JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Mass Spectrometric Evidence of Malonaldehyde and 4-Hydroxynonenal Adductions to Radical-Scavenging Soy Peptides

Jing Zhao,[†,∥] Jing Chen,[‡,∥] Haining Zhu,[‡] and Youling L. Xiong*[,†]

[†]Department of Animal and Food Sciences and [‡]Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40546, United States

ABSTRACT: Antioxidative peptides in food systems are potential targets of lipid oxidation-generated reactive aldehydes, such as malonaldehyde (MDA) and 4-hydroxynonenal (HNE). In this study, covalent modifications on radical-scavenging peptides prepared from soy protein hydrolysate by MDA and HNE were characterized by liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS/MS). MS/MS analyses detected the formation of Schiff base type adducts of MDA on the side-chain groups of lysine, histidine, arginine, glutamine, and asparagine residues as well as the N-termini of peptides. MDA also formed a fluorescent product with lysine residues. HNE adducted on lysine residues through Schiff base formation and on histidine, arginine, glutamine, and asparagine residues mainly through Michael addition. Despite the extensive MDA modification, peptide cross-linking by this potential mechanism was undetectable.

KEYWORDS: *radical-scavenging activity, peptides, malonaldehyde (MDA), 4-hydroxynonenal (HNE), mass spectrometry*

INTRODUCTION

Antioxidant peptides have been reported to behave as sacrificial radical scavengers and metal chelators in food systems, thus preventing other food components from being attacked. Our preliminary study showed that during the inactivation of hydroxyl radicals, some amino acid residues in soy protein hydrolysate, such as methionine, histidine, and lysine, were modified (unpublished data). The covalent modifications of amino acid residues during protein oxidation can be induced either directly by reactive oxygen species or indirectly by reaction with secondary products of lipid oxidation, for example, malonaldehyde (MDA) and 4-hydroxynonenal (HNE).[1] MDA and HNE, the two major reactive aldehyde species formed during polyunsaturated fatty acid peroxidation, are well-known for their detrimental effects on biomolecules (proteins, DNA, phopholipids, etc.) and involvement in human diseases, for example, neurodegenation.[2−4] These aldehydes are more stable than free radicals and, thus, can diffuse to a target far from the site of their formation,[3] resulting in different modification sites on peptides from those attacked by free radicals.

Lipid oxidation can exert various detrimental effects on food products. One of the most notorious effects is off-flavors generated from oxidation of unsaturated fatty acids. It also changes the type and concentration of molecular species present in a food,[5] thus influencing the quality attributes of foods, such as taste, texture, shelf life, appearance, and nutrition profile.[6] For example, the adduction of HNE on myoglobin induces the discoloration of meat, which leads to a marked loss of products' commercial value.[7] To prevent the deleterious effects of lipid oxidation in foods, antioxidative peptides have been applied, for example, in meat products and in emulsions in which tremendous lipid oxidation can occur.[8,9] Because by virtue of the inactivation of reactive oxygen species when used to protect other food components antioxidative peptides are sacrificed, it is of interest to understand the fate of these peptides following their reaction with reactive aldehydes generated from lipid oxidation.

The adduction of MDA and HNE on amino acids has been widely observed. MDA, a strong alkylating agent with two reactive aldehyde groups positioned on the opposing ends of the molecule, forms a Schiff base complex predominately with primary amino groups such as ε-amines of lysine residues and the N-termini of the peptide chains.[10] MDA has also been reported to react with histidine in bovine serum albumin.[11] In addition, MDA adduction can generate fluorescent compounds such as the dihydropyridine (DHP)-type adducts.

HNE is another highly reactive bifunctional aldehyde. The conjugation of the double bond with the aldehyde group and the electron-withdrawing 4-hydroxyl group in HNE makes the central carbon (C-3) extremely susceptible to nucleophilic attack by free amines. Thus, HNE forms Michael adducts with nucleophilic sites in proteins, such as histidine imidazole moieties, cysteine sulfhydryls, and ε-amino groups of lysine residues.[12] These Michael adducts are stabilized in the form of cyclic hemiacetals. Besides forming Michael adducts, the aldehyde group in HNE is able to form Schiff bases with the N-termini of peptide chains and ε-amino groups of lysine residues in proteins.[13] Because of the bifunctional nature of both MDA and HNE, they all have the potential to cross-link proteins.[14]

A previous study demonstrated an MDA concentration-dependent increase in the protein carbonyl content and decreases in the free sulfhydryl, disulfide, free amine, and lysine content of soy protein after reaction with MDA (0−100 mM, 25 °C, 24 h), indicating the adduction of MDA on cysteine, lysine, and the N-termini of soy protein polypeptide

Received: June 17, 2012
Revised: September 1, 2012
Accepted: September 4, 2012
Published: September 4, 2012

chains.[15] Uchida and Stadtman[16] suggested that the primary target of HNE on different proteins can vary, for example, lysine in low-density lipoproteins and glucose-6-phosphate dehydrogenase and histidine in insulin.

In the present study, mass spectrometry was applied to identify the MDA and HNE modification sites on chromatographically separated radical-scavenging soy peptides. Such an analytical approach has the merit of a high degree of confidence in the assignments and potential to identify the precise sites within the peptide chains that are modified or cross-linked.[17] To our knowledge, this was the first study in which mass spectrometry was used to reveal the modifications of radical-scavenging peptides by secondary lipid oxidation products.

MATERIALS AND METHODS

Materials. Soybeans were purchased from Bonnie Buhs Co. (Gibson, IN, USA). Soy protein isolate, protein hydrolysates, and peptide fractions were prepared in the laboratory. HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA), and 1,1,3,3-tetraethoxyproane (MDA precursor) was from Sigma Chemical Co. (St. Louis, MO, USA). Alcalase 2.4 L FG (2.4 AU/g) was obtained from Novo Nordisk (Bagsvard, Denmark). All other chemicals, all of reagent grade, were purchased from Fisher Scientific (Indianapolis, IN, USA) or Sigma Chemical Co.

Preparation of Soy Protein Hydrolysate (SPH). Soy protein was isolated from defatted soy flour using isoelectric precipitation as described by Jiang et al.[18] An SPH with a degree of hydrolysis of 5%, previously established to be strongly antiradical,[1] was prepared with Alcalase at pH 8, 50 °C, and a 1:100 enzyme/protein ratio. Degree of hydrolysis was controlled by the pH-stat method.[19] Prior to hydrolysis, the protein substrate was heat-treated at 90 °C for 5 min to improve enzyme accessibility. When a degree of hydrolysis of 5% was reached, the hydrolysate was heated at 80 °C for 15 min to inactivate Alcalase, adjusted back to pH 7, freeze-dried, and stored in a freezer until use.

Peptide Fractionation. SPH was subjected to peptide fractionation using low-pressure gel filtration chromatography with a 2.6 cm (diameter) × 70 cm (length) Sephadex G-25 fine column (Pharmacia XK 26/70, Piscataway, NJ, USA) according to the method of Ma et al.[20] SPH (1% protein) dissolved in deionized water was filtered through a 0.22 μm membrane, and an aliquot of 6 mL was loaded to the column for separation. Peptides were eluted with filtered deionized water at 4 °C and a flow rate of 0.9 mL/min. The eluents were collected with an automatic fraction collector with a collection time of 6 min for each collection tube. Protein concentration in each tube was measured with a UV−vis spectrometer at 280 nm. The corresponding peptide fractions from 20 chromatographic runs were pooled, lyophilized, and kept in a freezer for further analysis. The average molecular weight of each individual peptide fraction was estimated from a calibration curve generated from the elution volume of the following standards: cytochrome *c* (12327 Da), aprotinin (6512 Da), bacitracin (1423 Da), and tetrapeptide GGYR (452 Da), according to Ma et al.[20]

Measurement of Radical-Scavenging Capacity. Freeze-dried peptide fractions were dissolved in aqueous solutions. Protein concentration was measured with bicinchoninic acid (BCA) using a Pierce BCA protein assay kit purchased from Thermal Fisher Scientific Inc. (Rockford, IL, USA). Samples adjusted to the same protein concentration levels were subjected to the radical-scavenging activity assay using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radicals ($ABTS^{\bullet+}$) according to the method of Pellegrini et al.[21] The absorbance reading (734 nm) was taken after 10 min and converted to Trolox equivalent antioxidant capacity (TEAC, mM) based on a standard curve generated with Trolox.

Analysis of Amino Acid Composition. Acid hydrolysis of peptide samples prepared from the gel filtration fractionation was performed with 6 M HCl at 110 °C for 24 h in glass tubes purged with nitrogen gas before being sealed. The amino acid composition was analyzed by reverse-phase high-pressure liquid chromatography with a 3.9 mm × 300 mm Nova-Pak C18 column (Waters Co., Milford, MA, USA). Norleucine was added as the internal standard. Mobile phase A was prepared by mixing reagent 1 [1.9% (w/v) sodium acetate trihydrate, 0.05% (v/v) triethylamine, and 0.2 ppm ethylenediaminetetraacetic acid (EDTA) titrated to pH 6.40 with glacial acetic acid] and acetonitrile at a 47:3 (v/v) ratio. Mobile phase B was prepared by mixing acetonitrile, deionized water, and 1000 ppm EDTA at a 3000:2000:1 (v/v/v) ratio. Both eluent solutions were degassed before use.

Reaction with MDA. MDA was prepared by the hydrolysis of bis(1,1,3,3-tetraethoxypropane) (23 mg) in 0.1 M HCl (10 mL) at 40 °C for 40 min. The pH of the MDA solution was adjusted to 7.2 with 6 M NaOH. MDA concentration was measured at 245 nm in 1% H_2SO_4 and calculated using the molar extinction coefficient of 13700 M^{-1} cm^{-1}.[22] Peptide samples dissolved in 10 mM phosphate buffer (pH 7.2) and MDA were mixed at final concentrations of 1 mg/mL and 5 mM, respectively, and incubated at 37 °C for 24 h.

Reaction with HNE. HNE solution was prepared fresh by dilution to 4 mM with a 10 mM phosphate buffer (pH 7.2). Peptide samples (2 mg/mL) in the same buffer were mixed with the prepared HNE at a 1:1 ratio and incubated at 37 °C for 6 h. Half of the reacted sample was subjected to mass spectrometry (nonreduced), and the other half was treated with sodium borohydride ($NaBH_4$) at a concentration of 1.0 M to stabilize the reversible HNE−lysine adducts in the reduced form before mass spectrometry.[23]

Liquid Chromatography−Electrospray Ionization−Tandem Mass Spectrometry (LC-ESI-MS/MS). LC-MS/MS analysis was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with an Eksigent Nanoflex cHiPLC system (Eksigent, Dublin, CA, USA) through a nanoelectrospray ionization source. The peptide samples were separated using a reversed phase cHiPLC column (75 μm × 150 mm) at a flow rate of 300 nL/min. Mobile phase A was deionized water with 0.1% (v/v) formic acid, whereas mobile phase B was acetonitrile with 0.1% (v/v) formic acid. A 40 min gradient condition was applied: initial 3% mobile phase B was increased linearly to 50% B in 24 min and further to 85% B for 8 min before it was decreased to 3% B for re-equilibration. The eluted peptides were analyzed using data-dependent acquisition: all peptides eluted from the cHiPLC column at a particular elution time were analyzed by mass spectrometry using Orbitrap (m/z 100−1600) with a resolution of 60000. From each peptide MS spectrum, the seven most abundant peptides were subjected to collision-induced dissociation (CID) and MS/MS analysis in the LTQ linear trap.

Identification of Peptides and MDA/HNE Adduction Sites. The LC-MS/MS data were submitted to a local Mascot server for an MS/MS identification search against a customized database using Proteome Discoverer software. Peptide tolerance and MS/MS tolerance were set as 10 ppm and 0.8 Da, respectively. The database encompasses the sequences of five polypeptides from soy glycinin (G1, G2, G3, G4, and G) and three subunits of soy β-conglycinin (α, α′, and β chains) obtained from UniProtKB/Swiss-Prot (www.expasy.org/sprot). A decoy database was built and searched. Filter settings that determine false discovery rates (FDR) were used to distribute the confidence indicators for the peptide matches. Peptide matches that passed the filter associated with the strict FDR (with target setting of 0.01) were assigned as high confidence. Mass additions of 54 and 134 Da were searched to identify MDA Schiff base adduction and fluorescent DHP−lysine adduct, respectively. For HNE Michael adducts, the mass increments were 156 Da in nonreduced samples and 158 Da for the reduced adduction products. In the case of cysteine−HNE conjugate, a neutral loss of the HS−HNE moiety was examined according to the method of Wu.[24] HNE Schiff base formation was searched with mass additions of 138 and 140 Da in nonreduced and reduced samples, respectively. A reported stable pyrrole type HNE−lysine adduct, which has a mass addition of 120 Da, was examined in nonreduced samples.

Identification of MDA-Induced Cross-Linking. MDA-modified peptides were analyzed in an attempt to identify MDA-induced cross-linking. Reaction of peptides and MDA was done in the same way as

described above. After 24 h of incubation, the reaction was terminated by increasing the pH to 9. Samples were reduced with $NaBH_4$ before being subjected to acid hydrolysis in 6 M HCl at 110 °C for 24 h. The hydrolyzed samples were freeze-dried and redissolved in 10 mM phosphate buffer (pH 7.2) for LC-MS/MS analysis.

Statistical Analysis. Radical-scavenging activity tests were repeated three times as independent trials (replicates). The data were subjected to the analysis of variance using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). Significant ($P < 0.05$) differences between means were identified by least significant difference all-pairwise multiple comparisons. The LC-MS/MS experiments were repeated twice.

RESULTS AND DISCUSSION

Radical-Scavenging Capacity. Previous studies have shown that the size of peptides plays a role in their antioxidant activity.[25] Therefore, size exclusion gel filtration chromatography was applied to separate peptides in SPH (Figure 1),

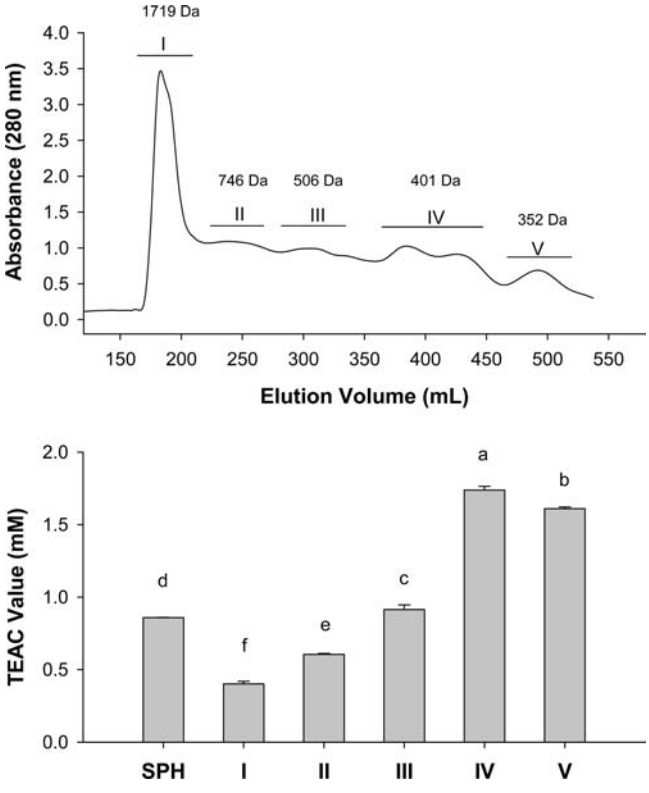


Figure 1. Radical-scavenging activity (lower panel) of soy peptide fractions prepared with gel filtration chromatography (upper panel). The result is expressed as Trolox equivalent antioxidant capacity (TEAC) with the means being different ($P < 0.05$) if marked by different letters (a–f). The labels above the peptide fractions (upper panel) indicate the estimated mean molecular weight of each fraction.

which were subsequently grouped into five molecular weight fractions. The antioxidant activity of the peptide fractions was estimated using the $ABTS^{\bullet+}$ scavenging test (Figure 1). The two large molecular weight fractions (I and II, at 1719 and 746 Da, respectively) exhibited lower radical-scavenging capability compared to SPH, whereas the scavenging activity was improved in small molecular weight fractions (III–V). Peptide fraction IV displayed a significantly higher ($P < 0.05$) antiradical potential than any other fractions and was 2-fold more potent than SPH. Hence, it was selected for subsequent oxidation analyses. The estimated mean molecular weight of fraction IV was 401 Da, corresponding to about four amino acids. However, the fraction could contain peptides with as many as six amino acid residues or as few as three amino acids when heading (461 Da) and tailing (369 Da) portions of the fraction were considered (Figure 1). The result agreed with literature reports that typical antioxidative peptides are composed of two to nine amino acids.[26]

Amino Acid Composition. The composition of amino acids in the above separated peptide fractions is presented in Table 1. The two fractions that exhibited the best radical-scavenging activity (IV and V) contained significantly higher ($P < 0.05$) amounts of histidine, tyrosine, and phenylalanine than SPH and other fractions. Histidine contents in fractions IV and V were 3.72 and 3.91 g/100 g protein, respectively, compared with 2.08–2.92 g/100 g protein in SPH and other fractions. The two aromatic amino acids tyrosine (17.14 g/100 g protein) and phenylalanine (18.31 g/100 g protein) were 2–6-fold more abundant in fraction IV, which exhibited the highest radical-scavenging activity of all, than in SPH and fractions I–III.

The relative abundances of histidine, tyrosine, and phenylalanine residues in the radical-scavenging peptide fraction (IV) supported previous findings that phenylalanine, tyrosine, and histidine were the main amino acids responsible for peptide antioxidant activity.[27−29] Interestingly, the total amounts of tyrosine and phenylalanine, which were 9.45, 6.21, 6.24, 9.72, 35.46, and 20.38 g/100 g protein in SPH and fractions I–V, respectively (Table 1), corresponded well (linear $R^2 = 0.84$; quadratic $R^2 = 0.92$; $P < 0.05$) with their radical-scavenging activities (Figure 1). Aromatic amino acids are believed to contribute to the antioxidant activity and radical-scavenging potential of several protein sources, for example, hydrolyzed potato protein,[27] egg yolk,[30] and pea seed protein hydrolysate.[31]

Peptide Identification. The selected radical-scavenging peptide fraction (IV) was subjected to LC-ESI-MS/MS to identify the peptide sequences. More than 100 peptides derived from glycinin (G1–4, G) and β-conglycinin (α′, α, and β) were identified from a Mascot search with high confidence. The peptides sequenced had a chain length of 5–14 amino acid residues with the majority of them consisting of 5–9 residues (results not shown). These peptides covered about 17% of the α and β subunits and 21% of α′ subunit of β-conglycinin, as well as 29% of each subunit of glycinin except glycinin G3 (16%). Lys-His-Glu-Trp-Gln-His-Lys and Leu-Arg-Asp-Tyr-Arg-Ile from β-conglycinin and Phe-Leu-Lys-Tyr-Gln from glycinin have been detected to be highly abundant. The average molecular weight of peptides identified by MS was larger than that estimated by the gel filtration (up to about six amino acid residues), probably due to the technical limitation of Sephadex gel filtration as an accurate tool to estimate the molecular weight of macromolecules.[32]

Identification of MDA/HNE Adduction Sites. *MDA Adduction.* Reaction of MDA with amino acid residues in peptides and proteins mainly took place in the form of Schiff base formation at the nucleophilic amine groups, such as lysine, histidine, arginine, and the amino termini.[23] In addition, MDA is capable of cross-linking proteins and producing fluorescent adducts such as DHP–lysine.[33,34] The predicted structures of MDA adduction products are summarized in Table 2.

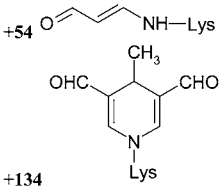
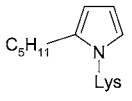
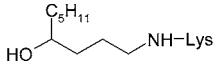
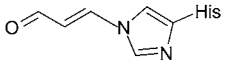
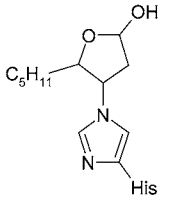
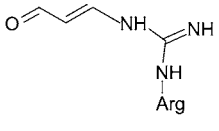
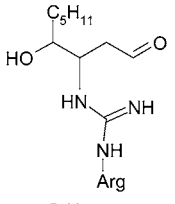
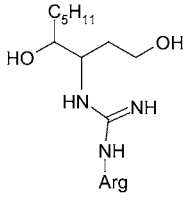
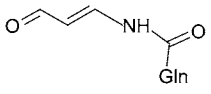
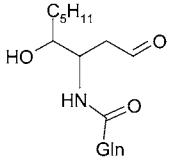
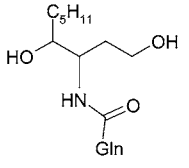
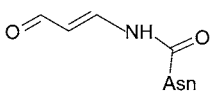
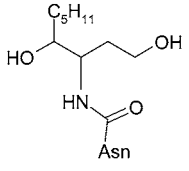
Mascot search with a mass increment of 54 was applied to identify MDA Schiff base formation with the side-chain groups of lysine, histidine, arginine, glutamine, and asparagine residues

Table 1. Amino Acid Composition of Soy Protein Hydrolysate (SPH) and Its Fractions (I–V) Separated by Sephadex Gel Filtration[a]

amino acid	SPH (g/100 g protein)	I (g/100 g protein)	II (g/100 g protein)	III (g/100 g protein)	IV (g/100 g protein)	V (g/100 g protein)
Asp + Asn	12.66 ± 0.08 a	10.86 ± 1.16 b	12.42 ± 0.59 a	10.79 ± 0.55 b	7.02 ± 0.25 c	5.84 ± 0.18 d
Glu + Gln	19.94 ± 0.39 bc	28.11 ± 5.27 a	23.07 ± 0.58 b	17.99 ± 0.66 c	10.48 ± 0.43 e	14.29 ± 0.38 d
Ser	4.78 ± 0.15 a	3.97 ± 0.64 b	3.79 ± 0.16 b	5.24 ± 0.16 a	4.11 ± 0.70 b	2.66 ± 0.08 c
Gly	3.50 ± 0.05 b	3.16 ± 0.28 c	3.44 ± 0.10 b	3.63 ± 0.07 b	3.60 ± 0.14 b	4.03 ± 0.08 a
His	2.63 ± 0.11 b	2.92 ± 0.42 b	2.08 ± 0.13 c	2.16 ± 0.16 c	3.72 ± 0.42 a	3.91 ± 0.42 a
Arg	8.60 ± 0.10 bc	10.39 ± 1.24 a	8.12 ± 0.08 c	7.78 ± 0.08 c	6.84 ± 0.73 d	9.16 ± 0.21 b
Thr	3.36 ± 0.07 b	2.68 ± 0.45 c	3.64 ± 0.07 ab	3.98 ± 0.13 a	2.17 ± 0.35 d	2.45 ± 0.05 cd
Ala	3.72 ± 0.04 b	2.54 ± 0.81 c	3.57 ± 0.10 b	5.41 ± 0.10 a	3.60 ± 0.16 b	2.66 ± 0.09 c
Pro	5.23 ± 0.03 c	7.23 ± 1.35 a	6.28 ± 0.08 b	4.01 ± 0.08 d	2.38 ± 0.04 e	5.05 ± 0.09 c
Tyr	3.84 ± 0.06 c	2.25 ± 1.00 d	2.34 ± 0.05 d	3.48 ± 0.07 c	17.14 ± 0.24 a	13.44 ± 0.06 b
Val	4.81 ± 0.07 b	3.26 ± 0.99 d	5.46 ± 0.06 a	5.50 ± 0.21 a	3.33 ± 0.15 d	4.07 ± 0.14 c
Met	1.21 ± 0.06 a	1.11 ± 0.04 a	0.92 ± 0.06 b	0.99 ± 0.09 b	0.74 ± 0.09 c	0.75 ± 0.04 c
Ile	5.02 ± 0.06 b	3.99 ± 0.66 c	6.02 ± 0.04 a	5.76 ± 0.12 a	3.51 ± 0.34 d	4.59 ± 0.10 b
Leu	8.06 ± 0.10 c	5.80 ± 1.44 d	7.54 ± 0.02 c	10.43 ± 0.18 b	8.40 ± 0.60 c	12.78 ± 0.10 a
Phe	5.61 ± 0.05 c	3.96 ± 1.07 d	3.90 ± 0.03 d	6.24 ± 0.07 bc	18.31 ± 1.31 a	6.95 ± 0.09 b
Lys	7.04 ± 0.07 bc	7.75 ± 0.54 a	7.42 ± 0.09 ab	6.62 ± 0.04 c	4.64 ± 0.42 d	7.37 ± 0.35 ab

[a]Means within the same row (same amino acid) sharing no common letter (a–e) differ significantly ($P < 0.05$).

Table 2. Predicted Structures of Detected Aldehyde Adducts to Amino Acid Residues in Soy Peptides[a]

	MDA	HNE non-reduced form	HNE reduced form
Lys	+54  +134	+120 	+140 
His	+54 	+156 	ND
Arg	+54 	+156 	+158 
Gln	+54 	+156 	+158 
Asn	+54 	ND	+158 

[a]MDA, malonaldehyde; HNE, 4-hydroxynonenal; ND, not detected.

dx.doi.org/10.1021/jf3026277 | J. Agric. Food Chem. 2012, 60, 9727–9736

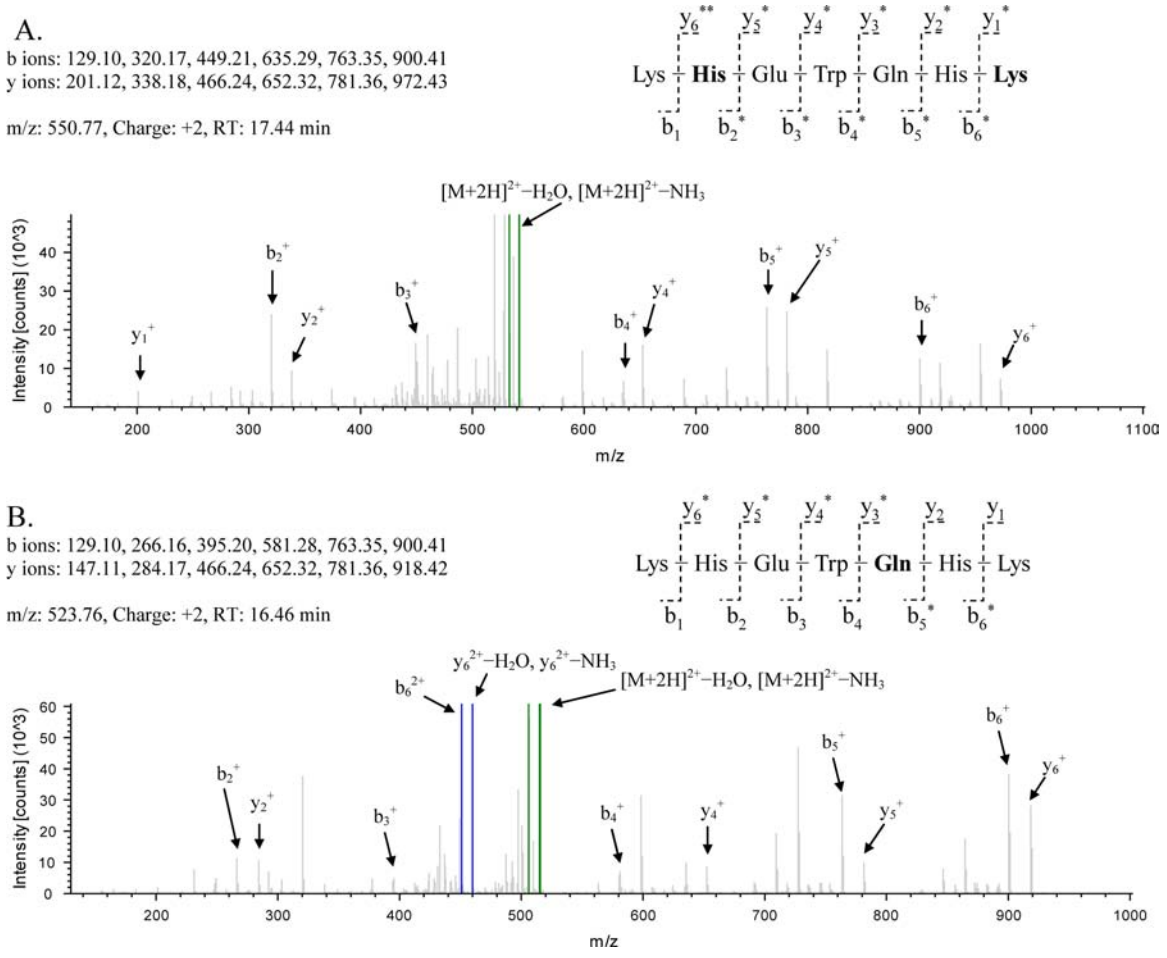


Figure 2. Malonaldehyde Schiff base adduction (∗, +54 Da; ∗∗, +108 Da) on lysine (A), histidine (A), and glutamine (B) side-chain groups in Lys-His-Glu-Trp-Gln-His-Lys.

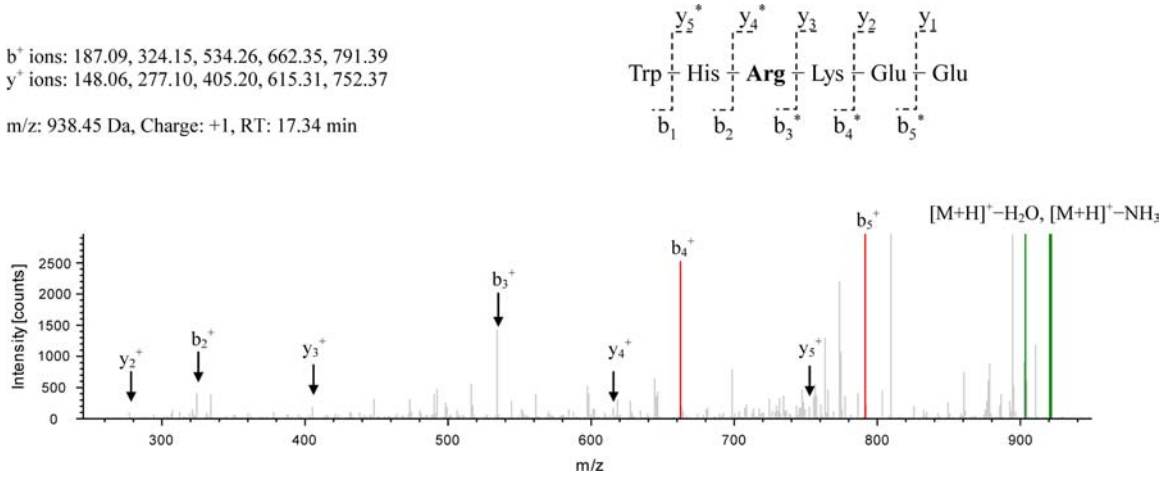


Figure 3. Malonaldehyde Schiff base adduction (∗, +54 Da) on arginine side-chain group in Trp-His-Arg-Lys-Glu-Glu.

as well as the N-termini of the peptide chains. Although the reactivity of glutamine and asparagine with aldehydes is expected to be low due to the reduced nucleophilicity of the amino group in an amide where the carbonyl group is present at the α position, both amino acid residues were included in the search. A total of 53 peptides were identified to react with MDA through Schiff base adduction. More than half of the modifications took place on the N-termini of the peptides. This could be a result of smaller steric hindrance at the terminus than within the sequence and the abundance of N-terminal residues as a result of protein hydrolysis (peptide bond cleavage). MDA Schiff base formation was detected at the side chains of lysine, histidine, arginine, asparagine, and glutamine. Surprisingly, MDA adduction on asparagine residues occurred in a large number of peptides.

dx.doi.org/10.1021/jf3026277 | J. Agric. Food Chem. 2012, 60, 9727–9736

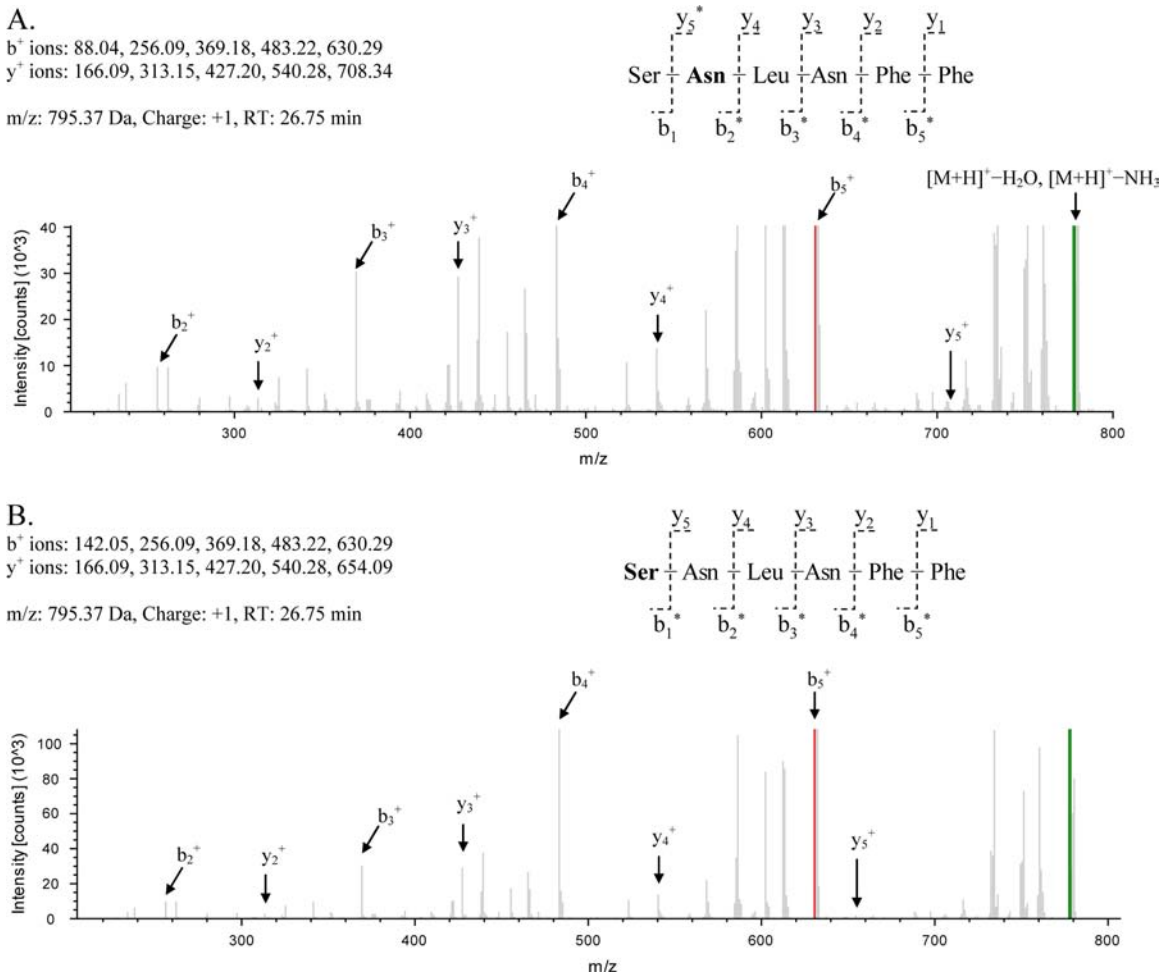


Figure 4. Malonaldehyde Schiff base adduction (∗, +54 Da) on asparagine side-chain group (A) and the N-terminus serine (B) in Ser-Asn-Leu-Asn-Phe-Phe.

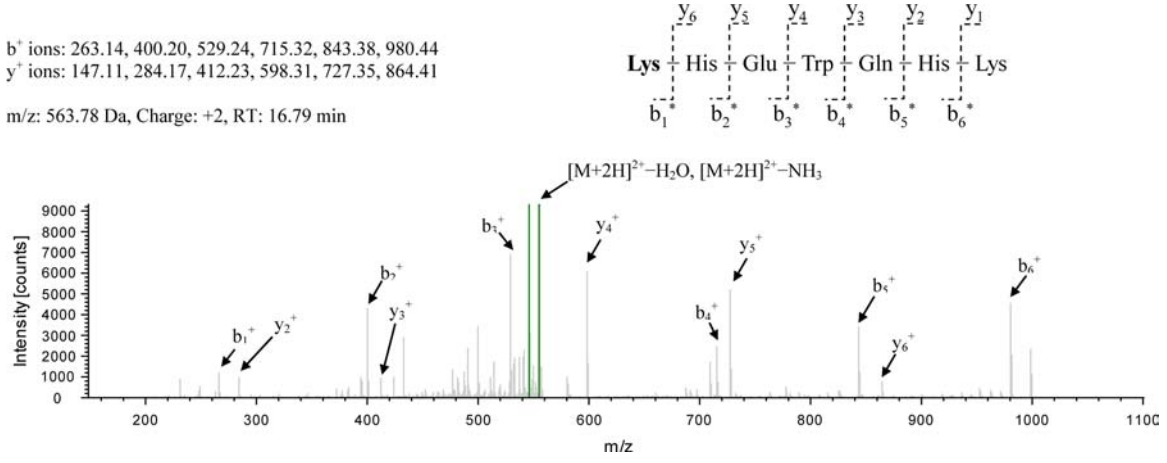


Figure 5. Dihydropyridine (DHP) type fluorescent malonaldehyde adduct (∗, +134 Da) on lysine residue in Lys-His-Glu-Trp-Gln-His-Lys.

The MS/MS results for some of these MDA adducts are shown in Figures 2−4. Both mono- and di-adductions adductions were observed on peptides with two or more nucleophilic sites. As an example, Figure 2 shows the MDA simultaneous adductions on histidine and lysine (A) and a mono-adduction on glutamine (B) in peptide Lys-His-Glu-Trp-Gln-His-Lys, which was one of the prominent peptides identified in the strongly antiradical fraction IV. Figure 3 is an example of MDA adduction on the arginine side-chain amino group in Trp-His-Arg-Lys-Glu-Glu. Figure 4 shows MDA Schiff base formation on the asparagine side chain (A) and on the N-terminal amino group serine (B) in Ser-Asn-Leu-Asn-Phe-Phe.

In addition to the Schiff base type adducts, a fluorescent DHP type adduct on lysine residues was detected in **Lys**-His-Glu-Trp-Gln-His-Lys, **Lys**-Tyr-Glu-Gly-Asn-Trp-Gly-Pro-Leu,

dx.doi.org/10.1021/jf3026277 | J. Agric. Food Chem. 2012, 60, 9727−9736

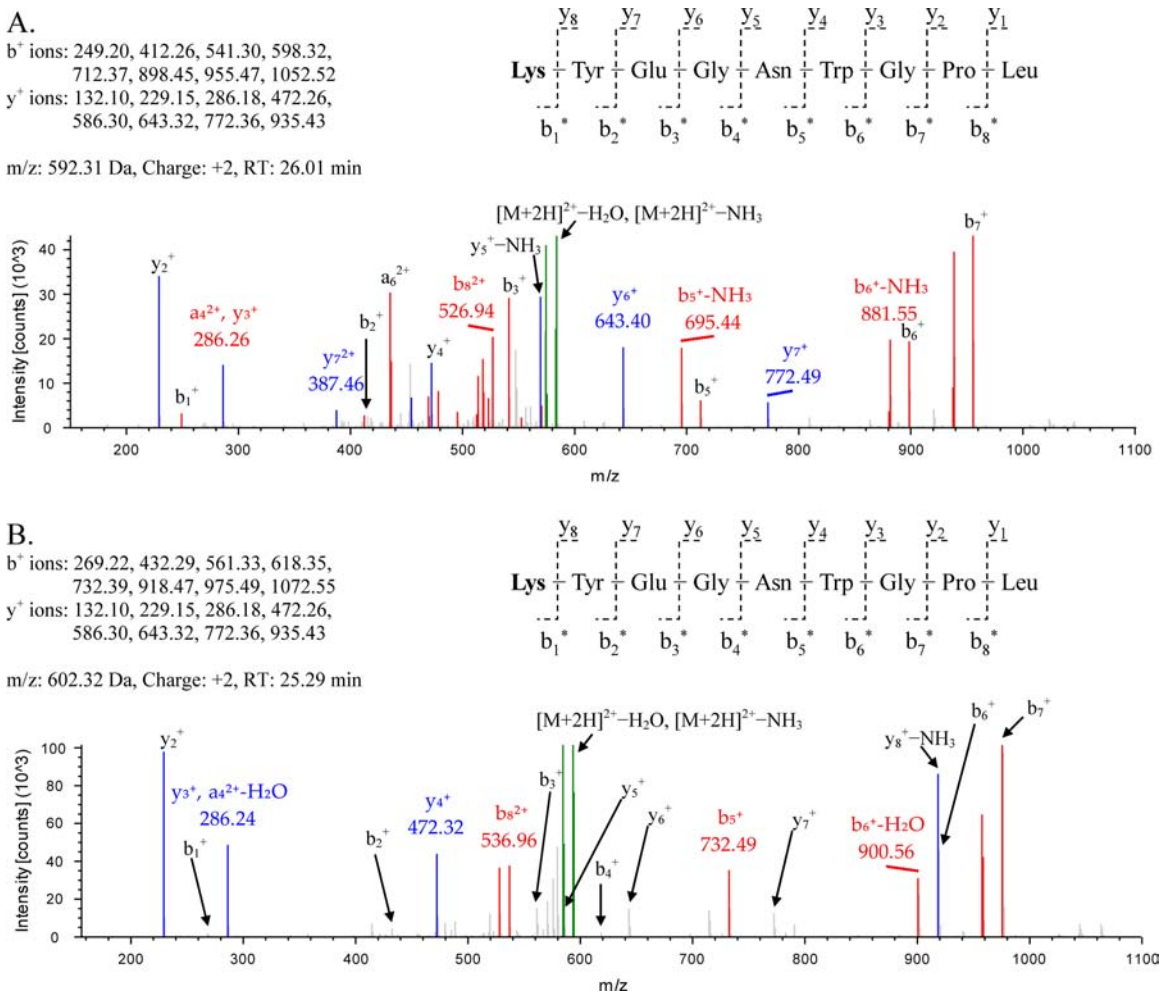


Figure 6. 4-Hydroxynonenal (∗) pyrrole type adduction (A, +120 Da) and Schiff base formation (B, +140 Da) on lysine residue detected from nonreduced and reduced Lys-Tyr-Glu-Gly-Asn-Trp-Gly-Pro-Leu, respectively.

Phe-**Lys**-Asn-Gln-Tyr-Gly-His-Val-Arg, Gly-Arg-**Lys**-Gln-Gly-Gln-His-Gln-Gln, and Gln-Lys-Gln-**Lys**-Gln-Glu-Glu-Glu. The MS/MS evidence of DHP−lysine formation in **Lys**-His-Glu-Trp-Gln-His-Lys is shown in Figure 5.

HNE Adduction. HNE is a readily diffusible and selective electrophile that is a key mediator of oxidative stress.[3] The Michael adducts of HNE on amino acid residues have a 156 Da (nonreduced form) or 158 Da (reduced form) mass increment. The aldehyde group in HNE is also capable of forming Schiff base adducts with nucleophilic amino groups, which have a 138 Da (nonreduced form) or a 140 Da (reduced form) mass increment. The detection of HNE adductions in the present study focused on lysine, cysteine, histidine, arginine, glutamine, and asparagine.

Mascot search yielded no match of Schiff base adducts with a mass addition of 138 Da (bound HNE) in nonreduced peptides. Previous studies have shown that HNE Schiff base adduction on lysine is reversible,[35,36] and the adduct is unstable during MS analysis.[37] However, the pyrrole-type HNE−lysine adduct is more stable[38,39] and was detected in peptide **Lys**-Tyr-Glu-Gly-Asn-Trp-Gly-Pro-Leu (Figure 6A). On the other hand, in samples reduced with $NaBH_4$, the reduced form of HNE−lysine Schiff base adduct was detected in Thr-Trp-Asn-Pro-Asn-Asn-**Lys**-Pro-Phe, Asn-Phe-Gly-**Lys**-Phe-Phe, and **Lys**-Tyr-Glu-Gly-Asn-Trp-Gly-Pro-Leu (Figure 7B). Michael addition products were successfully identified on histidine, arginine, glutamine, and asparagine residues but not on lysine. This observation suggested that HNE reacted with lysine residues in radical-scavenging peptides mainly through Schiff base formation. For all other amino acids, HNE modification occurred in the form of Michael addition.

In nonreduced samples, histidine residues were found to be the major targets of HNE. HNE−His Michael adducts were identified in 5 of 22 sequenced histidine-containing peptides, namely, Phe-Lys-Asn-Gln-Tyr-Gly-**His**-Val-Arg (Figure 7A), Gln-Tyr-Gly-**His**-Val-Arg, Phe-Val-Pro-**His**-Tyr-Asn-Lys-Asn, **His**-Phe-Leu-Ala-Gln, and Thr-Trp-Asn-Ser-Gln-**His**-Pro-Glu. In addition, HNE Michael addition was detected in Phe-Lys-Asn-Gln-Tyr-Gly-His-Val-**Arg** on the arginine side-chain group (from both nonreduced and reduced samples) (Figure 7B) and on the glutamine side chain groups in Thr-Trp-Asn-Ser-**Gln**-His-Pro-Glu (from nonreduced samples), Phe-Lys-Asn-**Gln**-Tyr-Gly-His-Val-Arg (from reduced samples) (Figure 8A), and Lys-His-Glu-Trp-**Gln**-His-Lys (from reduced samples). Previously, HNE Michael adduction on arginine residue has been reported in cytochrome *c* by Isom et al.[39] However, no HNE adduction on glutamine residue has been reported so far. Interestingly, the modified arginine and glutamine residues were all either beside a histidine residue or were one amino acid away from a histidine residue in the sequence. We assume that the detected HNE adduction on arginine and glutamine

dx.doi.org/10.1021/jf3026277 | *J. Agric. Food Chem.* 2012, 60, 9727−9736

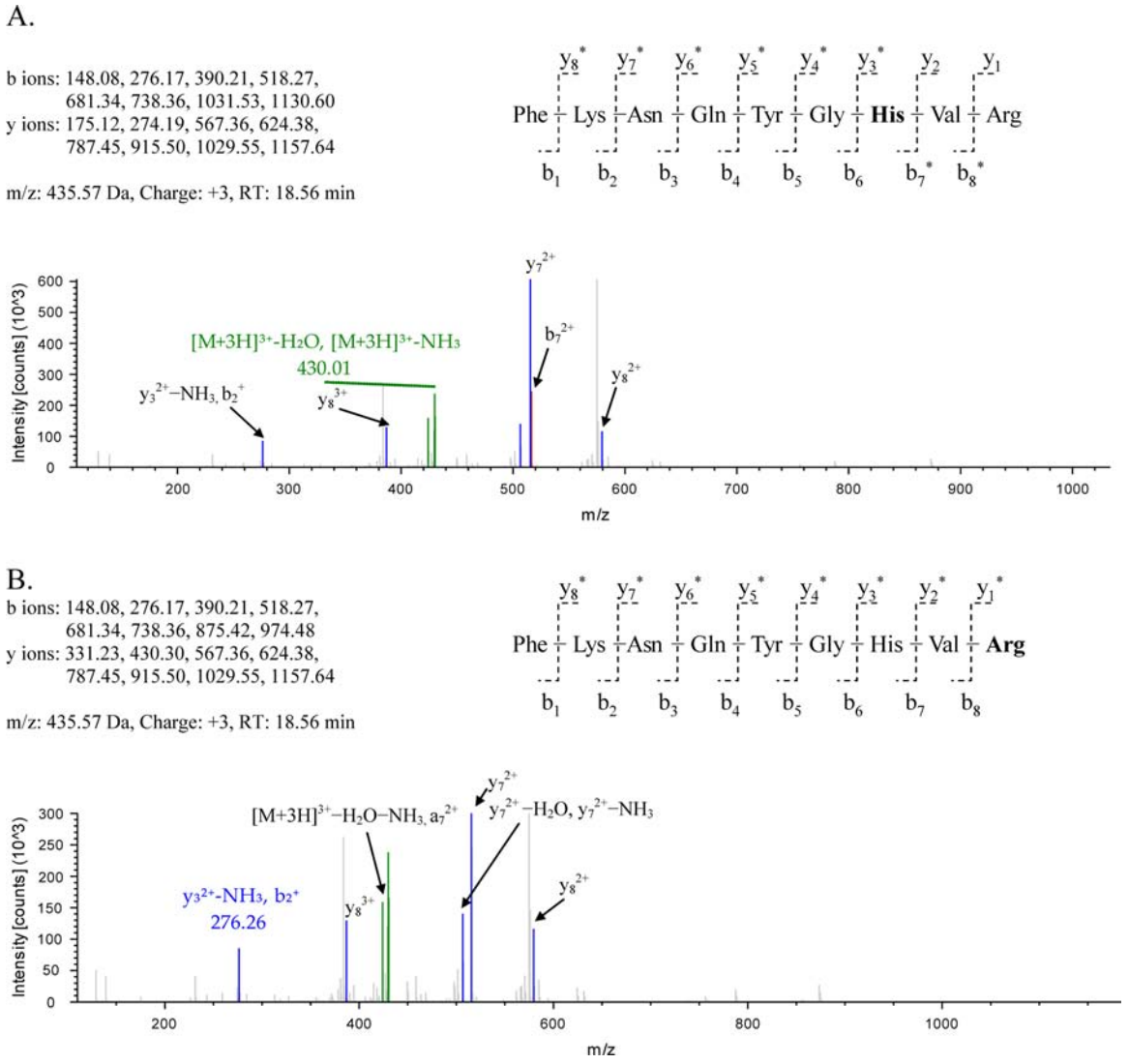


Figure 7. 4-Hydroxynonenal Michael adduction (∗, +156 Da) on histidine (A) and arginine (B) residues in Phe-Lys-Asn-Gln-Tyr-Gly-His-Val-Arg from nonreduced peptides.

residues might be a result of HNE transfer between amino acid side-chain groups.

In the reduced samples, HNE adduction on asparagine was found in Ser-**Asn**-Leu-Asn-Phe-Phe (Figure 8B), Phe-Lys-**Asn**-Gln-Tyr-Gly-His-Val-Arg, Gly-Val-Ala-Trp-Trp-Met-Tyr-**Asn**-Asn-Glu-Asp-Thr-Pro-Val-Val-Ala-Val, and Lys-Tyr-Glu-Gly-**Asn**-Trp-Gly-Pro-Leu, which was not identified in nonreduced samples. The results suggested that HNE−asparagine adducts were not stable during MS analysis and that the adduction reaction could be reversible.

It has been reported that HNE also reacts with cysteine residues, and sulfhydryl groups are the primary nucleophilic targets of HNE.[40] In our case, only one cysteine-containing peptide [Pro-Gln-Cys-Lys-Gly-Lys-Asp from glycinin G1 (A1a, Bx)] was detected from the radical-scavenging peptide fraction (IV) selected for mass spectrometry and HNE adduction study. Besides, according to the pK_a of the cysteine side-chain group (which is around 8.3), at the HNE reaction pH (pH 7.2), <10% of sulfhydryl groups would be in the deprotonated active form. Therefore, even though the reactivity of cysteine at neutral pH is the greatest among the nucleophilic amino acids (Cys, His, Lys, Arg),[41] it was not surprising that no HNE adduction on cysteine residues had been identified in our study.

Identification of MDA-Induced Cross-Linking. Because of the technical difficulty of performing Mascot search for cross-linking, an indirect approach was taken to verify MDA-induced peptide cross-linking. Because the Schiff base is unstable in acidic conditions, MDA-modified peptides were stabilized in their reduced form by reacting with $NaBH_4$. After acid hydrolysis, which released individual amino acids, the cross-linked complexes, for example, lysine−MDA−lysine, were searched in the MS spectrum. Although MDA adduction on individual amino acids (e.g., lysine and histidine) was successfully detected, no cross-linked compounds were found, demonstrating the lack of cross-linking by MDA in these radical-scavenging peptides. This seems understandable because peptides of 5−14 amino acid residues should be quite flexible, mobile, and elusive in solution when compared with large proteins, and the conceivable strong electrostatic repulsions could further prevent peptides from getting close enough to form complexes.

In conclusion, a strongly antiradical fraction from soy protein hydrolysate contained peptides (5−14 amino acid residues)

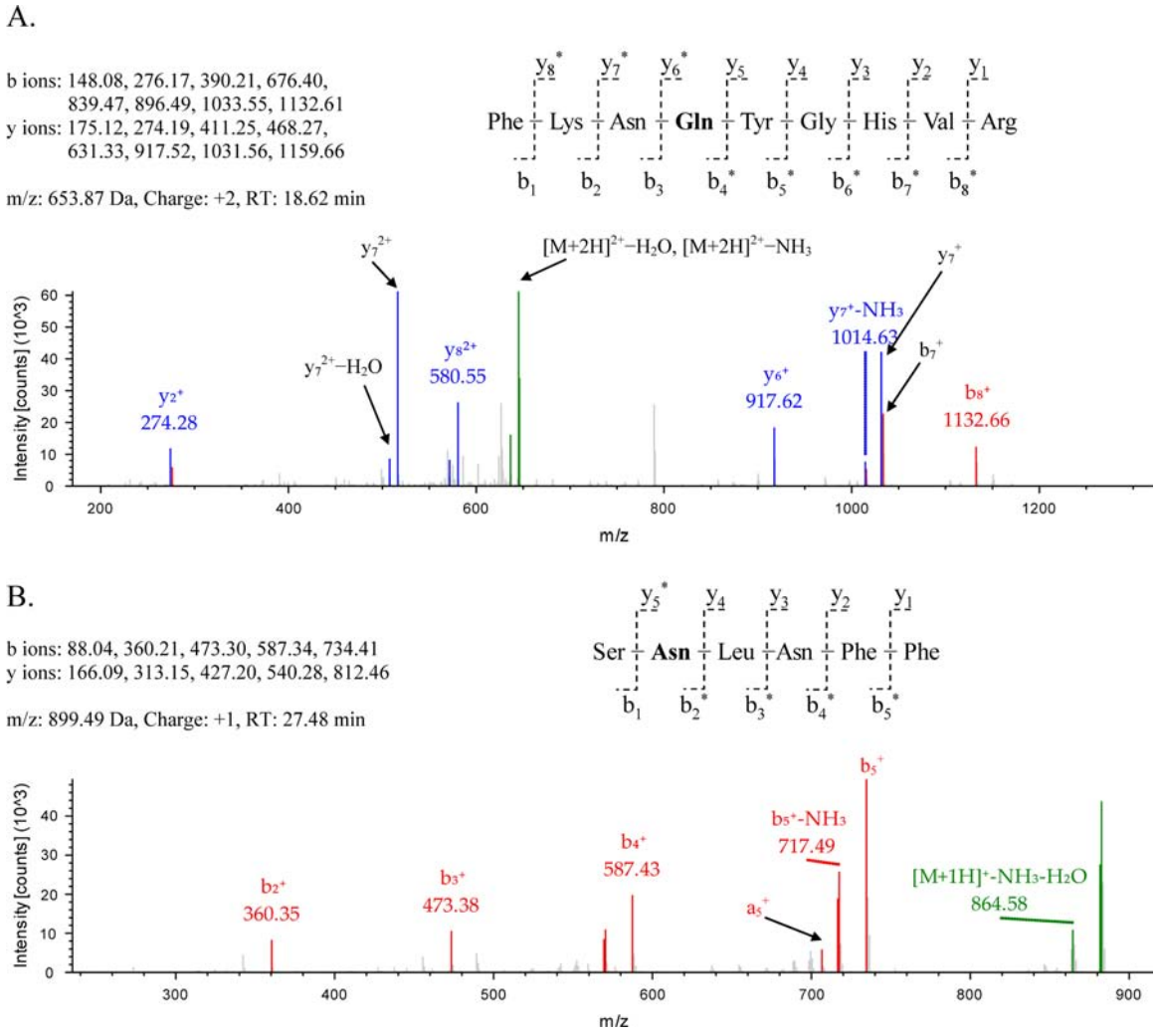


Figure 8. 4-Hydroxynonenal Michael adduction (∗, +158 Da) on glutamine residue in Phe-Lys-Asn-Gln-Tyr-Gly-His-Val-Arg (A) and asparagine residue in Ser-Asn-Leu-Asn-Phe-Phe (B) from peptides reduced with $NaBH_4$.

that were readily reactive with MDA and HNE through Schiff base formation or Michael addition. Besides the modification on lysine, histidine, arginine, and the N-terminal amino acid residues, MDA also adducted on glutamine and asparagine residues in a number of soy peptides. The modification of lysine by HNE was primarily through Schiff base formation, but the modification of other nucleophilic amino acid side-chain groups by HNE was via Michael addition. Nonetheless, MDA was unable to generate cross-linked products among small antiradical soy peptides, probably due to electrostatic repulsions between peptides, steric hindrances, and their high mobility in the aqueous solution. The radical-scavenging activity, coupled with the reactivity with potent secondary products from lipid oxidation by many of the peptides, may explain why enzymatic hydrolysis improves the antioxidant activity of soy protein as demonstrated in previous studies.

AUTHOR INFORMATION

Corresponding Author

*Phone: +1 (859) 257-3822. Fax: +1 (859) 257-5318. E-mail: ylxiong@uky.edu.

Author Contributions

‖These authors made equal contribution to this study.

Funding

This research was supported by NRI/CSREES/USDA (Grant 2008-35503-18790). We acknowledge the proteomics core that is partially supported by grants from the National Center for Research Resources (P20RR020171-09) and the National Institute of General Medical Sciences (P20GM103486-09) from the National Institutes of Health. The Orbitrap mass spectrometer was acquired by High-End Instrumentation Grant S10RR029127 (to H.Z.). Approved for publication as journal article 12-07-058 by the Director of the Kentucky Agricultural Experiment Station.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Shacter, E. Y. Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* **2000**, *32*, 307−326.

(2) Esterbauer, H.; Schaur, R. J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. *Free Radical Biol. Med.* **1991**, *11*, 81−128.

(3) Uchida, K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog. Lipid Res.* **2003**, *42*, 318−343.

(4) Rio, D. D.; Stewart, A. J.; Pellegrini, N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc.* **2005**, *15*, 316−328.

(5) Akoh, C. C.; Min, D. B. *Food Lipids: Chemistry, Nutrition and Biotechnology*; Dekker: New York, 1998; 816 pp.
(6) McClements, D. J.; Decker, E. A. Lipid oxidation in oil-in-water emulsions: impact of molecular environment on chemical reactions in heterogeneous food systems. *J. Food Sci.* **2000**, *65*, 1270−1282.
(7) Faustman, C.; Sun, Q.; Mancini, R.; Suman, S. P. Myoglobin and lipid oxidation interactions: mechanistic bases and control. *Meat Sci.* **2010**, *86*, 86−94.
(8) Wang, L. L.; Xiong, Y. L. Inhibition of lipid oxidation in cooked beef patties by hydrolyzed potato protein is related to its reducing and radical scavenging ability. *J. Agric. Food Chem.* **2005**, *53*, 9186−9192.
(9) Cheng, Y.; Chen, J.; Xiong, Y. L. Fractionation, separation, and identification of antioxidative peptides in potato protein hydrolysate that enhance oxidative stability of soybean oil emulsions. *J. Food Sci.* **2010**, *75*, C760−C765.
(10) Uchida, K.; Sakai, K.; Itakura, K.; Osawa, T.; Toyokuni, S. Protein modification by lipid peroxidation products: formation of malondialdehyde-derived *N*-(2-propenol)lysine in proteins. *Arch. Biochem. Biophys.* **1997**, *346*, 45−52.
(11) Slatter, D. A.; Avery, N. C.; Bailey, A. J. Identification of a new cross-link and unique histidine adduct from bovine serum albumin incubated with malondialdehyde. *J. Biol. Chem.* **2004**, *279*, 61−69.
(12) Smathers, R. L.; Fritz, K. S.; Galligan, J. J.; Shearn, C. T.; Reigan, P. Characterization of 4-HNE modified L-FABP reveals alterations in structural and functional dynamics. *PLoS ONE* **2012**, *7* (6), e38459.
(13) Requena, J. R.; Fu, M.; Ahmed, M. U.; Jenkins, A. J.; Lyons, T. J.; Thorpe, S. R. Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. *Nephrol. Dial. Transplant.* **1996**, *11* (Suppl. 5), 48−53.
(14) Rohn, T. T.; Nelson, L. K.; Waeg, G.; Quinn, M. T. U-101033E (2,4-diaminopyrrolopyrimidine), a potent inhibitor of membrane lipid peroxidation as assessed by the production of 4-hydroxynonenal, malondialdehyde, and 4-hydroxynonenal−protein adducts. *Biochem. Pharmacol.* **1998**, *56*, 1371−1379.
(15) Wu, W.; Zhang, C.; Hua, Y. Structural modification of soy protein by the lipid peroxidation product malondialdehyde. *J. Sci. Food Agric.* **2009**, *89*, 1416−1423.
(16) Uchida, K.; Stadtman, E. R. Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4544−4548.
(17) Schilling, B.; Row, R. H.; Gibson, B. W.; Guo, X.; Young, M. M. Ms2Assign, automated assignment and nomenclature of tandem mass spectra of chemically crosslinked peptides. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 834−850.
(18) Jiang, J.; Chen, J.; Xiong, Y. L. Structural and emulsifying properties of soy protein isolate subjected to acid and alkaline pH-shifting processes. *J. Agric. Food Chem.* **2009**, *57*, 7576−7583.
(19) Adler-Nissen, J. *Enzymic Hydrolysis of Food Proteins*; Elsevier Applied Science Publishers: London, UK, 1986; 427 pp.
(20) Ma, Y.; Xiong, Y. L.; Zhai, J.; Zhu, H.; Dziubla, T. Fractionation and evaluation of radical scavenging peptides from in vitro digests of buckwheat protein. *Food Chem.* **2010**, *118*, 582−588.
(21) Pellegrini, N.; Re, R.; Yang, M.; Rice-Evans, C. A. Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying the 2,2′-azobis(3-ethylenebenzothiazoline-6-sulfonic) acid radical cation decolorization assay. *Methods Enzymol.* **1999**, *299*, 379−389.
(22) Fenaille, F.; Mottier, P.; Turesky, R. J.; Ali, S.; Guy, P. A. Comparison of analytical techniques to quantify malondialdehyde in milk powders. *J. Chromatogr., A* **2001**, *921*, 237−245.
(23) Requena, J. R.; Fu, M. X.; Ahmed, M. U.; Jenkins, A. J.; Lyons, T. J.; Baynes, J. W.; Thorpe, S. R. Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. *Biochem. J.* **1997**, *322*, 317−325.
(24) Wu, J. *Tandem Mass Spectrometric Analysis of Protein and Peptide Adducts of Lipid Peroxidation-Derived Aldehydes*. D.Phil. dissertation, Oregon State University, Corvallis, OR, 2009; 217 pp.
(25) Ajibola, C. F.; Fashakin, J. B.; Fagbemi, T. N.; Aluko, R. E. Effect of peptide size on antioxidant properties of African yam bean seed (*Sphenostylis stenocarpa*) protein hydrolysate fractions. *Int. J. Mol. Sci.* **2011**, *12*, 6685−6702.
(26) Kitts, D. D.; Weiler, K. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Curr. Pharm. Des.* **2003**, *9*, 1309−1323.
(27) Cheng, Y.; Chen, J.; Xiong, Y. L. Chromatographic separation and tandem MS identification of active peptides in potato protein hydrolysate that inhibit autoxidation of soybean oil-in-water emulsions. *J. Agric. Food Chem.* **2010**, *58*, 8825−8832.
(28) Elias, R. J.; Bridgewater, J. D.; Vachet, R. W.; Waraho, T.; McClements, D. J.; Decker, E. A. Antioxidant mechanisms of enzymatic hydrolysates of β-lactoglobulin in food lipid dispersions. *J. Agric. Food Chem.* **2006**, *54*, 9565−9572.
(29) Wang, W. Y.; De Mejia, E. G. A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. *Comp. Rev. Food Sci. Food Saf.* **2005**, *4*, 63−78.
(30) Nimalaratne, C.; Lopes-Lutz, D.; Schieber, A.; Wu, J. Free aromatic amino acids in egg yolk show antioxidant properties. *Food Chem.* **2011**, *129*, 155−161.
(31) Pownall, T. L.; Udenigwe, C. C.; Aluko, R. E. Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) enzymatic protein hydrolysate fractions. *J. Agric. Food Chem.* **2010**, *58*, 4712−4718.
(32) Gruendling, T.; Guilhaus, M.; Barner-Kowollik, C. Fast and accurate determination of absolute individual molecular weight distributions from mixtures of polymers via size exclusion chromatography−electrospray ionization mass spectrometry. *Macromolecules* **2009**, *42*, 6366−6374.
(33) Ishii, T.; Kumazawa, S.; Sakurai, T.; Nakayama, T.; Uchida, K. Mass spectroscopic characterization of protein modification by malondialdehyde. *Chem. Res. Toxicol.* **2006**, *19*, 122−129.
(34) Lamore, S. D.; Azimian, S.; Horn, D.; Anglin, B. L.; Uchida, K.; Cabello, C. M.; Wondrak, G. T. The malondialdehyde-derived fluorophore DHP-lysine is a potent sensitizer of UVA-induced photooxidative stress in human skin cells. *J. Photochem. Photobiol. B* **2010**, *101*, 251−264.
(35) Nadkarni, D. V.; Sayre, L. M. Structural definition of early lysine and histidine adduction chemistry of 4-hydroxynonenal. *Chem. Res. Toxicol.* **1995**, *8*, 284−291.
(36) Lin, D.; Lee, H. G.; Liu, Q.; Perry, G.; Smith, M. A.; Sayre, L. M. 4-Oxo-2-nonenal is both more neurotoxic and more protein reactive than 4-hydroxy-2-nonenal. *Chem. Res. Toxicol.* **2005**, *18*, 1219−1231.
(37) Sayre, L. M.; Lin, D.; Yuan, Q.; Zhu, X.; Tang, X. Protein adducts generated from products of lipid oxidation: focus on HNE and ONE. *Drug Metab. Rev.* **2006**, *38*, 651−675.
(38) Sayre, L. M.; Arora, P. K.; Iyer, R. S.; Salomon, R. G. Pyrrole formation from 4-hydroxynonenal and primary amines. *Chem. Res. Toxicol.* **1993**, *6*, 19−22.
(39) Isom, A. L.; Barnes, S.; Wilson, L.; Kirk, M.; Coward, L.; Darley-Usmar, V. Modification of cytochrome *c* by 4-hydroxy-2-nonenal: evidence for histidine, lysine, and arginine−aldehyde adducts. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1136−1147.
(40) LoPachin, R. M.; Geohagen, B. C.; Gavin, T. Synaptosomal toxicity and nucleophillic targets of 4-hydroxy-2-nonenal. *Toxicol. Sci.* **2009**, *107*, 171−181.
(41) Doorn, J. A.; Petersen, D. R. Covalent modification of amino acid nucleophiles by the lipid peroxidation products 4-hydroxy-2-nonenal and 4-oxo-2-nonenal. *Chem. Res. Toxicol.* **2000**, *15*, 1445−1450.

NOTE ADDED AFTER ASAP PUBLICATION

There was an error in the caption of Figure 1 in the version of this paper published September 11, 2012. The correct version published September 13, 2012.