Effects of Alkali and Acid on the Solubility and Molecular Weight of Collagen Hydrolysates Extracted from Bovine Hide

by

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Abstract

Preparation of collagen hydrolysates with high molecular weight to meet its industrial demand is a crucial step for resource utilization of solid waste of animal skins/hides. However, it is very difficult to achieve both higher molecular weight and solubility of collagen hydrolysates through the traditional methods. In this study, we attempted to prepare bovine hide collagen hydrolysates with high molecular weight and solubility through the application of NaOH or different types of acid. Influences of the concentration of alkali and acid, hydrolysis temperature and time on the molecular weight and solubility were studied, respectively. The results showed that NaOH has a strong hydrolysis effect on bovine collagen, making it a suitable candidate for the preparation of collagen hydrolysates with medium or low molecular weight. Under these optimized NaOH treatment conditions, i.e., NaOH concentration of 0.13 mol/L, hydrolysis temperature of 60 - 70°C and time of 5 h, we achieved 96% of solubility for hide pieces and the molecular weight of collagen hydrolysates were in the range of 25 - 30 kDa. By contrast, the molecular weight of the hydrolysates prepared through H_2SO_4 hydrolysis method was higher than that of NaOH hydrolysis method. Under the optimized H_2SO_4 treatment conditions, i.e. H_2SO_4 concentration of 0.5 mol/L, hydrolysis temperature of 50°C and time in the range of 5 - 7 h, the solubility of hide pieces reached up to 80 - 97%. Additionally, in the H_2SO_4 hydrolysates, the proportion of macromolecular components with molecular weight of about 100 kDa was 41 - 55% and that of medium molecular components with molecular weight of about 20 kDa was 45 - 59%. This study showed that high solubility and high molecular weight collagen products can be obtained by H_2SO_4 hydrolysis under specific conditions. Thus, this study provided a useful scientific method and process parameters to guide the controlling of molecular weight and the industrial application of collagen from waste on a bigger scale.

Introduction

The demand for meat and dairy products has been increasing day by day, and has promoted the growth of livestock. About 300 million pieces of bovine hide come from the meat and dairy industries every year, of which about 60% are used for tanning, and 40% (about 120 million) collagen sheets are discarded. Discarded collagen sheets generate 3 million tons of landfill waste and release 2.7 million tons of greenhouse gases.[1] In addition, 350 kg of solid leather waste is produced and discarded during one ton of leather manufacturing, which also causes serious environmental pollution.[2] Therefore, it is a dire need of our time to find some high-value utilization of the aforementioned produced solid waste.

Animal skin is mainly composed of water-insoluble connective tissue woven by collagen fibers. About 85 - 90% of collagen is found in the dermis. Soluble collagen can be mainly obtained by acid, alkali, enzymatic and heat treatment methods. In the course of its preparation, natural collagen is denatured and hydrolyzed to varying degrees. Therefore, the properties and suitable fields of application of collagen hydrolysates depend mainly on its preparation methods. For example, collagen with a complete triple helix structure can be obtained by the application of a low concentration of acid and pepsin. Its molecular weight is about 300 kDa, which has biological activity[3] and fibril formation properties.[4] It can be used in tissue engineering, bone substitution, ophthalmic surgeries, drug delivery vehicle, immunity localization effects and sponges for burns or wounds, etc.[5] Gelatin and hydrolyzed collagen are the denatured products of native collagen obtained through the application of strong acid, alkali, enzyme or heat. However, these denatured products generally don't have any biological activity.[3] Collagen hydrolysates obtained through different methods exhibit different molecular weights and properties, i.e., hygroscopicity property, water holding capacity, oil holding capacity, emulsifying properties, foaming properties and antioxidant activity.[6] Therefore, the collagen hydrolysates with divergent properties are suitable for different applications, such as food additives, protein dietary supplements edible films, coatings,[7] cosmetics,[8] wood adhesives,[9] leather retanning agents[10,11]and paper sizing agents.[12]

The field and scope of application of hydrolyzed collagen are closely related to its molecular weight. For example, collagen peptides with molecular weights below 3 - 6 kDa don't have gelling and fibril formation properties, but have water-holding, moisture absorption, and anti-aging properties.[13] Therefore, they are the

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Manuscript received February 26, 2022, accepted for publication April 3, 2022.

most suitable candidates for their application in cosmetics,[8,13] food additives,[7,13] and facial skin essence materials.[14] The molecular weight of UVB-induced anti-photo aging materials is about 1.0 kDa.[15] Hydrolyzed collagen with high molecular weight is generally used in the industrial field, such as photosensitive materials,[16] packaging materials, paper surface sizing agents,[17] etc. For example, enzymatic hydrolysates of waste collagen proteins from current industrial manufacture (leather, edible meat product casings, etc.) of mean molecular mass 20 - 30 kDa by a reaction with dialdehyde starch, produce hydrogels applicable as biodegradable (or even edible) packaging materials for food, cosmetic and pharmaceutical products.[17] Both physical, mechanical properties and water resistance of the corrugated paper coated by GDESA (GDESA was prepared by modifying collagen hydrolysate with Glycol diglycidyl ether, then grafted with Butyl acrylate and styrene) were significantly improved when the molecular weight of collagen hydrolysates was about 10 kDa, and its emulsion exhibited robust stability in long-standing time.[12] Collagen protein with medium molecular weight (10 - 30 kDa detected by SDS-PAGE) from chrome leather scraps has been used as retarding agent in gypsum application, which produced a positive retarding effect in the prolongation of setting time.[18]

As mentioned above, preparation methods for collagen hydrolysates mainly include acid, alkali, enzymatic hydrolysis and heat extraction. To get intact collagen with a complete triple-helix structure, 0.5 mol/L of acetic acid or pepsin at low temperatures is usually applied, however, the solubility of these methods was too low, generally less than 50%.[19-21] Heating can accelerate the hydrolysis of collagen by destroying the hydrophobic interaction and hydrogen bond in collagen, and promoting the dissolving of collagen. Therefore, thermal extraction method is adopted as the traditional preparation method of gelatin[22] though this method always brings high energy consumption. Therefore, collagen can be easily hydrolyzed into low molecular weight soluble products under strong action of acid, alkali or enzymes. In this preparation process, collagen products with high molecular weight can be obtained under weaker hydrolysis conditions though the solubility is very low. Strong hydrolysis conditions will have the opposite effect on the molecular weight and solubility which limits its application on commercial scale.

Bovine hide contains about 89% of collagen fiber.[23] The production of soluble collagen is another effective utilization of bovine hide except for leather making. The preparation of hydrolyzed collagen or gelatin from bovine hide has been widely investigated. For example, yak hides were hydrolyzed by trypsin to produce collagen hydrolysates with 98.79% of solubility and 1000 - 2236 Da of molecular weight.[24] Tannery and slaughterhouse bovine hides were hydrolyzed by acid or alkali to produce gelatin, and the solubilities of all of these methods are lower than 31%.[23] Delimed bovine pelts were hydrolyzed by acetic acid at 70°C for 6 h to produce gelatin, however, the yield is only 4.78 % and the molecular weight is in the range of 6 - 38 kDa.[25] Calf split was respectively treated by acetic acid, pepsin and alkali to produce completed Type I and Type III collagen.[26]

Although animal skins/hides can be used to produce undenatured collagen and used in the field of medicine, food and cosmetics with high values, however, the requirement for the quality of collagen products is very high and the demand is limited. Moreover, it is difficult to balance the molecular weight and solubility of collagen hydrolysates at the same time because of the difficult controllability of the molecular weight. Therefore, to solve the problem of resource utilization of collagen waste from animal skins, the most effective way is to develop the industrial application of collagen on a bigger scale. Hence, establishing preparation methods for soluble collagen with controllable molecular weight is required to fulfill the requirements of industry application.

As mentioned previously, the preparation of small molecule collagen is relatively simple, but the industrial application needs macromolecular collagen. Therefore, this study focuses on the effects of alkali and acid treatment conditions on the solubility of delimed bovine split and the molecular weight of collagen hydrolysates. We attempted to establish a method for preparing collagen hydrolysates with large molecular weight along with high solubility.

Materials and Methods

Materials

Delimed bovine hide substrate (1 cm × 1 cm) with moisture of 58.09% and freeze-dried hide powder with moisture of 5.31% was obtained from our laboratory. Standard protein samples of ovalbumin (45000Da), myoglobin (17000Da), aprotinin (6700Da), neurotensin (1700Da) and angiotensin II(1000Da) were purchased from Agilent Technologies Inc., USA. Gelatin (Number-average molecular weight (Mn) = 65997, Weight-average molecular weight (Mw) = 94749, Mw/Mn = 1.44) was purchased from Chengdu Chron Chemicals Co., Ltd. All the chemicals used for the reaction and analysis were of analytical grade.

Preparation of bovine hide hydrolysates through NaOH hydrolysis method

20.0 g of intact pieces of bovine hide was weighed and mixed with 80.0 mL of NaOH solution (the concentration is in the range of 0.0 - 1.0 mol/L). Then, the mixture was stirred at different temperatures (50 - 80°C) for different times (1 - 9 h) at 200 r/min. After the reaction, samples were cooled to room temperature and centrifuged for 20 min at 4°C, 9460 r/min, then, filtered with constant weight filter paper. The filtrate was collected to determine the content of hydroxyproline (Hypro) by Chloramine T method and the molecular weight by Gel Permeation Chromatography (GPC) method, respectively. In addition, the filter residues were collected, washed with distilled water thoroughly and dried for determining the solubility.

Preparation of bovine hide hydrolysates through acid hydrolysis method

Effect of acid type

10.0 g of bovine hide powder was mixed with different acid solutions. The mixture was stirred at 50°C for 3 h at 200 r/min. After the reaction, samples were centrifuged and filtered. The solubility, the content of Hypro in the filtrate and the molecular weight of the hydrolysates were determined as the methods described above.

Preparation of bovine hide hydrolysates through H_2SO_4 hydrolysis method

20.0 g of intact pieces of bovine hide were weighed and mixed with 80.0 mL of H_2SO_4 solution (the concentration is in the range of 0.0 - 1.2 mol/L). Then, the mixture was stirred at different temperatures (50 - 80°C) for different times (1 - 9 h) at 200 r/min. After the reaction, samples were centrifuged and filtered. The solubility, the concentration of Hypro in the filtrate and the molecular weight of the hydrolysates were determined as the methods described above.

Solubility of bovine hide

The collected residues from bovine hide hydrolysates after filtering were constantly weighted at 102 ± 2°C. Quantification of solubility is calculated according to the ratio of the dry weight of residues after hydrolysis (M_1) and the dry weight of bovine hide pieces or powder ($M_0 \times S$). The calculation formula is as follows:

$$Solubility = (1 - \frac{M_1}{M_0 \times S}) \times 100\%$$

In the equation, S is the solid content of untreated bovine hide pieces.

Determination of Hypro by Chloramine T method

The concentration of Hypro was determined according to the modified colorimetric method.[27,28] First, 2 mL of filtrate was mixed with 2 mL of HCl (12 mol/L) and hydrolyzed at 120°C for 4 h. A colored soluble product was obtained based on the reaction of Hypro with *p*-dimethylaminobenzaldehyde. The absorbance of the colored mixture was measured at 560 nm to determine the amount of Hypro.

Determination of molecular weight of bovine hide hydrolysates by GPC method

The molecular weight of the bovine hide hydrolysates was determined through GPC method. The HPLC system used was an Agilent 1260 Infinity II HPLC system with an SEC separation column (130A 2.7 µm particle size, 7.8mm×300mm) and a diode array detector (Agilent Technology Inc.).[29] The GPC conditions were as follows: flow rate, 1 mL/min; injection volume, 10 µL; column temperature 25°C; mobile phase 150 mmol/L of NaH_2PO_4-Na_2HPO_4 buffer (pH 7.0); Detection wavelength, 220 nm.

A standard curve was plotted from the logarithm of molecular weight (Mw) and retention time (T) of the standard protein samples (LogMw= 6.914-0.4223T, R^2 = 0.9904). The molecular weight of the samples was obtained by comparing the retention time of the sample with the standard curve. The relative content of each peptide fraction was expressed as a percentage of the chromatographic peak area.

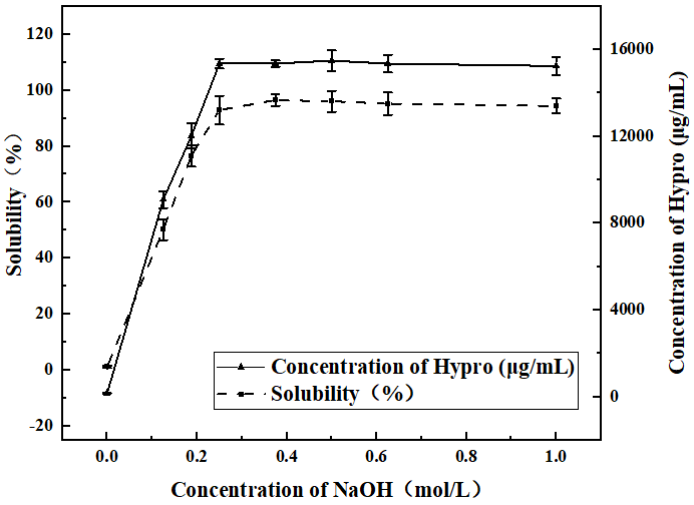

Figure 1. Effect of NaOH concentration on the solubility of bovine hide (50°C, 5 h)

Results and Discussion

Effect of NaOH hydrolysis conditions on the molecular weight of collagen hydrolysates

Effect of NaOH concentration

The results in Figure 1 showed that the solubility and concentration of Hypro in the reaction liquors were increased linearly with the increase of NaOH concentration in the range of 0.00 - 0.25 mol/L. The highest solubility of the bovine hide (about 93%) and the maximum content of Hypro in the hydrolysates (about 15000 µg/mL) were achieved at 0.25mol/L of NaOH at 50°C for 5 h. However, when the concentration of NaOH was higher than 0.25 mol/L, the solubility and content of Hypro have no significant increase.

The results in Figure 2 showed that the retention time of the hydrolysates was significantly shifted to the right along with the narrowing down of peak width with the increase of NaOH concentration, indicating the reduction in molecular weight and distribution of collagen hydrolysate. A linear negative correlation between the concentration of NaOH and the molecular weight of hydrolysates had been observed when the concentration of NaOH is no more than 0.25 mol/L. The most remarkable result to emerge from the data is that the M_W and the molecular weight distribution width (M_W/Mn) of the hydrolysates dropped off from 36 kDa to about 2.5 kDa and 3.64 to 1.69, respectively, as NaOH concentration increased from 0.13 mol/L to 1.00 mol/L. These results indicated that the proportion of low molecular weight components was increased with the increase of NaOH concentration and the preparation of low Mw collagen hydrolysates by the NaOH hydrolysis method is fairly simple.

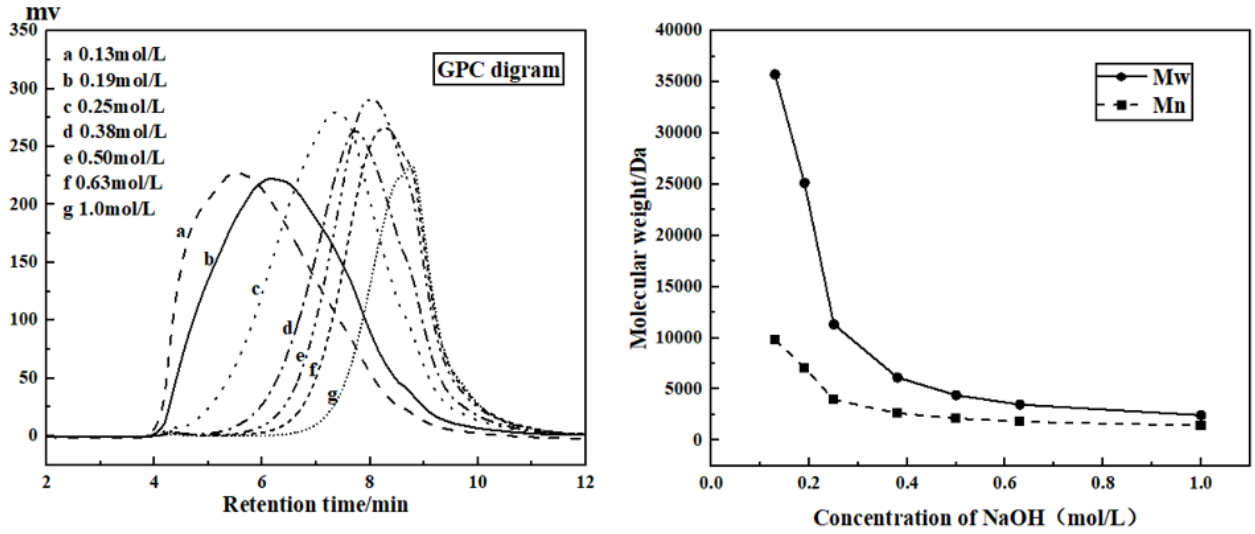

Figure 2. Effect of NaOH concentration on the molecular weight of collagen hydrolysates (50°C, 5 h)

In general, there is a negative correlation between the solubility of bovine hide and the molecular weight of the hydrolysates. When the concentration of NaOH is higher than 0.25 mol/L, more than 93% solubility of bovine hide had been observed, whereas, the molecular weight of the hydrolysates was lower than 12 kDa. Conversely, at a lower concentration of NaOH, i.e. 0.13mol/L, Mw of the hydrolysates was found to be higher than 30 kDa, however, the solubility of the bovine hide was only about 50%. These findings indicated that it is very difficult to prepare high molecular weight collagen hydrolysates along with high solubility from bovine hide. Therefore, from the perspective of preparing high molecular weight collagen hydrolysates, the effects of reaction time and temperature on the hydrolysis degree of bovine hide at low NaOH concentration were further investigated.

Effect of NaOH hydrolysis time

Results showed that the solubility of bovine hide was significantly increased with hydrolysis time and reached up to 50.2% ± 3.79% after reacting with 0.13 mol/L of NaOH at 50°C for 5 h. Then, with the consumption of NaOH, the hydrolysis of the bovine hide pieces gradually slowed down, and the solubility of the hide pieces was increased to 66.5% ± 2.34% at 9 h. The results in Figure 3 showed that the molecular weight of the collagen hydrolysates increased first and then decreased with hydrolysis time, which might be due to the heterogeneous reaction of the hydrolysis processes. In the early stage of hydrolysis (within 3 h), the hide pieces remained in a relatively intact state, and the solubility of low molecular hydrolysate components was faster than that of macromolecular components. The determined molecular weight of the collagen hydrolysates was about 36 kDa after 5 h of hydrolysis. Subsequently, the molecular weight of the hydrolysates was decreased to 32 - 36 kDa and the width of molecular weight distribution slightly increased (the M_W/Mn value is in the range of 3.62 - 4.23).

These results indicated that the high molecular weight hydrolysates (M_W > 30 kDa) can be prepared at low NaOH concentration and medium temperature. However, the solubility of the hide pieces was less than 70%, and it has little contribution to further improving the solubility by prolonging the hydrolysis time.

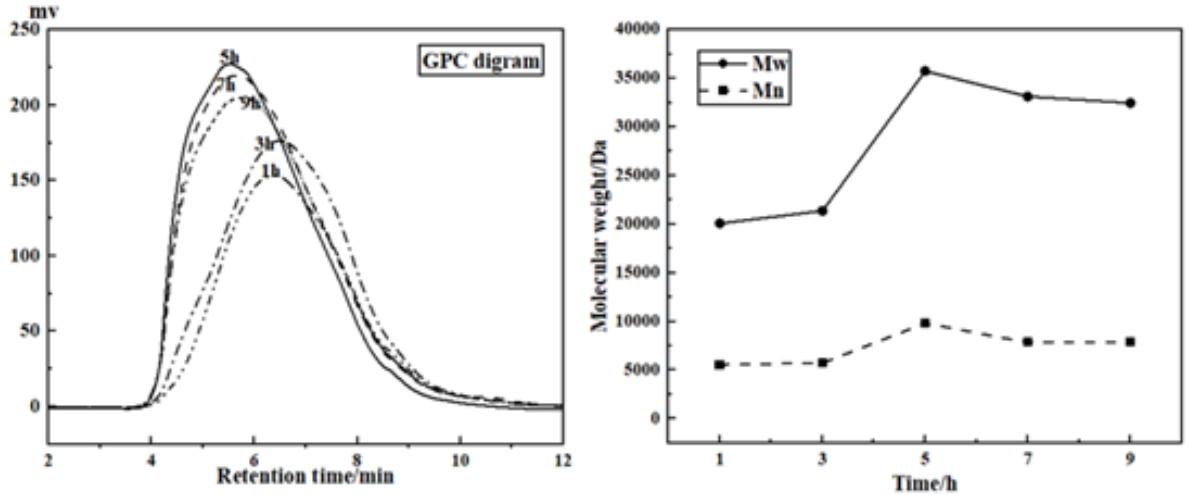

Figure 3. Effect of NaOH hydrolysis time on the molecular weight of collagen hydrolysates (C_{NaOH}=0.13 mol/L, 50°C)

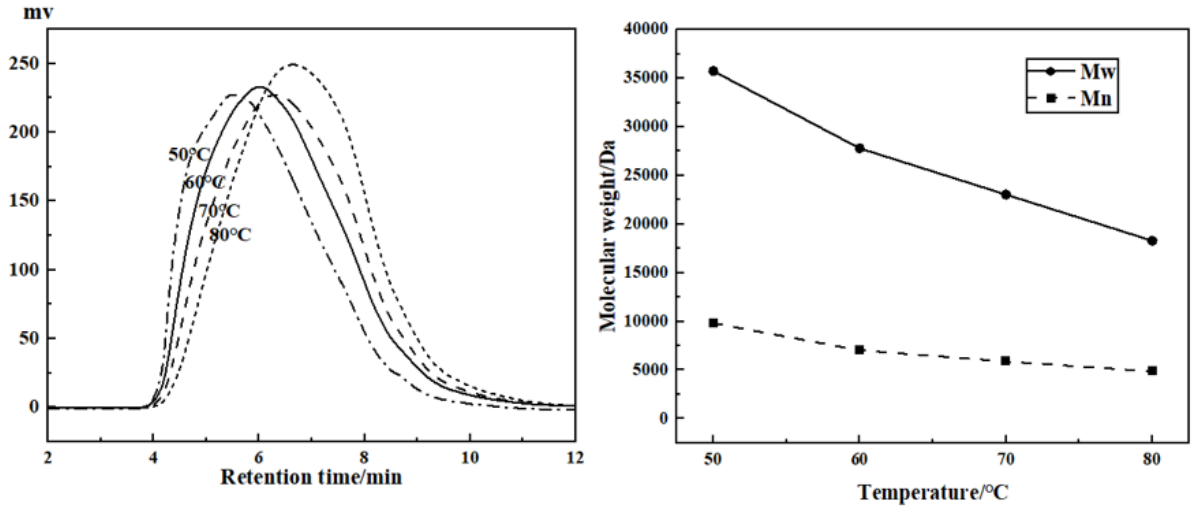

Figure 4. Effect of NaOH hydrolysis temperature on the molecular weight of collagen hydrolysates (C_{NaOH} =0.13 mol/L, 5 h)

Effect of NaOH hydrolysis temperature

In order to obtain high molecular weight hydrolysates with higher solubility, the effect of temperature on the hydrolysis of bovine hide at low NaOH concentration was further investigated. Results showed that the solubility of bovine hide was increased from 50.2% ± 3.79% to 96.6% ± 4.06% when the reaction temperature was increased from 50°C to 60°C at 0.13 mol/L of NaOH for 5 h. However, further increasing the reaction temperature just had a slight effect on the solubility of the bovine hide. The results in Figure 4 showed that the molecular weight of the collagen hydrolysates was decreased with the increase of hydrolysis temperature, e.g. the molecular weight of the hydrolysates was decreased to 18 kDa at 80°C from 36 kDa at 50°C. Additionally, hydrolysis temperature had little effect on the width of molecular weight distribution and the M_W/Mn value remained between 3.64 - 3.95.

In summary, NaOH solution has a strong hydrolysis effect on bovine hide, which is suitable for the preparation of collagen hydrolysates with low and medium molecular weight. The above results indicated that bovine hide pieces can be easily hydrolyzed into low molecular weight products (< 10 kDa) when the reaction conditions were reached or even higher than 0.25 mol/L of NaOH and 50°C. Oppositely, collagen hydrolysates with the molecular weight between 25 - 30 kDa can be produced when the concentration of NaOH is 0.13 mol/L and the hydrolysis temperature is in the range of 60 - 70°C for 5 h, and the solubility is reached up to 96%.

Effect of acid hydrolysis conditions on the molecular weight of collagen hydrolysates

Effect of acid type

Although the extraction of collagen hydrolysates from bovine hide pieces through NaOH method has higher solubility, the product has a lower molecular weight. Herein, the effect of acid hydrolysis on the molecular weight of the product was further studied.

Firstly, the effects of several typical acids on the solubility of bovine hide powder and the molecular weight of collagen hydrolysates were compared in a trial sample test to select optimal acids for further study (Table I). Results showed that, at a low concentration of acid (sulfuric acid 7.23 mmoL/g, other acids 14.46 mmoL/g, based on the dry weight of hide powder), the solubility of bovine hide powder by FA, AA, SA, MSA and NSA hydrolyzed at 50°C for 3 h were 29.18% ± 1.39%, 21.41% ± 1.18%, 74.8% ± 2.74%, 76.23% ± 2.18% and 74.99% ± 3.32%, respectively. The solubility of hide

Table I

Conditions of hydrolysis by different acids

Acid	Characterization	Liquor ratio	Content of acid (mmoL/g of hide powder)
Formic acid (FA)	Monoacid, Swelling acid	1:50	14.46
Acetic acid (AA)	Monoacid, Swelling acid	1:50	14.46
Sulfuric acid (SA)	Dibasic acid, Swelling acid	1:15	7.23
Methanesulfonic acid (MSA)	Monoacid, Swelling acid	1:15	14.46
2-Naphthalenesulfonic acid (NSA)	Monoacid, Non-swelling acid	1:15	14.46

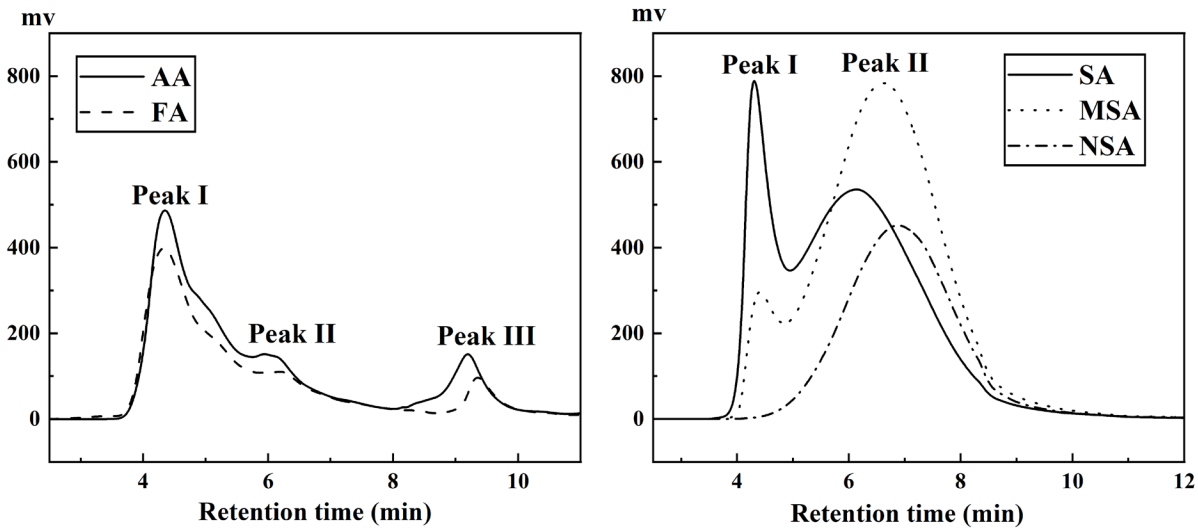

Figure 5. GPC diagrams of collagen hydrolysates by different acids
(AA: Acetic Acid, FA: Formic acid, SA: Sulfuric Acid, MSA: Methane Sulfonic Acid, NSA: 2-Naphthalene Sulfonic Acid)

Table II
Molecular weight of different acid hydrolyzed collagen hydrolysates

Acid	Peak	Mn (Da)	Mw (Da)	Mw/Mn	Peak area(%)
FA	I	75447	96196	1.28	73.28
	II	10543	14656	1.39	19.06
	III	<1000	<1000	-	7.66
AA	I	75826	92445	1.22	70.02
	II	15036	17994	1.20	17.85
	III	<1000	<1000	-	12.13
SA	I	104070	108339	1.04	26.93
	II	6994	21003	3.00	73.07
MSA	I	102481	104042	1.02	8.91
	II	6383	17071	2.67	91.09
NSA	II	5085	13666	2.47	100.00

powder was positively correlated with the strength of acids, e.g. weaker acid FA and AA with lower H^+ dissociation constant than strong acid SA, MSA and NSA, which produced lower strength of acid at the same theoretical acid concentration and result in low solubility. It is worth noting that compared with swelling acids, SA and MSA, there is no significant difference in the solubility of the bovine hide powder in non-swelling state when non-swelling acid NSA[30-31] was used.

The results in Figure 5 showed that the GPC chromatogram of the collagen hydrolysates produced by acid methods has obvious double-peak or multi-peak characteristics compared to NaOH hydrolysis method. For example, the collagen hydrolysates of FA and AA have triple peaks in a wide range, and the hydrolysates of SA, MSA and NSA have two peaks. Detailly, Peak I and II respectively represents the molecular weight of about 100 kDa and 15 - 20 kDa, and Peak III represent the molecular weight is lower than 1.0 kDa. The molecular weight of the Peak I component was larger than gelatin, indicating that the acid-soluble collagen might be in a complete structure. Table II showed that the proportion of Peak I in FA, AA, SA and MSA was 73.28%, 70.02%, 26.93% and 8.69%, respectively, indicating that weak organic acid is conducive to the dissolution of acid-soluble collagen in the bovine hide. Furthermore, there is no obvious Peak I component in the hydrolysates of the NSA treated sample, indicating that macromolecular acid-soluble collagen can be dissolved out at a non-swelling state of collagen fiber. To sum up, the treatment of hide powder by acids includes two stages at low concentrations of acid: the dissolution of macromolecular acid-soluble collagen and the hydrolysis of collagen; and the proportion of low molecular weight hydrolysates increased with the increase of acid strength.

Generally, the molecular weight of the collagen hydrolysates treated by H_2SO_4 is higher than 21 kDa, and the solubility is relatively high than other acids. Subsequently, the effect of H_2SO_4 concentration, hydrolysis temperature and time on the solubility of bovine hide and the molecular weight of hydrolysates were further investigated.

Table III

Molecular weight and distribution of collagen hydrolysates with varying H_2SO_4 concentrations

Concentration of H_2SO_4 (mol/L)	Content of H_2SO_4 per gram hide pieces (mmoL/g, dry weight)	Solubility (%)	Peak	Mn (Da)	Mw (Da)	Mw/Mn
0.3	2.86	67.33±2.52	I	117496	120620	1.03
			II	24008	29033	1.21
0.5	4.77	80.14±3.03	I	96146	108068	1.12
			II	11451	20430	1.78
0.7	6.68	93.60±2.40	I	97077	103546	1.07
			II	7770	18487	2.38
1.0	9.54	93.88±3.53	I	94024	99333	1.06
			II	6809	16384	2.41
1.2	11.45	92.88±2.42	I	94994	97673	1.03
			II	6169	10708	1.74

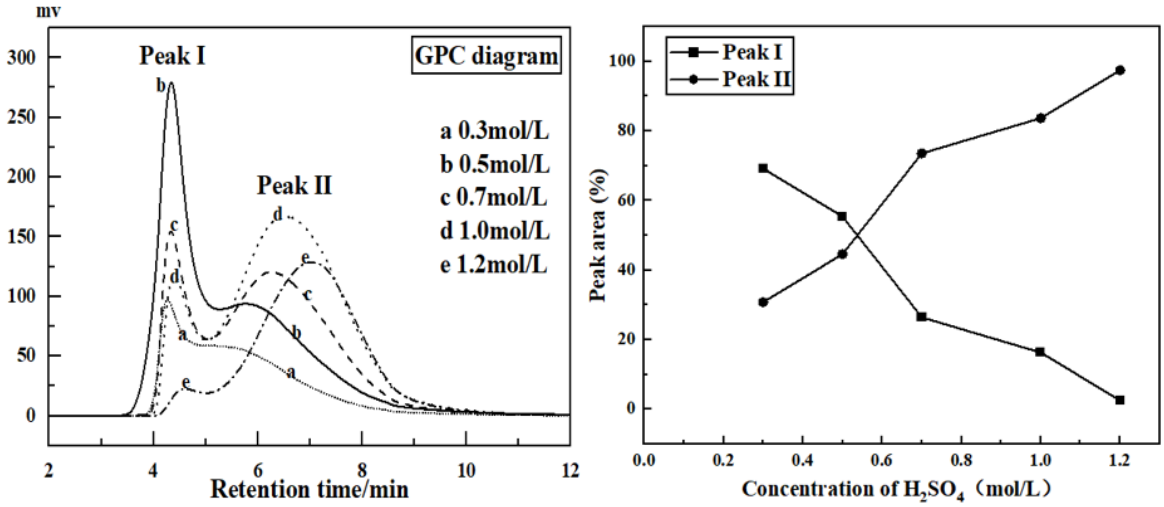

Figure 6. Analysis of GPC of collagen hydrolysates with varying H_2SO_4 concentrations (50°C, 5 h)

Effect of H_2SO_4 concentration

Bovine hide pieces were treated with different concentrations of H_2SO_4 at 50°C for 5 h. The results in Table III showed that when the concentration of H_2SO_4 was lower than 0.7 mol/L, the solubility of bovine hide increased obviously with the increase of acid concentration; When the concentration of H_2SO_4 reached up to 0.7 mol/L, the solubility of bovine hide tend to be the same at about 94%. At 0.7 mol/L of H_2SO_4, per gram of hide pieces (dry weight) had been hydrolyzed by 6.68 mmoL of H_2SO_4, which was close to 7.23 mmoL H_2SO_4 per gram of hide powder (dry weight) in the section of *Effect of acid type*. Therefore, the molecular weight and peak area ratio of collagen hydrolysates was very close to each other. However, the solubility at 7.23 mmoL of H_2SO_4 was lower than 6.68 mmoL of H_2SO_4 because of the shorter hydrolysis time.

The results in Table III and Figure 6 also showed that with the increase of H_2SO_4 concentration, the average molecular weight (Mn) of the macromolecular component (Peak I) changes little and the M_W is about 100 kDa; the Mn of the medium molecular component (Peak II) decreased obviously and the M_W is reduced from 30 kDa to 10 kDa. Besides, the area ratio of the two peaks changed apparently. With the increase of H_2SO_4 concentration from 0.3mol/L to 1.2mol/L, the area proportion of Peak I decreased from 69.21% to 2.51%, and the area proportion of Peak II increased from 30.79% to 97.49%. These results indicated that the hydrolysis degree of collagen products increased with the increase of H_2SO_4 concentration. Hence, the proportion of high molecular weight components in the product decreased, followed by the proportion of low and medium molecular weight components increased.

Compared with NaOH hydrolysis method, the molecular weight of the hydrolysates treated by H_2SO_4 method was obviously higher, and the molecular weight distribution was narrower, especially the M_W/Mn value of macromolecular compounds was about 1.0. When the bovine hide pieces were hydrolyzed by 0.3 mol/L of H_2SO_4 at 50°C, the proportion of macromolecular component (about 120 kDa) in the collagen hydrolysates was 69.21%, however, the solubility of the hide pieces was only about 67%. Although the molecular weight and the proportion of macromolecular components in the hydrolysates decreased when the concentration of H_2SO_4 solution increased to 0.5 mol/L, the solubility of the hide pieces was reached up to about 80%. Herein, the effects of hydrolysis time and temperature on the solubility and molecular weight of the hydrolysates were further investigated by using 0.5 mol/L of H_2SO_4.

Effect of H_2SO_4 hydrolysis temperature

The effects of temperature on the solubility of bovine hide and the molecular weight of collagen hydrolysates were investigated by using 0.5 mol/L of H_2SO_4 for 5 h, as shown in Figure 7 and Table IV.

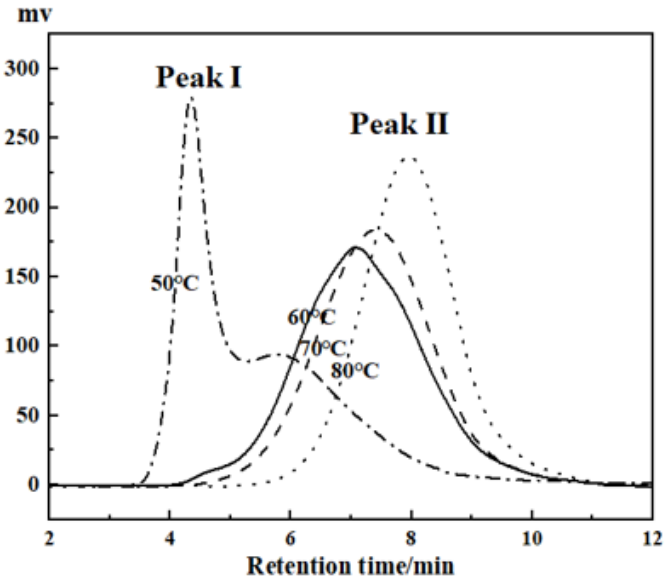

Figure 7. GPC diagrams of collagen hydrolysates by H_2SO_4 hydrolysis with varying temperatures (C_{H2SO4}= 0.5 mol/L, 5 h)

The results in Table IV showed that the solubility of bovine hide increased significantly with the increase of hydrolysis temperature and reached up to 96% when the temperature was 60°C. Further raising temperatures have no obvious effect on the solubility, oppositely, a significant reduction in the Mn of collagen hydrolysates and a considerable increase in the width of molecular weight distribution were presented. For example, the macromolecular component with M_W of about 100 kDa in the collagen hydrolysates was disappeared when the hydrolysis temperature was higher than 60°C, which has been further hydrolyzed into low molecular protein with M_W lower than 12 kDa. Results also showed that the major proportion of the collagen hydrolyzed into polypeptides with M_W less than 5 kDa at 80°C.

The above results indicated that the solubility of bovine hide and the molecular weight of the hydrolysates are inversely proportional to each other within a specific temperature range. For example, the solubility of bovine hide collagen was lower and the molecular weight of the hydrolysates was higher when the hydrolysis temperature was lower than 50°C; Although rising temperature could improve the solubility of collagen substrate, the molecular weight of the hydrolysates was significantly decreased. Furthermore, to overcome these limitations, the effects of H_2SO_4 hydrolysis time on the solubility of bovine hide and the molecular weight of hydrolysates were further investigated.

Effect of H_2SO_4 hydrolysis time

The effect of hydrolysis time on the solubility of bovine hides and the molecular weight of hydrolysate were investigated by using 0.5 mol/L of H_2SO_4 at 50°C, as shown in Figure 8 and Table V. The results in Table V showed that the solubility of bovine hide increased with the extension of hydrolysis time and reached up to about 97% at 7 h and then stabilized. The average molecular weight of both macromolecular and middle molecular components decreased slightly as time prolonged. The M_W of all the macromolecular components was more than 100 kDa, and the molecular weight distribution range was narrow (M_W/Mn value is about 1). The M_W of the middle molecular component was in the range of 20 - 26 kDa. On the other hand, by prolonging the hydrolysis time, it had been witnessed that the proportion of

Table IV

Molecular weight and distribution of collagen hydrolysates by H_2SO_4 hydrolysis with different H_2SO_4 hydrolysis temperatures (C_{H2SO4}=0.5 mol/L, 5 h)

Temperature (°C)	Solubility (%)	Peak	Mn (Da)	Mw (Da)	Mw/Mn	Peak area (%)
50	80.14 ± 3.03	I	96146	108068	1.12	55.41
		II	11451	20430	1.78	44.59
60	94.86 ± 2.39	I	4051	12114	2.99	100
70	93.98 ± 3.25	I	3568	9185	2.57	100
80	96.51 ± 2.34	I	2381	4727	1.99	100

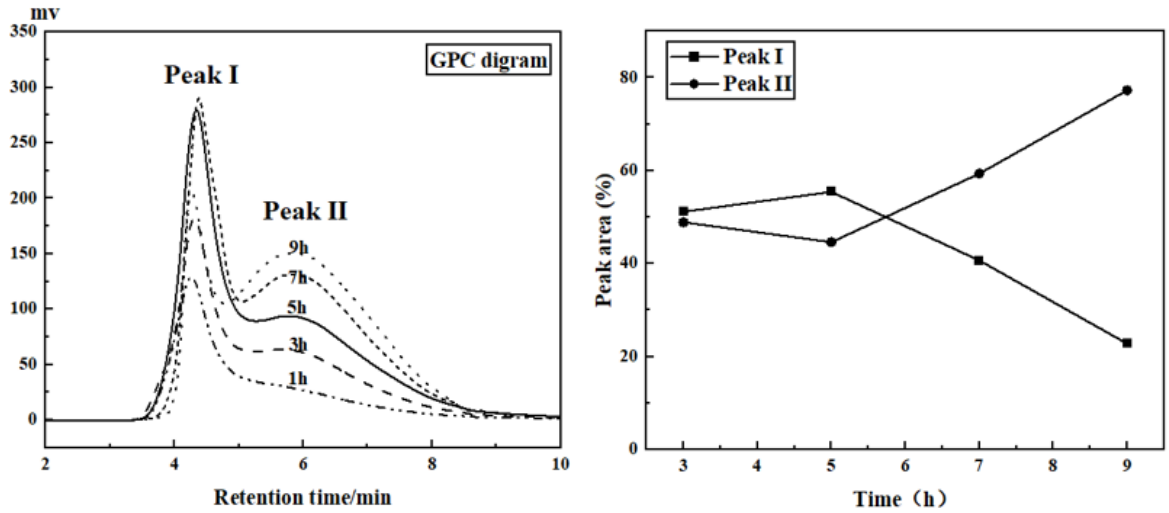

Figure 8. Analysis of GPC of collagen hydrolysates by H_2SO_4 hydrolysis with varying time (C_{H2SO4}= 0.5 mol/L, 50°C)

Table V

Molecular weight and distribution of collagen hydrolysates by H_2SO_4 hydrolysis with varying time (C_{H2SO4}=0.5 mol/L, 50°C)

Time (h)	Peak	Solubility (%)	Mn (Da)	Mw (Da)	Mw/Mn
3	I	49.77 ± 2.18	113586	122930	1.08
	II		14017	24637	1.76
5	I	80.14 ± 3.03	96146	108068	1.12
	II		11451	20430	1.78
7	I	97.12 ± 2.40	98295	105235	1.07
	II		10000	22587	2.26
9	I	96.36 ± 2.73	109947	114427	1.04
	II		10384	25791	2.48

macromolecular components decreased and the proportion of medium molecular components increased.

In the light of the above results, it may be assumed that the molecular weight of collagen hydrolysates prepared by H_2SO_4 hydrolysis method was significantly higher than that of NaOH hydrolysis method. The solubility of bovine hide could reach up to 80% - 97% after hydrolyzing at 50°C with 0.5 mol/L of H_2SO_4 for 5 - 7 h. The hydrolysates mainly had two molecular components, the proportion of macromolecular components with M_W of about 100 kDa was 55% - 41%, and the proportion of medium molecular components with M_W of about 20 kDa was 45% - 59%.

Conclusions

To prepare bovine hide collagen hydrolysates with high molecular weight and solubility, the effects of NaOH and acid treatment conditions on the solubility and molecular weight of collagen hydrolysates were studied. We have comprehensive results which show that NaOH is a suitable option for the preparation of low and medium molecular weight collagen hydrolysates owing to its strong hydrolysis effect. The bovine hide can be easily hydrolyzed into low molecular weight products (< 10 kDa) with 93% of solubility when the reaction conditions were reached or even higher than 0.25 mol/L of NaOH and 50°C. Oppositely, hydrolysates with the molecular weight between 25 - 30 kDa can be produced when the concentration of NaOH is 0.13 mol/L and the reaction temperature is in the range of 60 - 70°C, and the solubility is reached up to 96%. High molecular weight hydrolysates (M_W > 30 kDa) can only be prepared at low NaOH concentration and medium temperature (no more than 0.13 mol/L and 50°C), however, the solubility of the hide pieces was less than 70%.

Significantly, the hydrolysis effects of acid on bovine hide collagen were found to be weaker than that of alkali, and the molecular weight of collagen hydrolysates prepared by H_2SO_4 hydrolysis method was significantly higher than that of NaOH hydrolysis method. At lower acid concentrations, the hydrolysates had two main components: M_W about 100 kDa and 15 - 20 kDa. It is noteworthy to mention that with the increase in treatment intensity, a significant increase

in the hydrolysis degree of collagen was observed along with a considerable reduction in the proportion of macromolecular components. The solubility of bovine hide could reach up to 80% - 97% after hydrolyzing at 50°C with 0.5 mol/L of H_2SO_4 for 5 - 7 h, and the proportion of macromolecular components with M_W of about 100 kDa was 55% - 41%, the proportion of medium molecular components with M_W of about 20 kDa was 45% - 59%.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (21908149) and the National Key R&D Program of China (2017YFB0308402).

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