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**Studies on the efficient utilization of
bester sturgeon by-products
(ベステルチョウザメ由来廃棄物の有効利用)**

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Chapter I. General introduction

Fish by-products

Fishes are mostly used as a source of food for human consumption [1]. In recent years, the consumption of world fish products is increasing significantly. Between 1961 and 2016, the average annual increase in global food fish consumption (3.2%) outpaced population growth (1.6%) and exceeded that of meat from all terrestrial animals combined (2.8%). From 1961 to 2015, food fish consumption per capita grew from 9.0 kg to 20.2 kg, at an average rate of about 1.5% per year. The amount of aquatic resources captured from the wild has plateaued in the past 20 years, and instead, aquaculture production gradually replaced the fishing as the primary source of fish supply. Global aquaculture production in 2016 was 110.2 million tons, with the first-sale value estimated to be 243.5 billion United States dollar [1].

With increases in consumption of aquatic products, fish by-products have been attracted high attention. Fish by-products are fish tissues that are not suitable for consumption, including head, frames, fillet cut-offs, skin, scale, bone, cartilage, viscera, and so on. Olsen et al. [2] reported that fish by-products might constitute up to 70% of fish used in industrial processing. Thus, the present expansion of fish processing in the world is creating

increasing quantities of fish by-products [1]. In the past, fish by-products were often discarded as waste or used as material for direct feeding in aquaculture and raising of livestock and fur animals [1]. As known, discarding these fish by-products will aggravate the problem of environmental pollution.

The lack of effective use of fish by-products will result in a waste of aquatic resources because recent studies showed that fish by-products contained a considerable amount of useful substances. For example, Kotzamains et al. [3] noted that leftover of trout intestines from smoked fish was a good source of fatty acids. Fish by-products are indeed a great source of minerals and fat with abundant palmitic and oleic acid [4]. Fish by-products also contain a significant amount of useful proteins. For example, collagen is the most abundant protein in fish by-products. Due to its structure and functional characteristics, collagen has been widely used for various applications, including cosmetics, functional foods, and tissue engineering, etc. [5]. Also, diverse bioactive peptides can be produced by enzymatic or chemical hydrolysis from fish by-products. The bioactive peptides can be used in functional foods and cosmetics industries due to their excellent functions including antioxidant, antihypertensive, and antimicrobial activities [6].

In the present thesis, I focus on the collagen obtained from fish by-products and, in this section, review the characteristic features of fish collagen and two ways of its high-value-

added usage.

Biochemical and biophysical characteristics of fish collagen

Many studies have shown that collagens could be extracted from different fish species like shark, sturgeon, catfish, carp, and so on [7]. The non-edible part of these fish such as skin, bones, heads, fins, cartilage, and scales are good sources of collagens [7]. From these sources, mainly two types of collagen, types I and II, can be obtained. Type I collagen, whose molecule is a triple helical trimer of two identical $\alpha 1(I)$ and one $\alpha 2(I)$ chains, expressed as $[\alpha 1(I)]_2\alpha 2(I)$, is extracted from the skin, scales, and bones [7, 8]. In many fish species, another type of α chain named $\alpha 3(I)$ also exists [9]. In such fish, type I collagen molecules are a mixture of $[\alpha 1(I)]_2\alpha 1(I)$ and $\alpha 1(I)\alpha 2(I)\alpha 3(I)$. The $\alpha 3(I)$ chain is a characteristic feature of fish collagen and not observed in mammals [10]. Type II collagen is abundant in cartilage and notochord [7, 8], and its molecule consists of three identical $\alpha 1(II)$ chains ($[\alpha 1(II)]_3$) [11]. Such structure is the same as that of mammalian type II collagen.

As in the case of mammalian collagen, each peptide chain of the fish collagen molecule retained the repeating unit (Glycine (Gly)-X-Y), where X and Y are often proline (Pro) and hydroxyproline (Hyp), respectively [12, 13]. The monomer collagen molecule is approximately 300 nm in length and 1.5 nm in diameter in mammals [14]. From the

molecular weight and amino acid sequences of α chains in fish collagen, the size of a collagen molecule in fish should be similar to that in mammals [15]. The extracted collagen molecules of both mammals and fish can self-assemble into a supramolecular form (collagen fibril) *in vitro* [16] with a quarter-stagger package of molecules showing 67 nm D-periodic banding pattern [16, 17]. However, the amino acid composition between fish and mammalian collagens were different; especially the imino acids (Pro and Hyp) content in the mammalian collagens were higher than that of fish collagens [18]. This property is considered as the reason that the denaturation temperature of mammalian collagen is higher than fish collagen. Additionally, in the previous study, the fibril-forming ability of fish collagen was significantly higher than mammalian collagen at the same condition [8].

High-value-added usage of fish collagen-1: tissue engineering

One of the recent approaches to utilize collagen is tissue engineering research and application. Every year, many patients suffer the loss of organ or tissue as a result of disease or accidents. Organ or tissue transplantation is a general therapy to treat these patients. However, the transplantation therapy (regenerative medicine) is extremely limited because of the lesser number of donors, negative immune responses, pain to patients, load to donors, and the high price of the treatment, etc. [19]. Recently, tissue engineering has attracted many

scientists and surgeons with a hope to treat patients in a minimally invasive and less painful way [20]. The overall design of regenerative medicine using engineered tissues is as follows: isolate specific cells from patients, then grow them on a biomimetic scaffold under precisely controlled conditions, after that deliver the construct to the desired site in the injured part, finally direct new tissue formation into the scaffold [21].

Scaffold materials are the most crucial point for tissue engineering. Several requirements have been identified to be essential for scaffold materials: biocompatibility to prevent host tissue responses to implant, high cell adhesion properties to promote cell attachment and proliferation, adequate mechanical properties to maintain the structure and function immediately, and biodegradability to facilitate the formation of new tissue [22].

Collagen has been widely used as a material for scaffold due to its structure and biochemical characteristics. Collagen is the major protein in the extracellular matrix *in vivo* and plays as a cell scaffold in combination with other proteins and glycosaminoglycans in the tissues [23].

Collagen also gives particular mechanical property to the tissue. In bone, for example, collagen provides a framework for hydroxyapatite deposits and is a principal source of tensile strength [24]. In cartilage, type II collagen accounts for 60% of total dry weight and presents thin fibrils with a diameter less than 10 nm. These thin fibrils, together with proteoglycans which bind to the fibrils, contribute to the viscoelasticity and compressive function of

articular cartilage [25]. In tendon, collagen fibrils are generally oriented longitudinally to support high tensile stress exerted on the tissue [26].

Furthermore, collagen fibrils also interact with cells through specific receptors and regulate cell growth, differentiation, and migration [27]. Collagen fibrils also retain low antigenic activity, high cell adhesion properties, biocompatibility, and biodegradability [28]. In addition, the gels containing collagen fibrils with structural, mechanical, and biological properties similar to native fibrils *in vivo* can be fabricated through the self-assembling of extracted collagen molecules under suitable conditions [29-31]. These potential values of collagen as a biomaterial have led to numerous researches on collagenous scaffolds for tissue engineering. Many collagen materials with various morphologies, such as collagen sponge, nanofibrillar gel, and hydrogel, have already been applied as scaffolds in tissue engineering [32-34].

Till now, the most common collagen sources for biomedical usages are bovine skin and tendons, porcine skin, and rat tail [35]. However, concerns on these collagens are growing because of zoonoses such as bovine encephalopathy (BSE), foot-and-mouth disease (FMD), and African swine fever (ASF) [36]. Also, the products from porcine are repelled by Muslims due to religious barriers [37]. Therefore, alternative collagens extracted from fish sources, especially from fish by-products, have attracted increasing attention [38].

There are many reports on the fibril-forming ability of fish collagens including those from walleye pollock skin, tilapia skin, snakehead skin, shark skin, grass carp skin, and sturgeon skin and swim bladder [39-43]. However, only a few reports studied the application of fish collagen to biomedical materials. One of the reasons is lower thermal stability of fish collagen compared with mammalian one because low thermal stability of the molecules restricted the stability and strength of collagen materials. The denaturation temperatures of collagen molecules were about 40°C in mammals [44], 44°C in chicken [45], but 20-30°C in fish [46]. Low Hyp contents in fish collagen were considered as a reason for the low denaturation temperature. Previous reports found that Hyp contents of fish collagens were lower than those of mammalian collagens [8, 47]. The formation of water bridges and hydrogen bond are essential for stabilization of collagen molecules, and the hydroxyl group of Hyp provides for the bonding with a water molecule to stabilize the triple helical structure of collagen [48].

To increase the stability and strength of materials made of fish collagen, the cross-linking agents are generally applied. Yunoki et al. [49] successfully improved the thermal stability of a gel fabricated from salmon collagen by cross-linking the gel with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and found that salmon collagen gel promoted the human cell proliferation. Hoyer et al. [50] engineered a cytocompatible matrix with EDC

cross-linked jellyfish collagen and showed a potential of the material to support and maintain chondrogenic stimulation of human mesenchymal stem cells. These successful examples indicated the high potential of fish collagens as scaffold materials for tissue engineering when appropriate cross-linking was applied.

High value-added usage of fish collagen-2: functional peptides

In recent years, studies on fish-derived peptides attracted high attention of researchers because peptides showed various bioactivities, such as antioxidant, antihypertensive, antimicrobial, anticancer activities, and so on [51-54]. Due to these functionalities, fish peptides are applied for health foods and cosmetics. The functionalities of peptides depend on two factors: amino acid composition and molecular weight. For example, the binding of peptides to angiotensin-converting enzyme (ACE) is strongly influenced by the presence of tyrosine (Tyr), phenylalanine (Phe), tryptophan (Trp), Pro, lysine (Lys), isoleucine (Ile), valine (Val), leucine (Leu) and arginine (Arg) [55]. Amino acid residues such as histidine (His), Leu, Tyr, methionine (Met), and cysteine (Cys) are associated with radical scavenging activity [56]. Hydrophobic amino acids such as Pro and Hyp appear to play a role in the inhibition of lipid peroxidation [57]. As for the molecular weight of peptides, short peptides with a molecular weight below 3 kDa usually have higher bioactive properties than

long peptides [58].

In the Japanese food market, several products containing fish peptides as functional ingredients have been approved by Secretary General of the Consumer Affairs Agency of Japan as Food for Specified Health Uses. These products have been proved to be suitable for consumption by humans and launched into markets as dietary supplements or functional medicine [59]. However, the problem of digestion has been highly controversy of functional peptides. Since most of them are around 500–1500 Da in size, the majority of them may not have the ability to penetrate the intestinal cells and enter the blood circulation. From the parent peptide sequences, digestion may yield smaller peptides with the ability to reach target organs via the blood circulation, but bioactivities of such small peptides wait for further clarification.

Another possible application of peptides in the food industry is the prevention of oxidation of fatty acids and lipids. The reactive oxidative species (ROS)-mediated peroxidation of fatty acids and lipids can evoke color changes and flavor loss and generate toxic compounds in foods. At present, some synthetic antioxidants including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) are used as antioxidant additives to prevent lipid peroxidation in the food industry [60]. However, the use of these synthetic antioxidant additives is strictly regulated because of

their potential health hazards [60]. Previous research showed that the peptides with hydrophobic amino acids Pro and Hyp played a role in the inhibition of lipid peroxidation [59]. Since Pro and Hyp are rich in collagen, fish by-products, whose major protein is collagen, are the promised source of antioxidant peptides that can apply to prevent peroxidation of fatty acids and lipids in foods.

Anti-aging and moisturizing activities are the two major activities expected for functional cosmetics, and thus, materials that retain antioxidant activity and water holding capacity are the targets of the research in the cosmetic industry. In aerobic organisms, ROS with a high reaction activity, including hydroxyl radical, superoxide anion radical, hydrogen peroxide, and singlet oxygen, are generated during the metabolism [61]. In general, cellular antioxidases, such as superoxide dismutase, glutathione peroxidase, and catalase, can keep ROS in a state of balance [62]. Ultraviolet irradiation, aging, stress, environmental deterioration, and other reasons can disrupt the balance, and excess ROS in the body can induce enzyme inactivation, polysaccharide depolymerization, DNA cleavage, and cell membrane destruction [62]. These physiological damages caused by ROS result in skin aging, moisture loss, and wrinkles generation. Therefore, it is necessary to find the natural antioxidant additives for anti-aging cosmetic products. The previous research has shown that the peptides of molecular weight below 2 kDa with structural modification could pass through

the epidermal layer to a depth of 120 μm [63]. Actually, most of the antioxidant peptides reported from marine organisms showed a molecular weight below 2 kDa [64, 65]. It indicated that these antioxidant peptides are possible to exert their activities by percutaneous absorption. Also, these low molecular weight peptides have high water holding capacity [66]. For example, the presence of glutamic (Glu) and aspartic acids (Asp) with polar side chains in rainbow trout hydrolysate allows substantial water holding capacity [67]. Low-molecular-weight peptides show higher water-holding capacity than larger peptides because smaller peptide fragments are more hydrophilic, and higher ratio of polar groups, such as $-\text{COOH}$ and $-\text{NH}_2$ caused by enzymatic hydrolysis, significantly affect the amount of adsorbed water. According to these characteristics and functionalities, the peptides derived from fish protein hydrolysates are expected to apply for the cosmetics industry.

Sturgeons and their by-products

In the present thesis, I focused on the collagen obtained from by-products of sturgeon that is a highly valuable food fish and is especially famous for its caviar (ovary). However, because of the long culture period required to obtain caviar, the culture cost of sturgeon is higher than other fishes. The lack of efficient utilization of sturgeon tissues other than caviar constrains the profitability of the sturgeon aquaculture industry in some countries including

Japan. Therefore, I believe that the development of new commercial products from sturgeon by-products will support further development of the sturgeon aquaculture.

Previous studies in my laboratory have found that the quantity of collagens could be extracted from better sturgeon skin, swim bladder and notochord [8]. SDS-PAGE patterns and biochemical characterizations identified collagens from the skin and swim bladder as type I and from the notochord as type II. The extracted collagen molecules maintained the triple helical structure. Sturgeon skin and swim bladder type I collagens showed a better fibril-forming ability and better thermal stability than type I collagens obtained from other fish species [8]. Besides, for tissue engineering application, a novel technique to coat two types of highly oriented fibrils with different fibril diameter was developed using the swim bladder collagen in my laboratory (Miura, Moroi, and Tamura, unpublished observations). The two types of fibrils showed distinct influence on cell morphology, proliferation, and differentiation. These findings suggest that collagen obtained from sturgeon by-products have high potentials as scaffolds for tissue engineering. However, I found some tasks remained to be overcome to industrialize collagens from sturgeon by-products.

Research objectives

The first task to be overcome is a low yield of sturgeon collagens. The yields of

sturgeon collagen, 34.1% in skin and 5.1% in notochord (dry weight basis) [8], was lower than other fish collagen [68, 69]. Notably, the yield of type II collagen from notochord was very low. Such a low yield restricts its industrial application, although type II collagen is highly valuable in the market because of much lesser availability than type I collagen. Therefore, the first mission of the present study was to increase the yields of sturgeon collagens by improving extraction method. In Chapter II, I studied the effects of alkaline pretreatment on the yield, biochemical characteristics, and fibril-forming ability of skin type I and notochord type II collagens.

Second, factors that affect *in vitro* fibril formation and fibril morphology of sturgeon collagens have been clarified only in part. The regulation of fibril morphology is necessary when sturgeon collagens are applied for the cellular scaffolds of tissue engineering because the fibril morphology exerts potent effects on the cell behaviors as already described [27]. In the *in vitro* experiments of fibril formation in mammalian collagens, divalent anion strength of the fibril-forming fluid dramatically influences the process of fibril formation. However, such influences on the fibril-formation process of fish collagen are still unclear. It is necessary to investigate the influence of divalent anion strength on the fibril formation of sturgeon collagen and then achieve the regulation of fibril morphology. In this study, influences of phosphate ion concentrations on type I collagen fibril formation was clarified in Chapter III.

Meanwhile, the effects of different fibril morphologies on the morphology and proliferation of fibroblasts were examined.

Third, till now, researches in our laboratory on sturgeon by-products were all about triple-helical collagen molecules or collagen fibrils, and their application was mainly set for biomedical purposes. Medical collagen is the most expensive way of the usages but is required most strict safety tests for industrialization. Thus, a long time will be needed to realize commercial medical products using the sturgeon collagen. Given the current wide utilization of peptides obtained from fish by-products in health foods and cosmetics, the application of collagen peptides from sturgeon by-products is close to industrialization if the products show useful functionalities. Thus, the third objective of this study is to exploit the functionality of collagen peptides from sturgeon by-products for food and cosmetic industries. In Chapter IV, I investigated the antioxidant activity of collagen peptides produced from the sturgeon skin.

Finally, in Chapter V, based on the results of the experiments, I discuss the possible applications of sturgeon collagen in different forms: molecule, fibril, and peptides. I also discuss the practical application directions of three sturgeon by-products, notochord, swim bladder, and skin, according to the characteristics of each tissue.

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Chapter II. The effect of alkaline pretreatment on the biochemical characteristics and fibril-forming abilities of types I and II collagen extracted from bester sturgeon by-products

As already described in Chapter I, the previous study [1] showed that sturgeon contains large amounts of type I collagen in the skin and swim bladder, and small but industrially extractable amounts of type II collagen in the notochord. Moreover, the type I collagen extracted from sturgeon was quick to reassemble into fibrils, suggesting its suitability to fabricate biomedical materials. However, the low yields of collagen obtained from sturgeon by-products constitute a limiting factor to industrialize sturgeon collagens.

Alkaline pretreatment of tissues at low temperature is a common method to remove non-collagenous proteins to enhance collagen extraction and is widely used as a pretreatment for fish collagen purification [2, 3]. Alkaline pretreatment is also employed to reduce the endotoxin content of the collagen. Since endotoxins induce strong biological effects when they enter the human blood stream, even at very low concentrations [4], low-endotoxin collagen is desirable for biomedical use. After alkaline pretreatment of bovine hide type I collagen, however, Hattori et al. [5] have reported that collagen denaturation temperature was decreased, intramolecular cross-links involving proline and hydroxyproline were broken, and a portion of

the collagen lost its ability to form fibrils. In addition, the proposed alkali-soluble collagen theory [6] suggests that some loss of collagen would occur during alkaline pretreatment. However, the effects of alkaline pretreatment on the yield and nature of fish collagen are still unclear. Moreover, specific effects on different types of collagen having different biochemical and physicochemical properties are unknown, both for mammalian and fish collagens.

Because the extraction procedure employed in the previous study [1] did not include alkaline pretreatment, the biochemical nature of alkali-pretreated collagen was not addressed. In fact, as far as I know, the biochemical nature of alkali-pretreated and non-pretreated fish collagen has not been compared, even though alkaline pretreatment is frequently employed for the extraction of fish collagen. Therefore, the present study clarified the effect of alkaline pretreatment on the extraction process, yield, biochemical nature and fibril-forming ability of types I and II collagen from bester sturgeon skin and notochord.

Materials and methods

Isolation and purification of collagen

A live, cultured bester sturgeon (1.24 m, 6.00 kg) was procured from the Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan. Skin and notochord were removed and washed with chilled tap water, lyophilized in a

freeze dryer (FDU-830, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and stored at -30°C until use.

Skin fat was removed over 24 h in 99.5% ethanol (two solution-changes) at 4°C , with a sample:solution ratio of 1:10 (w/v). Defatted skin and notochord were cut into small pieces (approximately $0.5\times 0.5\text{cm}$) for collagen extraction. The tissues were then divided into alkali-pretreated and non-pretreated groups. In the alkali-pretreated group, the tissues were continuously stirred in a solution of 0.1 M NaOH for 12 h at 4°C , with a sample:solution ratio of 1:50 (dry w/v; two solution-changes). After alkaline pretreatment, the tissues were washed with cold distilled water until the pH value was neutralized and were then lyophilized.

The following extraction and purification procedures were conducted at 4°C . To extract collagen, the alkali-pretreated and non-pretreated tissues were stirred continuously in a solution of HCl (pH 2.0) containing 0.1% (dry w/v) porcine pepsin (EC 3.4.23.1, 1:10,000, Wako Pure Chemical Industries Ltd., Osaka, Japan) for 48 h, with a sample:solvent ratio of 1:100 (dry w/v). The mixtures were then centrifuged at $2,000\times g$ for 90 min (Model 6800, KUBOTA Manufacturing Corporation, Tokyo, Japan) to obtain supernatants. The supernatants were kept at 4°C until use, whereas the precipitates were re-extracted several times under the same conditions until no residue remained in the solutions obtained. Next, the supernatants were combined and salted-out by adding NaCl to a final concentration of 1 M. The precipitate

was collected by centrifugation at 10,000×g for 30 min, and re-dissolved in HCl solution (pH 2.0). The solution was dialyzed for 24 h with a 100-kDa dialysis membrane, against 50 volumes of distilled water, with two changes of the distilled water. Finally, the dialysate was lyophilized and stored at −30°C until use. The percentage of dry weight of collagen extracted in comparison with the dry weight of the initial tissues was calculated as the collagen yield. Experiments were conducted for 4 times, and the data were expressed as means ± standard errors (SE).

Amino acid analysis

The amino acid composition was analyzed at the Instrumental Analysis Division, Equipment Management Center, Creative Research Institution, Hokkaido University. In brief, samples were hydrolyzed in 6 M HCl at 110°C for 24 h. The hydrolysates were evaporated, and the remaining materials were dissolved in a citric acid buffer, and then analyzed using an automated amino acid analyzer (JLC-500 V, JEOL Ltd., Tokyo, Japan). Samples were assayed three times, and the means were calculated to obtain amino acid compositions.

Soluble-collagen quantification

The amount of solubilized collagen in each extraction step was measured using a

soluble collagen quantification kit (Sircol Soluble Collagen Assay, Biocolor life science assays, Northern Ireland U.K.), following the manufacturer's protocol. Purified types I or II collagen from the skin and notochord of baster sturgeon was used for standards. The amount of solubilized collagen in each extract was expressed as the percentage to the sum of the solubilized collagen of all extracts.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli [7]. The lyophilized collagens were dissolved in HCl (pH 2.0, 1 mg collagen/ml) and then mixed at a ratio of 1:1 (v/v) with sample buffer (0.5 M Tris–HCl buffer, pH 6.8, with 4% SDS and 20% glycerol) containing 10% β -mercaptoethanol. The mixed solution was boiled for 5 min. Ten micrograms of the mixture were loaded onto each lane. Electrophoresis was performed at 15 mA for the stacking gel and 20 mA for the 7.5% running gel. After electrophoresis, the gel was stained for 30 min with a 0.1% Coomassie Brilliant Blue R250 solution and destained with a mixture of 20% ethanol, 5% acetic acid, and 2.5% glycerin. Precision plus protein standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to estimate the molecular weight.

Circular dichroism (CD) measurement

CD spectra were measured using a JASCO model 725 spectrometer (JASCO, Tokyo, Japan). The measurement was performed following the method of Ikoma et al. [8].

Lyophilized collagens were dissolved in an HCl solution (pH 2.0) to 1 mg/ml and placed into a quartz cell. CD spectra were measured at 190–250 nm wavelengths at 10°C under a scan speed of 50 nm/min with an interval of 0.1 nm. The rotatory angle at a fixed wavelength of 221 nm was then measured at 10–50°C with a rate of 1°C/min to determine the denaturation temperature of the collagen molecules.

Collagen fibril formation *in vitro*

The fibril formation process of collagens was evaluated by the method of Zhang et al. [1] with some modifications. Lyophilized collagens were dissolved in an HCl solution (pH 2.0) to 0.3% (w/v). The skin and notochord collagen solutions were mixed with a 45 mM Na-phosphate buffer (pH 7.4). The ratio of collagen solution:Na-phosphate buffer was 1:2 (v/v). The mixed solution was placed into a cell, and the subsequent fibril formation at 21°C was monitored by measuring increased turbidity via optical absorbance at 320 nm, using a spectral monitor (Hitachi High-technology Corporation, Tokyo, Japan). Fibril formation was monitored for 1 h and 10 h for the skin and the notochord collagens, respectively.

Morphology of fibrils

The microstructure of skin and notochord collagen fibrils was observed using a scanning electron microscope (SEM; JSM6010LA, JEOL Ltd., Tokyo, Japan), as previously described by Zhang et al. [1]. Collagen fibrils were formed using the same conditions as described above. Sample suspensions were centrifuged at 20,000×g for 20 min at 4°C to obtain precipitates of collagen fibrils. The fibrils were fixed with 2.5% (v/v) glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature, rinsed with the phosphate buffer to remove the fixative, dehydrated with a graded series of ethanol solutions, and then soaked in two 30-min changes of t-butyl alcohol solution. Finally, collagen fibrils were freeze-dried in a t-butyl alcohol solution with a freeze-drying device (JFD-320; JEOL Ltd.) and coated with gold-platinum using an auto fine coater (JFC-1600; JEOL Ltd.).

Degree of fibril formation

The determination method of the fibril formation degree was followed by the method of Zhang et al. [1]. Skin and notochord collagen fibrils were formed as described above. After the centrifugation at 20,000×g for 20 min at 4°C, the protein concentration of the supernatant was measured following Lowry et al. [9] using bovine serum albumin as a standard. The degree of collagen fibril formation was defined as the percentage of the decrease of collagen

concentration in the solution after the experiment, which means the percent of collagen molecules that formed the fibrils.

Thermal stability of fibrils

The maximum transition temperature (T_m) of re-assembled fibrils of skin and notochord collagens was measured by differential scanning calorimetry (EXSTAR DSC6100, SII Nano Technology Inc., Chiba, Japan). Collagen fibrils were prepared using the same conditions as described above. The sample solutions were centrifuged at $20,000\times g$ for 20 min at 4°C to get precipitates of collagen fibrils. The precipitates were placed in an Al pan (70 μl), measured at $20\text{--}60^\circ\text{C}$ at a rate of $3^\circ\text{C}/\text{min}$ using Na-phosphate buffer pH 7.4 as a reference.

Statistical analysis

Data are expressed as means $\pm$ standard errors. Significance was set at $p < 0.05$. Statistical analyses were performed using student's t -test, in Microsoft Excel add-in statistical software (SSRI, Tokyo, Japan).

Results and discussion

Amino acid composition

The amino acid compositions of samples are shown in Table 1. The characteristic repeating unit of a collagen monomer is Glycine (Gly)-X-Y, where X and Y are often proline (Pro) and hydroxyproline (Hyp), respectively [10, 11]. Furthermore, collagen has abundant alanine (Ala) (approximately 10% of total amino acids) but low amounts of cysteine (Cys), methionine (Met), and threonine (Thr) [12, 13]. Results of the amino acid analysis show that both tissues contained Gly as the major amino acid (skin 32.2%, notochord 30.4%) and were rich in Pro, Hyp and Ala. These data support the relatively high collagen content of the sturgeon skin and notochord. Hyp is an imino acid, which is unique to collagen and often used to calculate collagen content [14]. From the Hyp content, the collagen contents of the tissues were calculated as 85.7% in the skin and 75.5% in the notochord.

After the alkaline pretreatment, the Hyp content in skin and notochord increased, and the collagen content of the alkali-pretreated tissues was calculated as 98.8% for the skin and 90.7% for the notochord. This result suggested that alkaline pretreatment efficiently removed non-collagenous proteins, especially from the skin. There were no significant differences in amino acid composition between the purified non-pretreated and alkali-pretreated collagens from either tissue. Thus, alkaline pretreatment did not affect collagen amino acid composition.

Soluble-collagen analysis

I conducted the analysis three times and obtained almost the same results. The typical results were shown in Fig. 1.

Three extractions were needed for the non-pretreated skin to fully solubilize the tissue. The second extract contained 49.4% of total solubilized collagen, which was more than the first (40.4%) and the third (10.1%) extracts. For the alkali-pretreated skin, 71.3% of total solubilized collagen was obtained in the first extraction, and all solubilized collagen was extracted after the second. Thus, the alkaline pretreatment improved the efficiency of collagen extraction from the skin.

For the non-pretreated notochord, only 0.1% of the total solubilized collagen was obtained in the first extraction. The second extract contained 75.0%. After that, the solubilized collagen content decreased with repeated extractions, and approximately 23.1% of the solubilized collagen was obtained in the third extraction. Thus, the notochord collagen was more difficult to extract than the skin collagen. The precise reason is as yet unknown, but it is possible that notochord contains more tightly-packed collagen tissue than skin, reflecting their different physiological roles in the body. Sturgeon insufficiently develops cartilaginous vertebrae and the notochord functions as the major axis of the body. Therefore, the notochord has a strong but flexible collagenous sheath that resists high pressure [15, 16].

After alkaline pretreatment of the notochord, only two extractions were needed to obtain all the solubilized collagen. Combined with the results of the skin collagen extraction, I conclude that alkaline pretreatment not only removed non-collagenous proteins but also accelerated the solubilized collagen extraction process. Hattori et al. [5] found that the extractability of bovine skin collagen, when treated with an alkaline solution, was much higher than that of non-treated tissue. In the present study, the same phenomenon was demonstrated for the first time for both types I and II fish collagens. Alkaline pretreatment might cause significant swelling of the collagen fibril structure [6]. After collagen fibrils are swollen, pepsin should more easily contact the collagen molecules to break their intermolecular cross-links.

Yields of collagens

The yields of purified collagen are summarized in Table 2. The yields of skin and notochord collagen were $63.9 \pm 0.19\%$ and $35.5 \pm 0.68\%$ (dry weight basis), respectively. These yields were much higher than the previous study (skin, 31.4%; notochord, 5.1%) in my laboratory [1]. Incomplete extraction might be the main factor that limited the yield in the previous study. In the present study, the increased number of extraction cycles increased

collagen yields and thus reduced waste. Also, a filtration process was avoided in the present study. This may be another reason for the reduced loss of solubilized collagen.

The collagen yields of alkali-pretreated tissues decreased to $56.2 \pm 0.84\%$ and $31.8 \pm 1.13\%$ for skin and notochord, respectively. This result suggests that collagens were lost during the alkaline pretreatment, and the loss of skin collagen was greater than that of notochord collagen. The existence of alkali-soluble collagen was previously reported for mammals [5]. The present results suggest that sturgeon skin contains more alkali-soluble collagen than notochord. Combined with the amino acid composition and the soluble-collagen analyses, it can be concluded that the alkaline pretreatment improved collagen extraction efficiency, but at the expense of greater collagen losses.

The yield of collagen from sturgeon skin ($63.9 \pm 19\%$) in the present study was significantly higher than that from catfish skin (44.8%), ocellate puffer fish skin (55.4%), and black drum skin (18.1%) [17-19]. It is difficult to compare the notochord collagen yield with other fishes or mammals because there have been few other studies on notochord collagen utilization. Even so, the yield of notochord collagen ($35.5 \pm 0.68\%$) was same levels or higher than that from cuttlefish skin (35%), black drum skin (18.1%), and rhizostomous jellyfish (35.2%) [19-21]. From these results, we conclude that sturgeon skin and notochord are suitable for large-scale industrial production of type I and type II collagen, respectively.

On the other hand, the collagen content of notochord was estimated as 75.5% of the tissue weight, but the yield of type II collagen was only $35.5 \pm 0.68\%$. I suppose that the loss was mainly generated during the salting-out process; the 1 M NaCl might not completely precipitate type II collagen from the extraction solution. In another study, a higher concentration of NaCl (4 M) was used to precipitate type II collagen from Amur sturgeon cartilage [22]. The salting-out using 2 M NaCl to obtain a greater yield was also tested, but the resulting collagen could not form a fibril structure (data not shown). Instead of the salting-out method, Cao and Xu [23] used a chromatographic separation method to purify collagen. Considering the cost of the method (especially on an industrial scale), I did not employ chromatographic separation in the present study. Studies on more efficient salting-out conditions should be pursued in the future.

SDS-PAGE

The SDS-PAGE patterns of collagens are shown in Fig. 2. As reported in the previous study [1], the non-pretreated skin collagen had two α -chains as the major constituents (approximately 120 kDa and 100 kDa), whereas the non-pretreated notochord collagen had only one α -chain (approximately 130 kDa), indicating that the skin and notochord collagen

were type I and type II, respectively. For both tissues, the alkaline pretreatment did not affect the band pattern.

High molecular weight bands, which might be cross-linked α -chains (that is, β - and γ -chains), showed lower intensities in the notochord collagens compared with the skin collagens, suggesting that the number of cross-links was much less in the notochord collagen. Also, the β - and γ -chain band intensities from bester sturgeon skin presented in the present study are much lower compared with those from carp and bigeye snapper skin [2, 24]. Therefore, the results suggest that inter- or intra-molecular cross-links in sturgeon skin collagen are less than those of other fishes.

Thermal stability of collagen molecule

CD spectra of skin and notochord collagens are shown in Fig. 3A. All collagens had a rotatory maximum at 221 nm and a crossover point (zero rotation) at approximately 212 nm, which are typical characteristics of the collagen triple-helical conformation [1]. Measurement of the collagen denaturation temperature is shown in Fig. 3B. As the temperature increased, CD values at 221 nm decreased following decomposition of the collagen triple-helical structure. The denaturation temperature of each collagen preparation was determined as the temperature with the fastest decrease in CD value. As previously shown by Zhang et al. [1],

the denaturation temperature of non-pretreated notochord collagen (31.8°C) and alkali-pretreated notochord collagen (32.0°C) were higher than those of non-pretreated skin collagen (27.0°C) and alkali-pretreated skin collagen (26.8°C).

No obvious differences were observed between the alkali-pretreated and non-pretreated collagens, suggesting that alkaline pretreatment did not affect the triple-helical structure. Bowes and Kenten [25] found that some peptide links involving proline and hydroxyproline were broken when bovine skin was treated with NaOH for 14 days at 20°C. Hattori et al. [11] reported that the denaturation temperature of bovine hide collagen decreased by 6°C after treatment with 0.75 M NaOH for several days at 20°C. Thus, the denaturation of collagen molecules may be induced by lengthy alkaline pretreatment at high temperature. In the present study, non-collagenous proteins were removed by pretreatment with 0.1 M NaOH for only 12 h at 4°C. Similar alkaline pretreatment conditions have been widely used in many other collagen and gelatin extraction studies [2, 3, 26].

Collagen fibril formation *in vitro*

Fibril formation during incubation at 21°C was monitored by optical absorbance change at 320 nm. The progression of fibril formation *in vitro* by skin and notochord collagen is shown in Fig. 4. For skin collagen, the non-pretreated preparation showed a higher rate of

turbidity increase than the alkali-pretreated preparation (Fig. 4A). The final turbidity of the non-pretreated preparation was approximately twice higher than that of the alkali-pretreated preparation. These data suggest that alkaline pretreatment inhibited fibril formation of the skin type I collagen. A similar inhibition of fibril formation by alkaline pretreatment was observed for bovine skin type I collagen [5]. Suzuki et al. [27] reported that alkaline pretreatment acted as a competitive inhibitor of fibril formation in steer hide type I collagen. The mechanisms by which alkaline pretreatment inhibits fibril formation have not been identified. One possible mechanism may be the modification of collagen amino acid side-chains, which might destroy intra- and inter-molecular cross-links [28].

Calf and human type II collagen have been reported to have a longer lag time for assembly and lower fibril formation rate than type I collagen [29, 30]. Notbohm et al. [31] investigated human type II collagen fibril formation in 30 mM phosphate buffer (pH 7.4) for 600 min. In the present study, a lower buffer concentration and a longer observation time were employed compared with the previous study [1], and fibril formation by type II collagen from a fish species was achieved for the first time (Fig. 4B). In contrast to the skin type I collagen, alkaline pretreatment showed little effect on fibril formation by the notochord type II collagen. The different effects of alkaline pretreatment may be explained by different characteristics and mechanisms of fibril formation between type I and type II collagen. This is

the first study to demonstrate that alkaline pretreatment has different influences on *in vitro* fibril formation by types I and II fish collagen.

Collagen fibril formation is affected by many factors, such as ionic strength, ionic species, pH value, temperature and others [32]. The present study included only a preliminary examination of the effects of alkaline pretreatment. For the industrial use of sturgeon skin type I and notochord type II collagen, it will be necessary to further optimize conditions for fibril formation.

Morphology of fibrils formed *in vitro*

SEM images of skin type I collagen fibrils after 1 h of incubation are shown in Fig. 5. The unordered, net-like appearance of fibrils in both non-pretreated and alkali-pretreated samples indicates that fibrillogenesis occurred under these experimental conditions. The fibril morphology was notably different between non-pretreated and alkali-pretreated collagens: thick fibril-bundle structures were formed only by the non-pretreated type I collagen. These bundles are likely to have been formed by the assembly of thin fibrils via spiral winding. Based on the proposed process of collagen fiber formation [33], I hypothesize that collagen molecules first assembled to form fibrils, then several fibrils coiled to form a fibril-bundle structure. In light of the results of the turbidity measurement experiment, the formation of

these bundles might increase the turbidity of the non-pretreated collagen. This result also indicates that alkaline pretreatment specifically inhibited bundle formation. Based on this observation, alkaline pretreatment is possibly a method that could be used to control type I collagen fibril morphology. The effect of alkaline pretreatment on the speed and morphology of fibril formation by mammalian type I collagen is already known. However, the present study is the first to report similar effects for piscine type I collagen.

The type II collagen formed many thin fibrils with a non-uniform network structure, for both non-pretreated and alkali-pretreated collagens (Fig. 6). There were no morphological differences between the two preparations. This finding is also consistent with the results of the turbidity measurement experiment: non-pretreated and alkali-pretreated type II collagens showed similar turbidity increases, indicating that alkaline pretreatment did not affect the fibril-forming ability of type II collagen. This is the first study to show the effects of alkaline pretreatment on the fibril-forming ability of type II collagen.

Bae et al. [34] showed that increasing NaCl concentration promoted fibril formation by red stingray type I collagen. In the previous study from my laboratory, similar phenomena were demonstrated using bester sturgeon skin and swim bladder type I collagens [1].

However, the preliminary study by Wang in my laboratory examined the effects on fibril formation by notochord type II collagen of increasing NaCl concentrations in the 0.1 M Na-

phosphate buffer (pH 7.4), but no collagen fibrils were formed when NaCl was added. The morphology of the type II collagen after 1 h was not a fibril structure (data not shown). In contrast to the type I collagen, high NaCl concentration seems to inhibit type II collagen fibril formation. Fibrils were visible when the buffer concentration was lowered and the fibril-forming time was prolonged compared with the previous study [1]. Thus, a lower buffer concentration and a longer reaction time were better for fibril formation by type II collagen than the type I collagen.

Degree of fibril formation

The degree of skin and notochord collagen fibril formation was assessed after 1 h and 10 h of fibrillogenesis, respectively, and the results are shown in Fig. 7. Alkali-pretreatment showed no significant influences on the degree of type I and type II collagen fibril formation: all samples showed values higher than 90%. These values were higher than those of barramundi and tilapia skin type I collagens, in which fibrils were formed for 24 h [35].

Thermal stability of collagen fibrils

DSC curves of re-assembled fibrils of types I and II collagen were obtained three times, and the typical curves are shown in Fig. 8. T_m was obtained as the endothermic peak of

the DSC curve. For type I collagen, non-pretreated collagen showed higher T_m ($40.14 \pm 0.31^\circ\text{C}$) than alkali-pretreated collagen ($37.26 \pm 0.08^\circ\text{C}$), which may be attributed to the thick fibril-bundle structure in the non-pretreated preparation. In addition, comparing with T_m of type I collagen molecules (27.0°C), the T_m of type I collagen fibrils was higher by $10\text{--}12^\circ\text{C}$. This result is consistent with the report that the thermal stability of collagen molecules is enhanced by molecular interaction [36]. This result might be of interest for its use as biomaterials for medical usages.

For type II collagen, no typical endothermic peaks were found on both non-pretreated and alkali-pretreated collagens. There were only slight changes in the curves at about $34\text{--}35^\circ\text{C}$ on both preparations. These temperatures were close to the denaturation temperatures of non-pretreated and alkali-pretreated type II collagen molecules ($31\text{--}32^\circ\text{C}$). These data suggested that although type II collagen fibrils were assembled, collagen fibril structures were not uniform or the thermally stable fibril structures were not formed in the present condition, and more suitable fibril formation condition need to be discussed in future.

Conclusion

This study demonstrated that a large amount of type I and type II collagen could be extracted from the skin and notochord of bester sturgeon and suggested that both tissues are

promising sources of fish collagen for industrial use. Alkaline pretreatment improved the extraction efficiency of both types of collagen, although it also reduced the yield of skin type I collagen. Although alkaline pretreatment did not affect the thermal stability of either type I or type II collagen molecules, it lowered the fibril-forming speed and T_m and changed the fibril morphology of the type I collagen. Since there has been no previous study on the effects of alkaline pretreatment on types I and II fish collagen, this study provides basic data describing the nature of these collagens as well as their suitability for industrial use.

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Figures and tables

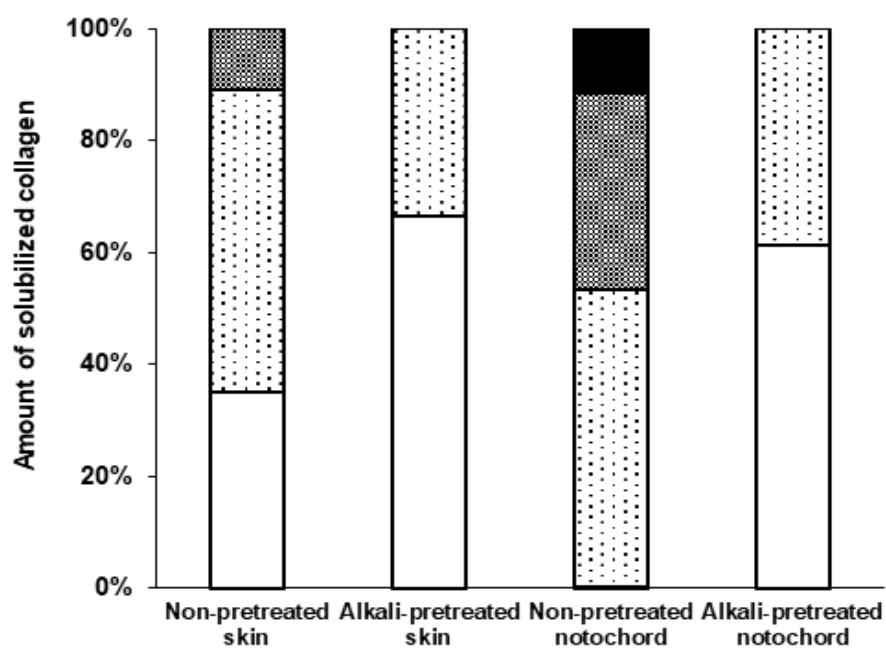


Fig. 1. Solubilized collagen content after each extraction step, expressed as a percentage of total solubilized collagen obtained. White, first extraction; spots, second extraction; stripes, third extraction; black, fourth extraction.

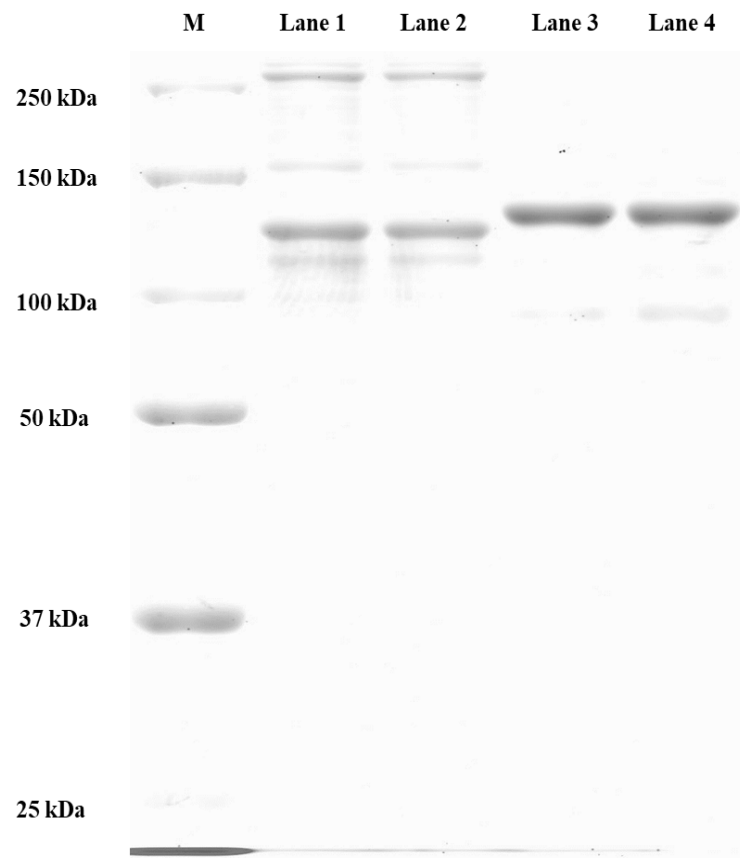


Fig. 2. SDS-PAGE of collagens from skin and notochord. M, molecular weight marker; lane 1: non-pretreated skin collagen; lane 2: alkali-pretreated skin collagen; lane 3: non-pretreated notochord collagen; lane 4: alkali-pretreated notochord collagen.

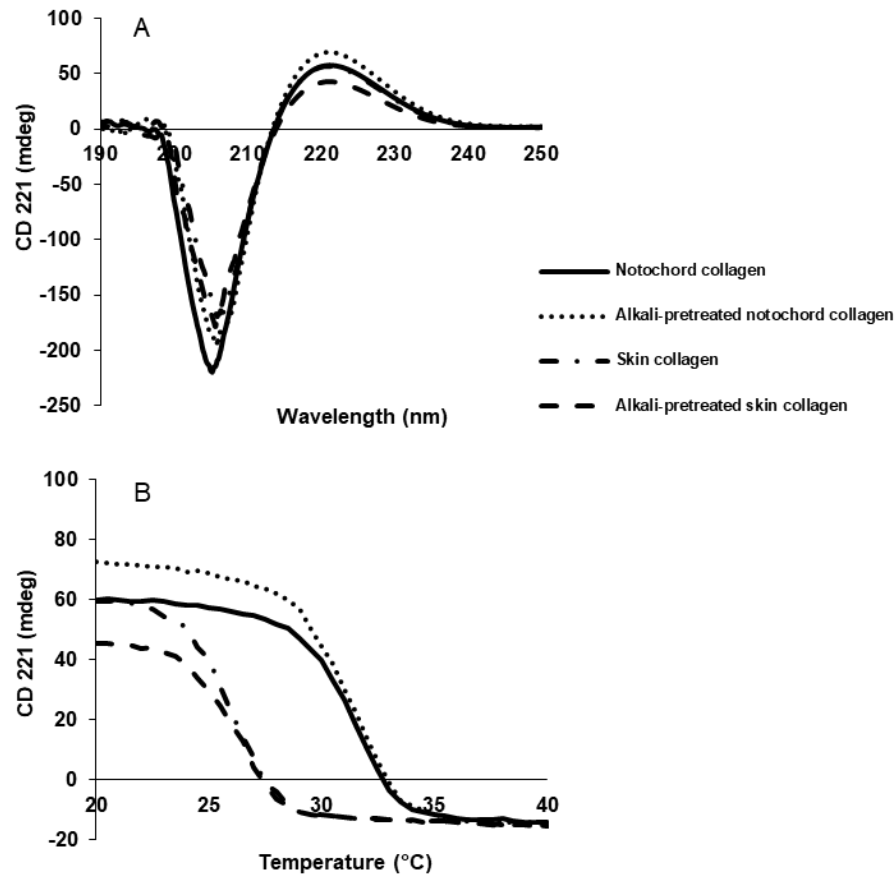


Fig. 3. (A) CD spectra of the collagens from non-pretreated and alkali-pretreated skin and notochord. (B) Temperature effect on the CD spectra at 221 nm of collagens from non-pretreated and alkali-pretreated skin and notochord.

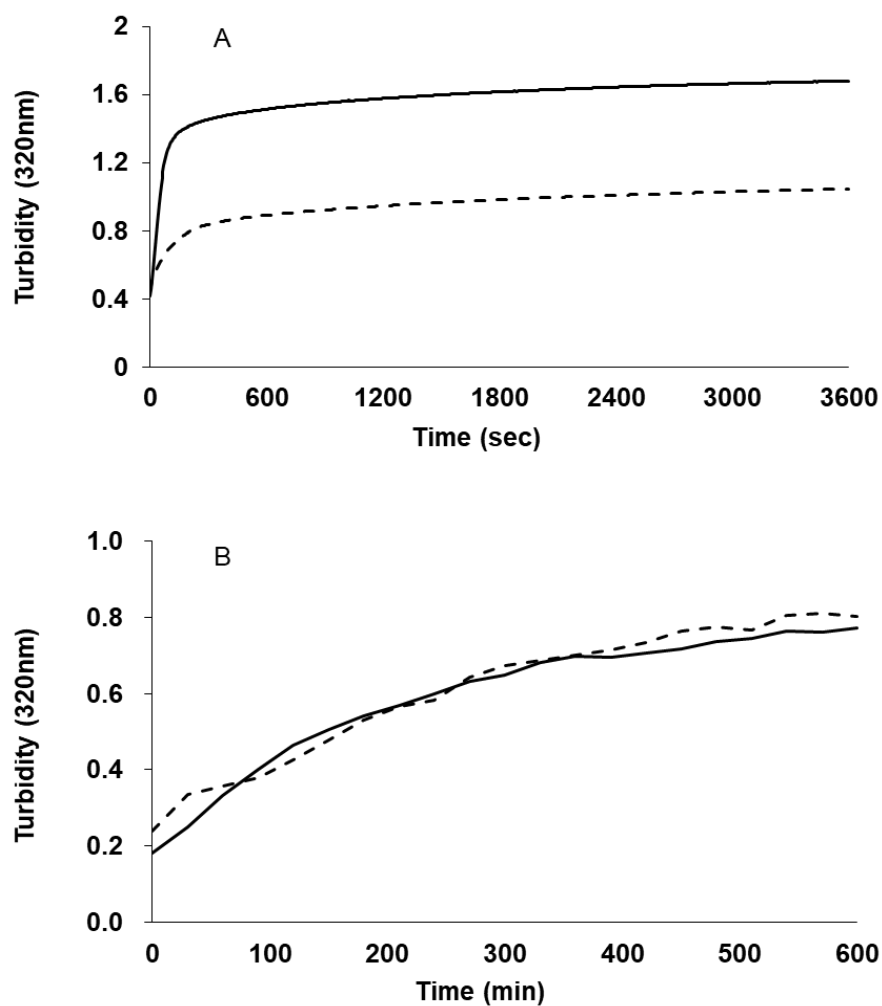


Fig. 4. Fibril formation *in vitro* by (A) skin type I collagen and (B) notochord type II collagen, measured by optical absorbance at 320 nm. Solid line, non-pretreated preparations; dotted line, alkali-pretreated preparations.

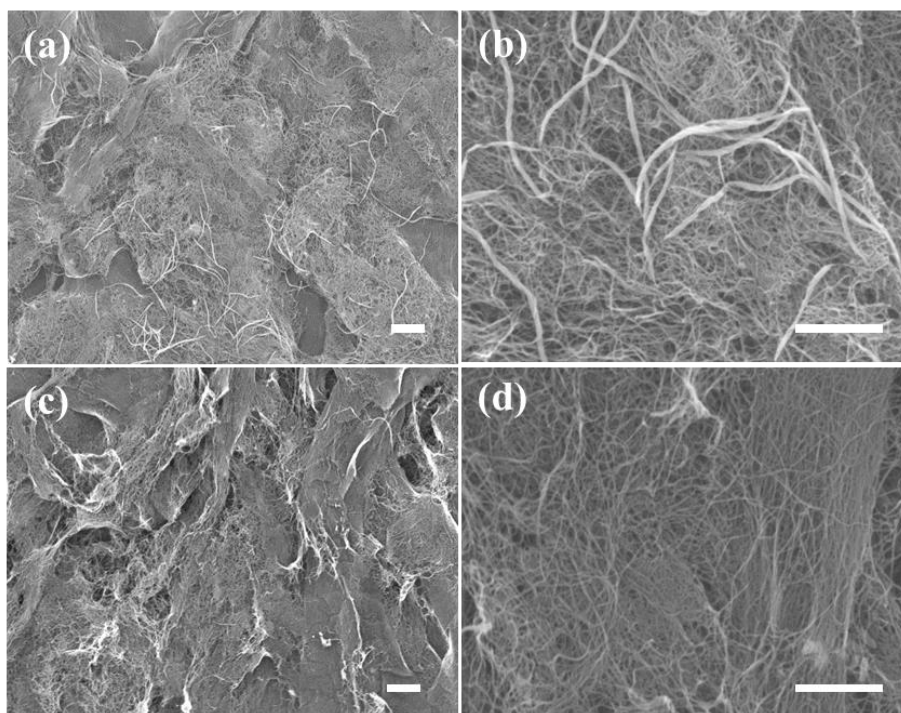


Fig. 5. Scanning electron micrographs of skin collagen fibrils formed at 21°C after 1 h. (a & b) non-pretreated skin collagen. (c & d) alkali-pretreated skin collagen. Scale bars: 10 μm in A & C, 5 μm in B & D.

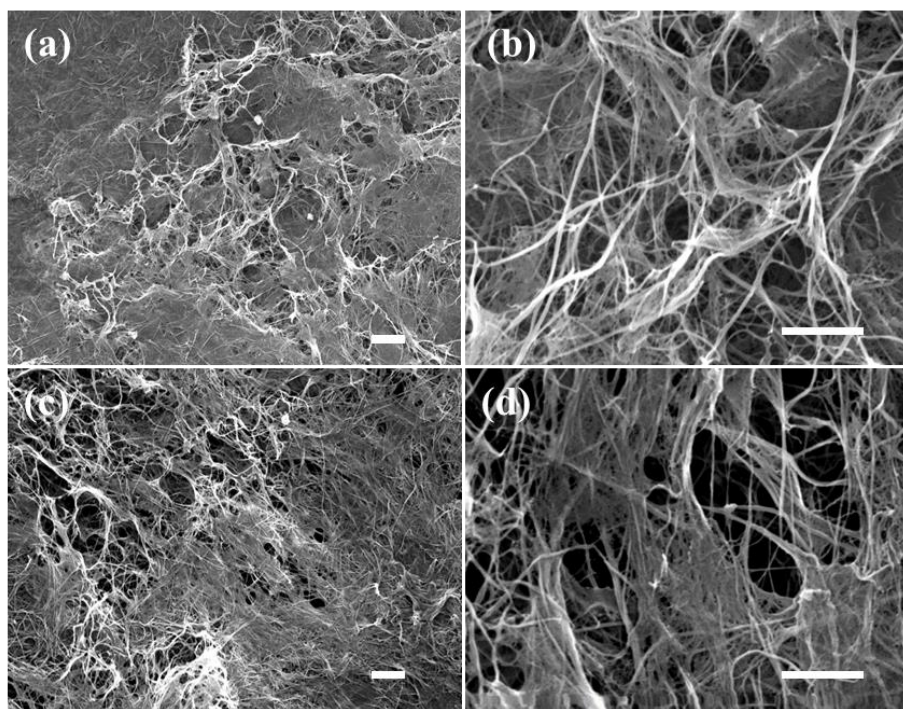


Fig. 6. Scanning electron micrographs of notochord collagen fibrils formed at 21°C after 10 h.

(a & b) non-pretreated notochord collagen. (c & d) alkali-pretreated notochord collagen. Scale

bars: 10 μm in A & C, 5 μm in B & D.

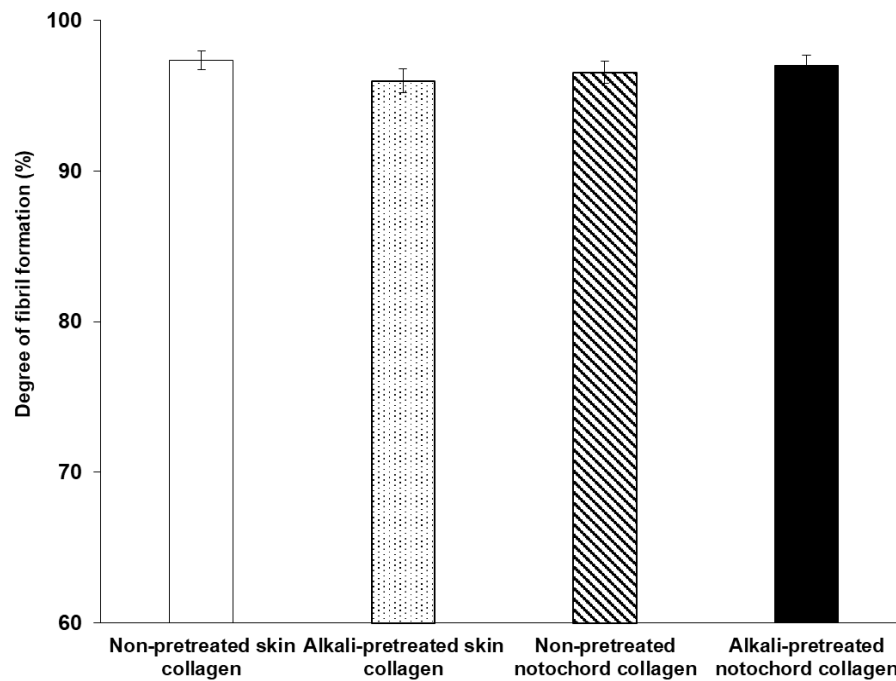


Fig. 7. The degree of fibril formation of skin collagen and notochord collagen of sturgeon.

White, non-pretreated skin collagen; spots, alkali-pretreated skin collagen; stripes, non-pretreated notochord collagen; black, alkali-pretreated notochord collagen. Columns and bars show mean and standard error of the means of three experiments.

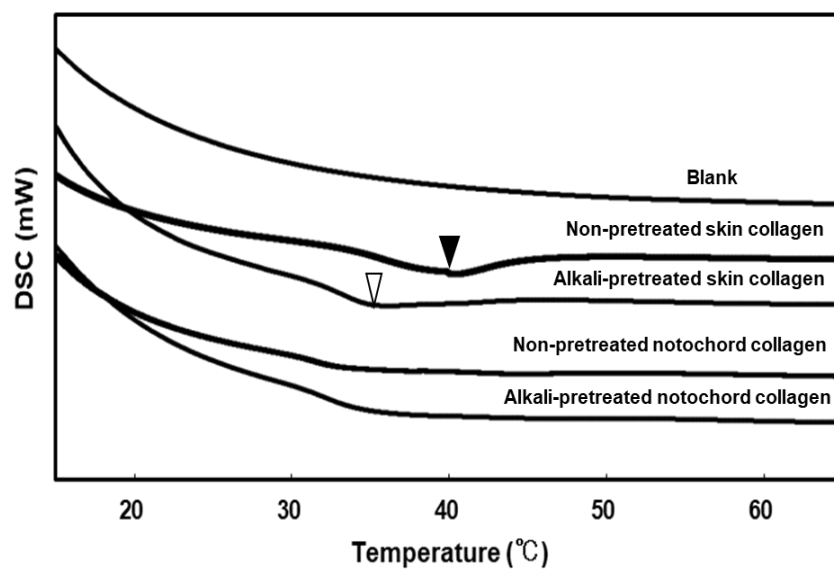


Fig. 8. DSC curves of re-assembled fibrils of skin and notochord collagens. The black and white arrowheads indicated the maximum transition temperatures of non-pretreated and alkali-pretreated skin collagen, respectively.

Table 1. Amino acid composition of non-pretreated tissues, alkali-pretreated tissues, and purified collagens (expressed as residues / 1000 total amino acid residues).

Amino acids	Skin	Alkali-pretreated skin	Skin collagen	Alkali-pretreated skin collagen	Notochord	Alkali-pretreated notochord	Notochord collagen	Alkali-pretreated notochord collagen
Asx	54	51	49	49	56	53	51	49
Thr	27	26	25	25	26	23	22	21
Ser	46	46	47	47	37	35	35	34
Glu	76	73	71	70	99	97	92	9
Gly	322	337	341	343	304	320	324	329
Ala	110	113	114	115	82	83	91	93
Cys	3	2	0	0	2	1	1	0
Val	22	20	17	17	25	22	18	17
Met	12	11	10	10	12	12	8	8
Ile	15	13	12	12	18	16	13	12
Leu	25	20	18	18	39	36	31	30
Tyr	7	5	2	2	8	5	4	2
Phe	16	15	13	13	15	14	14	13
Hylys	8	9	8	8	25	27	23	24
Lys	30	25	25	24	19	17	16	16
His	6	5	4	4	9	8	6	6
Arg	53	53	53	53	50	49	51	51
Hypro	58	64	70	71	64	69	77	79
Pro	110	113	120	120	111	113	123	124
Imino acid	168	177	190	191	175	182	210	213

Table 2. Extracted collagen yields (% , dry weight basis). All data are expressed as mean $\pm$ standard errors of four experiments. *Significantly different ($P < 0.05$) compared with non-pretreated skin (student's *t*-test).

Samples	Non-pretreated skin	Alkali-pretreated skin	Non-pretreated notochord	Alkali-pretreated notochord
Yields (%)	63.9 $\pm$ 0.19	56.2 $\pm$ 0.84*	35.5 $\pm$ 0.68	31.8 $\pm$ 1.13

Chapter III. Effects of the phosphate ion concentrations on sturgeon type I collagen fibrillogenesis

The extracted type I collagen molecules can form fibrils with structural, mechanical, and biological properties similar to native fibrils *in vivo* under the appropriate condition of the reaction fluid [1-3]. Because of its low antigenic activity, high cell adhesion properties, biocompatibility, and biodegradability type I collagen fibrils have high potential as biomedical materials such as cellular scaffolds for tissue engineering [4, 5]. As already described in Chapter I, the morphological factors including diameter, shape, and orientation of fibrils in the collagenous scaffolds exert strong effects on adhesion, alignment, proliferation, and differentiation of anchorage-dependent cells [6, 7]. Therefore, the optimization of fibril morphology in the scaffold is essential to obtain the best cellular performance for various applications in tissue engineering.

Previous studies indicated the ionic strength of the reaction fluid influenced collagen fibril formation *in vitro* [8-10]. Especially, divalent anions have a critical role in regulating fibril formation speed and fibril shape [11]. Wood [12] found that the speed of fibril formation and the fibril width decreased with increasing the ionic strength of the reaction fluid. Because collagen fibrillogenesis *in vitro* is a complex process influenced by many factors, such as

electrostatic interaction, hydrophobic interaction, and intermolecular force [13, 14], the mechanism that the ionic strength affects collagen fibrillogenesis remains to be clarified. Therefore, a better understanding of the effects of divalent anions of the fibril morphology will allow the rational design of collagen-based scaffolds.

Compared with the information of mammalian collagens, however, the knowledge of marine collagens on their fibrillogenesis is insufficient. Zhang et al. [15] showed increasing ionic strength by addition of NaCl accelerated the fibrillogenesis and promoted the formation of thicker fibrils in sturgeon skin and swim bladder collagens. Notably, the swim bladder collagen assembled into a unique fusiform structure. These findings suggested that sturgeon collagens had the potential to develop various fibril morphologies by control of the reaction-fluid environment.

In this study, I clarified the effects of phosphate ion (Pi) concentrations of the reaction fluid on the fibril formation of sturgeon swim bladder type I collagen (SBC) and skin type I collagen (SC). Phosphate ions are reported to bind to collagen molecules of mammalian sources, significantly affect electrostatic interactions of the molecules, and in turn, the interactions change self-assembly of collagen molecules to form fibrils [11, 13, 16]. I also applied the findings of the present study to coat different forms of SBC and SC fibrils on cell culture wells and examined fibroblast morphology and proliferation.

Materials and methods

Isolation and purification of collagen

Pretreatment and extraction of collagens were as already shown in Chapter II. No alkaline pretreatment was conducted in this experiment.

Collagen fibril formation *in vitro*

Freeze-dried SBC and SC were dissolved in an HCl solution (pH 2.0) at a concentration of 0.3% (w/v). Then, the SBC and SC solutions were mixed with pH 7.4 Na-phosphate buffer (PB) having a concentration of Na-phosphate at 22.5, 45, 90, or 180 mM. The ratio of collagen solution:PB was set at 1:2 (v/v), which resulted in the final PB concentration as 15, 30, 60, or 120 mM. The mixtures were kept at 21°C, and fibril formation was monitored for 1 h by the absorbance at 320 nm as the turbidity change using a spectral monitor (Hitachi High-technology Corporation, Tokyo, Japan).

Degree of collagen fibril formation

The degree of fibril formation was measured as described in Chapter II.

Morphology of fibrils

The method of fibril observation using SEM was shown in Chapter II. After the observation, digital images were obtained, and diameters of randomly selected 100 fibrils were determined with the ImageJ software. The measurements were conducted three times using different photos.

Thermal stability of collagen fibrils

The thermal stability of the fibrils was determined using DSC as described in Chapter II.

Preparation of collagen-fibril coated cell culture wells

A 200 μ l aliquot of 1% (w/v) SBC or SC solution (pH 2.0) was placed in each cell culture well (24-well cell culture plate; FALCON, Durham, NC), and wells were kept for 30 min. Then the solution of each well was changed to a 200 μ l aliquot of PB (15, 30, 60 or 120 mM; pH 7.4), and fibril formation was induced for 1 h. After removing the PB, the PB with same Na-phosphate concentration but containing 0.1 mM genipin as a crosslinker was added, and wells were kept for 12 h. Finally, the genipin-PB solution was removed, and wells were washed twice with Hank's balanced salt solution containing 100 U/ml penicillin and 100

µg/ml streptomycin and immediately used for cell culture experiments. The diluted HCl solution (pH 2.0) was used instead of the SBC or SC solution as a control. All procedures were conducted under the aseptic condition at 21°C.

Morphology of collagen fibrils on cell culture wells

After the collagen-fibril coating, the bottom surface of each well was observed by SEM. The methods of fixation, dehydration, and freeze-drying were same with those of fibril observation. After the freeze-drying, the bottom of each well was dissected out and cut into 1×1 cm using a heated scalpel. The surface was coated with gold-platinum and observed using SEM.

Cell culture

To study the cellular morphology and proliferation on the SBC and SC collagen fibrils, L929 mouse fibroblastic cells were maintained in the Eagle's minimum essential medium (Life Technologies Corporation, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Thermo Fisher Scientific, Carlsbad, CA, USA) and 1% penicillin/streptomycin mixture (Thermo Fisher Scientific, Grand Island, NY, USA). Cells were cultured for 5 days in a humidified incubator at 37°C and 5% CO₂, and the medium was

renewed after 3 days.

Cell morphology and proliferation

Morphology of the cells was observed with a phase contrast microscope (DMI600B, Leica, Wetzlar, Germany) over the cell culture time. The microstructure of the cells on 2 days after the seeding was observed by the SEM using the same technique with the observation of the fibrils on the cell culture wells.

The cell counting kit-8 (CCK-8, DOJIN, Mashiki, Japan) was used for the evaluation of the cell-proliferation activity. After a 5-day culture, the culture medium was removed, and the culture was washed with PBS twice. A 540 μ l of culture medium and 60 μ l of CCK-8 solution were added into each well, and cells were cultured for 30 min. The supernatant (100 μ l) was transferred to a 96-well plate, and the optical density at 450 nm was determined by a microplate reader (infiniteF50R, Wako, Osaka, Japan). Six parallel replicates were read for each sample.

Statistical analysis

Data are expressed as means $\pm$ standard errors. Significance was set at $p < 0.01$. Data were analyzed by ANOVA and Tukey–Kramer post hoc test for multiple comparisons using

Microsoft Excel add-in statistical software (SSRI, Tokyo, Japan).

Results

Collagen fibril formation

The influence of phosphate ion concentrations on the progression of SBC and SC fibril formation *in vitro* is shown in Fig.1. When PB concentrations were 15 and 30 mM, the turbidity value of SBC started over 0.5 with a short growth phase, and no lag phase was observed. As the PB concentration increased to 60 mM, a short lag phase appeared, and the growth phase followed. At 120 mM PB, the turbidity curve exhibited a sigmoidal shape with more extended lag phase with more gradual growth phase. The turbidity at the plateau phase also was affected by the Pi concentration: it was highest at 60 mM PB, followed by 120, 30 and 15 mM. The same trend was shown in SC turbidity curves. However, no turbidity increase was recorded at 120 mM PB. Additionally, the turbidity at the plateau of SC at 60 mM PB was lower than that of SBC.

The degree of fibril formation

The degree of fibril formation was assessed after 1 h (Fig. 2). The degrees were more than 95% when the Pi concentration was less than 30 mM in both SBC and SC. It decreased

to 89% in SBC and 60% in SC when the Pi concentration increased to 60 mM. At 120 mM Pi, the fibril formation degree decreased to 64% in SBC, and no collagen fibril was formed in SC, which was consistent with the SC turbidity curve.

Morphology of fibrils

The SEM images of SBC and SC fibrils after 1h incubation are illustrated in Fig.3, and the distribution of fibril diameters are summarized in Fig.4. Hereafter, fibrils were named using the origin of collagen (swim bladder or skin) with Pi concentration at the fibrillogenesis (15-120 mM), as SBC-15 fibrils, etc. At the lowest phosphate concentration (15 mM), thin fibrils curled together with high density in both SBC and SC (Fig. 3). The mean diameters were 77.7 nm in SBC-15 and 78.8 nm in SC-15, and variations of fibril diameter were small (Fig. 4). As the Pi concentration increased, both SBC and SC fibrils increased numbers of thicker fibrils (Fig. 3). As a result, the range of fibril diameter increased (Fig. 4). The thickest mean diameter was observed in SBC-60 (397 nm) and SC-60 (238 nm). Thick but short fusiform structures were found in SBC-30 (Fig. 3). As the phosphate concentration increased to 60 mM, the fusiform structures became larger and are characterized by a transverse periodic banding pattern (Fig. 3). These fusiform structures were interlaced in the thin fibrils, and the maximum diameter reached over 1600 nm (Fig. 4). The fusiform structures

disappeared in SBC-120, but the mean diameter of fibrils became thicker than that in SBC-60 (Figs. 3, 4). In SC fibrils, no fusiform structures were observed, while thick fibril-bundles were found in SC-30 (Fig. 3). Different from the fusiform structures in SBC, these bundles were likely to be formed by the spiral assembly of some thin fibrils (Fig. 3). The bundles disappeared in SC-60, but the diameter of fibrils became thicker than that in SC-30 (Figs. 3, 4).

Thermal stability of collagen fibrils

The DSC curves of SBC and SC fibrils are shown in Fig.5. The T_m values of SBC fibrils increased as the Pi concentrations increased to 60 mM and decreased at 120 mM; they were respectively 42.9, 43.3, 44.1, and 40.4°C in SBC-15, -30, -60, and -120. The T_m values of SC fibrils were tended to lower than those of SBC fibrils at the same Pi concentration; they were 37.7, 40.7, and 44.7°C, in SC-15, -30, and -60, respectively.

Collagen-fibril coating on cell culture wells

In this study, the 2-dimensional collagen-fibril coating was applied to study cell behaviors on the different collagen fibrils. SEM images of SBC- and SC-fibril coatings are illustrated in Fig.6. Both SBC- and SC-fibrils aligned into the same direction on the bottom of

culture wells. Although the trend of Pi-dependent increases in fibril diameter was the same as fibrils formed in the solution, the fibrils coated on the wells were tended to be thinner. Also, the SBC-fibrils showed more apparent changes of fibril diameter depending on the Pi concentration of the buffer. The fusiform structures were observed only in SBC-60. The bundle structures were not found in SC-fibrils.

Cell morphology

Figure 7 shows the morphology of L929 cells on SBC- and SC-fibrils. Six hours after the seeding, the cells stretched well on the fibrils except for SBC-60 and -120 fibrils. The cells on the SBC-60 and SBC-120 fibrils attached firmly to the fibrils but showed a round shape with higher cell height. Twenty-four hours after the seeding, the number of cells increased in all wells. Cells spread with no directionality on the control surface and the fibrils except for SBC-60 and -120. A part of cells on SBC-60 and -120 fibrils started to spread with apparent polarization along with the direction of the fibrils.

To further examine cell morphology, cells were observed by SEM at 2 days after the seeding (Fig. 8). Cells on the control surface were flat and exhibited a polygonal shape. In contrast, cells on both SBC and SC fibrils had higher cell height. Many cells extended two long protrusions into the counter directions. On SC fibrils, all cells stretched longer than those

on SBC fibrils and exhibited irregular directionality. Similarly, cells showed irregular directionality on the SBC-15 and -30 fibrils, but those on the SBC-60 and -120 fibrils displayed a higher degree of alignment along with the fibril direction.

Cell proliferation

Cell proliferation activities were assessed on day 5 using a CCK-8 kit (Fig. 9). The cells on the fibril-coated wells proliferated significantly slower than those on the control wells. The cells on the SC fibrils showed higher proliferation activity than those on the SBC fibrils. No significant differences were observed among SC-15, -30 and -60 groups. The proliferation on the SBC fibrils tended to decrease when the Pi concentrations increased from 15 to 60 mM, showed the lowest activity on SBC-60 fibrils, and increased tendency on SBC-120 fibrils.

Discussion

The present study clearly showed that Pi concentrations in the reaction fluid significantly affected the speed of fibril formation and morphology of fibrils formed *in vitro* in both SBC and SC. There are a few studies on the fibrillogenesis of type I collagen and morphology of fibrils formed *in vitro* in fish collagens such as salmon skin [17], shark skin

[18], red stingray skin [19], and bester sturgeon skin and swim bladder [15]. However, these studies all showed the influence of monovalent ions, Na^+ and Cl^- . Because divalent anions are at the higher order of the Hofmeister series, they have higher impacts on macromolecules and ions [19], and some reports on the fibrillogenesis of mammalian collagens found that the divalent anions showed more significant influences than monovalent anions [11, 20]. The present study is virtually the first report on the effects of Pi on the fibrillogenesis process and morphology of fibrils formed *in vitro* in fish collagen.

The collagen fibrillogenesis is a complex process of molecular assembly, which can be reflected by turbidity changes consisting of three phases: a lag phase with almost no turbidity change, a growth phase with a rapid increase in turbidity, and a plateau phase with constant turbidity. Ultrastructural studies on the fibrillogenesis using atomic force microscopy revealed that the lag and growth phases consisted of further multistep processes [21, 22]. The lag phase includes multiple co-assemblies of several collagen molecules (nucleation) and subsequent linear aggregation of nuclei to form subfibrillar units [23]. Subsequently, the subfibrillar units laterally aggregate to form fibrils, and then, fibrils grow to bundles of fibrils in the growth phase [24]. Silver and Trelstad [25] reported that fibril formation occurred at the end of the lag phase and only the mass of these fibrils increased during the growth phase. The subfibrillar units are important in dictating the mechanical properties of collagen fibers [24].

In this study, the addition of Pi significantly prolonged the lag phase of fibrillogenesis in both SBC and SC. At 15 and 30 mM Pi, the turbidities started at higher absorbance compared with those at 60 and 120 mM in both SBC and SC, suggesting that the lag phase was shorter than the time that took for the first measurement of the absorbance (less than 5 sec after the mixture of collagen solution and PB). As the Pi concentration increased, the lag phase became longer. Previous studies in mammalian collagens also indicated that Pi was influential in directing the linear and lateral growth of collagen molecules [11, 24, 25]. Therefore, I deduce that one of the main influences of Pi on the fibril formation of SBC and SC is in the process of subfibrillar unit formation at the lag phase. The shorter lag phase observed at lower Pi concentrations suggests acceleration of the nucleation and linear aggregation of nuclei into subfibrillar units. Under such condition, small but large number of subfibrillar units may be formed and the transition into the growth phase may occur rapidly. As Pi concentration increases, the nucleation and linear aggregation of nuclei may occur slowly, the lag phase is prolonged, and the larger subfibrillar units may be produced. Hayashi and Nagai [11] reported that the lag phase in polymerization is a unique feature which reflects the assemble mechanism, and the Pi concentration must markedly influence such molecular interactions of collagen. In addition, some studies in mammalian collagens showed that Pi had a substantial effect on the electrostatic interactions of collagen molecules [13, 26, 27], and the

assembly of subfibrillar units is controlled to a significant degree by electrostatic interactions [11, 13]. Thus, Pi may affect the lag phase of SBC- and SC- fibril formation through the electrostatic interactions of SC and SBC molecules.

Growth phase involves lateral aggregation of subfibrillar units to form higher molecular-weight fibrils. The slope of the turbidity reflects the speed of lateral aggregation, and the final turbidity expresses fibril diameter [8]. Zhu and Kaufman [28] reported that most of the increasing turbidity during the growth phase was due to increasing fibril diameter. In the present experiment, as the Pi concentration increased, the slope of the growth phase became gentle, and the curve reached high turbidity. These data suggested that larger subfibrillar units formed in the higher Pi environment need longer time to aggregate together to form thicker fibrils. Results of SEM observations on fibril morphology well supported this suggestion: the higher Pi concentration induced formation of thicker fibrils with an elongated morphology. This tendency was similar in both SBC and SC. Similar with the present results, Hayashi et al. [11] examined the fibril formation of calf skin collagen under 50-300 mM Pi and found that the fibril formation speed decreased as Pi concentration increased. Silver and Trelstad [25] reported that Pi concentrations lower than 30 mM showed little effects on the fibril formation speed of rat tendon collagen, but the speed slowed when Pi concentration

became over 30 mM. However, few studies directly reported the relationship of fibril morphology and Pi concentration.

Thick fibril-structures unique to SBC and SC, respectively, were produced at appropriate Pi concentrations. The fusiform fibril structures of SBC grew at 30 mM and 60 mM Pi. In contrast, thick fibril-bundles of SC were found at 30 mM Pi. These results indicated that the processes of fibril formation were different between type I collagens obtained from swim bladder and skin of sturgeon. The previous study found that hydroxyproline (Hyp) contents of SBC were higher than that of SC [15]. Since the hydroxyl group of Hyp forms a hydrogen bond to maintain collagen structure and stability, different Hyp contents in SBC and SC may be one of the reasons for the development of different structures. Such differences in Hyp contents of SBC and SC arise from a different composition of α chains in collagen molecules, which is characteristic in fish. The type I collagen molecule of mammals is formed as triple helical heterotrimer of two identical $\alpha 1(I)$ and one $\alpha 2(I)$ chains and expressed as $[\alpha 1(I)]_2\alpha 2(I)$. Although minor collagen molecules including type V molecules are usually found with type I collagen, the composition of the type-I α chain is stable in different tissues. In fish type I collagen molecule, however, another type of α chain named $\alpha 3(I)$ also exists [29]. Type I collagen molecules in fish are, therefore, a mixture of $[\alpha 1(I)]_2\alpha 2(I)$ and $\alpha 1(I)\alpha 2(I)\alpha 3(I)$, and the ratio of two types of the molecule seems

to be specific to each tissue. Zhang in my laboratory have already found the mRNA expression levels of $\alpha 1(I)$ chain and $\alpha 2(I)$ chain varied between the swim bladder and skin in Amur sturgeon [30]. Thus, the different ratio of $\alpha 1(I)$, $\alpha 2(I)$ and $\alpha 3(I)$ between SBC and SC should be the primary reason for different Hyp contents.

The ranges of fibril diameters obtained in the present *in vitro* fibrillogenesis experiments in SBC and SC were respectively 50-1600 nm and 50-350 nm. The thickness of collagen fibrils *in vivo* ranges from 0.5 to 3 μm [31, 32]. It indicated that sturgeon collagen could form fibrils with similar fibril diameter as those developed *in vivo* by adjusting the Pi concentration of the environment. Such a character is beneficial for sturgeon collagen to use as biomedical materials. Also, the T_m value of fibrils increased with the Pi concentration. The precise mechanism of the increased T_m should be clarified in the future, but the structural differences of fibrils may affect the T_m value.

The present study also showed that the higher Pi concentrations inhibited the amount of fibrils formed in 1 h (Fig. 2). The effects were more substantial in SC than in SBC, and no SC fibrils were formed at 120 mM Pi. It is possible that as the Pi concentration increased, more collagen molecules were used for linear aggregation to make large subfibrillar units, and the lateral aggregation might become insufficient. The subfibrillar units might be too narrow to be seen by turbidity and could not be collected as the collagen fibrils by centrifugation.

Presently, in situ observation of developing subfibrillar units is challenging, because it needs a resolution of the molecular level. The development of new techniques will facilitate the understanding of the mechanisms of how Pi changes fibril morphology.

I intended to apply SBC and SC fibrils as cellular scaffolds for tissue engineering since collagen fibril is the primary scaffold *in vivo*. Therefore, in the present study, I coated SBC and SC fibrils on the cell culture wells and examined the responses of fibroblastic L929 cells in morphology and proliferation. The L929 cells on the control wells exhibited polygonal shape and proliferated rapidly to be tiled on the bottom surface of the well. Compared with the cells on the control wells, all cells on the collagen fibrils showed higher cell height, and many cells extended two cellular processes to the counter direction. Notably, the cells on the SBC-60 and -120 fibrils showed polarized configuration: they spread the cellular processes along the fibrils and aligned the cell body to a similar direction.

Such a specific cell morphology on the collagen fibrils might be generated by accepting the information of fibrils through integrins, the cellular receptors to type I collagen fibrils [33]. Generally, the signaling pathways downstream of the integrins regulate different aspects of cell behaviors, such as cell morphology, orientation, proliferation and differentiation [34-36]. According to Friedrichs et al. [35], cells expressing integrins polarized

strongly along the collagen fibrils. In contrast, cells lacking integrins could attach to the substrate, but no polarization occurred.

The present study also revealed the existence of the appropriate range of fibril diameter for the polarization of cells. Since in some human tissues, such as bone and tendon, cells are arranged in parallel arrays [37], and thus, regulation of cell directions is essential for tissue engineering. The SBC-60 and -120 fibrils have high potential as scaffolds for the engineering of tissues having aligned cell arrays.

Cell proliferation became slower on the SBC and SC fibrils compared with the control, non-coated surface. On the SBC fibrils, effects of fibril diameter on the proliferation activity were apparent: as the fibril diameter increased, the cell proliferation activity decreased and reached to the minimum at 60 mM Pi. Thus, cells may get signals for cell proliferation distinctively from the fibrils having different diameters. Tsakonas et al. [38] found that signal transduction between cells also affected the cell proliferation. Thus, it is possible that a high degree of cell alignment on the SBC-60 and -120 fibrils cause the different signal transduction to reduce the proliferation. Mouse MC3T3-E1, ATDC5, 3T3-L1, and C2C12 cells also reduced their proliferation on SBC fibrils, especially when the fibril diameter was large (Moroi, Miura and Tamura, unpublished observations). Although the

precise reason has been unknown, the inhibitory activity of SBC fibrils is not specific for L929.

Conclusion

The Pi concentration showed significant influences on sturgeon type I collagen fibril formation speed, degree, and morphology as well as thermal stability of developed fibrils. Under the lower Pi concentration, thin fibrils having low thermal stability were formed. As Pi concentration increased, fibril formation speed slowed down, and the thick fibrils with higher thermal stability were formed. Also, fibrils having different morphology impacted the morphology and proliferation of fibroblasts. The cell culture experiments showed that thin fibrils were better for cell extension and proliferation, whereas thick fibrils restrained cell extension and proliferation but regulated cell orientation. This study is the first to show that the Pi concentration in the reaction fluid is an efficient way to regulate fibril morphology of sturgeon collagen for the tissue engineering application. Notably, the SBC is suitable to use for a new scaffold, because SBC fibrils respond to Pi and form more diverse morphology compared with SC fibrils.

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Figures

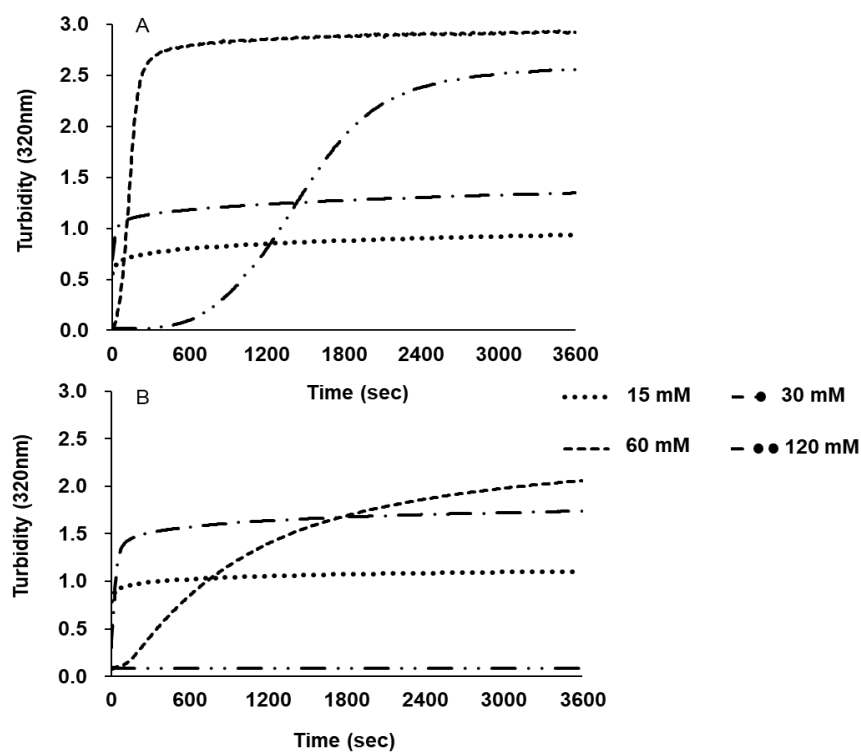


Fig. 1. Effects of phosphate ion concentrations on the in vitro progress of fibril formation of the swim bladder (A) and the skin (B) collagens of sturgeon.

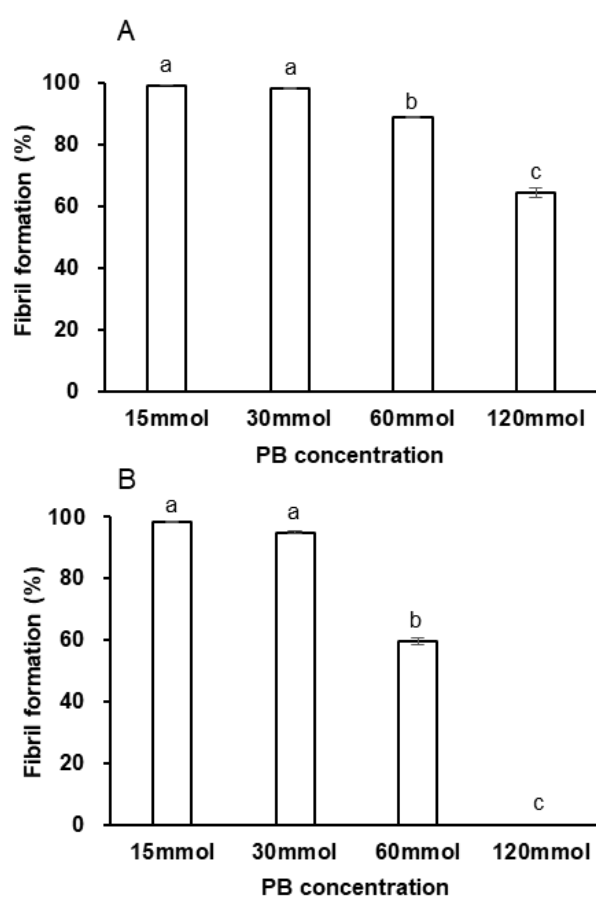


Fig. 2. Effects of phosphate ion concentrations on the degree of fibril formation of swim bladder (A) and skin (B) collagens of sturgeon. Columns and bars show mean and standard error of the means of three experiments. Differences between the groups with different alphabets are significant (Tukey–Kramer post hoc test, $P < 0.01$).

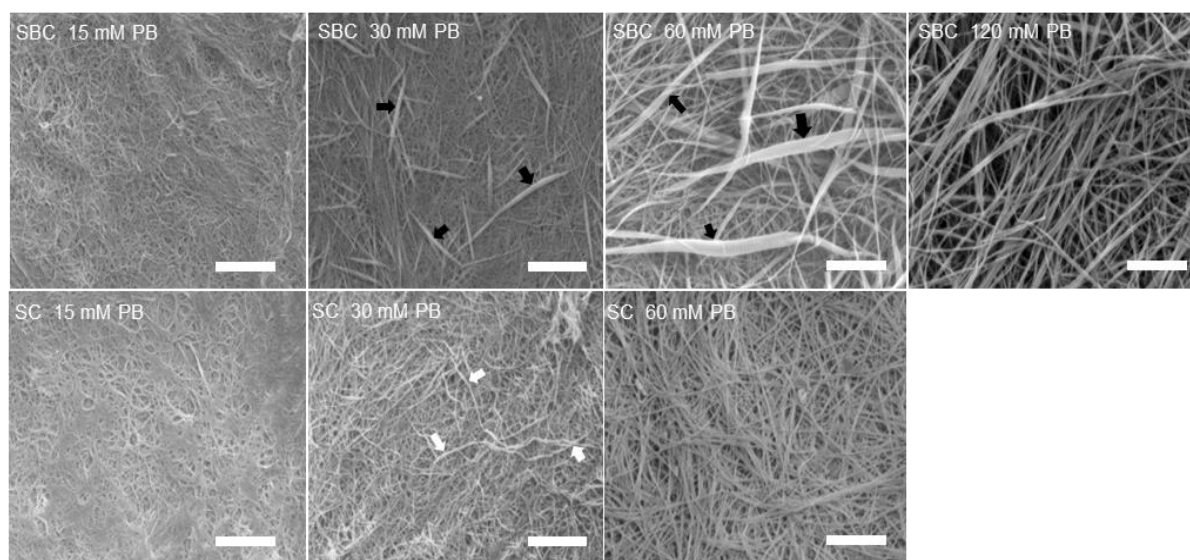


Fig. 3. Scanning electron micrographs of swim bladder and skin fibrils formed at 21°C for 1 h. Scale bars: 5 μ m. The black and white arrows indicated the fusiform and bundle structure in SBC and SC, respectively.

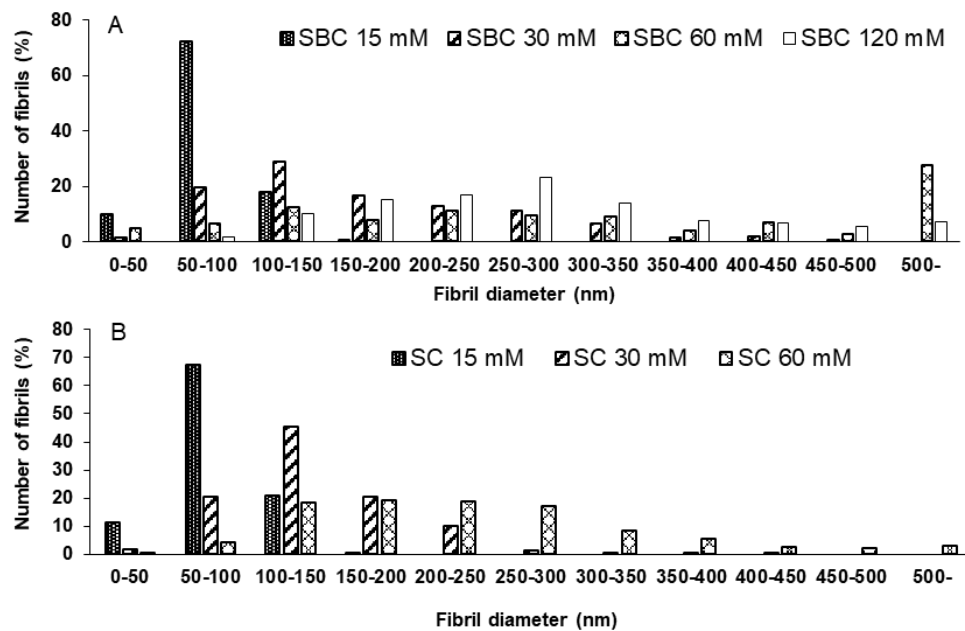


Fig. 4. Distributions of diameters of swim bladder and skin fibrils formed at 21°C for 1 h.

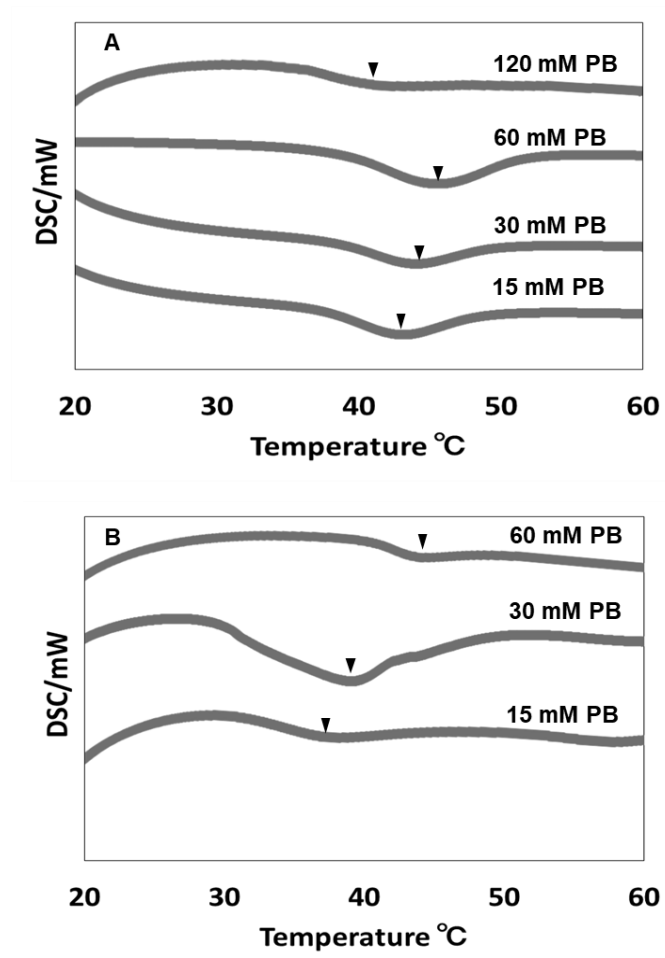


Fig. 5. DSC curves of swim bladder (A) and skin (B) fibrils formed at 21°C for 1 h. The black arrowheads indicated the maximum transition temperatures.

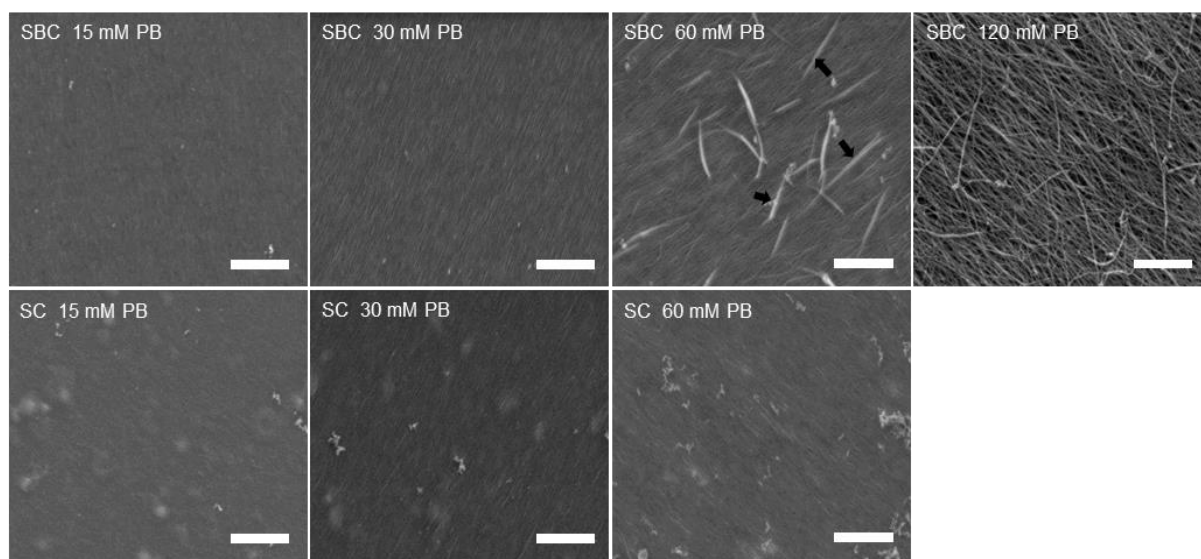
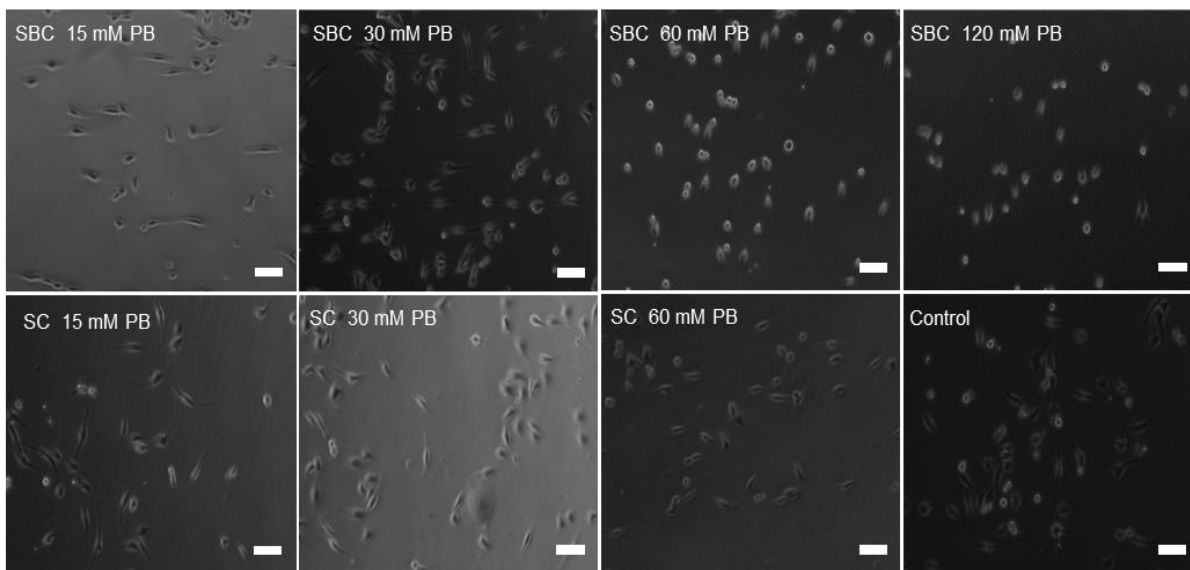


Fig .6. Scanning electron micrographs of the bottom surfaces of cell culture wells coated with swim bladder and skin fibrils. Scale bars: 5 μm . The black arrows indicated the fusiform structure in SBC coating wells.

6 hours



24 hours

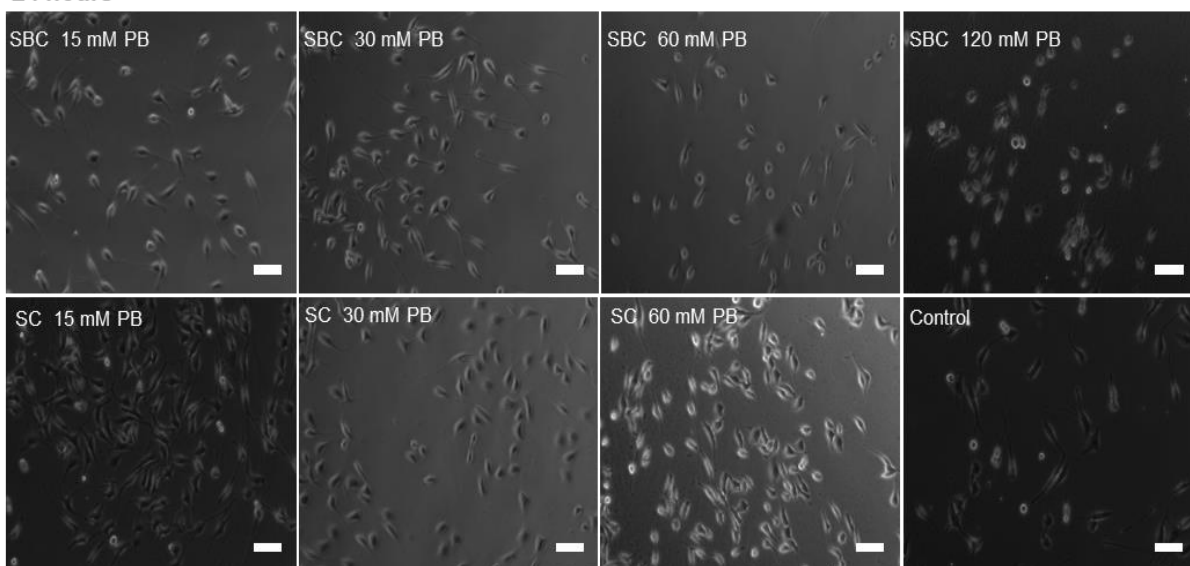


Fig. 7. Phase contrast microscope images of L929 cells cultured for 6 hours and 24hours.

Scale bars: 75 μm.

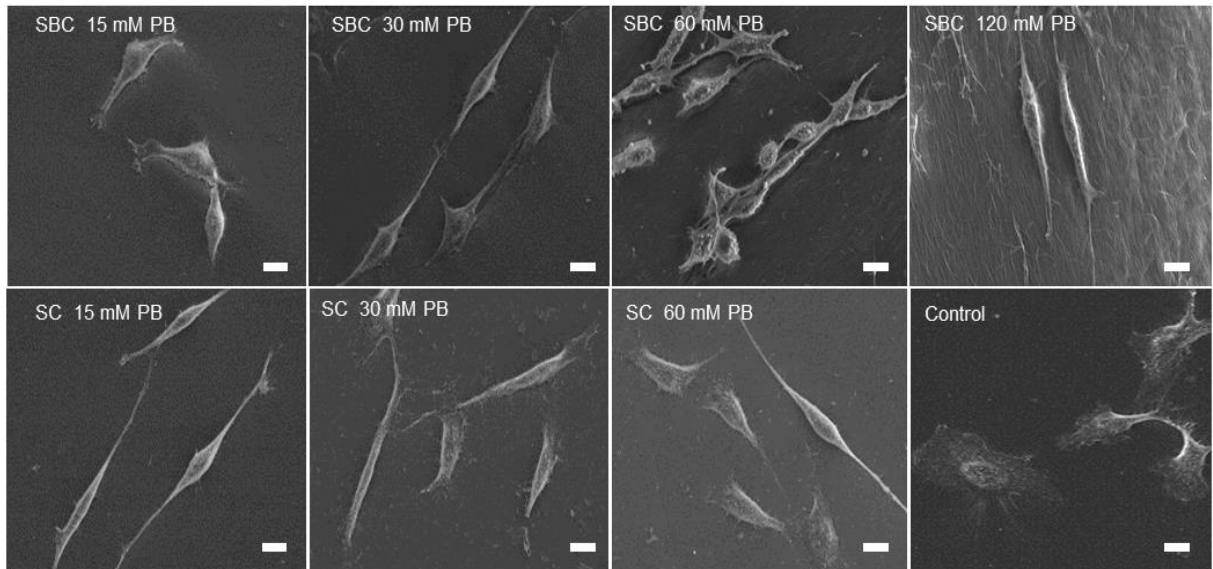


Fig. 8. Scanning electron micrograph images of L929 cells cultured for 2 days.

Scale bars: 10 µm.

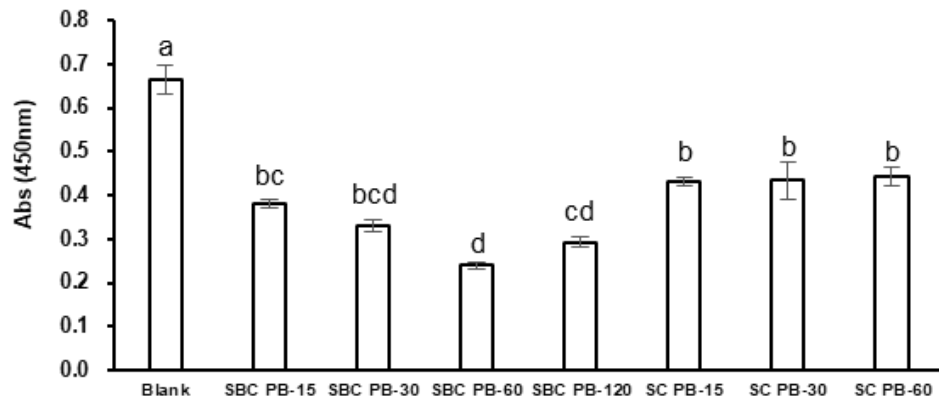


Fig. 9. The proliferation of L929 cells cultured for 5 days. Columns and bars show mean and standard error of the means of six measurements. Differences between the groups with different alphabets are significant (Tukey–Kramer post hoc test, $P < 0.01$).

Chapter IV. Comparison of antioxidant activity of collagen peptides and tissue

enzymatic hydrolysates from bester sturgeon by-products

In recent decades, antioxidant peptides have drawn intense research interest because of their advantages of high activity, easy absorption through our intestine into the bloodstream, and a lack of adverse side effects [1]. Fish by-products, which are rich in protein, are one of the most suitable sources to produce antioxidant peptides as described in Chapter I. The most abundant protein in fish by-products is collagen, which is widely distributed in tissues such as skin, bones, head, cartilage, and scales. Many collagen peptides derived from fish by-products have been demonstrated as antioxidant peptides [2, 3].

The antioxidant activity of peptides arises from the structure of amino acids. Davalos et al. [4] reported that among the amino acids, Trp, Tyr, and Met showed the highest antioxidant activity, followed by Cys, His, and Phe. Gly and Pro also play essential roles in the antioxidant activity of peptides because of the single hydrogen atom in the Gly side chain and the low ionization potential of the pyrrolidine ring in Pro allows it to serve as a proton-donating source [5]. According to the characteristic amino acid sequence (the repetitive Gly-X-Y sequence) of collagen, the antioxidant activity of collagen peptides is mainly attributed to the hydrophobic amino acids Gly and Pro because the levels of aromatic and sulfur-containing

amino acids are always low in collagen [6]. On the other hand, some studies have produced hydrolysates directly from fish by-products without gelatin extraction or removing noncollagenous proteins. The antioxidant peptides were Ile-Val-Ala-Gly-Pro-Gln from skate cartilage, Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser from tuna backbone, Gly-Val-Pro-Leu-Thr from bluefin leatherjacket head, and Val-Cys-Ser-Val and Cys-Ala-Ala-Pro from flounder muscle [7-10]. Based on the amino acid sequences of these peptides, they were derived from noncollagenous proteins. Therefore, to optimize production of active peptides with minimal loss of antioxidant activity, it is necessary to compare the antioxidant activities between the tissue hydrolysates and purified collagen hydrolysates. Few studies, however, have compared antioxidant activities of tissue hydrolysates and purified-collagen hydrolysates from fish by-products.

The antioxidant activity of peptides also relies on their molecular weight. Peptides containing 3–20 amino acid residues were reported to show high antioxidant activity. For example, Pro-Ile-Ile-Val-Tyr-Trp-Lys (Mw: 1004), Thr-Thr-Ala-Asn-Ile-Glu-Asp-Arg-Arg (Mw 1074), and Phe-Ser-Val-Val-Pro-Ser-Pro-Lys (Mw: 860) from the protein hydrolysate of blue mussel exhibited strong DPPH radical scavenging activity [11]. In another study, Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asn-His-Ala (Mw: 1076) and Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His (Mw: 1033) isolated from rotifer hydrolysate showed good antioxidant activity

[11]. Thus, it is also important to know the molecular weight range of the hydrolysates that have higher antioxidant activity.

In Chapter II, I already developed the efficient extraction method of type II collagen from sturgeon notochord. Compared with the yield of notochord collagen in the previous study [12], the yield increased from 5.1% to 37.5%. Thus, industrialization of notochord collagen has become possible, but there is no research on the effective utilization of sturgeon notochord. No way of notochord usage has been developed. Furthermore, while many studies have clarified the antioxidant activity of type I collagen peptides from fish skin and other tissues, there are few reports of the potential of peptides hydrolyzed from type II collagen.

The purpose of this study was to compare the antioxidant activities of purified collagen and tissue enzymatic hydrolysates from sturgeon skin and notochord with three free-radical scavenging assays. I also investigated the effect of different enzymes and determined the molecular weight ranges of hydrolysates with high antioxidant activity.

Materials and Methods

Pretreatment and extraction of collagens were as already shown in Chapter II. No alkaline pretreatment was conducted in this experiment.

Preparation of hydrolysates from purified collagen and tissues

Tissues (notochord and defatted skin) and purified collagen from both tissues were soaked in distilled water with a sample/solution ratio of 1:60 (w/v) at 60°C for 1 h for gelatinization. Papain, chymotrypsin, and trypsin were used to hydrolyze tissues and purified collagens with a concentration of 2.5% (w/w) for 4 h at the optimum temperatures (50, 37, and 37°C for papain, chymotrypsin, and trypsin, respectively). The reaction was terminated by boiling samples for 10 min. The reaction solution was filtered with a filter paper (pore size: 8 µm, Whatman, GE Healthcare Japan, Tokyo, Japan) to remove the unhydrolyzed residues. To remove the color, the skin and skin collagen hydrolysates were filtered with a cellulose acetate membrane filter (pore size 0.2 µm; Advantec Toyo, Tokyo, Japan). Then, all hydrolysates were lyophilized and stored at −30°C until use.

Fractionation of hydrolysates by ultrafiltration

Hydrolysates were fractionated by ultrafiltration using molecular weight cutoffs (MWCOs: Amicon Ultra centrifugal filter devices, Millipore Corporation, Billerica, USA) of 10 kDa and 3 kDa at 4000×g, 20 min, 4°C. Three fractions of over 10 kDa ($M_w > 10\text{ k}$), between 10 kDa and 3 kDa ($M_w 10\text{k}–3\text{ k}$), and below 3 kDa ($M_w < 3\text{ k}$) were collected, lyophilized, and stored at −30°C until use.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined according to the method of Pan et al. [10]. Briefly, a 1,10-phenanthroline solution (1.0 ml, 1.865 mM) was mixed with 2 ml of the sample solution, and then FeSO₄·7H₂O solution (1.0 ml, 0.75 mM) was pipetted into the mixture. The reaction was initiated by adding 1.0 ml of H₂O₂ (0.01% v/v). After incubation at 37°C for 60 min, the absorbance was measured at 536 nm against a reagent blank. The reaction solution without antioxidants was used as a negative control, and the mixture without H₂O₂ was used as the blank. The hydroxyl radical scavenging activity was calculated according to Eq. 1:

$$\text{Scavenging activity (\%)} = [(A_s - A_n) / (A_b - A_n)] \times 100\% \quad (1)$$

where A_s , A_n , and A_b are the absorbance values at 536 nm of the sample, negative control, and blank after the reaction, respectively.

ABTS radical scavenging activity

The ABTS radical scavenging activity was determined by the method of Re et al. [13]. In brief, ABTS radical cation was generated by mixing the ABTS stock solution (7 mM) with potassium persulfate (2.45 mM). The mixture was left in the dark at room temperature

for 16 h. The ABTS radical solution was diluted in 5 mM phosphate buffered saline at pH 7.4 to an absorbance of 0.7 at 734 nm. One milliliter of diluted ABTS radical solution was mixed with 1 ml of different concentrations of samples. Ten minutes later, the absorbance was measured at 734 nm against the corresponding blank. The ABTS radical scavenging activity of samples was calculated according to Eq. 2:

$$\text{Scavenging activity (\%)} = [(A_c - A_s) / A_c] \times 100\% \quad (2)$$

where A_s and A_c are the absorbances with and without a sample.

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined by the method of Williams et al. [14] with minor modifications. One milliliter of 0.1 mM DPPH radical (in 95% ethanol) was mixed with 1.0 ml of each sample. After being well mixed and kept at room temperature for 30 min in the dark, the mixture was transferred into cuvettes, and the absorbance was measured at 517 nm using an ultraviolet spectrophotometer (GE Healthcare). Deionized water was used instead of the sample solution for control measurement. The DPPH radical scavenging activity of the sample was calculated according to Eq. 3,

$$\text{Scavenging activity (\%)} = [(A_c - A_s) / A_c] \times 100\% \quad (3)$$

where A_c and A_s are the absorbances of the control and sample, respectively.

Mass distribution analysis

Mass spectra of the $M_w < 3$ k fractions of skin chymotrypsin, notochord chymotrypsin, and notochord papain hydrolysates (SC3, NC3, and NP3, respectively) were acquired by MALDI-TOF-MS (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA), operating in the positive ion delayed extraction reflector mode for the highest resolution and mass accuracy. Samples (0.5 μ l) were prepared using a dried-droplet preparation on a steel target plate, and 0.5 μ l of α -cyano-4 hydroxy-cinnamic acid was added as the matrix. Ions were generated by a YAG laser emitting at 355 nm.

Results and Discussion

Yields of hydrolysates

In previous studies, papain, trypsin, and chymotrypsin were used to obtain antioxidant peptides from fish by-products [7-9, 15]. Therefore, this study also used these enzymes to hydrolyze skin, notochord, and type I and II collagens; the hydrolysates were then tested for hydroxyl, ABTS, and DPPH radical scavenging activities. Vitamin C (a potent antioxidant) was used as a positive control to estimate the antioxidant activity of each sample.

The yield of each enzymatic hydrolysate is shown in Table 1. Among the enzymes

used, papain and chymotrypsin showed a tendency to give higher yields of hydrolysates.

Because the scales in the sturgeon skin could not be hydrolyzed, the yields of skin enzymatic hydrolysates were lower than notochord and collagen hydrolysates.

Hydroxyl radical scavenging activity

Among the ROS generated in an animal body, the hydroxyl radical is the primary active oxygen species with the strongest reactivity. Therefore, the scavenging of hydroxyl radicals is one of the most effective defense mechanisms of a living body against various diseases [5]. Hydroxyl radical scavenging activities of the hydrolysates are summarized in Fig. 1. The activities of all hydrolysates increased in concentration-dependent manners. The effects of enzyme species on the scavenging activities of purified type I and II collagen hydrolysates were significant, whereas those on skin and notochord hydrolysates were small. The chymotrypsin hydrolysates showed the highest scavenging activity in both types I and II collagen. All skin tissue hydrolysates showed higher scavenging activities than type I collagen hydrolysates, suggesting that the hydrolysates of the noncollagenous substances in the skin played an important role in scavenging hydroxyl radicals. Similarly, the trypsin hydrolysates of the type II collagen showed very low activity, suggesting that the trypsin hydrolysates of the noncollagenous substances in the notochord played an important role. In contrast, the

chymotrypsin hydrolysate of the type II collagen showed higher activity than notochord tissue hydrolysates, indicating that a large part of the action of the chymotrypsin hydrolysates of the notochord tissue was attributed to the hydrolysates of the type II collagen. Thus, the origin of antioxidants changes depending on the enzymes used for hydrolysis.

At the same concentrations, the hydroxyl radical scavenging activities of the skin hydrolysates were two times higher than those of the notochord hydrolysates. At the highest concentration tested (5 mg/ml), the activities of the chymotrypsin- and trypsin-hydrolyzed skin were close to the activity of the vitamin C positive control. Thus, the skin enzymatic hydrolysates are suitable to use in the pharmaceutical and food industries as a scavenger for reducing or eliminating the damage induced by hydroxyl radicals.

ABTS radical scavenging activity

The ABTS radical scavenging assay is commonly used for determining the antioxidant activities of peptides [13]. The oxidative power of ABTS radicals is lower than that of hydroxyl radicals, so it is possible to determine the antioxidant capacities of the hydrolysates at lower concentrations (Fig. 2). The results suggested that all enzymatic hydrolysates showed ABTS radical scavenging activity in a dose-dependent manner. Compared with the collagen hydrolysates, the tissue hydrolysates showed higher antioxidant

activities in both skin and notochord, again indicating that antioxidant substances were not only derived from collagens but also from noncollagenous constituents in the tissue. In general, the ABTS radical scavenging activities of notochord and type II collagen hydrolysates were higher than those of skin and type I collagen hydrolysates. Enzyme species also affected the activities except for the notochord tissue. The notochord trypsin hydrolysate showed the highest ABTS radical scavenging activity among the samples and showed a potency near that of the vitamin C positive control at the highest concentration tested. These data indicate that notochord enzymatic hydrolysates could be applied as electron or hydrogen atom donors to prevent free-radical damage in organisms.

DPPH radical scavenging activity

The DPPH radical scavenging activity is a quick, convenient, and efficient indicator of antioxidant activity and has been widely used to test the ability of compounds to act as free-radical scavengers. As shown in Fig. 3, the DPPH scavenging rate of all hydrolysates except for the type II collagen hydrolysates increased in a dose-dependent manner. Type II collagen hydrolysates showed almost no DPPH scavenging activity. Type I collagen chymotrypsin and papain hydrolysates showed apparent activities but were lower than those of the skin hydrolysates. These results indicated that the DPPH scavenger of notochord

hydrolysate was from noncollagenous components, whereas the actions of skin hydrolysates were derived from both type I collagen and noncollagenous substances. Enzyme species influenced the activities of skin hydrolysates, and the skin chymotrypsin hydrolysate had the highest activity. The influence of the enzyme species on the notochord hydrolysates was minor. Although notochord and skin hydrolysates showed scavenging activities against DPPH radicals, they were far lower than the vitamin C positive control (95% at 0.625 mg/ml). Thus, the data of vitamin C are not shown in Fig. 3.

Many researchers have used these three radical scavenging assays to evaluate the antioxidant activities of peptides, but different methods were applied in each case, including different concentrations of reactive solutions, different ratios of reactive solutions to samples, and different reaction times. Therefore, it is difficult to compare the antioxidant activities of peptides among the studies. In this study, to estimate the antioxidant activities of samples obtained from sturgeon by-product hydrolysates, the radical scavenging activity of vitamin C was measured as a positive reference. These results clearly show that tissue hydrolysates had higher antioxidant activity than collagen hydrolysates, indicating that the hydrolysates originating from noncollagenous substances in these tissues include active radical scavengers. It is known, extracellular matrix contains various kinds of proteins, such as elastin fiber, proteoglycans and fibronectins. Therefore, these non-collagenous proteins could be possible

sources of antioxidant peptides. The results also suggest that the direct hydrolysis of sturgeon skin and notochord is more useful to produce antioxidant peptides. Therefore, I used tissue hydrolysates for further analyses.

Fractionation of hydrolysates by ultrafiltration

The antioxidant capacity of peptides is closely related to their molecular size; the smaller molecular size fractions of protein hydrolysates had higher antioxidant activities [11, 16, 17]. To assess the influence of molecular size on the antioxidant activities, tissue hydrolysates were fractionated with 10 kDa and 3 kDa MWCO membranes. Three fractions were separated: those with molecular weight larger than 10 kDa ($M_w > 10\text{ k}$), between 10 kDa and 3 kDa ($M_w 10\text{--}3\text{ k}$), and less than 3 kDa ($M_w < 3\text{ k}$).

The hydroxyl, ABTS, and DPPH radical scavenging activities of each fraction are shown in Figs. 4-6. Although different radical scavenging mechanisms cannot make results agree with all samples and assays, the molecular weight range had a significant influence on the antioxidant activities of hydrolysates. The fractions of $M_w < 3\text{ k}$ generally showed the highest scavenging activities against hydroxyl, ABTS, and DPPH radicals, except for the DPPH radical scavenging activity of the notochord papain hydrolysate. These results agreed well with those of previous studies, which revealed the highest antioxidant activities of peptides in the range of 1000–3000 Da. For example, Val-Lys-Ala-Gly-Phe-Ala-Trp-Ala-Asn-

Gln-Gln-Lys-Ser (1519 Da) obtained from tuna backbone, Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala (1307 Da) purified from giant squid muscle, Leu-Leu-Gly-Pro-Gly-Leu-Thr-Thr-Asn-His-Ala (1076 Da), and Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His (1033 Da) extracted from marine rotifer demonstrated good antioxidant activities [7, 15, 18]. The high antioxidant activity of low molecular weight peptides may be attributed to their lower steric hindrance of when compared with long peptide chains, as well as the synergistic actions of high antioxidant amino acids.

It is also important to note that different proteases have different impacts on the antioxidant activities of hydrolysates. In this study, chymotrypsin was effective in producing high antioxidant hydrolysates in general. The chymotrypsin hydrolysate of skin with $M_w < 3$ k showed the highest scavenging activities against hydroxyl, ABTS, and DPPH radicals, with 95.0, 98.0, and 39.9% at 5, 0.25, and 5 mg/ml, respectively. Ko et al. [9] reported that flounder muscle was hydrolyzed by chymotrypsin to produce peptides with good antioxidant activity. Chymotrypsin cleaves aromatic amino acids such as Phe, Try, and Tyr, and these aromatic amino acids have been demonstrated to show high antioxidant activity [19]. The high antioxidant activities of the chymotrypsin hydrolysates are likely caused by exposure of aromatic amino acids at the peptide chain terminus where their antioxidant effects can be exerted.

Molecular weight distributions of Mw < 3 k fractions

To understand the molecular weight distributions of the Mw < 3 k fractions, which showed specifically high antioxidant activities, I conducted mass analyses of skin and notochord chymotrypsin hydrolysates and notochord papain hydrolysates by MALDI-TOF-MS (Fig. 7). Two distinct peaks at m/z 1578 and 2995 were evident in the Mw < 3 k fraction of the notochord chymotrypsin hydrolysate, suggesting that the antioxidant activity of this fraction was due mainly to these two peptides. On the other hand, the Mw < 3 k fractions of notochord papain hydrolysate and skin chymotrypsin hydrolysate had many peptides in the range of 1000–2500 and 1200–1800 Da, respectively. Therefore, the antioxidant activities of these two fractions seemed to be generated by the synergistic effect of many peptides.

From the viewpoint of industrial application of by-products, we did not conduct further isolation and purification of active peptides in this study.

Conclusion

In this study, I determined the antioxidant activities of hydrolysates of tissue and purified collagen obtained from sturgeon skin and notochord. Detailed analysis showed that the tissue enzymatic hydrolysates generally exhibited higher antioxidant activities. These data

indicated that the hydrolysates that originated from noncollagenous substances in these tissues are essential antioxidants. Therefore, the direct hydrolysis of sturgeon skin and notochord is useful for producing antioxidant peptides. To the best of my knowledge, the present study is the first to compare the antioxidant activities of tissue hydrolysates and purified-collagen hydrolysates. The tissue hydrolysates of $M_w < 3\text{ k}$ in both tissues showed higher radical scavenging activities, suggesting that smaller molecules of collagen and noncollagenous hydrolysates are the better antioxidants. In summary, the hydrolysates of sturgeon skin and notochord have better oxidation resistance than purified type I and II collagens and could be beneficial ingredients in cosmetic or food products.

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Figures and tables

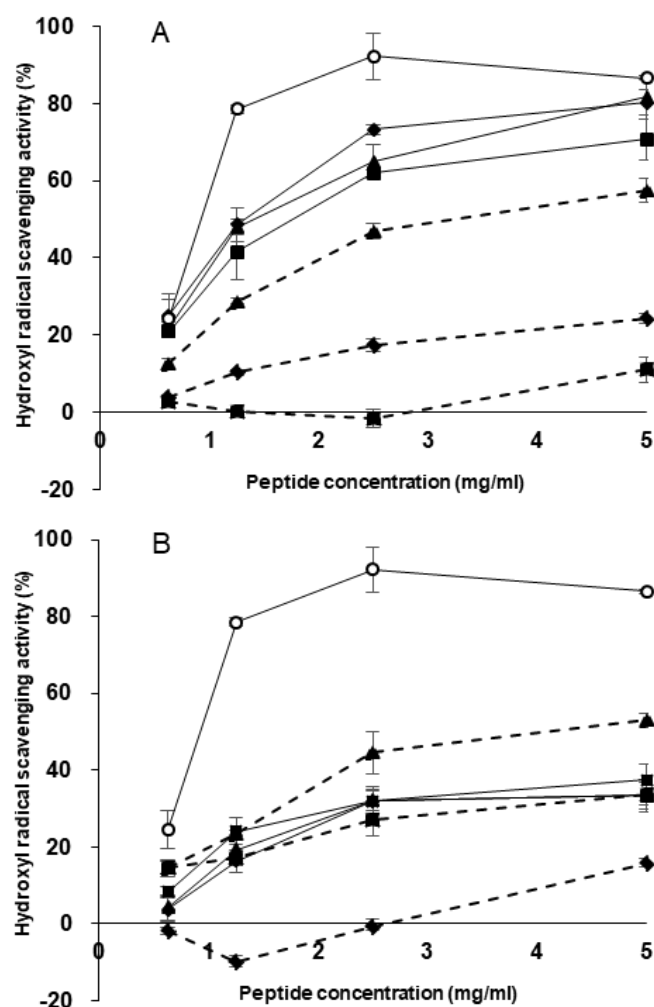


Fig. 1. Hydroxyl radical scavenging activities of tissue and purified collagen hydrolysates.

(A) Skin hydrolysates (solid line) and purified type I collagen hydrolysates (dotted line). (B)

Notochord hydrolysates (solid line) and purified type II collagen hydrolysates (dotted line).

Squares, papain hydrolysates; triangles: chymotrypsin hydrolysates; diamonds, trypsin

hydrolysates; circles, vitamin C. Values are mean $\pm$ SD of triplicate measurements.

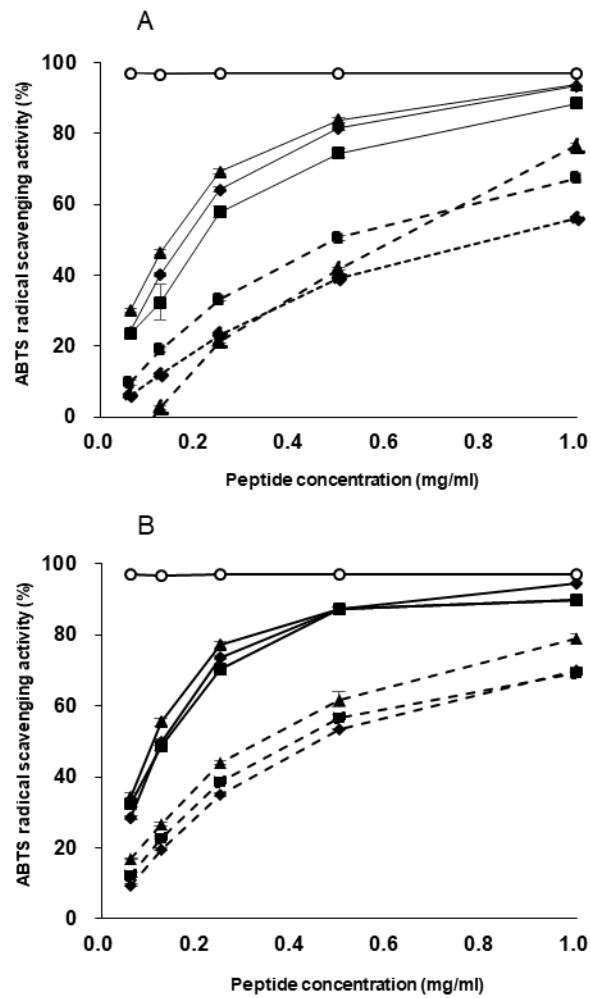


Fig. 2. ABTS radical scavenging activities of tissue and purified collagen hydrolysates. (A) Skin hydrolysates (solid line) and purified type I collagen hydrolysates (dotted line). (B) Notochord hydrolysates (solid line) and purified type II collagen hydrolysates (dotted line). Squares, papain hydrolysates; triangles: chymotrypsin hydrolysates; diamonds, trypsin hydrolysates; circles, vitamin C. Values are mean $\pm$ SD of triplicate measurements.

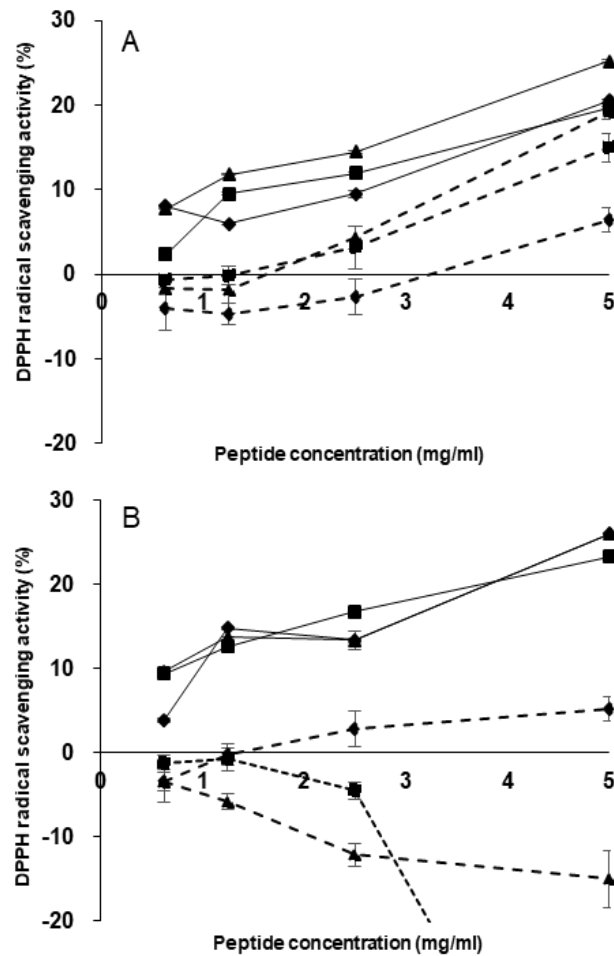


Fig. 3. DPPH radical scavenging activities of tissue and purified collagen hydrolysates. (A) Skin hydrolysates (solid line) and purified type I collagen hydrolysates (dotted line). (B) Notochord hydrolysates (solid line) and purified type II collagen hydrolysates (dotted line). Squares, papain hydrolysates; triangles: chymotrypsin hydrolysates; diamonds, trypsin hydrolysates. Values are mean $\pm$ SD of triplicate measurements.

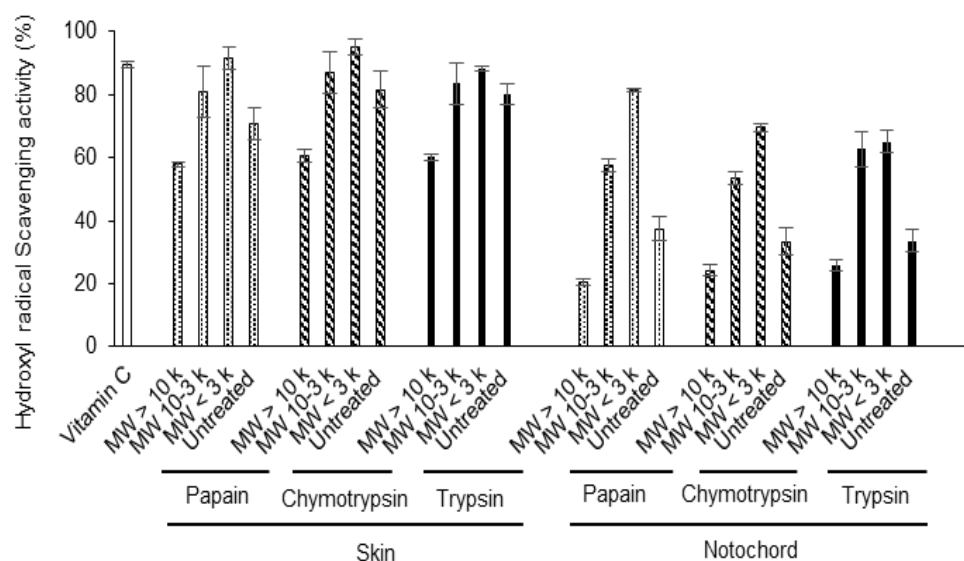


Fig. 4. Effect of molecular weight of tissue hydrolysates on the hydroxyl radical scavenging activity. Fractions of hydrolysates having a molecular weight over 10 k ($M_w > 10$ k), between 10 k and 3 k (M_w : 10–3 k), and below 3 k ($M_w < 3$ k) were used for the measurement of the hydroxyl radical scavenging activity at a concentration of 5 mg/ml. Left, skin hydrolysates; right, notochord hydrolysates. Values are mean $\pm$ SD of triplicate measurements.

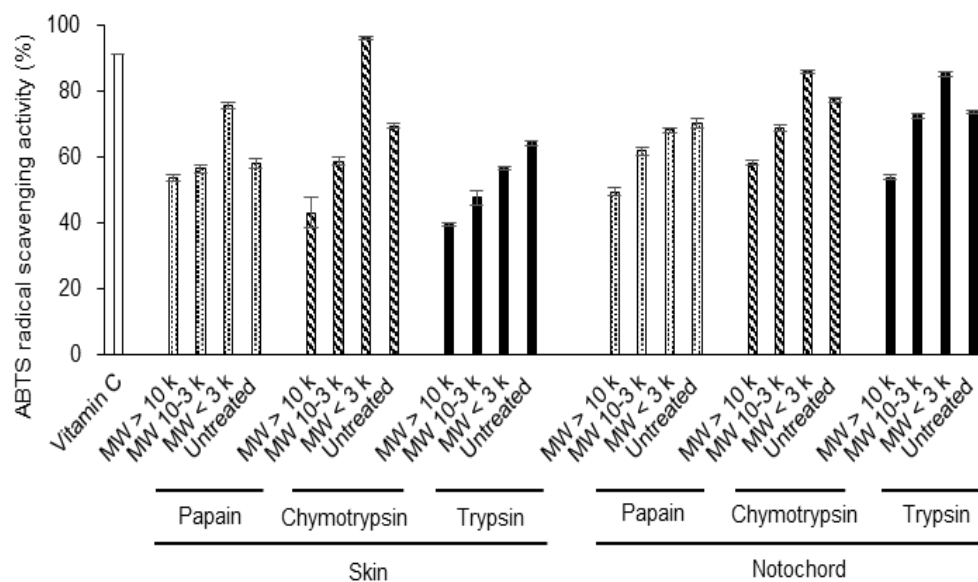


Fig. 5. Effect of molecular weight of tissue hydrolysates on the ABTS radical scavenging activity. Fractions of hydrolysates having a molecular weight over 10 k (Mw > 10 k), between 10 k and 3 k (Mw: 10–3 k), and below 3 k (Mw < 3 k) were applied for the measurement of the ABTS radical scavenging activity at a concentration of 0.25 mg/ml. Left, skin hydrolysates; right, notochord hydrolysates. Values are mean $\pm$ SD of triplicate measurements.

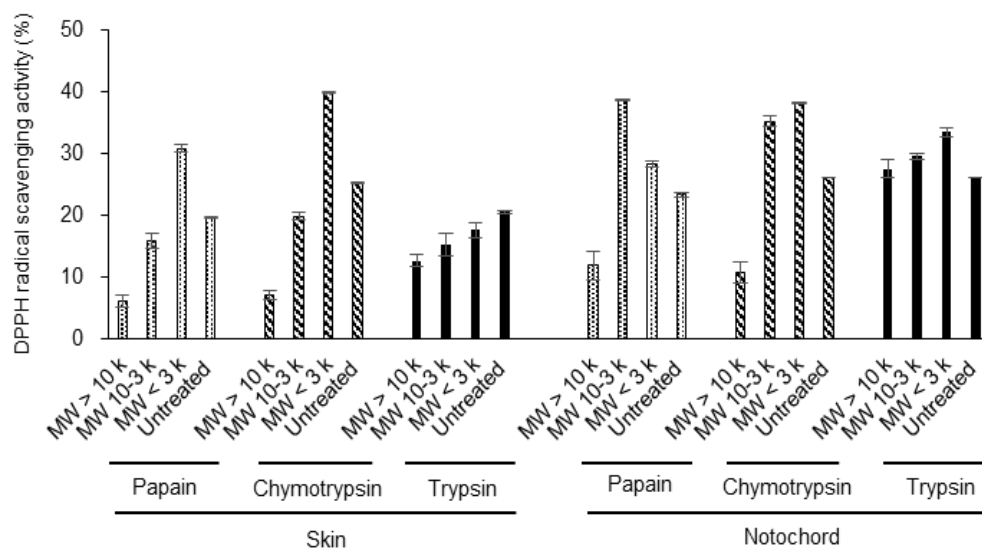


Fig. 6. Effect of molecular weight of tissue hydrolysates on the DPPH radical scavenging activity. Fractions of hydrolysates having a molecular weight over 10 k ($M_w > 10\text{ k}$), between 10 k and 3 k ($M_w: 10\text{--}3\text{ k}$), and below 3 k ($M_w < 3\text{ k}$) were applied for the measurement of the DPPH radical scavenging activity at a concentration of 5 mg/ml. Left, skin hydrolysates; right, notochord hydrolysates. Values are mean $\pm$ SD of triplicate measurements.

Table 1. Yields (% on dry basis) of tissue and collagen enzymatic hydrolysates.

Enzyme	Skin	Notochord	Type I collagen	Type II collagen
Papain	80.40	97.70	93.50	99.50
Chymotrypsin	83.80	96.12	94.30	99.60
Trypsin	77.90	98.86	83.77	79.82

Chapter V. General discussion

Previous studies in my laboratory have found a quantity of collagens could be extracted from better sturgeon skin, swimming bladder, and notochord [1]. The objective of this thesis was to overcome hurdles before the industrial applications of sturgeon collagen into two high-value-added ways: biomedical materials and functional peptides.

In the present thesis, I firstly improved the extraction processes with two methods, alkaline pretreatment and increasing cycles of extraction, to efficiently purify enough amount of collagen for large-scale industrialization. Then, I compared the biochemical characteristics and fibril-forming ability of triple helical collagen molecules. Secondly, as one of the high-value-added usages for sturgeon collagen, I developed the method to coat cell culture wells with collagen fibrils. I also studied the influence of phosphate ion concentrations on the fibril formation to regulate the fibril morphology. Finally, to exploit the functionality of collagen peptides from sturgeon by-products for food and cosmetic industries. I investigated the antioxidant activity of enzymatic hydrolysates of sturgeon collagen obtained from skin and notochord with three kinds of radical scavenging experiments.

The major outcomes are as follows:

1. The yields of collagens increased from 34.1% [1] to 64.2% in the skin type I collagen and

from 5.1% [1] to 37.5% in the notochord type II collagen.

2. By changing the phosphate ion concentrations in the reaction fluid, type I collagen fibrils with different morphologies could be coated on the bottom of the cell culture wells. The morphology of fibrils could regulate fibroblast morphology and proliferation.
3. Peptides obtained from purified skin and notochord collagen showed antioxidant activity. On the other hand, hydrolysates from whole skin and notochord showed higher antioxidant activity than collagen hydrolysates, indicating that the use of whole tissues is suitable for industrialization as a natural antioxidant agent.

As the general discussion, here I discuss the possible applications of sturgeon collagen in different forms, molecule, fibril, and peptides, based on the three findings of the present study.

Molecule

In Chapter II, I achieved the efficient method to purify types I and II collagen from sturgeon tissues. The high yield is a prerequisite to proceed researches on collagen fibrils because native molecules are used to fabricate fibrils. Firstly, as high-value-added usage of sturgeon collagens, I suggest using collagen molecules for manufacturing fibrils for

biomedical usages, and this point will be discussed later.

There is another high-value-added usage for type II collagen molecule: a health food to prevent and alleviate osteoarthritis (OA) and rheumatoid arthritis (RA). OA is the most common disease surrounding the cartilage joints, affecting an estimated 10% of men and 18% of women over 60 years of age [2]. According to the World Health Organization, OA is the sixth-leading cause of disability in the world [3]. RA is an autoimmune disease against type II collagen. In recent years, oral administration of undenatured type II collagen has been reported to be beneficial for the treatment of OA and RA. Lugo et al. [4] demonstrated that 40 mg of type II collagen in daily supplementation could make RA patient knee joint extension better. Mannelli et al. [5] found that oral administration of type II collagen could decrease pain and structural joint damage in the rat OA model. Gupta et al. [6] reported that type II collagen improved knee joint symptoms better than the same dose of glucosamine hydrochloride and chondroitin sulfate in knee OA subjects. Trentham et al. [7] demonstrated that type II collagen effectively enhanced joint comfort and flexibility thereby improving the quality of life in RA subjects. In these experiments, only non-denatured, triple helical type II collagen molecules are active. Till now, the precise mechanism of effects of orally administered type II collagen molecules on the joint function has not been resolved entirely. The most common theory is that oral type II collagen in RA patients involves in oral tolerance

against type II collagen autoimmunity and induces the regulatory T cells to produce IL-10 and TGF- β , and these immune adjusting factors play roles in the joint by a series of immunomodulatory response [4].

However, type II collagen in the market is much less than type I collagen. Furthermore, until now, only a few studies have reported about fish type II collagen, though fish collagen has several advantages such as biosafety and religious acceptance against the mammalian collagens (Chapter I). For example, Kittiphattanabawon et al. [8] extracted type II collagen from the bamboo shark and blacktip shark cartilage with yields of 9.59% and 10.30% (dry weight basis). Kobayashi in my laboratory extracted type II collagen from skate cartilage with the yield about 10% (Kobayashi, unpublished observation). In Chapter II, I got type II collagen with a yield of 35.5% from sturgeon notochord. This yield was much higher than that from other marine species. Through SDS-PAGE and CD spectra analyses, I also demonstrated that the extracted type II collagen maintained complete triple helical structure (Chapter II). Although the notochord is a small tissue (it occupies about 1.9% of sturgeon weight, unpublished observation), caviar producing females become more than 20 kg. Therefore, the expected yield of the notochord type II collagen from one caviar-producing fish is calculated to be over 130 g. Since recommended daily uptake of the type II collagen in the commercial product is 10 mg, the type II collagen extracted from sturgeon notochord has

a great potential to be used for OA and RA prevention or alleviation.

Fibril

Biomedical materials are the typical high-value-added way of the collagen usages. As already described in Chapter I, the engineered tissues are expected to be used for regenerative medicine. In recent years, the scaffolds made of fish collagen have attracted high attention due to their safety and no religious restrictions (Chapter I).

Several forms of fish-collagen scaffolds, such as nanofibrils [9] and porous scaffolds [10], have been developed. The scaffolds made of nanofibrils are usually created by the electrospinning technique. This technique uses electrostatic forces to draw polymers from a high-concentration solution to form continuous nanoscale fibrils onto the earthed collector [11]. Collagen nanofibers, which derived from the Hoki skin, jellyfish, and tilapia skin were successfully prepared by electrospinning [9, 11, 12]. Fish-collagen nanofibers were demonstrated with excellent bioactivity and biocompatibility by *in vitro* and *in vivo* experiments [11, 12]. Collagen porous scaffolds are produced by the lyophilization of the collagen suspension in water, leaving the porous structure in the scaffolds [13]. The pore size and structure significantly affect cell adhesion and migration *in vitro* and influence the rate and depth of cellular in-growth *in vivo* [14]. In both cases, pure collagen scaffolds are easy to

lose its contour structure and biodegrade quickly due to its low stability. Therefore, the cross-linking reagent is often used to modify the biodegrading rate and to optimize the mechanical property [15].

In Chapter III, I mimicked the mechanism of collagen self-assembly *in vivo* and synthesized collagen fibrils *in vitro*. The fibrils formed had a characteristic 67-nm D-periodic banding pattern, suggesting that the fibrils are close to their native form (Hatayama and Yunoki, unpublished observation). This is the most significant difference from other collagenous scaffolds made by electrospinning and lyophilization techniques. Also, I successfully controlled the collagen fibril morphology by changing the phosphate ion concentration in the reaction-fluid. The results suggested that swim bladder collagen has the better potential to develop various fibril morphologies compared with skin collagen. More importantly, I succeeded to make fibril-coated wells for cell culture and showed that the fibril morphology greatly influenced the fibroblast adhesion, morphology, and proliferation. These findings provided the essential information to develop the 2-D collagen coated materials and 3-D collagen hydrogel materials with optimized fibril morphology. Meanwhile, this research also opened a new window for utilization of sturgeon by-products to biomedical research and application.

Type II collagen is the main protein in the cartilage [16]. For cartilage engineering, it

is better to use type II collagen mimicking the cartilage matrix environment. In Chapter II, I succeeded to fabricate fibrils from the notochord type II collagen molecules, which was impossible in the previous study [6] and preliminarily discussed the fibril forming environment. Thus, the scaffold for cartilage engineering using type II collagen fibrils is another potential utilization field for sturgeon notochord.

Peptides

In Chapter IV, I showed a possibility to use peptides made of the purified collagen or whole tissues from sturgeon as a natural antioxidant agent. Previous studies on fish antioxidant peptides have been mainly conducted as ingredients for health foods. As I have already shown in Chapter I, orally administered peptides will get further enzymatic hydrolysis in our digestive system, and only small-sized peptides will be absorbed into the bloodstream. Therefore, a lot of active sequences determined in the previous studies (listed in Chapter IV) will not work in our body when they are orally administered. Therefore, here I propose antioxidant peptides obtained from sturgeon tissues to use as the percutaneous absorbent for cosmetic products. Antioxidant peptides are suitable for anti-aging cosmetics as described in Chapter I.

Cosmetics are applied to the human body for cleansing, beautifying, promoting

attractiveness, or altering the appearance [17]. In general, cosmetics products contain several forms, such as cream, lotion, and ointments [18]. The key ingredients of cosmetics often include vitamins, minerals, botanical extracts, and antioxidants [19]. Bioactive peptides have been increasingly used, and more than 25 different peptides are routinely found in a vast array of skin care products in the United States [20]. In recent years, the word "cosmeceuticals" is used for cosmetic products that contain bioactive ingredients.

The crucial factor of the bioactive compounds of cosmeceuticals is the molecular weight, because the molecules with molecular weight > 2 kDa have difficulty in penetrating the skin barrier [21]. In Chapter IV, I used the molecular weight cut-off to separate the peptides into three fractions and showed that the fraction with molecular weight < 3 kDa had the highest antioxidant activity. The MS analysis showed the molecular weights of most of the peptides of this fraction were below 2 kDa. These results suggested the antioxidant peptides from sturgeon have potential to be used as cosmeceutical ingredients.

As known, the outer layer of the skin is covered by a lipid bilayer called stratum corneum that plays a barrier role to protect the subcutaneous tissue [22]. By this barrier, hydrophilic and macromolecular substances are limited to enter into the skin [22]. In this study, we found that peptides with Mw below 3 k showed high antioxidant activity, but peptides are difficult to pass through the layer because they are hydrophilic. Therefore, to

achieve the functionality of peptides by percutaneous absorption, structural modification, surfactant modification, or other drug delivery techniques must be applied.

Industrial utilization of sturgeon by-products based on their characteristics

In this study, three sturgeon by-products, the notochord, swim bladder, and skin, were used for the experiments. Based on their properties, finally I suggest the utilization ways of three tissues.

1. Notochord

Notochord accounts for about 1.9% of the total weight of sturgeon (unpublished observation) but contains a significant amount of type II collagen. Antioxidant activity of peptides made of type II collagen and notochord tissue was lower than that made of type I collagen and skin tissue. Thus, it is better to use notochord as the ingredient for OA and RA treatment or cartilage-engineering materials. Fibrils of type II collagen may also suitable for cartilage engineering, but the cartilage engineering using fish type II collagen is yet unexploited research area.

2. Swim bladder

Swim bladder accounts for about only 1.5% of the total weight of sturgeon

(unpublished observation). Less quantity restrains its application for the food industry.

However, extraction of the swim bladder collagen is easy, and the collagen solution exhibits transparent liquid with low viscosity. Moreover, the fibril morphology shows a variety of forms. Due to these properties, the most suitable way of usage for sturgeon swim bladder is collagenous scaffolds for tissue engineering.

3. Skin

Skin account for about 4.9% of the total weight of sturgeon (unpublished observation). The large amount is an advantage for the large-scale application. However, the gray color of the purified collagen molecules limits their usage for cosmetics. In contrast, peptides made of skin collagen and tissue are white. Moreover, both collagen and tissue peptides of skin showed higher antioxidant activity than those of type II collagen and notochord tissue. Thus, skin is suitable to use as peptides for cosmeceuticals.

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