Processing Conditions to Inhibit Lysinoalanine Formation in Alkaline-Treated Proteins[1]

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ABSTRACT

Severe alkaline treatment of protein foods often results in the formation of amino acid degradation products such as lysinoalanine (LAL) in varying quantities depending on treatment conditions. Although several amino acid degradation products can be formed, the most easily measured is LAL. The presence of reducing anions such as bisulfite, bisulfide, and hypophosphite significantly reduced the formation of these products. Stirring and air incorporation were critical to formation of the intermediate compounds in LAL formation. Reduced air incorporation during treatment resulted in much lower LAL levels in final products.

Lysinoalanine (LAL) has been of growing concern to the food industry because it was shown to be nephrotoxic to rats (Gould and MacGregor 1977, Woodard et al 1975) and because it represents a corresponding decrease in available lysine (deGroot and Slump 1969). The increased cross-linking of the protein brought about by alkaline treatment also is thought to impair digestibility of the protein.

Alkaline treatment of food proteins has been used since ancient times (Katz et al 1974) and is being used increasingly in the food industry for several purposes, including preparation of textured vegetable protein (Hamdy 1974), peeling of fruits and vegetables (Hart et al 1974), and preparation of various protein isolates. Alkali and heat convert some of the amino acid residues in proteins to unnatural amino acids and also can cause racemization of amino acid residues (Asquith and Otterburn 1977, Finley and Friedman 1977, Provansal et al 1975). Sternberg et al (1975) reported that many kinds of heated protein-containing foods contain varying amounts of LAL residues. Karayiannis (1976) showed that proteins containing as little as 0.3 g of LAL/16 g of nitrogen can induce kidney changes. Because LAL poses a possible health hazard, LAL formation in processed foods should be controlled by careful control of processing conditions or, if necessary, by addition of blocking agents. deGroot and co-workers (1976) reported that only free lysinoalanine is nephrotoxic to rats and that free lysinoalanine is not toxic to other species; deGroot stated that protein-bound LAL is not toxic, which appears to disagree with earlier work by Karayiannis (1976) and Woodard et al (1975). It does not seem possible to resolve this question now, but one might speculate that digestibility of the protein might affect the results. The current work describes a procedure that reduces general protein damage and may improve protein quality regardless of the final correlation of LAL toxicity.

LAL presumably arises from the reaction of the ξ-amino group of lysine with the double bond of a dehydroalanine residue that is formed by the β-elimination of a cystine or serine residue. Dehydroalanine residues have been postulated as products from many different base-catalyzed elimination reactions of protein and glycoproteins (Asquith and Otterburn 1977). Finley et al (1978) reported that addition of mercaptoamino acids during alkaline treatment inhibits LAL formation. Although the precise mechanism of prevention is not clearly understood, it appears that an intermediate in dehydroalanine formation may be reduced by the mercapto groups. In this work we used reducing anions to inhibit dehydroalanine formation and subsequent formation of LAL. Because the intermediates are difficult to measure in proteins, the LAL product, which is easily measured, was used as an index of inhibition of alkaline damage to the protein.

MATERIALS AND METHODS

Materials

Soy isolate (Promine-D 200W) and soy flour (Soya Fluff) were obtained from Central Soya, Chicago, IL. Skim milk was obtained from a local dairy, and casein was obtained from National Dairy Products Corp, Norwich, NY. All of these materials were from lots that were very low in LAL.

Cysteine and both oxidized and reduced glutathione were obtained from Sigma Chemical Co., St. Louis, MO. Sodium meta-dithionite was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. Sodium bisulfite, sodium bisulfide, and sodium hypophosphite were obtained from Mallinckrodt Chemical Works, St. Louis, MO. LAL was synthesized from the copper complex of lysine and *N*-acetyldehydroalanine methyl ester as described by Finley and Snow (1977).

Methods

Amino Acid Analysis. Amino acid analysis was done on a Beckman 120 amino acid analyzer according to the method of Spackman (1963). LAL elutes before, and is well separated from, lysine. A color constant of 1.59 compared with 1.00 for leucine was used for calculating the LAL values.

Protein Isolates. Soy isolates were prepared from soy flour in the presence and absence of cysteine, mercaptoethanol mercaptoacetic acid, and the sodium salts of bisulfide, dithionite, bisulfite, and hypophosphite. Soy flour (100 g) was adjusted to pH 9 in 1 L of water in sodium hydroxide with 0.5 g of the appropriate reducing agent and stirred at room temperature for 1 hr. The mixture was centrifuged at 5,000 rcf for 20 min, and the supernatant was adjusted to pH 4.5 with *N* HCl. The precipitated protein was removed by filtration, washed three times with 1 L portions of water, redissolved in 500 ml of water at pH 7.0, and freeze-dried. A 10-mg sample of each preparation was hydrolyzed in 6*N* HCl for 20 hr at 110°C in vacuo and analyzed for basic amino acids.

Sodium Caseinate. Sodium caseinate was prepared from fresh pasteurized skim milk with and without added reducing agents. When added, 0.5 g of reducing agent was added to 1 L of skim milk before any treatment. The skim milk was adjusted to pH 4.6 with *N* HCl and centrifuged at 5,000 rcf for 10 min to recover the casein precipitate. The precipitated casein was suspended in 1 L of water and the pH adjusted to 8.5 to redissolve the casein, which was reprecipitated twice at pH 4.6, and finally redissolved at pH 7.0 and freeze-dried.

Alkaline Treatments. Commercial soy isolate or commercial casein (10 g) was dissolved in 100 ml of 0.1*N* NaOH. The solution was stirred at 60°C for 8 hr and adjusted to pH 4.5, and the entire solution was freeze-dried to assure recovery of any peptides formed

[1]Reference to a company or product does not imply approval or recommendation by the USDA to the exclusion of others that also may be suitable.

during treatment. Samples with a reducing agent were prepared by adding 0.1 g of each reducing agent to the NaOH solution before adding the soy isolate. The levels of reducing agent were varied in one experiment. To determine the importance of air incorporation, an experiment was conducted by bubbling air through the samples during alkaline treatment and by bubbling dry nitrogen through the samples during alkaline treatment.

RESULTS AND DISCUSSION

Both commercial soy isolate and commercial casein were subjected to rigorous alkaline treatment in the presence of the various reducing agents. The levels of LAL in the resulting products are shown in Table I. The results clearly show that the inorganic reducing agents are as effective as the organic reducing agents. Hypophosphite was slightly less effective than the other inorganic reducing agents but caused a significant reduction in the LAL level after alkaline treatment. Also, the materials treated with inorganic reducing agents had no off-odors, which were found when mercaptoethanol or mercaptoacetic acid was added. Addition of oxidized glutathione caused a substantial increase in the LAL level in the products after treatment. The increase is undoubtedly due to the increased levels of cystine, which could undergo a β-elimination furnishing more dehydroalanine and therefore more lysinoalanine (1977). Addition of cystine did not, however, increase the levels of LAL. The difference in the effect of oxidized glutathione and cystine on the formation of LAL is that the α-amino group of the cystine residue is blocked in glutathione and vulnerable in cystine. When degraded in alkali, any free dehydroalanine from cystine decomposition will further decompose to ammonia and pyruvic acid. Levels of LAL lower than 0.01 g/16 g N can be considered to be insignificant. Despite the harsh alkaline treatment the proteins received, therefore, the presence of reducing agents afforded excellent protection for the protein, and addition of a source of stable dehydroalanine significantly increased the LAL levels in the treated product.

Because these alkaline treatments far exceeded conditions normally used in food processing, the effectiveness of the reducing agents also was evaluated under somewhat milder conditions of alkaline treatment, such as those used in the preparation of protein isolates. These processes more closely emulate the treatment a protein might receive in isolation or processing. Sternberg et al (1975) reported that commercial casein and commercial soy can contain LAL levels ranging from 0.007 to 0.69 and from 0 to 0.03 g/16 g N, respectively. This was further substantiated by Finley et al (1978). Table II shows the LAL levels of proteins isolated in the presence of the various reducing agents. The results suggest that the reducing agents were all effective in protecting the protein from LAL formation. The isolation conditions were somewhat harsher than those in actual commercial operations, as shown by the relatively high LAL levels in the control samples, particularly in the soy isolate. When the various reducing agents were added, however, the LAL levels were reduced to the same range as that of commercial samples used as starting materials in Table I. To establish minimum effective levels of reducing agents for inhibition of LAL formation, various levels of bisulfide and cysteine were added before alkaline treatment of the proteins. The results in Table III show that, particularly with bisulfite, low levels of addition effectively reduce the formation of LAL. To reduce the LAL level to the desired range, however, it was necessary to add 1 g of reducing agent per 100 g of protein. These results suggest that, where strong alkaline treatment is to be used, the levels of reducing agent necessary to prevent LAL formation are within a practical working range.

In other work in this laboratory, attempts have been made to prepare large amounts of alkaline-treated protein; however, large variations in LAL levels were observed. An unconfirmed conclusion was that the variation seemed to be related to stirring. Based on the effectiveness of the reducing agents, the question of variation due to air incorporation and subsequent oxidations was studied. Samples were treated, as usual, with reducing agents except that mixing was varied and one set of samples was treated

TABLE I
Effect of Reducing Agents on Lysinoalanine Content of Alkali-Treated Proteins[a]

	Lysinoalanine Content (g/16 g N)	
Reducing Agent	**Casein**	**Soy**
Control (no reducing agent)	0.9	0.8
Cysteine	0.01	0.01
Cystine	0.85	0.81
Mercaptoethanol	0.04	0.06
Mercaptoacetic acid	0.003	0.002
Hypophosphite	0.03	0.05
Bisulfite	0.003	0.003
Dithionite	0.001	0.001
Bisulfide	0.002	0.002
Glutathione reduced	0.01	0.08
Glutathione oxidized	1.25	1.60
Untreated commercial material[b]	0.002	0.002

[a]Treatment at 60°C for 8 hrs in 0.1*N* NaOH.
[b]Commercial products that received no treatment.

TABLE II
Effect of Reducing Agents on Protein Lysinoalanine Content During Preparation of Soy and Casein Isolates

	Lysinoalanine Content (g/16 g N)	
Reducing Agent	**Casein**	**Soy**
Control (no reducing agent)	0.160	0.08
Cysteine	0.006	Trace
Mercaptoethanol	0.010	0.005
Mercaptoacetic acid	0.003	0.003
Hypophosphite	0.002	0.004
Bisulfite	0.002	0.002
Dithionite	0.003	0.001
Bisulfide	0.002	0.002

TABLE III
Effect of Level of Reducing Agent on Protein Lysinoalanine (LAL) Content During Alkaline Treatment of Soy Isolate

Reducing Agent	**Level Added (g/10 g protein)**	**LAL Content (g/16 g N)**
Control	0.0	0.9
Bisulfite	0.01	0.13
	0.05	0.09
	0.10	0.004
	0.50	0.003
Cysteine	0.01	0.09
	0.05	0.08
	0.10	0.003
	0.50	0.002

TABLE IV
Effect of Mixing, Air, and Nitrogen on Lysinoalanine Contents of Alkaline-Treated Soy Proteins in the Presence of Reducing Agents

Reducing Agent	**Air Bubbled**	**No Mixing**	**Nitrogen Mixing**
Control[a]	1.4	0.80	0.21
Bisulfite	0.4	0.002	0.003
Bisulfide	0.5	0.001	0.002
Cysteine	0.5	0.003	0.001
Glutathione (reduced)	0.9	0.008	0.005

[a]Control contained 0.81 lysinoalanine per 16 g N.

under nitrogen. The results in Table IV show the enormous variation in LAL levels.

Bubbling air through the solution during the treatment rapidly oxidized the reducing agents, causing them to be less effective in the prevention of LAL formation. The organic reducing agents also were much less effective than in previous experiments. When no mixing was used or in the case of nitrogen bubbling, the reducing agents were as effective as before. When no reducing agent was added and nitrogen bubbled through the solution, the LAL formation was only about 25% that observed when treated with no mixing or mixing at a moderate rate. From these results we conclude that oxygen is required for the formation of high levels of LAL. Careful control of the amount of air allowed in the system, combined with reducing agents, will effectively reduce the LAL formation to extremely low levels.

LAL formation can apparently be controlled, even when severe alkaline treatment is required. The presence of the reducing agents may alter the effectiveness of the alkaline treatment on the physical properties of the protein product. Furthermore, although the LAL levels obtained by addition of reducing agents are low, care must be taken to assure that other toxic by-products do not form. This should be done through extensive animal testing of these products.

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