https://doi.org/10.17113/ftb.60.03.22.7107 original scientific paper

Free Radical Scavenging, Redox Balance and Wound Healing Activity of Bioactive Peptides Derived from Proteinase K-Assisted Hydrolysis of *Hypophthalmichthys molitrix* Skin Collagen

Running head: Bioactive Peptides from *H. molitrix* Skin Collagen

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Received: 21 December 2020
Accepted: 28 April 2022

SUMMARY

Research background. Various protocols of enzymatic hydrolysis of fish by-products are increasingly tested to ensure value-added products with functional and biological properties important for food, cosmetic and medical applications. In addition, they target to minimize waste from industrial processing and environmental requirements. This study aimed to establish an efficient protocol based on two-step enzymatic hydrolysis of freshwater fish skin and to evaluate the effect of resulted bioactive peptides on free radicals scavenging, redox balance and regulation of fibroblast proliferation and migration processes.

Experimental approach. Pepsin-soluble collagen extracted from silver carp (*Hypophthalmichthys molitrix*) skin was hydrolyzed by proteinase K treatment, at specific sites, in controlled conditions. Ultrafiltration permeate was analyzed for molecular mass by gradient

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electrophoresis and gel filtration chromatography. The biologic activity of intermediate and small size bioactive peptides was evaluated in experimental models *in vitro* mimicking oxidative stress and skin wound conditions.

Results and conclusions. Enzymatic hydrolysis of extracted fish collagen was performed using proteinase K, the most efficient enzyme for the cleavage of the primary structure of the molecule, as previously found *in silico*. Established optimal conditions increased the enzyme specificity and the process yield. Bioactive peptides exerted significantly higher scavenging activity on free stable radicals and hydroxyl radicals often found *in vivo*, compared to fish collagen. They stimulated fibroblasts metabolism in a dose-dependent manner and up-regulated cell migration in a scratch-wound model. Pre-treatment of fibroblasts with induced oxidative stress using optimal concentrations of fish peptides prevented the increase of reactive oxygen species production. In conclusion, bioactive peptides from carp skin demonstrated valuable properties of redox balance maintaining and skin wound healing processes improvement, which indicated further potential applications in the development of pharmaceutic and nutraceutic formulations.

Novelty and scientific contribution. In this study the enzymatic hydrolysis was applied to isolated protein, in contrast to previous studies using waste tissue with variable composition. Recovered bioactive peptides acted not only as antioxidant agents, but also as regulators of oxidative stress and wound healing processes in skin cell models. Their nutritional and cosmetic application is recommended in novel formulations fighting skin aging phenomena.

Keywords: fish peptides; protease; antioxidant activity; cell migration; oxidative stress

INTRODUCTION

The nutritional value of fish by-products is almost identical to that of the edible parts so, nowadays, their reprocessing can lead to low cost, value-added food products with improved properties (*1*). Among the high grade products obtained by bioconversion of fish waste, bioactive peptides are important for their applications as alternative ingredients in functional food, nutraceuticals and pharmaceuticals, to prevent diseases and improve human health (*2*). As natural products with antioxidant activity, fish bioactive peptides can be used in food industry, as an alternative to synthetic antioxidants (butylated hydroxyanisole - BHA, butylated hydroxytoluene - BHT), which may have adverse effects on enzymes activity and DNA (*3*). Fish peptides are also recommended as ingredients in cosmetic industry owing to their antioxidant activity, cryoprotective, photoprotective and moisture-retention abilities (*4*). Antihypertensive potential through inhibition of the angiotensin-converting enzyme (ACE) and their anticoagulant, antimicrobial and immunomodulatory activities recommend them as high-value active ingredients for medical applications (*5,6*).

Besides the naturally occuring bioactive peptides, they can be produced by enzymatic hydrolysis of fish protein or by-products to smaller fragments, usually consisting of 2-20 amino acid residues. Various biotechnologies were proposed, at lab and pilot level, based on acid or alkaline hydrolysis of fish proteins, but final hydrolysates had low nutritional value due to degradation of essential amino acids, like Met, Trp, Thr, Lys, His, Ile and were valorized as fertilizers (*7*). Enzymatic hydrolysis used various commercial proteases to attain hydrolysates rich in valuable biologic active compounds, based on controlled hydrolysis degree in specific reaction conditions, according to the particularities of each enzyme type, *e.g.* pH, structure, specificity (*8*). Neutral enzymes of microbial origin (alcalase, flavourzyme) proved high efficiency in protein hydrolysis, as well as stability at pH and temperature variations, but they were mainly applied to waste tissues with variable content of protein (*9*). Sequential or combined enzymatic digestion of fish by-products was performed in a bioreactor system and followed by ultrafiltration, in order to separate functional peptides according to their molecular mass (MM) (*10*). Preliminary treatment is needed in case of fish by-products hydrolysis to avoid browning, unpleasant taste and odour of the final product, or formation of toxic compounds due to lipid oxidation products (*7*).

To date, numerous marine sources (sardines, tuna, Alaska pollock, Nile tilapia, sea bream or leatherjacket) have been documented for valorization of huge amounts of by-products (skin, scales and bones, viscera) in the form of bioactive peptides (*11,12*). Still, few data exist on production of bioactive peptides from by-products of aquaculture fish species, in particular from large cyprinidae, like common carp (*Cyprinus carpio*), bighead carp (*Arystichtys nobilis*), grass carp (*Ctenopharingodon idella*) and silver carp (*Hypophthalmichthys molitrix*) by neutral proteases action. A biotechnology was proposed based on bacterial protease hydrolysis directly applied to grass carp skin, yielding a fraction of peptides with MM below 1000 Da that favored *S. thermophilus* growth and the development of functional foods with probiotics (*13*). Hydrolysis of silver carp protein with flavourzyme facilitated the production of peptides with negatively-charged hydrophilic acidic amino acids, like aspartic and glutamic acids, for developing antibacterial agents used in food industry (*14*). Good recovery of low MM peptides was reported by alcalase treatment of silver carp protein and they played an important role in metal-chelation and inhibition of linoleic acid peroxidation (*15*). A study compared proteinase K and other endopeptidases treatment applied directly to grass carp skin and reported low degree of hydrolysis, but different working conditions were used for each enzyme and their specific activity was not provided (*16*). High yield of bioactive peptides with low MM were obtained by silver carp skin gelatin hydrolysis with collagenase or alkaline proteinase and applied for osteoporosis treatment in aged mice (*17*). According to *in silico* simulation, proteinase K and papain found numerous sites of action on collagen type I molecule and produced the most effective proteolysis to small peptides, but the studies included only the primary linear structure (*18*). The functional (solubility, water retention,

foaming, emulsifying) and biological (antioxidant, antihypertensive, antiproliferative) properties of bioactive peptides were found in significant correlation to their amino acid type and sequence, but also to their size (*19,20*).

The aim of this study was to obtain and characterize bioactive peptides by proteinase K-assisted hydrolysis of collagen extracted from freshwater silver carp skin and to evaluate their free radicals scavenging potential and also their effect on reactive oxygen species (ROS) production and cell migration in experimental models of fibroblast culture mimicking the oxidative stress and skin wound conditions.

MATERIALS AND METHODS

Materials

Silver carp skin was delivered on ice by a local fishery (Tulcea, Romania). Pepsin (E.C. 3.4. 23.1), proteinase K (E.C. 3.4.21.64), Sephadex G-75, bicinchonic acid (BCA) 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2'-azino'-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals of analytical grade used in experiments were purchased from Sigma-Aldrich (Taufkirchen, Germany), unless otherwise specified. A culture cell line of mouse fibroblasts NCTC clone L929 and Minimum Essential Medium Eagle (MEM) culture medium were purchased from ECACC (Sigma-Aldrich, Taufkirchen, Germany).

Extraction of carp skin collagen

Collagen was extracted from silver carp skin using the enzymatic method with pepsin in acetic acid, according to previously described protocol (*21*). Briefly, skin was thoroughly washed in cold tap water, minced and extracted in 0.5 M acetic acid containing pepsin (2500 U/mg), in a mass ratio of 1:10 enzyme:tissue, at 4 °C, with gentle stirring, for 24 h. The process was repeated twice. The gel was separated from the undigested tissue, purified by precipitation at 0.7 M NaCl concentration, centrifuged at 5000g, for 20 min and dialyzed against distilled water. The resulting carp skin collagen (CSC) gel had a dry mass (dm) of 1.2 %, pH=5.5 and it was stored at -18 °C for 2 months.

Enzymatic preparation of collagen peptides

Enzymatic hydrolysis of CSC was carried out under specific conditions of pH and temperature (Fig. 1). The treatment protocol consisted in diluted CSC incubation in 0.05 M Tris buffer, pH=8, containing 0.15 M NaCl and 5 mM $CaCl_2$, at 45 °C in a water bath, for 30 min. Then, proteinase K (10 U/mL) in the same buffer, pH=8, supplemented with 0.5 % SDS, as enzyme activator, was added and incubation continued at 55 °C, for 6 h. The pH of the reaction mixture was maintained at an optimum

value of 8 during the incubation period. Finally, the solution was heated at 95 °C, for 5 min to inactivate the enzyme, then cooled at room temperature and centrifuged at 8000g, 4 °C, for 15 min. The supernatant representing the enzymatic preparation (E-CSC) was subjected to centrifugal ultrafiltration through units provided with cellulose membrane at a MM cut-off of 3 kDa (Amicon, Taufkirchen, Germany). The permeate containing carp skin collagen peptides (CSCP) was collected and stored at -18 °C until analysis. Each experiment was performed on 10 samples of CSC. All samples were analyzed for protein content by BCA assay and a standard curve was built with pig skin gelatin in the range of concentrations 0.1-3 mg/mL, in order to calculate the process yield. Hydroxyproline (Hyp) content was determined in alkaline hydrolyzate using chloramine-T and Ehrlich's reagent, as previously described (*22*).

Fig. 1

Determination of the degree of hydrolysis

The concentration of free ε-amino groups was determined using TNBS assay, as previously described (*23*)**,** recording the absorbance (*A*) of the resulting complex at 346 nm using an UV-VIS spectrophotometer V650 (Jasco, Tokyo, Japan). The degree of hydrolysis (DH) was calculated as percentage relative to total hydrolysate preparared by CSC incubation in 6 M HCl, at 110 °C, for 18 h, using the following equation:

$$DH\ (\%) = (A_{sample} - A_{CSC})/(A_{total\ hydrolysate} - A_{CSC}) \times 100 \qquad /1/$$

SDS-polyacrylamide electrophoresis

CSC obtained by pepsin hydrolysis was analyzed by SDS-polyacrylamide electrophoresis, according to Laemmli method (*24*). The sample was denatured at 50 °C, for 30 min and loaded on 7.5 % SDS-PAA resolving gel provided with 5 % stacking gel. Migration was carried out at 30 V, for 4 h using a Biometra source (Jena, Germany). A sample of rat tail tendon collagen was migrated in the same conditions. The high MM marker (55-250 kDa) served to estimate the MM of separated bands based on their Rf.

E-CSC and CSCP samples were denatured at 37 °C, for 5 min and migrated in 10-20 % gradient tricine SDS-PAA gel (Invitrogen, Waltham, MA, USA), at 90 V, for 2.5 h (*25*). Low MM marker (1.7-40 kDa) was migrated in the same conditions. The migration patterns were visualized by gel staining with Coomassie Brilliant Blue.

Gel filtration chromatography

Gel filtration chromatography of the enzymatic preparations of E-CSC and CSCP was performed on a glass column (230 x 15 mm) packed with swollen Sephadex G-75 gel, equilibrated

with distilled water, as previously described (*26*). Samples (2 mg/mL) were loaded, eluted at a flow rate of 0.1 mL/min and monitored at 220 nm using an UV-VIS spectrophotometer V-650 (Jasco, Tokyo, Japan). Bovine serum albumin (66,000 Da), cytochrome c (12,384 Da), insulin (5,777 Da) and bacitracin (1,423 Da) were used as standards to build a MM calibration curve for the calculation of the MM of separated fractions.

Determination of the antioxidant activity

ABTS assay

The scavenging potential of CSCP against ABTS radicals was evaluated as previously described (*27*). Briefly, the stock solution of 7 mM ABTS containing 2.45 mM potassium persulfate was diluted to reach a value of 0.7±0.02 at 734 nm (control). Diluted CSCP (100 µL) were incubated with ABTS reagent (1 mL), at room temperature, in the dark, for 10 min. Then, the absorbance (*A*) of the reaction mixtures was recorded at an UV-VIS spectrophotometer V-650 (Jasco, Tokyo, Japan). The percentage of ABTS radical inhibition was calculated using the following equation:

$$\text{ABTS inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100 \quad /2/$$

A calibration curve was built using Trolox, an analog of vitamin E with known antioxidant activity, in the range of concentrations 0-150 µM. The antioxidant activity was expressed as µM Trolox equivalents (TE)/g protein.

DPPH assay

A colorimetric assay was used to evaluate the scavenging potential of CSCP against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, as previously described (*28*). Briefly, freshly prepared solution of 0.25 mM DPPH (1.35 mL) was mixed with 0.1 M Tris buffer, pH=7.4 (0.9 mL) and CSCP sample (0.15 mL), at room temperature, in the dark, for 30 min. Then, the absorbance (*A*) of the reaction mixtures was recorded at 517 nm using an UV-VIS spectrophotometer V-650 (Jasco, Tokyo, Japan). The percentage of DPPH radical inhibition was calculated using the following equation:

$$\text{DPPH inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100 \quad /3/$$

A calibration curve was built using Trolox, an analogue of vitamin E with known antioxidant activity, in the range of concentrations 0-100 µM. The antioxidant activity was expressed as µM TE/g protein.

Hydroxyl radical scavenging

The scavenging activity of hydroxyl (HO) radicals generated by Fenton reaction was determined as previously described (*26*). Diluted CSCP sample (80 µL) was mixed with 2 mM 1,10 phenanthroline (40 µL) in the wells of a 96-well microplate. Then, 40 µL of each 2 mM $FeSO_4$ solution

and 0.03 % (*V/V*) H_2O_2 were added into the mixture. The microplate was incubated at 37 °C, in the dark, for 1 h and the absorbance (*A*) of the resulting solution was measured at 536 nm using a Spectrostar nano microplate reader (BMG Labtech, Ortenberg, Germany). The percentage of HO radical inhibition was calculated using the following equation:

$$\text{HO inhibition (\%)} = (A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{control}} - A_{\text{negative control}})\text{x}100 \qquad /4/$$

where H_2O_2 was replaced by distilled water in control and the sample was replaced by distilled water in negative control.

The calibration curve was built using different concentrations of Trolox (0-500 μM). The antioxidant activity was expressed as mM TE/g protein.

Cell culture and cell viability assays

In vitro cytocompatibility of CSCP was evaluated according to the international standard SR EN ISO 10993-5 for medical devices by direct contact method. Briefly, mouse fibroblasts from L929 cell line were seeded in 96-well culture plate, at a cell density of $4\text{x}10^4$ cells/mL and cultivated in MEM culture medium supplemented with 10 % foetal bovine serum and 1 % mixture of antibiotics. The plates were incubated at 37 °C in 5 % CO_2 humidified atmosphere. After 24 h, the medium was replaced with fresh medium containing different concentrations of CSCP (10-400 μg/mL) incubation continued in standard conditions, for 24 h. Cells cultivated in MEM served as negative control, while cells treated with 0.5 mM H_2O_2 served as positive control. Cells were also treated with 15 μg/mL ascorbic acid, a known agent for stimulation of fibroblast proliferation. Cell viability was evaluated by MTT assay, as previously described (*29*). The absorbance values read at 570 nm were proportional to the number of viable cells, which reduced MTT to formazan salt. Cell viability was expressed as percentage relative to control, considered 100 %. The experiments were performed in triplicate.

Qualitative analysis consisted of light microscopy observations on cell morphology, in similar conditions as described above. Thus, L929 fibroblasts were cultivated in the presence of different concentrations of CSCP and incubated in standard conditions, for 24 h. After medium harvesting, cells were washed, fixed in methanol and stained with Giemsa solution. Micrographs were acquired at an Axio Observer D1 optical microscope equipped with digital camera (Carl Zeiss, Oberkochen, Germany).

In vitro scratch-wound assay

The effect of CSCP on cell migration during wound healing has been evaluated in an experimental model of scratch-wound achieved in a monolayer of fibroblasts, as previously described (*30*). Briefly, L929 fibroblasts were cultivated in 35 mm Petri dishes, in MEM culture medium, until confluence. Then, a mechanical scratch was made on the monolayer using a pipette tip and fresh

medium containing 100 µg/mL CSCP was added. Untreated cells served as negative control and cells treated with ascorbic acid (15 µg/mL) served as positive control. After 24 h of cultivation in standard conditions, cells were photographed using an AxioStar Plus phase contrast microscope (Carl Zeiss, Oberkochen, Germany). The rate of wound repair was calculated after image analysis using ImageJ software (*31*). Thus, the density of migrated cells within the wounded area was calculated by automatic measurement of cells as particles in the captured images and then, reported as percentage of the initial open area of the scratch.

Determination of cellular ROS production

The effect of CSCP on ROS production has been evaluated in an experimental model of oxidative stress induced in the fibroblast culture, as previously described (*32*). L929 cells were seeded in the wells of a 12-wells culture plate, at a density of $5x10^4$ cells/mL and cultivated in MEM, in standard conditions, for 24 h. Adhered cells were incubated with fresh medium containing 100 µg/mL CSCP, at 37 °C, in humidified atmosphere with 5 % CO_2, for 24 h (pre-treatment) and then, cells were treated with 50 µM *tert*-butyl hydroperoxide (t-BHP), for 30 min.

The assay used to measure ROS production at cellular level was based on the mechanism of hydrogen atom transfer (HAT) using a cell permeant fluorogenic dye of 2',7'-dichlorofluorescin diacetate (DCFH-DA). Upon reaction with free radicals, formation of a fluorescent product was monitored. The cells treated as above were incubated with 10 µM DCFH-DA, for 30 min and analyzed using a flow cytometer LSR II (Becton Dickinson, Franklin Lakes, NJ, USA). Acquired histograms were processed to calculate ROS production (%) in correlation to the fluorescence intensity using FlowJo version 10.6.1 (*33*) and BD FACSDiva version 6.1.3 (*34*) software. Cells incubated in normal medium and cells pre-treated with 12 µM ascorbic acid were processed in similar conditions and served as controls.

Statistical analysis

All experiments were carried out in triplicate. The results were expressed as mean value±standard deviation (SD) for three experiments. Statistical analysis of the data was performed on each pair of interest using two-tailed, paired Student's *t*-test (Microsoft Excel software) (*35*). Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

In this study, a two-step enzymatic process was applied for the extraction of CSC and, then, preparation of CSCP from carp skin residues by hydrolysis in controlled conditions (Fig. 1).

Extraction and characterization of CSC

The first step was the extraction of undenatured collagen from fish skin using pepsin treatment, in acidic conditions, as previously applied to mammalian tissues (*21*). After purification by salt precipitation, the pepsin-soluble collagen preparation with intact triple helix molecules was characterized by SDS-PAA electrophoresis under denaturing conditions (Fig. 2a). The pattern of CSC indicated the characteristic structure of type I collagen, consisting of three α chains, [$(\alpha1)_2\alpha2$], similar to that of collagen extracted, in a similar way, from rat tail tendon. The observed doublet, corresponding to two α1 chains and one α2 chain, had calculated MM of 130 and 116 kDa, respectively. Typical bands corresponding to β-dimers and γ-trimers were also observed in both collagen extracts. Previous studies reported similar electrophoresis pattern of pepsin-soluble collagen from grass carp (*36*) and marine giant croaker (*37*). The minor variation of MM values for α-, β- and γ-bands were observed within a narrow domain and correlated to the living environment (warm/cold water, salinity) and extraction method (acid, enzymatic), which could influence their structural characteristics (*37*). The chemical and structural characteristics of collagen extracted from fish species conferred them better rheological properties over mammalian collagen and recommended cosmetic applications (*38*).

Fig. 2

Preparation and characterization of CSCP

The second step of CSCP preparation consisted of CSC hydrolysis by proteinase K treatment, in specific and controlled conditions of pH, temperature and time of incubation, in order to maximize the process. After a short incubation to denature its triple-helical structure, the enzymatic hydrolysis of CSC followed the mechanism of action characteristic for an endo-peptidase, cleaving the amino acids chain at a specific site represented by peptide bonds adjacent to an aliphatic or aromatic amino acid. Thus, this enzymatic biotechnology was a more controlled process of fish collagen hydrolysis than a previously applied thermal treatment, which resulted in gelatin preparations with high MM (*39*).

The degree of CSC hydrolysis was 30.35 % after proteinase K treatment for 6 h. Close values of 28.85 and 30.27 % were obtained after 2 and 4 h of incubation, respectively, indicating that the enzymatic process reached a plateau. Previous studies have showed that the degree of hydrolysis varied between 11.50-33% for capelin protein hydrolysates and influenced their physicochemical properties and antioxidant activity (*40*).

After ultrafiltration, the obtained CSCP preparation was preliminary characterized, showing an average content of 87.5 % protein, 8.6 % Hyp and 2.1 % dm. The yield of CSCP preparation from carp skin using the two-step enzymatic biotechnology was 8 %, reported to the initial tissue dm, a

significantly increased value over previously reported 1.7-3.4 % (*16*), indicating optimal hydrolysis parameters.

Fig. 2b presents the electrophoretic migration pattern of the enzymatic preparations, before (E-CSC) and after ultrafiltration (CSCP) in Tricine-SDS-gradient gel. E-CSC preparation presented several bands distributed throughout the gel, indicating an efficient hydrolysis of collagen to fragments with MM below 40 kDa. Among them, a dense extended group of bands had calculated MM corresponding to a range of 16-22 kDa and two narrow bands were observed at 4 and 1 kDa, respectively. The CSCP preparation obtained after ultrafiltration presented a more pronounced band at around 10 kDa, while faint blue coloured bands below 10 kDa indicated the presence of low MM oligopeptides and peptides, considered bioactive compounds. Similar enzymatic hydrolysis biotechnology applied to grass carp skin using protease at 60 °C, pH=9, for 80 min resulted in separation of a fraction containing peptides with MM below 1 kDa, which proved to be very useful for functional food development (*41*).

The enzymatic preparations of E-CSC and CSCP were also subjected to gel filtration chromatography, in order to separate peptide fractions according to their MM. The chromatogram profile of samples obtained before and after ultrafiltration is presented in Fig. 2c. The E-CSC preparation showed two distinct peaks, one corresponding to low elution volumes containing high MM oligopeptides and proteins ranging between 28-112 kDa with a maximum intensity at 70 kDa. The second peak had higher intensity than the first peak, indicating high degree of CSC hydrolysis by proteinase K. CSCP preparation obtained after ultrafiltration presented a single peak, corresponding to peptides with a MM ranging between 1.4-18 kDa with a maximum intensity at 4.5 kDa. This confirmed the separation of low MM peptides with bioactive potential. Previous mathematical modeling showed that collagen was cleaved by proteinase K to small fragments of oligopeptides and bioactive peptides with low MM, which could exert significant biomedical activity (*18*).

The CSCP preparation containing small and intermediate size peptides was further investigated for *in vitro* antioxidant activity and biological activity in cell culture experimental models.

Effect of CSCP on free radical scavenging

The antioxidant capacity of low MM peptides fraction (CSCP) was determined as scavenging potential against the free radicals of ABTS, DPPH and HO, in comparison to that of the undenatured collagen (CSC). The results are presented in Table 1. The scavenging potential of CSCP against free ABTS (68.6 %) and DPPH (13.95 %) radicals was moderate, compared to Trolox, the synthetic analog of vitamin E. However, the values were significantly higher ($p<0.05$), compared to those of CSC (7.02 % and 0.54 %, respectively). Strong antiradical activity was recorded for CSCP incubated in the presence of HO radicals (71.04 %), representing highly encountered ROS within *in vivo* systems.

Thus, our results showed that small peptides and oligopeptides were more efficient antioxidant agents than collagen, reacting with free stable radicals by hydrogen or electron transfer (*42*).

Table 1

Similar studies analyzed the free radical scavenging potential of fish bioactive peptides, according to the fish species, enzyme used and hydrolysis parameters, showing different values between 30-90 %, but higher than those of undenatured fish collagen (*43*). The great variability was also due to the wide spectrum of tested concentrations and assays, from ABTS, DPPH, HO and superoxide to lipid peroxidation or metal chelating capacity (*44*). Previous studies on papain hydrolysate of catfish skin collagen showed an increase of DPPH scavenging capacity in the first 2.5 h of hydrolysis, but lower value (63.06 %), compared to the hydrolysate of bone tissue (71.55 %) (*45*). Fractions of marine fish oligopeptides with MM between 5-10 kDa presented higher antioxidant activity against DPPH radicals (55 %) than the fraction of low MM (<1 kDa) peptides (37 %) (*46*). The fractions with MM higher than 10 kDa presented good antihypertensive activity by inhibition of ACE activity (*7*), while those found in grass carp skin hydrolysates with MM lower than 1 kDa (Gln-Pro, Trp-Pro-Pro) were good antiaging agents by HO radical scavenging and regulating redox processes (*47*). Also, alcalase treated silver carp muscle proteins containing low MM peptides showed strong metal chelating capacity and lipid peroxidation inhibition (*15*). However, a recent study reported that no significant differences in ABTS scavenging potential were observed between peptide fractions separated according to MM from the alcalase treated fish skin gelatin (*48*). No studies on the antioxidant activity of proteinase K fish hydrolysate were found.

As previously reported in databases storing collagen peptide sequences, proteinase K favored the release of fish peptides with C-terminal hydrophobic amino acids and increased the number of amino and carboxyl groups in the CSCP preparation, favoring the free radicals scavenging (*49*). In contrast, the long polymeric chains of fish skin collagen have limited functional groups, show abundance of hydrophobic amino acids (Ala, Gly, Pro, Val, Leu, Ile, Met, Phe), a lesser proportion of hydrophilic amino acids with charged side chains (Glu, Asp, Arg, Lys) and polar amino acids (Hyp, Thr, Ser) that form hydrogen bonds (*50*).

Biologic activity in cell culture

The effect of CSCP on processes of skin wound healing and redox balance in oxidative stress conditions was investigated using *in vitro* experimental models developed in fibroblast culture. In this regard, it was first selected the optimal concentration to be tested.

In vitro cytocompatibility

Cell viability of fibroblasts cultivated in the presence of different concentrations of CSCP was evaluated by MTT assay. The results are presented in Fig. 3a. The values of cell viability varied between 112-119 %, in the range of CSCP concentrations of 10-200 µg/mL. The maximum value of 119.2 % was recorded for a concentration of 100 µg/mL CSCP and it was close to that of ascorbic acid (108.7 %). Higher concentrations of CSCP significantly ($p<0.05$) decreased the cell viability as low as 6.8 %, similar to hydrogen peroxide (10.5 %), used as positive control. These data indicated that CSCP were cytocompatible in fibroblast culture on a wide range of concentrations and had a similar effect to that of ascorbic acid, a known antioxidant agent involved in the up-regulation of fibroblasts proliferation.

Fig. 3

The results were confirmed by light microscopy observations on cell morphology in fibroblasts cultured in the presence of the same CSCP concentrations. Acquired micrographs showed that CSCP treatment maintained the normal cell morphology at concentrations between 10-200 µg/mL (Fig. 3b). CSCP-treated fibroblasts presented characteristic fusiform phenotype, euchromatic nuclei and clear cytoplasm, similar to that of untreated cells (control). Similar or higher cell density than in control culture was observed and cells were homogeneously distributed in the culture plate. Cell treatment at higher CSCP concentrations revealed morphological changes, degeneration and cell lysis, similar to cells treated with hydrogen peroxide, while the cell density decreased.

Quantitative and qualitative data indicated that CSCP were cytocompatible with cultured fibroblasts at a concentration of 100 µg/mL and stimulated cell metabolism, being subsequently tested in experimental models *in vitro*.

Effect of CSCP on wound healing

The effect of CSCP on cell migration was evaluated using an *in vitro* experimental model of scratch-wounded fibroblast monolayer. The results are presented in Fig. 4. Phase contrast micrographs showed that CSCP allowed cell migration to close the created wound after 24 h of cultivation, similar to the effect of ascorbic acid (Fig. 4a). Image analysis indicated that the scratch repair rate was significantly ($p<0.05$) higher in CSCP-treated fibroblasts (62.1 %) than in untreated cells (47.3 %). The percentage was similar to that in ascorbic acid-treated cells (56.7 %) (Fig. 4b).

Fig. 4

These observations indicated a significant involvement of CSCP in the stimulation of fibroblast proliferation and active cell migration process, as important phases during dermal wound healing process and tissue remodeling. Previous studies showed that fish bioactive peptides had low level of cytotoxicity in several fibroblast cell lines, indicating their potential for human health applications (*51,52*). Increased proliferation of NIH-3T3 mouse fibroblasts and human dermal fibroblasts was

reported for fish skin collagen peptides obtained by neutral protease hydrolysis, in accordance to our results (*4,53*). These data indicated that enzymatic-assisted hydrolysis of collagen favored the increase of bioactivity. In previous studies, collagen-derived dipeptides, such as Pro-Hyp, were emphasized showing that oral ingestion of hydrolyzates promoted fibroblast proliferation and wound healing (*54*). Also, collagen di- and tripeptides containing Hyp, such as Gly-Hyp, Pro-Hyp and Gly-Pro-Hyp stimulated human fibroblasts migration (*55*). Still, mixtures of fish collagen peptides could exert several activities as stimulators, mediators or cofactors of main growth factors and interacted with cell membrane and cytoplasmic components, according to their size, structure, hydrophobicity and charge, as previously discussed (*10,56*). Alcalase hydrolysate of carp gelatin containing mainly 1-3 kDa peptides with good antioxidant activity were easily absorbed during mice gastrointestinal digestion, exerting biological effects of skin photoprotection (*57*).

Effect on redox balance at cellular level

Involvement of fish protein hydrolyzates in regulating the antioxidant factors at cellular level was lately investigated (*20*). In our study, an experimental model of oxidative stress induced in CSCP-pretreated fibroblasts was used to evaluate their effect on the cellular redox state and balance of ROS production by reaction with DCFH-DA and flow cytometry. The results are presented in Fig. 5. The histogram showed that stressed cells presented high level of fluorescence as the peak shifted to the right (Fig. 5a). Decrease of fluorescence was observed in CSCP-pretreated cells and overlapped the histogram of ascorbic acid-treated cells. Results analysis using Diva software confirmed significantly ($p<0.05$) decrease of ROS level down to 11.86 % in fibroblasts pretreated with CSCP and 7.81 % in ascorbic acid treated cultures (Fig. 5b). All these data indicated effective redox signaling of CSCP and ascorbic acid, used as antioxidant agent in food and cosmetic products, to achieve the balance between ROS production and scavenging.

Fig. 5

Similar studies on inhibition of ROS production in hydrogen peroxide-stressed macrophages revealed the antioxidant activity of collagen-derived peptides from marine sponge by trypsin hydrolysis (*58*). Oral administration of products containing marine fish skin peptides supplemented with antioxidants, for 60 days increased plasma levels of oxidation markers (nitric oxide, malondialdehyde) in volunteers with aged facial skin, but within normal range, indicating no risk of oxidative damage (*59*).

CONCLUSIONS

A new protocol was proposed for proteinase K hydrolysis of collagen extracted from silver carp skin that was efficiently carried out, in controlled conditions and was followed by ultrafiltration to obtain

a preparation consisting of intermediate and small size peptides with hydrophobic C-terminal amino acids due to enzyme specificity. The potential to scavenge free radicals was higher than that of fish collagen, in particular against HO radicals, also found in biological systems. Dose-dependent stimulation of fibroblast proliferation and up-regulation of cell migration indicated their functions in skin wound healing processes. Moreover, they prevented excessive ROS formation induced by oxidative stress in fibroblast cultures, maintaining the redox balance. Therefore, freshwater fish by-products processing yielded value-added peptides with potential to improve human health. Further studies will focus on structure-activity correlation and *in vivo* experiments, in order to develop novel pharmaceutics and nutraceutics.

FUNDING

This work was supported by CCCDI–UEFISCDI, project no. PN-III-P1-1.2-PCCDI-2017-0569 (PRO-SPER) and funded by project no. PN-III-P2-2.1-PTE-2019-0181 (COLSTIM).

CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

AUTHORS' CONTRIBUTION

D.I. participated in carrying out hydrolysates preparation, performing HPLC and antioxidant analysis, data collection, data analysis, drafting the article. A.I. participated in performing cell culture experiments, data collection, data analysis, drafting the article. O.C. participated in design of the work, data analysis and interpretation, drafting the article, critical revision. AM.S.-G. participated in performing flow cytometry experiments, data collection, data analysis, drafting the article. C.S. participated in carrying out hydrolysates preparation, performing electrophoretic characterization, data collection, data analysis, drafting the article. F.O. participated in conception of the work, data analysis and interpretation, drafting the article, critical revision.

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Table 1. Antioxidant activity of carp skin collagen (CSC) and carp skin collagen peptides (CSCP) determined as capacity to scavenge free ABTS, DPPH and hydroxyl (HO) radicals. The results were expressed as inhibition percentage and Trolox equivalents (TE) and represented mean±SD (n=3). *p<0.05, compared to CSC

Sample	ABTS assay		DPPH assay		HO assay	
	% inhibition[a]	(μM TE/ g protein)	% inhibition[a]	(μM TE/ g protein)	% inhibition[a]	(mM TE/ g protein)
CSC	7.02±0.38	37.99±2.10	0.54±0.03	1.67±0.09	16.20±0.84	27.88±1.44
CSCP	68.60±3.26*	186.62±8.86*	13.95±1.18*	17.44±1.48*	71.04±4.02*	433.03±24.55*

[a] % inhibition determined at 1 mg/mL

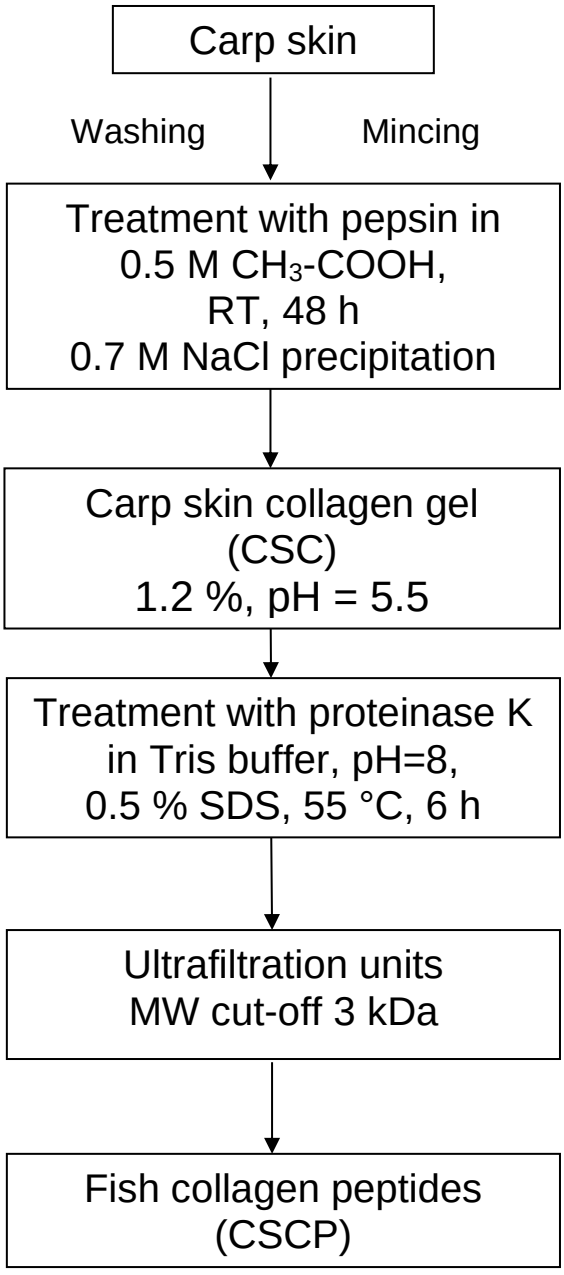


Fig. 1. Scheme of two-step preparation of carp skin collagen (CSC) by pepsin treatment and carp skin collagen peptides (CSCP) by enzymatic hydrolysis with proteinase K, in controlled conditions

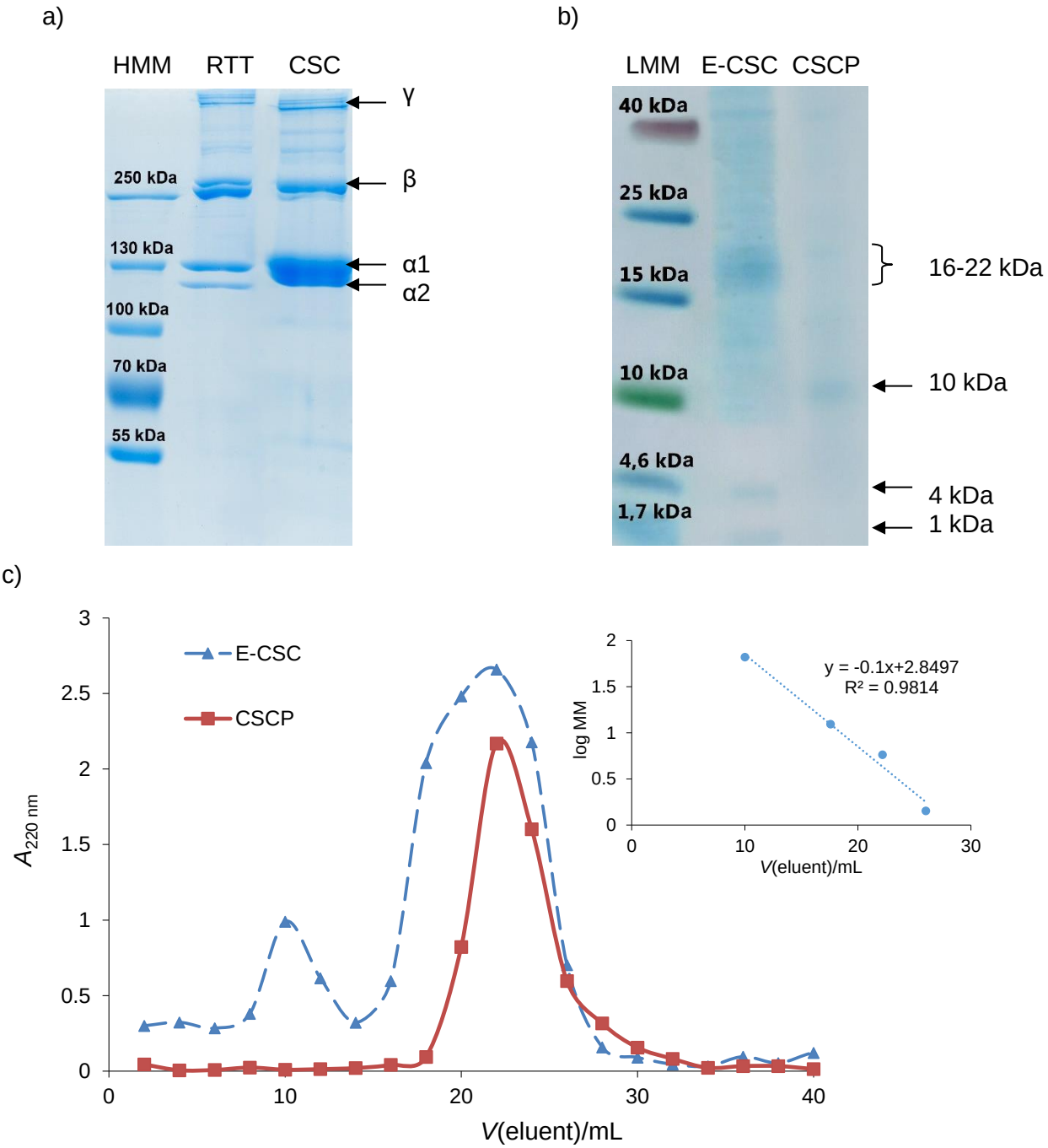
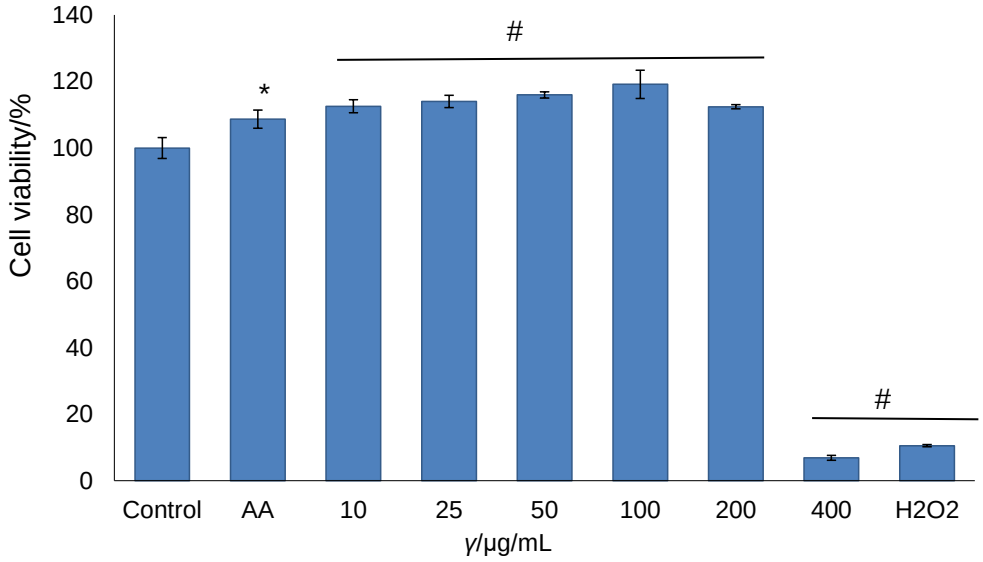


Fig. 2. Electrophoretic migration patterns of a) rat tail tendon (RTT) and carp skin collagen (CSC) in SDS-polyacrylamide gel showing the dublet characteristic pattern of α-chains β-dimers and γ-trimers (arrows) and b) enzyme hydrolysate of CSC (E-CSC) and carp skin collagen peptides (CSCP) in tricine SDS-polyacrylamide gel in gradient showing the corresponding MM of visualised bands (arrows). High MM (HMM) and low MM (LMM) markers were migrated in similar conditions. c) Gel filtration chromatography of E-CSC and CSCP on Sephadex G-75. Insert presenting the standard curve of MM as a function of the elution volume

a)



b)

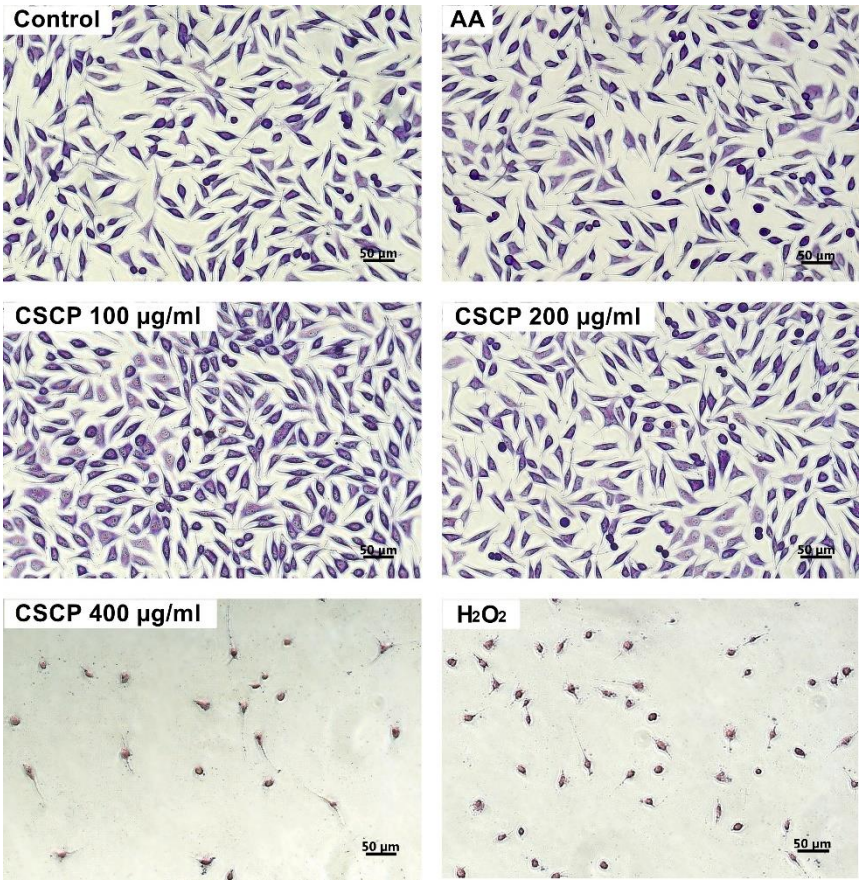
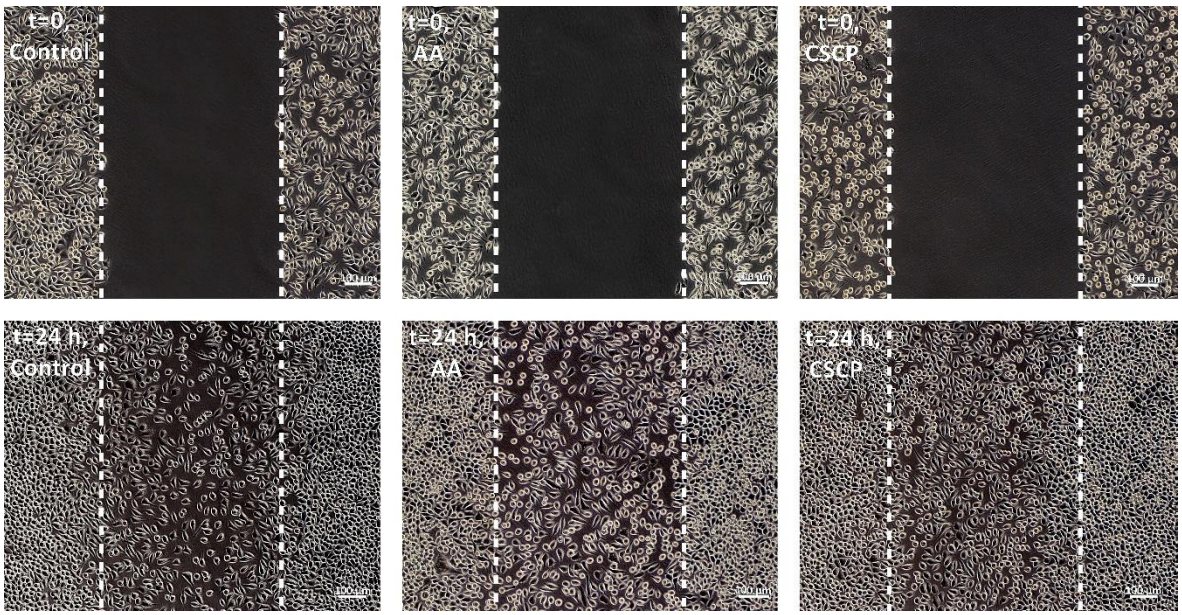


Fig. 3. Cell viability of L929 fibroblasts cultivated in the presence of different concentrations of carp skin collagen peptides (CSCP), for 24 h, determined by (a) MTT assay and (b) light microscopy (Giemsa staining). Scale bar=50 μm. Untreated cells (negative control), cells treated with ascorbic acid (AA), as regulator of cell proliferation and cells treated with hydrogen peroxide (positive control)

were cultivated in similar conditions. *p<0.05, compared to untreated cells (control); #p<0.01, compared to control

a)



b)

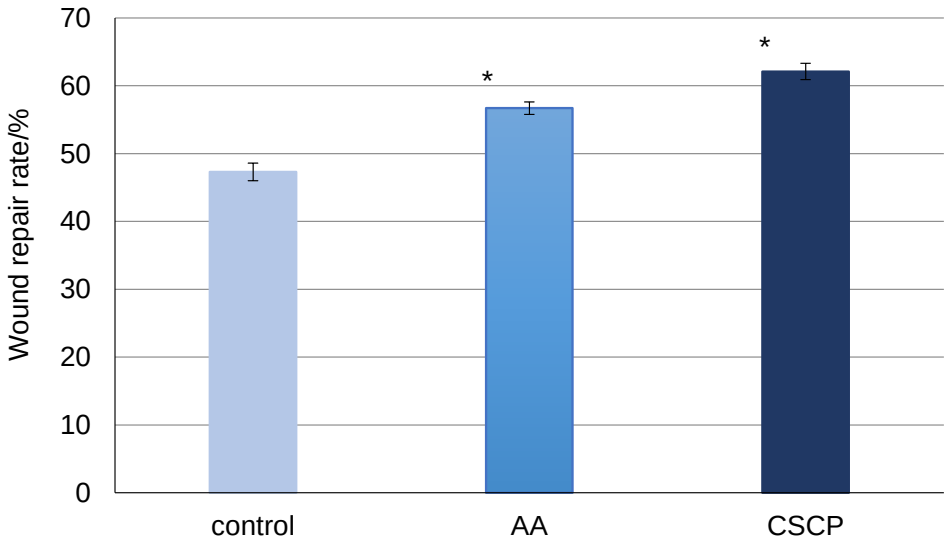
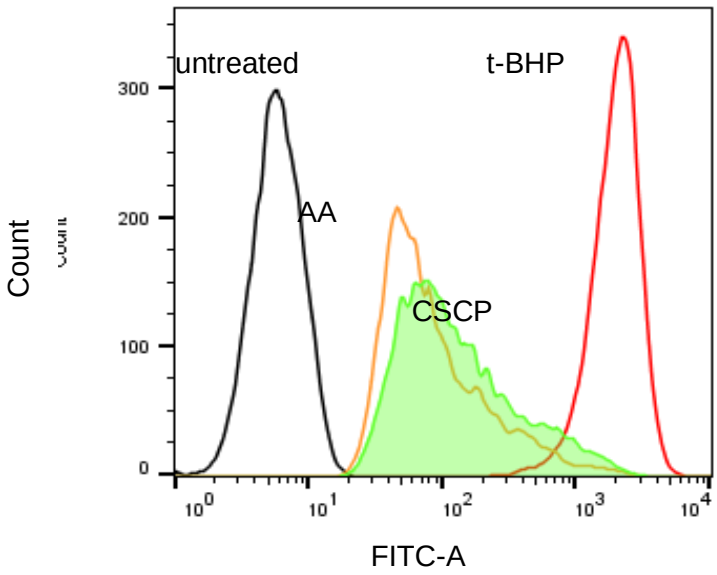


Fig. 4. Wound healing assay in scratched monolayer of untreated (control), treated with ascorbic acid (AA) and carp skin collagen peptides (CSCP) L929 fibroblasts, at initial time (t=0) and after 24 h, observed by a) phase contrast microscopy (scale bar=100 μm) and b) calculated as wound repair rate by image analysis using ImageJ software. *p<0.05, compared to untreated cells (control)

a)



b)

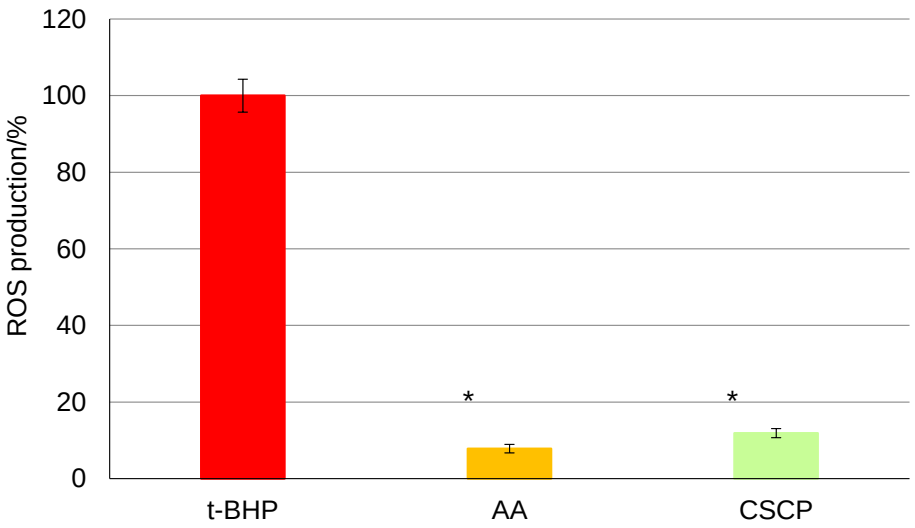


Fig. 5. Intracellular ROS production measured by DCFH-DA assay in untreated, t-BHP-stressed and pre-treated with 100 μg/mL carp skin collagen peptides (CSCP) and ascorbic acid (AA) L929 fibroblasts, presented as a) fluorescence intensity using flow cytometry and b) calculated as percentage from t-BHP-stressed cells using Diva software. *$p<0.05$, compared to t-BHP-stressed cells