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Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp (*Ctenopharyngodon idella*) skin

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ABSTRACT

This study investigated the effects of alkaline pretreatments and acid extraction conditions on the production of acid-soluble collagen (ASC) from grass carp skin. For alkaline pretreatment, 0.05 and 0.1 M NaOH removed non-collagenous proteins without significant loss of ASC at 4, 10, 15 and 20 °C; while 0.2 and 0.5 M NaOH caused significant loss of ASC, and 0.5 M NaOH caused structural modification of ASC at 15 and 20 °C. For acid extraction at 4, 10, 15 and 20 °C, ASC was partly extracted by 0.1 and 0.2 M acetic acid, while 0.5 and 1.0 M acetic acid resulted in almost complete extraction. The processing conditions involving 0.05–0.1 M NaOH for pretreatment, 0.5 M acetic acid for extraction and 4–20 °C for both pretreatment and extraction, produced ASC with the structural integrity being well maintained and hence were recommended to prepare ASC from grass carp skin in practical application.

1. Introduction

Collagen, with the unique right-handed triple superhelical structure, is the major structural proteins in both vertebrates and invertebrates, and it has been widely used in the food, pharmaceutical and cosmetic industries because of its excellent biodegradability and biocompatibility, and weak antigenicity (Liu, Li, Miao, & Wu, 2009; McCormick, 2009). Traditionally, collagen has been isolated from the skins and bones of land-based animals, such as cows, pigs and poultry. In recent years, the outbreaks of bovine sponge encephalopathy, foot-and-mouth disease and avian influenza have induced concerns among some consumers of these traditional collagen and collagen-derived products. In addition, porcine collagen and collagens from other animals that are not religiously slaughtered are unacceptable to develop kosher and halal food products (Regenstein, Chaudry, & Regenstein, 2003; Regenstein & Zhou, 2007). Therefore, there has been great interest regarding the use of fish processing by-products, such as skins, scales, bones and swim bladders, as the potential alternative sources of collagen (Duan et al., 2012; Kaewruang, Benjakul, Prodpran, & Nalinanon, 2013; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Liu, Liang, Regenstein, & Zhou, 2012; Nikoo et al., 2014; Niu et al., 2013).

The sensitivity of collagen to temperature is associated with its superhelical structure that is maintained by the conformational restrictions imposed by the pyrrolidine rings of the imino acids (hydroxyproline and proline). Moreover, the superhelical structure of collagen is also strengthened by the inter-chain hydrogen bonds formed through the hydroxyl group of hydroxyproline (Nagai, Suzuki, & Nagashima, 2008). Generally, collagens from warm-water fish have higher imino acid contents than those from cold-water fish, indicating that warm-water fish collagens possess higher heat resistance and greater structural stability. Grass carp is one of the "four major cultured fresh-water fish species" in China, and the total imino acid content and the corresponding thermostability of collagen from this fish were reported to be comparable to those of collagens from other warm-water fish species (Regenstein & Zhou, 2007; Zhang et al., 2007).

For producing collagen from cold-water fish, both the pretreatment and extraction conditions are established at a low temperature around 4 °C, and these low temperature processing conditions are commonly applied to warm-water fish (Ciarloa, Paredi, & Fraga, 1997; Nalinanon, Benjakul, & Kishimura, 2010; Regenstein & Zhou, 2007; Sadowska, Kołodziejska, & Niecikowska, 2003; Sato, Yoshinaka, Sato, & Shimizu, 1987; Singh, Benjakul, Maqsood, & Kishimura, 2011; Wang, An, Xin, Zhao, & Hu, 2007; Zhang et al., 2007). However, the thermosensitivity of collagen during preparation

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may differ between fish species with different habitat temperatures, leading to a variation of the optimal manufacturing conditions that are not only critical to obtain a desirable yield, purity and structural integrity of collagen, but also time-saving and cost-efficient in practical application. Therefore, this study was conducted to investigate the effects of NaOH concentration and temperature on the efficiency of removing non-collagenous proteins from grass carp (a typical warm-water fish) skins, the effects of acetic acid concentration and temperature on the efficiency of extracting ASC, and also the effects of combined conditions suggested from the above-mentioned various alkaline pretreatments and acid extraction processes on the structural integrity of the resulting ASC.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) and L-hydroxyproline were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The protein standard used for electrophoretic analysis of ASC consisted of the following proteins: myosin from rabbit muscle, 212 kDa; fusion of maltose-binding protein and β-galactosidase from *Escherichia coli*, 158 kDa; β-galactosidase from *E. coli*, 116 kDa; phosphorylase B from rabbit muscle, 97 kDa; serum albumin from bovine, 66 kDa; glutamic dehydrogenase from bovine liver, 56 kDa; maltose-binding protein from *E. coli*, 43 kDa; thioredoxin reductase from *E. coli*, 35 kDa; triosephosphate isomerase from *E. coli*, 27 kDa (New England Biolabs, Inc., Ipswich, MA, USA). Precision Plus Protein All Blue Standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) used for analysis of electrophoretic patterns of proteins which were removed by alkaline pretreatment contained a cocktail of recombinant proteins with the manufacturer's specified molecular weight of 100, 75 and 50 kDa. All the other chemicals used were of analytical grade.

2.2. Preparation of fish skins

Live farmed grass carps, with weights ranging from 3 to 4 kg, were obtained in the summer from a local market in Wuxi, Jiangsu Province, China. They were transported to the laboratory in water within 30 min. Upon arrival, the fish were stunned by a sharp blow to the head with a wooden stick in a walk-in chill room at a temperature around 4 °C, and the skins were manually removed with a filleting knife and then washed with cold distilled water. The cleaned skins were cut into small pieces (0.5×0.5 cm^2) using a scissor and then kept on ice prior to collagen extraction.

2.3. Production of ASC from fish skins

2.3.1. Effects of alkaline pretreatments

To determine the effects of alkaline pretreatments on collagen extraction, five following parameters were measured: total content of proteins removed during the alkaline pretreatment, loss of hydroxyproline during the alkaline pretreatment, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) patterns of proteins removed during the alkaline pretreatment, hydroxyproline content of ASC and SDS–PAGE patterns of ASC. The cleaned skins (10 g) were soaked in 20 volumes of NaOH solution, which was stirred using the C-MAG HS7 magnetic stirrer (IKA Werke GmbH & Co. KG, Staufen, Germany). NaOH concentration (0.05, 0.1, 0.2 or 0.5 M), temperature (4, 10, 15 or 20 °C) and time (1, 2, 4, 8 or 12 h) were chosen as the variables. After the alkaline pretreatment, the skin residues were drained and rinsed with cold distilled water; while the pretreatment solutions were collected and neutralised with HCl or NaOH, and then used directly for total protein determination by Biuret method (Gornall, Bardawill, & David, 1949) with weighed BSA as a standard.

The subsequent extraction procedures were done in a cold room at about 4 °C. The pretreated skins were suspended in 20 volumes of 10% (v/v) butyl alcohol to remove fat for 24 h with a change of solution every 12 h. After being thoroughly washed with cold distilled water, the residues were extracted with 40 volumes of 0.5 M acetic acid for 72 h. The suspension was then centrifuged at 10,000*g* for 20 min at 4 °C using an Avanti J-E centrifuge (Beckman Coulter, Inc., Indianapolis, IN, USA), and the supernatant was salted-out by adding NaCl to a final concentration of 2.0 M. After 12 h, the precipitate was collected by centrifugation at 10,000*g* for 20 min and then redissolved in 0.5 M acetic acid. The resulting solution was dialysed against cold distilled water using a dialysis bag with a molecular weight cut-off of 7 kDa (Shanghai Green Bird Science and Technology Development Co., Shanghai, China) and then lyophilised using the Labconco freeze dryer (Labconco Corp., Kansas, MO, USA).

2.3.2. Effects of acid extraction conditions

To determine the effects of acid extraction conditions on collagen extraction, two following parameters were determined: extraction yield of ASC and SDS–PAGE patterns of ASC. The cleaned skins (10 g) were firstly soaked in 20 volumes of 0.1 M NaOH for 36 h with a change of the alkaline solution every 12 h. After being washed with cold distilled water, the deproteinised residues were suspended in 20 volumes of 10% (v/v) butyl alcohol for 24 h, with the solution being changed every 12 h. The defatted residues were collected and washed with cold distilled water. All the pretreatments in these cases were done in a cold room at about 4 °C. The pretreated skins were then suspended in 40 volumes of acetic acid solution for 72 h. Acetic acid concentration (0.1, 0.2, 0.5 or 1.0 M) and temperature (4, 10, 15 or 20 °C) were chosen as the variables. After extraction, the collagens in the suspension were collected and lyophilised according to the methods described in Section 2.3.1.

2.3.3. Effects of combined conditions suggested from alkaline pretreatment and acid extraction

The suggested alkaline pretreatment conditions (NaOH concentration and temperature) in the section of "effects of alkaline pretreatments" and the suggested acid extraction conditions (acetic acid concentration and temperature) in the section of "effects of acid extraction conditions" were combined to determine the effects of combined conditions on the structural integrity of the resulting ASC, using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and differential scanning calorimetry (DSC). Moreover, extraction yield of ASC and SDS–PAGE patterns of ASC were also determined. The same temperature was used for both the alkaline pretreatment and acid extraction of each whole manufacturing process, which would be more convenient for practical and industrial application. The cleaned skins (10 g) were firstly soaked in 20 volumes of NaOH solution for 36 h with a change of the alkaline solution every 12 h. The deproteinised residues were suspended in 20 volumes of 10% (v/v) butyl alcohol for 24 h at 4 °C, with the solution being changed every 12 h. Then, the defatted residues were suspended in 40 volumes of acetic acid solution for 72 h. After extraction, the collagens in the suspension were collected and lyophilised according to the methods described in Section 2.3.1.

2.4. Analytical methods

2.4.1. Extraction yield of ASC

The undissolved skin residues after the ASC extraction were lyophilised and weighed (M_1), and the extraction yield of ASC

was calculated on the dry weight basis of the deproteinised and defatted skins (M_2) using the following equation.

$$\text{Yield} = (1 - M_1/M_2) \times 100\%$$

2.4.2. Determination of hydroxyproline

A 5 mg of ASC was hydrolysed in 8 mL of 6 M HCl in an evacuated and sealed tube with a volume of 15 mL at 110 °C for 22 h. In addition, a 4 mL of the alkaline pretreatment solution collected in Section 2.3.1 was mixed with 4 mL of 12 M HCl and then hydrolysed under the same condition. The hydroxyproline content in the resulting hydrolysate was determined according to the method of Woessner (1961) using L-hydroxyproline as the standard.

2.4.3. SDS–PAGE

SDS–PAGE was done with a Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, Inc.) according to the method of Laemmli (1970), using a 4% stacking gel and a 7.5% resolving gel with 1 mm thickness for ASC and a 4% stacking gel and a 12% resolving gel with 1.5 mm thickness for proteins in the alkaline pretreatment solution. ASC was dissolved in 1% (w/v) SDS to obtain a final concentration of 2 mg/mL and then mixed with an equal volume of sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 2% (w/v) SDS and 25% (v/v) glycerol), and a 10 μL of the sample was loaded in each well. The alkaline pretreatment solution was mixed with an equal volume of sample buffer and then a 40 μL of the sample was loaded. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 6.8% (v/v) glacial acetic acid for 5 h, and then destained using 7.5% (v/v) glacial acetic acid and 5% (v/v) methanol until clear bands could be observed.

2.4.4. ATR-FTIR

The ATR-FTIR spectra of ASC were obtained using a Nicolet Nexus 470 FT-IR spectrometer equipped with a Smart OMNI-Sampler accessory (Thermo Electron Corp., Madison, WI, USA). Lyophilised ASC were placed onto the single reflection germanium crystal cell with a 45° crystal angle. The signals were automatically collected for 64 scans over the range of 4000–400 cm^{-1} at a resolution of 2 cm^{-1} and were compared to a background spectrum collected from the clean empty cell.

2.4.5. DSC

DSC studies were done using the Q2000 Series DSC (TA Instruments, Inc., New Castle, DE, USA) that was calibrated for temperature and enthalpy using indium as the standard. ASC were rehydrated in 0.05 M acetic acid at a sample/solution ratio of 1:40 (w/v) for 2 days at 4 °C. Then, the rehydrated ASC samples (10 ± 0.5 mg) were accurately weighted into aluminium pans (TA Instruments, Inc.), hermetically sealed and scanned from 20 to 50 °C at a heating rate of 1 °C/min. An empty sealed aluminium pan was used as the reference. The maximum transition temperature (T_{max}) was recorded by the software as the peak temperature of each endothermic peak.

2.5. Statistical analysis

Statistical analysis was done using SAS version 8.0 (1999, SAS Institute, Inc., Cary, NC, USA). Analysis of variance (ANOVA) using the General Linear Model procedure and the difference between means using the Duncan test were determined at an α level of 0.05.

3. Results and discussion

Collagen constitutes more than 70% of fish skin on a dry weight basis (Liu et al., 2012). In the extracellular matrix of fish skin, collagen molecules align head-to-tail in a quarter stagger array, allowing the occurrence of crosslinking at the telopeptide between collagens, and the extent of crosslinking usually increases with age (McCormick, 2009). The collagens are almost insoluble in the alkaline solutions (Liu et al., 2012). In previous studies, both alkaline solutions and neutral salt solutions were used to remove non-collagenous proteins during pretreatment (Regenstein & Zhou, 2007). However, the newly synthesised uncrosslinked collagens could be removed by neutral salt solutions, leading to a low yield of collagen. Therefore, alkaline solutions such as NaOH were commonly used to remove non-collagenous proteins that are soluble in the alkaline pretreatment solutions with minimal loss of collagen. Organic acid solutions can not only solubilise the uncrosslinked collagens, but also break some inter-chain cross-linkages of collagens, such as the reducible aldimine condensation crosslinks, leading to further solubilisation of collagen during extraction (Regenstein & Zhou, 2007). Therefore, organic acid solutions such as acetic acid were commonly used to extract collagen from fish skins.

3.1. Effects of alkaline pretreatments

Before collagen extraction, alkaline pretreatments are often used to remove unwanted materials such as non-collagenous proteins and pigments, and also to exclude the effects of endogenous proteases on collagen during manufacturing (Sato et al., 1987; Yang et al., 2007). NaOH and $Ca(OH)_2$ are two alkalis that are frequently used for pretreatment, and both of them showed similar abilities to remove non-collagenous proteins from fish skins (Zhou & Regenstein, 2005). However, NaOH could cause a significant swelling of fish skins during pretreatment and thus facilitate the following acid extraction by promoting the mass transfer rate in the tissue matrix, whereas $Ca(OH)_2$ did not. Therefore, NaOH was used in this study to optimise the pretreatment conditions with maximal removal of non-collagenous proteins and minimal loss of collagen.

3.1.1. Proteins removed during alkaline pretreatments

The alkaline pretreatment solutions were collected to determine the non-collagenous proteins removed and the collagens lost during processing. As shown in Fig. 1, the total contents of proteins removed in the alkaline pretreatment solutions generally increased with time within 12 h of processing. After 12 h of pretreatment, the contents of proteins removed by 0.05 and 0.1 M NaOH varied slightly among the four temperatures, and slightly higher contents of proteins were removed by 0.1 M NaOH than by 0.05 M NaOH. At 0.2 M NaOH, a sharp increase in the contents of removed proteins was observed when the pretreatment temperature increased to 20 °C ($P < 0.05$). At 0.5 M NaOH, larger contents of proteins were removed than at lower concentrations of NaOH, and the contents of removed proteins increased significantly with pretreatment temperature ($P < 0.05$). Statistical analysis showed that the interactions of NaOH concentration and temperature, NaOH concentration and time, and temperature and time had significant effects on the removal of total contents of proteins in the alkaline pretreatment solutions ($P < 0.05$). As the classical extraction, the mass transfer rate within the skin was limited by the diffusion process, and the efficiency of protein removal was depended on pretreatment time and temperature (Wang, Yang, Du, Yang, & Liu, 2008).

After 12 h of pretreatment at the four temperatures, negligible contents of collagens that were expressed in terms of hydroxyproline were removed by 0.05 M NaOH and less than 0.14 mg hydroxyproline/g wet skins was removed by 0.1 M NaOH (Fig. 2A). At 0.2 M NaOH, about 0.16 mg hydroxyproline/g wet skins was removed by the pretreatment at 4 °C, and the contents of removed hydroxyproline increased sharply to 0.64 mg hydroxyproline/g wet skins when the pretreatment temperature reached

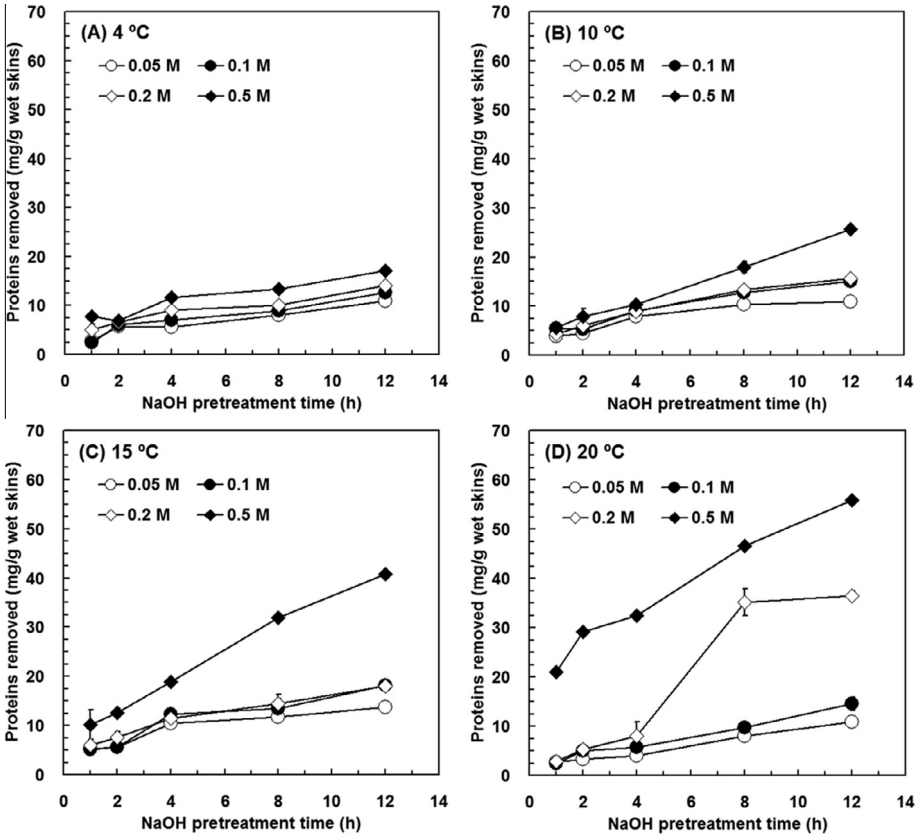

Fig. 1. Total content of proteins removed in the alkaline pretreatment solutions from grass carp skins at 4 °C (A), 10 °C (B), 15 °C (C) or 20 °C (D) as a function of pretreatment time. 0.05–0.5 M, molar concentrations of NaOH in the pretreatment solutions.

up to 20 °C ($P < 0.05$). At 0.5 M NaOH, the contents of removed hydroxyproline were much higher than at lower concentrations of NaOH, and reached up to 2.03 mg hydroxyproline/g wet skins at 20 °C ($P < 0.05$). These lost collagens might partly contribute to the higher contents of total proteins removed by 0.2 M NaOH at 20 °C and also by 0.5 M NaOH at the four temperatures (Fig. 1). Statistical analysis showed that the interaction of NaOH concentration and temperature had significant effects on the removal of contents of collagens in the alkaline pretreatment solutions ($P < 0.05$).

The electrophoretic analysis also confirmed that the total proteins removed after 12 h of pretreatment at the four temperatures were mainly non-collagenous proteins when 0.05 and 0.1 M NaOH were used. However, collagen related components together with non-collagenous proteins were observed when 0.2 and 0.5 M NaOH were used, and the intensity of collagen bands increased with pretreatment temperature (Fig. 3A). Moreover, diffused background and blurred non-collagenous bands were observed when NaOH concentrations increased to 0.2 and 0.5 M, especially at 20 °C, which might be due to the degradation of removed proteins in the alkaline pretreatment solutions. Taken together the above-mentioned results, the present study suggested that the removal of collagens from grass carp skins were triggered by 0.1 M NaOH and accelerated by increases in both the NaOH concentration and pretreatment temperature. Sato et al. (1987) also reported that collagens completely remained in the residues after carp muscle was pretreated with 0.01, 0.05 and 0.1 M NaOH at 5 °C for four days, while pretreatment with 0.5 and 1.0 M NaOH at 5 °C for four days removed a significant amount of collagens. For Alaska pollock, a cold-water fish, Zhou and Regenstein (2004) reported that alkaline pretreatment of the skins at 20 °C led to a high loss of collagens, and a pretreatment temperature lower than 10 °C was recommended for collagen extraction from cold-water fish. After 1 h of pretreatment at 2–4 °C, the amount of non-collagenous proteins removed from pollock skins increased with the increasing of NaOH concentration from 0.01 to 0.1 M and then remained almost unchanged when the NaOH concentration further increased to 0.5 M, and no significant amount of collagens were removed by NaOH in the concentration range of 0.01–0.5 M (Zhou & Regenstein, 2005). These results suggested that the efficiency of alkaline pretreatment varied depending on pretreatment temperature and time, NaOH concentration, and the differences in raw materials.

Hydroxyproline is a unique marker amino acid for collagen. Although elastin also contains hydroxyproline, the amount of elastin in skins is much less than that of collagen (Liu, Regenstein, Mulvaney, Boran, & Zhou, 2013). Therefore, based on the hydroxyproline content, the purities of ASC extracted after 12 h of various alkaline pretreatment were compared (Fig. 2B). In general, the hydroxyproline contents of ASC varied slightly in the range of 8.37–8.80%. Statistical analysis showed that NaOH concentration and temperature had no significant interaction effects on the hydroxyproline contents of ASC ($P > 0.05$). Traditionally, the alkaline pretreatment was done using 0.1 M NaOH at about 4 °C (Regenstein & Zhou, 2007), which resulted in an ASC with 8.41% hydroxyproline in the present study. At 0.05 and 0.1 M NaOH, no significant differences in hydroxyproline contents of ACS were observed between these two pretreatments at each temperature ($P > 0.05$), and the contents of hydroxyproline generally increased with temperature and reached up to about 8.60% at 20 °C ($P < 0.05$). After pretreatments by 0.2 and 0.5 M NaOH, slightly higher hydroxyproline contents were observed compared to pretreatments by 0.05 and 0.1 M NaOH at each temperature. Based

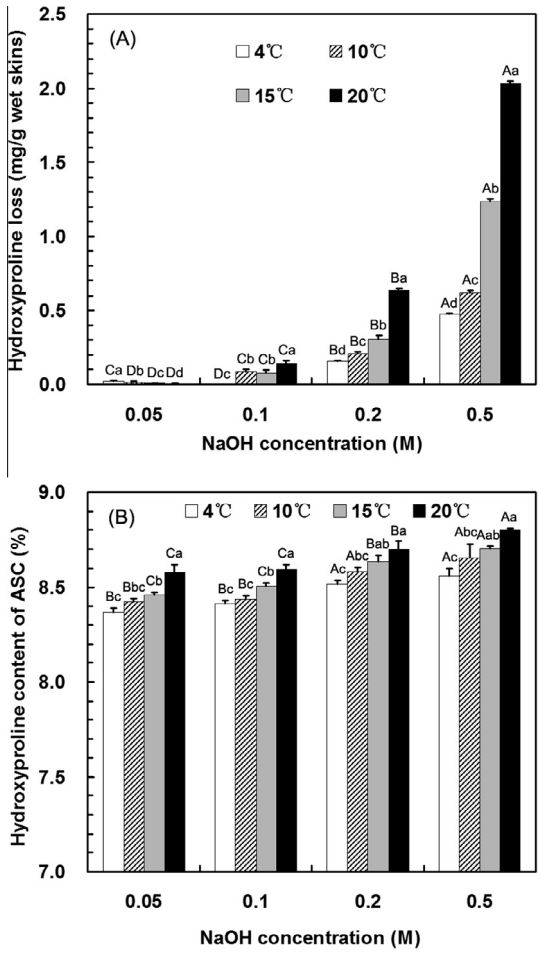

Fig. 2. Content of collagens removed that was expressed in terms of hydroxyproline lost in the alkaline pretreatment solutions from grass carp skins after 12 h of pretreatment at 4, 10, 15 or 20 °C as a function of NaOH concentration (A) and hydroxyproline content of ASC extracted from grass carp skins which were subjected to 12 h of alkaline pretreatment at 4, 10, 15 or 20 °C as a function of NaOH concentration (B). a–d, means at the same NaOH concentration labeled by different lower-case letters differ significantly among different pretreatment temperature ($P < 0.05$). A–D, means at the same pretreatment temperature labeled by different upper-case letters differ significantly among different NaOH concentration ($P < 0.05$).

on the hydroxyproline content of ASC, Sato et al. (1987) also indicated that a small amount of non-collagenous proteins were retained in the ASC extracted from carp muscle after pretreatment with 0.01 and 0.05 M NaOH at 5 °C, while such a small amount of impurities in the extracted ASC could not be detected using the electrophoretic analysis.

3.1.2. SDS–PAGE of ASC

As shown in Fig. 3B, ASC extracted after pretreatment with 0.05–0.5 M NaOH in the temperature range of 4–20 °C showed the typical electrophoretic patterns of type I collagen, including α-chains, β-chains and other high molecular weight aggregates. The mobility of each band was similar among pretreatments with 0.05, 0.1 and 0.2 M NaOH at the four temperatures, suggesting that these alkaline pretreatment conditions might not cause significant structural modifications of the extracted ASC. However, pretreatments with 0.5 M NaOH at 15 and 20 °C resulted in slightly lower mobility of α-chains, compared to other alkaline pretreatments, suggesting that pretreatments with 0.5 M NaOH at or above 15 °C caused structural modifications of the extracted ASC. Sato et al. (1987) also reported that ASC extracted from carp muscle after pretreatments with 0.5 and 1.0 M NaOH for four days showed band mobility different from that of ASC obtained after pretreatments with 0.01, 0.05 and 0.1 M NaOH for four days.

In previous studies on extraction and characterisation of collagen, the raw fish skins were commonly pretreated with fresh NaOH solution consecutively for three times, each time being 12 h (Liu, Zhou, Li, & Regenstein, 2014; Liu et al., 2012). In this section of the present study, only one time (12 h) of alkaline pretreatment was used to mainly study the effects of NaOH concentration and pretreatment temperature on the efficiency of removing non-collagenous proteins. For all alkaline pretreatment conditions, the total contents of proteins removed in the alkaline pretreatment solutions increased with time within 12 h of processing (Fig. 1). Taken together, three times of alkaline pretreatment, with each time being 12 h, were suggested for the following sections of the present study for the thorough removal of non-collagenous proteins.

From the above results, it suggested that pretreatments with 0.05 and 0.1 M NaOH in the temperature range of 4–20 °C could be used to remove non-collagenous proteins from grass carp skins, without significant simultaneous removal of collagens. In the following second section, one suggested mild alkaline pretreatment condition of 0.1 M NaOH and 4 °C that was commonly used in previous studies was applied (Liu et al., 2012, 2014). In the following third section, eight alkaline pretreatment conditions combined with 0.05 and 0.1 M NaOH and 4, 10, 15 and 20 °C were applied.

3.2. Effects of acid extraction conditions

Organic acids, such as acetic acid, citric acid and lactic acid, can be used for collagen extraction. Inorganic acids (eg., hydrochloric acid) can also be applied to extract collagen, however, their efficiencies are lower compared to the organic acids (Regenstein & Zhou, 2007; Wang et al., 2008). Acetic acid is the most commonly used organic solvent for collagen preparation due to the corresponding high extractability (Wang et al., 2008). The concentration of acetic acid determines the pH value of the extraction medium and thus regulates the charge density of collagen, which affects the electrostatic interaction and structure of collagen and finally determines the solubility and extractability of collagen from animal tissues. In addition, an increase of temperature to a certain extent may facilitate the extraction of collagen without affecting its native structure, while denaturation of collagen occurs when the temperature increases beyond a certain limit. Therefore, acetic acid was used in this study to optimise the extraction conditions with maximal yield ASC.

3.2.1. Extraction yield of ASC

At 0.1 M acetic acid, the extraction yields of ASC were less than 46% at 4 and 10 °C and then sharply increased to 81% and 91% at 15 and 20 °C, respectively (Fig. 4) ($P < 0.05$). At 0.2 M acetic acid, the extraction yield of ASC increased from 65% at 4 °C to 80% at 10 °C, and thereafter reached up to about 90% at 15 and 20 °C ($P < 0.05$). Traditionally, the acid extraction was done using 0.5 M acetic acid at about 4 °C (Regenstein & Zhou, 2007), which resulted in almost complete extraction of ASC in the present study. Moreover, complete extraction of ASC was maintained as the extraction temperature increased to 20 °C at 0.5 M acetic acid and also as the acetic acid concentration increased to 1.0 M at the four temperatures ($P > 0.05$). Statistical analysis showed that the interaction of acetic acid concentration and temperature had significant effects on the extraction yields of ASC ($P < 0.05$).

3.2.2. SDS–PAGE of ASC

As shown in Fig. 5, ASC extracted using 0.1–1.0 M acetic acid in the temperature range of 4–20 °C showed similar electrophoretic

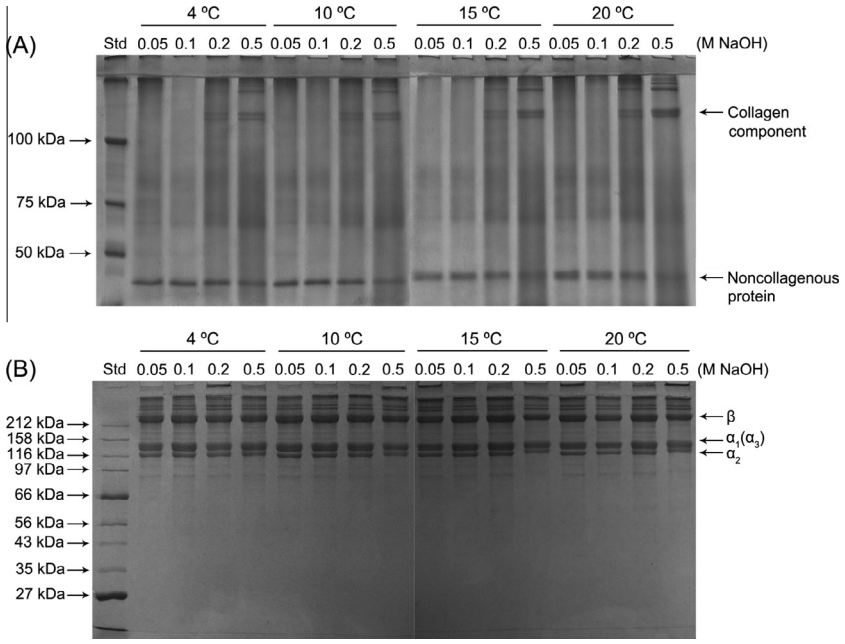

Fig. 3. SDS–PAGE patterns of proteins removed in the alkaline pretreatment solutions (0.05, 0.1, 0.2 or 0.5 M NaOH) from grass carp skins after 12 h of pretreatment at 4, 10, 15 or 20 °C (A) and ASC extracted from the skins after the corresponding alkaline pretreatment (B). The first lane is the protein standards (Std).

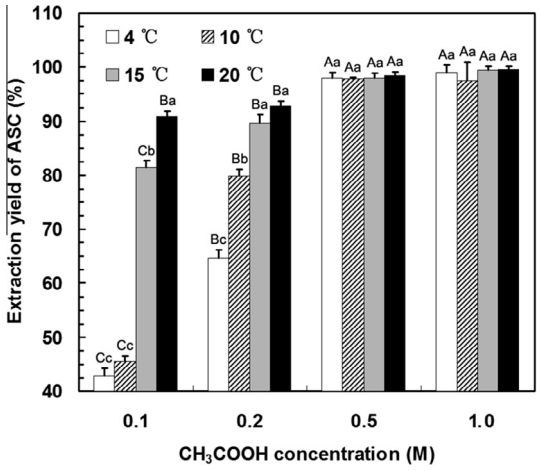

Fig. 4. Extraction yield of ASC from grass carp skins at 4, 10, 15 or 20 °C as a function of acetic acid concentration. a–c, means at the same acetic acid concentration labeled by different lower-case letters differ significantly among different extraction temperature ($P < 0.05$). A–C, means at the same extraction temperature labeled by different upper-case letters differ significantly among different acetic acid concentration ($P < 0.05$).

patterns, which consisted of the typical collagen derived components such as α-chains, β-chains and other high molecular weight aggregates, and the mobility of each band was similar among each acetic acid extraction process, suggesting that the present acid extraction processes might not cause significant structural modifications of the extracted ASC. Taken together, four acid extraction conditions combined with 0.5 M acetic acid and 4, 10, 15 and 20 °C were applied in the following third section.

3.3. Effects of combined conditions suggested from alkaline pretreatment and acid extraction

The suggested alkaline pretreatment conditions in the section of "effects of alkaline pretreatments" and the suggested acid extraction conditions in the section of "effects of acid extraction conditions" were combined in this third section as follows: temperature (4, 10, 15 or 20 °C), NaOH concentration (0.05 or 0.1 M) and acetic acid concentration (0.5 M). The same temperature was used for both the alkaline pretreatment and acid extraction of each whole manufacturing process, which would be more convenient for practical and industrial application. Therefore, eight combined conditions were studied.

As shown in Table 1, ASC was almost completely extracted from grass carp skin at the eight combined conditions. Moreover, ASC extracted using the eight combined conditions showed the typical electrophoretic patterns of type I collagen, and the mobility of each band was similar among each combined conditions (data not shown), suggesting that the present combined conditions might not cause significant structural modifications of the extracted ASC.

The FTIR spectra of ASC extracted using the eight combined conditions showed generally similar patterns (data not shown). An absorption ratio of approximately 1 between the amide III and the 1454 cm^{-1} band suggests that the triple helical structure of collagen is intact (Liu et al., 2012). ASC extracted using the eight combined conditions showed the absorption ratio in the range of 1.03–1.05 (Table 1), suggesting that the triple helical structures of ASC were well maintained. Moreover, ASC extracted using the eight combined conditions showed similar T_{max} values ($P > 0.05$), which further suggested that the structural integrity of ASC were well maintained at the present eight combined conditions.

For Nile tilapia, Potaros, Raksakulthai, Runglerdkreangkrai, and Worawattanamateekul (2009) reported that the yield of ASC from the skins was lower when both the alkaline pretreatment using 0.1 M NaOH and the acid extraction using 0.5 M acetic acid were performed at 22–23 °C, compared to the yield resulting from performing the whole manufacturing process at 4–6 °C; however, there were no significant differences in the molecular weight distribution, amino acid profile and denaturation temperature of ASC between these two processing temperatures. For Nile perch, another warm-water fish as Nile tilapia, a high yield of native ASC from the skins was also obtained using 0.5 M acetic acid at 15 °C (Muyonga, Cole, & Duodu, 2004). Wang, Yang, and Du (2009) reported that the yield of ASC from grass carp skins decreased as the extraction temperature increased above 20 °C

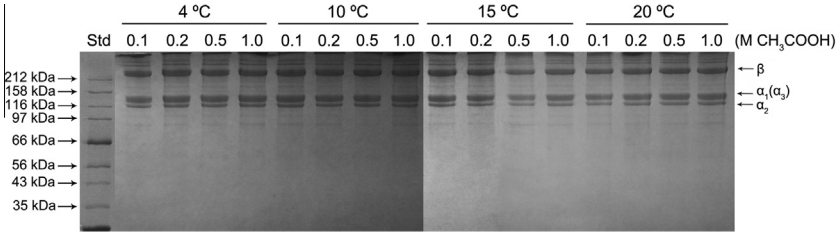

Fig. 5. SDS–PAGE patterns of ASC extracted from grass carp skins using 0.1, 0.2, 0.5 or 1.0 M acetic acid at 4, 10, 15 or 20 °C.

Table 1
Extraction yield of ASC, the absorption ratio between the amide III and the 1454 cm^{-1} band of the FTIR spectra of ASC, and the maximum transition temperature (T_{max}) of ASC.

Combined conditions			Yield (%)	Absorption ratio (amide III/1454 cm^{-1})	T_{max} (°C)
Temperature (°C)	NaOH concentration (M)	CH_3COOH concentration (M)			
4	0.05	0.5	98.0 ± 1.6[a]	1.05 ± 0.03[a]	36.1 ± 0.4[a]
	0.1		97.3 ± 3.2[a]	1.04 ± 0.02[a]	35.9 ± 0.5[a]
10	0.05		99.0 ± 0.5[a]	1.03 ± 0.01[a]	36.5 ± 0.1[a]
	0.1		97.8 ± 2.0[a]	1.03 ± 0.01[a]	36.0 ± 0.5[a]
15	0.05		98.2 ± 1.5[a]	1.05 ± 0.01[a]	35.5 ± 0.1[a]
	0.1		98.7 ± 1.1[a]	1.05 ± 0.02[a]	36.0 ± 0.5[a]
20	0.05		96.6 ± 3.5[a]	1.05 ± 0.01[a]	36.2 ± 0.5[a]
	0.1		98.0 ± 1.4[a]	1.04 ± 0.01[a]	35.9 ± 0.6[a]

[a] Means in a column followed by different lower-case letters differ significantly ($P < 0.05$).

when 0.5 M acetic acid was used. Taken together with these studies, the present study suggested that both alkaline pretreatment and acid extraction can be performed at a temperature in the range of 4–20 °C based on the processing season, instead of the commonly used low temperature around 4 °C, to produce ASC from grass carp skins without significant loss and structural modification of ASC, which would be more convenient in practical and industrial application, and these results may be applied to other warm-water fish species, including bighead carp, black carp and silver carp.

4. Conclusions

The production of ASC from grass carp skins was significantly affected by NaOH pretreatment concentration, acetic acid extraction concentration and processing temperature. In the temperature range of 4–20 °C, pretreatments by 0.05 and 0.1 M NaOH removed non-collagenous proteins without significant loss of collagens, while pretreatments by 0.2 and 0.5 M NaOH removed a significant amount of collagens, especially at high temperature. In the temperature range of 4–20 °C, ASC was partly extracted by 0.1 and 0.2 M acetic acid, while complete extraction was obtained at 0.5 and 1.0 M acetic acid. The combined conditions suggested from alkaline pretreatment and acid extraction, involving 0.05–0.1 M NaOH, 0.5 M acetic acid and 4–20 °C, produced ASC with the structural integrity being well maintained, and hence were recommended to produce ASC from grass carp skin, instead of the commonly used alkaline pretreatment with 0.1 M NaOH at about 4 °C and acid extraction with 0.5 M acetic acid at about 4 °C. Therefore, based on the processing season, a temperature for both the alkaline pretreatment and acid extraction could be adjusted in the range of 4–20 °C to produce ASC from the skins of grass carp and possibly other warm-water fish, which would be more convenient in practical and industrial application.

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