Evaluating molar CYP1A level in fish hepatic microsomes by competitive ELISA using recombinant membrane-free CYP1A standard protein

Moshe Tom a,*, C.R. Myers b, Michael R. Waterman c

a Israel Oceanographic and Limnological Research, PO Box 8030, 31080 Haifa, Israel
b Medical College of Wisconsin, Milwaukee, WI 53226, USA
c School of Medicine, Vanderbilt University, Nashville, TN 37232-0146, USA

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Abstract

Fish cytochrome P4501A (CYP1A) is a widely accepted environmental biomarker, detecting biological effects of several xenobiotic groups present in aquatic environments, when evaluated in target tissues of a biosensor species. However, appropriate utilization of its protein level as a routine environmental diagnostic tool requires evaluation of properly normalized molar levels, mitigating comparison among different laboratories, during a multi-annual time scale and over a variety of tested populations of the biosensor species. A competitive enzyme-linked immunosorbent assay (ELISA) was developed for determination of CYP1A of the striped sea bream, Lithognathus mormyrus, using our previously described antibody raised to a trout CYP1A synthetic peptide, and a recombinant L. mormyrus CYP1A as a competitor. The L. mormyrus CYP1A-cDNA was cloned and modified by truncating its 5' hydrophobic membrane anchor, as well as by addition of 4 × histidine tag, permitting its partial purification on a nickel–NTA column. The modified cDNA was ligated into the PCWOri + vector and heterologously produced in Escherichia coli as a cytosolic, membrane-free protein, retaining its immuno-affinity with the anti-CYP1A antibody in the presence of the detergent Triton X-100. The detergent was added to the ELISA solution during the competitive step, rendering the microsomal CYP1A more accessible to the antibody. ELISA components, including coated levels of the modified standard CYP1A, and the concentrations of the Triton X-100, CYP1A-specific antibody, and the range of dissolved CYP1A standard protein, were optimized. Hypothesized immuno-affinity differences between the microsomal and the recombinant CYP1As, and among microsomal samples, as well as assay accuracy, were examined and discussed. This ELISA can serve for more efficient utilization of fish CYP1A as a pollution biomarker, and also as a model for

Abbreviations: BSA, bovine serum albumin; CV, coefficient of variation; CYP1A, cytochrome P4501A; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani; OD, optical density; PBS, phosphate buffered saline; PMSF, phenyl methyl sulfonyl fluoride; PNPP, p-nitrophenyl phosphate; TB, teriffic broth; TPBS, 0.05% Tween 20 dissolved in phosphate buffered saline; X gal, 5-bromo-4-chloro-3-indolyl β-galactopyranoside.

* Corresponding author. Tel.: + 972-4-851-5202; fax: + 972-4-851-1911.

E-mail addresses: tom@ocean.org.il (M. Tom), cmyers@mcw.edu (C.R. Myers), michael.waterman@vanderbilt.edu (M.R. Waterman).
establishing competitive ELISAs aimed at quantification of many different microsomal P450 proteins. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The eucaryotic enzyme cytochrome P4501A (CYP1A), which belongs to the P450 gene superfamily, is a membrane-bound hemoprotein, located in the endoplasmic reticulum (microsomal fraction) of all examined vertebrates and carries out oxidation reactions related to xenobiotics bio-transformation (Omura et al., 1993; Whitlock and Denison, 1995; Nelson, 2001).

Enzyme-linked immunosorbent assay (ELISA) is an efficient tool for evaluating the level of a specific protein in a protein mixture, and many protocols have been developed to adapt the basic ELISA concept to different proteins and applications (Crowther, 2001). P450 ELISAs were used for screening of potential anti-P450 antibodies (e.g. Eliasson et al., 1998; Miyakawa et al., 1999; Sai et al., 1999; Yang et al., 1998), or for determining P450 levels (e.g. Kitawaki et al., 1989; Herrero and Castell, 1994; Eguchi et al., 1996; Amacher et al., 1997; Nilsen et al., 1997; Scholz et al., 1998; Harada et al., 1999).

CYP1A is induced by certain xenobiotic pollutants, therefore, suggested as an environmental biomarker of the aquatic environment when evaluated in a suitable biosensor fish species. Immunological tools, Western and ELISA, were used for estimating its relative levels in membrane preparations (Goksoyr, 1991; Bucheli and Fent, 1995; Nilsen et al., 1997; Scholz et al., 1998; Collier et al., 1998 and literature therein). However, appropriate utilization of CYP1A protein level as a routine diagnostic parameter, requires evaluation of properly normalized molar levels, mitigating the comparison among different laboratories, over a multi-annual time scale and a variety of tested populations of the biosensor species. Competitive ELISA (Crowther, 2001) is a method of choice for this application, and its establishment should include purification of solubilized CYP1A, required for ELISA well coating and as a calibration standard. It should include also examination of possible interference of the CYP1A associated membranes with CYP1A immuno-affinity. This interference should be reduced, if indicated, increasing measurement accuracy. Herrero and Castell (1994) have already developed a competitive ELISA for rat CYP1A and CYP2B1/2, using standard P450s purified from rat liver. Here we present the establishment of an improved competitive ELISA for fish CYP1A, taking advantage of a heterologously-produced, membrane-free, modified CYP1A, used as standard, and mild application of a detergent, disrupting the microsomal membrane and increasing the CYP1A immuno-affinity. The present ELISA protocol, its optimization procedure and the analysis of its results, is suggested as a model for establishing competitive ELISA aimed at accurate measurement of many different microsomal P450s.

2. Materials and methods

2.1. Materials and kits

Utilized enzymes were Superscript II reverse transcriptase, 200 U μl⁻¹ (Gibco-BRL); Taq DNA polymerase, 5 U μl⁻¹ (Sigma); high fidelity PFU turbo DNA polymerase, 2.5 U μl⁻¹ (Stratagene); solid lysozyme 73 000 U mg⁻¹ protein (Sigma). Utilized antibodies were anti-CYP1A IgG (24 μg ml⁻¹ total IgG) produced as described by Myers et al. (1993), and alkaline phosphatase-conjugated goat anti-rabbit IgG, used as secondary antibody (Jackson Immunoresearch Labs, PA; 0.6 mg ml⁻¹). Utilized kits were ABI-prism BigDye sequencing kit (Perkin–Elmer), pGEM-T easy T-A cloning kit (Promega), QIAGen kits for DNA extraction from agarose gels and polymerase chain reaction (PCR) solutions, Ni–NTA–agarose resin (Qiagen), and protein
determination kit by modified Lowry method (DC protein assay kit, BioRad). All other compounds were of analytical grade.

2.2. Fish sampling

Live *Lithognathus mormyrus* specimens were fished, by gillnet, at the Mediterranean coast of Israel. Livers were dissected out immediately following decapitation of live fish, snap frozen in liquid nitrogen and kept at −80 °C.

2.3. General molecular methods

General biochemical and molecular protocols followed Sambrook et al. (1989) or instructions provided with the commercial kits. These protocols include DNA, RNA, and protein electrophoresis, spectrophotometric evaluation of DNA, RNA and protein concentrations, plasmid manipulations, and selective growth of bacteria on ampicillin–isopropyl β-D-thiogalactopyranoside (IPTG)–5-bromo-4-chloro-3-indolyl β-galactopyranoside (X gal)–Luria–Bertani (LB)-agar plates. PCR procedures (Mullis et al., 1994) were done in a final solution of 0.2 mM dNTP and 10 μM (or 100 μM for degenerate primers) primer concentrations, using buffers supplied with the DNA polymerases. PCR designs and applied templates are detailed below for each particular reaction.

2.4. Total RNA and microsomal preparations

Total hepatic RNA was extracted using the TriReagent isolation kit (Molecular Research Center, USA). RNA quality was examined by electrophoresis on a 1% agarose–formaldehyde gel and its concentration was evaluated by spectrophotometry. Hepatic microsomes were prepared at 4 °C according to Nilsen et al. (1997). Briefly, livers were homogenized in 0.1 M sodium phosphate buffer, pH 7.4, including 0.15 M KCl, 0.1 mM of both ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) and 0.2 mM phenyl methyl sulfonyl fluoride (PMSF). Homogenates were centrifuged for 15 min at 8000 × g followed by ultracentrifugation of the resulting supernatant for 30 min at 150 000 × g for separation of the cytosol from the microsomal fraction. Microsomes were resuspended in the same buffer, in a 20% glycerol solution, and their protein content was determined by modified Lowry method (Bio Rad instructions).

2.5. Cloning of *L. mormyrus* CYP1A-cDNA

*L. mormyrus* CYP1A-cDNA was cloned by reverse transcription (RT)-PCR. RT of 3 μg RNA was performed using the superscript II reverse transcriptase (Gibco-BRL, manufacturer’s instructions), and a reverse oligo dT-linker primer (5’-GACTCGAGTCGACATCGAT17-3’). The procedure includes 15 min incubation at 37 °C followed by 50 min at 50 °C. Two microliters of the RT product were subjected to PCR (one cycle at 94 °C, 2 min, 35 cycles at 94 °C, 1 min, 58 °C, 1 min and 72 °C for 4 min, followed by final incubation at 72 °C for 15 min) using 1.25 U per 50 μl reaction of high fidelity DNA polymerase. The primer pair includes the reverse linker primer (5’-GACTCGAGTCGACATCG-3’) and a forward degenerate primer designed according to the conserved fish CYP1A translation start site (5’-ATGGTNYTNAATGATHYTNCC-3’). The resulting PCR band was extracted from agarose gel, T-A cloned, transfected to JM109 *Escherichia coli* strain and selected on ampicillin–IPTG–X gal LB-agar plates. A single appropriately transfected colony was sorted by PCR, using primers designed according to already known partial *L. mormyrus* CYP1A sequence (Tom, unpublished information). The CYP1A-cDNA was sequenced, revealing high similarity to other fish CYP1As, and was deposited in GenBank (Acc. No. AF264037).

2.6. Protein expression of modified *L. mormyrus* CYP1A

The coding region of *L. mormyrus* CYP1A-cDNA was modified following the general guidelines of Imai et al. (1993), Barnes (1996) as follows: (a) the second amino acid was replaced by alanine, amino acids 3–31 were deleted, and the A-T content of codons 32–38 was maximized. (b)
Four histidine residues were added to the 3' end of the coding region followed by the stop codon, permitting a one step protein purification on a nickel–NTA affinity column. The CYP1A-cDNA was modified by PCR, generally following Higuchi et al. (1988) (one cycle at 94 °C for 2 min, 35 cycles of 94 °C 1 min, 50 °C, 1 min, and 72 °C for 2.5 min, and final incubation at 72 °C for 15 min) using high fidelity DNA polymerase. The primer pair includes the forward primer 5'-TGTCTGGTCT ACCATATGGC TAAATTTTTT TT CGAACAGAAA TTCC-3' and the reverse primer 5'-ATACATAAGC TTCTAGTGAT GG-TGATGCTCT CCCTGCTCG-3'. The forward and the reverse primers contain NdeI and HindIII restriction sites, respectively, and their elongated 5' tails enabled efficient digestion by these enzymes. The modified coding region of the cDNA was purified from the PCR solution, digested by NdeI and HindIII, and cloned into the expression plasmid PCW0ori+. The produced construct was transfected into JM109 E. coli strain and an appropriate transformant bacterial colony was selected as described above for the CYP1A-cDNA cloning.

The protein was expressed following Barnes (1996). A freshly plated transfected bacterial colony was grown overnight in LB medium containing 100 μg ml⁻¹ ampicillin, followed by inoculation of 5 ml bacterial culture into 2.8-l Farenbach flasks containing 500-ml TB medium enriched by 1-mM thiamine, and containing 50 μg ml⁻¹ ampicillin. The culture was shaken at 37 °C, 240 rpm, until reaching absorbance of 0.8 OD units at 600 nm (3–4 h). The incubation conditions were changed at this point to 28 °C, 180 rpm, and the heme precursor δ-aminolevulinic acid and the inducer IPTG were added, both to a final concentration of 0.5 mM. The incubation was continued for another 24 h, and the bacteria were harvested by 15 min centrifugation at 3200 × g, 4 °C. The pellet was resuspended in 30 ml 0.1 M potassium phosphate buffer (K₂HPO₄, titrated to pH 7.4 with concentrated phosphoric acid) in 20% glycerol solution, supported with 0.1 mM of both EDTA and DTT. The resuspended bacteria were mildly tilted at 4 °C for half an hour in the presence of 0.5 mg ml⁻¹ lysosyme, and the resulting spheroplast suspension was sonicated on ice 8 times × 20 s. DNAaseI (1 μg ml⁻¹), and a protease inhibitor cocktail containing pepstatin A (final solution of 0.5 μg ml⁻¹), PMSF (0.2 mM), and leupeptin (0.1 μg ml⁻¹), was added to the spheroplast suspension before sonication. Preliminary experiments revealed no CYP1A in the bacterial membrane fraction, therefore, the sonicated lysate was centrifuged directly at 150,000 × g for half an hour, and only the CYP1A-containing cytosol saved. CYP1A concentration was determined by the CO difference spectrum method, summing the calculated concentrations of both 450 and 420-nm components of the spectrum, using the 91 and 110 cm⁻¹ mM⁻¹ extinction coefficients, respectively (Omura and Sato, 1964; and Fig. 1).

2.7. CYP1A purification on nickel–NTA agarose column

Cytosolic CYP1A, was purified on a nickel–NTA agarose column (Qiagen instructions manual), at a flow rate of 0.7 ml min⁻¹ cm⁻² column cross-section. The column was equilibrated and washed with 50 mM potassium phosphate buffer containing 0.2 M NaCl, 20 mM glycine, 20% glycerol and supported with 0.1 mM of both EDTA and DDT. The applied cytosol was adjusted to the same composition. The protein was eluted by 50 mM histidine dissolved in the washing buffer, and was 3-fold concentrated in comparison to the applied sample, as was determined by the CO difference spectrum method.

2.8. Western blot

The affinity and specificity of our anti-trout CYP1A antibody (Myers et al., 1993) with the modified CYP1A and with fish hepatic microsomal fractions was analyzed by western blot using the Protean II electrophoretic device (BioRad). Protein solutions were subjected to a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose. Immunoblot reactions (BioRad instruction) were carried out using 1:2000 dilution of the anti-CYP1A antiserum.
2.9. **ELISA procedure**

Competitive ELISA was carried out in 96-well microtiter plates (Nunc, Maxisorb), according to the general guidelines of Coligan et al. (1991). Plate wells were washed, when required, 3 times × 2 min by PBS (8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl) supplied with 0.05% Tween 20 (TPBS). General outline of our ELISA protocol includes: (a) Overnight coating of the wells, at 4 °C, with 100 μl of various concentrations of purified CYP1A diluted in 50 mM carbonate–bicarbonate buffer, pH 9.6. (b) One hour well blocking with 200 μl of PBS–2% BSA at 37 °C. (c) Overnight incubation of various dilutions of anti-CYP1A rabbit IgG (Myers et al., 1993), and gradually increasing purified standard CYP1A concentrations (calibration wells) or *L. mormyrus* hepatic microsomal samples, dissolved in 100 μl TPBS–1% BSA per well, supplied with various Triton X-100 concentrations. (d) One hour incubation, at 37 °C, with 1:10⁴ dilution of 100 μl alkaline phosphatase-conjugated anti-rabbit antibody in TPBS–1% BSA. (e) Incubation, at 37 °C, with color substrate–1 mg ml⁻¹ PNPP in 10% (w/w) diethanolamine in 0.01% MgCl₂ solution, pH 9.8. The solution was prepared immediately prior to its use. Color development was determined by measuring the well optical density (OD) at 405 nm, by a microplate reader (Tecan, Spectra Image, Austria). Each OD value represents an average of four replicate wells. Each plate contained control replicates, coated with coating buffer at stage (a), and incubated with TPBS–1% BSA, supplied with detergent at stage (c). Non-specific binding was evaluated from wells coated with coating buffer at stage (a) incubated with TPBS–1% BSA, supplied with detergent and primary antibody, at stage (c). The contribution of the non-specific binding to the OD was always low, therefore, the control wells OD was used as a background value subtracted from all other OD measurements.

2.10. **Analysis of the ELISA results**

All analyses were performed within a plate, assuming intra-plate uniformity of coating, there-
Fig. 2. An example of all constructed plots used for examination of the ELISA characteristics and for the calculation of CYP1A levels in microsomal preparations. (A) Calibration curves, using the linear–log (upper plate) and logit–log (lower plate) transformations. (B) Sample plots, using the respective transformations. Logit OD = log{(OD/OD₀)/(1 − OD/OD₀)}, when OD₀ is the maximum optical density obtained when no competing standard CYP1A was added to an ELISA calibration well, and OD is an optical density value of a calibration well containing specific standard CYP1A concentration. AU, absorbance units; CYP, standard CYP1A concentration (pM); P, total microsomal protein concentration (µg ml⁻¹). Example outlier values are pointed at by the arrows, and were eliminated from the analysis. Calibration and microsomal sample equations, presented for each plot, were calculated using the visually determined linear part of the plots, assisted by evaluating the calculated correlation coefficient values (not shown). Equality of the slope of each sample equation (e.g. −310.8 and −0.91; plate B) with the respective calibration equation (e.g. −279.4 and −1.026; plate A) was statistically tested (Sokal and Rohlf, 1995; Box 14.4, computation of regression with more than one value of Y per value of X; pp. 477–479), for evaluating hypothesized immuno-affinity differences between the recombinant and the microsomal CYP1As, as well as among different microsomal preparations. Microsomal CYP1A was calculated by substituting its OD or logit OD values (plate B) into the respective calibration equation (plate A), followed by calculation of the CYP value. This level was normalized per µg loaded total microsomal protein.

fore, each plate included its own calibration wells, with or without microsomal sample wells, as required. The calibration wells OD values were plotted against the standard CYP1A concentration, and the microsomal wells OD values, against the total protein concentration of the assayed microsomes. Plots were constructed using linear–log and/or logit–log transformations (Perlstein, 1987; Nix and Wild, 2000). An equation describing the linear part of each calibration curve or microsomal plot was calculated, excluding outlier values. An example of all constructed plots, including a description of the linear equations calculation procedure is presented in Fig. 2.

The intra-plate analysis includes (a) calculation of the CYP1A concentrations in the microsomal wells, by substituting their OD values into the calibration equation. Microsomal CYP1A was
finally expressed as pmol CYP1A per μg total loaded microsomal protein. (b) Testing the equality of slopes between the calibration equation and each sample plot, as well as among the microsomal sample plots (slopes were considered unequal when $F > F$ value of the appropriate degrees of freedom and which correspond to $P < 0.05$; Sokal and Rohlf, 1995; Box 14.7, tests for equality of slopes of several regression lines; pp. 495–498).

3. Results

3.1. Modified *L. mormyrus* CYP1A protein

Up to 200 nmol recombinant CYP1A were produced from the cytosolic fraction of 1-l bacterial culture. Partial CYP1A purification was carried out by affinity chromatography on Ni–NTA column, and a typical CO difference spectrum of the purified protein is presented in Fig. 1. The immuno-affinity of the cytosol, the purified protein and *L. mormyrus* hepatic microsomal preparation to the anti-CYP1A IgG (Myers et al., 1993) was examined by Western blot (Fig. 3), revealing a specific immunoreaction of one band at approximately 55 kDa. An additional larger molecular weight band appeared only in the crude bacterial lysate.

3.2. Optimization of competitive ELISA conditions

The ELISA optimization was aimed at maximizing both microsomal CYP1A access to the anti-CYP1A antibody, and the calibration curve linear range. The optimized parameters include the standard coated CYP1A level, anti-CYP1A antibody dilutions, and dissolved Triton X-100 and standard CYP1A concentrations. The optimization is an iterative procedure, and the examined parameters were adjusted repeatedly. The process began by setting the coating solution to 1050 pM standard CYP1A, the concentrations of the dissolved standard CYP1A to 15 000, 3750, 937, 234, 59, and 13 pM, and the primary antibody dilution to $1.2 \times 10^4$. ELISAs including construction of calibration curve and evaluation of CYP1A microsomal levels were carried out using 0.1, 0.2 and 0.4% Triton X-100 concentrations, and a detergent-less control, aiming at examining the effect of the detergent on the visualized microsomal CYP1A level. The samples were applied in a concentration of 20 μg total protein per ml, and maximal CYP1A levels were determined using 0.2% Triton X-100 (Fig. 4a). A second experiment, performed after the optimization of the coated standard CYP1A and the primary antibody dilution, revealed similar results (Fig. 4b). Therefore, 0.2% Triton X-100 was set as standard detergent concentration.

The linear range of the calibration curve was maximized, by optimizing the relative levels of coated standard CYP1A and primary antibody. A matrix of nine calibration curves, composed of three concentrations standard CYP1A coating solutions of 1050, 263 and 66 pM, and three primary antibody dilutions, $1.2 \times 10^4$, $1.4 \times 10^4$, and $1.8 \times 10^4$, was tested. The most wide and accurate range of linearity was achieved using $1.4 \times 10^4$ dilution of the primary antibody and 66 pM of the coated standard CYP1A (Fig. 5). Therefore, coating of the wells with 75 pM standard CYP1A, and competing solution, which includes $1.4 \times 10^4$ diluted primary antibody were used later as standard ELISA conditions.
3.3. Microsomal CYP1A measurements by ELISA

The performance of the optimized procedure was tested in 20 L. mormyrus hepatic microsomal preparations by evaluating two parameters, (a) accuracy of the average molar CYP1A level per μg microsomal protein, and (b) intra-plate equality of slopes between the calibration curve and each of the corresponding sample plots, and among the four sample plots, as a measure of immuno-affinity differences. The analyses were carried out using both the linear–log and the logit–log transformations (e.g. Fig. 2). A standard plate included a set of control wells, seven calibration curve values, 0, 139, 417, 1250, 3750, 11 250 and 33 750 pM standard CYP1A, and four different microsomal preparations, each of them in four 3-fold dilutions. The total protein range of each microsomal preparation was adjusted to roughly fall within the calibration curve OD range, through preliminary ELISAs, however, final average CYP1A values (Table 1) were calculated using the OD values obtained only from the 3- and 9-fold dilutions, as they were always located within both the linear part of the sample plots and that of the calibration curve.

Two sets of measurements were carried out:

1. inter-plate variability of results was evaluated in five replicate plates, using both the linear–log and the logit–log transformations. Only one of the 20 examined microsomal sample slopes was significantly unequal to the corresponding slope of the calibration curve. However, intra-plate significant difference among sample slopes was found in four out of the five examined plates. The microsomal CYP1A values were calculated for all four dilutions of each sample, in each plate, normalized per μg total microsomal protein, and are presented in Fig. 6. The intra-plate average CYP1A level of each preparation was calculated from the eight replicates of the 3- and 9-fold microsomal dilutions, excluding outlier values, and used for the calculation of the average among plates, presented in Table 1.

2. Evaluation of a wide range of CYP1A concentrations, carried out in duplicates, in 16 microsomal preparations. The results are presented in Fig. 7 and Table 1. Only two of the 32 examined microsomal sample slopes were significantly unequal to the corresponding slope of the calibration curve. Intra-plate significant inequality among sample slopes, was observed in four or five out of the eight examined plates, using the linear–log or the logit–log transformations, respectively. A significant positive correlation was revealed, when testing pairs of microsomal CYP1A per μg protein values and their respective coefficients of variation (Table 1), using both transformations ($R = 0.79$; $n = 16$, $P < 0.05$).

The coefficient of variation (CV) of the CYP1A level, among the four replicate wells of a specific value, reflecting the inaccuracy among wells, averaged $13.8 \pm 5.9$ or $15.6 \pm 10.3\%$ across the entire set of measurements, using the linear–log or the logit–log transformations, respectively.
4. Discussion

Purification of P450s from microsomal preparations requires membrane solubilization by detergents, rendering it difficult to coat ELISA wells. Several coating solutions were suggested, such as using a linker antibody, incubation of the coating solution with SM 2 Bio-beads (Kitawaki et al., 1989; Herrero and Castell, 1994), or sharp dilution of detergent-solubilized recombinant P450 microsomal preparations prior to coating (Amacher et al., 1997; Eliasson et al., 1998). The present approach is by far simpler, completely eliminating the detergent from the coating step. It is based on the ability to produce a soluble, membrane-free, modified CYP1A in *E. coli*. The solubilization was achieved by 5′ truncation of the hydrophobic membrane anchor, successfully applied for several microsomal P450s (Von Wachenfeldt et al., 1997; Cosme and Johnson, 2000; Tom and Waterman, unpublished information), although not fully generalized. The modified CYP1A maintained its immuno-affinity, and the transformed calibration curve was linear and usable, regardless of the application of the detergent Triton X-100 (Figs. 2 and 5). This detergent-insensitivity permitted detergent addition (0.2% Triton X-100) to the ELISA solution during the competitive step, disrupting the microsomal membranes, and resulting with an increased immuno-affinity of the microsomal CYP1A. Triton X-100 was chosen due to its successful utilization in P450 studies, disrupting membranes with minor denaturing of the enzyme.

Equality of slopes among a set of ELISA equations (Fig. 2) was assumed to indicate similar competition pattern among tested CYP1As, hence, signifying similar immuno-affinities to the CYP1A antibody, therefore, used to test similarity of immuno-affinities between competitor standard CYP1A and its solubilized microsomal homologue, or among different microsomal samples.

![Fig. 5. A matrix of ELISA calibration curves using various combinations of coated CYP1A levels and primary antibody concentrations.](image)
Table 1
CYP1A levels (pmol µg$^{-1}$ total microsomal protein) resulted from two sets of ELISA assays, an evaluation of inter-plate variability of four microsomal preparations among five replicate plates (fish 1–4), and the evaluation of CYP1A level-related variability, over a wide range of CYP1A levels, assayed in duplicate plates, in 16 microsomal preparations (fish 5–20).

<table>
<thead>
<tr>
<th>ELISA assay</th>
<th>Number of replicates</th>
<th>Fish #</th>
<th>CYP1A level (pmol µg$^{-1}$ protein)</th>
<th>Linear–log transformation</th>
<th>CV (%)</th>
<th>n</th>
<th>Logit–log transformation</th>
<th>CV (%)</th>
<th>n</th>
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<tbody>
<tr>
<td>Evaluation of inter-plate variability</td>
<td>Five replicate plates</td>
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<td>0.397 ± 0.127</td>
<td>32</td>
<td>36</td>
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<td>2</td>
<td>0.171 ± 0.056</td>
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<td>38</td>
<td>0.173 ± 0.047</td>
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<td>3</td>
<td>0.405 ± 0.143</td>
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<td>0.400 ± 0.119</td>
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<td>4</td>
<td>0.188 ± 0.060</td>
<td>32</td>
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<td>38</td>
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<td>Evaluation of CYP1A level-related Variability</td>
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<td>6</td>
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<td>0.524 ± 0.146</td>
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<td>13</td>
<td>0.092 ± 0.014</td>
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<td>0.099 ± 0.015</td>
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<td>14</td>
<td>0.112 ± 0.018</td>
<td>16</td>
<td>16</td>
<td>0.116 ± 0.016</td>
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<td>15</td>
<td>0.091 ± 0.013</td>
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<td>0.091 ± 0.013</td>
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<td>17</td>
<td>0.198 ± 0.024</td>
<td>12</td>
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<td>0.192 ± 0.033</td>
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<td>18</td>
<td>0.132 ± 0.017</td>
<td>13</td>
<td>16</td>
<td>0.127 ± 0.013</td>
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<td>19</td>
<td>0.157 ± 0.033</td>
<td>21</td>
<td>16</td>
<td>0.144 ± 0.038</td>
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<td>20</td>
<td>0.171 ± 0.037</td>
<td>22</td>
<td>16</td>
<td>0.164 ± 0.044</td>
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Intra-plate CYP1A levels (not presented) were calculated using the eight OD values obtained from the 3- and 9-fold microsomal dilutions, located within the linear part of both the microsomal plots and the respective calibration curve (Fig. 2), and n is the total number of ELISA wells used for the calculation of a CYP1A value (not uniform for each fish due to elimination of outlier values). The presented CYP1A levels and their corresponding CV are averages of intra-plate values, 5 or 2 values for the two sets of assays, respectively.

Hypothesized sources for differences in CYP1A immuno-affinity are (1) altered epitopes caused by the standard CYP1A modifications, assumedly a constant factor. However, the number of epitopes is limited here, since an anti-peptide antibody was used (Myers et al., 1993), and indeed, almost all of the microsomal plots were found to be equal to their respective calibration curves, permitting the conclusion that within a plate, the calibration equations are a valid tool for microsomal CYP1A evaluation. (2) The partition of the detergent among its micellar, monomer, lipid bound and protein bound fractions, is changed when applying constant detergent concentration to different membrane and protein concentrations (Helenius and Simons, 1975) and may change the CYP1A accessibility to the antibody, due to incomplete membrane disruption. Twenty-fold difference was observed in the present study between the highest and lowest microsomal CYP1A value (Table 1), leading to a variety of membrane and membrane-associated protein loads in ELISA-applied microsomal samples. The microsomal protein level of a specific sample (a measure of membrane amount), was adjusted to conform to the linear part of the calibration curve, resulting in four-dilution protein range of 0.5–13.5 µg ml$^{-1}$ for CYP1A-rich samples, reaching 2–54 µg ml$^{-1}$ in CYP1A-rich samples, reaching 2–54 µg ml$^{-1}$ in CYP1A-rich samples.
poor ones. This variable microsomal load is hypothesized to be the reason for the significant intra-plate slope inequality among microsomal plots, in more than half of the tested plates, indicating at least one differing slope in each of these plates.

The optimization procedure resulted with maximal immuno-affinity due to optimal Triton X-100 concentration. However, it may be that detergent type or concentration is still sub-optimal. Alternate solubilizers have to be chosen from the non-ionic or bile salt detergents, known to solubilize biological membranes with no apparent damage to the protein conformation at appropriate concentrations (Helenius and Simons, 1975). The optimization resulted also with maximal linear range of the calibration equation, however, it was noticed that different batches of ELISA plates revealed narrower linear range of the calibration curve, assumed to be a result of variable interbatch coating characteristics. Consequently, a repeated optimization of the calibration curve is recommended.

Evaluation of CYP1A level-related variability between two duplicates (Table 1) revealed positive correlation between CYP1A levels and their corresponding CVs. These CVs were ranged approximately between 10 and 50%, and averaged 24 ± 12 and 25 ± 11%, for the linear–log or the logit–log transformations, respectively. The increased variability at higher CYP1A levels is demonstrated also in Fig. 7, comparing CYP1A level among different microsomal dilutions. Both observations indicate yet unexplained effect of the membrane to CYP1A level ratio on the variability. The CV of the average CYP1A level of five replicates when evaluating inter-plate variability, was averaged 33 ± 1.4 and 28 ± 1.5% across the four examined microsomal samples, using the two transformations, respectively (Table 1). These values fell roughly within the CV range obtained for the CYP1A level-related variability measurement.

![Graph showing inter-plate variability assay results](image)

Fig. 6. Results of the inter-plate variability assay, obtained using the linear–log (A), or the logit–log (B) transformations. Each of the curves in the four pairs of coordinate systems depicts the CYP1A levels calculated for the four microsomal dilutions in separate plates. 1–4 are different microsomal preparations (similarly designated in Table 1). The appropriate undiluted microsomal protein concentration was determined by preliminary ELISA to roughly fall within the linear part of the calibration curve.
for similar CYP1A values, although more replicates were assayed (5 vs. 2).

The linear–log or the logit–log transformations revealed almost no difference, judged by both visual comparison of the relationship between microsomal dilution and CYP1A levels (Figs. 6 and 7), and by the compared molar CYP1A values (Table 1). The CV of the average CYP1A level, calculated from the results of the inter-plate variability assay (28 vs. 33%) was the only value showing slight improvement of the logit–log transformation performance over the linear–log one.

The within-wells CV (13.8 ± 5.9 or 15.6 ± 10.3% for the linear–log or the logit–log transformations, respectively) and the two sets of CYP1A measurements, demonstrated in Table 1, provide a general idea of the accuracy obtained, and it is our opinion that further elaboration of the procedure, including determination of suitable replicate number, repeated optimization of the calibration curve, and choosing the most adequate transformation for constructing the calibration equation, all for achieving higher accuracy, should be done as part of a quality control procedure, to be accomplished concurrent with routine assays.

Addition of histidine tag at the 3’ end of the recombinant protein permitted simple one-step partial protein purification on an affinity chromatography column. Recombinant CYP1A concentration was evaluated using the heme-related CO difference spectrum method. However, evaluating the contribution of hypothesized CYP1A apoprotein component, lacking the heme, is not included in this analysis, and if present in the bacterial preparation and bound to the Ni–NTA column, it may bias the results. It is suggested to check apoCYP1A by applying additional purification steps of the bacterial CYP1A product, ending with almost pure protein, as judged by sensitive electrophoretic analysis, followed by accurate measurement of total protein level.

To conclude, the presented competitive ELISA successfully responds to the need for molar evaluation of fish CYP1A. Moreover, it would serve as a model for the establishment of accurate ELISA for many different microsomal P450s, suggesting simple and general solution to the difficulties posed by P450–membrane association.

Acknowledgements

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References


