FUNCTIONAL AND BIOACTIVE PROPERTIES OF COLLAGEN AND GELATIN FROM ALTERNATIVE SOURCES: A REVIEW

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Abstract

The rising interest in the valorisation of industrial by-products is one of the main reasons why exploring different species and optimizing the extracting conditions of collagen and gelatin has attracted the attention of researchers in the last decade. The most abundant sources of gelatin are pig skin, bovine hide and, pork and cattle bones, however, the industrial use of collagen or gelatin obtained from non-mammalian species is growing in importance. The classical food, photographic, cosmetic and pharmaceutical application of gelatin is based mainly on its gel-forming properties. Recently, and especially in the food industry, an increasing number of new applications have been found for gelatin in products such as emulsifiers, foaming agents, colloid stabilizers, biodegradable film forming materials and micro-encapsulating agents, in line with the growing trend to replace synthetic agents with more natural ones. In the last decade, a large number of studies have dealt with the enzymatic hydrolysis of collagen or gelatin for the production of bioactive peptides. Besides exploring diverse types of bioactivities, of an antimicrobial, antioxidant or antihypertensive nature, studies have also focused on the effect of oral intake in both animal and human models, revealing the excellent absorption and metabolism of Hyp-containing peptides. The present work is a compilation of recent information on collagen and gelatin extraction from new sources, as well as new processing conditions and potential novel or improved applications, many of which are largely based on induced cross-linking, blending with other biopolymers or enzymatic hydrolysis.

Keywords: collagen, gelatin, bioactive peptides, fish, poultry, functional properties.

1.- Introduction

Gelatin is a soluble protein compound obtained by partial hydrolysis of collagen, the main fibrous protein constituent in bones, cartilages and skins; therefore, the source, age of the animal, and type of collagen, are all intrinsic factors influencing the properties of the gelatins (Johnston-Banks, 1990). Although to date, up to 27 different types of collagen have been identified, type I collagen is the most widely occurring collagen in connective tissue. Interstitial collagen molecules are composed of three α-chains intertwined in the so-called collagen triple helix. This particular structure, mainly stabilized by intra- and inter-chain hydrogen bonding, is the product of an almost continuous repeating of the Gly-X-Y- sequence, where X is mostly proline and Y is mostly hydroxiproline (Asghar & Henrickson, 1982). Only the very short N- and C-terminal regions, called telopeptides (15-26 amino acid residues), do not form triple helical structures as they are largely made up of lysine and hydroxylysine (Hyl) residues, as well as their aldehyde derivatives, in both intra- and inter-molecular covalent cross-links (Bateman, Lamandé & Ramshaw, 1996). Four to eight collagen molecules in cross-section are stabilized and reinforced by covalent bonds to constitute the basic unit of collagen fibrils. Thus, the typical strong, rigid nature of skins, tendons and bones is due to the basic structure formed by many of these cross-linked collagen fibrils.

Insoluble native collagen must be pre-treated before it can be converted into a form suitable for extraction, which is normally done by heating in water at temperatures higher than 45ºC. A chemical pre-treatment will break non-covalent bonds so as to disorganize the protein structure, thus producing adequate swelling and collagen solubilization (Stainsby, 1987). Subsequent heat treatment cleaves the hydrogen and covalent bonds to destabilize the triple helix, resulting in helix-to-coil transition and conversion into soluble gelatin (Djabourov, Lechaire & Gaill, 1993; Gómez-Guillén, Turnay, Fernández-Díaz, Ulmo, Lizarbe & Montero, 2002). The degree of collagen conversion into gelatin is related to the severity of both the pre-treatment and the warm-water extraction process, as a function of pH, temperature, and extraction time (Johnston-Banks, 1990). Two types of gelatin are obtainable, depending on the pre-treatment procedure and are known commercially as type-A gelatin (isoelectric point at pH~8-9) and type-B gelatin (isoelectric point at pH~ 4-5) obtained under acid and alkaline pre-treatment conditions respectively. Industrial applications call for one or the other gelatin type, depending on the degree of collagen cross-linking in the raw material. Because of the acid lability of cross-linking in immature collagens, such as in fish skins, reasonably mild acid treatment is enough to effect collagen solubilisation (Montero, Borderías, Turnay, & Leyzarbe, 1990; Norland 1990).

Gelatin quality for a particular application depends largely on its rheological properties (Stainsby, 1987). Apart from basic physico-chemical properties, such as composition parameters, solubility, transparency, colour, odour and taste, the main attributes that best define the overall commercial quality of gelatin are gel strength and thermal stability (gelling and melting temperatures). For

standardizing purposes, measurement of gel strength is determined using the so-called Bloom test, which consists of performing a well-defined protocol at a given gelatin concentration (6.67%), temperature (10ºC) and maturation time (17h), thus allowing gel strength to be expressed in the normalized "bloom value" (Wainewright, 1977). Both, gel strength and thermostability, are largely dependent on the molecular properties of gelatin, especially with respect to two main factors: (i) the amino acid composition, which is species specific, and (ii) the molecular weight distribution, which results mainly from processing conditions (Gómez-Guillén et al., 2002). The proline and hydroxyproline content is particularly important for the gelling effect. Triple helical structure stability in renatured gelatins has been reported to be proportional to the total content in pyrrolidine imino acids, given that it is the Pro + Hyp rich zones of the molecules that are most likely to be involved in the formation of nucleation zones (Ledward, 1986). However, although Pro is important, Hyp is believed to play a singular role in the stabilization of the triple-stranded collagen helix due to its hydrogen-bonding ability through its -OH group (Burjandze, 1979; Ledward, 1986). Moreover, it has also been observed that the total Gly-Pro-Hyp sequence content is one of the main factors affecting collagen thermostability (Burjandze, 2000). With regard to molecular weight distribution, gelatin consists of a mixture of polypeptides frequently presenting a band pattern distribution typical of type I collagen, with a characteristic α1/α2 chain ratio of around 2, the presence of β-and γ-components (covalently linked α-chain dimers and trimers, respectively), together with higher molecular weight forms as well as low molecular weight protein degradation fragments (Stainsby, 1987). A strong decrease in the prevalence of β- and γ-components and the near-disappearance of higher molecular aggregates, with an increased presence of degradation fragments, are normally the result of the application of more intense extracting conditions (pH, temperature, time), which is normal industrial practice for yield improvement (Johnston-Banks, 1990).

Physical properties of gelatin influence its quality and potential application, since they are related to gelatin structure (Yang and Wang, 2009). Structural properties of gelatin have been studied by different methodologies such as Size Exclusion Chromatography-Multi Angle Laser Light Scattering (SEC-MALLS) (Haug, Draget, & Smidsrød, 2004), viscoelasticity (Jamilah & Harvinder, 2002), confocal fluorescence microscopy (Nordmark & Ziegler, 2000), scanning and transmission electron microscopy (SEM and TEM) (Djabourov et al., 1993; Saxena, Sachin, Bohidar & Verma, 2005), circular dichroism (Gómez-Guillén et al., 2002), electrophoretic analysis (Zhou, Mulvaney & Regenestein, 2006), dielectric analysis (Lefebvre, Hart, Lipari, Long, McSwain & Wells, 2006), differential scanning calorimetry (Badii & Howell, 2006), Fourier transform infrared spectroscopy (FTIR) (Muyonga, Cole & Duodu, 2004b) and FT-Raman spectroscopy (Badii & Howell, 2006). Recently, nanotechnology has received much attention in studying structure at nanoscale level in food science, including gelatins. In this area, atomic force microscopy has been successfully applied to the study of gelatin structure obtained from mammalian sources (Benmouna & Johannsmann, 2004;

Saxena et al., 2005), and more recently from fish skins (Yang, Wang, Regenstein & Rouse, 2007; Wang, Yang & Regenstein, 2008; Yang, Wang, Zhou & Regenstein, 2008; Yang and Wang, 2009).

The most abundant sources of gelatin are pig skin (46 %), bovine hide (29.4 %) and pork and cattle bones (23.1 %). Fish gelatin accounted for less than 1.5 % of total gelatin production in 2007, but this percentage was double that of the market data for 2002, indicating that gelatin production from alternative non-mammalian species had grown in importance (Gómez-Guillén, Pérez-Mateos, Gómez-Estaca, López-Caballero, Giménez & Montero, 2009). Apart from the well-known socio-cultural and sanitary aspects, the rising interest in putting by-products from the fish industry to good use is one of the reasons why exploring different species and optimizing the extraction of fish gelatin has attracted the attention of researchers in the last decade (Gómez-Guillén et al., 2002; Karim & Bhat, 2009). The main drawback of fish gelatins is that gels based on them tend to be less stable and have worse rheological properties than gelatins from land mammals, and this may limit their field of application. Generally speaking, this is true in the case of cold-water fish species, such as cod, salmon, Alaska pollack, etc. Nevertheless, relatively recent studies have pointed out that tropical and sub-tropical warm-water fish species (tilapia, Nile perch, catfish) might have similar rheological properties and thermostability to that of mammal gelatins, depending on the species, type of raw material and processing conditions (Gilsenan & Ross-Murphy, 2000; Jamilah & Harvinder, 2002; Muyonga, Cole & Duodu, 2004b; Karim & Bhat, 2009; Gómez-Guillén et al., 2009; Rawdkuen, Sai-Ut, & Benjakul, 2010).

Scientific literature about different alternative sources and new functionalities of collagen and gelatin has experienced a boom in the last 10-15 years, in part due to the growing interest in the economical valorisation of industrial by-products (from the meat and fish industry), the environmental friendly management of industrial wastes, and the search for innovative processing conditions as well as potential novel applications. Many of these improved functional properties are largely based on chemical- and enzymatic-induced cross-linking, as well as blending with other biopolymers.

The classical food, photographic, cosmetic and pharmaceutical application of gelatin is based mainly on its gel-forming and viscoelastic properties. Recently, and especially in the food industry, an increasing number of new applications have been found for gelatin in products such as emulsifiers, foaming agents, colloid stabilizers, fining agents, biodegradable packaging materials and micro-encapsulating agents, in line with the growing trend to replace synthetic agents with more natural ones. Moreover, in many cases, these studies are dedicated to using collagens and gelatins from alternative sources to land-based animals.

On the other hand, enzyme-hydrolysed collagen plays an increasingly important role in various products and applications. Its different properties and functionalities benefit the end consumer now in ways which were not present ten years ago. Over the past decade, a large number of studies have investigated enzymatic hydrolysis of collagen or gelatin for the production of bioactive peptides. Besides exploring diverse types of bioactivity, studies focused on the effect of oral intake in both animal and human models have revealed the excellent absorption and metabolism of Hyp-containing peptides.

2.- Alternative sources of collagen and gelatin

The most common raw materials for collagen and gelatin extraction are skins or hides, bones, tendons and cartilages. Pigskin was the first raw material used for the manufacture of gelatin in the 1930s and continues to be the most important material for large-scale industrial production. Raw materials from fish and poultry have received considerable attention in recent years, but their still limited production makes them less competitive in price than mammalian gelatins. As far as fish gelatin is concerned, the huge number of species having very different intrinsic characteristics, has aroused the interest of the scientific community in optimising the extracting conditions as well as characterising the yields, and physico-chemical and functional properties of the resulting gelatins, obtained mainly from skin and bone residues. Examples of extracting procedures and gelling properties of gelatins from the skins of different typical cold-water species, such as cod, Atlantic salmon, haddock, Alaska pollack or hake; tropical or subtropical species, such as black or red tilapia, Nile perch, channel catfish, yellowfin tuna, sin croaker, shortfin scad, skate or grass carp; flat species, such as megrim, Dover sole; as well as cephalopods, such as giant squid have been reviewed by Karim & Bhat (2009) and Gómez-Guillén et al. (2009). Although strict comparisons are difficult since methodologies may differ considerably from one work to another, cold-water fish gelatins are characterised as generally having low gelling and melting temperatures (~4-12 ºC and <17 ºC, respectively), in contrast to the data reported for warm-water species (~18-19 ºC and ~24-29 ºC). Similarly, comparable gel strength values reported for cold-water species are frequently ~100g or even lower, whereas gelatins from warm-water species normally register values higher than 200 g. As a point of reference, commercial pork or bovine gelatins often present gel strength values in the range of 200-300 g and melting points higher than 30 ºC. The number of fish or marine species studied for gelatin extraction is continually growing. For example, in the last two years, gelatins with reasonably good gelling properties have been extracted from the skins and bones of bigeye snapper (*Priacanthus tayenus* and *Priacanthus macracanthus*) (Benjakul, Oungbho, Visessanguan, Thiansilakul, & Roytrakul, 2009), bigeye snapper (*Priacanthus hamrur*) (Binsi, Shamasundar, Dileep, Badii, & Howell, 2009), cuttlefish (*Sepia pharaonis*) (Aewsiri, Benjakul, & Visessanguan, 2009), greater lizardfish (*Saurida tumbil*) (Taheri, Abedian Kenari, Gildberg, & Behnam, 2009), grouper (*Serranidae sp*) (Rahman & Al-Mahruoqi, 2009), Hoki

(*Macruronus novaezelandiae*) (Mohtar, Perera, & Quek, 2010) and giant catfish (*Pangasianodon gigas*) (Jongjareonrak, Rawdkuen, Chaijan, Benjakul, Osako & Tanaka, 2010).

Although less versatile than gelatin, fish collagen has received considerable attention for its potential as an ingredient in processed functional food manufacturing, as well as for cosmetic, biomedical and pharmaceutical applications. Thus, extraction and functional characterization of acid- and/or pepsin-soluble collagen has also been reported for different fish species, like, trout and hake (Montero & Borderías, 1991), plaice (Pleuronectes platessa) (Montero, Alvarez, Martí & Borderías, 1995), squid *Illex coindetii* (Ruiz-Capillas, Moral, Morales & Montero, 2002), deep-sea redfish (Wang, An, Xin, Zhao & Hu, 2008), threadfin bream (Nalinanon, Benjakul, Visessanguan & Kishimura, 2008), walleye pollack (Yan et al., 2008), brownstripe red snapper (Jongjareonrak, Benjakul, Visessanguan, Nagai & Tanaka, 2005) or unicorn leatherjacket (Aluterus monoceros) (Ahmad, Benjakul & Nalinanon, 2010).

Scales constitute another important fish industry residue and may account for around 5% of the material contained in fish collagenous waste (Wang & Regenstein, 2009). Utilization of fish scales for collagen or gelatin extraction has been reported for sea bream and red tilapia (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003), black drum and sheepshead (Ogawa, Portier, Moody, Bell, Schexnayder, & Losso, 2004), sardine (Nomura, Sakai, Ishii, & Shirai, 1996; Harada, Kuwata, & Yamamoto, 2007), grass carp (Li, Zhong, Wan, Zhao, Gu, & Xiong, 2008), deep-sea redfish (Wang et al., 2008), Asian silver carp (Wang & Regenstein, 2009) and lizardfish (Wangtueai & Noomhorm, 2009). Unlike skins, scales are rich in Ca phosphate compounds such as hydroxyapatite and Ca carbonate; therefore, pre-treatment removal of Ca from fish scales is critical in order to obtain the final yield, purity, and gel strength of the gelatin (Wang & Regenstein, 2009). These authors found that pre-treatment with 0.2 mol/L EDTA produced decalcification >90%, with a gelatin yield of 22% and gel strength of 152 g, values considerably higher than those obtained with 0.20 mol/L HCl or 1.2 g/L citric acid.

Pre-cooked fin, an important waste product from canned tuna processing, has been proposed as a promising source for high performance gelatin extraction, though with a low yield (~2%) (Aewsiri, Benjakul, Visessanguan &Tanaka, 2008). Tuna fin presented an ash content as high as 40%, consequently an exhaustive demineralisation step was needed. Moreover, during the steam heating pre-cooking treatment, collagen might have undergone some denaturation, in detriment to the yield and the properties of the extracted gelatin, which exhibited inferior gelling, emulsifying, foaming and film-forming properties to those of commercial pigskin gelatin.

The solid waste from surimi processing, which may range from 50–70% of the original raw material (Morrissey, Lin, & Ismond, 2005), could also be the initial material for obtaining gelatin or collagen from under-utilized fish resources. More specifically, refiner discharge from the Pacific whiting surimi process, representing around 4-8% of whole fish, consisted of muscle (95%), skin (2.1%), bone (2.9%) and trace amounts of scale fragments. (Kim & Park, 2005). Crude collagen from refiner discharge, extracted in the form of acid-soluble collagen or recovered in a simpler way as partially purified collagen, was found to present higher functional properties (emulsifying activity, cooking stability, water and oil absorption capacity) compared with the other by-products (skins and frames) generated in the same manufacturing process (Kim & Park, 2004; 2005). In a later study, the acid soluble collagen from Alaska pollack surimi refiner discharge, having a thermal denaturation temperature slightly higher than that for Alaska pollack skin, was also proposed as a potentially functional food ingredient (Park, Lee, Kang, Park & Kim, 2007).

The offal from further processing of semi-processed fish products, such as skins from salted and marinated herring or cold-smoked salmon, has been studied as a source of gelatin (Kołodziejska, Skierka, Sadowska, Kołodziejski, & Niecikowska, 2008). Smoking did not significantly alter collagen susceptibility to thermal denaturation, as the resulting gelatin was considerably less degraded, and with higher gel strength than gelatins from the skins of marinated and salted herrings.

Farm-raised alligator bones (*Alligator mississippiensis*), which represent a highly significant part of the wastage from alligator meat processing industries in the southern states of the USA, China and Thailand, were also investigated in an effort to find additional non-land-based sources of highly thermostable collagen (Wood, Ogawa, Portier, Schexnayder, Shirley, & Losso, 2008). Most of the collagen from alligator bone was identified as type-I collagen, and the imino acid contents of the acid- and pepsin-solubilized collagen were 194 and 196 per 1000 residues, respectively, values which were very close to subtropical fish species (black drum and sheepshead) and slightly lower than that for calf skin.

Giant red sea cucumber (*Parastichopus californicus*) has been considered as a potential source of collagen for nutraceutical and pharmaceutical applications (Liu, Oliveira & Su, 2010). Type I collagen in the skin and connective tissue of giant red sea cucumbers was isolated by pepsin digestion procedures with yields of 20.8% from skin and 24.3% from connective tissue on a dry weight basis. The lower gel-forming ability of collagen from giant red sea cucumber in comparison to that from calf skin was attributed to the low content of imino acid residues in both skin and connective tissue (153 and 142 residues/1000 total, respectively), which were even lower than in walleye pollock or cod.

Poultry by-products are also increasingly processed into collagen-based high-value products. Pepsin or ethylene diamine was used for extracting type I and type III collagens from chicken skins, with a yield (collagen content in the solid phase) of 38.9% for pepsin extraction and 25.1% for extraction with ethylene diamine (Cliche, Amiot, Avezard & Gariépy, 2003). A pepsin-aided process was proposed to extract type II collagen from chicken sternal cartilage, which is another important by-product of the poultry processing industry, in order to eliminate the presence of immunogenic response-inducing telopeptides, while affecting only minimally collagen functional properties (Cao & Xu, 2008).

3.- Methods for species differentiation

Frequently, it may be necessary to differentiate between gelatins from different sources not only for safety and religious reasons, but because it has been reported that most gelatin-allergic patients develop allergic reactions to bovine and porcine gelatin, but do not react to fish gelatin (Sakaguchi et al., 2000). Despite labelling precautions, bovine and porcine gelatins pose a high degree of risk for sensitized patients because they are often present in commercial foods and food ingredients, due to cross-contamination during processing. Some studies have reported on differentiation between bovine and porcine gelatins based on amino acid analysis using principal component analysis (Nemati, Oveisi, Abdollahi & Sabzevari, 2004) or the pH drop method after calcium phosphate precipitation (Hidaka & Liu 2002). Nevertheless, more accurate methodologies have now been developed to trace the species origin of gelatin contained in commercial food products.

The close similarity between collagen amino-acid sequences from different species makes their immunochemical differentiation difficult when using polyclonal antibodies against the whole molecule. Venien & Levieux (2005) reported on the successful production of bovine specific antibodies by immunization of rabbits with synthetic peptides mimicking a short putative species-specific sequence of the bovine alpha 1(I) chain. Using these antibodies, an indirect ELISA was developed to allow a quick and easy differentiation between bovine and porcine gelatins and for the sensitive quantitation of their mixtures. Similarly, two sandwich ELISA methods, based on antibodies from rabbits and goats immunized with bovine gelatin were found to be highly specific for bovine and porcine gelatin, but had little reactivity with fish gelatin (Doi, Watanabe, Shibata & Tanabe, 2009).

Zhang et al. (2009) proposed a new method for species differentiation using high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS), based on detection and identification of specific marker peptides in tryptic-digested bovine and porcine gelatins, and found proline hydroxylation to be a key factor affecting peptide identification.

Recently, a method based on the application of Fourier transform infrared spectroscopy (FTIR) in combination with attenuated total reflectance (ATR), principal component analysis and discriminant

analysis, has been developed to provide simple, rapid qualitative differentiation of bovine and porcine gelatins (Hashim, Man, Norakasha, Shuhaimi, Salmah, & Syahariza, 2010). The IR-regions found to give information about the origin of the gelatin ranged from 3290-3208 cm^{-1} and from 1660-1200 cm^{-1}, and were related to N-H bond deformation.

4.- Functional properties of collagen and gelatin

Besides their basic hydration properties, such as swelling and solubility, the most important properties of collagen and gelatin can be divided into two groups: i) properties associated with their gelling behaviour, i.e. gel formation, texturizing, thickening and water binding capacity, and ii) properties related to their surface behaviour, which include emulsion and foam formation and stabilisation, adhesion and cohesion, protective colloid function, and film forming capacity (Shrieber & Gareis, 2007). Sikorski, Scott & Buisson (1984) reviewed the possibilities of fish collagen as a functional material, and Montero et al. (1990) and Montero & Borderías (1991) examined the effects of concentration, pH and ionic strength on optimization of its functional properties.

4.1.- Gelling and water binding properties

Gel formation, viscosity and texture are closely related properties determined mainly by the structure, molecular size and temperature of the system. Although collagen and gelatin are different forms of the same macromolecule, gelatin and collagen gels are often confused by non-specialists. The gelatin gelation mechanism as well as the formed gel network structure differs considerably from that of collagen, as described by Djabourov et al. (1993). During collagen gelation the process of collagen molecule aggregation and fibril formation takes place, induced by changes in ionic strength, pH and temperature. During the collagen gelation process, there is a lag phase where the primary aggregates (dimers and trimers of collagen molecules) are nucleated. Then, microfibrillar aggregation starts with the lateral aggregation of sub-units until equilibrium is reached. The self-assembly process of type-I collagen from vertebrates occurs when the gelation temperature is raised from 20 to 28ºC. In contrast, the basic mechanism of gelatin is related to the reverse coil-to-helix transition triggered by cooling solutions below 30ºC, during which the helices that are created are similar to the collagen triple-helix, but in this case, no equilibrium is reached. The gelation process for both collagen and gelatin is thermo-reversible, but in opposite directions: collagen gels melt by lowering the temperature, while gelatin gels melt by raising the temperature.

The most widespread single use of gelatin in food products is in water gel desserts, due to its unique melt-in-the-mouth property. Certain other hydrocolloids also have thermo-reversible characteristics, but they generally melt at higher temperatures. Gelatin desserts made from various gelatins may provide variety in texture and gel melting behaviour, offering new product development opportunities. By increasing gelatin concentrations or by using gelatin mixtures (of cold and warm

water fish), desserts made from fish skin gelatin were found to be more similar to desserts made from high bloom pork skin gelatin. Furthermore, the lower melting temperature in gel desserts made from fish gelatins may accelerate flavour release (Zhou & Regenstein, 2007).

New textures and appearance, offering ample versatility in product development, can be provided by introducing a gas phase into gelatin-gel-based products, such as fruit jellies or marshmallows. Moreover, the dispersed air makes portions less calorie-dense, by reducing the energy consumed per unit volume. The incorporation of gas (air, nitrogen or helium) in the form of bubbles has been found to weaken the structure of gelatin gels, resulting in unique mechanical properties and an opaque white appearance (Zúñiga & Aguilera, 2009). Among the three gases tested, air-filled gels presented the lowest densities, the largest bubble diameters and the weakest structures in comparison with the other gasified gels.

Gelatin or collagen chains in solution may be covalently cross-linked to form matrices capable of swelling in the presence of aqueous solutions, forming what are commonly known as gelatin hydrogels. Hydrogels, which are characterized by their hydrophilicity and insolubility in water, have the ability to swell to an equilibrium volume while preserving their shape. The chemical cross-linkers used may be either relatively small bifunctional molecules or polyfunctional macromolecules like, for instance, glutaraldehyde (Deiber, Ottone, Piaggio & Peirotti, 2009). At present, the use of natural polyampholytic hydrogels in applications such as gelatin-casings, with the ability to absorb large quantities of water, are becoming more important in the field of medicine, pharmacy, agriculture and biodegradable food packaging. Gelatin is particularly attractive for forming hydrogel packaging because it is relatively inexpensive and biodegradable, and its structure facilitates multiple combinations of molecular interactions. The properties of gelatin-pectin hydrogels have been shown to depend strongly on the pH in the reaction mixture and on the charge balance (determined by the gelatin-pectin ratio), which will influence the degree of electrostatic associations and ionic interactions in the gelling system (Farris, Schaich, Liu, Piergiovanni & Yam, 2009). These authors proposed the development of integrated hydrogel films from gelatin and pectin, based on the formation of permanent polyion-complex hydrogels composed of a primary entangled network of chemically-crosslinked gelatin chains that connect and encase physical hydrogel regions in which pectin is linked to gelatin by ionic interactions.

The super-swelling properties of gelatin hybrid hydrogels produced by mixing with synthetic polymers have been exhaustively revised, especially with reference to their swelling capacity, degradation rate and controlled release of drugs (Zohuriaan-Mehr, Pourjavadi, Salimi & Kurdtabar, 2009). In this work, a number of organic (PEG-dialdehyde, acrylamines, EDTAD, poly(acrylic acid)) and inorganic (kaolin, silica gel) compounds have been shown to produce modifications in the gelatin

backbone, thus affecting the strength, solubility, surface hydrophilicity and morphology of the composite hydrogels. The suitability of highly hydrolysed collagen (~2000-20000 Da) to synthesize superabsorbent hybrid hydrogels was also revised.

A possible means of manipulating the characteristics of a low-gelling gelatin to achieve greater similitude with the properties of mammalian gelatins is to induce enzymatic crosslinking through the action of an enzyme called transglutaminase (TGase), usually of microbial origin. The enzyme acts by catalysing an acyl transfer reaction between the γ-carboxamide group of glutamine residues and the ε-amine group of lysine residues of peptide chains (Folk, 1983). Extensive covalent crosslinking during the cooling of set gelation may cause an almost complete loss of thermo-reversibility in the resultant gelatin gel, depending on whether covalent crosslinking occurs predominantly before or after formation of the hydrogen-bonded triple-helix junction zones (Babin & Dickinson, 2001). The thermo-reversibility of the gelatin gel may be modulated depending on enzyme concentration, incubation time and the degree of enzyme heat-inactivation. Increasing concentrations of TGase were reported to raise the melting temperature, and to increase the elasticity and cohesiveness of megrim skin gelatin gels, but due to excessively rapid gel network formation there was a lowering of gel strength and hardness (Gómez-Guillén, Sarabia, Solas & Montero, 2001). For gelatin extracted from Baltic cod skins, the addition of TGase has proven to be effective for producing stable gels at room temperature, as well as after heating in boiling water for a period inferior to 30 min (Kolodziejska, Kaczorowski, Piotrowska & Sadowska, 2004).

Different polysaccharides, such as k-carrageenan and/or gellan (Haug, Draget, & Smidsrød, 2004; Pranoto, Lee, & Park, 2007), or hydroxypropylmethylcellulose (Chen, lin & Kang, 2009) have also been used to increase fish gelatin gel strength, setting time and thermostability in order to enhance their cold and thermal gelation properties, without the loss of their thermoreversible character. Similarly, gelatin or hydrolyzed collagen materials could be hardened by reaction with chemically modified polysaccharides, such as dialdehyde starch (Langmaier, Mokrejs, Kolomaznik & Mladek, 2008) or dextran dialdehydes (Schacht, Nobels, Vansteenkiste, Demeester, Franssen, & Lemahieu, 1993).

The high swelling and water binding capacity of solubilized collagen and gelatin makes them suitable materials for reducing drip loss and impairing juiciness in frozen fish or meat products when thawed or cooked, and where denatured protein has suffered a partial loss of its water holding capacity. Borderías, Martí & Montero (1994) used collagenous material from freeze-dried plaice skin to improve the water-holding ability and the sensory properties of cod mince during frozen storage. The improvements in sensory characteristics were due not only to the prevention of drip loss, but also

because upon heating the fish mince portion, the collagenous material gelatinized, giving it a much more desirable texture than portions without this material.

The application of fish gelatin as an additive in surimi processing to improve water retention in heat-set gels has also been examined. Alaska pollack surimi gels containing 7.5–15 g/kg of fish gelatin showed improved expressible moisture; however, when 15 g/kg were added it produced a disruptive effect which proved detrimental to the mechanical properties of the gel, more so when using surimi of maximum quality (Hernández-Briones, Velázquez, Vázquez & Ramirez, 2009). The gel-forming capability of threadfin bream (*Nemipterus japonicus*) mince was substantially increased by adding gelatin (0.5%) from the skin of bigeye snapper (Binsi, Shamasundar, Dileep, Badii, & Howell, 2009). In contrast, gelatin additions of 5% and 10% reduced the elastic modulus (G') values considerably, possibly due to water molecule entrapment by high gelatin concentrations making them unavailable for protein gelation. Accordingly, gelatin was considered to be an inactive binder with little or no interaction between filler particles and gel matrix. However, when examining the gelling profile of chicken myosin and pork skin gelatin mixtures upon heating from 25 to 80ºC, Yang, Zhou, Xu & Wang (2007) found that G' values were significantly higher than those of pure myosin, which meant that a possible myosin–gelatin interaction, likely of an electrostatic nature, had led to higher gel elasticity, suggesting the usefulness of this gelatin application in restructured (chicken) meat products. In a much more simplified application, washed and defatted poultry skin was included in the formulation of bologna (an emulsified meat product) (Bonifer, Froning, Mandigo, Cuppett & Meagher, 1996). The authors found that the incorporation of skin (10%) significantly improved sensory properties (texture, flavour and appearance) without affecting negatively the emulsion stability or textural parameters in the final product.

4.2.- Surface properties

Collagen and gelatin surface properties are based on the presence of charged groups in the protein side chains, and on certain parts of the collagen sequence containing either hydrophilic or hydrophobic amino acids. Both hydrophobic and hydrophilic parts tend to migrate towards surfaces, hence reducing the surface tension of aqueous systems and forming the required identically charged film around the components of the dispersed phase, which can be additionally strengthened by gel formation (Shrieber & Gareis, 2007).

Type A gelatins, with a relatively high isoelectric point (pI≥7.0), are suitable for creating oil-in-water emulsions with a positive charge over a wider range of pH values than is possible with conventional protein emulsifiers, such as soy, casein or whey proteins (Dickinson & Lopez, 2001). Early studies by Kim, Jeon, Lee & Lee (1996) reported that cod bone gelatin had emulsifying properties similar to those of a commercial emulsifier, such as Tween-80. As is often the case with gelling properties, the

emulsion capacity of gelatin from fish species is frequently lower than that from mammalians. Apart from the distribution of charge, an important criterion in selecting a suitable gelatin type is gel firmness, because, at the same temperature and concentration, the higher the gel firmness is, the firmer the gel-like protective sheath is around the oil droplets (Schrieber & Gareis, 2007). For example, the emulsion activity index of tuna fin gelatin was lower than for pig skin gelatin at the same protein concentration (Aewsiri et al., 2008). With both types of gelatin, the emulsion capacity increased with increasing protein concentration from 2 to 5%, with high protein concentrations facilitating more protein adsorption at the interface. However, oil-in-water emulsions could be prepared using a relatively low concentration of gelatin (0.05%), extracted from the skin of bigeye snapper fish (Binsi et al., 2009). The aforementioned authors attributed the higher value of emulsion capacity recorded by increasing gelatin concentrations (0.1 and 0.2%) to a higher degree of polypeptide unfolding during the shearing involved in the emulsifying process.

Besides protein concentration, the molecular weight could be a key factor influencing the ability of gelatin to form and stabilize oil-in-water emulsions. In this respect, low molecular weight fish gelatin (~55 kDa) emulsion contained more large droplets and exhibited more oil destabilization than high molecular weight fish gelatin (~120 kDa) (Surh, Decker & McClements, 2006). These authors also observed the presence of a small population of large droplets in the emulsions after homogenization, which was attributed to the relatively low surface activity of fish gelatin compared with globular proteins such as b-lactoglobulin.

Being an insoluble protein, collagen may be presumed to have little significance as an emulsifier. However, as early as 1973, Satterlee et al. (1973) pointed out that, when adequately processed, the use of collagen afforded advantages with respect to milk proteins. For this reason, isolated or partially purified collagen needs to be in a soluble form (acid-soluble or pepsin-soluble collagen) in order to display its surface active properties, which may differ considerably from one raw material to another. The emulsifying capacity of acid-soluble collagen from hake skin was found to be higher than that for trout skin, in both cases being lower than in the corresponding muscle connective tissues (Montero & Borderías, 1991). In a later study, the acid-soluble collagen from Pacific whiting surimi refiner discharge was found to present higher emulsifying activity than both acid-soluble collagen from skin and the commercial emulsifier, Tween-80 (Kim & Park, 2004).

Gelatin and soluble collagen exhibit suitable foaming properties, even without gelling, because they are able to reduce the surface tension at the liquid/air interface by increasing the viscosity of the aqueous phase (Shrieber & Gareis, 2007). Foaming properties depend to a great extent on the characteristics of the raw material. For adsorption at the air–water interface, molecules should contain hydrophobic regions which become more exposed upon protein unfolding, thus facilitating foam

formation and stabilisation (Townsend & Nakai, 1983). In this respect, the foam capacity of gelatin from farmed giant catfish was found to be higher than that from calf skin, the difference possibly being due to the higher content of hydrophobic amino acid residues in the former. Moreover, the almost 4 times greater viscosity of the farmed giant catfish skin gelatin was also one of the main reasons for its better foam stability (Jongjareonrak et al., 2010).

4.3.- Film-forming properties

Gelatin has been extensively studied on account of its film-forming ability and its usefulness as an outer film to protect food from drying and exposure to light and oxygen (Arvanitoyannis, 2002). Furthermore, there is an important trend in favour of the use of biodegradable films made from edible biopolymers from renewable sources to combat the environmental impact of plastic waste (Tharanathan, 2003). The highly hygroscopic nature of gelatin is its main drawback when considering the use of gelatin films as protective barriers, because they tend to swell or dissolve when in contact with the surface of foodstuffs with high moisture content. Consequently, the current trend in designing gelatin-based biodegradable materials for food packaging or biomedical applications is focused on developing films with improved mechanical and water resistance properties, by combining gelatin with biopolymers with different characteristics, such as lipids (Bertan, Tanada-Palmu, Siani, & Grosso, 2005; Pérez-Mateos, Montero & Gómez-Guillén, 2009; Limpisophon, Tanaka, & Osako, 2010), soy protein isolates (Cao, Fu, & He, 2007; Denavi, Pérez-Mateos, Añón, Montero, Mauri, & Gómez-Guillén, 2009), polysaccharides as gellan (Lee, Shim, & Lee, 2004), konjac glucomannan (Li, Kennedy, Jiang, Xie, 2006), chitosan (Arvanitoyannis, Nakayama & Aiba, 1998) pectins (Liu, Liu, Fishman & Hicks, 2007; Farris et al., 2009), new hydrophobic or hydrophilic plasticizers (Andreuccetti, Carvalho, & Grosso, 2009; Cao, Yang, & Fu, 2009), synthetic polymers such as poly(vinyl) alcohol (Carvalho et al., 2009) or polyethylene (Haroun, Beherei, & Abd El-Ghaffar, 2010a), as well as cross-linking agents, such as glutaraldehyde (Bigi, Cojazzi, Panzavolta, Rubini, & Roveri, 2001), TGase or 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) (Yi, Kim, Bae, Whiteside, & Park, 2006; Kolodziejska & Piotrowska, 2007).

The effect on film properties of gelatin attributes, especially from fish origin, was reviewed recently by Gómez-Guillén et al. (2009). The molecular weight distribution and amino acid composition, which are the main factors influencing the physical and structural properties of gelatin, are also believed to play a key role in the mechanical and barrier properties of the resulting films. Weaker and more deformable films are normally obtained when low-molecular weight fragments predominate in a given gelatin preparation, which may be caused by protein heat degradation during the water extraction step (Muyonga, Cole & Duodu, 2004a) or during the evaporation step (Carvalho et al., 2008). Furthermore, films made using a lower-molecular weight gelatin were found to be more plasticized, as a result of the higher plasticizer:biopolymer molar ratio (Thomazine, Carvalho &

Sobral, 2005). When gelatins come from different species, attention must also be paid to the amino acid composition, especially that of the most characteristic amino acids Gly, Pro and Hyp. A recent study compared films made from tuna skin gelatin and bovine hide gelatin, and found that the tuna gelatin films, having a lower amount of Pro+Hyp residues (185 *vs* 210), presented breaking deformation values approximately 10 times higher than those for bovine-hide gelatin films (Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009). The pyrrolidine rings of the imino acids may impose conformational constraints, imparting a certain degree of molecular rigidity that can affect film deformability. Films made from warm-water fish species, such as Nile perch or channel catfish, have exhibited mechanical and water vapour barrier properties comparable to those of films made from mammalian gelatin (Muyonga, Cole & Duodu, 2004b; Zhang, Wang, Herring & Oh, 2007). Avena-Bustillos et al. (2006) observed that the water vapour permeability (WVP) of cold-water fish-gelatin films was significantly lower than that of films made from warm-water fish gelatin or mammalian gelatin, and based their explanation for these differences on the higher amounts of hydrophobic amino acids and lower levels of hydroxiproline in cold-water fish gelatins. A higher hydrophobic amino acid content in blue shark gelatin has also been put forward as the main reason for the lower WVP in the resulting films, as compared to other films from fish and land-based animal gelatins (Limpisophon, Tanaka, Weng, Abe & Osako, 2009).

Gelatin has been reported to be one of the first materials used as a carrier of bioactive components (Gennadios, McHugh, Weller & Krochta, 1994). Enriching gelatin films with natural antioxidants and/or antimicrobial substances will extend the functional properties of these biodegradable films and provide an active packaging biomaterial. Because of "clean labelling" concerns, there is growing interest in using plant extracts as natural sources of polyphenolic compounds in the formulation of gelatin films. Aqueous extracts from leaves of murta (*Ugni molinae*) (Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007), oregano (*Origanum vulgare*) and rosemary (*Rosmarinus officinalis*) (Gómez-Estaca, Montero, Fernández-Martín, Alemán, & Gómez-Guillén, 2009b, Gómez-Estaca, Bravo, Gómez-Guillén, Alemán, & Montero, 2009c), as well as a water/alcohol extract from borage (*Borago officinalis*) seeds (Gómez-Estaca, Giménez, Montero, Gómez-Guillén, 2009d) have been used to add antioxidant capacity to fish gelatin films. In these studies, a slight decrease in either breaking force or breaking deformation was observed depending on the added extract, differences being attributed to the qualitative and quantitative composition of the plant extracts used, which might have influenced the degree of protein-polyphenol interactions (Gómez-Guillén et al., 2009). Furthermore, protein-protein interaction capacity specific to the type of gelatin, could also be a determinant factor influencing the polyphenol contribution to protein matrix reinforcement or disruption. Thus, Gómez-Estaca et al. (2009b) found that bovine-hide gelatin film properties were much less affected by the inclusion of oregano and rosemary aqueous extracts than by tuna-skin

gelatin films, in which protein-polyphenol interactions were more evident, causing a slight decrease in deformability and increased water solubility.

Based on their capacity to induce non-disulfide covalent interactions with gelatin polypeptide chains, oxidized phenols from oxygenated seaweed extracts have been proposed as natural non-toxic cross-linking agents for improving the elongation of fish gelatin films (Rattaya, Benjakul & Prodpran, 2009). In line with this, grapefruit seed and green tea extracts were found to increase the tensile strength and decrease WVP when included in blend films made of *Gelidium corneum*, red seaweed rich in agarose, and gelatin (Hong, Lim & Song, 2009). Another polyphenolic extract from *Acacia nilotica* bark, rich in catechin and gallic acid derivatives, has been reported to induce hydrogen bonding and hydrophobic interactions, the two major forces involved in the stabilization of cross-linked gelatin-poly(acrylamide)co-acrylic acid composite film (Haroun & El Toumy, 2010).The *A. nilotica* bark extract stabilized the gelatin molecules in the blended networks and promoted a reduction in both swelling and degradation of the polymeric biocomposite films.

4.4.- Microencapsulation

Gelatin coacervates complexed with anionic polymers in the form of microcapsules are of special interest as they can entrap functional components in a carrier and provide protection against oxidation or degradation during storage. Moreover, encapsulation can be used to control the release of functional components from the food product or the bioactive packaging when ingested in the body. Coacervates are formed when a mixed dilute solution of gelatin and an anionic polyelectrolyte are brought to a pH at which the polyelectrolytes have opposite net charges. Under these conditions the solution separates into a highly concentrated coacervate phase in the form of microdroplets and a dilute bulk phase. The liquid coacervate envelopes the liquid drops or solid particles that are present in the solution, encapsulating them on a micro-scale; hence the term “microencapsulation” (Schrieber & Gareis, 2007).

The use of complex systems and natural cross-linkers has also been reported as a means of reinforcing gelatin microcapsules. In this respect, plant-derived polyphenols and flavonoids (contained in instant coffee and grape juice) have been shown to react with gelatin-pectin-based microparticular coacervates under oxidizing conditions to form covalent cross-links. The resulting microparticles present a structure with greater mechanical strength and thermal stability, and less capacity to expand and absorb water, which could find a practical application as a reduced calorie fat replacer, flavour binder or texturizer (Strauss & Gibson, 2004). A complex coacervation microcapsule system has been used to encapsulate baking flavour oil in a complex of gelatin-gum Arabic system to improve the appeal of frozen baked foods upon heating by controlling the rate of oil release (Yeo, Bellas, Firestone, Langer & Kohane, 2005).

Lycopene extract from tomato pulp waste, which is highly susceptible to oxidation and isomerization reactions, could have a possible application in the manufacture of functional food formulations as it has been microencapsulated, producing an emulsion system with porcine skin type A gelatin and poly(γ-glutamic acid) as carriers (Chiu, Chiu, Chien, Ho, Yang & Chen, 2007). The release of lycopene from the microcapsules occurred rapidly at pH 5.5 and 7.0, while no lycopene was released at pH 2.0 and 3.5, which means that the unstable constituents can remain intact in the stomach and then be released into the intestine over a range of physiological pH values.

Incorporation of probiotic bacteria in functional food products has become an increasingly popular trend because of their ability to exert beneficial effects on intestinal microflora (Gobbetti, Di Cagno & de Angelis, 2010). Microcapsules based on gelatin, using extrusion and spray-drying technology, have also been developed to improve the survival of lactic acid bacteria (Weissbrodt & Kunz, 2007; Li, Chen, Cha, Park & Liu, 2009) and bifidobacteria (Lian, Hsiao & Chou, 2002) against harmful conditions during processing as well as against the aggressive conditions of the stomach.

Furthermore, encapsulation technology could be used for the development of active packaging materials based on the incorporation of bioactive compounds, for example, plant polyphenolic extracts in gelatin films. Apart from protecting the active potential of the natural extract and modulating its release into the covered food product, encapsulation may be useful for taste and odour masking purposes, since vegetal extracts often have very strong flavours. Bao, Xu & Wang (2009) incorporated chitosan nanoparticles containing tea polyphenol in fish skin gelatin films to provide them with antioxidant capacity, and found fish oil oxidation to be effectively retarded when packed with this composite material. The nanoparticles greatly reduced the tensile strength and oxygen permeability in the resulting films but increased its water vapour permeability. A gelatin/poly(L-lactide) (PLLA) composite has been suggested for producing homogeneous and well-shaped nanofibres by electrospinning, using gelatin extracted from channel catfish skins, as a first step in the development of new material with applications in the biomedical field (An, Liu, Guo, Kumar, & Wang, 2010). The addition of PLLA significantly increased the diameters of the e-spun gelatin fibres, as well as the strength and elastic behaviour of the composite fibrous material.

5.- Bioactive properties of hydrolysates

Dietary proteins are a source of biologically active peptides, which are inactive in the parent protein sequence but can be liberated during gastrointestinal digestion, food processing or fermentation. Once they are released, bioactive peptides can affect numerous physiological functions of the organism. Collagen and gelatin have been focused on as a source of biologically active peptides with promising health benefits for nutritional or pharmaceutical applications. Usually, collagen and gelatin

hydrolysates and peptides have been produced from pig skin or bovine hide (Jia, Zhou, Lu, Chen, Li & Zheng, 2010). However, outbreaks of mad cow disease and the banning of collagen from pig skin and bone in some regions for religious reasons have made it necessary to find new marine or poultry sources, that are safer and healthier for consumers. Normally discarded collagenous materials from the poultry and fish processing industries, have been found to be valuable sources of hydrolysates and peptides with bioactive properties (Cheng, Liu, Wan, Lin & Sakata, 2008; Nam, You & Kim, 2008; Saiga et al., 2008; Cheng, Wan, Liu, Chen, Lin & Sakata, 2009). These collagenous materials may include skins, tunics, bones, fins and scales. Furthermore, the isolation of peptides with important biological activities has been reported for collagen and gelatin obtained from other sources like jellyfish, bullfrog or sea cucumber (Zhuang, Sun, Zhao, Hou & Li, 2010; Zhuang, Sun, Zhao, Wang, Hou & Li, 2009; Zhuang, Zhao & Li, 2009; Wang et al., 2010; Zeng, Xiao, Zhao, Liu, Li & Dong, 2007; Zhao, Xue, Li, Tang, Wang & Wang, 2009; Qian, Jung & Kim, 2008).

Gelatin and collagen-derived hydrolysates and peptides are generally obtained by enzymatic proteolysis. A number of commercial proteases have been used for the production of these hydrolysates and peptides, including trypsin, chymotrypsin, pepsin, alcalase, properase E, pronase, collagenase, bromelain and papain (Kim, Kim, Byun, Nam, Joo & Shahidi, 2001; Mendis, Rajapakse, Byun & Kim, 2005a; Lin & Li, 2006, Yang, Ho, Chu, Chow, 2008). Besides commercial proteases, enzymatic extracts from fish viscera have been used to obtain bioactive hydrolysates from skin and bones of different fish species (Phanturat, Benjakul, Visessanguan and Roytrakul, 2010; Jung, Park, Byun, Moon & Kim, 2005). Protease specificity affects size, amount, free amino acid composition and, peptides and their amino acid sequences, which in turn influences the biological activity of the hydrolysates (Chen, Muramoto & Yamauchi, 1995; Jeon, Byun & Kim, 1999; Wu, Chen & Shiau, 2003). Alcalase, which is a commercial protease from a microbial source, has been used in numerous studies dealing with gelatin/collagen hydrolysis because of its broad specificity as well as the high degree of hydrolysis that can be achieved in a relatively short time under moderate conditions (Diniz & Martin, 1996; Benjakul & Morrisey, 1997). This enzyme manifested extensive proteolytic activity during the hydrolysis of skin gelatin from Alaska pollack, squid Todarodes pacificus and giant squid, producing hydrolysates with low average molecular weight which exhibited high antioxidant activity and ACE inhibitory capacity (Kim et al., 2001a; Nam et al., 2008; Giménez, Alemán, Montero, & Gómez-Guillén, 2009).

The average molecular weight of protein hydrolysates is one of the most important factors which determines their biological properties (Jeon et al., 1999; Park, Jung, Nam, Shahidi & Kim, 2001). Peptide fractions from protein hydrolysates may vary in their effectiveness for a given biological activity. An ultrafiltration membrane system could be a useful and industrially advantageous method for obtaining peptide fractions with a desired molecular size and a higher bioactivity, depending on

the composition of the starting hydrolysate and the activity being studied (Jeon et al., 1999; Korhonen & Pihlanto, 2003; Cinq-Mars & Li-Chan, 2007; Picot et al., 2010). This system has been successfully applied in the fractionation and functional characterization of gelatin hydrolysates from squid or cobia skins (Lin & Li, 2006; Yang et al., 2008); and also as a first step in the isolation and further purification of bioactive peptides from both non-collagenous and collagenous sources (Kim et al., 2001a; Mendis et al., 2005a; Zhao, Li, Liu, Dong, Zhao & Zeng, 2007; Saiga et al., 2008; Alemán, Giménez, Pérez-Santín, Gómez-Guillén & Montero, 2011b; Hernández-Ledesma, Amigo, Ramos & Recio, 2004; Hernández-Ledesma, Dávalos, Bartolomé & Amigo, 2005; Hai-Lun, Xiu-Lan,Cai-Yun, Yu-Zhong & Bai-Cheng, 2006).

Most of the studies about collagen and gelatin-derived peptides in the area of food science and technology have dealt with their antioxidant and antihypertensive/ACE inhibitory activity. These peptides have repeated unique Gly–Pro–Hyp sequences in their structure, and the observed antioxidative and antihypertensive properties have presumably been associated with this unique amino acid composition (Kim & Mendis, 2006). Moreover, collagen and gelatin-derived peptides have exhibited numerous other bioactivities, namely: antimicrobial activity, mineral binding capacity, the lipid-lowering effect, immunomodulatory activity and beneficial effects on skin, bone or joint health (Gómez-Guillén, López-Caballero, Alemán, López de Lacey, Giménez & Montero, 2010; Jung et al., 2005; Jung, Karawita, Heo, Lee, Kim & Jeon, 2006; Zhang, Kouguchi, Shimizu, Sato, Takahata & Morimatus, 2010; Hou et al., 2009; Moskowitz, 2000).

Some studies have been performed to confirm the *in vivo* biological activity of collagen and gelatin peptides, and some convincing data have been obtained for animal models. Moreover, human studies have also been carried out with positive results, especially those related to the capacity shown by collagen and gelatin hydrolysates to improve joint conditions. Because of this ability, collagen and gelatin hydrolysates, mainly obtained from mammalian sources, have long been used in pharmaceutical and dietary supplements (Krug, 1979; Oberschelp, 1985; Adam, 1991; Beuker & Rosenfeld, 1996; Moskowitz, 2000; Zuckley et al., 2004; Benito-Ruiz et al., 2009). Fish skin collagen hydrolysates have been reported to affect lipid absorption and metabolism in rats (Saito, Kiyose, Higuchi, Uchida & Suzuki, 2009). Besides the lipid-lowering effect, chicken bone collagen hydrolysates have been shown to reduce proinflammatory cytokine production in mice (Zhang et al., 2010). Bone mineral density in osteoporotic rats and joint disease in dogs were improved by ingesting chicken and porcine gelatin and collagen hydrolysates (Han et al., 2009; Watanabe-Kamiyama et al., 2010; Beynen, van Geene, Grim, Jacobs, van der Vlerk, 2010). In contrast, bovine collagen hydrolysate consumption did not produce any effects on bone metabolism as measured by biochemical indices of bone remodelling in postmenopausal women (Cúneo, Costa-Paiva, Pinto-Neto, Morais & Amaya-Farfan, 2010). Recently, some peptides from marine sources were reported to act

protectively against ultraviolet radiation-induced damage on mice skin (Hou et al., 2009; Zhuang, Hou, Zhao, Zhang & Li, 2009).

5.1.- Antimicrobial properties

Published information on the antimicrobial properties of hydrolysates or peptides from collagen or gelatin is very scarce. Gómez-Guillén, et al. (2010) reported antimicrobial activity in peptide fractions from tuna and squid skin gelatins within a range of 1-10 kDa and <1 kDa. The hydrolysates were tested using the agar diffusion assay against 18 strains of bacteria (both Gram-positive and negative), Lactobacillus acidophilus and Bifidobacterium animalis subp. Lactis, Shewanella putrefaciens and Photobacterium phosphoreum were found to be the most susceptible species. The reduced molecular weight in the peptide fractions, which was related to the elimination of aggregates, better exposure of the amino acid residues and their charges, as well as structure acquisition, were suggested as factors facilitating the interaction with bacterial membranes. On the other hand, Molinero, Julia, Erra, Robert & Infante (1988) synthesized dipeptides with a surfactant-like behaviour by condensation between Nα-lauroyl arginine and amino acids coming from a collagen hydrolysate. A higher antimicrobial activity against both Gram-positive and Gram-negative bacteria was found for those dipeptides having a more pronounced cationic character.

The relationship between peptide characteristics and antimicrobial activity has not yet been clearly stated. Several factors, such as amino acid composition, sequence, molecular weight and type of bacteria need to be taken into account (Di Bernardini et al., 2011). The hydrophobic character of amino acids would let peptides enter the bacterial membrane, as the positive charge would initiate the peptide interaction with the negatively charged bacteria surface (Wieprecht et al., 1997). On the other hand, the differences existing in membrane composition have implications in the mode of action and the specificity of the antibacterial compounds (Floris, Recio, Berkhout & Visser, 2003). Patrzykat & Douglas (2005) reported that the degree of lipopolysaccharide (LPS) binding is neither directly nor inversely proportional to peptide activity because, following the rupture of the outer membrane, peptide activity would depend on its ability to interact with the bacterial cytoplasmic membranes. Thus, both the sequence and concentration of the peptide and the composition of the bacterial membranes would influence the mode of interaction.

Collagen per se has also been described as a biomaterial with bactericidal and fungicidal properties when applied as a coating to wool-based materials (Jus, Kokol & Guebitz, 2009). However, as collagen takes part of the extracellular matrix it may be involved in binding some bacteria to the host tissues, for instance, *Pseudomonas aeruginosa*, which has been shown to readily adhere to a matrix rich in type I collagen (Plotkowski et al., 1991).

5.2.- Antioxidant activity

Since the antioxidant effect of peptides was first reported (Marcuse, 1960), numerous studies have investigated hydrolysate and peptide antioxidant properties from both plant and animal sources such as rice bran (Revilla et al., 2009), sunflower protein (Megias et al., 2008), alfalfa leaf protein (Xie, Huang, Xu & Jin, 2008), casein (Suetsuna, Ukeda & Ochi, 2000), egg-yolk protein (Sakanaka & Tachibana, 2006), mackerel muscle protein (Wu et al., 2003), squid skin gelatin (Mendis et al., 2005a), fish skin gelatin (Mendis, Rajapakse & Kim, 2005b), bovine skin gelatin (Kim, Kim, Byun, Park & Ito, 2001b), tuna backbone (Je, Qian, Byun & Kim, 2007), tuna cooking juice (Hsu, Lu & Jao, 2009), etc. Due to the enormous volume of fish processing waste generated annually, a great deal of attention has been paid to the production of antioxidant hydrolysates and peptides from skin gelatin of different fish species, such as Alaska pollack (Kim et al., 2001a), hoki (Johnius belengerri) (Mendis et al., 2005b), cobia (Rachycentron canadum) (Yang et al., 2008) and sole (Giménez et al., 2009), as well as from several squid species, such as giant squid (Dosidicus gigas) (Mendis et al., 2005a; Giménez et al., 2009), Jumbo flying squid (Dosidicus eschrichitii Streenstrup) (Lin & Li, 2006) or squid (Todarodes pacificus) (Nam et al., 2008). Some of the antioxidant peptide sequences isolated from fish skin gelatin and other collagenous sources are shown in Table 1.

The exact mechanism underlying the antioxidant activity of peptides is not fully understood, but various studies have shown that they are lipid peroxidation inhibitors, free radical scavengers and transition metal ion chelators. According to some studies, gelatin peptides could inhibit lipid peroxidation more efficiently than antioxidative peptides derived from many other protein sources (Kim et al., 2001b). In addition, it has been reported that collagen and gelatin antioxidative peptides may protect living cells against free radical mediated oxidative damage. Therefore, scavenging of free radical species is an important mechanism by which antioxidant peptides enhance cell viability against oxidation-induced cell death. Kim et al. (2001a) reported that a peptide isolated from Alaska pollack skin gelatin was able to protect rat liver cells from oxidant injury induced by organic hydroperoxide t-BHP. In the same manner, two purified peptides from squid skin gelatin exhibited a dose-dependent cell viability enhancement effect when their ability to overcome t-BHP-induced cytotoxicity was tested in human lung fibroblasts (Mendis et al., 2005a). Moreover, results from one study revealed that peptides isolated from hoki skin gelatin were capable of enhancing the expression of antioxidative enzymes such as glutathione peroxidase, catalase and superoxide dismutase in human hepatoma cells (Mendis et al., 2005b).

Peptide antioxidative properties are related to their amino acid composition, structure and hydrophobicity. The amino acid composition of collagen and gelatin hydrolysates is very similar to that of the parent proteins, being rich in residues of Gly, Ala, Pro, Hyp, Glx and Asx, but poor in Met, Cys, His and Tyr (Kim et al., 2001a; Mendis et al., 2005a; Gómez-Guillén et al., 2010; Alemán et al.,

2011b). Dávalos, Miguel, Bartolomé, and López-Fandiño (2004) studying individual amino acid activity reported that Trp, Tyr and Met showed the highest antioxidant activity, followed by Cys, His and Phe. The rest of the amino acids did not show any antioxidant activity. However, many peptides have been described as having antioxidant capacity without containing any of the above mentioned proton-donating amino acid residues in their sequences. Thus, Kim et al. (2001a) isolated two peptides composed of 13 and 16 amino acid residues, respectively from Alaska pollack skin, both of which contained a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp. Li, Chen, Wang, Ji and Wu (2007) identified the peptide which exhibited the highest antioxidant activity from porcine skin collagen hydrolysates as Gln-Gly-Ala-Arg. The peptide Asn–Gly–Pro–Leu–Gln–Ala–Gly–Gln–Pro–Gly–Glu–Arg was purified from squid skin gelatin with a valuable free radical quenching capacity (Mendis et al., 2005a). Furthermore, the antioxidant activity of collagen and gelatin peptides has been linked to the high content of hydrophobic amino acids, which could increase their solubility in lipids and therefore enhance their antioxidative activity (Kim et al., 2001a). Rajapakse, Mendis, Byun and Kim (2005) found that fish skin gelatin peptides showed higher antioxidant activity than peptides from meat protein, probably because of the higher percentage of Gly and Pro.

Not only is the presence of proper amino acids essential, but their correct positioning in the peptide sequence plays an important role too in antioxidant activity. Peptide conformation has also been claimed to influence antioxidant capacity, showing both synergistic and antagonistic effects, as far as the antioxidant activity of free amino acids is concerned (Hernández-Ledesma et al., 2005). As mentioned above, protease specificity used for hydrolysis may determine the size and the sequence of the peptides, and as a result their antioxidant activity. In different studies, it has been found that alcalase gelatin-derived hydrolysate antioxidant activity was higher than that of other enzyme hydrolysates such as those obtained by collagenase, pepsin, trypsin, chymotrypsin, papain or neutrase (Alemán, Giménez, Montero & Gómez-Guillén, 2011a; Qian et al., 2008). Moreover, antioxidant activity is strongly related to peptide molecular weight as demonstrated by Gómez-Guillén et al. (2010) who found antioxidant activity in all the peptide fractions from squid skin hydrolysate, but that it was higher in the fractions with lower molecular weight. Similarly, the peptide fraction from cobia skin hydrolysate, with most of the molecular mass values below 700Da showed the highest radical scavenging activity, being approximately 20% higher than that of the non-fractionated hydrolysate (Yang et al., 2008).

5.3.- Antihypertensive/ACE inhibitory activity

Antihypertensive peptides are peptide molecules which may lower blood pressure when ingested through inhibition of vasoactive enzymes such as the angiotensin converting enzyme (ACE). ACE plays an important role in the regulation of blood pressure by means of the rennin-angiotensin system,

and inhibition of this enzyme is considered to be a useful therapeutic approach in the treatment of hypertension (Ondetti, Rubin & Cushman, 1977; Chen et al., 2007).

Since the discovery of ACE inhibitory peptides in snake venom, many studies have tried to synthesize ACE inhibitors, such as captopril, enalapril, alacepril and lisinopril, which are used extensively in the treatment of hypertension and heart failure in humans (Ondetti, 1977; Patchett et al., 1980). However, synthetic ACE inhibitors are believed to produce certain side effects such as coughing, taste disturbances, skin rashes or angioneurotic edema (Atkinson & Robertson, 1979). Therefore, in the last 10 years many researchers worldwide have concentrated on finding natural sources of ACE inhibitors such as food proteins, which though less potent that the synthetic ones are without known side effects. Although the major natural source of ACE inhibitory peptides identified to date is milk, these peptides have been isolated from many other animal and plant protein sources, like blood proteins (Mito et al., 1996), ovalbumin (Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004), maize (Miyoshi, Ishikawa, Kaneko, Fukui, Tanaka & Maruyama, 1991), chickpea (Yust, Pedroche, Girón-Calle, Alaiz, Millán & Vioque, 2003), soy (Wu & Ding, 2001) and muscle proteins from pig, cattle, fish and chicken (Fujita & Yoshikawa, 1999; Fujita, Yokoyama & Yoshikawa, 2000; Katayama et al., 2003; Jang & Lee, 2005; Ahhmed & Muguruma, 2010). Collagen and gelatin have also been shown to be good sources of antihypertensive peptides by enzymatic digestion, despite not having been so extensively studied as other sources. Potent ACE inhibitory hydrolysates and peptides have been obtained from collagenous materials, not only from land-based sources such as porcine skin collagen (Anzai et al., 1997; Ichimura, Yamanaka, Otsuka, Yamashita & Maruyama, 2009), bovine skin gelatin (Kim, Byun, Park & Shahidi, 2001c), chicken legs and leg bone (Saiga et al., 2008; Cheng et al., 2009), but also from marine sources such as fish skins (Byun & Kim, 2001; Park, Kim, Kang, Park & Kim, 2009; Nagai, Nagashima, Abe & Suzuki, 2006), fish cartilage (Nagai et al., 2006), scales (Fahmi, Morimura, Guo, Shigematsu, Kida & Uemura, 2004), squid tunics (Alemán et al., 2011b) and sea cucumbers (Zhao et al., 2007). The peptide sequences identified from collagenous materials are shown in Table 2.

Although the relationship between the structure and the activity of ACE inhibitory peptides has not yet been established, these peptides have certain common features. Most of them are relatively short sequences with low molecular mass, as the active site of ACE cannot accommodate large peptide molecules. Binding to ACE is strongly influenced by the C-terminal tripeptide sequence, which may interact with subsites at the active site of the enzyme (Ondetti & Cushman, 1982). ACE prefers substrates or inhibitors that contain hydrophobic amino acid residues (aromatic or branched side chains) at each of the three C-terminal positions (Cheung, Wang, Ondetti, Sabo & Cushman, 1980; Murray & FitzGerald, 2007). The presence of Arg or Lys on the C -terminal position has also been reported to contribute substantially to the inhibitory activity (Cheung et al., 1980; Ariyoshi, 1993;

Meisel, 2003). The ACE inhibitory activity described for collagen and gelatin hydrolysates and peptides may be related to the high concentration of hydrophobic amino acids, as well as to high Pro levels. This amino acid seems to be one of the most effective for increasing ACE inhibitory activity and has been identified in many of the naturally occurring ACE peptide inhibitors (Gómez-Ruiz, Ramos & Recio, 2004ab; Quirós et al., 2007; Pihlanto, Akkanen & Korhonen, 2008; Contreras, Carrón, Montero, Ramos & Recio, 2009), especially in those derived from collagenous sources (Table 2) (Byun & Kim, 2001; Kim et al., 2001c; Saiga et al., 2008; Ichimura et al., 2009; Shimizu et al., 2010; Alemán et al., 2011b).

The *in vivo* effects of antihypertensive peptides are usually tested in spontaneously hypertensive rats (SHR), which constitute an accepted model for human essential hypertension (Fitz-Gerald, Murray, & Walsh, 2004). Many of the ACE inhibitory peptides isolated from collagenous sources have already been tested *in vivo*, and an antihypertensive effect has been reported. SHR experienced a significant decrease in blood pressure following oral administration of both a chicken leg collagen hydrolysate and the isolated octapeptide Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro (Cheng et al., 2009; Iwai, Saiga-Egusa, Hayakawa, Shimizu, Takahata & Morimatsu, 2008; Saiga et al., 2008). Furthermore, Faria, da Costa, Gontijo and Netto (2008) found that bovine and porcine collagen hydrolysates produced a considerable reduction in blood pressure in SHR after oral administration. In another study, the blood pressure of renal hypertensive rats was significantly reduced by administering the lowest fraction of sea cucumber gelatin hydrolysate (Zhao et al., 2007). ACE inhibitory peptides Gly-Pro and Gly-Phe-Hyp-Gly-Pro, isolated from porcine skin collagen hydrolysate also had an antihypertensive effect on SHR (Ichimura et al., 2009).

6.- Conclusions

Gelatin production from alternative non-mammalian species had grown in importance, largely as a way to valorise by-products from fish and poultry industrial processes. Regarding fish origin, tropical and sub-tropical warm-water species might have similar rheological properties and thermostability to that of mammal gelatins; therefore, they could be used for similar applications. Recently, an increasing number of new applications have been found for gelatin or collagen in products such as emulsifiers, foaming agents, colloid stabilizers, hydrogels, fining agents, biodegradable packaging materials, micro-encapsulating agents, as well as bioactive peptides, in line with the growing trend to replace synthetic agents with more natural ones. The main source for collagen or gelatin extraction from fish are skins and bones, however more recently they have been also extracted from scales and fins, processed fish offal, as well as from other aquatic organisms such as red sea cucumber or alligators. The huge number of available species had made necessary to adapt the extraction procedures in order to optimize the properties of the resulting material (in the form of collagen or gelatin). In the last decade, further improvement of the characteristics of low-gelling gelatins from

alternative sources has been achieved by using complex systems and natural cross-linkers. Additionally, the functional properties of collagen or gelatin hydrolysates have been focused on the production of bioactive peptides with a number of biological activities which have been previously described in other protein sources. On the other hand, the great development of modern analytical methods has allowed a deeper characterization of gelatin and collagen properties from all these new sources and of special interest could be their application for species identification.

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