Experiments
Учебен експеримент в природните науки

ISOLATION AND CHARACTERIZATION OF COLLAGEN FROM THE SKIN OF SYRIAN SHEEP

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Abstract. Acid soluble collagen (ASC) and Pepsin soluble collagen (PSC) from the skin of Syrian sheep were isolated and characterized. The yields of ASC and PSC were 6% and 14.4%, based on the weight of skin. ASC and PSC were exhibited a maximum absorbance at 230 and 240nm, respectively. Fourier transform infrared spectroscopy (FT-IR) showed regions of amides A,B, I, II and III for ASC and PSC. From Zeta potential analysis, isoelectric points (pI) of collagen from the skin of sheep were 5.2 and 5.6 for ASC and PSC respectively. The concentration of protein in extracted collagen ASC and PSC was 875, 930mg/g, respectively. Qualitative analysis of amino acids shows absence the cysteine and histidine from ASC and PSC and existence the amino acids distinguishing of collagen.

Keywords: acid soluble collagen (ASC), pepsin soluble collagen (PSC), zeta potential, FTIR, sheep skin.

Introduction

Collagen is the most abundant animal protein polymer in animal tissues and constitutes approximately 30% of total animal protein, a fibrous inextensible protein (Bama et al., 2010; Bolboacă & Jäntschi, 2008; Yan et al., 2008). It is widely distributed in skin, bones, cartilage, tendons, ligaments, blood vessels, teeth, cornea and all other organs of vertebrates (Yan et al., 2008; Potaros et al., 2009). In tendons, collagen has the strength equal to light steel wire. Collagen in the cornea is transparent, in heart valves it is fatigue-resistant, and in renal glomeruli it provides an excellent filtration system (O'Grady & Bordon, 2003).

Molecular structure of collagen contains three polypeptide a chains wound together in a tight triple helix. Each polypeptide called α chain consists of repeated sequence of triplet, (Gly-X-Y)n, where X and Y are often proline (Pro) and hydroxyproline (Hyp) (Senatatne et al., 2006).

Each peptide subunit is composed of about 1050 amino acid residues, which consist of approximately 33% glycine, 25% proline and hydroxyproline, and a relative abundance of lysine . The average length of each subunit is about of 300 nm and a diameter of 1.4 nm (Potaros et al., 2009; O'Grady & Bordon, 2003). Collagen has a repetitive primary sequence of which every third residue is glycine (Potaros et al., 2009).

There are 27 types of collagen presented in the literature. Each type of collagen has its distinctive amino acid sequence and molecular structure, and each plays a unique role in the tissue. Among these types, type I collagen generally comprises the highest proportion and is the most extensive in application (Cheng et al., 2009).

For industrial purposes, the main sources of collagen are limited to those of land based animals, such as bovine or porcine skin and bone (Bolboacă & Jäntschi, 2008). Highly infectious and contagious diseases like Mad Cow Disease (Bovine Spongiform Encephalopathy—BSE), Transmissible Spongiform Encephalopathy (TSE), and Foot and Mouth Disease (FMD) (Bama et al., 2010; Yan et al., 2008) limited the use of collagen derived from pigs and cattle for industrial purposes as there is a possibility to be transmitted those diseases from animals to human being via animal tissues. In addition, the collagen extracted from pigs cannot be used due to religious barriers. Therefore, many scientists have been focusing their experiments to find out alternative sources of collagen (Senaratne et al., 2006).

They have found that skin, bone, fin and scales of both fresh water and marine fish, chicken skin, mammals skin ,squid skin, octopus arms, marine sponge and bull frog skin can be used as alternatives (Senaratne et al., 2006; Yan et al., 2008; Suphatharaprateep et al., 2011).

Collagen is an important biomaterial which has a wide range of applications in leather and film industries, pharmaceutical applications including production of wound dressings, vitreous implants and as carriers for drug delivery, cosmetic because it has a good moisturizing property and biomedical materials, and food whereas heat denatured collagen called gelatin is important in food manufacturing (Senaratne et al., 2006; Bama et al., 2010; Yan et al., 2008; Potaros et al., 2009).

Materials and methods

Chemical reagents

Bovine serum albumin (BSA), pepsin from porcine stomach mucosa, anthrone reagent, Standard glucose, ninhydrin reagent, amino acids were purchased from Sigma-Aldrich Co. All other chemicals used were of analytical grade.

Raw material

Skin of sheep was caught from the commercial local butchery in Syria. The fur removal from the skin, then the skin minced and kept at -20°C.

Proximate analysis

The skin of sheep was subjected to proximate analysis including moisture and the sample of collagen also was subjected to proximate analysis including moisture and carbohydrate (Montero et al., 1990).

Extraction of collagen

The acid-soluble collagen (ASC) and pepsin- soluble collagen PSC were extracted from skins. Fifty grams of skin was precisely weighed. To remove non-collagenous proteins, the skin was mixed with 150ml of 0.1M NaOH at 4 °C. The mixture was stirred constantly for 5 days, while the NaOH solution was changed every day (Kumar et al., 2011).

After removal of supernatant, the sample was washed with distilled water till the pH value was neutral. The deproteinized skins were defatted with 150 ml of butyl alcohol for 24 h at 4 °C. The butyl alcohol was changed every 8 h. Defatted skins were washed with distilled water for three times (Jongjareonrak et al., 2005).

To extract ASC, the prepared skin was soaked in 0.5 M acetic acid with a solid/solution ratio of 1:6 (w/v) for 48 h at 4°C, this step repeat twice (Zelechowska et al., 2010).

The extract was filtered with whatman No.1 paper. The collagen was precipitated by adding NaCl to a final concentration of 0.9 M for 24 hr at 4°C (Yan et al., 2008; Cheng et al., 2009). 0.5 M acetic acid was applied to dissolve sediment and then dialyzed against 0.1 M acetic acid and distilled water sequentially. The collagen was obtained by freeze-drying. The yield of acid-soluble collagen was calculated on the basis of skin weight and expressed as percentage (g/g). To extract PSC, the same steps used in the extraction of ASC are applied in order to extraction of PSC, but added with acetic acid 0.1%pepsin (w/v) (Cheng et al., 2009).

UV–vis spectra

Collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/ml. The solution was then subjected to UV–Vis measurement. Prior to measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 200–600 nm with a scan speed of 50 nm/min at room temperature (Kittiphattanabawon et al., 2010; Hema et al., 2013).

Fourier transform infrared (FT-IR) spectroscopy

The FT-IR spectra were recorded using Fourier transform infrared spectrophotometer in the wave number region 4000 to 550 cm^{-1} at a data acquisition rate of 1 cm^{-1} per point at room temperature.

Zeta potential analysis

Collagen was dissolved in 0.5 M acetic acid to obtain the final concentration of 0.5 mg/mL. The mixture was continuously stirred at 4°C until the sample was completely solubilized. The collagen solution was titrated with 0.25M NaOH, and the Zeta potentials of collagen solution at the given PH from 2.87 to 7 were measured by (MARTINI

instruments). The titration temperature was 25°C and the increasing Ph intervals were 0.5 pH. Zeta potentials were plotted against PH and the PI of ASC was determined at the pH value where the Zeta potentials was zero (Kittiphattanabawon et al., 2010 ; Zhao et al., 2007).

Determination of collagen content

Collagen content was determination by biuret reaction, 0.1g collagen was dissolved in 25mL of 0.5M acetic acid, 5mL of biuret reagent add to 1mL of collagen solution and allows evolution color for 20-30min before reading absorbance at 540 nm. Bovine serum albumin (BSA) was used as a stander (Scopes, 2006).

Qualitative analysis of amino acids

Amino acids composition was analyzed using thin layer chromatography method, amino acid was dissolved in 0.01 M sodium hydroxide solution to a final concentration of 0.01M. Mobile phase was (5:3:2v/v/v) butanol: acetic acid: water (Qiu et al., 2010). 1.5g ninhydrin was dissolved in 100mL of n-butanol and then 3.0 ml of acetic acid was added (Quereshi et al., 2010). ASC and PSC samples were hydrolyzed under reduced pressure with 6M HCl at 100 °C for 24 h (Yan et al., 2008). The classical thin layer chromatography method was used to determine amino acids in collagen. Rf is calculated from the formula:

$$\text{Rf compound} = \frac{\text{distance traveled by compound}}{\text{distance traveled by solvent}}$$

Statistical analysis

All experiments were done in triplicates. Mean values were reported.

Result and discussion

Proximate analysis

Skin sheep contained the moisture (79.51g/100 g), the extracted collagen ASC and PSC contained the moisture (10.94 –11.53g/100g), respectively as a major component. Trace amount of carbohydrate (0.34 □ 0.23g/100g) was noticeable in ASC and PSC from skin sheep, respectively.

Collagen yield

Table 1 shows the yield of the collagen. The yield of acid-soluble collagen (ASC) was higher compared to and pepsin-soluble collagen (PSC) in sheep skin.

Table 1. Yield of the (ASC) and (PSC) from sheep skin

Collagen type	Yield(%)
Acid-soluble collagen (ASC)	6.0
Pepsin -soluble collagen (PSC)	14.4

In ASC, the skin was not completely solubilized with 0.5 M acetic acid even with two repetitions of extraction. This result suggested a high amount of cross-links at the telopeptide region as well as other intermolecular cross-links, leading to low solubility of collagen in acid.

UV–vis spectra

Figs. 1 and 2 show the UV-Vis spectra of the acid-soluble collagen from sheep skin. From UV–Vis spectra of the extracted collagen can be seen the distinct absorbance of the collagen was obtained near 220-240 nm, which is contributed by $n \rightarrow \pi^*$ transition of C=O in the peptide bond. Tyrosine and phenylalanine are sensitive chromophores and absorb UV light at 283 nm and 251 nm, where ASC and PSC have no evident absorbance. Therefore, acid-soluble collagen and pepsin- soluble collagen from sheep skin well support the property of collagen that there is absorbance at 220–240 nm, with little or no absorbance near 280 nm. Thus the protein is collagen (Yan et al., 2008; Kittiphattanabawon et al., 2010; Li et al., 2009).

FT-IR spectroscopy

Figs. 3 and 4 show the FTIR spectra of the acid-soluble collagen from sheep skin, The amide A band is associated with the N–H stretching frequency. A free N–H stretching vibration occurs in the range of 3400–3440 cm^{-1}, and when the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequency, usually near 3300 cm^{-1}, The amide A band of (ASC) and (PSC) from sheep skin was found at 3317.93 cm^{-1} and 3325.64 respectively, which shows that there were NH groups involved in hydrogen bonds. The amide B band of (ASC) and (PSC) was found at 2927.41cm^{-1} and 2888.84, respectively, which is related to asymmetrical stretch of CH_2.

The amide I band position was observed at 1633.41cm^{-1} for (ASC) and (PSC) from sheep skin, which is the absorption band of C=O stretching. It is associated with the secondary structure of the protein. The amide II band is associated with the N–H bending vibrations coupled to C–N stretching vibrations. Generally, the shift to the lower wave number showed the existence of hydrogen bonds in collagen, The amide II band of (ASC) and (PSC) from sheep skin was found at 1555.31cm^{-1} and 1556.27, respectively.

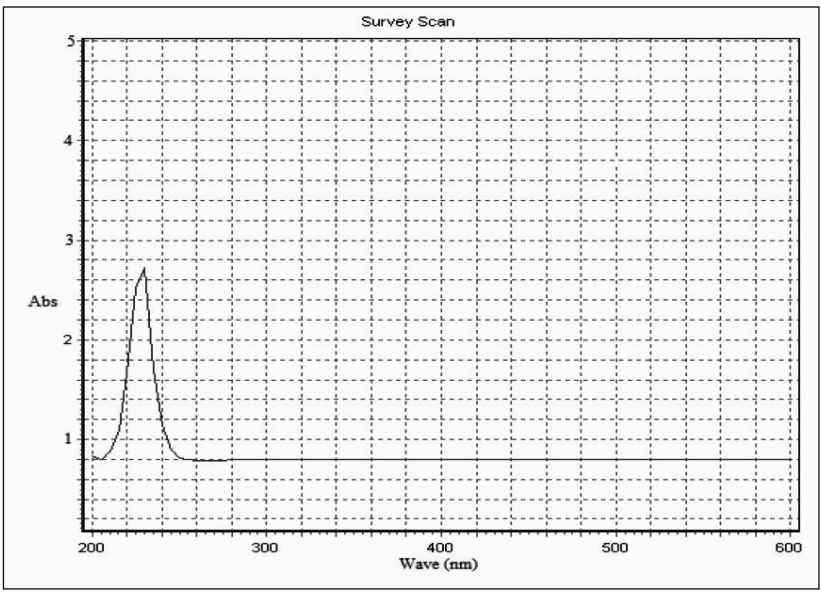

Fig. 1. UV–vis spectra of acid-soluble collagen from sheep skin

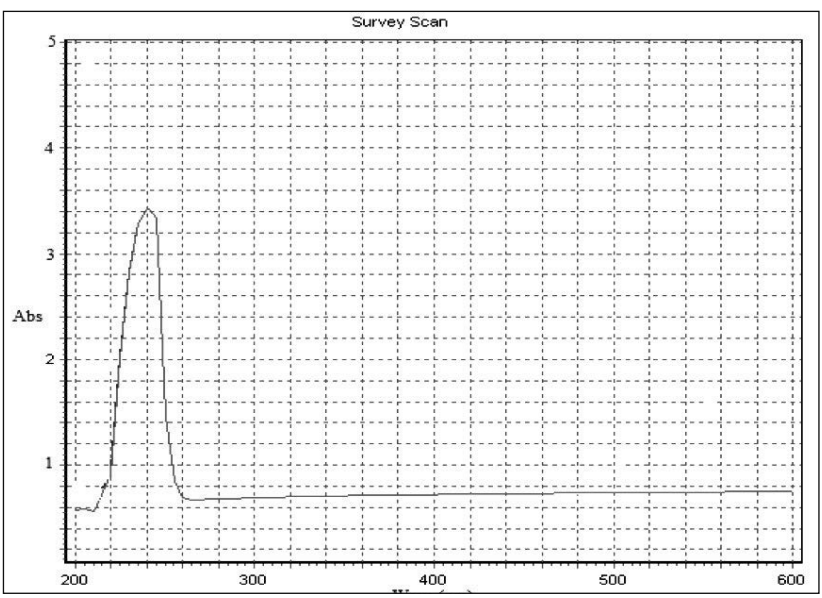

Fig. 2. UV–vis spectra of pepsin-soluble collagen from sheep skin

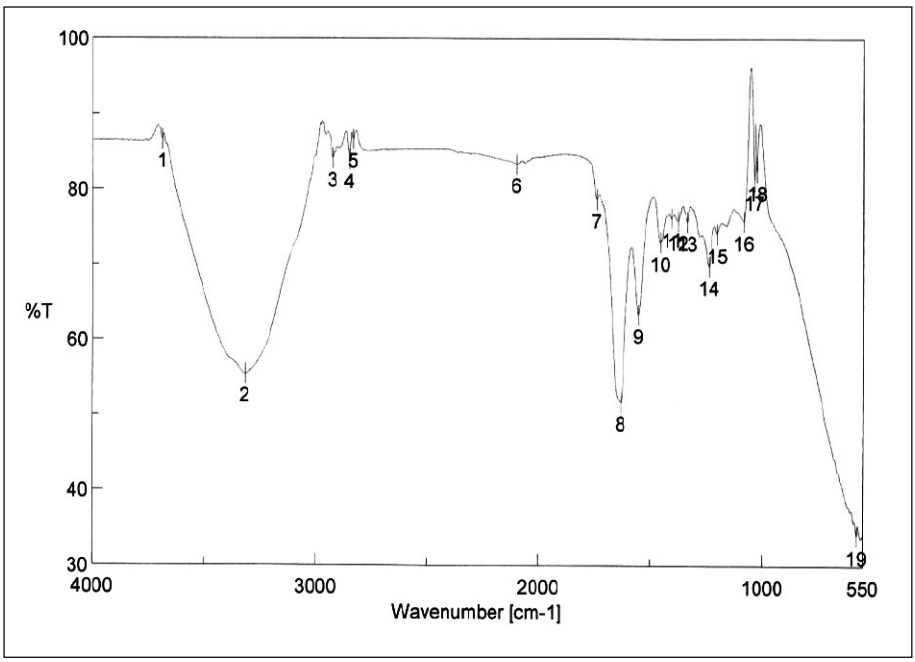

Fig. 3. FT-IR spectra of acid-soluble collagen from sheep skin

The amide III represented the combination peaks between N–H deformation and C–N stretching vibrations and the absorption between the 1236 cm^{-1} and 1452 cm^{-1} bands demonstrated the existence of helical structure of collagen. The amide III band of (ASC) and (PSC) from sheep skin was found at 1240.97cm^{-1} and 1241.93 respectively. Therefore, the FTIR investigations show the existence of helical arrangements sheep skin collagen (Yan et al., 2008; Kittiphattanabawon et al., 2010; Li et al., 2009; Ahmad & Benjakul, 2010; Cao & Hu, 2008).

Zeta potential analysis

The surface charges of ASC from the skin of sheep at different pHs are shown in Fig. 5. Isoelectric point is an important parameter of proteins, which is related to the proportion of acid amino residues and base amino residues in protein.

The pH at which the positive charges on a protein equal the negative charges or the pH at which the net charge of the protein is zero is defined as isoelectric point (pI).

For acid-soluble collagen (ASC) from the skin of sheep, the zero net charge was found at pH of 5.2 and it was lower compared to pepsin-soluble collagen (PSC) that was found at 5.6. At pHs above their pI, the sample possessed the negative charge, the

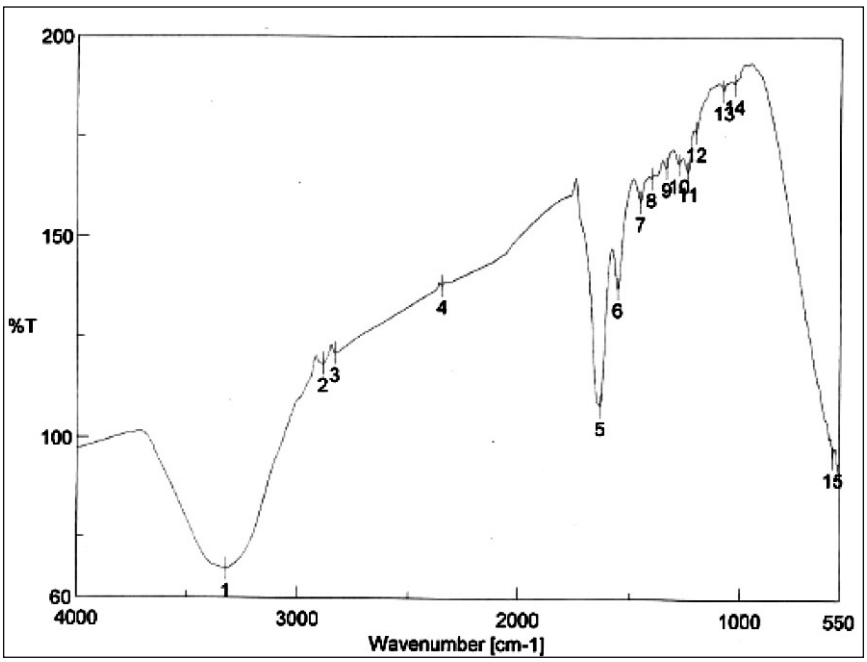

Fig. 4. FT-IR spectra of acid-soluble collagen from sheep skin

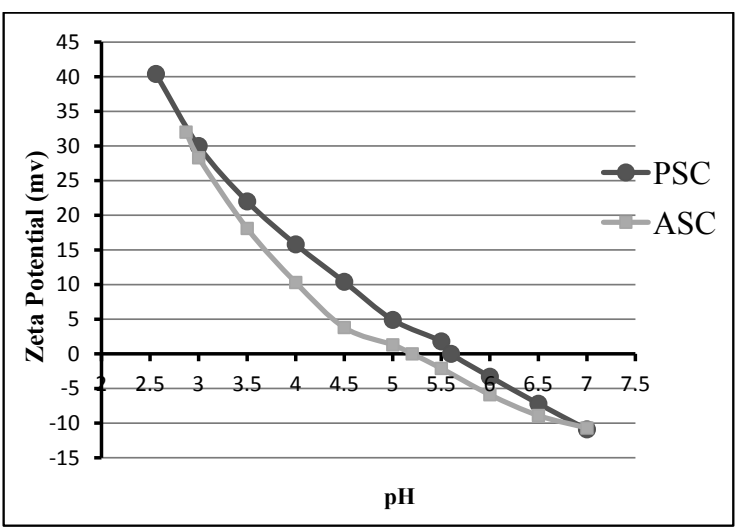

Fig. 5. Zeta potentials of ASC and PSC from the skin of sheep at different pHs

fall of the PI was attributed to decrease of some basic amino acids (Arg residues), and the increase of carboxyl groups as a result of deamination of the acid amide groups of the Asn and Gln residues (Kittiphattanabawon et al., 2010 ; Zhao et al., 2009).

Determination of collagen content

Table 2 shows collagen content of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from sheep skin collagen content of (PSC) was 930 (mg/g), and was higher compared to (ASC) that was 875(mg/g). This result suggested low solubility of collagen in acid because of existence telopeptide region.

Table 2. Yield of the collagen content for (ASC) and (PSC) from sheep skin

Collagen type	Yield(mg/g)
Acid- soluble collagen (ASC)	875
pepsin- soluble collagen (PSC)	930

Qualitative analysis of amino acids

Ninhydrin will react with the amino acid to produce a purple compound (Quereshi et al., 2010).

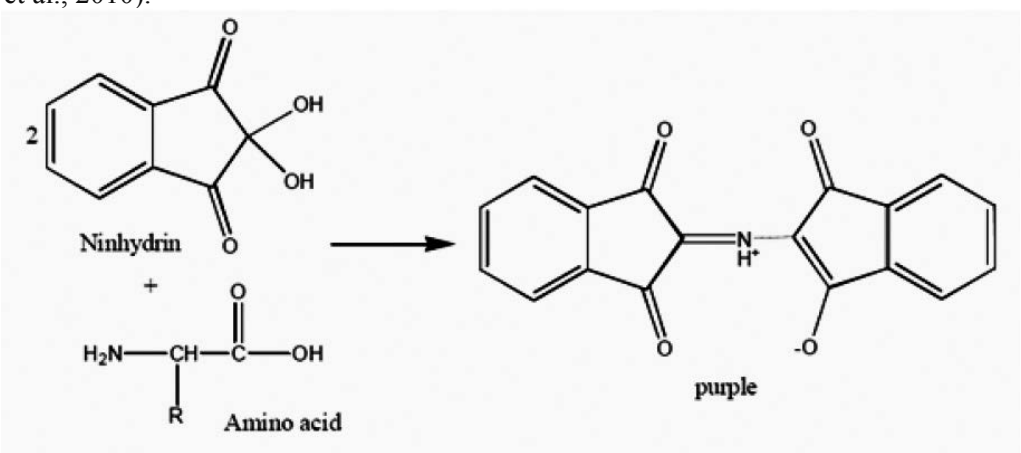

Fig. 6 shows TLC chromatogram of 12 amino acids, and table.3 shows the Rf values of this amino acids.

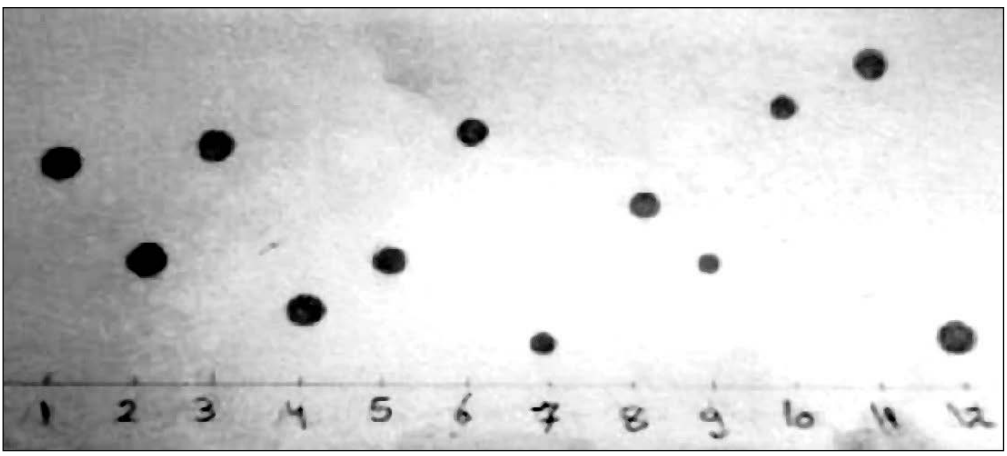

Fig. 6. The traditional TLC chromatogram of 12 amino acids. Line1: Alanine, 2: Lysine, 3: Glutamic Acid, 4: Asparagine, 5: Proline, 6: Cysteine, 7: Histidine, 8: Glycine, 9: Hydroxyroline, 10: Valine, 11: Isoleucine, 12: Arginine.

Table 3. Rf values of 12 amino acid

Amino acid	Alanine	Lysine	Glutamic Acid	Asparagine	Proline	Cysteine
Rf	0.30	0.23	0.33	0.21	0.24	0.39
Amio acids	Glycine	Hydroxyroline	Valine	Isoleucine	Arginine	Histidine
Rf	0.26	0.25	0.47	0.54	0.18	0.13

The experiment shows that the ASC and PSC from the sheep skin were containing of 10 from 12 amino acids studied. Whereas had not noticed the presence the amino acids Cysteine and Histidine. Also was become clear existence the amino acids distinguishing of collagen such as glycine, proline, lysine and hydroxyproline , this is shown by the Fig.7.

Conclusion

ASC and PSC could be extracted from the sheep skin, Much higher yield was obtained for PSC, in comparison with ASC. However, the extraction process with pepsin to increase the yield of collagen should be further improved, the collagen from sheep skin could be used as an alternative source with the wide applications in food, pharmaceutical, cosmetic and biomaterials.

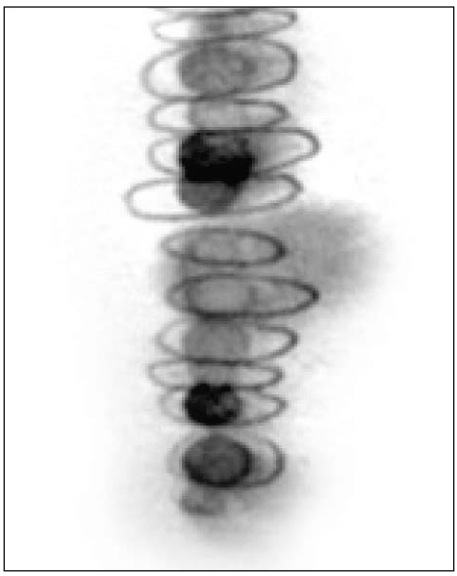

Fig. 7. TLC chromatogram of sheep's collagen

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