CHAPTER III

RESEARCH METHODOLOGY

Oligopeptides

Oligopeptides-derived from *B. mori* sericin protein were provided from Asst. Prof. Dr. Manote Sutheerawattananonda, School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakorn Ratchasrima, Thailand. The specific method of sericin extraction was described in Thai patent (Publication No. 70880)

Experimental animals

Male Wistar rats (200-250 g) were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom, Thailand. The animals were housed under a 12:12 h light–dark cycle conditions, maintained temperature (24±1 °C). The experimental protocols have been approved by the Animal Ethical Committee of Naresuan University, Phitsanulok, Thailand. The animals were allowed free access to rodent diet and tap water by the Animal Care facility centre, Chaingmai University, Thailand. All experimental animals were performed under the institutional guideline of Laboratory Animal Research (Research, et al., 1996).

Long term effects of oligopeptides; N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced hypertensive models

All experimental wistar rats were randomly divided and individually housed under control of standard conditions as previously described. The baseline of SBP and HR were measured before starting of each protocol. The procedure of non-invasive blood pressure measurement was tested by indirect (tail cuff) method. The rats were allowed to train in cage for 7 days before the experiment carrying out. All of animals were pre-warmed in a heating cage by overhead lamp that maintained temperature around 37 °C. The rat tail was put in a pneumatic cuff and attached to the distal part of the tail with pulse transducer sensor. SBP and HR were weekly recorded before and

each week until the end of each experiment. Pulse transducer is connected with Panlab NIPB LE5001 Controller (Panlab, Cornellà (Barcelona), Spain). SBP and HR were monitored and analyzed by averaging from 3 times.

The hypertensive model studies were proved the effects of oligopeptides both on protective and therapeutic effects as following:

1. The protective effects of oligopeptides against L-NAME-induced hypertensive rat

The rats were randomly divided into 9 experimental groups including:

1.1 Normotensive group, receive tap water (Ctr)

1.2 Normotensive + 200 mg/kg oligopeptides, receive the oligopeptides which is added into drinking tap water (Ctr + 200 mg/kg Oli)

1.3 Hypertensive group, receive 40 mg/kg/day L-NAME which is added into drinking tap water under the calculation of daily volume intake of tap water. (HT)

1.4 Hypertensive + 50 mg/kg oligopeptides, directly combined of 40 mg/ml L-NAME and 50 mg/ml oligopeptides into daily tap water (HT + 50 mg/kg Oli)

1.5 Hypertensive + 100 mg/kg oligopeptides (HT + 100 mg/kg Oli)

1.6 Hypertensive + 200 mg/kg oligopeptides (HT + 200 mg/kg Oli)

1.7 Hypertensive + 10 mg/kg enalapril (ACE blocker, as positive control, HT + 10 mg/kg Ena)

1.8 Hypertensive + 100 mg/kg Soy protein (Soy pro, Nutra Manufacturing, Inc., USA)(HT + 100 mg/kg soy)

1.9 Hypertensive + 100 mg/kg Bovine serum albumin (BSA, Sigma, St Louis, MO, USA)(HT+100 mg/kg BSA)

The experimental protocols were performed at 4 weeks duration of treatment of oligopeptides and other compounds as previous showing. The inducible hypertensive groups were received 40 mg/kg of L-NAME in daily drinking tap water and another normotensive was received only tap water. The treatment groups were received at each dosage compound that set on each above group (group 1-9) all the time since the experimental protocol started until the end. All of protocols were simultaneously added to daily drinking tap water. The SBP, HR and body weight were weekly recorded during the 4 weeks performing. After 4 weeks of each protocol, the animals were sacrificed. The aortic vessels were immediately collected, cleaned

connective tissue and kept in Krebs' solution until the vascular function was determined. Another part of aortic vessel was fixed into 10 % buffered formalin for histological assay of vascular structure later.

2. The therapeutic effects of oligopeptides on L-NAME-induced hypertensive rats

The experimental protocol was started and performed as the same of protective effects. However, the therapeutic protocol was induced animal to hypertensive symptoms (high pressure) for 4 weeks before treatment with each dosage of oligopeptides or other compounds with other 4 weeks later. The animals were randomly divided into 9 experimental groups as following:

2.1 Normotensive group

2.2 Normotensive + 200 mg/kg oligopeptides

2.3 Hypertensive group

2.4 Hypertensive + 50 mg/kg oligopeptides.

2.5 Hypertensive + 100 mg/kg oligopeptides

2.6 Hypertensive + 200 mg/kg oligopeptides

2.7 Hypertensive + 10 mg/kg enalapril

2.8 Hypertensive + 100 mg/kg Soy protein

2.9 Hypertensive + 100 mg/kg BSA

The experimental protocols begin with 4 weeks duration of pretreatment of 40 mg/kg BW of L-NAME in hypertensive group and another normotensive group was received only tap water. The experiments follow by 4 weeks of treatment of oligopeptides which added in daily drinking tap water in each dosage group and testing compound. The SBP, HR and body weight was weekly measured until the end of experiment. At the end of experiments, the animals were sacrificed. The aorta was collected as fast as possible for vascular function evaluation as the same of protective protocol. The some parts of aortic vessel were fixed to preserve in 10 % buffered formalin for histological assay.

3. Vascular function study.

After the experimental animals are sacrificed, the aorta was suddenly removed and cleaned their adhering connective tissue and cut into approximately 3 mm in segment length. The cleaned aortic ring was suspended in 2 ml containing

oxygenated Krebs's solution as followed composition (mM): NaCl, 122; KCl, 5; [N-(2-hydroxyethyl) piperazine N'-(2-ethanesulfonic acid)] (HEPE), 10; KH_2PO_4 , 0.5; NaH_2PO_4, 0.5; $MgCl_2$, 1; glucose, 11; and $CaCl_2$, 1.8 (pH 7.4). , and maintained the temperature at 37°C. The aortic ring was mounted between a pair platinum wire for measurement of isometric tension (Figure 12). Each aortic ring was performed under an initiation condition of resting tone at 1 g and allowed to equilibrate at least 60 minutes prior before the experimental protocols were carried out. The changes of isometric tension were recorded by isometric force transducer (Iworx System, Inc., NH 03820, USA) which connected to bridge amplifier (ADInstrument, Sydney, Australia). The analogue signal was converted to digital signal by MacLab 4/30 (ADInstrument, Sydney, Australia). The Data was recorded and monitored by chart 7 (ADInstrument, Sydney, Australia).

Each experimental aortic ring was allowed to equilibrate; phenylephrine (PE) at 1 μM was pre-contracted until sustained contraction, cumulative concentration-response curves for acetylcholine (Ach, 10^{-10}-10^{-5} M) was performed to determine the vasorelaxant responses. This relaxation was constructed on intact vascular segments that proved the endothelial functions. Furthermore, to confirm the endothelial function caused to relaxation by production of nitric oxide (NO), sodium nitroprusside (SNP, 10^{-12}-10^{-6} M) was performed by cumulative adding after pre-contraction with 1 μM of PE. The vasorelaxation was expressed to percentage of 1 μM PE contraction in both Ach and SNP protocols. Finally, the contractile response of vessel was generated by cumulative response-curve of PE (10^{-9}-10^{-5} M). The contractile response to PE was shown in percentage of contraction to 80mM KCl-induced maximal contraction.

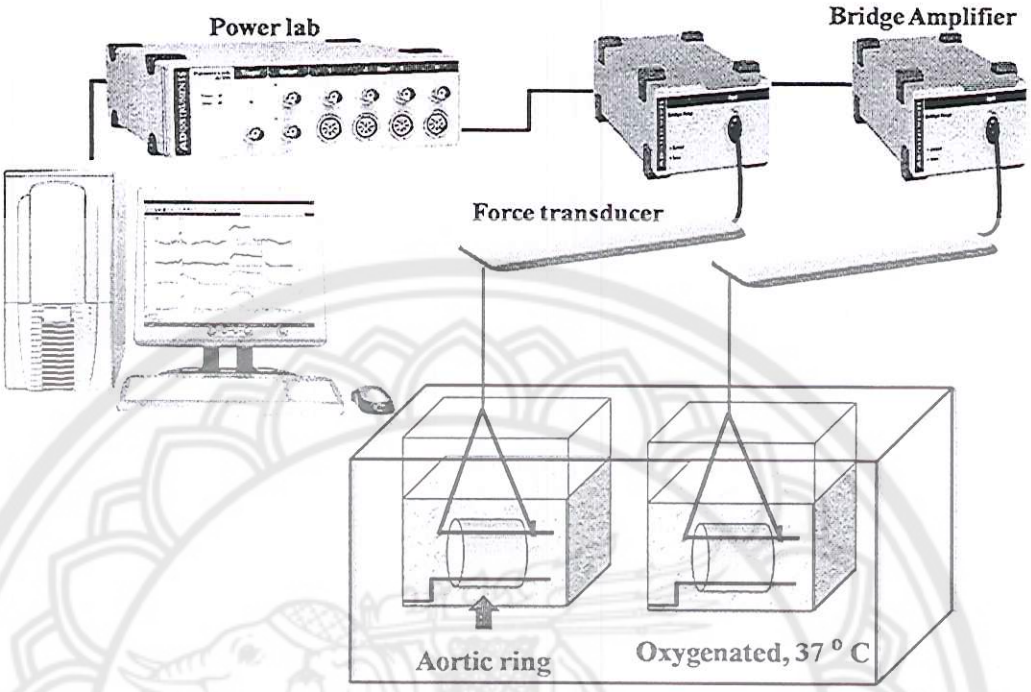

Figure 12 Diagram of organ bath for vascular study

Toxicity study of oligopeptides

1. **Acute toxicity study**

Acute toxicity study was performed according to OECD guideline (OECD, 2008). The rats and mice of each sex were randomly divided into 2 groups. The first group was considered to be the control group that consisted of 6 rats and 6 mice of each sex. The animals in control group were orally administrated with 1 and 0.5 ml of distilled water, respectively. The treated group was divided into 6 rats and 6 mice of each sex which orally administrated 1 and 0.5 ml, respectively, concomitant with oligopeptides at the dose of 2,000 mg/kg BW as a single dose. The animals were observed for lethality throughout the first 24 hr. The survived animals were kept for 14 days. During this period all recovering abnormal signs were recorded. After 14 days, all the survived animals were sacrificed and collected organs and blood for examination of hematology, biochemistry and histopathology.

2. **Chronic toxicity study of oligopeptides**

Male and female rats (8 weeks, 180-200 g) and mice (8 weeks, 20-30 g) were randomly divided into 4 groups of each species and gender including;

2.1 Control group

2.2 50 mg/kg oligopeptides administrated group

2.3 100 mg/kg oligopeptide administrated group

2.4 200 mg/kg oligopeptide administrated group

This experiment was carried out for 6 months consecutively. The control group was received daily drinking tap water. Whereas, at the same period of experiment, the treatment group was obtained oligopeptide in each dosage as above shown which added in daily drinking tap water. All animals were weekly determined body weight and monthly determined blood pressure (only rats). At the end of each experiment, all animals were fasted overnight (12 hours), sacrificed and immediately collected blood sample for determination of hematological, biochemical and lipid profile. The animal organs were preserved in 10% formalin until histological procedures are performed.

3. Hematological analysis

Animal blood was immediately collected in anticoagulation blood clot, with EDTA after the animal was sacrificed. Hematological parameters were analyzed which including white blood cell (WBC), monocyte, neutrophil, lymphocyte, eosinophil, basophil, hemoglobin, platelet and hematocrit (Hct) (MED STAR Lab,ISO/IEC 17025, 15189).

4. Biochemical analysis

As the same protocol for collecting blood sample in hematological assay, biochemical test was performed by autonomic biochemical analyzer (MED STAR Lab,ISO/IEC 17025, 15189).which including glucose, blood urea nitrogen (BUN), creatinine, uric acid, cholesterol, high density lipoprotein (HDL), triglyceride, total protein, bilirubin, aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT) and alkaline phosphatase.

5. Histopathological analysis

The animal organs include heart, kidney, aorta, lung, liver, epididymis and testis for male were evaluated. Whereas, in female including; heart, kidney, aorta, lung, liver and ovary were also determined. After preservation and fixation all of organs in 10% buffered formalin, previous description organs were processed in conventional paraffin embedding and stained with hematoxylin and eosin (H&E). The

morphology changes were observed under light microscope with 100X magnification for recording the changes of position, shape and size of these organs.

Vascular function analysis in normotensive rats

1. **Preparation of rat thoracic aorta**

Male wistar rat (200-250g) was anesthetized with 50 mg/kg by intraperitoneal injection (i.p.) of sodium pentobarbital. After complete unconsciousness, the rats was sacrificed and immediately removed of aorta for cleaning of adhering connective tissue and fat, cut vessel ring into 3 mm in length. In some aortic rings, the endothelial layer was removed by gently rubbing the intimal surface of vascular lumen for denuded endothelial ring. The aortic ring was immersed in 2 ml bath chamber which contained Krebs' solution between a pair of platinum wire (figure 12). All of chambers were continuously bubble with air and maintained 37 °C during experiment carry out. Every vessel segments were placed with initial resting tone at 1 g and allowed to 1 hour's period for equilibration of vessel before testing in each experimental protocol. The period of Krebs' solution replacement was sustained at every 15 minutes. After the equilibration period, the endothelial integrity or removal were verified with a sub-maximal concentration of PE (1 μM) pre-contraction in aorta. After the tension is stable, 10 μM of Ach was continuously added into chamber for determination of presence or absence of the endothelium-induced vasorelaxation, > 90 % relaxation was considered as endothelial intact ring, whereas, < 10 % relaxation was confirmed as endothelial-denuded ring. This verifying endothelium needed to do every ring before the experimental protocols were preformed. The exposed ring to chemical or testing compounds was washed out with Krebs' solution at least 30 minute before other procedure was generated.

2. **Vascular function study protocols**

2.1 Vasoconstrictor effects

Oligopeptides at 0.001-10 mg/ml was cumulatively added to resting tension of aorta. The contraction of aorta was expressed as percentage of contraction which compared to 80mMK-induced maximal contraction.

2.2 Vasodilator effects

Vasodilator effects of oligopeptides were performed in both endothelial intact and denuded ring. The aorta was pre-contracted with 1 μM PE or 80 mM KCl until the contracted tension is stable. Oligopeptides at 0.001-10 mg/ml was directly added to organ bath chamber in cumulative manner.

The underlying mechanisms of action of oligopeptides were investigated by using specific blocker. In endothelium-intact, aortic ring was exposed to 100 μM of L-NAME or 10 μM indomethacin (cyclooxygenase inhibitor) for 30 minutes before pre-contraction with 1 μM PE and concentration-response curve of oligopeptides.

For the endothelium-denuded ring, the possible mechanism of oligopeptides was determined by incubation of one of following inhibitors for 30 minute before initiated protocol; 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 μM, soluble guanylyl cyclase inhibitor), tetraethylammonium, (TEA,5 mM, BK_{Ca} inhibitor), 4-aminopyridine (4-AP, 1 mM, K_V inhibitor), glibenclamide (10 μM, K_{ATP} inhibitor) and $BaCl_2$ (1 mM, K_{IR} inhibitor). The response to oligopeptides was investigated in cumulative concentration manner as the same previously shown.

The relaxant data was shown as percentage of relaxation to sub-maximal contraction of PE.

2.3 Effect of oligopeptides on extracellular Ca^{2+} influx to smooth muscle cell (SMC)

To determine further mechanism of oligopeptides was constructed vasorelaxantion which involved inhibition of extracellular Ca^{2+} influx to vascular smooth muscle cell. The experimental protocol was performed under endothelial-denuded aortic ring. The free Ca^{2+} Krebs' solution was directly exposed to aortic ring and replaced to free Ca^{2+} 80 mM KCl solution for depolarizing construction of SMC. The concentration-response curve of $CaCl_2$ was cumulatively added to bath, then rinsed with Krebs' solution for 30 minutes and replaced with free Ca^{2+} Krebs' solution. When the tension went down to the basal, the ring was depolarized with free Ca^{2+} 80 mM KCl solution again. Either oligopeptides (1, 3, 5 and 10 mg/ml) or nifedipine (L-type calcium channel blocker, 1 μM) were pre-incubated for 30 minutes and $CaCl_2$ was re-challenged to construct the response-curve again. The contractile

response was expressed as percentage of contraction comparing with maximal contract of first $CaCl_2$-induced contraction.

2.4 Ca^{2+} release from intracellular stores sensitive to PE and Caffeine

To investigate whether oligopeptides can interfere with Ca^{2+} release from sarcoplasmic reticulum (SR), the experiment was carried out on endothelium-denuded aortic ring. The releasing of intracellular store was performed in free-Ca^{2+} Krebs' solution containing 1 mM EGTA. To confirm the complete constant refilling of Ca^{2+} to SR, 80 mM KCl was replaced for the SMC depolarizing and further opening voltage-operated calcium channel (VOCC) until steady state was performed. Next to wash out with free-Ca^{2+} Krebs' solution plus 1 mM EGTA for complete removal of extracellular Ca^{2+}. The aortic ring was stimulated transient contraction with 1 μM PE or 20 mM Caffeine. As the same manner, the previous procedure was repeated once time to confirm the transient contraction. The transient contractions of either PE or Caffeine were performed without (control) or with oligopeptide (1, 5 and 10 mg/ml) pre-incubation after challenging with PE or Cafeine.

Histological study

After the aorta and other organs are fixed in 10 % formalin in phosphate buffer, the processing of these tissues was carried out as following;

1. Fixation is the process to preserve the structure of these organs or tissue by inhibition of metabolism, enzymatic activity and termination of pathogenic microorganism.

2. Washing, before paraffin processing was started, these tissues needed to be cleaned out the fixative solution.

3. Dehydration was used to remove the water from tissue sample using serial alcohol solution; 70%, 80%, 90% and 100% Ethyl alcohol, respectively.

4. Clearing is the process of pushing out of dehydration solution. Xylene was used to remove the alcohol from dehydration process.

5. Infiltration, the tissue was processed in embedding medium, paraffin for maintenance intracellular components and cells structure.

6. Embedding is the process to make a solid phrase of tissue which is easier to section.

7. Sectioning, microtome was used to cut the embedded tissues into 5 μm thickness. Sectioned tissue was came off and further placed on slide for staining later.

8. Staining

8.1 Hematoxylin & Eosin (H&E) staining

The histopathology of these tissues including aorta and tissues form chronically toxicity test of oligopeptides were stained with H&E method.

8.2 Immunohistochemistry

For immunohistochemical analysis, CD31 (PCAM-1, 1:300) and endothelial nitric oxide synthase (eNOS, 1:100), the specific marker where expressed in endothelial cell was used as primary antibody. The section of aorta was cut, deparaffin with xylene and rehydration with serial ethyl alcohol before imunostaining was performed. After staining with primary antibody, the secondary antibody (1:1000) of each was incubated and finally visualized color in tissue slide by using diaminobenzidine (DAB, Envision, Dakocytomation) as chromogen. Slides were counterstained with hematoxylin.

9. Mounting is the process after staining to flow mounting medium between slide and cover slip without air bubble.

Data analysis

All data are expressed as means±standard error of the mean (S.E.M.). Statistical analysis was performed using one-way-analysis of variance (ANOVA) or student's t-test, followed by Dunnett's post hoc test by SPSS. The $p<0.05$ was considered to be significant. Concentration–response curves were plotted and adjusted by a nonlinear curves fitting program (GraphPad Prism 5).