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HELANE MARIA SILVA DA COSTA

PURIFICAÇÃO E CARACTERIZAÇÃO DE UMA TRIPSINA SENSÍVEL A METAL PESADO DO PEIXE *Caranx hippos*

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2007

Helane Maria Silva da Costa

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Dissertação apresentada ao **Programa de Pós-Graduação de Mestrado em Bioquímica** da Universidade Federal de Pernambuco, como parte dos requisitos necessários para a obtenção do grau de **Mestre em Bioquímica**.

Orientador: Prof. Dr. Ranilson de Souza Bezerra.
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Ata da defesa de dissertação da Mestranda Helane Maria da Silva Costa realizada em 10 de maio de 2007, como requisito final para obtenção do título de Mestre em Bioquímica.

Às 09:00 (nove horas), do dia 10 de maio de 2007, foi aberto, no "Anfitiatro 12 – Centro de Ciências Biológicas/UFPE, o ato de defesa de dissertação da mestranda Helane Maria da Silva Costa, aluna do Curso de Mestrado em Bioquímica/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. Vera Lúcia de Menezes Lima, Coordenadora do Curso supra citado, fez a apresentação da aluna, de seu orientador Prof. Dr. Ranilson de Souza Bezerra, dos co-orientadores Prof. Dr. Luiz Bezerra de Carvalho Júnior, Profa. Dra. Patrícia Maria Guedes Paiva e da Banca Examinadora composta pelos professores doutores: Ranilson de Souza Bezerra, na qualidade de Presidente, Maria Tereza dos Santos Correia, ambos do Depto. de Bioquímica/CCB/UFPE, Valdinete Lins da Silva, do Depto. de Engenharia Química/UFPE e Eurico Cabral de Oliveira Filho, do Depto. de Botânica da USP. Após as apresentações, a Profa. Dra. Vera passou a palavra para o Presidente que convidou a aluna para a apresentação de sua dissertação intitulada: "Purificação e caracterização da tripsina sensível a metal pesado do peixe tropical *Caranx hippos*" e informou que de acordo com o Regimento Interno do Curso, a candidata dispõe de até 50 (trinta) 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. A aluna procedeu a explanação e comentários acerca do tema em 40 (quarenta) minutos. Após a apresentação da mestranda, o Sr. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, o Prof. Dr. Eurico Cabral de Oliveira Filho, que agradeceu o convite, fez alguns comentários, deu sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Em seguida, o Sr. Presidente passou a palavra para Profa. Dra Maria Tereza dos Santos Correia que agradeceu o convite, fez alguns comentários e deu algumas sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Daí o Sr. Presidente passou a palavra para a Profa. Dra. Valdinete Lins da Silva, que agradeceu o convite, fez alguns comentários e deu algumas sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, o Sr. Presidente usou da palavra para tecer alguns comentários, agradecer à Banca Examinadora e parabenizar a candidata. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "Aprovada com Distinção". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 10 de maio de 2007.

A Deus pela dádiva da vida e todos os momentos vividos.

Aos meus pais pelo amor incondicional, dedicação por todos estes anos, incentivo, respeito e amizade.

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LISTA DE ABREVIATURAS

IUBMB	International Union of Biochemistry and Molecular Biology
pH	potencial Hidrogeniônico
DFP	diisopropil-fluorfosfato
PMSF	fenil-metil-sulfonil fluoreto
SBTI	Inibidores de tripsina de soja
BApNA	N-α-benzoil-L-arginina-p-nitoanilida
TAME	tosil-arginina-metil-éster
SDS	sulfato sódico de dodecila

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RESUMO

A tripsina-símile extraída do ceco pilórico de xaréu (*Caranx hippos*) foi purificada por um procedimento de três etapas: tratamento térmico, precipitação com sulfato de amônio e cromatografia de gel-filtração em Sephadex G-75. Os efeitos de vários íons metálicos e inibidores de proteases na atividade digestiva da enzima *in vitro* foram determinados. As propriedades físico-químicas e cinéticas da tripsina foram estabelecidas. O pH e temperatura ótima de reação encontrada foram 8,0 e 50°C, respectivamente. Após 30 minutos de incubação a 50ºC, foi detectada uma perda de 20% da atividade tríptica. A constante de Michaelis Menten foi 1,21 ± 0,38 mM usando benzoil-$_{DL}$-arginina-p-nitroanilida (BApNA) como substrato. Uma única banda (35,2 kDa) foi observada quando a amostra da enzima purificada foi aplicada em gel de eletroforese utilizando sulfato sódico de dodecila (SDS-PAGE, 12,5%). Atividade com substrato específico e inativação por inibidores de protease forneceram evidências adicionais que uma tripsina é responsável por esta atividade proteolítica. A tripsina de *C. hippos* apresentou sensibilidade a metais pesados. Utilizando soluções de íons na concentração de 1 mM, a tripsina foi inibida na ordem decrescente: Cd^{2+}=Al^{3+}>Zn^{2+}>Cu^{2+}>Pb^{2+}>Hg^{2+}. Menor efeito foi detectado com Co^{2+}, K^{+}, Li^{+}, Ba^{2+}, Mn^{2+}, Mg^{2+}e Ca^{2+}. Contudo, a concentração tão baixa quanto 0,01 ppm (10 µg/mL) dos íons Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+} e Al^{3+} foram suficientes para inibir 34%, 33%, 33%, 32%, 29% e 28%, da atividade proteolítica, respectivamente.

Palavras-chaves: espécies biomonitores, metal pesado, peixe marinho, tripsina, xaréu (*Caranx hippos*).

ABSTRACT

A fish trypsin-like enzyme extracted from pyloric caeca of crevalle jack (*Caranx hippos*) was purified by a three-step procedure previously described: heat treatment, ammonium sulfate precipitation and Sephadex G-75 filtration chromatography. The effect of various metal ions and protease inhibitors on the *in vitro* activity of digestive enzyme was determined. The physical-chemical and kinetics properties of a trypsin-like were characterized as well. The optimum pH found was 8.0, whereas the optimum temperature was 50^{o}C. After incubation at 50ºC for 30 min it was registered a loss of 20% the tryptic activity. The Michaelis-Menten constant was 1.21 ± 0.38 mM using in benzoyl-$_{DL}$-arginine-p-nitroanilide (BApNA) as substrate. Only one band (35.2 kDa) was observed when a sample of purified enzyme was applied in sodium dodecylsulf ate polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%). Specific substrate and protease inhibitors provided additional evidences that a trypsin-like enzyme is responsible for this proteolytic activity. This fish tryptic activity demonstrated to be very s ensitive to metal ions. Its activity was inhibited by the following ions (1mM) in decreasing order: Cd^{2+}=Al^{3+}>Zn^{2+}>Cu^{2+}>Pb^{2+}>Hg^{2+}. The effects of Co^{2+}, K^{+}, Li^{+}, Ba^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+} were noticeable but not extreme. Moreover the concentration as low as 0.01 ppm (10 µg/mL) of Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+} and Al^{3+} ions was enough to inhibit 34%, 33%, 33%, 32%, 29% and 28%, respectively.

Key words: Biomonitor species, Crevalle jack (*Caranx hippos*), Heavy metal, Marine fish, Trypsin.

1. INTRODUÇÃO

1.1 Xaréu (*Caranx hippos*).

Caranx hippos (Carangidae), conhecido regionalmente como xaréu e internacionalmente como crevalle jack (Fig.1), é um peixe marinho encontrado em águas tropicais e subtropicais, tem sua ocorrência da Nova Escócia até o Golfo do México e do Caribe ao Uruguai; ao Leste do Atlântico, de Portugal a Angola e no Oeste do Mediterrâneo (Fig.2). Podem ser encontrados em todo o litoral brasileiro. Habitam regiões de fundo duro, pedra ou areia, próximos a ilhas e costões. Grandes exemplares são encontrados em mar aberto, sendo que os pequenos podem ser capturados dentro de baías. Seu comprimento total médio é 100 cm, podendo alcançar 150 cm. É um peixe carnívoro alimentando-se principalmente de peixes, mas também de camarão e outros invertebrados (BERRY & SMITH-VANIZ, 1978). A maioria dos teleósteos não possui pâncreas definido, suas células pancreáticas estão difusas ao longo do trato digestivo. Nos peixes carnívoros o ceco pilórico geralmente é responsável pela síntese das enzimas digestivas (MARTINEZ & SERRA, 1989). Como um típico peixe de carnívoro, o sistema digestivo do xaréu é composto por um curto estômago seguido pelo ceco pilórico, e um pequeno intestino (ALENCAR *et al.*, 2003).

FIGURA 1. *Caranx hippos*. Fonte: www.mrmalin.com/fish/info-crevalle-jack.jpg

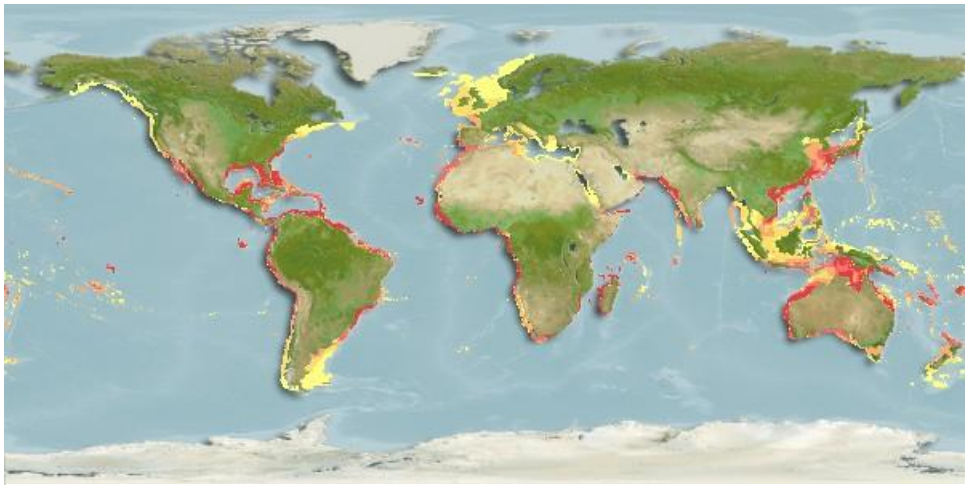

FIGURA 2. Distribuição geográfica do *C. hippos*. Fonte: www.fishbase.sinica.edu.tw/tools/aquamap/receive.php

1.2 Resíduos do processamento do pescado como fonte de biomoléculas.

Em 2005, aproximadamente, 4.900 toneladas de *C. hippos* foram capturadas na costa brasileira (IBAMA, 2007) gerando uma considerável quantia de resíduo pesqueiro, principalmente vísceras. O processamento de peixes gera grande quantidade de resíduos líquidos (águas residuais) e sólidos como: vísceras e cabeças. Estes geralmente são descartados no mar sem tratamento e não possuem valor comercial causando poluição ambiental (DOODE, 1996). A utilização de enzimas proteolíticas obtidas a partir das vísceras do peixe é uma alternativa para minimizar o ônus econômico e ecológico gerado pelo descarte destes resíduos da indústria pesqueira no ambiente marinho (BEZERRA *et al.*, 2001, 2005; SOUZA *et al.*, 2007). Este importante subproduto, em condições adequadas, pode ser utilizado como fonte alternativa de moléculas bioativas, como enzimas (BEZERRA *et al.*, 2001, 2005; CASTILLO-YÁÑES *et al.*, 2005; SOUZA *et al.*, 2007).

As enzimas digestivas presentes nas vísceras de peixes são as ácidas, encontra das no estômago e as alcalinas, encontradas no ceco pilórico. No que concerne às enzimas

alcalinas, tripsinas, quimiotripsinas e elastases, pertencentes à família das serino proteases, são as principais enzimas encontradas nesses tecidos (SIMPSON, 2000).

1.3 Proteases na indústria.

Proteases representam a classe mais importante de enzimas industriais, sendo responsáveis por 60% de toda enzima comercializada (Fig. 3) (DE VECHI & COPPES, 1996; JOHAVESLY & NAIK, 2001). Neste contexto, o uso de vísceras de peixes é citado como uma fonte alternativa de enzimas proteolíticas (BEZERRA *et al.*, 2001, 2005; RATHORE *et al.*, 2005; KURTOVIC *et al.*, 2006; KISHIMURA *et al.*, 2007; KLOMKLAO *et al.*, 2007; SIRINGAN *et al.*, 2007; SOUZA *et al.*, 2007). Alencar *et al.* (2003) em estudo prévio das proteases encontradas em diversos peixes, detectaram uma alta atividade de proteases alcalinas no ceco pilórico de *C. hippos*, sendo a tripsina responsável pela maioria desta atividade proteolítica.

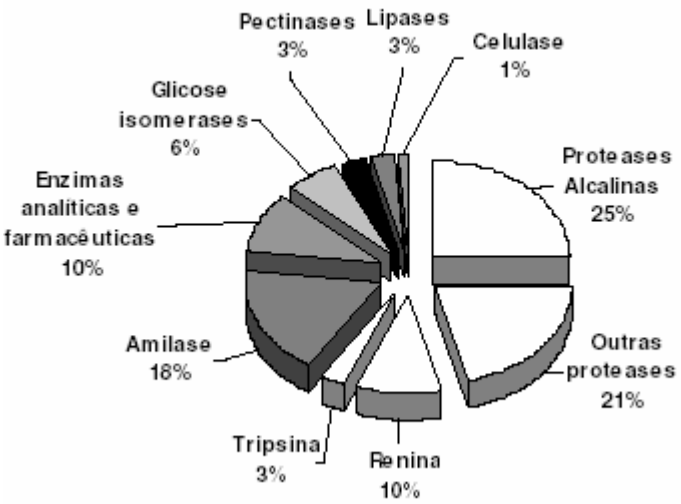

FIGURA 3. Enzimas no mercado mundial. Fonte: RAO *et al.*, 1998.

1.4 Tripsina

Do ponto de vista fisiológico, enzimas são catalisadores biológicos específicos, responsáveis pelo aumento da velocidade das reações bioquímicas. Com exceção de uns poucos RNAs catalíticos, todas as enzimas conhecidas são proteínas (NELSON & COX, 2004). Como são essenciais, tanto para a manutenção, como para o crescimento e a diferenciação celular, essas moléculas são encontradas em todos os organismos vivos (GUPTA *et al.,* 2002). As enzimas atuam de forma altamente específica com seus substratos. A reação enzimática ocorre no interior de uma cavidade na enzima chamada sítio ativo (Fig. 4), onde a molécula que se liga a essa região é contornada com resíduos de aminoácidos cujos grupos substituintes se ligam ao substrato e catalisam a sua transformação. As enzimas são divididas em seis grupos pela IUBMB de acordo com a reação específica que catalisa (Tabela 1) (NELSON & COX, 2004).

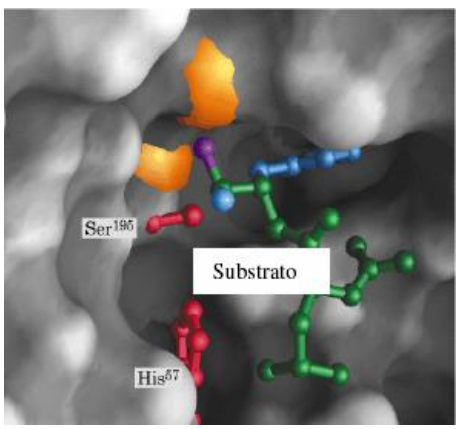

FIGURA 4. Ligação de um substrato ao sítio ativo de uma serinoproteinase. Fonte: NELSON & COX, 2004.

Tabela 1: Classificação das enzimas segundo a IUBMB.

CLASSE	REAÇÕES QUE CATALISAM
1. Oxidorredutases	Reações de oxidação-redução
2. Transferases	Reações de grupos contendo C, N ou P^-
3. Hidrolases	Clivagem das reações adicionando água
4. Liases	Clivagem de C-C, C-S e certas ligações de C-N
5. Isomerases	Racemização de isômeros ópticos ou geométricos
6. Ligases	Formação de pontes entre C e O, S, N acoplados a hidrólise de fosfatos de alta energia.

C, carbono; N, nitrogênio; P^-, íon fosfato; S, enxofre; O, oxigênio. Fonte: NELSON & COX, 2004.

Proteases são enzimas que catalisam a hidrólise das ligações peptídicas entre as proteínas (BERG *et al.*, 2004), podendo ser classificadas segundo o valor do pH no qual apresentam atividade máxima, diferenciando-se dessa forma em: proteases ácidas, neutras ou alcalinas (RAO *et al.*, 1998). De acordo com a IUBMB as proteases estão inseridas no subgrupo 4 do grupo 3 (Hidrolases), pois clivam a proteína adicionando uma molécula de água à ligação peptídica (Fig. 5) (BERG *et al.*, 2004).

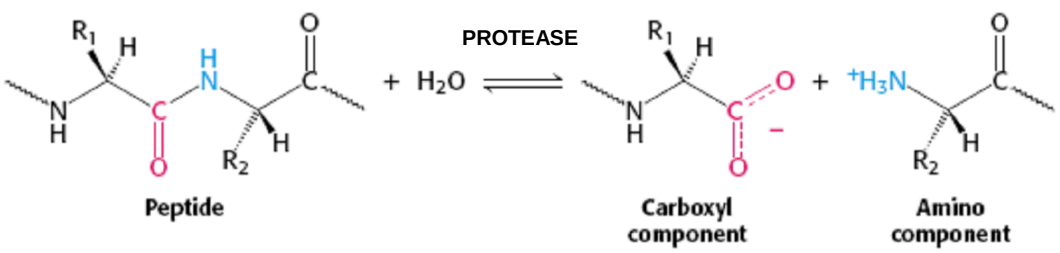

FIGURA 5. Hidrólise enzimática. Fonte: BERG *et al.*, 2004.

A tripsina (Fig. 6) é uma enzima chave na digestão protéica que apresenta atividade de endopeptidase, responsável também pela ativação do tripsinogênio e de outros zimogênios, como por exemplo, o quimiotripsinogênio (WHITAKER, 1994; KLOMKLAO *et al.*, 2007). Estão presentes em peixes como isoenzimas, apresentando essencialmente a mesma especificidade (CASTILLO-YÁÑEZ *et al.*, 2005). Essa enzima hidrolisa os peptídeos nos sítios carboxílico dos resíduos de aminoácidos carregados positivamente como: arginina e lisina (Fig. 7). Como outras serino proteases, a tripsina se caracteriza por possuir um resíduo de serina no sítio ativo, por apresentar o maior nível de atividade nos valores de pH entre 8,0 e 11,0 e em temperaturas de 35° - 45°C, inibição ou instabilidade em pH abaixo de 5,0 e acima de 11,0 e inibição por diisopropil-fluorfosfato (DFP), fluoreto fenil-metil-sulfonil (PMSF), inibidor de tripsina de soja (SBTI) e aprotonina. A tripsina hidrolisa substratos sintéticos como: N-α-benzoil-L-arginina-p-nitoanilida (BApNA) e tosil-arginina-metil-éster (TAME) (WHITAKER, 1994; SIMPSON, 2000).

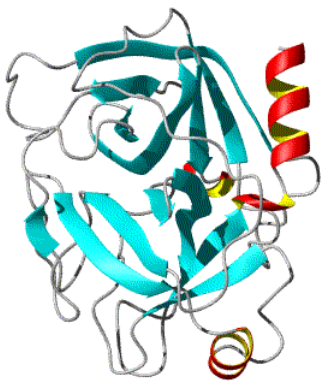

FIGURA 6. Estrutura terciária da tripsina.
Fonte: www.icp.csic.es/biocatalisis/web3/tripsina.g

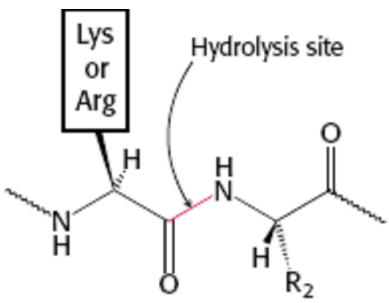

FIGURA 7. Sítio de hidrólise da tripsina.
Fonte: BERG *et al.*, 2004.

1.5 Purificação e caracterização de tripsinas

Com o objetivo de purificar tripsinas, se utilizam técnicas comuns a processos de isolamento de proteínas, onde a primeira etapa é o preparo de extratos em solução salina ou tampão, seguido de fracionamento salino com sulfato de amônio e diálise das preparações protéicas obtidas. O tratamento com o sal, salting-out, é capaz de precipitar proteínas por retirar a camada de solvatação que as tornam solúveis, este método é eficiente uma vez que separa moléculas de acordo com sua solubilidade (VOET & VOET, 1995; BRACHT & ISHII-IWAMOTO, 2002). Tripsinas de peixes tropicais têm sido parcialmente purificadas através deste procedimento (BEZERRA *et al.*, 2001, 2005; BOUGATEF *et al.*, 2007; SOUZA *et al.*, 2007).

Técnicas cromatográficas convencionais (Fig. 8) como, cromatografia de troca iônica (SIRINGAN *et al.*, 2007) e de gel filtração (BEZERRA *et al.*, 2001, 2005; BOUGATEF *et al.*, 2007; SOUZA *et al.*, 2007) que separam as tripsinas de acordo com a carga líquida da molécula ou por sua massa molecular, respectivamente, podem ser utilizadas. Além destas, a cromatografia de afinidade desenvolvida por CUATRECASAS *et al.* (1968) isola as tripsinas de acordo com a especificidade de interação via sítio ativo. Nesta técnica, as proteínas ligam-se ao suporte inerte para uma posterior dessorção, seja por competição bioespecífica pelo sítio protéico com o inibidor, sej a por alteração do pH e da força iônica (CASTILLO-YÁÑEZ *et al.*, 2005; KURTOVIC *et al.*, 2006).

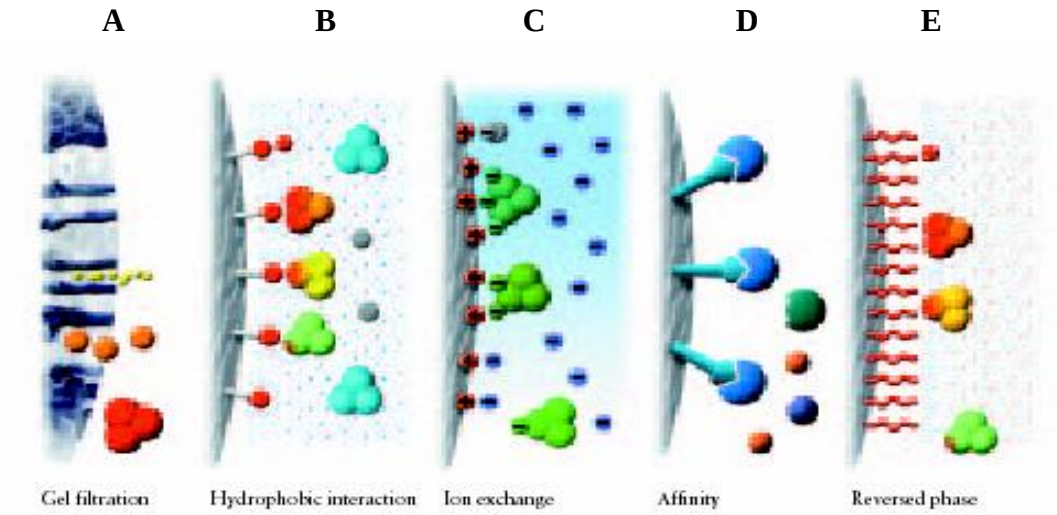

FIGURA 8. Técnicas cromatográficas. **(A)** Gel Filtração; **(B)** Interação Hidrofóbica; **(C)** Troca Iônica; **(D)** Afinidade; **(E)** Fase Reversa. Fonte: Amersham Bioscience Handbook. Gel Filtration. Principles and Methods.

As tripsinas podem ser caracterizadas por eletroforese; um método comumente utilizado para a determinação da pureza e da massa molecular de subunidades proté icas faz uso do detergente sulfato sódico de dodecila (SDS). Depois de realizada a eletroforese as proteínas são visualizadas pela adição de um corante, o azul de Coomassie (LAEMMLI, 1970). Eletroforese em gel de poliacrilamida para proteínas sob condições não desnaturantes é uma poderosa técnica de análise de pureza de estruturas moleculares nativas (NEMOTO & SATO, 1998), baseando-se em suas cargas resultantes totais positivas ou negativas (conteúdos relativos ácidos e básicos, respectivamente).

Tripsinas têm sido extraídas, purificadas e caracterizadas a partir de vísceras de peixes, incluindo: *Mallotus villosus* (HJELMELAND & RAA, 1982), *Gadus ogac* (SIMPSON & HAARD, 1984), *Colossoma macropomum* (BEZERRA *et al.*, 2001), *Engraulis japonica* (KISHIMURA *et al.*, 2005), *Sardinops sagax caerulea* (CASTILLO-YÁÑEZ *et al.*, 2005), *Oreochromis niloticus* (BEZERRA *et al.*, 2005), *Sardinops melanostictus* e *Pleurogrammus azonus* (KISHIMURA *et al.*, 2006), *Stolephorus spp.*

(SIRINGAN *et al.*, 2007), *Sebastes schlegelii* e *Alcichthys alcicornis* (KISHIMURA *et al.*, 2007), *Sardina pilchardus* (BOUGATEF *et al.*, 2007), *Katsuwonus pelamis* (KLOMKLAO *et al.*, 2007) e *Pseudupeneus maculatus* (SOUZA *et al.*, 2007).

Tripsinas de peixe tropical foram estudadas (GUIZANI *et al.*, 1991; BEZERRA *et al.*, 2001, 2005; KISHIMURA *et al.*, 2006; KLOMKLAO *et al.* 2007; SOUZA *et al.*, 2007). Estas enzimas demonstraram ser termoestável, com alta atividade em pH alcalino e sensível a metais pesados. O fato das tripsinas de peixes tropicais serem termoestáveis tem contribuído para sua purificação através do uso de tratamento térmico como etapa de purificação (BEZERRA *et al.*, 2001, 2005; BOUGATEF *et al.*, 2007; SOUZA *et al.*, 2007).

1.6 Metais pesados e Biomarcadores

Ao longo das décadas, a poluição ambiental por metais se tornou um dos problemas mais importantes no mundo (CHANDRAN *et al.*, 2005). O ambiente marinho está continuamente sujeito à poluição química proveniente das atividades antropogênicas que podem aumentar a descarga de metais pesados em várias concentrações no s ecossistemas aquáticos naturais (PAPAGIANNIS *et al.*, 2004) e prejudicar os organismos aquáticos que vivem naquele ambiente (ALINK, 1982). Esta contaminação ocorre devido ao uso extenso de metais nos processos agrícolas, químicos e industriais (PREGO & CO BELO-GARCIA, 2003; CHEUNG *et al.*, 2004). A contaminação de ecossistemas aquáticos por metais pesados aumentou mundialmente, esta poluição é menos visível e imediata que outros tipos, mas seus efeitos sobre o ecossistema e nos seres humanos são intensos e d e longo prazo (LANGSTON & SPENCE, 1995). A presença de metais pesados no ambiente é parcialmente devido aos processos naturais, mas é principalmente resultado dos resíduos industriais (MANSOUR & SIDKY, 2002). Os efeitos iniciais da poluição por metal pesad o

podem ser evidenciados em níveis celulares ou teciduais antes que mudanças significativas no comportamento dos peixes ou na aparência externa possam ser identificadas. Estas substâncias químicas podem afetar a biotransformação e a detoxificação das enzim as dos organismos aquáticos, seja como inibidores ou como moduladores de atividade enzimática (MOYLE & CECH, 1988).

Metais pesados são constituintes comuns do ambiente marinho e são encontrados em diversos níveis no solo e na água (NIEBOER & RICHARDSON, 19 80; MARTIN & COUGHTREY, 1982). Os contaminantes ambientais são aqueles que tendem a acumular -se em organismos, possuem estabilidade química ou baixa biodegradabilidade, e aqueles que são solúveis e então ambientalmente móveis (HELLAWELL, 1986; SANDERS, 199 7). Alguns metais pesados são elementos essenciais para o metabolismo normal de organismos; enquanto outros, não-essenciais, não possuem nenhum papel biológico significante (SANDERS, 1997; EC, 2001). Pelo menos, 11 são essenciais aos organismos marinhos: Co, Cu, Cr, Fe, Mn, Mo, Ni, Se, V e Zn (BRYAN, 1979). Estes metais sempre funcionam em combinação com moléculas orgânicas, geralmente proteínas (RAINBOW, 1992). Todos os metais, inclusive os essenciais, são tóxicos em altas concentrações, Ag, Cd, Cu, Hg e Pb são particularmente tóxicos (BRYAN, 1979; EC, 2001).

Os metais pesados devido a suas propriedades bioacumulativas e n ão biodegradáveis constituem o topo dos poluentes aquáticos (RAMESH, 2006). A concentração destes íons no ambiente marinho é variável e depende da entrada de efluentes no meio aquático (VENKATESWARA RAO *et al.*, 2006). Estudo comparativo da poluição por metais pesados nos ambientes aquáticos é possível através da análise da água, dos sedimentos e dos membros da biota como biomarcadores ou biomonitores (PHILLIPS & RAINBOW, 1993).

Os metais podem ser obtidos pelos peixes através da água, da comida, dos sedimentos, e a partir do material particulado suspenso (HARDERSEN & WRATTEN, 1998). Os metais pesados podem ser captados através de diferentes órgãos e em variadas concentrações nos peixes (RAO & PADMAJA, 2000). Sabe-se que bioacumulação é mediada por fatores abióticos e bióticos que influenciam na taxa de captação e de eliminação do metal (HAKANSON, 1984; RAJOTTE *et al.*, 2003) e pode ser evidenciada mesmo quando estes contaminantes estão presentes nas águas em concentrações não detectáveis (MACHADO *et al.*, 2002). O acúmulo de metais nos tecidos de organismos marinhos tem sido estudado como uma medida indireta da abundância e disponibilidade de metais no ambiente (LANGSTON & SPENCE, 1995). Estudos indicam que espécies diferentes de peixes viventes numa mesma área apresentam diferentes concentrações de metal em seus tecidos (CANLI & ATLI, 2003; MARCOVECCHIO, 2004; DURAL *et al.*, 2007) uma vez que estes podem usar diferentes estratégias para alcançar a homeostase na concentração do metal. Os mecanismos para reduzir a toxicidade o acúmulo de metal inclui a inibição da captação, aumento da excreção e detoxificação (FERNANDES *et al.*, 2007).

Nas análises ambientais, os poluentes como metais pesados e pesticidas são geralmente detectados pela inibição de uma atividade enzimática promovida por estes contaminantes tóxicos (MALITESTA & GUASCITO, 2005). Alterações na atividade enzimática de animais aquáticos podem servir como prévios indicadores de toxicidade por metais pesados, pesticidas e outros poluentes (RAMESH, 2006). O estudo da biotransformação das enzimas de peixes é importante em vários aspectos incluindo parâmetros evolucionários, ecológicos e toxicológicos (MOYLE & CECH, 1988).

O cádmio é um elemento-traço não-essencial, altamente tóxico aos ecossistemas aquáticos e não possui nenhum papel biológico (DWAF, 1996). É bastante utilizado nas industriais: em baterias de Cd, camada anticorrosiva de meta is, pigmentos, e estabilizadores para plástico (JARUP, 2003). Nos peixes, a contaminação por cádmio pode ser pela via alimentícia aquática, através do consumo direto da água ou biota; e ou por rotas não dietéticas como absorção epitelial. Além disso, as brânquias, a pele, e área digestiva são locais potenciais de absorção de Cd na água (HANDY, 1992).

Os peixes estão freqüentemente no topo da cadeia alimentar aquática e podem concentrar grandes quantias de Cd obtidos na água. Estudo da exposição de Cd em pe ixe detectou uma acumulação mais alta no intestino, rim e fígado (HANDY, 1992). A presença de Cd em comidas pode constituir um sério risco à saúde. Por exemplo, uma alta quantidade Cd acumulado no corpo humano pode prejudicar o rim ou fígado, com sintomas de toxicidade crônica incluindo prejuízo da função renal, tumores e deficiência hepática (MANSOUR & SIDKY, 2002).

Ruangsomboon & Wongrat (2006) em estudo sobre a bioacumulação de Cd na cadeia trófica, tendo como produtor o fitoplâncton, *Chlorella vulgaris*, como consumidor primário, o zooplâncton *Moina macrocopa* e as espécies de bagre *Clarias macrocephalus* e *Clarias gariepinus* como consumidor secundário; detectaram que as concentrações de Cd no músculo do peixe era diretamente proporcional ao aumento da con centração deste metal na comida e que após 60 dias de cultivo, as concentrações m édias de Cd no grupo analisado era de 1,04±0,06 μg/g (peso seco). O crescimento dos peixes também foi afetado quando comparado ao grupo controle.

O alumínio não é considerado um elemento essencial aos humanos, a exposição ao alumínio está implícita em inúmeras doenças humanas, incluindo Mal de Parkinson e Doença de Alzheimer (NARIN *et al.*, 2004). A dose de alumínio permitda para um adulto é de 60 mg /dia (WHO, 1989). Tuzen & Soylak (2007) detectaram as concentrações variantes de alumínio entre 0,45−1,50 μg/g em cinco espécies diferentes de peixes. A variação do conteúdo de alumínio encontrado em 3 espécies de peixes por Türkmen *et al.* (2005) foi de 0,02–5,41 mg kg^{-1} peso seco, enquanto Ranau *et al.* (2001) detectaram valores entre 0,032–5,346 μg/g peso seco.

Zinco é geralmente considerado um dos metais menos perigosos (DUFFUS, 1980; ROBINSON, 1996), mas freqüentemente encontra-se na natureza junto com outros metais, dos quais o Cd é um do mais comuns (DALLAS & DAY, 1993). Yang *et al.* (2007) detectaram as concentrações de 2,0 e 6,9 μg/g de Cu^{2+} e Zn^{2+}, respectivamente, em peixes *Gymoncypris namensis*.

O mercúrio é um metal pesado que além do seu estado elementar, se apresenta nos estados Hg^{1+} e Hg^{2+}. Pode ser proveniente de fontes naturais, como o intemperismo, atividades vulcânicas e a degaseificação da crosta e de fontes antropogênicas, incluindo a agricultura, a extração mineral e as atividades industriais. Em um ecossistema aquát ico, o mercúrio participa de múltiplas reações, uma delas é a metilação, que é a transformação de Hg^{2+} em metilmercúrio (MeHg), entrando dessa forma na cadeia alimentar. Quando entra na cadeia trófica, o MeHg apresenta biomagnificação, caracterizado pela t ransferência de MeHg acumulado no primeiro nível trófico (os produtores) para os consumidores, sendo que quanto mais longa for a cadeia, maior será a concentração acumulada pelo consumidor final (CABANA *et al.*, 1994). Desta forma, os maiores teores de MeHg são encontrados em

peixes que estão no topo da cadeia trófica, como os peixes carnívoros. A ingestão de peixes é a principal via de exposição de MeHg ao seres humanos, sendo este um reconhecido agente neurotóxico, em especial, sobre o sistema nervoso central de fetos humanos (ação teratogênica) (WHO, 1990).

O Pb e seus sais podem danificar o sistema nervoso, a hematose, os rins de peixe e seres humanos (CHUANG *et al.*, 2004). Mendil & Uluözlü (2007) em análise da concentração de metais em 5 espécies de peixes (*Cyprinus carpio*, *Capoeta tinca*, *Leiciscus cephalus*, *Carassius gibelio* e *Silurus glanis*) de lagos de Tokat (Turquia) encontraram as concentrações máximas de: 167; 48,6; 3,6; 2,8; 1,6; 64,3 e 5,6 μg/g, para os íons Fe, Zn, Cu, Pb, Cr, Mn e Ni, respectivamente.

O estudo da bioacumulação tem levado a adoção do conceito de bioindicador (LANGSTON & SPENCE, 1995). Uma vez que a análise direta de tais substâncias não fornece informações sobre seu efeito no ecossistema, o uso de biomonitores ou biomarcadores é uma opção altamente recomendada porque estes respondem especificamente a quantidade de contaminante biodisponível (RUELAS-INZUNZA & PÁEZ-OSUNA, 2000).

Biomarcadores são definidos como "indicadores de curto prazo" de efeitos biológicos a longo prazo (CAJARAVILLE *et al.*, 2000) que medem a interação entre um sistema biológico e um agente ambiental, que pode ser químico, físico, ou biológico (WHO/IPCS, 1993). O uso de marcadores biológicos ou biomarcadores a nível molecular ou celular foi proposto como ferramenta sensível de "primeira advertência" para mensurar qualidade ambiental (MCCARTHY & SHUGART, 1990).

Os biomarcadores podem ser usados para vários propósitos, dependendo da finalidade do estudo e do tipo da exposição química. Podem ter como objetivos avaliar a exposição (quantidade absorvida ou dose interna), avaliar os efeitos das substâncias químicas e avaliar a suscetibilidade individual. Além disso, podem ser utilizados independentemente da fonte de exposição, seja através da dieta, do meio ambient e geral ou ocupacional. A utilização de biomarcadores pode ter como finalidade elucidar a relação causa-efeito e dose-efeito na avaliação de risco à saúde; para fins de diagnóstico clíni co; e para fins de monitoramento biológico, realizada de maneira sistemática e periódica (WHO, 1993).

Independente da finalidade e aplicação dos biomarcadores, eles podem ser classificados em 3 tipos (WHO, 1993):

• ***Biomarcadores de Exposição*:** podem ser usados para confirmar e avaliar a exposição individual ou de um grupo, a uma substância exógena, seu metabólito ou o produto de interação de um agente xenobiótico com uma molécula ou célula, estabelecendo uma ligação entre a exposição externa e a quantificação da exposição interna.

• ***Biomarcadores de Efeito*:** podem ser usados para documentar as alterações bioquímicas, fisiológicas e outras alterações nos tecidos ou fluidos corpóreos decorrentes da exposição e absorção da substância química. Dessa forma, a ligação dos biomarcadores entre exposição e efeitos contribui para a defi nição da relação dose-resposta.

• ***Biomarcadores de Suscetibilidade*:** permitem elucidar os graus de resposta inerentes ou adquiridos de um organismo, a exposição a agentes xenobióticos específicos, incluindo fatores genéticos e as mudanças nos receptores que podem alterar a suscetibilidade do organismo ao que é exposto.

1.7 Enzimas de peixes como biomarcadores ambientais.

A presença de metais pesados e outros poluentes no ambiente aquático, seu acúmulo em peixes (OLSVIK *et al.*, 2000, 2001a, b; LUCKENBACH *et al.*, 2001; WATANABE & TANABE, 2003; AHMAD *et al.*, 2006; RUANGSOMBOON & WONGRAT, 2006; ASSIS *et al.*, 2007; LAMAS *et al.*, 2007; OLIVEIRA *et al.*, 2007; YANG *et al.*, 2007) e em outros organismos, tais como baleias (MENDEZ *et al.*, 2002) e moluscos (PÉREZ *et al.*, 2004; MARIANO *et al.*, 2006; JING *et al.*, 2006; RICCIARDI *et al.*, 2006) tem sido investigada nos últimos anos como indicadores de estresse ambiental.

Entre os biomonitores disponíveis, os peixes são freqüentemente usados, pois oferecem vantagens específicas em descrever as características naturais de sistemas aquáticos e na avaliação das mudanças do habitat (CHOVANEC *et al.*, 2003). Primeiramente, eles vivem muitos anos e integram e ou absorvem flutuações de contaminantes com o passar do tempo. Além disso, os contaminantes mais persistentes serão mais abundantes nos tecidos dos organismos mais velhos. Segundo, por serem organismos aquáticos, permitem uma monitoração contínua dos contaminantes e desempenham um importante papel na cadeia alimentar como o transportador de energia entre os níveis tróficos (BEYER, 1996; LAMAS *et al.*, 2007). E finalmente, a magnificação devido a bioacumulação melhora a precisão e baixa o custo da análise dos contaminantes, uma vez que permite a detecção dos contaminantes na faixa limite de detecção analítica se comparado às análises da água (RAINBOW & PHILLIPS, 1993). A compreensão da forma de captação, do comportamento e das respostas nos peixes possui uma grande relevância ecológica (STEGEMAN *et al.*, 1992).

Apesar de suas limitações, como mobilidade relativamente alta, os peixes geralmente são considerados os organismos mais viáveis para o monitoramento de poluição

em ecossistemas aquáticos (van der OOST *et al.,* 2003) e são extensamente usados como bioindicadores de poluição marinha por metais (EVANS *et al.,* 1993).

A fim de avaliar a ocorrência da exposição ou os efeitos dos poluentes de ecossistemas aquáticos, uma série de biomoléculas e parâmetros fisiológicos encontrados nos peixes pode ser utilizada como biomarcadores, como: enzimas de biotransformação (fase I e II), parâmetros de estresse oxidativo, produtos da biotransformação, proteínas de estresse, metalotioneínas (MTs), parâmetros hematológicos, imunológicos, reprodutivos, endócrinos, genotóxicos, neuromuscular, fisiológicos, histológicos e morfológicos (van der OOST *et al.,* 2003).

Dentre as enzimas de peixe utilizadas como bioindicadores, incluem-se: enzima citocromo P450, 7 ethoxiresorufinas-*O*deetilase (EROD), benzo(*a*)pireno monooxigenase (BaPMO), acetilcolinesterase (AChE), catalase e superóxido dismutase (SOD) (CORSI *et al.*, 2003; ATLI *et al.*, 2006; SEN & SEMIZ, 2006; KAVITHA & VENKATESWARA RAO, 2007; VEGA-LÓPEZ *et al.*, 2007).

Uma vez que o tubo digestivo é uma via importante de captação e entrada de metais pesados nos animais aquáticos, os efeitos adversos destas substâncias químicas podem prejudicar o transporte de nutrientes através das células epiteliais (XU & PASCOE, 1993). Embora o papel central de enzimas digestivas nos processos clivagem e absorção de alimentos, poucos estudos examinaram a atividade destas enzimas sob efeito de contaminantes e seu potencial como biomarcadores. A exposição de concentrações sub-letais de metais pesados pode interferir na atividade enzimática digestiva da espécie exposta, e promover uma redução da captação de energia, afetando a sobrevivência, o crescimento e a reprodução dos organismos (DE COEN & JANSSEN, 1997; DE COEN *et al.,* 1998). Estes efeitos no metabolismo energético podem ocorrer pois as células

digestivas estão conectadas às células de armazenamento de carboidratos e lipídios (BODAR *et al.,* 1990). Enzimas digestivas possuem inibição por metais pesados. Wang *et al.* (2006), isolaram e caracterizaram a pepsina do peixe *Scophthalmus maximus* L. que foi inibida por Cu^{2+} e Fe^{3+}. Como descrito em outras proteases de peixes tropicais (COHEN *et al.*, 1981; ARANISHI *et al.,* 1998; BEZERRA *et al.*, 2005; BOUGATEF *et al.*, 2007; SOUZA *et al.*, 2007), tripsinas têm demonstrado sensibilidade a metais pesados.

O uso de tripsinas de animais aquáticos foi proposto como biomarcador para monitoramento de metais pesados. A tripsina de *Daphnia magna* foi usada como um biomarcador para Cd, Cr e Hg (DE COEN & JANSSEN, 1997; DE COEN *et al.,* 1998). Alayse-Danet *et al.* (1979) detectaram uma redução do crescimento que coincidiu com uma clara diminuição da atividade enzimática da tripsina e amilase em *Artemia salina* exposta por 72h a concentrações sub-letais de cobre (2 ppm) e zinco (5 ppm). Sastry e Gupta (1979) avaliaram o efeito da concentração de 6,8 mg/L de $CdCl_2$ no sistema digestivo do peixe teleósteo *Heteropneustes fossilis* e detectaram uma diminuição na atividade da tripsina e um aumento da atividade da pepsina. O efeito da concentração de 0,3 mg/L de $HgCl_2$ na atividade da fosfatase alcalina, fosfatase ácida, glicose 6 fosfatase, amilase, maltase, lactase, lípase, tripsina, pepsina, aminotripeptidase, glicilglicina dipeptidase e glicil-L-leucina dipeptidase no sistema digestivo do peixe-gato *H. fossilis* após um período de 7, 15 e 30 dias de exposição foi avaliado por Gupta e Sastry (1981), uma diminuição significativa da atividade enzimática foi observada em todas as enzimas digestivas citadas, exceto a pepsina que se manteve inalterada.

2. JUSTIFICATIVA

Metais pesados são continuamente lançados no ambiente aquático por via natural ou antropogênica, ainda que em baixas concentrações, podem causar sérios efeitos tóxicos aos organismos e ao ecossistema. Tripsinas são enzimas-chave na digestão protéica nos organismos e uma alteração na sua atividade pode afetar o desenvolvimento dos organismos vivos. Devido ao fato da tripsina dos peixes poder ser obtida e purificada a partir de vísceras e apresentar sensibilidade a metais pesados, sugere-se que esta enzima possa ser empregada como biomarcador de metais pesados.

3. OBJETIVOS

3.1 Objetivo Geral

Extrair, purificar e caracterizar a tripsina presente no ceco pilórico do peixe tropical xaréu (*Caranx hippos*) e avaliar a sensibilidade desta enzima perante diferentes concentrações de metais pesados através de um ensaio enzimático *in vitro*.

3.2 Objetivos Específicos

- Detectar a presença de tripsina através de ensaio com substrato específico (BApNA) em preparações de ceco pilórico de *C. hippos*: extrato Bruto (EB), F 0-80% e pool de proteínas obtido a partir da coluna de gel-filtração;
- Purificar a tripsina através da metodologia previamente descrita por BEZERRA *et al.*, 2001;
- Caracterizar os parâmetros físico-químicos e cinéticos da tripsina purificada do ceco pilórico de *C. hippos*;
- Determinar o peso molecular aparente da tripsina purificada de *C. hippos* através de SDS-PAGE;
- Avaliar a sensibilidade da proteína purificada aos íons Al^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} e Zn^{2+} nas concentrações de 1 mM e 0,01, 0,001 e 0,0001 ppm (partes por milhão).

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TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO NO PERIÓDICO

CHEMOSPHERE

Metal-sensitive trypsin-like enzyme in the marine fish *Caranx hippos*

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Abstract

A fish trypsin-like enzyme (35.2 kDa) was extracted from the pyloric caeca of a crevalle jack (*Caranx hippos*) and purified. The effects of various metal ions and protease inhibitors on the *in vitro* activity of the digestive enzyme was then determined. The physical-chemical and kinetic properties of the trypsin-like enzyme were also characterized. The optimum pH and temperature were 8.0 and 50 ^{o}C, respectively. After incubation at 50ºC for 30 min, a 20% loss was registered in the tryptic activity. The Michaelis-Menten constant was 1.21 ± 0.38 mM using benzoyl-$_{DL}$-arginine-p-nitroanilide (BApNA) as substrate. Specific substrate and protease inhibitors provided additional evidence, which indicated that a trypsin-like enzyme is responsible for this proteolytic activity. This fish tryptic activity proved extremely sensitive to metal ions. This activity was inhibited by the following ions (1mM), in decreasing order: Cd^{2+}=Al^{3+}>Zn^{2+}>Cu^{2+}>Pb^{2+}>Hg^{2+}. Although certain effects were traced of Co^{2+}, K^{+}, Li^{+}, Ba^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+}, they were not to the extreme. Moreover, a concentration as low as 0.01 ppm (10 µg/mL) of Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+} and Al^{3+} ions was sufficient to inhibit activity in 34%, 33%, 33%, 32%, 29% and 28%, respectively. Trypsin activity inhibition may negatively affect the digestive physiology, thus causing a negative effect on the survival, growth and reproduction of this species.

Keywords: Marine fish, Crevalle jack (*Caranx hippos*), Biomonitor species, Metal, Trypsin.

1. Introduction

The Crevalle Jack (*Caranx hippos*) is a marine fish found throughout tropical and subtropical zones worldwide. On the American continent, it is found from Nova Scotia through to the entire Gulf of Mexico and Caribbean, and as far south as the Uruguayan coastline. It measures approximately 100 cm in length, but may reach 150 cm. It feeds primarily on fish, but also on shrimp and other invertebrates (Berry et al., 1978). In 2005, around 4,900 tons of this fish were caught along the Brazilian coast, and it is of significant commercial importance to the Brazilian fishery industry (IBAMA, 2007).

Most teleostei do not possess a defined pancreas gland, thus the pancreatic cells are spread along the digestive tract. In carnivore fish, the pyloric caeca is generally responsible for the synthesis of digestive enzymes (Zendzian and Barnarb, 1967; Martinez and Serra, 1989). Being a typical carnivorous fish, the crevalle jack digestive tract is composed of the stomach, leading to the pyloric caeca, and a short intestine. Previous study has revealed high amounts of alkaline proteases in the pyloric caeca of this fish. Trypsin was found to be largely responsible for most of the proteolytic activity (Alencar et al., 2003).

Trypsins are present in fish as isoenzymes and all have essentially the same specificity (Whitaker, 1994; Castillo-Yáñes et al., 2005), and constitute the key enzyme in the process of protein digestion. Moreover, it catalyses the hydrolysis of pancreatic zymogens. These enzymes are determined as thermostable, with high levels of alkaline pH activity, and are sensitive to many metals ions (Bezerra et al., 2001, 2005; Castillo-Yáñes et al., 2005; Bougatef et al., 2007; Kishimura et al., 2007; Klomklao et al., 2007; Siringan et al., 2007; Souza et al., 2007).

Over the past few decades, great concern has been shown regarding the increasing levels of environmental pollution from metals, due to the extensive use of metals in agricultural, chemical and industrial processes (Cheung et al., 2003; Prego and Cobelo - Garcia, 2003; Chandran et al., 2005). The marine environment is particularly vulnerable to the toxic effects of pollutants discharged into aquatic systems, and which may be detrimental to living aquatic organisms (Venkateswara Rao et al., 2006). These chemicals may affect the biotransformation and detoxification enzymes of aquatic organisms, either as inhibitors or as modulators of enzyme activity (Moyle and Cech, 1988).

Due to their bio-accumulative and non-biodegradable properties, metals constitute one of the main groups of aquatic pollutants (Ramesh, 2006). Metal content in seawater may vary from period to period, and depends very much on the discharge of effluents into the water (Venkateswara Rao et al., 2006). The bioaccumulation of heavy metals in fish is evident even in cases where they inhabit waters in which the concentration of heavy metals is so low that it can not be detected (Machado et al., 2002).

Fish are frequently used as biomonitors, since they offer several specific advantages for describing the natural characteristics of aquatic systems, and assessing changes to habitats (Chovanec et al., 2003; van der Oost et al., 2003). These animals play an increasingly important role in monitoring water pollution, due to their great sensitivity to aquatic environmental changes, since they employ several enzyme systems in the biotransformation of various xenobiotics. The impact of contaminants on aquatic ecosystems can be assessed by measuring the biochemical parameters in fish (e.g. enzyme activity) that respond specifically to the degree and type of contamination (presence of metals, pesticides and other pollutants) (Petrivalsky et al., 1997; Chovanec et al., 2003; van der Oost et al., 2003; Ramesh, 2006).

This study presents the *in vitro* effect of metals on a trypsin-like enzyme from the pyloric caeca of *C. hippos*. The use of specific inhibitors as well as certain physical-chemical and kinetics properties is also discussed.

2. Materials and Methods

2.1. Materials

Noronha Pescados (Recife-PE, Brazil) kindly donated the specimens of crevalle jack. Azocasein, benzoyl-$_{DL}$-arginine-p-nitroanilide (BApNA), phenyl-methyl-sulphonyl-fluoride (PMSF), tosyl-lysine chloromethyl ketone (TLCK) and molecular weight markers were acquired from Sigma Chemical Com. (St. Louis, MO, USA). All other reagents used were of an analytical grade.

2.2. Enzyme extraction

The *Caranx hippos* specimens used in this study were fresh (dissected within 24 h of being caught) with a total length of 74.7 ± 16.3 (mean ± SD). The pyloric caeca (55 g) was dissected, carefully cleaned with deionized water, and kept at 4ºC during tran sportation to the laboratory (~30 min). After this, the tissue were homogenized in 0.1 M Tris -HCl pH 8.0 (40 mg of tissue/mL buffer) by using a tissue homogenizer (4°C). The homogenate was then centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant (crude extract) was frozen at –20ºC and used for further purification steps.

116 *2.3. Enzyme purification*

117 The trypsin-like enzyme was purified with a three-step procedure. Crude extract
118 (100 mL) was incubated at 40ºC for 30 min and centrifuged at 10,000 *g* for 10 min at 4ºC.
119 The supernatant was collected and fractionated with ammonium sulfate (0 -80% saturation)
120 for 1 h at 4ºC. Afterwards, the precipitate containing trypsin activity was collected by
121 centrifugation and dialyzed against 0.1 M Tris-HCl pH 8.0. A dialyzed sample (5mg) was
122 applied to a Sephadex G75 column (1.2 x 42 cm), which was eluted with 0.1 M Tris -HCl
123 pH 8.0 at a flow rate of 0.34 $mL.min^{-1}$. Each fraction was tested for tryptic activity. The
124 protein peak with a higher specific proteolytic activity was pooled and used throughout the
125 enzyme characterization (Bezerra et al., 2001).

126

127 *2.4. Unspecific proteolytic activity*

128 In a micro centrifuge tube (quadruplicates), 1 % (w/v) azocasein (50 µL), prepared
129 in 0.1 M Tris-HCl pH 8.0 was incubated with the crude extract (30 µL) for 60 min at 25ºC.
130 Then, 240 µL of 10% (w/v) trichloroacetic acid (TCA) were added to stop the reaction.
131 After 15 min, centrifugation was carried out for 5 min at 8,000 *g*. The supernatant (70 µL)
132 was added to 1 M NaOH (130 µL) and the absorbance of this mixture was measured at 450
133 nm in a microtiter plate reader (Bio-Rad 680), against a similarly prepared blank, except
134 that 0.1 M Tris-HCl pH 8.0 replaced the crude extract sample. Previous experiments have
135 shown that this reaction follows a first order kinetic model for the first 60 min. One unit
136 (U) of enzymatic activity was defined as the amount of enzyme capable of producing a
137 0.001 change in absorbance per minute (Alencar et al., 2003).

138 *2.5. Tryptic activity*

139 30 µL of 4 mM N-α-benzoyl-$_{DL}$-arginine-p-nitroanilide (BApNA) prepared in
140 dimethylsulphoxide (DMSO) was incubated in microtiter wells with the enzyme (30 µL)
141 and 0.1 M Tris-HCl pH 8.0 (140 µL). The release of p-nitroaniline was followed by an
142 increase in absorbance at 405 nm in a microtiter plate reader (Bio-Rad 680). Controls were
143 performed without enzyme and substrate solution (Alencar et al., 2003).

144

145 *2.6. Protein determination*

146 The protein content was estimated by measuring sample absorbance at 280 nm and
147 260 nm, using the following equation: [protein] mg/mL = $1.5 \times A_{280\ nm} - 0.75 \times A_{260\ nm}$
148 (Warburg and Christian, 1941).

149

150 *2.7. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS –PAGE)*

151 SDS–PAGE was carried out according to the Laemmli (1970) method, using a 4%
152 (w/v) stacking gel and a 12.5% (w/v) separating gel. The molecular weight of the crevalle
153 jack trypsin-like enzyme was estimated using the molecular weight markers.

154

155 *2.8. Physical-chemical properties*

156 The influence of both temperature and pH on the trypsin activity of the crevalle jack
157 preparations were studied as follows: the purified enzyme was assayed (quadruplicates) as
158 described above, at temperatures ranging from 25ºC to 65ºC and pH values from 6.0 to 10.5
159 (Tris-HCl buffer) using 4 mM BApNA prepared in DMSO as substrate. The thermal

160 stability of the enzyme was determined by assaying (quadruplicates) its activity (25ºC) after
161 pre-incubation for 30 min at temperatures ranging from 30ºC to 60ºC (Souza et al., 2007).

162

163 *2.9. Effect of metal ions*

164 Samples of the purified enzyme (30 µL) were added to a 96-well microtiter plate
165 with 1 mM solution (70 µL) of $AlCl_3$, $BaCl_2$, $CaCl_2$, $CdSO_4$, $CoCl_2$, $CuSO_4$, $HgCl_2$, KCl,
166 LiCl, $MgCl_2$, $MnCl_2$, and $ZnSO_4$, and with 0.0001, 0.001, 0.01 ppm solutions (70 µL) of
167 $AlCl_3$, $CdSO_4$, $CuSO_4$, $HgCl_2$, $PbCl_2$ and $ZnSO_4$. Deionised water was used to prepare these
168 solutions. After 30 min of incubation, 0.1 M Tris-HCl buffer (70 µL), pH 8.0, and 4 mM
169 BApNA (30 µL) were added. The p-nitroaniline produced was measured in a microplate
170 reader at 405 nm after 30 min of reaction (Bezerra et al., 2005).

171

172 *2.10. Effect of protease inhibitors*

173 Purified crevalle jack trypsin (30 µL) was incubated during 30 min with protease
174 inhibitors (70 µL, 3mM): serine-protease inhibitor (PMSF) and trypsin-specific inhibitors
175 (TLCK). After incubation 4 mM BApNA was added and the release of *p*-nitroaniline was
176 followed by increasing absorbance at 405 nm. The enzyme and substrate blank were
177 similarly assayed without enzyme and substrate solution, respectively. The 100% values of
178 activities were those established in the absence of the inhibitors (Bezerra et al., 2001).

179

180 *2.11. Kinetic parameters*

181 BApNA prepared in DMSO was used as substrate (final concentration from 0.125
182 mM to 16 mM), in a total volume of 200 µL, at pH 8,0 (0.1 M Tris-HCl) in a 96-well

microtiter plate. The reaction (quadruplicates) was initiated by adding 30 μL of purified enzyme solution (112.5 μg protein/mL) and the release of p-nitroaniline was followed at 405 nm by using a microtiter plate reader. Blanks were similarly prepared without enzymes. The reaction rates were fitted to Michaelis-Menten kinetics using Origin 6.0 Professional (Bezerra et al., 2001).

2.12. Statistical analysis

All values are presented as mean ± standard deviations. These data were statistically analyzed by ANOVA, followed by a post-hoc (Tukey–Kramer) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($p < 0.05$).

3. Results

The results of the purification are detailed in Table 1. Following the three steps, the enzymatic preparation was purified around 100-fold, and a yield of 18% was achieved. Only one band (Fig. 1) with an apparent molecular weight of 35.2 kDa was observed when a sample of purified enzyme was separated on SDS-PAGE (12.5%). The optimum pH and temperature were 8.0 and 50ºC, respectively (Figure 2A and 2B). Thermal stability was almost unaltered after being incubated for 30 min at 45ºC (Figure 2C). This proteolytic activity was strongly inhibited by TLCK and PMSF, classic specific inhibitors of trypsin and serine-protease, respectively (Table 2). The Michaelis-Menten constant for the trypsin-like enzyme from pyloric caeca was 1.21 ± 0.38 mM, using BApNA as substrate.

The effects of the metal ions on the crevalle jack trypsin-like enzyme are also shown in Table 2. The effects of all metal ions were statistically different when compared to the activity in their absence ($p>0.05$). The presence of Cd^{2+} and Al^{3+} strongly inhibited the trypsin activity (>95%), while Zn^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+} resulted in inhibition between 50-85% of the enzyme activity. The effects of Co^{2+}, K^{+}, Li^{+}, Ba^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} were noticeable, but not extreme (46.2%, 36.7%, 34.6%, 32.6%, 30.6%, 24.6% and 21.1%, respectively). Contradictorily, Ca^{2+}, a known cofactor of mammalian trypsin, did not activate the studied trypsin. The concentration effect of the metal ions (those which presented higher inhibition degree) on the proteolytic activity is shown in the Figure 3. A concentration as low as 0.01 ppm (10µg/L) of ions Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+} and Al^{3+} was enough to inhibit 34%, 33%, 33%, 32%, 29% and 28% of crevalle jack trypsin-like enzyme, respectively. Moreover, the presence of 0.0001 ppm (0.1µg/L) of Zn^{2+} and Cu^{2+} was capable of inhibiting in the region of 20% and 30 %, respectively.

4. Discussion

The purification of trypsin-like enzymes from fish tissue deserves considerable attention, mainly due their characteristic properties (Bezerra et al., 2001, 2005; Castillo-Yáñes et al., 2005; Bougatef et al., 2007; Kishimura et al., 2007; Klomklao et al., 2007; Siringan et al., 2007; Souza et al., 2007). The Sephadex G-75 chromatograms for trypsin-like enzymes from tambaqui (Bezerra et al., 2001), Nile tilapia (Bezerra et al., 2005) and spotted goatfish (Souza et al., 2007) presented similar profiles to those found in the crevalle jack, with a protein peak of higher specific enzymatic activity. A similar methodology using Sephadex G-100 has been used to purify trypsin-like enzymes from sardine (Bougatef

et al., 2007). These studies have also demonstrated the efficiency of heat treatment as a step for purifying trypsins from tropical fish.

The optimum pH and temperature were 8.0 and 50ºC, respectively. Similar behavior was observed for the crude extract from the crevalle jack py loric caeca (Alencar et al., 2003), Nile tilapia (Bezerra et al., 2005), monterey sardine pyloric caeca trypsin (Castillo - Yáñes et al., 2005), elkhorn sculpin pyloric caeca trypsin (Kishimura et al., 2007). The thermal stability was almost unaltered after 30 min incubation at 45 ^{o}C.

Although the central role of digestive enzymes is encountered in the cleavage and absorption dietary processes, very few studies have examined either the activity of digestive enzymes under the effects of contamination, or its p otential as a biomonitor. It is known that sub-lethal concentrations of heavy metals can disturb the digestive enzymatic activity of the exposed species (De Coen and Janssen, 1997; De Coen et al., 1998). In the present study, *C. hippos* trypsin activity proved to be highly sensitive to Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+} and Al^{3+}. In fact, these enzymatic endpoints are likely to be ecologically relevant, as they play a crucial role in the overall food absorption process, i.e. reduced enzyme activity may be linked t o reduced energy uptake, which in turn affects the survival, growth and reproduction of the organism (Bodar et al., 1990; Xu and Pascoe, 1993; De Coen et al., 1998).

The use of trypsins from aquatic animals has already been proposed as biomarkers for monitoring heavy metals. They can be defined as practical, sensitive and cost -effective tools for monitoring environmental risks. Trypsin activity from *Daphnia magna* was used as a cadmium, chromium and mercury biomarker (De Coen and Janssen, 1997; De Coen et al., 1998). The effect of 48h and 96h exposure to sublethal concentrations of $CdCl_2$, $HgCl_2$ and $K_2Cr_20_7$ on the digestive enzyme activity of *D. magna* was assessed. Both ions, $CdCl_2$

(570 μg/L) and $HgC1_2$ (30μg/L) inhibited the enzyme activities after short-term (48h) exposure. The residual activity for $CdCl_2$ and $HgCl_2$ were 10% and 70%, respectively (De Coen and Janssen, 1997). De Coen et al. (1998) assayed the effect of Cd^{2+} (1 and 3 mg/L) and Hg^{2+} (0.05 and 0.10 mg/L) for 90 min. The residual activity was 40% a nd 20% for Cd^{2+} (1 and 3 mg/L, respectively) and 60% and 30% for Hg^{2+} (0.05 and 0.10 mg/L).

Alayse-Danet et al. (1979) reported a growth reduction coinciding with a distinct decrease in the trypsin and amylase activities in *Artemia salina* exposed for 72 h to sublethal concentrations of Cu (2 ppm) and Zn (5 ppm). Sastry and Gupta (1979) assayed the effect of concentration (6.8 mg/L) of $CdCl_2$ on the digestive system of the teleost fish, *Heteropneustes fossilis*. An elevation in the pepsin activity in the stomach was also recorded, but trypsin revealed inhibition in the intestine. Marked inhibition was also noticed in the activities of aminotripeptidase and glycylglycine dipeptidase. The results indicated that cadmium produces a decrease in digestive efficiency by inhibiting the activity of a number of enzymes. The effect of a sublethal concentration of (0.3 mg/l) $HgCl_2$ on the activities of alkaline phosphatase, acid phosphatase, glucose-6-phosphatase, amylase, maltase, lactase, lipase, trypsin, pepsin, aminotri peptidase, glycylglycine dipeptidase, and glycyl-L-leucine dipeptidase in the digestive system of a freshwater catfish, *H. fossilis*, after exposure for 7, 15, and 30 days was appraised by Gupta and Sastry (1981). These authors also observed a significant decrease in the activities of all the digestive enzymes except for pepsin, the activity of which remained unaltered.

As described for other tropical fish proteases (Bezerra et al., 2001, 2005; Bougatef et al., 2007; Souza et al., 2007) trypsin-like enzymes from tropical fish have demonstrated sensitivity to metal ions, particularly Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Hg^{2+} (1mM). It is

important to draw attention to the relatively low inhibitory effect of Co^{2+} and Mn^{2+} (about 44% and 30%, respectively) on crevalle jack trypsin, also recorded for Nile tilapia and spotted goatfish trypsins (Bezerra et al., 2005; Souza et al., 2007). Although it is known that calcium is required for trypsin activity, especially in mammalians, the same was not observed for this fish trypsin. Similar results regarding the effects of calcium on alkaline proteases from fish (Bezerra et al., 2005; Souza et al., 2007) and other aquatic animals (Kishimura and Hayashi, 2002; Saborowski et al., 2004) has been reported in the literature.

The ions Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+} and Hg^{2+} also inhibited the trypsin-like enzyme obtained from the intestine of the Nile tilapia (Bezerra et al., 2005) and from the intestine and pyloric caeca of the spotted goatfish (Souza et al., 2007). However, the inhibition of Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+} and Hg^{2+} (1mM) (99.7%, 99.6%, 82.3%, 76.2% and 55.1%, respectively) on *C. hippos* trypsin activity was higher than the inhibition found for *O. niloticus* trypsin (56.8%, 60.1%, 61.6%, 62.9% and 26.65). The effects of Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+} (1mM) on the crevalle jack pyloric caeca enzyme (respectively, 0.3%, 0.4%, 17.7% and 23.8%, residual activity) proved to be more severe than those registered for the pyloric caeca spotted goatfish enzyme (46.85%, 2.51%, 25.6% and 30.8%, respectively). Bougatef et al. (2007) obtained 31.7%, 51.1% and 62.2% (Mn^{2+}, Zn^{2+} and Cu^{2+} at 2mM, respectively) of inhibition trypsin activity. The inhibition of crevalle jack trypsin registered for Mn^{2+}, Zn^{2+} and Cu^{2+} (1mM) was 30.6%, 82.3% and 76.2%, respectively.

A concentration as low as 0.01 ppm of ions Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+} and Al^{3+} was enough to inhibit 34%, 33%, 33%, 32%, 29% and 28% of the crevalle jack trypsin, respectively. It is known that metals ions such as Cd^{2+}, Co^{2+} and Hg^{2+} act on sulfidryl residues in proteins, and are responsible for a breakdown of disulfides bounds generally causing an inhibition effect on the enzymatic activity (Aranishi et al., 1998). The inhibition

caused by these metal ions suggests the relevance of sulfidryl residues for the catalytic action of this protease.

Conclusion

In this work, the crevalle jack trypsin (35.2 kDa) proved to be easily purified from fish viscera. High sensitivity was also observed to metals such as Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+} and Al^{3+}. Given that trypsin is a key enzyme for protein digestion, these metals may negatively affect the digestive physiology, thus resulting in a negative effect on the survival, growth and reproduction of this species. Furthermore, this species is widely distributed around tropical and subtropical zones. Therefore, these facts suggest that crevalle jack may be used as a metal biomonitor species. This is an important contribution to the environmental monitoring of metals. However, further complementary studies need to be carried out in order to obtain a better understanding of the relationship enzyme/heavy metals, in order to support this application.

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Figure legends

Fig. 1. SDS-PAGE (12,5%) of *C. hippos* trypsin-like enzyme collected by Sephadex G-75 chromatography. (1) Pattern of stained bands produced by standard proteins. (2) *C. hippos* trypsin-like enzyme under denaturing and reduction conditions.

Fig. 2. The effect of (A) pH, (B) temperature, and (C) thermal stability for 30 min of crevalle jack purified trypsin. Samples (quadruplicates) of the purified enzyme (30 μL) was assayed (quadruplicates) at pH values (A) from 6.0 to 10.5 (Tris-HCl buffer), temperatures (B) ranging from 25ºC to 65ºC and the thermal stability (C) of the enzyme was determined by assaying (quadruplicates) its activity (25ºC) after pre-incubation for 30 min at temperatures ranging from 30ºC to 60ºC.

Fig. 3. Effect of heavy metals (ppm) on the trypsin-like enzyme of crevalle jack pyloric caeca. Samples (quadruplicates) of the purified enzyme (30 μL) were added to the metal ions solutions (70 μL). After 30 min of incubation, 0.1 M Tris–HCl pH 8.0 buffer (70 μL) and 4 mM BAPNA (30 μL) were added. The p-nitroaniline produced was spectrophotometrically measured at 405 nm after 30 min of reaction. Different letters mean statistically significant differences according to ANOVA followed by a post-hoc Tukey-Kramer test, $p<0.05$.

Table 1 – Purification of trypsin-like enzyme from crevalle jack pyloric caeca

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2,040. 0	1.863	0.9	100.0	1.0
Heating.	1,560. 0	1.743	1.1	85.4	1.2
Ammonium sulfate precipitation	85.6	387	4.5	20.8	5.0
Sephadex G-75 step	3.7	341	91.2	18.3	100.2

Protein and enzyme activity were established according to Warburg and Christian (1941) and Alencar et al. (2003), respectively.

474 **Table 2 – Effect of ions and protease inhibitors on the trypsin-like enzyme of**
475 **crevalle jack pyloric caeca.** Samples (n=4) of the purified enzyme (30 µL) were added
476 to the 1 mM metal ions solutions (70 µL). After 30 min of incubation, 0.1 M Tris-HCl,
477 pH 8.0 buffer (70 µL) and 4 mM BApNA (30 µL) were added. The p-nitroaniline
478 produced was measured at 405 nm after 30 min of reaction. According to ANOVA
479 followed by a post-hoc Tukey-Kramer test, $p<0.05$, the effects of all metals or
480 inhibitors were statistically significant different when compared to the proteolytic
481 activity in their absence.
482

Ion (1 mM) or Inhibitor (1 mM)	Residual activity (%)
Control*	100.0
Ions	
Cd^{2+}	0.3±0.1
Al^{3+}	0.4±0.0
Zn^{2+}	17.7±0.5
Cu^{2+}	23.8±1.2
Pb^{2+}	38.6±0.6
Hg^{2+}	44.9±0.7
Co^{2+}	53.8±0.1
K^{+}	63.3±0.8
Li^{+}	65.4±1.3
Ba^{2+}	67.4±1.7
Mn^{2+}	69.4±0.2
Mg^{2+}	75.4±0.9
Ca^{2+}	78.9±1.7
Inhibitors	
PMSF	22.4±1.7
TLCK	0±0

483 *Proteolytic activity without any of these ions and inhibitors solutions.
484

COSTA, HMS *et al*.

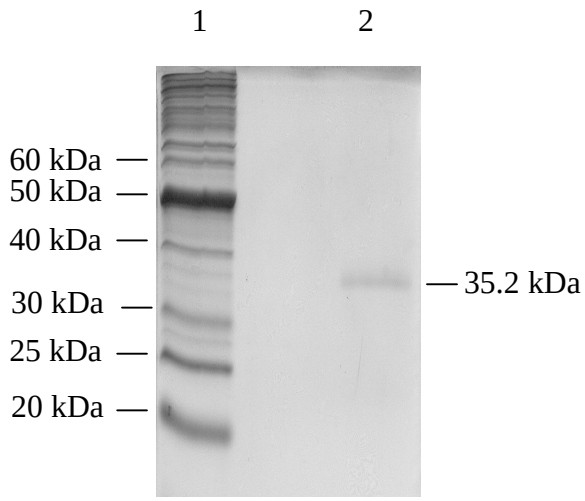

Figure 1

509 **COSTA, HMS *et al*.**
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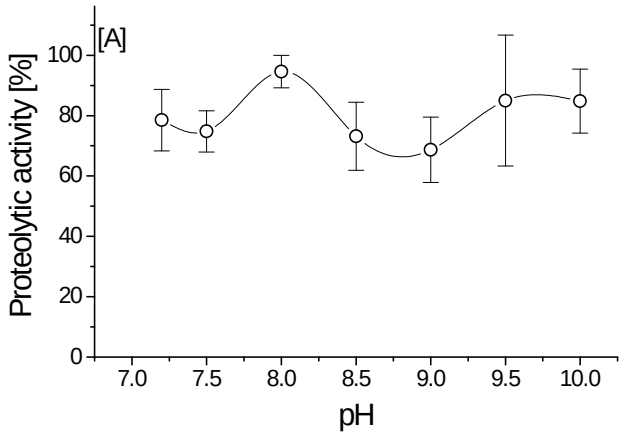

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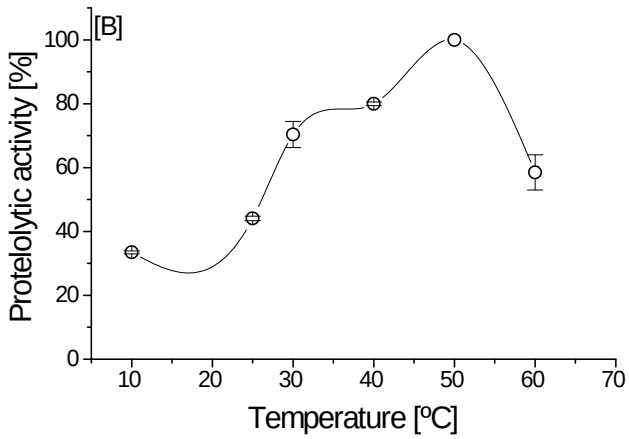

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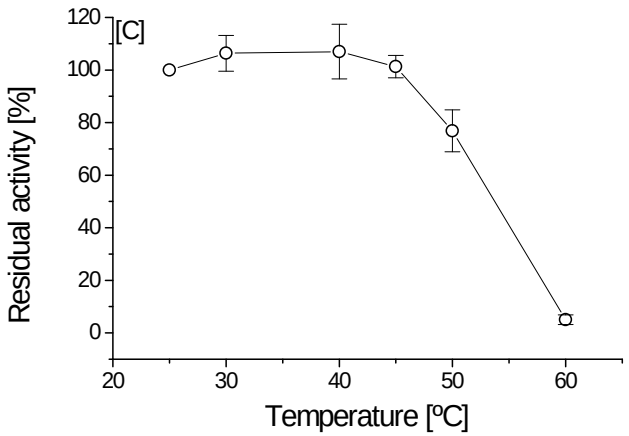

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515 **Figure 2**

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517 **COSTA, HMS *et al*.**

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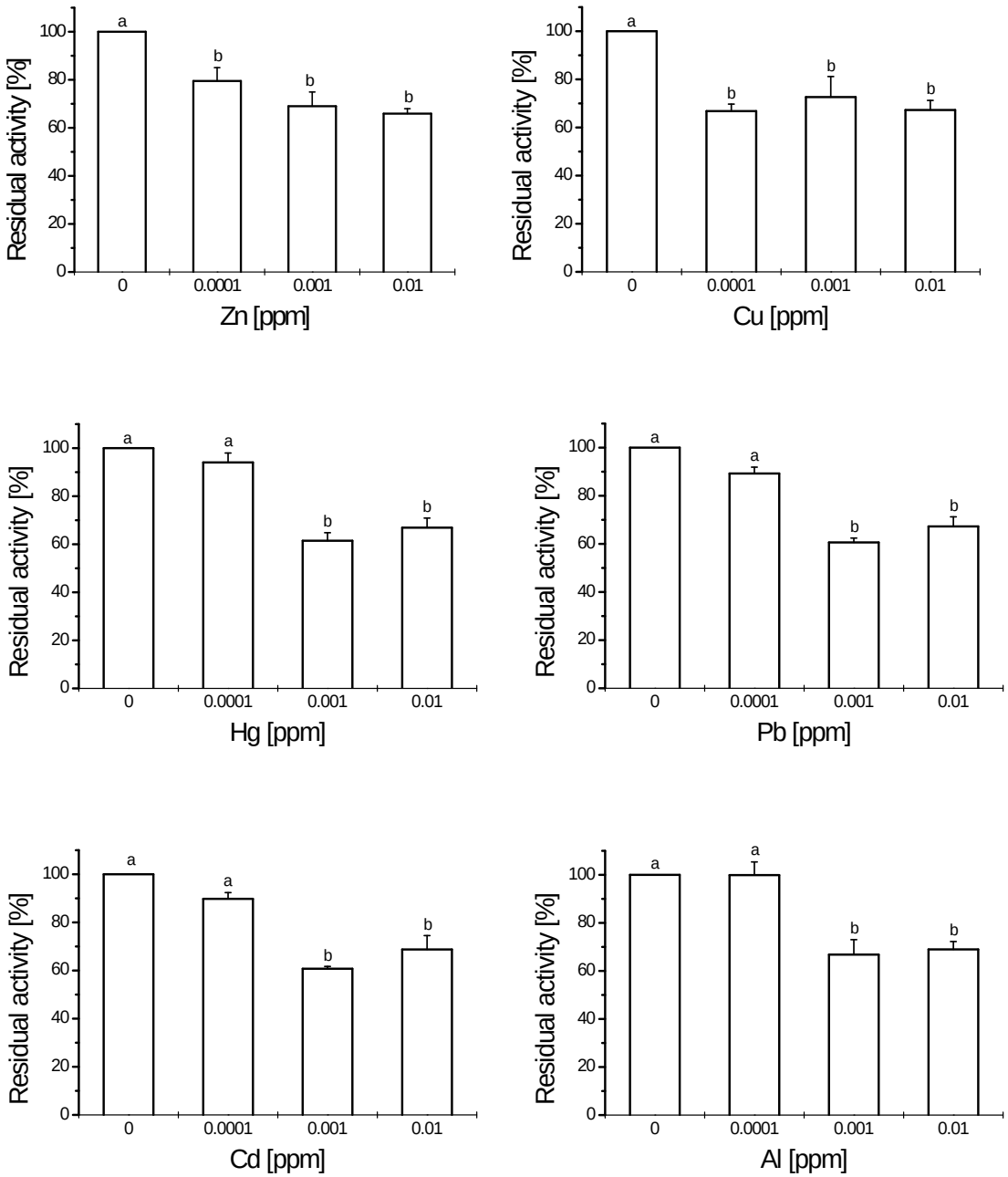

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522 **Figure 3**

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