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Effects of Malondialdehyde Modification on the in Vitro Digestibility of Soy Protein Isolate

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ABSTRACT: Soy protein isolate (SPI) was modified by lipid peroxidation product malondialdehyde (MDA), and the in vitro digestibility of modified SPI was investigated. Results indicated that incubation with increasing MDA concentration resulted in significant carbonyl group generation and loss of free amino groups of SPI. Fluorescence loss of natural tryptophan and formation of Schiff base were observed. Noncovalent interaction between molecules was enhanced and became the main force that led to the solubility reduction of MDA-modified SPI. Differential scanning calorimetry (DSC) indicated that SPI had higher thermal stability and lower total calorimetric enthalpy after MDA pretreatment. Electrophoresis showed that β-conglycinin was more sensitive to MDA modification. In vitro digestion indicated that MDA could induce non-disulfide covalent polymer of SPI, which could not be digested by pepsin and pancreatin. β subunits of β-conglycinin became more resistant to digestion with increasing MDA concentration. Evaluation of the free amino acid profile in the digests indicated that MDA-modified SPI had deteriorating nutritive quality.

KEYWORDS: *soy protein isolate, MDA, Schiff base, electrophoresis, in vitro digestibility, amino acid*

INTRODUCTION

Soy protein is an important food ingredient used in many protein-based food formulations, because it can improve the food quality and nutrition. Soy protein provides all of the essential amino acids needed to fulfill human nutritional requirements. Its protein value is essentially equivalent to that of food proteins of animal origin.[1]

Soy protein isolate (SPI) is widely used in lipid-enriched products such as meat products. Lipid is sensitive to oxidation under both enzymatic conditions, such as lipoxygenase, and nonenzymatic condition, such as ultraviolet light. Lipid peroxidation can produce free radicals as well as lipid hydroperoxides and reactive aldehydes, which can react with protein, leading to oxidative damage of protein.[2] Interaction of oxidizing soybean oil and soy protein resulted in the decrease of protein solubility, soluble protein hydrophobicity, and free amino groups of protein.[3] During the storage of whole milk powder and incubation of SPI with soybean oil, fluorescent compounds formed, which had an excitation wavelength around 350 nm and an emission wavelength around 450 nm.[3,4] These products may partly come from the reaction of lipid peroxidation product MDA with protein. However, it is difficult to specify the products, as lipid peroxidation is a very complex process, producing various kinds of byproducts. To better understand the effects of lipid peroxidation on food protein, it is necessary to understand how the main kinds of byproducts from lipid peroxidation react with protein and their effects on protein functional and nutritive quality. Wu et al. investigated the effects of various kinds of lipid peroxidation products (including MDA) on the gelling properties of SPI.[5,6] Chen et al. investigated the effect of AAPH-derived free radical on the emulsifying properties and digestibility of SPI.[7,8] All of these studies have given us a better understanding of the effects of lipid peroxidation on SPI.

Lipid hydroperoxides as well as their products of decomposition are potentially reactive substances that can cause deterioration of food proteins or amino acids.[9] The secondary products arising from hydroperoxide decomposition also readily damage protein and amino acids through formation of covalent bonds.[9] Among the secondary products, aldehydes have received the most attention because of their propensity to form Schiff bases with amino groups, and, in particular, the bifunctional MDA can cross-link protein via Schiff base formation.[9] Therefore, during lipid peroxidation, SPI may be vulnerable to MDA damage. For example, soy protein is widely used in meat products. MDA concentration in meat materials varies with the kind of animal. In raw meat materials (without processing), MDA concentration is low if kept in a refrigerator. On the contrary, heating and lighting will promote lipid peroxidation during processing. One study indicated that cooking chicken by microwave, conventional oven, deep-fat-frying, and boiling increased MDA concentrations by 60-, 55-, 17- 19-, and 22-fold, respectively.[10] Many meat products now are kept at room temperature. Thus, the relatively high storage temperature will accelerate lipid peroxidation. Therefore, the MDA concentration in real food products will vary over a large range because of different raw materials, processing methods, and storage conditions. Because of the importance of MDA as a lipid peroxidation product in vivo, the reactivity of various kinds of proteins with MDA under physiological conditions has been widely investigated.[11,12] However, the reaction of MDA with food proteins during food processing and storage is rarely investigated.

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An in vitro method using a multienzyme system for the estimation of protein digestibility has been widely used because of its high efficiency and low cost compared with in vivo bioassays.[13] Santé-Lhoutellier et al. had used a multienzyme system to investigate the in vitro digestibility of animal muscle protein under oxidative stress.[14,15] These investigations have shown that oxidation had either positive or negative effects on the protein digestibility depending on the oxidation extent.

The effects of chemical modification by lipid peroxidarion products on SPI's digestibility are not yet fully elucidated. The present study aims to evaluate the digestibility of SPI modified by different concentrations of MDA. The chemical characteristics of MDA-modified SPI and properties of its in vitro digests are presented and discussed. This information will be useful for the evaluation of soy protein as a nutritional source for lipid-enriched food products.

MATERIALS AND METHODS

Materials. Defatted soy flakes were purchased from Yuwang Group (Shangdong, China). 1,1,3,3-Tetramethoxypropane (TMP) was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Pepsin (104.75 units/mg) from porcine gastric mucosa, dithiothreitol (DTT), and bicinchoninic acid (BCA) protein assay kit were purchased from Dingguo (Beijing, China). Pancreatin (227.00 units/mg) from porcine pancreas and *o*-phthaldialdehyde (OPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent grade and obtained in China.

SPI Preparation. Defatted soy flake powder was mixed with 15-fold (in weight) deionized water, and the mixture (pH 6.8) was adjusted to pH 7.5 with 2.0 M NaOH. After stirring for 2 h, the resulting suspension was centrifuged at 8000*g* for 20 min at 4 °C to remove the insoluble material. Then the pH of the supernatant was adjusted to 4.5 with 2.0 M HCl, and the precipitate was collected by centrifugation at 8000*g* for 10 min at 4 °C. The precipitate was then redissolved with 5-fold (in weight) deionized water, and the pH was adjusted to 7.0 with 2.0 M NaOH. The neutral SPI solution was then freeze-dried and stored at 4 °C until use.

Preparation of MDA Stock Solution. A fresh MDA stock solution was prepared by hydrolyzing TMP according to the method described by Wu et al.,[5] with minor modification. Briefly, 8.4 mL of TMP was mixed with 10.0 mL of 5.0 M HCl and 31.6 mL of ultrapure water and shaken at 40 °C in the dark for 30 min to obtain MDA through acidic hydrolysis. After the TMP was hydrolyzed, the pH was adjusted to 7.4 with 6 M NaOH. The concentration of MDA in the stock solution was estimated by absorbance at 267 nm using a molar extinction coefficient value of 31500 M^{-1} cm^{-1}. MDA stock solution was made fresh daily.

Modification of SPI with MDA. Control and MDA-modified SPI were prepared according to the method described by Wu et al.[5] SPI suspension (40 mg/mL containing 0.5 mg/mL sodium azide, suspended in 10 mM sodium phosphate buffer, pH 7.4) was 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 concentrations of MDA were 0, 0.5, 3, 5, and 10 mM. The 0 mM MDA treated sample was control SPI, and the others were MDA-modified SPI. After that, the SPI suspension was dialyzed against deionized water at 4 °C for 72 h to remove free MDA and salt. Then the dialyzed solution was freeze-dried and stored at 4 °C until used.

Protein Carbonyl Group Measurement. Protein carbonyl group content in control and MDA-modified SPI was quantified according to the method described by Huang et al.,[16] using an SP-721 UV spectrophotometer (Shanghai, China). Samples were suspended in 10 mM sodium phosphate buffer (pH 7.0), stirred for 30 min at room temperature. Then the protein suspension was centrifuged at 10000*g* for 10 min. The supernatant was collected, and the protein concentration (approximately 1−3 mg/mL) in the supernatant was evaluated using a BCA protein assay kit. In 15 mL capped polyethylene centrifuge tubes, 1 mL of SPI solution was mixed with 3 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 M HCl and incubated at room temperature for 2 h. A matching aliquot of SPI solution was mixed with 3 mL of 2 M HCl as an absorbance blank. Then 4 mL of 20% trichloroacetic acid (TCA) was added to each tube and blended. After standing for 20 min, the mixture was centrifuged at 10000*g* for 10 min at 4 °C. The supernatant was discarded, and the pellet was washed three times with 5 mL of ethanol/ethyl acetate solution (1:1, v/v). The protein, free of DNPH, was then dissolved in 3 mL of 6 M guanidine hydrochloride in 0.1 M sodium phosphate buffer (pH 7.0). The absorbance at 367 nm was corrected by the absorbance in the HCl blank. The results were calculated using a molar extinction coefficient of 22000 M^{-1} cm^{-1} and expressed as nanomoles of carbonyl group per milligram of protein.

Free Amino Group Measurement. Free amino group content was determined by its derivatization with OPA. Fresh OPA reagent was prepared daily by the following steps:[17] (1) Disodium tetraborate decahydrate (7.620 g) and 200 mg of sodium dodecyl sulfate (SDS) were dissolved in 150 mL of deionized water. (2) OPA (160 mg) was dissolved in 4 mL of ethanol and transferred to (1). (3) DTT (176 mg) was added to (1) and brought to the final volume of 200 mL with deionized water. Control and MDA-modified SPI samples were suspended in 10 mM sodium phosphate buffer (pH 7.0) and stirred for 30 min at room temperature. Then the protein suspension was centrifuged at 10000*g* for 10 min. The supernatant was collected, and the protein concentration in the supernatant was evaluated using a BCA protein assay kit. Protein concentration was adjusted to 0.04 mg/mL for measurement. Four hundred microliters of each protein solution was mixed with 3 mL of OPA reagent for exactly 2 min. Then the fluorescence intensity was measured by the F7000 fluorescence spectrophotometer (Hitachi Co., Japan) with excitation and emission wavelengths set at 340 and 430 nm, respectively. The slit width was 5 nm. Free amino group content was calculated from a standard curve made by serine and expressed as micromoles of free amino group per milligram of protein. The linearity (R^2 = 0.9996; $p < 0.05$) between serine content (0.0−0.1 mM) was statistically significant.

Fluorescence Spectra of Tryptophan and Schiff base. Control and MDA-modified SPI samples were suspended in 10 mM sodium phosphate buffer (pH 7.0) and stirred for 30 min at room temperature. Then the protein suspension was centrifuged at 10000*g* for 10 min. The supernatant was collected, and the protein concentration in the supernatant was evaluated using a BCA protein assay kit. Protein concentration was adjusted to 0.04 mg/mL for tryptophan measurement and to 0.2 mg/mL for Schiff base measurement. To determine the fluorescence emission spectrum of tryptophan, the excitation wavelength was set at 290 nm, and the emission spectrum was recorded from 300 to 400 nm.[7] To determine the fluorescence emission spectrum of the Schiff base, the excitation wavelength was set at 350 nm, and the emission spectrum was recorded from 400 to 600 nm.[18] The slit width was set at 5 nm, and data were collected at 240 nm/min.

Determination of SPI Solubility in Various Solutions. Control SPI and MDA-modified SPI were dispersed in four types of solvent with the same concentration of 5 mg/mL: S1, 0.1 M, pH 7.5, sodium phosphate buffer (PB); S2, 1% SDS in PB; S3, 8 M urea and 1% SDS in PB; S4, 20 mM DTT, 8 M urea, and 1% SDS in PB. The dispersions were then incubated at 25 °C for 10 h. After incubation, the samples were centrifuged at 10000*g* for 10 min at 25 °C, and the protein concentration in the supernatant was measured according to the BCA method. To determine the solubility in the S4, supernatant was diluted with iodoacetamide solution to eliminate the influence of DTT on the BCA protein assay.[19] Solubility was calculated as the percentage of dissolved protein in the supernatant to the initial total protein content.

Differential Scanning Calorimetry. Differential scanning calorimetric study was performed using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE, USA). Approximately 1.5 mg of sample was weighed into an aluminum liquid pan, and 10 μL of 10 mM sodium phosphate buffer (pH 7.2) was added. The pan was hermetically sealed and equilibrated at 25 °C for >6 h. Then the pan was heated from 20 to 110 °C at a rate of 10 °C/min. A sealed empty pan was used as a reference. Peak transition or denaturation

temperature (T_d) and enthalpy change (ΔH) were computed from the curve by Universal Analysis 2000, version 4.1D (TA Instruments-Waters LLC). All experiments were conducted in triplicate.

In Vitro Digestion of SPI. SPI suspension (2% w/v, in deionized water) was adjusted to pH 2.0 with 2.0 M HCl, and then pepsin (4 units/mg, protein basis) was added. The suspension was incubated at 37 °C for 1 h. Then the pH was adjusted to 7.0 with 2.0 M NaOH, and pancreatin (4 units/mg, protein basis) was added. The suspension was incubated at 37 °C for 2 h and then submerged in a boiling water bath for 5 min to stop the digestion. The SPI digests were centrifuged at 10000*g* for 10 min at 4 °C, and the supernatant was collected and stored at −20 °C until used. Aliquots of SPI digests were removed at 0 h, 1 h pepsin digestion, and 2 h pancreatin digestion for further analysis. The digestion was replicated two times.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a discontinuous buffered system,[20] using 12% separating gel and 5% stacking gel. The digest samples (100 μL) were mixed with 400 μL of 0.06 M Tris-HCl buffer (pH 6.8), containing 2% (w/v) SDS, 25% (v/v) glycerol, 0.1% (w/v) bromophenol blue, and 5% (v/v) 2-mercaptoethanol (2-ME). The mixture was heated for 5 min in boiling water and centrifuged at 10000*g* for 10 min before electrophoresis. For each sample, 8 μL of the supernatant was loaded to each lane. A molecular weight marker made from phosphorylase (97 kDa), albumin (66 kDa), gultamic dehydrogenase (53 kDa), glyceraldehyde-3-phosphate (36 kDa), and trypsinogen (24 kDa) was used as reference. After the electrophoresis, the gel was stained using the Coomassie brilliant blue R-250 stain solution (45% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250) for 1.0 h and destained by destain solution (methanol/acetic acid/water, 1:1:8, v/v/v) for 24.0 h with two or three changes of destain solution.

Free Amino Acid (FAA) Analysis. For the determination of FAA, salicylic acid (400 μL) was added to 2 mL of digest sample to precipitate peptide or protein. After incubation for 2 h at 4 °C, the solution was centrifuged at 10000*g* for 15 min at 4 °C. The supernatant was passed through cellulose acetate membranes with pore size of 0.22 μm. The filtrate was subjected to an A300 auto amino acid analyzer (membra Pure, Bodenheim, Germany) equipped with a T263 column (4.6 mm × 100 mm) for amino acid separation. Postcolumn reaction with ninhydrin yielded amino acid derivatives. For calculation, a calibration curve was obtained with standard amino acid mixture (membra Pure), and quantification was made on the basis of retention time and peak area of standard compounds.

Statistical Analysis. Statistical calculations were performed using the statistical package SPSS 11.5 (SPSS Inc., Chicago, IL, USA) for one-way ANOVA. Least-squares difference was used for comparison of mean values among treatments and to identify significant differences ($p < 0.05$) among treatments.

RESULTS AND DISCUSSION

Chemical Characteristics of MDA-Modified SPI. Protein carbonyl group and free amino group content were used to characterize the interaction between SPI and lipid peroxidation byproduct MDA. Effects of MDA modification on the carbonyl group and free amino group content of SPI are given in Table 1. The carbonyl group level of control SPI was 5.26 nmol/mg. This value was close to that reported by Liu et al., who also investigated the carbonyl group content of SPI.[21] Carbonyl group content significantly increased ($p < 0.05$) as the MDA concentration increased. Simultaneously, free amino group content of SPI gradually decreased. Reaction with 10 mM MDA resulted in 41.11% loss of free amino group content. MDA, a lipoperoxidation-derived aldehyde, can bring about secondary oxidative damage to proteins.[22] Carbonyl derivatives can be introduced into the protein molecule as a consequence of reactions of MDA with nucleophilic side chain of Cys, His, and Lys residues, especially the ε-amino of lysine.[23,24] MDA is a molecule that includes two reactive carbonyl functional groups. These groups will react with free amino groups of protein forming Schiff base. Then previous "free" amino groups become "non-free" while the carbonyl functional groups in the MDA are introduced into the MDA-modified protein. Therefore, the results observed the increase of carbonyl group content and the decrease of free amino group content.

Table 1. Carbonyl Group and Free Amino Group Content of Control and MDA-Modified SPI[a]

MDA (mM)	carbonyl group (nmol/mg)	free amino group (μmol/mg)
0	5.26 ± 0.32 a	0.90 ± 0.04 a
0.5	9.04 ± 0.22 b	0.84 ± 0.01 a
3	26.68 ± 0.23 c	0.73 ± 0.07 b
5	35.5 ± 0.43 d	0.64 ± 0.05 b
10	50.33 ± 0.95 e	0.53 ± 0.02 c

[a]Values in the same column followed by different letters (a−e) are significantly different ($p < 0.05$).

Tryptophan residues are able to emit fluorescence in the range of 300−400 nm when excited at 290 nm. Schiff base is a conjugated fluorochrome with an excitation maximum around 350 nm and an emission maximum around 460 nm.[18] Figure 1 shows the typical tryptophan and Schiff base fluorescent emission spectra. Results indicated that with the increasing concentration of MDA, the fluorescence from tryptophan decreased while the fluorescence from Schiff base increased. This result was in accordance with the findings of some former investigations that also studied the effect of MDA on protein's fluorescence change.[22,25] The disappearance of tryptophan

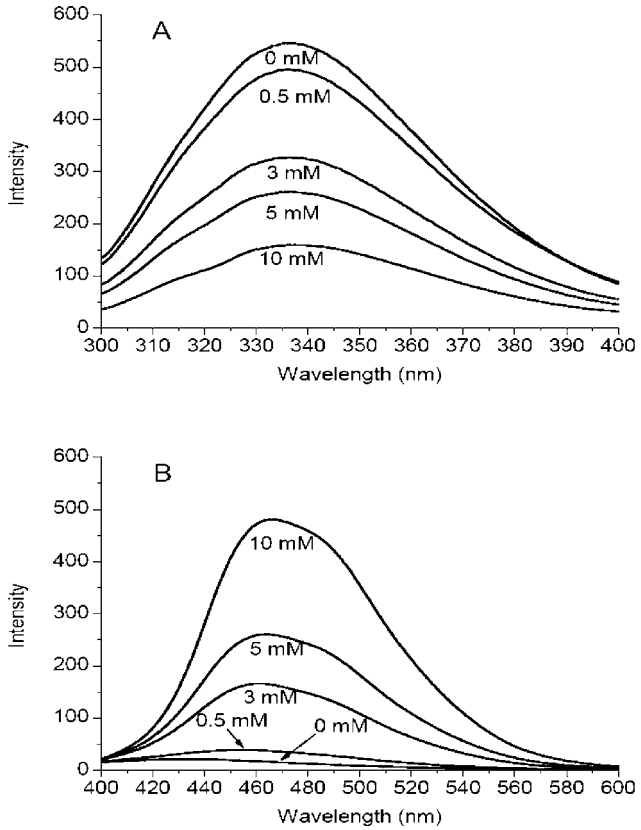

Figure 1. Fluorescent emission spectra of tryptophan (A) and Schiff base (B) of SPI modified by increasing concentration of MDA.

fluorescence could be attributed either to the destruction of tryptophan residues or to a change in the microenvironment in which tryptophan residues were located due to the binding of the aldehyde to other sites of the protein, leading to the lesser exposure of tryptophan to the aqueous environment.[22] The destruction of tryptophan was observed by Foettinger et al., who reported that the indole nitrogen of the tryptophan side chain would react with MDA.[26] The increasing fluorescence in 460 nm was attributed to the Schiff base formation. This further confirmed the interaction between MDA and free amino groups in protein.

To better understand the protein−protein interaction induced by MDA modification, solubility in four types of solvent was investigated (Figure 2). Among all of the solutions,

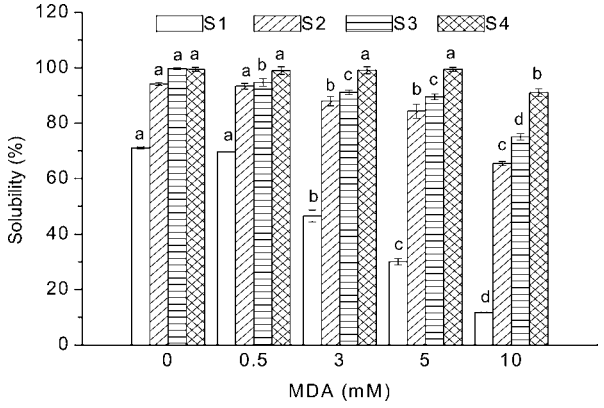

Figure 2. Solubility of control and MDA-modified SPI in four types of solution: S1, 0.1 M, pH 7.5 PB; S2, 1% SDS + PB; S3, 8 M urea + 1% SDS + PB; S4, 20 mM DTT + 8 M urea + 1% SDS + PB. Different letters (a−d) represent significant difference ($p < 0.05$) between different samples in the same kind of solution.

PB solubilized the least amount of proteins. Increasing MDA pretreatment resulted in decreasing solubility in PB, indicating that SPI was gradually denatured. The quantity of protein dissolved by SDS + PB was significantly higher than that of PB. Further addition of urea slightly increased the solubility. Urea and SDS are agents known to disrupt noncovalent interactions, such as hydrogen bonds and hydrophobic interactions.[27] Therefore, the noncovalent interaction increased as SPI was treated with increasing MDA concentration. The control sample was totally dissolved in the SDS + urea + PB solution. However, samples incubated with increasing MDA concentration still showed a decreasing solubility. The gap between these samples with the control might come from the covalent interaction between proteins. Urea + SDS + PB solution with added DTT totally dissolved control sample and SPI incubated with 0.5, 3, and 5 mM MDA. DTT is a strong reducing agent and has the ability to cleave disulfide bonds, which contributes to the main covalent interaction between protein molecules.[27] This observation indicated disulfide bonds gradually formed in the MDA-modified SPI. However, when SPI was incubated with 10 mM MDA, there were still particles that could not be dissolved in the DTT + urea + SDS + PB solution. The reason for these nonsoluble particles might be the covalent bonds formed by the Schiff base, which could not be disrupted by DTT. Even though, in lower MDA concentration, Schiff base also formed, they made no significant contribution to the decreasing solubility. We deduced that MDA first reacted with free amino groups, disrupting the native structure of SPI. This disruption unfolded the protein molecule and exposed hydrophobic groups. During this process, cross-linking between peptides also happened (through forming Schiff base or disulfide bond). When dissolved in water, exposed hydrophobic groups induced protein molecules to form insoluble aggregates. Notably, compared with the covalent interaction, noncovalent interaction appeared to play a more important role for the decreasing solubility.

DSC has been widely applied to characterize the thermal transition properties of the major globulins (β-conglycinin and glycinin) in SPI.[28] In this investigation, two peak transition temperatures (T_d) of about 75 and 90 °C, respectively, were observed. These two temperatures clearly corresponded to the thermal denaturation temperatures of β-conglycinin and glycinin components.[29,30] Table 2 lists the T_d data of the β-conglycinin (T_{d1}) and glycinin (T_{d2}) components, as well as the combined enthalpy change (ΔH) data of both β-conglycinin (ΔH_1) and glycinin (ΔH_2) components. Both T_{d1} and T_{d2} increased with increasing MDA concentration, accompanied by a gradual decrease of ΔH for both β-conglycinin and glycinin. It is acknowledged that the decrease of enthalpy is evidence for protein denaturation and the enthalpy value mainly comes from the undenatured protein.[31] One of the most obvious pieces of evidence of protein denaturation in this investigation was that the protein gradually became insoluble with the increasing MDA concentration. Therefore, decreasing enthalpy was observed with increasing MDA concentration because of the decreasing proportion of undenatured protein in MDA-modified SPI. Decreased solubility of SPI accompanied by decreased enthalpy value was also observed by other researchers.[32] T_d is usually an indicator of thermal stability (or tertiary conformational stability) of the proteins.[31] Compared to the control, MDA-modified SPI had higher T_d, which indicated that the undenatured protein possessed a more stable structure. The increased stability might come from the cross-linking between molecules caused by Schiff base and formation of disulfide bonds, as cross-linking of molecules usually resulted in an enhanced thermal stability.[28,33] The crossing-linking of soluble proportion was observed by the

Table 2. Peak Temperatures (T_d) and Enthalpy Changes (ΔH) for Control and MDA-Modified SPI[a]

MDA (mM)	T_{d1} (°C)	ΔH_1 (J g^{-1})	T_{d2} (°C)	ΔH_2 (J g^{-1})
0	73.72 ± 0.20 a	3.02 ± 0.11 a	90.57 ± 0.37 a	9.13 ± 0.04 a
0.5	74.06 ± 0.32 a	2.43 ± 0.22 b	92.07 ± 0.28 b	8.31 ± 0.23 b
3	76.20 ± 0.38 b	1.75 ± 0.07 c	92.60 ± 0.20 b	6.18 ± 0.04 c
5	77.30 ± 0.25 c	1.22 ± 0.10 d	92.62 ± 0.35 b	4.35 ± 0.25 d
10	77.75 ± 0.42 c	1.00 ± 0.07 d	92.82 ± 0.43 b	3.06 ± 0.35 e

[a] T_{d1} and T_{d2} represent the denaturation temperature of the β-conglycinin and glycinin components in the SPI samples; ΔH_1 and ΔH_2 represent enthalpy changes of β-conglycinin and glycinin. Values in the same column followed by different letters (a−e) are significantly different ($p < 0.05$).

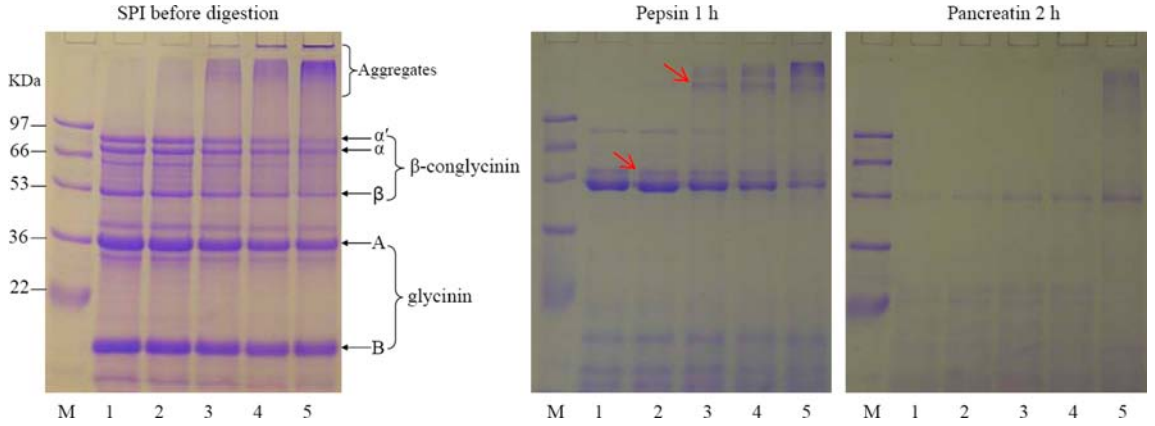

Figure 3. Electrophoretic patterns of control and MDA-modified SPI before digestion, after 1 h of pepsin digestion, and after 2 h of pancreatin digestion. Lanes: M, marker proteins; 1, 2, 3, 4, and 5, SPI treated with 0, 0.5, 3, 5, and 10 mM MDA, respectively. SPI constituents: α', α, and β, subunits for β-conglycinin; A and B, acidic subunits and basic subunits for glycinin.

Table 3. Free Essential Amino Acid Profile and Total Free Amino Acid Content of SPI Incubated with Increasing MDA, after in Vitro Gastrointestinal Digestion[a]

amino acid (mg/100 mL)	0 mM	0.5 mM	3 mM	5 mM	10 mM
Lys	13.66 ± 0.26 a	12.20 ± 0.79 ab	10.86 ± 0.44 bc	10.67 ± 0.77 bc	10.46 ± 0.50 c
Phe	40.85 ± 0.89 a	36.19 ± 0.27 b	34.65 ± 0.19 c	35.05 ± 0.64 bc	36.35 ± 0.33 b
Trp	10.27 ± 0.36 a	8.23 ± 0.35 b	8.27 ± 0.08 b	7.90 ± 0.46 b	8.57 ± 0.08 b
Leu	45.37 ± 0.20 a	42.94 ± 0.46 b	38.41 ± 0.24 c	40.02 ± 0.60 d	40.70 ± 0.31 d
Val	7.63 ± 0.51 a	5.77 ± 0.01 b	6.81 ± 0.05 ac	6.25 ± 0.08 bc	6.84 ± 0.55 ac
Ile	6.22 ± 0.20 a	5.34 ± 0.56 b	5.38 ± 0.05 b	5.49 ± 0.34 ab	5.84 ± 0.13 ab
Met	4.27 ± 0.22 a	3.59 ± 0.07 a	3.69 ± 0.27 a	3.55 ± 0.03 a	4.36 ± 0.71 a
Thr	2.66 ± 0.30 a	2.23 ± 0.22 a	3.00 ± 0.47 a	2.57 ± 0.04 a	2.81 ± 0.24 a
EAA	130.95 ± 4.93 a	116.49 ± 1.20 b	111.06 ± 4.95 b	111.50 ± 5.18 b	115.93 ± 2.94 b
TAA	184.05 ± 2.05 a	158.79 ± 1.88 b	160.41 ± 1.71 b	158.80 ± 2.85 b	166.99 ± 0.07 b

[a]Lys, Phe, Trp, Leu, Val, Ile, Met, and Thr represent lysine, phenylalanine, tryptophan, leucine, valine, isoleucine, methionine, and rhreonine, respectively. EAA represents essential amino acid. TAA represents total amino acid. Values in the same row followed by different letters (a–d) are significantly different ($p < 0.05$).

electrophoretic pattern of the soluble protein dissolved in PB (pH 7.5) (data not shown). Apart from the covalent interaction, the very strong noncovalent interaction in MDA-modified SPI might also contribute to the conformational stability. Enhanced covalent and noncovalent interaction with increasing MDA concentration made some protein denature and insoluble, but at the same time some soluble aggregates also formed and became the undenatured proportion contributing to the T_d and ΔH values.

Characteristics of in Vitro Digests. MDA modification significantly altered the electrophoretic pattern of SPI (Figure 3). The SDS-PAGE pattern of control SPI presented characteristic bands for the subunits of β-conglycinin and glycinin. No significant change of electrophoretic pattern was observed when SPI was incubated with 0.5 mM MDA, whereas incubation with 3, 5, and 10 mM MDA resulted in gradual degradation of β-conglycinin. Acid polypeptides of glycinin also became less intense with increasing MDA concentration but less significant than that of β-conglycinin subunits. The intensity change of basic polypeptides was negligible. A former study found that acid and basic subunits of glycinin would significantly degrade with further increasing MDA concentration (>10 mM).[34] These phenomena indicated that β-conglycinin was more vulnerable to MDA modification than glycinin. Simultaneously, aggregates gradually formed and accumulated on the top of the separating gel and stacking gel. These aggregates might come from the interaction of subunits through non-disulfide covalent bonds as this SDS-PAGE was performed under reducing condition with the addition of 2-ME, which would destroy the disulfide bonds. Because of the bifunctionality of MDA, cross-linking between free amino groups of proteins is possible, which is a non-disulfide bond leading to the polymerization of molecules.

SPI digested by gastrointestinal proteases exhibited distinctively different electrophoretic patterns, and these patterns were also affected by MDA concentration. After digestion by pepsin for 1 h, aggregates in the stacking gel degraded and disappeared. The aggregates on the top of the separating gel also degraded with the appearance of a new lower molecular fraction (the arrow). α' and α subunits of β-conglycinin also degraded after 1 h of pepsin digestion. Significantly, the band intensity of β subunits increased, which might indicate that some proportion of β subunits formed into aggregates that were then degraded by pepsin. These aggregates are not necessarily caused by MDA modification because the enhancing intensity was also observed in nonmodified SPI. They might come from the native SPI forming through other non-disulfide bonds. This indicated that compared with α' and α subunits, β subunits were more resistant to pepsin digestion. Besides, a new band was observed above the β subunits (the arrow), which might also come from the degradation of higher molecular composition. Glycinin was greatly digested, with the total

disappearance of the acid subunits and significant degradation of the basic subunits. This indicated that acid subunits were more vulnerable to pepsin digestion. The more significant disappearance of glycinin than β-conglycinin confirmed the former investigation by other researchers that β-conglycinin was more resistant to the proteolysis of pepsin than glycinin.[35] After 2 h of pancreatin digestion, aggregates at the top of the separating gel were almost digested except that of the SPI modified by 10 mM MDA. This indicated incubation with higher concentration of MDA would induce antidigestion aggregates. Basic subunits of glycinin were totally digested into lower molecular peptides. The overall intensity of β subunits reduced after 2 h of pancreatin digestion, but improved with increasing concentration of MDA, even though before digestion and at 1 h of pepsin digestion, the intensity of the β subunits decreased with the increasing concentration of MDA. We deduced that nonmodified β subunits were digested by pancreatin, whereas the remaining β subunits therefore might be MDA-modified. This result indicated that β subunits were very sensitive to MDA modification, which could change the physical recognition sites of pancreatin, thus decreasing their proteolytic susceptibility.

The free essential amino acid profile and total free amino acid content in each digest after in vitro gastrointestinal digestion are shown in Table 3. The availability of both essential and total FAA in the digests decreased after SPI was incubated with MDA. Contents of Lys, Phe, Trp, and Leu showed significant decreases. The degradation of Trp had been indicated in the fluorescence spectra of tryptophan. Incubation with 10 mM MDA brought 11.47 and 9.27% losses for essential and total FAA contents, respectively. Essential amino acid content is an important aspect to the value of the nutritive quality of protein,[36] and protein should be digested into FAA and small peptides, so that it can be absorbed by the human body.[36] Therefore, decreases in the essential and total FAAs implied the nutritional and absorbability deterioration of the MDA-modified SPI.

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Notes

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