Susceptibility of whey protein isolate to oxidation and changes in physicochemical, structural, and digestibility characteristics

Xianchao Feng,* Chenyi Li,* Niamat Ullah,* Jiqianrui Cao,* Yongli Lan,* Wupeng Ge,* Robert M. Hackman,† Zhixi Li,* and Lin Chen*¹

*College of Food Science and Engineering, Northwest A&F University, Yangling, Shaanxi, China 712100

†Department of Nutrition, University of California, Davis 95616

ABSTRACT

Oxidation is an important factor for denaturing of whey protein isolate (WPI) during food processing. We studied the effects of chemical oxidation on physicochemical and structural changes along with in vitro digestibility of WPI in this work. Evaluation of physicochemical changes showed that carbonyl level and dityrosine content increased, whereas total and free thiol group levels decreased for oxidized WPI samples. For the structural changes, protein aggregation was measured by surface hydrophobicity, turbidity, and particle diameter, which was increased for oxidized WPI samples. The increase of the secondary structure β -sheets and antiparallel β -sheet also supported the aggregation of oxidized WPI. A direct quantitative relationship between physicochemical and structural changes and protein digestibility indicated that oxidation-related damage restricts the susceptibility of WPI to proteases. In conclusion, WPI had high susceptibility to oxidative stress, and both physicochemical and structural changes caused by severe oxidative stress could decrease the rate of in vitro digestibility of WPI.

Key words: aggregation, in vitro digestion, physicochemical changes, whey protein isolates, structural changes

INTRODUCTION

Whey protein is an important component of milk, as it represents 20% of total protein and is much higher in human milk than in bovine milk (Hoffman and Falvo, 2004). During cheese production, whey is the main protein left over after coagulation of milk. Because of its high amounts of EAA and branched-chain AA, whey protein is an important functional food additive (Kimball and Jefferson, 2006). Beneficial functions of branched-chain AA are well documented (e.g., it

helps in prevention of cardiovascular disease, cancer, diabetes, weight gain, and so on), and they can also help in decreasing plasma insulin and improving lipid profile (Krissansen, 2007). Whey protein is commonly marketed as a dietary supplement and used to improve nutritional value and functional properties of formulated foods (e.g., dairy foods, bakery products, sports drinks; de Wit, 1998; Rawdkuen and Benjakul, 2008).

Reactive oxygen species and reactive nitrogen species, which are produced during food treatment (i.e., cooking, drying, storing, and so on), attack food protein very easily. Oxidation and denaturation of protein not only alter physicochemical, but also alter structural properties of protein during food treatments. Carbonyls, disulfide, and dityrosine bridges generated by oxidation of AA residues cause the polymerization and aggregation of proteins and change its secondary and tertiary structure (Sun et al., 2011; Cui et al., 2012). Structure is the base characteristic of protein, and characteristics such as hydrophobicity will be changed following the changes in structure and numerous biological processes will be affected. The effect of protein oxidation on neurological disorders has been studied and a strong association with neurodegenerative disorders, prion diseases, and with many forms of cancer has been noted (Blennow et al., 2006). In the food industry, protein oxidation happens in almost every food process, which reduces the digestibility of the protein by digestive tract enzymes (Bax et al., 2013).

When whey protein isolate (WPI) is used as a functional additive, foods are exposed to radical-mediated oxidative stress during processing and protein oxidation occurs; however, the association between oxidized WPI and digestibility is poorly documented. The relationship between digestion and physicochemical and structural changes caused by chemical oxidation of WPI has not been fully elucidated yet; that is, whether chemical oxidation has negative or positive effects has yet to be determined. The current study was designed to evaluate the physicochemical and structural changes of oxidized WPI, along with its nutritional quality, with the hypothesis that certain processes (drying, cooking,

Received May 13, 2015.

Accepted July 3, 2015.

¹Corresponding author: melody-sara@hotmail.com

and storing) could have a negative effect on the nutritional quality of WPI.

MATERIALS AND METHODS

WPI Oxidation

Whey protein isolate (Davisco Foods International Inc., Eden Prairie, MN), at 20 mg/mL (wt/vol) final concentration, was incubated for 3, 6, and 9 h at room temperature (25°C) in a free radical-generating system (20 mM PBS at pH 6.0, 0.1 mM FeCl₃, 5 mM H₂O₂, and 0.1 mM ascorbic acid). The oxidized WPI samples were recorded as 3-h hydrogen peroxide (**HP**), 6-h HP, and 9-h HP samples, separately. At each time point, to prevent subsequent oxidation, Trolox (MP Biomedicals LLC, Solon, OH), butylated hydroxyanisole (BHA; Aladdin Industrial Corporation, Fengxian, Shanghai, China), and EDTA (1 mM final concentration of each) was added. Control samples were dissolved with the same PBS buffer without the oxidizing agents. All the samples were lyophilized before further analysis.

Carbonyls Analysis

Modified method of Oliver et al. (1987) was used for carbonyls analysis. 2,4-Dinitrophenylhydrazine (**DNPH**) was used to detect carbonyl groups of WPI in the form of protein hydrazones. Two aliquots of 150 µL of WPI solution (20 mg/mL) were centrifuged at 10,000 × *g* at 4°C for 5 min. One precipitation was treated with 1 mL of 0.2% (wt/vol) DNPH in 2 *N* HCl, whereas the other was treated with an equal volume of 2 *N* HCl without DNPH. All the samples were incubated at room temperature under agitation for 1 h; then, the solutions were precipitated with TCA (20% TCA, wt/vol, final concentration). The precipitate was washed 3 times with ethyl acetate (1:1, vol/vol) to eliminate free DNPH. The WPI was dissolved by 1 mL of 6 *M* guanidine HCl at pH 6.5. The protein concentration of WPI was calculated in 2 *N* HCl control. The carbonyl concentration was measured in the treated sample by measuring the absorption of protein hydrazones at 370 nm with the molar extinction coefficient of 21,000 M⁻¹ cm⁻¹. The results were expressed as nanomoles of DNPH fixed per milligram of protein.

Dityrosine

The WPI samples were dissolved in 20 mM PBS (pH 6.0) to get a final concentration of 1 mg/mL. The contents of dityrosine in samples were measured using F-4600 FL spectrophotometer (Hitachi, Tokyo, Japan) with excitation at 325 nm and emission at 395 nm (ex-

citation slit = 5 nm; emission slit = 5 nm; Davies et al., 1987; Cui et al., 2012). The dityrosine contents were expressed as absorbance units per milligram of protein.

Total and Free Thiol Group Content

Thiol oxidation was measured according to a modified method of Ellman (1959) using 5,5'-dithiobis (2-nitrobenzoic acid) (Martinaud et al., 1997). The WPI was dissolved with 20 mM PBS buffer at pH 6.0 to get 10 mg/mL solution. One hundred microliters was diluted with 1 mL of 8 *M* urea in 100 mM PBS at pH 8.0 for total thiol group (**TT**); the other 100 µL was diluted with 1 mL of 100 mM PBS at pH 6.0 without urea for free thiol group (**FT**). Then, 0.5 mL of 4 mg/mL 5,5'-dithiobis (2-nitrobenzoic acid) was added and incubated in the dark at 25°C for 30 min. The absorbance at 386 nm was measured against a blank of 100 µL (20 mM PBS) using an absorption coefficient of 13,600 M⁻¹ cm⁻¹. The results are expressed as nanomoles of thiol per milligram of protein. Thiol group level embedded inside the protein (**TE**) was calculated as TE = TT content – FT group content.

WPI Surface Hydrophobicity

Surface hydrophobicity of WPI was determined using hydrophobic chromophore bromophenol blue (**BPB**) by following Cui et al. (2012) with some modifications. This method is based on fixing BPB with insoluble myofibrillar proteins and quantifying the amount of bound BPB. The BPB solution (50 µL, 1 mg/mL) was added to WPI solution (2 mg/mL) in 20 mM PBS at pH 6 and vortexed well. Then, the WPI solution was shaken at room temperature for 10 min in dark and centrifuged at 4,000 × *g* for 15 min at 4°C. The free BPB in the supernatant was measured at 595 nm. Control was prepared without WPI. The percent of bound BPB (µg) by WPI was calculated as an index of hydrophobicity; each determination was performed in duplicate:

$$\text{bound BPB } (\mu\text{g}) = 50 \mu\text{g} \times (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}},$$

where OD = optical density.

Turbidity

The formazin suspension prepared by mixing 7.5 mL of the hydrazine sulfate solution (10 mg/mL) and 7.5 mL of hexamethylenetetramine solution (100 mg/mL) with final volume of 100 mL, which was defined as a 600 formazin turbidity units (**FTU**) primary reference

standard. The turbidity of WPI solutions (10 mg/mL) were measured at room temperature by determination of absorbance at 600 nm with standards from 0 to 600 FTU. The turbidity of protein solution was expressed as FTU per milligram of protein.

Particle Diameter of WPI

The average particle diameter of WPI aggregates was measured by dynamic light scattering using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Samples were diluted with 20 mM PBS (pH 6.4) buffer to a final concentration of 1 mg/mL and measured. All measurements were performed in triplicate. The aggregates in the sample were expressed as the intensity measured by Nano ZS.

Fourier Transform Infrared Spectroscopy

The WPI samples were mixed well with potassium bromide powder before pressing into a tablet. Fourier transform infrared spectrum was investigated using a Bruker Vertex 70 (Bruker, Ettlingen, Germany) in the transmittance mode with a resolution of 4 cm^{-1} in the range of 400 to 4,000 cm^{-1} during 64 scans. Fourier self-deconvolution of the overlapping amide I band components (1,585–1,710 cm^{-1}) was made possible by using OMNIC Peak Resolve software (Thermo Fisher Scientific, Waltham, MA). The curve-fitting procedure was performed by the Gauss formula (Sakudo et al., 2009). Protein secondary structures (α -helix, β -sheet, β -turn, and random coil) were diagnosed accordingly to the previous reported studies (Sakudo et al., 2009; Carbonaro and Nucara, 2010). The area corresponding to each secondary structure was calculated and expressed as a percentage of the sum of areas.

In Vitro Digestibility

In vitro pepsin digestion of WPI was analyzed using the method of Bax et al. (2012) with little modification. The WPI samples (18.75 mg) were suspended in 25 mL of 33 mM glycine buffer (pH 1.82). Gastric pepsin was used to hydrolyze protein. The concentration of the gastric pepsin was made 40 U/mg and were applied at 37°C for 90 min. Trichloroacetic acid 45% was used to terminate the digestion with different time intervals (i.e., 0, 5, 10, 15, 20, 25, 30, 40, 60, and 90 min). After cessation of digestion, temperature of the samples was regulated by keep it on ice for 1 h. Hydrolyzed peptides were collected in supernatant after centrifugation at $4,000 \times g$ for 15 min at 4°C and was measured at 280 nm with 96-well plates using Epoch (Biotek, Winooski, VT). Analyses were repeated twice for reproducibility.

The same procedure was used for in vitro digestibility of WPI by trypsin and α -chymotrypsin after digested by pepsin for 1 h (Bax et al., 2012).

The digestion kinetics curves were described using the previous mathematical model (Gatellier and Santé-Lhoutellier, 2009). Primary parameters for the curve were estimated, such as initial optical density (initial OD), maximal digestion (ODmax), half-life time (is the time when the $\text{OD} = 1/2 \text{ ODmax}$), initial slope ($\Delta\text{OD}/\text{h}$), and maximal rate of digestion (Gatellier and Santé-Lhoutellier, 2009).

Statistical Analysis

We used SPSS (ver. 16.0, SPSS Inc., Chicago, IL) for all analyses. One-way ANOVA and the Student-Newman-Keuls (SNK) test were applied to determine the significance of the main effects ($P < 0.05$). All the data were expressed as mean $\pm$ standard deviation of duplicate determinations.

RESULTS AND DISCUSSION

Physicochemical Changes of Oxidized WPI

During processing, AA residues are subjected to attack by oxygen radicals and transformed into carbonyls groups, particularly those with NH or NH_2 , such as Asn, Gln, Arg, Lys, His, and Trp (Santé-Lhoutellier et al., 2007). Results of the current study showed that the content of protein carbonyls of oxidized WPI were higher than nonoxidized WPI, and carbonyl content of oxidized WPI samples increased in a time-dependent manner ($P < 0.05$; Table 1; Cui et al., 2012). These results were in agreement with previous observations on skeletal muscle proteins (Morzel et al., 2006; Bax et al., 2012). At each time point, carbonyl contents of oxidized WPI samples were significantly higher. The effect of oxidizing time on carbonyl content measured in the present study was fairly consistent with the effect of heating time (Santé-Lhoutellier et al., 2008). Actually, the formation of carbonyls can be caused by protein oxidation even without an oxidizing agent during storage (Bax et al., 2012). Thiol group level is another important parameter to estimate the denaturation of protein on the secondary structure, such as β -sheet structure and α -helices. Consequently, the thiol group level was usually used to monitor the susceptibility of protein to oxidation (Wu et al., 2006). The oxidized WPI had lower FT, TT, and TE compared with nonoxidized WPI ($P < 0.05$; Table 1). The decrease of FT and TT indicated the accessible cysteine residues located at both the protein surface and embedded inside the protein were attacked by free radicals.

Table 1. Effect of oxidation on the carbonyl content, dityrosine content, and thiol group levels of whey protein isolates (WPI) samples

WPI ¹	Carbonyls (nM/mg of protein)	Dityrosine (absorbance units/mg of protein)	Thiol groups ² (nmol/mg of protein)		
			FT	TT	TE
3-h control	1.74 ± 0.41 ^x	644.5 ± 30.9 ^x	21.13 ± 1.06 ^x	39.28 ± 1.81 ^x	19.68 ± 1.63 ^x
3-h HP	3.32 ± 0.30 ^{*a}	1,287.5 ± 81.3 ^{*a}	10.62 ± 0.92 ^{*a}	22.31 ± 1.98 ^{*a}	11.68 ± 1.06 ^{*a}
6-h control	2.14 ± 0.26 ^x	728.6 ± 31.0 ^x	23.98 ± 1.88 ^x	37.82 ± 2.69 ^x	13.85 ± 0.81 ^y
6-h HP	4.13 ± 0.27 ^{*b}	1,585.5 ± 91.2 ^{*b}	7.76 ± 0.85 ^{*b}	14.65 ± 1.77 ^{*b}	6.89 ± 0.92 ^{*d}
9-h control	2.50 ± 0.28 ^x	777.7 ± 11.8 ^x	25.78 ± 1.68 ^x	34.75 ± 2.61 ^x	8.97 ± 0.93 ^z
9-h HP	5.03 ± 0.41 ^{*c}	1,773.0 ± 52.3 ^{*c}	4.91 ± 0.63 ^{*c}	8.47 ± 1.36 ^{*c}	3.56 ± 0.73 ^{*c}

^{a-d}Different superscripts for WPI samples means significant difference ($P < 0.05$).

^{x-z}Different superscripts for nonoxidized WPI (control) means significant difference ($P < 0.05$).

¹HP = hydrogen peroxide.

²FT = free thiol; TT = total thiol; TE = thiol embedded in protein.

*Significant ($P < 0.05$) compared with nonoxidized WPI.

The decrease of thiol group supported the formation of disulfide bonds, which is very important for protein structure (Cui et al., 2012). The dityrosine could also be formed during protein oxidation. The dityrosine formation in oxidized WPI significantly increased with time, which had the similar changes as the carbonyl for

both oxidized and nonoxidized WPI samples (Table 1). Dityrosine was produced in all oxidized WPI samples (Cui et al., 2012). A previous study reported amounts of dityrosine detected when the protein samples were exposed to H₂O₂ to be sufficient (Morzel et al., 2006), which may be explained by the fact that the tyrosyl

Figure 1. Effect of oxidation on hydrophobicity (bound bromophenol blue, BPB/μg; A), turbidity (Formazin turbidity units, FTU; B), particle size distributions (%; C), and Z-average diameter (nm; D) of whey protein isolate (WPI) samples. An asterisk (*) means significant ($P < 0.05$) compared with nonoxidized WPI. Different lowercase letters (x,y) for nonoxidized WPI (control) means significant difference ($P < 0.05$); different lowercase letters (a-c) for oxidized WPI [hydrogen peroxide (HP)] means significant difference ($P < 0.05$). The analysis was repeated 3 times; errors bars indicate standard deviation. Color version available online.

radical could be formed and enhance the formation of dityrosine under H_2O_2 activation (Østdal et al., 2000).

Structural Changes of Oxidized WPI

Aggregates of proteins may arise by a covalent or noncovalent manner. Covalent aggregates arise when chemical bond is formed between 2 or more monomers. Formation of disulfide bonds resulting from previously unpaired free thiols is a common mechanism for covalent aggregation (Andya et al., 2003). Oxidation of Tyr also results in covalent aggregation by the formation of dityrosine (Giulivi and Davies, 1994). Compared with covalent aggregates, noncovalent protein interaction also contributes to protein aggregation (Cromwell et al., 2006). Protein turbidity, unlike dityrosine and disulfide bonds, could indicate the noncovalent protein-protein associations (Cromwell et al., 2006). In current study, the turbidity of WPI solutions increased drastically by 2.25-, 2.51-, and 3.27-fold when oxidized for 3, 6, and 9 h compared with nonoxidized WPI (Figure 1B). This proved that noncovalent protein-protein associations were also enhanced by oxidation. Changes in surface hydrophobicity reflect changes in chemical and physical characteristics of the protein structure (Sun et al., 2011). Consequently, protein hydrophobicity has been widely applied to assess the protein oxidation and denaturation (Sante-Lhoutellier et al., 2007). In current study, oxidized WPI had higher hydrophobicity than nonoxidized WPI ($P < 0.05$; Figure 1A). The mechanism of such changes could be explained by exposure of hydrophobic AA buried inside native protein during oxidation, which enhanced the surface hydrophobicity of protein (Grune et al., 2004). However, the surface hydrophobicity of oxidized WPI decreased with oxidizing time ($P < 0.05$; Figure 1A), whereas it increased for nonoxidized WPI (Figure 1A). This may have been caused by protein aggregation partially shielding the effect of unfolding in strongly oxidized WPI (Li et al., 2012). Protein oxidation under heating conditions breaks the hydrogen bonds and van der Waals forces and exposes the interior hydrophobic AA (Chelh et al., 2006). Likewise, smaller molecules from WPI hydrolysis caused by incubation at 25°C (3, 6, or 9 h) maybe the reason for the increased surface hydrophobicity of non-oxidized WPI (Yongsawatdigul and Park, 1999).

Dynamic light scattering has been reported as a parameter for complementary information of protein aggregates, which could detect even small aggregates of protein that cannot be detected by electrophoresis (Zhang and Vardhanabhuti, 2014). The particle size and Z-average diameter of oxidized WPI samples were higher and increased with oxidizing time ($P < 0.05$; Figure 1 C and D), which proved that the aggregates

Figure 2. Deconvolution and curve-fitted amide I region [A = composite of whey protein isolate; B = 3-h control; C = 3-h hydrogen peroxide (HP)]. The amide I region of Fourier transform infrared spectra (composite) was separated into 7 peaks (B and C; the sequence starting with 7, then 6, 5, 4, 3, 2, 1 in turn from 1,700 to 1,600 cm^{-1}). The ratio of area to composite represents the corresponding percentage of each structure; peaks 1 (aromatic ring vibration of tyrosine residues), 2 to 3 and 7 (β -sheet), 4 (random coil), 5 (α -helix), and 6 (β -turn) are indicated accordingly. Color version available online.

of oxidized WPI were enhanced. However, WPI aggregation of nonoxidized WPI samples increased slightly with time. This indicated that thermal denaturation at low temperature (25°C) mainly caused the increase of surface hydrophobicity of protein more than protein aggregates (Bax et al., 2012). The changes of particle size and Z-average diameter also supported the loss of thiol groups, the formation of disulfide bonds, dityrosine, and improvement of surface hydrophobicity protein, whereas all these changes can promote aggregation of WPI in radical-mediated oxidation. The aggregation measured by particle size and Z-average diameter also supported the increase of turbidity in oxidized WPI samples (Sun et al., 2011; Cui et al., 2012).

The amide I region is mainly associated with the C = O stretching vibration (70–85%) and C-N groups (10–20%; Carbonaro and Nucara, 2010). The range in area between 1,600 and 1,700 cm^{-1} could reflect the degree of oxidation. The larger area of the amide I region in oxidized WPI samples supported its higher carbonyl content compared with the nonoxidized samples (Figure 2A). The peaks 2, 3, and 7 are caused by the intermolecular β -sheets, which increased with time and was higher in oxidized WPI samples compared with that of nonoxidized WPI ($P < 0.05$; Table 2). The β -sheets are the indicator of protein aggregation (Sun et al., 2011); these results also supported the changes of particles size from dynamic light scattering. Moreover, peak 3 was caused by parallel β -sheets, whereas peaks 2 and 7 were generally considered antiparallel β -sheets (Pelton and McLean, 2000). The increased ratio of antiparallel β -sheets (peaks 2 and 7) and decrease of α -helix (peak 5) in oxidized WPI samples indicated that the protein rearranged the second structure during oxidation ($P < 0.05$; Table 2; Sakudo et al., 2009). Studies had reported the association of peak 1 with aromatic ring vibration of tyrosine residues. As a result, the decrease of ratio of peak 1 in oxidized WPI may be caused by formation

of dityrosine during oxidation process ($P < 0.05$; Table 2). The increased ratio of random coil and decreased α -helix may have occurred because of unfolded proteins (random coil) folded into β -sheet during rebuilding of secondary structure (Sun et al., 2011).

A correlation study was conducted to establish links between all parameters (Table 3). Results show that FT and TT embedded inside the protein were negatively correlated with carbonyl levels ($P < 0.05$), whereas dityrosine were positively correlated with carbonyl levels ($P < 0.05$). Carbonyls content is considered as an important indicator for the degree of oxidation. The decrease of thiol group was caused by disulfide bonds formation in oxidized WPI samples (Sun et al., 2011). Dityrosine formed from Tyr residues cross-linking had a similar tendency with disulfide bonds (Cui et al., 2012). Turbidity and particle diameter of WPI aggregation were positively correlated with carbonyls. However, no significantly positive correlations between turbidity and particle diameter with the surface hydrophobicity were observed. This could be explained by the fact that carbonyl groups generated by the formation of amide bonds with residual amino groups improved WPI polymerization, but protein aggregation partially shielded the effect of unfolding in strongly oxidized WPI (Sante-Lhoutellier et al., 2007). The significant correlations between carbonyls, TT, dityrosine, turbidity, and particle diameter with the second structure of WPI (aromatic ring vibration of tyrosine residues, β -sheets, and α -helix) indicated that rearrangement of second structure caused by oxidation could explain the physiochemical and structural changes of oxidized WPI samples (Sun et al., 2011; Cui et al., 2012).

In Vitro Digestibility of Oxidized WPI

Protein nutritional quality was assessed by the *in vitro* digestibility method. Gastric pepsin was used, which

Table 2. Secondary structural ratio of proteins (%) in different whey protein isolates (WPI) samples

	Aromatic ring vibration	β -Sheet	Random coil	α -Helix	β -Turn	β -Antiparallel
WPI ¹	Peak 1	Peak 2, 3, and 7	Peak 4	Peak 5	Peak 6	Peak 2 and 7
3-h control	2.82 $\pm$ 0.12 ^x	33.99 $\pm$ 0.49 ^x	18.62 $\pm$ 0.28 ^x	28.17 $\pm$ 0.42 ^x	16.39 $\pm$ 0.23 ^x	19.24 $\pm$ 0.57 ^x
3-h HP	2.12 $\pm$ 0.13 ^{*a}	36.16 $\pm$ 0.49 ^{*a}	18.59 $\pm$ 0.37 ^a	26.52 $\pm$ 0.34 ^{*a}	16.61 $\pm$ 0.40 ^a	20.92 $\pm$ 0.28 ^{*a}
6-h control	2.53 $\pm$ 0.13 ^{xz}	34.77 $\pm$ 0.41 ^x	18.44 $\pm$ 0.26 ^x	27.65 $\pm$ 0.35 ^x	16.60 $\pm$ 0.34 ^x	19.56 $\pm$ 0.48 ^x
6-h HP	1.83 $\pm$ 0.16 ^{*ab}	37.44 $\pm$ 0.35 ^{*b}	18.43 $\pm$ 0.81 ^a	25.82 $\pm$ 0.23 ^{*a}	16.47 $\pm$ 0.06 ^a	22.08 $\pm$ 0.29 ^{*b}
9-h control	2.48 $\pm$ 0.14 ^{yz}	34.76 $\pm$ 0.25 ^x	18.90 $\pm$ 0.28 ^x	27.44 $\pm$ 0.24 ^x	16.42 $\pm$ 0.14 ^x	19.83 $\pm$ 0.35 ^x
9-h HP	1.52 $\pm$ 0.15 ^{*b}	38.53 $\pm$ 0.48 ^{*c}	18.67 $\pm$ 0.76 ^a	24.94 $\pm$ 0.49 ^{*b}	16.34 $\pm$ 0.37 ^a	22.56 $\pm$ 0.35 ^{*b}

^{a-c}Different superscripts for WPI samples means significant difference ($P < 0.05$).

^{x-z}Different superscripts for nonoxidized WPI (control) means significant difference ($P < 0.05$).

¹HP = hydrogen peroxide.

*Significant ($P < 0.05$) compared with nonoxidized WPI.

Table 3. Correlation matrix between parameters of oxidative modification and protease activities¹

Item	CAR	FT	TT	TE	SH	TUR	DIT	PD	ARV	Sheet	Helix	ODP	ISP	MRP	HLTP	ODT α	IST α	MRT α	HLTT α
CAR	1																		
FT	-0.794*	1																	
TT	-0.892**	0.876**	1																
TE	-0.840*	0.577	0.767*	1															
SH	0.516	-0.674	-0.650	-0.477	1														
TUR	0.914**	-0.811*	-0.893**	-0.813*	0.503	1													
DIT	0.885**	-0.883**	-0.919**	-0.753*	0.661	0.890**	1												
PD	0.898**	-0.862**	-0.919**	-0.789*	0.648	0.895**	0.916**	1											
ARV	-0.908**	0.726	0.906**	0.725	-0.617	-0.806*	-0.904**	-0.909**	1										
Sheet	0.907**	-0.847**	-0.874**	-0.796*	0.589	0.813*	0.812*	0.910**	-0.916**	1									
Helix	-0.911**	0.838*	0.868**	0.713	-0.605	-0.869**	-0.908**	-0.814*	0.818*	-0.917**	1								
ODP	0.903**	-0.812*	-0.903**	-0.841*	0.638	0.888**	0.896**	0.911**	-0.907*	0.898**	-0.909**	1							
ISP	-0.773*	0.912**	0.856**	0.545	-0.665	-0.796*	-0.867**	-0.839*	0.815*	-0.837*	0.823*	-0.783*	1						
MRP	-0.773*	0.910**	0.853**	0.539	-0.644	-0.798*	-0.864**	-0.835*	0.812*	-0.836*	0.822*	-0.777*	0.919**	1					
HLTP	0.796*	-0.441	-0.615	-0.542	0.109	0.811*	0.606	0.713	-0.735	0.565	-0.751*	0.496	-0.338	-0.653	1				
ODT α	0.889**	-0.858**	-0.915**	-0.790*	0.683	0.881**	0.912**	0.918**	-0.905**	0.902**	-0.909**	0.913**	-0.834*	-0.829*	0.578	1			
IST α	-0.822*	0.902**	0.873**	0.600	-0.568	-0.847**	-0.880**	-0.858**	0.843*	-0.869**	0.853**	-0.807*	0.905**	0.909**	-0.742	-0.845**	1		
MRT α	-0.785*	0.906**	0.854**	0.546	-0.596	-0.813*	-0.865**	-0.836*	0.815*	-0.844**	0.825*	-0.776*	0.914**	0.916**	-0.493	-0.824*	0.915**	1	
HLTT α	0.879**	-0.843*	-0.872**	-0.694	0.342	0.890**	0.869**	0.867**	-0.863**	0.885**	-0.877**	0.838**	-0.835*	-0.842*	0.847**	0.848**	-0.886**	-0.858**	1

¹CAR = carbonyl; FT = free thiol group; TT = total thiol group; TE = thiol group level embedded inside the protein; SH = surface hydrophobicity; TUR = turbidity; DIT = dityrosine; PD = particle diameter; ARV = aromatic ring vibration of tyrosine residues; Sheet = β -sheets; Helix = α -helix; ODP = ODmax-Pepsin; ISP = initial slope-pepsin; MRP = maximal rate of digestion-pepsin; HLTP = half-life time-pepsin; ODT α = ODmax-trypsin and α -chymotrypsin; IST α = initial slope-trypsin and α -chymotrypsin; MRT α = maximal rate of digestion-trypsin and α -chymotrypsin; HLT α = half-life time-trypsin and α -chymotrypsin.

* $P < 0.05$, ** $P < 0.01$.

is most efficient in cleaving peptide bonds between hydrophobic and aromatic AA such as Phe, Trp, and Tyr (Sun et al., 2011). Proteolysis measured in oxidized WPI with gastric pepsin showed a biphasic response. That is why proteolysis rate of nonoxidized protein by gastric pepsin increased slightly with time, along with the related changes in initial slope and maximal rate of digestion (Figure 3). Conversely, the proteolysis rate of oxidized WPI by gastric pepsin decreased significantly with time (Figure 3). Proteolysis rates were highly and negatively correlated with carbonyl group level because

carbonyl groups were formed by amide bonds with residual of amino groups. In current study, the maximal digestion and half-life time were positively correlated with carbonyl contents, turbidity, and dityrosine (Table 3). These results proved that the degradation potential of WPI had been improved by oxidation but needed a longer digestion time (Figure 3; Bax et al., 2012). The proteolysis rate was also negatively correlated with turbidity and particle diameter (Table 3). This could be explained by the fact protein aggregation has a negative effect on proteolysis susceptibility (Sante-Lhoutellier et

Figure 3. Effect of oxidation on in vitro pepsin digestion parameters [A = initial optical density (OD); B = initial slope; C = maximal rate of digestion; D = half-life time; E = maximal degradation]. An asterisk (*) means significant ($P < 0.05$) comparing with nonoxidized whey protein isolate (WPI). Different lowercase letters (x,y) for nonoxidized WPI (control) means significant difference ($P < 0.05$); different lowercase letters (a-c) for oxidized WPI [hydrogen peroxide (HP)] means significant difference ($P < 0.05$). Color version available online.

Figure 4. Proposed mechanism of pepsin action on whey protein isolate (WPI) samples (A = nonoxidized WPI; B = denatured WPI; C = aggregated WPI). Color version available online.

al., 2007; Bax et al., 2012; Cui et al., 2012). Polymerization and aggregation have important effect on protein digestibility. Studies reported that heating at 90°C significantly increased the digestibility of β -LG, which is an important composition of whey protein (Peram et al., 2013). However, if heated at 90°C up to 24 h, the aggregates of β -LG become more resistant to enzymatic digestion (Pinto et al., 2014). Myofibrillar proteins had a similar response to pepsin as well (Sante-Lhoutellier et al., 2007; Sun et al., 2011); however, properly modified myofibrillar proteins by oxidation were susceptible to proteolysis (Kamin-Belsky et al., 1996). In the case of myosin from myofibrillar proteins, oxidation decreased its susceptibility to gastric pepsin, pancreatic trypsin, and chymotrypsin under nonreducing conditions (Liu and Xiong, 2000). In the current work, protein denaturation occurred in nonoxidized WPI at 25 (3, 6, or 9 h) and 37°C (120 min) in a water bath, characterized by visible but not significant protein conformation changes that maximized protein surface exposure to hydrophobic zones and, thus, promoted the bioaccessibility of pepsin to cleavage sites to a certain limit. Under free radical-generating systems, the aggregation process appears to outpace the thermal denaturation process; hence, the proteolysis rate of oxidized WPI decreased with oxidizing time (Bax et al., 2012).

Figure 4 proposes a mechanism of pepsin action based on the results of present study. Heating at 25°C in water bath (3, 6, and 9 h) produced subtle protein oxidation and proper conformational changes, enhancing the hydrolyzability by pepsin. Protein oxidized by free radical-generating systems led to protein aggregation and a decrease of protein hydrolyzability by pepsin. These results are in agreement with previous studies (Bax et al. 2012).

Both trypsin and α -chymotrypsin are serine endopeptidases (Asante et al., 2014). Trypsin exclusively

cleaves Arg or Lys residues on the C terminal, and α -chymotrypsin preferentially cleaves peptide amide bonds and prefers hydrophobic residues, such as Tyr, Trp, or Phe (Kim et al., 2013; Asante et al., 2014). These AA are sensitive to free radicals and transformed into carbonyl groups (Kim et al., 2013). The digestion profile with trypsin and α -chymotrypsin was similar with pepsin (Figure 5). For the nonoxidized protein, the initial slope, maximal rate of digestion, and maximal degradation increased (Figure 5). However, under free radical-generating systems, this initial slope and maximal rate of digestion of oxidized WPI decreased, whereas the half-time and maximal degradation increased (Figure 5). The mechanism of this phenomenon might be the same as that of pepsin digestion. Studies also reported the same results of the in vitro digestion of myofibrils protein (Sante-Lhoutellier et al., 2007; Sun et al., 2011; Bax et al., 2012). The initial slope, maximal rate of digestion, and maximal degradation with trypsin or α -chymotrypsin were higher compared with pepsin. This could be explained by the sensitivity of hydrophobic residues to dual-enzyme system containing α -chymotrypsin (Kim et al., 2013).

Pooling a full set of physicochemical, structural, and digestion results revealed that this data can be distinguished into 6 separate groups (Figure 6). On the first axis, nonoxidized WPI was opposed to the oxidized WPI. Carbonyl, turbidity, dityrosine, particle diameter, maximal rate of digestion, and half-life time were positively associated with the first axis (Bax et al., 2012). On the second axis, water bath time had a great effect on separation of WPI samples in both oxidation and non-oxidization conditions. Initial slope and degradation potential of both digestive proteases were associated positively to the second axis (Bax et al., 2012). This result indicated that both oxidizing agents and

oxidizing time had influence on the physicochemical, structural, and nutritional quality of WPI.

CONCLUSIONS

The results of the current work demonstrate the remarkable susceptibility of WPI to hydroxyl radical

oxidation at room temperature, which caused physicochemical and structural changes, such as the loss of thiol groups, formation of dityrosine and carbonyls, and increases in surface hydrophobicity, turbidity, and particle diameter. Oxidizing time had a great influence on oxidative changes; the aggregation and secondary structure changes had a negative effect on the nutri-

Figure 5. Effect of oxidation on in vitro trypsin and α -chymotrypsin digestion parameters [A = initial optical density (OD); B = initial slope; C = maximal rate of digestion; D = half-life time; E = maximal degradation]. An asterisk (*) means significant ($P < 0.05$) compared with non-oxidized whey protein isolate (WPI). Different lowercase letters (x-z) for nonoxidized WPI (control) means significant difference ($P < 0.05$); different lowercase letters (a-c) for oxidized WPI [hydrogen peroxide (HP)] means significant difference ($P < 0.05$). Color version available online.

Figure 6. Principal component analysis plot: factor 1 versus factor 2 of all treatments. WPI = whey protein isolate; HP = hydrogen peroxide oxidized WPI. Color version available online.

tional quality of WPI. This statement is supported by decrease of initial slope, maximal rate of digestion in both digestive proteases, and increase of half-life time. Although in vitro digestion model approaches have limitations, especially in terms of intestinal absorption factors, it is possible to grasp a dynamic dimension of the in vitro digestion model and to compare a broad series of processing conditions. Hence, this study elucidated the hypothesis that free radicals from technological processes could decrease the nutritional quality of WPI. As proteins are important and widely used food additives, processing technologies need to reduce levels of protein oxidation to preserve the nutritional quality of food products.

ACKNOWLEDGMENTS

The authors thank Wu Ding, Yayun Hu, Li Jiang and Xiaoli Liu from College of Food Science and Engineering, Northwest A&F University, for their technical support. This work was supported by National Natural Science Fund (Beijing, China) for Young Scholar (Grant No: 31401515).

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