UNIVERSITI PUTRA MALAYSIA

IMPROVING GELATIN EXTRACTION FROM HIDE USING PLANT ENZYME - ASSISTED PROCESS

TANBIR AHMAD

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By

TANBIR AHMAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

January 2018

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DEDICATION

Recite in the name of your Lord who created۞ Created man from a clinging substance۞ Recite, and your Lord is the most Generous۞ Who taught by the pen ۞ Taught man that which he knew not ۞

(Al-Quran Al-Kareem, Surah Al-'Alaq, 96:1-5)

Every challenging work needs self-efforts as well as the guidance of elders especially those who are close to your heart. Whose love, affection, encouragement and praise for day and night make me able to get such success and honor and the reason of what I become today. My humble effort, I dedicate to my honorable and loving

Father & Mother
Who have always been my epitomes of strength

My brothers
Who have been very supportive and encouraging

My wife and children
Who have been very understanding, patient and did not receive my love during the period due to hectic research work

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

IMPROVING GELATIN EXTRACTION FROM HIDE USING PLANT ENZYME - ASSISTED PROCESS

By

TANBIR AHMAD

January 2018

Chairman : Associate Professor Awis Qurni Sazili, PhD
Faculty : Agriculture

Gelatin is a high molecular weight biopolymer obtained by partial denaturation of collagen brought by hydrolysis. Collagen being a structural protein having three α-chains intertwined by hydrogen and covalent bonds form a very stable right-handed triple helix which requires pretreatment by acid or alkali to convert it into gelatin. This results in low production of gelatin. In the past, researchers have used various proteases to improve the gelatin yield and quality. Use of protease enzymes led to increase in gelatin yield but simultaneous reduction of gelatin quality mainly due to production of gelatin having short chain polypeptides. Gelatin with high molecular weight polypeptides (less degraded peptides) are reported to be better in functional properties. Therefore, it was proposed to use novel enzymes which could cut the collagen chains in such a way so that longer chain proteins could be obtained in the resulting gelatin. Additionally, scanty research works are available on use of ultrasound to extract gelatin and its effect on the yield and quality. Therefore, ultrasound assisted gelatin extraction in conjugation with enzymes was taken up as an objective. Endopeptidase enzymes present in the skin are also reported to be activated at high extraction temperature and cause the degradation of the gelatin protein. Therefore, autolysis study was carried out and suitable endopeptidase inhibitor was used to extract the gelatin.

Four plant enzymes namely actinidin (A), papain (P), bromelain (B) and zingibain (Z) were used to extract gelatin from bovine skin. The skins were pretreated for 48 h with these enzymes at the levels of 0, 5, 10, 15, 20 and 25 unit/g of skin at the respective optimum temperature and pH of the enzymes and the gelatin was extracted at 60 °C for 6 h. Significantly ($P<0.05$) higher gelatin of 22.67% was obtained from actinidin at the level of 20 unit/g (A20) than all other treatment levels of actinidin and control (17.90%). The yield from papain at level 20 (P20) was 23.59% yield. Gelatin extracted

using actinidin enzyme (GEA) showed significantly ($P<0.05$) higher gel strength than the control (283.35) and other treatment group samples. Bromelain at level 15 (B15) and 25 (B25) gave gelatin yield of 23.25 and 23.49%, respectively which was highest than all other samples of gelatin extracted using bromelain (GEB) whereas zingibain at level 15 (Z15) gave the highest yield of 23.52% within the gelatin extracted using zingibain (GEZ) group.

For A20, the gel strength was 366.39 g and least recorded gel strength in GEA samples was for A25 (348.56). Relatively low gel strength was recorded for the gelatin extracted using papain (GEP) as P10 exhibited the highest gel strength of 119.32 g within GEP treatment group. The yield and gel strength for B20 was 22.26% and 160.88 g, respectively and the least recorded gel strength was 111.56 g for B15 within the GEB group while the gelatin extracted using zingibain (GEZ) samples failed to form gel. Viscosities were significantly ($P<0.05$) higher for GEA samples compared to control gelatin (12.10 mPa.s) and all the treatment groups. Contrary to it, gelatin extracted using papain (GEP) showed lower viscosity than control. Viscosity of 13.53 mPa.s was noticed for A20 whereas within the GEP group, P25 exhibited the highest viscosity of 10.70 mPa.s. Viscosities of GEB were significantly ($P<0.05$) higher than GEZ samples. Among the treatment groups, lowest viscosities of 9.13 and 5.80 mPa.s were recorded in samples B15 and Z15, respectively. All the treatment samples and control showed complete degradation of β chains. GEA samples showed the presence of α chains and lower molecular weight peptides. GEP samples revealed complete degradation of β band along with α chains and lower molecular weight peptides were noticed in all GEP samples. All GEB and GEZ samples showed complete degradation of β and α chains. Low molecular weight peptides were observed in GEB samples. In contrast, GEZ samples revealed only smear bands with complete absence of protein band.

Actinidin and bromelain enzymes were used to pretreat the bovine skin for 48 h and ultrasonic wave (53 kHz and 500 W) was used for the time durations of 2, 4 and 6 h at 60 °C to extract gelatin samples (UA2, UA4 and UA6, respectively for actinidin pretreated gelatins and UB2, UB4 and UB6, respectively for bromelain pretreated gelatins). Control (actinidin and bromelain pretreated, UAC and UAB, respectively) gelatin was extracted using ultrasound for 6 h at 60 °C without enzyme pretreatment. Ultrasound treatment led to significant increase in the gelatin yield as the time duration of ultrasonic treatment increased. UA6 gave significantly higher yield (19.65%) than UAC (18.72%) and UB6 yield (19.71%) was significantly higher compared to UBC (18.67%).

The gel strength of UA6, UAC, UB6 and UBC were 502.16, 627.53, 595.51 and 603.24 (g), respectively. The corresponding viscosity was 15.60, 16.33, 16.37 and 16.33 mPa.s, respectively. Amino acid content was found to increase with the duration of ultrasound treatment and UAC and UBC showed the highest amino acid content in their respective treatment groups. Protein pattern of the extracted gelatin revealed progressive degradation with the time duration of ultrasound treatment. In ultrasound-

actinidin gelatins, fourier transform infrared (FTIR) spectroscopy revealed loss of molecular order and degradation in UA6. Amide I band of gelatins extracted by ultrasound-bromelain treatment suggested greater loss of molecular order in these samples than the commercial gelatin (CG). Longer duration of ultrasound treatment caused inter-connected network formation, protein aggregation and small particle size as revealed by scanning electron microscopy (SEM). Although decreased structural integrity of the gelatin samples were observed with increase in ultrasound extraction time, UBC did not lose its structural integrity.

Autolysis study of pretreated bovine skin (PS) showed that the maximal autolysis took place at 40 °C and pH 5. Various protease inhibitors were used to inhibit this autolysis and ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA) (10 mM) was found to inhibit the autolysis most effectively. EGTA being the preferentially calcium binder indicated that the metallocollagenases were the predominant endopeptidases present in the bovine skin. Subsequent to this, gelatin was extracted in the absence (GAI) and presence (GPI) of EGTA to study its effect on the extracted gelatin. PS was incubated at 10 °C for 24 h with 10 mM EGTA solution. Apart from this, one more gelatin sample was extracted using papain enzyme (GPIP). PS was treated with EGTA and then incubated with papain at 40 °C for 48 h. Gelatins were extracted in water bath at 60 °C for 6 h. The use of papain resulted in significantly ($P<0.05$) higher gelatin yield for GPIP (23.68%) than GAI (16.11%) and GPI (15.52%). Use of EGTA in GPI might led to non-significant ($P>0.01$) decrease in yield in GPI compared to GAI. GAI, GPI and GPIP exhibited gel strength of 554.90, 538.77 and 418.62 g, respectively. Molecular weight distribution showed complete degradation of β chains in all the samples and distinct inhibition of α chains in GPI was observed due to inhibitory effect of EGTA on endopeptidases. High intensities of α1 and α2 chains were noticed in GAI whereas there was complete degradation of α chains in GPIP gelatin. Molecular order was retained in GPI due to presence of EGTA and random coiled structure of GAI and GPIP was indicated as revealed by FTIR spectra.

Finally, it can be concluded that actinidin and bromelain enzymes, particularly at level 20 unit/g of skin, could be used to enhance the extraction yield and quality characteristics of gelatin from bovine skin. Among these two enzymes, actinidin was better in improving the yield and quality characteristics of gelatin from bovine skin. Ultrasound assisted extraction in conjugation with actinidin and bromelain enzymes resulted in higher gelatin yield and quality. The gelatin yield of bovine skin gelatin, its quality, molecular weight distribution and secondary structures were influenced by metallocollagenases. Papain enzyme pretreatment of bovine skin subsequent to EGTA incubation improved gelatin yield but reduced gel strength.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENAMBAHBAIKAN PENGEKSTRAKAN GELATIN DARIPADA KULIT LEMBU MELALUI PROSES BERBANTU ENZIM TUMBUHAN

Oleh

TANBIR AHMAD

Januari 2018

Pengerusi : Profesor Madya Awis Qurni Sazili, PhD
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Gelatin adalah biopolimer berat molekul yang tinggi yang diperolehi oleh denaturasi separa kolagen dari proses hidrolisis. Kolagen sebagai protein struktur yang mempunyai tiga rantaian α yang saling terhubung dengan ikatan hidrogen dan kovalen membentuk rantaian tiga helix yang sangat stabil yang memerlukan rawatan oleh asid atau alkali untuk mengubahnya menjadi gelatin. Ini menghasilkan pengeluaran gelatin yang rendah. Pada masa lalu, penyelidik telah menggunakan pelbagai protease untuk meningkatkan hasil dan kualiti gelatin. Penggunaan enzim protease menyebabkan peningkatan hasil gelatin tetapi pengurangan kualiti gelatin serentak terutamanya disebabkan oleh pengeluaran gelatin yang mempunyai polipeptida rantai pendek. Gelatin dengan polipeptida berat molekul yang tinggi (kurang peptida yang direndahkan) dilaporkan lebih baik dalam sifat fungsian. Oleh itu, ia dicadangkan untuk menggunakan enzim-enzim baru yang boleh memotong rantaian kolagen sedemikian rupa supaya rantaian protein yang lebih lama dapat diperoleh dalam gelatin yang terhasil. Selain itu, kerja-kerja penyelidikan kecil boleh didapati menggunakan ultrasound untuk mengeluarkan gelatin dan kesannya terhadap hasil dan kualiti. Oleh itu, ultrasound yang dibantu pengekstrakan gelatin dalam konjugasi dengan enzim telah diambil sebagai objektif. Enzim endopeptidase yang terdapat pada kulit juga dilaporkan diaktifkan pada suhu pengekstrakan tinggi dan menyebabkan penurunan protein gelatin. Oleh itu, kajian autolysis dijalankan dan inhibitor endopeptidase yang sesuai digunakan untuk mengeluarkan gelatin.

Empat enzim tumbuhan iaitu actinidin (A), papain (P), bromelain (B) dan zingibain (Z) digunakan untuk mengeluarkan gelatin dari kulit lembu. Kulit telah dipersiapkan selama 48 jam dengan enzim ini pada tahap 0, 5, 10, 15, 20 dan 25 unit / g kulit pada suhu optimum dan pH enzim masing-masing dan gelatin diekstrak pada suhu 60 ° C selama 6 jam. Gelatin yang lebih tinggi ($P < 0.05$) iaitu 22.67% diperolehi daripada

actinidin pada tahap 20 unit / g (A20) daripada semua tahap rawatan actinidin dan kawalan (17.90%). Hasil daripada papain pada tahap 20 (P20) adalah hasil 23.59%. Gelatin yang diekstrak menggunakan enzim actinidin (GEA) menunjukkan kekuatan gel yang lebih tinggi ($P < 0.05$) daripada kawalan (283.35) dan sampel kumpulan rawatan yang lain. Bromelain pada tahap 15 (B15) dan 25 (B25) memberikan hasil gelatin sebanyak 23.25 dan 23.49%, yang tertinggi daripada semua sampel gelatin lain yang diekstrak menggunakan bromelain (GEB) manakala zingibain pada tahap 15 (Z15) memberikan hasil sebanyak 23.52% dalam gelatin yang diekstrak menggunakan kumpulan zingibain (GEZ).

Bagi A20, kekuatan gel adalah 366.39 g dan kekuatan gel yang paling tinggi tercatat dalam sampel GEA iaitu A25 (348.56). Kekuatan gel yang relatif rendah direkodkan untuk gelatin yang diekstrak menggunakan papain (GEP) kerana P10 menunjukkan kekuatan gel tertinggi sebanyak 119.32 g dalam kumpulan rawatan GEP. Hasil dan kekuatan gel bagi B20 masing-masing adalah 22.26% dan 160.88 g, dan kekuatan gel yang paling kurang direkodkan ialah 111.56 g untuk B15 dalam kumpulan GEB manakala gelatin yang diekstrak dengan menggunakan zingibain (GEZ) gagal membentuk gel. Kelikatan adalah lebih tinggi ($P < 0.05$) bagi sampel GEA berbanding dengan gelatin kawalan (12.10 mPa.s) dan semua kumpulan rawatan. Bertentangan dengan keputusan itu, gelatin yang diekstrak menggunakan papain (GEP) menunjukkan kelikatan yang lebih rendah daripada kawalan. Kelikatan 13.53 mPa.s diperhatikan untuk A20 sedangkan dalam kumpulan GEP, P25 menunjukkan kelikatan tertinggi 10.70 mPa. Kelikatan GEB adalah ketara ($P < 0.05$) lebih tinggi daripada sampel GEZ. Antara kumpulan rawatan, viskositi terendah 9.13 dan 5.80 mPa.s masing-masing dicatatkan dalam sampel B15 dan Z15. Kesemua sampel rawatan dan kawalan menunjukkan kemerosotan lengkap rantaian β. Sampel GEA menunjukkan kehadiran rantaian α dan berat molekul peptida yang lebih rendah. Sampel GEP mendedahkan kemerosotan lengkap rantaian β bersama rantaian α dan berat molekul peptida yang lebih rendah telah diperhatikan dalam semua sampel GEP. Sebaliknya, sampel GEZ hanya menunjukkan band smear dengan ketiadaan band protein.

Enzim actinidin dan bromelain digunakan untuk rawatan kulit lembu dan gelombang ultrasonik (53 kHz dan 500 W) telah digunakan untuk tempoh masa 2, 4 dan 6 jam pada suhu 60 ° C untuk mengambil sampel gelatin (UA2, UA4 dan UA6) masing-masing untuk rawatan gelatin actinidin dan UB2, UB4 dan UB6, masing-masing untuk rawatan gelatin bromelain). Kawalan (pra rawatan actinidin dan bromelain, UAC dan UAB, masing-masing) gelatin diekstrak menggunakan ultrasound selama 6 jam pada suhu 60 ° C tanpa pra rawatan enzim. Rawatan ultrasound menyebabkan peningkatan ketara dalam hasil gelatin kerana tempoh masa rawatan ultrasonik meningkat. UA6 menghasilkan hasil yang lebih tinggi (19.65%) daripada UAC (18.72%) dan hasil UB6 (19.71%) jauh lebih tinggi berbanding UBC (18.67%).

Kekuatan gel UA6, UAC, UB6 dan UBC masing-masing adalah 502.16, 627.53, 595.51 dan 603.24 (g). Kelikatan yang sama ialah masing-masing 15.60, 16.33, 16.37 dan 16.33 mPa.s. Kandungan asid amino didapati meningkat dengan tempoh rawatan ultrasound dan UAC dan UBC menunjukkan kandungan asid amino tertinggi dalam kumpulan rawatan masing-masing. Corak gelatin protin yang diekstrak mendedahkan kemerosotan progresif dengan tempoh masa rawatan ultrasound. Dalam gelatin ultrasound-actinidin, spektroskopi transformasi inframerah empatier (FTIR) mendedahkan kehilangan urutan molekul dan degradasi dalam UA6. Amide I gelatin yang diekstrak oleh rawatan ultrasound-bromelain mencadangkan kehilangan molekul yang lebih besar dalam sampel ini daripada gelatin komersil (CG). Tempoh rawatan ultrasound yang lebih lama menyebabkan pembentukan rangkaian antara penyambung, agregasi protein dan saiz zarah kecil seperti yang diungkap oleh mikroskop elektron (SEM). Walaupun penurunan integriti struktur sampel gelatin diperhatikan dengan peningkatan masa pengekstrakan ultrasound, UBC tidak kehilangan integriti strukturnya.

Kajian autolysis pada kulit lembu yang dirawat (PS) menunjukkan bahawa autolysis maksima berlaku pada suhu 40 °C dan pH 5. Pelbagai inhibitor protease digunakan untuk menghalang autolysis ini dan etilena-bis (oxyethylenenitrilo) asid tetraacetic (EGTA) (10 mM) didapati menghalang autolysis paling berkesan. EGTA sebagai pengikat kalsium yang paling digemari menunjukkan bahawa metallocollagenases adalah endopeptidase utama yang terdapat dalam kulit lembu. Selepas itu, gelatin diekstrak tanpa kehadiran (GAI) dan kehadiran (GPI) EGTA untuk mengkaji kesannya terhadap gelatin yang diekstrak. PS yang diinkubasi pada 10 °C selama 24 jam dengan bersama dengan 10 mM EGTA. Selain itu, satu lagi sampel gelatin diekstrak menggunakan enzim papain (GPIP). PS dirawat dengan EGTA dan kemudian diinkubasi dengan papain pada suhu 40 C selama 48 jam. Gelatin diekstrak dalam tab air pada suhu 60 °C selama 6 jam. Penggunaan papain menghasilkan hasil gelatin yang lebih tinggi ($P < 0.05$) untuk GPIP (23.68%) daripada GAI (16.11%) dan GPI (15.52%). Penggunaan EGTA dalam GPI mungkin menyebabkan penurunan ($P > 0.01$) dalam hasil GPI berbanding dengan GAI. GAI, GPI dan GPIP masing-masing mempamerkan kekuatan gel 554.90, 538.77 dan 418.62 g. Pengagihan berat molekul menunjukkan kemerosotan lengkap rantai β dalam semua sampel dan perencatan yang berbeza rantaian α dalam GPI diperhatikan kerana kesan penghambatan EGTA pada endopeptidase. Keamatan yang tinggi rantaian α1 dan α2 telah dilihat dalam GAI sedangkan terdapat rantaian α yang lengkap dalam gelatin GPIP. Susunan molekul disimpan di GPI kerana kehadiran EGTA dan struktur gegelung rawak GAI dan GPIP ditunjukkan sebagai didedahkan oleh spektrum FTIR.

Akhirnya, dapat disimpulkan bahawa enzim actinidin dan bromelain, terutamanya pada tahap 20 unit / g kulit, boleh digunakan untuk meningkatkan hasil pengeluaran dan ciri-ciri kualiti gelatin dari kulit lembu. Di antara kedua-dua enzim ini, actinidin lebih baik dalam meningkatkan hasil dan ciri-ciri kualiti gelatin dari kulit lembu. Didapati bahawa pengekstrakan bantuan ultrasound dalam konjugasi dengan enzim actinidin dan bromelain menghasilkan hasil dan kualiti gelatin yang lebih tinggi. Dapat disimpulkan bahawa hasil gelatin kulit lembu, kualiti, pengagihan berat molekul dan

struktur sekunder dipengaruhi oleh metallocollagenases. Rawatan enzim papain kulit lembu selepas inkubasi EGTA meningkatkan hasil gelatin tetapi mengurangkan kekuatan gel.

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I certify that a Thesis Examination Committee has met on 5 January 2018 to conduct the final examination of Tanbir Ahmad on his thesis entitled "Improving Gelatin Extraction from Hide using Plant Enzyme-Assisted Process" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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Date: 27 February 2018

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by graduate student

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A	Amperage
a*	Redness
AA	Amino acids
AABA	L-α-amino-n-butyric acid
AC	Alternating current
APS	Ammonium persulfate
°A	Angstrom
AFC	Atomic force microscopy
b*	Yellowness
°C	Degree celcius
cP	Centi poise
Cm	Centimeter
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene-bis (oxyethylenenitrilo) tetraacetic acid
FAA	Free amino acids
FTIR	Fourier transformed infrared
g	Gram (weight)
g	gram (force)
GEA	Gelatin extracted using actinidin enzyme
GEP	Gelatin extracted using papain enzyme
GEB	Gelatin extracted using bromelain enzyme
GEZ	Gelatin extracted using zingibain enzyme
GAI	Gelatin extracted in the absence of endopeptidase inhibitor

GLM	Generalized linear model
GPI	Gelatin extracted in the presence of endopeptidase inhibitor without enzyme pretreatment
GPIP	Gelatin extracted in the presence of endopeptidase inhibitor with enzyme pretreatment
H	Hour
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HPLC	High performance liquid chromatography
HPU	High power ultrasound
H_2O_2	Hydrogen peroxide
IEP	Isoelectric point
IR	Infra red
kDa	Kilo Dalton
KCl	Potassium chloride
Kg	Kilogram
kHz	Kilohertz
L	Liter
L*	Lightness
LPU	Low power ultrasound
M	Mole
mA	Milli ampere
MMP	Matrix metalloproteinase
MT-MMP	Membrane type matrix metalloproteinase
MgCl2	Magnesium chloride
MgSO4	Magnesium sulphate
Min	Minute

µg	Micro gram
µl	Microliter
µl	Micro liter
µm	Micrometer
µM	Micromole
µmole	Micromole
ml	Milliliter
mM	Millimolar
MPa	Mega pascal
mPa.s	Millipascal seconds
Na	Sodium
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
%	Percent
PDB	Protein data bank
pmole	Pico mole
PMSF	Phenylmethanesulfonyl fluoride
PS	Pretreated skin
RMS	Response surface methodology
SBTI	Soybean trypsin inhibitor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	Standard error
SEM	Scanning electron microscopy

SHCAP	Smooth hound crude acid protease
Tris	2-amino-2-(hydroxymethyl)-propane-1, 3-diol
TEMED	Tetramethyl ethylenediamine
UAC and UBC	Control gelatin extracted using ultrasound without actinidin and bromelain pretreatment, respectively
UA2, UA4 and UA6	UA2, UA4 & UA6 refers to ultrasound assisted gelatin extracted for the time duration of 2, 4 and 6 h, respectively using actinidin enzyme pretreatment.
UB2, UB4 and UB6	UB2, UB4 & UB6 refers to ultrasound assisted gelatin extracted for the time duration of 2, 4 and 6 h, respectively using bromelain enzyme pretreatment.
UAE	Ultrasound-assisted extraction
UG	Gelatin extracted using ultrasound
USD	United States Dollar
UHP	Ultra high pressure
UB	Ultrasound bath
V	Voltage
v/v	Volume per volume
W	Watt
w/v	Weight per volume
WB	Waterbath
WPC	Whey protein concentrate
WPI	Whey protein isolate

CHAPTER 1

INTRODUCTION

Gelatin is a type of denatured fibrous protein extracted by partial thermal hydrolysis from collagen. It is a high molecular weight natural polymer possessing various functional characteristics like foaming and emulsifying properties, film-forming properties and water binding capacity which makes it an indispensable component in the photography, food, cosmetic, and pharmaceutical industries (Gimenez et al., 2005a; Gómez-Guillén et al., 2011). Gelatin shows enough flexibility to acquire a wide variety of conformations depending on temperature, solvent and pH conditions (Ahmad and Benjakul, 2011). It is amphoteric in nature having triple-stranded helical structure and interacts with water in a manner different than other synthetic hydrophilic polymers (Kozlov and Burdygina, 1983).

Gelatin is very versatile biopolymer that finds its wide range of application in the food, beverages, pharmaceuticals, nutraceuticals and photographic industries (Hao et al., 2009). Generally, gelatin acts as gelling agent as well as a film former, thickener, stabiliser, adhesive agent, emulsifier, protective colloid, foaming agent, and an agent for beverage fining in the food industry (Johnston-Banks, 1990; Segtnan et al., 2003). Therefore, rheological properties of gelatin for an intended application widely govern the quality of gelatin (Stainsby, 1987; Gomez- Guillen et al., 2002).

Cattle and pig skins and bones are the traditional source materials for gelatin with porcine skin derived gelatin holding the highest share (46%) of global gelatin production, followed by 29.4% from bovine hides and 23.1% from pig and cattle bones (Duconseille et al., 2015). The global annual production of gelatin is increasing due to its rising demand over the years. Although the annual world gelatin production was nearly 326 tons in 2007 (GME, 2009), it rose to 348.9 tons in 2011 (Transparency Market Research, 2013). Further, the global gelatin output in 2015 rose to 412.7 kilo tons and still expected to reach at 651.7 tons by 2024 amounting to 4.08 billion US dollars (Grand View Research, 2016). The worth of world gelatin market in 2011 was 1.77 billion US dollars and forecast to reach 2.79 billion US dollars in 2018 (Transparency Market Research, 2013). In 2015, the food & beverage sector had the largest application of gelatin utilizing 29.0% of the global volume, followed by the pharmaceutical segment (Grand View Research, 2016).

Principally, skin is made up of type I collagen and very small amount of type III collagen is also found (Bruckner, 2010). Gelatin is derived from collagen by its partial hydrolysis which involves destruction of native collagen structure (Fernandez-Diaz et al. 2001). Collagen being structural protein comprises three intertwined α-chains forming a right-handed triple helix that is highly stable (Bailey and Light, 1989) and inter-chain hydrogen bonding provides the stability to the triple helix structure (Johnston-Banks, 1990; Te Nijenhuis, 1997).

A pretreatment process using either acid or alkali is needed to turn insoluble collagen to a soluble form. This results in the disappearance of the native ordered structure of collagen and it becomes swollen but collagenous protein remains insoluble (Stainsby, 1987). For the separation of the proteins from the remaining raw materials, thermal treatment (gelatin extraction) is followed (Schrieber and Gareis, 2007). During the gelatin extraction process, hydrogen and covalent bonds are cleaved by heat leading to conversion of collagen as it turns into gelatin via a transition from helix to coil (Djabourov et al., 1993). Covalent and non-covalent bonds are degraded in adequate numbers to allow to release oligomers and free α chains (Johnston-Banks, 1990). Apart from this, several amide bonds found in the collagen molecules original chains go through hydrolysis (Bailey, 1985). Therefore, the gelatin obtained has lower molecular weight components compared to native collagen and is made up of a mixture of polypeptide fragments having molecular weight that range from 16–150 kDa (Asghar and Henrickson, 1982).

Collagen cross-linkages being resistant to acid and thermal treatment (Galea et al., 2000) result in low yield of gelatin (Nalinanon et al., 2008). Previously, some protein degrading enzymes that have the ability to break the cross-links of collagen were used to improve the recovery of gelatin (Nalinanon et al., 2008). Pepsin and proctase (isolated from *Aspergillus niger*) were used by Chomarat et al. (1994) to extract the gelatin from bovine skin. However, the relative amount of recovered gelatin, its gel strengths and viscosities were low.

Plant enzymes like papain and bromelain have also been utilised to extract gelatin from the collagenous source. The source materials of the gelatin were initially pre-treated with various proteases prior to gelatin extraction (Balti et al., 2011; Chomarat et al., 1994; Ktari et al., 2014; Lassoued et al., 2014; Nalinanon et al., 2008 and Zhang et al., 2011). On the other hand, Pitpreecha and Damrongsakkul (2006) and Damrongsakkul et al. (2008) extracted gelatin from bovine skin without pre-treating the skin with papain enzyme. Pitpreecha and Damrongsakkul (2006) made use of the crude proteolytic enzyme that was extracted from papaya latex and commercial papain enzyme to extract gelatin from the raw hide under optimum conditions (75 °C and pH 7) for the activities of both the enzymes. Higher gelatin recovery was achieved but the gel strength was relatively low and the α and β chains complete degradation in the recovered gelatin was observed in both types of samples. Papain was utilised to extract gelatin from rawhide splits but the recovered gelatin exhibited low gel strength and viscosity (Damrongsakkul et al., 2008). Norziah et al. (2014) pre-treated the surimi processing waste from ribbon fish (*Lepturacanthus savel*) before extracting gelatin with bromelain at 4°C but it was not the optimum activity temperature of the enzyme. Under optimum condition for gelatin extraction, the gelatin yield was observed to rise by almost 50% but the gel strength and viscosity were low. Faint presence of β-chain (α-chain dimmers) band was observed and α1and α2 chains regions were revealed scarcely. Lower molecular weight regions were also visible.

These examples clearly demonstrated that although a better gelatin yield was achieved with plant enzymes, the functional qualities of the obtained gelatin were compromised. Gelatin with high molecular weight polymers (less degraded peptides) is reported to be better in functional properties (Badii and Howell, 2006; Gómez-Guillén et al., 2002; Muyonga et al., 2004a; Zhang et al., 2011). This situation validates the search for new enzymes that can cleave long gelatin chains at few sites so that one can produce a long chain, high quality gelatin (Ahmad et al., 2017). Additionally, Ha et al. (2012) found that the actinidin protease had the most efficacy at hydrolysing the myofibril proteins of meat out of papain, actinidin, bromelain, and zingibain enzymes. They also reported that the zingibain enzyme had the most efficacy at hydrolysing the connective tissue proteins of beef among these four enzymes. Ginger extract used for meat tenderisation has also suggested that it has collagenolytic activity (Hashimoto et al., 1991). There is no published research work on gelatin extraction using actinidin and zingibain enzymes to the best of author knowledge. Therefore, in the first experimental study, these two enzymes actinidin and zingibain along with papain and bromelain were used at their optimum temperature and pH to extract gelatin from bovine skin and the yield of the extraction and different quality parameters of the recovered gelatin were analysed.

There is dearth of published research on ultrasound assisted extraction (UAE) from animal materials (Vilkhu et al., 2008). UAE can enhance extraction efficiency and extraction rate particularly for aqueous extraction and provides opportunity to extracted bioactive and heat sensitive food components even when the processing temperatures are lower (Vilkhu et al., 2008). Its promising effect in food science has attracted attention of the food industry (Jia et al., 2010). High power ultrasound (power >1W cm^{-2} and frequencies that range from 20 to 500 kHz) can be utilised to assist in the process of extraction for a wide range of food components (e.g. protein, herbal, oil, and polysaccharides) and even bioactive ingredients (e.g. antioxidants) from animal and plant sources (Vilkhu et al., 2008). Ultrasonic irradiation increased the yield of collagen from bovine tendon and significantly shortened the extraction time in comparison with the conventional pepsin isolation method (Li et al., 2009). The extraction yield of collagen increased with the ultrasonic treatment (Kim et al., 2012). Ultrasonic bath treatment of bighead carp scales resulted in better quality gelatin with high yield (30.94–46.67%) and the existence of α-and β-chains was noticed in the gelatin that was recovered (Tu et al., 2015).

Experiment 1 has showed encouraging result in terms of gelatin yield and quality when bovine skin was pre-treated with actinidin enzyme at a level of 20 units of enzyme per gram of skin. Therefore, actinidin enzyme had been included in the second experiment. Also, bromelain enzyme at level of 20 units of enzyme per gram of skin has shown better gelatin yield and quality in Experiment 1 compared to papain and zingibain enzymes. Thus, bromelain enzyme was also included in this study. There is no published research work on the extraction of gelatin using ultrasound as well as on the ultrasound-enzyme facilitated extraction of gelatin from bovine skin. Taking into consideration all the above facts, the objective of second experiment was to extract gelatin using ultrasound in conjugation with enzymes actinidin and bromelain

pretreatment and investigating their effects on the quality characteristics of the recovered gelatin.

Matrix metalloproteinases (MMPs) are a set of endopeptidases associated with mammalian skin which are dependent on zinc and have the ability to degrade essentially each of the components of the extracellular matrix (ECM) (Kähäri and Saarialho-Kere, 1997). They are capable of cleaving the native triple helical type I, II and III collagen chains after Gly in a specific sequence (Gln/Leu)–Gly---(Ile/Leu)–(Ala/Leu) (---the point of bond breakdown) (Daboor et al., 2010).

A collagen complex triple helix structure is broken down by very few enzymes (Visse and Nagase, 2003). Metallocollagenases are parts of the family of matrix Metalloproteinase (MMP) and like all MMP, they are enzymes that contain zinc and that usually need calcium for their optimum stability and activity (Raskovic et al., 2014). Metallocollagenases are capable of breaking down almost every component of the extracellular matrix (ECM) (Birkedal-Hansen et al., 1993; Woessner Jr, 1994). They have proteolytic activity against many native triple-stranded collagens including types I collagen (main constituent of skin collagen) (Freije et al., 1994). The serine proteases possess a uniquely reactive serine side chain and are present in numerous species of crab, fish, and other crustaceans (Daboor et al., 2010).

Heat-stable and heat activated endogenous proteases (matrix metalloproteinases) that are related to the skin matrix can bring destabilisation and degradation of the structure of collagen by cleaving the inter and intramolecular cross-links (Wu et al., 2008). Skin endogenous proteases may have a part in the drastic cleavage of collagen protein chains during the extraction of gelatin from the bigeye snapper (*Pricanthus tayenus*) skin at high temperature resulting in presence of low molecular weight polypeptides in protein pattern contributing to low bloom strength gelatin (Nalinanon et al., 2008). Bigeye snapper pepsin used to pretreat the skin was not responsible for degradation of gelatin protein chains. Further, they observed no degradation in gelatin extracted when the soybean trypsin inhibitor (SBTI) was present, which is a serine protease inhibitor, suggesting the involvement of endogenous proteases (most like serine proteases) in cleaving the gelatin chains. Further research has also strengthened the involvement of endogenous proteases in reducing the quality of the extracted gelatin. Heat activated serine proteases, most likely collagenase, resulted in the severe degradation of β and α gelatin protein chains that were extracted at 60°C from bigeye snapper skin and endogenous peptidases were inhibited by SBTI (Intarasirisawat et al., 2007). These aforementioned enzymes are associated with skin matrix parts like collagen (Woessner, 1991). These research works have established the existence of endogenous proteases found in the skin of various animal species and how they affect the extracted gelatin qualities. Such study was not found to be reported with regard to bovine skin gelatin in the good knowledge of the author. Henceforth, the third experiment aimed to examine the autolysis of bovine skin in light of endopeptidase enzymes and find out its inhibitor. Further experiment was performed to elucidate the consequence of such endopeptidase inhibitor on the extracted gelatin.

Hypotheses

Experiment 1

- The plant enzymes actinidin, papain, bromelain and zingibain utilised as pretreatment for the extraction of the gelatin will increase the yield and quality of the extracted gelatin.

Experiment 2

- Ultrasound treatment of the bovine skin in combination with enzyme pretreatment will improve the yield and quality characteristics of the recovered gelatin.

Experiment 3

- Endogenous peptidases are present in the hide of the cattle which deteriorate the quality of the extracted gelatin.

General objectives

To increase the gelatin yield and quality characteristics using different plant enzymes.

Specific objectives

1. To study the effects of plant enzymes (actinidin, papain, bromelain or zingibain) used as a pretreatment on the yield and quality of the extracted gelatin.
2. To assess the effects of ultrasound in combination with plant enzymes actinidin or bromelain on the quantity and quality of the extracted gelatin.
3. To investigate the autolysis of bovine skin and determine the effects of endopeptidase inhibitors on the extracted gelatin.

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