METHODS FOR OBTAINING OF KERATIN HYDROLYSATES FROM SHEEP WOOL

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ABSTRACT

Leather and textile production have the largest share in the generation of keratin-containing waste (hair, wool, etc.). For example, wool contains up to 95 % pure keratin, which can be extracted and used. However, a number of difficulties exist in the production of keratin hydrolysates due to the non-reactivity and stability of keratin. In addition to peptide bonds, the presence of disulfide bonds makes these processes extremely difficult. A number of technics have been studied to obtain keratin hydrolysates. Optimizing these processes and finding the best available technique is a major challenge for environmental protection.

The aim of the present study is to obtain and characterize keratin hydrolysates from sheep wool, respectively in native form and alkaline treated, using various oxidative and reductive methods. Three methods for hydrolysis were used, respectively: 1) hydrolysis with thioglycolic acid; 2) sulfitolysis with sodium pyrosulfate and urea; 3) hydrolysis with sodium hydroxide. The hydrolyzing ability of the three methods was compared, and the influence of the preliminary chemical treatment of the wool was taken into account. The obtained hydrolysates were characterized by qualitative reactions, spectrophotometric and FTIR analysis. It was proved that the method of hydrolysis with sodium hydroxide has the highest hydrolyzing effect and to a much greater extent for the alkaline treated wool than for the native one.

Keywords: sheep wool, keratin hydrolysates, extraction methods, analysis.

INTRODUCTION

The earliest use of keratin for medical purposes by the Chinese herbalist Li Shi Zhen dates back to the 16th century. The word "keratin" first appeared in the literature around 1850 to describe the material of which hard tissues are composed [1 - 3]. In 1905, John Hoffmeier's patent in the United States described the process of extracting keratins from animal horns using lime. The extracted keratins are used for the preparation of keratin gels, which can be reinforced by the addition of formaldehyde. These technologies were first used for animal horns and hooves, and were also used to extract keratin from wool and human hair. The development of techniques in the last century in terms of extraction process, purification and characterization of keratin proteins has led to the preparation of biomaterials based on keratin. Like many naturally occurring biomolecules, keratin has inherent biological activity and biocompatibility. These properties have led to the production of keratin biomaterials with medical applications: in wound healing, trauma, drug delivery, tissue engineering and for medical devices [1, 3, 4].

Significant interest is being shown in keratin as a new product for use in the pharmaceutical, medical, cosmetic and biotechnology industries. Wool keratin is a strong, insoluble biomaterial that can play a major structural role in biological systems [5 - 11].

There are a number of different keratins depending on the conditions of formation and the processes taking place in a living organism. They differ from each other both in their chemical composition and in some characteristic properties. The presence of a significant amount of cystine in keratin explains the presence of another type of cross-covalent bond between the main polypeptide chains in the direction of the side chains, namely the disulfide bond -S-S-. The disulfide bond is short and binds the polypeptide chain tightly, making keratin very resistant to various physicochemical effects. Due to the presence of a disulfide bond, keratins are sometimes called vulcanized proteins [12].

Unhairing process in the leather production is carried out using sodium sulfide, which reduces the disulfide bond and this leads to the destruction of the hair, due to the destruction of the cross-bridges between the polypeptide chains of keratin. In fact, the reduction itself does not cause the hair to dissolve. It only increases the solubility of reduced keratin in alkali. Thioglycolic acid and its salts have a strong reducing effect on the disulfide bond [13].

Many different methods have been used to obtain the keratin hydrolysates [14 - 20]. Keratin molecules are extremely stable and difficult to process, so they are processed under drastic conditions [14]. The hydrolysis of the peptide bonds proceeds relatively slowly in the presence of acids. The effect of the alkali is more pronounced. Under the alkali effect, the most vulnerable place is the disulfide bond, which leads to the formation of sulfonic acids and sulfhydryl derivatives [13, 20]. In long-term studies on the hydrolysis of keratins, some studies rely on long-term acid hydrolysis with concentrated sulfuric acid, other methods have been carried out using reducing agents - thioglycolic acid, sodium cyanide, sodium sulfide, and thirds use hydroxides - NaOH, KOH, $Ca(OH)_2$. There are also studies with the use of enzymes to denature keratin. All these methods have been known for many years, but the application of most of them is extremely difficult. During hydrolysis, in addition to breaking both types of bonds (disulfide and peptide), the resulting structure of keratin hydrolysates is different from the structure of keratin protein.

The aim of the present study is to obtain and characterize keratin hydrolysates from wool, respectively in native form and alkaline pre-treated, using different oxidative and reductive methods.

EXPERIMENTAL

Samples of native wool and pre-treated wool were used for the study. The wool, which is lime-sulphide pre-treated, is obtained after the process of unhairing of sheep leather under certain conditions.

Methods for hydrolysis of keratin-containing samples

Hydrolysis with thioglycolic acid

The samples were hydrolysed in an aqueous solution of 1M thioglycolic acid ($C_2H_4O_2S$) and 0.3 M urea at 30°C in reflux condenser and water bath during 6 hours [21].

Hydrolysis with sodium pyrosulphate (metabisulphate)

Keratin is extracted from wool by sulfitolysis with sodium metabisulfate [22]. Approximately 5 g of the cleaned and conditioned fibres are treated with 100 mL of a solution containing 8 M urea and 0.5 M sodium pyrosulphate ($Na_2S_2O_5$). The treatment continues until pH 6.5 with 5 N NaOH and it was reached by stirring for 2 h at 65°C.

Hydrolysis with sodium hydroxide

Cleaned and washed bird feathers are cut [3]. They were then dissolved in 5 % NaOH solution for 4 hours at 40°C. The resulting solution was dialyzed and precipitated with concentrated HCl at pH 4.2.

Methods for analysis of protein substances obtained after wool hydrolysis

Biuret method

Qualitative and quantitative determination of protein can be performed by a biuret reaction, which is characteristic for all proteins [23]. The protein solutions are coloured blue-violet in an alkaline medium in the presence of copper salts. The colouring is due to the formation of a complex compound of copper with the peptide groups. To a tube containing 1 mL of protein solution 1 - 2 mL of 10 % NaOH and 1 - 2 drops of 1 % $CuSO_4$ are added. When the mixture is shaken vigorously, a blue-violet or red-violet colour appears (an indicator of the presence of long or short polypeptide chains).

Spectrophotometric method

To each tube 4 mL of biuret reagent is added and left for 30 minutes at room temperature. The solutions are poured in a cuvette with a width of 10 mm wide at a wavelength λ = 540 nm. The apparatus used is type JENWAY 6300 Spectrophotometer.

Methods for determination of sulfur-containing amino acids

They are typical for proteins containing cystine and cysteine [23].

Nitroprusside reaction

In a test tube 0.5 mL protein solution is mixed with 0.5 mL of 10 % NaOH solution and heated during 3 min. After cooling 2 - 3 drops of sodium nitroprusside is added. A reddish-brown color appears.

Fol's reaction

A solution of NaOH was gradually added to 1 mL of lead acetate solution until the precipitated lead hydroxide is dissolved. Then 0,5 - 1,0 mL of the tested protein solution is added and heated until a black colour appears.

Use of infrared spectroscopy (FTIR) for analysis of the obtained hydrolysates

Infrared spectroscopy is one of the main instrumental methods for qualitative determination of functional groups, terminal group analysis as well as studying of configuration and rotational isomerism [24]. The analysis was performed using a Bruker Tensor 27 Spectrometer with a scanning speed of 10 kHz. The spectrum was recorded using an MCT detector (64 scans and 1 cm^{-1} resolution).

RESULTS AND DISCUSSION

The raw materials for the hydrolysis of keratin are samples from the hair cover (wool) from sheepskins, respectively in native form and lime-sulfide pre-treated. Each sample was finely cut and weighed to calculate the hydrolysis parameters. Wool samples were washed by surfactant and were defatted by means of a Soxhlet apparatus for extracting unbounded fats. Further they were conditioned in a desiccator for 24 hours, were cut, were weighed and prepared for hydrolysis.

The following methods were used for the hydrolysis of sheep wool:

1) hydrolysis with thioglycolic acid and urea;

2) sulphitolysis with sodium pyrosulphate and urea;

3) hydrolysis with 5 % NaOH.

Hydrolysis with 1M thioglycolic acid and 0.3M urea

Hydrolysis of native wool

Sample of 5 g conditioned native wool was hydrolyzed according to method [21] described above. Insufficient hydrolysis was observed on the first attempt. The experiment was carried out three times at the same conditions. The keratin hydrolyzate obtained is opaque, with wool particles, dark colored. After filtration and drying, no film formation was observed, but crystals. The filtered hydrolyzate was analyzed.

Hydrolysis of lime-sulfide pre-treated wool

A sample of 5 g conditioned wool, which obtained after unhairing processing by lime-sulfide method, was subjected to hydrolysis at the same conditions. After three treatments, the hydrolyzate obtained is opaque, with wool particles and has not been analyzed.

Hydrolysis with 0.5 M sodium pyrosulfate and 8 M urea

Hydrolysis of native wool

A sample of 5 g native wool is finely cut and subjected to sulfitolysis according to the method described in [22]. The hydrolysis process is triple repeated and result is not achieved.

Hydrolysis of lime-sulfide pre-treated wool

Sulfitolysis under the same conditions was performed for the pre-treated wool. After completion of the re-hydrolysis, the obtained solution is free of hair particles. The solution was filtered, dried and obtained in powder form.

Hydrolysis with sodium hydroxide

Hydrolysis of native wool

The sample of native wool was treated with a solution of 0.1 N HCl for 2 hours according to the methodology described in [3]. After washing, the sample was poured into 5 % NaOH for 4 hours at 40°C. After the second hour of treatment, the initial phase of rupture and hydrolysis of the wool has already been observed. After the duration of the treatment, the hydrolyzate was

filtered off and a very thin brittle film was obtained after drying. The keratin hydrolyzate was analyzed.

Hydrolysis of lime-sulfide pre-treated wool

A sample of 5 g of lime sulfide wool was hydrolyzed under the same conditions. The obtained hydrolyzate and keratin film were analyzed according to the methodical program.

The results of analysis of the keratin hydrolysates are shown in Table 1 and Figs. 1 - 4.

The results of the analysis of sample 1 (hydrolysis of native wool with thioglycolic acid) show that there are no polypeptide chains in the solution. An orange-yellow color, uncharacteristic of polypeptide chains, was observed during the Biuret reaction. The presence of a black coloring at the Fol's reaction showed that the disulfide bonds in the keratin were not affected during the treatment of the native sheep wool in the hydrolysis solution.

The Biuret reaction at sample 2 (hydrolysis of pre-treated wool by means of urea and sodium pyrosulfate) is light violet in color, which is evidence of the presence of polypeptides in the solution. Light color indicates the presence of very long polypeptide chains and the adsorption value A = 0.167. No sulfur-containing amino acids were detected in the solution, which correlates with the presence of long polypeptide chains. The Fol's reaction showed that the disulfide bonds in the keratin hydrolysate were broken because no characteristic black color was observed.

Sample 3 of hydrolyzed native wool (with 5 % NaOH) shows almost the same amount of polypeptides, i.e. absorption value A = 0.154. Biuret reaction also gives a light violet color. As a result of the achieved degree of hydrolysis, long polypeptide chains are present in the solution. A small amount of sulfur-containing amino acids is observed, which indicates the Nitroprusside reaction, a light brown color is obtained, which is not permanent. Destruction of disulfide bridges in the keratin hydrolysates was found.

The Biuret reaction at sample 4 (hydrolyses of pre-treated wool by means of NaOH) showed a high concentration of polypeptides in the Biuret reagent. Saturated violet coloring was observed and the absorption value A = 568. The absence of coloring at Fol's reaction showed destruction of disulfide bonds of the polypeptides. The red coloring at the Nitroprusside reaction is also an indication of the presence of sulfur-containing amino acids in the solution. Probably some of them have been hydrolyzed by the polypeptide chains during the sodium hydroxide treatment. This could be explained by the shorter polypeptide chains in the keratin hydrolysate.

FTIR spectroscopy of keratin hydrolysates is im-

Table 1. Results of the analysis of keratin hydrolysates from wool.

Type of sample Wool	Method of hydrolysis	Biuret reaction	Nitroprusside reaction	Fol's reaction
1. Native wool	thioglycolic acid and urea	Orange-yellow coloring	Does not give coloring	Black coloring
2. Lime-sulfide pre-treated wool	urea and sodium pyrosulfate	Light violet coloring A= 0,167	Does not give coloring	Does not give coloring
3. Native wool	5 % NaOH	Light violet coloring A= 0,154	Slight brown and then disappears	Does not give coloring
4. Lime-sulfide pre-treated wool	5 % NaOH	Violet coloring A= 0,568	Red coloring	Does not give coloring

portant to observe the changes in chemical structure as a result of different reagents effects. The amide or peptide bonds are represented by a set of characteristic maxima and fall in the range below 2000 cm^{-1}. Keratin shows characteristic bands associated primarily with peptide bonds (NH-CO). The vibrations at the peptide bonds are known as Amide I (in the range 1700 cm^{-1} - 1600 cm^{-1}), Amide II (1520 cm^{-1}) and Amide III (in the range 1200 cm^{-1} - 1300 cm^{-1}) [18].

Peaks responsible for Amide I - III are observed for our keratin hydrolysates. Amide I corresponds to the secondary structure of the polypeptide chains and refers to the vibrational state of C=O. In the spectra of keratin hydrolysates from the wool samples (Figs. 2 - 4), the absorption bands for Amide III (related to the vibrational states of C-N and N-H) and Amide I were observed. Absorption band at 1000 cm^{-1} - 1025 cm^{-1} indicates the presence of cysteine residues or sulfur-containing

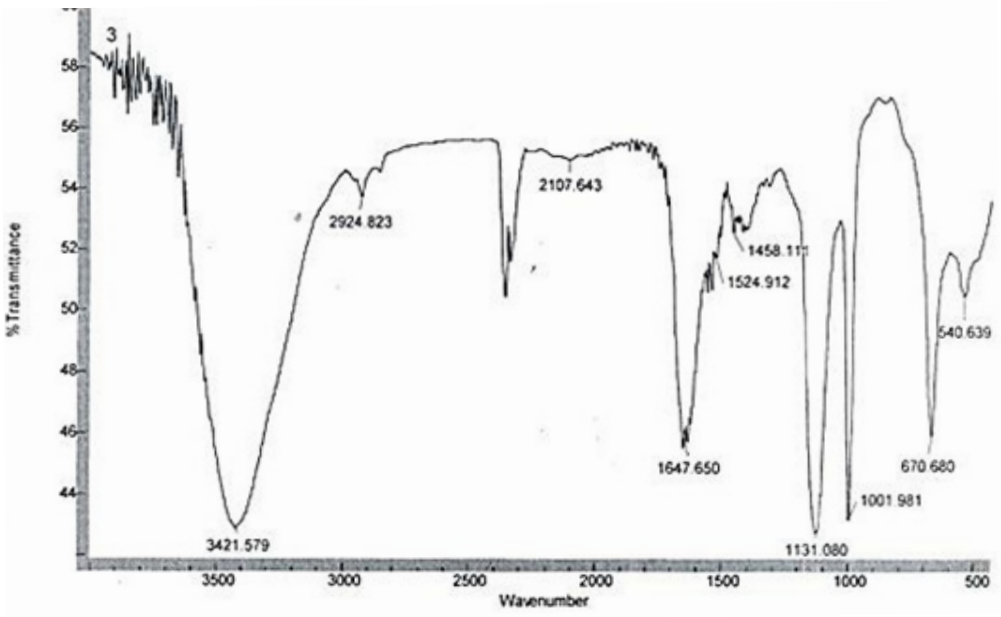

Fig.1. FTIR of keratin hydrolysate from native wool (with thioglycolic acid and urea).

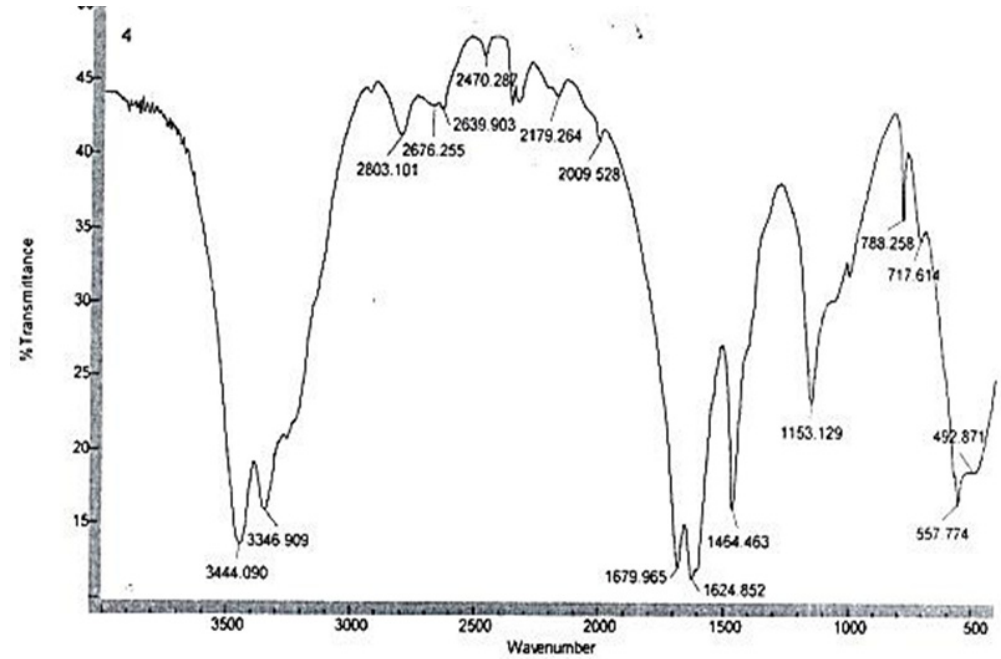

Fig. 2. FTIR of keratin hydrolysate from lime-sulfide pre-treated wool (with Na pyrosulfate and urea).

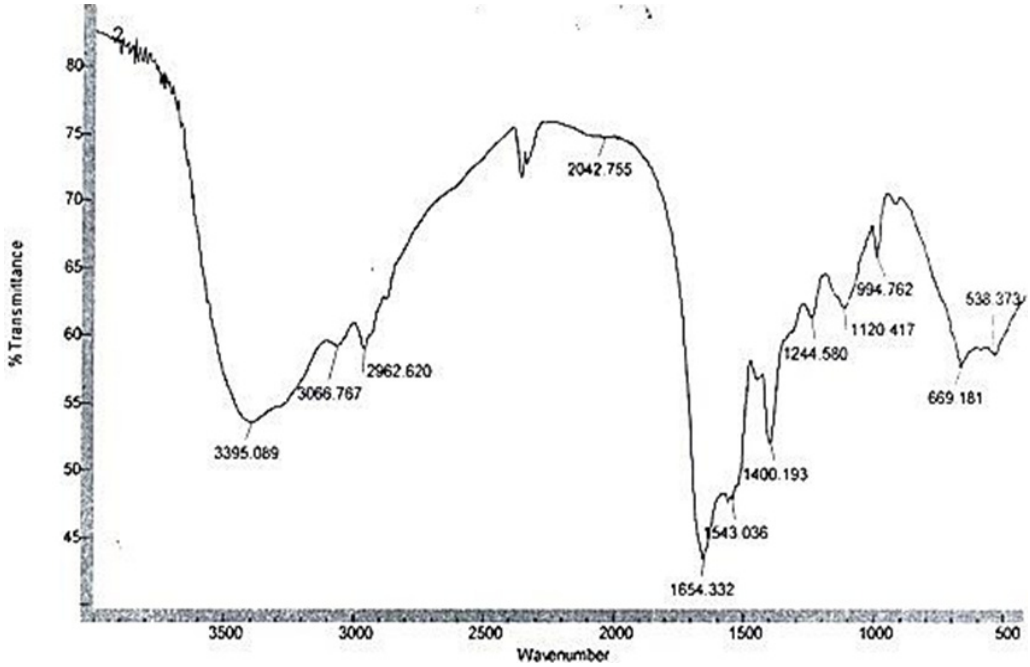

Fig. 3. FTIR of keratin hydrolysate from native wool (with NaOH).

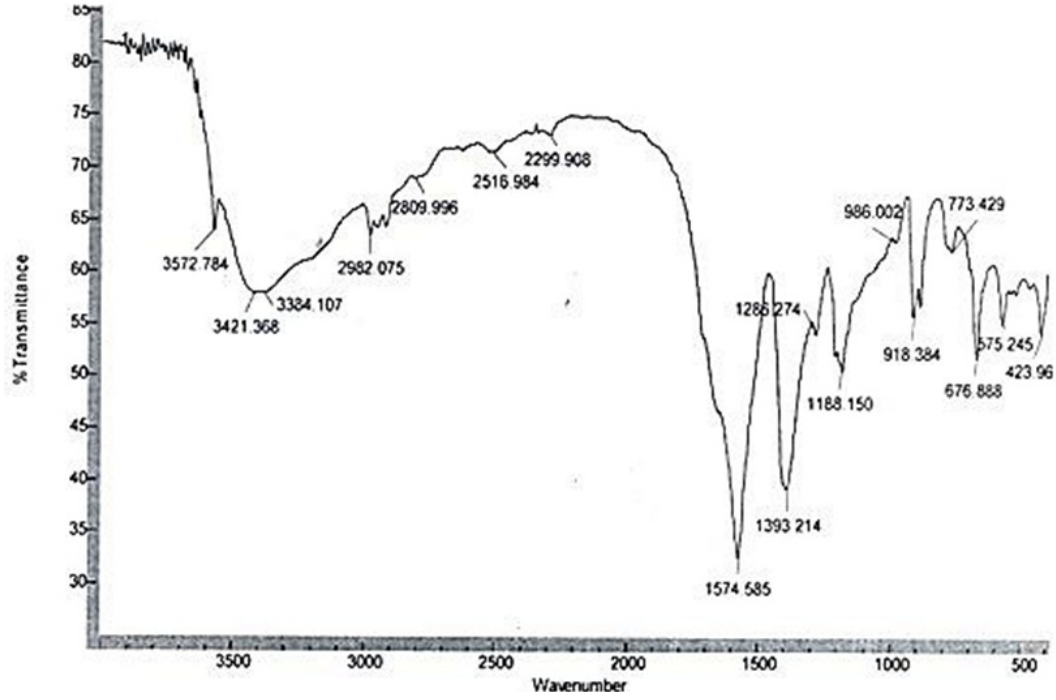

Fig. 4. FTIR of keratin hydrolysate from lime-sulfide pre-treated wool (with NaOH).

amino acids, and at 630 cm^{-1} - 625 cm^{-1} indicates the presence of S-S bonds. Destruction of the disulfide bonds was observed in the lime-sulfide pre-treated wool (Fig. 2 and Fig. 4), as well as at native wool with NaOH (Fig. 3), in contrast to the hydrolysis of the native wool with thioglycolic acid (Fig. 1). It is known that hydrolysis of the disulfide bond initially occurs under alkaline treatment. Observations from IR spectroscopic analyzes correlate with the qualitative and quantitative methods described above.

Based on the results from the hydrolyzes and the analyzes of the obtained keratin products, the following summary can be made. Finding the most suitable method for hydrolysis of keratin-containing waste is

essential both for solving the environmental problem of solid waste and for the utilization of this raw material, which is so valuable for many other industries.

In our study, the hydrolyzing ability of three methods was compared, as well as the influence of the preliminary chemical treatment of the wool. Sodium hydroxide has the strongest hydrolyzing effect on both the native and the wool obtained after lime-sulfide unhairing process. The wool pre-treatment during unhairing with $Ca(OH)_2$ and Na_2S greatly facilitates the hydrolysis. This is due to the preliminary rupture of part of the disulfide bridges during alkaline treatment in the presence of a reducer. This is confirmed by the literature data. Lime-sulfide treatment is a more suitable option for weakening the peptide and disulfide bonds, which is a widespread method in the leather industry for unhairing. Therefore, leather waste would be a suitable material for the production of keratin hydrolysates.

CONCLUSIONS

Three methods have been used to hydrolyze keratin from sheep wool, namely hydrolysis with thioglycolic acid, sulfitolysis with sodium pyrosulfate and urea, and hydrolysis with NaOH. The methods are applied respectively to the native wool and such obtained after lime-sulfide unhairing process. It has been proved that NaOH has the highest hydrolyzing effect on disulfide and peptide bonds. In addition, the pre-alkaline treatment with the presence of wool reducer increases the degree of hydrolysis compared to that of the native wool. It has been proved the presence of a keratin protein in some of the hydrolysates, as well as the destruction of the disulfide bonds.

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