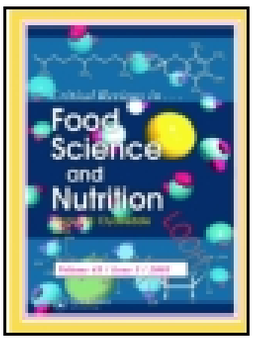

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Bioactive peptides derived from egg proteins: A review

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Abstract

Egg proteins have various functional and biological activities which make them potential precursor proteins for bioactive peptide production. Simulated *in vitro* gastrointestinal digestion and enzymatic hydrolysis using non-gastrointestinal proteases have been used as tools to produce these peptides. Bioactive peptides derived from egg proteins are reported to display various biological activities, including angiotensin I-converting enzyme (ACE) inhibitory (antihypertensive), antioxidant, antimicrobial, anti-inflammatory, antidiabetic and iron-/calcium-binding activities. More importantly, simulated *in vitro* gastrointestinal digestion has indicated that consumption of egg proteins has physiological benefits due to the release of such multifunctional peptides. This review encompasses studies reported to date on the bioactive peptide production from egg proteins.

Keywords

egg proteins; enzymatic hydrolysis; gastrointestinal proteases; non-gastrointestinal proteases; bioactive peptides, degree of hydrolysis, ultrafiltration

Abbreviations

GI gastrointestinal

non-GI non-gastrointestinal

ACE angiotensin I-converting enzyme

EW egg white

OVA ovalbumin

OT ovotransferrin

LYZ lysozyme

DH degree of hydrolysis

MWCOs molecular weight cut-offs

Ang I angiotensin I

Ang II angiotensin II

SBP systolic blood pressure

SHRs spontaneously hypertensive rats

HHL hippuryl-histidyl-leucine

ORAC oxygen radical absorbance capacity

DPPH 1,1-diphenyl-2-picrylhydrazyl

ABTS 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

FRAP ferric reducing-antioxidant power

BHT butylated hydroxytoluene

DPP-IV dipeptidyl peptidase-IV

1 Introduction

Hen egg is a rich source of functional and bioactive proteins. As an important dietary protein contributor, it provides various physiological benefits beyond normal nutrition value (Surai and Sparks, 2001). This has been attributed to the additional benefits provided by the production of bioactive peptides during protein digestion in the human gastrointestinal tract (Bhat et al., 2015a; Mine, 2007). Animal gastrointestinal (GI) proteases include pepsin, trypsin and chymotrypsin which hydrolyze proteins and release peptide sequences encrypted within the primary structure of the parent proteins. Many of these peptides are bioactive. Production of bioactive peptides from egg proteins has also been evaluated using non-gastrointestinal (non-GI) proteases from plant and microbial sources, such as papain, alcalase and thermolysin (Memarpoor-Yazdi et al., 2012). The use of non-GI proteases may create possibilities of obtaining more potent bioactive peptides, as they have different hydrolytic specificity, resulting in different peptide sequences exhibiting different bioactivities.

Egg protein derived peptides can exert diverse biological activities, including angiotensin I converting enzyme (ACE)-inhibitory, antihypertensive, antioxidant, antimicrobial, anti-inflammatory, antidiabetic and iron-/calcium-binding activities (Dávalos et al., 2004; Jahandideh et al., 2016; Jiang and Mine, 2000; Miguel et al., 2004; Palika et al., 2015; Pellegrini et al., 2004; Zambrowicz et al., 2015b). Bioactive peptides are normally enriched from the most potent hydrolysate fractions via ultrafiltration and/or chromatographic fractionation of the whole hydrolysate (Aluko, 2015). Numerous studies have only focused on particular identified bioactive peptides rather than the whole hydrolysate. However, from a global perspective, the whole hydrolysate is potentially of greater physiological relevance than individual identified

peptides, as there is the possibility that the biological effect manifested is complex and involves the whole hydrolysate. For commercial applications, the whole hydrolysates (unpurified bioactive peptides) are generally the main components of functional foods; while pure peptides find applications only in pharmaceuticals (Zambrowicz et al., 2013). Furthermore, the bioactivity of the total hydrolysate can act as an indicator for determining which GI and non-GI proteases (You and Wu, 2011), or a combination of different proteases are most suitable for generating hydrolysates (Huang et al., 2010; Memarpoor-Yazdi et al., 2012; Shen et al., 2010). In addition, the selection of proteases to generate hydrolysates can be important for the design and formulation of processed foods (Majumder and Wu, 2009, 2010).

From the literature, some non-GI proteases are reported to have an advantage over GI proteases in producing more potent bioactive peptide mixtures (Abeyrathne et al., 2016; Majumder and Wu, 2010; Memarpoor-Yazdi et al., 2012; You and Wu, 2011). However, these bioactive peptides when orally ingested will be challenged by the human GI digestion environment (Saadi et al., 2015). Thus, the stability of these peptides against further hydrolysis by GI proteases and peptidases is a consideration that needs to be studied (Majumder and Wu, 2011; Miguel et al., 2006a; Miguel et al., 2008; Miguel et al., 2007b; Yu et al., 2011b).

This work reviews the studies carried out on the production of bioactive peptides from egg proteins via enzymatic hydrolysis using GI and non-GI proteases. Of the various biological activities presented, the stability of ACE-inhibitory peptides against GI proteases, epithelial peptidases and pre-incubation with ACE is highlighted.

2 Egg proteins and peptide production by proteolysis using GI and non-GI proteases

Proteins in egg and particularly the proteins in egg white (EW) have a wide array of biological properties, making egg proteins useful agents for the nutraceutical and pharmaceutical industries (Abeyrathne et al., 2013; Mine, 1995, 2002). To expand the biological activities of egg proteins, enzymatic hydrolysis using GI and non-GI proteases has been carried out to obtain bioactive peptides with potential physiological benefits for human health. Among egg proteins, ovalbumin, ovotransferrin, ovomucin, lysozyme and phosvitin have most commonly been subjected to enzymatic hydrolysis. Therefore, the bioactivities of these precursor proteins are presented prior to the discussion of the derived bioactive peptides.

Ovalbumin (OVA), the most abundant EW protein, is a phosphoglycoprotein consisting of three subunits with different phosphate groups and a single carbohydrate moiety attached to its N-terminal (Li-Chan et al., 1995). It has a molecular weight of 45 kDa, 386 amino acid residues and belongs to the serpin family, despite lacking serpin-like protease-inhibitory activity (Yu et al., 2014). The biological function of OVA in EW has not been elucidated (Mine, 2007). However, when OVA is subjected to enzymatic hydrolysis using GI proteases, the hydrolysate has been reported to have ACE-inhibitory, antihypertensive, antioxidant and antimicrobial activities.

Ovotransferrin (OT) accounts for 12% of total EW proteins, and is a monomeric glycoprotein that contains a single oligosaccharide chain in the C-terminal part of the protein (Li-Chan et al., 1995). It has a molecular weight of 76 kDa, 686 amino acid residues and as it is a member of the transferrin family it has an iron-binding activity (Stevens, 1991). OT consists of two globular lobes (N- and C-lobes) interconnected by a 9 amino acid α-helix that can be released by tryptic

digestion (Giansanti et al., 2015). OT has been reported to have antibacterial, antiviral, antioxidant, anti-inflammatory and other biological activities (Giansanti et al., 2015; Kovacs-Nolan et al., 2005). Additionally, enzymatic hydrolysis using GI and non-GI proteases of OT generates peptides exhibiting ACE-inhibitory and antidiabetic activities.

Ovomucin accounts for ~ 3.5% of EW proteins, and is a sulfated glycoprotein that contains various carbohydrate moieties including sialic acid (Li-Chan et al., 1995; Watanabe et al., 1998a). The molecular weight of ovomucin ranges from 150 to 230 kDa (Abeyrathne et al., 2016). Due to its poor solubility after isolation, ovomucin is reduced or sonicated prior to use (Omana et al., 2010). However, such chemical or physical approaches could result in cleavage of disulfide bonds and the release of carbohydrate chains and may affect the biological activities of ovomucin (Watanabe et al., 1998a). Many studies have reported that ovomucin has antiviral, antitumor and anti-cholesterolemic activities (Nagaoka et al., 2002; Tsuge et al., 1996a, b, 1997; Yokota et al., 1999). Peptides derived from the enzymatic hydrolysis using non-GI proteases of ovomucin retain antiviral and antitumor activities and also show antioxidant and antibacterial potency.

Lysozyme (LYZ) accounts for ~ 3.5% of EW proteins, and is a monomeric protein that has a molecular weight of 14.4 kDa and 129 amino acid residues (Li-Chan et al., 1995). It can hydrolyze the β-linkages between N-acetylglucosamine and the N-acetylmuramic acid residues of the peptidoglycan of Gram-positive bacteria, but shows limited action against Gram-negative bacteria (Baron et al., 2016; Mine et al., 2004). However, following enzymatic hydrolysis using GI and non-GI proteases, LYZ-derived peptides have been reported to show antimicrobial

activities against both Gram-positive and Gram-negative (e.g., *E. coli K-12*, *B. bronchiseptica*, *S. enteritidis* and *H. pylori*) bacteria, as well as ACE-inhibitory and antioxidant activities.

Phosvitin, the major phosphoprotein in yolk, consists of 217 amino acid residues, and has a molecular weight between 36 and 40 kDa (Li-Chan et al., 1995; Samaraweera et al., 2014). Serine accounts for more than 55% of the total amino acids in phosvitin, and almost all of the serine residues are phosphorylated. This indicates that phosvitin may have a strong metal binding capacity and thus an inhibitory effect on metal ion-induced lipid peroxidation (Samaraweera et al., 2011). The phosphopeptides of phosvitin produced with trypsin have also demonstrated to have *in vitro* and *in vivo* metal binding activities.

2.1 Enzymatic hydrolysis for peptide production

Peptide production can be from both *in vitro* enzymatic hydrolysis and microbial fermentation (Korhonen and Pihlanto, 2006). The latter is based on hydrolysis of proteins in the medium by proteases secreted by bacteria or yeasts during fermentation. To date, *in vitro* enzymatic hydrolysis using purified proteases has been primarily used to produce peptides from egg proteins. Purified proteases used in these studies include animal GI proteases (e.g., pepsin, trypsin and chymotrypsin) and non-GI proteases (e.g., papain, alcalase and thermolysin) from plant and microbial sources. These proteases will differ not only in their specificities but the optimal conditions (i.e., pH and temperature) (Bankus and Bond, 2001).

As presented in the following sections, EW proteins are more frequently subject to peptide production than yolk proteins. Among EW proteins, OVA, OT and LYZ have been the most studied, as these three proteins account for ~ 70% of EW proteins in total (Li-Chan et al., 1995). Numerous studies on the *in vitro* GI digestion of EW proteins have demonstrated that trypsin,

chymotrypsin and pancreatin do not extensively hydrolyze OVA, OT, LYZ and ovomucin (Abeyrathne et al., 2014; Abeyrathne et al., 2016; Fu et al., 2002; Jiménez-Saiz et al., 2014; Mine et al., 2004; Odani et al., 1997; Sun et al., 2016). Pepsin, however, has been shown to hydrolyze these EW protein components. Various studies have also shown that proteolysis during *in vitro* GI digestion mostly occurs during the gastric phase (Nyemb et al., 2014; Nyemb et al., 2016). In accordance, peptide production from EW proteins has always incorporated pepsin with or without trypsin, chymotrypsin or pancreatin in the enzymatic hydrolysis. Hitherto, phosvitin is the only purified yolk component that has been used as a precursor protein for peptide production. Trypsin has been largely used to hydrolyze this protein, and the derived peptides are mainly studied for their antioxidant and calcium-/iron-binding activities.

Among non-GI proteases, papain, alcalase and thermolysin are widely used enzymes for peptide production from egg proteins. Reported studies have used considerably different enzymatic hydrolysis conditions, particularly incubation temperatures, even for the same enzyme. For instance, alcalase hydrolysis is largely carried out at 50°C (Chang et al., 2013; Liu et al., 2010; Nimalaratne et al., 2015; Park et al., 2001; You et al., 2010; You and Wu, 2011; Yu et al., 2012) but lower incubation temperatures, such as 37 and 45°C, have also been used for this enzyme (Abeyrathne et al., 2014; Abeyrathne et al., 2016; Moon et al., 2013). For thermolysin, the incubation temperature has been varied from 37 to 70°C during proteolysis (Fujita et al., 2000; Huang et al., 2010; Majumder and Wu, 2010; Shen et al., 2010; You and Wu, 2011). Incubation temperature is one of the most important factors influencing the enzyme activity. A high incubation temperature might also affect the structure of egg proteins, as some egg proteins start

to denature at 45°C (Dev et al., 2010). Therefore, the incubation temperature along with other digestion parameters may affect the peptide production.

Compared to those involving non-GI proteases, studies on peptide production via simulated *in vitro* GI digestion are of particular interest, as the potency and bioavailability of peptides measured *in vitro* may reflect the physiological benefits of egg proteins after oral intake and *in vivo* GI digestion. In this regard, the simulated *in vitro* GI digestion should be designed to closely mimic the human physiological condition. Several studies have mimicked the *in vivo* GI digestion process to prepare bioactive peptides from EW, yolk and whole egg (Majumder and Wu, 2009; Miguel et al., 2007a; Rao et al., 2012; You and Wu, 2011; Yousr and Howell, 2015). Results of these studies suggest that human GI digestion of egg proteins could produce bioactive peptides with antihypertensive and antioxidant activities.

2.2 Degree of hydrolysis and bioactivity of protein hydrolysates

It is generally accepted that for a single protease system the rate of proteolysis tends to decrease with increasing hydrolysis time, suggesting that the degree of hydrolysis (DH) will eventually reach a plateau and further increases in hydrolysis time will have little effect on peptide size or activity (Aluko, 2015). The DH of proteins plays an important role in controlling the composition and bioactivity of the obtained hydrolysates (Eckert et al., 2014). In addition to proteolysis conditions, protease specificities are important as this will affect the bioactive properties of the resulting protein hydrolysates (Ajibola et al., 2011; Aluko, 2015; Memarpoor-Yazdi et al., 2012).

The ACE-inhibitory activity of EW hydrolysates produced with pepsin, trypsin or chymotrypsin increased as the DH increased with hydrolysis time up to 24 h (Miguel et al., 2004). However, in

another study, while the OVA hydrolysates showed higher ACE inhibition as the DH increased during the initial 8 h of pepsinolysis, prolonged hydrolysis for 24 h resulted in a reduced ACE-inhibitory activity (Quirós et al., 2007). Similarly, Chen et al. (2012b) reported that the ACE-inhibitory activity of protein hydrolysates first increased and then decreased even though the DH of EW after hydrolysis with trypsin increased with hydrolysis time (up to 10 h). Inconsistent results for single protease systems have also been reported for the GI digestion of egg proteins. Majumder and Wu (2009) and Rao et al. (2012) hydrolyzed egg proteins (e.g., LYZ, EW, and whole egg) by successive gastric and duodenal digestions, and found the gastric hydrolysates were less potent than the duodenal counterparts in inhibiting ACE activity, suggesting that more ACE-inhibitors were released in the small intestine. Conversely, Miguel et al. (2007a) reported the gastric hydrolysate of EW had a higher ACE-inhibitory activity than the duodenal counterpart, which was due to the degradation of some ACE-inhibitors during the duodenal digestion.

The antioxidant activity of EW hydrolysates produced with different proteases (e.g., trypsin, papain, Flavourzyme, alcalase and neutrase) has been evaluated over 5 h of proteolysis (Chen et al., 2012a). The rate of DH increased most significantly during the first hour of hydrolysis and then the rate of change progressively slowed down (Chen and Chi, 2012; Chen et al., 2012b). Among these proteases, trypsin improved the antioxidant activity following an increase in the DH; however, the other four non-GI proteases resulted in an initial increase and then a decrease in the antioxidant activity during proteolysis. Similarly, Chang et al. (2013) reported that when Flavourzyme and Protamex were used to hydrolyze ovomucin, the antioxidant activity of both hydrolysates reached considerably higher levels after 2 h of proteolysis and remained nearly

unchanged until 24 h. These findings support the conclusion that protease specificity plays an important role in releasing bioactive peptides and that there is an optimum DH that leads to hydrolysates with high bioactivities (Chen et al., 2012a; Luo et al., 2014; Miguel et al., 2004; Quirós et al., 2007; Vermeirssen et al., 2003).

2.3 Ultrafiltration to refine bioactive peptides

Membrane ultrafiltration, particularly with low molecular weight cut-offs (MWCOs), has been routinely used to remove un-hydrolyzed proteins, proteases and large protein fragments from crude protein hydrolysates, so that lower molecular weight peptides with potentially higher bioactivity can be obtained (Aluko, 2015). In many cases, stepwise ultrafiltration using membranes with large to small MWCOs has been reported to refine bioactive peptides (Korhonen and Pihlanto, 2006).

Crude egg protein hydrolysates have been directly filtered using 3-kDa membrane as a refinery step prior to peptide purification (Baratzadeh et al., 2013; Chang et al., 2013; Majumder and Wu, 2009; Nimalaratne et al., 2015). A number of studies have demonstrated a positive correlation between low molecular weight peptides and high bioactivities; however, inconsistent findings have been reported. Table 1 lists studies that investigate the bioactivities of different fractions of egg protein hydrolysates subsequent to ultrafiltration with varied MWCOs. In general, the ACE-inhibitory and antioxidant activities of protein hydrolysates are improved following ultrafiltration with MWCOs of 10 kDa or lower. However, it is interesting to note that the ACE-inhibitory activity of the gastric but not the GI hydrolysates of LYZ and EW was enhanced by 3-kDa ultrafiltration (Miguel et al., 2007a; Rao et al., 2012). This indicated that some large peptides produced during the duodenal digestion also contribute to the inhibition against ACE.

In addition, as detailed in the following sections, some large peptides, although devoid of a high *in vitro* ACE-inhibitory activity, can exert significant antihypertensive potency *in vivo*. This has been attributed to the degradation of large non-effective peptides to smaller potent sequences that regulate blood pressure via mechanisms other than ACE-inhibition.

Ultrafiltration using low MWCO filters, however, may not always contribute to the enrichment of peptides with desired bioactivities. For example, for peptides to be antimicrobial, at least 7-8 amino acids are required to form amphipathic structures with hydrophobic and hydrophilic faces on opposite sides of a peptide molecule (Bahar and Ren, 2013). Therefore, for one-step ultrafiltration, a MWCO of 3 kDa might be considered as the initial target size prior to peptide purification.

The peptide yield is known to correlate with membrane MWCO filters, suggesting a lower yield upon the use of smaller MWCO filters. Compared to one-step ultrafiltration (e.g., 1 kDa), stepwise approaches (e.g., 10, 5, 3, 1 kDa) can obtain different molecular weight fractions, of which two or more fractions may not significantly differ in their bioactivities. In such case, further ultrafiltration will not be necessary, so that filtrates with both high yield and high bioactivity can be directly used for industrial applications.

The importance of ultrafiltration to obtain low molecular weight filtrates is evident, but other structural features may also influence the bioactivity of peptides produced. Indeed, several reviews on the structure-activity relationship of ACE-inhibitory (Aluko, 2015; Iwaniak et al., 2014), antioxidant (Sarmadi and Ismail, 2010), metal-chelating (Guo et al., 2014) as well antimicrobial (Bahar and Ren, 2013) peptides have pointed out that the bioactivity of peptides

crucially depends on multiple physiochemical factors, such as peptide molecular size, hydrophobicity, amino acid composition, sequence, configuration as well as peptide solubility.

2.4 Pretreatment of egg proteins prior to enzymatic hydrolysis

Food processing treatments, such as heating, are known to modify the structure of proteins resulting in protein denaturation and aggregation. Heat-induced structural changes may expose hydrolysis sites previously inaccessible to proteases, thereby producing novel peptides. As egg products are normally cooked before consumption, the majority of studies have preheated egg proteins prior to enzymatic hydrolysis for peptide production (Liu et al., 2010; Majumder and Wu, 2009; Nimalaratne et al., 2015; Rao et al., 2012; You et al., 2010; You and Wu, 2011). Majumder and Wu (2009) compared frying and boiling of egg products (EW, yolk and whole egg). They found a higher ACE-inhibitory activity present in fried protein GI hydrolysates than boiled protein GI hydrolysates. The authors attributed this to a higher heating temperature during frying than boiling. Moreover, Majumder and Wu (2010) pretreated OT with either sonication or reducing agents, and found the ACE-inhibitory activity of the protein hydrolysates was significantly enhanced by a sonication pretreatment.

Several studies have prepared bioactive peptides from defatted, phospholipid-extracted or lecithin-extracted yolk (Eckert et al., 2014; Pokora et al., 2013; Sakanaka et al., 2004; Yousr and Howell, 2015). The use of ethanol for delipidation would inevitably denature yolk proteins. Phosvitin isolated from yolk has been subject to partial alkaline dephosphorylation prior to enzymatic hydrolysis (Jiang and Mine, 2000; Katayama et al., 2006; Xu et al., 2007b). Jiang and Mine (2000) suggested that alkaline dephosphorylation could perturb the core conformation of phosvitin, thereby unmasking peptide bonds specifically vulnerable to proteases.

High hydrostatic pressure has been shown to induce the unfolding and aggregation of egg proteins (Hoppe et al., 2013; Van der Plancken et al., 2007a, b). Egg white proteins pretreated by high hydrostatic pressure exhibited enhanced susceptibility to peptic, tryptic and chymotryptic hydrolysis (Iametti et al., 1999; Iametti et al., 1998; Singh and Ramaswamy, 2014; Van der Plancken et al., 2004). In addition, Hoppe et al. (2013) identified several previously reported ACE-inhibitory and antioxidant peptides from the peptic hydrolysate of pressure-treated OVA.

Quirós et al. (2007) applied high hydrostatic pressure (up to 400 MPa) to the tryptic or chymotryptic digestion of OVA. Proteolysis under high hydrostatic pressure enhanced OVA hydrolysis and altered the proteolytic pattern. The authors found that proteolysis under high hydrostatic pressure did not improve the ACE-inhibitory activity of the OVA hydrolysate, but the release of several antihypertensive peptides (YAEERYPIL, FRADHPFL and RADHPFL) was accelerated. Similarly, López-Expósito et al. (2008) examined the peptic digestion of OVA under high hydrostatic pressure at 400 MPa. They found the proteolysis of OVA was enhanced and the hydrolysates showed lower antigenicity than those produced at atmospheric pressure. Proteolysis under high hydrostatic pressure results in a rapid hydrolysis of intact protein and the accumulation of intermediate peptides that are further degraded over time (Chicón et al., 2009).

3 ACE-inhibitory and antihypertensive peptides

The arterial blood pressure in the human body is regulated via several diverse physiological systems, such as the renin-angiotensin-aldosterone system. Renin in the blood initially hydrolyzes angiotensinogen to generate angiotensin I (Ang I, an inactive decapeptide). The dipeptide from the C-terminus of Ang I is then cleaved by the angiotensin converting enzyme (ACE; EC3.4.15.1, dicarboxy peptidase), which converts Ang I to the potent vasoconstricting

Ang II (Saleh et al., 2016; Yousr and Howell, 2015). The Ang I to Ang II conversion in the blood induces the release of adrenal aldosterone. This hormone enhances the reabsorption of renal tubular sodium and leads to a subsequent increase in blood pressure. Furthermore, ACE could hydrolyze bradykinin, a potent vasodilator, by cleaving its C-terminal dipeptide. This will inactivate metabolites in the depressor hormonal (kinin-kallikrein) system. Therefore, ACE inhibitors suppress the generation of vasoconstrictor Ang II and potentiate the action of the vasodilator bradykinin (Yousr and Howell, 2015). Due to the side effects of synthetic ACE-inhibitors (e.g., captopril), the development of natural ACE-inhibitory/antihypertensive peptides has received great interest. Apart from the widely studied dairy proteins, egg proteins have been shown to be another rich source of ACE-inhibitors.

The following two sections document both egg protein hydrolysates and identified peptides with ACE-inhibitory and/or antihypertensive activities. The ACE-inhibitory activity is expressed as an IC_{50} value (i.e., the concentration of an inhibitor required to inhibit 50% of the ACE activity) or a percentage of ACE-inhibition (i.e., the percentage inhibition achieved by a defined concentration of an inhibitor).

3.1 Protein hydrolysates with ACE-inhibitory/antihypertensive activity

The production of egg protein hydrolysates with potential ACE-inhibitory/antihypertensive activity by GI or non-GI proteases or a combination of both is summarized in Table 2. The use of GI proteases, particularly under simulated *in vitro* digestion models by mimicking human physiological conditions, is aimed to generate peptides that resemble the products of human GI digestion (Bah et al., 2015). However, on account of the limited number of proteases in the human GI tract as well as their largely defined specificity, the release of peptides following

protein digestion may be restrained, with weakly active or inactive functions (Udenigwe and Howard, 2013). In accordance, non-GI proteases from plant or microbial sources that possess different and often broader specificity have been under screening and exploitation in order to generate novel peptides from egg proteins with enhanced bioactivities (Abeyrathne et al., 2014; Abeyrathne et al., 2016; Eckert et al., 2014; Majumder and Wu, 2010; You and Wu, 2011).

In general, the production of ACE-inhibitory/antihypertensive peptides does not always require specific proteins or proteases, but digestible proteins and proteases that generate small-size peptides (< 10 amino acid residues) are preferable candidates for this purpose (Aluko, 2015). In this regard, both proteases and substrate proteins are factors that influence the release of bioactive peptides. It therefore should be possible to select appropriate proteases for obtaining hydrolysates with specific and preserved peptides (Tavano, 2013). As the interest in the production of ACE-inhibitory peptides with potential antihypertensive activity from egg proteins increases, numerous studies have investigated the impact of different proteases on the activities of egg protein hydrolysates. The ACE-inhibitory activities of egg protein hydrolysates produced by GI and various non-GI proteases are compared and present in Table 3.

Among GI proteases, pepsin hydrolysates of egg proteins demonstrate a higher ACE-inhibitory activity than those produced with chymotrypsin and trypsin. Furthermore, when egg proteins are subjected to successive actions of pepsin and pancreatic enzymes, protein hydrolysates appear to have a greater ACE-inhibitory activity. Studies have shown that pepsin, at enzyme/substrate (E/S) ratios resembling a physiological situation (1:20 w/w) or even lower, can efficiently hydrolyze major EW proteins such as OVA (López-Expósito et al., 2008; Martos et al., 2010; Martos et al., 2011; Odani et al., 1997), LYZ (Ibrahim et al., 2005; Mine et al., 2004),

ovomucoid (Kovacs-Nolan et al., 2000; Martos et al., 2011) and ovomucin (Abeyrathne et al., 2016; Sun et al., 2016). In contrast, pancreatic enzymes, at similar or higher E/S ratios, show limited proteolytic action on these major EW proteins (Abeyrathne et al., 2014; Fu et al., 2002; Mine et al., 2004; Odani et al., 1997; Sun et al., 2016; Takagi et al., 2003). Pepsinolysis that favors the cleavage of protein polypeptide chains would accordingly generate peptides with various molecular sizes and amino acid compositions and thus greater ACE-inhibitory activities.

Compared to GI proteases, non-GI proteases, such as thermolysin and alcalase can liberate peptides from egg proteins that show higher ACE-inhibitory activities (Majumder and Wu, 2010; You and Wu, 2011). Thermolysin is a thermostable endopeptidase that catalyzes hydrolysis of the peptide bonds specifically of large hydrophobic residues, in particular Leu, Ile and Phe (Pelmenschikov et al., 2002). Thermolysin retains its conformation even up to 70°C (Kunugi et al., 1982). As egg proteins are prone to heat-induced unfolding and denaturation, thermolysin-catalyzed hydrolysis of egg proteins at high temperatures would likely proceed more extensively than at low temperatures. Alcalase has a broad specificity and is used for the release of potent antihypertensive peptides from food proteins (Udenigwe and Howard, 2013). However, studies conducted by Fujita et al. (2000) and Abeyrathne et al. (2014) showed that OVA hydrolysates produced with thermolysin and alcalase had lower ACE-inhibitory activities than those produced with pepsin or chymotrypsin. Such discrepancies, however, are believed due to the unfavorable reaction temperatures i.e., 37°C used for both thermolysin and alcalase in these two studies. Alcalase-catalyzed proteolysis has been shown to be slower at 35°C than 55°C (Ovissipour et al., 2009), as the optimal temperature for alcalase could be up to 65°C according to Novozymes®.

In addition, neutrase and a plant serine protease isolated from *Cucurbita ficifolia* fruit pulp have also been used to hydrolyze EW and yolk. Both proteases produced protein hydrolysates that showed an ACE-inhibitory activity (IC_{50} = 245-9,071 μg protein/mL, 59-482 μg protein/mL, respectively) (Eckert et al., 2014; Pokora et al., 2013; Pokora et al., 2014). These studies suggested that some non-GI proteases can cleave peptide bonds, inaccessible to GI proteases, to produce more potent ACE-inhibitors. However, it should be noted that upon oral intake these bioactive peptides released by non-GI proteases will be challenged by the human GI digestion environment. Therefore, the proteolytic stability of these ACE-inhibitory peptides should be thoroughly examined, as discussed below.

When EW and yolk proteins are hydrolyzed by GI and non-GI proteases, the ACE-inhibitory activity of protein hydrolysates will depend on the proteases used. Majumder and Wu (2009) compared the ACE-inhibitory activity of the GI hydrolysates of EW and yolk, and found that the EW hydrolysate had a higher ACE-inhibitory activity than the yolk hydrolysate. Whereas, after hydrolysis with neutrase, the EW hydrolysate showed a lower ACE-inhibitory activity than yolk hydrolysate (Pokora et al., 2013).

As aforementioned, ultrafiltration of crude protein hydrolysates can enrich bioactive peptides. When low molecular size filtrates or even whole hydrolysates were subsequently fractionated by ion exchange or gel filtration chromatography, followed by reversed-phase HPLC, peptide fractions with the highest ACE-inhibitory activity could be separated and the bioactive peptides could be identified (Eckert et al., 2014; Pokora et al., 2014; You and Wu, 2011; Yousr and Howell, 2015; Zambrowicz et al., 2015b), as discussed in the section below.

3.2 ACE-inhibitory/antihypertensive peptides

Identified ACE-inhibitory peptides and their antihypertensive potency, as evaluated by measuring the decrease in systolic blood pressure (SBP, Δ mmHg) of spontaneously hypertensive rats (SHRs) after a single intravenous or oral dose are shown in Table 4. The ACE-inhibitory and antihypertensive activities of egg peptides are often compared with those of captopril, a known ACE-inhibitor. Captopril has an IC_{50} value of 0.02 μM (Fujita and Yoshikawa, 1999) with a noticeable antihypertensive activity (e.g., − 16 mmHg after 5 h of a single oral administration at 10 mg/kg body weight, or − 55 mmHg after 6 h of a single oral administration at 50 mg/kg body weight) (Miguel et al., 2007a; Yu et al., 2017). By comparison, the determined IC_{50} values of egg peptides ranged from 0.4 to 102 μM and are thus potent ACE-inhibitors. Although egg peptides show a lower *in vitro* ACE-inhibitory activity than captopril, they exert a comparable or even higher antihypertensive activity than captopril when orally or intravenously administered to SHRs. For instance, at a single oral dose of 2 mg/kg body weight, RADHPFL, YAEERYPIL and IVF decreased the SBP by over 30 mmHg, while captopril decreased the SBP by 54 mmHg at 50 mg/kg body weight (Miguel et al., 2005).

Egg peptides have been reported to have a dose-dependent antihypertensive activity (Lee et al., 2006; Majumder et al., 2013b; Matoba et al., 1999; Miguel et al., 2005; Scruggs et al., 2004). In one study, a single high-dose (50 mg/kg body weight) oral administration of peptide QIGLF to SHPs suppressed the recovery of SBP to an even larger extent than captopril at a dose of 10 mg/kg body weight (Yu et al., 2017). The authors postulated that a different absorption and metabolism or different transport rate to sites of action was involved. Yu et al. (2017) also reported that peptide QIGLF exerted both short- and long-term antihypertensive effects that were

similar to captopril. Additionally, Fujita et al. (1995) found that the blood pressure-lowering effect of the peptide ovokinin (FRADHPFL), when emulsified with egg yolk, at a low dose (25 mg/kg body weight) was more pronounced than at a high dose (100 mg/kg body weight) in saline solution. Ovokinin is potentiated by its emulsification with yolk, which might be due to an improved intestinal absorption of this peptide or a yolk-induced protective effect on ovokinin from peptidases (Fujita et al., 1995).

Degradation of ACE-inhibitory peptides by GI proteases and epithelial peptidases

It is generally believed that ACE-inhibitory peptides have antihypertensive potency; however, several studies have reported a lack of correlation between the *in vitro* ACE inhibition and the *in vivo* antihypertension activities of egg peptides (López-Fandiño et al., 2006). For instance, some OVA-derived ACE-inhibitory peptides (e.g., FFGRCVSP and ERKIKVYL) failed to exert an antihypertensive activity (Fujita et al., 2000). This result is likely due to the degradation or modification of susceptible egg peptides in the gut, the vascular system or the liver (Hartmann and Meisel, 2007). Fig. 1 depicts the possible enzymatic degradation of ACE-inhibitory peptides in the GI tract, enterocytes and blood vessels.

The resistance of bioactive egg peptides to proteases or peptidases has been evaluated by simulated *in vitro* GI digestion or *in vivo* studies to validate their physiological effects (Miguel et al., 2006a; Miguel et al., 2008; Miguel et al., 2007b). To determine the antihypertensive activity, hypertensive animals are administered test peptides orally or intravenously (Fujita et al., 2000). Upon oral administration, ACE-inhibitory peptides firstly pass animal GI tract before being absorbed in an intact form from the intestine. In order to inhibit ACE in blood vessels, peptides

should resist the degradations by both GI proteases and plasma peptidases (Korhonen and Pihlanto, 2006).

RADHPFL and YAEERYPIL are two OVA-derived ACE-inhibitory peptides identified from an EW pepsin hydrolysate (Miguel et al., 2004). These two peptides also exhibited a strong antihypertensive activity (Table 4). Miguel et al. (2006a) subjected both peptides to simulated *in vitro* GI digestion and found that RADHPFL and YAEERYPIL were degraded into smaller peptides (e.g., YAEER, YPI, RADHPF and RADHP) which showed substantially reduced ACE-inhibitory activities (IC_{50} = > 1000, > 1000, 382 and 153 μg/mL, respectively). However, the degradation products RADHP and YPI, showed a similar maximum SBP reduction (− 30 and − 37 mmHg, respectively) at the same dose (2 mg/kg body weight) but after shorter (2-4 h) oral administration (Miguel et al., 2006a). These findings suggest that: 1) the ACE-inhibitory peptides RADHPFL and YAEERYPIL are susceptible to GI digestion; 2) the degradation products of both peptides are ineffective ACE-inhibitors, and 3) these degradation products reduce blood pressure through other regulatory pathways, which are the underlying antihypertensive mechanism for RADHPFL and YAEERYPIL.

The antihypertensive activity of food-originating peptides is generally due to their ACE inhibition potency (Miguel and Aleixandre, 2006). Peptides may exert antihypertensive effects via other mechanisms, such as renin inhibition, endothelium-derived nitric oxide generation, Ang II receptor blockage, inhibition of the release of endothelin-1 (a vasoconstricting peptide) by endothelial cells, potentiation of bradykinin or binding to opioid receptors (Aluko, 2015; Erdmann et al., 2008; Korhonen and Pihlanto, 2006). Peptic digestion of EW produced several OVA-derived peptides, such as YRGGLEPINF and ESIINF that are devoid of an ACE-

inhibitory activity *in vitro* (IC_{50} > 1000 μM) (Miguel et al., 2004), but these peptides were later found to have a strong antihypertensive activity (Miguel et al., 2007b). The authors suggested that following oral ingestion by SHRs, YRGGLEPINF and ESIINF exerted a blood pressure-lowering effect by acting as vasodilators. As both peptides might have been further degraded in the rat GI tract in the experiment, their degradation products were evaluated for ACE-inhibitory, vasodilatory and antihypertensive activities. All of the main degradation products (e.g., YRGGLEPI, GGLEPI, ESI, YR and NF) retained a vasodilatory activity but were not effective as ACE-inhibitors (IC_{50} > 1000 μM), except for YR which displayed a moderate ACE-inhibitory activity (IC_{50} = 191 μM) (Miguel et al., 2007b). This implied that OVA-derived peptides, even devoid of an ACE-inhibitory activity *in vitro*, could suppress hypertension through their vasodilatory degradation products.

Some ACE-inhibitory peptides derived from EW proteins are reported to be resistant to the simulated *in vitro* GI digestion. Yu et al. (2011b) obtained three OVA-derived ACE-inhibitors, DHPFLF, HAEIN and QIGLF, after alcalase hydrolysis of EW. When these three peptides were subject to hydrolysis by pepsin or trypsin, DHPFLF and HAEIN showed reduced ACE-inhibitory activities after trypsinolysis. QIGLF retained its ACE-inhibitory activity after either pepsinolysis or trypsinolysis. Majumder and Wu (2011) hydrolyzed OT using thermolysin followed by pepsin and obtained three ACE-inhibitory tripeptides, IRW, IQW and LKP. The stability of these ACE-inhibitors against successive *in vitro* digestion by pepsin, pancreatin and mucosal peptidase was examined. It was found that IQW resisted GI and mucosal digestions (unchanged IC_{50} = 1.6 μM). The ACE-inhibitory potency of IRW dramatically decreased after GI digestion (IC_{50} from 0.6 to 63 μM), due to degradation into IR and W. In contrast, LKP,

survived the GI digestion but was subsequently degraded by mucosal peptidase into KP and L, resulting in significantly attenuated ACE-inhibitory activity (IC_{50} from 2.9 to 113 μM).

Miguel et al. (2008) used a Caco-2 cell culture model to study the stability of several OVA-derived antihypertensive peptides against degradation by brush-border aminopeptidases. These authors also examined the transepithelial transport of the peptides, FRADHPFL, RADHPFL, RADHPF, RADHP, YAEERYPIL, YAEER and YPI. Caco-2 cells derived from a human intestinal adenocarcinoma provide a useful cell culture model of the small intestinal epithelium (Shimizu et al., 1997), as they undergo similar differentiation and polarization, and express brush-border hydrolases (Hidalgo et al., 1989). They have therefore been widely used to study the bioavailability (e.g., epithelial peptidase resistance) and transepithelial transport of food-originating bioactive peptides (Picariello et al., 2010; Picariello et al., 2013a; Quirós et al., 2008; Zhu et al., 2008). Hitherto, only Miguel et al. (2008) have studied the resistance of egg protein-derived antihypertensive peptides against epithelial peptidases as well as their transepithelial transport routes. Fig. 2 illustrates the stability of these bioactive peptides against degradations by brush-border peptidases and, possibly, intracellular peptidases. The potent antihypertensive tripeptide YPI was resistant to cellular peptidases and transferred across the Caco-2 cell monolayer via peptide transporter 1-mediated transcytosis. This implied that YPI exerted its blood pressure-lowering effect at tissue level.

Stability of ACE-inhibitory peptides against pre-incubation with ACE

Intravenous administration prevents ACE-inhibitory peptides from enzymatic degradation in the GI tract and enterocytes, but in the cardiovascular system ACE could possibly hydrolyze these ACE-inhibitors to become less or more potent. In an *in vitro* ACE-inhibition assay, hippuryl-

histidyl-leucine (HHL) is the substrate for ACE which cleaves the hippuryl-histidine bond to release hippuric acid. When test peptides are present in the ACE/HHL mixture, the release of hippuric acid may be inhibited, and thus the assay enables measurement of effectiveness of an ACE-inhibitor. However, in the *in vitro* ACE-inhibition assay, when HHL is absent and a potential ACE-inhibitor alone is incubated with ACE, this peptide might be a substrate for ACE, resulting in peptide degradation. The degradation products will be isolated and assayed again to determine their ACE-inhibitory activity using HHL as the substrate. Hence, the stability of some potential ACE-inhibitory peptides against pre-incubation with ACE can be determined. The significance of peptide pre-incubation with ACE lies in the fact that some poor ACE-inhibitors can be hydrolyzed by ACE to generate smaller peptides with higher potency. Based on the fate of pre-incubation with ACE, ACE-inhibitory peptides have been classified into three groups, namely, inhibitor type, substrate type and prodrug type (Fujita and Yoshikawa, 1999).

For instance, in Table 4, peptic digestion of OVA produced two ACE-inhibitory nonapeptides FFGRCVSP and ERKIKVYL (Fujita et al., 2000). Both peptides were shown to be substrates for ACE, meaning they were hydrolyzed by ACE and their IC_{50} values increased (from 0.4 to 4.6 μM and from 0.2 to 6 μM, respectively) after pre-incubation with ACE. On the other hand, dipeptide LW was a true ACE-inhibitor, with an unchanged IC_{50} value (6 μM) after pre-incubation with ACE (Fujita et al., 2000). Lee et al. (2006) obtained a prodrug type ACE-inhibitory peptide KVREGTTY from the chymotryptic digestion of OT. Subject to pre-incubation with ACE, KVREGTTY was hydrolyzed into KVREGT, resulting in a considerably decreased IC_{50} value (from 108 to 17 μM). Further, Lee et al. (2006) compared the antihypertensive potency between KVREGTTY and KVREGT after intravenous administration

to SHRs. The maximal blood pressure-lowering effect of KVREGTTY and KVREGT occurred at 40 and 20 min respectively, after injection. The authors suggested that the 20 min delay in the antihypertensive effect of KVREGTTY might be due to ACE-induced degradation of KVREGTTY in the bloodstream to generate KVREGT, as the latter is a true ACE-inhibitor that has an immediate effect.

In another study, Majumder and Wu (2011) pre-incubated OT-derived tripeptides IRW, IQW and LKP (IC_{50} = 0.6, 1.6 and 2.9 μM, respectively) with ACE and found that their IC_{50} values did not significantly change and thus were true ACE-inhibitors. Later, Majumder et al. (2013b) and Majumder et al. (2015a) examined the long-term antihypertensive effect of these three ACE-inhibitors. SHRs were orally administered IRW, IQW and LKP at 15 mg/kg body weight daily for 18 days. At the end of the trial, the blood pressure of the hypertensive animals was significantly reduced, in comparison to the control. It should be noted that, unlike IQW, the peptides IRW and LKP have been shown to be degraded by simulated *in vitro* GI and mucosal digestion (Majumder and Wu, 2011). Therefore, upon oral administration, IQW might be the only peptide absorbed in its intact form by enterocytes to exert a blood pressure-lowering effect. It is possible that the antihypertensive effects of IRW and LKP were essentially due to their degradation products (e.g., IR and KP), as the dipeptide IR has been shown to be a strong ACE-inhibitor (Li and Aluko, 2010).

4 Antioxidant peptides

Another group of bioactive peptides are the antioxidant peptides. They are potent inhibitors of the oxidation of biomolecules (e.g., proteins, lipids, and DNA) owing to their effectiveness at scavenging free radicals and reactive oxygen species, inactivating reactive intermediaries and

chelating prooxidative transition metals (Elias et al., 2008). Enzymatic hydrolysis of proteins enables antioxidative amino acids, such as Cys, Met, Trp, Tyr, Phe, and His, to be exposed and to function more effectively (Shen et al., 2010). The antioxidant activities of egg protein hydrolysates and identified peptides are presented in Table 5. The *in vitro* antioxidant activity has been examined by many techniques due to the different mechanisms potentially involved (Rao et al., 2012). These methods include the oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation decolorization, ferric reducing-antioxidant power (FRAP), lipid peroxidation and metal chelating assays (Alam et al., 2013).

4.1 Peptides derived from EW proteins

Xu et al. (2007a) hydrolyzed OVA using pepsin and demonstrated that the 3-kDa filtrate showed scavenging activities against superoxide anion and hydroxyl radical (•OH) as well as an inhibitory effect on lipid peroxidation in a linoleic acid model system. Abeyrathne et al. (2014) hydrolyzed OVA using GI or non-GI proteases (pepsin, trypsin, chymotrypsin, papain and alcalase), either individually or in combination. They observed that all of the hydrolysates showed a significant antioxidant activity in an oil-in-water emulsion system as well as metal (Fe^{2+}, Cu^{2+}) chelating activities, except for the pepsin hydrolysate which was shown not to effectively chelate ferrous ion.

Shen et al. (2010) investigated the antioxidant activity of OT hydrolysates produced with pepsin and thermolysin, and found the pepsin hydrolysate was more potent than thermolysin-pepsin and thermolysin hydrolysates. Furthermore, Huang et al. (2010) and Shen et al. (2010) pretreated OT with sonication prior to the thermolysin hydrolysis. They found that the antioxidant capacity of

the thermolysin hydrolysate was markedly enhanced and the four most potent antioxidant peptides (WNIP, GWNI, IRW and LKP) were identified. IRW and LKP have also been shown to possess ACE-inhibitory, antihypertensive and anti-inflammatory activities (Majumder et al., 2013a; Majumder et al., 2013b, 2015a; Majumder et al., 2015b; Majumder and Wu, 2010), which indicates that these peptides exhibit multiple bioactivities.

LYZ is known to possess antimicrobial activities, but several studies investigating the proteolysis of this protein showed that LYZ hydrolysates acted as strong antioxidants. Rao et al. (2012) reported the GI hydrolysate of LYZ showed a significant radical scavenging activity at a low concentration (2 mg/mL), as well as a marked reducing power and lipid peroxidation inhibitory activity. When the hydrolysate was ultrafiltrated, the 3-kDa filtrate showed intensified antioxidant activities. Memarpoor-Yazdi et al. (2012) compared the LYZ hydrolysates produced with trypsin and papain. They found the action of trypsin-papain resulted in a hydrolysate with a higher radical scavenging activity than those produced from papain or trypsin individually, and all of the hydrolysates exhibited Fe^{2+}-chelating and lipid peroxidation-inhibitory activities. You et al. (2010) hydrolyzed LYZ with a non-GI protease, alcalase, and observed that the fractionated peptides showed higher radical scavenging activities, as determined by ORAC and ABTS assays, than the hydrolysate. But the DPPH radical scavenging activity of the hydrolysate was not significantly affected by fractionation.

Measuring lipid peroxidation inhibition and metal chelating, Abeyrathne et al. (2016) reported that ovomucin hydrolysates produced with trypsin, papain or alcalase showed strong prooxidant potency and were not suitable as metal chelators. Similar to this, Shen et al. (2010) has previously identified two prooxidant peptides from the OT thermolysin hydrolysate. Whereas,

when Chang et al. (2013) hydrolyzed ovomucin using microbial proteases including alcalase, all of the hydrolysates showed a pronounced $ABTS^{\bullet+}$ radical scavenging activity. In this study two peptides (LDEPDPL and NIQTDDFRT) were identified and their antioxidant activities were shown to be higher by the DPPH assay than by the ABTS assay (Table 5). Therefore, the discrepancy in the antioxidant activity of ovomucin alcalase hydrolysates between these two studies likely originated from various aspects such as proteolysis conditions and different assays i.e., lipid peroxidation assay (Abeyrathne et al., 2016) and radical scavenging assay (Chang et al., 2013).

These studies show that enzymatic hydrolysis, particularly pepsinolysis, can considerably enhance the radical scavenging activity of EW, and the use of ultrafiltration or fractionation can significantly intensify the antioxidant activity of the hydrolysate (Table 5). Following peptic digestion of EW, Dávalos et al. (2004) obtained four OVA-derived peptides (YAEERYPIL, SALAM, YQIGL and YRGGLEPINF) that exhibited high ORAC antioxidant capability. The nonapeptide YAEERYPIL also showed an inhibitory activity against Cu^{2+}-induced lipid oxidation of low-density lipoprotein. YAEERYPIL has previously been reported to be a potent ACE-inhibitor (Miguel et al., 2004), suggesting that this peptide is a multifunctional peptide with potential for the control of cardiovascular disease, and hypertension in particular. Sun et al. (2014) reported that ultrafiltration enriched for the antioxidant activity of EW pepsin hydrolysates. The 2-5 kDa filtrate showed stronger antioxidant activities than other fractions, as assayed by DPPH, hydroxyl and superoxide anion radical scavenging as well as lipid peroxidation inhibition. Further, You and Wu (2011) compared GI (pepsin-pancreatin) and non-GI (thermolysin and alcalase) digestion on the antioxidant activities of EW hydrolysates. The

authors reported that GI digestion resulted in either higher, similar or lower antioxidant activities than non-GI treatment depending on the assays (Table 5). In line with this result, several other non-GI proteases were also utilized to hydrolyze EW and the hydrolysates generated showed varied antioxidant activity, and several identified peptides released by protease P showed considerable antioxidant activity (Nimalaratne et al., 2015).

4.2 Peptides derived from yolk proteins

Proteolysis of yolk with GI or non-GI proteases also produces protein hydrolysates and peptides with noticeable antioxidant activities (Table 5). Zambrowicz et al. (2015b) hydrolyzed yolk with pepsin and the hydrolysate was subsequently fractionated. As measured by DPPH and FRAP assays, fractionation enriched for the antioxidant activities of the hydrolysate, and four peptides with pronounced antioxidant activities were identified. These peptides were derived from apolipoprotein B, vitellogenin-2 and apovitellenin-1, and displayed both antioxidant and ACE-inhibitory activities, and were thus multifunctional peptides. In another study, Yousr and Howell (2015) ultrafiltrated the GI hydrolysate of yolk into three molecular weight ranges (10, 5 and 2 kDa). The lipid peroxidation-inhibitory activity of these filtrates followed the order of 2, 5 and 10 kDa, implying that low-molecular weight peptides were more potent antioxidants. The authors further identified three active peptides (WYGPD, KLSDW and KGLWE) which showed DPPH radical scavenging activity similar or even higher than butylated hydroxytoluene (BHT). However, when similar filtrates of 10, 5 and 1 kDa were prepared from yolk alcalase hydrolysate, Park et al. (2001) reported the highest antioxidant activity for 5-kDa filtrate. From the 5-kDa filtrate, two antioxidant peptides (LMSYMWSTSM and LELHKLRSSHWFSRR) were identified and LELHKLRSSHWFSRR showed a lipid peroxidation-inhibitory activity 61%

higher than α-tocopherol. You and Wu (2011) also assessed the antioxidant activity of yolk hydrolysates produced by GI and non-GI proteases with three different assays. They noticed that the antioxidant activity varied in accordance with the protease used (Table 5). It seems that different proteases vary in their hydrolysis actions on proteins in EW and yolk, and the antioxidant activity of protein hydrolysates is also closely related to the measurement methods. Indeed, Pokora et al. (2013) found yolk neutrase hydrolysate had higher antioxidant activities (i.e., DPPH radical scavenging, ferric reducing power, and Fe^{2+}-chelating) than the neutrase-treated EW. Other non-GI proteases such as orientase (EC 3.4.21.62) and a protease (EC 3.4.11.12) from *Bacillus* sp. also hydrolyzed yolk to produce hydrolysates that showed scavenging activities against DPPH, superoxide, and hydroxyl radicals as well as a lipid peroxidation-inhibitory activity (Sakanaka and Tachibana, 2006; Sakanaka et al., 2004). In addition, Zambrowicz et al. (2015a) applied a non-commercial protease obtained from *Cucurbita ficifolia* to yolk hydrolysis. They reported considerable antioxidant activities for the hydrolysate and a 5-kDa filtrate as well as several identified peptides (e.g., LAPSLPGKPKPD). These peptides have also been shown to effectively inhibit ACE, as detailed above (Eckert et al., 2014). Yolk phosvitin has a marked iron binding capacity, which imparts this protein with strong antimicrobial and antioxidant activities (Castellani et al., 2004). Many studies investigating the proteolysis of phosvitin have firstly dephosphorylated this protein, due to the structural resistance of phosvitin to common proteases such as trypsin (Samaraweera et al., 2011). It has been reported that tryptic hydrolysates of metal-free phosvitin can inhibit •OH formation in the Fe(II)/H_2O_2 Fenton reaction system more effectively than •OH scavengers such as mannitol (Ishikawa et al., 2004). Xu et al. (2007b) found that the phosphopeptides derived from tryptic

hydrolysis of dephosphorylated phosvitin displayed significantly high DPPH radical scavenging and lipid peroxidation-inhibitory activities. These phosphopeptides further exhibited protective effects on Caco-2 cells against the H_2O_2-induced oxidative stress to a similar extent as that of glutathione (Katayama et al., 2006). And when further subject to simulated *in vitro* GI digestion, these phosphopeptides showed high stability and retained their antioxidative stress bioactivity (Young et al., 2011).

4.3 Protease dependence of the antioxidant activity of protein hydrolysates

The protease dependence of the antioxidant activity of different egg protein hydrolysates has been studied using several antioxidant activity assays (Table 6). As these methods differ in their mechanisms, the antioxidant activity of protein hydrolysates appears to be dependent on not only proteases but also how the antioxidant activity is determined. For instance, You and Wu (2011) hydrolyzed EW using pepsin-pancreatin, thermolysin and alcalase, and found the GI hydrolysate had higher ORAC and DPPH radical scavenging activity but lower ABTS radical scavenging activity, compared to thermolysin and alcalase counterparts. These authors also prepared yolk hydrolysates using these three proteases, and found pepsin-pancreatin only improved the ORAC value but alcalase considerably enhanced the DPPH and ABTS radical scavenging activities. These findings were in agreement with other studies, which suggest that when assessing the production of antioxidant hydrolysates using different proteases, the analysis method must be taken into consideration (Tang et al., 2009).

5 Antimicrobial peptides

EW contains several proteins with reported antimicrobial activities, such as antibacterial proteins like LYZ, avidin and OT (Baron et al., 2016) as well as antiviral proteins such as ovomucin

(Omana et al., 2010). Proteolysis of these known antimicrobial molecules and other egg proteins (e.g., OVA) has been carried out to produce peptides with strong antimicrobial properties (Table 7).

To date, no studies have shown that OVA has antimicrobial activity (Baron et al., 2016). But Pellegrini et al. (2004) reported a strong antibacterial activity of OVA-derived peptides against *Bacillus subtilis*, and, for some peptides a weak fungicidal activity against *Candida albicans*.

Ibrahim et al. (1998) hydrolyzed OT with trypsin and obtained a 92-residue peptide (Leu109-Asp200, within the N-lobe) that displayed bactericidal activity against *Staphylococcus aureus* and *Escherichia coli* K-12. However, reduction of the three interchain disulfide bridges of this peptide could completely abolish its bactericidal activity. Using *E. coli* K-12 as a model, Ibrahim et al. (2000) later discovered that this peptide exhibited a cell membrane permeation-mediated bacteriostatic action. Giansanti et al. (2005) chemically synthesized two OT peptides (DQKDEYELL and KDLLFK) that shared sequence and/or structural homologies with antiviral peptides obtained from bovine lactoferrin and reported that both synthesized peptides showed a significant inhibitory activity against Marek's disease virus, but it is unknown if such peptides can be obtained through specific enzymatic hydrolysis of OT.

With regard to LYZ, it is most effective against some specific Gram-positive bacteria but largely ineffective against Gram-negative bacteria (Mine et al., 2004). Accordingly, attempts have been made to broaden the bacteriolytic action of LYZ to Gram-negative bacteria and food-borne pathogens, typically by protease-nicking of bactericidal domains in LYZ. Ibrahim et al. (2005) reported that the pepsinolysis of LYZ under conditions mimicking the newborn stomach condition (pH 4) generated peptides with potent bactericidal activity against several strains of

Gram-negative and Gram-positive bacteria in a dose-dependent manner. These bactericidal peptides were exclusively generated from an N-terminal helix-loop-helix domain (Lys1-Phe38) with the first two β-strands (Asn39-Leu56) of the β-domain of LYZ. Mine et al. (2004) hydrolyzed LYZ with successive actions of pepsin and trypsin. Two peptides IVSDGDGMNAW and HGLDNYR that inhibited *E. coli* K-12 and *S. aureus* 23-394 respectively were identified. Memarpoor-Yazdi et al. (2012) identified an antioxidant peptide, NTDGSTDYGILQINSR, in the LYZ hydrolysate produced with trypsin-papain, which also showed an antibacterial activity against both *E. coli* and *Leuconostoc mesenteroides*. In another study, Pellegrini et al. (1997) hydrolyzed LYZ with clostripain, which yielded a pentadecapeptide, IVSDGNGMNAWVAWR (98-112). This peptide is part of a helix-loop-helix domain (87-114) located at the upper lip of the active site cleft of LYZ (Ibrahim et al., 2001). Compared to LYZ, this peptide was active against fewer bacterial species with a considerably weaker bactericidal action on a molar basis. It was most effective against *B. subtilis* but showed no muramidase activity. Pellegrini et al. (1997) also chemically modified this peptide by substituting amino acids in the sequence, and observed that some modified peptides (e.g., RAWVAWR) had more potent bactericidal activity of some modified peptides compared to the parent peptide or native LYZ. Similarly, Masschalck et al. (2001) modified IVSDGNGMNAWVAWR (98-112) by adding amino acids to both N- and C-terminals to obtain KKIVSDGDGMNAWVAWRNRCK (96-116). These authors examined whether high hydrostatic pressure treatment (up to 300 MPa) could sensitize a test panel of bacteria for this peptide. They found six Gram-negative bacteria (*E. coli*, *P. fluorescens*, *S. enteritidis*, *Salmonella* serovar Typhimurium, *S. flexneri* and *S. sonnei*) were sensitized for this peptide.

Native ovomucin has been shown to have both antibacterial and antiviral activities (Omana et al., 2010) which were retained when the protein was subjected to proteolysis. Kobayashi et al. (2004) separated glycopeptides from pronase-treated ovomucin and found that these glyopeptides showed a strong binding affinity to *E. coli* O157:H7. The authors attributed this to the presence of sialic acid residues that act as specific binding site on the peptides for *E. coli* O157:H7. Sialic acids can act as biological recognition sites for a variety of molecules (Schauer, 2009). Compared to intact ovomucin, hydrolysates of this protein have been generated using various proteases (e.g., protease A, protex, alcalase, flavourzyme, etc.). A hydrolysate produced with pronase showed the highest content of sialic acid (Sun et al., 2016). Similarly, Watanabe et al. (1998b) prepared ovomucin glycopeptides (120 kDa and 220 kDa) with pronase and observed antitumor effects on Meth-A tumor cells. But the 120-kDa glycopeptide fraction showed a direct antitumor activity that was independent of the sialic acid residues.

6 Other bioactive peptides

In addition to the aforementioned ACE-inhibitory, antihypertensive, antioxidant and antimicrobial activities, other bioactive properties have been reported to be present in egg protein hydrolysates (Table 7). For instance, some antioxidant peptides (e.g., GWNI) released from thermolysin hydrolysis of OT lack an *in vitro* anti-inflammatory activity (Jahandideh et al., 2016). But when these peptides were subject to GI digestion, some of the degradation products, such as GW, showed a strong *in vitro* anti-inflammatory activity. Moon et al. (2013) examined the *in vitro* cytotoxicity of OT hydrolysates towards various human cancer cell lines. Among the proteases used (neutrase, alcalase, protamex, protex 6L, flavorzyme, α-chymotrypsin, trypsin and

collupulin MG), trypsin produced a hydrolysate showing a potent cytotoxic effect on cervix (HeLa) and stomach (AGS) cancer cell lines.

Similar to the *in vitro* cytotoxicity of OT peptides against cancer cell lines, ovomucin peptides have been shown to be able to potentiate macrophages, thereby enhancing their tumor cytotoxicity. In the human immune system, tumor necrosis factor and soluble effectors contribute to the tumor cytotoxicity of macrophages, which have been shown to be activated by ovomucin glycopeptides produced with pronase and papain, but not with trypsin (Tanizaki et al., 1997). These glycopeptides have been shown to induce morphological alteration of macrophages and to enhance the H_2O_2 generation and interleukin-1 production from the cells.

In addition to the potential anti-tumor effects of ovomucin peptides, they have exhibited other health promoting benefits. For instance, Sun et al. (2016) observed that, among 14 different proteases, the pepsin-pancreatin digestion of ovomucin produced peptides that could significantly promote the growth of *Bifidobacterium infantis*, a probiotic strain found in the human gut. In this regard, human GI digestion of ovomucin can benefit the human gut microbiota.

Palika et al. (2015) obtained an iron-binding peptide, DKLPGFGDS$^{(PO)}{}_4$IEAQ (OVA 61-73), from the simulated *in vitro* GI digestion of EW. This peptide showed an iron-binding activity in a dose-dependent manner, but dephosphorylation of the peptide completely abolished its iron-binding activity. Phosphate retention also played a critical role in the calcium-binding ability of phosphopeptides derived from tryptic hydrolysis of phosvitin (Jiang and Mine, 2000). But the phosphate content of peptides did not correlate with the calcium-binding activity. In line with this finding, Choi et al. (2005) reported that phosvitin peptides produced with trypsin could

increase both calcium absorption in intestine and calcium accumulation in bones when added to a diet for rats. Later, Feng and Mine (2006) used a Caco-2 cell monolayer model to confirm that these phosvitin phosphopeptides could also increase iron uptake by the cells.

Antidiabetic peptides have also been obtained through proteolysis of egg proteins. Yu et al. (2011a) isolated eight peptides from EW hydrolysate produced with alcalase. Among them RVPSLM and TPSPR showed the highest inhibitory activity against α-glucosidase, but none of these eight peptides inhibited α-amylase. Recently, Zambrowicz et al. (2015b) identified three peptides from the yolk pepsin hydrolysate. These peptides showed an inhibitory activity against both α-glucosidase and dipeptidyl peptidase-IV (DPP-IV), but their antidiabetic action was much weaker than that reported by (Yu et al., 2011a). Furthermore, Zambrowicz et al. (2015a) used a protease from *Cucurbita ficifolia* to produce antidiabetic peptides (RASDPLLSV, RNDDLNYIQ and LAPSLPGKPKPD) from yolk. These peptides exerted a DPP-IV inhibitory activity but only RNDDLNYIQ showed a α-glucosidase inhibitory activity.

7 Challenges of the commercial production of bioactive egg peptides

The most common method used to carry out enzymatic hydrolysis is in batch mode, where the enzymes and substrate proteins are mixed in a container and incubated for a certain period of time, followed by the proteases being inactivated by either heat, pH adjustment or adding inhibitors to stop the hydrolysis, and the end products are recovered. For commercial peptide production, conventional batch processes have several challenges, including: difficulties in controlling the pH of the proteolysis, the impact of removing the digestion products on process kinetics and the challenge of recycling the enzymes (Agyei and Danquah, 2011; Picariello et al., 2013b).

Some of the challenges associated with conventional batch reactors can be overcome by using reactors in the form of dialysis bags with specific MWCOs, which have been used to study protein digestibility in a physiological situation (Mouécoucou et al., 2003). This approach might also enable a continuous peptide production process, in which the protease remains in the reactor while the substrate protein can be continuously supplied to the reactor with simultaneous removal of short peptides via dialysis. In addition, immobilized enzymes may be more advantageous than soluble enzymes for industrial-scale peptide production, since the enzymes can be easily removed from the reaction medium rather than being inactivated (Agyei and Danquah, 2011; Tavano, 2013). Immobilization of enzymes on a solid support offers several advantages, such as increasing the possibility of recycling, better stability, reduced inhibition and less enzyme chain aggregation and/or autolysis (Tavano, 2013). The use of enzymatic membrane reactors is therefore attracting attention (Rios et al., 2004), as they would allow continuous peptide production process which integrates enzymatic hydrolysis, product separation and catalyst recovery into a single operation (Bhat et al., 2015b). Enzymatic membrane reactors, thus, favor the production of consistently uniform peptides, with improved protease-catalyzed bioconversion efficiency owing to the simultaneous separation of the proteolytic products.

8 Future research directions

In addition to their nutritive value, egg proteins have been shown to be a rich source of bioactive peptides. *In vitro* enzymatic hydrolysis of egg proteins using GI and non-GI proteases have produced protein hydrolysates with various biological activities, and a number of peptides exhibiting bioactivity have been characterized. EW proteins mainly OVA, OT and LYZ have

been more frequently investigated for bioactive peptide production than yolk proteins, but research on bioactive peptides derived from yolk proteins is increasing.

Simulated *in vitro* GI digestion of egg proteins has been shown to produce bioactive peptides that may be physiologically beneficial. Bioactive egg peptides produced by enzymatic hydrolysis with non-GI proteases have considerable potential for commercial application. These bioactive peptides could be incorporated as ingredients into nutraceuticals and even pharmaceuticals to exert their biological activities, such as ACE-inhibitory activity. The stability of these *in vitro* ACE-inhibitory peptides must, however, be examined further using either simulated *in vitro* GI digestion or *in vivo* animal models in order to validate their antihypertensive activity if orally ingested. It must be taken into account that these peptides should be protected and survive through the human GI tract. For example, effective protective delivery approaches, such as microencapsulation, may be required. Nevertheless, studies are required to examine their transepithelial stability and transport mechanisms as well as resistance to plasma peptidases. A better understanding of how these bioactive compounds function in the human GI tract and cardiovascular system, will enable the potential health promoting activities of egg protein hydrolysates to be fully utilized.

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Table 1. Effects of ultrafiltration on the bioactivity of different egg protein hydrolysate fractions.

Egg proteins	Bioactivity of whole hydrolysate and different fractions after ultrafiltration		References
	ACE-inhibitory activity	Antioxidant activity	
Lysozyme (LYZ)	Pepsin digest: 3-kDa > whole hydrolysates Gastrointestinal digest: whole > 3-kDa hydrolysates	Gastrointestinal digest: 3-kDa > whole hydrolysates	Rao et al. (2012)
Egg white (EW)	Pepsin digest: 3-kDa > whole > larger 3-kDa hydrolysates		Miguel et al. (2004)
	Pepsin digest: 3-kDa > whole hydrolysates Gastrointestinal digest: whole > 3-kDa hydrolysates		Miguel et al. (2007a)
	Trypsin digest: 3-kDa > whole hydrolysates	Papain/trypsin digest: 3-kDa > whole hydrolysates	Chen et al. (2012a); Chen et al. (2012b)
	Plant protease digest: 30-kDa > whole hydrolysates		Pokora et al. (2014)
		Pepsin digest: 10-30-kDa > 1-kDa > 1-10-kDa hydrolysates Trypsin digest: 10-30-kDa > 1-kDa > 1-10-kDa	Lin et al. (2012)

		hydrolysates Alcalase digest: 1-kDa > 10-30-kDa > 1-10-kDa hydrolysates	
		Pepsin digest: 2-5-kDa > larger 5-kDa > 1-kDa > 1-2-kDa hydrolysates	Sun et al. (2014)
Yolk	Plant protease digest:5-kDa > whole > 5-30-kDa hydrolysates		Eckert et al. (2014)
		Alcalase digest: 5-kDa > 1- and 10-kDa hydrolysates	Park et al. (2001)
		Gastrointestinal digest: 2-kDa > 5-kDa > 10-kDa hydrolysates	Yousr and Howell (2015)
		Plant protease digest: 5-kDa > whole hydrolysates	Zambrowicz et al. (2015a)

Table 2. Studies on egg protein hydrolysates with ACE-inhibitory and antihypertensive activities.

Egg proteins	Enzyme used	Hydrolysates	ACE inhibitor (IC_{50}, μg protein/mL)	Antihypertensive activity (Δ mmHg; time; and dose, mg/kg body weight)	References
Ovalbumin (OVA)	Pepsin, chymotrypsin, trypsin, thermolysin	Pepsin digest	45.3		Fujita et al. (2000)
		Thermolysin digest	83		
		Chymotrypsin digest	> 1,000		
		Trypsin digest	> 1,000		
	Pepsin, chymotrypsin, trypsin	Pepsin digest	9		Quirós et al. (2007)
		Chymotrypsin digest	62.5		
		Trypsin digest	NA		
Ovotransferrin (OT)	Thermolysin, pepsin	Pepsin digest	320		Majumder and Wu (2010)
		Thermolysin digest	201		
		Thermolysin-pepsin digest	198		
Lysozyme(LYZ)	Pepsin, chymotrypsin, trypsin	Pepsin digest	160.2		Rao et al. (2012)
		3-kDa pepsin filtrate	75.2		
		Gastrointestinal digest	12.6		
		3-kDa gastrointestinal filtrate	28.6		
Egg white	Pepsin, chymotrypsin,	Pepsin digest	55.3	− 44.3; 6 h;	Miguel et al. (2005, 2006b);

(EW)	trypsin			400 mg/kg (o)	Miguel et al. (2004)
		3-kDa pepsin filtrate	34.5	− 39.1; 6 h; 100 mg/kg (o)	
		Chymotrypsin digest	454.1		
		Trypsin digest	> 750		
	Pepsin, Corolase PP	Pepsin digest	44		Miguel et al. (2007a)
		3-kDa pepsin filtrate	20	− 28; 6 h; 100 mg/kg (o)	
		Gastrointestinal digest	68		
		3-kDa gastrointestinal filtrate	74	− 20; 2-4 h; 100 mg/kg (o)	
	Pepsin, pancreatin	Pepsin digest of boiled EW	142		Majumder and Wu (2009)
		Gastrointestinal digest of boiled EW	98		
		Pepsin digest of fried EW	63		
		Gastrointestinal digest of fried EW	76		
	Pepsin, pancreatin, thermolysin, alcalase	Gastrointestinal digest	268		You and Wu (2011)
		Gastrointestinal digest fractions	171-345		
		Thermolysin digest	96		
		Thermolysin digest fractions	47-336		

		Alcalase digest	155		
		Alcalase digest fractions	59-273		
	Trypsin	Trypsin digest	50% (5 h of digestion)		Chen et al. (2012b)
	Papain	Papain digest	1,676		Chen and Chi (2012)
	Protease from *Cucurbita ficifolia*	Digest	9,071		Pokora et al. (2014)
		3-kDa filtrate	8,001		
		Fractions of 3-kDa filtrate	68.7-1,424		
		Sub-fractions of 3-kDa filtrate	27.4-425		
	Neutrase	Digest	245.7		Pokora et al. (2013)
Yolk	Pepsin	Digest	623		Zambrowicz et al. (2015b)
		Digest fractions	249.1-475.8		
		Digest sub-fractions	35-423		
	Pepsin, pancreatin	Pepsin digest of boiled yolk	1,118		Majumder and Wu (2009)
		Gastrointestinal digest of boiled yolk	846		
		Pepsin digest of fried yolk	459		
		Gastrointestinal digest of fried yolk	168		
	Pepsin, pancreatin	10-kDa filtrate	33.1%		Yousr and Howell (2015)

		5-kDa filtrate	42.2%		
		2-kDa filtrate (total 60 fractions)	49.7% (5,440)		
		Fraction 56 from 2-kDa filtrate	69.2% (3,350)		
	Pepsin, pancreatin, thermolysin, alcalase	Gastrointestinal digest	210		You and Wu (2011)
		Gastrointestinal digest fractions	92-398		
		Thermolysin digest	144		
		Thermolysin digest fractions	86-659		
		Alcalase digest	140		
		Alcalase digest fractions	52-144		
	Protease from *Cucurbita ficifolia*	Digest	482.5		Eckert et al. (2014)
		5-kDa filtrate	470.0		
		5-kDa filtrate fractions	47.8-288.8		
		5-kDa filtrate sub-fractions	4.5-9.0		
	Newlase F from *Rhizopus niveus*	1-kDa filtrate	1,220		Yoshii et al. (2001)
	Neutrase	Digest	59.2		Pokora et al. (2013)
Whole egg (WE)	Pepsin, pancreatin	Pepsin digest of boiled WE	19		Majumder et al. (2013c); Majumder and Wu (2009)
		Gastrointestinal digest of boiled WE	25		
		Pepsin digest of fried WE	25		

		Gastrointestinal digest of fried WE	9	− 24; 3 days; 1,000 mg/kg (o)	

ACE: angiotensin I-converting enzyme; IC_{50}: concentration of ACE inhibitor required to inhibit 50% of ACE; NA: non-effective; (o): oral administration; (iv): intravenous administration

To determine the acute antihypertensive activity, spontaneously hypertensive rats (SHRs) were orally or intravenously administered test hydrolysate at a single given dose and the maximum reduction in systolic blood pressure (SBP) after certain period of time was measured.

To determine the short- or long-term antihypertensive activity, SHRs were orally or intravenously administered test hydrolysate at a single given dose daily for a number of days and the reduction in SBP was measured on the last day.

Table 3. Protease dependence of the ACE-inhibitory activity of egg protein hydrolysates.

Egg proteins	Proteolysis conditions	ACE-inhibitory activity of protein hydrolysates following proteolysis by different proteases	References
Ovalbumin (OVA)	E/S ~ 1:100 (w/w) at pH 2 (pepsin), pH 7.5 (trypsin, chymotrypsin, thermolysin) at 37°C for 3 h	Pepsin > Thermolysin > Chymotrypsin, Trypsin hydrolysates	Fujita et al. (2000)
	E/S = 1:100 (w/w) at pH 2.5 (pepsin), E/S = 1:16 (w/w) at pH 8 (trypsin, chymotrypsin) at 37°C for 1 h	Pepsin > Chymotrypsin > Trypsin hydrolysates	Quirós et al. (2007)
	E/S = 1:2 (w/w) at pH 2.5 (pepsin), pH 7.6-7.8 (trypsin, chymotrypsin), pH 6.5 (alcalase, papain) at 37°C for 3 h	Pepsin > Chymotrypsin > Alcalase-Trypsin > Pepsin-Alcalase > Pepsin-Papain hydrolysates, but no significant difference	Abeyrathne et al. (2014)
Ovotransferrin (OT)	pH 8 (thermolysin) at 55°C for 3 h, pH 2 (pepsin) at 37°C for 3 h	Thermolysin-Pepsin > Thermolysin > Pepsin hydrolysates	Majumder and Wu (2010)
Lysozyme(LYZ)	E/S = 1:100 (w/w), pH 2 (pepsin) at 37°C for 2 h; E/S = 1:2 (w/w), pH 7.5	Pepsin-Chymotrypsin/Trypsin > Pepsin hydrolysates	Rao et al. (2012)

	(trypsin and chymotrypsin) at 37°C for 4 h		
Ovomucin	E/S = 1:100 (w/w) at pH 2.5 (pepsin), pH 7.8 (trypsin), pH 6.5 (alcalase, papain) at 37°C for up to 24 h	Papain > Alcalase > Trypsin hydrolysates, but no significant difference	Abeyrathne et al. (2016)
Egg white (EW)	E/S = 1:100 (w/w), pH 2 (pepsin); E/S = 1:100 (w/w), pH 7.8 (trypsin); E/S = 1:20 (w/w), pH 8 (chymotrypsin) at 37°C for up to 24 h	Pepsin > Chymotrypsin > Trypsin hydrolysates	Miguel et al. (2004)
	E/S = 1:100 (w/w), pH 2 (pepsin) at 37°C for 3 h; E/S = 1:50 (w/w), pH 7-8 (Corolase PP) at 37°C for 4 h	Pepsin > Pepsin-Corolase PP (a mixture of trypsin, chymotrypsin and numerous amino and carboxypeptidases) hydrolysates	Miguel et al. (2007a)
	E/S = 1:25 (w/w), pH 2 (pepsin) at 37°C for 3 h; E/S = 1:50 (w/w), pH 7.5 (pancreatin) at 37°C for 3 h	Pepsin-Pancreatin > Pepsin hydrolysates	Majumder and Wu (2009)
	E/S = 1:50 (w/w), pH 2 (pepsin) at 37°C for	Thermolysin > Alcalase > Pepsin-Pancreatin hydrolysates	You and Wu (2011)

	3 h, pH 7 (pancreatin) 37°C for 3 h, pH 8 (thermolysin) at 70°C for 3 h, pH 7.5 (alcalase) at 50°C for 3 h		
Yolk	E/S = 1:25 (w/w), pH 2 (pepsin) at 37°C for 3 h; E/S = 1:50 (w/w), pH 7.5 (pancreatin) at 37°C for 3 h	Pepsin-Pancreatin > Pepsin hydrolysates	Majumder and Wu (2009)
	E/S = 1:50 (w/w), pH 2 (pepsin) at 37°C for 3 h, pH 7 (pancreatin) 37°C for 3 h, pH 8 (thermolysin) at 70°C for 3 h, pH 7.5 (alcalase) at 50°C for 3 h	Alcalase ≥ Thermolysin > Pepsin-Pancreatin hydrolysates	You and Wu (2011)
Whole egg (WE)	E/S = 1:25 (w/w), pH 2 (pepsin) at 37°C for 3 h; E/S = 1:50 (w/w), pH 7.5 (pancreatin) at 37°C for 3 h	Pepsin-Pancreatin > Pepsin hydrolysates	Majumder and Wu (2009)

E/S, enzyme/substrate ratio

Table 4. Studies on egg protein-derived peptides with ACE-inhibitory and antihypertensive activities.

Egg proteins	Enzyme used	Peptides	ACE inhibitor (IC_{50}, μM)	Antihypertensive activity (Δ mmHg; time; and dose, mg/kg body weight)	References
Ovalbumin (OVA)	Pepsin	FRADHPFL (358-365), ovokinin	3.2	− 18; 4 h; 25 mg/kg (o)	Fujita et al. (1995)
	Chymotrypsin	RADHPF (359-364), ovokinin (2--7)	> 1,000	− 15; 6 h; 20 mg/kg (o) − 20; 20 min; 5 mg/kg (iv)	Matoba et al. (1999); Scruggs et al. (2004)
	Pepsin	FGRCVSP	6.2		Fujita et al. (2000)
		ERKIKVYL	1.2	0; 10 mg/kg (iv) 0; 60 mg/kg (o)	
		FFGRCVSP	0.4	0; 10 mg/kg (iv) 0; 60 mg/kg (o)	
		LW	6.8	− 45; 2 h; 10 mg/kg (iv) − 22; 2 h; 60 mg/kg (o)	
		FCF	11		
		NIFYCP	15		
Ovotransferrin (OT)	Pepsin, thermolysin	IRW	0.6	− 40; 18 days; 15 mg/kg (o)	Majumder et al. (2013b, 2015a); Majumder and Wu (2010)
		IQW	1.6	− 19; 18 days;	

				15 mg/kg (o)	
		LKP	2.9	− 30; 18 days; 15 mg/kg (o)	
	Chymotrypsin	KVREGTTY	102.8	− 21; 40 min; 1 nmol/kg (iv)	Lee et al. (2006)
		KVREGT	17.2	− 24; 20 min; 1 nmol/kg (iv)	
Lysozyme(LYZ)	Pepsin, chymotrypsin, trypsin	KVF (1-3)	14		Rao et al. (2012)
		MKR (12-14)	25.7		
		AMK (11-13)	94.2		
		AKF (32-34)	6.5		
		GIL (54-56)	53.8		
		RGY (21-23)	61.9		
		WIR (123-125)	88.5		
		VAW (109-111)	2.8		
Egg white (EW)	Pepsin	FRADHPFL (ovokinin)	3.2		Miguel et al. (2005); Miguel et al. (2004)
		RADHPFL (OVA 359-365)	6.2	− 34.1; 6 h; 2 mg/kg (o)	
		YAEERYPIL (OVA 106-114)	4.7	− 31.6; 6 h; 2 mg/kg (o)	
		IVF (OVA 178-180)	33.9	− 31.7; 6 h; 4 mg/kg (o)	
		YRGGLEPINF (OVA 125--134)	> 1,000	Strong vasodilator	
		ESIINF (OVA 256--261)	> 1,000	Strong vasodilator	
		RDILNQ (OVA	435.7	Strong	

		84--89)		vasodilator	
		SALAM (OVA 36--40)	229.1	Weak vasodilator	
		ELIN (OVA 144--147)	> 1,000	Weak vasodilator	
		YQIGL (OVA 212--216)	173.8	Weak vasodilator	
		VLLPDEVSGL (OVA 243--252)	> 1,000	Weak vasodilator	
		FSL (OVA 99--101)	172.9	Weak vasodilator	
		VALDGGL (OT 392--398)	> 1,000	Weak vasodilator	
		DGSRQPV (OT 230--236)	> 1,000	Weak vasodilator	
	Protease from *Cucurbita ficifolia*	SWVE (OVA 148--151)	33.9 µg/mL		Pokora et al. (2014)
		DILN (OVA 86--89)	73.4 µg/mL		
	Alcalase	RVPSL (OT 328-332)	20		Liu et al. (2010)
	Alcalase	DHPFLF (OVA 362-367)	35%		Yu et al. (2011b)
		HAEIN (OVA 332-336)	5%		
		QIGLF (OVA 214-218)	47% (75 µM)		
	Alcalase	RVPSL	20		Yu et al. (2012); Yu et al. (2017)
		QIGLF	75	− 13; 5 h; 50	

				mg/kg (o)	
		TNGIIR	70		
Yolk	Pepsin	YINQMPQKSRE	10.1		Zambrowicz et al. (2015b)
		YIEAVNKVSPR AGQF	9.4		
		YINQMPQKSRE A	12.6		
		VTGRFAGHPAA Q	27.3		
	Pepsin, pancreatin	MPVHTDAD	40%		Yousr and Howell (2015)
		YPSPV	80%		
		SDNRNQGY	60%		
	Protease from *Cucurbita ficifolia*	ITMIAPSAF	3.24		Eckert et al. (2014)
		RASDPLLSV	4.7		
		RNDDLNYIQ	3.72		
		LAPSLPGKPKP D	1.97		
		AGTTCLFTPLA LPYDYSH	8.08		

ACE: angiotensin I-converting enzyme; IC_{50}: concentration of ACE inhibitor required to inhibit 50% of ACE; (o): oral administration; (iv): intravenous administration

To determine the acute antihypertensive activity, spontaneously hypertensive rats (SHRs) were orally or intravenously administered test peptide at a single given dose and the maximum reduction in systolic blood pressure (SBP) after certain period of time was measured.

To determine the short- or long-term antihypertensive activity, SHRs were orally or intravenously administered test peptide at a single given dose daily for a number of days and the reduction in SBP was measured on the last day.

Table 5. Studies on egg protein hydrolysates and derived peptides with antioxidant activities.

Egg proteins	Enzyme used	Hydrolysates/ peptides	Free radical-inhibitor (μmol TE/mg protein)			Lipid peroxidation-inhibitor	Metal-chelator [μg Fe^{2+}(Cu^{2+})/mg]	References
			ORAC	DPPH	ABTS			
Ovotransferrin (OT)	Pepsin, thermolysin	Pepsin digest	1.42					Huang et al. (2010); Shen et al. (2010)
		Thermolysin digest	0.49					
		Thermolysin-pepsin digest	1.14					
		WNIP	15.5 μmol TE/μmol					
		GWNI	13.9 μmol TE/μmol					
		IRW	6.6 μmol TE/μmol					
		LKP	1.3 μmol TE/μmol					
Lysozyme (LYZ)	Pepsin, trypsin, chymotrypsin	Gastrointestinal digest		25.7%		76.4%		Rao et al. (2012)
		3-kDa filtrate		63.2%		85.2%		
	Trypsin, papain	Trypsin digest		37.2%	1.91	Linoleic acid model system	Fe^{2+}-chelator	Memarpoor-Yazdi et al. (2012)
		Papain digest		50.4%	2.57			
		Trypsin-papain digest		64.2%	2.82			

	Alcalase	Digest	0.45	0.15	1.69			You et al. (2010)
		Digest fractions	0.39-2.77	0.16-0.24	1.40-3.78			
Ovomucin	Protamex, flavourzyme, alcalase	Protamex digest			90.4%			Chang et al. (2013)
		Flavourzyme digest			89.4%			
		Alcalase digest			88.6%			
		LDEPDPL		51.8%	19.7%			
		NIQTDDFRT		24.7%	1.0%			
Egg white (EW)	Pepsin	Pepsin digest	0.38					Dávalos et al. (2004)
		3-kDa filtrate	2.78					
		3-kDa filtrate fractions	1.81-3.73					
		YAEERYPIL (OVA 106-114)	3.8 μmol TE/μmol			Cu^{2+}-induced lipid oxidation		
		SALAM (OVA 36--40)	2.7 μmol TE/μmol					
		YQIGL (OVA 212--216)	1.7 μmol TE/μmol					
		YRGGLEPINF (OVA	1.2 μmol					

		125--134)	TE/μmol					
	Pepsin	> 5-kDa filtrate		75 %		45%		Sun et al. (2014)
		2-5-kDa filtrate		98 %		85%		
		1-2-kDa filtrate		34 %		27%		
		< 1-kDa filtrate		65 %		39%		
	Pepsin, pancreatin, thermolysin, alcalase	Gastrointestinal digest	0.91	0.19	2.01			You and Wu (2011)
		Thermolysin digest	0.66	0.14	2.14			
		Alcalase digest	0.62	0.16	2.13			
	Trypsin, papain, flavourzyme, alcalase, neutrase	Trypsin digest		55 %				Chen et al. (2012a)
		Papain digest		73 %				
		Flavourzyme digest		44 %				
		Alcalase digest		60 %				
		Neutrase digest		44 %				
	Protease P, protease M, protease S, protex 51FP, alcalase	Protease P digest	1.28		1.61			Nimalaratne et al. (2015)
		Protease M digest	1.30		1.94			

		Protease S digest	0.90		1.24			
		Protex 51FP digest	0.96		1.24			
		Alcalase digest	1.14		1.29			
		EERYP (OVA 109-113)	1.53 μmol TE/μmol					
		AEERYP (OVA 108-113)	3.32 μmol TE/μmol					
		SVDSQTAF (OVA 166-173)	0.48 μmol TE/μmol					
		DEDTQAMP (OVA 190-198)	3.14 μmol TE/μmol					
		LPDEVSG (OVA 245-251)	0.44 μmol TE/μmol					
		PVDENDEG (cystatin 36-43)	2.46 μmol TE/μmol					
		QPSSVDSQTAM (OVA 163-173)	1.24 μmol TE/μmol					
Yolk	Pepsin	Pepsin digest		0.5			405.0	Zambrowicz et al. (2015b)
		Pepsin digest fractions		0.54-2.9			13.2-250.1	
		YINQMPQKSRE		2.3			37.0	
		YIEAVNKVSPRAGQF		2.2			25.2	
		YINQMPQKSREA		1.8			8.5	
		VTGRFAGHPAAQ		1.5			NA	

	Pepsin, pancreatin, thermolysin, alcalase	Gastrointestinal digest	2.51	0.26	2.17			You and Wu (2011)
		Thermolysin digest	1.18	0.19	1.59			
		Alcalase digest	1.44	0.35	3.62			
	Protease from *Cucurbita ficifolia*	Digest		0.42			695.76	Zambrowicz et al. (2015a)
		5-kDa filtrate		3.63			440.68	
		ITMIAPSAF		2.97			231.43	
		RASDPLLSV		4.71			26.36	
		RNDDLNYIQ		2.28			196.96	
		LAPSLPGKPKPD		6.03			179.33	
		AGTTCLFTPLALPYDYSH		2.59			27.6	

TE: Trolox equivalent; NA: non-effective

Table 6. Protease dependence of the antioxidant activity of egg protein hydrolysates.

Egg proteins	Antioxidant activity of protein hydrolysates following proteolysis by different proteases	References
Ovalbumin (OVA)	Lipid peroxidation inhibition: Alcalase-trypsin, pepsin-alcalase > pepsin-papain, chymotrypsin > pepsin hydrolysates Fe^{2+}-chelating: Chymotrypsin, alcalase-trypsin > pepsin-papain, pepsin-alcalase > pepsin hydrolysates Cu^{2+}-chelating: No significant differences among protein hydrolysates	Abeyrathne et al. (2014)
Ovotransferrin (OT)	ORAC: Pepsin > thermolysin-pepsin > thermolysin hydrolysates	Shen et al. (2010)
Lysozyme(LYZ)	DPPH radical scavenging: Trypsin-papain > papain > trypsin hydrolysates ABTS radical scavenging: Trypsin-papain > papain > trypsin hydrolysates Lipid peroxidation inhibition: No significant difference among protein hydrolysates Metal-chelating: Trypsin-papain > papain > trypsin hydrolysates	Memarpoor-Yazdi et al. (2012)
Ovomucin	ABTS radical scavenging: Protamex > flavourzyme > alcalase hydrolysates	Chang et al. (2013)
Egg white (EW)	ORAC: Gastrointestinal > thermolysin > alcalase hydrolysates DPPH radical scavenging: Gastrointestinal > alcalase > thermolysin hydrolysates	You and Wu (2011)

	ABTS radical scavenging: Alcalase > thermolysin > gastrointestinal hydrolysates	
	DPPH radical scavenging: Papain > alcalase > trypsin > flavourzyme, neutrase hydrolysates	Chen et al. (2012a)
	ORAC: Protease M > protease P > alcalase > protex 51FP > protease S hydrolysates ABTS radical scavenging: Protease M > protease P > alcalase > protex 51FP, protease S hydrolysates	Nimalaratne et al. (2015)
Yolk	ORAC: Gastrointestinal > alcalase > thermolysin hydrolysates DPPH radical scavenging: Alcalase > gastrointestinal > thermolysin hydrolysates ABTS radical scavenging: Alcalase > gastrointestinal > thermolysin hydrolysates	You and Wu (2011)

Table 7. Studies on egg protein hydrolysates and derived peptides with antimicrobial and other activities.

Egg proteins	Enzyme used	Hydrolysates/peptides	Antimicrobial activity	Antidiabetic activity		Other activities	References
				a-glucosidase inhibitor (IC_{50}, μM)	DPP-IV inhibitor (IC_{50}, μM)		
Ovalbumin (OVA)	Trypsin, chymotrypsin	SALAM (36--40) SALAMVY (36--42) YPILPEYLQ (111--119) ELINSW (143--148) NVLQPSS (159--165) AEERYPILPEYL (127--138) GIIRN (155--159) TSSNVMEER (268--276)	Strong bactericidal activity against *Bacillus subtilis*; weak fungicidal activity against *Candida albicans*				Pellegrini et al. (2004)
Ovotransferrin (OT)	Trypsin	Leu109-Asp200 (of the N-lobe)	*Staphylococcus aureus*, *E. coli* K-12				Ibrahim et al. (1998); Ibrahim et al. (2000)
	Pepsin, thermolys	IRW, IQW, LKP				Anti-inflammator	Majumder et al. (2013a); Majumder et

	in					y activity	al. (2013b, 2015a); Majumder et al. (2015b)
Lysozyme(LYZ)	Pepsin	Hydrolysate	Gram-positive (*S. aureus, S. epidermidis, B. subtilis* and *M. luteus*) and Gram-negative bacteria (*E. coli K-12, B. bronchiseptica, S. enteritidis* and *H. pylori*)				Ibrahim et al. (2005)
	Pepsin-trypsin	IVSDGDGMNAW (98--108)	*E. coli* K-12				Mine et al. (2004)
		HGLDNYR (15--21)	*Staphylococcus aureus* 23-394				
	Trypsin-	NTDGSTDYGILQINS	*E. coli*				Memarpoor-Yazdi et al.

	papain	R (46-61)	and *L. mesenteroides*				(2012)
	Clostripain	IVSDGNGMNAWVAWR (98--112)	Most effective against *Bacillus subtilis*				Pellegrini et al. (1997)
Ovomucin	Pronase	Glycopeptides	*E. coli* O157:H7 binding activity				Kobayashi et al. (2004)
	Pronase	Glycopeptides (70, 120 and 220 kDa)				Antitumor effect on Meth-A tumor cells	Oguro et al. (2000); Watanabe et al. (1998b)
	Pronase-papain	Sulfated glycopeptides				Macrophage-stimulating activity	Tanizaki et al. (1997)
Egg white (EW)	Pepsin, pancreatin	DKLPGFGDS$^{(PO)}_4$IEAQ (OVA 61-73)				Iron-binding ability	Palika et al. (2015)
	Alcalase	RVPSLM (OT 328--333)		23		No α-amylase inhibitory activity	Yu et al. (2011a)
		TPSPR (OT 356--360)		40			
		DLQGK, AGLAPY, RVPSL, DHPFLF, HAEIN, QIGLF		> 150			
Phosvitin	Trypsin	Phosphopeptides (1-3 kDa)				Calcium-binding	Feng and Mine (2006); Jiang and Mine (2000)

						ability, iron uptake by Caco-2 cells	
	Trypsin	Hydrolysate				Calcium-binding ability	Choi et al. (2005)
Yolk	Pepsin	YINQMPQKSRE		1694.3 μg/mL	222.8 μg/mL		Zambrowicz et al. (2015b)
		YINQMPQKSREA		454.6 μg/mL	355.8 μg/mL		
		VTGRFAGHPAAQ		365.4 μg/mL	1402.2 μg/mL		
	Protease from *Cucurbita ficifolia*	RASDPLLSV		NA	361.5-426.25		Zambrowicz et al. (2015a)
		RNDDLNYIQ		NA			
		LAPSLPGKPKPD		1,065.6			

DPP-IV: dipeptidyl peptidase-IV; IC_{50}: concentration of an inhibitor required to inhibit 50% of the enzyme; NA: non-effective

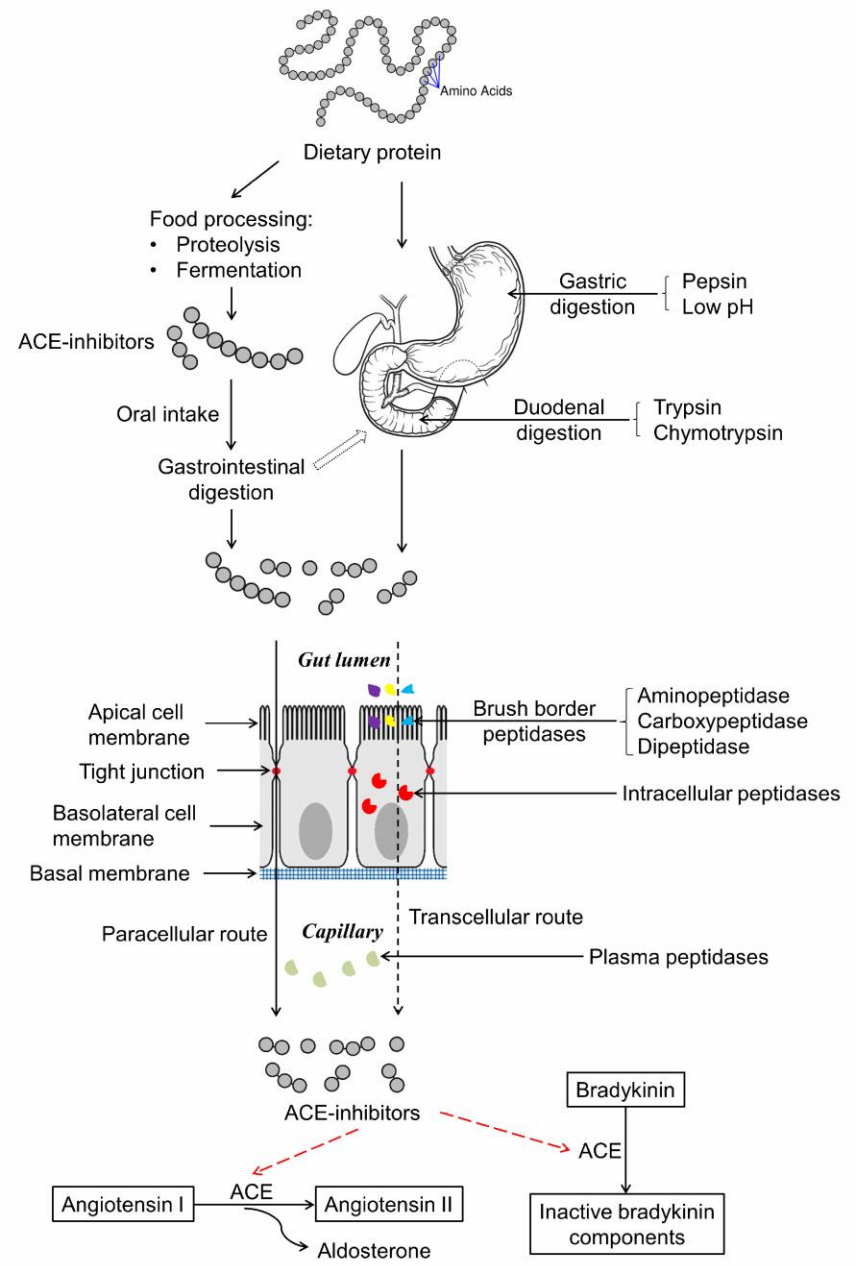

Figure 1. The potential degradation of angiotensin I converting enzyme (ACE) inhibitory peptides by gastrointestinal proteases, epithelial peptidases and plasma peptidases in human body.

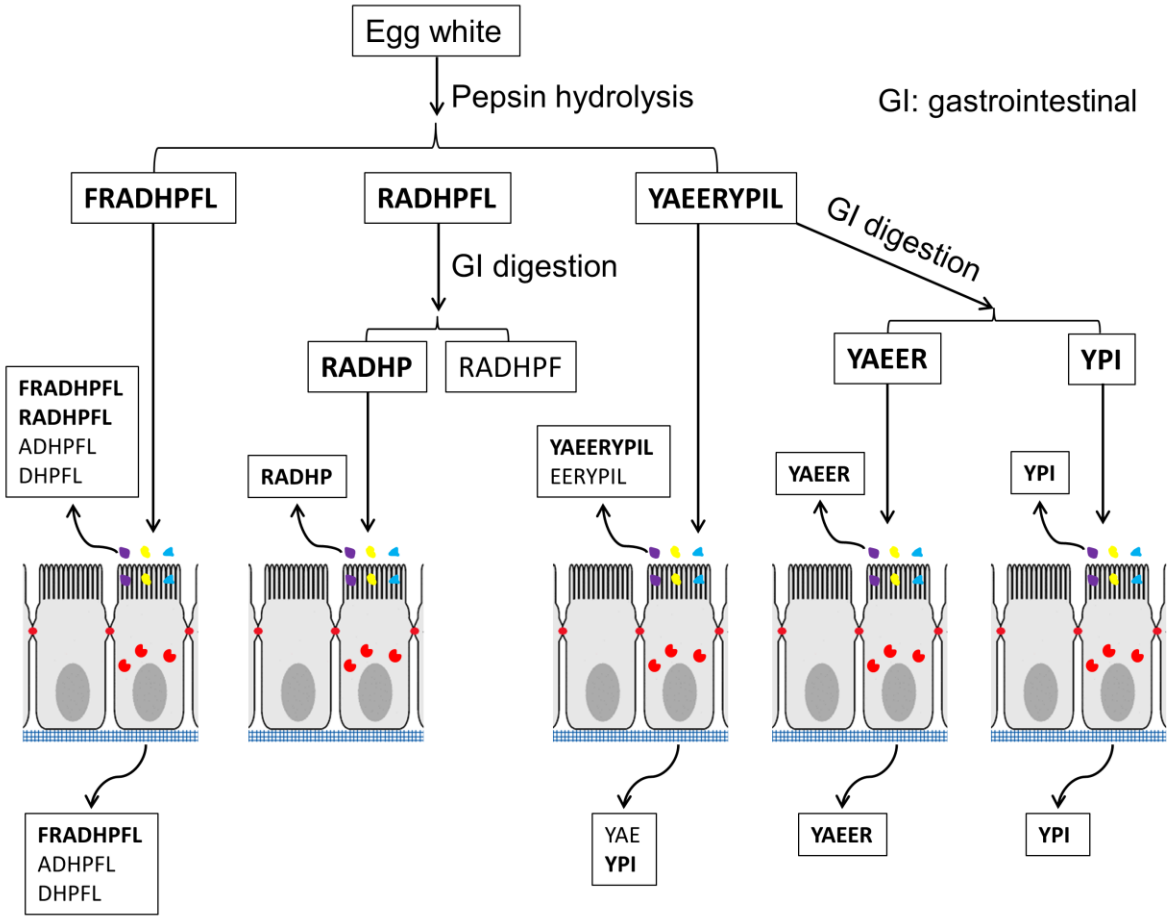

Figure 2. Examples of several antihypertensive peptides (bold letters) degraded by brush-border peptidases and intracellular peptidases in a Caco-2 cell monolayer model.