Resource Utilization of Bovine Hair Recycling from Enzymatic Unhairing during Leather Manufacturing: Alkali-Protease Synergistic Preparation of Keratin and its in Vitro Antioxidant Activity

by

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Abstract

With the development of enzymatic unhairing technology in the leather industry, enhancing the recycling of bovine hair is a new issue emerging in leather-making. In this work, the effects of alkali concentration, time, and temperature in the pretreatment, as well as enzyme dosage, time, temperature, and pH in the following treatment on the dissolution performance and keratin yield of bovine hair were investigated. Hence, the extraction process of keratin from bovine hair by the alkali-enzyme method was optimized. The results show that the optimal conditions for the keratin extraction are as follows: for the pretreatment with alkali, the alkali concentration is 0.4 M, the pretreatment time is 1.0 h, the pretreatment temperature is 65°C, and for the following enzymatic treatment, the enzyme dosage is 3324 U/g bovine hair, the action time is 4.0 h, action temperature is 45°C, and the pH is controlled between 8.5-11.5. Compared with the alkali method, the alkali-enzyme method increases the keratin yield from 24.4% to 58.1%, and the alkali dosage is reduced by 50%. The prepared keratin had high in vitro antioxidant activity, which provided a new idea for the resource utilization of bovine hair waste.

Introduction

As the raw material of the leather industry is obtained from animal husbandry, leather making is the most feasible and sustainable way to solve the problem of recycling about 300 million pieces of bovine hide every year. In traditional leather processing, to achieve the purpose of unhairing and dispersing collagen fibers, a large amount of sulfide and lime is applied to remove hairs from hides and make hides swell.[1,2] This method has the advantages of convenient operation, easy control, and steady effect, and is widely used in the leather industry. However, due to the heavy use of sulfide and lime, the pollutant content generated by the dissolution of bovine hair in wastewater is high, and the emission loads of chemical oxygen demand (COD), sulfide, ammonia nitrogen, and total nitrogen in traditional unhairing are more than 30% of the whole leather processing.[3,4] With the advantages of being sustainable and eco-friendly, enzymatic unhairing technology is considered an effective way to reduce pollutant discharge in traditional unhairing. Moreover, after years of research and development, the safety and controllability of enzymatic unhairing technology have been effectively broken through, and it has gradually been widely applied in practical production.[5] Enzymatic unhairing technology can achieve a recovery of 80%–90% of bovine hair; and how to improve the high-value utilization efficiency of bovine hair is a new challenge for the leather industry.

Bovine hair is rich in keratin.[6] With good biocompatibility, skin friendliness, biodegradability, and physical and mechanical property, keratin has broad application prospects in protein composite film, regenerated fibers, food packaging material, leather functional material, cosmetic, and biomedical material.[7-9] In recent years, animal hair keratin extraction technology has developed rapidly. Wool, rabbit hair, pig hair, feather, and human hair have been applied for keratin extraction,[10, 11] but reports about keratin extraction using bovine hair as raw material are few. At present, the commonly used keratin extraction methods include mechanical grinding,[12] acid hydrolysis,[13] alkaline hydrolysis,[14] reduction,[15] oxidation,[16] enzymatic hydrolysis,[17] ionic liquid assisted method,[18] metal salt assisted method and copper–ammonia solution assisted method. The solubility of hair, the keratin yield, and the molecular weight, group, and chemical properties of keratin obtained from hair vary with different raw materials and extraction methods. In general, the keratin obtained by mechanical grinding under heating and pressurizing has a smaller molecular weight and is mainly used in the production of animal feed; the acid and alkaline hydrolysis method treat hair with a low solubility and obtain small molecular weight, while producing more neutral salts;[19] the keratin prepared by the reduction method has a large relative molecular weight with a narrow distribution and remains active sulfhydryl groups of cysteine to the maximum extent, but the shortcoming of this method is that the reducing agents used are mostly sodium sulfide, sodium bisulfite, sodium metabisulfite, and sulfhydryl compounds, all of which are toxic and easily cause secondary pollution to the environment.[20] The oxidation method has a relatively high solubility and a small relative

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molecular weight, and the common oxidants are mainly hydrogen peroxide, peroxyformic acid, peroxyacetic acid, and sodium hypochlorite, which also cause some environment problems, and the products generally need to be reduced with reducing agents;[16] the ionic liquid assisted method also has a high extraction rate and generally needs to be used with reducing agents.[18] The enzymatic hydrolysis method has the advantages of green, high efficiency, and mild reaction condition, which is one of the key research directions of keratin extraction technology from animal hair.

Nonspecific proteases and keratinases are used for the enzymatic hydrolysis method to obtain keratin from hair. Keratinase can break the disulfide bond. Thus, the extraction efficiency of keratin can be improved, but commercial keratinase is rare and the price is high.[21] Nonspecific proteases, commonly used for enzymatic extraction of hair keratin, enhance the efficiency of keratin extraction mainly by catalyzing the hydrolysis of peptide bonds in the keratin. However, nonspecific proteases do not break the disulfide bond of keratin, and the presence of the lipid-like layer outside the scale layer on the hair surface prevents the enzyme from contacting and penetrating the hair, which affects the catalytic action of the enzyme and decreases the extraction efficiency of keratin. Therefore, before enzymatic treatment, the hair is generally pretreated by oxidizing agents, reducing agents, alkalis, or other substances to destroy the lipid-like substances on the surface of the hair, improving the efficiency of enzymatic action.[22] The pretreatment protocols and enzymatic action conditions directly affect the solubility of hair, the extraction rate of keratin, and the relative molecular weights and structural characteristics of the extracted keratin samples.

In this work, the effects of alkali pretreatment conditions and enzymatic action conditions on the solubility and keratin yield of bovine hair were investigated to construct an efficient alkali–enzyme extraction. The research would provide a reference for improving the comprehensive utilization ratio of the bovine hair recovered by enzymatic unhairing in leather processing.

Materials and Methods

Materials

Alkaline protease (LKT, 280000 U/g, Shandong Lonct Enzymes Co., Ltd., China); bovine hair was obtained from Xuzhou Nanhai Tannery Co., Ltd., China, by the enzymatic unhairing process, and was washed with deionized water three times to remove dirt and blood, and dried at 105°C to constant weight; analytical grade chemicals were used in biochemical analysis.

Methods

Pretreating the bovine hair with differing concentration of NaOH before enzymatic hydrolysis

A 1.0 g sample of bovine hair was shake-incubated with 25.0 mL of NaOH solutions with concentrations of 0.2, 0.4, 0.8, 1.6, and 2.0 M at 65°C for 2.0 h, respectively. Then, the pH value of the mixture was adjusted to 11.5 with 6.0 M HCl solution. To this mixture, 3324 U of LKT protease per gram of bovine hair was added, and the reaction was kept at 55°C for 4.0 h by shaking incubator (water bath) (SHA-BA, Changzhou Aohua Instruments Co., Ltd., China). Subsequently, the enzyme was inactivated at 95°C for 10 min. The hydrolysate was rapidly cooled to room temperature and centrifuged at 10000 × g at 4°C for 20 min with high-speed refrigerated centrifuge (TGL-16M, Changsha Xiangyi Centrifuge Instruments Co., Ltd., China). The supernatant was dialyzed in deionized water for 48 h using a dialysis bag (350 Da, Millipore Co., Ltd. USA), and the deionized water was replaced every 12 h. After dialysis was completed, the dialysate was lyophilized to obtain the keratin (EKE) prepared by the alkali-enzyme method, using vacuum lyophilizer (FD-113-50, Beijing Boyikang Experimental Instruments Co., Ltd., China). The undissolved hair was repeatedly washed three times with deionized water and dried at 40°C to constant weight.

Effect of alkaline pretreating time on enzymatic hydrolysis of bovine hair

A 1.0 g portion of bovine hair was shake-incubated with 25.0 mL of NaOH solutions with concentrations of 0.4 M at 65°C for 0.5, 1.0, 2.0, 3.0 and 4.0 h, respectively. Then, the pH value of the mixture was adjusted to 11.5 with 6.0 M HCl solution. The other operations were performed as above.

Effect of alkaline pretreating temperature on enzymatic hydrolysis of bovine hair

A 1.0 g portion of bovine hair was shake-incubated with 25.0 mL of NaOH solutions with concentrations of 0.4 M at 35°C, 45°C, 55°C, 65°C and 75°C for 2.0 h, respectively. Other operations were the same as above.

Effect of protease dosage on enzymatic hydrolysis of bovine hair

A 1.0 g portion of bovine hair was shake-incubated with 25.0 mL of NaOH solutions with concentrations of 0.4 M at 65°C for 2.0 h. Then, the pH value of the mixture was adjusted to 11.5 with 6.0 M HCl solution. To this mixture, 416 U, 832 U, 1664 U, 3324 U, and 6648 U of LKT protease per gram of bovine hair was added, respectively, and the reaction was kept at 55°C for 4.0 h. Other operations were the same as above.

Effect of pH on enzymatic hydrolysis of bovine hair

After pretreatment with alkali, the pH value was adjusted to 8.5, 9.5, 10.5, 11.5, and 12.5, respectively, and the enzyme dosage was controlled to 3324 U/g for hydrolysis. The other operations were the same as above.

Effect of time on enzymatic hydrolysis of bovine hair

After the bovine hair was pretreated with alkali, the pH was adjusted to 11.5 and treated with 3324 U/g of protease at 55°C for 2.0, 3.0, 4.0, 5.0, and 6.0 h, respectively. Other operations were the same as above.

Table I

Horizontal table of orthogonal experimental factors

Level	Alkali concentration (M)	Alkali pretreatment time (h)	Enzyme dosage (U/g)	Enzymatic treatment time (h)
	A	B	C	D
1	0.2	1.0	1662	2.0
2	0.4	2.0	3324	3.0
3	0.8	3.0	6648	4.0

Effect of temperature on enzymatic hydrolysis of bovine hair

After the bovine hair was pretreated with alkali, the pH was adjusted to 11.5 and treated with 3324 U/g of protease at 35°C, 45°C, 55°C, 65°C, and 75°C, and the other operations were the same as above.

Orthogonal experiments for hydrolysis of bovine hair by alkali-enzyme synergy

According to the optimization results of the single-factor test, a four-factor and three-level orthogonal experiment was conducted to further optimize the alkali-enzyme method conditions. The extraction ratio of keratin was considered as the response value, and the alkali concentration, enzyme dosage, enzymatic treatment time, and enzyme treatment temperature were considered as the response factors.

Determination of bovine hair solubility and keratin extraction rate

$$\text{Bovine hair solubility (\%)} = \frac{W_1}{W_0} \times 100\%$$

$$\text{Keratin extraction rate (\%)} = \frac{W_0 - W_2}{W_0} \times 100\%$$

W_0: weight of initial bovine hair, g; W_1: weight of bovine hair keratin after freeze-drying, g; W_2: weight of insoluble material after hydrolysis of bovine hair, g.

Determination of molecular weight

The molecular weight distribution of bovine hair hydrolysate was measured by gel filtration chromatography (GPC) and Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

GPC analyzation: 100.00 mg freeze-dried bovine hair hydrolysate was dissolved in 10.00 mL deionized water in volumetric flask, filtered through a 0.22 um filter. Then, the molecular weights of the hydrolysates were analyzed by Agilent 1260 Infinity II HPLC system and TSK gel G2000 SWXL column (7.8 mm × 300 mm) as the conditions reported before,[23] by using the mobile phase: 0.05 M, pH=7.0 NaH_2PO_4-Na_2HPO_4+0.15 M NaCl buffer.

SDS-PAGE analyzation: The hydrolysates solution was prepared at 10 mg/m L, and SDS-PAGE was performed using 12% separating gel, and after the end of the process, it was stained with Kormas Brilliant Blue R250 and decolorized with acetic acid-ethanol solution.

Analysis of amino acid composition of bovine hair keratin

A 50 mg portion of freeze-dried keratin powder was taken in a digestion tube, and 4 mL of 6 M HCl was added in it. After digesting at 120°C for 24 h, the sample solution was concentrated and evaporated to dryness.[24] After diluting to 100.00 mL with deionized water, the solution was filtered through the 0.22 μm filter membrane. The concentration of each amino acid in the hydrolyzate was determined with an amino acid analyzer.

Determination of DPPH scavenging activity

A 2.0 mL aliquot of ethanol solution containing 25.0 μg/mL DPPH was well-mixed with 2.0 mL of keratin solutions with different concentrations, respectively. The reaction was carried out in the dark at room temperature for 60 min, then measuring the absorbance of the mixture at 517 nm.[25] Meanwhile, 2.0 mL of ethanol solution and 2.0 mL of keratin solutions were mixed as blank, and 2.0 mL of ethanol solution containing DPPH and 2.0 mL of deionized water were mixed as control. The DPPH free radical scavenging activity was calculated according to the following equation.

$$DPPH\ (\%) = 1 - \frac{A_s - A_c}{A_b} \times 100\%$$

A_s is the absorbance of the keratin solution mixed with DPPH solution; A_c is the absorbance of the keratin solution mixed with ethanol; A_b is the absorbance of the DPPH solution mixed with deionized water.

Determination of ABTS scavenging activity

A 0.4 ml volume of keratin solutions with different concentrations were mixed with 3.6 mL of fresh 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) diammonium salt (ABTS) solution with an absorbance value of 0.7±0.02 (OD_{734}), and the mixture was kept in the dark at room temperature for 5 min, measuring the absorbance of the mixture at 734 nm.[26] Deionized water and reduced glutathione were alternatives of samples as the negative and positive control, respectively. The ABTS free radical scavenging activity was calculated according to the following equation.

$$ABTS\ (\%) = 1 - \frac{A_b - A_S}{A_b} \times 100\%$$

A_b is the absorbance of deionized water mixed with ABTS solution; A_s is the absorbance of the keratin solution mixed with ABTS solution.

Unless otherwise indicated, three replicate assays were conducted in all experiments, and the results were expressed as the average ± standard.

Results and Discussion

The alkali–enzyme extraction of keratin from bovine hair consisted of two steps: pretreatment with alkali and enzymatic treatment. The extraction was optimized by investigating the effect of process conditions on the solubility and the yield of keratin.

Optimization of alkali pretreatment parameters

Effect of alkali concentration

The NaOH concentrations were 0.2, 0.4, 0.8, 1.6, and 2.0 M. After pretreatment at 65°C for 2.0 h, the pH of the reaction mixture was adjusted to 11.5, and 3324 U of LKT protease per gram of bovine hair was added. Then, the reaction was kept at 55°C for 4.0 h. The results are shown in Figure 1.

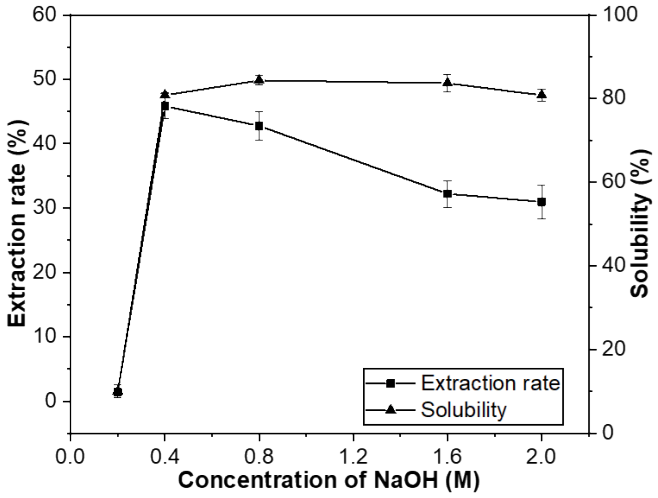

Figure 1. Effect of NaOH concentration on the solubility and keratin yield

Figure 1 shows the solubility and the keratin yield increases gradually as the NaOH concentration increases. When the NaOH concentration increases to 0.4 M, the solubility stabilizes, but the keratin yield decreases. This is mainly due to the scale layer of bovine hair contains many lipid-like materials and disulfide bonds with the hard structure, which makes the hair hydrophobic and resistant to enzymatic hydrolysis.[27] The pretreatment with NaOH can destroy the scale layer of the bovine hair surface and make it more hydrophilic, which is beneficial to improving the efficiency of the enzymatic action. However, as shown in Figure 2 and Table II, both the molecular weight of the bovine hair hydrolysates and their distributions (PD) would decrease when the concentration of sodium hydroxide reached more than 0.4 M in alkaline treatment. This could be attributed to that, when the concentration of alkali is too high, the hydrolysis of the peptide bonds in proteins would be accelerated and the higher molecular keratin would be further hydrolyzed into the low molecular components, producing the smaller molecule peptides, amino acids. Moreover, the dialysis treatment removed most of the small molecular hydrolysates, including amino acids, increasing the loss rate of keratin and decreasing the keratin yield. Therefore, the NaOH concentration should be about 0.4 M during pretreatment.

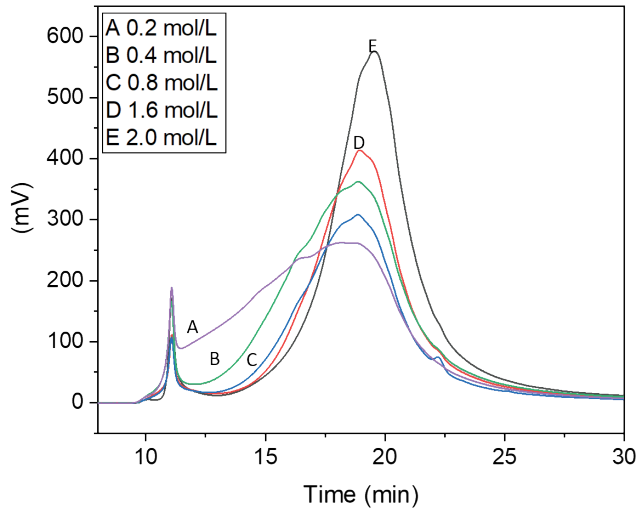

Figure 2. GPC gel chromatogram of keratin with differing NaOH concentration

Table II

Molecular weight distribution of keratin with differing NaOH concentration*

Concentration (M)		Percentage (%)	Mn/Da	Mw/Da	PD
0.2	1	5	67927	68017	1.00
	2	95	7392	35872	4.85
0.4	1	1	68620	68620	1.00
	2	99	7293	27189	2.66
0.8	1	3	66910	67025	1.00
	2	97	12304	23195	1.17
1.6	1	3	66731	66907	1.00
	2	97	7757	20693	1.99
2.0	1	3	66046	66143	1.00
	2	97	8308	18075	1.88

*: Mn is the number-average molecular weight, Mw is the weight-average molecular weight, PD is the molecular weight distribution coefficient, representing Mw/Mn

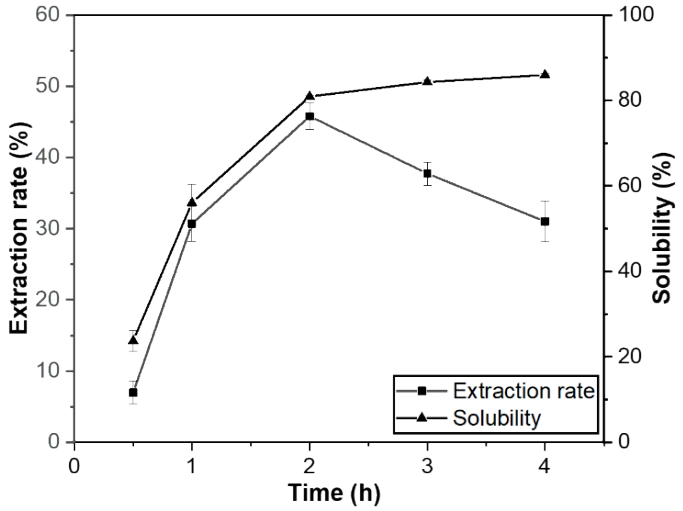

Figure 3. Effect of pretreatment time with NaOH on the solubility and keratin yield

Effect of pretreatment time with alkali

To investigate the effect of pretreatment time with alkali, the NaOH concentration was 0.4 M, and reactions were conducted at 65°C for 0.5, 1.0, 2.0, 3.0, and 4.0 h. The results are shown in Figure 3.

Figure 3 shows when the pretreatment time with alkali reaches 2.0 h, the solubility of bovine hair gradually stabilizes, and the yield of keratin gradually decreases. When the pretreatment time exceeds 2.0 h with 0.4 M NaOH solution, the peptide bond of keratin begins to be hydrolyzed by alkali, and the yield of keratin is affected by the formation of hydrolysis products with low molecular weights.

Effect of pretreatment temperature with alkali

To investigate the effect of pretreatment temperature with alkali, the NaOH concentration was 0.4 M, and reactions were conducted at 45°C, 55°C, 65°C, 75°C, and 85°C for 2.0 h. The results are shown in Figure 4.

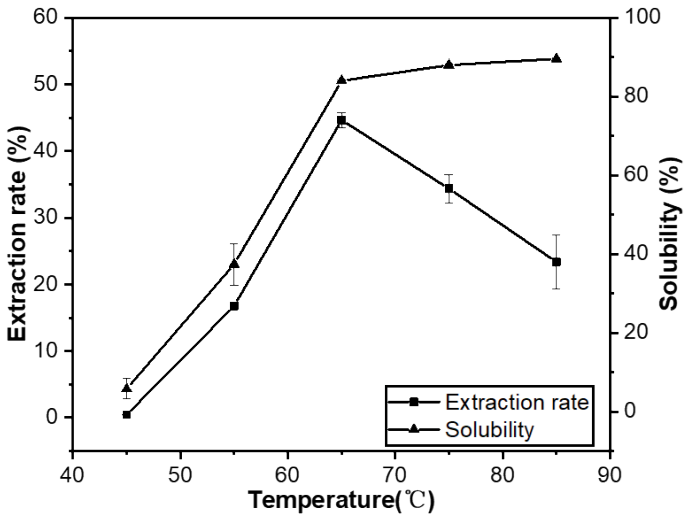

Figure 4. Effect of pretreatment temperature with NaOH on the solubility and keratin yield

Figure 4 shows that with the increase in pretreatment temperature, the solubility of bovine hair shows a trend of increasing and then stabilizing, but the keratin yield shows a trend of increasing and then decreasing. When the temperature is higher than 65°C, the keratin yield decreases sharply. On the one hand, the increase in pretreatment temperature helps make the keratin fiber swell fully. On the other hand, it causes the intense hydrolysis of the keratin peptide bond, resulting in a sharp decrease in keratin yield. Moreover, the excessive temperature makes controlling the temperature in the later enzymatic treatment more difficult. Therefore, the pretreatment temperature with alkali should be 65°C.

Optimization of enzymatic hydrolysis parameters

Effect of enzyme dosage

After pretreatment with 0.4 M NaOH solution at 65°C for 2.0 h, the pH of the reaction mixture was adjusted to 11.5 with 6.0 M HCl solution. Then, to investigate the effect of enzyme dosage, protease was added at different concentrations of 416, 832, 1664, 3324, and 6648 U per gram of bovine hair, and reactions were conducted at 55°C for 4.0 h. The results are shown in Figure 5.

Figure 5 shows the increase of enzyme dosage from 416 U/g to 6648 U/g has no substantial effect on the solubility of bovine hair, but the keratin yield shows a trend of increasing and then decreasing because the protease can effectively enter the interior of the bovine hair fiber and hydrolyze the peptide bond after pretreatment with alkali. When the enzyme concentration increases to about 3324 U/g, it can make the reaction of the enzyme and keratin reach better conditions. When the enzyme dosage further increases, it causes the excessive hydrolysis of the peptide bond and affects the final yield of keratin. As the dosage of enzyme increases, the enzyme protein content in the keratin also increases, which affects the purity of keratin. Therefore, the enzyme concentration should be 3324 U/g.

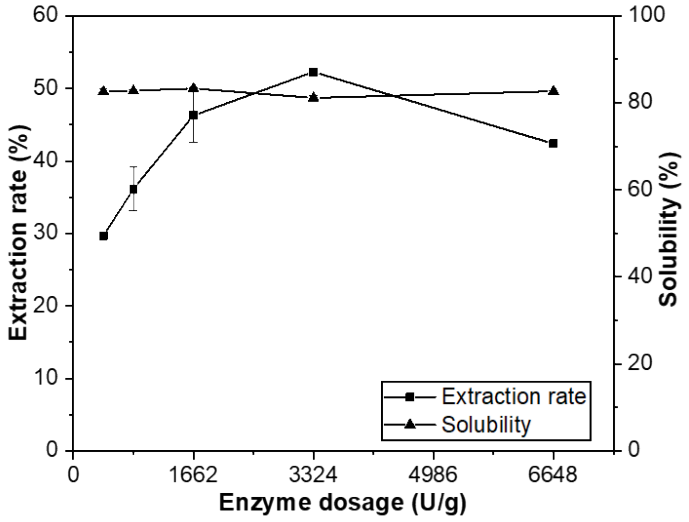

Figure 5. Effect of enzyme dosage on the solubility and keratin yield

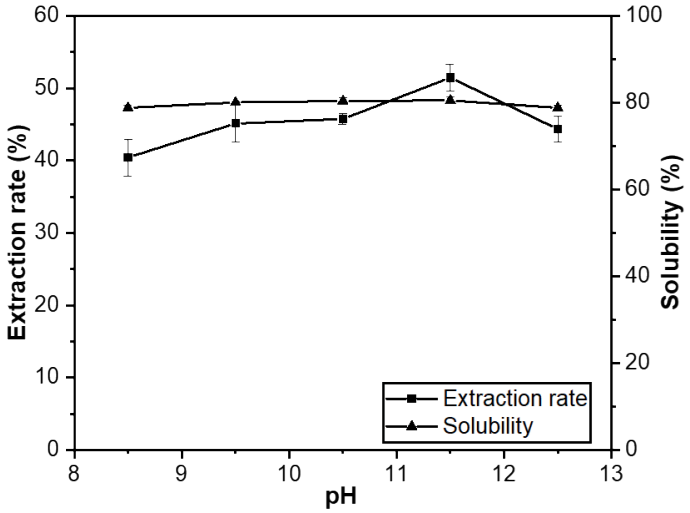

Figure 6. Effect of pH of enzymatic treatment on the solubility and keratin yield

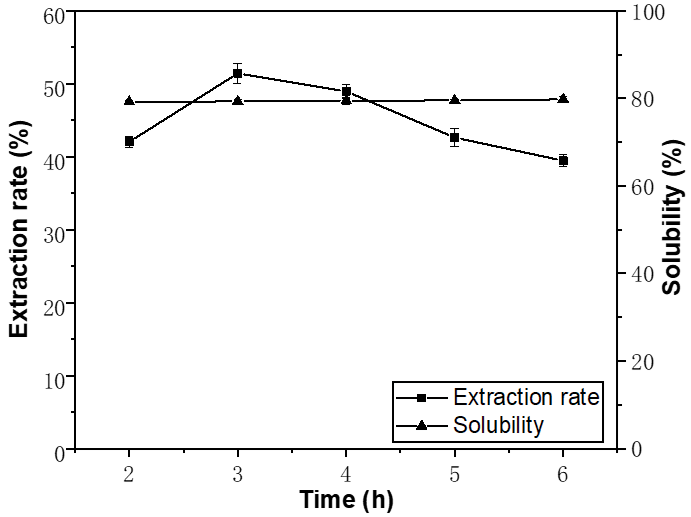

Figure 7. Effect of time of enzymatic treatment on the solubility and keratin yield

Effect of pH of enzymatic treatment

After the pretreatment with alkali, the pH values of reaction mixtures were adjusted to 8.5, 9.5, 10.5, 11.5, and 12.5 with 6.0 M HCl solution, and then treated with 3324 U/g (based on the enzyme activity at pH 11.5, and the same below) of protease at 55°C for 4.0 h. The results show that when the enzyme dosage is 3324 U/g, the change in pH does not have a considerable effect on the solubility of bovine hair and keratin yield, which are stable at about 80% and 45%, respectively, because the stability of the enzyme activity is better in the range of pH 8.5–12.5, and the enzyme dosage is high. Therefore, the pH has no remarkable effect on keratin hydrolysis.

Effect of time of enzymatic treatment

After pretreatment with alkali, the pH values of reaction mixtures were adjusted to 11.5, and each mixture was added 3324 U/g of the enzyme. Subsequently, reactions were conducted at 55°C for 2.0, 3.0, 4.0, 5.0, and 6.0 h. The results are shown in Figure 7. Under the above conditions, the increase of enzymatic treatment time has no substantial effect in the solubility of bovine hair, but the extraction rate of keratin increases slightly and then decreases gradually because the enzymatic hydrolysis of bovine hair is carried out in steps. In the initial stage of enzymatic treatment, the enzyme is affected by the adsorption of keratin fibers and mainly hydrolyzes the peptide bonds of undissolved fibrillar keratin, thus enhancing the solubility of bovine hair. When the hair is completely dissolved, the enzyme continues to act on the soluble keratin and further hydrolyzes keratin peptide bonds, which further reduces the molecular weight of soluble keratin and even generates peptides with low molecular weights and amino acids. Therefore, when the enzymatic treatment time exceeds about 3.0 h, the yield of keratin starts to decrease remarkably. Therefore, to ensure a high yield of keratin, the enzymatic treatment time should be about 3.0 h.

Effect of temperature of enzymatic treatment

After pretreatment with alkali, the pH of the reaction mixture was adjusted to 11.5, and 3324 U/g of the enzyme was added. To investigate the effect of temperature on the enzymatic treatment further, reactions were conducted at 35°C, 45°C, 55°C, 65°C and 75°C for 3.0 h. The results are shown in Figure 8.

Figure 8 shows that the temperatures below 45°C have no substantial effect on the solubility and the keratin extraction rate. Temperatures higher than 45°C also have no considerable effect on the dissolution rate, but the keratin yield gradually decreases. When the temperature is higher (under higher pH conditions), although the enzyme activity is inhibited or even inactivated, the protein becomes alkaline hydrolyzed. Therefore, the keratin further hydrolyzes to form peptides with low molecular weights and amino acids, affecting the keratin yield. The maximum keratin yield is obtained when the enzymatic treatment temperature is about 45°C.

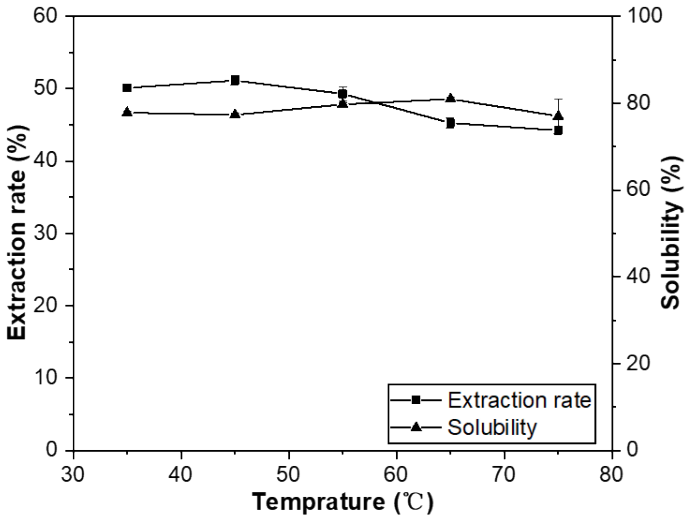

Figure 8. Effect of temperature of enzymatic treatment on the solubility and keratin yield

Optimization of orthogonal experiment

According to the result of the single-factor experiment, the optimal conditions for the keratin extraction of bovine hair by the alkali-enzyme method are as follows: For the pretreatment with alkali, the NaOH concentration is 0.4 M, and the pretreatment time and temperature cannot exceed 2.0 h and 65°C, respectively. For the following enzymatic treatment, the enzyme dosage, pH, and time are 3324 U/g, 8.5–12.5, and around 3.0 h, respectively, and the treatment temperature cannot exceed 45°C. Four factor variables were further selected as follows: alkali concentration (A), alkali treatment time (B), enzyme dosage (C), and enzyme treatment time (D). A four-factor and three-level orthogonal experiment was conducted to investigate the optimal conditions for keratin preparation. The results in Table III show the main order of the four key factors affecting the keratin extraction rate is alkali concentration of pretreatment > alkali pretreatment time > enzyme dosage > enzyme treatment time. The optimal conditions of the experiment can be obtained as follows: A2B1C2D3, that is, the NaOH concentration, pretreatment time, enzyme dosage, and enzymatic treatment time are 0.4 M, 1.0 h, 3324 U/g, and 4.0 h, respectively. Thus, the keratin extraction rate is 61.21%, which is similar to the results obtained from the single-factor optimization experiment. The best combination of enzymatic hydrolysis by calculating the K value is A2B1C3D3, that is, the NaOH concentration, pretreatment time, enzyme dosage, and enzymatic treatment time are 0.4 M, 1.0 h, 6648 U/g, and 4.0 h, respectively. The keratin extraction rate is 62.46%, which is greater than the results of the nine experiments in the orthogonal experiment. Owing to the double amounts of enzyme, the extraction rate only increases by 1.0%. Therefore, A2B1C2D3 is selected as the optimal reaction condition.

Table IV shows that compared with the conventional alkaline hydrolysis method reported in the literature[28] and the optimized alkaline pretreatment conditions lacking only the enzyme, the alkali–enzyme synergistic hydrolysis method can reduce the amount of alkali from 80.0% to 40.0%, and the acid dosage used for adjusting pH also decreases from 73% to 43.0%. Although the bovine hair solubility of the alkali–enzyme method is lower than that of the alkaline hydrolysis method, the extracted keratin yield increases from 24.4% to 58.1% because the dissolved keratin is easily hydrolyzed into amino acids and other products with low molecular weight when the alkali concentration is high, resulting in the loss of keratin.

Table III

Results of orthogonal experiment of keratin preparation obtained from bovine hair by alkali-enzyme method

Number	A	B	C	D	Extraction rate /%
1	1	1	1	1	1.21
2	1	2	2	2	1.59
3	1	3	3	3	2.92
4	2	1	2	3	61.21
5	2	2	3	1	58.35
6	2	3	1	2	55.36
7	3	1	3	2	55.29
8	3	2	1	3	51.26
9	3	3	2	1	48.26
K1	5.72	127.71	117.83	117.82	
K2	174.92	121.20	121.06	122.24	
K3	154.81	116.54	126.56	125.39	
k1	1.91	42.57	39.28	39.27	
k2	58.31	40.40	40.35	40.75	
k3	51.60	38.85	42.19	41.80	
Range	52.90	3.72	2.91	2.52	
Order-Sequence	A>B>C>D				
Optimal Level	A2	B1	C3	D3	
Optimal Combination	A2B1C3D3				

Table IV

Comparison of keratin extraction of alkali-enzyme and alkaline hydrolysis method

Method	Alkali dosage /%	Acid dosage /%	Solubility/%	Keratin yield/%
Alkaline hydrolysis*	80.0	73.0	90.0	24.4
Alkali-Enzyme hydrolysis**	40.0	43.0	72.1	58.1
Alkaline pretreatment***	40.0	43.0	33.9	16.8

*Alkaline hydrolysis conditions (AKE): 0.8 M NaOH, 1:25 solid-liquid ratio of, 65°C, 4.0 h.[28]

**Alkali-enzyme hydrolysis conditions (EKE): for pretreatment, 0.4 M NaOH, 1:25 solid-liquid ratio, 65°C, and 1.0 h are selected; for enzymatic treatment, when the reaction mixture is cooling down to 45°C, the pH of the mixture is adjusted to 11.5, and the reaction is conducted for 4.0 h by adding 3324 U/g of the protease.

***Alkaline pretreatment: 0.4 M NaOH, 1:25 solid-liquid ratio, 65°C, and 1.0 h are selected; then, when the reaction mixture is cooling down to 45°C, the pH of the mixture is adjusted to 11.5, and the reaction is conducted for 4.0 h.

Analysis and evaluation of the keratin extracted from bovine hair

Molecular weight and distribution of keratin

Studies have shown that the molecular weight of bovine hair hydrolyzed keratin affects its application properties, therefore the molecular weight and distribution of bovine hair hydrolyzed products were analyzed by gel filtration chromatography (GPC) after dialysis, concentration and drying, and the results are shown in Figure 9 and Table V.

Bovine hair keratin has many intermolecular crosslinks and is a large molecular protein. Upon alkali or alkali-enzyme hydrolysis, keratin undergoes cleavage of peptide bonds. Both bovine hair hydrolysis products (after dialysis) have two main fractions, consisting of proteins with molecular weights (Mw) in the range of 35.6 kDa-66.7 kDa Da. The content of fractions around 65.9 kDa was 18.55% in AKE and 81.45% in Mw of 43.5 kDa; the content of fractions around 66.7 kDa was 2.95% in AKE and 97.05% in Mw of 35.6 kDa. The proportion of higher molecular weight fractions in both bovine hair keratin was low. In alkali-hydrolyzed keratin, the content of the large molecular weight fraction was significantly higher than that of the enzymatic digestion product, probably because of specific cleavage and efficient catalysis by the enzyme. The low molecular weight fraction of EKE accounted for the majority of the fraction and had a lower molecular weight than the AKE. The results of SDS-PAGE analyzation in Figure 9 further show that the final keratin produced from bovine hair by both alkaline and alkaline-enzyme method contains protein components with continuous molecular weight distribution. This is consistent with the

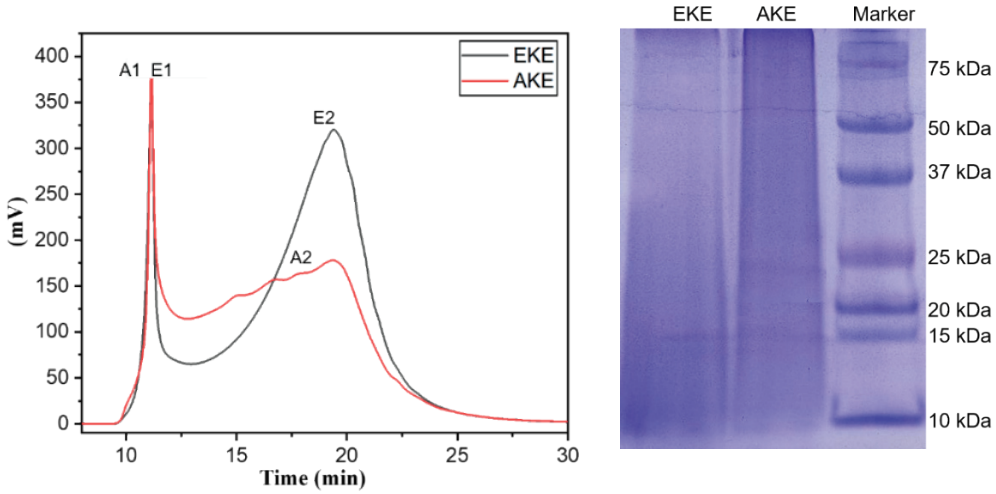

Figure 9. GPC gel chromatogram and SDS-PAGE of keratin

Table V

Molecular weight distribution of keratin

Sample		Percentage (%)	Mn/Da	Mw/Da	PD
AKE	1	18.55	65817	65913	1.00
	2	81.45	37642	43548	1.16
EKE	1	2.95	66643	66714	1.00
	2	97.05	25181	35639	1.41

Table VI

Analysis of amino acid composition of bovine hair and keratin (mg/100 mg)

Amino acid type	HAA*	BAA**	AAA***	TAA****
Bovine hair	35.67	12.97	5.18	53.82
EKE	32.55	11.94	4.20	48.69
AKE	31.52	15.36	3.93	50.81
Cottonseed Protein Peptide[30]	22.58	12.19	5.99	40.76
Okra Protein Peptide[31]	32.00	9.22	1.02	42.24

*HAA-total hydrophobic amino acids (Gly, Ala, Val, Pro, Phe, Leu, Ile, Met);

**BAA-total alkaline amino acids (Arg, Lys, His);

***AAA-total aromatic amino acids (Phe, Tyr);

****TAA-total amino acids affecting antioxidant properties.

results of GPC, for the PD was larger than 1.0. This could be attributed to the fact that the hydrolysis sites of the protein peptide bonds by alkaline are random, and the LKT protease can catalytic hydrolysis for the peptide bonds formed by most amino acids.

Amino acid composition of bovine hair keratin

Antioxidants are an important area of application for protein hydrolysis products. Studies have shown that hydrophobic, alkaline and aromatic amino acids can act as good electron donors to scavenge free radicals and enhance the antioxidant properties of protein products.[29] Therefore, the content of amino acids related to antioxidant properties in the prepared bovine hair keratin was analyzed and compared with protein peptides with good antioxidant properties reported. Table VI shows that the amino acid content of bovine hair hydrolysates related to antioxidant properties ranged from 48.96 mg/100 mg to 50.81 mg/100 mg, which was significantly higher than the cottonseed protein peptide and okra Protein peptides.[30,31] It can be speculated that bovine hair keratin products have higher antioxidant properties.

DPPH free radical scavenging ability

1,1-diphenyl-2-trinitrophenylhydrazine (DPPH) is a stable free radical and is widely used to assess the free radical scavenging ability of protein hydrolysates, peptides, and other substances.[32]

As seen in Figure 10 and Table VII, both AKE and EKE show a good free radical scavenging ability and the scavenging rate increases with the increase in peptide concentration. EKE and AKE have the IC_{50} value of 1.16 mg/mL and 1.51 mg/mL, respectively, and the IC_{50} of positive control GSH was 0.39 mg/mL. Although the DPPH free radical scavenging capacity of keratins were lower than the GSH, it is significantly higher than elastin peptides, whose IC_{50} value is 4.86 mg/mL.[33] In addition, the scavenging capacity of EKE for DPPH radicals was higher than AKE, mainly because AKE contains more randomly curled structures, which affects its antioxidant properties.[31]

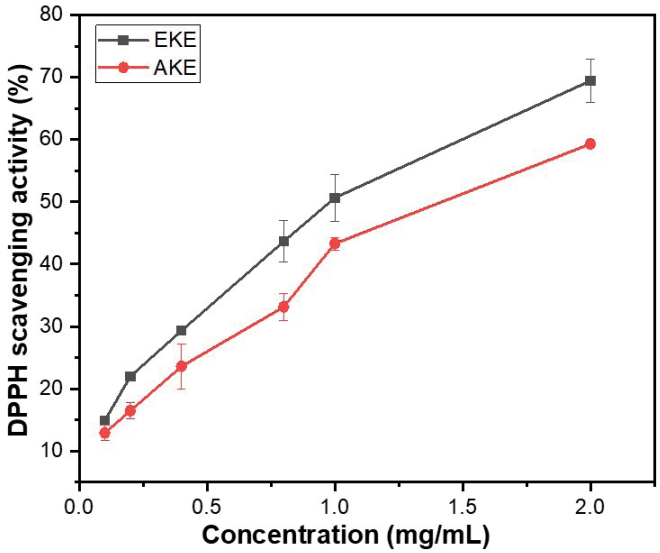

Figure 10. DPPH free radical Scavenging abilities of keratin under different concentrations

Table VII

DPPH free radical Scavenging abilities of keratin under different concentrations

Sample	Regression equation	R^2	IC_{50}(mg/mL)
AKE	y = 0.2462x + 0.1298	0.9676	1.51
EKE	y = 0.2813x + 0.1723	0.9572	1.16

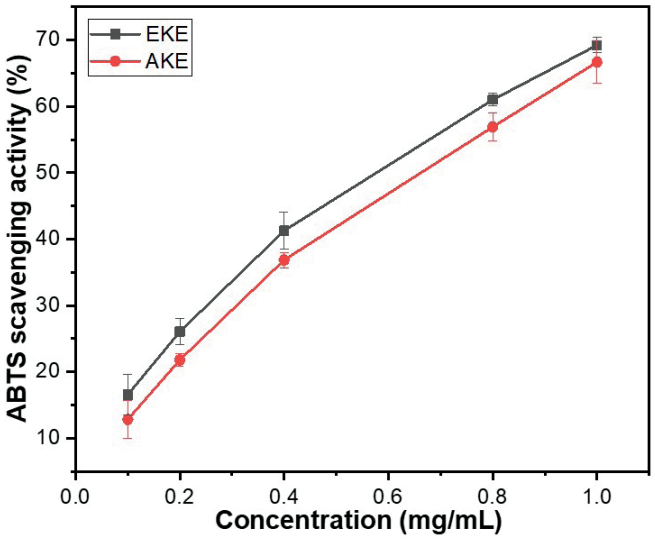

Figure 11. ABTS free radical Scavenging abilities of keratin under different concentrations

Table VIII

ABTS free radical Scavenging abilities of keratin under different concentrations

Sample	Regression equation	R^2	IC_{50}(mg/mL)
AKE	y = 0.5471x + 0.0951	0.9869	0.74
EKE	y = 0.5724x + 0.1421	0.9826	0.63

ABTS free radical scavenging ability

2,2-biazo-bis (3-ethyl-benzothiazole-6-sulfonic acid) diammonium salt (ABTS) is a positively charged free radical that can acquire an electron from the antioxidant molecule to form a stable neutral molecule and has been widely used for the antioxidant activity assay.

As seen in Figure 11 and Table VIII, both bovine hair keratin AKE and EKE have significant scavenging effects on ABTS radicals, and their scavenging ability increase in a dose-dependent way when the concentration of keratin increases to 1.0 mg/mL, the ABTS free radical scavenging rate of EKE and AKE is 65%-70%. The IC_{50} values of the scavenging capacity of keratin for ABTS obtained from the two hydrolysis methods were 0.74 mg/mL and 0.63 mg/mL, both higher than the glutathione (0.54 mg/mL). The scavenging capacity of keratin for ABTS was weaker than the glutathione, but both were stronger than that of the bovine hair hydrolysate reported.[34]

Conclusion

Enzymatic unhairing technology have been gradually widely applied in practical production, and the tanneries are concerned about how to improve the solubility and keratin yield of the recycled bovine hair, and the basic application properties such as the antioxidant properties of the resulting keratin. The work in this paper reports the synergistic effect of enzyme and alkali in keratin extraction from hair wastes generated by leather industry. The optimal conditions for the bovine hair keratin preparation by the alkali-enzyme method were obtained by single-factor and orthogonal experiments: the NaOH concentration, pretreatment time, pretreatment temperature, enzyme dosage, enzymatic treatment time, enzymatic treatment temperature, and enzymatic treatment pH are 0.4 M, 1.0 h, 65°C, 3324 U/g, 4.0 h, 45°C, and pH 11.5, respectively. Under these conditions, the keratin extraction rate was 61.21%. Compared with the alkali treatment, the alkali-enzyme treatment reduced the alkali dosage and improved the dissolution rate, which complied with the solid waste principle. Moreover, the prepared keratin had high in vitro antioxidant activity. The synergistic method successfully increases the bovine hair, the keratin yields and the antioxidant activity of keratin, which provided a new idea for the resource utilization of bovine hair waste.

Acknowledgements

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