_2$ in 100 mM Tris/HCl pH 7.5) according to standard protocols.

**ELISA for glucose oxidase binding of scFv.** The culture supernatant, periplasmic fraction and osmotic shock fraction were tested for active *scFv* in an enzyme linked immuno sorbent assay (ELISA).

**Table 1.** Sequences of primers used for amplification of genes for variable antibody regions according to Rohde *et al.* (1998). The recognition sites for the restriction enzymes are in bold (NcoI for H1-5, HindIII for G, MluI for L1-4 and BamHI for K)

Heavy Chain

H1 CAG CCG G**CC ATG G**CG CAG GTS CAG CTG CAG SAG
H2 CAG CCG G**CC ATG G**CG GAG GTG AAG CTG CAG GAG TCA GGA CCT AGC CTG GTG
H3 CAG CCG G**CC ATG G**CG CAG GTS MAR CTG CAG SAG TCW GG
H4 CCG G**CC ATG G**CG CAG GTG CAG CTG CAG CAG TCT GG
H5 CAG CCG G**CC ATG G**TC CAR CTK CTC GAG TCW GG

G ACC AGG GGC CAG TGG ATA GAC **AAG CTT** GGG TGT CGT TTT

Light Chain

L1 GAA GC**A CGC GT**A GAT ATC KTG MTS ACC CAA WCT CCA
L2 GAA GC**A CGC GT**A GAT ATC GTG ATR ACM CAR GAT GAA CTC TC
L3 GAA GC**A CGC GT**A GAT ATC WTG MTG ACC CAA WCT CCA CTC CT
L4 GAA GC**A CGC GT**A GAT ATC GTK CTC ACY CAR TCT CCA GCA AT

K GAA GAT **GGA TCC** AGC GGC CGC AGC ATC AGC

Goat anti-mouse immunoglobulin (Dako, Glostrup, Denmark) in a concentration of 10 µg/ml in PBS was adsorbed to the solid phase of a microtitration plate overnight at 4°C. After blocking with PBS/NCS (neonatal calf serum, one hour, room temperature), anti myc-*tag* antibody 9E10 (1 µg/ml in PBS/NCS) was added and incubated for one hour at room temperature. The different *scFv* containing fractions were then applied with 175 µg/ml GOD-solution (Sigma, Taufkirchen, Germany). As control samples, periplasmic and osmotic shock fractions of a *scFv* binding horseradish peroxidase (previously unpublished) or simply PBS/NCS were used instead of the anti-GOD *scFv*. For another control experiment GOD was replaced by HRP in this step. After incubation for one hour the plates were washed with 0.5 M NaCl, 0.1% Triton X-100, 50 mM Tris-HCl pH 7.5. Detection of bound GOD was performed with a substrate solution containing 5 µg/ml horseradish peroxidase (Fluka, Taufkirchen, Germany), 280 mM glucose and OPD (o-phenylene diamin, 1 mg/ml) in sodium dihydrogen phosphate buffer, pH 5.6, while GOD oxidizes glucose the synthesized hydrogen peroxide is then used for the HRP-reaction. The reaction was stopped with 1 M $H_2SO_4$ containing 50 mM $Na_2SO_3$ and absorption was measured at 492 nm.

## Results and Discussion

Following three different immunization schemes cell fusions for the generation of monoclonal antibodies against glucose oxidase from *Aspergillus niger* were performed. Out of about 400 positive wells, we obtained 62 monoclonal antibodies. A representative selection of these antibodies is shown in Table 2. There were 18 monoclonal antibodies from fusion LU2 where heat denatured GOD was used as the antigen for immunization. Although all of these antibodies bound GOD adsorbed to the solid phase, none of the antibodies was able to bind native and active GOD. The remaining 44 antibodies from fusions E9 and E13, where the immunization and screening was performed with active GOD were all binding to native GOD, but surprisingly none of them bound GOD adsorbed to the solid phase. These results were not observed for other monoclonal antibodies against GOD (Samoszuk & Yang, 1994; Karmali & Oliveira, 1999). Most of the generated monoclonal antibodies were of the IgG class, but two antibodies were of IgA class, which is unusual for hybridomas generated from spleen cells. Such IgA antibodies could be useful for the production of bispecific IgA/IgM-antibodies (Behrsing *et al.*, 1992).

**Table 2.** Monoclonal antibodies against glucose oxidase obtained from three different cell fusions

| Antibody | Class/Subclass | Binding to native GOD | Binding to adsorbed GOD |
|---|---|---|---|
| E9AA6 | IgG2b | ++ | -- |
| E9AF8 | IgG1 | ++ | -- |
| E9AG12 | IgG1 | ++ | -- |
| E9BG10 | IgG2a | ++ | -- |
| E9CF2 | IgG1 | ++ | -- |
| E9GC5 | IgG2a | ++ | -- |
| E9HA11 | IgG1 | ++ | -- |
| E9HH12 | IgG1 | ++ | -- |
| E13AA8 | IgA | ++ | -- |
| E13AD12 | IgG1 | ++ | -- |
| E13BC8 | IgG1 | ++ | -- |
| E13EH12 | IgG1 | ++ | -- |
| E13GC11 | IgG1 | ++ | -- |
| E13JC4 | IgG1 | ++ | -- |
| E13FF7 | IgA | ++ | +/- |
| LU2AC12 | IgG2b | -- | ++ |
| LU2EA3 | IgG1 | -- | ++ |
| LU2HF5 | IgG2b | -- | ++ |
| LU2KE1 | IgG1 | -- | ++ |

| | | |
|---|---|---|
| pelB | | MKYLLPTAAAGLLLLAAQPAMA |
| $V_H$ | FR I | EVKLQESGPSLVAPSQSLSITCTVSGFSLT |
| | CDR I | NDGVD |
| | FR II | WVRQPPGKGLEWLG |
| | CDR II | VIWGGGSTNYNSALMS |
| | FR III | RLSINKDNSKSQVFLKMNSLQTDDTAMYYCAK |
| | CDR III | HELGRGFAY |
| | FR IV | WGQGTLVTVSA |
| Yol | | AKTTPKL**EEGEFSEARV** |
| $V_L$ | FR I | DILMTQSPLSLPVSLGDQTSISC |
| | CDR I | RSSQSIVHSNGNTYLE |
| | FR II | WYLQKPGQSPKLLIY |
| | CDR II | KVSNRFS |
| | FR III | GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYC |
| | CDR III | FQGSHFPWT |
| | FR IV | FGGGTKLEIK |
| *Tags* | | RADAAS EQKLISEEDL S HHHHHH |

**Fig. 1.** Amino acid sequence of *scFv* E13BC8 (pelB: signal peptide from pectate lyase B, FR: framework regions, CDR: complementarity determining regions, Yol: linker region containing the epitope for the tubulin monoclonal antibody Yol1/34 (bold), *Tags*: C-terminal region containing the c-myc *tag* (underlined) and 6 histidine *tag*.

One of the generated monoclonal antibodies binding native GOD was chosen to clone the corresponding *scFv*. The DNA encoding the variable regions ($V_L$ and $V_H$) of the murine monoclonal antibody E13BC8 was amplified by PCR from the hybridoma cell line. We used four different degenerated primers for $V_L$ and five different degenerated primers for $V_H$ (Table 1). We found amplificates with all primer pairs. The best results were obtained with the pair H2/G vor $V_H$ and L3/K for $V_L$. PCR products from that reaction were used to prepare *scFv* gene fusion constructs for cloning into the prokaryotic expression vector *pOPE101*. This plasmid contains the synthetic promoter P/A1/04/03, which is inducible with IPTG and repressed by glucose. The two antibody chain genes were cloned step by step into the expression vector by using different restriction sites for each PCR product. All steps were checked by gel electrophoresis where the V-fragments were about 350 *bp*, respectively and the full length construct approximately 750 *bp*. The antibody gene is preceded by a pelB bacterial leader sequence for secretion into the periplasmic space. The genes for the heavy- and light chain variable domains are linked together by a peptide linker containing the yol epitope of alpha-tubulin recognized by the monoclonal antibody Yol1/34 (Breitling & Little, 1986). Downstream the *scFv* gene contains a c-myc *tag* and a $(His)_6$-*tag*. The cloned *scFv* was sequenced and the deduced amino acid sequence is shown in Fig. 1. The framework and complementarity determining region (CDR) sequences were determined in accordance to Kabat *et al*. (1991). There were no unusual amino acids in the heavy chain and only the threonin in position 19 of the light chain is rarely observed in that position in the framework I region (Martin, 1996). The *scFv* possessed a heavy chain that was classified as a member of the subgroup IB and a kappa light chain from the family II, subgroup I (Kabat *et al*., 1991). A database search using the BLAST algorithm (Altschul *et al*., 1990) against the SWISS-PROT, PDB and Kabat database (Berman *et al*., 2000; *Bergholz et al*., 2001; Boeckmann *et al*., 2003) revealed 79% identity to the *Fv* region for the heavy chain of the monoclonal anti-hen egg white lysozyme antibody D1.3 (Bhat *et al*., 1990) and even 93% identity to the light chain of an Fab fragment binding to the histidine-containing protein of the phosphoenolpyruvate-sugar phosphotransferase system of *E. coli* (Prasad *et al*., 1988).

To analyze the GOD binding capacity of the *scFv* fragments, soluble proteins were used from culture supernatant and from the periplasm of *E. coli* transformed with *pOPE101-E13BC8* and induced with 100 µM IPTG. GOD-binding *scFv* were concentrated in the periplasmic and osmotic shock fraction of *E. coli*. Both fractions gave high signals with glucose oxidase as antigen (Fig. 2). There was almost no active recombinant protein in the culture supernatant. Probably, the expression rate is low. The pelB signal sequence leads to secretion of the recombinant protein into the periplasmic space. Binding to glucose oxidase seems to be specific as unrelated *scFv*

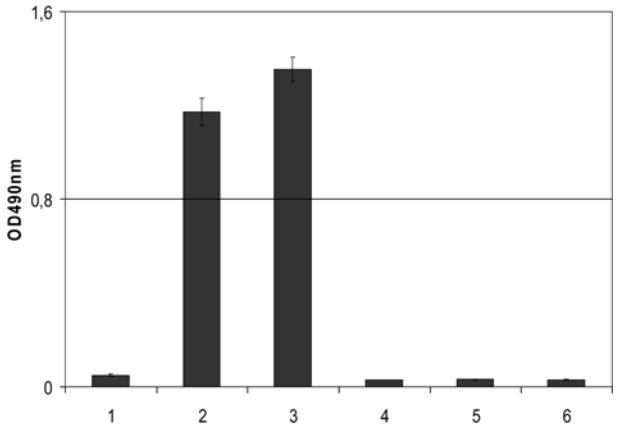


**Fig. 2.** Binding of soluble *scFv* to glucose oxidase. A 96 well plate was coated with antibody 9E10 and fractions of *scFv* E13BC8 were applied together with glucose oxidase. The test was developed with OPD, the reaction stopped with 1M $H_2SO_4/Na_2SO_3$ and measured at 490 nm. Tested probes were *scFv*-E13BC8 culture supernatant (1), periplasmic fraction (2), osmotic shock fraction (3), periplasmic (4) or osmotic shock fraction (5) of an unrelated anti-HRP *scFv* and PBS/NCS (6). The experiment was replicated three times, the figure represents typical results.

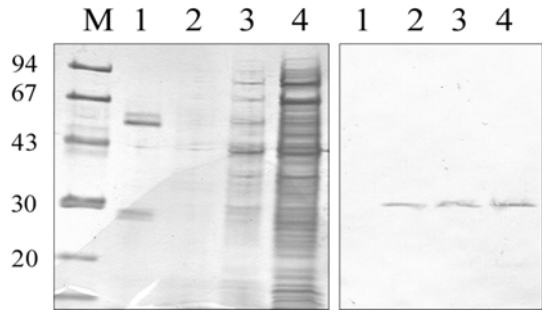


**Fig. 3.** SDS-PAGE and Immunoblot of the different *scFv* fractions. Fractions were separated in a gradient (7.5-15%) SDS-PAGE and stained with Coomassie Blue (left side) or blotted to a nitrocellulose membrane and stained with anti-myc antibody 9E10 followed by HRP conjugated goat anti mouse IgG and DAB (right side). Lane 1 represents the monoclonal antibody E13BC8 as a control, the *E. coli pOPE101-scFv-E13BC8* culture supernatant (lane 2), periplasmic fraction (lane 3) or osmotic shock fraction (lane 4). M is the Low Molecular Weight Marker (numbers in kDa) (Amersham Pharmacia).

fragments did not bind to GOD (Fig. 2) and the anti-GOD *scFv* did not bind to horseradish peroxidase in a control ELISA experiment (data not shown). The recombinant *scFv* was not able to detect denaturated or adsorbed glucose oxidase (data not shown), as it was already shown for the parental monoclonal antibody. In other publications, this phenomenon was not described. Monoclonal (Samoszuk & Yang, 1994) and recombinant antibodies (Ascione *et al*., 2004) were selected against adsorbed GOD and supposed to bind also the active native enzyme. As GOD is highly glycosylated we propose that these antibodies might recognize carbohydrate epitopes (Jan & Husain, 2004) and our *scFv* should detect a conformational protein epitope.

Further characterization in a western blot revealed a unique band for the periplasmic or osmotic shock fraction after overnight expression (Fig. 3). This band at about 30 kDa corresponds to the molar mass determined from the protein sequence of the *scFv*. Surprisingly, in the immunoblot we could detect the *scFv* in the culture supernatant, although in ELISA there was only a minority of active protein.

Here we describe the first *scFv* cloned from a mouse monoclonal antibody directed against glucose oxidase from *Aspergillus niger*. The *scFv* was cloned and easily expressed in *E. coli*. This expression format offers the possibility of fast and cheap production of large amounts of recombinant protein. The correct folding of *scFv* is often a problem when cloning antibodies from established hybridoma cells. This could be overcome by applying a phage display round after cloning if the pure antigen is available in sufficient amounts (Pan *et al*., 2006). Interestingly, we could show that our *scFv* is able to bind native glucose oxidase without inhibiting the enzyme activity. This is especially important for analytical applications. Humanized anti-GOD *scFv* fused to anti-tumor *scFv* in a bispecific construct could also offer a possibiliy as therapeutic agent.

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