J. Dairy Sci. 98:1–12
http://dx.doi.org/10.3168/jds.2015-9814

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

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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 physiochemical, 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.
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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 m*M* PBS at pH 6.0, 0.1 m*M* $FeCl_3$, 5 m*M* H_2O_2, and 0.1m*M* 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 m*M* 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 m*M* 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 (excitation 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 m*M* 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 m*M* PBS at pH 8.0 for total thiol group (**TT**); the other 100 μL was diluted with 1 mL of 100 m*M* 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 m*M* 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 m*M* 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:

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

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