Deamidation Studies on Selected Food Proteins

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Complete deamidation (amide bond hydrolysis) of soy protein and some other food proteins was achieved by acid hydrolysis. The amide content of the proteins was determined based on the amount of ammonia generated. When soy protein was treated with commercial proteases, deamidation was found to occur to the free glutamine in the hydrolysates. The deamidation was nonenzymatic and was accelerated by anions such as phosphate and bicarbonate. Ammonia was also generated during the proteolysis from sources other than deamidation. The generation of nonamide ammonia was most likely from enzymatic deamination (cleavage of amino groups) because of the reaction of microorganisms.

KEY WORDS: Ammonia electrode, casein, cottonseed, gluten.

Protein deamidation has been widely investigated recently because it is an effective way to improve the functional properties of food protein (1-4). For deamidation analysis, the extent of amide bonds hydrolyzed is invariably monitored by measuring the ammonia generated. However, the sources of ammonia could be glutamine and/or asparagine deamidation. The deamidation could occur to the amide amino acid residues in the protein molecule or to the free amide amino acids in the hydrolysate. Different types of deamidation effect protein functionality in different ways. Furthermore, given the right reaction conditions, all nitrogen-containing groups in a protein molecule could be converted to ammonia. There is a possibility that some ammonia generated during protein hydrolysis could be from non-amide sources. Obviously, the nature of the ammonia needs to be examined further and protein deamidation should be more clearly characterized and evaluated.

In this investigation, soy protein and some other food proteins were hydrolyzed chemically and enzymatically under various conditions. The ammonia generated was related to different types of deamidation, as well as to sources other than deamidation. A novel electrode method for ammonia analysis was compared to a well known enzyme method. The effects of pH, temperature, and anions on glutamine deamidation were examined. The occurrence of deamination and its effect on the deamidation analysis were also investigated.

EXPERIMENTAL

Materials. Soy protein (Mira-Pro 111) was obtained from A.E. Staley Mfg. Co. (Galesburg, IL). Protease 2A and Prozyme 6 were from Amano International Enzyme Co. (Troy, VA) pronase E was from Sigma Chemical Co. (St. Louis, MO). Glutamate dehydrogenase (GIDH), 2-oxoglutarate, and reduced nicotinamide-adenine dinucleotide (NADH) were from Boehinger Mannheim Biochemicals (Indianapolis, IN). Cottonseed protein was prepared by 10% NaCl extraction of defatted glandless cottonseed flour, after water extraction to remove nonstorage proteins, according to Zarins and Cherry (5). Casein was from J.D. Baker Chemical Co. (Phillipsburg, NJ), and gluten was from Ogilvie Mills Ltd. (Montreal, Quebec, Canada). Amino acids and Thymersal (sodium ethylmercuric thiosalicylate) were from Sigma Chemical Co.

Complete protein deamidation. Hydrochloric acid (5.0 mL, 8.0 N) was added to a suspension of 0.2 g protein in 15 mL water. The mixture was stirred at 100°C for 3 hr, and then analyzed for the ammonia generated.

Enzymatic proteolysis. Eighteen mg enzyme was added to a suspension of protein (0.3 g) in sodium phosphate buffer solution (0.2 M, pH 8.0, 30 mL). The mixture was stirred at 37°C for 3 or 18 hr before being analyzed.

Deamination of amino acid. Eighteen mg of enzyme was added to a solution of amino acid (2 mmoles) in sodium phosphate buffer (0.2 M, pH 8.0, 30 mL). The mixture was stirred at 37°C for 18 hr and then analyzed for ammonia.

Electrode method for ammonia determination. To an aliquot of the reaction mixture (10 mL) was added trichloroacetic acid (TCA) solution (36% TCA, 5.0 mL). The precipitate was removed by centrifugation. Aliquots of the TCA-soluble solution (3.0 mL) were each diluted to 24 mL with water before being analyzed for ammonia by an ammonia electrode (Orion Research Inc., Boston, MA). A reference curve was prepared using standard ammonium chloride solutions (10^{-4} M to 10^{-3} M).

Enzyme method for ammonia determination. The analysis was conducted based on the Bergmeyer procedure (6). Mix 1.0 mL each of the following solutions, which were prepared in phosphate buffer (0.2 M, pH 8.0): Sample solution (ammonia in the range of 10^{-4} to 4.0×10^{-4} M); 2-oxoglutarate (5.0 mg/mL); NADH (0.5 mg/mL); and GIDH (50 mg/mL). After 1 hr at room temperature, the decrease in absorbance of the solution measured at 340 nm was estimated. A reference curve was prepared using standard ammonium chloride solutions (10^{-4} to 4.0×10^{-4} M). For acid deamidation, the protein solution was evaporated to dryness in a round bottom flask by a rotary evaporator. The residue was redissolved in phosphate buffer (0.2 M, pH 8.0) to make a solution of 1.0% in protein concentration before being analyzed.

Calculation of percent deamidation. Percent deamidation was normally calculated as the percent ratio of ammonia generated to the ammonia generated at complete deamidation. However, for reactions that produced both amide and non-amide ammonia (see Results and Discussion for detailed information), percent deamidation was calculated as follows: % deamidation = $100 \times [a - (c - b)]/b$, where, a = amount of ammonia found in the TCA-soluble solution at a given reaction time during proteolysis; b = amount of ammonia generated

by the complete deamidation of the sample which had not been affected by the enzyme activity (at 0 hr enzymatic reaction time); and c = amount of ammonia generated by the complete deamidation of the sample at a given reaction time during proteolysis.

RESULTS AND DISCUSSION

Ammonia from deamidation. Complete protein deamidation is normally achieved by hydrolyzing the protein in 2 N HCl at 100°C for 3 hr. Under these conditions, the ammonia generated represents the amide content of the protein because the amino acid residues of asparagine and glutamine, both in the protein and its hydrolysates, are completely deamidated. This was demonstrated, as shown in Table 1, in the determination of amide contents of several food proteins. Ammonia was determined by both the electrode method and the enzyme method. Values of amide content for soy protein, cottonseed protein, casein, and gluten, ranging from 0.92–2.70 mmole/g, were obtained. There was very little difference between these two methods in accuracy and reliability. However, the enzyme method has an optimum pH of 8.0, and sample pretreatments are often required to remove reaction components that may interfere with the analysis. On the other hand, the electrode method is fast, reliable, and almost totally unaffected by interference.

Glutamine deamidation. When soy protein was treated with protease in phosphate buffer for 3 hr, ammonia was also generated. In this case, deamidation occurred to the free glutamine in the hydrolysates and it is non-enzymatic. No ammonia was generated when free asparagine was treated under the same conditions, whereas glutamine deamidation was found to occur to the same extent with or without protease. The effects of reaction conditions on glutamine deamidation are shown in Table 2. The deamidation increased with increased pH, higher temperature, and the catalysis of certain anions, i.e., phosphate, bicarbonate, and borate. In a relatively short period of time (3 hr), substantial deamidation (>35%) could be obtained by either raising the temperature to 80°C in water or using phosphate buffer (pH 8) as the reaction medium at a relatively mild temperature of 37°C. The result of anion effect on glutamine deamidation confirmed the findings of Gilbert *et al.* (10), who reported that phosphate and bicarbonate anions enhanced the non-enzymatic deamidation of glutamine.

Ammonia from non-amide sources. When soy protein was treated with proteases for 18 hr at 37°C, unusually large quantities of ammonia were generated. The results are shown in Table 3. For a given enzyme reaction, the values listed under complete deamidation at 0 hr reaction time (column 2) are the amount of ammonia generated by completely deamidating the sample before the enzymatic activity could occur. These values represent the amount of ammonia that can be produced by the deamidation of the total amide groups available. The corresponding values that were obtained after 18 hr of enzymatic activity are listed under complete deamidation at 18 hr reaction time (column 3). Since the 18 hr values in column 3 are consistently higher than the 0 hr values in column 2, additional ammonia must be produced from nonamide sources.

Deamidation analysis. For reactions with deamidation as the only ammonia generator, as shown in Tables 1 and 2, the extent of deamidation was equivalent to the amount of ammonia generated during the reaction. For reactions that produced both amide and non-amide ammonia (referring to the data in Table 3) the ammonia listed under enzymatic deamidation (column 4) represents the total ammonia generated in the system. The difference in value between columns 2 and 3 accounts for the non-amide ammonia, which should be substracted from the amount in column 4 to obtained the true value of ammonia from deamidation.

Deamination of amino groups. The non-amide ammonia was obviously generated during proteolysis by the deamination of the amino groups mostly in the protein hydrolysates. The amino group in an amino acid is very resistant to acid deamination even under the severe conditions of 6 N HCl at 110°C for 24 hr, which are commonly used for complete protein hydrolysis. However, deamination could occur during enzymatic proteolysis when deaminating enzymes such

TABLE 1

Amide Content of Food Protein as Estimated Using the Electrode and the Enzyme Methods

Protein	Amide N, mmole/g protein		
	(Electrode)[a]	(Enzyme)[a]	(Conway)[b]
Soy protein	1.15 ± 0.25	1.23 ± 0.35	1.12[c]
Cottonseed protein	1.33 ± 0.15	1.34 ± 0.09	1.33[d]
Casein	0.93 ± 0.45	0.97 ± 0.40	0.95[d]
Gluten	2.70 ± 0.50	2.69 ± 0.25	2.26[c]

[a]Mean of triplicate ± standard deviation.
[b]The Conway microdiffusion method (7).
[c]From Arntfield and Murray (8).
[d]From Hagenmaier (9).

TABLE 2

Effects of pH, Temperature, and Anions on Glutamine Deamidation[a]

pH	Temperature, °C	Anions, 0.2 M	Deamidation, %[b]
6.0	37	Phosphate	9.6
7.0	37	Phosphate	20.6
8.0	37	Phosphate	41.3
9.0	37	Phosphate	55.5
7.0	37	(Water)	0.0
7.0	60	(Water)	3.0
7.0	80	(Water)	35.0
7.0	100	(Water)	78.5
8.0	37	Bicarbonate	28.3
8.0	37	Borate	2.9

[a]Thirty mg glutamine was dissolved in 30 mL buffer with pH and anion content as indicated. The solution was heated at temperature as indicated for 3 hr before being analyzed for ammonia.
[b]Percent deamidation was calculated as the ratio of moles of ammonia generated to moles of amide groups available in the glutamine solution.

TABLE 3

Ammonia Generated in Solution of Soy Protein Treated with Protease[a]

Enzyme	Complete deamidation[b] (μmoles/mL) 0 hr	18 hr	Enzymatic deamidation[c] (μmoles/mL) 18 hr	Percent deamidation (%) 18 hr
Protease 2A	0.96	1.15	0.51	33.7
Prozyme 6	0.97	1.24	0.67	41.5
Pronase E	0.99	1.03	0.45	40.9

[a]Soy protein (0.3 g) was treated with enzyme (18 mg) in phosphate buffer (0.2 M, pH 8.0, and 30 mL) at 25°C.
[b]Aliquots of reaction mixture (10 mL), withdrawn at 0 hr and 18 hr, were each reacted with HCl (6 N, 5.0 mL) at 100°C and complete deamidation was achieved in 3 hr.
[c]Reaction mixture (10 mL) was precipitated by TCA solution (36%, 5.0 mL). Separated by centrifugation, the TCA-soluble solution was analyzed for ammonia.

TABLE 4

Ammonia Generated in Solutions of Free Amino Acid Treated with Protease[a]

Enzyme	Ammonia generated (μmoles/mL) Aspartic acid	Serine	Arginine
Protease 2A	3.05	6.88	3.51
Prozyme 6	2.66	7.15	4.70
Pronase E	0.37	1.34	0.59
Prozyme 6-I[b]		0.25	
Control	0.25	0.33	0.35

[a]Amino acid (2.0 mmoles) was treated with protease (18 mg) in phosphate buffer (0.2 M, pH 8.0, and 30 mL) for 18 hr at 25°C. The control was conducted under the same reaction conditions, but without the addition of enzyme.
[b]The reaction with Prozyme 6 was inhibited by Thimerosal (10 mg).

as dehydrogenases were present as impurities or being generated by the reaction of microorganisms. When treated under the same proteolytic conditions, serine was found to be by far the most readily deaminated amino acid, followed by arginine and aspartic acid (Table 4). The non-amide ammonia was totally eliminated, as expected, by the addition of agents such as Thymursal which inhibited the growth of microorganisms.

When proteins were hydrolyzed in acid, deamidation occurred to both the carboxamide residues in the protein and the free amide amino acids in the hydrolysates. The amount of ammonia generated was equivalent to the total deamidation. When proteins were treated with proteases, deamidation could occur (particularly when accelerated by the presence of certain anions, such as phosphate and bicarbonate), but only to the free glutamine in the hydrolysates. However, the ammonia thus generated may or may not represent a true measure of the deamidation, because deamination of amino groups could also occur and result in the generation of non-amide ammonia.

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