

## Research Article

### Study on Mechanism of Soy Protein Oxidation Induced by Lipid Peroxidation Products

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**Abstract:** Oxidative modification of soy protein by lipid peroxidation products, which was potentially present in a lipoxygenase-catalyzed polyunsaturated fatty acid peroxidation system, was investigated in this study. 13S-Hydroperoxy-9Z, 11E-Octadecadienoic acid (HPODE), malondialdehyde and acrolein were selected as representative primary product and secondary byproducts of lipid peroxidation and 2, 2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-derived peroxy radical were chosen to simulate lipid peroxidation-derived free radical. Incubation of soy protein with increasing concentration of AAPH, HPODE, malondialdehyde and acrolein resulted in gradual generation of protein carbonyl derivatives, loss of free sulphhydryl groups, total sulphhydryl groups, free amine, available lysine, surface hydrophobicity and formation of oxidation aggregates. The average distribution model of protein accessible groups could explain majority mechanism of lipid peroxidation products-mediated soy protein oxidation. Primary oxidation aggregates further developed into insoluble aggregates by covalent cross-linking also may provide a partial mechanism of lipid peroxidation products-mediated soy protein oxidation.

**Keywords:** Aggregation, lipid peroxidation product, mechanism, protein oxidation, soy protein

## INTRODUCTION

Lipoxygenase-catalyzed lipid peroxidation can lead to oxidation modification of soy protein during preparation and storage, which resulted in deterioration of nutritional value and loss of functional properties of soy protein (Hua *et al.*, 2005). Lipid peroxidation is a very complex free radical reaction with lipid hydroperoxides as major initial products and eventual formation of reactive aldehydes, ketones and other oxygenated compounds. Lipid peroxidation could induce protein oxidation in two ways: attack by lipid peroxidation-derived free radical and modification by reactive products of lipid peroxidation (lipid hydroperoxides and reactive aldehydes) (Esterbauer *et al.*, 1991). Lipid peroxidation-derived free radical could attack backbone of protein and side chains of specific amino acid residues, resulting in fragmentation and cross-linking of polypeptide chain. Reactive products of lipid peroxidation react with specific side chain groups of protein, resulted in cross-linking of protein. Meat protein oxidation which induced by lipid peroxidation products has received the most attention, while less attention has been given to lipid peroxidation products-mediated soy protein oxidation, let alone to compare soy protein oxidation by different lipid

peroxidation products and investigate mechanism of lipid peroxidation products-mediated soy protein oxidation.

Recently, we have shown that lipid peroxidation-derived free radical, lipid hydroperoxides and reactive aldehydes could covalently modify soy protein molecule and change structural characteristics of soy protein (Wu *et al.*, 2009a, b, c; Wu *et al.*, 2010). However, our previous research about structural characteristics of oxidized soy protein is not comprehensive and a comparison of soy protein oxidation by different lipid peroxidation products is also neglected. To systemically study the mechanism of lipid peroxidation products-mediated soy protein oxidation, the roles of lipid peroxidation-derived free radical, lipid hydroperoxides and reactive aldehydes in the course of soy protein oxidation should be comprehensively and contrastively studied.

Peroxy radical is a major initiator free radical of lipid peroxidation and an important reactive oxygen species which could induce protein oxidation (Gieseg *et al.*, 2003). Thermal decomposition of 2, 2'-Azobis(2-Amidinopropane) dihydrochloride (AAPH) under aerobic condition lead to formation of peroxy radicals. Therefore, AAPH-derived peroxy radicals were chosen to simulate lipid peroxidation-derived free radicals. 13-

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Hydroperoxyoctadecadienoic acid (HPODE) was selected as a representative of lipid hydroperoxides because linoleic acid is the richest unsaturated fatty acid in soybean. Malondialdehyde (MDA) represents the most abundant reactive aldehyde resulting from lipid peroxidation and is widely used as an index of oxidative deterioration of lipids in food (Adams *et al.*, 2008), while acrolein is one of the strongest  $\alpha$ ,  $\beta$ -unsaturated aldehydes resulting from lipid peroxidation (Uchida *et al.*, 1998), MDA and acrolein are used as two representatives of lipid peroxidation-derived reactive aldehydes. Therefore, 13S-Hydroperoxy-9Z, 11E-octadecadienoic acid (HPODE), malondialdehyde and acrolein were selected as representative primary product and secondary byproducts of lipid peroxidation while AAPH-derived peroxy radicals were chosen to simulate lipid peroxidation-derived free radicals to investigate the mechanism of lipid peroxidation products-mediated soy protein oxidation in this study. The present study was undertaken as an extension of our previous study on the comparison of soy protein oxidation by different lipid peroxidation products and mechanism of lipid peroxidation products-mediated soy protein oxidation.

## MATERIALS AND METHODS

**Materials:** Soybean was purchased from a local market, which was harvested from Heilongjiang Province, P.R. China. 1, 1, 3, 3-Tetramethoxypropane (TMP), AAPH was obtained from Wako Pure Chemical Co., Ltd (Osaka, Japan). Soybean lipoxygenase type I-B (70,800 units/mg), linoleic acid (assay  $\geq 99.0\%$  (GC)), 2, 4, 6-trinitrobenzenesulfonic acid and 5, 5'-dithiobis (2-nitrobenzoate) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

**Methods:** Preparation of lipid free-soybean protein isolates. Lipid free-soybean protein isolates was prepared by the method of Wu *et al.* (2009a) using soybean as raw material.

**Preparation of MDA:** A fresh MDA stock solution (200 mmol/L) was prepared by hydrolyzing TMP, which was modified according to the method described by Adams *et al.* (2008). Briefly, 8.4 mL (50 mmol/L) TMP was mixed with 10.0 mL 5.0 mol/L HCl and 31.6 mL ultra pure water and shaken at 40°C in dark for 30 min to obtain MDA through acidic hydrolysis. After the TMP 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. The concentration of MDA in the stock solution was estimated by absorbance of diluted to  $10^5$  at 267 nm using a molar extinction coefficient value of 31,500 M/cm. MDA solutions were made fresh daily.

**Preparation of HPODE:** HPODE was made from linoleic acid by soybean lipoxygenase I-B according to the method described by Kato *et al.* (1999). The linoleic acid was dissolved in 0.1 mol/L borate buffer (pH 9.0). Lipoxygenase was added to the solution and reacted under  $O_2$  with stirring at room temperature. After 90 min, the pH was adjusted to 4.0 with 0.1 mol/L HCl for the termination of the oxidation. The peroxide formed was extracted with a 2-fold volume of chloroform-methanol (1:1, v/v). The extract was evaporated, dissolved in chloroform and then applied to a thin-layer chromatography plate. The chromatography was performed using a development with n-hexane-diethyl ether (4:6, v/v). The band of HPODE was visualized by a UV lamp and scraped off. The HPODE was extracted with chloroform and evaporated. The purified HPODE was dissolved in ethanol and stored under argon at -20°C before use. The concentration of HPODE was determined by absorbance at 234 nm using  $\epsilon = 25000$  M/cm.

**Incubation soy protein with acrolein, MDA and HPODE:** 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 acrolein (0.1-100  $\mu$ mol/L), MDA (0.01-100 mmol/L) and HPODE (0.01-100 mmol/L). Followed incubation by a 3-day dialysis at 4°C against deionized water, the oxidized soy protein solutions were then freeze-dried and stored at 4°C.

**Incubation soy protein with AAPH-derived peroxy radicals:** 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 AAPH and then incubated by continuous shaking under air at 37°C in dark for 24 h. The final concentration of AAPH was 0 (control), 0.04, 0.2, 1, 5 and 25 mmol/L. The reaction was stopped by immediately cooling the solution to 0-4°C by ice-bathing. After centrifuging at 10,000 g for 1 h at 4°C to remove small quantity of insoluble substances which was formed during solution cooling, the protein solution was dialyzed against deionized water at 4°C for 72 h to remove residual AAPH and the oxidized soy protein solution were freeze-dried and stored at 4°C.

**Protein carbonyl content measurement:** Protein carbonylation was quantified according to the method described by Huang *et al.* (2006) which uses the reaction of 2, 4-Dinitrophenylhydrazine (DNPH) with the carbonyl groups of native and oxidized soy proteins. The results were expressed as nmoles of carbonyl groups per milligram of soluble protein with molar extinction coefficient of 22,000 M/cm. Soluble protein concentration was evaluated by the bicinchoninic acid method with bovine serum albumin as the standard.

Sulfhydryl and disulfide content measurement. Contents of sulfhydryl (free and buried SH) and total disulphide/sulfhydryl groups were determined using Ellman's procedure modified by Huang *et al.* (2006). The nmoles of SH per milligram protein were calculated by using the extinction coefficient of 13,600/M/cm.

**Free amine measurement:** Free amines were measured as described by Liu *et al.* (2000) which calculated as  $\mu\text{mol}$  free amines per mg protein using a standard curve constructed from glycine.

**Available lysine content measurement:** Available lysine was determined by the method of Hall *et al.* (1973) which used the reaction of 2, 4, 6-trinitrobenzenesulfonic acid with amino groups in soy protein. Pure L-lysine was used as a standard.

**Measurement of surface hydrophobicity:** The surface hydrophobicity of non-oxidized and oxidized soy protein were determined using 1-anilino-8-naphthalene-sulfonate as described by Huang *et al.* (2006).

**High Performance Size Exclusion Chromatogram (HPSEC):** Soy protein solution (5 mg/mL) was centrifuged at 10,000 g for 10 min (25°C). The obtained supernatant was filtered through a cellulose acetate membrane with a pore size of 0.45  $\mu\text{m}$  (Sartorius Co., Ltd., Gottingen, Germany). A Waters 2690 liquid chromatogram 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 to determine the molecular weight

distribution. The flow rate was 1 mL/min using phosphate buffer (0.05 mol/L, 0.3 mol/L NaCl, pH 7.0) as the mobile phase. About 10  $\mu\text{L}$  protein solutions were injected into the column and the eluent was monitored at 280 nm.

**Statistical analysis:** Analysis of Variance (ANOVA) was performed using the General Linear Model procedures of the SAS package v.8.1 (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at ( $p < 0.05$ ). The results are presented as mean  $\pm$  standard deviation.

## RESULTS AND DISCUSSION

**Effects of oxidative modification on soy protein carbonyl, free sulfhydryl and total disulphide /sulfhydryl contents:** Effects of AAPH, HPODE, MDA and acrolein on soy protein carbonyl contents are given in Table 1. Exposure of soy protein to increasing concentration of AAPH, MDA and acrolein led to a significant increase in protein carbonyl content ( $p < 0.05$ ). A statistically significant increase ( $p < 0.05$ ) of protein carbonyl contents in oxidized soy protein which modified by HPODE occurred at the higher HPODE concentrations (0.1-100 mmol/L). Peroxyl radical could attack side chains or backbone of protein under aerobic condition, resulting in structural modification of side chains and oxidative cleavage of protein backbone, finally led to protein carbonylation (Headlam and Davies, 2004). The reaction of HPODE with  $\epsilon$ -amino groups of lysine residues in protein could yield amide-type adducts, which contributed to protein carbonylation (Kato *et al.*, 1999). Carbonyl groups may

Table 1: Protein carbonyl, free sulfhydryl, total disulphide and sulfhydryl, free amine content of soy protein which induced by AAPH-derived proxyl radicals, HPODE, MDA and acrolein

Lipid peroxidation products	Concentration (mmol/L)	Protein carbonyl (n mol/mg)	Free sulfhydryl (n mol/mg)	Total disulphide / sulfhydryl (n mol/mg)	Free amine ( $\mu\text{mol/mg}$ )
AAPH	0	1.68 (0.12)a	3.97 (0.05)a	82.74 (2.34)a	0.425 (0.009)a
	0.04	2.12 (0.13)b	3.38 (0.07)b	76.39 (1.75)b	0.424 (0.010)a
	0.20	2.83 (0.13)c	2.51 (0.09)c	70.59 (2.19)c	0.421 (0.009)a
	1	4.11 (0.13)d	1.78 (0.10)d	63.05 (2.28)d	0.412 (0.006)a
	5	6.32 (0.15)e	1.18 (0.08)e	55.88 (2.16)e	0.392 (0.009)b
	25	10.14 (0.18)f	0.63 (0.09)f	43.78 (1.56)f	0.353 (0.009)c
HPODE	0	1.65 (0.08)a	4.05 (0.09)a	78.68 (0.92)a	0.425 (0.009)a
	0.001	1.81 (0.07)a	3.95 (0.09)a	78.11 (0.88)a	0.424 (0.007)a
	0.01	2.12 (0.09)b	3.78 (0.07)a	77.44 (0.88)a	0.421 (0.010)a
	0.1	2.52 (0.10)c	3.49 (0.09)b	75.43 (0.82)a	0.414 (0.010)a
	1	2.86 (0.10)d	3.08 (0.06)c	73.04 (0.88)b	0.401 (0.007)b
	10	3.21 (0.09)e	2.54 (0.06)d	69.64 (1.09)c	0.382 (0.006)c
MDA	0	1.65 (0.06)a	4.05 (0.09)a	77.56 (1.32)a	0.425 (0.009)a
	0.01	2.25 (0.11)b	3.42 (0.09)b	76.18 (1.16)a	0.416 (0.006)a
	0.1	3.27 (0.13)c	2.65 (0.12)c	73.99 (1.23)a	0.409 (0.007)a
	1	5.95 (0.14)d	2.11 (0.12)d	69.73 (1.27)b	0.393 (0.007)b
	10	9.15 (0.11)e	1.77 (0.06)e	63.48 (0.96)c	0.325 (0.011)c
	100	16.55 (0.20)f	1.38 (0.09)f	48.37 (1.30)d	0.126 (0.011)d
Acrolein	0	1.62 (0.07)a	4.15 (0.06)a	78.46 (0.98)a	0.425 (0.009)a
	0.001	2.07 (0.09)b	3.98 (0.07)b	74.33 (1.02)b	0.422 (0.009)a
	0.01	2.68 (0.11)c	3.81 (0.09)c	70.83 (1.01)c	0.412 (0.007)a
	0.1	4.31 (0.10)d	2.68 (0.09)d	68.60 (0.94)d	0.385 (0.007)b
	1	8.33 (0.12)e	1.84 (0.09)e	67.24 (0.95)d	0.331 (0.008)c

All values are the mean of triplicate determinations; Values in the parenthesis are standard errors; The same letter in column indicates no different difference at ( $p \geq 0.05$ )

Table 2: Available lysine, surface hydrophobicity and aggregates content of soy protein which induced by AAPH-derived peroxy radicals, HPODE, MDA and acrolein

Lipid peroxidation products	Concentration (mmol/L)	Available lysine (g/100 g)	Surface hydrophobicity	Aggregates content (%)
AAPH	0	5.87 (0.18)a	81.13 (1.10)a	0
	0.04	5.86 (0.16)a	78.92 (1.06)a	0.54
	0.20	5.82 (0.11)a	75.89 (1.03)b	0.59
	1	5.71 (0.11)a	61.78 (1.04)c	0.93
	5	5.47 (0.11)a	44.87 (0.66)d	2.96
	25	4.99 (0.11)b	24.68 (1.21)e	3.17
HPODE	0	5.87 (0.18)a	84.22 (1.04)a	0
	0.001	5.85 (0.11)a	82.36 (1.10)a	1.05
	0.01	5.80 (0.11)a	79.14 (1.11)b	2.89
	0.1	5.72 (0.11)a	75.43 (1.11)c	4.80
	1	5.61 (0.10)a	69.98 (1.10)d	5.94
	10	5.48 (0.11)a	60.26 (1.12)e	7.54
MDA	0	5.87 (0.18)a	84.24 (1.10)	0
	0.01	5.74 (0.15)a	81.73 (1.34)a	0.03
	0.10	5.67 (0.13)a	76.33 (1.41)b	0.08
	1	5.39 (0.10)b	69.06 (1.32)c	0.27
	10	4.64 (0.14)c	53.44 (0.91)d	31.84
	100	2.11 (0.13)d	21.93 (1.36)e	37.59
Acrolein	0	5.87 (0.18)a	84.22 (1.25)a	0
	0.001	5.71 (0.19)a	77.84 (1.04)b	1.38
	0.01	5.36 (0.16)a	65.51 (1.23)c	3.52
	0.1	4.27 (0.15)b	51.67 (1.09)d	7.42
	1	3.08 (0.10)c	32.23 (1.06)e	9.68

All values except the content of aggregates are the mean of triplicate determinations. Values in the parenthesis are standard errors. The same letter in column indicates no different difference at ( $p \geq 0.05$ )

be also introduced into protein by reaction of nucleophilic side chain of His and Lys residues and protein primary amino groups with acrolein and MDA (Esterbauer *et al.*, 1991). Reaction of soy protein with 25 mmol/L AAPH-derived peroxy radicals, 10 mmol/L HPODE, 100 mmol/L MDA and 1 mmol/L acrolein resulted in 6-fold, 2-fold, 10-fold, 5-fold increases in carbonyl contents compared to control, respectively. Compared to other three oxidants, acrolein is the most active, but concentration of acrolein employed in the present study was 100-fold lower than that of MDA, therefore, carbonyl contents of acrolein modified soy protein was lower than that of MDA modified soy protein.

**Effects of oxidative modification on soy protein free sulphhydryl and total disulphide/sulphhydryl contents:**

Cysteines are reactive because of the presence of sulphhydryl groups and reactivity of cysteines often makes them vulnerable to oxidative damage. The oxidation state of cysteine residues was evaluated by sulphhydryl assay and the results are given in Table 1. Incubation of soy protein with increasing concentration of AAPH, MDA and acrolein resulted in a gradual decrease of free sulphhydryl groups ( $p < 0.05$ ) and only higher HPODE concentrations (1-100 mmol/L) could led to a statistically significant decrease ( $p < 0.05$ ) of free sulphhydryl content of soy protein. In addition, oxidized soy protein with increasing concentration of AAPH, MDA, HPODE and acrolein also resulted in decline of total disulphide/sulphhydryl. Compared to other three oxidants, peroxy radicals exhibit very high rate of sulphhydryl group modification, because peroxy

radicals could react with protein sulphhydryl groups to form sulphinyl radicals and thiol peroxy radicals which accelerated the decay of sulphhydryl groups (Platt and Gieseg, 2002). HPODE, MDA and acrolein could react with sulphhydryl groups of protein, resulting in the formation of stable adducts and loss of sulphhydryl groups (Esterbauer *et al.*, 1991).

**Effects of oxidative modification on free amine and available lysine contents of soy protein:**

Free amine and available lysine contents of soy protein decreased with increasing concentration of oxidants (Table 1 and 2). The extent of free amine and available lysine contents decrease in MDA modified soy protein was the most significant among four oxidants employed, that of acrolein modified soy protein followed and the reactivity between HPODE and amino groups was the lowest. Reaction of peroxy radicals with  $\epsilon$ -amino group resulted in the formation of carbonyl group which could further react with amino groups of soy protein to form Schiff's base, leading to a decrease of free amino and available lysine content (Liu *et al.*, 2000). HPODE and MDA could react with amino groups of protein to form amino-type adduct, resulting in a decrease of amino and available lysine content of soy protein (Kato *et al.*, 1999). Carbonyl groups of acrolein could modified amino groups of protein and the double bond of acrolein could form covalent adducts with  $\epsilon$ -amino group via Michael-type additions, which contributed to the decrease of available lysine content (Esterbauer *et al.*, 1991). In addition, the available lysine content in protein is also affected by the extent of protein aggregation which resulted in a

lower measured available lysine value than those of the native protein because of the smaller accessibility of the test reagent.

**Effects of oxidative modification on surface hydrophobicity of soy protein:** As shown in Table 2, the surface hydrophobicity of soy protein steadily decreased with increase of oxidants concentration. The observed decrease in surface hydrophobicity of modified soy protein could be attributed to the formation of hydrophilic groups (protein carbonyls groups), protein aggregation via hydrophobic interactions and structural modification of exposed hydrophobic residues of soy protein. The hydrophobic surface of globular protein was modified into a hydrophilic surface by oxidative modification and then solvent of water and oxidants enter the interior of globular protein which resulted in structural modification of interior amino acid residues, leading to protein unfolding and exposure of hydrophobic groups. The exposed hydrophobic groups interact with each other by hydrophobic interaction to form aggregates and cross-linking resulting from oxidative modification accelerated the development of aggregation (Grune *et al.*, 2003).

**Effects of oxidative modification on soy protein aggregation:** Size-exclusion chromatography chromatogram is an effective way to analyze soy protein aggregation and results of size-exclusion chromatography chromatogram analysis are shown Table 2. Molecular weight was estimated from the calibration curve of standard protein for the column (Fig. 1a). Non-modified soy protein presented a polydisperse distribution with two major peaks of 150 kDa (molecular weight distributed from 20 to 630 kDa), 14.1 kDa (molecular weight distributed from 2 to 20 kDa) and one little shoulder of molecular weight below 2.0 kDa (molecular weight distributed from 1 to 2 kDa) (Fig. 1b). The peak of 150 kDa probably corresponded to the  $\beta$ -conglycinin, glycinin and 15 S. The peak of 14.1 kDa probably corresponded to the 2S as well as subunits of  $\beta$ -conglycinin and glycinin. The observed peak of the lower molecular weight (below 2.0 kDa) was probably corresponded to the low molecular weight polypeptides. Incubation of soy protein with AAPH-derived peroxy radical, HPODE, MDA and acrolein resulted in a new peak of molecular weight higher than 1000 kDa formation (corresponding retention time was about 5.9 min). The new peak corresponded to the aggregate of  $\beta$ -conglycinin and glycinin and the percentage area of the peak could indicate the contents of soluble soy protein aggregates. The contents of soluble soy protein aggregates steadily increased as exposure of soy protein to increasing concentration of AAPH-derived peroxy radical, HPODE, MDA and

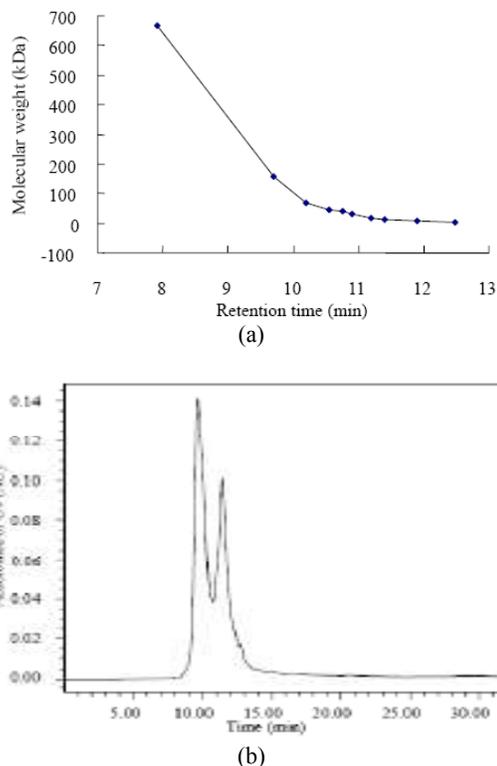
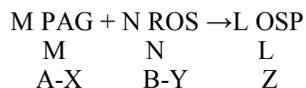


Fig. 1: High-performance size-exclusion chromatograph of (a) the calibration curve of standard protein (b) native soy protein

acrolein. The phenomena implied that oxidative modification of soy protein resulted in gradual aggregation.

**Mechanism of peroxy radicals, HPODE, MDA and acrolein-mediated soy protein oxidation:** In order to further investigate the mechanism of lipid peroxidation products-mediated soy protein oxidation, protein carbonyl contents, free sulfhydryl and total disulphide/sulfhydryl content, free amino content, available lysine content, surface hydrophobicity and content of soluble aggregate were defined as oxidative marker of oxidative modification. The reaction of soy protein with lipid peroxidation products could be assumed to reach equilibrium state after 24 h incubation and lipid peroxidation products could modify protein accessible groups after exposure of potential protein accessible groups. The reaction of soy protein with oxidants could be described as follows:



- PAG : Protein accessible groups
- ROS : Oxidants (Reactive oxygen species)
- OSP : Oxidized soy protein
- A : Initial content of accessible protein groups
- B : Initial content of oxidants

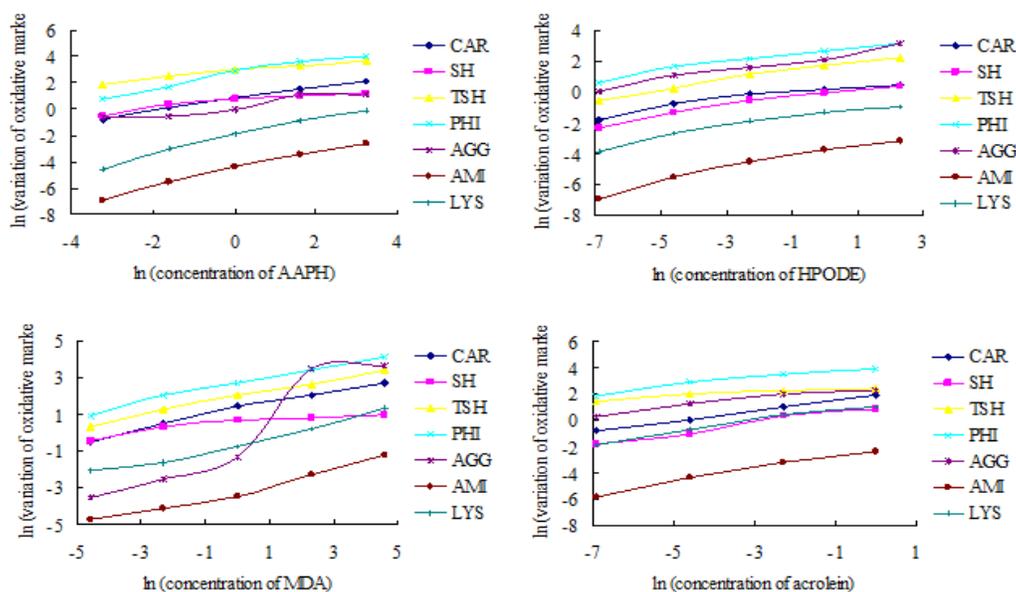


Fig. 2: The relationship between logarithmic value of oxidant content and logarithmic value of the variation of protein carbonyl, free sulfhydryl, total disulphide and sulfhydryl, free amine, available lysine, soluble aggregates content, and surface hydrophobicity of oxidized soy protein

CAR: Protein carbonyl content; SH: Free sulfhydryl content; TSH: Total disulphide/sulfhydryl content; PHI: Surface hydrophobicity; AGG: Content of aggregates; AMI: Free amine; LYS: Available lysine

X : Content of the reacted accessible protein groups in the equilibrium state

Y : Content of the oxidants in the equilibrium state

Z : Content of the oxidized soy protein in the equilibrium state

The reaction (1) reached equilibrium state after 24 h incubation and the equilibrium constant was:

$$K = Z^L / [(A-X)^M (B-Y)^N] \quad (1)$$

Concentration of oxidants in the reaction system was excessive compared to accessible protein groups. Therefore,  $B-Y \approx B$  and the Eq. (1) could be rewritten as:

$$L \ln Z = \ln K + M \ln (A-X) + N \ln B \quad (2)$$

Assuming three adjacent concentration of oxidants were  $B_{n-1}$ ,  $B_n$  and  $B_{n+1}$  ( $B_{n-1} < B_n < B_{n+1}$ ), respectively. The parameters of the corresponded equilibrium state were  $(A_{n-1}, B_{n-1}, X_{n-1}, Z_{n-1})$ ,  $(A_n, B_n, X_n, Z_n)$  and  $(A_{n+1}, B_{n+1}, X_{n+1}, Z_{n+1})$ , respectively. Substituting the three groups' parameters into Eq. 2 and then rearranged to Eq. (3):

$$L \left[ \ln \left( \frac{Z_{n+1}}{Z_n} \right) - \ln \left( \frac{Z_n}{Z_{n-1}} \right) \right] = \ln \left( \frac{K_{n+1}}{K_n} \right) - \ln \left( \frac{K_n}{K_{n-1}} \right) + M \left[ \ln \left( \frac{A_{n+1} - X_{n+1}}{A_n - X_n} \right) - \ln \left( \frac{A_n - X_n}{A_{n-1} - X_{n-1}} \right) \right] + N \left[ \ln \left( \frac{B_{n+1}}{B_n} \right) - \ln \left( \frac{B_n}{B_{n-1}} \right) \right] \quad (3)$$

The temperature of the reaction kept constant, therefore, the equilibrium constant  $K$  also kept constant and  $L [\ln (K_{n+1}/K_n) - \ln (K_n/K_{n-1})] = 0$ . In addition, the concentration of oxidants was geometric sequence, therefore,  $\ln (B_{n+1}/B_n) - \ln (B_n/B_{n-1}) = 0$ . The sum of protein accessible groups which involved in the reaction was related to the concentration of oxidants and the proportion of protein accessible groups were assumed to keep constant in order to facilitate the discussion, namely,  $X_1 = \alpha A_1$ ,  $X_2 = \alpha A_2$ ,  $X_3 = \alpha A_3$ . The Eq. (3) could rearrange to Eq. (4):

$$\ln \left( \frac{Z_{n+1}}{Z_n} \right) - \ln \left( \frac{Z_n}{Z_{n-1}} \right) = \frac{M}{L} \left[ \ln \left( \frac{A_{n-1}}{A_n} \right) - \ln \left( \frac{A_n}{A_{n+1}} \right) \right] \quad (4)$$

The relationship between logarithmic value of oxidant content and logarithmic value of the variation of oxidative marker are shown in Fig. 2 in order to explain Eq. (4).

If the curve in Fig. 2 bend downwards with increasing concentration of oxidants, the left side of Eq. (4) can be written:  $\ln (Z_{n+1}/Z_n) - \ln (Z_n/Z_{n-1}) < 0$ , namely,  $(A_n - A_{n-1}) / A_n > (A_{n+1} - A_n) / A_{n+1}$ . The situation indicates that extent of oxidative markers variation steadily decreased with increasing concentration of oxidants. The curve of free sulfhydryl and total disulphide/sulfhydryl of AAPH-derived peroxy radicals, HPODE and MDA modified soy protein belong to this situation. The situation also could apply

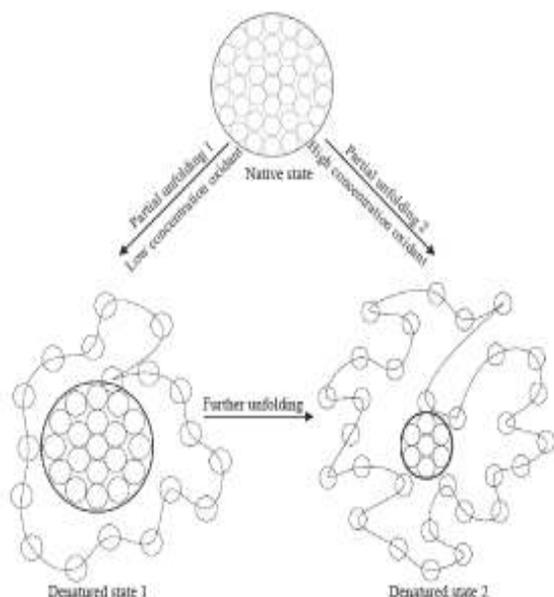


Fig. 3: A schematic diagram of partial unfolding of the globular structure of soy protein by oxidative modification

Circle: Reactive groups of soy protein; Denatured state 1: Low concentration oxidant; Denatured state 2: High concentration oxidant

to the curve of free amine and available lysine of AAPH-derived peroxy radicals, HPODE and acrolein modified soy protein as well as curve of all surface hydrophobicity. In order to explain the phenomenon, protein accessible groups were assumed to be evenly distributed and globular protein was made up of several layers of protein accessible groups which shown in Fig. 3. The outer layers contain the largest number of protein accessible groups and protein accessible groups in the interior of protein exist as potential state which modified in the condition of exposure to the oxidants. Surface protein accessible groups were preferentially modified by oxidant, resulted in protein unfolding and oxidative modification of interior protein accessible groups (Grune *et al.*, 2003). The number of protein accessible groups in globular protein decrease from outside to inside, therefore, the number of protein accessible groups increased with increasing of concentration of oxidants, but the extent of increase was smaller as concentration of oxidants increased, resulting in curve bend downwards. A majority of curves in Fig. 2 bend downwards with increasing concentration of oxidants, therefore, the average distribution model of protein accessible groups could explain majority mechanism of lipid peroxidation products-mediated soy protein oxidation.

If the curve in Fig. 2 is linear, the left side of Eq. (4) can be written:  $\ln(Z_{n+1}/Z_n) - \ln(Z_n/Z_{n-1}) = 0$ , namely,  $Z_{n+1} = Z_n = Z_{n-1}$ . The situation indicates that the number of protein accessible groups keep constant. The curve

of protein carbonyl content of acrolein modified soy protein belongs to this situation. The situation could be contributed to the high reactivity of acrolein because both double bonds and carbonyl groups in acrolein could modify protein. In addition, the molecule of acrolein is the smallest among the oxidant which employed, so acrolein could quickly penetrate into the interior of globular protein and simultaneously modify internal and external protein accessible groups.

If the curves in Fig. 2 bend upwards with increasing concentration of oxidants, the left side of Eq. (4) can be written:  $\ln(Z_{n+1}/Z_n) - \ln(Z_n/Z_{n-1}) > 0$ . The curve of soluble aggregate content of AAPH-derived peroxy radicals, HPODE and MDA modified soy protein when the concentration of oxidants is low belongs to this situation. Oxidative modification resulted in partial unfolding of protein, the exposed groups were further modified on the one hand and simultaneously form oxidation aggregates by hydrophobic and electrostatic interactions on the other hand, the oxidation aggregates could further aggregate by covalent cross-linking. When the concentration of oxidants was low, extent of covalent cross-linking was not significant, oxidation aggregates were soluble and made up of secondary interactions (hydrophobic and electrostatic interactions) and the corresponded aggregation could defined as primary aggregation. When concentration of oxidant was high, the primary aggregates could further aggregate by covalent cross-linking, resulting in rapid formation of soluble aggregates and primary aggregates could eventually develop into insoluble aggregates as extent of covalent cross-linking was significant (Grune *et al.*, 2003). Therefore, the curve of soluble aggregates content bend upwards when concentration of oxidants was low and curve of soluble aggregates content slowly changed as high concentration of oxidants employed. Primary oxidation aggregates further developed into insoluble aggregates by covalent cross-linking also may provide a partial mechanism of lipid peroxidation products-mediated soy protein oxidation.

## CONCLUSION

Soy protein was vulnerable to lipid peroxidation product. Exposure of soy protein to increasing concentration of AAPH-derived peroxy radicals, HPODE, MDA and acrolein resulted in gradual generation of protein carbonyl derivatives, loss of protein sulfhydryl groups, free amine groups and available lysine content. Oxidative modification of soy protein also resulted in decline of protein surface hydrophobicity as well as formation of protein oxidation aggregates. The average distribution model of protein accessible groups could explain majority mechanism of lipid peroxidation products-mediated soy protein oxidation. Primary oxidation aggregates further developed into insoluble aggregates by covalent cross-

linking also may provide a partial mechanism of lipid peroxidation products-mediated soy protein oxidation. Effects of lipid peroxidation products on the functional properties of soy protein need be studied further. Detailed research on the soybean proteins oxidative modified by lipid peroxidation products may uncover the role of products of lipid peroxidation during soy protein oxidation process.

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