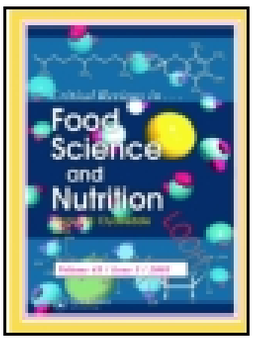

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Exploration of collagen recovered from animal by-products as a precursor of bioactive peptides: Successes and challenges

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Abstract

A large amount of food-grade animal by-products is annually produced during industrial processing and they are normally utilized as animal feed or other low-value purposes. These by-products are good sources of valuable proteins, including collagen or gelatin. The revalorization of collagen may lead to development of a high benefit-to-cost ratio. In this review, the major approaches for generation of collagen peptides with a wide variety of bioactivities were summarized, including antihypertensive, antioxidant and antidiabetic activities, and beneficial effects on bone, joint and skin health. The biological potentials of collagen peptides and their bioavailability were reviewed. Moreover, the unique advantages of collagen peptides over other therapeutic peptides were highlighted. In addition, the current challenges for development of collagen peptides as functional food ingredients were also discussed. This article discusses the opportunity to utilize collagen peptides as high value-added bio-functional ingredients in the food industry.

Keywords: Collagen; Gelatin; Bioactive peptides; Bioavailability; Functional foods

Abbreviations:

ACE: Angiotensin I-converting enzyme

DPP-IV: Dipeptidyl peptidase-IV

GIP: Glucose-dependent insulinotropic polypeptide

GLP-1: Glucagon-like peptide-1

IC_{50}: Half-maximal inhibitory concentration

ROS: Reactive oxygen species

QSAR: Quantitative structure-activity relationship

T2D: Type 2 diabetes

Introduction

Industrial processing of land and aquatic animal produces tons of by-products. In the European Union, it is estimated that over 20 million tons of animal by-products are generated annually (Henchion et al., 2016; Martínez-Alvarez et al., 2015; Pal and Suresh, 2016). Nevertheless, these by-products are normally utilized for production of low-value products, including fertilizers, animal feed, pet food, etc (Alao et al., 2017). Therefore, alternative utilization of animal by-products for high-value products not only reduces the environmental impact but also provides new food ingredients and increases the potential revenue for food industry (Henchion et al., 2016). Mammalian skins, bones, connective tissue, and fish bone, fins and scales as main components of animal by-products can serve as promising sources of collagen and gelatin (Alemán and Martínez-Alvarez, 2013). Gelatin can be obtained by partial hydrolysis of collagen. In this regard, conversion of collagen/gelatin into high value-added food ingredients, e.g. collagen hydrolysates or peptides with potential bioactivities, has gained a great attention, which leads to a high benefit-to-cost ratio (Toldrá et al., 2016).

Bioactive peptides are defined as certain peptide fragments with 2-20 amino acid residues that can exert a positive impact on human health (Kitts and Weiler, 2003). They are inactive within the sequences of their parent proteins but exhibit the beneficial health-promoting effects once they are released. Enzymatic hydrolysis of collagen or gelatin can release encrypted bioactive peptide sequences with various physiological functions (Gómez-Guillén et al., 2011). Collagen peptides can be released from their precursors during food processing, gastric digestion and enzymatic hydrolysis through exogenous and endogenous proteases (Fu et al., 2017b; Udenigwe, 2014). The past decade has witnessed a growing number of studies on collagen peptides with antihypertensive, antioxidant, antidiabetic, bone, joint and skin health-promoting effects (Alemán and Martínez-Alvarez, 2013; Gómez-Guillén et al., 2011). Furthermore, a growing body of evidence supports the excellent bioavailability of collagen peptides (Liu et al., 2015; Zague, 2008). Although some review

papers focus on the functional and bioactive properties of collagen/gelatin peptides (Gómez-Guillén et al., 2011; Pal & Suresh, 2016), the major approaches for generating collagen peptides with various bioactivities are classified and the biological potentials of collagen peptides and their underlying mechanisms are highlighted in the present review. More importantly, unique advantages of collagen peptides and the current challenges for exploitation of collagen peptides-based functional food ingredients are summarized. The present work contributes to development of collagen peptides from animal by-products as functional food ingredients and provides some future directions and strategies to overcome these challenges.

Structure and physicochemical characteristics of collagen

Collagen, a major structural protein, can be found in animal processing by-products, including mammalian skin, bones and connective tissue, and also fish skin, bones and scales (Ferraro et al., 2016; Pal and Suresh, 2016). Collagen accounts for approximately 25-35% of all vertebrate body proteins (Banerjee and Shanthi, 2016). So far, 29 different types (type I–IXXX) of collagen have been identified (Liu et al., 2015), among which type I collagen is the most abundant. Collagen molecule mainly consists of three almost identical α-chains, namely collagen triple helix. This triple helix is composed of the continuous repetitive motif, Gly-X-Y, where X is proline (Pro) and Y is hydroxyproline (Hyp). These two imino acids are specific amino acids of collagen, which contributes to the helical structure of each chain. Compared with mammalian collagen, a lower amount of imino acids (proline and hydroxyproline) is present in marine collagen, which leads to lower melting point and weaker gel strength (Karim and Bhat, 2008, 2009).

Collagen can be extracted from land and marine animal by-products through chemical or biochemical pretreatment, followed by extraction process by warm water (above 40 °C) (Gómez-Guillén et al., 2011). This process can cleave the covalent and hydrogen bonds, leading to destabilizing the triple-helix and further conversion to soluble gelatin (Gómez-Guillén et al., 2011).

Dependent on the pH, temperature and extraction time used, the extracted gelatin products vary in the functional properties. However, gelatin as a denatured product of collagen shares similar structures with collagen (Schrieber and Gareis, 2007). In general, there are two types of gelatin, namely type A and type B. Type A gelatin is derived from the acid-extracted raw material, while type B gelatin is obtained through an alkaline extraction process. Even though mammalian gelatins have been extensively used in different fields, the utilization of gelatin derived from marine sources is increasingly attractive, as it is not involved in the risk of diseases, such as bovine spongiform encephalopathy and also meet certain religious requirements (Karim and Bhat, 2009).

Collagen peptides prepared from hydrolyzed collagen and low-molecular-weight gelatin products (0.3 - 8 kDa) are water-soluble, readily digestible and easily absorbed by the body (Sibilla et al., 2015). These characteristics coupled with the unique Gly-X-Y motif confer collagen peptides with versatile functionalities and various applications as food ingredients and supplements in the form of powders, drinks as well as liquid sprays (Hayes, 2011; Venkatesan et al., 2017). The structural features and functionalities of collagen and collagen peptides have been reviewed by Gómez-Guillén et al. (2011), as summarized in Fig. 1.

Generation of collagen-derived bioactive peptides

In recent years, there has been an increasing number of investigations on bioactive collagen peptides derived from different animal by-products. Several approaches for generation of collagen peptides with different bioactivities have been employed, such as *in vitro* and *in silico* hydrolysis. In addition, the integrated approach (peptidomics combined with *in silico* prediction) can also be used to identify novel collagen peptides with diverse bioactivities. The major research approaches on collagen-derived bioactive peptides are summarized in Fig. 2.

Discovery of collagen peptides by the in vitro approach

In vitro enzymatic hydrolysis of collagen or gelatin is one of the most common and empirical approach to release bioactive collagen peptides with the aid of different commercial proteases or their combinations, such as Alcalase®, papain, pepsin, trypsin, chymotrypsin and other food-grade proteases (Fu et al., 2015; Fu and Zhao, 2013; Gómez-Guillén et al., 2011; Zhang et al., 2013). The classic *in vitro* method is primarily initiated by collagen or gelatin extraction from animal by-products, followed by enzymatic hydrolysis to produce collagen hydrolysates. Then, depending on different bioactivities, *in vitro* bioassays are conducted to screen the most active hydrolysates. The subsequent fractionation and purification by a range of chromatography technologies are implemented to screen the most active peptide fractions (Udenigwe and Aluko, 2012). Afterwards, the most active peptide fractions can be further analyzed by LC-MS/MS to identify amino acid sequences, which enables their bioefficacy validation using synthetic peptides. In recent years, *in vitro* classic approach has been successfully employed to isolate and identify novel bioactive collagen peptides (Chen et al., 2017b; Choonpicharn et al., 2016; Guo et al., 2015). Nevertheless, several drawbacks of this approach include the time-consuming procedure (especially, isolation and purification process) and the relatively low yield of the target peptides. In addition, some potent peptides may be excluded during the process of fractionation and purification due to the synergistic or antagonistic effects of other peptides within the whole hydrolysate (Udenigwe, 2014).

In silico approach

In order to overcome the drawbacks and challenges caused by the *in vitro* approach, *in silico* approach has been proposed and used to release bioactive peptide sequences from collagen (Fu et al., 2016b; Lafarga et al., 2014; Yu et al., 2017). Compared to empirical experiments, it is much more time-saving and economical to screen and identify novel bioactive collagen peptides. Several bioactive peptides have been identified with the aid of *in silico* analysis from a variety of protein sources, especially meat or meat by-products (Lafarga et al., 2014), cereal proteins (Udenigwe et

al., 2013) and milk proteins (Nongonierma and FitzGerald, 2014) and potato proteins (Fu et al., 2016a). There are several frequently-used tools of *in silico* analysis, including BIOPEP "enzyme action" (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), ExPASy PeptideCutter (http://web.expasy.org/peptide_cutter), MEROPS (https://www.ebi.ac.uk/merops/) and MS-Digest (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest, Protein Prospector). Typically, this approach is associated with hydrolysis of a protein sequence obtained from a protein database using the known enzymatic cleavage sites. In addition, the QSAR (quantitative structure-activity relationship) model, a regression model that correlates structural characteristics of molecules with their bioactivities, can be employed to predict activities of unidentified collagen peptides. The peptide sequence with the highest predicted potency can be further chemically synthesized in order to validate their actual *in vitro* potencies. Furthermore, identification of collagen peptide structures with certain bioactivity contributes to structure-function relationship studies and use of specific protease or protease cocktail to generate specific hydrolysates (Udenigwe, 2014). Molecular docking, a computational simulation method that can help predict binding sites between receptor (enzyme) and ligand (inhibitor) and assess binding energy based on the scoring system (Pripp and Ardö, 2007), has been applied to better understand specific binding of collagen peptides to enzymes, e.g. Angiotensin I-converting enzyme (ACE) (Fu et al., 2017a; Fu et al., 2016c).

However, variable results between *in vitro* and *in silico* hydrolysis have been observed. Fu et al. (2016b) reported the discordance in ACE-inhibitory peptides from a papain digest of bovine collagen between *in silico* and *in vitro* protein digestions. Similarly, pepsin-catalyzed hydrolysis of potato proteins was found to be different by *in vitro* hydrolysis compared to *in silico* hydrolysis according to certain enzymatic cleavage sites (Rajendran et al., 2016). The differences between *in vitro* and *in silico* predictions may be due to different reasons. *In silico* hydrolysis assumes that all

cleavable peptide bonds would be accessible to the enzyme and be readily hydrolyzed (Udenigwe, 2014). Thus, it is not guaranteed that virtually-generated peptides can be experimentally released, as the cleavage of peptide bonds during enzymatic hydrolysis is dependent on their accessibility as well as the enzyme activity, which is influenced by the external experimental hydrolysis conditions. Also, in terms of the complicated interaction of protease-proteins, the complex spatial structure of collagen might hinder enzyme access to cleavage sites of native proteins, leading to a mismatch between predicted peptides and experimentally released peptides (Fu et al., 2016b; Nongonierma and FitzGerald, 2017). Furthermore, post-translational modifications (PTMs) and amino acid modifications (e.g. high content of Hyp in collagen) of peptides during food storage and processing is not considered during *in silico* hydrolysis (Rajendran et al., 2016). Overall, the *in silico* approach can serve as an alternative approach for exploration of possible bioactive collagen peptides, but the limitations should not be ignored.

Integrated approaches

Given that the limitations of the methods discussed above, the integrated approach may be promising to discover bioactive peptides from collagen. The integrated approach involves screening the optimal enzyme based on *in silico* prediction. The subsequent *in vitro* hydrolysis using the optimal protease is implemented to release collagen peptides. The peptide profile of hydrolysates can be characterized through peptidomics (LC-MS/MS) analysis (Sasaki et al., 2010). Thereafter, the identified sequences can be further predicted for their *in vitro* potential of bioactivities by an *in silico* method. The PeptideRanker server (http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php) can rank peptide sets and assign scores ranging from 0 to 1 based on structure-function relationships (Mooney et al., 2012). The closer the predicted score is to 1, the higher possibility that the peptide is bioactive (Mooney et al., 2012). Eventually, *in vitro* bioactivities of collagen peptides can be further

confirmed by synthetic peptides. However, it should be noted that the relevant knowledge on the structure-function pattern is necessary to predict meaningful information (Udenigwe, 2014), as PeptideRanker cannot predict the particular type or potency of bioactivity of the peptides. More important, experimental hydrolysis is essential to apply the integrated approach (Udenigwe, 2014).

Health beneficial potentials of collagen peptides

In recent years, an increasing number of research findings have indicated a variety of bioactivities for collagen hydrolysates or peptides (Fig. 3), such as antihypertensive, antioxidant and antidiabetic activities in addition to beneficial effects on bone, joint and skin health. Furthermore, the bioactivities of some collagen peptides have been confirmed by *in vivo* animal models and human clinical trials.

Antihypertensive and ACE-inhibitory activity

Hypertension, a serious global health issue, represents one of the major risk factors for developing cardiovascular diseases (Bhat et al., 2015; Organization, 2013). Angiotensin-I converting enzyme (ACE) plays a pivotal physiological role in regulation of blood pressure and electrolyte homeostasis (Perazella and Setaro, 2003). Effective inhibition of ACE has been considered the main target for treatment of hypertension (Udenigwe and Mohan, 2014). However, long-term administration of synthetic ACE inhibitors, such as Captopril, Lisinopril and Enalapril, has been reported to have adverse side effects (Flather et al., 2000). Thus, there has been an increasing interest in development of the natural antihypertensive and ACE-inhibitory peptides as a safer alternative for lowering blood pressure. It has been reported that collagen is a good precursor of ACE-inhibitory peptides (Minkiewicz et al., 2011). A number of identified collagen peptides with ACE-inhibitory activities are mainly derived from fish skins (Lee et al., 2011; Ngo et al., 2015; Ngo et al., 2014), squid gelatin (Alemán et al., 2011), chum salmon gelatin (Lee et al., 2014), Pacific cod gelatin (Himaya et al., 2012), bovine connective tissue (Fu et al., 2016c) and jellyfish collagen (Zhuang et

al., 2010). Some identified collagen peptides with ACE-inhibitory and antihypertensive activities are listed in Table 1.

It is widely documented that the ACE-inhibitory properties of collagen peptides are closely related to their unique amino acid compositions (Aluko, 2015b; Martínez-Alvarez et al., 2015). The ACE-inhibitory activities of collagen peptides can be strongly influenced by the occurrence of hydrophobic (aromatic or branched side chains) amino acid residues, such as Pro, Phe, and Val, at the C-terminal positions (Aluko, 2015a, b). Therefore, the high proportion of Pro residues within the sequence of ACE-inhibitory peptides, to a large extent, contributes to higher potency (Gómez-Guillén et al., 2011; Martínez-Alvarez et al., 2015). In addition, the identified collagen peptides tend to be short and of low-molecular-weight, which potentiates higher activities (Herregods et al., 2011), as small peptides are more efficiently embedded into the active sites of ACE in order to impair its activity (Iwaniak et al., 2014).

Currently, several collagen peptides with ACE-inhibitory activities have been confirmed for their *in vivo* antihypertensive effects. Oral administration of collagen hydrolysates derived from squid skin gelatin, salmon skin gelatin and jellyfish collagen can successfully lower blood pressure in spontaneously hypertensive rats (SHR) (Lee et al., 2014; Lin et al., 2012; Zhuang et al., 2010). Ingestion of a collagen-derived dipeptide Gly-Pro at 500 mg/kg can also induce an antihypertensive effect in SHR after 24 h (Ichimura et al., 2009). Zhuang et al. (2012) evaluated antihypertensive effect of long-term oral administration (100 mg/kg for 30 days) of jellyfish collagen peptides on renovascular hypertension rats (RVH) and found that both systolic blood pressure and diastolic blood pressure of RVH were significantly decreased by 34.8 and 52.6 mmHg. Lee et al. (2014) suggested the antihypertensive effect in SHR of Gly-Leu-Pro (20 mg/kg), an ACE-inhibitory peptide from chum salmon skin gelatin, by decreasing 31 mm Hg systolic blood pressure after 6 h. However, further study on the *in vivo* antihypertensive effects of collagen peptides based on animal

and human clinical trials is still needed for further development of new antihypertensive collagen peptides.

Antioxidant activity

Oxidative stress is an imbalance between excessive production and quenching of reactive oxygen species (ROS). Oxidative stress is implicated in a diversity of diseases, such as aging, hypertension, neurodegenerative diseases, inflammation, gastric ulcers and diabetes mellitus (Fu and Zhao, 2015; Valko et al., 2006). Nowadays, due to safety concerns and consumer preferences, there is an increasing interest in the utilization of natural antioxidants (Sarmadi and Ismail, 2010). In this regard, a wide array of peptides that possess antioxidant activity have been found from various collagenous sources and verified in different oxidative systems (Gómez-Guillén et al., 2011). In recent years, the collagen-derived antioxidant peptides have been identified from blue shark skin gelatin (Weng et al., 2014), Amur sturgeon skin gelatin (Nikoo et al., 2014), grass carp skin (Cai et al., 2015), duck skin gelatin (Lee et al., 2012), croceine croaker scales (Wang et al., 2013), cod skin gelatin (Ngo et al., 2011) and tilapia skin gelatin (Zhang et al., 2012). The identified collagen peptide sequences with antioxidant activities are listed in Table 2.

To date, the exact mechanism responsible for the antioxidant activity of collagen peptide has not been fully elucidated. The antioxidant capacities of collagen peptides have been reported to involve free radical scavenging, singlet oxygen quenching or chelation of metal ion (Kitts and Weiler, 2003). Chen et al. (1998) suggested that the total antioxidant effect of peptides is probably associated with the synergistic effects of the above mechanisms. It is demonstrated that the sequence, composition, peptide conformation, positioning of amino acids, hydrophobicity and molecular weight exert some impacts on antioxidant activities (Alemán and Martínez-Alvarez, 2013; Martínez-Alvarez et al., 2015). The radical-scavenging activity of collagen peptides is attributed to the presence of certain amino acids within their sequences, which can donate protons to

electron-deficient radicals (Samaranayaka and Li-Chan, 2011). The presence of Tyr, Phe, Trp, His, Met and Pro in the peptide sequences has been suggested to enhance chelating and free radical scavenging properties (Banerjee and Shanthi, 2016). Recently, collagen peptides isolated from cod and skate skin gelatin have been demonstrated to enhance cell viability and protect living cells against free radical-mediated oxidative damage (Ngo et al., 2014; Ngo et al., 2011). Two collagen peptides, Thr-Cys-Ser-Pro and Thr-Gly-Gly-Gly-Asn-Val derived from Pacific cod skin can protect H_2O_2-mediated DNA damage and ROS production in RAW 264.7 cells in a dose-dependent manner. At the low peptide concentration (50 μg/mL), DNA damage was attenuated more than 90% and ROS (over 60%) was largely scavenged after 3h (Ngo et al., 2011). Similarly, 10 μM of skate skin gelatin peptides (Met-Val-Gly-Ser-Ala-Pro-Gly-Val-Leu and Leu-Gly-Pro-Leu-Gly-His-Gln) can scavenge intracellular ROS in EA.hy926 cells, evaluated by flow cytometry and light microscope analysis (Ngo, Ryu and Kim 2014). Meanwhile, the mRNA and protein expression levels of antioxidant enzymes (superoxide dismutase and glutathione) were up-regulated with the treatment of Met-Val-Gly-Ser-Ala-Pro-Gly-Val-Leu and Leu-Gly-Pro-Leu-Gly-His-Gln in a dose-dependent manner (10-100 μM) (Ngo, Ryu and Kim 2014). Therefore, the underlying mechanism responsible for antioxidant activities of collagen peptides in cell models may be due to the ability to enhance the expression of antioxidant enzymes in cultured cells by treatment with collagen peptides (Cheung et al., 2015; Fu and Zhao, 2015). In addition, collagen peptides have been reported to retard lipid peroxidation, which is promising for future application in food preservation for the enhanced shelf life and oxidative stability (Sohaib et al., 2016).

Dipeptidyl peptidase-IV (DPP-IV) inhibitory activity

Type 2 diabetes (T2D), a chronic metabolic disorder, is increasing at an alarming rate worldwide. It is characterized by hyperglycemia resulting from inadequate insulin secretion, resistance to insulin action and pancreatic beta-cell dysfunction (Jao et al., 2015; Patil et al., 2015). For T2D

management, one of the latest strategies is associated with inhibition of dipeptidyl peptidase-IV (DPP-IV) activity (Lacroix and Li-Chan, 2016; Nongonierma and FitzGerald, 2016). DPP-IV inhibitors can play a key role in regulation of glucose level through retarding degradation of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), further leading to insulin secretion (Power et al., 2014). Enzymatic hydrolysis of dietary proteins has been reported to release DPP-IV inhibitory peptides (Nongonierma & FitzGerald, 2015). Among the different sources of proteins, collagen is predicted to be one of the optimal potential precursors of DPP-IV inhibitory peptides based on *in silico* analysis (Lacroix and Li-Chan, 2012). In the past years, a range of collagen-derived DPP-IV inhibitory peptides has been discovered. Table 3 shows a summary of sequences and potencies of DPP-IV inhibitory peptides from different collagenous sources, such as bovine collagen (Hatanaka et al., 2014, Lafarga et al., 2014), Atlantic salmon gelatin (Li-Chan et al., 2012), barbel fish gelatin (Sila et al., 2015), deer collagen (Jin et al., 2015), porcine skin gelatin (Huang et al., 2014) and silver carp collagen (Zhang et al., 2016). Interestingly, a common feature of the identified collagen-derived DPP-IV inhibitory peptides is that most of them share the similar repeating motif, Gly-X-Y sequence.

The structure-activity relationship and the underlying mechanisms responsible for collagen peptide-induced DPP-IV inhibition have not been fully elucidated. Nevertheless, some structural characteristics of DPP-IV inhibitory peptides have been observed in recent studies. Typically, several potent DPP-IV inhibitory peptides contain 2-7 amino acid residues with Pro or Ala as the penultimate amino acid located at the N-terminal (Power et al., 2014). Furthermore, it has been demonstrated that DPP-IV can preferably cleave Pro, Ala, Ser and Hyp in protein sequence as the second N-terminal residue (Davy et al., 2000), so collagen hydrolysates with high proportions of these peptide motifs, such as Xaa-Pro and Xaa-Ala, may have the potential to be DPP-IV inhibitors. This fact is recently confirmed based on *in silico* and *in vitro* analyses (Nongonierma and

FitzGerald, 2013; Nongonierma and FitzGerald, 2014; Wang et al., 2017). In addition to amino acid composition, the peptide sequence can also exert an impact on DPP-IV inhibitory activity. A practical example is that DPP-IV inhibitory dipeptides Ile-Pro and Trp-Val exhibit no inhibitory activity once the peptide sequences are reversed (Hatanaka et al., 2014; Nongonierma and FitzGerald, 2017; Power et al., 2014).

Although *in vitro* DPP-IV inhibitory activity of collagen peptide is widely reported, a limited amount of *in vivo* studies using diabetic animals or humans have been performed to evaluate their bioefficacy. Zhu et al. (2010) claimed that supplement of marine collagen hydrolysates (13 g/day for 3 months) in Chinese patients with T2D may benefit glucose and lipid metabolism, insulin sensitivity and renal function in Chinese patients with T2D. Recently, Hsieh et al. (2015) reported that oral administration of Atlantic salmon skin gelatin hydrolysate at the dose of 300 mg/day for 5 weeks could inhibit GLP-1 degradation by DPP-IV, leading to the enhanced insulin secretion and improved glycemic control in streptozotocin-induced diabetic rats. Similarly, oral administration of porcine skin gelatin hydrolysate (300 mg per day for 42 days) can also improve the glucose tolerance in streptozotocin-induced diabetic rats through inhibition of plasma DPP-IV activity by 63.4% (Huang et al., 2014). However, the molecular mechanisms responsible for DPP-IV inhibition by collagen peptides remains to be unraveled. Besides, clinical trials are needed to validate and confirm the efficacy and bioavailability of collagen peptides.

Bone and joint health

With the increasingly aging population, osteoporosis and osteoarthritis have become major age-related musculoskeletal diseases (Dequeker et al., 2003). Osteoporosis is a systemic skeletal disease characterized by low bone mass, deterioration of skeletal microarchitectural and increased risk of bone fragility and fracture (Inderjeeth and Poland, 2010). Likewise, osteoarthritis is the most prevalent type of joint disease with chronic musculoskeletal pain and disability mainly due to the

imbalance between synthesis and degradation of the articular cartilage (Bello and Oesser, 2006). Over the years, a large and growing body of evidence indicates that collagen hydrolysates/peptides play a positive role in management of osteoporosis, joint disorders and osteoarthritis (Bello and Oesser, 2006; Daneault et al., 2017).

A number of *in vitro* studies have confirmed that collagen peptides can stimulate proliferation and differentiation of osteoblasts and improve calcium absorption (Fu and Zhao, 2013; Guillerminet et al., 2012). Fu and Zhao (2013) demonstrated that salmon skin gelatin hydrolysates (0.1 mg/mL for 48h) were capable of inducing osteoblast proliferation, accelerating cell cycle progression and inhibiting cell apoptosis (from 6.9% to 4.5%) in human hFOB1.19 cells. In parallel, collagen hydrolysates (0.1-6.0 mg/mL for 7 days) have been confirmed to stimulate osteoblast proliferation and differentiation (Daneault et al., 2017; Kim et al., 2014; Liu et al., 2014). Moreover, it has been shown that collagen peptides (0.1 mg/mL for 7 days) can significantly up-regulated expression of the bone-related genes in osteoblasts, including type I collagen, alkaline phosphatase, osteocalcin and osteopontin (Han et al., 2009; Kim et al., 2014; Liu et al., 2014; Yamada et al., 2013). However, the related mechanisms underlying the beneficial actions on osteoblasts are not completely clarified. Currently, there are potential reasons that help shed lights upon underlying beneficial effects. Firstly, collagen peptides can activate some related receptors on the cell membrane and exert an anabolic effect on bone cells (Daneault et al., 2017). It has been documented that collagen hydrolysates can induce the expression of bone-related genes in osteoblasts (Daneault et al., 2017). Furthermore, due to its richness in Pro and Hyp, collagen hydrolysate may exhibit the beneficial effects on osteoblasts through inducing insulin-like growth factor 1 (IGF1) secretion and activating the calcium sensing receptor (Conigrave et al., 2008; Daneault et al., 2017; Dawson-Hughes et al., 2007). In addition, it is claimed that the source and

molecular weight of collagen hydrolysates may be also key factors for mediating beneficial actions on bone metabolism (Daneault et al., 2017).

Daneault et al. (2017) recently summarized some evidence from different animal models based on bone growth, bone loss and bone healing models and implied that collagen hydrolysates/peptides can help maintain a balanced bone turnover in different physiological conditions (growth, bone loss and healing) via promoting bone formation and development. To date, a limited number of clinical studies have been implemented to evaluate beneficial effects of collagen hydrolysates on bone metabolism. Supplement of calcium-collagen chelate has been shown to improve bone mass and prevent excessive bone loss and turnover (Elam et al., 2015; Hooshmand et al., 2013). A 4-month randomized double-blind trial executed by Martin-Bautista and colleagues revealed that the daily intake of collagen hydrolysates (250 mL hydrolyzed gelatin products) can exert a positive effect on bone remodeling by significantly increasing the levels of IGF1 and bone alkaline phosphatase (Martin-Bautista et al., 2011). Nevertheless, future standardized clinical trials remain to be conducted to strengthen this scientific evidence.

Scientific evidence suggests that collagen hydrolysates exert a positive therapeutic effect on osteoarthritis (Bello and Oesser, 2006; Kumar et al., 2015). *In vitro* cell studies have revealed that collagen hydrolysates/peptides stimulate synthesis of proteoglycans and collagen in human chondrocytes (Oesser and Seifert, 2003; Porfírio and Fanaro, 2016; Schadow et al., 2017). Meanwhile, the clinical studies have demonstrated that intake of collagen peptides (5 g/day for 12 weeks) can lead to the relief of joint pain (Bruyere et al., 2012; Zdzieblik et al., 2017) and stimulate regeneration of type II collagen and the biosynthesis of proteoglycans in cartilage tissue osteoarthritis patients (McAlindon et al., 2011). Trč and Bohmová (2011) claimed that ingestion of collagen hydrolysate (1.5g/day) for 24 weeks resulted in improvement of joint conditions. These

findings provide a theoretical basis for exploitation of collagen hydrolysate as a regenerative agent for osteoarthritis patients.

Skin health

Skin characteristics are subject to deterioration by chronological aging, dermatological disorders and environmental conditions, which can be exacerbated by photo-aging or other undesirable lifestyle issues (Sibilla et al., 2015; Zague, 2008; Zague et al., 2011). An overview of the beneficial impacts of collagen peptides on the skin by Sibilla et al. (2015) indicates that collagen peptides can stimulate proliferation and motility of fibroblasts (Chai et al., 2010), increase the density and diameter of collagen fibers (Matsuda et al., 2006), elevate production of hyaluronic acid (Matsuda et al., 2006), promote the expression of type I and IV collagen and protect against photoaging (Sibilla et al., 2015; Sun et al., 2013). A number of controlled clinical trials have evidenced the bioefficacy and benefits of collagen peptides on skin properties, including hydration (Choi et al., 2013), skin elasticity (Proksch et al., 2014) and reduction of wrinkles (Schwartz and Park, 2012).

Other potential health benefits

Apart from the above-mentioned bioactivities, collagen peptides have been reported to exhibit some other physiological activities, including renin-inhibitory activity, metal chelating capacity, immunostimulatory activity, lipid-lowering activity, neuroprotective effect and wound-healing activity (Pal and Suresh, 2016). In addition to ACE inhibition, several recent investigations have revealed the inhibitory activity of renin by collagen peptides (Fu et al., 2017b; Lafarga et al., 2014), which is an emerging target to better lower the blood pressure (Udenigwe and Mohan, 2014). Collagen peptides have been demonstrated to display the excellent metal chelating capacity, which can improve the absorption of mineral ions in the human body, including calcium (Guo et al., 2015), copper (Zhuang et al., 2009) and ferrous (Nakchum and Kim, 2016) ions. Zhang et al. (2011) and Wang et al. (2015a) reported that oral administration of chum salmon collagen peptides could

accelerate wound healing. Niu et al. (2016) indicated that cod skin collagen peptides exerted a protective action on the acetic acid-induced gastric ulcer in rats. It has been reported that collagen hydrolysates show immunostimulatory activity in RAW 264.7 macrophage cells (Sae-leaw et al., 2016), Caco-2 cells (Chen et al., 2017a) and C57BL/6.KOR-ApoE^{shl} mice (Zhang et al., 2010). In addition, the beneficial effects of on lipid metabolism have been reported. Oral administration of collagen hydrolysates (0.4 g for 2h) can significantly lower the levels of total lipids and triglycerides in plasma (Saito et al., 2009). Minaguchi et al. (2012) claimed that Pro-Hyp (200 ng/mL), collagen-derived dipeptide significantly reduced the size of lipid droplets in mouse 3T3-L1 adipocytes after 14 days. Besides, the neuroprotective effect of collagen peptides (3.0 g/kg for 90 days) was reported to benefit learning and memory in the rat brain via attenuating oxidative damage and acetylcholinesterase activity in the brain (Pei et al., 2010; Xu et al., 2015). According to Lima et al. (2015), collagen hydrolysates (5 mg/mL) exhibited antibacterial activity against Gram-positive bacteria and Gram-negative bacteria. The low-molecular-weight of collagen peptides may facilitate interactions with bacterial membranes due to better structure acquisition of amino acid residues (Lima et al., 2015). It has been documented that Pro-rich peptides are potential antimicrobial peptides (Reddy et al., 2004), which is compatible with collagen structure with abundant Pro in its repeating Gly-X-Y motif. The present interest in the collagen peptides can guide exploration of new bioactivities in future research activities.

Unique advantages of collagen peptides over other therapeutic peptides

Bioavailability of collagen peptides and underlying mechanisms

A growing amount of recent studies have indicated that collagen peptides, especially those with C-terminal Pro or Hyp residue, can be transported across the intestinal epithelial monolayer, enter the bloodstream and exhibit their bioactive properties (Alemán and Martínez-Alvarez, 2013). An increasing number of collagen-derived peptides has been identified in the blood after oral ingestion

of collagen/gelatin hydrolysates, among which Pro-Hyp and Hyp-Gly account for a major percentage (Ohara et al., 2007; Shigemura et al., 2011; Shigemura et al., 2014). The presence of collagen-derived oligopeptides with higher concentrations in the blood suggests a high resistance to digestion by the enzymes in plasma and gastrointestinal tract and blood protease (Taga et al., 2014, 2016; Wang et al., 2015b). The unique amino acid composition and structure confer collagen peptides with excellent stability and less protease cleavage sites. The high Pro level exists in collagen peptides (10-30% abundance) may increase their stability towards digestive enzymes and intestinal peptidases in comparison to other therapeutic peptides (Banerjee and Shanthi, 2016). This is because the presence of a Pro residue adjacent to the cleavage site prevents proteases from cleaving the normally susceptible peptide bond (Liu et al., 2015). Examples of detection of collagen peptides in the plasma of animals and humans after the ingestion of collagen/gelatin hydrolysates are listed in Table 4.

In vitro, animal experiments and human clinical trials have highlighted the beneficial effects of collagen peptides, especially Hyp-containing peptides (Matsuda et al., 2006; Ngo et al., 2015; Tsuruoka et al., 2007; Wu et al., 2004). Collagen peptides can stimulate the growth of fibroblasts in the skin as well as the synthesis of hyaluronic acid (Ohara et al., 2010; Shigemura et al., 2009) and improve skin barrier dysfunction (Shimizu et al., 2015). Pro-Hyp and Pro-Hyp-Gly exhibit chemotactic activities in human fibroblast, peripheral blood neutrophils and monocytes (Zague, 2008). Furthermore, oral intake of collagen peptides (2.5 g/day for 8 weeks) with beneficial effects on dermal matrix synthesis have been confirmed by human clinical trials (Proksch et al., 2014) and animal experiments (Zague et al., 2011). The reported evidence has indicated that collagen peptides, as a biological messenger, can trigger the synthesis of new collagen and modulate fibroblast cells reorganization in the extracellular matrix (Wang et al., 2015b). Iwai et al. (2009) examined oral ingestion of chicken collagen hydrolysates and detected a range of Hyp-containing collagen

peptides in human blood with ACE-inhibitory activity, among which Ala-Hyp had the strongest ACE-inhibitory activity (IC_{50}=177 μM). Several collagen peptides identified in the plasma, such as Gly-Pro-Y and X-Hyp-Gly types, may have significant antidiabetic effects *in vivo* (Taga et al., 2016).

The transepithelial transport mechanisms of collagen peptides have been extensively investigated. Aito-Inoue et al. (2007) investigated the mechanism for the transcellular transport of Gly-Pro-Hyp based on porcine brush border membrane vesicles. Gly-Pro-Hyp was further degraded into Gly and Pro-Hyp in the apical side, while the intact Gly-Pro-Hyp was not identified on the basolateral side, leading to failure of transport across the intestinal epithelial apical membrane. By contrast, two collagen peptides (Val-Gly-Pro-Val and Gly-Pro-Arg-Gly-Phe) have been reported to show stability against epithelial cell peptidases and transported across Caco-2 cell monolayers via paracellular pathway (Fu et al., 2016c). Similarly, low-molecular-weight (<2 kDa) collagen hydrolysates can be efficiently transported across the Caco-2 cell monolayer (Feng and Betti, 2017). The difference might be due to collagen type and source as well as the variations in substrate specificities peptidases of animal species on the intestinal epithelial apical membrane (Liu et al., 2015). In addition, Watanabe-Kamiyama et al. (2010) investigated ingestion of low-molecular-weight (approximately 800 Da) collagen hydrolysates containing a high amount of Gly-Pro-Hyp (288 mg for 0.5-6h) and reported that Gly-Pro-Hyp could be absorbed into the bloodstream through the intestinal brush border membrane in an intact form using Wistar rats as the animal model. These above-mentioned results may provide some evidence for the excellent bioavailability of collagen peptides.

Sustainable sources and biocompatibility of collagen peptides

The utilization of inexpensive protein sources, especially animal by-products as the raw materials to prepare bioactive peptides cannot only reduce production costs but also by-products. Collagen is

one of the most abundant proteins in the world that is widely available and inexpensive to recover from animal by-products (Banerjee and Shanthi, 2016). Currently, utilization of collagen as high value-added ingredients via enzyme technology has been the top trend in the meat industry, leading to a high benefit-to-cost ratio and deriving value from slaughterhouse by-products (Lafarga and Hayes, 2014). The resultant collagen peptides have an enormous commercial potential as food ingredients or nutraceuticals. In addition, collagen peptides are biocompatible and safe due to their unique biological and structural characteristics. Regardless of different collagen types, they all share the nearly identical sequence and structure, which guarantee weak *in vivo* immunogenicity (Banerjee and Shanthi, 2016; Lee et al., 2001).

Neutral taste of collagen peptides

Peptides have a wide array of tastes, including sweet, bitter, umami, sour or salty taste (Temussi, 2012). However, bitter peptides are frequently released during enzymatic protein hydrolysis (Li-Chan, 2015), which influences consumer acceptability and impedes the further development of peptide-based food products (Udenigwe, 2014). Humans can distinguish the bitter taste of peptides mainly through taste receptor cells of taste buds on the surface of the tongue (Maehashi and Huang, 2009). The bitter taste is suggested to be mediated by taste receptor type 2 (T2Rs), a large family of G-protein-coupled receptors. The interaction of bitter peptides with T2Rs through binding and stimulating units can trigger the signaling cascades (Lafarga and Hayes, 2016; Maehashi and Huang, 2009).

Bitter peptides have been widely identified in various protein sources, including milk protein (Singh et al., 2005), soy protein (Cho et al., 2004), wheat gluten protein (Liu et al., 2016) and bovine hemoglobin (Aubes-Dufau et al., 1995). To our knowledge, however, it has not been reported that collagen hydrolysates possess bitter tastes. Moreover, hydrolyzed collagen contains a high content of glycine, which also contributes to neutral taste. During the industrial production of collagen

hydrolysates, release of bitter peptides is low when compared to the amount formed from other hydrolyzed proteins (Schrieber and Gareis, 2007). Several approaches have been employed to predict the peptide bitterness. The 'Q rule', which was first proposed by Ney (Ney, 1979), can help estimate the bitterness of peptides based on amino acid composition, where Q value is calculated from the average hydrophobicity of peptide sequences. According to the Ney's Q rule, the peptides (under 6 kDa) with Q values more than 1400 cal/mol are estimated to be bitter. However, the average hydrophobicity (Q) of collagen is 1280 cal/mol (Ney, 1979). This value is much lower than casein (1605 cal/mol) and soy protein (1540 cal/mol). The low Q value implies that collagen hydrolysates/peptides tend to be less bitter and with neutral taste, which promotes its potential as a functional ingredient in the food industry.

The main hurdles for commercialization of collagen peptides

The past decade has witnessed an increasing number of studies on bioactive collagen peptides, which contributes to development of functional foods with health-promoting functions to combat the growing global burden of chronic diseases. Even with the unique advantages of collagen peptides as compared to other therapeutic peptides, conversion to functional foods has confronted several challenges, such as inadequate evidence of clinical bioefficacy, industrial-scale production as well as food processing and storage.

Inadequate clinical trials

Even though collagen peptides with various bioactivities have been confirmed on the basis of *in vitro* or animal models, adequate human clinical studies are scanty to substantiate bioefficacy and support future health claims. A number of nutritional intervention studies on the detection of Hyp-containing peptides in human plasma or tissues after ingestion of collagen or gelatin hydrolysates have been reported (Shigemura et al., 2014; Sugihara et al., 2012; Taga et al., 2014; Zague, 2008). Given that the bioavailability of bioactive peptides in humans is relatively very low (Foltz et al.,

2010; Nongonierma and FitzGerald, 2017), the amount of the collagen peptides that should be ingested to exhibit an *in vivo* effect remains to be explored. Thus, utilization of dose-response approaches in humans may allow assessment of their *in vivo* potency. Recently, Shigemura et al. (2014) reported ingestion of three different doses (30.8, 153.8 and 384.6 mg/kg for 15-360 min) of cod skin gelatin hydrolysates by humans and demonstrated a dose-response pattern between the amount of hydrolysate ingested and the level of Hyp and Hyp-containing peptides in plasma. There was no absorption limit of Hyp and Hyp-containing peptide, suggesting that higher doses would lead to elevated plasma level. In contrast, a well-known health claim on milk-derived antihypertensive tripeptides (Ile-Pro-Pro and Val-Pro-Pro) was rejected by the European Food Safety Authority (EFSA), suggesting lack of cause and effect relationship for consumption of Ile-Pro-Pro and Val-Pro-Pro at the proposed dosage (EFSA Panel on Dietetic Products and Allergies, 2011). Although several clinical studies of collagen peptides have been executed to demonstrate efficacy and benefits on skin properties (Sibilla et al., 2015), more clinical trials are still needed to confirm other *in vivo* bioactive properties of collagen peptides.

Industrial scale production

Novel strategies, such as *in silico* and the integrated approaches can predict and optimize generation of bioactive peptides with targeted activities. However, a major challenge is industrial-scale production of pure collagen peptides. Chemical synthesis of peptides involves the consumption of large amounts of solvents and leads to the generation of waste, which is environmentally unfriendly. Recombinant expression of peptides may serve as an alternative way for the production of longer peptides (more than 25 amino acids in length) (Lafarga and Hayes, 2016; Rodríguez et al., 2014). Unfortunately, this method is not suitable for the generation of smaller peptides with short amino acid sequences. Therefore, industrial-scale production of crude protein hydrolysates containing bioactive peptide sequences is a more practically feasible strategy, given that the peptides retain

their bioactivities within the hydrolysates (Agyei and Danquah, 2011; Udenigwe, 2014). From a viewpoint of industrial-scale production, the use of immobilized enzymes and membrane separation techniques (e.g. ultrafiltration) can offer several benefits, including higher efficiency and lower operating costs for the large-scale production. Integration of complementary processes to establish economical and efficient industrial-scale processes for fractionation and continuous production of bioactive peptides has been proposed (Wu et al., 2013). For example, electrodialysis with ultrafiltration membrane technology based on the separation of molecules according to the charge and molecular mass may be compatible with large-scale production of peptides (Doyen et al., 2012). Therefore, the exploration of novel methodologies to generate bioactive collagen peptides in an inexpensive and efficient way is imperative.

Food processing and storage

The stability of collagen peptides during food processing and storage is crucial for their application as functional ingredients, as peptides are prone to suffer from chemical modifications of the backbone or side chains. These chemical reactions include disulfide bond formation, dehydration, glycation and aromatic ring oxidation (Udenigwe and Fogliano, 2017), giving rise to changes in structure and bioactivity of peptides (López-Fandiño et al., 2006).

During food processing and storage, interactions between peptides and food matrix may bring about some physicochemical reactions, such as hydrophobic interactions, disulfide interactions and Maillard reaction (Rao et al., 2016). Moreover, peptide aggregates formed by covalent bond can be widely detected in different powder-based protein hydrolysates (Rao et al., 2016), leading to impaired quality of products. Maillard reaction has been frequently observed during the storage and thermal food processing in the presence of reducing sugar (Jiang et al., 2013; Rao et al., 2016). These processes can exert some deleterious impacts on the sensory profile, solubility, color and bioactivity of the peptide-based functional food products (Udenigwe, 2014). However, it is worth

noting that the altered bioactivities of peptide-based Maillard reaction products can be partly attributed to the formation of new compounds (Udenigwe & Fogliano, 2017). Therefore, the unwanted Maillard reaction during processing and storage of peptide-rich products could be minimized through application of sugar substitute (e.g. sugar alcohol). Recently, collagen peptides have been demonstrated to retain potent ACE-inhibitory activity after exposure to different processing temperatures (20-100 °C) and pH values (2-10) (Fu et al., 2015). Unfortunately, the information on the food matrix-collagen peptides interaction and its impact on availability is extremely limited.

In addition, oxidation of amino acids via endogenous production of oxidative compounds and exogenous oxidizing agents during processing and storage may also lead to the changes in molecular structure and bioactivity (López-Fandiño et al., 2006). For instance, Met is easily oxidized to methionine sulfone and homocysteic acid in the presence of peroxides, which makes the oxidized products biologically unavailable (López-Fandiño et al., 2006). Therefore, the appropriate packaging materials and conditions should be used to prevent oxidative damage in peptide-based products.

Conclusion

Over the years, there has been an increasing interest in exploration of collagen peptides as bioactive ingredients. This review indicates that collagen from animal by-products can serve as an excellent precursor to release bioactive peptides. Collagen peptides with various bioactivities and health benefits have a promising potential as functional food ingredients. The integration of interdisciplinary approaches may result in improved generation and identification of novel bioactive collagen peptides. The sustainable sources of collagen, good bioavailability and neutral taste render collagen peptides with exclusive health benefits over other therapeutic peptides. Further advances in effective large-scale production and recovery processes are crucial, and the standardized clinical

trials need to be executed to evaluate and validate the bioefficacy of collagen peptides based on human intervention studies. Finally, to fully explore the benefits of collagen peptides, their interactions with the food matrix during food storage and processing need to be considered as part of holistic approach for functional food development.

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Figure captions:

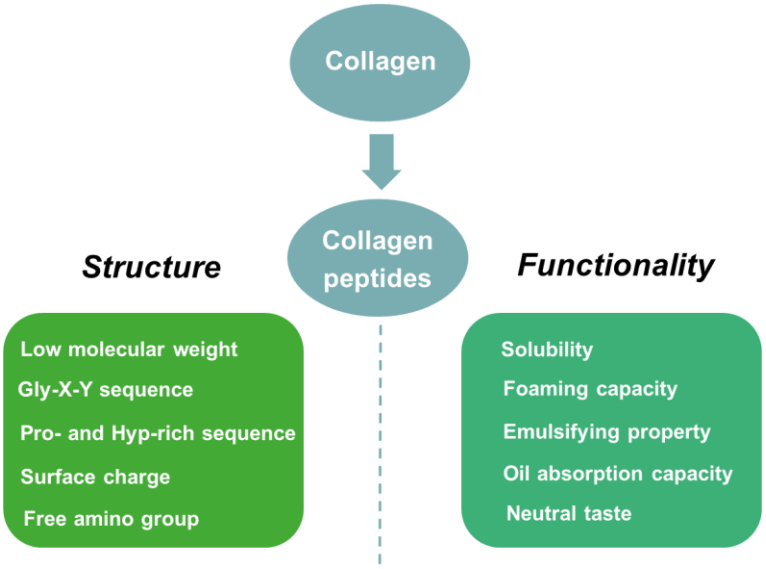

Fig. 1 Structural features and functionalities of collagen peptides.

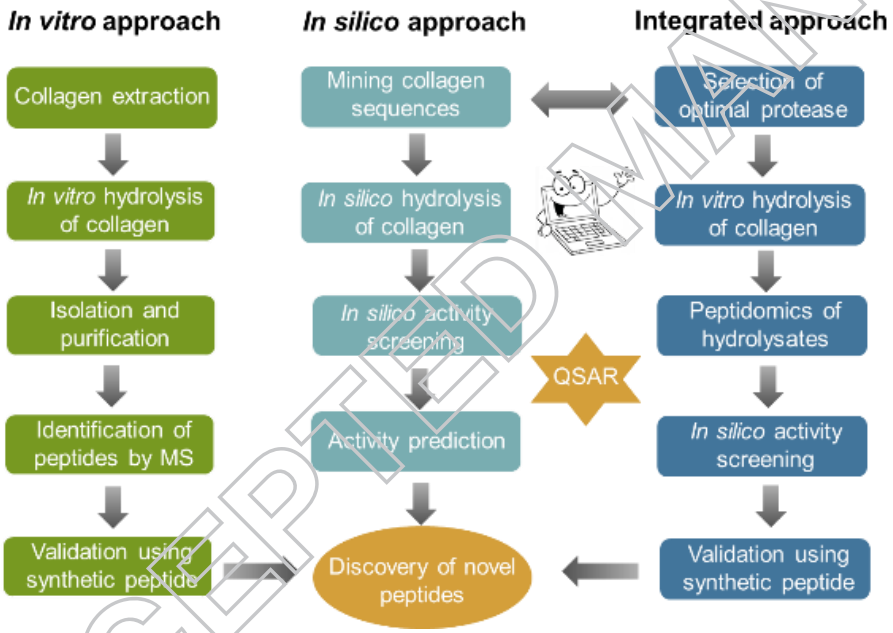

Fig. 2 The approaches for the discovery and production of bioactive collagen peptides.

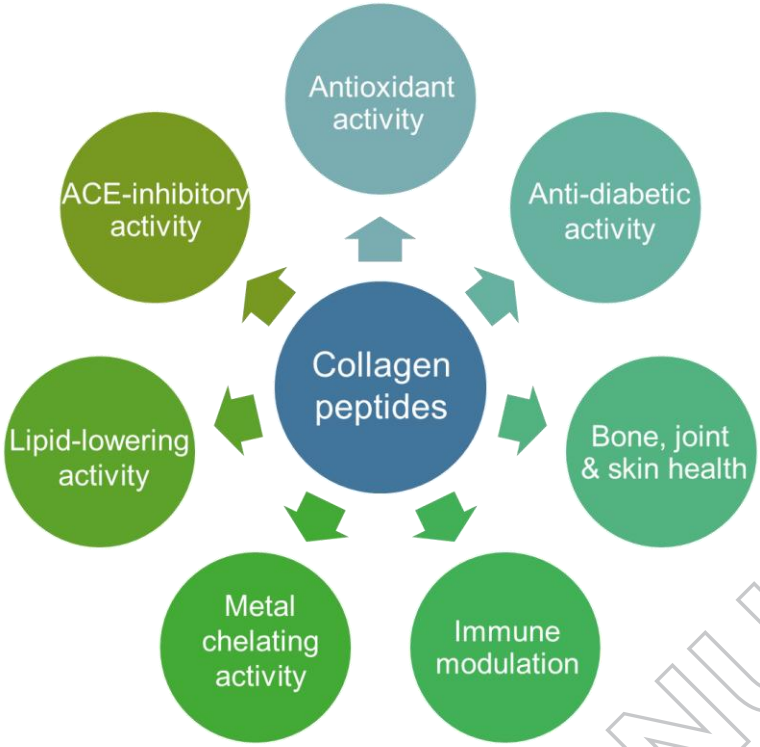

Fig. 3 Biological activities of collagen peptides.

Table 1 Antihypertensive/ACE inhibitory peptides different sources of collagen.

Source	Enzyme used	Sequence	In *vitro*/*in vivo* bioefficacy	Reference
Squid tunic gelatin	Alcalase	CP**P**GP**P**GF**P**GP**P**G**P**S	IC_{50}=90.03 μM	(Alemán et al. 2011)
Skate skin	α-Chymotrypsin	PGPLGLTGP QLGFLGPR	IC_{50}= 95 μM IC_{50}= 148 μM	(Lee et al. 2011)
Nile Tilapia gelatin	Alcalase	DPALATEPDPMPF	IC_{50}= 62.2 μM	(Vo et al. 2011)
Pacific cod gelatin	Pepsin, Trypsin & α-Chymotrypsin	LLMLDNDLPP	IC_{50} = 35.7 μM	(Himaya et al. 2012)
Jellyfish collagen	Alcalase	N/A	IC_{50}= 43 μg/mL Decreased blood pressure in RVH, 100 mg/kg	(Zhuang et al. 2012)
Squid collagen	Pepsin & Pancreatin	GRGSVPA**P**GP	IC_{50}=47.78 μM	(Alemán et al. 2013)
Mackerel gelatin	Pepsin	FGN	N/A	(Khiari et al. 2013)
Chum salmon skin	Trypsin	GLPLNLP	IC_{50}= 18.7 μM Decreased blood pressure in SHR (20 mg/kg)	(Lee et al. 2014)

Skate gelatin	Alcalase	MVGSAPGVL LGPLGHQ	IC_{50}= 3.09 μM IC_{50}= 4.22 μM	(Ngo et al. 2014) (Ngo et al. 2015)
Bovine collagen	Alcalase Papain	VGPV GPRGF	IC_{50}= 405.12 μM IC_{50}= 200.92 μM	(Fu et al. 2016a) (Fu et al. 2016b)

Note: P with boldface denotes Hyp (hydroxylated proline). N/A: not available. SHR, spontaneously hypertensive rats. RHV, renovascular hypertensive (RVH) rat.

Table 2 Antioxidant peptides identified from different sources of collagen.

Source	Enzyme used	Sequence	Antioxidant activity tests	IC_{50} (μM)	Reference
Pacific cod gelatin	Papain	TCSP TGGGNV	DPPH and hydroxyl radical scavenging, DNA damage and ROS	Scavenging activities of DPPH and hydroxyl radicals, 72% and 56% at 1 mg/mL	(Ngo et al. 2011)
Skate skin gelatin	Alcalase, Flavourzyme, Neutrase and Protamex	MVGSAPGVL LGPLGHQ	ROS, glutathione and superoxide dismutase	N/A	(Ngo et al. 2014)
Scales of croceine coaker	Trypsin & Pepsin	GFRGTIGLVG GPAGPAG GFPSG	Hydroxyl, DPPH, superoxide and ABTS radical scavenging	Hydroxyl radical (0.293, 0.240, and 0.107 mg/mL), DPPH radical (1.271, 0.675, and 0.283 mg/mL), superoxide radical (0.463, 0.099, and 0.151 mg/mL) and ABTS radical (0.421, 0.309, and 0.210 mg/mL).	(Wang et al. 2013)
Alaska pollock skin collagen	Typsin & Flavourzyme	GPAGPHGPPG	Ca, Fe and Cu chelating activity	11.52 ± 2.23, 1.71 ± 0.17 and 0.43 ± 0.02 μmol/μmol	(Guo et al. 2015)
Duck skin gelatin	Collagenase & pepsin	HTVQCMFQ	Hydroxyl, DPPH and alkyl radical scavenging	32.6, 22.7, 55.1, and 49.8 μg/mL	(Lee et al. 2012)

			ROS		
Spanish dry-cured ham	-	GLAGA	DPPH radical scavenging and ferric-reducing antioxidant power	0.5 units of absorbance at 1 mg/mL	(Escudero et al. 2013)
Amur sturgeon skin gelatin	Alcalase	PAGT	DPPH, ABTS and hydroxyl radical scavenging	5.38, 0.008 and 0.89 mg/mL	(Nikoo et al. 2014)
Blue shark skin gelatin	Protamex	-	DPPH and hydroxyl radical scavenging	0.57 and 2.04 mg/mL	(Weng et al. 2014)
Tilapia skin gelatin	Properase E	LSGYGP	Hydroxyl radical scavenging	22.47 μg/mL.	(Sun et al. 2013)
Collagen from Nile tilapia	Pepsin, trypsin & chymotrypsin	GPM	DPPH radical	25.64 μg/mL	(Liang et al. 2011)
Tilapia skin gelatin	Properase E	EGL YGDEY	Hydroxyl radical scavenging	4.61 and 6.45 μg/mL	(Zhang et al. 2012)
Grass carp skin	Alcalase	PYSFK GFGPEL VGGAP	DPPH, hydroxyl, and ABTS radical scavenging	DPPH radical (2.459, 3.634 and 6.063 mM), hydroxyl radical (3.563, 2.606 and 4.241 mM), ABTS radical (0.281, 0.530 and 0.960 mM)	(Cai et al. 2015)

Table 3 DPP-IV inhibitory peptides from different sources of collagen.

Source	Enzyme used	Sequence	IC_{50} (μM)	Reference
Bovine collagen	Papain, Ficain or Bromelain	PPG	2252.68	(Lafarga et al., 2014)
Atlantic salmon gelatin	Alcalase, Bromelain or Flavourzyme	GPAE GPGA	49.6 41.9	(Li-Chan et al., 2012)
Barbel fish gelatin	Esperase, Savinase, Alcalase,	-	-	(Sila et al., 2015)

	Trypsin, Izyme G, Protamex, Neutrase, Peptidase			
Deer collagen	Pepsin, Pepsin/Alcalase, Pepsin/Trypsin	GPGSPGGPL GPVGXAGPPGK GPM(O)GPXGVK GPVGPSGPXGK GPAGPXGVXGL	1638.3 83.3 226.9 93.7 318.1	(Jin et al., 2015)
Halibut, hake, tilapia, milkfish	Flavourzyme	SPGSSGPQGFTG GPVGPAGNPGANGLN PPGPTGPRGQPGNIGF IPGDPGPPGPPGP LPGERGRPGAPGP GPKGDRGLPGPPGRDGM	101.6 81.3 146.7 65.4 76.8 89.6	(Wang et al., 2015)
Pork, cattle, fish, chicken feet	Streptomyces or Collagenase	GAX GPA GPX	>20,000 5030 2510	(Hatanaka et al., 2012)
Porcine skin gelatin	Alcalase	GPX GPAG	45.3 41.1	(Hsu et al., 2013)
Silver Carp collagen	Neutrase	APGPAGP LPIIDI	229.14 105.44	(Zhang et al., 2016)
Alaska pollock skin collagen	Pepsin, Corolase PP & Trpsin	-	-	(Guo et al., 2014)

Table 4 Summary of animal and human intervention studies on the identification and quantification of collage peptides *in vivo*.

Source	Peptides	Locations	Maximal concentration	Bioactivity	Reference
Commercial fish scale gelatin hydrolysate	Hyp containing di- and tri-peptides	Plasma of adults	60.65 nmol/mL Pro-Hyp	Fibroblast proliferation	(Ichikawa et al., 2010)
Commercial fish scale gelatin	Pro-Hyp and Hyp-Gly	Plasma of adults	120 nmol/mL	Fibroblast proliferation and improved skin	(Shigemura et al., 2011) (Shimizu et

hydrolysate				barrier dysfunction	al., 2015) (Haratake et al., 2015)
Porcine skin gelatin hydrolysate	Hyp-Gly	Plasma of adults	4.2 nmol/mL	Beneficial effects on bone tissue	(Sugihara et al., 2012)
Commercial fish scale gelatin hydrolysate	Hyp and Hyp containing di- and tri-peptides	Plasma of adults	0.663 nmol/mL Pro-Hyp-Gly 163 nmol/mL Pro-Hyp	Fibroblast proliferation	(Taga et al., 2014)
Bovine skin gelatin hydrolysates	Tripeptides (X-Hyp-Gly)	Plasma of rats	0.78 nmol/mL Ala-Hyp-Gly	Chondroprotective effect and DPP-IV inhibitory activity	(Taga et al., 2016)
Porcine skin Collagen tripeptides	Gly-X-Y tripeptide	Plasma of rats	130 nmol/mL Gly-Pro-Hyp	Interaction with platelets and the central nervous system	(Yamamoto et al., 2015)
Chicken collagen hydrolysate	Ala-Hyp	Plasma of adults	2.27 nmol/mL	ACE-inhibitory activity	(Iwai et al. 2009)
Porcine skin collagen hydrolysates	Tripeptides (Gly-X-Y)	Plasma of adults	Ala-Hyp (26.01 nmol/mL) Gly-Pro-Hyp (21.13 nmol/mL) Pro-Hyp (16.84 nmol/mL)	Diverse activities	(Yazaki et al., 2017)