UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

ENZIMAS DIGESTIVAS DO CAMARÃO BRANCO *Litopenaeus vannamei* CULTIVADO COM DIETAS À BASE DE CONCENTRADO PROTÉICO DE SOJA EM SUBSTITUIÇÃO À FARINHA DE PEIXE

DOUGLAS HENRIQUE DE HOLANDA ANDRADE

RECIFE, 2011

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco como pré-requisito para a obtenção do grau de mestre em Ciências Biológicas

Orientador: Prof. Dr. Ranilson de Souza Bezerra
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Esta dissertação foi julgada para a obtenção do título de Mestre em Ciências Biológicas e aprovada em 11 /02/2011 pelo Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco em sua forma final.

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“Quer você ache que pode, quer você ache que não pode, em ambos os casos você está certo.”

Henry Ford

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Dedico aos meus pais e meus irmãos, pelo incentivo e apoio para enfrentar os desafios da vida.

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RESUMO

Nos últimos anos, a aquicultura tem apresentado um rápido desenvolvimento, sendo a carcinicultura um dos segmentos mais lucrativos e crescentes. Apesar do progresso dessa atividade econômica, o custo com a alimentação dos animais ainda representa um dos principais problemas para os produtores. Com isso, a substituição da farinha de peixe, ingrediente mais caro da dieta dos camarões, por fontes protéicas alternativas tem sido cada vez mais frequente. Desta forma, objetivou-se avaliar o efeito da substituição da farinha de peixe por concentrado protéico de soja (SPC) nos níveis de 0% (C), 30% (S_{30}), 60% (S_{60}) e 100% (S_{100}) sobre o desempenho das enzimas digestivas do *Litopenaeus vannamei*. Para tanto, espécimes com 2,02±0,51g foram submetidos às dietas experimentais ao longo de dez semanas. Após esse período, foi realizada a biometria dos animais. Hepatopâncreas de quinze camarões de cada tratamento foram coletados, homogeneizados em tampão Tris-HCl 10mM, pH 8,0 com adição de NaCl 15mM e centrifugados para obtenção dos extratos enzimáticos. Para a análise das enzimas digestivas presentes nos extratos enzimáticos realizou-se ensaios *in vitro* na presença dos substratos de cadeia longa (azocaseína 1% e amido 2%), p-nitroanilide (BApNA, SApNA e Leu-p-Nan) e β-naphthylamide (alanina, arginina, leucina, tirosina, serina, glicina, isoleucina e histidina). Além disso, foram realizados SDS-PAGE e zimogramas de atividade proteolítica e amilolítica. Dentre os grupos experimentais o S_{100} apresentou maior ação enzimática quando empregado os substratos azocaseína 1% (1,18±0,01 $U.mg^{-1}$) e amido 2% (5,04±0,33 $U.mg^{-1}$) para a determinação da atividade proteolítica e amilolítica total, respectivamente. Maiores atividades de enzimas quimotripsina (13,78±1,61 $U.mg^{-1}$) e leucino aminopeptidase (0,45±0,03 $U.mg^{-1}$) utilizando os respectivos substratos SApNA e Leu-p-Nan foram observadas para o grupo controle (C). Enquanto que a mais elevada atividade tríptica (13,13±0,53 $U.mg^{-1}$), usando BApNA como substrato, foi constatada para o tratamento S_{30}. Entre os substratos β-naphthylamide analisados, verificou-se valores mais altos de atividade aminopeptídica para arginina e alanina em todos os tratamentos, principalmente no S_{30} que também obteve maior atividade na presença da glicina (1,05±0,08 $U.mg^{-1}$). Notou-se que para a serina, a atividade das aminopeptidases sofreu uma redução gradativa à medida que aumentou o nível de SPC na dieta dos camarões. O tratamento S_{60} apresentou maior atividade aminopeptídica para isoleucina (0,69±0,02 $U.mg^{-1}$) e histidina (0,85±0,04 $U.mg^{-1}$). Em relação à leucina e tirosina, a atuação das aminopeptidases mostrou-se indiferente estatisticamente às variações dietárias. De acordo com o perfil eletroforético dos extratos enzimáticos através de SDS-PAGE, foram observadas vinte e seis bandas protéicas, compreendidas entre 6,9 e 198,8 KDa, para todos os tratamentos. O zimograma de protease exibiu dois perfis semelhantes, um com dezoito (C e S_{30}) e outro com doze bandas proteolíticas (S_{60} e S_{100}). Enquanto que o zimograma de amilase revelou cinco bandas com atividade amilolítica para todos os tratamentos. A análise do ganho de peso corporal médio dos camarões cultivados mostrou valor mais elevado com o uso da dieta S_{30} (8,48±1,03 g), entretanto não foram evidenciadas diferenças significativas ($P<0,05$) entre os tratamentos. Os resultados expostos concluíram que a substituição da farinha de peixe por SPC em 30, 60 e 100% nas dietas dos camarões cultivados proporcionou um efeito positivo na performance dos animais. Esses resultados fornecem informações importantes quanto ao potencial do camarão-branco (*L. vannamei*) em utilizar formulações de alimentos alternativos com baixos níveis de fontes de proteína animal.

Palavras - chave: *Litopeneaus vannamei*, ração, proteína de soja, proteases, amilase.

ABSTRACT

In the last few years, aquaculture went through a rapid development, being shrimp farming one of the most profitable and growing segments. Despite the progress of this economical activity, the cost of animal feed still represents a major financial problem for producers. Thus, the replacement of fishmeal, most expensive ingredient of the diet, by alternative protein sources have been increasingly frequent. Therefore, the objective of the present study was to evaluate the effect of the replacement from fishmeal by soybean protein concentrate (SPC) at levels of 0% (C), 30% (S_{30}), 60% (S_{60}) and 100% (S_{100}) on the performance of the digestive enzymes of *Litopenaeus vannamei*. For this, specimens with 2.02 ± 0.51 g were subjected to experimental diets for ten weeks. After this period was performed the biometry of the animals. Then fifteen shrimp midgut glands of each treatment were randomly collected, homogenized in 10 mM Tris-HCl, pH 8.0 with 15 mM NaCl and centrifuged to obtain the crude extracts. For the analysis of the digestive enzymes present in the crude extracts there were carried out several *in vitro* assays, in the presence of long-chain substrates (1% azocasein and 2% starch), p-nitroanilide (BApNA, SApNA and Leu-p-Nan) and β-naphthylamide (alanine, arginine, leucine, tyrosine, serine, glycine, isoleucine, and histidine). Moreover, there were performed SDS-PAGE and zymograms of proteolytic and amylolytic activities. Among the experimental groups, the S_{100} showed higher enzyme activity when the substrates 1% azocasein (1.18 ± 0.01 $U.mg^{-1}$) and 2% starch (5.04 ± 0.33 $U.mg^{-1}$) were employed for the determination of total proteolytic and amylolytic activities, respectively. Major activities of chymotrypsin enzymes (13.78 ± 1.61 $U.mg^{-1}$) and leucine aminopeptidase (0.45 ± 0.03 $U.mg^{-1}$) using their respective substrates SApNA and Leu-p-Nan were observed for the control group (C). While the highest trypsin activity (13.13 ± 0.53 $U.mg^{-1}$), using BApNA as substrate, was observed for the S_{30} treatment. Among the β-naphthylamide substrates analyzed, there were higher levels of aminopeptidasic activity for arginine and alanine in all treatments, mainly in the S_{30} that also showed increased activity in the presence of glycine (1.05 ± 0.08-$U.mg^{-1}$). It was noted that for serine, the activity of aminopeptidases was reduced gradually as the level of SPC was increased in the diets. The treatment S_{60} showed higher aminopeptidasic activity for isoleucine (0.69 ± 0.02 $U.mg^{-1}$) and histidine (0.85 ± 0.04 $U.mg^{-1}$). In relation to leucine and tyrosine, the action of aminopeptidases was unmoved statistically dietary variations. According to the SDS-PAGE profile of the crude extracts, there were found 26 protein bands between 6.9 and 198.8 kDa for all treatments. The zymogram of protease exhibited two similar profiles, one with eighteen (C and S_{30}) and another with twelve proteolytic bands (S_{60} and S_{100}). While the zymogram of amylase revealed five bands with amylolytic activity for all treatments. The average body weight gain of shrimps showed the highest value when used the S_{30} diet (8.48±1.03 g), however did not evidenced significant differences ($p<0.05$) between treatments. The above results concluded that the substitution of fishmeal by SPC at 30, 60 e 100% in the diets of farmed shrimps provided a positive effect on animals performance. These results provide important information about the potential use of lower levels of protein from animal sources while formulating feeds for white shrimp.

Keywords: *Litopeneaus vannamei*, feed, soybean protein, proteases, amylase.

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LISTA DE ABREVIATURAS

AA-NA – aminoacil-β-naftilamida

AA-Nan – aminoacil-p-nitroanilida

ABCC – Associação Brasileira de Criadores de Camarão

BApNA – benzoil arginina ρ-nitroanilida

DFP – diisopropil-fluorfosfato

EC – Enzyme Commission

ES – complexo Enzima-Substrato

FAO – Food and Agriculture Organization

IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

IUBMB – União Internacional de Bioquímica e Biologia Molecular

KDa – quilo Daltons

Leu-p-Nan – aminoacil de β - naftilamida

PB – proteína bruta

PMSF – fluoreto fenil-metil-sulfonil

RNA – ácido ribonucleico

SApNA – N-succinil-Ala-Ala-Pro-Phe-p-nitroanilida

SBO – óleo de soja

SBTI –inibidor de tripsina de soja

SDS-PAGE – eletroforese em gel de poliacrilamida utilizando Dodecil sulfato de sódio

SPC – concentrado protéico de soja

TAME – tosil-arginina-metil-éster

TCA – Ácido Tricloroacético

SUMÁRIO

1. INTRODUÇÃO

A pesca extrativa mundial encontra-se no máximo de seu potencial e, em contraste, a produção da aquicultura vem assumindo uma importância cada vez maior, sendo apontada como a principal opção para aumentar a oferta de pescado por todo o mundo (FAO, 2008). Além de ser uma atividade econômica bastante relevante, a aquicultura tem apresentado um constante crescimento devido não só ao aumento na demanda por produtos pesqueiros, mas também por representar uma alternativa para amenizar a exploração dos recursos naturais (GOLDBURG & NAYLOR, 2005).

Segundo dados da FAO (2008), foram produzidos cerca de 144 milhões de toneladas de pescado em 2006, das quais 92 milhões foram oriundos da pesca extrativa e aproximadamente 52 milhões, da aquicultura. Apesar da captura de organismos aquáticos ainda ser responsável por cerca de 63% do total de pescado fornecido, a atividade vem apresentando estabilidade desde a década de 80 do último século. Ainda de acordo com a FAO (2008), no período de 2002 a 2006, a captura diminuiu de 93 para 92 milhões de toneladas, enquanto que a aquicultura cresceu 30%, passando de 40 para 52 milhões de toneladas.

Entre os diversos segmentos da aquicultura, o cultivo de camarão ou a carcinicultura é um dos setores mais lucrativos, apresentando crescimento acelerado desde a década passada. Esta atividade, que surgiu no sudoeste da Ásia no século XV com a captura de larvas marinhas (ARANA, 1999), apresentou no ano de 2006 uma produção global de camarões marinhos de 6,6 milhões de toneladas. Desse total, 52,23% foram provenientes da pesca e 47,77% da aquicultura. Ainda relacionado a essa produção, 45,82% corresponderam à captura e cultivo de apenas duas espécies de peneídeos: o *Litopenaeus vannamei* (BOONE, 1931) e o *Penaeus monodon* (FABRICIUS, 1798), principais espécies das Américas e Ásia, respectivamente (FAO, 2008).

Desde o surgimento da carcinicultura, pacotes tecnológicos vêm sendo desenvolvidos com objetivo de ampliar a sua produtividade. No entanto, entre os desafios encontrados por parte dos produtores destacam-se os gastos com a alimentação, uma vez que a proteína é o componente mais oneroso da ração, alcançando cerca de 50% do custo total da produção (AKIYAMA et al., 1992; SHIAU, 1998; HERTRAMPF e PIEDAD-PASCUAL, 2000; LEMOS, 2003).

A formulação de uma ração é baseada nos requerimentos nutricionais dos organismos cultivados. Para camarões a principal fonte protéica é a farinha de peixe que também apresenta um balanço de aminoácidos e ácidos graxos adequado para o rápido crescimento desses organismos marinhos (CRUZ-SUÁREZ et al., 2000; HERTRAMPF e PIEDAD-PASCUAL, 2000). Entretanto, o emprego da farinha de peixe é afetado por fatores econômicos, ecológicos e de mercado, os quais elevam seu custo e restringem a sua utilização (GUZMAN, 1996). Com isso, a substituição por

fontes protéicas alternativas tem sido cada vez mais utilizada em formulações de rações comerciais (EAPA, 2006; SWICK, 2007). Podem ser citados como fontes alternativas, os subprodutos da pesca e da pecuária e ingredientes de origem vegetal. Muito embora, são necessários estudos que evitem o fornecimento de alimentos que possam apresentar fatores antinutricionais e deficiência de aminoácidos essenciais (LONGAS, 1996).

No entanto, nem sempre a aplicação de uma ração nutricionalmente balanceada irá produzir o crescimento esperado, o que pode consequentemente, comprometer o retorno do investimento empregado (LEE & LAWRENCE, 1997). Tal fato pode ser referido à falta de conhecimento da fisiologia digestória dos animais cultivados, sobretudo das suas enzimas digestórias. Segundo Fernández 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. Além disso, a atividade específica das 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).

2. OBJETIVOS

2.1. Geral

Avaliar o efeito da substituição da farinha de peixe por concentrado protéico de soja (SPC) sobre o desempenho das enzimas digestivas do camarão branco *Litopenaeus vannamei.*

2.2. Específicos

- Determinar a atividade de endoproteases e exoproteases do hepatopâncreas do *L. vannamei* submetidos a dietas com diferentes níveis de concentrado protéico de soja em substituição à farinha de peixe;
- Avaliar a atividade de amilase total do hepatopâncreas do *L. vannamei* submetidos a essas dietas;
- Analisar o perfil protéico das enzimas digestivas dos camarões através de SDS-PAGE e verificar a atividade dessas enzimas mediante zimogramas.

3. REVISÃO DA LITERATURA

3.1. Histórico e situação atual da carcinicultura marinha no Brasil

O desenvolvimento da produção de camarões marinhos em cativeiro no Brasil pode ser dividido em três fases principais, as quais 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 1970 a 1984, com o cultivo da espécie exótica *Marsupenaeus japonicus* em sistemas extensivos (ROCHA, 2001). Apesar de ser uma das espécies mais importantes cultivadas no continente asiático, na época, sua produção foi inviabilizada no Nordeste brasileiro, devido a problemas na qualidade da água, decorrentes de períodos chuvosos.

Este fato levou os produtores a investirem nas técnicas de maturação, reprodução e larvicultura das espécies nativas *Litopenaeus schmitti*, *Farfantepenaeus subtilis*, *F. paulensi* e*, F. brasiliensis*, caracterizando assim, a segunda fase da carcinicultura nacional (MAIA, 1993). Novamente a produtividade foi baixa, principalmente devido à falta de informações sobre os requerimentos nutricionais das espécies e à inexistência de rações que atendessem a suas exigências nutricionais (BRASIL, 2001).

No início dos anos 90, ocorreu uma revolução na carcinicultura marinha no Brasil com a introdução da espécie *Litopenaeus vannamei* (BARBIERI JUNIOR e OSTRENSKY, 2002). Nessa terceira fase o cultivo de camarões se tornou uma atividade importante e bastante rentável (BURGOS-HERNÁNDEZ et al., 2005), especialmente no período que vai de 1998 a 2003, no qual a atividade apresentou um incremento de 1244% (Figura 1). Dentre os fatores que proporcionaram o sucesso no desenvolvimento do cultivo do *L. vannamei*, destacam-se o domínio da técnica de criação, a disponibilidade de ração adequada e a elevada capacidade de adaptação da espécie às condições de cultivo semi-intensivo e intensivo (IBAMA, 2010). Esse crescimento foi mais perceptível nos estados do Nordeste, devido a essa região apresentar um litoral com condições ideais para o cultivo de camarão marinho, possibilitando a criação desses crustáceos o ano todo (NUNES, 2001; LOPES, 2006).

Esse aumento na produtividade sofreu uma retração no ano de 2004, principalmente devido ao surgimento de doenças como o vírus da mionecrose infecciosa, a queda no câmbio do dólar e a ação antidumping movida pelos EUA contra o camarão brasileiro (ABCC, 2008). A participação do camarão foi reduzida de 244,79 para 74,86 milhões de dólares na Balança Comercial de Pescado do Brasil entre 2003 e 2007 (ABCC, 2009). A crise na carcinicultura brasileira se estendeu durante o período de 2004 a 2007 e provocou uma interrupção no crescimento exponencial de 71% ao ano (ROCHA, 2008).

Os reflexos da crise na produção de camarões no Brasil geraram muitas incertezas no setor, evidenciadas pela perda de competitividade das suas exportações e ineficiente cadeia de comercialização interna. Segundo Rocha (2007), a valorização do Real e o aumento dos custos de produção superaram todas as demais adversidades e se constituíram como os principais entraves para a sustentabilidade econômica do setor.

Recentemente, de acordo com os dados do IBAMA (2010), o cultivo de camarões marinhos no Brasil retomou seu crescimento e a produção se manteve nos patamares de 70251,2t em 2008 e de 65189,0t em 2009 (Figura 1). Os investimentos e o aprimoramento de tecnologias no setor da carcinicultura impulsionaram o desenvolvimento da atividade e colocaram o Brasil numa posição de destaque na área de produção de camarões marinhos. Os avanços na área da genética, alimentação, reprodução, doenças e o aprimoramento do sistema de manejo estão amplamente referenciados no acervo tecnológico elaborado e organizados pela ABCC (MARTINS, 2006). Porém, apesar da superação dos principais problemas, ainda observa-se certa fragilidade na carcinicultura brasileira, em consequência, dentre outros fatores, de estar baseada praticamente em uma única espécie de camarão, o *Litopenaeus vannamei*.

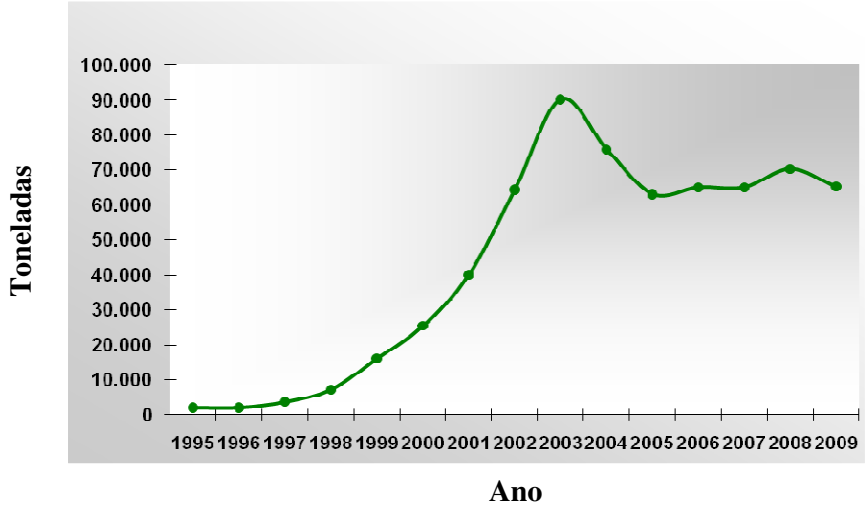

Figura 1. Evolução da produção (em toneladas) da carcinicultura no Brasil entre os anos de 1995 a 2009. Fonte: (IBAMA, 2010).

3.2. *Litopenaeus vannamei*

O camarão branco *Litopenaeus vannamei* (Figura 2) é uma espécie que está distribuída desde o leste do Oceano Pacífico, a altura de Sonora, no México, até a altura de Thumbes, norte do Peru. Com preferência por fundos lamosos, a espécie pode habitar desde a região do infralitoral, até

profundidades de 72 metros. Na natureza pode chegar a 23 cm de comprimento e apresenta hábito alimentar onívoro (BARBIERI JR e OSTRENSKY NETO, 2001).

Figura 2. Camarão exótico *Litopenaeus vannamei*

Pertencente a família *Penaeida*, o *L. vannamei* apresenta ciclo de vida semelhante aos demais membros, com desenvolvimento dos estágios (Figura 3): larva (náuplio) com cinco sub-estágios (N1 a N5) e duração de 36 horas, protozoea com três sub-estágios (Z1 a Z3) e duração de 48 horas, misis com três sub-estágios (M1 a M3) e duração de cerca de três dias, pós-larva, juvenil e adulto (ALFONSO; COELHO, 1997; DALL et al., 1999; PRIMAVERA, 1984; ANDREATTA e BELTRAME, 2004). Nos estágios de pós-larvas os camarões apresentam anatomia e fisiologia semelhante a um camarão adulto, diferindo apenas em alguns detalhes. Os juvenis, por sua vez, são exatamente iguais aos adultos, porém sem atingir a maturação gonadal (BARBIERI JR e OSTRENSKY NETO, 2001).

O ciclo de vida dos camarões peneídeos no habitat natural é migratório e tem como finalidade única, incrementar as chances de sobrevivências da prole (NUNES, 2001). As três primeiras fases de vida ocorrem no oceano, mais precisamente na região planctônica. A partir da fase pós-larval, os animais são encontrados em zonas estuarinas com salinidade moderada e na última fase, a adulta, retorna ao ambiente marinho para o processo de maturação e desova (VALLES-JIMENEZ et al., 2005).

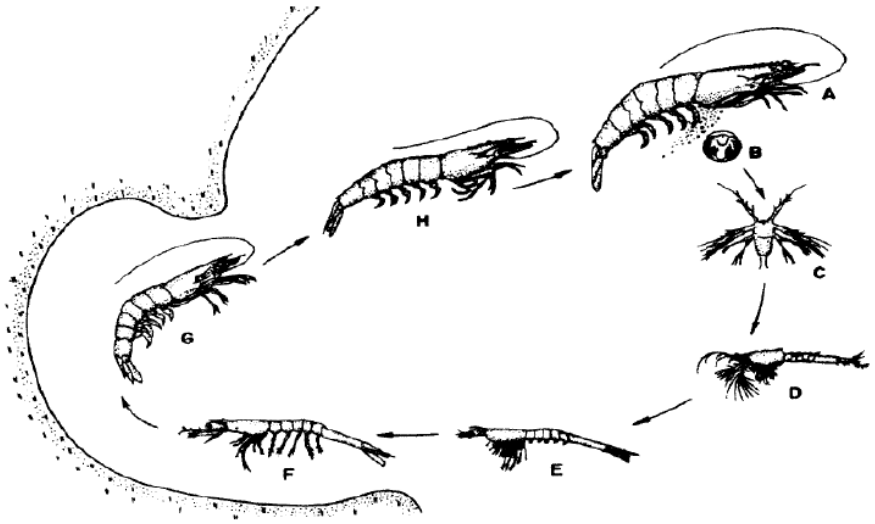

Figura 3. Ciclo de vida do camarão marinho. A, reprodutor desovando; B, ovo; C, náuplio; D, zoea; E, misis; F, pós-larva; G, juvenil; H, Adulto. Fonte: (FREITAS, 2003).

A capacidade de adaptação às mais variadas condições de cultivo, aliada aos altos índices zootécnicos como elevadas taxas de crescimento e conversão alimentar posicionaram o camarão branco do Pacífico como a principal espécie cultivada em toda a América Latina, onde é empregado em sistemas semi-intensivo e intensivo (WAINBERG e CÂMARA, 1998).

De acordo com Sá (2003), o *L. vannamei* tem uma excelente performance em cultivo, se desenvolvendo muito bem em uma salinidade entre 15 e 30‰, com temperatura entre 23 e 30 °C. O requerimento alimentar para o cultivo em confinamento, em termos de ração peletizada, contempla uma carga de proteínas que pode variar entre 22 e 40%, em dependência da intensificação do cultivo nos viveiros; da capacidade de tolerância em alta densidade de estocagem; do baixo requerimento protéico da sua dieta alimentar; e da produtividade natural das águas em uso. Quanto à aceitação comercial da espécie, que garante o custeio de todo o ciclo produtivo, o *L. vannamei* é significativamente preferido entre as demais espécies no mercado nacional e internacional, com forte demanda compradora.

3.3. Características Morfológicas dos Camarões

3.3.1. Anatomia Externa

O corpo dos camarões é dividido em duas regiões distintas compostas por cefalotórax e abdômen. No cefalotórax, o qual é formado pela fusão entre a cabeça e o tórax e localizado na região anterior, são encontradas estruturas de grande importância funcional para o animal. Dentre elas, a carapaça cuja função é recobrir e proteger as brânquias e os órgãos vitais, os olhos pedunculados, responsáveis pela visão e o rostro que é uma estrutura pontiaguda com função de proteger o animal contra os predadores (Figura 4). Nesta região, também se encontram apêndices profundamente modificados. Os dois primeiros pares de apêndices são antenas e estão situadas numa posição pré-oral responsáveis basicamente pela função sensorial. Os três últimos pares de apêndices localizam-se atrás da boca (um par de mandíbulas e dois pares de maxilas) úteis na alimentação do animal. A mandíbula possui bordas capazes de moer e cortar os alimentos, enquanto que as maxilas ajudam as mandíbulas na manipulação do alimento. Ainda no cefalotórax encontram-se cinco pares de patas conhecidas por pereiópodes (apêndices ambulatórios) que desempenham a função de locomoção, cópula (nos machos) ou ainda o transporte de óvulos (nas fêmeas). Na região abdominal encontram-se os pleiópodos, responsáveis pela locomoção natatória do animal. Já no final desta região está presente o Telson, estrutura pontiaguda que juntamente com os urópodes formam o último segmento abdominal. O telson auxilia nos ataques de defesa e os urópodes são responsáveis por direcionar o animal durante o deslocamento natatório (BARBIERI JR; OSTRENKSKY NETO, 2001).

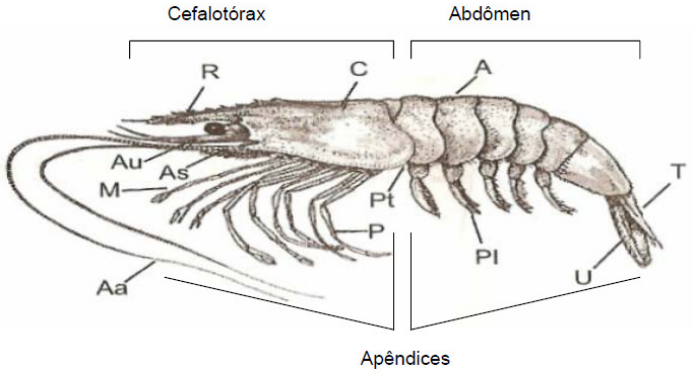

Figura 4. Vista lateral de um camarão *L. vannamei* macho. A, abdômen; Aa, antena; As, escama antenal; Au, antênula; C, carapaça; M, terceiro maxilípide; P, pereiópode; Pl, pleópodo; Pt, petasma; R, rostro; T, telson; U, urópodo. Fonte: (BARBIERI JR; OSTRENSKY NETO, 2001).

3.3.2. Anatomia interna

A anatomia interna dos camarões se assemelha aos representantes do grande grupo dos artrópodes. No cefalotórax encontram-se vísceras importantes como o cérebro, coração, hepatopâncreas, estômago e as gônadas, enquanto parte do intestino e a maior parte da musculatura dos peneídeos encontra-se na região do abdômen (Figura 5) (BARBIERI JR e OSTRENKY NETO, 2001; ANDREATTA e BELTRAME, 2004).

Estes animais possuem órgãos excretores pareados e compostos de um saco terminal, um canal excretor e um duto de saída, todos localizados na cabeça, sendo chamados de glândulas antenais ou maxilares, pois os poros excretores encontram-se na base das antenas ou das maxilas. As brânquias excretam amônia e são os órgãos responsáveis pelo equilíbrio salino. O estômago possui muitos músculos permitindo que só seja repassado ao hepatopâncreas o que está totalmente liquefeito. O hepatopâncreas é uma glândula de suma importância, assumindo um papel fundamental no metabolismo destes organismos, interagindo com os processos fisiológicos de muda, além de produzir respostas rápidas a alterações induzidas por fatores endógenos e ambientais. É também responsável pelo armazenamento de substâncias de reservas e produção de enzimas digestivas. O sistema circulatório é aberto, possuindo hemolinfa (sangue) onde circulam os hemócitos. Os hemócitos são produzidos pelo tecido hematopoiético localizado próximo ao estômago. A hemolinfa passa por todo o corpo retornando sempre para o coração, principal órgão propulsor, pequeno e constituído por três partes de óstio. O órgão linfóide é o órgão responsável pela defesa, tornando-se hipertrofiado em algumas enfermidades. O sistema nervoso dos camarões marinhos é bem rudimentar e apresenta um cordão nervoso direcionado para todos os segmentos (RUPPERT e BARNES, 1996).

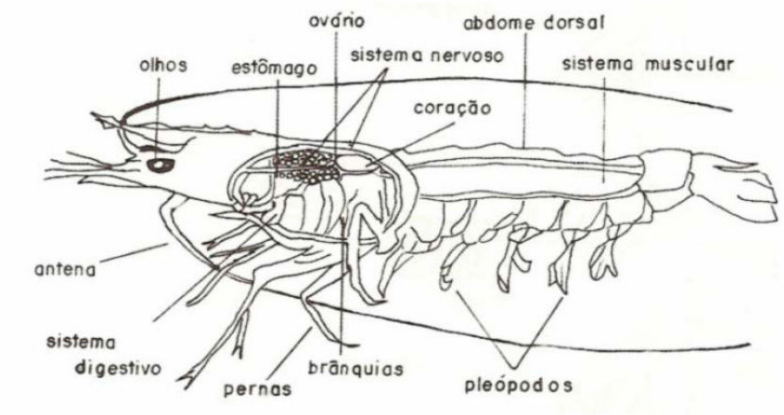

Figura 5. Principais órgãos internos do camarão marinho segundo Andreatta e Beltrame (2004).

3.4. Aparelho digestório dos camarões

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).

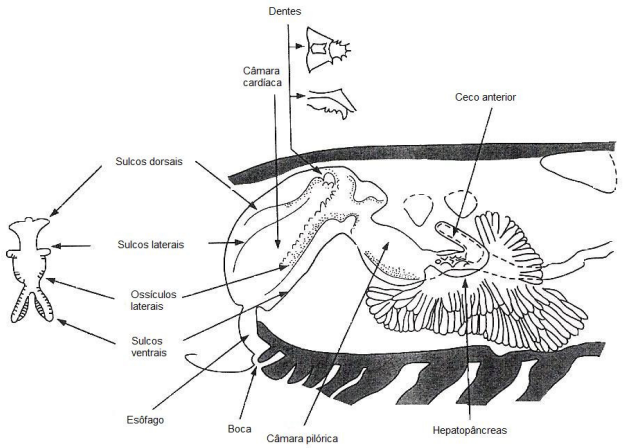

Figura 6. Esquema da anatomia do aparelho digestório de camarões (adaptado de Ceccaldi, 1997).

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 cárdio-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 fluído gástrico passam por essa rede de cerdas.

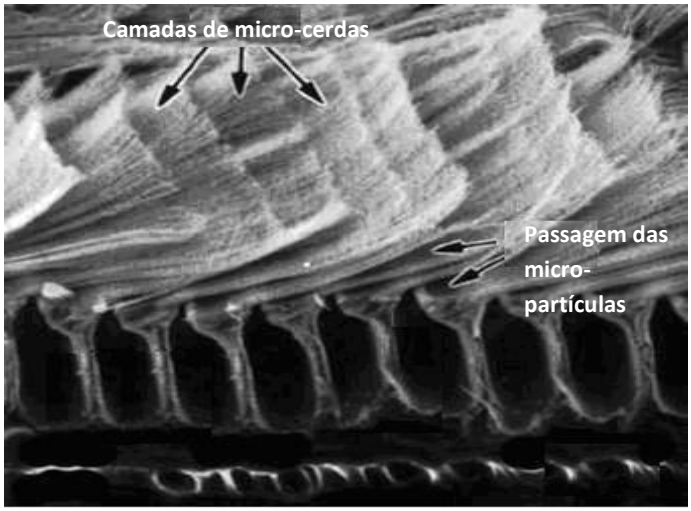

Figura 7. Filtro-prensa do estômago de *Penaeus monodon* (adaptado de Lin, 2000).

A glândula digestiva ou hepatopâncreas dos peneídeos 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 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 ânus 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 fluído gástrico e alimento no estômago de decápodas. 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 fluído 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.

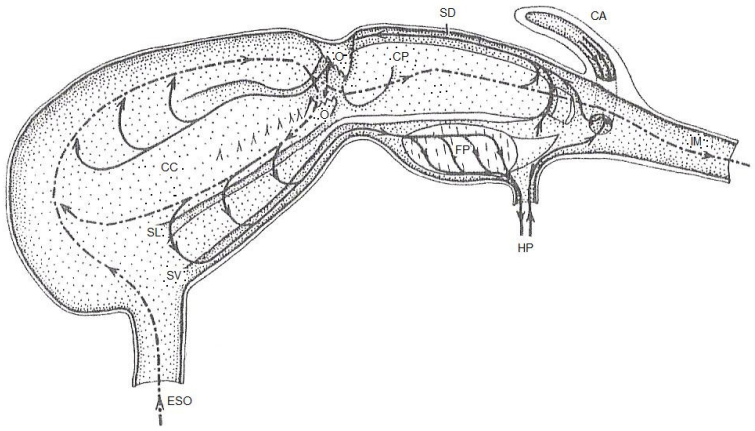

Figura 8. Diagrama da circulação do fluido gástrico e alimento no estômago de decápodas. Linhas pontilhadas: fluxo do alimento sólido; Linha contínua: fluxo do fluído; ESO: Esôfago; CC: Câmara cardíaca; O: ossículos do moinho gástrico; SL: sulcos laterais; SV: sulcos ventrais; CP: Câmara pilórica; SD: Sulcos dorsais da câmara pilórica; CA: Ceco anterior; HP: abertura do hepatopâncreas; FP; filtro-prensa; IM: intestino médio (DALL e MORIARTY, 1983).

3.5. Proteína de soja como fonte alternativa de alimento

A alimentação consiste num dos fatores mais importantes do cultivo de camarão. Através do alimento, os animais obtêm a energia necessária para sintetizar moléculas requeridas para o desenvolvimento, sobrevivência e realizar ações tais como: locomoção, reprodução e defesa.

Segundo Guillaume (1997), os crustáceos exigem uma suplementação equilibrada de aminoácidos essenciais. De acordo com Holmes et al. (2009) os aminoácidos essenciais na dieta dos crustáceos são arginina, histidina, isoleucina, leucina, lisina, metionina, fenilalanina, treonina, triptofano e valina. Outros aminoácidos como tirosina e cisteína podem ser considerados semiessenciais, já que a sua presença na dieta reduz a exigência de fenilalanina e metionina, respectivamente (GUILLAUME, 1997).

A farinha de peixe é a principal fonte protéica dietária que satisfaz as exigências dos aminoácidos essenciais e não essenciais na produção de ração para a aquicultura, sendo o maior

constituinte em rações para espécies onívoras/detritívoras de camarões marinhos (TACON, 2006; FAO, 2007). Uma das vantagens do seu uso é o alto teor de lisina e metionina comparados a outras rações. Além disso, outros componentes como as vitaminas do complexo B e os minerais, cálcio e fósforo dos ossos, e ainda iodo, zinco, ferro, selênio e flúor, levam à escolha da farinha de pescado para uso em formulações especiais (GUILLAUME, 1997).

A maioria das farinhas comerciais de peixe é produzida a partir de várias espécies de peixes e pode ser rotulada em função da cor (branca ou marrom), espécie de pescado, procedimento de manufatura ou país de origem. A qualidade destas farinhas depende de vários fatores, tais como, temperatura no momento da captura do pescado, método de captura, temperatura e tempo de estocagem antes do processamento, e composição do pescado capturado (OLIVEIRA, 2002). Apesar de ser um ingrediente de alto valor protéico, a sua grande participação na composição dos custos das rações tem conduzido ao interesse contínuo na identificação e desenvolvimento de novas fontes alternativas de proteínas.

A utilização de fontes protéicas de origem vegetal na formulação de rações para camarões marinhos já vem sendo realizada com sucesso (DAVIS E ARNOLD, 2000; SUDARYONO et al. 1999). Dentre as fontes de proteína de origem vegetal, a soja *Glycine Max* (L) é considerada a nível global como a opção com maior potencial para substituir a farinha de peixe na formulação das rações comerciais pois apresenta um alto teor de proteínas, baixo teores de carboidratos e fibras, alta digestibilidade, e bom padrão de aminoácidos essenciais quando comparados a outras fontes de proteína vegetal (ALAN et al., 2005).

No entanto, de acordo com Samocha et al. (2004), a soja tem uma utilização comercial limitada devido a problemas potenciais associados com níveis insuficientes de aminoácidos essenciais como lisina e metionina. Além disso, a presença de determinados carboidratos afetam a sua palatabilidade, e fatores antinutricionais comprometem a sua digestibilidade. Porém, durante o processamento da soja muito desses fatores podem ser removidos com a aplicação de solvente (álcool aquoso) ou através de lixiviação isoelétrica, produzindo um produto com até 65% de proteína bruta (STOREBAKKEN et al.,2000). Tais procedimentos tornam o emprego na carcinicultura promissor, uma vez que fica mais acessível aos animais.

3.6. Enzimas

Enzimas são biomoléculas catalisadoras que atuam diminuindo o nível de energia de ativação, implicando no aumento da velocidade das reações bioquímicas (HARVEY et al., 2009). Todas as enzimas conhecidas, com exceção de certos RNAs catalíticos, são proteínas (NELSON e COX, 2004), e estão presente em todos os organismos vivos, sendo essenciais, tanto para a manutenção, como para o crescimento e a diferenciação celular (GUPTA et al., 2002).

As enzimas agem em sequências organizadas e catalisam centenas de reações sucessivas, pelas quais as moléculas de nutrientes são degradadas. Essas biomoléculas catalisadoras 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. Provavelmente, apenas uma fração da molécula denominada sítio ativo é a responsável pela ligação da enzima ao substrato, e essa fração determina a especificidade enzimática (NELSON e COX, 2004).

Uma vez que a reação química catalisada por uma enzima é a propriedade específica que distingue uma enzima de outra, a IUBMB (União Internacional de Bioquímica e Biologia Molecular) dividiu as enzimas em seis grandes classes (Tabela 1).

Tabela 1: Classificação das enzimas segundo a IUBMB.

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

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

3.6.1. Enzimas digestivas

Conhecer e compreender o metabolismo das enzimas digestivas é necessário para a escolha de ingredientes a serem introduzidos nas dietas de organismos aquáticos. O êxito no cultivo depende, em grande parte, de uma nutrição adequada e de um bom manejo alimentar.

As proteases estão entre as enzimas de crustáceos que recebem maior atenção (FERNÁNDEZ GIMENEZ et al., 2002), pois são responsáveis pela digestão de proteínas dos alimentos ingeridos, os componentes mais caros da alimentação de camarões (SÁNCHEZ-PAZ et al., 2003).

De acordo com a IUBMB as proteases estão inseridas no subgrupo 4 do grupo 3 (Hidrolases), pois por uma reação de hidrólise, clivam a proteína adicionando uma molécula de água à ligação peptídica (BERG et al., 2004) (Figura 9).

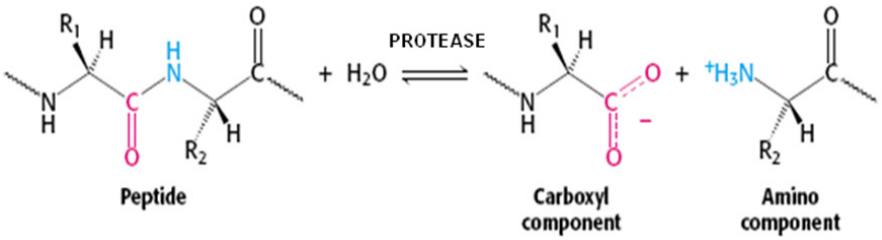

Figura 9. Hidrólise enzimática de uma proteína hipotética. (Fonte: BERG et al., 2004).

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 10), isto é, clivam resíduos de aminoácidos na posição N-terminal da proteína (GONZALES e ROBERT-BAUDOUY, 1996).

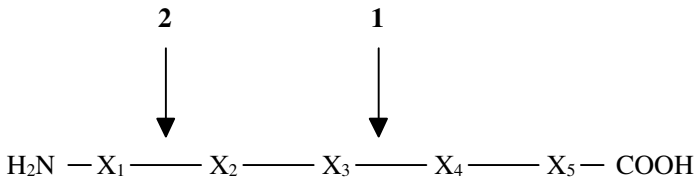

Figura 10. Classificação das proteases: Endoproteases clivam ligações peptídicas dentro da proteína (1). Exoproteases, mais especificamente as aminopeptidases, clivam resíduos localizados na posição N-terminal da proteína (2). Figura modificada de Gonzales e Robert-Baudouy (1996).

A tripsina é a protease mais abundante no sistema digestivo de crustáceos e sua contribuição para a digestão protéica em peneídeos é em torno de 60% (FERNANDEZ GIMENEZ et al., 2002). Ela faz parte da família das serinoproteases, caracterizadas por apresentar um mecanismo 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 (KOMKLAO et al., 2007) (Figura 11).

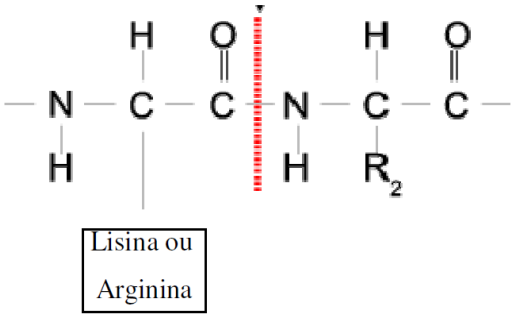

Figura 11. Sítio de hidrólise específico para tripsina.

A atuação da tripsina é importante em vários processos biológicos como: digestão protéica propriamente dita, ativação de zimogênios e mediação entre a ingestão do alimento e a assimilação dos nutrientes (SAINZ et al., 2004). Devido à extrema relevância funcional da tripsina, associada a uma ampla aplicabilidade industrial, esta enzima é uma das mais estudadas em organismos aquáticos (KLEIN et al., 1996).

A tripsina se caracteriza por apresentar o maior nível de atividade nos valores de pH entre 8,0 e 11,0 e em temperaturas de 35 °C a 45 °C. Esta enzima pode ainda ter sua atividade alterada em pH abaixo de 5,0 e acima de 11,0 ou pela presença de alguns inibidores como diisopropil-fluorfosfato (DFP), fluoreto fenil-metil-sulfonil (PMSF), inibidor de tripsina de soja (SBTI) e aprotonina. Dentre os substratos sintéticos hidrolizados pela tripsina e usados em pesquisas científicas destacam-se: N-α-benzoil-L-arginina-p-nitoanilida (BApNA) e tosil-arginina-metil-éster (TAME) (WHITAKER, 1994; SIMPSON, 2000).

Conforme a atividade proteolítica, a quimotripsina é considerada a segunda enzima mais abundante no sistema digestório de crustáceos (GARCIA-CARREÑO et al., 1994). Esta endopeptidase, solúvel em água, catalisa a hidrólise de ligações peptídicas de proteínas na porção carboxila de aminoácidos aromáticos como: fenilalanina, tirosina e triptofano (Figura 12) e também substratos sintéticos, tais como SApNA (DE VECCHI e COPPES, 1996; VIPARELLI et al., 2001; ABUIN et al., 2004; CASTILLO-YAÑEZ et al., 2006).

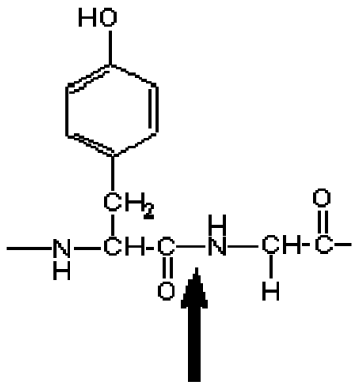

Figura 12. Sítio de hidrólise específica para quimotripsina

As principais enzimas responsáveis pela liberação dos aminoácidos livres são as aminopeptidases. Além dos aminoácidos, as aminopeptidases liberam também pequenos peptídeos através da hidrólise das ligações peptídicas na posição N-terminal de proteínas (GONZALES e ROBERT-BAUDOUY, 1996). Essas enzimas, geralmente inespecíficas, estão amplamente distribuídas na natureza, presentes em vários organismos, e apresentam importâncias biológicas e médicas por causa da sua função na degradação de proteínas (OLIVEIRA et al., 1999). As aminopeptidases vêm sendo amplamente investigadas por estudos bioquímicos e a viabilidade potencial de sua dosagem constitui-se em uma medida diagnóstica ou preventiva em algumas patologias relacionadas com seu papel fisiológico. Essas enzimas atuam também catalisando a hidrólise de substratos artificiais tais como aminoacil-β-naftilamida (AA-NA) e aminoacil-p-nitroanilida (AA-Nan).

Para a realização da digestão do amido há a atuação de diversas enzimas. A α-amilase [EC 3.2.1.1] é uma endocarboidrase encontrada na saliva e no trato digestivo de animais vertebrados (SALEH 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 et al., 2000; ROSAS et al., 2000).

Ao contrário de mamíferos e outros vertebrados, os crustáceos decápodas 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 et al., 1994; ROSAS et al., 2000).

3.6.1.1. Enzimas digestivas em *Litopenaeus vannamei*

Investigações sobre os processos digestivos em camarões peneídeos têm sido realizadas com o intuito de avaliar a capacidade dos organismos para hidrolisar, absorver e assimilar os principais nutrientes da dieta (GUZMAN et al., 2001). Estudos sobre a atividade das enzimas digestivas do camarão *Litopenaeus vannamei* vêm se tornando frequente, pois a indução dessas enzimas sintetizadas e secretadas no hepatopâncreas desses crustáceos tem influência direta na adaptação dos animais às variações na composição dietária (Le MOULLAC et al., 1997).

Vários trabalhos têm enfocado a atuação de enzimas como tripsina, quimotripsina, aminopeptidases, lipases e carboidrases no sistema digestivo do *L. vannamei*, (LE BOULAY et al., 1996; VAN HORMHOUDT e SELLOS, 1996; VAN HORMHOUDT et al., 1995) sendo esse estudo essencial para a compreensão do mecanismo de digestão e um melhor conhecimento das necessidades nutricionais (Le MOULLAC et al., 1997). Em conjunto, essas enzimas digestivas presentes nos hepatopâncreas de *L. vannamei* são capazes de hidrolisar uma variedade de substratos e vários fatores estão implicados em sua regulação. Entre esses fatores destacam-se a dieta (LE MOULLAC et al. 1996; GUZMAN et al., 2001; BRITO et al., 2001), variações ontogênicas (LOVETT e FELDER, 1990; LEMOS e RODRIGUEZ, 1998), tamanho corporal (LEE e LAWRENCE, 1985), ritmo circadiano (GONZALEZ et al., 1995; MOLINA et al., 2000), fases da muda (MOLINA et al., 2000; SANCHEZ-PAZ et al., 2003) e até mesmo um efeito estimulante da água de tanques tem sido reportado (MOSS et al.,2001).

A atividade tríptica em *L. vannamei* foi primeiramente evidenciada por Lee e Lawrence (1982). Em estudos posteriores, extratos enzimáticos da glândula digestiva do camarão branco exibiram três isoformas de tripsina (KLEIN et al., 1996; LE MOULLAC et al., 1996; EZQUERRA et al., 1997; MUHLIA-ALMAZÁN et al., 2003). De acordo com Van Wormhoudt et al. (1996) a eficiência catalítica da tripsina é maior em crustáceos peneídeos comparada aos vertebrados e em *L. vannamei* é a enzima mais ativa de todas as proteases caracterizadas (LEMOS et al., 2000).

A maior parte do conhecimento sobre a enzima quimotripsina é baseado em fontes de mamíferos, embora a pesquisa sobre as enzimas de outros grupos de organismos já esteja disponível. As propriedades catalíticas dessas enzimas, como a hidrólise de substratos sintéticos e os efeitos de alguns inibidores da protease, são semelhantes aos dos mamíferos. Van Wormhoudt et al. (1992) relata a purificação de duas isoformas de quimotripsina nas glândulas do intestino médio de *L. vannamei*. Em estudos anteriores, a atividade de quimotripsina não foi detectada. Por

exemplo, não foi detectada por Gates e Travis (1973) em *L. setiferus* e nem por Lee et al. (1984) em *L. vannamei*, provavelmente devido à falta de substratos sensíveis e altamente específicos. Tsai et al. (1986) evidenciaram atividade de quimotripsina e tripsina nas glândulas intestinais, estômago e intestino de *P. monodon, P. penicillatus, M. japonicus, Metapenaeus monoceros* e *Macrobrachium rosenbergii*. Estes autores concluíram que a quimotripsina foi tão importante quanto a tripsina nos processos digestivos destes decápodes.

Entre as carboidrases dos camarões peneídeos, a α-amilase (Van WORMHOUDT et al., 1995, FERNÁNDEZ et al., 1997), é uma das enzimas digestivas mais estudadas em *L. vannamei*, representando 1% do extrato bruto do hepatopâncreas desses animais (Van WORMHOUDT et al. 1996). Três isoformas da enzima amilase foram determinadas em *L. vannamei* (Wormhoudt Van et al. 1996). Os estudos sobre a digestão de carboidratos são importantes porque são frequentemente incluídos em rações comerciais para a redução dos custos de alimentação (WIGGLESWORTH e GRIFFITH, 1994).

Em relação às exoproteases, as mais altas atividades de aminopeptidases no hepatopâncreas do camarão branco (*Penaeus vannamei*) foram encontradas quando as espécimes foram alimentadas com proteínas de farinha de peixe de baixa qualidade nutricional (EZQUERRA et al., 1999). De acordo com Guillaume (1997), foi observado alto teor de hidrólise de substratos contendo aminoácidos necessários em altas concentrações na dieta de camarões, principalmente para arginina (5,8% de proteína bruta-PB), leucina (5,4% PB) e lisina (5,3% de PB).

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5. ARTIGO CIENTÍFICO

The results of the experimental work of this dissertation are presented in the article entitled **"Digestive enzymes of the white shrimp *Litopenaeus vannamei* fed under diets based on soy protein concentrate in replacement of fishmeal"** (manuscript), which is attached and will be submitted to the Journal Animal Feed Science and Technology (ISSN: 0377-8401).

Digestive enzymes of the white shrimp *Litopenaeus vannamei* fed under diets based on soy protein concentrate in replacement of fishmeal

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ABSTRACT

This work aimed to evaluate the effect of replacing fishmeal by soybean protein concentrate (SPC) at levels of 0% (C), 30% (S_{30}), 60% (S_{60}) and 100% (S_{100}) on the performance of digestive enzymes from *Litopenaeus vannamei*. Juvenile specimens (2.02 ± 0.51 g) were subjected to experimental diets during ten weeks. Then midgut glands from shrimps of each treatment were collected and enzyme activities were analyzed by *in vitro* assays, using long-chain substrates (1% azocasein and 2% starch), p-nitroanilide (BApNA, SApNA and Leu-p-Nan) and β-naphthylamide (alanine, arginine, leucine, tyrosine, serine, glycine, isoleucine, and histidine). Moreover, there were performed SDS-PAGE and proteolytic and amylolytic zymograms. The S_{100} group showed higher enzyme activity using 1% azocasein (1.18 ± 0.01 $U.mg^{-1}$) and 2% starch (5.04 ± 0.33 $U.mg^{-1}$). Major activities of chymotrypsin (13.78 ± 1.61 $U.mg^{-1}$) and leucine aminopeptidase enzymes (0.45 ± 0.03 $U.mg^{-1}$) using SApNA and Leu-p-Nan, respectively, were observed for the control group. While the highest trypsin activity (13.13 ± 0.53 $U.mg^{-1}$), using BApNA, was observed for the S_{30} treatment. Among the β-naphthylamide substrates analyzed, there were higher levels of aminopeptidasic activity for arginine and alanine in all treatments, mainly in the S_{30} that also showed increased activity in the presence of glycine (1.05 ± 0.08 $U.mg^{-1}$). It was noted that for serine, the aminopeptidasic activity was reduced gradually as the level of SPC in the shrimps diets were increased. The S_{60} treatment showed higher aminopeptidasic activity for isoleucine (0.69 ± 0.02 $U.mg^{-1}$) and histidine (0.85 ± 0.04 $U.mg^{-1}$). In relation to leucine and tyrosine, the aminopeptidasic activity was unmoved statistically dietary variations. SDS-PAGE revealed 26 protein bands between 6.9 and 198.8 kDa for all treatments. The protease zymogram exhibits two similar profiles, one with eighteen (C and S_{30}) and another with twelve proteolytic bands (S_{60} and S_{100}). While the amylolytic zymogram revealed five bands for all treatments. The average body weight gain of shrimps showed the highest value using the S_{30} diet (8.48±1.03 g), however did not evidenced significant differences ($p<0.05$) between treatments. Analysing the results above, it was possible to determine the influence of diet on digestive physiology of *L. vannamei*. The substitution

of fishmeal by SPC at 30, 60 e 100% in the diets of farmed shrimps provided a positive effect on animals performance. These results provide important information about the potential use of lower levels of protein from animal sources while formulating feeds for white shrimp.

Keywords: *Litopeneaus vannamei*, feed, soybean protein, proteases, amylase

1. Introduction

The production of aquatic organisms in captivity has increased substantially in recent decades due to increasing demand for new food sources. Among the activities that most developed, shrimps farming are highlighted and associate to high commercial market value attained by shrimps has been established worldwide (FAO, 2003). In Latin America, about 90% of the penaeid cultivated corresponds to the white shrimp *Litopenaeus vannamei* (Boone, 1931), a shrimp native of the Pacific Ocean (Wurmann et al., 2004). The quest for increased productivity has stimulated numerous studies aimed at determining various ideal zootechnical parameters for optimal performance in captivity of this shrimp (Nunes et al, 2006; Araneda et al., 2008, Esparza-Leal et al., 2010, Neal et al., 2010).

However, the feed remains the main obstacle for producers, since about 60% of the total cost of shrimp production are related to feed (Roy et al., 2009), being protein the most expensive component of the animals' diet (Lemos et al., 2003). The main feed source for shrimp is the fishmeal, which is rich in quality protein and has a balance of amino acids and fatty acids composition, that is suitable for the rapid growth of marine organisms (Cruz-Suárez et al. 2000). However, the use of fishmeal is affected by economic, ecological and market factors, raising its cost and restricting its use (Amaya et al., 2007). Thus, the substitution of fishmeal by alternative protein sources such as: by-products fisheries, livestock or animal and plant ingredients have been increasingly common in commercial diets formulations (Samocha et al., 2004; EAPA, 2006; Amaya et al., 2007; Swick, 2007; Roy et al., 2009). However, the presence of anti-nutritional factors or

deficiency of some essential amino acids may represent a negative point in the use of these raw materials in shrimp feeds (Davis et al., 2004).

In turn, the replacement of fishmeal by alternative components in diet does not always produce the expected growth due to the fact that certain dietary components are not properly absorbed by the animal. According to Fernández et al., (2001), biochemical information about the enzymatic arsenal of an organism can be useful in selecting ingredients for use in animal feed, since their enzymatic profile is closely related to feeding habits and the diets that are submitted. Furthermore, the specific activity of enzymes in the digestive tract can be used to illustrate the ability of crustaceans to explore various diets in order to supplement their nutritional requirements (Johnston and Freeman, 2005).

In this sense, the study aimed to evaluate the effect of replacing fishmeal by soybean protein concentrate (SPC) on the performance of the digestive enzymes of *L. vannamei*.

2. Material and Methods

2.1. Reagents

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

2.2. Cultivation Experimental

Specimens of *L. vannamei*, weighing 2.2 ± 0.51 g, were farmed in 50 circular tanks with a capacity of 500 L each, under a continuous water recirculation and density of 70 animals / m² (40 shrimp / tank). The cultivation was conducted at the Institute of Marine Sciences at the Federal University of Ceará, Brazil (LABOMAR - UFC) for a period of 10 weeks. For the feeding of

shrimps four isonitrogenous diets (38% crude protein) and isoenergetic (15.9 MJ / kg, dry matter basis) (Tables 1 and 2) were produced in the laboratory. For the group of four diets with the same level of inclusion of fish oil, the fishmeal was gradually replaced by soy protein concentrate (SPC) in 0% (control), 30%, 60% and 100%. The treatments were performed in triplicate. As inclusion of SPC increased, the level of dietary soybean oil (SBO) was also increased in order to balance the lipids and energy content of diets. Experimental diets were supplemented with synthetic sources of methionine and lysine. The diets were offered twice a day according to the appetite of the animals. At the end of cultivation, was performed biometry using fifteen shrimp/tank for each treatment. The length measurement was limited to distance from the eyeball until the end of telson. To assess the body weight of shrimp subjected to four treatments, was adopted the model: Average weight gain (WG) in grams obtained by the difference between the final average weight (AW_f) and the initial weight (W_I): $WG = AW_f - W_i$.

2.3. Preparatio of crude extract and determination of total soluble protein

Fifteen shrimps per treatment were collected for the removal of the midgut glands. The midgut glands were packed in dry ice and transported to the Laboratory of Enzymology at the Federal University of Pernambuco, Brazil (Labenz-UFPE), where they were thawed and homogenized in 5 mg / mL concentration (w / v) of tissue in a solution of 0.01 M Tris-HCl, pH 8 0, with the addition of 0.15 M NaCl. Then the homogenate was centrifuged at 10,000 g for 25 min at 4 °C to remove tissue debris. The supernatants obtained (crude extracts) were collected and stored at -25 °C for further analysis. The dosage of total soluble protein in crude extracts was determined as described by Bradford (1976), using bovine serum albumin as standard protein.

2.4. Enzymatic assays

2.4.1. Total proteolytic activity

The total enzymatic activity of proteases present in crude extracts was performed using 1% azocasein as substrate, prepared in 10 mM Tris-HCl, pH 8 0. Aliquots containing 30 µL of the crude extract were incubated with 50 µL of substrate solution for 1 hour at 25 °C. Then it was added 240 µL of 10% trichloroacetic acid to stop the reaction. After 15 minutes the mixture was centrifuged at 8,000 xg for 5 minutes. The supernatant was collected and 70 µL of it was mixed in 130 µL 1M sodium hydroxide solution (revealing solution) in microplates. The absorbance was measured on a microplate reader (Bio-Rad 680) at a wavelength of 450 nm. A negative control (blank) was performed, replacing the enzyme extract by a solution of 10 mM Tris-HCl, pH 8.0 with added 0.15 M NaCl. The activities were carried out in triplicate and one unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change of 0.001 units of absorbance per minute.

2.4.2. Specific proteolytic activities

The enzymatic activities of trypsin, chymotrypsin and leucine aminopeptidase, were determined in microplates with the use of Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), succinyl phenylalanine proline alanine aminotransferase pnitroanilide (SApNA) and pnitroanilide-leucine (Leu-p-Nan) as specific substrates, respectively (Bezerra et al., 2005). These substrates were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 8 mM. All assays were performed in triplicate. The enzyme extracts (30 µL) were incubated with 140 µL of buffer Tris-HCl 0.1 M, pH 8.0, and 30 µL of the substrate for a period of 15 minutes. Soon after, the absorbance readings were measured and recorded by using a microplate reader (Bio-Rad 680). The wavelength used in the measurements was 405 nm. One unit (U) of activity was defined as the

amount of enzyme required to produce one mole of p-nitroaniline per minute. The specific activity was expressed as units per milligram of protein.

For the determination of aminopeptidasic activities 8 amino acids were used as specific substrates (Alanine, Arginine, Glycine, Histidine, Isoleucine, Leucine, Serine, Tyrosine.) First, a time kinetic was performed for each substrate to determine the their reaction time. Then the assay was performed in microcentrifuge tubes at 37 °C. The substrate (40 µL) was incubated with 40 µL of distilled water, 40 µL of Tris-HCl buffer 0.1 M, pH 8.0 and 480 µL of sodium phosphate buffer 0.05 M, pH 7.0. After incubation, the reaction was stopped by adding 200 µL of Garnet reagent prepared in sodium acetate buffer 0.2 M, pH 4.2, containing 10% Tween 20 (v / v). Posteriorly 200 µL of the mixture was transferred to a microplate. The absorbance was measured at 525 nm with a microplate reader (Bio-Rad 680). The activities were expressed as units per milligram of protein.

2.4.3. Amylolytic activity

The total amylase activity was based on the method of Bernfeld (1955), using 2% starch solution (w / v) as substrate. The reaction consisted in the incubation of 20 µL of the crude extract with 125 µL of buffer 0.1 M Tris-HCl, pH 8.0 and 125 µL of the substrate at 37 °C for 10 minutes. Then 30 µL of incubated solution was added to 300 µL of 3,5-dinitrosalicylic acid (DNSA) at 100 °C for 10 minutes to stop the reaction. Soon after its cooling, 200 µL of the solution were transferred to microplate and the absorbance was measured at 570 nm using a microplate reader (Bio-Rad 680). One unit of enzyme activity was expressed as mg released maltose at 37 °C per minute per milligram of protein. To determine the concentration of released maltose, a calibration curve was prepared using comercial maltose.

2.5. SDS-PAGE

The polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE) was performed according to the methods of Laemmli (1970). The separation gel was 12.5% (w / v) and the concentration was 4% (w / v). Samples containing 100 μg of protein were applied into the gel, along with a standard solution of defined molecular mass containing the following proteins: Myosin (198.8 kDa), β-galactosidase (115.7 kDa), Bovine serum albumin (96.7 KDa) , ovalbumin (53.5 kDa), Carbonic anhydrase (37.1 kDa), Soybean trypsin inhibitor (29.1 kDa), Lysozime (19.5 kDa), Aprotinin (6.9 kDa). The gel was stained with a solution composed of Coomassie Brilliant Blue 0.01% (w / v), methanol 25% (v / v) and acetic acid 10% (v / v) and after 24 hours was bleached in solution with the same composition but devoid of the dye for visualization of bands.

2.6. Zymograms

Zymograms were performed to determine the proteolytic activity (Garcia-Carreño et al., 1993) and amylolytic activity (Fernández et al., 2001). Both zymograms were initiated by electrophoresis (SDS-PAGE) under immersion in an ice bath. Separation gels were used at 12.5% (w / v) and concentration gels at 4% (w / v). Enzyme preparations (30 μg of protein) were applied to the concentration gel. After electrophoresis, the gels were immersed in 100 mL of Triton X-100 2.5%, diluted in Tris-HCl 0.1 M, pH 8.0, for a period of thirty minutes at 4 ° C to remove the SDS. Then Triton X-100 was removed by washing the gels with Tris-HCl 0.1 M, pH 8.0. One of the gels was incubated in 100 mL of casein 3% (w / v) diluted in Tris-HCl 0.1 M, pH 8.0, for 30 minutes at 4° C to determine the proteolytic activity. Soon after the gel was kept in the same casein solution at 25° C for 90 minutes to allow the digestion of casein by active fractions. Finally the gel was stained with a solution composed of Coomassie Brilliant Blue 0.01%, methanol 25% and acetic acid 10% and after 24 hours was bleached in a solution with the same composition but devoid of the dye. To determine the activity of α-amylase, another gel was incubated with starch solution 2% (w / v) containing phosphate buffer 10 mM, pH 8.0 and $CaCl_2$ 1mM for a period of 60 minutes at 37 °C to

allow the digestion of starch by enzymes. Then the gel was washed with distilled water, stained with solution of potassium iodide / iodine (10%) for 5 minutes and added acetic acid solution (13%) to stop the reaction. The final procedure was to visualize the intensity and number of bands on gels that showed proteolytic and amylolytic activities.

2.7. 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 program MicrocalTM OriginTM version 8.0 (Software, Inc, U.S.).

3. Results

In vitro assays were performed with the use of long-chain substrates, determining the action of enzymes present in extracts of the midgut glands of *L. vannamei* cultured with different diets. The results related to these activities are shown in Figure 1. The three dietary treatments, that concisted on the replacements of 30% (S_{30}), 60% (S_{60}) and 100% (S_{100}) of fishmeal by soybean protein concentrate (SPC), did not show any significant differences ($p <0.05$) in the total proteolytic activity, using 1% azocasein as substrate, between them. However, it was observed that the experimental diets differed significantly ($p <0.05$) of the control group (0.90 ± 0.03 U.mg^{-1}) (Figure 1A). Regarding the performance of amylase, the treatment S_{100} (5.04 ± 0.33 U.mg^{-1}) was more efficient in the hydrolysis 2% starch solution, differing significantly ($p <0.05$) of the control (4.01 ± 0.32 U.mg^{-1}). The shrimps from S_{30} and S_{60} did not provide statistical differences between them and were indifferent also the other two diets (C and S_{100}) (Figure 1 B).

Analyzing the specific activities of these proteases in the presence of p-nitroanilide substrates (Figure 2) it was revealed that the S_{30} (13.13 ± 0.53 mU.mg^{1}) and S_{60} (11.82 ± 0.21

233 mU.mg^{-1}) treatments had the highest trypsin activity. These groups did not show significant
234 differences. Moreover, these treatments were statistically different of the control (9.23 ± 0.52
235 mU.mg^{-1}), and S_{100} diet (9.09 ± 0.40 mU.mg^{-1}) (Figure 2A). With the SApNA substrate was
236 assessed the activity of enzymes chymotrypsin and showed that animals submitted to diet composed
237 only with fish protein (C) showed higher activity for chymotrypsin (13.78 ± 1.61 mU.mg^{-1}) and was
238 significantly different ($p < 0.05$) compared to S_{60} and S_{100} treatments. The lowest chymotrypsin
239 activity was found in the S_{60} treatment (4.28 ± 0.64 mU.mg^{-1}), which exhibited no statistical
240 differences in relation to diet with 100% SPC. The S_{30} treatment (10.77 ± 1.26 mU.mg^{-1}) showed no
241 statistical difference to both the control group and the S_{100} treatment (Figure 2B). The activity of
242 leucine aminopeptidase using Leu-p-Nan substrate was the highest in C treatment (0.45 ± 0.03
243 mU.mg^{-1}). This control group was significantly different ($p < 0.05$), when compared to experimental
244 diets, which proved to be similar among them. Thus, it was observed that the catalytic action of this
245 enzyme decreased with the increase of the soy protein concentration in the diets (Figure 2C).

246 Variations in nutrients of animal and vegetable origin in the diets of shrimp also affected the
247 activity of aminopeptidase from them. The assays were performed in the presence of β-
248 naphthylamide substrates, noting activity for all amino acids used (Figure 3). The total replacement
249 of fish protein for soy in the diet of penaeid provided a decrease in aminopeptidasic activity when
250 using the nonpolar amino acid (Ala-) as substrate (Figure 3A). Using the basic substrate (Arg-), the
251 highest aminopeptidasic activity was found for S_{30} diet, but it did not statistically differed ($p < 0.05$)
252 from the control group. The increase in the level of substitution of animal protein by vegetable (S_{60}
253 and S_{100}) also resulted in decreased aminopeptidasic activity (Figure 3B). For nonpolar (Leu-) and
254 neutral polar (Tyr-) substrates, the action of aminopeptidases was unmoved statistical variations
255 diets (Figure 3C and 3D). It was noted that for neutral polar (Ser-) substrate the activity of the
256 aminopeptidase of shrimps subjected to experimental diets gradually decreased (Figure 3E). The
257 aminopeptidasic activity of cultured animals, when the neutral polar amino acid (Gly-) were used as
258 substrate, reached the highest value in the S_{30} treatment (1.05 ± 0.08 U.mg^{-1}), revealing significant

differences ($p < 0.05$) when compared to the control group (0.80 ± 0.02 U.mg^{-1}) (Figure 3F). With the use of nonpolar amino acid (Ile-), the aminopeptidasic activity was higher in S_{60} (0.69 ± 0.02 U.mg^{-1}) followed by the control group (0.68 ± 0.01 U.mg^{-1}). Both were not statistically different between them, but showed significant differences with the other treatments (Figure 3G). For the basic amino acid (His-), the S_{60} diet showed the highest value of aminopeptidasic activity (0.85 ± 0.04 U.mg^{-1}). The group C showed statistical differences when compared to the experimental diets (Figure 3H).

Proteins from the midgut glands of cultured *L. vannamei* were analyzed by SDS-PAGE (Fig. 4 A). A common pattern was observed in the number of bands in each treatment. There were detected twenty-six bands ranging from 6.9 kDa to 198.8 kDa. The proteolityc zymogram revealed differences in the number and intensity of bands. Eighteen bands (C and S_{30}) were seen, these with greater intensity, and twelve for both S_{60} and S_{100}. The zymogram of amylase revealed five bands with amylase activity for all treatments (Figure 4).

The analysis of average body weight gain of shrimps showed the highest value when used the S_{30} diet (8.48 ± 1.03 g), however did not evidenced significant differences ($p<0.05$) between treatments (Figure 5).

4. Discussion

Since one of the premises of sustainable aquaculture is to minimize the use of resources of limited availability, several studies evaluating the replacement of the fishmeal by alternative protein sources in the production of feeds for aquatic organisms has been reported (Tidwell et al., 1993; Webster and Lim, 2002). The effect of alternative protein sources on digestive enzymes of penaeid has also been reported (Gimenez et al., 2009).

In this study, assays employing of long-chain substrates (azocasein and starch) showed increased enzymatic activity as the fishmeal was replaced by SPC in the diets. Although fishmeal contain a supply of high quality protein and a balance of fatty acids and amino acids suitable for the

285 rapid growth of marine organisms (Cruz-Suarez et al., 2000; Hertrampf; Piedad-Pascual, 2000), the
286 inclusion of SPC in diets for *L. vannamei* showed a positive effect on digestion of both proteins and
287 carbohydrates. As is well known, the presence of a high content of endo and exoproteases renders
288 protein digestion more efficient. A digestive adaptation to new food preferences may be occurring
289 in this period.

290 The analysis of specific proteolytic activities in the presence of p-nitroanilide substrates,
291 revealed high values for both trypsin and chymotrypsin, compared to the activity using the substrate
292 Leu-p-Nan. These results are consistent with the literature, because generally, the crustacean
293 digestive system presents a high concentration of serine proteases, mainly trypsin and chymotrypsin
294 (Fernández et al., 1997). Trypsin also plays an important role in digestion through the activation of
295 zymogens of both itself and other endopeptidases (Natalia et al., 2004).

296 Despite the intense trypsin activity observed in the midgut glands of cultured animals,
297 occurred a variation of these activities due to a change in diet composition. The replacement of
298 fishmeal by soy protein concentrate at 30 and 60% provided an increase of trypsin activity
299 compared to other treatments (C and S_{100}). As the literature reports, the trypsin activity in *L.*
300 *vannamei* can be strongly modulated by the quality and quantity of dietary protein (Lee et al.,
301 1984). The increase of trypsin activity can be suggested as a consequence of an adjustment
302 mechanism to low protein content of the diet or low availability of dietary protein because of
303 relatively poor digestibility. (Le Vay et al., 1993; Rodríguez et al., 1994; Kumlu and Jones, 1995;
304 Lemos and Rodríguez, 1998).

305 The chymotrypsin and leucine aminopeptidase activities from midgut glands of cultured *L.*
306 *vannamei* decreased as fishmeal was replaced by soybean protein concentrate in diets. These results
307 indicate the adaptation in *L. vannamei* of theses digestive enzymes to the quality of dietary protein.
308 However, possible factors limiting enzymatic hydrolysis may be suggested, as the presence of
309 inhibitors or deficiency of certain nutrients in the diet. The effect of alternative sources of protein
310 on the activity of chymotrypsin in penaeid was also reported by Gimenez et al., (2009), highlighting

311 the achievements in researchs, involving the replacement of fishmeal by soy protein in diets for
312 shrimp.

313 Several authors have reported the study of aminopeptides in fish (Sabapathy and Teo, 1993;
314 Tengjaroenkul et al., 2000; 2002; Natalia et al., 2004; Refstie et al., 2006). This demonstrates the
315 importance of understanding the role of these enzymes in the digestion of aquatic organisms.
316 However, there is little information available on aminopeptidases in shrimp.

317 In this study also were analyzed the aminopeptidasic activities of midgut glands of the
318 farmed shrimp, through β-naphthylamide substrates. Elevated levels of aminopeptidasic activity
319 were observed in presence of arginine and alanine. This may be related to the efficient digestion and
320 incorporation of these essential nutrients (Lemos and Nunes, in press). Moreover the results
321 corroborate the requirements described in the literature for arginine, since this essential amino acid
322 is described as one of the most limiting in commercial shrimp diets (Fox et al., 1995). Heu et al.
323 (2003) also found high activities of aminopeptidases to arginine in the residues of processing in
324 *Pandalus borealis* and *Trachypena curvirostris*.

325 Although the enzymatic activity for substrates (Ala- and Arg-) to be considered high, its
326 values decreased as the fishmeal was gradually replaced by levels of SPC. A similar result was
327 observed with the use of serine as substrate. Studies Ezquerra et al. (1999) demonstrated the
328 influence of diet composition on aminopeptidasic activity in *L. vannamei.* In their experiments, the
329 activity of aminopeptidase also decreased when the shrimps were subjected to the diet with soy
330 protein.

331 As is known, the nutritional value of protein ingredients, usually defined by protein and
332 amino acids in the composition may influence the enzymatic hydrolysis of aminopeptidases.
333 However, other nutritional parameters such as availability of minerals, carbohydrates, lipids and
334 presence of antinutritional factors could also affect the digestive system of shrimp.

335 The analysis of the extracts of midgut glands of cultured *L. vannamei* showed no differences
336 by SDS-PAGE in the number of proteolytic bands between treatments. However, the zymogram of

proteases showed a decrease in the intensity of proteolytic bands as fishmeal was gradually replaced by soy protein in diets. Although the amylase activity to have revealed significant differences between treatments, the zymogram of amylase was not able to highlight those differences.

The levels of substitution of fishmeal by SPC at 30, 60 and 100% in diets for *L. vannamei* provided a positive effect on animals performance mainly relationship to body weight gain. Similar result was found by Samocha et al. (2004), where *L. vannamei* were fed practical diets containing 32% CP (crude protein) and 100% of the fishmeal was replaced by co-extruded soybean poultry by-product meal. Commercial shrimp feeds are commonly reported to include fishmeal at levels between 25% and 50% of the total diet (Dersjant-Li, 2002; Tacon and Barg, 1998). However, recent studies have shown that commercial shrimp feeds containing 30 - 35% crude protein can include levels as low as 7.5 - 12.5% fishmeal without compromising shrimp performance (Fox et al., 2004). The successful replacement of animal protein sources with plant proteins in shrimp feeds also has been achieved by Davis et al. (2004).

5. Conclusion

It was possible to determine the influence of diet on the *L. vannamei* digestive enzymes. The differences in enzyme activities of midgut glands of the farmed shrimp provided important information about the potential of white shrimp (*L. vannamei*) to use alternative food formulations with lower levels of animal protein sources. Given the results above, it was concluded that the substitution of fishmeal by SPC at levels of 30, 60 e 100% in diets for *L. vannamei* offered a positive effect on shrimps performance. This fact corroborates with the information that *L. vannamei* can be fed with vegetable protein sources to replace fishmeal without affecting the development of the animal. It is expected, with determining the feasibility of partial or total substitution of animal protein for vegetable protein, contribute to reducing the cost of feed, without

362 reducing the productivity of production systems. Also, are expected ecological benefits, such as
363 preservation of species of marine fish and recovery of the balance of the marine environment.
364
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366
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369
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490 **Figure captions**

491

492 Figure 1. Proteolytic (A) and amylase activity (B) in the midgut glands of the *Litopenaeus*
493 *vannamei* using long-chain substrates, 1% azocasein and 2% starch, respectively. The shrimps were
494 fed diets with gradual replacement of fishmeal by soybean protein concentrate in 0% (C), 30%
495 (S_{30}), 60% (S_{60}) and 100% (S_{100}). Different letters show statistical differences ($p < 0.05$).

496

497 Figure 2. Specific proteolytic activities in the midgut glands of the *L. vannamei* in the presence of
498 p-nitroanilide substrates. The enzymatic activities of trypsin (A), chymotrypsin (B) and leucine-
499 aminopeptidase (C) were determined with the use of Nα-benzoyl-DL-arginine-p-nitroanilide
500 (BApNA), succinyl phenylalanine proline alanine aminotransferase p-nitroanilide (SApNA) and p-
501 nitroanilide-leucine (Leu-p-Nan) as substrates, respectively. The specimens cultured had changes in
502 their diets where fishmeal was gradually replaced by soy protein at concentrations of 0% (C), 30%
503 (S_{30}), 60% (S_{60}) and 100% (S_{100}). Different letters show statistical differences ($p < 0.05$).

504

505 Figure 3. Aminopeptidase activities in the midgut glands of the *L. vannamei*, using β-
506 naphthylamide substrates. Eigth amino acids were employed as specific substrates: Ala (A), Arg
507 (B), Leu (C), Tyr (D), Ser (E), Gly (F), Ile (G), Hist (H). The diet established for cultured penaeid
508 was based on the gradual replacement of fishmeal by soybean protein concentrate in 0% (C), 30%
509 (S_{30}), 60% (S_{60}) and 100% (S_{100}). Different letters show statistical differences ($p < 0.05$).

510

511 Figure 4. Polyacrylamide gel electrophoresis - SDS-PAGE of crude extracts in the midgut glands of
512 cultured *L. vannamei* (A). The diet established for cultured penaeid was based on the gradual
513 replacement of fishmeal by soybean protein concentrate in 0% (C), 30% (S_{30}), 60% (S_{60}) and 100%

514 (S_{100}). A standard molecular weight (P) was applied to gel. In (B) zymogram of protease activity
515 and (C) amylase zymogram in the midgut glands of the cultured *L. vannamei*. Both electrophoresis
516 and zymograms was used in an electric current of 11mA.
517
518 Figure 5. Average body weight gain of the reared *L. vannamei* for ten weeks in an experimental
519 clearwater system. The shrimps were fed diets with progressive replacement of anchovy fishmeal
520 by soy protein concentrate at fish oil inclusion level of 2%. The shrimps showed initial weight
521 2.02±0.51g.
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Table 1. Ingredient composition of practical diets for *L. vannamei* used to evaluate the replacement of fishmeal by soy protein concentrate.

Ingredients	Experimental groups			
	C	S_{30}	S60	S100
Soybean meal, 46% CP (Bunge)	33.00	33.00	33.00	33.00
Wheat flour	25.00	25.00	25.00	25.00
Poultry meal, 61% (Nordal)	15.00	15.00	15.00	15.00
Fishmeal, Anchoveta 67% (Copeinca)	12.00	8.50	5.00	0.00
Soy protein concentrate, 62% (Selecta)	0.00	3.84	7.75	13.32
Soybean oil	2.04	2.30	2.79	3.45
Fish oil	1.00	1.00	1.00	1.00
Broken rice	4.15	3.54	2.59	1.27
Vitamin mineral premix, Shrimp SI (DSM)	2.00	2.00	2.00	2.00
Soy lecithin	1.50	1.50	1.50	1.50
Monodicálcico phosphate, 20% (Serrana)	1.30	1.30	1.30	1.30
Salt	1.00	1.00	1.00	1.00
Potassium chloride	1.00	1.00	1.00	1.00
Synthetic binder, Pegabind (Bentoli)	0.70	0.70	0.70	0.70
L-Lysine (Degussa)	0.12	0.13	0.15	0.17
DL-Methionine 99% (Degussa)	0.00	0.04	0.08	0.14
Magnesium sulfate	0.12	0.07	0.07	0.08
Rovimix Stay-C 35% (DSM)	0.07	0.07	0.07	0.07

566 **Table 2.** Nutritional composition of experimental diets offered to the
567 shrimp *L.vannamei.*

Ingredients	Experimental groups			
	C	S_{30}	S_{60}	S_{100}
Basic Nutrients				
Ash	5.87	5.52	5.16	4.65
Crude Fat	8.00	8.02	8.25	8.55
Crude Protein	36.00	36.00	36.00	36.00
Crude Fiber	1.61	1.77	1.93	2.16
Moisture	8.70	8.65	8.59	8.50
Aminoacids (%)				
Met + Cys	1.1497	1.1541	1.1589	1.1658
Methionine	0.6706	0.6700	0.6700	0.6700
Lysine	2.2508	2.2508	2.2508	2.2508
Phe + Tyr	2.7711	2.8137	2.8567	2.9180
Alanine	0.0000	0.1044	0.2107	0.3623
Arginine	2.4284	2.4728	2.5177	2.5818
Histidine	0.8392	0.8445	0.8500	0.8578
Phenylalanine	1.6152	1.655	1.6953	1.7528
Isoleucine	1.5658	1.5846	1.6040	1.6316
Leucine	2.6806	2.6998	2.7193	2.7470
Cystine	0.4791	0.4841	0.4889	0.4958
Threonine	1.3246	1.3247	1.3251	1.3257
Tryptophan	0.4215	0.4260	0.4305	0.4370
Tyrosine	1.1541	1.1569	1.1596	1.1634
Valine	1.7354	1.7443	1.7534	1.7663
TSSA	1.1497	1.1541	1.1589	1.1658
Lipids (%)				
Arachidonic (C20:4n6)	0.0177	0.0128	0.0080	0.0010
Docosahexaenoic (C22:6n3)	1.5509	1.1242	0.6975	0.0880
Eicosapentaenoic (C20:5n3)	0.4360	0.3584	0.2808	0.1700
Linoleic (C18:2n6)	1.7172	1.8402	2.0795	2.4056
Linolenic (C18:3n3)	0.2589	0.2542	0.2638	0.2756
Sum n3 EFA	5.6373	4.1427	2,6627	0.5464
Sum n6 EFA	1.2683	1.3526	1.5470	1.8098
Cholesterol	0.2513	0.2513	0.2513	0.2513
Phospholipid	1.4250	1.4250	1.4250	1.4250
Minerals (%)				
Calcium	1.9939	1.9124	1.8308	1.7142
Magnesium	0.1373	0.0800	0.0800	0.0800
Manganese	0.0005	0.0004	0.0002	0.0000
Potassium	1.3301	1.3842	1.4384	1.5157
Sodium	0.5401	0.5243	0.5085	0.4858
Total Phos.	1.1643	1.1378	1.1108	1.0722
Avail. Phos.	1.0185	0.9755	0.9322	0.8705
Chlorine	1.2672	1.2485	1.2296	1.2025
Energy (KJ/kg)				
Gross Energy (Kcal/kg)	4.282	4.266	4.261	4.251
Metabolizable Carbohydrate	5.964	5.925	5.845	5.735
Metabolizable Fat	3.024	3.032	3.119	3.232
Metabolizable Protein	6.048	6.044	6.040	6.034
Metabolizable, Energy	15.036	15.001	15.003	15.000
Other (%)				
Vitamin C (Ascorbic Acid)	0.025	0.025	0.025	0.025

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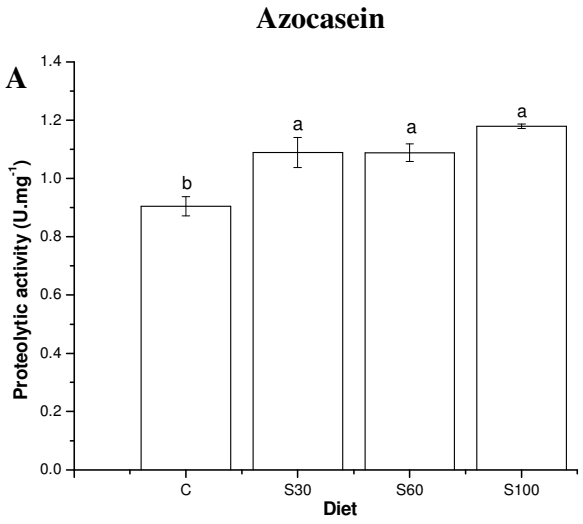

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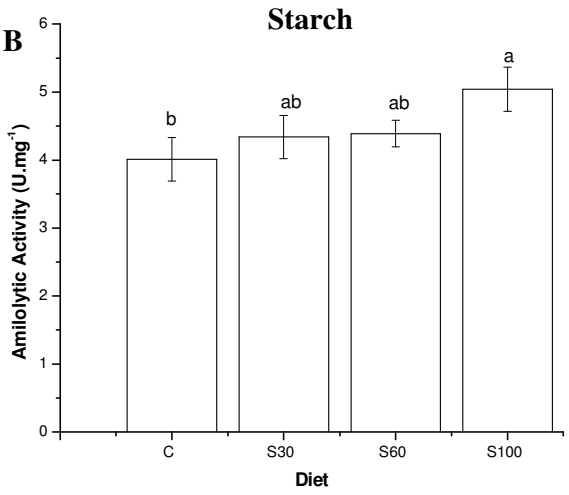

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572 **Figure 1**

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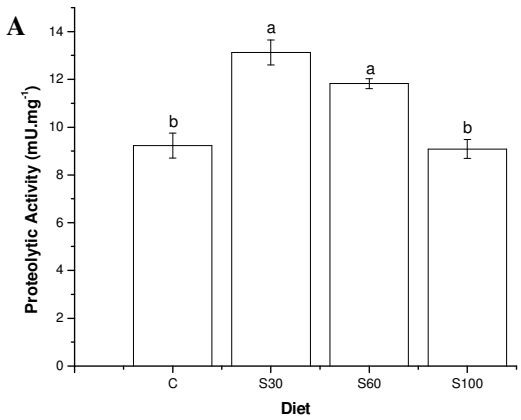

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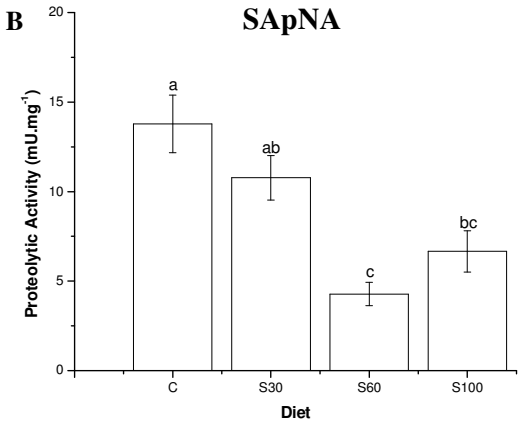

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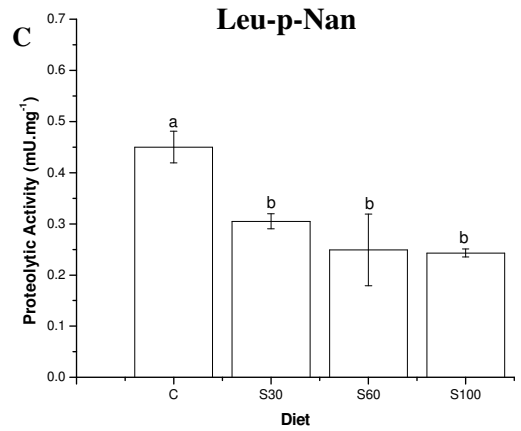

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578 **Figure 2**

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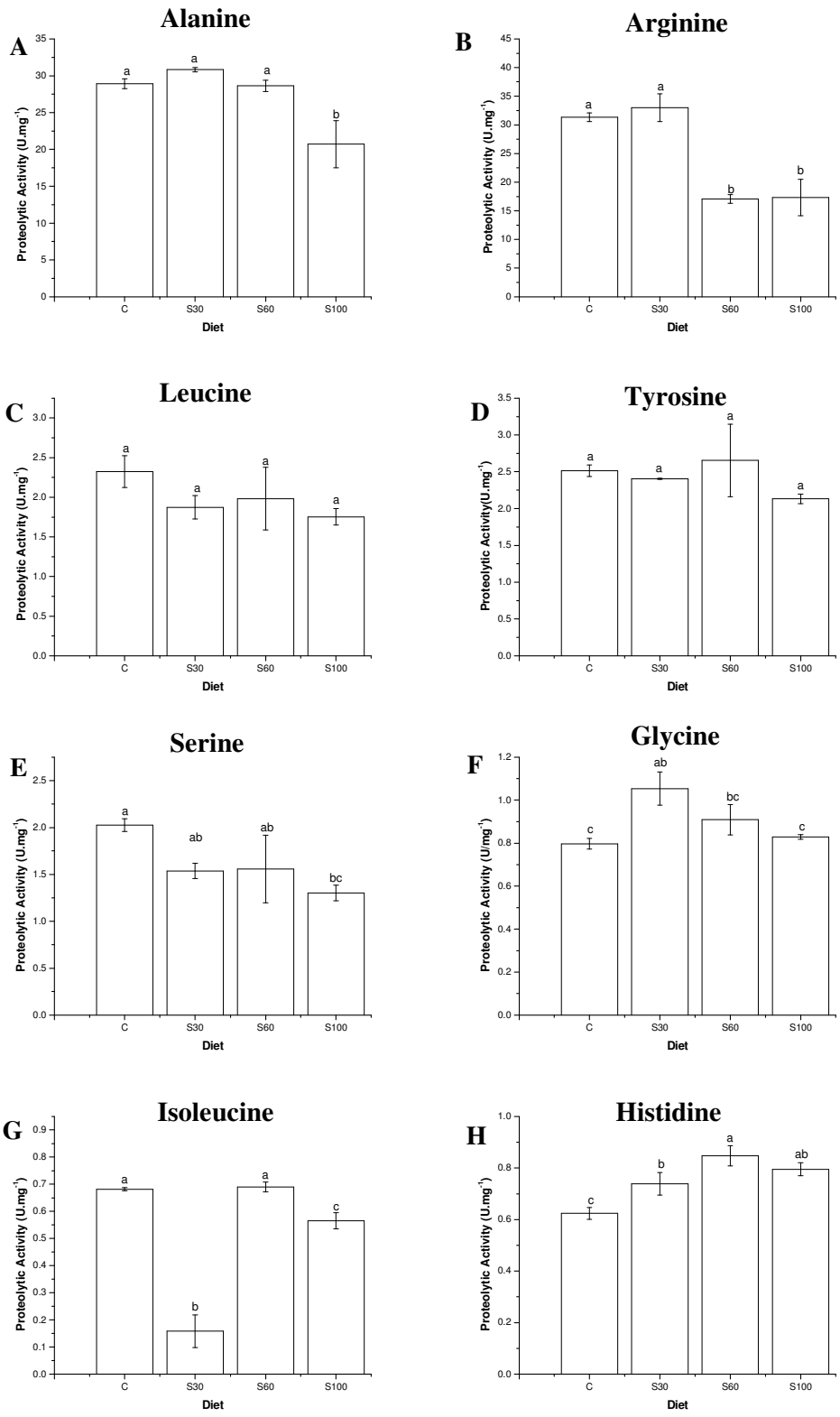
A
Alanine
Proteolytic Activity (U.mg-1)
a
a
a
b
C
S30
S60
S100
Diet
B
Arginine
Proteolytic Activity (U.mg-1)
a
a
b
b
C
S30
S60
S100
Diet
C
Leucine
Proteolytic Activity (U.mg-1)
a
a
a
a
C
S30
S60
S100
Diet
D
Tyrosine
Proteolytic Activity(U.mg-1)
a
a
a
a
C
S30
S60
S100
Diet
E
Serine
Proteolytic Activity (U.mg-1)
a
ab
ab
bc
C
S30
S60
S100
Diet
F
Glycine
Proteolytic Activity (U/mg-1)
c
ab
bc
c
C
S30
S60
S100
Diet
G
Isoleucine
Proteolytic Activity (U.mg-1)
a
b
a
c
C
S30
S60
S100
Diet
H
Histidine
Proteolytic Activity (U.mg-1)
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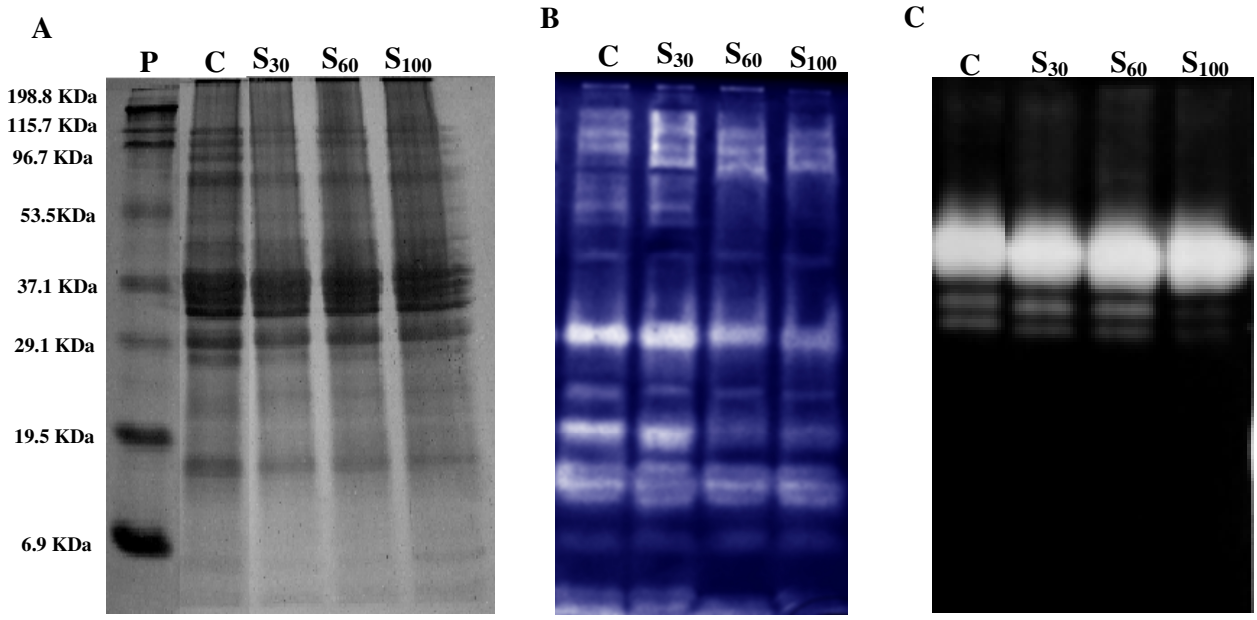
b
a
ab
C
S30
S60
S100
Diet

Figure 3


A
P
C
S30
S60
S100
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115.7 KDa
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53.5KDa
37.1 KDa
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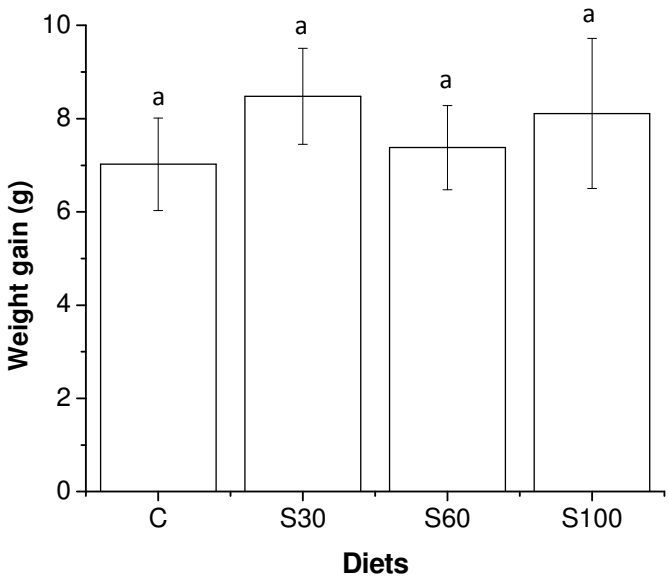
C
S30
S60
S100

Figure 4



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613 **Figure 5**

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6. CONSIDERAÇÕES FINAIS

Foi possível determinar a influência da dieta sobre a atividade das enzimas digestivas do *L. vannamei*. A substituição da farinha de peixe por SPC em níveis de 30, 60 e 100% nas dietas para *L. vannamei* evidenciaram um efeito positivo na performance dos camarões. As diferenças nas atividades enzimáticas dos hepatopâncreas dos camarões cultivados forneceram informações importantes quanto ao potencial do camarão-branco (*L. vannamei*) em utilizar formulações de alimentos alternativos com baixos níveis de fontes de proteína animal. Espera-se, com a determinação da viabilidade da substituição parcial ou total da proteína animal por proteína vegetal, contribuir para a diminuição do custo da ração, sem diminuir a produtividade dos sistemas de produção. Além disso, são previstos benefícios ecológicos, como a preservação de espécies de peixes marinhas e recuperação do equilíbrio do meio ambiente marinho.

7. ANEXO

7.1 Normas da revista: Animal Feed Science and Technology

Guide for Authors

1. Original Research Papers (Regular Papers)
2. Review Articles
3. Short Communications
4. Book Reviews

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

Review Articles should cover subjects falling within the scope of the journal which are of active current interest.

A *Short Communication* is a concise but complete description of a limited investigation, which will not be included in a later paper. Short Communications should be as completely documented, both by reference to the literature and description of the experimental procedures employed, as a regular paper. They should not occupy more than six printed pages (about 12 manuscript pages, including figures, tables and references).

Book Reviews will be included in the journal on a range of relevant books which are not more than two years old. Book reviews will be solicited by the Book Review Editor. Unsolicited reviews will not usually be accepted, but suggestions for appropriate books for review may be sent to the Book Review Editor:

Professor G. Flachowsky
Federal Research Centre of Agriculture
Institute of Animal Nutrition
Bundesallee 50
D-38116 Braunschweig
Germany

Manuscripts describing the use of commercial feed products are welcome, but should include the following information: major components, contents of active ingredients (for example enzyme activities). Independent verification, as opposed to a manufacturers guarantee, is always desirable and often avoids difficulties in the review process, especially where there are no, or few, treatment impacts. The Editors reserve the right to reject any manuscript employing such products, wherein this information is not disclosed.

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Submissions concerning NIRS may be suitable when more accurate, precise or robust equations are presented. Mathematical, technical and statistical advancement, may constitute the foundation for acceptance. For more details see the editorial in Vol. 118/3-4.

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Manuscripts should have **numbered lines**, with wide margins and **double spacing** throughout, i.e. also for abstracts, footnotes and references. **Every page of the manuscript, including the title page, references, tables, etc., should be numbered continuously.** However, in the text no reference should be made to page numbers; if necessary, one may refer to sections. Avoid excessive usage of italics to emphasize part of the text.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.
If reference is made to AOAC, ISO or similar analytical procedure(s), the specific procedure identification number(s) must be cited. A number of references for neutral and acid detergent fibre (NDF, ADF) assays exist, and an alternative reference to the now out-of-print USDA Agriculture Handbook 379 must be used. There are many options for NDF and ADF assays (e.g. sodium sulfite, alpha amylase, residual ash), which must be specified in the text. For more details see the editorial in Vol. 118/3-4.

The following definitions should be used, as appropriate:
a. aNDFom-NDF assayed with a heat stable amylase and expressed exclusive of residual ash.
b. NDFom-NDF not assayed with a heat stable amylase and expressed exclusive of residual ash.
c. aNDF-NDF assayed with a heat stable amylase and expressed inclusive of residual ash.
d. NDF-NDF assayed without a heat stable amylase and expressed inclusive of residual ash.
e. ADFom-ADF expressed exclusive of residual ash.
f. ADF-ADF expressed inclusive of residual ash.
g. Lignin (sa)-Lignin determined by solubilization of cellulose with sulphuric acid.
h. Lignin (pm)-Lignin determined by oxidation of lignin with permanganate.
While expressions of NDF and ADF inclusive of residual ash will continue to be acceptable (i.e., the terms aNDF, NDF and ADF above), the Editors-in-Chief highly recommend reporting all fibre values, including digestibilities, on an OM basis. Silica is partially soluble in ND, is quantitatively recovered in AD, and so may contribute to the 'fibre' values and to subsequent digestibility coefficients.
Reporting 'hemicellulose' values as the difference between NDF and ADF is generally only acceptable if the analyses have been sequential on the same sample. Crude fibre (CF), nitrogen-free

extract (NFE) and total digestible nutrients (TDN) are not acceptable terms for describing feeds and should only be referred to in a historical context.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature. Combined 'Results and Discussion' sections are only acceptable for 'Short Communications', except under compelling circumstances.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

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• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a "Present address" (or "Permanent address") may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

The abstract should be clear, descriptive and not longer than 400 words. It should contain the following specific information: purpose of study; experimental treatments used; results obtained, preferably with quantitative data; significance of findings; conclusions; implications of results if appropriate.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

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Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

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Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI. You are urged to consult IUB: Biochemical Nomenclature and Related Documents: http://www.chem.qmw.ac.uk/iubmb/ for further information.

Authors and Editors are, by general agreement, obliged to accept the rules governing biological nomenclature, as laid down in the *International Code of Botanical Nomenclature*, the *International Code of Nomenclature of Bacteria*, and the *International Code of Zoological Nomenclature*. All biotica (crops, plants, insects, birds, mammals, etc.) should be identified by their scientific names when the English term is first used, with the exception of common domestic animals. All biocides and other organic compounds must be identified by their Geneva names when first used in the text. Active ingredients of all formulations should be likewise identified.

SI or SI-derived units should be used throughout (e.g. MJ and not Kcal for energy concentrations). Concentrations should be expressed on a 'per kg' basis (w/w); however, w/v, v/v, mol/mol or M may be accepted depending on the circumstances. In addition, 'units' and 'equivalents' are

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Percent is *only* used to indicate relative changes. For composition, both w/w (often solids composition g/kg) and w/v (e.g. g/L), v/v (e.g. m/L), mol/mol or M can be accepted depending on the circumstances. Specify units (e.g. g/L) and never as percent. Digestibility/metabolisability and degradability should always be expressed as a coefficient (not %), and the content of, for example, the digestible component should be expressed as g/kg: thus, the coefficient of digestibility of dry matter is 0.8, while the content of digestible dry matter is 800g/kg. A distinction between true and apparent digestibility should be made, as well as between faecal and ileal (e.g. coefficient of total tract apparent digestibility - CTTAD). The terms 'availability' and 'bioavailability' should be avoided without definition in context.

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Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

If differences between treatments are statistically significant, this should be indicated by adding the actual 'P' value obtained. If $0.10 > P > 0.05$, then differences can be considered to suggest a trend, or tendency, to a difference, but the actual 'P' value should be stated. Further information on this issue can be found in *Animal Feed Science and Technology* Vol. 129/1-2.

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Strunk Jr., W., White, E.B., 1979. The Elements of Style, third ed. Macmillan, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 1999. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

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