Valorization of Cyprinus Carpio Skin for Biocompatible Collagen Hydrolysates with Potential Application in Foods, Cosmetics and Pharmaceuticals

Elena Dănîlă¹ · Raluca Stan¹ · Mădălina Albu Kaya² · Georgeta Voicu¹ · Maria Minodora Marin¹,² · Alina Moroșan¹ · Irina Titorencu³ · Raluca Țuțuiianu³

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Abstract
Fish collagen is reported with an increased bioavailability as compared to other sources, the extraction being performed on secondary sources as skin, bones, scales, or fins resulted after fish processing. The aim of the present study was to obtain biocompatible collagen hydrolysates from waste Cyprinus carpio skin, the main aquaculture species in Romania using an inexpensive and “green” neutral hydrolysis process. Neutral hydrolysis of pretreated fish skins performed for 6 h at a temperature of 135 °C and a pressure of 315 kPa produced collagen hydrolysates in 24.6–35.5% yields depending on the adopted pretreatment procedure. The extensive characterization of hydrolysate samples revealed a high purity degree (98% protein content, undetected ash content, pH value in the range 6–7), also confirmed by the absence of undesired aggregates in the characteristic fibril structure as determined by electronic microscopy. A specific collagen hydrolysate random coil structure and the absence of triple helix was determined by FTIR analysis and sustained by CD spectroscopy and X-Ray diffraction. The biocompatibility assessment for the obtained fish collagen hydrolysates revealed no cytotoxic effect on Human keratinocytes, with an 80% cell viability, superior as compared to conventional bovine collagen hydrolysate. Neutral hydrolysis of waste Cyprinus carpio skin yielded collagen hydrolysates with determined characteristics and biocompatibility superior to bovine collagen, suitable for application in foods, cosmetics and pharmaceutical industry.

Graphic Abstract

Keywords Fish collagen hydrolysates · Cyprinus carpio skin · Neutral hydrolysis · Biocompatibility

Statement of Novelty

An inexpensive procedure, neutral hydrolysis in mild conditions, was applied on waste Cyprinus carpio skin, the major type of fish consumed in Romania with 8000 t/year production, to obtain collagen hydrolysates. The targeted starting material, waste carp skin represents 6–9% of the total fish
mass, and according to literature data may provide collagen hydrolysates with a lower molecular mass and increased bioavailability being up to 1.5 times more efficiently absorbed by the body than bovine or porcine collagen. Prior to neutral hydrolysis, several pretreatment procedures involving affording materials were investigated, and biocompatibility of resulted samples with a high purity degree was assessed as compared to commercial bovine collagen hydrolysate proving a superior cell viability on human keratinocytes.

Introduction

Collagen is the most abundant fibrillar protein (approximately 30%) in the body of vertebrates, found in various organs and tissues: tendons, skin, bones, cartilage, blood vessel walls, muscles, heart, liver, kidneys, central nervous system, basement membrane, etc. In the human body, collagen represents one third of the total protein, based on the dry weight of the skin and is the most important component of the extracellular matrix (ECM) [1]. Collagen is a natural polymer consisting of 20 amino acids, arranged in characteristic sequences for the collagen molecule, which has a unique conformational structure of triple helix. In the collagen composition the amino acids with the highest percentage are: Glycine (Gly)—33%, Proline (Pro)—12–13%, Alanine (Ala)—11% and Hydroxyproline (Hypro)—9–10%, the latter being the amino acid considered as characteristic [2, 3].

Collagen is part of a family of so-called “isotypes”, genetically different. The term isotype refers to the fact that there are several types of macromolecular substances thatstructurally have both common and different elements, in order to be individualized. Presently, 29 types of collagen are known encoded by at least 44 genes. The types of collagen were grouped into eight classes, depending on the structural similarities and the formed quaternary, polymeric exchanges [4]. The most important natural sources of collagen are the skin, tendons, cartilage and bones, nowadays collagen extraction being performed predominantly on starting materials of bovine and porcine nature. Alternative extraction sources have been explored over the past 10–15 years such as fish collagen, thus reducing the risk of the transference of zoonotic diseases such as BSE (bovine spongiform encephalopathy), TSE (transmissible spongiform encephalopathy), and FMD (Foot and Mouth Disease) or even avoiding religious constraints [5].

Fish collagen presents an increased bioavailability and is up 1.5 times more efficiently absorbed as compared to porcine and bovine collagen, due to the lower molecular mass of its component polypeptides [6], specific applications in biomaterials used for wound healing, drug delivery and tissue engineering being recently reported [7, 8]. Nowadays, fish collagen has a wide range of applications in the health-related sectors, namely in cosmetics as an effective ingredient to improve dermal health, with antiaging effects [9], in the pharmaceutical industry and in medical care (including plastic surgery, orthopedics, ophthalmology, dentistry etc.) [10] and in the food sector (food processing, as additive and nutraceuticals) [11]. Multiple biological activities (antioxidative, lipid homeostasis modulation, anti-inflammatory, antihypertensive, etc.) and functional properties (emulsifying, foaming, gelling) are reported for fish protein hydrolysates which are considered as promising nutraceutical or functional ingredients for foods [12]. Moreover, fish collagen hydrolysates play important roles in cartilage metabolism [13] and stimulation of the collagen type I mRNA production by fibroblasts main cell types involved in the collagen synthesis [14]. The fish collagen extraction is performed using secondary raw materials such as skin, bones, scales or fins resulted after fish processing, which require specific pretreatment and complex extraction techniques with an extensive time schedule, lower extraction efficiency and higher costs than for other sources of collagen extraction. Fish collagen Type I is obtained from the skin and bones, type II collagen from cartilage, and type IV collagen can be extracted from the sea sponges [15].

Fish skin collagen hydrolysates are mainly obtained by acid and enzymatic hydrolysis, extensive studies upon the nature of the obtained peptides and method optimization being reported in the literature [11]. Enzymatic hydrolysis leads to superior extraction yields than acid procedure but requires a subsequent purification by ultrafiltration, gel filtration, desalination, etc. [16, 17].

As an alternative, neutral hydrolysis of proteins, referred as subcritical water hydrolysis (SWH) using water at a temperature of 100–374 °C and a pressure of 0.1–22 MPa is a greener processing technique that does not introduce chemicals or produce toxic waste and it has a short reaction time. The mechanism that underlies SWH is the formation of hydronium (H3O+) and hydroxide (OH−) ions that act as catalysts due to the subcritical condition of water. The method was successfully applied on different protein containing food wastes and parameters (temperature, pressure) were optimized to control the conversion of the proteins into peptides or free aminoacids [18]. Tuna skin collagen hydrolysates with antioxidant and antimicrobial potential have been successfully obtained by SWH working at temperatures of 150–300 °C and pressure of 5–10 MPa [19].

The aim of the present study was to obtain biocompatible collagen hydrolysates from Cyprinus carpio skin, the main aquaculture species in Romania, with a 8000 t/year production, using a neutral inexpensive hydrolysis process involving lower temperature (135 °C) and pressure values (315 KPa) and by employing various pretreatment procedures. The targeted starting material, waste carp skin represents 6–9% of the total fish mass, being an important
source of fish collagen hydrolysates. The performance of the employed procedures was estimated by an extensive characterization of the obtained samples (physical–chemical analyses, infrared spectroscopy, optical and scanning electron microscopy, circular dichroism spectroscopy, X-Ray diffraction, isoelectric point, biocompatibility), in order to provide helpful information for the potential application of carp skin collagen hydrolysates in cosmetics, food and pharmaceutical industry.

**Materials and Methods**

**Chemicals**

All the reagents and solvents of analytical grade were purchased from Sigma-Aldrich and used with no further purification. Distilled water was used in all the experiments.

**Fish Collagen Hydrolysates Extraction**

Romanian carp (*Cyprinus carpio*) skin was purchased from a local market and kept at – 25 °C until processing. First the fish skin was thawed, cut into smaller pieces and washed with a 2% organic detergent solution for 2–3 h. Throughout the extraction process the fish skin was kept at a temperature below 10 °C, because the denaturation temperature of the fish collagen is lower than the bovine one [20]. Further skin fish was subjected to the following pretreatment procedures:

**Acid Treatment**

The fish skin was divided into three samples and subjected to acid treatment by soaking in acid solution for 24 h at 4 °C, skin: acid solution ratio 1:3 (m/v), for removal of the meat, scales and some of the fat attached to the skin. Sample 1 was treated with a 3% lactic acid solution, sample 2 was treated with 3% citric acid, and sample 3 was treated with 3% citric and lactic acid mixture in 1:1 ratio, respectively.

**Alkaline Treatment**

The acid treated fish skin samples 1–3 were firstly washed with cold water for 2–3 h until the wash water had faintly acid or neutral pH and then subjected to an alkaline or saline treatment, by soaking for 24 h at 4 °C to remove non-collagenous proteins, resulting HO4P1s–HO4P5s pretreated carp skin samples as presented in Table 1.

**Ethanol 15% Treatment**

The HO4P1s–HO4P5s carp skin samples were cold water-washed for 6 h, changing the water every hour, until the wash water had faintly alkaline or neutral pH and then were subjected to a treatment with 15% ethanol in order to remove fats, by soaking for 24 h at a skin: 15% ethanol ratio 1:10 (w: v).

**Hydrogen Peroxide 1% Treatment**

The skin samples were cold water washed for 6 h, changing the water every hour, and then were subjected to a treatment with 1% hydrogen peroxide, by soaking for 24 h at a skin:1% hydrogen peroxide ratio 2:5 (w: v), in order to remove pigments.

**Neutral Hydrolysis**

After washing with cold water by soaking for 12 h, the carp skin samples were subjected to a neutral hydrolysis process using an autoclave 3850 ELV CGP (Tutttnauer). A mixture of

| Skin sample | Acid pretreatment skin: acid solution ratio (m/v) | Alkaline or saline pretreatment skin: solution ratio (m/v) | Collagen hydrolysate sample | Yield, % |
|-------------|-----------------------------------------------|------------------------------------------------|-----------------------------|----------|
| HO4P1s      | 3% citric and lactic acid 1:1 mixture 1:3      | 1 M NaOH 1:10                                   | HO4P1                       | 35.5     |
| HO4P2s      | 3% lactic acid solution 1:3                    | NaCl 1 M 1:5                                    | HO4P2                       | 28.1     |
| HO4P3s      | 3% lactic acid solution 1:3                    | 1 M NaHCO₃ 1:10                                 | HO4P3                       | 24.6     |
| HO4P4s      | 3% citric acid 1:3                             | 1 M NaOH 1:10                                   | HO4P4                       | 29.3     |
| HO4P5s      | 3% lactic acid solution 1:3                    | 1 M NaOH 1:10                                   | gelatin                     | –        |

HO4P1s–HO4P5s represent pretreated skin samples
HO4P1–HO4P4 represents fish skin collagen hydrolysates samples
pretreated fish skin and distilled water in 1:3 ratio was placed in a metal recipient and heated for 6 h at a temperature of 135 °C and a pressure of 315 kPa. Liquid hydrolysates were separated by filtration.

**Freeze-Drying**

Liquid hydrolysates were freeze-dried using a DELTA 2–24 LSC freeze dryer (Christ, Germany) obtaining yellowish–white powders, slightly pearlescent of fish collagen hydrolysate., samples HO4P1–HO4P4.

The yield of collagen was calculated and expressed as percentage by Eq. (1) [21]:

\[ \text{Yield of collagen} = \frac{\text{Weight of collagen, g}}{\text{Weight of dry skin, g}} \times 100 \]  

(1)

**Physical–Chemical Characterization**

Multiple physical–chemical analyses using standardized methods were performed for the characterization of the carp skin collagen hydrolysates as follows: gravimetric methods for dry substance content (SR EN ISO 4684:2006) and total ash content (SR EN ISO 4047:2002) by drying at constant mass at 102 ± 2 °C and by calcining at 800 °C, respectively; volumetric methods for total nitrogen content (Kjeldahl method) and protein substance (SR ISO 5397:1996), aminic nitrogen according to a Leather and Footwear Research Institute (ICPI) internal protocol; potentiometric method for pH value (SR EN ISO 4045:2008).

**Degree of Hydrolysis**, DH of carp skin collagen hydrolysates was estimated using the values of total nitrogen, \( N_t \) and amino nitrogen (\( NH_2 \)) according to literature data (Eq. 2) [22]:

\[ \text{DH(%) = } \frac{\text{Amino nitrogen (NH}_2\text{)}}{\text{Total Nitrogen (N}_t\text{)}} \times 100 \]  

(2)

**Infrared Spectroscopy (FT-IR)**

The determinations were made using an FTIR spectrometer (Jasco, model 4200, Jasco Europe S.R.L., Milan, Italy) by the method of attenuated total reflection (FT-IR ATR) under the following conditions: wavelength range, 4000–600 cm\(^{-1}\); resolution, 4 cm\(^{-1}\); radiation angle of incidence, 45°; the IR radiation is directed through the germanium crystal towards the diamond sensor, on the surface of which is placed the sample to be analyzed. For deconvolution experiments, FTIR spectroscopic analysis was performed on a Nicolet iS 50 FT-IR spectrometer using an integral diamond ATR unit; the measurements were carried out in the range of 4000–400 cm\(^{-1}\), using the resolution 8 cm\(^{-1}\) and co-adding 64 scans per each spectrum. Fourier self-deconvolution was conducted on the average spectra for the amide I band using Fityk 1.3.1 (Function type: Voight).

**Circular Dichroism Spectroscopy (CD)**

The secondary structure of fish collagen hydrolysates was evaluated by CD. Spectra acquisition was performed on a Jasco J-1500 Circular Dichroism Spectrophotometer model, Japan using a quartz cell with l = 1 mm. For each determination, 500 µL of aqueous collagen hydrolyzed solution of 0.025% concentration was used. CD spectra were obtained by triplicate scanning, using the spectral range 170–260 nm, with a scan speed of 100 nm/min.

**X-Ray Diffraction (XRD) Analysis**

The composition and crystallinity degree of these materials were assessed by X-Ray diffraction, which was performed using a Shimadzu XRD 6000 diffractometer, with Ni-filtered CuK\( \alpha \) radiation (\( \alpha = 1.5406 \) Å), 2 theta in 5–30° range by a step of 0.02° and an angular speed of 2°/min.

**Determination of Isoelectric Point (pI)**

The isoelectric point was measured by determining the electrophoretic mobility of the systems at different pH values, using the ZetaSizer Nano, Malvern, Zeta Protein module. A sample of 3 mg of collagen was solubilized in 3 mL PBS (Phosphate-buffered saline) with different pH values (2–8), then 1 mL of 5 mM NaCl solution (electrolyte role) was added. Collagen hydrolysates samples solubilized with electrolyte content were placed in special cells and subjected to analysis in 3 cycles of 30 measurements.

**Optical Microscopy Analysis**

**Optical Microscopy Analysis** was performed using a LEICA optical microscope model S8AP0, with Increase Power: 20–160×. The images were captured by an attached Nikon Coolpix MCD camera (Japan). The results are presented in supporting information section figure S2.

**Scanning Electron Microscopy (SEM)**

The morphology of the fish collagen hydrolysates samples was investigated by scanning electron microscopy (SEM), using a HITACHI S2600N equipment coupled with EDX spindle. Powder samples for SEM analyses were covered with a thin silver layer deposited by dc-sputtering.
Biocompatibility Assessment

Human adult keratinocytes (HaCaT cell line) were cultured in DMEM 4.5 g/L glucose, with 10% fetal bovine serum (FBS), at 37 °C and 5% CO2. Cells were seeded at 10,000 cells/cm², and after 24 h, the medium was changed with complete medium supplemented with 1% collagen hydrolysates. After 6 days, with one medium changed at day 3, the cellular morphology was investigated using phase contrast microscopy (Zeiss Axio Vert.A1, Germany) and the cell viability was quantified using the XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) method according to the manufacturer's specifications (Thermo Scientific). Briefly, the cells were incubated with warm culture medium containing 0.25 mg/mL XTT and 6.25 µM PMS (N-methyl dibenzopyrazine methyl sulfate, the electron coupling agent) for 4 h at 37 °C, after which the absorbance of the samples was measured at 450 nm with reference at 650 nm.

The experiments were performed in triplicate and the results reported as percentage of control (cells grown in complete medium) and expressed as mean ± standard deviation [23].

Statistical Analyses

All physico-chemical experiments were performed in triplicate (n = 3). The data are expressed as mean ± Standard Deviation SD. Statistical analysis was performed using GraphPad Prism software also for biocompatibility assessment. The significance of differences was evaluated by single factor ANOVA test and it was considered significant if P < 0.05.

Results and Discussion

Fish Collagen Hydrolysate Extraction

Most reported methods for extraction of collagen hydrolysate from fish skin use enzymatic or acid hydrolysis through several stages, with the disadvantage that in the skin processing step, residues such as scales, bones, meat or fat are manually removed, a process that can take a long time and may be inefficient for the total removal of these residues. Other disadvantages reside in the cost of enzymes, the long hydrolysis time, and the difficulty of purification of the final product, residues of acids or enzymes affecting the properties of the final product.

The method proposed in the present paper eliminates some of these disadvantages by using neutral hydrolysis, employing water in subcritical conditions at 135 °C and 315 kPa. Several pretreatment procedures were developed for removing residues (scales, bones, meat or fat), non-collagenous proteins and pigments involving food and cosmetic compatible organic acids (lactic and citric acid), mild alkaline or saline treatment, diluted ethanol and hydrogen peroxide, respectively, as presented in Fig. 1. The solvent concentrations involved in the extraction process were chosen based both on our previous experience in obtaining collagen and on other studies reported in the literature [5].

After the hydrolysis process, the samples were stored at 4 °C for 24 h. In most of the cases, stable solid hydrolysates, in moderate yields (Table 1) were obtained with exception of the sample HO4P5, resulted after employing 3% lactic acid and NaOH in the separation process which yielded gelatin, proving this treatment unsuitable for obtaining fish skin collagen hydrolysate. Reported literature data showed that gelatin extraction yields from mackerel skin [24] are higher for lactic acid treatment as compared to citric acid, respectively, and depend on molar concentration of the acid. A possible explanation for the lower extraction yields of collagen hydrolysates after 3% lactic acid pretreatment (28.1% for HO4P2 and 24.6% for HO4P3, respectively) may be the increase amount of gelatin obtained in this process which was removed by subsequent washings. The pre-hydrolysis treatment with the mixture of organic acids and NaOH 1 M (sample HO4P1, 35.5% extraction yield) proved to be the most efficient procedure for extraction of fish skin collagen hydrolysates.

The obtained extraction yields are in good agreement with acid soluble collagen obtained from carp skin, Cyprinus carpio – 41.3% [25] and Ctenopharyngodon idellus – 25.5% [26]. Literature data report different extraction yields for acid soluble fish skin collagen (red Snapper – 5.71%, milkfish – 4.00%, paper nautilus – 55.2%, Japanese seabass – 51.4%, chub mackerel – 49.8%, bullhead shark – 50.1%) explained as a difference in solubility in acid solutions due to the degree of cross-linking of collagen molecules [25].

Fig. 1 Extraction process for fish skin collagen hydrolysates
Physical–Chemical Characterization

Physical–chemical parameters and the estimations of collagen content are important data in evaluating the potential value of fish collagen hydrolysates. Thus, the obtained *Cyprinus carpio* skin collagen hydrolysates HO4P1–HO4P4 were subjected to a standard physical–chemical characterization, the results are presented in Table 2.

Examining the data from Table 2 we may conclude that all the obtained fish collagen hydrolysates samples present a high degree of purity according to the very high percentage of protein (about 98%) and undetected values for ash content respectively, an important advantage of the extraction method employed. By comparison, reported literature data for acid soluble *Cyprinus carpio* skin collagen hydrolysate shows a percentage of protein of 27–27.9% and an ash content of 0.22–1.21% [27]. The proposed procedure for the separation of collagen hydrolysates yielded samples with pH values in the range 6–7, which indicates the suitability of obtained carp skin hydrolysates for food supplements or cosmetics. Additional information resulted by examining the values for total nitrogen, N, and amino nitrogen (NH₂) to obtain an estimation of the degree of hydrolysis, DH. As the degree of hydrolysis increases, the average molecular mass of the hydrolysate decreases, an estimation of the latter based on the amine nitrogen content being possible according to previous reported results [28, 29]. Thus, HO4P2 is the hydrolysate with the highest molecular weight (DH value 3.16%, estimated average molecular mass > 25,000 Da) whilst the estimated molecular mass for the other samples ranges in the 13,000–20,000 Da. This may be explained by the differences in the applied treatment, pKa value of the acid involved in the pretreatment step and the nature of non-collagenous protein removal agent: sample HO4P2 was obtained after treatment with lactic acid (pKa = 3.85, proved to favor hydrolysis to gelatin) and a saline, mild treatment with NaCl 1 M for removing non-collagenous proteins.

### Infrared Spectroscopy (FT-IR)

The structure of collagen hydrolysates samples was confirmed by FT-IR spectroscopy as revealed by comparative IR spectrum for collagen hydrolysates in Fig. 2, similar with other fish collagen hydrolysates reported in the literature [30]. The identified absorption bands for collagen hydrolysates samples HO4P1–HO4P4 (Table 3) are characteristic for the functional groups specific to collagen:

![FTIR spectrum for fish collagen hydrolysates](image)

### Table 2 The physical–chemical properties of fish skin collagen hydrolysates HO4P1–HO4P4

| Characteristics       | HO4P1  | HO4P2  | HO4P3  | HO4P4  |
|-----------------------|--------|--------|--------|--------|
| Dried substance, %    | 92.89±0.39 | 91.18±0.38 | 92.13±0.38 | 91.06±0.38 |
| Ash content, %        | Undetectable | Undetectable | Undetectable | Undetectable |
| Total nitrogen, % (N) | 17.41±0.11 | 17.38±0.11 | 17.44±0.11 | 17.55±0.12 |
| Protein substance, %  | 97.84±2.61 | 97.68±2.59 | 97.99±2.61 | 98.62±2.62 |
| pH                    | 6.94±0.1  | 6.24±0.1 | 6.15±0.1 | 6.18±0.1 |
| Amine nitrogen, % (NH₂)| 0.71 | 0.55 | 0.96 | 0.77 |
| DH, %                 | 4.08    | 3.16    | 5.5     | 4.38    |

HO4P1–HO4P4 represents fish skin collagen hydrolysated samples

Last column bold character represents year of approval of the referred standard

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amide A—secondary amine N–H stretching band, amide B—asymmetrical stretching of methylene group, amide I—carbonyl stretching band, amide II and amide III—N–H bending vibrations coupled with C–N stretching vibration and C–H stretching, respectively, no significant differences between samples being observed.

Deconvolution of amide band I (Fig. S1, supplementary information) and quantitative band fitting analysis for the main component peaks (Table 4) provided interesting information on protein conformational changes in the obtained fish skin hydrolysates. According to literature data [31, 32] the component peaks obtained after deconvolution of amide band I for the examined collagen hydrolysate samples may be attributed as follows:

- Peak 1, 1625–1628 cm\(^{-1}\)—intramolecular beta-sheets
- Peak 2, 1651–1656 cm\(^{-1}\)—random coil conformations, imide residues and \(\alpha\)-like helix conformations
- Peak 3, 1665–1681 cm\(^{-1}\)—\(\beta\)-turns of the C- and N-telopeptides in collagen
- Peak 4, 1691–1694 cm\(^{-1}\)—gelatin and helices of aggregated collagen-like peptides

An intense absorption at 1660 cm\(^{-1}\) is generally attributed to the triple helix state of collagen [30], a decrease of the band intensity accompanied by enhancement of bands around 1630 cm\(^{-1}\) being generally associated with heat denaturation of collagen. Examining the deconvolution component peaks of amide I for the fish collagen hydrolysates samples, no triple helix characteristic absorption is observed, the most intense component being the one due to random coil conformations, imide residues and \(\alpha\)-like helix conformations (Peak 2 at 1651–1656 cm\(^{-1}\)). This conclusion was also sustained by the circular dichroism and XRD experiments, no triple helix structure being evidenced for all the obtained fish collagen hydrolysates.

### Circular Dichroism Spectroscopy (CD)

Mechanical properties of collagen are greatly influenced by the existing triple helical structure and maintaining the integrity of this structure influence the further applications of the fish collagen hydrolysates. Information about the existence of triple helix structure were obtained by Circular Dichroism Spectroscopy, a spectroscopic method based on the differential absorption of left–right polarized circular light of chiral macromolecules, typically used to evaluate the secondary structure of proteins [33]. CD spectra of the analyzed fish collagen hydrolysates samples are presented in Fig. 3.

All analyzed samples showed a pronounced negative band around 196 to 200 nm, typical of a random coil structure and no positive maximum at the wavelength 220 nm, characteristic for the triple helix was observed. Thus, a specific structure characteristic for the denatured collagen (hydrolyzed collagen) was attributed to all fish collagen extracts HO4P1–HO4P4 [34]. Prevalence of random coil structure and no triple helix characteristic band are in good agreement with FT-IR spectra and intensity of component bands measured after deconvolution of amide I band, the highest intensity being observed for band 2, characteristic for random coil conformations. (Fig. S1, Table 4).

### Table 3 Characteristic FT-IR absorption bands for collagen hydrolysates samples HO4P1–HO4P4

| Characteristic band | Peak location, wavenumber (cm\(^{-1}\)) |
|--------------------|------------------------------------------|
| Amide A            | 3282                                    |
| Amide B            | 2929                                    |
| Amide I            | 1629                                    |
| Amide II           | 1532                                    |
| Amide III          | 1235                                    |

### Table 4 Location and percent area contribution of FTIR amide I band component peaks for fish skin hydrolysates HO4P1–HO4P4

| Sample | Component peak location (cm\(^{-1}\)) and percent area, % (in brackets) contribution of total band |
|--------|-------------------------------------------------------------------------------------------------|
| HO4P1  | 1625.88 1.243 (29.10) 1656.32 2.551 (59.00) 1681.87 0.0074 (0.22) 1691.08 0.2331 (5.40) |
| HO4P2  | 1627.21 3.059 (28.50) 1651.54 6.9176 (59.60) 1664.41 0.5039 (4.4) 1693.67 0.4182 (3.60)  |
| HO4P3  | 1626.36 3.8727 (26.80) 1652.03 8.5713 (59.40) 1674.88 1.3539 (9.40) 1693.7 0.2671 (1.90) |
| HO4P4  | 1628.31 3.5362 (28.20) 1651.27 7.8129 (62.50) 1661.25 0.7015 (5.60) 1694.06 0.1671 (1.30) |
X-Ray Diffraction (XRD) Analysis

X-ray diffraction is often used to assess collagen fibril distribution and orientation in fish mineralized tissues. There are two peaks characteristic of collagen molecule and considered as a signature. The first one (~7°) and sharpest is related to the triple helix conformation and distance between molecular chains, and the second peak (~20°) is related to the distance between the skeletons. The X-Ray spectra of lyophilized fish collagen hydrolysates samples presented in Fig. 3b reveal no sharp peak associated with the triple helical structure, due to the loosing of the native conformations of collagen during the hydrolysis process as confirmed also by FT-IR characteristic amide I band analysis and CD spectra. The second peak is present at 21.19° (HO4P1), 19.18° (HO4P2), 19.28° (HO4P3) and 19.84° (HO4P4), respectively. The Bragg equation $d(\text{Å}) = \frac{\lambda}{2\sin \theta}$ (where $\lambda$ is the X-ray wavelength (1.54 Å), and $\theta$ is the Bragg diffraction angle), was used to calculate the minimum value of the repeated spacings. The distance between skeletons were 4.18 Å (HO4P1), 4.62 Å (HO4P2), 4.59 Å (HO4P3) and 4.47 Å (HO4P4) respectively, in good agreement with the reported values for collagen extracted from other fish species [35].

Determination of Isoelectric Point (pI)

Possible applications of fish collagen hydrolysates are influenced by their solubility and by the effect of pH on protein solubility determined by its isoelectric point (pI) value. Isoelectric point (pI) is the pH of the collagen molecule at zero charge. Zeta potential measurements for each fish collagen hydrolysate sample at variable pH were performed (Fig. 4) allowing determination of the isoelectric point considered as the pH value at which PZ intersects the x axis (equals zero). The calculated values for pI vary between 3.9 and 2.9 as follows: pI = 2.9 for sample HO4P1, pI = 2.9 for sample HO4P2; pI = 3.7 for sample HO4P3 and pI = 3.9 for sample HO4P4, respectively. Similar values of pI for acid and pepsin-soluble collagen were reported in the literature on the extraction of collagen from fish skin and bones. The pI value differences of collagen hydrolysates samples may be attributed to the ratio of acidic and basic amino acid residues, which was more likely governed by the removal of telopeptides after acidic treatment. Hydrolyzed collagen is an amphoteric macromolecule [36] composed of both acidic (COOH) and basic (NH$_2$) functional groups and the pI decrement could be due to the deamination process. When hydrolyzed collagen was treated at high temperature, the asparagine groups transformed to aspartic acid and the
glutamine groups into glutamic acid. This leads to a loss of amino groups and a large relative increase in the carboxyl groups, or a higher content of acidic amino acids, which become dominant, shifting the pI to lower values.

**Scanning Electron Microscopy**

Morphology of the obtained fish collagen hydrolysates was investigated using Optical microscopy and Scanning electron microscopy. The optical microscopy results (Fig. S2 supplementary information) confirm the presence of fibrils structure of collagen similar to other reported by literature data [37]. More detailed visual insights were obtained from SEM micrographs (Fig. 5), for all collagen hydrolysate samples a characteristic highly entangled structure, like sheets folded and wrinkled was identified. Both SEM and optical microscopy observations revealed a randomly distributed fibril pattern and confirmed the absence of undesired aggregates (cell debris, skeletal parts or undissociated collagen fibers) between the fibrils of collagen [38].

**Biocompatibility Assessment**

Human adult keratinocytes, HaCaT cell line proliferation experiments cultured for 6 days on medium supplemented with 1% fish collagen hydrolysates and investigated using phase contrast microscopy (Fig. 6a) revealed no obvious differences in cell morphological aspect for the fish collagen hydrolysates and control, with the exception of the cells cultured in the presence of sample HO4P2 which was not associated with a confluent layer at the end of the incubation period. This result suggests that, at 1% concentration in culture medium, most of the fish collagen hydrolysates samples sustained cell proliferation.

This first observation was supported by the quantification of cell viability using the XTT assay (Fig. 6b) at the end of the 6 days cultivation period. The percentage of viable cells for the fish collagen hydrolysates was above 80%, except for the sample HO4P2. Furthermore, all 3 samples—HO4P1, HO4P3 and HO4P4, showed a higher viability compared to the control bovine hydrolysate, suggesting a superior

![Fig. 5 Scanning electron microscopy images for fish skin collagen hydrolysates (a HO4P1, b HO4P2, c HO4P3, d HO4P4)]
effect for biomedical applications. An explanation for low biocompatibility of fish sample hydrolysate HO4P2 may be the highest molecular mass of its component polypeptides as compared to other samples (> 25 kDa as estimated from amine nitrogen content and DH). According to literature data, collagen hydrolysates with a molecular weight between 5 and 13 kDa [39] used in biological in-vitro essays promotes cell proliferation and collagen I secretion in fibroblast, together with a better penetration of skin. This may be an explanation for the superior cell viability observed for the sample HO4P3, with the lowest estimated molecular mass (~ 13 kDa) in the range of the reported literature data (Fig. 6).

Conclusions

Biocompatible fish collagen hydrolysates were obtained from waste carp skin by neutral hydrolysis, using several pretreatment procedures involving organic acids (lactic, citric acid and a 1:1 mixture of both) for removal of residues (scales, bones, meat or fat), followed by removing of non-collagenous proteins with 1 M NaOH, 1 M NaCl, or 1 M NaHCO₃ respectively and subsequent defatting and removal of pigments. The highest extraction yield (35%) was obtained for the sample HO4P1, resulted after pre hydrolysis treatment with the mixture of organic acids and NaOH.

A high degree of purity of fish collagen hydrolysates given by percentage of protein (about 98%), undetected ash content and the pH value in the range 6–7, show that all hydrolysates samples can be safely used in food supplements or cosmetics. The purity was confirmed by the absence of undesired aggregates in the characteristic fibril structure as determined by optical and scanning electron microscopy.

A random coil structure, specific of the denatured collagen (hydrolyzed collagen) with no triple helical structure characteristics was attributed to all neutral hydrolysis obtained collagen hydrolysate samples by examining FT-IR spectra after deconvolution of characteristic amide I band, conclusion sustained by Circular Dichroism Spectroscopy and X-Ray diffraction. Moreover, fibril distribution and orientation determined by XRD analyses revealed similar values of distance between skeletons to those measured for collagen hydrolysates extracted from other fish species.

The biocompatibility assessment revealed that the obtained fish collagen hydrolysates had no cytotoxic effect on Human keratinocytes, except for the sample with the lower degree of hydrolysis, HO4P2. Furthermore, the cell viability for these samples was 80%, higher than that for the conventional bovine hydrolysate.

In conclusion, carp skin collagen hydrolysates obtained by neutral hydrolysis with proved biocompatibility provides attractive alternative source to bovine collagen, with characteristics suitable for potential application in foods, cosmetics, and pharmaceutical industry.

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Declarations

Conflict of interest The authors declare no competing financial interest.

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Authors and Affiliations

Elena Dănilă1 · Raluca Stan1 · Madălina Albu Kaya2 · Georgeta Voicu1 · Maria Minodora Marin1,2 · Alina Moroșan1 · Irina Titorencu3 · Raluca Țuțuianu3

1 Faculty of Applied Chemistry and Materials Science, Bucharest, University Politehnica of Bucharest, 1-7, Gh. Polizu, 011061 Bucharest, Romania

2 Collagen Department, INCDTP – Leather and Footwear Research Institute, 93, Ion Minulescu, 031215 Bucharest, Romania

3 Nicolae Simionescu Institute of Cellular Biology and Pathology of the Romanian Academy, 8, B. P. Hasdeu, 050568 Bucharest, Romania