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APLICAÇÕES FISIOLÓGICAS

PATRÍCIA FERNANDES DE CASTRO

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ENZIMAS DE ORGANISMOS AQUÁTICOS E SUAS
APLICAÇÕES FISIOLÓGICAS

PATRÍCIA FERNANDES DE CASTRO

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas, área de concentração Biotecnologia

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A comissão examinadora, composta pelos professores abaixo, sob a presidência do primeiro, considera APROVADA a candidata PATRICIA FERNANDES DE CASTRO

Recife, 04 de março de 2009

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RESUMO

Nos últimos anos, tem-se observado um aumento na demanda por produtos pesqueiros, seguido por um significativo incremento na produção mundial de organismos aquáticos. O crescimento da indústria pesqueira gera, como consequência, uma grande quantidade de resíduos e subprodutos ricos em biomoléculas, que são indevidamente descartados no ambiente. Esses resíduos podem ser usados em diversos estudos relacionados à fisiologia digestiva de espécies aquáticas de interesse comercial e ao desenvolvimento de ingredientes alternativos que possam ser usados como componentes de rações animais. O objetivo do presente trabalho foi investigar enzimas digestivas do hepatopâncreas de camarões penêdeos e avaliar o efeito de dietas com diferentes concentrações de hidrolisado protéico de camarão (HPC) sobre o crescimento, a composição corporal e a fisiologia digestiva de juvenis de Oreochromis niloticus. Amilases do hepatopâncreas dos camarões Farfantepenaeus subtilis (13g), Litopenaeus schmitti (23g) e L. vannamei (com 11 e 25g) e proteases do F. subtilis (6 e 13g) e F. paulensis (8g) foram caracterizadas a partir de estudos de atividade enzimática, incluindo pH e temperatura ótima, estabilidade térmica, efeito de íons metálicos e de inibidores, eletroforeses e zimogramas. Amilases de todas as espécies estudadas apresentaram atividade residual superior a 85% em pH alcalino (7,0 a 8,0), em temperatura entre 40 e 50°C e não foram termoestáveis em temperaturas superiores a 55°C. O íon Ca²⁺ ativou as amilases apenas na concentração de 1mM, mas inibiu a enzima nas concentrações de 5 e 10mM. Os demais íons empregados inibiram fortemente as enzimas independentemente da concentração utilizada. O inibidor Tipo I de α-amilase foi altamente eficiente sobre as enzimas de F. subtilis e L. schmitti, mas os mesmos resultados não foram observados para o L. vannamei. O zimograma de atividade revelou nove isoformas de amilases para o F. subtilis, oito para o L. schmitti e sete para o L. vannamei de 11 e 25g, respectivamente. Atividades de tripsina, quimotripsina e leucino aminopeptidase foram detectadas em F. subtilis e F. paulensis. As maiores atividades de todas as proteínases também ocorreram em pH alcalino (7,5 a 9,0) e temperatura de 45 a 55°C. O zimograma de atividade revelou oito bandas proteolíticas para F. paulensis e seis para F. subtilis. As atividades de aminopeptidases (Arg, Leu, Lys, Phe, Val) e os níveis recomendados desses aminoácidos essenciais em dietas de camarões penêdeos apresentaram correlação positiva (P<0,05). Os efeitos de diferentes níveis de inclusão de hidrolisado protéico de camarão (HPC) em dietas sobre a atividade de enzimas digestivas de juvenis da tilápia do Nilo foram avaliados e correlacionados com parâmetros de crescimento com a composição corporal. O HPC foi incluído nas dietas em concentrações de 0, 1,5, 3 e 6%. Uma dieta comercial foi usada como referência. Hemoglobina, azocaseína, BAPNA, SApNA, α-β naftilamidas e amido foram usados como substratos. Apesar de terem sido observadas algumas diferenças, não houve correlação entre atividade enzimática e as diferentes
concentrações de HPC das dietas. O zimograma de atividade foi realizado para analisar as mudanças no perfil das proteases digestivas causadas pela inclusão do hidrolisado protéico. Foram observadas 12 bandas proteolíticas, oito das quais responderam à incorporação do HPC. O zimograma de inibição indicou uma diminuição na atividade de três enzimas com atividade de tripsina quando o nível de inclusão do HPC aumentou, enquanto que o inverso ocorreu para uma aminopeptidase. Perfis distintos de proteases foram encontrados para cada tratamento, sugerindo uma alta adaptabilidade da tilápia do Nilo às diferentes dietas. Atividades de tripsina e de aminopeptidase tiveram correlação positiva com parâmetros de crescimento e com o teor de proteína da carcaça e correlação negativa com a taxa de conversão alimentar e com o teor de lipídeos da carcaça. Uma vez que a digestão envolve a ação conjunta de diferentes enzimas, a compreensão adequada das características das enzimas envolvidas é necessária para que a capacidade digestiva das diferentes espécies possa ser estabelecida. O estudo das enzimas pode gerar informações básicas importantes para futuras pesquisas sobre nutrição das espécies, bem como servir como ferramenta para análise de diferentes dietas.

Palavras-chave: Enzimas digestivas, fisiologia digestiva, camarões marinhos, hidrolisado protéico de camarões, rações, tilápia do Nilo.
ABSTRACT

In the last years, there has been a general increase in human demand for fishery products, followed by a significant increase in the world production of aquatic organisms. The growth of fishery industry generates, as consequence, large amounts of wastes and by-products rich in biomolecules, which are discharged into environment. Fishery wastes can be used in several physiological studies related to digestive physiology of marketable aquatic species and to the development of alternative ingredients which can be used as component of animal feeds. The aim of the present work was to investigate digestive enzymes from the hepatopancreas of some peneaid shrimps and evaluate the effects of diets with different shrimp protein hydrolysate (SPH) concentrations on growth, body composition and digestive physiology of Oreochromis niloticus juveniles. Amylases from the hepatopancreas of Farfantepenaeus subtilis (13g), Litopenaeus schmitti (23g) and L. vannamei (11 and 25g) and proteases from F. subtilis (6 and 13g) and F. paulensis (8g) were characterized according the following properties: optima pH and temperature, thermal stability, effect of inhibitors and metallic ions, electrophoresis and zymograms. Amylases from all species presented residual activity above 85% at alkaline pH (7.0 – 8.0) and at 40 – 50°C and were not thermostable at temperatures greater than 55°C. Amylases of all species were activated by calcium chloride at 1mM but were inhibited at 5 and 10mM. All other ions employed showed inhibitory effect at any employed concentration. A strong inhibition of alpha-amylase Type I inhibitor on enzyme activity was observed for F. subtilis and L. schmitti, but the same result was not observed for L. vannamei. The substrate SDS-PAGE zymograms revealed nine isoforms of amylase for F. subtilis, eight for L. schmitti and seven and ten for L. vannamei of 11 and 25g, respectively. Trypsin, chymotripsyn and leucine-aminopeptidase activity was detected for F. subtilis and F. paulensis. The greatest activity of all proteinases also occurred at alkaline pH (pH 7.5 – 9.0) and temperature from 45 to 55 °C. The substrate SDS-PAGE zymograms revealed eight proteinase bands for F. paulensis and six ones for F. subtilis. Aminopeptidase activity (Arg, Leu, Lys, Phe, Val) and recommended concentrations of these essential amino acids in penaeid shrimp diets were positively correlated (p<0.05). The effects of different dietary inclusion levels of shrimp protein hydrolysate (SPH) on digestive enzyme activity of Nile tilapia juveniles were evaluated and correlated with growth parameters and body composition. SPH was included in diets at concentrations of 0, 1.5, 3 and 6%. A commercial diet was used as reference. Hemoglobin, azocasein, BApNA, SAPNA, AA-β naphthylamide and starch were used as substrates. Despite some differences, there was no correlation between enzyme activity and different SPH concentrations in the diets. Substrate-SDS-PAGE zymogram was also performed for the analysis of changes in the profile of Nile tilapia digestive proteases caused by the inclusion of protein hydrolysate. Zymograms revealed 12 proteolytic bands, eight of which responded to incorporation of SPH. Inhibition zymograms
indicated that there was a decrease in the activity of three enzymes with trypsin activity as SPH increases, whereas the opposite occurred for one aminopeptidase. Distinct protease profiles were also found for each treatment, suggesting adaptability of the Nile tilapia to the different diets. Trypsin and aminopeptidase activity was positively correlated with growth parameters and carcass protein content and negatively correlated with feed conversion ratio and carcass lipid content. Since digestion involves the joint action of different enzymes, adequate comprehension of enzyme characteristics is required for the assessment of the digestive capability of the different species. Enzyme studies may provide basic information useful for further nutritional researches, as well as be an efficient tool for analysis of different diets.

Keywords: Digestive enzymes, digestive physiology, shrimps, shrimp protein hydolysate, feeds, Nile tilapia.
INTRODUÇÃO

O aumento na demanda por produtos pesqueiros para consumo humano tem resultado em um constante crescimento da produção aquícola mundial, a qual apresentou um aumento de aproximadamente 21,6%, no período de 1997 a 2006. Em 2006, foram produzidas 159 milhões de toneladas de pescado, das quais 93 milhões foram oriundas da pesca e 66 milhões, da aquicultura (FAO, 2008).

Embora em termos percentuais a captura de organismos aquáticos ainda seja responsável por cerca de 60% do total de pescado produzido, essa atividade vem apresentando sinais de estabilização desde a década de 80. De acordo com dados da FAO (2008), no período de 1997 a 2006, a captura diminuiu de 95 para 93 milhões de toneladas, enquanto que a aquicultura cresceu 86%, passando de 35 para 66 milhões de toneladas.

Com o aumento da produção pesqueira e, conseqüentemente, do volume de pescado processado mundialmente, grande quantidade de resíduos e de subprodutos tem sido gerada. Tal fato representa um grande desafio para empresários e comunidade científica interessados em buscar estratégias para que a produção de organismos aquáticos, seja oriunda do extrativismo, seja da aquicultura, torne-se uma atividade sustentável. Segundo Arruda (2004), cerca de 50% do pescado mundial produzido em 2000 transformou-se em resíduo. Supondo que esse percentual tenha se mantido ao longo dos anos, das 159 milhões de toneladas de pescado produzidas em 2006, 79,5 milhões teriam sido descartadas, constituindo-se em uma fonte significativa de desperdício de recursos e de contaminação ambiental.

Além de poder ser utilizado para a produção de hidrolisado protéico, de farinha e óleo de peixe, os resíduos da indústria pesqueira são uma importante fonte de biomoléculas de interesse comercial. Dentre elas podem-se destacar enzimas como proteases, lipases e carboidrases, passíveis de aplicação em diversos segmentos do setor industrial (GILDBERG e STENBERG, 2001; SHAHIDI e JANAKKAMIL, 2001; GUPTA, BEG et al., 2002; ESPOSITO, AMARAL et al., 2009a; ESPOSITO, AMARAL et al., 2009b). Estudos com vísceras de pescado podem ser direcionados ao conhecimento da fisiologia digestiva de organismos aquáticos. O entendimento da capacidade enzimática digestiva de um determinado organismo pode facilitar a elaboração de dietas adequadas ao bom desenvolvimento de espécies com potencial para o cultivo comercial (KUZ’MINA, GLATMAN et al., 2003), uma vez que o aproveitamento dos nutrientes dietários depende da capacidade do organismo de digerir o alimento consumido (CHISTY, HASHIM et al., 2008).
1.1. **ENZIMAS**

As enzimas são biomoléculas protéicas capazes de catalisar, ou seja, de acelerar a velocidade das reações químicas nos organismos, diminuindo o nível de energia de ativação das reações (HARVEY, CHAMPE *et al.*, 2009). Na ausência da catálise, a maioria das reações nos sistemas biológicos ocorreria de forma tão lenta que a formação de seus produtos não atenderia, em tempo hábil, às exigências ou necessidades de um determinado organismo (BERG, TYMOCZKO *et al.*, 2004). A eficiência das enzimas em catalisar reações é tal que a velocidade de uma reação pode ser aumentada em até $10^{20}$ vezes (CAMPBELL e FARRELL, 2007).

As enzimas não reagem quimicamente com as substâncias sobre as quais atuam, nem alteram o equilíbrio das reações. De uma maneira geral, uma enzima liga-se ao seu substrato formando um complexo Enzima-Substrato (ES), de caráter transitório. Essa ligação ocorre em uma região específica da enzima denominada sítio ou centro ativo localizada em uma fenda ou bolsão tridimensional formado por grupamentos que vêm de diferentes partes da sequência linear de aminoácidos da enzima (BERG, TYMOCZKO *et al.*, 2004).

A maioria dos substratos forma, pelo menos, três pontos de ligação com as enzimas através de ligações fracas não covalentes tais como, interações eletrostáticas, pontes de hidrogênio, força de van der Waals e interações hidrofóbicas. A partir da formação do complexo ES existem então dois caminhos a serem seguidos: ou ocorre a dissociação em E e S ou a formação do produto (P) e a liberação da enzima (MURRAY, GRANNER *et al.*, 2002).

A atividade catalítica de uma enzima se constitui em um meio sensível e específico para sua mensuração. Assim, para se medir a quantidade de uma enzima em uma amostra, mede-se a velocidade de reação catalisada pela enzima. Os resultados são geralmente expressos em unidades enzimáticas e as quantidades relativas de enzima em diferentes extratos podem ser então comparadas. Uma unidade de enzima pode ser definida como a quantidade de substrato que reage ou do produto formado por minuto (MURRAY, GRANNER *et al.*, 2002).

Alguns fatores como, por exemplo, a temperatura, a concentração dos reagentes e o pH, afetam a velocidade das reações alterando a atividade enzimática. A elevação da temperatura pode aumentar a velocidade de uma reação catalisada por uma enzima até o limite no qual essa temperatura seja responsável pelo rompimento das ligações fracas que conferem a estrutura secundária-terciária da enzima e cause sua desnaturação. Embora haja enzimas que trabalhem bem em pH alcalinos, neutros ou ácidos, valores extremos podem desnaturá-las ou causar alterações em sua carga elétrica, alterando sua atividade.

As enzimas podem ainda ter sua atividade alterada pela presença de inibidores. A inibição pode ser irreversível, quando o inibidor se liga ao sítio ativo da enzima causando alterações estruturais. Os inibidores reversíveis podem ser de dois tipos: competitivos e não
competitivos. O primeiro apresenta semelhança estrutural com o substrato e se liga ao sítio ativo, formando um complexo enzima-inibidor e impedindo a ligação da enzima ao substrato. Diferentemente do inibidor irreversível, não há alteração na estrutura da enzima. O inibidor não competitivo se liga a outra região da enzima provocando uma alteração em sua estrutura, principalmente em torno do sítio ativo, impedindo a reação de catálise mesmo quando o substrato está ligado a ela.

O estudo das enzimas tem uma grande relevância prática. A determinação da atividade enzimática no plasma sanguíneo ou em tecidos, por exemplo, é importante no diagnóstico de certas doenças, e muitas drogas exercem seu poder biológico através de interações com enzimas. Essas moléculas são importantes ferramentas práticas não só na medicina, mas também na indústria química, alimentícia, têxtil, do couro, do papel, na agricultura (MURRAY, GRANNER et al., 2002; NELSON e COX, 2005b; DAMHUS, KAASGAARD et al., 2008) e na pecuária (SOUZA, LINDEMANNN et al., 2007)

Segundo Fernández, Moyano et al. (2001), informações bioquímicas sobre o arsenal enzimático de um organismo podem ser úteis na seleção de ingredientes a serem usados em rações, uma vez que seu perfil enzimático tem estreita relação com hábitos alimentares e com a dieta a que estão submetidos.

1.2. CAMARÕES MARINHOS

1.2.1 Histórico da produção de camarões marinhos e as espécies estudadas

Segundo dados da FAO (2008), no ano de 2006 a produção global de camarões marinhos foi de 6,625 milhões de toneladas (52,23% provenientes da pesca e 47,77%, da aquicultura). Desse total, 45,82% referem-se à captura e cultivo de apenas duas espécies de peneídeos: o *Litopenaeus vannamei* e o *Penaeus monodon*, principais espécies das Américas e Ásia, respectivamente.

No Brasil a situação se inverte, sendo a carcinicultura responsável por 62,82% das 103,46 mil toneladas de camarões produzidas em 2006 (IBAMA, 2008). Na pesca extrativa, os maiores representantes são os camarões rosa (*Farfantepenaeus paulensis, Farfantepenaeus subtilis* e *Farfantepenaeus brasiliensis*), o branco ou vila franca (*Litopenaeus schmitti*) e o sete barbas (*Xiphopenaeus kroyeri*). Apesar da representatividade desses peneídeos nas estatísticas da pesca nacional (76,07% do total capturado), a espécie exótica *L. vannamei* (camarão cinza) responde pela quase totalidade dos camarões cultivados.

O desenvolvimento da carcinicultura marinha no Brasil pode ser dividido em três fases principais, que se baseiam no cultivo de diferentes espécies e na adoção de diferentes práticas de manejo e de tecnologias. A primeira etapa corresponde ao período de 1978 a 1984 e foi representada pelo cultivo da espécie exótica *Marsupenaeus japonicus* em sistemas extensivos
Apesar de resultados iniciais bastante promissores, a espécie mostrou-se susceptível às variações na qualidade da água decorrentes de períodos chuvosos, o que inviabilizou sua produção no Nordeste do País.

Técnicos e produtores envolvidos no setor passaram então a avaliar as espécies nativas *F. subtilis*, *F. paulensis*, *F. brasiliensis* e *L. schmitti*, caracterizando assim, a segunda fase da carcinicultura nacional (MAIA, 1993). Durante aproximadamente dez anos de trabalho para domesticação dessas espécies, foi constatada a viabilidade dos processos de maturação, reprodução e larvicultura, entretanto, as produtividades obtidas nos cultivos eram tão baixas que não justificavam o investimento financeiro necessário. Segundo Brasil (2001), as observações resultantes desses trabalhos indicaram que o principal fator limitante ao crescimento dos camarões nativos esteve relacionado à falta de informações sobre os requerimentos nutricionais das espécies e à inexistência de rações que atendessem a suas exigências. Nessa etapa, ficou evidenciada a necessidade de um programa de pesquisa básica e aplicada para investigar os aspectos biológicos, reprodutivos e nutricionais desses camarões.

A terceira fase é finalmente caracterizada pelo cultivo do *L. vannamei*, espécie encontrada no norte do Peru até o norte do México e introduzida no país ainda na década de 80 (BARBIERI JÚNIOR e OSTRENSKY NETO, 2001), mas que teve seu cultivo estabelecido apenas em meados dos anos 90. Na Figura 1 pode ser vista a evolução da carcinicultura brasileira nessa fase. Em apenas cinco anos (1998 a 2003), a atividade apresentou um crescimento de 1244%. A partir de 2004, no entanto, eventos como o surgimento do vírus da mionecrose infecciosa, a queda no câmbio do dólar e a ação antidumping movida pelos EUA contra o camarão brasileiro provocaram uma queda na produção (ABCC, 2008).


*F. paulensis* (Pérez-Farfante, 1967) encontra-se distribuído ao longo da costa atlântica da América do Sul, de Ilhéus, na Bahia, até Mar del Plata, na Argentina (D'INCAO, VALENTINI et al., 2002). Essa espécie, também conhecida como camarão-rosa (Figura 3) é um dos crustáceos de maior importância para as regiões sul e sudeste do Brasil. Assim como diversos outros penédeos, em ambiente natural apresentam-se como onívoros oportunistas (DALL, HILL et al., 1990), entretanto, quando em cativeiro, demonstram um comportamento altamente carnívoro (SOARES, PEIXOTO et al., 2008).
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![Figura 1 - Desempenho da carcinicultura marinha brasileira no período de 1998 a 2007 (Fonte: ABCC, 2008).](image)

*L. schmitti* (Bukenroad, 1936) é conhecido no Brasil como camarão branco ou camarão Vila Franca (Figura 4). A espécie é comumente encontrada em fundos lamosos e às vezes com areia, em profundidades que variam de 2 a 47 metros, embora sejam mais abundantes dos 15 aos 30m. São indivíduos de grande porte, atingindo comprimento de 235mm, habitando o oceano atlântico, na região das Antilhas, e na costa das Américas Central e do Sul, de Honduras até o sul do Brasil (PÉREZ-FARFANTE, 1970; HOLTHUIS, 1980).

*L. vannamei* (Boone, 1931) é originária do oceano Pacífico (Figura 5), ocorrendo em fundos lamosos e profundidades de até 72m, de Sonora, no México, até o norte do Peru encontrando-se distribuída de Sonora, no México, até o norte do Peru, em profundidades máximas de aproximadamente 72m (HOLTHUIS, 1980). Embora seja endêmico dessa região, a espécie, de hábito alimentar onívoro, foi introduzida em países de todo o mundo, onde é cultivada em sistemas semi-intensivo e intensivo. *L. vannamei* apresenta hábito alimentar onívoro e é mundialmente conhecida como camarão branco do Pacífico, tendo recebido o nome de camarão cinza no Brasil.
Figura 2 – Camarão nativo *Farfantepenaeus subtilis*

Figura 3 – Camarão nativo *Farfantepenaeus paulensis* (Fonte: www.pesca.sp.gov.br/imagens/244)
Figura 4 – Camarão nativo *Litopenaeus schmitti*

Figura 5 – Camarão exótico *Litopenaeus vannamei*
1.2.2 Aparelho digestório dos camarões

A nomenclatura das estruturas que compõem o sistema digestório em crustáceos apresenta divergências. Denominações diferentes para as mesmas estruturas podem ser vistas em diversos trabalhos que descrevem a morfologia do aparelho digestório dos camarões peneídeos (DALL e MORIARTY, 1983; DALL, HILL et al., 1990; GUILLAUME e CECCALDI, 1999) Essa controvérsia se baseia no fato de que alguns termos derivam de analogia feita com vertebrados, entretanto, determinadas classificações não se aplicam aos crustáceos, pois algumas das estruturas não apresentam as mesmas funções (DALL e MORIARTY, 1983)

O aparelho digestório de crustáceos (Figura 6), de uma maneira geral, está dividido em três partes: o intestino anterior, que engloba, o esôfago e o estômago ou proventrículo; o intestino médio onde se encontra o hepatopâncreas ou glândula do intestino médio e o intestino posterior, constituído pelo reto e ânus. Tanto o intestino anterior quanto o posterior são revestidos por uma camada quitino-protéica renovada a cada ciclo de muda (GUILLAUME e CECCALDI, 1999). O intestino anterior tem início na boca formada por um labro rígido e circundada por vários pares de apêndices especializados na quimiorecepção e apreensão dos alimentos (maxilas, maxílulas, mandíbulas e maxilípedes).

Figura 6 - Esquema da anatomia do aparelho digestório de camarões (adaptado de Ceccaldi, 1997).
O esôfago constitui-se em um tubo curto, reto e contrátil, revestido por uma camada quitino-protéica (GUILLAUME e CECCALDI, 1999), cuja função básica é conduzir o alimento ao estômago. O estômago ou proventrículo é uma estrutura mais complexa e se apresenta dividido em uma porção anterior (câmara cardíaca) e uma posterior (câmara pilórica), separadas por uma válvula cardio-pilórica. As duas câmaras são providas por peças calcáreas articuladas movidas por músculos específicos localizados na parede externa. Essas peças possuem funções diversas, segundo sua localização. Algumas peças são mais fortes e mais calcificadas (ossículos, discos e dentes) e localizam-se na câmara cardíaca, formando o moinho gástrico, cuja função é triturar os alimentos. Na câmara pilórica, encontram-se peças menores e menos calcificadas, que participam do processo de filtração. A ação combinada dessas peças possibilita a maceração do alimento e impede a passagem de partículas grandes para o intestino médio. A câmara pilórica está, por sua vez, dividida em uma porção dorsal, com sulcos laterais, que levam ao intestino médio, e outra ventral, onde se localiza o filtro-prensa. Essa estrutura é composta por um sistema de inúmeras micro-cerdas que filtram as partículas que passam para a glândula digestiva (Figura 7). Somente partículas menores que 1µm e fluido gástrico passam por essa rede de cerdas.

Figura 7 – Filtro-prensa do estômago de Penaeus monodon (adaptado de Lin, 2000).

A glândula digestiva ou hepatopâncreas dos peneídeo é constituída por dois lóbulos simétricos e pode representar de 2 a 6% da massa corporal. Ela é formada por uma centena de túbulos cegos que desembocam em câmaras que se abrem na porção pilórica do
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estômago. No interior dos túbulos se distinguem zonas de diferenciação celular, zonas responsáveis pela secreção de enzimas e pela absorção de nutrientes. Segundo Ceccaldi (1997), o hepatopâncreas apresenta diversas funções biológicas que incluem síntese e secreção de enzimas digestivas, digestão e absorção dos nutrientes da dieta, manutenção de reservas minerais e substâncias orgânicas, metabolismo de lipídeos e carboidratos, distribuição das reservas estocadas durante o período de intermuda e catabolismo de alguns compostos orgânicos.

O intestino médio se estende dorsalmente do final do estômago pilórico ao longo dos segmentos abdominais, terminando no reto e ánus que compõem o intestino posterior. Suas paredes apresentam cecos ou divertículos volumosos, onde se distinguem células nervosas, hemócitos e células endócrinas. Nessa região são secretados o muco e a película de quitina que envolve as fezes, mas essa membrana não impede a absorção dos nutrientes residuais presentes nas fezes.

Na Figura 8 encontra-se um diagrama da circulação do fluido gástrico e alimento no estômago de decápodos. De maneira sintética, o alimento é capturado pelos apêndices que circundam a boca, passa pelo esôfago e entra na câmara anterior do estômago, onde imediatamente se mistura com o fluido gástrico liberado pela glândula digestiva. O alimento circula repetidamente pelo estômago, sendo triturado pelas placas, dentes e ossículos do moinho gástrico. Após a trituração, o bolo alimentar segue para os sulcos ventrais e passa pelo filtro-prensa que exclui partículas superiores a 1µm, entrando por fim no lúmen da glândula digestiva.

1.2.3 Enzimas digestivas

Os peneídeos têm sido descritos como onívoros oportunistas, assim, o tipo de alimento ingerido pode variar em função de fatores como idade, tamanho e disponibilidade dos itens alimentares (DALL, HILL et al., 1990). De uma maneira geral, os peneídeos alimentam-se de grande variedade de invertebrados, detritos e algas, justificando uma ampla diversidade enzimática constituída por proteases, carboidrases e lipases (DALL e MORIARTY, 1983).

A digestão tem sido uma das áreas mais intensamente estudadas na nutrição de camarões (FERNÁNDEZ GIMENEZ, GARCÍA-CARREÑO et al., 2001), pois as propriedades das enzimas associadas a informações fisio-morfológicas do trato digestivo podem ajudar no entendimento e na determinação da capacidade digestiva destes organismos (VEGA-VILLASANTE, NOLASCO et al., 1995). Por causa da importância das enzimas digestivas na utilização dos nutrientes (FERNÁNDEZ, OLIVA et al., 1997), a compreensão do modo como elas atuam podem fornecer subsídios para uma correta formulação de rações com os
componentes adequados para o cultivo de camarões marinhos (MUHLIA-ALMAZÁN, GARCIA-CARREÑO et al., 2003).


Segundo Rodriguez, Le Vay et al. (1994), há um relacionamento próximo entre o hábito alimentar e as enzimas digestivas produzidas nos crustáceos, sendo a presença e a concentração destas um indicador da importância relativa de cada componente na dieta. A atividade específica dessas enzimas do trato digestivo pode ser usada para ilustrar a capacidade dos crustáceos de explorar várias dietas, com o intuito de suprir suas exigências nutricionais (JOHNSTON e FREEMAN, 2005).

Geralmente, crustáceos que apresentam um hábito alimentar tendendo a carnívoria possuem enzimas proteolíticas em concentrações elevadas, que condizem com sua habilidade em digerir os elevados níveis de proteína de sua dieta (JOHNSTON, HERMANS et al., 1995; JOHNSTON e YELLOWLEES, 1998). Por sua vez, as espécies que possuem um hábito alimentar mais onívoro e herbívoro apresentam concentrações mais elevadas de carboidrases, consistentes com sua habilidade de digerir carboidratos de origem animal e vegetal (BÄRLOCHER e PORTER, 1996).

As proteases estão entre as enzimas de crustáceos que recebem maior atenção (FERNÁNDEZ GIMENEZ, GARCIA-CARREÑO et al., 2002), pois são responsáveis pela digestão de proteínas dos alimentos ingeridos, liberando os aminoácidos essenciais ao crescimento (SÁNCHEZ-PAZ, GARCIA-CARREÑO et al., 2003). Dentre as proteases de maior
importância encontram-se a tripsina, a quimotripsina e as aminopeptidases. A tripsina e a quimotripsina são endoproteases, ou seja, clivam as ligações peptídicas dentro da proteína, enquanto que as aminopeptidases são exoproteases (Figura 9), isto é, clivam resíduos de aminoácidos na posição N-terminal da proteína (GONZALES e ROBERT-BAUDOUY, 1996).

A tripsina é a protease mais abundante no sistema digestivo de crustáceos (FERNÁNDEZ GIMENEZ, GARCIA-CARREÑO et al., 2002). Alguns autores têm enfatizado a importância desta enzima em peixeídos e estimam que sua contribuição para a digestão protéica seja em torno de 60% (SÁNCHEZ-PAZ, GARCIA-CARREÑO et al., 2003). Ela é um membro da família das serino proteases, as quais são caracterizadas por um mecanismo catalítico comum, envolvendo a presença de uma tríade catalítica composta de resíduos específicos: serina, histidina e ácido aspártico. Esta enzima cliva as ligações peptídicas no lado carboxila de resíduos de aminoácidos carregados positivamente como arginina e lisina (KOMKLÃO, BENJAKUL et al., 2007), sendo importantes em muitos processos biológicos como: digestão protéica propriamente dita, ativação de zimogênios e mediação entre a ingestão do alimento e a assimilação dos nutrientes (SAINZ, GARCIA-CARREÑO et al., 2004). Vale também destacar a ampla aplicabilidade industrial de tripsinas (KLEIN, LE MOULLAC et al., 1996). Tais características têm feito das tripsinas as enzimas mais estudadas em organismos aquáticos.

A quimotripsina é considerada a segunda enzima mais abundante no sistema digestivo de crustáceos considerando a atividade proteolítica (GARCIA-CARREÑO, HERNÁNDEZ-CORTÉZ et al., 1994). Esta protease é uma endopeptidase solúvel em água que catalisa a hidrólise de ligações peptídicas de proteínas na porção carboxila de aminoácidos aromáticos como: fenilalanina, tirosina e triptofano e também substratos sintéticos, tais como SAPNA (DE VECCHI e COPPES, 1996; VIPARELLI, ALFANI et al., 2001; ABUIN, LISSI et al., 2004; CASTILLO-YAÑEZ, PACHECO-AGUILAR et al., 2006).
As aminopeptidases são enzimas geralmente inespecíficas que catalisam a hidrólise em ligações peptídicas na posição N-terminal de proteínas liberando pequenos peptídeos e aminoácidos livres (GONZALES e ROBERT-BAUDOY, 1996). Essas enzimas atuam também catalisando a hidrólise de substratos artificiais tais como aminoacil-β-naftilamida (AA-NA) e aminoacil-p-nitroanilida (AA-Nan). Elas estão amplamente distribuídas na natureza e apresentam importâncias biológicas e médicas por causa da sua função na degradação de proteínas (OLIVEIRA, FREITAS et al., 1999).

Os polissacarídeos de reserva mais importantes são o amido, nos vegetais, e o glicogênio, nos animais, ambos com alto peso molecular. São polímeros de glicose em ligações α(1,4) nas cadeias principais e ligações α(1,6) nos pontos de ramificação, sendo o glicogênio mais compacto por apresentar mais ramificações em sua molécula (KAUSHIK, 1999).

Os efeitos da inclusão de carboidratos em dietas sobre o desempenho zootécnico de camarões penêdeos vêm sendo estudados há algumas décadas. As pesquisas demonstram que esses animais são capazes de digerir melhor carboidratos na forma de dissacarídeos e amido, do que na forma de glicose (SICK e ANDREWS, 1973; PIEDAD-PASCUAL, COLOSO et al., 1983; SHIAU e PENG, 1992). Em sua forma direta, a ingestão da glicose provoca uma saturação imediata na hemolinfa resultante de sua rápida absorção ao longo do trato digestivo, reduzindo a ingestão de alimentos, e pode ainda inibir a absorção de aminoácidos no intestino (SANTOS e KELLER, 1993; SHIAU, 1998). Por essas razões, sugere-se a utilização de moléculas mais complexas, como o amido e seus derivados (amilose e amilopectina) na elaboração de rações, uma vez que a absorção da glicose produzida só acontece após a hidrólise enzimática (ROSAS, CUZON et al., 2001).

Uma vez ingerido, o amido sofre a ação de diversas enzimas. A α-amilase [EC 3.2.1.1] é uma endocarboxidrase encontrada na saliva e no trato digestivo de animais vertebrados (SALEH, AFAF et al., 2005), responsável pela hidrólise de ligações glicosídicas α(1,4), no amido e glicogênio. Nesse processo são produzidos oligossacarídeos, α-dextrinas e maltose (VAN WORMHOUDT e FAVREL, 1988), que são hidrolisados à glicose pela ação complementar da α-glicosidase [EC 3.2.1.20], da sacarase-isomaltase [EC 3.2.1.48] e da α-dextrinase [EC 3.2.1.20]. Dentre essas, a α-glicosidase está diretamente relacionada à exo-hidrólise de ligações glicosídicas α(1,4) da maltose e demais oligossacarídeos formados após a atuação da α-amilase (LE CHEVALIER e VAN WORMHOUDT, 1998; DOUGLAS, MANDLA et al., 2000; ROSAS, CUZON et al., 2000).

Ao contrário de mamíferos e outros vertebrados, os crustáceos decápodos não utilizam carboidratos e lipídeos como fonte primária de produção de energia. Entretanto, alguns trabalhos já revelam que a inclusão de carboidratos nas dietas de algumas espécies de camarão promove um bom crescimento e eficiência alimentar, indicando que essas moléculas
apresentam a característica de poupar a proteína ("protein sparing"), liberando-a para o crescimento (CRUZ-SUÁREZ, RICQUE-MARIE et al., 1994; ROSAS, CUZON et al., 2000).

Para a escolha adequada de ingredientes a serem utilizados nas dietas de camarões, informações sobre o arsenal enzimático das espécies alvo são necessárias. Estudos nutricionais específicos para camarões marinholos nativos são ainda escassos, embora o êxito no cultivo de organismos aquáticos dependa, em grande parte, de uma nutrição adequada e de um bom manejo alimentar. Se considerarmos que o gasto com alimentação pode chegar a 50% dos custos de produção, é importante estudar os processos fisiológicos do organismo que afetam sua capacidade de consumo e de digestão dos alimentos, entre os quais a atividade enzimática desempenha um papel de vital importância.

1.3. A TILAÍA-DO-NILÓ (Oreochromis niloticus Linnaeus, 1758).

As tilápias são peixes nativos da África, Jordânia e Israel, representados por aproximadamente 70 espécies e cultivados em todo o mundo. As de importância comercial estão divididas em três gêneros, distintos pelo comportamento reprodutivo: Tilapia (incubam seus ovos em substratos); Sarotherodon (os ovos são incubados na boca dos machos e fêmeas) e Oreochromis, cujos ovos são incubados na boca das fêmeas (TREWAVAS, 1982).

A ampla distribuição do gênero, principalmente nas regiões tropicais e subtropicais, está associada à alta tolerância que apresenta às variações das condições ambientais. Apontada como a principal espécie da piscicultura brasileira devido a características biológicas e mercadológicas relevantes (FARIA, HAYASHI et al., 2001), a tilápiá é produzida em diferentes sistemas de cultivo e escalas, com maior ou menor dependência de insumos, principalmente ração.

No Brasil, a produção de tilápiá cultivada cresceu muito entre os anos de 2000 e 2006, passando de 32.459 para 71.253 toneladas, com um aumento médio de 19,92% ao ano no período (IBAMA, 2000; 2008). Embora ainda sejam encontrados cultivos com a tilápiá vermelha, híbridos de Oreochromis, a espécie mais cultivada no país é sem dúvida a tilápiá-do-niló, O. niloticus (Figura 10). De toda a produção da aquicultura continental brasileira em 2006, a tilápiá respondeu por 37,5% do volume produzido, sendo o peixe com maior representatividade. Sua importância se torna maior quando analisada sua participação na produção da Região Nordeste, onde correspondeu a 73,5% da produção da aquicultura continental, totalizando 26,4 mil toneladas.
1.3.1 Aparelho digestório de peixes

De uma forma geral, a compreensão da anatomia do sistema digestório dos organismos aquáticos facilita o entendimento de sua fisiologia digestiva. Os peixes podem ser classificados de acordo com o hábito alimentar em quatro categorias principais: detritívoros, herbívoros, onívoros e carnívoros. Em cada uma dessas categorias, os organismos podem ser eurímafagos (ingerem grande variedade de alimentos), estenómafagos (pequena variedade de itens alimentares) ou monómafagos (consomem apenas um tipo alimentar). A maioria dos peixes cultivados são carnívoros eurímafagos (salmão, linguado), herbívoros eurímafagos (carpas, milkfish) e onívoros eurímafagos, tais como bagre canal e tilápias (RUST, 2002). Embora haja exceções, a anatomia digestiva entre espécies de uma mesma classe é bastante similar, mas diferenças marcantes são observadas entre as classes (Figura 11).

As tilápias são peixes altamente oportunistas e sua alimentação pode variar em função de características como a idade, o sexo e a disponibilidade do alimento. Essa é uma característica chave para o enorme sucesso desses peixes na colonização de novos habitats (LOWE-MCCONNELL, 2000). As espécies do gênero *Oreochromis* são todas onívoras, alimentando-se de algas, plantas aquáticas, pequenos invertebrados, material detrital e bactérias associadas a biofilmes (FITZSIMMONS, 1997).

A digestão é uma ação coordenada de atividades físicas, químicas e enzimáticas que tem início tão logo o alimento entra na boca, e termina com a excreção das fezes. Incluídos nos processos físicos estão a apreensão, a trituração e amistura; o ácido clorídrico produzido pelo estômago é um processo químico que auxilia a hidrólise e quebra de compostos; os processos enzimáticos são mais específicos e envolvem um grande número de enzimas digestivas (RUST, 2002).

![Figura 11 – Sistema digestório de peixes com diferentes hábitos alimentares (SMITH, 1989b).](image-url)
Em geral, três processos são usados para a captura de alimentos pelas tilápias: a alimentação por sucção, na qual a presa é sugada para a cavidade bucofaringeal, com o peixe em estado estacionário; a mordida, no qual os dentes das mandíbulas superior e inferior são usados na captura, e a visual, no qual o peixe localiza e seleciona o alimento a ser ingerido. A alimentação por sucção e a visual são utilizadas para a ingestão de zooplânctons maiores, enquanto que a sucção sozinha, é usada na ingestão de bactérias livres, fitoplâncton e zooplâncton menores (BEVERIDGE e BAIRD, 2000). Para a mordida, as tilápias possuem de uma a cinco fileiras de dentes mandibulares utilizados na ingestão de alimentos como macrófitas, pequenos invertebrados, perifiton, detritos (LANZING e HIGGINBOTHAM, 1976; NORTHCOTT e BEVERIDGE, 1988; DEMPSTER, BEVERIDGE et al., 1993).

Independentemente do item alimentar a ser ingerido, o alimento é forçado através da cavidade bucofaringeal por fortes correntes de água geradas por áreas específicas da cavidade bucal, criando um mecanismo de transporte, posicionamento e reposicionamento da presa ou item alimentar, conhecido como língua hidrodinâmica (LIEM, 1991). O movimento de vai-e-vem dos ossos e dentes faringeanos permitem a quebra do alimento em fragmentos menores, a ruptura das paredes celulares de materiais de origem vegetal, aumentando a área superficial dos alimentos e facilitando a digestão (BOWEN, 1982).

Segundo Rust (2002), os órgãos digestivos em peixes são aqueles onde os alimentos são transformados em nutrientes e absorvidos pelos organismos. Esses órgãos incluem o esôfago posterior, o estômago (nas espécies gástricas) ou bulbo intestinal (nas agástricas), o intestino superior e inferior e os cecos pilóricos. Como órgãos de suporte encontram-se o pâncreas, a vesícula biliar e o fígado.

Após passar por um esôfago muito curto, o alimento entra no estômago, o qual apresenta três regiões distintas, correspondendo às porções cardíaca (initial), fúngica (mediana) e a pilórica (terminal) nos mamíferos. Quatro camadas de tecido distintas podem ser encontradas nos órgãos digestivos de peixes (CACECI, EL-HABBACK et al., 1998; RUST, 2002): partindo-se do lúmen distinguem-se a mucosa, a submucosa, a muscular e a serosa. A mucosa é composta por uma variedade de células colunares epiteliais que formam as rugosidades; a submucosa é composta por tecido conectivo que serve de suporte para a mucosa; a muscular é composta por duas camadas de músculo, uma longitudinal externa e outra circular interna, que ajudam no movimento do conteúdo do lúmen; a serosa é uma camada de células mesoteliais sobre tecido conectivo frouxo (Figura 12). Na mucosa, sobretudo da porção fúngica, encontram-se as glândulas gástricas formadas por células oxintopépticas responsáveis pela secreção de ácido clorídrico, enzimas digestivas, mucos e hormônios (RUST, 2002; ROTTA, 2003). A função do estômago é começar a transformação do alimento em digesta, liberando nutrientes solúveis. Seus músculos misturam o conteúdo
estomacal para facilitar o contato entre o bolo alimentar e o suco gástrico (BEVERIDGE e BAIRD, 2000).

O esfincter pilórico define o limite posterior do estômago, cuja função é controlar a taxa de passagem da digesta altamente ácida para a parte superior do intestino, o que é importante não só porque auxilia no controle do tempo de contato do alimento com o suco gástrico, como no controle da quantidade de material ácido que entra no intestino. Secreções produzidas no pâncreas e na vesícula biliar contendo bicarbonato de sódio entram no intestino, via ducto biliar, elevando o pH para níveis mais neutros ou ligeiramente alcalino (RUST, 2002).

![Figura 12 – Porção do estômago cardíaco de juvenis de linguado: a) camada serosa; b) camada muscular longitudinal externa; c) camada muscular circular interna; d) músculo estriado; e) submucosa; f) glândulas gástricas; g) lâmina própria; h) epitélio da mucosa i) lúmen (Adaptado de RUST, 2002)](image)

O intestino é um órgão relativamente simples e, embora nem sempre haja uma separação distinta como nos mamíferos, é considerado como tendo duas partes: uma porção anterior superior, denominada intestino ascendente ou delgado, e uma porção posterior inferior, conhecida por intestino descendente ou grosso (RUST, 2002). Possui glândulas digestivas e um suprimento abundante de vasos, onde se completa a digestão iniciada no estômago. No
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O intestino é onde ocorre a maior parte da absorção dos nutrientes, íons e água oriundos da dieta, sendo os produtos da digestão mantidos em solução, o que facilita a absorção. Nos peixes, além da função de digestão e absorção, o intestino pode desempenhar outras funções, como auxiliar na osmorregulação ou na respiração (ROTTA, 2003).

Histologicamente, o intestino superior difere do inferior na quantidade de células globet que são mais escassas e na camada muscular que é mais fina no intestino inferior. Há uma mudança de um epitélio colunar de células secretoras e de absorção para um epitélio cuboidal escamoso que secreta basicamente muco no intestino inferior (SMITH, 1989a).

Nos peixes, o pâncreas pode se apresentar como uma estrutura individualizada ou em forma difusa, com nódulos pancreáticos espalhados no tecido adiposo, mesentério, fígado, duto biliar, vesícula biliar, cecos pilóricos, intestino, etc (SMITH, 1989b).

As tilápias não apresentam os cecos pilóricos, que nos demais peixes, são circundados por tecido pancreático e são responsáveis pela produção de enzimas digestivas como proteases alcalinas, amilases e lipases e pela secreção de hormônios como a insulina, glucagon. Em contrapartida, possuem um intestino muito longo, (cerca de 7 a 14 vezes o comprimento padrão do corpo), o que pode compensar a ausência de cecos pilóricos (SHELTON e POPMA, 2006). As principais funções do intestino estão ligadas à secreção de enzimas e hormônios e à absorção de nutrientes.

1.3.2 Enzimas digestivas em peixes

a) Fluidos e enzimas gástricas

Segundo Rotta (2003), as secreções do estômago, produzidas na região fúndica, incluem água, sais inorgânicos, hormônios, muco (mucina), ácido clorídrico a 0,1 N, pepsinogênio e lipase gástrica. A secreção ácida nas tilápias pode ter um pH 1,0, o que parece auxiliar a ruptura das paredes celulares das algas. A secreção de muco e suco gástrico estão condicionadas à presença de alimentos na luz do estômago, a hormônios e a estímulos neurais do nervo vago. O muco, alcalino, protege a mucosa estomacal da ação do ácido clorídrico e também da irritação mecânica dos alimentos.

A pepsina é, sem dúvida, a principal protease ácida dos peixes, produzida na forma inativa de pepsinogênio (FÄNGE e GROVE, 1979). A enzima é ativada inicialmente em condições ácidas, através da remoção de um peptídeo de baixo peso molecular pelo ácido clorídrico e continua o processo de transformação por autocatálise. O ácido clorídrico estimula ainda a estimula a liberação do hormônio secretina, responsável pela descarga do suco pancreático rico em íons bicarbonato que irão auxiliar na neutralização do pH no intestino (ROTTA, 2003).
Algumas enzimas não proteolíticas como amilase, lipase, esterase, quitinase, celulase, têm sido registradas no estômago de peixes (FÄNGE e GROVE, 1979; GUILLAUME e CHOUBERT, 1999; RUST, 2002). As tilápia apresentam lipase e amilase gástrica, mas essa enzima hidrolisa apenas as gorduras de baixo ponto de fusão e já emulsificadas. A ação sobre as gorduras da dieta é mais completa e mais eficaz pela lipase pancreática (FÄNGE e GROVE, 1979; ROTTA, 2003).

b) Enzimas pancreáticas

O suco pancreático é rico em enzimas que atuam na digestão de proteínas, carboidratos, gorduras e nucleotídeos. Tripsina, quimotripsina, carboxipeptidases e elastase são armazenadas nas células pancreáticas na forma de zimogênios. Ao chegar ao lúmen intestinal, a tripsina é ativada pela enteroquinase produzida por células da mucosa intestinal e, em seguida, a própria tripsina ativa outras enzimas como quimotripsina, colagenase, elastase e lipase (RUST, 2002). Além das citadas anteriormente, as enzimas pancreáticas incluem: amilases, quitinases, lipases, esterases, aminopeptidases e ribonucleases (FÄNGE e GROVE, 1979; GUILLAUME e CHOUBERT, 1999; RUST, 2002).

c) Enzimas intestinais

Dados divergentes são encontrados na literatura sobre a secreção de enzimas digestivas por células intestinais. Fänge e Grove (1979) citam que enzimas intestinais são produzidas nas membranas da borda em escova do epitélio intestinal. Segundo Guillaume e Choubert (1999), o intestino de peixes não secreta enzimas digestivas. Os autores concordam, contudo, que a atividade enzimática detectada no fluido intestinal pode ser de origem alimentar, bacteriana, estomacal ou principalmente pancreática, visto que enzimas pancreáticas tendem a se ligar ao glicocálix das células epiteliais. As enzimas ditas como sendo produzidas pela mucosa intestinal incluem aminopeptidases, di e tripeptidases, nucleosidades alcalinas e ácidas, polinucleosidases, lectinases, lipases e outras esterases, amilases, maltase, isomaltase, sacarase, lactase, trealase e laminarinase.

1.3.3 Ingredientes alternativos em rações para peixes

A produção de organismos aquáticos em cativeiro requer rações de alta qualidade, com alto conteúdo protéico (LARA-FLORES, OLVERA-NOVOA et al., 2003) e a farinha de peixe ainda permanece como a principal fonte protéica, compreendendo entre 20 e 60% das rações para peixes (WATANABE, 2002). A farinha é uma excelente fonte de aminoácidos e ácidos
graxos essenciais, vitaminas, minerais e, geralmente, aumenta a palatabilidade da ração (El-SAYED, 1999; DAVIS e ARNOLD, 2000). Por ser uma “commodity” de oferta limitada e demanda crescente, a sua disponibilidade e a constante flutuação nos seus preços podem afetar seriamente a sustentabilidade e rentabilidade da aquicultura (FARIA, HAYASHI et al., 2001; OLVERA-NOVOA, OLIVERA-CASILLO et al., 2002). Em longo prazo, muitos países em desenvolvimento serão incapazes de manter a utilização da farinha de peixe como a principal fonte de proteína em rações aquáticas.

A determinação de fontes protéicas de menor custo e que promovam bom crescimento é vantajoso tanto para a indústria de rações como para os aquicultores (COYLE, MENGEL et al., 2004). Assim, pesquisas têm sido cada vez mais direcionadas a fontes protéicas alternativas que sejam de baixo custo e prontamente disponíveis, como substitutas para a farinha de peixe (WEE e WANG, 1987; WATANABE, 2002).

Diversos produtos têm sido utilizados com o propósito de substituir total ou parcialmente a farinha de peixe em rações aquáticas, incluindo subprodutos de pescado ou de animais terrestres, sementes oleaginosas, plantas aquáticas, concentrados protéicos, proteína de organismos unicelulares (single cell protein) e subprodutos de leguminosas e cereais (OLVERA-NOVOA, PEREIRA-PACHECO et al., 1997; PLASCÊNCIA-JATOMEA, OLVERA-NOVOA et al., 2002; EL-SAIDY e GABER, 2003; GABER, 2006).

O farelo de soja tem sido preconizado como a principal fonte protéica de origem vegetal em rações para peixes (FURUYA, HAYASHI et al., 2001a), por apresentar alto teor de proteína, um bom perfil de aminoácidos, preço razoável e composição e disponibilidade constantes. Entretanto, seu percentual de metionina é baixo e, além disso, esse farelo pode conter 30% de carboidratos indigestíveis e vários compostos ou fatores antinutricionais que podem prejudicar os processos digestivos (HERNÁNDEZ, MARTÍNEZ et al., 2006). Assim, a utilização desse ingrediente em rações animais exige um adequado processo de fabricação do produto, como o aquecimento para inativação dos fatores antinutricionais (FURUYA, PEZZATO et al., 2001b), ou a necessidade de suplementação de aminoácidos para complementar o seu perfil de aminoácidos (FURUYA, PEZZATO et al., 2001c).

De forma geral, a viabilidade da substituição da farinha de peixe por ingredientes vegetais estará condicionada ao hábito alimentar do animal que se pretende alimentar. Em oposição aos peixes carnívoros, os peixes onívoros possuem adaptações morfológicas e fisiológicas que possibilitam a utilização de rações com elevadas percentagens de ingredientes vegetais, pois utilizam melhor os carboidratos e a proteína (aminoácidos) dessas fontes (FURUYA, PEZZATO et al., 2001b).

O grau de inclusão de fontes protéicas vegetais em rações aquícolas parece ser limitado pela presença de fatores antinutricionais, a deficiências em aminoácidos essenciais (FRANCIS, MAKKAR et al., 2001) e à disponibilidade do fósforo. Nos alimentos de origem vegetal, cerca
de 70% deste mineral está complexado na forma de fitato, que não é utilizado pelos monogástricos e que promove também a redução na disponibilidade de outros elementos, como zinco, cálcio, ferro e manganês (FARIA, HAYASHI et al., 2001).

Além de fontes protéicas alternativas oriundas de vegetais, diversos subprodutos animais vêm sendo testados como ingredientes na composição de dietas para peixes. No processamento de camarões, por exemplo, são geradas grandes quantidades de resíduos na forma de cabeça, apêndices e carapaças, as quais têm sido descartadas no ambiente de forma inadequada. A farinha produzida a partir desses resíduos vem sendo identificada como uma fonte de proteína animal de grande potencial para a aquicultura (FANIMO, ODUGUWA et al., 2000).

O resíduo do processamento do camarão é rico em proteína e quitina. A fermentação deste resíduo por ácido lático tem sido relatada como uma técnica eficaz e econômica de proteger esta biomassa da decomposição bacteriana, formando uma silagem que contém um líquido rico em proteínas e uma fração lipídica e quitina insolúvel (LÓPEZ-CERVANTES, SÁNCHEZ-MACHADO et al., 2006). Entretanto, o uso destes subprodutos pode ser restringido devido aos altos conteúdos de fibras e cinzas, resultando na formação de peletes fracos, com baixa hidroestabilidade (CAVALHEIRO, SOUZA et al., 2007).

Fanimo, Oduguwa et al. (2000) determinaram a qualidade protéica de uma farinha de subprodutos de camarão (cabeça, apêndices e exoesqueleto) e demonstraram que o valor biológico da proteína é inferior ao da proteína da farinha de peixe; porém, sua qualidade protéica pode ser melhorada mediante suplementação com lisina e metionina. López-Cervantes, Sánchez-Machado et al. (2006) identificaram tirosina, treonina, leucina e glicina como os aminoácidos mais abundantes na fração protéica liofilizada do hidrolisado de resíduo de camarão. Estes autores afirmam, ainda, que aminoácidos livres são uma das mais importantes frações não-protéicas e alguns deles, como alanina, ácido glutâmico e glicina, são responsáveis pelo sabor e odor característicos destes produtos. Outros trabalhos relatam que produtos obtidos a partir do processamento de resíduos de camarão podem servir como uma adequada fonte de proteína e flavorizante em formulações alimentares, devido principalmente aos seus teores de aminoácidos livres (HEU, KIM et al., 2003).

Os resíduos do processamento da indústria camaroneira podem ser utilizados para a produção de hidrolisado protéico (HP). Esse produto é resultado da solubilização das proteínas do pescado que pode ser obtida através da utilização de ácidos e de solventes orgânicos ou ainda ser catalisada por enzimas proteolíticas endógenas, ou seja, presentes no próprio organismo (autólise enzimática) e/ou por enzimas de origem vegetal, animal ou microbianas adicionadas à matéria-prima (KRISTINSSON e RASCO, 2000; MARTONE, BORLA et al., 2005). O processo consiste da quebra de longas cadeias de moléculas protéicas e resulta em duas frações, uma contendo proteínas não hidrolisadas e outros materiais insolúveis (fração
insolúvel) e outra, rica em proteínas, peptídeos de cadeia curta e aminoácidos livres (fração solúvel). Além de ser um meio de reduzir a poluição ambiental causada pelo descarte indevido de resíduos a produção do HP pode ainda fornecer um alimento com propriedades funcionais e de alto valor nutritivo (SLIZYTE, DAUKSAS et al., 2005), uma vez que pode apresentar um perfil de aminoácidos similar ao da matéria-prima utilizada para sua produção.

Silva (2004) elaborou um hidrolisado protéico a partir de cabeças do camarão marinho Litopenaeus vannamei, por meio da trituração deste resíduo e da digestão por autólise (45°C), seguida por desativação enzimática (100°C), separação e centrifugação, o qual foi considerado como uma excelente fonte alimentar, sobretudo de aminoácidos, com ácido glutâmico, ácido aspártico, leucina, lisina, tirosina e arginina como os mais abundantes.

A obtenção de ingredientes alternativos, como farinhas ou hidrolisados protéicos obtidos de resíduos do processamento industrial de alimentos, que atuem como fontes de proteína e que sejam mais baratos e acessíveis que a farinha de peixe, pode resultar em rações aquícolas de menor custo, mantendo ou melhorando a qualidade nutricional das rações destinadas à alimentação animal. Entretanto, mudanças na origem e quantidade de nutrientes utilizados na elaboração de rações, podem modificar a atividade, concentração e o perfil enzimático no trato digestivo dos animais (LUNDSTEDT, MELO et al., 2004), que podem se traduzir em adaptações para uma melhor assimilação destes nutrientes (MORAES e BIDINOTTO, 2000).
2. OBJETIVOS

2.1. OBJETIVO GERAL

- Identificar e caracterizar enzimas provenientes de organismos aquáticos visando ao entendimento da fisiologia digestiva de espécies de interesse comercial, bem como à utilização dessas moléculas como indicadoras da qualidade de dietas.

2.2. OBJETIVOS ESPECÍFICOS

- Caracterizar as alfa-amilases presentes no hepatopâncreas das espécies *F. subtilis*, *L. schmitti* e *L. vannamei*, visando fornecer subsídios para o conhecimento da digestão de carboidratos em camarões peneídeos;
- Caracterizar proteinases e peptidases digestivas visando ao conhecimento da digestão protéica dos camarões nativos *F. subtilis* e *F. paulensis*;
- Investigar a utilização do hidrolisado protéico de camarão como fonte protéica em rações para juvenis da tilápia-do-nilo *O. niloticus*, avaliando-se o desempenho zootécnico dos peixes;
- Avaliar o perfil de enzimas digestivas da tilápia-do-nilo, submetida a dietas com diferentes concentrações de hidrolisado protéico de camarões, correlacionando-as com os parâmetros de crescimento e composição corporal.
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4. CAPÍTULO 1: FISIOLOGIA DIGESTIVA DE CAMARÕES MARINHOS
4.1 Artigo 1: Comparative study of amylases from the midgut gland of three penaeid shrimps
COMPARATIVE STUDY OF AMYLASES FROM THE MIDGUT GLAND OF THREE PENAEID SPECIES

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Running Title: Amylases from penaeid shrimps

Keywords: Amylase characterization; midgut gland; penaeid shrimp; Farfantepenaeus subtilis; Litopenaeus schmitti; Litopenaeus vannamei.
Abstract

Amylases and A/P ratio from the midgut gland of wild *Farfantepenaeus subtilis* and *Litopenaeus schmitti* and of farmed *Litopenaeus vannamei* were characterized. Total amylolitic activity of farmed shrimps was twice higher than that of wild specimens. Amylases of all species presented residual activity above 85% at alkaline pH (7.0-8.0) and optimal temperature between 40 and 50°C. The enzymes of all species were not thermal stable at temperatures greater than 55°C. Substrate zymograms revealed nine, eight, ten and seven amylolitic bands from *F. subtilis*, *L. schmitti*, adult (25g) and juvenile (11g) *L. vannamei*, respectively. Alpha-amylase activity of *F. subtilis* and *L. schmitti* was totally inhibited by inhibitor Type I at 50 and 100µg.mL\(^{-1}\) inhibitor concentrations, while adult *L. vannamei* enzymes retained 43.5±1.98 and 22.5±0.65% of its activity at the same concentrations, respectively. Ca\(^{2+}\) increased amylase activity of all species only at 1mM concentration, but inhibited the activity at 5 and 10mM. All other ions employed (Cd\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\), Cu\(^{2+}\) and Al\(^{3+}\)) strongly inhibited amylase activity regardless the concentration used. The great number of isoforms observed in the midgut gland of all species suggests shrimp capability for carbohydrate digestion. Feeds with different concentrations of carbohydrate should be tested.

Keywords: Amylase characterization, midgut gland, penaeid shrimp, *Farfantepenaeus subtilis*; *Litopenaeus schmitti*; *Litopenaeus vannamei*. 
Introduction

Penaeid shrimps are among the most cultured crustaceans in the world. In Brazil, fishery statistics revealed a total shrimp production of 103,460 tonnes in 2006, of which 62.82% were provided by aquaculture (IBAMA, 2008). Despite the existence of native shrimps with attractive market features, such as Farfantepenaeus subtilis, F. brasiliensis, F. paulensis and Litopenaeus schmitti, the exotic species L. vannamei responds for most of the farmed shrimp in the country.

In fact, years of study on reproduction, nutrition and feeding, genetics, physiology and diseases provided information used to develop complete diets, genetically improved postlarvae or even a growth-out technology package, which enabled the spreading out of L. vannamei outside the Eastern Pacific coast.

The lack of basic information about biology and more specifically about digestive physiology of Brazilian native shrimps have led to the adoption of improper feeds and management in growth-out systems which resulted in both poor feed conversion rates (2.88 to 3.44 for F. subtilis) and yields (Maia & Nunes, 2003). The formulation of specific diets depends upon information about nutritional requirements and digestive capability of the target species.

The study of digestive physiology in aquatic organisms is important since enzyme profile of a given species is closely related to its feeding habit and to its capability of digest a wide range of food items (Fernández et al., 2001). There are a number of works about nutrition and feeding of penaeid shrimps, but studies on Brazilian native species are limited. Some authors deal with semi-intensive culture and feeding studies of F. subtilis (Nunes et al., 1996; 1997; Nunes & Parsons, 2000) and F. paulensis (Abreu et al., 2007; Ballester et al., 2007; Fróes et al., 2007; Soares et al., 2008). Digestive enzyme studies are related to proteases of L. schmitti (Lemos et al., 2002) and of F. paulensis (Lemos et al., 1999; Buarque et al., 2009) but little is available about carbohydrases of these species.

Among carbohydrases of penaeid shrimps are alpha-amylases (Van Wormhoudt et al., 1995, Fernández et al., 1997), maltase (Omondi e Stark, 1995; Aguilar-Qualesma e Sugai, 2005), chitinase, laminarinase and cellulase (Guillaume e Ceccaldi, 1999). Studies on carbohydrate digestion are important because are often included in commercial diets for reducing feeding costs (Wigglesworth & Griffith, 1994; Aguilar-Quaresma & Sugai, 2005). Thus is necessary to understand the profile of enzymes involved in this process.

The aim of the present work was to characterize amylases present in the midgut gland of two native penaeid shrimps (F. subtilis and L. schmitti) and of the exotic species L. vannamei.
Materials and methods

Materials

Specimens of *F. subtilis* (12.77±0.78g) and *L. schmitti* (23.30±2.89g) were obtained from commercial fishery and *L. vannamei* (25.17±1.47 and 11.13±0.48g) from a commercial farm at Pernambuco State, Brazil. All reagents used in enzymatic assays were from analytical grade purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Enzyme extraction and total soluble protein determination

Eighteen shrimps of each species were transported alive to a commercial shrimp hatchery where they were weighed and sacrificed by thermal shock. The hepatopancreas were readily dissected out, weighed (0.59±0.16g – *L. schmitti*; 0.79±0.22g and 0.46±0.05g – *L. vannamei*; 0.39±0.04g – *F. subtilis*), placed in cryotubes and transported to the Laboratório de Enzimologia (LABENZ) of the Universidade Federal de Pernambuco. Six groups of three glands of each species were homogenized (40 mg mL\(^{-1}\)) in chilled 10mM Tris-HCl buffer, pH 7.5, with 0.15M NaCl, using a Potter-Elvehjen tissue homogenizer (Bodine Electric Company – Chicago, USA) at 40-50 rpm for 3 min at 4\(^{\circ}\)C. Homogenates were centrifuged at 10,000 xg for 25 min at 4\(^{\circ}\)C, to remove lipid and tissue debris, and supernatants (crude enzyme extract) were stored at −20\(^{\circ}\)C for further utilization. The total soluble protein was determined as described by Bradford (1976), using bovine serum albumin as standard protein.

Amylase and proteinase assays

Total amylase activity was determined based on the method of Bernfeld (1955) using 2% (w/v) starch solution as substrate. The reaction consisted of 60µL of crude extract, 375µL of starch solution and 375µL of 10mM phosphate buffer pH 7.5. After 10 min of incubation at 37\(^{\circ}\)C, 100µL of this mixture were added to 1mL of 3.5-dinitrosalicylic acid (DNSA) solution and kept in a boiling water bath for 10 min in order to stop the reaction. The absorbance was recorded at 570nm. A blank of substrate and another of enzyme were similarly prepared, except that 10mM phosphate buffer replaced substrate or enzymatic extract, respectively. All assays were carried out in quadruplicate. One unity of amylase activity was expressed as mg of maltose released at 37\(^{\circ}\)C per min per mg of protein.

Total proteinase activity was assayed using azocasein as substrate in a microcentrifuge tube. Triplicate samples of each enzyme extract (30µL) were incubated with 10gL\(^{-1}\) azocasein (50µL) dissolved in 0.1M Tris-HCl, pH 8.0, for 60 min at 25\(^{\circ}\)C (Bezerra, Lins et al., 2005). Following, 100gL\(^{-1}\) trichloroacetic acid (240µL) was added to stop the reaction and the mixture was centrifuged at 8000 xg for 5 min. The supernatant (70µL) was mixed with 1M NaOH (130µL) and absorbance was measured in a microtiter plate reader (Bio-Rad 680) at 450nm against a similarly prepared blank (0.1M Tris-HCl, pH 8.0, in place of the crude extract).
Protease activity was expressed as units per mg of protein. One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change in absorbance of 0.001 mL\(^{-1}\) min\(^{-1}\).

**The effect of pH and temperature on amylase activity**

The effects of pH and temperature on amylase activity were evaluated as described above, using a series of different 10mM buffers (Citrate-phosphate: pH 5 – 7.5; Tris-HCl: pH 7.5 – 9.0 and Glycine-NaOH: pH 9.0 – 11.5) and temperatures ranging from 25 to 70°C, respectively. Thermal stability was evaluated by assaying enzyme activity at 25°C after pre-incubation for 30 min at temperatures ranging from 25 to 70°C at 5 ºC intervals (Bezerra et al., 2005).

**Inhibition assays**

The effect of α-amylase inhibitor Type I (Sigma A1520) from *Triticum aestivum* on amylase activity was recorded. Equal volumes (60 µL) of enzyme extract and inhibitor in different concentrations (50 and 100 µg.mL\(^{-1}\)) were incubated at 25°C for 20 min. Following, 375 µL of 2% starch solution and 315 µL 10mM phosphate buffer pH 7.5 were added and the mixture was incubated for 10min at 37°C. The amylase activity was evaluated as previously described and the results expressed as percentage of the activity recorded in control tubes without inhibitor. Commercial α-amylase from *Bacillus subtilis* was also submitted to the inhibitor.

**Effect of ions**

The effect of metallic ions on amylase activity was evaluated according to method adapted from Souza et al. (2007). The methodology followed the same steps described in inhibition assays except that inhibitor was replaced by different ion solutions (1, 5 and 10mM). The ions employed were Ca\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\), Al\(^{3+}\), Cu\(^{2+}\) and Zn\(^{2+}\).

**Electrophoresis SDS-PAGE and zymograms**

Crude enzyme extracts from shrimps were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% (w/v) stacking gel and a 15% (w/v) separating gel (Laemmli, 1970). Enzyme preparations (150 µg of protein) and 10 µL of molecular weight marker (8–233kDa) were applied to tracks of a vertical electrophoresis device (BIO-RAD). The gel was stained for protein overnight in 1,8gL\(^{-1}\) Comassie Brilliant Blue R250 prepared in water, acid acetic and methanol (65:10:25) and the background of the gel was distained in the same solution without dye. Electrophoresis was performed at a constant current of 15mA per gel.
Substrate SDS-PAGE Zymograms were carried out following methodology described by Garcia-Carreño et al. (1993). Enzyme preparations (50 µg of protein) and 0.006 U of α-amylase of *Bacillus subtilis* (used as reference) were applied to a 13.5% (w/v) separating gel. After electrophoresis performed at 4 °C, the gel was immersed in 2.5% (100 mL) Triton X-100 in 0.1M Tris-HCl pH 8.0 for 30 min at 4 °C to remove the SDS. The Triton X-100 was removed by washing the gel three times with 100mL of 0.1M Tris-HCl buffer, pH 8.0. Then, the SDS-free, Triton X-100 free gel was incubated with starch solution (2% w/v) containing 10mM phosphate buffer pH 8.0 and 1mM CaCl$_2$ for 60min at 37 °C, to allow the digestion of starch by the active fractions. Finally, the gel was washed with distilled water and stained with an iodine/KI solution (10%) for 5 minutes. Gel was washed with distilled water to stop the reaction and then with an acetic acid solution (13%).

**Statistical analysis**

Data of enzyme activity were analyzed using one-way analysis of variance (ANOVA) complemented with Tukey’s test. Differences were reported as statistically significant when $P<0.05$, using the Jandel Scientific SigmaStat software program (version 2.0).

**Results**

Table 1 displays the proteolytic activity, amylase activity and amylase:protease ratio of the *Farfantepenaeus subtilis*, *Litopenaeus schmitti* and *L. vannamei* specimens. *F. subtilis* showed the greater protease activity ($p<0.05$) while *L. vannamei* the greater amylase activity.

Please, insert Table 1 here

Assays about the effects of pH on enzyme activity revealed that amylases of all species showed maximal activity at pH ranging from 7.0 to 8.0 (Fig. 1). Figure 2 displays the effect of temperature on amylase activity. The enzyme activity reached maximal values at 40 °C for *L. schmitti* and *L. vannamei* 11g, 45 °C for *F. subtilis*, and 50 °C for *L. vannamei* 25g, respectively. *F. subtilis* and *L. vannamei* presented residual activity upper to 85% from 30 to 55 °C, while for *L. schmitti* this range was narrower (35 to 45 °C). Thermal stability of amylases of the three species is displayed in Fig. 3. Enzymes of *L. vannamei* showed no remarkable change (residual activity $\geq$ 80%) up to 45 °C. Amylase activity of *L. schmitti* and *F. subtilis* increased even after the 30 min heat treatment up to 40 – 45 °C. Enzyme from *F. subtilis* showed great activity (75%) at 55 °C but all shrimps were extremely affected after this temperature.
A strong inhibition of amylase Type I inhibitor on enzyme activity was observed for *F. subtilis* and *L. schmitti* at concentrations of 50 and 100 µg inhibitor mL\(^{-1}\). Adult *L. vannamei* amylase retained 43.5±1.98 and 22.5±0.65 of its activity at the same concentrations, respectively. Commercial α-amylase of *Bacillus subtilis* showed 41.3±1.79 at 50µg.mL\(^{-1}\) and no activity at 100µg.mL\(^{-1}\).

The effect of ions on activity of amylases is displayed in Table 2. Enzymes of all species were activated by calcium chloride at 1mM but were inhibited at 5 and 10mM. All other ions showed inhibitory effect at any employed concentration.

Crude enzymatic extracts of the three species were studied through SDS-PAGE electrophoresis (Fig. 4). Adult *L. vannamei* showed 19 bands ranging from 8 to 118 kDa while *L. schmitti* and *F. subtilis* presented 11 (8 to 42kDa) and 13 bands (8 to 233kDa), respectively. Substrate zymograms revealed 9 bands with amylase activity for *F. subtilis*, 8 bands for *L. schmitti*, 10 bands for adult *L. vannamei* and 7 bands for juvenile *L. vannamei* (Fig. 5).

**Discussion**

Specific amylase activity of reared *L. vannamei* hepatopancreas was twice greater than that observed for wild *L. schmitti* and *L. subtilis*. The proteic profile of crude extract of the hepatopancreas shrimps also exhibited a great number of protein bands for reared *L. vannamei* when compared to wild species. In farmed conditions pond are fertilized for stimulating natural productivity and shrimps are commonly fed diets with 30-37% crude protein content. This fact, allied to probable genetic characteristic of species could explain the observed differences.

According to Johnston & Freeman (2005), digestive enzyme activity is closely related to dietary components. Thus, high proteinase, carbohydrase and lipase activity reflects a diet rich in protein, starch or cellulose and lipid, respectively (Johnston & Yellowlees, 1998; Johnston, 2003).

In the present work, the amylase:protease ratio were the same for *L. schmitti* and *L. subtilis* and twice higher for *L. vannamei*. Even though all shrimps species exhibited an omnivorous opportunistic habit, *F. subtilis* and *L. schmitti* show preference for animal protein (Dall *et al.*, 1990; Nunes and Parsons, 2000). Researches about digestive enzymes of aquatic organisms postulate that amylase activity depends on the natural diet of the species,
herbivorous and omnivorous animals presenting greater activity than carnivorous (Hidalgo et al., 1999; Jonhston & Freeman, 2005).

Crustacean hepatopancreas is responsible for synthesis of enzymes as well as food digestion and nutrient absorption. The synthesized enzymes are released into the midgut gland lumen and flows to stomach to initiate the hydrolysis of the ingested food (Guillaume & Ceccaldi, 1999; Verri et al., 2001). Dall & Moriarty (1983) and Bickmeyer et al. (2008) reported that pH in the gastric fluid and midgut gland of crustaceans ranges from 5.0 to 7.0 and from 4 to 5.5, respectively. Regardless the enzyme, if proteases (Fernández Gimenez et al., 2001; 2002), carbohydrates (Omondi & Stark, 1995; Figueiredo et al., 2001; Pavasovic et al., 2004) or lipases (López-López et al., 2003), crustacean digestive enzymes generally show optimum activity close to this range or at pH more alkaline as it was observed for amylases of the three species studied in the present work.

Amylase in the hepatopancreas of Farfantepenaeus subtilis, Litopenaeus schmitti and L. vannamei showed maximal activity at 40-50°C, as recorded for other crustaceans and fishes (Mayzaud, 1985; Fernández et al., 2001; Pavasovic et al., 2004). Despite the peak of amylase activity has been observed at this temperature range, enzyme from F. subtilis and L. vannamei maintained over 80% of its activity at 30-55°C. Enzyme from L. schmitti seemed to be more thermal sensitive since at 30 and 55°C, residual activity was about 40 and 60% respectively. Omondi & Stark (1995) studied the simultaneous effect of pH and temperature on amylase activity of Penaeus indicus and found an enhancement of about 50% at pH 6.8 when temperature increased from 22 to 37°C, while the inverse was recorded in P. vannamei.

Regarding thermal stability, amylase from F. subtilis were more resistant and maintained 75% of its activity at 55°C. At this temperature, none or very low activity was recorded for the other two species, suggesting that enzymes must have been denaturated.

The commercial inhibitor Type 1 from Triticum aestivum (Sigma A1520) has been used as α-amylase inhibitor of fishes (Fernández et al., 2001) and its effectiveness seems to change according to the species. The authors described inhibition rates ranging from “not detected” to 61% of α-amylases of five sparid fishes. Product information released by Sigma-Aldrich (product manufacturer) states that inhibition concentration of 200 and 50µg.mL-1 mL support inhibition rates from 40 to 60% against porcine and salivary human α-amylases, respectively. Shrimp α-amylase of the shrimp species herein studied seemed to be more sensitive. The enzymes of the wild species F. subtilis and L. schmitti were totally inhibited even at 50µg.mL-1 mL concentrations, while farmed species L. vannamei retained 43% of enzyme activity at the same concentration.

Some enzymes require other chemical groups besides their amino acid residues for catalytic activity. These groups should be complex organic or metallorganic molecules (coenzymes) or simply additional chemical component (cofactor) such as inorganic ions (Nelson
& Cox, 2005a). According to Wigglesworth & Griffith (1994), calcium ions are necessary to maintain the secondary and tertiary stability of \( \alpha \)-amylase molecules of penaeid shrimps. A number of metallic ions, such as \( \text{Ca}^{2+}, \text{Ba}^{2+}, \text{Ag}^{3+}, \text{Mn}^{2+}, \text{Hg}^{2+}, \text{Cu}^{2+} \) are important because their presence or absence may regulate enzyme activity (Dutta et al., 2006).

Amylases from the penaeid shrimps studied in the present work were strongly inhibited by \( \text{Zn}^{2+}, \text{Cd}^{2+}, \text{Hg}^{2+}, \text{Cu}^{2+} \) and \( \text{Al}^{3+} \) at 1, 5 or 10 mM concentrations. Calcium enhanced amylase activity only at 1 mM concentration. It seems that the effect of metallic ions on enzyme is species-specific since results in literature are sometimes diverse. While \( \text{Zn}^{2+} \) herein inhibited more than 50% of shrimp amylase activity, it had no effect on \text{Heliodiaptomus viidus}, a Crustacea Copepoda (Dutta et al., 2006). Figueiredo et al. (2001) reported an enhancement of 342% in amylase activity of redclaw crayfish \text{Cherax quadricarinatus} when enzyme was incubated with CaCl\(_2\) 15 mM. These studies are important because some ions are commonly included in mineral and vitaminic mixtures, what may interfere with food digestion.

Substrate SDS-PAGE zymogram is an interesting tool for analysis of differences in digestive enzymes of different species, allowing the identification of active enzyme forms. Herein, a great number of isoforms were identified for all shrimp species (\textit{L. vannamei} 25 g – 10; \textit{L. vannamei} 11 g – 7; \textit{L. schmitti} – 8 and \textit{F. subtilis} – 9). Polymorphism of \( \alpha \)-amylase is a common subject among vertebrates, insects and was already related for crustaceans (Van Wormhoudt & Favrel, 1988; Le Moullac et al., 1996; Perera et al., 2008), fishes (Fernández et al., 2001), mollusks (Huvet et al., 2008). Differences in number of crustacean isoforms have been associated with differences between populations or in gene expression during intermoult cycles. Some authors suggest that production of amylase is not related to food but is family-specific (Chakrabarti et al., 1995). Others suggest that amylase activity is at some extent influenced by diet composition. In fact, the ability for digesting different food items is related to the enzyme profile of a given species. The presence of numerous amylases isoforms may represent an ecological advantage and may indicate that species are able to better beneficiate from carbohydrate in the diets.

Acknowledgements

The authors would like to express their thanks to Mr. Otaviano Tavares da Costa, Rafael Padilha, Albérico Espírito Santo and João Virgínio for their technical assistance. This study was supported by Financiadora de Estudos e Projetos (FINEP/RECARCINE), Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Secretaria Especial de Aquicultura e Pesca – (SEAP/PR), Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq), Fundação de Apoio à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and Petróleo do Brasil S/A (PETROBRAS).
References


Figure legends

Figure 1: Effects of pH on amylase activity of *Litopenaeus vannamei* 25g (○), *L. vannamei* 11g (●), *L. schmitti* (□) and *Farfantepenaeus subtilis* (■) using starch as substrate. Values are shown in mean of six crude extracts obtained from three hepatopancreas each. Values were expressed as percentages of the highest activity (100%).

Figure 2: Effects of temperature on amylase activity of *Litopenaeus vannamei* 25g (○), *L. vannamei* 11g (●), *L. schmitti* (□) and *Farfantepenaeus subtilis* (■), using starch as substrate. Values are shown in mean of six crude extracts obtained from three hepatopancreas each. Values were expressed as percentages of the highest activity (100%).

Figure 3: Effects of thermal stability on amylase activity of *Litopenaeus vannamei* 25g (○), *L. vannamei* 11g (●), *L. schmitti* (□) and *Farfantepenaeus subtilis* (■), using starch as substrate. Values are shown in mean of six crude extracts obtained from three hepatopancreas each. Values were expressed as percentages of the highest (100%). Thermal stability was determined by assaying enzyme activity (25°C) after pre-incubation for 30 min at the indicated temperatures. Values were expressed as percentages of the highest activity (100%).

Figure 4: Electrophoresis (SDS-PAGE) from the midgut gland of *Farfantepenaeus subtilis*, *Litopenaeus schmitti*, *L. vannamei* 25g and *L. vannamei* 11g.

Figure 5: Zymogram of amylase activity from the midgut gland of *Farfantepenaeus subtilis*, *Litopenaeus schmitti*, *L. vannamei* 25g and *L. vannamei* 11g. α-amylase from *Bacillus subtilis* was used just as reference.
Table 1: Proteolytic and amilolytic activity in the hepatopancreas of *Farfantepenaeus subtilis*, *Litopenaeus schmitti* and *Litopenaeus vannamei*, using azocasein and starch as substrates. Amylase:protease ratio is also displayed.

<table>
<thead>
<tr>
<th></th>
<th><em>Farfantepenaeus subtilis</em></th>
<th><em>Litopenaeus schmitti</em></th>
<th><em>Litopenaeus vannamei</em> (11g)</th>
<th><em>Litopenaeus vannamei</em> (25g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic activity</td>
<td>6.26±0.67 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.60±0.75 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.47±0.15 &lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.38±0.71 &lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amylolytic activity</td>
<td>0.62±0.05 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.51±0.08 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.03±0.09 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.28±0.07 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amylase:protease ratio</td>
<td>0.10±0.01 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.12±0.03 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.19±0.02 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.24±0.02 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are shown as mean ± Standard Deviation (SD) of triplicates of six crude extracts obtained from three hepatopancreases each. Different italic letters (in lines) denotes statistical differences (p<0.05). Enzyme activity is expressed as U mg-1 of protein.
Table 2: Effect of different concentrations of ions (mM) on amylase activity of *Farfantepenaeus subtilis*, *Litopenaeus schmitti* and *L. vannamei*.

<table>
<thead>
<tr>
<th>Ions</th>
<th><em>F. subtilis</em></th>
<th></th>
<th><em>L. schmitti</em></th>
<th></th>
<th><em>L. vannamei</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>10</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1 +</td>
<td>1 -</td>
<td>2 -</td>
<td></td>
<td>2 +</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>3 -</td>
<td>4 -</td>
<td>4 -</td>
<td></td>
<td>4 -</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>1 -</td>
<td>3 -</td>
<td>3 -</td>
<td></td>
<td>2 -</td>
</tr>
<tr>
<td>Al³⁺</td>
<td>4 -</td>
<td>4 -</td>
<td>4 -</td>
<td></td>
<td>4 -</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>4 -</td>
<td>4 -</td>
<td>4 -</td>
<td></td>
<td>4 -</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>4 -</td>
<td>4 -</td>
<td>4 -</td>
<td></td>
<td>4 -</td>
</tr>
</tbody>
</table>

Activation: 1 + (1-25%); 2 + (26-50%)
Inhibition: 1 – (1-25%); 2 - (26-50%); 3 – (51-75%); 4 – (76-100%)
Figure 1
Figure 2
Amylase activity (%) vs Temperature (°C)

- L. vannamei 25g
- L. vannamei 11g
- L. schmitti
- F. subtilis

Figure 3
Figure 4
Figure 5
4.2  Artigo 2: Digestive peptidases and proteinases in the midgut gland of the pink shrimp *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeidae)
Digestive peptidases and proteinases in the midgut gland of the pink shrimp *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeidae)

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Abstract

Proteases from the midgut gland of the *Farfantepenaeus paulensis* juveniles were assessed. Enzyme activity was determined using protease substrates and inhibitors. The effect of pH, temperature and calcium on proteolytic activity was assayed. Caseinolytic activity was assayed in substrate-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Trypsin, chymotrypsin and leucine aminopeptidase activity was detected. Proteolytic activity was strongly inhibited by the specific trypsin inhibitors, Tosyl-phenylalanine chloromethylketone inhibited 99.1% of chymotrypsin activity. The greatest trypsin-like activity occurred at pH 8.0 and 45 °C. Chymotrypsin-like activity reached maximal values at alkaline pH (7.2-9.0) and 55 °C. CaCl₂ did not increase trypsin-like activity, but rather inhibited it at concentrations of 30 (20%) and 50 (20%) and 100 mM (50%). The substrate-SDS-PAGE zymogram revealed eight proteinase bands. Two possibly thermal-resistant (85 °C, 30min) chymotrypsin isoenzymes were found, which were inhibited by phenyl-methyl-sulphonyl fluoride. Aminopeptidase activity of enzyme extracts (Arg, Lys, Gln, Phe and Val) and the recommended concentrations of these essential amino acids in penaeid shrimp diets were positively correlated (P<0.05). Because protein digestion involves the combined action of different enzymes, adequate knowledge of shrimp digestion and enzyme characteristics is required for the assessment of the digestive potential of different feed sources and development of in vitro digestibility protocols.

Keywords: trypsin, chymotrypsin, aminopeptidase, protein digestion, substrate-SDS-PAGE, *Farfantepenaeus paulensis* subtilis

Introduction

Shrimp use energy and monomers obtained from their diet to synthesize the molecules needed for growth, survival, reproduction, tissue repair and defense. Ingested food is subjected to enzymes that break it down into compounds, which are absorbed by cells in the gut (Shih 1998; Gordoa-Muñoz, García-Carrero & Navarrete del Toro 2003).

Digestion is a rather well-studied subject in the field of shrimp nutrition (Fernández-Gimenez, Garcia-Carrero, Navarrete del Toro & Fenacci 2001), and is mainly focussed on enzyme properties that help determine digestive capabilities (Vega-Vilasante, Nolasco & Civera 1995). An understanding of digestive enzymes is important for the rational use of feed resources in shrimp farming (Fernández, Oliva, Carrillo & Van Wormhoudt 1997; Muhlau-Almazán, García-García, Sánchez-Parra, Pérez-Ploescuencio & Peregirino-Unarre 2005). Proteases are the most assessed digestive enzymes in crustaceans and play a key role in the overall assimilation of nutrients (Fernández-Gimenez, García-Carrero, Navarrete del Toro, 2003).

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Toro & Fennaci 2002). These enzymes are also very important for the metabolism and growth of penaeids due to their fundamental role in providing essential amino acids. Thus, the release of essential amino acids may be dependent on the effective hydrolysis of proteins by digestive proteases (Sánchez-Paz, García-Correio, Mahlia Almazán, Hernandez-Saavedra & Yepiz-Plascencia 2003).

The pink shrimp *Farfantepenaeus paulensis* is a highly valued fishery resource on the southern coast of Brazil (Peixoto, Soares, Wasielewski, Cavalli & Jensen 2004). Reproduction and hatchery techniques are rather well established for seed production (Vinaterra & Andreatto 1997). The species is considered to be a potential alternative for the currently cultured species *Litopenaeus vannamei* in sub-tropical and temperate areas. However, *F. paulensis* farming has been restricted by a lack of information on nutrition and feeds for sustaining suitable growth at the commercial level (Lemos, Navarrete del Toro, Córdova-Martueza & García-Correio 2004). The aim of the present study was to assess the basic functional characteristics of proteases in the midgut gland of *F. paulensis* juveniles, which is essential information for further application of enzymes in nutritional studies.

Materials and methods

Materials

All reagents were of analytical grade and purchased from Sigma (St Louis, MO, USA) and Merck (Darmstadt, Germany).

Organisms and sampling

*Farfantepenaeus paulensis* postlarvae were obtained from a public hatchery and reared for 190 days until reaching 6–10 g of live weight. The shrimp were raised at a temperature of 24–28 °C and fed a commercial feed with 35% crude protein. Following sacrifice, midgut glands were excised from healthy individuals predominantly in intermolt (Dall, Hill, Rothlisberg & Staples 1990). The glands (0.12–0.2 g wet weight) were pooled and immediately stored in liquid nitrogen (−190 °C). Recovery of enzyme extracts began with homogenization of the midgut glands (40 mg/mL) in chilled 0.15 M NaCl using a Potter–Elvehjem tissue homogenizer (Bodine Electric, Chicago, IL, USA) at 40–50 rpm for 3 min at 4 °C. Crude enzyme extracts were obtained from superna-

tants after centrifugation at 10000 × g for 25 min at 4 °C and lipid removal, followed by storage at −20 °C for later use. The total soluble protein content of the enzyme extracts was determined using bovine serum albumin as the standard protein (Bradford 1976).

Enzyme activity assays

The total protease activity was assayed through hydrolysis of 1% azocasein dissolved in 0.1 M Tris-HCl buffer, pH 8.0 (Garcia-Correio 1992). Triplicate samples (30 μL) of enzyme extract were incubated with substrate solution (30 μL) for 60 min at 25 °C in a microtube (Brezza, Lins, Alencar, Paiva, Chavez, Coelho & Carvalho Jr 2005). The reaction was stopped with the addition of 10% trichloroacetic acid (240 μL) and the mixture was centrifuged at 8000 × g for 5 min. Unhydrolysed substrate was sedimented and the supernatant was recovered and then further mixed (70 μL) with 1 M NaOH (130 μL). The absorbance of supernatants was measured in a microtitre plate reader (Bio-Rad 680, Japan) at 490 nm. Changes in absorbance over time were calculated by the difference from reactions stopped at zero (blank controls) and after 10 min. One unit of total protease activity was expressed as the amount of enzyme required to hydrolyse azocasein and produce a 0.001 change in absorbance per millilitre per minute. Specific protease activity was expressed in units per milligram of protein.

Trypsin activity was determined using benzoyl-

**nitropeptidase** activity was determined using l-arginine-**p*-nitroanilide (BAPNA). Chymotrypsin activity was measured using either succinyl-alanine-alanine-p-nitroanilide (SAPPN) or N-succinyl-l-phenylalanine-p-nitroanilide (Suc-Phe-p-Na) as specific substrates. Leucine aminopeptidase activity was detected using leucine-**p*-nitroanilide (Leu-p-Na). All substrates were dissolved in dimethylsulphoxide (DMSO) to a final concentration of 0.6 mM (Brezza et al. 2005). The change in absorbance at 405 nm was recorded for 15 min using a microtitre reader (Bio-Rad 680). One unit of activity was defined as the amount of enzyme required to produce 1 μmol of **p*-nitroaniline per minute (ε = 9400 M−1 cm−1). Specific activity was expressed as units per milligram of protein.

Aminopeptidase activity was also evaluated using aminocaproyl **β*-naphthylamide (AANA) with the following substrates: Arg, Leu, Phe, Val and Lys. The procedure adapted from Oliveira, Freitas Jr and Alves (1999) was carried out by incubating 4.2 mM

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substrate (50 µL), 50 mM sodium phosphate buffer, pH 7.0 (600 µL) and dionized H₂O (50 µL) at 37 °C. After temperature equilibration, the enzyme extract (50 µL) was added. After 120 min, the reaction was stopped by adding 1 mg mL⁻¹ fresh Garnet reagent (250 µL) prepared in 0.2 M sodium acetate buffer, pH 4.2, containing 10% v/v Tween 20. Absorbance was measured at 525 nm and the amount of β-naphthylamine was determined using a standard β-naphthylamine curve. Activity was expressed as protease milliunits per milligram of protein. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1 µmol β-naphthylamine/min⁻¹.

**Inhibition assays**

To determine the effect of specific inhibitors on protease activity, equal volumes (25 µL) of crude extract and inhibitor were incubated for 30 min at 25 °C before the determination of residual activity. Phenyl-methyl-sulphonyl-fluoride (PMSF) was used as an inhibitor for serine proteases; tosyl-lysine chloromethyl ketone (TLCK) and benzamidine were used as inhibitors for trypsin; and tosyl-phenylalnine chloromethyl ketone (TPCK) was used as an inhibitor for chymotrypsin. Volumes were then adjusted to 170 µL with 0.1 M Tris-HCl buffer, pH 8.0, and the respective substrate (30 µL). Trypsin assays: BAPNA with PMSF, TLCK or benzamidine. Chymotrypsin assays: SAPNA with PMSF and TPCK. All inhibitors were prepared in DMSO to a final concentration of 1 mM (Bezerra, Santos, Paiva, Coelho, Vieira & Curvalho. Jr 2001). The change in absorbance at 405 nm was recorded for 15 min using a microtitre reader (Bio-Rad 680). Inhibitory effects on enzyme activity were expressed in relation to controls (without specific inhibitors).

**The effects of pH, temperature and concentration of calcium on enzyme activity**

To evaluate the effects of pH and temperature on proteolytic activity, crude extracts were assayed with Tris-HCl buffer, with pH ranging from 7.2 to 9.0 and temperature ranging from 25 to 85 °C. Enzyme activity was determined as described above, using specific substrates. Thermal stability was evaluated by recording enzyme activity at 25 °C after pre-incubation for 30 min at temperatures ranging from 25 to 95 °C.

The effect of calcium on trypsin activity of the midgut gland extract was determined. CaCl₂ was added to the standard reaction assay to obtain final concentrations ranging from 1 to 100 mM. Residual activity was determined as described above, using BAPNA as the substrate. The effect on enzyme activity was expressed in relation to the control (without CaCl₂).

**Enzyme characterization in substrate-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Zymograms were prepared according to Garcia-carreno, Dimes and Haard (1993). Crude enzyme extracts were dialysed and loaded onto gels at 10 µg protein per lane, corresponding to 60–80 U of enzyme activity following electrophoresis of crude extracts in SDS-PAGE (12% acrylamide, 0.1% SDS), gels were immersed in 0.1 M buffer Tris–HCl, pH 8.0 containing 2.5% Triton X-100 (100 mL) for 30 min at 4 °C to remove the SDS. The Triton X-100 buffer was removed by washing the gels three times with 100 mL of 0.1 M Tris-HCl buffer, pH 8.0. The SDS-free and Triton X-100-free gels were then incubated with 100 mL of 3% casein (w/v) in 0.1 M Tris-HCl buffer, pH 8.0, for 30 min at 4 °C. The temperature was raised to 25 °C and maintained for 90 min to allow the digestion of casein by active enzyme fractions. Gels were then stained with 0.18% (w/v) Coomassie brilliant blue and destained in 10% (v/v) acetic acid and 25% (v/v) methanol. For the determination of enzyme thermal stability in the zymogram, samples of enzyme extract were incubated for 30 min at temperatures ranging from 25 to 85 °C before loading onto the gel. For the enzyme inhibition zymogram, enzyme extracts were pre-incubated with specific inhibitors of serine proteases, trypsin and chymotrypsin, as described above (inhibition assays). These enzyme inhibition zymograms were compared with those with no inhibitor (control).

**Statistics**

Data (mean ± standard deviation) were processed using the MICROCAL OLIPIAN (A) software program. Differences between means (effect of CaCl₂) were analysed using one-way ANOVA followed by Tukey’s multi-comparison test and considered to be significant when P<0.05 (Zar 1984).
Table 1 Proteolytic activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles using different substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Enzyme activity (mU mg⁻¹ protein ± SD; n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azocasein</td>
<td>6.49 ± 0.20</td>
</tr>
<tr>
<td>BAPNA</td>
<td>5.13 ± 0.56</td>
</tr>
<tr>
<td>SAPNA</td>
<td>12.20 ± 1.29</td>
</tr>
<tr>
<td>Suc-Phe-p-Nan</td>
<td>Not detected</td>
</tr>
<tr>
<td>Leu-p-Nan</td>
<td>0.20 ± 0.02</td>
</tr>
</tbody>
</table>

BAPNA: benzoyl arginine p-nitroanilide; SAPNA, succinylalalnine-alamine-proline-phenylalanine-p-nitroanilide; Suc-Phe-p-Nan: N-succinyl-L-phenylalanine-p-nitroanilide; Leu-p-Nan: leucine p-nitroanilide.

Table 2 Effect of specific inhibitors on protease activity in midgut gland extracts from *Farfantepenaeus paulensis* juveniles

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Activity inhibition (% SD; n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSE</td>
<td>37.3 ± 0.43</td>
</tr>
<tr>
<td>PMSE</td>
<td>27.1 ± 0.94</td>
</tr>
<tr>
<td>TLCK</td>
<td>85.3 ± 0.06</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>89.9 ± 0.14</td>
</tr>
<tr>
<td>TPCK</td>
<td>89.3 ± 0.60</td>
</tr>
</tbody>
</table>

Maximal specific proteolytic activity (100%) was 5.46 mU mg⁻¹ protein and 11.65 mU mg⁻¹ using BAPNA and SAPNA as substrates, respectively.

*PMSF inhibition using BAPNA as substrate.
**PMSF inhibition using SAPNA as substrate.
PMSF: phenylmethylsulphonyl fluoride; serine protease inhibitor; TLCK: tosyl l-lysine chloromethyl ketone, trypsin inhibitor; TPCK: tosyl phenylalalnine chloromethylketone, chymotrypsin inhibitor; BAPNA, benzoylarginine p-nitroanilide; SAPNA, succinylalalnine-alamine-proline-phenylalanine-p-nitroanilide.

Results

Table 1 displays the activity of digestive proteases in *F. paulensis*. Trypsin-like activity (BAPNA) was observed in midgut gland extracts whereas no activity was detected using Suc-Phe-p-Nan as the specific chymotrypsin substrate. However, enzyme activity was verified with the SAPNA chymotrypsin substrate, which contains more than one amino acid. Table 2 displays the effects of four different inhibitors on digestive proteases in the midgut gland of *F. paulensis*. Proteolytic activity using BAPNA was 37.3% and was inhibited by PMSE, which is a serine protease inhibitor. Tosyl-lysine chloromethyl ketone and benzamidine (both synthetic trypsin inhibitors) demonstrated a strong inhibitory effect (85.1% and 89.9% respectively) using the same substrate. Phenylmethylsulphonyl-fluoride was also capable of inhibiting chymotrypsin activity by 27.1%, as determined by SAPNA hydrolysis. Proteolytic activity was also inhibited by TLCK (50.3%), which is a specific bovine chymotrypsin inhibitor, using SAPNA as the substrate. Activity with the Leu-p-Nan substrate revealed the presence of leucine aminopeptidase-like enzymes (Table 1). Furthermore, plotting aminopeptidase activity using specific AA-NA (Arg, Leu, Lys, Phe and Val β-naphthylamide) versus recommended concentrations of respective essential amino acids in penaeid shrimp diets resulted in a significant positive correlation (*P* ≤ 0.05) (Fig. 1).

The effect of pH on enzyme activity revealed the most trypsin-like activity at pH values ranging from 8.0 to 9.0 (Fig. 2a), whereas chymotrypsin-like activity was greatest at a slightly lower pH range (7.5–8.0) (Fig. 2b). Temperature exerted pronounced effects on the proteolytic activity of *F. paulensis* enzyme extracts (Fig. 3). Enzyme activity reached maximal values at 45 °C (A) and 55 °C (B) in assays with BAPNA (trypsin-like activity) and SAPNA (chymotrypsin-like activity) respectively. Trypsin-like activity was ≥ 60% of maximal activity between 25 and 65 °C, whereas chymotrypsin-like activity was ≥ 60% of maximal activity between 45 and 65 °C. Figure 4a shows the influence of heat treatment for 30 min on trypsin-like stability. The activity exhibited no marked change up to 35 °C, but was drastically re-
duced after 45 °C, with nearly no activity detected at temperatures above 55 °C. Chymotrypsin-like activity demonstrated lesser stability in comparison with trypsin (Fig. 4b). However, overall higher thermal stability was noticed at temperatures between 5 and 65 °C. No significant difference (\( P > 0.05 \)) was observed in trypsin activity between 1 and 10 mM CaCl\(_2\). At these concentrations, activity remained between 80% and 110% of controls. Decreasing activity was recorded at concentrations of 30, 50 and 100 mM (\( P < 0.05 \)) (Fig. 5).

The caseinolytic activity of F. puellaads midgut gland extracts revealed eight active bands in substrate-SDS-PAGE at 25 °C (Fig. 6). The activity of heat-treated extracts (35–55 °C) exhibited a band pattern similar to the control (25 °C). However, there was a less intense band at 55 °C (Fig. 6, lane 4, white dashed arrow). Surprisingly, two bands remained active at temperatures up to 85 °C (Fig. 6, white arrow). Enzyme extracts demonstrated specific inhibition in substrate-SDS-PAGE (Fig. 7). Proteolytic bands were strongly or slightly inhibited by PMSE, with the exception of bands with a lower molecular weight (Fig. 7, lane 2, double white arrow). Tosyl-lysine chloromethyl ketone (Fig. 7, lane 3) and benzamidine (Fig. 7, lane 4) were responsible for inhibiting five and three proteolytic bands, respectively. The band with the lowest molecular weight was only inhibited by TLCK (Fig. 7, lane 3). In contrast, TLCK was unable to inhibit a band previously affected by PMSE (Fig. 7).
Figure 4 Thermal stability of proteolytic activity in midgut gland extracts from *F. patens* juvenile. CaCl₂ and NaCl were added for final concentrations of 1–300 mM. Proteolytic activity was determined using BAPNA as substrate. Values expressed as mean ± SD (n = 5). BAPNA, benzoyl arginine p-nitroanilide; SAPNA, succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide.

Figure 5 Effect of CaCl₂ concentration on tryptic activity in midgut gland extracts from *F. patens* juveniles. CaCl₂ and NaCl were added for final concentrations of 1–100 mM. Trypsin activity was determined using BAPNA as substrate. Values expressed as mean ± SD (n = 5). BAPNA, benzoyl arginine p-nitroanilide.

Figure 6 Thermal stability of digestive proteinases in midgut gland extracts from *F. patens* juvenile. Proteinase activity after incubating crude extract for 30 min at each respective temperature. White dashed arrow: less intense bands at 55°C. White arrow: thermoresistant bands at 85°C. Blue arrow: Thermoresistant bands at 55°C. Before electrophoresis the samples were previously dialyzed. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Discussion

Studies on digestive physiology and enzyme activity provide important basic knowledge for the assessment of nutritional status in farmed shrimp (Jones, Kumlu, LeVay & Fletcher 1995). The digestion of feed tested in vitro with specific enzyme extracts may indicate differences in the digestive potential of feed.
Figure 7 Inhibition of protease activity in midgut gland extracts from *Eirfanophyton paulensis* juveniles in substrate-SDS-PAGE using specific inhibitors. Lanes: 1 – control (without inhibitors); 2 – PMSE; 3 – TLCK; 4 – Ben- zamidine; 5 – TPCK. Thermoresistant bands inhibited by PMSE and TPCK (white dashed arrows). Band inhibited by PMSE but not by TLCK (white arrow). Band with the lowest molecular weight not inhibited by PMSE (double white arrow). Before electrophoresis the samples were previously dialysed. TLCK, tosyl-phenylalanine chloromethyl ketone; PMSE, phenylmethyl-sulphonyl-fluoride; TLCK, tosylyl lysine chloromethyl ketone; SDE-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

between species (Lemos, Ezquerro, & García-Carreño, 2000; Lemos et al., 2004). Most digestive proteases from decapods are reported to be serine proteases (more recently called serine endopeptidases), including trypsin and chymotrypsin, which seem to be the most important crustacean digestive enzymes (García-Carreño, Hernández-Cortés, & Haard, 1994; Fernández et al., 1997). Trypsin- and chymotrypsin-like enzyme activity has been reported previously throughout the ontogenetic development of *E. paulensis* (Lemos, Hernández-Cortés, Navarrete del Toro, Garcia-Carreño, & Phan, 1999). The present study indicates that the midgut gland of *E. paulensis* juveniles contains trypsin, chymotrypsin and aminopeptidases. The presence of proteinases and peptidases in the same compartment is an important adaptive advantage. In fact, it makes the protein digestion more efficient. Following hydrolysis of proteins by proteases into long-chained peptides, peptidases such as aminopeptidases further degrade it into smaller peptides and free amino acids, thus enhancing the absorption of these nutrients. It is also important to note that a better understanding of the digestive physiology of this species is essential for an adequate shrimp feed formulation, a central topic in aquaculture. Also, a misplanned dietary management may lead to excessive feed loss and metabolic excretion, therefore generating environmental pollution.

Considering the inhibition of trypsin and chymotrypsin for protein digestion in penaeid shrimp, these findings may have nutritional relevance regarding the use of less complex molecules such as protein hydrolysates (Zambonino-Infante & Calu, 2007). Proteases from the midgut gland of *E. paulensis* exhibited properties from the serine class. These proteases were inhibited by PMSE, which is consistent with the presence of serine and histidine residues at the catalytic site (Salin, García-Carreño, & Hernández-Cortés, 2004) and agrees with previous findings on the inhibition of decapod crustacean serine proteases by PMSE (Lemos, García-Carreño, Hernández & Navarrete del Toro, 2002). The inhibition of three trypsin isoforms from the Pacific white shrimp *L. vannamei* has been reported with TLCK and benzamidine when BAPNA was used as the substrate (Salin et al., 2004). Despite inhibiting 68% of the chymotrypsin activity in *E. paulensis* in the present study, TPCK has not proven effective on chymotrypsin activity in other penaeid species (Tsai, Liu, & Chung, 1986; García-Carreño et al., 1994; Lemos et al., 1999).

The greatest trypsin-like activity in *E. paulensis* was found in the pH range 8.0–9.0 for the hydrolysis of BAPNA, thereby indicating a slightly higher optimal pH for the species (Jang, Moody, & Chen, 1991) found optimal pH values of 7.0–8.0 for three trypsins from the midgut gland of *Penaeus monodon* using p-toluenesulphonyl-L-arginine methyl ester as substrate. The highest hydrolysis of trypsin in other decapods, such as crayfish, has also been found to be at pH values between 7.0 and 8.0 (Dionysius, Hoek, Milne, & Slattery, 1993). The highest chymotrypsin-like activity in *E. paulensis* at pH values between 7.5 and 8.0 are also in contrast to values of around 7.0 and between 8.0 and 8.5 reported for this enzyme in the gastric fluid of the marine crab *Cancer pagurus* (Saborowski, Sahling, Navarrete del Toro, Walter, & García-Carreño, 2004) and the gut of *Diphipus* (Ebert, Agiawul, Gebauer, Jareusch, Bauer, & Zitz, 2004) respectively. In view of such specificities in enzyme functioning, the determination of the optimal pH is fundamental to the assessment of the digestive capacity of different feeds (e.g. degree of protein hydrolysis) in assays considering the relationship between
peptide bond breakage and changes in pH values (Enquerra, García-Carreño, Čivera & Haard 1997). Trypsin-like activity exhibited the greatest BAPNA hydrolysis at 45°C, which is a lower optimal temperature than that found for other crustacean species, such as the digestive tract of P. monodon (55-65°C) (Jiang et al. 1991); L. vannamei (50°C) (Sainz et al. 2004); and Triops sp. (50-60°C) (Mendoza-Martínez, Obregón-Barboza, Navarrete del Toro, Obregón-Barboza & García-Carreño 2000). The thermostability of trypsin in F. paedensis at temperatures higher than 45°C contrasts trypsin from C. pagurus, which is reported to retain 70% of its initial activity after 60 min at 50°C (Saborowski et al. 2004). On the other hand, chymotrypsin in F. paedensis had the highest activity at 55°C, but lost 75% of this activity after incubation at this temperature for 30 min. Although the literature reports chymotrypsin-like activity in the digestive system of shrimp (Vega-Villanueva et al. 1998; Fernandez et al. 1997; Fernandez-Gaume et al. 2003; Cervera-Morente, García-Carreño & Navarrete del Toro 2004), information on its physicochemical characterization remains scarce. A similar study has reported chymotrypsin activity in the gastric fluid of C. pagurus extinguished after incubation at 60°C for 20 min (Saborowski et al. 2004).

Thermal effects on proteolytic enzymes were also determined in substrate gel electrophoresis. All bands remained active up to a pre-treatment of 55°C, although the enzymatic tube assay of proteolytic activity was reduced at this temperature. Indeed, the zymogram method seems to be more sensitive than the quantitative assay when using soluble substrates (Lemos et al. 2000). The fact that only one caseolidetic band (with the lowest molecular weight) was not inhibited by PMSF indicates that most of the proteases belong to the serine class. The protease with the lowest molecular weight in F. paedensis was inhibited by TLCK, which is a specific trypsin inhibitor, but was not inhibited by benzamidane, which is also a trypsin inhibitor. This suggests that its activity centre may not be homologous to the mammal trypsin (benzamidane-sensitive). On the other hand, a caseolidetic band (Fig. 7, lane 3, white arrow) that was inhibited by PMSF and benzamidane, but not by TLCK, possibly represents a trypsin-like enzyme. It is noteworthy that two bands (Fig. 7, lane 2, white dashed arrow) were only inhibited by PMSF. By exclusion, this finding suggests the presence of a thermal-stable chymotrypsin. Through inhibition by PMSF, chymotrypsin isoforms have been reported previously in early and juvenile stages of F. paedensis (Lemos et al. 1999).

Calcium chloride is used in different concentrations as an enzyme stabilization factor for the determination of trypsin and chymotrypsin activity in protocols developed for mammal enzymes (Erlander, Kokowsky & Cohen 1964). Trypsin activity in F. paedensis seemed to be sensitive to concentrations usually used in conventional assays (>20 mM CaCl2), exhibiting significantly reduced enzyme activity. On the other hand, although the effect of certain calcium concentrations on enzyme activity may be non-significant, the hydrolytic potential of the activity of standardized enzyme extracts may be improved at some Ca2+ concentrations, as observed in assays for protein hydrolysis (Pedersen & Eggum 1983). This may be particularly relevant to the formulation of shrimp feed, considering the variety of ingredients with different mineral contents and their potential effects on the digestive capacity in shrimp (Lemos 2004).

The significant correlation found between aminopeptidase activity (Arg, Leu, Lys, Phe and Val) and the recommended concentrations of these essential amino acids in shrimp diets (Guillaume 1997) possibly indicates a physiological response to protein hydrolysis based on the required concentrations of essential amino acids. To some extent, the data from the present study corroborate reported recommended concentrations for shrimp, with arginine and lysine requiring relatively high concentrations (Fox, Lawrence & Li-Chan 1995). Thus, increased hydrolytic efficiency is dependent on the composition of essential amino acids in commercial shrimp diets (Lemos & Nunes 2008). Further determination of aminopeptidase activity with additional essential AA-NA substrates (e.g. methionine, cystine and threonine) may contribute towards an understanding of the relationships between digestive enzyme activity, hydrolytic potential and diet composition in terms of nutrient requirements.

Enzyme technology has been useful in the development of specific in vitro methods for the quality control of diets and ingredients in the livestock feed industry (Fuller 1991). However, the different nature in the functioning, affinity and catalytic performance of enzymes from terrestrial versus aquatic animals entails the development of methods based on specific enzymatic digestion features (Dimes, Haard, Dong, Rasco, Forster, Fairgrieve, Arndt, Hardy, Barrows & Higgs 1994). The set-up of in vitro assay conditions for proper hydrolysis is mostly dependent on basic
conditions such as pH and temperature. Thus, the characterization of the functional properties of digestive enzymes in aquatic animals is a necessary starting point for further use of enzyme extracts as tools in the search for the proper nutrient sources in aquaculture.

Acknowledgments

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4.3 Artigo 3: Digestive proteinases and peptidases in the hepatopancreas of the southern brown shrimp (*Farfantepenaeus subtilis*) in two sub-adult stages
Digestive proteinases and peptidases in the hepatopancreas of the southern brown shrimp (*Farfantepenaeus subtilis*) in two sub-adult stages

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Abstract

The aim of this study was to examine proteinases and peptidases from the hepatopancreas of two sub-adult stages of *Farfantepenaeus subtilis*: SAS₁ (5.93 ± 0.69 g wet weight) and SAS₂ (13.26 ± 0.60 g wet weight). Trypsin and chymotrypsin activity was higher in the extract from the SAS₁ individuals (*P* < 0.05). The highest activity among aminoacyl-β-naphthylamide substrates was found using alanine-, arginine-, leucine- and lysine-β-naphthylamide. There was a positive correlation between the recommended concentration of essential amino acids in penaeid shrimp feed and aminopeptidase activity in both sub-adult stages. Proteolytic activity of *F. subtilis* was strongly inhibited by specific trypsin inhibitors. The optimal temperature for trypsin, chymotrypsin and leucine aminopeptidase activity was between 45 and 55 °C. Six and seven bands were found in caseinolytic zymograms for SAS₁ and SAS₂, respectively. All bands were inhibited by phenylmethyldesulfonyl fluoride in both sub-adult stages. The use of tosyl-lysine-chloromethyl-ketone and benzamidine caused strong inhibition of the proteolytic bands. Trypsin and chymotrypsin activity was the main difference observed between the protease pattern of SAS₁ and SAS₂ *F. subtilis*.

**KEY WORDS**: aminopeptidases, chymotrypsin, digestive enzymes, *Farfantepenaeus subtilis*, southern brown shrimp, trypsin

Introduction

The southern brown shrimp, *Farfantepenaeus subtilis*, is native to the Atlantic coast of Central and South America, from Cuba down to Rio de Janeiro, and was one of the first species to be farmed in Brazil, along with *Farfantepenaeus brasiliensis*, *Farfantepenaeus paulensis* and *Litopenaeus schmittii*. The southern brown shrimp exhibits benthic omnivorous opportunistic feeding habits under semi-intensive conditions, although polychaetes and calanoid copepods seem to be favoured during all growth stages ( Nunes & Parsons 2000).

Despite its farming potential and attractive market features, the culture of *F. subtilis* in semi-intensive conditions in Brazil has failed mainly due to low yields. Studies carried out by Brazilian farmers report a food conversion ratio ranging from 2.88 to 3.44 and an atypical growth performance, thus generating low productivity. The growth rate slows after the shrimp reach 6 g of body weight. This suggests that the poor results may be related to nutritional problems and ontogenetic changes in the digestive enzyme metabolism (Maia & Nunes 2003).

Comprehension of digestion physiology and nutrient digestibility remains a problem for the culture of *F. subtilis*. Knowledge concerning the digestive system of this species can provide information applicable to food utilization. Thus, the identification and characterization of digestive enzymes during shrimp growth is an important step towards understanding the digestive mechanisms and formulating diets that promote better growth responses, as feed can be designed according to the digestion capacity ( López-Lopez et al. 2005).

A number of studies have indicated properties of digestive enzymes in shrimp and other crustaceans, such as proteases, carbohydrates, lipases and the digestibility of
feed ingredients (Lemos et al. 2000, 2004; Córdova-Murucutu et al. 2003; Mathia-Almazán et al. 2003; Gaxiola et al. 2005; López-López et al. 2005). However, synthesis regulation and enzyme activity are species-specific (Fernández Giménez et al. 2002) and it is therefore not possible to extrapolate characteristics from one species to another. This study describes the investigation of proteases and peptidases as well as certain properties of these digestive proteases from the hepatopancreas of the southern brown shrimp, *F. subtilis*, in two sub-adult stages. These findings provide basic information on protein digestion and will be useful in further nutritional research.

**Materials and methods**

**Materials**

*Farfantepenaeus subtilis* specimens were obtained from a commercial fishery on the coast of Barra de Sirinhaem (8°36'S; 35°11'W), 100 km from the city of Recife in the state of Pernambuco, Brazil. All reagents used in the enzyme assays were of analytical grade, purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

**Enzyme extraction and determination of total soluble protein**

The specimens were transported alive to the Enzymology Laboratory of the Universidade Federal de Pernambuco. Sixty-litre plastic bags were used for temporary storage at a density of two specimens per bag in 12 L of salt water. The water was saturated with oxygen and a ratio of one-third water to two-thirds oxygen. The shrimp were classified in two sub-adult stages: SAS, (approximately 6 g of wet weight) and SAS1 (approximately 13 g of wet weight). The hepatopancreas from 20 SAS, (5.93 ± 0.69 g wet weight) and 20 SAS1 (13.26 ± 0.60 g wet weight) specimens were dissected immediately after killing by decapitation. For each sub-adult stage, four sets of five hepatopancreases were homogenized (40 mg mL⁻¹) in chilled 0.15 M NaCl, using a Potter-Elvehjem tissue homogenizer (Bodine Electric Company, Chicago, IL, USA) at 30–50 rpm for 3 min at 4 °C. Homogenates were centrifuged at 10 000 g for 25 min at 4 °C to remove lipid and tissue debris, and supernatants (crude enzyme extract) were stored at −20 °C for further use. The total soluble protein was determined following the procedure described by Bradford (1976), using bovine serum albumin as the standard protein.

**Non-specific enzyme assays**

Non-specific proteolytic activity was assayed using azocasein as substrate in a microcentrifuge tube. Triplicate samples of each enzyme extract (30 μL) were incubated with 10 g L⁻¹ azocasein (50 μL) dissolved in 0.1 m Tris-HCl, pH 8.0, for 60 min at 25 °C (Bezerra et al. 2005). Next, 100 g L⁻¹ trichloroacetic acid (120 μL) was added to stop the reaction and the mixture was centrifuged at 9000 g for 5 min. The supernatant (70 μL) was mixed with 1 m NaOH (130 μL) and absorbance was measured in a microplate reader (Bio-Rad 680) at 450 nm against a similarly prepared blank (with 9 g L⁻¹ of NaCl in place of the crude extract). Precautions demonstrated that, for the first 60 min, the reaction carried out under the above-mentioned conditions follows first-order kinetics. Protease activity was expressed as units per mg of protein. One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change in absorbance of 0.001 mL⁻¹ min⁻¹.

**Specific enzyme assays**

Trypsin, chymotrypsin and leucine aminopeptidase activity was determined in a 96-well microtiter plate, using benzoyl-DL-arginine-p-nitroanilide (BAPNA), succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAPNA) and leucine-p-nitroanilide (Leu-p-Nan) as specific substrates respectively (Bezerra et al. 2005). Triplicate samples of enzyme extracts (30 μL) were incubated with either 4 mm of BAPNA, SAPNA or Leu-p-Nan (30 μL) dissolved in dimethylsulphoxide (DMSO) and 0.1 m Tris-HCl (140 μL), pH 8.0. The reactions occurred at 25 °C for 15 min and were recorded at 405 nm using a microplate reader. Activity was expressed as protease mU mg⁻¹ of protein. One unit (U) of activity was defined as the amount of enzyme required to produce 1 μmol p-nitroaniline min⁻¹.

Aminopeptidase activity was also evaluated using aminoacyl-β-naphthylamide as substrate. The substrates used were Ala, Arg, Leu, Phe, Val, Ser, Glu, Ile, Tyr, His, Lys and Gln. The procedure was carried out by incubating 4.2 mm of substrate (50 μL), 50 mm of sodium phosphate buffer, pH 7.0, (600 μL) and H₂O (50 μL) at 37 °C. After temperature equilibration, the enzyme (50 μL) was added. After 120 min, the reaction was stopped by adding fresh Garnett reagent (250 μL) prepared in 0.2 m of sodium acetate buffer, pH 4.2, containing 100 mL⁻¹ of Tween 20. Absorbance was measured at 225 nm and the amount of β-naphthylamide was determined using a standard β-naphthylamide curve. Activity
was expressed as protease mU mg⁻¹ of protein. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of β-naphthylamine min⁻¹ (Oliveira et al. 1999).

Inhibition assays

The following inhibitors prepared in DMSO at a final concentration of 1 mM were used: phenylmethanesulfonyl fluoride (PMSF – serine protease inhibitor), tosyl-lysyl-chloromethyl ketone (TLCK) and benzamidine (both trypsin inhibitors); Tosyl-phenylalanine chloromethyl ketone (TPCK – chymotrypsin inhibitor; and bestatin (aminopeptidase inhibitor) (Bezerra et al. 2005). Triplicate samples of enzyme extract (25 μL) and inhibitors (25 μL) were placed in a well of a microtitrate plate and incubated at 25 °C for 15 min. Volumes were then adjusted to 170 μL with 0.1 M Tris- HCl, pH 8.0, and the respective substrate (BAPNA – PMSF, TLCK and benzamidine; SAPNA – PMSF and TPCK; Leu-p-Nan – bestatin) and the proteolytic activity were determined as described above. The enzyme and substrate blank were similarly assayed without enzyme and substrate solution respectively. The 100% values were established using DMSO without inhibitors.

Physical chemical properties

The effects of pH and temperature on proteolytic activity of the F. subtilis enzyme extract were evaluated as described above, using 0.1 M Tris- HCl buffer, with pH ranging from 7.2 to 9.0 and temperature ranging from 25 to 85 °C. Thermal stability was evaluated by assaying enzyme activity at 25 °C after pre-incubation for 30 min at temperatures ranging from 25 to 85 °C (Bezerra et al. 2005).

Electrophoresis sodium dodecyl sulphate polyacrylamide gel electrophoresis and zymograms

Proteases from F. subtilis were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using a 4% (w/v) stacking gel and 12.5% (w/v) separating gel (Laemmli 1970). Enzyme preparations and molecular weight markers (15 μL) (ovovalbumin – 46 kDa, glyceraldehyde 3-phosphate dehydrogenase – 36 kDa, carbonic anhydrase – 29 kDa, trypsinogen – 24 kDa and α-lactalbumin – 14.2 kDa) were applied to each track of a vertical electrophoresis device (Bio-Rad). The gels were stained for protein overnight in 1.8 g L⁻¹ Coomassie Brilliant Blue R250 prepared in water, acetic acid and methanol (65 : 10 : 25) and the background of the gel was de-stained in the same solution without dye. Electrophoresis was performed at a constant current of 15 mA per gel at 4 °C.

Zymograms were also carried out, following the procedure described by Garcia-Carrasco et al. (1993). After electrophoresis, the gels were immersed in 2.5 mL L⁻¹ (100 mL) Triton X-100 in 0.1 M Tris- HCl, pH 8.0, for 30 min at 4 °C to remove the SDS. The Triton X-100 was removed by washing the gels three times with 100 mL of 0.1 M Tris- HCl buffer, pH 8.0. The SDS-free, Triton X-100-free gels were then incubated with 100 mL of 3 g L⁻¹ casein in 0.1 M Tris- HCl, pH 8.0, for 30 min at 4 °C. The temperature was raised to 25 °C and maintained for 90 min to allow the digestion of casein by the active fractions. Finally, the gels were stained and de-stained as described previously. Thermal stability was also determined using the caseinolytic zymogram. The same methodology described above was employed, except that samples were pre-incubated at temperatures ranging from 25 to 75 °C. For the zymogram of enzyme inhibition, samples were pre-incubated with serine protease and trypsin inhibitors as described above (inhibition assays). Samples incubated with proteinase inhibitors were compared with control (without inhibitors). Decrease of the intensity or evanishment of the bands indicated inhibition of proteolytic activity, identifying the type of enzyme.

Statistical analysis

Data (mean ± standard deviation) processing was carried out using the MICROCAL ORIGIN 6.0 software program. Differences between mean values were analysed using the Student’s t-test. Differences were considered significant when P < 0.05 (Zar 1984).

Results

Table 1 displays the proteolytic activity of the SAS8 and SAS13 F. subtilis specimens. Total proteolytic activity (azocasein as substrate) did not differ (P > 0.05) between SAS8 and SAS13. The use of specific substrates revealed the presence of trypsin-, chymotrypsin- and leucine aminopeptidase-like enzymes. Trypsin-like (BAPNA) and chymotrypsin-like (SAPNA) activity was significantly higher (P < 0.05) in SAS8 than SAS13. Chymotrypsin-like activity was almost twice higher in SAS8 than SAS13. The presence of aminopeptidases was more evident with β-naphthylamide substrates (Table 1). Higher activity was found for basic (Arg-, Lys-) and non-polar (Ala-, Leu-) substrates. Lower activity occurred with aromatic (Tyr), uncharged polar (Ser-) and
Table 1: Proteolytic activity in the hepatopancreas of *Furunculus officinalis* SAS₆ and SAS₇ using specific and non-specific substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>SAS₆ (µU mg⁻¹)</th>
<th>SAS₇ (µU mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-specific substrate</td>
<td>12.20 ± 0.63</td>
<td>11.97 ± 0.74</td>
</tr>
<tr>
<td>Azocasein</td>
<td>5.13 ± 0.08</td>
<td>4.41 ± 0.19</td>
</tr>
<tr>
<td>p-Nitroanilide</td>
<td>6.82 ± 0.14</td>
<td>4.04 ± 0.07</td>
</tr>
<tr>
<td>BAPNA</td>
<td>0.33 ± 0.02</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>Leu-P-Nan</td>
<td>100.34 ± 12.01</td>
<td>100.25 ± 5.40</td>
</tr>
<tr>
<td>β-Naphthylamide</td>
<td>100.97 ± 10.01</td>
<td>100.42 ± 8.61</td>
</tr>
<tr>
<td>Alanine</td>
<td>60.04 ± 5.68</td>
<td>60.17 ± 4.01</td>
</tr>
<tr>
<td>Arginine</td>
<td>60.08 ± 8.08</td>
<td>56.68 ± 7.95</td>
</tr>
<tr>
<td>Lysine</td>
<td>20.33 ± 2.24</td>
<td>19.56 ± 1.93</td>
</tr>
<tr>
<td>Serine</td>
<td>20.28 ± 1.62</td>
<td>18.08 ± 2.16</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.35 ± 1.06</td>
<td>9.98 ± 1.12</td>
</tr>
</tbody>
</table>

BAPNA, benzoyl-α-arginine-p-nitroanilide — trypsin-specific substrate; SAPNA, succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide; Leu-P-Nan, leucine-p-nitroanilide — leucine aminopeptidase-specific substrate. SAS₆ approximately 6 g of wet weight; SAS₇, 13 g of wet weight. Values shown are mean ± standard deviation (SD) of triplicates of four crude extracts obtained from five hepatopancreases each. Different italic superscript letters denote statistical differences (P < 0.05).

Non-polar (Gly-) substrates. However, activity was observed for all aminocetyl-β-naphthylamide substrates used, some of which (Glu, Ile, Phe, His and Val-β-naphthylamide) revealed negligible activity and were not listed. There were no statistical differences in the proteolytic activity (P ≥ 0.05) between SAS₆ and SAS₇, using aminocetyl-β-naphthylamide as substrates. A positive correlation between the recommended concentration of essential amino acids for penaeid feed and aminopeptidase activity was detected in both sub-adult stages, using aminocetyl-β-naphthylamide as substrates (Fig. 1).

Digestive proteases were partially inhibited by PMSF (using BAPNA and SAPNA as substrates) in both sub-adult stages (Table 2). TLCK and benzamidine exhibited a strong effect of trypsin-like activity inhibition on SAS₆ (91.70 ± 0.58% and 89.81 ± 0.21% respectively) and SAS₇ (92.20 ± 0.21% and 89.91 ± 0.15% respectively) specimens. TPCK inhibited chymotrypsin activity in both sub-adult stages. Leucine aminopeptidase activity was inhibited by bestatin at a rate of 81.49 ± 0.02% for SAS₆ and 85.21 ± 0.01% for SAS₇.

The highest trypsin-like activity was obtained in a pH range from 8.0 to 9.0; optimal pH was 8.5 for both SAS₆ and SAS₇. Maximum chymotrypsin-like activity was observed at pH 8.5 for SAS₆ and 8.0 for SAS₇. Optimal pH for leucine aminopeptidase was 8.0 in both sub-adult stages (Fig. 2).

Figure 1: Correlation between aminopeptidase activity of *Furunculina annulifera* SAS₆ (a) and SAS₇ (b) using aminocetyl-β-naphthylamide as substrates and recommended concentration of essential amino acids for penaeid shrimp feed. Values are shown as mean ± SD of triplicates of four crude extracts obtained from five hepatopancreases each. Concentration of amino acids expressed in % of crude protein: arginine (5.8), leucine (5.4), lysine (5.3) and phenylalanine (4.0), valine (4.0). *Based on Guillaume (1997).
Table 2 Effect of specific inhibitors on proteases of the hepatopancreas of *Farfantepenaeus subtilis* SAS₆ and SAS₁₃

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Enzyme inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SAS₆</td>
</tr>
<tr>
<td>PMSF¹</td>
<td>52.35 ± 0.34</td>
</tr>
<tr>
<td>PMSF²</td>
<td>55.23 ± 0.72</td>
</tr>
<tr>
<td>TLCK</td>
<td>91.73 ± 0.58</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>85.54 ± 0.21</td>
</tr>
<tr>
<td>TPCK</td>
<td>42.40 ± 0.48</td>
</tr>
<tr>
<td>Bestatin</td>
<td>81.49 ± 0.02</td>
</tr>
</tbody>
</table>

PMSF, phenylmethylsulphonyl fluoride; TLCK, tosyl lysine chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethylketone; SAS₆, approximately 6 g of wet weight; SAS₁₃, approximately 13 g of wet weight.

Values are shown as mean ± SD of triplicates of four crude extracts obtained from five hepatopancreases each. Different italic superscript letters denote statistical differences (P < 0.05). Maximal specific proteolytic activity (100%) was 7.03 μM mg⁻¹ for SAS₆ and 6.09 μM mg⁻¹ for SAS₁₃ using BAPNA as substrate. 100% was 4.40 μM mg⁻¹ for SAS₆ and 4.94 μM mg⁻¹ for SAS₁₃ using SAPNA as substrate. 100% was 0.89 μM mg⁻¹ for SAS₆ and 0.23 μM mg⁻¹ for *F. subtilis* adults using Leu-β-Nan as substrate.

1 PMSF inhibition using BAPNA as substrate.
2 PMSF inhibition using SAPNA as substrate.

Residual trypsin-like activity was reduced to 15% after a 30-min heat treatment at 55 °C (optimal temperature) in both SAS₆ and SAS₁₃ (Fig. 4a). A similar profile was obtained for the thermal stability of leucine aminopeptidase activity in both SAS₆ and SAS₁₃ specimens (Fig. 4c). Chymotrypsin-like enzymes demonstrated higher heat resistance than trypsin- and leucine aminopeptidase-like enzymes (Fig. 4b). No significant loss of activity appeared up to 35 °C. At 60 °C, activity decreased to 60% of the initial value.

Proteins from the hepatopancreas of SAS₆ and SAS₁₃ *F. subtilis* were analysed by electrophoresis (Fig. 5). A common pattern was observed concerning the number of bands in both sub-adult stages. Six bands ranging from 15.3 to 42.2 kDa were detected. Three bands were found to have molecular weights higher than 45 kDa and one band had a molecular weight lower than 14.2 kDa.

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Figure 3. Effects of temperature on proteolytic activity from *Fusarium oxysporum* subtilis SAS1 (●) and SAS3 (○) using BAPNA (a), SAFNA (b) and Leu-p-nitroanilide (c) as substrates. Values are shown as mean ± SD of triplicates of four crude extracts obtained from five hepatopancreases each. The crude extract was incubated with the above substrates at different temperatures for 15 min and reactions were measured at 405 nm. Values are expressed as percentage of the highest (100%) and were 7.41 mU mg⁻¹ for SAS1 and 2.49 mU mg⁻¹ for SAS3 using BAPNA as substrate; 40.69 mU mg⁻¹ for SAS1 and 28.02 mU mg⁻¹ for SAS3 using SAFNA; and 0.26 mU mg⁻¹ for SAS1 and 0.42 mU mg⁻¹ for SAS3 using Leu-p-nitroanilide as substrate, respectively. SAS1: approximately 6 g wet weight; SAS3: approximately 13 g wet weight.

Dashed arrow, Fig. 6a, lane 1). This proteolytic band is also evident in Fig. 7a (black arrow, lane C). From 35 to 55 °C, the number and intensity of bands were similar for both subadult stages. One band remained active in SAS1 even at temperatures as high as 65 °C (white arrow, Fig. 6a, lane 5).

Figure 7 displays the enzyme inhibition zymogram. All proteolytic bands were either totally or partially inhibited by PMSF (lane 1) in the SAS1 and SAS3 extracts, indicating that most bands must be serine proteases. No reaction was recorded in two bands using TLCK (lane 2) and in three bands using benzamidine (lane 3), suggesting a strong presence of trypsin-like activity in the *F. subtilis* enzyme extract. By comparing these results to those recorded in Fig. 6, it is evident that the thermostable proteolytic band in SAS1 (white arrow, Fig. 7a, lane C) was inhibited by PMSF, TLCK and benzamidine. The proteolytic bands that were not inhibited by trypsin inhibitors exhibited greater intensity (white dashed arrows, Fig. 7a, b, lanes 2 and 3).

Discussion

Studies on the characterization of digestive enzymes in shrimp are important to understanding their digestive physiology in comparative studies and represent basic information for further use of enzyme extracts as tools in the search for the proper nutrient sources in aquaculture (Buarque et al. in press). The fact that proteases and peptidases are present in the hepatopancreas of *F. subtilis* is a relevant physiological advantage. Following hydrolysis of proteins by proteases, peptidases such as aminopeptidases break long-chain peptides down into smaller peptides and free amino acids, thereby enhancing the absorption of these nutrients. According to Zaumbebaio-Ino et al. (2007), the activity of peptidases facilitates the assimilation of amino acids in the larvae of marine fishes. A better understanding of the digestive physiology of this species is essential to the formulation of an adequate shrimp feed (Buarque et al. in
Digestive proteases in *Farfantepenaeus subtilis*

Figure 4. Effects of thermal stability on proteolytic activity from *Farfantepenaeus subtilis* SAS (○) and SAS₂ (■) using BAPNA (a), SAPNA (b) and Leu-p-Nan (c) as substrates. Values are shown as mean ± SD of triplicates of four crude extracts obtained from five hepatopancreases each. Thermal stability was determined by assaying its activity (25 °C) after pre-incubation for 30 min at the indicated temperatures. Values are expressed as percentage of the highest (100%) and were 7.14 μU mg⁻¹ for SAS, and 4.93 μU mg⁻¹ for SAS₂ using BAPNA as substrate, 9.89 μU mg⁻¹ for SAS, and 4.26 μU mg⁻¹ for SAS₂ using SAPNA, and 0.28 μU mg⁻¹ for both sub-adult stages using Leu-p-Nan as substrate respectively. SAS: approximately 6 g of wet weight, SAS₂: approximately 13 g of wet weight.

Digesting protein from diets with a high concentration of basic or aromatic amino acid.

The structure and morphology of digestive systems of decapod crustaceans are generally similar. However, differences are often observed on the biochemical level, two of which are related to the cell pH of the midgut gland and enzyme expression (Szamowietz et al. 2008). According to these authors, while crustaceans with a lower pH produce cysteine and aspartic proteases, those with a higher pH (such as penaeid shrimp) predominantly express serine proteases.

Trypsin- and chymotrypsin-like enzymes were identified in both sub-adult stages of *F. subtilis*. The higher trypsin- and chymotrypsin-like activity in the SAS₂ specimens may be related to the faster metabolism of younger organisms. The crustacean digestive system generally exhibits a high concentration of serine proteases, mainly trypsin and chymotrypsin (Fernández et al. 1997). Trypsin also plays an important role in digestion through the activation of zymogens of both itself and other endopeptidases (Natalia et al. 2004).

In this study, aminopeptidases were also observed in both sub-adult stages. As little information is available on aminopeptidases in shrimp, Leu-p-Nan and aminocetyl-β-naphthylamide substrates were used to provide a greater understanding of these enzymes. Aminopeptidases in the hepatopancreas of *F. subtilis* SAS, and SAS₂ were capable of strongly hydrolyzing alanine, arginine, leucine, lysine- and serine-β-naphthylamide substrates. Greater hydrolysis of substrates containing amino acids was required at greater concentrations in the shrimp diets, such as arginine, leucine and lysine. According to Guillaume (1997), the requirements of these amino acids for penaeids are 5.8%, 5.4% and 5.3% of crude protein respectively. These amino acids correlated with the aminopeptidase activity using some β-naphthylamide substrates (Arg-, Leu-, Lys-, Phe- and Val-). The results of this study corroborate the requirements reported in literature.
as lysine and arginine are described as the most limiting essential amino acids in commercial shrimp feeds (Fox et al. 1995). The correlation between recommended concentrations of some dietary components, such as lysine and arginine, and high aminopeptidase activity may be related to the efficient digestion and incorporation of these key nutrients (Lemos & Nunes 2008). This interesting correlation has also been observed for _F. panulirus_ (Buarque et al. in press).
The most important proteases in decapod crustaceans belong to the serine class and are inhibited by PMSF (Lemos et al., 2002), as observed with the proteases in hepatopancreatic tissue of *F. subtilis* in this study. The strong inhibition by TLCK and benzamidine (trypsin inhibitors) on the proteolytic activity in both sub-adult stages indicates classical trypsin activity (traditional mammalian trypsin). However, TPCK was not efficient in inhibiting chymotrypsin activity in the crude extract of both sub-adult stages. This inhibitor has also demonstrated a low effect on chymotrypsins in other crustaceans (García-Carreño et al., 1994; Lemos et al., 1999; Fernández Giménez et al., 2002).

Classical protease inhibitors, such as PMSF, TLCK, TPCK and benzamidine, are generally synthesized based on the mammalian enzyme mechanism. However, some studies have found a low inhibitory effect by these molecules on crustacean proteases (García-Carreño et al., 1994; Lemos et al., 1999; Fernández Giménez et al., 2002). This is a strong evidence of the low compatibility of these enzymes with mammalian proteases. According to Dall & Moriarty (1983), the digestive system of crustaceans is so different from that of mammals that differences in the activity of their enzymes are to be expected. This may be important information for aquaculture, mainly because the quality of shrimp feeds is commonly evaluated using digestibility assays employing mammalian enzymes (i.e. pepsin test). In fact, these results reinforce the advantage of the use of methodologies such as pH-STAT, which evaluates digestibility by employing enzyme extracts from the target shrimp species.

Crustacean proteases generally exhibit the greatest activity in the pH range from 5.5 to 9.0; trypsin activity is greatest between pH 7.0 and 9.0 (Jiang et al., 1991; Maeda-Martínez et al., 2000) and chymotrypsin activity is greatest between pH 7.0 to 10.0 (Saborowski et al., 2004; Von Elert et al., 2004). The optimal pH for trypsin enzymes in *F. subtilis* falls within this interval. Chymotrypsin-like enzymes also exhibited maximal proteolytic activity in the alkaline range. According to Dall & Moriarty (1983), pH in gastric fluid during feeding may fluctuate around neutrality and all digestive enzymes may be active enough in this range for adequate digestion to occur.

Trypsin-like enzymes in the hepatopancreas of both *F. subtilis* SASb and SASa exhibited the highest proteolytic activity at similar temperatures: 55 °C at pH 8.0. These values correspond to those recorded for other crustaceans (from 30 to 60 °C) (Jiang et al., 1991; Maeda-Martínez et al., 2000). However, trypsin-like enzymes in *F. subtilis* SASa and SASb retained about 15% of their activity after incubation for 30 min at 55 °C (Fig. 4). Although trypsin exhibited maximal activity at 55 °C in both sub-adult stages, its thermal stability was low at the same temperature, suggesting that most of this enzyme must have been denatured.

There is little information concerning the heat treatment and temperature resistance of chymotrypsins from crustaceans. Chymotrypsin activity from the gastric fluid of the crab *Cancer pagurus* was extinguished after incubation at 60 °C for 20 min (Saborowski et al., 2004). Therefore, chymotrypsin of *C. pagurus* seems to be less thermostable than the same enzyme in *F. subtilis* (about 40% of initial value after a 30-min heat treatment at 60 °C).

A number of authors have studied aminopeptidases in fish (Refstie et al., 2006). This demonstrates the importance of understanding the role of aminopeptidases in the protein digestion of aquatic organisms. Galgani et al. (1984) report the presence of leucine aminopeptidase in crude extract from *Penaeus kerathurus*. However, there is a lack of information on aminopeptidases in shrimp with regard to their physicochemical characterization. Further studies are required to compare the physicochemical effects on aminopeptidases in different shrimp species. Regarding the properties of leucine aminopeptidase in the hepatopancreas of *F. subtilis* SASb, and SASa, optimal pH and temperature were 8.0 and 30–55 °C, respectively, and indicate a similar temperature denaturation profile being completely inactivated at 80 °C.

In the electrophoresis (SDS-PAGE) of the extracts from the hepatopancreas of *F. subtilis* SASb, and SASa, a similar pattern was observed in both samples (Fig. 5). Two bands were well visualized in the molecular weight range from 24 to 29 kDa, which is equivalent to the trypsin enzyme molecular weight reported in the literature for other aquatic animals (Kolodziejka & Sikorski, 1998).

The thermal stability of proteolytic enzymes from *F. subtilis* is also shown in zymograms and the results demonstrated a similar band profile in both sub-adult stages studied. However, one slight band with proteolytic activity at 65 °C was observed in SASa (white arrow, Fig. 6), suggesting that this enzyme remains active even under adverse temperature conditions. Moreover, an extra band was also observed in the crude extract from SASa specimens, indicating the presence of one more protease in the hepatopancreas of *F. subtilis* (white dashed arrow, Fig. 6). This band was inhibited by PMSF, TLCK and benzamidine (white arrow, Fig. 7), suggesting that it is a trypsin enzyme. All bands remained active until 55 °C in both sub-adult stages, even though the quantitative determination of proteolytic activity was low at the same temperature. In fact, the zymogram technique is more sensitive than the quantitative assays using soluble substrates (Lemos et al., 2000).

Proteolytic bands inhibited by both PMSF (serine protease inhibitor) and TLCK or benzamidine (trypsin inhibitors)
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correspond to trypsin, which is a key enzyme in proteolytic digestion. Inhibition by PMSF alone indicates the presence of chymotrypsin, another proteolytic enzyme present in the hepatopancreases of penaeids (Lemos et al. 2002).

Conclusions

This study demonstrated a large diversity of proteases in the hepatopancreas of *F. subtilis*, with the presence of trypsin, chymotrypsin and aminopeptidases. The presence of a high content of proteases and proteinases renders protein digestion more efficient. The most striking difference between sub-adult stages was the greater chymotrypsin activity in the crude extract of SAS. In wild animals, however, it was not possible to associate the protease metabolism with the previously observed slowdown in the growth rate in cultured specimens (after reaching 6 g of body weight). Moreover, a considerable large diversity of aminopeptidase was found in both sub-adult stages. The highest aminopeptidase activities were observed using alanine, arginine, lysine and leucine-$\beta$-naphthylamide as substrates. The proteolytic enzymes studied revealed optimal $\text{pH}$ within the expected range for decapod crustaceans, as described in the literature. While the trypsins and leucine aminopeptidases found in this study exhibited an optimal temperature of about 55 °C, they were not thermostable at this temperature. These findings provide additional relevant information and could help to elucidate the relationship between diet and digestive potential of *F. subtilis*. This study may be used as a comparative reference for further feeding and nutrition studies on this species under farming conditions.

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References


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5. CAPÍTULO 2: FISIOLOGIA DIGESTIVA DE PEIXES
5.1. Artigo 4: Utilization of shrimp protein hydrolysate in Nile tilapia (*Oreochromis niloticus*) feeds
Shrimp protein hydrolysate in tilapia feed

Utilization of shrimp protein hydrolysate in Nile tilapia

*Oreochromis niloticus, L.* feeds

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Abstract

A 45-day feeding trial was carried out to evaluate the use of shrimp protein hydrolysate (SPH) in diets to Oreochromis niloticus, L. SPH was included in isonitrogenous diets at levels of 0, 5, 10 and 20% of fish meal protein replacement and offered to juvenile Nile tilapia (1.7±0.4g) stocked in 40-L glass aquaria. The inclusion of SPH did not produce significant differences (P≥0.05) on final weight, survival, weight gain (WG), average daily gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and apparent net protein utilization (ANPU). The inclusion of SPH in diets for Nile tilapia significantly affected (P<0.05) the final fish body composition. Protein and ash contents decreased and fat content increased slightly with SPH inclusion levels. This study has demonstrated that SPH could be included up to 6% in diets for Nile tilapia without adverse effects on growth and nutrient utilization.

Keywords: growth, protein utilization, shrimp protein hydrolysate, tilapia.
Introduction

Aquaculture requires high-quality feeds with high protein content. In general, protein is the most expensive nutrient in aquafeeds. Marine protein sources (mainly fish and by-products meals) generally enhance aquafeeds palatability and are excellent sources of essential amino acids and fatty acids, vitamins and minerals (Sudaryono et al 1995, El-Sayed 1999, Hardy et al 2007).

At present, fish meal still remains as the major dietary protein source, comprising between 20 and 60% of fish feed (Watanabe 2002). On the long-turn, many developing countries will be unable to depend on fish meal as a major protein source in aquafeeds. The determination of less-expensive sources of protein which provide good growth is advantageous for diet manufacturer and aquaculture producers alike (Coyle et al 2004).

Nile tilapia, Oreochromis niloticus, Linnaeus (1758) is one of the most cultured fish in tropical and subtropical regions of the world. Tilapia is an omnivorous species that has a digestive system that differs both from those of carnivorous and from many herbivorous fish: utilize a wide spectrum of foods (Sklan et al 2004a) use dietary carbohydrate efficiently (Boscolo et al 2002) and has a great ability to digest plant protein (Olvera-Novoa 2002, Shelton and Popma 2006, Gatlin III et al 2007).

Hence, many products has been tested as protein source to tilapia: soybean meal; Leucaena leaf meal (Wee and Wang 1987); feather meal (Bishop et al 1995); shrimp, blood, meat and bone and poultry by-product meals (El-Sayed 1998); cottonseed meal (Mbahinzireki et al 2001); sunflower cakes, anchovy meal and wheat bran (Maina et al 2002); a mix of soybean meal, cottonseed meal, sunflower meal and linseed meal (El-Saidy and Gaber 2003); distillers dried grains with solubles (Coyle et al 2004); corn gluten, rapeseed meal, sorghum and barley (Sklan et al 2004b); soybean meal, maize gluten meal, dehulled flax, pea and canola protein concentrates (Borgeson et al 2006). Despite of this, the inclusion of plant protein sources in aquafeeds is limited by antinutritional factors, associated to amino acids imbalances (Francis et al 2001), and fiber levels (Olvera-Novoa et al 1997).

Stimulated by increasing shrimp production from catches and farming, the shrimp waste meal has been identified as an animal protein source with great potential (Fanimo et al 2000), and
could reduce environmental problems as a result of improper deposition of inedible parts of shrimp, such as heads, shells and tails (Heu et al 2003). Nevertheless, the use of shrimp waste meal could be restricted due to its high fiber and ash contents (Cavalheiro et al 2007). Accordingly, ash and fiber contents reduced crustacean meal digestibility in tilapia (Koprucu and Ozdemir 2005), decreased lipid absorption and increased water content in Atlantic salmon *Salmo salar* L. feces (Olsen et al 2006).

Silva (2004) produced a shrimp protein hydrolysate (SPH) from Pacific white shrimp, *Litopenaeus vannamei*, Boone (1931) heads, which was considered an excellent protein source due to its amino acids profile and low fiber content. Products obtained from shrimp processing wastes may serve as an useful source of protein and flavorings in food formulations, mainly due to its free amino acids levels (Heu et al 2003, Ruttanapornvapeesakul et al 2005). The present study aimed to evaluate the nutritional quality of SPH by assessing growth performance and protein utilization of juvenile Nile tilapia.

**Materials and Methods**

**Shrimp Protein Hydrolysate (SPH)**

The shrimp protein hydrolysate (SPH) was produced by enzymatic autolysis, according to Bezerra (2000) Firstly, the raw material (shrimp heads) was washed and ground in distilled water (1:1). The blend was submitted to digestion in water-bath (45±2°C, 3 hours and slight agitation), with posterior temperature elevation (100°C, 10 minutes) for enzymatic deactivation. The solid and liquid fractions are separated by filtration (1mm² mesh) and then centrifuged at 10,000 *x*g for 40 minutes. The obtained supernatant was defined as shrimp protein hydrolysate (SPH) (Figure 1). The product was sent to Centro de Química de Proteínas of Faculdade de Medicina de Ribeirão Preto, São Paulo, Brazil, for amino acid profiles, and to the Empresa Pernambucana de Pesquisa Agropecuária for proximate composition.
**Diets**

Four isonitrogenous (37% crude protein) and isocaloric (440 kcal 100 g⁻¹) experimental diets were formulated to feed *Oreochromis niloticus* juvenile (Tables 1 and 2). SPH was included in the diets at 0 (as control), 1.5, 3 and 6% inclusion levels, which corresponded to 0, 5, 10 and 20% of fish meal protein replacement. The SPH was incorporated to soybean meal and the dough was dried (65°C for 24h).

The ingredients were mixed and the diets prepared by extrusion under industrial conditions to obtain 1-mm diameter pellets. A commercial diet (COM, 36% crude protein) was used as a reference.

**Animals and Experimental Conditions**

Juvenile sex-reversed Nile tilapia were obtained from Universidade Federal Rural de Pernambuco Aquaculture Station. Groups of eight fish were stocked in each of fifteen 40-L glass aquaria equipped with biological filter and continuous aeration. After a 7-day acclimatization period, diets were randomly assigned to three groups of fish. Fish were individually weighed (1.7±0.4 g) and measured (4.7±0.4 cm) before the onset of feeding trial. Diets were offered four times a day (800, 1100, 1400 and 1700 h) at an initial feeding rate of 15% of aquaria biomass. As it is generally recognized that younger or smaller fish consume more feed on a percent weight bases than larger one (Lim, Webster et al 2006), feeding rate was gradually reduced from 15 to 6% of biomass. A sample of five fish per aquarium was weighed each nine days for adjusting feeding rates.

Despite no significant feed scrap had been observed, aquaria were siphoned twice daily and submitted to a 66% water exchange due to faeces accumulation and turbidity of water. Water temperature, dissolved oxygen, pH, ammonia and nitrite were monitored and averaged 28.7±0.59°C (mean±sd), 3.5±0.92 mg L⁻¹, 8.1±0.19, 0.14±0.22 mg L⁻¹ and 0.08±0.02 mg L⁻¹, respectively.
Growth and Nutrient Utilization

Fish performance was evaluated through weight gain rate (WG), average daily gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and apparent net protein utilization (ANPU), according to the following formulae:

\[
\begin{align*}
\text{WG} (g) &= \text{BW}_f - \text{BW}_i \\
\text{ADG} &= \frac{\text{WG} (g)}{\text{time (days)}} \\
\text{SGR} &= \frac{100}{\text{time (days)}} \left( \ln \text{BW}_f - \ln \text{BW}_i \right) \\
\text{FCR} &= \frac{\text{dry feed offered (g)}}{\text{wet weight gain (g)}} \\
\text{PER} &= \frac{\text{wet weight gain (g)}}{\text{protein fed (g)}} \\
\text{ANPU} &= \frac{100}{\text{TF} \times \text{CP}} \left( \text{BW}_f \times \text{BCP}_f - \text{BW}_i \times \text{BCP}_i \right)
\end{align*}
\]

where \( \text{BW}_i \) and \( \text{BW}_f \) = average initial and final body weight (g) of fish, respectively; \( \text{BCP}_i \) and \( \text{BCP}_f \) = initial and final body crude protein (g 100g\(^{-1}\)) respectively; \( \text{TF} \) = total amount of diet fed (g), and \( \text{CP} \) = crude protein of diet (g 100g\(^{-1}\)).

Fish length and weight data were plotted (X and Y, respectively) to allow analysis of length-weight relationship, using the mathematical model \( W_t = \Phi \text{Lt}^\theta \) to adjust the tendency of these plots (Santos 1978).

Analytical methods

At the end of trial, all fish were weighed and two fish from each aquarium were sampled and frozen for determination of body composition. Initial body composition analyses were performed on a pooled sample of eight fish which was frozen prior to the study. Moisture, lipid, protein and ash contents were determined using standard methods (AOAC 1990).

Statistical Analysis

A one-way analysis of variance (ANOVA) was used to test the effects of SPH inclusion in the diets on fish performance. Tukey’s test was used at \( \alpha = 0.05 \) to test for differences among treatment means when \( F \)-values from the ANOVA were significant. The models of length-weight relationship were confronted using the statistic \( W \), which was compared to chi-square distribution at \( \alpha = 0.05 \) (Mendes 1999). Data obtained from commercial diet were not used in statistical analysis.
Results

Shrimp protein hydrolysate resulted in a product with 9.7% moisture, 43.63% crude protein, 6.25% ether extract, 7.32% ash and 3,633 kcal/kg gross energy, and a total amino acids content of 46.79 g/100g (41.2% essential and 58.8% non-essential), mainly glutamate, aspartate, leucine, lysine, tyrosine and arginine.

The effects of SPH inclusion on tilapia performance and nutrient utilization are given on Table 3. The level of SPH incorporated on diets (0, 1.5, 3 or 6%) did not affect (P≥0.05) final fish weight (27.18, 29.46, 26.02 and 25.19 g), weight gain (25.51, 27.73, 24.29 and 23.39g), average daily gain (0.57, 0.62, 0.54 and 0.52 g day⁻¹) and specific growth rate (7.15, 7.38, 6.85 and 6.73% day⁻¹). Feed conversion ratio (1.15, 1.09, 1.13 and 1.17), protein efficiency ratio (2.26, 2.33, 2.20 and 2.14) and apparent net protein utilization (39.31, 40.39, 38.57 and 34.72) also were not affected by SPH inclusion.

Fish fed actively on all diets. Even though diets had been offered four times a day, it was observed a territorial behavior related to feeding competition, but no mortality was recorded during feeding trial.

The parameters of the mathematical models to evaluate length-weight relationships of fish fed different diets are shown in Table 4. Evaluation of these models revealed statistical differences (P<0.05) on fish growth. Fish fed SPH5 (1.5% inclusion rate) presented the best length-weight relationship. Higher SPH inclusion levels (3 and 6%) did not contribute for fish growth, resulting in similar or worse growth performance to that provided by the SPH0 diet.

The evolution of mean weight of Nile tilapia fed diets containing 0, 5, 10 and 20% of SPH protein as partial substitute for fish meal protein and commercial diet is presented in Figure 2. Mean weight of fish linearly enhanced throughout feeding trial. The experimental diets provided equal (P≥0.05) growth performances among themselves.

Initial and final body compositions of whole fish are shown in Table 5. The inclusion of SPH in diets for Nile tilapia significantly affected (P<0.05) final fish body composition. Protein content decreased (p<0.05) when SPH replaced 20% of fish meal. Fish fed SPH 10 and SPH 20 presented greater fat contents (58.4 and 59.8 g kg⁻¹, respectively) than fish fed diets without (51.2 g
kg\(^{-1}\)) or with the lowest SPH inclusion level (50.3 g kg\(^{-1}\)). Fish fed the diet with no SPH presented higher ash content (40.5 g kg\(^{-1}\)) than the others ones (P<0.05).

**Discussion**

A number of authors described the feasibility of using fishery by-catch and by-product as sources of animal protein for aquatic feeds (Goddard et al 2003, Li et al 2004, Goddard and Perret 2005, Whiteman and Gatlin III 2005). It was reported by Plascencia-Jatomea et al (2002) that shrimp protein hydrolysate produced by fermentative silage could be included in tilapia diets at concentrations as high as 15%, improving fish growth rate. The present study demonstrated that 6% of SPH produced by autolysis can be included in diets for Nile tilapia without reducing growth performance.

In fact, there is a remarkable difference between these two methods of production of the shrimp silage used by the authors above cited and the shrimp protein hydrolysate used in the present study. Herein, SPH was produced by autolysis, without introduction of any chemical or biological exogenous additive, what is common in silage process. Plascencia-Jatomea et al (2002) reported that the acidic conditions in which shrimp hydrolysate by fermentative silage is produced causes loss of labile nutrients such as tryptophan, what was not observed in the present study. Although the two products have a similar essential amino acid (EAA) composition, the SPH herein produced proved to be an interesting source of tryptophan, showing 3.5, 3.5 and 1.75 to 2.4 fold higher levels of tryptophan than fish, shrimp and soybean meals, respectively (Table 6). At the same way, SPH seemed to be a good source of other EAA, with emphasis to lysine, leucine, arginine, phenylalanine and valine. Methionine levels (an important limiting amino acid for fishes) showed to be 4.5 to 5.7, 3 and 1.5 to 2.5 fold higher than soybean, shrimp and fish meals, respectively (Table 6). Results concerning methionine and lysine levels are particularly important in aquaculture because most of times, a supplementation of diets with these amino acids is required when alternative sources of protein are used for fish meal replacement (Cheng et al 2003, Alam et al 2005, Forster et al 2006, Sardar et al 2008). The inclusion of only 6% of SPH in the diet (Table 6) can supply 6 to 13% of EAA tilapia requirement described by Santiago and Lovell (1988).
The separation of shrimp carapaces promoted by filtration step during SPH production (Fig 1) removes the chitin, a significant anti-nutritional factor associated to poor fish growth, which is present in large amounts in products derived from crustacean and insects (Shiau and Yu 1999, Ogunji et al 2008). The supernatant provided after filtration and centrifugation steps (SPH) contains high levels of small peptides (unpublished data) which render the product highly soluble. It is possible that high loss of protein by lixiviation process could happen at inclusion levels of SPH in the diets greater than 6%. This concentration of SPH corresponds to about 20% of fish meal replacement in the diets which is important since fish meal is a limiting ingredient in aquatic feeds, while SPH is produced from shrimp processing waste.

Nutrient utilization and growth performance of fish fed the experimental feeds were not significantly different (P≥0.05), meaning that the four experimental diets had enough quality to assure good growth of the fish. The maximum percentage of survival registered also reflected adequate handling and experimental conditions. Even though data obtained from commercial diet were not used in statistical analysis, the experimental diets produced evident better results of growth and feed utilization. Comparing these results to those reported by Plascencia-Jatomea et al (2002), and Nwanna et al (2004), who worked with shrimp head silage as fish meal replacer in Nile tilapia and *Clarias gariepinus* diets, respectively (Table 7), it was observed the better performance of fingerlings fed SPH in the present work. These authors also found that shrimp silage can replace 20% of fish meal without adverse effects on growth and feed efficiency. Even when the protein source is fish meal produced from fisheries by-catch and fish processing waste (Goddard et al 2008), the same results could be observed.

Fish feeds should be formulated based on the nutritional requirement of the target species, but this characteristic is not the only one to be followed. Feed acceptance depends upon other important aspects such as appearance, particle size and organoleptic properties related to smell, taste and texture (Jobling et al 2001) and these characteristics can be influenced by the choice of feed ingredients and processing conditions. According to Higuera (2001), the feeding stimulants that cause the greatest behavioral responses in fishes are those composed of free amino acids, nucleotides and nucleosides and quaternary ammonium bases. Stimulant products should present
properties like: low molecular weight, nitrogen-containing, non-volatile, amphoteric, water-soluble, stable to heat treatment and broad biological distribution. Alanine, glycine, proline, valine, tryptophan, tyrosine, phenylalanine, lysine and histidine appear to be major components of feeding stimulants for many fish species. Based on amino acid composition of shrimp protein hydrolysate (Table 6) and on fish behavior when SPH based diets were offered (diets were avidly consumed), it was concluded that SPH could also be used as flavoring in tilapia feeds.

The territorial behavior observed in all treatments could have caused heterogeneous growth of tilapia. Fernandes and Volpato (1993) reported that increase in heterogeneous growth as a result of grouping in Nile tilapia can be associated to social stress imposed by the dominant fish on the subordinates. Such stress may decrease energy available for growth. Social hierarchy generally induces to a different access of individual fish to available feeds (Alanärä and Brännäs 1996, Hakoyama and Iguchi 1997). According to Kestemont and Baras (2001) better competitors usually have early access to food, digest their first meal and feed again before the end of feeding period, whereas subordinate fish do not have this opportunity, resulting in growth heterogeneity.

Although SPH inclusion has resulted in carcass fat deposition (Table 5), even the greatest values (59.8 g kg\(^{-1}\)) was smaller than that observed in fish fed the commercial diet. These results are similar to those reported by Plascencia-Jatomea et al (2002) who found body crude lipid content ranging from 53.6 to 67.2 in Nile tilapia fed diets containing shrimp head hydrolysate by fermentative silage. Whole body composition reflected diet composition only regarding ash contents. As SPH inclusion increased in experimental diets, the ash body contents decreased. It is a result of combined effects of low ash content in SPH and gradual reductions on amounts of fish meal employed.

**Conclusion**

Results of shrimp protein hydrolysate amino acid composition and growth data suggest that shrimp protein hydrolysate is a promising protein feedstuff for Nile tilapia and could be included up to 6% in the diets for juvenile Nile tilapia (20% of fish meal replacement) without adverse effects on
growth and nutrient utilization. Further research will be required to evaluate higher shrimp protein hydrolysate inclusions and its influence on digestive enzyme profile and economical values.

Acknowledgments

We express our thanks to Poytara Indústria e Comércio de Rações Ltda. for the manufacturing of the experimental diets and to Laboratório de Análise de Plantas & Rações (LAPRA) of the Empresa Pernambucana de Pesquisa Agropecuária (IPA) for proximate composition determinations. Recognition is given to all the technical staff of the Laboratório de Enzimologia for their assistance in conducting the feeding trial.

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SHRIMP HEADS
WASHING
GRINDING
DIGESTION
ENZYMATIC DEACTIVATION
FILTRATION

SOLID FRACTION

LIQUID FRACTION

CENTRIFUGATION

SPH (SUPERNATANT)

PRECIPITATE
Time (days)

SPH5 a*
SPH10 a
SPH20 a
COM
Figure 1. Scheme of shrimp protein hydrolysate production (modified from Bezerra, 2000).

Figure 2. Mean weight evolution of Nile tilapia fed diets with increasing shrimp protein hydrolysate (SPH) inclusion levels and commercial diet (COM) over a 45-days feeding trial (*different superscript letters differ significantly, P<0.05). Data obtained from commercial diet were not used in statistical analysis.
Table 1. Composition of the experimental diets.

<table>
<thead>
<tr>
<th>Ingredients (%)</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPH0</td>
</tr>
<tr>
<td>Fish meal (57% CP)</td>
<td>23.0</td>
</tr>
<tr>
<td>Shrimp protein Hydrolysate (SPH)</td>
<td>0.0</td>
</tr>
<tr>
<td>Soybean meal (40.4% CP)</td>
<td>47.0</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>16.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>10.5</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.0</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Mineral and vitamin mix&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.0</td>
</tr>
<tr>
<td>Salt</td>
<td>0.5</td>
</tr>
<tr>
<td>Antioxidant BHT</td>
<td>0.02</td>
</tr>
</tbody>
</table>

CP = crude protein. BHT = butylated hydroxytoluene.

<sup>1</sup> Mineral and vitamin mix (quantity kg<sup>-1</sup> premix): vitamin A (20,000 UI), vitamin D<sub>3</sub> (5,000UI), vitamin E (250 mg), vitamin K<sub>3</sub> (25 mg), vitamin B<sub>1</sub> (37.5 mg), vitamin B<sub>2</sub> (37.5 mg), vitamin B<sub>6</sub> (25 mg), vitamin B<sub>12</sub> (0.053 mg), vitamin C (250 mg), niacin (200 mg), pantothenic acid (100 mg), biotin (1,25 mg), choline (1,000 mg), inositol (250 mg), Fe (100 mg), Cu (12 mg), Zn (125 mg), Mn (37,5 mg), Se (0,25 mg), I (1,25 mg), Co (0,25 mg).
Table 2. Proximate analysis of the commercial and experimental diets.

<table>
<thead>
<tr>
<th>Proximate analysis (as-fed basis, g kg(^{-1}))</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COM</td>
</tr>
<tr>
<td>Dry matter</td>
<td>918.0</td>
</tr>
<tr>
<td>Crude protein</td>
<td>345.6</td>
</tr>
<tr>
<td>Ether extract</td>
<td>65.0</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>34.9</td>
</tr>
<tr>
<td>Ash</td>
<td>67.5</td>
</tr>
<tr>
<td>Nitrogen-free extract</td>
<td>487.0</td>
</tr>
<tr>
<td>Calcium</td>
<td>14.3</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>11.3</td>
</tr>
<tr>
<td>Gross energy (kcal 100 g(^{-1}))(^1)</td>
<td>461.5</td>
</tr>
<tr>
<td>P/GE ratio (mg kcal(^{-1}))</td>
<td>74.9</td>
</tr>
</tbody>
</table>

\(^1\) Estimative based on 5.65, 4.2 and 9.5 kcal g\(^{-1}\) for protein, carbohydrate and lipid, respectively.
Table 3. Growth performance and nutrient utilization in Nile tilapia fed diets with increasing substitution of fish meal by shrimp protein hydrolysate (SPH) and a commercial diet (COM).

<table>
<thead>
<tr>
<th>Diets</th>
<th>SPH0</th>
<th>SPH5</th>
<th>SPH10</th>
<th>SPH20</th>
<th>COM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>1.67±0.14</td>
<td>1.73±0.07</td>
<td>1.73±0.09</td>
<td>1.80±0.06</td>
<td>1.57±0.07</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>27.17±2.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.46±1.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.02±3.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.09±2.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.40±1.10</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>WG (g)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>25.51±2.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.73±1.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.29±3.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.39±22.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.83±1.12</td>
</tr>
<tr>
<td>ADG (g day&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.57±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>SGR (% day&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>7.15±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.38±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.85±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.73±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.29±0.22</td>
</tr>
<tr>
<td>FCR&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.15±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.09±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.17±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.28±0.03</td>
</tr>
<tr>
<td>PER&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.26±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.33±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.21±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.14±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.08±0.05</td>
</tr>
<tr>
<td>ANPU&lt;sup&gt;6&lt;/sup&gt;</td>
<td>39.31±6.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.39±6.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.57±3.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.72±3.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.08±1.27</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of mean. Within a row, means with different superscript letters differ significantly (P<0.05) by Tukey test. Data obtained from commercial diet were not used in statistical analysis.

<sup>1</sup>Weight gain rate; <sup>2</sup>Average daily gain; <sup>3</sup>Specific growth rate; <sup>4</sup>Feed conversion ratio; <sup>5</sup>Protein efficiency ratio; <sup>6</sup>Apparent net protein utilization.
Table 4. Parameters of the mathematical models ($Wt = \Phi Lt^\theta$) adjusted to length-weight data of fish fed diets with increasing shrimp protein hydrolysate (SPH) inclusion levels and commercial diet (COM) over a 45-days feeding trial.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\Phi$</th>
<th>$\theta$</th>
<th>$R^2$</th>
<th>n</th>
<th>C.S.¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPH0</td>
<td>0.0134</td>
<td>3.1035</td>
<td>0.9919</td>
<td>129</td>
<td>b</td>
</tr>
<tr>
<td>SPH5</td>
<td>0.0132</td>
<td>3.1163</td>
<td>0.9950</td>
<td>126</td>
<td>a</td>
</tr>
<tr>
<td>SPH10</td>
<td>0.0142</td>
<td>3.0682</td>
<td>0.9924</td>
<td>128</td>
<td>b,c</td>
</tr>
<tr>
<td>SPH20</td>
<td>0.0149</td>
<td>3.0412</td>
<td>0.9933</td>
<td>125</td>
<td>c</td>
</tr>
<tr>
<td>COM</td>
<td>0.0163</td>
<td>2.9995</td>
<td>0.9889</td>
<td>127</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Comparative statistic using statistic $W$, compared to $\chi^2$ distribution at $\alpha = 0.05$. Data obtained from commercial diet were not used in statistical analysis.
Table 5. Initial and final proximate composition (g kg\(^{-1}\) on as-fish basis) of whole body of Nile tilapia fed diets with increasing shrimp protein hydrolysate (SPH) inclusion levels and commercial diet (COM) over a 45-days feeding trial.

<table>
<thead>
<tr>
<th>Fish</th>
<th>Dry matter</th>
<th>Protein(^{1})</th>
<th>Fat(^{1})</th>
<th>Ash(^{1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>260.4</td>
<td>150.4</td>
<td>45.3</td>
<td>34.3</td>
</tr>
<tr>
<td>Final body composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPH0</td>
<td>270.3</td>
<td>163.2±0.2(^{a})</td>
<td>51.2±0.1(^{a})</td>
<td>40.5±0.1(^{b})</td>
</tr>
<tr>
<td>SPH5</td>
<td>266.2</td>
<td>161.9±0.0(^{a})</td>
<td>50.3±0.0(^{a})</td>
<td>37.4±0.0(^{a})</td>
</tr>
<tr>
<td>SPH10</td>
<td>274.0</td>
<td>163.0±0.5(^{a})</td>
<td>58.4±0.0(^{b})</td>
<td>35.9±0.0(^{a})</td>
</tr>
<tr>
<td>SPH20</td>
<td>272.3</td>
<td>153.7±0.0(^{b})</td>
<td>59.8±0.0(^{b})</td>
<td>36.2±0.0(^{a})</td>
</tr>
<tr>
<td>COM</td>
<td>282.5</td>
<td>142.8±0.1</td>
<td>89.0±0.2</td>
<td>30.5±0.1</td>
</tr>
</tbody>
</table>

\(^{1}\)Each value is the mean (± standard error of mean) of two replicates. Mean with common superscript in the same column are not significantly different (P<0.05). Data obtained from commercial diet were not used in statistical analysis.
Table 6 - Amino acid (AA) composition of shrimp protein hydrolysate (SPH) and other ingredients used in aquatic feeds expressed as percentage of dietary protein

<table>
<thead>
<tr>
<th>Essential amino acids</th>
<th>Fish Meal a</th>
<th>Soybean meal a</th>
<th>Shrimp meal b</th>
<th>Shrimp head silage c</th>
<th>SPH e</th>
<th>Tilapia AA requirement f</th>
<th>% DV g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>3.78</td>
<td>4.45</td>
<td>3.05</td>
<td>3.11</td>
<td>2.79</td>
<td>6.26</td>
<td>7.50</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.40</td>
<td>1.21</td>
<td>1.25</td>
<td>1.12</td>
<td>1.07</td>
<td>2.33</td>
<td>1.61</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.15</td>
<td>1.84</td>
<td>1.75</td>
<td>2.42</td>
<td>1.86</td>
<td>3.16</td>
<td>2.64</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.77</td>
<td>3.55</td>
<td>2.91</td>
<td>2.85</td>
<td>2.98</td>
<td>6.65</td>
<td>4.11</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.98</td>
<td>4.03</td>
<td>2.57</td>
<td>2.67</td>
<td>2.41</td>
<td>7.36</td>
<td>7.35</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.80</td>
<td>1.10</td>
<td>0.47</td>
<td>0.60</td>
<td>0.91</td>
<td>1.86</td>
<td>2.62</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.81</td>
<td>1.93</td>
<td>1.91</td>
<td>2.33</td>
<td>1.76</td>
<td>4.20</td>
<td>2.35</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.99</td>
<td>2.32</td>
<td>1.51</td>
<td>1.88</td>
<td>1.58</td>
<td>6.30</td>
<td>2.13</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.39</td>
<td>-</td>
<td>0.80</td>
<td>0.58</td>
<td>0.40</td>
<td>-</td>
<td>0.96</td>
</tr>
<tr>
<td>Valine</td>
<td>2.59</td>
<td>2.47</td>
<td>1.90</td>
<td>2.24</td>
<td>2.03</td>
<td>4.23</td>
<td>2.98</td>
</tr>
</tbody>
</table>

* a - Halver (1995) (from INFIC data base)
  b - Embrapa (1989) (from Brazilian feedstuffs)
  f - Santiago and Lovell (1988), based in a 28% crude protein diet
  g – Percent daily values of amino acid tilapia requirement with the inclusion of 6% of SPH in the diet
Table 7 – Growth performance of fishes fed diets with different marine protein sources as fish meal replacers

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SPH 1</th>
<th>SHS 2</th>
<th>SHS 3</th>
<th>FM 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight Gain (g)</td>
<td>23.4 – 27.7</td>
<td>-</td>
<td>16.5 – 20.7</td>
<td>-</td>
</tr>
<tr>
<td>Weight Gain (%)</td>
<td>1,301 – 1,624</td>
<td>429 - 616</td>
<td>-</td>
<td>534 - 659</td>
</tr>
<tr>
<td>Average Daily Gain (g day-1)</td>
<td>0.52 – 0.62</td>
<td>0.22 – 0.37</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Specific Growth Rate (% day-1)</td>
<td>6.7 – 7.4</td>
<td>1.3 – 1.5</td>
<td>1.0 – 1.2</td>
<td>2.7 – 3.1</td>
</tr>
<tr>
<td>Feed Conversion Ratio</td>
<td>1.1 – 1.2</td>
<td>1.9 – 2.2</td>
<td>2.5 – 2.8</td>
<td>1.3 – 1.4</td>
</tr>
<tr>
<td>Protein Efficiency Ratio</td>
<td>2.1 – 2.3</td>
<td>1.6 – 1.9</td>
<td>0.4 – 0.52</td>
<td>1.8 – 1.9</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>100</td>
<td>93 - 100</td>
<td>-</td>
<td>97 - 99</td>
</tr>
</tbody>
</table>

1 Shrimp protein hydrolysate: Present study
2 Shrimp head silage: as fish meal replacer in Nile tilapia diet (Plascencia-Jatomea et al 2002)
3 Shrimp head silage: as fish meal replacer in Clarias gariepinus diet (Nwanna et al 2004)
4 Fisheries by-catch and processing waste meals in Nile tilapia diet (Goddard et al 2008)
5.2. Artigo 5: Profile of digestive enzymes from Nile tilapia (Oreochromis niloticus) submitted to diets with different concentrations of shrimp protein hydrolysate and its correlation with growth parameters

SUBMETIDO AO PERIÓDICO
JOURNAL OF FISH BIOLOGY
Artigo em revisão
Title: Digestive enzyme activity in Nile tilapia (Oreochromis niloticus L.) submitted to diets with different inclusion levels of shrimp protein hydrolysate and its correlation with growth parameters and body composition

Short Title: Effects of SPH on tilapia digestive enzymes

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Abstract: The effects of different dietary inclusion levels of shrimp protein hydrolysate (SPH) on digestive enzyme activity of Nile tilapia juveniles were evaluated and correlated with growth parameters and body composition. SPH was included in diets at concentrations of 0, 1×5, 3 and 6%. A commercial diet was used as reference. Hemoglobin, azocasein, BApNA, SApNA, AA-β naphthylamide and starch were used as substrates. Despite some differences, there was no correlation between enzyme activity and different SPH concentrations in the diets. Substrate-SDS-PAGE zymogram was also performed for the analysis of changes in the profile of Nile tilapia digestive proteases caused by the inclusion of protein hydrolysate. Zymograms revealed 12 proteolytic bands, eight of which responded to incorporation of SPH. Inhibition zymograms indicated that there was a decrease in the activity of three enzymes with trypsin activity as SPH increases, whereas the opposite occurred for one aminopeptidase. Distinct protease profiles were also found for each treatment, suggesting adaptability of the Nile tilapia to the different diets. Trypsin and aminopeptidase activity was positively correlated with growth parameters and carcass protein content and negatively correlated with feed conversion ratio and carcass lipid content.
Digestive enzyme activity in Nile tilapia (*Oreochromis niloticus* L.) submitted to diets with different inclusion levels of shrimp protein hydrolysate and its correlation with growth parameters and body composition


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Running headline: Effects of SPH on tilapia digestive enzymes

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ABSTRACT

The effects of different dietary inclusion levels of shrimp protein hydrolysate (SPH) on digestive enzyme activity of Nile tilapia juveniles were evaluated and correlated with growth parameters and body composition. SPH was included in diets at concentrations of 0, 1.5, 3 and 6%. A commercial diet was used as reference. Haemoglobin, azocasein, BApNA, SApNA, AA-β naphthilamide and starch were used as substrates. Despite some differences, there was no correlation between enzyme activity and different SPH concentrations in the diets. Substrate-SDS-PAGE zymogram was also performed for the analysis of changes in the profile of Nile tilapia digestive proteases caused by the inclusion of protein hydrolysate. Zymograms revealed 12 proteolytic bands, eight of which responded to incorporation of SPH. Inhibition zymograms indicated that there was a decrease in the activity of three enzymes with trypsin activity as SPH increases, whereas the opposite occurred for one aminopeptidase. Distinct protease profiles were also found for each treatment, suggesting adaptability of the Nile tilapia to the different diets. Trypsin and aminopeptidase activity was positively correlated with growth parameters and carcass protein content and negatively correlated with feed conversion ratio and carcass lipid content.

Keywords: Nile tilapia, shrimp protein hydrolysate, growth parameters, digestive enzymes, SDS-PAGE zymograms.
INTRODUCTION

Tilapia production has increased significantly on a global scale in the last decade. This development has been followed by an increase in feed consumption and has stimulated the search for new ingredients for use in diet formulas (Schulz et al., 2007). The growth of the aquaculture industry has generated large amounts of waste and by-products, which represents a challenge to the sustainability of the activity (Bezerra et al., 2001). Shrimp processing waste, for instance, is discharged into the environment and is a significant source of water and land pollution. One possible solution to this problem is the transformation of waste into suitable ingredients for use as components in animal feeds. Shrimp processing waste has been identified as an animal protein source of great potential (Fanimo et al., 2000). A simple protocol for producing protein hydrolysate from white shrimp Litopenaeus vannamei (Boone) processing waste through autolysis has recently been designed at the Universidade Federal de Pernambuco. This method renders a protein concentrate that is considered to be an excellent nutrient source of amino acids, with high levels of glutamate, aspartate, leucine, lysine, tyrosine and arginine (Silva, 2004). In fact, crustacean protein silage and hydrolysate has been used in fish feeds both as a new protein source (Plascência-Jatomea et al., 2002) and, in small amounts, as flavouring to enhance the attractiveness of feeds (Kolkovski et al., 2000).

Variations in the quality and quantity of nutrients used in diet formulation may modify the enzymatic profile and activity in the digestive tract of animals (Lundstedt et al., 2004). Thus, feed composition could induce biological adaptations, including an increase in nutrient absorption (Morais & Bidinotto, 2000). Digestive enzymes have been investigated for many years as a way of understanding nutritional requirements and the effects of diet composition on enzyme activity in order to reduce feeding costs in fish farms (Caruso et al., 1996). Most studies thus far have evaluated the effect of feeds with different concentrations of protein,
carbohydrates and lipids, correlating these results with enzyme activity. Combined with
growth parameters, such results may contribute to the establishment of the appropriate
quantities of nutrients to be included in newly developed feeds. Moreover, differences in
enzyme quality profile may be related to nutrient levels in the diet (Fountoulaki et al., 2005).
The present survey focuses on the following hypotheses: a) the inclusion of shrimp
protein hydrolysate at different concentrations in tilapia feeds could promote detectable
changes in the activity of the main digestive enzymes; b) the use of substrate-SDS-PAGE
zymograms could be an effective tool for improving the analysis of these changes; and c)
there is a correlation between digestive enzyme activity and growth parameters and body
protein and fat contents.

MATERIALS AND METHODS

MATERIALS
All reagents were of analytical grade and purchased from Sigma (St. Louis, MO, USA)
and Merck (Darmstadt, Germany). The diets were prepared in Poytara LTDA (Araraquara –
São Paulo – Brazil).

DIET PREPARATION
Four isonitrogenous (37% crude protein - CP) and isocaloric (1,842 kJ 100 g⁻¹)
experimental diets were formulated to feed Nile tilapia (Oreochromis niloticus L.) juveniles.
Shrimp protein hydrolysate (SPH) was included in the diets at concentrations of 0 (control –
SPH0), 1.5 (SPH1.5), 3 (SPH3) and 6% (SPH6). A 1:2 animal:plant protein ratio in the diets
was established. SPH was incorporated to soybean meal and the dough was dried at 65°C for 24 h. The ingredients were then mixed and extruded under industrial conditions. A commercial diet for omnivorous fish (36% CP) was used as reference.

SPH used in experimental diets was produced according to a methodology adapted from Bezerra (2000) for tambaqui, Colossoma macropomum (Cuvier). Approximately 20 kg of L. ramosei heads were collected, weighed, washed and crushed in distilled water (1:1 w/v). The mix was placed in a water bath at 45 ± 2°C for 150 min under agitation. The solution was then submitted to 100°C for 10 min in order to deactivate the enzymes and the solid portion was strained through a 1.0mm sieve. The resulting material was centrifuged at 10,000 x g for 10 min and the supernatants (SPH) were stored in plastic bottles at -20°C until the preparation of the diets. Tables I and II display the proximate composition and total amino acid content of the shrimp hydrolysate, respectively. Table III displays the formulation and proximate composition of the experimental diets.

Please insert tables I, II and III here

ANIMALS AND CULTURE CONDITIONS

Juvenile sex-reversed Nile tilapias were obtained from the Aquaculture Station of the Universidade Federal Rural de Pernambuco. The fish were stocked in fifteen 40-L glass aquaria (8 ind. per aquarium) equipped with a biological filter and continuous aeration and were submitted to a 7-day acclimatization period both for diets and experimental conditions in a completely randomized design, with five treatments and three replicates. Prior to the feeding trial, the fish were weighed (1.7±0.4g) and measured (4.7±0.4cm). The fish were fed four
times a day at rates ranging from 15% to 6% of biomass, adjusted every nine days over a 45-

day period.

The aquaria were siphoned twice daily, with 66% water exchange. Temperature, 

dissolved oxygen, pH, ammonia and nitrite were monitored and averaged (mean ± SD)

28.7±0.59°C, 3.5±0.92 mg L⁻¹, 0.1±0.19, 0.14±0.22 mg L⁻¹ and 0.08±0.02 mg L⁻¹,

respectively.

DIGESTIVE ENZYME EXTRACTION

At the end of trial, six individuals from each replicate aquarium were removed after 

fasting for 24 hours and sacrificed in an ice bath for biometric measurements and tissue 

removal. Stomach and intestines were immediately collected and homogenized (40 mg 

tissue/mL) in 0.01M Glycine-HCl pH 3.0 and 0.01M Tris-HCl pH 8.0 buffers, respectively, 

containing 0.15M NaCl, using a tissue homogenizer. The resulting preparations were 

centrifuged at 10,000 x g for 10 min at 4°C to remove cell debris and nuclei. The supernatants 

(crude enzyme extracts) were frozen at -20°C and used in further assays (Bezerra et al., 2005). 

Protein concentration was determined according to Bradford (1976) using bovine serum 

albumin (BSA) as the standard and reported as mg protein equivalent to BSA.

ENZYMATIC ASSAY

Acid proteolytic activity

Acid protease activity was evaluated using haemoglobin (Hb) as the substrate as follows:

In microcentrifuge tubes, 100 μL of 2% Hb in 0.06M Glycine-HCl buffer pH 3.0 was mixed 

with 50 μL stomach crude extract and 350 μL 0.5M Glycine-HCl buffer pH 3.0 for 60 min at
25°C. Five hundred µL of 10% trichloroacetic acid (TCA) were then added to stop the reaction. After 15 min, centrifugation was carried out at 8,000 x g for 10 min. The absorbance of supernatant (70 µL) was measured at 280 nm (Bio-Rad SmartSpec 3000, USA) against a similarly prepared blank in which 0.01M Glycine-HCl buffer pH 3.0 replaced the crude extract sample, based on a methodology adapted from Díaz-López et al. (1998). Previous experiments showed that, for the first 60 min, the reaction carried out under the conditions described above followed first order kinetics. One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing haemoglobin to produce a 0.001 change in absorbance per minute per milligram of protein.

Alkaline proteolytic activity

In a microcentrifuge tube (performed in triplicate), 1% azocasein prepared in 0.1M Tris-HCl buffer pH 8.0 was incubated with intestine crude enzyme extract (30 µL) for 60 min at 25°C. Two hundred forty µL of 10% trichloroacetic acid (TCA) was then added to stop the reaction. After 15 min, centrifugation was carried out at 8,000 x g for 5 min. The supernatant (70 µL) was added to 1 M NaOH (130 µL) in a 96-well microtiter plate and the absorbance of this mixture was measured in a microtiter plate reader (Bio-Rad 680, Japan) at 450 nm against a similarly prepared blank in which 0.01M Tris-HCl pH 8.0 replaced the crude extract sample. One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001 change in absorbance per minute per milligram of protein (Bezerra et al., 2005).

Trypsin and chymotrypsin activity

The activity of trypsin and chymotrypsin was determined using 8mM BApNA (Nα-benzoyl-DL-arginine-p-nitroanilide) and 8mM SApNA (Suc-Ala-Ala-Pro-Phe p-nitroanilide)
in DMSO (Dimethyl sulfoxide), respectively. Intestine crude enzyme extract (30 µL) was incubated with 0.1M Tris-HCl buffer pH 8.0 (140 µL) and respective substrates (30 µL) in a microtiter plate reader (Bio-Rad 680, Japan). The absorbance was measured at 405 nm against a similarly prepared blank in which 0.1M Tris-HCl pH 8.0 replaced the crude extract sample. Enzyme activity was determined in triplicate. Trypsin and chymotrypsin units of activity were expressed as a change in absorbance per minute per milligram of protein.

Aminopeptidase activity

Aminopeptidase activity was evaluated using aminoacyl of β-naphthylamide (AA of arginine) as substrate. The procedure adapted from Oliveira et al. (1999) was carried out in triplicate, by incubating 4.2mM substrate (50 µL), 50mM sodium phosphate buffer pH 7.0 (600 µL) and deionised H2O (50 µL) at 37 °C. After temperature equilibration, the crude enzyme extract (50 µL) was added and 30 minutes later, the reaction was stopped by adding 1mg mL⁻¹ fresh Garnet reagent (250 µL) in 0.2M sodium acetate buffer pH 4.2 containing 10% Tween 20 (v/v). After 10 minutes, absorbance was measured at 525 nm (Bio-Rad SmartSpec 3000, USA) and the amount of β-naphthylamine was determined using a standard β-naphthylamine curve. Activity was expressed as protease mU mg⁻¹ of protein. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze one µmol of p-nitroaniline per minute per milligram of protein.

Amylase activity

Amylase activity was evaluated according to Bernfeld (1955) using 2% starch as substrate: 60 µL intestine crude extract were incubated with 375 µL starch solution and 375 µL 10mM phosphate buffer pH 8.0 containing 15mM NaCl at 25 °C. After 20 minutes, 3.5-dinitro salicylic acid (DNSA) was added and the solution was submitted to 100 °C for 10 min.
After temperature equilibration, absorbance was measured at 570 nm (Bio-Rad SmartSpec 3000, USA) against a similarly prepared blank in which 10 mM phosphate buffer replaced the crude extract sample. Enzyme activity was determined in triplicate. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 mg of maltose per milligram of protein per min.

HYDROLYSIS PROFILE OF SPH

The hydrolysis profile of shrimp hydrolysate was evaluated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using stacking gel at 4% (w/v) and separation gel at 17% (Laemmli, 1970). SPH was produced as previously described and enzymatic hydrolysis was followed at sampling times of 0, 30, 60, 90, 120, 150 and 180 minutes.

ENZYME CHARACTERIZATION IN SUBSTRATE-SDS-PAGE

Proteases from intestine crude extract of O. niloticus were studied in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using stacking gel at 4% (w/v) and separation gel at 12.5% (Laemmli, 1970). Zymograms were carried out based on García-Carreño et al. (1993). After electrophoresis, the gels were immersed in 2.5% Triton X-100 dissolved in 0.1 M Tris-HCl buffer pH 8.0 to remove the SDS and incubated with 3% casein (w/v) in 0.1 M Tris-HCl buffer pH 8.0 for 30 min at 4°C. The temperature was raised to 37°C and maintained for 90 min to allow the digestion of casein by the active fractions. Finally, the gel was stained overnight for protein in 0.18% (w/v) Coomassie Brilliant Blue R250 prepared in acid acetic and methanol (10:25% v/v) and the background of the gel was distained in
acetic acid and methanol (10:25% v/v). Clear bands in blue background denoted protease bands by digestion of casein substrate.

INHIBITION ASSAYS

The following inhibitors prepared in DMSO at a final concentration of 2 mM were used: Tosyl phenylalanine chloromethyl ketone (TPCK - chymotrypsin inhibitor); Phenyl-methyl-sulfonyl-fluoride (PMSF - serine proteases inhibitor); benzamidine and tosyl-lysine chloromethyl ketone (TLCK), both trypsin inhibitors; and bestatin (leucine aminopeptidase inhibitor) (Bezerra et al., 2005). Samples of enzyme extract and inhibitors were incubated at 25°C for 30 min and zymogram was performed as described above. The 100% values (control) were established using DMSO without inhibitors.

GROWTH PARAMETERS

Fish performance was evaluated through final mass (FM), average daily gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER), based on the following formulae: MG (Mass gain) = 100 (final mass - initial mass) / initial mass; ADG = mass gain (g) / time (days); SGR = 100 (Ln Mf − Ln Mi) / time (days); FCR = dry feed offered (g) / wet mass gain (g); PER = wet mass gain (g) / protein fed (g).

PROXIMATE BODY COMPOSITION

Compositional analyses were performed on individuals at the end of the experiment. Pooled samples of fish were frozen before and after the feeding trial (two fish from each
aquarium). Lipid and protein content of whole fish were determined using standard methods (AOAC, 1984).

STATISTICAL ANALYSIS

Data on enzyme activity were analyzed using one-way analysis of variance (ANOVA) complemented with Tukey’s test. Chymotrypsin and acid proteolytic activity data, which failed normality or equal variance test, were transformed (ln) and submitted to parametric ANOVA. Correlations were tested using Pearson’s Product Moment Correlation following regression analysis. Differences were reported as statistically significant when P < 0.05, using the Jandel Scientific SigmaStat software program (version 2.0).

RESULTS

Figure 1 displays the hydrolysis profile of SPH. Enzymatic autolysis visibly promoted the digestion of proteins with the greatest molecular weight over time. At Time 0, most proteins had molecular weight between 25 and 100 kDa, but there were also proteins with weight greater than 220 kDa. At 150 min most proteins were smaller than 25 kDa.

Table IV displays the enzyme activity in the crude extracts. Acid proteolytic activity was the only enzyme activity assayed in crude extract from stomach tissues. Statistical differences (P<0.05) between animals fed the experimental diets (SPH0, SPH1.5, SPH3 and SPH6) were observed for chymotrypsin, trypsin and total alkaline proteases, but the greatest differences
were always between the experimental and commercial diets. Lower enzymes values were
always related to the commercial diets (Table IV).

The zymograms revealed caseinolytic activity in crude extracts of fish from all
treatments (Fig. 2). Twelve caseinolytic bands were found in animals fed SPH0: P1, P2, P3,
P4, P5, P6, P7, P8, P9, P10, P11 and P12. In all treatments, the proteins P1 to P4 were not
significantly affected by the inclusion of the protein hydrolysate, but P5 to P12 underwent
alterations in the caseinolytic pattern. As the concentration of hydrolysate increased, there
was a slowdown in the activity of proteases P6, P7 and P8. The opposite occurred for P5, P9,
P10, P11 and P12, in which the caseinolytic bands proved more intense.

Figure 3 displays the inhibition of alkaline proteases performed with the SPH0 crude
extract. SPH0 without inhibitors (control) revealed 12 caseinolytic bands, as also
demonstrated in Fig 2. TPCK did not significantly affect the caseinolytic activity of O
niloticus enzymes except for P11 that was the only enzyme inhibited. PMSF revealed high
inhibition of P9 and P11 caseinolytic bands. Benzanidine completely inhibited P6 and P8
bands. One band between P5 and P6 enzymes (white arrow) revealed activity in the presence
of benzanidine, but did not appear in the control. TLCK strongly inhibited P3, P4, P6, P7, P8
and P11. Bestatin totally inhibited seven digestive enzyme bands of O. niloticus (P3, P4, P5,
P6, P7, P8 and P9). Inhibitors have no effect on P1, P2, P10 and P12 caseinolytic bands.

No correlation was found between growth parameters (FM, MG, ADG, SGR, FCR,
PER), body composition (protein, and lipid contents), acid proteolytic activity, chymotrypsin,
amylase and total alkaline proteases. Positive correlations were found between growth parameters (FM, MG, ADG and SGR), body protein content and trypsin activity (Figure 4). Aminopeptidase activity was positively correlated with all the above-listed parameters, except with MG (Fig. 5). FCR and body lipid content were negatively correlated with both trypsin and aminopeptidase (Figures 4 and 5).

Please, insert Figs 4 and 5 here.

DISCUSSION

The nutritional quality of protein hydrolysate may be related to the high concentration of small peptides and essential amino acids as well as compounds that stimulate immune response in fish, thereby promoting growth and resistance to disease (Gildberg & Stenberg, 2001). These peptides are also accepted as attractive food due to chemical stimulation in larvae and juveniles, which contributes to the consumption of artificial diets in fish hatcheries.

A number of authors have analyzed digestive enzyme activity in aquatic organisms and dietary composition, reporting divergent results. Nagase (1964) and Kohla et al. (1992) found enhanced trypsin activity, corresponding to an increase in feeding rates for the species Mozambique tilapia, Oreochromis mossambicus (Peters) and C. macropomum, respectively. Papoutsoglou & Lyndon (2006) found an increase in chymotrypsin activity in Spotted wolfish, Anarhichas minor (Olafsen), when the protein concentration was reduced, indicating the adaptation of this species to low protein concentrations as a way to better absorb nutrients from the diet.

There is little information available in the literature regarding the specific effects of hydrolysate protein inclusion in diets on digestive enzymes, growth parameters and body
composition in fish. In the present study, no correlation was found between different concentrations of hydrolysate in the diets and the activity of any of the enzymes studied. Evaluating amylase and trypsin activity in European seabass, *Dicentrarchus labrax* (L.) larvae fed diets with 10 and 19% protein hydrolysate, Kotsamanis *et al.* (2007), also found no statistical differences between treatments. However, aminopeptidase activity was greater at a concentration of 10% protein hydrolysate.

Although there were differences in enzyme activity (total alkaline proteases, trypsin and chymotrypsin) between the fish fed different experimental diets in the present work, the greatest differences were between the commercial and experimental diets, which must be related to the content of fish meal in the formulated diets (about 20%), as its concentration in commercial feeds for tilapia is generally lower than 5% in Brazil. Quantitative studies on digestive enzymes appeared not be helpful in distinguishing the effects of different concentrations of shrimp protein hydrolysate (SPH) in diets. However, the use of substrate-SDS-PAGE revealed interesting results. This is a biochemical tool several times more sensitive than other methods for detecting the protease composition of crude extracts from tissues and allows the determination of enzyme activity zones caused by protease inhibitors (García-Carreño *et al.*, 1993).

Through analyses of inhibition zymograms (Fig. 3), it was possible to determine the following: one aminopeptidase-like inhibited only by Bestatin (P5) and probably another aminopeptidase (P9) inhibited by Bestatin and by PMSF; P3, P4, P6, P7, P8 and P11 seemed to be proteases of low specificity, with trypsin/aminopeptidase activity (P3, P4, P6, P7 and P8 which were inhibited by trypsin inhibitors and by Bestatin) and chymotrypsin/trypsin activity (P11 which was inhibited by PMSF, TPCK and TLCK). It was not possible to identify P1, P2, P10 and P12, as none of the inhibitors had an inhibitory effect over these proteases. Comparing the inhibition zymogram to Fig. 2, with the increase in the concentration of
shrimp protein hydrolysate, there was a slowdown of proteases with trypsin activity (P6, P7 and P8). On the other hand, aminopeptidase activity identified in P5 and P9 was increased with the increase in SPH. Moreover, proteases P10 and P12 (not identified) and P11 (with chymotrypsin-like/trypsin-like activity) also exhibited an increase in activity with the inclusion of SPH. Cahu et al. (2004) found that trypsin secretion was high in larvae of the D. labrax fed diets with 14% protein hydrolysate and was reduced at high concentrations (46%).

According to zymograms (Figs. 2 and 3) it was observed that the classical protease inhibitors herein employed were not effective over some enzymes (P1, P2, P10 and P12), and over other ones the inhibitory effect was not conclusive (P3, P4, P6, P7, P8, P9 and P11), which suggests a low compatibility of these enzymes with mammalian proteases. In fact, these commercial inhibitors are generally synthesized based on the mammalian enzyme mechanism and as the digestive system of fishes are different from that of mammals, probably there are also differences in the mechanisms of their enzymes. Even thought in the present work classical trypsin inhibitors were not enough effective on enzymes of tilapia crude extract, Bezerra et al. (2005) showed that purified Nile tilapia trypsin was strongly inhibited by TLCK and Benzanidrine. On the other hand, PMSF was able to inhibit only about 50% of the trypsin activity.

Fish use energy and monomers obtained from their diets for the synthesis of functional proteins and muscle growth. Ingested food is subjected to enzymes that break it down into compounds, which are absorbed by cells in gastrointestinal tract. Thus, it is very tempting to think that there may be a correlation between digestive enzyme activity and fish growth parameters or body composition. Indeed, the present study found that the activity of trypsin and aminopeptidase was correlated with growth parameters (final mass, mass gain, average daily gain, feed conversion ratio and specific growth rate) and body protein content. The results indicate the important role of these enzymes in the regulation of tilapia growth and
incorporation of proteins. Similar results were found by Lemieux et al. (1999), who describe positive correlations of trypsin and chymotrypsin with growth rates in the Atlantic cod, Gadus morhua (L.). Trypsin and aminopeptidase were negatively correlated with body fat content. As muscle growth (synthesis of protein) increases the energy demand in the cell, there may be a decrease in the concentration of precursors of endogenous lipid synthesis (Nelson & Cox, 2005), which could be related to the low concentration of lipids in the body.

Although there were differences in digestive enzyme activity between fish fed different experimental diets, there was no logical correlation between enzyme activity and different concentrations of shrimp protein hydrolysate in these diets. Substrate-SDS-PAGE zymogram proved to be an efficient tool for the detection of changes in enzyme activity in fish submitted to different diets. Through this method, different protease profiles were determined for each experimental diet. These data reinforce the known ability of tilapia to adapt to different food sources. Actually, as an omnivorous opportunistic fish, tilapia is able to assimilate nutrients from different sources, like benthic algae, phytoplankton, macrophytes, zooplankton, small invertebrates, detritus, cyanobacteria. The feed choice is mainly related to the availability of food items in the environment (Bowen, 1982, Fitzsimmons, 1997, Stickney, 1997, Beveridge & Baird, 2000, Lowe-McConnell, 2000). To benefit from all these sources, the enzyme arsenal of tilapia should be diverse enough to digest the wide range of ingested food. The activity of trypsin and aminopeptidase in the fish fed the experimental diets was significantly positively correlated with some growth parameters and body protein content, which demonstrates the importance of these enzymes in growth regulation.

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REFERENCES


525 Figure legends

526
527 Fig. 1. Hydrolysis profile of shrimp protein hydrolysate (SPH) in sodium dodecyl sulphate
528 polyacrylamide gel electrophoresis (SDS-PAGE) using stacking gel at 4% (w/v) and
529 separation gel at 17%. Lanes correspond to: molecular weight marker (MW), 0, 30, 60, 90,
530 120, 150 and 180 minutes of enzymatic hydrolyze. SPH was produced through autolysis of
531 Litopenaeus vannamei heads.

532
533 Fig. 2. Zymogram of digestive proteases (3% casein as substrate) of intestine enzyme extracts
534 from Nile tilapia Oreochromis niloticus fed different diets. Lanes correspond to: commercial
diet, SPH 0, SPH 1.5, SPH 3 and SPH 6. Further details in Material and Methods.

536
537 Fig. 3. Inhibition zymogram of digestive proteases (3% casein as substrate) of intestine
enzyme extracts from Nile tilapia Oreochromis niloticus fed SPH 0 diet. Lanes correspond to:
control without inhibitors, TPCK, PMSF, Benzamidine, TLCK and Bestatin. Further details in
Material and Methods.

540
541 Fig. 4. Relationship between trypsin activity (8mM BAPNA as substrate) and (A) final mass,
potential regression \(y = 0.0067x^{1.364}; R^2 = 0.9754; P = 0.0042\); (B) mass gain, polynomial
regression \(y = -4E-06x^2 + 0.0128x - 7.5996; R^2 = 0.864; P = 0.0325\); (C) average daily gain,
potential regression \(y = 7.6697x^{0.3167}; R^2 = 0.9731; P = 0.0050\); (D) specific growth rate,
potential regression \(y = 0.006x^{2.486}; R^2 = 0.9869; P = 0.0091\); (E) feed conversion ratio,
polynomial regression \(y = -6.6978x^2 + 4.364x + 6.5296; R^2 = 0.9825; P = 0.0011\); (F)
[Protein], potential regression \(y = 9E-15x^{2.9356}; R^2 = 0.8938; P = 0.0231\); (G) [Lipid],
exponential regression \(y = 13.931e^{-0.008x}; R^2 = 0.9251; P = 0.0192\) of Nile tilapia.
Oreochromis niloticus fed different diets; commercial diet (■), SPH 0 (□), SPH 1:5 (▲), SPH 3 (▲) and SPH 6 (▲). Error bars represent S.E. of the mean trypsin activity (n=3).

Fig. 5. Relationship between aminopeptidase activity (AA of arginine as substrate) and (A) final mass, potential regression \( y = 0.3655x^{14.409}; R^2 = 0.9324; P = 0.0177 \); (B) average daily gain, polynomial regression \( y = -297.04x^2 + 366.64x - 68.151; R^2 = 0.9313; P = 0.0201 \); (C) specific growth rate, polynomial regression \( y = -5.8787x^2 + 85.885x - 269.22; R^2 = 0.9257; P = 0.0204 \); (D) feed conversion ratio, polynomial regression \( y = -617.7x^2 + 1327x - 666.24; R^2 = 0.9718; P = 0.0107 \); (E) [Protein], polynomial regression \( y = -0.0025x^2 + 3.0128x - 865.28; R^2 = 0.956; P = 0.0154 \); (F) [Lipid], polynomial regression \( y = -0.0017x^2 + 0.6826x - 23.545; R^2 = 0.9258; P = 0.0202 \) of Nile tilapia Oreochromis niloticus fed different diets; commercial diet (■), SPH 0 (□), SPH 1:5 (▲), SPH 3 (▲) and SPH 6 (▲). Error bars represent S.E. of the mean aminopeptidase activity (n=3).
Table I. Proximate composition (%) and energy of lyophilized shrimp protein hydrolysate (SPH, from L. vannamei) (Silva, 2006).

<table>
<thead>
<tr>
<th>Composition</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>9.7</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>43.6</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>6.2</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>7.3</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>33.1</td>
</tr>
<tr>
<td>Energy (KJ 100 g⁻¹)</td>
<td>1520.8</td>
</tr>
</tbody>
</table>
Table II. Amino acid composition of the shrimp protein hydrolysate (SPH from *Litopenaeus vannamei*) (Silva, 2006).

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>SPH</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg 100g(^{-1})</td>
<td></td>
</tr>
<tr>
<td><strong>Essential</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>3400 ± 0.043</td>
<td>7.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>1060 ± 0.005</td>
<td>2.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2000 ± 0.021</td>
<td>4.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>3490 ± 0.021</td>
<td>7.4</td>
</tr>
<tr>
<td>Lysine</td>
<td>3350 ± 0.000</td>
<td>7.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>1290 ± 0.005</td>
<td>2.7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2370 ± 0.002</td>
<td>5.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>2120 ± 0.031</td>
<td>4.5</td>
</tr>
<tr>
<td>Thryptophan</td>
<td>670 ± 0.016</td>
<td>1.4</td>
</tr>
<tr>
<td>Valine</td>
<td>2250 ± 0.012</td>
<td>4.8</td>
</tr>
<tr>
<td><strong>Non-essential</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3370 ± 0.004</td>
<td>7.2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>4270 ± 0.031</td>
<td>9.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5780 ± 0.003</td>
<td>12.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>2890 ± 0.005</td>
<td>6.2</td>
</tr>
<tr>
<td>Serine</td>
<td>2030 ± 0.001</td>
<td>4.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>3070 ± 0.017</td>
<td>6.6</td>
</tr>
<tr>
<td>Proline</td>
<td>2970 ± 0.024</td>
<td>6.3</td>
</tr>
<tr>
<td>Cystine</td>
<td>410 ± 0.015</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>46790</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>
Table III. Composition and proximate analysis of the experimental diets.

<table>
<thead>
<tr>
<th>Ingredients (%)</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Commercial</td>
</tr>
<tr>
<td>Fish meal</td>
<td>23.0</td>
</tr>
<tr>
<td>Shrimp protein hydrolysate (SPH)</td>
<td>0.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>47.0</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>16.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>10.5</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.0</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Mineral and vitamin mix¹</td>
<td>1.0</td>
</tr>
<tr>
<td>Salt</td>
<td>0.5</td>
</tr>
<tr>
<td>Antioxidant BHT</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Proximate analysis (on as-fed basis)

<table>
<thead>
<tr>
<th></th>
<th>Commercial</th>
<th>SPH0</th>
<th>SPH1.5</th>
<th>SPH3</th>
<th>SPH6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (g kg⁻¹)</td>
<td>918.0</td>
<td>914.8</td>
<td>935.9</td>
<td>936.5</td>
<td>946.7</td>
</tr>
<tr>
<td>Crude protein (g kg⁻¹)</td>
<td>345.6</td>
<td>371.9</td>
<td>374.3</td>
<td>376.2</td>
<td>380.6</td>
</tr>
<tr>
<td>Ether extract (g kg⁻¹)</td>
<td>65.0</td>
<td>48.1</td>
<td>56.2</td>
<td>52.1</td>
<td>35.9</td>
</tr>
<tr>
<td>Crude fibre (g kg⁻¹)</td>
<td>34.9</td>
<td>39.7</td>
<td>38.8</td>
<td>41.1</td>
<td>46.6</td>
</tr>
<tr>
<td>Ash (g kg⁻¹)</td>
<td>67.5</td>
<td>105.7</td>
<td>102.9</td>
<td>101.6</td>
<td>101.9</td>
</tr>
<tr>
<td>Nitrogen-free extract</td>
<td>487.0</td>
<td>434.6</td>
<td>427.8</td>
<td>429.0</td>
<td>435.0</td>
</tr>
<tr>
<td>Calcium (g kg⁻¹)</td>
<td>14.3</td>
<td>22.2</td>
<td>21.7</td>
<td>20.0</td>
<td>17.2</td>
</tr>
<tr>
<td>Phosphorus (g kg⁻¹)</td>
<td>11.3</td>
<td>12.4</td>
<td>12.5</td>
<td>12.6</td>
<td>12.8</td>
</tr>
<tr>
<td>Gross energy (kJ 100 g⁻¹)</td>
<td>1931.8</td>
<td>1834.7</td>
<td>1860.7</td>
<td>1851.0</td>
<td>1807.5</td>
</tr>
</tbody>
</table>
1 Mineral and vitamin mix (kg⁻¹ premix): vitamin A (20,000UI), vitamin D₃ (5,000UI), vitamin E (250mg), vitamin K₃ (25mg), vitamin B₃ (37.5mg), vitamin B₁₂ (37.5mg), vitamin B₆ (25mg), vitamin B₁₂ (0.053mg), vitamin C (250mg), niacin (200mg), pantothenic acid (100 mg), biotin (1.25mg), choline (1,000mg), inositol (250mg), Fe (100mg), Cu (12mg), Zn (125mg), Mn (37.5mg), Se (0.25mg), I (1.25mg), Co (0.25mg).

2 Based on 5.65, 4.2 and 39.77 kJ g⁻¹ protein, carbohydrate and fat, respectively.
Table IV. Digestive enzyme activity on crude extracts of *Oreochromis niloticus* fed commercial and experimental diets containing increasing shrimp protein hydrolysate (SHP) levels.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Acid proteolytic activity</th>
<th>Alkaline proteolytic activity</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Aminopeptidase</th>
<th>Amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial</td>
<td>45.72±0.22 <em>a</em></td>
<td>8.20±1.06 <em>a</em></td>
<td>1.13±0.14 <em>a</em></td>
<td>11.96±2.13 <em>a</em></td>
<td>26.49±3.65 <em>a</em></td>
<td>25.24±3.74 <em>a</em></td>
</tr>
<tr>
<td>SHP 0</td>
<td>155.84±24.63 <em>a</em></td>
<td>11.30±1.23 <em>b</em></td>
<td>2.59±0.21 <em>b</em></td>
<td>22.70±2.52 <em>b</em></td>
<td>41.88±6.65 <em>b</em></td>
<td>45.47±8.78 <em>b</em></td>
</tr>
<tr>
<td>SHP 1.5</td>
<td>111.38±39.56 <em>a</em></td>
<td>15.35±1.70 <em>c</em></td>
<td>3.38±0.49 <em>c</em></td>
<td>39.74±7.88 <em>c</em></td>
<td>45.34±3.66 <em>c</em></td>
<td>53.50±10.41 <em>c</em></td>
</tr>
<tr>
<td>SHP 3</td>
<td>162.49±28.91 <em>b</em></td>
<td>13.60±1.64 <em>d</em></td>
<td>2.82±0.45 <em>d</em></td>
<td>36.89±5.41 <em>d</em></td>
<td>47.66±4.16 <em>d</em></td>
<td>48.79±9.64 <em>d</em></td>
</tr>
<tr>
<td>SHP 6</td>
<td>132.10±22.74 <em>c</em></td>
<td>15.23±1.78 <em>a</em></td>
<td>2.62±0.30 <em>a</em></td>
<td>36.66±7.20 <em>a</em></td>
<td>30.85±5.22 <em>a</em></td>
<td>56.77±8.63 <em>a</em></td>
</tr>
</tbody>
</table>

*Acid and alkaline proteolytic activity expressed as U mg of Protein (P)-1, trypsin, chymotrypsin and aminopeptidase activity expressed as mg of P-1 mg of P-1, and amylase activity expressed as mg of maltose min-1 mg of P-1. Different superscripts in the same column denote statistical differences (P < 0.05) (mean ± S.D. of three replicates).*
Figure 1
Figure 4
Figure 5
6. CONCLUSÕES

O presente estudo indicou que:

- Amilases dos camarões marinhos estudados apresentaram propriedades físico-químicas similares às de outros crustáceos descritos na literatura. Embora as três espécies sejam descritas como onívoras oportunistas, *Farfantepenaeus subtilis* e *Litopenaeus schmitti* apresentam uma tendência maior à carnivoria, o que foi corroborado pelas taxas A:P mais baixas. Foi identificado um grande número de isoformas de amilases no hepatopâncreas das três espécies, o que pode representar uma vantagem ecológica e sugerir que as espécies são capazes de se beneficiar com os carboidratos da dieta;

- O hepatopâncreas de *F. subtilis* e *F. paulensis* apresentam tripsina, quimotripsina e aminopeptidases, com características gerais próximas a de outros camarões peneideos. Essas enzimas parecem diferir estruturalmente das enzimas de mamíferos, visto que não respondem de forma semelhante aos inibidores clássicos de proteases utilizados. A correlação entre as aminopeptidases e as concentrações recomendadas dos respectivos aminoácidos na dieta de camarões pode ser indicativo de uma resposta fisiológica das espécies à hidrólise protéica;

- O hidrolisado protéico de camarão demonstrou ser uma boa fonte de aminoácidos para juvenis da tilápia do Nilo, podendo ser incluído nas dietas até o nível de 6%, sem causar efeitos adversos na utilização do alimento e no crescimento dos peixes;

- Não foi constatada correlação entre diferentes níveis de hidrolisado protéico de camarão nas dietas e a atividade enzimática das tilápias. Entretanto, essas diferenças puderam ser observadas através da análise de zimogramas de atividade, sugerindo que a técnica pode ser uma boa ferramenta para identificação de diferentes tratamentos alimentares. Os inibidores clássicos de proteases utilizados para identificação das enzimas de tilápias ou não tiveram efeito ou os efeitos não foram conclusivos, sugerindo baixa similaridade das enzimas com proteases de mamíferos.
7. ANEXOS
7.1. **NORMAS DO PERIÓDICO AQUACULTURE RESEARCH**

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Edited by:
Ronald W. Hardy, Marc Verdegem and Lindsay Ross

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5. Use of active voice (usually the first person). The passive voice should be used.
6. References, *e.g.* lack of match between text and list and wrong format.
7. Figure and caption, *e.g.* keys should be on the caption, not on the figure.
8. Variables not defined correctly. Normally these should be single letters qualified with subscripts if required, *e.g.* $LT$ for total length.
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1. Buarque, Diego Souza, Castro, Patrícia Fernandes, Santos, Fábio Marcel Silva, Lemos, Daniel, Júnior, Luiz Bezerra Carvalho, Bezerra, Ranilson Souza

2. ASSIS, C. R. D., AMARAL, I. P. G., CASTRO, P. F., CARVALHO JUNIOR, L. B., BEZERRA, R. S.

3. LEGAT, Jefferson Francisco Alves, PUCHNICK, Angela, CASTRO, P. F., PEREIRA, Alitiene Moura Lemos, GOES, J. M., GOES, L. C. F.

**Artigos completos aceitos para publicação**


**Capítulos de livros publicados**

1. BEZERRA, R. S., BUARQUE, D. S., AMARAL, I. P. G., CASTRO, P. F., ESPOSITO, T. S., CARVALHO JUNIOR, L. B.

**Trabalhos publicados em anais de eventos (resumo)**

   Alkaline proteases from pyloric caeca of bijupirá (Rachycentrun canadum) In: XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq e XI Congresso da PABMB, 2008, Águas de Lindóia. 
   Anais do XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq e XI Congresso da PABMB. 2008.

   Anais do XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq e XI Congresso da PABMB. , 2008.


   Anais do XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq e XI Congresso da PABMB. , 2008.


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*[Anais do XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq e XI Congresso da PABMB.]*, 2008.

*[Anais do XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq e XI Congresso da PABMB.]*, 2008.

*[Anais do XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq e XI Congresso da PABMB.]*, 2008.

Alkaline proteases from tropical fish processing waste as heavy metal In: XXXVI Reunião Anual da SBBq, 2007, Salvador.
*[XXXVI Reunião Anual da SBBq.]*, 2007.

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