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REVIEW

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Structural and functional properties of food protein-derived antioxidant peptides

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Abstract

The aim of this work is to provide a timely examination of the structure–activity relationship of antioxidative peptides. The main production approach involves enzymatic hydrolysis of animal and plant proteins to produce protein hydrolyzates, which can be further processed by membrane ultrafiltration into size-based peptide fractions. The hydrolyzates and peptide fractions can also be subjected to separation by column chromatography to obtain pure peptides. Although the structural basis for enhanced antioxidant activity varies, protein hydrolyzates and peptide fractions that contain largely low molecular weight peptides have generally been shown to be potent antioxidants. In addition to having hydrophobic amino acids such as Leu or Val in their N-terminal regions, protein hydrolyzates, and peptides containing the nucleophilic sulfur-containing amino acid residues (Cys and Met), aromatic amino acid residues (Phe, Trp, and Tyr) and/or the imidazole ring-containing His have been generally found to possess strong antioxidant properties.

Practical applications

High levels of reactive oxygen species (ROS) in addition to the presence of metal cations can lead to oxidative stress, which promotes reactions that cause destruction of critical cellular biopolymers, such as proteins, lipids, and nucleic acids. Oxidative stress could be due to insufficient levels of natural cellular antioxidants, which enables accumulation of ROS to toxic levels. A proposed approach to ameliorating oxidative stress is the provision of exogenous peptides that can be consumed to complement cellular antioxidants. Food protein-derived peptides consist of amino acids joined by peptides bonds just like glutathione, a very powerful natural cellular antioxidant. Therefore, this review provides a timely summary of the in vitro and in vivo reactions impacted by antioxidant peptides and the postulated mechanisms of action, which could aid development of potent antioxidant agents. The review also serves as a resource material for identifying novel antioxidant peptide sources for the formulation of functional foods and nutraceuticals.

KEYWORDS

antioxidants, FRAP, lipid peroxidation, metal chelation, peptides, protein hydrolyzates, radical scavenging

[Correction added on 30 January 2019: this article has been added to the issue after an inadvertent omission.]

1 | INTRODUCTION

At normal physiological concentrations, reactive oxygen species (ROS) play essential roles in biological systems including modulating cell cycle progression and intracellular signal transduction pathways in addition to functioning as signaling molecules for gene expression systems (Verbon, Post, & Boonstra, 2012; Wang et al., 2018). When produced in the normal course of everyday physiological processes, ROS such as hydroxyl radicals, singlet oxygen, superoxide anions, and hydrogen peroxide can be efficiently neutralized and eliminated by living (aerobic) organisms. This is because these organisms have been adequately equipped by evolution with highly advanced endogenous antioxidant defense systems (Harnedy, O'Keeffe, & Fitzgerald, 2017; Wu et al., 2017). These defense systems include enzymatic (glutathione peroxidase, catalase, and superoxide dismutase) and non-enzymatic (glutathione, tocopherol, ascorbic acid, melatonin) antioxidants (Görlach et al., 2015; Lobo, Patil, Phatak, & Chandra, 2010; Wu et al., 2017). However, under pathological or extreme environmental conditions, excess ROS are often generated, which overwhelm the cellular natural antioxidant defense capacity. This imbalance towards high ROS concentration could result in oxidative damage to critical cellular biopolymers (proteins, lipids, and nucleic acids), especially if the free radicals are produced and accumulate unchecked for a prolonged period (Aluko, 2012; Wu et al., 2017). The sustained oxidative damage of these biological macromolecules is linked to the development of chronic diseases like cancer, cardiovascular diseases, Alzheimer's, Parkinson's, and arthritis (Aluko, 2012; Lobo et al., 2010; Wu et al., 2017).

The possibility of complementing the body's natural antioxidant defense system with exogenous antioxidants in the form of antioxidative protein hydrolyzates and peptides has continued to receive significant research attention as a result of their potential for wide applications (He et al., 2012; Samaranayaka & Li-Chan, 2011; Zou, He, Li, Tang, & Xia, 2016). For instance, antioxidative peptides could serve as ingredients in food product formulations for the prevention, delay, control, and/or management of oxidative stress-related disease conditions. In addition, antioxidant peptides could also serve as additives in foods in order to curb the oxidation of food products (Qiu, Chen, & Dong, 2014). This is especially useful to prevent oxidative rancidity of foods containing oils where the formation of ketones and aldehydes could impair the food's organoleptic properties, shorten its shelf life and result in considerable economic losses (He et al., 2012; Nwachukwu & Aluko, 2018; Qiu et al., 2014). The ability of such protein-derived antioxidative agents to play a role in the prevention and/or amelioration of ROS-induced oxidative damage has been increasingly linked to their structural properties (Aluko, 2012; Samaranayaka & Li-Chan, 2011; Zou et al., 2016). Thus the aim of this review is to summarize recent developments with a focus on the relationship between peptide structure and antioxidant function.

2 | PRODUCTION OF ANTIOXIDANT ENZYMATIC PROTEIN HYDROLYZATES

Although certain antioxidative peptides, such as anserine, ophidine, carnosine, and glutathione naturally occur in foods (Samaranayaka & Li-Chan, 2011), the vast majority of food protein-derived antioxidative hydrolyzates and peptides that have been studied for their bioactivity are typically obtained following enzymatic hydrolysis, fermentation, (simulated) gastrointestinal tract (GIT) digestion, food processing, or chemical synthesis (Aluko, 2012). Simulated in vitro GIT digestion mimics protein degradation in the stomach and small intestines (Phongthai, D'Amico, Schoenlechner, Homthawornchoo, & Rawdkuen, 2018; Sun et al., 2018; Vilcacundo, Miralles, Carrillo, & Hernández-Ledesma, 2018; Zhang, Tong et al., 2018) and has continued to grow in popularity given its capacity to estimate bioactive peptides that could potentially be released from a food protein source when ingested as part of the diet (Aluko, 2012). Studies have shown increased peptide antioxidant activity and even the generation of novel peptides following GIT digestion (Gallego, Mora, & Toldrá, 2018). Although performing actual in vivo GIT digestion of proteins is possible, its use in peptide research remains impractical due to the ethical, logistical, and fiscal constraints connected to such an endeavor as well as the ease and relative lower cost of replicating GIT digestion conditions in vitro (Aluko, 2012). Useful antioxidant peptides have also been obtained through fermentation (He et al., 2012) or by chemical synthesis based on the sequences of peptides identified from food proteins (Gallego et al., 2018; Jin, Liu, Zheng, Wang, & He, 2016). In a couple of recent reports, microbial enzyme preparations were employed for the liberation of antioxidative cryptides (specific amino acid sequences encrypted within the primary structure of various food proteins) from their parent vegetable (Babini, Tagliazucchi, Martini, Dei Più, & Gianotti, 2017) and fish muscle (Jemil et al., 2017) proteins.

In addition to being the most frequently used method for bioactive protein hydrolyzate and peptide production, the enzymatic hydrolysis of food proteins is generally recognized as efficient, safe, quick, and relatively inexpensive (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). Hydrolysis conditions such as temperature, pH, pressure, reaction time as well as other factors like enzyme-substrate ratio and degree of hydrolysis are known to influence the specific chemical properties of such enzyme-derived peptides (Kilara & Chandan, 2011; Udenigwe & Aluko, 2012). Given the growing interest in using underutilized foods as inexpensive sources for the production of bioactive peptides, there has been an upsurge in the number of studies examining the antioxidant properties of peptides generated from hitherto conventionally inedible or non-conventional foods in recent years. These include hydrolyzates and peptides from the red alga *Palmaria palmata* (Harnedy et al., 2017), processed fish wash water (Zhou et al., 2016), chicken blood cells (Zheng, Si, Ahmad, Li, & Zhang, 2018), squid pen chitosan (Shavandi et al., 2017), abalone viscera (Je, Park, Hwang, & Ahn, 2015), pearl

oysters (Ma, Wu, & Li, 2018), rice bran (Phongthai et al., 2018), mulberry leaf (Sun et al., 2018), and silkworm (Liu, Wan, Liu, Zou, & Liao, 2017). Others include microorganisms and single cell organisms (Alzahrani, Perera, & Hemar, 2018), blood clam (Chi, Hu, Wang, Li, & Ding, 2015), and echinoderm byproducts (Mamelona, Saint-Louis, & Pelletier, 2010). As summarized in Table 1, the enzymes used for the production of antioxidative hydrolyzates and peptides from food proteins are as diverse as the sources themselves and range from food grade proteases such as Alcalase, Flavourzyme, Neutrase, and Protamex to the pepsin/pancreatin enzyme system used for simulated GIT digestion. In addition to the wide variation in food protein sources and enzymes used for hydrolysis, Table 1 also contains detailed information on hydrolysis conditions and specific in vitro methods used for the measurement of hydrolyzate and peptide antioxidant property in selected recent studies.

3 | ANTIOXIDANT PROPERTIES

Overall, the antioxidant properties of peptides are determined based mostly on in vitro ability to scavenge free radicals (2,2-diphenyl-1-picrylhydrazyl, hydroxyl, superoxide), reduce ferric iron to ferrous, bind metals (metal chelation), and inhibit lipid oxidation. However, there are also few reports that have used cell culture and animal experiments to estimate the antioxidant potential of various food protein-derived peptides.

3.1 | Radical scavenging effects

The antioxidative capacity of protein hydrolyzates is known to be influenced by structural properties, such as amino acid composition and peptide size or molecular weight (Ketnawa, Wickramathilaka, & Liceaga, 2018). For instance, protein hydrolyzates containing a high amount of hydrophobic amino acids such as those found in chickpea protein hydrolyzates and peptide fractions after sequential pepsin and pancreatin digestion (Torres-Fuentes, Contreras, Recio, Alaiz, & Vioque, 2015) are known to possess strong free radical scavenging properties. This was illustrated by previous works, which reported a direct relationship of hydrophobicity with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of cod (Girgih et al., 2015) and salmon (Girgih, Udenigwe, Hasan, Gill, & Aluko, 2013) peptide fractions. Similarly, protein hydrolyzates containing negatively charged acidic amino acids (Glu and Asp) could serve as potent scavengers of free radicals or reducers of metal cations due to the excess electrons that are readily donated by such amino acids (Aluko, 2012; He et al., 2012; Zou et al., 2016). High Glu and Asp content (~22%) contributed to the strong DPPH radical scavenging activity of protein hydrolyzates produced through the solid state fermentation of rapeseed proteins by *Bacillus subtilis* (He et al., 2012). Protein hydrolyzates and peptides containing a high proportion of Ala, Leu, Pro as well as the aromatic amino acids Trp, Phe, Tyr, and His have been linked with strong free radical scavenging activities through direct transfer of electrons (Ketnawa et al., 2018). In particular, the powerful radical scavenging and metal chelating ability of His has been credited to its imidazole ring since the aromatic side chain can participate in hydrogen atom transfer (HAT) and single electron transfer reactions (Aluko, 2012; Lin, Deng, & Huang, 2014; Udenigwe & Aluko, 2011). In addition, the enrichment of cationic and anionic rainbow trout peptide fractions with hydrophobic amino acids and peptides possessing electron or hydrogen donating ability following electrodialysis with filtration membrane treatment was similarly linked to at least a twofold increase in DPPH and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging abilities (Suwal, Ketnawa, Liceaga, & Huang, 2018).

Low molecular weight peptides are generally associated with enhanced antioxidant activity in contrast to larger peptides (Jin et al., 2016; Yang, Li, Lin, Zhang, & Chen, 2017). For example, the DPPH radical scavenging activity of African yam bean protein hydrolyzates was inversely related to peptide size (Ajibola, Fashakin, Fagbemi, & Aluko, 2011). A previous study of the antioxidant activities of flaxseed protein hydrolyzates used a crude protease preparation from *Bacillus altitudinis*. The work reported higher ABTS radical scavenging ability of low molecular weight (<1 kDa and 1–3 kDa) flaxseed peptide fractions in comparison to the lower scavenging capacity of fractions containing larger peptides (Hwang, Chen, Luo, & Chiang, 2016). The lower scavenging power of the bigger peptides was attributed to possible steric hindrance due the size and increased peptide repulsion of the bulkier peptides. Similarly, low molecular weight (1–3 kDa) fractions obtained following the ultrafiltration of rice protein hydrolyzates prepared with microbial (*Aspergillus oryzae* and *Bacillus licheniformis*) proteases were found to possess the highest cation (ABTS) and oxygen (ORAC) radical quenching abilities (Zhou, Canning, & Sun, 2013). The DPPH radical scavenging activity of hemp seed peptides was also reported to be stronger for low molecular weight peptides when compared to bigger peptides (Girgih, Udenigwe, & Aluko, 2011). Although low molecular weight peptides are generally known to be better antioxidants (Zou et al., 2016), a recent study of acid protease-hydrolyzed soybean proteins indicates this is not always the case. This non-conformity was attributed to the type of processing, which utilized high solid or substrate concentrations (8%, 16%, 24%, and 32%) during enzymatic hydrolysis. The higher substrate levels produced greater amounts of peptides thus resulting in stronger DPPH radical scavenging activity by higher molecular weight soybean protein hydrolyzates and peptide fractions (Chen et al., 2018). The increased level of amino acids such as His, Met, Val, Asp, and Glu, which are associated with strong antioxidant properties is thought to have contributed to the higher DPPH radical quenching ability of the high solid concentration samples in spite of their larger molecular weight distributions (Chen et al., 2018). However, the soybean protein hydrolyzates and peptide fractions with higher degree of hydrolysis (low molecular weight) were shown to possess greater hydroxyl radical scavenging ability. He, Girgih, Malomo, Ju, and Aluko (2013) also reported stronger radical scavenging ability for bigger rapeseed peptides when compared to the <1 kDa peptides. Overall, these results suggest that individual contributions of amino acids within a peptide sequence may be more

TABLE 1 Selected studies on the antioxidant properties of food protein hydrolyzates and peptides

Protein source	Protease(s)	Hydrolysis conditions				Antioxidant properties measured[f]	References
		pH ratio	Temp (°C)	Time (h)	E/S		
Male silkmoth	Alcalase + Alkaline protease	9.5	55	2.16	1:20 (w/w)	ORAC, DPPH	Liu et al. (2017)
Seabass skin	Alcalase	8.0	55	6	1 U/g	ABTS	Sae-Leaw et al. (2017)
Soy bean protein	Acid protease	3.0	55	V[a]	1:100	DPPH, OH, Fe^{2+} chelation, FRAP	Chen et al. (2018)
Microalgae	Trypsin, Alcalase, Flavorzyme	8.5	–	–	1:100	ORAC, ABTS, DPPH, SRSA	Alzahrani et al. (2018)
Pearl oyster (*Pinctada fucata*)	Alcalase	Ultrasonic wave treatment at 58 ± 2°C, 300 W for 17 min followed by 90 min incubation at 58 ± 2°C				DPPH, OH, SRSA, FRAP	Wu et al. (2018)
Walnut		4.0	37	3	1:50 (w/w)	DPPH, Fe^{2+} chelation, Inhibition of lipid peroxidation	Su et al. (2018)
Sea snail (*Rapana venosa*)	Pepsin + Pancreatin	2.0; 8.0	37	4	1:50; 1:14	DPPH, FRAP, OH	Luo, Xing, Wang, Yang, and Li (2018)
Rainbow trout (*Oncorhynchus mykiss*)	Pepsin; Alcalase	2.0; 8.0	37; 55	2	1:50	DPPH, FRAP	Rajabzadeh, Pourashouri, Shabanpour, and Alishahi (2018)
Mulberry leaf	Pepsin + Pancreatin	2.0; 7.5	37	4	1:25 (w/w)	DPPH, ABTS, SRSA, FRAP, Fe^{2+} chelation	Sun et al. (2018)
Hazelnut	Alcalase	8.0	54	2.5	10,000 U/g	ABTS, DPPH	Liu et al. (2018)
Rice bran protein	Pepsin + Trypsin	1.5; NP[b]	37	4	1:100 (w/w)	FRAP, DPPH, ABTS, Fe^{2+} chelation	Phongthai et al. (2018)
Bigeye tuna (*Thunnus obesus*)	Alcalase	6.5	54	5.6	100:3.8 (w/w)	DPPH, OH, SRSA, FRAP	Yang, Ke, Hong, Zeng, and Cao (2011)
Stingray (*Dasyatis kuhlii*)	Alcalase	8.5	60	3	1:10	ABTS, OH, inhibition of lipid peroxidation	Wong et al. (2019)
Blood clam (*Tegillarca granosa*) muscles	Neutrase	7.0	60	6	1:67	DPPH, ABTS, SRSA, OH, inhibition of lipid peroxidation	Chi et al. (2015)
Cowpea (*Vigna unguiculata*)	Pepsin + Pancreatin	2.0; 7.0	37	4	1:1,000	ORAC	Marques et al. (2015)
Abalone viscera	Alcalase, Flavorzyme, Neutrase, Protamex	7.0	50	8	1:25	ORAC, FRAP, OH, Fe^{2+} chelation, inhibition of lipid peroxidation	Je et al. (2015)
Sea lettuce flakes (*Palmaria palmata*)	Corolase PP	7.0	50	4	1:100 (w/w)	ORAC, FRAP	Harnedy et al. (2017)

(*Continued*)

Table 1 (Continued)

Protein source	Protease(s)	Hydrolysis conditions				Antioxidant properties measured[f]	References
		pH ratio	Temp (°C)	Time (h)	E/S		
Sardinelle (*Sardinella aurita*)	*Bacillus subtilis* A26 proteases	8.0	45	0.08–5	3:1	ORAC, FRAP	Jemil et al. (2017)
Echinoderm tissues	Alcalase	8.0	55	16	1:133	ORAC, inhibition of lipid peroxidation	Mamelona et al. (2010)
Hempseed protein hydrolyzates	Pepsin + Pancreatin	2.0; 7.5	37	6	1:25 (w/v)	DPPH, OH, SRSA, FRAP, ORAC, Fe^{2+} chelation, inhibition of lipid peroxidation	Girgih, Udenigwe, and Aluko (2013)
Flaxseed	Thermoase GL-30	8.0	37	4	1:40 and 1:33	DPPH, OH, SRSA, FRAP, Fe^{2+} chelation	Nwachukwu and Aluko (2018)
Chia (*Salvia hispanica* L.)	Papain	7.0	45	3	10 U/mg	DPPH, ABTS	Cotabarren et al. (2019)
Pear millet (*Pennisetum glaucum*)	Trypsin	6.5	37	3	1:100	FRAP, OH, DPPH, ABTS, Fe^{2+} chelation	Agrawal et al. (2016)
Quinoa (*Chenopodium quinoa* Willd.)	Pepsin + Pancreatin	3.0; 7.0	37	V[c]	1:1	ORAC	Vilcacundo et al. (2018)
Australian canola	Alcalase, chymotrypsin, pepsin, trypsin, pancreatin	V[d]	V[d]	4	1:20	FRAP, SRSA, DPPH, ABTS, inhibition of lipid peroxidation	Alashi et al. (2014)
Rapeseed	Alcalase, Flavorzyme, Proteinase K, Thermolysin, Pepsin + Pancreatin	V[e]	V[e]	4	1:25	DPPH, SRSA, FRAP, inhibition of lipid peroxidation, Fe^{2+} chelation	He et al. (2013)
Cumin seeds (*Cuminum cyminum*)	Protamex	8.0	42.6	1.83	1:20	DPPH, FRAP	Siow and Gan (2016)
Chinese chestnut (*Castanea mollissima* Blume)	Alcalase	10.0	55	4	1:33	FRAP, OH, DPPH, ABTS	Feng, Ruan, Jin, Xu, and Wang (2018)

[a]Various times. [b]Not provided. [c]0 and 120 min for pepsin; 180 and 240 min for pancreatin for a total hydrolysis duration of 4 hr. [d]Alcalase pH 8.0, 60°C; chymotrypsin pH 8.0, 37°C; pepsin pH 3.0, 37°C; trypsin pH 8.0, 37°C; pancreatin pH 8.0, 40°C. [e]Alcalase pH 8.0, 50°C; Flavorzyme pH 6.5, 50°C; Proteinase K pH 7.5, 37°C; Thermolysin pH 8.0, 50°C; Pepsin + Pancreatin pH 2.0, 37°C + pH 7.5, 37°C. [f]DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; FRAP, Ferric reducing antioxidant power; OH, hydroxyl radical; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical; SRSA, superoxide radical scavenging activity; ORAC, oxygen radical absorbance capacity.

important than the additive effects of amino acids in the quenching of this radical by soybean protein hydrolyzates.

Although certain aspects of the structure-function relationship of antioxidant peptides are still poorly understood (Harnedy et al., 2017), amino acid type and sequence are thought to be important determinants of antioxidative property in peptides (Aluko, 2012; Torres-Fuentes et al., 2015; Zou et al., 2016). Thus, the strong ABTS radical scavenging property of GPH-E21 (PYSWK), the main peptide present in a reverse phase-HPLC fraction of grass carp skin protein hydrolyzates, was credited to the indole and benzene rings of its aromatic amino acids (Cai et al., 2015). For example, KTFCGRH (a fish protein-derived peptide) displayed very strong DPPH and hydroxyl radical scavenging effects and also prevented hydroxyl-radical-induced DNA damage (Nazeer, Kumar, & Ganesh, 2012). Similarly, the presence of amino acids with aromatic and indole side groups may be responsible for the strong radical scavenging activity reported for PIIVYWK, TTANIEDRR, and FSVVPSPK, which were purified from mussel (*Mytilus edulis*) proteins (Park, Kim, Ahn, & Je, 2016).

Furthermore, the strong DPPH radical scavenging ability of CSQAPLA purified from corn protein hydrolyzates is thought to be related mostly to the APLA portion of the peptide chain since hydrophobic interactions among non-polar amino acid residues have been correlated with improved antioxidant ability (Jin et al., 2016). In addition, the difference in antioxidant activity of two other peptides (YPKLAPNE and YPQLLPNE) obtained from corn proteins in the same study was attributed to the repetitive LL sequence in the latter peptide given that repetitive di- or tri- amino acid residues within a peptide have been linked to enhanced antioxidant activity (Jin et al., 2016). However, it should be noted that differences in the overall peptide conformation could also have contributed to the observed antioxidant properties of YPKLAPNE and YPQLLPNE. The positive role of peptide hydrophobicity in enhancing radical scavenging through increased interaction with ROS was further illustrated by the strong DPPH scavenging ability of WVYY, a hemp seed protein-derived peptide when compared to other less hydrophobic peptides such as WSY, WYT, SVYT, and PSLAPA (Girgih, He et al., 2014). The role of peptide chain length is not fully understood but results from a previous work suggest a positive effect. For example, the Trolox equivalent antioxidant capacity of IE, SDK, and AYPS was reported to be 6.86, 69.00, and 389.02 μM, respectively (Xie, Liu, Wang, & Li, 2014). However, it should be emphasized that peptide chain length for these type of short sequences is likely less important than the type of amino acid residues present.

In addition to chain length, amino acid type, amino acid composition, and amino acid sequence, the location of specific amino acids in a peptide chain could be critical to its antioxidant property (Gallego et al., 2018). Essentially, the steric, hydrogen bonding, electronic, and hydrophobic properties of amino acids at the C- and N-terminals, which are determined by specific peptide structures have been correlated with antioxidant capacity. Thus, the carboxyl group of Glu next to Tyr in the octapeptide AEEEYPDL from Spanish dry-cured ham is believed to play a role in the peptide's antioxidant capacity since it could induce the donation of hydrogen atom from the phenolic hydroxyl in the tyrosine residue (Gallego et al., 2018).

Additionally, the contributions of spatial conformation and amino acid position to antioxidant activity have also been studied. For example, the occurrence of Arg (R) at the C-termini of GLFGPR and GATGPQGPLGPR was correlated with their high ABTS radical scavenging ability, when compared to other peptides similarly synthesized from the protein hydrolyzate sequences of seabass skin (Sae-Leaw et al., 2017). This is because the C-terminal Arg residue has been linked to high antioxidant activity of certain peptides. Moreover, the spatial conformation of two mutated homologous series of peptides (named NPFMAP and MPFMAP) were found to confer increased antioxidant (DPPH, superoxide, and hydroxyl radical scavenging) ability (Ma et al., 2018). Using a site-directed mutagenesis strategy in which His was selected as the site-directed mutant amino acid residue to replace other amino acids, investigators found that although the peptides shared similar amino acid compositions with slightly varying primary structures, the conformation or spatial structure of the peptide chain had a greater effect on antioxidant activity (Ma et al., 2018). However, for peptide YWDHNNPQIR, C-terminal Arg residue was not as important as the N-terminal bulky residues, tyrosine (Y), and tryptophan (W). This is because the DPPH radical scavenging of YWDHNNPQIR (IC_{50} = 0.64 mg/ml) was similar to that of WDHNNPQIR (IC_{50} = 0.69 mg/ml) and YWDHNNPQ (IC_{50} = 0.70 mg/ml) with either or both bulky groups occupying the N-terminal (Xu, Wang et al., 2017). In contrast, peptide DHNNPQIR, which retained the Arg residue at the C-terminal but without the N-terminal bulky groups was a weaker scavenger with IC_{50} = 1.29 mg/ml. A recent work also reported the importance of dissociation bond energy in influencing the radical scavenging activity of peptides (Leung, Venus, Zeng, & Tsopmo, 2018). The authors showed that CQV had stronger hydroxyl radical scavenging activity than QCV, QVC, QCA, and glutathione (GSH). The higher activity of CQV was attributed to presence of the Cys residue (C) at the N-terminal where the S–H bond had the least dissociation energy (334.8 kJ/mol) when compared to 339.3, 337.7, 339.7, and 342.0 for QCV, QVC, QCA, and GSH, respectively. The lower bond dissociation energy then facilitates ability of CQV to undergo a faster HAT to neutralize the hydroxyl radical.

3.2 | Metal chelation

Superoxide radicals produced in the course of normal physiological processes could be converted to hydroxyl radicals in the presence of transition state metal ions, such as Cu^{2+} and Fe^{2+}, possibly resulting in the oxidative damage of biological macromolecules like proteins and nucleic acids (Jin et al., 2016). Therefore, antioxidative peptides with the capacity to chelate metal ions could prove vital in preventing oxidative damage-related chronic disease conditions. The ability of ultrafiltration fractions of rice bran protein hydrolyzates to chelate metal ions seemed to not only depend on peptide size but also on the presence of specific peptide structures, such as the presence of Glu, Asp, His, and Lys residues all of which interact

with metal ions (Phongthai et al., 2018). For pea protein-derived peptides, hydrophobicity contributed to enhanced metal chelation (Pownall, Udenigwe, & Aluko, 2010). Increased content of charged amino acid (Glu) residues following peptic digestion was correlated with enhanced metal ion chelating ability of mulberry leaf (Sun et al., 2018) and fish (Lin et al., 2014) peptides. The fish peptides with highest level of histidine were bound to the most amount of ferrous ion. Also, the 3–5 kDa rice bran peptide fractions were found to be highly potent metal chelators while the smallest peptides tested surprisingly showed the weakest metal chelating ability. Conversely, low molecular weight (<1 kDa and 1–3 kDa) peptides purified from flaxseed proteins showed very strong metal chelating ability (Hwang et al., 2016). However, ultrafiltration led to reduced metal chelation capacity of hemp seed peptides but potency was directly related to peptide size (Girgih et al., 2011). In contrast, ultrafiltration did not improve or diminish the metal chelating ability of African yam bean peptides (Ajibola et al., 2011). These contrasting results are difficult to explain but could be due to variations in the amino acid composition and sequence of active peptides present within the samples.

In another study highlighting the importance of amino acid sequence to antioxidant activity, Egusa Saiga and Nishimura (2013) found that the metal ion chelation ability of an antioxidative peptide is affected by the side chain composition of its terminal amino acid residues. Although the peptides DAQEK (S35-C2) and QEKLE (S35-N2) have the same chain length, the latter showed stronger metal chelating ability as a result of the Gln residue present at its N-terminus. Since Gln contains a carbamoyl (–$CONH_2$) group in its structure, the carbonyl group (CO–) is able to function as a ligand molecule, thus facilitating the formation of a stable complex with the metal ion and enabling S35-N2 to trap metal ions more powerfully (Egusa Saiga & Nishimura, 2013).

3.3 | Ferric reducing antioxidant power (FRAP)

The FRAP assay is routinely used to determine ability of antioxidative food protein-derived hydrolyzates and peptides to serve as reductants by donating their excess electrons to the Fe^{3+}-2,4,6-tripyridyl-S-triazine complex, and reducing it to the more stable divalent ferrous ion, Fe^{2+} (He et al., 2012; Nwachukwu & Aluko, 2018; Torres-Fuentes et al., 2015). In a study of protein hydrolyzates and peptides from Corolase PP-hydrolyzed sea lettuce flakes (*P. palmata*), the high reducing power of the decapeptide, SDITRPGGNM was linked to the terminal Met residue (Harnedy et al., 2017) because sulfur-containing amino acids, such as Cys and Met are known to be highly effective in reducing the Fe^{3+}-ferricyanide complex (Udenigwe & Aluko, 2011). In addition, the presence of hydrophobic amino acids such as Ile, Pro, Gly, and Met within the sequence of the decapeptide may have contributed to its overall antioxidative property due to the high electron density. A similar work showing enhanced FRAP with increased hydrophobicity of HPLC peptide fractions has been reported (Pownall et al., 2010). Conversely, Lys has been reported to negatively affect FRAP activity of protein hydrolyzates (Udenigwe & Aluko, 2011). Thus, it is instructive to note that 9 out of the 13 peptides with low FRAP values obtained from the sea lettuce flake proteins contained a Lys residue with eight of the residues appearing at the C-terminal of the peptides (Harnedy et al., 2017). This is consistent with the report on pea protein-derived peptide fractions, which showed decreased FRAP as the cationic property increased (Pownall, Udenigwe, & Aluko, 2011). In rice bran protein membrane fractions, the proportion of Tyr and Trp proved to be a key difference in FRAP activity as the fraction (F3) with the least content of both aromatic amino acid residues also possessed the lowest FRAP value (Phongthai et al., 2018).

3.4 | Inhibition of linoleic acid oxidation

The ability of peptides and protein hydrolyzates to inhibit linoleic acid oxidation during in vitro assays could provide a useful estimation of their capacity to reduce lipid peroxidation rate in biological systems. It is therefore, a critical antioxidative property since the oxidative damage of membrane lipids could potentiate a breach of membrane integrity and ultimately lead to impaired membrane transport channels (Wu et al., 2017). Lipid peroxidation in foods also generates peroxides and degradation products that reduce food quality and overall shelf life through increased production of undesirable taste and smell. It was reported that the hydrophobic (Val, Leu, and Pro) and aromatic (Tyr and Phe) amino acid residues present in the GPH-E4, GPH-E5, and GPH-E21 grass carp skin protein hydrolyzate HPLC fractions contributed to their strong inhibition of linoleic acid oxidation (Cai et al., 2015). For antioxidative peptides obtained from papain hydrolyzates of porcine myofibrillar proteins, amino acid charge, hydrophobicity, and peptide length proved to be important in determining linoleic acid peroxidation inhibition capacity (Egusa Saiga & Nishimura, 2013).

For instance, the dramatic difference in the lipid peroxidation inhibition capacity of the two peptides DAQEKL (S35-C1) and DAQEK (S35-C2) was linked to the absence of the hydrophobic Leu residue from the C-terminus of S35-C2. Since hydrophobicity increases fatty acid accessibility and could thus enhance lipid peroxidation inhibition, it is thought that the possible interaction of Leu with oxidized lipids may have contributed to the greater anti-peroxidation activity of S35-C1 (Egusa Saiga & Nishimura, 2013). In other antioxidative peptides derived from the papain hydrolyzates (Egusa Saiga & Nishimura, 2013), the replacement of Glu (E) in IEAEGE with Ala (A) (to obtain IAAAGA) resulted in weaker capacity to counteract lipid peroxidation. Since Fe (II) was used to induce lipid peroxidation in this study, anions from the negatively charged and acidic Glu may have interacted with Fe (II) cations to result in the greater lipid peroxidation inhibitory activity of IEAEGE. Similarly, the replacement of Lys, Asp and Glu in DAQEKLE with Ala to obtain AAQAALA also led to a weaker ability to inhibit linolenic acid oxidation (Egusa Saiga & Nishimura, 2013). This could be as a result of the absence of the charged acidic amino acid residues (Asp and Glu) and the positively charged Lys (contains the antioxidative ε–amino group) from AAQAALA sequence (Egusa Saiga & Nishimura, 2013). The role of amino acid charge was further demonstrated when it was reported

that positively-charged casein-derived peptides had stronger inhibition of low density lipoproteins (LDL) oxidation when compared to peptides with negative charge (Wang, Wang, Huo, & Li, 2016). Specifically, the peptide MPFPK, which contained no negatively-charged amino acid was the most effective in prolonging the lag time for LDL oxidation at ~479 min when compared to 102 min for the control. In comparison, KEMPFPK (1 negatively-charged amino acid) and KNQDKTEIPT (2 negatively-charged amino acids) had lag times of ~410 and 249 min, respectively. While peptides with negatively-charged amino acids could bind metal cations to prevent lipid oxidation, the stronger activity of the positively charged peptides could be attributed to the termination of the free radical chain reaction and prevention of LDL oxidation propagation through HAT and scavenging of lipid radicals (Wang et al., 2016). A previous work also reported that KTFCGRH, a fish peptide with positively charged amino acids inhibited linoleic acid oxidation at levels equivalent to the activity of α-tocopherol (Nazeer et al., 2012).

Further evidence of the effect of amino acid sequence on antioxidant capacity can be seen in the milk protein-derived hexapeptide ISELGW, which strongly inhibited the oxidation of linoleic acid (Tsopmo et al., 2011). When Trp (W) was replaced with Tyr (Y) at the C-terminus, there was no decrease in the ability of the hexapeptide to inhibit the formation of hydroperoxide but the substitution of Trp with Phe led to a considerable decrease in linoleic acid oxidation inhibitory capacity. It is therefore, likely that the additional OH group (and electrons) in the Tyr residue made it a more effective inhibitor of lipid peroxidation relative to the structurally similar Phe.

3.5 | Ex vivo antioxidant effects

The in vitro antioxidative properties of food protein-derived enzymatic hydrolyzates and peptides have been widely reported and do provide an insight into the potential behavior of these antioxidant agents in physiological conditions. However, such in vitro properties cannot be directly extrapolated to their effects within in vivo environments given the differences in peptide stability, bioavailability, and reactivity between the two environments (Samaranayaka & Li-Chan, 2011; Sarmadi & Ismail, 2010). Nevertheless, a number of studies have demonstrated the capacity of protein hydrolyzates and peptides to function as antioxidants in vivo as well as in cellular studies. The demonstration of such antioxidative properties in cell culture work constitutes an important and necessary step prior to animal studies and human clinical trials. For example, an antioxidative tetrapeptide AKRA from Chinese Baijiu was recently shown to protect HepG2 cells against 2,2′-Azobis(2-methylpropanimidamidine) or AAPH-induced oxidative stress (Wu et al., 2017). In addition to scavenging intracellular ROS and preventing AAPH-induced decreases in cellular catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), and glutathione peroxidase (GSH-Px) levels, AKRA inhibited increases in malondialdehyde and oxidized glutathione (GSSG) levels (Wu et al., 2017). In a similar study, the pepsin digest of blue mussel was subjected to fractionation and purification to isolate PIIVYWK, TTANIEDRR, and FSVVPSPK as the main antioxidant peptides (Park et al., 2016). The three peptides prevented H_2O_2-induced hepatotoxicity in cultured hepatocytes, which was due to ability to upregulate heme oxygenase-1 expression, a phase II detoxifying enzyme. It is interesting to note that the antioxidant peptides from these two studies have lysine and arginine in common, two amino acids that have strong free radical scavenging properties.

Furthermore, a novel antioxidant peptide (YD1) purified from a strain of *Bacillus amyloliquefaciens* has also been reported to demonstrate strong antioxidant properties by reducing nitric oxide and ROS generation and increasing the mRNA and protein levels of antioxidant enzymes such as SOD1, CAT, and GPx-1 in RAW 264.7 murine macrophage cells (Rahman et al., 2018). The alcalase hydrolyzate of bovine lung had strong in vitro radical scavenging and iron reducing effects but could not prevent H_2O_2-induced DNA damage (O'Sullivan, Lafarga, Hayes, & O'Brien, 2017). However, the lung hydrolyzate presented significant anti-inflammatory activity when tested in RAW264.7 macrophages by reducing nitric oxide production in addition to decreasing the levels of IL-6 and IL-1b, which are pro-inflammatory cytokines. Using similar macrophage cells, it was reported that a <3 kDa rice bran protein hydrolysate fraction suppressed the mRNA levels of pro-inflammatory cytokines, such as iNOS, IL-6, and TNF-α after treatment with lipopolysaccharide and rmIFN-γ (Saisavoey, Sangtanoo, Reamtong, & Karnchanatat, 2016). The effectiveness of the rice protein hydrolysate fraction was due to the high level of free radical scavenging activity, which was potentiated by the high levels of bulky amino acids such as His, Trp, and Tyr.

In another cellular study, genes involved in antioxidant defense and ROS metabolism such as GPX3, GPX5, SOD3, CYGB, SEPP1, and MT3 were downregulated in HePG2 cells exposed to hydrogen peroxide-induced oxidative damage but were found to be upregulated in similar HepG2 cells pretreated with low molecular weight (<1 kDa) peptide fractions from corn gluten Alcalase hydrolyzate (Wang et al., 2018). A recent work also reported ability of DLEE (a peptide isolated from ham) to prevent H_20_2-induced generation of ROS in Caco-2 cells (Xing et al., 2018). In fact, the relative antioxidant capacity of DLEE (88.17%) during the cell culture test was similar to that of glutathione (90.83%), the natural cellular antioxidant. Thus, DLEE could function effectively as an exogenous antioxidant in preventing excessive intracellular accumulation of ROS.

Caco-2 cells were also used to confirm the antioxidative effects of phosphopeptides derived from hen's egg yolk. Cells pretreated with the phosphopeptides had increased expression of genes associated with antioxidant defense such as glutathione peroxidase, myeloperoxidase, lactoperoxidase, superoxide dismutase, and the peroxiredoxins (Young, Nau, Pasco, & Mine, 2011). Therefore, the peptides can work as antioxidants not only through direct scavenging of ROS but through upregulation of cellular biopolymers that promote reductions in oxidative stress. Using human neuroblastoma SH-SY5Y cells, the antioxidant properties of a fish protein hydrolyzate (containing FYY and DW as active peptides) was demonstrated. Specifically, the fish hydrolyzate enhanced cellular levels of antioxidant factors such as CAT, SOD, and GSH-Px in the absence

or presence of H_2O_2 (Chai, Wu, Yang, Li, & Pan, 2016). The pepsin digest of blue mussel (*Mytilus edulis*) was subjected to fractionation and purification to isolate PIIVYWK, TTANIEDRR, and FSVVPSPK as the main antioxidant peptides (Park et al., 2016). The three peptides prevented H_2O_2-induced hepatotoxicity in cultured hepatocytes, which was due to ability to upregulate heme oxygenase-1 expression, a phase II detoxifying enzyme.

3.6 | In vivo antioxidant effects

One of the earlier works examined the ability of corn peptides to protect mice from carbon tetrachloride-induced liver injury. Following alcalase hydrolysis of the corn proteins, the <5 kDa peptides were isolated by ultrafiltration and orally administered at different doses to mice for 14 consecutive days before carbon tetrachloride (CCl_4) treatment (Yu, Lv, He, Huang, & Han, 2012). Their results showed mice that received 200 mg/kg body wt had significantly less hepatocellular injury than the control group. The peptide treatment also led to significant increases in liver SOD and glutathione levels, which indicate stronger antioxidant capacity against free radicals that were generated by the CCl_4. The hepatoprotective effect of the corn peptides was also consistent with the associated strong in vitro scavenging of hydroxyl radicals.

In a recent animal study, the daily administration of a novel casein-derived peptide (VLPVPQK) for 8 weeks was responsible for alleviating osteoporosis in ovariectomized rats through the inhibition of oxidative damage (Mada et al., 2017). The peptide ingestion led to increased bone mineral density, reduced bone resorbing cytokines and other antiosteopenic effects in the osteoporotic rat model by enhancing antioxidant activity. This was evidenced through effects on various oxidative stress biomarkers including reduced serum malondialdehyde level as well as increased serum SOD and CAT levels (Mada et al., 2017). Daily oral administration of corn gluten meal hydrolyzate to mice led to significant antioxidant benefits that was mostly dose-dependent and superior to the effects produced by vitamin E (Liu et al., 2015). For example, rats that received the 300, 700, and 1,000 mg kg^{-1} day^{-1} for 10 days had 399, 409, and 423 U/mg protein, respectively of liver GSH-Px in comparison to the control (311 U/mg protein) and vitamin E (374 U/mg protein) groups. The mice that received the corn protein hydrolyzate also had reduced (9.7–10.4 nmol/ml) plasma levels of malondialdehyde (a marker of oxidative stress) when compared to the control (18.5 nmol/ml) and vitamin E-fed (12.2 nmol/ml) groups. The superior antioxidant effect of the protein hydrolyzate could be due to the presence of several peptides with a synergistic effect that beats vitamin E, a single antioxidant molecule. Using D-galactose-induced aged mice, it was demonstrated that oral administration of a fish protein hydrolyzate (FYY and DW as active peptides) led to increased level of glucose-6-phosphate dehydrogenase (G6PDH) as well as reduced levels of lipid peroxidation and endothelial nitric oxide synthase (Chai et al., 2016). High levels of D-galactose increases formation of advanced glycation end products from proteins and peptides, which leads to rapid aging and senescence. In addition to the antioxidant effects, the fish protein hydrolyzate ameliorated the D-galactose-induced memory impairment in the treated mice. The improved memory was attributed to enhanced levels of brain-derived neurotrophic factor (BDNF), which regulates short-term synaptic function along with increased long-term potentiation of synaptic plasticity (Chai et al., 2016). In a similar work, oral feeding of scopolamine-induced amnesic mice with a radical scavenging anchovy enzymatic protein hydrolyzates led to amelioration of brain injury (Zhao et al., 2017). Using the Morris water maze test, escape latency reduced from 42 s for the untreated mice to 30–34 s for the mice treated with anchovy protein hydrolyzates. The number of times the mice committed errors during the step-down test reduced from 1.40 for the untreated mice to 0.29–0.80 for the hydrolyzates, which is similar to the 0.42 for the normal mice and 0.46 for the disease mice treated with piracetam (anti-amnesia drug). Thus, the results suggest that antioxidative properties of the peptides may have prevented extensive oxidative damage to vital brain cell compounds in the D-galactose or scopolamine-induced memory impaired mice. While the authors did not determine the presence of peptides within the brain, the positive effects suggest that some of the antioxidant peptides may have crossed the blood–brain barrier.

In another study, hemp seed meal protein hydrolyzates obtained through consecutive pepsin and pancreatin hydrolysis increased plasma CAT and SOD levels while reducing the level of total peroxides in both young and old spontaneously hypertensive rats following an 8 weeks feed supplementation period (Girgih, Alashi et al., 2014). The results suggest that some of the active hemp seed peptides were absorbed into the blood circulatory system. Other researchers reported that oral administration of a fish peptide KTFCGRH to Wistar rat led to improved antioxidant status through enhanced activities of cellular antioxidant enzymes (Nazeer et al., 2012). The fish peptide-treated rats had levels of CAT (283.6 U/min/mg of protein), glutathione-S-transferase (4.3 U/min/mg of protein) and SOD (28.42 U/min/mg of protein) that were significantly higher than the 196.4, 1.3, and 15.1, respectively, for the negative control. A fish liver protein hydrolyzate with strong DPPH radical scavenging ability displayed anti-fatigue bioactive effects when fed to mice (Xu, Li, Regenstein, & Su, 2017). After 6 weeks of consuming the hydrolyzate, there was a dose-dependent significant increase in longset climbing time of the mice. Treated mice also had reduced levels of malondialdehyde but higher SOD activity when compared to the untreated mice. The anti-fatigue and antioxidant effects of the fish liver hydrolyzate was attributed to the high content (~83%) of hydrophilic amino acids, which would have provided surplus electrons for ROS scavenging. In an earlier work, Pan, Guo, and Jiang (2011) showed that daily oral administration of an enzymatic milk protein hydrolyzate fraction (P3) to mice for 28 days led to increased swimming time, reduced serum nitrogen, increased hepatic glycogen and reduced blood lactic acid contents. The beneficial effects of P3 was attributed to its role as a potent scavenger of hydroxyl and superoxide radicals, which would have reduced the degradation of muscle components normally associated with a strong physical exercise such as swimming.

4 | FUTURE RESEARCH NEEDS

The search for natural antioxidants to replace synthetic equivalents continues to be an important activity within the international research community. The importance of antioxidants is due to the potential use in multiple applications, especially in food preservation and human health (Girgih, Alashi et al., 2014; He et al., 2012; Qiu et al., 2014). The antioxidant peptide research is being fueled by the abundance of plant proteins as well as animal by-products that provide suitable substrates for enzymatic conversion (Babini et al., 2017; Cai et al., 2015; Chai et al., 2016; Gallego et al., 2018; Hwang et al., 2016; Park et al., 2016; Phongthai et al., 2018). However, progress has been limited due to the unrefined nature of protein hydrolyzates, which limits utilization in foods or human health because of low potency and possibility of unknown negative side effects. While purified peptides could provide better potency and greater specificity of action, the cost associated with large-scale peptide purification is a limiting factor. Therefore, future works that produce food protein hydrolyzates with stronger antioxidant potency and reduced potential for undesirable side reactions are required. Moreover, increased knowledge of the role of specific amino acids and their interactions in promoting potent antioxidant effects will assist in developing effective antioxidant peptide products (Girgih, He et al., 2014; Udenigwe & Aluko, 2011). This will also enable development of cost-effective large-scale separation methods to remove inactive peptides or free amino acids as a means of enhancing antioxidant potency of protein hydrolyzates. The use of purified peptides could provide for better control of dose and target use but stronger scientific evidence is required to clarify the relationship between amino acid sequence and antioxidant potency. Therefore, future studies dealing with quantitative structure-activity relationships of antioxidant peptides are needed. More so, additional knowledge is required to clarify the stability of peptides during long-term storage of foods. Such information is needed to provide confidence in the use of protein hydrolyzates to promote better shelf life, eating quality and safety of formulated products.

Although the ability of protein hydrolyzates and bioactive peptides to act as antioxidative agents has been roundly demonstrated during in vitro (Agrawal, Joshi, & Gupta, 2016; Alashi et al., 2014; Harnedy et al., 2017; Lin et al., 2014; Rahman et al., 2018; Wong et al., 2019) and in vivo (Girgih, Alashi et al., 2014; Liu et al., 2015; Mada et al., 2017; Xu, Li et al., 2017; Zhao et al., 2017) tests, there is a dearth of human clinical trials replicating the results. Given the challenge often encountered in translating results from animal and chemical-based assays to clinical outcomes, future work should be designed to include tests in human subjects. Moreover, with regards to human health promotion, the bioavailability, persistence within the body and in vivo specificity of antioxidant peptides require further studies. Additionally, the bitter taste of protein hydrolyzates remains a formidable hurdle for the incorporation of amino acids and peptides into functional food products or therapeutic formulations intended for the management and control of oxidative damage-related chronic diseases (Humiski & Aluko, 2007; Ishibashi et al., 1988; Kim, Choi, & Lee, 1999; Zhang, Alashi et al., 2018). Since the bitter taste of protein hydrolyzates has actually been attributed to hydrophobic and aromatic amino acid residues (Cheung, Aluko, Cliff, & Li-Chan, 2015; Edens et al., 2005) to which antioxidant properties are also credited, cost-effective strategies for masking the bitter taste of antioxidative peptides without removing the hydrophobic residues must form an integral part of the design of peptide-based antioxidant ingredients and formulations. Furthermore, the interaction of antioxidative peptides with other components in the food matrix, which could influence their activity, reduce food eating quality or lead to the production of undesirable products deserves the attention of investigators. Lastly, given the advances in bioinformatics and gene editing, more research efforts should be directed toward the use of site-directed mutagenesis and other molecular biology techniques in examining options for direct enhancement of antioxidant peptide levels in foods.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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