Oxidation of Free Methionine and Methionine Residues in Protein Involved in the Browning Reaction of Phenolic Compounds

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The oxidation of methionine to its sulfoxide, as a possible cause of decrease in the biological value of red clover during drying with aeration, was examined using various model systems, in the presence or absence of polyphenol oxidase. The effects of catalase were also examined. Results indicated hydrogen peroxide as a possible intermediate that directly oxidizes methionine. The methionine oxidation can be one of the causes of the decrease in biological value of red clover during drying, beside the known damage of lysine in the same process.

The biological value of red clover is known to decrease upon air-drying because of the loss of available lysine, which is brought about by browning reactions of proteins and phenolic compounds.^{1~4)} However, the loss of methionine or methionine residues in protein may likewise be a cause of the decrease in biological value, especially since the oxidation of methionine to methionine sulfoxide has been reported to lower its growth promotive effect on rats by 40%.⁵⁾

Recent studies have shown that methionine sulfoxide can be gotten by the oxidation of methionine using autooxidating ascorbic acid,⁶⁾ oxidizing agents such as chloramine-T and *N*-chlorosuccinimide,⁷⁾ and oxidized lipids.⁸⁾ However, no mention has been made of methionine oxidation brought about by browned phenolic compounds such as flavonoids (which are abundant in red clover and grasses). We therefore examined the possibility of methionine oxidation by browning reactions of phenolic compounds in red clover. This report first describes oxidation of methionine (and amino acids) in free and bound states by nonenzymatically browned solutions of phenolic compounds and phenolic compounds undergoing nonenzymatic oxidation, and secondly the effects of catalase on the methi-

onine oxidation.

MATERIALS AND METHODS

Chemicals. Phenolic compounds and casein (Hammersten, Merck) were the same as those used in our previous work.⁹⁾ A standard amino acid mixture for amino acid analysis was purchased from Wako Pure Chemical Industries Ltd. Catalase (ovine liver, 150,000 units per ml) was purchased from P. L. Biochemicals, Inc.

Preparation of polyphenol oxidase. Crude oxidase was prepared from acetone powder of *Petastites japonicus* Miq. as described by Horigome *et al.*¹⁰⁾ Acetone powder (2.5 g) was mixed with 75 ml of 0.05 M phosphate buffer (pH 7.0) containing 1 M NaCl and 1% sodium ascorbate, constantly stirred for 2 hr at 5°C. An extract was obtained by filtration of the mixture through cheesecloth and subsequent centrifugation at 10,000 × *g* for 30 min at 5°C. The extract was chromatographed on a column of Sephadex G-25 (3 × 28 cm) equilibrated with 0.05 M phosphate buffer (pH 7.0). The column was developed with the same buffer. Fractions with oxidase activity were pooled and used in the experiment.

Amino acid analysis. For amino acid analysis we used an Atto amino acid analyzer (MLC-703). The protein sample was first hydrolyzed with 6N HCl at 110°C in an evacuated, sealed tube for 24 hr. Methionine and methionine sulfoxide were measured as described by Kobayashi *et al.*¹¹⁾ and Neuman *et al.*,¹²⁾ respectively. In the former procedure, however, the addition of 8.7 M acetic acid was omitted. Available lysine was measured as described by Carpenter.¹³⁾ Half cystine was quantified as cysteic acid as

described by Moore.¹⁴⁾

Experiment 1

Reaction of amino acid with browned solutions of phenolic compounds. A mixture of 0.5 ml ethyl alcohol solution containing 3.3 μ mol of phenolic compound and 1 ml of 0.067 M phosphate buffer (pH 6.8) was incubated at 80°C for 5 hr. After cooled, 0.5 ml of the standard amino acid mixture (0.4 μ mol of individual amino acids/ml) was added to the mixture, and again incubated at 37°C for 3 hr. The mixture was diluted with 1 ml of 0.2 M sodium citrate buffer (pH 2.2) and centrifuged. The supernatant was used for amino acid analysis. A reaction mixture without addition of phenolic compounds was used for the control experiment.

Experiment 2

Reaction of amino acid with browned solution of quercetin. A solution of 0.1 mmol of quercetin in 75 ml of 0.067 M phosphate buffer (pH 6.8) was mixed with 20 ml of H₂O and kept at 80°C for 24 hr, in a stoppered conical flask. The brown solution resulting was concentrated and filled up to 25 ml. One milliliter aliquots of the solution was taken in each capped test tube and mixed with 0.5 ml of amino acid mixture (0.4 μ mol of individual amino acids/ml). The mixture was incubated at 37°C. The incubation time was 3, 6, 12, and 24 hr. After the incubation, the mixture was diluted with 1.5 ml of 0.2 M sodium citrate buffer (pH 2.2), centrifuged, and used for amino acid analysis.

Experiment 3

Reaction of amino acid with phenolic compounds. Phenolic compound (each 3.3 μ mol) dissolved in 0.5 ml of ethyl alcohol was mixed with 0.5 ml of amino acid mixture (0.2 μ mol of individual amino acids/ml) and was kept at 80°C for 1 hr. The solution was evaporated to dryness. The residue was dissolved in 1.5 ml of 0.2 M sodium citrate buffer (pH 2.2), and the solution was centrifuged. The supernatant was used for amino acid analysis.

Experiment 4

Reaction of amino acid with enzymatically oxidized phenolic compounds. Phenolic compounds (caffeic acid, chlorogenic acid, or quercetin) (each 10 μ mol) dissolved in 0.5 ml of ethyl alcohol were added to 0.5 ml of 0.067 M phosphate buffer (pH 6.8), 0.5 ml of amino acid mixture (0.4 μ mol of individual amino acids/ml) and 0.5 ml of polyphenol oxidase solution, and were incubated at 37°C for 3 hr. After incubation, 0.1 ml of 20% aqueous sulfosalicylic acid was added to the mixture, and after 6 hr the mixture was centrifuged. The supernatant was used for amino acid analysis.

Experiment 5

Reaction of casein with phenolic compounds. Casein (100 mg) was dissolved in 1.5 ml of 0.05 M NaOH and

7.5 ml of 0.067 M phosphate buffer (pH 6.8). The solution was mixed with 10 μ mol of a phenolic compound (caffeic acid, chlorogenic acid, or quercetin) dissolved in 0.5 ml of ethyl alcohol, and incubated at 80°C for 24 hr. The solution was acidified (pH 4.2~4.4) by adding 10% acetic acid, and centrifuged. The supernatant was decanted. The precipitate was washed three times with ethyl alcohol and two times with diethyl ether by centrifugation and dried in a dessicator. The control experiment was done without adding any phenolic compound.

Experiment 6

Reaction of casein with enzymatically oxidized phenolic compounds. The procedure was the same as in experiment 5, except that 2 ml of polyphenol oxidase solution was included in the reaction mixture. The control experiment was done without adding any phenolic compound.

Experiment 7

Reaction of amino acid with catalase-treated browned solution. Quercetin (15 μ mol) dissolved in 2.5 ml of ethyl alcohol was mixed with 5 ml of 0.067 M phosphate buffer (pH 6.8) and incubated at 80°C for 24 hr. A 0.75 ml aliquot of this browned mixture was preincubated at 30°C for 3 hr with added catalase solution of 0.1 ml (15,000 units), and again incubated at 37°C for 3 hr with addition of 0.25 ml of amino acid mixture (0.4 μ mol of each amino acid/ml). The solution was mixed with 0.4 ml of 7.5% sulfosalicylic acid and centrifuged after 6 hr of standing. The supernatant was analyzed for amino acids. For the control experiment, catalase solution was replaced by distilled water.

Experiment 8

Reaction of casein with catalase-treated browned solution. Quercetin or caffeic acid (10 μ mol) dissolved in 2 ml of ethyl alcohol was mixed with 7.5 ml of 0.067 M phosphate buffer (pH 6.8) and incubated at 80°C for 24 hr. A 4.75 ml aliquot was mixed with 0.2 ml of catalase solution and preincubated at 30°C for 3 hr. Then 50 mg casein (in 0.75 ml of 0.05 M NaOH) was added to the solution and incubated at 37°C for 24 hr. Subsequent procedures were the same as described in experiment 5. For the control experiment, catalase solution was replaced by distilled water.

RESULTS

Effects of browned solutions of phenolic compounds on free methionine

Effects of 14 phenolic compounds on methionine damage are shown in Table I. The results show that browned solutions of phenolic compounds oxidize methionine to methionine sulfoxide, because their sum is rough-

TABLE I. EFFECTS OF NONENZYMATICALLY BROWNE SOLUTIONS OF PHENOLIC COMPOUNDS ON FREE METHIONINE (EXPERIMENT 1).

The values are the average of duplicate measurements. See text for experimental procedures (experiment 1).

Phenolic compound	Met remained (nmol/0.1 ml)	Met(O) ^a formed (nmol/0.1 ml)	Met + Met(O) (nmol/0.1 ml)
Control	6.68	0	6.68
Caffeic acid	4.23	1.89	6.12
Catechin	4.40	1.95	6.35
Tannic acid	4.44	1.61	6.05
Myricetin	4.61	2.25	6.86
Chlorogenic acid	4.91	1.14	6.05
Dihydroquercetin	4.97	1.29	6.26
Quercetin	5.12	1.87	6.99
Rhamnetin	5.32	1.38	6.70
Rutin	5.36	0.62	5.98
Protocatechuic acid	5.42	0.98	6.40
Luteolin	5.64	0.76	6.40
Azaleatin	5.86	0.93	6.79
Kaempferol	6.00	0.65	6.65
Phloroglucinol	6.45	0	6.45

^a Methionine sulfoxide.

FIG. 1. Reaction of Methionine with Nonenzymatically Browened Solution of Quercetin (Experiment 2).

See the text for experimental procedures (experiment 2). —○—, methionine; —●—, methionine sulfoxide; --○--, methionine + methionine sulfoxide.

ly equal to the original amount of methionine. The extent of oxidation was increased with the number of OH groups adjacent to the 4'-OH group of 3,5,7,4'-tetrahydroxyflavone. The extent of methionine oxidation by browened solutions of protocatechuic acid and phloroglucinol was less than that by a browened solution of their parent compound quercetin.

Figure 1 shows conversion of methionine to methionine sulfoxide by the incubation with a

TABLE II. AMINO ACIDS REMAINING AFTER INCUBATION WITH FLAVONOIDS (EXPERIMENT 3)

The values are the average of duplicate measurements. See the text for experimental procedures.

Amino acid	Flavonoids used			
	Without flavonoid	With		
		Kaempferol	Quercetin	Myricetin
Amino acid content (nmol/0.1 ml)				
Asp	6.58	6.70	6.54	6.66
Thr	6.56	6.16	6.02	5.21
Ser	6.80	6.90	6.42	5.98
Glu	6.47	6.39	6.28	6.69
Pro	6.53	6.30	6.41	6.41
Gly	6.82	6.79	6.22	5.17
Ala	6.63	6.72	6.46	6.19
Cys	6.17	4.30	3.06	4.05
Val	6.69	6.66	6.42	5.81
Met	6.42	4.31	1.46	0
Met(O)	0	1.24	4.73	5.22
Ile	6.74	6.58	6.38	5.55
Leu	6.74	6.43	6.39	5.53
Tyr	6.65	6.54	6.12	5.10
Phe	6.70	6.43	6.12	4.81
Lys	6.51	6.56	4.87	2.62
His	6.77	6.51	5.09	2.75
Arg	6.67	6.56	5.54	3.52

browened solution of quercetin. The result also shows the sum of methionine and methionine sulfoxide almost unchanged.

Effects of nonenzymatic oxidation of phenolic compounds on free amino acids

The results of experiment 3 are shown in Table II. Similar results for cystine, methionine, methionine sulfoxide, lysine, and histidine are summarized in Fig. 2.

Methionine was converted to methionine sulfoxide in the presence of phenolic compounds undergoing oxidation, with the exception of luteolin and phloroglucinol. The order of the extent of methionine oxidation was myricetin > quercetin > kaempferol. Beside the methionine oxidation, cystine, histidine, and arginine were also decreased by the presence of quercetin and myricetin undergoing nonenzymatic oxidation. The decrease in cystine was marked especially in the case of

FIG. 2. Amino Acid Remaining after Incubation with Phenolic Compounds Undergoing Nonenzymatic Oxidation (Experiment 3).

See the text for experimental procedures. □, 1 hr; ▨, 3 hr.

quercetin and myricetin, and the decrease in lysine and histidine, which occurred in the presence of quercetin, myricetin, and caffeic acid, was less significant (Fig. 2).

Effects of enzymatic oxidation of phenolic compounds on free amino acids

The results are summarized in Table III, which showed significant decreases in proline, cystine, tyrosine, phenylalanine, lysine, histidine, and arginine. For methionine, the extent of decrease (9 to 24%) was generally less than that for the above amino acids. The decrease in methionine in this system was much less than that in Table I (23 to 37%).

Amino acid composition of enzymatically and nonenzymatically browned casein

The results are shown in Tables IV and V. The methionine content of casein was hardly decreased by enzymatic browning reaction, and the amount of methionine in casein browned by nonenzymatic browning reaction decreased by 70, 74, and 44% for caffeic acid, chlorogenic acid, and quercetin, respectively. In both experiments, the sum of methionine and methionine sulfoxide was approximately equal. In nonenzymatically browned casein (Table IV), the lysine content decreased by 7,

TABLE III. AMINO ACIDS REMAINING AFTER INCUBATION WITH PHENOLIC COMPOUNDS IN THE PRESENCE OF POLYPHENOL OXIDASE (EXPERIMENT 4)

The values are the average of duplicate measurements. See the text for experimental procedures.

Amino acid	Phenolic compounds used			
	Without phenolic compound	With		
		Caffeic acid	Chlorogenic acid	Quercetin
Amino acid content (nmol/0.1 ml)				
Asp	9.87	9.43	9.74	9.77
Thr	10.12	8.18	8.47	9.90
Ser	10.12	8.56	8.94	10.06
Glu	9.65	8.88	9.69	9.61
Pro	10.12	6.63	7.23	9.76
Gly	9.71	8.13	8.42	10.00
Ala	10.12	10.35	10.32	10.43
Cys	9.49	3.05	2.92	8.80
Val	9.89	9.40	9.26	10.06
Met	9.87	7.90	7.52	8.96
Met(O)	0	0.30	0.45	0.24
Ile	9.83	8.81	8.51	9.42
Leu	10.37	9.61	9.38	9.59
Tyr	9.72	6.98	6.10	8.25
Phe	9.97	7.06	6.80	9.01
Lys	10.31	6.49	6.14	9.18
His	9.40	3.40	4.07	7.79
Arg	9.68	7.27	7.18	9.25

TABLE IV. AMINO ACID COMPOSITION OF CASEIN INCUBATED WITH PHENOLIC COMPOUND (EXPERIMENT 5)

See the text for experimental procedures.

Amino acid	Phenolic compounds used			
	Without phenolic compound	With		
		Caffeic acid	Chloro- genic acid	Quercetin
Amount (g/15.67 g N)				
Asp	7.79	8.02	7.70	7.97
Thr	4.48	4.57	4.40	4.58
Ser	6.18	6.14	5.58	6.37
Glu	26.71	26.79	25.69	25.28
Pro	12.30	12.38	12.82	12.24
Gly	2.28	2.12	2.06	2.28
Ala	3.48	3.37	3.23	3.41
1/2 Cys	0.71	0.96	0.87	0.87
Val	8.49	8.54	8.00	7.31
Met	2.93	0.87	0.75	1.65
Met(O)	0.28	2.35	1.99	1.52
Ile	5.79	5.66	5.48	5.87
Leu	10.88	10.56	10.31	10.81
Tyr	6.80	6.14	6.07	6.56
Phe	5.90	5.74	5.66	5.92
Lys	8.85	8.25	8.18	8.73
avl. Lys ^a	8.78	7.81	6.42	7.72
His	2.91	2.62	2.60	2.80
Arg	3.84	3.56	3.64	3.75

^a Available lysine.

TABLE V. AMINO ACID COMPOSITION OF CASEIN INCUBATED WITH PHENOLIC COMPOUND IN THE PRESENCE OF POLYPHENOL OXIDASE (EXPERIMENT 6)

See the text for experimental procedures.

Phenolic compounds used				
Amino acid	Without phenolic compound	With		
		Caffeic acid	Chlorogenic acid	Quercetin
Amount (g/15.67 g N)				
Asp	7.90	7.73	7.70	7.98
Thr	4.51	4.50	4.49	4.64
Ser	6.25	6.15	6.29	6.21
Glu	25.80	25.07	25.81	25.94
Pro	13.21	13.21	12.46	13.29
Gly	2.17	2.15	2.12	2.20
Ala	3.30	3.25	3.26	3.38
1/2 Cys	0.80	0.73	0.83	0.87
Val	7.18	7.04	6.96	7.30
Met	2.87	2.81	2.88	2.74
Met(O)	0.35	0.21	0.21	0.27
Ile	5.67	5.62	5.49	5.74
Leu	10.32	10.11	10.24	10.28
Tyr	6.18	6.11	5.88	6.06
Phe	5.49	5.45	5.59	5.49
Lys	9.18	9.15	8.80	8.97
avl. Lys	8.28	7.69	7.69	7.65
His	2.97	2.73	2.68	2.92
Arg	4.03	3.83	3.81	3.96

slight decrease in the other amino acids was observed when catalase was added.

Amino acid composition of browned casein, which was prepared by the reaction of casein with nonenzymatically browned solution or with that preincubated with catalase, are shown in Table VII. The loss of methionine and its conversion to methionine sulfoxide were suppressed by pretreatment with catalase.

DISCUSSION

Free methionine decreased in the order myricetin, quercetin, kaempferol, by incubation with nonenzymatically browned solutions of phenolic compounds or the phenolic compounds themselves (experiments 1 and 3).

8, and 1% for caffeic acid, chlorogenic acid, and quercetin, respectively, and the available lysine content decreased by 11, 27, and 12% for these phenolic compounds, respectively. In enzymatically browned casein (Table V), the available lysine content decreased by 7, 7, and 8% for caffeic acid, chlorogenic acid, and quercetin, respectively.

Effects of catalase treatment of browned solution on amino acid damage

As seen in Table VI, free methionine was almost completely converted to methionine sulfoxide by the nonenzymatically browned solution of quercetin, while addition of catalase to the browned solution prevented the formation of methionine sulfoxide. Only a

TABLE VI. EFFECTS OF CATALASE ON THE ACTION OF NONENZYMATICALLY BROWNE SOLUTION OF QUERCETIN ON THE AMINO ACID MIXTURE (EXPERIMENT 7)

Values indicate nmol/0.1 ml amino acid mixture that remained after reaction. Initial amino acid: 6.67 nmol/0.1 ml each. See the text for experimental procedures.

Amino acid	Without catalase	With catalase
	Amino acid content (nmol/0.1 ml)	
Asp	5.77	6.04
Thr	5.56	5.94
Ser	5.50	5.42
Glu	5.50	6.19
Pro	4.76	5.03
Gly	5.42	5.87
Ala	5.36	6.14
Cys	5.60	5.96
Val	5.47	5.99
Met	0.23	5.62
Met(O)	5.50	0
Ile	5.43	5.70
Leu	5.33	5.78
Tyr	5.61	5.90
Phe	5.47	5.84
Lys	4.97	5.05
His	5.34	5.27
Arg	5.48	5.37

Correspondingly, methionine sulfoxide was formed. Luteolin (lacking the 3-OH of quercetin), rutin (the 3-*O*-glycoside of quercetin), and rhamnetin (the 7-methyl ether of quercetin) seemed equally or slightly less active than quercetin. These results suggested that the oxidizing activity increase with the number of OH groups adjacent to the 4'-OH group of 3, 5, 7, 4'-tetrahydroxyflavone.

It is seen from Table I that a nonenzymatically browned solution of phloroglucinol, which is one of the decomposition products of quercetin, was inactive in the oxidation of methionine. This result indicated the possibility that the dominant cause of the decrease in methionine by nonenzymatically browned solutions of quercetin is the oxidation of protocatechuic acid or quercetin itself. In casein or the amino acid mixture that was incubated with the nonenzymatically browned solution of quercetin, the decrease in methionine and

TABLE VII. EFFECTS OF CATALASE ON AMINO ACID DAMAGE BY THE PRESENCE OF NONENZYMATICALLY BROWNE SOLUTION OF PHENOLIC COMPOUND (EXPERIMENT 8)

See the text for experimental procedures.

Amino acid	Phenolic compounds used			
	Quercetin		Caffeic acid	
	Without catalase	With catalase	Without catalase	With catalase
	Amount (g/15.67 g N)			
Asp	7.64	7.39	7.40	7.61
Thr	4.57	4.42	4.46	4.52
Ser	6.19	5.53	6.04	5.83
Glu	23.65	23.00	22.97	24.08
Pro	13.66	13.25	12.87	13.37
Gly	1.94	1.92	1.96	2.06
Ala	3.29	3.01	2.99	3.23
Val	7.40	6.98	6.92	7.21
Met	2.13	3.08	1.14	2.80
Met(O)	0.96	0	0.88	0
Ile	5.46	4.95	5.10	5.30
Leu	9.95	9.14	9.27	9.68
Tyr	6.45	6.14	6.08	6.30
Phe	5.63	5.36	5.26	5.51
Lys	9.05	8.29	8.55	9.04
His	3.05	2.84	2.85	3.02
Arg	4.67	4.52	4.80	4.81

the formation of methionine sulfoxide were observed [experiments 1 and 8 (without catalase)]. On the other hand, when casein or the amino acid mixture was incubated with quercetin in the presence of polyphenol oxidase, the extent of the decrease in methionine and the formation of methionine sulfoxide was very small (experiments 4 and 6). Therefore, the decrease in free methionine and methionine in protein observed in feed containing kaempferol, quercetin, and/or myricetin seems to be due to the nonenzymatic browning of these flavonoids.

Suppression of the methionine oxidation by catalase (experiments 7 and 8) suggests that hydrogen peroxide produced by the nonenzymatic browning reaction of quercetin is the immediate active agent in methionine oxidation. This supposition is supported by the reports of methionine oxidation by hydrogen

peroxide,¹⁵⁾ and of production of hydrogen peroxide by autooxidation of 5,6-dihydroxyindole, an intermediate in melanin formation.^{16,17)}

The decrease in available lysine caused by caffeic acid, chlorogenic acid and quercetin undergoing nonenzymatic oxidation was generally less than that in methionine (experiment 5). This supports the hypothesis that the decrease in biological value of red clover by air-drying is due to the methionine oxidation by flavonoids and other phenolic compounds as well as the decrease in available lysine.

One reason that the oxidation of methionine to methionine sulfoxide has not attracted attention so far may be that browned proteins have usually been analyzed after 6N HCl hydrolysis, in which methionine sulfoxide is reduced back to methionine.^{11,12,18)}

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