Inhibition of Browning by Sulfur Amino Acids. 1. Heated Amino Acid–Glucose Systems

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Amino acids interact with carbohydrates to form Maillard browning products. Such reactions reduce the nutritional value of foods containing amino acids and carbohydrates and may lead to the formation of compounds that are mutagenic and clastogenic or chromosome-damaging. A need therefore exists to inhibit these heat-induced interactions. To demonstrate whether SH-containing sulfur amino acids minimize nonenzymatic browning, β-alanine, N^{α}-acetyl-L-lysine, glycylglycine, and a mixture of amino acids were each heated with glucose in the absence and presence of the following potential inhibitors: *N*-acetyl-L-cysteine, L-cysteine, reduced glutathione, sodium bisulfite, and urea. Inhibition was measured as a function of temperature, time of heating, and concentration of reactants. The extent of browning was estimated by absorbance measurements at 420 nm. Inhibition was independent of the amino group containing reactant. The minimum concentrations for optimum inhibition, in moles of inhibitor per mole of D-glucose, were as follows: sodium bisulfite, 0.02; L-cysteine, 0.05; *N*-acetyl-L-cysteine, 0.2; reduced glutathione, 0.2; urea, 8. An "index of prevention" (IP) was used to calculate the inhibition at the optimum mole ratio range, where IP = 100 − [molar absorptivity value (MAV) of the amine compound + glucose + inhibitor] × 100/(MAV of the amine compound + glucose). The calculated values were about 90% in all cases. Possible mechanisms of browning prevention are discussed.

INTRODUCTION

Sulfur-containing amino acids such as cysteine, *N*-acetylcysteine, and the tripeptide glutathione actively participate in the detoxification of xenobiotics in vivo. These sulfur-containing compounds also inhibit the action of mutagens, carcinogens, and other toxic compounds by direct interaction. These antioxidant and antitoxic effects are due to several mechanisms including the ability to act as (a) reducing agents, (b) scavengers of reactive oxygen (free-radical species), (c) strong nucleophiles that can trap electrophilic compounds and intermediates, (d) precursors for intracellular reduced glutathione, and (e) inducers of cellular detoxification. For example, we (Friedman, 1984; Friedman et al., 1982a) have shown that cysteine and related thiols inactivate the mutagenicity of aflatoxin. Other examples include (a) the demonstration by De Flora (1989) that coadministration of *N*-acetylcysteine dramatically decreased urethane-induced tumor formation in mice, (b) the report by Troll (1986) that the sulfur-rich protein called the Bowman–Birk protease inhibitor suppresses nitrosamine-induced carcinogenicity in the digestive tract of rats, (c) the reported protection in sheep against bitterweed (*Hymenoxys odorata*) poisoning by dietary components that stimulate the formation of SH-containing compounds in vivo (Calhoun et al., 1989), (d) the reported reduction of mutagen formation in fried beef by adding cottonseed flour (Rhee et al., 1987) or soy protein concentrate (Wang et al., 1982), and (e) the observed inhibition of lysinoalanine formation by cysteine (Finley et al., 1978; Friedman, 1978).

For these reasons, fruitful results are expected from evaluation of the effectiveness of sulfur amino acids and sulfur-rich proteins in (a) preventing the formation of antinutritional and toxic browning products by trapping intermediates and (b) reducing the toxicity of browning products in animals by preventing transformation of such compounds to biologically active forms.

This study examines the relative potencies of three SH-containing amino acids, sodium bisulfite, and urea in inhibiting browning of amino acids or peptides heated with glucose. Since sulfites are reported to induce asthmatic crises in 4–8% of exposed asthmatics (Gifon et al., 1989), a need exists to develop sulfite substitutes that can inhibit food browning. In two companion papers we cover related studies on the inhibition of browning in apples, potatoes, fruit juices, and protein-rich foods (Molnar-Perl and Friedman, 1990a,b).

MATERIALS AND METHODS

Matrials. L-Cysteine (free base) was obtained from U.S. Biochemical Corp., Cleveland, OH. All other amino acids, reduced glutathione, and urea came from Sigma, St. Louis, MO. Sodium bisulfite came from Malinckrodt, St. Louis, MO.

Instruments. Browning of amino acids was assayed by absorbance measurements with a Beckman DB spectrophotometer. A Radiometer pHM 26 meter and a Beckman 39030 thin-probe combination electrode were used for pH measurements.

Browning of Amino Acids with Glucose. Stock solutions of D-glucose (2 M), β-alanine (2 M), N^{α}-acetyl-L-lysine (0.5 M), mixed amino acids (0.6 M), glycylglycine (0.8 M), *N*-acetyl-L-cysteine (0.5 M), L-cysteine (0.4 M), glutathione (0.4 M), sodium bisulfite, (0.1 M), and urea (5 M) were adjusted to pH 6.5 before use. The solution of mixed amino acids was made up as follows (moles per liter): α-alanine (0.02), L-arginine (0.04), glycine (0.04), L-histidine (0.04), L-isoleucine (0.04), L-ornithine (0.04), L-phenylalanine (0.04), L-proline (0.04), L-serine (0.04), L-threonine (0.04), and L-valine (0.04) or 0.06 mol/L mixed amino acids.

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Table I. Molar Absorptivity Values (Am/M·cm) Obtained from the Reactions of Alanines (AL) and N^{α}-Acetyl-L-lysine (*N*-ALL) with D-Glucose (D-Glu)[a]

reactants	Am/(M·cm) 420 nm	Am/(M·cm) 300 nm	Am_{300}/Am_{420}
DL-Al–D-Glu	0.28 ± 0.015	7.0	25
L-Al–D-Glu	0.28 ± 0.015	7.0	25
D-Al/D-Glu	0.46 ± 0.010	11.0	24
β-Al–D-Glu	3.50 ± 0.20	52.5	15
N-ALL–D-Glu	1.43 ± 0.20	41.0	29

[a] Conditions: β-AL/D-Glu = 0.4 M/0.2 M; N-ALL/D-Glu = 0.10 M/0.2 M; temperature, 100 °C; reaction time, 90 min; initial pH 6.1–6.2; final pH 5.7–5.8.

To the mixture of 0.5 mL of D-glucose and 1.0–2.0 mL of one of the amino group containing solutions were added different amounts of the inhibitors in matched vials with screw caps (Kimble, Division of Owens, IL, No. 60910-1). The final volume of the solutions was adjusted to 5.0 mL with distilled water. The vials were placed in a boiling water bath and heated at 100 °C for periods up to 120 min. At the end of the heating period the vial rack was placed in a cold water bath. The absorbances of the solutions at 420 nm against distilled water blanks were determined using matched 1-cm cells. The standard error in preliminary experiments was estimated to be ±5%.

RESULTS AND DISCUSSION

Electron spin resonance studies (ESR) and related studies on the browning reaction (Feather and Huang, 1986; Friedman, 1982; Friedman et al., 1990; MacGregor et al., 1989; Montgomery, 1983; Namiki and Hayashi, 1983; Yen and Lai, 1987) revealed that both α- and β-alanines react with D-glucose under the influence of heat and that β-alanine is a much more reactive precursor of Maillard products than is α-alanine. For this reason, we selected β-alanine as the major model compound to assess the effectiveness of several potential inhibitors of nonenzymatic browning reactions. Table I compares molar absorptivity values of D-glucose heated with L-alanine, D-α-alanine, DL-α-alanine, β-alanine, and N^{α}-acetyl-L-lysine. The results at 420 nm show that β-alanine browning was about 12 times greater than that of α-alanine and about twice that of N^{α}-acetyl-L-lysine, a model for proteins. The values at 300 nm roughly parallel those at 420 nm. Figure 1 illustrates the absorbance maxima of the heated alanine–glucose mixtures. The results suggest that Maillard products produced by the alanines are probably similar in structure and that the structure of the alanine influences the concentrations of specific products in the equilibrium mixture.

Table II lists the molar absorptivity values at 420 nm of the browning products derived from various concentrations of β-alanine–D-glucose and N^{α}-acetyl-L-lysine–D-glucose heated at 100 °C for various times. The results strikingly illustrate that browning increases progressively as a function of concentration and time.

Figure 2 shows the pH profile of the molar absorptivities of β-alanine–D-glucose with initial pH ranging from 4.4 to 10.0, heated at 100 °C for 90 min. The sigmoid-shaped curve suggests that browning is strongly catalyzed by pH. Although initial and final pH values of the reaction mixtures were similar up to about pH 7, the pH decreased significantly in the final values for initial values beginning at about 8. The cause of this decrease is not immediately apparent.

On the basis of these results, we chose the following conditions to assess the effectiveness of several potential browning inhibitors: temperature 100 °C, reaction time 90 min, and pH 5.8–6.1 at a constant level of inhibitor. The following mole ratios of reactants were generally used:

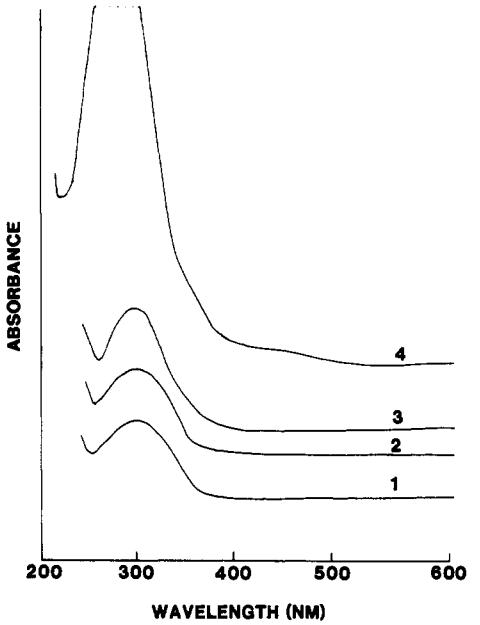

Figure 1. Absorbance spectra of the reaction products of alanines with D-glucose. Conditions: AL/D-Glu = 0.4 M/0.2 M; spectra taken with solutions diluted 1:10; 100 °C; 90 min; initial pH 6.1–6.2; final pH 5.7–5.8. Spectra: (1) L-AL–D-Glu; (2) D-AL–D-Glu; (3) DL-AL–D-Glu; (4) β-AL–D-Glu.

Table II. Molar Absorptivity Values (Am/M·cm) at 420 nm Obtained from the Browning Reactions at 100 °C of β-AL–D-Glu and N^{α}-Acetyl-L-lysine (*N*-ALL)–D-Glu as a Function of Concentrations and Mole Ratios of Reactants and Reaction Time[a]

β-AL:D-Glu mole ratio	β-AL:D-Glu concn, mol:mol	Am/(M·cm) at 420 nm 15 min	30 min	60 min	120 min
1:1	0.5:0.5	0.28	1.63	0.75	
1:2	0.5:1.0	0.47	1.90	2.25	
1:3	0.5:1.5	0.81	3.70	5.00	
2:1	1.5:0.5	1.02	3.60	5.00	
3:1	1.5:0.5	3.00	9.50	11.50	
3:1	0.75:0.25	0.88	3.40	5.00	22.80
	0.375:0.125	0.16	0.50	0.96	5.60
	0.150:0.050	0.10	0.16	0.24	0.90
	0.075:0.025	0.0	0.0	0.0	0.60
3:1	0.60:0.020			4.40	
6:1	0.60:0.10			3.40	
12:1	0.60:0.05			3.00	
24:1	0.60:0.025			2.92	

β-AL or *N*-ALL:D-Glu mole ratio	β-AL or *N*-ALL:D-Glu concn, mol:mol	β-AL:D-Glu (90 min)	*N*-ALL:D-Glu (90 min)
0.25:1	0.06:0.2	0.55 ± 0.05	1.08 ± 0.08
0.5:1	0.1:0.2	0.61 ± 0.04	1.43 ± 0.02
1:1	0.2:0.2	0.90 ± 0.03	3.25
1.5:1	0.3:0.2	2.07 ± 0.05	5.28 ± 0.20
2:1	0.4:0.2	3.50 ± 0.20	8.50
3:1	0.6:0.2	7.93 ± 0.35	
4:1	0.8:0.2	15.30 ± 0.40	
6:1	1.2:0.2	18.50 ± 0.10	

[a] Conditions: See Table I.

β-alanine/D-glucose, 0.4 M/0.2 M; N^{α}-acetyllysine/D-glucose, 0.1 M/0.2 M; glycylglycine/D-glucose, 0.16 M/0.2 M; and mixture of amino acids/D-glucose, 0.12 M/0.2 M.

Since oxygen may influence the extent of free-radical reactions as well as the extent of browning, mixtures of β-alanine–D-glucose with and without the inhibitor *N*-acetylcysteine were each saturated with nitrogen and oxygen, respectively, before heating. Since the extent of browning

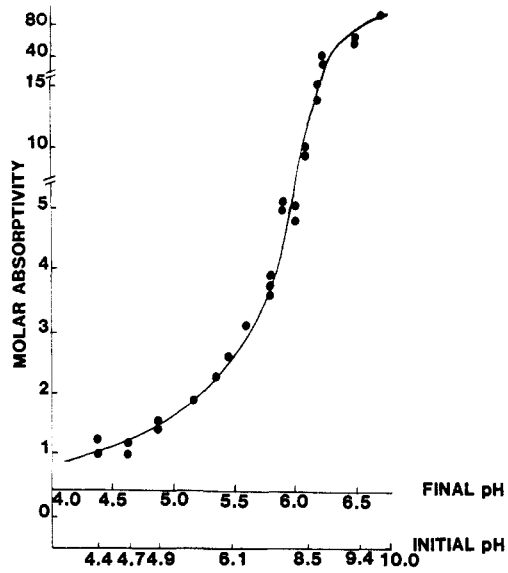

Figure 2. Molar absorptivity value–pH curve for the reaction of β-AL–D-Glu performed in solutions of various pH values. Conditions: β-AL/D-Glu = 0.4 M/0.2 M; 100 °C; 90 min. The initial (pH 4.40–10.0) and the final (pH 4.40–6.85) values are both indicated on the axis.

Table III. Effectiveness of Inhibitors on Browning Reactions (I–IV) of D-Glucose with β-Alanine (I), N^{α}-Acetyl-L-lysine (II), a Mixture of Amino Acids (III), and Glycylglycine (IV)

reaction	inhibitors	IP,[a] %	stoichiometry of inhibition,[b] mol of inhibitor/mol of MP
I	*N*-acetyl-L-cysteine	70	0.2
II	*N*-acetyl-L-cysteine	83	0.4
III	*N*-acetyl-L-cysteine	91	2.0
IV	*N*-acetyl-L-cysteine	89	0.4
I	L-cysteine	79	0.05
II	glutathione	83	0.08
I	sodium bisulfite	79	0.02
II	sodium bisulfite	96	0.12
III	sodium bisulfite	74	0.16
IV	sodium bisulfite	91	0.05
I	urea	91	12
II	urea	88	25
III	urea	95	12
IV	urea	89	12

[a] Index of prevention (IP) = 100 − (molar absorptivity value of the amine compound + glucose + inhibitor) × 100/(molar absorptivity value of the amine compound + glucose). [b] Minimum mole ratios needed to achieve the corresponding IP value. MP, Maillard reaction product precursor.

of the two solutions was the same, oxygen does not seem to influence the Maillard reactions.

Next, we used ultraviolet–visible and nuclear magnetic resonance spectroscopy (NMR) to systematically evaluate the relative suppression of browning by the following inhibitors: L-cysteine, *N*-acetyl-L-cysteine, reduced glutathione, sodium sulfite, and urea. The UV–visible spectra were plotted as molar absorptivities (Am) as a function of the molar ratios of the heated amino acid–D-glucose reaction mixtures in the absence and presence of each inhibitor (not shown). The tables and plots reveal the following information about browning and its prevention.

(1) Browning inhibition, as defined by an index of prevention (IP in Table III) varied between 70% and 96%. However, the amount of inhibitor needed to achieve this degree of inhibition varied. The minimum concentrations of each inhibitor needed for optimum inhibition per mole of D-glucose were as follows: sodium bisulfite, 0.02; L-cysteine, 0.05; *N*-acetylcysteine, 0.2; reduced glutathions, 0.2; urea, 8. For the first three, the concentration needed to inhibit browning was less than the theoretical amount needed to interact stoichiometrically with either partner in the browning reaction.

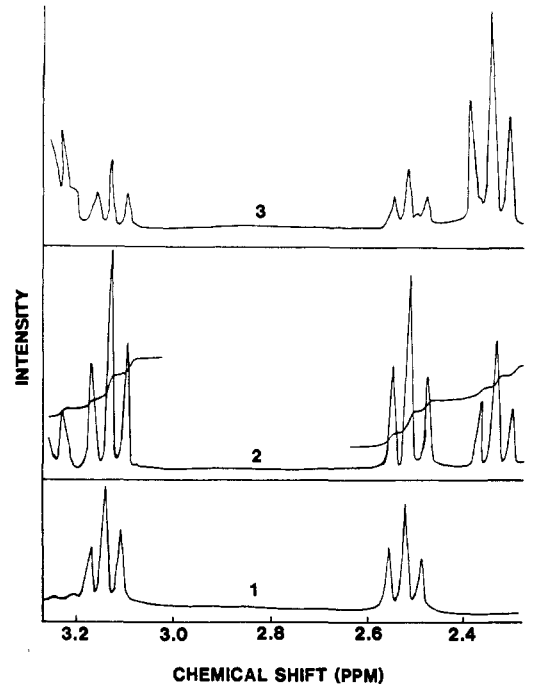

Figure 3. NMR spectra in the range 2.30–3.35 ppm of β-AL (0.4 M) (spectrum 1), β-AL (0.4 M) plus urea (3.0 M) (spectrum 2), and β-AL (0.4 M) plus urea (3.0 M) plus D-Glu (0.2 M) (spectrum 3).

(2) The molar absorptivity values for β-alanine–D-glucose and the inhibited Maillard reactions were surprisingly high. Mole ratios of 0.2, 0.5, and 0.6 of sulfur amino acids or sodium sulfite to Maillard product precursors resulted in absorptivity values that increased with inhibitor concentration. This suggests that autoxidation products of the inhibitors may contribute to the spectra.

(3) Preliminary NMR studies show that the addition of inhibitors to β-alanine–D-glucose caused no spectral changes. This result suggests that there are no apparent chemical interactions between cysteine, *N*-acetylcysteine, reduced glutathione, or sodium bisulfite and the Maillard product precursors. However, the NMR spectra do reveal possible interactions between urea and D-glucose. Thus, Figure 3, spectrum 1, shows two triplets associated with heated β-alanine. In the presence of urea, a third triplet appears at around 2.35 ppm, presumably arising from the interaction between urea and β-alanine (Figure 3, spectrum 2). The ratio of intensities of the two triplets centered at 2.70 and 2.35 ppm is 6:4. The spectrum of heated β-alanine plus D-glucose plus urea (Figure 3, spectrum 3) shows that D-glucose changes this ratio to about 1:4. The figure also shows that the initial triplets due to β-alanine at 2.53 and 3.14 ppm are further split as a result of reaction with urea, forming two additional triplets centered at 2.35 and 3.27 ppm. These observations suggest that urea inhibits browning by chemically interacting with β-alanine, thus preventing its reaction with glucose.

(4) Figures 4–7 show the absorption spectra in the region 200–600 nm for the Maillard product β-alanine–D-glucose (spectrum 1), for D-glucose plus inhibitors (spectrum 2), for β-alanine plus inhibitors (spectrum 3),

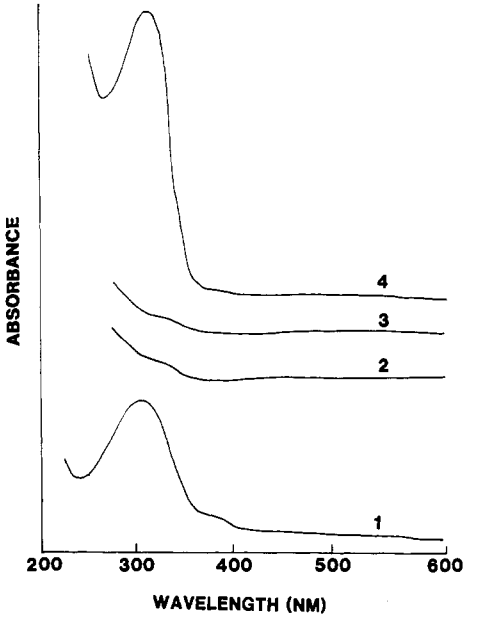

Figure 4. Absorbance spectra in the region 600–200 nm of solutions of β-AL/D-Glu = 0.4 M/0.2 M (spectrum 1), *N*-acetylcysteine (NAC)/D-Glu = 0.2 M/0.2 M (spectrum 2), NAC/β-AL = 0.2 M/0.4 M (spectrum 3), and β-AL/D-Glu/NAC = 0.4 M/0.2 M/0.2 M (spectrum 4), all four treated at optimum condition. Spectra have been taken at dilutions 1:50.

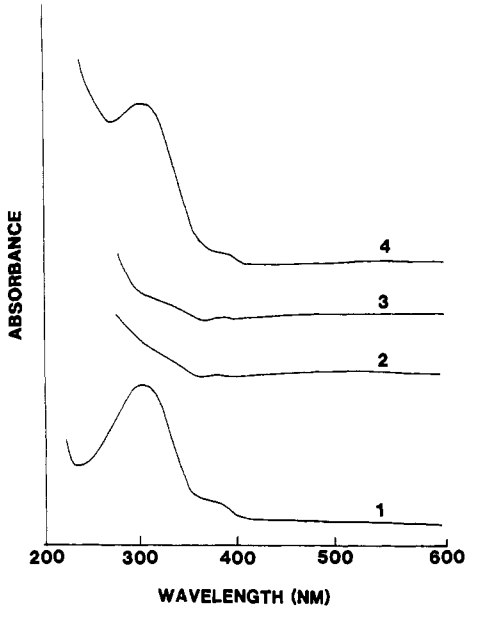

Figure 5. Absorbance spectra of solutions of β-AL/D-Glu = 0.4 M/0.2 M (spectrum 1), GSH/D-Glu = 0.05 M/0.2 M (spectrum 2), GSH/β-AL = 0.05 M/0.4 M (spectrum 3), and β-AL/D-Glu/GSH = 0.4 M/0.2 M/0.05 M (spectrum 4). Conditions as in Figure 1.

and for the inhibited Maillard products (spectrum 4). As with the NMR data, the electronic spectra show changes only for the urea-inhibited reaction (compare spectrum 1 to spectrum 4 in Figure 7), in which the characteristic absorption spectrum at 300 nm observed with Maillard product is decreased considerably. This difference may be due to the fact that any maximum absorption of the inhibited Maillard product is in a different part of the overlapping spectra. Evaluation of the spectra depicted in Figures 4–6 suggests the following: (a) the products of the inhibited reactions have absorption maxima at the same wavelength as the Maillard product precursors (Figure 4, spectra 1 and 4); and (b) the molar absorptivity values of the inhibited Maillard reaction are nearly the same as

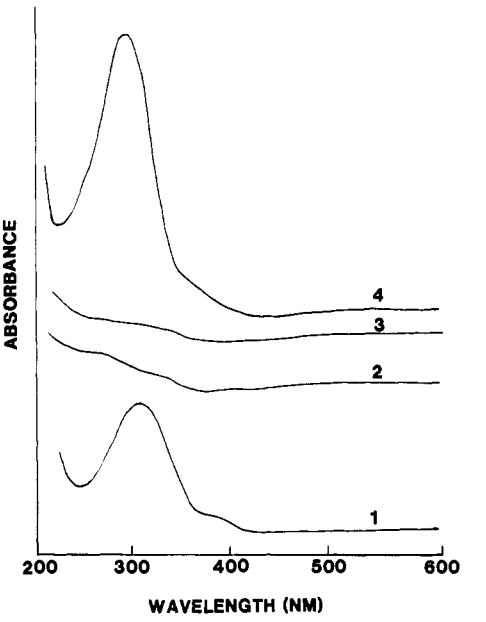

Figure 6. Absorbance spectra of solutions of β-AL/D-Glu = 0.4 M/0.2 M (spectrum 1), sodium bisulfite/D-Glu = 0.01 M/0.2 M (spectrum 2), sodium bisulfite/β-AL (spectrum 3), and β-AL/D-Glu/sodium bisulfite = 0.4 M/0.2 M/0.01 M (spectrum 4). Conditions as in Figure 1.

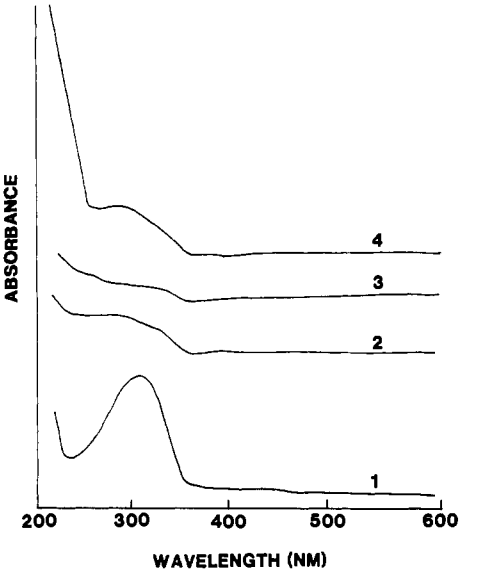

Figure 7. Absorbance spectra of solutions of β-AL/D-Glu = 0.4 M/0.2 M (spectrum 1), urea/D-Glu = 3.0 M/0.2 M (spectrum 2), urea/β-AL = 3.0 M/0.4 M (spectrum 3), and β-AL/D-Glu/urea = 0.4 M/0.2 M/3.0 M (spectrum 4). Conditions as in Figure 1.

(Figure 4 and 5) or somewhat higher than (Figure 6) that of the uninhibited Maillard reaction mixture. The spectral studies were repeated several times to validate the above conclusions.

Chemistry of Inhibition. Although the nature of the inhibition processes is not well understood, possibilities include (a) suppression of free-radical formation, whereby the formed radicals during heating are abstracted by and localized on the sulfur moiety of the thiol; and (b) interaction of the sulfhydryl compounds with intermediates formed during browning, thus trapping them and preventing them from forming the final browning product(s). Because of their strong nucleophilic reactivity and ability to dissipate free radicals, sulfur amino acids are especially capable of participating in the cited transformations on the basis of their extensively studied chemical properties. Thus, as previously noted (Fried-

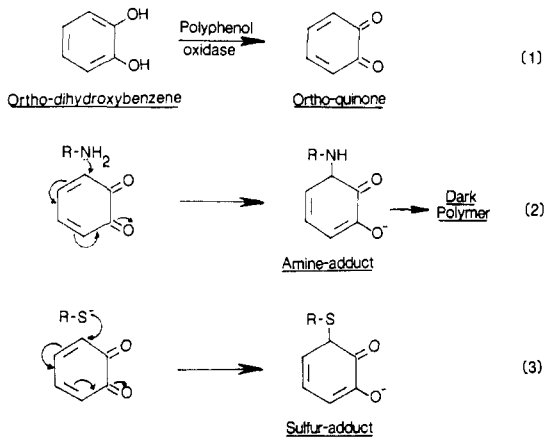

Figure 8. Postulated mechanism for the inhibition of polyphenol oxidase induced enzymatic browning by thiols: oxidation of a dihydroxybenzene to an *o*-quinone that can then participate in nucleophilic addition reactions with amino groups, leading to the formation of dark browning products, or competitively with mercaptide ions to form sulfur adducts, thus blocking polymer formation.

man, 1973), sulfhydryl groups in amino acids, peptides, and proteins participate in anionic, cationic, and free-radical reactions both in vitro and in vivo. The reactivity of the thiolate anion is much greater than would be expected from its basicity. Indeed, the thiolate anion appears to be one of the strongest nucleophiles known. This great reactivity presumably results from the polarizability of sulfur electrons and the availability of empty d orbitals, permitting d-orbital overlap. Another possibility is that the SH-containing compounds reduce carbonyl groups or react with carbonyl groups and double bonds in brown products to form colorless materials. These properties of the SH group can be taken advantage of to improve the quality and safety of our food supply.

Some of these concepts will be briefly illustrated with the aid of Figure 8, which schematically depicts the oxidation of a dihydroxy(poly)phenol by polyphenol oxidase to an *o*-quinone. This highly reactive intermediate then reacts with amino acids, peptides, and proteins to form dark polymers (Deshpande et al., 1984; Golan-Goldhirsh and Whitaker, 1984; Hurrell and Finot, 1984). In the presence of SH groups, which are known to react 200–300 times faster than amino groups in related nucleophilic addition reactions (Friedman et al., 1965), the quinone intermediate may be preferentially trapped as the sulfur adduct, thus preventing enzymatic browning.

Similar equations can be written for the preferential trapping of aldehyde or keto groups of reducing sugars to suppress nonenzymatic browning (Friedman, 1973). For example, in the pathway toward Maillard browning, it has been postulated that an aldehyde group of a reducing sugar interacts with an amino group to form a hemiketal adduct, which then dehydrates rapidly to form an aldimine (Schiff base). After an Amadori rearrangement, the latter is further transformed to dark browning products (eq 1). In the presence of a thiol, the aldehyde can com-

$$\underset{\text{carbohydrate}}{\text{R—CHO}} + \underset{\text{amine}}{\text{R'—NH}_2} \overset{\text{H}^+}{\rightleftharpoons} \underset{\text{hemiketal (carbinolamine)}}{\text{R—CH(OH)N}^+\text{H}_2\text{—R'}} \xrightarrow{-\text{H}_2\text{O}}$$

$$\underset{\text{aldimine (Schiff base)}}{\text{RCH=NH—R'}} \rightarrow \text{dark products} \quad (1)$$

$$\text{R—CHO} + \underset{\text{thiol}}{\text{R'—SH}} \rightleftharpoons \underset{\text{thiohemiketal (carbinol sulfide)}}{\text{R—CH(OH)S—R'}} \quad (2)$$

$$\text{R—CH(OH)S—R'} + \text{R'SH} \xrightarrow{-\text{H}_2\text{O}} \underset{\text{thioketal}}{\text{R—CH—(SR')}_2} \quad (3)$$

petitively interact with one or two SH groups to form a thiohemiketal or thioketal, thus blocking Maillard browning (eqs 2 and 3).

Relative reactivities of aldehyde or ketone groups with SH or NH_2 groups in structurally different environments will dictate the extent of inhibition of Maillard browning by various thiols. More work is needed to define the exact chemistry of the browning inhibition, as was done for sulfur dioxide (Wedzicha, 1987).

CONCLUSIONS

Our results indicate that SH-containing amino acids such as cysteine, *N*-acetylcysteine, and reduced glutathione are nearly as effective as sodium bisulfite in preventing nonenzymatic browning of heated amino acid–glucose mixtures. Urea is less effective, but it may be of special value in feed products for animal consumption, where it can also serve as a nutritional source of nitrogen for ruminants such as cattle and sheep (Friedman et al., 1982b).

Conditions were defined to evaluate the extent of browning inhibition in terms of an index of prevention (IP). If widely adopted, this index could serve as a well-defined measure of the relative effectiveness of browning inhibitors. This would facilitate comparing results from different studies in terms of a single parameter of browning prevention. Our findings also suggest that SH-containing sulfur amino acids may be of practical value to prevent browning in various food products. This has been successfully demonstrated in two companion papers (Molnar-Perl and Friedman, 1990a,b).

Finally, a special need exists to prevent browning in solution used for parenteral nutrition, containing both amino acids and carbohydrates (Kies, 1989; Rassin, 1989; Neuhauser-Berthold, 1989). The described studies on inhibition of browning in heated amino acid–carbohydrate solutions may provide a basis for designing parenteral solutions containing both of these food ingredients, so that a single intravenous feeding could replace two separate feedings containing either amino acids or carbohydrates.

ACKNOWLEDGMENT

We thank H. R. Bolin and S. Schwimmer for helpful comments.

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Received for review June 12, 1989. Revised manuscript received March 21, 1990. Accepted April 3, 1990.

Registry No. Glucose, 50-99-7; β-alanine, 107-95-9; *N*-α-acetyl-L-lysine, 1946-82-3; glycylglycine, 556-50-3; *N*-acetyl-L-cysteine, 616-91-1; L-cysteine, 52-90-4; glutathione, 70-18-8; sodium bisulfite, 7631-90-5; urea, 57-13-6.