FABRICATION OF COLLAGEN HYDROLYSATE NANOFIBERS BY THE ELECTROSPINNING METHOD

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ABSTRACT. Nanotechnology is a ground-breaking technology which has found applications on many areas of daily life. Fibers with nanometer diameters are the most suitable candidates for a large number of applications since they have high surface area to volume ratio and different surface characteristics. Among the various nanofiber production techniques, the most advanced and efficient is the electrospinning technique. In our study, nanofibers were obtained from collagen hydrolysate by the electrospinning method. For this purpose, collagen hydrolysate was dissolved at different concentrations in 2,2,2-trifluorethanol and stirred for six hours at room temperature. The turbidity, viscosity, conductivity and pH of the solutions were determined. According to the results obtained, it was observed that viscosity and conductivity values rose as the amount of collagen hydrolysate increased. Nanofibers of collagen hydrolysate with 103 nm and 384 nm obtained by the electrospinning method were examined by SEM, and their morphological characteristics were discussed.

KEY WORDS: electrospinning, collagen hydrolysate, nanofiber, leather

FABRICAREA NANOFIBRELOR DIN HIDROLIZAT DE COLAGEN PRIN METODA ELECTROSPINNING

REZUMAT. Nanotehnologia este o tehnologie de ultimă oră, care a găsit aplicaţii în multe domenii ale vieţii de zi cu zi. Fibrele cu diametru de ordin nanometric sunt cele mai potrivite candidate pentru un număr mare de aplicaţii, deoarece au raport suprafaţă/volum ridicat şi caracteristici diferite ale suprafeţei. Printre diferitele tehnici de producţie a nanofibrelor, cea mai avansată şi eficientă este tehnica electrospinning. În studiul nostru, s-au obţinut nanofibre din hidrolizat de colagen prin metoda electrospinning. În acest scop, hidrolizatul de colagen a fost dizolvat la diferite concentraţii în 2,2,2-trifluoretanol şi agitat timp de şase ore la temperatura camerei. S-au determinat turbiditatea, vâscozitatea, conductivitatea şi pH-ul soluţiilor. Conform rezultatelor obţinute, s-a observat că valorile vâscozităţii şi conductivităţii au crescut odată cu creşterea cantităţii de hidrolizat de colagen. Nanofibrele din hidrolizat de colagen cu 103 nm şi 384 nm obţinute prin metoda electrospinning au fost examinate prin SEM şi s-au discutat caracteristicile morfologice ale acestora.

CUVINTE CHEIE: electrospinning, hidrolizat de colagen, nanofibră, piele

LA FABRICATION DE NANOFIBRES D'HYDROLYSAT DE COLLAGÈNE PAR LE PROCÉDÉ D'ÉLECTROFILAGE

RÉSUMÉ. La nanotechnologie est une technologie révolutionnaire qui a trouvé des applications dans de nombreux domaines de la vie quotidienne. Les fibres de diamètre nanométrique sont les candidats les plus appropriés pour un grand nombre d'applications car elles ont un rapport surface/volume élevé et des caractéristiques de surface différentes. Parmi les différentes techniques de production de nanofibres, la plus avancée et la plus efficace est la technique d'électrofilage. Dans notre étude, les nanofibres ont été obtenues à partir d'hydrolysat de collagène par la méthode d'électrofilage. Dans ce but, l'hydrolysat de collagène a été dissous à différentes concentrations dans le 2,2,2-trifluoréthanol et agité pendant six heures à température ambiante. La turbidité, la viscosité, la conductivité et le pH des solutions ont été déterminés. Selon les résultats obtenus, on a observé que les valeurs de viscosité et de conductivité augmentaient à mesure que la quantité d'hydrolysat de collagène augmentait. Les nanofibres d'hydrolysat de collagène à 103 nm et 384 nm obtenues par la méthode d'électrofilage ont été examinées par SEM, et leurs caractéristiques morphologiques ont été discutées.

MOTS CLÉS : électrofilage, hydrolysat de collagène, nanofibre, cuir

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INTRODUCTION

Nanotechnology is a ground-breaking technology which has found applications in many areas of daily life. The basic reason for the rapidly growing interest in nanotechnology is that studies of nanotechnology have shown materials to have different characteristics at very small dimensions, thus completing deficiencies in basic knowledge. Fibers with nanometer diameters are the most suitable candidates for a large number of applications since they have high surface area to volume ratio and different surface characteristics and have shown much better mechanical performance than materials in other forms in terms of hardness and mechanical strength [1].

Nanofibers fabrication can be performed by bicomponent extrusion, template synthesis, self-assembly, phase separation, melt-blowing, drawing, centrifugal spinning and electrospinning [2]. However, the electrospinning method is easy and cheap, and fibers can be produced from many polymers, making it the most flexible and most preferred method [3]. In the electrospinning method, a polymer solution or a molten polymer is exposed to a high voltage, which gives it an electric charge. A jet of the polymer solution from a fine nozzle is directed towards a target with the opposite charge, placed opposite the nozzle. During this process the solvent evaporates, or if molten polymer is being used it begins to solidify. The jet of polymer is scattered as very fine fibers, and in this way fibers with a diameter on the nano scale can be obtained [4-6].

A large number of studies on the production of nanofibers by the electrospinning method deal with the use of many different polymers for various purposes. These studies have shown that polyurethane [7, 8], PVA/sodium alginate [9] and PEO/sodium alginate [10], chitin [11], cellulose [12], PVA [13], polyacrylic acid [14], PMMA [15], polystyrene [16], nylon-6 [17] and all other polymers which can be used in nanofiber production techniques are suitable for electrospinning.

The main source of natural polymer collagen is the skins of various animals. Among the fibrous proteins in the skin structure, the amount of collagen is 98% [18]. Collagen hydrolysate (CH) is produced by the controlled hydrolysis of collagen and consists of a mixture of polypeptides that are dispersed by molecular weight [19]. CH shows technological advantages such as good resolution, heat stability and high resistance to precipitation with metal ions or pH [20]. It is used in different fields such as food, medicine, pharmaceuticals, cosmetics and biomaterials due to its bioactivity, biocompatibility and penetration as well as its excellent digestibility and high consumer tolerance [19].

Today, as well as intense research into nanofibers, more importance is being accorded to studies on developing new products and materials containing collagen to be used for different purposes, in order to increase the opportunities for making use of collagen-based products. In a scan of the literature, it was found that gelatin, a biopolymer with known advantages for use especially in the biomedical field [21, 22], collagen [23-25], and mixtures of collagen with other polymers had been used in a number of studies of electrospinning [26-29]. Collagen and chitin derivate nanofibers were obtained by electrocapillary, electrocentrifugal, Nanospider™ methods [30]. Also, it was found that in these studies, different cross-linkers or hardening polymers had been used in the production of collagen-based nanofibers. In this study therefore, the topic of the production of collagen hydrolysate nanofibers by the electrospinning method without the use of cross-linkers or hardening polymers was considered, and the morphological characteristics of the collagen hydrolysate nanofibers obtained were discussed.

MATERIALS AND METHODS

Materials

Collagen hydrolysate was provided by Sigma-Aldrich and the TFE (2,2,2-trifluorethanol) used as a solvent was supplied by Fluka (Germany).

Method

Setting up the Apparatus for Electrospinning

The main apparatus used in this study was an electrospinning unit used in the production of nanofibers. The most important parts of this

were the feed unit and spinning collector cylinder, designed by the Küçüker company (Turkey) with the support of the Scientific Research Projects Coordinatorship of Pamukkale University, a high voltage source (Simco, UK), and a syringe perfusion pump (New Era 1100, USA), obtained from the distributor companies.

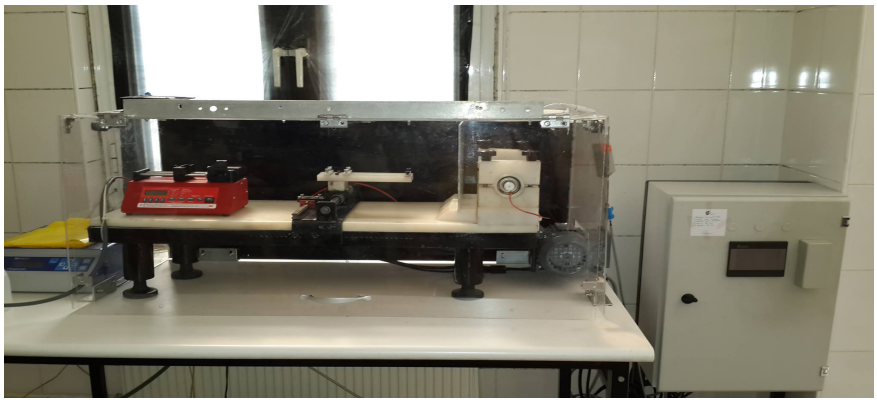


Figure 1. Electrospinning apparatus used in nanofiber production

Preparation of Solution, Optimization and Nanofiber Production

Collagen hydrolysate (CH) at various concentrations (10%, 15%, 20%, 25%, 30% w/v) was dissolved in 2,2,2-trifluoroethanol (10% v/v) and stirred for six hours at room temperature (relative humidity 40%). The resulting solution was loaded into a plastic syringe (Ayset Plastik, Turkey) of volume 10 mL and diameter 0.9 mm with a metal syringe needle tip, and optimization of the parameters for the production of nanofibers by electrospinning was carried out at room temperature.

First of all, the distance between the end of the syringe and the collector plate were examined in the range of 8-12 cm. The parameters in the electrospinning process to obtain nanofibers of the voltage applied and the flow rate of the feed solution were accepted as experimental variables. In order to determine the effect on nanofiber yield of these parameters, different voltages and different flow rates were applied for concentrations of collagen hydrolysate (Table 1). Optimization studies showed that optimum values were a concentration of 20%, 25% and 30% w/v of CH, a stirring time of 6 hours, a voltage value of 20kV and a flow rate of 0.8 mL/hour. The distance between the metal tip of the syringe and the collector plate was determined as 12 cm. The duration of all experiments was set at 120 and 180 minutes (for obtaining thicker nanofiber mats).

Table 1: Electrospinning parameters

Electrospinning parameters	
Different voltages, kV	13, 15, 17, 20
Flow rates, mL/h	0.8, 1.0, 1.5, 2.0
Distance, cm	8, 10, 12
Duration, min	120, 180
Solution parameters	
Concentration, % (w/v)	10, 15, 20, 25, 30

Characterization of Solution

The pH measurements of the working solutions were performed using a digital pH meter (WTW, Germany). The viscosity of the working solutions was determined by using a rheometer (Fungilab, Spain). The turbidity of the working solutions was measured with a turbidimeter (Velp TB1, Italy). The conductivity of the solution was determined by testing its ability to carry an electric current, and conductivity affects the rate of movement of a polymer solution during electrospinning. The electrical conductivities of the solutions to be fed to the electrospinning device were determined with an electrical conductivity meter (WTW MULTI 9310, Germany). Measurement of the viscosity, turbidity and conductivity of the feed solutions were conducted at room temperature (25˚C) triplicate. The results are given as the mean ± standard deviation of the three measurements.

FTIR Analysis of Nanofibers

A Fourier Transform Infrared (FTIR) spectrophotometer (Perkin Elmer, USA) was used in order to determine the chemical structure of the surfaces of the nanofibrous matrices. Spectra were recorded with a resolution of 4 cm^{-1} and at wavelengths of 400-4000 cm^{-1}, and were analyzed using an FTIR software program.

Scanning Electron Microscope Analysis of Nanofibers

A SEM device (JEOL 840, USA) was used to examine the morphological properties of the nanofibers and to obtain information about their diameters. Small sections of nanofibers on aluminum foil obtained by electrospinning were taken and gold-plated, after which SEM images were obtained. SEM images with 40 kX enlargement were taken for analysis of the dimensions and morphological characteristics of the nanofibers. The mean diameter value was determined by performing 50 different measurements from the SEM images of each sample.

RESULTS AND DISCUSSIONS

Analysis of Solutions

pH Value

The acidic characteristics and the degree of reactivity of a solution as well as other important characteristics can be determined by a correctly measured pH value. In addition, many chemical characteristics and processes such as the solubility of a compound are to a large extent connected to the pH value of the solution. Table 2 shows the pH values of 20%, 25% and 30% solutions of collagen hydrolysate. The results of tests performed with a pH meter showed that solutions of collagen hydrolysate had a weak acidic character with pH values of between 5.4 and 5.6.

At the isoelectric point of protein there is no net charge on it, whereas above the isoelectric point, the protein should carry a net negative charge. It was observed that fibers with protein are obtained if the pH of the electrospinning solution is adjusted to the isoelectric point of the protein [31]. The pH values of the collagen hydrolysate solutions (pH 5.4-5.6) used in this study were in the range of the isoelectric point of collagen peptides (pH 5-6).

Turbidity Results

Turbidity test results were used to ascertain whether the solutions had dissolved before electrospinning, and further tests furnished information on the morphology of the nanofibers. Turbidity was measured between 0 and 1000 NTU. The production of homogeneous, continuous and fine solutions is directly related to a turbidity value of 0 NTU. Table 2 gives the turbidity values of collagen hydrolysate solutions. It was determined from tests conducted using a turbidimeter that the solutions were very transparent in appearance and homogeneous. If solutions contain undissolved solid materials, a clear solution is not produced, and the morphology of the nanofibers obtained will not be regular. This can give unwanted results. Turbidity values of between 19.4 and 22.7 NTU were determined for the solutions in the study.

Viscosity Results

Gelling occurs under the effect of van der Waals forces, with aggregation between particles or molecules in a liquid. The gelling process is studied using rheological measuring techniques. The viscosity characteristics of the solutions in our study were determined in this way. As seen in the Table 2, mean viscosity values of the solutions at room temperature were found to be generally between 15.4 and 21.0 mPa.s. The viscosity values of the collagen hydrolysate solutions were found to increase with increasing CH concentration, which was resulted in a greater number of polymer chain entanglements within the solution [32]. An increase in the viscosity values of the solutions is undesirable because they become lumpy in the nozzle of the electrospinning apparatus, and this causes the formation of non-homogeneous beaded nanofibers. The viscosity values determined in the study are showed the suitability of solutions with low viscosity values for entanglement to be electrospun.

Conductivity Results

Electrical conductivity is a parameter which greatly affects the diameter of the nanofibers. Table 2 gives the conductivity values

of the collagen hydrolysate solutions. These were found to vary between 3.3 and 4.6 mS/cm. In our study, it was observed that conductivity increases with the increase of solution concentration. This situation can be explained by the increase in the total amount of ions in solution. It was thought that an increase in conductivity values by an increase in collagen hydrolysate concentration would make the diameters of the nanofibers obtained low and in this way these values would provide an advantage in the electrospinning process. It has been seen in some studies that solutions with high conductivity produced nanofibers with smaller diameters [33, 34]. However, some other studies support the hypothesis that high conductivity results in fibers with large diameters [35]. The conductivity results obtained in this study contributed to the formation of CH nanofibers without formation of beads.

Table 2: Properties of collagen hydrolysate solutions

Concentration	pH	Turbidity (NTU)	Viscosity (mPa/s)	Electrical conductivity, (mS/cm)
%20	5.4	19.4±6.7	15.4±0.3	3.3±0.4
%25	5.5	20.2±1.7	17.2±0.8	4.4±0.2
%30	5.6	22.7±3.2	21.0±0.2	4.6±0.2

Characterization of Nanofibers

FTIR Results

Figure 2 gives the FTIR spectra of nanofibers obtained from 20%-30% collagen hydrolysate. The graphics obtained are very close to the FTIR spectra of collagen obtained by many other researchers [36-38]. Examining spectra obtained after spectral scans, it was determined that the bands emerging in connection with tension vibration of the (0) C-O, (1) C-O-C, (2) OR-C-C, (3) $COO-CH_3$ (asymmetric), (4) C=C and (5) C=O groups on the structure chain of the collagen hydrolysate nanofiber samples were at wavenumbers of (0) 1035 cm^{-1}, 1082 cm^{-1} (1) 1064 cm^{-1}, (2) 1140 cm^{-1}, 1174 cm^{-1}, (3) 1454 cm^{-1}, (4) 1633 cm^{-1}-1647 cm^{-1}, and (5) 1723 cm^{-1}-1735 cm^{-1} respectively (Figure 2).

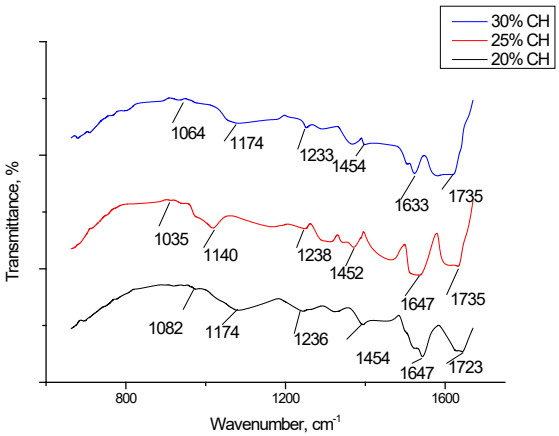


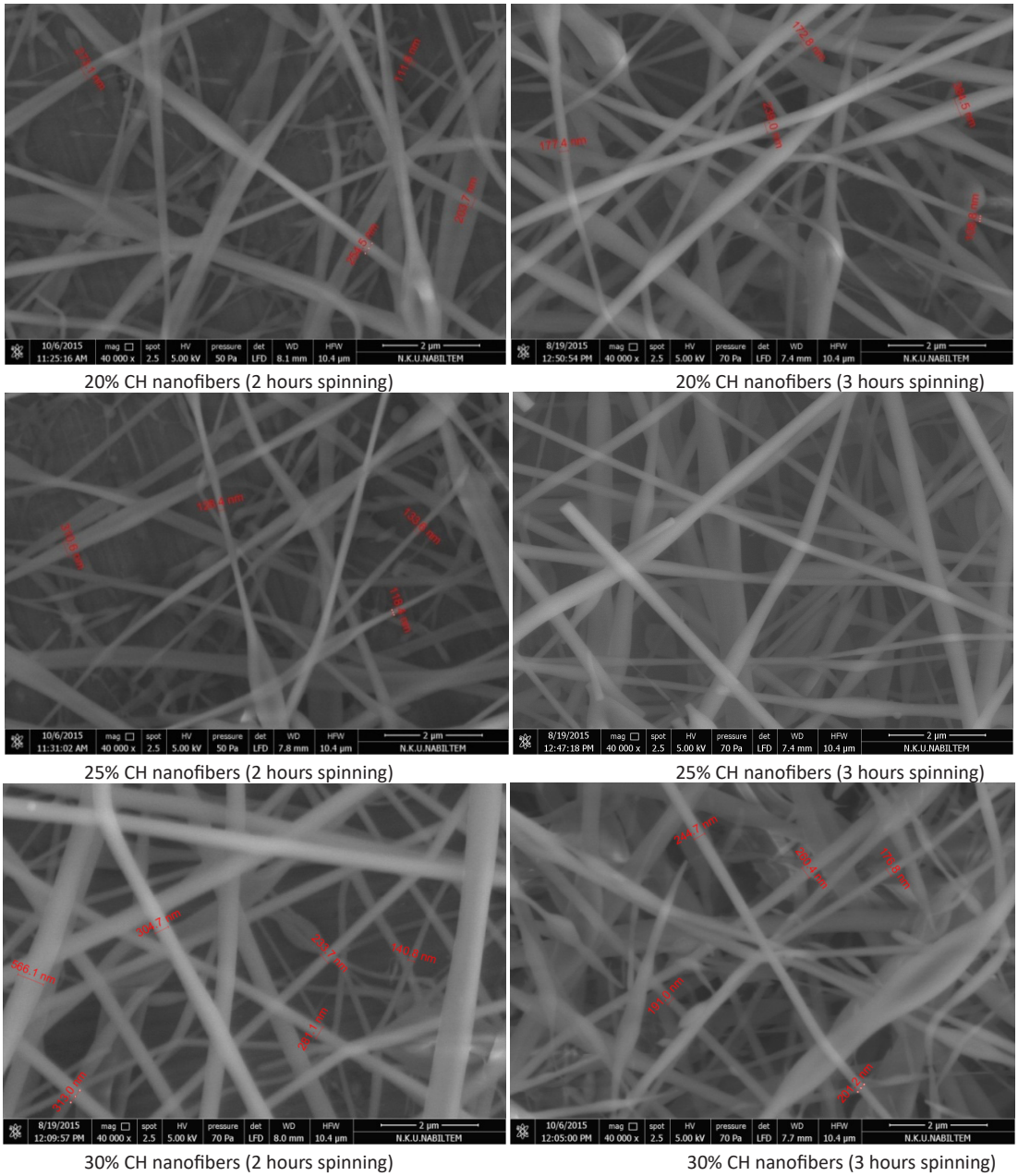
Figure 2. FTIR spectra of collagen hydrolysate nanofibers

In addition, it was determined that in all samples peaks between wavenumbers of 1233 cm^{-1} and 1236-1237 cm^{-1} belonged to the amide I band, and those between 1452 cm^{-1} and 1454 cm^{-1} belonged to the amide II band. It was observed that the carboxyl band which appeared in connection with the C=O absorption band was at wavenumbers of 1723 cm^{-1}-1735 cm^{-1} in all samples. Absorption bands between 1238 cm^{-1} and 1452 cm^{-1} are bands which appear in relation to the existence of a helix structure [37]. Thus FTIR examination showed that the

helix structures in CH nanofibers obtained could be preserved without destruction.

Results from the Scanning Electron Microscope

Figure 3 shows 40 kX magnified SEM images of nanofibers obtained from 20%, 25% and 30% collagen hydrolysate solutions. It was seen that the diameters of the nanofibers obtained as a result of 2 hours spinning of a 20% collagen hydrolysate solution varied between 111 nm and 273 nm, while the nanofibers obtained as a result of 3 hours' spinning of a 20% solution of CH had diameters of 103 nm to 384 nm. The nanofibers obtained from a 25% collagen hydrolysate solution after 2 hours of electrospinning had diameters of 116 nm-133 nm, and after 3 hours of electrospinning 157 nm-235 nm. With a 30% CH solution, the diameters of the nanofibers obtained were 140 nm-313 nm with 2 hours of electrospinning and 176 nm-260 nm with 3 hours. It was determined from SEM images that the smallest diameter of nanofibers obtained from CH solutions of 20%-30% was 103 nm, and the largest was 384 nm.



20% CH nanofibers (2 hours spinning) | 20% CH nanofibers (3 hours spinning)

25% CH nanofibers (2 hours spinning) | 25% CH nanofibers (3 hours spinning)

30% CH nanofibers (2 hours spinning) | 30% CH nanofibers (3 hours spinning)

Figure 3. SEM images of nanofibers obtained from 20%, 25% and 30% solutions of CH

In previous studies conducted on collagen, nanofibers in the range of 100-700 nm in diameter were obtained [23, 24, 39, 40], but when collagen hydrolysate mixed with chitin derivate and polyvinyl alcohol was used, nanofibers of between 200 nm and 600 nm were obtained [30]. In the present study, collagen hydrolysate nanofibers with small diameters of 103 nm-384 nm were obtained using no other material than a solvent. It is thought that more homogeneous nanofibers could be obtained in future studies by adding salt and large-molecule polymers or a plasticizer.

CONCLUSIONS

In conclusion, collagen hydrolysate nanofibers were successfully produced by the electrospinning method. First, it was determined that the 10%, 15% CH solutions beaded, 20%, 25% and 30% CH solutions used had pH values of 5.4-5.6, viscosities of 15.4-21.0 mPa.s, turbidities of 19.4-22.7 ntu, and conductivities of 3.3-4.6 mS/cm according to concentration. This clearly showed that nanofibers can be obtained from collagen hydrolysate at concentrations of between 20% and 30%. Studies are being carried out in many different scientific fields today in connection with the potential advantages of using collagen hydrolysate to develop new products and materials containing collagen which can be put to various different uses. In the light of the results of the present study, nanofibers obtained by the use of collagen hydrolysate can be accepted as a new collagenic material, and after improvement and development in future studies, will form a new material of industrial and economic importance. This also provides an alternative opportunity to make use of collagen hydrolysate, a solid waste of the leather industry, which will also provide ecological benefits.

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