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Analytical methods and effects of bioactive peptides derived from animal products: A mini-review

Abstract

Peptides with bioactive effects are being researched for various purposes. However, there is a lack of overall research on pork-derived peptides. In this study, we reviewed the process of obtaining bioactive peptides, available analytical methods, and the study of bioactive peptides derived from pork. Pepsin and trypsin, two representative protein digestive enzymes in the body, are hydrolyzed by other cofactors to produce peptides. BCA assay, SDS-PAGE, chromatography, and *in vitro* digestion simulation systems are utilized to analyze bioactive peptides for protein digestibility and molecular weight distribution. Pork-derived peptides mainly exhibit antioxidant and antihypertensive activities. The antioxidant activity of bioactive peptides increases the accessibility of amino acid residues by disrupting the three-dimensional structure of proteins, affecting free radical scavenging, reactive oxygen species inactivation, and metal ion chelating. In addition, the antihypertensive activity decreases angiotensin II production by inhibiting ACE and suppresses blood pressure by blocking the AT1 receptor. Pork-derived bioactive peptides, primarily obtained using papain and pepsin, exhibit significant antioxidant and antihypertensive activities, with most having low molecular weights below 1 kDa. This study may aid in the future development of bioactive peptides and serve as a valuable reference for pork-derived peptides.

Keywords: Pork, Bioactive peptide, Angiotensin converting enzyme inhibitory peptide, Antioxidative peptide

Introduction

In general, peptides have a smaller molecular structure than proteins, consisting of 2-50 amino acids. Certain peptides play a role in regulating the activity of other molecules. Bioactive peptides consist of 2–20 amino acids and have a relatively small molecular weight compared to proteins (Lafarga and Hayes, 2014). The market for bioactive peptides is expanding with the growth of functional food and beverage products, and they are widely applied in functional foods, natural health products, health foods, and cosmetics (Chalamaiah et al., 2019). This growth can be attributed to the fact that consumers are becoming more health-conscious, and industries are utilizing functional ingredients to develop new products. Bioactive peptides are found in food proteins, especially in milk, meat, fish, and legumes. They are utilized as ingredients in functional foods and pharmaceuticals due to their beneficial effects on the human digestive, endocrine, nervous, and cardiovascular systems, among others, and their role in health (Heres et al., 2021a; Sánchez and Vázquez, 2017). The efficacy of bioactive peptides is often determined by their molecular weight and amino acid sequence because the amino acids that comprise the peptide sequence can have varying properties and effects. Livestock-derived bioactive peptides have been reported to have antioxidant, antihypertensive, antithrombotic, and antimicrobial activities, which have positive effects on disease prevention and blood circulation (Aluko, 2015; Lafarga and Hayes, 2014; Kim et al., 2023; Rubak et al., 2022). Previous studies have confirmed that bioactive peptides that modulate various biological actions can be obtained from pork (Arihara, 2006). In particular, antioxidant and antimicrobial active peptides isolated from pork muscle proteins provide important health benefits to humans and can be utilized as functional ingredients in foods (Di Bernardini et al., 2011). However, the effective utilization of pork-derived bioactive peptides has not been adequately studied. Therefore, in this study,

we introduced a method for protein digestion analysis that can be utilized to obtain peptides and categorize various potencies and types of bioactive peptides derived from pork.

Process of protein digestion by enzymes

Proteins consumed by humans must be hydrolyzed by proteolytic enzymes secreted by the stomach, pancreas, and small intestine in order to be digested and absorbed by the body. After proteins digestion, peptides present in the intestinal lumen typically consist of 2–6 amino acids, which account for about 80% of the total amino acids (Bhutia and Ganapathy, 2018). In the intestinal lumen, the amount of amino acids present in peptides is higher than that of free amino acids (Adibi and Mercer, 1973). Consequently, most peptides and free amino acids are transported across the intestinal epithelium into the digestive tract through the brush border membrane transport system. The majority of peptides are then hydrolyzed to free amino acids, which make up about 90% of the total amino acids (Bhutia and Ganapathy, 2018).

Ingested proteins are broken down by a variety of enzymes secreted by the body's digestive system. The initial step in this process is performed by pepsin, a proteolytic enzyme secreted by the stomach. Pepsin is initially secreted as an inactive precursor, called pepsinogen, which is produced by the chief cells of the stomach (Gupta, 2018). This inactive precursor, pepsinogen, is then activated through an autocatalytic reaction in the acidic pH environment of the stomach, resulting in the production of pepsin (Gupta, 2018). Protein hydrolysates processed by pepsin are mostly in the form of polypeptides, and only a small amount of free amino acids is released through hydrolysis (Hinsberger and Sandhu, 2004). After undergoing digestion in the stomach, gastric contents pass through the duodenum and jejunum, where they stimulate cells in the intestinal mucosa to produce cholecystokinin

(Liddle, 1997). Cholecystokinin then triggers the secretion of pancreatic juice, which is rich in proteolytic enzymes, and causes the gallbladder to contract and release bile (Liddle, 1997). In addition, when the gastric contents reach the small intestine, the acidic pH environment created by gastric acid prompts S-cells in the duodenum to release secretin, which is produced by these cells (DiGregorio and Sharma, 2019). Secretin increases the secretion of bicarbonate from the pancreas and biliary tract. This neutralizes the acidic pH environment in the duodenum caused by stomach acid to a pH level of around 6–8 and reduces the secretion of stomach acid (Bhutia and Ganapathy, 2018; DiGregorio and Sharma, 2019).

The pancreas is a vital digestive organ that produces and secretes proteolytic enzymes into the small intestine to digest ingested protein. The major pancreatic proteolytic enzymes have been identified as trypsin, chymotrypsin, elastase, and carboxypeptidase (Whitcomb and Lowe, 2007). Similar to pepsin, these enzymes are initially secreted as inactive precursors, including trypsinogen, chymotrypsinogen, proelastase, and procarboxypeptidase (Whitcomb and Lowe, 2007). Among them, trypsinogen is first activated to trypsin by enteropeptidase in the small intestine. Activated trypsin then acts on chymotrypsinogen, proelastase, and procarboxypeptidase to form active chymotrypsin, elastase, and carboxypeptidase (Bhutia and Ganapathy, 2018). Trypsin is highly reactive towards peptides containing the basic amino acids arginine and lysine, while chymotrypsin hydrolyzes peptides containing the aromatic amino acids tyrosine, phenylalanine, and tryptophan (Whitcomb and Lowe, 2007). In addition, elastase acts on peptide binding sites formed by the non-polar amino acids glycine and alanine (Whitcomb and Lowe, 2007). Consequently, the proteolytic enzymes in the pancreas hydrolyze proteins that are mostly present in polypeptide form during small intestinal digestion into oligopeptides and free amino acids consisting of 6-8 amino acids (Bhutia and Ganapathy, 2018). These oligopeptides are then hydrolyzed into smaller forms of peptides, such as tripeptides and dipeptides, by brush border peptidases found in the

105 microvilli composed of small intestinal enterocytes (Hooton et al., 2015). Finally, the myriad
106 of tripeptides and dipeptides are absorbed into the small intestine where they are broken
107 down into amino acids by cytoplasmic peptidases and released into the bloodstream (Boron
108 and Boulpaep, 2016).
109

110 ## Analysis methods for protein digestibility

111 Methods such as the bicinchoninic acid (BCA) assay, sodium dodecyl sulfate-
112 polyacrylamide gel electrophoresis (SDS-PAGE), gel permeation chromatography (GPC),
113 and *in vitro* digestion system have been utilized to analyze protein digestibility and molecular
114 weight distribution, which are relevant for peptide acquisition (Li et al., 2017; Rezvankhah et
115 al., 2021; Wen et al., 2015).
116 The BCA assay is a highly sensitive method for quantifying proteins by comparing their
117 chromogenic reactions. The principle behind this method is that Cu^{2+} ions are reduced to Cu^{+}
118 ions by peptide binding of proteins in an alkaline environment. The Cu^{+} ions then combine
119 with BCA to form a purple complex. This assay is similar to the Lowry assay but has the
120 advantage of being relatively simple and resistant to compounds that may interfere with the
121 results (Walker, 2009). Once the reaction is complete, the complex can be analyzed using a
122 spectrophotometer to measure the amount of protein at the maximum absorption wavelength
123 of 562 nm (He, 2011). This reaction is mainly influenced by the presence of four amino acid
124 residues (cysteine, cystine, tyrosine, and tryptophan) in the protein molecule (Fischer et al.,
125 1999). Therefore, the BCA assay can be utilized to determine the initial protein content of a
126 sample.
127 SDS-PAGE is an electrophoresis technique that analyzes the movement of charged
128 protein molecules in an electric field. It is commonly used to separate proteins by size and

analyze them qualitatively (Rajput and Sharma, 2011; Roy et al., 2012). Sodium dodecyl sulfate (SDS), an anionic surfactant with a strong protein denaturing effect, binds to proteins at a constant rate. During this process, the proteins are transformed into a linear chain structure and become negatively charged (Farrell, 2009; Rajput and Sharma, 2011). The proteins then move in the electric field containing the polyacrylamide gel according to their molecular size, with smaller proteins migrating and separating faster than larger proteins (Rajput and Sharma, 2011). Therefore, SDS-PAGE separates polypeptides based on molecular size, making it the best experimental method for analyzing protein digestibility as a function of protein molecular weight (Righetti, 2005). Li et al. (2017) and Wen et al. (2015) used SDS-PAGE to screen for changes in molecular weight and compare the digestibility of pork protein before and after *in vitro* digestion. Specifically, Li et al. (2017) compared the molecular weight of four types of pork proteins (cooked, emulsion-type sausage, dry-cured, and stewed) before digestion, after pepsin digestion, and after pepsin and trypsin digestion. Additionally, Wen et al. (2015) compared the differences in molecular weight of proteins from four types of cooked meat (pork, beef, chicken, and fish) before digestion, after pepsin digestion, and after pepsin and trypsin digestion. In both studies, compared to undigested samples, samples treated with pepsin alone lost protein bands greater than 150 kDa, and a relatively greater amount of protein bands between 50–100 kDa was identified (Li et al, 2017; Wen et al, 2015). These results indicate that high molecular weight proteins were digested and decomposed into small sizes. In samples treated with pepsin and trypsin together, proteins were decomposed more effectively, and the degraded proteins were confirmed to be clustered in the range between 2–10 kDa (Li et al, 2017; Wen et al, 2015).

Size exclusion chromatography (SEC), a chromatography method developed in 1955, is the most commonly method used to separate polymers such as proteins and peptides according to their molecular size. SEC is utilized for various purposes including adsorption,

desalting, and determining molecular weight distribution (Sorci and Belfort, 2014; Wang et al., 2017). As the polymer moves through the column, larger molecules elute faster because they cannot penetrate the pores of the gel, while smaller molecules can penetrate the pores and move freely, increasing the elution time (Boone and Adamec, 2016; Deb et al., 2019). SEC is often used interchangeably with gel permeation chromatography (GPC) or gel filtration chromatography (GFC). GPC is a method of separating molecules by size through elution from a column composed of porous gels such as dextran, agarose, and polyacrylamide. This method can be utilized for extracting specific proteins and analyzing the molecular weight distribution of hydrolysates (Jia et al., 2010; Lee et al., 2022; Ting et al., 2013).

Protein digestibility can be assessed through both *in vivo* and *in vitro* experiments. Kjeldahl assay can be used after feeding experimental animals to determine the crude protein content in feed samples and feces, as well as to calculate feed intake for examining the digestibility of apparent proteins and peptides (AOAC, 2000; Kumar et al., 2019). *In vivo* digestion experiments can provide the most accurate results, but they are time-consuming, costly, and subject to ethical constraints (Boisen and Eggum, 1991; Guerra et al., 2012).

In vitro digestion simulation systems are more efficient compared to *in vivo* digestion experiments and are widely used to evaluate protein digestibility and physiological properties. *In vitro* models have been proposed as an alternative to the financial and ethical challenges of *in vivo* experiments involving humans or animals (Bohn et al., 2018). These systems can be utilized to rapidly screen various food structures and ingredients. In particular, meat (18%) has been identified as the most commonly studied food product using *in vitro* digestion simulation systems after plant foods (45%) (Coles et al., 2005; Hur et al., 2011). In a previous study, an *in vitro* digestion simulation system was utilized to identify biochemical indicators of digestibility and nutritional properties of pork muscle protein

following different meat processing methods (Bax et al., 2012). Lee *et al.* (2020) investigated the digestibility and antioxidant properties of beef protein according to aging period and cooking conditions by simulating the digestive conditions of infants. Gallego *et al.* (2020) also employed an *in vitro* digestion simulation system to evaluate the antioxidant activity of peptides detected after digestion in dry-brined pork hindquarters. However, further testing is needed to confirm the similarity of results obtained from studies using these *in vitro* digestion simulation systems when applied to *in vivo* models.

Utilizing an *in vitro* digestion mimicry system, researchers have identified peptides in fibrillar protein hydrolysates from porcine loin muscle that exhibit partial sequence homology to peptides found through *in vivo* digestion experiments (Escudero et al., 2010a). For example, the peptide AGDDAPR, identified in pork actin, has been found to share partial sequence homology with AGDDAPRAVF and AGFAGDDAPR identified in the duodenum or jejunum of pigs after consuming beef and trout (Bauchart et al., 2007; Escudero et al., 2010a). However, the digestive enzymes, conditions, and other factors in for each stage of digestion in the *in vitro* simulation system can vary based on age and sex, making it challenging to replicate results from *in vivo* animal experiments. Therefore, comprehensive research is needed to achieve similar outcomes as *in vivo* experiments.

The applicable bioavailability methods for bioactive peptides

Antioxidative activities

The antioxidant activity of proteins and peptides is manifested through mechanisms such as free radical scavenging, inactivation of reactive oxygen species, chelation of metal ion, and antioxidant enzyme activity (Elias et al., 2008; Yan et al., 2020).

Free radicals are atoms, molecules, or ions that possess an unpaired electron, making them unstable and highly reactive with other organic compounds (Lobo et al., 2010). These free radicals and other reactive oxygen species derived from oxygen are generated in the body through normal cell metabolism or exposure to external factors such as smoking, ultraviolet light, ozone, and X-rays (Bagchi and Puri, 1998; Carocho and Ferreira, 2013). Reactive oxygen species include the free radicals superoxide ion (O_2^-), hydroxyl radical (HO), hydroperoxyl radical (HO_2), and nitric oxide (NO), as well as other reactive oxygen species such as singlet oxygen (O_2), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid (HClO) (Carocho and Ferreira, 2013; Lobo et al., 2010). These reactive species are neutralized by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and various antioxidants (Rock et al., 1996). However, when the balance between reactive species and antioxidants is disrupted, the overabundance of reactive species causes oxidative stress (Rock et al., 1996). Free radicals and other reactive oxygen species exhibit high reactivity with most cellular molecules, including amino acids, sugars, and lipids, causing cellular damage and disruption of homeostasis (Lobo et al., 2010; Young and Woodside, 2001). In addition, excessive oxidative stress can contribute to the development of cancer, liver, kidney, cardiovascular, and neurodegenerative diseases (Carocho and Ferreira, 2013; Soomro, 2019; Tönnies and Trushina, 2017). Chelation is the formation of chelate compounds through the coordination bonding of organic substances with metal ions such as iron and copper (van Lith and Ameer, 2016). Metal ions can trigger redox reactions, leading to oxidative stress and the generation of free radicals that damage biomolecules (van Lith and Ameer, 2016; Yan et al., 2020). Furthermore, an imbalance of metal ions such as iron, copper, zinc, and calcium, along with oxidative stress, can contribute to the development of Alzheimer's disease, a neurodegenerative condition (Wang et al.,

2020). Therefore, it is important for antioxidants to effectively inhibit and reduce the interactions of reactive oxygen species with DNA, proteins, lipids, and sugars.

Proteins represent the three-dimensional structure of polypeptides, and most peptides with antioxidant activity are located inside this structure. Therefore, disrupting the three-dimensional structure of proteins through methods such as heat treatment can increase the solvent accessibility of amino acid residues in peptides, thereby enhancing their antioxidant activities (Elias et al., 2008). Furthermore, enzymatic hydrolysis can increase antioxidant activity by breaking peptide bonds to expose amino acid residues. Studies have shown that the antioxidant activity of enzymatically hydrolyzed peptides is higher than that of undigested proteins (Elias et al., 2008; Park and Chin, 2011). In addition, proteins lacking metal ion storage or transport capabilities can chelate metal ions. Proteins with exposed histidine, glutamic acid, and aspartic acid on their surface have been shown to chelate metal ions (Elias et al., 2008). Therefore, there is a need for chelators derived from natural sources with minimal side effects that can bind to these metal ions to form chelate compounds. Peptides derived from pork skeletal muscle proteins have been found to chelate ferrous ions (Fe^{2+}), and their chelating ability is enhanced through *in vitro* digestion (Zhu et al., 2016). Previous studies have also confirmed that peptides derived from pork proteins and collagen can chelate metal ions (Li et al., 2007; Saiga et al., 2003; Xing et al., 2016).

Experimental methods for measuring the antioxidant activity of proteins and peptides include the ABTS (2,2-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid) radical scavenging assay, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, iron-chelating assay, and reducing power assay (Acharya, 2017; Bhalodia et al., 2013; Zhong and Shahidi, 2015).

The ABTS assay is considered more responsive and less sensitive to pH than the DPPH assay. Additionally, ABTS assay saves time, money, and sample volume, making it a more efficient option (Moniruzzaman et al., 2012; Shalaby and Shanab, 2013).

The DPPH assay is widely used to evaluate antioxidant activity and is an electron transfer-based assay (Huang et al., 2005; Zhong and Shahidi, 2015). DPPH is a stable nitrogen radical with a dark purple color. Unlike the ABTS assay, the DPPH assay does not require the generation of radicals before conducting the test (Prior et al., 2005).

Iron-chelating assays are used for analyzing antioxidant activity by measuring the capacity of proteins and peptides to chelate Fe^{2+}. This is determined by the level of chromogenicity, as proteins and peptides chelate Fe^{2+} to form chelate compounds, and ferrozine binds to the unchelated Fe^{2+} (Santos et al., 2017). Therefore, low chromogenicity indicates a strong ability of proteins and peptides to bind and chelate Fe^{2+}, indicating high antioxidant activity (Gülçin, 2005).

The reducing power assay is another method that can be utilized to measure the antioxidant activity of proteins and peptides. Since antioxidants also function as reducing agents, reducing power is an important indicator of antioxidant activity (Shahidi and Zhong, 2015). This assay measures reducing power by detecting the reaction of a substance with potassium ferricyanide to form potassium ferrocyanide, which then reacts with ferric chloride to form a ferric-ferrous complex. This reaction results in a yellowish discoloration as the ferric form of potassium ferricyanide is reduced to the ferrous form (Bhalodia et al., 2013; Park et al., 2015).

Experimental methods for measuring antioxidant enzyme activity in proteins and peptides *in vitro* include the superoxide dismutase (SOD) assay, catalase (CAT) assay, and peroxidase (POD) assay (Dasgupta and Klein, 2014; Haida and Hakiman, 2019).

SOD, an antioxidant enzyme, plays an important role in protecting biomolecules from oxidative stress induced by reactive oxygen species (Boguszewska et al., 2010). The SOD assay measures the activity of SOD, which catalyzes the conversion of the free radical

275 superoxide ion (O_2) into hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) (McCord and
276 Fridovich, 1969).
277 CAT, another antioxidant enzyme, is found in most tissues including the liver and
278 stomach of animals. The CAT assay measures the activity of CAT, which catalyzes the
279 conversion of H_2O_2 to O_2 and H_2O (Liu and Kokare, 2017; Miranda-Bautista et al., 2017).
280 CAT can inhibit cellular damage caused by oxidative stress by reducing the amount of H_2O_2,
281 a reactive oxygen species produced *in vivo* (Catalán et al., 2018). This is based on the
282 principle that the amount of resorufin, a product that is reduced to O_2 and H_2O by CAT, and
283 the remaining H_2O_2 reacts with horseradish peroxidase (HRP) and a non-fluorescent probe,
284 which is then analyzed by fluorescence or absorbance measurements (Pinto et al., 2011).
285 Similarly, the POD assay determines the activity of POD by measuring the amount of
286 resorufin produced by the reaction of H_2O_2 with HRP and a non-fluorescent probe. Therefore,
287 these antioxidant assays can be used to predict the mechanism of antioxidant activity of
288 bioactive peptides by considering their principles.
289
290 **Angiotensin converting enzyme (ACE) inhibitory assay**
291 The antihypertensive activity of proteins and peptides can be measured through the
292 angiotensin converting enzyme (ACE) inhibitory assay. ACE plays a crucial role in the renin-
293 angiotensin system (RAS), which regulates blood pressure (Gurley and Coffman, 2007)
294 (Figure 1). Gurley and Coffman (2007) have shown that renin in the blood converts
295 angiotensinogen produced by the liver to angiotensin I, which is then converted to
296 angiotensin II by ACE. Within the RAS, ACE converts angiotensin I, an inactive
297 decapeptide, to angiotensin II, an octapeptide with vasoconstrictor activity, and inactivates
298 bradykinin, which exhibits vasodilator activity (Mora et al., 2018; Zhuo et al., 2013).
299 Angiotensin II binds to two G protein coupled-receptors (GPCRs), the AT1 receptor and AT2

receptor, to carry out its biological functions (Wu et al., 2017). The AT1 receptor is associated with a variety of physiological functions, including vasoconstriction, secretion of hormones such as noradrenalin and aldosterone, and sodium reabsorption, while the AT2 receptor promotes vasodilation and sodium excretion (Carey, 2017; Contreras et al., 2003; Kaschina and Unger, 2003; Wu et al., 2017). In this context, antihypertensive functional peptides can reduce angiotensin II production by inhibiting ACE and lower blood pressure by blocking the AT1 receptor (Contreras et al., 2003; Ferrario et al., 2005). In addition, antihypertensive functional peptides play a role in balancing the vasoconstrictor and dilator effects of angiotensin I and bradykinin (Mora et al., 2018).

On the other hand, ACE2, which has been identified as a homologue of ACE, is known to play a physiological role in the regulation of homeostasis (Turner, 2015). In addition, ACE2 cleaves the amino acids at the C-terminus of angiotensin II to form angiotensins 1-7, which bind to the Mas receptor and exert anti-inflammatory, vasodilatory, and antifibrotic effects (Barroso et al., 2015; Shenoy et al., 2015; Simões e Silva et al., 2013). Similarly, ACE2 can hydrolyze angiotensin I to produce angiotensin 1-9, which can be converted to angiotensin 1-7 by ACE action (Donoghue et al., 2000). Previous studies have reported that angiotensin 1-9 can exhibit vasodilatory functions, reducing blood pressure and preventing cardiomyocyte hypertrophy (Gonzalez et al., 2018; Sotomayor-Flores et al., 2020). Therefore, the antihypertensive activity of the peptide may be mainly determined by ACE inhibition.

Bioactive peptides in pork

It has been confirmed that bioactive peptides exhibit little bioactivity in their normal protein-bound state, and their activity is triggered by protein degradation through processes such as ripening, fermentation, enzymatic hydrolysis, and digestion (Arihara and Ohata,

2008; Xing et al., 2019). Previous studies have shown that plant-derived bioactive peptides are extracted using digestive enzymes such as trypsin, chymotrypsin, and pepsin, or plant-derived proteolytic enzymes papain, bromelain, and ficin (Ryan et al., 2011; Singh et al., 2019). Additionally, alkaline proteases from microbial fermentation processes have been identified to be used to produce highly nutritious protein hydrolysates (Sharma et al., 2019; Sumantha et al., 2006). The use of commercialized proteolytic enzymes, including Protamex® and Flavourzyme® , for the production of ACE inhibitory active peptides has been previously documented (Mirdhayati et al., 2016). Furthermore, it has been reviewed that bioactivities, such as antioxidant and ACE inhibitory activities of protein hydrolysates formed by using alcalase with other proteolytic enzymes are increased (Tacias-Pascacio et al., 2020). In addition, large amounts of bioactive peptides have been produced from pork after *in vitro* digestion, confirming that pork can be a major source of bioactive peptides (Escudero et al., 2010a). The process of bioactive peptide formation from proteins in meat is shown in Figure 2.

Table 1 displays the peptides with antioxidant activity derived from porcine proteins. All of these peptides have a small molecular weight, mostly less than 1 kDa. In addition, bioactive peptides with high DPPH radical scavenging and metal ion chelating activities were extracted from protein hydrolysate obtained from pork source fiber protein (Saiga et al., 2003). Carnosine and anserine, representative peptides with antioxidant activity, were also obtained from porcine loin muscle (Simonetti et al., 2019). Furthermore, bioactive peptides predicted to be generated after hydrolysis from pork myofibrillar proteins were identified through in silico analysis as potentially exhibiting a variety of bioactivities, including antioxidant, antihypertensive, antithrombotic, and dipeptidyl peptidase-IV (DPP-IV) inhibition (Kęska and Stadnik, 2017). DPP-IV is an enzyme that degrades incretin, a blood sugar-regulating hormone released when food is consumed. Inhibition of DPP-IV increases

incretin content, stimulating the release of insulin and inhibiting the release of glucagon, which regulates blood sugar (Drucker, 2007). Peptides extracted from dry-cured pork ham with a molecular weight of less than 1 kDa exhibit the highest antioxidant activity (Xing et al., 2018). Meanwhile, meat-derived bioactive peptides are considered to have higher antioxidant activity as they contain more hydrophobic amino acids (leucine, isoleucine, and valine) and aromatic amino acids (tryptophan, tyrosine, and phenylalanine) (Peighambardoust et al., 2021).

Among the bioactive peptides, the most extensively studied are angiotensin-converting enzyme inhibitory peptides (Arihara and Ohata, 2008).

According to the World Health Organization, approximately 1.13 billion people worldwide have high blood pressure (WHO, 2013; WHO, 2021). High blood pressure can weaken the heart, damage artery walls, alter blood flow, and lead to complications such as stroke, heart disease, kidney failure, vision loss, and hardening of the arteries (Williams et al., 2018). Due to the severe side effects of various synthetic drugs used to treat hypertension, there has been extensive research on bioactive peptides derived from food proteins that can effectively treat hypertension without causing adverse reactions (Toldrá et al., 2018). Table 2 displays the antihypertensive functional peptides derived from porcine proteins, with most originating from fibrillar proteins such as myosin, actin, and troponin. Previous studies have shown that peptides with a molecular weight of less than 10 kDa have superior antioxidant and antihypertensive properties compared to larger peptides with relatively larger molecular weights. Some peptides obtained from pork proteins through *in vitro* digestion have shown ACE inhibitory activity (Escudero et al., 2010b; Escudero et al., 2012). For example, peptides (MYPGIA and VIPEL) derived from pork actin and GAPDH, and peptides (KRVITY and VKAGF) isolated from pork myosin heavy chain and actin exhibit ACE inhibitory activity (Escudero et al., 2010b ; Muguruma et al., 2009). Peptides KAPVA and PTPVP from titin,

and peptide RPR from neblin in pork enzymatic hydrolysate, show strong ACE inhibitory activity (Escudero et al., 2012). Furthermore, differences in the amino acid composition of bioactive peptides may affect ACE-inhibitory activity. For example, differences in the composition of amino acids that make up peptides, such as acidic amino acids (aspartic acid and glutamic acid), and the presence of positively charged amino acids in the carboxyl group can affect the increase in ACE-inhibitory activity (Daskaya-Dikmen et al., 2017; Peighambardoust et al., 2021).

Conclusion

In this study, we presented a protein digestion analysis method and a peptide bioactivity analysis method that can be utilized for peptide acquisition. The digestive enzymes present in the intestinal tract include pepsin, trypsin, chymotrypsin, and procarboxypeptidase, with cholecystokinin and secretin playing auxiliary roles in protein digestion. Proteins are hydrolyzed in the body to generate peptides. Methods such as BCA assay, SDS-PAGE, and chromatography have been used to analyze protein digestibility and molecular weight distribution, which are applicable to peptide acquisition. In recent years, *in vitro* digestion simulation systems have been utilized to evaluate protein digestibility and changes in activity. In addition, the ACE inhibitory and antioxidant properties of bioactive peptides derived from pork suggest potential industrial applications. In particular, papain has been primarily used as a hydrolyzing agent for antioxidant peptides in pork. Actomyosin and tropomyosin are found in myofibrillar proteins, and they have molecular weights below 1 kDa. The antihypertensive activity is often attributed to the use of pepsin as a hydrolyzing agent in pork, with most peptides identified having a molecular weight of lower than 1 kDa. Therefore, this study can serve as a basis for the effective utilization in the development of pork-derived bioactive

peptides and exploration of their bioactivity in the future. Furthermore, the advancement of pork-derived bioactive peptides may aid in promoting domestic pork consumption.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Author Contributions

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Ethics Approval

This work does not require IRB/IACUC approval because there are no human or animal participants.

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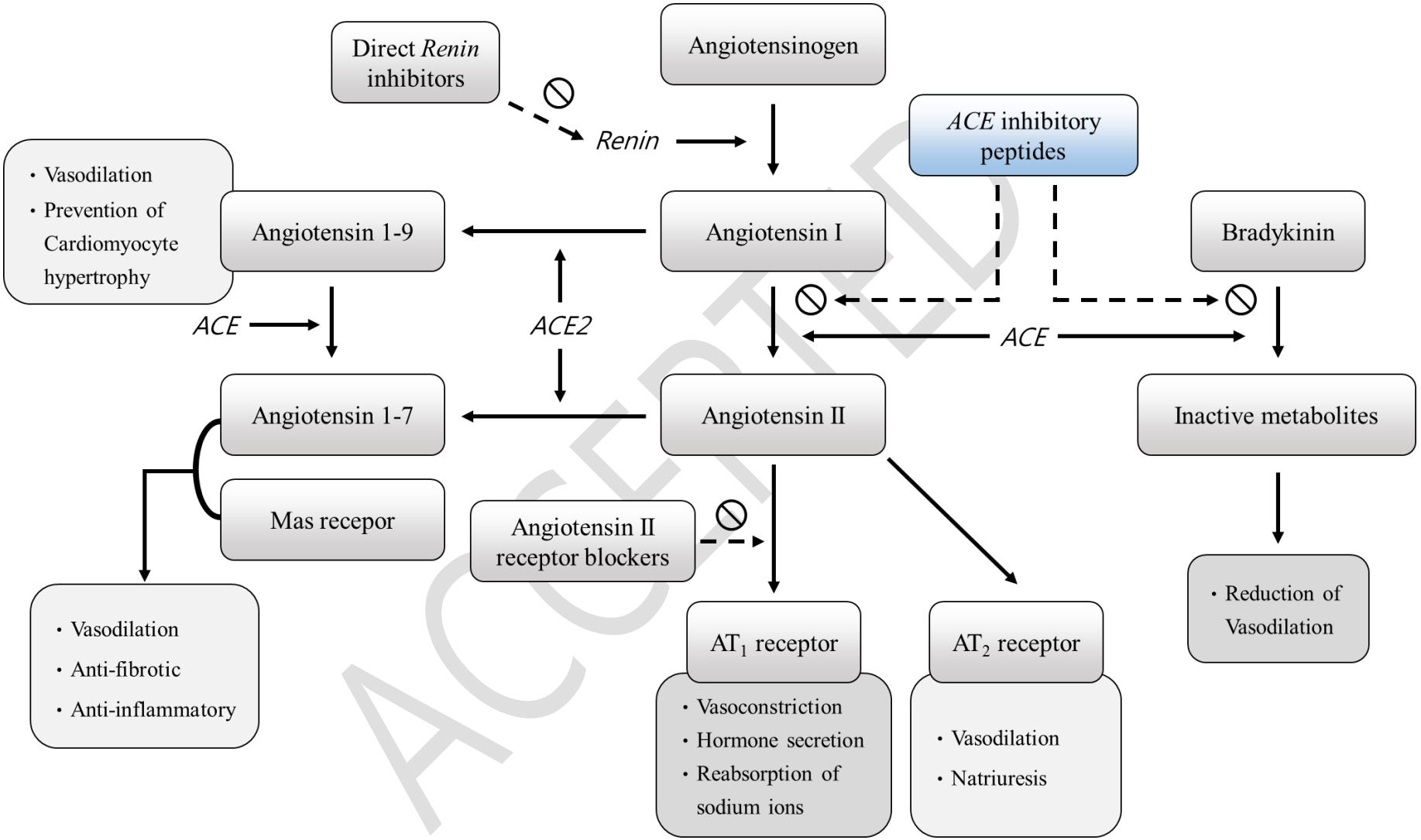

Fig 1. The renin-angiotensin system.

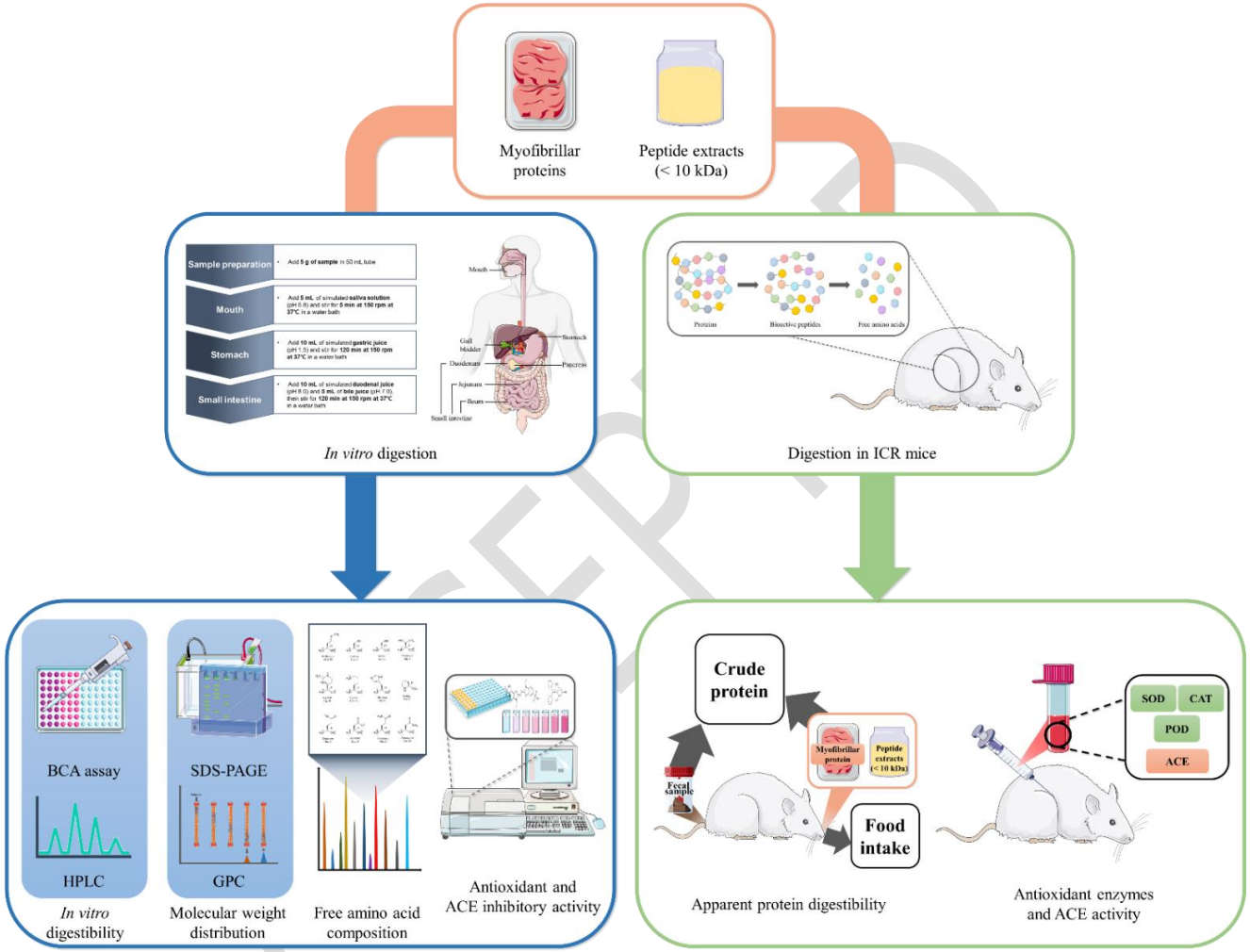

Fig 2. The analytical process for assessing the digestibility and bioactivities of myofibrillar proteins and peptide extracts from Jeju black pigs and three-way crossbred pigs (Landrace x Yorkshire x Duroc, LYD).

Table 1. Antioxidant peptides in pork

Protein Source	Peptide Sequence	Treatment	MW (Da) [a]	Reference
Porcine myofibrillar protein (Actin)	DSGVT	Papain	650.3	Saiga et al., 2003
Porcine myofibrillar protein (Unknown)	IEAEGE	Papain	646.4	Saiga et al., 2003
Porcine myofibrillar protein (Tropomyosin)	DAQEKLE	Papain	832.5	Saiga et al., 2003
Porcine myofibrillar protein (Tropomyosin)	EELDNALN	Papain	916.9	Saiga et al., 2003
Porcine myofibrillar protein (Myosin heavy chain)	VPSIDDQEELM	Papain	1275.0	Saiga et al., 2003
Porcine muscle (Actomyosin)	DLYA	Papain	480.5	Arihara, 2006
Porcine muscle (Actomyosin)	SLYA	Papain	452.5	Arihara, 2006
Porcine muscle (Actomyosin)	VW	Papain	303.4	Arihara, 2006
Porcine ham skeletal muscle proteins	GKFNV, HA, LPGGGT, LPGGGHGDL	Dry-cured; Pepsin + Trypsin	-	Zhu et al., 2016
Porcine *Biceps femoris* muscle proteins	GLAGA, SAGNPN	Dry-cured	-	Escudero et al., 2013
Porcine fresh ground ham	QYP	Fermentation	-	Ohata et al., 2016
Porcine *Biceps femoris* muscle proteins	DLEE	Dry-cured	504.2	Xing et al., 2016

Porcine ham muscle proteins	MDPKYR, TKYRVP	Dry-cured	-	Gallego et al., 2019
Porcine *longissimus dorsi* muscle	VW, LW	*In vitro* gastro-intestinal digestion	< 3,000	Martini et al., 2019
Porcine ham muscle proteins (Actin)	EAGPSIVHR, ALPHAIMR, AGFAGDDAPR, VAPEEHPTL, DEAGPSIVH, AGPSIVHRK	Dry-cured	908.1-992.1	Wang et al., 2021
Porcine ham muscle proteins (Tropomyosin)	MDAIKKK, DPIIQDR	Dry-cured	833.0-856.0	Wang et al., 2021

[a] Molecular weight measured in Daltons (Da).

Table 2. Angiotensin I-converting enzyme (ACE)-inhibitory peptides in pork

Protein Source	Peptide Sequence	Treatment	MW (Da) [a]	Reference
Porcine muscle (Myosin)	MNPPK	Thermolysin	585.7 [b]	Nakashima et al., 2002
Porcine muscle (Myosin)	ITTNP	Thermolysin	-	Nakashima et al., 2002
Porcine muscle (Myosin light chain)	VKKVLGNP	Pepsin	854.0	Katayama et al., 2007
Porcine muscle (Troponin)	KRQKYDI	Pepsin	950.1 [b]	Muguruma et al., 2009
Porcine muscle (Myosin heavy chain)	KRVITY	Pepsin	805.97	Muguruma et al., 2009
Porcine muscle (Actin)	VKAGF	Pepsin	520.62	Muguruma et al., 2009
Porcine muscle (Nebulin)	RPR	Pepsin + Pancreatin	-	Escudero et al., 2012
Porcine muscle (Titin)	KAPVA	Pepsin + Pancreatin	-	Escudero et al., 2012
Porcine muscle (Titin)	PTPVP	Pepsin + Pancreatin	-	Escudero et al., 2012
Porcine muscle (Actin)	MYPGIA	Pepsin + Pancreatin	-	Escudero et al., 2010a
Porcine muscle (GAPDH)	VIPEL	Pepsin + Pancreatin	-	Escudero et al., 2010a

Porcine *longissimus dorsi* muscle (Actin)	VFPS, LKYPI, AVF, MYPGIA	*In vitro* gastro-intestinal digestion	< 3,000	Martini et al., 2019
Porcine *longissimus dorsi* muscle	VW, IW, VF, WL, LW, VIP, LGI, LPF, IVP, IL, LLF, WM, FIV, LR, ILP, VLP, PL, LF, IAIP, IR, IF, GLx, AV, AI, DL, NIIPA	*In vitro* gastro-intestinal digestion	< 3,000	Martini et al., 2019
Porcine ham muscle proteins	GGVPGG, TKYRVP, HCNKKYRSEM	Dry-cured	-	Gallego et al., 2019
Porcine ham muscle proteins	EL, EV, RL, EEL, ESV	Dry-cured	-	Hao et al., 2020
Porcine ham muscle proteins	GA, VF	Dry-cured	-	Heres et al., 2021b; Heres et al., 2022

[a] Molecular weight measured in Daltons (Da). [b] The peptide molecular weight was derived from the PubChem.