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JULIETT DE FÁTIMA XAVIER DA SILVA

PRODUÇÃO, CARACTERIZAÇÃO E MÉTODOS DE CONSERVAÇÃO DE HIDROLISADO PROTEICO PROVENIENTES DE RESÍDUOS DO PROCESSAMENTO DE TILÁPIA (*Oreochromis niloticus*)

Recife
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco como pré-requisito para a obtenção do grau de doutor em Ciências Biológicas.

Orientador: Prof. Dr. Ranilson de Souza Bezerra
Co-orientadora: Prof. Dra. Karina Ribeiro

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Aprovado em 28/02/2014

BANCA EXAMINADORA

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Dr. Janilson Felix da Silva/UFPE

“... De tudo ficou um pouco

Do meu medo. Do teu asco.

Dos gritos gagos. Da rosa

ficou um pouco.

(...) Pois fica um pouco de tudo...

Ficou um pouco de tudo

No pires de porcelana,

Dragão partido, flor branca,

ficou um pouco

de ruga na vossa testa,

retrato.

(...) E de tudo fica um pouco”.

(Resíduo)

Carlos Drummond de Andrade

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A todos meu MUITO OBRIGADA.

LISTA DE ABREVIATURAS

ANOVA - Analysis of Variance

AOAC **-** Official methods of analysis

BApNA - Benzoyl-DL-arginine-*p*-nitroanilide

BHAP- Bactéria heterotrófica aeróbica psicrotrófica

CFR - Code of Federal Regulations

(C12) – Ácido Láurico

(C14:0) - Ácido Mirístico

(C15:0) - Ácido Pentadecanóico

(C16:0) - Ácido Palmítico

(C17:0) - Ácido Heptadecanóico

(C18:0) - Ácido Esteárico

(C20:0) - Ácido Araquídico

(C22:0) - Ácido Behênico

(C14:1) - Ácido Miristoleico

(C16:1) - Ácido Palmitoleico

(C18:1n9c) - Ácido Oléico

(C20:1) - Ácido cis-Eicosenóico

(C22:1n9) - Ácido Erucico

(C24:1) - Ácido Nervonico

(C18:2n6c) - Ácido Linolêico

(C18:3n6) - Ácido Gama Linolênico

(C18:3n3) - Ácido Linolênico

(C20:2) - Ácido Cis-Eicosadienóico

(C20-3n3) - Ácido cis- Eicosatrienóico

(C20:3n6) - Ácido Cis-Eicosatrienóico

(C20:4n6) - Ácido Araquidonico

(C20:5n3) - Ácido cis-Eicosapentaenóico

(C22:6n3) - Ácido cis-Docosahexaenóico

(C18:1n9t) - Ácido Elaidico

CFU - Colony forming units

DHA - Docosahexaenoic acid

DMSO - Dimethyl sulfoxide

Gy – Gray

GH - Degree of hydrolysis
Grau de hidrólise

EAA - Essential amiono acids
Aminoácidos essenciais

EC - Enzyme Commission
Comissão de enzimas

EPA- Eicosapentaenoic acid

ES - Enzyme-substrate complex
Complexo enzima-substrato

FAO - Food and Agriculture Organization
Organização para a Alimentação e Agricultura

FDA - Food and Drug Administration

FPH – Fish protein hydrolysate

HPP – Hidrolisado proteico de peixe

HUFA - Highly unsaturated fatty acids

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

IOEA - International Organization of energy Agency

IUBMB - União Internacional de Bioquímica e Biologia Molecular

kDa - Kilodaltons

KGy – Kilogray

Leu-*p*-Nan - Leucine-*p*-nitroanilide

mM - milimolar

NMP - Número mais provável

MPA - Ministério da Pesca e Aquicultura

NRC - Nutrient Requirements of Fish and Shrimp

ONp_{100} - Intestine crude extract (100mg/mL)

ONp_{600} - Intestine crude extract (600mg/mL)

PCA - Plate Count Agar

PV – Valor de peróxido

REP - Relação de eficiência protéica

SApNA - Succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide

SDS-PAGE - Dodecyl sulfate polyacrylamide gel electrophoresis

TCA - Trichloroacetic acid

TBARS - Thiobarbituric acid reactive substances

TBA - Ácido tiobabitúrico

U – unidade de atividade enzimática (1 μM de substrato processado por minuto)

UFC – Unidades formadoras de colônias

WHO – World Health Organization

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RESUMO

Carcaças e vísceras constituem um importante resíduo de processamento de peixes, podendo representar cerca de 70% do peso corporal da tilápia (*Oreochromis niloticus*). Esse material é uma fonte de biomoléculas, dentre elas proteínas e proteases, com propriedades interessantes para processos biotecnológicos como a produção de hidrolisado proteico de peixe (HPP). O objetivo do primeiro estudo foi avaliar o uso de resíduos do processamento de tilápia para produzir HPP. Assim, três condições de produção foram avaliadas: duas utilizando hidrolise enzimática com enzimas extraídas do intestino da tilápia em diferentes concentrações ($HPP_{100,}$ 100 mg de tecido/mL e HPP_{600}, 600 mg de tecido/mL) e o terceiro utilizando 0.5% (v/v) de Alcalase (HPP_{com}), uma enzima comercial. Os extratos experimentais revelaram a presença de proteases totaias, tripsina, quimotripsina e leucinoaminopeptidase. O teor de proteínas, aminoácidos e ácidos graxos foram calculados em matéria seca. Depois de 4 horas de reação, o grau de hidrolise máximo (GH) do $HPP_{com,}$ $HPP_{100,}$ HPP_{600} foram 34.73 ± 1.44%, 29.21 ± 0.79%, e 41.66 ± 1.33%, respectivamente. O perfil eletroforetico demonstrou bandas de 190 a 20 kDa (HPP_{100}), 54 kDa (HPP_{com}) e 53 a 20 kDa (HPP_{600}). O resultados dos teores proteicos foram, 584.8 g/kg, 492.3 g/kg, e 508.2 g/kg para HPP_{com}, HPP_{100}, e HPP_{600}, respectivamente. Metionina e lisina foram identificados em níveis de 32.0 e 77.0 g/kg (HPP_{com}), 31.0 e 64.0 g/kg (HPP_{100}), e 33.0 e 69.0 g/kg (HPP_{600}), respectivamente. O conteúdo de ácidos graxos polisanturados do HPP_{com}, HPP_{100} e HPP_{600} foram 101.0 g/kg, 138.0 g/kg e 70 g/kg, respectivamente, com predominância do ácido linoleico (C18:2n-6). O valor do IAAI foi de 1066.07, 688.4 e 738.51 no HPP_{com}, HPP_{100} e $HPP_{600,}$ respectivamente. A composição de aminoácidos, perfil lipídico e escore químico sugerem que todos os HPPs testados podem ser empregados como fonte proteica em dietas para organismos aquáticos. O segundo estudo comparou a eficiência de enzimas do intestino de tilápia (200 mg de tecido/mL) bruto e parcialmente purificados por precipitação salina (0 – 80%, v/v) e etanólica (0-30%, v/v), (30 – 70%, v/v) com duas enzimas comerciais Alcalase 0.5% (v/v) e Flavourzyme 0.5% (v/v) na hidrolise da carcaça da tilápia. O extrato enzimático semi-purificado com $(NH_4)_2SO_4$ mostrou rendimento de 62.6% (ativ. esp. de 9.3 U/mg de tecido) e com etanol um rendimento de 42.6% (ativ. esp. de 37.0 U/mg de tecido) e 68.4% (33.9 U/mg de tecido). Após 4 horas de reação o $HPP_{EC(200)}$ mostrou maior GH (37.8%), seguidos por HPP_A (35.3%), HPP_{ET} (33.2%), HPP_{AS} (24.6%) e HPP_F (18.5%). O perfil eletroforético demosntrou que o peso molecular dos HPPs variaram entre 116.25 a 29.05 KDa. O extrato bruto e os extratos semi-purificados foram mais eficientes na hidrolise da carcaça de tilápia que a enzima comercial Flavourzime. O terceiro estudo testou os efeitos da esterilização térmica, acidificação e irradiação na conservação de HPP. Foram realizadas análises físico-quimicas (TBARS, pH, composição centesimal), microbiológicas (mesófilos, piscicrófilos e microorganismos específicos) e sensorial (coloração) durante 60 dias. As análises mostraram que os HPPs possuem alto teor proteico e lipídico e são susceptíveis a mudanças de pH, oxidação lipídica e descoloração. A partir do décimo dia de estocagem todos os HPPs tiveram teores de TBARS aumentados e sofreram descoloração. O conteúdo proteico e lipídico foi alterado, mas não comprometeu o valor nutricional dos HPPs. A esterilização térmica com adição de ácidos e esterlização por irradiação foram eficientes na à eliminação dos microorganismos garantindo a segurança do produto por 60 dias de armazenamento. No quarto trabalho obteve-se uma patente referente à separação da parte lipídica da proteica do hidrolisado proteico de peixe a partir da irradiação.

Palavras chaves: hidrólise proteica, purificação parcial de proteases alcalinas, tratamentos de esterilização.

ABSTRACT

Fish viscera and carcasses represent about 70% of body weight of tilapia (*Oreochromis nilotic*), and are known sources of biomolecules such as protein and proteases. These enzymes can be employed in various biotechnological processes, e.g. preparation of fish protein hydrolysates. In this way, the aims of the first study were to evaluate the use of processing waste from Nile tilapia (*Oreochromis niloticus*) as a source of protein and proteases to produce FPH. Three FPH production conditions were evaluated: two conditions used autolysis with enzymes extracted from the tilapia intestine at different concentrations ($FPH_{100,}$ 100 mg of tissue/mL and FPH_{600}, 600 mg of tissue/mL) and the third used 0.5% (v/v) Alcalase (FPH_{com}), a commercial protease preparation. The experimentais extracts showed total proteolytic activities, trypsin, chymotrypsin, and leucine aminopeptidase activities proteases. Protein, amino acids and fatty acids content were calculated as DM basis. After a 4-h reaction, maximum hydrolysis percentages (DH) from FPH_{com}, FPH_{100}, and FPH_{600} systems were 34.73 ± 1.44%, 29.21 ± 0.79%, and 41.66 ± 1.33%, respectively. The protein content in the resulting FPS were 584.8 g/kg, 492.3 g/kg, and 508.2 g/kg for FPH_{com}, FPH_{100}, and FPH_{600}, respectively. Methionine and lysine were found at levels of 32.0 and 77.0 g/kg (FPH_{com}), 31.0 and 64.0 g/kg (FPH_{100}), and 33.0 and 69.0 g/kg (FPH_{600}), respectively. Polyunsaturated fatty acid contents of FPH_{com}, FPH_{100}, and FPH_{600} were 101.0 g/kg, 138.0 g/kg, and 70 g/kg, respectively, with a predominance of linoleic acid (C18:2n-6). IAAI reached a value of 1066.07 in FPH_{com}, 688.4 in FPH_{100} and 738.51 in $FPH_{600.}$ Amino acid composition, lipid profile, and amino acid score suggested that all of the experimental FPHs could be employed as a protein source in diets for aquatic organisms and other farmed animals. The second study compared the efficiency of the enzymes from tilapia intestine (200 mg tissue/mL) crude and partially purified by salting-in (0 - 80% v/v) and ethanol (0-30%, v/v), (30 - 70% v/v) with two commercial enzymes Alcalase 0.5% (v/v) and 0.5% Flavourzyme (v/v) hydrolysis of the carcass tilapia. The partial purified enzyme extract with $(NH_4)_2SO_4$ showed a yield of 62.6% (act. esp. of 9.3 U/mg of tissue) and ethanol a yield of 42.6% (act. esp. of 37.0 U/mg tissue) and 68.4% (33.9 U/mg tissue). After 4 hours the reaction, FPH_{EC} (200) showed a higher DH (37.8%), followed by FPH_A (35.3%), FPH_{ET} (33.2%), FHP_{AS} (24.6%) and FPH_F (18.5%). The electrophoretic profile of FPHs showed molecular weight ranged from 116.25 to 29.05 kDa. The crude extract and partial purified extracts were more effective in the hydrolysis of tilapia carcasses than commercial enzyme Flavourzime. The third study tested the effects of heat sterilization, irradiation and acidification in the conservation of FPH. They were carried out physical-chemical analysis (TBARS, pH, chemical composition), microbiological (mesophilic, piscicrophilic and specific microorganisms) and sensorial (color) for 60 days. Analyzes have shown that FPHs have high protein and lipid content and FPHs are susceptible to changes in pH, lipid oxidation and discoloration. From the 10^{th} day of storage all FPH had increased TBARS levels and discoloration. The protein and lipid content has changed, but did not compromise the nutritional value of FPHs. Heat treatment with citric acid and gamma irradiation were effective in the removal of microorganisms pathogenic securing of the product for 60 days of storage. In the fourth work we obtained a patent for the separation of the lipid portion of the protein fish protein hydrolyzate from the irradiation.

Keywords: protein hydrolysis, partial purification of alkaline proteases, sterilization treatments

SUMÁRIO

1. INTRODUÇÃO

O aumento na demanda por produtos pesqueiros para consumo humano tem resultado principalmente em um constante crescimento da produção aquícola mundial e como consequência ocorre aumento na quantidade de resíduos gerados a partir do processamento do pescado pelas unidades de beneficiamento e indústrias de pescado.

Segundo Arruda (2004), cerca de 50 % do pescado mundial produzido em 2000 transformou-se em resíduo. Supondo-se que esse percentual tenha-se mantido, dos 154 milhões de toneladas de pescado produzidos em 2011 (FAO, 2012), 77 milhões teriam sido descartados ou subutilizados, constituindo-se em uma fonte significativa de desperdício de recursos e de contaminação ambiental.

O termo resíduo de pescado refere-se a todos os subprodutos e sobras do processamento de organismos aquáticos de valor comercial relativamente baixo. Em relação ao peixe, o material residual pode ser constituído por peixes fora do tamanho ideal para consumo e restos obtidos do processamento de filés ou outros cortes que resulta em carcaças (restos de carne, cabeça, pele, ossos, escamas) e vísceras (Oetterer, 2002).

Dentre os variados tipos de cortes, o filé é o item mais nobre e de maior valor econômico, e seu rendimento varia de acordo com o com o tamanho dos peixes e com o domínio tecnológico das empresas processadoras. No caso da tilápia nilótica (*Oreochromis niloticus* L.), umas das espécies mais cultivadas no mundo, seu rendimento pode atingir entre 30 e 40% do peso do animal sendo o restante considerado resíduo e com baixo valor comercial. Desta forma, os resíduos da industrialização da tilápia, que representam de 60 a 70% da matéria-prima são atualmente subutilizados ou descartados pelas indústrias processadoras de filés (Boscolo et al., 2007).

Entretanto, estes resíduos são fontes de proteína (carcaça) e proteases (vísceras), podendo ser utilizados em processos biotecnológicos e transformados em produtos alternativos passíveis de utilização tanto na nutrição humana quanto na nutrição animal (Martone et al., 2005).

Dentre os produtos obtidos através da transformação desses resíduos destacam-se os hidrolisados proteicos de peixes (HPP), produto obtido através da solubilização do pescado inteiro ou residual, onde a quebra das proteínas se converte em peptídeos solúveis e aminoácidos livres. Estas proteínas podem ser obtidas a partir da utilização de enzimas proteolíticas endógenas, ou seja, presentes no próprio peixe, extraídas principalmente do trato digestório adicionadas à matéria-prima. É um produto com alto teor de proteína e aminoácidos essenciais, além de altamente digerível, podendo ser utilizado como suplemento

alimentar e como substituto parcial da farinha de peixe, principal fonte proteica inserida em rações para organismos aquáticos.

Sendo o HPP destinado a alimentação, é de grande importância a inocuidade do mesmo. Portanto, para a obtenção de um alimento seguro é necessário que este esteja isento de microorganismos patógenos e deteriorantes, os quais estando presentes poderão comprometer a qualidade nutricional do produto e diminuir o tempo de prateleira do mesmo (Oetterer, 2001).

Porém, existe uma grande lacuna a ser preenchida quanto a produção e caracterização do hidrolisado proteico de peixe líquido a partir do processamento de tilápia, bem como a determinação de métodos de conservação eficientes que permitam a inocuidade do HPP e que sejam capazes de preservar as características nutricionais e sensoriais do produto.

2. REVISÃO DA LITERATURA

2.1 Panorama da Tilápia nilótica

O aumento na demanda por produtos pesqueiros tem resultado em um constante crescimento da produção aquícola mundial. Em 2011, foram produzidos 154 milhões de toneladas de pescado, das quais 90,4 milhões foram oriundos da pesca e 63,6 milhões, da aquicultura. Aproximadamente 130 milhões foram destinados ao consumo humano e 23,2 milhões a produção de farinha e óleo de peixe (FAO, 2012).

Embora em termos percentuais a captura de organismos aquáticos ainda seja responsável por quase 59% do total de pescado fornecido, essa atividade vem apresentando estabilidade desde a década de 80, onde no período de 2002 a 2010, de 93 para 88,6 milhões de toneladas. Entretanto, nas últimas três décadas (1980-2010), a produção mundial da aquicultura se expandiu por quase 12 vezes mais, com uma taxa média de crescimento anual de 8,8 % (FAO, 2012).

Atualmente, a aquicultura é um dos sistemas de produção de alimentos com maior taxa de crescimento no mundo, o que coloca esta atividade em foco pela grande oportunidade de produção de alimentos, geração de postos de trabalho e desenvolvimento de negócios (Howarth, 1996). Com destaque para produção de peixes de água doce, que em 2010 representaram 56,4% (33,7 milhões de toneladas), dos quais a tilápia é o segundo maior gênero mais cultivado do mundo (FAO, 2012).

O século 20 firmou o cultivo de tilápias. Este desenvolvimento se deu principalmente pelo cultivo na África e América do Sul, o melhoramento genético, a expansão do cultivo em águas salinas nas Filipinas e pela demanda do filé pelos Estados Unidos, onde a China se tornou o maior produtor e grande fornecedor dos EUA (GUERRERO, 2008). Sua produção mundial passou de aproximadamente 0,4 milhões de toneladas em 1991 para cerca de 3,0 milhões de toneladas em 2010, sendo o continente asiático o maior produtor (72%), especialmente a China e o Sudeste da Ásia, seguidos pelos continentes africano (19%) e americano (9%) (FAO, 2012).

De acordo com Wing-Keong e Hanin (2007), *Oreochromis niloticus* (Figura 1) representa 80% das espécies de tilápia cultivadas no mundo, sendo considerada a mais importante.

A tilápia nilótica, pertence à família Cichlidae, são nativas da África, mas foram introduzidas mundialmente em muitas regiões tropicais, subtropicais e temperadas durante a segunda metade do século 20 (EL-SAYED, 2006).

Devido as suas características biológicas e mercadológicas como rápido crescimento, rusticidade, alimentação em baixos níveis tróficos, tolerância a variações ambientais, resistência a doenças, ausência de espinhos intra-musculares, dentre outros, a tilápia do Nilo é considerada uma das principais espécies da piscicultura mundial e a principal espécie brasileira.

Figura 1. Tilápia (*Oreochromis nilotucus*)

Fonte: Silva, J.F.X., (2010).

No Brasil, em 2011 a piscicultura continental representou 86,6% (544.490 t) da produção total de pescado nacional, sendo a tilápia o principal grupo cultivado com produção de 253.824,1 t, (46,62%) (MPA, 2011).

A tilapicultura firmou-se como atividade empresarial no Brasil a partir de 1980, quando surgiram os empreendimentos pioneiros, devido dentre outras características, ao excelente desempenho em ganho de peso e fácil adaptação às diversas condições de cultivo nas regiões brasileiras, principalmente no nordeste e sudeste.

Ressalta-se que a produção de tilápia apresenta um padrão de crescimento contínuo desde 1994 (IBAMA, 2009), com incremento de mais de 2000% entre os anos de 1994 (11.500 t,) a 2011 (253.000 t) (MPA, 2011). Assim, o crescimento dessa atividade no país, impulsionou a instalação de unidades de processamento de filé, e despertou o interesse de investidores nacionais.

2.2 Produção e aproveitamento de resíduos de tilápia

Nas diferentes etapas da cadeia produtiva da piscicultura, desde a produção até a comercialização no varejo, é gerada uma quantidade significativa de resíduos orgânicos. Os tipos e as quantidades de resíduos gerados na industrialização variam conforme o

processamento empregado: peixe inteiro eviscerado, eviscerado e descabeçado, filetado, espalmado, dentre outras. As quantidades estão relacionadas ao rendimento da carcaça dos peixes, que varia em função do processamento, da espécie, do peso, tamanho do peixe, formato do corpo e destreza do filetador..

Atualmente, a espécie de peixe de água doce mais industrializada no Brasil é a tilápia, processada para obtenção de filés frescos e congelados. O rendimento do filé varia de acordo com o com o tamanho dos peixes e com o domínio tecnológico das empresas processadoras, podendo render entre 30 e 40% do peso do animal, sendo o restante, cerca de 60 – 70% considerado resíduo e sem valor comercial (restos de carne, cabeça, pele, ossos, escamas e vísceras) (Figura 2) (BOSCOLO et al., 2007). As vísceras podem representar de 8 a 12%, a pele de 3 a 4%, as escamas de 2 a 3%, a cabeça de 14 a 18% e os restos de carne aderida ao esqueleto de 28 a 30% (KUBITZA e CAMPOS, 2006).

Figura 2. Peixe após processamento (*Oreochromis nilotucus*)

Fonte: Ferreira, A.C.M. (2013)

As vísceras são excelentes fontes de enzimas digestivas, dentre elas as proteases, com propriedades interessantes para processos biotecnológicos como, por exemplo, na degradação de subprodutos ricos em proteínas (SHAHIDI et al.,1995). Enquanto a carcaça é um material rico em proteína (BOSCOLO et al., 2007).

Entretanto, no Brasil, o aproveitamento de resíduos de pescados é pequeno. Aproximadamente 50% da biomassa no Brasil são descartadas durante o processo de enlatamento ou em outras linhas de produção, como a filetagem (PESSATTI, 2001). Muitas vezes estes resíduos são descartados sem nenhum tratamento prévio gerando um problema ambiental (VAZQUEZ e MURADO, 2008). O uso de aterros sanitários e lagoas de tratamento

de efluentes, por exemplo, não são alternativas recomendáveis, devido ao odor desagradável que provocam nas áreas costeiras ou continentais (LUSTOSA NETO, 1994).

Portanto, existe a necessidade do aperfeiçoamento de sistemas de aplicação e gerenciamento destes resíduos através de tecnologias capazes de tratá-los ou de recuperar compostos orgânicos de interesse econômico, antes de serem descartados, para minimizar a poluição (QUITAIN et al., 2001).

Nesse sentido, é importante a utilização da matéria-prima em toda sua extensão, recuperando os subprodutos e evitando a formação do próprio resíduo (MAIA et al., 1998). Ao longo dos anos, diversos esforços têm sido os empreendidos por pesquisadores em todo o mundo para desenvolver métodos que possibilitem a transformação desses resíduos em produtos passíveis de utilização tanto na nutrição humana quanto na animal (ARMENTA e GUERREROLEGARRETA, 2009; CHEN et al., 2011; CAHÚ et al., 2012).

Uma alternativa viável para estes resíduos seria a obtenção de produtos obtidos por meio de tratamentos biotecnológicos, que podem ser incorporados como ingredientes na formulação de dietas de organismos aquáticos, tais como farinha, silagens e hidrolisados proteicos (GILDBERG E STENBERG, 2001; FORSTER, 2008).

Os resíduos do processamento de filé de tilapia, por exemplo, são identificados como potencial fonte proteica animal, com níveis de proteína bruta entre 14 a 18% além de lipídios totais (14 a 15%) (SOUZA et al., 1999).

A farinha de peixe elaborada com resíduos possui qualidade inferior em comparação com a farinha produzida a partir de peixes pelágicos, e normalmente contem menor teor de proteína (< 48 – 60%), menor disponibilidade de aminoácidos essenciais (± 10%), maior teor de lipídeos (> 10%) e maior quantidades de cinzas (> 20%), material que quando em excesso prejudica a digestibilidade de outros nutrientes, pois implica em menores teores de proteína e aminoácidos. (NUNES e SÁ, 2010).

A silagem consiste em uma forma de preservação da matéria prima pela adição de ácidos orgânicos ou inorgânicos (silagem ácida) ou de microorganismos (silagem biológica) (VIDOTTI et al., 2003).

Outra forma de aproveitamento destes resíduos é o hidrolisado proteico de peixe, produto obtido através de processo da hidrolise das proteínas do pescado, cuja degradação ocorre através da atuação de enzimas proteolíticas que convertem as proteínas em peptídeos solúveis e aminoácidos livres, consequentemente, em uma maior digestibilidade para os peixes (BERGE e STOREBAKKEN, 1996). A presença de moléculas de baixo peso

molecular e aminoácidos livres, por sua vez, estimulam o consumo pelos organismos aquáticos, garantindo aos hidrolisados o poder de estimulantes alimentares (NRC, 2011).

2.3 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).

No catabolismo as enzimas agem em sequências organizadas e catalisam centenas de reações sucessivas, pelas quais as moléculas de nutrientes são sintetizadas. 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 União Internacional de Bioquímica e Biologia Molecular (IUBMB) dividiu as enzimas em seis grandes classes (Tabela 1).

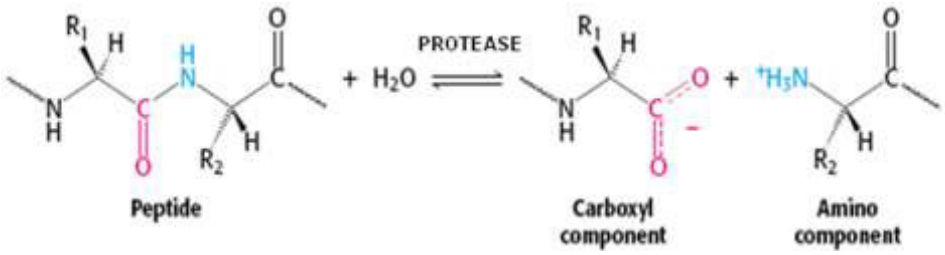
Tabela 1. Classificação das enzimas segundo a IUBMB.

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)

Enzimas proteolíticas, proteases, proteinases ou peptidases são sinônimos para as enzimas que hidrolisam ligações peptídicas (BERG et al.,2004) (Figura 3). De acordo com a IUBMB as proteases estão inseridas no subgrupo 4 do grupo 3 (Hidrolases). Esses biocatalisadores podem ser divididos em dois principais grupos: exopeptidases e endopeptidases.

Figura 3. Hidrólise enzimática de uma proteína hipotética.



(Fonte: BERG et al., (2004)

As exopeptidases atuam próximo às extremidades das cadeias polipeptídicas em uma extremidade N-terminal liberando um resíduo de aminoácido (aminopeptidases; EC 3.4.11), de um dipeptídeo ou de um tripeptídeo (dipeptidase e tripeptidases, EC 3.4.14). As exopeptidases que atuam no C-terminal livre libera um único resíduo (carboxipeptidases, EC 3.4.16-18), ou um dipeptídeo (peptídeo-dipeptidases; EC 3.4.15). As carboxipeptidases são divididas em três grupos com base no mecanismo catalítico: as serinocarboxipeptidases (EC 3.4.16), os metalocarboxipeptidases (EC 3.4.17) e as cisteínocarboxipeptidases (EC 3.4.18).

Enquanto que as endopeptidases atuam preferencialmente nas regiões internas das cadeias polipeptídicas. As endopeptidases são divididas em sub-subclasses, com base no mecanismo catalítico, e a especificidade é utilizada apenas para identificar enzimas individuais dentro dos grupos. As subsubclasses são: serina endopeptidases (serinoproteases) (EC 3.4.21), cisteína endopeptidases (cisteínoprotease) (EC 3.4.22), aspartato endopeptidases (aspartatoproteases) (EC 3.4.23), metaloendopeptidases (metaloproteases) (EC 3.4.24) e treonina endopeptidases (treoninoprotease) (EC 3.4.25).

Levando-se em conta o valor do pH no qual apresentam atividade máxima, estas enzimas podem ser classificadas em: proteases ácidas ou alcalinas (RAO et al., 1998). Neste segundo grupo encontram-se as principais proteases industriais. A tabela 2 baseada em Rao et al. (1998) demonstra a classificação e divisão das proteases

Tabela 2. Classificação e divisão das proteases

Local de clivagem do substrato	Sítio ativo da enzima	Número de resíduos de aminoácidos removidos
Exopeptidases	Aminopeptidases	Aminopeptidases
		Aminodipeptidases
		Aminotripeptidases
		Sítio ativo da carboxipeptidase
	Carboxipeptidases	Serinocarboxipeptidase
		Metalocarboxipeptidase
		Cisteínocarboxipeptidase
Endopeptidases	Serinoproteases	
	Aspartatoproteases	
	Cisteinoproteases	
	Metaloproteases	

Fonte: Rao et al. (1998).

2.3.1 Enzimas digestivas em peixes

Fluídos e enzimas gástricas

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

A pepsina é, sem dúvida, a principal protease ácida dos peixes, produzida na forma inativa de pepsinogênio (BALDISSEROTTO, 2009). A enzima é ativada inicialmente em condições ácidas, através da remoção de um peptídeo de baixo peso molecular pelo ácido clorídrico e continua o processo de transformação por autocatálise. O ácido clorídrico estimula ainda a estimula a liberação do hormônio secretina, responsável pela descarga do suco pancreático rico em íons bicarbonato que irão auxiliar na neutralização do pH no intestino (ROTTA, 2003).

Algumas enzimas não proteolíticas como amilase, lipase, esterase, quitinase, celulase, têm sido registradas no estômago de peixes (FÄNGE e GROVE, 1979; GUILLAUME e CHOUBERT, 1999; RUST, 2002, BALDISSEROTTO, 2009). As tilápias apresentam lipase e amilase gástrica, mas essa enzima hidrolisa apenas as gorduras de baixo ponto de fusão e já emulsificadas. A ação sobre as gorduras da dieta é mais completa e mais eficaz pela lipase pancreática (FÄNGE e GROVE, 1979; ROTTA, 2003)

Enzimas pancreáticas

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

Dentre as proteases de maior importância encontram-se a tripsina, a quimotripsina e as aminopeptidases. A tripsina e a quimotripsina são endoproteases enquanto que as aminopeptidases são exoproteases (GONZALES e ROBERT-BAUDOUY, 1996). A tripsina é a protease mais abundante no sistema digestivo de organismos aquáticos, esta enzima cliva as ligações peptídicas no lado carboxila de resíduos de aminoácidos carregados positivamente como arginina e lisina (KOMKLAO et al., 2007). 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).

A quimotripsina é considerada a segunda enzima mais abundante no sistema digestório de organismos aquáticos (KOMKLAO et al., 2007). Esta enzima catalisa a hidrólise de ligações peptídicas de proteínas na porção carboxila de aminoácidos aromáticos como: fenilalanina, tirosina e triptofano e também substratos sintéticos, tais como Succinil-alanina-alanina-prolina-fenilalanina-*p*-nitroanilida (SAPNA) (ABUIN et al., 2004; CASTILLO-YAÑEZ et al., 2006).

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). Estas enzimas atuam também catalisando a hidrólise de substratos artificiais *in vitro* como aminoacil-β-naftilamida (AA-NA). Dentre as aminopeptidades destaca-se a leucinoaminopeptidase, que também atua hidrolisando substrato sintético como o leucina-*p*- nitroanilida (Leu-*p*-Nan).

Enzimas intestinais

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

2.3.2 Semi-purificação e avaliação de proteases alcalinas de peixes

O processo de purificação de uma proteína requer primeiramente a separação desta dos componentes celulares. Os tecidos e células são rompidos em solução tampão, obedecendo a critérios que evitam a desnaturação da proteína de escolha, de modo que se forma uma mistura denominada extrato bruto (BRACHT; ISHIIIWAMOTO, 2002). Estas biomoléculas

podem ser purificadas de acordo com diferentes métodos que se baseiam em diferenças físicas como, tamanho da molécula protéica, carga elétrica e afinidade com outras moléculas (NELSON; COX, 2004).

Não existe uma sequência exata dos métodos de purificação a serem usados em todas as proteínas. Devlin (1998) e Voet (2005) relatam que se deve escolher uma sequência de técnicas de purificação que resulte em um elevado grau de purificação e alto rendimento. A obtenção de métodos sensíveis e específicos para distinguir e medir quantitativamente a proteína que se pretende isolar é também indispensável.

Há um grande interesse no estudo das proteases alcalinas das vísceras de peixes, seja com o objetivo de conhecer a fisiologia digestiva do peixe ou para demonstrar o potencial biotecnológico destas moléculas. Para estudar estas proteases é necessário primeiramente separá-las dos outros componentes celulares ou possíveis contaminantes encontrados nas vísceras destes animais. Vários trabalhos têm sido publicados com diferentes técnicas para esta finalidade (BEZERRA et al., 2001b; GARCIA-CARREÑO et al. 2002; KOMKLAO et al., 2004; BEZERRA et al., 2005; CASTILLO-YÁNEZ ET al. 2005; KISHIMURA et al., 2005, 2006, 2007; BOUGATEF et al., 2007; SOUZA ET al., 2007). As técnicas mais comuns utilizadas (e que tem apresentado melhores resultados quando utilizadas combinadas) por estes autores para purificar proteases alcalinas de vísceras de peixes são: Centrifugação, tratamento térmico, fracionamento por "salting-out", gel filtração e cromatografia de afinidade.

Centrifugação

A centrifugação é um processo de separação em que a força centrífuga relativa gerada pela rotação da amostra é usada para sedimentar partículas em suspensão (células, organelas ou moléculas) de diferentes densidades, separando-os (DEVLIN, 1998). A centrifugação diferencial separa proteínas solúveis de materiais insolúveis; a força centrífuga e a duração da centrifugação são ajustadas para assegurar que os materiais insolúveis sedimentem, formando precipitados, de forma que as proteínas solúveis permaneçam no líquido sobrenadante. As proteínas aí contidas podem ser então separadas por outros métodos de purificação (COX, 2004).

Tratamento térmico

O tratamento térmico do extrato bruto têm sido uma ferrramenta inicial ou intermediária eficiente no processo de purificação de proteases alcalinas de peixes. Esta

técnica foi primeiramente testada para enzimas de peixes por Bezerra et al. (2001b), com o propósito de diminuir a quantidade de proteases, eliminando aquelas termolábeis e convertendo grãos de zimogênios em enzimas ativas presentes na amostra.

Fracionamento salino e com etanol: Purificações fundamentadas nas diferenças de Solubilidade

Vários métodos têm sido usados para purificar as enzimas de peixes. Um método comum que tem sido adotado por vários autores é submeter o extrato a tratamentos que separem a proteína em diferentes frações, baseados em propriedades como tamanho ou carga.

Muitos solventes orgânicos miscíveis em água são capazes de precipitar enzimas. Devido a sua baixa constante dielétrica (quando comparado com a água), solventes orgânicos aumentam a atração entre as moléculas de proteínas, formando agregados, até que as partículas assumam proporções macroscópicas e precipitem. Este fenômeno consiste na remoção da água de solvatação da proteína, permitindo que forças eletrostáticas induzam regiões de cargas opostas da proteína a se atraírem. Neste caso, a água é removida tanto pelo solvente orgânico, como pela estruturação ao redor da molécula orgânica. Como consequência, a constante dielétrica é diminuída (SCOPES, 1988; WANG et al., 1979; HARRISON, 1993).

Os álcoois - metanol, etanol e isopropanol - são os mais importantes precipitantes industriais. O etanol, no entanto, apresenta o balanço ideal entre o efeito na solubilidade e características hidrofóbicas adequadas para reduzir a desnaturação. A precipitação com etanol é uma técnica promissora que pode ser aplicada para muitos tipos de proteínas em escala industrial. O etanol é, depois da água, o mais importante dos solventes, por possuir boas características físico-químicas, como uma completa miscibilidade com a água, baixo ponto de fusão, ausência de risco de misturas explosivas, alta volatilidade, inércia química, baixa toxicidade e baixo custo, especialmente no Brasil (CORTEZ; PESSOA Jr., 1999).

Os sais neutros têm efeito pronunciado sobre a solubilidade de proteínas. Para Nelson; Cox (2004) os sais de íons divalentes, tais como $MgCl_2$ e $(NH_4)_2SO_4$, são muito mais eficientes na solubilização do que os sais de íons monovalentes como o NaCl, NH_4Cl e KCl. Com o uso dos sais ocorre o aumento de solubilidade (*salting in*) ou perda de solubilidade (*salting out*) das proteínas. O sulfato de amônio é o sal mais usado para a precipitação, pois

tem solubilidade acentuada e produz força iônica elevada (BRACHT; ISHII-IWAMOTO, 2002).

2.4 Hidrolisado proteico de peixe

Hidrolisado protéico de peixe (HPP) é um produto constituído por aminoácidos livres e peptídeos que apresentam uma vasta gama de massas moleculares resultantes do maior ou menor grau de hidrolise das proteínas do pescado (GONÇALVES, 2011).

Na hidrólise das proteínas ocorre a ruptura das ligações peptídicas entre os aminoácidos, a qual pode verificar-se num maior ou menor número de ligações, levando a formação de peptídeos com diferentes massas moleculares. A extensão da hidrolise pode ser avaliada pelo grau de hidrólise, definido como a percentagem das ligações peptídicas quebradas em relação ao número total de ligações peptídicas por unidade de massa (GONÇALVES, 2011). Existem vários métodos para medir o grau de hidrólise, que variam em complexidade, precisão e exatidão, como por exemplo: a medida de solubilidade em ácido tricloroácetico (TCA), determinação do nitrogênio amino através da titulação com formaldeído, reação com ácido trinitrobenzenosulfônico (TNBS) e pH-Stat (MAHMOUD, 1994; HOLANDA, 2004).

Os hidrolisados podem ser obtidos por hidrólise química (ácida ou alcalina) e por hidrólise enzimática (enzimas endógenas e exógenas) (MARTONE et al., 2005).

A hidrólise ácida pode ser realizada com uso de ácidos inorgânicos, orgânicos ou por uma mistura de ambos. Os ácidos inorgânicos, como o ácido clorídrico e o ácido sulfúrico, embora de baixo custo, têm a desvantagem de necessitar de neutralização, antes do alimento ser consumido (OETTERER, 2001). Este processo resulta em uma considerável quantidade de sal nos produtos, decorrente da adição de NaOH para sua neutralização, o que pode tornar o produto não palatável e interferir na funcionalidade dos alimentos. Outro inconveniente é a destruição do triptofano, que é um aminoácido essencial (HOLANDA, 2004).

A hidrólise alcalina é realizada com soluções de bases fortes, como NaOH e KOH, sob aquecimento e agitação, resultando em produtos com baixa funcionalidade, além de poder afetar o valor nutritivo dos hidrolisados protéicos (KRISTINSSON e RASCO, 2000a). As quatro reações químicas que descrevem o que ocorre com as proteínas quando se utiliza álcali (base) são: (1) hidrólise da ligação peptídica, (2) destruição de alguns aminoácidos (3) cross-linking e (4) racemização de resíduos de aminoácidos do L-aminoácidos para D-aminoácidos (HAYASHI e KAMEDA, 1980).

A hidrólise enzimática pode ser realizada com enzimas endógenas, proteases extraidas do próprio pescado ou com enzimas exógenas (enzimas de origem vegetal, animal ou microbianas) (ASPMO et al., 2005).

A hidrólise enzimática de pescado é um método que objetiva a recuperação de proteínas de espécies subtilizadas ou de resíduos de processamento que seriam desperdiçados através do emprego de enzimas proteolíticas extraídas do trato digestório do próprio peixe ou através de enzimas comerciais, para solubilização da proteína do pescado, resultando em duas frações: solúvel e insolúvel. A fração insolúvel é desprezada e a fração solúvel, que contém a proteína hidrolisada, pode se constituir em ingrediente a ser incorporado aos alimentos elaborados e destinados ao consumo humano e animal (SILVA et AL., 2014).

A hidrólise enzimática resulta na liquefação do tecido do pescado. Essa metodologia possui distintas vantagens sobre as demais técnicas, incluindo: 1. a especificidade de ação da enzima, que torna possível o controle das características do produto final; 2. Digestão sob condições moderadas, evitando pH e temperaturas extremas que poderiam comprometer a qualidade nutritiva do hidrolisado; 3. taxa de hidrólise controlada através da desativação da enzima por aquecimento; 4. atrativas propriedades funcionais, como solubilidade e dispersibilidade, e nenhuma destruição dos aminoácidos, retendo o valor nutritivo da proteína (DINIZ e MARTIN, 1999).

Na seleção da enzima mais adequada para a preparação de HPP, têm sido seguidos vários processos, não havendo, todavia, uma metodologia padrão. As aplicações pretendidas para os HPP vão determinar as diferentes opções seguidas. Assim, os HPP com elevado valor nutricional ou terapêutico devem possuir peptídeos com baixa massa molecular e poucos aminoácidos livres. Porem, se o objetivo for melhorar as propriedades funcionais, então os HPP devem apresentar peptídeos com grandes massas moleculares. As proteases podem ser endopeptidases ou exopeptidases. As primeiras promovem a ruptura de ligações peptídicas no meio das cadeias proteicas, dando origem a peptídeos com maior ou menor massa molecular, exibindo maior atividade funcional, e a relativamente poucos aminoácidos livres. As exopeptidades catalizam a hidrolise de ligações nas extremidades das cadeias polipeptídicas produzindo muitos aminoácidos livres responsáveis por sabores acentuados e poucos peptídeos (OETTERER, 2001).

Em relação às enzimas endógenas, é de suma importância o conhecimento sobre a caracterização enzimática da fonte visceral que será utilizado no processo hidrolítico, devido a sazonalidade das atividades enzimáticas entre as diversas espécies de peixes, visto que diferentes atividades podem levar à formação de diferentes frações de peptídeos, com

diferentes propriedades físico-químicas. (BENJAKUL e MORRISSEY, 1997; KRISTINSSON e RASCO, 2000c). A maioria das proteases comerciais pode ser usada para solubilizar a proteína do pescado e podem ser produzidas a partir de plantas, animais ou microorganismos. Os microorganismos proteolíticos parecem ser as mais promissoras fontes de proteases, por produzirem uma maior variedade de enzimas específicas, em comparação às plantas ou aos animais (Diniz & Martin, 1999). Entretanto, para serem eficientes as mesmas precisam atuar sob condições favoráveis de pH, temperatura e concentração de substrato (DINIZ e MARTIN, 1999).

Aproximadamente 60% do total das enzimas industriais são proteases, amplamente empregadas na produção de couro e na indústria de alimentos. Na Tabela 3 estão listadas algumas enzimas utilizadas em hidrólise protéica. Dentre elas a alcalase, uma endopeptidase alcalina produzida pela fermentação submersa do microrganismo *Bacillus lincheniformis*, bastante utilizada pela indústria por ser considerada uma das melhores enzimas para o preparo de hidrolisados, pois o produto apresenta gosto suave mesmo quando tem elevado grau de hidrólise (BENJAKUL e MORRISSEY, 1997; KRISTINSSON e RASCO, 2000a; CENTENARO et al. 2009).

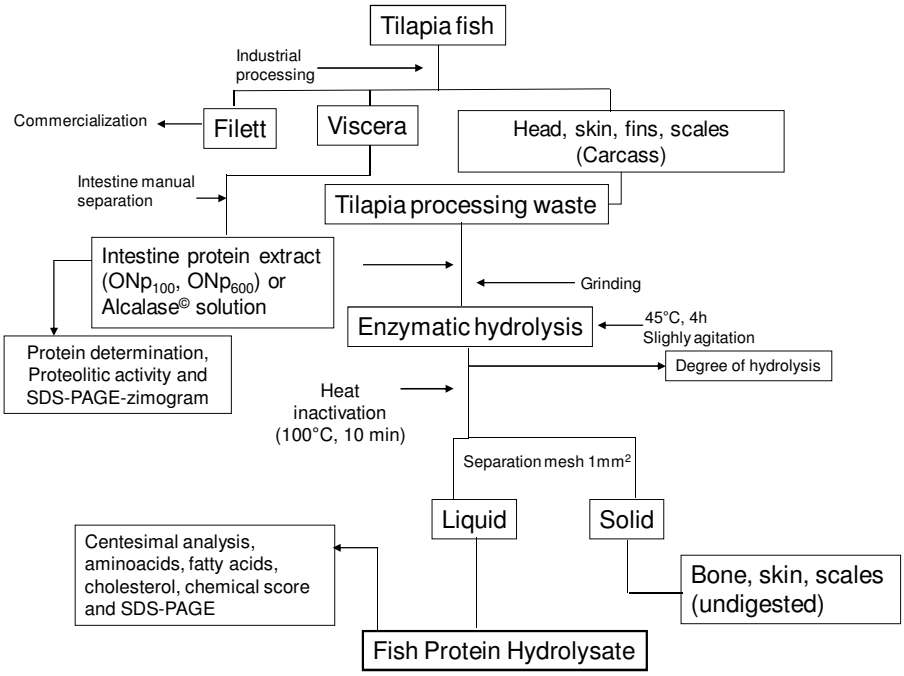
Tabela 3. Enzimas usadas em hidrólise enzimática de proteínas

PROTEASES	**ORIGEM**	**pH de máxima atividade proteolítica**	**Temperatura (ºC) de máxima atividade proteolítica**
Microbriana			
Alcalase	*Bacillus licheniformis*	6,5-8,5	55-70
Neutrase	*Bacillus subtilis*	5,5-7,5	45-55
Pronase	*Streptomyces griseus*	7,0-9,0	37
Animal			
Quimiotripsina	Bovina/suína	7,0-9,0	40
Pancreatina	Bovina/suína	7,0-9,0	40
Pepsina	Bovina/suína	2,0-4,0	40
Tripsina	Bovina/suína	7,0-9,0	40
Planta			
Bromelina	Caule do abacaxi	5,0-8,0	50-60
Ficina	Látex de figos	5,0-8,0	30-50
Papaina	Mamão	5,0-7,0	65-80

Fonte: Diniz e Martin (1999)

O processo de obtenção do HPP é relativamente simples e rápido. Após a trituração e a digestão durante 2 horas a 45ºC, a suspensão de proteína é retirada do digestor e filtrada, para eliminação de ossos e peles não digeridos, seguindo-se a pasteurização durante 10 minutos a 100ºC, com a conseqüente desativação das enzimas (FURLAN e OETTERER, 2002). O fluxograma para obtenção do hidrolisado protéico de pescado por hidrólise enzimática desenvolvida por Bezerra (2000) pode ser visto na Figura 4.

Figura 4. Procedimento para a obtenção do hidrolisado protéico de peixe (SILVA et AL., 2014).



O hidrolisado proteico pode ser mantido líquido ou desidratado por qualquer um dos métodos convencionais como a liofilização, secagem, dentre outros (OETTERER, 2001). O "Spray-Dryer" é um dos equipamentos mais empregados na conversão do produto líquido para a forma de pó, com a vantagem adicional de ser de fácil manipulação e maior estabilidade, porém a elevada temperatura empregada pode causar alguns efeitos na qualidade do produto final, sobretudo no conteúdo protéico, umidade e teor de todos os aminoácidos essenciais, exceto a metionina. (ABDUL-HAMID et al., 2002).

As propriedades funcionais dos HPP são particularmente importantes quando estes se destinam a ser utilizados como ingredientes em produtos alimentares. Segundo Cândido (1998), propriedade funcional é uma propriedade físico-química que afeta o processamento e o comportamento das proteínas em um sistema alimentar, julgada pelos atributos de qualidade do produto final. Freqüentemente, os HPP mostram excelente dispersibilidade em água e alta solubilidade sob um largo intervalo de pH, principalmente no ponto isoelétrico da proteína. Essa característica é resultado da degradação enzimática da proteína nativa em unidades peptídicas menores.

A composição do HPP em geral reflete a composição da matéria-prima que lhe deu origem. A composição típica de um hidrolisado protéico produzido a partir de músculos de

um peixe magro (não gorduroso) com base no peso seco é de 85-90% de proteína, 2-4% de lipídios e 6-7% de cinza (FURLAN e OETTERER, 2002).

A maioria das pesquisas realizadas tem demonstrado que o HPP é uma excelente fonte de lisina, arginina, glicina, alanina e prolina, que são importantes flavorizantes em produtos de crustáceos (NILSANG et al., 2005 além de apresentar propriedades funcionais úteis para a indústria alimentícia, tais como retenção de líquido e alta solubilidade (OETTERER, 2001). Como produto para consumo humano, serve como suplemento em certos tipos de biscoitos, como os butterscotch, barras de nozes e produtos tipo hambúrguer, e como flavorizante de sopas e na fabricação de pães, bolachas e massas (OETTERER, 2001). O HPP também pode ser adotado em dietas para pessoas com problemas de digestão ou de má absorção de proteínas, graças à sua elevada digestibilidade e aos aminoácidos essenciais disponíveis (FURLAN e OETTERER, 2002).

Goldhor e Regenstein (1988) enumeram várias características e/ou qualidades que o HPP apresenta na nutrição animal, como a melhoras na palatabilidade de alimentos para animais monogástricos e na digestibilidade do alimento para larvas de peixes, o que promove melhores taxas de crescimento e sobrevida (Cahu *et al.*, 1998). Além do alto teor protéico e baixo teor de cinza. Essa última característica é de suma importância no preparo de produtos destinados à aquicultura.

Em geral, o HPP possui conteúdo de aminoácidos essenciais similares ou até superior ao da proteína referência sugerida pela FAO, onde testes biológicos de digestibilidade, como a relação de eficiência protéica (REP), comparam seu valor nutritivo ao da caseína do leite (DINIZ e MARTIN, 1999; OETTERER, 2001, KECHAOU et al., (2009).

2.5 Métodos de conservação em pescado

O pescado é um produto altamente perecível devido as características intrínsecas da sua carne, como elevada quantidade de água nos tecidos, fatores microbiológicos, rápida instalação da fase de rigidez *post mortem*, liberação de muco, tecido rico em proteínas que servem de substrato para bactérias, pH neutro e presença de ácidos graxos poli-insaturados altamente susceptíveis a oxidação (GONÇALVES, 2011).

A deterioração do pescado se instala logo após a morte e avança com o tempo de exposição e estocagem do produto, sendo que a velocidade de decomposição depende de fatores exógenos (manipulação, manejo de abate, processamento e condição de estocagem) e endógenos (características físico-químicas do peixe) (OETTERER, 1998). Esses processos

deteriorativos envolvem atividades enzimáticas, a rancificação de gorduras e a ação de micro-organismos (FORSYTHE, 2002; SETEVANATO et al, 2007).

Desta forma, a viabilidade de se produzir subprodutos elaborados do pescado, está diretamente relacionado à qualidade dos resíduos gerados nas linhas de produção, desde a captura até a comercialização (PESSATTI, 2001). Entretanto, para prevenir alterações nesses alimentos, sugerem-se formas de acondicionamento adequado (OETTERER, 2002).

De maneira geral, há dois pontos críticos que devem ser levados em consideração durante a produção e o armazenamento do hidrolisado proteico de peixe: a oxidação de lipídeos e a contaminação por microorganismos. Estes fatores podem causar efeitos indesejáveis no produto e diminuição na vida de prateleira.

A oxidação lipídica é a deterioração oxidativa de ácidos graxos, que pode ser iniciada por via enzimática (ação da lipoxigenase) ou por fatores como calor, luz, presença de hemopigmentos da mioglobina e hemoglobia, oxigênio, traços de metais, etc. Os principais efeitos da oxidação sobre o valor nutritivo do pescado são a degradação lipídica (principalmente de ácidos graxos poliinsaturados) a produtos primários como os hidroperóxidos que alteram diversas propriedades importantes, como qualidade sensorial (sabor, textura, aroma). Isto se deve ao fato da oxidação gerar uma grande variedade de compostos carbonílicos, ácidos graxos de cadeia curta, sendo o principal deles o malonaldeído. (PAPAS, 1999). Além disto, a degradação também altera o valor nutritivo do pescado, diminuindo o valor biológico das proteínas. Tais mudanças podem ter origem durante a produção, processamento, armazenamento e preparo dos produtos (Araújo, 2001). Para avaliar a oxidação lipídica são utilizados o valor de peróxido (PV) e o teste do ácido tiobarbitúrico (TBA).

Quanto a contaminação com microrganismos, estes podem está presentes na própria microbiota do pescado ou através de manipulação. Os peixes de águas tropicais tendem a ter uma microbiota mais rica em bactérias mesófilas Gram positivas como o *Micrococcus spp*. e *Bacillus*, enquanto os peixes de água fria abrigam mais bactérias psicotróficas Gram negativas, dentre elas *Pseudomonas* e *Enterococcus*. A presença de *Pseudomonas* é muito mais pronunciada em pescado marinho independente das condições de temperatura da água. Entretanto, a presença de *Aeromonas* e *Enterobacteria* é mais acentuada em pescado de água doce (GONÇALVES, 2011).

Gelli (1988) classifica os contaminates de pescado da seguinte forma: (a) microrganismos deteriorantes: apesentam metabolismos que provocam a deterioração do pescado (os que apresentam capacidade proteolítica, lipolítica, etc), exemplo: *Pseudomonas*;

(b) microrganismos indicadores de higiene e/ou processamento: são indicados para avaliar as condições higiênicas do pescado, exemplo: bactérias mesófilas, coliformes totais, bolores e leveduras; (c) microrganismos indicadores de contaminação fecal: Coliformes termotolerantes com destaque para *Escherichia coli*, *Salmonella* e *Enterococcus faecalis*; (d) microrganismos indicadores de manipulação inadequada: indicam falhas nos pontos críticos de controle, exemplo, *Staphylococcus aureus*; (e) microrganismos capazes de causar doenças veiculadas ao pescado: *Vibrio cholerae*, *Listeria*, *Salmonella*, *E. coli*. (f) microrganismos capazes de liberar histamina: *Morganella morganii*, *Klebsiella pneumoniae* e *Hafnia alvei*; e (g) toxinas biológicas: Tetrodotoxina, Ciguatera, Toxinas paralisantes, Toxinas diarreticas, Neurotoxinas e Toxinas amnésicas.

Na indústria de processamento de pescado, os procedimentos de higienização são fundamentais para assegurar a qualidade dos produtos. Assim, a utilização de cuidados rigorosos de higiene, seguindo normas adequadas, favorece o controle da qualidade, viabiliza os custos de produção, satisfaz os consumidores e os protege contra riscos à sua saúde, além de respeitar as normas e padrões microbiológicos recomendados pela legislação vigente (GERMANO & GERMANO, 2001).

A Resolução-RDC nº 12 de 02/01/01, da Agência de Vigilância Sanitária, estabelece padrões microbiológicos sanitários para alimentos e determina os critérios para conclusão e interpretação de resultados das análises microbiológicas de alimentos destinados ao consumo humano. Como limite de tolerância para amostra indicativa para pescado *in natura*, resfriado ou congelado não consumido cru, fixa os seguintes valores: 103UFC g^{-1} para *Staphylococcus* coagulase positiva, 102 NMP/g para coliformes e ausência de *Salmonella sp.,* sendo esta última classificada como perigo de severidade moderada (SILVA e FILHO, 1999). O limite máximo de bactéria heterotrófica aeróbica psicrotrófica (BHAP) para frutos do mar é de 107UFC g^{-1} (ICMSF, 1986).

A deterioração microbiológica é o principal problema para alimentos com elevada atividade de água e pH neutro. A extensão da deterioração por microrganismos varia de acordo com a temperatura, em geral microrganismos psicotróficos predominam a T < 8º C, enquanto que os mesófilos predominam a T > 15º C. Os princípios para seu controle são: diminuição de temperatura (inibe o crescimento), aumento da temperatura (destruição térmica), remoção da água livre, diminuição do pH pela adição de ácidos ou por fermentação, controle de O_2 ou CO_2 e manipulação da composição do produto removendo nutrientes que sejam essenciais ao microrganismo, por exemplo, desengordurandu-o (GONÇALVES, 2011).

Existem várias de técnicas de conservação que podem ser utilizadas para evitar ou inibir a deterioração microbiológica e prolongar a vida de prateleira como, refrigeração (resfriamento e congelamento), secagem, embalagem a vácuo, embalagem de atmosfera modificada, acidificação, fermentação e adição de conservantes, esterilização, pasteurização e irradiação.

A refrigeração é uma prática baseada no abaixamento da temperatura e compreende os processos de resfriamento e congelamento. O resfriamento pode manter as características do pescado fresco em seu estado original, desde que esteja eviscerado o que diminui a taxa de deterioração (GONÇALVES, 2011). Segundo Jay (2005), temperaturas de resfriamento compreendem entre 0 ºC e 7 ºC. No congelamento, o desenvolvimento de microrganismos é bruscamente inibido devido ao aumento da concentração relativa de soluto e abaixamento da atividade de água nos tecidos (OGAWA e MAIA, 1999).

Alguns ácidos orgânicos podem ser utilizados como aditivos em alimentos. Em produtos de pescado, podem ser usados como: acidificantes, saborizantes, conservantes, aceleradores de cura, fixadores de cor, antioxidantes e sequestradores de metais, (GOLÇALVES, 2011).

Ácidos orgânicos de baixo peso molecular e seus sais atuam como conservantes microbianos, seja pela redução do pH ou pela atuação de suas formas não dissociadas (no caso de ácidos) ou seus ânions (sais) em solução (GOLÇALVES, 2011). Para a preservação ácida, os mais utilizados são os ácidos lático, acético, fórmico e cítrico. A ação preservativa ácida é influenciada por vários fatores, como a concentração, temperatura, tipo e tamanho da população de microrganismos contaminantes (OGAWA e MAIA, 1999).

As leveduras são muito menos susceptíveis aos efeitos de grandes concentrações hidrogeniônicas do que as bactérias. Muitas espécies de bactérias se desenvolvem bem em pH neutro e são incapazes de crescerem em meios com pH abaixo de 4,5, com exceção do *Lactobacillu*s e *Clostridium butryricum*. Entretanto alguns fungos se desenvolvem bem numa faixa de pH ligeiramente ácida (5,0 a 6,0) e podem tolerar meios com pH 2,0 (OGAWA e MAIA, 1999).

A esterilização é um dos métodos mais utilizados em alimentos, consiste na eliminação de microorganismos através da submissão de altas temperaturas (esterelização térmica) ou irradiação, e pode ser avaliado através de técnicas de plaqueamento ou contagem de bactérias (JAY, 2005).

Segundo a International Organization of Energy Agency (IOEA), irradiação é uma forma de energia emitida quando ocorre a excitação de uma partícula (prótons, nêutrons e

elétrons) em um átomo ocasionando uma desestabilização eletrônica, e para estabilizar energeticamente o átomo, é emitida uma energia eletromagnética que se propaga em forma de ondas. Esta energia eletromagnética e sua emissão são chamadas de radiação (GONÇALVES, 2011).

A radiação ionizante age sobre as bactérias contaminates através da lesão de seus ácidos nucleicos, na desnaturação enzimática e na membrana celular microbiana dependendo da dose utilizada, o que explica a inibição de crescimentio microbiano (AYMERICH et al., 2008).

Segundo Brewer (2009), a tecnologia da irradiação é aprovada e regulamentada pelo Food and Drug Administration (FDA) e pelo Code of Federal Regulations (CFR), regulamentando para uso em alimentos, os raios gama emitidos pela fonte cobalto 60 (^{60}Co) e o césio 137 (^{137}Cs).

A dose de radiação é a quantidade de energia absorvida pelo alimento quando este atravessa um campo de radiação, podendo esta ser medida em Gray (Gy)**,** onde 1 Gy equivale a energia de 1 joule absorvido por 1 kg de material. A dose máxima absorvida por uma alimento submetido à irradiação não deve exceder 10 kGy, exceto quando houver necessidade de alcançar uma finalidade tecnológica. (FORSYTE, 2002).

Para a irradiação do pescado, podem ser utilizados cortes como filés e postas de peixes acondicionados em embalagens de poliestireno (isopor) embalados por filme plástico.

Em pescado, a utilização da radiação ionizante associada com outros métodos de conservação como o resfriamento ou congelamento, tratamento térmico, cura, aditivos e embalagem a vácuo; pode aumentar consideravelmente sua vida útil e eliminar patógenos em pescado fresco, resfriado ou congelado além de higienizar individualmente as embalagens de pescado congelado e permitir o desenvolvimento de um produto com maior estabilidade. Todavia, o mesmo cuidado é necessário durante o manuseio da matéria-prima para que não ocorra uma nova contaminação, uma vez que o alimento irradiado estará isento de qualquer flora microbiológica (GONÇALVES, 2011).

2.6 Substituição de farinha de peixe por fontes proteicas alternativas em dietas para organismos aquáticos

O cultivo de organismos aquáticos se baseia no fornecimento de dietas com alto conteúdo proteico. Peixes e crustáceos geralmente apresentam uma maior exigência de proteína nas dietas quando comparado aos animais terrestres, devido a menor exigência em

energia (KAUSHIK e SEILIEZ, 2010). Segundo o NRC (2011), a exigência em proteína da maioria das espécies de camarão marinho é de 35 a 40%. Ingredientes de origem marinha, principalmente a farinha de peixe, são os mais utilizados por serem ótimas fontes de nutrientes.

As características das farinhas de peixe variam amplamente de acordo com a origem, que pode ser a partir de pequenos peixes pelágicos inteiros ou a partir dos resíduos da indústria de beneficiamento (HARDY e BARROWS, 2002; NRC, 2011). No geral, a farinha de peixe possui um teor de proteína que pode variar entre 54 a 72% (FURUYA, 2010; NRC, 2011), sendo rica em nutrientes essenciais, como os aminoácidos metionina e lisina, e os ácidos graxos poli-insaturados EPA (ácido eicosapentanóico) e DHA (ácido docosahexanóico). Além disso, a farinha de peixe tem alta digestibilidade, que garante uma boa conversão alimentar e com isso a redução de resíduos nitrogenados no ambiente de cultivo, além de alta palatabilidade (WATANABE, 2002; NRC, 2011). De modo geral, a farinha de peixe compreende entre 17 a 65% da composição das dietas utilizadas na alimentação de peixes de altos níveis tróficos e crustáceos e entre 2 a 10% em dietas para peixes de baixos níveis tróficos, com execeção de algumas espécies de tilápias e bagres onde alguns países utilizam até 25% (FAO, 2012).

Em 2010, 20,2 milhões de toneladas de pescado foram destinados a fins não alimentares, dos quais 75% (15 milhões de toneladas) foi reduzido a farinha e óleo de peixe. Aproximadamente 60% da farinha de peixe produzida em 2008 foram direcionadas para o uso da aquicultura (FAO, 2012). Muitas espécies diferentes são usadas para a produção de farinha e óleo de peixe. No entanto, pequenos pelágicos, em especial, anchoveta, são os principais grupos de espécies utilizadas, porém o volume destes produtos varia anualmente de acordo com as flutuações nas capturas destas espécies. O fenômeno do El Niño tem efeitos consideráveis sobre as capturas de anchovetas, com uma série de picos e quedas drásticas nas últimas décadas, passando de 12,5 milhões de toneladas em 1994 para 4,2 milhões de toneladas em 2010. Como resultado, a produção de farinha e óleo de peixe exibiram tendências semelhantes.

A produção de farinha de peixe atingiu o pico em 1994 com 7,48 milhões de toneladas e seguiu uma tendência flutuante desde então. Em 2009, caiu para 5,74 milhões de toneladas, devido às capturas reduzidas de anchoveta (FAO, 2012). A análise dos últimos 15 anos (1994-2009) indica que a produção mundial de pesca marinha destinada a produção de farinha e óleo de peixe têm sido diminuindo a taxas médias anuais de 1,7% a 2,6% (FAO, 2012).

Diante do exposto, a aqüicultura mundial deve buscar reduzir a dependência quanto ao uso de farinha de peixe, que apesar de ser a principal fonte protéica inserida em rações, possui qualidade às vezes duvidosa, possui oferta limitada, demanda crescente, variabilidade da disponibilidade e constante flutuação nos preços, o que pode afetar a sustentabilidade e rentabilidade da aqüicultura. Estes entraves promovem limitações ao uso da farinha de peixe em rações e vem motivando a busca por potenciais substitutos à mesma (FARIA et al., 2001).

Diversos produtos têm sido utilizados com o propósito de substituir total ou parcialmente a farinha de peixe em rações aquáticas, incluindo subprodutos de pescado ou de animais terrestres, sementes oleaginosas, plantas aquáticas, concentrados protéicos, proteína de organismos unicelulares e subprodutos de leguminosas e cereais (ALAM et al., 2005). A determinação de fontes proteicas de menor custo e que promovam bom crescimento é vantajosa tanto para a indústria de rações como também para os aquicultores (COYLE et al., 2004). Sendo assim, vários estudos têm sido realizados para avaliar novas fontes proteicas que poderiam ser usadas na fabricação de rações (OLVERA-NOVOA ET al., 1997; OLVERA-NOVOA et al., 2002; PLASCENCIA-JATOMEA et al., 2002; EL - SAIDY e GABER, 2003; GABER, 2006, LEAL et al., 2009). Entretanto, tais recursos devem garantir os mesmos ou melhores resultados de produção, sanidade do animal, crescimento e qualidade do produto.

O farelo de soja tem sido preconizado como a principal fonte protéica de origem vegetal em rações aquícolas (FURUYA et al., 2001a). Embora possua alto teor de proteína e um bom perfil de aminoácidos, seu nível de metionina é baixo, contêm aproximadamente 30% de carboidratos indigeríveis, vários compostos ou fatores antinutricionais que podem prejudicar os processos digestórios e limitação na disponibilidade do fósforo (HERNÁNDEZ et al., 2006). Nos alimentos de origem vegetal, cerca de 70% deste mineral está complexado na forma de fitato, que não é utilizado pelos monogástricos e que promove também a redução na disponibilidade de outros elementos, como zinco, cálcio, ferro e manganês (FARIA et al., 2001). Com relação aos teores de fibras indigeríveis, OLVERA-NOVOA et al. (1997) e FONSECA (2004) recomendam o uso de concentrados protéicos como uma forma de eliminar as fibras indigeríveis do ingrediente integral, permitindo o uso de maiores níveis de materiais vegetais nas rações para peixes. MENTE et al. (2002) demostraram que os aminoácidos constituintes da proteína do peixe são particularmente importantes quando incorporados nas dietas contendo proteína vegetal. Assim, a utilização desse produto em rações animais exige um adequado processo de fabricação (FURUYA et al., 2001b). De forma geral, a viabilidade da substituição da farinha de peixe por ingredientes vegetais estará condicionada ao hábito alimentar do animal a ser cultivado.

Neste sentido, a substituição da farinha de peixe por alimentos alternativos é importante para a redução dos custos de produção. Uma vez que se estimam os gastos com alimentação entre 40 a 50% do custo total da produção, visto que a proteína é o nutriente mais oneroso da dieta. Uma boa alimentação deve satisfazer as necessidades nutricionais das espécies cultivadas, como também ser de fácil digestão e com uma boa perspectiva custo-benefício (LEMOS et al., 2004).

Subprodutos da indústria pesqueira também vêm sendo estudados como alternativa na substituição da farinha de peixe. A produção de hidrolisado protéico a partir de resíduos das indústrias de pescado representa uma excelente alternativa para o incremento da oferta de proteína animal (KLOMKLAO et al., 2005; CENTENARO, 2009). Hidrolisados proteicos de peixes tem sido relatado como potenciais substitutos à farinha de peixe em dietas para pós-larvas de *Litopenneus vannamei* (SOUZA DO VALLE et al., 2013), assim como hidrolisados proteicos de camarão são relatados como potenciais substitutos à farinha de peixe em dietas para *Oreochromis niloticus* (LEAL et AL., 2009). Em geral, devido à sua elevada solubilidade e ao seu balanço em aminoácidos os HPP apresentam vantagem sobre a farinha de resíduo de peixe (FURLAN e OETTERER, 2002).

Além da qualidade nutricional, os hidrolisados também contêm compostos que estimulam a resposta imune não específica em peixes (MURRAY et al., 2003; LIANG et al., 2006). Frações de baixo peso molecular podem agir como imunomoduladores, aumentando a atividade dos macrófagos de peixes (BOGWALD et al., 1996; GILDBERG et al., 1995). Assim, a suplementação de dietas com hidrolisados proteicos pode aumentar o crescimento dos peixes e camarões, bem como aumentar a resistência a doenças.

3. OBJETIVOS

3.1 Geral

- Avaliar o uso de resíduo do processamento da tilápia *Oreochromis niloticus* como fonte protéica e enzimática na produção de hidrolisado protéico de peixe (HPP).

3.2 Específicos

- Obter proteases a partir de extratos brutos do intestino de tilápias;
- Produzir HPP a partir de resíduos do processamento da tilápia;
- Definir o grau de hidrólise dos HPP utilizando duas fontes enzimáticas;
- Identificar o perfil eletroforético da hidrólise das proteínas presentes nos HPP;
- Obter o perfil nutricional dos HPP, mediante análise bromatológica, composição de aminoácidos, ácidos graxos e escore químico baseado nos requerimentos nutricionais de camarões juvenis carnívoros e onívoros;
- Semi-purificar proteases de intestino de tilápia utilizando metodologias como: precipitação com sulfato de amônio e precipitação com etanol;
- Obter hidrolisado protéico de carcaça de tilápia utilizando as enzimas semi-purificadas e uma comercial como referência;
- Avaliar diferentes metodos de conservação (acidificação, esterilização térmica e irradiação Gamma) e monitorar a dinâmica microbiológica e química das amostras de hidrolisado.

CAPÍTULO 1

Utilization of tilapia processing waste for the production of fish protein hydrolysate

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Utilization of tilapia processing waste for the production of fish protein hydrolysate

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ABSTRACT

Viscera and carcasses represent 60–70% of whole fish body weight, and this processing waste is a known source of bioactive molecules, including proteases. These enzymes can be employed in various biotechnological processes, such as, for preparing fish protein hydrolysate (FPH). Therefore, the objective of this study was to evaluate the use of processing waste from Nile tilapia (*Oreochromis niloticus*) as a source of protein and proteases to produce FPH. Three FPH production conditions were evaluated: two conditions used autolysis with enzymes extracted from the tilapia intestine at different concentrations (FPH_{100}, 100 mg of tissue/mL and FPH_{600}, 600 mg of tissue/mL) and the third used 0.5% (v/v) Alcalase (FPH_{com}), a commercial protease preparation. Protein, amino acids and fatty acids content were calculated as DM basis. After a 4-h reaction, maximum hydrolysis percentages from FPH_{com}, FPH_{100}, and FPH_{600} systems were 34.73 ± 1.44%, 29.21 ± 0.79%, and 41.66 ± 1.33%, respectively. The protein content in the resulting FPS was 584.8 g/kg, 492.3 g/kg, and 508.2 g/kg for FPH_{com}, FPH_{100}, and FPH_{600}, respectively. Methionine and lysine were found at levels of 32.0 and 77.0 g/kg (FPH_{com}), 31.0 and 64.0 g/kg (FPH_{100}), and 33.0 and 69.0 g/kg (FPH_{600}), respectively. Polyunsaturated fatty acid contents of FPH_{com}, FPH_{100}, and FPH_{600} were 101.0 g/kg, 138.0 g/kg, and 70 g/kg, respectively, with a predominance of linoleic acid (C18:2*n*−6). Amino acid composition, lipid profile, and amino acid score suggested that all of the experimental FPHs could be employed as a protein source in diets for aquatic organisms and other farmed animals.

1. Introduction

Nile tilapia, *Oreochromis niloticus* (L.), is the second most cultivated freshwater fish worldwide, and the filet is the main processed product, yielding 30–40% of whole fish wet weight. Therefore, approximately 60–70% of the fish body is processing waste, which includes meat remains, head, skin, bones, scales, and viscera (Clement and Lovell, 1994). While a fish carcass

Abbreviations: FPH, fish protein hydrolysate; E/S, enzyme:substrate ratio; AA, amino acid; BApNA, benzoyl-DL-arginine-*p*-nitroanilide; SApNA, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; Leu-*p*-Nan, leucine-*p*-nitroanilide; DH, degree of hydrolysis; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMSO, dimethylsulphoxide; BSA, bovine serum albumin; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

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has high protein content, fish viscera (mainly stomach, pyloric caeca, and intestine) is an important source of proteases (Bezerra et al., 2001, 2005; Souza et al., 2007; Espósito et al., 2009; Marcuschi et al., 2010; Silva et al., 2011).

Large amounts of protein-rich by-products from the fishery and aquaculture industry are discarded or processed into fish meal. However, the use of these processing wastes to produce fish meal may be restricted, mainly due to its high ash content. In contrast, enzymes are an important tool for the food industry due to their ability to transform raw materials into improved food products. For example, proteases are used to hydrolyze proteins and polypeptides to produce low molecular weight peptides and free amino acids, increasing the digestibility of the product (Shahidi and Kamil, 2001). Therefore, enzymatic hydrolysis is an alternative to recovering protein from fishery and aquaculture processing waste, resulting in a more soluble product known as fish protein hydrolysate (FPH) (Martone et al., 2005; Leal et al., 2009). In this way, production of FPH by proteolytic treatment may be a way to transform cheap pelagic fish, by-catch from trawlers, and fish processing waste into products with improved quality and functional characteristics (Shahidi 1994; Kristinsson and Rasco, 2000).

In this study, we evaluated the use of tilapia processing waste as a source of protein and proteases to produce FPHs. FPHs were also characterized regarding their degree of hydrolysis (DH), molecular weight range of the peptides, proximate chemical composition, amino acid and lipid profiles, and chemical score on the basis of whole-egg protein. Additionally, tilapia proteases extract results were compared with those of a commercial enzyme extract (Alcalase).

2. Materials and methods

2.1. Raw-materials, enzyme extraction and protein determination in intestine crude extracts

By-products (viscera and carcass) from farmed tilapia were kindly donated by Noronha Pescados Ltd., a local fish processing plant in Pernambuco State, Brazil. The materials were brought immediately to the laboratory on ice. By-products comprised tilapia heads, meat remains, skin, bones, and viscera. All other reagents used in enzymatic assays were of analytical grade and purchased from Sigma (St. Louis, MO, USA).

The intestinal were collected and homogenized in distilled water (4 °C) at concentrations of 100 and 600 mg of tissue/mL and these solutions were named ONp_{100} and ONp_{600}, respectively. The resulting preparation was centrifuged (Herolab Unicen MR Centrifuge, Germany) at 10,000 × *g* for 15 min at 4 °C to remove cell debris and nuclei. The supernatant (crude extract) was frozen at −20 °C for subsequent use in enzymatic assays. Soluble protein concentration was determined by the Folin phenol reagent method (Lowry et al., 1951), and absorbance was measured at 750 nm in a microplate reader with bovine serum albumin (BSA) as the standard. This protein measurement method was found to be more suitable for the enzyme extract in this work.

2.2. Enzymatic assay

2.2.1. Total proteolytic activity assay

Total proteolytic activities were determined in all extracts using 1% (m/v) azocasein as the substrate prepared in 0.1 M Tris–HCl, pH 8. The assay was performed by mixing 30 μL of sample with 50 μL of 1% azocasein for 60 min at 25 °C, and the reaction was stopped by adding 240 μL of 10% trichloroacetic acid (TCA) for 15 min. The mixture was centrifuged at 8000 × *g*, for 5 min. The supernatant (70 μL) was mixed with 1 M NaOH (130 μL) and absorbance was measured at 450 nm in a microplate reader (Bio-Rad X-Mark spectrophotometer, Hercules, CA, USA). A blank control was prepared by replacing the sample with 0.1 M Tris–HCl, pH 8.0 (Bezerra et al., 2005). Previous experiments showed that the enzymatic reaction performed under the conditions mentioned above followed first order kinetics during the first 60 min. One unit (U) of enzymatic activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001 absorbance per minute change. Specific enzyme activity was calculated by dividing the enzyme activity (U) per amount of protein in the sample (mg/mL) and expressed as U/mg.

2.2.2. Specific enzyme assays

Trypsin, chymotrypsin, and leucine aminopeptidase activities were determined in 96-well microtiter plates using benzoyl-DL-arginine-*p*-nitroanilide (BApNA), succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SApNA), and leucine-*p*-nitroanilide (Leu-*p*-Nan) as specific substrates, respectively, prepared in dimethyl sulfoxide. The assay was performed by mixing 30 μL of sample with 140 μL of 0.1 M Tris–HCl, pH 8.0 and 30 μL of 8 mM BApNA, SApNA, or Leu-*p*-Nan for 10 min at 25 °C. Release of p-nitroaniline (product) was measured at 405 nm in a microplate reader (Bio-Rad X-Mark spectrophotometer). A blank control was prepared by replacing the sample with 0.1 M Tris–HCl, pH 8.0 (Souza et al., 2007). One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing 1 μmol of BApNA, SApNA, or Leu-*p*-Nan per minute under the established conditions, using a molar coefficient of 9100 mM^{-1} cm^{-1} (Bezerra et al., 2005). Enzymatic specific activity is expressed as mU/mg.

2.3. Fish protein hydrolysates (FPHs)

FPHs ($n = 3$) were produced by fish enzyme hydrolysis adapted from the method described by Cahú et al. (2012). A commercial enzyme (Alcalase, Product Code P4860, supplied by Sigma–Aldrich, St. Louis, MO, USA). at 0.5% (m/v) was adopted

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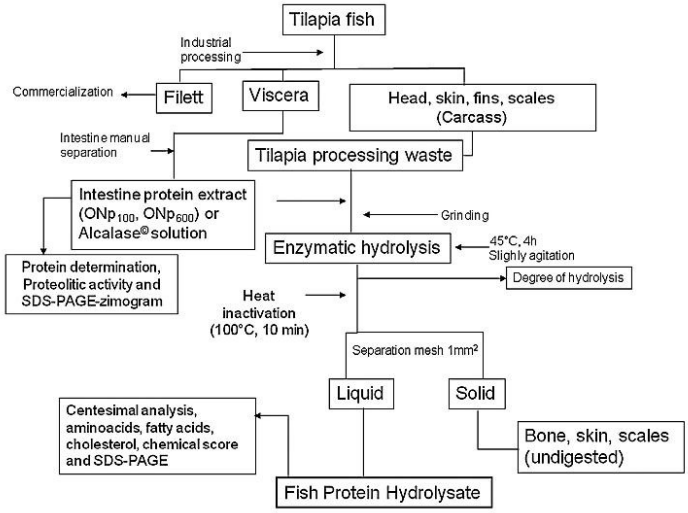

Fig. 1. Schematic of fish protein hydrolysate production.

as a comparative standard. The carcasses (200 g, without visceras) were washed in distilled water prior to hydrolysis and were mixed together in an industrial blender with 0.5% (w/v) Alcalase solution (200 mL), fish enzyme at 100 mg/mL (200 mL) and 600 mg/mL (200 mL) to produce FPH_{com}, FPH_{100}, and FPH_{600}, respectively. The mixture was digested in a water bath at 45 °C for 240 min. Then, the temperature was raised to 100 °C for 10 min to stop the reaction. Solid and liquid fractions were separated by filtration (1 mm^2-mesh), and the filtrate was defined as FPH (Fig. 1).

2.4. Degree of hydrolysis (DH)

DH is defined as the percentage of free amino groups obtained by the cleavage of a protein, which was calculated from the ratio of α-amino nitrogen and total nitrogen (Nilsang et al., 2005). During the hydrolysis process ($n = 3$), one 6-mL sample was collected at 0, 10, 20, 30, 60, 90, 120, 150, 180, 210, and 240 min from each of the hydrolysis conditions. The reaction was heat inactivated as before and added of 4 mL of 6.25% (w/v) TCA. After resting for 15 min, these samples were centrifuged (8000 × *g* for 10 min at room temperature) and the supernatant proteins were quantified using the method of according to Smith et al. (1985) by the measurement using bicinchoninic acid, and absorbance was measured at 565 nm in a microplate reader (Bio-Rad X-Mark spectrophotometer, Hercules, CA, USA), this process determined the soluble protein in each sample using bovine albumin as standard (Sigma-Aldrich, St. Louis, MO, USA) This method proved to be more sensible for determination of soluble proteins and peptides. DH was measured according to the method described by Hoyle and Merritt (1994) and Liceaga-Gesualdo and Li-Chan (1999), calculated according to the equation:

$$\%DH = \left(\frac{6.25\%TCA\ soluble\ protein\ in\ the\ sample}{total\ protein\ in\ the\ sample}\right) \times 100.$$

Total protein was determined by the Kjeldahl method (AOAC, 1995a) and the conversion factor used was 6.25.

2.5. Electrophoresis and zymograms

At the end of 240 min of hydrolysis, the samples were centrifuged and dialyzed (molecular weight cut-off 14 kDa), each sample (100 μg of protein) was concentrated by lyophilization and used for electrophoresis, according to Laemmli (1970), on a 4% (w/v) stacking gel and 14.0% (w/v) separating gel. The gel was stained with silver (Yaobo, 2002). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted at 11 mA using a vertical electrophoresis system (Vertical Electrophoresis System, Bio-Rad). Molecular mass of the purified protein band was estimated by comparison with a molecular mass standard (Amersham Biosciences, Little Chalfont, UK) containing myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), transferrin (80 kDa), BSA (66 kDa), glutamate dihydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21 kDa). Zymograms were also performed following

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Table 1
Protein determination and total proteolytic and specific activity of the proteases from protein hydrolysis using non-specific and specific substrates.

Enzymes	Total protein* (mg/mL)	Alkaline protease activity (Azocasein) (U/mg)	BApNA (mU/mg)	SApNA (mU/mg)	Leu-p-Nan (mU/mg)
Alcalase	11.95	5.55 ± 0.09^a	0.70 ± 0.03^a	2.62 ± 0.35^a	0.32 ± 0.02^a
ONp_{100}	13.47	5.71 ± 0.04^b	2.59 ± 0.06^b	2.75 ± 0.08^a	1.85 ± 0.02^b
ONp_{600}	20.69	6.92 ± 0.01^c	8.18 ± 0.14^c	9.64 ± 0.13^b	6.10 ± 0.04^c

BAPNA, benzoyl-DL-arginine-p-nitroanilide – trypsin-specific substrate; SAPNA, succynil-alanine-alanine-proline-phenylalaninep-nitroanilide; Leu-p-Nan, leucine-p-nitroanilide – leucine aminopeptidase-specific substrate. Values are shown as mean ± standard deviation (SD) of triplicates. Different italic superscript letters denote statistical differences by Tukey's test ($P<0.05$).

* Measured with the Folin phenol method (Lowry et al., 1951), and with bovine serum albumin (BSA) as standard.

the procedure described by García-Carreño et al. (1993), on a 4% (w/v) stacking gel and 14.0% (w/v) separating gel. After electrophoresis, the gels were washed in 0.1 M Tris–HCl and immersed in 2.5 mL/L (100 mL) Triton X-100 in 0.1 M Tris–HCl, pH 8.0, for 30 min at 4 °C to remove SDS. Triton X-100 was removed by washing the gels three times with 100 mL of 0.1 M Tris–HCl buffer, pH 8.0. Afterward, the gel was then incubated with 100 mL of 3 g/L casein in 0.1 M Tris–HCl, pH 8.0, for 30 min at 4 °C. The temperature was raised to 25 °C and maintained for 90 min to allow digestion of casein by the active fractions. The gel was then stained with 0.1% (w/v) Coomassie Blue for 2 h and destained in 10% (v/v) acetic acid and 25% (v/v) methanol for 12 h.

2.6. Proximate composition, composition of amino acids, fatty acids and cholesterol

Analyses of proximate composition, amino acid and fatty acid profiles, and cholesterol from the FPHs were performed at the Laboratory of Experimental and Food Analysis, Department of Nutrition, Federal University of Pernambuco. In summary: Moisture (AOAC, 1996, method 926.12) content was carried out by weighting 5 g of prepared sample and drying to constant weight in vacuum oven at uniform temperature 20–25 °C above boiling point of H_2O at working pressure and cool in efficient desiccator for 30 min. Constant weight is attained when successive 1 h drying; Crude protein (AOAC, 1995a, method 991.20) was measured by the Kjeldhal method and 6.25 as N-protein conversion factor; Ether extract (U.K. FEEDING STUFFS, 1982, pp. 9–11), sample was extracted with petroleum ether and dried for 3–4 h. The contents were finely ground, and the material was again extracted for another hour. After evaporation of the solvent, the oil was dried at 100 °C and weighed; Ash (AOAC, 1995b, method 920.39) an amount of 2 g sample was dried and pre-extracted with water, extracted in Soxhlet with dry ethyl ether for 4 h, following evaporate the ether, cooled and weighted; Essential amino acids, non-essential amino acids (White et al., 1986, pp. 170–177; Hagen et al., 1989, pp. 912–916), samples were hydrolyzed in 6 M HCl/phenol solution for 24 h to obtain free amino acids, following derivatization with phenylisothiocyanate (PITC), separated by reversed-phase HPLC and detected by UV at 254 nm; Fatty acids, and cholesterol (AOAC, 2005, 2007, method 996.06) samples were hydrolyzed with HCl in ethanol in a warm bath (70–80 °C) for 40 min and then extracted with ethyl ether and petroleum ether solution (1:1), the solvent was removed and the content methylated and analyzed by GC-FID. The results were converted to DM basis.

2.7. Chemical score of FPHs and Indispensable Amino Acid Index (IAAI)

Indispensable Amino Acid Index (IAAI) and chemical scores of FPHs were calculated according to Hardy and Barrows (2002), relative to the EAA profile based on the reference amino acid in whole-egg protein. Indispensable Amino Acid Index (IAAI) is the ratio of the indispensable amino acid in the test protein (TP) divided by the indispensable amino acids in whole-egg protein (WEP) a follows:

$$\text{IAAI} = \frac{\text{HIS(TP)}}{\text{HIST(WEP)}} + \frac{\text{ISO(TP)}}{\text{ISO(WEP)}} + \frac{\text{LEU(TP)}}{\text{LEU(WEP)}} + \cdots + \frac{\text{ARG(TP)}}{\text{ARG(WEP)}} \times 100$$

Chemical scores were calculated using the following equation:

$$\text{Chemical score} = \frac{\text{EAA in test protein(g/kg)}}{\text{EAA amino acid in whole} - \text{egg protein(g/kg)}} \times 100$$

2.8. Statistical analysis

Data from enzyme activities of different preparations were compared and analyzed using one-way analysis of variance (ANOVA) and Tukey's test. $P<0.05$ was considered as statistically significant. Microcal Origin version 8.0 was used for the analysis (Software, Inc., USA) (Zar, 1984).

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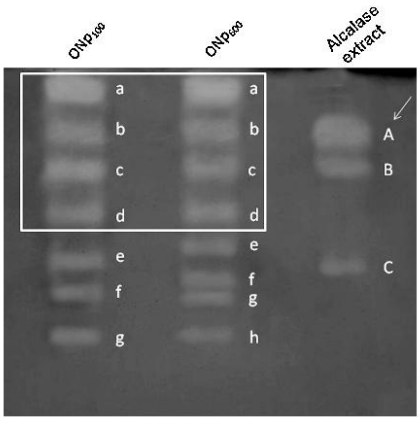

Fig. 2. Protease activity of *Oreochomis niloticus* intestine extract and Alcalase extract (native-PAGE with Acrylamide/bis). *Lane 1*: ONp_{100}, *Lane 2*: ONp_{600}, *Lane 3*: Alcalase extract.

3. Results

Three protease extracts including Alcalase, ONp_{100}, and ONp_{600} were employed to hydrolyze fish processing waste. Table 1 shows the proteolytic activity of the proteases used in this study. Total proteolytic activity (azocasein as the substrate) was different among the extracts ($P<0.05$). ONp_{600} showed the highest specific activity (6.92 ± 0.01 U/mg), followed by ONp_{100} (5.71 ± 0.04 U/mg), and Alcalase (5.55 ± 0.09 U/mg). Use of specific substrates revealed the presence of trypsin, chymotrypsin, and leucine aminopeptidase. In the experimental extracts, trypsin (BApNA as the substrate) and leucine aminopeptidase (Leu-*p*-Nan as the substrate) activities were significantly different among treatments ($P<0.05$), whereas ONp_{600} (8.18 ± 0.14 and 6.10 ± 0.04 mU/mg) showed the highest specific activity, followed by ONp_{100} (2.59 ± 0.06 and 1.85 ± 0.02 mU/mg), and alcalase (0.70 ± 0.03 and 0.32 ± 0.02 mU/mg). Chymotrypsin (SApNA as the substrate) activity was not significantly different from Alcalase (2.62 ± 0.02 mU/mg) and ONp_{100} (2.75 ± 0.08 mU/mg). ONp_{600} (9.64 ± 0.13 mU/mg) showed higher specific chymotrypsin activity compared with those of ONp_{100} and Alcalase (Table 1).

A caseinolytic zymogram was prepared to compare the proteolytic activities of the enzymes extracts (Fig. 2). Similar patterns were observed for ONp_{100} and ONp_{600}. Seven caseinolytic bands were observed for ONp_{100} (a–g) and eight were observed for ONp_{600} (a–h). Four bands were common to both extracts (a–d). The main difference in these extracts was found between bands e and h (see the highlight in Fig. 2). These different bands are due the different concentration in the extracts, which may appear splitted or additional probably in consequence of enzyme activation/concentration (enzyme per 50 μg of protein applied). Alcalase preparation showed a lower number of caseinolytic bands, three (A, B, and C), and one of them with higher intensity (A).

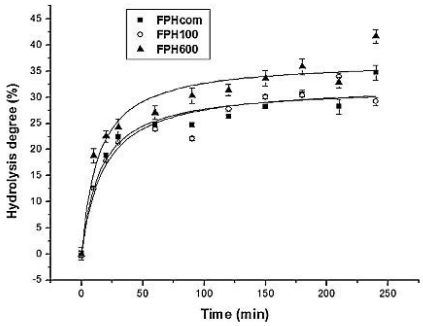

Fig. 3. Degree of hydrolysis of fish protein hydrolysates (■ HPP_{com} $R^2 = 0.97708$), (HPP_{100}, $R^2 = 0.95775$) (▲ HPP_{600} $R^2 = 0.98462$).

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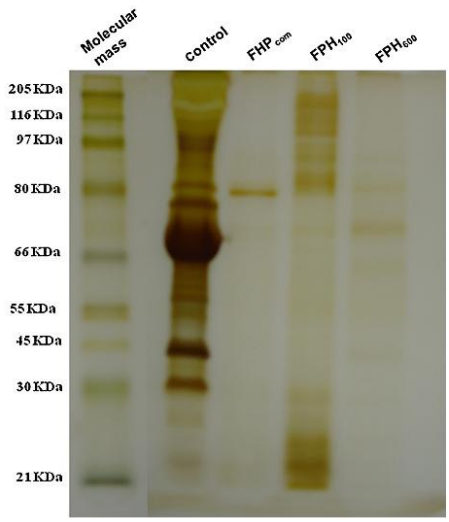

Fig. 4. Polyacrylamide gel electrophoresis – SDS-PAGE of FPH at the end of 240 min. *Lane 1* (standard markers: myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), transferrin (80 kDa), BSA (66 kDa), glutamate dihydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21 kDa), lane 2 (control-carcass without enzyme), lane 3 Fish protein hydrolyzate$_{com}$ (FHP$_{com}$), lane 4 Fish protein hydrolyzate $_{100}$ (FPH$_{100}$), lane 5 Fish protein hydrolyzate $_{600}$(FPH$_{600}$).

Table 2
Comparison of proximate composition and calorific value of fish protein hydrolysates (FPH) and carcass of tilapia (*Oreochromis niloticus*) with fish meal (DM basis).

Components	Carcass	FPH$_{com}$	FPH$_{100}$	FPH$_{600}$	Menhaden meal (NRC, 1993)
Crude protein (g/kg)	423.60	584.80	492.30	508.20	645.00
Ether extract (g/kg)	428.40	374.70	407.00	445.10	96.00
Ash (g/kg)	99.40	26.70	52.10	30.40	190.00
Crude fiber (g/kg)	12.80	18.70	33.2	17.50	7.00
Calorie (kcal/100 g)	205.07	106.00	136.11	103.38	406.00

Fig. 3 shows a comparison of the hydrolysis curves for the enzymatic extracts. The curves exhibited an initial fast reaction. The hydrolysis curves for FPH$_{com}$ and FPH$_{100}$ after 60 min were quite similar and reached a plateau. Maximum DH obtained after 240 min at a 1:1 enzyme extract/cascass ratio was 41.66% ± 1.33 for FPH$_{600}$, 34.73% ± 1.44 for FPH$_{com}$, and 29.21% ± 0.79 for FPH$_{100}$.

Electrophoretic profiles of the protein from the control (carcass protein extract obtained without enzymatic treatment), FPH$_{com}$, FPH$_{100}$, and FPH$_{600}$ at the end of 240 min are displayed in Fig. 4. Different protein patterns were observed between the control and all experimental hydrolysates. The control showed a diversity of bands ranging mainly from 30 to 205 kDa (lane 2). Compared with the control profile, it was evident that the three enzymatic treatments were efficient for producing FPHs. Only one band (80 kDa) was found (lane 3) for FPH$_{com}$. When intestine extracts were employed for hydrolysis, few bands between 190 and 30 kDa were observed for FPH$_{100}$ treatment (lane 4) and bands between 80 and 30 kDa were observed for FPH$_{600}$ treatment (lane 5).

Proximate composition of FPHs in DM basis and a comparison between tilapia carcass and menhaden meal are given in Table 2. All FPHs in contained a low quantity of dry matter (170–190 g/kg), ash (26–31 g/kg), and a high amount of protein (490–590 g/kg).

Saturated fatty acid contents of FPH$_{com}$, FPH$_{100}$, and FPH $_{600}$ were 165.0, 254.0, and 309.0 g/kg, respectively, and were predominantly myristic (C14:0), 19.0 g/kg (FPH$_{com}$), 25.0 g/kg (FPH$_{100}$) and 35.0 g/kg (FPH$_{600}$) and palmitic (C16:0), 115.0 (FPH$_{com}$), 177.0 (FPH$_{100}$) and 204.0 (FPH$_{600}$) and stearic acids (C18:0) 24.0 (FPH$_{com}$), 43.0 (FPH$_{100}$), 46.0 (FPH$_{600}$). Monounsaturated fatty acids were present at 190.0 g/kg (FPH$_{com}$), 178.0 g/kg (FPH$_{100}$), and 299.0 g/kg (FPH$_{600}$) and were predominantly oleic (C18:1*n*−9c), 148.0 g/kg (FPH$_{com}$), 157.0 g/kg (FPH$_{100}$), 224.0 g/kg (FPH$_{600}$) and palmitoleic acids (C16:1), 33.0 g/kg (FPH$_{com}$), 21.0 g/kg (FPH$_{100}$), 50.0 g/kg (FPH$_{600}$). Polyunsaturated fatty acid contents of FPH$_{com}$, FPH$_{100}$, and FPH$_{600}$ were 101.0, 70, and 138.0 g/kg, with a predominance of linoleic acid (C18:2*n*−6), 70.0 g/kg (FPH$_{com}$), 60.0 g/kg (FPH$_{100}$), 107.0 g/kg (FPH$_{600}$). Furthermore, Omega-3, 7.0 g/kg (FPH$_{com}$), 10.0 g/kg (FPH$_{100}$), 10.0 g/kg (FPH$_{600}$), Omega 6, 84.0 g/kg (FPH$_{com}$),

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Table 3
Fatty acid composition of fish protein hydrolyzates (FPH) (DM basis).

Fatty acids (g/kg)	FPH_{com}	FPH_{100}	FPH_{600}
(C12)	1.0	ND	1.0
(C14:0)	19.0	25.0	35.0
(C15:0)	2.0	ND	5.0
(C16:0)	115.0	177.0	204.0
(C17:0)	2.0	ND	6.0
(C18:0)	24.0	43.0	46.0
(C20:0)	1.0	ND	2.0
(C22:0)	1.0	ND	7.0
Saturated fat	**165.0**	**245.0**	**309.00**
(C14:1)	1.0	ND	2.0
(C16:1)	33.0	21.0	50.0
(C18:1*n*9c)	148.0	157.0	224.0
(C20:1)	7.0	ND	11.0
(C22:1*n*9)	0.0	ND	9.0
(C24:1)	0.0	ND	0.0
Monounsaturated	**190.0**	**178.0**	**299.0**
(C18:2*n*6c)	70.0	60.0	107.0
(C18:3*n*6)	4.0	ND	8.0
(C18:3*n*3)	5.0	9.0	10.0
(C20:2)	4.0	ND	2.0
(C20-3*n*3)	4.0	ND	ND
(C20:3*n*6)	1.0	ND	2.0
(C20:4*n*6)	4.0	ND	ND
(C20:5*n*3)	1.0	ND	1.0
(C22:6*n*3)	6.0	ND	8.0
Polyunsaturated fats	**101.0**	**70.0**	**138.0**
(C18:1*n*9t)	2.0	ND	4.0
Trans fat	2.0	ND	4.0
Omega 3	7.0	10.0	10.0
Omega 6	84.0	60.0	117.0
Cholesterol	1.0	ND	5.0

(ND) Not detected by the methods used by AOAC (2005, 2007).

60.0 g/kg (FPH_{100}), 117.0 g/kg (FPH_{600}) and cholesterol 1.0 g/kg (FPH_{com}), 5.0 g/kg (FPH_{600}) were present in all hydrolysates (DM basis) (Table 3).

Methionine and lysine were found at levels of 32.0 and 77.0 g/kg (FPH_{com}), 31.0 and 64.0 g/kg (FPH_{100}), and 33.0 and 69.0 g/kg (FPH_{600}), respectively (Table 4, DM basis). Amino acid chemical scores based on the amino acids of whole-egg protein were given in Table 5. EAA scores from FPH_{com} ranged from 50.65 to 137.87%, FPH_{100} ranged from 40.25 to 100% and FPH_{600} ranged from 45.97 to 109.09%. IAAI reached a value of 1066.07 in FPH_{com}, 688.4 in FPH_{100} and 738.51 in FPH_{600}, while fishmeal and soybean meal reached a value of 409.72 and 346.23, respectively. All these values were higher than those found in fish and soybean meal.

4. Discussion

Proteolytic enzyme hydrolysis is a process used to increase the nutritional value of proteins by improving their digestibility (Li et al., 2010). Most bioactive peptides from food proteins have been obtained by hydrolysis with commercial proteases, such as Alcalase, which is an alkaline bacterial protease produced from *Bacillus licheniformis* (Hoyle and Merritt, 1994; Bhaskar et al., 2008). However, industrial applications of commercial proteases are limited by their high cost, and because of increasing demand for protein hydrolysates, a cheaper resource and simpler method for preparing these enzymes are required.

Fish viscera is a known by-product of the fishery and aquaculture industries and has been proposed as a potential source of enzymes for biotechnological applications (Bezerra et al., 2001, 2005; Leal et al., 2010; Marcuschi et al., 2010). In this study, higher trypsin, chymotrypsin, and leucine aminopeptidase activities were found in both fish enzyme preparations tested, whereas trypsin and leucine aminopeptidase activities were low in alcalase, as the major enzyme component is subtilisin (Corîci et al., 2011). Fish digestive tract generally has a high concentration of serine endoproteases, mainly trypsin and chymotrypsin (Klomklao et al., 2007). Trypsin cleaves the peptide bonds on the carboxyl side of positively charged amino acid residues, such as arginine and lysine, whereas chymotrypsin catalyzes the hydrolysis of peptide bonds more efficiently in the carboxyl portion of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan (Klomklao et al., 2007). Exopeptidase activity (leucine aminopeptidase) was also detected in all extracts, and higher leucine aminopeptidase activities were observed in ONp_{600} and ONp_{100}. In fact, the presence of endoproteases and exoproteases may make proteolytic hydrolysis more efficient. After the action of endoproteases, such as trypsin and chymotrypsin on protein, exoproteases, such as leucine aminopeptidase, cleave long-chain peptides into smaller peptides and free amino acids, thereby enhancing

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Table 4
Comparison of the amino acid profiles of fish protein hydrolysates (FPH) and other ingredients used in feeds for aquatic organisms (DM basis).

Amino acids	FPH_{com}[a]	FPH_{100}[a]	FPH_{600}[a]	Fishmeal[b]	Soybeanmeal[b]	Reference protein 1[c]	Reference protein 2[d]	Reference protein 3[e]
Essential (g/kg)								
Histidine	22.0	17.0	19.0	14.0	12.5	8.5	6.2	16.0
Isoleucine	39.0	31.0	35.4	21.5	17.5	13.1	9.5	13.0
Leucine	67.0	62.0	68.0	37.7	29.1	26.9	19.6	19.0
Lysine	77.0	64.0	69.0	39.8	25.7	28.3	20.6	16.0
Methionine	32.0	31.0	33.0	18.0	4.7	10.4	7.6	17.0[f]
Phenylalanine	42.0	34.0	37.0	18.1	19.1	14.8	10.8	–
Threonine	46.0	36.0	38.0	19.9	15.1	18.5	13.4	9.0
Trytophan	42.0	8.0	7.0	3.9	8.0	5.2	3.8	–
Valine	50.0	36.0	40.0	25.9	19.0	16.4	11.9	13.0
Arginine	91.0	66.0	72.0	37.8	30.5	29.8	21.7	–
Non essential ($g\,kg^{-1}$)								
Tyrosine	29.0	23.0	26.0	–	–	15.0	10.9	–
Ac. Aspartic	104.0	81.0	82.0	–	–	–	–	–
Ac. Glutamic	167.0	123.0	133.0	–	–	–	–	–
Glycine	150.0	101.0	109.0	–	–	–	–	–
Alanine	93.0	66.0	70.0	–	–	–	–	–
Proline	85.0	61.0	67.0	–	–	–	–	–
Cystine	17.0	13.0	23.0	–	–	5.2	3.8	–
Taurine	11.0	10	14.0	–	–	–	–	–
Serine	48.0	38.0	42.0	–	–	–	–	–

(–) Data not provided by the authors.
[a] Present work.
[b] Halver (1995).
[c] Recommended dietary nutrient levels for carnivorous juveniles shrimp (FAO, 1989).
[d] Recommended dietary nutrient levels for omnivorous juveniles shrimp (FAO, 1989).
[e] Suggested profile of essential amino acid requirements for adult humans by FAO/WHO (1990).
[f] Methionine + cysteine.

Table 5
Indispensable Amino Acid Index (IAAI) and chemical score of amino acids of FPHs compared with fishmeal and soybean meal (DM basis).

Aminoacids	Chemical scores = limiting amino acid in test protein/amino acid in whole-egg protein (%)					Whole egg amino acids (g/kg)
	FPH_{com}	FPH_{100}	FPH_{600}	Fishmeal[a]	Soybean meal[a]	
Histidine	91.66	70.83	79.16	58.33	52.08	24.0
Isoleucine	50.65	40.25	45.97	27.92	22.72	77.0
Leucine	72.82	67.39	73.91	40.97	31.63	92.0
Lysine	110.0	91.42	98.57	43.26	36.71	70.0
Methionine	80.0	77.5	82.50	45.0	11.75	40.0
Phenylalanine	66.66	53.96	58.73	28.73	30.31	63.0
Threonine	106.97	83.72	88.37	46.27	35.11	43.0
Trytophan	280.0	53.33	46.66	26.0	53.33	15.0
Valine	69.44	50.0	55.55	35.97	26.38	72.0
Arginine	137.87	100.0	109.09	57.27	46.21	66.0
IAAI[b]	1066.07	688.4	738.51	409.72	346.23	–

[a] Data from Halver (1995).
[b] According to Hardy and Barrows (2002).

proteolytic hydrolysis and producing FPHs with a higher concentration of small peptides (Buarque et al., 2009; Li et al., 2010).

The zymogram technique is used as an important tool for the identification of active enzyme forms, thus allowing analysis of digestive enzymes. In the present study, the proteolytic zymograms showed bands of caseinolityc activity in all extracts (ONp_{100}, ONp_{600} and Alcalase extract). However, the bands are in greater number in samples obtained from ONp_{100}, ONp_{600}, because these extracts presented larger variety of proteases than Alcalase solution. Similar results were also described by Santos et al. (2013), which shows that tilapia intestine extract is composed by a mixture of endo- and exoproteases, with broad cleavage specificity. The presence of these enzymes allows a more efficient digestion (Buarque et al., 2009).

Proteolytic enzymes solubilize or break down fish residual muscle protein, resulting in two distinguishable fractions, soluble and insoluble. The insoluble fraction comprises bones and other undigested components, while the soluble contains hydrolyzed protein that can be converted and incorporated into animal feed. DH depends on the E/S ratio, temperature, pH, hydrolysis time, and the enzymes used in the process. In the present study, ONp_{600} had the highest capacity for hydrolysis of the carcass, when compared to ONp100 and Alcalase, which may be due to differences in both content and diversity of enzymes (Hoyle and Merritt, 1994). Additionally, enzymes such as proteases are native as zymogens and need other active

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enzymes to become fully activated. The increase of enzyme amount can be responsible for further extract activation and also a more suitable enzyme/substrate ratio for this reaction.

Several studies, which employed different conditions and enzyme sources, have shown that a huge variation in DH is observed depending on the reaction conditions (Kristinsson and Rasco, 2000). Hoyle and Merrit (1994) reported that the rate of hydrolysis tends to decrease after an initial rapid phase and enters a stationary phase. In the present study, ONp_{100} and ONp_{600} were similar in terms of Alcalase reaction rates, and there was an initial rapid rate of hydrolysis followed by a decreased rate. Such shapes in the hydrolysis curves were similar to those previously published for enzymatic hydrolysis of yellowfin tuna (Nguyen et al., 2011), casein and soybean protein hydrolysates (Li et al., 2010), Persian sturgeon viscera (Ovissipour et al., 2009), salmon head (Sathivel et al., 2005), fish soluble concentrate (Nilsang et al., 2005), and herring muscle and head (Sathivel et al., 2003). DH of FPHs was also analyzed by the electrophoretic profile, showing that the proteins found in the carcass were partially hydrolysed by the experimental proteases. Hydrolysis was more efficient using ONp_{600} and Alcalase, as almost all proteins were hydrolyzed in both treatments.

It was seen that ONp_{600} was efficient on the hydrolysis of the substrate. The electrophoretic pattern showed few bands between 80 and 30 kDa. Most other carcass proteins were removed. It has been proved that the proteases in ONp_{600} were able to break down proteins into peptides and amino acids after 240 min of hydrolysis. According Bhaskar et al. (2008), for the fish protein hydrolysates to be of high nutritional value, it has been shown that the dietary protein should be rich in low molecular weight peptides with as low as possible amounts of free amino acids. Ingested proteins are broken down during digestion through hydrolysis to form free amino acids and low-molecular weight peptides by enzymes in the gastointestinal tract. These products are absorbed by the mucosal cells wherein intracellular digestion of small peptides occurs; thus only amino acids appear to be released into the portal vein as products of protein digestion. The amounts involved are not quantitatively significant but this process may be a physiologically important mechanism, possibly for modulation of the immune system through antigen sampling (NRC, 2011), in addition contributing for protein synthesis and energy balance.

Proximate composition (DM basis) of experimental FPHs were based in dry matter and revealed a protein content of 584.8 g/kg for FPH_{com}, 492.3 g/kg for FPH_{100} and 508.2 g/kg for FPH_{600}. These values were below compared with Menhaden meal (NRC, 1993). However, their values were within the range of protein content reported by previous studies (Nguyen et al., 2011; Ovissipour et al., 2009; Santos et al., 2009; Bhaskar et al., 2008; Pacheco-Aguilar et al., 2008; Nilsang et al., 2005; Sathivel et al., 2005).

High lipid content (above 37.47%) was observed in all experimental FPHs. Similar results have been reported in literature (Sathivel et al., 2005). High lipid content was attributed to the carcass, mainly because of the high lipid content of head and viscera. The enzymatic extracts ONp are based on viscera (intestines) homogenate in water, therefore adding lipid content in FPH. The ether extract (g/kg) was 374.7 for FPH_{com}, 407.0 for FPH_{100} and 445.1 for FPH_{600} in DM basis. EFAs exert a great influence on growth and development of fish, shrimp, catfish, rainbow trout, and Pacific salmon, which require these nutrients in their diet (NRC, 2011). In shrimp and other crustaceans, weight gain responses to different levels of dietary oils, either alone or in combination, indicate that highest gains generally achieved at dietary levels of 5–6% inclusion. Higher levels (>10%) often retard growth (NRC, 2011). However, they do require polyunsaturated fatty acids, phospholipids, and sterols. Shiau (1998) showed that four fatty acids are essential in *M. japonicus*, such as linoleic (18:2 $n-6$), linolenic (18:3 $n-3$), eicosapentaenoic acid (EPA; 20:5 $n-3$), and docosahexaenoic acids (DHA; 22:6 $n-3$). NRC (2011) shows that growth of *F. chinensis* has been improved by the addition of 18:3 $n-3$ to the diet and that optimum growth has been attained with diets containing 0.7 to 1.0% 18:3 $n-3$, while *Penaeus monodon* requires 1.2% 18:3 $n-3$ and 1.2% 18:2 $n-6$. According NRC (2011) EPA and DHA are highly unsaturated fatty acids (HUFA) in the $n-3$ family and are indispensable. The optimal level of EPA and DHA in the diet for juvenile *Metanephrops japonicus* is 1% (Shiau, 1998), for *Litopenaeus vannamei* is 0.25 to 0.50% and for *P. monodon* is 0.90% (NRC, 2011). Rees et al. (1994) concluded that while *P. monodon* post-larvae can grow well on an *Artemia* diet containing low amounts of $n-3$ HUFA, a high dietary supply of HUFA (12–22 mg/g dry weight lipid) considerably enhances their ability to sustain stress and improves survival. However, excess dietary $n-3$ HUFA ($\geq$31.2 mg/g dry weight) may lead to detrimental effects on both growth and survival of post-larvae. Furthermore, cholesterol is essential for penaeids and this is probably the most unique aspect of lipid nutrition in crustaceans, according NRC (2011), *M. japonicus* and *L. vannamei* require 0.2% and 0.13%, respectively. Therefore, the three hydrolysates produced during our study met this criterion and can be employed in the production of shrimp feed.

Ash content found in all FPHs was similar to Kechaou et al. (2009) and lower than the values found for fish meal as reported by several authors (Nguyen et al., 2011; Ovissipour et al., 2009; Pacheco-Aguilar et al., 2008; Nilsang et al., 2005). Higher ash content suggests a high inorganic residue content and low recovery of organic matter. According to Cho et al. (1985), a good quality diet to aquatic organisms must contain ash content less than 13%. Thus, the low levels showed in the FPHs suggest that these hydrolysates are potential ingredients that can be used to replace fishmeal in feed of aquatic organisms.

Amino acid composition is a relevant aspect to be evaluated in FPHs, mainly because it can be used as a potential protein source for animal diet formulations based on its nutritional value and high EAA content (Leal et al., 2009). In fact, several authors have reported that FPH is a good source of EAA, supplying the nutritional requirements for adult humans considering EAAs in reference protein as described by FAO/WHO (1990). This result indicates that FPH can be supplied to carnivorous and omnivorous juvenile shrimp according to the recommended dietary nutrient levels for shrimp (FAO, 1989). However, the nutritive value of any protein ingredient depends on its capacity to fulfill the needs of organisms with respect to EAAs. Methionine and lysine are limiting amino acids in fish and shrimp feed because fish meal is a common source of these amino

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acids (Leal et al., 2009). Amino acid compositions of the protein hydrolysates prepared in this study were similar to those reported by Ovissipour et al. (2009), Bhaskar et al. (2008), Nilsang et al. (2005), and Sathivel et al. (2005).

Based on the essential amino acid content, indispensable amino acid and index (IAAI) and chemical score is usually used to evaluate the nutritive value of a protein source. This parameter compares the amino acids in a test protein with those in whole-egg protein (Hardy and Barrows, 2002). Chemical score next to 100% is considered of high nutritional value. Chemical score revealed that the amino acid profiles of the three FPHs tested were higher in EAAs compared with the chemical scores of fishmeal and soybean meal. The results of fishmeal chemical score revealed that tryptophan and isoleucine were the most limiting amino acid, while soybean meal chemical score of isoleucine, valine and methionine being the most limiting amino acids. However, all other amino acids are present in adequate or excess quantities in FPHs. Amino acid composition of FPHs is according the values of whole-egg protein, a complete protein. Therefore, it can be concluded that, FPHs prepared in this study have good nutritional value and can be considered a potential ingredient for a balanced shrimp diet.

5. Conclusions

We demonstrated that protein hydrolysates derived from tilapia and its by-products could serve as a good source of amino acids. Hydrolysis of tilapia processing waste and using fish enzymes resulted in 42% DH. Tilapia FPHs had desirable amino acid compositions, which were comparable to the hydrolysate produced with Alcalase. The hydrolysate prepared from tilapia viscera waste has potential applications in aquaculture/animal feed production.

Conflict of interest

There is no any conflict of interest in this study.

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CAPÍTULO 2

Fish protein hydrolysis by protease partial purified from Nile tilapia (*Oreochromis niloticus)* intestine

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Fish protein hydrolysis by protease partial purified from Nile tilapia (*Oreochromis niloticus)* intestine

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Abstract

Proteases were extracted from the viscera of *Oreochromis nilotucus* and precipitated with ammonium sulfate (0 – 80%, v/v) and ethanol (0-30%, v/v), (30 – 70%, v/v). The enzymatic extract with $(NH_4)_2SO_4$) was partially purified with a yield of 62.6% (sp.act. of 9.3 U/mg of tissue) and with ethanol a yield of 42.6% (sp.act of 37.0 U/mg of tissue) and 68.4% (33.9 U/mg of tissue). Seven caseinolytic proteases bands were observed in zymogram. The potential application of proteases in the protein hydrolysis of tilapia processing waste was investigated in comparison to the use of two commercial proteases, Alcalase® and Flavourzyme™ under their optimal hydrolysis conditions. The effects of enzymes to substrate ratio on the degree of hydrolysis (DH) were studied. $FPH_{EC(200)}$ showed the highest DH (37.8%), following by FPH_A (35.3%), FPH_{ET} (33.2%), FPH_{AS} (24.6%) and FPH_F (18.5%). A native-PAGE was employed to show the molecular weight from fish protein hydrolysate by proteases and were estimated between 116.25 and 29.05 KDa. The present study proved that crude extract and partial purified extract from the tilapia waste by ammonium sulfate and ethanol precipitation can be used for protein hydrolysis as well as other commercial proteases.

Keywords: Fish processing waste*;* digestive protease; commercial enzymes; partial purification.

Abbreviations: FPH, fish protein hydrolysate; E/S, enzyme:substrate ratio, DH, degree of hydrolysis; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

1. Introduction

Enzymatic hydrolysis of fish protein has been employed as an alternative approach for converting underutilized fish biomass, which is commonly used in making animal feed or even fertilizer, into edible protein products (Bueno-Solano et al., 2009).

Recently, protein hydrolysates, extracted from marine by-products, have become popular in the food industry due to the high protein content (Cordova-Murueta et al., 2007). Protein hydrolysis refers to any process in which the protein is broken down by protein-digesting enzymes (Vidotti et al., 2003). Hydrolysis decreases the peptide size, making hydrolysates the most available amino acid source for protein biosynthesis (Gildberg and Stenberg, 2001).

Both chemical and enzymatic methods have been adopted for protein hydrolysis. Chemical methods involve alkali, such as NaOH and acid, such as HCl. However, enzymatic hydrolysis is greatly preferred because chemical hydrolysis may oxidize cysteine and methionine and destroy serine and threonine, then reduce protein quality and biological value (Li et al., 2010). Enzymatic hydrolysis involves minimum side reactions and leads to limited hydrolysis of selected peptide bonds. The hydrolysis is more specific and can eliminate many of the problems associated with chemical hydrolysis (Kumar et al, 2004). A number of commercially available proteases have been applied for specific modification of protein and production of peptides, such as Alcalase, Flavourzyme, Neutrase and Protamex (Sathivel et al., 2005, Kechaou et al., 2009, Nguyen et al., 2011).

However, industrial applications of commercial proteases are limited by the high cost of these enzymes. On the other hand, enzymes contained in fishing wastes have caught more attention recently. A large amount of processing discards and byproducts is created from fishery industry annually. For example, in the processing of fillet of nile tilapia (*Oreochromis*

niloticus) one of the most cultured fish in tropical and subtropical regions in the world, approximately 70% corresponds to residue (meat remains, head, skin, bones, scales and viscera) (Clement and Lovell, 1994). Previous works proved that tilapia are the potential source of protein (Abdul-Hamid et al., 2002, Foh et al, 2012).

Utilization of these wastes could not only provide a large and cheap resource for production of enzymes but also reduce the pollution loading to the environment. Studies of separation and utilization of enzymes from the waste from fishery industry have been reported, as serine proteases from pyloric caeca of Atlantic salmon (*Salmo salar*) (Kristinsson and Rasco, 2000), trypsin from the pyloric ceca of walleye pollock (Kishimura et al., 2008) and proteases from the spleen of tuna (Li et al., 2010). So far, few results have been reported about hydrolysis efficiency of partial purified protease from tilapia intestine extract.

Ammonium sulfate precipitation and ethanol precipitation is an alternative technology which have been used on partial purification of proteases from various fish species, such as *Colossoma macropomum* (Bezerra et al., 2001; Esposito et al, 2009, Marcuschi et al., 2010), *Oreochromis niloticus* (Bezerra et al., 2005), *Lutjanus synagris* (Esposito et al, 2010) and *Diapterus rhombeus* (Silva et al., 2011). Therefore, the aim of the present work was to investigate the potential application of the proteases separated from tilapia intestine by crude extract and ammonium sulfate and ethanol precipitation in the hydrolysis of tilapia processing waste, where both commercial enzymes were adopted in comparison (Alcalase® and Flavourzyme™).

2. Materials and methods

2.1. Materials

By-products from farmed tilapia were kindly donated by Noronha Pescados Ltd., a local fish processing plant in Pernambuco State, Brazil. The materials were brought immediately to the laboratory on ice. By-products were comprised of tilapia heads, meat remainders, skin, bones, and viscera. All other reagents used in enzymatic assays were of analytical grade and purchased from Sigma (St. Louis, MO, USA).

2.2. Enzyme extraction

Intestines of *O. niloticus* were collected and homogenized in distilled water (4 ºC) at concentration of 200 mg of tissue/mL of 0.1M Tris-HCl buffer. The resulting preparation was centrifuged (Herolab Unicen MR Centrifuge, Germany) at 10,000 × g for 15 min at 4 °C to remove cell debris and nuclei. The supernatant (crude extract) was frozen at −20 °C and used for partial purification steps (Bezerra et al., 2005) and for posterior hydrolysis process.

2.3. Enzyme partial purification

2.3.1 Ethanol precipitation

The crude extract was incubated for 30 min at 45ºC and centrifuged at 10,000g for 15 min at 4º C. The supernatant (300 mL) obtained was then fractionated with iced ethanol by adapted methodology of Cortez and Pessoa (1999). The ethanol concentration values employed in the precipitation experiments were 0–30% (Fraction F1) and 30–70% (Fraction F2). For each fraction, the ethanol was slowly added to the extract under agitation (200 rpm) at 4ºC with a burette in a cold chamber. After the addition of ethanol, the agitation was stopped for 30 minutes and the mixture was centrifuged at 10000g for 15 min at 4º C. The pellets (Fraction F1 and F2) was mixed to form the pool of proteases (0-70%) were dissolved in 100 mL 0.1 M Tris–HCl buffer (pH 8.0) at 4ºC and dialyzed at 4 L of 0.05 M Tris–HCl

buffer (pH 8.0) for 3 h. All the fraction of ethanol precipitation were used for posterior hydrolysis process.

2.3.2 Ammonium sulfate precipitation

The crude extract was incubated for 30 min at 45ºC and centrifuged at 10,000g for 15 min at 4º C. The precipitate was discarded and the supernatant was collected. Precipitation was then performed with ammonium sulphate, yielding fraction of 0–80% salt saturation. The salt was slowly added to the extract under agitation. After the total dissolution of the salt, the extract was kept at 4ºC for 4 h. Salt saturation fraction was centrifuged at 10,000g for 15 min at 4ºC and the precipitate was resuspended in 100mL 0.1 M Tris–HCl, pH 8.0 and dialyzed at 4 L of 0.05 M Tris–HCl buffer (pH 8.0) for 12 h. The fraction was used posterior hydrolysis process.

2.4. Enzymatic assay

Proteolytic activities were determined in the crude extract and the fractions using 1% azocasein as substrate, according to Bezerra et al. (2005). Triplicate samples of each enzyme extract (30 µ) were incubated with 1% azocasein (50 µL) dissolved in 0.1 M Tris–HCl pH 8.0, for 60 min at 25ºC. Following, 10% trichloroacetic acid (120 µL) was added to stop the reaction and the mixture was centrifuged at 8000g, for 5 min. The supernatant (70 µL) was mixed with 1 M NaOH (130 µL) and absorbance was measured in a microtiter plate reader (Bio-Rad 680) at 450 nm against a similarly prepared blank contend 0.1 M Tris–HCl pH 8.0, replaced the crude extract sample. Previous experiments showed that for the first 60 min the reaction carried out under the above mentioned conditions follows first order kinetics. A unit (U) of enzymatic activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001 change in absorbance per minute. Protein content was measured

according method by Lowry et al., (1951). Specific enzyme activity was calculated by dividing the enzyme activity (U) per amount of protein in the sample (mg/mL) and expressed as U/mg of protein.

2.5. Electrophoresis

At the end of 240 min of hydrolysis, the samples were centrifuged and dialyzed, the samples (100 µg of protein) were concentrated by lyophilization and used for electrophoresis, according to Laemmli (1970), on a 4% (w/v) stacking gel and 12.5% (w/v) separating gel. The gel was stained with 0.1% (w/v) Coomassie Blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted at 11 mA using a vertical electrophoresis system (Vertical Electrophoresis System, Bio-Rad). Molecular mass of the purified protein band was estimated by comparison with a molecular mass standard (Amersham Biosciences, Little Chalfont, UK) containing myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), transferrin (80 kDa), BSA (66 kDa), glutamate dihydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21 kDa).

For the zymogram, samples were centrifuged and dialyzed (50µg of protein). It was performed following the procedure described by Garcia-Carreño et al. (1993), on a 4% (w/v) stacking gel and 14.0% (w/v) separating gel. After electrophoresis, the gels were washed in 0.1 M Tris-HCl and immersed in 2.5 mL/L (100 mL) Triton X-100 in 0.1 M Tris-HCl, pH 8.0, for 30 min at 4 °C to remove SDS. Triton X-100 was removed by washing the gels three times with 100 mL of 0.1 M Tris-HCl buffer, pH 8.0. Afterward, the gel was then incubated with 100 mL of 3 g/L casein in 0.1 M Tris-HCl, pH 8.0, for 30 min at 4 °C. The temperature was raised to 25 °C and maintained for 90 min to allow digestion of casein by the active

fractions. The gel was then stained with 0.1% (w/v) Coomassie Blue for 2 h and destained in 10% (v/v) acetic acid and 25% (v/v) methanol for 12 h.

2.6. Fish protein hydrolysate (FPH)

FPHs (n = 3) were produced by fish enzyme hydrolysis adapted from the method described by Cahú et al. (2012). A commercial enzyme (Alcalase® Prod Code P4860). at 0.5% (w/v) and (Flavourzyme™ Prod Code 6110) were adopted as a comparative standard. The carcasses (200 g, without visceras) were washed in distilled water prior to hydrolysis and were mixed together in an industrial blender with 0.5% (w/v) Alcalase solution (200 mL), Flavourzyme solution (200 mL), fish enzyme at 200 mg/mL (200 mL), fish enzyme by ammonium sulphate purification fraction 0 - 80% (200 mL), fish enzyme by ethanol purification fraction 0 - 70% (200 mL) to produce FPH_A, $FPH_{F;}$, $FPH_{CE(200),}$ FPH_{AS} and FPH_{ET}, respectively. The mixture was digested in a water bath at 45 °C for 240 min. Then, the temperature was raised to 100 °C for 10 min to stop the reaction. Solid and liquid fractions were separated by filtration (1 mm^2- mesh), and the filtrate was defined as FPH.

2.7. Degree of hydrolysis (DH)

Degree of hydrolysis was estimated according to the method of Hoyle and Merritt (1994) and Liceaga-Gesualdo and Li-Chan (1999). During the hydrolysis process (n = 3), one 6-mL sample was collected at 0, 10, 20, 30, 60, 90, 120, 150, 180, 210, and 240 min from each of the hydrolysis conditions. The reaction was inactivated by adding 4 mL of 6.25% (m/v) TCA. After resting for 15 min, these samples were centrifuged (8,000 g for 10 min at room temperature) and the supernatant proteins were quantified using the method of Lowry et

al. (1951), and absorbance was measured at 562 nm in a microplate reader (Bio-Rad X-Mark spectrophotometer, Hercules, CA, USA), this process determined the soluble protein in each sample using bovine albumin as standard (Sigma-Aldrich, St. Louis, MO, USA). DH was expressed as the ratio between the solubilized protein and the total protein present in the initial substrate by Kjeldahl method (AOAC, 1995) in %, according to Equation 1.

% DH = (6.25% TCA soluble N2 in the sample/Total N2 in the sample) * 100

2.8 Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) and Tukey's test. $P < 0.05$ was considered as statistically significant. Microcal Origin version 8.0 was used for the analysis (Software, Inc., USA) (Zar 1984).

3. Results and discussion

3.1. Purification partial of Nile tilapia intestine proteases

In the present study alkaline proteases were partial purified from the intestine of the nile tilapia to posterior use in the hydrolysis of fish processing waste (Table 1) by using a two-step procedure: (1) heat treatment and (2) ammonium sulphate fractionation (0 - 80%, v/v fraction) and (2) ethanol fraction 1 (0 - 30%, v/v) (F1), fraction 2 (30 - 70%, v/v) (F2). The specific activity in the initial enzyme extract (200 mg/mL) was 9.8 U/mg, whilst total activity was 1997.5 U. The first step (heat treatment) resulted in a slight increase in the specific activity, generating a purification factor of 1.8-fold and a yield of 76.9 %.

In the second step, the fraction with highest specific activity was 0 - 30% of ethanol saturation, in which it was observed a 3.8-fold increase, with a yield of 42.6%. In the Fraction

F2 (30 – 70%, v/v) the purification was 3.5-fold and the yield was 68.4%. However, the second highest enzymatic recovery was observed in Fraction 0 – 80% (v/v) (ammonium sulphate precipitation), presenting purification and yield of 0.9-fold and 62.6%, respectively. This same protocol has been successfully used in the purification of trypsins from tropical fish as, *Oreochromis niloticus* (Bezerra et al., 2005), *Pseudupeneus maculatus* (Souza et al., 2007), *Lutjanus synagris* (Esposito et al., 2010), *Colossoma. macropomum* (Marcuschi et al., 2010) and *Diapterus rhombeus* (Silva et al., 2011). Bezerra et al. (2001) reported the importance of the heat treatment in the purification of a trypsin from *C. macropomum*. Despite the low purification factor obtained in this stage, heating eliminates thermolabile proteins and promotes the hydrolysis of the thermostable contaminating proteins. This property improves the performance in the subsequent stages of ammonium sulphate fractionation and ethanol fractionation.

The ethanol is known as an important protein industrial precipitant (Esposito et al., 2009). This technique was able to purify a protease from the viscera of *Colossoma macropomum* for use in the laundry detergent by Esposito et al., (2009). Furthermore, the industrial use of this solvent for enzymes concentration has some advantages such as: the process does not need interruption, requires simple equipment, and can be easily scaled up (Cortez & Pessoa, 1999). In this study, the proteases recovered by ethanol precipitation F1 and F2 were together to concentrate the most of protease recovered to potencies the hydrolysis process.

The zymogram of the crude extract and treatments are presented in Fig. 1. This technique is utilized as an important tool for the identification of active enzyme forms. Their proteolytic activities on casein are shown in lane 1 (crude extract), lane 2 (heated crude extract), lane 3 (0 - 30%), lane 4 (30 - 70%) and lane 5 (0 -80%). Similar patterns were observed for crude extract and the others treatments. Seven caseinolytic bands were observed

for all treatments (a - g). The main difference between the extracts was found in (0 -30%), these bands showed slight decrease intensity when compared to crude extract and heat crude extract (d – g). In general, it was suggested that protease could be partial purified and recovered from tilapia intestine spleen by ammonium sulphate precipitation and ethanol precipitation.

3.2 Effect of enzymes on degree of hydrolysis

Degree of hydrolysis (DH) is defined as the percentage of free amino groups obtained by the cleavage of a protein, which was calculated from the ratio of α-amino nitrogen and total nitrogen (Nilsang et al., 2005). The variation of degree of hydrolysis dependent on both (E/S) ratio, the type of enzymes, temperature, pH and time. Several studies, which employed different conditions and enzyme sources, have shown that a huge variation in DH is observed depending on the reaction conditions (Kristinsson and Rasco, 2000).

The changes of DH during the hydrolysis of tilapia carcass by the proteases solutions during 240 min, at 45º C at 1:1 E/S ratio were shown in Figure 2. According Hoyle and Merrit (1994), after an initial rapid phase of hydrolysis, the rate of hydrolysis tends to decrease, entering a stationary phase. In this study, the curves exhibited an initial fast reaction and were similar until 90 min. However, after 90 min observed increase in the DH of the FPHs followed by a stationary phase which reached a plateau. In crescent order the maximum DH obtained after 240 min was 37.8 ± 1.94 for $FPH_{CE(200)}$, 35.3% ± 1.00 for FPH_A, 33.2% ± 3.59 for FPH_{ET}, 24.6% ± 1.10 FPH_{AS} and 18.5% ± 1.46 FPH_F, which only the FPH_F differed significantly ($p < 0.05$). The shapes of these curves were similar to those reported for enzymatic hydrolysis of other fish by-product substrates as enzymatic hydrolysis of salmon (Liaset et al., 2003), enzymatic hydrolysis of Atlantic cod (Aspmo et al., 2005), fish soluble

concentrate (Nilsang et al., 2005), enzymatic hydrolysis of yellowfin tuna (Nguyen et al., 2011) and Persian sturgeon viscera (Ovissipour et al., 2009).

In the present study, although the FPH_A had higher DH than FPH_{AS} and FPH_{ET}, but not differ statistically ($p > 0.05$), the alkaline proteases from intestine of tilapia had great capacity for hydrolysis of the carcass. $FPH_{EC(200)}$ performed the best in terms of DH for the substrate. The third best performed was FPH_{ET}, probability due the combination of F1 and F2 with action from different enzymes The FPH_{AS} had higher DH than FPH_F and lower DH than the others FPHs. The fish enzymes was more efficient in the hydrolysis, may be due to differences in both content and diversity of enzymes (Hoyle and Merrit, 1994). In addition, may be attributed to their broader specificities in cleaving various peptide bonds (Klomklao, 2007). It corroborate with the total proteolytic activities by the crude extract (1997.5 U), ammonium sulphate extract (1250.8 U) and ethanol extract (850.0 U) (F1) and (1366.1 U) F2. According Santos et al., (2013), tilapia intestine is composed by a mixture of endoprotease and exoprotease activities, mainly trypsin and chymotrypsin. Trypsin hydrolyses peptide bonds whose carbonyl function is donated by basic amino acids such as lysine or arginine. Chymotrypsin catalyses most effectively the hydrolysis of the peptide bonds on carboxyl side of amino acid residues with large hydrophobic side chains (e.g. phenylalanine, tryptophan or tyrosine) (Kumar et al., 2004). By a mixture of trypsin and chymotrypsin, the enzymatic specificities are complementary. Exopeptidases, such as leucine aminopeptidase, cleave long-chain peptides into smaller peptides and free amino acids (Li et al, 2010). More extensive hydrolysis of protein could be achieved by mixture of trypsin and chymotrypsin rather than a single enzyme.

On the other hand, Alcalase is a bacterial endopeptidase produced by *Bacillus licheniformis* widely used in the hydrolysis process (Li at al., 2010). The main enzymatic component of Alcalase, subtilisin A is a serine endo-protease. It has been proved that

hydrolysates of protein produced with single proteases (e.g. trypsin, pepsin, chymotrypsin, or papain) often show a limited degree of hydrolysis (Cao et al, 2000). Flavourzyme is also used in the hydrolysis process, is a fungal protease complex produced by submerged fermentation of a strain of *Aspergillus oryzae*. Although exhibits both endoprotease and exoprotease activities (Kechaou et al., 2009), this enzyme wasn't able for hydrolysis of the fish carcass, probability due the low concentration E/S.

In general, in the hydrolysis condition, the hydrolysis was more efficient using proteases from intestine of tilapia and Alcalase because almost all proteins were hydrolyzed in these treatments.

3.3 Analysis of hydrolysates by SDS-PAGE

The effect of enzymatic hydrolysis on protein breakdown in fish carcass protein after hydrolysis for 240 min was shown by SDS-PAGE (Figure 3). It can be noticed that the protein profiles were substantially changed according the treatments.

Electrophoretic patterns indicated hydrolysis was more extensive in the $FPH_{CE(200)}$ with bands between 37.41 to 29.05 kDa (Lane 5), following by FPH_A with only one band (53.89 kDa) (Lane 4), FPH_{ET} with bands between 84.59 to 37.41 kDa (Lane 3), FPH_{AS} with bands between 116.25 to 37.41 kDa (Lane 2) and FPH_F with bands between 84.59 to 53.89 kDa (Lane 1). Similar result was showed by Li et al, (2010), that obtained molecular weight around 27 kDa in the soybean hydrolysis by tuna spleen extract purified after 180 minutes of hydrolysis.

It was seen that DH of FPHs was also demonstrated by the electrophoretic profile. It proved that the intestine crude extract and alkaline proteases partial purified from the intestine of the nile tilapia were such efficient as the commercial enzyme Alcalase on the hydrolysis of

the fish carcass substrate. It has been proved that the proteases in the intestine extracts were able to broke down proteins in peptides and amino acids after 240 minutes of hydrolysis, what show a high nutritional value in the product.

4. Conclusion

Partial purification of proteases from intestine by-product provided a possible low-cost source of animal-derived protease containing alkaline proteases activity. The comparative study between commercial enzymes (Alcalase and Flavourzyme), crude extract (200 mg/mL) and proteases purified from the intestine of nile tilapia by ammonium sulphate precipitation and ethanol precipitation showed that these extracts could provide an equal or greater degree of hydrolysis of tilapia carcass comparing to Alcalase. Its hydrolysis efficiency on carcass was higher than Flavourzyme. The present results proved that a low-cost protease could be obtained from tilapia processing waste by methods of partial purification and this protease has the proteolytic activity as other commercial proteases.

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6. Tables

Table 1. Purification of digestive proteases from the intestine of Nile tilapia (*O. niloticus*).

Purifn steps	Total act. (U)	Total protein (mg/mL)	Sp. Act. (U/mg)	Purify (fold)	Yield (%)
Crude extract	1997,5	203,6	9,8	1,0	100
Heated Crude Extract	1536,7	86,5	17,8	1,8	76,9
Ammonium sulfate precipitation $(NH_4)_2SO_4$ F (0-80%)	1250,8	135,1	9,3	0,9	62,6
Ethanol precipitation					
F1 (0-30%)	850,0	23,0	37,0	3,8	42,6
F2 (30-70%)	1366,1	40,4	33,9	3,5	68,4

7. Figures

Figure 1: Zymogram in polyacrylamid gel - SDS-PAGE of alkaline protease from the viscera of *O. niloticus* obtained before and after partial purification with ethanol and ammonium sulfate precipitation (Lane 1: Crude extract, Lane 2: Crude extract heated, Lane 3: fraction 0 - 30% ethanol, Lane 4: fraction 30 - 70% ethanol; Lane 5: fraction 0 -80% ammonium sulfate).

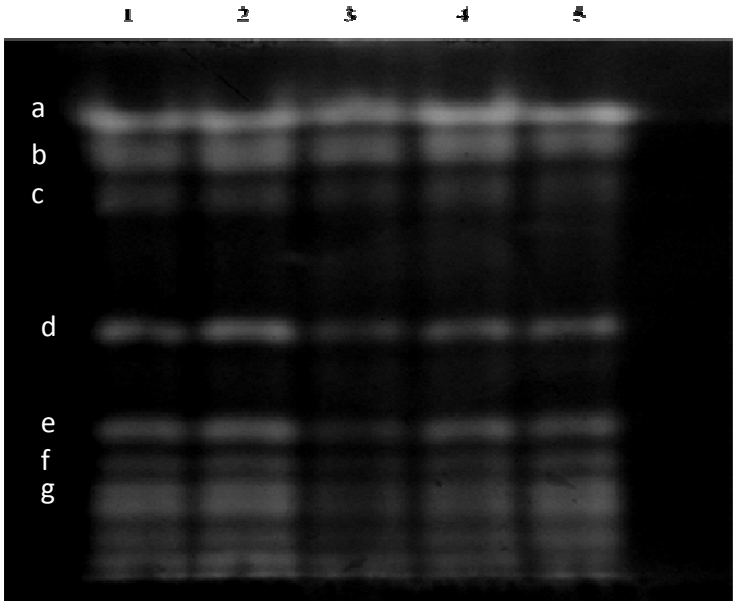


Figure 2: Electrophoretic analysis of hydrolysates after hydrolysis for 240 min (SDS-PAGE with Acrylamide/bis in stacking gel, stained by Coomassie blue staining; Lane M, standard markers; Lane 1, Fish protein hydrolysate (FPH_F); Lane 2, Fish protein hydrolysate (FPH_{AS}); Lane 3, Fish protein hydrolysate, (FPH_{ET}); Lane 4, Fish protein hydrolysate (FPH_A); Lane 5, Fish protein hydrolysate ($FPH_{CE(200)}$).

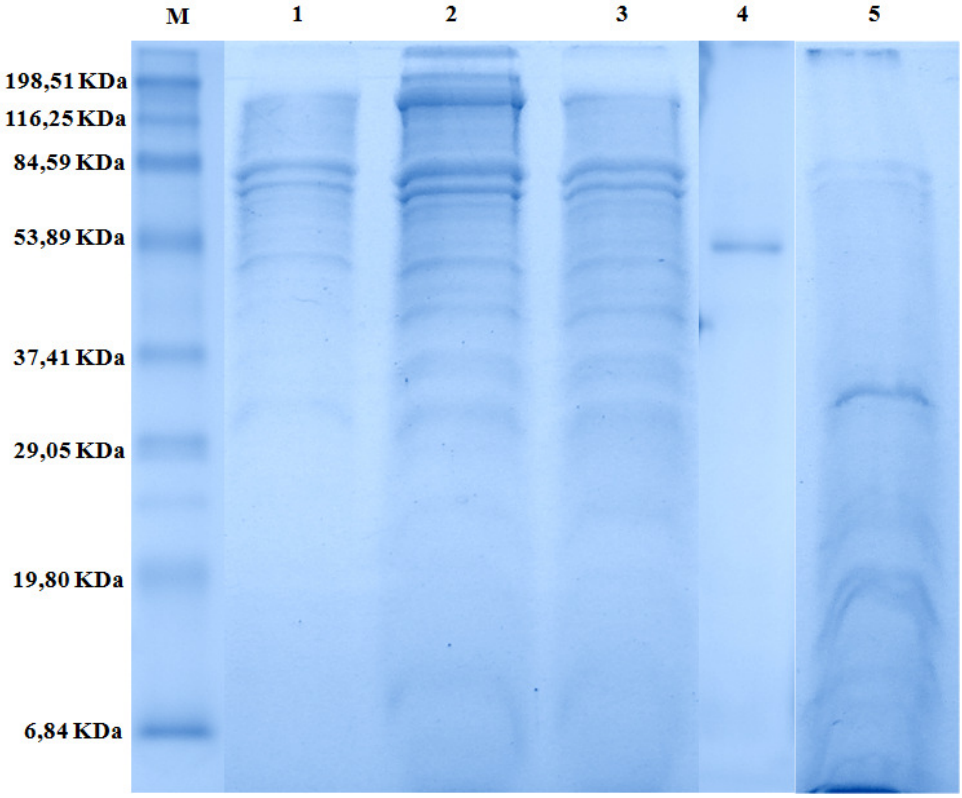
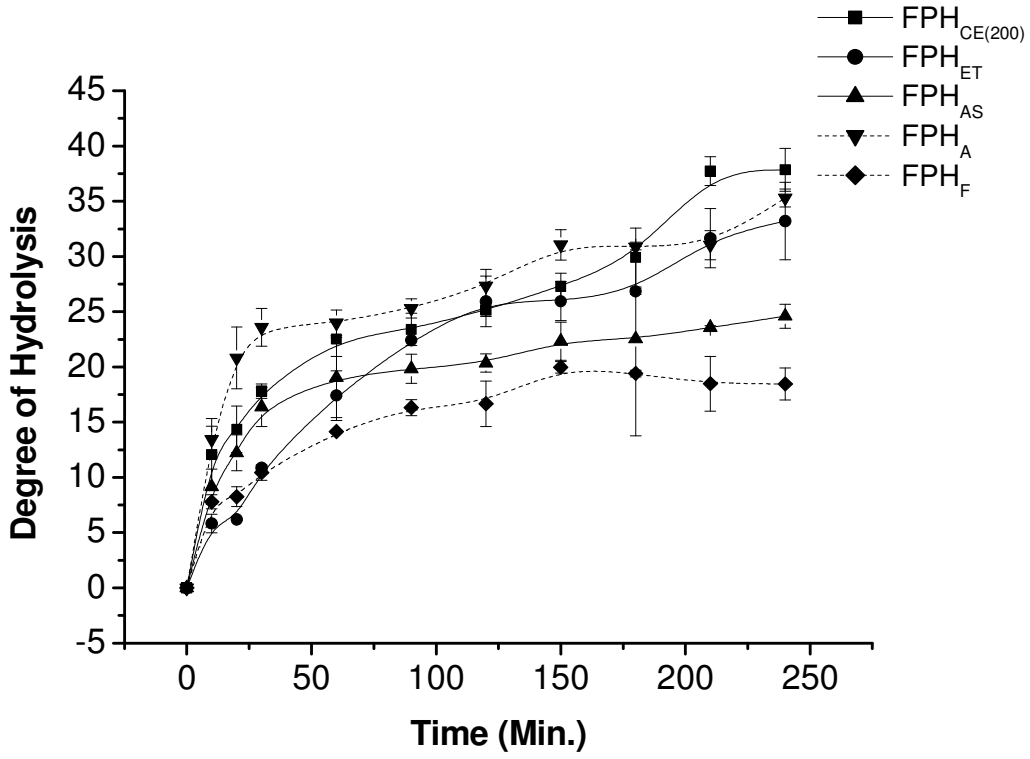


Figure 3: Effect of enzyme on degree of hydrolysis profile with carcass of fish as substrate (■ $FPH_{CE(200)}$), (● FPH_{ET},), (▲FPH_{AS}), (▼FPH_A), (♦FPH_F).



CAPÍTULO 3

Use of different methods of conservation on the fish protein hydrolysates obtained from tilapia processing waste

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Use of different methods of conservation on the fish protein hydrolysates obtained from tilapia processing waste

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Abstract

The effects of heat sterilization in combination with acidification, gamma irradiation and acidification on the conservation of fish protein hydrolysate (FPH) obtained from tilapia (*Oreochromis niloticus*) processing waste was examined, during 60 days of storage. The control and the treated FPH were analyzed periodically for chemical (TBARS, pH, proximate composition) and microbial characteristics. Of the chemical indices determined, TBARS values were variable in FPH samples, indicative of no specific oxidative rancidity trend, colour values were also variable, pH increase and content protein were satisfactory. Based on chemical and microbial data, $FPH_{Acid\ 30^{\circ}\ C}$ was improper in extending the product for 9 days. In the 9^{th} day, occurred the presence of mesophiles (5.0×10^2 CFU/mL) and in the 60^{th} the presence fungi (8.7×10^2 CFU/mL). Combination of sterilization with acidification storage at refrigeration and gamma irradiation at refrigeration storage was appropriate during 60 days of storage.

Keywords: tilapia processing waste; irradiation; combination processes of sterilization

1. Introduction

Fish is known a rich source with high digestiblity and also provides protein, polyunsaturated fatty acids, vitamins, and minerals for human nutrition. However, a large proportion of fish processing waste remains unused. Although some species are used industrially for fish meal manufacture, a need for their conservation and utilization for animal consumption has been recognized in order to prevent post-harvest fishery losses. Recovery of fish processing waste by hydrolysis is probably the most promising approaches. Protein hydrolysates have a diversity of industrial applications, including pharmaceuticals, human and animal nutrition, cosmetics and as a nitrogen source in the growth media for microorganisms (Silva et al, 2014; Duarte de Holanda and Netto, 2006).

Earlier studies have shown that fish processing waste from Nile tilapia (*Oreochromis niloticus*) have been identified as an animal proteins and proteases sources to produce fish protein hydrolysate (FPH) liquid with potential applications in aquaculture/animal feed production (Silva et al., 2014, Valle et al., 2013). However, a few number of studies have been reported on the conservation methods of fish protein hydrolysate in form liquid.

Several techniques are applied to fish products to prevent microbiological deterioration and to conserve the organoleptic characteristics of them, e.g. refrigeration, freezing, drying, acidification, fermentation, modified atmosphere packaging, addition of preservatives, sterilizing, pasteurizing and gamma irradiation (Gould, 1996).

Most established methods of food preservation constitute certain combinations of various preservative factors. Combination of radiation treatment with heat treatment seems also to be of potential importance in enhancing the effectiveness and reducing the energy or dose requirement of the food preservation while retaining/improving product quality (Mbarki et al., 2009). On the other hand, artificial preservatives such as sodium acetate, sodium

lactate, sodium citrate have been used to avoid microorganism proliferation on refrigerated fish slices (Fernández et al., 2009).

Thus, the objective of the present work was to determine the effect of heat treatment with the addition of citric acid, acidification and gamma irradiation during 60 days by monitoring microbiological, biochemical and sensory changes in the fish protein hydrolysates as a function of treatment.

2.3 Fish protein hydrolysate (FPH)

FPHs (n = 3) were produced by a commercial enzyme (Alcalase® Prod Code P4860) at 0.5% (m/v) hydrolysis from the method described by Silva et al., (2014). The carcasses (200 g, without visceras) were washed in distilled water prior to hydrolysis and were mixed together in an industrial blender with 0.5% (w/v) Alcalase solution (200 mL), to produce FPHs. The mixture was digested in a water bath at 45 °C for 240 min. Then, the temperature was raised to 100 °C for 10 min to stop the reaction. Solid and liquid fractions were separated by filtration (1 mm^2- mesh), and the filtrate was defined as FPH.

2.4 Conservation methods

FPH (n = 3) were stored in sterilized glasses and subjected to different conservation (acidification, heat treatment followed by acidification and gamma irradiation). FPHs control (FPH 30º C) was stored at room temperature (approximately 30 ºC) during 24 hours.

2.5 Acidification

FPH (n = 3) were acidified by adding 2.5% of citric acid and were stored at 4 °C (FPH_{Acid} 4º C) and 30 °C (FPH_{Acid} 30º C) during 60 days.

2.6 Heat sterilization

FPH (n = 3) were subjected at dry heat sterilization (150° C/2 hours) in a heater (TE 394/L Tecnal), stored at 30 ºC (FPH_{Heater} 30º C) during 60 days and were subjected at moister heat (120° C/15min) in Autoclave Vertical Phoenix Luferco, stored at 30º C (FPH_{Auto} 30º C)

and 4º C (FPH_{Auto} 4º C), stored at 30º C and 4º C, respectively during 60 days. In these FPHs was added citric acid (2.5%) as an additive.

2.7 Gamma irradiation

FPH (n = 3) were irradiated with cobalt 60 gamma irradiation (source gamma cell ^{60}Co) in the Energy Nuclear Department of Federal University of Pernambuco, Brazil. The doses were 2.5 kGy at 30 minutes ($FPH_{2.5\ kGy}$), 5.0 kGy at 60 minutes ($FPH_{5.0\ kGy}$) and 7.0 kGy at 90 minutes ($FPH_{7.0\ kGy}$). Then the FPHs were stored at 4 ºC during 60 days.

2.8 Microbiological analyses (Total mesophilic and psychotropic)

Analysis of total mesophilic and psychotropic was performed according to methodology Portuguese standard NP 4405 (2002). FPHs were monitored at 0, 12, 24, 36, 48 and 60th day. Only the $FPH_{Acid30°\ C}$ was monitored at 0, 2, 5 and 9th day and the $FPH_{30°\ C}$ (Control) was monitored at 0 and 1st day.

The assay was performed by mixing 500 µL of each FPH sample with 950 µL of peptone water (0.1% in 0.9% w/v NaCl solution) by serial dilution (1:10, 1:100, 1:1000) inside safety biological cabin (Pachane Pc 410). Aliquots from 1 mL (n=3) of each diluted sample were collected and inoculated into petri dishes with 15 mL of plate count agar (PCA). Then, the Petri dishes ($FPH_{30°\ C}$, $FPH_{Auto30°\ C}$, $FPH_{Heater\ 30°\ C}$, $FPH_{Acid\ 30°\ C}$) were submitted at 30 º C for 48 hours in an heater (TE 394 / 1 Tecnal) for evaluation of mesophiles and the the Petri dishes ($FPH_{Auto\ 4°\ C}$, $FPH_{Acid\ 4°\ C}$, $FPH_{2,5\ kGy}$, $FPH_{5.0kGy}$, $FPH_{7.0kGy}$ were submitted at 4 º C for 72 hours for analysis of psychrotrophic microorganisms.

2.9 Microbiological analyses (specific microorganisms)

Microbiological analyses were performed according to the AOAC methods (1995), 46.030, 46.062, 46.115, 46.011, and 4.030 for coliforms at 45 ºC; coagulase-positive *staphylococci*; *Salmonella spp*; fungi and *Pseudomonas aeruginosa*, respectively. $FPH_{Heater30\ °C}$, $FPH_{Auto\ 30\ °\ C}$, $FPH_{Auto\ 4\ °\ C}$, $FPH_{2.5\ kGy}$, $FPH_{5.0\ kGy}$ and $FPH_{7.0\ kGy}$ were analysed after 60

days of storage; $FPH_{30^{o}\,C}$ after 24 hours of storage; $FPH_{Acid\ 30\ ^{\circ}C}$ in the 9th day of storage and $FPH_{Acid\ 4^{\circ}\,C}$, in the 48th of storage. They were performed at the Laboratory of Experimental and Food Analysis, Department of Nutrition, Federal University of Pernambuco.

2.10 Colour

The colour of the FPHs were measured during 30 days using a colorimeter (Konica Minolta, Model Chroma Meter CR – 400) and reported in the CIE colour profile system as L* (lightness), measures the variation in brightness between black (0) and white (100), a* (redness), set the color red for values positives and green for values negatives and b* (yellowness), set the color yellow for values positives and blue for values negatives. Values were recorded at four different locations of the surface of the FPH.

2.11 pH measurement

The pH of FPHs was measured using a pH-meter (Analon PM 608). pH was measured by dipping the pH electrode into a homogenised sample.

2.12. Measurement of thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Samples (2.0 g) were mixed with 10 mL of a TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixtures were heated in a boiling water for 10 min to develop a pink colour, cooled with running tap. The mixture was then centrifuged at 3600g at 25º C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,1,3,3-tetramethoxypropane (TEP) and TBARS were expressed as mg of malondialdehyde Eq/L sample.

2.13. Proximate composition

Analyses of proximate composition from the FPHs were performed at the Laboratory of Experimental and Food Analysis, Department of Nutrition, Federal University of

Pernambuco. In summary:Moisture (AOAC, 1996, method 926.12) content was carried out by weighting 5 g of prepared sample and drying to constantweight in vacuum oven at uniform temperature 20–25◦C above boiling point of H2O at working pressure and cool in efficientdesiccator for 30 min. Constant weight is attained when successive 1 h drying; Crude protein (AOAC, 1995a, method 991.20)was measured by the Kjeldhal method and 6.25 as N-protein conversion factor; Ether extract (U.K. FEEDING STUFFS, 1982,pp. 9–11), sample was extracted with petroleum ether and dried for 3–4 h. The contents were finely ground, and the materialwas again extracted for another hour. After evaporation of the solvent, the oil was dried at 100◦C and weighed; Ash (AOAC,1995b, method 920.39) an amount of 2 g sample was dried and pre-extracted with water, extracted in Soxhlet with dry ethylether for 4 h, following evaporate the ether, cooled and weighted.

2.14. Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) and Tukey's test. $P < 0.05$ was considered as statistically significant. Microcal Origin version 8.0 was used for the analysis (Software, Inc., USA) (Zar 1984).

3. Results

3.1. Microbiological changes

The present study focused on the monitoring of the following species of microorganisms: Total mesophilic and psychotropic, coliforms at 45 ºC; coagulase-positive *staphylococci*; *Salmonella spp*; fungi and *Pseudomonas aeruginosa*. The initial (day 1) $FPH_{30\ ^{\circ}C}$ was 3.0 x 10^7 CFU/mL $FPH_{30\ ^{\circ}C}$ (Table 1). On day 9 ($FPH_{Acid\ 30\ ^{\circ}C}$) was 5.0 x 10^2 cfu/g (Table 1). On day 48 and 60 ($FPH_{Acid\ 4^{o}\ C}$) was 1.3 x 10^2 and 2.5 x 10^2 cfu/mL, respectively (Table 1). Coliforms at 45 °C, Coagulase-positive *staphylococci, Salmonella spp* and *Pseudomonas aeruginosa* haven't been observed after 60 days (Table 2). Finally, fungi

produced counts of 9.5 x 10^3 ($FPH_{Acid\ 4°C}$) and 2.5 x 10^2 cfu/mL ($FPH_{Acid\ 30\ °C}$) after 60 days of storage (Table 2).

3.2 Colour Measurement

Colour characteristics of FPHs after applying the conservation methods during 30 days of storage is given in Figure 1. $FPH_{30°\ C}$, only was measured after 24 h (L* 38.92, a* 2.98, b* 6.98). Lightness L* of the FPHs is marked ($P > 0.05$) in all FPH process, redness a* ($P < 0.05$) and Yellowness b* ($P < 0.05$). $FPH_{2.5kGy}$ (L*55.34 – 60.00) was the darkest, $FPH_{ACID\ 4°\ c}$ (L*40.82 –61.93) manifested higher variation in the Lightness. $FPH_{2.5kGy}$ manifested lower variation in the yellowness (b* 9.16 – 9.12), while $FPH_{ACID\ 30°\ C}$ (b* 5.13 – 21.21) manifested higher variation in the yellowness and $FPH_{Auto\ 4°\ C}$ (a* 0.87 – 0.47) less variation in redness.

3.3 Chemical analysis

Values of pH for $FPH_{30°\ C}$ (control) was 6.5 on day one, similar to the pH of fresh fish (6.5). Samples on day one and day 60, respectively were in the range of 3.01 - 3.39 ($FPH_{Heater\ 30°\ C}$), 2.97 - 3.34 ($FPH_{Auto\ 30°\ C}$), 3.03 - 3.44 ($FPH_{Auto\ 4°\ C}$), 3.19 - 3.28 ($FPH_{Acid\ 4°\ C}$), 3.18 - 3.39 ($FPH_{Acid\ 30°\ C}$), 6.61 - 6.64 ($FPH_{2.5\ kGy}$), 6.60 - 6.70 ($FPH_{5.0\ kGy}$), 6.65 - 6.68 ($FPH_{7.0\ kGy}$). FPHs submitted to the acidification treatment did not differ significantly ($p > 0.05$) during 60 days and remained uniform due the acid. FPH submitted to the Gamma irradiation were significant difference ($p < 0.05$) and showed value of pH over the other FPHs.

Lipid oxidation in all FPHs was monitored by measuring TBARS, the secondary products of lipid oxidation. TBARS were measured and expressed as mg of malonaldehyde/kg of tissue. Changes in TBARS samples during the 30-day storage period are shown (Figure 2). As expected, TBARS levels were initially low, for $FPH_{Auto\ 4°\ C,}$ (0.24 mg mgMAEq/L) for $FPH_{Acid\ 4°\ C,}$ (0.72 mgMAEq/L), for $FPH_{Heater\ 30°\ C}$ (0.58 mgMAEq/L), for

$FPH_{Auto\ 30^{o}\ C,}$ (0.58 mgMAEq/L), for $FPH_{Acid\ 30^{\circ}\ C}$, (0.53 mgMAEq/L), for $FPH_{2.5kGy}$, (2.74 mgMAEq/L), for $FPH_{5.0\ kGy}$ (2.29 mgMAEq/L), and for $FPH_{7.0\ kGy}$ (3.02 mgMAEq/L). These values increased steeply from the 10^{th} day to 30^{th} of storage, producing lower final values, for $FPH_{Auto\ 4^{\circ}\ C}$ (38.81 mgMAEq/L), for $FPH_{Acid\ 4^{\circ}\ C,}$ (47.52 mgMAEq/L), for $FPH_{Heater\ 30^{\circ}\ C}$, (24.84 mgMAEq/L), for $FPH_{Auto\ 30^{o}\ C,}$ (35.38 mgMAEq/L), for $FPH_{Acid\ 30^{\circ}\ C}$, (56.34 mgMAEq/L), for $FPH_{2.5kGy}$, (17.68 mgMAEq/L), for $FPH_{5.0\ kGy}$ (22.96 mgMAEq/L) and for $FPH_{7.0\ kGy}$ (23.96 mgMAEq/L).

Proximate composition of FPHs in DM basis on day one and day 60 are given in Table 3. On day one all FPHs contained a low quantity of dry matter (11.74 – 23.22 g/100g), ash (1.34 – 4.03 g/100g), a high amount of protein (54.15 - 71.03 g/100g) and a high amount of lipid (22.90 - 37.47 g/100g). On day 60 all FPHs remained with a low quantity of dry matter (13.32 – 24.68 g/100g), ash (1.5 – 4.13 g/100g), a high amount of protein (52.34 – 66.16 g/100g) and a high amount of lipid (30.83 - 50.35 g/100g).

4. Discussion

4.1 Microbiological analyses

The presence of certain microorganisms in a food product indicates the consumption safety of the product. The microflora of temperate fresh water fish is dominated by mesophiles Gram-positive bacteria belonging to the genera *Aeromona* and *Enterobacter*, however it is well documented that Gram-negative bacteria such as *Pseudomonas* can grow at Cooling Temperature. Most of the available literature on freshwater fish (sea bass, tilapia, rainbow trout, silver perch) reports bacterial counts of 102–106 cfu/g (Gelman et al., 2001)On the other hand the contamination also occurs due to failures in the handling and processing of fish that can happen from the collection, transport, or through contact surface and utensils as polyethylene boxes used for transporting fish.

In this study, the results of microbiological analyses from $FPH_{30^{o}C}$ exceeded the value of 7 log cfu/g, which is considered as the upper acceptability limit for fresh water and marine species as defined by ICMSF (2002). Bueno-Solano et al., (2009) reported total mesophilic of 1730 cfu in shrimp protein hydrolysate dried. $FPH_{Acid\ 30\ ^{\circ}C}$ and $FPH_{Acid\ 4^{o}C}$ was below of 7 log cfu/g. According Kilinc et al., (2005) Several bacteria grow up at neutral pH, but some bacteria may develop at pH below 4.5.

Colifoms at 45 °C, Coagulase-positive *staphylococci, Salmonella spp* and *Pseudomonas aeruginosa* haven't been observed in all FPHs, except fungi that were found in the $FPH_{Acid\ 30\ ^{\circ}C}$ and $FPH_{Acid\ 4^{o}C}$. The inhibitory effects of the acids on bacteria and enzymes increase with concentration, while some fungi grow up well in acidic pH (Kilinc et al., 2005).

The sterilization heat in combination with acidification ($FPH_{Heater\ 30\ ^{\circ}C}$, $FPH_{Auto\ 30\ ^{\circ}C}$ and $FPH_{Auto\ 4^{\circ}C}$) and the Gamma irradiation ($FPH_{2.5kGy}$, $FPH_{5.0kGy}$ and $FPH_{7.0kGy}$) applied to the FPHs, showed higher inhibitory effect on the growth of bacteria during 60 days when compared to $FPH_{30^{o}C}$ (control) and $FPH_{Acid\ 30\ ^{\circ}C}$ and $FPH_{Acid\ 4^{o}C}$ that were submitted only to the acidification method. Similar results were found by Bueno-Solano et al. (2009) and Pantazi et al. (2008).

The combination of conservation methods are being widely used due to the rigor and demands of consumers in relation to the consumption of healthy foods, and is most often more efficient regarding the elimination of microorganism. The FPHs submitted to heat sterilization and Gamma irradiation combined with cooling, were free of spoilage microorganisms. Similar results were found by Mbarki et al., (2009), that used a vacuum packaging low-dose irradiation in *Scomber japonicus* and Pantazi et al. (2008), that evaluated various packaging conditions on the shelf-life of chilled Mediterranean swordfish (*Xiphias gladius)* under refrigeration (4 1C). The heat treatment of fish marinates constitute the most appropriate method to destroy pathogenic microorganisms (Kilinc et al., 2005).

Furthermore, was observed the synergy between irradiation and refrigeration (4º C). The principal action of radiation in aiding the preservation of food products is to control spoilage due to microbiological effects or to reduce the microbial population sufficiently to delay spoilage (Hammad, et al. 2000). On the other hand, the cold is an agent that decreases slowly the enzymatic reactions and inhibits bacterial action even temporarily.

In the case of fungi, heat treatment preceding irradiation usually results in a greater antimicrobial effect of the combination process compared with heating after irradiation (Jozsef Farkas, 1990). In the last step process to produce FPH the temperature was raised to 100 °C for 10 min to stop the enzymatic reaction.

In bacterial spores the radiation-induced heat sensitivity persists for a holding period of at least 24 h at room temperature in aqueous suspensions (Farkas, 1990), and for at least several months in dry media such as flour or spices (Farkas, 1990). The mechanisms synergistic of the effect of heat/radiation combinations are not yet clear. They may be due to heat inactivation of enzymes repairing DNA injuries (Aymerich et al., 2008). Farkas, 1990 suggested that radiation-induced breaks in, or decarboxylation of, cortex peptidoglycan may be responsible for the heat-sensitization of bacterial spores by ionizing radiation, including the loss of osmoregulatory or dehydrating mechanisms

Ouattara et al. (2002) have observed that Enterobacteriaceae and other Gram-negative bacteria (total coliforms and *Pseudomonas* spp.) exhibited a greater sensitivity toward the irradiation treatment than the Gram-positive bacteria. Hence, irradiation is an interesting method of preservation to reduce qualitatively and quantitatively the microbial population in fish and fishery products (Mendes et al., 2005). Besides, storage at temperatures below 3ºC can preclude the possibility of growth and toxin production of *Clostridium botulinum* type E, under prolonged storage conditions (Chouliara et al., 2004).

On this way, most established methods of food preservation constitute certain combinations of various preservative factors. However, current trends towards improvement in safety, quality and convenience of foods, and saving energy in food processing and distribution are increasing interest in developing new combination methods for food preservation. Combination of radiation treatment with other preservative agents seems also to be of potential importance in enhancing the effectiveness of the food preservation while retaining/improving product quality.

4.2 Colour Measurement

Factors such as light, oxygen, temperature and water may affect colour stability. In this study, FPHs storage for 30 days showed gradual increase in the lightness and yellowness, however occurred decreased in redness. Yields higher L* values could be attributed to the retention of connective tissue, similar results was found by Foh et al., (2011). The slight decrease in redness (a*) is likely attributed to the degradation of hemoproteins in the protein hydrolysate. Dekkers et al., (2011) reported decrease in the redness of mahi mahi red muscle dipped in tilapia protein hydrolysates, indicating that could lead to loss of haem pigments from the muscle into the solution. More yellowness (b*) in all FPHs could be in part due to more retention of lipids in the FPHs. A positive correlation between lipid oxidation (TBARS) and yellow colour (b*) formation was seen in all the FPHs. Free radicals and carbonyl compounds, produced from oxidation of the highly unsaturated fatty acids in the cell membranes, can react with free amino groups in proteins with a subsequent condensation to polymeric brown pigments (Thanonkaew et al., 2006). The soluble brown pigments substances were produced by interaction of hydroperoxide decomposition products with primary and secondary amino groups of protein (Thanonkaew et al., 2006). The pigments are probably formed by ionic condensation of primary amino groups of protein with conjugated

unsaturated aldehydes or similar reactive lipid-oxidation products that are produced by cleavage of unsaturated hydroperoxides. FPHs became discoloured probability because the lipid oxidation induced the formation of yellow fluorescent pigments in the FPHs.

4.3 Chemical analysis

During the storage period (60 days) pH value of FPHs increased slightly according to storage time. It has to be supported by other chemical and sensory analyses (Klinc et al., 2005). The same authors reported that ammonia production by bacterial desamination of aminoacids has been assumed to be the reason for the increase of pH observed in the spoiled marinade. Gokoglu et al. (2004) reported that pH values in anchovy marinated with 4% acetic acid increased from 3.95 to 4.13 during 150 days. Poligne and Collignan (2000) found that the pH levels of anchovies pickled with acetic acid increased from 3.90 to 4.21 after 20 d of storage and then remained constant until the end of the storage. These results were similar to our findings on the increase of pH value during storage.

Lipids performed an important role in the quality of food products, particularly in relation to organoleptic properties that make them desirable to and provide nutritive value of food, a source of metabolic energy, essential fatty acids and fat soluble vitamins. However, during storage period can occur the lipid oxidation due the presence of polyunsaturated fatty acid contents. In this study, lipid oxidation in all FPHs was monitored by measuring TBARS, the secondary products of lipid oxidation. TBARS values obtained in the present study were variable, indicative of no specific trend in FPHs samples, irrespective of conservation conditions during storage. These variations can be explained as a result of the different phases of peroxides decomposition, formation of carbonyls, and the interaction compounds with nucleophilic molecules present in the muscle (Aubourg et al., 2004). The initial values of TBARS in all FPHs were low (24 h) and these values increased from 10^{th} until the final of

storage period (30 days), including the FPHs submitted to gamma irradiation, that is a excellent sterilization method, but the irradiation can promote the formation of free radicals, resulting in the possibility of changes in color, lipid oxidation and generation of foul odor (Brewer, 2004; Brewer, 2009). Similar results were related for Thanonkaew et al., (2006), Mbarki et al., (2009), and Dekkers et al., 2011 In fact, major fish processing treatments increased the lipid oxidation, during subsequent storage. Goktepe and Moody (1998) observed a twofold increase in TBARS value of raw catfish after smoking process. In this work, lipid oxidation occurred mainly due the high lipid content in the FPHs and it is known that lipid oxidation in muscle foods is one of the major deteriorative reactions causing losses in quality during frozen storage (Thanonkaew et al., 2006). An alternative to decrease TBARS value could be the use of antioxidants.

Proximate composition (Table 4) of experimental FPHs were based in dry matter and revealed on one day and 60 days of storage, respectively a protein content of 63.78 - 57.32 g/100g for $FPH_{Heater\ 30^{o}\ C,}$ 54.15 – 52.34 g/100g for $FPH_{Auto\ 30^{o}\ C,}$ 54.54 – 53.63 g/100g for $FPH_{Auto\ 4^{o}\ C,}$ 61.89 - 54.18 g/100g for $FPH_{Acid\ 4^{o}C,}$ 66.24 – 50.35 g/100g for $FPH_{Acid\ 30^{o}\ C,}$ 71.03 – 66.24 g/100g for $FPH_{2.5kGy,}$ 69.73 – 60.14 g/100g for $FPH_{5.0kGy}$ and 68.76 – 66.16 g/100g for $FPH_{7.0kGy.}$ On 60 days of storage, observed a decrease in protein. The main group of fish muscle proteins is represented by the myofibrils, with easy desnaturation. In this study, some factors influenced the desnaturation of protein, as the low pH, heat and degradation of lipids. Compounds from oxidation of lipids react with protein causing desnaturation, particularly carbonyl compounds such as malondialdehyde. All these factors promote the cleavage of the hydrogen bonds, favoring protein desnaturation. Furthermore, the dose radiation could promove coagulation proteins, unfolding and dividing of amino acids. Usually the main effects occur in sulfuric bonds and hidrogen bonds. However, these values were within the

range of protein content reported by previous studies (Silva et al., 2014, Nguyenet al., 2011; Santos et al., 2009; Bhaskar et al., 2008; Nilsang et al., 2005 and Sathivel et al., 2005).

High lipid content (above 22.90 g/100g) was observed in all experimental FPHs on one day of storage. Similar results have been reported in literature for Silva et al., (2014) and Sathivel et al., (2005). High lipid content was attributed to the carcass, mainly because of the high lipid content of head and viscera. On 60 days of storage the values increased (above 30.83 g/100g), probably due the decrease of content protein.

Ash content found in all FPHs was similar to Silva et al., (2014) and Kechaou et al. (2009) and lower than the values found for fish meal as reported by several authors (Nguyen et al., 2011; Ovissipour et al., 2009; Pacheco-Aguilar et al., 2008; Nilsang et al., 2005). Higher ash content suggests a high inorganic residue content and low recovery of organic matter.

5. Conclusions

It could be concluded that fish protein hydrolysate liquid derived from the processing waste have a high content of protein and lipid and was a susceptible pH changes, lipid oxidation and discoloration. TBARS and colour indices data showed that FPH is good for use until 10^{th} day. Proximate composition and microbiological indices data showed that FPH can be use until 60^{th} day. Treatments of heat sterilization in combination with citric acid at 30 ºC and 4 ºC and gamma irradiation at 4 ° C proved to be a potential microbial conservation method for FPH liquid due the effective in inhibiting the growth or development of pathogenic microorganisms for 60 days.

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6. Tables

Table 1: Total count of mesophilic and psychrotrophics microorganisms in the FPHs after different treatments during 60 days.

	Storage days									
Conservation Methods	0	1	2	5	9	12	24	36	48	60
Mesophilics (CFU/mL)[a]										
Control[b]	20	$3.0x10^7$								
FPH_{Auto}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FPH_{Heater}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FPH_{Acid}	ND	ND	10	10	$5.0x10^2$	-	-	-	-	-
Psychrotrophics (CFU/mL)[a]										
FPH_{Auto}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FPH_{Acid}	ND	ND	ND	ND	ND	ND	ND	ND	$1.3x10^2$	$2.5x10^2$
$FPH_{2.5\ kGy}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
$FPH_{5.0\ kGy}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
$FPH_{7.0\ kGy}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Colony forming units (CFU) per milliliter

[b] The control experiment was carried out without any additive at the temperature of 30^o C. Also, any conservation method was employed

(ND) Not detected by the methodology Portuguese standard NP 4405 (2002)

Table 2: Microbial counts in the FPHs after different treatments after 60 days and storage at 30º C and 4ºC.

Conservation Methods	Colifoms at 45 °C	Coagulase-positive *Staphylococcus*	*Salmonella spp*	Fungi	*Pseudomonas aeruginosa*
$FPH_{30ºC}$	< 3.0	< 10	Absence	8.7×10^2	ND
$FPH_{Auto\ 30\ ºC}$	< 3.0	< 10	Absence	< 10	ND
$FPH_{Heater\ 30\ ºC}$	< 3.0	< 10	Absence	< 10	ND
$FPH_{Auto\ 4\ ºC}$	< 3.0	< 10	Absence	< 10	< 1.1
$FPH_{Acid\ 4\ ºC}$	< 3.0	< 10	Absence	9.5×10^3	< 1.1
$FPH_{Acid\ 30\ ºC}$	< 3.0	< 10	Absence	2.5×10^2	< 1.1
$FPH_{2.5\ kGy}$	< 3.0	< 10	Absence	< 10	< 1.1
$FPH_{5.0\ kGy}$	< 3.0	< 10	Absence	< 10	< 1.1
$FPH_{7.0\ kGy}$	< 3.0	< 10	Absence	< 10	< 1.1

Results representing no growth considering the limits of the methods.

CFU: colony forming unit per milliliter (<10 CFU/g).

MPN = Most Probable Number (<3.0 MPN/g).

(ND) Not detected.

Table 3: Comparison of proximate composition of fish protein hydrolysates (FPHs) between one day and 60 day of storage (g per 100 g DM basis).

Conservation Methods	Dry matter		Crude Protein		Ether extract		Ash	
	1st day	60th day	1st day	60th day	1st day	60th day	1st day	60th day
Control	18.68	-	58.48	-	37.47	-	2.67	-
$FPH_{Heater\ 30\ ^{o}C}$	14.58	17.83	63.78	57.32	22.90	39.20	3.77	3.42
$FPH_{Auto\ 30\ ^{o}C}$	19.72	21.36	54.15	52.34	37.67	38.25	2.84	3.51
$FPH_{Auto\ 4\ ^{o}C}$	15.29	21.76	54.54	53.63	37.54	39.01	3.07	3.58
$FPH_{Acid4\ ^{o}C}$	21.90	22.35	61.89	54.18	34.73	36.76	1.36	3.13
$FPH_{Acid30\ ^{o}C}$	23.22	16.21	66.24	55.64	31.31	50.35	1.55	3.27
$FPH_{2.5\ KGy}$	11.74	24.68	71.03	66.24	24.64	37.07	4.03	1.5
$FPH_{5.0\ KGy}$	12.17	13.32	69.73	60.14	26.57	35.73	3.70	4.13
$FPH_{7.0\ KGy}$	13.75	16.61	68.76	66.16	27.23	30.83	4.00	3.01

. Figures

Figure 1: Changes in L* [A], a* [B] and b* [C] values of fish protein hydrolysates submitted to acidification and heat sterilization, stored at 4º C and 30º C and gamma irradiation stored at 4º C. Error bars indicate the standard deviations from the means of triplicate determinations.

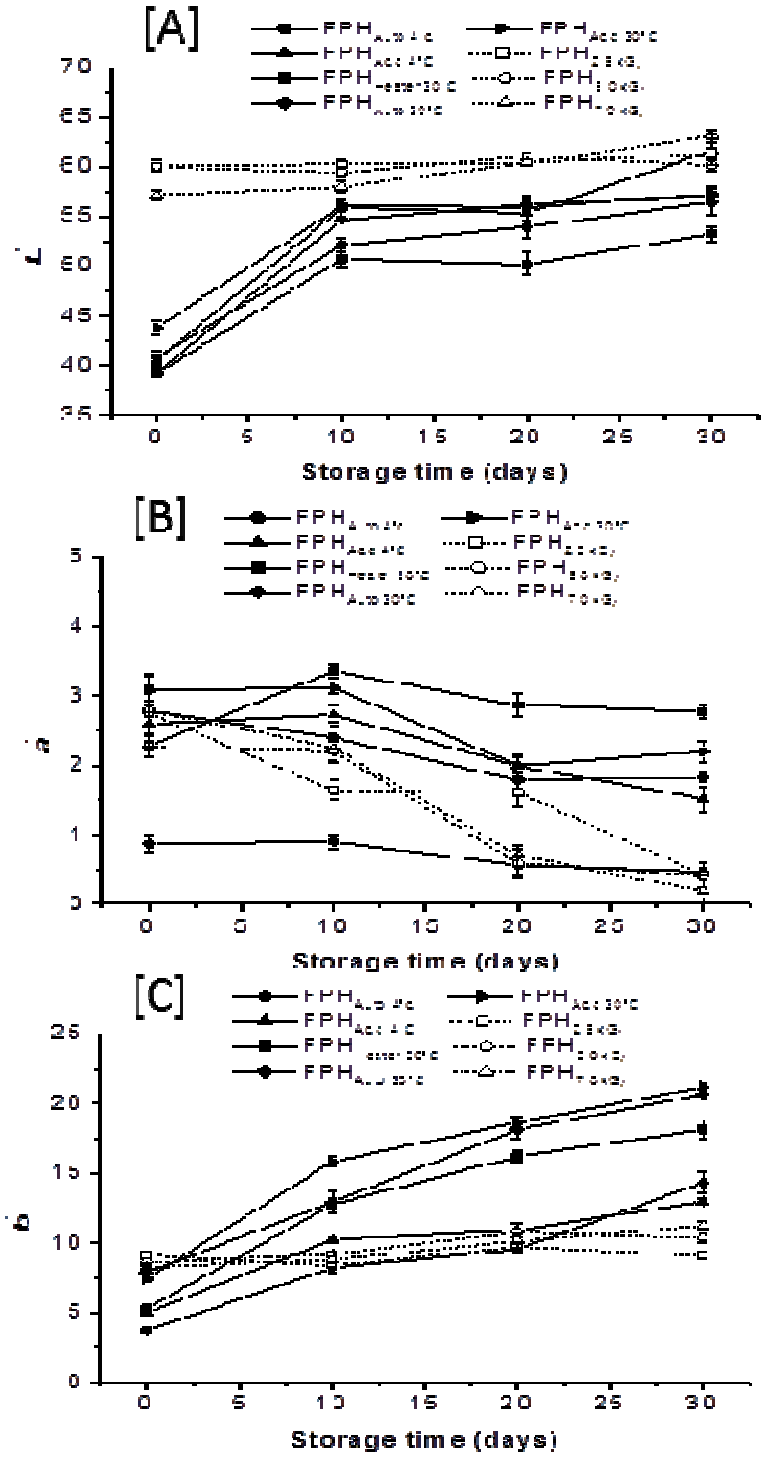
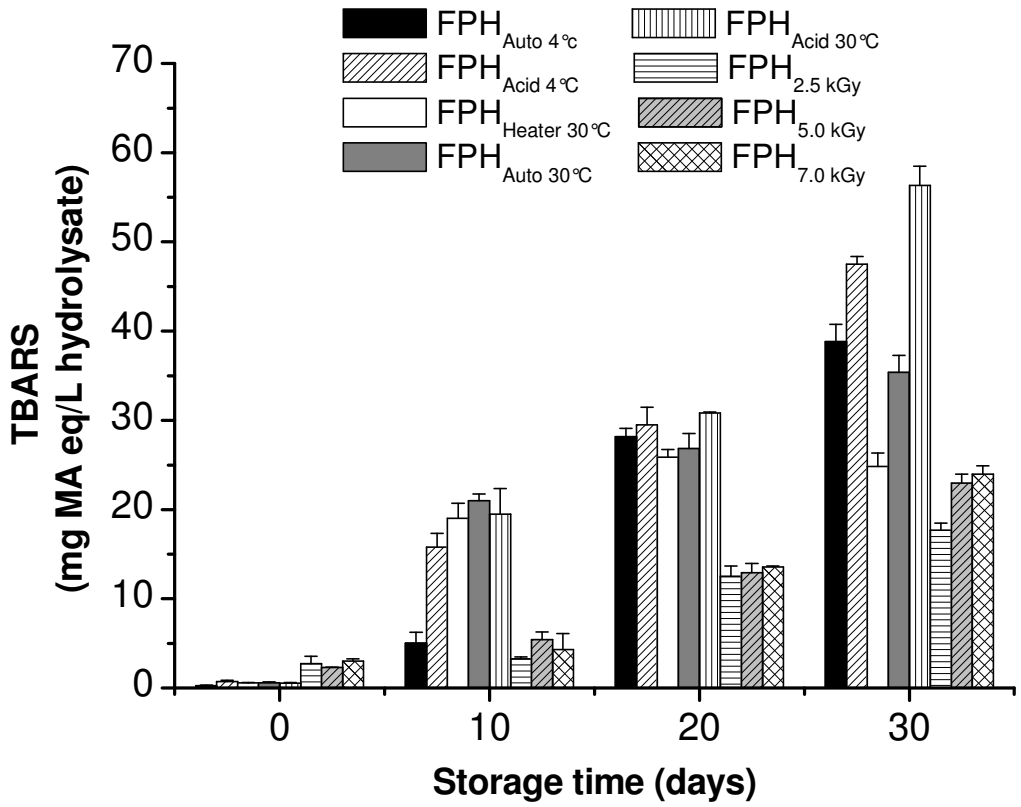


Figure 2: Thiobarbituric acid reactive substance (TBARS) measurement of fish protein hydrolysate subjected to acidification and heat sterilization, stored at 4º C and 30º C and gamma irradiation stored at 4º C. All studies were carried out in triplicate.



CAPÍTULO 4

PATENTE

PROCESSO PARA PRODUÇÃO DE UM PRODUTO PROTEICO E LIPÍDICO A PARTIR DA IRRADIAÇÃO DO HIDROLISADO PROTEICO DE PEIXE.

PROCESSO Nº BR 10 2012 032931 0.

RELATÓRIO DESCRITIVO DA PATENTE DE INVENÇÃO

PROCESSO DE OBTENÇÃO DE PRODUTO PROTEICO E LIPÍDICO A PARTIR DA IRRADIAÇÃO DO HIDROLISADO PROTEICO DE PESCADO

CAMPO DA INVENÇÃO

A presente invenção diz respeito a um processo para a produção de hidrolisados proteicos de pescados e dos seus óleos de forma estéril. O produto é obtido a partir de resíduos da indústria pesqueira e pode ser utilizado pela as indústrias de cosmética, alimentícia e nutricêutica. A presente invenção refere-se, mais particularmente, a aplicação de um processo de separação e conservação com a aplicação de radiação para aumentar o tempo de prateleira de hidrolisados proteicos e óleos de pescado de forma estéril com aplicações biotecnológicas.

DESCRIÇÃO DO ESTADO DA TÉCNICA

O filé é o item de maior valor econômico, o seu rendimento varia de acordo com o domínio tecnológico das empresas processadoras de pescado e pode chegar entre 30 e 40% do peso do animal dependendo da espécie, a outra parcela é considerado como resíduo sendo composto por restos de carne, cabeça, pele, ossos, escamas e vísceras. Logo, os resíduos da indústria de processamento de pescado, por exemplo, da tilápia, representam de 60 a 70% da matéria-prima. Atualmente, estes resíduos gerados são ainda subutilizados e muitas vezes descartados de forma inadequada ocasionando danos ao meio ambiente. Diante dessa problemática, pesquisadores em todo o mundo vêm desenvolvendo diversos esforços para obtenção de métodos que possibilitem a transformação desses resíduos em produtos passíveis de utilização tanto na nutrição humana quanto animal (MARTONE et al., 2005).

Os resíduos gerados pela indústria de processamento de pescado podem ser utilizados na obtenção de variados produtos como biofertilizantes, biogás, farinha de peixe, couro, gelatina, óleo e hidrolisado proteico (PI0604910-9 A; PI1000529-3 A2; PI0506315-9 A; PI0804954-8 A2; PI0516797-3 A; PI1004335-8 A2).

O hidrolisado proteico de peixe é resultado da solubilização das proteínas do pescado gerando proteínas menores, peptídeos e aminoácidos. Pode ser obtido a partir da hidrólise química (hidrólise ácida e alcalina), por hidrólise enzimática através de enzimas de origem

vegetal, animal ou microbianas adicionadas à matéria-prima a ser catalisada ou ainda por enzimas proteolíticas endógenas, ou seja, presentes no próprio organismo (KRISTINSSON e RASCO, 2000; MARTONE et al., 2005).

A hidrólise enzimática é um método baseado na adição de enzimas para clivagem das proteínas, sendo um processo usado para aperfeiçoar ou modificar as propriedades químicas, funcionais e sensoriais da proteína sem prejudicar o seu valor nutricional. O processo enzimático ocorre sob condições brandas, sem produzir produtos de degradação, observados nas hidrólises ácida e alcalina (FONKWE e SINGH, 1996). Enquanto que, a hidrólise por autólise enzimática é um método alternativo, em que se empregam as enzimas proteolíticas (proteases das vísceras do próprio peixe) para solubilização da proteína do pescado.

O processo de hidrólise é utilizado para modificar propriedades funcionais de alimentos e, em alimentos dietéticos, como fonte de pequenos peptídeos e aminoácidos. Podem ser incorporados a uma série de produtos, como fórmulas balanceadoras para atletas, regimes de emagrecimento, e utilizados na alimentação animal como, por exemplo, em porcos e bezerros, e na forma de suplementos proteicos em rações para animais. Na alimentação humana, servem como suplementos de biscoitos e produtos tipo hambúrguer, nuggets, entre outras, com possibilidade de também serem adotados para pessoas com problemas de digestão ou de má-absorção de proteínas, graças à sua elevada digestibilidade e aos aminoácidos essenciais disponíveis. Além do mais, estudos evidenciam que hidrolisado proteicos e peptídeos obtidos de organismos marinhos podem promover a saúde humana por meio da prevenção de doenças crônicas (KIM et al., 2007; KIM e WIJESEKARA, 2010). Juntamente com isto, estudos têm demonstrado que hidrolisados proteicos de peixe, obtidos por hidrólise enzimática, podem ser utilizados como potenciais antioxidantes naturais (YOU et al., 2010; LEE, JEON E BYUN, 2011).

Dependendo da matéria-prima, além de proteínas pequenas, peptídeos e aminoácidos os HPP também podem conter elevado teor de lipídio que podem ser separados e removidos em uma etapa seguinte, resultando na formação de um hidrolisado proteico uniforme e com baixo teor de gordura e de um óleo de peixe concentrado. O óleo de peixe apresenta aplicações diversas, não apenas, na indústria de cosméticos, mas também na de alimentos como item na composição de nutricêuticos, como pode ser visto nas patentes: PI0901392-0 A2; PI 0414818-5 A; PI 0509878-5 A e PI 0516797-3 A.

A hidrólise dos triacilgliceróis e consequentemente o aumento do teor de ácidos graxos livres formados durante armazenamento e processamento, é um dos fatores que determinam a vida de prateleira e produzem características organolépticas indesejáveis ao

alimento. (STEVANATO, 2007). As alterações na qualidade dos alimentos frescos podem ocorrer devido à oxidação de lipídeos e pigmentos contidos em alimentos gordurosos, resultando na liberação de sabor indesejável e na formação de compostos que possuem efeitos biológicos adversos ou que favoreçam a descoloração (FORSYTHE, 2002). VIEGAS (2000), estudando hidrolisado proteico de peixe a partir de resíduos do processamento de filés de tilápias, obteve um produto praticamente isento de lipídios, o que lhe conferiu excelente qualidade, devido ao fato de não apresentar problemas de rancidez oxidativa durante a estocagem.

Outro fator de grande importância, uma vez que, o hidrolisado proteico de peixe é destinado à alimentação humana e animal é da sua inocuidade. Uma vez que para a obtenção de um alimento seguro é necessário que este esteja isento de microrganismos patógenos e deteriorantes, os quais estando presentes poderão comprometer a qualidade nutricional do produto. Para tanto é necessário à aplicação de formas de conservação e esterilização, garantindo a qualidade e uma maior vida de prateleira deste produto.

A conservação consiste em manter o alimento o mais estável possível no que se refere ao aspecto microbiológico. Sendo necessário retardar a proliferação dos microrganismos com o controle de variáveis como a temperatura, o pH e a umidade. Dentre os métodos baseados na redução do crescimento microbiano estão os tratamentos com aplicação de sais, aplicação térmicos, acidificação, utilização de embalagens com atmosfera modificada e irradiação (FORSYTHE , 2002).

Neste contexto existem patentes voltadas para produção de hidrolisado proteico de peixe utilizando enzimas comerciais no seu processo de hidrólise (WO2009/101134A1; WO2009/101146A1; PI0705220-0A2), utilização de hidrolisado proteico na composição de nutricêuticos (PI1000331-2 A2), na conservação do hidrolisado pela aplicação de sais, ácidos, ou desidratação (CN102125173 - A), e na utilização de óleos de peixes para composição de nutricêuticos e óleo diesel (PI0314100-4A; PI9611826-1A; PI0509878-5A; PI0516797-3A). Porém, ainda não foi desenvolvido de forma conjunta um processo que envolvesse a produção e separação de um hidrolisado proteico de peixe do seu óleo de forma estéril.

APRESENTAÇÃO DOS PROBLEMAS EXISTENTES

A Separação de lipídios do hidrolisado proteico comumente envolve técnicas de custo elevado tais como centrifugação e ultrafiltração, além do uso de solventes como etanol e

acetona que produzem resíduos e não são recomendados pela ANIVISA (BR), quando o produto é destinado ao uso farmacêutico, alimentício ou cosmético.

Para tornar economicamente viável a produção de hidrolisado proteico de peixe em larga escala são necessários esforços no sentido de melhorar o rendimento do produto principal e de aproveitar outros subprodutos gerados como o óleo, agregando valor ao processamento do filé de peixes.

A esterilização destes produtos requerem métodos que inibam temporariamente a proliferação de microrganismos patogênicos. Tradicionalmente utiliza-se aplicação de sais ou ácidos e tratamentos térmicos. No entanto, esses métodos são utilizados apenas para a conservação e não para separar a parte proteica do óleo do hidrolisado proteico de peixe.

APRESENTAÇÃO DA SOLUÇÃO EM LINHAS GERAIS

A presente invenção vem solucionar os problemas citados, não apenas quando propõe um processo de produção de hidrolisado proteico de peixe que proporciona um aproveitamento dos resíduos da indústria pesqueira, mas quando também propõe um processo caracterizado pela simplicidade e pelo baixo custo para recuperação de biomoléculas. Por outro lado, o processo de irradiação (2,5 KGy), objeto do presente pedido de patente, além de esterelizar o produto, permite a separação da parte proteica e dos lipídios, não sendo necessário assim o uso de técnicas laboriosas para a acessibilidade do produto.

Outra vantagem da irradiação a 2,5 KGy é que é está dentro do limite da faixa de irradiação para alimentos de acordo com as normas da ANVISA, e essa técnica de conservação permite que o produto fique armazenado a 4ºC por até 60 dias sem o uso de nenhum conservante. Sendo assim o produto é passível de ser utilizado tanto para a nutrição humana quanto para animal.

A irradiação (cobalto 60) promove a esterilização do produto e proporciona a separação dos lipídios sem necessariamente ter que passar por outro processo de separação mecânica, transformando o mesmo em um concentrado proteico.

A presente invenção vem agregar valor ao processamento de filés de tilápias e de outros pescados devido ao aproveitamento de resíduos que antes era descartado contribuindo com o desenvolvimento sustentável do processo.

DESCRIÇÃO DETALHADA DO INVENTO

A presente invenção tem o objetivo de apresentar um método de obtenção de um produto proteico (hidrolisado proteico) e lipídico estéril, a partir da irradiação de hidrolisados proteicos de pescado. Este método permite a conservação do produto por 60 dias. O processo de obtenção compreende as seguintes etapas:

1. Preparação dos hidrolisados

a) Lavagem do resíduo do processamento de pescado

b) Trituração da carcaça com extrato de intestino de peixe (600mg/mL) ou Alcalase (0,5%) na proporção 1:1.

c) Hidrólise enzimática (digestão em banho-maria por 2 horas a 45ºC)

d) Desativação enzimática (aquecimento a 100ºC/10min)

e) Separação da parte sólida da parte líquida

A produção do hidrolisado proteico de peixe da presente invenção compreende a utilização de carcaças e com adição de enzimas endógenas (extrato de intestino de peixe de 200 a 600mg/mL) ou enzima comercial Alcalase (0,5%), sem ajuste de pH, de acordo com o método desenvolvido por Bezerra (2000) modificado. Os resíduos (carcaça adicionado ao extrato ou enzima comercial) são triturados na proporção de 1:1. A mistura é submetida à digestão entre temperatura de 35 e 50 ºC onde permanece por 2 horas a uma pequena agitação contínua para otimizar o contato enzima/substrato. Posteriormente o hidrolisado é fervido a 100°C, durante 10 minutos, para desativação enzimática. Ao final do processo a parte líquida (hidrolisado proteico de peixe) é separada da parte sólida através de uma peneira.

2. Obtenção de um produto proteico e lipídico estéril a partir da irradiação do hidrolisado proteico de pescado

A presente invenção compreende o acondicionamento do hidrolisado proteico de peixe em recipientes autoclavavéis, posteriormente são submetidos ao processo de irradiação em Fonte Gama Cell 220 (^{60}Co), nas doses entre 2 KGy a 8 KGy de 29 a 93 minutos. Em seguida armazenados sob refrigeração a ± 4º C, onde se inicia o processo de separação do óleo da parte proteica. A presente invenção consiste também em uma segunda opção de extração do óleo do hidrolisado proteico de pescado, podendo ser aplicada anterior ao processo de

irradiação, neste caso a irradiação seria apenas um tratamento de esterelização do produto. Esta outra forma compreende um processo que utiliza um equipamento que permite a aplicação de uma força centrífuga no qual o hidrolisado proteico de pescado é separado em duas fases, onde a gordura que é mais leve segue para o centro do equipamento saindo do seu rotor por tubulação.

A seguir serão expostos exemplos específicos da invenção.

Exemplo 1:
Análises microbiológicas

As análises microbiológicas foram realizadas com a finalidade de comprovar a eficiência dos métodos de conservação.

a) Contagem total de microorganismos psicrotróficos

A contagem total de microorganismos psicrotróficos para os irradiados foram realizados nos dias 0, 12, 24, 36, 48, 60. Sendo coletadas amostras de 1mL de cada hidrolisado proteico de peixe em triplicata e submetidas a dilução seriada (1:10; 1:100 e 1:1000) em tubos de ensaios esterelizados contendo 9 mL de solução (0.1% de água peptonada em 0,9% de Nacl de solução) e homogeneizadas sob condições estéreis em cabine de segurança biológica Pachane(Pc 410). Foi coletado 1mL de cada amostra diluída e inoculada em 3 placas de Petri. Para cada placa foi adicionado 15 mL de Plate Count Agar (Standard Methods Agar Acumedia). As amostras foram homogeinizadas imediatamente após ser vertido o meio através de rotações das placas de Petri, de forma a obter a dispersão uniforme das colônias. Após a completa solidificação as placas são invertidas e incubadas a 4ºC por 5 dias para análise de microorganismos psicrotróficos.

b) Análises microbiológicas de microrganismos específicos

Para verificar se o hidrolisado proteico de peixe atende a regulamentação (Anvisa/7d da RDC 12/2001) e obedece a padrões de segurança alimentar foram realizadas análises microbiológicas (bolores e leveduras, coliformes a 45ºC, estafilococos coagulase positiva, *Salmonella ssp e Pseudomonas*). O hidrolisado proteico de peixe foi submetido a irradição e foi analisado após 60 dias e o hidrolisado proteico de peixe controle foi analisados após 2 dias de armazenamentode de acordo com metodologia da “Bacteriological Analytical Manual" da

Food and Drug Administration , editado por Association of Official Analytical Chemists (FDA/AOAC) em suas últimas edições e ou revisões (BRASIL, 2001) e Standard Methods for Examination of Water and Wastewater (APHA, 1998). As análises foram realizadas no Laboratório de Experimento e Análise de Alimentos do Departamento de Nutrição da Universidade Federal de Pernambuco.

Exemplo 2:

A contagem total de microrganismos psicrotróficos, expressos em unidades formadora de colônia por mL (UFC/mL), dos hidrolisados proteicos de peixe irradiados foram expressos em 0 UFC/mL. Os resultados comprovam a conservação o produto sob o ponto de vista microbiológico. Desta forma os hidrolisados permaneceram livres de contaminação por microrganismos psicrotóficos e apresentaram-se conservados por 60 dias de armazenamento sob refrigeração a ± 4ºC.

Para avaliar a segurança alimentar dos hidrolisados proteicos de peixe foi realizada a pesquisa de microrganismos específicos. A presença em níveis pré-determinados de alguns microrganismos em um produto alimentício avalia seu estado de segurança alimentar e se o mesmo em sua produção obedece às normas de boas práticas de fabricação e manipulação, os princípios do HACCP, dentre outras normas que possam garantir o consumo do produto.

A tabela 1 fornece os resultados das pesquisas de microrganismos nos hidrolisados submetidos aos métodos de conservação a 4ºC durante 60 dias.

Tratamentos	Colifomes a 45°C	Estafilococos coagulase(+)	*Salmonella*	Fungos	*Pseudomonas aeruginosa*
Controle	< 3,0	< 10	Ausência	$8,7 \times 10^2$	-
2,5 KGy	< 3,0	< 10	Ausência	< 10	< 1,1
5,0 KGy	< 3,0	< 10	Ausência	< 10	< 1,1
7,0 KGy	< 3,0	< 10	Ausência	< 10	< 1,1

Os resultados apresentados na tabela 1 atendem ao item 7d da RDC 12/2001 – ANVISA quanto aos parâmetros obrigatórios para as amostras.

Os tratamentos realizados nos hidrolisados foram eficientes quanto à presença de microrganismos patógenos e/ou deteriorantes estando livres destes mantendo-se assim conservados a 4ºC até o prazo de 60 dias.

O produto obtido pela presente invenção apresenta a vantagem de ser uma forma de conservação e extração de óleo mais estável por apresentar uma baixa atividade microbiológica não sendo necessário s tratamentos e adição de conservantes.

REIVINDICAÇÃO

1. Apresente invenção é caracterizada por um processo de produção compreendendo a separação e conservação por meio da aplicação de radiação do hidrolisado proteico de pescado para produção de um produto composto por um hidrolisado proteico e óleo de pescado estéril.

2. Processo de acordo com a reivindicação 1, caracterizado por envolver uma hidrolise proteolítica por autólise utilizando as enzimas de pescado como enzimas das vísceras de peixes, ou enzimas comerciais como Alcalase.

3. Processo de acordo com a reivindicação 1, caracterizado pelo a acondicionamento do hidrolisado proteico de pescado em recipiente autoclavável seguido pelo processo de irradiação por Fonte Gama Cell 220 (^{60}Co) ou outro, nas doses entre 2 KGy a 8 KGy de 29 a 93 minutos .

4. Processo de acordo com a reivindicação 3, caracterizado pelo armazenamento do referido hidrolisado irradiado sob uma refrigeração a ± 4ºC, para separação do óleo da parte proteica.

5. Processo de acordo com a reivindicação 1 a 4, caracterizado por utilizar um equipamento antes do processo referente à reivindicação de 3 a 4 o qual aplica uma força centrífuga separando diferentes fases (hidrolisado proteico e óleo), de forma que, o hidrolisado proteico de pescado suba pela área externa de pratos presentes no referido equipamento e que o óleo siga para o centro do equipamento saindo do seu rotor por tubulação.

6. Produto, de acordo com a reivindicação de 1 a 5, caracterizado por um hidrolisado proteico e óleo de pescado estéril por irradiação.

RESUMO

A presente invenção diz respeito a um processo para a separação do óleo da parte proteica de hidrolisados proteicos de pescado de forma estéril. O processo de acordo com a presente invenção primeiramente refere-se ao ajuste das condições simples de hidrólise que tornem favoráveis a produção aumentada do hidrolisado proteico de pescado a partir de resíduos da indústria pesqueira. Em um segundo momento a aplicação de um processo de separação e conservação para aumentar o tempo de prateleira de hidrolisados proteicos e óleos de pescado estéreis com aplicações biotecnológicas.

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4. **CONSIDERAÇÕES FINAIS**

Aproveitando o resíduo do processamento de filés de tilápia do Nilo, foi possível elaborar extratos enzimáticos com intestino da própria tilápia, onde a concentração de 600mg de tecido/mL provou ser mais eficiente na hidrólise da carcaça da tilápia do que a enzima comercial Alcalase para a produção do hidrolisado proteico de peixe. As atividades proteolíticas totais e específicas deste extrato foram comprovadas mediante ensaios com substratos inespecífico e específicos e zimograma.

Proteases alcalinas na concentração de 200 mg/mL parcialmente purificadas com etanol e sulfato de amônio também demonstraram eficiência na hidrólise da carcaça da tilápia em igual e maior grau de hidrólise comparadas com as enzimas comerciais Alcalase e Flavourzyme, respectivamente.

O hidrolisado proteico de peixe produzido a partir destes resíduos constitui-se de um produto com elevado teor de proteína e aminoácidos, com destaque para metionina e lisina, os quais são limitantes em dietas para organismos aquáticos. Alto teor de lipídio apresentando ácidos graxos poli-insaturados com predominância do ácido linoleico, além de ômega 3 e 6. Além do baixo teor de cinzas, o que confere uma boa qualidade ao produto devido ao maior aproveitamento da matéria orgânica.

Durante trinta dias de estocagem em temperatura ambiente e sob-refrigeração, os HPP sofreram efeitos da oxidação lipídica, comprovado através dos altos valores de TBARS a partir do décimo dia até o final. O que teria influenciado também na mudança da coloração dos produtos.

Do ponto de vista microbiológico, os tratamentos de esterilização térmica em combinação com ácido cítrico, mantidos a 30º C e 4º C e os tratamentos com irradiação gama combinados com refrigeração (4º C) provaram ser eficientes em detrimento ao desenvolvimento de micro-organismos patogênicos e deteriorantes, conferindo aos produtos segurança alimentar durante 60 dias de estocagem.

O método de irradiação não apenas estereliza o produto como promove a separação da parte proteica da parte lipídica, transformando o mesmo em um concentrado proteico.

Devido as suas características nutricionais o hidrolisado proteico de peixe é um produto passível de utilização em dietas para humanos e animais.

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ANEXOS

ANEXO 1:

Aquaculture Nutrition

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Replacement of fishmeal by fish protein hydrolysate and biofloc in the diets of *Litopenaeus vannamei* postlarvae

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Abstract

This study aimed to evaluate the effect of the gradual replacement of fishmeal with fish protein hydrolysate (FPH) and biofloc flour (BF) in the diets of white shrimp *Litopenaeus vannamei* postlarvae (2 mg). Five diets (420 g kg^{-1} of crude protein) were formulated, and these replaced 0 (control), 10, 20, 30 and 40% (T0, T10, T20, T30 and T40, respectively) of the fishmeal. A commercial diet was used as an external control. The alternative ingredients FPH and BF were added at a ratio of 1 : 1. After 42 days, the shrimp survival was higher than 99% in all the treatment groups. A regression test indicated that the ideal fishmeal substitution level to obtain optimal zootechnical parameters (final weight, weight gain and protein efficiency) is between 15.16 and 16.5%. In this study, we demonstrated that BF and FPH are potential ingredients that can be used to replace fishmeal in *L. vannamei* postlarvae diets.

KEY WORDS: enzymatic hydrolysis, fishmeal, microbial flocs, nursery phase, protein food, shrimp

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Introduction

Feeding corresponds to approximately 50% of the total cost of intensive shrimp farming (Naylor *et al.* 1998). This cost is mainly due to the use of fishmeal as a main protein source in aquafeeds. Fishmeal has high palatability and is an excellent source of amino acids, fatty acids, vitamins and minerals (Cruz-Suárez *et al.* 2009). However, increasing demand places pressure on the supplies of fishmeal supply increase its cost.

Aquaculture is currently growing faster than any other animal food production sector (FAO 2010), and this growth must to be accompanied by efforts to promote sustainability. Fish and marine shrimp may consume more fish as fishmeal than it produces (Tacon & Metian 2009). Thus, a reduction in the use of fishmeal has been widely researched.

A range of alternative ingredients, including plant products, such as soybeans, canola, cotton and corn (Lim *et al.* 1997; Alvarez *et al.* 2007; Suárez *et al.* 2009), have been studied. However, antinutritional factors, amino acid deficiencies, low palatability and low digestibility restrict the use of these ingredients (Davis & Arnold 2000; Nunes *et al.* 2006; Gatlin *et al.* 2007).

Residues from the fishery industries are also an alternative to fishmeal. The fish protein hydrolysate (FPH) produced from these residues has a high protein content, a good balance of amino acids and fatty acids, a low ash volume, and high palatability and digestibility (D'Abrowski 1984; Goldhor & Regenstein 1988; Sgarbirei 1996; Oetterer 2001). Thus, the FPH contains features for aquafeed production, and some researchers consider FPH to be a good substitute for fishmeal. In a previous study, the growth of *Penaeus monodon* was improved by replacing only 3% of the fishmeal with FPH (Anggawati *et al.* 1990).

More recently, the bioflocs produced in intensive farming systems, which are known as BFT (Biofloc Technology), have been studied (Abreu *et al.* 2007; Kuhn *et al.* 2010; Xu & Pan 2013). In this system, the shrimp actively consumes the bioflocs, which can represent 20–30% of assimilated protein (Burford *et al.* 2003, 2004). The bioflocs are formed by bacteria, fungi, invertebrates

ANEXO 2

ANIMAL FEED SCIENCE AND TECHNOLOGY (ISSN: 0377-8401)

Guide for Authors
1. Original Research Papers (Regular Papers)
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3. Short Communications
4. Book Reviews

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Professor G. Flachowsky
Federal Research Centre of Agriculture
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Germany

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b. NDFom-NDF not assayed with a heat stable amylase and expressed exclusive of residual ash.
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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.
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ANEXO 3

Guide foe authors

LWT- Food Science and Technology

INTRODUCTION

LWT - Food Science and Technology is an official journal of the Swiss Society of Food Science and Technology (SGLWT/SOSSTA) and the International Union of Food Science and Technology (IUFoST). *LWT - Food Science and Technology* is an international journal that publishes innovative papers in the fields of food chemistry, biochemistry, microbiology, technology and nutrition. The work described should be innovative either in the approach or in the methods used. The significance of the results either for the science community or for the food industry must also be specified. Contributions that do not fulfil these requirements will not be considered for review and publication. Submission of a paper will be held to imply that it presents original research, that it has not been published previously, and that it is not under consideration for publication elsewhere.

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e. Total number of figures ≤ 5.
f. Maximum number of references (including those cited in tables and figures) not to exceed 50.
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