Preparation collagen and collagen peptides from Bluefin tuna abdominal skin and their action on HepG2 cell

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These days consumption of fish was increased worldwide and the Japanese especially, consume a wide range of fish species daily. Particularly, Bluefin tuna (*Thunnus Orientails*) is one of the famous tunas in Japan and its consumption and culture has been increasing. However, great quantities of these wastes like skin, bone, and fin are produced in many fish shop and fish-processing factories. These wastes are dumped in which they caused pollutions emit with an offensive odor. However, fish bone, skin, scale and fins are good source of collagen. Collagen is a common protein that makes up a significant part of the living body, whether human and animal. As a structural protein, collagen is essential to build the body's physical structure, and as an extracellular matrix it acts as a supporting framework over which our cells are arranged. Also, collagen is widely and diversely used in food, medicine, and cosmetics; the consumption of collagen has increased with the development of new industrial application. Aquatic animals have been increasing attention as a backup collagen resource since bovine spongiform encephalopathy (BSE). But the study about collagen of bluefin tuna and their action on cancer cell is not sufficient from the tuna wastes.

In this study, we report preparation of type I collagen and collagen peptides and that of action on HepG2 cell.

Materials and Methods

Collagen preparation was followed. Bluefin tuna (*Thunnus Orientails*) was obtained from Okinawa campus, Kinki University, Japan. The skin of bluefin tuna was dissected out from the body and stored at -20°C. Bluefin tuna type I collagen was isolated according the reported researches and it was slightly modified. (Fig.1). The salmon skin type I collagen was purchased from Wako Pure Chemical Industries, Ltd., Japan. Collagen recovery rate was estimated by using Bradford method and collagen yield (dry basis) of bluefin tuna abdominal skin was calculated as;

(Weight of final collagen sample, g) /(weight of bluefin tuna sample, g) × 100

The bluefin tuna skin collagen and salmon skin collagen were dissolved in distilled water with various concentration (0.1~50 mg/mL) and their protein content were determined by Bradford assay with centrifugation (1,500 rpm, 5 min, 4°C) or without centrifugation.

SDS-PAGE was performed by the method of Laemmli, using the Tris-HCl/glycine buffer system with 7.5% resolving gel and 4% stacking gel. The collagen sample was dissolved in a sample buffer (0.5 M Tris-HCl, pH 6.8, containing 8% SDS, 30% glycerol, 0.2% bromophenolblue) with 20% β

-ME and then boiled for 5 min. The samples were loaded and electrophoresed. Also, for detection of bluefin tuna abdominal skin collagen, we prepared anti-fish collagen antibodies. Salmon skin collagen type I (2 mg/mL) were immunized into guinea pig with fish collagen antibody 5 times. Then, the serum was drawn 4 times at 0, 24, 31, and 45 days and checked titers and reactivities. For western blotting, the sample was loaded onto SDS-PAGE gels, electrophoresed, and then transferred onto PVDF membrane and for 1 hr incubation with a primary antibody (1:150) at 25°C. Blots were washed with washing PBS buffer (3 × 5 min) and incubated for 1 hr at 25°C with secondary antibody conjugated with peroxidase (1: 1000). The protein band on the membrane was detected on X-ray film using the standard enhanced chemiluminiscent (ELC) method (Amersham-Pharmacia Japan, Tokyo, Japan).

Preparation of collagen peptides was performed by the method of Zhang *et al.* The extracted bluefin tuna abdominal skin collagen and salmon skin collagen were dissolved in 0.1 M sodium phosphate buffer (pH 7.8) at a concentration of 6 mg/mL. After adding trypsin (EC.3.4.21.4., 11600 units/mg, Sigma Chemical Co.) with an enzyme substrate 70 μg to collagen solution, the reaction mixture was incubated at 37°C for 5 min. In order to stop the reaction, the mixture was heated immediately at 100°C for 10 min.

The degree of hydrolysis was determined by using 2,4,6,-trinitrobenzene sulfonic acid (TNBS) methods. One milliliter of 0.1% TNBS solution was added to 1 mL of sample solution (0.15 mg/mL) containing 1% sodium dodecyl sulfate (SDS) and 4% $NaHCO_3$ buffer (pH9.5), and the solution was mixed rapidly, held to react at 40°C for 2 hrs in a water bath, and finally the reaction was stopped by adding 0.5 mL of 1 N HCl and 10% SDS. The absorbance of the sample was read at 490 nm against a blank. The total number of amino group was determined in a sample that had 100% hydrolyzed at 110°C for 24 hrs in 6 N HCl.

HepG2 cells were grown in Dulbecco's-modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplement with 1% Non-Essential Amino acids, 7.5% $NaHCO_3$, 100 units/mL penicillin, 100 μg/mL streptomycine, 200 mM glutamine and 100 mM pyruvic acid in 5% CO_2 incubator at 37°C. HepG2 cell (1.0×10^4) were cultured in each well of 96 wells plate for 24 hrs in 37°C CO_2 incubator. After incubation, the collagen and its collagen peptides samples (2 μg/mL) with 10 μℓ were added to each well and sterilized water was added to control well. Then, the 96 wells plates were incubated above same condition for 24 hrs. After that, MTT method was performed (Fig. 5A). On the other hand, samples of same concentration were added when the HepG2 cell seeded in 96 wells plates. The 96 wells plates were incubated for 24 hrs and 48 hrs, respectively at 37°C CO_2 incubator and MTT methods to assay for the result were performed MTT assay for relative cell growth (Fig. 5B).

Results and Discussion

The collagen recovery rate and yield of bluefin tuna abdominal skin were shown in Fig.2. The protein recovery rate was decreased as the preparation step of type I collagen from bluefin tuna abdominal skin increased from step 3 to step 7. However, the protein yield at step 3 was 2.0 g/ 100 g and that of at step 7 was 1.8 g/100 g. Therefore, the protein recovery rate was good when type I collagen of bluefin tuna abdominal skin collagen

were prepared. The yield of bluefin tuna abdominal skin type I collagen was 4.1%, on the dry basis. This yield was similar to those of the report by researchers

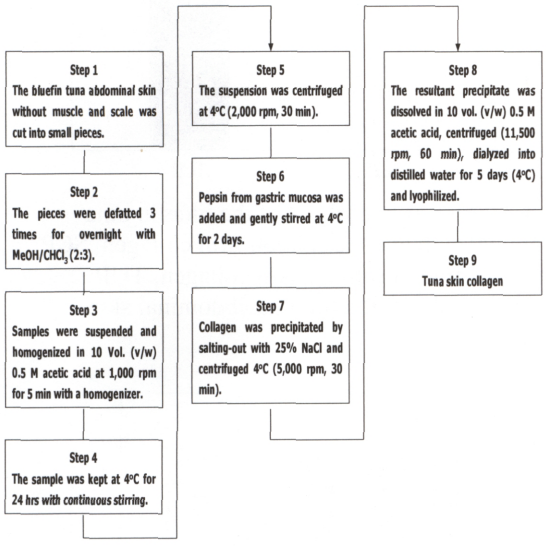

Fig.1. Diagram of extracting method of type I collagen from bluefin tuna abdominal skin.

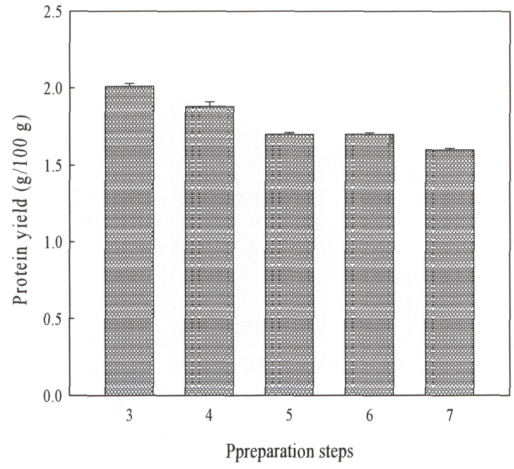

Fig.2. The protein recovery rate in preparing type I collagen from bluefin tuna abdominal skin.

Solubility of type I collagen from bluefin tuna abdominal skin and salmon skin in distilled water decreased as concentration of collagen increased.

Two collagen samples showed high solubility under a concentration of 0.1 mg/mL but the solubility reduced markedly after 5 mg/mL concentration. The bluefin tuna abdominal skin collagen was high solubility than the salmon skin collagen within all range of concentration in distilled water. This seems to the results from different imino acid contents.

The collagen samples from the bluefin tuna abdominal skin and the salmon of reagent grade were analyzed by polyacrylamaide gel electrophoresis in the presence of SDS, using 7.5% gel (Fig. 3). SDS-PAGE pattern

Table 1. Solubility of type I collagen of bluefin tuna abdominal skin and salmon skin collagen in distilled water

Samples	Concentration (mg/ml)	Solubility (%) (No centrifugation; suspension state)	Solubility (%) (Supernatant after centrifugation)
Salmon skin collagen	0.1	100.0±3.9	63.4±1.7
	0.3	55.9±0.6	25.6±2.0
	0.5	54.5±0.4	13.4±0.5
	1.0	31.7±2.9	14.5±0.5
	5.0	8.5±0.1	3.4±0.1
	10.0	4.4±0.1	1.8±0.1
	20.0	4.2±0.1	1.0±0.0
	30.0	3.7±0.1	1.3±0.0
	50.0	2.1±0.0	0.6±0.0
Bluefin Tuna abdominal skin collagen	0.1	93.8±2.2	35.5±1.1
	0.3	68.6±1.5	36.5±1.0
	0.5	59.8±0.8	31.7±0.2
	1.0	52.8±0.8	18.0±0.1
	5.0	16.6±0.4	5.2±0.1
	10.0	10.8±0.2	3.4±0.0
	20.0	4.6±0.1	2.3±0.0
	30.0	3.1±0.6	1.3±0.1
	50.0	2.3±0.1	1.3±0.0

showed that bluefin tuna abdominal skin collagen and salmon skin collagen were composed of two different α chains (α 1 and α 2) and β chain. The density of α 1 is higher than that of α 2 of bluefin tuna abdominal skin collagen and salmon skin collagen and this result were similar pattern with the previous report of other fish species and is typical of type I collagen. The estimated molecular weight for α □□and α 2 chains, using marker standard were approximately 120 kD and 112 kD. From the result, α 1, α 2, and β chain of bluefin

tuna abdominal skin collagen was lower than those of salmon skin collagen. The β chain is a dimmer and molecular weight was approximately 205 kD of samples. This SDS-PAGE pattern seems to be similar pattern regardless with different fish species. The prepared antisera against fish collagen were reacted with bluefin tuna abdominal skin collagen and salmon skin collagen. The reactivity of salmon collagen seemed to be high but the reactivity of bluefin tuna abdominal skin collagen was not relatively high.

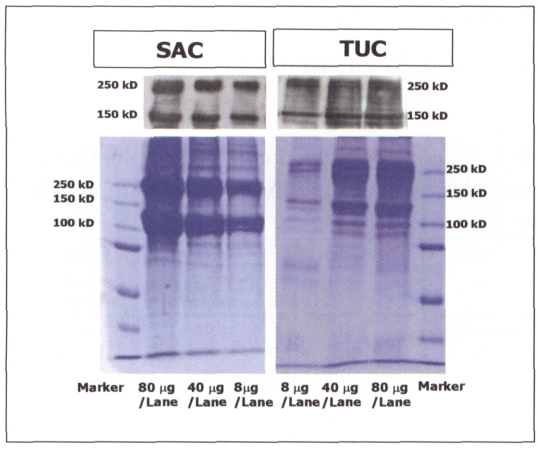

Fig.3. SDS-PAGE (lower) of fish skin collagen and western blot (upper) analysis using the antisera. SAC: salmon skin collagen; TUC: bluefin tuna abdominal skin collagen.

Degree of hydrolysis of bluefin abdominal skin collagen and salmon skin collagen showed in Fig.4. The peptides of bluefin tuna abdominal skin collagen and salmon skin collagen were prepared by enzyme, trypsin. Degree of hydrolysis of salmon skin collagen peptide was 53.1% whereas degree of hydrolysis of bluefin tuna abdominal skin collagen peptide was 96.2%. The degree of hydrolysis of the prepared salmon skin collagen peptide was lower than that of collagen peptides from bluefin tuna abdominal skin by 43%. This results maybe seems to be difference of amino acid content and imino acid content between the bluefin abdominal skin collagen and the salmon skin collagen.

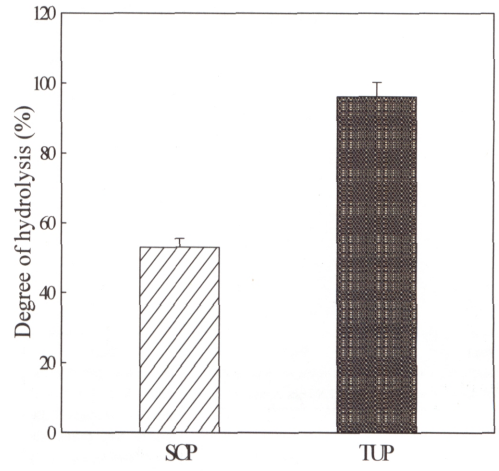

Fig.4. Degree of hydrolysis of the bluefin tuna abdominal skin and the salmon skin collagen. SCP: peptide sample of salmon skin collagen; TUP: peptide sample of bluefin tuna abdominal skin collagen.

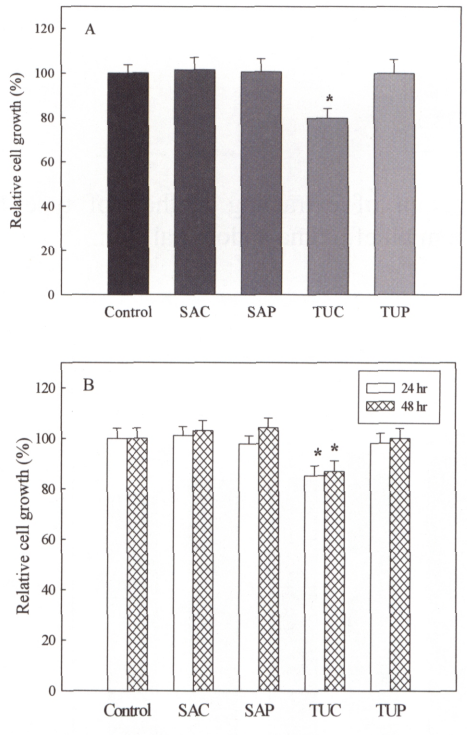

Fig.5. Effect of the salmon skin collagen, the bluefin tuna abdominal skin collagen, and their peptides on HepG2 cell growth by MTT assay. A: the cells incubated for 24 hrs at 37°C in CO_2 incubator and the samples were added to cells; B: the cells did not incubated and the samples were added when cells plated. Then, the cells were incubated for 24 hrs and 48 hrs at 37°C incubator. Control: No addition sample; SAC: salmon skin collagen; SAP: salmon skin collagen peptides; TUC: bluefin tuna abdominal skin collagen; TUP: bluefin tuna abdominal skin collagen peptides. *, $p<0.05$ compared with control.

The collagen and their peptides of bluefin tuna abdominal skin collagen and salmon skin collagen were treated on HepG2 cell by 20 μg/1.0×10^4 cells (Fig. 5A). The bluefin tuna abdominal skin collagen had 22% reduction of cell growth but the salmon skin collagen did not reduce on the cell growth, incubated for 24 hrs and 48 hrs at 37°C CO_2 incubator. The relative cell growth of this has similar trend with above data regardless with sample adding timing. This interesting result suggested that utilization value of collagen relatively. The action of both prepared collagen peptides did not reduce on the cells growth. Also, samples of same concentration were added when the HepG2 cell seeded in 96 wells plates and the 96 wells plates from bluefin tuna abdominal skin waste as a functional component on cancer cell. Moreover, studies of the mechanism for reduction effect of cell proliferation and the effect of bluefin tuna abdominal collagen on other cancer cells will be needed.

Acknowledgment

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