UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
MESTRADO EM BIOQUÍMICA E FISIOLOGIA

Caracterização da acetilcolinesterase cerebral do tambaqui (*Colossoma macropomum*) e efeito de pesticidas organofosforados e carbamatos sobre sua atividade

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RECIFE, 2008

Assis, Caio Rodrigo Dias de

Caracterização da acetilcolinesterase cerebral do tambaqui (*Colossoma macropomum*) e efeito de pesticidas organofosforados e carbamatos sobre sua atividade / Caio Rodrigo Dias de Assis. – Recife: O Autor, 2008.

63 folhas : Il., fig., tab.
Orientadora: Ranilson de Souza Bezerra.
Co-Orientadora: Luiz Bezerra de Carvalho Júnior.
Dissertação (Mestrado) – Universidade Federal de Pernambuco. CCB. Bioquímica e Fisiologia, 2010.

Inclui bibliografia e anexos.

1. Enzimas 2. Pesticidas – Aspectos ambientais 3. Peixe – Efeito da poluição da água I. Título.

572.7 CDD (22.ed.) UFPE/CCB-2010-128

Ata da defesa de dissertação da Mestranda **Caio Rodrigo Dias de Assis,** realizada em 09 de outubro de 2008, como requisito final para obtenção do título de Mestre em Bioquímica e Fisiologia da Universidade Federal de Pernambuco.

Às 09:10 horas, do dia 09 de outubro de 2008, foi aberto, no Auditório Prof. Marcionilo Lins – Depto. de Bioquímica/CCB/UFPE, o ato de defesa de dissertação do mestrando **Caio Rodrigo Dias de Assis**, aluno do Programa de Pós-Graduação em Bioquímica e Fisiologia (Mestrado), do Centro de Ciências Biológicas, da Universidade Federal de Pernambuco. Iniciando os trabalhos a *Profa. Dra. Patrícia Maria Guedes Paiva, em substituição a Coordenadora do* Programa supra citado, fez a apresentação do aluno, de seu orientador, Prof. Dr. Ranilson de Souza Bezerra e da Banca Examinadora composta pelos professores doutores: Ranilon de Souza Bezerra, na qualidade de Presidente, Prof. Dr. Silvio José de Macedo, do Depto. de Oceanografia/CTG/UFPE, Dra. Karina Ribeiro, Pesquisadora do Depto. de Bioquímica/CCB/UFPE e Profa. Dra. Patrícia Maria Guedes Paiva, do Depto. de Bioquímica/CCB/UFPE . Após as apresentações, o Sr. Presidente convidou o aluno para a apresentação de sua dissertação intitulada: **"Caracterização da acetilcolinesterase cerebral do tambaqui (*Colossoma macropomum*) e efeito de pesticidas organofosforados e carbamatos sobre sua atividade**", e informou que de acordo com o Regimento Interno do Programa, o candidato dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluno para responder às perguntas será de 30 (trinta) minutos. O aluno procedeu a explanação e comentários acerca do tema em 40 (quarenta) minutos. Após a apresentação do mestrando, o Sr. Presidente convidou a Banca Examinadora para ocupar seus lugares na mesa e passou a palavra o Prof. Dr. Silvio José de Macedo, que agradeceu ao convite, fez alguns comentários, deu algumas sugestões e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Daí o Sr. Presidente passou a palavra para a Dra. Karina Ribeiro, que agradeceu ao convite, fez alguns comentários, deu algumas sugestões e iniciou sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, o Sr. Presidente passou a palavra para a Profa. Dra. Patrícia Maria Guedes Paiva, que agradeceu ao convite, fez alguns comentários, deu algumas sugestões e iniciou sua argüição. Ao final, a referida doutora deu-se por satisfeita. Continuando os trabalhos, o Sr. Presidente, na qualidade de orientador, usou da palavra para tecer alguns comentários sobre o trabalho do aluno e agradeceu à Banca Examinadora Examinadora. Daí o Sr. Presidente suspendeu a sessão para o julgamento pela Banca Examinadora a qual se reuniu na Secretaria da Pós-Graduação. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovado com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 09 de outubro de 2008.

CAIO RODRIGO DIAS DE ASSIS

"Caracterização de acetilcolinesterase cerebral do tambaqui (*Colossoma macropomum*) e efeito de pesticidas Organofosforados e carbamatos sobre sua Atividade"

Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

Aprovado por: _____

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Data: 09 / 10 / 2008

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AGRADECIMENTOS

A Deus, pela minha vida e por tudo o que tenho recebido durante esses anos;

À minha mãe, Célia Dias dos Santos, pelo seu amor, por tudo que me ensinou e pelo estímulo constante;

Ao meu avô, José Dias dos Santos e a minha avó Helena Barbosa dos Santos (*In memoriam*) por tudo o que representaram e representam na minha vida.

Aos meus familiares de Recife, Belo Horizonte e Brasília, em especial, Carlos Dias dos Santos, Floripes Rodrigues dos Santos, Lídia Dias dos Santos, Anita Dias, Themer Bastos e Cláudio Dias dos Santos;

Aos meus orientadores, Ranílson de Souza Bezerra e Luiz Bezerra de Carvalho Junior, pelo auxílio no desempenho de minhas tarefas no Laboratório de Enzimologia da UFPE e pela paciência com que aguardaram a conclusão deste trabalho e com que agüentaram a minha "cabeça-dura";

Às minhas grandes amigas Amanda Guedes Linhares, Daniela de Souza Paiva, Juliana Ferreira dos Santos, Elba Verônica Maciel de Carvalho e Patrícia Fernandes de Castro, por tudo;

Ao grande amigo Carlos Dâmaso, pelas conversas e instruções de sempre;

Ao amigo Robson Liberal (*In memoriam*) pela amizade constante;

Aos amigos e colegas de trabalho, Marina Marcuschi, Diego Buarque, Thiago Cahú, Ian Porto, Rosiely Felix, Cynarha Cardoso, Karina Ribeiro, Karollina Lopes, Werlayne Mendes, Talita Espósito, Suzan Diniz, Renata França, Helane Costa, Augusto Vasconcelos, Robson Coelho, Mirela Assunção, Felipe César, Anderson Henriques, Gilmar Cezar, pelos momentos de descontração, amizade e pela consideração.

Aos amigos da turma do Mestrado, em especial Belisa Duarte, Fernando Antônio Vaz, Ana Paula Amaral, Ricardo Guedes, Ana Linda Soares pelos estudos em conjunto e pelo companheirismo;

Aos professores e funcionários do Departamento de Bioquímica da UFPE, em especial a Neide Fernandes, Patrícia Guedes de Paiva, Márcia Vanusa da Silva, Albérico Espírito Santo, João Virgínio, Miron Oliveira e Djalma Gomes;

Aos amigos do CELEC, da Capemi e das maravilhosas Campanhas de domingo: Frank e Mônica Moneta, Elaine Cristina Silva e sua maravilhosa família, William Guterres, Priscila

Batista, Felippe Maciel, Rosa Saraiva, Camila e Bruno Sanchez, Emanuela e Anderson, Francimar Bezerra, Sra. Marlene Nipo, Deyvid Galindo e Carol Brito, Ângelo Borba, Mauro e Cláudia Costa, Sérgio e ao Sr. Hildo dos Santos.

Aos amigos da Agronomia Liliana Ramos, Adriana Dornelas, Carlos Gilberto Barbalho Júnior, Kleison Dantas, Herman Okasaki, Magda Mendonça, André "Baleia" Vasconcelos e Manoel Bandeira pela grande amizade que nos une;

À Mar Doce do Nordeste Piscicultura e Projetos Ltda., em especial, na pessoa de seu Gerente, Niraldo Melo, pela amizade empenhada, orientação de estágio em piscicultura e fornecimento de peixes para a realização da pesquisa;

Aos companheiros de Gestão Ambiental da Escola Politécnica da UPE, Mônica de Moraes Barbosa, Carlos Eduardo Costa Lopes, Marcos Veras Reis, Isabel Fonseca Faro e Bruno Elldorf, pela amizade e companheirismo no decorrer do curso;

A João Almeida e Alda Maria dos Santos pela hospitalidade, confiança e estímulo.

Muito obrigado! Que Deus abençoe a todos nós!

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RESUMO

Organofosforados e carbamatos são as principais classes de pesticidas no mercado mundial. Sua rápida degradação e baixa estabilidade no meio ambiente fizeram com que substituíssem rapidamente outras classes importantes. Todavia, sua alta toxicidade em relação aos mamíferos e outros organismos não-alvo aliada às grandes quantidades utilizadas constituem uma ameaça à saúde humana e ambiental. O modo de ação de ambas as classes baseia-se na inibição de enzimas colinesterases. Metodologias vêm sendo desenvolvidas utilizando colinesterases de organismos aquáticos para detectar a presença de pesticidas em amostras ambientais, uma vez que seus habitats são constantemente contaminados por esses compostos. Neste trabalho, a acetilcolinesterase presente no extrato bruto (AChE; EC 3.1.1.7) de cérebro de tambaqui (*Colossoma macropomum*) foi exposta a concentrações de 0,001 a 10 ppm de um pesticida comercial, cujo princípio ativo é o organofosforado diclorvós. Os resultados demonstraram inibição de aproximadamente 18% da atividade enzimática referente à concentração de 0,01 ppm (0,0452 µmol/L) do princípio ativo. Em seguida, a enzima foi caracterizada e exposta a cinco pesticidas organofosforados e dois carbamatos: diclorvós, diazinon, clorpirifós, temefós, TEPP, carbaril e carbofuran, respectivamente. Foram determinados parâmetros físico-químicos e cinéticos como pH ótimo (7,0 a 8,0), temperatura ótima (40 a 45ºC) e estabilidade térmica (60% da atividade retida até 50ºC). As concentrações dos pesticidas foram de 0,001 a 10 ppm. A concentração de 0,001 ppm causou decréscimo na atividade enzimática em 34,4% (dichlorvos), 17,1% (clorpirifós) e 16,3% (carbofuran). A CI50 estimada para cada composto foi 0,0451 µmol/L (diclorvós), 7,583 µmol/L (clorpirifós), 3,734 µmol/L (TEPP), 33,86 µmol/L (carbaril), 0,9202 µmol/L (carbofuran). Esses resultados contribuem para a determinação de condições ótimas experimentais e sugerem a utilização da acetilcolinesterase de tambaqui no monitoramento ambiental de alguns desses pesticidas.

Palavras-chaves: Organofosforados, carbamatos, biomarcador, acetilcolinesterase, tambaqui *Colossoma macropomum*, peixe

ABSTRACT

Organophosphorus and carbamate are the major classes of pesticides in use around the world. Their relatively fast hydrolysis and low persistence in environment allow them to quickly replace other important classes. However, their high toxicity to mammals and other non-target organisms allied to the large amounts used are a threat for human and environmental health. Both classes are cholinesterase inhibitors and several methodologies have been developed, using these enzymes from various species, in order to monitor their presence in natural samples. Aquatic species are commonly chosen for it, since their environments are being contaminated with those compounds. Here, acetylcholinesterase (AChE; EC 3.1.1.7) from brain of the Amazonian fish tambaqui (*Colossoma macropomum*) was partially characterized, and its activity was assayed in presence of five organophosphate and two carbamate insecticides: dichlorvos, diazinon, chlorpyrifos, temephos, TEPP, carbaryl and carbofuran, respectively. The optimum pH (between 7.0 and 8.0), temperature (ranged from 40 to 45ºC) and thermal stability (up to 60% activity retained until 50ºC) were determined. The inhibitory assays were performed at insecticide concentrations from 0.001 to 10 ppm. The concentration as low as 0.001 ppm of dichlorvos, chlorpyrifos and carbofuran was capable to inhibit 34.4 %, 17.1 %, 16.3 % the AChE activity from tambaqui brain, respectively. The IC50 determined for each compound were 0.045 µmol/L (dichlorvos), 7.583 µmol/L (chlorpyrifos), 3.734 µmol/L (TEPP), 33.86 µmol/L (carbaryl) and 0.92 µmol/L (carbofuran). These results suggest that AChE from tambaqui brain could be useful for routine organophosphorus and carbamate screening.

Key words: Organophosphorus, Carbamates, Biomarker, Acetylcholinesterase, *Colossoma macropomum*, fish.

1 - INTRODUÇÃO

1.1 Enzimas colinesterases

Em 1914, Dale sugeriu o possível envolvimento de uma enzima que degradava ésteres de colina na transmissão de impulsos nervosos. Essas enzimas foram, pela primeira vez, chamadas de colinesterases por Stedman e colaboradores, em 1932. Alles e Hawes (1940) relataram discrepâncias na atividade dessas enzimas em relação à taxa de degradação de alguns substratos no plasma e nos eritrócitos, dando origem a estudos que concluíram que não poderia ser apenas um tipo de enzima a realizar essas tarefas (MASSOULIÉ e BONN, 1982).

Atualmente, são aceitos dois tipos de colinesterases, a acetilcolinesterase (AChE; EC 3.1.1.7) e a butirilcolinesterase (BChE; EC 3.1.1.8). A primeira, presente principalmente no tecido nervoso, muscular e eritrócitos, hidrolisa acetilcolina enquanto a segunda, presente principalmente no fígado e plasma, hidrolisa butirilcolina e acetilcolina. Estas enzimas pertencem à família das serino-esterases, que hidrolisam especificamente ésteres de colina e são classificadas como globulares ou assimétricas associadas a lipídeos, glicofosfolipídeos e colágeno (fig. 1). As formas globulares apresentam-se como monômeros, dímeros e tetrâmeros que podem estar solúveis ou ligados à lâmina basal ou ainda ancorados à membrana celular no sistema nervoso, músculos estriados e cardíacos, plasma, eritrócitos, fígado e outros órgãos onde não são sintetizadas e aos quais chegam através da circulação nos vertebrados e invertebrados. Sua principal e clássica função é a desativação de neurotransmissores nas sinapses colinérgicas e junções neuromusculares, modulando os impulsos nervosos responsáveis pela comunicação neuronal (QUINN, 1987; TÕUGU, 2001). Evidências também apontam para um possível papel dessas enzimas no desenvolvimento do sistema nervoso, particularmente na diferenciação neuronal (BRIMIJOIN e KOENIGSBERGER, 1999).

As colinesterases têm sido extensivamente estudadas pelo seu polimorfismo intra e interespecífico e por serem os alvos primários de diversos compostos utilizados em agropecuária, medicina, campanhas de saúde pública e armas químicas (FORGET, LIVET e LEBOULENGER, 2002).

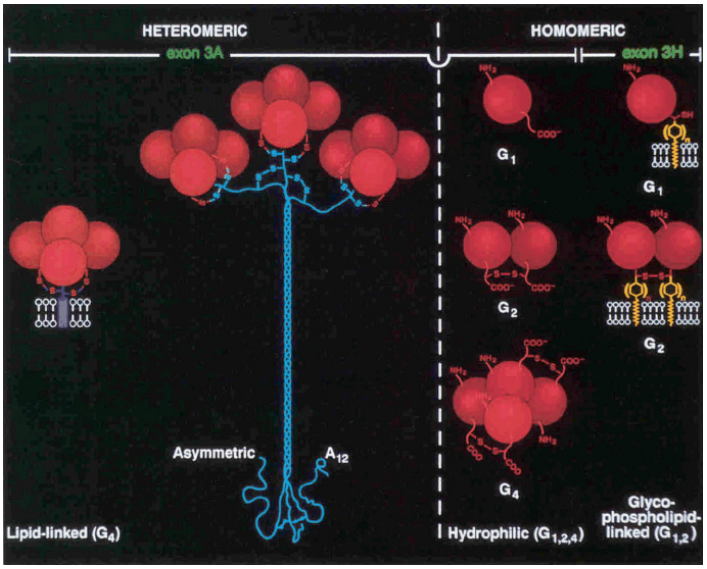


Figura 1 – Formas das colinesterases encontradas em vertebrados (Massoulié e Bonn, 1982)

1.2 Acetilcolinesterase

A acetilcolinesterase (Fig. 2) age na desativação do principal neurotransmissor do sistema nervoso, na maioria das espécies: a acetilcolina. A AChE hidrolisa rapidamente esse neurotransmissor, na fenda sináptica, encerrando sua ação e garantindo a intermitência dos impulsos nervosos (Fig. 3). (QUINN, 1987; TÕUGU, 2001; SILMAN e SUSSMAN, 2005). A AChE é freqüentemente descrita como uma enzima perfeita porque suas propriedades catalíticas se conjugam para aproximar sua atividade do limite máximo de velocidade permitido pela própria difusão do substrato no meio circundante (TÕUGU, 2001; SILMAN e SUSSMAN, 2005). Uma molécula de acetilcolinesterase é capaz de degradar 300 mil moléculas de acetilcolina por minuto.

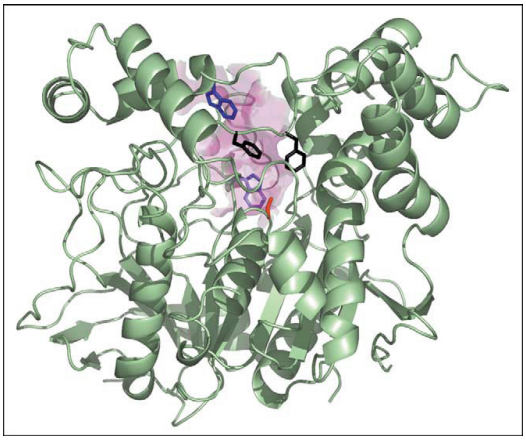
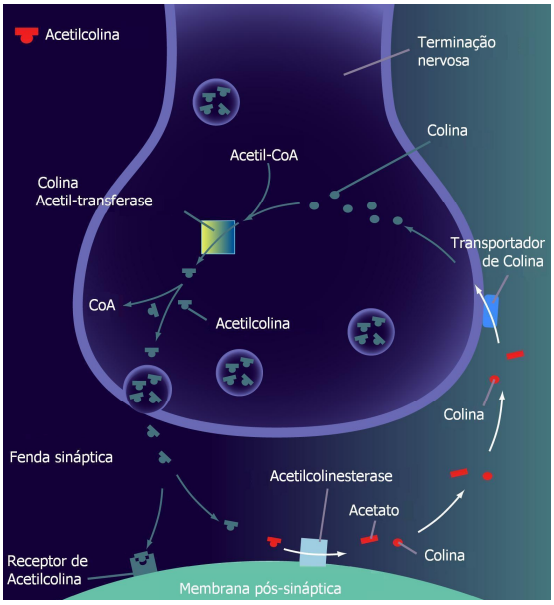


Figura 2 – Estrutura tridimensional da AChE da arraia elétrica do Pacífico *Torpedo californica* (Silman e Sussman, 2005)

A acetilcolinesterase contem dois sítios catalíticos, um sítio esterásico e um sítio aniônico. O sítio esterásico da acetilcolinesterase situa-se dentro de uma cavidade estreita (active site gorge) e é constituído de uma tríade catalítica formada pelos resíduos dos aminoácidos serina 200, histidina 440 e glutamato 327 (podendo variar ligeiramente suas posições). Na catálise, o sítio aniônico, situado às bordas da cavidade, atrai fortemente o nitrogênio quaternário, carregado positivamente, da acetilcolina. Uma vez dentro da fenda catalítica, a acetilcolina sofre o ataque nucleofílico da serina (desprotonada pelo resíduo histidina) ao seu carbono carbonílico, criando um intermediário tetraédrico estabilizado por pontes de hidrogênio o qual, num primeiro momento, forma serina acetilada e libera colina. Ao final do processo de clivagem da ligação éster, o grupo acetila é liberado pela adição de água, formando ácido acético e regenerando o sítio catalítico (TAYLOR et al., 1995; VIEGAS Jr et al., 2004). A AChE apresenta inibição por excesso de substrato, através da ligação do mesmo a um sítio periférico formado por resíduos de aminoácidos que margeiam a entrada do sítio ativo central (MASSOULIÉ e BONN, 1982; EASTMAN et al., 1995).



Adaptado de: CNSforum.com

Figura 3 – Desenho esquemático do ciclo da acetilcolina onde é possível observar o papel da acetilcolinesterase desativando o excesso desse neurotransmissor.

A inibição desse mecanismo resulta no acúmulo do neurotransmissor nas sinapses do sistema nervoso central, nas junções neuromusculares, nas terminações nervosas parassimpáticas e simpáticas. Alta concentração de acetilcolina é então liberada aos seus

receptores (TÕUGU, 2001). Essa inibição é uma reação específica, considerada o principal efeito da exposição aos pesticidas organofosforados (TAYLOR et al., 1995) e carbamatos (JARRARD et al., 2004). Uma vez iniciada, a inibição tende à irreversibilidade, gerando quadros de intoxicação aguda ou crônica, dependendo do grau de exposição à substância. Um indivíduo agudamente intoxicado por qualquer inibidor de acetilcolinesterase pode morrer, pela superestimulação de seu sistema nervoso, convulsões e parada respiratória (TÕUGU, 2001). Segundo dados da Food and Agriculture Organization (2007), uma inibição da atividade da AChE a partir de 20% caracteriza a ação de agentes anti-colinesterásicos, porém sinais clínicos geralmente aparecem após 50% de inibição e morte após 90%.

1.3 Organofosforados e carbamatos

O uso excessivo de pesticidas na agricultura, desde a preparação do cultivo, até o armazenamento de produtos, é um fator determinante para a contaminação dos alimentos de origem vegetal. Os níveis de resíduos encontrados no meio ambiente e na alimentação refletem a freqüência de aplicação desses compostos, a qual varia com a cultura, estágio de desenvolvimento, nível de infestação da praga-alvo e fatores climáticos como pluviosidade e umidade relativa do ar.

Organofosforados (OP) e carbamatos (CB) são as classes de pesticidas mais utilizadas em todo mundo, juntos respondem por mais de 50% do que é comercializado (Tabela 1). São largamente utilizados nos países em desenvolvimento, de economia predominantemente agrícola, para o controle de pragas e em campanhas de combate a vetores de doenças (WHO, 1986a; 1989; USDA, 1997; ATSDR, 2005). Entretanto, alguns representantes da classe dos organofosforados constituem o princípio ativo de armas químicas como os gases neurotóxicos tabun, sarin, soman e VX (RILEY, 2003; KELLAR, 2006).

Tabela 1 – Modo de ação dos 100 inseticidas/acaricidas mais vendidos no mundo e sua participação no mercado mundial (Nauen e Bretschneider, 2002)

Modo de Ação	1987 %	1999 %	Mudança %
Acetilcolinesterase*	71	52	- 20
Canais de Na^+ voltagem-dependente	17	18	+ 1,4
Receptores de acetilcolina	1,5	12	+ 10
Canais de Cl^- GABA-dependente	5,0	8,3	+ 3,3
Biossíntese de quitina	2,1	3,0	+ 0,9
NADH desidrogenase	0,0	1,2	+ 1,2
Desacopladores	0,0	0,7	+ 0,7
Receptores de octopamina	0,5	0,6	+ 0,1
Receptores de ecdisona	0,0	0,4	+ 0,4

* Organofosforados e carbamatos

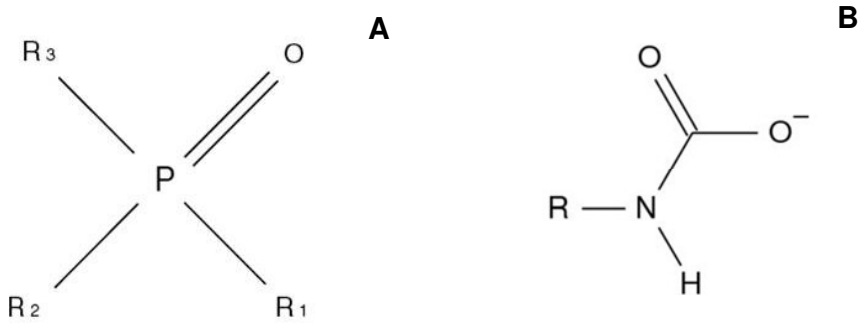


Figura 4 – Fórmula estrutural geral dos organofosforados (A) e carbamatos (B).

Os OPs são ésteres, amidas ou derivados tióis dos ácidos fosfórico, fosfônico, fosforotióico ou fosfonotióico, enquanto os CBs são ésteres ou derivados N-substituídos do ácido carbâmico (Fig. 4). Ambos apresentam baixa solubilidade em água e são, em geral, facilmente hidrolizáveis em ambientes alcalinos. Em geral, os OPs necessitam de biotransformação (dessulfuração por ação das monoxigenases do complexo citocromo P450) para se tornarem toxicologicamente ativos (Fig. 5), enquanto os CB já são bioativos (WHO, 1986a; 1986b). Esses pesticidas são inibidores típicos das enzimas colinesterases

(ALDRIDGE, 1950; ALDRIDGE e DAVIDSON, 1952; WHO, 1986a; b). Alguns são utilizados como medicamento no tratamento de doenças como *miastenia gravis*, glaucoma e mal de Alzheimer (FRANCIS et al., 1999; VIEGAS Jr. et al, 2004; CASIDA e QUISTAD, 2005; POPE, KARANTH e LIU, 2005; ALBUQUERQUE et al., 2006).

Seu mecanismo de ação se dá através da ligação com o sítio esterásico da acetilcolinesterase (Fig. 6), com fosforilação para organosfosforados e carbamilação no caso dos carbamatos, produzindo a inibição da enzima (QUINN, 1987). A inibição por carbamatos é reversível e a regeneração da enzima pode levar de alguns minutos a horas. Já a inibição por organofosforados tende à irreversibilidade se não houver tratamento. Contudo, existe uma taxa de regeneração da enzima, que varia de composto para composto, enquanto a fração restante sofre o processo chamado de "envelhecimento" e não mais se regenera, podendo resultar em um efeito cumulativo ante exposições seguidas a esses compostos. A diferenciação entre as inibições promovidas por diferentes compostos se dá não apenas pela intensidade de inibição, mas também pela taxa de regeneração (WHO, 1986a; b).

Esses pesticidas tiveram seu uso intensificado depois da proibição de utilização da maioria dos compostos organoclorados (ECOBICHON, 1996; USDA, 2002; MUKHERJEE e GOPAL, 2002), os quais são menos tóxicos, porém com maior bioacumulação no meio ambiente (NUNES e TAJARA, 1998; USDA, 2002).

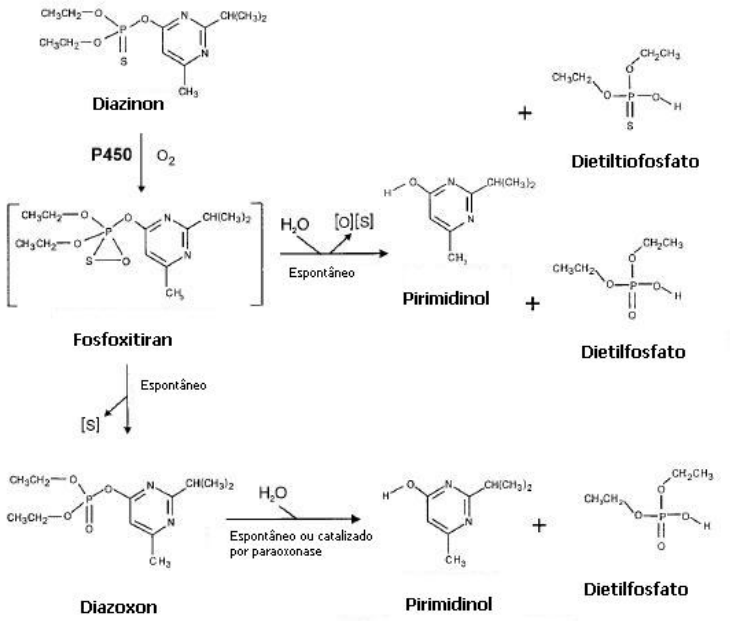


Figura 5 – Ativação do OP diazinon em fígado humano adaptado de Kappers et al. (2001)

Os organofosforados e carbamatos são absorvidos pelo organismo por via oral, respiratória e cutânea, sendo a oral, a maior causa de internações hospitalares de emergência e a cutânea, a causa mais comum de intoxicações ocupacionais (UFF, 2000). O tratamento mais freqüente de intoxicações por agentes anticolinesterásicos, sobretudo os organofosforados, é feito através do uso de atropina em combinação com oximas. A primeira bloqueia os receptores muscarínicos, impedindo que os mesmos sejam superestimulados pelo excesso de acetilcolina na fenda sináptica e a segunda, aplicada o mais cedo possível, reativa as enzimas fosforiladas por ter maior afinidade com as moléculas do pesticida, impedindo a irreversibilidade da inibição (KELLAR, 2006).

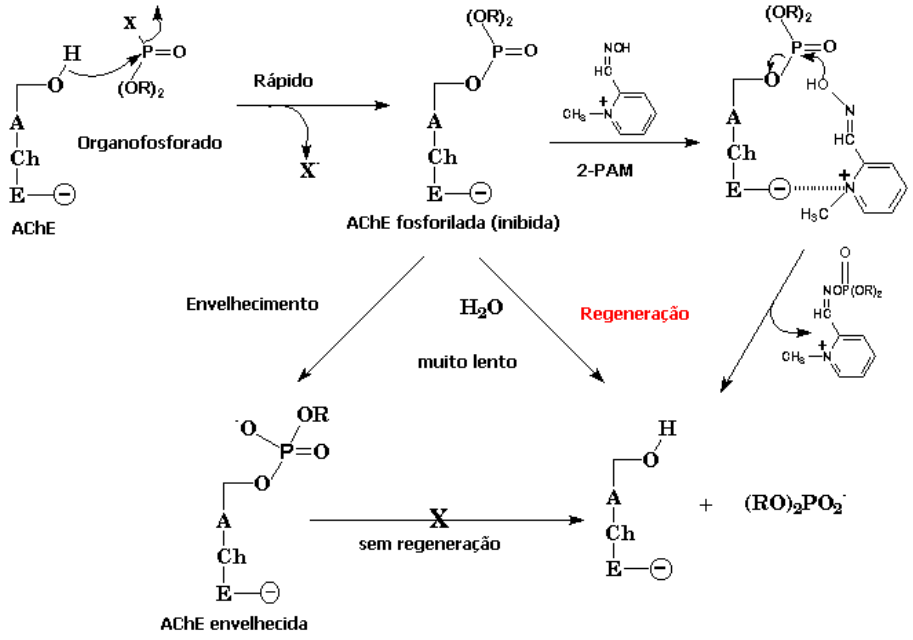


Figura 6 – Esquema da atuação dos OPs sobre a AChE e sua regeneração ou "envelhecimento"

1.4 Organofosforados e carbamatos no meio ambiente e alimentos

Apenas 0,1% dos pesticidas aplicados atingem as pragas-alvo, de forma que o restante desse material contendo o princípio ativo se espalha pelas imediações, contaminando o ar e o solo (YOUNG, 1987). OPs e CBs podem atingir os ecossistemas aquáticos e lençois freáticos (Fig. 7), carreados pelo escoamento superficial e lixiviação das águas da chuva, irrigação e

drenagem, bem como através de pulverizações (USEPA, 1990 e 1999; DUBUS et al., 2000; MÜLLER et al., 2002, TOMITA e BEYRUTH, 2002). Uma vez presente no ambiente aquático, eles podem se associar ao material em suspensão, aos sedimentos no leito do corpo d'água ou ser absorvidos pelos organismos onde sofrerão bioacumulação ou detoxificação (NIMMO, 1985).

No prosseguimento da cadeia alimentar, os pesticidas chegam até os alimentos e demais produtos de origem agroindustrial utilizados pelos homens. A ingestão diária e durante longo prazo de alimentos contaminados com tais agentes, mesmo em pequenas doses, pode levar a quadros de intoxicação de diversos graus (UFF, 2000), tornando-se clara a necessidade de se monitorar tanto o meio ambiente quanto a qualidade dos alimentos. Particularmente pela alta toxicidade desses pesticidas em relação aos organismos aquáticos, os recursos hídricos devem ser continuamente monitorados (BEAUVAIS et al., 2002).

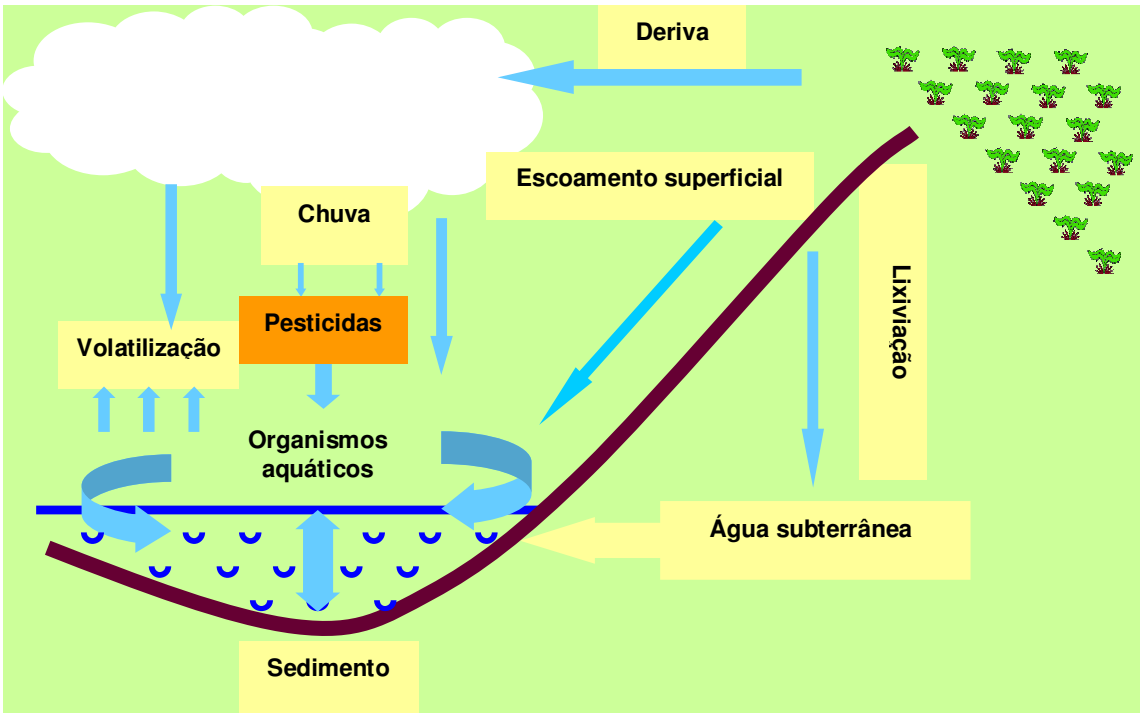


Figura 7 – Processos de entrada dos pesticidas em ambientes aquáticos (Adaptado de Tomita e Beyruth, 2002).

1.5 Esterases no monitoramento de pesticidas

O monitoramento ambiental pode ser definido como o contínuo acompanhamento e mensuração dos impactos, bem como, reações ambientais às atividades e interferências humanas (IBAMA/GTZ, 2000). Uma aplicação prática do monitoramento ambiental seria a comparação temporal entre as condições ambientais de uma dada área, sujeita a variações devido à ação humana ou natural.

Normalmente, o monitoramento ambiental é dividido em químico e biológico. Monitoramento químico é o conjunto de análises químicas que quantificam resíduos de contaminantes em um compartimento ou matriz ambiental (água, ar, solo, sedimentos e organismos animais ou vegetais) em uma escala temporal ou espacial. Por outro lado, quando o enfoque dado está na determinação da magnitude dos efeitos de tal contaminação sobre os organismos em nível individual, populacional ou de comunidade biológica, temos o monitoramento biológico (HENRÍQUEZ-PÉREZ e SÁNCHEZ-HERNÁNDEZ, 2003).

Diversas ferramentas de monitoramento ambiental e alimentar vêm sendo avaliadas quanto à eficácia, praticidade e viabilidade econômica. Dentre elas, destacam-se as metodologias que utilizam moléculas provenientes de seres vivos como indicadores de substâncias nocivas, tendo em vista sua alta especificidade em relação a esses compostos (MARCO e BARCELÓ, 1996; ARIAS *et al.*, 2007; MONSERRAT, 2007).

Unir os enfoques metodológicos dos monitoramentos químico e biológico é uma tarefa de importância para a avaliação da contaminação ambiental e seus efeitos sobre o ecossistema. Nisso fundamenta-se o conceito de bioindicadores.

As substâncias conhecidas como bioindicadores conseguem unir as duas abordagens, pois são compostos de origem animal, vegetal, fúngica e microbiológica que, além de permitirem caracterizar quimicamente os poluentes e determinar suas concentrações, também podem estimar o impacto causado por esses poluentes aos organismos bioindicadores, que fornecem as substâncias em questão (WIJESURIYA e RECHNITZ, 1993; WATSON e MUTTI, 2003). Dentre essas substâncias, as enzimas representam papel importante, pelo alto grau de especificidade e rapidez na resposta às alterações pertinentes às substâncias-alvo. O uso de enzimas como bioindicadores baseia-se na interferência negativa ou inibitória, causada pelas substâncias-alvo, em sua atividade catalítica (MARCO e BARCELÓ, 1996).

A enzima acetilcolinesterase tem sido testada, em diversos estudos, como bioindicador da presença de organofosforados e carbamatos na água ou da exposição de diversas espécies de animais a esses compostos. Sánchez-Hernández e Moreno-Sánchez (2002) utilizaram o lagarto *Gallotia galloti*, típico das Ilhas Canárias, como fonte da enzima para estudar a contaminação pelos pesticidas naquela localidade, tendo em vista que seu estudo em aves tornava-se bastante problemático devido ao tamanho das áreas percorridas pelas mesmas e pela dificuldade de captura de indivíduos contaminados e não contaminados.

Estudos utilizando peixes como a tilápia do Nilo, *Oreochromis niloticus* (RODRÍGUEZ-FUENTES e GOLD-BOUCHOT, 2000), o centrarquídeo norte-americano Bluegill, *Lepomis macrochirus* (BEAUVAIS et al., 2002), o salmão-prateado *Oncorhynchus kisutch* (JARRARD et al., 2004), a carpa comum *Cyprinus carpio* (CHANDRASEKARA e PATHIRATNE, 2005) e a correlação de alterações comportamentais com indicadores fisiológicos de várias espécies (SCOTT e SLOMAN, 2004) têm confirmado os peixes como uma fonte prática e economicamente viável de acetilcolinesterase, capazes de tornar rotineiros os procedimentos de biomonitoramento de recursos hídricos (BOCQUENÉ, GALGANI e TRUQUET, 1990).

Silva (1997) estudou a exposição aos inseticidas de trabalhadores na atividade de desinsetização doméstica em Belo Horizonte, Minas Gerais e encontrou parâmetros físico-químicos de utilização da acetilcolinesterase extraída do sangue humano para maior confiabilidade dos resultados. A busca por essa caracterização físico-química é corroborada por Rodríguez-Fuentes e Gold-Bouchot (2004) e por Sturm et al. (1999a; b), como forma de se obter uma resposta confiável das reações químicas.

Existe uma necessidade de se caracterizar a atividade dos diversos tipos de colinesterases, uma vez que a variabilidade de formas apresentadas por diferentes espécies e diferentes indivíduos é muito alta. Weill et al. (2003) encontraram um mecanismo de resistência à ação dos organofosforados, em populações de mosquitos *Anopheles gambiae* e *Culex pipiens*, que consistia na substituição de um único aminoácido na cadeia da acetilcolinesterase sintetizada por esses insetos, demonstrando que a enzima apresenta diferenças intraespecíficas de grande importância. Além disso, segundo Silman e Sussman (2005), o provável motivo para a acetilcolinesterase apresentar-se em uma série de formas moleculares em um mesmo indivíduo seria o de atender aos diversos tipos de sinapses colinérgicas presentes no tecido nervoso. Marques, Nunes e Marty (2001) e Sotiropoulou et

al. (2005), utilizaram acetilcolinesterases mutantes ou geneticamente modificadas como biodetectores da presença de inseticidas organofosforados.

Os efeitos primários dos organofosforados e carbamatos não se restringem às colinesterases: outras esterases do sistema nervoso central e periférico sofrem inibição, como a 'esterase-alvo' da neuropatia tardia por organofosforados (antiga 'esterase neurotóxica', ainda sem número na *Enzyme Commission*) (LOTTI, 1984; JOHNSON, 1990; JOHNSON e GLYNN, 1995; GLYNN, 1999) e algumas carboxilesterases (CEs; EC 3.1.1.1), as quais catalisam a hidrólise de ésteres carbâmicos e carboxílicos presentes nos inseticidas carbamatos e piretróides, respectivamente (COHEN e EHRICH, 1976; SOGORB e VILANOVA, 2002). As esterases do plasma sanguíneo foram divididas em A e B (ALDRIDGE, 1953a), ambas com a capacidade de hidrolisar carbamatos e piretróides, mas diferindo quanto à interação com organofosforados (ALDRIDGE, 1953a, 1953b; SOGORB e VILANOVA, 2002). Enquanto as do tipo A não sofrem inibição por organofosforados, as esterases B são inibidas por essa classe de compostos (ALDRIDGE, 1953a). As colinesterases são enquadradas no tipo B.

1.6 Tambaqui como fonte de acetilcolinesterase

O tambaqui (*Colossoma macropomum*), peixe da família Characidae (Fig. 8), também conhecido como tetra gigante, pacu preto ou cachama negra, é originário das bacias dos rios Solimões e Orinoco. É a terceira espécie de peixe mais cultivada no país, sendo a primeira dentre as espécies nativas. Sua produção nacional em 2006 foi de 26.662 t (IBAMA, 2008). O tambaqui apresenta fácil adaptação ao consumo de alimentos artificiais, excelente conversão alimentar, rápido crescimento, fácil reprodução artificial, produção massiva de alevinos e a possibilidade de se fazer várias desovas durante o ano (SANTIS, RESTREPO e ÁNGEL, 2004). Todos esses atributos convertem-no em uma espécie promissora também para o manejo ambiental, fonte abundante de biomoléculas para estudos de monitoramento.

Figura 8 – tambaqui, *Colossoma macropomum* (fonte: wikipédia)

No presente trabalho, foram utilizados juvenis dessa espécie como forma de reduzir custos e aumentar substancialmente o número de indivíduos estudados, possibilitando maior abrangência e confiabilidade aos resultados. Além disso, há evidências de que o mecanismo de ação dos pesticidas difere quando age no cérebro, em relação ao resto do sistema nervoso, afetando a maturação das células nervosas cerebrais, suas sinapses e portanto, tornando os animais jovens mais susceptíveis ao seu poder tóxico (KARANTH e POPE, 2000; SLOTKIN, LEVIN e SEIDLER, 2006).

Segundo o Governo Federal (IBAMA, 2002), ainda existe uma grande lacuna a ser preenchida em relação ao diagnóstico de áreas contaminadas por pesticidas, principalmente em ecossistemas aquáticos. No Brasil, poucos trabalhos foram realizados na área, voltados para o biomonitoramento in vitro utilizando peixes.

Nesse contexto, a caracterização físico-química e cinética, bem como o efeito de pesticidas sobre a acetilcolinesterase em tecido nervoso de tambaqui fazem-se necessários para identificá-la como uma provável ferramenta de utilização no monitoramento ambiental e alimentar.

2. OBJETIVOS

2.1. Geral

Caracterizar físico-química e cineticamente a acetilcolinesterase da espécie *Colossoma macropomum* e investigar o efeito de pesticidas organofosforados e carbamatos sobre sua atividade.

2.2. Específicos

- Definir as propriedades físico-químicas e cinéticas da acetilcolinesterase do tambaqui;

- Calcular a Concentração Inibitória Mediana (CI_{50}) referente aos pesticidas de inibição significativa e;

- Analisar o efeito de cinco pesticidas organofosforados e dois carbamatos sobre a atividade da enzima em questão, comparando os resultados de inibição com a legislação nacional e internacional vigente.

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4. CAPÍTULO I – EFFECT OF DICHLORVOS ON THE ACETYLCHOLINESTERASE FROM TAMBAQUI (*COLOSSOMA MACROPOMUM*) BRAIN

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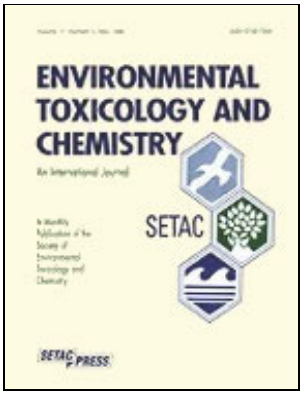

Environmental Toxicology and Chemistry, Vol. 26, No. 7, p. 1451–1453, 2007

CAPÍTULO I – EFFECT OF DICHLORVOS ON THE ACETYLCHOLINESTERASE FROM TAMBAQUI (*COLOSSOMA MACROPOMUM*) BRAIN

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 26, No. 7, pp. 1451–1453, 2007

Printed in the USA
0730-7268/07 $12.00 + .00

Short Communication

EFFECT OF DICHLORVOS ON THE ACETYLCHOLINESTERASE FROM TAMBAQUI (*COLOSSOMA MACROPOMUM*) BRAIN

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(*Received* 26 *September* 2006; *Accepted* 24 *January* 2007)

Abstract—Dichlorvos is an acutely toxic organophosphorous pesticide that is known as a classical acetylcholinesterase (AChE; EC 3.1.1.7) inhibitor. Here, the brain AChE from the important Amazonian fish tambaqui (*Colossoma macropomum*) was assayed in the presence of this insecticide and also of deltamethrin, a classical sodium and potassium channel inhibitor (negative control). Four tissue homogenates were analyzed in triplicate for AChE activity using acetylthiocholine as the substrate and 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) as the color-developing agent. Each tissue homogenate represented pooled brains from five fish. The inhibitory effect of dichlorvos on AChE activities was determined at concentrations from 0.001 to 10 ppm and compared to controls. This effect followed an exponential decay model ($y = 9.420 + 26.192e^{(-x/5.380)}$; $r^2 = 0.989$), presenting IC50 (the concentration of dichlorvos that is required for 50% of AChE inhibition) of 0.081 ppm (0.368 μmol/L). No effect was observed for the deltamethrin, and the concentration 0.0452 μmol/L of dichlorvos was significantly different from this control. These results suggest that tambaqui brain AChE can be proposed as a biomarker for dichlorvos and can be used as a tool for aquatic environment monitoring.

Keywords—Organophosphorous pesticide Dichlorvos Biomarker Acetylcholinesterase Tambaqui (*Colossoma macropomum*)

INTRODUCTION

Environmental monitoring is of paramount importance for the management of any area. Several methodologies have been recently developed in order to monitor aquatic environments [1–4], and the use of fish or molecules extracted from them is amongst the in vivo or in vitro detection methods for this objective [3,5,6]. Acetylcholinesterase (AChE; EC: 3.1.1.7) is responsible for degrading acetylcholine in synaptic gaps (cholinergic synapses) and neuromuscular junctions. This enzyme has proved to be a good biomarker for monitoring contaminant concentrations in aquatic environments [7–9]. It has been widely used as a biomarker to detect the occurrence of organophosphorous and carbamate pesticides in the environment [10,11], mainly due to the good correlation observed between the AChE activity and toxic effects [9].

Dichlorvos is an organophosphorous pesticide widely used to combat outdoor and in-home mosquito vectors of several tropical diseases, and it is also used in tropical aquaculture to control ectoparasitic infections, generating contamination in aquatic environments. This pesticide is a direct-acting inhibitor of AChE that provokes an accumulation of acetylcholine in synapses with disruption of the nerve function causing parasympathetic disorders and death of the organism [11].

In this context, fish and their enzymes are good biomarkers and have widely been employed for environmental monitoring [2,4,8,10–13]. Here, brain from juveniles of tambaqui (*Colossoma macropomum*), one of the most important Amazonian fish and a native species in Brazilian aquaculture, was proposed as a source of AChE for sensitive dichlorvos monitoring. The use of this fish may represent an important contribution to environmental monitoring and therefore conservation.

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MATERIALS AND METHODS

Materials

Acetylthiocholine iodide, bovine serum albumin, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane and HCl were from Merck (Darmstadt, Germany). Dichlorvos (Mafu®; 1.0%) and deltamethrin (Penetrol®; 0.01%) were acquired from Bayer (São Paulo, SP, Brazil) and Otto Baumgart (São Paulo, SP, Brazil), respectively. The juveniles specimens of *C. macropomum* were supplied by Mar Doce Piscicultura e Projetos (Camaragibe-PE, Brazil). Specimens of tambaqui 17.3 ± 3.2 cm in length and 16.5 ± 2.3 g in weight were captured from 600-m² outdoor tanks (dissolved oxygen 6.2 ± 0.3 ppm, temperature 26.5 ± 0.2°C, pH 8.26 ± 0.2) and kept at 4°C during transportation to the laboratory (~30 min).

Methods

Enzyme extraction. Five juvenile fish were sacrificed through immersion during 10 min in an ice bath (0°C) and their brains immediately removed and homogenized to a final concentration of 40 mg/ml in 0.1 mol/L Tris-HCl buffer, pH 8.0. Afterwards, this homogenate was centrifuged for 10 min at 5,000 *g* (4°C) to remove cell debris. The supernatants, from now on called crude extract, were frozen at −20°C (not exceeding a storage time of 48 h) and then used for further assays [8,14].

Protein determination. Protein content was estimated ac-

cording to a modified dye binding method [15] using bovine serum albumin as standard protein.

Enzyme activity. The crude extract (60 μl) was added to 1 ml of 0.25 mM DTNB (dissolved in 0.5 mol/L Tris-HCl buffer, pH 7.4) and the reaction started by the addition of 0.125 mol/L acetylthiocholine iodide (60 μl) [16]. The enzymatic activity (triplicate) was monitored in a spectrophotometer at 405 nm for 3 min during which the reaction followed a first order kinetics pattern. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 μmol of substrate per minute. The blank assay was similarly prepared except that 0.1 mol/L Tris-HCl buffer, pH 7.4, replaced the crude extract sample.

Inhibition assay. The assays for AChE inhibition by two commercially available insecticides were carried out using Mafu and Penetrol in which the active components are dichlorvos (an organophosphate) and deltamethrin (a pyrethroid), respectively. The last one was used as a negative control because it does not have any effect on the AChE activity [17]. The insecticides were first dissolved in DMSO and then diluted in distilled water to a final concentration of 0.001, 0.005, 0.01, 0.1, 1.0, and 10.0 ppm. The insecticide solutions (20 μl) were incubated with the crude extract (40 μl) for 1 h and the residual activity (%) was determined as previously described, using the enzymatic activities in the absence of pesticide as 100%. All the enzymatic and inhibition assays (triplicate) were carried out at room temperature (25°C). Four tissue homogenates pools from five fish brains were analyzed in triplicate for each insecticide concentration and data were expressed as mean ± standard deviation of 12 assays. These data were statistically analyzed and fitted to exponential decay by using the software MicroCal™ Origin™ Version 6.0 (MicroCal, Northampton, MA, USA).

RESULTS AND DISCUSSION

The crude extract of *C. macropomum* brain presented a specific activity of 38.3 mU/mg of protein. This enzyme preparation was exposed to increasing concentrations of dichlorvos and deltamethrin to assess the inhibition of AChE activity. There was no difference ($p > 0.05$) between the AChE activities in the presence and absence of deltamethrin (negative control), which is expected since this pesticide does not have anticholinesterase effects [18]. This result also demonstrates that DMSO, used as a solvent for the deltamethrin and dichlorvos, does not have any effect on the acetylcholinesterase activity from tambaqui brain.

In contrast, dichlorvos was capable of inhibiting AChE extracted from tambaqui even at concentrations as low as 0.005 ppm (0.226 μmol/L) when 18% of inhibition was detected. An exponential decay ($r^2 = 0.989$; $y = 9.420 + 26.192e^{(-x/5.380)}$, where x is the natural log of dichlorvos concentration in parts per million) of activity was obtained when the enzyme activity was measured after incubation with increasing concentrations of dichlorvos (Fig. 1).

The assay herein described demonstrated high sensitivity for the presence of dichlorvos. This sensitivity was confirmed by estimating the IC50 of AChE from this source in presence of this pesticide, which was found to be 0.081 ppm (0.368 μmol/L). The IC50 value reported for the AChE activity from European sea bass (*Dicentrarchus labrax*) was approximately 90-fold higher (7.34 ppm or 30.3 μmol/L) [11,13]. In contrast to the present study, the experiment with European sea bass was carried out using acetone as the dichlorvos solvent [11].

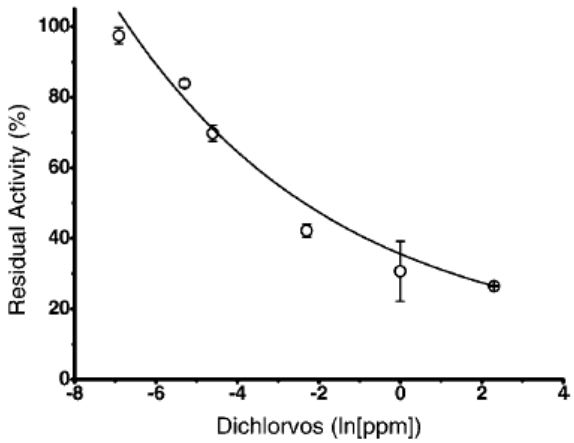

Fig. 1. Effect of increasing concentrations of dichlorvos on acetylcholinesterase (AChE) extracted from brain of juvenile *Colossoma macropomum*. The assay was performed at 25°C as described in the *Materials and Methods* section and the experimental points are the mean ± standard deviation of triplicate of four crude extracts obtained from five brains each ($y = 9.420 + 26.192e^{(-x/5.380)}$; $r^2 = 0.989$).

However, no statistically differences ($p > 0.05$) were found to tambaqui brain AChE inhibition by dichlorvos dissolved either by DMSO or acetone (data not shown).

Although several methodologies have been described for pesticide monitoring in aquatic environment those based on in vitro procedures are by far cheaper, more sensitive, less time-consuming, and less laborious than in vivo assays. Furthermore, the enzyme sensor rather than either tissue or animal sensor directly interacts with the inhibitor, excluding several features that can interfere in the use of latter biomaterials.

According to these results, the enzyme from brain of juvenile tambaqui was shown to be very sensitive and useful as a tool for aquatic environment monitoring.

Acknowledgement—The authors would like to thank Financiadora de Estudos e Projetos (FINEP/RECARCINE), Petróleo do Brasil S/A (PETROBRAS), Secretaria Especial de Aqüicultura e Pesca—(SEAP/PR), Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq), and Fundação de Apoio à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for financial support. Mar Doce Piscicultura e Projetos is also thanked for providing tambaqui juveniles specimens. C.R.D. Assis presented part of this contribution as his manuscript to fulfill a postgraduation degree at the Escola Politécnica de Pernambuco, Universidade de Pernambuco, Brazil.

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5. CAPÍTULO II – CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM THE BRAIN OF THE AMAZONIAN TAMBAQUI (*Colossoma macropomum*) AND IN VITRO EFFECT OF ORGANOPHOSPHORUS AND CARBAMATE PESTICIDES

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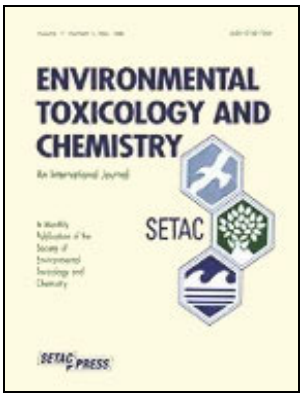

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Running header: Acetylcholinesterase from tambaqui brain.

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Total number of words (text, references, figure legends and tables): 3,986 words

Characterization of acetylcholinesterase from the brain of the Amazonian tambaqui (*Colossoma macropomum*) and in vitro effect of organophosphorus and carbamate pesticides

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(Submitted 11 January 2010; Returned for Revision 12 March 2010; Accepted 23 April 2010)

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Abstract

In the present study, acetylcholinesterase (AChE) from the brain of the Amazonian fish tambaqui (*Colossoma macropomum*) was partially characterized and its activity was assayed in the presence of five organophosphates (dichlorvos, diazinon, chlorpyrifos, temephos and tetraethyl pyrophosphate) and two carbamates (carbaryl and carbofuran) insecticides. Optimal pH and temperature were found to be 7.0 to 8.0 and 45ºC, respectively. The enzyme retained approximately 70% of activity after incubation at 50ºC for 30 min. The insecticide concentration capable of inhibiting half of the enzyme activity (IC50) for dichlorvos, chlorpyrifos and temephos and tetraethyl pyrophosphate (TEPP) were calculated as 0.04 µmol/L, 7.6 µmol/L and 3.7 µmol/L, respectively. Diazinon and temephos did not inhibit the enzyme. The IC50 values for carbaryl and carbofuran were estimated as 33.8 µmol/L and 0.92 µmol/L, respectively. These results suggest that AChE from juvenile *C. macropomum* brain could be used as an alternative biocomponent of organophosphorus and carbamate biosensors in pesticide routine screening in the environment.

Key words: Organophosphorus pesticide, Carbamate pesticide, Acetylcholinesterase, Biomarkers, *Colossoma macropomum*.

Introduction

Organophosphorus and carbamate are major classes of pesticides in use throughout the world. Together, they share about 50% of the world market of insecticides/acaricides. Their relatively fast hydrolysis and low persistence in the environment have supported their increasing use. However, their toxicity to mammals and other non-target organisms, together with the large amounts used, constitute a threat to human health and the environment. Both classes are cholinesterase inhibitors and several methodologies have been developed using these enzymes from various species in order to monitor their environmental presence. These neurotoxic agents have been distributed throughout the world without control in recent decades and, due to misuse and a lack of specificity, have become a serious problem to both humans and the environment [1]. Therefore, methods for organophosphorus and carbamate detection using either organisms or their enzymes as bioindicators and biomarkers, respectively, have been evaluated [2, 3]. The cholinesterase group stands out among such molecules [4-6].

Acetylcholinesterase (AChE; EC 3.1.1.7) is widely known as a specific biomarker of organophosphorus and carbamate pesticides due to the inhibition of its activity [7]. This enzyme is responsible for modulating neural communication in the synaptic cleft by hydrolyzing the ubiquitous neurotransmitter acetylcholine. A lack of AChE activity causes central and peripheral nervous system disorders and death [8].

Studies have confirmed cholinesterases as suitable for monitoring the occurrence of these pesticide classes in environmental compartments [6, 9-11]. For instance, biosensors have been proposed based on AChE from electric eel and both genetically engineered (B394) and wild type strains of *Drosophila melanogaster* [12]. However, the high inter-specific and intra-specific polymorphism of these enzymes cause varied responses to insecticide compounds, thereby hindering the evaluation and comparison of results from different studies

[13]. Consequently, it is necessary to characterize AChE activity in each species and type of tissue.

In previous work, AChE from brain of the juvenile Amazonian fish tambaqui (*Colossoma macropomum*) was shown to be sensitive to dichlorvos [14]. This enzyme source could be proposed as a feasible alternative for setting up biosensors once it is located in a discarded tissue (brain) of this fish, which is the third most farmed species in Brazil (30,598 tons in 2007, according to the Brazilian Ministry of Environment at http://www.ibama.gov.br/recursos-pesqueiros/documentos/estatistica-pesqueira/).

The aims of the present study were to partially characterize some kinetic and physicochemical parameters of this enzyme and evaluate the effect of seven relevant organophosphorus and carbamate pesticides on its activity in order to propose it as the biocomponent for in vitro biosensor.

Materials and Methods

Materials

Acetylthiocholine iodide, bovine serum albumin, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), Tris (hydroxymethyl) aminomethane and dimethyl sulfoxide (DMSO) were purchased from Sigma. Analytical grade dichlorvos (98.8%), diazinon (99.0%), chlorpyrifos (99.5%), temephos (97.5%) and tetraethyl pyrophosphate (97.4%), carbofuran (99.9%) and carbaryl (99.8%) were obtained from Riedel-de-Haën, Pestanal®. Disodium hydrogen phosphate and HCl were obtained from Merck. Trisodium citrate was acquired from Vetec (Rio de Janeiro, RJ, Brazil). Glycine was acquired from Amersham Biosciences. The spectrophotometer used was Bio-Rad Smartspec™ 3000. The juvenile specimens of *C. macropomum* were supplied by Mar Doce Piscicultura e Projetos (Camaragibe, PE, Brazil).

Tambaqui specimens 16.5 ± 3.7 cm in length and 93.8 ± 7.9 g in weight were captured from a 750-m^3 pond.

Methods

Enzyme Extraction

Twenty juvenile fish were acclimatized in 100 L aquaria (dissolved oxygen 8.04 ± 0.05 mg/L, temperature 26.04 ± 0.07ºC, pH 6.93 ± 0.22, salinity 0.17 g/L) for one week and then sacrificed by immersion in an ice bath (0ºC). The brains were immediately removed, joined in pairs and homogenized in 0.5 mol/L Tris-HCl buffer, pH 8.0, maintaining a ratio of 20 mg of tissue per ml of buffer using a Potter-Elvehjem tissue disrupter. The homogenates were centrifuged for 10 min at 1000 x *g* (4ºC) and the supernatants (crude extracts) were frozen at -20ºC.

Enzyme activity and protein determination

The crude extract (30 µl) was added to 500 µl of 0.25 mmol/L DTNB dissolved in 0.5 mol/L Tris-HCl buffer, pH 7.4, and the reaction started by the addition of 0.125 mol/L acetylthiocholine iodide (30 µl) [14]. Enzyme activity (quadruplicate) was spectrophotometrically determined by following the absorbance at 405 nm for 180 s, in which the reaction exhibited a first-order kinetics pattern [14]. A unit of activity (U) was defined as the amount of enzyme capable of converting 1 µmol of substrate per minute. A blank assay was similarly prepared except that 0.5 mol/L Tris-HCl buffer, pH 8.0, replaced the crude extract sample. Protein content was estimated according to a modified dye-binding method [15], using bovine serum albumin as the standard.

Optimal pH and temperature

Assays were performed using DTNB solutions in a pH range from 2.5 to 9.5 by using citrate-HCl (2.5 to 4.5), citrate-phosphate (4.0 to 7.5), Tris-HCl (7.2 to 9.0) buffers. Substrate

non-enzymatic hydrolysis (in basic pH) was corrected by subtracting their values from the activities. Optimum temperature was established by assaying the enzyme activity at temperatures ranging from 5 to 70ºC for 180 s.

Thermal stability

Thermal stability of juvenile *C. macropomum* AChE was evaluated by exposing crude extract samples for 30 min at temperatures ranging from 25 to 80ºC and assaying the activity retained after 5 min of equilibration at 25ºC (room temperature).

Inhibition Assay

Acetylcholinesterase inhibition was assayed using five organophosphates (dichlorvos, diazinon, chlorpyrifos, temephos and TEPP) and two carbamates (carbaryl and carbofuran). The insecticides were first dissolved in DMSO and then diluted in distilled water to five final concentrations ranging from 0.001 to 10 ppm, with each subsequent concentration 10-fold higher than the previous concentration. These concentrations correspond respectively: dichlorvos, 0.0045 to 45.2 µmol/L; diazinon, 0.0032 to 32.8 µmol/L; chlorpyrifos, 0.0028 to 28.5 µmol/L; 0.0021 to 21.4 µmol/L (temephos); 0.0034 to 34.5 µmol/L (TEPP); 0.0061 to 61.3 µmol/L (carbaryl); and 0.0045 to 45.2 µmol/L (carbofuran). The insecticide solutions (10 µl) were incubated with crude extract (20 µl) for 1 h [14] and the residual activity (%) was determined as previously described, using the absence of pesticide as 100% activity. All enzymatic and inhibition assays were carried out at room temperature (25ºC). Five crude extracts from 10 fish brains were analyzed in triplicate for each insecticide concentration and data were expressed as mean ± standard deviation. These data were statistically analyzed by non-linear regression fitted to polynomial or exponential decay ($\rho > 0.05$) modeling using the software MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA). The concentration capable of inhibiting half of the enzyme activity (IC50) was estimated for each pesticide.

Results and discussion

Optimum pH for juvenile *C. macropomum* AChE was found to be in the range 7.0 to 8.0 (Fig. 1A) similar to those described in the literature for other fishes (Table 1): *Solea solea* (7.0), *Scomber scomber* (8.0) and *Pleuronectes platessa* (8.5) [9]; *Cymatogaster aggregate*[16] and *Hypostomus punctatus* (between 7.0 and 7.2) [17]. Optimum temperature was estimated as 45ºC (Fig. 1B). Bocquené et al. [9] found in the range 32 to 34ºC for *Pleuronectes platessa;* Beauvois et al. [4] at 25ºC for *Lepomis macrochirus* and Hazel [18] at 35ºC for *Carassius auratus*. In the present study, AChE from juvenile *C. macropomum* after being incubated for 30 min at 50ºC retained about 70% of its activity at 35^{o}C (Fig. 1B). Zinckl et al. [19] reported absence of cholinesterase activity in the brain of *Oncorhyncus mykiss* (formerly known as *Salmo gairdneri*) subjected to temperatures higher than 45ºC.

The Michaelis-Menten kinetics is displayed in Figure 2, from which the maximal velocity (V_{max}) and apparent bimolecular constant (K_m) were 129.7 ± 5.3 mU/mg protein and 0.434 ± 0.025 mmol/L, respectively, using acetylthiocholine iodide as substrate. The Lineweaver-Burk plot is also presented. Varó et al. [20] reported acetylthiocholine iodide inhibition at concentrations greater than 5.12 mmol/L in brain tissue from *Sparus aurata*, in contrast to muscle tissue, for which inhibition occurred at 20.48 mmol/l. Rodríguez-Fuentes and Gold-Bouchot [5] found acetylthiocholine inhibition at 4.89 mmol/L in AChE from the brain of *Oreochromis niloticus*. However, in the present study, no substrate inhibition was observed even at the 15 mmol/L acetylthiocholine iodide. According to Table 1, the apparent Michaelis-Menten constant of the juvenile *C. macropomum* AChE was lower than that estimated for *Pleuronectes vetulus* muscle and higher than *Pleuronychtis verticalis* muscle and *Oreochromis niloticus* brain whereas the maximum velocity was smaller than those reported for these mentioned tissues.

Among the anticholinesterasic agents, organophosphates and its analogues play a different role into the metabolic paths before reaching sites of neuronal transmission. Some of them are produced in a less toxic form (thion form, P=S) which is more stable in the environment. When absorbed by an organism, this form of pesticide undergoes bioactivation to a more toxic form (oxon form, P=O) by monooxigenases from the cytochrome P450 complex present in some organs/tissues including liver, kidneys, lungs and brain. Therefore, this phenomenon and the diverse effect of the resulting products on the AChE can determine differences in the thion form of these pesticides.

The Food and Agriculture Organization [21] recommends that 20% inhibition is the relevant end-point to determine acceptable daily intakes of an anticholinesterasic compound. In the present study, some of the compounds analyzed were highly toxic to tambaqui AChE and the inhibition they caused could rapidly reach the above-mentioned levels.

Results from inhibition assays are displayed in Fig. 3 and Table 2 and summarizes the IC50 values estimated from these data for the five organophosphates (dichlorvos, diazinon, chlorpyrifos, temephos and tetraethyl pyrophosphate - TEPP) and two carbamate insecticides (carbaryl and carbofuran). Dichlorvos as previously demonstrated [14] was shown to strongly inhibit the juvenile *C. macropomum* AChE. Among the investigated pesticides in the present study, this insecticide presented the lowest IC50 value (0.04 µmol/L; 0.01 ppm) and the lowest value compared with those reported in the literature for other fish species. Chuiko [22] estimated the IC50 value of 0.31 µmol/L for *Leuciscus idus* and *Esox lucius* and 0.63 µmol/L for *Alburnus alburnus*. Dichlorvos is a direct inhibitor of AChE. It is an oxon organophosphate compound [23] and does not require bioactivation for enzyme inhibition in contrast with thion compounds, for which only a fraction of the total amount is activated in the tissues [24, 25]. Chlorpyrifos also displayed lower IC50 value (7.6 µmol/L) than that reported for *Cyprinus carpio* [26]. Diazinon and temephos did not show inhibition effect on

the juvenile *C. macropomum* AChE under the experimental conditions used in the present. According to a number of studies, acute toxicity from phosphorothionate pesticides such as diazinon and chlorpyrifos is strongly influenced by differences in the activity of cytochrome P450-mixed oxidase systems, which bioactivate these compounds [27, 28]. Nevertheless, these influences only determine toxic effects through the balance between activation and detoxification pathways: P450 dearylation, carboxylesterase and butyrylcholinesterase phosphorylation, oxonase-mediated hydrolysis [29]. Thus, the contrast between high sensitivity to oxons and apparent lower oxidation activity possibly could be a *C. macropomum* enantiostatic mechanism when facing xenobiotic threats [30]. Another condition that may cause discrepancies, particularly in case of chlorpyrifos, is that this compound accumulates in tissues, which likely affects other results. Antwi [31] also found no statistical differences in four fish species (*Oreochromis niloticus*, *Sarotherodon galilaea*, *Alestes nurse* and *Schilbe mystus*) between controls and individuals living in areas treated weekly with temephos over a six-year period. Temephos is also a thion compound, but the reasons for such results are not only caused by the circumstances mentioned for diazinon and chlorpyrifos. This pesticide is known to exhibit moderate to low toxicity to mammals and other non-target organisms, and is commonly used in potable water treatment against mosquito larva vectors of diseases in public health campaigns [31]. Tetraethyl pyrophosphate (TEPP) displayed IC50 value of 3.7 µmol/L. This is an organophosphorus known to be highly toxic to mammals and is the biotransformation product of another pesticide, which is classified as an extremely hazardous by the World Health Organization [32]. Table 3 displays its in vivo LC50 for other fish species provided by from the U.S. Environmental Protection Agency Ecotoxicology Database (http://cfpub.epa.gov/ECOTOX/), which reflects the high toxicity of this compound (6.8 h at 25 ºC) [33]. Tetraethyl pyrophosphate is currently classified as an obsolete pesticide [32], but in fact is responsible for part of the toxic action in

some organophosphate products, such as diazinon, chlorpyrifos, parathion, demeton, where it appears as an impurity or breakdown product due to the manufacturing process or unsuitable storage conditions [33]. The two analyzed carbamate insecticides carbaryl and carbofuran presented IC50 values of 33.8 µmol/L and 0.99 µmol/L respectively. The latter IC50 value is similar to that reported by Dembélé, Haubruge and Gaspar for in vitro, *Cyprinus carpio* [26], namely, 0.45 µmol/l (0.1 ppm).

The monitoring of pesticides such as organophosphates and carbamates can be evaluated by using organisms in aquatic environments (in vivo assays). In these cases, tanks, animal manipulation, feeding demands and specially trained personnel are required. Otherwise, animals can be collected from their environment and these toxic components analyzed in their tissues. The use of enzymes, namely, cholinesterases, allows in vitro procedures that are less costly, less time-consuming, less laborious and more sensitive. The analysis of reactions can take place without interfering compounds present in tissues or animal sensors that could interact with anticholinesterasic agents, thereby causing false positives or negatives. Moreover, biosensors based on these enzymes can be built and used in environmental monitoring. The findings described here confirm previous findings [14] related to the sensitivity of AChE from the brain of the juvenile Amazonian tambaqui towards dichlorvos and its possible use as the biocomponent of in vitro sensor for this pesticide and also for chlorpyrifos, carbaryl and carbofuran.

Acknowledgement — The authors would like to thank Financiadora de Estudos e Projetos (FINEP), Petróleo do Brasil S/A (PETROBRAS), Ministério da Aqüicultura e Pesca (MAP), Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq) and Fundação de Apoio à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for financial support.

Mar Doce Piscicultura e Projetos also deserves our thanks for providing tambaqui juveniles specimens.

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Tables

TABLE 1 – Kinetics and physicochemical parameters of AChE from some freshwater and marine species.

Species [Reference]	Km [mmol/L]	Vmax [U/mg of protein]	Optimum pH	Optimum temperature [ºC]	Source	Life stage
Oreochromis niloticus [5]	0.10 ± 0,03	0.229 ± 0.014	ND	ND	Brain	Juvenile 48.2 ± 3.9 g
Pleuronectes vetulus [6]	1.69 ± 0.26	0.482 ± 0.034	ND	ND	Muscle	Juvenile 13.5-29.5cm
Pleuronychtis verticalis [6]	0.30 ± 0.07* 0.23 ± 0.06**	0.524 ± 0.032* 0.120 ± 0.08**	ND	ND	Muscle	Juvenile
Solea solea [9]	ND	ND	7.5	ND	Brain	ND
Pleuronectes platessa [9]	ND	ND	8.5	33	Brain	ND
Scomber scomber [9]	ND	ND	8.0	ND	Brain	ND
Colossoma macropomum [Present work]	0.43 ± 0.02	0.13 ± 0.05	7.5	45	Brain	Juvenile 16.6 ± 3.7 cm

Km – Michaelis-Menten constant; Vmax – Maximum velocity of enzyme activity;
* Female specimens; ** Male specimens; ND - not determined.

TABLE 2 – Pesticide* IC50▲ values for *in vitro* freshwater fish.

Species [Reference]	IC50 (µmol/L)
Dichlorvos	
Alburnus alburnus [23]	0.63
Leuciscus idus [23]	0.31
Esox lucius [23]	0.31
Colossoma macropomum [14]**	0.36
Colossoma macropomum [present study]	0.04
Chlorpyrifos	
Cyprinus carpio [27]	810
Colossoma macropomum [present study]	7.6
Diazinon	
Oncorhynchus mykiss [26]	2.5
Danio rerio [26]	20.0
Poecilia reticulata [26]	7.5
Cyprinus carpio [26]	0.2
Colossoma macropomum [present study]***	No effect
Temephos	
Oreochromis niloticus, Sarotherodon galilaea, Alestes nurse and *Schilbe mystus* [32]	No effect.
Colossoma macropomum [present study]	No effect.
TEPP	
Colossoma macropomum [present study]	3.7
Carbaryl	
Colossoma macropomum [present study]	33.8
Carbofuran	
Cyprinus carpio [27]	0.45
Colossoma macropomum [present study]	0.92

* - Purity degree varied from 97.4% to 99.9%;
** - Commercial formulation and
*** - up to 1.0 ppm.
▲ – Concentration capable of inhibiting 50% of enzyme activity

TABLE 3 – TEPP LC50 in several fish species.

Species	TEPP [%]	LC50 [mg/L]
Carassius auratus	40.0%	21.00
Gambusia affinis	40.0%	2.84
Ictalurus punctatus	40.0%	1.60
Lepomis macrochirus	40.0%	0.79
Pimephales promelas	40.0%	1.00
Poecilia reticulata	40.0%	1.80
Oncorhynchus tshawytscha	40.0%	0.31

Source: U.S. Environmental Protection Agency ECOTOX Database.
TEPP **-** Tetraethyl pyrophosphate;
LC50 - Concentration resulted in death for half of the animals.

Figure legends

FIGURE 1 – Effect of pH (A) and temperature (B) on the AChE from brain of juvenile *C. macropomum*. The pH range was attained by using citrate-HCL, citrate-phosphate, tris-HCl buffers whereas the temperature effect was investigated either on the enzyme activity (optimum temperature; ■) or on the enzyme preparation (thermal stability; ○) for 30 min that after 5 min (25ºC equilibrium) its activity was estimated.

FIGURE 2 - Michaelis–Menten plot of the AChE from brain of juvenile *C. macropomum* acting on acetylthiocholine. Data are expressed as the mean ± standard deviation of three replicates from four homogenates. The insert shows the Lineweaver–Burk plot.

FIGURE 3 – Effect of organophosphates and carbamates on the activity of AChE from brain of juvenile *C. macropomum*. Dichlorvos (A), diazinon (B), chlorpyrifos (C), temephos (D), TEPP (E), carbaryl (F) and carbofuran (G) concentrations ranged from 0.001 to 10 ppm. All the assays were performed at 25ºC and the experimental points are the mean ± standard deviation of triplicate of four crude extracts.

Figure 1

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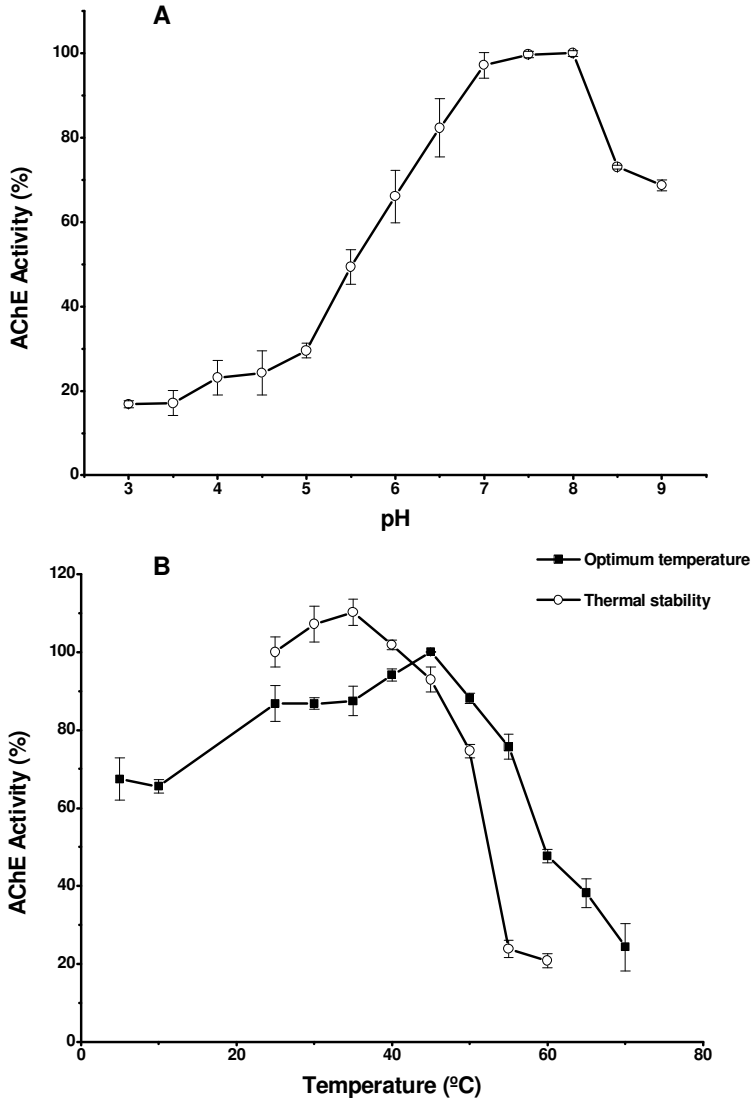

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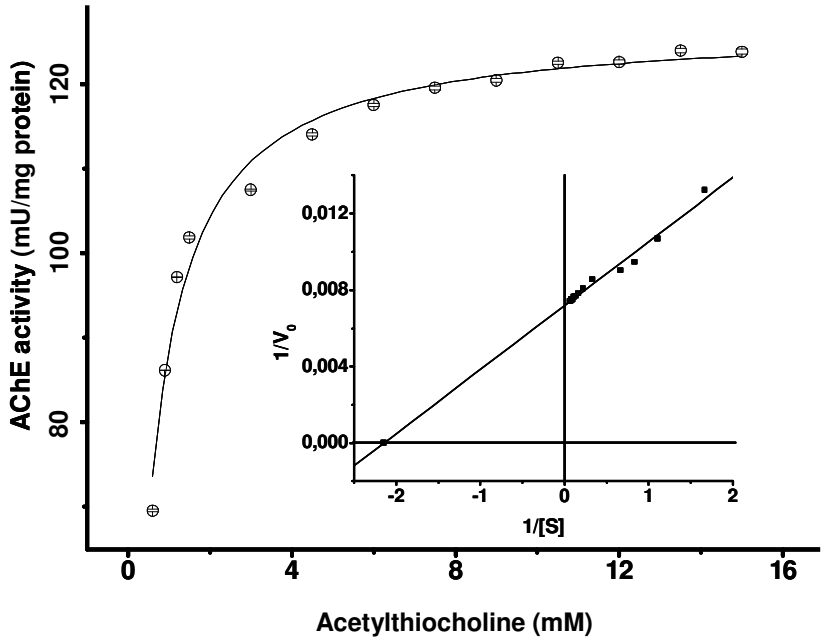

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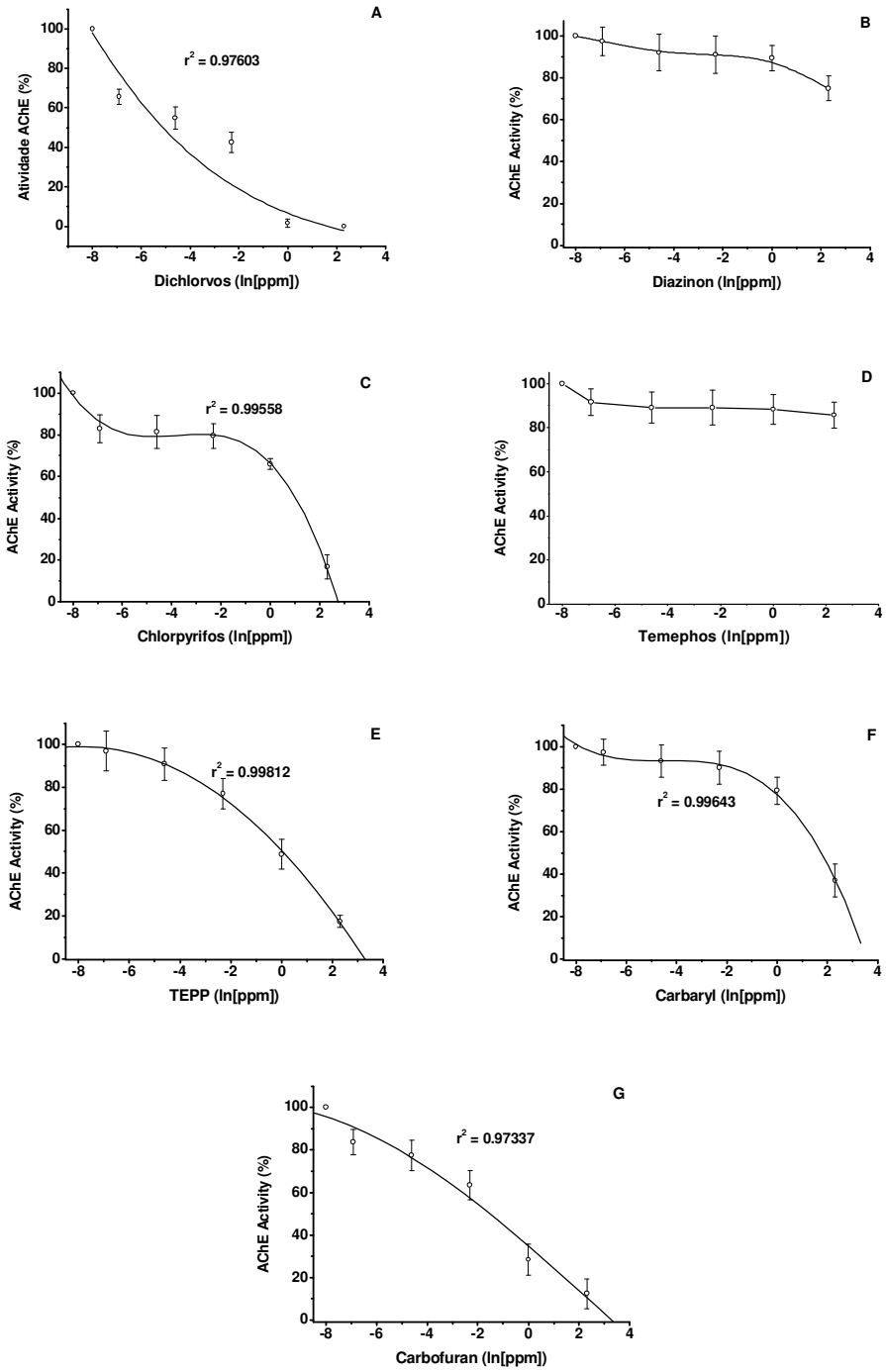

6. CONCLUSÕES

No presente trabalho, alguns dos pesticidas analisados foram altamente tóxicos em relação à acetilcolinesterase de tambaqui. Níveis relevantes de inibição da atividade enzimática foram alcançados em concentrações abaixo dos limites máximos de resíduos (LMRs) para esses pesticidas, previstos na legislação nacional e internacional em vigor.

Os limites de detecção do método observados para os pesticidas diclorvós e carbofuran situaram-se abaixo do LMR adotado para organofosforados e carbamatos em águas das classes 1 e 2, de acordo com a Resolução do CONAMA nº. 20 de 1986 e, no caso específico do carbofuran, também abaixo dos limites da legislação americana previstos no *USEPA National Primary Drinking Water Standards*. Para o organofosforado TEPP, seu efeito sobre a acetilcolinesterase alcançou altos níveis de inibição em concentrações ainda aceitáveis em águas da classe 3, segundo a mesma norma nacional.

De acordo com esses resultados, a acetilcolinesterase cerebral de tambaqui se mostra como uma ferramenta promissora para utilização em programas de monitoramento ambiental de pesticidas organofosforados e carbamatos.

7. ANEXOS

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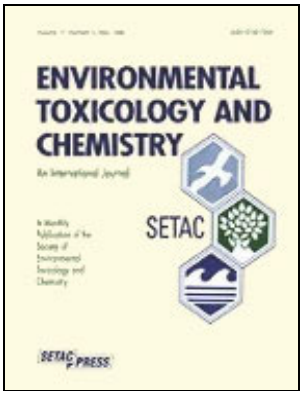

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- *Journal Article.* Author AB, Author CD. 2007. Title of article. *Environ Toxicol Chem* 26:2200-2204.
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Tables are frequently overused in scientific publications, and the first step in constructing a table should be to decide if it is needed. Presenting all data collected is rarely necessary, and printing tables is very costly. Tables should not duplicate information in the text or data presented in graphic forms and should stand alone without reference back to the text, as they are often reprinted.

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- Size and Proportion
- Shading
- Symbols and Lines

Well-chosen and carefully executed illustrations will aid in the comprehension of the text. They are costly to reproduce and consume much space, however, and should be limited to six per article. Illustrations should not duplicate information in tables or text; be certain that all are necessary to explain your research. Fitting the text around numerous illustrations is awkward and sometimes confuses the reader.

Include titles and brief explanatory legends for all illustrations on a separate page placed before the figures. Place long lists of symbol definitions in the figure legend, not on the figure itself. Label multipart figures with consecutive letters of the alphabet.

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In this section the X indicating multiplication should be consistent throughout.

Use acronyms and abbreviations sparingly to avoid impeding comprehension of the text, and use only those that are well known.

Define each at first introduction in the abstract and again at first mention in the text and on each table and figure legend, giving the abbreviation or acronym in parentheses. Spell out those that begin a sentence. The following may be used without definition: RNA, DNA, DDT, standard chemical symbols (such as Ca, NaOH), and those generally used for units of measure.

Technical Information

Equations, mathematical formulas, flow diagrams

Simple equations should be written as A/B on one line. Decimals are preferred to fractions. Write out and hyphenate simple fractions (two-thirds), except in figures, graphs, legends, and in parentheses.

Gases Express parts per million (ppm) as microliters per liter µl/L) or parts per billion (ppb) as nanoliters per liter (nl/L). Use metric system only.

Ions should be represented in the following way: Na+, Mn3+, Br-, and PO^{3-}

Isotopes An isotopically labeled compound is indicated by placing the isotopic symbol in square brackets attached to the name or the formula [^{14}C]ethanol; [^{32}P]ATP; [^{2}H]C_2H_2; [^{3}H]DNA. The specific position of the isotope should be given at the time of first mention; thereafter, it can be abbreviated to the less specific notation. The symbol indicating configuration should precede the isotopic symbol, and the position of isotopic labeling is indicated by Arabic numerals as in D-[^{14}C]lactate;D-[^{14}C]glucose 6-P; sodium D-[^{14}C]acetate; L-[1,2-^{14} C]alanine.

The term U indicates uniform labeling, as in [U-^{14}C]sucrose, where the isotope is uniformly distributed among all 12 carbons. Preference is given to [$^{14}C_2$] and ^{32}Pi rather than to [^{14}C]CO_2 or [^{14}C]CO_2 and [^{32}P]Pi.

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The metric system is standard, and SI units should be used as far as possible. Spell out all numbers or fractions that begin a sentence. If this is awkward, rephrase the sentence to avoid beginning with a numeral. Do not use a hyphen to replace the preposition "to" between numerals: 13 to 22 min, 3 to 10°C. Exception: The dash may be used in tables, figures, graphs and in parentheses. Write out numerals one through nine except with units of measure.

Check tabular data, as well as numerical values, reported in the text for the proper number of significant figures. For decimals smaller than one, insert a zero before the decimal point: 0.345.

Powers in tables and figures Care is needed in tables and figures to avoid numbers with many digits. The unit should be followed by the power of 10 by which the actual quantity was multiplied to give the reported quantity; the unit may be changed by the use of prefixes such as "m" or "m." For example, an entry "5" under the heading "g X 10^{-3}" means that the value of g is 0.005; and entry "5" under the heading "g X 10^{-3}" means that the value of g is 5,000. A concentration of 0.0015 M may be expressed as 1.5 under the heading "concn. (mM)" or as 1,500 under the heading "concn. (mM)" as 15 under the heading "10^{-4} x concn. (M)."

Ratios Mixtures use "to" when general words are used, i. e., "the chloroform to methanol" ratio. Always use a colon with words when numerical ratio is provided, i. e., chloroform:methanol (2:1,v/v). Always use colon with number ratio. Use a hyphen with mixture only if numerical value is not given, i. e., "used in chloroform-methanol."

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Solutions Solutions of common acids and bases should be described in terms of normality (N) , and salts in terms of molarity (M), thus 1 N NaOH, O.1 N acetic acid and O.1 M Na_2SO_4. Fractional concentrations should be expressed in the decimal system: 0.1 N acetic acid and not N/10 acetic acid. The term % must be defined as w/w, w/v, or v/v; 10% (w/v) signifies 10 g/100 ml. Express concentrations as ng/L, mg/L, mg/L, ng/g, mg/g, etc.

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Site Information

7.2 Indicadores de produção 2007-2008

7.2.1. Resumos em congressos

Resumo na SBBq 2007

L-72 FREITAS JR., A. C. V. ; CASTRO, P. F. ; ESPOSITO, T. S. ; AMARAL, I. P. G. ; ASSIS, C. R. D. ; MARCUSCHI, M. ; COSTA, H. M. S. ; CARVALHO JUNIOR, L. B. ; BEZERRA, R. S. Characterization of digestive amylases from marine shrimp *Litopenaeus schmitti*. In: XXXVI Reunião Anual da SBBq, 2007, Salvador. Resumos da XXXVI Reunião Anual da SBBq, 2007. v. único.

Palavras-chave: Carbohydrases characterization; Digestive physiology; Litopenaeus schmitti.

Referências adicionais: Classificação do evento: Nacional; Brasil/ Português; Meio de divulgação: Vários

Resumo na SBBq 2008

L - 44 CASTRO, P. F.; FREITAS Jr, A. C. V.; SANTANA, W. M.; ASSIS, C. R. D.; COSTA, H. M. S., FRANÇA, R. C. P.; CARVALHO JUNIOR, L. B.; BEZERRA, R. S. Characterization of amylases from the hepatopâncreas of the brown shrimp *Farfantepenaeus subtilisi.* In: XXXVII Reunião Anual da SBBq, 2008, Águas de Lindóia. Resumos da XXXVII Reunião Anual da SBBq, 2008. v. único.

Palavras-chave: Carbohydrases characterization; Digestive physiology; Farfantepenaeus subtilisi.

Referências adicionais: Classificação do evento: Nacional; Brasil/ Português; Meio de divulgação: Vários