UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO

DEPARTAMENTO DE PESCA E AQÜICULTURA

PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQÜICULTURA

CARACTERIZAÇÃO DA ATIVIDADE TRÍPTICA DO COPÉPODO HARPACTICOIDA *Tisbe biminiensis*

RENATA CRISTINA DA PENHA FRANÇA

- RECIFE- PE -

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RENATA CRISTINA DA PENHA FRANÇA

CARACTERIZAÇÃO DA ATIVIDADE TRÍPTICA DO COPÉPODO HARPACTICOIDA *Tisbe biminiensis*

A comissão examinadora, composta pelos professores abaixo, sob a presidência do primeiro, considera a candidata **RENATA CRISTINA DA PENHA FRANÇA** como: **APROVADA**
Recife, 08 de maio de 2007

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MENSAGEM

Eu sou o Senhor, o teu Deus que te ensina o que é útil e te guia pelo caminho em que deves andar.

Isaías: 48:17

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A Deus por me dar forças quando sou fraca;

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Dedico aos meus pais Ozires França e Sebastião França (*in memorian*), pela educação, pela oportunidade que me ofereceram e por hoje eu ser quem eu sou!

LISTA DE ABREVIATURAS

kDa - quilo Daltons

SDS - dodecil sulfato de sódio

SDS - PAGE - eletroforese em gel de poliacrilamida utilizando SDS

PMSF - fluoreto de fenil-metil-sulfonil

TPCK – tosil - fenilalanina clorometil cetona

TLCK – tosil - lisina clorometil cetona

BApNA – benzoil- DL- ρ-nitroanilida

SBTI - inibidor de tripsina da semente de soja

EDTA – ácido etilenodiaminotetracético

DHA – ácido docosaexaenóico

EPA – ácido ecosapentaenóico

PUFA – ácidos graxos altamente insaturados

RESUMO

Os copépodos harpacticóides são microcrustáceos que servem como alimento vivo preferencial na natureza para diversas espécies de larvas de organismos aquáticos sendo nutricionalmente superiores quando comparados a outros alimentos vivos comumente utilizados nas larviculturas. O alimento vivo é uma importante fonte de enzimas exógenas que contribui para o processo digestivo dessas larvas que por não estarem com o sistema digestivo completamente formado, dependem das enzimas provenientes do alimento vivo para um melhor aproveitamento dos ingredientes da dieta, apresentando consequentemente melhores desempenhos na taxa de crescimento e sobrevivência. Este trabalho teve como objetivo caracterizar as proteases alcalinas do copépodo harpacticóide *Tisbe biminiensis* através de parâmetros cinéticos e físico-químicos como: pH e temperatura ótima, efeito de inibidores, estabilidade térmica, além da caracterização eletroforética por SDS-PAGE e zimograma. O extrato bruto do *T. biminiensis* apresentou uma atividade proteolítica total de 0,39 U/mg de proteínas sendo a atividade tríptica de 2,33 U/mg. A atividade enzimática apresentou uma temperatura ótima de 55ºC e pH ótimo de 9,0 e mostrou-se estável termicamente no intervalo de 25 a 50ºC. A atividade enzimática foi fortemente inibida por inibidores específicos de tripsina, TLCK (100±1%), SBTI (100±2%), benzamidina (91±10%), e não houve inibição significativa com os inibidores: EDTA (35±1%), PMSF (35±1%), β-mercaptoetanol (7±11%). Estes resultados demonstraram que a principal protease alcalina presente no extrato bruto do *T. biminiensis* foi a tripsina, exercendo importante papel na digestão protéica destes animais e este copépodo pode contribuir como fonte de enzimas exógenas para o processo digestivo nas larvas de organismos aquáticos.

ABSTRACT

Tisbe biminiensis is a potential live prey for many species of aquatic animals since its nutritional value is better than those found in other feed organisms commonly used in crustacean and fish larvivultures. Live food seems to be a source of exogenous enzymes which eases food digestion of fish and crustacean early life stages. The aim of this work was to study alkaline proteases from the harpacticoida copepod *Tisbe biminiensis* by evaluating the effects of pH, temperature and specific inhibitors on the proteolytic activity. Proteases were also studied by SDS-PAGE and zymograms. Crude extract from *T. biminiensis* showed a total proteolytic activity of 0.39 mU/mg of protein and tryptic activity of 2.33 mU/mg. Optima pH and temperature were 9.0 and 55^{o}C, respectively. The enzymes were thermostable at temperature ranging from 25 to 50^{o}C. The enzymatic activity was strongly inhibited by the trypsin specific inhibitors TLCK (100%), SBTI (100%) and benzamidine (91%). However, EDTA, PMSF and β-mercaptoethanol caused only a slight inhibition (35, 35 and 7%, respectively). The results show that alkaline proteases are present on crude extract of *Tisbe biminiensis*. The highest effects of trypsin inhibitors on enzyme activity suggest that this enzyme plays an important role in protein digestion. *T. biminiensis* is an important source of live prey to fish and crustacean larvae in nature and partially replace others live food commonly used in aquaculture because they contribute with both exogenous enzymes for the digestion processes and other nutrients required during development of aquatic animals and contribute to development of aquaculture.

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1. INTRODUÇÃO

A aqüicultura, definida como o "cultivo de organismos com hábito predominantemente aquático", inclui principalmente os peixes, moluscos, crustáceos, anfíbios e plantas aquáticas (VALENTI, 2000) e é atualmente a atividade de maior crescimento entre os setores de produção animal (FAO, 2002). Dentre os produtos oriundos da aqüicultura, o camarão marinho apresenta um alto valor econômico**.** Os países asiáticos são os principais produtores mundiais deste crustáceo e o Brasil, em 2004/2005, ocupou o nono lugar a nível mundial, com produção de 65.000 toneladas produzidas em 2005 (GAA, SRHIMP OUTLOOK 2005, GLOBEFISH).

A alimentação de animais marinhos, bem como carnívoros de água doce, criados em larga escala, apresenta-se como o problema central no cultivo de espécies aqüícolas. Sabe-se que o sucesso do setor começa com a produção de sementes, ou seja, larvas e/ou pós-larvas de qualidade e em quantidade suficiente, que por sua vez dependem da alimentação e da nutrição (BERGER, 2000).

De maneira geral, as altas mortalidades ocorrentes em larviculturas de peixes e crustáceos estão relacionadas, entre outros fatores, às deficiências nutricionais e também da dependência das espécies marinhas em utilizar os componentes dietários de acordo as enzimáticas digestivas existentes em seus tratos digestivos (BARRETO E CAVALCANTI, 1997; ALARCON, DIAZ, MOYANO e ABELLAN, 1998).

Denomina-se zooplâncton o conjunto de organismos aquáticos que não têm capacidade fotossintética (heterotróficos) e que vivem dispersos na coluna d´água. O zooplâncton é o segundo elo da cadeia alimentar dos ecossistemas aquáticos: estes organismos alimentam-se do fitoplâncton e do

bacterioplâncton - são consumidores primários, apesar de haver neste grupo alguns predadores - e, por sua vez, servem de alimentação a organismos maiores. Os principais constituintes desse grupo são: microcrustáceos, rotíferos, cladóceros e copépodos (NAYAR, 1998).

Segundo Kibria *et al*., (1997), o zooplâncton é uma valiosa fonte de aminoácidos, lipídeos, ácidos graxos, minerais e enzimas.

Vários autores comprovaram que a disponibilidade de alimento vivo com alto valor nutricional é um fator relevante para o crescimento dos peixes e larvas de crustáceos (IMMANUEL, *et al*., 2007; RAJKUMAR, KUMARAGURU VASAGAM, 2006).

Na natureza, os copépodos harpacticóides fazem parte da dieta de diversos grupos economicamente importantes como juvenis e larvas de peixes e camarões (HICKS E COULL, 1983).

Apesar dos copépodos servirem de presas vivas para larvas de diversos organismos aquáticos, o seu uso na aqüicultura ainda permanece de forma esporádica. Os rotíferos (*Brachionus sp*.) e os náuplios de *Artemia* continuam mantendo-se predominantes na aqüicultura apesar de seus baixos valores nutricionais em comparação com os copépodos, principalmente pela relativa facilidade na produção destes animais (STØTTRUP, 2000).

A *Artemia* é um microcrustáceo branquiópodo próprio de ambientes aquáticos de elevada salinidade e que se encontra distribuído em todo o mundo. Este animal vem desempenhando um papel fundamental no desenvolvimento da aqüicultura, como alimento vivo para estágios iniciais do desenvolvimento de peixes e crustáceos. Este fato se deve a grande facilidade de estocagem dos seus cistos de resistência (LAVENS E SORGELOOS, 1998).

As enzimas atuam como catalisadores biológicos de alta especificidade, desempenhando um papel fundamental na manutenção da vida. São as enzimas proteolíticas secretadas no lume do trato digestivo dos animais que degradam as proteínas da dieta, de sorte que os aminoácidos e peptídeos possam ser melhor aproveitados por estes. Enzimas proteolíticas, proteases, proteinases ou peptidases são sinônimos para as enzimas que hidrolisam ligações peptídicas.

De maneira geral, as proteases podem ser divididas em dois principais grupos: exopeptidases e as endopeptidases. As do primeiro grupo atuam próximo das extremidades das cadeias e as endopeptidases atuam preferencialmente nas regiões internas das cadeias polipeptídicas (Figura 1) (RAO *et al*., 1998; BEZERRA, *et al*., 2001).

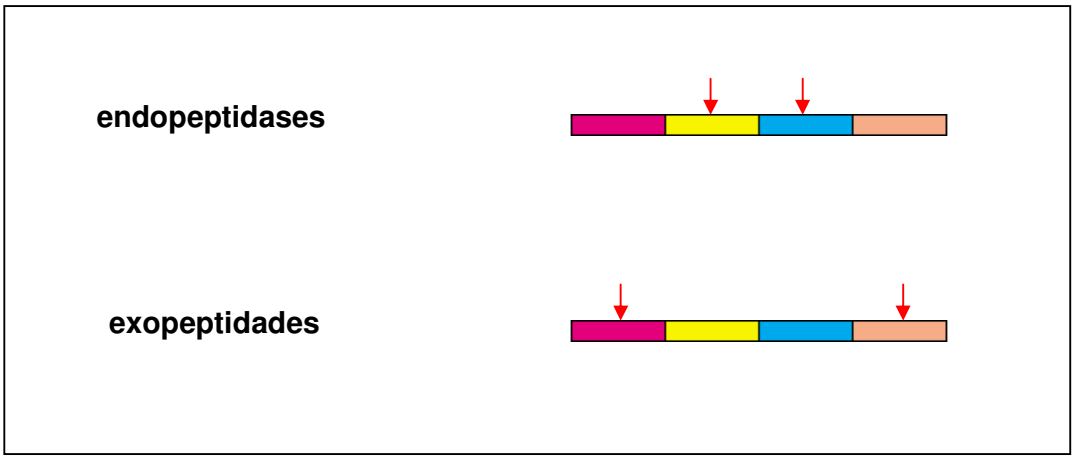

Figura 1. Representação esquemática figurativa da ação de hidrólise em uma cadeia polipeptídica realizado por uma endopeptidase e por uma exopeptidase.

A investigação da atividade das proteases em diversas espécies marinhas tem sido realizada com o intuito de se conhecer melhor o potencial digestivo de determinadas espécies e, conseqüentemente, desenvolver dietas

eficazes para cultivos intensivos (BEZERRA, 2001; BEZERRA *et al*., 2001b; BEZERRA, *et al*., 2005, SOUZA, *et al*., 2007). A atividade enzimática propriamente dita reflete o potencial para digerir vários tipos de matérias orgânicas podendo indicar diferentes adaptações a diversas fontes alimentares (LE VAY *et al*., 2001).

Esses conhecimentos são relevantes, principalmente porque os animais podem ajustar seus metabolismos variando a secreção de enzimas de forma a permitir a utilização dos ingredientes dos alimentos. Sabe-se que as larvas de peixes e crustáceos possuem deficiências enzimáticas ainda assim, são numerosos os estudos existentes referentes ao grau de contribuição de enzimas derivadas do zooplâncton ao processo digestivo dos peixes e crustáceos em seus estágios larvais (KOVEN *et al*., 2001; KOLKOVISKI, 2001; KUZ´MINA e GOLOVANOVA, 2004).

2. REVISÃO BIBLIOGRÁFICA:

2.1. Alimentação x Alimento vivo na aqüicultura:

O potencial do Brasil para o desenvolvimento sustentável da aqüicultura é imenso. O setor é dividido em carcinicultura, piscicultura, malacocultura, ranicultura, mitilicultura e algocultura. Constituído por 8,4 mil quilômetros de costa marítima, 5,5 milhões de hectares de reservatórios de águas doces e aproximadamente, 12% da água doce disponível no planeta, o país também possui clima favorável, disponibilidade de terras, mão-de-obra e crescente demanda por pescados no mercado interno e externo (VALENTI, 2000).

Dentre os aspectos relacionados à aqüicultura, os custos com alimentação podem representar até 60% em sistemas de cultivo intensivo, por isso o conhecimento da fisiologia digestiva de animais cultiváveis é importante para o desenvolvimento e o fornecimento de dietas adequadas e racionais (KUBITZA, 2000).

A nutrição balanceada das larvas de peixes e crustáceos é um dos principais elementos para o sucesso da aqüicultura (CURNOW, *et al*., 2006). Para muitas espécies de peixes, a qualidade do alimento é mais importante do que a quantidade de alimento fornecido para a promoção da saúde. Muitos estudos sobre peixes marinhos relatam que a primeira alimentação das larvas de peixes e crustáceos requer uma quantidade suficiente de n - 3 HUFA, particularmente ácido eicosapentaenóico (EPA; 20:5n – 3) e ácido docosahexaenóico (DHA; 22:6n – 3) (IMMANUEL, *et al*., 2007; RAJKUMAR, KUMARAGURU VASAGAM, 2006; STØTTRUP e ATTRAMADAL, 1992; KOVEN *et al*., 1993, KRAUL, *et al*., 1993). Os copépodos marinhos têm alto teor de n - 3 (EFA – ácidos graxos essenciais), carboidratos e enzimas (amilases, proteases, exonucleases e esterases) que são essenciais para a

sobrevivência, o crescimento, a digestão e metamorfose (EVJEMO, *et al*., 2003; KLEPELL *et al*., 2005).

Dabrowski, (1979) demonstrou que a morfologia da estrutura dos tratos digestivos das larvas de peixes é bastante simples e estão relacionadas com baixa produção de enzimas resultando em digestão deficiente das dietas artificiais causando a dependência de organismos vivos como fonte de alimentos, no entanto, estudos gerais realizados por Eshel, *et al*., (1993) demonstraram que 40 – 80% da atividade enzimática foram estimuladas pelos alimentos vivos em várias espécies de peixes. A taxa de ingestão e absorção de aminoácidos durante a proteólise pode ser determinada pelo conhecimento das propriedades funcionais das atividades das proteases.

Organismos vivos como rotíferos (*Brachionus sp*.) e *Artemia* tem sido considerados essenciais para o sucesso dos cultivos (SORGELOOS, 2001). As técnicas existentes para o desenvolvimento larval são predominantemente dependentes de alimentos vivos, que requerem cuidados especiais e monitoramento freqüente, tornando-se muito dispendiosas. Várias fontes de alimento animal (vivo ou congelado) têm sido experimentadas. Entretanto, a mais utilizada atualmente é o náuplio de *Artemia* graças principalmente à praticidade de seu uso (SORGELOOS *et al*., 1983). Na aqüicultura, o uso de *Artemia* tem causado alguns transtornos, principalmente pela reduzida disponibilidade de cistos, em função da limitação de locais para sua produção e processamento (SORGELOOS, 1980). Isto contribui para a queda em sua produção, tornando os preços elevados, conforme demanda (LAVENS *et al*., 2000). Além disso, há considerável variação na qualidade dos cistos quanto à

taxa de eclosão e ao valor nutricional do náuplio eclodido (SORGELOOS e LÉGER, 1992).

Outro problema relevante é que grande parte da produção mundial de *Artemia* ocorre no Great Salt Lake (GSL), no estado de Utah nos EUA. Tal lago vem enfrentando uma queda na salinidade da água devido ao fenômeno do El–Niño, que provocou fortes nevascas e o derretimento do gelo das montanhas da região. Atualmente, a salinidade na região sul do lago está em torno de 70 - 80 ppt enquanto que a salinidade ótima para a produção de cistos de *Artemia* fica em torno de 100 – 150 ppt (SORGELOOS, 2001).

No Brasil, a *Artemia* é encontrada principalmente nas salinas do Rio Grande do Norte em conseqüência de inoculações feitas em 1977 com cistos provenientes da baía de São Francisco (Califórnia, EUA). Ao longo dos anos ela se dispersou por toda a região salineira do Rio Grande do Norte e exerceu papel importante no desenvolvimento e consolidação da carcinocultura do nordeste do Brasil, especialmente em relação à provisão de cisto e biomassa utilizados nas larviculturas e nos laboratórios de maturação (CÂMARA, 2004).

Os copépodos apresentam um tamanho similar aos náuplios de *Artemia*, com variações entre 150 - 350µm, tamanho este, apropriado para alimentar cultivos de larvas de peixes durante seus estágios juvenis (SHANSUDIN *et al.*, 1997), diversos estudos mostraram diferenças nos níveis das taxas de aminoácidos, classes de lipídeos e pigmentos entre os copépodos e os outros organismos que servem de presas vivas (STØTTRUP, 2000).

Os copépodos são bem representados na base da cadeia pelágica do ecossistema marinho onde cerca de 10.000 espécies já são conhecidas e

ainda existe um número similar de espécies desconhecidas e amplamente distribuídos neste ecossistema (HAIRSTON e BOHONAK, 1998).

Esses animais contribuem significantemente para a transferência de matéria e energia entre os níveis tróficos, no entanto, o detalhamento de funções de algumas espécies em particular e seus estágios de desenvolvimento na cadeia alimentar pelágica não está completamente esclarecido devido à grande variedade de espécies e as possíveis interações tróficas entre elas (KNOTZ, *et al*., 2006).

Larvas de diversas espécies marinhas alimentadas apenas com dietas artificiais apresentam baixas taxas de crescimento e sobrevivência. Apesar de tais dietas serem ricas em nutrientes e com alta tecnologia de processamento, seus componentes nutricionais não são bem absorvidos pelos animais (KUZ´MINA e GOLOVANOVA, 2004; CAHU e ZAMBONINO INFANTE, 1994). Sugere-se que uma das razões para este melhor aproveitamento dos nutrientes seja pela incorporação das enzimas derivadas dos zooplânctons ao intestino das larvas, fato que amplia a digestibilidade e a absorção dos alimentos (LAUFF e HOFFER, 1984). Segundo Brunet *et al*., (1994), a utilização bioquímica dos alimentos é facilitada por uma série de enzimas digestivas que são sintetizadas na região média do intestino dos copépodos.

Fatores negativos como o alto número de bactérias patogênicas que estão associadas aos diversos tipos de alimentos vivos, podem afetar direta e negativamente na saúde dos peixes (OLSEN *et al*., 2000; OLAFSEN, 2001). Muitos pesquisadores têm tentado substituir completa ou parcialmente o alimento vivo por outros tipos de dietas, mas não têm obtido resultados satisfatórios (CURNOW, *et al*, 2006; CAHU, *et al*., 2001).

Os organismos vivos estimulam a alimentação das larvas através de seus movimentos e a liberação de metabólitos (aminoácidos, peptídeos e sais de amônia, entre outros) servem de atrativos (KOLKOVISKI *et al*., 1997a; 1977b; TESSER e PORTELLA, 2006). Estas substâncias agem sinergicamente estimulando a atividade de ingestão das microdietas artificiais pelas larvas. Planas e Cunha (1999) afirmam que um dos maiores problemas na utilização do alimento artificial é a falta de estímulo visual das dietas, pois as larvas ingerem principalmente organismos vivos (que se movimentam) durante os primeiros dias de desenvolvimento. Pedersen e Hjelmeland (1988) comprovaram que a utilização de alimento vivo nas dietas induziu um aumento na secreção de tripsina endógena na larva do arenque *Clupea harengus*.

Através do entendimento do processo digestivo e da assimilação de componentes específicos das dietas, é possível identificar o tipo de presa viva preferida dos animais e quais apresentam um melhor arsenal para digerir essas presas. Por exemplo, espécies carnívoras possuem alta atividade de enzimas proteolíticas para digerir proteínas das dietas, enquanto que os herbívoros e os onívoros que ingerem grandes quantidades de carboidratos em sua alimentação possuem altas atividades de carboidrases (JOHNSTON e FREEMAN, 2005). Estudos prévios com crustáceos mostram que existe uma relação direta entre a composição alimentar e a presença de enzimas digestivas (FIGUEIREDO *et al*., 2001).

Delbare *et al.,* (1996) estudaram e relataram várias vantagens no uso de copépodos como fonte de alimento vivo: a) a grande variação no tamanho do corpo entre a forma de náuplios e as formas adultas; b) o movimento realizado por estes, que constitui um estímulo visual para as larvas; c) a alta quantidade

de ácidos graxos poliinsaturados (PUFA); d) os altos níveis de enzimas digestivas, que podem exercer um importante papel na nutrição das larvas e, no caso dos copépodos harpacticóides, contribuição para a manutenção das paredes dos tanques limpos, através da alimentação dos detritos que se formam nestes tanques e de utilizarem diferentes fontes de alimento. Os copépodos já vêm sendo utilizados semi - extensivamente na escala industrial (SØRENSEN, *et al*., 2004).

Segundo Lavens e Sorgeloos (1996), entre os copépodos, os harpacticóides bentônicos, principalmente os gêneros *Tisbe* e *Tigriopus*, possuem características que facilitam o seu cultivo, tais como: alta fecundidade e curto tempo de geração, limites extremos de tolerância às mudanças ambientais (salinidade 15 – 70 ppt e temperaturas de 17 – 30ºC), usam uma grande variedade de alimentos, como microalgas, fermento, rações secas e chegam a alcançar altas densidades populacionais com até 100 indivíduos/ml para *Tisbe sp.*

Vários autores pesquisaram o cultivo de copépodos. *Tisbe clodiensis* e *T. holothruriae* (FAVA e CROTTI, 1979), *Amphiascoides atopus* (SUN e FLEEGER, 1995). Tseng e Hsu (1984) estudaram o cultivo do copépodo *Tigriopus japonicus* e seu valor como alimento para juvenis do peixe *Acanthopagrus latus.* Vilela (1984) estudou a produção do copépodo harpacticóide *Tigriopus brevicornis* criado em vários regimes de alimentação. Støttrup e Norsker (1997) estudaram a produção em massa e uso de *Tisbe holothuriae* na larvicultura de peixes marinhos. Altas densidades populacionais de *Tisbe biminiensis* (Figura 2) foram conseguidas, em volumes de 500 ml

(2003) com a utilização de técnicas de cultivo relativamente simples (Figura 3 e 4) (SOUZA-SANTOS, *et al*., 2006).

Figura 2. Fêmea ovada de *Tisbe biminiensis*.

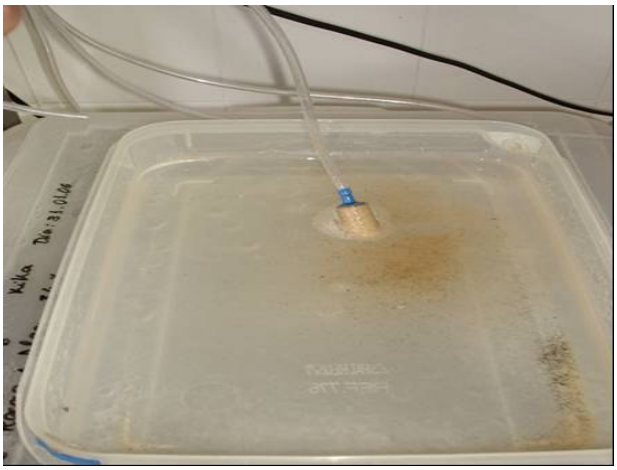

Figura 3. Caixa plástica utilizada para o cultivo do *T. biminiensis* neste experimento. Os animais foram cultivados em condições laboratoriais controladas: água do mar filtrada, salinidade (32 – 36 ppt), aeração contínua, fotoperíodo (12h luz/12hescuro).

Figura 4. Cultivo de microalgas (*Thalassiosira weis e Chaetoceros muelerii*) utilizado para a alimentação dos copépodos deste experimento.

O cultivo intensivo de copépodos pode representar uma alternativa aos alimentos vivos já utilizados nas larviculturas, podendo gerar futuramente uma independência mesmo que parcial, do fornecimento de cistos de *Artemia* (RIBEIRO, 2005).

Apesar do ótimo potencial como alimento vivo para a aqüicultura do copépodo harpacticóide *T. biminiensis*, existe ainda uma lacuna com respeito a estudos sobre sua composição bioquímica, sobretudo de sua fisiologia digestiva, podendo representar uma fonte alternativa de proteases exógenas para larvas de animais aquáticos com potencial aqüícola.

3. OBJETIVOS

3.1 Geral

- Caracterizar as proteases digestivas visando à contribuição para o conhecimento da biologia da espécie *Tisbe biminiensis*.

3.2 *Específicos*

- Identificar as enzimas digestivas em exemplares adultos;
- Caracterizar, as endo e exoproteases utilizando SDS-PAGE e zimogramas;
- Definir parâmetros físico-químicos e cinéticos dessas enzimas;

4. REFERÊNCIAS BIBLIOGRÁFICAS

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Characterization of trypsin-like enzymes from harpacticoid copepod (*Tisbe biminiensis*)

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Aquaculture

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ABSTRACT

The harpacticoid copepod *Tisbe biminiensis* is a potential alternative of live prey in marine crustacean and fish larvicultures. Trypsin activity was assayed in the crude extract prepared by homogenization of specimens reared under controlled laboratory conditions, fed on diatoms and commercial fish food. The physical-chemical and kinectics parameters were determined using Nα-benzoyl–DL-arginine-p-nitroanilide (BApNA) as substrate. The influence of pH (7.2-10.5), temperature (25-75ºC) and trypsin inhibitors on the tryptic activity was also studied. For thermostability, samples were incubated during 30 min at temperatures ranging from 25 to 75ºC. Optima pH and temperature were 9.0 and 55ºC, respectively. This enzyme was thermostable at 25 to 50ºC. Michaelis-Menten constant was 0.69mM. Moreover, it was strongly inhibited by specific trypsin inhibitors: TLCK (100%), benzamidine (91%) and SBTI (100%). SDS-PAGE of crude extract from *T. biminiensis* showed the presence of various protein bands whose molecular weight ranged from 14.0 to 116 kDa. These results show that *T. biminiensis* produces trypsin-like enzymes.

KEYWORDS: Proteases, Exogenous enzymes, Trypsin, Live prey, Copepod, *Tisbe biminiensis*

Introduction

Nutrition is of paramount importance during fish farming. Different types of diets are available, amongst them live food plays a key role during growth of many aquatic animals. The main problem in most marine fish culture is related to the high mortality rates associated with first larval feeding (Nanton and Castell, 1997; García-Ortega, *et al*., 2000), moreover, for many fish species, live food still gives better results in terms of growth and survival when compared to fish fed artificial diets (Dabrowski, 1984). Nowadays, the commercial production of larvae, post larvae and juvenile of fish and crustacean still depends on the supply of live prey, mainly the rotifers *Brachionus sp*. and *Artemia* nauplii (Koven *et al*., 2001; Kolkovski, 2001; Sorgeloos, P., 2001). Despite of these animals are of low nutritional, their production are relatively easy what ensure their predominance (Støttrup, 2000).

Copepods could be an alternative to this problem. Brine shrimp, when compared to copepods, synthesize or incorporate significantly less amount of EFA 22:6 - 3 (essential fatty acids), and have lower DHA (docosahexaenoic acid):EPA (eicosapentaenoic acid) ratios even when fed diets rich in DHA (McEvoy *et al*., 1995), These fatty acids (EPA, DHA and EFA) are important to successfull development of aquatic animals because they promote growth, pigmentation, stress resistance and improvement of larval quality (Laven *et al*., 1995).

Despite copepods is recognized as an important source of nutrients, they are poorly used in the aquaculture because brine shrimp presents an advantage in relation to copepods which is the storage of their cysts of resistance. (Drillet *et al*., 2006). However, they are promising candidates for mass culture as live food for fish and crustacean larvae for the following reasons: the wide range of body size beetwen nauplii and adults; their movement, which constitutes a visual stimulus for the larvae; they have

high fecundity and short generation time; they tolerate a wide range of environmental changes; they can use a large variety of food sources such as yeast and algae and reach high population density (Delbare *et al*., 1996).

The digestion of food during the early stages of these fish and shrimp groups can be improved by a set of enzymes that are synthesized in the midgut region of copepods (Brunet, 1994). Jancarik (1964) assumed that these exogenous enzymes, which are present in live food organisms, may support the digestive processes in fish. Understanding the enzymatic activity in aquatic animals is important to the choice of specific ingredients for artificial diets which can lead to better food conversion ratio, attempts to reduce the dependence of live food as a starter food and established the importance of trypsin as the key enzyme in protein digestion processes has been done and these researches foccus on digestive physiology of aquatic animals (Castillo-Yáñez, *et al*., 2005; Lemos, 2002; Muhlia-Almazán, 2003; Garcia-Carreño, 2002; Alencar, 2003; Natalia, 2004).

One of the main digestive proteases detected in the pyloric caeca, and intestine of fish and crustaceans hepatopancreas is trypsin. This enzyme is a member of a large family of serine proteases and cleaves proteins and peptides at the carboxyl side of arginine and lysine and is the most important enzyme of animals and humans because of its role in activating other enzymes (Kishimura *et al*., 2007, Klomklao *et al*., 2007). The aim of this study was to investigate the tryptic activity of harpacticoid copepod (*Tisbe biminiensis*) as well as some physical-chemical properties and characterization by SDS-PAGE and zymograms.

2. Materials and Methods

2.1. Reagents

Nα-benzoil-DL-arginine-ρ-nitroanilide (BApNA), N, N, N, Tetramethylethylenediamine (TEMED), Nα-p-tosyl-$_{L}$-lysine chloromethyl ketone (TLCK), N-tosyl- $_{L}$- phenylalanine chrolomethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), benzamidine, bestatine, casein, azocasein and soy bean trypsin inhibitor (SBTI) were purchased from SIGMA (St. Louis, MO, USA). Dimethyl sulfoxide was from BAKER. Protein molecular weight marker containing galactosidase (116.0 kDa), phosphorilase b (97.4 kDa), bovine serum albumine (66.2 kDa), alcohol dehydrogenase (37.6 kDa), carbonic anhydrase (28.5 kDa), myoglobin (18.4 kDa) and lysozyme (14.0 kDa) was purchased from Molecular Biologische Technologie (German). All other reagents used in this work were of analytical grade.

2.2. Copepods cultivation

Ten thousands specimens of *Tisbe biminiensis* were cultured at Departamento de Oceanografia (Universidade Federal de Pernambuco) for several generations in 500 mL or 5 L vessels in 25 and 3 μm filtered seawater (34 - 36‰). They were fed on both *Thalassiosira weis* and *Chaetoceros muelerii* microalgae and a commercial fish food (Souza-Santos *et al*., 2006). Microalgae were cultivated in f/2 medium (Guillard, 1975) (for medium preparation, Tris–HCl buffer (pH 7.8) and f/2 nutrient stocks were added before medium sterilization in autoclave (121ºC for 15 min). Vitamin solution was sterilized by filtration (0.2 μm) and added to the medium just before algal inoculation). Cultures of copepods and microalgaes were incubated at 28–30ºC temperature and 12 h light/dark photoperiod.

2.3. Preparation of crude extract

About 10,000 specimens of *Tisbe biminiensis* were individually collected and homogenized in 0.01 M Tris-HCl buffer pH 8.0 (10 mL) prepared in 0.15 mM NaCl using a tissue homogenizer. The homogenate was centrifuged at 10,000 x *g* for 5 min at 4ºC to remove cell debris and nuclei. The supernatant (crude extract) was frozen at -20ºC and used for further studies of characterization.

2.4. Non-specific enzymatic assays

Unspecific proteolytic activity was assayed using azocasein as substrate in a microcentrifuge tube (Alencar *et al*., 2003). Duplicate samples of enzyme extract (30 µL) were incubated with 1% azocasein (50 µL) dissolved in 0.1 M Tris-HCl pH 8.0, for 60 min at 25^{o}C. Then, 10% trichloroacetic acid (240 µL) was added to stop the reaction and the mixture was centrifuged at 8,000 *x g* for 5 min. Supernatant (70 µL) was mixed with 1 M NaOH (130 µL) and its absorbance was measured in a microtiter plate reader (Bio-Rad 680) at 450 nm against a blank similarly prepared except that 0.15 mM NaCl replaced the crude extract sample. Previous experiment showed that for the first 60 min the reaction carried out under the conditions described above follows a first order kinetics. One unit (U) of enzymatic activity was defined as the amount of enzyme required to hydrolyze azocasein and to produce a 0.001 increase in absorbance per min. Specific activity was expressed as units per mg of protein.

2.5 Specific enzyme assays

Trypsin activity was determined in a 96 well microtiter plate using Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA) as specific substrate (Bezerra *et al*., 2001). Duplicate samples of enzyme extracts (30 µL) were incubated with 8 mM BApNA (30 µL)

dissolved in dimethylsulphoxide (DMSO) and 0.1 M Tris-HCL pH 8.0 (140 µL). The reactions occurred at 25ºC for 60 min and were recorded at 405 nm using a 96-well microplate reader. One unit (U) of enzymatic activity was defined as the amount of enzyme required to hydrolyze BApNA and to produce a 0.001 increase in absorbance per min. The specific activity was expressed as protease units per mg of protein.

2.6 Protein determination

The protein content was estimated by measuring sample absorbance at 280 and 260 nm and using the following equation: [protein] mg/mL = $A_{280\ nm}$ x 1,55 – $A_{260\ nm}$ x 0,76 (Warburg and Christian, 1941).

2.7 SDS-PAGE and zymograms

Proteases from *T. biminiensis* were studied by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% (w/v) stacking gel and a 15% (w/v) separating gel (Laemmli, 1970). Briefly, enzyme preparations and protein molecular weight markers were applied to a vertical electrophoresis unit (BIO-RAD) and the gel was silver stained. Electrophoresis was performed at constant current of 15 mA per gel, at 4°C.

Zimograms were carried out according the method described by Garcia-Carreño *et al.* (1993). Samples for zimograms were prepared using a buffer without 2-mercaptoethanol and were not boiled prior to eletrophoresis. After electrophoresis (4°C), gels were immersed in 2.5% Triton X-100 in 0.1 M Tris-HCl pH 8.0 to remove SDS and incubated with 3% casein (w/v) prepared in 0.1 M Tris-HCl pH 8.0 for 30 min at 4°C. The temperature was raised to 37ºC and kept for 90 min to allow the digestion of casein by the active fractions. Finally, gels were stained for protein in 0.18% (w/v)

Coomassie Brilliant Blue R250 prepared in acetic acid:methanol (10:25% v/v) and the background of the gel was destained in acetic acid:methanol (10:25% v/v).

2.8 Physical-chemical characterization

The influences of temperature and pH on the proteolytic activity of the enzyme preparation were evaluated as follows: the crude extract was assayed (duplicate) as described above at temperatures ranging from 25 to 75ºC and pH from 7.2 to 10.5 (Tris-HCl and NaOH-Glycin buffers) using 8mM BApNA as substrate. Thermostability was evaluated by assaying enzyme activity at 25ºC after pre-incubation for 30 min at temperatures ranging from 25 to 75ºC (Bezerra *et al*., 2001).

2.9 Effects of inhibitors

Samples of crude extract (30 µL) and 0.1 mM Tris-HCl pH 8.0 (115 µL) were poured in a 96-well microtiter plate with the following inhibitors (25 µL), prepared in DMSO: 8mM PMSF, TPCK, TLCK, benzamidine, β-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), and 40mg/mL SBTI. After incubation for 15 min 8mM BApNA (30 µL) was added. Increasing in absorbance at 405 nm was monitored using a microtiter plate reader. Controls were performed without enzyme and substrate solution (Bezerra *et al*., 2001).

2.10 Kinetics parameters

Michaelis-Menten constant (K_m) was calculated by assaying trypsin activity using various substrate concentrations: 140 µL of 0.1 M Tris-HCl pH 8.0 and 30 µL of BApNA (0.25 to 32 mM) were added to a 96-well microtiter plate. The reaction was started by addition of 30 µL of crude extract (3,247 µg protein/mL). The increase in

absorbance at 405 nm was followed using a microtiter plate reader. The reaction rates obtained were fitted using Microcal Origin 6.0™. Each reaction was performed in duplicate. The blanks were similarly prepared without enzyme (Bezerra *et al*., 2005).

2.11 Statistics

Microcal Origin 6.0™ software was used for statistical analysis.

3. Results and Discussion

Currently, trypsin activity has been characterized by several authors based on physical-chemical properties, namely optima pH and temperature, thermostability, SDS-PAGE and zymogram (Kumar *et al*., 2005; Casanova, *et al*., 2006). Variations in trypsin properties occur because different types of dietary protein and environments require different types of enzymes for its digestion (Cahu and Zambonino Infante, 1994; García-Ortega *et al*., 2001).

The effect of pH on crude extract from *T. biminiensis* is presented in Figure 1. Despite there is no reports about the effects of pH on copepods proteases, several studies in fish and shrimp trypsins demonstrated maximun activities at pH ranging from 6.0 to 10.0 (El-Shemy and Levim, 1997; Bezerra *et al*., 2001; Souza *et al*., 2007; Oh, *et al*., 2000).

Enzymatic activity of crude extract of *T. biminiensis* was stable in temperatures ranging from 25 to 50ºC (Figure 2). A further increase to 55ºC caused a decrease of 50% of trypsin activity. This activity was almost negligible in temperatures higher than 60ºC. Optimum temperature was 55˚C (Figure 3). Similar results were found for Nile tilapia and tambaqui enzymes (50 and 55ºC). García-Carreno *et al*., (2002) found an optimum temperature at 60ºC for piracanjuba (*Brycon orbignyanus*). *Penaeus californiensis* have an optimum temperature of 50ºC (Veja-Villasante *et al*., 1995). (García-Ortega *et al*., 2000) submitted *Artemia* decapsulated cysts to heat treatment for 5 minutes and had a lower total alkaline protease and specific trypsin activity. Over 60ºC heat treatment total alkaline proteases and trypsin activity were negligible and at 96ºC heat treatment trypsin activity was almost zero. The reduction of enzymes activities in cysts heated at high temperature implies less exogenous enzymes for fish and crustacean digestive process.

Crude extract of *T. biminiensis* showed unspecific proteolytic activity of 0.39 U/mg using azocasein as substrate. The specific tryptic activity was of 2.33 mU/mg of protein using BApNA as substrate. Lauff (1983) found a specific tryptic activity of 9.3 mU/mg of protein for *Artemia salina*, which is the main live food used in aquaculture. *Daphnia carinata* have a trypsin activity of 0.21 ± 0.02 U/mg of protein (Kumar *et al*., 2005). In *Monia sp*. (important member of pond cladocerans), trypsin and chymotrypsin activities were 47.3 and 3.9 mU/mg of protein, respectively (Lauff and Hofer, 1984).

Michaellis-Menten constant for trypsin like enzyme from *T. biminiensis* was 0.69 mM (Figure 4). This value is higher than those reported for other tropical fishes: common carp, *Cyprinus carpio* – 0.039mM (Cohen *et al*., 1981); mullet, *Mugil cephalus* – 0.49 mM (Guizani *et al*., 1991) and lower than Nile tilapia (*Oreochromis niloticus* – 0.755 mM (Bezerra *et al*., 2005).

Molecular weights of protein bands of crude extract from *T. biminiensis* ranged from 14.0 to 116 kDa (Figure 5). Kumar *et al*., (2005) found similar results for proteins from *Daphnia carinata* that ranged from 20 to 93 kDa. SDS-PAGE of carnivorous ornamental fish (*Scleropages formosus*) showed at least six to eight different alkaline proteases with molecular weight ranging from 18.6 to 97 kDa in both intestine and pancreas samples (Natalia, *et al*., 2004). Five caseinolytics bands were observed in zymogram from *T. biminiensis* (Figure 5). The thermostability of proteolytic enzymes from *T. biminiensis* was also assayed in zymograms (Figure 6). All bands were active until 55°C. However at 65ºC the first and the last bands disappeared and at 75ºC all five caseolytics bands lost their activities. In fact, zymograms are more sensitive than electrophoresis and usually bands of enzyme activity are observed where no bands of protein are seen (Lemos *et al*., 2000).

A first characterization of alkaline proteases present in the rotifer *Brachionus plicatilis* was performed by Hara *et al*., (1984a). These authors found two fractions with alkaline proteinase activities, classified as a serine protease and a trypsin-like protease (Hara *et al*., 1984b). Wethmar and Kleinow (1993a) characterized the proteolytic activity of rotifer extracts by zymograms finding proteases bands ranging from 45 kDa to 90 kDa. Díaz *et al*., (1997) also found two caseinolytic bands of 53 kDa and 71 kDa.

The effects of various proteases inhibitors on the activity of crude extract from *T. biminiensis* were determinated (Table 1). It was inhibited by serine protease inhibitor, such as PMSF (35%±1%), TLCK (100% ± 1%), benzamidine (91% ± 10%), SBTI (100% ± 2%). β-mercaptoethanol (7% ± 11%) and EDTA (3% ± 20%) showed a slight inhibition. These results indicated that most of the enzymes present in the crude extract of *T. biminiensis* are serine proteases, with a high content of trypsin-like enzymes. Hatching enzyme (HE) from *Penaeus chinensis* was inhibited by SBTI, PMSF, bestatin and TLCK which indicate that shrimp HE might be a kind of trypsin-like (Li, *et al*., 2006).

Alkaline proteases are present in crude extract from *T. biminiensis* and were strongly inhibited when a specific trypsin inhibitor was used showing that trypsin plays an important role in protein digestion in *T. biminiensis*. This enzyme was found to have physical-chemical parameters similar to those described for shrimps. This, in turn, suggests that these shrimp may well use the trypsin from *T. biminiensis* as an exogenous enzyme source in its digestion processes. Further researches are necessary to find out others properties of *T. biminiensis* enzymatic set and its application in the aquaculture industry. Studies about the effects of *T. biminiensis* as live prey on tryptic activity of shrimps and fish are also important in order to evaluate the use of this copepod for replacing others live food commonly offered in fish and crustacean larviculture.

4. Acknowledgments

The authors acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), FINEPE and RECARCINE to financial support and wish to thank Albérico Espírito Santo for technical assistance.

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TABLES

Table 1. Enzymatic inhibition from *T. Biminiensis* by specific inhibitors

Inhibitors	Inhibition (%)± SD (Standard derivation)
PMSF	35% ± 1
TLCK	100% ± 1
Benzamidine	91% ± 10
SBTI	100% ± 2

FIGURES CAPTIONS

França, R.C.P. et al.

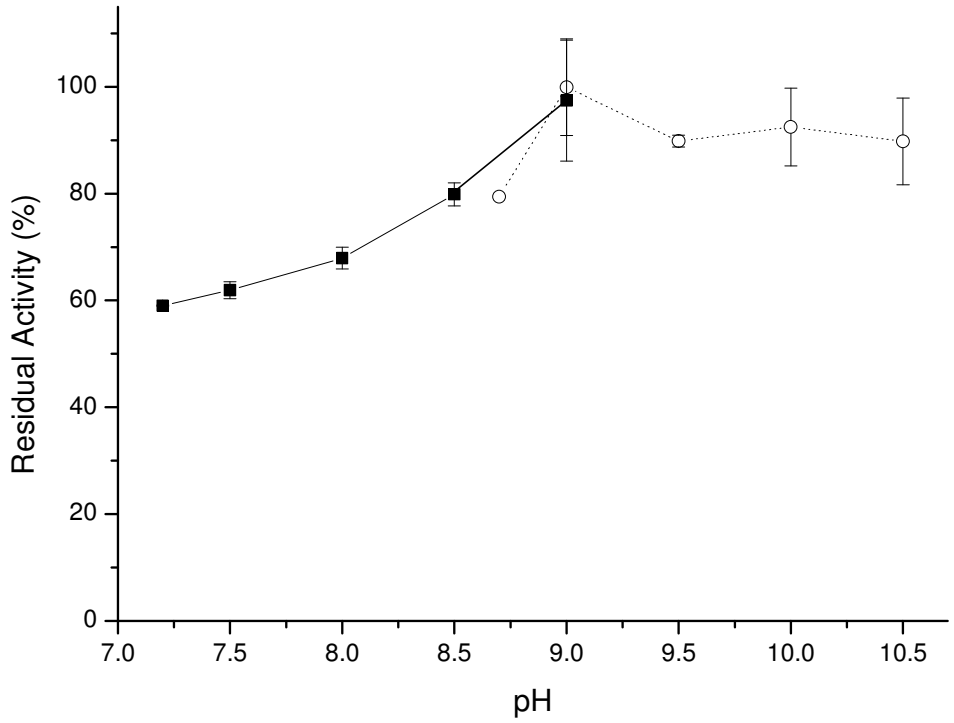

FIGURE 1

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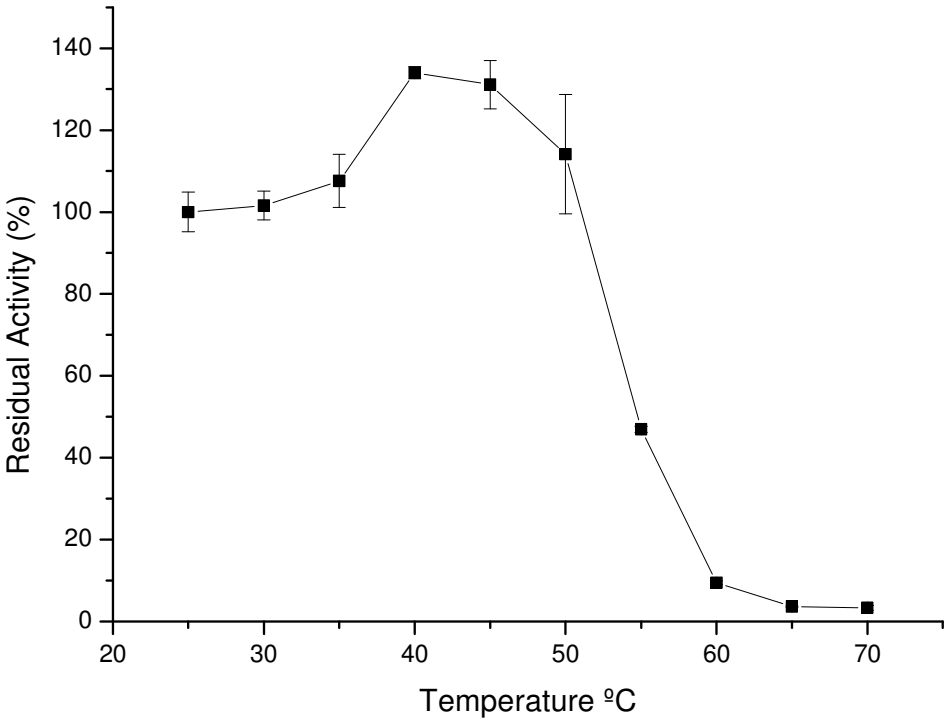

FIGURE 2

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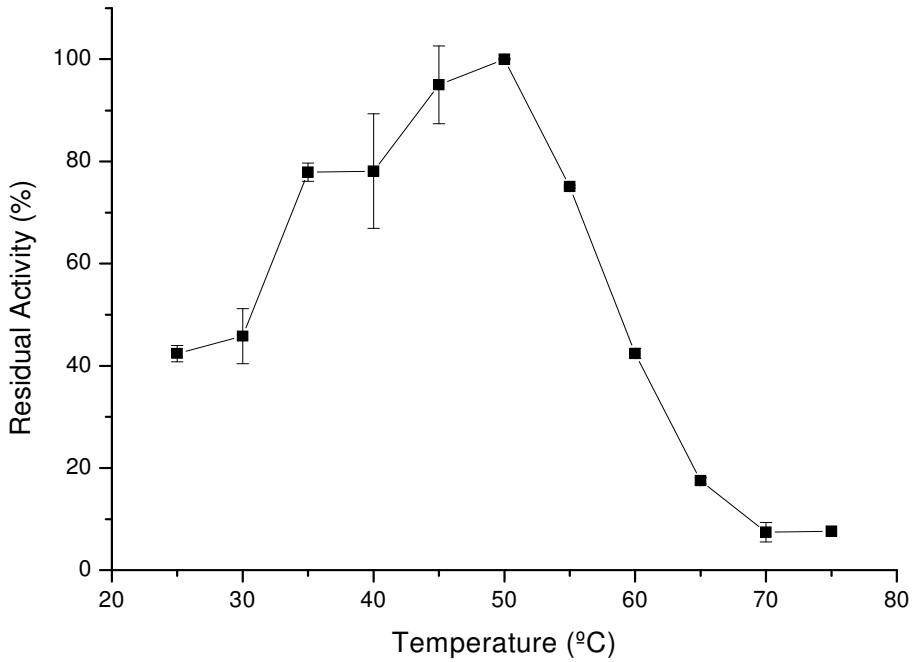

FIGURE 3

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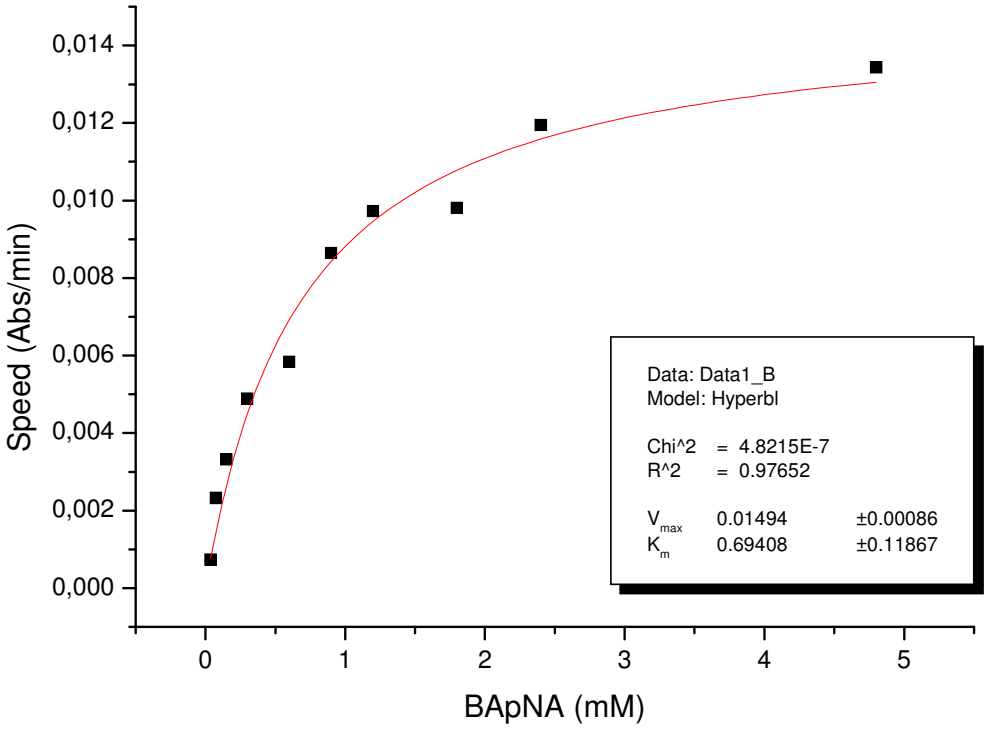

FIGURE 4

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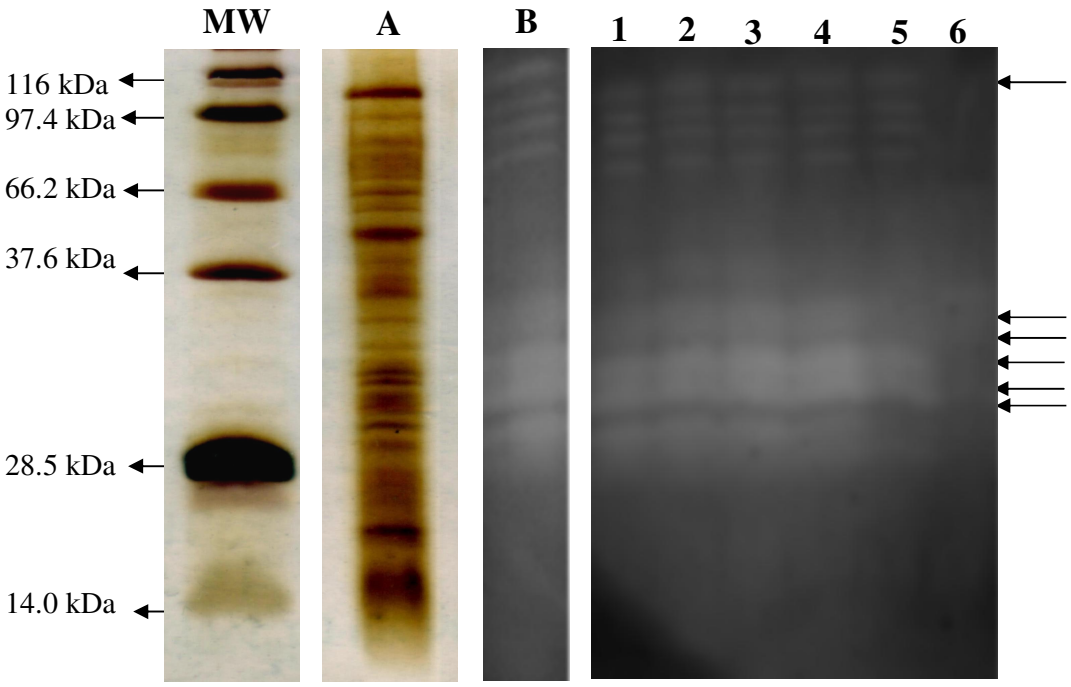

FIGURE 5

FIGURES CAPTIONS

Figure 1 - Effects of pH on crude extract form *T. biminiensis*. Activity was measured using two buffers (■) Tris-HCl buffer and (o) NaOH-glicin buffer. 8mM BApNA was used as substrate. Percentage of enzyme activity was estimated considering 100% the highest activity detected in this assay.

Figure 2 – Thermostability was determinated by assaying (duplicates) its activity (25 ˚C) after pre- incubation for 30 min at the indicated temperatures. 8mM BApNA was used as substrate.

Figure 3 – The effect of temperature in crude extract of *T. biminiensis* was evaluated using 8mM BApNA as substrate and temperatures ranging from 25ºC to 75ºC. Percentage of enzyme activity was estimated based on the highest activity detected in this assay as 100%.

Figure 4 – Michaelis-Menten kinetics plot for trypsin-like Kinetics. BApNA initial concentration (0.25 to 32mM): amount of enzyme used in this assay (30µL); buffer: 0.1mM Tris-HCl pH 8.0 (140µL); R^2 = 0.97 V_{max}= 0.014 ± 0.00086 and K_m= 0.69 ± 0.11867

Figure 5 – SDS-PAGE, zymogram and thermostability of crude extract from *T. biminiensis*. MW (Molecular weight markers): galactosidase (116.0 kDa), phosphorilase b (97.4 kDa), bovine serum albumine (66.2 kDa), alcohol dehydrogenase (37.6 kDa), carbonic anhydrase (28.5 kDa), myoglobin (18.4 kDa) and lysozyme (14.0 kDa) A- Crude extract of *T. biminiensis* electrophoresis, B -Zymogram of crude extract from *T. biminiensis* showed many activity bands zymogram: Line 1: 25 ºC; line2: 35ºC; line 3: 45ºC; line 4: 55ºC; line 5: 65ºC and line 6: 75ºC

5. ANEXOS

6.1 Guide for Authors (Aquaculture)

Types of contribution
1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communications
4. Technical Papers
5. Letters to the Editor
6. Book Reviews

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere, except in a preliminary form.

Review Articles can cover either narrow disciplinary subjects or broad issues requiring interdisciplinary discussion. They should provide objective critical evaluation of a defined subject. Reviews should not consist solely of a summary of published data. Evaluation of the quality of existing data, the status of knowledge, and the research required to advance knowledge of the subject are essential.
Short Communications are used to communicate results which represent a major breakthrough or startling new discovery and which should therefore be published quickly. They should *not* be used for preliminary results. Papers must contain sufficient data to establish that the research has achieved reliable and significant results.

Technical Papers should present new methods and procedures for either research methodology or culture-related techniques.

The *Letters to the Editor* section is intended to provide a forum for discussion of aquacultural science emanating from material published in the journal.

Book Reviews will be solicited by the Book Review Editor. Unsolicited reviews will not usually be accepted, but suggestions for appropriate books for review may be sent to the Book Review Editor: Mrs. A.A.C. de Groot, Brederoodseweg 49, 2082 BS Santpoort-Zuid, The Netherlands.

Submission of manuscripts

Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. Submission also implies that all authors have approved the paper for release and are in agreement with its content. Upon acceptance of an article by the journal, the author(s) will be asked to transfer the copyright of the article to the publisher. This transfer will ensure the widest possible dissemination of information.

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Nutrition:
R. P. Wilson

Husbandry and Management:
B.Costa-Pierce
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E.M. Donaldson
Diseases:
D.J. Alderman
Genetics:
G. Hulata

Preparation of manuscripts

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2. The preferred medium of submission is on disk with accompanying manuscript (see `Electronic manuscripts' above). Submit the original and two copies of your manuscript. Enclose the original illustrations and two sets of photocopies (three prints of any photographs).
3. Manuscripts should be typewritten, typed on one side of the paper (with numbered lines), with wide margins and double spacing throughout, i.e. also for abstracts, footnotes and references. **Every page of the manuscript, including the title page, references, tables, etc. should be numbered in the upper right-hand corner.** However, in the text no reference should be made to page numbers; if necessary, one may refer to sections. Underline words that should be

in italics, and do not underline any other words. Avoid excessive usage of italics to emphasize part of the text.

4. Manuscripts in general should be organized in the following order:

Title (should be clear, descriptive and concise)

Name(s) of author(s)

Complete postal address(es) of affiliations

Full telephone and fax number and E-mail address of the corresponding author

Present address(es) of author(s) if applicable

Abstract
Keywords (indexing terms), normally 3-6 items.

Introduction
Material studied, area descriptions, methods, techniques

Results
Discussion
Conclusion
Acknowledgements and any additional information concerning research grants, etc.
References
Tables
Figure captions

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6. SI units should be used

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Gaugh, Jr., H.G., 1992. Statistical Analysis of Regional Yield Trials. Elsevier, Amsterdam, 278 pp.

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4. Give the meaning of all symbols immediately after the equation in which they are first used.

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6. Equations should be numbered serially at the right-hand side in parentheses. In general only equations explicitly referred to in the text need be numbered.

7. The use of fractional powers instead of root signs is recommended. Also powers of e are often more conveniently denoted by exp.

8. Levels of statistical significance which can be mentioned without further explanation are $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

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