PROTEIN COMPOSITES FROM COLLAGEN BY-PRODUCTS FOR SAFE USE IN CIRCULAR ECONOMY

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Sustainable economy means reducing the carbon footprint and minimizing the amount of waste released from productive activities. This paper presents the characterization of composites obtained from by-products of the leather industry based on collagen and keratin extracts. The protein composites have specific properties for the agricultural field and industrial applications in accordance with the current recommendations for a sustainable economy. Chemical-enzymatic hydrolysis of leather and wool by-products was performed for protein extraction. The composites were obtained by addition and crosslinking of collagen and keratin extracts with tannins from vegetable by-products. The characterization of composites was performed based on the results of analytical investigations by physico-chemical methods: volumetry, potentiometry, Texture Analysis, Dynamic Light Scattering, colorimetry, microscopy. It has been found that new collagen and keratin extracts contain small and medium components size, useful for the biostimulation of agricultural crops, but also contain large size components which give adhesive and film-forming properties, useful in industrial applications.

Keywords: by-products, composites, biocompatibility

INTRODUCTION

The circular economy is a model of production and consumption, which involves extending the life cycle of products and reducing waste to a minimum. When a product reaches the end of its life, its materials are kept within the economy wherever possible and they can be productively used again, thereby creating further value.

The leather processing industry (cattle, sheep, pigs, birds, fish) as well as the food industry generate significant amounts of by-products. As a result of biotechnological processing, industrial protein waste generates amino acids and peptides, which constitute alternative sources of nutrients for agriculture, as biostimulators and fertilizers (Epure *et al*., 2018), but can also be used in various other applications: adhesives (Sun *et al*., 2020), packaging (Ahmed *et al*., 2022), surfactants (Li *et al*., 2021), auxiliaries for leather processing (Ammasi *et al*., 2020), or as a metal chelating agent in decontamination actions (Lin *et al*., 2021). Furthermore, collagen from protein waste from the fish industry can be successfully used in various biomedical applications, including wound healing, tissue engineering and regeneration, drug delivery and other therapeutic applications (Subhan *et al*., 2021). Based on the proteins extracted from the by-products, we can generate materials with added value without using substances obtained through chemical synthesis, if we use extracts from vegetable waste that contain significant amounts of phenolic and polyphenolic acids, which function as crosslinking agents for protein compounds (peptides, polypeptides, amino acids) recovered from waste (Kaczmarek *et al*., 2020).

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The intelligent use of secondary protein resources is an ecological alternative to synthetic materials and contributes to reducing the carbon footprint, in the context of the circular economy.

This paper presents the characterization of new composites obtained from by-products of the leather industry based on collagen and keratin extracts for the agricultural field and industrial applications in accordance with the current recommendations for a green economy. For the safety of contact during the use of this composites, a cytotoxicity study was carried out, which will be presented in this work.

EXPERIMENTAL PART

Materials

The bovine leather by-products were collected from the Leather and Footwear Research Institute Division, Romania and wool was purchased from sheep farmers, for collagen and keratin extraction, as gelatin with average molecular weight over 35 kDa and collagen and keratin hydrolysate with average molecular weight 16 - 24 kDa.

The pomegranate peel was collected from food industry to obtain tannin extract.

Chemical reagents of analytical grade, like ammonia (25%), sodium carbonate, potassium hydroxide, tartric acid, oxalic acid, were purchased from Chimreactiv SRL (Bucharest, Romania). Ethoxylated alkyl non-ionic detergent from Borron SE for wool degreasing. Alcalase 2.4 L (protease from Bacillus licheniformis with 2.4 U/g) were purchased from Sigma-Aldrich (Bucharest, Romania). Glycerol were products of SC Chimopar SA Romania. Polyvinyl alcohol from SC REMED PRODIMPEX SRL.

Stabilized mouse connective tissue fibroblast cell line (NCTC clone 929) from the European Collection of Cell Cultures (ECACC - Sigma-Aldrich, USA).

Procedures

Bovine gelatin (GPU-B) was obtained by thermal hydrolysis of semi-processed leather by-products at 80°C temperature and pH 5.5-6.0. The collagen hydrolysate (HPU-B) was obtained by chemical-enzymatic hydrolysis at 63±2°C and pH 8.0-9.0. The keratin hydrolysate (KHU-B) was obtained by alkaline-enzymatic hydrolysis at pH 8.0-8.5 for 24-32 hours.

Pomegranate peel extract was used for crosslinking. The pomegranate peel extract (with 6% dry substance, 1.5% tanning substances) was obtained by hydrolyzing the pomegranate peel powder in water at pH=3.0-4.0, centrifugation and vacuum filtration (Cano-Lamadrid *et al.*, 2022).

The composites based on collagen or collagen and keratin were made by continuously stirring the gelatin, collagen or keratin hydrolysate additivated-crosslinked with glycerol, pomegranate peel extract at 40°C for 90-120 minutes, mixing with 5% polyvinyl alcohol solution and ultrasonication for 30 minutes at 40°C, casting as films and drying in vacuum at 55°C. The following types of films were made: PH1 collagen film, PH2 collagen and keratin film, PGA gelatin film. Also, PM control film, were made only of polyvinyl alcohol.

For the characterization of gelatin GPU-B, other films were cast: film FGS only from gelatin at the standard concentration 6.67% (GS) and film FGC only from gelatin at a concentration three times higher than the standard (GC).

The cytotoxic potential was determined by *in vitro* tests by colorimetric determination of cell viability using the standard MTT test (3-(4.5-dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide). The plates were incubated for 24 and 48 hours at

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37°C in an air atmosphere with 5% CO_2. Finally, the absorbance was measured at the wavelength of 570 nm. To study the morphology, the culture of mouse fibroblast cells (NCTC), fixed in methanol and stained with Giemsa solution, was analyzed microscopically 48 hours after the addition of the protein compositions.

Analytical Methods

The collagen and keratin extracts and their complexes were analysed by gravimetric methods, dry substance (SR EN ISO 4684:2006) and total ash (SR EN ISO 4047:2002), by volumetric methods, in terms of total nitrogen and protein substance (SR ISO 5397:1996), aminic nitrogen (ICPI protocol) by potentiometric method for pH measurement (SR EN ISO 4045:2008).

Texture tests of gelatin and composites based on collagen and keratin were carried out using a TEX'AN texture analyser.

Dynamic Light Scattering was used for size particle determination and distribution by ZetaSizer device Nano ZS (Malvern, UK).

To evaluate biocompatibility, the absorbance was measured at a wavelength of 570 nm, using a Mithras LB 940 plate reader (Berthold Technologies). The morphology of mouse fibroblast cell cultures was observed with a Zeiss AxioStar Plus microscope equipped with a digital camera driven by AxioVision 4.6 software (Carl Zeiss, Germany).

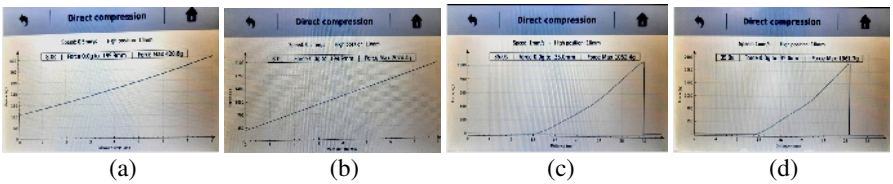
RESULTS AND DISCUSSIONS

To obtain collagen and keratin composites, the following protein extracts were prepared: GPU-B gelatin, HPU-B collagen hydrolysate, KHU-B keratin hydrolysate, with the chemical characteristics presented in Table 1.

Table 1. Characteristics of protein extracts

Characteristics	MU	Gelatin	Hydrolysates	
		GPU-B	HPU-B	KHU-B
Volatile matter	%	7.29	4.25	7.54
Total ash	%	0.88	1.44	7.80
Total nitrogen	%	16.33	16.77	12.60
Protein substance	%	91.71	94.24	76.35
Amino nitrogen	%	0.45	0.85	0.62
pH analytical solution	-	5.60	7.54	7.68

An important characteristic for the industrial applications of gelatin is the strength of the gelatin. The strength of gelatin with standard concentration (GS), compared to gelatin approximately 3 times more concentrated (GC) was determined using a TEX'AN texture analyzer. The results of the analyses are presented in Figure 1 (a) and (b).



(a) (b) (c) (d)

Figure 1. Determining the strength of gelatin: (a) GS, (b) GC and of gelatin films: (c) FGS, (d) FGC

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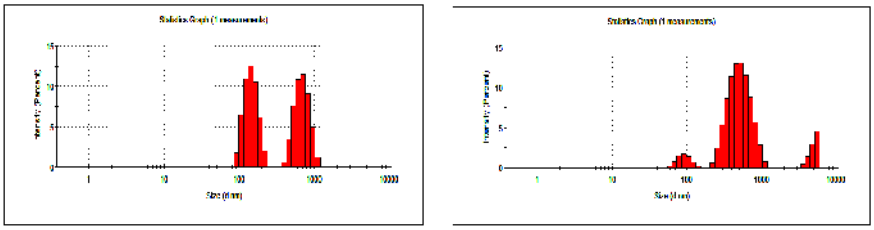
Analytical data indicate that concentrated gelatin (GC) has a strength, expressed in grams, almost 5 times higher than that of gelatin with standard concentration (GS), respectively 2074.4 g versus 420.8 g. Both tests highlight the fact that a higher concentration, compared to the standard concentration, confers a higher resistance, and for the films associated with them, FGS and FGC the deformation force up to the breaking point is almost double, namely 1961 g compared to 1052 g.

The analysis of collagen and keratin hydrolysates by Dynamic Light Scattering (DLS) presented in Figure 2, (a) for HPU-B collagen hydrolysate and (b) for HKU-B keratin hydrolysate, highlights the existence of small peptide fragments, in the specific "nano" field in this case for free amino acids and oligopeptides.



(a) (b)

Figure 2. Particle size in protein hydrolysates: (a) collagen; (b) keratin

The particle size distribution in collagen and keratin hydrolysates is presented in Table 2:

Table 2. Particle size distribution in protein hydrolysates

Sample	Particle size share		
	10-100 nm	100-1000 nm	1000-10000 nm
Collagen hydrolysate, HPU-B	1.8	97.0	1.2
Keratin hydrolysate, HKU-B	4.2	85.6	10.2

The reflected light intensity measurements indicate both populations of small particles, in the range of 10-100 nm and mainly populations of medium particles, located in the range of 100-1000 nm, dominant for collagen hydrolysates, but also large particles, in the range of 1000-10000 nm, spread over a wider range in keratin hydrolysates. DLS analysis corresponds with the higher content of amino nitrogen, which indicates a lower average molecular weight (Sörensen method) of collagen hydrolysate compared to keratin hydrolysate.

In this research, films were made from collagen and keratin extracts by additivating and crosslinking them with polyvinyl alcohol, glycerol, pomegranate peel extract, in three variants: with gelatin (PGA); with collagen hydrolysate (PH1); with collagen and keratin hydrolysates (PH2). The deformation forces up to the breaking point were determined in comparison with the control film, made of polyvinyl alcohol (PM). The results of determining the breaking point for these films are presented in Figure 3.

It is observed that although the appearance of the curves differs from one sample to another, the values of the deformation forces up to the breaking point are located near the maximum detection limit of the device, from 4995.2 g for the control film, to 5008.2 g for the film with additive-crosslinked collagen hydrolysate.

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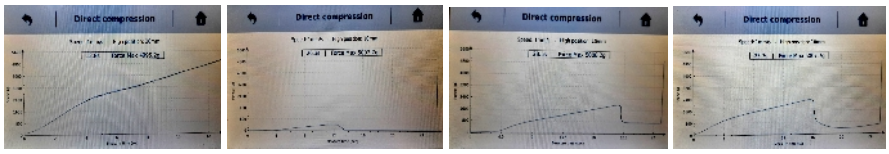


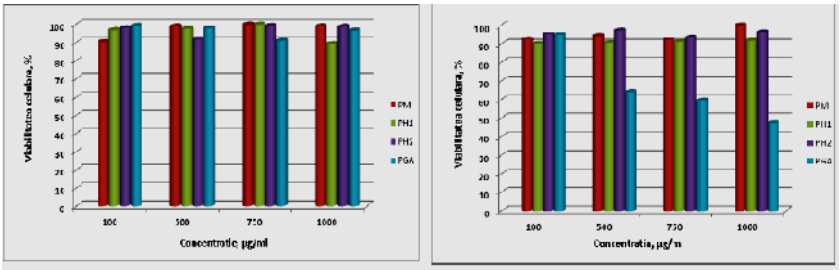
(a) (b) (c) (d)

Figure 3. Determining the strength of films: (a) PM; (b) PGA; (c) PH1; (d) PH2

It is also noted that the additivation-crosslinking of gelatin, Figure 3(b) causes an increase in the deformation forces up to the breaking point by approximately 2.5 times compared to the film formed from concentrated gelatin, without additivation-crosslinking, Figure 1(d).

The biocompatibility of the composites was quantified by cell viability, shown in Figure 4, (A) after 24 hours and (B) after 48 hours. The results were reported as viability percentages depending on the control sample (cells incubated without solution from the analysed sample) considered to have 100% viability.

It is found that the samples analyzed after 24 hours have no cytotoxic effect, remaining viable between the values of 96.84% and 89.28% in the concentration range 100-1000 g/ml for sample PH1; 98.00% and 98.94% in the concentration range 100-750 g/ml for sample PH2; 99.05% and 96.63% in the concentration range 100-1000 g/ml for the PGA sample and 90.33% and 98.74% in the concentration range 100-1000 g/mL for the PM sample. After 48 hours of incubation, over the entire concentration range, 100-1000 g/ml, samples PH1, PH2, PM do not have a cytotoxic effect, but the PGA sample has a non-cytotoxic effect at a concentration of 100 g/ml, slightly cytotoxic at in the range of concentrations 500-750 g/ml and moderately cytotoxic at a concentration of 1000 g/ml.



(A) (B)

Figure 4. Cell viability: (A) after 24 hours; (B) after 48 hours

The microscopic evaluation led to the following observations: (i) the culture control (MC) shows the specificity of the cell line of mouse fibroblasts, NCTC-type, clone 929, with normal, elongated cells, having 2-3 extensions and monochrome cytoplasm; the positive control (M+), namely 3% oxygenated water, added to the culture medium (MEM) in an amount of 2 µl/ml generates cytotoxicity on the cells; (ii) for the PH1 sample in the concentration range 100-1000 g/ml, the cells are uniform, monochrome cytoplasm, without cellular debris, with a cell density comparable to the culture control, without cytotoxicity; (iii) for the PH2 sample in the concentration range 100-1000 g/ml, the cells have a normal appearance, without cytotoxicity; (iv) for the PGA sample at the concentration of 100 g/ml cells are uniform, comparable to the culture control, without cytotoxic effect, at the concentration of 500-750 g/ml cells are uniform, but rare, slight

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cellular debris appears, with slightly cytotoxic effect, at a concentration of 1000 g/ml, cells are uniform, rounded, rarer, small cellular debris is present, the effect being moderately cytotoxic; (v) for the control sample PM, in the concentration range 100-1000 g/ml, the cells have a normal appearance, without cytotoxicity.

CONCLUSIONS

The new collagen and keratin extracts contain small and medium components size, useful for the biostimulation of agricultural crops and contain large size components which give adhesive and film-forming properties, for industrial applications.

The protein extracts processed or not by additivation, crosslinking or just by simple physical conditioning, lead to obtaining composites with specific properties for the production of plant growth biostimulators, nutrients for crops and agricultural land, adhesives, for agriculture and industrial applications alike (wood, paper, leather, etc.).

The biocompatibility tests demonstrate the fact that the collagen and keratin hydrolysates from the composites do not have cytotoxic potential, but some composites with gelatin at concentrations higher than 500 g/ml may show a slight cytotoxic effect in comparison with a control that does not have such an effect.

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