

Full Length Research Paper

Comparison of the properties of collagen extracted from dried jellyfish and dried squid

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With increased recent interest in the utilization of industrial by-products, finding different sources, optimizing extracting conditions and characterization of collagen extracts have recently become important research topics. This study addresses the isolation of acid-soluble and pepsin-soluble forms of collagen from dried jellyfish and squid, and their partial characterization. The properties of these proteins have been studied and a comparison made of the protein patterns of collagen extracted from marine organisms with those from other organisms, to determine which collagen subtypes are present, and in what proportions. Pepsin-soluble collagen (PSC) from dried jellyfish and dried squid contained a collagen form classified as type I, of molecular composition comparable with that of collagen type I from rat tail. Peptide maps of collagens digested by achromopeptidase were slightly different, indicating some differences in amino acid sequence or conformation. The collagen showed high solubility at acidic pH (4-5) but its solubility markedly decreased in the presence of sodium chloride (NaCl) up to 2%. Collagen type I from dried jellyfish and dried squid could be a useful alternative to mammalian collagen, with potential use in the biomedical, pharmaceutical and nutraceuticals industries.

Key words: Collagen, pepsin-soluble form, acid-soluble form, partial characterization.

INTRODUCTION

Collagen is a major class of structural proteins in bone, skin, cartilage and connective tissue (Liu et al., 2007; Ogawa et al., 2003; Bateman et al., 1996). It plays an important role in tissue development and is the most abundant protein in vertebrates, constituting about 30% of the total. Collagen finds a wide range of applications in the food, cosmetic, biomedical, pharmaceutical, leather

and film industries. The collagens from different tissues vary considerably in polypeptide chain composition, amino acid composition and physiochemical characteristics, thereby meeting the specific functional requirements of the tissues. Collagen is unique in its ability to form insoluble fibres ('fibrils') of high tensile strength (Gelse et al., 2003). Some 28 distinct vertebrate

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collagen types have been identified (Gordon and Hahn, 2010; Birk and Bruckner, 2005), although not all of these form fibrils. The structure of mature collagen, known as tropocollagen, consists of three polypeptide chains, called α -chains, intertwined in the so-called collagen triple-helix, each chain being coiled in a left-handed helix, and wound around two others to form a right-handed triple super-helix. After secretion from cells, collagen fibrils are further polymerized through the subsequent formation of covalent cross-links. The major collagenous component of vertebrate bones and tendons is type I, of cartilage type II, of skin type III and of basement membranes type IV; in each case several minor collagen types are also present (Kimura et al., 1976; Mayne and Zettergen, 1980). With the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD), there has been an increased demand for non-mammalian collagen for food markets, as well as for a wide range of other applications. As a consequence, alternative sources of collagen, especially from aquatic animals (Nagai and Suzuki, 2000; Sathivel et al., 2003), including freshwater and marine fish, have received increasing attention.

Many reports focus on the isolation and determination of the biochemical and physicochemical properties of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) extracted from sea organisms such as squid (Mario et al., 2010; Ilona et al., 1999; Mingyan et al., 2009; Thanonkaew et al., 2006; Nagai et al., 2001), bigeye snapper fish (Nalinanon et al., 2007), jellyfish (Sourour et al., 2011; Nagai et al., 2000; Miura and Kimura, 1985; Zhuang et al., 2009; Krishnan and Perumal, 2013; Thanonkaew et al., 2006; Addad et al., 2011) and others (Zelechowska et al., 2010; Byun and Kim, 2001; Je et al., 2007). Squid and jellyfish have been used as food for thousands of years, and represent a renewable marine resource with a high nutritional value. If a suitable extraction procedure can be developed, squid could potentially become a significant source of collagen. From the medical point of view, the analysis of collagens is of great importance; the use of collagen in biochemical applications has also grown rapidly and expanded into biomaterials with biocompatibility and biodegradability. From the analytical point of view, there are several methods for determining collagen and collagen types, the most common being based on the quantitation of hydroxyproline, which accounts for approximately 10% of the collagen molecule. The amino acid composition can also be analyzed by High Performance Liquid Chromatography (HPLC). Determination of the molar ratios of particular collagen types involves separation of the peptide mixture produced by enzymatic digestion, using various separation methods, such as Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) or HPLC, and their detection by Mass Spectrometry (MS) or

Liquid chromatography/electrospray ionization-mass spectrometers (LC/ESI-MS) (Guifeng et al., 2006) and High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) (Pataridis et al., 2008) which enable the analysis of marker peptides in peptide mixtures produced by cyanogen bromide/trypsin digestion. Another analytical method involves the radioactive labeling of proline and enzyme immunoassay by collagen-specific antibodies (Van der et al., 1994).

The aim of the present study is to compare the acid-soluble and pepsin-soluble isolated forms of collagen from dried jellyfish and dried squid, and their properties. It should be possible to determine which collagen subtypes are present in collagen extracted from dried jellyfish and dried squid, and the functional properties of these proteins will be studied and a comparison made of collagen extracted from marine organisms with those from other organisms, as marine collagens could be used as alternative sources of high quality collagen in applications within the pharmaceutical and nutrition industries.

MATERIALS AND METHODS

Experimental samples

Dried squid and dried jellyfish bought from a local market, Bangsae, Chonburi, Thailand were used in this study.

Preparation of collagen

Extraction of acid-soluble collagen (ASC)

Duplicate samples were washed in distilled water at 4°C with continuous stirring for 3 days, and the flesh cut into small pieces and soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) at 4°C for a further 3 days. The mixture was filtered through two layers of cheesecloth and the residue re-extracted under the same conditions. The two filtrates were combined. Collagen was precipitated by adding 0.9M NaCl with 0.05M Tris (hydroxymethyl) aminomethane buffer, pH 7.0. The resultant precipitate was collected by centrifugation at 20,000xg for 60 min, then dissolved in a minimal volume of 0.5 M acetic acid and dialyzed against 50 volumes of 0.1 M acetic acid for 3 days, followed by the dialysis against the same volume of distilled water for another 3 days. The dialysate was freeze-dried and is referred to as acid-soluble collagen (ASC). The yield of ASC was calculated from the dry weight of collagen extraction in comparison with the wet weight of the initial fresh squid used.

Extraction of pepsin-soluble collagen (PSC)

The undissolved residue obtained after ASC extraction was used for further extraction with the gastric proteinase pepsin. The residue was soaked in 0.5M acetic acid with a solid/solvent ratio of 1:15 (w/v) and pepsin (20 U/g residues) added. The mixture was continuously stirred at 4°C for 3 days, followed by filtration through two layers of cheesecloth. The filtrate was subjected to precipitation and the pellet dialyzed, as previously described for ASC. The dialysate was freeze-dried and is referred to as pepsin-soluble collagen (PSC). The yield of PSC was calculated in the same

manner as ASC. Additionally, the accumulated yield of collagen was calculated from the total yield of ASC and PSC.

Protein quantitation and electrophoresis

Quantitation of total protein

The protein contents of the clear protein samples were determined using the Coomassie Plus (Bradford) protein assay kit (Thermo scientific, USA), with bovine serum albumin as a standard (Bradford, 1976).

Molecular weight determination

All samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), 5 μ g of each protein sample being separated according to the method of Laemmli (1970). The separation was performed with a 10% separating gel and a 5% stacking gel using the miniVE Vertical electrophoresis system (GE Healthcare, U.S.A). The relative molecular mass (M_r) of each protein was determined using a standard curve generated from standard set of 12 pre-stained proteins in the range 3.5-260 kDa (Novex Sharp Protein Standard, Invitrogen, USA).

The gels were visualized by staining with Colloidal Coomassie Brilliant Blue G-250 (CBB) as described by Neuhoff et al. 1988. After gel electrophoresis, the gel was transferred into a fixative solution (50% ethanol, 2% Phosphoric acid in ddH₂O) for 1 h at room temp. The gel was stained with CBB (0.2% w/v) for 12-24 h by agitation on a shaker overnight. The staining solution was then removed. The gel was destained with several changes of ddH₂O.

Peptide mapping of collagen

Peptide mapping of ASC and PSC was performed according to the method of Kittiphattanabawon et al. (2005) with slight modification. A collagen sample (6 mg) was dissolved in 1 ml of 0.1 M sodium phosphate, pH 7.2 containing 0.5% (w/v) SDS, the mixture preheated at 45°C for 3 h and 300 μ l of the preparation transferred to test tubes for digestion. To initiate the digestion, 20 μ l of lysyl endopeptidase from *Achromobacter lyticus*, (5.0 μ g/ml) was added to the mixture, which was then incubated at 37°C for 5 min. Proteolysis was stopped by boiling for 3 min. SDS-PAGE was performed by the method of Laemmli (1970) in 10% acrylamide NuPAGE Bis-Tris Mini Gels with MOPS buffer, followed by staining and destaining as previously described.

Collagen solubility

Collagen was dissolved in 0.5 M acetic acid to a final concentration of 3 mg/ml and the mixture stirred at 4°C until completely solubilized (Kittiphattanabawon et al., 2005).

Effect of pH on solubility

Four ml collagen solution (3 mg/ml) was transferred to 15 ml centrifuge tubes and either 6 N NaOH or 6 N HCl added to obtain the final pH, ranging from 1 to 10. The volume of solution was made up to 10 ml with deionized water previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20,000 g for 30 min and the protein content of the supernatant determined. Protein solubility was calculated using the following equation.

Solubility= (protein content of the supernatant)/(total protein content

of the sample)

Relative solubility= (solubility at given pH)/(highest solubility in the range of pH)

Effect of NaCl on solubility

Four ml collagen solution (3 mg/ml) was mixed with 1 ml of NaCl in 0.5 M acetic acid to give final concentrations of 0 to 12%. The mixture was stirred for 30 min, followed by centrifugation at 20,000 g for 30 min. Protein content in the supernatant was measured and the relative solubility calculated as previously described.

Statistical analysis

All experiments are replicated three times, and the results are presented as means \pm standard deviation (SD). Analyses of variance (ANOVA) were performed and mean comparison was done by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Electrophoretic characterization of collagen is shown in Figure 1. The electrophoretic patterns of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were examined by SDS-PAGE using 10% acrylamide Bis-tris gels. In the case of collagen extracted from jellyfish, the ASC fraction contained no high-molecular weight bands, although there was a band of molecular weight about 21 kDa, which was also found in the PSC fraction and bands of α_1 and α_2 chains were visible near 116 kDa in the PSC fraction. Bands below 116 kDa represent the products of enzymatic hydrolysis of collagen. In the case of squid, the PSC showed the typical SDS-PAGE pattern of type I collagen with two different α bands, α_1 and α_2 . It also contains β and γ chains as well as other higher molecular weight cross-linked components, together with some higher molecular weight protein bands. In ACS extracts α_1 and α_2 chains were found. The band intensity of α_2 was approximately 2-fold higher than that of α_1 , suggesting a [α_2]₂ α_1 in collagen triple helix. We suggest that the collagen in both marine organisms are different type I collagens.

Peptide mapping

The peptide maps of both forms of extracted collagen from dried jellyfish and squid, after digestion by Achromopeptidase (37°C for 5 min), are shown in Figure 2, with collagen type I from rat tail for comparison. The band intensities of molecular weight cross-linked components β and γ component, of rat tail collagen (control collagen) slightly decreased with the appearance of 116 kDa peptide fragment. The results suggested that in both ASC and PSC, the α_1 , α_2 , β and γ chains were more resistant to digestion by Achromopeptidase than rat-tail collagen. It appears that more stringent conditions or different proteases will be required to degrade these

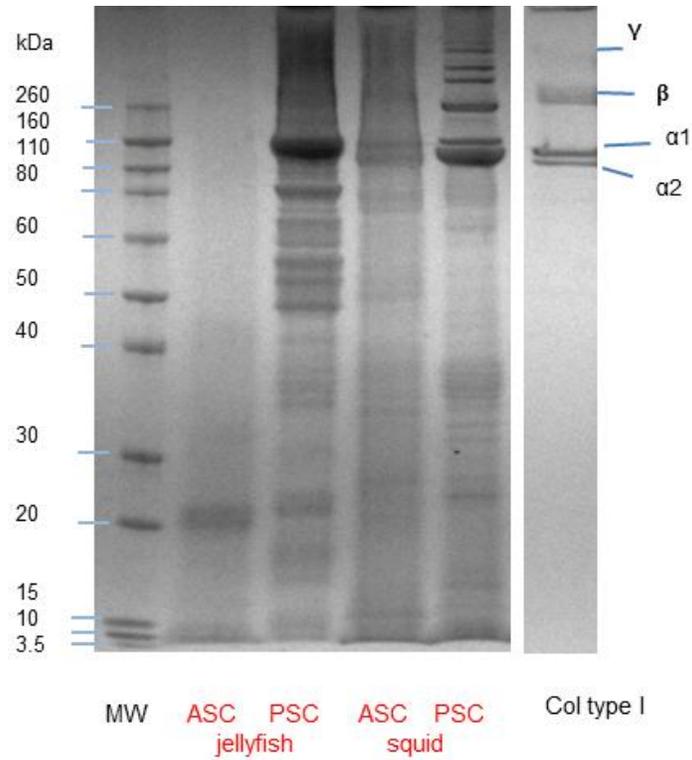


Figure 1. SDS-PAGE profiles of ASC and PSC extracted from jellyfish and squid.

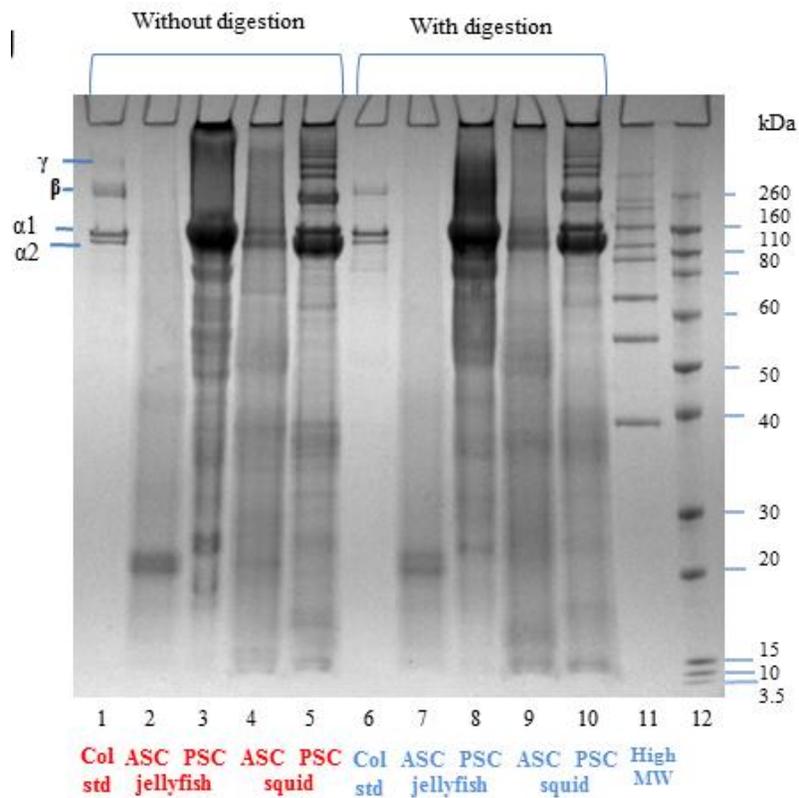


Figure 2. Comparison of jellyfish and squid collagen with/out digested.

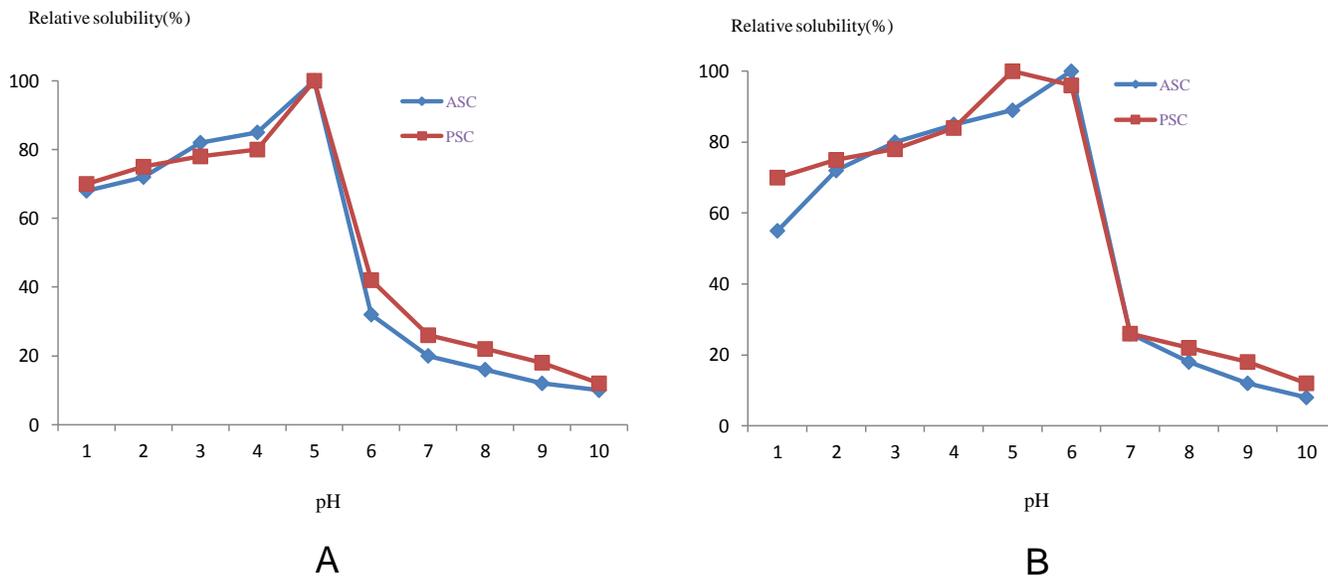


Figure 3. Relative solubility (%) of ASC and PSC extracted from jellyfish (A) and (B) at different pH values.

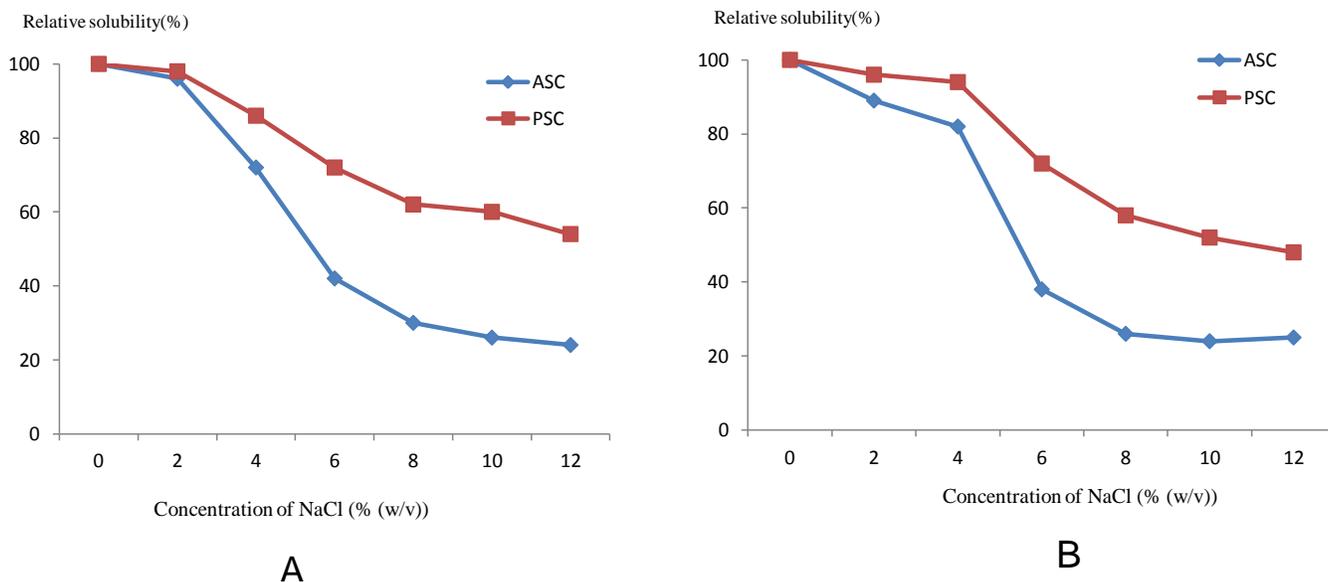


Figure 4. Relative solubility (%) in 0.5M acetic acid of ASC and PSC extracted from the Jellyfish (A) and squid (B) in the presence of NaCl at different concentration.

collagens. Thus, it was presumed that primary structures of collagens from the organism were quite similar in term of amino acid composition.

Solubility of collagens

The effects of pH and NaCl concentrations on collagen solubility are shown in Figures 3 and 4. The highest

solubility of ASC and PSC from jellyfish and PSC from squid was at pH 5, whereas ASC from squid had a higher solubility at pH 6. A sharp decrease in solubility was observed at neutral pH. However, solubility was also slightly decreased at very acidic pH values. The solubility in 0.5 M acetic acid of both collagen fractions from jellyfish was maintained in the presence of NaCl up to 2%, whereas ASC and PSC fraction of squid maintained its solubility up to 4%. A marked decrease in solubility

was observed with increasing NaCl concentration, the well-known salting out effect. From these data, collagen from squid was more tolerant to salt than collagen from jellyfish.

In conclusion, PSC isolated from dried jellyfish and dried squid contained a collagen form classified as type I, of molecular composition comparable with collagen type I from rat tail. Squid collagen is a heteropolymer of α_1 , α_2 , β and γ chains, but, jellyfish PSC constitute mainly α_1 and α_2 chains. Peptide mapping of these collagens by digestion with Achromopeptidase was attempted but was largely unsuccessful, indicating some differences in amino acid sequence or conformation from rat-tail collagen. The marine collagens showed high solubility at acidic pH (4 to 5) and the solubility markedly decreased in the presence of NaCl up to 2%. Type I collagen has also been extracted from skin, bone, fins, and scales of fresh water and marine fishes, chicken skin and different marine animals such as squid, octopus, jellyfish, starfish and fish (Swatschek et al., 2002; Sadowska et al., 2003; Nagai et al., 2004; Falguni et al., 2010). Collagens from these sources were evaluated for their potential applications as alternatives to mammalian collagen. However, the properties of collagen vary markedly with the type of marine organism, indicating a need for characterization of this protein from different sources (Nagai and Suzuki, 2002; Kittiphattanabawon et al., 2005). In future work, collagen from jellyfish and squid will be separated by 2DE following development of the extraction protocols. Then the protein spots of interest will be excised from the polyacrylamide gels, digested with trypsin and analyzed by Matrix-assisted-laser-desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). In the final step, the acquired peak lists will be analyzed by searching NCBI database with Mascot software.

Conflict of Interests

The authors have not declared any conflict of interests.

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