Loss of Methionine in Casein during Storage with Autoxidizing Methyl Linoleate

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A model system consisting of casein (nine parts) and methyl linoleate (one part) has been used to study changes in methionyl residues of proteins as a consequence of lipid oxidation. A modified McCarthy-Sullivan procedure was developed which could distinguish methionine from its oxidation products, since acid hydrolysis partially converts methionine sulfoxide back to methionine. The concentration of unreacted methionine was followed in the model system at storage relative humidities of 0, 33, and 75%; in each case the loss of methionine was proportional to the amount of protein-bound nonenzymatic browning pigment. Methionyl residues may act as peroxide decomposers with concomitant carbonyl compound formation which in turn would lead to nonenzymatic browning.

Oxidation of small thiols and protein sulfhydryl groups by lipid peroxides is a well-known phenomenon (Little and O'Brien, 1966, 1967). The corresponding oxidation of sulfides has been demonstrated for methionine methyl ester in the presence of cod liver oil (Njaa *et al.*, 1968) and methionine in the presence of methyl linoleate (Karel and Tannenbaum, 1966). In neither case was nonenzymatic browning a significant problem, and in both cases the product of oxidation was methionine sulfoxide.

During an investigation on nonenzymatic browning of protein induced by lipid oxidation, the authors decided to determine whether, and to what extent, methionine was co-oxidized. A previous study (Tannenbaum *et al.*, 1968) indicated that methionine sulfoxide disproportionated to methionine and methionine sulfone during esterification for gas chromatographic analysis. Subsequent experiments confirmed the previous observation of Njaa (1962) that methionine is regenerated from its sulfoxide under conditions used for acid hydrolysis of proteins (6*N* HCl, 110° C., 20 hours). Therefore, it was necessary to develop the analytical methodology which would allow distinction between methionine and methionine sulfoxide in intact proteins.

EXPERIMENTAL

Materials and Model System. Methyl linoleate (Hormel Institute, Austin, Minn.) was vacuum distilled before use and was less than 0.5% oxidized. Casein was of Hammersten quality (Nutritional Biochemicals Corp., Cleveland, Ohio). Casein, methyl linoleate, and water were mixed in a high speed blender at 0° C. under a nitrogen blanket in proportions of 9 to 1 to 8. The resultant paste was extruded into Erlenmeyer flasks, freeze-dried, and stored for varying lengths of time in air at 37° C. with humidity controlled by an appropriate saturated salt solution: NaCl for RH 75%, and $MgCl_2$ for RH 33%.

Analysis. The concentration of methionine and the extent of nonenzymatic browning were determined in the protein portion of the model system following extraction of the lipid portion with methanol to chloroform (3 to 1). Browning was determined in enzyme hydrolysates of the protein by measurement of absorptivity at 420 mμ. Enzyme hydrolysis was effected by *Streptomyces griseus* protease (Pronase, Calbiochem, New York) which was capable of total solubilization of the reacted protein. Acid hydrolysis was effected in 6*N* HCl at 110° C. for 20 hours in evacuated sealed ampules.

Methionine was determined by a modification of the procedure of McCarthy and Sullivan (1941) as follows: 1 to 2 ml. of sample containing 0.1 to 1 mg. of methionine is added to a 5-ml. volumetric flask containing 0.5 ml. of 5*N* NaOH; 0.8 ml. of 1% sodium nitroprusside and 1.6 ml. of concentrated HCl are added in that order. The solutions are thoroughly mixed after each addition and allowed to cool to room temperature after addition of HCl and before bringing to volume with water. The solution is then vigorously shaken for 1 minute and the absorbance measured within 5 minutes at 510 mμ against a reagent blank. For highly colored samples it is also essential to prepare a sample blank with substitution of water for sodium nitroprusside. This modified procedure omits the use of glycine to eliminate interference from histidine and uses instead an optimal concentration of acid which has been found to avoid histidine interference entirely. Interference from tryptophan can still be a problem, but tryptophan reacts more slowly than methionine and its presence can be minimized by reading the samples shortly after mixing. Neither methionine sulfoxide, methionine sulfone, nor any of the common sulfur amino acids produce interfering pigments under the specified conditions.

Table I. Concentration of Methionine and Nonenzymatic Browning in Stored Casein-Linoleate Samples

Rel. Humidity	Days Stored	Browning[a]	Methionine in Hydrolysate[b]	
			Enzyme	Acid
0	32	3.8	2.5	2.5
0	46	3.7	2.4	2.4
0	67	4.2	2.4	2.2
0	113	15.0	0.5	1.8
33	32	3.4	2.4	2.2
33	46	8.9	1.5	2.0
33	67	10.5	1.2	2.3
33	113	13.0	0.8	1.8
75	32	6.1	2.2	2.2
75	46	12.5	1.5	1.9
75	67	13.4	1.3	1.9

[a] Absorptivity (420 mμ)/gram protein.
[b] Per cent of protein.

Results and Discussion. The concentration of methionine in stored casein-linoleate samples and the extent of browning of these samples is given in Table I. The concentration of

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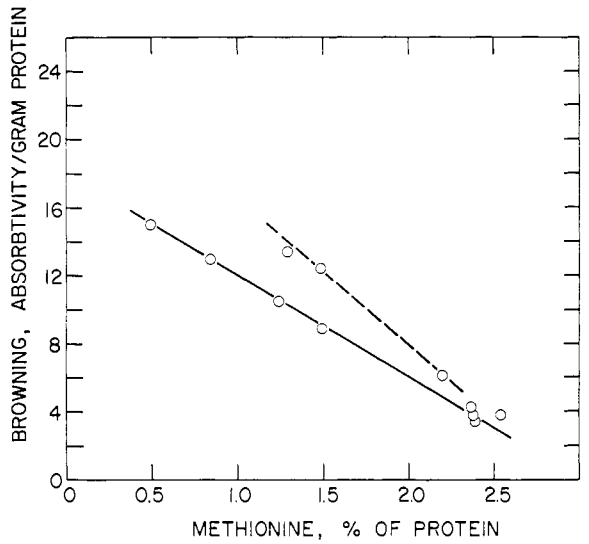

Figure 1. Relation between browning and loss of methionine in oxidized casein-linoleate system

75% RH - - - - -
0 or 33% RH —

Table II. Concentration of Methionine in Stored Casein[a]

Treatment	Methionine in Hydrolysate[b]	
	Enzyme	Acid
Control	2.9	2.7
45° C. storage	2.8	2.6
55° C. storage	2.3	2.6

[a] 115 days at 75% RH.
[b] Per cent of protein.

methionine in the acid hydrolysate is almost always higher than in the enzyme hydrolysate and the difference is ascribed to the presence of methionine sulfoxide. Under the conditions of acid hydrolysis, the sulfoxide would disproportionate to form free methionine and its sulfone, and the additional free methionine would contribute to the McCarthy-Sullivan value.

The loss of methionine in these samples is mainly a consequence of oxidation of the linoleate. As seen in Table II, casein alone can be stored under considerably more severe conditions with only minor changes in methionine concentration. Mildly browned casein-glucose model systems and nonfat dry milk show little if any change in methionine. It was not possible to examine very brown samples because the brown pigment increasingly interfered with the McCarthy-Sullivan pigment.

The methionine data are plotted against the extent of browning in Figure 1. A clear linear relationship can be seen in which the samples stored at 0 and 33% RH form a distinctly separate pattern from the condition of 75% RH. For this model system, a water activity of 0.33 is only slightly above a calculated Brunauer-Emmett-Teller monolayer, so that most of the water would be adsorbed at specific sites on the protein (Labuza, 1968). At a water activity of 0.75, there is a considerable quantity of liquid water condensed in multilayers and capillaries. Thus, the physical nature of the protein-lipid-water system could be very similar for storage conditions of 0 and 33% RH and vastly different from 75% RH. Similar reasoning could be used to analyze the environmental condition of the lipophilic methionyl side chain.

Figure 1 should not be interpreted to mean that methionine may be oxidized as a direct consequence of nonenzymatic browning reactions. Dehydrated protein foods which have undergone lipid oxidation usually give low peroxide values because the rate of peroxide decomposition approximates the rate of formation (Martinez and Labuza, 1968). Thus, the methyl sulfide side chain of methionine, which may be soluble in the lipid phase or aligned at the water-lipid interface, could decompose linoleate hydroperoxides and form carbonyl compounds. The carbonyl compounds could then react with other sites on the protein (*e.g.* ε-aminolysyl residues) to produce brown pigments, and the methionyl residues would be oxidized to the sulfoxide as a consequence of the peroxide decomposition.

The nutritional significance of this change is outside the scope of this discussion and has been extensively treated elsewhere (Carpenter *et al.*, 1963; Rao *et al.*, 1963; Miller *et al.*, 1965). However, the chemical and microbiological assays used in the investigations cited above could not distinguish between methionine and methionine sulfoxide, either because of the use of acid hydrolysis, or because most auxotrophic microorganisms can use both methionine and its sulfoxide interchangeably. The modified McCarthy-Sullivan procedure used in this investigation can distinguish between the two forms of the amino acid but has limited use (for low methionine contents) because of the low sensitivity of the nitroprusside reaction and the difficulties associated with interfering pigments in the sample.

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