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Oxidative modification of soy protein by peroxyl radicals

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ABSTRACT

Oxidative modification of soy protein by peroxyl radicals generated in a solution containing 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) under aerobic condition was investigated. Incubation of soy protein with increasing concentration of AAPH resulted in gradual generation of protein carbonyl derivatives and loss of protein sulphydryl groups. Circular dichroism spectra indicated that exposure of soy protein to AAPH led to loss of α-helix structure. Effect of oxidation on tertiary structure was demonstrated by surface hydrophobicity and tryptophan fluorescence. Surface hydrophobicity steadily decreased, accompanied by loss and burial of some tryptophan residues, indicating that soy protein gradually aggregated. The results of the size exclusion chromatogram (SEC) implied that incubation caused an AAPH-dose-dependent increase of fragmentation and aggregation of oxidised soy protein. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) indicated that non-disulphide linkages were involved in aggregate formation, and β-conglycinin was more vulnerable to peroxyl radicals than glycinin.

1. Indroduction

Soy protein is one of the most important food ingredients in the world. The functional properties of soy proteins are affected by many factors, one of which is the oxidation of protein structure (Hua, Cui, Wang, Mine, & Poysa, 2005). Huang, Hua and Qiu (2006a) found that the oxidation extent of soy protein was related to the content of residual lipids and activity of lipoxygenase in commercial low denatured defatted soybean flours. Further studies showed that the Lipoxygenase – catalysed linoleic acid peroxidation products were highly reactive substances that could change the structural characteristics of soy protein according to a free radical transfer mechanism (Huang et al., 2006a; Huang, Yu, Hua, & Qiu, 2006b). As the protein oxidation proceeded, a gradually lower gelling ability of soy protein was noticed (Kong, Li, Wang, Hua, & Huang, 2008).

Protein oxidation is the covalent modification of a protein induced either directly by reactive oxygen species (ROS) or indirectly by reaction with secondary by-products of oxidative stress (Shacter, 2000). Proteins are major targets for oxidants because of their high abundance in biological systems and high rate constants for reaction of oxidants with proteins (Davies, 2005). Oxidative stress results in multiple structural changes of target proteins. These can include oxidation of side chain groups, backbone fragmentation, cross-linking, unfolding, and changes in conformation. Ultimately, the structural changes lead to decrease or loss of biological function, nutritional value, and functional properties of the target proteins (Dean, Fu, Stocker, & Davies, 1997; Hawkins & Davies, 2001).

The rate of protein oxidation is dependent on the concentration of target and the rate constants for reaction of oxidant with protein (Davies, 2005). Hydroxyl radical (·OH)-mediated protein oxidation has received the most attention because hydroxyl radicals show the highest rate constants for the reactions with protein (Hawkins & Davies, 2001). Less attention has been given to peroxyl radical (ROO·)-mediated protein oxidation. Since peroxyl radicals can derive from a variety of oxidative environments (Sies, 1997), peroxyl radicals also play an important role in protein oxidation. In fact, peroxyl radicals can be considered as an important linkage between protein oxidation and lipid peroxidation because the oxidant is regarded as a major initiator factor of lipid peroxidation chain reactions and a wide ROS for protein oxidation (Duggan, Rait, Platt, & Gieseg, 2002; Gieseg, Pearson, & Firth, 2003). In many food and biological systems, protein oxidation and lipid peroxidation occur simultaneously because they are interdependent phenomena to some extent (Gatellier, Mercier, Rock, & Renerre, 2000).

Since peroxyl radicals are intermediates in lipid peroxidation and protein oxidation (Gieseg, Duggan, & Gebicki, 2000), the study of peroxyl radicals-mediated oxidation of soy protein is very important for us to understand the mechanisms of lipoxygenase – catalysed lipid peroxidation and protein oxidation during storage and processing.

Thermal decomposition of the water-soluble radical initiator 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) leads to the formation of carbon-centred radicals, which under aerobic conditions yield peroxyl radicals (Gieseg et al., 2000). Oxidative modification of proteins by AAPH-derived peroxyl radicals has

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been extensively studied in recent years (Chao, Ma, & Standman, 1997; Kang, Kim, Choi, Kwon, & Won, 2001; Kwon, Choi, Won, Kang, & Kang, 2000; Ma, Chao, & Stadtman, 1999). However, little information is available on the oxidation of soy proteins by peroxyl radicals. In this paper, the mechanism of peroxyl radical-mediated soy protein oxidation was investigated in order to characterise oxidative markers of peroxyl radical-modified soy protein and to determine the structural change of soy protein modified by increase concentration of AAPH-peroxyl radicals.

2. Materials and methods

2.1. Materials

Soybean was purchased from a local market, which was harvested from Heilongjiang Province, PR China. 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) was obtained from Wako Pure Chemical Co., Ltd (Osaka, Japan). Acrylamide, *N*,*N*′-methylenebisacrylamide, 1-anilino-8-naphthalene-sulphonate, and 5,5'-dithiobis (2-nitrobenzoate) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lower molecular mass protein standards with molecular mass range of 14.4–97.4 kDa for SDS–PAGE were bought from Shanghai Biochemical Institute (Shanghai, China). All other chemicals were of analytical reagent grade.

2.2. Preparation of lipid free-soybean protein isolates (SPI)

To control the oxidative level of the starting SPI and to obtain the low oxidatively modified SPI, defatted soybean flour and SPI were prepared in our laboratory in a unique way.

Soybean was cleaned and frozen at −18 °C for 12 h, and then freeze-dried for 72 h. The dried soybean was dehulled, and blended in deoxygenated water for 12 h. After vacuum filtrating, the wet soybeans were homogenised with five-fold of chilled acetone three times at −20 °C. The solid residue was vacuum dried at 20 °C and defatted using hexane three times with ratio of 1:4 (w/v) at 20 °C. After grinding to pass through an 80 meshes (size of 0.198 mm), the defatted soybean flour was incubated with hexane and ethanol with ratio of 1:2:4 (w/v/v) at 4 °C for 1.0 h. The slurry was vacuum filtered and the filter cake was immersed in 95% (v/v) ethanol at 20 °C for 1.0 h with a flour solvent ratio of 1:5 (w/v). After vacuum filtering, the cake was vacuum dried at 20 °C. The dried material was ground to pass through an 80 meshes (size of 0.198 mm) and stored at 4 °C. The SPI was subsequently prepared from the defatted flour. Aqueous alcohol washed soy flake precipitate were suspended in distiled water in a ratio of 1:15 (w/v) of flake to water, and pH was adjusted to 7.0 with 2 mol L^{-1} NaOH. After stirring for 1 h at 20 °C, the suspension was centrifuged at 15,900*g* for 30 min at 4 °C to recover the supernatant. Soy protein was precipitated by adjusting pH to 4.5 with 2 mol L^{-1} HCl and centrifuged at 6,000*g* for 30 min at 4 °C. After washing the curd with distiled water, the protein precipitate was re-suspended in distiled water with a ratio of 1:5 (w/v) of precipitate to water, and neutralised to pH 7.0 with 2 mol L^{-1} NaOH. After centrifuging at 15,000*g* for 30 min at 4 °C to remove small quantity of insoluble substances, the supernatant was freeze-dried, and then stored at 4 °C. The composition of the obtained SPI was (g kg^{-1}) as follows: protein (953.46, as determined by micro-Kjeldahl method), residue lipid (0.21, total lipids of samples extracted with a solvent of chloroform/methanol (2:1, v/v), ash (17.69), and moisture (28.25).

2.3. Protein oxidation

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 zero (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 10,000g for 1 h at 4 °C to remove small quantity of insoluble substances which was formed during solution cooling, the protein solution was dialysed against deionised water at 4 °C for 72 h to remove residual AAPH and the oxidised soy protein solution were freeze-dried and stored at 4 °C.

2.4. Protein carbonyl content measurement

Protein carbonylation was quantified according to the method described by Huang et al. (2006a) which uses the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl groups of native and oxidised soy proteins. The results were expressed as nmoles of carbonyl groups per milligram of soluble protein with molar extinction coefficient of 22,000 M^{-1} cm^{-1}. Soluble protein concentration was evaluated by the bicinchoninic acid method with bovine serum albumin as the standard.

2.5. Sulphydryl and disulphide content measurement

Contents of sulphydryl (free and buried SH) and total disulphide/sulphydryl groups in the soy protein were determined using Ellman's procedure modified by Huang et al. (2006a), and soluble protein concentration was evaluated by the bicinchoninic acid method with bovine serum albumin as the standard. The nmoles of SH per milligram soluble protein were calculated by using the extinction coefficient of 13,600 M^{-1} cm^{-1}.

2.6. Circular dichroism (CD) spectra measurement

CD spectra were scanned at the far-UV range (250–190 nm) with a CD spectropolarimeter (Jasco J-715, Jasco Corp., Tokyo, Japan) in a 0.1 cm quartz CD cuvette (Hellma, Muellheim, Baden, Germany) at 25 °C. The concentration of protein for CD analysis was 50 μg/mL. Distiled water used to dissolve soy protein was used as blank solution for all of the samples. The values of scan rate, response, bandwidth, and step resolution were 100 nm/min, 0.25 s, 1.0 nm, and 0.2 nm, respectively. Five scans were averaged to obtain one spectrum. The CD data were expressed in terms of mean molar ellipticity [θ] (deg cm^2 $dmol^{-1}$).

2.7. Measurement of surface hydrophobicity

The surface hydrophobicity of non-oxidised and oxidised soy protein were determined using 1-anilino-8-naphthalene-sulphonate as described by Huang et al. (2006a).

2.8. Intrinsic fluorescence measurement

Intrinsic fluorescence of non-oxidised and oxidised soy protein were determined at 25 °C in phosphate buffer (0.01 mol/L, pH 7.0) with an RF-5301PC fluorescence spectrometer (Shimadzu, Kyoto, Japan) at Ex 295 nm (slit width was 3 nm), Em 300–380 nm (slit width was 3 nm) and 10 nm/s of scanning speed. Phosphate buffer was used as blank solution for all of the samples.

2.9. High-performance size exclusion chromatogram (HPSEC)

Non-oxidised and oxidised soy protein solutions (5 mg/mL) were 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 μm (Satorious Company, Germany). A

Waters 2690 liquid chromatogram system equipped with a Shodex protein KW-804 column (Shodex Separation and HPLC Group, Tokyo, Japan) and a Wasters 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 and the eluent was monitored at 280 nm.

2.10. Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of non-oxidised and oxidised soy protein samples were run using the method of Huang et al. (2006a) with modification. A stacking gel with 40 g/L acrylamide and a running gel with 125 g/L acrylamide were used.

2.11. Statistical analysis

Analysis of variance (ANOVA) was performed using the General Linear Models procedures (GLM) of the Statistical Analysis System (SAS package, version 8.1). Statistical significance was set at $p < 0.05$. The results are presented as mean ± standard deviation.

3. Results and discussion

AAPH is a water-soluble thermolabile radical generator. Peroxyl radicals are considered to be the major ROS responsible for oxidation of protein induced by AAPH. Decomposition of AAPH in an aqueous phase at pH 7.4 and 37 °C produces peroxyl radical at a constant rate. Therefore, the amount of peroxyl radicals formed in this paper is proportion to the concentration of AAPH.

3.1. Characterisation of the oxidative markers of AAPH-mediated soy protein oxidation

Protein carbonyl is used to evaluate soy protein oxidation in this paper because protein carbonyl content is the most general indicator and by far the most commonly used marker of protein oxidation. Effect of AAPH concentration on soy protein carbonyl contents are given in Table 1. Exposure of soy protein to increasing concentration of AAPH led to a significant increase of protein carbonyl formation ($P < 0.05$). After incubated with 25 mmol/L AAPH, the oxidised soy protein showed a six-fold increase in carbonyl groups compared to control. Protein carbonylation is a common phenomenon of peroxyl radicals-mediated protein oxidation. Previous study showed that exposure of glutamine synthetase to 15 mM AAPH at 37 °C for 4 h led to almost 14-fold increase in the protein carbonyl content (Ma et al., 1999). AAPH treatment caused a dose-dependent and a time-dependent manner increase of protein carbonyl content for rat liver proteins and human ceruloplasmin, respectively (Chao et al., 1997; Kang et al., 2001).

It is well established that proteins can be oxidatively modified, giving rise to protein carbonyl groups through direct attack by ROS. Reactions of peroxyl radicals with protein molecule result in initial formation of carbon-centred radicals at either amino acid side chains or backbone, which under aerobic conditions yield protein peroxyl radicals. The subsequent reactions of amino acid side chain peroxyl radicals result in structural modification of side chain groups and carbonylation of some amino acid residues (Headlam & Davies, 2004). Side chain groups of cysteine, tryptophan, methionine, proline, valine, histidine, tyrosine, and lysine residues were vulnerable to peroxyl radicals (Kang et al., 2001; Kwon et al., 2000). Oxidation of lysine, arginine, proline and threonine residues yielded carbonyl derivatives (Mayo et al., 2003). Protein carbonyl derivatives can also be generated through oxidative cleavage of protein backbone at the site of α-carbon peroxyl radicals via α-amidation pathway or through oxidation of glutamine side chains, resulting in the formation of a peptide in which the N-terminal amino acids is blocked by an α-ketoacyl derivative (Davies, 2005; Dean et al., 1997; Hawkins & Davies, 2001). Therefore, protein carbonyls may be formed either by oxidation of vulnerable amino acid residues or oxidative cleavage of protein backbone by peroxyl radicals.

Cysteine residues were in many cases the most susceptible amino acid residues of proteins, but the carbonyl contents could not reflect oxidation state of cysteine residues. The oxidation state of cysteine residues in soy protein was evaluated by sulphydryl assay in this paper (Table 1). Oxidised soy protein with increasing concentration of AAPH resulted in a significant declining ($P < 0.05$) in free sulphydryl and total disulphide/sulphydryl. It was noteworthy that incubation with 25 mmol/L AAPH resulted in 84% and 47% loss of sulphydryl groups and total disulphide/sulphydryl, respectively. Peroxyl radicals could react with protein sulphydryl groups to form sulphinyl radicals. Sulphinyl radicals reacted with molecular oxygen to form thiol peroxyl radical that were capable of involving further oxidation of protein, resulting in a decrease of sulphydryl groups and accelerating protein oxidation. Platt and Gieseg (2002) found that free sulphydryl within BSA was very reactive, which could act as an antioxidant to scavenge aqueous peroxyl radicals and prevent protein oxidation products formation. Protein sulphydryl can be oxidised to reversible form (protein disulphide and sulphenic acid) or irreversible form (sulphinic and sulphonic acid) in different oxidative environments (Eaton, 2006; Thomas & Mallis, 2001). The simultaneous decrease of free sulphydryl and total disulphide/sulphydryl could be attributed to the formation of sulphur oxidation products other than disulphide bonds. Soybean glycinin contains 48 mole of cysteine per mole of protein but β-conglycinin is very low in sulphydryl group content. The major sulphydryl oxidation is therefore occurred on the soybean glycinin.

3.2. Effects of oxidative modification by peroxyl radicals on the secondary structure of soy protein

Far-ultraviolet CD spectra of a protein is a direct reflection of its secondary structure. Fig. 1 shows the CD spectra of native and oxidised soy protein. The spectra of native soy protein exhibited one strong positive peak in the vicinity of 194 nm, one negative shoulder at 218 nm, two negative troughs at 207 nm as well as 222 nm, and one weak positive peak between 220 and 230 nm. The positive

Table 1
Protein carbonyl, free sulphydryl, and disulphide groups of soy protein incubated with increasing concentration of AAPH for 24 h at 37 °C.

AAPH (mmol/L)	Protein carbonyl (nmole/mg)	Free sulphydryl (nmole/mg)	Total disulphide and sulphydryl (nmole/mg)
0	1.68(0.12)a	3.97(0.05)a	82.74(2.34)a
0.04	2.12(0.13)b	3.38(0.07)b	76.39(1.75)b
0.2	2.83(0.13)c	2.51(0.09)c	70.59(2.19)c
1	4.11(0.13)d	1.78(0.10)d	63.05(2.28)d
5	6.32(0.15)e	1.18(0.08)e	55.88(2.16)e
25	10.14(0.18)f	0.63(0.09)f	43.78(1.56)f

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 $p \geqslant 0.05$.

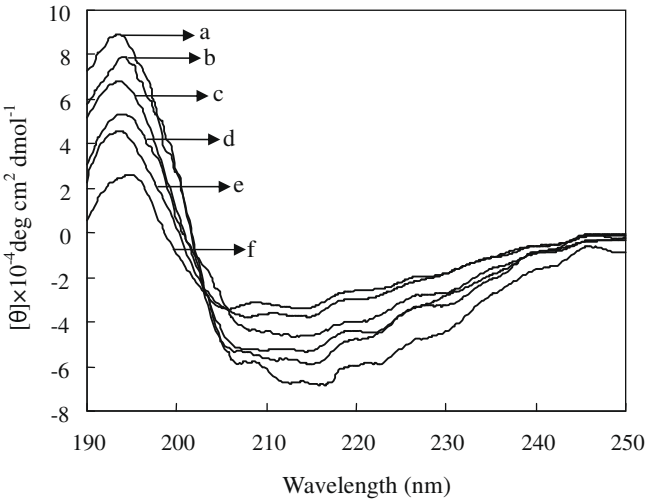

Fig. 1. Far-UV CD spectra of soy protein incubated with increasing concentration of AAPH at 37 °C for 24 h (a, control; b, 0.04 mmole/L AAPH; c, 0.2 mmole/L AAPH; d, 1 mmole/L AAPH; e, 5 mmole/L AAPH; f, 25 mmole/L AAPH).

peak at 194 nm and negative shoulder at 218 nm are both indicative of presence of β-sheet structure. The two negative troughs are caused by a negative Cotton effect characteristic of α-helical structure, and the weak positive peak between 220 and 230 nm is characteristic of disorder structure of protein (Choi & Ma, 2007). The results deduced from the CD spectra revealed that secondary structure of native soy protein was composed of four different conformations including α-helix, β-turn, and with a dominance of β-sheet and random structure. However, oxidation by peroxyl radicals affected the CD spectrum of soy protein significantly. As AAPH concentration increased from 0.04 to 25 mmol/L, intensity of the negative peak at 208 nm and 222 nm, positive peak at 194 nm, and negative shoulder at 218 nm simultaneously declined. The phenomena demonstrated that oxidation resulted in a gradual loss of α-helical structure with a concomitant decrease of β-sheet structure.

Peroxyl radicals-mediated protein oxidation was not always accompanied with significant change in the protein secondary structure. Only very minor differences were observed between the CD spectra of the native sticholysin I and peroxyl radicals-mediated oxidised sticholysin I (Campos et al., 1999). The different results between oxidised soy protein and oxidised sticholysin I may attribute to the difference of oxidative environments and protein structure.

3.3. Effects of oxidative modification by peroxyl radicals on tertiary structure of soy protein

Surface hydrophobicity of protein is one of the structural characteristics used to evaluate the change in protein conformation. Surface hydrophobicity of modified soy proteins are shown in Fig. 2A. Oxidised soy protein with increase of AAPH concentration led to a gradual decrease in surface hydrophobicity of soy protein. Boatright and Hettiarachchy (1995) and Liang (1999) also reported that oxidation of soy protein resulted in decrease of surface hydrophobicity. The observed decrease in surface hydrophobicity can be explained as combined effects of conformational change of soy protein. Aggregation via hydrophobic association, covalent modification of exposed hydrophobic residues (tryptophan residue), and introduction of new hydrophilic components (protein carbonyls groups) were related to the decline of surface hydrophobicity.

Oxidation of tryptophan residues of proteins has been shown to form a variety of products in varying yields, depending upon the kind of ROS used (Ma et al., 1999). Oxidative damage of tryptophan

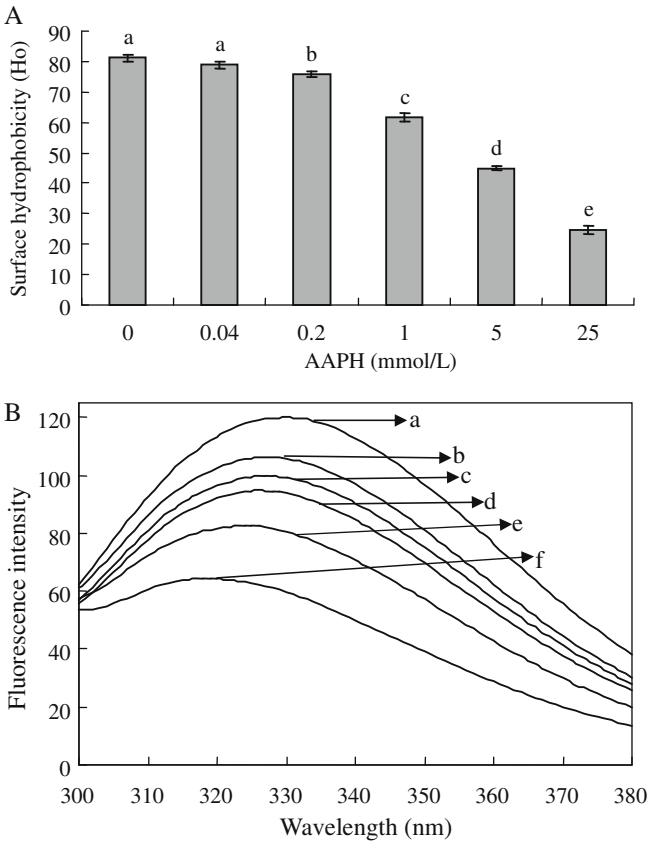

Fig. 2. Effect of 24 h incubation with different concentration AAPH at 37 °C for 24 h on the (A) surface hydrophobicity (columns with different letters are significantly different ($p < 0.05$)) and (B) intrinsic fluorescence of in phosphate buffer (0.01 mol/L, pH 7.0) of soy protein (a, control; b, 0.04 mmole/L AAPH; c, 0.2 mmole/L AAPH; d, 1 mmole/L AAPH; e, 5 mmole/L AAPH; f, 25 mmole/L AAPH).

residue can not be evaluated by protein carbonyl content because new carbonyl groups are not introduced in majority of oxidation products of tryptophan. Tryptophan fluorescence is not only used as a marker for detecting conformational change of protein, but also used as an efficient way to evaluate oxidative loss of tryptophan residues (Campos et al., 1999). Intrinsic fluorescence spectra of tryptophan residues in oxidised soy protein were obtained to track conformational changes and loss of tryptophan residues which induced by peroxyl radicals in this paper. As shown in Fig. 2B, the fluorescence spectrum of native soy protein excited at 295 nm had a broadband with a maximum at 330 nm in 0.01 mol/L phosphate buffer (pH 7.0). However, exposure of soy protein to increasing concentration of AAPH led to a decrease of tryptophan fluorescence intensity and a blue-shifted wavelength of the maximum emission (from 330 to 319 nm). Tryptophan residue is vulnerable to peroxyl radicals, and loss of intrinsic tryptophan fluorescence is an early phenomenon of peroxyl radicals-mediated protein oxidation (Campos et al., 1999; Chao et al., 1997; Ma et al., 1999). The decrease of intrinsic fluorescence intensity indicated that tryptophan residues of soy protein were destroyed by peroxyl radicals. Tryptophan residues have the lowest one-electron oxidation potentials of all of the amino acid residues in proteins. Therefore, peroxyl radicals could act as an initiator to convert tryptophan residues into metastable tryptophan carbon-centred free radicals, which under aerobic conditions yielded tryptophan peroxyl radicals that were capable of accelerating damage to protein (Simat & Steinhart, 1998). The wavelength of maximum emission of tryptophan fluorescence (λ_{max}) is sensitive to its local environment. λ_{max} shorter than 331 nm indicated that most of the tryptophan residues were inside

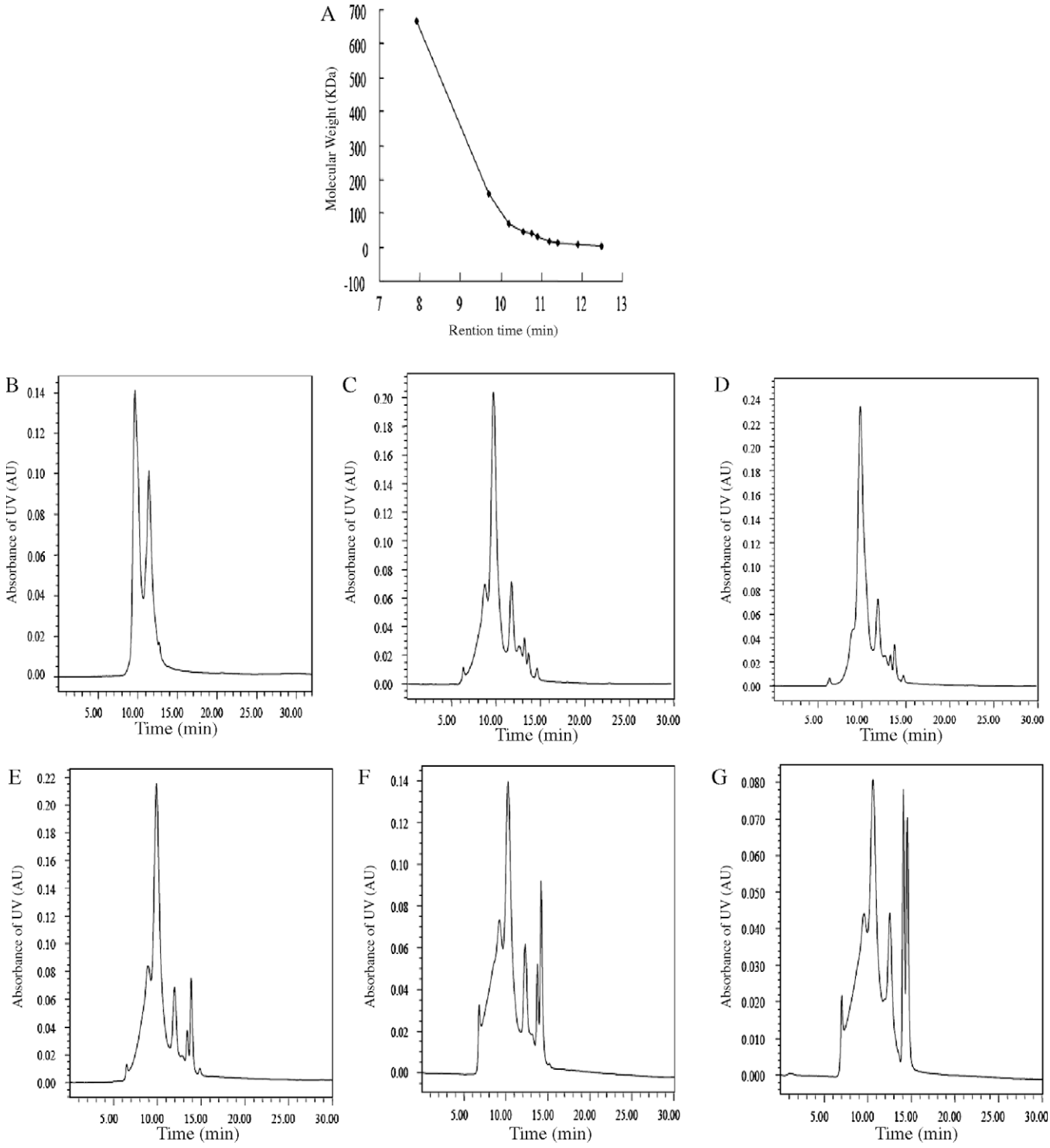

Fig. 3. High-performance size exclusion chromatograph of soy protein incubated with different concentration AAPH (A) the calibration curve of standard protein, (B) control, (C) incubated with 0.04 mmole/L AAPH, (D) incubated with 0.2 mmole/L AAPH, (E) incubated with 1 mmole/L AAPH, (F) incubated with 5 mmole/L AAPH, (G) incubated with 25 mmole/L AAPH. A calibration curve of 10 standard proteins was used for interpreting the results. Ten standard proteins were thyroglobulin (MW: 669, 000), aldolase (MW: 158,000), BSA (MW: 67,000), ovalbumin (MW: 43,000), peroxidase (MW: 40,200), adenylate kinase (MW: 32, 000), myoglobin (MW: 17,000), ribonuclease A (MW: 13,700), aprotinin (MW: 6500), and vitamin B12 (MW: 1350), respectively.

of "non-polar" environment of protein molecule. In contrast, λ_{max} longer than 330 nm implied that tryptophan residues were assigned a "polar" environment (Vivian & Callis, 2001). Therefore, blue shift (from 330 to 319 nm) of the maximum emission in this paper demonstrated that the previously exposed tryptophan residues of native soy protein were buried into the inner of oxidised soy protein, resulting from soy protein aggregation.

3.4. Effects of oxidative modification by peroxyl radicals on soy protein aggregation

The size exclusion chromatogram using a high-performance liquid chromatogram system was used to study molecular weight distribution profiles of the oxidised soy protein, and the results are shown in Fig. 3 and Table 2. Molecular weight was estimated

Table 2
Percentage area of peaks of high-performance size exclusion chromatograph of soy protein incubated with increasing concentration of AAPH for 24 h at 37 °C.

AAPH mmolL^{-1}	Percentage area of peak (%) corresponding retention time of				
	6.0 min (>1000 kDa)	9–10 min 150 kDa	11–12 min 14.1 kDa	12–13 min <2 kDa	13–15 min <1 kDa
0	0	56.80	39.87	3.33	0
0.04	0.54	74.63	15.62	3.60	5.61
0.2	0.59	76.77	13.35	3.65	5.64
1	0.93	77.38	7.57	4.91	9.21
5	2.96	71.46	0	12.88	12.7
25	3.17	63.35	0	16	17.48

from the calibration curve of standard protein for the column, as shown in Fig. 3A. Native soy protein presented a polydisperse distribution with two major peaks of 150 kDa (56.80% area, molecular weight distributed from 20 to 630 kDa), 14.1 kDa (39.87% area, molecular weight distributed form 2 to 20 kDa), and one little shoulder of molecular weight below 2.0 kDa (3.33% area, molecular weight distributed from 1 to 2 kDa) (Fig. 3B). The continuous distribution of molecular weight indicated that a series of different molecular weight proteins existed in soy protein. Soy protein can be fractionated into four main groups on the basis of sedimentation velocity as 2S (<20 kDa), 7S (150–190 kDa, β-conglucinin), 11S (320–360 kDa, glycinin), and 15S (dimer of glycinin). Therefore, the peak of 150 kDa probably corresponded to β-conglucinin, glycinin and 15S, and the peak of 14.1 kDa probably corresponded to the 2S as well as subunits of β-conglucinin and glycinin. The observed peak of the lower molecular weight (below 2.0 kDa) was probably corresponded to the low molecular weight polypeptides. The chromatogram changed as soy protein incubated with increase concentration of AAPH. At AAPH treatment concentration of 0.04 mmol/L, a new peak of molecular weight higher than 1,000 kDa emerged which probably corresponded to the aggregation of β-conglucinin and glycinin (Fig. 3C), and the percentage area of the peak steadily increased as exposure of soy protein to increase concentration of AAPH. The phenomena implied that oxidation induced soy protein gradual aggregation (Fig. 3C–G). As concentration of AAPH increased from 0 to 25 mmol/L, percentage area of the peak corresponded to molecular weight below 2 kDa and below 1 kDa simultaneously increased (Fig. 3B–G). In contrast, percentage area of the peak corresponded to molecular weight of 14.1 kDa dramatically declined, reaching the minimum (0% area) when AAPH concentration was 5–25 mmol/L (Fig. 3B–G). The phenomena implied that peroxyl radicals simultaneously induced subunits of 7S and 11S fragmentation and aggregation. Moreover, percentage area of the peak corresponded to molecular weight of 150 kDa increased with increase of AAPH concentration, reaching the maximum (77.38% area) when AAPH concentration was 1 mmol/L, and then decreased with further increase of AAPH concentration to 25 mmol/L. The phenomenon was probably due to fragmentation significantly increased with increase of AAPH concentration. In conclusion, the results of SEC indicated that fragmentation and aggregation simultaneously happened, and extent of fragmentation and aggregation were increased in an AAPH-dose-dependent manner.

SDS–PAGE was employed to monitor structural changes of soy protein subunits after oxidation. Fig. 4 shows SDS–PAGE patterns of native and oxidised soy protein. The SDS–PAGE patterns of

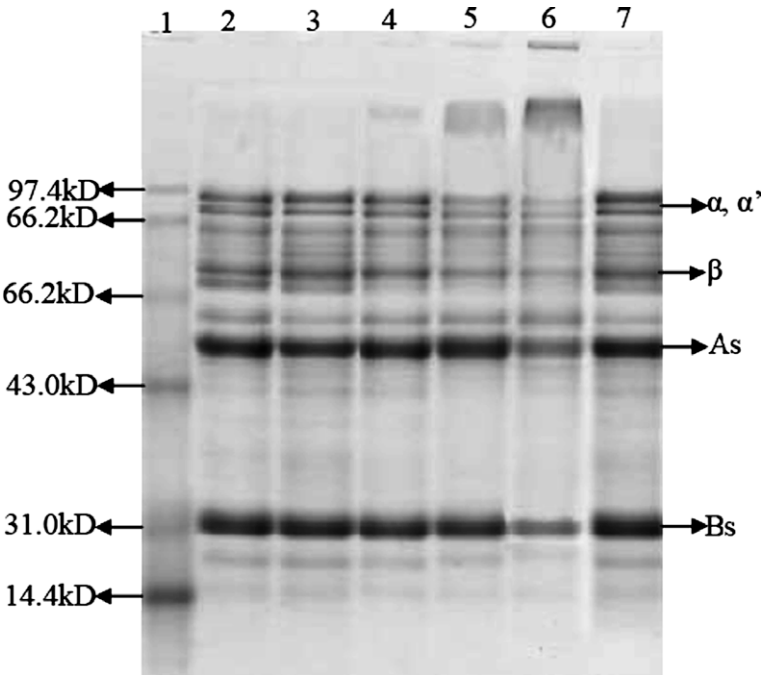

Fig. 4. SDS–PAGE analysis of soy protein incubated with different concentration AAPH for 24 h and standard low molecular weight markers. α', α and β indicate subunits of β-conglycinin (7S). As and Bs indicate acidic and basic polypeptides of glycinin (11S), respectively. Lane 1-the molecular weight markers to which be pointed with arrows in the left: hen egg white lysozyme (14.4 kDa), trypsin inhibitor (20.1 kDa), bovine carbonic anhydrase (31.0 kDa), rabbit actin (43.0 kDa), bovine serum albumin (66.2 kDa), and rabbit phosphorylase b (97.4 kDa), respectively; Lane 2, 0.04 mmol/L AAPH; Lane 3, 0.2 mmol/L AAPH; Lane 4, 1 mmol/L AAPH; Lane 5, 5 mmol/L AAPH; Lane 6, 25 mmol/L AAPH; Lane 7, control.

native soy protein presented the characteristic bands for the subunits of β-conglycinin (α', α, and β) and glycinin (As and Bs) (Fig. 4 lane 7). Electrophoretic patterns of oxidised soy protein were related to AAPH concentration. When concentration of AAPH was 0.04 and 0.2 mmol/L, the electrophoretic patterns of modified soy protein were identical to that of control (Fig. 4 lane 2–3). The phenomena indicated that concentration of peroxyl radicals changed the overall protein structure and had no significant impact on subunits. However, intensity of α', α, β and As bands gradually declined with increase of AAPH concentration from 1 to 25 mmol/L (Fig. 4 lane 4–6), and that of Bs bands steadily decreased with increase of AAPH concentration from 5 to 25 mmol/L (Fig. 4, lane 5–6). The phenomena indicated that β-conglycinin were more vulnerable to peroxyl radicals than glycinin. In addition, one band of aggregate protein appeared on the top of running gel when concentration of AAPH was 1 mmol/L, and intensity of the band gradually increased as AAPH concentration increasing from 1 to 25 mmol/L (Fig. 4, lane 4–6). Another band of aggregate protein appeared on the top of stacking gel when concentration of AAPH was 5 mmol/L, and intensity of the band increased when concentration of AAPH was 25 mmol/L (Fig. 4 lane 5–6). In this condition, β-mercaptoethanol was added to the sample to break the disulphide bonds in soy protein prior to loading of the protein samples into gel. Therefore, the bands on the top of running gel and stacking gel were attributed to the aggregation of subunits via non-disulphides linkage. Results of electrophoresis indicated that peroxyl radicals caused soy protein aggregation which non-disulphide covalent was involved in aggregate formation, and subunits of β-conglycinin were more vulnerable to peroxyl radicals than glycinin. Aggregation is a common phenomenon for peroxyl radical-mediated protein oxidation (Kang et al., 2001; Ma et al., 1999; Mayo et al., 2003). Aggregation of oxidised soy protein probably resulted from cross-linking of protein radicals derivatives which induced by peroxyl radicals (Chao et al., 1997).

4. Conclusions

Soy protein was vulnerable to peroxyl radicals. Exposure of soy protein to increase concentration of AAPH resulted in accumulation of protein carbonyls and diminution of sulphydryl. Oxidation of soy protein was accompanied by gradual loss of α-helix structure, backbone fragmentation, and aggregation. Non-disulphide covalent was involved in aggregate formation, and β-conglycinin was more susceptible to oxidative modification than glycinin. Effect of peroxyl radicals on the functional properties of soy protein needs be studied further. Detailed researches on the soybean proteins oxidative modified by peroxyl radicals may uncover the role of products of lipid peroxidation during soy protein oxidation process.

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