CHAPTER III
MATERIAL AND METHODS

A. Fish skin and scale preparation

Skin and scale of giant gouramy (Osphronemus guramy) purchased from the supermarket were obtained after the fish was washed with tap water for several times. Then, the skin was cut into small pieces approximately 1-4 cm^2 and pulverized, using pulverizer (Freezer/Mill®, model 6850). The scale was directly subjected to pulverization after being removed from fish. The minced samples were then lyophilized and kept at -20°C until used.

B. Extraction of collagen from skin and scale

Collagens were prepared by the method described by Kittiphattanabawon et al (2005) with slight modification. All extraction steps were performed at 4°C with continuous stirring. In order to remove non-collagenous protein, the lyophilized skin and scale were mixed with 0.1 N NaOH at a sample/alkali ratio of 1:30 (w/v). The mixture was stirred for 6 hours with the change of alkali solution every 2 hours.

After centrifugation at 3,000 rpm, 30 mins, the deproteinized samples were washed with cold water for several times until neutral or faintly basic pH of wash water was obtained.

Deproteinized skin was defatted in 10% butanol using a solid/solvent ratio of 1:30 (w/v) for 18 hours with the change of solvent every 6 hours. Defatted skin was centrifuged at 3,000 rpm, 30 mins and washed with cold water a couple of times. The skin was then soaked in 0.5 M acetic acid containing 20% pepsin (1:10,000) with a solid/solvent ratio of 1:90 (w/v) for 24 hours. The supernatant was collected after centrifugation at 15,000xg for 60 mins. The pellet was re-extracted and the supernatant of two extractions were pooled. The collagen in the supernatant was precipitated by adding NaCl to the final concentration of 2.6 M in the presence of 0.05 M Tris (hydroxymethyl) aminomethane, pH 7.0. The collagen obtained by centrifugation at 15,000xg for 60 mins was dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid and finally sterile distilled water. Dialyzed collagen was lyophilized afterwards and kept at -20°C.

Deproteinized scale was demineralized with 0.5 M ethylenediaminetetracetic acid (EDTA) (pH 7.4) at the ratio of 1:30(w/v) for 40 hours with the solvent changed every 8 hours. The residues was centrifuged at 3,000 rpm, 30 mins and washed thoroughly with cold water. The scale collagen was then extracted in the same manner as that used for skin.

C. Determination of collagen concentration

Collagen concentration was determined by modified Lowry's method (Komsa-Penkova et al., 1996). Briefly, 200 μl of collagen solution in 0.1 M acetic acid were incubated with 180 μl of reagent A, consisting of 0.4% potassium-sodium tartrate, 10% Na_2CO_3 and 0.5M NaOH, at 50°C for 20 mins. After cooling down to room temperature, 20 μl of reagent B (2% potassium-sodium tartrate, 3% $CuSo_4\,5H_2O$ and 0.1M NaOH) was added and the mixture was incubated for 10 mins at room temperature. Then, 600 μl of diluted Folin-Ciocalteau reagent (1:15) was added. After being shaken vigorously, the mixture samples were incubated at 50°C for 10 mins. The samples were cooled and their absorbances were measured at 650 nm against the reference.

The collagen standard curve was drawn using bovine skin collagen dissolved in 0.1 M acetic acid at different concentrations of 0.1, 0.3, 0.5 mg/ml. The samples and standards were performed in duplicate.

D. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method described elsewhere (Kittiphattanabawon et al., 2005) with slight modifications. In brief, Four mg of lyophilized collagen samples were dissolved in 0.02 M sodium phosphate containing 1% SDS and 3.5 M urea at pH 7.2 with continuous stirring at room temperature. The solubilized samples were then mixed with sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) at the ratio of 1:1 (v/v) and heated at 100°C for 5 mins. After cooling down to room temperature, ten μl of sample was loaded onto 6% acrylamide gel and subjected to electrophoresis at constant voltage of 120 volt. The gel was then stained with 0.05% (w/v) Coomassie brilliant blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. The high molecular weight marker (Sigma®) was used to estimate

molecular weight of collagen proteins. Porcine tendon collagen type I (Nitta Gelatin Inc., Japan) was used as standard collagen.

E. Analysis of subunit composition

The separation of subunits in collagen samples were performed by using 1x5 cm HiTrap-CM-FF column chromatography. Approximately 40 mg of collagen samples were dissolved in 1 ml of 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea at 4°C for 24 hours and then denatured at 45°C for 30 mins. After centrifugation at 12,000xg at room temperature for 45 mins, the clear supernatant was applied to 1x5 cm HiTrap-CM-FF column chromatography connected to HPLC instrument and previously equilibrated with the same buffer for 1 hours. Elution was achieved by similar buffer comprised of linear gradient of 0-0.2 M NaC1 at a flow rate of 1.5 ml/min. Fraction of 1 ml was collected. Each subunit was detected by monitoring the absorbance at 230 nm of each fractions. In addition, fractions indicated by number were examined by SDS-PAGE on 6% gel. The staining and destaining procedures were done as previously described. Molecular weight marker (BIORAD) was used as protein marker.

F. Peptide mapping

Lyophilized collagen samples (approximately 3.0 mg) were dissolved in 1 ml of 0.1 M sodium phosphate buffer (pH7.2) containing 0.5% (w/v) SDS and heated at 100°C for 5 mins. After cooling on ice, the digestion with *Staphylococcus aureus* V 8 protease (EC 3.4.21.19, Sigma Chemical Co., St. Louis, Mo., USA) was carried out at 37°C for 30 min using the substrate:enzyme ratio of 30:1. Then, the digested sample was mixed with sample buffer to the final collagen concentration at 1.67 μg/μl and then heated at 100°C for 5 mins before applying the collagen sample on SDS-PAGE of 10% gel concentration. The electrophoresis was done as previously described with broad range molecular weight marker as protein markers.

Digestion of collagens by lysyl endopeptidase enzyme was performed by adding the proteolytic enzyme to the denatured samples at the ratio of substrate:enzyme at 2,000:1. The samples were incubated at 37°C for 5 mins. After proteolysis, the digested collagen was examined by the procedure used for *Staphylococcus aureus* V 8 protease digestion.

G. Analysis of thermal stability

The thermal stability of gouramy's collagens was carried out by the method described by Kittiphattanabawon et al (2005). The lyophilized samples were rehydrated by adding the sterile distilled water at a sample:solution ratio of 1:40 (w/v) and kept at 4°C for 2 days.

The rehydrated skin and scale collagen of 9.1 mg and 9.83 mg, respectively were analyzed for the denatured temperature using differential scanning calorimetry (DSC) model NETZSCH DSC 204F1. The both samples were put into aluminum pan and sealed then scanned by increasing of temperature at 1°C/min over the range 20-50°C. The empty pan was used as the reference. The temperature indicating melting temperature (T_m) of each collagens was determined from each DSC thermogram.

H. Amino acid analysis

Amino acid composition of collagen from skin and scale of giant gouramy were determined by amino acid analyzer, using the method written elsewhere (Van Wanderlen and Cohen, 1997). Collagen samples hydrolysed with 6N hydrocholic acid (HCl) and placed in heating block at 110°C for 22 hours. Then, internal standard were added in the hydrolysates. The mixture were diluted with deinoinized water and mixed with AccQ. Samples were incubated at 55°C for 10 min. Five μl of each samples was applied in HPLC system (WATERS Alliance 2695), using AccQ Tag column (3.9x 150 mm). AccQ Tag eluent A and 60% acetonitrite were used as eluents. The amino acid was reported as mg in 100 mg total amino acid analysed.

I. Proliferation assay

Subculture of human dermal fibroblast

Primary cell culture of human normal skin fibroblasts (HDFs) (CCL-110, ATCC, USA) at the passage of 18th was kindly provided by Dr. Neeracha Sanchavanakit, Faculty of Dentistry, Chulalongkorn University. Upon arrival, the HDFs maintaining in standard medium, Dulbecco's minimal essential medium (DMEM, Gibco, NY, USA) supplemented with 10% heat inactivated fetal bovine

serum (FBS, Gibco, NY, USA) 1% L-glutamine, 1% non essential amino acid, 1% Penicillin-Streptomycin solution and insulin 1 μg/ml (Sigma, St. Louis, USA) were plated on 100 mm culture dish. For maintaining the cell culture, cells were placed in culture incubator (NuAir, Plymouth, USA) at 37 °C in a humidified atmosphere with 5% CO_2/95% air. The cells were trypsinized with 0.25% trypsin-EDTA every 1-2 weeks after reaching the confluence and then subcultured at 1:2-1:4 spit ratio. The standard culture medium was changed every two days.

Preparation of substrates for cell culture

Giant gouramy skin and scale collagen prepared as described above, a 1 mg/ml stock and commercial collagen-Type I porcine tendon, a 3 mg/ml stock obtained from Nitta Gelatin Inc. was used as extracellular matrix substrate.

All substrates were diluted to a concentration of 1 mg/ml with sterile distilled water. In making collagen film, all type of collagen substrates (200 μl/well) was applied to 48-well Costar culture dishes bottoms prior to adding the cells. Collagen film was allowed to form even surface on the well bottom by placing the coated plate with lid in shaker cabinet at 100 rpm, 30°C, 24 hours.

MTT** [3-(4,5-dimethy-2-thiazolyl)-2,5-dimethyltetrazolium bromide] **assay

Viable cells attached to cell culture dishes coated with or without collagen were quantified at the 6, 24 and 48 h after plating using MTT metabolic assay. The MTT assay is based on mitochondrial metabolic reduction of soluble MTT to insoluble formazan crystals by mitochondrial dehydrogenase of viable cells. Relative cell numbers were suggested as a proportional to the absorbance of formazan products (Alley et al., 1988; Scudiero et al., 1988). The MTT assays were performed in triplicate for each passage. Briefly, HDFs at their 19th or 24th passage (viability more than 90% determined by tryphan blue exclusion method) were counted and plated ($1x10^4$ cells/well) into 48 well collagen coated- or non-coated-plate containing DMEM supplements with substance as shown above except heat inactivated fetal bovine serum. In addition, the control of normal HDFs growth were done by plating cells into non-coated plate containing standard culture medium with 5% FBS.

At each time point 6, 24 or 48 hours, MTT solutions at a concentration of 0.5 mg/ml (125 μl/ well) were added into sample and incubated for 15 min in CO_2 incubator. After incubation, the excess MTT solution was then removed. Subsequently, the formazan crystal trapped within cells was dissolved out with 100 μl

dimethysulfoxide (DMSO) incubated in CO_2 incubator for 15 min. The DMSO-solubilized cell solutions (100 μl) were transferred to a 96-well plate. The absorbance of all sample solution was measured in a microplate reader (Tecan®, Sunrise Absorbance plate reader, Hayward California, USA) at wavelength of 570 nm (OD_{570}). Non-specific background absorbance reading at wavelength of 620 nm (OD_{620}) was corrected for by subtracting the absorbance unit of the specimen data. The correct OD unit, OD_{570}/OD_{620} was used. The effects of collagen substrate on the viability of attached cell were then calculated as percent change by the following equation.

$$\% \text{ change} = \frac{OD_{570/620} \text{ of Exp sample - Avg } OD_{570/620} \text{ of sample grew in standard medium (5\%FBS)}}{\text{Avg } OD_{570/620} \text{ of Exp sample grew in standard medium (5\% FBS)}}$$

Statistical Analysis

All data were expressed as raw data ($OD_{570/620}$) and means $\pm$ standard error of mean and analysed by one way ANOVA and Student Newman's Keul test. Statistical significance was considered at $p < 0.05$.