UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION, ENCAPSULATION AND CHARACTERIZATION OF BROMELAIN-GENERATED ANGIOTENSIN I-CONVERTING ENZYME (ACE)-INHIBITORY HYDROLYSATES FROM STONE FISH (Actinopyga lecanora Jaeger)

SHEHU MUHAMMAD AUWAL

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By

SHEHU MUHAMMAD AUWAL

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

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DEDICATION

To my beloved parents

Thank you for all your supports and sacrifices

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

OPTIMIZATION, ENCAPSULATION AND CHARACTERIZATION OF BROMELAIN-GENERATED ANGIOTENSIN I-CONVERTING ENZYME (ACE)-INHIBITORY HYDROLYSATES FROM STONE FISH (*Actinopyga lecanora* Jaeger)

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June 2018

Chairman : Professor Nazamid Saari, PhD
Faculty : Food Science and Technology

Stone fish is an under-utilized sea cucumber with many nutritional and ethno-medicinal values. Proteases-aided hydrolysis of stone fish protein was found to generate hydrolysates with strong antioxidant and ACE-inhibitory effects. However, the direct use of stone fish-derived hydrolysates as potential antihypertensive molecules is limited by their low stability against digestive enzymes and unpleasant fishy odor. Therefore, the present study aimed to optimize hydrolysis of stone fish protein for the production of angiotensin I-converting enzyme (ACE)-inhibitory hydrolysates using bromelain and improve the stability and ACE-inhibitory activity of the resulting hydrolysates against gastrointestinal digestion by nanoencapsulation. Response surface methodology (RSM) based on a central composite design (CCD) was used to model and optimize the degree of hydrolysis (DH) and ACE-inhibitory activity. A pH of 7.0, temperature of 40^{o}C, E/S ratio of 2% and reaction time of 240 min were determined using a response surface model as the optimum levels to obtain the hydrolysates with maximum ACE-inhibitory activity of 84.26% at 44.59% degree of hydrolysis. The hydrolysates were further profiled in which five novel ACE-inhibitory peptides including ALGPQFY, KVPPKA, LAPPTM, EVLIQ and EHPVL were identified with their respective IC_{50} values of 0.012 mM, 0.980 mM, 1.31 mM, 1.44 mM and 1.68 mM. The ACE-inhibitory hydrolysates were then nanoencapsulated in chitosan nanoparticles and optimized based on 3-factor 3-level Box–Behnken experimental design. The optimized nanoparticles showed a good physicochemical stability following their twelve weeks of storage at 4^{o}C. The result of *in vitro* efficacy indicated significantly higher ($p<0.05$) ACE-inhibitory activity of 51.96% for the nanoparticles compared to 36.84% for the free hydrolysates following their simulated gastrointestinal digestion. The *in vivo* antihypertensive effect of the optimized nanoparticles was also evaluated on spontaneously

hypertensive rats within 24 h following single oral administration at 200 mg/kg, 400 mg/kg and 800 mg/kg. The results demonstrated significant systolic blood pressure lowering effects of the nanoparticles at all the three doses compared to the group treated with the unencapsulated hydrolysates. The nanoparticles were then evaluated for acute and sub-acute toxicity on liver and kidney of Wistar Kyoto rats. The result indicated no death with a safe dose limit of the nanoparticles greater than 2000 mg/kg. Serum biochemical analysis and histomorphological examination following 28 days of repeated exposure of the rats to the 200 mg/kg, 400 mg/kg and 800 mg/kg doses of the nanoparticles, revealed no alteration in the function and architecture of both liver and kidney with no occurrence of cellular necrosis. This indicated the safe therapeutic application of the chitosan nanoparticles loaded with stone fish-derived ACE-inhibitory hydrolysates to be utilized as food bio-ingredient for long term management of hypertension.

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

OPTIMISASI, ENKAPSULASI DAN PENCIRIAN HIDROLISAT PERENCAT ENZIM PENUKAR ANGIOTENSIN -1 (ACE) DARIPADA IKAN BATU (*Actinopyga lecanora* Jaeger) YANG DIHASILKAN MELALUI TINDAKAN BROMELAIN

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Ikan batu adalah sejenis timun laut yang kurang dimanafatkan walaupun mempunyai pelbagai nilai pemakanan dan etno-ubat. Protease-berbantu hidrolisis protein ikan batu didapati menghasilkan hidrolisat dengan antioksidan dan kesan perencatan ACE yang kuat. Walau bagaimanapun, penggunaan langsung hidrolisat ikan batu sebagai molekul antihipertensi yang berpotensi adalah terhad disebabkan oleh kestabilan yang rendah terhadap enzim pencernaan dan bau hanyir yang tidak menyenangkan. Oleh itu, kajian ini bertujuan untuk mengoptimumkan hidrolisis protein ikan batu untuk penghasilan hydrolisat perencat enzim penukar angiotensin I (ACE) dengan menggunakan bromelain dan meningkatkan kestabilan dan aktiviti hidrolisat perencat ACE terhadap pencernaan gastrointestinal melalui nanoenkapsulasi. Kaedah tindak balas permukaan (RSM) berdasarkan reka bentuk komposit pusat (CCD) digunakan untuk memodel dan mengoptimumkan darjah hidrolisis (DH) dan aktiviti perencatan ACE. Nilai pH 7.0, suhu 40^{o}C, nisbah E/S sebanyak 2% dan masa tindak balas 240 min ditentu menggunakan model tindakbalas permukaan adalah tahap optimum untuk mendapatkan hidrolisat dengan aktiviti perencatan ACE maksimum sebanyak 84.26% pada tahap darjah hidrolisis 44.59%. Hidrolisat kemudian diprofilkan dimana lima peptida perencatan ACE baru termasuk ALGPQFY, KVPPKA, LAPPTM, EVLIQ dan EHPVL telah dikenalpasti dengan nilai IC_{50} masing-masing 0.012 mM, 0.980 mM, 1.31 mM, 1.44 mM dan 1.68 mM. Hidrolisat perencat ACE kemudiannya di nanoencapsulasi dalam nanopartikel kitosan dan dioptimum berdasarkan reka bentuk eksperimen Box-Behnken 3-faktor 3-peringkat. Nanopartikel yang dioptimumkan menunjukkan kestabilan fizikokimia yang baik berikutan penyimpanan selama dua belas minggu pada 4^{o}C. Dapatan daripada kajian keberkesanan secara *in vitro* menunjukkan peningkatan lebih tinggi yang signifikan ($p<0.05$) aktiviti perencatan ACE

sebanyak 51.96% bagi hidrolisat nanopartikel berbanding dengan 36.84% bagi hidrolisat bebas berikutan pencernaan gastrointestinal. Kesan antihipertensif secara *in vivo* bagi nanopartikel yang dioptimumkan juga dinilai ke atas tikus hipertensi secara spontan dalam masa 24 jam berikut administrasi oral tunggal hidrolisat pada dos 200 mg/kg, 400 mg/kg dan 800 mg/kg. Dapatan menunjukkan kesan penurunan tekanan darah sistolik yang signifikan bagi nanopartikel pada tahap ketiga-tiga dos berbanding dengan kumpulan tikus yang dirawat dengan hidrolisat yang tidak dinanoenkapsulasikan. Nanopartikel tersebut kemudiannya dinilai untuk ketoksikan akut dan sub-akut ke atas hati dan ginjal tikus Kyoto Wistar. Dapatan menunjukkan tiada kematian dengan had dos selamat nanopartikel lebih besar daripada 2000 mg/kg. Analisis biokimia serum dan pemeriksaan histomorfologi berikutan pendedahan berulang tikus selama 28 hari kepada nanopartikel pada dos 200 mg/kg, 400 mg/kg dan 800 mg/kg, menunjukkan tiada perubahan dalam fungsi dan susunan struktur hati dan buah pinggang dengan tiada kejadian nekrosis selular**.** Ini menunjukkan aplikasi terapeutik yang selamat bagi nanopartikel kitosan yang dimuatkan dengan hidrolisat perencat ACE dari ikan batu dan boleh seterusnya digunakan sebagai bioramuan makanan untuk pengurusan jangka panjang tekanan darah tinggi.

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

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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ACE	Angiotensin I-Converting enzyme
AOAC	Association of analytical communities
ACN	Acetonitrile
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ARBs	Angiotensin II Receptor Blockers
AST	Aspartate aminotransferase
AT_1	Angiotensin II receptor type 1
BCA	Bicinchoninic Acid
BD	Bile duct
BP	Blood pressure
BS	Bowman's space
BW	Body weight
CDD	Central composite design
CID	Collision induced dissociation
CV	Central vein
CVDs	Cardiovascular diseases
DBP	Diastolic blood pressure
DH	Degree of hydrolysis
DHM	Direct hydration method
DT	Distal tubules
DTT	Dithiothreitol
E/S	Enzyme-substrate ratio
FOSHU	Foods for Specified Health Uses
GI	Gastrointestinal

GIT	Gastrointestinal tract
GM	Glomerulus
HA	Hepatic artery
HCL	Hydrochloric acid
H&E	Haematoxylin and eosin
HHL	Hippuryl histidyl leucine
HPLC	High performance liquid chromatography
H_2SO_4	Sulphuric acid
IC_{50}	Concentration of an inhibitor where the response is reduced by half
IEF	Isoelectric focusing
IPG	Immoblized pH gradient gel strip
L-NAME	L-nitro-arginine methyl ester
LC/MS	Liquid chromatography tandem mass spectrometry
LD_{50}	Amount of an ingested substance that kills 50 percent of a test sample
LFH	Lipid film hydration
MS/MS	Tandem mass spectrometry
MWCO	Molecular weight cut off
NO	Nitric oxide
NOS	Nitric oxide synthase
NPs	Nanoparticles
OPA	O-phthaldialdehyde
PC	Phosphatidyl choline
PT	Proximal tubules
PV	Portal vein
Q-SAR	Quantitative structure–activity relationship
Q-TOF	Quadrupole time-of-flight
RASS	Renin Angiotensin Aldosterone System
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography

RSM	Response surface methodology
SBP	Systolic blood pressure
SHRs	Spontaneously hypertensive rats
SHs	Stone fish derived ACE-inhibitory hydrolysates
SN	Sinusoids
TFA	Trifluoroacetic acid
TPP	Tripolyphosphate
WHO	World health organization
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background

Angiotensin I-converting enzyme (ACE) acts as a key enzyme in blood pressure regulation via its dual effects on the renin angiotensin as well as the kinin-kallikrein pathways. The Zn-containing peptidyl dipeptide hydrolase triggers the conversion of Angiotensin I to a potent vasopressor, Angiotensin II (Ariyoshi, 1993), and promotes the inactivation of bradykinin, an active vasodilator, resulting in elevated blood pressure or hypertension (Barbana & Boyce, 2011). The mechanism of the vasopressor effect involves the release of aldosterone, a sodium-retaining steroid from the adrenal cortex (Wijesekara & Kim, 2010).

Hypertension is defined as a sustained increase in systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure less than 90 mmHg. It is inferred as a common public health risk of global concern that is associated with cardiovascular related diseases such as heart failure, kidney failure, stroke and disability (Sun et al., 2014; WHO, 2013). Cardiovascular diseases have been identified as the major cause of mortality with a global death rate of greater than 17.30 million cases in 2013 which accounted for 31% of all the reported deaths during the year (Naghavi et al., 2015; Roth et al., 2015). The rate of death incidence due to poorly controlled CVDs is expected to exceed 23.60 million worldwide by 2030 (Go et al., 2013). However, it has been previously projected that a 34% decrease in premature death due to CVDs in the vulnerable group (30 to 70 yrs) could be achieved by the year 2025 through specifically targeting smoking, alcoholism, obesity, elevated blood pressure, glucose and salt intake as the major risks factors (Kontis et al., 2014).

More importantly, suppression of hypertension/elevated blood pressure alone among other risk factors could sufficiently reduce mortality due to CVDs by 30.40% and 38.00% in males and females respectively (Patel et al., 2015). In this regard, inhibition of ACE by decreasing angiotensin II formation and increasing bradykinin production is among the necessary strategies to control elevated blood pressure or hypertension (Girgih et al., 2016; Van der Ven et al., 2002). Presently, ACE-inhibitors, renin inhibitors, aldosterone inhibitors, beta blockers and angiotensin receptor blockers are the five major classes of antihypertensive drugs with specific target site inhibitory action on renin-angiotensin-aldosterone system (RAAS) that are used in the management of hypertension. However, the therapeutic potential of such drugs is limited by their cost and related adverse effects including insulin resistance and diabetes, skin rashes and taste disturbance etc (Geng et al., 2016; Soltani et al., 2015).

Alternatively, ACE-inhibitory hydrolysates are generated as mixture of peptides known as biopeptides through enzymatic hydrolysis of food proteins and have been reported to exhibit blood pressure lowering effects in hypertensive animals with little or no side effects (Adams et al., 2016; Beltrán-Barrientos et al., 2016; Norris et al., 2013; Sharma et al., 2011). In contrast to synthetic drugs, the food derived antihypertensive hydrolysates / biopeptides can be cheaply produced from certain proteins obtained from locally available food sources. Furthermore, the protein hydrolysates and their isolated constituent peptides with ACE-inhibitory activities are the most studied among the different classes of food biopeptides (Iwaniak et al., 2014). Food derived antihypertensive peptides have been associated with safe effects and exhibit their role through the inhibition of ACE (Chakrabarti et al., 2014; Udenigwe & Aluko, 2012). Thus, the food biopeptides can be used as alternative to synthetic drugs such as enalapril and lisinopril which are often accompanied with certain adverse effects such as thirst, diarrhea, skin rashes, fever and headache (Meresa et al., 2017; Kim et al., 2013). Food biopeptides occur as inactive sequences within the primary structure of their parent protein and are released via properly-designed enzymatic hydrolysis.

Among other potential sources, marine invertebrates are naturally endowed with valuable proteins that can be exploited in the production of functional hydrolysates and their constituent's peptides. Many peptides exhibiting one or more biological activities including those for the production of antimicrobial, antioxidant, antithrombotic, anti-Alzheimer, opiate and antihypertensive activity originating from marine invertebrates have been reported (Lee et al., 2012). Antihypertensive/ACE-inhibitory biopeptides have been generated from both animals and plant proteins including those of marine invertebrates (Wang et al., 2017; Li et al., 2016; Kim et al., 2013; Lee et al., 2012).

Similarly, the stone fish (*Actinopyga lecanora*) used in this study is a marine invertebrate that belongs to the phylum echinoderm and class Holothuroidea. It is categorized among the edible species of sea cucumber that are common to Malaysia and other south-Asian sea shores where it is collected by hand picking, free-diving and as by-catch of the fishing industry (Ghanbari et al., 2012; Conand et al., 2010). Despite being abundantly available, easy to propagate and with great commercial potential, the species is still under-utilized. Several biological effects, including antioxidant- (Bordbar et al., 2013), antibacterial- and antihypertensive (Ghanbari e al., 2015; Ghanbari et al., 2012) effects have been ascribed to stone fish hydrolysates produced using different proteases. *In vitro* and *in vivo* experiments with bromelain-generated hydrolysates of stone fish indicated their significant inhibitory capacity against the angiotensin I-converting enzyme activity (Vishkaei et al., 2016; Ghanbari et al., 2015). However, most of the ACE-inhibitory hydrolysates derived from marine invertebrates were reported to exhibit low bioactivity that varies with the type of hydrolyzing enzyme being used (Ghanbari et al., 2015). Thus, the choice of an appropriate enzyme is critical for obtaining inhibitory hydrolysates/biopeptides with strong activity against ACE. As previously reported, alcalase and bromelain-generated hydrolysates yielded the highest ACE-inhibitory activity when stone fish

was hydrolyzed by six different proteases whereas the activity decreased with trypsin, papain, pepsin, and flavourzyme generated hydrolysates (Ghanbari et al., 2015).

A series of chromatographic techniques including reverse phase high performance liquid chromatography, isoelectric focusing electrophoresis and LC/MS mass spectrometry are used in the profiling of protein hydrolysates/biopeptides (complex mixture of peptides) to ease the identification of the potent ACE-inhibitory peptide sequences (Zarei et al., 2015; Yea et al., 2014). In this regard, enzyme-generated protein hydrolysates with the highest ACE-inhibitory activity are selected and profiled to identify the peptide sequences present. The purified peptides can be synthesized as food grade or non-food grade. The production of both forms is costly and not economical. In addition, the non-food grade peptides cannot be directly used as functional ingredient or incorporated into foods. Thus, the natural forms of the peptides or their mixture (protein hydrolysates/biopeptides) which preserve the nutritional quality and constitute the net activity of the whole peptides present are safely and cheaply used for food and commercial purposes. Therefore, the profiling protocol including the identification, synthesis and characterization of the purified peptides are to provide evidence about the suitability of their protein hydrolysates/biopeptides mixture as antihypertensive bioactive ingredients for application in functional foods.

Functional food products enriched with bioactive ingredients such as the ACE-inhibitory hydrolysates are intended for oral consumption to aide in the management of hypertension. The oral route is more preferred for being safe, non-toxic and inexpensive. However, orally administered protein hydrolysates/biopeptides are susceptible to degradation by gastrointestinal enzymes and need to be protected for effective oral-colon delivery and biological efficacy. This can be achieved through encapsulation to safe guard their structural and functional integrity and to improve their stability against gastrointestinal proteases and peptidases (Mohan et al., 2015). Hence, encapsulation has become a relevant and important technology to promote the utilization of bioactive protein hydrolysates as functional food bioingredients for human health promotion. It protects the biopeptides against physicochemical modifications and enhances their bioavailability both *in vitro* and *in vivo* (Patel et al., 2014).

Several approaches including permeation enhancers, enzyme inhibitors, lipid based delivery and different polymeric carrier systems have been used to overcome these challenges (Sajeesh et al., 2015; Renukuntla et al., 2013). In this regard, nanoencapsulation become the most desirable approach to protect the protein hydrolysates and peptides against digestive enzymes, enhance their cellular uptake and controlled release for effective oral-colon delivery and biological efficacy (Niu et al., 2016; Renukuntla et al., 2013). By the virtue of their large surface area to volume ratio, the small size nanocapsules have been reported to circumvent first pass effect and improve the solubility, gastrointestinal stability, target site delivery, bioavailability and biological role of the encapsulated bioactive agent (Shegokar et

al., 2010). In due course, the nanoparticles prolong the half-life or retention time and reduce the dosing frequency of the incorporated antihypertensive peptides (Alam et al., 2017). The selection of appropriate biocompatible and biodegradable wall material is necessary to meet safety requirements for health concern about their possible toxicity especially when nanocarriers are used for the delivery of the bioactive agents (Aggarwal et al., 2009; Bystrzejewska-Piotrowska et al., 2009).

In order to overcome the above mentioned limitations, the production of the ACE-inhibitory hydrolysates was initially optimized by response surface methodology (RSM) using bromelain to determine the best conditions to produce stone fish protein hydrolysates with maximum inhibitory effects on ACE. It is hoped that improving the target activity will expand the utilization of *A. lecanora* as a bio resource abundantly available in many countries for various applications in functional foods development. The hydrolysates were then profiled and the potent ACE-inhibitory peptides present were identified. These peptides were synthesized and evaluated for their inhibitory effect against ACE.

In an attempt to improve their *in vitro* gastrointestinal stability, the bromelain-generated ACE-inhibitory stone fish-hydrolysates were encapsulated by different methods including nanoliposome formed by lipid film hydration and direct heating methods and chitosan nanoparticles fabricated via ionotropic gelation method. The chitosan nanoparticles showed higher *in vitro* gastrointestinal stability and residual ACE-inhibitory efficacy compared to nanoliposome and were selected and optimized by Box-Behnken design. The optimized chitosan nanoparticles containing the hydrolysates were characterized for physicochemical properties and then for residual ACE-inhibitory activity following their digestion under *in vitro* stimulated gastrointestinal condition. The *in vivo* antihypertensive efficacy of the nanoparticles was also evaluated on spontaneously hypertensive rats.

The selection of the chitosan as the coating material was based on a preliminary study. Chitosan have been safely used as a pH sensitive, biodegradable, biocompatible and mucoadhesive polymer in the fabrication of polymeric nanoparticles for oral-colon delivery and controlled release of bioactive peptides and proteins (Rizwan et al., 2017; Ahmed & Aljaeid et al., 2016; Luo et al., 2016). Chitosan based nanoparticles have been reported to enhance oral delivery and bioavailability of anti-cancer agent (Liang et al., 2017; Yang et al., 2017; Khdair et al., 2016; Zhang et al., 2016) and antidiabetic agent (Agrawal et al., 2015) among others. The cationic amino residues of chitosan form complex via ionic gelation with oppositely charged non-toxic polyanionic molecules, like sodium tripolyphosphate (Dambies et al., 2001). The method is simple and doesn't involve harsh treatment such as the use of organic solvents and high temperatures, hence applied for the encapsulation of fragile molecules, including peptides and proteins (Rampino et al., 2013; Al-Qadi et al., 2012; Nasti et al., 2009; Berger et al., 2004; Xu & Du, 2003).

Chitosan have been widely used as a natural polymer to improve the stability and preserve the biological efficacy of bioactive compounds. However, the biochemical and cellular toxicity of chitosan nanoparticles containing ACE-inhibitory hydrolysates/biopeptides have not been investigated. Like other polymers, the use of chitosan in the fabrication of nanoparticles for oral delivery of therapeutic agents has become a major concern about their possible tissue toxicity. The toxicity may result from direct accumulation and adverse interaction of non-biodegradable nanoparticles or due other unwanted molecules that enter the cells a long with the nanoparticles via tight junctions. Consequently, it is imperative to investigate tissue compatibility of the chitosan nanoparticles for safe therapeutic applications. Thus, acute toxicity test was carried out and the safe dose limit of the fabricated chitosan nanoparticles loaded with the ACE-inhibitory hydrolysates determined. The long term sub-acute toxicity effects of three different doses of the nanoparticles was then studied on some serum biochemical parameters and histomorphological changes on liver and kidneys of Wistar Kyoto rats.

1.2 Problem Statement

Food protein derived ACE-inhibitory hydrolysates exhibit varying degree of efficacy based on the type of enzyme used in their generation. Thus, the selection of an appropriate enzyme and optimizing its condition is necessary for obtaining inhibitory hydrolysates with strong activity against ACE. Furthermore, the ACE-inhibitory hydrolysates are preferentially administered via oral route for being cheap, non-toxic and safe. However, orally administered protein hydrolysates are susceptible to degradation by gastrointestinal enzymes which decrease their bioavailability and efficacy. Hence, there is a need for the ACE-inhibitory hydrolysates to be protected via encapsulation to safeguard their structural and functional integrity for effective oral-colon delivery and biological efficacy. Moreover, in view of safety concern, the toxicity of the resulting capsule containing the ACE-inihibitory hydrolysates need to be verified especially when nanocarriers are used.

1.3 Objectives

The work was designed to achieve the following objectives;

1. To optimize the production of stone fish hydrolysates with maximum ACE-inhibitory activity using bromelain
2. To profile the ACE-inhibitory hydrolysates for the identification of the potent ACE-inhibitory peptides sequences
3. To improve the stability of the ACE-inhibitory hydrolysates by nanoencapsulation
4. To evaluate the *in vivo* efficacy and toxicity effects of the nanoparticles containing the ACE-inhibitory hydrolysates on rats

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