CHARACTERISATION OF BUFFALO (*Bubalus bubalis*) BONE GELATIN EXTRACTED USING ACID AND PAPAIN PRE-TREATMENT

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FACULTY OF FOOD SCIENCE AND NUTRITION
UNIVERSITI MALAYSIA SABAH
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I hereby declare that the material in this thesis is my own except for quotations, equations, summaries, and references, which have been duly acknowledged.

23 March 2023

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ABSTRACT

Gelatin is a versatile biopolymer with numerous industrial uses, but traditional sources like bovine and porcine raise safety and religious concerns. Alternatives like fish gelatin often require further refining for quality enhancement. Buffalo bone gelatin offers a promising solution as it is free from diseases and religious restrictions. The extraction process, however, requires a thorough understanding of the complex bone structure to recover collagen for gelatin production. The extraction of gelatin from buffalo bone has yet to be extensively explored and holds great potential. This study explored the effects of pre-treatment conditions using different acids and concentrations, with and without papain, on the properties and extractability of gelatin derived from buffalo bone. The buffalo bone was pre-treated with hydrochloric acid (HCl) and citric acid at 0.05 M and 0.025 M concentrations, respectively, with and without papain (100 U/g), prior to hot water extraction. The study compared the physicochemical and functional properties of buffalo bone gelatin with standard bovine gelatin (SBG). The highest yield (6.36 %, dry weight) and hydroxyproline content (26.98 g/100g) was obtained from gelatin extracted using 0.025 M HCl pre-treatment without papain. Pre-treatment with 0.05 M citric acid without papain resulted in the lowest yield (3.79 %, dry weight) with hydroxyproline content of 12.49 g/100g. Papain significantly influenced the yield of gelatin in citric acid but not in HCl treatment when used at 100 U/g. Buffalo bone gelatin had significantly ($p<0.05$) lower moisture content than SBG and had a pH range of 5.11 to 5.44, indicating it is Type A gelatin. The gelatin was whiter with HCl pre-treatment and slightly yellow with citric acid pre-treatment. Low ash content (<2%) confirmed effective removal of minerals. Gelatin treated with 0.025 M citric acid without papain had the highest emulsion activity index (9.52 m^2/g), emulsion stability index (30.29 min), foaming expansion (13.66 %), foaming stability (10.64 to 12.46 min), water holding capacity (11.68 mL/g), and fat binding capacity (8.88 mL/g). The Fourier Transform Infrared Spectroscopy (FTIR) results of all gelatin shown a similar secondary structure to SBG with the presence of Amide A, B, I, II and III. Papain used in HCl and citric acid pre-treatment resulted in degradation of α and ß components, which produced the lowest gel strength (20.35 g by 0.05 M citric acid with papain). The highest gel strength (50.64 g) was produced from 0.025 M citric acid without papain treatment. Buffalo bone gelatin shows potential as an alternative source of halal gelatin manufacturing.

Keywords : Buffalo, bone, papain-aided, pre-treatment, physicochemical properties, functional properties

ABSTRAK

PENCIRIAN GELATIN TULANG KERBAU (Bubalus bubalis) YANG DIEKSTRAK DENGAN PRA-RAWATAN ASID DAN ENZIM PAPAIN

Gelatin ialah biopolimer serba boleh dengan pelbagai aplikasi industri, tetapi sumber konvensional seperti lembu dan babi menimbulkan kebimbangan keselamatan dan kekangan agama. Alternatif seperti gelatin ikan sering memerlukan pemprosesan lanjut untuk menjamin kualiti gelatin yang baik. Gelatin tulang kerbau menawarkan penyelesaian yang menjanjikan kerana ia bebas daripada penyakit dan sekatan agama. Proses pengekstrakan, bagaimanapun, memerlukan pemahaman yang menyeluruh tentang struktur tulang yang kompleks untuk memperoleh kolagen untuk penghasilan gelatin. Pengekstrakan gelatin daripada tulang kerbau masih belum diterokai secara meluas dan mempunyai potensi yang tinggi. Kajian ini meneroka kesan pra-rawatan menggunakan asid dengan kepekatan yang berbeza, dengan dan tanpa papain, terhadap ciri-ciri dan kebolehekstrakan gelatin yang diekstrak daripada tulang kerbau. Tulang kerbau telah dirawat terlebih dahulu dengan asid hidroklorik (HCl) dan asid sitrik pada kepekatan 0.05 M dan 0.025 M, masing-masing, dengan dan tanpa papain (100 U/g), sebelum pengekstrakan terma. Kajian ini membandingkan sifat fizikokimia dan berfungsi gelatin tulang kerbau dengan gelatin lembu piawai (SBG). Hasil tertinggi (6.36 %, berat kering) dan kandungan hidroksiprolin (26.98 g/100g) diperoleh daripada gelatin yang diekstrak menggunakan pra-rawatan 0.025 M HCl tanpa papain. Pra-rawatan dengan asid sitrik 0.05 M tanpa papain menunjukkan hasil yang paling rendah (3.79 %, berat kering) dengan kandungan hidroksiprolin sebanyak 12.49 g/100g. Papain mempengaruhi hasil gelatin secara signifikan dalam pra-rawatan asid sitrik tetapi tidak dalam pra-rawatan HCl apabila digunakan pada 100 U/g. Gelatin tulang kerbau mempunyai kandungan lembapan yang jauh lebih rendah ($p<0.05$) daripada SBG dan mempunyai julat pH 5.11 hingga 5.44, menunjukkan ia adalah gelatin Jenis A. Gelatin lebih cerah dengan pra-rawatan HCl dan sedikit kuning dengan pra-rawatan asid sitrik. Kandungan abu yang rendah (<2%) menunjukkan penyingkiran mineral yang berkesan. Gelatin yang dirawat dengan asid sitrik 0.025 M tanpa papain mempunyai indeks aktiviti emulsi tertinggi (9.52 m^2/g), indeks kestabilan emulsi (30.29 min), pengembangan berbuih (13.66 %), kestabilan berbuih (10.64 hingga 12.46 min), kapasiti pegangan air (11.68 mL/g), dan kapasiti mengikat lemak (8.88 mL/g). Keputusan Fourier Transform Infrared Spectroscopy (FTIR) semua gelatin menunjukkan struktur sekunder yang serupa dengan SBG dengan kehadiran Amida A, B, I, II dan III. Papain yang digunakan dalam pra-rawatan HCl dan asid sitrik mengakibatkan degradasi komponen α dan ß, yang menghasilkan kekuatan gel yang paling rendah (20.35 g dirawat dengan 0.05 M asid sitrik dengan papain). Kekuatan gel tertinggi (50.64 g) dihasilkan daripada asid sitrik 0.025 M tanpa papain. Gelatin tulang kerbau menunjukkan potensi sebagai sumber alternatif penghasilan gelatin halal.

Kata Kunci : Kerbau, tulang, papain, pra-rawatan, sifat fizikokimia, sifat berfungsi

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LIST OF ABBREVIATIONS

AABA	-	Aminobutyric acid
ANOVA	-	Analysis of Variance
AOAC	-	Association of Official Analytical Collaboration
BSE	-	Bovine Spongiform Encephalopathy
DFP	-	di-isopropyl fluorophosphate
DNA	-	Deoxyribonucleic Acid
EAI	-	Emulsion Activity Index
EDTA	-	Ethylenediaminetetraacetic acid
ES	-	Emulsion Stability
ESI	-	Emulsion Stability Index
FAOSTAT	-	Food and Agriculture Organization Corporate Statistical Database
FBC	-	Fat Binding Capacity
FC	-	Foaming Capacity
FD	-	Fluorescence detector
FE	-	Foaming Expansion
FMD	-	Foot and Mouth Disease
FS	-	Foaming Stability
FTIR	-	Fourier transform infrared
GME	-	Gelatin Manufacturers of Europe
GMIA	-	The Gelatin Manufacturers Institute of America
HPLC	-	High Performance Liquid Chromatography
IBM	-	International Business Machines
MOA	-	Ministry of Agriculture
NC	-	Not conducted
ND	-	Not documented
NM	-	Not mentioned

PAGE	-	Polyacrylamide gel electrophoresis
PCMB	-	p-chloromercuribenzoate
PRNP	-	Prion protein gene
RBF	-	Raw buffalo bone
RM	-	Ringgit Malaysia
RNA	-	Ribonucleic acid
RSM	-	Response surface methodology
SBG	-	Standard bovine gelatin
SD	-	Standard deviation
SDS	-	Sodium dodecyl-sulfate
SPSS	-	Statistical Package for Social Sciences
TA	-	Texture analyser
TSE	-	Transmissible Spongiform Encephalopathy
UK	-	United Kingdom
USA	-	United States of America
USD	-	United States Dollar

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Gelatin is a protein produced primarily from skin and bone and is partially hydrolysed and denatured by thermal treatments (Mulyani *et al.,* 2021). The global demand for gelatin has been increasing in the market for the past decade. It accounts for gelatin's unique characteristics that make it useful in various industrial applications. In 2020, the global gelatin market was valued at USD 2.43 billion and was expected to reach USD 3.44 billion in 2026 (Expert Market Research, 2021). Meanwhile, in 2021, the global food gelatin market was valued at USD 3.32 billion and is projected to reach USD 4.05 billion in 2026 (Marketwatch, 2021).

Gelatin is one of the most widely used natural high molecular weight biopolymers in industries derived from collagen because of its unique functional and technological properties (Alfaro *et al.,* 2014). This led to the application of gelatin in various industries such as food, cosmetics, pharmaceutical, photography, technical and medical sector. Gelatin unique properties make it effective as an emulsifying, foaming, stabilising, and microencapsulating agent, make it important to the industry.

In food, gelatin is used as an ingredient to enhance food properties, particularly the elasticity, consistency, and stability of the food products. For instance, gelatin act as a gelling and emulsifying agent in jellies, cakes, aspics, marshmallow, yoghurt, ice cream and sweets (Zilhadia *et al.,* 2018). In addition, gelatin can improve the chewiness, texture and foam stability in confectionery products (Santoso *et al.,* 2019). Other than that, gelatin can exert creaminess and mouth-feel sensation in low-fat spreads (Choe & Kim, 2018), stabilise the batter for baked goods (Aisyah *et al.,* 2014) and provide water-binding capabilities in meat products (Karim & Bhat, 2009). In medical sectors, gelatin provides medicinal delivery capacity, which regulates the release of active ingredients and microencapsulation properties (Karim & Bhat, 2009). In line with the rising market values and industrial applications in gelatin, gelatin production is also soaring globally.

Gelatin can be extracted from the skins and bones of mammals, poultry, marine animals, and insects (Mariod & Adam, 2013). Industrially, gelatin is produced using acid and alkali in the pre-treatment process, resulting in Type A from acidic treatment and Type B gelatin from alkaline treatment (GMIA, 2019). In general, acidic treatment is used when extracting gelatin from the skin matrix, and alkaline treatment is applied to a more complex matrix such as bone (GMIA, 2019). Conventionally, the acidic pre-treatment of porcine skins will take up around 10 – 45 hours of soaking time (Ahmad *et al.,* 2017). Besides, the alkaline treatment of bones and hides usually consumes a more prolonged duration of 30 – 100 days (Damrongsakkul *et al.,* 2008). The insoluble collagen needs to be transformed into a soluble form by exposure to acid or alkali during the pre-treatment process. This will cause the breakdown of the ordered structure of native collagen, which will induce the swelling of the structure. Then, the conversion into gelatin happened during the extraction process. The extraction process involves the cleaving of hydrogen and covalent bond by the thermal treatment, which promotes the helix-to-coil transition and releases free α-chains and oligomers (Ahmad *et al.,* 2018). Meanwhile, the hydrolysis of some amide bonds in the collagen molecules simultaneously occurs during the extraction process and produces gelatin that possesses a mixture of polypeptide fragments with molecular weight ranging from 16-150 kDa (Ahmad *et al.,* 2018). Since the process of producing gelatin takes a long time and can be quite tedious, the application of protease enzymes to assist the extraction process has been of interest.

Generally, gelatin extractability and properties depend upon the nature of the raw material such as the age of animal, type, and matrix of tissues, which can be manipulated through the extraction parameters and conditions. Serial chemical and enzymatic treatment steps at diverse concentrations and exposure times have been studied diligently to extract the gelatin from various animal tissues. The use of acid pre-treatment in gelatin extraction from multiple sources has proven to be effective in several research for gelatin from pecking duck skin (Teng *et al.,* 2021), fish bone waste from shark, tuna and milkfish (Hapsari *et al.,* 2018), duck feet (Nik Muhammad et al., 2017), buffalo hide (Mulyani *et al.,* 2017ab), and bovine bone (Arioui *et al.,* 2018). Meanwhile, for the alkaline treatment, few research in the extraction using NaOH has shown a good yield recovery and properties of gelatin produced such as gelatin from buffalo hide and skins (Rabiatul Amirah *et al.,* 2020), mechanically deboned meat residue (Erge & Zorba, 2018), *Alaska pollock* skin (Zhou & Regenstein, 2005), and yak skin (Xu *et al.,* 2017a:2017b).

According to Wang *et al.* (2017), protein enzymatic hydrolysis using proteolytic enzymes is quite popular in the food industry. One of the unique properties of enzymes is their ability to hydrolyse proteins more gently than acids and alkalis. Enzymatic proteolysis from animal and plant sources has been extensively studied and described by several authors over the last decades, and it is the most commonly used method for adding value to underutilised species. Commercial enzymes for preparing collagen hydrolysates include papain, α-chymotrypsin, bromelain, pepsin, and trypsin. They do not require high temperatures and typically cleave specific peptide bonds, which may increase yield recovery and shorten extraction time (Motyan *et al.,* 2013).

Several proteases have been incorporated in gelatin extraction, such as pepsin from fish by-products like Thornback ray skin (Lassoued *et al.,* 2014) and bigeye snapper skin (Nalinanon *et al.,* 2008). Besides, pepsin is also used in gelatin extraction from bovine bone (Cao *et al.,* 2020), ovine bones (Gao *et al.,* 2018) and bovine skins (Ahmad *et al.,* 2021). Other than that, plant enzymes such as papain are actively used in gelatin production from buffalo bones (Samatra *et al.,* 2020), *Barramundi* fish skin (Jamilah *et al.,* 2013), Alaska pollock and yellowfin sole fish bones (Mi *et al.,* 2019). However, the enzymatic treatment needs to be monitored closely to avoid over hydrolysis of the gelatin structure that could compromise the properties of gelatin produced due to enzymes are easily affected by experimental conditions such as hydrolysis pH, temperature, duration, and ionic strength.

Many previous works had reported of extracting gelatin from bovine, marine sources and poultry. Gelatin were successfully extracted from the hides and bones of bovine, buffalo, and yak has been reported by many scientists worldwide (Wulandari *et al.,* 2016; Ahmad *et al.,* 2021; Ahmad *et al.,* 2019; Bahar *et al.,* 2018; Arioui *et al.,* 2018; Cao *et al.,* 2020; Mulyani *et al.,* 2017a,2017b; Mulyani *et al.,* 2019; Rabiatul Amirah *et al.,* 2020; Samatra *et al.,* 2020; Xu *et al.,* 2017a; Li *et al.,* 2009). Other than that, the production of gelatin from marine sources also gained lots of interest such as fish skins and bones, squid, and octopus (Abdelmalek *et al.,* 2016; Atma & Taufik, 2021; Hapsari *et al.,* 2018; Haryati *et al.,* 2019; Jridi *et al.,* 2015; Kusumaningrum *et al.,* 2018). Besides, there are several works on the extraction of gelatin from poultry such as chicken skin (Kim *et al.,* 2012; Rasli & Sarbon, 2015), chicken feet (Choe & Kim, 2018; Widyasari & Rawdkuen, 2014), duck skin (Kim *et al.,* 2020; Teng *et al.,* 2021), duck feet (Teng *et al.,* 2021; Park *et al.,* 2013; Kuan *et al.,* 2016). Other alternative resources to extract gelatin has been explored from insect sorghum bug (Mariod & Fadul, 2015) and amphibian for instance, frog (Tümerkan *et al.,* 2019).

The quality of gelatin extracted from all sources has its own advantages and disadvantages related to their properties. The quality of gelatin produced from mammals exerts superior properties compared to marine gelatin which possesses low physicochemical properties and a bad odour (Lin *et al.,* 2017). However, safety and religious constraints associated with the sources of gelatin open new opportunities to explore other alternative sources that meet consumer demand and at the same time, could improve the properties of gelatin.

The buffalo meat production was valued at 936 million in 2017 (MOA, 2017). According to Khalex *et al.* (2021), almost half (46.7 %) of the total buffalo population in Malaysia was found to be located in Sabah. Sabah harbours a diverse range of mammalian species, including the water buffalo (*Bubalus bubalis*) (Zamri-Saad *et al.,* 2017). While this animal is a popular source of meat and bones in local cuisine, its true potential remains untapped possibly due to inadequate infrastructure and processing facilities for its by-products such as skins and bones (Mohd Azmi *et al.,* 2021). As a result, the water buffalo is still considered as underutilised in Sabah (Rabiatul Amirah *et al.,* 2020). In this regard, buffalo can be considered a potential alternative source for gelatin production especially in Malaysia.

The outbreak of prion diseases such as bovine spongiform encephalopathy (BSE) has never been reported about the occurrence in buffalo, as buffalo is genetically unique and less susceptible to prion diseases such as BSE (Zhao *et al.,* 2016). Thus, buffalo is suitable to be explored as an alternative source of gelatin. However, limited studies have managed to extract gelatin from buffalo. Up to our knowledge, only Mulyani *et al.* (2017a; 2017b; 2019) and Rabiatul Amirah *et al.* (2020) extracted gelatin from buffalo skin, and only Samatra *et al.* (2020) reported to extract gelatin from buffalo bone as a preliminary study. Therefore, this study aimed to extract gelatin from buffalo bone using different types of acids and concentrations, with and without the addition of papain during the pre-treatment process. The physicochemical and functional properties of the extracted gelatin were evaluated and compared with the commercial gelatin sold on the market.

1.2 Problem Statement

Until now, gelatin has been considered one of the most widely used biopolymer in various industries. It is owing to gelatin's unique functional and technological properties that make it a beneficial ingredient to be incorporated into various food products. Subsequently, this has caused the soaring demand for gelatin over the years. Generally, leading gelatin sources in the market are derived from porcine and bovine by-products. Porcine skins make up 46 % of global gelatin production, 29.4 % from bovine hides, 23.1 % from bovine bones and 1.5 % from other sources (GME, 2020). This has become a restriction for Muslims and Jewish consumers as they are only allowed to consume halal food (diet for Muslim consumers) and kosher food (diet for Jewish consumers), respectively and the choices of gelatin are limited in the market (Kuan *et al.,* 2016). Besides, gelatin from bovine sources has become of great concern as it has been linked with the spread of bovine spongiform encephalopathy (BSE) and foot and mouth disease (FMD) (Abedinia *et al.,* 2020).

There has been a substantial effort to produce gelatin from marine animals such as fish by-products, as an alternative source of gelatin. However, fish gelatin often needs further refining due to its inferior quality compared to its mammalian counterpart because fish gelatin is commonly associated with lower gelation and melting temperature due to lower hydroxyproline content (Otoni *et al.,* 2012). This has resulted in the attempt to discover gelatin from the underexploited species which might have better quality. Also, the waste from buffalo such as hides and bones contains an ample amount of collagen that can be hydrolysed into gelatin.

The extraction process of gelatin is crucial in ensuring high quality and yield. Despite extensive research on gelatin extraction, there is still a need to improve the process and better understand the role of pre-treatment and extraction methods. This study focuses on the utilisation of acids and enzymes in the extraction process, specifically papain, to evaluate their impact on the yield and the properties of gelatin extracted from buffalo bone. The results of this study may provide a new source of gelatin to substitute for commercial gelatin in the market and highlight the importance of optimised extraction techniques.

1.3 Justification of the study

The extraction process of gelatin is crucial in ensuring high quality and yield. Despite extensive research on gelatin extraction, there is still a need to improve the process and better understand the role of pre-treatment and extraction methods. This study focuses on the utilisation of acids and enzymes in the extraction process, specifically papain, to evaluate their impact on the yield and the properties of gelatin extracted from buffalo bone. The results of this study may provide a new source of gelatin to substitute for commercial gelatin in the market and highlight the importance of optimised extraction techniques.