Modification of Histidine Residues in Bovine Serum Albumin by Reaction with (*E*)-2-Octenal

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Reaction of bovine serum albumin (BSA) with the lipid peroxidation product (*E*)-2-octenal leads to the generation of (*E*)-2-octenal–BSA adducts. Amino acid analysis of the modified protein (after reduction with $NaBH_4$) showed that histidine residues were major targets of the reaction and showed the formation of new amino acid derivatives. The same products were detected in acid hydrolysates of poly(L-histidine) and *N*-(carbobenzoxy)-L-histidine after their reactions with (*E*)-2-octenal and $NaBH_4$. The reaction of *N*-(carbobenzoxy)-L-histidine with (*E*)-2-octenal led to the production of isomeric forms of *N*-(carbobenzoxy)-1(3)-[1′-(formylmethyl)hexyl]-L-histidine dihydrate. Upon acid hydrolysis, these compounds yielded stoichiometric amounts of histidine. However, after reduction with $NaBH_4$, acid hydrolysis led to a mixture of amino acid derivatives [presumably, isomeric forms of 1(3)-[1′-(hydroxyethyl)hexyl]-L-histidine] that were indistinguishable from those obtained from BSA, poly(L-histidine), and *N*-(carbobenzoxy)-L-histidine after similar treatment. Although other possibilities are not excluded, it is suggested that the modification of histidine residues in BSA by (*E*)-2-octenal involves a Michael-type addition of the imidazole nitrogen atom of histidine to the α,β-unsaturated bond of (*E*)-2-octenal. The reaction of histidine residues with (*E*)-2-octenal provides the basis for methods by which the contributions of (*E*)-2-octenal in the modification of proteins can be determined.

Keywords: *Alkenals; histidine residues; bovine serum albumin; lipid peroxidation*

INTRODUCTION

Lipid oxidation is one of the major causes of food spoilage and is undesirable not only from an acceptability and economic point of view but also because oxidative reactions can decrease the nutritional quality of food and generate oxidation products that are potentially toxic (Matsuo, 1962; Morton, 1977; Nawar, 1985; Richardson, 1984). Among the negative effects produced by peroxidizing lipids in foods, their chemical interactions with proteins have received considerable attention (Desai and Tappel, 1963; Gardner, 1983; Karel et al., 1975; Pokorny et al., 1988). This is an important deteriorative mechanism in the processing and storage of foods causing loss in flavor, color, functional properties, and nutritive value and, also, causing changes in biological tissues and is a basic pathological process in vivo (Funes et al., 1982; Kanner and Karel, 1976; Kanner et al., 1987; Tappel, 1973).

Exposure of proteins to peroxidizing lipids of their secondary breakdown products can produce changes in proteins, including loss of enzyme activity, polymerization, insolubilization, scission, formation of lipid–protein complexes, some of which are fluorescent (Chio and Tappel, 1969; Funes et al., 1982; Gardner, 1979; Pokorny and Janicek, 1975; Hidalgo and Kinsella, 1989), and destruction of labile amino acid residues—namely, histidine, lysine, cysteine, and methionine (Yanagita et al., 1976; Chiba et al., 1976; Horigome et al., 1974; Braddock and Dugan, 1973; Roubal and Tappel, 1966; Buttkus, 1967). In the presence of peroxidizing lipids, methionine residues are oxidized to methionine sulfoxide (Tannenbaum et al., 1969; Njaa et al., 1968), lysyl ε-amino groups readily form Schiff base condensation products with aldehydes (Buttkus, 1967; Kuusi et al., 1975; Davidkova et al., 1975), and cysteinyl sulfhydryl groups add to the double bonds in linoleate hydroperoxides via a free radical mechanism (Gardner et al., 1985) or to aldehydes via condensation reactions (Esterbauer et al., 1976). In the degradation of histidine by peroxidizing lipids, Yong and Karel (1978a) theorized the following sequence of events: (1) formation of an α-carbon radical by deamination, (2) hydroperoxidation of the α-carbon, and (3) hydroperoxide homolysis, which led to the observed products imidazolelactic acid, and imidazoleacetic acid. Additionally, the imidazole side chain was susceptible to attack as determined with the model compound 4-methylimidazole (Yong and Karel, 1978b) or with α-amino derivatives of histidine, such as *N*-acetylhistidine (Yong and Karel, 1979).

Little is known, however, about the reactions leading to the degradation of histidyl residues by 2-alkenals (aldehydes produced by lipid oxidation). Histidyl residues in proteins with (*E*)-2-octenal were substantially altered (Alaiz et al., 1994). Histidine is an essential amino acid for infants (Altschul, 1974) and has been observed to exhibit relatively strong antioxidative activities in emulsified or lyophilized model systems (Tjhio and Karel, 1969; Marcuse, 1962). Moreover, because of the acid–base characteristics of the imidazole side chain at physiological pH, histidyl residues are often associated with the active sites of enzymes (Bisby et al., 1974; Miles, 1974) and, at the same time, are important metal-binding groups in many metalloproteins (Harding and Long, 1968; Candlin and Harding, 1970). Thus, elucidation of the mechanisms leading to the degradation of histidyl residues by 2-alkenals will have important nutritional as well as biological implications.

In the present paper, we find that histidine residues in bovine serum albumin (BSA) (model protein) are major targets for reaction with the lipid peroxidation product (*E*)-2-octenal. In addition, the results of model studies with *N*-(carbobenzoxy)-L-histidine and poly(L-histidine) suggest that (*E*)-2-octenal reacts with the imidazole nitrogen atoms of histidine residues in BSA by a Michael-type addition reaction.

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Table 1. Amino Acid Composition of Bovine Serum Albumin Modified by (*E*)-2-Octenal after Reduction with Sodium Borohydride

amino acid	no. of residues	bovine serum albumin native	incubated with none (control)	incubated with (*E*)-2-octenal
Ala	46	47.81	46.42	47.90
Arg	23	22.50	23.73	24.72
Asp	48	49.11	50.52	51.63
Cys	35	35.00	32.43	35.30
Gly	15	16.61	15.12	15.22
Glu	75	75.14	77.60	79.90
His	17	17.70	17.50	8.30
Ile	14	14.43	14.90	14.92
Leu	61	63.71	64.52	63.82
Lys	59	56.54	57.00	50.60
Met	4	5.21	4.61	4.53
Phe	26	28.61	29.14	28.92
Pro	28	26.82	26.58	29.21
Ser	28	28.21	27.85	28.52
Thr	34	33.72	33.84	34.90
Trp	2	–[a]	–	–
Tyr	19	21.50	20.31	19.59
Val	36	35.10	36.32	36.91

[a] –, not determined.

MATERIALS AND METHODS

Materials. (*E*)-2-Octenal was obtained from Aldrich Chemie (Steinheim, Germany). *N*-(Carbobenzoxy)-L-histidine, poly(L-histidine), and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). MN-Kieselgel 60 (0.063–0.2 mm particle size) for column chromatography and Alugram analytical plates (20 × 20 cm) with fluorescent indicator for TLC were obtained from Macherey Nagal (Duren, Germany). A glass column (40 × 1.8 cm) for absorption chromatography was from Afora (Barcelona, Spain). All other reagents were of the highest grade commercially available.

Instrumentation. The HPLC system (Waters) consisted of a Model 600E multisolvent delivery system, a Wisp Model 712 automatic injector, a Model 484 UV–vis detector, and an APC IV NEC personal computer. Data acquisition and processing were effected with Maxima 820 version 3.3 software (Waters). Separations were attained using a 300 × 3.9 mm i.d. reversed-phase column (Nova-Pak C_{18}, 4 μm; Waters). The column was maintained at 18 °C by a temperature controller (Julabo F 10).

Reaction of Bovine Serum Albumin and Poly(L-histidine) with (*E*)-2-Octenal. The reaction mixture containing 4 mg of bovine serum albumin or 1.5 mg of poly(L-histidine) was incubated with 3 mM (*E*)-2-octenal in 4 mL of 100 mM sodium phosphate (pH 7.0) for 4 h at 37 °C. After incubation, the reaction mixture was treated with $NaBH_4$ (6 mg, 40 mM) at 37 °C for 15 min and then dialyzed against 100 mM sodium phosphate (pH 7.0) for measurement of amino acid analysis.

Reaction of *N*-(Carbobenzoxy)-L-histidine with (*E*)-2-Octenal. The reaction mixture (4 mL) containing 7 mM *N*-(carbobenzoxy)-L-histidine, 7 mM (*E*)-2-octenal, and 100 mM sodium phosphate (pH 7.0) was incubated at 37 °C for 4 h. After incubation, the reaction mixture was treated with $NaBH_4$ (6 mg, 40 mM) at 37 °C for 15 min and then extracted with diethyl ether (4 × 2 mL) to remove the lipids. The aqueous phase was assayed for amino acid composition.

Amino Acid Analysis. A sample (2 mL) of the reaction mixture, with D,L-α-aminobutyric acid as internal standard, was hydrolyzed with 12 M HCl (2 mL) for 20 h at 110 °C under nitrogen atmosphere. The hydrolyzed sample was evaporated to dryness and dissolved in 25 mL of 1 M sodium borate (pH 9.0) containing 0.02% sodium azide. A sample aliquot (5 mL) was treated with 4 μL of diethyl ethoxymethylenemalonate (DEEMM) for 50 min at 50 °C with vigorous shaking. The resulting mixture was cooled to room temperature, and 15 μL was injected into the chromatograph for determination of amino acid composition by reversed-phase HPLC (Alaiz et al., 1992). Resolution of the amino acid derivatives was routinely

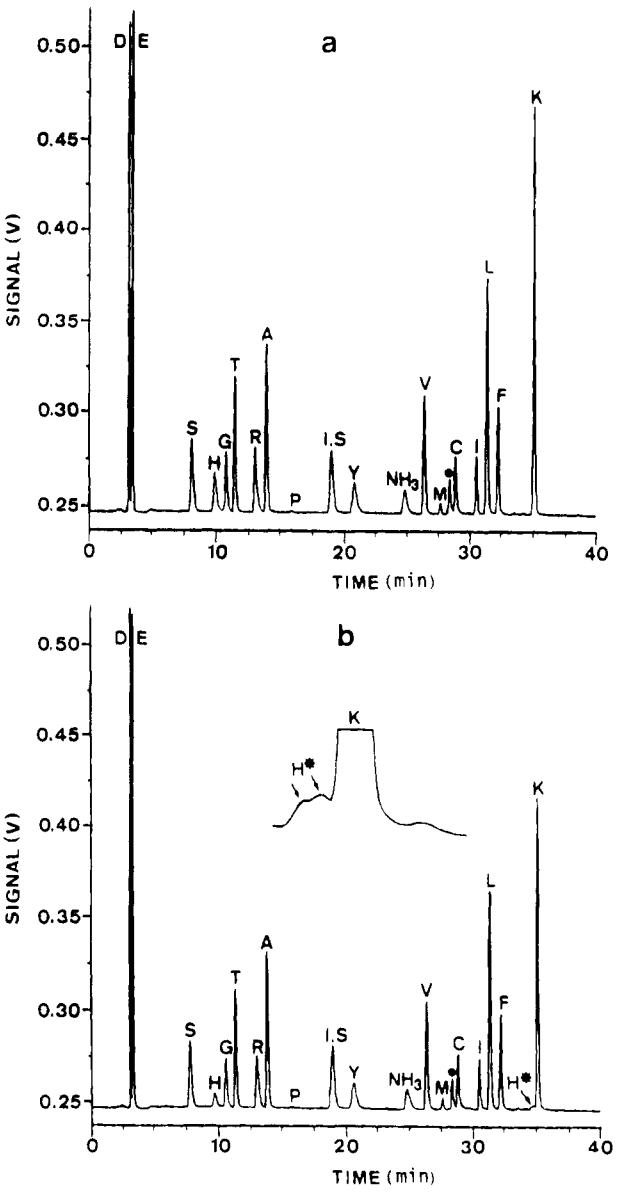

Figure 1. HPLC profile of amino acid analysis of unmodified BSA (a) and of BSA modified with (*E*)-2-octenal (b). Samples were prepared as described under Materials and Methods. Peaks are labeled with single-letter notations for amino acids. I.S., D,L-α-aminobutyric acid (internal standard); ●, unidentified peak; H*, (*E*)-2-octenal–histidine adducts.

accomplished using a binary gradient system. The solvents used were (A) 25 mM sodium acetate containing 0.02% sodium azide (pH 6.0) and (B) acetonitrile. Solvent was delivered to the column at a flow rate of 0.9 mL/min as follows: time 0.0–3.0 min, linear gradient from A–B (91:9) to A–B (86:14); 3.0–13.0 min, elution with A–B (86:14); 13.0–30.0 min, linear gradient from A–B (86:14) to A–B (69:31); 30.0–40.0 min, elution with A–B (69:31). Detection was by UV at 280 nm.

Synthesis of *N*-(Carbobenzoxy)-1(3)-[1′-(formylmethyl)hexyl]-L-histidine Dihydrate (Isomeric Mixture). These Michael adducts were obtained by reaction between *N*-(carbobenzoxy)-L-histidine and (*E*)-2-octenal under physiological medium (Alaiz and Girón, 1994). A reaction mixture (100 mL) containing 25 mM *N*-(carbobenzoxy)-L-histidine, 25 mM (*E*)-2-octenal, and 100 mM sodium phosphate (pH 7.0) was incubated at 37 °C for 24 h. After 24 h, the mixture was lyophilized and the residue was treated with 5 mL of methanol. The inorganic salts formed were filtered off, and the solution was concentrated under N_2 and subjected to Kiesegel 60 (50 g) column chromatography, eluting with chloroform–methanol (2:1). The collected fractions were examined by TLC, developing with 1-propanol–water (16:1), and spots were visualized

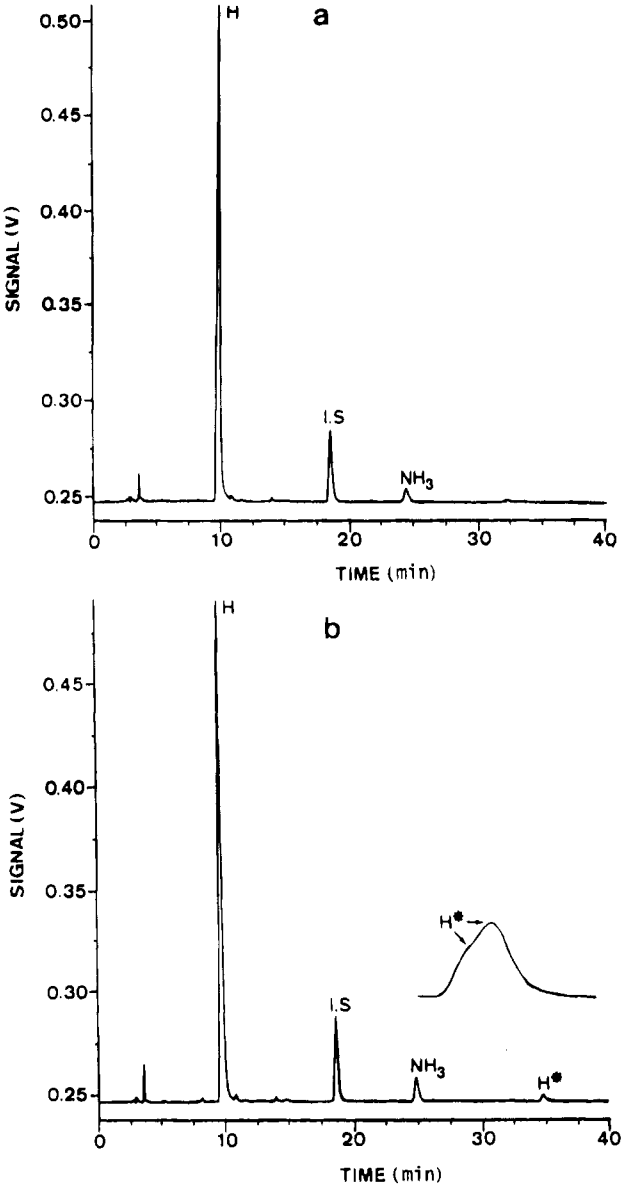

Figure 2. HPLC profile of amino acid analysis of unmodified poly(L-histidine) and of poly(L-histidine) modified with (*E*)-2-octenal (b). Samples were prepared as described under Materials and Methods. H, histidine; I.S., D,L-α-aminobutyric acid (internal standard); H*, (*E*)-2-octenal–histidine adducts.

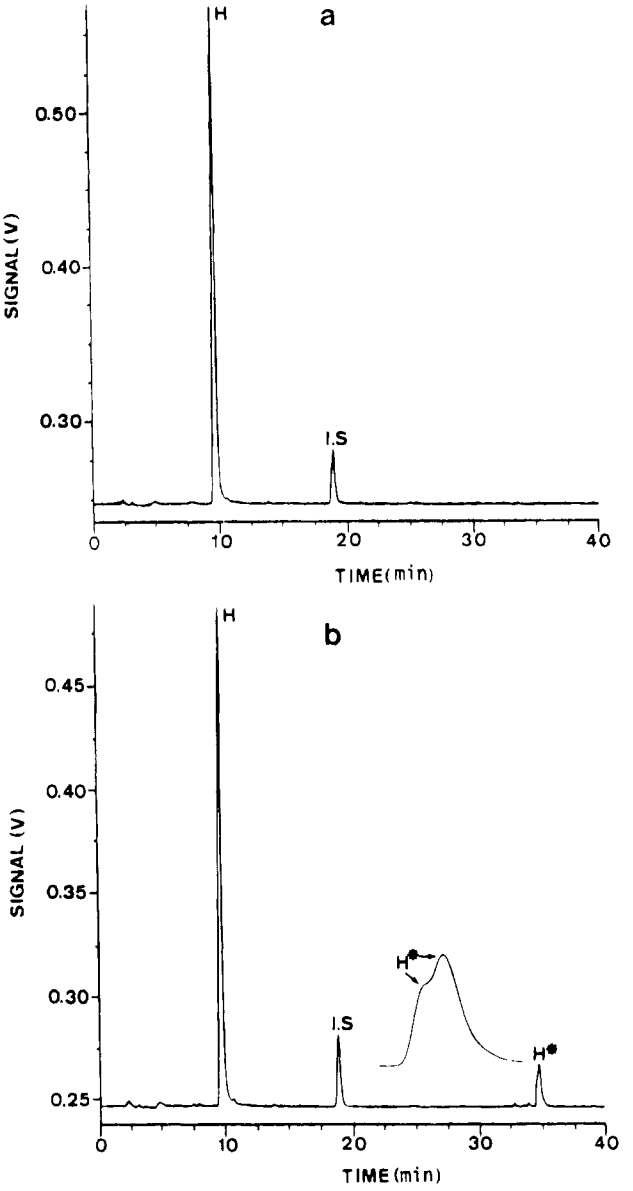

Figure 3. HPLC profile of amino acid analysis of unmodified *N*-(carbobenzoxy)-L-histidine and of *N*-(carbobenzoxy)-L-histidine modified with (*E*)-2-octenal (b). Samples were prepared as described under Materials and Methods. H, histidine; I.S., D,L-α-aminobutyric acid (internal standard); H*, (*E*)-2-octenal–histidine adducts.

by exposure to UV light at 254 nm or to 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole spray reagent (Dickinson and Jacobsen, 1970). TLC pure crystalline Michael adducts were obtained with a yield of 14.4%, after the solvent was evaporated under N_2 and those fractions that afforded a single spot of R_f 0.30 were dried under high vacuum. These Michael adducts were characterized by elemental analysis and UV, IR, and NMR spectroscopy (Alaiz and Girón, 1994).

Preparation of Calibration (*E*)-2-Octenal–Histidine Adducts. Standard solutions of *N*-(carbobenzoxy)-1(3)-[1′-(formylmethyl)hexyl]-L-histidine dihydrate with D,L-α-aminobutyric acid as internal standard were treated with 40 mM $NaBH_4$ for 15 min at 37 °C. After incubation, the mixtures were hydrolyzed with 6 N HCl for 20 h at 110 °C under nitrogen atmosphere. After incubation, the mixtures were evaporated to dryness, and then 1 M sodium borate (pH 9.0) containing 0.02% sodium azide was added. The mixtures were treated with DEEMM for 50 min at 50 °C and were chromatographed as described (Alaiz et al., 1992). The calibration curve was obtained by plotting peak response (peak response = peak area/internal standard area × internal standard amount) versus standard amount, using D,L-α-aminobutyric acid as internal standard.

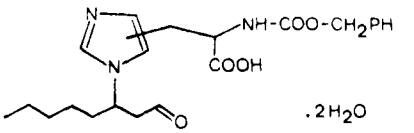

Figure 4. Structure of *N*-(carbobenzoxy)-1(3)-[1′-(formylmethyl)hexyl]-L-histidine dihydrate.

RESULTS AND DISCUSSION

In preliminary studies with several proteins, it became evident that, in addition to lysine residues, the reaction with (*E*)-2-octenal leads to a significant loss of histidine residues (Alaiz et al., 1994). To facilitate characterization of the (*E*)-2-octenal–histidine adduct, BSA was selected for further studies. The (*E*)-2-octenal-modified BSA was prepared by incubating BSA (1 mg/mL) with 3 mM (*E*)-2-octenal for 4 h at 37 °C. After incubation, a sample of the mixture was treated with $NaBH_4$ and then subjected to acid hydrolysis, and after

Table 2. Loss of Histidine and Concomitant Formation of (*E*)-2-Octenal–Histidine Adducts

sample[a]	histidine, mol/mol of subunit		(*E*)-2-octenal–histidine formed, mol/mol of subunit
	before	after	
bovine serum albumin	17.50	8.30	8.95
poly(L-histidine)	132.30	115.36	16.84
N-(carbobenzoxy)-L-histidine	1.0	0.74	0.26

[a] Each sample was incubated with (*E*)-2-octenal in 100 mM sodium phosphate (pH 7.0) for 4 h at 37 °C. Histidine and (*E*)-2-octenal–histidine adducts before and after incubation were determined (see Materials and Methods).

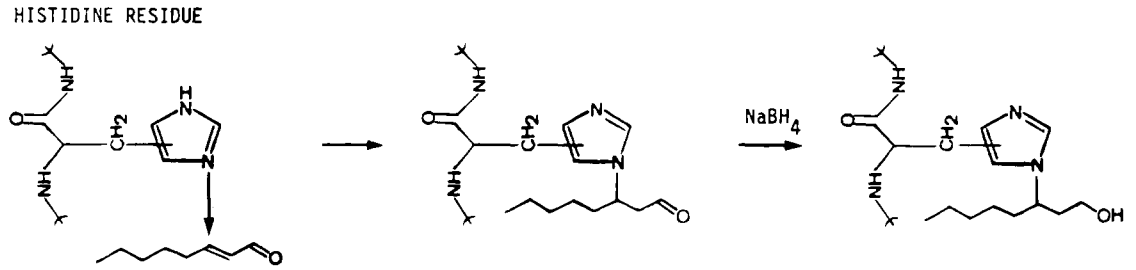

Figure 5. Proposed reaction of histidine residue with double bond of (*E*)-2-octenal and subsequent reduction of (*E*)-2-octenal–histidine adducts with $NaBH_4$.

derivatization with DEEMM, the amino acid composition of the mixture was determined by HPLC (Alaiz et al., 1992). The analysis showed that (*E*)-2-octenal modification is associated with the loss of 11.22% lysine residues and 52.57% histidine residues (Table 1) and the formation of two new amino acid derivatives whose DEEMM derivatives (peaks H*) were eluted between 34.60 and 34.80 min (Figure 1). The same amino acids were detected in acid hydrolysates of poly(L-histidine) and *N*-(carbobenzoxy)-L-histidine that had been treated with (*E*)-2-octenal and subsequently reduced with $NaBH_4$ (Figures 2 and 3).

In recent studies, Alaiz and Girón (1994) showed that the reaction of *N*-(carbobenzoxy)-L-histidine with (*E*)-2-octenal led to the production of isomeric forms of *N*-(carbobenzoxy)-1(3)-[1′-(formylmethyl)hexyl]-L-histidine dihydrate (Figure 4). Upon acid hydrolysis, these Michael adducts lead to quantitative release of the histidyl moiety as free histidine. However, borohydride reduction followed by acid hydrolysis and then DEEMM derivatization of the Michael adducts gave the same peaks H* in amino acid analysis as those detected in the (*E*)-2-octenal-modified BSA. This confirms that the same adducts were generated in BSA exposed to (*E*)-2-octenal.

In the HPLC system used, the (*E*)-2-octenal–histidine adducts can be separated from all other normal amino acids. Therefore, the procedure used here can be used to quantitate the amount of (*E*)-2-octenal-modified histidine residues in proteins. The response factor for DEEMM–(*E*)-2-octenal–histidine adducts was calculated from calibration runs employing 10–2500 pmol of *N*-(carbobenzoxy)-1(3)-[1′-(formylmethyl)hexyl]-L-histidine dihydrate, using D,L-α-aminobutyric acid as an internal standard. Before derivatization with DEEMM, the standard solutions were treated with $NaBH_4$ and then hydrolyzed with HCl. Linearity of the response was ascertained in this range (correlation coefficient 0.980), and response factor was 0.26. The detection limit was below 5 pmol. As shown in Table 2, 9.20 histidine residues in BSA and 16.94 histidine residues in poly(L-histidine) were modified by (*E*)-2-octenal under our experimental conditions. In the reaction between *N*-(carbobenzoxy)-L-histidine and (*E*)-2-octenal, 26.00% of the histidine residues were modified.

The proposition that (*E*)-2-octenal reacts with the imidazole nitrogen atoms of histidine residues, by a Michael addition mechanism (Figure 5), is not without precedence. α,β-Unsaturated sugar aldehydes have been shown to react with purines by a Michael-type mechanism to yield isonucleosides (Kaluza et al., 1989), and 4-hydroxynonenal has been shown to attack histidine residues in proteins by Michael-type addition (Uchida and Stadtman, 1992).

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