Effects of oxidative modification on thermal aggregation and gel properties of soy protein by malondialdehyde

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Abstract Malondialdehyde (MDA) was selected as a representative of lipid peroxidation products to investigate the effects of oxidative modification on thermal aggregation and gel properties of soy protein by lipid peroxidation products. Incubation of soy protein with increasing concentration of MDA resulted in gradual decrease of particle size and content of thermal aggregates during heat denaturation. Oxidative modification by MDA resulted in a decrease in water holding capacity, gel hardness, and gel strength of soy protein gel. An increase in coarseness and interstice of MDA modified protein gel network was accompanied by uneven distribution of interstice as MDA concentration increased. The results showed that degree of thermal aggregation of MDA-modified soy protein gradually decreased as MDA concentration increased, which contributed to a decrease in water holding capacity, gel hardness, and gel strength of MDA-modified soy protein gel.

Keywords Soy protein · Oxidative modification · Malondialdehyde · Thermal aggregation · Gelation

Introduction

Soy proteins are widely used in many protein-based food formulations because the gelation of soy protein in food processing provides various textural properties (Kuraishia et al. 2001; Hefnawy and Ramadan 2011). The ability of soy protein to form thermally induced gel is generally considered as one of the most important functional properties of soy protein, and it is worthwhile to study factors which could influence the gel properties of soy protein. Hua et al. (2005) proposed that gel properties of soy protein could be affected by the oxidative modification of soy protein structure in the course of storage and processing procedures. Soybeans are rich in lipoxygenase and linoleic acids. Huang et al. (2006) reported that an increase of lipoxygenase-mediated linoleic acids peroxidation was accompanied by oxidative modification of soy protein, and Kong et al. (2008) indicated that gel properties of soy protein isolates were related to the lipoxygenase activity in defatted soybean flour. Furthermore, our recent studies implied that free radicals and reactive oxidation products of lipid peroxidation could oxidatively modify side chains and polypeptide backbone of soy protein, resulting in cross-linking, fragmentation, aggregation, and conformational changes, which finally could affect gel properties of soy protein (Wu et al. 2009a, b, c, 2010). Therefore, oxidative modification of soy protein by lipid peroxidation products could influence gel properties of soy protein. However, mechanism of lipid peroxidation products-mediated changes of gel properties of soy protein still poorly understood.

Thermal gelation of soy protein is a complex process which involves protein unfolding and aggregation prior to formation of three-dimensional network structure (Hermansson 1986). Heat denaturation of soy protein in solution resulted in

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protein unfolding, exposing functional groups (carbonyl groups and amine groups of peptide bonds, side chain amine groups, and hydrophobic groups), which then would interact each other by intermolecular hydrogen bonding, electrostatic interactions, and hydrophobic interactions, leading to aggregation and subsequent formation of gel network structure (Subirade et al. 1998). Gelation and aggregation are closely related. Size and molecular weight of aggregates were related to the thickness of the strands of gel network and gel properties (Doe 1993; Lakemond et al. 2003). Hence, protein aggregation plays an important part in gelation (Tay et al. 2005). Effects of pH, ionic strength, and protein concentration on the thermal aggregation behavior of soy protein have been intensively studied (Puppo and Anon 1998; Renkema et al. 2002). However, effects of oxidative modification on thermal aggregation properties of protein have been ignored, and studies of the effects of oxidative modification on thermal aggregation properties of soy protein could reveal the mechanism of effects of oxidative modification on gel properties of soy protein.

Malondialdehyde (MDA) is one of the most abundant reactive products resulting from lipid peroxidation and has been widely used as an index of oxidative deterioration of lipids in foods (Adams et al. 2008), and our previous studies indicated that oxidative modification of soy protein by MDA could result in changes of structural characteristics and aggregation behavior of soy protein at room temperature (Wu et al. 2009a, b, c). Therefore, MDA was selected as a representative of lipid peroxidation-derived reactive products to investigate the effects of oxidative modification on thermal aggregation and gel properties of soy protein by lipid peroxidation products in this paper. Our objective was to reveal the mechanism of effects of oxidative modification on gel properties of soy protein by lipid peroxidation products.

Materials and methods

Materials and chemical reagents Soybean was purchased from a local market, which was harvested from Heilongjiang Province, P.R. China. 1,1,3,3-tetramethoxypropane was obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). All other chemicals were analytical reagent grade.

Preparation of lipid free-soybean protein isolates Lipid free-soybean protein isolates were prepared by the method of Wu et al. (2009a, b, c, 2010) using soybean as raw material.

Preparation of MDA solution A fresh MDA stock solution (200 mmol/L) was prepared by hydrolyzing 1,1,3,3-

tetramethoxypropane, which was modified according to the method described by Wu et al. (2009c). The concentration of stock solution was chosen to avoid possible self-polymerization of MDA under the reaction conditions used. Briefly, 8.4 mL (50 mmol/L) 1,1,3,3-tetramethoxypropane was mixed with 10.0 mL 5.0 mol/L HCl and 31.6 mL ultrapure water, and incubated at 40 °C in the dark for 30 min to obtain MDA through acidic hydrolysis. After 1,1,3,3-tetramethoxypropane was hydrolyzed, the pH was adjusted to 7.4 with 6 mol/L NaOH and the stock solution was diluted to 250 mL with 0.05 mol/L sodium phosphate buffer, pH 7.4. Concentration of MDA in the stock solution was estimated by absorbance at 267 nm when the solution was diluted to 10^{-5} using a molar extinction coefficient value of $31,500 \text{ mol}^{-1} \text{ cm}^{-1}$ (Adams et al. 2008). MDA solutions were made fresh daily.

Preparation of MDA-modified soy protein MDA-modified soy protein was prepared by the method of Wu et al. (2009c). Briefly, soy protein solution (10 mg/mL containing 0.5 mg/mL sodium azide, suspended in 0.01 mol/L sodium phosphate buffer, pH 7.4) were mixed with a serial concentration of MDA, and then incubated by continuous shaking under air at 25 °C in dark for 24 h. The final concentration of MDA was 0 (control), 0.01, 0.1, 1, 10, and 100 mmol/L. Free MDA was removed from the soy protein following incubation by a 72 h dialysis at 4 °C against distilled water. The MDA-modified soy protein solution were then freeze-dried and stored at 4 °C.

Particle size distributions measurement of MDA-modified soy protein during thermal denaturation Particle size distributions were measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., UK) to monitor the growth of the aggregates and to determine size of thermal aggregates of MDA-modified soy protein. Briefly, MDA-modified soy protein was suspended in 0.01 mol/L sodium phosphate buffer (pH7.4) at a concentration of 1 mg/mL and magnetic stirred thoroughly, and then the suspensions were centrifuged at 59,700g for 30 min and the supernatants were filtered through cellulose acetate membranes with pore size of 0.45 μm to remove any insoluble particles. The obtained soy protein solutions were injected into screw-cap test tubes and heated in a water bath at 50–90 °C for 30 min, with an interval of 10 °C, and then immediately measured the particle size distribution of soy protein at corresponding temperature.

Particle size distributions and molecular weight distributions measurement of MDA-modified soy protein after heat treatment MDA-modified soy protein solutions (1 mg/mL in 0.01 mol/L sodium phosphate buffer, pH7.4) were injected into screw-cap test tubes and heated in a water

bath at 100 °C for 30 min, and then cooled to room temperature in an ice bath for 5 min, followed by particle size distributions measurement and molecular weight distribution measurement at 25 °C. The molecular weight distributions were determined by a Waters 2,690 liquid chromatography system equipped with a Shodex protein KW-804 Column (Shodex Separation and HPLC Group, Tokyo, Japan), and a Waters 996 photodiode array detector was used. The peak with retention time of 5.9 min in molecular weight distributions corresponded to the molecular weight of 1,000 kDa, which could indicate the contents of soluble thermal aggregates of soy protein (Wu et al. 2009a, b, c, 2010).

Measurement of water holding capacity (WHC) of MDA-modified soy protein gel WHC was measured according to the method of Pares et al. (1998). Briefly, soy protein gel was prepared by heating soy protein dispersion (120 mg/g) in cylindrical glass moulds with an inner diameter of 25 mm and a height of 60 mm. The moulds were filled three-quarters full to enable air bubbles to escape, the air bubbles were removed using a vacuum pump and then placed vertically in a water bath. Sample was heated at 95 °C for 30 min and then cooled to room temperature by immersing in an ice-bath. Soy protein gel was cut into 3–5 mm cubes from different areas of each gel and then packed by filter paper with weight W_1 . The weight of the package of gel after centrifuge at 4,000g for 10 min at 15 °C was W_2 , and weight of filter paper was W_3 . WHC was defined by the following equation:

$$\text{WHC} = 1 - \frac{W_1 - W_2}{0.88 \times (W_1 - W_3)}$$

Gel hardness measurement of MDA-modified soy protein gel Gel hardness of soy protein gel was measured by Texture Analyzer (TA-XT2i, Stable Micro Systems, Godalming, Surrey, UK). Before analysis, soy protein gel (as the way of WHC measurement) was removed from the container and transferred to a Texture Analyzer. A cylinder probe with diameter of 10 mm was chosen and the probe speed was set up to 1 mm/s. The tests were performed at 25 °C and by compressing the sample until rupture.

Rheological measurement of MDA-modified soy protein gel Thermal gelation of MDA-modified soy protein dispersions (120 mg/g) was induced by heating from 25 °C to 95 °C at a heating rate of 2 °C/min, holding the temperature at 95 °C for 30 min, and then cooled down to 25 °C at a cooling rate of 2 °C/min. Dynamic rheological measurements were performed in a rheometer (AR1000, TA Instruments, New Castle, DE, USA) using the parallel plate

(20 mm radius, 1 mm gap). The storage modulus (G') and loss modulus (G'') were measured at a constant strain of 0.01, which was within the linear region, and at an angular frequency of 0.63 rad/s. To prevent drying, samples were covered with a cap, sealed with a thin layer of paraffin oil during the procedure. Frequency sweeping measurement was followed temperature sweeping with range changing from 0.1 Hz to 10 Hz at 25 °C.

Scanning electron microscopy (SEM) of MDA-modified soy protein gel MDA-modified soy protein gel (as the way of WHC measurement) were cut into 3–5 mm cubes from different areas of each gel and fixed in a 3% glutaraldehyde in 0.05 mol/L sodium phosphate buffer solution (pH 7.0) at 4 °C for 2 h. Three 20 min rinses with cold 0.05 mol/L sodium phosphate solution (pH 7.0) followed primary fixation. A secondary fixation followed for 1.5 h in 1% osmium tetroxide in the 0.05 mol/L sodium phosphate solution (pH 7.0) at 4 °C, succeeded with three 20 min rinses. Samples were dehydrated for 20 min each in 30%, 50%, 70%, and 95% ethanol. Samples were immediately dried by the method of supercritical fluid drying of CO₂. Dried samples were fractured. The specimens were attached to stubs with silver conducting paint and coated with a layer of gold-palladium approximately 30 nm thick. The coated specimens were measured with SEM (Quanta-200, FEI Ltd., Netherlands).

Results and discussion

Effect of oxidative modification by MDA on particle size distributions of soy protein during thermal denaturation Particle size distributions of MDA-modified soy protein during thermal denaturation are shown in Fig. 1. Size of MDA-modified soy protein gradually increased as heating temperature increased from 50 °C to 90 °C, which could be ascribed to thermally induced aggregation of soy protein. Size of MDA-modified soy protein at 50 °C slowly increased with increasing concentration of MDA, reaching the maximum when MDA concentration was 1 mmol/L, and then progressively decreased with further increase of MDA concentration to 100 mmol/L. In contrast, size of MDA-modified soy protein at 90 °C dramatically increased with increasing concentration of MDA, reaching the maximum when MDA concentration was 0.01 mmol/L, and then slowly decreased with further increase of MDA concentration. Our recent study indicated that oxidative modification of soy protein by increasing concentration of MDA resulted in gradual formation of soluble oxidation aggregates, and solubility of modified soy protein significantly decreased as concentration of 0.1–100 mmol/L MDA was employed (Wu et al. 2009c). Therefore, a slow increase

Fig. 1 Particle size distributions of MDA-modified soy protein under different temperature in the process of heat-induced denaturation (**a** control; **b** 0.01 mmol/L MDA; **c** 0.1 mmol/L MDA; **d** 1 mmol/L MDA; **e** 10 mmol/L MDA; **f** 100 mmol/L MDA; **g** Z-average of diameter of MDA-modified soy protein during heat-induced denaturation)

in size of modified soy protein at 50 °C followed by progressive decrease in modified soy protein size as MDA concentration gradually increased from 0.01 to 100 mmol/L. The observed phenomena of decrease in size of modified soy protein at 90 °C as MDA concentration increased from 0.01 to 100 mmol/L indicated that degree of thermal aggregation of modified soy protein gradually decreased with increasing of MDA concentration.

Effect of oxidative modification by MDA on particle size distribution and molecular weight distribution of soy protein after heat treatment Particle size distribution and molecular weight distribution of MDA-modified soy protein after heat treatment at 100 °C for 30 min are shown in Figs. 2 and 3. Particle size distribution of MDA-modified soy protein after heat treatment moved to lower diameter as MDA concentration increased (Fig. 2), and percentage of

Fig. 2 Particle size distributions of MDA-modified soy protein after heat treatment in 100 °C water bath for 30 min

soluble thermal aggregates of modified soy protein also gradually decreased as MDA concentration increased (Fig. 3). Both the phenomena implied that oxidative modification by MDA resulted in decreasing degree of

soy protein thermal aggregation. Our previous studies showed that MDA could oxidatively modify amino groups, sulfhydryl groups, and other functional groups of soy protein, leading to soy protein cross-linking and formation of oxidation aggregates (Wu et al. 2009c). The decrease of functional groups (amino groups, sulfhydryl groups, and hydrophobic groups) of protein contributed to the decrease degree of thermal aggregation. In addition, the soluble oxidation aggregates could develop into insoluble aggregates during heat denaturation (Wu et al. 2009c), which could also decrease percentage of soluble thermal aggregates after heat treatment. The molecular weight of soluble thermal aggregates has a significant effect on gel strength of protein gel. Wang and Damodaran (1990) showed that size and molecular weight of soluble thermal aggregates which incorporate into gel network structure had a positive correlation with gel strength, and globular protein which had a weight-average molecular weight less than 23,000 Da

Fig. 3 Molecular weight distributions of MDA-modified soy protein after heat treatment in 100 °C water bath for 30 min (a control; b 0.01 mmol/L MDA; c 0.1 mmol/L MDA; d 1 mmol/L MDA; e

10 mmol/L MDA; f 100 mmol/L MDA; g percentage of soy protein thermal aggregates after heat treatment)

Table 1 Water holding capacity and gel hardness of MDA-modified soy protein gel

MDA (mmol/L)	Water holding capacity (%)	Gel hardness (N)
0	82.2±3.61a	146.6±2.27a
0.01	80.7±2.83a	133.7±2.33b
0.1	78.1±3.16a	103.3±2.37c
1	68.3±2.61b	53.8±2.28d
10	60.4±2.19c	31.5±1.72e
100	40.5±2.42d	15.4±2.02f

All values are the mean of triplicate determinations, and the same letter in column indicates no difference at $P \geq 0.05$. MDA, Malondialdehyde

could not form self-supporting gel network at any reasonable concentration.

Effects of oxidative modification by MDA on WHC and gel hardness of soy protein gel WHC is a quantitative indication of the amount of water retained within protein gel network structure, which could reflect spatial structure of protein gel, and WHC of MDA-modified soy protein gel are shown in Table 1. WHC of modified soy protein gel gradually decreased as MDA concentration increased, and only oxidative modification by high concentration of MDA (≥ 1 mmol/L) could result in a significant decrease of WHC ($P < 0.05$). The results implied that oxidative modification by MDA increased coarseness of gel structure of soy protein. Our previous study confirmed that oxidative modification by MDA could decrease solubility and sulfhydryl groups of soy protein (Wu et al. 2009c), and Xiong (2000) suggested that decrease of WHC of myofibrillar protein by oxidative modification was accompanied by decrease of protein solubility and sulfhydryl groups.

Gel hardness of soy protein gel is also shown in Table 1. Oxidative modification of soy protein by MDA resulted in a significant decrease of gel hardness ($P < 0.05$). Alting et al. (2003) indicated that size of soluble thermal aggregates had a positive correlation with gel strength of protein gel. Therefore, the observed decrease of gel hardness of

modified soy protein gel could be attributed to decreasing degree of protein thermal aggregation.

Effects of oxidative modification by MDA on rheological properties of soy protein gel Figure 4 shows the evolution of G' of MDA-modified soy protein dispersion as a function of time and temperature. A progressive increase of G' value after initial lag time was observed for all soy protein samples at initial-holding temperature period, and then G' value reaches a plateau region at post-holding temperature period, finally, value of G' quickly increased again at subsequent cooling period. The curve of G' of all soy protein samples was similar to the report of Renkema and van Vliet (2003). Initial G' value of MDA-modified soy protein at heating stage steadily increased as MDA concentration increased, and the phenomena was attributed to the presence of oxidation aggregates resulting from oxidative modification by MDA (Wu et al. 2009c). Kong et al. (2008) found that soy protein isolates which derived from high lipoxigenase activity defatted soybean flours exhibited higher initial G' value than that of derived from low lipoxigenase activity defatted soybean flour at heating stage.

The point of sudden increase of G' value at initial-holding temperature period could be defined as the starting point of the gelling process, which could be used to measure the gelation time of MDA-modified soy protein (Kavanagh et al. 2000). As shown in Fig. 4, gelation time of MDA-modified soy protein gradually increased as MDA concentration increased. In contrast, degree of progressive increase of G' of MDA-modified soy protein steadily decreased when concentration of MDA increased from 0 to 100 mmol/L. Protein gelation occurred only when the degree of thermal aggregation approached a critical point, and degree of thermal aggregation was determined by cross-linking bond formation among functional groups. A large degree of cross-linking bond formation means short gelation time and large value of G' . Our previous studies showed that MDA could modify amino groups, sulfhydryl groups, and hydrophobic groups of soy protein (Wu et al. 2009c). Therefore, the reduction number of bonding site

Fig. 4 Storage moduli (G') as a function of heating time and temperature in the process of soy protein gel formation

resulting from oxidative modification of soy protein by MDA could be ascribed to the increase of gelation time and decrease degree of progressive increase of G' . A slow increase of G' value of MDA-modified soy protein at post-holding temperature period was contributed to the rearrangement of the gel network which sulfhydryl/disulfide exchange reaction shift to the formation of disulfide bonds (Shimada and Cheftel 1989). Since sulfhydryl groups of soy protein was modified by MDA (Wu et al. 2009c), therefore, the increase extent of G' value of MDA-modified soy protein gradually decreased as MDA concentration in-

creased at post-holding temperature period. The value G' of soy protein samples dramatically increased again at the subsequent cooling period because three-dimensional network of protein gel was reinforced by hydrogen bonds which strengthen at low temperature (Nagano et al. 1994).

Besides the above mentioned temperature sweep, there is another way to analyze gel properties of MDA-modified soy protein which both G' and G'' and their frequency dependence (frequency sweep) was employed, and the results are shown in Fig. 5. The phenomena indicated that all MDA-modified soy protein could form true protein gel because Winter and

Fig. 5 G' , G'' and $\tan \delta$ (G''/G') as a function of frequency of MDA-modified soy protein gel after temperature sweep (**a** control; **b** 0.01 mmol/L MDA; **c** 0.1 mmol/L MDA; **d** 1 mmol/L MDA; **e** 10 mmol/L MDA; **f** 100 mmol/L MDA; **g** $\tan \delta$ as a function of frequency)

Chambon (1986) proposed that curves of G' and G'' of a typical protein gel were independent of the oscillation frequency and parallel to each other with $G' > G''$. However, the loss tangent $\tan \delta$ (G''/G') gradually increased as MDA concentration increased. The results indicated that oxidative modification by MDA led to a decrease of gel strength of soy protein gel. Kong et al. (2008) found that gel strength of soy protein gel decreased as lipoxygenase activity of defatted soybean flour was inactivated by dry heating, and proposed that oxidative modification by lipid peroxidation products had a negative effect on the gel strength of soy protein gel. Inhibition of lipid peroxidation by antioxidants could result in a increase of gel strength of protein gel, and the phenomena was observed in the study of oxidative modification of myofibrillar protein by lipid peroxidation products (Wan et al. 1994; Liu and Xiong 1996; Decker et al. 1993). Ooizumi and Xiong (2004) proposed that decrease of gel strength of modified myofibrillar protein by lipid peroxidation products was attributed to the decrease of disulfide bonds and formation of oxidation aggregates. Our previous study showed that MDA could modify sulfhydryl groups of soy protein, resulting in a decrease of disulfide bonds and formation of oxidation aggregates (Wu et al. 2009c). Therefore, decrease of disulfide bonds and formation of

oxidation aggregates resulting from oxidative modification by MDA could be ascribed to decrease of gel strength of MDA-modified soy protein gel.

Effect of oxidative modification by MDA on microstructure of soy protein gel Microstructure of protein gel was related to the gel properties of soy protein, and SEM micrographs of native soy protein gel and MDA-modified soy protein gel are shown in Fig. 6. Native soy protein gel exhibited a dense network with homogeneous distribution of small pores (Fig. 6a). Oxidative modification of soy protein by MDA resulted in an increase in coarseness of gel and uneven distribution of gel pores (Fig. 6b, c). The phenomena indicated that oxidative modification by MDA has negative effects on gel structure of soy protein, which was consistent with the results of WHC measurement, large deformation test, and rheological measurements.

Conclusions

Soy protein was vulnerable to MDA. Exposure of soy protein to increase concentration of MDA resulted in decrease in particle size and content of thermal aggregates. Oxidative

Fig. 6 Scanning electron microscope micrographs of MDA-modified soy protein gel (**a** control; **b** 0.1 mmol/L MDA; **c** 100 mmol/L MDA)

modification of soy protein by MDA was accompanied by gradual decrease in WHC, gel hardness and gel strength. An increase in coarseness and interstice of the gel network was accompanied by uneven distribution of interstice as extent of oxidation of soy protein increased. A decrease in disulphide content and formation of oxidation aggregate in the process of oxidative modification resulted in a decrease in particle size and content of thermal aggregates, leading to a decrease in WHC, gel hardness and gel strength of soy protein.

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