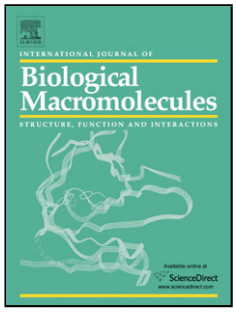

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Evaluation of alternative sources of collagen fractions from *Loligo vulgaris* squid mantle

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Abstract

Acid-Solubilized Collagen (ASC) and Pepsin-Solubilized Collagen (PSC) were extracted from the mantle of the common European squid, and were comparatively characterized. ASC and PSC were isolated with an extraction yield of 5.1 and 24.2% (on dry weight basis), respectively. SDS-PAGE showed that the ASC was mostly comprised of α_1- and α_2-chains; while the PSC presented relevant β- and γ-components. GPC analysis confirmed that both the ASC and the PSC consisted of fractions characterized by different molecular weight. Thermal denaturation behavior of ASC and PSC were followed by calorimetric and rheological analyses; denaturation temperature was estimated to be 22°C for ASC and 21°C for PSC. Amino acid composition and solubility of collagen were also investigated. Finally, the cytotoxicity of the isolated collagen was evaluated *in vitro* and no cytotoxic activity caused by the collagen extracts was observed. This study demonstrated that squid mantle has potential as an alternative source of collagen-derived materials.

Keywords: Collagen; *Loligo vulgaris*; Mantle; Acid-Solubilized Collagen (ASC); Pepsin-Solubilized Collagen (PSC); Physico-chemical properties.

1. Introduction

Collagen material is a biopolymer used in a wide range of applications, in food, cosmetic and pharmaceutical industries [1]. It is frequently used as scaffold also in the field of tissue engineering due to its biocompatibility, biodegradability, low immunogenicity and cell-adhesive properties [2]. In particular, among the fibril-forming collagens, type I and V collagen fibrils have been extensively used as biomaterial for the development of tissue engineering constructs since they contribute to the structural backbone of bone [3].

Nowadays, the main sources of collagen and collagen-derived products, such as gelatin and collagen hydrolysates, are limited to bovine skin and tendon, and porcine dermis [4]. Collagen is however present not only in mammals, but throughout the entire animal kingdom including birds and fishes. Sources, extraction methods and pre-treatments affect the final characteristics of collagen, such as composition, rheological properties, solubility and thermal stability [5] and consequently its biological activity.

In the last years, concerns have been expressed about the use of collagen derived from land-based animals due to the risk of infection and diseases such bovine sponge encephalopathy,

transmissible spongiform encephalopathy, foot-and-mouth disease and avian influenza [6] [7] and increasing interest has been paid to alternative collagen sources. Marine organisms could be a valuable collagen source being highly available, with no risks of disease transmission [8][9].
Each member of the collagen family is characterized by the repetitions of the proline-rich tripeptide Gly-X-Y involved in the formation of trimeric collagen triple helices [3]. Fibrillar collagens are of two types, acid soluble or pepsin soluble. Neutral salt-extraction and low concentration acid-extraction are the most commonly used methods to isolate collagen from natural tissues [10][11]. Dilute acid solvents are more efficient than neutral salt solutions since they dissociate the intermolecular cross-links of the aldimine type causing the swelling of the fibrillar structures [12]. However, dilute acids will not disassociate less labile cross-links such as keto-imine bonds. Much higher yields can be achieved using proteases, i.e. pepsin that cleaves peptides in the telopeptide region [13]. Extraction and characterization of acid and pepsin-solubilized collagen have been reported for different fish species and fish collagen started to became a potential ingredient for cosmetic, food, pharmaceutical and biomedical applications [14][15][16]. The amino acid profiles of the two types of collagen may vary, depending on the source, as well as molecular weight and denaturation temperature. During the processing of the material, the presence of covalent cross-links between molecules represents the major impediment to dissolution of collagen from tissues. Therefore, native collagen must be pre-treated before it can be converted into a form suitable for extraction [17]. In addition, as a protein, collagen conformations are sensitive to different factors during the extraction process, which can induce the destruction of the native form and consequently cause the loss of specific functions. For example heat can induce the triple helix collapse and the thermal denaturation into gelatin [18].
In view of the large number of potential sources to be studied and the large number of parameters related at the extraction process, the chemical and biological characterization of isolated collagen is necessary and efficient systems of evaluation need to be developed [19].
Loligo vulgaris is a neritic, semipelagic species that occurs abundantly in coastal water from the North Sea to the west coast of Africa [20]. This species is commercially very important. In fact, it is extensively exploited by commercial fisheries during the whole year, with annual catches over 15,000 tons [21]. Due to the good availability, easy and fast storage conditions, there is the possibility to use this species for the extraction of collagen and as a model for the development of a simple and efficient system for study the quality of resulting protein. Each anatomical part of a live organisms has a specific function that can affect the amount of collagen and its degree of aggregation. Squid are fast-growing species and very active during their lifetimes. In particular, their mantle needs to be elastic to perform its propulsive function. Due to its peculiar musculature and the high degree of protein turnover [22], squid mantle presents a promising protein composition and collagen is present in a considerably large amounts inside the tissue (up to about 11% of total protein in the muscle of some squid species, like *Illex argentinus*) [23]. However, at present, there has been no investigation on the collagen extracted from *Loligo vulgaris*.
Therefore, in the present work, first acid-solubilized collagen (ASC) and then pepsin-solubilized collagen (PSC) from squid mantle (*Loligo Vulgaris*) were isolated and characterized in order to provide a parallel comparison of these collagens. A complete characterization of the physico-chemical properties of the resulting proteins was performed, exploiting different techniques. The extracted collagen was characterized using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Fourier Transform Infrared Spectroscopy (FTIR) and Gel Permeation Chromatography (GPC). Amino acid composition and solubility of collagen were also evaluated. Denaturation temperature was measured by viscosity change and confirmed with thermal analyses using Differential Scanning Calorimetry (DSC). No cytotoxic effect of isolated collagens was observed after the extraction process.

2. Materials and methods

2.1. Materials

Acetic acid, Sodium hydroxide (NaOH), Sodium chloride (NaCl), Bovine collagen type I, Pepsin, Disodium hydrogen phosphate (Na_2HPO_4) and high molecular weight markers were purchased from Sigma-Aldrich (St. Louis, MO, USA).
All the chemicals and reagents were of analytical grade and were used without further purifications.

2.2. Squid mantle preliminary treatment

Squid *Loligo vulgaris* caught in the northern Adriatic Sea was purchased at Trento local market and kept in ice using a solid/ice ratio of 1:2 (w/w). Squid was washed with iced tap water (0–2°C), then skin and tentacles were discarded. The squid mantle was cut into small pieces (0.5 x 0.5 cm^{-1}). Squid mantle fragments were soaked in 0.1 M NaOH for 2 days to remove non-collagenous proteins, and then washed with DI water for 1 day. All the procedures were carried out at a temperature lower than 4°C.

2.3 Isolation of acid- and pepsin-solubilized collagen fractions

2.3.1 Extraction of acid-solubilized collagen

Acid-solubilized collagen fraction (hereinafter ASC) was extracted according to [24] with some modifications. Briefly, pre-treated mantle fragments were finely minced and then treated with a 0.5 M acetic acid solution for 3 days under continuous stirring. Extraction was carried out at a temperature of 4°C. The mixture was later centrifuged at 80,000 x *g* for 2 h to pellet non-solubilized collagen fractions. Solid residues underwent a second extraction process under the same conditions. The filtrates obtained in the two processes were later mixed and collagenous molecules were precipitated by adding NaCl to a final concentration of 0.9 M. Precipitated proteins were recovered by centrifugation at 20,000 x *g* for 30 min at 4°C and re-dissolved in a minimum volume of 0.5 M acetic acid. The solution was dialyzed against 0.1 M acetic acid for 2 days and then against DI water for 1 day in a Slide-A-Lyzer Cassette (MWCO 3,500 Da from Pierce, Rockford, Illinois, USA). The resulting dialysate was freeze-dried to obtain the ASC fraction.

2.3.2 Extraction of pepsin-solubilized collagen

After ASC extraction, the remaining insoluble collagen was washed with DI water and treated with 0.5 M acetic acid with 0.1% (w/v) pepsin for 3 days at 4°C under continuous stirring. The mixture was centrifuged at 80,000 x *g* for 2 h to remove residues and dissolved collagen molecules in the supernatant were salted-out by addition of NaCl to a final concentration of 0.9 M. Precipitate proteins were then separated by centrifugation at 20,000 x *g* for 30 min at 4°C, dissolved in 0.5 M acetic acid and dialyzed against 0.02 M Na_2HPO_4 solution for 1 day to inactivate pepsin and against 0.1 M acetic acid for 2 days. Finally, the solution was dialyzed against DI water for 1 day, and the resulting dialysates were freeze-dried to obtain the pepsin-solubilized collagen fraction (hereinafter PSC).

2.4. Characterization of extracted collagen fractions

2.4.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

All SDS-PAGE kit and reagents were purchased from Invitrogen (Carlsbad, CA, USA). First, the lyophilized collagen powders were re-dissolved in NuPAGE® LDS Sample Buffer at a concentration of 0.5 mg/ml and incubated at 70°C for 10 min. Samples (15 µg protein) were analyzed by one-dimentional SDS-PAGE, with a XCell4 SureLock™ Midi-Cell (Carlsband, CA, USA), using NuPAGE® Tris-Acetate SDS Running Buffer, with a constant voltage of 150 V. Acrylamide SDS-PAGE NuPAGE® Novex Tris-Acetate Gels (3%-8% gradient) were used for electrophoresis. The acrylamide gels were stained using a Coomassie stain (Imperial Protein Stain). Gel separation patterns were digitalized by a GEL LOGIC 200 (Kodak Scientific Imaging Systems, Rochester, NY, USA) imaging system. SeeBlue® Plus2 Pre-Standard was used as Molecular Weight (MW) reference.

2.4.2 Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC) analysis of the isolated collagens was conducted with a Shodex SB-805HQ column (Shodex OH pak®, Showa Denko, Munich, Germany). Freeze-dried ASC and PSC samples were re-dissolved in 0.5 M acetic acid to obtain a protein concentration of 0.5 mg/ml. The obtained collagen solutions were dialyzed against DI water, using a cellulose membrane (MWCO 3,500 Da from Pierce, Rockford, Illinoys, USA). The chromatography system was operated with a flow rate of 1 ml/min and elution was detected with a Jasco UV-1570 detector set (Jasco, Bouguenais, France) at 224 nm. Calibration curve was obtained with Low/High Molecular Weight Gel Filtration Calibration Kit (GE Healthcare Europe, Freiburg, Germany).

2.4.3 Amino acid analysis

The amino acid content of ASC and PSC lyophilized samples was determined with Waters AccQ-Fluor™ Reagent Kit using the AccQ-Tag™ amino acid analysis method (Waters Corp., Milford, MA, USA). Briefly, ASC and PSC samples were frozen in LN and freeze-dried using a Lio-5P lyophilizer (5Pascal, Milan, Italy). About 5 mg of lyophilized powders were hydrolyzed with 6 N HCl at 114 ± 2 °C in a silicone oil bath for 24 h. Air-dried hydrolysates were later reconstituted with 20 mM HCl to obtain a solution at a concentration in the range 4-200 pmol and then derivatized with Water AccQ Flour Reagent to obtain stable amino acids. The amino acid content was determined by Reversed-phase high-performance liquid chromatography (RP-HPLC) using an AccQ-Tag™ column (Waters Corp.) with a gradient of Waters AccQ·Tag™ Eluent A, Milli-Q water and Acetonitrile (HPLC grade) at a flow rate of 1 ml/min. The amino acids were detected with Jasco UV-1570 detector set (Jasco, Bouguenais, France) at 254 nm. The chromatograms obtained were compared with Water amino acid hydrolysate standard to identify single amino acid residues. Hydroxyproline content was determined with the Hydroxyproline Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). ASC and PSC lyophilized powders were first hydrolyzed with 6 N HCl at 120°C for 3 h. The hydroxyproline concentration was measured by the reaction of hydroxyproline with 4-(Dimethylamino)benzhaldehyde (DMAB, Sigma-Aldrich, St. Louis, MO, USA), which results in a colorimetric (560 nm) product proportional to the hydroxyproline present.
All samples and standards were run in triplicate.

2.4.4 Fourier transform-infrared spectroscopy (FT-IR)

FT-IR spectra of lyophilized collagen powders were collected using Spectrum One spectrometer with ATR correction (Perking Elmer, Waltham, MA, USA) with Zinc Selenide crystal. Sample spectra were averaged over 4 scans, ranging from 400 to 4,000 cm^{-1} at a resolution of 4 cm^{-1}.

2.4.5 Denaturation Temperature

The denaturation temperature of ASC and PSC fractions was evaluated exploiting two different techniques: differential scanning calorimetry (DSC) and viscosity measurements.

Differential scanning calorimetry (DSC) was conducted using a Mettler DSC 30 (Mettler-Toledo, OH, USA) to analyze the thermal stability of collagen. The ASC and PSC collagen powders were dissolved in 0.5 M acetic acid solution (sample/solution ratio of 1:40 w/v), and the mixtures were stored at 4°C for 2 days. For the DSC analysis the samples were accurately weighed into aluminum pans and sealed. The scan was performed over a range of 5-50°C, with a heating rate of 1°C/min. The denaturation temperature (T_d) was calculated from the peak of the DSC transition curve. The denaturation temperature was determined also using a Viscosimeter Physica MCR 301 (Anton Paar GmbH, Rivoli, Italy) according to the method of Zhang et al. (2007) with slight modifications [25]. A 2 mg/ml collagen solution in 0.5 M acetic acid was used to measure the viscosity. The thermal determination curve was obtained over a range of 16 to 30°C. The measurement was performed five times at each point. The fractional viscosity for each designated temperature was calculated with the following equation:

$$\boldsymbol{v} = \frac{(\boldsymbol{v_{max}} - \boldsymbol{v}(\boldsymbol{T}))}{(\boldsymbol{v_{max}} - \boldsymbol{v_{min}})}$$

These values were plotted against the temperatures and the dinamic denaturation temperature (T_d) was evaluated as the temperature at which the fractional viscosity was 0.5.

2.4.6 Solubility of extracted collagen

The solubility of ASC and PSC extracted collagen in 0.5 M acetic acid was evaluated as a function of NaCl concentration and pH. Lyophilized collagen were re-dissolved in 0.5 M acetic acid at 4°C at a concentration of 3 mg/ml. The effect of NaCl on collagen solubility was evaluated by preparing various 0.5 M acetic acid solutions with different concentrations of NaCl (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 M). The effect of pH on collagen solubility was evaluated by adjusting the pH of the 0.5 M acetic acid solution with 6 M HCl or 6 M NaOH to obtain a final pH values ranging from 1 to 10. The mixtures were kept at 4°C under stirring for 30 min and then centrifuged at 20,000 x *g* at 4°C for 30 min to remove un-dissolved debris. Total proteins content in the supernatants was determined by means of Bradford Protein Assay (Sigma-Aldrich, St. Louis, MO, USA), using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) as standard. The relative solubility of the collagen samples was calculated as a percentage of the total collagen in acetic acid solution 0.5 M.

2.4.7 Cytotoxicity

Cytotoxicity of extracted collagens was evaluated following ISO 10993-5 (1999), by indirect cultivation of cells with collagen extracts. Embryonic mouse fibroblast NIH/3T3 cells (3T3) were expanded and cultured at 37ºC with 5% CO_2 in Dulbecco's Modified Eagle medium (DMEM) (Euroclone, Pero, Italy), supplemented with 10% fetal bovine serum (Gibco, NY, USA), 2mM L-glutamine, 1mM sodium pyruvate and 0.1% antibiotics (Gibco, Eggenstein, Germany). Culture medium was changed every 2 days until cells reached confluency, then cells were detached with 0.1% trypsin and re-suspended in culture medium. Later, 3T3 cells were seeded in polystyrene 24-well plate at a density of 1×10^4 cells per well and incubated under standard culture conditions. When cells reached about 70-80% of confluency, culture medium was removed and replaced with conditioned media containing collagen extracts. Conditioned media were prepared by soaking freeze-dried ASC and PSC samples in reduced medium at a concentration of 4 g of dry collagen per 20 ml of medium, as prescribed by the ISO standard (24 h of soaking at 37°C with 5% CO_2). Reduced medium was prepared with Dulbecco's Modified Eagle medium without phenol red with 10% heat inactivated serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 0.1% antibiotics. Fibroblast 3T3 cells were cultured in medium conditioned with collagen samples in standard

conditions for 72 h. Five independent samples were tested for both ASC and PSC. Cells cultured in reduced medium were used as negative control. Lactate dehydrogenase assay (LDH) (TOX7, Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate the cytotoxicity impact of collagen extracts on the cells. An aliquot of 100 µm of culture medium was then collected from all wells and mixed with the LDH mixture, following manufacturers' instructions. Cells cultured in reduced medium and treated for 30 min with Triton x-100 were used as positive controls. Absorbance was measured using a Tecan Infinite 200 microplate reader (Tecan Group, Männedorf, Switzerland) at 490 nm, background absorbance was measured at 690 nm. Results were presented as mean ± standard deviation (n = 5).

3. Results and discussion

3.1. Collagen extraction yield

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) were subsequently isolated from *Loligo vulgaris* squid mantle with yields of 5.1% and 24.2% (on dry weight basis), respectively. The low yield of ASC suggests that a large amount of the collagen presents inside the squid mantle was not solubilized by 0.5 M acetic acid solution. A higher yield was obtained with pepsin extraction. During the extraction process, the main impediment to collagen dissolution from tissues is related to the presence of covalent cross-links between molecules that decrease the solubility of collagen [26]. It was reported that it is possible to solubilize approximately 2% of the tissue collagen using dilute salt or acid solutions [13]. For example, dilute acetic acid solutions dissociate the intermolecular cross-links and induce swelling of fibrillar collagen structures. It is well known that the remaining insoluble collagen can be further extract, without damage the triple helix-structure, using strong alkali or enzymes [27]. According to Nalinanon et al. the enzymatic treatment can provide much higher yields compared with acid extraction but the characteristics of the extracted collagen, such as the size of the molecules and molecular weight distribution, strongly depend on the time of treatment and the enzyme concentration [28]. In particular, it was found that pepsin cleaved the cross-linked collagen molecules at the telepeptide region, thus allowing extraction with higher yield and without damaging the integrity of the triple helix [29]. Furthermore, the resulting material, so called atelocollagen, benefits from the removal of the antigenic P-determinant located on the non-helical protein sections and seems to provoke a milder immune response [30] [31]. In the present work we proved that pepsin can be used to increase the extraction yield of collagen from the *Loligo vulgaris* squid mantle. The low yield of ASC suggests that the squid mantle has a high percentage of keto-imine bonds. In fact, dilute acids will not disassociate less labile crosslinks such as keto-imine bonds and consequently the amount of ASC inside the tissue is strongly related to the percentages of these bonds [32].
The total extractable yield (sum of yield of ASC and PSC) of squid mantle collagens 29.3% (on dry weight basis) was higher than that of bigeye snapper skin (7.5%) and brownstripe red snapper (13.7%) [33][6].

3.2 Electrophoretic characterization and molecular weights determination

The electrophoretic profiles of ASC and PSC collagens are reported in Fig.1(a). The SDS-PAGE protein patterns of the isolated collagens were compared with the profile of commercial bovine type I collagen (Gibco Life Technologies Inc., Grand Island, NY, USA) which was used as a reference for the collagen type I protein pattern. The profiles of ASC and PSC showed the presence of two bands in correspondence of the α_1 and α_2 bands of collagen type I. These results suggested that both extracted collagens mainly consisted of two different α chains, α_1 and α_2. For both, ASC and PSC, the band associated with α_2 chain was more intense than the α_1 chain band. In addition, SDS-PAGE analysis of PSC revealed the presence of β- and γ-chain components, which are considered respectively a dimer and a trimer of the α-chains [34]. The protein profiles of

ASC and PSC are typically associated with types I and V collagens that usually presented an heterotrimer structure in the forms $[\alpha_1(I)]_2\alpha_2(I)$, $[\alpha_1(V)]_2\alpha_2(V)$ or $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ [35] and were already detected in the mantle of other squid species [36][37][9].
Elutions profiles and molecular weight distributions of ASC and PSC measure by GPC are reported in Fig. 1(b). The chromatograms showed that both the extracted collagens have a wide molecular weights distribution and ASC presented a more broad and complex elution profile than PSC. From the figure it is possible to observe that severe peaks overlapping occurred and not all peaks are clearly distinguishable from the experimental data points. Therefore, the experimental curves were deconvoluted in order to analyze the molecular weight distributions of the different collagen components. Deconvolution of the GPC profiles was performed by the fitting of the chromatograms into separate curves corresponding to the different molecular weights of the collagens fractions. The experimental data points were deconvoluted using the Multipeaks Fit deconvolution function of OriginPro software (OriginLab, OriginPro 8.5, USA). The peak fit analysis resulted in five peaks for ASC and four peaks for PSC, with a correlation > 0.997 between the experimental and the theoretical curves obtained by deconvolution. Peak I corresponds at the higher molecular weights peptides and it was found at M_n = 677 kDa (pdi = 5.51) for ASC and M_n = 154 kDa (pdi = 2.44) for PSC. In addition, ASC showed a second peak (Peak 2) at M_n = 95 kDa (pdi = 2.02) and three further peaks (Peak 3, Peak 4 and Peak 5) at M_n < 10 kDa. Peak 2 of PSC was found at M_n = 32 kDa (pdi = 3.21) and in this case, two peaks corresponding at the low molecular weights (Peak 3 and Peak 4, M_n < 10 kDa) were detected. These results suggested that both, ASC and PSC, are composed by peptides with different range of molecular weights. In particular, ASC seems to be composed of more complex components since it had a wider molecular weight distribution and comprised higher molecular weight aggregates than PSC. Is interesting to note that ASC and PSC collagens presented a peak, respectively Peak 2 at M_n = 95 kDa and Peak 1 at M_n = 154 kDa, that are probably related at the α chains. In fact, the presence of the α_1 and α_2 chains were clearly detected also in the SDS–PAGE pattern of the two collagens, which highlights two marked bands in correspondence with the same range of molecular weights. Furthermore, the electrophoretic profile of PSC showed some bands at low MW that are not present in the pattern of ASC and collagen type I. This result was confirmed by the presence of Peak 2 at M_n = 32 kDa in the GPC curve and could be due at the action of pepsin that might induce the partial hydrolysis of high MW cross-linked molecules in PSC collagen. The combination of gel electrophoresis and GPC can provide a deeper understanding of size, charge and polarity of the isolated proteins. Protein separations by SDS-PAGE are commonly used to determine the approximate molecular weights of a protein and the relative abundance of major proteins in a sample [38][39]. In fact, SDS-PAGE has a higher resolution in the bands separation and consequently allows the characterization of more narrowly distributed weights fractions [40]. However GPC enabled to investigate a wider range of molecular weights than SDS-PAGE and to gives some important information about the molar mass distribution and the construction of the molecules.

3.3. Amino acid composition of collagen from squid mantle

The amino acid compositions of ASC and PSC extracted from the squid mantle were determined by HPLC and are reported in Table 1. The content of single amino acids was expressed as number of residues per 1,000 amino acid residues.
The amino acid composition found for both ASC and PSC were consistent with the amino acid composition of marine-derived collagens reported by other studies [6][41]. Glycine was the most abundant amino acid both for ASC and for PSC, representing 256 and 262 residues/1,000 residues, respectively. In addition, HPLC analysis found relatively high amounts of alanine, proline and glutamic acid. Both ASC and PSC presented a low content of methionine, cysteine, histidine; no tyrosine was detected. Also these results were in agreement with past analyses of collagens from different fish species [15][42]. The total amount of proline and hydroxyproline was higher for PSC than ASC (159 residues/1,000 residues and 152 residues/1,000 residues, respectively).
The amino acids were also functionally divided into three groups: (a) hydrophobic; (b) charged; and (c) polar amino acids. Both the collagens present a relatively high amount of hydrophobic amino acids, 591 residues/1,000 residues for ASC and 585 residues/1,000 residues for PSC. ASC

presented a higher amount of polar amino acids than PSC (109 residues/1,000 residues and 76 residues/1,000 residues, respectively) and a lower amount of charged amino acid (239 residues/1,000 residues for ASC and 266 residues/1,000 residues for PSC).
The high glycine content found in both the isolated collagens is due to the particular feature of collagen. In fact glycine regularly occurs in the helical part of the molecule in every third position, except for the first 14 amino acid residues from N-terminus and the first 10 residues from the C-terminus [43]. About 35% of the non-glycine positions in the repeating unit Gly-X-Y are occupied by proline or hydroxyproline [13]. Hydroxyproline is derived from proline by post-translational hydroxylation mediated by prolylhydroxylase [44]. The total amount of proline and hydroxyproline is an important factor because it is strongly related to thermal stability of the helix structure of collagen, and in particular, the higher is this content, the more stable are the helices [45]. In fact the pyrrolidine rings of proline and hydroxyproline stabilize the secondary structure of the polypeptide chain, preventing possible changes in its conformation. The structure is also maintained by the hydrogen bonds through the hydroxy groups of hydroxyproline. Consequently, the higher amount of proline and hydroxyproline in PSC suggested that PSC extracted from squid mantle presents a more stable conformation than ASC. However, in the ASC it was found a larger content of hydrophobic amino acids compare with PSC. This group includes amino acids with aliphatic side chains, such as alanine, isoleucine, leucine, proline and valine, that are non-reactive and rarely involved directly in protein function but play an important role in substrate recognition [46]. Therefore these largely non-reactive side chains can confer more stability at the collagen conformation and at the same time increase his capacity to bind/recognize specific ligands, such as lipids.

3.4. Fourier transform infrared (FTIR) spectra of collagen from squid mantle

FTIR spectra of collagen extracted from the squid mantle are presented in Fig.2. The major peaks in the spectra of ASC and PSC were similar to those of collagen isolated from others fish species. Similar FTIR spectra were observed between ASC and PSC. The amide I band was centered at 1643 for ASC and at 1648 cm^{-1} for PSC sample. As it is possible to see in the figure, the amide I peak is assigned with C=O stretching vibration or hydrogen bond coupled with COO^-. The amide II band of PSC (1530 cm^{-1}) was found at lower wavenumbers than ASC band (1547 cm^{-1}) and represent N–H bending vibrations coupled with CN stretching vibration. The amide III band, assigned to C-H stretching, was found to be centered at 1236 cm^{-1} for the ASC and at 1240 cm^{-1} for PSC collagen. The band of amide A is typically assigned to N-H stretching mode and was detected at 3380 for ASC and 3378 cm^{-1} for PSC. Finally, the amide B band of ASC and PSC were found at 2960 cm^{-1} and is associated at the asymmetrical stretch of CH_2.
As previously reported by Payne et al. [47] collagen dry films show a characteristic FT-IR spectrum, with absorption bands of amide I at ~1650 cm^{-1}, amide II at ~1560 cm^{-1}, and a set of three weaker bands that represent amide III vibration modes centered at ~1245 cm^{-1}. Both ASC and PSC showed the characteristic peaks observed in collagen. In particular, the position of amide I band of ASC and PSC was similar to that reported for others marine-derived collagens, usually found in the range of 1600 - 1700 cm^{-1} [48] [49]. The amide I peak is associated to the stretching vibration of the carbonyl groups (C=O bond) along the polypeptide. When collagen is heated at higher temperature this peak undergoes a decrease in absorbance, becomes more broad and presents additional shoulders. Due to the similarity in the position, shape and amplitude, both collagens appeared not denatured after the extraction process. This was reconfirmed by the ratio between amide III and the peak observed at 1440 cm^{-1} for ASC and 1449 cm^{-1} for PSC. In fact, a ratio of approximately 1 between these two peaks can be considered as an index of the triple-helical structure of collagen [50]. The position of amide II band for both the collagens was found at lower wave numbers if compare with the characteristic position of this peak in collagen [51]. This band represent N–H bending vibrations and a shift to lower wave numbers can be explained with a decrease in molecular order in the extracted collagens. Amide A band is typically assigned to N-H stretching mode and can prove that the NH groups in both extracted collagens were involved in hydrogen bonding, probably with a carbonyl group of the peptide chain [52]. In fact, N-H stretching vibration usually is observed in the range 3400–3440 cm^{-1}; however, amide A peak can shift to

lower frequencies when the NH group of a peptide is involved in a hydrogen bond [49]. Finally, the amide B band of both ASC and PSC were found at 2960 cm^{-1} and is associated at the asymmetrical stretch of CH_2 [49].
The similarity between the IR-spectra of ASC and PSC suggested that pepsin hydrolysis had no apparent effects on the triple-helical structure of PSC. However, it is well known that pepsin extraction can cause some differences between ASC and PSC especially at the telopeptide region or in the secondary structural components (e.g., α-helix, β-sheet) of these collagens [53].

3.5. Thermal denaturation temperature of collagen from squid mantle

The thermal stability of extracted collagen samples was investigated with DSC and viscosity measurements. Dynamic viscoelasticity measurement was performed using a cone/plate system (40 mm diameter). The temperature was controlled by a circulating water bath and the collagen samples were heated from 16 to 30°C. The measurements were performed over a shear rate range of 12.5 – 54.5 s^{-1}. In Fig.3(a) we reported the change in viscosity of ASC and PSC solutions in 0.5 M acetic acid with temperature. During the helix-coil transition the intact trimers (γ) in the collagen solutions turn into individual chains (α) or dimers (β) causing the decrease of viscosity with the increase of temperature. The temperature at which the change in viscosity was half the maximum value was taken as the denaturation temperature of analyzed collagens solutions. In particular, the denaturation temperature (T_d) of ASC and PSC collagen from squid mantle was calculated to be about 22°C and 21°C, respectively.
The collagen denaturation process occurs with appreciable heat absorption. For this reason, differential scanning calorimetry (DSC) was also used to define collagen T_d, by measuring the endothermic heat flow. The thermograms of PSC and ASC are shown in Fig.3(b). The thermal denaturation process of extracted collagens was studied through the enthalpy change (ΔH) estimated from the denaturation endothermic peak of collagen and the minimum endothermic temperature (T_d of ASC and PSC were 21°C and 24°C, respectively and ΔH about 0.9 J/g for the ASC and 0.8 J/g for PSC). Thermal denaturation temperature of extracted collagen in solution is an important parameter to understand the thermal stability of the material and to design the processing conditions. Indeed, thermal denaturation causes the irreversible transformation of the native triple helical structure into a more random (coiled) structure, thus changing several physical properties of the material, such as viscosity, elasticity, sedimentation, diffusion light scattering and optical activity [55]. Both the collagen extracted from squid mantle had a T_d value lower than mammalian-derived collagen; for example, collagen extracted from calf skin has a T_d of 37°C [56]. Denaturation temperature of collagen can be correlated with the different amino acids composition, but also at the environmental and body temperatures of fish species [57]. It was reported that the total amount of proline and hydroxyproline have a strong influence on thermal stability of collagen and in particular, a higher content of proline and hydroxyproline is associated at a higher thermal stability [58]. In addition, it was found that the difference content of proline and hydroxyproline amongst collagens isolated from different animals is correlated with the difference in the living environments of their sources, particularly habitat temperature [7]. For example, *Loligo vulgaris* typically lives in temperate seas and the T_d of collagen extracted from the squid mantle was lower than the T_d of tropical fish species, such as Brownstripe red snapper (30.5°C) [6] and bigeye Snapper (28.7°C) [59]. On the other side, the T_d of the squid collagens were higher than those of collagen extracted from cold-water fishes, such as Argentine hake (10°C), Baltic cod (15°C) [60] and Alaska pollack (17°C) [34]. These results confirm that some properties of isolated collagens, between others the T_d, are strongly related to the nature and living conditions of the organism used for the extraction and can be considered species-dependent. In addition, it is interesting to note that even if ASC and PSC collagens do not exist as separate forms inside the natural tissue, they presented different characteristics in relation to their specific amino acid compositions and as a consequence of to the extraction method. For these reasons, a comparative characterization of

ASC and PSC is necessary to evaluate how the different compositions can affect the physicochemical properties of the isolated proteins.

The heat transformation of collagen to gelatin is interpreted as a disintegration of the collagen triple helical structure into random coils. The overall endothermic helix to coil transition process of solubilized collagen has been monitored also by DSC. In the DSC thermogram of PSC a secondary endothermic peak was observed at T' = 15°C. A similar behavior is generally observed in the calorimetric curves of bovine hide collagen [61]. The minor peak was related to the breaking of hydrogen bonds among collagen molecules [25]. The presence of this minor peak can suggest that in the PSC the interactions between the collagen molecules are stronger than those of ASC. On the other side, the main endothermic peak in ASC curve was sharper compare with that of PSC, since ASC is composed of more complex components. The PSC collagen showed a denaturation temperature slightly lower than the ASC. Probably it is due to the action of pepsin, used during the extraction process. In fact, pepsin cleaves the cross-linked collagen molecules at the telepeptide region and this fact can facilitate the thermal denaturation process of PSC. However, DSC and viscosity measurements showed the helix-coil transition for both the extracted collagens, indicating that pepsin digestion did not affect the triple-helical structure of the protein. Previous studies have demonstrated that the use of solutions containing traces of acetic acid during the extraction process has the effect to shift the denaturation temperature of collagen to a lower temperature; most marine collagen in solutions have a T_d under 30°C [62]. These results suggested that when collagen is solubilized in a solution containing acetic acid, some of the intra-molecular hydrogen bonds that stabilize the triple helical structure can be disrupted for the repulsion between the collagen molecules dispersed in the acidic solution [14]. Therefore, a possible strategy to increase the thermal stability in order to extend the application area of collagen after the extraction process is to increase the degree of cross-linking inside the material [63] [64]. After processing marine collagen could be partially denaturate, thus resulting in reduced chemical and mechanical stability. However, cross-linked fish collagen matrices have shown adequate mechanical and thermal stability for several research and clinical applications. Different ways to introduce exogenous cross-linking into the molecular structure of reconstituted collagen have been previously reported, most notably the use of aldehydes with formaldehyde and glutaraldehyde [65][66]. In addition, it has been demonstrated that denatured collagen matrices can present advantages for some biomedical applications. For example, such structures showed better tissue regeneration potential in comparison with native collagen matrices [67].

3.6. Solubility of collagen from the from squid mantle

The solubility behaviour of ASC abd PSC with changes in pH and NaCl concentrations play a crucial role in their further processability and applications. Fig.4(a) reported the solubility of ASC and PSC in a 0.5 M acetic acid solution with different NaCl concentrations. Both collagen samples showed similar solubility behavior. The solubility of ASC and PSC remained at a high level (more than 90%) in the presence of NaCl at concentrations lower than 0.4 M, and decreased sharply when NaCl concentration was between 0.4 and 0.6 M NaCl. At higher NaCl concentration, for both collagen fractions, the solubility remained relatively stable but very low (around 10%). It is well known that the decrease of collagen solubility at high NaCl concentration is due to the so-called “salting out” effect. [68] With the increase of NaCl concentration, the ionic strength of the solution increases, this enhancing hydrophobic–hydrophobic interactions between protein chains. As a consequence, competition of salt ionic in water, results in protein precipitation. [1]

The effect of the pH of the solution on collagen solubility is shown in Fig.4(b). Also in this case, both ASC and PSC were fully solubilized in 0.5 M acetic acid, and then the pH of the solution was adjusted to obtain a final pH ranging from 1 to 10. Both, the ASC and PSC had a high solubility in acid conditions and they reached the greatest solubility at pH between 2 and 3. A sharply decrease of the solubility was registered for both the collagens between pH 3 and pH 4 and between pH 4 and pH 5. At pH values higher than 5, the collagen solubility became low (under 20%) but relatively stable. ASC and PSC showed the lowest solubility at pH between 5 and 6. It is well known that a protein dissolved in buffer at its pI has no net charge, and thus, hydrophobic–hydrophobic

interaction increases, and protein precipitation and aggregation are induced. Therefore, these results suggest that the extracted collagens had isoelectric points in the interval of pH between 5 to 6. This value was slightly lower than the values of pI previously reported for collagen, ranging from pH 6 to 9 [69]. The lower pI of ASC and PSC was probably related to a higher density of carboxyl groups inside the isolated collagens caused by the hydrolysis of some of the side amide groups during the extraction process.
Finally, we can observed that PSC showed a higher solubility than ASC for different NaCl concentrations and at pH higher than 5. This result confirmed that the content of the high MW cross-link of ASC was higher than in PSC and that ASC is composed of more complex components. The electrophoretic profiles showed that PSC presented a number of bands corresponding to the fractions with MW lower than those of the monomeric collagen chains. It could be due at the action of pepsin that might induce the partial hydrolysis of high MW cross-linked molecules, resulting in a greater solubility of PSC than ASC.

3.7. In vitro cytotoxicity evaluation of extracted collagens: LDH analysis

Cells were cultivated with culture medium conditioned with ASC and PSC powders. As shown in Fig.5, there was no significant difference in LDH release among the experimental groups at 72 hours. The results of LDH assay showed that the squid-derived collagens have no cytotoxic effect after the extraction process.

4. Conclusion

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) from squid mantle (*Loligo Vulgaris*) were successfully isolated and characterized. The results showed that the use of pepsin during the extraction process increase the yield of isolated collagen (by 4.7-fold) without damaging the integrity of the triple helix. DSC and viscosity measurements, showed the helix-coil transition for both the extracted collagens, indicating that pepsin digestion did not affect the conformation of the proteins. The SDS-PAGE patterns of ASC and PSC presented a protein profile characteristic of type I and V collagens, consisting of two α-chains (α_1 and α_2), β- and γ-components. GPC analysis showed that the extracted collagens are composed of different families of peptides which different molecular weight distributions. In particular ASC was composed by a higher content of high-molecular weight cross-links than PSC. These results were supported by the thermal analysis and the solubility behaviors that reported a lower solubility of ASC compare with PSC. Both the collagens had high solubility at acidic pH and lost solubility when the NaCl concentration increased above 0.4 M. The denaturation temperature of extracted collagens, in a 0.5 M acetic acid solution, was found to be lower than those of mammalian collagens since it is strongly influenced by the content of proline and hydroxyproline, environmental and body temperatures conditions. Finally, the *in vitro* cytotoxicity tests proved that after the extraction process the material did not release cytotoxic substances. The results of this study demonstrated that squid mantle has a potential as an alternative source of collagen and could be considered for a further use in food, pharmaceutical or biomedical applications.

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Evaluation of alternative sources of collagen fractions from *Loligo vulgaris* squid mantle

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TABLE 1

Amino acid residues	Samples	
	ASC	**PSC**
Hydrophobic amino acids		
Glycine (Gly)	256	262
Alanine (Ala)	106	98
Proline (Pro)	91	86
Valine (Val)	7	7
Isoleucine (Ile)	50	52
Leucine (Leu)	50	60
Phenylanine (Phe)	24	14
Methionine (Met)	7	6
Total content	**591**	**585**
Charged amino acids		
Aspartic Acid (Asp)	38	41
Glutamic acid (Glu)	95	125
Arginine (Arg)	69	73
Lysine (Lys)	37	27
Total content	**239**	**266**
Polar amino acids		
Serine (Ser)	39	36
Histidine (His)	16	7
Threonine (Thr)	46	33
Cysteine (Cys)	8	0
Tyrosine (Tyr)	0	0
Total content	**109**	**76**
Hydroxyproline	**61**	**73**
Total residues	**1,000**	**1,000**

TABLE 1

Table 1. Amino acid composition of ASC and PSC extracted from the mantle of squid *Loligo vulgaris* (number of residues per 1,000 residues).

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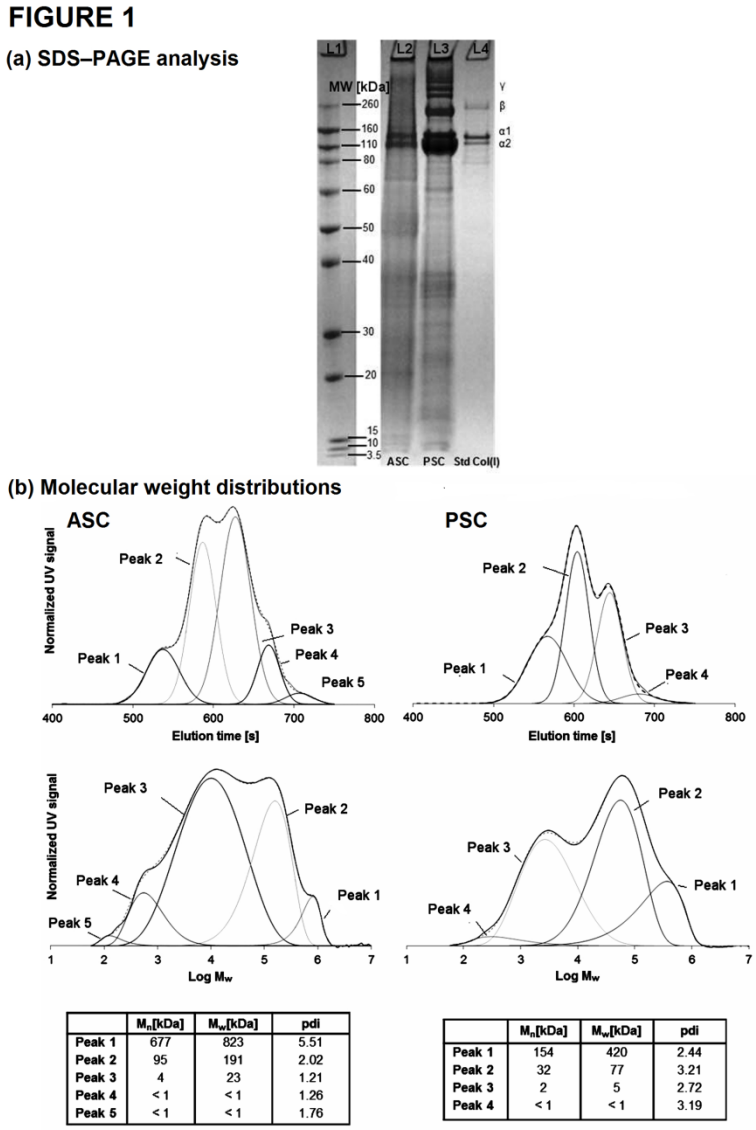
FIGURE 1



	M_n[kDa]	M_w[kDa]	pdi
Peak 1	677	823	5.51
Peak 2	95	191	2.02
Peak 3	4	23	1.21
Peak 4	< 1	< 1	1.26
Peak 5	< 1	< 1	1.76

	M_n[kDa]	M_w[kDa]	pdi
Peak 1	154	420	2.44
Peak 2	32	77	3.21
Peak 3	2	5	2.72
Peak 4	< 1	< 1	3.19

Evaluation of alternative sources of collagen fractions from *Loligo vulgaris* squid mantle

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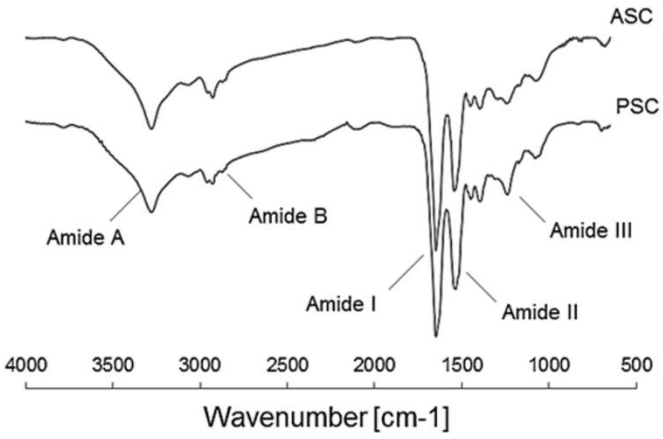
FIGURE 2

FIGURE 2



Properties	Peak wavenumber (cm^{-1})		Assignment	Reference
	ASC	PSC		
Amide A	3380	3378	NH stretch and hydrogen bond	*Payne & Veis (1988)*
Amide B	2960	2960	CH_2 asymmetrical stretch	*Abe & Krimm (1972)*
Peak	2873	2929	CH_3 asymmetric stretch mainly protein	*Abe & Krimm (1972)*
Amide I	1643	1648	C=O stretch/hydrogen bond and COO-	*Abe & Krimm (1972)*
Amide II	1547	1530	NH bend and CN stretch	*Payne & Veis (1988)*
Peak	1440	1449	CH_2 bending vibration	*Jackson et al. (1995)*
Peak	1408	1407	COO- symmetrical stretch	*Jackson et al. (1995)*
Peak	1394	1394	CH_2 wagging of proline	*Jackson et al. (1995)*
Amide III	1236	1240	NH bend and CN stretch	*Payne & Veis (1988)*
Peak	1172	1080	COO-C asymmetric stretch	*Jackson et al. (1995)*
Peak	1066	850	C-O stretch/C-O band	*Jackson et al. (1995)*
Peak	600	600	Skeletal stretch	*Abe et al. (1972)*

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FIGURE 3

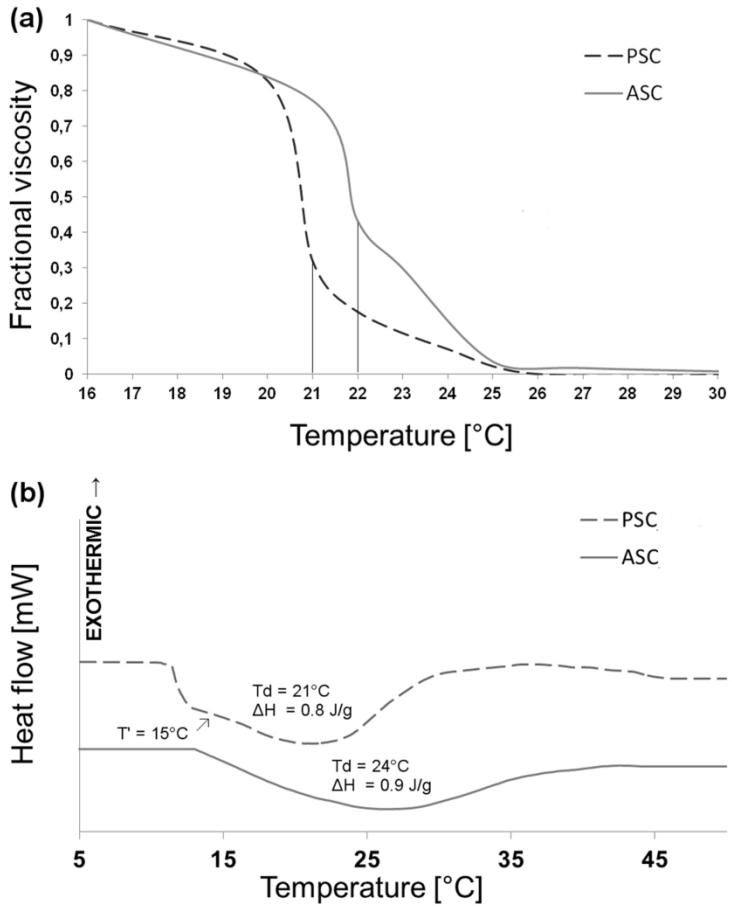

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FIGURE 4

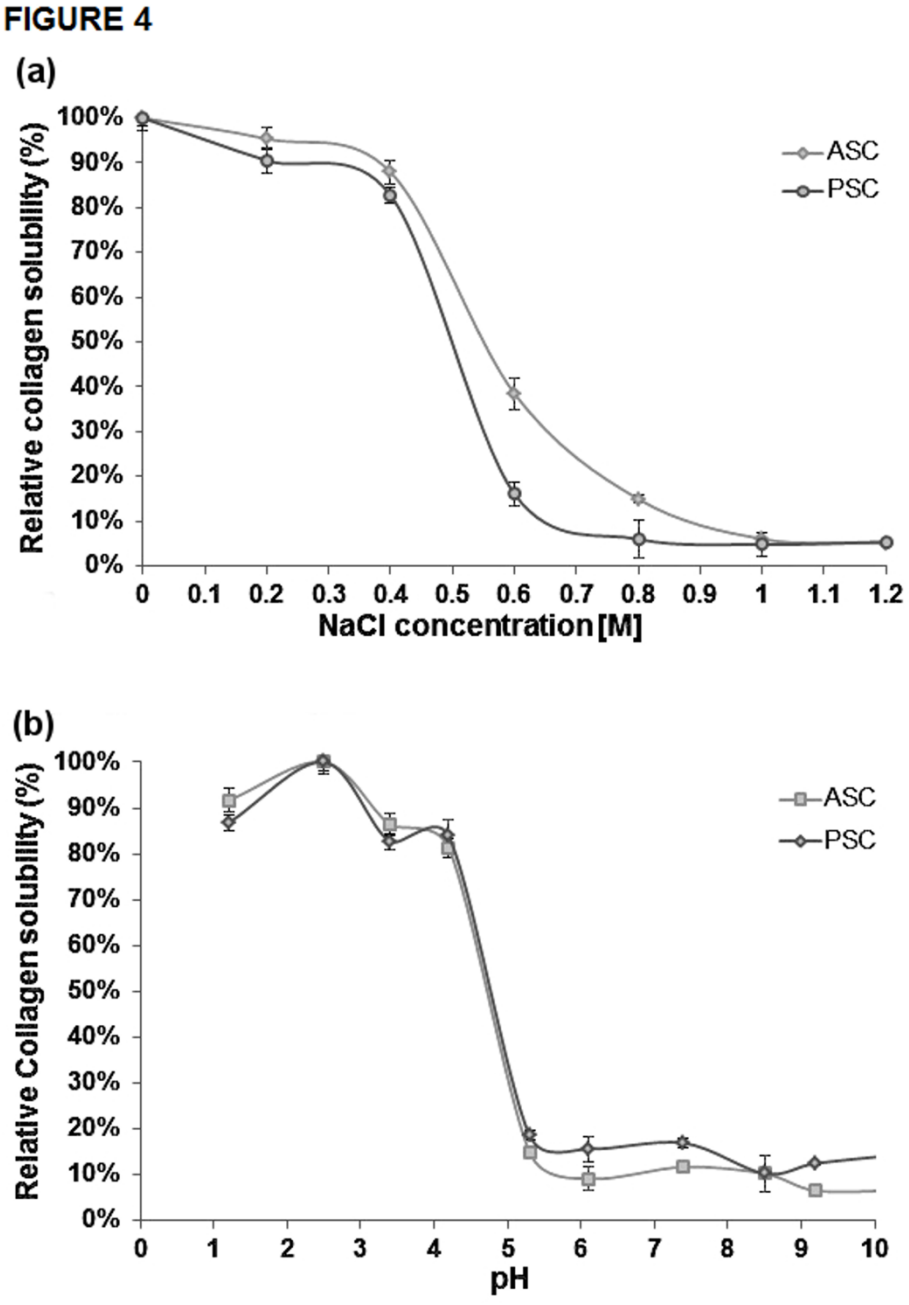

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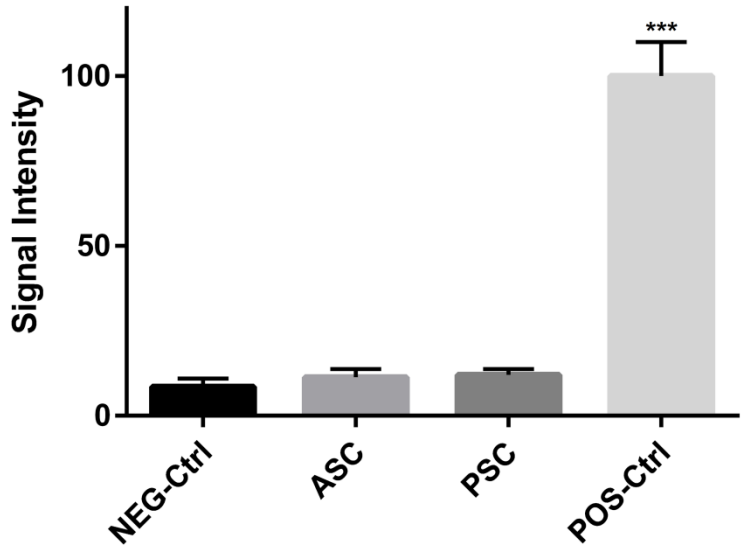
FIGURE 5

FIGURE 5



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Captions to illustrations:

FIGURE 1
Fig.1(a). SDS–PAGE pattern of collagens from the squid mantle (Loligo vulgaris). Lane 1(L1): high molecular weight marker; lane 2 (L2): ASC collagen from squid mantle; lane 3 (L3): PSC collagen from squid mantle; lane 4 (L4): commercial bovine collagen type I.
Fig.1(b). Deconvoluted GPC curves of ASC and PSC from the squid mantle (Loligo vulgaris): elution profiles and differential molecular weight distributions. The curves represent the envelope smoothing function calculated from the experimental data; the picks are the Gaussian best-fit curves denoting the deconvolved populations. For each peak, the number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity index (pdi) are reported in tables.

TABLE 1
Table 1. Amino acid composition of ASC and PSC extracted from the mantle of squid *Loligo vulgaris* (number of residues per 1,000 residues).

FIGURE 2
Fig.2. Fourier transforms infrared spectra of ASC and PSC from the squid mantle.

FIGURE 3
Fig.3. Thermal denaturation temperature of collagens (ASC, PSC) from squid mantle. (a) Thermal denaturation curves of ASC and PSC as shown by changes in fractional viscosity; (b) Thermograms of PSC and ASC dispersed in 0.5 M acetic acid.

FIGURE 4
Fig.4. Solubility of collagen solution from squid mantle in 0.5 M acetic acid. (a) Solubility with different NaCl concentrations; (b) Solubility at different pH values.

FIGURE 5
Fig.5. In vitro cytotoxicity evaluation of extracted collagens using the lactate dehydrogenase assay (LDH). NEG-Ctrl: standard culture medium; ASC: medium containing ASC collagen extracts; PSC: medium containing PSC collagen extracts; POS-Ctrl: medium containing Triton X-100. *** $p<0.001$, $n=4$.