UFRRJ

INSTITUTO DE TECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

Tese

Produção de peptídeos bioativos através da otimização do processo de hidrólise enzimática da proteína obtida da pele do peixe tilápia (oreochromis niloticus, LINNAEUS 1758) e purificação dos peptídeos utilizando resina macroporosa

SIRAJ SALMAN MOHAMMAD

2024

UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
INSTITUTO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

THE PRODUCTION OF BIOACTIVE PEPTIDES BY OPTIMIZATION THE PROCESS OF ENZYMATIC HYDROLYSIS OF PROTEIN OBTAINED FROM TILAPIA FISH SKIN (*Oreochromis niloticus*, LINNAEUS 1758) AND PURIFICATION THE PEPTIDES USING MACROPOROUS RESIN

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Sob a orientação do professor
José Lucena Barbosa Junior

Co orientação da professora
Nathalia Rodrigues

Tese submetida como requisito parcial para obtenção do grau de **Doutor em Ciência e Tecnologia de Alimentos**, no curso de Pós-Graduação em Ciência e Tecnologia de Alimentos, Área de concentração em Tecnologia de Alimentos.

Seropédica, RJ
Fevereiro de 2024

Mohammad, Siraj Salman, 1987-
MM697s Produção de peptídeos bioativos através da otimização
p do processo de hidrólise enzimática da proteína obtida
da pele do peixe tilápia (oreochromis niloticus,
LINNAEUS 1758) e purificação dos peptídeos utilizando
resina macroporosa / Siraj Salman Mohammad. -
Tartous, 2024.
125 f.

Orientador: José Lucena Barbosa Junior.
Tese(Doutorado). -- Universidade Federal Rural do Rio
de Janeiro, Programa de pós graduação em ciência e
tecnologia de alimentos, 2024.

1. Antioxidante. 2. purificação. 3. peptídeo
bioativo. I. Barbosa Junior, José Lucena, 1975-,
orient. II Universidade Federal Rural do Rio de
Janeiro. Programa de pós graduação em ciência e
tecnologia de alimentos III. Título.

O presente trabalho foi realizado com apoio da Cooredenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES) – Código de financiamento 001.

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

TERMO Nº 34/2024 - PPGCTA (12.28.01.00.00.00.00.41)

Nº do Protocolo: 23083.005642/2024-11

Seropédica-RJ, 07 de fevereiro de 2024.

SIRAJ MOHAMMAD

Tese submetida como requisito parcial para obtenção do grau de **Doutor em Ciência e Tecnologia de Alimentos**, no Curso de Pós-Graduação em Ciência e Tecnologia de Alimentos, área de Concentração em Ciência de Alimentos.

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AGRADECIMENTOS

O maior graças a Deus o maior do mundo que não tem limite para sua generosidade e amor, obrigado deus por me dar essa grande oportunidade de estudar durante o mestrado na UFRRJ, você esteve sempre comigo em todas as etapas do meu estudo, na sala de estudos, laboratório, restaurante universitário, alojamento e em todos os lugares sempre foi a melhor proteção, obrigado deus a facilitar minha vida e conseguir esse grande sucesso no meu estudo de mestrado.

Á minha família. Palavras não podem expressar o quanto sou grato a minha mãe e pai por todos os sacrifícios que você fez em meu nome. Sua oração por mim foi o que me sustentou até agora. Eu sou muito grato meu filho presente de Deus e fonte de alegria e minha esposa meu amor Doaa, pela compreensão e por me estimular a perseverar sempre, e especialmente eu não posso agradecer o suficiente por me encorajar durante toda essa experiência. Também gostaria de agradecer pelo grande apoio a minha irmã querida Elfat, meu irmão querido Rawad e família dele.

Á meu orientador, professor Luçena, por me orientar e apoiar ao longo dos anos. Você definiu um exemplo de excelência como pesquisador, mentor, instrutor e modelo. Obrigado pela paciência, por acreditar em mim, por tanto tempo investido em tantas explicações, por nunca ter se negado a me ajudar.

À minha co-orientadora, Nathalia Rodrigues, por toda paciência e auxílio e por estar sempre disposta a ajudar;

À professora Mari Ivone para ficar ao meu lado, por sua orientação e me ajudar a ter sucesso.

Ao professor Luís Calçada e sua família Ana e Gabriel pelo grande amizade.

Aos técnicos de DTA Vinicius, Paulo Cezar, Ivaniuda e Fernando, que foram essenciais nos laboratórios;

Ao pesquisador Dr. Ronoel, e Monaliza, pois com eles as análises foram mais divertidas na Embrapa.

Aos pessoas que trabalhar na restaurante da universidade que facilita muito a vida da pesquisa.

Aos amigos da universidade e do alojamento: Gepatrick, David, Jose Fernando, Lucas, Kenedy, Ronel, Jonatthan, Alan, e Arturo que dividiram comigo todos os momentos do mestrado e doutorado, tentando superar juntos os obstáculos que sempre aparecem, dando-me todo o suporte ao longo destes quatro anos de doutorado;

Por fim, a CNPq por quase 4 anos de auxílio financeiro.

Muito obrigado!

RESUMO

MOHAMMAD, Siraj Salman. **PRODUÇÃO DE PEPTÍDEOS BIOATIVOS ATRAVÉS DA OTIMIZAÇÃO DO PROCESSO DE HIDRÓLISE ENZIMÁTICA DA PROTEÍNA OBTIDA DA PELE DO PEIXE TILÁPIA (*Oreochromis niloticus*, LINNAEUS 1758) E PURIFICAÇÃO DOS PEPTÍDEOS UTILIZANDO RESINA MACROPOROSA, 2022.** Thesis (PhD in Food Science and Technology). Instituto de Tecnologia, Departamento de Tecnologia de Alimentos, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2022.

A hidrólise enzimática (EH) de proteínas baseia-se, essencialmente, no aumento das propriedades funcionais e nutricionais das proteínas, tais como atividade antioxidante, solubilidade, capacidade de retenção de óleo e capacidade de retenção de água, emulsificação, propriedades espumantes e propriedades sensoriais. Neste estudo, os hidrolisados proteicos obtidos de resíduos de pele de peixe tilápia (HPRPPT) foram otimizados utilizando alcalase 2.4.L por desenho composto central (CCD). A cinética da reação foi considerada em relação à concentração inicial do substrato, concentração inicial da enzima e tempo de hidrólise. Os hidrolisados proteicos foram separados usando três resinas macroporosas. Grau de hidrólise (GH%), atividade sequestrante de radicais (DPPH) e poder antioxidante redutor férrico (FRAP) foram utilizados como variáveis dependentes. temperatura, pH e proporção enzima para substrato (PE%) como variáveis independentes. O grau ótimo de hidrólise GH, DPPH e FRAP foram alcançados na temperatura de 58,4 ℃, pH de 8,7 a menos que DPPH que estava em pH de 7,0. O modelo cinético mostrou que a GH aumentou com o aumento da concentração inicial da enzima E_0, sob uma concentração de substrato constante à medida que o tempo de hidrólise se prolongou para 120 min. Além disso, a GH diminuiu com o aumento da concentração inicial de substrato S_0 sob uma concentração constante de enzima à medida que o tempo de hidrólise se prolongou para 120 min. No entanto, além de 120 min, o GH dessas amostras não variou significativamente. XAD-7HP apresentou as maiores capacidades de adsorção e dessorção. Os testes isotérmicos indicaram que o mecanismo de adsorção foi melhor explicado usando o modelo de Freundlich que proporcionou grande precisão na separação de HPRPPT para resina XAD-7HP. O extrato purificado e normal de HPRPPT apresentou alta capacidade antioxidante em ambos os métodos de FRAP e DPPH. Este HPRPPT purificado poderia potencialmente encontrar aplicação como compostos bioativos naturais brutos nas preparações da indústria cosmética e farmacêutica.

Palavras-chave: antioxidante, teste isotérmico, purificação, adsorção, polímero, peptídeo bioativo, hidrolisados, colágeno.

abstract

MOHAMMAD, Siraj Salman. **THE PRODUCTION OF BIOACTIVE PEPTIDES BY OPTIMIZATION THE PROCESS OF ENZYMATIC HYDROLYSIS OF PROTEIN OBTAINED FROM TILAPIA FISH SKIN (*Oreochromis niloticus,* LINNAEUS 1758) AND PURIFICATION THE PEPTIDES USING MACROPOROUS RESIN**2022. Tese (Doutorado em Ciência e Tecnologia de Alimentos). Instituto de Tecnologia, Departamento de Tecnologia de Alimentos, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2022.

Enzymatic hydrolysis (EH) of proteins relies, essentially, in the enhancing of the functional and nutritional properties of proteins, such as antioxidant activity, solubility, oil holding capacity and water holding capacity, emulsification, foaming properties and sensory properties. In this study, the protein hydrolysates obtained of tilapia fish skin waste (PHTFSW) was optimized using alcalase 2.4.L by central composite design (CCD). Kinetics of the reaction was considered in relation to initial substrate concentration, initial enzyme concentration, and hydrolysis time. Protein hydrolysates were separated using three macroporous resin. Degree of hydrolysis (DH), radical scavenging activities (DPPH) and ferric reducing antioxidant power (FRAP) were used as dependent variables. temperature, pH and proportion enzyme to substrate (PE%) as independent variables.. The optimum degree of hydrolysis DH%, DPPH and FRAP were achieved at temperature 58.4 ℃, pH of 8.7 unless DPPH which was at pH of 7.0. Kinetic model presented that DH increased with increasing the initial enzyme concentration E_0, under a constant substrate concentration as the hydrolysis time prolonged to 120 min. Furthermore the DH decreased with increasing the initial substrate concentration S_0 under a constant enzyme concentration as the hydrolysis time prolonged to 120 min. However beyond 120 min, the DH of these samples did not vary significantly. XAD-7HP showed the highest adsorption and desorption capacities. The isotherm tests indicated that the adsorption mechanism was better explained using Freundlich model which provided great accuracy in the separation of PHTFSW for XAD-7HP resin. Purified and normal extract of PHTFSW showed high antioxidant capacity in both methods of FRAP and DPPH. This purified PHTFSW could potentially find application as raw natural bioactive compounds in the cosmetic and pharmaceutical industry preparations.

Key words: antioxidant, isotherm test, purification, adsorption, polymer, bioactive peptide, hydrolysates, collagen.

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Nenhuma entrada de índice de ilustrações foi encontrada.

CAPÍTULO V

LISTA DE ABREVIATURAS

MARs	Macroporous resins
PHTFSW	protein hydrolysates of tilapia fish skin waste
TFSW	Tilapia Fish Skin Waste
CCD	central composite design
EH	Enzymatic hydrolysis
AKT	alkaline-thermal
AT	acid-thermal
EDTA	ethylenediaminetetraacetic acid
ASC	Acid-solubilized collagen
PSC	pepsin-solubilized collagen
HPP	high pressure processing
IEX	Ion exchange chromatography
PTFSW	protein obtained from tilapia fish skin waste
EHP	Enzymatic hydrolysis process
QPH	quinoa protein hydrolysates
RSA	radical scavenging activity
DCGP	hydrolysis of defatted corn germ protein
AKT	Alkaline-thermal
AT	Acid-thermal
EDTA	ethylenediaminetetraacetic acid
PtH	Protein hydrolysates
OHC	Oil holding capacity
WHC	water holding capacity
BSSG	Blue shark skin gelatin
ROS	Reactive oxygen specieS
DH	Degree of hydrolysis
EDFM	Electrodialysis with filtration membranes
Phe	Phenylalanine
PE%	Ratio enzyme/substrate
BSA	Bovine serum albumin
C_0	The initial concentration
V_b	The amount of base of normality
N_b	Base of normality
m_p	The mass of protein
h_{TDT}	The total number of peptide bonds
α	The average degree of dissociation
pK	The average value of the α-NH2 amino groups
S	The concentration of substrate
E	The concentration of enzyme
P	The product (g/L)
ES	The concentration of enzyme substrate complex
V	The hydrolysis velocity
S_0	The initial substrate concentration
K_m	The Michaelise-Menten constant (g/L)
C_0	The initial concentration of the total peptides (mg/mL)
C_e	The equilibrium concentration of the total peptides (mg/mL)
V_d	The volume of the desorption solution (ml)
V_0	The volume of (PHTFSW) solution added into the flasks (ml).

D	The desorption ratio (%);
q_d	The desorption capacity (mg/ g dry resin);
C_d	The concentration of the total peptides in the desorption solu (mg/L);
R	The recovery of the total peptides %
A	The adsorption ratio %
K_L	Langmuir parameter
K_f	Freundlich parameter

Sumário

ESTRUTURA DA TESE

A tese está estruturada conforme descrito a seguir: Inicialmente no Capítulo I é apresentado introdução geral e revisão bibliográfica sobre a importância econômica dos resíduos de pescado, além de mostrar o processo de hidrólise enzimática como um método adequado e útil devido às características de melhorar as propriedades funcionais e biológicas das proteínas e manter seu valor nutricional.

Capítulo II, é apresentada uma revisão de literatura acerca dos principais artigos científicos sobre diferentes características das hidrolisados proteicos, incluindo a capacidade antioxidante dos hidrolisados proteicos e o efeito de várias condições de hidrólise no grau de hidrólise e propriedades funcionais dos hidrolisados proteicos, bem como apresentar técnicas de extração e separação de proteínas.

No Capítulo III estão apresentados os resultados do estudo de otimização dos parâmetros da hidrólise, incluindo (pH, temperatura e proporção enzima/substrato) utilizando o desenho composto central (CCD), além de determinar DPPH, FRAP e grau de hidrólise DH% como respostas do processo.

No Capítulo IV são apresentados resultados do modelo cinético das melhores condições de hidrólise enzimática de proteína de resíduos de pele de peixe tilápia (PRPPT) para obter maior grau de hidrólise de proteína usando Alcalase 2.4.L. Além disso, o efeito das concentrações de substrato e enzima no grau de hidrólise de PRPPT foi determinado.

No Capítulo V são apresentados os resultados da adsorção e dessorção de hidrolisados proteicos de resíduos de pele de peixe tilápia HPRPPT em MARs e identificar o melhor tipo de resina para separar e purificar peptídeos com base no teste estático de adsorção e dessorção, e teste termodinâmico de adsorção, além de apresentar a capacidade antioxidante de HPRPPT purificado.

CAPÍTULO I: Introdução geral e revisão bibliográfica

1. INTRODUÇÃO GERAL

Os resíduos de peixes são fontes significativas de proteínas e outros componentes, como ácidos graxos poliinsaturados, fosfolipídios, vitaminas solúveis e compostos bioativos (SHIRAHIGUE et al., 2016), que tornam esses resíduos atrativos para diversas aplicações tecnológicas que promovem o desenvolvimento de produtos e avanços significativos na indústria pesqueira (FELTES et al., 2010). Consequentemente, há estudos e investigações direcionados para encontrar novas formas de aproveitamento dos resíduos pesqueiros, a fim de mitigar os problemas ambientais. (SHERIFF et al., 2014). O colágeno da pele de tilápia apresentou efeito claro na preparação de microestruturas eletrofiadas/eletropulverizadas, proporcionando assim uma maneira de maximizar a utilização de recursos de resíduos de processamento de tilápia (BI et al., 2019).

Os hidrolisados proteicos têm ganhado grande interesse nos últimos anos (KTARI et al., 2020), uma vez que a modificação por enzimas ou produtos químicos melhora as propriedades funcionais das proteínas nativas e a sua utilidade como ingredientes intermediários nos setores cosmético, farmacêutico, alimentar e nutracêutico (ŠLIŽYTĖ et al., 2005; KLOMPONG et al., 2008; SARMADI ANDISMAIL, 2010). Além disso, a hidrólise de proteínas tem sido a forma mais comum de produção de peptídeos bioativos. (NWACHUKWU ANDALUKO, 2019). As vantagens do uso da hidrólise enzimática em comparação ao método de hidrólise química podem ser um produto proteico com melhores propriedades biológicas, digestíveis e funcionais, pois o método de hidrólise enzimática tem seletividade para quebrar ligações peptídicas especificas e liberar peptídeos com sítios ativos enquanto método de hidrólise química não tem essa seletividade, onde esse método funciona para quebrar as ligações peptídicas nos lugares dos sítios ativos liberando peptídeos com menos propriedades biológicas, além disso, hidrólise enzimática usa uma temperatura de hidrólise mais baixa (50–60 °C), consequentemente a temperatura baixa pode proporcionar peptídeos e óleo de maior qualidade e estabilidade. (CARVAJAL AND MOZURAITYTE, 2016).

O controle sobre os parâmetros da hidrólise enzimática permite a aquisição de polipeptídeos de tamanho específico e propriedades biológicas melhoradas. A atividade biológica dos hidrolisados proteicos depende de vários fatores, incluindo: tipo de enzima protease, proporção enzima-substrato, concentração de substrato, duração da incubação, temperatura e pH. (HALIM et al., 2016). Hidrolisados de proteínas derivadas de alimentos e peptídeos bioativos são considerados nutracêuticos e ingredientes alimentares funcionais promissores, esses compostos são essenciais para a saúde humana na prevenção e manejo de doenças crônicas devido às suas diversas atividades biológicas. Os hidrolisados proteicos também são considerados para diversas atividades biológicas, como anti-hipertensiva, anti-inflamatória, antimicrobiana, antioxidante, imunomoduladora, osteoprotetora, opioide, entre outras atividades (CAPRIOTTI et al., 2016). Os resíduos de peixe, compostos em média por cabeça, cauda, pele, intestino, barbatanas e estrutura, contêm 58% (p/p) de proteína (FANG et al., 2015). Proteínas de resíduos de peixes podem ser extraídas por hidrólise enzimática usando proteases como alcalase (BASTIDAS-OYANEDEL et al., 2016). O hidrolisado de proteína de peixe é a principal forma de subproduto de frutos do mar com aplicações na alimentação humana e na alimentação animal (CAVALHEIRO et al., 2007; KIM ANDKIM, 2014).

A esse respeito, Fanimo et al. (2000) descobriram que uma dieta protéica à base de farinha de peixe mostrou melhor utilização líquida de proteína em ratos do que uma dieta à base de farinha de camarão (FANIMO et al., 2000). As proteínas dos resíduos de peixes também podem ser convertidas em aminoácidos, por hidrólise enzimática usando alcalase, e em peptídeos bioativos com propriedades anti-hipertensivas, antitrombóticas, imunomoduladoras e antioxidantes, propriedades antidiabéticas, anticancerígenas, de ligação ao cálcio e hipocolesterolêmicas também foram relatadas (GHALY et al., 2013). Além disso, os hidrolisados de proteínas podem levar a diferentes derivatizações peptídicas, especialmente a perda de grupos SH devido a interações com derivados carbonílicos reativos (MOHAN AND UDENIGWE, 2015). Portanto, a separação de

hidrolisados proteicos merece destaque na obtenção de peptídeos bioativos com mais pureza e valor nutricional a fim de evitar a deterioração destes compostos devido à interação química com diferentes componentes presentes no complexo sistema biológico.

Separação por membrana (QU et al., 2015), ultrafiltração e filtração em gel (AGRAWAL et al., 2019) e extração líquido-líquido (ELAGLI et al., 2016) são métodos diferentes para separação de proteínas hidrolisadas. No entanto, estes métodos apresentam problemas associados à baixa pureza, baixo rendimento de peptídeos alvo e baixa taxa de conversão da proteína substrato. Resinas macroporosas, especialmente o tipo de adsorção comumente usado e troca iônica que corresponde ao mecanismo de adsorção física inespecífica e troca iônica respectivamente, são amplamente utilizadas como materiais de separação (HASHIMOTO, 1991). As resinas de adsorção macroporosas (MARs) são frequentemente aplicadas no isolamento e purificação de produtos naturais, como polifenóis (JIN et al., 2015), flavonoides (MA et al., 2013), outros antioxidantes (ZOU et al., 2017), alcaloides (LIU et al., 2014) e poliaminoácidos (ZHEN et al., 2015). Resinas macroporosas de troca iônica são frequentemente usadas na remoção de íons de metais pesados em solventes orgânicos (SHI et al., 2009), separação de aminoácidos (RUIZ et al., 2007), e descoloração (Xiong et al., 2019).

Além disso, não há modelo cinético relatado para condições otimizadas de hidrólise de proteínas obtidas de resíduos de pele de peixe tilápia utilizando alcalase 2.4.l e purificação de hidrolisados proteicos utilizando resina macroporosa. Portanto, o objetivo deste estudo é identificar as melhores condições de hidrólise enzimática da proteína dos resíduos de pele de peixe tilápia incluindo (pH, temperatura e relação enzima/substrato) para obter maior grau de hidrólise proteica utilizando Alcalase 2.4.L. e identificar o melhor tipo de resina para separar e purificar hidrolisados proteicos de resíduos de pele de peixe tilápia HPRPPT e apresentar as características de adsorção/dessorção deste processo.

2. LITERATURE REVIEW

2.1. Tilapia fish

Tilapia is becoming principal kind of fish for aquaculture with farms and the most important food fish in the world. Tilapia is sometimes described as aquatic chicken due to their fast growth speed, adaptability to a wide range of environmental conditions, ability to grow and reproduce in captivity, easy feed on low trophic level, and easy processing to fish fillets (EL-SAYED, 2006). Tilapia culture has been spread and grown in a lot of countries around the world. Processing waste of bones, meat remains, head, skin, scale, and viscera consist approximately 60-70% of the fish body (SILVA et al., 2014), of these, approximately 30% consists of skin and bones with high nutritional quality for obtaining different products (HARATI et al., 2020).Tilapia skin can be used as a good resource to produce collagen as value-added product, thus solving the problem of waste disposal. Several methods have been successfully used to extract collagen from tilapia skin such as acetic acid methods and pepsin hydrolysis (SUN et al., 2017).

To extract proteins and collagen, biochemical, chemical, physical methods and combined techniques have been developed. The physical methods include centrifugal, thermal, ultrasonic, and membrane filtration (GARCÍA et al., 2017; SHU et al., 2018). The chemical methods include alkaline-thermal (AKT), pH adjustment, acid-thermal (AT), ethylenediaminetetraacetic acid (EDTA) and aldehydic reagent (BOLEIJ et al., 2018; FENG et al., 2019). However, the biochemical methods include enzymatic and assisted enzymatic treatment (WU et al., 2016) are commonly used for producing peptides in terms of safety, production rate, and high efficiency (JANG et al., 2008).

2.2. Tilapia wastes gelatine extraction

Raw material from food source, low costs and enzyme commercially available are so important factors to produce bioactive peptides by enzymatic hydrolysis of protein (TAVANO et al., 2018). Biological activity of hydrolysates could be different according to critical parameters of hydrolysis process as Temperature, pH and time of hydrolysis (NONGONIERMA et al., 2017).

Hemker et al. (HEMKER et al., 2020) used pressure-assisted enzymatic hydrolysis to obtain functional peptides transformed from tilapia fish wastes. This method accelerated the hydrolysis process and facilitated the release of free amino acids. Other properties like solubility, and emulsifying were also considerably improved. However it was noticed a decrease of water holding capacity. Comparing to the hydrolysate produced with alcalase, desirable amino acid compositions were significantly produced from tilapia waste in protein hydrolysis process using fish enzymes and degree of hydrolysis reached to 42% (SILVA et al., 2014).

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) are the main methods of collagen extraction where producing ASC requires the use of an organic acid and PSC requires the use of enzyme (ZHU et al., 2019). Menezes et al. (MENEZES et al., 2020) identified the ideal conditions for the efficient extraction of intact triple-helix collagen ASC from Nile tilapia skin using acetic acid with higher melting and denaturation temperatures was temperature at 20 °C, 0.35 mol/L acetic acid and time of 65 hours. Sun et al. (2017) used the skin of Nile tilapia (Oreochromis niloticus) to extract acid-soluble (ASC) and pepsin-soluble collagen (PSC) to be as raw materials suitable for food and cosmetic preparation (SUN et al., 2017).

2.3. The importance of enzymatic hydrolysis of protein

Enzymatic hydrolysis as mild process has been used to prepare food protein hydrolysates, with less undesirable side effects. Enzymatic treatment increases the extraction yield and improve the content of bioactive compounds. These bioactive peptides obtained from food proteins can be used in food applications as potential natural antioxidants (WANG et al., 2021). Protein

hydrolysates with antioxidant activities can inactivate scavenging free radicals acting as hydrogen donors or electron donors, decreasing reactive oxygen species and hydroperoxides (PHONGTHAI et al., 2018). Alcalase is considered as an endopeptidase which work to break peptide bonds from C-terminal amino acids and has the highest efficacy resulting in the highest antioxidant activity, whereas flavourzyme is considered as an endo- and exopeptidase that breaks the N-terminal of peptide chains (AMBIGAIPALAN et al., 2015). Hydrolysis by alcalase results in the production of peptides with a bitter taste as a result of the hydrophobic residues being present at the end of the protein hydrolysates (LIU et al., 2022).

2.4. Functional and bioactive properties of fish by-product protein hydrolysates

Recently, fish protein content has been interested to produce biologically active peptides which are inactive within the sequences of the parent proteins. However, these peptides tend to exhibit various bioactivities such as inhibition of the angiotensin-I-converting enzyme, cytomodulatory, immune-modulatory and antioxidant when produced by enzymatic hydrolysis (HALIM et al., 2016). Protein hydrolysates presented antioxidant capacities identified by deferent methods (DPPH, FRAP, ORAC, hypochlorite ion, hydroxyl radical, peroxynitrite, and hydroxyl radical) (MATSUI et al., 2018), Wheat germ proteins hydrolysed with Proleather FG-F, a protease from Bacillus subtilis, scavenged 81% DPPH (1.6 mg/mL) and 75% O2- (0.6 mg/mL) radicals (CHENG et al., 2006), functional roles were presented in cultured cells and in vitro by some antioxidant peptides (HIMAYA et al., 2012), for instance, AREGETVVPG peptide which isolated from whole wheat products was demonstrated protective role against high glucose-induced oxidative stress in vascular smooth muscle cells (CHEN et al., 2017), protein of egg white hydrolysed by trypsin also showed an increasing effect in plasma radical scavenging in spontaneous hypertensive rats (MANSO et al., 2008)

In addition, the hydrolysis process of protein molecules can improve their functional properties such as solubility, binding properties, surfactant and foaming properties, etc (QUEIRÓS et al., 2018). Currently, it is so necessary and required to innovate a new advanced hydrolysis technology able to convert fish waste into functional peptides, especially in presence of many limitations of hydrolysis process such as extended reaction time and nonselective hydrolysis.

Food wastes represent a very interesting strategy to convert protein into bioactive peptides because of low cost and need to reduce industrial residues. In this regard, fish waste with their content of high quality proteins are considered as an important source of these peptides (PEREZ-GALVEZ et al., 2016).Bioactive properties of peptides might vary during different steps of processing (VAN LANCKER et al., 2011). For instance, enzymatic glycosylation of proteins result in Maillard compounds which tend to improve bioactivity of protein hydrolysates and oxidative stability (HONG et al., 2015; Zhu et al., 2019). Beyond thermal treatments, a new commercial sterilization techniques, such as high pressure processing (HPP) or ultrasound treatment, have been developed to be less aggressive concerning sensory and nutritional loss (UTRILLA-COELLO et al., 2013). HPP and Ultrasound sterilization techniques were reported to different effect on functional properties of protein and peptides where HPP can cause lower dimensions, a different structure (AMBROSI et al., 2016), denaturation and potential aggregation or precipitation. (GALAZKA et al., 2000). It was also found that ultrasound reduces hydrodynamic volume of the proteins and presents better physical-chemical and emulsifying properties (JIANG et al., 2014).

2.5. The biological importance of bioactive peptides

Research has presented that bioactive peptides show a wide range of potentially beneficial biological properties, which has an important application as therapeutic agents. In the United States, more than 59 peptides showing therapeutic activities have been approved for human consumption since 2018. since the 1920s, insulin is one of the most significant peptide-based medications

licensed and commercialized (LAU AND DUNN, 2018). The World Health Organization (WHO) has declared that "non-communicable illnesses including hypertension, diabetes, and cancer cause 36 million fatalities annually" (ORGANIZATION, 2014).

Studies have exhibited that bioactive peptides may be able to decrease the risk of a range of these chronic illnesses, including diabetes (MIRZAPOUR-KOUHDASHT et al., 2021), hypertension, and cancer (MIRZAPOUR-KOUHDASHT et al., 2020; TAGHIZADEH ET AL., 2020). Consequently, the development of supplements, functional foods or drugs containing bioactive peptides may be able to enhance the health of the general population and strategies for the production of these compounds from different protein sources, such as macroalgae, have been proposed. However, any bioactive peptides formulation intended for oral administration should be appealing to consumers that mean it should not have an undesirable mouth feel or flavor profile. The bitter taste of many bioactive peptides is one of the largest hurdles to their widespread use in supplements, functional foods, and drugs intended for oral ingestion (MAEHASHI AND HUANG, 2009). Many animals, including humans, perceive peptides as having an unpleasant taste due to millions of years of evolution, since peptides are often associated with harmful substances (MEYERHOF, 2005).

2.6. The separation and purification process for protein hydrolysates using macroporous resins

Macroporous resins are widely used as adsorption and ion-exchange materials characterized by mechanism of nonspecific physical adsorption and ion exchanges respectively (HASHIMOTO, 1991). MARs are frequently applied in the separation and purification of natural products such as polyphenols (JIN et al., 2015), antioxidants (ZOU ET AL., 2017), alkaloids (LIU et al., 2014), flavonoids (MA et al., 2013), and poly amino acids (ZHEN et al., 2015), in addition to their commonly used in separation of amino acids (RUIZ et al., 2007), removal of heavy metal ions (SHI et al., 2009) in organic solvents and decolouring (XIONG et al., 2019). Zhuang et al (2016) effectively separated the peptides of soy sauce using resins XAD-16 resin due to its high adsorption and total desorption capacities and the particle diffusion kinetics model was suitable for describing the whole exothermic (DH < 43 kJ/mol) adsorption process on the SP-825 and HP-20 resins (ZHUANG et al., 2016) . Zhang et al. (2018) successfully separated L-valine (L-val) from a aqueous solution using ion exchange resins where adsorption capacity was increased in proportion with solution concentration and the temperature but decreased with increasing the diameter of resin (ZHANG et al. 2018).

Ion exchange chromatography IEX is considered one of the most common separation and purification techniques for proteins. in the presence of larger proteins IEX is specific useful for small biomolecules isolation based on ionic interactions between charged residues in the proteins and immobilized charged ligands of opposite charge (GONZÁLEZ-ORTEGA et al., 2012). Due to the nature of the interaction, the adsorption process by ion exchange is reversible and easily controlled to separate proteins once they are retained by the adsorbent.

This simplicity in operation makes ion exchange chromatography the most common separation and purification technique for proteins (BONNERJEA et al., 1986).

Briskot et al. (2020) explicated the effect of pH and ionic strength on protein adsorption using the linear Poisson-Boltzmann equation and a colloidal representation of the protein which described the non-stoichiometric electrostatic interactions in IEX chromatography (BRISKOT et al., 2020).

2.7. Conclusion

The enzymatic hydrolysis process is considered as an appropriate and useful method because of characteristics for improving the functional properties of proteins and maintaining their nutritional value. The efficient of this process is affected by several factors including enzyme type, substrate, and hydrolysis conditions such as enzyme concentration, temperature, pH, and time. Enzyme activity is extremely dependent on these different factors which make hydrolysis process is highly controllable. Proteins obtained by enzymatic hydrolysis with control of reaction conditions can be modified to enhance their quality and functional properties such as solubility, oil holding capacity and water holding capacity, emulsification, foaming properties, and sensory properties.

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CAPÍTULO II: Characteristics of enzymatic hydrolysis of protein from different food sources and potential separation techniques

Artigo de revisão foi publicado na revista Journal of Current Nutrition and Food Science, citescore 1.7

Characteristics of Enzymatic Hydrolysis of Protein from Different Food Sources and Potential Separation Techniques

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Abstract

Enzymatic hydrolysis (EH) of proteins relies essentially upon enhancing the functional and nutritional properties of proteins, such as antioxidant activity, solubility, oil holding capacity, water holding capacity, emulsification, foaming properties, and sensory properties. There is a big challenge for protein separation and purification due to the high production cost, the large number of amino acids, and the complex biological system of proteins. These biological structures are always presented in a multi-component mixture in native environments, which are usually similar to other molecular weights of other components, such as protein-lipid complexes. These difficulties present the importance of a combination of the hydrolysis process of protein with adequate technology for separation and purification to achieve better bioactive peptide recovery. There are many studies conducted dealing with the characteristics of the hydrolysis process of proteins, and other implications, such as the separation of protein and bioactive peptides. Therefore, this study aims to review the important and recent research papers that investigated the effect of various conditions of the enzymatic hydrolysis process (EHP) (type of enzyme, enzyme to substrate ratio, temperature, pH, and time) on the antioxidant capacity of protein hydrolysates and degree of hydrolysis, as well as to assess the recent studies about protein purification and potential separation techniques.

Keywords: bioactive peptide, hydrolysates, collagen, purification, resin, filtration.

PRACTICAL APPLICATIONS

Recently, bioactive peptides have been explored due to their significant biological and antioxidant properties; protein hydrolysates can be a natural substitute for the former to prevent lipid oxidation, particularly in food products and emulsions, which would minimize the potential risks of synthetic ones. The protein compounds also represent a source of energy and are considered essential nutrients. Proteins also have the characteristic of fulfilling an

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important role in the quality and sensory properties of food. Therefore, it is so important to present the results of recent studies about the effect of various hydrolysis conditions on the degree of hydrolysis and functional properties of a protein hydrolysate, as well as to exhibit protein extraction and separation techniques. The final product of purified protein hydrolysate and bioactive peptides can be used as raw materials for further applications in the cosmetic, pharmaceutical, and food areas.

1- Introduction

The 21st century has brought functional compounds to the top trend regarding compounds that help the human body in many positive effects. In light of this, protein hydrolysates are considered one of these compounds [1]. Compared to synthetic antioxidants (BHA, BHT, and propyl gallates), protein hydrolysates can be a natural substitute for the former to prevent lipid oxidation, particularly in food products and emulsions, which would minimize the potential risks of synthetic ones [2]. Protein compounds also represent a source of energy and are considered essential nutrients. Proteins also have the characteristic of fulfilling an important role in the quality and sensory properties of food. There are many studies in the literature that have used several methods for the recovery of proteins from animal and plant sources [3, 4].

EH of proteins is known for enhancing the functional and nutritional properties of proteins by three distinct modifications of protein molecule: the decrease in molecular weight of protein, an increase in the number of ionizable groups, and exposure of hydrophobic groups [5]. Several studies have reported the radical-scavenging activities in different products of protein hydrolysates. [6] showed that the antioxidant activity of the quinoa protein hydrolysates (QPH) produced by papain and a microbial papain-like enzyme was approximately twice as higher as the sample control of quinoa protein isolate.

With the antioxidant activities of protein hydrolysates, there are many studies performed to look into the effect of various conditions of EHP (type of enzyme, temperature, pH, etc.) on the antioxidant capacity of protein hydrolysates [7, 8]. The effect of EH on the antioxidant activities of concentrated protein hydrolysates from black beans was investigated. EVANGELHO et al. (2016) concluded that a higher antioxidant capacity measured by ABTS was obtained using alcalase enzyme [9]. Similarly, a higher level of radical DPPH scavenging was observed in hydrolysates obtained using the pepsin enzyme. Mao X-Y et al. (2011) evaluated the anti-inflammatory properties and capacity to inhibit free radicals (hydrogen peroxide, DPPH, and superoxide) in yak milk casein before and after subjecting it to

enzymatic hydrolysis [10]. Compared with other treatments, the casein hydrolyzed by alcalase showed the highest radical DPPH scavenging. Zhao XH et al. (2010) assessed the radical scavenging activity RSA of papain-catalyzed casein plasteins, the results indicated an increase in RSA for all cases in plasteins [11].

To extract proteins, biochemical, chemical, physical, and combined methods have been developed. Examples of physical methods include centrifugal, thermal, ultrasonic, and membrane filtration [12, 13]. The chemical methods can be listed: alkaline-thermal (AKT), pH adjustment, acid-thermal (AT), ethylenediaminetetraacetic acid (EDTA), and aldehydic reagent [14, 15]. On the other hand, there are biochemical methods including enzymatic and assisted enzymatic treatment [16], which are commonly used for producing peptides in terms of safety, production rate, and high efficiency.

The objective of this review is to present recent and important studies about the different characteristics of protein hydrolysates (PtH) including the antioxidant capacity of protein hydrolysates and the effect of various hydrolysis conditions on the degree of hydrolysis and functional properties of a protein hydrolysate, as well as to exhibit protein extraction and separation techniques.

2. Effects of the parameters of EHP on the degree of hydrolysis

The hydrolysis process and degree of hydrolysis of a protein depend on different conditions of pH, temperature, ratio of solid to liquid, enzyme-substrate ratio, and time as shown in Fig. 1 [8, 17-19]. The elevation of temperature and hydrolysis time can cause an increase in the cleavage sites which leads to produce more free amino acids and peptides of small molecular weight and increase the rate of the hydrolysis reaction, which consequently, gets a higher degree of hydrolysis, however, high temperatures more than critical level can cause the thermal deactivation of the enzyme [12, 20, 21].

In addition to temperature and time, pH also significantly affects the EHP and, as a result, the final degree of hydrolysis of protein [22]. The effect of pH on enzyme activity varies depending on the type of enzyme, for instance, free trypsin presented the highest activity in the pH range (7.8–8.1) [23]. In this context, the secondary and tertiary structures of the protein substrate, as well as the enzyme, can be changed because of variations in pH which led to holding up hydrogen bonding and affect salt bridges [23, 24]. Butré et al, (2015) Studied the hydrolysis of whey protein isolate by Bacillus licheniformis and proved the influence of pH on enzyme selectivity, different levels of pH 7.0, 8.0, and 9.0 caused

differences in enzyme selectivity for different cleavage sites of β-Lg [25]. EHP of protein generates peptides that possess different kinetics of release depending on conditions of reaction, this can also be mostly explained because of the changes in the ratio enzyme/substrate which led to different accessibility of the active sites of the enzyme to the substrate [26, 27]. Therefore, many studies suggested increasing the efficiency of agitation to achieve a bigger contact area between enzymes and substrates of protein compounds, which consequently, inhibits the non-specific adsorption and avoids the decrease in the degree of hydrolysis [28, 29].

The maximum hydrolysis degree of protein obtained from the muscles of Chinese sturgeon (Acipenser sinensis) was one at solid-to-liquid 1:1; under the following conditions: enzyme-substrate ratio of 3% (w/w); pH 6; temperature 70 °C and time 6 h [17]. The utilization of PtH in many applications in the food industry, such as emulsions, gels, and foams is dependent on the solubility that increases after the hydrolysis process [30, 31]. Parameters of EHP can also improve this property, for instance, the range of pH 2-10 was proved to increase the solubility of PtH obtained from pink perch (Nemipterus japonicus) muscle [32] and Chinese sturgeon (Acipenser sinensis) by using papain enzyme [17]. Ramadhan AH, et al. (2018) obtained a 24.89% of yield for the degree of hydrolysis of protein from the muscles of Chinese sturgeon (Acipenser sinensis) by using papain at the optimum conditions of solid-to-liquid (1:1), Enzyme-substrate ratio (3%) (w/w), pH (6), temperature (70 °C) and time (6 h) [17]. These conditions resulted in fish protein hydrolysates FPH with appropriate contents of protein and amino acids, high solubility, and good properties of emulsification. In addition, the generated FPH provided good oil holding capacity (OHC) and water holding capacity (WHC),

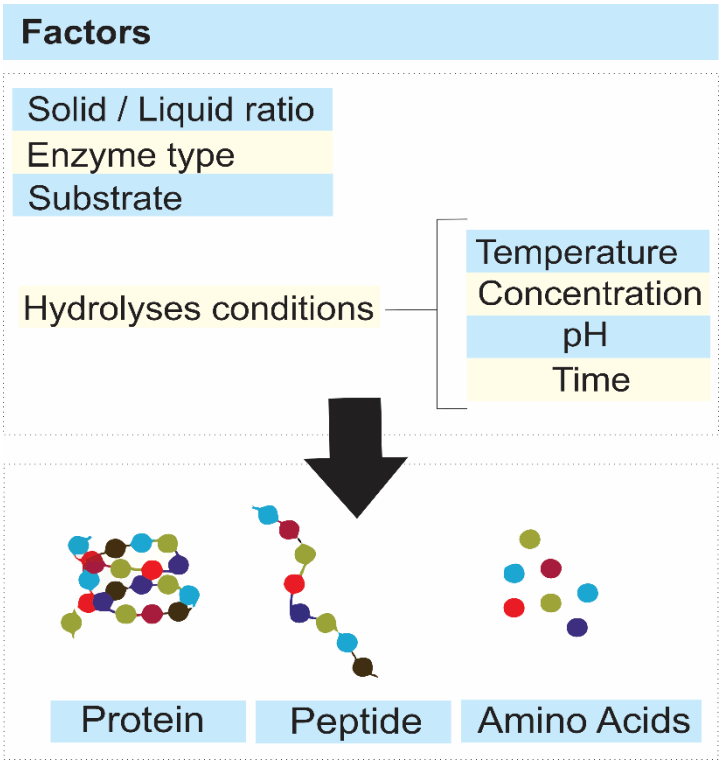

Fig 1. Schematic diagram of enzymatic hydrolysis of protein under specific parameters.

3. Effects of the EHP on the antioxidant properties of protein hydrolysates

The bioactive peptide can be produced effectively by EHP from different food sources Fig. 2 [33, 34]. The controlled parameters and the conditions of hydrolysis are essential to obtain polypeptides with specific properties and high antioxidant activities.

The factors of protease enzyme type, enzyme to substrate ratio, substrate concentration, incubation duration, and pH influence the final degree of biological activity of protein hydrolysates [35]. To acquire a hydrolysate, which could be utilized as a food additive, it has to be produced using commercially available enzyme or enzyme preparation, and the price has to be acceptable for the manufacturer [36]. Temperature, pH, and duration have been identified as critical parameters of the hydrolysis process, and according to different combinations of those parameters, the obtained product may have various biological activities [37]. According to the FRAP test, the antioxidant activity of peptides and amino acids produced by enzymatic hydrolysis can be explained by their ability to donate an electron to free radicals and terminate the oxidation chain reaction [7, 38]. DPPH radical scavenging activity also presented more confirmation of antioxidant activity of peptides and amino acids explained by their ability to pair the single electron of DPPH [7, 39].

The effect of optimized conditions of pH, temperature, and incubation duration on antioxidant properties of the hydrolysates obtained from blue shark skin gelatin (BSSG) was observed [40], ferric reducing antioxidant power (FRAP) values were at lower levels and varied in the range of 0.12–0.62 μM of Trolox/mg of protein which met the concentration of acquired hydrolysates of 50 mg/mL.

Based on the ferric ion reducing power, bioactive peptides obtained from hydrolysis of different protein sources presented antioxidant capacity. For instance, bioactive peptides obtained from PtH of brown stripe red snapper skin using pyloric caeca (part of the fish's intestine) extract presented lower ferric ion reducing power (1.9–2.4 μM Trolox/mg) [41], the higher the reducing power, the better abilities to donate electron or hydrogen which represent a significant indicator for compounds that can be used as antioxidant [38].

Tkaczewska J et al. (2020) studied the best conditions, such as pH, temperature, and type of enzyme to produce hydrolysates of carpio fish skin gelatin with the highest antioxidant properties, the study confirmed that the Cyprinus carpio skin gelatin hydrolysate obtained at pH 7 and temperature of 50 °C using Protamex enzyme preparation showed high antioxidant properties [7]. During the hydrolysis process, protein is broken down into bioactive peptides, where the types of amino acids affect the antioxidant capacity of the hydrolysates [42]. Thus whey peptides acquire antioxidant properties derived from their content of amino acids, such as cysteine, methionine, lysine, histidine, tyrosine, and tryptophan [43, 44].

Various conditions of the hydrolysis process can affect the antioxidant capacity of protein hydrolysates, at a high level of pH the ability of the donor groups in peptides is generally improved [7, 45]. However, pH also can change the conformation of peptides which consequently causes an increase or decrease in the activity of peptides. Antioxidant capacity can also be enhanced by increasing the enzyme hydrolysis time because of the release of more peptides and amino acids with antioxidant activity (such as methionine, lysine, and tyrosine) [8, 46].

Recently, the hydrolysis process was used to produce bioactive peptides from plant sources, these bioactive peptides presented characteristics as being pharmacologically and biologically active compounds [33, 43], Shahi Z. (2020) also presented that controlled conditions of enzymatic hydrolysis of proteins obtained from defatted Bunium persicum Bioss (black cumin) press cake increase the degree of hydrolysis which consequently enhance the antioxidant activities determined by DPPH radical scavenging activity [8].

Quinoa protein, for instance, can be used to produce either hydrolysates and acid-induced gels characterized by functional and antioxidant properties, at a low degree of hydrolysis, quinoa protein hydrolysates could also be used to prepare semi-solid foods that combine both antioxidant and gel attributes [47]. The antioxidant features of radical scavenging activity DPPH and ABTS inhibitory effect for bioactive peptides derived from defatted Bunium persicum bioss were affected by different parameters of EH including time and the enzyme-substrate ratio [8].

In that sense, bioactive peptides have been demonstrated to be powerful antioxidant compounds with the ability to protect the human body from ROS effects. Furthermore, they can improve body functions through different mechanisms of action, for instance, aromatic amino acids such as phenylalanine present the ability to neutralize the electron-deficient radicals by the mechanism of proton donating which increases the antioxidant capacity of protein hydrolysates [48, 49]. Bioactive peptides can be produced through several different routes, such as enzymatic hydrolysis, chemical hydrolysis, microbial fermentation, and digestive processes. The most extensively studied route was in vitro enzymatic hydrolysis employing pepsin, chymotrypsin, and especially trypsin [50].

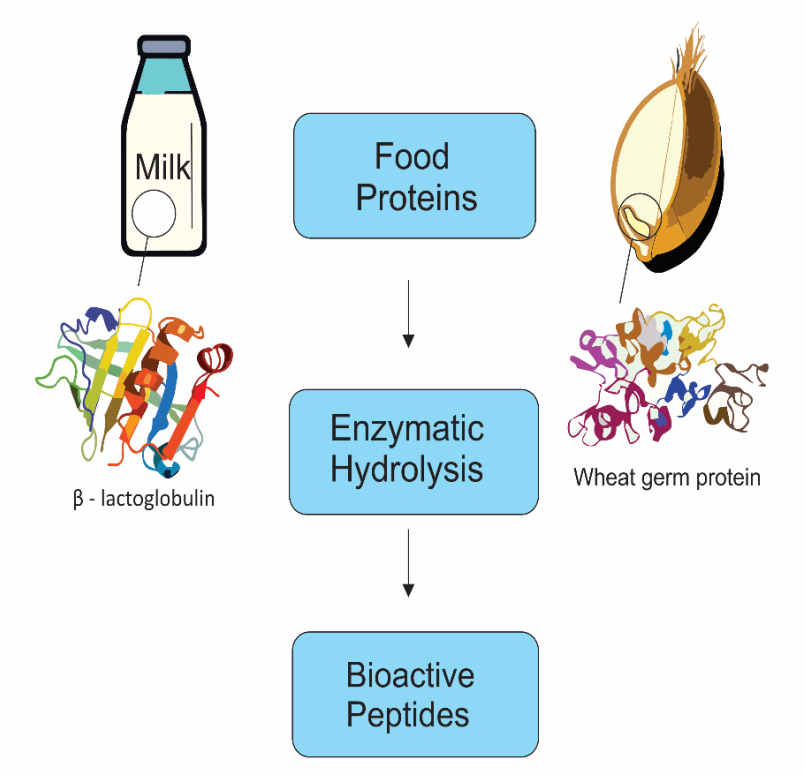

Fig 2 Schematic diagram of bioactive peptides obtained from various food sources.

4. Effects of the parameters of EHP on the functional properties of protein hydrolysates

The use of enzymatic hydrolysis is often considered an appropriate and useful method for improving the functional properties of proteins and maintaining their nutritional value Fig. 3 [47, 51, 52]. The process depends on several factors including enzyme type, substrate, and hydrolysis conditions, such as enzyme concentration, temperature, pH, and time. These factors work cooperatively to make the enzyme activity and hydrolysis process more manageable [8, 17, 53, 54]. The controlled conditions of hydrolysis can modify and enhance the quality and functional properties of the protein, such as solubility, oil and water holding capacity, emulsification, foaming properties, and sensory properties, which are shown in table 1 [55, 56]. The functional properties of fish protein hydrolysates were significantly subjected to the substrate and enzyme used and to the degree of hydrolysis [57-59].

The method of hydrolysis process can also affect the bioactive properties of hydrolyzed products in addition to the other conditions used during hydrolysis [60]. Pressure-assisted enzymatic hydrolysis method presented a significant effect in improving solubility, which has to do with the increase in the trichloroacetic acid-solubility index, also the type and nature of protein molecules together with the method used in hydrolysis can affect its solubility [61, 62] . However, high pressure (> 400 MPa) caused a reduction in the solubility of proteins. Pressure-assisted protein unfolding has been shown to reduce hydrolysis time [61], and enhance proteolysis via increased exposure of susceptible peptide bonds to enzymatic cleavage [63]. High pressure stabilizes and increases the activity of some enzymes during the hydrolysis of proteins [64]. Pressure treatment also increases protein digestibility [65], facilitates the enzymatic release of antioxidant peptides [63], and enhances the formation of antioxidant peptides in the hydrolysates [66]. Moreover, some antimicrobial peptides are only active under high pressure [67]. These results suggest the feasibility of producing unique, bioactive peptides via pressure-assisted enzymatic proteolysis [68].

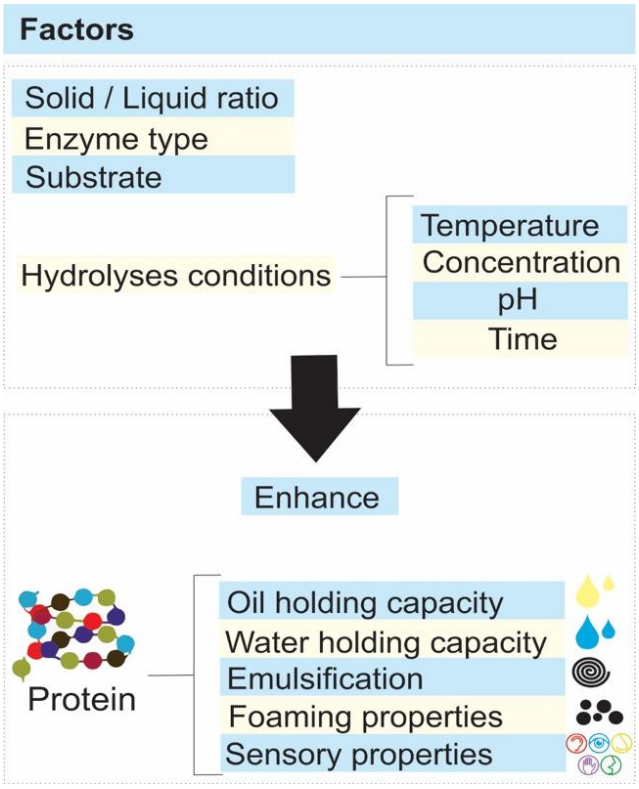

Fig 3. Schematic diagram of functional properties of protein hydrolysates under specific parameters.

Table 1 Effect of the conditions of hydrolysis on the functional properties of protein hydrolysates. (The author Siraj Salman Mohammad prepared the table 1 to present more clarification of the text).

Table 1 Effect of the conditions of hydrolysis on the functional properties of protein hydrolysates.

Enzyme	Protein	Investigated parameters	Functional properties	References
pancreatic	Quinoa protein	Time and pH	Improve the solubility, emulsifying, and foaming properties	[69]
Trypsin	Rice bran protein	pH and temperature (pH=8, 37 °C), (enzyme/substrate=1/100)	Increase its flexibility and fluidity, change its surface hydrophobicity, and increase its solubility	[56]
Pancreatin powder	Soy protein isolates	Extrusion pre-treatment, 20% v/v oil, 1.6% w/v emulsifier, and pH 7.0	Increase the protein solubility, improve the emulsifying capability	[70]
Bromelain	Soy protein	Enzyme-to-substrate ratios were selected to reach 2% or 4% DH values, 50 ºC, and pH 7.0	Modify and enhance the rheological properties	[71]
Alcalase	Hypoallergenic soybean protein	Different degrees of hydrolysis (2.5–10.0%)	Improve the flavor and techno-functional properties of hypoallergenic soybean protein hydrolysates (SPHs)	[72]

Pepsin, trypsin, Alcalase	Faba bean protein	pH and temperature	Increasing solubility and rheological properties such as foaming capacity and stability	[73]
Trypsin	Soy protein	pH, degrees of hydrolysis	Improve the solubility, and emulsifying properties of soy protein hydrolysates	[74]
Papain	The protein of muscles of Chinese sturgeon	The solid-to-liquid mixing ratio of 1:1, enzyme–substrate ratio of 3%, pH 6, the temperature of 70 °C, and incubation time of 6 h.	Improve the solubility, emulsifying activity, emulsion stability, water holding capacity, oil holding capacity, and foam capacity	[17]
Alcalase and papain enzymes	Fish egg protein (*Cirrhinus mrigala*)	pH, temperature, and time	Increase the protein solubility, fat absorption capacity, foam, and emulsifying capacity	[51]
Alcalase	Protein from tilapia fish by-products	Pressure-assisted enzymatic hydrolysis (38–462 MPa) and hydrolysis time (6–35 min)	Increase trichloroacetic acid-solubility index, improve soluble protein content and antioxidant activity	[60]

5. Protein recovery and potential separation techniques

5.1. Centrifugal method

Proteins and bioactive peptides can be separated effectively after enzymatic hydrolysis using centrifugal methods [75-77]. These techniques separated oil and purified proteins from soybean after enzymatic hydrolysis and provided good separation with a protein yield of 58.7 %. Rovaris ÂA et al. (2012) investigated the possibility of using soybean waste as a source of protein after oil extraction using enzymatic hydrolysis (r Alcalase/Viscozyme L) followed by a successful centrifugal process [78]. The centrifugal method is a promising green and safe alternative to separate protein from synthetic and complex biological systems. Qian J et al. (2019) also isolated protein from soybean after 90% of oil separation using a hydrolysis process followed by a centrifugal force of 1960×g [77].

Proteins from Thai brown rice (Khao Dawk Mali 105) were hydrolyzed using bromelain and the hydrolysate was separated by centrifugation at 5,000 × g for 15 min, the precipitated proteins (albumin, globulin, glutelin, and prolamin) were also separated using the centrifugal method at 5,000 × g for 20 min at 4 °C [79]. The protein of silkworm pupae was hydrolyzed at 170 ºC for 1 h using superheated water, at the end of the hydrolysis process the protein hydrolysate was extracted and separated from the precipitated solid material using the centrifugal method, the process was repeated three times, then the separated protein was successfully used in silk fabric for color application [80].

The protein of tilapia fish obtained from waste was hydrolyzed using pepsin enzyme, after the hydrolysis process the protein hydrolysate was separated from fine solids and oil by centrifugation at 10,000 rpm for 15 min. The purified protein presented promising results to be used for dietary supplementation [75]. Casein was fermented using Lactobacillus helveticus to produce extracellular and intracellular peptides, such as bioactive compounds. The obtained peptides were isolated and purified using two steps of the centrifugation process; normal centrifugation at 4500 rpm/min and ultrafiltration centrifugation using centrifugal filter units. The process played a significant role in removing the macromolecule casein from fermented casein [81].

5.2. Membrane filtration method

The method of continuous coupling of enzymatic hydrolysis and membrane separation (CEH-MS) was used to obtain bioactive peptides from wheat germ protein (DWGP) [82] and Porphyra yezoensis protein. The results showed compatibility between steady state theory and the ongoing CEH-MS kinetics study where the kinetic model was successfully established with Km of 8.163 g/L and Vmax of 0.790 g/L min. In this hydrolysis process, the substrate concentration of protein significantly affected the conversion rate and degree of hydrolysis [82-84]. The steady state of protein conversion rate was reached at 90 min after it was gradually increased by raising the time according to the kinetic model prediction. Similarly, it was observed that by increasing the substrate concentration the conversion rate of protein decreased ($p < 0.05$) [82, 85]. It was also proven that ultrafiltration enzymatic membrane reactors can increase the yield of hydrolysates because it reduces the inhibition caused by product accumulation [82, 86]. The continuous process facilitated enzyme-catalyzed degradation reaction together with a feature by constantly removing inhibitors and avoiding potential restriction of mass transfer [87].

The membrane filtration presented a high capability to separate Lactoferrin and lactoperoxidase from Sweet whey, at least those with molecular weights of ~80 and ~66 kDa [88]. Colostrum whey was also separated by two steps of ultrafiltration membrane [88, 89], the first with the membrane of 100 kDa MWCO and the second with the membrane of 10 MWCO.

Electrodialysis with filtration membranes (EDFM) improved the capacity of bioactive peptide separation [90]. After enzymatic hydrolysis of protein, EDFM worked to attract peptide compounds according to their charge and size of molecules [90-92]. Bioactive peptides obtained from enzymatic hydrolysis of flaxseed protein were separated efficiently using high hydrostatic pressure and electrodialysis with ultrafiltration membranes EDFM. These compounds were rich in arginine and presented biological activities that benefit systolic blood pressure and anti-diabetic activity [90].

5.3. Macroporous resins

Macroporous resins are widely used as separation materials, which correspond to the mechanism of nonspecific physical adsorption and ion exchanges respectively [93]. The

chemical and physical characteristics of macroporous resins can affect the capacity of the adsorption and desorption process including chemical matrix, polarity, surface area, pore diameter, and particle size of macroporous resins [94, 95].

Macroporous adsorption resins are frequently applied in the isolation and purification of natural products, such as polyphenols [95], flavonoids [96], antioxidants [97], alkaloids [98] and poly amino acids [99]. Macroporous resins are frequently used in the removal of heavy metal ions in organic solvents [100], separation of amino acids [101], and decoloring agents [102]. PtH from soy sauce was separated using macroporous resin and the particle diffusion kinetics model successfully described the whole exothermic (DH < 43 kJ/mol) adsorption process on the SP-825 and HP-20 resins. In addition, they presented XAD-16 resin as the most effective one for the enrichment of peptides due to its high adsorption and total desorption capacities which belong to its large surface area [103].

Macroporous resin, such as DA201-C has been used to separate grass carp fish scale peptides, DA201-C as a hydrophobic macroporous resin presented significant results for dynamic adsorption and gradient desorption of hydrophobic amino acids which, in turn, showed an ability to prevent angiotensin-I converting enzyme in vitro [104]. 8-amino-1-naphthol-3,6-disulfonic acid (H-acid) was separated using four types of macroporous resin (GMDS resin, NDA-88, the anion-exchange resin D-301, and XAD-4). GMDA resin presented a better capacity to recover H-acid because of its wide pore diameter with higher adsorption capacity, large surface area, and the functional groups on the surface of the GMDA resin. It also showed a significant effect on adsorbing H-acid [105].

XAD 16 resin presented a significant capacity to separate bioactive peptides from wheat gluten hydrolysates because of their characteristics as nonpolar resin and large surface area, which increased the capacity to recover bioactive peptides [103, 106]. PtH was obtained from whey protein by hydrolysis process using pancreatin combined with Protease and can be incorporated into the diet for phenylketonuria patients. It released more than 90% Phenylalanine, and the separation process was done by a magnified D101 resin column which recovered 98.38% of Phe [107]. The macroporous resin of XAD-16 also presented the ability to recover Phe from whey Pancreatin hydrolysate, in addition to XAD-4 which adsorbed around 95% of Phe from whey hydrolysate [108, 109].

Macroporous cation resin D72 presented the best recovery of L-methionine from fermentation broth because of the non-polar interaction between L-methionine and resin [102, 110]. The pseudo-second-order model was more suitable for the adsorption kinetics test of L-methionine on D72 resin and the adsorption isotherm was well described by the Sips model [102].

5.4. Restricted access media and chromatographic separation techniques

Several methods have been explored in the literature to reduce undesired protein interaction at binding sites. The concept of combining polymer restriction (say, size exclusion) with other ways of interaction (hydrophobic interactions and ion exchange reaction for example) is well established Fig. 4 [111, 112]. Restricted access media (RAM) systems are not new; such RAM matrices for analytical applications have been in existence for quite some time. Generally, RAM possum hydrophilic–hydrophobic properties are characterized as material packings for the direct analysis of drugs that can be found as small analytes in protein solutions [113].

In recent years, restricted-access media (RAM) columns have become an important tool for direct high throughput analysis of biological fluid samples [114]. For instance, to reduce adsorption of large proteins while allowing adsorption of small ones as shown in Fig. 4. LIU et al. (2016) reported that surface-initiated activator generated by electron transfer (AGET) atom transfer radical polymerization (ATRP) led to the controlled grafting and achieved high-capacity adsorption of peptides and high-effective separation with new hydrophobic charge-induction chromatography (HCIC) resins [115], which had a potential application for large scale antibody purification. YOSHIDA et al. (1984) developed protein-coated porous silica for drug analysis in plasma [116], it introduced with this approach new ways to isolate smaller proteins, a method now known as restricted access media (RAM) analysis [117, 118]. As RAM serves, the controlled access polymer permeation adsorption (CAPPA) was also developed as an effective method to separate protein hydrolysates with low molecular weight (LMW) of peptides, González-ortega et al. GONZÁLEZ-ORTEGA et al. (2012) reported that CAPPA media consisted of agarose beads modified with amino-PEG-methoxy and with trimethyl ammonium groups, having chloride capacities between 20 and 40 eq/mL, CAPPA presented high ability to restrict and adsorb small proteins and peptides while refusing the high molecular weight ones [119].

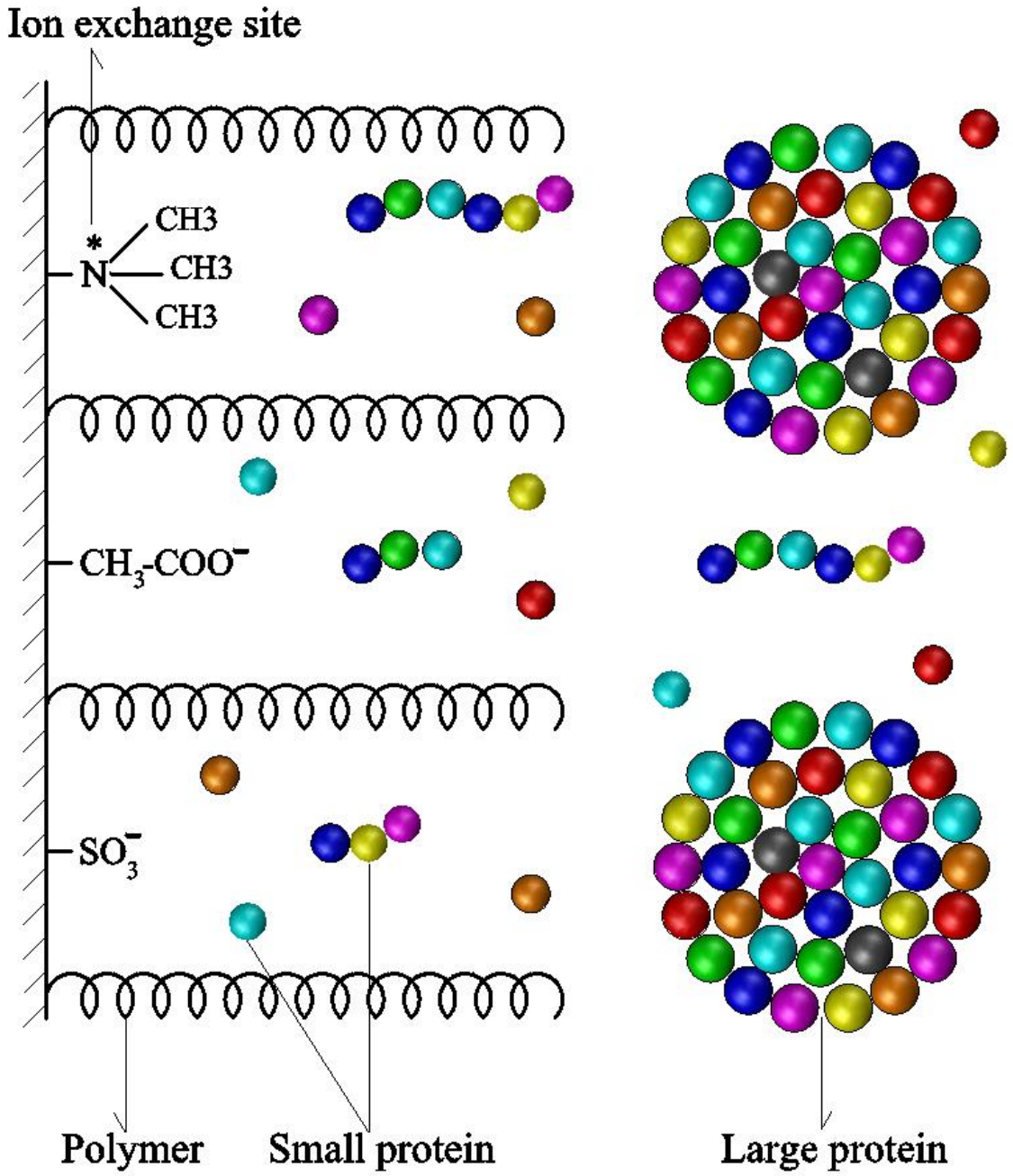

Fig 4. Selective adsorption and size restriction schematic concept of protein compounds using the resin of restricted access media (RAM) with ion exchange sites.

The remarked advances in the pharmaceutical industry presented more interest in the peptide that is basically used to produce peptide-based drug therapies, including cyclic peptides, monoclonal antibodies, bioconjugates, and vaccines [120]. Ion exchange

chromatography, gel chromatography, and reversed-phase high-performance liquid chromatography (RPLC) are commonly used as separation and purification methods of polypeptides [121, 122]. However, despite the advanced innovations in the field of chromatography, this method is still mostly used for analysis purposes for bioactive peptides [123, 124].

Gel chromatography is considered an effective method to separate and purify the polypeptides for several reasons, such as mild separation conditions, economical equipment, high sample recovery, and high experimental repeatability [125]. Shuangqi Tian. (2022) obtained purified wheat germ albumin hydrolysates with a high antioxidant activity using membrane separation and gel chromatography, wheat germ albumin was hydrolyzed by papain [126]. The exceptional robustness, separation efficiency, and selectivity make RPLC a popular peptide separation technique [127]. Furthermore, RPLC is easily compliant with coupling with mass spectrometry and can be a useful technique for separating complex mixtures in the pharmaceutical industry [120]. The unified chromatography (UC) analysis method was compared with RPLC to separate and analysis of 43 peptides, including 10 linear peptides and 33 cyclic ones, the results showed that RPLC method sensitivity was better with RPLC than UC. However, the two methods presented the same operational costs [128]. RPLC is commonly used to control the quality of synthetic and therapeutic peptides with the C18 stationary phase, (Ryan et al, 2020) enhancing the ability of this method and resolving the problem of removing the structurally closely related impurities with similar sequences in preparative RPLC, this was performed by developing generic 2D-LC impurity profiling method [129].

6. Conclusion

The use of EH is often considered an appropriate and useful method for improving the functional properties of proteins and maintaining their nutritional value. The EH process depends on several factors including enzyme type, substrate, enzyme concentration, temperature, pH, and time. These factors together have a direct effect on the final level of the enzyme activity, thus making the hydrolysis process more manageable. EH can be used efficiently to improve proteins' solubility and functional properties. Proteins obtained by EH with control of reaction conditions can enhance their quality and functional properties, such as solubility, oil holding capacity and water holding capacity, emulsification, foaming, and sensory properties. EH can also improve the

capacity of the separation process, which results in more recovery of bioactive peptides, specifically using the centrifugal method, membrane filtration method, and macroporous resins. The final product of purified protein hydrolysate and bioactive peptides can be used as raw materials for further applications in the cosmetic, pharmaceutical, and food areas.

FUNDING

This study was financed in part by the The Coordination of Improvement of Higher Education Personnel – Brazil (CAPES) – finance code 001; National Council for Scientific and Technological Development – Brazil (CNPq) – Finance codes 443310/2016-5 and 443243/2016-6; and the fundação carlos chagas filho de amparo à pesquisa do estado do Rio de Janeiro – Brazil (FAPERJ) – financial code e-26/010.001507/2015.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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CAPÍTULO III: The Production Of Bioactive Peptides By Optimization Of Enzymatic Hydrolysis Process Of Protein From Tilapia Fish Skin Waste (Oreochromis Niloticus, Linnaeus 1758) Using Alcalase 2.4.L

Artigo de experimento foi publicado na revista Journal of Current bioactive compounds, citescore 2.0.

The production of bioactive peptides by optimization of enzymatic hydrolysis process of protein from tilapia fish skin waste (*Oreochromis niloticus*, Linnaeus 1758) using alcalase 2.4.L

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ABSTRACT

Aims: The production of bioactive peptides by optimization of enzymatic hydrolysis process of protein from tilapia fish skin waste (Oreochromis niloticus, Linnaeus 1758) using alcalase 2.4.L

Background: The importance of natural bioactive peptides belongs to low toxicity and their therapeutic properties as antioxidants.

Objective: The optimized conditions of protein hydrolysis obtained from tilapia fish skin waste (oreochromis niloticus, linnaeus 1758) using alcalase 2.4.l

Methods: In this study the hydrolysis of protein obtained from tilapia fish skin waste (TFSW) was optimized using alcalase 2.4.L by central composite design (CCD). Degree of hydrolysis (DH), radical scavenging activities (DPPH) and ferric reducing antioxidant power (FRAP) were used as dependent variables. temperature, pH and proportion enzyme to substrate (PE%) as independent variables

Result: The optimum degree of hydrolysis DH%, DPPH and FRAP were achieved at temperature 58.4 ℃, pH of 8.7 unless DPPH which was at pH of 7.0.

Conclusion: The present work showed that TFSW can be used as source to produce bioactive peptides with significant antioxidant activities under specific condition of enzymatic hydrolysis.

Key words: Enzymes, Bioactive peptides, Antioxidants, Amino acids, Collagen. Hydrolysates.

1. Introduction

Fish protein hydrolysates obtained by enzymatic hydrolysis are considered as one of the most important bioactive peptides for human health because of their properties as antioxidant against free radicals, antihypertensive pharmacological agents [1, 2], anti- inflammatory, anti-proliferative [3, 4] immuno-modulatory physiological reactions [5]. Bioactive peptides that do not have toxicity effects tend to be used more in the food industries as natural antioxidant

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instead of synthetic ones [6]. Bioactive peptides presented also significant effect to improve the technological properties of foods across emulsifying and foaming characteristics [7], solubility[8], water holding capacity [9] and oil binding capacity [10].

In this context, large amounts of fisheries and aquaculture products reach about 75 megatons are discarded and lost every year [11]. Depending on the kind of food processing, fishing industry produces between 30% to 70% of wastes which includes viscera, skin, leftover meat, heads, blood, skin and bone [12]. Fish waste generation and discarding can cause a critical problem and deteriorate the environmental system. However, fish waste is considered as source rich in protein [13] which can be processed to use as high quality protein source for animal feeding using silage technology [14] and as source of bioactive peptides obtained by hydrolysis process for food and pharmaceutical applications [6]. Consequently, fish waste processing can be used as effective way to reduce the risks of environment pollution in addition to obtain value-added products which are cheaper and higher nutritional value

Different methods were used to process fish wastes such as chemical hydrolysis and extraction by organic solvent; however these traditional methods presented a lot of drawbacks like low nutritional quality and low-term conservation [15]. Enzymatic hydrolysis is considered as distinct method to produce bioactive peptides from fish wastes with a lot of benefits for human health including cardio protective effect and immuno-modulatory physiological reactions [5, 16]. Enzymatic hydrolysis can improve the functional and biological properties of bioactive peptides, protein hydrolysates obtained from plant sources using different enzymes like alcalase, trypsin or papain presented enhancement in the solidity [17-19]. Enzymatic hydrolysis has the ability to modify the origin protein, for instance, decreasing the allergenicity of whey proteins by the cleavage of antigenic regions and the reduction of protein across breaking the corresponding peptide [20].

Bioactive peptides obtained by enzymatic hydrolysis process can be then separated and isolated using different method such as macroporous resins MARs and Membrane filtration method [21, 22]. The enzymatic hydrolysis process of protein will decrease the molecular weights of peptides to reach ~80 and ~66 kDa and result in different ionic charges, consequently, the Electrodialysis with filtration membranes (EDFM) presented a high capability to separate and attract peptide compounds according to their charge and size of molecules [23], EDFM efficiently separated the hydrolyzed flaxseed protein which is rich in anti-diabetic compounds like arginine [24]. MARs also used to separate and purify hydrolyzed protein, enzymatic hydrolysis process can produce different hydrophobic and hydrophilic peptides which correspond to be adsorbed in hydrophobic and hydrophilic MARs. For instance, hydrophobic amino acids of grass carp fish scale peptides were successfully separated by hydrophobic MARs such as DA201-C [25]. Among these hydrolysates, the bioactive peptides obtained from fish skin (BPFS) have increasing importance for the food and pharmaceutical areas, BPFS presented antioxidant activity to protect skin cells toward ROS, which can be attributed to aromatic amino acids and hydrophobic peptides rich in NH2 group [26, 27]. BPFS also showed anti-inflammatory activity because of hydrophobic and positively charged amino acids, in addition to presence the branched-chain amino acids (valine, isoleucine and leucine) which suppress nitric oxide (NO) production in lipopolysaccharide-treated cells [28].

Recently, various studies looked into the optimization of enzymatic hydrolysis of protein and studied the effect of different parameters on the hydrolysis process including temperature, pH, time, type of enzyme, type of substrate and ratio enzyme to substrate [29, 30], Type of enzyme play an important role in the sequence of peptides and amino acids which are generated during hydrolysis process [29, 31], Sierra-Lopera & Zapata-Montoya. (2021) reported that bioactive peptides were obtained under optimized condition of pH 8.1,

temperature 58.5 ºC, substrate 45 g/L and enzyme 4.42 g/L using Alcalase 2.4 L and presented high level of antioxidant activity [29]. Plasma of bovine was also hydrolysed and the optimum conditions were studied using response surface methodology with dependent variables of degree of hydrolysis and antioxidant capacity [32]. de Castro & Sato. (2015) studied the effect of different condition of hydrolysis of egg white proteins on their antioxidant activity and presented that maximum antioxidant capacity was at degree of hydrolysis 50% [33].

However, none studied the optimized conditions of protein hydrolysis obtained from tilapia fish skin waste (oreochromis niloticus, linnaeus 1758) using alcalase 2.4.l. Therefore the aim of this paper was to optimize the parameters of hydrolysis process including (pH, temperature and proportion of enzyme to substrate) using central composite design (CCD), in addition to determine DPPH, FRAP and degree of hydrolysis DH% as response variables.

2. MATERIALS AND METHODS

2.1. Chemicals

Sulfuric Acid 98% for the determination of nitrogen, Hydrogen Peroxide 30% w/v (100 vol.) for analysis, Sodium Hydroxide, Boric Acid or Hydrochloric Acid or Sulfuric Acid, Methyl Red, Bromocresol Green, Methylene Blue, methanol P.A, sodium deoxycholate, Copper sulfate pentahydrate, potassium tartrate, bovine serum albumin (BSA), Protease from Bacillus licheniformis (ALCALASE 2.4.L), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and other antioxidant reagents will be purchased from Sigma (St. Louis, MO, USA).

2.2. Materials

Tilapia fish skin (Oreochromis niloticus, Linnaeus 1758) was obtained from (Peixesul Cooperativa dos Aquicultores e Pescadores do Sul Fluminense), the raw materials were

cooled to 4 ºC and transported to laboratory. The skin mass was treated at 90 °C for 20 min to inactivate the endogenous enzymes [34] followed by freeze-dried in a Labconco freeze drying system (Seropédica City, RJ, BR). The powder product was stored in the freezer at -20 ºC.

2.3. Methods

2.3.1. Biochemical analysis

Official methods from Association of the Official Analytical Chemists [35] were used to determine the proximate composition of the TFSW in fresh and dry form, gravimetrical way was used to measure both the moisture and mineral content at 105 ºC and 550 ºC, respectively. The Kjeldahl method was used to determine the nitrogen content followed by measured the crude protein by employing the conversion factor of 6.25 g of protein per gram of nitrogen, **annex A** [36]. Soxhlet semi-continuous method [37] was used to extract and determine lipid content.

2.3.2. Determination of DPPH radical-scavenging activity

The scavenging activity of samples against the DPPH radical was determined using a previously described method [38]. 0.06 mM DPPH solution was prepared by dissolving 0.0024 g of Dpph in methyl alcohol and making up to 100 ml. the solution absorbance should be between 680-700 nm. trolox was prepared by dissolving 0.02g in 50% ethanol and making up to 10 ml and then sonicating in an ultrasonic bath for approx.5 min. 2.85 ml of a DPPH radical solution was added to 150 ml of sample solution, then homogenized and kept to rest under absence the light at room temperature for 60 minutes and read at 517 nm. Results were calculated using a standard Trolox curve and expressed in mg equivalent of TE/g sample. more details about the method are presented in **annex A.**

2.3.3. Determination of ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power of samples was measured according to a previously described method [39]. The FRAP reagent was obtained from the combination of 25 ml of 0.3M acetate buffer, 2.5 ml of a 10 mM TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) solution and 2.5 ml of a 20 mM aqueous ferric chloride solution, FRAP solution has to be used immediately after preparation. In a dark environment, dilute an aliquot of 90 µl of sample solution in 270 µl of distilled water and add 2.7 ml of the previously prepared FRAP reagent, homogenize in a sealed tube and place in a water bath at 37°C for 30 minutes, cool and read at 595 nm, the FRAP reagent was used as a blank to calibrate the spectrophotometer. Results were calculated using a standard Trolox curve and expressed in mg equivalent of TE/g sample. more details about the method are presented in **annex A**

2.3.4. Enzyme and hydrolysis procedure

Alcalase 2.4.L (subtilisin, EC 3.4.21.62) is a protease extracted from Bacillus licheniformis and purchased from Sigma (St. Louis, MO, USA) to produce the protein hydrolysates,. Hydrolysis experiments were carried out in a jacketed stirred tank reactor of 250 mL for 150 min. The dried powder of fish skin was homogenized with distilled water to obtain a suspension of 10% (w/w) protein. A volume of 30 mL were used for hydrolysis process and the conditions of pH, temperature and proportion enzyme to substrate (PE, %) were adjusted prior to the hydrolysis.

The degree of hydrolysis (DH %) can be defined as the percentage ratio of the number of peptide bonds cleaved to the total number of peptide bonds available in the substrate, DH% was monitored throughout the reaction. DH % was calculated according to the volume of base NaOH 1 N consumed during the reaction to keep the pH constant [40] using the following equation :

$$DH = \frac{V_b \cdot N_b}{a \cdot m_p . h_{TOT}} \cdot 100 \qquad \text{Eq (1)}$$

where V_b (mL) is the amount of base of normality N_b (eq/L) consumed during the reaction, m_p (g) is the mass of protein fed to the reactor (protein, determined as N = 6.25.), h_{TOT} is the total number of peptide bonds available for proteolytic hydrolysis assumed to be 8.6 milliequivalents of peptide bonds per gram of protein, α is the average degree of dissociation of the α-NH_2 amino groups released during hydrolysis, which can be related to reaction pH and temperature by Eq. (2):

$$\alpha\frac{10^{pH-pK}}{1+10^{pH-pK}} \qquad \text{Eq (2)}$$

The average pK value of the α-NH2 amino groups was estimated according to [41] (STEINHARDT & BEYCHOK, 1964) [41]

$$pK = 7.8 + \frac{298-T}{298 \cdot T} \qquad \text{Eq (3)}$$

The hydrolysis process and the reaction was stopped after 150 min by heating the solution at 100 ºC for 15 min achieving the thermal deactivation of the alcalase enzyme. The resulting hydrolysate were cooled down to room temperature and frozen till next analysis.

2.3.5. Experimental design, optimization and statistical analysis

The input variables described above were assayed at different levels according to a central composite design (CCD) of 17 experimental runs, including 3 replicates of the central point. The complete CCD design matrix and coded independent variables was shown in table 1 and table 4. The levels of pH (3.5–10.5), temperature (33 °C– 67 °C), and proportion of alcalase (PE, %) for protein content (0.32 – 3.68%) were chosen considering the intervals of activity reported for Alcalase. Degree of hydrolysis (DH; %) DPPH and FRAP were optimized as responses variables (Y) according to the input factors defined above. The degree of hydrolysis DH% was calculated by Eq. (1) and expressed as percentage.

According to the response surface methodology, the responses DH, DPPH and FRAP are related to the input factors (pH, T and PE) by second order polynomials. The regression models were expressed by the general quadratic Eq. (4):

$$(DH, DPPH, FRAP) = b_0 + b_1.pH + b_2.T + b_3.PE + b_{11}.pH^2 + b_{22}.T^2 + b_{33}.PE^2 + b_{12}.pH.T + b_{13}.pH.PE + b_{23}.T.PE \quad \text{Eq (4)}$$

The intercept b_0 and the coefficients b_1 to b_{34} are the model parameters that are estimated and define the second order polynomial regression, these parameters were estimated by multiple regression.

Table. 1 Table 1 Coded independent variables.

Factor	**Levels**				
	-α	**-1**	**0**	**+1**	**+α**
Temperature (°C), X_1	33	40	50	60	67
PE, (%, W/W), X_2	0.32	1	2	3	3.68
pH, X_3	3.5	5	7	9	10.5

3. RESULTS AND DISCUSSION

3.1. Proximate Composition of TFSW

As shown in table 2, the chemical compositions of the fresh TFSW were (30.4, 5.2, 62.2, 0.67) % for protein, fat, moisture, and ash contents respectively and the chemical compositions of dry powder of TFSW were (89.7, 9, 1, 0.98) % for protein, fat, moisture, and ash contents respectively. A large content of protein indicates the importance of TFSW as source to produce bioactive peptides.

Table. 2 Proximate analysis of fresh and dry powder of TFSW.

Proximate analysis	Fresh sample %	Dry sample %
Protein	30.488 ± 0.139	89.711 ± 0.376
Lipid	5.212± 0.114	9 ± 0.102

Moisture	62.296 ± 0.12	1± 0.003
Ash	0.673 ± 0.0068	0.98 ± 0.001

3.2. A quadratic polynomial model

As shown in table 4 the selected combinations of three independent variables: temperature, pH and enzyme to substrate ratio on dependent variable revealed that the results of DH varied from 8% to 23.7%, DPPH varied from 25 µg/ml to 56.6 µg/ml and FRAP varied from 44 µg/ml to 75.8 µg/ml. The results were analyzed according to the second-order polynomial model consisting of a constant, linear, quadratic and interaction terms as shown by the eq (9) and parameters of the model for each response variable were presented in table 3.

$$(DH, DPPH, FRAP) = b_0 + b_1.pH + b_2.T + b_3.PE + b_{11}.pH^2 + b_{22}.T^2 + b_{33}.PE^2 + b_{12}.pH.T + b_{13}$$

Temperature and pH showed a high significant effect ($p < 0.01$) in quadratic term but the proportion enzyme to substrate PE% (X3) showed the significant influence ($p < 0.05$) on DH, DPPH and FRAP in linear terms, This predictive model was obtained by non-linear regression and reduced by backward elimination, where the terms with associated p-value higher than 5% were removed. The goodness of fit of the reduced model was confirmed by the coefficient of determination R^2 values (0.963, 0.937 and 0.898) for DH%, DPPH and FRAP respectively, values of R^2 revealed that the models could be used to explain the relationship between dependent and independent variables. Furthermore, the predicted values by regression model and experimental values were plotted and found to vary uniformly around the diagonal (Fig. 1) which further confirmed the validation of the model.

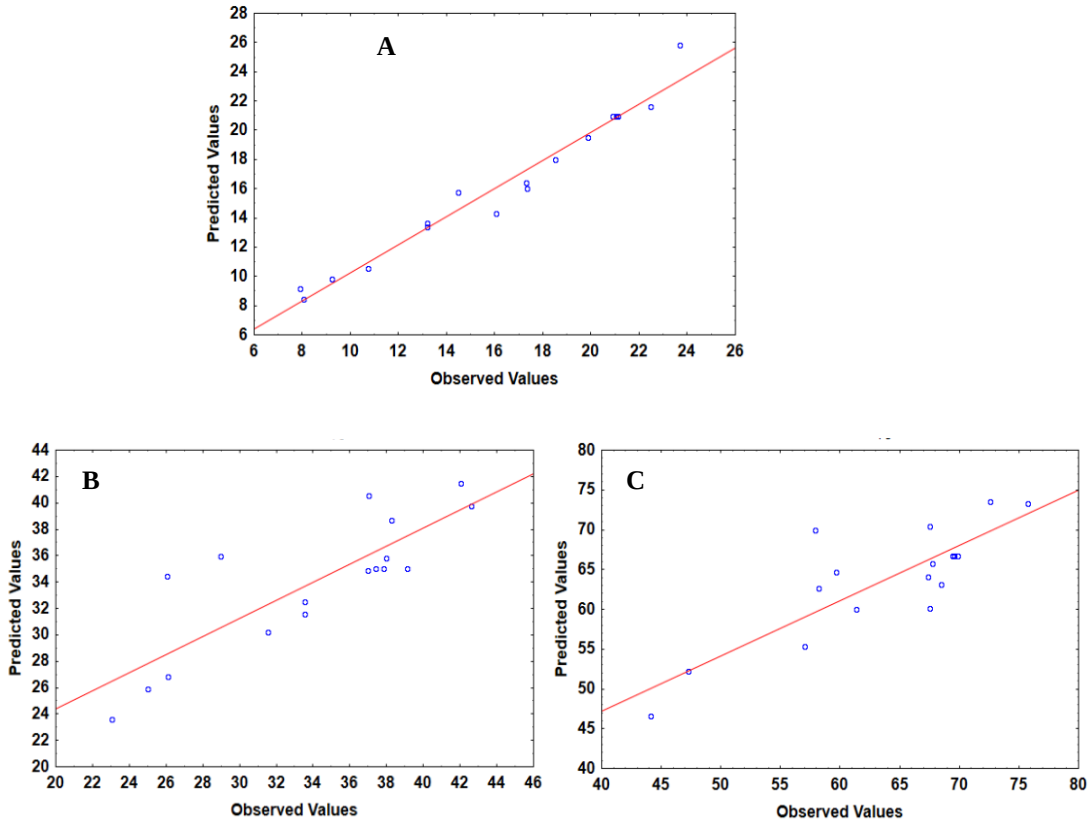


Figure 1. Relationship between the observed and predicted values of the degree of hydrolysis (A), DPPH (B) and FRAP (C) (Parity plot) using alcalase.

Table. 3 Coefficient of determination R^2 and regression parameters for predicting different responded variables.

Response variable	b_0	b_1	b_2	b_3	b_{11}	b_{22}	b_{33}	b_{12}	b_{13}	b_{23}	coefficient of determination R^2
DH%	-33.190	9.459	0.258	-0.042	-0.637	0.100	0.735	0.000	-0.007	0.002	0.963
DPPH	-83.893	9.636	2.575	8.135	-0.792	-0.020	0.221	0.031	0.182	-0.149	0.937
FRAP	-155.177	15.314	5.603	10.505	-0.905	-0.047	0.919	-0.014	-0.443	-0.143	0.898

3.3. Analysis the graphs of central composite design

3.3.1. The influence of operation conditions on degree of hydrolysis

The effect of temperature, pH and ratio enzyme to substrate on the degree of hydrolysis by alcalase was determined using CCD. The observed values for DH% at different combinations of the operation conditions are presented in Table 4. As shown in the ANOVA table 5 and 3D response surface plots of fig. 2A, the independent variables temperature and pH presented relatively higher quadratic significant effect ($p < 0.01$) while ratio E/S presented only linear significant effect. The interactions between the different independent variables did not significantly influence the dependent variable ($p > 0.05$).

Table. 4 Final degree of hydrolysis (DH, %), DPPH and FRAP of the tilapia skin hydrolysates as a function of the reaction parameters.

Standard run	pH	T °C	PE%	DH%	DPPH (µg/ml)	FRAP (µg/ml)
1	5.0	40.0	1.0	8.062	25.0	47.282
2	5.0	40.0	3.0	16.048	33.571	61.373
3	5.0	60.0	1.0	13.189	36.978	58.236
4	5.0	60.0	3.0	19.876	46.099	67.509
5	9.0	40.0	1.0	10.720	26.099	57.055
6	9.0	40.0	3.0	17.287	39.835	68.509
7	9.0	60.0	1.0	14.492	37.967	67.782
8	9.0	60.0	3.0	22.484	56.648	72.600
9	3.636	50.0	2.0	9.241	26.044	59.691
10	10.364	50.0	2.0	13.205	35.989	57.918
11	7.0	33.18	2.0	7.926	23.077	44.145

12	7.0	66.81	2.0	18.513	41.813	67.373
13	7.0	50.0	0.31	17.325	40.824	67.509
14	7.0	50.0	3.68	23.693	50.275	75.782
15	7.0	50.0	2.0	21.043	49.396	69.600
16	7.0	50.0	2.0	20.903	48.516	69.873
17	7.0	50.0	2.0	21.131	48.297	69.418

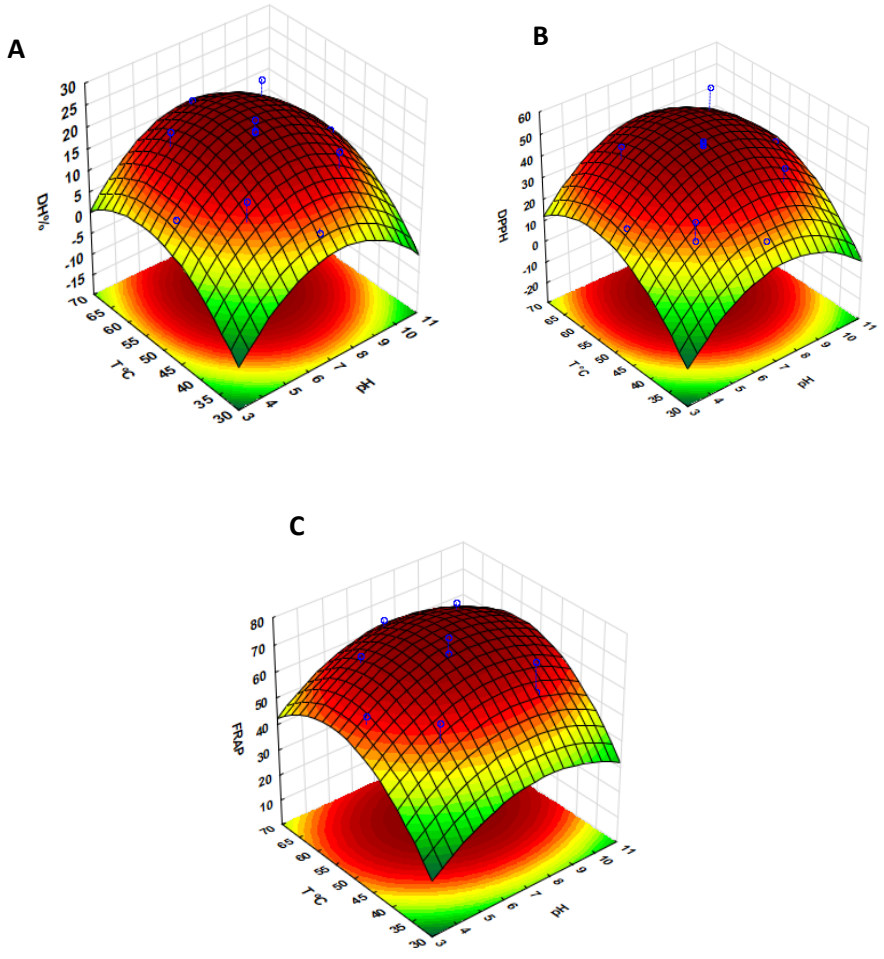


Figure 2. Fitted surface of independent variables on degree of hydrolysis DH% (A), antioxidant activities DPPH (B) and FRAP (C) as a function of various hydrolysis conditions: temperature, pH and ratio enzyme/substrate PE% (v/v).

The response surface graph for DH% as a function of temperature and pH of hydrolysis (Fig. 2A) indicates that the DH% increased with increase in temperature of hydrolysis up to 58.4 ºC before reducing considerably. The effect of temperature on enzymatic hydrolysis is well studied. Generally, the increase of DH% with temperature can be attributed to the exposed of peptide bonds, DH% decreases at higher temperature because of the thermal denaturation of protease which decrease the exposure of peptide bonds to the effect of enzymes [30], similar results were reported by da Silva Bambirra Alves et al. (2021) and showed the highest DH% was at 50 ºC for chicken blood protein using alcalase [42].

Likewise, DH% increases with an increase in pH up to pH 8.6 and then decreased prominent beyond that pH. Many studies reported significant effect of pH on enzymatic hydrolysis of protein [30, 43, 44] because of the direct effect of pH on the spatial structure and active sites of protease, the capacity of protease increases and more active sites is created at the best level of pH that led to break more peptide bonds, and consequently, increase the degree of hydrolysis. On the other hand, inadequate value of pH can slow down or obstruct the hydrolysis process because of the change in the ionic charge of substrate and the dissolving of protein in the solution depending on the iso electric point, and consequently decrease the binding ability between enzyme and protein.

Table. 5 ANOVA for the effect of operation conditions of protein hydrolysis and their interactions on degree of hydrolysis DH%, DPPH and FRAP]

Factor	Degree of Hydrolysis				DPPH scavenging				FRAP				
	Sum of squares	degrees of freedom	Mean sum of squares	F Value	Sum of squares	Degrees of freedom	Mean sum of squares	F Value	Sum of squares	degrees of freedom	Mean sum of squares	F Value	Sum of squares
X_1	93.46	1	93.46	66.48**	525.27	1	525.27	57.42*	368.83	1	368.83	34.33**	0.03
X_1^2	83.74	1	83.74	59.57**	319.29	1	319.29	34.9**	317.26	1	317.26	29.53**	0.02
X_2	15.34	1	15.34	10.91	92.93*	1	92.93	10.16*	87.67	1	87.67	8.16*	0.007
X_2^2	135.29	1	135.29	96.24	385.88**	1	385.88	42.18**	139.16	1	139.16	12.95*	0.03
X_3	116.81	1	116.81	83.09	318.99**	1	318.99	34.87**	209.97	1	209.97	19.54*	0.017
X1*X3				NS	-	-	-	NS					0.003
Error	15.46	11	1.40		100.61	11	9.14		118.15	11	10.74		0.006
Total SS	420.93	16			1612.81	16			1165	16			0.108

X1: Temperature, X2:pH and X3 proportion enzyme to substrate PE%

**P < 0.001; *P < 0.05.

NS (P < 0.05)

3.3.2. The influence of operation conditions on DPPH and FRAP

The DPPH radical scavenging activities and ferric reducing antioxidant power FRAP of protein hydrolysates of TFSW are shown in Fig. 2B and C. DPPH and FRAP activities were influenced by temperature and pH ($p< 0.01$), An increase in hydrolysis temperature up to 58.4 ºC increased the DPPH and FRAP of the protein hydrolysate, DPPH and FRAP decreased with further increase in temperature as shown in fig. 2B and C, the highest level of antioxidant capacity confirmed by FRAP and DPPH was also demonstrated at pH 8.6 and pH 7 respectively as shown in fig. 2B and C. As shown in the ANOVA (Table 5), ratio E/S presented linear effect on DPPH and FRAP which can be attributed to liberate more bioactive peptides and amino acids that have antioxidant activities such hydrophobic amino acid [29, 45].

Structurally, tilapia skin collagen is confirmed as collagen , the kinetics of hydrolysis process of collagen showed that the concentration of peptides increased dramatically at first and then tended to be stable, the antioxidant capacity of these bioactive peptides can be partially attributed to the proline at position C3 [46].

Similarly, DPPH and FRAP increased at high level of DH% which can be explained by large amount of small peptides that might work as effective radical scavengers [47, 48]. Similar results were reported by Cui, Sun, Cheng, & Guo. (2022) when extensive protein hydrolysates obtained from milk protein using Alcalase-Flavourzyme exhibited high radical-scavenging capacity [49]. These results were also extremely close to the study reported by Korkmaz & Tokur. (2021) when higher degree of hydrolysis of trout and whiting wastes was achieved at temperature of 60 ºC and enzyme ratio of 1% and 1.27% respectively and presented high antioxidant capacity [50]. Song et al. (2020) also reported that protein hydrolysates of Agrocybe aegerita had the highest antioxidant activities at temperature of 51.1 ºC, pH value of

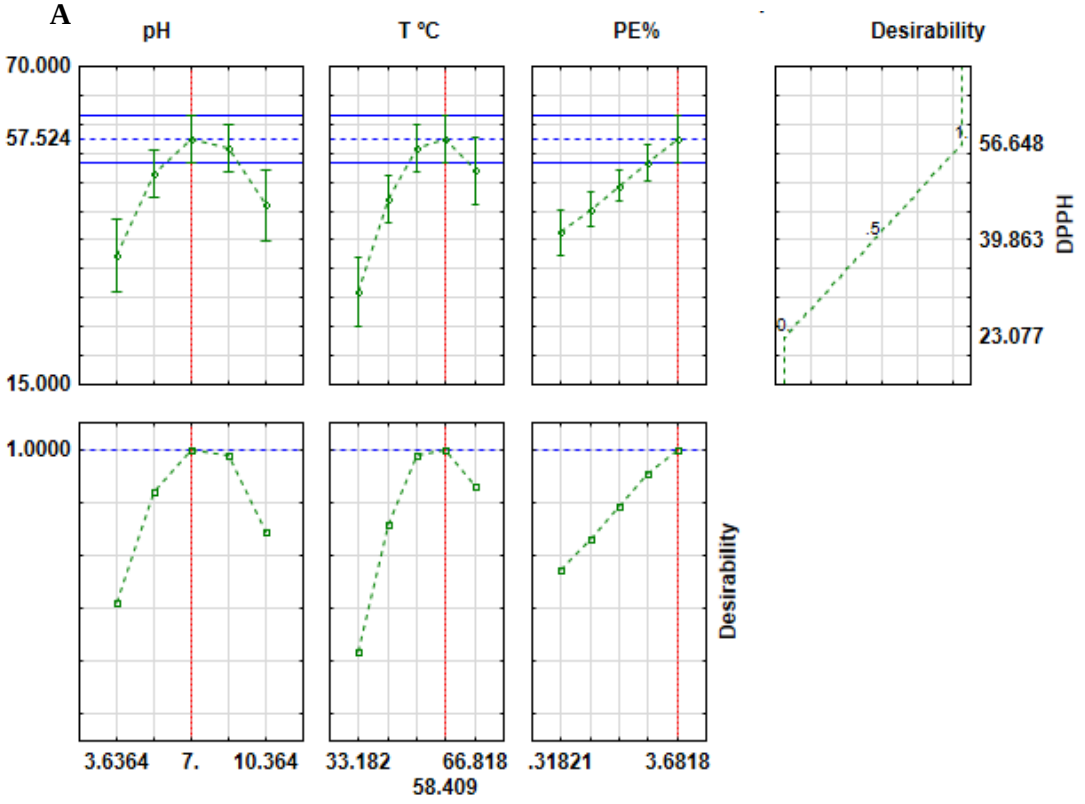
8.5 and E/S ratio of 3% because of distinct sequence of bioactive peptides released from hydrolysis process [51].

3.3.3. Optimization the independent variables of enzymatic hydrolysis using desirability function

Desirability function determines the relationship between predicted dependent variables and its desirability. The optimization and the profiles of desirability function can be obtained by the least square method which fit the responses variables at the observed high and low values for the corresponding desirability of 1 and 0, respectively. As shown in fig. 3 of desirability profile, the predicted independent variables and the desirability levels of DH%, DPPH and FRAP indicated that the desirability 1.0 was obtained at pH of 7.0-8.6 , hydrolysis temperature of 50-58.4 ºC and proportion enzyme to substrate PE% (v/v) 3.681. All responses variables increased with increasing of proportion enzyme to substrate PE%.

The combination of the optimal regions for all independent variables (temperature, pH and PE%) showed that the optimum degree of hydrolysis DH%, DPPH and FRAP were achieved at temperature 58.4 ℃ and pH 8.681 unless DPPH at pH 7, proportion enzyme to substrate PE% of 3.681% (v/v). Song *et al.* (2020) also found that the antioxidant capacity increased with higher degree of protein hydrolysis when studied the operation conditions to bioactive peptides derived from *Agrocybe aegerita* proteins by enzymatic hydrolysis [51], the results showed that the highest degree of hydrolysis and antioxidant capacities were determined at temperature of 51.1 ◦C, pH of 8.5 and ratio enzyme to substrate of 3% using neutral protease.

A


pH
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PE%
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70.000
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15.000
56.648
39.863
23.077
DPPH
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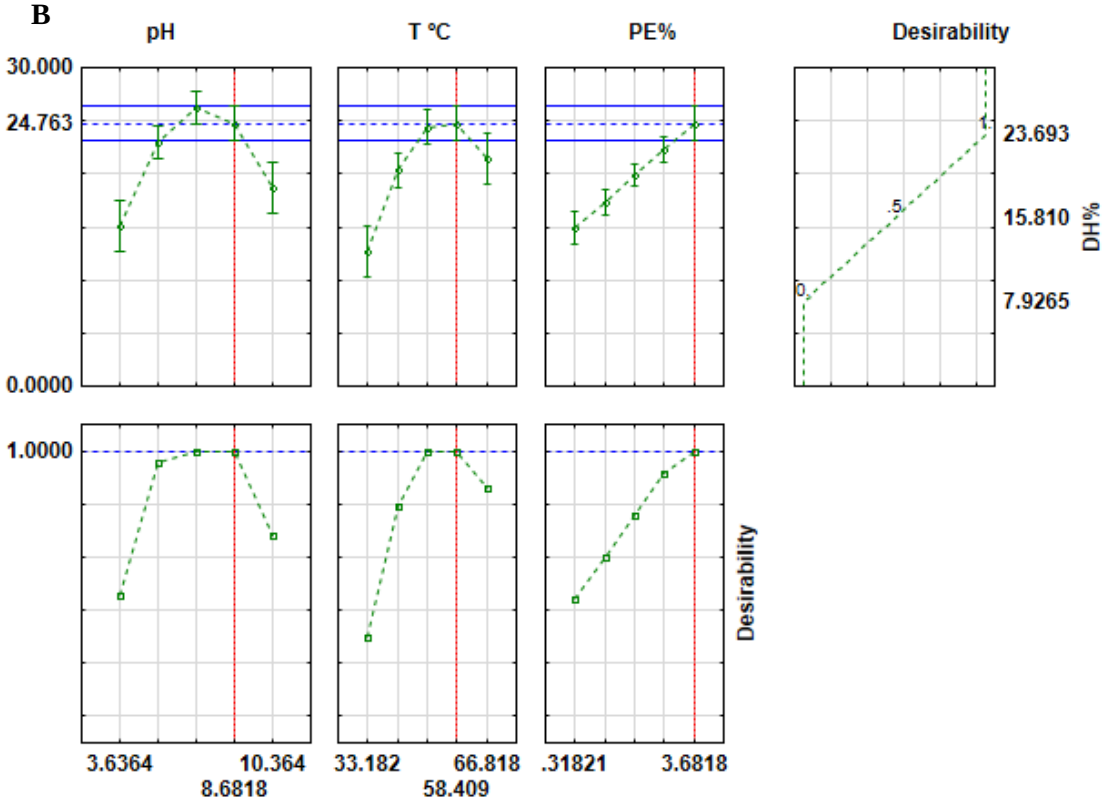
B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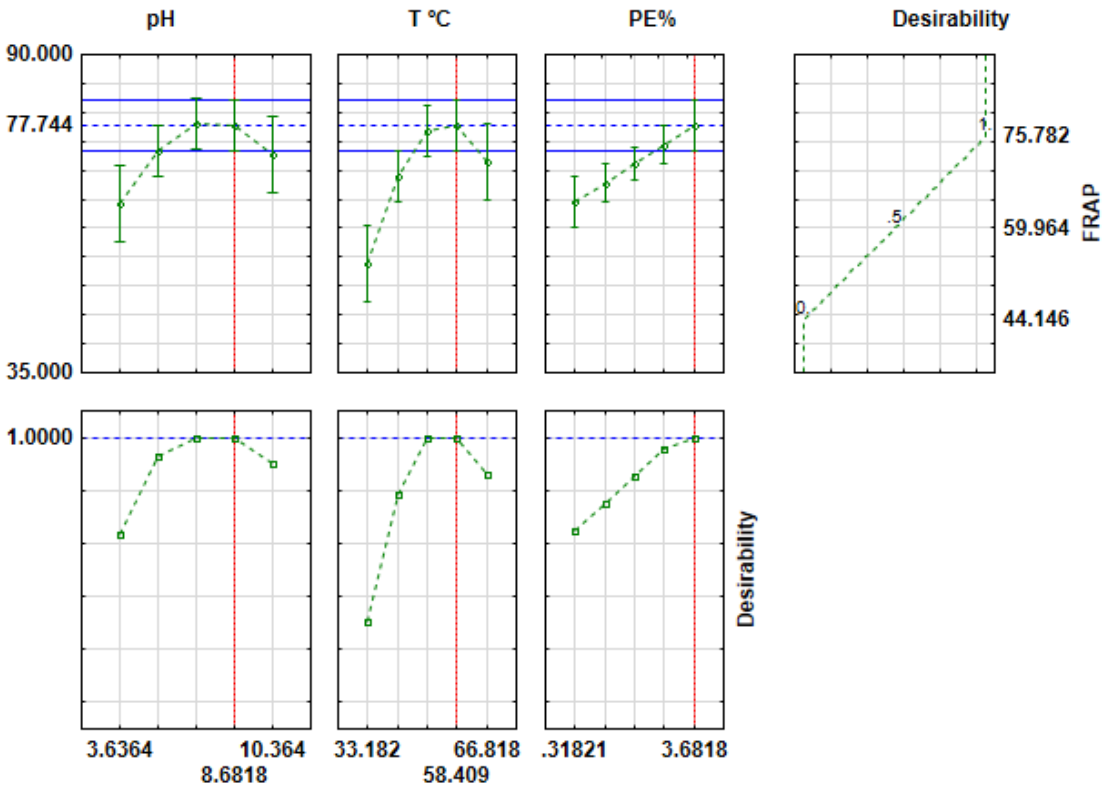

Figure 3. Desirability profile of (A) degree of hydrolysis, (B) DPPH scavenging and (C) ferric reducing antioxidant power FRAP.

4. Conclusion

Protein hydrolysates obtained from TFSW presented higher antioxidant activity at optimized condition of enzymatic hydrolysis. Central composite design (CCD) was adopted to optimize the independent variables. The results showed that the temperature and pH as an important factors which could affect the degree of hydrolysis and antioxidant activity of hydrolysates. The optimal conditions to obtain peptides with the highest degree of hydrolysis and antioxidant activity were at temperature of 58.4 ºC and pH 8.7 unless DPPH which was at pH of 7.0. The present study indicated the useful of alcalase 2.4.l to produce bioactive peptides from TFSW, these hydrolysates had a potential to be used as bioactive compounds for the food and pharmaceutical applications.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001; the Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brasil (CNPq) – Finance Codes 443310/2016-5 and 443243/2016-6; and the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro – Brasil (FAPERJ) – Financial Code E-26/010.001507/2015.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CONFLICT OF INTEREST

None.

ACKNOWLEDGEMENTS

None declared.

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CAPÍTULO Ⅳ: KINETIC MODEL FOR OPTIMIZED CONDITIONS OF PROTEIN HYDROLYSIS OBTAINED FROM TILAPIA FISH SKIN WASTE USING ALCALASE 2.4.L

Artigo de experimento foi submetido na revista da ciência rural, citescore 1.7

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MODELO CINÉTICO PARA CONDIÇÕES OTIMIZADAS DE HIDRÓLISE DE PROTEÍNAS OBTIDAS DE RESÍDUOS DE PELE DE PEIXE DE TILÁPIA USANDO ALCALASE 2.4.L

KINETIC MODEL FOR OPTIMIZED CONDITIONS OF ENZYMATIC HYDROLYSIS TO PRODUCE BIOACTIVE PEPTIDES FROM TILAPIA FISH SKIN USING ALCALASE 2.4.L

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Abstract

Protein hydrolysates have been gaining great interest in recent, since the modification by enzymes or chemicals improves functional properties of native proteins and their usefulness as intermediate ingredients in the cosmetics, pharmaceutical, food and nutraceutical sectors. Therefore, the aim of this study is to identify the kinetic model for optimized conditions of protein hydrolysis obtained from tilapia fish skin waste (TFSW) using alcalase 2.4.l. The reaction kinetics was realized according to hydrolysis time, initial substrate concentration and initial enzyme concentration. The optimized conditions of enzymatic hydrolysis process of protein obtained from TFSW were pH 8.7 and temperature 58.4 ºC, degree of hydrolysis (DH) increased with increasing the initial enzyme concentration (E_0), under a constant substrate concentration (S) as the hydrolysis time prolonged to 120 min. Furthermore the DH decreased with increasing the initial substrate concentration S_0 under a constant enzyme concentration as the hydrolysis time prolonged to 120 min. However beyond 120 min, the DH of these samples did not vary significantly. The importance of this work is that kinetic model can provide prevision of hydrolysis process and degree of hydrolysis based on the time and initial concentration of enzyme and substrate.

Keywords: Antioxidant, hydrolysates, bioactive peptides, enzyme, waste.

1. INTRODUCTION

Fish co-products are considered as a significant source of proteins, and other nutrients, such as phospholipids, polyunsaturated fatty acids, soluble vitamins and bioactive compounds (Shirahigue *et al.*, 2016), which make them as desirable components in applications of food technology and promote product development in fish industry and pharmaceutical companies (Feltes *et al.*, 2010). Consequently, many studies and investigations have been directed towards finding new forms of exploitation of fish waste in order to mitigate environmental problems and produce bioactive compounds. (Sheriff *et al.*, 2014).

Fish skin can be a valuable source of collagen that can be used to produce gelatine – a multifunctional component used in the food industry. Fish skin gelatine can be a source of bioactive peptides with various biological functions obtained through enzymatic hydrolysis (Gómez-Guillén *et al.*, 2011; Mohammad *et al.*, 2023). In the literature, it has been reported that fish hydrolysates demonstrated extremely valuable physicochemical and functional properties (Nalinanon *et al.*, 2011; Galla *et al.*, 2012). Furthermore, fish hydrolysates can act as antioxidants against free radicals (Nasri *et al.*, 2013).

Tilapia is belonging to the *Cichlidae* family. They are native to Africa, they were also introduced into many tropical, subtropical and temperate regions of the world, tilapia are an ideal candidate for aquaculture because of their attributes such as fast growth, tolerance to a wide range of environmental conditions (including, low dissolved oxygen, temperature, salinity, etc.), resistance to stress and disease and ability to reproduce in captivity and short generation time (El-Sayed, 2006). Approximately 60 a 70% of the fish body is processing waste, which includes meat remains, skin, scale, head, bones and viscera.

Tilapia skin can be applied to extract collagen which had obvious effect on the preparation of electrospun/electrosprayed microstructures of tilapia skin collagen and provide a way to maximize resource utilization of tilapia processing waste (Bi *et al.*, 2019).

Recently, Protein hydrolysates have been gaining more interest (Šližytė *et al.*, 2005; Karkouch *et al.*, 2017) since the modification caused by enzymes enhances characteristics of native proteins and their usefulness as intermediate ingredients in the food, nutraceuticals, cosmetics and pharmaceutical sectors (Šližytė *et al.*, 2005; Klompong *et al.*, 2008; Sarmadi andIsmail, 2010). Furthermore, hydrolysis of proteins has been the most common way of producing bioactive peptides (Nwachukwu andAluko, 2019; Mohammad *et al.*, 2023). The controlled factors and parameters of enzymatic hydrolysis including type of protease enzyme, enzyme to substrate ratio, substrate concentration, temperature, time and pH are essential to produce polypeptides of improved biological activities and specific size (Halim *et al.*, 2016).

Indeed, the enzymatic hydrolysis of protein in a heterogeneous system is consisted of highly complex reactions, because of product inhibition, substrate inhibition, and enzyme deactivation (Valencia *et al.*, 2015). Generally, Many studies confirmed that enzyme deactivation during the enzymatic hydrolysis process can slowdown the hydrolytic rate (Gan *et al.*, 2003; Zhou *et al.*, 2016). The kinetic models are usually used in order to set mathematical equation that help to predict the optimal proteolysis conditions, for instance, Michaelis model is classical modeling of enzymatic hydrolysis applied in a homogeneous system (Yang andFang, 2015). Kinetics of the operation of enzymatic hydrolysis of protein can help to optimize the bio-reactor conditions. However these kinetics parameters are difficult to be assessed because of a large number of peptides and polypeptide that are released during the hydrolysis process (Wali *et al.*, 2018).

(Wu *et al.*, 2016) Established a kinetic model to describe the enzymolysis process of sweet sorghum grain protein (SSGP), according to experimental data, the kinetic parameters, a and b, were determined and indicated that kinetic model was well founded to describe the kinetic behaviour for SSGP hydrolysis by alcalase. (Musa *et al.*, 2019) studied the effect of

ultrasound pretreatment on the enzymatic hydrolysis of defatted corn germ protein (DCGP) and related kinetics and thermodynamics, the results found that the First-order kinetic model was well described the traditional, SFU and MFU pretreated enzymatic hydrolysis of DCGP.

According to our recent work, we optimized the conditions of (pH, proportion enzyme to substrate E/S and temperature (Salman Mohammad *et al.*, 2023a), the optimized conditions were applied to set kinetic model of hydrolysis process and describe the relationship between the degree of hydrolysis and two reaction conditions (initial enzyme concentration and initial substrate concentration). Therefore, the purpose of this study is to establish kinetic model of the best conditions of enzymatic hydrolysis of protein from tilapia fish skin waste (PTFSW) which determined in our previous work to obtain higher degree of protein hydrolysis using Alcalase 2.4.L. Furthermore, the effect of substrate and enzyme concentrations on the degree of hydrolysis of PTFSW was determined.

MATERIALS AND METHODS

Chemicals

Sulfuric Acid 98% for the determination of nitrogen, Hydrogen Peroxide 30% w/v (100 vol.) for analysis, Sodium Hydroxide, Boric Acid or Hydrochloric Acid or Sulfuric Acid, Methyl Red, Bromocresol Green, Methylene Blue, methanol P.A, sodium deoxycholate, Copper sulfate pentahydrate, potassium tartrate, bovine serum albumin (BSA), Protease from Bacillus licheniformis (ALCALASE 2.4.L), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and other antioxidant reagents will be purchased from Sigma (St. Louis, MO, USA).

Materials

Tilapia fish skin (*Oreochromis niloticus*, Linnaeus 1758) was obtained from (Peixesul Cooperativa dos Aquicultores e Pescadores do Sul Fluminense), the raw materials were cooled to 4 ºC and transported to laboratory. The skin mass was treated at 90 °C for 20 min to inactivate the endogenous enzymes (Benhabiles *et al.*, 2012) followed by freeze-dried in a Labconco freeze drying system (Seropédica City, RJ, BR). The powder product was stored in the freezer at -20 ºC.

Methods

Biochemical analysis

Official methods from Association of the Official Analytical Chemists (A.O.A.C, 2012.) were used to determine the proximate composition of the TFSW in fresh and dry

form, gravimetrical way was used to measure both the moisture and mineral content at 105 ºC and 550 ºC, respectively. The Kjeldahl method was used to determine the nitrogen content followed by measured the crude protein by employing the conversion factor of 6.25 g of protein per gram of nitrogen (Adler-Nissen, 1986). Soxhlet semi-continuous method (Morales-Medina *et al.*, 2016) was used to extract and determine lipid content.

Enzyme and hydrolysis procedure

Alcalase 2.4.L (subtilisin, EC 3.4.21.62) is a protease extracted from *Bacillus licheniformis* and purchased from Sigma (St. Louis, MO, USA) to produce the protein hydrolysates. Hydrolysis experiments were carried out in a jacketed stirred tank reactor of 250 mL for 150 min. The dried powder of fish skin was homogenized with distilled water to obtain a suspension of 10% (w/w) protein. A volume of 30 mL were used for hydrolysis process and the optimized parameters of enzymatic hydrolysis process (pH 8.7 and temperature 58.4 ºC) were adjusted according to our recent work of this thesis (Salman Mohammad *et al.*, 2023b)

The degree of hydrolysis (DH %) can be defined as the percentage ratio of the number of peptide bonds cleaved to the total number of peptide bonds available in the substrate, DH% was monitored throughout the reaction. DH % was calculated according to the volume of base NaOH 1 N consumed during the reaction to keep the pH constant (Rutherfurd, 2010) using the following equation :

$$DH = \frac{V_b \cdot N_b}{a \cdot m_p . h_{TOT}} \cdot 100 \qquad \text{Eq (1)}$$

where V_b (mL) is the amount of base of normality N_b (eq/L) consumed during the reaction, m_p (g) is the mass of protein fed to the reactor (protein, determined as N = 6.25.), h_{TOT} is the total number of peptide bonds available for proteolytic hydrolysis assumed to be 8.6 milliequivalents of peptide bonds per gram of protein, α is the average degree of dissociation of the α-NH_2 amino groups released during hydrolysis, which can be related to reaction pH and temperature by Eq. (2):

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \qquad \text{Eq (2)}$$

The average pK value of the α-NH2 amino groups was estimated according to (Steinhardt andBeychok, 1964) (STEINHARDT & BEYCHOK, 1964) (Steinhardt andBeychok, 1964)

$$pK = 7.8 + \frac{298-T}{298 \cdot T}$$ Eq (3)

The hydrolysis process and the reaction was stopped after 150 min by heating the solution at 100 ºC for 15 min achieving the thermal deactivation of the alcalase enzyme. The resulting hydrolysate were cooled down to room temperature and frozen till next analysis.

Kinetics analysis – modelling the enzymatic hydrolysis of tilapia skin protein:

After optimization the parameters of enzymatic hydrolysis process (pH 8.7 and temperature 58.4 ºC) as described at our recent study (Salman Mohammad *et al.*, 2023b), the kinetic equation was explained by modeling the hydrolysis as a zero order reaction and inactivation of the enzyme as a second order reaction (Zhang *et al.*, 2012). According to the enzyme reaction intermediate complex theory, the process of enzymatic hydrolysis of a protein was expressed as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where, S is the concentration of substrate (g/L), E is the concentration of enzyme (g/L), P is the product (g/L), ES is the concentration of enzyme substrate complex (g/L), k_1, k_{-1},k_2 are the reaction rate constants of enzyme adsorption (L/U min), enzyme desorption (g/U min), and product formation (g/U min), respectively.

At a constant pH and temperature, the hydrolysis velocity can be determined by the irreversible stage (Márquez andVázquez, 1999).

$$V = S_0 \frac{d(DH)}{dt} = k_3[ES]$$ Eq (4)

Where, V is the hydrolysis velocity (g/L min), S_0 is the initial substrate concentration (g/L), DH is the degree of hydrolysis (%), and k_3 is the reaction rate constant for inactivation (L/U min).

According to the mechanism and kinetic model of enzymatic hydrolysis of protein, the hydrolysis velocity also can be expressed by the following equation:

$$V = \alpha . S_0 . exp[-b(DH)]$$ Eq (5)

In Eq (4) , $\alpha = k_2 . \frac{E_0}{S_0}, b = \frac{k_3 K_m}{k_2} = \frac{k_3(k_{-1}+k_2)}{k_2 k_1}$

where, a is the kinetic parameter (min^{-1}); b is the kinetic parameter, dimensionless and K_M is the Michaelise-Menten constant (g/L). The relationship between the hydrolysis velocity and DH is expressed in Eq (6) that derived from Eq. (5) and Eq. (4):

$$\frac{d(DH)}{dt} = \alpha . exp[-b(DH)]$$ Eq (6)

Eq. (7), which expresses the relationship between the degree of hydrolysis and the hydrolysis time, is obtained by integrating Eq.(6):

$$DH = \frac{1}{b} ln(1 + abt) \quad \text{Eq (7)}$$

t is the hydrolysis time, degree of hydrolysis was calculated every 10 minutes for 2 hours and every 15 min for the rest 30 min, the kinetic parameters a and b were obtained by linear regression to fit Eq. (7). Therefore, a_m (the fitted value for a), V and DH will be determined, and accordingly enzymolysis kinetic equation can be obtained under certain conditions.

RESULTS AND DISCUSSION

Proximate Composition of TFSW

As shown in table 1, the chemical compositions of the fresh TFSW were 30.4, 5.1, 62.3 and 0.76% for protein, fat, moisture, and ash contents respectively and the chemical compositions of dry powder of TFSW were 88.5, 8.2, 1, 0.99% for protein, fat, moisture, and ash contents respectively. A large content of protein indicates the importance of TFSW as source to produce bioactive peptides.

Table 1 Proximate analysis of fresh sample and dry powder of TFSW.

Proximate analysis	Fresh sample %	Dry sample %
Protein	30.456 ± 0.112	88.523 ± 0.351
Lipid	5.115± 0.102	8.21 ± 0.11
Moisture	62.358 ± 0.172	1± 0.002
Ash	0.763 ± 0.0038	0.99 ± 0.001

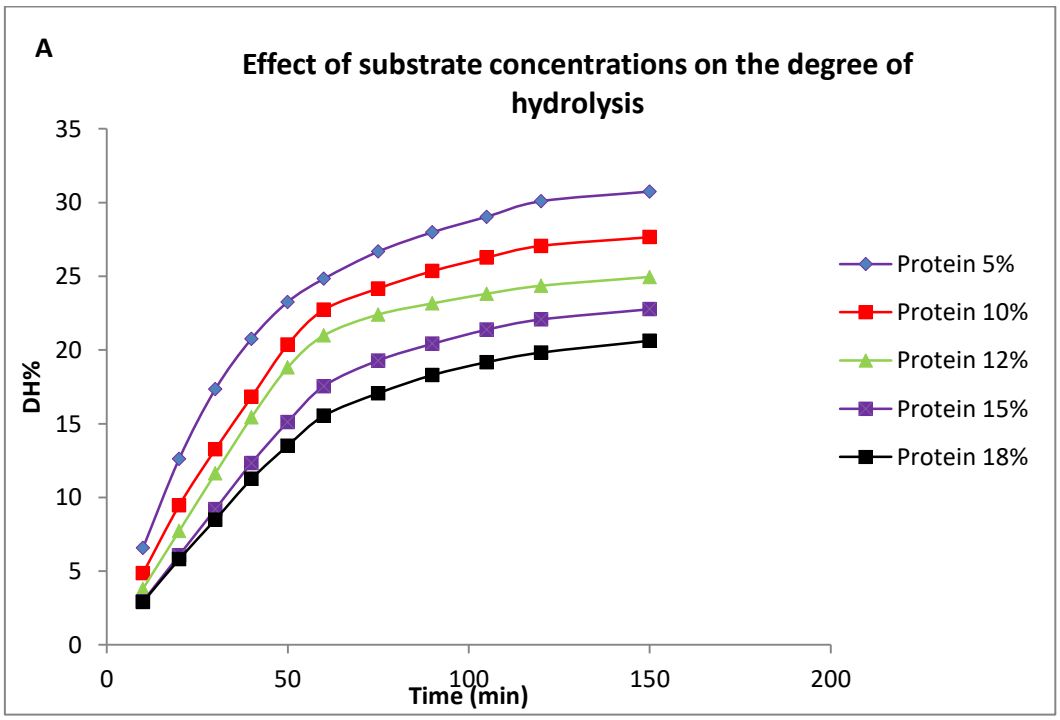
Effect of substrate and enzyme concentrations on the degree of hydrolysis of PTFSW

Based on our previous study (Salman Mohammad *et al.*, 2023b), Protein hydrolysates obtained from TFSW presented higher antioxidant activity and degree of hydrolysis at optimized condition of enzymatic hydrolysis of temperature 58.4 ºC and pH 8.7. This enzyme concentration is involved as a constant factor (5 g/l) in studying the effect of different levels of substrate concentrations on DH during a time. Similarly, 100 g/L of substrate is selected as a constant factor on studying the effect of substrate concentrations on the DH during a time.

Substrate concentration

At a constant level of alcalase (5 g/L), low concentrations of the substrate (<180 g/L) increased the DH, while substrate concentrations higher than 100 g/L decreased the DH (Fig.

1A). After 10 min of hydrolysis, the DH increased steadily, with significant magnitudes ($p \leq 0.05$), as the substrate concentration increased from 50 g/L to 180 g/L. This increasing order in the DH of samples with all concentrations continued as the hydrolysis time prolonged to 105 min. Beyond 105, the DH of these samples did not vary significantly unless the sample with substrate concentration 50g/l the samples presented increase the DH as the hydrolysis time prolonged to 120 min, beyond 120 min, the DH of these samples did not vary significantly, and thereafter the DH of the substrates with concentrations of 50 g/L and 100 g/L surpassed that of 120, 150 and 180 g/L. Accordingly, the substrate with concentration of 50 g/L resulted in the highest DH (32.06%) at a reaction time of 150 min. As can be seen (Fig. 1A and B), the enzymolysis reaction rate decreased under certain conditions of the substrate and enzyme concentrations. As shown at Fig. 1A degree of hydrolysis DH % decreased at higher concentration of substrate from 5 % to 15 % protein concentration, and therefore, at higher substrate concentration than the optimal, the reaction rate decreased, suggesting an inhibitory effect on enzyme activity which can be explained by higher concentration of peptide bonds that are not exposed to contact with enzyme molecules at active sites



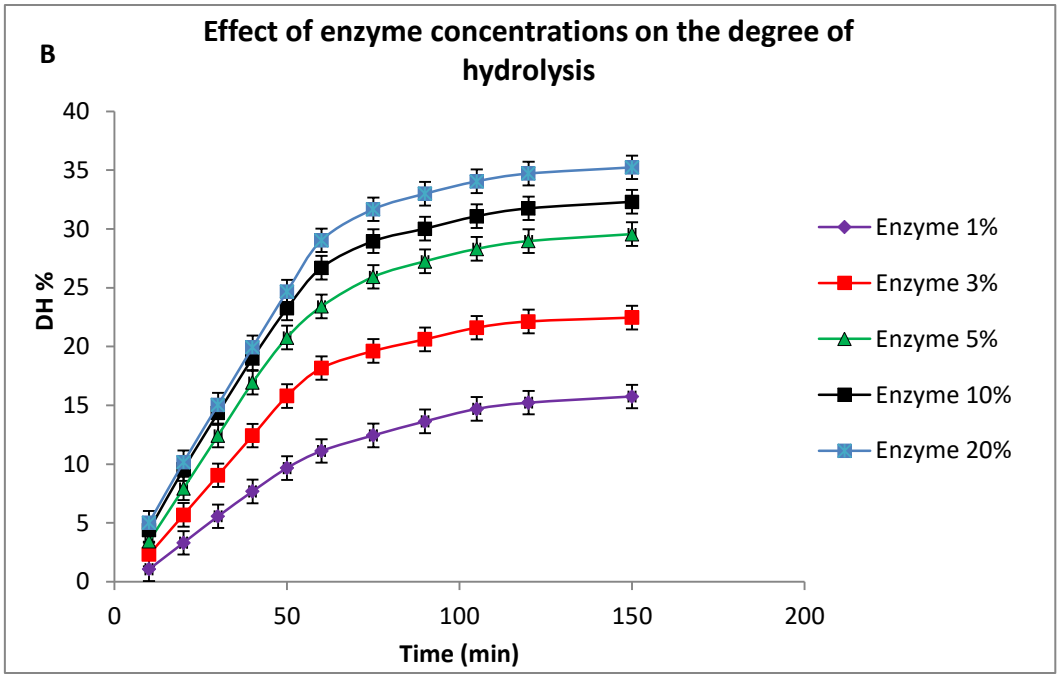


Fig. 1. The effect of initial substrate concentration (S_0) A and initial alcalase concentration (E_0) B on the degree of hydrolysis (DH),

Enzyme concentration

In **Fig. 1B**, at the initial stage of the reaction, the DH of the substrate (constant concentration 100 g/L) increased rapidly for all alcalase enzyme concentrations (1 g/L to 20 g/L) and subsequently the rate of increase in the DH decreased as the hydrolysis time prolonged. Moreover, the DH increased with increasing the concentration of the enzyme from 1 g/L (lowest DH) to 20 g/L (highest DH) $p \leq 0.05$. For all enzyme concentrations, the DH of samples continued as the hydrolysis time prolonged to 105 min. Beyond 105, the DH of these samples did not presented a significant difference except the sample with enzyme concentration 20 g/l the samples presented increase the DH as the hydrolysis time prolonged to 120 min, beyond 120 min, the DH of these samples did not vary significantly. According to these results, it can be suggested that the hydrolysis rate showed increasing trend the increase of enzyme concentration and decreasing when the period prolonged at level higher than critical. Therefore, the hydrolysis process would be inhibited, and as a consequence the DH decreased rapidly. This might be related to the release a big number of peptide bonds which are exposed to be hydrolysed; this reflects decreasing the proportion enzyme/peptide bonds and results in a lowering of enzyme activity.

Kinetic model of enzymatic hydrolysis of PTFSW

From the mechanism and the kinetic equation, the proposed kinetic parameters for enzymatic hydrolysis of the rapeseed meal can be calculated. The experimental data in Fig.1A and B were fitted into Eq. (6) using linear regression analysis, and the kinetic data are shown in Table 2. The influence of the initial substrate concentration (S_0) and the initial enzyme (E_0) on α and b parameters was checked. However, parameter b changed when S_0 and E_0 changed.

Table 2 Kinetic parameters of of enzymatic hydrolysis of PTFSW using alcalase.

S_0 g/L	E_0 g/L	E/S	b (min^{-1})	α (min^{-1})	$α_m$ (min^{-1})
50	5	0.1	0.025	1.620	0.356
100	5	0.05	0.044	1.451	0.268
120	5	0.041	0.049	0.992	0.203
150	5	0.033	0.050	0.736	0.167
180	5	0.027	0.073	0.599	0.128
100	1	0.01	0.066	0.396	0.100
100	3	0.03	0.053	0.965	0.194
100	5	0.05	0.042	1.443	0.272
100	10	0.1	0.023	1.639	0.368
100	20	0.2	0.021	2.914	0.542

S_0, Initial substrate concentration; E_0, Initial enzyme concentration; α, The kinetic parameter; b, The kinetic parameter, dimensionless; $α_m$, The fitted value for α.

The parameter b values lies within a very small range and therefore an average value of 0.045 was used for further analysis.

Parameter α are presented in (Table 2) showed a clear dependence on S_0. Whereas, the values of α increased with increasing S_0, reaching its highest level (2.91 min^{-1}) at 100 g/L for S_0. Thereafter, the α values decreased as S_0 increased. Similarly, the α values increased when the initial enzyme concentration increased.

The average value of b was brought into Eq. (7) and yields the expression:

$DH = 22.17\ ln(1 + 0.045\ at)$

which applied in calculation of a_m values (Table 2).

A plot (Fig. 2) of a_m values and $[E_0]/[S_0]$; with R^2= 0.944; led to Eq. (8):

$a_m = 2.299\ \frac{E_0}{S_0} + 0.1108$…… …….. Eq. (8):

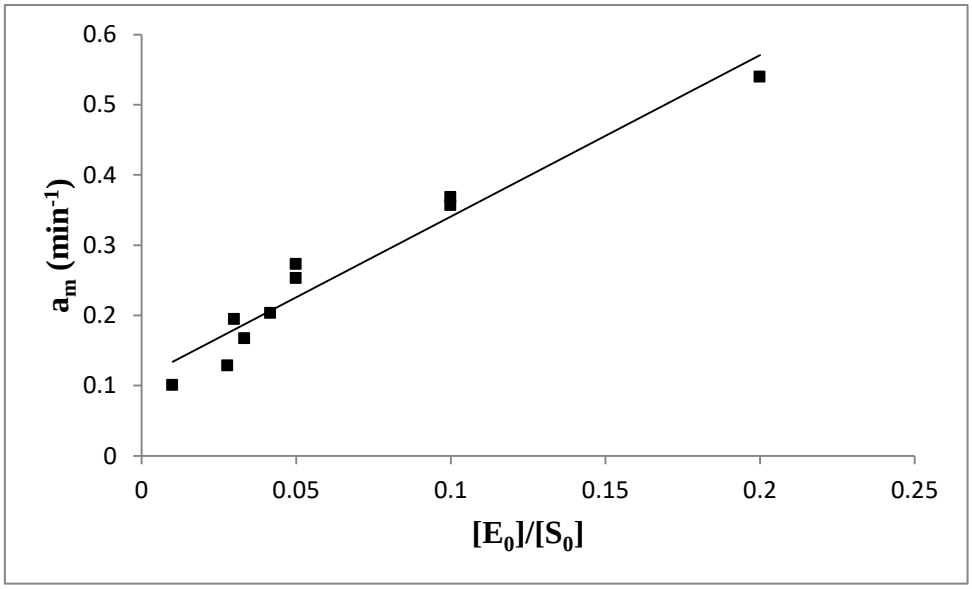


Fig. 2. The linear relationship between a_m and $[E_0]/[S_0]$. Regression equation, α_m = 2.2993$[E_0/S_0]$ + 0.1108, R^2= 0.9444.

The kinetic parameters b from Table 2 and a_m from Eq. (8) were brought into Eq. (7), and the kinetic equation of enzymatic hydrolysis of protein of **TFSW** under optimized condition of temperature 58.4 ◦C and pH 8.7 was obtained in Eq. (9):

$$DH = 22.22 \ln\left(1 + \left(\frac{0.103E_0}{S_0} + 0.0049\right)t\right) \quad \text{.....Eq (9)}$$

Eq. (9) showed that the degree of hydrolysis is related to the initial enzyme concentration, E_0, initial substrate concentration, S_0, and hydrolysis time, t. That is, the DH increased with increasing the E_0, under a constant substrate concentration. In contrast, the DH decreased with increasing the S_0, under a constant enzyme concentration. These facts agreed with the results depicted in Fig. 1A and B, indicating adequacy between the calculated values of the DH versus time and the experimental conditions.

Conclusion

According to the best hydrolysis conditions of protein from tilapia fish skin waste (temperature 58.4 ºC and pH 8.7), a mathematical model of the hydrolysis kinetics of the PTFSW (Eq. (9)) was established based on the initial alcalase concentration, initial substrate concentration and hydrolysis time was established numerically. DH increased with increasing the E_0, under a constant substrate concentration as the hydrolysis time prolonged to 120 min. Furthermore the DH decreased with increasing the S_0 under a constant enzyme concentration as the hydrolysis time prolonged to 120 min. However beyond 120 min, the DH of these

samples did not vary significantly. The hydrolysis kinetic equation was obtained to guide and optimize the hydrolysis reaction process. Thus it gives the possibility of estimation of the critical concentrations of the enzyme and substrate, the reaction rate and the degree of hydrolysis.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001; the Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brasil (CNPq) – Finance Codes 443310/2016-5 and 443243/2016-6; and the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro – Brasil (FAPERJ) – Financial Code E-26/010.001507/2015.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CONFLICT OF INTEREST

None.

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CAPÍTULO V: Purification of bioactive peptides obtained from tilapia fish skin (Oreochromis niloticus, Linnaeus 1758) using macroporous resins.

O artigo foi submetido ao Journal of Iranian Chemical Society A4

Purification of bioactive peptides obtained from tilapia fish skin (*Oreochromis niloticus*, Linnaeus 1758) using macroporous resins

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Abstract

Fish and fish product consumption has presented a huge increase, resulting in a massive generation of fish waste with valuable proteins. Thus, the production of fish protein hydrolysates is a suitable strategy to reduce environmental impacts and obtain functional food rich in bioactive peptides. Therefore, regarding the relevance of purification steps, this study investigated the adsorption and desorption characteristics of protein hydrolysates of tilapia fish skin waste (PHTFSW) using three macroporous absorbent resins (XAD 4, XAD 16, and XAD 7HP). XAD-7HP showed the highest adsorption and desorption capacities, as well as the greatest recovery. The adsorption data were analyzed by the Langmuir and Freundlich isotherm models, indicating that the Freundlich model best fitted the experimental data and provided high accuracy modeling PHTFSW separation using XAD-7HP resin. Moreover, the effects of purification on the amino acid profile and antioxidant capacity of PHTFSW were also evaluated. Chromatographic analyses revealed that the separation process of PHTFSW

using XAD 7HP resins had no significant effect on the amino acid structures. Regarding the antioxidant capacity, purified PHTFSW showed higher values for both FRAP and DPPH assays compared to PHTFSW not treated with the resin. Therefore, the overall results underscore the potential of XAD 7HP resin as a feasible and promising technology to separate and purify bioactive peptides from PHTFSW.
Keywords: antioxidant, isotherm test, purification, adsorption, polymer.

1 Introduction

Tilapia (Oreochromis niloticus, Linnaeus 1758), which belongs to the Cichlidae family, is a freshwater fish native to Africa that was introduced into many tropical, subtropical, and temperate regions of the world [1]. This species presents suitable characteristics for aquaculture, including fast growth, tolerance to a wide range of environmental conditions (e.g. temperature, salinity, and low dissolved oxygen), resistance to stress, ability to reproduce in captivity, and short generation time [2]. Moreover, tilapia is valued due to its nutritional properties, which are mainly attributed to its protein content and essential amino acid composition [3,4].

In contrast, increasing aquaculture and fish markets have led to a large generation of fish waste [5]. Approximately 60 to 70% of the fish body is processing waste, which includes meat remains, head, bones, skin, scale, and viscera [6]. It creates a global environmental concern, but the conversion of fish waste into valuable materials has been underscored and is currently in high demand [7,5]. For example, fish skin is a valuable source of collagen that can be used for its production, in addition to resulting in value-added products, playing a role in solving the waste disposal issue [8].

Fish protein hydrolysates (FPH), which may be produced from fish waste such as skin, are nutritious feed ingredients and potential fish meal replacers in aquaculture diets [9]. Apart from their nutritional importance, FPH been reported to exhibit functional properties essential for a healthy living being, as bioactive peptides with various biological functions are obtained through enzymatic hydrolysis [10]. Fish hydrolysates can act as antioxidants against free radicals [11], as well as antihypertensive and anti-obesity agents [12,13]. Besides, studies have reported -great physicochemical and functional properties for FPH [14].

Macroporous resins are porous cross-linked polymer beads that have been developed as useful adsorbents [15], showing promising results concerning the purification of natural materials. Therefore, using macroporous absorbent resins

(MARs) during protein hydrolysate obtention may be a suitable strategy as purification is an important step. They are considered more applicable than common adsorbents due to their physical and chemical stability, large surface areas, fast adsorption rate, strong adsorption capacity, high adsorption selectivity, and easy elution and recycling [16]. Thus, MARs have shown great applicability with polyphenols [17], alkaloids [18], dianthrones [19], melanoidins [20], and amino acids [21].

Conventional methods such as membrane separation, ultrafiltration, gel filtration, and liquid–liquid extraction [22] have been applied for protein hydrolysate separation. However, they present disadvantages due to their low effectiveness concerning purity, yield of target peptides, and conversion rate of substrate protein. Therefore, using MARs can be highlighted as a promising tool, where studies on their application in fish protein hydrolysates are lacking [21].

In this context, this work studied the adsorption and desorption of protein hydrolysates of tilapia fish skin waste (PHTFSW) treated with MARs to determine the most effective type of resin in terms of separation and purification of peptides. Furthermore, this paper aims to present an innovative method that can be used to prepare protein samples for more effective separation and identification of proteins from their complex system by HPLC.

2 Materials and methods

2.1 Chemicals

Sulfuric acid, potassium sulfate, sodium hydroxide, hydrochloric acid, boric acid, bovine serum albumin (BSA), Trolox, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and other reagents for antioxidant analyses, free amino acids (Ref. 03835), acetonitrile, and other solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Amino acid standard (H, Ref. WAT088122), 6-aminoquinolyl-succimidyl-carbamate (AQC), borate buffer (AccQ-Fluor Borate Buffer), and free amino acids (Ref. 03835, Sigma Aldrich, St. Louis, USA) were acquired from Waters Corporation (Milford, USA).

2.2 Materials

Tilapia fish skin (*Oreochromis niloticus*, Linnaeus 1758) was obtained from a cooperative (Peixesul Cooperativa dos Aquicultores e Pescadores do Sul Fluminense, Pirai, Rio de Janeiro, Brazil). The skin mass was treated at 90 °C for 20 min to

inactivate the endogenous enzymes, followed by freeze drying in a Labconco system (Kansas, Missouri, USA). PHTFSW powder was obtained using the optimal conditions determined in a previous study by Mohammad et al. (2023) [23]. Thus, the highest degree of protein hydrolysis was achieved using Alcalase 2.4.L at temperature 58.4 ºC and pH 8.6, this was according to the results of optimization process that was explicated at chapter 3, the hydrolysis process was realized in a laboratory pilot scale bioreactor. The proximate composition of PHTFSW powder was determined according to the AOAC procedures [24]. The powder was stored at -20 ºC for further purification.

Amberlite resins (XAD 4, XAD 16, and XAD 7HP) were purchased from Sigma-Aldrich (St. Louis, Missouri, EUA). Their chemical and physical properties are summarized in Table 1.

Table 1

Table 1 Chemical and physical properties of resins

Amberlite Resins	**Chemical matrix**	**Polarity**	**Surface área (m^2/g)**	**Pore envelope (Ã)**
XAD 16	Styrene-divinylbenzene	Nonpolar	800	200
XAD 7HP	Acrylic ester	Polar	500	450
XAD 4	Styrene-divinylbenzene	Nonpolar	750	100

2.3 Pre-treatment of resins

The resins (5 g) were soaked in distilled water and treated with ethanol (140 mL, 95%). Then, they were washed with distilled water until the eluent is clear and eluted with 140 mL of 4% hydrochloric acid, followed by the treatment with distilled water until pH 7.0 (neutral). Subsequently, the resins were washed with 5% sodium hydroxide (140 mL), followed by other washing with distilled water to achieve pH 7.0. Finally, the resins were dried at 60 ˚C in an oven for 24 h to achieve a constant weight [25]. **Annex B.**

2.4 Static adsorption and desorption tests

Pre-treated resins (0.5 g) were added to 12.5 mL of a PHTFSW solution prepared by dissolving 1 g of dried PHTFSW in 12.5 mL of distilled water **annex B**. The mixtures were homogenized in a shaker 100 rpm, at 30 °C for 24 hours and the pH was adjusted to 3, 5, 7, and 9 using 0.1 N of HCl (0.1 N) and 0.1 N of NaOH (0.1 N).

For the static desorption tests, the resins were filtered, washed with distilled water, and combined with 25 mL of ethanol (99.8%). The flasks were kept in a shaker (100 rpm) at 50 °C for 24 hours. Adsorption and desorption ratios and capacities were determined according to the equations bellow:

Adsorption ratio: $A\ (\%) = \frac{(C_0 - C_e)}{C_o}$ Equation (1)

Adsorption capacity: $q_e = (C_0 - C_e) \times \frac{(V_i)}{m}$ Equation (2)

Where A is the adsorption ratio (%) and q_e is the adsorption capacity (mg/g dry resin) at equilibrium; C_0 and C_e are the initial concentration and the equilibrium concentration of the total peptides (mg/mL), respectively; m is the resin initial weight (g); V_i is the volume of the PHTFSW solution (mL).

Desorption ratio: $D\ (\%) = C_d \frac{V_d}{(C_d - C_e)V_0} \times 100$ Equation (3)

Desorption capacity: $q_d = C_d \times \frac{V_d}{m}$ Equation (4)

Recovery: $R\ (\%) = \frac{C_d V_d}{C_o V_o} \times 100$ Equation (5)

Where D is the desorption ratio (%), q_d is the desorption capacity (mg/g dry resin), and R is the recovery after complete desorption; C_d is the concentration of total peptides in the desorption solution (mg/L); V_d is the volume of the desorption solution (mL); C_o, C_e, m, and V_0 are the same as those in Equations 1 and 2.

2.5 Adsorption isotherm of PHTFSW solution on macroporous resins

The procedures below were performed for the resin that presented the highest recovery. After being activated and washed, five aliquots of the resin (0.5 g per aliquot) were placed into conical flasks. Then, 10 mL of PHTFSW purified solutions with different nitrogen concentration (0.125%, 0.25%, 0.5%, 1.0% or 2.0%) were added. The adsorption was carried out at three temperatures (30, 40, and 50 ˚C) in a shaker at 120 rpm. The absorption equilibrium time was determined by the adsorption kinetics study on macroporous resins. Total peptide concentrations were quantified after the adsorption equilibrium time. The equilibrium adsorption isotherms for peptide were calculated using the Langmuir and Freundlich equations:

The Langmuir equation:

$$\frac{C_e}{q_e} = \frac{1}{q_m} \cdot C_e + \frac{1}{q_m K\_L} \quad \text{Equation (6)}$$

The Freundlich equation:

$$\ln q_e = \frac{1}{n} \cdot \ln C_e + \ln K_f \quad \text{Equation (7)}$$

2.6 Protein assay

The Kjeldahl method was used to determine the nitrogen content in the initial solution of PHTFSW [26].

The methodology described by Simonian and Smith (2001) [27] was applied to determine the protein concentration of the PHTFSW solution during static, kinetics, and isotherm tests using a spectrophotometer with UV lamp at 280 nm. The results were assessed by using a calibration curve prepared with a standard protein solution of bovine serum albumin (BSA).

2.7 Amino acid composition of PHTFSW

2.7.1 Separation and quantification of amino acids by acid hydrolysis

The methodology reported by Pacheco (2014) [28] and the acid hydrolysis step according to AOAC method number 994.12 [26] were used for the separation and quantification of the main amino acids present in PHTFSW [29] **annex B**. Determination of free amino acids (Ref. 03835, Sigma Aldrich, St. Louis, USA) in PHTFSW was carried out by performing an acid extraction step with subsequent derivatization with 6-aminoquinolyl-succimidyl-carbamate (AQC). For the acid extraction step, a mixture containing 1 g of the sample and 10 mL of 0.1 M HCl was vortexed for 1 min. Then, it was extracted in ultrasound for 10 min and centrifuged at 6000 rpm for 10 min, followed by filtration through a 0.45 μm filter. Subsequently, the derivatization reaction was carried out by adding 60 μL of borate buffer (AccQ-Fluor Borate Buffer, Waters Corporation, Milford, USA) to 20 μL of extract. Chromatographic analysis was also performed as described above, with a flow of 0.8 mL/min [30].

2.8 Antioxidant capacity

The antioxidant capacities were determined for the purified PHTFSW treated with XAD 7HP, and the PHTFSW not submitted to the treatment with the resins (control).of both purified and normal extracts of PHTFSW were determined by DPPH and FRAP assays. The scavenging activity of samples against the DPPH radical was determined using a previously described method He et al., 2013 [31]. The extract (150 mL) was added to a 0.06 mM methanolic DPPH solution (2.85 mL). The mixture was homogenized and left resting for 1 h in the dark at room temperature (23 to 25 ºC). The absorbance was measured at 517 nm and the results were expressed as mg Trolox equivalent (TE)/ g wet sample.

For the Ferric Reducing Antioxidant Power (FRAP) assay, 2.7 mL of freshly prepared FRAP reagent (TPTZ, $FeCl_3$, and acetate buffer) was mixed with the extract (90 µL) and distilled water (270 µL). The mixture was heated at 37 °C for 30 minutes. The absorbance was determined at 595 nm and the results were expressed as mg TE/g wet sample (Pulido, Bravo, and Saura-Calixto, 2000).

2.9 Statistical analysis

All tests were performed in triplicate. The experimental data was expressed as mean ± standard deviation and was submitted to analysis of variance (ANOVA) and the means compared by the Tukey test at 5% of significance. The experimental models of kinetics and the adsorption / desorption isotherm were adjusted using the non-linear regression method using the Statistica 7.0 program. The adequacy of the model between the experimental and predicted data were verified based on the coefficient of determination (R^2).

3 Results and discussion

3.1 Proximate composition of PHTFSW powder

The proximate composition of PHTFSW powder is presented in Table 2. The high protein content (89.71 ± 0.37%) indicates the importance of PHTFSW as a source to produce bioactive peptides. The levels of total lipids and ash were 9.42 ± 0.38% and 0.98 ± 0.01¨%, respectively, while 1.11 ± 0.38 was found for moisture. These values are in agreement with the ones determined tilapia waste in other studies (Roslan *et al.*, 2014; Tejpal *et al.*, 2021).

Table 2 Proximate composition of protein hydrolysates from tilapia fish skin waste

Parameter	Content (%)
Protein	89.71 ± 0.37
Lipid	9.42 ± 0.38
Moisture	1.11 ± 0.38
Ash	0.98 ± 0.01

Results presented as mean ± standard deviation. n=3.

3.2 Static adsorption and desorption tests

Figures 1 and 2 show adsorption and desorption parameters of PHTFSW, respectively, regarding the different resins evaluated (XAD 7HP, XAD 4, and XAD 16). Regarding the different pH tested, the results demonstrate that pH 3 was the most suitable for adsorption, showing higher adsorption capacity, regardless of the resin used. This fact may be due to the isoelectric point of most proteins that ranges from 4 to 7 [34]. Therefore, PHTFSW bioactive peptides are dissolved at pH 3, resulting in an increased contact area between macroporous resin surface and peptides molecules.

XAD 7HP presented the highest adsorption capacity (337.90 mg/g) and adsorption ratio (96.37%) (Figure 1), which can be attributed to its higher pore size (Table 1) and electrostatic interactions between charged bioactive peptides and XAD 7HP, which showed more similar polarity. These results are in accordance with previous studies that demonstrated the influence of polarity and pore size on adsorption capacity [35,36].

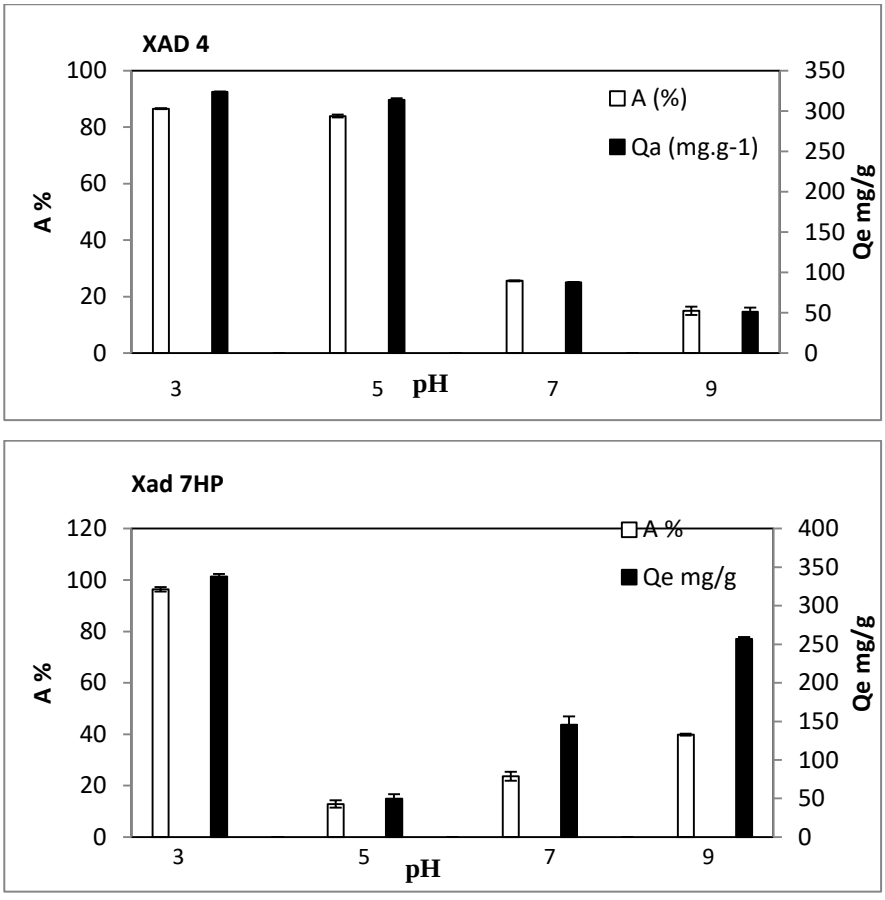
Electrostatic interactions can be derived from negative and positive charged amino acids like lysine, arginine, glutamic acid, and aspartic acid [37], which increases the adsorption capacity of bioactive peptides on XAD 7HP. Thus, polarity can be affected by unique sequence of polar amino acids liberated or available in smaller peptides chains, including serine, threonine, cysteine, asparagine, glutamine, and tyrosine

In contrast, XAD 4 showed the lowest adsorption capacity (323.61 mg PHTFSW/g), with an adsorption rate of 86.48% (Figure 1). As shown in Table 1, the

small pore size and non-polarity characteristic of XAD 4 resins can explain their slightly low adsorption capacity.

Regarding desorption parameters, the lowest desorption ratio (75.35%) of PHTFSW was observed for XAD 4, while the highest values (91.48%) was determined when XAD 7HP was used (Figure 2). For desorption capacities, the values ranged from 242.07 (XAD 16) to 305.23 mg/g (XAD 7HP).

The highest recovery of PHTFSW was found for XAD 7HP (87.05%) ($p \leq 0.05$); however, there was no significant difference for this parameter between XAD 4 and XAD 16 ($p > 0.05$) (Figure 3). Similar results were reported by Zhuang M et.al. (2016) [36], who showed that XAD-16 was the most effective resin for the enrichment of umami peptides.



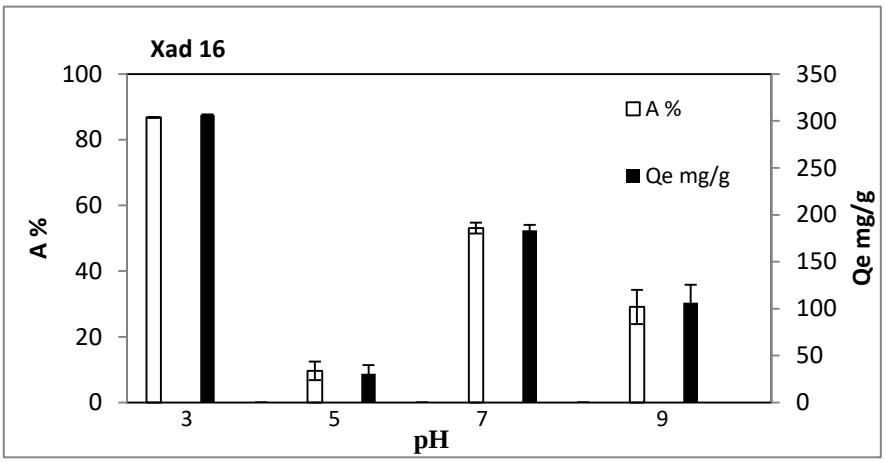


Fig.1 Static adsorption capacity Qa ($mg.g^{-1}$) and adsorption ratio A (%) of protein hydrolysates from tilapia fish skin waste using different macroporous resins. Results are mean of two determinations.

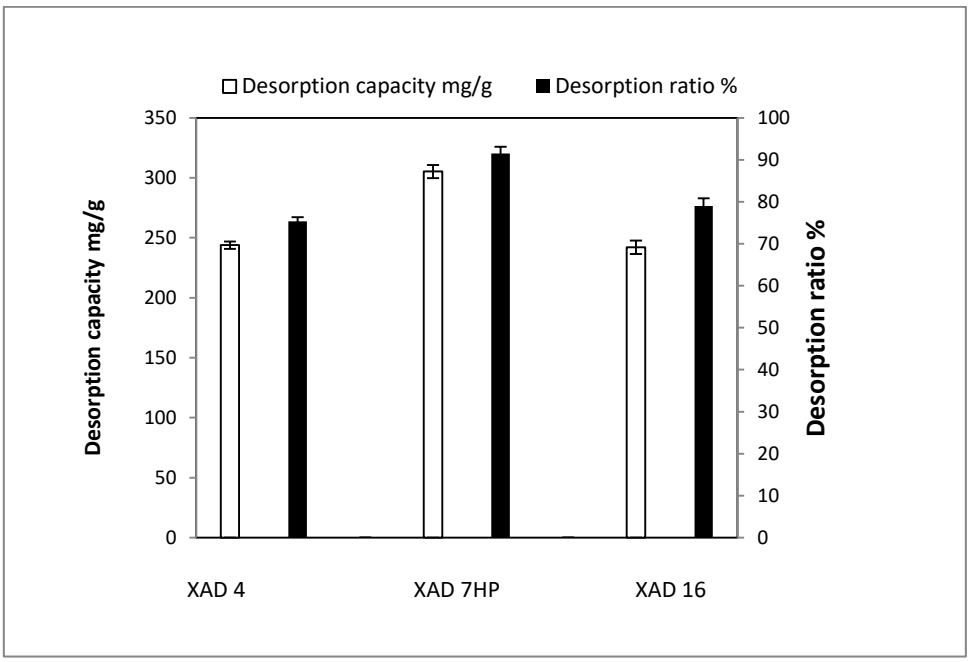


Fig. 2 Static desorption capacity and desorption ratio of protein hydrolysates from tilapia fish skin waste using different macroporous resins.

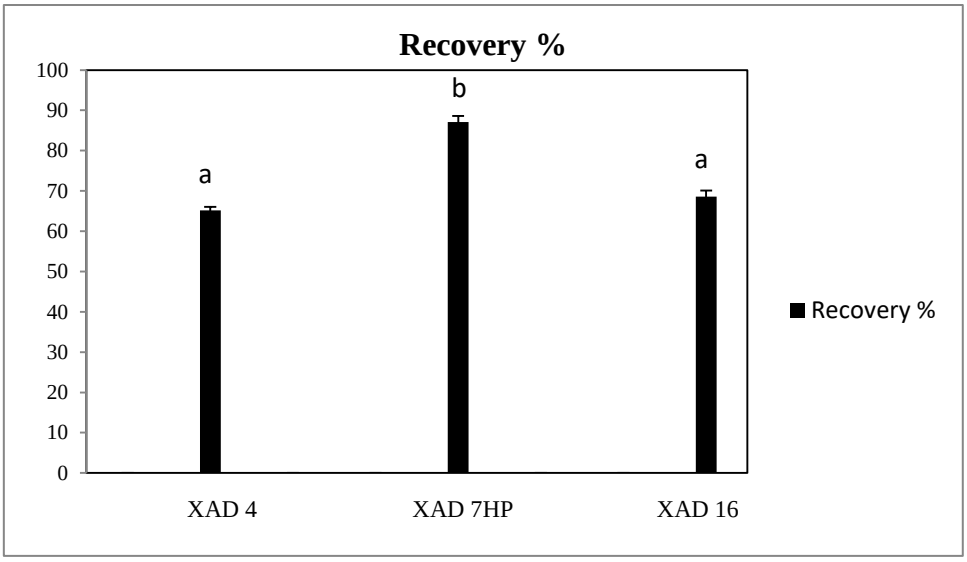


Fig. 3 recovery ratio of protein hydrolysates from tilapia fish skin waste using different macroporous resins. Different lower- indicate significant differences of related bars ($p \leq 0.05$).

3.3 Adsorption isotherm tests

As XAD 7HP showed the highest recovery percentage, adsorption isotherm tests were performed with this resin. The adsorption capacities of PHTFSW increased with the initial concentration of 0.125 % of nitrogen. This is in accordance with other reports which studied the adsorption process of another bioactive compounds [38,39]. Nevertheless, for XAD 7HP, the adsorption capacity decreased as the temperature increased from 40 °C to 60 °C (Figure 4), which is related to the decreasing observed for the predicted values of maximum adsorption capacity (Q_{max}) (Table 3). Therefore, the results suggest that the adsorption process of PHTFSW on XAD-7HP was exothermic, which is in conformity with results determined in thermodynamic studies [40,35].

To set up the optimum model, the experimental data of equilibrium were fitted to the Langmuir and Freundlich models according to the parameters and regression coefficients (R^2) presented in Table 3. The highest results for regression coefficients indicate that the models were suitable to describe the tested adsorption system in the concentration range studied. According to the values of Langmuir constant (K_l), the exothermic process is favorable if $0 < K_l < 1$, linear if $K_l = 1$, and unfavorable if $K_l > 1$. Therefore, the values of K_l at different temperatures summarized in Table 3 suggest that the isotherm of XAD-7HP was unfavorable.

Compared with the Langmuir isotherm model, the Freundlich model reflects the adsorption process on a heterogeneous surface and is suitable to describe adsorption in a narrow range of solute concentrations [41]. K_F is a Freundlich affinity parameter for a hetero-disperse system, the n value is related to the magnitude, which is related to the sorption driving force and the energy distribution of the sorption sites, and the value of 1/n indicates the type of isotherm [42]. Considering the values of 1/n, the adsorption is favorable ($0 < 1/n < 1$) or unfavorable if 1/n value exceeds 1. As shown in Table 3, the values of 1/n of the Freundlich equation reflect that the adsorption of PHTFSW on XAD-7HP was favorable to occur at the selected temperature. Furthermore, the R^2 values of the Freundlich model on XAD 7HP were high and may confirm to the promising potential of this resin for PHTFSW purification [40].

Table 3 Langmuir and Freundlich constants of protein hydrolysates from tilapia fish skin waste on Amberlite XAD 7HP

Table 3 Langmuir and Freundlich constants of protein hydrolysates from tilapia fish skin waste on Amberlite XAD 7HP

Model	**T (°C)**	**K_l**	**Q_{max}**	**(R^2)**
Langmir				
XAD 7HP	40	1.012	873.24	0.995
	50	1.777	818.899	0.994
	60	4.098	805.46	0.997
Model	**T (°C)**	**1/n**	**K_f**	**(R^2)**
Freundlich				
XAD 7HP	40	0.518	691.89	0.987
	50	0.627	534.21	0.985
	60	0.712	454.61	0.999

Q_{max} - maximum adsorption capacity. R^2 - regression coefficient. K_l - Langmuir constant.. K_f - Freundlich constant. n – adsorption intensity.

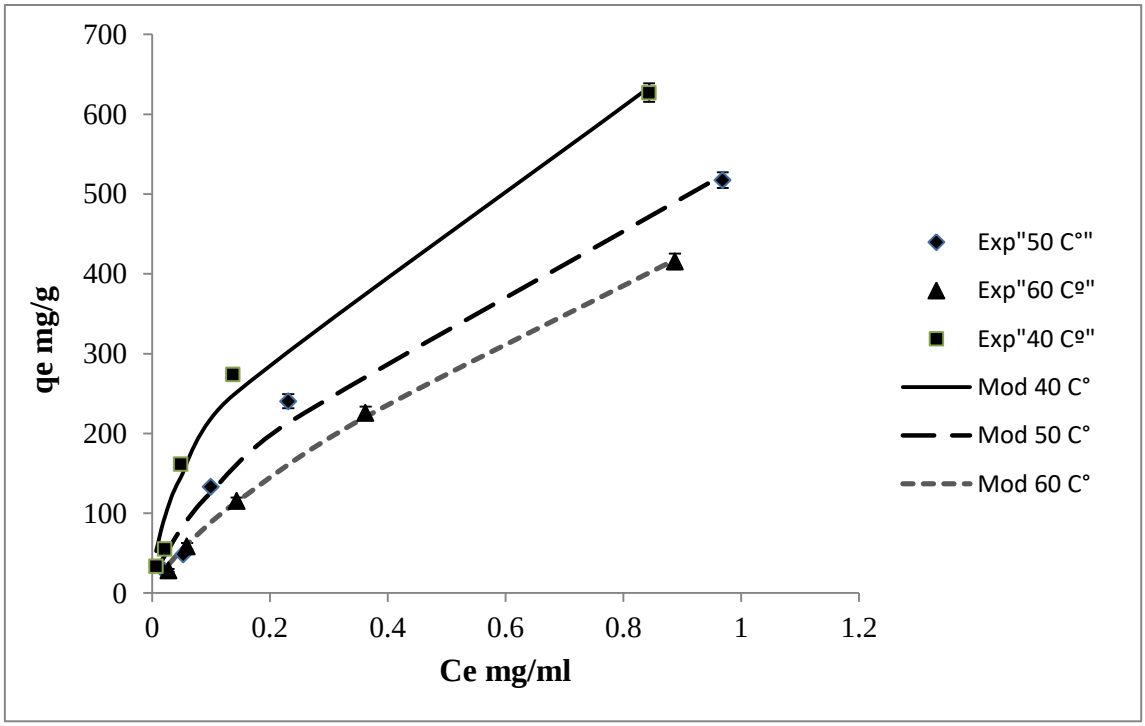


Fig.4 Adsorption isotherms of PHTFSW based on Freundlich equation on XAD 7HP resin, qe is the adsorption capacity (mg/g dry resin) and C_e is the equilibrium concentration of the total peptides (mg/mL), (Mod= model values, Exp= experimental values). Results are mean of two determinations.

3.4 Amino acids profile of PHTFSW

To investigate the effects of purification on the amino acids profile of PHTFSW, chromatography analyses were carried out with the purified extract, treated with NAD 7HP, and the extract not submitted to the treatment with the resins (control). As presented in Table 4, the separation process of PHTFSW using XAD 7HP resin had no significant effect on the structure of amino acids for initial and purified PHTFSW extract. The chromatographic analyses allowed the identification of sixteen amino acids: HYP, ASP, SER, GLU, GLY, HIS, ARG, THR, ALA, PRO, TYR, VAL, LYS, ILE, LEU and PHE, which were determined in both control and purified PHTFSW.

XAD 7HP presented high capacity for amino acids recovery. Therefore purified extract of PHTFSW using XAD 7HP resin can be used as source of amino acids and bioactive peptides for food and pharmaceutical applications.

The purified bioactive peptides and separated amino acids have an important availability to loaded them to other nanomaterial like gold nanoparticles (Au NPs), consequently these purified peptides can be considered as promising therapeutic agents [43], these biological properties of purified peptides belong to various interactions of

the unique sequence of amino acids across peptides including hydrophobic, electrostatic, π-π stacking to form 2 or 3D structures and hydrogen bonding [44].

Table 4 Chromatographic and spectroscopic characteristics, and composition of amino acids from normal and purified extract of PHTFSW obtained by HPLC–FLD.

Sample	Concentration (g/100 g)															
	HYP	ASP	SER	GLU	GLY	HIS	ARG	THR	ALA	PRO	TYR	VAL	LYS	ILE	LEU	PHE
PHTFSW purified extract	6.28	2.23	1.76	3.58	7.18	0.13	4.07	1.46	3.76	5.14	0.05	1.11	1.39	0.70	1.58	1.39
PHTFSW normal extract	2.03	1.05	0.76	1.54	2.59	0.13	1.28	0.61	1.69	2.35	0.06	0.65	0.50	0.47	0.94	0.87

HYP - Hydroxyproline; ASP - Aspartic; SER - Serine; GLU - Glutamic; GLY – Glycine; HIS - Histidine; ARG – Arginine; THR - Treonine; ALA - alanina; PRO - Proline; TYR – Tyrosine; VAL - Valine; LYS – Lysine; ILE- Isoleucine; LEU - Leucine; PHE – phenylalanine.

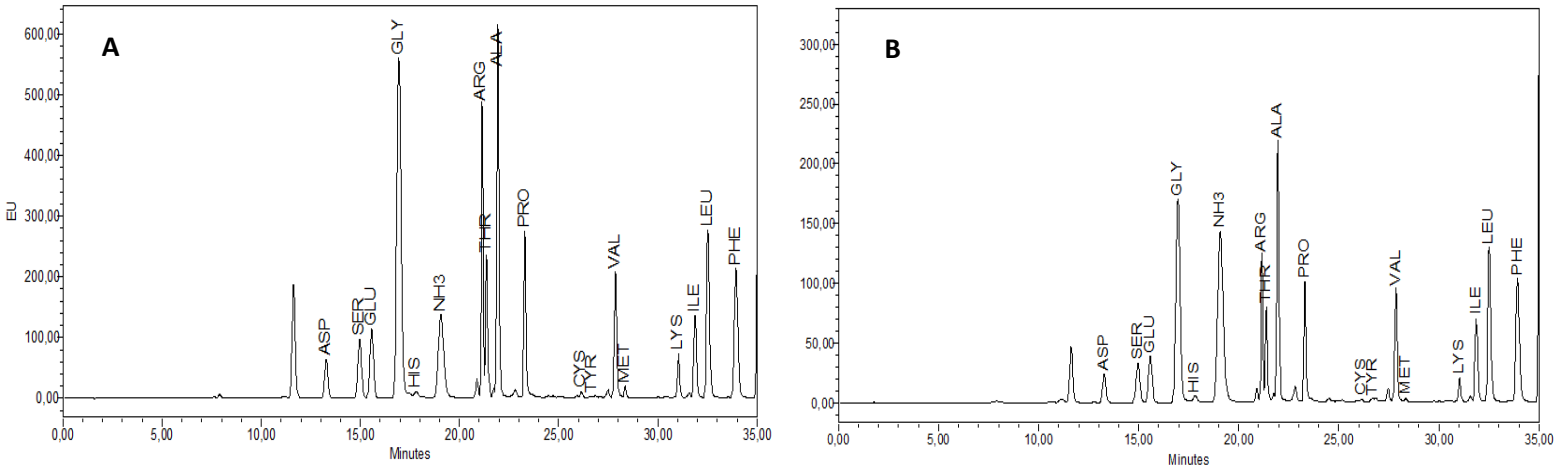


Fig.5 High-performance liquid chromatography separation of amino acids from normal (A) and purified extract (B) of PHTFSW.

3.5 Antioxidant capacity of PHTFSW

DPPH and FRAP assays were employed to evaluate the antioxidant capacities of purified and control PHTFSW, as shown in Figure 6. The results indicate that purified and normal extract of PHTFSW showed high antioxidant capacity in both methods of FRAP and DPPH. Antioxidant capacity measured by DPPH was 25.54 ± 0.77 (mg TE g^{-1}), 31.15 ± 1.7 (mg TE g^{-1}) in normal and purified extract of PHTFSW respectively. Antioxidant capacity measured by FRAP was 24.82 ± 0.7 (mg TE g^{-1}), 31.91 ± 0.03 (mg TE g^{-1}) in normal and purified extract of PHTFSW. According to results in Fig. 6 there was no significant difference ($p > 0.05$) in the antioxidant value of the two methods FRAP and DPPH. However, significant deference in antioxidant capacity was observed between purified and normal extract of PHTFSW, purified extract presented higher antioxidant capacity than normal one, which means the useful of separation process of bioactive peptides using XAD 7HP resin to produce peptides with more biological activity.

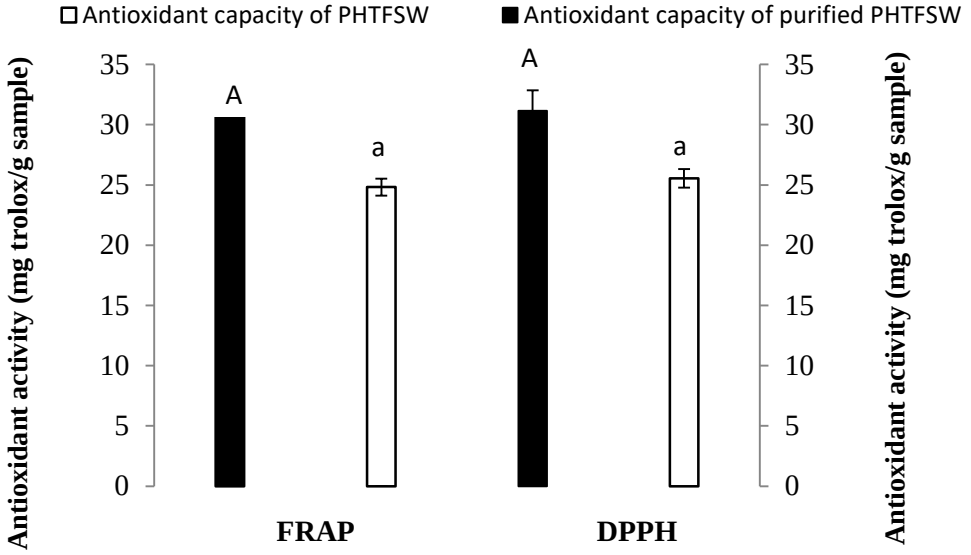


Fig.6 Antioxidant activity of initial and purified PHTFSW using XAD 7HP. Results are mean of three determinations. Similar upper-case letter and similar lower- case letter indicate no significant differences between both methods of FRAP and DPPH ($p > 0.05$).

4. Conclusions

This study has contributed to the understanding on the separation and purification PHTFSW using different kinds of macroporous resins, including XAD 4,

XAD-7HP and XAD 16.The purification process of bioactive peptides using macroporous resins serve to create the first method for protein sample preparation for more effective HPLC analysis. The highest recovery of PHTFSW (87.05%) was presented using XAD 7HP resins due to their similarity to the polarity of PHTFSW. Furthermore, Freundlich model provided great accuracy in the separation of PHTFSW for XAD-7HP resin. The exothermic feature of the adsorption process of PHTFSW was confirmed based on the results of adsorption isotherm tests. The same structure of all amino acids for initial and purified PHTFSW extract was presented by HPLC-FLD analysis which confirms the possibility to use macroporous resins to separate and purify of bioactive peptides, furthermore, XAD 7HP resin can be used to prepare protein samples for HPLC analysis by separate the peptides from complex system. In comparing with normal extract of PHTFSW, the purified extract of PHTFSW showed higher antioxidant capacity in both methods of FRAP and DPPH. Therefore, purified PHTFSW could potentially find application as natural bioactive compounds, dietary supplement, antioxidant ingredient for functional foods, and as a raw material in the cosmetic and pharmaceutical industry preparations.

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Conclusões Gerais

No capitulo I apresentou que os hidrolisados proteicos têm ganhado grande interesse nos últimos anos, uma vez que a modificação por enzimas ou produtos químicos melhora as propriedades funcionais das proteínas nativas e sua utilidade como ingredientes intermediários nos setores cosmético, farmacêutico, alimentício e nutracêutico.

No capítulo II. O artigo de revisão apresentou os estudos recentes sobre importância de hidrólise enzimática da proteína com controle das condições de reação para melhorar sua qualidade e propriedades funcionais, como solubilidade, capacidade de retenção de óleo e capacidade de retenção de água, emulsificação, formação de espuma e propriedades sensoriais. Além disso, os estudos mais recentes sobre separação dos hidrolisados proteicos foram apresentados, incluindo método centrífugo, método de filtração por membrana, resinas macroporosas, meios de acesso restrito e técnicas de separação cromatográfica.

No capítulo III, os hidrolisados proteicos obtidos a partir de resíduo de pele de peixe da tilápia apresentaram maior atividade antioxidante na condição otimizada de hidrólise enzimática. O desenho composto central foi adotado para otimizar as variáveis independentes. Os resultados mostraram que a temperatura e o pH (58.4 ℃, pH 8.7) são fatores importantes que podem afetar o grau de hidrólise e a atividade antioxidante dos hidrolisados.

No capítulo IV, a cinética da reação foi considerada em relação à concentração inicial do substrato, concentração inicial da enzima e tempo de hidrólise. As condições otimizadas do processo de hidrólise enzimática da proteína obtida a partir de resíduos de pele de peixe da tilápia RPPT foram pH 8,7, temperatura 58,4 ºC e tempo 120 min, DH aumentou com o aumento da concentração inicial da enzima E_0, sob concentração constante de substrato conforme o tempo de hidrólise se prolongou para 120 min. Além disso, a DH diminuiu com o aumento da concentração inicial de substrato S_0 sob uma concentração constante de enzima à medida que o tempo de hidrólise se prolongou para 120 min.

No capítulo V, adsorção e dessorção de hidrolisados proteicos obtidos de resíduos de pele de peixe da tilápia (HPRPT) utilizando três MARs (XAD 7HP, XAD 4 e XAD 16) foram avaliados. XAD-7HP apresentou as maiores capacidades de adsorção e dessorção. Os testes isotérmicos indicaram que o mecanismo de adsorção foi melhor explicado usando o modelo de Freundlich que proporcionou grande precisão na separação de HPRPT para resina XAD-7HP. A mesma estrutura de todos os aminoácidos para o extrato HPRPT inicial e purificado foi apresentada pela análise HPLC-FLD que confirma a possibilidade de usar resinas macroporosas para separar e purificar peptídeos bioativos, além disso, a resina XAD 7HP pode ser usada para preparar amostras de proteínas para HPLC análise pela separação os

peptídeos do sistema complexo. Em comparação com o extrato normal de HPRPT, o extrato purificado de HPRPT apresentou maior capacidade antioxidante em ambos os métodos de FRAP e DPPH. Este HPRPT purificado poderia potencialmente encontrar aplicação como compostos bioativos naturais brutos nas preparações da indústria alimentícia, cosmética e farmacêutica.

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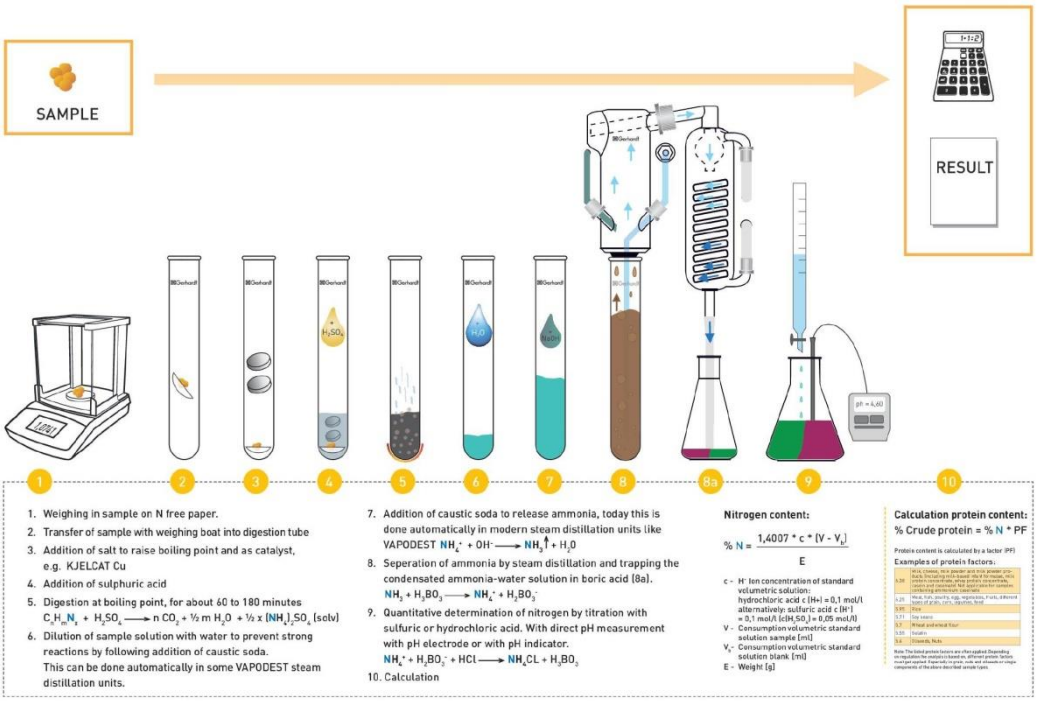
Annexes

Annex A

1. Metodologias detalhadas utilizadas no Capítulo III

1.1. The Kjeldahl method was used to determine the nitrogen and protein



1.2. DPPH assay

Extracts (150 µL) of PHTFSW reacted with 2.85 mL of the DPPH* solution (0.06 mM) for 1 h in the dark. Then the absorbance was taken at 517 nm, in spectrophotometer (*Spectrophotometer Model NOVA 2000 UV*).

Trolox standard solutions were analyzed for the calibration curve construction. Free radical scavenging was expressed in percentual (%FRS) according to Equation 1.

$$\%\,FRS = \frac{(Abs_B - Abs_A)}{Abs_B} \times 10 \quad \text{Equação (1)}$$

Where:

Abs_B = spectrophotometer absorbance of control read at 517 nm;

Abs_A = spectrophotometer absorbance of PHTRSW extract read at 517 nm;

The antioxidant capacity was expressed by μM of Trolox equivalent per gram of wet weight (μM TE. g^{-1}) from the regression coefficient obtained of calibration curve.

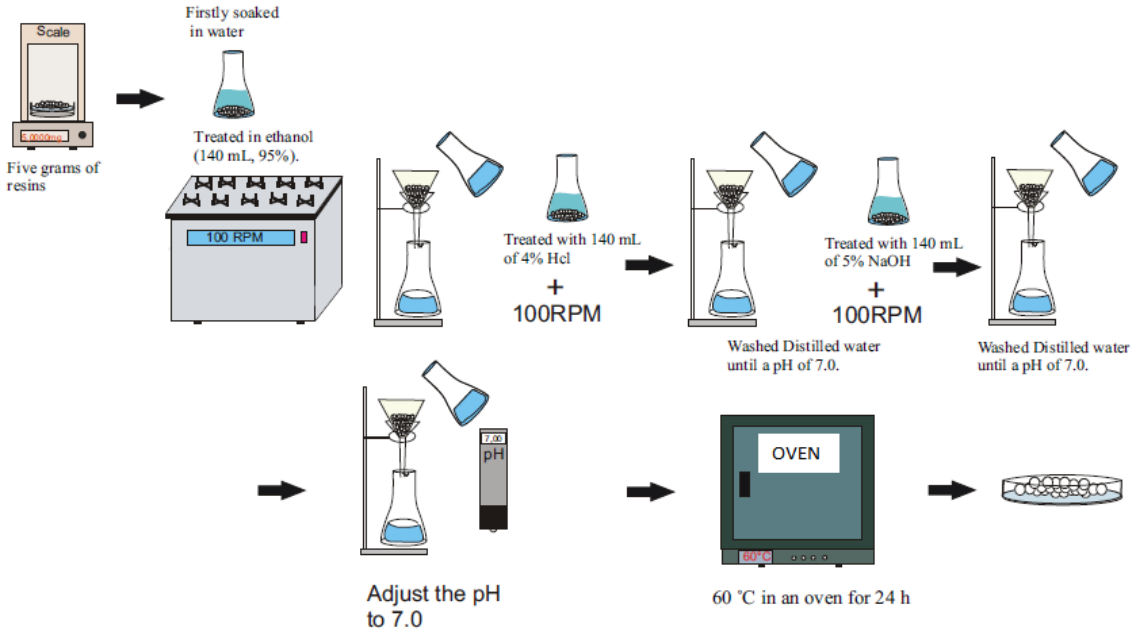
1.3. FRAP assay

90 μL of PHTFSW extract was diluted in distilled water (270 μL) and allowed to react with 2.7 mL of FRAP, wich were mixture by vortex and held in a water bath at 37ºC for 30 minutes in absence of light. Readings of the colored product were taken at 595 nm. The standard curve was linear between 100 and 1000 μM of Trolox equivalent. Results were expressed in μM of Trolox equivalent per gram of wet weight (μM TE. g^{-1}).

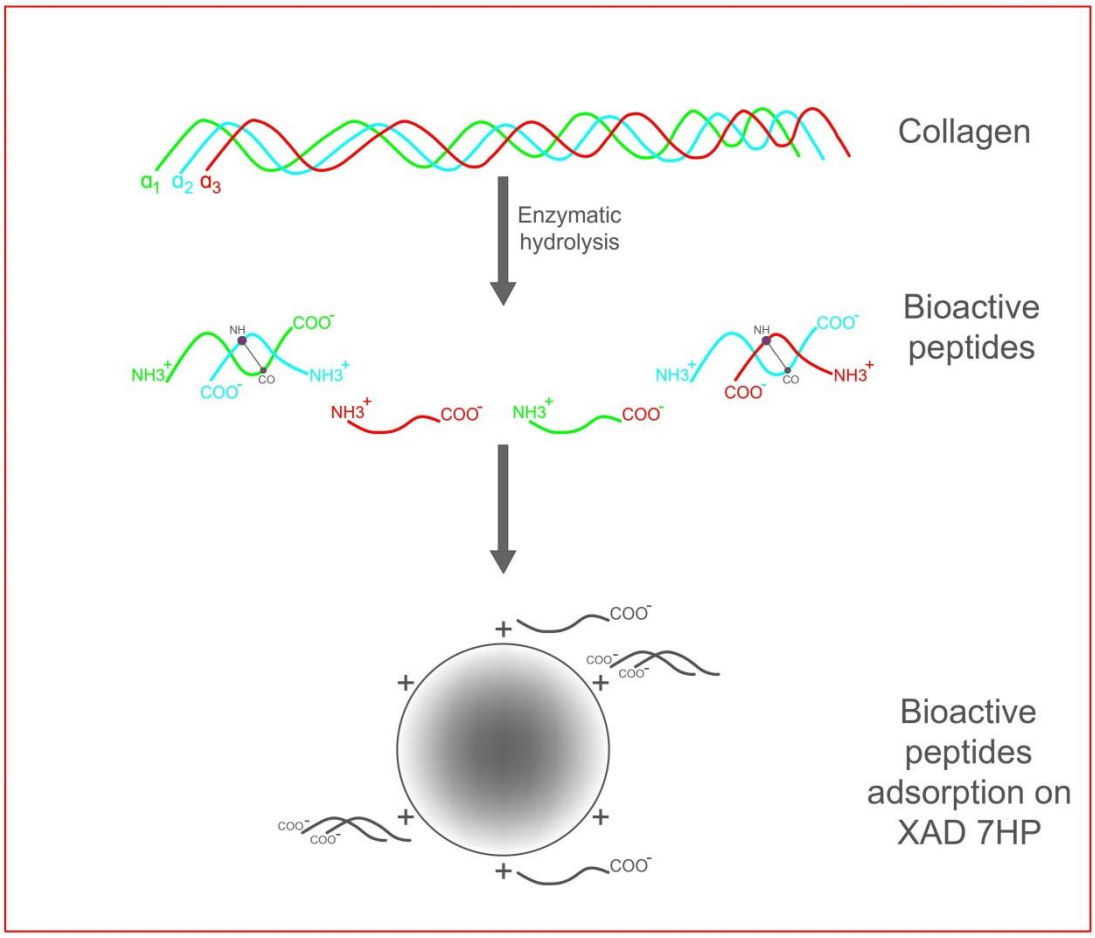
Annex B

1. Metodologias detalhadas utilizadas no Capítulo V

1.1. Pre-treatment of resins



1.2. Static adsorption and desorption tests



1.3. Separation and quantification of amino acids by acid hydrolysis

The methodology reported by Pacheco (2014) [28] and the acid hydrolysis step according to AOAC method number 994.12 [26] were used for the separation and quantification of the main amino acids present in PHTFSW. Subsequently, an aliquot (50 µL) was removed and placed in a vial to be dried in a vacuum desiccator for 24 h with subsequent derivatization reaction [29]. Chromatographic analysis was carried out by using an HPLC system (Waters® Alliance model 2690/5) equipped with a fluorescence detector HPLC-FLD (λexc = 250 nm and λem = 395 nm) and Empower® software (2002) (Waters Corporation, Milford, USA). A Thermo BDS HYPERSIL C18 column (100 × 4.6 mm; 2.4 µm) (Thermo Fisher Scientific, Waltham, USA) was used at 37°C, and elution in gradient mode with AccQ Tag® (Waters Corporation, Milford, USA); water (1:10; v/v) (Phase A), acetonitrile (Phase B), and water (Phase C) with a

flow of 1.0 mL/min [30]. Quantification was performed by external standardization using an analytical curve of standards (Amino Acid Standard H, Ref. WAT088122) also submitted to the derivatization step with 6-aminoquinolyl-succimidyl-carbamate (AQC).