Development of a monoclonal antibody for ELISA of CYP1A in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes

S. Scholz, I. Behn, H. Honeck, C. Hauck, T. Braunbeck and H. Segner

Induction of cytochrome P4501A (CYP1A) in cultured cells can be used to determine the induction potencies of xenobiotics or complex environmental samples. This report describes the development of an enzyme-linked immunosorbent assay (ELISA) for measurement of CYP1A expression in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Juvenile rainbow trout were injected with β-naphthoflavone (BNF) (25 mg kg^{-1} body weight) to induce the synthesis of CYP1A. The CYP1A isoenzyme was purified, characterized by immunological cross-reactivity and N-terminal sequencing and used to prepare a monoclonal antibody in Balb-C mice. The specificity of the antibody for CYP1A was proved by Western blotting of samples from control and BNF-injected fish. Two ELISA methods, a direct and a competitive one, were evaluated, with both methods being of comparable sensitivity. Rainbow trout hepatocytes, maintained as monolayers in serum-free, chemically defined medium, were exposed to β-naphthoflavone, and the induction response was measured both by 7-ethoxyresorufin-*O*-deethylase (EROD) activity and the direct ELISA method. Comparison between EROD activity and immunodetectable CYP1A protein can provide information on the catalytic efficiency of CYP1A.

Keywords: *cytochrome P4501A, primary culture, induction, ELISA, monoclonal antibody.*

Abbreviations not explained in the text: *ABTS, 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); DTE, dithioerithrol; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; HEPES, N-(2-hydroxethyl)piperazine-N´-(2-ethanesulphonic acid); MAB, monoclonal antibody; PBS, phosphate buffered saline; P450, cytochrome P450; PMSF, phenylmethylsulphonylchloride.*

Introduction

Teleost fish are equipped with a cytochrome P450 enzyme system that is involved in the metabolism of endogenous compounds such as steroids and exogenous compounds such as polycyclic aromatic hydrocarbons (PAHs) or halogenated aromatic hydrocarbons (HAHs; Goksøyr and Förlin 1992). Metabolism via cytochrome P450 monooxygenases is an important factor to control activation or detoxification of drugs and organic xenobiotics (Goldstein and Faletto 1993). Function and regulation of these proteins determines the susceptibility of a species to the toxic action of many xenobiotics (Goksøyr *et al.* 1987, Braunbeck and Voelkl 1991) as it takes influence on their bioaccumulation and metabolism (Lech and Bend 1980).

Exposure of fish to PAHs and HAHs results in the induction of CYP1A, a particular isoenzyme of the cytochrome P450 enzyme system (Zhang *et al.* 1990, van der Weiden *et al.* 1993). Planar PAHs – or those which are able to take a planar shape – bind to an intracellular cytosolic protein referred to as the Ah-receptor. The inductor–ligand complex is coupled to a second protein called Arnt (Aryl hydrocarbon nuclear translocator), which transfers the complex to the nucleus where it interacts with xenobiotic-responsive DNA elements (XRE) in the promotor region of the CYP1A gene and activates transcription. The activation process finally leads to elevated levels of CYP1A protein and, concomitantly, to enhanced catalytic activity (Bock 1993).

There are different methods for the measurement of CYP1A induction. Beside catalytic assays, which utilize the oxidative dealkylation of 7-ethoxyresorufin or other artificial substrates (Ullrich and Weber 1972, Burke and Mayer 1974), specific antibodies for the detection of the CYP1A protein or the cDNA probes to analyse the amount of CYP1A mRNA have been developed (Haasch *et al.* 1989, Förlin and Celander 1993). Measuring the induction of CYP1A solely by 7-ethoxyresorufin-*O*-deethylase (EROD) activity could lead to biased results, since (1) the postmicrosomal protein supernatant of rainbow trout liver seems to contain an activity-inhibiting substance (Achazi *et al.* 1994) and (2) various environmental chemicals have been shown to inhibit enzyme activity. Several polychlorinated biphenyl congeners result in an inhibition of EROD activity, regardless of the fact that the amount of enzyme protein increases (Gooch *et al.* 1989, Goksøyr *et al.* 1991b, Boon *et al.* 1992, Hahn *et al.* 1996). This situation has prompted the development of immunochemical methods as a supplement to the catalytical detection of CYP1A (Goksøyr *et al.* 1991b, Brüchschweiler *et al.* 1996).

The use of cell cultures to detect the CYP1A-inducing potential of chemicals or environmental samples attracts growing attention in the field of aquatic toxicology (Pesonen *et al.* 1992, Clemons *et al.* 1994, Hahn *et al.* 1996). *In vitro* bioassays not only allow for rapid and sensitive screening of CYP1A-inducing compounds under highly standardizable conditions, but they also provide an experimental basis for the study of structure–activity relationships in CYP1A induction or for the analysis of mixture effects.

Whereas most *in vitro* studies on CYP1A induction in fish have made use of cell lines (Clemons *et al.* 1994, Brüschweiler *et al.* 1996, Hahn *et al.* 1996) the present study employs primary hepatocyte cultures from rainbow trout. We explore the possibility of using primary cultures for the parallel assessment of catalytic EROD activity and immu

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of CYP1A protein, since the induction response at these two levels can be different (Gooch *et al.* 1989, Hahn *et al.* 1996). For this purpose, we developed a monoclonal antibody against rainbow trout CYP1A and applied it in an ELISA technique.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss*) of 200–300 g body weight were obtained from a local hatchery and caged in a flow-through tank system at a temperature between 15 and 17°C for microsome preparation and 12 and 15°C for cell isolations, respectively. Fish were fed daily with commercial trout pellets.

Balb-C mice were used for the preparation of syngenal peritoneal macrophages. Mice were fed *ad libitum*.

Preparation of rainbow trout liver microsomes

Rainbow trout were injected intraperitoneally with 25 mg BNF kg^{-1} body weight dissolved in cod liver oil (7.5 mg ml^{-1}). After 5 days, fish were killed by an overdose of 4-ethylaminobenzoate (4 mg ml^{-1}) and the liver was perfused via the heart with 0.9% NaCl. After clearing of blood, the liver was minced into small pieces in ice-cold homogenization buffer (50 mM Tris/HCl pH 7.8, 0.25 M sucrose, 2 mM EDTA, 150 mM KCl, 1 mM DTT, 0.25 mM PMSF) using 4 ml buffer per g liver and then gently homogenized with five or six strokes in a Potter-Elvehjem homogenizator at 300 rpm while cooling with ice.

For the preparation of the microsomes, the homogenate was first centrifuged at 2500 *g* for 10 min (Heraeus Biofuge 17RS). The microsomal pellet was obtained from the supernatant by centrifugation for 90 min at 100 000 *g* (Beckman L5-65 ultracentrifuge) and was suspended in 50 mM potassium phosphate (pH 7.4), 20% glycerol, 1 mM EDTA, 0.1 mM DTT using 0.5 ml buffer per 1 g original liver weight. The microsomal fractions of 58 fish were collected, pooled, frozen with liquid nitrogen and stored at –80°C.

Purification of CYP1A

Microsomes were solubilized while stirring at 4°C in 100 mM potassium phosphate (pH 7.4), 20% glycerol, 1 mM EDTA, 0.1 mM DTE and 0.25 PMSF at a protein concentration of 10 mg ml^{-1} protein in the presence of 0.8% cholate. After 30 min the solution was centrifuged at 100 000 *g* for 60 min and the pellet was discarded.

The solubilized cytochrome P450 was loaded on an ω-aminooctyl-Sepharose 4B column (2–3 nmol P450 ml^{-1} ω-aminooctyl-Sepharose 4B; Pharmacia, Freiburg, Germany) equilibrated with 100 mM potassium phosphate (pH 7.4) containing 20% glycerol, 0.25 M KCl, 1 mM EDTA, 0.1 mM DTE and 0.4% cholate. The P450 solution was adjusted to the same concentrations as the equilibration buffer. After a washing step with the equilibration buffer P450 was eluted with 0.3% emulgen 911 (Kao Corporation, Tokyo) in equilibration buffer. Fractions containing the P450 isoenzymes were collected and concentrated by ultrafiltration over a PM30 membrane (Amicon, Witten, Germany).

An NAP-column (Pharmacia, Freiburg, Germany) was used to change the buffer to 10 mM Tris/acetate (pH 7.5) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTE and 0.4% emulgen 911. Further purification was performed by loading the P450 solution on a DEAE–Toyopearl 550 column (60 nmol P450 per 7 ml gel in a 1 cm × 10 cm column equilibrated with the Tris/acetate buffer using FPLC apparatus (Pharmacia). P450 was eluted with a step gradient (4, 10, 40 and 100%) leading to a final concentration of 0.8 M acetate. Fractions containing the CYP1A isoenzyme were pooled and concentrated by ultrafiltration. The subsequent purification step was performed with Pharmacia FPLC apparatus employing a hydroxylapatite column equilibrated with 10 mM potassium phosphate (pH 7.2), 20% glycerol, 0.3% emulgen 911, 0.1% cholate, 0.1 mM EDTA and 0.1 mM DTE. After changing the buffer the P4501A fraction (40 nmol P450 on 11 ml hydroxylapatite gel) was loaded on the column. The column was washed with equilibration buffer and developed with a linear gradient of 0.15 M potassium phosphate in equilibration buffer (total volume 100 ml).

A last purification step was performed to remove the detergents, which were necessary to solubilize the P450 from the microsomal membranes. The CYP1A solution was concentrated and then diluted 1:10 in hydroxylapatite column buffer. After loading with CYP1A, the column was rinsed with 100–200 ml emulgen- and cholate-free buffer, while the effluent was monitored at 280 nm. CYP1A was eluted with a buffer containing 0.4 M potassium phosphate (pH 7.2), 20% glycerol, 0.1 mM EDTA and 0.1 mM DTE.

Identification of the CYP1A isoenzyme

During purification, cytochrome P450 and total protein were detected by measuring the on-line absorption of the effluent at 417 nm (P450) and 280 nm (protein). The fractions containing fish CYP1A isoenzyme were identified by cross-reaction with a polyclonal rat CYP1A1 antibody (Amersham, Braunschweig, Germany). The purified trout P4501A showing only a single band in an SDS polyacrylamide gel electrophoresis was further characterized by N-terminal protein sequencing.

Preparation of antibodies

The preparation of antibodies was performed according to the method of Köhler and Milstein (1975) as modified by Behn and Fiebig (1984). A 100–150 μg sample of the antigen diluted in 200 μl PBS was injected intraperitoneally in mice. Hybridoma clones secreting a CYP1A antibody could be identified with an ELISA, probing the supernatant of the hybridoma cultures against microtitre plates (Becton Dickinson, Heidelberg, Germany) coated with 10 μg ml^{-1} of the purified rainbow trout CYP1A.

The antibody subtype was determined with the mouse hybridoma subtype kit from Boehringer (Mannheim, Germany).

Isolation and culture of liver cells from rainbow trout

Liver cells were isolated and cultured based on descriptions by Blair *et al.* (1990) as modified by Braunbeck and Storch (1993) with the collagen perfusion technique.

Cells were seeded at a density of 1.6×10^6 ml^{-1} in Primaria culture dishes (Becton Dickinson, Heidelberg, Germany) in HMEM (Hank's buffered Minimum Essential Medium; Biochrom, Berlin) supplemented with 2 mM glutamine, 10 U ml^{-1} Penicillin and 10 μg streptomycin. Every 20-cm^2 dish contained 5 ml of the suspension. Cells were cultured for 3 days and the medium was changed daily. For CYP1A induction 0, 3.9, 31, 125, 500 and 2000 μM β-naphthoflavone (BNF) dissolved in DMSO were added to the medium to give a final concentration of 0.1% DMSO. BNF was added after 24 h when cells had fully attached to the culture plates.

Preparation of microsomes from trout liver cell cultures

Approximately 1×10^6 cells were used to prepare the microsomes. The cells were scratched off the culture dishes, resuspended in 100 μl PBS and centrifuged at 200 *g*. The pellet was resuspended with 100 μl homogenization buffer, sonified for 10 s and then centrifuged at 10 000 *g* for 10 min. The supernatant was centrifuged at 100 000 *g* for 90 min in a Beckman Optima TL ultracentrifuge and the resulting pellet was resuspended in coating buffer (50 mM sodium bicarbonate, pH 9.5) and diluted to a final concentration of 10 μg protein ml^{-1}.

Analytical procedures

Protein concentration

Protein was determined according to Bradford (1976). Total contents of cytochrome P450 were assayed by the spectral method of O

EROD activity

Erod activity was measured at 25 °C by the conversion of 7-ethoxyresorufin to the fluorescent resorufin as described by Burke and Mayer (1974) and Pluta (1992) with a Perkin Elmer LS50B fluorimeter (Perkin Elmer, Überlingen, Germany) or with an SLT Fluostar fluorescence microtitre plate reader (SLT, Crailsheim, Germany). For measuring EROD activity in cultured hepatocytes, cells were scratched off the culture plates and resuspended in 300 µl PBS per 8×10^6 cells. The suspension was centrifuged at 50 *g* for 3 min. The pellet was resuspended in 300 µl homogenization buffer, sonified for 10 s and stored at –80 °C after quick freezing with liquid nitrogen. For analysis of enzyme activity, samples were thawed, centrifuged for 10 min at 10 000 *g*, and activities were determined in the supernatants.

Inhibition of EROD activity by antibodies

Prior to the inhibition test, antibodies were purified with a protein G column (Mab Trap G II kit, Pharmacia, Freiburg, Germany) to avoid unspecific effects of serum proteins of the hybridoma culture supernatant. Microsomal protein (0.004–0.4 mg ml^{-1}) of livers of induced and control rainbow trout were incubated with the same volume of a 0.01–0.03 mg protein ml^{-1} solution of antibodies. As an unspecific control antibodies against B-cell surface protein CD 22 (provided by Dr I. Behn, University of Leipzig) was used. After 30 min incubation EROD activities of the microsomes/antibody mixture were measured.

Western blotting

Samples of homogenates and microsomes of rainbow trout liver containing 0.05–10 µg protein were separated by electrophoresis in a 10% polyacrylamide gel. The gel was stained with Coomassie Blue (Serva, Heidelberg, Germany) to detect protein bands. For the detection of CYP1A, proteins were transferred to nitrocellulose (0.45 µm, Sartorius, Göttingen, Germany) in Hoefer Western blot apparatus (Serva, Heidelberg, Germany) with a constant current of 0.5 A for 6 h (blot-buffer: 25 mM Tris, 0.193 M glycine). Bands were detected by staining the peroxidase activity of the secondary antibody with chloronaphthol.

Direct ELISA

Homogenate and microsome samples were diluted to 10 µg ml^{-1} protein in coating buffer (50 mM Na-bicarbonate, pH 9.5). Standard concentrations of purified CYP1A were prepared between 5 and 60 ng ml^{-1} in coating buffer. Microtitre plates (96-well) (Becton Dickinson, Heidelberg, Germany) were coated with 100 µl of these solutions and incubated overnight at 4 °C. After washing the plates three times with washing buffer (0.1% Tween 20 in PBS), 200 µl of the blocking solution (1% BSA in PBS) were added to each well and incubated for 1–2 h. Prior to adding 100 µl of the specific anti-CYP1A (hybridoma culture supernatant, diluted 1:10 in PBS) antibody, plates were rinsed again three times in washing buffer. Plates were incubated for 1 h with the specific antibody, then rinsed again with washing buffer and incubated for 1 h with the conjugated antibody (goat anti-mouse Ig, horse-radish peroxidase, DAKO, Hamburg, Germany, dilution 1:500). After a final washing step the colour was developed in ABTS-reagent (100 mM ABTS, 115 mM Na-acetate-3-hydrate, 50 mM Na_2HPO_4, 1 µl ml^{-1} H_2O_2 added immediately before use; pH adjusted with acetic acid to 4.2). The absorbance was read after 60 min at 490 nm with a reference wavelength of 405 nm in an SLT microtitre plate reader (SLT, Crailshaim, Germany).

Competitive ELISA

Plates were first coated with 0.1 µg ml^{-1} CYP1A and incubated overnight at 4 °C. Plates were then washed and blocked as in the direct ELISA. The primary antibody was diluted 1:10 with PBS and mixed with an equal volume of microsomes, homogenate or CYP1A solution in reaction tubes and incubated for 1 h (total volume 400 µl). The mixtures were then added to the microtitre plates in a volume of 100 µl per well. After another incubation for 1 h, the secondary antibody was added at a dilution of 1:500. Development of colour was performed as described for the direct ELISA.

Determination of enzyme activity and ELISA detection of CYP1A was done in triplicate.

Curve fitting of dose–response curves

Dose–response curves were calculated by approximation of the concentration/activity values – where *x* represents the concentration of the inducer and *y* the CYP1A concentration or catalytic activity, respectively – to a sigmoidal curve. The curve was described by the following equation:

$$y = a_0 + \frac{a_1}{1 + e^{\left(\frac{x - a_2}{a_3}\right)}}$$

Calculation was done by the iterative approximation of the constants a_0 to a_3 with the computer program SlideWrite Plus (Advanced Graphics Software, Carslbad, USA).

Statistics

Two independent cell isolations were performed. Within each isolation data were measured in triplicate. Dose–response curves were calculated from the mean of both cell isolations.

Results

Preparation of the antibody

BNF-injected rainbow trout used for the purification of CYP1A protein showed a significant induction of hepatic CYP1A. EROD activity in the 10 000 *g* supernatant of homogenates of control rainbow trout increased more than 10-fold from 14 ± 10 pmol min^{-1} mg^{-1} protein in control animals to 240–300 pmol min^{-1} mg^{-1} in induced animals. Activities in microsomes elevated up to 100-fold from 21 ± 10 pmol min^{-1} mg^{-1} protein for control microsomes to 420–2920 pmol min^{-1} mg^{-1} in BNF-treated animals.

In the purification procedure, the CYP1A isoenzyme could first be separated from other P450 enzymes after the purification on DEAE–Toyopearl 550 (Figure 1). The third peak could be identified as CYP1A by cross-reactivity in Western blots with a polyclonal antibody against rat CYP1A1 and by the N-terminal sequence of the protein, which was identified as L-M-I-L-P-I-I-G-S-V-. This sequence was identical to that of rainbow trout CYP1A published by Heilmann *et al.* (1988).

Purification yielded less than 27% and 15%, respectively of the initial amount of cytochrome P450 after the detergents had been removed, respectively. However, specific contents were increased more than 15-fold as was analysed by carbon monoxide difference spectra (Table 1). SDS gel electrophoresis of the purified protein showed only a single band after staining with Coomassie Blue. The molecular weight of this protein was in the range between 45 000 and 67 000 (Figure 2).

Two hybridoma clones were identified (denoted as HCRT1 and HCRT2), which produced IgG1 antibodies against purified rainbow trout CYP1A. Both antibodies were able to detect a single band in microsome and homogenate samples of BNF-injected rainbow trout and purified C

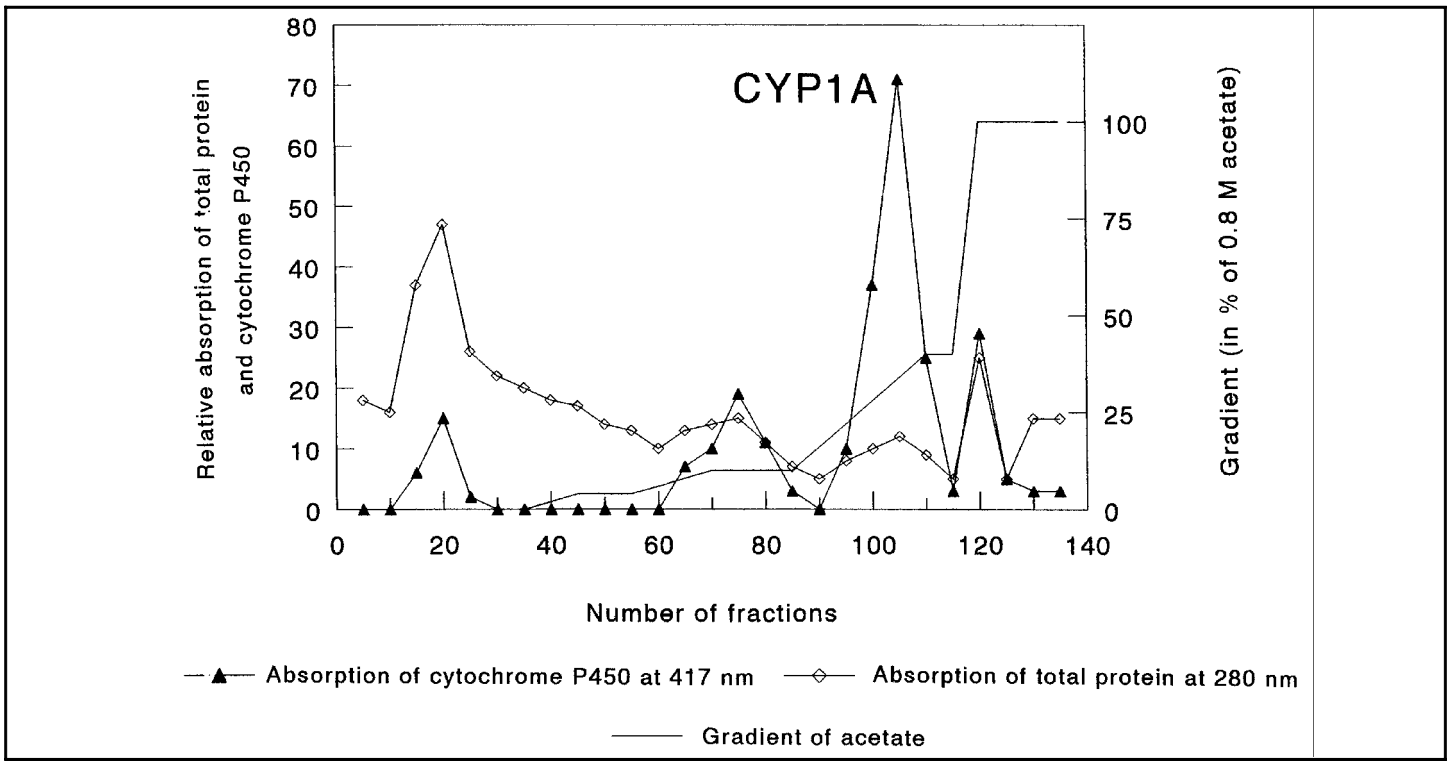

Figure 1. Chromatogram of the CYP1A purification on a DEAE–Toyopearl 550 column. Fractions containing cytochrome P450 were detected by measuring the absorption at 417 nm. Total protein was measured by 280 nm absorption. The fractions containing the CYP1A isoenzyme were identified by Western blotting against an anti-rat CYP1A1 polyclonal antibody. Proteins were eluted from the column with a step gradient leading to a final concentration of 0.8 M acetate (= 100%, line without symbols). Each fraction contained between 1.6 and 1.9 ml of effluent. The column was loaded with 61 nmol cytochrome P450.

(Figure 2). In samples of control animals, no band could be detected (control lane not included in Figure 2). The optimal dilution range for the two hybridoma culture supernatants containing the antibodies was 1:10.

Depending on the epitope recognized by an antibody, it should be expected to inhibit the activity of the antigenic enzyme. Inhibition studies with the purified antibodies of the supernatant of the two clones developed in this study showed no inhibitory effect on enzyme activity. Since affinity of the two antibodies differed, only the antibody with higher affinity was used for the detection of CYP1A protein.

Purification step	Specific cytochrome P450 content (nmol mg^{-1} protein)	Yield (percent of P450 content in liver microsomes)
Liver microsomes	1.01	100
Solubilization	1.1	95
ω-Amino-octyl-sepharose 4B	3.7	85
DEAE-Toyopearl	5.8	42
Hydroxylapatite	16.95	27

Table 1. Purification of CYP1A from BNF-injected rainbow trouts.

Specific P450 content was measured as total cytochrome P450 content per mg of total sample protein. The yield represents the recovery of cytochrome P450, if compared with the initial amount of the microsomes. Total recovery after removal of the detergents was 15%.

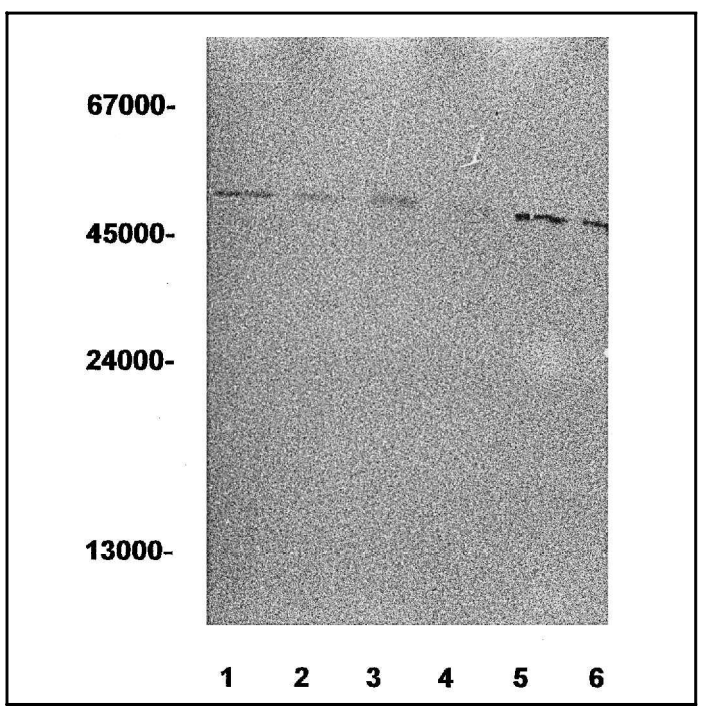

Figure 2. Western blot of liver homogenate and microsomes of BNF-induced rainbow trout. Lanes 1 and 2: 1 μg and 0.5 μg homogenate protein; lanes 3 and 4: 0.5 μg and 0.25 μg microsomal protein; lanes 5 and 6: 0.05 μg and 0.025 μg CYP1A. All lanes probed with supernatant of the hybridoma clone HCRT1, diluted 1:10 (homogenates and microsomes of control animals showed no bands and were not included in the figure).

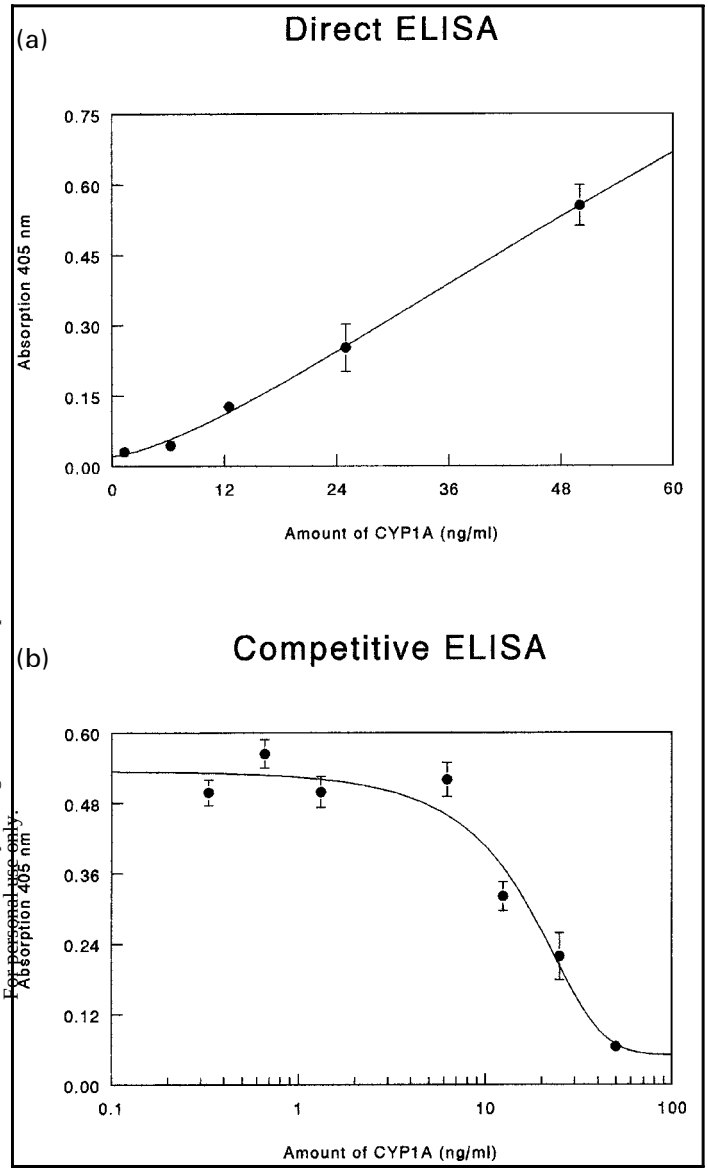

Figure 3. ELISA standard curves of CYP1A using antibodies of the hybridoma clone HCRT1. (a) Standard curve of a direct ELISA. (b) Standard curve of a competitive ELISA. The standard deviation is the intra-assay variation.

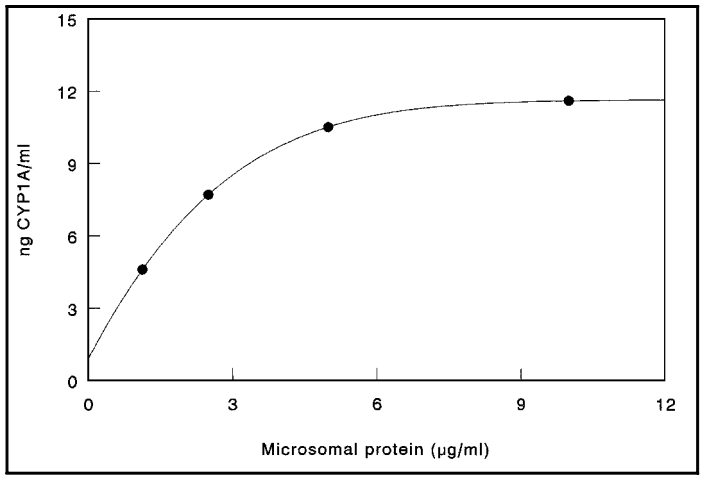

Figure 4. Dependence of CYP1A concentration measurement on microsomal protein concentrations. Microtitre plates were coated with different concentrations of liver microsomes of induced rainbow trout. CYP1A was measured by a standard curve with an ELISA.

Development of an ELISA against rainbow trout CYP1A

In developing an ELISA against trout CYP1A, a direct and a competitive ELISA were compared. A dilution series of purified CYP1A ranging from 3.1 to 50 ng ml^{-1} was used to determine optimal concentrations of antibodies and the detection level. Dilutions of 1:10 from the supernatant of antibodies secreting hybridomas were found to give optimal results in both ELISAs. Under these conditions the sensitivity of both ELISAs was at 6.25 ng CYP1A ml^{-1} (Figure 3). In all further investigations, we therefore used the direct ELISA, which saved purified CYP1A protein.

No linear relationship between the amount of microsomal protein used for the coating of the ELISA plates and the corresponding CYP1A concentrations detected by ELISA was observed (Figure 4). At higher protein concentrations CYP1A showed a saturation effect, which had been also found for ELISAs with monoclonal antibodies against cod CYP1A1 (Goksøyr 1991). Due to the missing linearity, we took care to always use exactly the same amount of protein in induction ELISA measurements (10 µg protein ml^{-1}).

Induction of CYP1A in primary cultures of rainbow trout liver cells

Exposure of cultured hepatocytes to a concentration series between 3.1 and 2000 nM BNF led to an induction of CYP1A, shown by EROD activity and the ELISA technique (Figure 5). Sigmoidal dose–response relationships could be established for the catalytic as well as the immunological detection method. Differences between the two independent cell isolations were greater for immunochemical measurements of CYP1A, compared with kinetic measurements. Moreover, CYP1A showed a lower induction factor: while EROD activity in 10 000 *g* homogenates of liver cells increased by a factor of 9 for 2000 nM BNF after 2 days incubation, ELISAs showed only an induction factor lower than 3. Since homogenates showed a high signal to noise ratio, ELISA had to be performed with microsomes of cultured liver cells.

Discussion

The aim of this study was to develop a monoclonal-based ELISA technique to detect the induction of CYP1A in primary cultures of fish hepatocytes. Since rainbow trout is the most widely used teleost model (cf. Segner 1997), the development of a monoclonal antibody was regarded as important.

Evidence for the specificity of the prepared monoclonal antibody came from several observations. First, the antibody reacted only with a single protein band in Western blots prepared from homogenates and microsomal preparations of livers of BNF-induced rainbow trout. The detected band in Western blots had the same molecular weight as the band of the purified CYP1A detected in gels stained for proteins (data not shown). Second, a parallel increase of the immunochemically recognizable CY

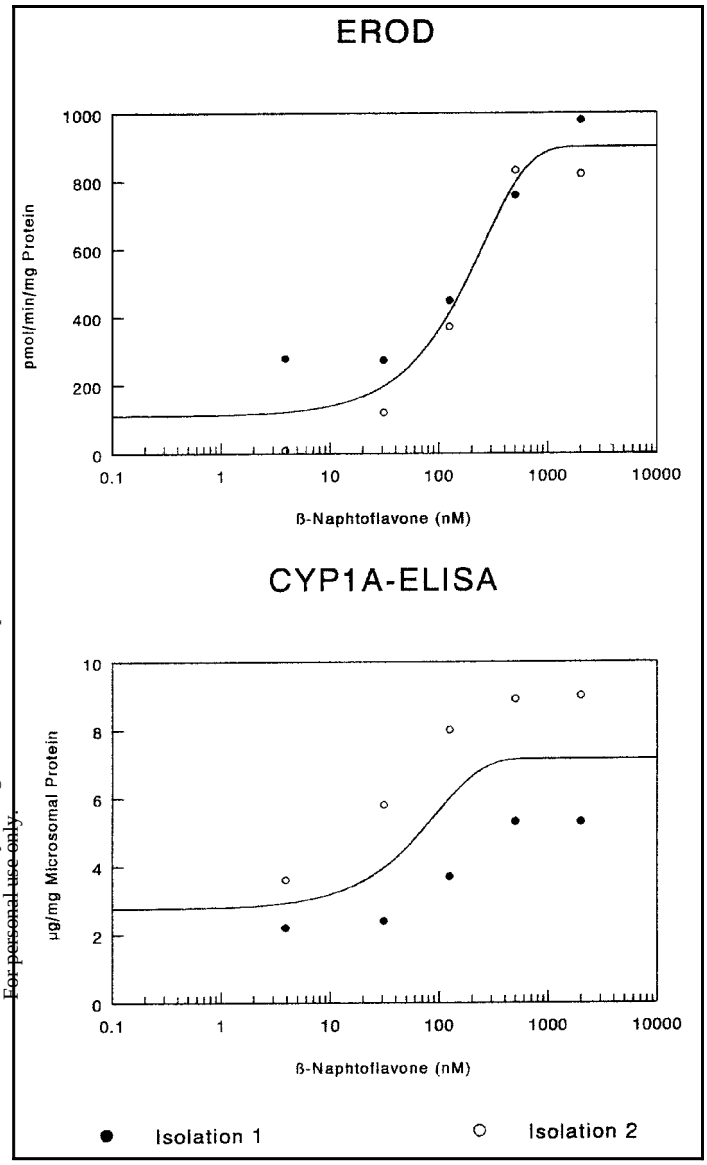

Figure 5. Induction of CYP1A in primary cultures of rainbow trout hepatocytes incubated for 2 days in medium containing 0, 3.9, 31, 125, 500 and 2000 nM of BNF. Induction of CYP1A was measured by EROD activity or by immunochemical detection (CYP1A–ELISA) of the protein. Two hepatocyte isolations were performed, where each datum point represents the result of one isolation. The dose–response curve was calculated from the mean of both cell isolations.

increase in EROD activity could be shown for both BNF-injected animals and for liver cell cultures. Since BNF has been reported as a specific inducer of CYP1A and the corresponding EROD activity (Stegeman *et al.* 1986, Celander and Förlin 1991, Ioannides and Parke 1993), this finding supports the conclusion that the antibody is specific for CYP1A. The fact that the antibody could not inhibit EROD activity is no argument against its specificity, since possibly the epitope detected by the antibody is not located in the active centre of CYP1A.

The CYP1A antibodies were used to establish an ELISA. Values obtained by CYP1A-ELISA represent relative rather than absolute values (Goksøyr 1991). This is partly due to the amount of variable non-CYP1A protein present in the sample, which may interfere with immunochemical detection of specific CYP1A proteins. Also, overloading of the plates could not be excluded – although common protocols suggest to use up to 10 µg protein per well (Peters and Baumgarten 1988). However, in standard solutions of purified CYP1A with protein concentrations about 60 ng ml^{-1} this interference could not be observed.

To date, only a few authors have reported the use of antibodies for the detection of CYP1A in primary cultures of fish hepatocytes. Miller *et al.* (1993a, b) measured the induction of CYP1A by acetaminophen over a culture period of 96 h. Contrary to our findings, these authors could not detect CYP1A immunochemically in control cells, but only in induced cells. This difference might be related to different baseline levels of CYP1A in the experimental animals. Pesonen *et al.* (1992) investigated the time course of CYP1A mRNA, protein and EROD activity in trout hepatocytes exposed to single doses of BNF or TCDD. In the present study, dose–response curves of the ELISA and the EROD assay for BNF were compared. Both methods showed a dose–response relationship for CYP1A activity/concentration according to BNF concentration. The curves were in parallel, but the induction factor was lower with the ELISA than with the EROD. This could be due in part to different sample preparations techniques: while EROD was measured in homogenates, ELISA had to be performed with microsomes, since homogenates gave only values close to the background level. These results conflict with the EROD activities in the liver of the BNF-injected rainbow trout: in microsomes – a concentrated source of cytochrome P450 isoenzymes – EROD showed a 10-fold higher induction if compared with homogenates.

Despite this lower sensitivity, when defined as an increase of response per increase of dose, the ELISA offers the advantages that (1) CYP1A can be measured in the presence of inhibiting substance and chemicals or if high concentration of the inducer became inhibitory (Goksøyr *et al.* 1991b, Achazi *et al.* 1994, Brüschweiler *et al.* 1996), (2) the ELISA requires only small amounts of protein, which is usually a limiting factor in cell cultures, and (3) immunochemical detection is less sensitive to storage conditions of CYP1A protein. Another interesting point is from the work of Pesonen *et al.* (1992): whereas EROD activity dropped to control levels immediately after removal of the inducer, CYP1A protein remained elevated for a prolonged post-exposure period. Such behaviour may become important in biomonitoring studies or in situations of repeated exposure.

Differences between the two independent hepatocyte isolates were greater for ELISA detection of CYP1A than for EROD activity. This higher variation might result from the preparation of microsomes. Differences in microsomal protein contents in individual animals could lead to altered CYP1A levels, if values are expressed as per mg microsomal protein.

The measurement of CYP1A induction with primary liver cell cultures could be more sensitive if compared with different permanent cell lines. While dose–response relationships on the induction of EROD and CYP1A immunodetectable protein can be obtained with the relative weak inducer β-naphthoflavone in liver cell cultures, similar results for permanent cell lines have been reported only with strong inducers such as dioxins (Lorenzen and Okey 1990, Clemons *et al.* 1994).

Recording of dose–response curves of EROD and CYP1A for additional inducers and the measurement of environmental samples for CYP1A induction in trout liver cell cultures have been initiated in our laboratory. The combination of EROD activity measurement and immunochemical detection of CYP1A can be a promising approach to reveal structure–activity relationships for the induction of piscine CYP1A as well as for the screening on the presence of inducing compounds in environmental samples.

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