Research Article
Physicochemical Changes of Antioxidant Peptides Hydrolyzed From Porcine Plasma Protein Subject to Free Hydroxyl Radical System

[1]Hehong Yang, [1]Yanqing Li, [1]Peijun Li, [1]Qian Liu, [1]Baohua Kong, [2]Xu Huang and [3]Zengbao Wu
[1]Department of Food Science, Northeast Agricultural University, Harbin, Heilongjiang, 150030, China
[2]Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan, 430060, China
[3]Department of Neurosurgery, Renji Hospital of Shanghai Jiao Tong University,
Shanghai, 200127, China

Abstract: Antioxidant peptides have attracted much attention for potential application as natural food ingredients but the fate of them, as well as oxidized proteins in foods during processing, is still poorly understood. Physicochemical changes in antioxidant peptides hydrolysated from porcine plasma protein were discussed in a free hydroxyl radical-mediated oxidation system. Porcine Plasma Protein Hydrolysates (PPH) was prepared by hydrolyzing porcine plasma protein with Alcalase for 5 h at pH 8.0, 55°C. The content of carbonyl groups increased significantly at various degrees when PPH exposed to free radical-mediated oxidation for different time and different concentrations of H_2O_2, while total sulfhydryls, reactive sulfhydryls and free amines contents decreased. It was concluded that PPH played an antioxidant role in the radical-mediated oxidation system. This provides a potential way for antioxidation in food production.

Keywords: Aantioxidant peptide, free radical-mediated oxidation, physicochemical changes, porcine plasma protein

INTRODUCTION

It is known that proteins are essential nutrients for humans and also play an important functional role in food processing. Protein can be hydrolyzed with some kinds of enzymes. When partially hydrolyzed with appropriate proteases, many of the protein fragments known as peptides have antioxidant activity, which has the ability to inhibit lipid and protein oxidation thereby protecting food products' flavor and textural characteristics during storage. Natural antioxidant peptides can be produced from a variety of protein sources, including porcine plasma protein (Liu *et al*., 2010), whey protein (Peña-Ramos *et al*., 2004), egg protein (Davalos *et al*., 2004), potato protein (Wang and Xiong, 2005), maize zein (Zhu *et al*., 2008) and buckwheat protein (Ma and Xiong, 2009). An exemplary food system where antioxidants are widely used to preserve the products' shelf-life is muscle foods.

Despite the demonstrated antioxidant potential of protein hydrolysates, the fate of the antioxidants, as well as oxidized proteins in food systems during processing and subsequent storage, is poorly understood. For example, it is not clear that how antioxidant peptides would survive during food processing under various heating, pH, and ionic conditions, or how these processing conditions would affect the physicochemical properties of antioxidant peptides. So, free radical-mediated oxidation of the peptides under different conditions should be discussed.

Porcine blood is an important by product in meat industry and considered to be a potential source of nutritional and functional protein source. However, the utilization of porcine plasma protein has not been developed widely till now. It was reported that the porcine plasma protein hydrolysates were of antioxidant activity and improved functional properties (Benkakul *et al*., 2001).

What we hypothesize is that when exposed to small-sized radials, for example, hydroxyl radicals, antioxidant peptides will undergo major structural changes and form aggregates among themselves and finally, alter the physicochemical characteristics. The objective of this study was to investigate the physicochemical changes of antioxidant peptides hydrolyzed from porcine plasma protein as a result of exposure to free radical-mediated oxidation.

MATERIALS AND METHODS

5′-Dithiobis-2-Nitrobenzoic acid (DTNB), 2, 4-Dinitrophenylhydrazine (DNPH), 6-hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid (Trolox), Butylated Hydroxyanisole (BHA), Ethylene Diamine Tetraacetic Acid (EDTA), 1-Anilino-8-naphthalenesulfonate Mag

Nesium Salt (ANS), N-dimethyl-2-mercaptoethy lammonium chloride, L-leucine, Ortho-Phthaldi Aldehyde (OPA) and sodium ascorbate were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Sodium Dodecyl Sulfate (SDS) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals and reagents were of analytical grade.

Methods: Preparation of porcine Plasma Protein Hydrolysates (PPH). PPH was prepared with the method of Liu *et al*. (2009), with minor adjustments. Porcine plasma protein solution (40 mg protein/mL) was heat pretreated (55ºC, 5 min) and then hydrolysed with Alcalase at 55ºC. The enzyme to substrate ratio (E/S) was 2:100 (g/g). The pH of the protein solution was adjusted to 8.0, which was the optimal value for Alcalase. During 5h-hydrolysis, the pH was readjusted to the optimal value every 15 min with 1 M NaOH solution. After hydrolysis, the pH of the solution was brought to 7.0, and then the solution was heated to 95ºC and holding for 5 min to inactivate the protease.

PPH oxidation: PPH was oxidized in a free radical-mediated oxidation system, as described by Kong *et al*. (2011). Stock solution of the components of the Hydroxyl Radical-Generating Systems（HRGS）was prepared before using. The components of the HRGS were mixed in the order of 50 mM phosphate buffer (pH 6.0), $FeCl_3$, H_2O_2, and then ascorbic acid. Immediately the ascorbic acid was mixed with the other components of the HRGS, the PPH was added to the solution and mixed thoroughly, while the control sample contained buffer without the components of the HRGS. The PPH suspension (20 mg protein/mL) was oxidized at 20±1°C for 1, 3, or 5 h in the HRGS which consisted of 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, and five different levels of H_2O_2 (0.1, 0.5, 1, 2.5, 5, 10 mM). The PPH Oxidation was terminated by adding Trolox (1 mM each).

Carbonyls contents: Carbonyl groups in PPH samples were measured by reacting with DNPH to form protein hydrazones, using the method of Oliver *et al*., (1987), with slight modifications. Carbonyl groups were determined by reaction of 1 mL PPH samples (10 mg/mL protein) with 1 mL of 10 mM DNPH in 2 M HCl. Control was the same of the 1 mL PPH mixed with 1 mL of 2 M HCl only. After reaction under agitation at room temperature for 1 h, 2 mL of the reacted PPH solutions were precipitated with 1 mL of 20% trichloroacetic acid (w/v). The samples were centrifuged at 10,000×g for 5 min. The precipitates were washed three times with 1 mL of ethanol: ethyl acetate (1:1, v/v) to eliminate free DNPH. PPHs were then dissolved in 3 mL of 6 M guanidine HCl with a 20 mM potassium phosphate buffer at pH 2.3. In order to dissolve the proteins completely, the samples were bathed at 37°C for 15 min. Any insoluble material was removed by centrifugation for 3 min at 10,000×g. Protein concentration in the supernatant was measured using the biuret method (Gornall *et al*., 1949). Carbonyl concentration was determined from the absorbance at 370 nm and was expressed as nmol per mg of protein.

Total and reactive sulfhydryls: Total Sulfhydryl (SH) content was measured using the Ellman's method with DTNB as described by Simplicio *et al*. (1991). 1 mL PPH samples (20 mg/mL protein) was treated with 8 mL Tris-glycine buffer (10.4 g of Tris-HCl, 6.9 g of glycine, 480 g of urea, and 1.2 g of EDTA/L at pH 8.0) and 1 mL Ellman's reagent (4 mg DTNB/mL) in the dark at room temperature for 30 min. Reference blank contained 9 mL of Tris-glycine buffer and 1 mL Ellman's reagent without protein. Then the absorption at 412 nm of it was measured on a TU-1800 spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., Beijing, China). The concentration of SHs was calculated using a molar extinction coefficient of 13,600/M/cm. The results were expressed as nmol of total thiol per mg of protein.

Reactive sulfhydryl content was determined with the method of Xia *et al*. (2009), with little modifications. Samples were solubilized in Tris-glycine buffer (0.086 M Tris, 0.09 M glycine, 4 mM EDTA, 8 M Urea, pH 8) and then centrifuged at l0,000×g for 15 min to remove insoluble protein. Reactive SH content of the supernatant was determined. 0.5 mL of 10 mM DTNB was added to 4.5 mL of the supernatant. The absorbance at 412 and 540 nm was measured using a TU-1800 Spectrophotometer and the reactive sulfhydryl value was calculated to the following equation:

Reactive sulfhydryl contente (μmol SH/g PPH)
$$= 73.53 \times (A_{412} - 1.6934 \times A_{532} + 0.009932) \qquad (1)$$

A_{41} = The absorbance 412 nm
A_{532} = The absorbance 532 nm

Determination of free amines: The quantity of free amines was determined by the modified Ortho-Phthaldi Aldehyde (OPA) method (Chevalier *et al*., 2001). The OPA reagent was prepared by mixing with 40 mg OPA (dissolved in 1 mL of methanol), 50 mL of 0.1 M sodium borate buffer (pH 9.3), 100 mg N-Dimethyl-2-Mercaptoethylammonium Chloride (DMMAC) and 1.25 mL of 20% (w/w) SDS in water. Two hundred μL PPH solution (10 mg/mL protein) was added to 4 mL of OPA reagent. The absorbance was read at 340 nm after a minimal delay of 2 min at 35ºC. Nonoxidized PPH contented 100% free amines. A calibration curve was obtained by using 0.25-2 mM L-leucine as a standard.

Statistical analysis: All specific experiments were repeated at three times. Statistical analysis was performed using Statistix 8.1. Analysis of Variance (AOV) was done to determine the significance of the difference. Significant differences ($p<0.05$) between means were identified using Least Significant Difference procedures.
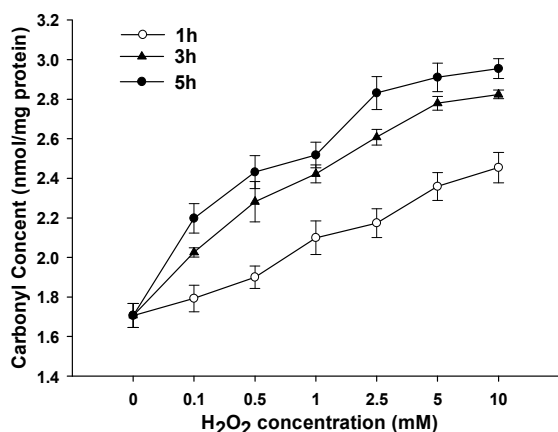
Fig. 1: Changes in carbonyl content of PPH at 20°C for different concentrations of H_2O_2 at 1 h (○), 3 h (▲) and 5 h (●)
The "0" sample was the one without any oxidants ($FeCl_3$, H_2O_2, or ascorbic acid); Results are the means from three replicates
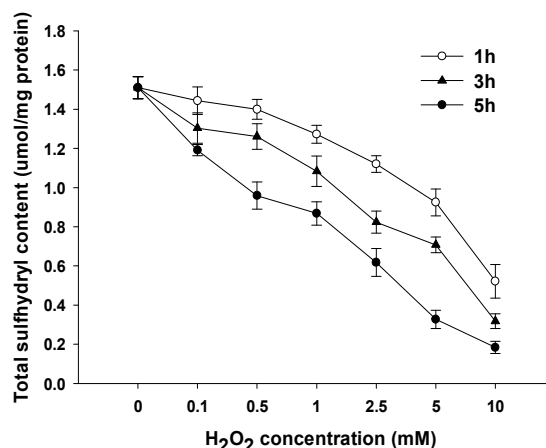

Fig. 2: Changes in total Sulfhydryl (SH) content of PPH at 20°C for different concentrations of H_2O_2 at 1 h (○), 3 h (▲) and 5 h (●)
The "0" sample was the one without any oxidants ($FeCl_3$, H_2O_2, or ascorbic acid); Results are the means from three replicates

RESULTS AND DISCUSSION

Carbonyls contents: Amino acids with NH or NH_2 moiety on their side chains can react with free oxygenated radical from lipolysis, then transforming into carbonyl group (Sun, 2010), which was an indicator for protein oxidation. The content of protein carbonyls in PPH samples generally increased with increasing oxidant concentrations and reaction time (Fig. 1). Non-oxidized PPH (0 h) contained 1.71 nmol carbonyls/mg proteins. The amount of carbonyl groups significantly increased ($p<0.05$) from 1.71 to 2.45, 2.82, and 2.95 nmoL/mg protein when incubation at 1, 3 and 5 h, respectively. The carbonyl content of protein samples over-h incubation period increased from 1.71 to 2.95 nmoL/mg protein when the H_2O_2 concentration was increased from 0.1 to 10 mM at 0.1 mM $FeCl_3$. Compared with PPH of 1 h, the carbonyl contents of the samples incubation for 3 and 5 h were much greater. The results indicated that the formation of carbonyls in PPH was sensitive to H_2O_2 concentration changes.

Carbonyls could result from oxidative peptide scission, which are transformed to carbonyl groups during protein oxidation, therefore increasing protein carbonyl content (Stadtman, 1993). It is known that amino acid side chain groups are sensitive when hydroxyl radical attacked, particularly for those with NH-or NH_2 (Sante-Lhoutellier *et al.*, 2007). So when the porcine plasma protein was hydrolysed to PPH, carbonyls were readily to be formed since free NH_2-increased.

Total sulfhydryls and reactive sulfhydryls: The susceptibility of PPH to oxidation during hydroxyl radical-generating processing was also monitored by
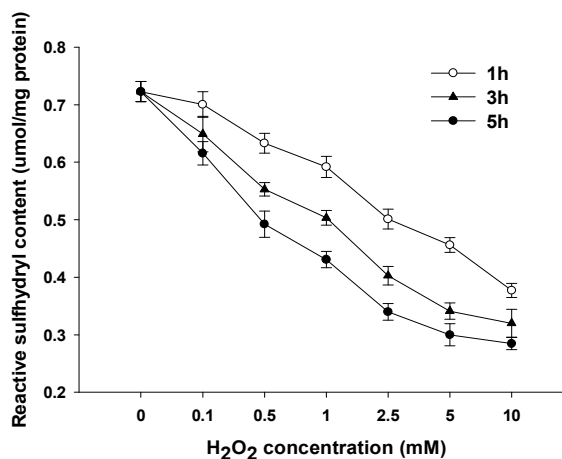

Fig. 3: Changes in reactive Sulfhydryl (SH) content of PPH at 20°C for different concentrations of H_2O_2 at 1 h (○), 3 h (▲) and 5 h (●).
The "0" sample was the one without any oxidants ($FeCl_3$, H_2O_2, or ascorbic acid); Results are the means from three replicates

the estimation of total sulfhydryl and reactive sulfhydryls levels. Results are shown in Fig. 2 and 3. The total sulfhydryl and reactive sulfhydry levels in PPH at 0 h were 1.51 and 0.72 nmoL/mg, respectively. There were significant changes in the total sulfhydryl and reactive sulfhydry levels during the process. As processing time proceeded, both of the total sulfhydryl and reactive sulfhydry levels decreased gradually. The levels of 1, 3 and 5 h reaction system were 34.5, 29.9, and 12.18%, respectively, for total sulfhydryl and 52.22, 44.32, and 39.34%, respectivly for reactive sulfhydry with 10 mM H_2O_2 at 0.1 mM $FeCl_3$.
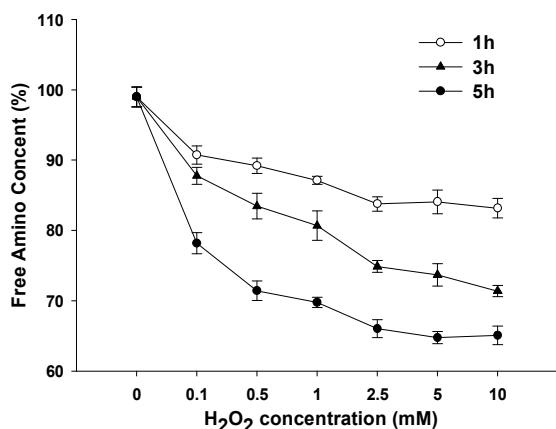
Fig. 4: Changes in free amines content of PPH at 20°C for different concentrations of H_2O_2 at 1 h (○), 3 h (▲), and 5 h (●)

The "0" sample was the one without any oxidants ($FeCl_3$, H_2O_2, or ascorbic acid); Results are the means from three replicates

It is known that disulphide bonds can stabilize the folded conformation of proteins and decrease the conformational entropy, thus, improving their thermodynamic stability (Sun, 2010). The conversion of sulfhydryl groups into disulfides is one of the earliest observable events in the radical-mediated oxidation of proteins (Dean *et al.*, 1997). The loss of the SH groups may be used for the formation of disulfide bonds either within polypeptides or between polypeptides. Also, the decrease in total sulfhydryl and reactive sulfhydry levels may also be due to degradation reactions (Kong *et al.*, 2011).

Free amines contents the content of free amines was often used as the indicator of the degree of protein oxidation. It was measured in the present study with growing oxidant concentrations and reaction time (Fig. 4). The content declined dramatically as H_2O_2 concentrations increasing and oxidant time prolonged, especially when the concentration of H_2O_2 rose to 10 mM and oxidant time reacted 5 h. In this study, PPH sample (10 mM, 5 h) showed a 30.03% decrease in amines compared to the control (non-oxidised PPH). To be more specific, when PPH was oxidized in the presence of 10 mM H_2O_2 for 1, 3 and 5 h at 22°C, the free amines content went down to 16.83, 28.61, 34.92%, respectively.

The significant decline of free amines indicated that the extent of PPH oxidation increased with growing oxidant concentrations at various degrees. Lysine residue is known as one of the most sensitive targets for hydroxyl radical attack. This contributes to the increase of carbonyls content, for the amino groups (NH-or NH2) can be broken down through deamination during the radical-mediated oxidation and converted to carbonyls in the PPH samples (Liu and Xiong, 2000). It was in good accordance with the results from carbonyls

content. Besides, amino groups could react with non-protein and protein carbonyls, which also leads to the loss of free amines content (Morzel *et al.*, 2006). Therefore, the overall loss of the free amines content was considered to be the deamination during radical-mediated oxidation, as well as its reaction with other components.

CONCLUSION

PPH was prepared by hydrolyzing porcine plasma protein with Alcalase for 5 h at pH 8.0, 55°C. The amount of carbonyl groups increased significantly at various degrees when PPH exposed to free radical-mediated oxidation for different time and different concentrations of H_2O_2, while total sulfhydryls, reactive sulfhydryls and free amines contents decreased. It was concluded that PPH played an anti-oxidation role in the radical-mediated oxidation system.

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