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Purification and Characterization of Antioxidative Peptides Prepared from Pea Protein with Strong Inhibitory Activity on Lipid Oxidation

Wenjun Zhu, Shi Sun, Fei Yang, and Kequan Zhou

Department of Nutrition and Food Science, Wayne State University, Detroit, MI 48202, USA

Correspondence: kzhou@wayne.edu (K.Z.)

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ABSTRACT | This study was to evaluate the potential for developing superior antioxidative peptides derived from pea protein isolates that could be used to prevent meat lipid oxidation and improve its shelf-life. Pea protein isolate was hydrolyzed with individual microbial proteases and further fractionated by sequential ultrafiltration (molecular weight cut-off of 3 and 10 kDa) to a total of 12 hydrolysate fractions. Their antioxidant activities were evaluated and compared. The hydrolysate fractions exerted significant oxygen radical absorbance capacity ranging from 24.5 to 81.6 μmol Trolox equivalents /g dry weight and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{+\bullet}$) scavenging activity ranging between 22.4 and 50.2 μmol TE/g dry weight. Three hydrolysate fractions with superior antioxidant activities were selected for further evaluation on the inhibition of lipid peroxidation using a food model (ground beef) during a 15-day storage period. At the end of the treatment, NP-F4 produced by neutral protease from *Bacillus subtilis* at both doses (250 and 500 μg/g beef) exerted significant inhibitory activities against lipid oxidization which were comparable to butylated hydroxyanisole (200 μg/g beef), a commonly used synthetic antioxidant with the thiobarbituric acid reactive substances reduced by 31.9%, 38.8%, and 45.7%, respectively, as compared to the negative control. Our results suggest that the specific antioxidative hydrolysates produced from pea protein with commercial proteases and ultrafiltration separation could be used to replace synthetic ones to inhibit lipid oxidation and improve shelf-life of lipid-rich food products.

KEYWORDS | Antioxidant activity; Lipid peroxidation; Microbial proteases; Pea protein hydrolysates

ABBREVIATIONS | AAPH, 2,2′-azobis(2-amino-propane) dihydrochloride; $ABTS^{+\bullet}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl; HPLC, high pressure liquid chromatography; MDA, malondialdehyde; MWCO, molecular-weight-cut-off ; ORAC, oxygen radical absorbance capacity; TBARS, thiobarbituric acid reactive substances; TE, Trolox equivalents

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1. INTRODUCTION

Lipid autoxidation is one of the major reasons reducing nutritional value and shortening shelf-life of many food products which results in the development of rancidity and a number of other potential toxic compounds that deteriorate food quality and safety. Lipid oxidation is initiated by free radicals and antioxidants both natural and synthetic ones have been widely used in lipid-rich food products. However, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) have been under scrutiny not only because of the consumers' preference on natural antioxidants but also due to the rise of safety concerns. For example, BHA has been potentially linked to carcinogenic effects [1]. For this reason, there is a strong interest in developing superior natural antioxidants that can potentially replace synthetic ones that can be applied in many food products without harmful safety issues. Antioxidant peptides or hydrolysates are ideal candidates for this development because they bear unique physical, chemical, and sensorial properties suitable for applications in different food products. Indeed, there is a growing body of publications utilizing food proteins for the development of food-based hydrolysates with strong antioxidant activity attempting to be used as natural preservatives.

Food protein hydrolysates in fact have been gaining popularity in research and food product development as promising functional food ingredients [2]. Enzymatic hydrolysis of food proteins can be conducted via commercial enzymes, fermentation, and other food processing methods to improve their nutritive values, functional properties such as gelling, foaming, or emulsifying activities, and specific bioactivities such as antioxidant, anti-inflammatory, or anti-hypertensive activities [3]. Plant proteins such as soy, rice, pea, cowpea proteins have been investigated for potential production of functional hydrolysates, mostly via digestive enzymes or commercial proteases [3–10].

Pea (*Pisum sativum*) contains high quality proteins which are particularly rich in essential branched-chain amino acids important in muscle protein synthesis [11–13]. Pea proteins are globulins consisting of two fractions, 7S (convicilin and vicilin) and 11S (legumin) [14, 15]. Due to its unique functionalities, pea protein has been utilized as wall materials in encapsulations of food ingredients such as ascorbic acid, α-tocopherol and conjugated linoleic acid [16, 17]. Pea protein has also been considered as a safe, nutritional, satiety-inducing food ingredient, as previous studies showed that pea protein ingestion could stimulate satiety relevant signaling in animals and humans [18–20]. In fact, pea protein hydrolysates have been shown to stimulate significant peptide release in murine intestinal tissues after co-incubation [21]. A previous study investigated functional properties of pea protein hydrolysates via chymosin hydrolysis and showed improved foaming stability,

solubility, and emulsifying activity [22]. Antioxidant properties of pea protein hydrolysates via thermolysin hydrolysis were also investigated [3]. Still, there are limited research on the preparation of pea protein hydrolysates and their functional properties. This study aimed to determine the potential of developing antioxidative hydrolysates/peptides from pea protein via three commercial enzymes: neutral protease from *Bacillus subtilis*, validase from *Aspergillus oryze*, and alkaline protease from *Bacillus licheniformis*. The hydrolysates after incubation were separated via sequential ultrafiltration to obtain a series of fractions and their antioxidant properties were assessed by various methods including assays for oxygen radical absorbance capacity (ORAC), and 2,2-diphenyl-1-picrylhydra-zyl (DPPH$^{\bullet}$) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{+\bullet}$) scavenging activities. Three fractions with the strongest antioxidant activities were chosen for further evaluation of inhibiting lipid peroxidation on a food model during a 15-day storage period. The ultimate goal of this study is to develop antioxidative food protein hydrolysates (replace synthetic antioxidants) for improving food quality and shelf-life by inhibiting lipid oxidation.

2. MATERIAL AND METHODS

2.1. Materials

The pea protein isolate was obtained from Biotics Research (Rosenberg, TX, USA). Protease validase® FP concentrate and alkaline protease concentrate were kindly provided by Valley Research (South Bend, IN, USA). Neutral protease was provided by BIO-CAT (Troy, VA, USA). Validase is a fungal peptidase complex possessing both endoprotease and exopeptidase activities which is generated by *Aspergillus oryzae* fermentation. The alkaline protease possessing endopeptidase activity is prepared from *Bacillus licheniformis*. The neutral protease as an extracellular endopeptidase is produced from *Bacillus subtilis*. These proteases were chosen because they are thermostable and commonly used industrial proteases. Fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and DPPH$^{\bullet}$ were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2,2′-Azobis(2-amino-propane) dihydrochloride (AAPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ATBS) diammonium salt were purchased from Wako Chemicals (Richmond, VA, USA). All other chemicals and solvents were of analytical or high pressure liquid chromatography (HPLC) grade. The ultrafiltration system (model No. 8050) and cellulose membranes for the preparation of protein hydrolysates were from Millipore (Billerica, MA, USA).

2.2. Preparation of Pea Protein Hydrolysates

Pea protein isolate was hydrolyzed by three microbial proteases individually according to the processing guidelines from the manufacturers with slight modification. The protein was first suspended and homogenized in water at 50 g/500 g and then hydrolyzed by the individual proteases at a concentration of 1.0 g/50 g (enzyme/substrate). The enzymatic hydrolysis was conducted for 6 h in a water bath at 55ºC under shaking. For validase and neutral protease, the reaction mixture was adjusted to pH 7.0 using 0.5 M NaOH or 0.6 M HCl during hydrolysis. For alkaline protease, the solution was adjusted to the optimum pH 10. The enzymatic reactions were terminated by boiling the mixtures for 5 min. Each mixture was centrifuged at 15,000 *g* and the soluble fraction was then filtered using filtration paper for further fractionation.

2.3. Fractionation of Protein Hydrolysates by Ultrafiltration

The resulting protein hydrolysates were ultra-filtered sequentially using a Millipore 8050 ultrafiltration unit through cellulose membranes with different molecular weight (MW) limits. The hydrolysates were first diluted with water and ultra-filtered through a membrane with 10 kilodalton (kDa) molecular-weight-cut-off (MWCO) under 276 kPa nitrogen gas to yield two fractions: retentate (fraction 1, F1, represented hydrolysates > 10 kDa) and filtrate (MW < 10 kDa). The filtrate was further ultra-filtered through a 3 kDa MWCO membrane to obtain the second retentate (fraction 2, F2, represented hydrolysates between 3 and 10 kDa) and the second filtrate. The filtrate was further ultrafiltered through a 1 kDa MWCO membrane to yield the third retentate (fraction 3, F3, represented hydrolysates between 1 and 3 kDa) and the third filtrate (fraction 4, F4, represented hydrolysates < 1 kDa). All retentates and filtrates

were lyophilized and stored at –20°C until further analysis.

2.4. Measurement of Oxygen Radical Absorbance Capacity

The ORAC assay was performed using fluorescein as a protein probe to evaluate the antioxidant protection of the generated pea hydrolysates against the peroxyl radicals. The radicals were generated using AAPH in 75 mM phosphate buffer (pH 7.4) [23]. Trolox was used an antioxidant standard and ORAC values of the hydrolysates were expressed in micromoles of Trolox equivalents per gram dry weight hydrolysates (μmol TE/g).

2.5. Measurement of DPPH$^{\cdot}$ Scavenging Activity

The assay was performed according to a previously reported protocol [24]. The DPPH$^{\cdot}$ scavenging activities of the pea hydrolysates were evaluated by mixing 100 μl of sample with 100 μl of 0.2 mM DPPH$^{\cdot}$ solution and after 40-min incubation, the absorbance was measured at 517 nm against a blank of deionized water. Ascorbic acid and BHT were used for comparisons.

2.6. Measurement of ABTS$^{+\cdot}$ Scavenging Activity

The assay was performed in a reaction mixture containing 1.0 ml of ABTS$^{+\cdot}$ and 80 μl of the protein hydrolysates [25]. The absorbance at 734 nm was measured following the reaction. Trolox was used as an antioxidant standard and the ABTS$^{+\cdot}$ scavenging activities of the pea hydrolysates were expressed in micromoles of Trolox equivalents per gram dry weight hydrolysates (μmol TE/g).

2.7. Determining the Effects of the Selected Protein Hydrolysates on Meat Lipid Oxidation

Three hydrolysate fractions (NP-F4, AP-F4, and Val-F3) that possess the strongest free radical scavenging activities were selected for further evaluation on inhibition of lipid peroxidation using a food model (ground beef) according to a published protocol [23]. Briefly, fresh beef trims obtained from the local grocery store were ground through a plate. The beef samples (250 g) were further homogenized with or without the protein hydrolysates by a macro-ultrafine generator and then distributed into 50 ml centrifuge tubes, weighed, and centrifuged to remove trapped air. To promote lipid oxidation, the beef mixtures were heated to an internal temperature of 71°C in a water bath. The internal temperature was monitored by a needle-size thermocouple inserted into the thermal center of the tubes during cooking. After cooling to room temperature, the homogenized and cooked beef samples were placed in trays, covered with PVC film, and stored at 4°C. The extent of lipid oxidation in each meat sample was determined by the thiobarbituric acid reactive substances (TBARS) assay on days 1, 8, and 15 post cooking. The final TBARS value was calculated with the standard curve and expressed as mg of malondialdehyde (MDA) equivalents per kg of sample (mg MDA equivalent/kg).

2.8. Statistical Analysis

The mean values within each test were compared using a two-sample t-test. Data is presented as mean ± SD (standard deviation). Difference was considered statistically significant when the P value was < 0.05 (SPSS for Windows, version 10.0.5, 1999). A two-tailed Pearson correlation test was conducted to determine the correlations among means.

3. RESULTS AND DISCUSSION

3.1. ORAC of Pea Protein Hydrolysates

As shown in **Figure 1**, all the pea protein hydrolysate fractions showed remarkable peroxyl radical scavenging activities ranging from 24.5 to 81.6 μmol TE/g dried hydrolysates. Their ORAC values are comparable with those of hydrolysates prepared from other food proteins and vegetables extracts such as broccoli and carrot [8, 9, 23]. In general, antioxidant activities are negatively associated with their molecular weights; small molecular hydrolysate fractions (molecular weight between < 1 kDa) possessed significantly higher ORAC values (55.2–81.6 μmol TE/g) than the fractions with MW above 10 kDa did (24.5–35.4 μmol TE/g). The phenomenon was also seen in other food protein hydrolysates where smaller molecular hydrolysates contained higher ORAC activities [9, 23]. Specifically, NP-F4 (81.6 μmol TE/g) and AP-F4 (73.5 μmol TE/g) contained the

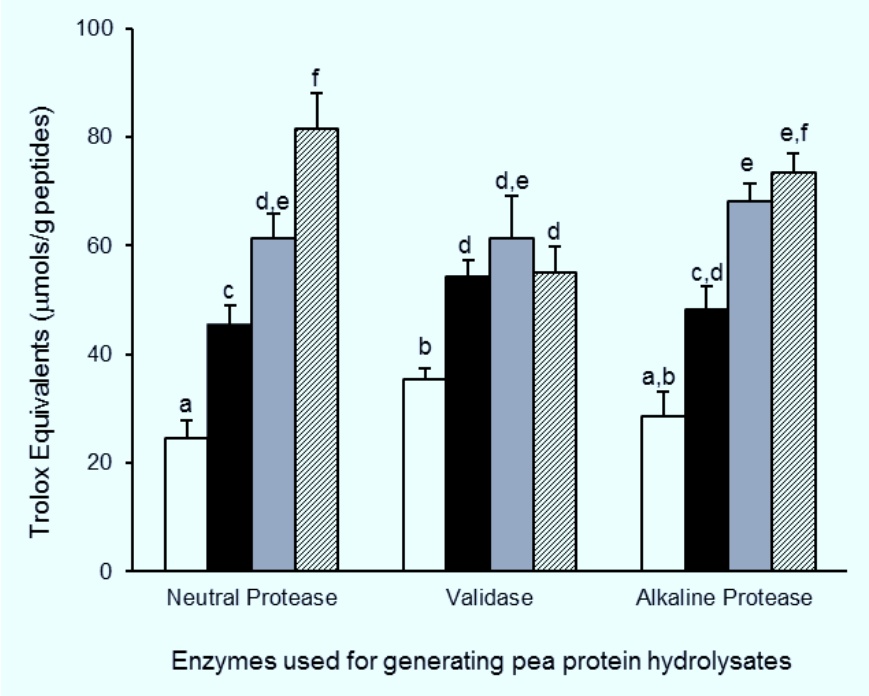

FIGURE 1. Oxygen radical absorbance capacity (ORAC) of pea protein hydrolysate fractions. Each pea hydrolysate fraction of different molecular weights (F1, F2, F3, and F4 correspond to samples > 10, 3–10, 1–3, or < 1 kDa, respectively) was tested in triplicate with ORAC values expressed in Trolox equivalents per gram of dry weight of pea hydrolysates (µmol TE/g). The data marked by different letters are significantly different ($P < 0.05$). Open bar represents the hydrolysate fraction F1, black bar represents F2, grey bar represents F3, and texture bar represents F4.

highest ORAC activities followed by AP-F3 (68.1 µmol TE/g), NP-F3 (61.4 µmol TE/g) and Val-F3 (61.3 µmol TE/g). The lowest ORAC values were found in the fractions of NP-F1 (24.5 µmol TE/g) and AP-F1 (28.5 µmol TE/g). In comparison, the ORAC value of NP-F4 was over three times of that of NP-F1, suggesting that molecular size of the hydrolysates could significantly affect their ORAC activities and hydrolysate fractionation based on their molecular weight is effective to obtain specific fractions with superior antioxidant activity.

3.2. DPPH˙ Scavenging Activity of Pea Protein Hydrolysates

The DPPH˙ scavenging activities of the pea hydrolysate fractions at 100 mg/mL in the reaction were compared and varied from 26.3% to 70.5% of the radicals being quenched (**Figure 2**). Among the fractions, AP-F4 and Val-F3 showed the strongest activities by scavenging 70.5% and 68.5% DPPH˙, respectively. In comparison, the fractions of Val-F1 and NP-F1 quenched 26.3% and 33.4% radicals, respectively. In contrast to the above observed ORAC values in the hydrolysates, the DPPH˙ scavenging activities were not associated with their molecular weight. DPPH˙ scavenging assay has been commonly used for antioxidant evaluation for protein hydrolysates due to its stability and simplicity [26, 27]. However, the comparison of DPPH˙ activities is not meaningful if the samples are not conducted in the same conditions due to lack of antioxidant standard. We did not detect a significant correction between ORAC values and DPPH˙ scavenging activities of the hydrolysate fractions which is normal since the hydrolysates may interact with peroxyl radicals and

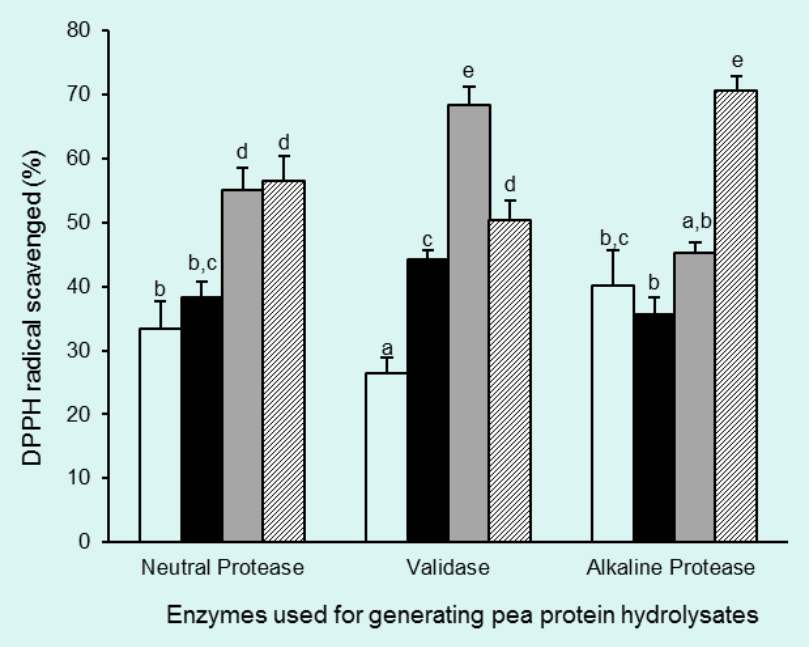

FIGURE 2. DPPH• scavenging capacity of pea protein hydrolysate fractions. Each pea hydrolysate fraction of different molecular weights (F1, F2, F3, and F4 correspond to samples > 10, 3–10, 1–3, or < 1 kDa, respectively) was tested in triplicate with ORAC values expressed in Trolox equivalents per gram of dry weight of pea hydrolysates (μmol TE/g). The data marked by different letters are significantly different ($P < 0.05$). Open bar represents the hydrolysate fraction F1, black bar represents F2, grey bar represents F3, and texture bar represents F4.

DPPH• in different mechanisms [28]. However, AP-F4 and Val-F3 were among the strongest activities in both assays.

3.3. $ABTS^{+\bullet}$ Scavenging Activity of Pea Protein Hydrolysates

$ABTS^{+\bullet}$ scavenging activity of the pea hydrolysates varied from 22.4–50.2 μmol TE/g dry weight (**Figure 3**). Among the fractions, AP-F4 (52.5 μmol TE/g) and NP-F4 (50.2 μmol TE/g) showed the highest activities, followed by Val-F3 (48.6 μmol TE/g), AP-F3 (44.2 μmol TE/g), and NP-F2 (44.2 μmol TE/g). While the fractions of Val-F1 (22.4 μmol TE/g) and AP-F1 (28.3 μmol TE/g) had the lowest $ABTS^{+\bullet}$ scavenging activities. In general, the fractions with higher molecular weight possessed the lower $ABTS^{+\bullet}$ scavenging activities but no significant correction was detected. The cation radical $ABTS^{+\bullet}$ has been widely used in antioxidant activity evaluation since it interacts with most antioxidants. In our experiments, the fraction AP-F4 exhibited the highest DPPH• and $ABTS^{+\bullet}$ scavenging activities and the second highest ORAC value; while NP-F4 possessed the highest ORAC value and the second highest $ABTS^{+\bullet}$ scavenging activity and Val-F3 had the second highest DPPH• scavenging activity and the third highest $ABTS^{+\bullet}$ scavenging activity. Therefore, these three pea hydrolysates were selected for further antioxidant activity evaluation on lipid peroxidation using a real food model.

3.4. Lipid Peroxidation in Ground Beef

Natural antioxidants possess unique advantages over synthetic ones being used as food preservatives for inhibiting lipid peroxidation in food products and improving their shelf-life. Previous studies have explored the potential of food protein hydrolysates to be utilized for this purpose [29–31]. Indeed, specific

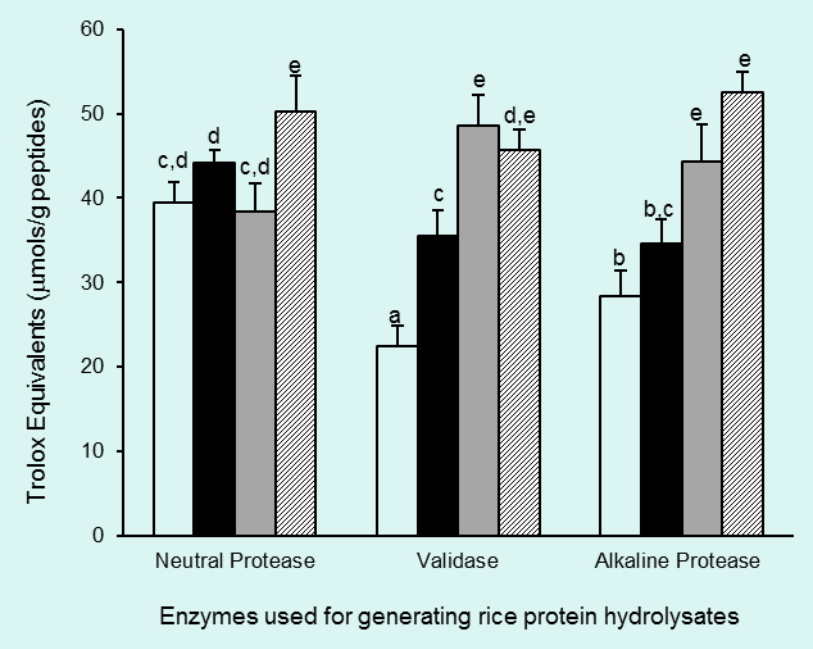

FIGURE 3. ABTS$^{+\bullet}$ scavenging activity of pea protein hydrolysate fractions. Each pea hydrolysate fraction of different molecular weights (F1, F2, F3, and F4 correspond to samples > 10, 3–10, 1–3, or < 1 kDa, respectively) was tested in triplicate with ORAC values expressed in Trolox equivalents per gram of dry weight of pea hydrolysates (µmol TE/g). The data marked by different letters are significantly different ($P < 0.05$). Open bar represents the hydrolysate fraction F1, black bar represents F2, grey bar represents F3, and texture bar represents F4.

peptides or hydrolysates exerted potent antioxidant activity (comparable to synthetic antioxidants) against lipid oxidation in various food models, suggesting it is possible to develop safe, cost-effective food protein-derived, antioxidative hydrolysates to replace synthetic antioxidants for food applications [9, 23, 32]. In the current study, three selected pea protein hydrolysate fractions (NP-F4, AP-F4, and Val-F3) and with strong antioxidant properties were evaluated for their ability in inhibiting meat lipid peroxidation in cooked ground beef at 3 different storage times (days 1, 8, and 15). As shown in **Table 1**, meat lipid peroxidation reflected by TBARS values was gradually increased with the increase of the storage period. A significant degree of lipid oxidation started at storage day 1 with the TBARS values being 2.26–9.03 MDA equivalent/kg beef. Among the samples, NP-F4 (250 and 500 µg/g beef), Val-F3 (500 µg/g beef), and BHA (200 µg/g beef) significantly inhibited lipid peroxidation with TBARS reduced by 62.4%, 72.6%, 29.5%, and 60.8%, respectively, as compared to negative control. Other selected hydrolysates did not exhibit significant activity. At storage day 8, the lipid oxidation was remarkably increased with TBARS values changed from 8.24 to 26.24 MDA equivalent/kg beef for the negative control. NP-F4 (250 and 500 µg/g beef), Val-F3 (500 µg/g beef), and BHA (200 µg/g beef) again significantly inhibited lipid peroxidation with TBARS reduced by 34.3%, 40.1%, 34.7% and 47.4%, respectively, as compared to negative control. Other fractions showed no significant activities on beef lipid oxidation. At storage day 15, the control's TBARS value was 38.55 MDA equivalent/kg. BHA, NP-F4, and Val-F3 at higher dose showed consistency in inhibiting beef lipid oxidation. NP-F4 (250 and 500 µg/g beef), Val-F3 (500 µg/g beef), and BHA (200 µg/g beef) again significantly inhibited lipid peroxidation with TBARS reduced by 31.9%, 38.8%, 33.2% and 45.7%, respectively, as compared to the

TABLE 1. Lipid peroxidation determined by TBARS assay in cooked beef after treatments at three different storage times post cooking

Protein Hydrolysate Fractions	Concentration in the Meat (μg/g)	TBARS at Different Storage Times (mg MDA equivalent/kg)		
		Day 1	Day 8	Day 15
Control	0	8.24 ± 0.35	26.42 ± 1.68	38.55 ± 2.55
NP-F4	250	3.10 ± 0.24*	17.37 ± 2.34*	26.27 ± 1.69*
NP-F4	500	2.26 ± 0.78*	15.83 ± 2.05*	23.59 ± 1.63*
AP-F4	250	8.64 ± 1.31	28.54 ± 0.57	36.38 ± 2.34
AP-F4	500	6.92 ± 0.26	26.60 ± 2.50	34.47 ± 1.58
Val-F3	250	9.03 ± 0.88	24.44 ± 2.16	33.35 ± 1.99
Val-F3	500	5.81 ± 0.49*	17.26 ± 1.32*	25.76 ± 1.46*
BHA	200	3.23 ± 0.66*	13.89 ± 1.22*	20.93 ± 1.37*

Note: The microbial enzymes used for pea protein digestion were validase from aspergillus oryze (Val), neutral protease from Bacillus subtilis (NP), and alkaline protease from Bacillus licheniformis (AP). The final unit of TBARS value was calculated with the standard curve and expressed as mg of malondialdehyde equivalent per kg of sample (mg MDA equivalent/kg). The data marked by an asterisk is significantly different as compared to the control meat sample ($P < 0.05$).

control. In general, NP-F4 of pea protein hydrolysates at both doses showed potent inhibitory activities against beef lipid oxidization which were comparable to BHA, a commonly used synthetic antioxidant. Our results showed that the potential of developing superior natural antioxidants from pea protein isolate with commercial proteases and ultrafiltration separation. The specific antioxidative hydrolysates such as NP-F4 could be used to replace synthetic ones to inhibit lipid oxidation and improve shelf-life of lipid-rich food products.

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