Thermal Deamidation of Proteins in a Restricted Water Environment

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Nonenzymatic deamidation reactions of soy protein, egg lysozyme, milk casein, and wheat gliadin at moisture contents ranging from 0 to 80% at 115 °C for 2 h were studied. The deamidation percentage varied from 3.20 to 18.48% for all four proteins. All proteins had a maximum deamidation rate at the limited water content, approximately 50% for soy protein, 50% for lysozyme, 60% for casein, and 6% for gliadin.

INTRODUCTION

In recent years, there has been considerable interest in modifying proteins by selectively hydrolyzing amide bonds of the protein molecule (deamidation) to improve their functional properties (Shih, 1987, 1990, 1991; Ma et al., 1986; Ma and Khanzada, 1987; Matsudomi et al., 1985a,b; Kato et al., 1987, 1989). Proteins such as soy protein and wheat protein contain a large quantity of glutamine (Gln) and asparagine (Asn) which could be either enzymatically or chemically hydrolyzed to acidic groups. The deamidated protein has a lower isoelectric point and, therefore, has better solubility in many mildly acidic food application systems (Finley, 1975). It has been reported that even small levels of deamidation (e.g., 2–6%) could significantly improve protein functionalities (Matsudomi et al., 1986; Hamada and Marshall, 1989).

When a peptide model reaction system was used, many factors were found to influence the deamidation rate of protein, among which are pH, temperature, ionic strength, anions, buffer concentration, peptide sequence, secondary and tertiary protein structure, and the presence of other reactants such as ascorbic acid and anion species (Robinson, 1974).

In a recent review, Wright (1991) stated that deamidation is a hydrolytic reaction catalyzed by acid and base and requires a water molecule. However, it was demonstrated that the deamination of Asn-containing peptides at neutral to alkaline pH values involved an intramolecular nucleophilic reaction between the peptide amide nitrogen and the carbonyl group of the side-chain amide and formed a cyclic imide intermediate which then hydrolyzed to yield the isoAsp and Asp peptides. On the other hand, deamidation involved mainly direct hydrolysis of the side chain at acidic conditions (Patel and Borchardt, 1990a,b). Similarly, Gln residues can also have a deamidation reaction via formation of a six-membered cyclic imide but often at a slower rate than for the Asn residues due to the greater distance from adjacent main-chain amide NH groups to the Gln side-chain amide group compared to that of Asn (Wright, 1991). This implies that the existence of a water molecule and its physical state could be important in controlling deamidation.

Most foods are processed and stored in a restricted water environment. The limitation of water to protein not only influences the protein structure, which will indirectly affect the deamidation rate, but also directly influences the availability of the water molecule to the hydrolytic deamidation reaction. Understanding how the water content affects deamidation of food protein under food processing or storage conditions would be essential to monitor the functionalities and shelf life of protein-containing foods.

EXPERIMENTAL PROCEDURES

Material. Soy protein isolate (ARDEX DHV) was purchased from Archer Daniels Midland Co. (Decatur, IL); the protein content of the isolate was about 91.5% (oven dry basis) with 0.2% fiber content and 6.0% moisture content (wet basis). Lysozyme, casein, and gliadin were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium chloride standard solution (0.1 M) and ionic strength adjustment solution for electrode ammonia determination were obtained from Orion Research Inc. (Boston, MA). Deionized water was used for all reaction mixtures, and freshly made distilled water was used in all of the ammonia determinations. Phosphorus pentoxide was from Fisher Scientific, Inc. (Piscataway, NJ).

Determination of Moisture Content of the Proteins. An oven-drying method was used for moisture determination (AOAC). About 2 g of each protein was placed in a convective oven at a constant temperature of 105 °C until constant weight was obtained.

Preparation of Moisture-Free Proteins. A thin layer of about 2 g of each protein was deposited into a desiccator in which 500 g of phosphorus pentoxide (P_2O_5) was also placed. The protein was kept in the desiccator at room temperature for 1 week to assure that all free water in the protein had been evaporated into the free space and chemically absorbed by phosphorus pentoxide. The proteins prepared here were used as zero-moisture proteins in the deamidation studies.

Protein Deamidation in a Restricted Water Environment. Deamidation of each protein at the moisture content ranging from 0 to 80% (wet basis) was conducted in an 18-mL Kimax brand glass tube with black phenolic screw cap in which there was a PTFE-faced temperature and pressure resistant rubber liner (Fisher Scientific). To further prevent ammonia vapor loss, an additional 1 mm thick and temperature-resistant PTFE-faced silicone rubber flat disk septa (Fisher Scientific) was placed into the cap to secure the airtightness of the reaction tube. In each glass tube 1 gram of protein was thoroughly mixed with a preweighed amount of water to reach the required moisture content. The mixture was then capped tightly and placed in a refrigerator overnight to assure uniform moisture distribution within each protein sample. The reaction tubes were then heated in a convective oven at a constant temperature of 115 °C for 2 h. The deamidation reaction ceased with the addition of ice water after 2 h. Eight milliliters of 1 N HCl was injected into the glass tube reactor through the septa to absorb the ammonia released during the deamidation reaction, and then the samples were tightly capped and stored in a freezer before ammonia analysis was conducted.

Ammonia Determination. An electrode method was used for ammonia determination (Shih, 1990). A 4-mL aliquot of the HCl–protein mixture in the reactor was transferred to a centrifuge tube that contains an equal volume of 10% trichloroacetic acid (TCA). The mixture was well-shaken to make sure all soluble

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Table I. Amide Contents of Four Food Proteins As Measured with Electrode Method

protein	protein content[a] (% oven-dry basis)	amide N[b] (mmol/g of protein)	moisture content[c] (%, wet basis)
soy isolate	91.5	1.06 ± 0.06	7.26
gliadin	>90.0	3.84 ± 0.14	6.25
casein	>90.0	0.90 ± 0.05	6.50
lysozyme	>98.0	1.12 ± 0.04	5.80

[a] Data provided by suppliers. [b] Mean of triplicate ± standard deviation. [c] Average value of duplicates.

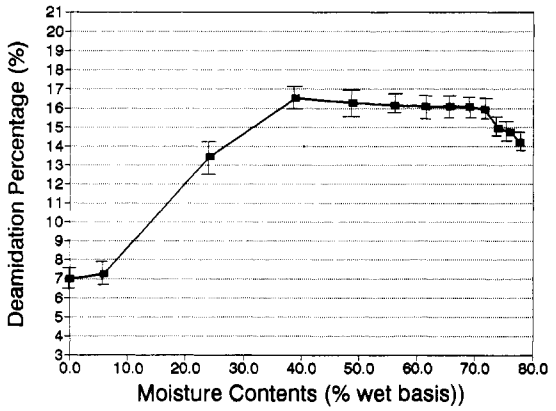

Figure 1. Effects of moisture contents on deamidation in soy isolate.

protein precipitated to protect the hydrophobic membrane on the electrode. The mixture was then centrifuged. A 5-mL clear solution from the centrifuged sample was diluted to 100 mL with distilled water before it was analyzed by an ammonia ion-selective electrode (Orion Research). A calibration curve was prepared using standard ammonium chloride solutions (10^{-6}–10^{-2} M).

Determination of Total Amide Content. Total deamidation was conducted to determine the total amide content of each protein investigated. Five grams of each protein was suspended in 100 mL of 2 N HCl. The mixture, which was heterogeneous due to the low solubility of proteins, except casein and lysozyme at acidic conditions, was refluxed for 3 h, and then the total ammonia released was measured. For each measurement, duplicates were run.

Deamidation Percentage Calculation.

$$\text{deamidation percentage (\%)} = [(C_{A0} - C_A)/C_{A0}] \times 100$$

where C_A (millimoles per gram of protein) is the concentration of the remaining amide groups and C_{A0} (millimoles per gram of protein) is the initial total amide group concentration at time zero for the deamidation reaction. The remaining amide concentration C_A was obtained by subtracting the ammonia released from the initial amide concentration C_{A0}.

RESULTS AND DISCUSSION

The total amide content as measured by hydrolyzing the protein in 2 N HCl at 100 °C for 3 h is shown in Table I, ranging from 0.90 to 3.84 mmol/g of protein for casein, soy protein, lysozyme, and gliadin. The difference in amide content among casein, soy protein, and lysozyme was not substantial, while gliadin had a considerably higher amount of amide content. The results were very comparable to literature values reported by Shih (1990).

In Figures 1–4 the deamidations of four proteins at a moisture content ranging from 0 to 80% (wet basis) are shown. The deamidation percentage varied from 3.20 to 18.48% for the four proteins under our experimental conditions. It is interesting to note that all four proteins had a maximum deamidation rate at the limited water content, approximately 50% for soy protein, 6% for gliadin, 60% for casein, and 50% for lysozyme. Soy protein

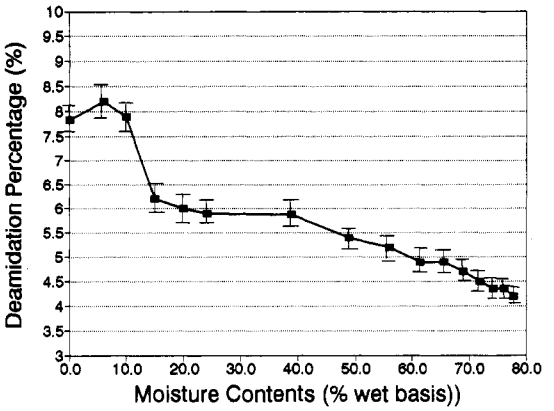

Figure 2. Effects of moisture contents on deamidation in gliadin.

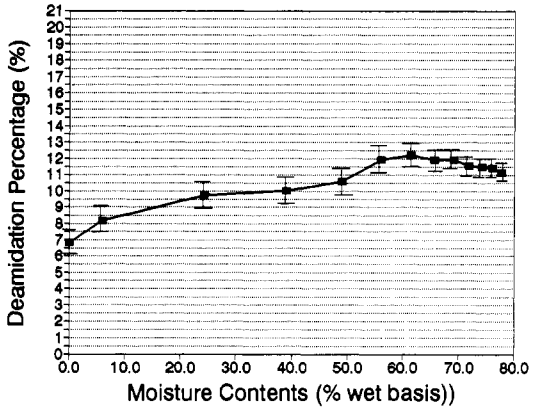

Figure 3. Effects of moisture contents on deamidation in casein.

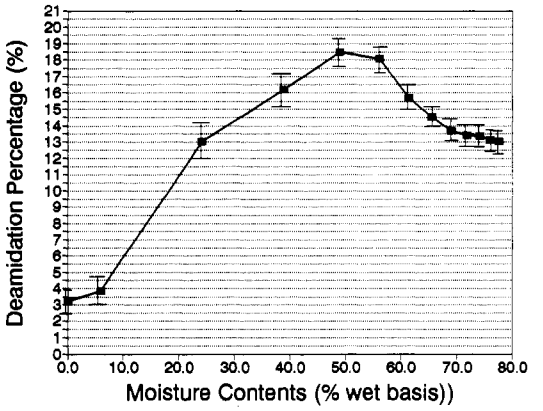

Figure 4. Effects of moisture contents on deamidation in lysozyme.

underwent only slight deamidation at the lower moisture contents; however, the deamidation rate increased rapidly from 7 to ~16.5% as the moisture content increased from 0 to ~50%. When the moisture content of soy protein further increased above 50%, the percentage of the deamidation gradually declined. Zhang et al. (1993) reported that in an aqueous solution soy protein had a deamidation percentage of about 9.5 when heated at 115 °C for 2 h. This value was considerably lower than the maximum deamidation percentage for soy protein in this study.

Similar to soy protein, slight deamidation was observed for lysozyme at moisture contents lower than 6% (Figure 4), and the deamidation of lysozyme reached the highest rate at a moisture content of ~45–55%. For casein, deamidation increased gradually as its moisture content increased from 0 to ~50%. Further increase in moisture content seemed to have very little effect on its deamidation

rate (Figure 3). It was most interesting to find that gliadin had the highest deamidation and occurred at a very low moisture content of below 10%. As the moisture content further increased, the deamidation gradually declined.

Soy protein, casein, and lysozyme used in this investigation are soluble in water at neutral pH; therefore, their three-dimensional structures are dependent on the total available water present. The three-dimensional structure has been regarded as an important factor governing the deamidation rate of a protein (Wright, 1991). When studying trypsin deamidation with neutron crystallography, Kosslakoff (1988) reported that all deamidated Asn residues were clearly distinguished by a distinct local conformation and hydrogen-bonding structure in contrast to those observed for the nondeamidated Asn residues.

It is known that the flexibility of proteins, measured in terms of mean-square displacements of various probes, is significantly impacted by the level of hydration and temperature (Frauenfelder and Gratton, 1986; Parak, 1986; Parak et al., 1986). Studies on trypsin indicated increased protein mobility as water mobility increased as the result of the plasticizing effect of water (Parak, 1986). Since the ring-formation mechanism for deamidation involves an intramolecular nucleophilic attack and requires substantial flexibility of the protein backbone structure, the increase in water content for proteins not only increased the water concentration, which would accelerate the hydrolytic deamidation reaction, but also increased protein flexibility. Manning et al. (1989) mentioned that the moisture dependence of the rates of acid- and base-catalyzed protein deamidation could differ due to different mechanisms. Direct hydrolysis (acid-catalyzed) deamidation may only require side-chain mobility, while the deamidation via ring formation requires a significant degree of segmental flexibility (Hageman, 1992). Therefore, an increase in protein flexibility should enhance deamidation through both direct hydrolysis and ring-formation mechanism.

Gliadin has a large number of amide side chains which can have hydrogen bonding that likely contributes to the stability of α-helices. Also, gliadin has many hydrophobic side chains with high probability of α-helical segments embedded in hydrophobic regions. Low levels of basic amino acids such as lysine, arginine, histidine, and free carboxyl groups make gliadin one of the least charged proteins known. Gliadin is, therefore, very hydrophobic and absorbs very little water (Il et al., 1991). It was observed during this investigation that the gliadin became a very aggregated, rubber-like material which was precipitated from the majority of the bulk water. The hydrophobicity effect, which aggregated the gliadin three-dimensional structure and thus reduced the protein backbone as well as side-chain flexibility, could be adverse to the ring-formation and side-chain catalysis effects. This may be the factor causing more deamidation to occur for gliadin at very limited water content.

It seemed to be contradictory to the hydrolytic nature of deamidation, which requires a water molecule for reaction, to find that a substantial degree of deamidation still occurred for all proteins when the moisture content was assumed to be zero, especially for gliadin, soy protein, and casein. However, as already discussed, the backbone peptide amide can undergo nucleophilic attack on the side-chain amide through a β-aspartyl (glutuminyl) shift mechanism to form a five- or six-membered cyclic imide intermediate and release a molecule of ammonia (Lura and Schirch, 1988). Therefore, deamidation could occur without the participation of water, only to yield the cyclic imide intermediate, which in the absence of water might be stable. Furthermore, it is impossible to prepare a protein of zero moisture content using the chemical-absorption drying method in this study. It is well-known that a protein has some physically and chemically bonded water molecules at its most polar groups. The residual water in proteins might have been another factor contributing to the deamidation observed for all proteins at zero moisture content.

In summary, our results indicate that for reasonably soluble proteins such as soy isolate, casein, and lysozyme, moisture contents of 50–60% (w/w) are found to give maximal levels of deamidation. Gliadin, a hydrophobic protein, which apparently aggregates and forms a rubbery gelatin on this treatment, shows a low level of deamidation (8%) which decreases on increasing hydration. This is probably a consequence of gelatin formation during the heating process in the presence of water.

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