Use of waste from the leather industry for the production of biotechnological products based on collagen

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Abstract Leather industry generates vast quantities of by-products which could be a cheap and available source of collagen and collagen peptides. These peptides have been widely used in cosmetic, biomedical, and pharmaceutical industries. The main objective of present study was to obtain a biotechnological product based on collagen and to study its properties.

Lime fleshings from hides were fragmented into small pieces with size ≤ 0.5 cm². Collagen was extracted from trimming waste using 0.5 M acetic acid in the presence of 5 mM EDTA. The purity of extracted collagen was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Collagen was enzymatically hydrolyzed by pepsin. To stop the reaction, the mixture was heated immediately at 100°C and then centrifuged at 4000 g for 30 min. The supernatant was filtered through ceramic membrane for separation of collagen peptides fraction (26≤kDa). Collagen peptides were prepared from the obtained collagen. The generated collagen peptides was analyzed by SDS-PAGE using 18% resolving gel.

The influence of collagen peptides on antioxidant enzymes and lipid peroxidation were explored. Collagen peptides supplementation also lowered serum level of pro-inflammatory cytokines such as IL-1β, IL-12, while level of anti-inflammatory cytokine IL-4 was within the control value.

1. Introduction

The problem of processing and rational use of waste products of industries in recent years became particularly relevant. Wastes by leather industries have raised serious concerns on account of their environmental impacts [1]. This waste should be recycled to overcome the pollution issues which would ultimately cause health problems for human and other living beings. Therefore it is important to develop a simple and available method for the optimal utilization of this waste to provide a practically feasible and economically viable solution. The leather industry waste has a huge amount of protein-rich by-products, which after extraction could be used for different purposes. The trimmings contain high amount of fibrous protein [2], particularly collagen, which is actually a valuable bioresource due to its biological safety, excellent biocompatibility, low antigenicity, high biodegradability, and cell growth potential. Generally,
collagen has been applied in different fields, among the best known are the food, cosmetic, biomedical, pharmaceutical, and film industries [3].

Nowadays, special attention is given to research into collagen peptides (CP) due to a broad spectrum of their bioactivities. CP has been found to have a wide range of functional and biological properties, including antimicrobial, anti-inflammatory, anti-ulcer, lipid-lowering, wound-healing and anti-skin-aging activities [4,5]. These bioactivities and great health-enhancing potentials have led to the use them as ingredients of functional foods or pharmaceuticals. Majority of the studies on collagen peptides focused on their anti-oxidative activity which is very important taking into account that many human diseases are known to be caused by generation of free radicals and development of oxidative stress. Collagen peptides can reduce the peroxidation of lipids or fatty acids, scavenge free radicals and chelate transition metal ions [6], which has led to the hypothesis that these peptides could be suitable supplement agents for several types of illness associated with oxidative stress.

The main objective of this study was to isolate the collagen fraction from the trimming waste of leather industry, prepared collagen peptides and then evaluated the effect of CP administration on oxidative stress markers and cytokine profile in obese rats.

2. Materials and methods

2.1. Extraction of collagen
Collagen was extracted according to the method described in [7]. Extraction of collagen from raw material of leather industry was done in two steps. At first, lime flashings from hides were washed in cold water, poured with 20 % NaCl and left for a day to precipitate non-collagen proteins, then washed with distilled H₂O to neutral pH and dried. Extraction of the collagen from the dried material was carried out using 0.5 M of acetic acid containing 5 mM EDTA for 24 h. The extract was centrifuged at 10000 g for 30 min and the residues were re-extracted with the same solution for 24 h with further centrifugation. The supernatant was combined and salted out by adding NaCl to a final concentration of 0.9 M. The precipitated collagen was separated by centrifugation at 10000 g for 30 min, redissolved in 0.5 M of acetic acid and precipitated again with NaCl. The obtained precipitate was dialyzed against distilled water and lyophilized.

2.2. Preparation of collagen peptides
The procedure of CP preparation was performed as described [8]. The obtained collagen was enzymatically hydrolyzed by pepsin (3000 U g protein⁻¹) at 37°C, pH 2.0 for 8 h. To stop the reaction the mixture was heated to boiling for 5 min and then centrifuged at 4000 g for 30 min. The supernatant was filtered through a ceramic membrane (200 μm) to separate the collagen peptides fraction with molecular weight below 26 kDa.

2.3. Estimation of extracted collagen and collagen peptides by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-PAGE was done as described by the [9] 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.

2.4. Animals and experimental design
A total of 30 adult male Wistar rats weighing 170±5 g were used in present study. The study protocol was approved by the Ethical Committee of ESC “Institute of Biology and Medicine” Taras Shevchenko
National University of Kyiv. All animals were housed under controlled conditions with temperature of 25±2°C, relative humidity of 60±10 %, room air changes 12–18 times/h, and a 12-h light/dark cycle. Animals were allowed free access to food and water. The potential antioxidant and anti-inflammatory effects of collagen peptides were investigated on obesity model induced by high-calorie diet [10].

2.5. Sample collection
Blood samples were collected in standard biochemical test tubes. Serum for the determination of biochemical parameters was prepared by centrifugation at 1000 g of previously incubated blood samples for 30 min at 37°C. The serum was separated and kept at -20°C until analysis. Protein concentration was determined according to the method of Bradford [11], using crystalline bovine serum albumin as a standard.

2.6. Measurement of lipid peroxidation
The lipid peroxidation (LPO) was characterized by the spectrophotometric assessment of thiobarbituric acid reactive substances (TBARS). An aliquot of 0.4 mL of blood serum was added to 1.6 mL an aqueous solution of 25 mM Tris-HCl and 175 mM KCl (pH 7.4). Total protein fraction was separated from the mixture by precipitation with 20% trichloroacetic acid and further centrifugation for 15 min at 5000 g. After addition of 1 mL of 0.8 % aqueous solution of thiobarbituric acid to 2 mL of obtained supernatant, the samples were heated for 30 min in a boiling water bath. After cooling, the optical density of the samples was determined with a spectrophotometer (Smart Spec™Plus, BioRad, USA) at 532 nm. The amount of LPO products was calculated using the molar extinction coefficient $\epsilon_{532}=1.56 \times 10^5$ M$^{-1}$cm$^{-1}$. The TBARS level was expressed as nmol (mg protein)$^{-1}$ [12].

2.7. Antioxidant enzyme activity assays
Superoxide dismutase (SOD) activity was assayed by the method [13] based on the capability of SOD to inhibit the autooxidation of adrenaline. Catalase (CAT) activity was measured by the method [14].

2.8. Cytokine assay
Cytokine measurements in liver tissue were done by enzyme-linked immunosorbent assay according to the standard instructions [15].

2.9. Statistical analysis
Data entry and analysis were performed using StatSoft Statistica ver. 7.0 for Windows. After testing for normality (by Shapiro-Wilk), one-way analysis of variance (ANOVA) was used to compare the means among different groups. Differences were considered to be statistically significant when $p<0.05$. Data were reported as means±standard deviation (SD).

3. Results and discussion
The purity of obtained collagen was estimated by SDS-PAGE. It's known that collagens type I are constituted of two or more different chains (heterotrimers): typically two alpha 1, one alpha 2 chains of similar molecular weight about 100-110 kDa and a beta component structure [16]. Figure 1 showed that extracted collagen consisted of both $\alpha_1$- and $\alpha_2$-chains, and the band of $\alpha_1$-chain was about twice as intense as that of $\alpha_2$-chain. The presence of band at the region of 200 kDa could be result of dimmerization of $\alpha$-chains. Thus, on the basis of subunit composition and SDS-PAGE pattern, it could be speculated that collagen extracted from waste of leather industry belongs to collagens type I.

Many researches have demonstrated that collagen peptides exhibited favorable functional properties and biological activities, which had become a topic of great interest for health food and processing/preservation industries. Enzymatic hydrolysis is one of the widely used approaches for the
effective release of bioactive peptides from protein sources. During hydrolysis, a wide variety of smaller peptides are generated, depending on enzyme specificity and the hydrolysis time. Hydrolysis by pepsin was used as the most suitable approach for producing of collagen peptides. Figure 2 represented the composition of the obtained collagen peptides estimated by SDS-PAGE.

It has been widely accepted that oxidative stress plays a critical role in initiating and driving the cascade of events that results in development of serious pathologies [17]. Obesity is one of the diseases which appear to be strongly associated with increased generation of free radicals and LPO reactions [18].

![Figure 1. SDS-PAGE pattern of collagen extracted from waste of leather industry on 6 % separating gel. Lane M - high molecular weight (MW, kDa) protein markers; lane 1-2 - extracted collagen](image1)

![Figure 2. SDS-PAGE pattern of collagen peptides on 18 % separation gel. Lane M - low molecular weight (MW, kDa) protein markers; lane 1 - fraction of collagen peptides](image2)

The main parameter that allows to assess the intensity of oxidative processes and the stage of the oxidative stress is the amount of lipid oxidation products, in particular thiobarbituric acid reactive substances (TBARS). Normally, endogenous antioxidant enzymes are able to scavenge the excessive ROS to protect tissues from oxidative injuries. Superoxide dismutase (SOD, EC 1.15.1.1.) and catalase (CAT, EC 1.11.1.6.) are two important antioxidant enzymes that inactivate superoxide anions and hydrogen peroxide, respectively. However, when oxidative stress persists for a long time, the antioxidant resources are depleted due to a decrease in the activity of antioxidant enzymes, as well as inhibition of their synthesis.

According to our results the systemic oxidative stress manifested by the accumulation of TBARS and decrease activity of main antioxidant enzymes SOD and CAT was observed in obese animals (table 1).

We found that long-term administration of the collagen peptides to animals which were on a high-calorie diet resulted in a significant decrease in TBARS content and partly normalization activities of both SOD and CAT. This may be due to the ability of collagen peptides influence the passing of free radical processes. The exact mechanism through which peptides display antioxidant activity is not fully understood. However, several explanations have been proposed to elucidate their antioxidant properties. The antioxidant activity of bioactive peptides is related to their amino acid composition, structure, and hydrophobicity. The presence of hydrophobic amino acids also increases the affinity of peptides to the lipid system, favoring their distribution at the water-lipid interface and enhancing the radical-scavenging activity at the lipid phase [19]. It also has been demonstrated [20] that peptides could be involved in the induction of the expression of genes for nonenzymatic antioxidant components.
Table 1. The activity of antioxidant enzymes (superoxide dismutase SOD, catalase CAT, and the content of thiobarbituric acid reactive substance (TBARS) in blood serum of control rats and rats with diet-induced obesity (DIO) with or without collagen peptides administration

| The experimental groups          | Control                  | Control with collagen peptides administration | DIO                  | DIO with collagen peptides administration |
|----------------------------------|--------------------------|-----------------------------------------------|----------------------|-------------------------------------------|
| SOD activity, U∙(mg protein)^{-1}∙min^{-1} | 48.2±3.3                 | -                                             | 23.5±4.1*            | 33.6±4.2*#                                |
| CAT activity, mmol H₂O₂∙(mg protein)^{-1}∙min^{-1} | 71.6±5.6                 | -                                             | 13.8±3.5*            | 37.2±6.4*#                                |
| TBARS, nmol∙(mg protein)^{-1}     | 93.5±11.5                | -                                             | 187.4±18.6*          | 356.0±18.5*#                              |

Values are expressed as mean±SD (n=10); *p<0.05 significantly different from the control; #p<0.05 significantly different from the DIO

Substantial evidences indicate that obesity is linked to a state of chronic low-grade inflammation [21]. Adipocytes have been identified as sources of pro-inflammatory cytokines, which are involved in the triggering of chronic local inflammation in adipose tissue as well as systemic inflammation [22]. In addition, oxidative stress is associated with an irregular production of adipokines which, in turn, are potent stimulators for the production of reactive oxygen and nitrogen species by macrophages and monocytes, generating oxidative stress and, a major, irregular production of other adipokines [23]. These changes may interact among themselves and amplify, producing, in this manner, the set of metabolic and vascular alterations. Considering the relationship between oxidative stress and cytokine profile, as well as the positive effect of the CF treatment on oxidative stress biomarkers, we tested whether CP could improve the cytokine balance in obese rats.

According to the results (table 2) the increase in the pro-inflammatory cytokines IL-1β, IL-6 concentration as well as a decrease in the IL-4 concentration in blood serum of animals with obesity was observed. A slight increase in the IL-10 concentration can be considered as a compensatory reaction of the organism in response to the changes of metabolic status.

Table 2. The concentration of cytokines in blood serum of control rats and rats with diet-induced obesity (DIO) with or without collagen peptides administration

| The experimental groups                        | Control                  | Control with collagen peptides administration | DIO                  | DIO with collagen peptides administration |
|-----------------------------------------------|--------------------------|-----------------------------------------------|----------------------|-------------------------------------------|
| **Pro-inflammatory cytokines, rel.units∙mL^{-1}** |                          |                                               |                      |                                           |
| IL-1β                                         | 19.22±2.34               | 20.23±2.54                                    | 24.21±2.71           | 14.55±3.28#                               |
| IL-6                                          | 21.91±3.10               | 21.21±2.10                                    | 26.82±3.54           | 17.67±4.12#                               |
| **Anti-inflammatory cytokines, rel.units mL^{-1}** |                          |                                               |                      |                                           |
| IL-4                                          | 63.77±7.63               | 60.14±8.54                                    | 38.64±5.27*          | 52.34±5.17#                               |
| IL-10                                         | 47.44±4.96               | 49.45±5.46                                    | 49.76±4.12           | 52.63±6.51                                |

Values are expressed as mean±SD (n=10); *p<0.05 significantly different from the control; #p<0.05 significantly different from the DIO
When collagen peptides were administrated to obese animals we noticed that pro-inflammatory cytokine (IL-1β, IL-6) levels were significantly decreased while concentration of anti-inflammatory cytokines (IL-4, IL-10) were increased. These results could indicate a potential application of collagen peptides against inflammatory conditions.

Conclusions
Waste of leather industry could be a profitable resource for collagen extraction and subsequent obtaining of collagen peptides. Our findings on the rat model of diet-induced obesity provide preliminary indication that CP administration can influence metabolic status under pathologies associated with oxidative stress development. This positive effect is most likely mediated by collagen peptide-induced up-regulation of antioxidant capability particularly enhancing the activities of antioxidant enzymes and reducing lipid peroxidation process that, in turn, help prevent oxidative stress and inflammation development. Taking into account results on anti-oxidant and anti-inflammatory activities of collagen peptides extracted from waste of leather industry, these peptides can be used for production of pharmacological products against diseases associated with oxidative stress.

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