Effect of Cystine Oxidation on Lysinoalanine Formation in Proteins

J. W. Finley,*[1] E. L. Wheeler, H. G. Walker, Jr., and A. J. Finlayson

Several different proteins (soy, safflower, alfalfa leaf protein, and lactalbumin) were treated with hydrogen peroxide prior to alkaline treatment in a study of the effect of oxidation on the formation of lysinoalanine (LAL). The oxidation state of cysteine appeared to be the determining factor in the production of LAL. When cysteine was oxidized to either the sulfinic acid or cysteic acid, no LAL formation was observed. LAL formation was observed, however, at intermediate oxidation states. The relationship of the oxidation state of cysteine, as induced by lipid hydroperoxides, to the tendency to form LAL in oilseed products is discussed.

Lysinoalanine (LAL) is formed in food proteins when the food is subjected to severe processing conditions that include high pH and temperature. For example, very high pHs are encountered when ropes are prepared for spinning protein. LAL is presumably formed in intact proteins at high pH through a β elimination of cystine or serine to yield dehydroalanine (DHA), followed by a Michael addition of lysine to the DHA to yield the lysinoalanine residue in the protein. LAL has been a subject of controversy because it causes kidney lesions (nephrocytomeglia) in rats but does not appear to cause similar lesions in other species (Woodard, 1969; Woodard and Short, 1973; deGrott et al., 1976; Karayiannis et al., 1979; Gould and MacGregor, 1977. Although the hazard of lysinoalanine to humans may not be as serious as initially thought, it is still desirable to limit its occurrence. The LAL content of proteins, however, varies enormously in foods receiving similar heat or alkaline treatments (Sternberg et al., 1975; Finley et al., 1976), making it difficult for processors to make products with uniformly low levels of LAL.

In studying this problem we observed an increased LAL content in soy isolates mixed with air at high pH compared with isolates not mixed with air, suggesting that oxidation is involved in the formation of LAL. On the basis of this, we (Finley et al., 1976) inhibited LAL formation by adding reducing agents, such as cysteine, or by processing under anaerobic conditions. This indicated that oxidized forms of cystine, rather than the sulfhydryl or disulfide forms, are most likely to undergo β elimination and form LAL.

The purpose of this work was to determine the influence of sulfur amino acids in various oxidation states on the formation of lysinoalanine in proteins. To this end, solutions of proteins were oxidized with hydrogen peroxide and lipid hydroperoxide, both of which occur naturally in goods; this was followed by alkaline treatment. Alkaline treatments were also carried out on partially oxidized glutathione derivatives that served as model peptides. Tendencies of the oxidized compounds to form dehydroalanine and subsequently lysinoalanine were studied.

MATERIALS AND METHODS

Reduced glutathione (GSH), lysozyme, bovine serum albumin, and N^{α}-acetyllysine were obtained from Sigma Chemical Co. (St. Louis, MO). Soy isolate (Promine-D) was obtained from Centra Soya Inc., Chicago, IL. α-Lactalbumin was obtained from United States Biochemical Co., Cleveland, OH. Safflower isolate and leaf protein concentrate were gifts from Cameron Lyon and Benny E. Knuckles of this laboratory (USDA–WRRC, Berkeley, CA). Glutathione in the disulfide monoxide form (GSOSG) was prepared by the procedure (Savige et al., 1964) used to make cystine monoxide from cystine. Similar procedures were used throughout, substituting glutathione for cystine in the material. Product was precipitated by making the final solution 90% ethanol at pH 6.0. The glutathionesulfinic (GSO_2H) and sulfonic acid (GSO_3H) derivatives were prepared as described by Calam and Waley (1962), but a pure disulfide dioxide GSO_2SG could not be obtained by their procedure. HPLC analysis of the product indicated gross contamination with oxidzed glutathione (GSG) and GSOSG. Lipid hydroperoxide was prepared from linoleic acid as previously described (Finley et al., 1981). Protein oxidations were carried out as previously reported (Finley et al., 1981). Alkaline treatments of oxidized protein were carried only for 4 h in 0.1 N sodium hydroxide at 65 °C.

Table I. Dehydroalanine Formation in Partially Oxidized Glutathione Derivatives

glutathione derivative	rate of dehydroalanine formation, $M\ s^{-1}$, at 65 °C in 0.1 N NaOH	
	bubbled with air	bubbled with N_2
reduced glutathione (GSH)	0	0
oxidized glutathione (GSSG)	2.3×10^{-4}	1.0×10^{-4}
glutathione monoxide (GSOSG)	5.6×10^{-4}	5.3×10^{-4}
glutathione dioxide (GSO_2SG)		
glutathionesulfinic acid (GSO_2H)	0	0
glutathionesulfonic acid (GSO_3H)	0	0

The kinetics of dehydroalanine formation were followed spectrophotometrically at 240 nm according to Nashef et al. (1977). Initial rate studies were carried out on 10 mM solutions of dimeric forms of glutathione and 20 mM solutions of monomeric glutathione products.

Hydrolysis for amino acid analysis was carried out with 10 mg of protein ($N \times 6.25$) under N_2 or in evacuated flasks for 24 at 110 °C in 6 N hydrochloric acid. Amino acid analysis of the hydrolyzed protein solution was carried out on a Durrum amino acid analyzer; analysis of the glutathione samples used a Beckman 120 C amino acid analyzer according to Spackman (1963).

RESULTS AND DISCUSSION

Glutathione derivatives (see Scheme I) with the sulfur amino acid portion in various oxidation states were heated with alkaline conditions at 65 °C in both N_2 and air (Table I). In both cases, only the GSOSG monoxide and GSSG forms yielded DHA in significant amounts. The major difference was under air, the rate of formation of DHA was more than twice the rate observed under N_2 for GSSG, but

Western Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, Berkeley, California 94710.

[1]Present address: Ralston Purina Company, St. Louis, MO 63188.

Table II. Products of Reaction of Equimolar Oxidized Glutathione and N^{α}-Acetyllysine (One Hour in 0.1 N NaOH)

	mmol/mL amino acid recovered[a]						
	Lys	LAL	Glu	Gly	Cys	$CYSO_3H$	LAN
GSH + Lys[b]	19.0	0	21.0	20.1	17.2	0	0
GSH + Lys	18.7	Tr[c]	19.7	19.9	16.6	0.2	0.4
GSSG + Lys	17.1	2.0	19.1	18.4	14.6	2.8	0.5
GSOSG + Lys	18.3	1.7	18.9	20.2	8.4	10.3	0.5
GSO_2SG + Lys	19.1	Tr	19.0	19.6	11.6	5.8	3.7
GSO_2H + GSO_3H	20.0	0	20.0	19.3	0	16.4	0
GSO_2H + GSO_3H	19.3	0	19.7	19.6	0	19.8	0

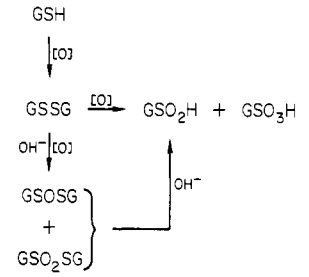
[a] Average of duplicate determinations. [b] No alkaline treatment. [c] Tr, trace.

Table III. Effect of Oxidation with Hydrogen Peroxide on LAL Formation in α-Lactalbumin

ratio of H_2O_2 to protein sulfur[a]	amino acid recovery,[b] g/16 g of N			
	lysine	cysteic/cystine	methionine	lysinoalanine
0	7.1	0	1.64	0.055
0.5	6.9	0.08	1.63	0.084
1	7.4	0.12	1.57	0.032
2	7.8	0.40	1.45	0
4	7.7	0.58	1.29	0

[a] Oxidations were carried out by direct addition of the appropriate amount of 10% hydrogen peroxide to 1% solutions of the protein in 0.1 M phosphate buffer at pH 6.5. After the solutions were stirred overnight and exhaustive dialysis, the samples were freeze-dried. Freeze-dried proteins were treated for 4 h at 65 °C in 0.1 N NaOH. [b] Average of triplicate samples.

Scheme I. Forms of Glutathione as Oxidized Cysteine Residues[a]



[a] GSH, reduced glutathione; GSSG, oxidized glutathione (disulfide); GSOSG, glutathione monoxide; GSO_2SG, glutathione dioxide; GSO_2H, glutathionesulfinic acid; GSO_3H, glutathionesulfonic acid.

there was essentially no difference for GSOSG.

When the alkaline reaction of the glutathione derivatives was carried out in the presence of N^{α}-acetyllysine, analysis of the hydroysates (Table II) indicated that the disulfide and monoxide forms of glutathione produced LAL but the more highly oxidized forms did not. These results are consistent with the results in Table I showing that dehydroalanine, the precursor of LAL, is not formed by any of the highly oxidized forms of glutathione. It is also interesting to note that small amounts of lysinoalanine were produced and that oxidized sulfur amino acids yielded large amounts of what appeared to be cysteic acid in the hydrolysate. Our chromatographic procedure does not, however, separate alaninesulfinic acid from cysteic acid, so hydrolysis products of the various oxidized glutathione derivatives cannot be well-defined, as previously reported by Finley and Lundin (1980). We conclude that the cystine monoxide moiety undergoes a β-elimination reaction to yield dehydroalanine, followed by a Michael addition reaction that produces LAL. Because oxygen increases the rate of LAL formation from GSH, a substantial portion of the cystine moiety may be oxidzed to the monoxide prior to the β-elimination step. The formation of some dehydroalanine by the cystine moiety under anaerobic conditions indicates that it can also undergo β elimination, but more slowly that the monoxide.

Dehydroalanine may also be formed by different routes. Nashef et al. (1977), for example, discussed several nonoxidative pathways and products from alkaline treatment of proteins. The possible oxidation of the cysteinyl residues prior to β elimination adds one more significant pathway for dehydroalanine formation. Most likely there is a mixture of pathways in food systems.

To determine if oxidation prior to alkaline treatment caused increased lysinoalanine formation in a pure protein such as α-lactalbumin, we analyzed amino acids in samples of α-lactalbumin (Table III) that were treated with H_2O_2 before being given the standard alkali treatment. It can be seen that as the degree of oxidation prior to alkaline treatment increases, the amount of LAL formed first increases and decreases. In fact, at the highest ratios of hydrogen peroxide to sulfur amino acid in the protein, little or no LAL was formed. It is interesting that the amount of methionine decreased as oxidation increased. Table III gives cysteic/cystine ratios because cystine is destroyed by HCl hydrolysis. Oxidation with performic acid prior to hydrolysis would not add any information concerning the oxidation state because the sulfur amino acids would be oxidized to cysteic acid and methionine sulfone. The results strongly suggest increased oxidation of cysteine residues to cysteic acid or cysteinesulfinic acid (they are not separated by our ion-exchange technique) with increasing amounts of hydrogen peroxide. They also indicate

Table IV. Effect of Various Oxidants on LAL Formation in Various Proteins[a]

	LAL, g/16 g of N[b]				
	untreated	unoxidized (alkali only)	benzoyl peroxide	potassium bromate	lipid hydroperoxide
lysozyme	0	0.67	0.057	0.061	0.094
bovine serum albumin	0	0.024	0.037	0.032	0.072
α-lactalbumin	0	0.055	0.093	0.072	0.110
soy isolate	0.01	0.039	0.052	0.065	0.084
leaf protein content	0	0.032	0.066	0.084	0.110
safflower isolate	0	0.060	0.038	0.052	0.036

[a] Same conditions as in Table III. [b] Average of triplicate determinations.

that a more complete study of the oxidative states of cysteine in the protein is warranted.

As previously reported (Finley and Lundin, 1980), when hydrogen peroxide reacts with peptide cysteine, a variety of products is formed. Excess hydrogen peroxide produces large amounts of GSO_2SG and GSO_2H. The sulfinic acid derivative does not yield dehydroalanine on alkaline hydrolysis, but we suspect that the disulfide dioxide does. This is based on the observation (Kice and Rogers, 1974; Savige et al., 1964) that the active dehydroalanine-producing disulfide monoxide form is a prominent intermediate in the alkaline hydrolysis of related disulfide dioxides. Lysinoalanine was determined after several proteins and protein isolates were oxidized with benzoyl peroxide, potassium bromate, or linoleic acid hydroperoxide followed by alkaline treatment (Table IV). With the exception of the safflower isolate, proteins oxidized by lipid hydroperoxide yielded the most lysinoalaanine. Results of Finley and Lundin (1980) indicate that lipid hydroperoxide caused formation of more cystine monoxide and cystine dioxide in oxidizing glutathione than did hydrogen peroxide. This suggests that the stronger, more rapid oxidant may oxidize cysteine residues directly to the sulfinic acid without going through the disulfide but that oxidation by lipid hydroperoxide probably goes through the disulfide intermediate. In Table IV the safflower samples which had oxidant added have lower lysinoalanine contents than control samples. This may be due to the fact that the safflower isolate was slightly oxidized. Although the amount of oxidized lipid present was probably small, it gave the safflower isolate a moderately rancid smell, indicative of some oxidation.

In conclusion, it appears that partial oxidation of cysteine residues in proteins to the disulfide or monoxide form increases the susceptibility of the residue to dehydroalanine formation. This dehydroalanine can yield a variety of products, including lysinoalanine. Cysteine residues that are oxidized to the sulfinic acid or cysteic acid stages appear to be more stable to alkali, at least to the extent that they do not form significant amounts of dehydroalanine or lysinolanine.

LITERATURE CITED

Calam, D. H.; Waley, S. G. *Biochem. J.* **1962**, *85*, 417-419.
deGroot, A. P.; Slump, P.; Feron, V. J.; Van Beek, L. *J. Nutr.* **1976**, *106*, 1527-1538.
Finley, J. W.; Lundin, R. E. In "Autoxodiation and Antioxidants"; Simic, M.; Karel, M., Eds.; Plenum Press: New York, 1980.
Finley, J. W.; Snow, J. T.; Johnston, P. H.; Friedman, M., presented at the 36th Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 6-9, 1976, Abstract No. 236.
Finley, J. W.; Wheeler, E. L.; Witt, S. C. *J. Agric. Food Chem.* **1981**, *29*, 404.
Gould, D. H.; MacGregor, J. T. In "Protein Crosslinking-B Nutritional and Medical Consequences"; Friedman, M., Ed.; Plenum Press: New York, 1977; p 29.
Karayiannis, N. I.; MacGregor, J. T.; Bjeldanes, L. F. *Food Cosmet. Toxicol.* **17**, 591-604.
Kice, J. L.; Rogers, T. E. *J. Am. Chem. Soc.* **1974**, *96*, 8009-8015.
Nashef, A. S.; Osaga, D. T.; Lee, H. S.; Ahmed, A. I.; Whitaker, J. R.; Feeney, R. E. *J. Agric. Food Chem.* **1977**, *25*, 245-251.
Savige, W. E.; Eager, J.; Maclaren, J. A.; Roxburgh, C. M. *Tetrahedron Lett.* **1964**, *44*, 3289-3293.
Spackman, D. H. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1963**, *22*, 244.
Sternberg, M.; Kim, C. F.; Schwende, F. J. *Science (Washington, D.C.)* **1975**, *190*, 992-994.
Woodard, J. C. *Lab. Invest.* **1969**, *20*, 9-16.
Woodard, J. C.; Short, D. D. *J. Nutr.* **1973**, *103*, 569-574.

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Lysine Content of Triticale Protein Increased by Germination

Y. Victor Wu

Triticale, a cross between wheat and rye, was germinated for 1-8 days. Lysine content of germinated triticale increased after 8 days from 3.5 to 5.9 g/16 g of nitrogen. A large increase in water-soluble nitrogen (rich in lysine) and a large decrease in 70% ethanol soluble nitrogen (low in lysine) accompanied sprouting. The percent protein in triticale germinated for 3 days or more is greater than in the initial grain as a result of dry matter loss in the grain during germination, but the absolute amount of protein per kernel is decreased.

Triticale, a cross between wheat and rye, is the first man-made cereal. The protein content of the best triticales has held at about 13% in CIMMYT since 1973 when their yields first closely approached those of the best bread wheats (CIMMYT, 1978). The average content of lysine in triticale protein was 3.4% in 1972 and 3.7% in 1973 (Villegas and Bauer, 1974). The comparable figures for most bread wheat would be 10-12% protein and 2.7% lysine, whereas the average rye has 13% protein and 3.7% lysine (Bushuk, 1976). International triticale yield nursery testing data confirmed the high-yield potential of two triticales in 1979-1980, with up to 10% more average yield across all locations than the bread wheat check variety (CIMMYT, 1981). Lorenz (1974) reviewed the history, development, and utilization of triticale; Hulse and Laing (1974) reported the nutritive value of triticale protein; and Wu et al. (1978) summarized the food uses of triticale.

Although triticale has higher lysine content than wheat, it is still deficient in lysine—the first limiting amino acid. Dalby and Tsai (1976) reported an increase in lysine content expressed as percent of dry weight of triticale

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604.