Collagen and Gelatin

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Abstract

Collagen and gelatin have been widely used in the food, pharmaceutical, and cosmetic industries due to their excellent biocompatibility, easy biodegradability, and weak antigenicity. Fish collagen and gelatin are of renewed interest, owing to the safety and religious concerns of their mammalian counterparts. The structure of collagen has been studied using various modern technologies, and interpretation of the raw data should be done with caution. The structure of collagen may vary with sources and seasons, which may affect its applications and optimal extraction conditions. Numerous studies have investigated the bioactivities and biological effects of collagen, gelatin, and their hydrolysis peptides, using both in vitro and in vivo assay models. In addition to their established nutritional value as a protein source, collagen and collagen-derived products may exert various potential biological activities on cells in the extracellular matrix through the corresponding food-derived peptides after ingestion, and this might justify their applications in dietary supplements and pharmaceutical preparations. Moreover, an increasing number of novel applications have been found for collagen and gelatin. Therefore, this review covers the current understanding of the structure, bioactivities, and biological effects of collagen, gelatin, and gelatin hydrolysates as well as their most recent applications.

INTRODUCTION

Collagen is the most abundant structural protein in both vertebrates and invertebrates, and constitutes approximately 30% of an animal's total proteins. Collagen and collagen-derived products are now widely used in the food, pharmaceutical, and cosmetic industries for their nutritional and functional properties. This article provides an overview of the literature for five main topics as follows: the structure of the collagen molecule and its determination using modern technologies, the variations of collagen with sources and seasons, collagen- and gelatin-derived peptides and their bioactivities, the potential biological effects of oral ingestion of collagen and collagen-derived products on skin physiology and the corresponding underlying mechanism, and nonconventional applications of gelatin.

THE STRUCTURE OF THE COLLAGEN MOLECULE

As demonstrated by its nomenclature, a collagen molecule consists of three α-chains, each of which contains approximately 1,000 amino acids and has a molecular weight of approximately 100 kDa. The average amino acid molecular weight is lower than that for most proteins because of the high Gly content of the amino acids. The three α-chains of collagen may be identical (a homotrimer) or different (a heterotrimer), depending on the types and sources. Type I collagen, the first collagen to be identified, is commonly known as a heterotrimer, with two identical α_1-chains and one α_2-chain in the molecular form of $[\alpha_1(I)]_2\alpha_2(I)$. In addition, another heterotrimer of type I collagen, $\alpha_1(I)\alpha_2(I)\alpha_3(I)$, has also been identified in the skin and muscles of rainbow trout and contains three different α-chains, with the α_3-chain having a similar molecular weight to the α_1-chain (Saito et al. 2001). However, skin collagen from the chordate giant Red Sea cucumber was reported to mainly consist of a $[\alpha_1(I)]_3$ homotrimer (Liu et al. 2010). The ratio of different chains is frequently estimated by the corresponding staining ratio on an electrophoretic gel. However, the resulting ratio should be corrected further by the molecular weight of each chain, given the staining intensity is generally proportional to the protein size at equal molar concentrations. In addition, all bands being quantitated should be in the Beer-Lambert region, but this is sometimes not the case.

Type I collagen is widely known as the major fibrillar collagen due to its unique ability to form insoluble fibers in vitro with high tensile strength and stability. Each α-chain of collagen forms a left-handed helix by itself, and then three α-chains further intertwine to form a right-handed triple superhelical structure. This is in agreement with one of the fundamental theories in modern protein chemistry that the architecture of a protein is determined by its primary sequences. The amino acid sequences of the helix domain, i.e., the main structure of the molecule, are characterized by the Gly-X-Y repeating triplets, with the X and Y positions being occupied predominantly by Pro and Hyp, respectively. Collagen biosynthesis begins with the mRNAs specific for each α-chain, followed by posttranslational hydroxylation of specific Pro by 3- or 4-Pro hydroxylases, both intra- and extracellularly. The 4-Pro hydroxylase is the major form of the enzyme in most tissues.

The normal occurrence of Gly at every third residue is a critical requirement for the formation of the collagen superhelical structure (Bae et al. 2008). Gly, as the smallest amino acid, with only a hydrogen atom side chain, can become part of the center of the superhelix without any steric hindrance, thus allowing the three helical α-chains to pack together tightly to form the final superhelix with a hydrophobic core (Regenstein & Zhou 2007). The superhelical structure is further strengthened by the conformational restrictions imposed by the pyrrolidine rings of the imino acids and also partly maintained by the interchain hydrogen bonds formed with the hydroxyl group of Hyp (Nagai et al. 2008).

The helix ends of each chain are capped by a short peptide extension, the telopeptide, which does not contain the Gly-X-Y repeats (George et al. 1999). This telopeptide domain determines the intermolecular interactions that contribute to and stabilize normal fibril assembly. The carboxyl terminus of the α_1(I) chain's telopeptide consists of 25 amino acid residues, and the amino terminus has 16 residues. The amino acid sequences of these telopeptides may vary between species, whereas certain cross-linking regions that are involved in fibril formation are highly conserved. Both the carboxyl and amino termini of each molecule have two cross-linking sites (McCormick 2009). Once secreted into the extracellular matrix, collagen molecules align head to tail in a quarter stagger array; therefore, the cross-linking site of one collagen molecule is close to that of another, allowing the occurrence of cross-linking at the telopeptide by oxidative deamination by the enzyme lysyl oxidase, which is specific for Lys or Hly.

The extent of cross-linking between collagens in vivo usually increases with age (McCormick 2009). On the basis of the extraction methods, collagens can be classified into salt-soluble, acid-soluble, and pepsin-soluble collagens. Salt-soluble collagen refers to the newly synthesized collagens that are not cross-linked in tissues and can be extracted using a cold neutral salt solution, usually leading to a low collagen yield and purity. An organic acid solution not only solubilizes the uncrosslinked collagens, but also breaks some interchain cross-linkages of collagens, such as the reducible aldimine condensation cross-links, leading to a further solubilization of collagens during extraction. Furthermore, a limited proteolysis at the telopeptides, where collagens are highly cross-linked by nonreducible trivalent bonds, will not affect the structural integrity of the super triple helix; therefore, various enzymes such as pepsin are used to facilitate solubilization of collagens from some raw materials where collagens are hard to extract with only an acid process (Regenstein & Zhou 2007, Heu et al. 2010).

For a direct and complete determination of the structure, collagen must be crystallized and then analyzed using X-ray crystallography. However, the structure of collagen has frequently been studied using various modern technologies such as circular dichroism (CD) and infrared spectroscopy, due to the smaller required sample sizes and the lower equipment costs. However, the results obtained using these techniques are indirect and must be used appropriately. The interpretation of the CD must take into account that there are no α-helixes, β-sheets, or β-turns in collagen. Thus, the normal software that comes with most CD machines may not be appropriate for collagen. The typical triple superhelical structure of native collagen molecules can be characterized with CD by the presence of a positive peak at approximately 220 nm and the negative peak at approximately 199 nm (Ogawa et al. 2004).

The specific wavenumbers of the amide I, II, and III peaks on the infrared spectra are directly associated with the conformation of collagen (Benjakul et al. 2010, Heu et al. 2010). Broadening of the amide I peak; an increase in the intensity of the amide III peak; and the shifts of the amide I, II, and III peaks to lower wavenumbers correspond to increased intermolecular hydrogen bonding. In addition, an absorption ratio of approximately one between the amide III peak and the 1,454-cm^{-1} band suggests that the triple superhelical structure of native collagen after isolation is well maintained.

The strong amide I peak of the infrared spectra, in the range of 1,600–1,700 cm^{-1}, is associated mainly with the C = O stretching vibration along the polypeptide backbone. Deconvolution of the amide I peak has been used to analyze the secondary structure of proteins, and the corresponding results have a positive correlation with those obtained by X-ray analysis (Muyonga et al. 2004a). However, great care must be taken when assigning each subpeak generated from deconvolution of the amide I peak. Using this method, the typical secondary structures that frequently occur in globular proteins, including α-helixes, β-sheets, β-turns, and random coils, have been reported to occur in the fibrillar native collagen and even in the corresponding limited pepsin

digested collagen. Using infrared spectroscopy, the secondary structure of a synthetic rat α_1(I)-chain amino telopeptide with 16 amino acid residues has been demonstrated to exist mainly in a random configuration at 4°C and changes to a β-sheet with a β-turn around 30°C (George et al. 1999). The occurrence of typical secondary structures in the telopeptide is reasonable; however, there are serious doubts concerning those structures reported in native collagen and especially in the pepsin digested collagen, given the main structure of the collagen molecule is a triple superhelix. Wang et al. (2008) reported that deconvolution of the amide I peak resulted in three components for collagen from the skins, scales, and bones of deep-sea redfish. The components at 1,633 and 1,660 cm^{-1} were attributed to the unwinding of the peptide chain and hydrogen bonds, respectively; the component at 1,696 cm^{-1} was sensitive to the extent of the intermolecular cross-linking.

Secondary structure analysis can also be done using the amide III peak (1,220–1,330 cm^{-1}), as water and water vapor have a negligible impact in this spectral region, whereas the absorption peak of the H-O-H bend vibration overlaps with the amide I peak. Moreover, the amide III peak region has the advantage that structural transitions can be determined easily without deconvolution, given the absorption peaks of α-helixes, β-sheets, and other secondary structures are usually well separated (Flores-Fernández et al. 2009). However, the amide III peak position and intensity are sensitive to both the composition of the side chains and the conformation of the backbone, and thus must be applied with caution to characterize protein secondary structures (George et al. 1999).

VARIATIONS OF COLLAGEN DEPENDING ON SOURCES AND SEASONS

At present, at least 29 distinct types of collagen, the products of more than 30 genes, have been detected in vertebrates and invertebrates (McCormick 2009). Each type of collagen varies in sequence, structure, and function, and therefore each one is distributed differently in the skins, bones, tendons, vascular systems, or intramuscular connective tissues, where they act to maintain the stability and structural integrity of the corresponding tissues and organs. Type I collagen, in particular, is identified as the major fibrillar collagen in almost all vertebrae connective tissues.

Traditionally, most commercial collagen and collagen-derived products are isolated from the processing by-products of land-based animals, mainly cows and pigs. These products have been used widely in the food, pharmaceutical, and cosmetic industries given their excellent biocompatibility, easy biodegradability, and weak antigenicity. However, the outbreaks of bovine spongiform encephalopathy and foot-and-mouth disease have caused some anxiety among health-conscious consumers of these traditional collagen and collagen-derived products. In addition, collagen and collagen-derived products from pigs and even cows that were not religiously slaughtered are unacceptable to Jews and Muslims (Regenstein et al. 2003). Beef gelatin is also prohibited for Hindus.

Recent research and commercialization of collagen from fish processing by-products have provided potential alternative sources of collagen. Sources for such products include the scales, skins, bones, and swim bladders. Scale collagen has a unique niche in the kosher market in that only scaled fish are regarded as kosher, and thus scale collagen is always derived from kosher fish (Regenstein et al. 2003). With the rapid development of the fish processing industry, large quantities of by-products are produced, accounting for 50–70% of the original raw materials. Optimal use of these by-products can avoid environment problems, produce value-added products to increase the revenue of the fish processing industry, and even create new job/business opportunities.

As in other vertebrates, type I collagen is identified as the major fibrillar collagen in most organs of fish. However, type V collagen was also identified in carp and spotted mackerel muscles (Sato et al. 1988, 1989), snakehead scales (Liu et al. 2009), and Japanese flounder muscles and skins (Nishimoto et al. 2005). Moreover, Mizuta et al. (2003) reported that collagen from the pectoral fin cartilage of skate consisted of three types of collagens, with type I and type II being the major ones and type XI being a minor one.

Imino acid content, especially Hyp, has been positively correlated with the thermal stability of collagens, which is one of the most important characteristics determining their potential use (Regenstein & Zhou 2007). The imino acid contents of collagens from fish species are generally lower than those of collagens from land-based animals (Regenstein & Zhou 2007, Bae et al. 2008). Therefore, there is an interest in seeking fish collagens with imino acid contents that are closer to those of collagens from land-based animals, in that the high content of imino acids is a key factor determining the feasibility of using fish collagen as a potential commercial substitute for its mammalian counterpart.

Currently, more than 20,000 different fish species have been identified. On the basis of their living environments, fish are usually classified into four groups: hot-water fish, warm-water fish, cold-water fish, and ice-water fish (Zhou & Regenstein 2009). In general, the collagens from hot- and warm-water fish have higher imino acid and Hyp contents than those from cold- and ice-water fish. Moreover, the imino acid compositions may also vary among collagens produced from different tissues and with different methods. For bighead carp, the imino acid contents of collagens from the internal tissues (swim bladder and bones) were only slightly higher than those of collagens from the external tissues (fins, scales, and skin) (Liu et al. 2012). During studies on skins from Nile perch, Muyonga et al. (2004a,b) indicated that the maturation stage of fish species did not significantly affect the imino acid compositions of collagens.

In addition, the imino acid compositions of collagens are similar among some closely related fish species, such as the skin and scale collagens of the typical freshwater fish: grass carp, black carp, silver carp, and bighead carp, all of which belong to the same family of *Cyprinidae* (P. Zhou, unpublished data). In some developing countries, these fish species are sometimes processed together, and the processing by-products are often not separated. These results suggest a need to develop techniques for processing by-products from multiple closely related fish species together in such a way as to retain product consistency between different batches.

In a recent study on collagens from skins and swim bladders of grass carp and bighead carp, no clear changes in imino acid contents were observed due to seasonal variation, although the year-round local environmental temperatures varied from an average of 4°C in January to 30°C in July (P. Zhou, unpublished data). The seasonal consistency of these fish collagens may allow the corresponding by-products from processing to be used as alternative sources for producing collagens on a year-round basis, without the concern that it will be necessary to modify the extraction process or the end use due to seasonal variation. However, Duan et al. (2012) reported that the denaturation temperatures of scale collagens from carp and silver carp in the summer were higher by 2°C than those of the winter equivalents, which may be attributed to the higher ratio of α_1-chains to α_2- and α_3-chains in the collagens from the summer carp. The higher thermal stability of scale collagens from the summer in the outermost part of the fish's body may be induced by the physiological adaptation of the cold-blooded fish to their environmental temperature, in that expression of structurally more stable collagens may be required for the survival of fish in the summer season (Duan et al. 2010). These results suggest that the seasonal variation of fish collagens may depend on their sources and on the extraction and analysis methods; therefore, comparisons and interpretation of results from different research groups should be done with caution.

GELATIN HYDROLYSATES, GELATIN-DERIVED PEPTIDES, AND BIOACTIVITIES

Development of Gelatin Hydrolysates and Gelatin-Derived Peptides

Gelatin is a heterogeneous mixture of peptides derived from the parent protein collagen by procedures involving the destruction of cross-linkages between the polypeptide chains along with some breakage of polypeptide bonds. A further more extensive enzymatic degradation of gelatin results in gelatin hydrolysates (Zhou & Regenstein 2004, 2005; Zhou et al. 2006).

Enzymatic hydrolysis is widely used to improve the functional and nutritional properties of food proteins (Samaranayaka & Li-Chan 2011). During hydrolysis of collagen and gelatin, the α- and β-chains are gradually degraded into smaller peptides with increases in the degree of hydrolysis (DH) (Khantaphant & Benjakul 2008, Klompong et al. 2008, Haug & Draget 2009). Electrophoretic analysis of fish gelatin hydrolysate showed peptide bands in the molecular weight range of 1.4–26 kDa (Khantaphant & Benjakul 2008, Alemán et al. 2011a). Commercial proteases such as alcalase, pepsin, trypsin, α-chymotrypsin, neutrase, papain, properase E, protamex, savinase, and NS37005 (Kim et al. 2001; Mendis et al. 2005a,b; Ngo et al. 2010; Alemán et al. 2011a; Himaya et al. 2012a,b; Zhang et al. 2012) as well as the endogenous proteases of fish (Khantaphant & Benjakul 2008, Phanturat et al. 2010) have been used for the production of fish gelatin hydrolysates. The molecular weight distribution of the gelatin hydrolysates depends on gelatin sources, enzyme types, and hydrolysis conditions. Squid gelatin hydrolysates prepared using alcalase, neutrase, or esperase contained higher proportions of peptides with molecular weights below 1,400 Da, whereas those produced using trypsin or NS37005 contained higher proportions of peptides with molecular weights above 1,400 Da (Alemán et al. 2011a).

Structure of Gelatin-Derived Peptides

Gly, Pro, and Hyp together account for slightly more than half of the approximately 1,000 amino acid residues in each of the three α-chains of collagen (Hulmes et al. 1973). The Gly-Pro-Hyp sequences may be up to approximately 10% of the molecule, whereas the Pro or Hyp residues of the typical repeating triplets are sometimes substituted by any of the other amino acid residues within the collagen sequences (Nimni & Harkness 1988, Damodaran 2007).

The sequences of a few gelatin-derived peptides have been identified. A peptide (His-Gly-Pro-Leu-Gly-Pro-Leu) isolated from hoki skin gelatin contained two repeating doublets (Gly-Pro) with Leu or His at the third position of the typical Gly-X-Y repeating triplets of gelatin (Mendis et al. 2005b). Another peptide (Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly) derived from Alaska pollock skin gelatin showed the typical gelatin sequence with Gly at every third residue position (Kim et al. 2001). A peptide (Gly-Pro-X-Gly-X-X-Gly-Phe-X-Gly-Pro-X-Gly-X-Ser) was isolated from giant squid gelatin and showed the gelatin tripeptide sequence with the X positions being occupied by either Hyp or Leu (Alemán et al. 2011a). The peptide Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu isolated from jumbo squid gelatin has two Gly-X-Y repeating sequences, although the first three residues would have difficulty forming the typical collagen superhelix (Mendis et al. 2005a). The tetrapeptide Pro-Ala-Gly-Tyr isolated from farmed Amur sturgeon skin gelatin contained both Pro and Gly, which was in agreement with the general gelatin tripeptide sequence (Nikoo et al. 2014). Zhang et al. (2012) purified two antioxidative peptides (Tyr-Gly-Asp-Glu-Tyr and Glu-Gly-Leu) from tilapia skin gelatin. Some peptides without the typical sequence of Gly-X-Y were also identified from crude gelatin preparations, including Gly-Gly-Phe-Asp-Met-Gly from Japanese flounder skins (Himaya et al. 2012b), Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro from Pacific cod skins (Himaya et al. 2012a), Gly-Pro-Ala-Glu

and Gly-Pro-Gly-Ala from Atlantic salmon skins (Li-Chan et al. 2012), Tyr-Gly-Asp-Glu-Tyr from tilapia skins (Zhang et al. 2012), Thr-Gly-Gly-Gly-Asn-Val from Pacific cod skins (Ngo et al. 2011), Leu-Ser-Gly-Tyr-Gly-Pro from tilapia skins (Sun et al. 2013), and Ser-Cys-His from Alaska pollock skins (Guo et al. 2013). Some of these peptides might actually come from impurities that were not removed during the extraction process. **Table 1** summarizes all of this information.

The Health Promoting Benefits of Gelatin Hydrolysates and Gelatin-Derived Peptides

The growing demands for foods with additional health promoting properties, i.e., functional foods, have led to the proposed use of food constituents with presumed safety given their origins. These then have the potential to improve human health after being tested for functional properties (Kim & Wijesekara 2010, Mills et al. 2011, Harnedy & FitzGerald 2012). Gelatin hydrolysates and gelatin-derived peptides are among those materials that have been studied for their potential biological benefits, such as antioxidant, antihypertensive, anticancer, antiphotoaging, and cholesterol-lowering effects.

Peptides produced during hydrolysis of collagens and gelatins might serve as potential natural antioxidants (Phanturat et al. 2010, Harnedy & FitzGerald 2012, Senphan & Benjakul 2014). The currently used synthetic antioxidants may have negative consequences on human health (Lobo et al. 2010, Shahidi & Zhong 2010). Therefore, with the increasing need for clean labels, natural antioxidants may be preferred to reduce the need for synthetic antioxidants in foods and pharmaceuticals (Velasco et al. 2010). Some peptides produced from fish skins have been shown to scavenge free radicals and reactive oxygen species, and also to chelate metal ions (Mendis et al. 2005a,b; Ngo et al. 2010; Himaya et al. 2012a,b; Kittiphattanabawon et al. 2012b; Zhang et al. 2012; Nikoo et al. 2014).

For the peptide His-Gly-Pro-Leu-Gly-Pro-Leu prepared from hoki skin gelatin hydrolysates using trypsin, the presence of both Gly and Pro and the locating of Leu at the C-terminus and His at the N-terminus were demonstrated to be associated with radical scavenging activity (Mendis et al. 2005b). According to Giri & Ohshima (2012), the presence of hydrophobic amino acids such as Pro, Ala, Val, and Leu at the N-terminus and the amino acids Tyr, Val, Met, Ile, Leu, Glu, and Trp at the C-terminus may contribute to the high radical scavenging activity of the corresponding peptides. In agreement with this principle, the peptide Pro-Ala-Gly-Tyr produced from Amur sturgeon skin gelatin hydrolysates using alcalase had Pro as the N-terminus and Tyr as the C-terminus, and therefore showed high radical scavenging activity (Nikoo et al. 2014). Gelatin generally contains very limited amounts of Tyr. However, this amino acid was identified in the peptides Tyr-Gly-Asp-Glu-Tyr (Zhang et al. 2012), Leu-Ser-Gly-Tyr-Gly-Pro (Sun et al. 2013), and Pro-Ala-Gly-Tyr (Nikoo et al. 2014), although the two former peptides did not have the typical gelatin tripeptide sequence. The antioxidant activity of Tyr-containing peptides may be attributed to the phenolic group of Tyr, which serves as a hydrogen donor to terminate the radical-mediated chain reaction (Sarmadi & Ismail 2010).

Many gelatin peptides have been reported to scavenge free radicals, whereas few have been identified that chelate metal ions. An exception is the Ser-Cys-His peptide produced from Alaska pollock skins, although this peptide might not be derived from gelatin (Guo et al. 2013). These results suggested that free radical quenching is the main antioxidation mechanism for gelatin-derived peptides (Mendis et al. 2005b).

Angiotensin I-converting enzyme (ACE) plays a key role in regulating blood pressure. ACE inhibitors can prevent ACE from synthesizing the potent vasoconstrictor, angiotensin-II, and can

Table 1 Marine gelatin hydrolysates and gelatin-derived peptides and their biological activities

Source	Enzyme	Biological Activity	MW (Da)	Peptide sequence	References
Alaska pollock skin gelatin	Pronase E	Antioxidant activity		Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly	Kim et al. 2001
Jumbo squid skin gelatin	Trypsin	Antioxidant activity	880 1242	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg	Mendis et al. 2005a
Hoki skin gelatin	Trypsin	Antioxidant activity	797	His-Gly-Pro-Leu-Gly-Pro-Leu	Mendis et al. 2005b
Brownstripe red snapper skin gelatin hydrolysate	Trypsin-like proteases from pyloric caeca extract	Antioxidant activity			Khantaphant & Benjakul 2008
Nile tilapia scale gelatin	Alcalase	Antioxidant activity	1382.57	Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe	Ngo et al. 2010
Squid inner and outer tunics gelatin hydrolysate	Protamex, trypsin, neutrase, savinase, NS37005, esperase, alcalase	Antihypertensive, anticancer and antioxidant activities			Alemán et al. 2011
Pacific cod skin gelatin	Papain	Antioxidant and ACE-inhibitory activity	388 485.5	Thr-Cys-Ser-Pro Thr-Gly-Gly-Gly-Asn-Val	Ngo et al. 2011
Blacktip shark skin gelatin hydrolysate	Papain	Antioxidant activity			Kittiphattanabawon et al. 2012
Squid skin gelatin hydrolysate	Pepsin	Antihypertensive activity			Lin et al. 2012

Atlantic salmon skin gelatin	Flavourzyme	Dipeptidyl-peptidase IV enzyme inhibitory activity	372.4 300.4	Gly-Pro-Ala-Glu Gly-Pro-Gly-Ala	Li-Chan et al. 2012
Pacific cod scale gelatin	Pepsin, trypsin, α–chymotrypsin	Antioxidant and antihypertensive activity	1301	Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro	Himaya et al. 2012b
Japanese flounder skin gelatin	Pepsin	Antioxidant activity	582	Gly-Gly-Phe-Asp-Met-Gly	Himaya et al. 2012a
Tilapia skin gelatin	Properase E, multifect neutral	Antioxidant activity	645.21 317.33	Tyr-Gly-Asp-Glu-Tyr Glu-Gly-Leu	Zhang et al. 2012
Blacktip shark skin gelatin hydrolysate	Papain	Antihypertensive activity, inhibition of human LDL cholesterol and DNA oxidation			Kittiphattanabawon et al. 2013
Chum salmon skin gelatin hydrolysates	Papain, alcalase	Cell proliferation, cycle progression and apoptosis			Fu et al. 2013
Tilapia skin gelatin	Properase E	Antiphotoaging activity	592.26	Leu-Ser-Gly-Tyr-Gly-Pro	Sun et al. 2013
Amur sturgeon skin gelatin	Alcalase	Antioxidant and cryoprotective effects	405.99	Pro-Ala-Gly-Tyr	Nikoo et al. 2014

therefore prevent hypertension (Mills et al. 2011, Ngo et al. 2011). ACE-inhibitory activity was observed for gelatin hydrolysates prepared from blacktip shark skins using papaya latex crude enzymes (Kittiphattanabawon et al. 2013); giant squid tunics using protamex, trypsin, neutrase, savinase, NS37005, esperase, or alcalase (Alemán et al. 2011a,b); and squid skin using pepsin (Lin et al. 2012). The peptide Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro produced from Pacific cod skin gelatin also has ACE-inhibitory activity, although it does not have a gelatin-like sequence (Himaya et al. 2012a). Again lacking a typical gelatin sequence, the purified peptides Thr-Cys-Ser-Pro and Thr-Gly-Gly-Gly-Asn-Val, prepared from Pacific cod skin using papain, inhibited ACE activity by 81 and 68%, respectively, at 500 μg/ml (Ngo et al. 2011). The presence of Pro and Hyp at the C-terminus is associated with ACE-inhibitory activity (Ichimura et al. 2009). Gelatin is rich in Gly-Pro-Hyp repeating sequences; therefore, peptides containing Pro and Hyp are highly likely to be present in gelatin hydrolysates (Kittiphattanabawon et al. 2013). Peptides without typical sequences, however, are also identified in gelatin preparations, and these peptides may be derived from impurities, probably elastin.

The peptides Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala produced from Atlantic salmon skin gelatin hydrolysates using flavourzyme inhibited the activity of dipeptidyl-peptidase IV, and thus showed the potential to be used in functional foods or pharmaceuticals against type 2 diabetes (Li-Chan et al. 2012). Alemán et al. (2011b) investigated the effects of squid gelatin hydrolysates prepared using protamex, trypsin, neutrase, savinase, NS37005, esperase, or alcalase against cancer cell lines MCF-7 (human breast carcinoma) and U87 (glioma). The esperase hydrolysates showed the highest cytotoxic effect on cancer cells and a relatively high cytotoxic effect was observed for alcalase hydrolysates.

Gelatin hydrolysates, with a DH ranging from 10 to 40%, were prepared from blacktip shark skins using papaya latex crude enzymes. They inhibited the oxidation of human low-density lipoprotein (LDL) cholesterol by 8.3–39.2%, mainly through their free radical scavenging and metal ion chelating activities (Kittiphattanabawon et al. 2013). The hydrolysate with 40% DH also partly inhibited hydroxyl- and peroxyl-radical-induced DNA scission, and 35 and 74% of the supercoiled DNA strands were retained in the peroxyl and hydroxyl radical systems, respectively. Sun et al. (2013) investigated the effect of the peptide Leu-Ser-Gly-Tyr-Gly-Pro from tilapia skin gelatin on UV-induced damage to mice skin. These peptides protected the skin cells' antioxidative systems, including superoxide dismutase, catalase, glutathione peroxidase, and glutathione, and thus less damage occurred to lipids and collagen. Ngo et al. (2010, 2011) investigated the antioxidant activity of the tilapia scale gelatin hydrolysates and the corresponding peptide of Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe as well as the Pacific cod skin gelatin hydrolysates and the corresponding peptides of Thr-Cys-Ser-Pro and Thr-Gly-Gly-Gly-Asn-Val in free radical–mediated oxidative systems. Both the gelatin hydrolysates and peptides without typical gelatin sequences effectively scavenged intracellular free radicals and inhibited hydroxyl radical–induced DNA damage. These hydrolysates and peptides were noncytotoxic to mouse macrophages (RAW 264.7) and human lung fibroblasts (MRC-5). Himaya et al. (2012b) reported that the peptide Gly-Gly-Phe-Asp-Met-Gly produced from Japanese flounder skin gelatin upregulated the expression of the antioxidative enzymes dismutase-1, glutathione, and catalase at 10–50 μM, although this peptide did not have a typical gelatin sequence.

Potential Application of Gelatin Hydrolysates and Gelatin-Derived Peptides in Foods

Gelatin hydrolysates and gelatin-derived peptides can be used as potential functional ingredients for their potential antioxidative and cryoprotective properties. For surimi subjected to freeze-thaw

cycles, a blacktip shark skin gelatin hydrolysate showed a cryoprotective effect that was comparable to that of a commercial cryoprotectant, a mixture of sucrose and sorbitol (1:1, w/w), which adds unwanted sweet flavor to the surimi (Kittiphattanabawon et al. 2012a). After the freeze-thaw cycle, the highest Ca^{2+}-ATPase activity and the lowest surface hydrophobicity were obtained by the addition of gelatin hydrolysate with 10% DH. The loss of total sulfhydryl groups was prevented, whereas no differences in the contents of disulfide bonds were observed between surimi with an added sucrose/sorbitol mixture and the gelatin hydrolysate, suggesting that gelatin hydrolysates can also prevent the denaturation of the surimi proteins. Blacktip shark skin gelatin hydrolysates with a 40% DH slowed the formation of thiobarbituric acid reactive substances in a cooked comminuted pork model system at 500 and 1,000 ppm during refrigerated storage (Kittiphattanabawon et al. 2012b). The inhibition of oxidation was attributed to the radical scavenging activity of the gelatin hydrolysates as well as its chelating of Fe^{2+}, a pro-oxidant present in meat systems.

A bovine gelatin peptide fraction, with molecular weights in the range of 700–1,400 Da, was reported to reduce ice crystal growth by a factor of 10 in an ice cream mixture (Wang et al. 2009). The repeating sequence of Gly-Pro-Y and Gly-X-Y in gelatin hydrolysates was associated with their effects on inhibiting ice crystal growth (Damodaran 2007). As demonstrated by low-field nuclear magnetic resonance, a tetrapeptide Pro-Ala-Gly-Tyr isolated from Amur sturgeon skin gelatin prevented water loss of a Japanese sea bass mince subjected to different freeze-thaw cycles (Nikoo et al. 2014). After six freeze-thaw cycles, both a larger population of myofibrillar water (T_{21}) and a smaller population of free water (T_{22}) were obtained with the minces supplemented with 25 ppm of this peptide, compared to minces not supplemented with the peptide. In addition, lipid oxidation in the minces was decreased when the peptide was incorporated at 25 ppm, as evidenced by the lower hydroperoxides observed in the Fourier transform infrared spectra. The gelatin-derived cryoprotective tetrapeptide Pro-Ala-Gly-Tyr also showed a combined effect with a nonpeptide antioxidant (caffeic acid) in retarding quality losses in minces resulting from freezing and thawing (Nikoo et al. 2015). Minces supplemented with a combination of this cryoprotective tetrapeptide (12.5 ppm) and 50 ppm caffeic acid had the greatest effect in lowering lipid and protein oxidation and in maintaining the myofibrillar water pools in the frozen/thawed minces. In addition, a higher thermal stability of myosin was observed in the minces using differential scanning calorimetry.

POTENTIAL BIOLOGICAL EFFECTS OF ORAL INGESTION OF COLLAGEN AND COLLAGEN-DERIVED PRODUCTS ON SKIN PHYSIOLOGY

In recent years, various nutritional and functional foods containing collagen and collagen-derived products such as gelatin and collagen peptide have been marketed for use in the field of skin care. These products have gained commercial popularity in the marketplace, at least partly due to the claimed beneficial biological effects on skin conditions (e.g., anti-aging effects). However, scientific validation of these skin claims is currently limited. It was commonly assumed that, as is the case for other protein and protein-derived products, collagen and collagen-derived products would be degraded into amino acids during the digestion and absorption steps in the gastrointestinal tract. As such, there are serious doubts concerning the claimed biological efficacy of collagen and collagen-derived products. This section aims to describe the current understanding of the metabolic fate of orally ingested collagen and collagen-derived products and the corresponding effect this might have on skin physiology.

Physiological Functions of Collagen and Collagen-Derived Products on Skin

The skin, the human body's largest organ, protects the body against various external insults, and it comprises an epidermis and dermis. The epidermis consists of a stratified squamous epithelium; the dermis contains two major extracellular matrix components, collagen and glycosaminoglycan, which are produced mainly by fibroblasts (Matsuda et al. 2006). Collagen is the major structural constituent of the connective tissues such as the dermis and the tendons in the extracellular matrix.

On the basis of their conformations, the currently identified collagens can generally be classified into one of the following three groups: fibrous collagen, fibril-associated collagen, and basement membrane collagen (Matsuda et al. 2006). In the dermis, type I collagen is the most abundant collagen, and it assembles into collagen fibrils that further polymerize into collagen fibers to form the backbone structure of the dermis in association with other types of fibrous and fibril-associated collagens. Thus, the size of the collagen fibril determines the biomechanical and physiological properties of the skin, and it is reportedly regulated by the content of type III or V collagen or other types of nonfibrous collagens and also by the rate of collagen synthesis and degradation. Type IV collagen forms a highly cross-linked network that maintains the mechanical stability of the basement membrane; for example, its content seems to be lower at the bottom of a wrinkle. Therefore, collagen atrophy is widely regarded as the major characteristic of skin aging (Zague et al. 2011). Glycosaminoglycans consist of alternating two sugar units and exist in the skin mainly as dermatan sulfate and hyaluronic acid (Matsuda et al. 2006, Ohara et al. 2010). Dermatan sulfate is located on the surface of the collagen fibril in the form of the proteoglycan decorin, which transmits force among collagen fibrils by covalently interconnecting them. Decorin also acts to resist compression, to promote fibril elongation, and to regulate the size of the collagen fibril. Hyaluronic acid has a high molecular weight in the approximate range of 10^5–10^7 Da and also a large hydrodynamic volume and a strong hydrophilicity, which consequently allows hyaluronic acid to interact with a large amount of water to form a highly viscous and elastic solution that serves to provide space-filling, lubricating, and filtering functions. The synthesis of hyaluronic acid is independently regulated by the *hyaluronan synthase genes 1*, *2*, and *3*; for example, the type 1 gene produces low molecular weight hyaluronic acid, and only the type 2 gene is responsible for hyaluronic acid synthesis in cultured dermal fibroblasts (Itano & Kimata 2002). Hyaluronic acid injection has now been proven to be minimally complicated and among the safest operations of the many injection practices in reconstructive and cosmetic medicine. Moreover, hyaluronic acid has been suggested to be involved in many biological processes; for example, hyaluronic acid levels are significantly elevated during wound healing, cell migration, tissue turnover, malignant transformations, and embryogenesis (Chen & Abatangelo 1999). These observations suggest that the diameter of the collagen fibril, and the amount and ratio of dermatan sulfate and hyaluronic acid, may work in synergy to determine the skin's condition and resist various external mechanical stresses.

Skin characteristics, including appearance and integrity, are known to be influenced by various endogenous and exogenous factors, including aging, hormones, nutrition, and UV radiation, which may impair skin metabolism, such as upregulating synthesis of matrix metalloproteinases that can degrade almost all components of the skin's extracellular matrix including collagen, elastin, and proteoglycan (Zague et al. 2011). Additionally, skin hydration and elasticity are largely influenced by the metabolism of dermal collagen fibers.

Matrix metalloproteinases, comprising a family of structurally associated molecules, includes interstitial collagenases (MMP1 in humans and MMP13 in rodents) and gelatinases (MMP2 and MMP9). MMP1 and MMP13 in particular can initiate the degradation of type I collagen, and MMP9 further degrades the resulting collagen fragments. However, MMP2 degrades

type IV collagen, contributing to collagen deficiency and the formation of skin wrinkles and furrows. Besides injuring the dermis by directly degrading collagen, matrix metalloproteinases can also indirectly inhibit collagen synthesis by producing collagen fragments, which serve as negative regulators for type I collagen biosynthesis. However, MMP9 can diminish this inhibition by further breaking down collagen fragments. Taken together, matrix metalloproteinases may play a potentially important role in the metabolism and aging process of skin. A healthy skin condition largely reflects a person's general health status and is an important factor in the quality of life.

Skin condition is affected by the daily consumption of dietary substances, especially by imbalanced and incomplete diets. Therefore, increasing attention is paid to the influence of nutritional factors on skin physiology, and several studies have investigated the effect of dietary supplementation on the skin's condition, using cultured cells, animal models, and human trials. Collagen and collagen-derived products have low protein nutritional values due to a deficiency or even complete absence of some essential amino acids; however, they have long been used in food supplements and pharmaceuticals given their beneficial biological functions, especially maintaining and improving skin conditions. Some preclinical human trials and animal experiments have indicated that the oral ingestion of gelatin might have beneficial effects similar to those of collagen hydrolysates.

In a preclinical trial, Matsumoto et al. (2006) investigated the effects of daily intake of 7 g of a commercially available collagen hydrolysate mixture containing 5 g of fish type I collagen hydrolysates on the skin properties of 25 Japanese female volunteers (35 ± 5 years old) who tended to have dry and rough skin in winter. After 6 weeks of ingestion, the moisture contents in the stratum corneum of the face cheek, forearm, and back of the neck increased, suggesting skin condition improved, in that the presence of an adequate amount of water in the stratum corneum is a prerequisite for the general appearance of smooth and soft skin. The viscoelastic properties including pliability and elasticity also significantly improved. Microscopic analysis also showed a significant improvement in the skin surface properties of the face cheek, such as greater smoothness, fewer wrinkles, and less roughness. Moreover, the skin surface pH remained unchanged at a weakly acidic pH of 5.9 during the whole study period, indicating the maintenance of good skin conditions.

During chronological aging, quantitative and qualitative changes occur in the skin, including loss of elasticity, formation of wrinkles and xerosis, and reduction in epidermal thickness and collagen content, especially for postmenopausal women. In a double-blind and placebo-controlled (maltodextrin) trial, Proksch et al. (2014) investigated the effects of daily ingestion of 2.5 or 5 g of porcine type I collagen hydrolysates with an average molecular weight of 2.0 kDa on the skin biophysical parameters for cutaneous aging of 69 women aged 35 to 55 years. After 8 weeks of ingestion, a significant increase in skin elasticity was observed in both dosage groups compared to the placebo-controlled group. At 4 weeks after the last ingestion for the treatment group, a longer lasting positive physiological effect on skin elasticity was still observed with the elderly women over 50 years old. Moreover, a positive effect of collagen hydrolysate ingestion on skin moisture and evaporation was also observed with the elderly women. These findings are opposite to those of the topical skin care products, which increased skin elasticity predominantly by enhancing epidermal hydration (Xhauflaire-Uhoda et al. 2008). The classically applied creams and lotions did not penetrate deeply into the skin barrier, and the skin moisturizing effects disappeared once treatment stopped; the slight increase in skin hydration was due to the newly formed corneocytes from the deeper skin layer. Moreover, long-term topical treatment with a hydrocarbon cream caused damage to the skin barrier and increased transepidermal water loss (Buraczewska et al. 2007). However, in a double-blind clinical trial with 20 healthy women volunteers aged 40 to 62 years, a gel formula containing 3% of the collagen-like hexapeptide was topically applied on skin in the eye zone area twice a day for 4 weeks. This resulted in significant reductions in the total

surface area of wrinkles, the number and average depth of the wrinkles, and the total and average length of the wrinkles, suggesting that the collagen-like peptide may penetrate the wrinkles to achieve its physiological effect on skin (Bauza et al. 2004).

Oral ingestion of collagen and collagen-derived products may also have a beneficial effect on ultraviolet (UV) radiation–induced skin damage. On the basis of wavelength, UV is classified into three categories: UV-A (400–315 nm), UV-B (315–280 nm), and UV-C (<280 nm). Ultraviolet B radiation causes the generation of reactive oxygen species that damage the antioxidative defense systems of the skin, resulting in immune inhibition and cancer formation through the oxidation of intra- and extracellular components. Repeated skin exposure to UV-B radiation usually leads to photoaging of skin, as characterized by wrinkles, laxity, roughness, irregular pigmentation, and the formation of small dilated blood vessels near the skin surface. In an animal experiment with 6-week-old male Hos:HR-1 hairless mice, Tanaka et al. (2009) investigated the effect of daily administration of collagen peptides (0.2 g/kg body weight) prepared from fish scales on skin damaged by repeated exposure to UV radiation (UV-B, 0.3 mW/cm^2) over 6 weeks. The oral administration of the collagen peptides suppressed the UV exposure–induced epidermis hyperplasia that resulted from proliferation of keratinocyte, and also suppressed the decrease in both the stratum corneum hydration and soluble type I collagen content. This suggests that collagen peptides ingested as a dietary supplement can protect skin from UV exposure–induced damage and photoaging of both the dermis and epidermis. The beneficial effects of oral ingestion of collagen and collagen-derived products on UV-B radiation–induced skin damage may be explained by the potential antioxidative and biological activity of the corresponding food-derived collagen peptides (Yaar & Gilchrest 2007, Tanaka et al. 2009). Some collagen-derived peptides have been demonstrated to be antioxidative in vitro (Kim et al. 2001); however, whether their concentrations in the skin after oral ingestion are high enough to show the antioxidative activity in vivo remains to be further elucidated.

With the aim to determine whether the effect of ingestion of collagen and collagen-derived products on skin is collagen specific or is due to the ingestion of protein and protein-derived products themselves, Minaguchi et al. (2005) evaluated the effects of daily administration of two doses (0.2 and 1.0 g/kg body weight) of collagen peptides on the extracellular matrix of the Achilles tendon. As a control, they administered lactalbumin or water alone to rabbits for 56 days. The ingestion of collagen peptides affected the size of the collagen fibrils and the composition of the glycosaminoglycans in a dose-dependent and protein-specific manner so as to improve the mechanical properties of the Achilles tendon. Although the experiment was done using only tendons, this observation may also be extended to skin effects, given both tissues contain type I collagen as the major component of their extracellular matrixes.

Bioavailability of Collagen and Collagen-Derived Products

Before speculating about the potential mechanism underlying the beneficial effects of ingested collagen and collagen-derived products on skin, their bioavailability after oral ingestion must be clarified. To be biologically active on skin, orally ingested collagen and collagen-derived products must be able to cross the intestinal barrier to get into the blood circulation system in some form and be transported to the skin in sufficient quantity so as to play a role in metabolic processes, or even just to be incorporated in the skin.

In an animal experiment with male mice (C57/BL, 21–25 g body weight), a ^{14}C–labeled gelatin hydrolysate (10 mg and 580 Bq/g body weight) with a mean molecular weight of 6.1 to 6.8 kDa was administered, and the time course of gelatin hydrolysate absorption and its subsequent distribution in various organs were evaluated (Oesser et al. 1999). The control mice were administered

^{14}C-labeled Pro along with an unlabeled gelatin hydrolysate. Within the first 6 h after oral administration of the ^{14}C-labeled gelatin hydrolysate, more than 90% of the enterally administered radioactivity had been absorbed from the gastrointestinal tract, and the radioactivity in the plasma simultaneously attained its peak value. In addition, the radioactivity in the skin reached maximal concentration 12 h after administration and maintained 58% of the maximal value up to 192 h, in contrast to plasma for which more than 85% of the radioactivity disappeared after 24 h. In a further absorption experiment using gut-sac prepared from 4–6-cm pieces of the small intestines of C57/BL mice, Oesser et al. (1999) showed that peptides with molecular weights ranging from 1 to 10 kDa were able to transit from the mucosa to the serosa side of the intestine, suggesting that gelatin hydrolysates were to some extent absorbed as relatively high molecular weight components.

In another animal experiment, 5-week-old Wistar rats were administered 288 mg of a chicken feet collagen hydrolysate (800 Da average molecular weight) containing Gly-[^{14}C]Pro-Hyp and [^{14}C]Pro as radioactive tracers for the peptide and the amino acid, respectively (Watanabe-Kamiyama et al. 2010). The low molecular weight collagen hydrolysate was partially absorbed into the blood in the peptide form. The radioactivity in the plasma reached a maximal value 3 h after administration, and the corresponding maximal value in the skin was observed during the following 3 h. On the fourteenth day after administration, 70% of the maximal radioactivity was still found in the skin, whereas radioactivity for the most part disappeared in other tissues such as the liver, kidney, spleen, cartilage, femur, tibia, brain, and muscle, suggesting a final lasting preferential accumulation of absorbed collagen hydrolysate in the skin of rat.

It is generally believed that collagen and collagen-derived products are hydrolyzed into amino acids in the gastrointestinal tract prior to being absorbed into the blood circulation system. However, increasing evidence indicates that peptides might also be absorbed directly. Using chromatography methods, Iwai et al. (2005) found that the gelatin hydrolysate–derived Hyp in the peptide form appeared in the blood of healthy human volunteers who ingested 9.4–23 g of gelatin hydrolysates from porcine skin, chicken feet, and cartilage after 12 h of fasting, and reached a maximum concentration of 20–60 nmol/ml plasma within 1–2 h subsequent to ingestion and then was reduced to half of the maximum value at 4 h after oral ingestion. The ratio of Hyp in the peptide form to the free form was approximately 1:3. Porcine skin and chicken feet mainly consist of type I collagen, whereas chicken cartilage predominantly consists of type II collagen. In the case of ingestion of type I collagen–derived gelatin hydrolysates, more than 90% of the corresponding peptides in the blood were identified to be Pro-Hyp. Pro-Hyp also accounted for 70% of the peptides for subjects who ingested type II collagen–derived gelatin hydrolysates, with 19% of the peptides being Pro-Hyp-Gly. Additionally, small but significant quantities of Ala-Hyp, Ala-Hyp-Gly, Pro-Hyp-Gly, Leu-Hyp, Ile-Hyp, and Phe-Hyp were also detected, whereas peptides larger than tripeptides were not detected. Using a chromatography method with precolumn derivatization with phenyl isothiocyanate, Shigemura et al. (2011) identified Hyp-Gly as another major peptide besides Pro-Hyp in the blood of healthy human volunteers after ingestion of a fish scale collagen hydrolysate (25 g/kg body weight) with an average molecular weight of 1 kDa. The ratio of Hyp-Gly to Pro-Hyp ranged from 0 to 5.0, depending on the subject. Hyp-Gly cannot be resolved by conventional chromatography methods without precolumn derivatization due to its hydrophilic nature and small molecular weight (Iwai et al. 2005). In contrast to other conventional derivatives, peptides derivatized with phenyl isothiocyanate can be directly applied to a peptide sequencer based on the Edman degradation reaction.

Previous methods to estimate the concentration of Hyp-containing peptides in the plasma involved the subtraction of the free form of Hyp from the total Hyp to get the total Hyp in the peptide form. Subsequently, the concentration of individual collagen-derived, Hyp-containing

peptides was determined semiquantitatively by identifying the recovery of Hyp in each peptide peak on the chromatographic elution curves. Using a more sensitive and convenient liquid chromatography–tandem mass spectrometry method, the detection and quantification limits were 0.01 and 12.5–1,000 pmol/ml in plasma, respectively (Ichikawa et al. 2010). In addition, nine Hyp-containing peptides were simultaneously quantified in the blood of five healthy male volunteers (33.0 ± 6 years old) after oral ingestion of a fish scale gelatin hydrolysate (0.385 g/kg body weight) with an average molecular weight of 5 kDa. At 1–2 h after ingestion, Pro-Hyp was identified to be the major component with maximal concentration of 60 ± 6 nmol/ml; the minor components were Ala-Hyp, Phe-Hyp, Leu-Hyp, Ile-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Gly-Pro-Hyp, and Pro-Hyp-Gly with maximal concentrations in the range of 0.67–23.8 nmol/ml. Moreover, the concentrations of Hyp-containing peptides were significantly higher than those of other peptides after oral ingestion. Using the liquid chromatography–tandem mass spectrometry method, high plasma levels of Pro-Hyp and Hyp-Gly were also quantified in five healthy male volunteers who ingested 8 g of porcine skin collagen hydrolysate with an average molecular weight of 1.2 kDa and the Hyp-Gly–to–Pro-Hyp ratio varied in the range of 0.063–0.221 (Sugihara et al. 2012). At 1 h after ingestion, the Hyp-containing peptides increased to a maximal concentration of 32 nmol/ml in the plasma.

In a single blind crossover study, five healthy male volunteers (33 ± 6 years old) ingested type I collagen hydrolysates (0.385 g/kg body weight) with an average molecular weight of 5 kDa from fish scales, fish skins, and porcine skins after 12 h of fasting (Ohara et al. 2007). Within 24 h after ingestion, the total area under the concentration-time curve of Hyp-containing peptides in the plasma in different cases decreased in the following order: fish scales > porcine skins ≥ fish skins. Hyp in the peptide form comprised approximately 30% of all detected Hyp. In the cases of the fish scale group and the fish skin group, Pro-Hyp accounted for 39 and 42% of the collagen-derived peptides in the plasma, respectively, with Ala-Hyp and Leu-Hyp also found in relatively high concentrations; in the case of the porcine skin group, however, 95% of the collagen-derived peptides were identified to be Pro-Hyp.

The above-mentioned studies suggested that the structure and quantity of collagen-derived peptides in human blood depended on the collagen type and source as well as the subject, in that the amino acid sequence of collagen differs with origin, and polymorphism may exist in both the endo- and exopeptidase activity and in the intestinal peptide transporter (PEPT) selectivity between subjects. These results may offer clues to the potential mechanism underlying the beneficial biological effects of oral ingestion of collagen and collagen-derived products.

Most of the collagen-derived peptides in the blood contained Hyp, and Pro-Hyp were identified as the major peptides in most cases, with Hyp-Gly simultaneously reaching a relatively high level in some cases. It has been difficult to measure and isolate small quantities of food-derived peptides containing no marker amino acids or amino acids with posttranslational modifications (e.g., Hyp) from animal and human blood, which is rich in complex interfering constituents after oral ingestion, owing to the sensitivity and resolution limitation of the current analytical methods (Ichikawa et al. 2010). Moreover, the triple helix domain of a collagen molecule has a high abundance of the Gly-Pro-Hyp repeating motif, which may partly explain the high levels of collagen-derived, Hyp-containing peptides in blood, especially Pro-Hyp. On the basis of the primary structures of type I collagen subunits, approximately 1.7 g of Pro-Hyp potentially could be released from 25 g of gelatin (Iwai et al. 2005).

Some peptides are frequently degraded within a short time by host peptidases in the blood. For example, half-lives of 0.8, 2, 2.5, and 8.6 min, respectively, were reported for Ala-Tyr, Gly-Tyr, Ala-Gln, and Gly-Gln after intravenous injection in human blood (Hubl et al. 1989, Druml et al. 1991). However, it was assumed that Hyp-containing peptides were highly stable in organisms,

given enzymatic systems that could hydrolyze the corresponding peptide bonds rarely occurred (Ashmarin 2007). More than 75% of the synthetic Pro-Hyp remained even after 24 h of in vitro incubation with human serum at 37°C, suggesting that Pro-Hyp is resistant to digestion by human serum peptidases (Iwai et al. 2005). Pro-Hyp has been shown to be excreted in urine in an intact form. Shigemura et al. (2011) also reported that the degradation rates of synthetic Pro-Hyp and Hyp-Gly were only 5.5 and 0.5%, respectively, after in vitro reaction with human serum for 3 h at 37°C, indicating that Hyp-Gly is more resistant to peptidases in human blood than Pro-Hyp.

The small intestinal mucosa comprises a layer of epithelial cells compartmentalized into villi and crypt regions by the proliferation and differentiation of enterocytes. It provides the primary site for absorption of nutrients such as amino acids and oligopeptides, requiring the following two transmembrane processes: uptake by the epithelia from the lumen across the brush border membrane and subsequent transfer to the blood across the basolateral membrane (Cheng & Leblond 1974). There are three potential mechanisms for the transcellular transport of oligopeptides across the intestinal epithelial cells, including a transporter-mediated pathway, a transcytotic route, and an intracellular passive transport. PEPT1, localized on the brush border membrane, is an electrogenic protein and peptide symporter that is able to transport almost all potential di- and tripeptides. It has a different proton-to-substructure stoichiometry when transporting different peptides whose charges were affected by pH. The acidic pH gradient across the small intestinal epithelial apical membrane is maintained in part by the Na^+/H^+ antiporter activity on the brush border membrane (Daniel 2004).

The Caco-2 cell line, derived from a human intestinal adenocarcinoma, has been used to generate a cell culture model for the small intestinal epithelia, because of its spontaneous enterocyte-like differentiation to show morphological polarity and brush border functions such as the expression of peptidases and transporters (Hidalgo & Brochardt 1989). However, the activities of transcytosis and PEPT1 in Caco-2 cells are lower than those of epithelia isolated from animal intestines. Porcine brush border membrane vesicles are preferred for in vitro models to assess di- and tripeptide uptake, given these vesicles can structurally and functionally mimic the in vivo biology of the human intestinal apical membrane (Aito-Inoue et al. 2007).

Using porcine brush border membrane vesicles prepared from the jejunal mucosa of Yorkshire grower pigs (70–75 days old), Aito-Inoue et al. (2007) investigated the mechanism for the transcellular transport of Gly-Pro-Hyp, which shows potential bioactivity. The resulting vesicle had an intravesicular space of 1.1–1.6 μL/mg of membrane proteins. Gly-Pro-Hyp was gradually degraded into Gly and Pro-Hyp on the apical side of the vesicle, accompanied by the occurrence of Pro-Hyp in the intravesicular space. However, free forms of Hyp and Pro were not detected. The transepithelial transport of Pro-Hyp across the brush border membrane was faster within the first 30 min of incubation in the presence of a pH gradient of 5.5 on the apical side to 7.4 on the basolateral side, and was partly prevented by the addition of Gly-Pro, a known strong competitive substrate for PEPT1. This indicated the presence of other potential routes for the uptake of Pro-Hyp, such as simple diffusion given the leaky nature of brush border membrane vesicles in vitro. These results suggested that Gly-Pro-Hyp could be partially degraded to remove Gly by the brush border membrane bound aminopeptidase N, and the resulting Pro-Hyp was, at least in part, transported into the small intestinal epithelia via a proton-coupled PEPT1-mediated pathway. Moreover, intact Gly-Pro-Hyp could not cross the intestinal epithelial apical membrane, and Pro-Hyp was highly resistant to intestinal mucosal apical peptidases. However, by doing animal experiments with Wistar rats administered low molecular weight collagen hydrolysates containing a high amount of Gly-Pro-Hyp, Watanabe-Kamiyama et al. (2010) demonstrated that Gly-Pro-Hyp was transported to the blood through the intestinal brush border membrane in an intact

form. This difference might be attributed to the variations in substrate specificities between rat and porcine aminopeptidases bound on the intestinal epithelial apical membrane.

Potential Mechanisms to Explain the Effects of Collagen and Collagen-Derived Products on Skin

From a nutritional point of view, supplementation with collagen and collagen-derived products may potentially stimulate extracellular matrix anabolic metabolism. This leads to an increase of collagen biosynthesis in the skin using the corresponding absorbed degradation products as substrates, and to a promotion of the repair processes in dermal wounds. These positive effects might result from the biochemical similarities of exogenous collagen, such as the unique amino acid and peptide profiles, to the endogenous collagen in connective tissue and in particular to the major type I collagen in the dermis. Watanabe-Kamiyama et al. (2010) substantiated this hypothesis by hydrolyzing the skin of Wistar rats on the fourteenth day after administration of 288 mg of low molecular weight collagen hydrolysate with 570 kBq Gly-[^{14}C]Pro-Hyp; they then analyzed the samples with thin layer chromatography. The resulting autoradiograph of the thin layer chromatography profile showed two spots corresponding to Pro and Hyp, suggesting that an orally administered collagen hydrolysate was absorbed and then used for the synthesis of skin proteins.

Given that type I collagen levels gradually decrease during the course of a lifetime and hence induce skin aging, an increase of type I collagen synthesis after oral ingestion of collagen and collagen-derived products is desired for its anti-aging benefits. Zague et al. (2011) investigated the effect of continuous daily ingestion of a collagen hydrolysate on skin extracellular matrix proteins, in an animal experiment with 4-week-old male Wistar rats that were administered for 4 weeks a (*a*) modified AIN-93 diet (isocaloric and isoproteic) containing 12% casein as the reference group, a (*b*) bovine hide collagen hydrolysate with an average molecular weight of 3,600 Da as the treatment group, or a (*c*) nonprotein modified AIN-93 diet as a negative control group. Compared to the reference and control group, ingestion of the collagen hydrolysate significantly increased the relative expression of type I and IV collagens as evaluated using an immunoblot; both the proenzyme and active forms of MMP2 were significantly suppressed, as zymography shows (Zague et al. 2011). The activity of MMP9 was not significantly influenced by the collagen hydrolysates. On the basis of these results, Zague et al. (2011) hypothesized that the collagen-derived peptides appeared in the skin after oral ingestion of a collagen hydrolysate. This might also simulate their effect in injured skin, which might prevent further collagen breakdown by matrix metalloproteinases; it may also mitigate the indirect inhibition of collagen biosynthesis by collagen fragments generated by the matrix metalloproteinases. These results suggested in addition that daily ingestion of collagen and collagen-derived products may reduce aging-associated changes in the extracellular matrix by stimulating anabolic processes in the skin in a collagen-specific manner. Moreover, this anti-aging effect was not simply dependent on a general increase in amino acid intake.

Generally, a biologically active peptide screened by in vitro assays from food protein hydrolysates cannot be used in feeding experiments because of the difficulty in obtaining a quantity large enough of the active peptide. Moreover, unlike other functional compounds, some active peptides in food protein hydrolysates are further degraded by the host peptidases during the courses of ingestion, digestion, and absorption, leading to the loss of their potential biological activities measured using in vitro assay after ingestion. Alternatively, some inactive peptides measured using in vitro assay can be changed to their active forms by further limited degradation. Consequently, peptide activity measured using an in vitro assay does not always directly express

the corresponding biological activity for humans after ingestion. Therefore, the potential activity of a peptide should be screened with a feeding experiment aimed at isolating and identifying the food-derived peptides in the blood and target organs (Aito-Inoue et al. 2006, Sato et al. 2008). Various peptides identified from these in vivo experiments can be chemically synthesized for use in both in vivo and in vitro experiments to investigate their transportation mechanism, metabolic fate, and potential biological activity.

An in vitro study using a cell culture system showed that Pro-Hyp and Pro-Hyp-Gly had chemotactic activity toward human fibroblasts, peripheral blood neutrophils, and monocytes in Boyden chemotaxis chambers (Postlethwaite & Kang 1976, Postlethwaite et al. 1978, Laskin et al. 1986). The collagen-derived peptides generated by digesting type I, II, and III collagens using a bacterial collagenase were chemoattractants for fibroblasts. Moreover, some Hyp-containing synthetic di- and tripeptides were also chemotactic. Under physiological conditions, fibroblasts are quiescently buried in collagen fibrils. Platelet-derived growth factor released from damaged tissues can stimulate fibroblasts in the collagen gel and enhance their mobility; the activated fibroblasts can then synthesize collagen and other components of the extracellular matrix (Li et al. 2004). Migration of these cells has been confirmed to play a significant role in the early stages of wound healing and inflammation. Given that collagen is degraded and remodeled at the sites of tissue damage and inflammation, these findings indicated that the resulting collagen-derived peptides might act as biological messengers of degradation of the extracellular matrix and initiate the wound healing process by playing a role in stimulating the migration of fibroblasts and other cells in vivo to effectively repair the injured tissues. As such, it seems likely that the potential beneficial biological effects of oral ingestion of collagen and collagen-derived products may be obtained by humans normally along with additional benefits in the case of a pathological condition from food-derived peptides, including Pro-Hyp, a major collagen-derived peptide regardless of the sources. These food-derived collagen peptides may function as biological messengers for the underlying cell signaling pathway to help stimulate fibroblasts and trigger the biosynthesis of new collagen fibers and hence the reorganization of the extracellular matrix (Zague 2008). However, the millimolar concentrations of collagen-derived peptides needed for in vitro chemotactic activity were significantly higher than plasma micromolar concentrations (50–200 μM) of food-derived collagen peptides. In addition, fibroblasts will not grow spontaneously in the extracellular matrix or on a collagen gel, whereas they grow so rapidly in plastic wells that they reach confluence within a few days (Kono et al. 1990). Thus, it is possible that the in vitro response of fibroblasts in the Boyden chemotaxis chambers may not reflect what occurs in vivo.

Shigemura et al. (2009) investigated the effect of Pro-Hyp on the migration of mouse skin fibroblasts using 3-mm diameter mouse skin discs that were punched out from 5-week-old male Balb/c mice and then cultured on a 24-well plastic plate. The fibroblasts migrated from the mouse skin discs were collected and then cultured on a collagen gel, using Dulbecco's modified Eagle's medium in the presence or absence of fetal bovine serum at 37°C. They studied the effect of Pro-Hyp on their growth. In the culture medium containing fetal bovine serum, which is rich in platelet-derived growth factor and fibroblast growth factor, large numbers of fibroblasts migrated from the skin to the plate after 72 h of incubation regardless of the presence of Pro-Hyp, which consequently allows for the collection of fibroblasts that have migrated from the skin. When the fibroblasts recovered from the skin were cultured in plastic plates in the presence of fetal bovine serum, the Hyp contents in the HCl hydrolysates of the culture medium including the cells and their products increased significantly in a time-dependent manner, indicating the biosynthesis of collagen, a typical characteristic of fibroblasts. In the absence of fetal bovine serum, a smaller number of fibroblasts migrated from the skin when endogenous platelet-derived growth factor and fibroblast growth factor were secreted into the medium regardless of the presence of

Pro-Hyp. Without the presence of exogenous growth factors, this culture system could be considered a model for the wound healing process of skin. Under these conditions, the addition of Pro-Hyp (200 nmol/ml) significantly increased the number of fibroblasts. However, this effect was inhibited by mitomycin C, which can suppress the cell cycle in the G2 phase without affecting cell migration, indicating that Pro-Hyp enhanced the growth of fibroblasts without affecting their mobility. In the presence of fetal bovine serum, fibroblasts grew rapidly on the plastic plate regardless of the presence of Pro-Hyp, whereas the growth of fibroblasts was inhibited on the collagen gel even in the presence of fetal bovine serum. This is analogous to the quiescently buried fibroblasts in the dermis. Therefore, this culture system coated with a collagen gel may better mimic the biological response of the skin fibroblasts in the injured tissues than the conventional model system using plastic plates. The addition of Pro-Hyp (200–1,000 nmol/ml) abolished the inhibition of the growth of fibroblasts on the collagen gel and significantly increased their growth in a dose-dependent manner in the presence of fetal bovine serum rather than acting as a growth factor. However, the equivalent constituent amino acids of Pro-Hyp had no significant effects on either migration or growth of fibroblasts. Therefore, these results suggested that the food-derived collagen peptide Pro-Hyp in human blood might stimulate fibroblast proliferation in the skin and hence increase the number of fibroblasts migrating from the skin, leading to the promotion of wound healing in the presence of growth factors.

In a subsequent study by the same authors (Shigemura et al. 2011), Hyp-Gly, another major food-derived collagen peptide in blood, was demonstrated to stimulate the growth of mouse primary fibroblasts on collagen gels to a greater extent than Pro-Hyp at equal concentration (200 nmol/ml) in a medium containing fetal bovine serum at 37°C. This suggests that Hyp-Gly plays a more significant role in exerting the biological effects associated with the ingestion of collagen and collagen-derived products. Later, Shigemura et al. (2012) identified Pro-Gly as the major food-derived elastin peptide in human blood, with a maximum concentration of 18 nmol/ml at 30 min after ingestion. The addition of Pro-Gly significantly increased the elastin biosynthesis of normal human dermal fibroblasts cultured in plastic plates for 72 h in a dose-dependent manner from 0.1 to 10 μg/ml. However, the proliferation of neither normal human dermal fibroblasts cultured in plastic plates nor mouse skin primary fibroblasts cultured on collagen gels was affected by the presence of Pro-Gly. These results suggested that food-derived collagen and elastin peptides in human blood caused different biological responses with fibroblasts, even though Hyp-Gly and Pro-Gly vary only in the occurrence of a hydroxyl group. However, Nakaba et al. (2007) reported that human skin elasticity and subjective perception of skin conditions were significantly improved by daily supplementation of 1 g of a collagen hydrolysate with 0.1 g of an elastin hydrolysate compared to a collagen hydrolysate alone; this was attributed to the potential synergetic biological effects of food-derived collagen and elastin peptides.

Using in vitro cultured human dermal fibroblasts on a plate during passages 5–7, Ohara et al. (2010) investigated the cell proliferation and modulation of dermal extracellular matrix components as affected by eight collagen-derived Hyp-containing peptides, including Pro-Hyp, Ala-Hyp, Leu-Hyp, Ile-Hyp, Phe-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, and Pro-Hyp-Gly. Pro-Hyp was the most effective in enhancing the proliferation of fibroblasts and expression of hyaluronan synthase 2 mRNA. At a dose of 200 nmol/ml Pro-Hyp, a maximal increase of 1.5- and 2.3-fold was observed for fibroblasts and hyaluronan synthase 2 mRNA, respectively, with a concomitant 3.8-fold increase in hyaluronic acid synthesis. Hyaluronic acid may not induce mitogenic activity directly but instead may enhance hydration of the extracellular space so as to promote fibroblast proliferation. A small interfering RNA-mediated knockdown of the *hyaluronan synthase 2* gene in human skin fibroblasts suppressed Pro-Hyp-induced transcription of hyaluronan synthase 2 mRNA and also fibroblast proliferation. Epidermal growth factor triggers a signaling pathway where activated

epidermal growth factor receptors stimulate phosphorylation of a signal transducer and activator of transcription 1, 3, and 5 via the Tyr kinase Src. The phosphorylated signal transducer and activator of transcription have been demonstrated to enhance transcription of hyaluronan synthase 2 mRNA (Darnell 1997, Goi et al. 2000, Saavalainen et al. 2005). As with the epidermal growth factor signaling, Pro-Hyp markedly and rapidly enhanced phosphorylation of the signal transducer and activator of transcription 3. However, both genistein, a Tyr kinase inhibitor, and H7, a Ser/Thr kinase inhibitor, antagonized the Pro-Hyp-induced stimulation of *hyaluronan synthase 2* mRNA. These results suggested that Pro-Hyp might be involved, at least partly, in the phosphorylation of the signal transducer and activator of transcription by the activated kinase, although the presence of a specific receptor for Pro-Hyp remains to be established. Proksch et al. (2014) also reported that supplementation of a porcine type I collagen hydrolysate with an average molecular weight of 2.0 kDa for primary human dermal fibroblast cultures led to pronounced increases in the expression of the extracellular matrix macromolecules including type I collagen and proteoglycans such as decorin, biglycan, and versican.

Pigs, like humans, are omnivorous, and their skins are used clinically as human skin substitutes. In an animal experiment using a litter of 9 healthy 66-day-old piglets, Matsuda et al. (2006) investigated the fibroblast density and the extracellular matrix in the dermis as affected by 62 days of daily oral administration of a porcine skin-derived collagen hydrolysate (0.2 g/kg body weight) with a molecular weight in the range of 3–5 kDa, in comparison with lactalbumin or a water control. Compared to the control group, oral ingestion of collagen hydrolysate led to significant increases in both the diameter and density of the collagen fibrils, which might be achieved, at least in part, by an increase in fibroblasts, given their densities also increased significantly. Moreover, the ratio of dermatan sulfate to hyaluronic acid was largest in the collagen hydrolysate group, and this was in agreement with the observation that the amount of collagen fibrils was also highest in this group. These results suggested that ingestion of a collagen-derived peptide improved the mechanical properties of the skin in a collagen-specific manner.

Although animal experiments and preclinical human trials have suggested beneficial biological effects, the clinical efficacy of long-term ingestion of collagen and collagen-derived products on skin conditions remains to be studied. Additionally, the biological responses of other minor collagen-derived peptides (except Pro-Hyp) also suggest the need for further studies, due to the potential occurrence of high biological activity even at low plasma concentrations, as well as the potential synergistic effects of various peptides. The mechanisms of action or the cell signaling pathways underlying the collagen peptide–mediated enhancement of fibroblast growth and consequently biosynthesis of collagens and other extracellular matrix components are still obscure. It is possible that food-derived collagen peptides may function by binding with the cell surface receptor or transporter, if present, or by affecting the interactions between the extracellular matrix and fibroblasts. In addition, the effects of food-derived collagen peptides on the biological response of the epidermal keratinocytes require further investigation.

Technological advances in the food industry such as the appearance of more sensitive and convenient equipment for large-scale screening of bioactive peptides, together with extensive well-designed clinical human trials, will establish or refute the credibility of the commercial skin claims and ensure consumer confidence in the nutritional and functional foods containing collagen and collagen-derived products.

NONCONVENTIONAL USES OF GELATIN

Gelatin has been a multifunctional component in the manufacture of many foods, pharmaceuticals, and cosmetics for more than a century. After a long history of traditional uses, some

nonconventional applications of gelatin have recently been added to the gelatin literature. Some of these applications are discussed in this section based on the studies published in the past few decades.

Uses in the Food Industry as Aroma Carriers

Gelatin is generally used in the food industry in two fundamental ways: in the formulation of the food itself as a gelling agent, stabilizer, and/or emulsifier, and in the formulation of packaging films and coatings to preserve foods and extend their shelf life. When gelatin is used in food formulations, interactions between gelatin and the aroma compounds are inevitable. These interactions affect the aroma profile of the product and alter its sensory perception. Several factors might affect the intensity and the rate of the flavor release in these products. The viscosity of the product is often changed by the addition of gelatin, and this may be the most important factor involved. Changed viscosity alters the transport of volatile compounds from the interior of the food product to its surface (Pangborn & Szczesniak 1974, Baines & Morris 1987, Secouard et al. 2003, Terta et al. 2006). In addition, the interactions between these aroma compounds and the gelatin also affect the flavor release and thus consumer perception. The concentration of gelatin is another factor affecting flavor release. Studies showed that firmer gels release fewer aroma compounds (Jaime et al. 1993). Finally, the surface characteristics of food products and the changes in the surfaces during mastication are of great importance with respect to flavor release and perception.

When gelatin is used in packaging films and coatings, it is again important to study how aroma compounds migrate through the film or coating. There are only a few studies reported so far on gelatin films or coatings carrying various aroma compounds, which may also have antioxidant and/or antimicrobial characteristics. These aroma compounds are mainly constituents of essential oils, which are highly concentrated volatile chemicals generally obtained from aromatic plants. These chemicals have many applications in foods as flavoring, coloring, and texturing agents and for their antimicrobial and antioxidant activity (Zivanovic et al. 2005). Recent research concluded that the release of antimicrobial and antioxidant compounds from a composite gelatin film significantly reduced the number of some food related microorganisms (Gómez-Estaca et al. 2010, Giménez et al. 2012). In other research, the incorporation of aromatic plant extracts with various gelatin films has been reported to significantly improve the antioxidant and light barrier properties of the films, consequently extending the shelf life of the food (Gómez-Estaca et al. 2009a,b). Another purpose for the incorporation of these functional compounds within gelatin films is to protect them from evaporation and reaction with or migration into foods, so as to keep them active at their effective levels (Madene et al. 2006).

Gelatin Composites for Packaging Purposes

Petroleum-based polymers, such as polyethylene and polystyrene, are currently being replaced by polymers of various animal and plant origins because of environmental and health related concerns. Gelatin is one such polymer being explored for this use. Gelatin films are prepared by first dissolving gelatin in hot water, then casting the solution on a plate or in a tray, and finally drying the solution in an oven to obtain a film (**Figure 1**). Many studies have investigated the mechanical, barrier, and moisture aging properties of these films prepared using gelatin alone or in combination with various plasticizers. The most important properties of gelatin films are water uptake, water aging (amount of water loss with time), mechanical properties, especially resistance, thermomechanical properties (change in mechanical resistance at elevated temperatures), and barrier properties with respect to water vapor, oxygen, and other gas migrations (Shubhra 2013). When

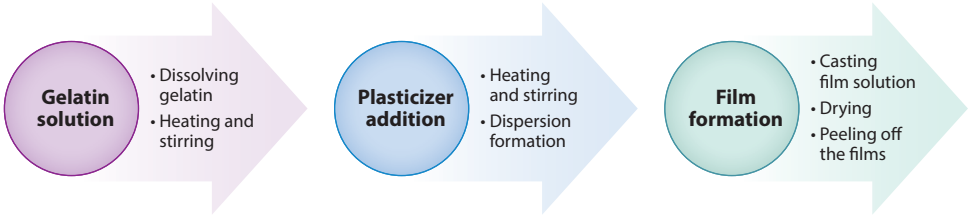

Figure 1

General flow scheme for the manufacture of gelatin films.

used in packaging films and/or coatings, gelatin has some difficulty with regard to its permeability and mechanical resistance. Although gelatin films have comparatively good oxygen barrier properties and satisfactory mechanical resistance at low or medium levels of relative humidity, these characteristics become problematic at high relative humidity levels due to the hydrophilic nature of gelatin (Krochta et al. 1994, Arvanitoyannis et al. 1998). There have been many studies in the past decade on improving the mechanical resistance and the permeability of gelatins containing packaging and/or coating materials (Vanin et al. 2005, Andreuccetti et al. 2009, Cao et al. 2009, Rivero et al. 2010). The use of hydrophilic plasticizers in combination with gelatin improves flexibility and creates less brittle films. Chemical cross-linking using formaldehyde and glutaraldehyde was effective in improving the thermal and mechanical properties (Bigi et al. 2001; Martucci et al. 2006, 2012; Alves et al. 2011). Because of the toxicity risk of these aldehydes, alternative materials such as natural phenolic compounds were used in gelatin films, which again resulted in water repellent gelatin films with improved mechanical properties (Cao et al. 2007, Peña et al. 2010) compared to untreated films. In addition, several plasticizers, including glycerol and sorbitol, are often used in manufacturing gelatin-based films, yielding transparent films with improved mechanical resistance (Vanin et al. 2005, Andreuccetti et al. 2009). The key strategy in strengthening the gelatin films is to modify the hydrophilic nature of gelatin, which can be achieved with the use of either covalent cross-linking agents or plasticizers. Processes such as lamination and the formation of multilayer films can also be utilized to provide mechanical resistance.

Laboratory Applications in Microbiological Methods

Gelatin has many applications in microbiological studies as a substrate for measurement of proteolytic activity and gelatinase production, specifically as an inducer of protease production and as a preservation and transportation medium, among others. The tube method, for example, is still used in general microbiology for differentiating bacteria according to their ability to produce gelatinase, which can be useful for species identification (Lima et al. 2008). In this method, gelatin is used in the culture medium as a gelling agent. After inoculation of bacteria into the liquid gelatin-containing medium in the tubes, the tubes are incubated for several days and then placed in a refrigerator to determine whether gel formation can be observed. Those tubes that do not form gels upon cooling indicate the presence of gelatinase (**Figure 2**). The cup plate method is another way to easily determine protease and/or gelatinase activity by bacteria and fungi, directly on the plate (Vermelho et al. 1996, Silva Neves et al. 2006). In this method, gelatin is used as a substrate for those bacteria or fungi with an ability to produce proteases or gelatinase. Protease or gelatinase secretion in the culture medium causes gelatin hydrolysis and formation of a transparent halo around the bacteria or fungi colony, whereas the rest of the medium remains opaque.

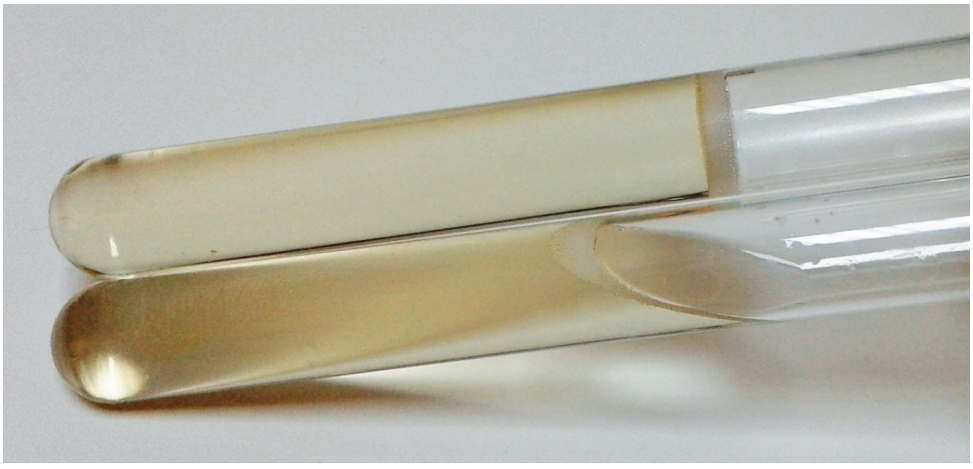

Figure 2

Gelatinase activity. (*bottom*) Gelatinase (+) (sample is fluid). (*top*) Gelatinase (−) (sample retains its shape).

Gelatin is also used in air filters to retain, detect, and analyze airborne microorganisms causing allergic reactions (Gelatin Filter cat. no. 225–9551 and 225–9552; see **http://www.skcinc.com/catalog/pdf/instructions/40060.pdf**). Gelatin filters are completely water soluble, gamma radiation sterilized, and individually wrapped and packaged in polyethylene bags. These filters are very effective in capturing submicron particles. Gelatin is also used in gelatin discs for preserving microorganisms. These provide a simple and inexpensive method, which is reported to be superior in terms of longer storage times and ease of application (Stamp 1947, Obara et al. 1981). In this method, a gelatin-containing medium is inoculated with microorganisms intended for storage, and then the gelatin media are dried on filter paper and stored for future use.

Use of Gelatin in Print Lifting

Although this review focuses on the use of gelatin as a food, there are other new and interesting uses for gelatin and also potential for new applications of gelatins. One such use of gelatin is in forensic science and, particularly, in shoeprint lifting. These gelatin lifters are used to remove impressions found at crime scenes. Gelatin lifters consist of a thick layer of gelatin cast on a flexible woven material that is then placed over the item whose impression is sought. When the gelatin lifter (see **http://www.bvda.com/**) is placed on the desired target, all the dust and/or powder at the site adheres to the gelatin lifter when it is removed from the surface. As the gelatin lifter is thick but flexible, it penetrates all the fine cracks and holes, lifting and holding the site's arrangement of such dust and powder intact. Some pressure is needed for the most effective lifting, and a pneumatic press may be used for this purpose. However, pressure may also cause some problems, especially when the item is imprinted using fibrous materials such as paper, cloth, and cardboard, as loose fibers may interfere with the imprint (Glattstein et al. 2000). However, such fibers can be removed using adhesive strips to clean the gelatin lifter, clarifying the imprint on the gelatin lifter (Glattstein et al. 1996, Vinokurov et al. 1998). Although pressure can be used to lift marks and/or imprints effectively, excessive pressure should be avoided, as the shape of these lifters can be distorted easily. Gelatin lifters have been used successfully in lifting not only shoeprints, but also fingerprints and fabric imprints.

Use of Gelatin in Surgery

Hemostasis (i.e., preventing bleeding) is critical in surgery. There are only a few hemostatic agents made from collagen or gelatin reported so far. One example is FloSeal (**http://www.floseal.com/us/**), a ready-to-use disposable syringe consisting of a sterile, nonpyrogenic gel composed of gelatin, bovine thrombin, and glutaraldehyde as a stabilizer (Oz et al. 2000, Bak et al. 2004). Both gelatin and thrombin act on the bleeding and form a stable clot at the bleeding site. Swelling gelatin particles limit blood flow and form a mechanically stable matrix around this bleeding site, providing a site for a fibrin clot to form. FloSeal is FDA approved and its components when incorporated into the clot are completely resorbable by the body within 6 to 8 weeks. Another example of a gelatin containing hemostatic material is Surgiflo (**http://www.ethicon.com/healthcare-professionals/products/biosurgery/surgiflo-hemostatic-matrix**), which differs from FloSeal only with respect to the source of the gelatin: Surgiflo contains porcine gelatin, whereas FloSeal contains bovine gelatin. When used in appropriate amounts, it is claimed that Surgiflo is easily absorbable by the body and that it can be prepared for use more quickly than FloSeal. In addition to these hemostatic tools, gelatin-based sponges have also been in use for decades under different trademarks. These hemostatic sponges can be used alone or in combination with thrombin during various types of surgery, including cranial and spinal surgeries, for controlling and/or stopping bleeding safely and quickly (Gazzeri et al. 2013).

CONCLUSIONS

This review compiles currently available information regarding the structure, bioactivities, and biological effects of collagen and collagen-derived products along with their new applications. Type I collagen, the major fibrillar collagen in both vertebrates and invertebrates, has a unique right-handed triple superhelical structure, and the determination of its structure using various modern technologies that are frequently used for globular proteins should be done with caution. The structure of collagen may vary with sources and seasons, which may affect its physicochemical properties and hence its applications and optimal extraction conditions. Although animal experiments and preclinical human trials have suggested the beneficial biological effects of collagen and collagen-derived products, it is still too early to make general clinical recommendations regarding the appropriate intakes in dietary and functional foods that may affect human health. The technological advances in the food industry, together with extensive well-designed in vitro and in vivo assay, will allow deeper characterization and better understanding of the physicochemical properties, bioactivities, and biological effects of collagen, gelatin, and their derived peptides. As such, their applications in various fields will be expanded on the basis of this fundamental research.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding or financial holdings that might be perceived as affecting the objectivity of this review.

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