



## The extraction of collagen protein from pigskin

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### ABSTRACT

*As the collagen protein market increasingly grows, it becomes a priority to explore a economic, stable, and environmentally-friendly extraction method to realize the high-value transformation of pigskin. In this paper, the structure of collagen protein is introduced first. Then five methods, which extract collagen protein from pigskin or a kind of leather industry waste-chrome shavings, are reviewed. These methods include the salting out method, alkali method, acid method, enzyme method and hybrid method. They are contrasted in several ways, and thus provide evidence for better extracting collagen protein.*

**Keywords:** Collagen protein; extraction; pigskin; chrome shavings

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### INTRODUCTION

With the consistent development of production level and scientific technology in China, the production of meat is changing rapidly. According to data collected by the National Bureau of Statistics in February 2013, the total yield of meat in 2012 reached 83.84 million tons. Of all meat, the total yield of pork, beef and lamb were 53.35, 6.62, and 4.01 million tons, respectively. This indicates that the production of pork far exceeds that of other meat. Pork products are predominant in our country's meat industry. Pigskin, as a by-product of pork production, has not been well utilized except for a fraction of 20%~30%, which are made into leather products [1]. Thus, it is meaningful to extract collagen protein, which has high added values, from pigskin resources or leather industry wastes. This will greatly benefit the pig-breeding industry financially.

Collagen protein is a biopolymer. It is a white, opaque, free of branched chain fibrous protein. It can be found in animal's skin, bone, cartilage, tooth, tendon, ligament, and blood vessel. It is an extremely important structural protein in connective tissues, and it is often of a network or fiber structure. Collagen protein functions as a supporter for organs, and a protector of the body. In animal cells, it plays an important part to connect tissues together. Collagen protein is the most abundant protein in mammals, making up from 25% to 30% of the whole body protein content, which is equivalent to 6% of body weight [2]. Collagen protein has various advantages: it has wide raw material resources; it is renewable, degradable, and biocompatible; its structure is adjustable. Thus, it is extensively used in making health care products, food [3], cosmetics [4], medical materials [5] [6] [7], fodder, animal feed, and paper. The market of collagen protein is huge.

### The structure of collagen protein

Collagen protein is a structural protein of extracellular matrix. Its molecules gather in extracellular matrix and become a super molecular structure, with a molecular weight of 300KDa. There are three types of common collagen protein: type-I, type-II, and type-III. Type-I collagen protein makes up approximately 90% of the whole collagen protein in organisms. It is mostly type-I collagen protein in animal's skin, tendon, and bone. Type-I collagen protein is composed of two equivalent  $\alpha_1$  chains and one  $\alpha_2$  chain. Two  $\alpha$  chains form into a peptide chain dimer, which is known as the  $\beta$ -peptide chain. Three  $\alpha$  chains form into a peptide chain trimer; which is known as the  $\gamma$ -peptide chain. The  $\gamma$ -peptide chain is the tropocollagen molecule. The molecular weight of each peptide chain is about

100KDa. It is composed of 1052 amino acid residues. The diameter and length are measured to be around 1.5nm and 300nm under electron microscopy [8]. Repeat units of Gly-X-Y (Gly stands for glycine; X usually is proline; and Y usually is hydroxyproline or hydroxylysine) form its polypeptide chain. The most significant difference between the collagen protein and other proteins is that collagen protein has a special amino acid composition. In collagen protein, the total contents of glycine (~20%), proline and hydroxyproline (~25%), and glutamic acid (~11%) exceed 50% [9]. Additionally, there is a large amount of hydroxylysine and alanine. The contents of proline and hydroxyproline are the highest in type-I collagen, while hydroxylysine only exists in collagen protein but not other proteins [10]. The most typical structural characteristic of collagen protein is the triple helix structure. Triple helix structure is a stable right-handed superhelix structure formed by three  $\alpha$ -chains, i.e., three left-handed helix peptide chains. The diversity and complexity of the collagen protein's structure has determined its important role and good application prospect in many fields [11].

### Study on the extraction of collagen protein

Collagen protein is a component of extracellular matrix. It exists in body in an insoluble macromolecular structure, and combines with proteoglycan, glycoprotein, and etc. Thus, the preparation of collagen protein includes: selection of the materials, preprocess, extraction, separation, purification and so on. As the extraction techniques of protein develop, there are more and more methods to extract collagen protein. Generally, there are two categories: methods that extract from animal tissues and methods that synthesize artificially. For synthesis methods, there are three major approaches: chemical synthesis, DNA recombination synthesis, and enzymatic synthesis. However, the high expense and various associated by-products of synthesis methods make it difficult to realize mass-produce. DNA recombination synthesis method is the most promising one out of all synthesis methods.

At present, most of collagen proteins in the market mainly come from animal tissues; however, there are some that are extracted from the solid waste in leather industry. Because of the homology between pigs and human beings, there are fewer disease sources. Thus, we study how to extract collagen protein from pigskin. Commonly used methods are: salting out method, alkaline method, acid method, and enzyme method.

### The salting out method

Similar to the general protein, collagen proteins also have the properties of salt soluble and salting out. Then different types of collagen proteins can be separated using the relationship between different collagen proteins and salt concentrations. Salting out method is mainly used in extracting the soluble collagen protein from tissues. Neutral salt solution is usually used, such as the solution of NaCl, Tris-HCl, phosphate, or citrate. Controlling the concentration of salt is the key in this method. For example, when the concentration of sodium chloride is less than 1.0mol/L in the neutral solution, it is good for the dissolution of type-I collagen; when the concentration is bigger than 1.0mol/L, it is good for the precipitate of type-I collagen [12]. However, there are less soluble collagen proteins in adult animal tissues, because most collagen protein molecules are cross-linked to each other. To conclude, the salting out method to extract collagen protein is not stable, and its usage is limited.

### The alkali method

Alkali has strong hydrolysis ability to collagen fibers. Through hydrolysis, large amounts of hydrolyzed collagen proteins are produced. Alkali method is generally used to extract collagen protein from leather wastes. Specifically, alkaline matters (such as lime, sodium hydroxide, sodium carbonate, and magnesium oxide) are used for impregnation. Treating chromium-containing leather wastes with the alkali method not only extracts collagen protein, but can also de-chromes chrome shavings. In chrome shavings, chromium coordinates and combines with the carboxyl of collagen. When hydrolyzing with alkali method, hydroxyl and chromium begin to coordinate because the coordination power between hydroxyl and chromium is stronger; and thus the carboxyl of collagen is freed. Chrome shavings are now de-chromed. Hydroxyl and chromium combined into  $\text{Cr}(\text{OH})_3$  precipitate. After separation the solution of collagen protein is obtained. The dechroming fraction of alkali method is higher than that of acid method, but it has some disadvantages as well. For example, the hydrolysis level of the obtained collagen protein is high; the molecular weight is small; ash content and chrome content are relatively high; the alkali method will destroy all amino acids that contain hydroxyl and sulfhydryl; the final products are racemized. Thus, collagen proteins that are extracted through the alkali method are not suitable for edible or medical purposes. To overcome these weaknesses, Kunyu Wang *et al.* [13] proposed a new fractionation method to extract high quality collagen protein from chrome shavings. In Kunyu's paper, chrome shavings were firstly handled under mild extraction conditions (for example, relatively low temperature, low alkali dosage and short extraction time), so that collagen products, which were easier to dissolve, were obtained first. Then extraction conditions were gradually strengthened to extract collagen products that were relatively difficult to dissolve. Repeating 2-4 times, extraction was completed. This fractionation method can extract collagen protein that outperforms products from the general alkali method in every evaluation index. For the general alkali method, collagen extraction fraction is 25.9%; ash content is 10.3%; and chromium content is 23.2mg/kg. For the fractionation method, these numbers were 43.3%; less than 2%; and

less than 2mg/kg, respectively. Moreover, the molecular weight of collagen protein obtained from the fractionation method was 85KDa. A chromium content that is lower than 2mg/kg indicated that collagen protein obtained from the fractionation method met standards of edible gelatin. Wenwei Liang *et al.* [14] used NaOH 9% to hydrolyze chrome shavings under temperature of 120°C for 6 hours. The extraction fraction turned out to be 87.45%. The obtained collagen protein was mixed with plant fibers without any further processing, and physical properties of the resulting paper, especially the tensile properties, were improved. Experiments concluded that the optimal adding amount of collagen protein was 4%.

The alkali method has various advantages. For example, the techniques are simple; it is easy to control; involved reactions are mild. Nowadays, this method is used often when producing collagen protein hydrolysis product – gelatin; however, this method causes heavy pollution for water, soil and air. In addition, it consumes a huge amount of water, and has a long production cycle. The techniques await further improvements.

#### ***The acid method***

Acid of low concentration can destroy salt bonds between molecules and Schiff bases, and cause collagen fibers to expand and dissolve. Thus, acetic acid, citric acid or hydrochloric acid of 0.5mol/L and with pH 2-3 can be used to extract collagen [15]. Faxing Luo *et al.* [16] discussed the influences of citric acid, acetic acid, and hydrochloric acid solutions on the dissolution rate of collagen protein extracted from pigskin. They concluded that citric acid has the best effects, and then acetic acid, and lastly hydrochloric acid.

Hydrolyzing by acid can also de-chrome leather wastes that originally contain chromium. Because organic acids like oxalic acid and citric acid have stronger coordination capabilities with chrome than carboxyl of collagen, acid radical ions can directly go into chrome coordination complexes and coordinate with chrome. Collagen carboxyl will be replaced, and dechroming is completed. The detached chromium is at a dissolved state under acid condition, and will be difficult to be completely separated from collagen protein. Thus, the dechroming rate under the acid method is relatively low.

The extraction fraction of obtained collagen protein under the acid method is much higher than that under the alkali method or enzyme method. The hydrolysis in both alkali and acid methods happens fast. Amino acids are easy to be destroyed and racemized. The hydrolysis of collagen chains is non-selective, and the resulting molecular weights of hydrolyzed collagen proteins are continuously distributed. In order to gain products with high added values, purification must be performed in the follow-up process at a great cost [17]. Because the degradation level of collagen molecules is exceptionally high, the resulting products will have relatively small molecular weights. Besides, both alkali and acid methods are strongly corrosive to equipment; secondary pollution is also severe. When applying alkali or acid methods to leather wastes that contain chromium, we not only can extract collagen protein, but also realize dechroming. In a view of dechroming effect, alkali method outperforms acid method. Thus in industry, alkali method is used more often when extracting collagen protein from leather wastes that contain chromium.

#### ***The enzyme method***

Using enzyme to extract collagen protein is one of the relatively ideal methods currently. Enzyme has a better reaction selectivity, and it is less destructive to collagen protein. We can gain active protein whose triple helix structure is better reserved. The resulting product will have a high purity, and stable physical and chemical properties. Thus, enzyme method is often used in extracting active collagen protein. In addition, this method has other advantages. For example, the reaction conditions are mild; it does not corrode equipment; it has low energy consumption. However, enzyme method does have some disadvantages. For example, hydrolysis is not complete enough; reaction time is long and etc. [18]

Commonly used enzymes to extract collagen protein are: pepsin [19], papain, trypsin [20] and etc. These enzymes only act on non-helix peptide chains of collagen protein, and usually have no effect for helix peptide chains. It is identified by the immunoblotting test that pepsin cut the collagen short at the position of N-terminal 3/4 [21]. This kind of enzyme retains the triple helix section in collagen protein, so that it is still biologically active. Thus, it is widely used in the extraction of collagen protein. Various kinds of enzymes in animal tissues have an optimal temperature between 35°C to 40°C. Most enzymes have an optimal pH between 5 and 8, for example, papain's best pH is between 5 and 5.5; trypsin's best pH is 7.5; pepsin has a lower optimal pH between 1.5 and 2.5 [22]. Chuan Wang *et al.* [23] compared the effects of extracting pigskin collagen protein between pancreatin, pepsin, and papain. Pancreatin was found to have the highest extraction rate and best operability; however, it caused relatively severe structural damage; molecular weight of the hydrolyzed collagen protein was the smallest, between 10 and 70 KDa; color of the lyophilized collagen protein sample is slightly yellow. Pepsin was found to best maintain the structure; the resulting molecular weight was around 100KDa; and color of the lyophilized sample was white; however, the

extraction rate was the smallest among all three enzymes. Papain's extraction rate and resulting molecular weight was found to be in between the other two enzymes. Because water is the solvent, it has the mildest reaction conditions. From this study, the order of hydrolysis ability was proved to be: pancreatin > papain > pepsin. The triple helix structure of the collagen protein obtained from pepsin hydrolysis was maintained relatively well.

Feng Zhang *et al.* [24] used neutral protease to treat leather materials. During enzymatic hydrolysis, reaction temperature was 40°C; reaction time was between 8 to 10 hours; dose of enzyme was 2.0g/(kg raw-hide). Intermittent stir was required during the procedure. Gelatin extraction was carried out under three different temperature conditions: 70°C for 1h, 80°C for 2h, and 90°C for 3h. The starting pH value was set to be 5. Under these conditions, the yield of gelatin could reach 23.05%. Compared with the traditional method, this method has a greatly shortened production cycle (7~10 days), little environmental pollution, and a good application prospect.

Using enzyme to extract collagen protein from chromium-containing leather wastes was studied previously in literature. Common de-chroming enzymes are neutral protease, alkaline protease, cellulose and so on. Xiujin Chen *et al.* [25] utilized alkaline protease to hydrolyze de-chromed shavings and prepared collagen hydrolysate. It was found in the study that the optimal hydrolysis conditions were: alkaline protease 0.16%, pH 9.5, 65°C, 40 minutes, and ratio of water to chrome shavings 5:1. Under such conditions, the extraction rate of collagen hydrolysate was 66.2%, and the chromium content was 4.20mg/kg.

Bi Wang *et al.* [26] used single and complex enzymes to extract collagen protein and its polypeptides with different ranges of molecular weights from leather scraps. The functional properties of the samples were determined. Enzymatic de-chroming has high specificity and high yield. It does not damage amino acids, and the resulting hydrolysate is relatively stable. However, de-chroming is required before applying the method; there are too many steps; and the cost is high.

#### **The hybrid method**

The salting out method, acid method, alkaline method, and enzyme method all have their own advantages and disadvantages. Thus, it becomes a hot topic in research exploring a hybrid method to achieve better outcomes.

#### *The acid-enzyme hybrid method*

Surui Liu *et al.* [27] used acid-enzyme hybrid method to extract type-I collagen protein. Specifically, pigskin was soaked in the solution of hydrochloric acid for 10~16h (pH=2.0~2.5). After the skin swelled, double distilled water was added for beating. Then pepsin was added for hydrolysis for 24h under the temperature of 4°C. After isolation and purification, the obtained type-I collagen protein kept well its triple helix structure of the natural collagen and could make great biomedical materials.

#### *The alkali-enzyme hybrid method*

Xihuai Qiang *et al.* [28] used alkali-enzyme hybrid method to extract collagen protein from chrome shavings. Firstly, they used 3% CaO, 3% MgO, 300% H<sub>2</sub>O, a temperature of 80°C, and 4 hours to extract gelatin. The extraction rate of gelatin was more than 40%. Then the residue was hydrolyzed with 1398 neutral proteinase, an enzyme dose of 0.125% and a temperature of 46°C, the yield of protein polypeptide reached 20%. The contents of Cr<sub>2</sub>O<sub>3</sub> were lower than 50mg/kg in both obtained gelatin and protein polypeptide.

#### *The acid-alkali oxidation hybrid method*

Using acid or alkali alone to treat chrome shavings can only give us elementary collagen protein products. However, using alternating acid-alkali method can result in improvement in de-chroming rate. Plus, collagen products that are of good stability, high quality, and thus high added values can be obtained. Zeen Shao *et al.* [29] proposed a four-step de-chroming method: soak with lime milk, soak with dilute sulfuric acid, soak with 0.5% NaOH solution, and soak with hydrogen peroxide. These four steps were used to treat chrome shavings to extract collagen protein and prepare pet feed. In the final product, the content of chromium was 15mg/kg, which was lower than the standard requirement of less than or equal to 25mg/kg for animal feed and formula feed by the National Feeding Law.

#### *The ultra-high pressure acid pretreatment method*

Liqing Chen *et al.* [30] proposed a novel pretreatment method using ultra-high pressure and acid to extract gelatin from pigskin. The pressure was 250 MPa; time was 10 minutes; and HCl concentration was 0.75%. Under such conditions, the extraction rate and gel strength of gelatin were up to 88.62% and 384.43g, respectively. It was seen a 20% increase in gel strength of gelatin compared to the traditional acid method without pretreatment. The pretreatment time was shortened significantly (from 20h of the traditional method to 15min). The quantity of acid wastewater was greatly reduced (concentration of acid was reduced from 1% to 0.75%, and the ratio of raw pigskin to the solution of HCl was reduced from 1:10g/mL to 1:1.5g/mL). Pollution was highly reduced, and the production

cycle was greatly shortened.

### CONCLUSION

Pigskin and solid leather waste contain a rich amount of collagen proteins. Transforming the resource and realizing its high utilization are the requirements of environmental protection, sustainable development, and economic growth. Collagen fibers can be easily damaged while extracting and processing. Thus, reaction conditions are not to be too strong. This causes a not very high extraction rate of collagen protein. At the same time, there are issues such as pollution and cost in the extraction of collagen protein. Thus, further research is needed to study how to extract collagen protein from pigskin and solid leather waste. Because collagen protein has various applications in food supplement, cosmetics, biomedical materials, animal feed, and etc. we believe that the potential development of the market for extracting collagen protein from pigskin is huge.

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