COLLAGEN MATRICES FROM LEATHER INDUSTRY WASTES FOR BIOMEDICAL APPLICATION

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Modern biomedical science is challenged to develop new wound healing drugs. The collagen-containing wastes of leather industry could be the rich source of collagen products for further use in biomedical science. The aim of this research was to find the best source of collagen between limed pelt, delimed pelt and fleshings of cattle hides, and to prepare it for the use as a matrix for further microbiological studies. Collagen was extracted with 0.5 M acetic acid and 5 mM EDTA. The purity of the extracted collagen was checked by gel-electrophoresis (SDS-PAGE). The rate of growth and crystal violet assay of laboratory strains (*S. aureus*, *P. aeruginosa*) were used for microbiological evaluation of obtained collagen matrices. The delimed pelt provided the highest concentration of collagen and the greatest volume of collagen products. All obtained collagen products were applicable as matrices for microbial cells growth. The applicability of collagen products from leather industry wastes for biomedical studies in Ukraine was shown.

Keywords: Collagen, biomedical application, leather industry wastes.

INTRODUCTION

Collagen can be extracted from different species of animals as it is usually obtained from by-products of slaughter. The main sources of collagen are the skin, tendons, cartilage and bones. Some studies were focused on obtaining collagen from such animal sources as fish and birds, as an alternative to bovine collagen (cattle) due to the risk of zoonosis, and as an alternative to pig-derived collagen for use in countries with restrictions because of ethical or religious reasons.

In the pharmaceutical and biomedical fields collagen is used as microparticles, injectable dispersions, ophthalmic shields, drug delivery systems, skin substitutes, blood vessels, and human ligaments. This is due to its characteristics such as weak antigenicity, ability to attach to cells, biodegradability and biocompatibility. Type I collagen is considered the gold standard for tissue engineering due to its high biocompatibility. It is used as the main matrix for the cell culture system. Collagen-based biomaterials, such as injection matrices, scaffolds for bone regeneration etc. are widely used (Parenteau-Bareil *et al.*, 2010). In modern medicine, scaffolding based on collagen plays a vital role. It helps in the reconstruction of cartilage and bones. During vascular and cardiac reconstruction, patients are successfully treated with collagen in the form of tissue engineered vessels. Collagen-based bandages are used in the form of sponges, films and powders for wounds or burns, surgical sutures, for urogenital disorders, corneal defects, study of nerve migration, in dentistry, bone transplantation, arthritis and obesity (Sanz-Herrera and Reina-Romo, 2011). Collagen has a variety of applications in cardiology (heart valve), dermatology (for skin replacement, soft tissue augmentation, skin engineering), surgery (as a hemostatic agent, in wound healing and dressing, nerve repair, blood vessel prostheses), orthopedics (tendon, bone and ligament repair, cartilage reconstruction), ophthalmology (horn grafts, contact lenses), urology (hemodialysis membranes, sphincter restoration) (Rose and Oreffo, 2002).

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Only fully or partially soluble collagen can be used in the production of matrices, powders, sponges, fibers or threads for tissue engineering. The distribution of molar mass, structure and composition, as well as functional properties of collagen depend on the extraction process and conditions of the raw material (Prestes, 2013). So it is needed to get the proper extraction parameters for each type of raw material to obtain the best characteristics of the extracted collagen. Due to the extraordinary diversity of tissues and types of collagen, it is difficult to develop a standard method of its extraction. The number of covalent intermolecular interactions in the structure of collagen increases over time and often determines the almost complete insolubility of the obtained product (Mocan *et al.*, 2011). It is required to remove numerous covalent intra- and intermolecular cross-links primarily including lysine and hydroxylysine residues, ether bonds, and other polysaccharide bonds for collagen extraction.

OBJECTIVES OF THE STUDY

The objects of this study were collagen samples obtained from leather production waste of the footwear uppers from raw materials of the cattle tannery “Chinbar” (Kyiv, Ukraine). To find the best option, different types of waste were selected such as limed pelt after trimming, delimed pelt after trimming, limed fleshings.

Chemical Analysis of Leather Production Waste

It was determined the content of the main components in the waste samples: moisture, minerals, total Nitrogen and calcium hydroxide (Table 1).

Table 1. Chemical composition of leather production wastes

Indicator	limed pelt	delimed pelt	fleshings
Mass fraction, %:			
moisture	82.1	80.4	84.2
minerals *	10.7	3.3	31.7
total Nitrogen*	14.3	15.0	5.4
calcium hydroxide*	2.6	0.4	6.7

Note: * on absolutely dry matter

The increased content of minerals and calcium hydroxide in the limed pelt and limed fleshings samples can be explained by the peculiarities of the technological process of leather production, which involves the processing of raw materials in a calcium hydroxide concentrated solution. As the main purpose of the deliming process is the extraction of calcium hydroxide from the dermis, the content of minerals and calcium hydroxide in the delimed pelt after trimming was 3.2-9.6 and 6.5-16.8 times lower, respectively. The lowest content of total Nitrogen, which indirectly indicated the amount of collagen in the structure, was found in the limed fleshings samples.

METHODS

The content of moisture, minerals and total Nitrogen in the waste of leather production was determined by standard methods ISO 1666:1996, ISO 3593:1981, ISO

5397:1984. The concentration of collagen in the solution was determined by biuret method on Spectrophotometer ULAB 102, China (540 nm).

Method of Collagen Extraction

The method of (Savchuk *et al.*, 2017) with changes was chosen as a basis.

First Extraction

1. Deliming process with an ammonium sulfate consumption of 3% by weight of the samples, duration 1 h, 38-40 °C (only to obtain samples of delimed pelt).
2. Samples grinding to the size of 3×3 mm.
3. Weighing.
4. Washing with water at 20 °C for 45 minutes with water change every 15 minutes.
5. Deposition of non-collagen proteins with 10% sodium chloride solution, duration 1 h of shaking at 20 °C, then 22 h of rest at 4 °C and again 1 h of shaking at 20 °C.
6. Washing the samples with distilled water to pH = 6.5.
7. Extraction of collagen with 0.5 M acetic acid solution in the presence of 5 mm EDTA in a ratio of 1 : 10 (weight : volume), 2 h of shaking at 20 °C, 20 h of rest at 4 °C and again 2 h of shaking at 20 °C.
8. Filtering through a paper filter. The samples were used for the 2nd extraction.
9. Precipitation of collagen from the filtrate with dry sodium chloride (with a final concentration of NaCl 1M) for 24 h at 4 °C.
10. Centrifugation for 30 min at 3000 rpm
11. Dissolution of precipitated collagen in the least amount of 0.5 M acetic acid.
12. Re-precipitation of collagen with dry sodium chloride (with a final concentration of NaCl 0.9 M) for 24 h at 4 °C.
13. Centrifugation for 30 min at 3000 rpm
14. Dissolution of precipitated collagen in the smallest amount of 0.1 M acetic acid.

Second Extraction

Collagen samples remaining after the 1st extraction (item 8) were processed according to items 7-14.

Third Extraction

Collagen samples remaining after the 2nd extraction (item 8) were processed according to items 7-14.

Estimation of Extracted Collagen and Collagen Peptides by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was done as described by the (Laemmli, 1970) using 4 % (w/v) stacking gel, 6 % (w/v) separating gel for estimation of purity of extracted collagen and 18 % (w/v) separating gel for collagen peptides. SDS-PAGE was performed using Mini-Protean Tetra System (Bio Rad, USA) at 19 mA for stacking and 36 mA for separating gels. After electrophoresis, the gels were stained with 2.5 % coomassie brilliant blue R-250 in 10 % (v/v) ethanol, 10 % (v/v) acetic acid, 15 % (v/v) isopropanol and the background of the gel was destained with 7 % (v/v) acetic acid for 30 min.

Microbial Assay

Collagen samples after extraction and washing with distilled water (for pH 5.5) in sterile conditions was used as matrices for bacterial cell growth. Laboratory strains and hospital isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used. The OD and attachment rate were evaluated with microtiter plate assay (630 nm) and crystal violet assay (570 nm) on Spectrophotometer UV-Vis, Russia (O'Toole, 2011).

RESULTS

The main criteria for evaluating the extraction efficiency of collagen obtained from collagen-containing wastes from the leather industry was the amount of extracted collagen, which was determined by the protein content in the solution after extraction and subsequent dissolution in acetic acid. Collagen was re-extracted twice from the supernatant obtained after the first and second extraction stages to determine the economic feasibility of re-extraction. The changes in pH of the solutions at different stages of extraction are presented (Table 2).

Table 2. The pH values of collagen-containing solutions

Extraction method item	limed pelt	delimed pelt	fleshings
it. 4 after 15 min	10.55	6.80	7.95
it. 4 after 30 min	7.85	6.40	6.60
it. 4 after 45 min	6.50	5.90	6.40
it. 5	8.00	7.50	9.80
it. 14	2.60	2.60	2.80

Data on the collagen amount obtained after the first and further extractions from different leather waste were presented in Table 3.

Table 3. Collagen amount obtained after extraction

	limed pelt	delimed pelt	fleshings
The amount of total Nitrogen after the 1 st extraction, mg	7.6	50.4	3.3
The amount of total Nitrogen after the 2 ^d extraction, mg	39.2	71.4	11.5
The amount of total Nitrogen after the 3 ^d extraction, mg	3.3	1.0	0.5
The total yield of total Nitrogen after three extractions, %	2.79	5.97	2.60
The amount of collagen after the 1 st extraction, mg	10.1	25.3	3.0
The amount of collagen after the 2 nd extraction, mg	5.0	11.4	1.4
The amount of collagen after the 3 rd extraction, mg	2.2	4.7	0.6

The largest amount of collagen was extracted from samples of delimed pelt. It was found that the third extraction was not efficient due to the low amount of extracted collagen in all samples. The less amount of collagen in limed pelt and fleshings could be explained by the fact that part of the acetic acid from the solution was spent on neutralizing the calcium hydroxide excess found in the samples of these groups. The fleshings samples were also characterized by a lower initial content of total Nitrogen (Table 1).

It is known that type I collagen consists of two or more different chains (heterotrimers): usually two α -1, one α -2 chain with a similar molecular weight of about 100-110 kDa and the structure of the beta component (Kimura *et al.*, 1987). It was shown that extracted collagen consisted of both α 1 and α 2 chains (Fig.1). Thus, based on the SDS-PAGE results collagen extracted from the waste of the leather industry belongs to type I collagen.

Figure 1. SDS-PAGE pattern of collagen extracted from waste of leather industry on 6% separating gel. Lane *K* – control collagen (Savchuk *et al.*, 2017), *M* - high molecular weight (MW, kDa) protein markers; 1 - limed pelt, 2 - delimed pelt, 3 - fleshings

After microbiological studies it was found that the optical density of *S. aureus* strains was two times less while the attachment was two times higher in the presence of collagen samples. This tendency was also kept in case of *P. aeruginosa* with the only difference - the indexes were about 30%. The highest bacterial growth and attachment were observed on the collagen matrices from 1st extraction samples of delimed pelt and limed pelt. The 2nd extraction samples were about 25-30% slightly less effective. According to studied parameters the first choice collagen matrices for bacterial growth was collagen from delimed pelt, the second choice matrices - collagen from limed pelt.

CONCLUSIONS

Collagen-containing leather industry wastes could be the great source of collagen for biomedical application. The greatest amount of collagen was obtained from delimed pelt after the first and second extractions. Such kind of collagen was classified as type I and pure. It was also the perfect matrix for microbial cell grow which could be the evidence of its matching for further using in skin wound treatment.

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