UNIVERSIDADE ESTADUAL DE CAMPINAS

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"UMA ABORDAGEM PROTEOMICA NA IDENTIFICAÇÃO DO CITOCROMO P450 EM *PROCHILODUS SCROFA*: UMA NOVA FERRAMENTA EM ENSAIOS ECOTOXICOLÓGICOS"

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Funcional e Molecular, na área de Bioquímica.

Orientador: Prof. Dr. José Camillo Novello

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"Toda ciência seria supérflua se a forma de manifestação e a essência das coisas coincidissem imediatamente"

Karl Marx

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RESUMO

Citocromos P450 (CYP) constituem uma superfamília de heme proteínas envolvidas na fase I da biotransformação que participa da detoxificação de compostos endógenos e exógenos. Para que esta atividade seja exercida existe a necessidade da associação do CYP com o citocromo b5 e com a enzima NADPH citocromo P450. Esta associação só ocorre graças ao ambiente de membrana. Qualquer alteração na uniformidade do ambiente lipídico leva à alterações na funcionalidade do sistema CYP.

Baseado nesta informação e no fato de que vários poluentes têm sua solubilidade aumentada pela adição destes compostos, realizamos testes *in vitro* utilizando microssomas hepáticos de Curimbatá (*Prochilodus scrofa)* - um peixe tropical brasileiro pertencente a família Prochilodontidae - e observamos que dois surfactantes normalmente utilizados na solubilização de pesticidas, o Triton X- 100 e o Tween 80. O conteúdo total de CYP e a atividade da EROD foram fortemente inibidos pelo Triton X-100 e pelo Tween 80 de uma maneira dose dependente; o conteúdo de CYP foi reduzido à zero. A atividade da EROD foi completamente inibida pelo Triton X-100 e inibida em mais de 40% na presença do Tween 80. A estrutura molecular do surfactante influencia na alteração que o sistema irá sofrer visto que ambos são hábeis em interagir com as proteínas do sistema microssomal, em especial as monooxigenases, alterando sua conformação e, consequentemente, destruindo sua função. Os resultados desta primeira parte mostraram que os surfactantes interagem com os componentes do sistema microssomal hepático levando a inibição do sistema. A atividade do CYP, que foi usada como biomarcadora de exposição ao xenobiótico pode ser utilizada como marcadora em associação com outras enzimas.

Estes resultados levantaram a hipótese de que os surfactantes também poderiam alterar a atividade do CYP quando utilizados em processos de purificação. Por ser uma proteína de membrana, o CYP precisa ser solubilizado antes de ser purificado. No entanto os surfactantes utilizados durante o processo poderiam levar a destruição do CYP, mascarando a real concentração do CYP obtido durante o processo. Sendo assim, na segunda parte deste trabalho desenvolvemos um novo método de purificação que exclui a necessidade de surfactantes durante o processo de purificação.

A purificação do CYP1A foi feita utilizando-se uma coluna C18 associada à HPLC de fase reversa. O CYP purificado foi caracterizado em relação as suas propriedades eletroforéticas, imunoquímica e atividade biocatalítica. A fração isolada como CYP produz uma única banda uniforme com massa molecular ao redor de 58.000 Da em SDS - PAGE e mostra uma forte reatividade cruzada com anticorpos produzidos contra o CYP1A de trutas. A fração isolada também foi encapsulada em dois tipos de sistemas reconstituídos, um composto por lipídeos neutros e outro por lipídios negativamente carregados. Em ambos os sistemas foi possível detectar a atividade da EROD, mas não a atividade da PROD, confirmando que a fração purificada corresponde a CYP1A e teve todas as suas características enzimáticas conservadas. Houve um

aumento da atividade quando a o CYP1A foi encapsulado nas vesículas contendo lipídeos negativamente carregados, confirmando que a carga do lipídeo é essencial para a manutenção das características enzimáticas do CYP. O processo se mostrou eficaz para a purificação do CYP1A mantendo todas a suas características conservadas. No entanto não permitiu a separação das isoformas CYP1A.

A terceira parte do projeto teve como objetivo a identificação das isoformas CYP1A1 e CYP1A3 através de eletroforese bidimensional associada à espectrometria de massas do tipo MALDI-TOF. Foram identificados 3 *spots* com a mesma massa molecular, mas com pIs variando entre 5,5 e 6,5, sugerindo a presença de três isoformas do CYP1. Os *spots* foram selecionados e tratados com tripsina. O mapa de peptídeos obtidos através da digestão dos *spots* selecionados teve suas massas identificadas por espectrometria de massas do tipo MALDI-TOF e através da digestão teórica de seqüências de CYP já existentes no banco de dados. Houve a identificação de 10 picos que correspondem a 31% do total de aminoácidos traduzidos para a família CYP1A de peixes e mamíferos. Todos os três *spots* tiveram o mesmo padrão de fragmentação confirmando que eles são isoformas CYP1A1 e CYP1A3. Dentre estes peptídeos, dois demonstraram maior importância, o pico T3, que representaria um domínio conservado do CYP1A e o pico T2, que representaria uma região conservada apenas em peixes. Os resultados sugerem fortemente que este novo procedimento seria eficiente para identificar, simultaneamente, diferentes isoformas do CYP1A hepático e suas regiões conservadas.

Dada a sensibilidade das técnicas de proteômica, nesta ultima fase do projeto propomos o uso da eletroforese bidimenisonal associada à espectrometria de massas na identificação de isoenzimas de P450 diferencialmente expressas em microssomas hepáticos de Curimbatás tratados com 3 – metilcloranteno (3-MC). A avaliação das proteínas expressas em cada tratamento foi feita através de eletroforese uni e bidimensional e a identificação feita através da análise de massa de peptídeos. Ambas as eletroforeses permitiram a identificação de proteínas induzidas de maneira diferencial pelo 3-MC, embora a eletroforese tenha sido mais efetiva na identificação de proteínas altamente homólogas, como a CYP1A1 e CYP1A3. Também foi possível a identificação de outras proteínas atuantes na detoxificação de xenobióticos, dentre elas o citocromo b5 (~15kDa) e a glutationa – S – Transferase microssomal (~20kDa).

Os dados demonstrados no projeto mostram claramente que o sistema associado a abordagem proteômica têm um grande potencial para serem utilizados na identificação de proteínas expressas diferencialmente conforme a situação ambiental.

ABSTRACT

Cytochromes P450 constitute a superfamily of the phase I enzymes whose primary task is the detoxification of both endogenous and xenobiotic compounds. This activity is only possible through the association of CYP with cytochrome b5 and the enzyme NADPH cytochrome P450 reductase. This association is possible because of membrane environment. Any alteration in this environmet leads to altereation on the functionality of CYP system.

Based on this information and in the fact that various pollutants have its solubility increased by the surfactant addition, we had done *in vitro* tests using hepatic microsomes of Curimbatá - a Brazilian fish belong the family Prochilodontidae – and observed the effects of Triton X-100 and Tween 80 in CYP system. Both surfactants are commonly used in pesticides solubilization. Total CYP and EROD were strongly inhibited by Triton X-100 and Tween 80 in a concentration-dependent way; the content of CYP was reduced until zero while EROD activity was completely inhibited in the presence of Triton X-100 and more than 40% inhibited in the presence of Tween 80. Each surfactant causes a different effect on each antioxidant enzyme. No effect was detected in SOD activity in the presence of even Triton X-100 or Tween 80. Triton X-100 increase catalase activity, while Tween 80 decreases this enzyme activity. The molecular structure of the surfactants causes the alteration of this system, since they are able to interact with the microsomal protein, especially with monooxigenase's components, altering their conformation and, consequently destroying their function. Our results in this first part suggest that surfactants can interact with components of the microsomal system leading to inhibition of CYP. Therefore, CYP activity, which has been used as a biomarker of xenobiotic exposure, should be used as a marker in association with other enzymes.

With those results we suggest the hypothesis that surfactants that are commonly used in the purification process could alter the CYP activity. Since that CYP is a membrane protein, the solubilization process is necessary before the purification process to separated membrane lipids of proteins. The use of surfactants in the process could mask the real concentration of CYP obtained. Then, in the second part of this project we develop a new method to of purification that excludes the necessity of surfactants.

The purification of CYP1A was done by Reverse Phase HPLC on a C18 column. Purified CYP1A was characterized with respect to electrophoretic, immunochemical and biocatalyst properties. CYP1A fractions produced a single uniform band on SDS-PAGE with an apparent molecular mass of 58 kDa. Purified CYP1A of P. scrofa showed strong cross-reactivity with antibodies directed against CYP1A from trout. The fraction was also encapsulated in two different reconstituted systems; one composed of neutral lipids and another of negatively charged lipids. In both of them, we could detect EROD activity but not PROD activity, which confirms that the CYP1A was purified with all its enzyme activity. There was an increase of activity when CYP1A and NADPH cytochrome P450 (CYP) reductase were encapsulated in negatively charged lipids, which confirms

that the charge of lipid is essential to CYP1A activity. All these characteristics strongly suggest that this new procedure is efficient for purifying hepatic CYP1A from P. scrofa, showing that the CYP1A isoform of this fish has a highly conserved protein region. However the process was not efficient to separate the isoforms of CYP1A.

In the third part of project we aimed the identification of CYP1A1 and CYP1A3 isoforms through bidimensional electrophoresis and MALDI – TOF mass spectrometry. Three major spots were detected. These spots had the same molecular weight, but presented pI in a range of 5.5 to 6.0, suggesting the presence of 3 isoforms of CYP1. Spots were collected, and treated with Trypsin and the resultant peptides were measured by MALDI-TOF Mass Spectrometry. Tryptic peptide mass fingerprint of CYP1A showed the presence of 10 masses that matched the expected tryptic peptides obtained through theory digestion of the database sequence. These values corresponded to 31% of the translated amino acids of CYP1A family of other fishes and mammals. All spots had the same profile of fragmentation, which confirms that they are isoforms. Among these peptides, two demonstrated major importance T3, which is a conservative domain of CYP1A and T2, which could represent a more conservative region that occurs only in fishes. All these characteristics strongly suggest that this new procedure efficiently identifies, simultaneously, different isoforms of hepatic CYP1A from P. scrofa and its conservative region of protein.

Due to the sensibility of the technique, in this last part of the project we try to identify the CYP isoenzymes that are expressed in hepatic microsomes of Curimbatá using one – dimensional (1 – DE) and two dimensional (2 – DE) gel electrophoresis followed by tryptic peptide mapping (PMF). Both 1 –DE and 2 – DE showed that 3 – MC induces not only CYP1A1 and CYP1A3, but other biotransformation enzymes presents in endoplasmic reticulum like cytochrome b5 (~15kDa), NADPH cytochrome P450 reductase (~75kDa) and microsomal Glutathione – S – Tranferase (~20kDa). There were 25 proteins that appeared only in 3 – MC treated group and a matching of 38% between control and 3 – MC gel.

The results demonstrated here show that CYP system associated to the proteomic approach have great potential to be utilized in the identification of proteins differentially expressed according to environmental situation.

INDICE

1 - INTRODUÇÃO

1.1 – BIOMARCADORES

A crescente expansão demográfica e o desenvolvimento industrial comprometeram a qualidade dos diferentes ecossistemas do planeta, em especial os aquáticos, onde ocorre o despejo de diferentes produtos químicos de origem industrial e doméstica. O Brasil, assim como a maioria dos países, é deficiente em recursos que previnam e tratem os problemas causados pelo despejo de resíduos tóxicos no ambiente. Contudo a preocupação crescente em encontrar meios que contenham a poluição ainda em níveis iniciais e evitar seu contato com a população fez crescer, a partir de 1950, as pesquisas em tecnologias para a prevenção da contaminação ambiental (GOLDAFARB et al., 1998).

Os efeitos das atividades humanas sobre o ambiente representam uma grande preocupação para as autoridades. A contaminação ambiental por compostos químicos é a conseqüência direta do rápido processo de industrialização, do crescimento agrícola, da mineração, da emissão de poluentes orgânicos etc., representando um grande risco para o ambiente e para a saúde humana. Estes compostos orgânicos estranhos que são liberados pelas comunidades urbanas e industriais recebem o nome de **xenobióticos**.

A *Ecotoxicologia* é a ciência que alerta para os danos causados por substâncias químicas despejadas indiscriminadamente no ambiente e descreve os efeitos desses compostos sobre o ecossistema e seus componentes não – humanos, incluindo sua interação com o ambiente e os caminhos da transferência desses agentes dentro de um ecossistema definido (de FERNICOLA et al., 2003). Sendo assim, a ecotoxicologia é uma ciência geradora de conhecimentos básicos e essenciais que servirão de base para formulações legais, normas de programas e diretrizes gerenciais em análises de risco ecotoxicológico, potencial ou real determinado pelo uso de compostos xenobióticos no ambiente.

A sociedade tem estado, cada vez mais preocupada com os efeitos nocivos dos xenobióticos em seres humanos e no ambiente em virtude do aumento significativo de doenças, como leucemia, cancro de pulmões e testículos,

cuja origem vem sido atribuída a exposição constante do homem a contaminantes ambientais. Em resposta a esta preocupação a sociedade internacional vem exigindo medidas capazes de prever, avaliar e gerenciar os efeitos nocivos de compostos xenobióticos ao homem e ao ecossistema ao seu redor (MOORE, 2002).

A abordagem clássica de uma avaliação de risco é medir a quantidade do contaminante e relaciona-lo com dados experimentais de cobaias de laboratório, junto aos efeitos adversos causados por uma dada concentração do contaminante. Porém esta abordagem tem limitações. Poucos compostos já têm definidos quais são os níveis críticos para o organismo, visto que, no ambiente, uma grande variedade de organismos são expostos a uma complexa mistura de poluentes no ecossistema, levando a necessidade de um monitoramento biológico seja feito de maneira complementar ao monitoramente químico (HACON, 2003, VAN DER OOST et al, 2003).

A preocupação com o meio ambiente intensificou a procura por meios de monitoramento ambientais mais rápidos e eficientes. Ao enfocar apenas a análise química dos níveis de poluentes na água ou no sedimento, os métodos tradicionais forneciam pouco ou nenhum indício do efeito dos poluentes na fauna e flora, pois tais procedimentos não levam em conta as alterações sofridas pelo organismo em relação às mudanças químicas no meio (GOLDFARB et al., 1998). A fim de solucionar estes problemas, pesquisas procurando indicadores biológicos de qualidade de água têm crescido exponencialmente nos últimos anos.

Os processos de avaliação de risco ambiental utilizam os bioindicadores na avaliação do risco ecológico e dos biomarcadores na avaliação do risco à saúde humana; ambos são utilizados em conjunto para avaliar os riscos ecológicos em populações humanas e ecossistemas em diferentes níveis organizacionais.

Entende-se como bioindicadores medidas que incluem todos os níveis de organização ecológica; desde uma espécie até as populações de um ecossistema. Já os biomarcadores incluem qualquer medida que reflita a interação entre um agente estressor e organismo (HACON, 2003). Na Figura 1 podemos

observar a seqüência de respostas de um sistema biológico na presença de um poluente.

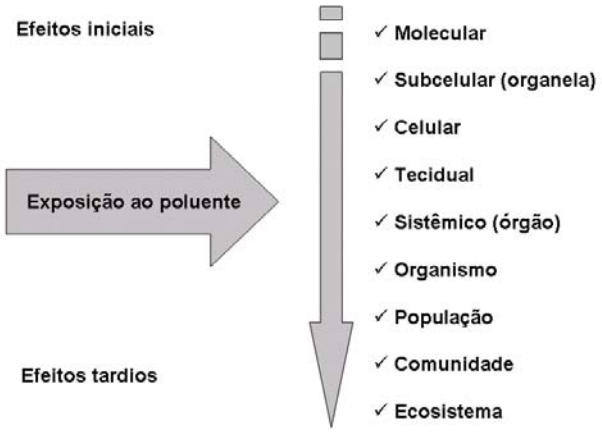

Figura 1 – Representação esquemática da seqüência de respostas de um sistema biológico na presença de um poluente (modificado de VAN DER OOST et al, 2003).

O termo biomarcador foi definido pela primeira vez em 1987 pelo Committee on Biological Markers of National Research Council (NRC) como "indicadores/sinalizadores de eventos em sistemas e/ou amostras biológicas cujo objetivo é determinar: (i) exposição, (ii) efeitos adversos e (iii) susceptibilidade de indivíduos e/ou população com o objetivo de prever e prevenir seres humanos dos efeitos tóxicos de algum composto" (SCHLENK, 1999, VAN DER OOST et al, 2003). Em 1990, Adams modificou esta definição incluindo características do organismo, populações ou comunidades capazes de responder a mudanças no meio (ADAMS, 1990). Peakall (1992) sugeriu utilizar o termo biomarcador para discutir efeitos relacionados com o organismo individual e o bioindicador em relação aos efeitos medidos em níveis hierárquicos maiores, como populações e comunidades.

Sendo assim, estabeleceu-se o uso do termo biomarcador para qualquer resposta biológica que possa ser medida "dentro" do organismo (atividade enzimática) ou em seus produtos (urina, fezes etc.); por outro lado, ficou estabelecido que o termo bioindicador seria definido como as informações tiradas de um organismo que revelam as condições ambientais medidas no seu habitat natural (ADAMS, 1990, DEN BESTEN, 1998, GOLDFARD et al., 1998, SCHLENK,

1999, VAN DER OOST et al, 2003). Atualmente, o termo biomarcador pode ser definido como *"qualquer resposta biológica correspondente a uma exposição, efeito, ou susceptibilidade dos indivíduos aos agentes químicos e/ou estressores ambientais"* (HACON, 2003, VAN DER OOST et al, 2003). Estas medidas geram respostas funcionais, fisiológicas, bioquímicas em nível celular ou de interação molecular. Baseado nesta definição estabeleceu-se três classes de biomarcadores:

a) **Biomarcadores de exposição –** capaz de detectar a presença de um xenobiótico, seu metabólito ou a interação do xenobiótico com algumas moléculas ou células alvo através das características farmacodinâmicas e físico-químicas do composto no organismo (SCHLENK, 1999, VAN DER OOST et al, 2003).

b) **Biomarcadores de efeito –** incluem respostas observadas ao nível molecular (bioquímico, fisiológico e histológico) que podem ser relacionadas com o observado no nível ecológico. Por exemplo, redução na população de uma dada espécie de peixes pode estar relacionada com o aumento da incidência de tumores nesta espécie em conseqüência da exposição desta população a PAH (SCHLENK, 1999).

c) **Biomarcadores de susceptibilidade –** indicam a habilidade adquirida por certo organismo para responder a mudanças de exposição a um dado xenobiótico, seja por fatores genéticos (mutação) ou bioquímicos (alteram a susceptibilidade do organismo) (DEN BESTEN, 1998, SCHLENK, 1999, VAN DER OOST et al, 2003).

Um dos principais critérios que fez explodir as pesquisa sobre novos biomarcadores, em especial os de exposição em ambientes aquáticos, foram os custos–benefícios proporcionados por estes modelos. Comparado aos custos dos métodos químicos de análise de resíduos de compostos como dioxinas e policlorados bifenis, o uso de biomarcadores reduz significativamente os gastos e também o tempo de análise.

Os biomarcadores são ferramentas de monitoramento em qualquer ecossistema, em especial no aquático. No entanto, as respostas são

potencializadas quando se usa o biomarcador certo e no local certo para que a resposta mensurável seja real (ISOMAA e LILIUS, 1995, DEN BESTEN, 1998).

No contexto ambiental, os biomarcadores são indicadores sensíveis e capazes de demonstrar a presença de um xenobiótico no organismo. O biomarcador mais utilizado é o biomarcador de exposição, e os efeitos vão refletir a conseqüência da distribuição de um xenobiótico ou seu metabólito no organismo.

Os principais parâmetros analisados são:

a) Bioacumulação – xenobióticos hidrofóbicos se acumulam no organismo através das brânquias ou pele (bioconcentração), captação de partículas em suspensão (ingestão) e consumo de alimento contaminado (biomagnificação). No entanto alguns dos efeitos só são perceptíveis nos estágios finais de vida, multigeração ou ainda no topo mais alto da cadeia alimentar. A bioacumulação é pré-requisito para efeitos adversos no ecossistema (VAN DER OOST et al, 2003).

b) Bioconcentração – é a razão entre a concentração do xenobiótico no organismo e a água durante o estado de equilíbrio. Isto é possível, pois a captação de poluentes ocorre através da difusão passiva no ambiente aquático, gerando uma relação linear entre a exposição e os resíduos teciduais.

c) Biomagnificação – é a razão entre a captação do xenobiótico e sua liberação.

d) Bioavaliação – é a fração do volume do xenobiótico presente no solo/sedimento e na água que pode ser captado pelo organismo (VAN DER OOST et al, 2003).

e) Biotransformação – transformação de um xenobiótico lipofílico em um composto mais hidrofílico que pode ser mais facilmente excretado.

A maioria dos xenobióticos atinge o organismo através da via gastro-intestinal e, neste sentido, o fígado é o principal órgão de biotransformação devido à sua posição, suprimento de sangue e função. As enzimas envolvidas na biotransformação estão localizadas principalmente no retículo endoplasmático liso e no citossol das células hepáticas. Em número menor estão presentes ainda nas

mitocôndrias e em outras organelas. (VROLIJK et al, 1994, BAINY, 1996, GOKSφYR e HUSφY, 1998, MACHALA et al, 1998, QUABIUS et al, 1998, AU et al, 1999, COUSINOU et al, 2000, TIMBRELL, 2000).

A biotransformação também pode alterar a toxicidade de um composto, tornando – o benéfico ou maléfico para o organismo. São chamadas de **reações de detoxificação** as reações que reduzem a toxicidade de um compsoto permitindo sua excreção. Já a **bioativação** são as reações que transformam o composto xenobiótico em um metabolito reativo mais tóxico que o composto parental

A biotransformação dos xenobióticos ocorre em duas fases:

-**Fase I –** Alteração não sintética, isto é oxidação, redução ou hidrólise. O metabolismo oxidativo é a primeira etapa do processo de biotransformação, sendo catalisado por dois grupos de monooxigenases: as flavoproteínas monooxigenases e o sistema microssomal P450. Nesta primeira fase ocorre a alteração da estrutura da molécula original através da adição de grupos funcionais (-OH, COOH, etc) que a tornam mais hidrofílicos (BAINY, 1996, STEGEMAN e LECH, 1991). A inserção destes grupos leva a produção de metabólitos com alta solubilidade, podendo provocar não só a inativação do composto, mas também a ativação de compostos pro-carcinogênicos (COUSINOU et al, 2000, STEGEMAN e LECH, 1991, KOBLYAKOV, 1998, GRAHAM e PETERSON, 1999).

- **Fase II –** O composto gerado na fase I é conjugado com compostos endógenos, como as glutationas. Na fase II esta molécula estruturalmente diferente será conjugada com compostos endógenos e polares, tornando-a mais hidrofílica, menos tóxica e facilmente excretável (VROLIJK et al., 1994, IYER e SINZ, 1999, TIMBRELL, 2000).

A Figura 2 resume os dois processos acima descritos.

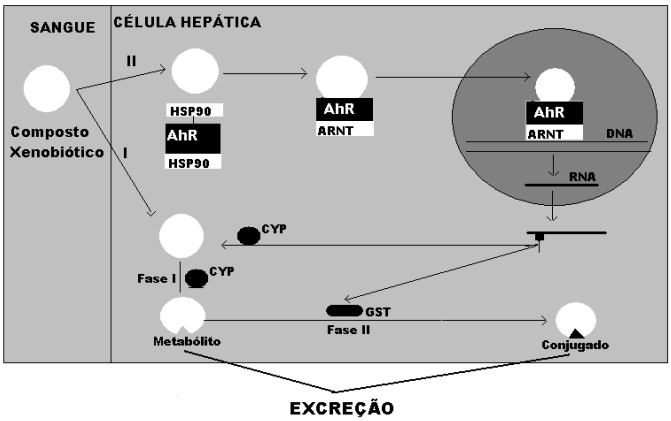

Figura 2 – Mecanismo simplificado de biotransformação de xenobióticos no fígado (modificado de VAN DER OOST et al, 2003).

1.2 –SISTEMA MICROSSOMAL HEPÁTICO - P450

A Fase I da biotransformação envolve a oxidação, redução ou hidrólise do composto xenobiótico original. Tais reações são catalisadas pelas enzimas do sistema microssomal hepático, também conhecidas como as oxidases de função mista (MFO - *mixed function oxidase*). Este sistema é composto pelo citocromo P450 (CYP), citocromo b5 (cit b5), e a enzima NADPH citocromo P450 redutase (NCR). A função mais importante do MFO está relacionada a sua habilidade em facilitar a excreção de certos compostos transformando-o em um composto mais hidrofílico (VAN DER OOST et al., 2003). O MFO é um sistema sensível a certos poluentes ambientais, fazendo com que sua atividade sirva como monitor biológico de exposição.

Embora tenha sido observado pela primeira vez no final dos anos 40, o termo “citocromo P450” foi usado pela primeira vez em 1962 para identificar uma hemeproteína com características únicas, valendo-lhe o nome de pigmento microssomal ligante ao monóxido de carbono (“microsomal carbon monoxide-binding pigment”) (OMURA e SATO, 1964a, OMURA e SATO, 1964b, BERNHARDT, 1995, OMURA, 1999, GUENGERICH, 2000). A característica clássica dos P450 é a posição peculiar do seu pico de Soret em 450 nm como resultado da formação do complexo Fe(II)-CO – surgindo desta constatação o

nome P450 (OMURA e SATO, 1964a, OMURA e SATO, 1964b, MANSUY, 1998, RAUCY e ALLEN, 2001). O grupamento heme do citocromo P450 é derivado da protoporfirina IX, o que parece contribuir para as suas características espectrais únicas (MANSUY, 1998, OMURA, 1999, GUENGERICH, 2000). Tais características espectrais também são atribuídas à presença da cisteína e histidina ligadas ao ferro-heme. O 5º ligante do ferro-heme é o responsável por gerar o espectro característico do P450 reduzido e complexado com monóxido de carbono e, graças aos avanços das técnicas de cristalografia de proteínas e da biologia molecular, hoje se sabe que este quinto ligante do ferro é o ânion tiolato de um resíduo de cisteína (OMURA, 1999).

A reação clássica do P450 (Figura 3) envolve a transferência dos seus átomos de oxigênio para o substrato – daí ser denominada monooxigenação. A ativação deste sistema necessita de dois equivalentes redutores que chegam até o P450 por dois passos distintos. Os passos do ciclo catalítico do citocromo P450 são: (1) Ligação do substrato (RH) ao citocromo P450 (Fe^{+3}); (2) redução monoeletrônica do complexo enzima-substrato pela enzima NADPH citocromo P450 redutase (NCR); (3) ligação do oxigênio molecular (O_2) ao complexo enzima-substrato. Enquanto um átomo do oxigênio molecular é introduzido no substrato, o outro é reduzido até água gerando espécies reativas do oxigênio, tais como o ânion superóxido ($O_2^{\cdot -}$) e o peróxido de hidrogênio (H_2O_2); (4) transferência do segundo elétron para o complexo enzima-substrato através do citocromo b5; (5) protonação do complexo enzima-substrato com liberação de peróxido de hidrogênio; (6) clivagem da ligação O-O gerando um intermediário reativo; (7) inserção de um átomo de oxigênio no substrato (monooxigenação); (8) dissociação do produto e liberação do citocromo P450; (9) reação de hidroxilação sem a necessidade de equivalentes redutores (COON et al., 1992, GUENGERICH et al., 1995, GUENGERICH et al., 1998, KOBLYAKOV, 1998, OMURA, 1999)

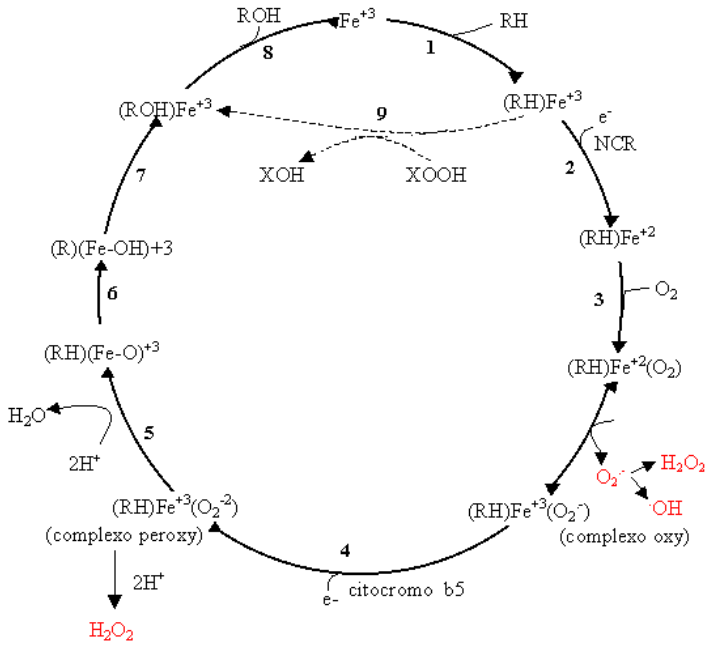

Figura 3 - Ciclo catalítico do citocromo P450. Em destaque as espécies reativas geradas pelas reduções do ciclo (adaptado de BENHARDT, 1995). NCR – NADPH citocromo P450 redutase; Fe+3 – o citocromo P450; RH – Substrato.

Não existe uma regra para a distribuição tecidual dos CYPs. Visto que suas funções são muito variadas, estas isoenzimas podem ser encontradas em todos os tipos de tecidos em todos os tipos de organismos. Embora tenham sido descritos primariamente em mamíferos, onde são relativamente abundantes, já foram descritas isoformas de CYPs em diferentes organismos.

As pesquisas iniciais sobre P450 eram relacionadas ao metabolismo de carcinógenos, drogas e esteróides, no entanto com o passar dos anos, a atividade deste grupo de enzimas também passou a ser relacionada à biotransformação (detoxificação/ ativação) de pesticidas, metabolismo de vitaminas hidrosolúveis e compostos endógenos como os eicosanóides e prostaglandinas (OMURA, 1999, GUENGERICH, 2000).

Os citocromos P450 estão distribuídos por toda natureza, incluindo formas específicas de microorganismos, plantas, vertebrados e invertebrados. Acredita-se que a origem desta superfamília seja nos procariotos antes ainda do

acúmulo do oxigênio molecular na atmosfera (WERCK-REICHHART e FEYEREISEN, 2000). No caso de microorganismos, como as bactérias e os fungos, o papel dos CYPs no organismo está relacionado à biotransformação de compostos endógenos através de reações de β-hidroxilação (URLACHER et al., 2004), já em plantas, a função do CYP está relacionada ao metabolismo de pigmentos coloridos e defesas contra parasitas; acredita-se ainda que neste grupo de organismo a origem dos CYPs seja polifilética (GUENGERICH, 2000, WERCK-REICHHART e FEYEREISEN, 2000). Em animais (vertebrados e invertebrados) o papel do CYP está relacionado ao catabolismo de produtos naturais, síntese de eicosanoides e esteróides, além da biotransformação (detoxificação/ativação) de xenobióticos (BERNHARDT, 1995, OMURA, 1999, GUENGERICH, 2000). Dentre as diversas classes animais, os mamíferos são os organismos que utilizam o menor *set* de proteínas e funções.

Embora a maioria das isoformas esteja presente no fígado (1A, 1B e 3A), podem existir outras isoformas em tecidos extras hepáticos. As isoformas podem existir ancoradas no retículo endoplasmático – relacionadas ao metabolismo de xenobióticos e síntese de eicosanóides e prostaglandinas – ou na membrana interna da mitocôndria – relacionada a sínteses endógenas (BERNHARDT, 1995, OMURA, 1999, GUENGERICH, 2000, RAUCY e ALLEN, 2001). As isoformas microssomais (aquelas ancoradas no retículo endoplasmático), são facilmente reconhecidas por uma região rica em prolinas que formam uma estrutura semelhante á um “cotovelo” que antecede um grupo de aminoácidos básicos entre o segmento hidrofóbico - responsável pelo ancoramento da membrana - e a porção globular da proteína (WERCK-REICHHART e FEYEREISEN, 2000).

Acredita-se que em eucariotos esta superfamília tenha se diversificado de uma única proteína ancestral; as últimas famílias a terem aparecido teriam sido as envolvidas no metabolismo de xenobióticos (CYPs1 -4), a cerca de 600 milhões de anos atrás; coincidentemente quando os animais evoluíram do ambiente aquático para o terrestre facilitando a evolução destas famílias graças às interações animais – plantas (RAUCY e ALLEN, 2001).

Embora todos os CYPs tenham o interior hidrofóbico da sua estrutura semelhantes, cada isoforma apresenta substratos específicos e mecanismos únicos de reconhecimento (RAUCY e ALLEN, 2001). Todas apresentam várias hélices conservadas nomeadas de A - L, sendo que as hélices I e L são as regiões de ligação ao grupamento heme e resíduos de aminoácidos presentes nas hélices B e L são responsáveis pelo reconhecimento do substrato (GUENGERICH, 2000, RAUCY e ALLEN, 2001). Existem também duas folhas β conservadas responsáveis pelo ancoramento da proteína na membrana do retículo endoplasmático e da mitocôndria (RAUCY e ALLEN, 2001).

1.3 – BIOLOGIA MOLECULAR DO CYP1A

Ao longo dos anos houve um aumento do número de pesquisas sobre o citocromo P450, culminando em um vasto banco de dados e informações sobre estrutura, famílias gênicas, atividade catalítica e especificidade por substratos de aproximadamente 17 isoformas do citocromo P450 em diferentes espécies (COON et al., 1992, BUHLER e WANG-BUHLER, 1998, GUENGERICH et al., 1998, KOBLYAKOV, 1998, OMURA, 1999, WAXMAN, 1999, RAUCY e ALLEN, 2001). Quatro destas famílias (CYPs 1-4) expressam enzimas hepáticas que metabolizam compostos xenobióticos (drogas, poluentes químicos) e substratos endógenos lipofílicos como os esteróides, prostaglandinas, ácido araquidônico etc (MANSUY, 1998).

Os avanços na área de biologia molecular permitiram o enriquecimento no conhecimento da estrutura e da seqüência de aminoácidos de diferentes P450, exigindo a criação de uma nomenclatura particular para especificar os diferentes tipos de P450s baseada na homologia estrutural. P450s de diferentes fontes, P450 com mais de 40% de homologia entre as seqüências são incluídos na mesma família, que é identificada por um algarismo arábico. Quando a homologia é superior a 55% as proteínas são incluídas na mesma subfamília, identificada por uma letra capital. Por exemplo: *CYP1A*, identifica os genes dos citocromos P450 da família 1 e subfamília A, a identificação da enzima é a mesma, mas sem o itálico (COON et al., 1992).

Algumas isoformas de CYP são induzidas por substrato, propriedade que permite a célula se adaptar as mudanças no ambiente químico. Desta forma, a indução seria um mecanismo protetor contra compostos químicos lipofílicos que tenderiam a acumular-se em níveis danosos ao organismo. No entanto, a indução pode ser desvantajosa em alguns casos. Devido ao fato dos diferentes CYPs apresentarem diferentes especificidades por diferentes substratos, a indução de uma isoforma pode levar ao aumento do metabolismo de um determinado metabólito que teria efeitos danosos ao organismo. Assim, ao invés de inibir a indução de carcinógenos químicos os CYPs estariam aumentando a concentração de compostos potencialmente carcinogênicos.

Esta rápida resposta indutiva do citocromo P450 torna - o uma das melhores ferramentas para o estudo de metabolismo de drogas dependentes do P450, farmacocinética, interações droga-droga e droga-P450, toxicidade e carcinogenicidade de xenobióticos e na ativação e distribuição de hormônios. A indução do P450 em resposta a exposição à xenobióticos é freqüentemente associada ao aumento da formação de metabólitos reativos que podem desencadear resposta patofisiológicas, como o dano celular e tissular e o desenvolvimento de tumores (KOBLYAKOV, 1998, MANSUY, 1998, RAUCY e ALLEN, 2001).

A maioria dos genes que codificam as famílias CYPs 1-4 podem ser ativados por xenobióticos que induzem a expressão gênica do P450 através de um mecanismo dependente de receptor. Hoje já se sabe que há proteínas receptoras específicas para cada xenobiótico que irá induzir apenas uma ou duas isoformas diferentes (BENHARDT, 1995, KOBLYAKOV, 1998, GRAHAM e PETERSON, 1999, DELESCLUSE et al., 2000).

Dentre estas isoformas, a mais estudada tem sido a CYP1A. Esta isoforma é expressa em nível muito baixo em vários tecidos como fígado, pele, rins e pulmões. No entanto, na presença de indutores, a expressão desta proteína fica extremamente aumentada. A indução da enzima CYP1A reflete os aumentos nas taxas de transcrição do gene *CYP1A*. A indução é rápida e ocorre mesmo na

ausência de síntese de proteína, visto que o fator protéico necessário para a indução já existe previamente na célula (WHITLOCK, 1999).

Um destes receptores, o Ah ("Aryl Hidrocarbon") é uma proteína pertencente à família PAS dos fatores de transcrição e estimula a transcrição dos genes CYP1A (Figura 4). A indução ocorre quando o indutor (xenobiótico) atravessa a membrana plasmática graças a sua lipofilicidade e se liga ao AhR. Este receptor fica situado no citossol em sua forma heterotetramérica. Esta forma é composta pelo receptor AhR propriamente dito, duas Hsp90 (que funcionam como chaperonas, evitando a ativação transcricional do receptor, mas mantendo-o na sua conformação ótima para a ligação do xenobiótico) e uma proteína não identificada (P) (DELESCLUSE et al., 2000). Após a ligação do indutor, as Hsp90 são liberadas junto com a proteína P e o receptor ativado é translocado para o núcleo, formando um heterodímero com o fator de transcrição Arnt que se liga à seqüência 5'- GCGTC-3'do DNA; esta seqüência é designada como XRE (elemento responsivo de xenobiótico) (NEBERT, 1989, KOBLYAKOV, 1998, WAXMAN, 1999, WHITLLOCK, 1999, DELESCLUSE et al., 2000). A ligação do AhR/Arnt no DNA altera a estrutura da cromatina ativando o processo de transcrição gênica através da perda da configuração nucleossomal, facilitando o acesso dos fatores de transcrição ao promotor (WHITLLOCK, 1999). Este processo parece ser conservado tanto entre diferentes tipos celulares quanto entre diferentes espécies e parece ser estimulado por diferentes classes de policíclicos aromáticos (WAXMAN, 1999).

A indução da resposta do CYP1A a um dado xenobiótico, em especial aos policíclicos aromáticos em sistemas *in vivo*, é mediada através do receptor Ah, contudo, algumas situações como hiperóxia e diferenciação, ativam a translocação do receptor mesmo na ausência de ligante (DELESCLUSE et al., 2000). O tempo de indução varia entre os organismos e o agente indutor, o que torna pertinente um estudo prévio do organismo que se pretende utilizar como bioindicador (COON et al., 1992, OMURA, 1999, DELESCLUSE et al., 2000). As subfamílias 1A e 2B parecem ser as principais responsáveis pelo metabolismo de xenobióticos. Estas isoformas estão localizadas nos tecidos que ficam em contato direto com as

substâncias vindas do exterior, como o trato gastro-intestinal, o fígado, a pele, os rins, etc (VROLIJK et al., 1994, IYER e SINZ, 1999).

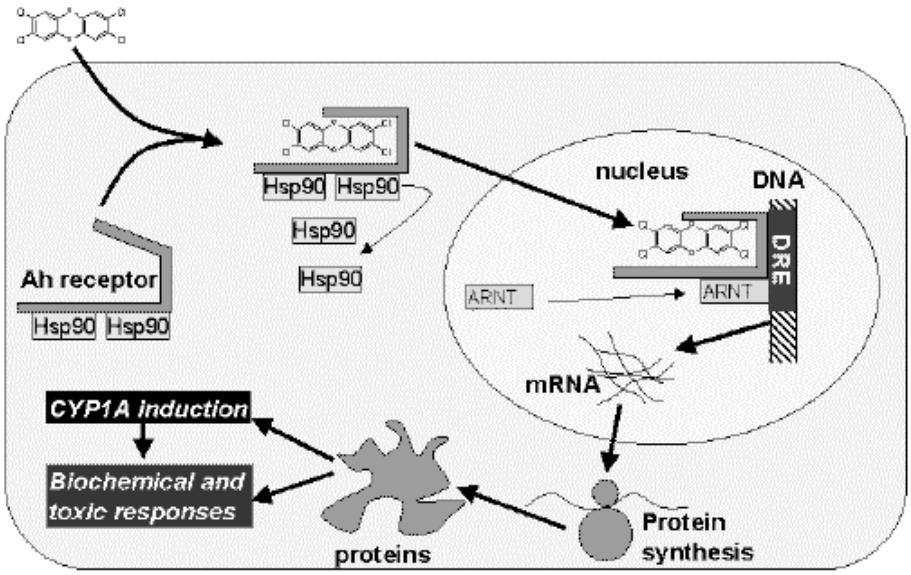

Figura 4 – Mecanismo de indução, transcrição e tradução do CYP1A (adaptado de Delescluse et al., 2000).

A CYP1A é uma enzima que pode apresentar polimorfismo genético, sendo que um dos alelos leva a diminuição da atividade. Esta é uma característica muito importante visto que é uma enzima que pode ser expressa mesmo na ausência de indutores em mamíferos (ANZENBACHER e ANZENBACHEROVÁ, 2001). Esta mesma característica já foi descrita pra peixes e parece estar relacionada às características ambientais (SEAN e ARINÇ, 1998).

1.4 – CYP1A EM PEIXES

Peixes representam aproximadamente metade das espécies de vertebrados existentes, divididos em 200 famílias, 32 ordens e 3 classes dentro do subfilo Vertebrata. Esta grande diversidade permite que eles ocupem um número muito grande de nichos no ambiente aquático, além de representarem uma significativa fonte de proteína para os humanos (WOOTTON, 1992, RABERGH et

al., 2000). Há aproximadamente 25.000 espécies de teleósteos descritos até hoje e que compõem aproximadamente 70% do ambiente. Além disto, peixes representam uma importante fonte de proteínas para os seres humanos. No entanto, menos de 5% das espécies descritas são bem estudadas e/ou utilizadas como modelos biológicos (KULLMAN et al., 2000).

Peixes foram escolhidos como modelo biológico por estarem expostos a níveis subletais de contaminantes, temperatura e concentração de oxigênio que, quando são desfavoráveis e/ou flutuantes, causam um estresse fisiológico e comportamental no organismo impedindo a realização de algumas funções importantes, como o fenômeno da piracema. A maioria dos estudos com peixes enfatiza o uso destes animais em estudos sobre impacto ambiental, uma vez que a maioria dos poluentes está sempre em contato com o ambiente aquático, associado ao sedimento, à coluna de água, à biota etc. (BUHLER e WANG-BUHLER, 1998, GOKSϕYR e HUSϕY, 1998, COUSINOU, 2000, KULLMAN et al, 2000, VEIGA et al.2002).

Os citocromos P450 desempenham um papel essencial na biotransformação de compostos exógenos e endógenos. Muitos poluentes são capazes de induzir a síntese de P450 em peixes, onde o substrato lipofílico gerado é convertido pelas enzimas de conjugação em um produto mais polar e solúvel em água e facilmente excretado através da urina e das brânquias (STEGEMAN e LECH, 1991, VROLIJK et al, 1994, KRISHNAKUMAR et al., 1995, GOKφYR e HUSφY, 1998). Em estudos paralelos desenvolvidos neste laboratório, observamos que peixes coletados em locais poluídos apresentavam níveis de P450 e b5 até 7 vezes maiores quando comparado com peixes coletados em criadouros artificiais (LEITÃO et al., 2000).

Diversos estudos demonstraram que as alterações dos níveis de indução do P450 em peixes refletem muitas vezes os níveis de contaminantes no ambiente. No entanto, durante o processo de biotransformação, o xenobiótico pode ser ativado, aumentando seu potencial carcinogênico (JAMES, 1989, LIVINGSTONE et al., 1989, STEGEMAN, 1989, STEGEMAN e LECH, 1991, BEYER e GOKφYR, 1993, WASHBURN et al., 1994, VROLIJK et al., 1994,

BUHLER e WANG-BUHLER, 1998, GOKφYR e HUSφY, 1998, WATSON et al., 1998, WILLIAMS et al., 1998, OMURA, 1999, PESONEM et al., 2000).

Embora a estrutura dos componentes e o modo de indução do sistema P450 em peixes sejam bastante semelhantes a mamíferos, a regulação nos dois organismos é bem diferente (KLOTZ et al., 1983, STEGEMAN e LECH, 1991). Enquanto em mamíferos a regulação é influenciada primordialmente por fatores endógenos (NEBERT e GONZALEZ, 1987, HALPERT, 1995), em peixes os níveis de P450 são mais baixos e influenciados tanto por fatores endógenos como por fatores exógenos. Temperatura, fotoperíodo, mudanças sazonais, dieta, estágio de desenvolvimento e maturação sexual influenciam na magnitude da resposta do P450 e parecem ser essenciais para as defesas contra a toxicidade de compostos químicos (BUHLER e RASMUSSON, 1968, RIVIERE e CABANNE, 1987, STEGEMAN, 1989, BUHLER e WANG-BUHLER, 1998, GOKφYR e HUSφY, 1998, QUABIUS et al., 1998).

Atualmente há 12 subfamílias do citocromos P450 descritos para peixes, incluindo as CYP1A, 3A, 4A, 1B, 2B, E, K, M, N, P, 11, 17 e 19. A atividade catalítica e a seqüência de nucleotídeos indicam que muitos dos citocromos P450 encontrados em diferentes espécies de peixes são similares aos de mamíferos (NERBERT e GONZALEZ, 1987, BENHARDT, 1995, MORRISON et al., 1995, MORRISON et al., 1998, KULLMAN et al., 2000). A principal isoforma do citocromo P450 presente em peixes é a CYP1A, provavelmente homóloga á CYP1A1 de mamíferos. De fato, diversos autores sugerem que a família CYP1A de peixes é composta por uma proteína híbrida, cujos genes seriam ancestrais da isoformas CYP1A1 e CYP1A2 de mamíferos (BUHLER e WANG-BUHLER, 1998, MORRISON et al., 1995, GOKSϕYR e HUSϕY, 1998, MORRISON et al, 1998, RABERGH et al., 2000).

A estrutura geral das diferentes isoformas do citocromo P450 parece ser bem conservada, no entanto são encontradas variações em suas seqüências primárias, secundárias e terciárias responsáveis pela especificidade das diferentes isoformas pelos substratos e pares redox, além de influenciarem na ancoragem do P450 na membrana.

Estudos anteriores em nosso laboratório (Processo FAPESP 98/13798-9 e 99/11329-4) demonstraram que Curimbatás são bons modelos para o estudo da interação de P450 com diferentes xenobióticos para testes *in vitro* do metabolismo de diferentes xenobióticos, análise da interação dos xenobióticos com a membrana e efeitos oxidativos. Além disto, Curimbatás - assim como outros peixes brasileiros - apresentam um componente extra no sistema microssomal hepático que parece influenciar no metabolismo de xenobióticos tanto *in vitro* como *in vivo* (DEGTEREV at al., 1999, LEITÃO et al., 2000).

A determinação da atividade metabólica de diferentes isoformas do citocromo P450 pode ser determinada por diferentes técnicas, incluindo a imunoinibição, inibição química, substratos para isoformas específicas e estudos de atividade usando enzimas purificadas. Recentemente, dois novos métodos têm sido usados na determinação do papel do citocromo P450 no metabolismo de xenobióticos, a expressão de proteínas recombinantes e os sistemas reconstituídos, ambos visando enfocar a relação estrutura-função do citocromo P450 e os efeitos de diversos xenobióticos estruturalmente diferentes (BUHLER e WANG-BUHLER, 1998, GRAHAM e PETERSON, 1999, GUENGERICH et al., 2000, KULLMAN et al., 2000, dentre outros).

O citocromo P450 é um bom modelo para estes estudos devido a sua capacidade de metabolizar uma grande variedade de substratos. A escolha da isoforma CYP1A se dá principalmente por dois motivos, (i) esta é a isoforma presente em maior abundância no fígado de peixes, sendo a principal responsável pela metabolização de compostos xenobióticos, tais como os poluentes; (ii) é induzida por compostos altamente lipofílico e de estrutura química planar, tais como os Policíclicos Aromáticos, Policíclicos Bifenóis, Dioxinas, Organoclorados e Fosforados.

1.5 – USO DA ESPECTROMETRIA DE MASSAS NA IDENTIFICAÇÃO DE PROTEÍNAS.

O monitoramento das alterações nos níveis de proteínas é crucial para se obter um panorama completo dos diferentes processos biológicos do

organismo. Contudo, estas alterações não são conseqüências apenas de processos transcricionais, mas também traducionais e pós-traducionais, visto que muitos estudo demonstraram discrepância entre os níveis de mRNA e proteínas em uma mesma célula; demonstrando que o importante não é a quantidade absoluta de proteína, mas a comparação dos níveis de uma mesma proteína em duas situações distintas (STEEN e PANDEY, 2002). Nos últimos anos, a espectrometria de massas tem se tornando uma das ferramentas mais úteis para a comparação e identificação de uma mesma proteína em duas situações distintas.

A espectrometria de massas pode ser definida como a técnica capaz de determinar a massa molecular de um determinado composto químico através da separação de íons moleculares de acordo com a razão massa – carga (*m/z*). A geração destes íons ocorre através da perda e/ou ganho de carga.

O primeiro espectrômetro de massas (MS) foi desenvolvido em 1912 por J.J.Thompson (*appud* SIUZDAK, 1996). Até a década de 80, o uso da espectrometria de massas era restrito a um pequeno número de moléculas capazes de sobreviver ao fenômeno de ionização. Moléculas maiores, como as proteínas não podiam ser analisadas através de MS sem a presença de grupos protetores para um aumento da volatidade e da estabilidade. Apenas da década de 90 é que se começou a utilizar a espectrometria de massas nas análises de (poli) peptídeos, metabólitos de drogas, oligonucleotídeos e polissacarídeos, graças ao desenvolvimento de técnicas de ionização mais suaves (SIUZDAK, 1996). Esta nova forma de análise só foi possível graças ao desenvolvimento de duas novas metodologias de MS: a ***Matrix-assisted laser desorption/ionization (MALDI)*** e a ***Electrospray ionization (ESI)***. Ambas as técnicas associadas ao uso de software permitiram o estudo de macromoléculas de importância biologia de forma rápida, sensível e com alta resolução. A partir de 1993, graças a estes programas computacionais, passou a ser possível correlacionar os dados obtidos em um espectro de massas com as seqüências existentes nos bancos de dados da rede mundial de computador (MANN e WILM, 1995, MANN et al., 2001). Era o início da era proteômica

A espectrometria de massas tem desempenhado um importante papel nas ciências biológicas nos últimos dez anos por medir uma propriedade intrínseca da molécula: sua massa. Até 1990, os estudos proteômicos eram restritos as análises de eletroforese em géis de poliacrilamida em uma e/ou duas dimensões. No entanto, a identificação de cada spot/banda era restrita as proteínas mais abundantes. Hoje, a sensibilidade conquistada pelos espectrômetros de massas permite que mesmo aquelas proteínas presentes em baixíssimas concentrações possam ser analisadas e identificadas através do uso da MS associada aos bancos de dados, aumentando a rapidez e a robustez das análises. A Figura 5 mostra como ocorre à associação entre a MS e os bancos de dados.

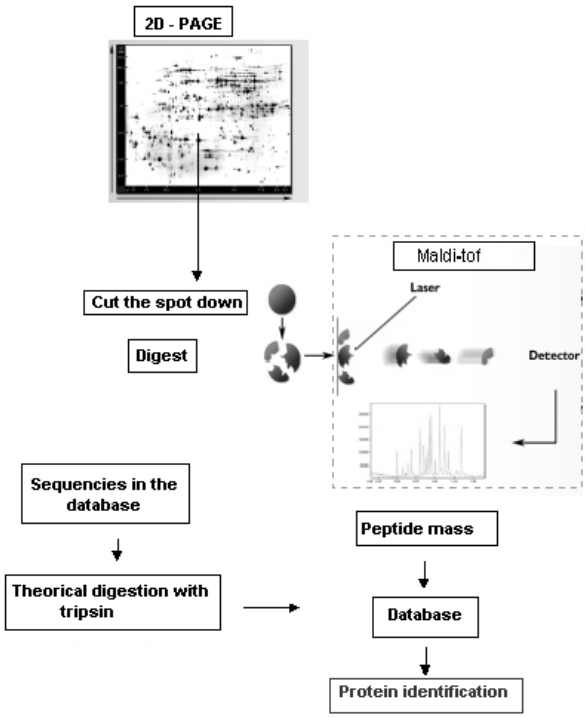

Figura 5 – Interação entre a espectrometria de massas (MS) e os bancos de dados na identificação de proteínas.

Desde então a espectrometria de massas passou a desempenhar várias funções em pesquisa biológicas. Dentre os usos mais freqüentes da

espectrometria de massas podemos citar (i) o uso da MS para o monitoramento de proteínas recombinantes (EASTERLING et al., 1998, VILLANUEVA et al., 2001, entre outros); (ii) identificação de interações entre macromoléculas (DNA e proteínas) entre si e/ou com drogas (BAKHTIAR e NELSON, 2000; ZHANG et al., 2001, entre outros); (iii) identificação de proteínas envolvidas em doenças de alta incidência, como o câncer, doenças cardíacas e neuronais (ALFONSO et al., 2004, FRIEDMAN et al., 2004, HAYMAN e PRZYBORSKI, 2004); identificação de proteínas envolvidas no metabolismo de drogas (GALEVA e ALTERMANN, 2002, GALEVA et al., 2003); (iv) projetos de proteômica em larga escala (SMOLKA et al., 2003) (v) na caracterização de modificações pós – traducionais como as glicosilações e fosforilações (KÜSTER e MANN, 1998, SICKMANN et al., 2002) e (vi) sequenciamento de proteínas/peptídeos (CHAURAND e LUETZENKIRCHENM, 1999, BAKHTIAR e NELSON, 2000).

Este crescimento de utilidades do espectrômetro de massas vem aumentando nos últimos anos e têm levado ao desenvolvimento de novos instrumentos e estratégias de aplicação da tecnologia, como a quantificação de proteína, identificação de grupos funcionais, identificação do NH_2 – terminal e do C – terminal, identificação de ácidos nucléicos entre outras.

1.5.1 – MATRIX – ASSISTED LASER IONIZATION TIME OF FLIGHT (MALDI – TOF)

Introduzido em 1988 por KARAS e HILLENKAMP (*appud* SIUZDAK, 1996), o MALDI é uma técnica de ionização suave baseada na utilização de matrizes orgânicas sólidas que absorvem o laser, permitindo a vaporização e ionização de moléculas biológicas não voláteis (SIUZDAK, 1996, CHAURAND e LUETZENKIRCHENN, 1999, MANN et al., 2001).

Este processo produz moléculas ionizadas de maneira descontinua daí a associação com analisadores descontínuos como o TOF. Nestes aparelhos, o processo de formação, separação, e detecção de íons ocorrem em condições de vácuo. Todos os íons são acelerados com a mesma energia cinética através de

um campo elétrico. Estes íons têm seu "tempo de chegada" e sua intensidade gravados por um detector e separados segundo uma ordem crescente de massa/carga m/z (SIUZDAK, 1996, BAKHTIAR e NELSON, 2000). A Figura 6 mostra esquematicamente o processo acima descrito.

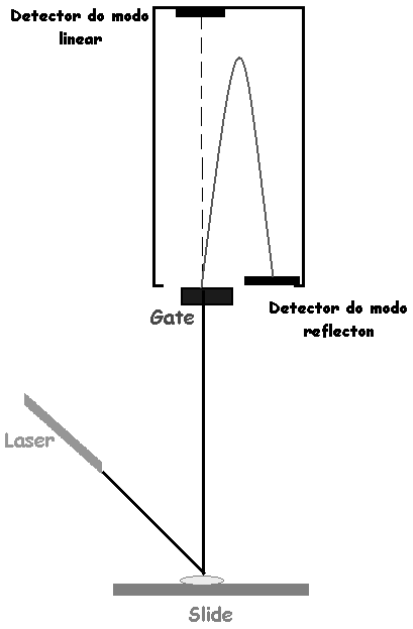

Figura 6 – representação esquemática de um espectrômetro de massas do tipo MALDI-TOF.

As matrizes empregadas são, na sua maioria, ácidos orgânicos sólidos, cujo comprimento de absorção de onda é próximo ao do laser utilizado (MANN et al., 2001).

O papel da matriz é evitar que a amostra a ser analisada seja degradada pela força do laser. Esta prevenção é possível devido ao fato da matriz ser cristalizada junto com a macromolécula a ser analisada. A matriz absorve a energia do laser vaporizando a amostra, minimizando o dano, contribuindo para o processo de ionização.

O conjunto de características do MALDI-TOF permite a detecção de amostras na faixa do fentomole (10^{-18} mole). No entanto, a técnica só é útil se a

macromolécula alvo (proteína/peptídeo) tem sua seqüência disponível no banco de dados (KÜSTER e MANN, 1998).

A identificação de proteínas através do MALDI-TOF é sempre associada ao uso de eletroforese em gel de poliacrilamida de uma e/ou duas dimensões. Após o gel ser corado (preferencialmente com comassie blue), as bandas/spot de interesse são recortados e enzimaticamente degradados com tripsina. Os peptídeos gerados vão compor o "mapa de massas de peptídeos" (do inglês *peptide mass fingerprinting* – PMF). O conjunto de massas obtido é comparado ao da "digestão teórica" da proteína cuja seqüência já esteja disponível no banco de dados (Figura 5).

Embora seja mais segura a análise de massas de uma proteína cuja seqüência já esteja disponível em bancos de dados, o PMF permite a identificação de proteínas homólogas entre espécies próximas desde que haja aproximadamente 80% de identidade na seqüência (LUSKA e SHEVCHENKO, 2003). A identificação através do MALDI-TOF permite a caracterização detalhada da composição de aminoácidos, estrutura e homologia de uma proteína desconhecida.

1.5.2 – ELECTROSPRAY IONIZATION MASS SPECTROMETRY (ESI-MS)

Utilizada pela primeira vez por FENN e colaboradores em 1989 para amostras biológicas (*appud* SIUZDAK, 1996), a ESI-MS tem como base o uso de um campo elétrico que força a vaporização da amostra através de uma agulha eletricamente carregada (aproximadamente 4-5kV) (BAKHTIAR e NELSON, 2000, MANN et al., 2001). O único requerimento da técnica é a necessidade de que a molécula seja suficientemente polar para receber as cargas (MANN e WILM, 1995, SIUZDAK, 1996).

Não existe um limite máximo de massas para a análise do ESI-MS. Em moléculas grandes, bem como em íons maiores, o espectro obtido apresenta moléculas multicarregadas, permitindo a análise de amostras maiores mesmo que

a capacidade do aparelho seja para baixas razões m/z (BAKHTIAR e NELSON, 2000, MANN et al., 2001). No entanto esta facilidade do aparelho faz com que os espectros gerados sejam mais difíceis de serem analisados.

Variações do método já foram feitas no sentido de aumentar a sensibilidade da técnica. Algumas destas adaptações associam a cromatografia líquida com a espectrometria de massas, permitindo o uso de poucos microlitros de amostra que, sendo devidamente preparada, é analisada em aproximadamente uma hora resultando no sequenciamento da amostra com sinais de alta intensidade e resolução (MANN et al., 2001).

1.5.3 – USO DA ESPECTROMETRIA DE MASSAS NO ESTUDO DO CITOCROMO P450.

Até o momento não existe nenhuma informação sobre a estrutura do CYP em organismos superiores. Isto ocorre devido ao fato de CYPs serem proteínas de membrana cuja estrutura é altamente dependente do ambiente lipídico, dificultando a obtenção de cristais para a determinação da estrutura por raio – X. Sendo assim, os estudos sobre estrutura e interação de droga como sítio ativo da proteína são obtidos através de métodos indiretos.

Neste sentido, a espectrometria de massas se fez muito útil, pois permite não só o estudo de diversas formas de interação entre o CYP e vários xenobióticos, mas também ajuda a elucidar algumas informações sobre a estrutura da proteína.

Graças ao desenvolvimento de aparelhos do tipo MALDI – TOF, ROBERTS e colaboradores (1998) conseguiram mostrar que compostos como o 5-fenil –1 pentino são capazes de inativar os CYP2E1 e CYP2B1 através de um ataque eletrofílico ao grupamento hidroxila do resíduo Thr302, conservado em todas as isoformas.

A espectrometria de massas associada à cromatografia líquida de alta precisão (LC/MS) permitiu que fossem identificadas as isoformas de CYP responsáveis pelo metabolismo do Iloperidone. Os resultados rápidos e precisos

obtidos com este estudo permitiram grandes avanços na descoberta de fatores que influenciam na farmacodinâmica de drogas em grupos específicos de pacientes (MUTLIB e KLEIN, 1998). Outro ensaio utilizando a LC/MS, desta vez realizado por KOENIGS e TRAGER (1998), demonstrou que os metabólitos do 8 – metoxiporalen (8 – MOP) e outros furanocoumarins de estrutura semelhante ligam-se covalentemente ao CYP2B1 e ao CYP2A6 levando a um aumento de massa de aproximadamente 240 Da de diferença conforme o metabólito gerado.

Em 2001, ZHANG e colaboradores usaram a espectrometria de massas (ESI - MS) para uma análise quantitativa de isoformas específicas do CYP utilizando a wafarina como substrato – sonda. Os resultados obtidos neste trabalho contribuíram para esclarecer o mecanismo de inibição de várias isoformas do CYP, incluindo a CYP1A1/2, CYP2C/19 e CYP3A4. Estas são as isoformas responsáveis pelo metabolismo de drogas em humanos.

Em 2002, GALEVA e ALTERMANN usaram o PMF e o MALDI-TOF na identificação de proteínas da fração microssomal hepática de ratos. O objetivo do estudo era comparar a eficiência das eletroforeses uni – e bi – dimensional em gel de poliacrilamida. Dentre as proteínas identificadas estavam ao menos três isoformas de CYP em cada tipo de gel. Em 2003, o mesmo grupo (GALEVA et al., 2003) usou a técnica para identificar o CYP2B em amostras purificadas e de microssomas brutos, obtendo informações sobre outras subfamílias da família CYP2B em uma amostra não purificada.

Podemos, portanto, perceber que a espectrometria de massas é usada prioritariamente para estudos sobre mecanismos de inibição de várias famílias de CYPs, deixando a o uso da técnica para identificação de proteínas – uma abordagem mais proteômica – de lado.

A maioria dos avanços na toxicologia durante as últimas duas décadas está focado na identificação de caracterização de proteínas específicas expressas em resposta a metais tóxicos e xenobióticos (MOORE, 2002). A proteômica seria a ligação entre os genes, as proteínas e a função celular, permitindo a diferenciação entre um estado saudável e um patológico como, por exemplo, as patologias induzidas por poluentes ambientais.

Segundo MOORE (2002), hoje podemos dizer que a ecotoxicologia possui quatro objetivos:

a) Entender os mecanismos moleculares e subcelulares das interações de poluentes sob aspectos proteômicos e genômicos;
b) Desenvolver modelos capazes de prever os efeitos tóxicos de poluentes em processos fisiológicos e celulares;
c) Fazer a ligação entre os efeitos iniciais (níveis moleculares) e as conseqüências finais (ecossistema) da exposição à xenobióticos;
d) Prevenir os efeitos danosos de poluentes no ambiente através do desenvolvimento de novos processos industriais.

Peixes, assim como outros organismos superiores, possuem aproximadamente 50.000 – 60.000 genes, que são expressos seletivamente em células individuais gerando proteínas com funções específicas em cada estado, ou seja, embora o número de genes seja fixo para toda vida, a foram como eles são expressos em peixes variam conforme a situação ambiental.

A proteômica tem a função de identificar apenas aquelas proteínas expressas em uma determinada situação, auxiliando na explicação de como os genes são regulados e como esta regulação é alterada por adaptações ou distúrbio ambientais induzidos por poluentes, viabilizando seu uso como técnica auxiliar na identificação de ambientes poluídos (CUNNINGHAM, 2000, MOORE, 2002).

Hoje já foram identificadas cerca de 1925 isoformas de CYP (dnelson. utmem.Edu/CytochromeP450.html) utilizando métodos tradicionais como identificação das isoformas por anticorpos mono e policlonais, sondas específicas para RNAm e inibidores/substratos específicos. Estes métodos são eficientes para a identificação de isoformas conhecidas, mas tornam –se impreciso quando se trata de uma nova isoforma. Neste sentido, o uso do MALDI-TOF associado ao PMF se torna extremamente útil por permitir a análise de diferentes proteínas em uma mesma amostra. Se associado aos dois estiver à eletroforese bi-dimensional, a identificação se torna mais precisa em virtude da sua precisão na separação de

isoformas com a mesma massas molecular, mas com diferentes pIs ou alteraçãos pós – traducionais.

Sendo assim, a abordagem proteômica pode e deve ser aplicada na identificação, definição da composição e da estrutura de diferentes isoformas do CYP em diferentes espécies.

1.6 – ECOLOGIA DO *Prochilodus scrofa* (PISCES, CHARICIFORME, PROCHILODONTIDAE).

Pertencente a família Prochilodontidae, os Curimbatás (Fig.7) têm distribuição restrita à América do Sul, em especial nas bacias hidrográficas brasileiras das regiões Sudeste e Centro-Oeste.

Curimbatás são peixes iliófagos, isto é, ingerem substratos formados por lodo e areia que por si só não é alimento, mas sim o local de vida das diastomáceas, uma talófita que compõe sua maior fonte de nutrientes. Em decorrência do seu hábito alimentar, estes peixes apresentam estômago musculoso capaz de digerir a carapaça de sílica das diastomáceas (GODOY, 1975, FERNANDES et al., 1995).

Figura 7 - *Prochilodus scrofa* (Steinndachner, 1881).

Estes peixes são encontrados em ambientes lodosos com baixas pressões de oxigênio e, portanto, muito resistentes a situações de hipóxia, apresentando um "regulador de oxigênio", cuja função é aumentar o volume respiratório. É um peixe reolífico, ou seja, que sobe os rios para se reproduzir quando atinge a maturidade sexual, o que lhe exige numerosas adaptações fisiológicas. Sua resistência também é notada em situações de baixas temperaturas, tolerando viver entre 5 e 10º C quando no seu habitat a temperatura normal é 25ºC (GODOY, 1975, FERNANDES et al., 1995).

Outra peculiaridade do gênero *Prochilodus* é a presença de um número diplóide de cromossomos 2n= 54, enquanto que o normal é 2n=40, sendo que os cromossomos extras são todos metacêntricos e submetacêntricos. Esta característica ocorre principalmente nos exemplares da Bacia do Paraná que, segundo GODOY (1975), é uma população isolada de *Prochilodus scrofa*, explicando ainda que o número extra de cromossomos associada às alterações histológicas, seriam características populacional no gênero *Prochilodus* (PAULS e BERTOLLO, 1990, HATANAKA e GALETTI, 2003).

Curimbatás já vendo sendo utilizados como bioindicadores de poluição ambiental devido a sua alta sensibilidade a xenobióticos. Estudos demonstraram que os rins têm sua morfologia afetada pela exposição ao Trichlorfon. A conseqüência direta desta alteração seriam alterações nas taxas de filtração glomerular e nas defesas imunológicas do animal; alterando toda homestasia do animal (VEIGA et al.2002). Brânquias, fígado e rins são os principais órgãos a acumulares metais pesados, mesmo quando o período de exposição é curto, visto que fatores como o pH e a temperatura também afetam a taxa de captação de metais pesados como o cobre (MAZON et al., 2002, CARVALHO et al., 2004).

Estudos anteriores já demonstraram que o sistema P450 é ativo em Curimbatás. Estudos realizados neste laboratório demonstraram que o CYP de Curimbatás apresenta peculiaridades na sua caracterização espectral, como um pico em 420nm em conjunto com o pico clássico em 450nm. Este novo pico não estaria representando a forma inativa da proteína e sim uma nova hemeproteína

que auxiliaria o CYP em processo de detoxificação (DEGTEREV et al., 1999, LEITÃO et al., 2000).

Ensaios *in vitro* demonstraram que o sistema pode ser inibido por substâncias com características surfactantes tanto através de inibição seletiva, onde existiria a interação direta do xenobiótico com o sítio ativo da hemeproteína, bem como pela alteração do ambiente lipídico da membrana (DA SILVA e MEIRELLES, 2004).

O conjunto de características acima descritas mostra que a utilização do Curimbatá como o organismo modelo para estudos de ecotoxicológico é válido e promete resultados promissores.

2 – OBJETIVOS

Este trabalho teve como objetivo geral o estudo do citocromo P4501A (CYP1A1 e CYP1A3) em *Prochilodus scrofa* com a finalidade explorar seu uso como um biomarcador de poluição ambiental.

Como objetivos específicos para este projeto temos:

- Avaliar a interação de compostos xenobióticos com microssomas hepáticos através de estudo *in vitro*.
- Desenvolvimento de uma técnica de purificação da CYP1A que exclua a necessidade de compostos com características surfactantes
- Identificação do CYP1A (1A1 e 1A3) através de mapa de digestão tríptica associada à espectrometria de massas com análise de homologia através de bancos de dados.
- Investigação do uso de técnicas proteômicas em ensaios ecotoxicológicos.
- Comparação das eletroforeses Uni- e Bi-dimensional na identificação de proteínas microssomais relacionadas a biotransformação.
- Uso da espectrometria de massas (MALDI – TOF) na identificação das proteínas expressas de maneira diferencial em cada tratamento.

3 – RESULTADOS

O corpo dos resultados obtidos compõem o material de 4 artigos publicados e ou submetidos a revistas de política editorial seletiva e estão incluídos a seguir:

3.1 – **da Silva, M.E.F.**, Meirelles, N.C. (2004) Interaction of non - ionic surfactants with hepatic CYP in *Prochilodus scrofa*. **Toxicology in vitro** (no prelo)

3.2 - **da Silva, M.E.F.**, Silva, J. A., Marangoni, S., Novello,J.C., Meirelles, N.C. (2004) A new method to purify the hepatic CYP1A - of *Prochilodus scrofa*, a brazilian freshwater fish. **Comparative Biochemistry and Physiology Part C** 138(2004) 67-74

3.3 - **da Silva, M.E.F.,** Silva, J. A., Martins, D., Marangoni, S., Meirelles, N.C., Novello,J.C. (2004) Use of 2D – electrophoresis and MALDI – TOF mass spectrometry in the identification of hepatic CYP1A isoforms of *Prochilodus scrofa*, a brazilian freshwater fish. Submetido para **Ecotoxicoly Environmental Safety**

3.4 - **da Silva, M.E.F.,** Silva, J. A., Winck, F.V. , Marangoni, S., Meirelles, N.C., Novello,J.C. (2004) Proteomic approach to identify cytochrome P450 isozymes – a new tool to access the environmental pollution. Submetido para **Environmental Pollution**.

3.1 - INTERACTION OF NON - IONIC SURFACTANTS WITH HEPATIC CYP IN *Prochilodus scrofa*

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Interaction of non-ionic surfactants with hepatic CYP in *Prochilodus scrofa*

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Abstract

Cytochromes P450 (CYP) constitute a superfamily of hemeproteins that play a vital role in the metabolism of a wide variety of endogenous and xenobiotic compounds. Xenobiotic metabolism and the role of CYP are of particular interest in studies regarding the prevention of the damage caused by chemical pollutants. We investigated, in this study, the interaction of Triton X-100 and Tween 80 with CYP and antioxidant defenses in Curimbatá, a Brazilian fish. Aiming to clarify the effects of non-ionic surfactants in the monooxigenase system of fish through in vitro study, the effects of Triton X-100 and Tween 80 were analyzed using monooxygenases and antioxidant system as experimental model. Total CYP and EROD were strongly inhibited by Triton X-100 and Tween 80 in a concentration-dependent way; the content of CYP was reduced until zero while EROD activity was completely inhibited in the presence of Triton X-100 and more than 40% inhibited in the presence of Tween 80. Each surfactant causes a different effect on each antioxidant enzyme. No effect was detected in SOD activity in the presence of even Triton X-100 or Tween 80. Triton X-100 increase catalase activity, while Tween 80 decreases this enzyme activity. The molecular structure of the surfactants causes the alteration of this system, since they are able to interact with the microsomal protein, especially with monooxigenase's components, altering their conformation and, consequently destroying their function. Our results suggest that surfactants can interact with components of the microsomal system leading to inhibition of CYP. Therefore, CYP activity, which has been used as a biomarker of xenobiotic exposure, should be used as a marker in association with other enzymes.

Keywords: Fish; P450; EROD; Triton X-100; Tween 80; Antioxidant defenses

1. Introduction

Agricultural, industrial and household practices have led to the contamination of terrestrial and aquatic environments with a range of inorganic and organic xenobiotics. Among them, the surfactants have been extensively used for many years and are, therefore, widely dispersed in the nature. In the aquatic environment, surfactants appear associated with the sediment, where they may potentiate effects of organic compounds such as hydrocarbons, PCBs, PAHs and pesticides (Piscureanu et al., 2001; Krogh et al., 2003).

Many researchers have shown that non-ionic surfactants inhibit CYP activity not only through time- and concentration-dependent mechanisms, but also through species-dependent mechanisms (Lee et al., 1996; Yun et al., 1996, 1997; Guengerich et al., 1998; Hosea and Guengerich, 1998; Mountfield et al., 2000; Inouye et al., 2001). Since all CYP in eukaryotic organisms are membrane bound proteins, protein–lipid and protein–protein interactions are responsible for the functional control of CYPs, and the lipid environment contributes to the interaction of CYP/reductase/cytochrome b5 (Benhardt, 1995).

CYP, whose name is derived from the characteristic peak at 450 nm in the carbon monoxide difference spectrum of reduced CYP (Omura and Sato, 1964), constitutes a superfamily of hemethiolate enzymes involved in the phase I of oxidative biotransformation of hydrophobic compounds, including pesticides, dioxins, drugs and polycyclic hydrocarbons (Benhardt, 1995; Omura and Sato, 1964; Mansuy, 1998; Omura, 1999).

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CYP and NADPH-cytochrome P450 reductase are the key enzymes of this process. The reductase is responsible for the transference of two electrons from NADPH to CYP, which inserts one atom of molecular oxygen into the substrate and reduces another atom with two electrons to water; these electrons may be supplied by cytochrome b5 (Omura, 1999; Halkier, 1996). These electron transferences are largely responsible for the generation of reactive oxygen species (ROS) even in the steady state or in the biotransformation process.

Some CYP enzymes are substrate inducible, a property that allows the cell to adapt itself to the chemical changes in the environment. One of the major proteins responsible for the metabolism of surfactants xenobiotic in liver is the CYP1A family, which is mainly regulated by the aromatic aryl hydrocarbon receptor (AhR), AhR nuclear translocator and estrogen receptor α (hERα) (Whitlock, 1999; Lewis et al., 2002).

Two xenobiotics with surfactant characteristics are analyzed in this study; the chemical structures of these compounds have each a hydrophilic and a lypophilic group (Attwood and Florence, 1983; Haigh, 1996; Berthod et al., 2001). Triton X-100 and Tween 80 (Fig. 1A and B, respectively) are non-ionic surfactants widely used in the agricultural industrial and household products.

The present study characterized the interactions between Triton X-100 and Tween 80 with CYP from Curimbatás liver microsomes using an in vitro assay. *Prochilodus scrofa*, known as Curimbatá, is a Brazilian tropical fish belonging to the Prochilodontidae family. Fish was chosen as biological model since most part of pollutants is dispersed in water and fish are one of the most important protein sources for humans. Curimbatás were chosen by their: (a) seasonal migratory behavior for reproductive purposes (Godoy, 1975), which put them in contact with pollutants distributed in different parts of the water column; (b) high tolerance to hypoxia, exhibiting critical oxygen consumption when oxygen levels drop (Fernandes et al., 1995), (c) detritivorous feeding, meaning that the fish are in contact with xenobiotics that interact with algaes upon stone and in sediment grown algae. For these reasons, Curimbatá may be considered a model for ecotoxicology studies, since xenobiotics are able to act upon any of these characteristics. The aim of this study was to determine the effects of non-ionic surfactants in the proteins that could be used as biomarker of xenobiotic exposure; especially CYP protein. Our hypothesis is that the surfactants Triton X-100 and Tween 80 could interact with both proteins and membrane lipids, destroying the CYP activity and affecting the biotransformation process. To understand the potential for the impact of surfactants like Triton X-100 and Tween 80 on wildlife and on human health, the knowledge of the effects of those on the enzymes involved in their metabolism is essential, especially because there is a lack of information about those effects in microsomal system.

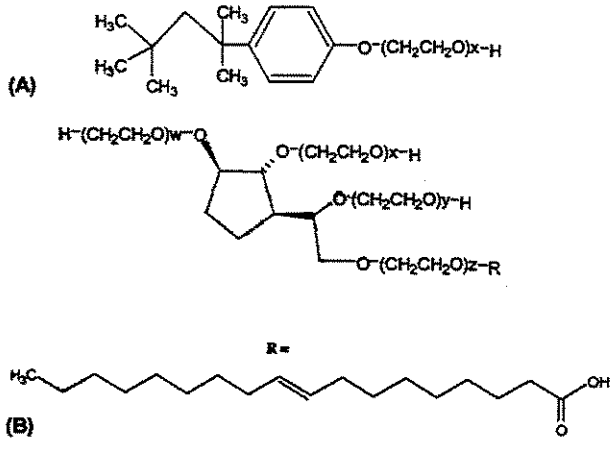

Fig. 1. Chemical structures of (A) Triton X-100 and (B) Tween 80. In A, $X = 9$–10 and in B, $X + Y + Z + W = 20$.

2. Material and methods

2.1. Material

Bovine serum albumin; glycine, adrenalin, hydrogen peroxide, NADPH; glutathione reductase, 7-ethoxyresorufin, *tert*-butylhydroperoxide, Triton X-100 (polyetoxyethanol-*t*-octiphenoxy) and Tween 80 (polyoxyethylene-sorbitol monooleate) were purchased from SIGMA Co.

2.2. Fish

Twenty-four male and female *Prochilodus scrofa* fish (Steindachnner, 1881) (Pisces, Characiforme, Prochilodontidae), out of the reproductive cycle were obtained from the Center of Aquiculture Research and Training (CEPTA, Pirassununga, SP, Brazil). They were raised in artificial open-air reservoirs with pollutant-free water. In the laboratory, fish were anesthetized with spike oil and sacrificed by decapitation. Livers were removed and washed with saline solution. Microsomes were prepared in pools of 4–5 livers.

2.3. Microsome preparation

Microsomal fractions were prepared by the standard procedure: tissues were homogenized in ice-cold 0.1 M potassium phosphate, pH 7.5, containing 0.15 M KCl and 0.1 mM EDTA. Crude homogenate was centrifuged at 12,000×*g* for 20 min and the supernatant centrifuged at 105,000×*g* for 60 min. The pellet was re-suspended in potassium buffer and re-centrifuged at 105,000×*g* for

60 min. Microsomes were isolated in the pellet. The microsomal fraction was re-suspended in the same buffer solution containing 20% glycerol and frozen at −70 °C (Vrolijk et al., 1994). Proteins were determined according to the Lowry method (1951). CYP content was assayed in microsomes according to the method of Omura and Sato (1964), and expressed as nmol/mg protein on the basis of the millimolar extinction coefficient of 91 $mM^{-1} cm^{-1}$.

2.4. Biochemical assay

Essays were conducted at 25 °C using surfactants in a range of 0–500 μM. The protein concentration was 1.0 mg/ml over a 20 min period in 0.1 M potassium phosphate buffer pH 7.4 and a NADPH regenerator system (10 mM glucose 6-phosphate, 1 mM NADP+ and 0.5 U/ml glucose 6-phosphate desidrogenase). The control consisted of a sample without the surfactant but with the NADPH regenerator system. Three different concentrations of each surfactant were used. In the case of Triton X-100, it was used a concentration less than CMC (100 μM), one near to the CMC (200 μM) and one above the CMC (500 μM). In the case of Tween 80, all the concentrations used were over to CMC, since that it was 12 μM. Estimate of P450 inactivation was performed according to the method described by Omura and Sato (1964) after different incubation times. Results were expressed as percentages of initial content. Absorbance was measured on a Hitachi U-2010—UV/Vis Spectrophotometer (Hitachi Instruments, Inc, Japan) and the concentration of P450 was determined using the millimolar extinction coefficient of 91 $mM^{-1} cm^{-1}$.

7-Ethoxyresorufin-*O*-deethylase activity was monitored by the continuous spectrofluorimetric procedure of Prough et al. (1978). Reactions (1 ml, 37 °C) contained 1.0 mg/ml microsomal protein, 10 μM 7-ethoxyresorufin and NADPH regenerator system (10 mM glucose 6-phosphate, 1 mM NADP+ and 0.5 U/ml glucose 6-phosphate desidrogenase) in 0.1 M Tris–HCl buffer, pH 7.4. Resorufin fluorescence at different concentration was used as standard. Fluorescence intensity was measured on a Hitachi F4500 spectrofluorometer (Hitachi Instruments, Inc., Japan).

The interaction effects of surfactants with the CYP system were analyzed via change in tryptophan fluorescence; all experiments were performed in 100 μM potassium phosphate buffer, at room temperature, containing 1 mg/ml of microsomal protein (Yun et al., 1996). For tryptophan fluorescence, the mixture was excited at 295 nm and the emission recorded at 305–580 nm. Fluorescence intensity was measured on a Hitachi F4500 spectrofluorometer (Hitachi Instruments, Inc., Japan). The protein concentration was 1.0 mg/ml. The excitation wavelength was 295 nm. Native-page electrophoresis was performed according to Lammeli (1970) to separate proteins according to the pI. If the surfactant interacts with any protein of the microsomal system changing the charge of the system, the protein migration will be changed when compared to the control.

Superoxide dismutase activities were estimated by the adrenochrome formation rate by measuring the absorption changes at 480 nm (Misra and Fridovich, 1972; Boveris et al., 1983). SOD activities were expressed in pmol SOD $mg\,prot.^{-1}\,min^{-1}$. Catalase activities were determined from the decrease of hydrogen peroxide concentration, by the decrease in the absorbance at 240 nm (Beers and Sizer, 1952; Aebi, 1984). Catalase activities were expressed in pmol $mg\,prot.^{-1}\,min^{-1}$. The total glutathione peroxidase activities were measured in a coupled enzyme system by measuring the decrease in NADPH at 365 nm (Flohé and Gunzler, 1984). The activities were expressed as pmol $mg\,prot.^{-1}\,min^{-1}$ (1 pmol of GPx is equivalent to 6 mmol oxidized NADPH) (Flohé and Gunzler, 1984). Results are expressed as means ± SD; all samples were performed in triplicate.

Determination of TBARS was used to access endogenous lipid peroxidation in microsomes through the products derived from the lipid oxidation with thiobarbituric acid (TBA) (Ohkawa et al., 1979). Microsomal suspension was diluted up to a concentration of 0.5 mg/ml in phosphate buffer. The mixture was incubated with different concentrations of each xenobiotic in the presence and absence of NADPH regenerator system for 20 min as described before. The suspensions were treated with 500 μl of trichloroacetic acid (TCA) 25% to precipitate the microsomal proteins. The microsomal suspension was centrifuged and 1 ml of the supernatant was removed and re-suspended in 1 ml of TBA (0.1 g TBA/9 ml NaCl 0.5 M). The mixture was maintained in boiling water for 60 min, cooled in ice, and then measured spectrophotometrically at 535 nm. Absorbances are reported as nmol/TBARS/mg prot.

Correlation among distinct antioxidant enzymes, as well as the contents of cytochrome P450 were performed and tested statistically using linear regression assuming a 95% confidence interval ($p < 0.05$) (Zar, 1996). All experiments were done with an $n = 10$ and in triplicate. Differences among treatment and control groups were verified through ANOVA and Turkey test.

3. Results

3.1. CYP Content

The initial concentration of total CYP was 22 nmol/mg prot., which is in agreement with the literature. At 500 μM no spectral CYP was detectable after 20 min of incubation, suggesting that Triton X-100 (Table 1) and

Table 1
Decrease of CYP content in the presence of Triton X-100

Time (min)	% of CYP content[a]			
	Triton X-100 (mM)			
	0.0	0.1	0.2	0.5
0	100.0	100.0	71.47	85.12
3	48.63	30.70	0.0	0.0
9	18.18	9.10	0.0	0.0
15	7.39	6.02	0.0	0.0
20	7.39	2.27	0.0	0.0

[a] Related to the 0.0 mM of surfactant in time of 0 min.

Table 2
Decrease of CYP content in the presence of Tween 80

Time (min)	% of CYP content[a]			
	Tween 80 (mM)			
	0.0	0.1	0.2	0.5
0	100.0	100.0	100.0	93.86
3	72.86	58.72	86.38	33.00
9	52.93	35.28	14.60	2.64
15	47.14	20.12	7.14	0.0
20	39.00	13.53	0.0	0.0

[a] Related to the 0.0 mM of surfactant in time of 0 min.

Tween 80 (Table 2) significantly destroyed the hepatic CYP in Curimbatá compared to the control (0.0 μM of surfactant).

The activity of CYP1A was measured through the EROD activity. This enzyme catalyzes the CYP1A reaction and its activity could be measured by an increase in the fluorescence intensity, since resorufin (the product of the enzyme reaction) is fluorescent. According to Fig. 2A, the presence of Triton X-100 decrease the EROD activity, a behavior similar to the one observed in the total CYP content. This was confirmed by the strong correlation found between CYP content and EROD activation ($r = 0.9783$, $p < 0.0001$). Tween 80 (Fig. 2B) also decreases EROD activity; however, the effects of this surfactant are less intense than those caused by Triton X-100, possibly due to the physical chemical characteristics of this surfactant.

3.2. Antioxidant enzymes

Microsomal samples were incubated with different concentrations of Triton X-100 or Tween 80 and a slight increase in superoxide dismutase activity was seen after 20 min of incubation, but this difference was not significant. In this case, the basal levels of SOD could be sufficient to react with the oxygen free radicals produced.

Triton X-100 led to an increase in CAT activity, while the presence of Tween 80 in the microsomal suspension decreased the activity of CAT after 20 min of incubation (Fig. 3A), what suggests that hydrogen peroxide was not generated by CYP cycle, since the cytochrome was completely destroyed.

The effects of each xenobiotic on the activity of glutathione peroxidase are shown in Fig. 3B. Triton X-100 caused an increase in GPx activity, compared to the control, in a concentration-dependent manner. A high GPx activity was found in the microsomal suspension after 20 min of incubation with 0.2 mM of Tween 80. The absence of any correlation between CYP content and GPx activity confirms the hypothesis that the hydroperoxides attacked by GPx were not produced by the CYP cycle, since this cytochrome was not detectable after 20 min of incubation. A positive correlation between CAT and GPx ($r = 0.8987$, $p = 0.010$) was found in the presence of Triton X-100 and suggests that both enzymes act in the destruction of hydroperoxides generated by the microsomal suspension in the presence of Triton X-100. No significant correlation between CAT and GPx was found in treatments with Tween 80.

After incubating microsomal suspensions during 20 min with Triton X-100, no peroxyl radicals ($LOO^{\cdot}$) were detected by the TBARS method, even in the presence or in the absence of SRNADPH. In the presence of Tween 80, the microsomal suspension caused a slight decrease in lipid peroxidation in the presence and absence of SRNADPH, but the difference was not significant. These results show that lipid peroxidation is not a factor responsible for the CYP destruction.

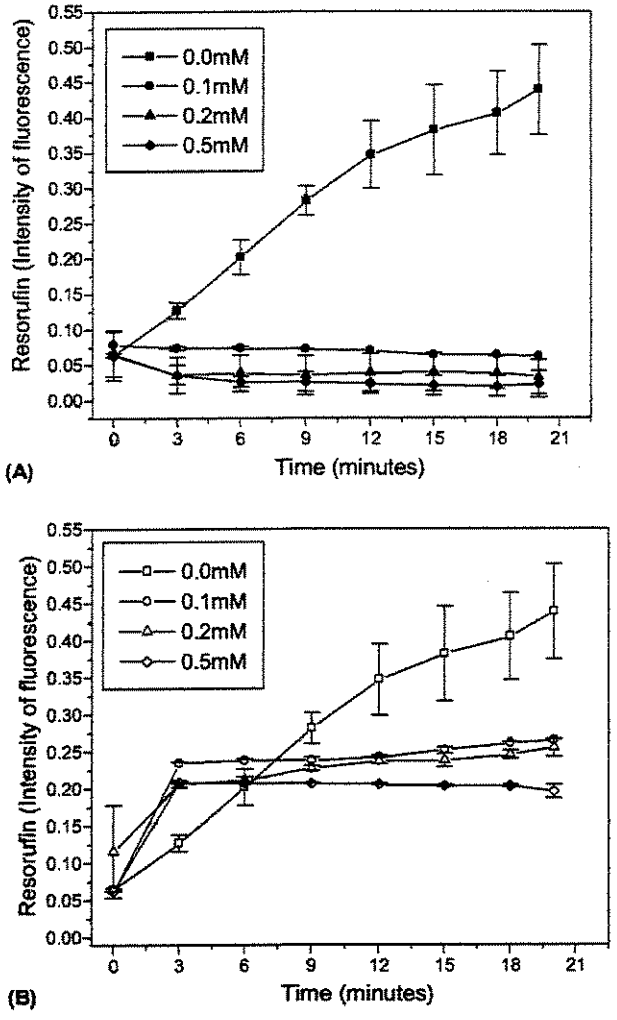

Fig. 2. EROD activity in microsomal suspension incubated with Triton X-100 (A) and Tween 80 (B). (■) Control; (●) 0.1 mM; (▲) 0.2 mM and (◆) 0.5 mM. Solid symbols represent the data of Triton X-100, while open symbols represent Tween 80 data. Values represent mean ± SD of measurement from triplicate enzymes analysis of nine microsomal sample.

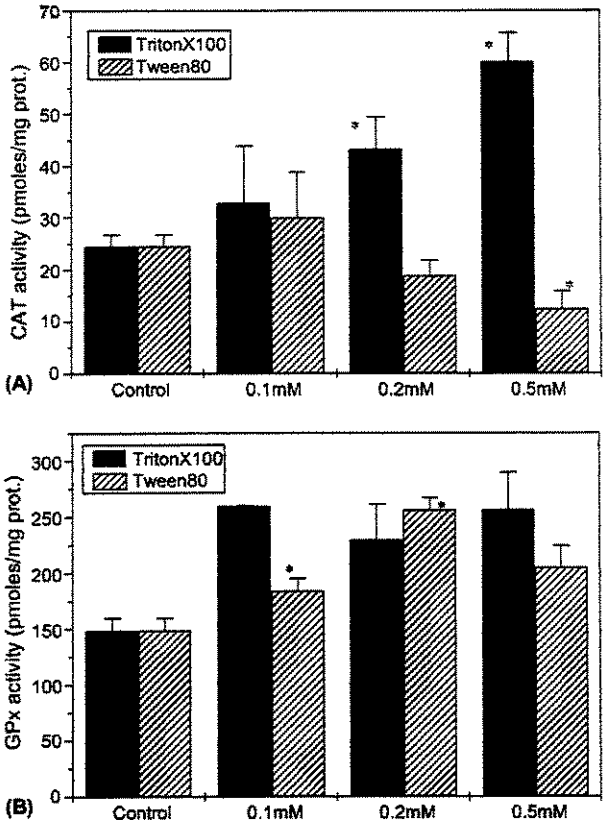

Fig. 3. Activity of antioxidant enzymes in microsomal sample after a 20 min incubation with Trion X-100 or Tween 80. (A) Catalase activity and (B) gluthatione peroxidase activity in hepatic microsomes of *Prochilodus scrofa*. The samples were treated with 100, 200 and 500 μM of each xenobiotic. $^{*}p < 0.05$ related to the control. Values represent mean ± SD of measurement from triplicate enzymes analysis of nine microsomal sample.

The main hypothesis of this study was that surfactants interact with both membrane and CYP in microsomal samples. In order to prove this hypothesis, we analyzed the effects of surfactant upon tryptophan after incubating microsomal sample during 20 min. Triton X-100 led to an increase in the tryptophan fluorescence, which indicated that the aminoacid is being exposured to an apolar environment, signifying that the proteins are enclosed by surfactants or lipids and explaining why the proteins are more negative in the presence of surfactants (Fig. 4A). A contrasting behavior of tryptophan was observed in the presence of Tween 80. This surfactant caused a decrease in fluorescence, indicating a quenching and suggesting that the aminoacid was exposed to a hydrophilic environment (Fig. 4B). These results suggest that both surfactants change the microsomal environment, exposing the CYP system to a different medium that could alter the protein conformation.

According to the migration of the proteins in the native-page electrophoresis, incubation of microsomes with Triton X-100 (Fig. 4A—inset) gave a negative charge to the proteins of microsomal system, since they migrated to the positive pole. This result suggests an interaction of Triton X-100 with the microsomal system proteins, including CYP, cytochrome b5 and NADPH cytochrome P450 reductase. No detectable change in the protein charge was detected in the samples incubated with Tween 80, which could indicate a weak interaction of proteins with the surfactant (Fig. 4B, inset). Unfortunately, all proteins of microsomal system migrate to the positive pole together, what suggest that surfactants were acting in all of them in the same manner and that

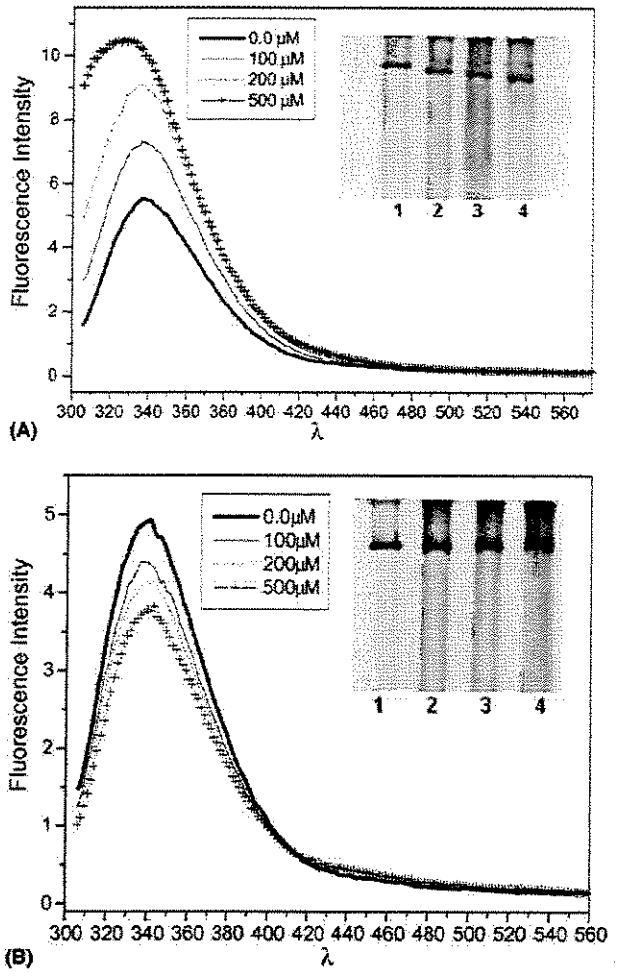

Fig. 4. Effects of Triton X-100 (A) and Tween 80 (B) in the fluorescence spectrum of triptophan after a 20 min incubation. (—) Control; (——) 100 µM; (···) 200 µM and (-+-) 500 µM. Inset shows the native-page of microsomal suspension after a 20 min incubation with each surfactant. Line 1—control; Line 2—100 µM; Line 3—200 µM and Line 4—500 µM of surfactant.

native-page is not a good method to study protein surfactant interaction.

4. Discussion

CYP1A is a member of the superfamily of enzymes that catalyze the biotransformation of both endogenous and xenobiotic compounds in eukaryotes. Activation of CYP1A through its catalytic cycle involves two monoelectronic reductions of oxygen. These reactions are responsible for the generation of superoxide anion ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Benhardt, 1995; Koblyakov, 1998). Both radicals are able to produce the most reactive radical, the hydroxyl radical, which has the capacity to link to proteins and DNA.

Triton X-100 causes the destruction of CYP1A, even in monomers. These results are in disagreement with the literature. The critical micelle concentration (CMC) of Triton X-100 is around 250 µM. The destruction of the system was not expected in concentrations lower than this, since the surfactant's molecules are present in the monomer and the interaction between the surfactant and the membrane occurs through micelle (Haigh, 1996; Hosea and Guengerich, 1998; Inouye et al., 2001). The results demonstrate that, as monomers, there is a direct interaction of surfactant with CYP. In this case, the inhibition occurred via an interaction between Triton X-100 and CYP, which may explain why the decrease in CYP content is drastically (de Montellano and Correa, 1983; Halpert, 1995; Lee et al., 1996; Hosea and Guengerich, 1998; Mountfield et al., 2000; Inouye et al., 2001). This interaction could alter the conformation of the active site and may inhibit the CYP activity via a competitive inhibitions mechanism, already described by other authors (de Montellano and Correa, 1983; Halpert, 1995). When the surfactant concentration in the system was above the CMC, CYP content decreases more drastically. In these conditions, the surfactant acts by disrupting the lipid environment of the microsomal membrane.

The effects of Tween 80 in the system were caused by the micelles themselves, since all concentrations evaluated in the experiments were above the CMC of approximately 12 µM. Thus, the main factor responsible for the system inhibition is the micelles interaction with the microsomal membrane, which affects the lipid organization and the electron transport among the CYP system's proteins (De Pierre and Dallner, 1975; Guengerich, 1977; Yun et al., 1996, Mountfield et al., 2000). The initial increase of CYP activity may be due to this new lipid organization that, in a first moment, propitiated an increase of electron transport among the proteins of microsomal system. The increase of Tween 80 concentration affects drastically this transport through microsomal components.

Triton X-100 was the most harmful to the microsomal membrane due to the high delipidation rate, that substitutes phospholipid molecules for surfactant molecules (de Foresta et al., 1989). The delipidation rate of the microsomal membrane caused by Tween 80 is lower than that of Triton X-100 due to the structural characteristics of the molecule. Tween 80 has a large apolar head, compared to Triton X-100, and the disposition of its polyethyleneglycol chain impedes this surfactant insertion in the membrane, decreasing the delipidation rates (Robinson and Tanford, 1975; Garda and Brenner, 1984; Attwood and Florence, 1983; de Foresta et al., 1989; Haigh, 1996; Jones, 1999).

According to Black (1992), tryptophan is an aminoacid, which could indicate the interaction of any xenobiotic with the CYP, NADPH cytochrome P450

reductase and cytochrome b5, since it is localized in the cytoplasmatic region of these proteins. Any alteration in this region of the protein implies CYP inactivation (Black, 1992; Neunaber and Achazi, 1999). Triton X-100 led to an increase in fluorescence intensity of tryptophane, indicating a conformational change in the protein. The main hypothesis is that the aminoacid is being exposed to a hydrophobic environment. Triton X-100 is a surfactant able to delipidate membranes in a process that substitutes endogenous lipids (among them phosphatidylcholine) for surfactants molecules. Phosphatidylcholine is not able to form micelles in the aqueous environment. This pool of lipids generated by delipidation links to the CYP surface, blocking it from the hydrophobic environment and inhibiting its activity (de Foresta et al., 1989; Oteiza et al., 1993; Ohyshiki et al., 1998).

However the effects of Triton X-100 also affect the hydrophobic region of CYP since the delipidation process exposes the NH_2-terminal of protein, responsible for the protein anchorage of in the membrane. According to Yun and collaborators (Yun et al., 1996, 1997), CYP activity depends on the presence of phospholipids that maintain the protein in the membrane and facilitate the electron rates through CYP, NADPH–CYP–reductase and cytochrome b5. Any compound able to change these interactions could change the conformation of CYP impeding the activation of the system.

Tween 80 is able to expose triptophan residues to a hydrophilic environment. The quenching of fluorescence is concentration dependent and suggests a smaller degree of charge transference between microsomal proteins and the surfactant, being the chemical structure of Tween 80 responsible for this. The large apolar head and the disposition of polyethyleneglycol chain decreased the membrane delipidation level compared to Triton X-100 (Attwood and Florence, 1983; de Foresta et al., 1989; Haigh, 1996).

The physical–chemical characteristics of Triton X-100 are also mainly responsible for the increase in CAT and GPx activity. Triton X-100 caused the disruption of the membrane at concentrations above 100 μM, therefore the hydroperoxides generated by the lipid peroxidation pathway were not responsible for the increase of Catalase, nor was Glutathione Peroxidase (Ohkawa et al., 1979; Oteiza et al., 1993). The presence of Triton X-100 in the microsomal suspension may create a hydrophobic environment due to the formation of mixed micelles, which are composed of microsomal proteins, phospholipids and Triton X-100 molecules. In addition, hydrophobic environments may enhance enzyme specificity, activity, and stability as well as shifting the thermodynamic equilibrium. Our hypothesis suggests that catalase and gluthathione peroxidase are encapsulated inside these micelles and covalently attached the surfactant, which, in this medium, may stabilize the enzyme/surfactant complex (Jene et al., 1997; Lamb et al., 1998; Sekhar et al., 1999; Matés, 2000). Once the affinity of catalase for its substrate—hydrogen peroxide—is not high, this new environment stabilizes the complex and increases the hydrogen peroxide degradation velocity (Lamb et al., 1998; Sekhar et al., 1999; Matés, 2000).

The presence of Tween 80 in the medium suggests an inhibition of Catalase activity and an accumulation of hydrogen peroxide, which could aggravate the oxidative stress. Activation of GPx could be a strategy of the organism to avoid the damage caused in the microsomal membrane by hydroperoxides, since these low levels of catalase could deprive the microsomal membrane of defenses against hydroperoxides radicals (Wilhelm-Filho and Marcon, 1996; Dwivedi et al., 1998; Farombi, 2000). Once the concentration of Tween 80 used in the assay was higher than the CMC, the process of micelles formation was intensified. Tween 80 is derived from oleic acid and is able to donate monounsaturated fatty acid to the membrane. However, these fatty acids are not a good substrata for membrane lipid peroxidation due to their low unsaturation, explaining the low levels of TBARS in the assay (Ohkawa et al., 1979; Attwood and Florence, 1983; Kapich et al., 1999).

In conclusion, this study demonstrated that compounds with surfactant characteristics, which are commonly used in soluble pesticides and industrial processes, are able to inactivate the components of the CYP system, even in the monomer form—through interaction with CYP—and in micelle form by disrupting the integrity of the membrane. The inactivation of the CYP system may alter important biological functions such as the seasonal reproduction and fetal development. The inhibition of the CYP1A catalytic activity by those surfactants may have important physiological and pharmacological implications. Since CYP plays an important role in the metabolic activation and/or disablement of xenobiotics, the effects of Triton X-100 and Tween 80 in CYP system requires additional studies.

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References

Aebi, H., 1984. Catalase in vitro. Meth. Enzymol. 105, 121–126.
Attwood, D., Florence, A.T. (Eds.), 1983. Surfactants Systems—Their Chemistry, Pharmacy and Biology. Chapman & Hall Publisher, London, New York.

Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140.

Benhardt, R., 1995. Cytochrome P450: structure, function and generation of reactive oxygen species. Rev. Physiol. Biochem. Pharmacol. 127, 138–221.

Berthod, A., Tomer, S., Dorsey, J.G., 2001. Polyoxyethylene alkyl ether nonionic surfactants: physicochemical properties and use for cholesterol determination in food. Talanta 55, 69–83.

Black, S.D., 1992. Membrane topology of mammalian P450 cytochromes. FASEB J. 6, 680–685.

Boveris, A., Fraga, C.G., Varsavsky, A.I., Koch, O.R., 1983. Increased chemiluminescence and superoxide production in the liver of chronically ethanol-treated rats. Arch. Biochem. Biophys. 227, 534–541.

de Foresta, B., le Maire, M., Orlowski, S., Champeil, P., Lund, S., Müller, J.V., Michelangeli, F., Lee, A.G., 1989. Membrane solubilization by detergent: use of brominated phosphilipids to evaluate the detergent-induced changes in Ca+2-ATPase/lipid interaction. Biochemistry 28, 2558–2567.

de Montellano, P.R., Correa, M.A., 1983. Suicidal destruction of cytochrome P450 during oxidative drug metabolism. Ann. Rev. Pharmacol. Toxicol. 23, 481–503.

De Pierre, J.W., Dallner, G., 1975. Structural aspects of the membrane of endoplasmatic reticulum. Biochim. Biophys. Acta 415, 411–472.

Dwivedi, P.D., Das, M., Khanna, S.K., 1998. Role of cytochrome P450 in quinalphos toxicity: effect on hepatic and brain antioxidant enzymes in rats. Food Chem. Toxicol. 36, 437–444.

Farombi, E.O., 2000. Influence of amodiaquine treatment on microsomal lipid peroxidation and antioxidant defenses systems of rats. Pharmacol. Toxicol. 87, 249–254.

Fernandes, M.N., Barrionuevo, W.R., Rantin, F.T., 1995. Effects of the thermal stress on respiratory responses to hypoxia of a South American Prochilodontid fish, *Prochilodus scrofa*. J. Fish Biol. 46, 123–133.

Flohé, L., Gunzler, A., 1984. Assays of glutathione peroxidase. Meth. Enzymol. 105, 114–121.

Garda, H.A., Brenner, R.R., 1984. Short-chain aliphatic alcohols increase rat-liver microsomal membrane fluidity and affect the activities of same microsomal membrane bound enzymes. Biochim. Biophys. Acta 769, 160–170.

Godoy, M.P. (Ed.), 1975. Peixes do Brasil—Subordem Characiforme. Editora Franciscana, Piracicaba, SP.

Guengerich, F.P., 1977. Separation and purification of multiples forms of microsomal cytochrome P450. J. Biol. Chem. 252, 3570–3970.

Guengerich, F.P., Hosea, N.A., Parikh, A., Bell-Parikh, C., Johnson, W.W., Gillam, E.M.J., Shimada, T., 1998. Twenty years of biochemistry of human P450. Purification, expression, mechanism and relevance to drug. Drug Met. Disp. 26, 1175–1178.

Haigh, S.D., 1996. A review of the interaction of surfactants with organic contaminants in soil. Sci. Total Environ. 185, 161–170.

Halkier, B.A., 1996. Catalytic reactivities and structure/function relationship of cytochrome P450 enzymes. Phytochemistry 43, 1–21.

Halpert, J.R., 1995. Structural basis of selective cytochrome P450 inhibition. Ann. Rev. Pharmacol. Toxicol. 35, 29–53.

Hosea, N.A., Guengerich, F.P., 1998. Oxidation of nonionic detergents by cytochrome P450 enzymes. Arch. Biochem. Biophys. 353, 365–373.

Inouye, K., Kondo, S., Yamamura, M., Nakanishi, D., Sakaki, T., 2001. Inhibitory effects of detergents rat CYP1A1-dependent monooxygenase: comparison of mixed and fused system consisting of rats CYP1A1 and yeast NADPH-P450 reductase. Biochem. Biophys. Res. Commun. 280, 1346–1351.

Jene, Q., Pearson, J.C., Lowe, C.R., 1997. Surfactant modifies enzymes: solubility and activity of surfactant modified catalase in organic solvents. Enzyme Microbiol. Technol. 20, 69–74.

Jones, M.N., 1999. Surfactants in membrane solubilization. Int. J. Pharm. 177, 137–159.

Kapich, A., Hofrichter, M., Vares, T., Hatakka, A., 1999. Coupling of manganese peroxidase-mediated lipid peroxidation with destruction of nonphenolic lignin model compounds and 14C-labeles lignins. Biochem. Biophys. Res. Commun. 259, 212–219.

Koblyakov, V.A., 1998. Inducers of the cytochrome P450 superfamily as promoters of carcinogenesis. Biochemistry 63, 885–898.

Krogh, K.A., Halling-Sørensen, B., Mogensen, B.B., Vejrup, K.V., 2003. Environmental properties and effects of nonionic surfactant adjuvant in pesticides: a review. Chemosphere 50, 871–901.

Lamb, S.B., Lamb, D.C., Kelly, S.L., Stuckey, D.C., 1998. Cytochrome P450 immobilization as a rout to bioremediation/biocatalysts. FEBS Lett. 431, 343–346.

Lammeli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lee, P.C., Patra, S.C., Stelloh, C.T., Lee, W., Struve, M., 1996. Interaction of nonylphenol and hepatic CYP1A in rats. Biochem. Pharmacol. 52, 885–889.

Lewis, D.F.V., Jacobs, M.N., Dickins, M., Lake, B.G., 2002. Quantitative structure–activity relationship for inducers of cytochromes P450 and nuclear receptor ligands involved in P450 regulation within the CYP1, CYP2 CYP3 and CYP4 families. Toxicology 176, 51–57.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurements with Folin-phenol reagent. J. Biol. Chem. 193, 265–275.

Mansuy, D., 1998. The great diversity of reaction catalysed by cytochrome P450. Comp. Biochem. Physiol. 121C, 5–14.

Matés, J.M., 2000. Effects of antioxidants enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 153, 83–104.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autooxidation of epinephrine and a simple assay to superoxide dismutase. J. Biol. Chem. 247, 3175–3179.

Mountfield, R.J., Senepin, S., Schleimerm, M., Walter, I., Bittner, B., 2000. Potential inhibitory effects of formulation ingredients on intestinal cytochrome P450. Int. J. Pharmac. 211, 89–92.

Neunaber, R., Achazi, R.K., 1999. A novel endogenous inhibitor of 7-ethoxyresorufin-O-deetylase activity isolated from liver cytosolic fraction of bream (Abramis brama L.). Comp. Biochem. Physiol. 124B, 89–100.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Ohyshiki, T., Suzuki, S., Satoh, E., Uemori, Y., 1998. A marked stimulation of Fe+2-initiated lipid peroxidation in phospholipid liposomes by lypophilic aluminum complex, aluminum acetylacetonate. Biochim. Biophys. Acta 1389, 141–149.

Omura, T., 1999. Forty years of cytochrome P450. Biochem. Biophys. Res. Commun. 266, 690–698.

Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. I—Evidence for its heme protein nature. J. Biol. Chem. 239, 2370–2378.

Oteiza, P.I., Fraga, C.G., Keen, C.L., 1993. Aluminum has both oxidant and antioxidant effects in mouse brain membranes. Arch. Biochem. Biophys. 300, 517–521.

Piscureanu, A., Pop, T., Dogaru, M., Piscureanu, M., Manaila-Maximean, D., 2001. Influence of non-ionic surfactants on surface activity of pesticide colloidal systems. Colloids Surf.—A: Physicochem. Eng. Aspects 178, 129–133.

Prough, R.A., Burke, M.D., Mayer, B.T., 1978. Direct fluorimetric methods for measuring mixed-function oxidase activity. Meth. Enzymol. 52, 374–377.

Robinson, N.C., Tanford, C., 1975. The binding of Deoxycholate, Triton X-100, Sodium Dodecyl Sulfate and Phosphatidylcholine vesicles to cytochrome b5. Biochemistry 14, 369–377.

Sekhar, S., Bhat, N., Bhat, S.G., 1999. Surfactant of detergent permeabilized Bakers' yeast whole cell catalase. Process Biochem. 34, 349–354.

Vrolijk, N.H., Targett, N.M., Woodin, B.R., Stegman, J.J., 1994. Toxicological and ecological implication of biotransformation enzymes in the tropical Teleost Chaetodos capistratus. Mar. Biol. 119, 151–158.

Whitlock, J.P., 1999. Induction of CYP1A1. Annu. Rev. Pharmacol. Toxicol. 39, 103–125.

Wilhelm-Filho, D., Marcon, J.L., 1996. Antioxidant defense in fish of the amazon. In: Val, A.L., Almeida-Val, V.M.F., Randal, D.J. (Eds.), Physiology and Biochemistry of the Fishes of the Amazon. INPA, Manaus, AM. Brasil, pp. 299–312.

Yun, C.H., Song, M., Ahn, T., Kim, H., 1996. Conformational changes of cytochrome P4501A2 induced by sodium chloride. J. Biol. Chem. 271, 31312–31316.

Yun, C.H., Song, M., Kim, H., 1997. Conformational changes of cytochrome P4501A2 induced by phospholipids and detergents. J. Biol. Chem. 272, 19725–19730.

Zar, J.H., 1996. Biostatistical Analysis. Prentice Hall, EUA.

3.2 - A NEW METHOD TO PURIFY THE HEPATIC CYP1A - OF *Prochilodus scrofa*, A BRAZILIAN FRESHWATER FISH.

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A new method to purify hepatic CYP1A of *Prochilodus scrofa*, a Brazilian freshwater fish

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Abstract

Cytochromes *P*450 constitute a superfamily of the phase I enzymes whose primary task is the detoxification of both endogenous and xenobiotic compounds. Fish, among non-mammalian species, have received great interest because they are a direct food source for humans as well as conveyors of toxic chemicals to human beings. The aim of the present study was the purification of the hepatic isoform of CYP1A in *Prochilodus scrofa* (Prochilodontidae), a Brazilian fish, using only one chromatographic step. The purification of CYP1A was done by Reverse Phase HPLC on a C18 column. Purified CYP1A was characterized with respect to electrophoretic, immunochemical and biocatalyst properties. CYP1A fractions produced a single uniform band on SDS-PAGE with an apparent molecular mass of 58 kDa. Purified CYP1A of *P. scrofa* showed strong cross-reactivity with antibodies directed against CYP1A from trout. The fraction was also encapsulated in two different reconstituted systems; one composed of neutral lipids and another of negatively charged lipids. In both of them, we could detect EROD activity but not PROD activity, which confirms that the CYP1A was purified with all its enzyme activity. There was an increase of activity when CYP1A and NADPH cytochrome *P*450 (CYP) reductase were encapsulated in negatively charged lipids, which confirms that the charge of lipid is essential to CYP1A activity. All these characteristics strongly suggest that this new procedure is efficient for purifying hepatic CYP1A from *P. scrofa*, showing that the CYP1A isoform of this fish has a highly conserved protein region.

Keywords: CYP; Purification; Fish; CYP1A; HPLC; EROD; Curimbatá; NADPH cytochrome *P*450 reductase

1. Introduction

The hepatic cytochrome *P*450 (CYP) is responsible for the oxidative metabolism of structurally unrelated compounds, including both xenobiotic and endogenous compounds. This system plays different roles in an organism: (a) it is the major route by which living organisms convert lipophilic compounds into more soluble ones; a process which facilitates the elimination of xenobiotics from the body—this process is called detoxification; (b) this enzyme system can convert certain carcinogenic compounds into more toxic products that can bind covalently to macromolecules such as DNA and proteins; (c) finally, this system can also metabolize numerous endogenous compounds, including steroids, fatty acids, vitamins, leukotrienes and others (Ryan and Levin, 1990; Buhler and Wang-Buhler, 1998; Omura, 1999; Raucy and Allen, 2001).

CYPs are found in both prokaryote and eukaryotic organisms and are divided into 281 families (http://drnelson.utmem.edu/CytochromeP450.html). Apart from the clear importance of those families to the metabolism of xenobiotics compounds, the CYP1A family code forms monooxygenases that are chiefly responsible for the detoxification and activation of xenobiotic compounds in fish (Morrison et al., 1995, 1998). Among the non-mammalian species, fish have received great attention since that they are a direct food source for humans, acting as conveyors of toxic compounds to the human population.

Initially, it was thought that fish lacked any CYP-linked detoxification system; however, in the 1960s, Buhler and Rasmusson (1968) demonstrated that there is a CYP-dependent system present in the liver of many marine and freshwater fishes. The CYP system in fish is structurally similar to that in mammals, but some differences are found;

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for example, the enzyme activity of fish liver microsomes is lower than in mammals, and the system could be regulated by exogenous factors (Klotz et al., 1983; Stegeman and Lech, 1991; Buhler and Wang-Buhler, 1998). In recent years, research on fish CYP1A has increased with the attempt to provide data to biomonitoring and pollutant metabolism.

Prochilodus scrofa (Curimbatá) is a Brazilian tropical fish belonging to the Prochilodontidae family. Curimbatás were chosen because of their: (a) seasonal migratory behavior for reproductive purposes (Godoy, 1975), which puts them in contact with pollutants distributed in different parts of the water column; (b) high tolerance to hypoxia, exhibiting critical oxygen consumption when oxygen levels drop (Fernandes et al., 1995); (c) detritivorous habit, meaning that the fish are in contact with xenobiotics that interact with algae that grow upon stone and in sediment. For these reasons, Curimbatá may be considered a model for ecotoxicology studies since xenobiotics are able to act upon any of these characteristics.

Traditional methods of purification of membrane proteins like CYP1A are known to use many solubilization, chromatographic and dialysis steps. The main consequences of this are: (i) necessity of large amount of liver sample, (ii) loss of catalytic activity of CYP due to factors such as time and temperature, and (iii) decrease of recovery in each step. In this article, we are describing a new method to purify CYP1A in a single chromatographic step with the exclusion of solubilization and dialysis steps, increasing the recovery of purified CYP1A and the percentage of CYP1A biocatalyst activity.

2. Materials and methods

2.1. Fish

Twenty-four male and female *P. scrofa* (Steindachner, 1881) (Pisces, Characiformes, Prochilodontidae), not in reproductive cycle, were obtained from the Center of Aquaculture Research and Training (CEPTA, Pirassununga, SP, Brazil). The fish collected were of comparable masses (300–500 g) and the monooxygenase activity was induced with BHT (butylhydroxytoluene) by a dose of 200 mg/kg diluted in corn oil. The animals were raised in artificial open-air reservoirs of pollutant-free water. After 7 days, fish were anesthetized with spike oil and sacrificed by decapitation. Livers were removed and washed with saline solution. Microsomes were prepared in batches from four to five livers.

2.2. Microsome preparation

Microsomes were prepared in the standard procedure: tissues were homogenized in ice-cold 0.1 M potassium phosphate, pH 7.5, containing 0.15 M KCl and 0.1 mM EDTA (1:5 w/v). Crude homogenate was centrifuged at $12,000 \times g$ for 20 min and the supernatant centrifuged at $105,000 \times g$ for 60 min. The pellet was re-suspended in potassium phosphate buffer and re-centrifuged at $105,000 \times g$ for 60 min. Microsomes were isolated in the pellet. The microsomal fraction was re-suspended in the same buffer solution containing 20% glycerol and frozen at −70 °C (Vrolijk et al., 1994). Proteins were determined according to the Lowry method (1951). CYP content was assayed in microsomes according to the method of Omura and Sato (1964), and expressed as nmol/mg protein on the basis of the millimolar extinction coefficient of 91 mM^{-1} cm^{-1}.

2.3. Purification of cytochrome P4501a

The experimental procedure described above was developed for the isolation of CYP1A from *P. scrofa* liver microsomes. The procedure was optimized in order to purify this heme protein in only one chromatographic step with the highest content and specific activity in the catalytic form. All procedures were carried out at 0–4 °C.

The crude microsomes were diluted in 0.1 M potassium phosphate, pH 7.5, containing 0.15 M KCl and 0.1 mM EDTA (1:5 w/v) until they reached a concentration of 5–6 mg/ml. In any step of purification, microsomes were solubilized with detergents. The sample was applied to a Reverse Phase HPLC system (Waters 515 Pumps; Waters 2487 Dual λ absorbance detector, Waters, USA) with an analytical C18 column (μ-Bondapack 3.9 × 300 mm, Waters). Trifluoracetic acid was used to equilibrate the column while acetonitrile was used as the elution buffer. The elution profile is described in Table 1. The elution of samples was followed by the absorbance at 280 nm (total proteins) and 418 nm (heme protein). Purification procedures were carried out at 4 °C to prevent any protein denaturation by temperature. Each peak was collected and lyophilized for further procedures. Lyophilized samples were stored at −8 °C and resuspended in 50 mM Tris–HCl buffer pH 7.4 just prior to use. Protein concentration of purified samples was measured according to the method of Lowry et al. (1951).

Table 1
Elution gradient used in the purification of *P. scrofa* CYP1A using a Reverse Phase HPLC with a μ-Bondapack C18 column

Time (min)	Flow (ml/min)	Trifluoroacetic acid (%)	Acetonitrile (%)
0	2.0	100	0
5	2.0	100	0
20	2.0	50	50
40	2.0	30	70
50	2.0	25	75
60	2.0	0	100
70	2.0	0	100
71	2.0	100	0
81	2.0	100	0
82	2.0	100	0

2.3.1. Activity assay

Ethoxyresorufin-*O*- and pentoxyresorufin-*O*-deethylase activity were measured according to the method of Prough et al. (1978). Reactions (1 ml, 37 °C) contained microsomal protein or purified protein in reconstituted system, 10 μM 7-ethoxyresorufin, or pentoxyresorufin, and NADPH regenerator System (10 mM glucose-6-phosphate, 1 mM $NADP^+$ and 0.5 U/ml glucose-6-phosphate dehydrogenase) in 0.1 M Tris–HCl buffers, pH 7.4. Resorufin fluorescence at different concentrations was used as standard. Fluorescence intensity was measured on a Hitachi F4500 spectrofluorimeter (Hitachi Instruments, Japan). NADPH cytochrome *P*450 reductase activity was measured by a spectrophotometric method described by Vrolijk et al. (1994) using cytochrome *c* as substrate. All these experiments were done at room temperature (25–28 °C) and assay conditions were optimized for liver microsomes or purified protein of *P. scrofa*.

In reconstituted studies, two types of vesicles were used; one contained only egg phosphatidylcholine, cholesterol and α-tocopherol in a ratio of 4:3:1 (this vesicle was called PC) and the other contained egg phosphatidylcholine, phosphatidylethanolamine and dioleoyl-L-α-phosphatidic acid, and α-tocopherol in a ratio of 2:1:1:0.07 (this vesicle was called PC/PE/PA). Synthetic lipid, cholesterol and α-tocopherol were diluted in chloroform and dried in nitrogen until the formation of a lipidic film. This film was put in a vacuum system for 2 h and completely dried. Samples were stored at −8 °C. This film was resuspended in 1.25 ml of 50 mM Tris–HCl buffer pH 7.4 and vortexed for 5 min. This suspension was separated into 250 μl aliquots and used to dilute the purified CYP1A to a concentration of 10.27 pmol for use in the EROD assay and 3.376 pmol for the NADPH cytochrome *P*450 reductase assay. These suspensions were mixed for 60 min at 4 °C (Kisselev et al., 2002).

2.3.2. Electrophoretic and immunochemical properties

Molecular weight estimations were performed by using polyacrylamide gel electrophoresis (12.5%) in the presence of sodium dodecyl sulfate in a discontinuous buffer system as described by Laemmli (1970) and silver stained (Blum et al., 1987). The presence of CYP1A homologue in hepatic microsomes of *P. scrofa* and in the purified fraction was detected by Western blot essentially as described by Towbin et al. (1979) using the monoclonal antibodies *P*450LM4b against trout liver (kindly provided by Dr. D.R. Buhler).

3. Results

3.1. Purification of CYP1A

Purification of CYP1A was done using Reverse Phase HPLC on a C18 column. A summary of results is shown in Table 2. The recovery of CYP1A was 9.4%, an increase of 10 times, compared to classic purification methods. This increase in recovery is a result of decreasing the waste material, since we are proposing the exclusion of some chromatographic steps in the purification process. The steps that are being excluded are solubilization of membrane lipids, use of more than one column and dialysis of the sample. These steps can be cut out of the purification process due to the use of a C18 column.

Acetonitrile is an apolar solvent used in the elution process of the C18 column. This solvent is responsible for the separation of membrane lipids and proteins. This process is possible because the concentration of acetonitrile necessary to elute the molecule is proportional to the hydrophobicity. Molecules that are highly hydrophobic need a high concentration of acetonitrile. The use of a nonlinear elution profile associated with the low rate of the analytical column (flow of 1.0 ml) increases the number of proteins eluted from the column and avoids two or more proteins being eluted in the same fraction. Another property of the C18 column is the capacity of desalting the sample at the beginning of process, which replaces the dialysis process (Villanueva et al., 2001).

Fig. 1A shows the chromatographic profile of liver microsomes of *P. scrofa* following the absorbance at 280 nm. It is possible to see that the salt (P_1) is the first fraction to be eluted, even in the absence of acetonitrile. Peaks P_2 and P_3 were eluted with low percentage of acetonitrile and represent proteins with low molecular weights as shown by SDS-PAGE (data not presented).

Two peaks were eluted with high concentration of acetonitrile. They were called UT_1 and UT_2, respectively. However, the UT_2 peak showed three small peaks together, which suggests the presence of isoforms, since their hydrophobic degrees are close. Following the absorbance of both peaks at 417 nm (Fig. 1B), we confirm the hypothesis that UT_2 is the major heme protein, suggesting that it is CYP1A, the main isoform of cytochrome *P*450 in the liver of fish.

UT_1 peak, eluted with 75% acetonitrile, suggesting a high degree of hydrophobicity, showed two bands in an SDS-PAGE (Fig. 3). One of them has an apparent molecular mass of 15,000 Da and corresponds to cytochrome *b*5. The

Table 2
Purification of CYP1A from *P. scrofa* liver microsomes

Fraction	Protein (mg/ml)	CYP (nmol/mg protein)	Recovery (%)
Crude microsomes	31.507 ± 5.67 (10)	3.58 ± 0.412* (6)	100
C18-HPLC	9.125 ± 0.593 (8)	0.3376 ± 21.91** (8)	9.43

The number enclosed in parenthesis shows the number of samples analyzed and the values are expressed as mean ± S.D.

*This protein value corresponds to total CYP.

**This value corresponds to CYP1A recovery after the purification in C18-HPLC.

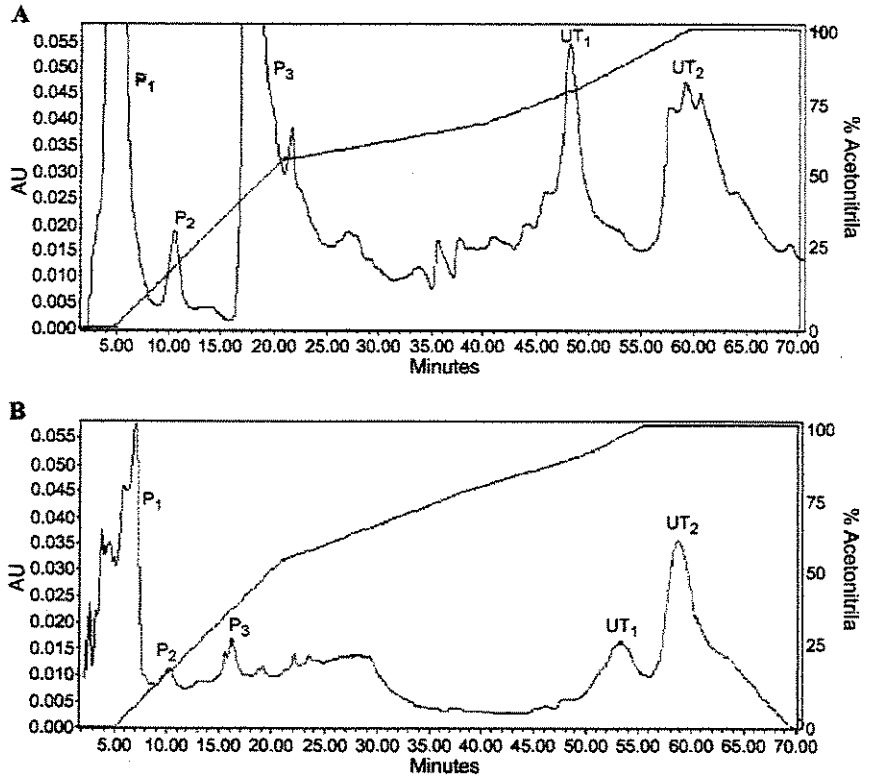

Fig. 1. (A) Elution profile of Reverse Phase HPLC-C18 following the absorbance at 280 nm. (B) Chromatographic profile following the absorbance at 417 nm. Microsomal protein (6.3 mg) was applied to the column. P_1-salt; P_2 and P_3-low molecular mass proteins not identified; UT_1-NADPH cytochrome *P*450 reductase and UT_2-CYP1A.

second band has an apparent molecular mass of around 70,000 Da, corresponding to NADPH cytochrome *P*450 reductase. This hypothesis that the UT_1 peak corresponds to the NADPH cytochrome *P*450 reductase was confirmed by the capacity of the enzyme to reduce cytochrome *c* in the presence of synthetic lipids (Fig. 2). The separation of this fraction is necessary since both cytochrome *b*5 and NADHP cytochrome *P*450 reductase are responsible for the transference of electrons to CYP1A during the catalysis (Bernhardt, 1995).

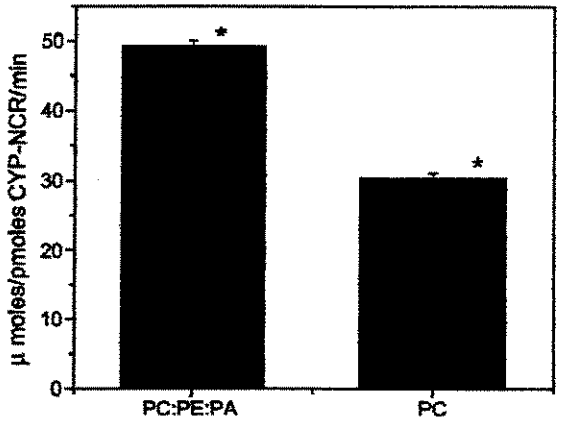

Fig. 2. Activity of purified NADPH cytochrome *P*450 reductase present in the UT_1 fraction encapsulated in two reconstituted systems; a negatively charged one (PC/PE/PA) and a neutral one (PC). Reaction was followed by the reduction of cytochrome *c*. The difference between the reconstituted systems was determined through ANOVA.

UT_2 peak was eluted with 95% acetonitrile; confirming that the protein has a hydrophobic character. Following the absorbance at 417 nm, we can see in Fig. 1B that the three peaks that are present in UT_2, disappear at 280 nm, confirming that they are isoforms of the same protein; however, the isoforms cannot be separated in an SDS-PAGE because their molecular masses are very close.

3.2. Electrophoretic and immunochemical properties

As shown in Fig. 3, a protein band with molecular mass around 58,000 Da was observed when the UT_2 peak was subjected to an SDS-PAGE. In addition, Western blot analysis using the LM4b anti-trout CYP1A1 also produced a single band which corresponded to a silver stained protein band on SDS-PAGE (Fig. 4), showing that CYP1A of *P. scrofa* is an orthologue of trout CYP1A1 (Sen and Arinç, 1997, 1998; Bárány et al., 1998; Buhler and Wang-Buhler, 1998). Since it was not possible to separate both peaks obtained in UT_2 in an SDS-PAGE, we believe that it could represent CYP1A3 that could also be an orthologue of trout CYP1A3.

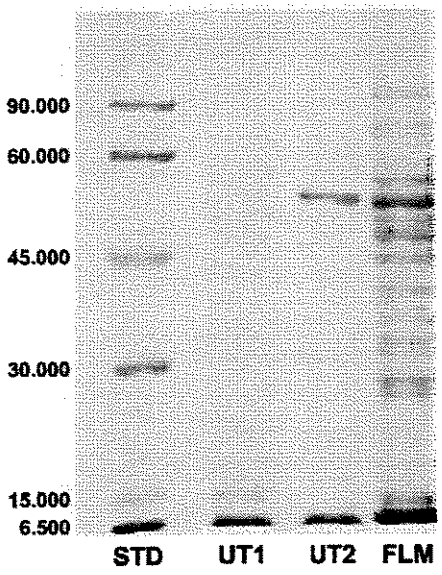

Fig. 3. SDS-PAGE of purified *P. scrofa* liver CYP1A. Samples were prepared as described by Laemmli (1970). Molecular masses are listed on the left. UT_1-NADPH cytochrome *P*450 reductase (70,000 Da) and cytochrome *b*5 (16,000); UT_2-CYP1A purified (58,000 Da); FLM-crude microsome. SDS-PAGE was silver stained.

3.3. Activity of purified P. Scrofa liver microsomal CYP1A

Biocatalyst activity of CYP1A purified from *P. scrofa* was measured by its ability to catalyze various monooxygenase reactions in the reconstituted system containing NADPH cytochrome *P*450 reductase and synthetic lipids. NADPH cytochrome *P*450 reductase was purified using a C18 HPLC column and was identified in the peak UT_1 by its ability to oxidize cytochrome *c* in a reconstituted system with synthetic lipids (Fig. 2).

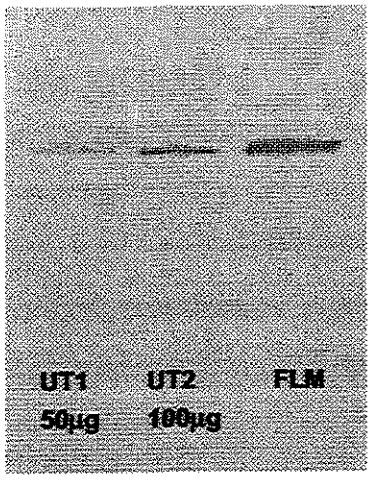

Fig. 4. Western blot analysis of sample obtained of Reverse Phase HPLC-C18. Immunochemical detection of CYP1A was accomplished by the use of antibody LM4b raised against purified trout CYP1A. UT_2 (50 µg) and UT_2 (100 µg) represent purified CYP1A; FLM represents crude microsomes before purification.

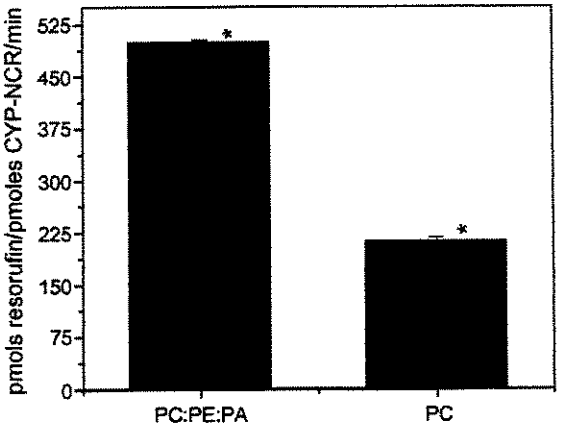

Fig. 5. Activity of purified CYP1A present in the UT_2 fraction and encapsulated in two reconstituted systems; a negatively charge one (PC/PE/PA) and a neutral one (PC). Reaction was followed through the deethylation of 7-ethoxyresorufin. The difference between the reconstituted systems was determined by ANOVA. Measured fluorescence in the fraction UT_1 and deethylation of 7-pentoxyresorufin in fraction UT_2 was between 0.005 and 0.01, which was at the lowest detection limit of the fluorimeter and was equal to zero times the blank value. EROD activity was established through a standard curve of resorufin and the difference between the two reconstituted systems was determined by ANOVA.

Fig. 5 shows the biocatalyst activity of purified CYP1A of *P. scrofa* towards two types of reconstituted systems, a neutral one, that is constituted only by egg phosphatidylcholine (PC) and a negatively charged one, that is constituted by egg phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid (PC/PE/PA). It was clearly shown that CYP1A does not catalyze the *O*-deethylation of pentoxyresorufin, since the fluorescence intensity that was recorded was equal to zero times the blank value. On the other hand, it does catalyze the *O*-deethylation of ethoxyresorufin at high rates, which is the chief and prototype substrate for CYP1A homologues.

Figs. 2 and 5 show an increase in the activity of NADPH cytochrome *P*450 reductase and EROD when the fractions UT_1 (reductase) and UT_2 (CYP1A) were encapsulated in negatively charged vesicles (49.18 ± 0.88 µmol/pmol NCR/min and 499.53 ± 3.42 µmol/pmol CYP1A-NCR/min, respectively). In neutral vesicles, the activity of NADPH cytochrome *P*450 reductase and EROD were 30.32 µmol/pmol NCR/min and 212.42 ± 5.45 µmol/pmol CYP1A-NCR/min, respectively. Those results show that the activity of CYP is enhanced by the negative charge of lipids, confirming that CYP activity can be regulated at the level of membrane lipids (De Pierre and Ernster, 1977; Bernhardt, 1995).

4. Discussion

The new method of CYP1A purification described here shows that the use of the C18 column in conjunction with Reverse Phase HPLC is an effective method to purify

CYP1A and NADPH cytochrome *P*450 reductase in different fractions in only one chromatographic step. These proteins that constitute the smooth endoplasmic reticulum are characterized by a hydrophobic region responsible for their anchorage to the membrane, which allowed us to separate them using a hydrophobicity column and following the elution profile at 280 and at 417 nm for heme proteins.

Since those cytochromes *P*450 are membrane proteins with different molecular masses depending on the isoforms and the degree of aggregation, their purification could be affected by different chromatographic conditions such as ionic strength of the elution buffer, pH and concentration of surfactant used in the membrane solubilization. All those factors make it difficult to reproduce the process in other laboratories (Funae and Imaoka, 1985). The use of a chromatographic column associated with HPLC can diminish those problems due to the automation of the equipment, allowing the reproducibility of the CYP1A elution profile.

The traditional techniques of protein purification include membrane solubilization with non-ionic surfactants and further surfactant elution using buffers with different ionic strengths (Haugen et al., 1975; Guengerich, 1977; Roos, 1996; Sen and Arinç, 1997, 1998; le Maire et al., 2000). Polyethyleneglycol (PEG) is the most common surfactant used in the purification and one of the consequences of this is an increase of CYP1A molecular mass in SDS-PAGE through the interaction of PEG and CYP1A that decreases the electrophoresis mobility of CYP1A (Nakhgevany et al., 1996).

Nakhgevany et al. (1996) shows that this interaction does not affect the biocatalyst activity of CYP1A, even if the surfactant is associated to the heme group. Many authors have shown that the interaction of non-ionic surfactants with the active site of CYP1A leads to the inactivation of the cytochrome through the process of alkylation of the heme site (Lee et al., 1996; Yun et al., 1996, 1997; Guengerich et al., 1998; Hosea and Guengerich, 1998). Our method of purification excludes the necessity of surfactant and, consequently, increases the recovery of catalytically active CYP1A.

The use of Reverse Phase HPLC associated with a hydrophobic column is not common in the purification of CYP (Kotake and Funae, 1980; Roos, 1996; Buhler and Wang-Buhler, 1998). Roos (1996), in a review work about CYP purification, showed that many authors had a homogeneous preparation of CYP in only one chromatographic step using a phenyl-Sepharose column with a sample of microsomes that had been precipitated with ammonium sulphate and solubilized with Lubrol 1%. However, there was a decrease of catalytically activity CYP due to the presence of Lubrol. One of the biggest problems of the use of a chromatographic column could be the elution of CYP and cytochrome *b*5 in the same fraction, since both proteins could link to the column with the same affinity (Roos, 1996). In our work, we obtained CYP, NADPH cytochrome *P*450 reductase and cytochrome *b*5 in different fractions (UT_2 and UT_1), through the use of a nonlinear gradient along with an analytical column.

The nonlinear gradient of acetonitrile in the elution of protein allowed the separation of three proteins in UT_2 peak. The analysis of electrophoresis properties showed that they migrate as a single band of 58,000 Da—the molecular mass of CYP1A (Guengerich, 1977), suggesting that they correspond to CYP1As that are genetically polymorphic; an event that had already been reported for fish (Buhler and Wang-Buhler, 1998; Sen and Arinç, 1998; Raucy and Allen, 2001) or the isoforms CYP1A1 and CYP1A3. CYP1A was identified through its strong immunochemical cross-reactivity with LM4b, an antibody raised against CYP1A of trout, showing that the CYP1A of *P. scrofa* contains the most conserved region of CYP1A proteins.

The results also show that perhaps the acetonitrile is a solvent that can cause some alteration in the structure of proteins, as when UT_1 and UT_2 are reconstituted in lipids vesicles, they recover their native structure and catalytic activity. This recovery was possible because (i) the interaction between CYP1A and NADHP cytochrome *P*450 reductase was intensified since they belong to the same organism (Buhler and Wang-Buhler, 1998; Sen and Arinç, 1998). However, further studies are necessary to elucidate the molecular basis of this species-specific interaction; (ii) interaction of CYP1A and NADPH cytochrome *P*450 reductase was stimulated by lipids that are not in the bilayer (Imaoka et al., 1992; Kisselev et al., 2002) and (iii) interaction of CYP1A and the substrate (7-ethoxyresorufin).

Smooth endoplasmic reticulum is composed of 60–70% protein and 30–40% lipids. Lipids (55% phosphatidylcholine, 20–25% phosphatidylethanolamine; 5–10% phosphatidylserine, 5–10% phosphatidylinositol and 4–7% sphingolipid) in a ratio of 1:200 (CYP/lipid) will stimulate an interaction between CYP1A and NADPH cytochrome *P*450 reductase (De Pierre and Ernster, 1977; Bernhardt, 1995). Our results show that lipids could work as regulators of CYP activity at two levels: (a) they stabilize the catalytically active conformation of CYP and reductase and (b) they modulate the electron transference between CYP and reductase through the negative charge of phosphatidylethanolamine and phosphatidic acid (Imaoka et al., 1992; Bernhardt, 1995; Yun et al., 1997; Kisselev et al., 2002).

The necessity of negatively charged lipids is related to the conformational change that occurs in CYP1A when the substrate interacts with the active site of the protein. Many authors have shown, through circular dichroism, that those alterations that occur in the high and low spin of heme-iron of CYP1A in the presence of negatively charged lipids are associated with the an increase of the α-helix and a decrease of the β-sheet. All those changes are influenced by phosphatidylethanolamine, but not by phosphatidylcholine, explaining the increase of CYP1A activity in the negatively charged vesicle (Bernhardt, 1995; Yun et al., 1997).

Our results describe that the use of HPLC-C18 can be a useful method to purify CYP1A. However, further studies are being conducted associating 2D-PAGE and MALDI-TOF mass spectrometry in order to elucidate if the CYP1A of *P. scrofa* is divided into different isoforms, since we could identify in the chromatographic profile that the peak that corresponds to CYP1A is comprised of three different small peaks which strongly suggests the presence of, at least, the isoforms CYP1A1 and CYP1A3.

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References

Bárány, M., Bárány, K., Giometti, C.S., 1998. Gel electrophoresis for studying biological function. Anal. Chim. Acta 372, 33–66.

Bernhardt, R., 1995. Cytochrome *P*450: structure, function, and generation of reactive oxygen species. Rev. Physiol., Biochem. Pharmacol. 127, 137–221.

Blum, H., Beier, H., Gross, H.J., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93–99.

Buhler, D.R., Rasmusson, M.E., 1968. The oxidation of drugs by fishes. Comp. Biochem. Physiol. 25, 223–239.

Buhler, D.R., Wang-Buhler, J.L., 1998. Rainbow trout cytochrome *P*450s: purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. Comp. Biochem. Physiol. C121, 107–137.

De Pierre, J.W., Ernster, L., 1977. Enzyme topology of intracellular membranes. Ann. Rev. Biochem. 46, 201–262.

Fernandes, M.N., Barrionuevo, W.R., Rantin, F.T., 1995. Effects of the thermal stress on respiratory responses to hypoxia of a South American Prochilodontide fish, *Prochilodus scrofa*. J. Fish Biol. 46, 123–133.

Funae, Y., Imaoka, S., 1985. Simultaneous purification of multiples forms of rat liver microsomal cytochrome *P*-450 by high-performance liquid chromatography. Biochim. Biophys. Acta 842, 119–132.

Godoy, M.P., 1975. Peixes do Brasil-Subordem Characiforme, 1st ed. Editora Franciscana, Piracicaba, SP.

Guengerich, F.P., 1977. Separation and purification of multiple forms of microsomal cytochrome *P*450. Activities of different forms of cytochrome *P*450 towards several compounds of environmental interest. J. Biol. Chem. 252, 3970–3979.

Guengerich, F.P., Hosea, N.A., Parikh, A., Bell-Parikh, C., Johnson, W.W., Gillam, E.M.J., Shimada, T., 1998. Twenty years of biochemistry of human *P*450. Purification, expression, mechanism and relevance to drugs. Drug Metab. Dispos. 26, 1175–1178.

Haugen, D.A., Van Der Hoeven, T.A., Coon, M.J., 1975. Purified liver microsomal cytochrome *P*450. J. Biol. Chem. 250, 3567–3570.

Hosea, N.A., Guengerich, F.P., 1998. Oxidation of nonionic detergents by cytochrome *P*450 enzymes. Arch. Biochem. Biophys. 353, 365–373.

Imaoka, S., Imai, Y., Shimada, T., Funae, Y., 1992. Role of phospholipids in reconstituted cytochrome *P*4503A form and mechanism of their activation of catalytic activity. Biochemistry 31, 6063–6069.

Kisselev, P., Schwarz, D., Platt, K.L., Schunk, W.H., Roots, I., 2002. Epoxidation of benzo[*a*]pyrene-7,8-dihydrodiol by human CYP1A1 in reconstituted membranes. Effects of charge and nonbilayer phase propensity of the membrane. Eur. J. Biochem. 269, 1799–1805.

Klotz, A.V., Stegeman, J.J., Walsh, C., 1983. An aryl hydrocarbon hydroxylating cytochrome *P*450 from the marine fish *Stenotomus chrysops*. Arch. Biochem. Biophys. 226, 578–592.

Kotake, A.N., Funae, Y., 1980. High-performance liquid chromatography technique for resolving multiples forms of hepatic membrane-bound cytochrome *P*450. Proc. Natl. Acad. Sci. U. S. A. 77, 6473–6475.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lee, P.C., Patra, S.C., Stelloh, C.T., Lee, W., Struve, W., 1996. Interaction of nonylphenol and hepatic CYP1A in rats. Biochem. Pharmacol. 52, 885–889.

le Maire, M., Champeil, P., Møller, J.V., 2000. Interaction of membrane proteins and lipids with solubilizing detergents. Biochim. Biophys. Acta 1508, 86–111.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Morrison, H.G., Oleksiat, M.F., Cornell, N.W., Sogin, M.L., Stegeman, J.J., 1995. Interaction of cytochrome *P*4501A (CYP1A) genes from two teleost fish, toadfish (*Opsanus tau*) and scup (*Stenotomis chrysops*) and phylogenetic analysis of CYP1A genes. Biochem. J. 308, 97–104.

Morrison, H.G., Weil, E.J., Karchner, S.I., Sogin, M.L., Stegeman, J.J., 1998. Molecular cloning of CYP1A from the estuarine fish *Fundulus heteroclitus* and phylogenetic analysis if CYP1 genes: update with new sequences. Comp. Biochem. Physiol. C121, 231–240.

Nakhgevany, R., Bonsu, O., Barber, J., Mabrouk, P.A., Ueng, Y.F., Bell, L.C., Guengerich, F.P., 1996. Preparation, purification, and spectrophotometric characterization of cytochrome *P*4501A2 conjugated with polyethyleneglycol monomethyl ether. Biochem. Biophys. Res. Commun. 222, 406–409.

Omura, T., 1999. Forty years of cytochrome *P*450. Biochem. Biophys Res. Commun. 266, 690–698.

Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its heme protein nature. J. Biol. Chem. 239, 2370–2378.

Prough, R.A., Burke, M.D., Mayer, B.T., 1978. Direct fluorimetric methods for measuring mixed-function oxidase activity. Methods Enzymol. 52, 374–377.

Raucy, J.L., Allen, S.W., 2001. Recent advances in *P*450 research. Pharmacogenetics J. 1, 178–186.

Roos, P.H., 1996. Chromatographic separation and behavior of microsomal cytochrome *P*450 and *b*5. J. Chromatogr. 684, 107–131.

Ryan, D.E., Levin, W., 1990. Purification and characterization of hepatic microsomal cytochrome *P*-450. Pharmacol. Ther. 45, 153–239.

Sen, A., Arinç, E., 1997. Separation of three *P*450 isoenzymes from liver microsomes of gilthead seabream treated with β-NF and partial purification of cytochrome *P*4501A1. Biochem. Mol. Biol. Int. 41, 131–141.

Sen, A., Arinç, E., 1998. Preparation of highly purified cytochrome *P*4501A1 from leaping mullet (*Liza saliens*) liver microsomes and its biocatalyst, molecular and immunochemical properties. Comp. Biochem. Physiol. C121, 249–265.

Stegeman, J.J., Lech, J.J., 1991. Cytochrome *P*450 monoxygenase system in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ. Health Perspect. 90, 101–109.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of protein from polyacrylamide gel to nitrocellulose sheets: procedure and some application. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.

Villanueva, J., Canals, F., Querol, E., Avilés, F.X., 2001. Monitoring the expression and purification of recombinant proteins by MALDI-TOF mass spectrometry. Enzyme Microb. Technol. 29, 99–103.

Vrolijk, N.H., Targett, N.M., Woodin, B.R., Stegeman, J.J., 1994. Toxicological and ecological implication of biotransformation enzymes in tropical teleost *Chaetodon capistratus*. Mar. Biol. 119, 151–158.

Yun, C.H., Songs, M., Ahn, T., Kim, H., 1996. Conformational changes of cytochrome *P*4501A2 induced by sodium chloride. J. Biol. Chem. 271, 31312–31316.

Yun, C.H., Songs, M., Kim, H., 1997. Conformational changes of cytochrome *P*4501A2 induced by phospholipids and detergents. J. Biol. Chem. 272, 19725–19730.

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Table 2

Purification of CYP1A from *P.scrofa* liver microsomes

Fraction	Protein (mg/ml)	CYP (nmol/mg protein)	Recovery (%)
Crude Microsomes	31.507 $\pm$ 5.67 (10)	3.58 $\pm$ 0.412* (6)	100
C18 – HPLC	9.125 $\pm$ 0.593 (8)	0.3376 $\pm$ 0.02191** (8)	9.43

The number enclosed in parenthesis shows the number of samples analyzed and the values are expressed as mean $\pm$ S.D.

* This protein value corresponds to total CYP.

** This value corresponds to CYP1A recovery after the purification in C18 - HPLC

3.3 - USE OF 2D – ELECTROPHORESIS AND MALDI – TOF MASS SPECTROMETRY IN THE IDENTIFICATION OF HEPATIC CYP1A ISOFORMS OF *Prochilodus scrofa*, A BRAZILIAN FRESHWATER FISH.

USE OF 2D – ELECTROPHORESIS AND MALDI – TOF MASS SPECTROMETRY IN THE IDENTIFICATION OF HEPATIC CYP1A ISOFORMS OF Prochilodus scrofa, A BRAZILIAN FRESHWATER FISH.

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ABSTRACT

Cytochromes P450 constitute a superfamily of the phase I enzymes whose primary task is the detoxification of both endogenous and xenobiotic compounds. Fish, among the non-mammalian species, receive great interest because they are direct food source for humans as well as conveyors of toxic chemicals to human beings. The aim of the present study is the identification of hepatic isoforms of CYP1A in Prochilodus scrofa, a Brazilian fish belonging the family Prochilodontidae and more commonly known as Curimbatá. Purification of CYP1A was performed by Reverse Phase HPLC on a C18 column. The fractions collected were applied in 2D-PAGE and three major spots were detected. These spots had the same molecular weight, but presented pI in a range of 5.5 to 6.0, suggesting the presence of 3 isoforms of CYP1. Spots were collected, and treated with Trypsin and the resultant peptides were measured by MALDI-TOF Mass Spectrometry. Tryptic peptide mass fingerprint of CYP1A showed the presence of 10 masses that matched the expected tryptic peptides obtained through theory digestion of the database sequence. These values corresponded to 31% of the translated amino acids of CYP1A family of other fishes and mammals. All spots had the same profile of fragmentation, which confirms that they are isoforms. Among these peptides, two demonstrated major importance T3, which is a conservative domain of CYP1A and T2, which could represent a more conservative region that occurs only in fishes. All these characteristics strongly suggest that this new procedure efficiently identifies, simultaneously, different isoforms of hepatic CYP1A from P. scrofa and its conservative region of protein.

INTRODUCTION

The hepatic cytochrome P450 (CYP) is responsible for the oxidative metabolism of structurally unrelated compounds, including both xenobiotic and endogenous compounds. This system plays different roles in different organisms, for example it converts lipophylic compounds into more soluble compounds, and can convert certain carcinogenic compounds into more toxic products that bind covalently to macromolecules such as DNA and proteins and metabolize numerous endogenous compounds, including steroids, fatty acids, vitamins, leukotrienes and others [1 – 4].

CYPs are found in both prokaryotic and eukaryotic organisms and are divided into 281 families (http://drnelson.utmem.edu/CytochromeP450.html). Among them, the CYP1A family form monooxygenases that are the main proteins responsible for the detoxification and activation of xenobiotic compounds in fish [5-6].

The CYP system in fish is structurally similar to that of mammals, but some differences are found; for example, enzyme activity of fish liver microsomes are lower than in mammals and the regulation of CYP may be performed by exogenous factors [2, 7-8]. In recent years, research on fish CYP1A has increased in an attempt to provide data for the biomonitoring of pollutants metabolism.

Current research to identify the different isoforms of CYP employs specific inhibitors or substrates, antibody-based identification and mRNA – based analysis. However, this kind of identification can limit the number of isoforms. Tryptic peptide mass fingerprinting (PMF) in conjunction with MALDI – TOF mass spectrometry has become one of the most important analytical tools in protein

identification [9-10]. This increase in interest in this technique is the consequence of the ability to analyze picomoles quantities of protein separated in gel, or by HPLC, in a short time [10 -13]. The use of PMF and MALDI – TOF are important since they (a) allow the identification of several peptides in one protein and (b) the identification of variation in the amino acid sequence in different CYP isoforms.

Based on these data, the aim of the present study is the identification of hepatic isoforms of the CYP1A family, in Brazilian fish, using 2D - PAGE electrophoresis associated with MALDI- TOF analysis by PMF.

Prochilodus scrofa, (Curimbatá), is a Brazilian tropical fish belonging to the Prochilodontidae family. Curimbatás were chosen due to their: (a) seasonal migratory behavior for reproductive purposes [14]; which puts them in contact with pollutants distributed in different parts of the water column, (b) high tolerance to hypoxia, exhibiting critical oxygen consumption when oxygen levels drop [15], (c) being detritivorus fish, meaning that the fish are in contact with xenobiotics that interact with algaes that grow upon stone and in sediment. For these reasons, Curimbatá may be considered a model for ecotoxicology studies, since xenobiotics are able to act upon any of these characteristics.

2 - MATERIAL AND METHODS

2.1 - MATERIAL

All reagents were of the highest purity. DTT, iodoacetamide, sodium phosphate (dibasic) were purchased from Sigma Chemical Co. (St. Louis, MO); trifluoroacetic acid (TFA) from Fluka Chemical Corp. (Milwaukee, WI); modified trypsin, clostripain from Promega, Madison, WI, U.S.A. Ammonium acetate,

ammonium bicarbonate, sodium phosphate (monobasic), glacial acetic acid, acetonitrile, and methanol were acquired from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All solutions were prepared with the in-house reverse osmosis purified water. All other reagents and solvents were of analytical or HPLC grade.

2.2 - FISH

Twenty-four male and female Prochilodus scrofa fish (Steindachnner, 1881) (Pisces, Chariciforme, Prochilodontidae), out of the reproductive cycle, were obtained from the Center of Aquiculture Research and Training (CEPTA, Pirassununga, SP, Brazil). The fish collected were of comparable weigh (300 – 500g) and the monooxygenase activity was induced with BHT (butylhydroxidetoluene) in a dose of 200mg/kg diluted in corn oil. The animals were raised in artificial open-air reservoirs of pollutant-free water. After seven days, fish were anesthetized with spike oil and sacrificed by decapitation. Livers were removed and washed with saline solution. Microsomes were prepared in pools of 4-5 livers.

2.3 - MICROSOME PREPARATION

Microsomal fractions were prepared by the standard procedure: tissues were homogenized in ice-cold 0.1M potassium phosphate, pH 7.5, containing 0.15M KCl and 0.1mM EDTA. Crude homogenate was centrifuged at 12,000Xg for 20 min and the supernatant centrifuged at 105,000Xg for 60 min. The pellet was re-suspended in potassium buffer and re-centrifuged at 105,000Xg for 60 min.

Microsomes were isolated in the pellet. The microsomal fraction was re-suspended in the same buffer solution containing 20% glycerol and frozen at –70ºC [16]. Proteins were determined according to the Lowry method [17]. CYP content was assayed in microsomes according to the method of Omura and Sato [18], and expressed as nmol/mg protein on the basis of the millimolar extinction coefficient of 91 mM^{-1} cm^{-1}.

2.4 – PURIFICATION OF CYTOCHROME P4501A

The crude microsomes were diluted until a concentration of 5 mg/mL with 0.1M Potassium Phosphate buffer pH7.4 and applied in a Reverse – Phase HPLC on a C18 column using acetonitrile as elution buffer. The elution profile was non-linear and was followed through the absorbance at 280 nm (total proteins) and 418 nm (hemeprotein). Each peak was colleted and lyophilized for further procedures and stored at -8ºC and re-suspend in 50mM Tris-HCl buffer pH 7.4 at the time of use. All procedures were carried out at 0 – 4ºC as soon as possible. The protein concentration of purified samples was measured according to the method of Lowry et al. [17]. Molecular weight estimations were performed by polyacrylamide gel electrophoresis (12.5%) in the presence of sodium duodecyl sulfate in a discontinuous buffer system, as described by Laemmli [19]. The presence of CYP1A homologue in hepatic microsomes of P. scrofa and in the purified fraction was detected by Western blot essentially as described by Towbin et al. [20] using the P450LM4b monoclonal antibody against trout liver (kindly provide by Dr. D. R. Buhler).

2.5 – TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (2D – PAGE)

Approximately 50μg of CYP1A was added to 340 μL of sample buffer containing 8M urea, 4% w/v, CHAPS 2% v/v, carrier ampholytes pH 3-10, 70 mM DTT, and 0.001% w/v bromophenol blue (all reagents were from Amersham Biosciences - Uppsala - Sweden, except for DTT, which was from Biorad). The samples were applied to IPG gel strips with a pH 3-10 nonlinear separation range (catalog number 17-1235-01, Amersham Biosciences - Uppsala - Sweden). After a 10-hour rehydration, isoelectric focusing was performed at 20ºC for 1 hour at 500V, 1 hour at 1000 V, and 10 hours at 8000V in an IPGphor apparatus (Amersham Biosciences - Uppsala - Sweden). The limiting current was 50 μA per strip. Strips were subjected to reduction and alkylation steps to second dimension. Strips were soaked for 8 minutes in a solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% v/v, glycerol, 2% w/v, SDS, and 2% w/v DTT, followed by an additional 12 minutes in the same solvent containing 2.5% w/v iodoacetamide instead of DTT.

Second dimension electrophoresis (SDS-PAGE) was performed with an SE-600 system connected to a Multitemp II refrigerating system (Amersham Biosciences - Uppsala - Sweden). After laying the strip on the top of a 12.5% polyacrylamide gel and sealing it with agarose, the gels were run for 1 hour at 60 V, after which constant amperage of 30 mA per gel was applied until the migration front reached the lower end of the gel. Proteins were detected by Comassie Blue staining.

2.6 – IN-GEL ENZYMATIC DIGESTIONS

Spots A, B and C resulting from the 2D electrophoresis were excised from the gel. Peptides were generated and extracted from the gel-separated proteins following established in-gel digestion protocols using sequencing grade modified trypsin (Madison, WI, USA).

The digestions were carried out using trypsin as follows: gel slices were washed twice for 45 min at 37ºC in 0.5 ml of 200mM NH_4HCO_3/50% (v/v) acetonitrile with agitation and shrunk in pure acetonitrile for 15 min. After removal of acetonitrile and drying under vacuum, the samples were reswollen with 40mM NH_4HCO_3 containing sequencing-grade modified trypsin at a ca. 10:1 molar ratio of the protein to trypsin (but not less than 6μg/ml trypsin).

The volume of buffer was ca. 1.5 times that of the excised gel band. After the absorption of trypsin, additional buffer (30–50μL) was used to cover the gel pieces to keep them wet during overnight digestion (16–18 h) at 37ºC.

After drying down, the samples were resuspended in 10 μL 0.1% aqueous TFA and cleaned with ZipTips (Millipore, Oxford, MA, USA) according to the protocol supplied by the manufacturer. The samples were diluted 1:200 before mixing the peptide solution with equal volumes of saturated matrix solution and spotting 2μL of the mixture onto the MALDI target plate (Micromass, Manchester, UK) for analysis.

2.7 - MALDI-TOF

The peptides were analyzed on a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Prior to their application to the sample plate the samples were purified using C18 Zip Tips (Millipore, Oxford, MA, USA), and eluted directly with a matrix solution of 2% w/v α-cyano-4-hydroxycinnamic acid (Sigma), 60% acetonitrile and 0.1% v/v TFA. Internal mass calibration was performed using trypsin auto digestion products (842.509, 1045.563 and 2211.104 Da).

2.8 – PROTEIN IDENTIFICATION

The SWISS-PROT database (Swiss Institute of Bioinformatic, http://us.expasy.org) and EMBL (Bioanalytical Research Group, http://www.mann.embl-heidelberg.de/GroupPages/) was used to identify the protein. The parameters used in the search were as follows: peptide mass tolerance 25Da, 1 missed cleavage, carboxymethylated cystein, 9 peptides used in the search and 5 peptides requires for match.

3 – RESULTS

Results of 2D separation of possible isoforms of CYP1A are presented in Figure 1. Our particular attention was focused on the spot between 58,000Da and 59,000 Da. In this case, only three spots were shown in the 2-DE pattern, since a sample of purified protein was used; they show the same molecular weight and pI average of between 5.5 and 6.0. Analysis of spectrum of spots A, B and C

demonstrated that the same fingerprinting was generated after trypsin digestion and confirms that they are isoforms of CYP1A.

(FIGURE 1)

Differential identification of CYP homologues using MALDI – TOF is greatly dependent on the achievement of maximal sequence coverage. To this end, we compared peptides patterns of spots A, B and C. The results of peptide mass fingerprinting of Prochilodus scrofa obtained by trypsin digestion are shown in Table 1. An expanded peptide mass fingerprint view of spot A is presented in Figure 2. No significance differences in the peptide profiles of spots A, B and C were found

(TABLE 1)

(FIGURE 2)

MALDI-TOF detected 19 masses of resultant peptides from protein digestion. Ten of these peptide masses matched with another theoretical peptide mass from CYP1A digest of other fishes and mammalian species.

The mass difference among these peptides was lower than 25Da. Table 2 shows some species with similar patterns of theoretical digestion. The values

found in these 10 peaks demonstrate 31% of the translated amino acids found adjust to the CYP1A sequence in different fish species.

(TABLE 2)

The T3 peptide has a mass with 36 units lower than the theoretical mass of CYP1A (Table 1). This region has the same amino acid composition of all species analyzed and corresponds to a conserved domain of all CYP1A isoforms in fish and mammals. The T2 peptide corresponds to the amino acids 44-73 and shows a high homology with CYP1A only in fish. It is a conserved sequence that may differentiate the CYP1A of fish and mammals. The T5, T6 and T8 Peptides are also present only in fish, but their degrees of homology are lower than the T2 peptide. Peptides T1, T4 and T7 are conserved regions in fish and mammals, but with a smaller degree of similarity than that of T3.

4 - DISCUSSION

2D-PAGE was first used to identify CYP at the beginning of the 1980's years in a study by Vlasuk and Walz [21-22] and more recently by Galeva and Altermann [23-24]. The results obtained by all these authors were not satisfactory, mainly due to the artifacts generated during the first dimension of the 2D-PAGE. These artifacts are the consequence of the influence of the lipids of the membrane bilayer upon protein charge. We, herein, demonstrate the successful employment

of this technique, since the sample was freed of lipids by the use of acetonitrile in the purification process.

CYP1As are expressed in varying amounts under different circumstances. The isoforms that are found in many species (CYP1A1, 1A2 and 1A3) differ very little in their amino acid sequences, catalytic properties and post – translational modifications and, as such, these differences can be detected only by sensitive methods such as silver stained 2D-PAGE [11, 23, 25]. The present study identifies the isoforms CYP1A1 and CYP1A3 (homologue to CYP1A2 in mammals) and a third spot that could represent a third isoform of the CYP1 family, CYP1B, which was recently described in fish that have the same catalytic properties and a high homology with the CYP1A family [26] – or an isoform caused by lack of post – translational modification [11]

In addition to the fact that many authors have unsatisfactorily analyzed the expression of CYP by 2D-PAGE, our results identifying CYP isoforms using 2D-PAGE could be considered useful, since they employ purified samples that are free of lipids. The use of traditional SDS - PAGE in the identification of isoforms of CYP1A in Prochilodus scrofa were not used by us, since the isoforms differed only in 21 amino acids, which not change their molecular weights.

Just one spot was expected in the 2D-PAGE studies, since two isoforms of CYP1A are described only for Salmonides [5-6]. Morrison [5-6] established that CYP1A1 and CYP1A2 in trout are not orthologous to the mammalian isoforms, since the duplication event in trout is a more recent event that of mammalian CYP1A duplication. As such, the terminology used to identify CYP1A isoforms are CYP1A1 and CYP1A3, however, CYP1A1, CYP1A2 and CYP1A3 contain the

same amino acid sequence at position 283-291 and 97% nucleotide identity [2, 5-6, 27].

Differences found suggest that this inconsistence in P450 isozymes composition might be due to some environmental or physiological conditions that are specific to each species.

Data obtained here, show that the use of proteomic analysis has a great potential in the rapid and accurate characterization and identification of CYP1A of purified or crude samples, when compared with others methodologies.

In conclusion, results presented herein, concerning Prochilodus scrofa, strengthen the evidence that multiple CYP1A proteins exist in some fish, implying orthology to other CYP1A in others species. The function and the origin of these genes require further elucidation through phylogenetic studies.

5 - BIBLIOGRAFY

[1] D.E. Ryan, W. Levin, Pharmac. Ther. 45 (1990) 153 - 239.

[2] D.R. Buhler, J.L. Wang-Buhler, Comp. Biochem. Physiol. 121C (1998) 107 - 137.

[3] T. Omura, Biochem. Biophys Res. Comm. 266 (1999) 690 - 698.

[4] J.L. Raucy, S.W. Allen, Pharmacogenetics J. 1 (2001) 178 - 186.

[5] H.G. Morrison, M.F. Oleksiak, N.W. Cornell, M.L. Sogin, J.J. Stegeman, Biochem. J. 308 (1995) 97 - 104.

[6] H.G Morrison, E.J. Weil, S.I. Karchneer, M.L. Sogin, J.J. Stegeman, Comp. Biochem. Physiol. 121C (1998) 231 - 240.

[7] A.V. Klotz, J. J. Stegeman, C. Walsh, Arch. Biochem. Biophys. 226(1983) 578 - 592.

[8] J.J. Stegeman, J.J. Lech, Environ Health Perspec. 90 (1991) 101 - 109.

[9] M. Mann, M. Wilm, TIBS 20 (1995) 219 - 224.

[10] M. Mann, R.C. Hendrickson, A. Pandey, Annu. Rev. Biochem. 70 (2001) 437 - 473.

[11] B. Kuster, M. Mann, Curr. Opinion Struct. Biol. 8 (1998) 393 - 400.

[12] P. Chaurand, F, Luetzenkirchen,. J. Am. Soc. Mass Spectrom. 10 (1999) 91 - 103.

[13] R. Bakhtiar, R.W. Nelson, Biochem. Pharmacol. 59 (2000) 891 - 905.

[14] M. P. Godoy, Peixes do Brasil – Subordem Charici forme, Editora Franciscana. Piracicaba, S.P , 1975.

[15] M. N. Fernandes, W. R. Barrionuevo, F. T. Rantin, J. Fish. Biol. 46 (1995) 123 - 133.

[16] N.H. Vrolijk, N. M. Targett, B.R. Woodin, J.J. Stegeman, Mar. Biol. 119 (1994) 151 - 158.

[17] O.H. Lowry, N.J Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265 - 275.

[18] T.Omura, R. Sato, J. Biol. Chem. 239 (1964) 2379 - 2385.

[19] U.K. Laemmli, Nature 227 (1970) 680 - 685.

[20] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350 - 4354.

[21] G.P. Vlasuk, F.G. Walz Jr, Anal. Biochem. 105 (1980) 112 - 120.

[22] G.P. Vlasuk, F.G. Walz Jr, Arch. Biochem. Bioph. 214 (1982) 248 – 259.

[23] N. Galeva, M. Altermann, Proteomics 2 (2002) 713 - 722.

[24] N.Galeva, D. Yakovlev, Y. Koen, T. Duzhak, M. Alterman, J. Drug Metab. Disp. 31(2003) 351 - 355.

[25] M.P. Molloy, Anal. Biochem. 280 (2000)1 - 10.

[26] M.J. Leaver, S.G. George, Gene 256 (2002) 83 - 91.

[27] C. M. I. Rabergh, N.H. Vrolijk, M.M. Lipsky, T.T. Chen, Toxicol. Appl. Pharmacol. 165 (2000) 195 – 205.

FIGURE AND TABLE CAPTION

Table 1 – Identification of CYP1A tryptic peptides

Table 2 – Species used in peptide identification.

Figure 1 – Bi – dimensional electrophoresis of purified CYP1A of Prochilodus scrofa.

Figure 2 - Peptide mass fingerprint of 2D – PAGE (spot – A) contained an isoform of CYP1A.

Figure 1

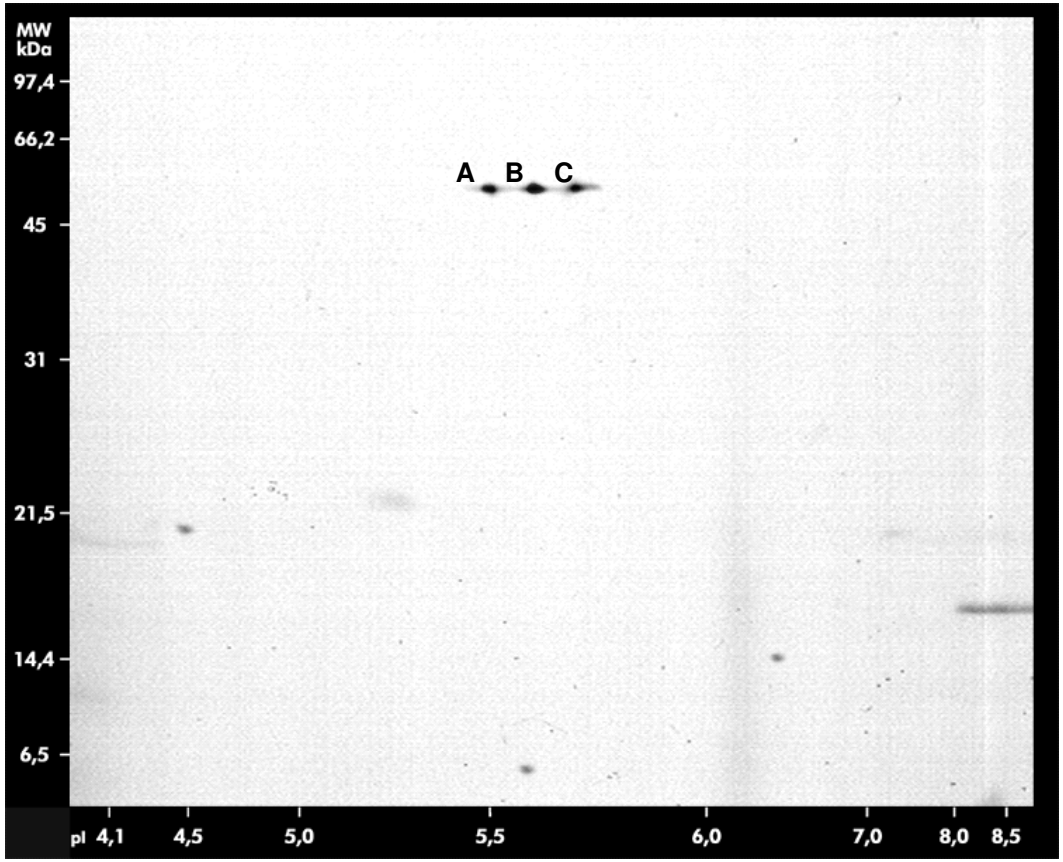

Table 1

Theor. AVG	Tryptic peptide	Sequence position (start – end)	Theoric Aminoacid sequence	AV observed (Spot A, B and C)
3457.1±61.26	T1	107-137	*GRPDLYTSTLITDGQSLTFSTDSGPVWAARR*	3354.9±1.002
3131.2±82.13	T2	44-73	QLPGPKPLPIIGNVLEVGSK PHLSLTAMSK	3103.03±0.648
2849.64±6.49	T3	74-98	RYGNVFQIQIGMRPVVVLSG NETVR	2813.50± 0.95
2372.5±13.53	T4	144-165	SFSIASDPASSTSCYLEEHVSK	2340.76±0.93
1804.90±0	T5	437-452	FLSCNGTEVNKQEGEK	1792.03±0.38
1577.7±14.05	T6	349-361	LYQELKENVGLDR	1585.89± 11.45
1376.11± 44.80	T7	33-44	SFQDKIPEGLSR	1385.64±0.32
1045.86±1.869	T8	428-436	EPSSFNPDR	1041.42±0.53
1016.868±0.97	T9	462-470	RCIGEVIAR	1016.9430±0.508
851.45	T10	453-460	CIGEVIAR	844.21±1.19

Figure 2

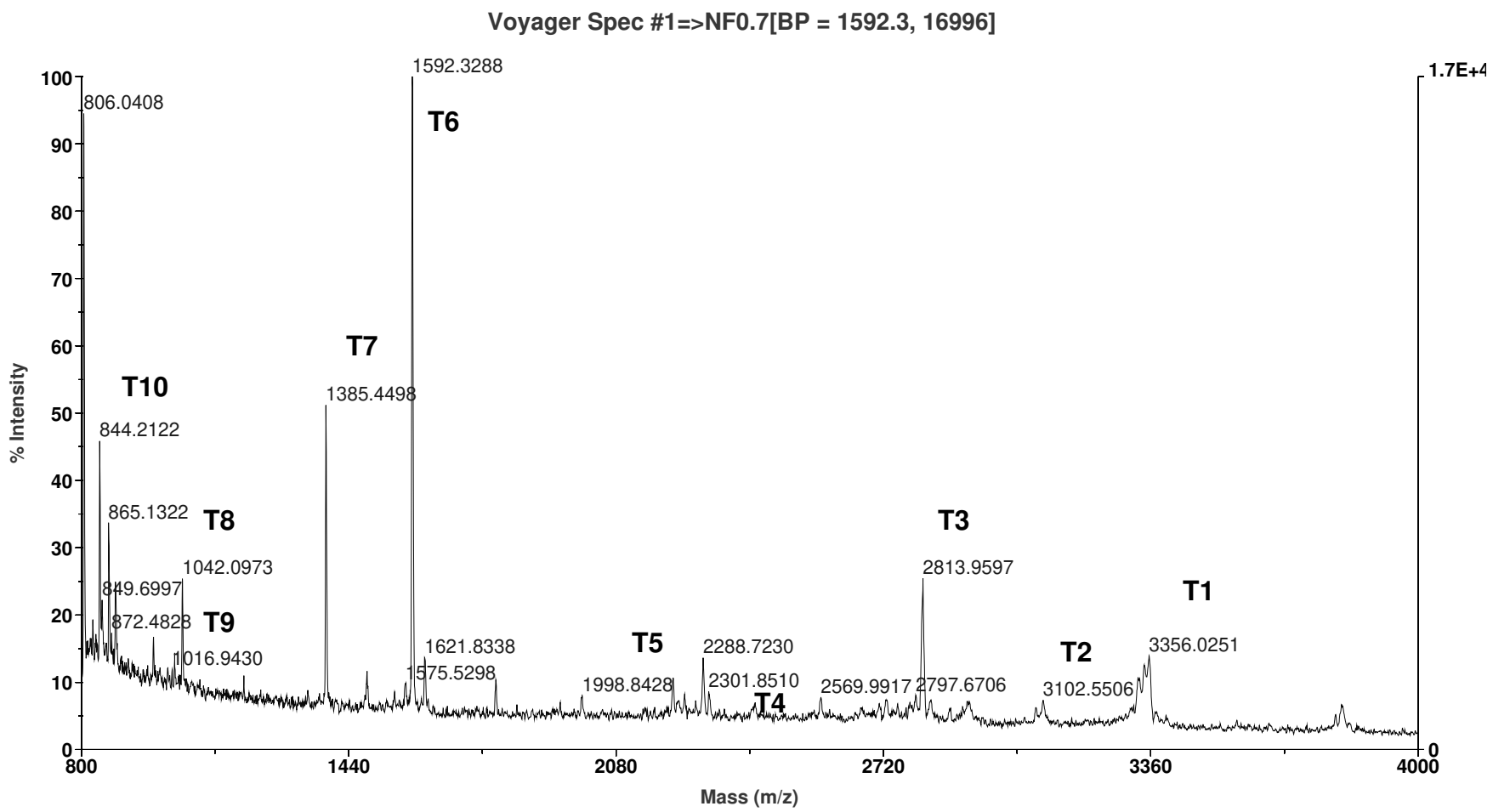
Voyager Spec #1=>NF0.7[BP = 1592.3, 16996]
% Intensity
Mass (m/z)
1.7E+4
0
100
90
80
70
60
50
40
30
20
10
0
800
1440
2080
2720
3360
4000
1592.3288
806.0408
T6
T7
T10
1385.4498
844.2122
865.1322
T8
T3
1042.0973
2813.9597
849.6997
T1
872.4828
T9
1621.8338
T5
2288.7230
T2
3356.0251
1016.9430
1575.5298
1998.8428
2301.8510
T4
2569.9917
2797.6706
3102.5506

Table 2

Specie	Acession number	CYP isoform	Number of pepetides with similar weight of P. scrofa peptides.
Chaetodon capistratus*	Q92039	1A1	8
Macaca fascicularis^{O}	P33616	1A1	8
Oryctolagus cuniculus^{O}	P00187	1A2	8
Mesocricetus auratus^{O}	P24453	1A2	8
Spauratus aurata*	O42457	1A1	8
Platichthy flesus*	Q9YH64	**1A1**	8
Limanda limanda*	O42430	1A1	8
Mesocritus auratus*	Q00557	1A1	8
Opsanus tau*	Q92095	1A1	8
Homo sapiens^{O}	P04798	1A1	7
Lisa saliens*	Q9W683	1A1	7
Oryctolagus cuniculus^{O}	P05176	1A1	7
Ovis aries^{O}	P56591	1A1	7
Oncorhynchus mykiss*	Q92109	1A1	7
Rattus norvegicus^{O}	P04799	1A2	6
Mus musculus^{O}	P00186	1A2	6
Homo sapiens^{O}	P05177	1A2	6
Cavia porcellus^{O}	Q64391	1A2	6
Pagrus major*	P98181	1A1	6
Cavia porcellus^{O}	Q06367	1A2	6
Dicentrarchus labrax*	P79716	1A1	5

*** fish species; ^{O} mammalian species**

3.4 - PROTEOMIC APPROCH TO IDENTIFY CYTOCHROME P450 ISOZYMES – A NEW TOOL TO ACCESS THE ENVIRONMENTAL POLUTTION.

PROTEOMIC APPROCH TO IDENTIFY CYTOCHROME P450 ISOZYMES – A NEW TOOL TO ACCESS THE ENVIRONMENTAL POLUTTION.

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Keyword – CYP, fish, MALDI -TOF, 2D – PAGE, isoforms

ABSTRACT

The cytochromes P450 (CYP) constitute a multigene family of enzymes playing a critical role in the oxidation of many endogenous and xenobiotic substrates. The CYP1 family is of particular interest in environmental toxicology because its members are dominant in the metabolism of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and aryl amines This system plays different roles in different organisms, for example it converts lipophylic compounds into more soluble compounds, and can convert certain carcinogenic compounds into more toxic products that bind covalently to macromolecules such as DNA and proteins. The objective of this study was identify the CYP isoenzymes that are expressed in hepatic microsomes of Curimbatá using one – dimensional (1 – DE) and two dimensional (2 – DE) gel electrophoresis followed by triptic peptide mapping (PMF). Both 1 –DE and 2 – DE showed that 3 – MC induces not only CYP1A1 and CYP1A3, but other biotransformation enzymes presents in endoplasmatic reticulum like cytochrome b5 (~15kDa), NADPH cytochrome P450 reductase (~75kDa) and microsomal Glutathione – S – Transferase (~20kDa); however, 2DE was more efficient as 1DE to separate microsomal enzymes with molecular weigh around 50,000 – 70,000Da. There were 25 proteins that appeared only in 3 – MC treated group and a matching of 38% between control and 3 – MC gel. Those results shows that 2 –DE, associated to PMF, is a tool that allowed us to observe the down/enhancer of biotransformation enzymes, which viable this technique to ecotoxicological studies. This new tool has a great potential to be utilized to identify CYP isoenzymes that are expressed in different environmental studies.

INTRODUCTION

The hepatic cytochrome P450 (CYP) is responsible for the oxidative metabolism of structurally unrelated compounds, including both xenobiotic and endogenous compounds. They are the key enzymes of the hepatic drug – metabolizing system (Omura, 1999, Benhardt, 1995).

CYPs are found in both prokaryotic and eukaryotic organisms and are divided into 281 families (http://drnelson.utmem.edu/CytochromeP450.html). Among them, the CYP1A family form monooxygenases that are the main proteins responsible for the detoxification and activation of xenobiotic compounds in fish (Morrison et al, 1995, Morrison et al, 1998).

The CYP system in fish is structurally similar to that of mammals, but some differences are found; for example, enzyme activity of fish liver microsomes are lower than in mammals and the regulation of CYP may be performed by exogenous factors such as temperature (Klotz et al. 1983, Stegeman and Lech, 1991, Omura, 1999). In recent years, research on fish CYP1A has increased in an attempt to provide data for the biomonitoring of pollutants metabolism.

Current research to identify the different isoforms of CYP employs specific inhibitors or substrates, antibody-based identification and mRNA – based analysis (Buhler and Wang-Buhler, 1998, Gokφyr and Husφy, 1998, Cousinou et al., 2000, Craft et al., 2001). However, this kind of identification can limit the number of isoforms. Gel electrophoresis in combination with peptide mass fingerprinting (PMF) in conjunction with MALDI – TOF mass spectrometry has become one of the most important analytical

tools in protein identification (Galeva e Altermann, 2002, Galeva et al., 2003, Sickmann et al., 2002, Bakhtiar e Nelson, 2000). The separation capacity of modern two-dimensional gel electrophoresis (2 – DE) is extremely high and allows the separation of more than 1,000 proteins on one gel. However, the method has a disadvantage: 2 – DE has a low performance in separation and analysis of membrane proteins, due to the propensity of hydrophobic proteins to aggregate (Ghaim et al, 1997, Molloy, 2000).

In this study we have used MALDI – TOF – based peptide mass fingerptinting (MLADI – TOF/PMF) to perform a direct identification of distinct proteins involved in the biotransformation process, among than the CYP isoforms and have shown that the identification of those enzymes by proteomic technique offers advantages over other methods and should become a new tool for identification of biomarkers of environmental pollution.

Prochilodus scrofa, (Curimbatá), is a Brazilian tropical fish belonging to the Prochilodontidae family. Curimbatás were chosen due to their: (a) seasonal migratory behavior for reproductive purposes; which puts them in contact with pollutants distributed in different parts of the water column, high tolerance to hypoxia, exhibiting critical oxygen consumption when oxygen levels drop, (c) being detritivorus fish, meaning that the fish are in contact with xenobiotics that interact with algaes that grow upon stone and in sediment (Godoy, 1975). For these reasons, Curimbatá may be considered a model for ecotoxicology studies, since xenobiotics are able to act upon any of these characteristics.

2 - MATERIAL AND METHODS

2.1 - MATERIAL

All reagents were of the highest purity. DTT, iodoacetamide, sodium phosphate (dibasic), 3 – metylclorantrene (3 - MC) were purchased from Sigma Chemical Co. (St. Louis, MO); trifluoroacetic acid (TFA) from Fluka Chemical Corp. (Milwaukee, WI); modified trypsin, clostripain from Promega, Madison, WI, U.S.A. Ammonium acetate, ammonium bicarbonate, sodium phosphate (monobasic), glacial acetic acid, acetonitrile, and methanol were acquired from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All solutions were prepared with the in-house reverse osmosis purified water. All other reagents and solvents were of analytical or HPLC grade.

2.2 - FISH

Twenty-four male and female Prochilodus scrofa fish (Steindachnner, 1881) (Pisces, Charici forme, Prochilodontidae), out of the reproductive cycle, were obtained from the Center of Aquiculture Research and Training (CEPTA, Pirassununga, SP, Brazil). The fish collected were of comparable weigh (300 – 500g) and were divided in two groups: (i) control group, that receive a intra peritoneal injection so corn oil and (ii) 3 – MC group, where the monooxygenase activity was induced with 3 – metylcloranthrene (3 –MC) in a dose of 150mg/kg diluted in corn oil. The animals were raised in artificial open-air reservoirs of pollutant-free water. After two hours, fish were anesthetized with spike oil and sacrificed by decapitation. Livers were removed and washed with saline solution. Microsomes were prepared in pools of 4-5 livers.

2.3 - MICROSOME PREPARATION

Microsomal fractions were prepared by the standard procedure: tissues were

homogenized in ice-cold 0.1M potassium phosphate, pH 7.5, containing 0.15M KCl and 0.1mM EDTA. Crude homogenate was centrifuged at 12,000Xg for 20 min and the supernatant centrifuged at 105,000Xg for 60 min. The pellet was re-suspended in potassium buffer and re-centrifuged at 105,000Xg for 60 min. Microsomes were isolated in the pellet. The microsomal fraction was re-suspended in the same buffer solution containing 20% glycerol and frozen at –70ºC (Vrolijk et al., 1994). Proteins were determined according to the Lowry method (1954). CYP content was assayed in microsomes according to the method of Omura and Sato (1969), and expressed as nmol/mg protein on the basis of the millimolar extinction coefficient of 91 mM^{-1} cm^{-1}.

2.4 – ONE DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (1- DE)

Samples of Prochilodus scrofa liver microsomes (100μg) were diluted with the denaturating buffer (70mM DTT, 2% SDS, 0.1M Tris – HCl pH6.8, 10% glycerol) and heated at 95ºC for 10 min. Samples were subjecting to SDS – PAGE using 12.5% gel). Electrophoresis was performed at 60 V for 60 minutes, followed by 30mA for 2 – hours. Gels were stained with 0,08% Coomassie Brilliant Blue in methanol.

2.5 – TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (2 - DE)

Approximately 100 - 150μg of microsomes was added to 340 μL of sample buffer containing 8M urea, 4% w/v, CHAPS 2% v/v, carrier ampholytes pH 3-10, 70 mM DTT, and 0.001% w/v bromophenol blue (all reagents were from Amersham Biosciences

- Uppsala - Sweden, except for DTT, which was from Biorad). The samples were applied to IPG gel strips with a pH 3-10 nonlinear separation range (catalog number 17-1235-01, Amersham Biosciences - Uppsala - Sweden). After a 10-hour rehydration, isoelectric focusing was performed at 20ºC for 1 hour at 500V, 1 hour at 1000 V, and 10 hours at 8000V in an IPGphor apparatus (Amersham Biosciences - Uppsala - Sweden). The limiting current was 50 μA per strip. Strips were subjected to reduction and alkylation steps to second dimension. Strips were soaked for 8 minutes in a solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% v/v, glycerol, 2% w/v, SDS, and 2% w/v DTT, followed by an additional 12 minutes in the same solvent containing 2.5% w/v iodoacetamide instead of DTT.

Second dimension electrophoresis (SDS-PAGE) was performed with an SE-600 system connected to a Multitemp II refrigerating system (Amersham Biosciences - Uppsala - Sweden). After laying the strip on the top of a 12.5% polyacrylamide gel and sealing it with agarose, the gels were run for 1 hour at 60 V, after which constant amperage of 30 mA per gel was applied until the migration front reached the lower end of the gel. Proteins were detected by Comassie Blue staining.

2.6 – IN-GEL ENZYMATIC DIGESTIONS

Spots/bands resulting from the 1 – DE and 2 - DE electrophoresis were excised from the gel. Peptides were generated and extracted from the gel-separated proteins following established in-gel digestion protocols using sequencing grade modified trypsin (Madison, WI, USA).

The digestions were carried out using trypsin as follows: gel slices were washed twice for 45 min at 37ºC in 0.5 ml of 200mM NH_4HCO_3/50% (v/v) acetonitrile with agitation and shrunk in pure acetonitrile for 15 min. After removal of acetonitrile and drying under vacuum, the samples were reswollen with 40mM NH_4HCO_3 containing sequencing-grade modified trypsin at a ca. 10:1 molar ratio of the protein to trypsin (but not less than 6μg/ml trypsin).

The volume of buffer was ca. 1.5 times that of the excised gel band. After the absorption of trypsin, additional buffer (30–50μL) was used to cover the gel pieces to keep them wet during overnight digestion (16–18 h) at 37ºC.

After drying down, the samples were resuspended in 10 μL 0.1% aqueous TFA and cleaned with ZipTips (Millipore, Oxford, MA, USA) according to the protocol supplied by the manufacturer. The samples were diluted 1:200 before mixing the peptide solution with equal volumes of saturated matrix solution and spotting 2μL of the mixture onto the MALDI target plate (Micromass, Manchester, UK) for analysis.

2.7 - MALDI-TOF

The peptides were analyzed on a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Prior to their application to the sample plate the samples were purified using C18 Zip Tips (Millipore, Oxford, MA, USA), and eluted directly with a matrix solution of 2% w/v α-cyano-4-hydroxycinnamic acid (Sigma), 60% acetonitrile and 0.1% v/v TFA. Internal mass calibration was performed using trypsin auto digestion products (842.509, 1045.563 and 2211.104 Da).

2.8 – PROTEIN IDENTIFICATION

The SWISS-PROT database (Swiss Institute of Bioinformatic, http://us.expasy.org) and EMBL (Bioanalytical Research Group, http://www.mann.embl-heidelberg.de/GroupPages/) was used to identify the protein. The parameters used in the search were as follows: peptide mass tolerance 25Da, 1 missed cleavage, carboxymethylated cystein, 9 peptides used in the search and 5 peptides requires for match. The gel matching was done using the software Imagem Master 2D Elite.

3 – RESULTS

A 1 – DE electrophoresis pattern of total microsomal proteins from untreated and 3 – MC treated fish is presented in Figure 1.

(Figure 1)

Analyzing Figure 1, we can observe that few proteins are expressed in only one treatment. Those results show that those proteins have their expression modulated by the presence of xenobiotic; since those results were repeted in Bi – dimensional electrophoresis, we confirm that it was not artefacts. Most part of the proteins are presented in both treatment, but in different concentration, suggesting that their transcription activity are modulated by the xenobiotics. Most part of those proteins are localized in the region of 40 – 70kDa and may be related to CYP families.

In this work we identify only the proteins with differential pattern of expression in two states, however we could see that 1 – DE is not an efficient technique to separate protein in the region of 45 – 60kDa, where proteins have close rate of migration.

Results of 2 – DE separation and subsequent identification of microsomal proteins are presented in Figure 2 – 3. The protein pattern of untreated and 3 – MC treated group were clearly different, especially in protein between 50-60kDa, where our attention was focused, since that in this region are located most part of CYP isoforms.

(Figure 2)

(Figure 3)

It was possible identify around 72 spots in untreated group against around 92 in 3 – MC treated group. Five regions were common in both groups and represents proteins that are presents in both treatment, excluding the hypothesis that they are artifacts of the technique. Those regions were denominated A – E and the spots intensity vary among them, suggesting a differential pattern of expression in both states. There was a matching of 38% between the gel of control and 3 – MC group, showing the induction of the CYP system and also the other enzymes (Figure 4).

Spots marked as 43, 44, 45 have a molecular weigh around 60,000 Da and represents the CYP1 family according to the peptide profile mapping (Figure 5). The same pattern of distribution was observed in a 2 – DE of CYP1A purified (data non

shown). However in the purified sample the spots were localized in an acid region, while in microsomal sample the spots are localized in an alkaline region.

DISCUSSION

Nowadays, the complexity of eukariotic cells leads to a shift of global proteomicas to subcellular proteomics. The advantage of two dimensional gel electrophoresis is its capacity in separated proteins by charge (first dimension) and by mass (second dimension), allowed the isolation of proteins that have close molecular weight , like highly homologues isoforms and the identification of proteins with post translational changes (fosforilation and glicosilation).

Monitoring the proteins levels is crucial to analyze in a complete manner one biological event. The protein level found in our results shown that endoplasmatic proteins are regulated by translational and post – translational, explaining the discrepancy between mRNA levels and proteins (data non shown) (Steen and Pandey, 2002). The aim of this work was not quantify/identify a specific proteins – CYP1A for example – but compare the abundance and the pattern of expression of a group of proteins in two different states.

Analyzing Figures 2 – 3 we could see a great number of horizontal “riscos” that do not have a spot resolution. Those results are common in 2 – DE when the sample correspond to membrane proteins. Those are not artifacts but the consequence of a strong interaction between membrane proteins and ampholytes (Perdew et al, 1983). The regions A – E have already been described by the literature by Kaderbhai and Freedman (1980) and Vlasuk e Walz (1980)

According to literature, it was expected a bigger number of spot in the acid region of both control and 3 -MC gel, since that membrane proteins are rich in glutamate and aspartate residues (Kaderbhai e Freedman, 1980). In our results we observe the opposite: a great number of spots were observed in the region of pH 5.0 – 8.0. These result was similar to the one found by Vlasuk e Walz (1980) e Galeva e Altermann (2002); and may be related to the use of a discontinuous pH gradient during the isoeletric focalization. Besides of that,

The quantitative application of 1 – DE as a proteomic tool is questionable especially in studies of endoplasmatic reticulum where most part of the proteins are located in the same region (45 – 75 kDa) and are not satisfactory separated by the analytical performance of 1 – DE. Perhaps of this, in 1 – DE gel we identify more proteins in 3 – MC treated group than in control group, confirm the hypothesis that many proteins are induced by xenobiotic.

The ability of 3 – MC to induce xenobiotic metabolism by CYP system has been known since 70's year. In 1982, Vlasuk and Walz (1980 and 1982) identified through peptide mass fingerprinting the presence of CYP isoformas in IFSDS – gel electrophoresis, but there is no data in literature about the induction of other microsomal protein than CYP system. Our work shows that besides CYP1A, proteins like cytochrome b5, heat shock proteins, protein disulfide isomerase among other were also induced by the xenobiotic and resolved into several spots in 2 – DE. It is possible to conclude that the final pattern of proteins in liver endoplasmatic reticulum after 3 – MC treatment results from the interplay of both inductive and repressive processes.

Microsomal membranes do not represent a single organelle. Fragmetn of Golgi membrane and nuclear membrane could be present. Nevertheless, the material

analyzed here is what is generally referred as microsomal fraction. Its major component is undoubtedly vesicle derived from the endoplasmic reticulum that consist of CYP system (Kaderbhai and Freedman, 1980, Galeva and Altermann, 2002).

In our study we observed the presence of only CYP1A1 and CYP1A3. That fact had already been described by other authors and may be related to the genetic polymorphism of CYP1A in fishes due to some environmental and physiological conditions (Sean and Arinç. 1998). The shift found between purified and microsomal CYP1A1 is a consequence of conformational changes in CYP1A structure. Those changes occurs because of the absence of membrane responsible by a strong electrostatic interaction among CYP and NADPH cytochrome P450 reductase through lysine and arginine residues (Shen e Strobel, 1992, Shen e Strobel, 1993, Izumi et al., 2003) creating a stable complex that associated itself to lipids membrane through hydrophobic interaction (Vlasuk e Walz, 1980, Vergères et al., 1989, Shen e Strobel, 1993, Benhardt, 1995, Yun et al., 1996, Omura, 1999).

This study shows that the technique could be used as a new tool to access biomarkers since that the identification of each spot could reveal the molecular mechanism of action of pollutants. The proteomic approach is expected to liberate us from the need to guess the best biomarkers and let the data tell us which are the best indicators of each environmental pollutant. Information may also be gleaned about the pathobiology of the process and allowed us to prevent in initial stages the effects of pollutants in living organism.

CONCLUSION

The data presented here demonstrate that proteomics analysis of endoplasmatic reticulum focused in biotransformation enzymes can be used as a new tool to direct identification of differentially expressed enzymes - including CYP isoformas - and should became a standard technique for characterizing biomarkers of environmental pollution.

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FIGURE SUBTITLE

FIGURE 1 - Total Curimbatá liver mmicrosomal proteins separated by 1 – DE. (C) Control group; (3 – MC) 3 – methylcloranthrene treated group; (MW) mass standard.

FIGURE 2 – 2 –DE pattern of control Curimbatá Liver microsomes. Arrows shows groups of proteins that appear only in this treatment.

FIGURE 3 – 2 – DE pattern group of 3 –MC induced Curimbatá liver microsomes. Arrows shows groups of proteins that appear only in this treatment.

FIGURE 4 - Gel matching of control group (red) and 3 – MC induced group (blue) with vectors in green. There was find 38% of matching of both gel.

FIGURE 5 – Peptide mass finger printing of 2 – DE spot containing CYP1A

REFERENCES

Bakhtiar, R., Nelson, R.W., 2000. Electrospray ionization and Matrix – assited laser desorption ionization mass spectrometry. Emerging technologies in biomedical sciences. Biochem. Pharmacol. 59, 891-905.

Bernhardt, R., 1995. Cytochrome P450: Structure, Function, and generation of reactive oxygen species. Rev. Physiol. Biochem. Pharmacol. 127, 137-221.

Buhler, D. R., Wang-Buhler, J.L., 1998. Rainbow trout cytochrome P450s: purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. Comp. Biochem. Physiol. 121C, 107-137.

Cousinou, M., Nilsen, B., Lopez-Barea, J., Dorado, G., 2000. New methods to use cytochrome P4501A1 to assess marine organic pollutants. Sci. Total Environ. 247, 231-225.

CRAFT, J.A., ROBERTSON, F.E., Mcphail, M.E., BROWN, E., STAGG, R.M., 2001. Measurement of cytochrome P4501A induction in dab (*Limanda limanda*) and other teleosts with species-specific cDNA probes: isolation and characterization of dab cDNA and its use in expression studies with β-naphtoflavone-treated fish. Comp. Biochem. Physiol. 129C, 115-127.

Galeva, N., Altermann, M., 2002. Comparasion of one – dimensional and two – dimensional gel electrophoresis as a separation tool for proteomic analysis of rat liver microsomes: cytochorme P450 and other membrane proteins. Proteomics 2,713 - 722.

Galeva, N., Yakovlev, D., Koen, Y., Duzhak, T., Alterman, M., 2003. Directed Identification of cytochorme P450 isoenzymes by Matrix – assisted laser desorption / Ionization time of flight – based proteomic approach. Drug Metab. Dispos. 27, 188 – 192.

Ghaim, J.B., Tsatsos, P.H., Katsonouri, A., Mitchell, D. M., Salcedo – Hernandez, R., Gennis, R.B., 1997. Matrix – assisted laser desorption ionization mass spectrometry of membrane proteins: Demonstration of a simple method to determine

subunit molecular weights of hydrophobic domains. Biochim. Biophys. Acta 1330, 113 – 120.

Godoy, M. P.,1975. Peixes do Brasil – Subordem Charici forme. 1ª ed. Editora Franciscana. Piracicaba, S.P.

GOKφYR, A., HUSφY, A. M., 1998. Immunochemical approaches to studies of CYP1A localization and induction by xenobiotics in fish. In, **Fish Ecotoxicology**. Ed. By T. Braunbeck; D. E. Hinton & B. Street. pp 165-202.

Izumi, S., Kaneko, H., Yamazaki, T., Hirata, T., Kominami, S., 2003. Membrane topology of guinea pig cytochrome P450 17α revealed by a combination of chemical modifications and mass spectrometry. Biochemsitry 42, 14663 – 14669.

Kaderbhai, M.A., Freedman, R.B., 1980. Two – dimensional gel electrophoresis of rat liver microsomal membrane protein. Biochim. Biophys Acta 601, 11 – 21.

Klotz, A.V., Stegeman, J. J., Walsh, C., 1983. An aryl hydricarbon hydroxylating cytochrome P450 from the marine fish *Stenotomus chrysops*. Arch. Biochem. Biophys. 226, 578-592.

Lowry, O. H.; Rosebrough, N. J; Farr, A. L.; Randall, R. J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Molloy, M.P., 2000. Two – dimensional electrophoresis of membrane proteins using immobilized pH gradients.Anal. Biochem. 280:1-10.

Morrison, H. G., Oleksiak, M. F., Cornell, N. W., Sogin, M. L., Stegeman, J.J., 1995. Identification of cytochrome P4501A (CYP1A) gene from two teleost fish toadfish

(*Opsanus tau*) and scup (*Stenotomus chrysops*), and phylogenetic analysis of CYP1A genes. Biochem. J. 308, 97-104.

Morrison, H.G., Weil, E.J., Karchneer, S.I., Sogin, M.L., Stegeman, J.J., 1998. Molecular cloning of CYP1A from the estuarine fish *Fundulus heteroclitus* and phylogenetic analysis of CYP1 genes: update with new sequences. Comp. Biochem. Physiol. 121C, 231-240.

Omura, T., 1999. Forty years of cytochrome P450. Biochem. Biophys Res. Comm. 266:690-698.

Omura, T., Sato, R. , 1964a. The Carbon Monoxide-binding pigment of liver microsomes. I - Evidence for its heme protein nature. J. Biol. Chem. 239, 2370-2378.

Perdew, G. H., Schaaup, H.W., Selivonchick, D. P., 1983. The use of a zwitterionic detergent in two dimensional gel electrophoresis of trout liver microsomes. Anal. Biochem. 135, 453 – 455.

Sen, A., Arinç, E., 1998. Preparation of highly purified cytochrome P4501A1 from leaping mullet (*Liza saliens*) liver microsomes and its biocatalytic, molecular and immunochemical properties. Comp. Biochem. Physiol. 121C, 249-265.

Shen, S., Strobel, H.W., 1992. The role of Cytochrome P450 lysine residues in the interaction between cytochrome P4501A1 and NADPH cytochrome P450 reductase. Arch. Biochem Bioph. 294, 83-90.

Shen. S., Strobel, H.W., 1993. Role of lysine and arginine residues of cytochrome P450 in the interaction between cytochrome P4502B1 and NADPH – cytochrome P450 reductase. Arch. Biochem. Biophys. 304, 257 – 265.

Sickmann, A., Mreyen, M., Meyer, H.E., 2002. Identificationof modified proteins by mass spectrometry. Life 54, 51 – 57.

Steen, H., Pandey, A., 2002. Proteomics goes quantitative: measuring protein abundance. TRENDS Biotechnol. 20, 361 – 364.

Stegeman, J. J., Lech, J. J., 1991. Cytochrome P450 monoxigenase system in aquatic species: Carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ Health Perspec. 90,101-109.

Vergères, G., Winterhalter, K.H., Richter, C., 1989. Microsomal cytochrome P450: sybstrate binding, membrane interactions and topology. Mut. Res. 213, 83-90.

Vlasuk, G. P., Walz, F.G. Jr, 1980. Liver endoplasmatic reticulum polypeptides by two – dimensional gel electrophoresis. Anal. Biochem. 105, 112 – 120.

Vlasuk, G. P., Walz, F.G. Jr, 1982. Liver microsomal polypeptides from Fischer – 344 rats affected by age, sex, and xenobiotic induction. Arch. Biochem. Biophys. 214, 248 – 259.

Vrolijk, N.H.; Targett, N. M.; Woodin, B.R., Stegeman, J.J., 1994. Toxicological and ecological implication of biotransformation enzymes in tropical teleost Chaetodon capistratus. Mar. Biol. 119,151-158.

Yun, C.H., Songs, M., Ahn, T., Kim, H., ,1996. Conformational changes of cytochrome P4501A2 induced by sodium chloride. J. Biol. Chem. 271, 31312-31316.

FIGURE 1

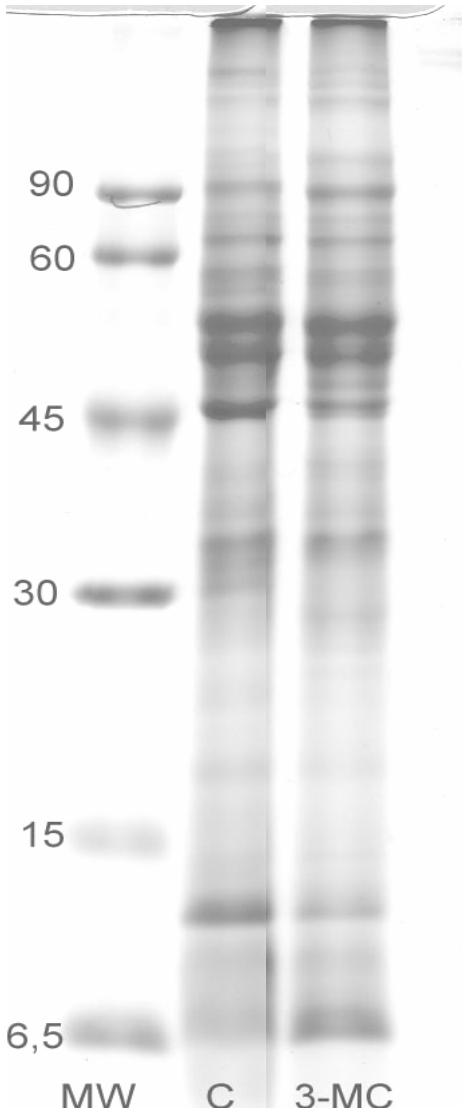


FIGURE 2

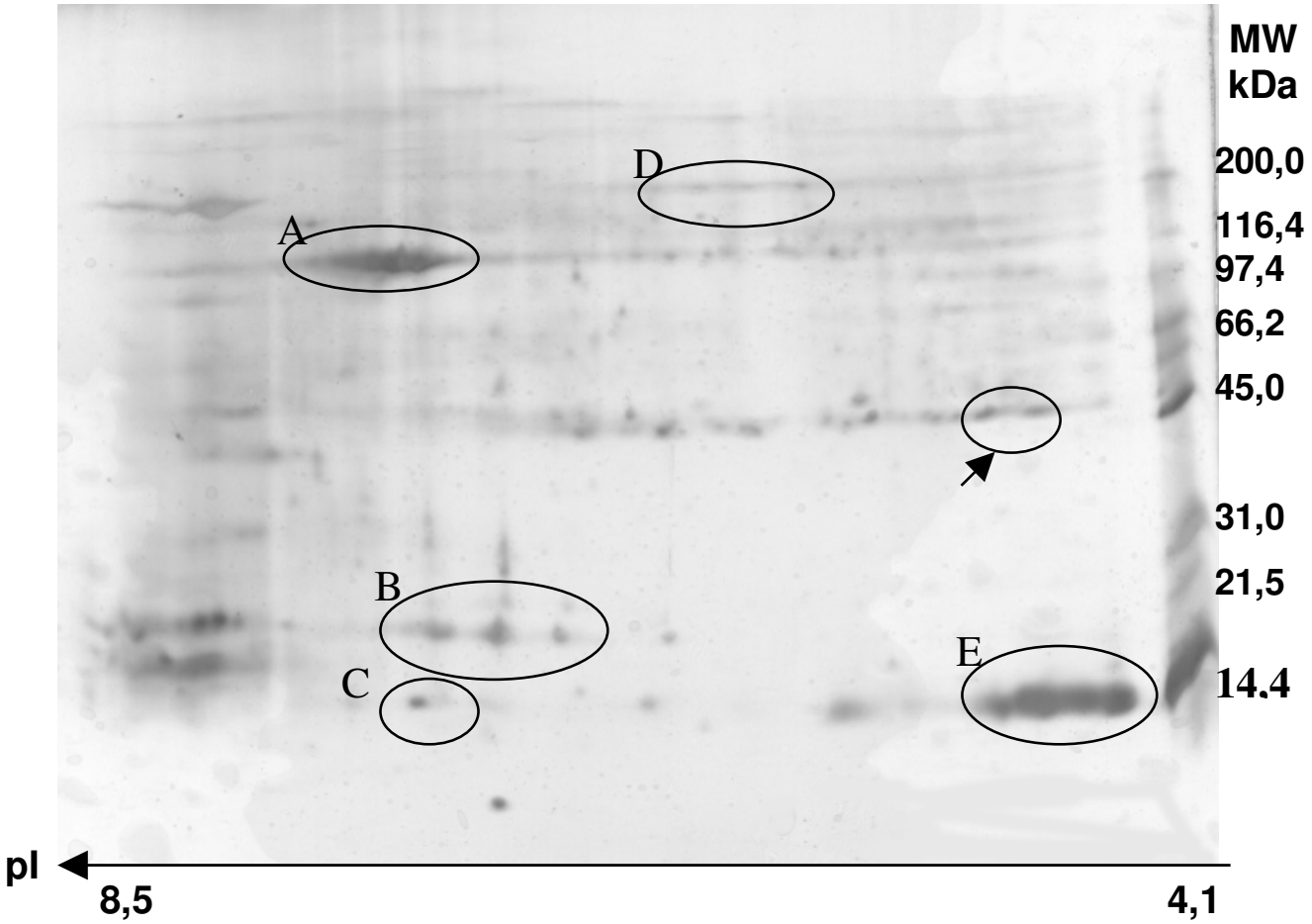


FIGURE 3

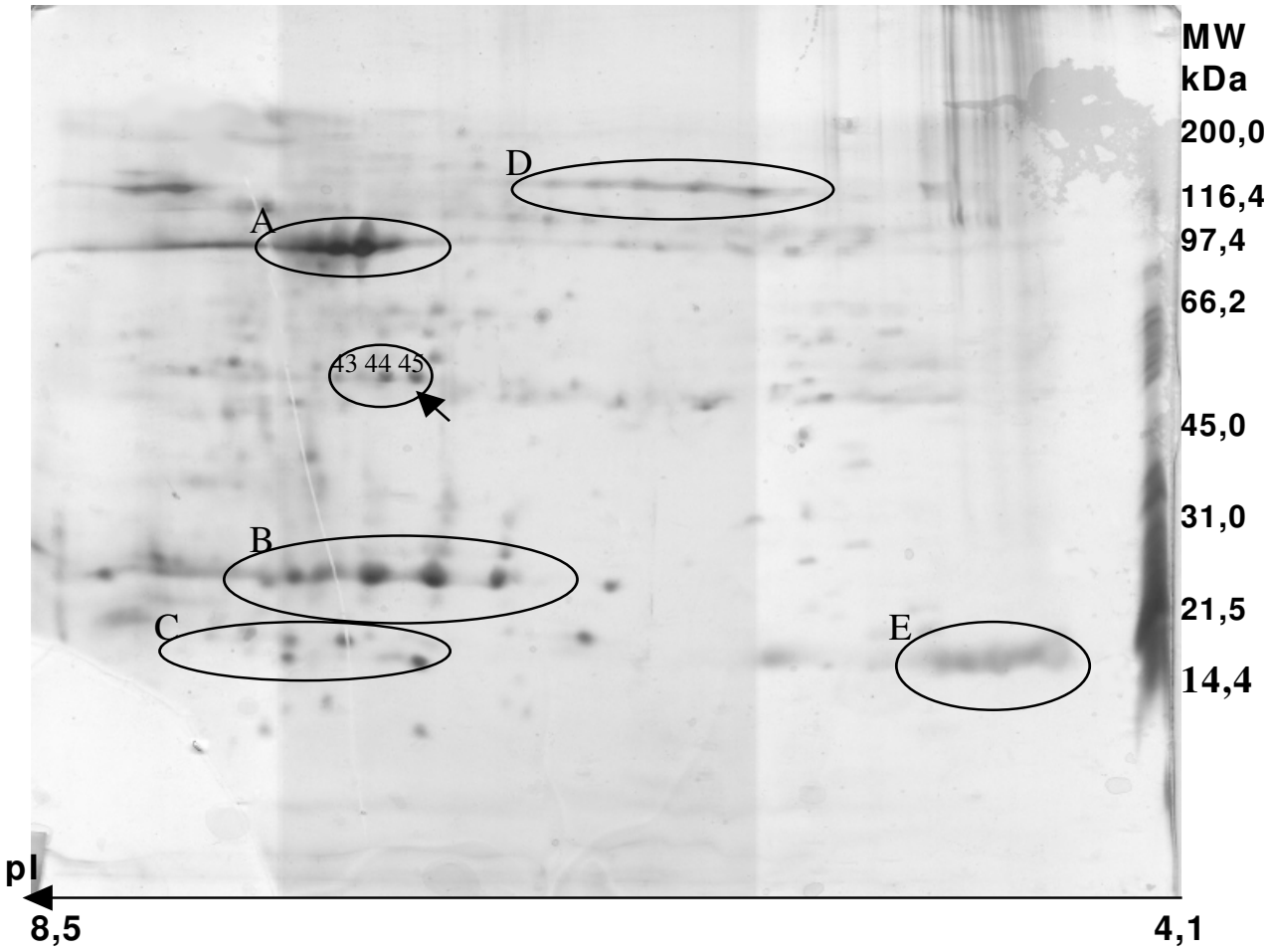


Figure 4

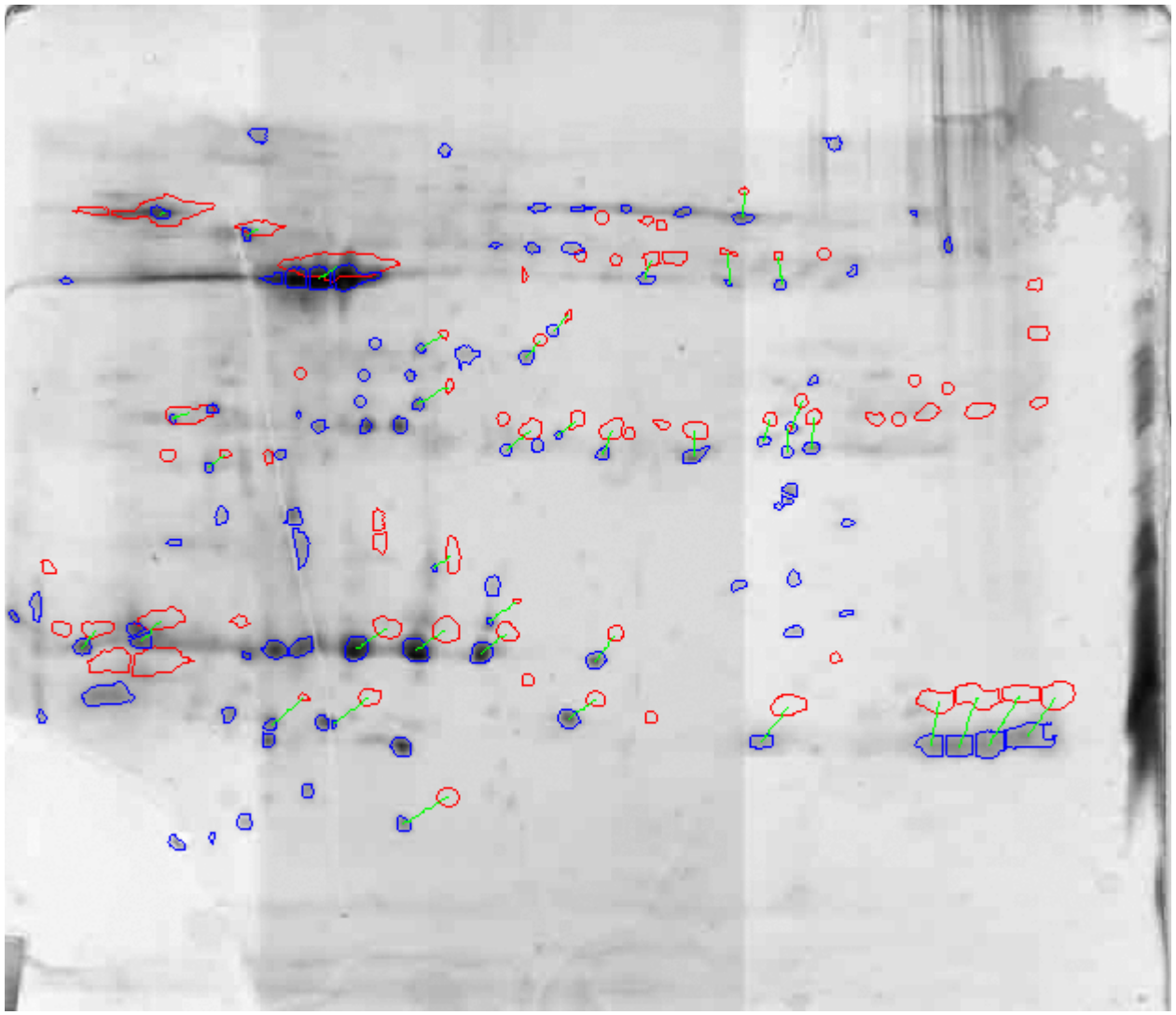
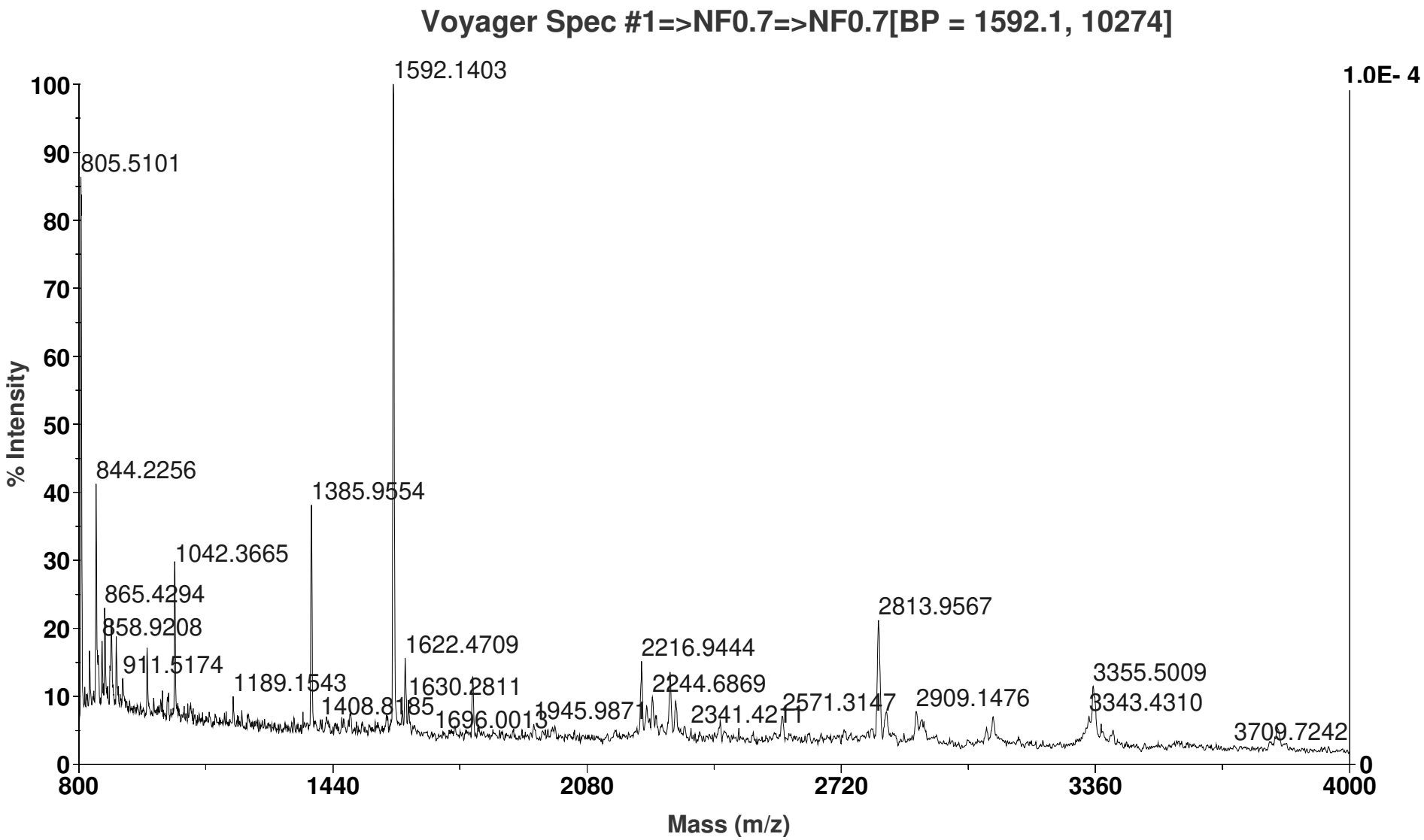


Figure 5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2244.6869
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2571.3147
2813.9567
2909.1476
3355.5009
3343.4310
3709.7242
% Intensity
Mass (m/z)
100
90
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70
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3360
4000

4 – CONCLUSÃO

O uso do sistema CYP como biomarcador de poluição ambiental vem sendo estudado há anos. Tais estudos baseiam-se na avaliação da atividade enzimática do CYP. Mais recentemente, tais estudos visam avaliar os níveis de indução das diferentes isoformas através de ensaios com RNA mensageiro. No entanto, tais métodos encontram obstáculos no limitado número de P450 caracterizados segundo: (i) especificidade de substrato, (ii) afinidade de anticorpos e (iii) compostos inibidores. Além disto, nem sempre existe uma correlação positiva entre os níveis de proteína encontrado na célula e os de RNA mensageiro expressas.

A partir dos resultados obtidos neste trabalho podemos ressaltar os seguintes aspectos que podem enriquecer os estudos a respeito do uso do CYP como biomarcador de poluição ambiental:

1) Interações de xenobióticos com características surfactantes e o CYP.

2) Uso da espectrometria de massas na identificação de isoformas do CYP1A

3) A tecnologia proteômica em estudos de Ecotoxicologia.

4.1 – INTERAÇÃO DE XENOBIÓTICOS COM CARACTERÍSTICAS SURFACTANTES E O CYP

Os estudos com xenobióticos com características surfactantes questionou os resultados que mostram que os efeitos nocivos de surfactantes sobre o CYP ocorreriam apenas quando o surfactante encontrava-se acima da sua Concentração Micelar Crítica (CMC), quando ocorre a desestruturação do ambiente de membrana.

Nossos resultados mostraram que os efeitos dos xenobióticos sobre o CYP podem ocorrer quando eles se encontram em concentrações abaixo deste valor. Neste caso podemos observar a existência de interações diretas entre o sistema CYP e os surfactantes tão forte capazes de alterar a conformação do sistema.

Através destas informações desenvolvemos um método de purificação mais rápido e eficiente que os anteriores por estar baseado no uso da cromatografia de fase reversa associada a colunas de hidrofobicidade. Desta forma aumentamos o rendimento e a atividade da enzima em sistemas reconstituídos.

Estes resultados também permitiram a purificação do CYP1A em um peixe exclusivamente brasileiro, o que ainda não havia sido descrito na literatura. Mesmo sendo um peixe dotado de inúmeras peculiaridades, o CYP1A de Curimbatás conserva as mesmas características dos CYPs de outros peixes.

Nossos resultados abrem perspectivas para novos estudos sobre a interação de poluentes com os CYPs, visto que vários destes compostos químicos tem sua solubilidade aumentada pela adição de surfactantes. Além disto, esta nova forma de purificação, que exclui a necessidade de passos como solubilização e diálise, também pode ser aplicada a outras proteínas de membrana.

4.2 – USO DA ESPECTROMETRIA DE MASSAS NA IDENTIFICAÇÃO DAS ISOFORMAS DA CYP1A

O grande problema dos processos de purificação dos CYPs estão relacionados à inabilidade destes em separar as isoformas do CYP1A, visto que as isoformas CYP1A1 e CYP1A3 se diferem em apenas 20 aminoácidos posicionados na porção C-terminal. Tal diferença não pode ser observada em gel de SDS – PAGE e nem sempre existem anticorpos monoclonais ou policlonais disponíveis que consigam distinguir entre as isoformas de determinadas espécies.

Neste ponto a eletroforese bi-dimensional associada à espectrometria de massas do tipo MALDI – TOF se tornou uma ferramenta eficaz na identificação de isoformas altamente homólogas devido a sua capacidade de identificar concentrações em picomoles de proteínas separadas em gel.

Os três spots isolados a partir do CYP1A purificado correspondiam não apenas as isoformas CYP1A1 e CYP1A3, mas também a isoformas que ainda não sofreram modificações pós traducionais.

Assim, os dados demonstrados claramente mostraram que esta abordagem tem um grande potencial para a identificação direta de isoformas do CYP. A eletroforese bidimensional associada á digestão triptica e á espectrometria de massas (PMF/MALDI-TOF) abre um novo campo de identificação de proteínas mais rápido e preciso.

4.3 – TECNOLOGIA PROTEÔMICA EM ESTUDOS DE ECOTOXICOLOGIA

Os estudos ecotoxicológicos atuais tem se baseado apenas em ensaios de bioacumulação, bioconcentração, biomagnificação e biotransformação. Estes estudos nem sempre permitem fazer a correlação entre os níveis de proteínas presentes e de RNA mensageiro expresso. Nosso trabalho foi capaz de demonstrar que a eletroforese em duas dimensões pode se tornar uma técnica padrão para a caracterização de microssomas permitindo a identificação de proteínas que se expressam de maneira diferencial conforma e situação ambiental. Os níveis de proteínas encontrados em nossos resultados, não são resultados apenas da regulação transcricional das proteínas, mas também da regulação traducional e pós traducional.

O objetivo desta parte do trabalho não é centrado na determinação da quantidade de uma dada proteína – no caso o CYP1A e suas isoformas – e sim na comparação da sua abundância relativa em dois estados. Monitorar os níveis de proteínas é crucial quando se pretende analisar de maneira completa e precisa um dado evento biológico. Assim, propomos que a abordagem proteômica também tem um grande potencial em se tornar uma ferramenta padrão não apenas para a identificação de proteínas expressas, ou silenciadas, na presença de um poluente, mas também para a elucidação do mecanismo de ação de novos xenobióticos.

5 – REFERÊNCIAS BILIOGRÁFICAS

ADAMS, S.M., 1990. Status and use of biological indicators for evaluating the effects of stress on fish. Am. Fish. Soc. Sym. 8,1-8.

ALFONSO, P., CATALA, M., RICO – MORALES, M.L., DURANTE – RODRIGUEZ, G., MORO – RODRIGUEZ, E., FERNANDEZ – GARCIA, H., ESCRIBANO, J.M., ALVAREZ – FERNANDEZ, E., GARCIA – POBLETE, E., 2004. Proteomic analysis of lung biopsies: Differential protein expression profile between peritumoral and tumoral tissue. Proteomics 4, 442 – 447.

ANZENBACHER, P., ANZENBACHEROVÁ, E., 2001. Cytochrome P450 and metabolism of xenobiotics. Cell. Mol. Life Sci. 58, 737 – 747.

AU, D. W. T., WU, R.S.S., ZHOU, B. S., LAM, P.K. S., 1999. Relationship between ultrastructural changes and EROD activies in liver of fish exposed to Benzo[a]pyrene. Environ. Pollut. 104: 235-247.

BAINY, A.C. D., 1996. Oxidative stress as a biomarker of polluted aquatic site. In: Physiology and Biochemistry of the fishes of the Amazon. Edited by A. L. Val, V. M. F. Almeida-Val & D. J. Randal. Manaus, Brasil.

BAKHTIAR, R., NELSON, R.W., 2000. Electrospray ionization and Matrix – assited laser desorption ionization mass spectrometry. Emerging technologies in biomedical sciences. Biochem. Pharmacol. 59, 891-905.

BERNHARDT, R., 1995. Cytochrome P450: Structure, Function, and generation of reactive oxygen species. Rev. Physiol. Biochem. Pharmacol. 127, 137-221.

BEYER, J., GOKSϕYR, A., 1993. Cytochrome P450 observation in gulf fish. Mar. Pollut. Bull. 27, 293 – 296.

Braz. Arch. Biol. Technol. 45, 171 – 175.

BUHLER, D. R. & RASMUSSON, M. E., 1968. The oxidation of drugs by fishes. Comp. Biochem. Physiol. 25:223-239.

BUHLER, D. R., WANG-BUHLER, J.L., 1998. Rainbow trout cytochrome P450s: purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. Comp. Biochem. Physiol. 121C, 107-137.

CARVALHO, C. S., ARAUJO, H.S.S., FERNANDES, M.N., 2004. Hepatic metallothionein in a teleost (*Prochilodus scrofa*) exposed to copper at pH 4.5 and pH 8.0 Comp. Biochem. Physiol. 137B, 225–234

CHAURAND, P., LUETZENKIRCHEN, 1999. Peptide and prote[in identification by matrix-assisted laser desorption ionization (MALDI) and MALDI – post-source decay time – of flight mass spectrometry. J. Am. Soc. Mass Spectrom. 10, 91-103.

COON, M. J., DING, X., PERNECKEY, S. J. & VAZ, A. D. N., 1992. Cytochrome P450: progress and prediction. FASEB J. 6:669-673.

COUSINOU, M., NILSEN, B., LOPEZ-BAREA, J. & DORADO, G., 2000.New methods to use cytochrome P4501A1 to assess marine organic pollutants. Sci. Total Environ. 247:231-225.

CUNNINGHAM, M.J., 2000. Genomics and Proteomics. The new millennium of drug discovery and development. J. Pharmacol. Toxicol. Meth. 44, 291-300.

DA SILVA, M.E.F., MEIRELLES, N.C., 2004. Interaction of non - ionic surfactants with hepatic CYP in *Prochilodus scrofa*. Toxicol. In vitro (no prelo)

DE FERNICOLA, N.A.G.G., BOHRER-MOREL, M.B.C., BAINY, A.C.D., 2003. Ecotoxicologia. In: As bases Toxicológicas da Ecotoxicologia. Coordenadores F.A. de Azevedo, A.A. M. Chasin. Rima Editora e InterTox. São Carlos, SP, Brasil.

DEGTEREV, I.A.; MEIRELLES, N.C.; VERCESI, A. E. & DA SILVA, M.E.F., 1999. Redox properties of hepatic cytochromes from Prochilodus scrofa (Curimbatá), a freshwater fish. In: Biology of tropical fishes. Edited by A. L. Val e V. M. F. Almeida-Val. INPA, Manaus, AM. Brasil. pp 253-261.

DELESCLUSE, C., LEMAIRE, G., DE SOUSA, G., RAHMANI, R., 2000. Is CYP1A1 induction always related to AHR signaling pathway. Toxicology 153, 73 – 82.

DEN BESTEN, P. J., 1998. Concepts for the implementation of biomarkers in environmental monitoring. Mar Environ. Res. 46, 253-256.

EASTERLING, M.L., COLANGELO, C.M., SCOTT, R.A., AMSTER, I.J., 1998. Monitoring proteinexpression in whole bacterial cells with MALDI time –of-flight mass spectrometry. Anal. Chem. 70, 2704-2709.

FERNANDES, M. N., BARRIONUEVO, W. R., RANTIN, F. T.,1995. Effects of the termal stress on respiratory reponses to hipoxia of a South American Prochilodontid fish, *Prochilodus scrofa*. J. Fish. Biol. 46, 123-133.

FRIEDMAN, D.B., HILL, S., KELLER, J.W., MERCHANT, N.B., LEVY, S.E., COFFEY, R.J., CAPRIOLI, R.M., 2004. Proteome analysis of human, colon cancer by two – dimensional difference gel electrophoresis and mass spectrometry. Proteomics 4, 793 – 811.

GALEVA, N., ALTERMANN, M., 2002. Comparasion of one – dimensional and two – dimensional gel electrophoresis as a separation tool for proteomic analysis of rat liver microsomes: cytochorme P450 and other membrane proteins. Proteomics 2,713 - 722.

GALEVA, N., YAKOVLEV, D., KOEN, Y., DUZHAK, T., ALTERMAN, M., 2003. Directed Identification of cytochorme P450 isoenzymes by Matrix – assisted laser desorption / Ionization time of flight – based proteomic approach. Drug Metab. Dispos. 27, 188 – 192.

GODOY, M. P.,1975. Peixes do Brasil – Subordem Charici forme. 1ª ed. Editora Franciscana. Piracicaba, S.P.

GOKφYR, A. & HUSφY, A. M., 1998. Immunochemical approaches to studies of CYP1A localization and induction by xenobiotics in fish. In, Fish Ecotoxicology. Ed. By T. Braunbeck; D. E. Hinton & B. Street. pp 165-202.

GOLDFARB, P.; LIVINGSTONE, D.; BURMELIN, C., 1998. Biomonitoring in the aquatic environment: use of molecular biomarkers. Biochem. Soc. Transac. 26,690-694.

GRAHAM, S. E.,PETERSON, J. A., 1999. How similar are P450s and what their differences teach us. Arch Biochem. Biophys. 369: 24-29.

GUENGERICH FP, PARIKH A, YUN CH, KIM D, NAKAMURA K, NOTLEY LM, GILLAM EM., 2000. What makes P450s work? Searches for answers with known and new P450s. Drug Metab Rev., 32, 267-81.

GUENGERICH, F. P. , BELL, L. C., OKAZAKI, O., 1995. Interpretation of cytochrome P450 mechanisms from kinetics studies. Biochimie 77:573-580.

GUENGERICH, F.P., HOSEA, N. A., PARIKH, A., BELL-PARIKH, C., JOHNSON, W. W., GILLAM, E. M. J. & SHIMADA, T., 1998. Twenty years of biochemistry of human P450. Purification, expression, mechanism and relevance to drug. Drug Met. Disp. 26: 1175-1178.

GUENGERICH, P. F., 2000. Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity, Chem. Res. Toxicol. 14, 612 – 650.

HACON, S.S., 2003. Ecotoxicologia. In: As bases Toxicológicas da Ecotoxicologia. Coordenadores F.A. de Azevedo, A.A. M. Chasin. Rima Editora e InterTox. São Carlos, SP, Brasil.

HALPERT, J. R.; 1995. Structural basis of selective cytochrome P450 inhibition. Ann. Rev. Pharmacol. Toxicol. 35:29-53.

HATANAKA, T, GALETTI, P. M. JR., 2003. RAPD markers indicate the occurrence of structured populations in a migratory freshwater fish species. *Gen.Mol. Biol.* 26, 19-25

HAYMAN, M.W., PRZYBORSKI, S.A., 2004.Proteomic identification of biomarkers expressed by human pluripotent stem cells. Biochem. Biophys. res. Commun. 316, 918-923.

ISOMAA, B., LILIUS, H., 1995. The urgent need for *in vitro* tests in ecotoxicology. Toxic. In Vitro 9, 821-825.

IYER, K. R. & SINZ, M. W., 1999. Characterization of phase I and phase II hepatic drug metabolism activities in a painel of human liver preparation. Chem. Biol. Interac. 118:151-169.

JAMES, M.O., 1989. Cytochrome P450 monooxigenases in crustaceans. Xenobiotica 19: 1063-1076.

KOBLYAKOV, V. A., 1998. Inducers of the cytochrome P450 superfamily as promoters of carcinogenesis. Biochemistry 63: 885-898

KOENIGS L. L., TRAGER, W.F., 1998. Mechanism – based inactivation of cytochrome P450 2B1 by 8 – methoxypsoralen and several other furanocoumarin. Biochemistry 37, 13184 – 13193.

KRISHNAKUMAR, P. K., CASILAS, E. & VARANASI, U. , 1995. Effects of chemical contaminants on the health of *Mytilus edulis* from Puget Sound, Washington. II. Cytochemical detection of subcellular changes in digestives cells. Mar. Biol. 125:251-259.

KULLMAN, S.W., HAMM, J. T. & HINTON, D. E., 2000. Identification and characterization of a cDNA encoding cytochrome P4503A from the freshwater teleost medaka (Oryzias litipes). Arch. Biochem. Biophys. 380: 29-38.

KÜSTER, B., MANN, M., 1998. Identifying proteins and post – translational modification by mass spectrometry. Curr. Opin. Struc. Biol. 8, 393 – 400.

LEITÃO, M.E.S.; AFFONSO, E. G.; DA SILVA, M. E. F.; MEIRELLES, N.C.; RANTIN, F.T.; VERCESI, A. E.; JUNQUEIRA, V.B.C. & DEGTEREV, I. A., 2000. The liver monooxigenese system of Brazilian freshwater fish. Comp. Biochem. Physiol. 121C:29-38.

LIVINGSTONE, D. R.; KUCHEN, M. A. & WISEMAN, A., 1989. Cytochrome P450 and oxidative metabolism in molluscs. Xenobiotica 19: 1041-1062.

LUSKA, A. J., SHEVCHENKO, A., 2003. Expanding the organismal scope of proteomics: cross –s pecies proteins identification by mass spectrometry and its implications. Proteomics 3, 19 – 29.

MACHALA, M., NECA, J., DRÁBECK, P., ULRICH, R., SABATOVÁ, V., NEZVEDA, K., RASZYK, J., GAJDUSKOVA, V., 1998. Effects of chronic exposure to PCBs on cytochrome P450 system and steroidogenesis in liver and testis of bulls (*Bos taurus*). Comp. Biochem. Physiol. 120A:65-70.

MANN, M., HENDRICKSON, R. C., PANDEY, A., 2001. Analysis of proteins ans proteomes by mass spectrometry. Annu. Rev. Biochem. 70, 437-473.

MANN, M., WILM, M., 1995. Electrospray mass spectrometry for protein characterization. TIBS 20, 219-224.

MANSUY, D., 1998. The great diversity of reaction catalysed by cytochrome P450. Comp. Biochem. Physiol. 121C: 5-14.

MAZON, A. F., MONTEIRO, E. A. S., PINHEIRO, G. H. D., FERNANDES, M. N., 2002. HEMATOLOGICAL AND PHYSIOLOGICAL CHANGES INDUCED BY SHORT-TERM EXPOSURE TO COPPER IN THE FRESHWATER FISH, *Prochilodus scrofa. Braz. J. Biol. 62*, 621-631,

MOORE, M.N., 2002. Biocomplexity: the post-genome challenge in ecotoxicology. Aquat Toxicol 59,1-15.

MORRISON, H. G., OLEKSIAK, M. F., CORNELL, N. W., SOGIN, M. L., STEGEMAN, J.J., 1995. Identification of cytochrome P4501A (CYP1A) gene from two teleost fish toadfish (*Opsanus tau*) and scup (*Stenotomus chrysops*), and phylogenetic analysis of CYP1A genes. Biochem. J. 308, 97-104.

MORRISON, H.G., WEIL, E.J., KARCHNEER, S.I., SOGIN, M.L., STEGEMAN, J.J., 1998. Molecular cloning of CYP1A from the estuarine fish *Fundulus heteroclitus* and phylogenetic analysis of CYP1 genes: update with new sequences. Comp. Biochem. Physiol. 121C, 231-240.

MUTLIB, A. E., KLEIN, J. T., 1998. Application of liquid chromatography/mass spectrometry in accelerating the identification of human liver cytochrome P450 isoforms involved in the metabolism of Iloperidone. J. Pharmacol. Exp. Ther. 286, 1285 – 1293.

NEBERT, D. W., GONZALEZ, F. J. , 1987. P450 genes, structure, evolution and regulation. Ann. Rev. Biochem. 56, 945-993.

OMURA, T. & SATO, R.; 1964B. The carbon Monoxide-binding pigment of liver microsomes. II – Solubilization, purification and properties. J. Biol. Chem. 239: 2379-2385.

OMURA, T. , 1999. Forty years of cytochrome P450. Biochem. Biophys Res. Comm. 266:690-698.

OMURA, T.; SATO, R. , 1964a. The Carbon Monoxide-binding pigment of liver microsomes. I - Evidence for its heme protein nature. J. Biol. Chem. 239, 2370-2378.

PAULS, E., BERTOLLO, L. A. C., 1990. Distribution of supernumerary chromosome system and aspects of karyotypic evolution in the genus *Prochilodus* (Pisces, Prochilodontidae). Genetica 81, 117-123.

PEAKALL, D., 1992. Animal biomarkers as pollution indicators. Chapman & Hall, London.

PESONEN, M., KORKALAINEM, M., LAITINEN, J. T., ANDERSSON, T. B. & VAKKURI, O., 2000. 2, 3, 7, 8 – Tetrachlorodibenzo-*p* – dioxin alters melatonin metabolism in fish hepatocytes. Chem. Biol. Interac. 126:227-240.

Prochilodus lineatus Valenciennes, 1836 Characiformes, Prochilodontidae) Induced by sublethal Concentration of Trichlorfon Exposure

QUABIUS, E. S., NOLAN, D. T., BALM, P. H. M. & BONGA, S. E. W., 1998. The influence of polychlorinated biphenyl 126 on tilapia (*Oreochromis mossambicus*) liver. Comp. Biochem. Physiol. 120A:57-63.

RABERGH, C. M. I., VROLIJK, N. H., LIPSKY, M. M., CHEN, T.T., 2000. Differential expression of two *CYP1A* gene in Rainbow trout (*Oncorhynchys mykiss*). Toxicol. Appl. Pharmacol. 165, 195 – 205.

RAUCY, J.L., ALLEN, S.W., 2001. Recents advances in P450 reserch. Pharmacogenetics J. 1, 178-186.

RIVIERE, J. L. & CABANNE, F., 1987. Animal and plant cytochrome P450 systems. Biochimie 69:743-752.

ROBERTS, E. S., ALWORTH, W.L., HOLLENBERG, P.F., 1998. Mechanism – based inactivation of cytochrome P450 2E1 and 2B1 by 5 – Phenyl – 1 – pentyne. Arch. Biochem. Biophys. 354, 295 – 302.

SCHELENK, D., 1999. Necessity of defining biomarker for use in ecological risk assessments. Mar. Pol. Bull. 39, 48-53.

SEN, A., ARINÇ, E., 1998. Preparation of highly purified cytochrome P4501A1 from leaping mullet (*Liza saliens*) liver microsomes and its biocatalytic, molecular and immunochemical properties. Comp. Biochem. Physiol. 121C, 249-265.

SICKMANN, A., MREYEN, M., MEYER, H.E., 2002. Identificationof modified proteins by mass spectrometry. Life 54, 51 – 57.

SIUZDAK, G., 1996. Mass spectrometry for biotechnology. Academic Press. San Diego, California, USA. 161p.

SMOLKA, M.B., MARTINS, D., WINCK, F.V., SANTORO, C.E., CASTELLARI, R.R., FERRARI, F., BRUM, I.J., GALEMBECK, E., DELLA COLLETA FILHO, H., MACHADO, M.A., MARANGONI, S., NOVELLO, J.C., 2003. Proteome analysis of the plant pathogen *Xylella fastidiosa* reveals major cellular and extracellular proteins and a peculiar codon bias distribution. Proteomics 3, 224-237.

STEEN, H., PANDEY, A., 2002. Proteomics goes quantitative: measuring protein abundance. TRENDS Biotechnol. 20, 361 – 364.

STEGEMAN, J. J. & LECH, J. J., 1991. Cytochrome P450 monoxigenase system in aquatic species: Carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ Health Perspec. 90:101-109.

STEGEMAN, J.J., 1989. Cytochrome P450 form in fish: catalytic, immunological and sequence similarities. Xenobiotica. 19:1093-1110.

TIMBRELL, J., 2000. Principles of Biochemical Toxicology. 3ª edição. Taylor & Francis. Londres, 394p.

URLACHER, V. B., LUTZ-WAHL, S., SCHMID, R. D., 2004. Microbial P450 enzymes in biotechnology. Appl Microbiol Biotechnol 64, 317–325

VAN DER OOST, R.; BEYER, J.; VERMEULEN, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Envir. Toxicol. Pharmacol. 13, 57-149.

VEIGA, M.L., RODRIGUES. E.L., PACHECO, F.L., RANZANI-PAIVA, M.J.T., 2002. Histopathologic changes in the kidney tissue of *Prochilodus lineatus* Valenciennes, 1836 (Characiformes, Prochilodontidae) induced by sublethal concentration of Trichlorfon exposure. Braz. Arch. Biol. Technol. 45, 171 – 175.

VILLANUEVA J., CANALS, F., QUEROL, E., AVILÉS, F. X., 2001. Monitoring the expression and purification of recombinant proteins by MALDI – TOF mass spectrometry. Enz. Microb.Technol. 29, 99 – 103.

VROLIJK, N.H.; TARGETT, N. M.; WOODIN, B.R., STEGEMAN, J.J., 1994. Toxicological and ecological implication of biotransformation enzymes in tropical teleost Chaetodon capistratus. Mar. Biol. 119,151-158.

WASHBURN, B. S., BADEN, D. G., GASSMAN, N. J. & WALSH, P.J., 1994. Brevetoxin: tissue distribution and effect on cytochrome P450 enzymes in fish. Toxicon 32:799-805.

WATSON, D. E., REICHERT, W. & DI GIULIO, R. T, 1998. Induction of hepatic CYP1A in channel catfish increases binding of 2-aminoanthracene to DNA *in vitro* and *in vivo*. Carcinogenesis 19: 1495-1501.

WAXMAN, D., J., 1999. P450 gene induction by structurally diverse xenochemicals: central role of nuclear factores CAR, PXR, and PPAR. Arch. Biochem. Bioph. 369: 11-23.

WERCK-REICHHART, D., FEYEREISEN, R., 2000. Cytochrome P450: a success story. Gen. Biol. 1, 1-9.

WHITLOCK, J.P. Jr, 1999. Induction of cytochrome P4501A1. Annu. Ver. Pharmacol. Toxicol. 39, 103 – 125.

WILLIAMS, D. E., LECH, J. J. & BUHLER, D. R., 1998. Xenobiotics and xenoestrogens in fish: modulation of cytochrome P450 and carcinogenesis. Mut. Res. 399: 179-192.

WOOTTON, R. J., 1992. Ecology of Teleost Fishes. 3ª ed. Chapman & Hall. Londres, 404p.

ZHANG, Z. Y., KING, B. M., WONG, Y.N., 2001. Quantitative liquid chromatogrphy/mass spectrometry/ mass spectrometry Warfarin assay for *in vitro* cytochrome P450 studies. Anal. Biochem. 298, 40-49.

6– APENDICE – TRABALHOS PARALELOS

6.1 – da Silva, M.E.F., Meirelles, N.C., 2004. Effects of Trifluoperazine in Cytochrome P450 of Prochilodus scrofa – the interaction of a surfactant – like compound in CYP1A system. **Submetido para Toxicology in vitro em setembro de 2004.**

EFFECTS OF TRIFLUOPERAZINE IN CYTOCHROME P450 OF PROCHILODUS SCROFA – THE INTERACTION OF A SURFACTANT – LIKE COMPOUND IN CYP1A SYSTEM.

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RUNNING TITLE – TRIFLUOPERAZINE AND CYP SYSTEM

ABSTRACT

Cytochromes P450 (CYP) constitute a superfamily of hemeproteins that play a vital role in the metabolism of a wide variety of endogenous and xenobiotic compounds. Xenobiotic metabolism and the role of CYP are of particular interest in studies regarding the prevention of the damage caused by xenobiotics. We investigated, in this study, the interaction of Trifluoperazine (TFP) with hepatic CYP system in Curimbatá, a Brazilian fish. Aiming to clarify the effects of surfactants - like compounds in the fish monooxigenase system, through *in vitro* study, the effects of TFP were analyzed using CYP system enzymes as experimental model. Despite the fact that CYP content was reduced in the presence of TFP, we can say that this inhibition is selective since it was possible to detect an increase in the activity of EROD, which showed that TFP was being metabolized by the system. Our hypothesis was that TFP, or its metabolites, is able to interact with CYP system leading to the inhibition of the process. This hypothesis was confirmed through experiments that showed a decrease in the absorption of P450 – CO complex peak (450nm) and the increase of the absorption of a P450 – TFP complex (456nm). This peak is a characteristic of N – oxidation metabolites, principal pathway of phenotiazines compounds metabolism. This interaction is able to change the protein structure, since the experiment with tryptophan fluorescence showed that the system was being exposed to a hydrophilic environment (quenching at 350nm) and there is a shift from 350nm to 470nm, confirming the interaction of N – metabolites with CYP system. Tryptophan fluorescence also showed that the membrane environment is affected by TFP, what was confirmed by the results of lipid peroxidation.

Since CYP plays an important rule in the metabolic activation and/or disablement of xenobiotics, the effects of nitro compounds in CYP system requires additional studies.

KEYWORDS: fish, P450, EROD, TFP

1 - INTRODUCTION

CYP, whose name is derived from the characteristic peak at 450nm in the carbon monoxide difference spectrum of reduced CYP (Omura and Sato, 1964), constitutes a superfamily of heme-thiolate enzymes involved in the phase I of hydrophobic compounds oxidative biotransformation. It includes pesticides, dioxins, drugs and polycyclic hydrocarbons (Benhardt, 1995, Omura and Sato, 1964, Mansuy, 1998, Omura, 1999). CYP and NADPH – cytochrome P450 reductase are the key enzymes of this process. The reductase is responsible for the transference of two electrons from NADPH to CYP, which inserts one atom of molecular oxygen into the substrate and reduces another atom with two electrons to water; these electrons may be supplied by cytochrome b5 (Omura, 1999, Halkier, 1996). The electrons transferences are largely responsible for the generation of reactive oxygen species (ROS), even in the steady state or in the biotransformation process.

Some CYP enzymes are substrate inducible, a property that allows the cell to adapt itself to the chemical changes in the environment. One of the major proteins responsible for the metabolism of xenobiotic in liver is the CYP1A family, which is mainly regulated by the aromatic aryl hydrocarbon receptor (AhR), by the AhR nuclear carrier and the estrogen receptor α (hERα) (Whitlock, 1999, Lewis et al, 2002).

The present study characterized the interactions between Trifluoperazine with CYP from microsomes of Curimbatás' liver, using *in vitro* assay.

Trifluoperazine (TFP) (Figure 1) is a phenothiazine derivative used in the treatment of psychiatric disorder. This family of compounds induces the aryl

hydrocarbon receptor and, consequently, the administration of TFP could affect CYP1A isoenzyme (Tateishi et al, 1999). Many researchers have shown that surfactants – like compounds inhibit CYP activity, not only through time- and concentration-dependent mechanisms, but also through species-dependent mechanisms (Lee et al, 1996, Yun et al, 1996, Yun et al, 1997, Guengerich et al, 1998, Hosea and Guengerich, 1998, Mountfield et al, 2000, Inouye et al, 2001). Since all CYP in eukaryotic organisms are membrane bound proteins, the protein – lipid and protein – protein interactions are responsible for the functional control of CYPs, and the lipid environment contributes to the interaction of CYP/reductase/cytochrome b5 (Benhardt, 1995).

(Figure 1)

Prochilodus scrofa, known as Curimbatá, is a Brazilian tropical fish belonging to the Prochilodontidae family. Fish was chosen as biological model since most part of pollutants is dispersed in water and fish are one of the most important protein sources for humans. Curimbatás were chosen by their: (a) seasonal migratory behavior for reproductive purposes (Godoy, 1975), which put them in contact with pollutants distributed in different parts of the water column; (b) high tolerance to hypoxia, exhibiting critical oxygen consumption when oxygen levels drop (Fernandes et al, 1995), (c) detritivorous feeding, meaning that the fishes are in contact with xenobiotics that interact with upon stone algae and in sediment grown ones. For these reasons, Curimbatá may be considered a model for ecotoxicology studies, since xenobiotics are able to act upon any of these characteristics.

Our hypothesis is that Trifluoperazine could interact mainly with proteins, but also with membrane lipids, affecting the biotransformation process catalyzed by CYPs. Induction or inhibition of CYP activity by TFP might contribute to elucidate the pharmacokinetics of TFP when it is metabolized by CYP system.

2 - MATERIAL AND METHODS

2.1 - MATERIAL

Bovine serum albumin; glycine, adrenalin, hydrogen peroxide, NADPH; glutathione reductase, 7-ethoxyresorufin, ter-butylhydroperoxide and Trifluoperazine were purchased from SIGMA Co.

2.2 - FISH

Twenty-four, male and female, Prochilodus scrofa (Pisces, Chariciforme, Prochilodontidae), out of the reproductive cycle, were obtained from the Center of Aquiculture Research and Training (CEPTA, Pirassununga, SP, Brazil). They were raised in artificial open-air reservoirs with pollutant-free water. In the laboratory, fish were anesthetized with spike oil and sacrificed by decapitation. Livers were removed and washed with saline solution. Microsomes were prepared in pools of 4-5 livers.

2.3 - MICROSOME PREPARATION

Microsomal fractions were prepared by the standard procedure: tissues were homogenized in ice-cold 0.1M potassium phosphate, pH 7.5, containing 0.15M KCl and 0.1mM EDTA. Crude homogenate was centrifuged at

12,000Xg for 20 min and the supernatant centrifuged at 105,000Xg for 60 min. The pellet was resuspended in potassium buffer and re-centrifuged at 105,000Xg for 60 min. Microsomes were isolated in the pellet. The microsomal fraction was resuspended in the same buffer solution containing 20% glycerol and was frozen at –70ºC (Vrolijk et al, 1994). Proteins were determined according to Lowry et al. (1951). CYP content was assayed in microsomes according to the method of Omura and Sato (1964), and expressed as nmol/mg protein based on the millimolar extinction coefficient of 91 mM-1 cm-1. However, the concentration of P450 – TFP complex was expressed based in the millimolar extinction coefficient of 75 mM-1 cm-1, as described by Werringloer and Estabrook (1975).

2.4 - BIOCHEMICAL ASSAY

Trifluoperazine (0 –500μM) metabolism assays were performed at 25-28ºC. The assay consisted of protein, which concentration was 1.0mg/mL, 0.1M Potassium Phosphate buffer, pH7.4 and NADPH regeneration System (10mM Glucose 6 – Phosphate, 1mM NADP+ and 0.5 U/ml Glucose 6 – Phosphate Desydrogenase) over a 20 min period. The control consisted of a sample with no surfactant but with NADPH regeneration system in zero minutes' time. After the recording of the P450 content, of the TBARS levels and the Tryptophan fluorescence, TFP was added to the system. Three minutes later, a sample of the suspension was separated and the biochemical parameters, analyzed.

Estimate of P450 inactivation was performed according to the method described by Omura and Sato (1964), after different periods of

incubation. Results were expressed as percentages of the initial content. Absorption was measured on a Hitachi U- 2010 – UV/Vis Spectrophotometer (Hitachi Instruments, Inc, Japan).

7-Ethoxyresorufin – O – deethylase activity was monitored by the continuous spectrofluorimetric procedure of Prough et al. (1978). Reactions (1mL, 37ºC) contained 1.0mg/mL of microsomal protein, 10μM 7 – etoxyresorufin and NADPH regeneration System (10mM Glucose 6 – Phosphate, 1mM NADP+ and 0.5 U/ml Glucose 6 – Phosphate Desidrogenase) in 0.1M Tris-HCl buffer, at pH 7.4. Fluorescence intensity was measured on a Hitachi F4500 spectrofluometer (Hitachi Instruments, Inc, Japan).

Determination of TBARS was used to reach endogenous lipid peroxidation in microsomes through the products derived from the lipid oxidation with tiobarbituric acid (TBA) (Ohkawa et al., 1979). Microsomal suspension was diluted to a concentration of 0.5mg/ml in phosphate buffer. The mixture was incubated with different concentrations of each xenobiotic in the presence and absence of NADPH Regeneration System for 20 minutes, as described before. The suspensions were treated with 500 μl of tricloroacetic acid (TCA) 25%, so the microsomal proteins could precipitate. The microsomal suspension was centrifuged and 1ml of the supernatant was removed and resuspended in 1 ml of TBA (0.1g TBA/9 ml NaCl 0.5M). The mixture was maintained in boiling water for 60 minutes, cooled in ice, and then measured spectrophotometrically at 535nm. Absorption was reported as nmoles/TBARS/mg prot.

The effects of surfactants interaction with the CYP system were analyzed by changing the Tryptophan fluorescence; all experiments were

performed in 100μM potassium phosphate buffer, at room temperature, containing 1mg/ml of microsomal protein. For the tryptophan fluorescence assay, the mixture was excited at 295nm and the emission was recorded at 305-580nm. Fluorescence intensity was measured on a Hitachi F4500 spectrofluorometer (Hitachi Instruments, Inc, Japan). The protein concentration was 1.0 mg/mL.

All experiments were done considering n=10 and in triplicate. Correlation between P450 content and EROD activity was done through linear regression and differences among treatment and control groups were verified through ANOVA and Tukey tests (Zar, 1996).

3 – RESULTS

3.1 – CYP CONTENT

The initial concentration of total CYP was 22nmoles/mg prot., which is in agreement with the literature.

When microsomal suspension was mixed with different concentrations of TFP, absorbance spectra indicative of NADPH consumption was obtained (data not shown), which indicated that the xenobiotic was being metabolized. With increasing concentration of TFP, the total content of CYP decreases when compared to the control (Figure 2). At TFP 500 μM, no spectral CYP was detectable after 20 minutes of incubation, suggesting that TFP significantly destroyed the hepatic CYP in Curimbatá, compared to the control (0,0 μM of TFP).

(FIGURE 2)

The activity of CYP1A was measured through the EROD activity. This enzyme catalyzes the CYP1A reaction and its activity could be measured by an increase in the fluorescence intensity, once resorufin (the product of the enzyme reaction) is fluorescent. According to Figure 3, the presence of TFP increases the EROD activity, an opposite behavior to the one observed in the total CYP content, what suggests a selective inhibition of CYP1A. Those results were confirmed by the negative correlation found between P450 content and EROD activity ($r = -0.8 \pm 0.078$; $p<0.001$), which shows that the decrease of total CYP content was not accompanied by the decrease of CYP1A (determined by EROD activity).

(FIGURE 3)

In the absence of TFP, the spectrum shows a peak of absorbency at 450nm. Increasing TFP concentration, the peak at 450 shifts to 456nm (Figure 4). This shift does not represent the P450 – CO complex, but a P450 – TFP complex that could inhibit some isoforms of CYP. This complex arrangement depends on the TFP concentration ($r = 0.9969$, $p = 0.0031$) and is not a consequence of the CYP destruction. Since the experiments were done using varying concentrations of TFP to alter the amount of product formed, it was observed that 75 –85% of CYP could be complex.

(FIGURE 4)

To discard the hypothesis that a disrupting in microsomal membrane could be the responsible by the decrease of the CYP content when TFP was present in the system, peroxyl radicals ($LOO^{\cdot}$) were searched by the TBARS method. According to Figure 5, only at high concentration of TFP in the system, levels of $LOO^{\cdot}$ increase and can contribute to disrupt the membrane environment and destroy the CYP catalytic activity.

(FIGURE 5)

The main hypothesis of this study was that TFP interact with CYP in microsomal samples. In order to prove this hypothesis, we analyzed the effects of surfactant upon tryptophan, after incubating microsomal samples during 20 minutes. Increasing TFP concentration progressively decreases the tryptophan emission at 350nm, suggesting that tryptophan residues are exposed to the aqueous phase (Figure 6, peak A). A decrease in the intensity of this band occurs as a result of quenching and a second peak appears at 460nm, proposing an interaction of TFP, or its metabolites, with the MFO components.

4 - DISCUSSION

The phenotiazines are important drugs in the treatment of schizophrenia and other psychoses (Murray and Reidy, 1989). Trifluoperazine is a phenothiazine derivative that acts as a calmodulin antagonist and is able to prevent necrosis induced by CCl4 (Malheiros et al., 1998, Malheiros et al, 2000). Numerous studies have demonstrated that the main group of enzymes involved in phenotiazynes metabolism was the cytochrome P450 (CYP)

subfamilies. This class of antipsychotics induces the aryl hydrocarbon receptor (Murray and Reidy, 1989, Tateishi et al., 1999).

Our hypothesis was that TFP and its metabolites may interact with microsomal membrane proteins, including P450 system, altering the biotransformation process.

Initial experiments revealed that TFP appeared to destroy the CYP content; what was in agreement with literature that shows the inhibition of CYP by chlorpromazine and thioridazine, considered classical phenotiazines (Breyer, 1972, Murray and Reidy, 1989, Murray, 1992).

The first explanation was that TFP may interact with the lipids of the endoplasmic reticulum membrane, altering lipid – CYP – reductase interaction, once, at pH 7.4, TFP is protonated and can act as a surfactant in the membrane system (Ruggieiro and Meirelles, 1998, Malheiros et al, 1998, Malheiros et al, 2000). Once the membrane integrity is essential to CYP activity, any compound that disrupts this system can destroy the CYP activity. However, the present finding showed that CYP1A was not markedly inhibited by TFP, suggesting that this phenotiazinic may inhibit the activity of specific CYPs in liver (Werringloer and Estabrook, 1975, Murray, 1992, Tateishi et al, 1999). A second suggestion was that oxidative damage was responsible by the CYP inhibition. Nevertheless, our results showed that TFP has a two-phase effect in the production of peroxyl radicals. When TFP is present in low concentration, it is able to prevent these events, which was in agreement with literature; but when TFP is present in high concentration, it could disrupt the system through the saturation of the membrane environment (Malheiros et al, 1998).

The major pathways *in vitro* of phenothiazine metabolism are N-dealkylation, hydroxylation, sulfoxidation and N – oxidation of the aromatic ring system; this last process plays a dominant role in *in vitro* process at high concentration of TFP (Breyer, 1972, Gaertner et al, 1974). This process forms a nitro compound that exhibits a strong interaction with other reduced heme proteins, like hemoglobin and myoglobin (Mansuy et al, 1976). Some compounds are able to form, during oxidative metabolism by CYP system, complexes with Fe (II). They absorb maximally at 455 nm. The TFP nitrogen may have the ability to donate electrons and form a coordinate covalent bond with CYP (James and Franklin, 1975, Werringloer and Estabrook, 1975, Mansuy et al, 1976, Mansuy et al, 1989). Our results suggest that some metabolic intermediaries of the reactions that take place around the nitrogen were joined to CYP, forming a 455nm complex.

According to Black (1992), tryptophan is an aminoacid, which is placed in the cytoplasmatic region of CYP, NADPH cytochrome P450 reductase and cytochrome b5, so it could indicate the interaction of any xenobiotic with them. Any alteration in this region of the protein implies CYP inactivation (Black, 1992, Neunaber and Achazi, 1999). Nitro compounds must bind close to one or more tryptophan residues, exposing them to the aqueous environment and leading to changes in protein structure and in membrane environment, thus altering the lipid – protein interaction (Ruggiero and Meirelles, 1998). This interaction could also alter the conformation of the active site and may inhibit the CYP activity via a competitive inhibition mechanism, already described by other authors (de Montellano and Correa, 1983, Halpert, 1995).

The experiment also suggests that the peak observed at 456nm is a result of the complex formation with the reduced cytochrome P4450 and N – oxidation metabolites. Werringloer and Estrabook (1975) had shown that this interaction occurs through the N – oxidation products with an unpaired electron that is shared with the ferric cytochrome P450' heme.

Most part of chemical pollutants is nitro compounds, so those interactions previously discussed alter the xenobiotic detoxification process. The inactivation of the CYP system may alter important biological functions, such as the seasonal reproduction and fetal development. The inhibition of the CYP1A catalytic activity by those compounds may have important physiological and pharmacological implications. Since CYP plays an important rule in the metabolic activation and/or disablement of xenobiotics, the effects of nitro compounds in CYP system requires additional studies.

In conclusion, these preliminary results should lead to a correlation between the structure of some nitro compounds and their ability to produce a 455 nm absorbing CYP – complex, after the metabolism process, that could alter the catalytic activity of this protein. The chemistry of the product formed during the Trifluoperazine metabolism is still to be defined.

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5 – BIBLIOGRAPHY

Benhardt, R. (1995) Cytochrome P450: structure, function and generation of reactive oxygen species. Rev. Physiol. Biochem. Pharmacol. 127, 138-221.

Black, S.D. (1992) Membrane topology of mammalian P450 cytochromes. FASEB J. 6, 680-685.

Breyer, U., 1972. Perazine, Chlorperazine and Imipramine as inducers of Microsomal Drug metabolism. Arch. Pharmacol. 272, 277 – 288.

de Montellano, P.R. and Correa, M,A. (1983) Suicidal destruction of cytochrome P450 during oxidative drug metabolism. Ann. Rev. Pharmacol. Toxicol. 23, 481 – 503.

Fernandes, M.N., Barrionuevo, W.R and Rantin, F.T. (1995) Effects of the thermal stress on respiratory responses to hypoxia of a South American Prochilodontid fish, Prochilodus scrofa. J. Fish. Biol. 46, 123-133.

Gaertner, H., J., Breyer, U., Liomin, G., 1974. Metabolism of trifluoperazine, fluophenazine, prochlorperazine and perphenazine in rats: *in vitro* and urinary metabolites. Biochem. Pharmacol. 83, 303 – 311.

Godoy, M. P. (Eds) (1975). Peixes do Brasil – Subordem Charíciforme. Editora Franciscana. Piracicaba, S.P.

Guengerich, F.P, Hosea, N.A, Parikh, A., Bell-Parikh, C., Johnson, W.W., Gillam, E.M.J and Shimada, T. (1998) Twenty years of biochemistry of human P450. Purification, expression, mechanism and relevance to drug. Drug Met. Disp. 26, 1175-1178.

Halkier, B. A. (1996) Catalytic reactivitieses and structure/function relationship of cytochrome P450 enzymes. Phytochemistry 43, 1-21.

Halpert, J.R. (1995) Structural basis of selective cytochrome P450 inhibition. Ann. Rev. Pharmacol. Toxicol. 35, 29-53.

Hosea, N.A. and Guengerich, F.P. (1998) Oxidation of nonionic detergents by cytochrome P450 enzymes. Arch. Biochem. Bioph. 353, 365-373.

Inouye, K., Kondo, S., Yamamura, M., Nakanishi, D. and Sakaki, T. (2001) Inhibitory effects of detergents rat CYP1A1- dependent monooxygenase: comparison of mixed and fused system consisting of rats CYP1A1 and yeast NADPH – P450 reductase. Biochem. Bioph. Res. Comm. 280, 1346-1351.

James, R. C. and Franklin, M. R. (1975). Comparison of the formation of cytochrome P-450 complexes absorbing at 455nm in rabbit and rat microsomes. Biochem. Pharmacol. 24, 835 – 838.

Lee, P.C., Patra, S.C., Stelloh, C.T., Lee, W. and Struve, M. (1996) Interaction of nonylphenol and hepatic CYP1A in rats. Biochem. Pharmacol. 52, 885-889.

Lewis, D.F.V., Jacobs, M.N, Dickins, M. and Lake, B.G. (2002) Quantitative structure-activity relationship for inducers of cytochromes P450 and

nuclear receptor ligands involved in P450 regulation within the CYP1, CYP2 CYP3 and CYP4 families. Toxicology 176, 51-57.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurements with Folin-phenol reagent. J. Biol. Chem. 193, 265 – 275.

Malheiros, S. V., P., de Paula, E., Meirelles, N.C., 1998. Contribution of trifluoperazine/lipid ration and drug ionization to hemolysis. Biochim. Biophys. Acta 1373, 332 – 340.

Malherios, S. V., P., de Paula, E., Meirelles, N.C., 2000. Pathways involved in trifluoperazine - , dibucaine – and praziquantel – induced hemolysis. Biophys. Chem. 83, 89 – 100.

Mansuy, D. (1998) The great diversity of reaction catalysed by cytochrome P450. Comp. Biochem. Physiol. 121C, 5-14.

Mansuy, D., Battioni, P., Battioni, J.P. (1989). Chemical model system for drug – metabolizing cytochrome P450 – dependent monooxygenases. Eur. J. Biochem. 184, 267 – 285.

Mansuy, D., Beaune, P., Chottard, J. C., Bartoli, J. F., Gans, P. (1976). The nature of the “455nm absorbing complex” formed during the cytochrome P450 dependent oxidative metabolism of amphetamine. Biochem. Pharmacol. 25, 609 – 612.

Mountfield, R.J, Senepin, S., Schleimerm, M., Walter, I., Bittner, B. (2000) Potential inhibitory effects of formulation ingredients on intestinal cytochrome P450. Int. J. Pharmac. 211, 89-92.

Murray, M., 1992. Inhibition of cytochrome P450 2B1 in rat liver promazine and chlorpromazine. Biochem. Pharmacol. 44, 1219 – 1222.

Murray, M., Reidy, G. F. (1989). *In vitro* inhibition of hepatic drug oxidation by thiorodazinr. Biochem. Pharmacol.38, 4359 – 4365.

Murray, M., Reidy, G.F., 1989. In vitro inhibition of hepatic drug oxidation by thioridazine. Biochem. Pharmaco. 38, 4359 – 4365.

Neunaber, R. and Achazi, R.K. (1999) A novel endogenous inhibitor of 7-ethoxyresorufin-O-deetylase activity isolated from liver cytosolic fraction of bream (Abramis brama L.). Comp. Biochem. Physiol. 124B, 89-100.

Ohkawa, H., Ohishi, N. and Yagi, K. (1979) Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. Anal. Biochem. 95, 351-358.

Omura, T. (1999) Forty years of cytochrome P450. Biochem. Biophys Res. Com. 266, 690-698.

Omura, T. and Sato, R. (1964) The Carbon Monoxide-binding pigment of liver microsomes. I - Evidence for its heme protein nature. J. Biol. Chem. 239, 2370-2378.

Prough, R.A., Burke, M.D. and Mayer, B.T. (1978) Direct fluorimetric methods for measuring mixed-function oxidase activity. Meth. Enzymol. 52, 374-377.

Ruggieiro A.C., Meirelles, N. C. (1998).Effects of Trifluoperazine on the conformation and dynamics of membrane proteins in human erythrocytes. Mol. Gen. Metab. 64, 148 – 151.

Tateishi, T., Kumai, T., Watanabe, M., Tanaka, M., Kobayshi, S. (1999). A comparison of the effects of five phenothiazines on hepatic CYP isoenzymes in rats. Pharmacol. Toxicol. 85, 252 – 256.

Vrolijk, N.H., Targett, N.M., Woodin, B.R. and Stegman, J,J. (1994) Toxicological and ecological implication of biotransformation enzymes in the tropical Teleost Chaetodos capistratus. Mar. Biol. 119, 151-158.

Werringloer, J., Estabrook, R.W. (1975). Heterogeneity of liver microsomal cytochrome P450: the spectral characterization of reactants with reduced cytochrome P450. Arch. Biochem and Biophys. 167, 270 – 286.

Whitlock, J.P. (1999) Induction of CYP1A1. Annu. Rev. Pharmacol. Toxicol. 39, 103-125.

Yun, C.H., Song, M. and Kim, H. (1997) Conformational changes of cytochrome P4501A2 induced by phospholipids and detergents. J. Biol. Chem. 272, 19725-19730.

Yun, C.H., Song, M., Ahn, T. and Kim, H. (1996) Conformational changes of cytochrome P4501A2 induced by sodium chloride. J. Biol. Chem. 271, 31312-31316.

Zar, J.H. (1996) Biostatistical Analysis. Prentice Hall, EUA.

FIGURE SUBTITLES

Figure 1 – Trifluoperazine Chemical Structure

Figure 2 – Cytochrome P450 content in microsomal suspension incubated with Trifluoperazine. (■) Control; (●) 0.1mM; (▲) 0.2mM and (◆) 0.5mM. Values represent mean ± SD of measurement from triplicate enzymes analysis of 9 microsomal sample. All sample were statistically significant ($p<0.05$) related to the control

Figure 3 – EROD activity in microsomal suspension incubated with Trifluoperazine (TFP). (■) Control; (●) 0.1mM; (▲) 0.2mM and (◆) 0.5mM. Values represent mean ± SD of measurement from triplicate enzymes analysis of 9 microsomal sample.

Figure 4 – Formation rate of the 455 nm complex after 20 minutes-incubation with Trifluoperazine. Values represent mean ± SD of measurement from triplicate enzymes analysis of 9 microsomal sample.

Figure 5 - Effects of Trifluoperazine in the formation of peroxyl radicals. Values represent mean ± SD of measurement from triplicate enzymes analysis of 9 microsomal sample; * $p<0.05$.

Figure 6 - Effects of Trifluoperazine in the fluorescence spectrum of Tryptophan after 20 minutes-incubation. (_____) Control; (____) 100µM; (-----) 200µM and (+++) 500µM. Values represent one measurement from the analysis of 9 microsomal samples.

FIGURE 1

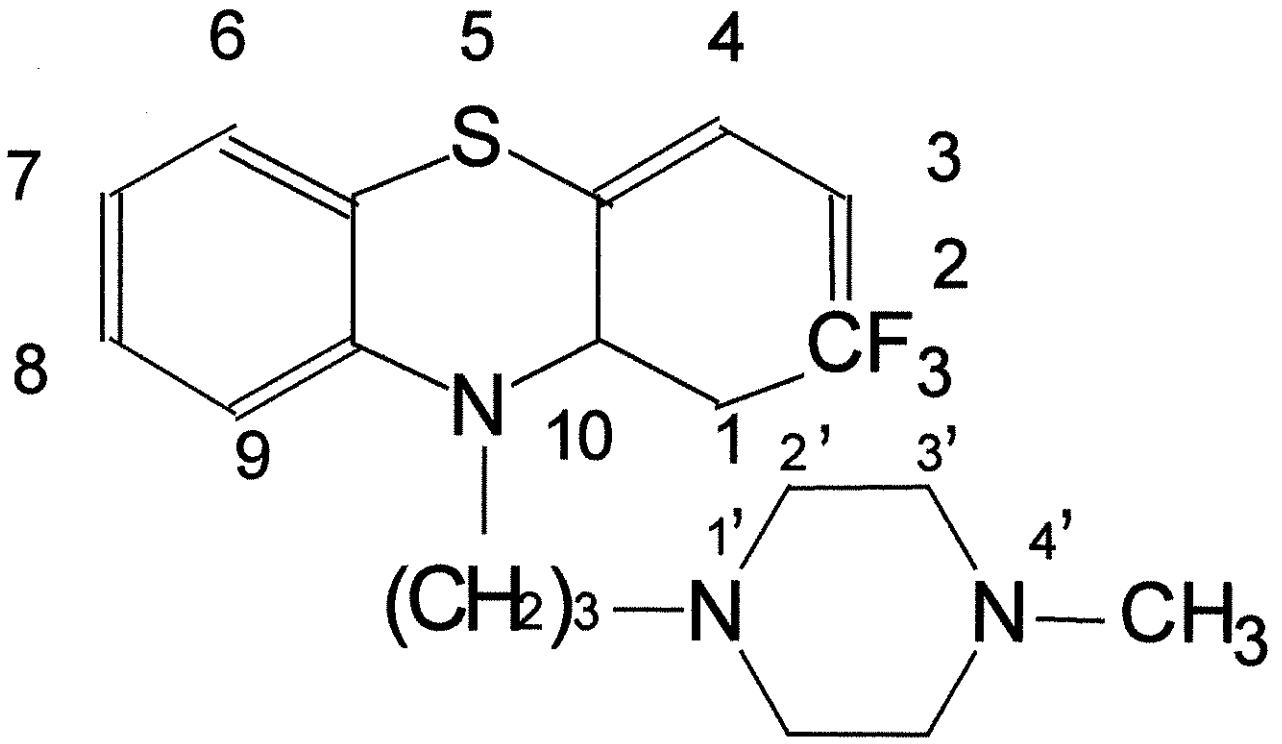


FIGURE 2

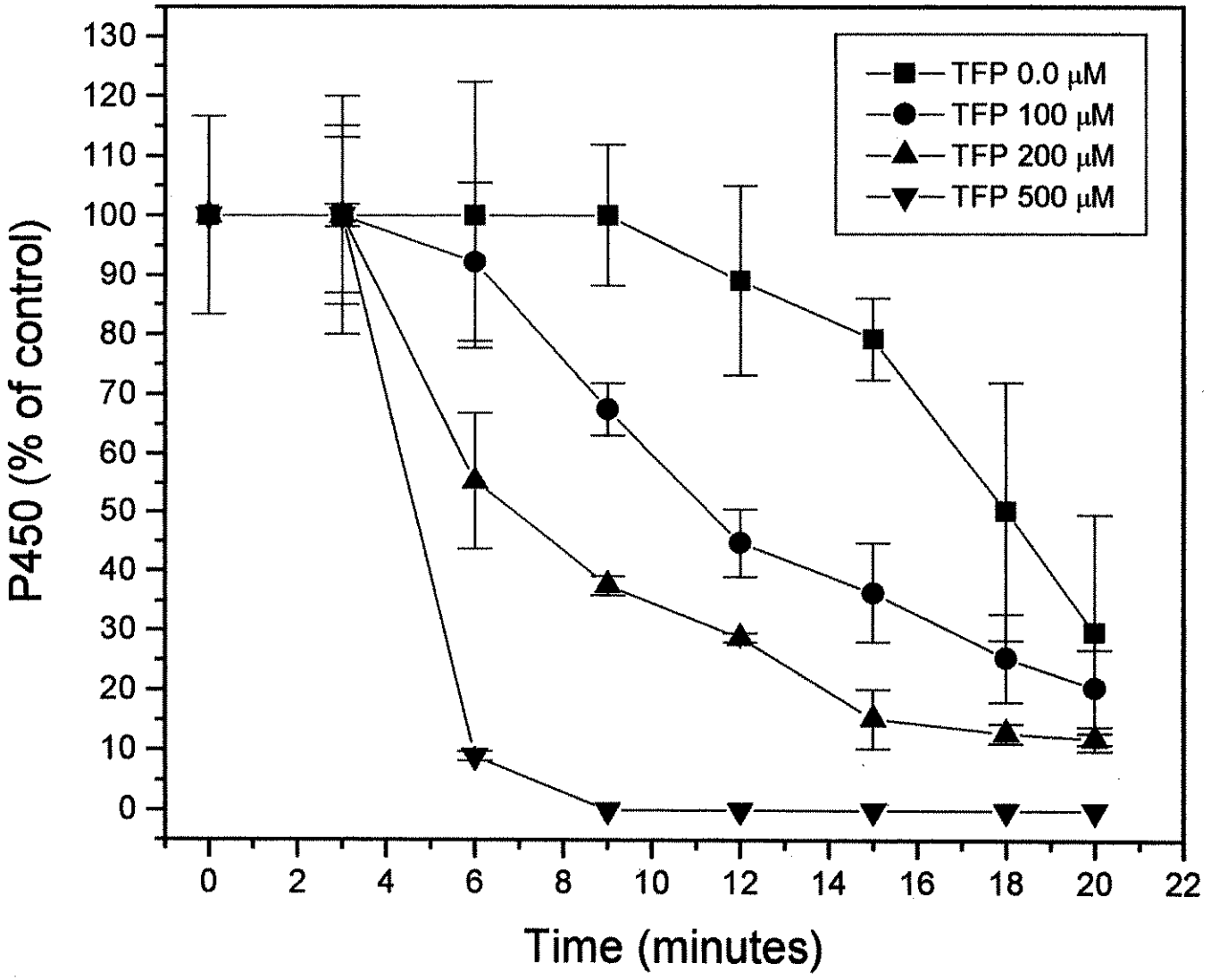

TFP 0.0 μM
TFP 100 μM
TFP 200 μM
TFP 500 μM
P450 (% of control)
Time (minutes)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
0
2
4
6
8
10
12
14
16
18
20
22

FIGURE 3

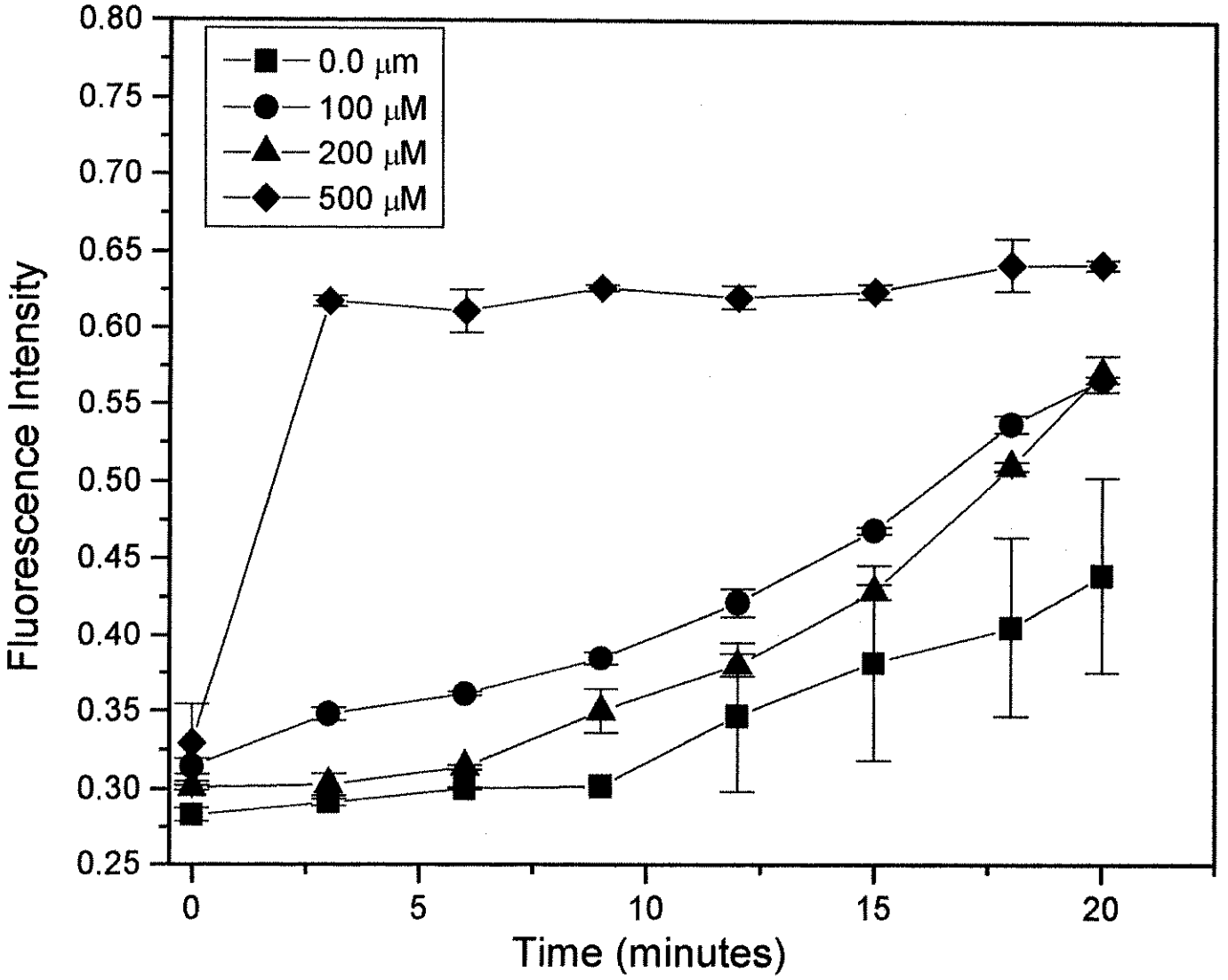
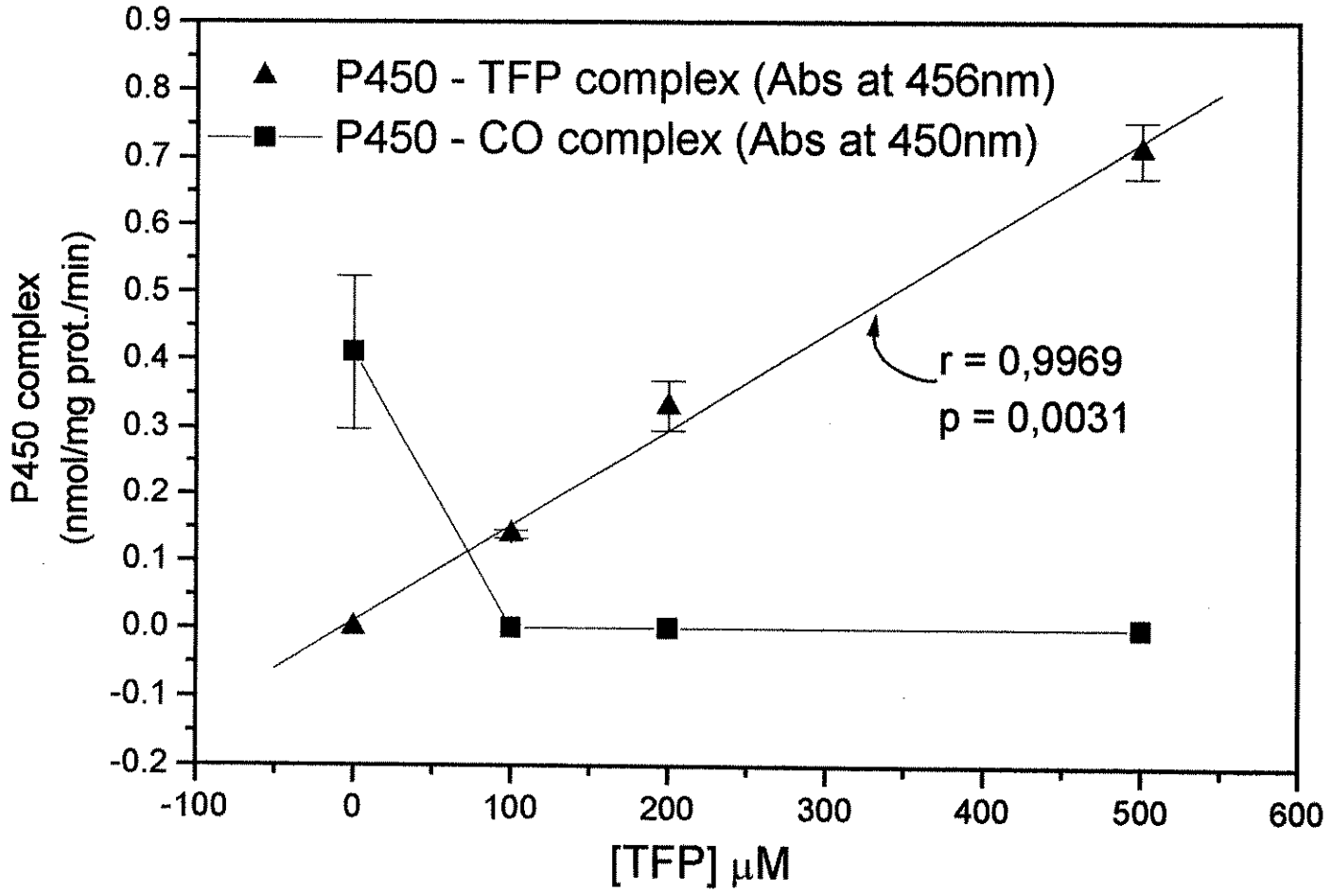

0.0 μm
100 μM
200 μM
500 μM
Fluorescence Intensity
Time (minutes)
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0
5
10
15
20

FIGURE 4


P450 - TFP complex (Abs at 456nm)
P450 - CO complex (Abs at 450nm)
r = 0,9969
p = 0,0031
P450 complex
(nmol/mg prot./min)
[TFP] μM
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-100
0
100
200
300
400
500
600

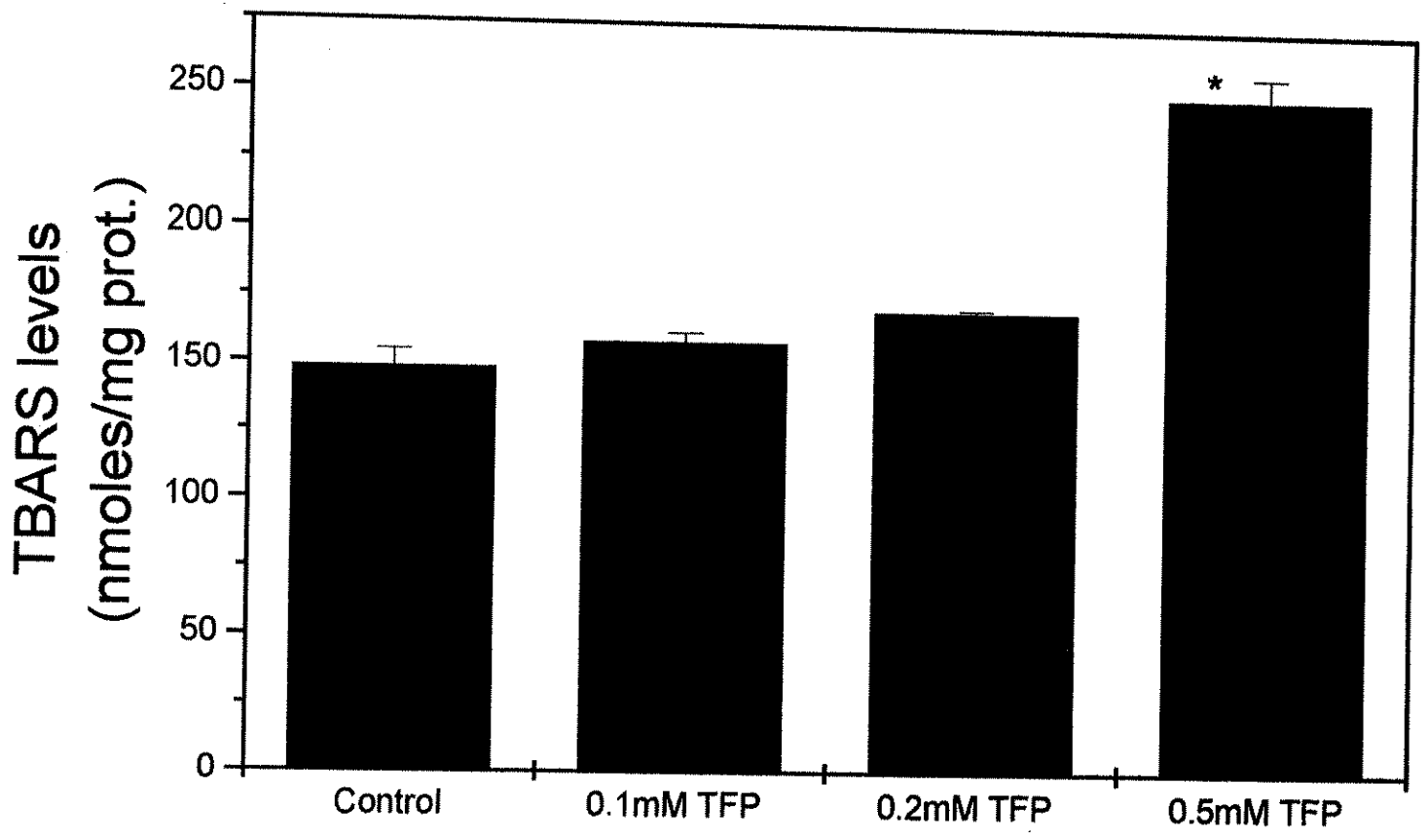
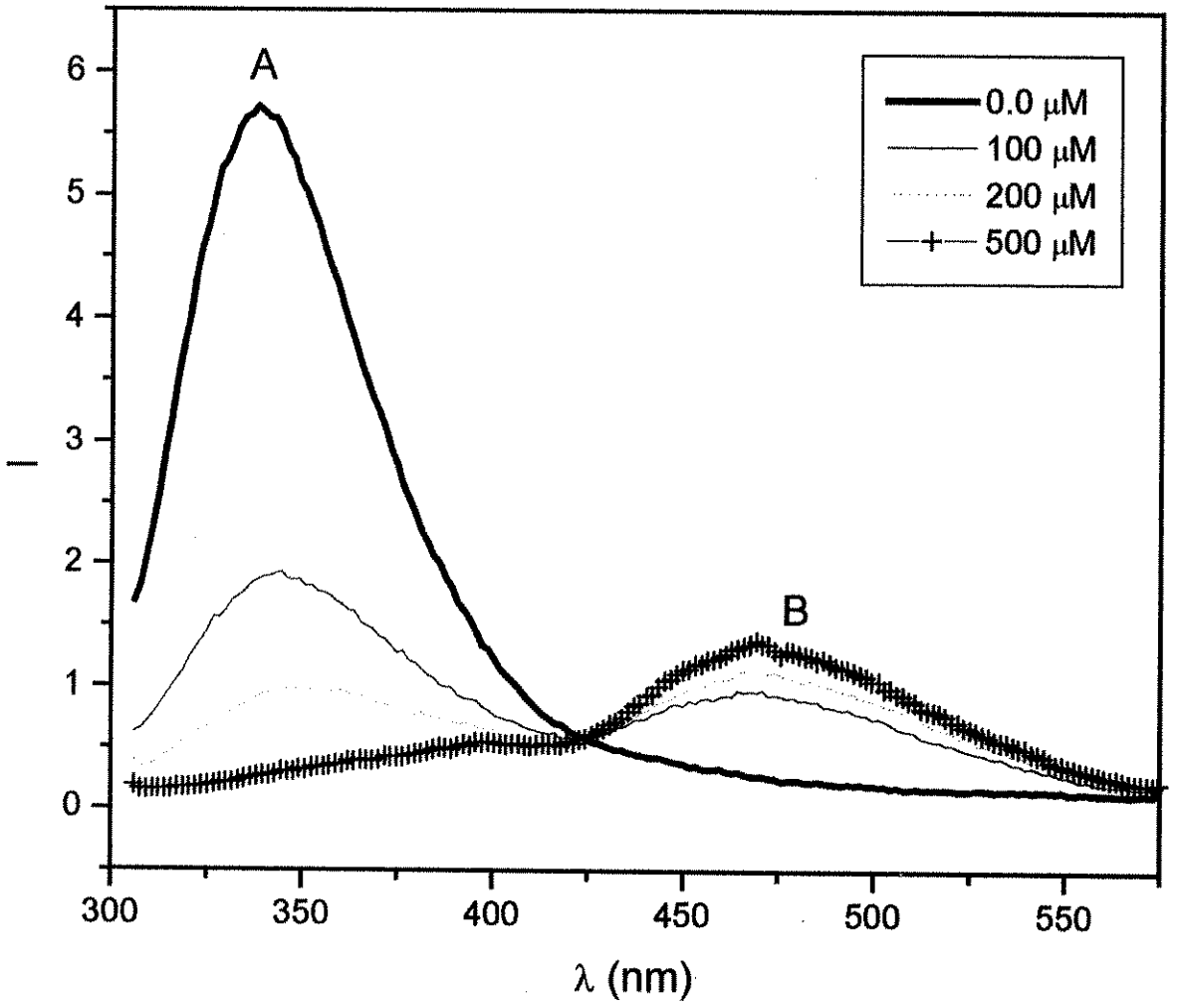

TBARS levels
(nmoles/mg prot.)
250
200
150
100
50
0
*
Control
0.1mM TFP
0.2mM TFP
0.5mM TFP

FIGURE 5

FIGURE 6


A
B
0.0 μM
100 μM
200 μM
500 μM
I
λ (nm)
0
1
2
3
4
5
6
300
350
400
450
500
550