International Food Research Journal 23(3): 913-922 (2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

Mini Review

Collagen extraction process

[1]Schmidt, M. M., [1*]Dornelles, R. C. P., [1]Mello, R. O., [1]Kubota, E. H., [2]Mazutti, M. A., [3]Kempka, A. P. and [4]Demiate, I. M.

[1]Department of Technology and Food Science, Federal University of Santa Maria, Santa Maria, RS, Brazil.
[2]Department of Chemical Engineering, Federal University of Santa Maria, Santa Maria, RS, Brazil.
[3]Department of Food Engineering, State University of Santa Catarina (UDESC), Pinhalzinho, SC, Brazil.
[4]State University of Ponta Grossa (UEPG), Graduate Program in Food Science and Technology (PPGCTA), Ponta Grossa, PR, Brazil.

Article history

Received: 18 March 2015
Received in revised form: 30 September 2015
Accepted: 13 November 2015

Keywords

Hydrolysis
Proteases
Ultrasound
By-products

Abstract

Collagen is a fibrous protein which is dominant in the connective tissue of animals; it has a wide range of applications in the food, pharmaceutical, cosmetic and photographic industries, among others. There is a growing interest in the extraction process of collagen and its derivatives due to the growing tendency to use this protein to replace synthetic agents in various industrial processes, which results in a greater appreciation of the by-products from animal slaughter. Collagen's characteristics depend on the raw material and the extraction conditions, which subsequently determine its application. The most commonly used extraction methods are based on the solubility of collagen in neutral saline solutions, acid solutions, and acid solutions with added enzymes. Recently, the use of ultrasound, combined with these traditional processes, has proven effective in increasing the extraction yield. The objective of this review is to address the different collagen extraction processes, from raw materials to the use of combinations of chemical and enzymatic processes, as well as the use of ultrasound. The information outlined in this article have been collected from different national and international journals in Agricultural Sciences e Science and Food Technology, using four bibliographic databases and also some books of renowned authors. Were selected articles published between 2000 and 2015, to address the different collagen extraction processes have been studied.

Introduction

Collagen is the dominant protein in connective tissue, and it is found in various forms in tissues of all species of multicellular organisms; it exercises various functions, depending on its location (Shimokomaki *et al*., 2006; Damoradan *et al*., 2010). It can be extracted from various animal species and it is generally derived from slaughter by-products. The main sources of collagen are the skin, tendons, cartilage and bones. Some studies have addressed ways of obtaining collagen from different animal sources, such as fish and birds, as alternatives to bovine collagen because of the risk of bovine spongiform encephalopathy (Kawwdang *et al*., 2014; Wang *et al*., 2014), and also as an alternative to collagen derived from pigs for use in Muslim countries.

There is growing interest in the processes used to extract collagen and its derivatives due to the growing tendency to use this protein in place of synthetic agents in various industrial processes, and also to provide a greater appreciation of the by-products of animal slaughter (Karim and Bhat, 2008; Gómez-Guillén *et al*., 2011).

The distribution of molar mass, structure and composition, and the subsequent functional features and properties of collagen, depend on the processing conditions of the raw materials from which it is derived and the specificity of the enzyme used in the extraction process (Prestes, 2013). Thus, it is necessary to determine the appropriate extraction process for each raw material in order to obtain the best performance and the best collagen characteristics for the desired application.

Collagen is considered to be one of the most useful biomaterials because it has a wide range of industrial applications (Lafarga and Hayes, 2014). There is great demand in the food industry for collagen and gelatin because of their high protein content and their functional properties, such as water absorption capacity, gel formation, and the ability to form and stabilize emulsions. In the biomedical

*Corresponding author.
Email: *rosacrisprestesdornelles@outlook.com*
Tel: +55 55 32208254

and pharmaceutical fields, collagen has several applications; it is used as a vehicle for drugs, proteins and genes, as well as a substitute for human skin, blood vessels and ligaments (Kim and Mendis, 2006; Gómez-Guillén *et al*., 2011). Several studies have examined collagen in order to obtain bioactive compounds with antimicrobial, antioxidant and anti-hypertensive properties (Saiga *et al.*, 2008; Jia *et al.*, 2009; Wang *et al*., 2010; Ding *et al*., 2011; Gómez-Guillén *et al.*, 2011; Bernardini *et al*., 2012; Barzideh *et al*., 2014; Abedin *et al*., 2015).

The objective of this review is to address the different collagen extraction processes, from raw materials to the use of combinations of chemical and enzymatic processes, as well as the use of ultrasound. The information outlined in this article have been collected from different national and international journals in Agricultural Sciences e Science and Food Technology, using four bibliographic databases and also some books of renowned authors. Were selected articles published between 2000 and 2015, to address the different collagen extraction processes have been studied.

Raw materials for collagen extraction

Meat is the main product derived from the slaughter of animals, while all other entrails and offal are classed as by-products (Bhaskar *et al.*, 2007), including bones, tendons, skin, fatty tissues, horns, hooves, feet, blood and internal organs. The yield of by-products that is generated depends, among other factors, on the species, sex, age and body weight of the animal. The yield varies from 10% - 30% in cattle, pigs and sheep and from 5% - 6% in poultry (Nollet and Toldrá, 2011). According to Bhaskar *et al.* (2007) about 40% of these by-products are edible and 20% are inedible.

Depending on the culture and the country, edible by-products can be considered as waste or as delicacies that command high prices (Toldrá *et al*., 2012). However, the majority of by-products are not suitable for human consumption due to their characteristics. As a result, a potential source of income is lost, and the cost of disposal of these products has become increasingly high (Jayathilakan *et al*., 2012). Nevertheless, there is a growing awareness that these by-products can represent valuable resources if they are used properly.

Generally, inedible by-products are used in the manufacture of fertilizers, animal feed and fuel but there is also a growing market in using them to obtain minerals, fatty acids, and vitamins and to obtain protein hydrolysates and collagen. Obtaining those products, which have high added value, is a better alternative to use these by-products, which would otherwise be discarded.

The main sources for collagen extraction are by-products from the slaughter of pork and beef (Jia *et al*., 2010; Silva and Penna, 2012). Several of these by-products have been studied, including the Achilles tendon (Li *et al*., 2009), pericardium (Santos *et al*., 2013), bovine inner layer of skin (Moraes and Cunha, 2013) and bovine bones (Paschalis *et al*., 2001), porcine skin (Yang and Shu, 2014) and porcine lung (Lin *et al*., 2011).

Recent research has examined alternative sources for the extraction of collagen, with particular emphasis on fish by-products (Muralidharan *et al*., 2013; Kaewdang *et al*., 2014; Ninan *et al*., 2014; Wang *et al.*, 2014; Mahboob, 2015; Tang *et al*., 2015). This is mainly due to religious restrictions, regarding the non-consumption of pork by Muslims and Jews, and also the risk of bovine spongiform encephalopathy (BSE) (Kaewdang *et al*., 2014). The latter belongs to a family of diseases known as transmissible spongiform encephalopathies, which are caused by the accumulation of the pathological prion protein (PrPSc) in the brain and central nervous system, which affects adult bovines (Callado and Teixeira, 1998; Toldrá *et al*., 2012).

The extraction of collagen from fish has been carried out in several species using different by-products, such as Japanese sea bass skin *(Lateolabrax japonicus)* (Kim *et al.*, 2012), skin of clown featherback *(Chitala ornata)* (Kittiphattanabawon *et al.*, 2015), bladder of yellow fin tuna *(Thunnus albacares)* (Kaewdang *et al*., 2014), skin and bone from Japanese seerfish *(Scomberomorous niphonius)* (Li *et al*., 2013), cartilage from Japanese sturgeon *(Acipenser schrenckii)* (Liang *et al*., 2014), and the fins, scales, skins, bones and swim bladders from bighead carp *(Hypophthalmichthys nobilis)* (Liu *et al*., 2012). Despite the extraction of marine collagen is easy and safe this collagen presents some limitations in their application, due to its low denaturation temperature (Subhan *et al*., 2015).

The extraction of collagen from poultry slaughter waste has also been researched, but with less emphasis because of the risk of the transmission of avian influenza (Saito *et al*., 2009). Studies have been performed regarding emu skin *(Dromaius novaehollandiae)* (Nagai *et al*., 2015), and chicken feet (Saiga *et al*., 2008; Almeida *et al.*, 2012a; Hashim *et al*., 2014), chicken sternal cartilage (Cao and Xu, 2008), chicken skin (Cliche *et al.*, 2003; Munasinghe *et al*., 2015) and chicken tarsus (Almeida *et al*., 2012b) etc.

The processing of by-products can convert a

product with low value, or one that requires costly disposal, into a product that is able to cover all the costs of processing and disposal, with consequent higher added value and reduced environmental damage (Toldrá *et al*., 2012).

Collagen

Collagen is a fibrous protein found in all multicellular animals (Voet *et al*., 2006). It is an important component in the support structures in vertebrates and invertebrates. It is the most abundant protein in mammals, corresponding to approximately 25% of the weight of all proteins (Ward and Courts, 1977; Voet *et al*., 2006), and is the major constituent protein of skin, tendons, cartilage, bones and tissues in general. In poultry and fish it plays a similar role to that of invertebrates and is an important component of the body wall (Ward and Courts, 1977).

Collagen molecules are about 280 nm long, with a molar mass of 360,000 Da; they are stabilized by hydrogen bonds and intermolecular bonds (Silva and Penna, 2012), which are composed of three helical polypeptide chains, each with about 1000 amino acids, which are called an α chain. The chains become entangled, forming a stable triple helix which is varied in size. The triple helix molecules have terminal globular domains and are called procollagen. These globular regions are cleaved in varying degrees to give a polymerized structure (tropocollagen), which is the basic unit of collagen. The tropocollagen molecules are stabilized by hydrophobic and electrostatic interactions (Nelson and Cox, 2004; Damoradan *et al*., 2010).

There are different kinds of collagen in vertebrates; they typically contain about 35% glycine (Gly), 11% alanine (Ala) and 21% proline (Pro) and hydroxyproline (Hyp). The amino acid sequence in collagen is generally a repetitive tripeptide unit (Gly-X-Y), where X is frequently Pro and Y is Hyp (Nelson and Cox, 2004).

At least 29 different types of collagen have been reported, which are classified according to their structure into: striatum (fibrous), non-fibrous (network forming), microfibrillar (filamentous) and those which are associated with fibril (Damoradan *et al.*, 2010).

Type I collagen is the most common, primarily in connective tissue, in tissues such as skin, tendons and bones. It consists of three polypeptide chains, two of which are identical, which are called chain α1 (I) and α2 (I), and which are composed of different amino acids. Type II collagen occurs almost exclusively in cartilage tissue and it is believed that the α1 (II) subunit is similar to the α1 (I) subunit. Type III collagen is strongly dependent on age: very young skin can contain up to 50%, but with the passage of time that percentage can be reduced to 5-10%. Other types of collagen are only present in very small quantities, mainly in specific organs such as the basement membranes, cornea, heart muscle, lungs and intestinal mucosa (Schrieber and Gareis, 2007; Karim and Bhat, 2009).

Collagen extraction process

Collagen can be basically obtained by chemical hydrolysis and enzymatic hydrolysis (Zavareze *et al*., 2009). Chemical hydrolysis is more commonly used in industry, but biological processes that use the addition of enzymes are more promising when products with high nutritional value and improved functionality are required (Martins *et al*., 2009). Moreover, enzymatic processes generate less waste and may reduce the processing time, but they are more expensive. To extract collagen it is necessary to remove numerous covalent intra- and intermolecular cross-links, which primarily involves residues of lysine and hydroxy-lysine, ester bonds and other bonds with saccharides, all of which makes the process quite complex (Ran and Wang, 2014).

Before the collagen can be extracted a pre-treatment is performed using an acid or alkaline process, which varies according to the origin of the raw material. The pre-treatment is used to remove non-collagenous substances and to obtain higher yields in the process. The most commonly used extraction methods are based on the solubility of collagen in neutral saline solutions, acidic solutions, and acidic solutions with added enzymes. Table 1 presents a summary of the procedures employed in the extraction of collagen from animal by-products.

Pre-treatment

Due to the nature of the cross-linked collagen that is present in the connective tissue of animals, it dissolves very slowly, even in boiling water. As a result, a mild chemical treatment is necessary to break these cross-links before extraction (Schreiber and Gareis, 2007). To this end, diluted acids and bases are employed, and the collagen is subjected to partial hydrolysis, which maintains the collagen chains intact but the cross-links are cleaved (Prestes, 2013).

In the acidic form of pre-treatment the raw material is immersed in acidic solution until the solution penetrates throughout the material. As the solution penetrates the structure of the skin at a controlled temperature it swells to two or three times its initial volume and the cleavage of the non-covalent inter-

Table 1. Some procedures used in the extraction of collagen from animal waste reported in the literature.

Raw Material	Pre-treatment	Extraction procedure	Reference
Emu skin (*Dromaius novaehollandiae*)	Homogenization with 10% ethanol for 4 days. Extraction with 0.1 M NaOH for 2 days. Washing with distilled water for 2 days. Lyophilization.	Consecutive extractions with 0.5 M acetic acid for 48 h, 0.9 M NaCl in 0.5 M acetic acid and pepsin (10%) in 0.5 M acetic acid for 4 days.	Nagai *et al.* (2015)
Japanese sturgeon skin (*Acipenser schrenckii*)	Homogenization with 20% NaCl three times at 4°C.	Extraction with 0.45 M NaCl 1:100 (w/v) for 24 h. Followed by acid extraction with 0.5M acetic acid twice for 24 h. Followed by extraction with pepsin (0.1%) in 0.01 M HCl for 48 hours. All operations were performed at 4°C.	Wang *et al.* (2014)
Brownbanded bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Carcharhinus limbatus*) cartilage	Fat removal with 0.1 M NaOH 1:10 (w/v) for 6 h and 0.5 M EDTA decalcification, 1:10 (w/v) for 40 h, both at 4°C.	Acid hydrolysis with 0.5 M acetic acid 1:15 (w/v) for 48 h followed by enzymatic hydrolysis with porcine pepsin (40 units/g of residue) in 0.5 M acetic acid 1:15 (w/v) for 48 h, both at 4°C.	Kittiphatanabawon *et al.* (2010)
Bovine Achilles tendon	Washing with 0.15 M NaCl and acetone.	Enzymatic hydrolysis with pepsin in 0.5 M acetic acid for 2 days at 20°C. With and without ultrasound (40 kHz, 120 W, pulsed 30/30 minutes).	Li *et al.* (2009)
Yellowfin tuna (*Thunnus albacares*) swim bladder	Extraction with 0.15 M NaOH 1:10 (w/v) for 2 h at 4°C and 10% butyl alcohol, 1:10 (w/v) for 12 h. Washing with cold distilled water three times.	Extraction with 0.5 M acetic acid, 1:10 (w/v) for 48 h at 4°C. Enzymatic hydrolysis with extract of stomach of yellowfin tuna (20 units/g of swim bladder) in 0.5 M acetic acid 1:10 (w/v) for 48 h at 4°C.	Kaewdang *et al.* (2014)
Bovine pericardium	Immersion in 0.1 M NaOH for 48 h at 4°C.	Extraction with pepsin in 10 mM hydrochloric acid 1:20 (w/v) for 12 h at 4°C.	Santos *et al.* (2013)
Japanese seerfish (*Scomberomorous niphonius*) skin and bones	Skin: Fat removal with 0.1 M NaOH 1:10 (w/v) for 2 days at 4°C. Washing with cold water until neutral pH. Extraction with 10% butyl alcohol for 2 days. Bones: Extraction with 0.1 M NaOH 1:20 (w/v) for 48 h. Washing with cold water until neutral pH. Descaling with 0.5 M EDTA-2Na for 5 days. Extraction with 10% butyl alcohol for 2 days.	Skin: Extraction with 0.5 M acetic acid, 1:15 (w/v) for 24 h, followed by extraction with porcine pepsin (750 U/mg dry weight) in 0.5 M acetic acid, 1:15 (w/v) at 4°C for 2 days. Bones: Extraction with 0.5 M acetic acid, 1:15 (w/v) for 3 days. Subsequent extraction with porcine pepsin (20 U/g of residue) in 0.5 M acetic acid at 4°C for 2 days.	Li *et al.* (2013)

and intra-molecular bonds occurs (Ledward, 2000). The acidic process is more suitable for more fragile raw materials with less intertwined collagen fibers, such as porcine and fish skins (Almeida, 2012b).

The alkaline process consists of treating the raw material with a basic solution, typically sodium hydroxide (NaOH), for a period that can take from a few days to several weeks (Prestes, 2013). This process is used for thicker materials that require a more aggressive penetration by the treatment agents, such as bovine ossein or shavings (Ledward, 2000). NaOH and Ca $(OH)_2$ are often used for pre-treatment,

but NaOH is better for pre-treating skins because it causes significant swelling, which facilitates the extraction of collagen by increasing the transfer rate of the mass in the tissue matrix (Liu *et al*., 2015).

A study by Liu *et al.* (2015) evaluated the effect of alkaline pre-treatment on the extraction of acid soluble collagen (ASC) from the skin of grass carp *(Ctenopharyngodon idella)*. Concentrations of NaOH from 0.05 to 0.1 M were effective in removing non-collagenous proteins without losing the ASC and structural modifications at temperatures of 4, 10, 15 and 20°C. However, 0.2 and 0.5 NaOH M caused a significant loss of ASC, and 0.5 M NaOH resulted in structural modification in the collagen at 15 and 20°C. In addition to the use of acids and bases, enzymes or chemicals may also be used to cleave the cross-linked bonds to obtain products with different characteristics (Schrieber and Gareis, 2007).

Chemical hydrolisis

In the extraction of collagen which is soluble in salt, neutral saline solutions are used, such as sodium chloride (NaCl), Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride), phosphates or citrates. Caution is required in this process in order to control the concentration of salt, but considering that the majority of collagen molecules are cross-linked, the use of this method is limited (Yang and Shu, 2014).

Acid hydrolysis can be performed by using organic acids such as acetic acid, citric acid and lactic acid, and inorganic acids such as hydrochloric acid. However, organic acids are more efficient than inorganic acids (Skierka and Sadowska, 2007; Wang *et al.*, 2008). Organic acids are capable of solubilizing non-crosslinked collagens and also of breaking some of the inter-strand cross-links in collagen, which leads to a higher solubility of collagen during the extraction process (Liu *et al*., 2015). Therefore, acidic solutions, especially acetic acid, are commonly used to extract collagen.

For the extraction of acid-soluble collagen, the pre-treated material is added to the acid solution, usually 0.5 M acetic acid, and maintained for 24 to 72 hours under constant stirring at 4°C, depending on the raw material (Wang *et al.*, 2014; Nagai *et al*., 2015; Kaewdang *et al*., 2014).

After the extraction stage, a filtering is performed to separate the supernatant (residue) from the collagen, which is in the liquid phase. To obtain collagen powder, the filtrate is usually subjected to precipitation with NaCl. The precipitate is then collected by centrifugation and subsequently re-dissolved in a minimum volume of 0.5 M acetic acid and then dialyzed in 0.1 acetic acid for 2 days, and distilled water for 2 days, with replacement of the solution on average every 12 hours.

Moraes and Cunha (2013) analyzed collagen from the inner layer of bovine hide that was extracted under different temperature conditions (50, 60 or 80°C) and pH (3, 5, 7 or 10) under stirring for 6 hours. The hydrolysates that were produced in different conditions showed distinct properties. The highest levels of soluble proteins were obtained from treatments at a temperature of 80°C and a pH below the isoelectric point. The products obtained in conditions of extreme pH (3 and 10) or high temperatures (60 and 80°C) were completely denatured. The extractions with acidic pH and high temperature produced collagen with reduced molar mass. In general, the hydrolysates obtained with acidic pH formed firmer gels. The water retention capacity of the gels was approximately 100%, except for the hydrolysates that were obtained at high pH (7 and 10) and above the denaturation temperature (80°C).

Wang *et al.* (2008) optimized the conditions for extraction of acid-soluble collagen in skin from grass carp *(Ctenopharyngodon idella)*, having evaluated the effects of the concentration of acetic acid (0.3, 0.5 and 0.8 M), temperature (10, 20 and 30°C) and extraction time (12, 24 and 36 hours). The three tested variables showed a significant effect on collagen extraction and a positive relationship was found between time and the collagen yield. Increased temperature and concentration of acetic acid increased the yield to a certain value, which then decreased. The optimal conditions to obtain the highest yield of acid-soluble collagen in skin from grass carp were: an acetic acid concentration of 0.54 M at a temperature of 24.7°C for 32.1 hours.

Acid-soluble collagen from the skin and swim bladder of barramundi *(Lates calcarifer)* was extracted by Sinthusamran *et al.* (2013). The pre-treated raw materials were extracted with 0.5 M acetic acid for 48 hours at 4°C. The acid-soluble collagen from the swim bladder showed a higher yield (28.5%) compared to that which was obtained from the skin (15.8%). In both cases the collagen was identified as type I, with some differences in the primary structure. Both the skin and the swim bladder of barramundi showed potential for collagen extraction.

A study by Liu *et al.* (2015) suggested that alkaline pre-treatment and extraction with acetic acid for grass carp *(Ctenopharyngodon idella)* can be performed at a temperature range of 4 to 20°C instead of the commonly used low temperature of 4°C. In this case, there was no significant loss of collagen and structural integrity was maintained,

which represents greater convenience in terms of practical and industrial applications.

In general, chemical hydrolysis processes seek optimum conditions for obtaining higher yields by controlling process variables such as concentration, pH, temperature, and process time.

Enzymatic hydrolysis

For the extraction of collagen by enzymatic hydrolysis, the raw material, which can be the residue of acidic extraction, is added to 0.5 M acetic acid solution containing selected enzymes such as pepsin, Alcalase® and Flavourzyme® (Novozymes®, Araucária PR, Brazil). The mixture is continuously stirred for about 48 hours at 4°C followed by filtration (Li *et al*., 2009; Li *et al*., 2013; Wang *et al*., 2014). The filtrate is subjected to precipitation and dialysis under the same conditions as for obtaining acid-soluble collagen.

Woo *et al.* (2008) optimized the extraction of collagen from the skin of yellowfin tuna *(Thunnus albacares)*. Pre-treatment was performed with NaOH (0.5 to 1.3 N) at 9°C for 12 to 36 hours for the removal of non-collagenous protein. Subsequently, digestion with pepsin (0.6 to 1.4% (w/v) was performed in hydrochloric acid (HCl) solution (pH 2.0) at 9°C for 12 to 36 hours. The optimal extraction conditions were obtained with a pre-treatment of 0.92 N NaOH for 24 hours and digestion with pepsin at a concentration of 0.98% (w/v) for 23.5 hours.

Wang *et al*. (2014) isolated and characterized collagen from the skin of Japanese sturgeon *(Acipenser schrenckii)* using NaCl, acetic acid and pepsin for extraction. Initially, the skin was pre-treated with NaCl and Tris-HCI and then the saline-soluble collagen was extracted (SSC) in 0.45 M NaCl at pH 7.5 for 24 h with continuous stirring; this was performed six times. After the extraction with salt, the residue was suspended in 0.5 M acetic acid for the extraction of acid-soluble collagen (ASC); the procedure was carried out for 24 hours, twice. The material that was insoluble in acetic acid was used to extract pepsin-solubilized collagen (PSC) by using 0.1% (w/v) pepsin in 0.01 M HCl for 48 hours. The yields of SSC, ASC and PSC were 4.55%, 37.42% and 52.80%, respectively. All the isolated collagens maintained a triple helix structure and were mainly type 1 collagen, with similar morphology and amino acid profiles. The spectroscopic analysis in the mid-infrared region using Fourier transform spectroscopy (FTIR) showed more hydrogen bonds in the PSC and more intermolecular cross-linking in the ASC. The different collagens also showed some differences in terms of thermal stability, which could have been due to the hydration level, as well as the number and type of covalent cross-links.

Kittiphattanabawon *et al.* (2010) extracted collagen from the cartilage of brownbanded shark *(Chiloscyllium punctatum)* and blacktip shark *(Carcharhinus limbatus)*. Pre-treatment was performed using NaOH and ethylenediamine tetraacetic acid (EDTA). The extraction was initially performed with acetic acid for 48 hours at 4°C. Thereafter, the residue that was not dissolved by the acidic extraction was extracted with porcine pepsin in acetic acid for 48 hours at 4°C. The collagen extracted by pepsin had a much higher yield than the acid-extracted collagen. Furthermore, the spectra of both collagens that were obtained by FTIR were very similar; suggesting that hydrolysis with pepsin does not affect the secondary structure of collagen, especially the triple helix structure.

The method of extraction can influence the length of the polypeptide chains and the functional properties of collagen, such as viscosity, solubility, as well as water retention and emulsification capacity. This varies according to the processing parameters (enzyme, temperature, time and pH), the pre-treatment, method of storage and the properties of the initial raw material (Karim and Bhat, 2009).

Thus it is necessary to perform a partially controlled hydrolysis of the cross-linked bonds and the peptide bonds of the original structure of the collagen in order to obtain the ideal distribution of molar mass for a given application (Schreiber and Gareis, 2007). This factor has emphasized the use of selected animal or vegetable proteolytic enzymes, such as trypsin, chymotrypsin, pepsin, pronase, alcalase, collagenases, bromelain and papain (Gómez-Guillén *et al*., 2011; Khan *et al.*, 2011) because these permit the control of the degree of cleavage of the substrate protein. In addition, enzymatic hydrolysis presents some advantages compared with chemical hydrolysis, such as specificity, the control of the degree of hydrolysis, moderate conditions of action, and lower salt content in the final hydrolyzate. Furthermore, enzymes can be generally employed at very low concentrations and it is not necessary to remove them from the medium (Zavareze *et al*., 2009). Despite the high cost of enzymatic hydrolysis, the fact that it results in lower levels of waste, better control of the process and higher yield justifies the use of enzymes.

The use of ultrasound in the collagen extraction process

Ultrasound is widely used to improve the transfer of mass by wet processes, which are of importance

in terms of mixture, extraction and drying (Li *et al*., 2009). Ultrasound has been used successfully in collagen extraction by reducing the processing time and increasing the yield (Kim *et al.*, 2012; Kim *et al*., 2013; Ran and Wang, 2014; Tu *et al*., 2015).

Ultrasound is a process that uses the energy of sound waves which are generated at a higher frequency than the hearing capacity of human beings (higher than 16 kHz) (Chemat and Khan, 2011). The effects of ultrasound in liquid systems are mainly due to the phenomenon known as cavitation (Hu *et al*., 2013). During sonication, cavitation bubbles are quickly formed, which then suffer a violent collapse, resulting in extreme temperatures and pressures. This leads to turbulence and shearing in the cavitation zone (Chemat and Khan, 2011).

In a study by Kim *et al*. (2012), the extraction of acid-soluble collagen from the skin of Japanese sea bass *(Lateolabrax japonicus)* showed increased yield and reduced extraction time after ultrasonic treatment at a frequency of 20 kHz in 0.5 M acetic acid. Extraction with ultrasound did not alter the major components of the collagen, more specifically the α1, α2 and β chains.

Ran and Wang (2014) compared the extraction of collagen from bovine tendon with and without the use of ultrasound (20 kHz pulsed 20/20 seconds). Conventional extraction was performed with pepsin (50 Umg^{-1} of sample) in acetic acid for 48 hours. For the extraction with ultrasound the same conditions were used, but the times of extraction with ultrasound (3 to 24 h) and pepsin (24 to 45 hours) were varied, resulting in a total of 48 hours of treatment. The combination of ultrasound with pepsin resulted in a greater efficiency of collagen extraction, reaching a yield of 6.2%, when the conventional extraction yield was 2.4%. The adequate time for extraction using ultrasonic treatment was 18 h. The collagen that was extracted from bovine tendon showed a continuous helical structure, as well as good solubility and fairly high thermal stability. The use of ultrasound in conjunction with pepsin improved the efficiency of the extraction of natural collagen without damaging the quality of the resulting collagen.

Li *et al*. (2009) utilized ultrasound (40 kHz, 120 W) to extract collagen from bovine tendon using the enzyme pepsin. The results showed that ultrasound increased extraction by up to 124% and reduced the process time. These results were explained by the increased activity and dissolution of the substrate because irradiation allows for a greater dispersion of pepsin and opening of collagen fibrils, which facilitates the action of the enzyme. The use of circular dichroism analysis, atomic force microscopy and FTIR showed that the triple helix structure of the collagen remained intact, even after the ultrasonic treatment.

According to Kim *et al.* (2013) the use of ultrasound in the extraction of collagen generated a higher rate of yield than the conventional extraction method with 0.5 M acetic acid, even when using a low concentration of acid (0.01 M). In addition, the yield of collagen from the skin of Japanese sea bass *(Lateolabrax japonicus)* increased greatly with increased treatment time and amplitude of ultrasound.

However, studies of the effect of ultrasound on enzyme activity are still very limited (Li *et al*., 2009; Yu *et al*., 2014). Yu *et al*. (2014) suggested that the activity of the enzymes papain and pepsin can be modified by ultrasound treatment, mainly due to changes in their secondary and tertiary structures. The activity of papain was inhibited, and the activity of pepsin was activated by the ultrasound treatment that was tested.

The application of ultrasound for a long period of time may give rise to elevated temperatures and shear strength, as well as high pressures within the medium because of cavitation. It can also break the hydrogen bonds and van der Waals forces in polypeptide chains, leading to the denaturation of the protein/enzyme (Ran and Wang, 2014).

Conclusions

This review examined differences in collagen extraction processes because this is a key step in achieving higher yield, purity and structural integrity of this protein, as well as saving time and reducing process costs. Among the methods of extraction that were discussed, extraction by saline solution has lower yields and some limitations; for those reasons it is less commonly used. Acidic extraction can be efficient, but enzymatic extraction has some advantages, such as specificity, degree of control of hydrolysis, moderate action conditions and less waste; for those reasons it is more widely used. Nevertheless, extraction methods may favorably and unfavorably affect the characteristics of collagen, including thermal stability, molar mass, water-retaining capacity and gel-forming capacity.

The use of ultrasound has recently increased due to higher yields and reduced extraction time, without damaging the quality of collagen. However, further studies are needed to assess the effect of ultrasound on enzyme activity and the benefits of the use of ultrasound combined with other methods, mainly with a view for future industrial use. The re-use of industrial by-products is of great importance in the

search for cleaner and more sustainable production. In this context, collagen extraction is of great interest because it can add value to the by-products of animal slaughter.

References

Abedin, M.Z., Karim, A.A., Gan, C.Y., Ghazali, F.C., Barzideh, Z., Zzaman, W. and Zaidul, I.S.M. 2015. Identification of angiotensin I converting enzyme inhibitory and radical scavenging bioactive peptides from sea cucumber *(Stichopus vastus)* collagen hydrolysates through optimization. International Food Research Journal 22(3): 1074-1082.

Almeida, P.F., Araújo, M.G.O. and Santana, J.C.C. 2012a. Collagen extraction from chicken feet for jelly production. Acta Scientiarum. Technology 34(3): 345-351.

Almeida, P.F., Vanalle, R.M. and Santana, J.C.C. 2012b. Produção de Gelatina: Uma perspectiva competitiva para a cadeia produtiva de frango de corte. Produto and Produção 13(2): 22-39.

Barzideh, Z., Latiff, A.A., Gan, C.Y., Abedin, M. and Alias, A.K. 2014. ACE inhibitory and antioxidant activities of collagen hydrolysates from the ribbon jellyfish (*Chrysaora* sp.). Food Technology and Biotechnology 52(4): 495-504.

Bernardini, R.D., Mullen, A.M., Bolton, D., Kerry, J., O'neill, E. and Hayes, M. 2012. Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions. Meat Science 90(1): 226-235.

Bhaskar, N., Modi, V.K., Govindaraju, K., Radha, C. and Lalitha, R.G. 2007. Utilization of meat industry by products: protein hydrolysate from sheep visceral mass. Bioresource Technology 98(2): 388-394.

Callado A.K.C. and Teixeira M.F.S. 1998. Encefalopatias espongiforme transmissíveis e considerações sobre o agente etiológico. Ciência Animal 8(1): 13-21.

Cao, H. and Xu, S.Y. 2008. Purification and characterization of type II collagen from chicken sternal cartilage. Food Chemistry 108(2): 439-445.

Chemat, F. and Khan, M.K. 2011. Applications of ultrasound in food technology: processing, preservation and extraction. Ultrasonics Sonochemistry 18(4): 813-835.

Cliche, S., Amiot, J., Avezard, C. and Gariepy, C. 2003. Extraction and characterization of collagen with or without telopeptides from chicken skin. Poultry Science 82(3): 503-509.

Damodaran S., Parkin, K. and Fennema, O.R. 2010. Química de alimentos de fennema. p. 726-730. 4ª ed. São Paulo: Artmed.

Ding J.F., Li Y.Y., Xu J.J., Su X.R., Gao X. and Yue F.P. 2011. Study on effect of jellyfish collagen hydrolysate on anti-fatigue and anti-oxidation. Food Hydrocolloids 25(5): 1350-1353.

Gómez-Guillén, M.C., Giménez, B., López-Caballero, M.A. and Montero, M.P. 2011. Functional and bioactive properties of collagen and gelatin from alternative sources: A review. Food Hydrocolloids 25(8): 1813-1827.

Hashim, P., Ridzwan, M.M. and Bakar, J. 2014. Isolation and characterization of collagen from chicken feet. International Journal of Biological, Veterinary, Agricultural and Food Engineering 8(3): 242-246.

Hu, H., Fan, X., Zhou, Z., Xu, X., Dan, G., Wang, L., Huang, X., Pan, S. and Zhu, L. 2013. Acid-induced gelation behavior of soybean protein isolate with high intensity ultrasonic pre-treatments. Ultrasonic Sonochemistry 20(1): 187-195.

Jayathilakan, K., Sultana, K., Radhakrishna, K. and Bawa, A.S. 2012. Utilization of by-products and waste materials from meat, poultry and fish processing industries: a review. Journal of Food Science and Technology 49(3): 278-293.

Jia, J., Zhou, Y., Lu, J., Chen, A., Li, Y. and Zheng, G. 2010. Enzymatic hydrolysis of Alaska pollack *(Theragra chalcogramma)* skin and antioxidant activity of the resulting hydrolysate. Journal of the Science of Food and Agriculture 90(4): 635-640.

Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of collagens from the swim bladders of yellowfin tuna *(Thunnus albacares)*. Food Chemistry 155: 264-270.

Karim, A.A. and Bhat, R. 2009. Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. Food Hydrocolloids 23(3): 563–576.

Khan, M.I., Arshad, M.S., Anjum, F.M., Sameen, A., Rehman, A. and Gill,W. 2011. Meat as a functional food with special reference to probiotic sausages. Food Research International 44(10): 3125-3133.

Kim, H.K., Kim, Y.H., Kim, Y.J., Park, H.J. and Lee, N.H. 2012. Effects of ultrasonic treatment on collagen extraction from skins of the sea bass *Lateolabrax japonicus*. Fisheries Science 78(2): 485-490.

Kim, H.K., Kim, Y.H., Park, H.J. and Lee, N.H. 2013. Application of ultrasonic treatment to extraction of collagen from the skins of sea bass *Lateolabrax japonicus*. Fisheries Science 79(5): 849-856.

Kim, S.E. and Mendis, E. 2006. Bioactive compounds from marine processing byproducts – a review. Food Research International 39(4): 383-393.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2010. Isolation and characterization of collagen from the cartilages of brownbanded bamboo shark *(Chiloscyllium punctatum)* and blacktip shark *(Carcharhinus limbatus)*. LWT-Food Science and Technology 43(5): 792-800.

Kittiphattanabawon, P., Benjakul, S., Sinthusamran, S. and Kishimura, H. 2015. Characteristics of collagen from the skin of clown featherback *(Chitala ornata)*. International Journal of Food Science and Technology 50(9): 1972-1978.

Lafarga, T. and Hayes, M. 2014. Bioactive peptides from meat muscle and by-products: generation,

functionality and application as functional ingredients. Meat Science 98(2): 227-239.

Ledward, D.A. 2000. Gelatin. In Handbook of hydrocolloids, ed G. O. Philips, P.A. Williams. p. 67-86. Woodhead Publishing Limited.

Li, D., Mu, C., Cai, S. and Lin, W. 2009. Ultrasonic irradiation in the enzymatic extraction of collagen. Ultrasonics Sonochemistry 16(5): 605-609.

Li, Z.R., Wang, B., Chi, C.F., Zhang, Q.H., Gong, Y.D., Tang, J.J., Luo, H.Y. and Ding, G.F. 2013. Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel *(Scomberomorous niphonius)*. Food Hydrocolloids 31(1): 103-113.

Liang, Q., Wang, L., Sun, W., Wang, Z., Xu, J. and Ma, H. 2014. Isolation and characterization of collagen from the cartilage of Amur sturgeon *(Acipenser schrenckii)*. Process Biochemistry 49(2): 318-323.

Lin, Y.K., Lin, T.Y. and Su, H.P. 2011. Extraction and characterisation of telopeptide-poor collagen from porcine lung. Food Chemistry 124(4): 1583-1588.

Liu, D., Wei, G., Li, T., Hu, J., Lu, N., Regenstein, J. M. and Zhou, P. 2015. Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp *(Ctenopharyngodon idella)* skin. Food Chemistry 172: 836-843.

Liu, D., Liang, L., Regenstein, J.M. and Zhou, P. 2012. Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp *(Hypophthalmichthys nobilis)*. Food Chemistry 133(4): 1441-1448.

Martins, V.G., Costa, J.A.V. and Hernandez, C.P. 2009. Hidrolisado protéico de pescado obtido por vias química e enzimática a partir de corvina. Química Nova 32(1): 61-66.

Mahboob, S. 2015. Isolation and characterization of collagen from fish waste material- skin, scales and fins of *Catla catla* and *Cirrhinus mrigala*. Journal of Food Science and Technology-Mysore 52(2): 4296-4305.

Moraes, M.C. and Cunha, R.L. 2013. Gelation property and water holding capacity of heat-treated collagen at different temperature and pH values. Food Research International 50(1): 213-223.

Munasinghe, K.A., Schwarz, J.G. and Whittiker, M. 2015. Utilization of Chicken By-Products to Form Collagen Films. Journal of Food Processing 2015: 1-6.

Muralidharan, N., Shakila, R.J., Sukumar, D. and Jeyasekaran, G. 2013. Skin, bone and muscle collagen extraction from the trash fish, leather jacket *(Odonus niger)* and their characterization. Journal of Food Science and Technology 50(6): 1106-1113.

Nagai, T. 2015. Characterization of collagen from emu *(Dromaius novaehollandiae)* skins. Journal of Food Science and Technology 52(4): 2344-2351.

Nelson, D.L. and Cox, M.M., 2005. Lehninger. Principles of Biochemistry. 4 ed. New York: W. H. Freeman. 1119 p.

Ninan, G., Joseph, J. and Aliyamveettil, Z.A. 2014. A comparative study on the physical, chemical and functional properties of carp skin and mammalian gelatins. Journal of Food Science and Technology 51(9): 2085-2091.

Nollet, L.M.L. and Toldrá, F. 2011. Handbook of Analysis of Edible Animal By-Products. p. 3-11. CRC Press: Gent, Belgium.

Paschalis, E.P., Verdelis, K., Doty, S.B., Boskey, A.L., Mendelsohn, R. and Yamauchi, M. 2001. Spectroscopic characterization of collagen cross-links in bone. Journal of Bone and Mineral Research 16(10): 1821-1828.

Prestes, R.C. 2013. Colágeno e seus derivados: características e aplicações em produtos cárneos. Revista Unopar Científica Ciências Biológicas e da Saúde 15(1): 65-74.

Ran, X.G. and Wang, L.Y. 2014. Use of ultrasonic and pepsin treatment in tandem for collagen extraction from meat industry by-products. Journal of the Science of Food and Agriculture 94(3): 585-590.

Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., Kitamura, S., Nishimura, T. and Morimatsu, F. 2008. Angiotensin I-converting enzyme inhibitory peptides obtained from chicken collagen hydrolysate. Journal of Agricultural and Food Chemistry 56(20): 9586-9591.

Saito, M., Kiyose, C., Higuchi, T., Uchida, N. and Suzuki, H. 2009. Effect of collagen hydrolysates from salmon and trout skins on the lipid profile in rats. Journal of Agricultural and Food Chemistry 57(21): 10477-10482.

Santos, M.H., Silva, R.M., Dumont, V.C., Neves, J.S., Mansur, H.S. and Heneine, L.G.D. 2013. Extraction and characterization of highly purified collagen from bovine pericardium for potential bioengineering applications. Materials Science and Engineering: C 33(2): 790-800.

Schrieber R. and Gareis H. 2007. Gelatine handbook: theory and industry practice. Hardcover. 348p.

Shimokomaki M, Olivo R, Terra N.N. and Franco B.D.G.M. 2006. Atualidades em ciência e tecnologia de carnes. São Paulo: Varela. 236p.

Sinthusamran, S., Benjakul, S. and Kishimura, H. 2013. Comparative study on molecular characteristics of acid soluble collagens from skin and swim bladder of seabass *(Lates calcarifer)*. Food Chemistry 138(4): 2435-2441.

Skierka, E. and Sadowska, M. 2007. The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod *(Gadus morhua)*. Food Chemistry 105(3): 1302-1306.

Silva, T.F. and Penna, A.L.B. 2012. Colágeno: Características químicas e propriedades funcionais. Revista do Instituto Adolfo Lutz 71(3): 530-539.

Subhan, F., Ikram, M., Shehzad, A. and Ghafoor, A. (2015). Marine Collagen: An Emerging Player in Biomedical applications. Journal of Food Science and Technology 52(8): 4303-4307.

Tang, L., Chen, S., Su, W., Weng, W., Osako, K. and Tanaka, M. 2015. Physicochemical properties and film-forming ability of fish skin collagen extracted from different freshwater species. Process Biochemistry 50(1): 148-155.

Toldrá, F., Aristoy, M., Mora, L. and Reig, M. 2012. Innovations in value-addition of edible meat by-products. Meat Science 92(3) 290-296.

Tu, Z.C., Huang, T., Wang, H., Sha, X.M., Shi, Y., Huang, X.Q.,Man, Z.Z. and Li, D.J. 2015. Physico-chemical properties of gelatin from bighead carp *(Hypophthalmichthys nobilis)* scales by ultrasound-assisted extraction.Journal of food science and technology 52(4): 2166-2174.

Voet, D., Voet, J. and Pratt, C.F.D.B. 2006. Fundamentos de bioquímica. 3ª Ed. Porto Alegre: Armed.

Wang, J., Wang, Y., Tang, Q., Wang, Y., Chang, Y., Zhao, Q. and Xue, C. 2010. Antioxidation activities of low-molecular-weight gelatin hydrolysate isolated from the sea cucumber *Stichopus japonicus*. Journal of Ocean University of China 9(1): 94-98.

Wang, L., Liang, Q., Chen, T., Wang, Z., Xu, J. and Ma, H. 2014. Characterization of collagen from the skin of Amur sturgeon *(Acipenser schrenckii)*. Food Hydrocolloids 38: 104-109.

Wang, L., Yang, B., Du, X., Yang, Y. and Liu, J. 2008. Optimization of conditions for extraction of acid-soluble collagen from grass carp *(Ctenopharyngodon idella)* by response surface methodology. Innovative Food Science and Emerging Technologies 9(4): 604-607.

Ward, A.G. and Courts, A. 1977. The Science and Technology of Gelatin – A Series of Monographs. London: Academic Press.

Woo, J.W., Yu, S.J., Cho, S.M., Lee, Y.B. and Kim, S.B. 2008. Extraction optimization and properties of collagen from yellowfin tuna *(Thunnus albacares)* dorsal skin. Food Hydrocolloids 22(5): 879-887.

Yang, H. and Shu, Z. 2014. The extraction of collagen protein from pigskin. Journal of Chemical and Pharmaceutical Research 6(2): 683-687.

Yu, Z. L., Zeng, W.C., Zhang, W.H., Liao, X.P. and Shi, B. 2014. Effect of ultrasound on the activity and conformation of α-amylase, papain and pepsin. Ultrasonics Sonochemistry 21(3): 930-936.

Zavareze, E.R., Silva, C.M., Mellado, M.S. and Prentice-Hernández, C. 2009. Funcionalidade de hidrolisados proteicos de cabrinha *(Prionotus punctatus)* obtidos a partir de diferentes proteases microbianas. Química. Nova 32(7): 1739-1743.