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Extraction of Type I Collagen from Tilapia Scales Using Acetic Acid and Ultrafine Bubbles

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Abstract: Type I collagen is commonly used in medical materials and cosmetics. While it can be extracted from the skin and bones of mammals, marine collagen has attracted attention recently, since the use of mammalian collagen could result in zoonosis, and products containing mammalian collagen are avoided due to some religious beliefs. Chemical extractions using strong acids and alkalis, thermal extractions, and other nonconventional methods have been used for collagen extraction. However, there are few reports on environmentally friendly methods. Although heat extractions provide higher yields of collagen, they often cause collagen denaturation. Therefore, dilute acetic acid and ultrafine bubbles of oxygen, carbon dioxide, and ozone were used to extract type I collagen from tilapia scales. The extraction performance of the different conditions employed was qualitatively analyzed by SDS-PAGE electrophoresis, and the collagen concentration was quantified using circular dichroism spectroscopy by monitoring the peak intensity at 221 nm, which is specific to the triple helix of type I collagen. Collagen was extracted from tilapia scales with a yield of 1.58% by the aeration of ultrafine bubbles of carbon dioxide gas in a 0.1 M acetic acid solution for 5 h.

Keywords: collagen; circular dichroism; tilapia scales; ultrafine bubbles; Sirius Red

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1. Introduction

The extracellular matrix is commonly defined as noncellular component tissue that provides both biochemical and essential structural support to its cellular constituents [1]. Collagen is an extracellular protein that accounts for 25–30% of the total protein content within the human body [2]. In fact, most invertebrates [3] and vertebrates [4] are rich in collagen. Collagen is actively involved in cell differentiation and proliferation, and is synthesized in the human body by fibroblasts and osteoblasts [5,6]. Human skin is primarily composed of type I collagen (about 75–80%), though it also contains a small amount of type III collagen [7]. Type I collagen is also present in tendons and ligaments [8,9], while the majority type found in human cartilage is type II collagen [10]. Furthermore, collagen is present in other mammals, such as those belonging to bovine and porcine species [4], and in fish. Based on the organism of origin, collagen is classified as either mammalian or marine; however, the triple-helix structure consisting of three polypeptide chains containing repeating units of three amino acids—$(Gly\text{-}Pro\text{-}Xaa)_n$—is common to both mammalian and marine collagen [11]. The levels of proline and hydroxyproline in the collagen of different fish species vary significantly depending on the environmental temperature in which the fish lives, as the amounts of these amino acids affect the thermal stability of the collagen [12,13]. Collagen also has a slightly different amino-acid composition depending on its site of origin. As a result, 28 different types of collagen have been identified thus far, the most common types being types I–V, and can be categorized by their macromolecular structures [14–16]. For example, the type I collagen molecule has a triple-helix structure composed of two α1 chains and one α2 chain. A cross-link between two α chains is called a β chain, which is a dimer of chains, while a cross-link between three chains is called a γ chain (trimer) [17]. From the perspective of materials science, collagen is one of the most common polymeric biomaterials and is used as such in a wide variety of

fields ranging from foods to cosmetics to drugs [18,19]. Compared to synthetic polymers, collagen has a high biological affinity, prompting its recent use in the field of regenerative medicine as a scaffolding material for cell culturing [20,21]. Type I collagen is the most abundant collagen type and is suitable as a raw material for cosmetics and biomaterials. The main source of type I collagen is bovine or porcine dermis [4], although, type I collagen of fish origin, commonly extracted from Atlantic cod [22], salmon [23], sea bream [24], and tilapia [25,26], has recently attracted attention. Marine collagen is inexhaustible and is safer than mammalian collagen because it is not exposed to zoonotic diseases. Furthermore, employing discarded fish scales from food processing for the extraction of type I collagen is a particularly attractive means to effectively recycle organic resources and limit waste. However, most of the methods for extracting type I collagen from fish scales are enzymatic [26,27], require heat [28], or use chemical reagents [29], resulting in a mixture of gelatin (denatured collagen) and collagen. Although the enzymatic method is convenient, it is not suitable for processing large amounts of raw materials due to the cost of enzymes. While chemical reagents are cheaper, some substances can be harmful or toxic. Recently, a collagen extraction method using pressurized CO_2 gas has been reported [30], and more environmentally friendly extraction methods are also gaining increasing attention.

Therefore, to develop an environmentally friendly method for extracting collagen from fish scales, the present work employed a combination of dilute acetic acid (0.1 M and 0.5 M) with ultrafine bubbles in place of conventional, toxic substances like hydrochloric acid and sodium hydroxide. Gas-based extraction may be useful in chemical-effluent treatment processes, as it does not require the removal of chemicals and enzymes. Furthermore, oxygen and ozone gas are strong oxidizers, with the former being easily soluble in water, and carbon dioxide gas can acidify aqueous solutions. Therefore, the effectiveness of each of these three gases for collagen extraction were determined and compared. Ultrafine bubbles, or nanobubbles, are defined as gas-filled spherical bubbles with diameters of less than 1000 nm. The International Standards Organization is currently evaluating standards for ultrafine bubbles (ISO/TC 281) [31], and many details related to the mechanism of ultrafine bubbles remain unresolved. However, various application technologies, such as sterilization [32] and material decomposition [33], have been developed by applying cavitation generation. Studies on the extraction of collagen with dilute acetic acid solutions have been previously reported [5,34–37], but an extraction method using a combination of dilute acetic acid and ultrafine bubbles has not been reported thus far. In this study, type I collagen was extracted from tilapia scales using dilute acetic acid in combination with ultrafine bubbles formed using different gases. An improved collagen quantification method involving circular dichroism (CD) spectroscopy was also developed, which was used to assess the extracted type I collagen.

2. Materials and Methods

2.1. Materials

Acid-demineralized tilapia (*Oreochromis niloticus*) scales were provided by the Shinryo Corporation (Fukuoka, Japan) for use in this study. General reagents were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). WIDE-VIEW™ Prestained Protein Size Marker III from FUJIFILM Wako Pure Chemicals (Osaka, Japan) was used as a molecular-weight marker for electrophoresis. The β-mercaptoethanol sample treatment for Tris-SDS, the running buffer for SDS-Tris-glycine (10X), and Page Blue 83 Stain Reagent (CBB-R250) were purchased from Cosmo Bio Co. The running buffer for SDS-Tris-glycine was diluted from 10X to 1X before use. The final concentration of the running buffer (1X) was 25 mM Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4. Ready-to-use gel MULTIGEL® II mini 7.5, 13 W (7.5% gel) from Cosmo Bio Co., Ltd. was used for SDS-PAGE. The quantitative analysis of collagen was conducted using the Sirius Red Total Collagen Detection Kit from Chondrex, Inc., (Woodinville, WA, USA). Nitrogen, carbon dioxide, and oxygen gases were obtained from Fukuoka Oxygen Corporation (Fukuoka, Japan). Cellcampus AQ-03A (from

tilapia scales, 0.3% type I collagen solution, Taki Chemical Co., Ltd.) was used to prepare the calibration curve for the CD analysis.

2.2. Extraction of Collagen from Fish Scales

The acetic acid solution (5 L, 0.1 M or 0.5 M) was added to 125 g of tilapia scales, and the immersed scales were then stored at 25 °C for 12 h, during which neither mechanical stirring nor gas-aeration treatment were performed. The scales were then supplied with O_2, CO_2, or O_3 in the form of ultrafine bubbles at a gas flow rate of 3 L/min for 6 h. Each sample was monitored every hour for up to 6 h at 25 °C. An ozone cube, SK-W005L, manufactured by Shoken Corporation (Hiroshima, Japan) was used to generate ozone. In addition, ultrafine bubbles were generated using a small, desktop-model ultrafine bubbler (0.1 kW) manufactured by ZEC FIELD.INC (Fukuoka, Japan). To separate the scales from the solution after the reaction, natural filtration was performed with a nonwoven cloth commonly used for food preparation. The filtrate samples were immediately stored at −20 °C and then analyzed after thawing.

2.3. SDS-PAGE of Extracted Collagen Samples

SDS-PAGE analysis employing the Laemmli method [38] was used to estimate the molecular weight of the proteins present in the marine collagen isolated from the tilapia scales. The pH values of the samples were adjusted to neutral pH by adding the appropriate amount of 1.0 M NaOH. The sample solution was mixed with β-mercaptoethanol solution of the reducing reagent in a ratio of 1:1 (*v*/*v*) and heated at 95 °C for 10 min to completely denature the sample. The Cassette Electrophoresis Unit Model DPE-1020 from Cosmo Bio Co., Ltd. was used. The sample solutions (10 μL) and 10 μL of protein marker were loaded onto the gel, and electrophoresis was performed at 30 mA for 1 h. The gels were stained with a Page Blue 83 Stain Reagent (CBB-R250) solution for 1 h and then de-stained via shaking in a solution containing water, methanol, and acetic acid (875:50:75, *v*/*v*/*v*).

2.4. Quantitative Analysis of Soluble Collagen Using the Sirius Red Total Collagen Detection Kit

The quantitative analysis of soluble collagen was performed according to the manual of detection kit. In a microtube, 100 μL of the extraction sample described in Section 2.2 and 900 μL of ultrapure water were mixed. Next, 500 μL of Sirius Red solution included in the kit was added to the mixed solution, and the tubes were stirred in a vortex mixer and then stored at room temperature for 20 min. After centrifugation (10,000 rpm, 3 min, 4 °C), the supernatant was removed, and the red pellet at the bottom of the tube was stored. A washing solution (500 μL) was added to the pellet, which was then stirred in a vortex mixer before being re-suspended in the washing solution. After centrifugation (10,000 rpm, 3 min, 4 °C), the supernatant was removed. The extraction buffer (250 μL) was added to the pellet, which was completely dissolved using a vortex mixer. The absorbance of the solution was measured at 540 nm using a UV-vis spectrophotometer. The standard of the detection kit, Bovine type I collagen (0.5 mg/mL), was used to construct a calibration curve for calculating the sample concentrations.

The yield of type I collagen by the Sirius Red method was calculated according to the protocol supplied with the collagen detection kit; likewise, a calibration curve was prepared. Using the calibration curve, the weight concentration (g/mL) of type I collagen per 1 mL of the acetic acid-extracted sample solution was calculated. In addition, the yield (%) based on the weight of the raw material (dried tilapia scales) was calculated using the following formula:

$$\text{Yield (\%)} = [\text{concentration of collagen (g/mL)} \times \text{total volume of 0.1 M acetic acid (mL)/total weight of immersed fish scales (g)}] \times 100 \quad (1)$$

Here, acetic acid refers to the aqueous acetic acid solution used in the preparation of the immersion. The 0.1 M solution is listed here, but the calculation when using 0.5 M acetic acid is the same. The denominator of this equation is the total weight of the scales

immersed in acetic acid, and the numerator is the product of the sample concentration and the total volume of the acetic acid immersion solution.

2.5. Circular Dichroism Measurements

To analyze the secondary structure of collagen solubilized in acetic acid, CD measurements were performed using a J-805 CD spectrophotometer (JASCO Corp., Tokyo, Japan) at 25 °C. Measurements were obtained at wavelengths of 200–250 nm, and a cylindrical quartz cell with an optical path length of 1 mm was used. Due to the large baseline associated with the 0.5 M acetic acid solution, only the 0.1 M acetic acid solution samples were measured.

3. Results and Discussion

3.1. Analysis of Collagen by SDS-PAGE Profiles

The electrophoretic pattern of collagen exhibited two thick bands and two thin bands. There are several trimers of α chains in tilapia skin that are $(\alpha1)_2\alpha2$ type [39]. The α1 and α2 bands are 136 and 127 kDa, respectively [40], and the band intensity of α1 is twice that of α2, indicative of type I collagen. One of the three chains that comprise the collagen triple helix is called the α chain and has a molecular weight of approximately 100–130 kDa. Other bands representing γ chains (300 kDa) and β chains (200 kDa) were also identified, further supporting the presence of type I collagen. A γ chain is a heterotrimer formed from two α1 chains and one α2 chain, and a β chain is a dimer of α chains. These data are in agreement with the results obtained from tilapia, carp [41], zebrafish [42], and other fish species [24].

When comparing the gas types, thick bands were observed for oxygen and carbon dioxide, with both gases efficiently extracting collagen after 1 h of aeration, as shown in Figure 1a,b. However, no thick bands were observed with ozone, suggesting that collagen was not extracted by ozone gas, as indicated in Figure 1e,f. The thin band observed at 0 h may have been decomposed by the strong oxidizing power of ozone. When evaluating the effect of acetic acid concentration on the effectiveness of extraction, it is clear that more collagen was extracted in a shorter amount of time by 0.5 M acetic acid than by 0.1 M acetic acid. The bands became thicker as the gas aeration time increased, indicating that a larger amount of collagen was being extracted. However, it is difficult to determine from the SDS-PAGE profile, alone, whether it is gelatin or collagen. [43] In general, SDS-PAGE band images provide only a qualitative evaluation; therefore, other methods are needed to quantitatively analyze collagen extraction.

3.2. Evaluation of Collagen Extraction Rate Using the Sirius Red Total Collagen Detection Kit

The Sirius Red method was used to determine the content of the extracted collagen. This method for the quantitative estimation of collagen was first reported by Marotta et al. [44]. Sirius Red is also frequently used to distinguish between type I and type III collagen in tissue sections. According to previous reports, type I to type III collagen in fish, amphibian, reptile, bird, and mammalian organs can be stained by Sirius Red and identified using polarized light microscopy experiments [45]. Picrosirius Red, a mixture of picric acid and Sirius Red, also stains collagen fibrils in cellular tissue [46]. Specifically, the sulfonate groups of the Sirius Red molecules bind to the side chains of the basic amino acids of the collagen [47].

Figure 2 summarizes the yield of collagen extracted with respect to the time each gas was supplied as ultrafine bubbles. When 0.5 M acetic acid and CO_2 were used, the yield increased to approximately 0.2% after 0 h and 0.4% after 1 h, but the yield rapidly decreased after bubbling for longer periods of time. When 0.1 M acetic acid was used, the yields of collagen extracted in the presence of CO_2 and O_2 gases after 0 h and 1 h were approximately 0.1%, exhibiting lower yields than those obtained with 0.5 M acetic acid. In addition, the yield of type I collagen decreased further with prolonged bubbling. The results of the Sirius Red method did not agree with the results of the SDS-PAGE profile, where the bands had become thicker with increasing bubbling time. The reason for the decrease in yield was

presumed to be due calcium ions derived from the fish scales. The fish scales used as the raw material in this study were demineralized products. However, it was inferred that any calcium remaining in the scales was eluted by gas bubbling. Therefore, it is likely that a large excess of calcium ions leached from the scales inhibited the binding of collagen to the sulfonic acid groups of the Sirius Red molecules. Although type I collagen may have been extracted as the reaction time increased, the apparent yield decreased with time, owing to the large excess of calcium ions present.

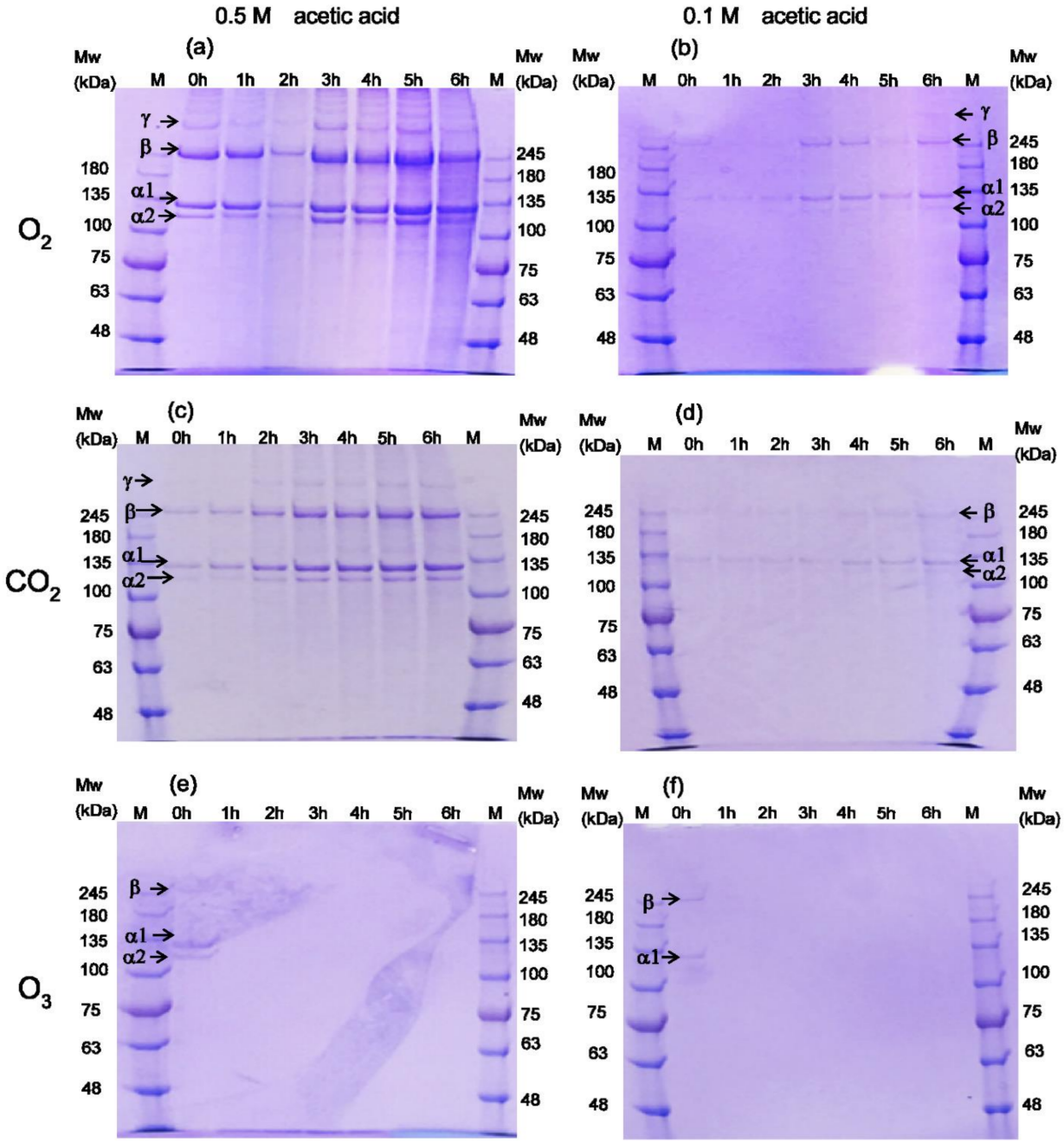

Figure 1. SDS-PAGE analysis of collagen extracted from tilapia scales using different acetic acid concentrations and gases: (**a**) 0.5 M acetic acid, O_2; (**b**) 0.1 M acetic acid, O_2; (**c**) 0.5 M acetic acid, CO_2; (**d**) 0.1 M acetic acid, CO_2; (**e**) 0.5 M acetic acid, O_3; and (**f**) 0.1 M acetic acid, O_3. The outer lanes are markers, and the inner lanes labeled from 0 to 6 h indicate the gas bubbling time. The α1, α2 chains, β chain, and γ chain are shown in panel (**a**–**c**). The α1, α2 chains and β chain are shown in panel (**d**,**e**). The α1 chains and β chain are shown in panel (**f**).

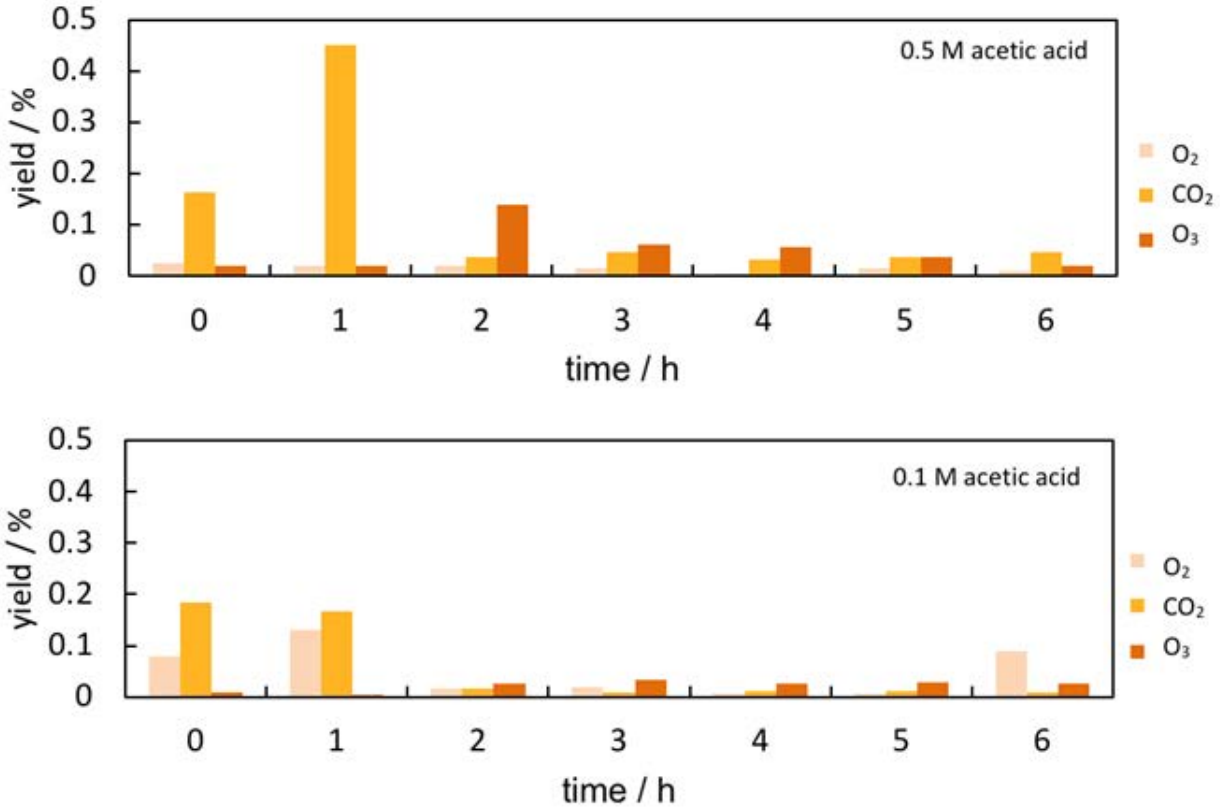

Figure 2. Yield of collagen extracted from tilapia scales using different acetic acid concentrations and gases. Yields were calculated using Equation (1), with concentration values obtained by the Sirius Red method.

3.3. Yield of Collagen from Tilapia Fish Scales Determined by CD Spectral Change

Figure 3a shows the CD spectrum of the aqueous collagen standard from the tilapia scales. The CD spectrum of type I collagen exhibits a positive maximum at 221 nm and a negative minimum at 198 nm. This CD spectral pattern was derived from the triple-helix structure of type I collagen and other collagen types [35,48]. Collagen is denatured by heating, where the triple helix becomes unstable and changes to gelatin, which results in the CD spectral pattern changing from that of collagen to that of gelatin. The CD values at 221 nm for the collagen concentrations of the standards are plotted in Figure 3b. The CD value at 221 nm increased linearly with increasing collagen concentration. From the linear approximation of Figure 3b, the concentration of triple-helical collagen was calculated using Equation (2).

$$\text{Concentration of collagen (mg/mL)} = \text{CD}_{221}/84.468 \quad (2)$$

Figure 4 shows the CD spectra of the samples extracted using 0.1 M acetic acid and each gas type. However, the absorption wavelength of dissolved collagen could not be detected in 0.5 M acetic acid because of the strong absorption of acetic acid. For this reason, we measured CD spectra of samples extracted only under 0.1 M acetic acid conditions. Comparing each spectral pattern, the spectrum of the collagen triple helix was most similar in the case of CO_2. The spectral intensity increased with the aeration time of the gas. On the other hand, the spectrum obtained when O_2 was used was weaker than that obtained with CO_2. In the case of O_3 gas, the spectral pattern was similar to that observed for the random structure of normal proteins. Thus, it is possible that collagen may have been transformed into gelatin in the presence of O_3 or fragmented into peptides.

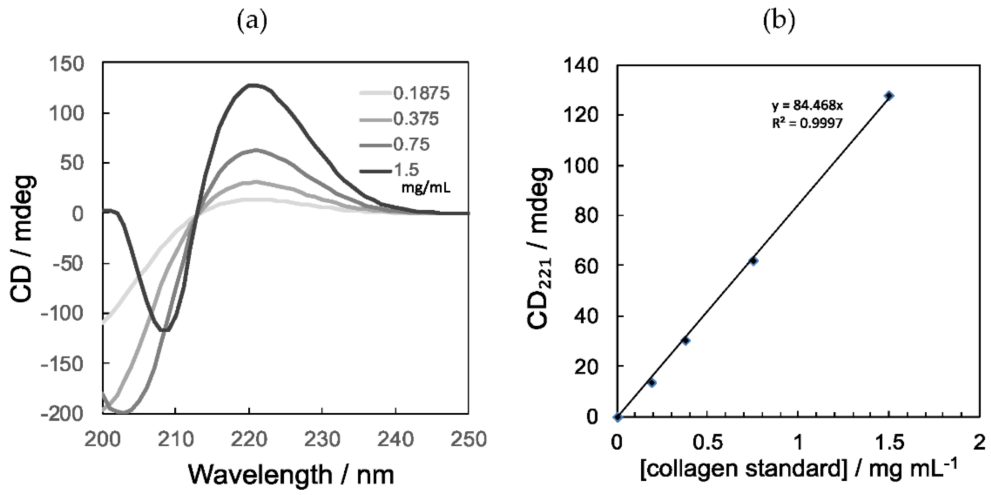

Figure 3. (**a**) Variation in the circular dichroism (CD) spectral pattern as the concentration of commercial, standard type I collagen changes; (**b**) Plot of the CD maxima (mdeg) at 221 nm against the collagen concentration of the standard sample, and the resulting equation of the linear approximation.

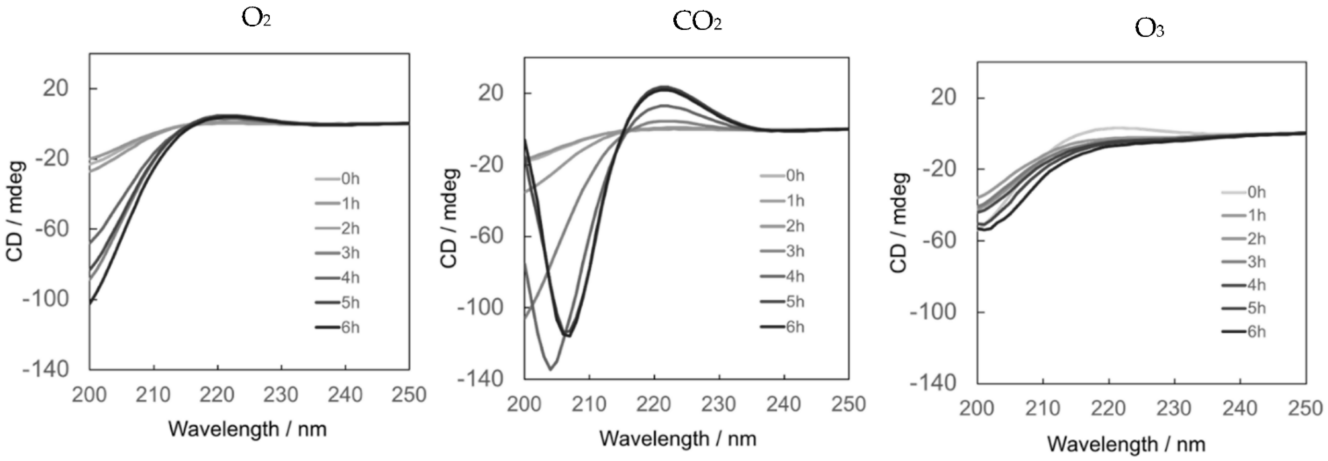

Figure 4. Changes in the circular dichroism (CD) spectral pattern of the collagen solution extracted with 0.1 M acetic acid over the course of 6 h when supplying ultrafine bubbles of each gas species.

The results of these CD spectral patterns are consistent with the results of the SDS-PAGE analysis. Equation (2) was used to calculate the concentration of collagen (g/mL). Furthermore, this value of collagen concentration was applied to Equation (1). The yield (%) of collagen extracted from dried tilapia fish scales immersed in dilute acetic acid was thus obtained. The results are shown in Figure 5. In particular, it was found that optimal collagen extraction could be achieved by continuous gas bubbling with CO_2 for 5 h. Other researchers have reported that the yield of collagen from marine sponges extracted in a high-pressure reactor with carbon dioxide ranged from 9.0% to 17.3%; however, the purity was low, containing approximately 50% gelatin [34]. On the other hand, Sousa et al. [30] used CO_2 gas under high pressure to extract collagen from the skin of Atlantic cod. The yield was 13.8%, and only type I collagen was obtained. Although the yields from these studies are higher than those presented here, it is difficult to compare fish scales and marine sponges, because marine sponges contain lower levels of calcium than fish. Another researcher shredded tilapia scales and extracted them by immersion in 0.5 M acetic acid, resulting in a 3.2% yield of collagen [49]. This method gave a yield approximately two times greater than that obtained by the method presented here. However, this previously reported pre-shredding method may have resulted in some collagen being transformed into gelatin by

the frictional heat produced during the shredding process. Therefore, employing ultrafine bubbles of CO_2 may be more suitable than physically cutting scale tissue for extracting collagen, as it preserves the collagen structure while minimizing losses to gelatin formation.

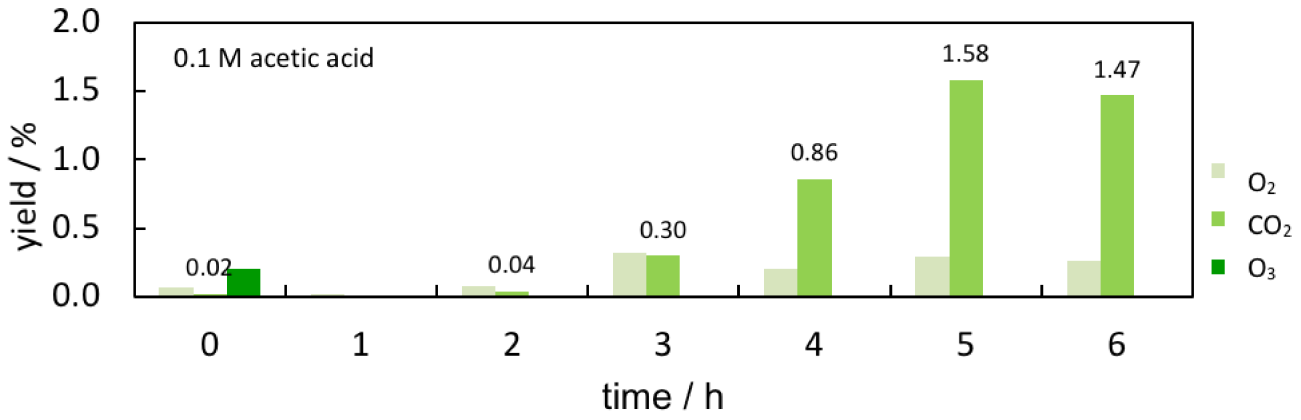

Figure 5. Yield of collagen extracted from tilapia scales using 0.1 M acetic acid and different gases. Yields were calculated using Equations (1) and (2) based on the concentration values obtained from circular dichroism (CD) values at 221 nm.

4. Conclusions

In this experiment, type I collagen was extracted from tilapia scales by bubbling ultrafine bubbles of three separate gases (O_2, CO_2, and O_3) into an acetic acid solution. SDS-PAGE analysis of the extracted samples displayed bands indicating the presence of three chains of type I collagen, namely α1, α2, and β, when O_2 and CO_2 were used. The thickness of these bands increased with the aeration time of the ultrafine bubbles. Since SDS-PAGE does not provide quantitative results, we decided to obtain the concentration of type I collagen directly from the extraction solution. In order to determine the extraction efficiency, the concentration of type I collagen in the extraction solution was checked using the Sirius Red Total Collagen Detection Kit; however, calcium ions in the scales likely interfered with the binding of collagen chains to Sirius Red. Therefore, CD spectroscopy was used to evaluate the change in the peak value of the absorption maximum at 221 nm, which is characteristic of type I collagen. As a result, it was shown that the use of ultrafine bubbles of CO_2 in a 0.1 M acetic acid solution was the most effective method to obtain type I collagen in a relatively high yield (1.58%).

Using these optimal conditions, we first extracted collagen chains forming the characteristic triple-helix structure from dried tilapia scales. The extracted samples were then directly analyzed via CD, and the amount of type I collagen forming a triple helix was calculated from the change in the CD value at 221 nm. If denatured gelatin were present in the samples, those bands might also have been identified. Although the Sirius Red method for the quantification of type I collagen was inadequate for this system, likely due to the presence of calcium ions from the tilapia scales, the CD spectral method that was employed was able to quantify the amount of collagen chains forming the triple-helix structure based on the calibration curve of the standard sample. This collagen quantification method by CD is suitable for the analysis of large-scale extraction samples. Furthermore, if calcium-ion chelating agents such as EDTA are added, collagen extracted from scales may also be quantifiable using the Sirius Red method. However, further investigation is needed to determine how EDTA affects the conformation of the triple helical structure of collagen. As reported by Seixas et al., type II collagen was extracted without affecting the triple helix conformation after demineralization of cartilage [50].

Therefore, a simple, mild, cost-effective, and environmentally friendly method for extracting type I collagen from inedible Tilapia scales was developed, which obtained collagen in 1.58% yield after 5 h. Type I collagen can be used in pharmaceuticals and cosmetics, and the use of dilute acetic acid and carbon dioxide ultrafine bubbles for its

extraction ensures that these collagen-containing products are free of harsh chemicals. Furthermore, the use of processed fish scales promotes resource recycling, making this extraction method more environmentally friendly than other methods while obtaining a reasonable yield of type I collagen. In the future, the author plans to investigate the effects of the flow rate and diameter of the CO_2 ultrafine bubbles on the yield of collagen, as well as to clarify the extraction mechanism.

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Data Availability Statement: Data from this study can be made available upon request.

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