Optimization of Extraction of Gelatin from the Head of Kalamtra Sturgeon (*Huso dauricus* × *Acipenser scherenkii* × *Acipenser transmontanus*)

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Received: 30 June 2020; Accepted: 21 September 2020; Published: 23 September 2020

Featured Application: Gelatins extracted from the sturgeon head would be applicable in the foods, cosmetics, and biomedical industries.

Abstract: To develop a new method for extracting gelatin from the sturgeon head, the conditions for pretreatment and extraction were optimized. Treatment at 65 °C (3–3.5 h) was enough to separate the head into mixed tissues (skin, scales, pectoral fins, muscle, bones, gills, and small cartilage pieces), skull cartilage, and liquid. From the intensities of α-, β- and γ-bands and yields, the optimized pretreatment conditions for type A and type B gelatin were 0.05 M HCl (1 h) and 0.1 M NaOH (1 h), respectively. The best extraction conditions were: (1) for type A gelatin: with distilled water (DW) (w/v 1:5) at 35 °C, pH 7 when stirring at 200 rpm for 3 h, and (2) for type B gelatin: with DW (w/v 1:5) at 50 °C, pH 8 when stirring at 200 rpm for 1 h. After the decalcification of extracted residues with 0.05 M HCl for 3 h, re-extraction of gelatin was possible. Under the best extraction conditions, yields of type A and B gelatins were 5.01 and 7.25% (dry gelatin weight/wet sample weight), respectively. Thus, it is possible to extract an industrial amount of gelatin from sturgeon heads, making them valuable by-products.

Keywords: sturgeon head; tissue separation; pretreatment; extraction conditions

1. Introduction

Aquaculture farming of sturgeon was initiated globally at the end of the 20th century. By 2017, a total of 2329 commercial sturgeon farms were reported, with biomass production of 102,327 tons [1]. The present sturgeon aquaculture industry generates a large quantity of by-products. The primary commercial product is the ovary (caviar), with the meat often being discarded as a by-product of caviar processing [1]. The suitable utilization of all parts of sturgeon by-products could enhance the profits of the aquaculture farming and processing industry, while concurrently contributing to the Sustainable Development Goals adopted by all United Nations Member States in 2015.

Fish by-products are tissues and organs that are not directly used as foods [2] but are rich in collagen or gelatin. A preliminary study showed that by-products of commercial sturgeon (>2 kg) include approximately 1.9% notochord, 2.1% digestive tract, 4.2% fins, 5.7% vertebrae, 6.1% skin, 0.54% swim bladder, and 17.1% head of the total body weight. There are some earlier reports on the utilization of sturgeon skin for gelatin extraction [3–6]. However, no study exists on the utilization of the...
sturgeon head, despite it representing a substantial part of the by-products. Consequently, the present study focused on extracting gelatin from the head of Kalamtra sturgeon, which is a hybrid species of *Huso dauricus* × *Acipenser schrenkii* × *Acipenser transmontanus*, for which aquaculture production is currently expanding in Hokkaido, Japan.

The properties and functionality of fish gelatin depend on the sources of fishes, organs, pretreatments, and extraction conditions. Typically, alkali- or acid-treated fish tissues are used to extract gelatin under a hot-water extraction process. When acid is used in the final pretreatment of tissues, the extracted gelatin is known as type A. Alternatively, if alkali is used in the last pretreatment step, the gelatin is called type B [7]. Both acid and alkali pretreatments are required to remove the unwanted smell and color, swell the tissues by weakening the triple helix of collagen molecules, and reduce extraction time [8,9]. However, it is difficult to use the sturgeon head directly for gelatin extraction using conventional methods because of its large size, hardness, and complexity of the multiple tissue composition. Thus, to utilize the sturgeon head, it is first necessary to develop a new method that makes the head soft and manipulatable for tissue separation. After that, suitable pretreatment and extraction processes are required to obtain the desired properties and functionalities of head gelatin. Therefore, the present study aimed to develop suitable: (1) tissue separation and pretreatment techniques and (2) extraction processes by manipulating pH, temperature, and processing time for the head of Kalamtra sturgeon fish.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents were of special grade unless otherwise noted. Hydrochloric acid, sodium hydroxide, sodium dodecyl sulfate (SDS), β-mercaptoethanol, Coomassie Brilliant Blue R-250, and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). The Precision Plus protein standard was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

2.2. Tissue Separation

The heads of Kalamtra sturgeon were supplied from an aquaculture farm (Bifuka Shinko Kosha, Bifuka, Japan) and stored at −20 °C. These heads were by-products of fillet production. In the farm, usually, males with a body weight of >2.0 kg are harvested. Each head was thawed overnight at 4 °C, washed with deionized water (DW), weighed, put into a polyethylene bag (34 cm × 24 cm) with a tight seal, and incubated at 65 °C for 3–3.5 h. After incubation, the skull cartilage was manually removed from the head, weighed, and stored at −30 °C. The remaining tissues of the head were considered as the mixed tissue, composed of skin, scales, pectoral fins, muscle, bones, gills, and small cartilage pieces. These tissues were weighed separately, cut into small pieces, thoroughly mixed, and stored at −30 °C. Only the mixed tissue was used to extract gelatin.

2.3. Optimization of the Pretreatment Process

Sturgeon head is a complex mixture of soft tissue (skin and muscle), semi-soft tissue (small cartilage pieces), and hard tissue (bone, gill, and scale). Gelatin was to be extracted firstly from the soft-tissue, and then, from the semi-soft tissue and the hard tissue. For these purposes, the head skin, which was the most abundant soft tissue of the head, was used as a representative sample of the soft tissue to optimize the pretreatment and extraction conditions. Then, residues after the first extraction, which were mainly composed of the semi-soft tissue and the hard tissue, were used to optimize the decalcification process for re-extraction.

Following previous articles [6,10], the effects of pretreatment with HCl (0.05 M), NaOH (0.1 M), or their combination (NaOH → HCl) with modifications of treatment time (1, 3, and 6 h) on the
intensities of α-, β-, and γ-bands of gelatin in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and yield were tested.

After separating the tissues of the head, the skin was cut into small pieces (10–12 mm × 10–12 mm), weighed, and pretreated with 0.05 M HCl (w/v 1:10) or with 0.1 M NaOH at 4 °C while stirring at 150 rpm for 1, 3, and 6 h. For the combined pretreatment (NaOH → HCl), the sample was first treated with 0.1 M NaOH for 1 h and subsequently with 0.05 M HCl for 1 h.

After the pretreatment, the supernatants were separated by centrifugation (10,000×g for 15 min at 4 °C) and stored for further analysis. The precipitates were cut into smaller pieces (5–6 mm × 5–6 mm) and used to extract gelatin with DW (w/v 1:5) at 65 °C and pH 7 for 1 h when stirring at 200 rpm. As a control, the mixed tissue without pretreatment was used. The extracted solution was centrifuged (10,000×g for 15 min at 21 °C), the supernatant was collected, and vacuum filtered (Advantec No. 6 filter paper, with a pore size of approximately 3 µm, Advantec, Tokyo, Japan). The extracted and pretreated solutions were directly used to observe the intensities of α-, β-, and γ-bands of gelatin in SDS-PAGE, as described in Section 2.7. Finally, the yield (% dry gelatin weight/wet sample weight) was calculated after freeze-drying the extract. Here, the wet weight of the sample before the pretreatment was used as the wet sample weight.

2.4. Optimization of the Extraction Process

The optimal extraction process was determined using the head skin for both type A and B gelatins. Before the extraction, the skin was cut into small pieces and pretreated under the best conditions for type A and B gelatins.

To determine the optimum pH, type A gelatin was extracted with DW (w/v 1:5) at 65 °C for 1 h when stirring at 200 rpm after adjusting the pH to 2, 6, 7, 8, or 12. Similarly, type B gelatin was extracted after adjusting the pH to 2, 6, 7, 8, 9, 10, or 12. Then, the extracted solutions were collected by centrifugation and filtered as indicated in Section 2.3. The extracted solutions were directly used to observe the intensity of α-, β-, and γ-bands of gelatin in SDS-PAGE, as described in Section 2.7. The optimum extraction pH was determined by evaluating the yield as described in Section 2.3 and the intensities of the α-, β-, and γ-bands of gelatin, as described in Section 2.7.

Next, a suitable temperature and time were determined using the best extraction pH. Type A gelatin was extracted with DW (w/v 1:5, pH 7) at 35, 50, and 65 °C for 0.5, 1, and 3 h. Type B gelatin was extracted with DW (w/v 1:5, pH 8) at 35, 50, and 65 °C for 0.5, 1, and 3 h. Then, the optimum extraction temperature and time were determined by evaluating the yield and intensities of α-, β-, and γ-bands of gelatin in SDS-PAGE, as described above.

2.5. Optimization of Decalcification of the Semi-Soft and the Hard Tissue for Re-Extraction

The residues of the first extraction were decalcified with 0.05 M HCl (w/v 1:10, 4 °C, 150 rpm) for 1, 3, and 6 h, largely following the previous report [11]. After decalcification, samples were centrifuged (10,000×g for 15 min at 4 °C), and the supernatant was retained to determine the loss of gelatin. The residues were cut into small pieces (2–3 mm × 2–3 mm). Re-extraction was completed using DW (w/v 1:5) at 35 °C, pH 7, when stirring at 200 rpm for 3 h (for type A residues) or at 50 °C, pH 8, when stirring at 200 rpm for 1 h (for type B residues). The re-extracted solutions were collected by centrifugation and filtration, as described in Section 2.4. The optimum decalcification period was determined by evaluating the yield as described in Section 2.4 and intensities of α-, β-, and γ-bands of gelatin in SDS-PAGE, as described in Section 2.7.

2.6. Gelatin Extraction and Re-Extraction Using the Optimized Condition

Using the optimized conditions, Type A and type B gelatin were extracted from the mixed tissue. Then, the residues were decalcified using the optimized conditions, and gelatins were re-extracted. The yields and the intensities of α-, β-, and γ-bands were measured as described in Sections 2.4 and 2.7, respectively.
2.7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted using 7.5% gel, following the methods of Laemmli [12] and Zhang et al. [13] with some modifications. During the optimization process, 1–3 µL each of extracts and 5 µL each of pretreated solutions were used directly. The intensities of the α-, β- and γ-bands of gelatin show the concentration of gelatin in the solution; hence, they are the indicators of the amount of gelatin extracted (in case of extracts) or lost (in case of pretreated solutions). After the extraction and re-extraction using the optimized conditions, freeze-dried gelatins were dissolved in DW, heated at 50 °C for 15–20 min, and loaded in SDS-PAGE (10 µg/lane) for qualitative assessment of gelatin. In both cases, samples were mixed (v/v 1:2) with the sample buffer (0.5 M Tris-HCl buffer at pH 6.8 with 4% SDS and 20% glycerol) containing 10% β-mercaptoethanol, boiled for 3 min, and loaded onto the gel. A Precision Plus Protein Standard (Bio-Rad Laboratories, Inc.) was used to assess the molecular weight. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 solution for 50 min and de-stained with a mixture of 20% ethanol, 5% acetic acid, and 2.5% glycerin for 2–3 h. Using captured images of the gels, the intensities of the α-, β-, and γ-bands and low molecular weight fragments were quantified using the Image-J software (Version 1.52, National Institutes of Health, Bethesda, MD, USA).

2.8. Statistical Analysis

All experiments were replicated three times, and data were presented as the mean ± standard error (SE). Student’s t-test or one-way ANOVA and the Tukey-Kramer test were performed using Microsoft Excel add-in statistical software (version 2.12, Social Survey Research Information Co., Ltd., Tokyo, Japan). The statistical significance was set at p < 0.05.

3. Results and Discussion

3.1. Tissue Separation

Sturgeon heads are tough and difficult to manipulate. This makes sturgeon heads impossible to use in industries. To make the sturgeon head as soft and manipulatable, we adopted a strategy whereby sturgeon heads were lightly heated, thus avoiding the high temperatures that tend to break gelatin molecules into smaller peptides. After the incubation of the head (585.50 ± 86.99 g, n = 3) at 65 °C for 3–3.5 h (depending on the size of each head), sturgeon heads became soft and manipulatable. After the incubation, a small amount of liquid was obtained in the polyethylene bag. This liquid component comprised 9.02 ± 0.28% of the head weight. The heated-head tissues were manually dissected. The skull cartilage that comprised 13.73 ± 0.17% of the head weight was stored at −30 °C for future production of rarely available type II gelatin or chondroitin sulfate. The remaining head tissue was considered as the mixed tissue, comprising 70.56 ± 0.38% of the head weight. Approximately 6.68 ± 0.71% of the head weight was lost during the dissection. In terms of the weight of the head, the mixed tissue was composed of 7.68 ± 0.06% skin, 5.56 ± 0.47% scales, 26.19 ± 0.63% muscle, 6.25 ± 0.37% pectoral fins, 11.38 ± 0.59% gills, 6.72 ± 0.29% bones, and 6.79 ± 0.30% small cartilage pieces. To separate complex tissues constructing the head, heads were heated at a relatively low temperature for a short time to prevent unnecessary gelatin breakdown.

3.2. Determining Optimum Pretreatment Conditions

In this study, the quality of gelatin was defined as the relative amount of un-hydrolyzed, complete form of gelatin. Therefore, intensities of α-, β-, and γ-bands as well as low molecular-weight bands (smaller than 100 k) in SDS-PAGE are the good indicators of the quality of gelatin.

For type A gelatin, intensities of α-, β-, and γ-bands of gelatin were highest in the 1 h pretreated group and decreased with the pretreatment time (Figure 1a and Figure S1a). Similarly, the highest yield, 6.81% (dry gelatin weight/wet tissue weight), was recorded in the 1 h treatment group (Table 1). No gelatin bands were observed in the supernatant of pretreated solution in the 1 h treatment group.
was obtained for type B gelatin, where the sample pretreated with 0.1 M NaOH for 1 h had the strongest gelatin bands (Figure 1b and Figure S1b) with maximum yield (10.56%, Table 1). Again, no loss of gelatin was observed in this condition (Figure S1b). The intensity of low molecular-weight bands in SDS-PAGE increased with the pretreatment time for both types of gelatins (Figure 1a,b and Figure S1a,b). The intensity of low molecular-weight bands was higher in type B gelatin than in type A (Figure S1a,b); thus, acid pretreatment produces higher quality but lower yields of gelatin than does the alkali pretreatment.

Pretreatments might also swell the tissues, leading to higher yields of gelatin after the partial breakdown of the crosslinks of collagen [3,14,15]. However, gelatin yield decreased with increasing acid- or alkali-pretreatment time, as shown by Nikoo et al. [6] and Yang et al. [16]. The longer pretreatment of tissues might hydrolyze more collagen molecules, destroying α-, β-, and γ-bands into fragments that are dissolved in the pretreatment solution, resulting in lower yields (Figure 1a,b and Figure S1a,b and Table 1). Actually, after HCl and NaOH pretreatment for 6 h, the supernatants had weak bands that were smaller than 150 k (Figure S1a,b); thus, non-collagenous proteins, as well as broken gelatin, were probably removed by the pretreatments.

Figure 1. Effects of the pretreatments on the intensities of α-, β-, γ-, and low molecular-weight (<100 k) bands of gelatin in the extracts and the pretreatment solutions in SDS-PAGE (7.5% gel). Gelatins were extracted (65 °C, 1 h, pH 7) from the head skin after being pretreated with (a) 0.1 M HCl for 1, 3, and 6 h, (b) 0.1 M NaOH for 1, 3, and 6 h, (c) the combined pretreatment (NaOH, 1 h → HCl, 1 h), and (d) without any pretreatments as the control. Each lane contained 1 µL extract. Columns and bars represent means and standard errors of three replicates, respectively. Different letters in each band indicate a significant difference (p < 0.05).
Table 1. Yields (% dry weight gelatin/wet weight sample) of gelatins extracted from the head skin of Kalamtra sturgeon after different pretreatment conditions.

| Gelatin Pretreatments | Extraction Conditions | Yields (%) |
|-----------------------|-----------------------|------------|
|                       | Reagents              | pH | Temperature (°C) | Time (h) | |
| Type A                | 0.05 M HCl            | 7  | 65             | 1        | 6.81 ± 0.56 |
|                       |                       | 6  |                | 1        | 5.60 ± 0.40 |
|                       |                       |    |                | 6        | 3.81 ± 0.32 * |
| Type B                | 0.1 M NaOH            | 7  | 65             | 1        | 10.56 ± 0.39 |
|                       |                       | 3  |                | 1        | 9.79 ± 0.68 |
|                       |                       | 6  |                | 1        | 8.92 ± 0.52 |
| Combined              | 0.1 M NaOH ↓ 0.05 M HCl | 7  | 65             | 1        | 5.38 ± 0.60 |
| Control               | -                     | 7  | 65             | 1        | 3.97 ± 0.14 |

All values are the mean ± standard error of three replicates. Control samples were extracted without any pretreatments. * Significantly different (p < 0.05) compared with 1 and 3 h.

Combined pretreatment produced lower intensities of gelatin bands with higher intensities of low molecular-weight bands in SDS-PAGE and lower yields (5.38%), when compared with HCl and NaOH pretreatments (Figure 1c and Figure S1c and Table 1). Lower yields might have been caused by over hydrolysis and loss of collagen by the combined pretreatment (NaOH → HCl). The control samples without pretreatment had the lowest gelatin bands (Figure 1d and Figure S1d) and the lowest yield (3.97%, Table 1). Thus, optimized acid and alkaline pretreatments increase the efficiency of gelatin extraction.

In summary, the optimum pretreatment period was determined as 1 h in both acid and alkaline pretreatments. Combined pretreatment was not recommended. The optimum pretreatment period does not lead a loss of gelatin, but over-pretreatment evokes loss of gelatin and a decrease in yield in both acid and alkaline pretreatments.

3.3. Determining the Optimum Extraction Conditions

Extraction processes such as extraction pH, temperature and time strongly affect the yield and quality of gelatin [9]. The effects of extraction conditions were assessed based on the intensities of the α-, β-, and γ-bands of gelatin in SDS-PAGE (Figure 2 and Figure S2) and the yields (Table 2) for type A and type B gelatins. Gelatin is a denatured, uncoiled form of collagen but sometimes contains hydrolyzed collagen (peptides) and other substances simultaneously extracted from the material tissues. Therefore, the intensities of α-, β-, and γ-bands in SDS-PAGE reflect amount of un-hydrolyzed, complete form of gelatin, which shows gelatin purity of the extracts.
Table 2. Yields (% dry weight gelatin/wet weight sample) of gelatins extracted from the head skin of Kalamtra sturgeon using different extraction pH, temperature (°C), and time (h).

| Factors          | Gelatins | Extraction Conditions | Yields (%) |
|------------------|----------|-----------------------|------------|
|                  | pH       | Temperature (°C) | Time (h) |                 |
| Type A           | 2        | 65                   | 1         | 5.22 ± 0.17 b    |
|                  | 6        | 6.99 ± 0.54 a,b      |           | 5.04 ± 0.22 b    |
|                  | 7        | 8.17 ± 0.25 a,b      |           | 5.24 ± 0.79 a,b  |
|                  | 8        | 8.41 ± 0.28 a,b      |           | 7.74 ± 0.66 a    |
|                  | 12       | 8.23 ± 0.53 a,b      |           | 9.05 ± 1.15 a,b  |
| Extraction pH    | 2        | 10.60 ± 0.60 a       |           | 7.25 ± 0.25 b    |
| Type B           | 6        | 8.29 ± 0.41 a,b      |           | 8.29 ± 0.41 a,b  |
|                  | 7        | 8.17 ± 0.25 a,b      |           | 8.17 ± 0.25 a,b  |
|                  | 8        | 8.41 ± 0.28 a,b      |           | 8.41 ± 0.28 a,b  |
|                  | 9        | 8.23 ± 0.53 a,b      |           | 8.23 ± 0.53 a,b  |
|                  | 10       | 10.60 ± 0.60 a       |           | 9.05 ± 1.15 a,b  |
|                  | 12       | 10.60 ± 0.60 a       |           | 10.60 ± 0.60 a   |

Figure 2. Effects of the extraction pH (2–12) (a,b) and extraction temperature (35 °C–65 °C) and time (0.5 h–3 h) (c,d) on the intensities of α-, β-, γ-, and low molecular-weight (<100 k) bands of gelatin in the extracts in SDS-PAGE (7.5% gel). Gelatins were extracted from the head skin at different conditions after 0.05 M HCl for 1 h (Type A gelatin) or 0.1 M NaOH for 1 h (Type B gelatin). Each lane contained 1 µL extract. Columns and bars represent means and standard errors of three replicates, respectively. Different letters in each band indicate a significant difference (p < 0.05).
Table 2. Cont.

| Factors | Gelatins | Extraction Conditions | Yields (%) |
|---------|---------|-----------------------|------------|
|         |         | pH | Temperature (°C) | Time (h) | |
|         |         | 0.5 | 35 | 1 | 7.30 ± 1.30 a |
|         |         | 0.5 | 50 | 1 | 6.17 ± 0.70 a |
|         |         | 0.5 | 65 | 1 | 6.85 ± 1.22 a |
| Type A  |         | 3  | 35 | 1 | 7.26 ± 0.99 a |
|         |         | 3  | 50 | 1 | 6.24 ± 0.76 a |
|         |         | 3  | 65 | 1 | 7.56 ± 0.70 a |
|         |         | 3  | 50 | 3 | 6.18 ± 0.92 a |
|         |         | 3  | 65 | 3 | 7.08 ± 0.13 a |
|         | 8       | 0.5 | 35 | 1 | 8.07 ± 0.39 a |
|         | 8       | 0.5 | 65 | 1 | 8.99 ± 0.50 a |
| Type B  |         | 3  | 35 | 1 | 8.21 ± 0.54 a |
|         |         | 3  | 50 | 3 | 8.83 ± 0.60 a |
|         |         | 3  | 65 | 3 | 8.79 ± 0.56 a |
|         |         | 3  | 65 | 3 | 8.98 ± 0.11 a |

All values are the mean ± standard error of three replicates. Different letters in the superscript of each column show significant difference (p < 0.05) for each extraction process at different conditions.

3.3.1. Extraction pH

For type A gelatin, significantly thicker α-, β-, and γ-bands were obtained when extraction was conducted at pH 7 (Figure 2a and Figure S2a). The yield was the highest at pH 12 (7.74%), followed by pH 7 (6.99%, Table 2). The gelatin bands of type A were more extensively destroyed under both highly acidic and basic conditions than under neutral pH (Figure 2a and Figure S2a). The low molecular-weight bands were visible at pH 12 where β- and γ-bands were completely hydrolyzed (Figure S2a). This finding suggested that strong alkaline conditions more easily destroyed higher gelatin bands than acidic conditions. Thus, pH 7 was identified as the optimum pH for extracting type A gelatin. Previous studies on the effects of pH for the suitable extraction of fish type A gelatin have not been conducted, except for that by Díaz-Calderón et al. [17]. The authors found that gelatin extracted at pH 5 had a higher protein content and molecular weight than that extracted at pH 3. Most other studies [5,6,18] used neutral pH to extract type A gelatin, but comparisons with other pH levels were not shown.

In contrast, the strongest intensities of gelatin bands were recorded at pH 8 for type B gelatin (Figure 2b and Figure S2b). The yield was the highest at pH 12 (10.60%, Table 2). Similar to type A gelatin, type B gelatin also showed weak α-, β-, and γ-bands under both highly acidic and basic conditions (Figure 2b and Figure S2b). The low molecular-weight bands were stronger at pH 2 and 12 (Figure S2b). Similar to type A gelatin, β- and γ-bands were also completely hydrolyzed at pH 12. Thus, pH 8 was considered as the optimum condition to extract type B gelatin. The highest yield at pH 4, but the highest quality at pH 6–7 was reported in type B gelatin from skate skin [19]. This previous study also documented that highly acidic or basic extraction conditions destroy the gelatin bands.

In comparison, the band intensities of gelatin, especially those of β-bands, were higher in type A than type B (Figure 2a,b and Figure S2a,b). A tendency of higher α-band intensities than β-band intensities was clear in type B gelatin. This finding suggested that β-band was more strongly affected by alkali-pretreatment than acid-pretreatment. Moreover, the intensities of the low molecular-weight
bands were always lower in type A gelatin than type B, suggesting the higher quality of the type A gelatin.

3.3.2. Extraction Temperature and Time

In SDS-PAGE, a slight tendency towards dominant gelatin bands, especially in $\beta$-bands, were observed at 35 °C compared to 50 and 65 °C for type A gelatin (Figure 2c and Figure S2c). Effects of extraction time were small, except for the extraction at 65 °C, at which 3 h extraction clearly decreased the intensities of gelatin bands (Figure 2c and Figure S2c). The low molecular-weight bands were obvious when gelatin was extracted at 65 °C (Figure 2c and Figure S2c), suggesting that the quality of type A gelatin was low when extracted at this temperature.

In type B gelatin extraction, a tendency of stronger gelatin bands was observed at 50 °C (Figure 2d and Figure S2d). Moreover, the band intensities showed a tendency to increase as the extraction time extended at this temperature (Figure 2d and Figure S2d). The low molecular-weight bands were observed at all the examined conditions in type B gelatin extraction (Figure 2d and Figure S2d). The variation of extraction temperature between type A and type B gelatins might be due to pretreatment effects; the acid-pretreatment increases H$^+$ that promote water access to the tissues and evokes better swelling of the tissues [20], resulting in easier gelatin extraction at lower temperatures than alkali-pretreatment.

When the extraction temperature increased from 35 to 65 °C, the impact on gelatin yield was minimal (Table 2). However, slightly higher yields were obtained at lower temperatures for type A gelatin, and at higher temperatures for type B gelatin. This difference might be attributed to the effects of acid and alkali pretreatments. Compared to the alkali-pretreatment, the acid-pretreatment increases the loss and breakdown of collagen molecules, even at low concentrations and low temperature [14]. This may affect the extractability of gelatin at lower temperatures. The broken collagen fragments with non-collagenous fractions were probably absorbed into the acid solution during pretreatment; consequently, type A gelatin contained fewer low molecular-weight bands in SDS-PAGE (Figure S2c,d) and showed lower yields compared to type B gelatin.

Yields tended to increase when extraction time increased for both types of gelatin. The optimum extraction temperature and extraction time were 35 °C for 3 h for type A gelatin and 50 °C for 1 h for type B gelatin, in which relatively higher yields and stronger gelatin bands were recorded. Higher yields but weaker bands of gelatin, suggesting lower gelatin quality such as lower intensity of $\alpha$-, $\beta$- and $\gamma$-bands as well as higher content of low molecular-weight bands, were obtained when extraction temperature and time were increased in squid [21] and the skin of featherback [22].

Based on these experiments, the best extraction conditions were: (1) for type A gelatin, DW ($w/v$ 1:5) at 35 °C, pH 7 when stirring at 200 rpm for 3 h, and (2) for type B gelatin, DW ($w/v$ 1:5) at 50 °C, pH 8 when stirring at 200 rpm for 1 h.

3.4. Decalcification of Extracted Residues

After extracting the gelatin from the mixed tissues of the Kalamtra sturgeon, the residues were composed of a mixture of semi-soft (small cartilage pieces) and hard tissues (bones and scales). Decalcification methods for the re-extraction of gelatin were investigated. The $\alpha$- and $\beta$-bands of gelatin were significantly more intense after decalcifying the extracted residues with 0.05 M HCl for 3 h for both types of gelatin (Figure 3a,b and Figure S3a,b). The yields for both types of gelatin were also higher in the 3 h decalcified groups (1.64% in type A and 1.59% in type B, Table 3). In SDS-PAGE, no low molecular-weight bands were observed in either type of gelatin in the 3 h groups (Figure S3a,b); thus, gelatin did not seem to break down under this treatment. Thus, 3 h is the best decalcification period.
The optimized methods of tissue separation, pretreatment, extraction, decalcification, and re-extraction of type A and type B gelatin from the mixed tissues of Kalamtra sturgeon are summarized in Figure 4.

Table 3. Yields (% dry weight gelatin/wet weight sample) of gelatins after decalcification of extracted residues from the mixed tissue of the Kalamtra sturgeon head using 0.05 M HCl at different times.

| Gelatin | Decalcification Time (h) | pH | Temperature (°C) | Time (h) | Yields (%) |
|---------|-------------------------|----|------------------|----------|------------|
| Type A  | Control                 | 7  | 35               | 3        | 1.10 ± 0.02 |
|         | 1                       |    |                  |          | 1.53 ± 0.15 |
|         | 3                       |    |                  |          | 1.64 ± 0.03 * |
|         | 6                       |    |                  |          | 1.31 ± 0.17 |
| Type B  | Control                 | 8  | 50               | 1        | 0.95 ± 0.03 |
|         | 1                       |    |                  |          | 1.28 ± 0.13 |
|         | 3                       |    |                  |          | 1.59 ± 0.03 * |
|         | 6                       |    |                  |          | 1.26 ± 0.08 |

All values are the mean ± standard error of three replicates. Control samples were extracted without decalcification. * Significantly different from control group.

After re-extraction, the re-extracted residues were decalcified for 3 h and gelatin was extracted again. Although the yield was similar to the re-extracted samples, SDS-PAGE showed faint gelatin bands in both types of gelatin (data not shown). Thus, gelatin from the re-extracted residues was not suitable for future studies and applications.

The optimized methods of tissue separation, pretreatment, extraction, decalcification, and re-extraction of type A and type B gelatin from the mixed tissues of Kalamtra sturgeon are summarized in Figure 4.
Table 3. Yields (%, dry weight gelatin/wet weight sample) of gelatins after decalcification of extracted residues from the mixed tissue of the Kalamtra sturgeon head using 0.05 M HCl at different times.

| Decalcification | Extraction Conditions | Yields (%) |
|-----------------|-----------------------|------------|
| Time (h)        | pHTemperature (°C)    |            |
| 1               | 735                   | 1.10 ± 0.02|
|                 | 1                    | 1.53 ± 0.15|
|                 | 3                    | 1.64 ± 0.03*|
|                 | 6                    | 1.31 ± 0.17|
| 1               | 850                   | 0.95 ± 0.03|
|                 | 1                    | 1.28 ± 0.13|
|                 | 3                    | 1.59 ± 0.03*|
|                 | 6                    | 1.26 ± 0.08|

All values are the mean ± standard error of three replicates. Control samples were extracted without decalcification. *Significantly different from control group.

Figure 4. An overview of protocols used for the extraction and re-extraction of type A and type B gelatins from the head of Kalamtra sturgeon.

3.5. Final Extraction and Re-Extraction of Mixed Tissues

Using the optimized methods, type A and type B gelatins were finally extracted and re-extracted using the mixed tissues from the head of Kalamtra sturgeon. The final yields and band patterns of gelatin in SDS-PAGE are presented in Table 4 and Figure 5. The yield of type B extracted gelatin was higher than that reported in the previous type B gelatin extracted from tiger tooth croaker head (1.67%), whereas that of type A extracted gelatin was similar to that of the type A gelatin extracted from mackerel had (3.3–3.7%) [23,24]. Type B extracted gelatin had the highest yield, while type A extracted gelatin had the significantly strongest α- and β-bands than the others. The low molecular-weight bands were significantly higher in type B extracted gelatin. These results again indicate that type A extracted gelatin is of the best quality. In other words, type B gelatin may contain more non-gelatinous substances (like non-gelatinous proteins, lipids, sugar, and ash) and result in a higher yield. As the head is a mixture of collagenous and non-collagenous tissues, alkali pretreatment might allow more gelatinous and non-gelatinous materials to be extracted than in the acid pretreatment. This phenomenon might arise because the alkali-pretreatment lowers the isoelectric point and destroys the amide groups of proteins, resulting in a weaker collagen matrix [25] than the acid-pretreatment. However, acid- and
alkali-pretreatments affect the physicochemical characteristics and purity of gelatin is now under investigation. The quality of type A extracted gelatin, indicated from the low molecular-weight bands in SDS-PAGE, was better than that of type A gelatin extracted from mackerel had [24].

Table 4. Yields (%a, Dry weight gelatin/wet weight sample) of gelatins extracted from the head of Kalamtra sturgeon.

| Gelatins  | Extraction (%) | Re-Extraction (%) | Total (%) |
|-----------|----------------|-------------------|-----------|
| Type A    | 3.42 ± 0.11    | 1.59 ± 0.11       | 5.01 ± 0.02 |
| Type B    | 5.71 ± 0.06 *  | 1.54 ± 0.06       | 7.25 ± 0.11 * |

*Significantly different from the value of type A gelatin (p < 0.05).

![Figure 5. SDS-PAGE (7.5% gel) patterns (a) and the intensity of α-, β-, and γ- and low molecular-weight (<100 k) bands (b) of gelatins extracted from the head of Kalamtra sturgeon. M, molecular marker; 1, type A extracted gelatin (AE); 2, type A re-extracted gelatin (ARE); 3, type B extracted gelatin (BE); and 4, type B re-extracted gelatin (BRE). All gelatins were loaded as 10 µg/lane. Positions of α1-, α2-, β-, and γ-bands of gelatin were shown by arrows in (a). Columns and bars represent means and standard errors of three replicates, respectively, in (b). Different alphabets in each band show significant difference (p < 0.05), in (b).](image)

The yields of type A and type B re-extracted gelatins were lower than those of the extracted gelatins but were similar to those of perch bone (1.3–2.4%) [26]. The intensity of gelatin bands of re-extracted gelatins was lower than that of type A extracted gelatin (Figure 5); thus, the quality of re-extracted gelatins was likely to be lower than extracted gelatins.

The total yields (extraction + re-extraction) of type A (5.01%) and type B (7.25%) gelatins from the sturgeon head in the current study were higher than those previously reported for fish head gelatins; examples include 1.67% type B gelatin in tiger tooth croaker head [23] and 3.3–3.7% type A gelatin in mackerel head [24]. In contrast, the present yields were close to those of fish skin type A gelatins of pink perch and croaker (5.57–7.56%) [27] but lower than that of the leatherjacket fish (5.23–11.54%) [15] and sturgeon (5.04–24.11%) [4–6]. As the skin is the most dominant collagenous tissue, it is logical that the sturgeon head had lower gelatin yields than sturgeon skin. However, the head is the major by-product of commercial sturgeon, as its weight is about three times greater than that of skin. Thus, the sturgeon head is a suitable raw material for extracting both high-quality type A and high-yield type B gelatins at an industrial scale. Consequently, this novel extraction method from the head could open new opportunities for by-product utilization and enhance the economic benefits of aquaculture farming of sturgeon.
4. Conclusions

This study developed a novel optimized process of gelatin extraction from an unutilized resource: the head of Kalamtra sturgeon fish. A three-step method, tissue separation, acid/alkaline pretreatment, and extraction, was employed (Figure 4). In tissue separation, incubation of head at 65 °C (3–3.5 h) is essential to make the head soft and manipulable for gelatin extraction. The yields were 5.01% in type A gelatin and 7.25% in type B gelatin, both of which were higher than the yields reported for the gelatins of the heads of other fish species but were similar to those reported for the skin of other fish species. The quality of gelatin, indicated from the intensities of gelatin bands and low molecular-weight bands in SDS-PAGE, were also higher. Such high yields and high quality are the value-added points of the present method. However, the re-extraction yield was low; thus, only type A and type B extracted gelatins were recommended for industrialization. Also, the remaining residues after extraction still have a possibility to use in the future for zero discard. The optimized conditions developed by the current study could promote the use of fish head by-products and the aquaculture of sturgeon, contributing to the Sustainable Development Goals adopted by all United Nations Member States in 2015. However, for the industrialization of the sturgeon head gelatin, simplification of each step adapting the method to large-scale gelatin extraction is required. In addition, further studies on the purity, properties, and functionality of both type A and type B gelatins are essential, and they are now in progress in this research group.

**Supplementary Materials:** The following are available online at [http://www.mdpi.com/2076-3417/10/19/6660/s1](http://www.mdpi.com/2076-3417/10/19/6660/s1).

Figure S1: SDS-PAGE (7.5% gel) patterns of gelatin extracted (65 °C, 1 h, pH 7) from the head skin after being pretreated with (a) 0.05 M HCl, (b) 0.1 M NaOH, (c) the combined pretreatment (NaOH → HCl), and (d) without any pretreatments as the control. M, molecular maker, p, pretreated gelatin-extracted solution (1 µL/lane); S, supernatant of the pretreated solution (5 µL/lane); and WP, gelatin-extracted solution (1 µL/lane) without any pretreatments. Pretreatment time is shown in the subscripts. Positions of α1-, α2-, β-, and γ-bands of gelatin are shown by arrows. Figure S2: SDS-PAGE (7.5%) patterns of type A (a,c) and type B (b,d) gelatins after extracted at different pH (a,b, extracted at 65 °C for 1 h), and temperature and time (c,d) using the head skin. M, molecular maker; pH, extraction pH was shown in subscripts; and 35, 50 and 65, extraction temperature where time was shown in subscripts. All extracted gelatins were loaded as 1 µL/lane. Positions of type I collagen α1-, α2-, β-, and γ-bands were shown by arrows. Figure S3: SDS-PAGE (7.5%) patterns of type A and type B re-extracted gelatins after the decalcification of extracted (a,b) residues with 0.05 M HCl, and their supernatant at different times. M, molecular maker; WD, gelatin-extracted solution (3 µL/lane) without any decalcifications as the control; D, gelatin-extracted solution (3 µL/lane) after decalcification; and S, supernatant of the decalcified solution (5 µL/lane). The decalcification time is shown in the subscripts. Positions of α1-, α2-, β-, and γ- bands of gelatin are shown by arrows.

**Author Contributions:** Conceptualization, M.R.I. and Y.T.; methodology, M.R.I. and T.Y.; software, M.R.I.; data curation, M.R.I.; writing—original draft preparation, M.R.I.; writing—review and editing, M.R.I.; Y.T. and K.U.; supervision, Y.T. and K.U.; funding acquisition, Y.T. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was partly supported by a Grant-in-Aid for Scientific Research (B) 18H02273 from the Japan Society for the Promotion of Science.

**Conflicts of Interest:** The authors declare no conflict of interest.

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