Effect of Temperature, Feed Moisture, and pH on Protein Deamidation in an Extruded Wheat Flour

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The effect of three parameters on deamidation levels during twin-screw extrusion of wheat flour was studied: temperature, feed moisture content, and pH. This was accomplished by generating three sets of extrudates. The first set varied temperature (130, 155, and 170 °C), the second set varied feed moisture content (20%, 25%, and 30%), and the third set varied pH of the liquid feed (3.0, 7.0, and 12.0). In each set all other parameters were held constant. It was found that increases in temperature and feed moisture enhanced deamidation and that deamidation was also favored at extremes of pH. Possible explanations for these trends are discussed.

INTRODUCTION

The nonenzymatic deamidation of amide residues in proteins has been a topic of research in the fields of biochemistry and molecular biology for several years. Deamidation is described as the loss of the amide of a glutamine or asparagine residue resulting in the liberation of ammonia, transforming these amino acids to glutamic acid and aspartic acid. Deamidation is a hydrolytic reaction and thus requires only water to form products. The details and mechanisms of this reaction have been reviewed by Robinson and Rudd (1974) and Wright (1991). The majority of the biochemical studies they reviewed focus on the chemistry and mechanism of deamidation and have been done in reference to biologically significant peptides and proteins, specifically focusing on the modification of the more labile asparagine residue (Aswad, 1984; Murray and Clarke, 1984; Johnson and Aswad, 1985; Johnson et al., 1987; Stephenson and Clarke, 1989). In vivo deamidation is commonly viewed as a labile process which is theorized to be linked to protein and cell aging (Lowenson and Clarke, 1988).

Investigations on protein deamidation are not limited only to the realms of biochemical research. Studies on the deamidation of food proteins have recently been of great interest to the food industry since it provides an effective way to improve functional properties of proteins (Matsudomi et al., 1985; Shih, 1987; Shih and Kalmar, 1987; Finley, 1975). The deamidation of wheat gluten appears to be of particular interest because of its relatively high glutamine content, approximately one-third of the total amino acids (Whitaker, 1977).

Gluten modification via deamidation can be carried out in two ways. Chemical deamidation of gluten or "acid solubilization" is carried out under acidic conditions and high temperature (Wu et al., 1976). Enzymatic treatment of gluten proteins has also been proposed as a method of protein deamidation (Kato et al., 1987; Bollecker et al., 1990; Popineau and Thebaudin, 1990). Whether chemically or enzymatically induced, the deamidation of gluten proteins has been shown to result in an increased charge density on the protein, causing changes in protein conformation due to electrostatic repulsion. These charge-induced conformational changes result in enhanced surface hydrophobicity due to an unfolding of the protein exposing more hydrophobic residues to the protein surface (Matsudomi et al., 1982). It is the increase in surface hydrophobicity coupled with the presence of more negatively charged polar groups which provides the resultant modified protein with its amphiphilic characteristics. This makes for an ideal surface active agent for use as an emulsifier or foam stabilizer. Even though surface hydrophobicity increases, solubility is enhanced due to decreased protein–protein interactions. Levels of deamidation as low as 2–6% have been shown to enhance the functional properties of proteins (Matsudomi et al., 1985; Hamada and Marshall, 1989).

One can see that deamidation of food proteins results in a variety of molecular transformations. Presently, the research done on deamidation of food proteins has focused on the investigation of new fields of protein utilization exploiting this chemical modification. However, no work has been done to investigate the extent of this reaction in a complex food system during a food-processing step. Deamidation has been shown to occur as a result of heat, acidic or basic conditions (Wu et al., 1976; Matsudomi et al., 1981, 1982; Ma et al., 1986), changes in ionic strength (Shih, 1991), or enzymatic hydrolysis (Bollecker et al., 1990; Hamada and Marshall, 1989; Popineau and Thebaudin, 1989; Kato et al., 1987; Shih, 1990). These parameters are very common environmental factors present during food processing.

If deamidation can so dramatically alter the functional properties of gluten proteins by altering structure and charge, then perhaps an insight into the extent of this chemical modification in complex food systems will be of interest. The general consensus is that the functional properties of wheat flour doughs and the resultant product quality can be attributed to the gluten proteins (Pomeranz, 1988). A process such as extrusion, which is so widely used to produce a variety of products from wheat (i.e., breakfast cereals, snack foods, and pasta products), exposes food systems to high temperatures in the presence of water. If the deamidation of gluten proteins occurs during extrusion processing, it may alter the extent and the chemistry of gluten complexation with other macromolecules such as starch and lipids. It is this complexation that leads to food matrix formation and dictates changes in physical attributes such as food texture.

A popular way to measure the degree of deamidation of a protein is to hydrolyze the amides with 2 N HCl at high temperatures and directly measure the amount of ammonia released spectrophotometrically using a sensitive enzymatic method (Kun and Kearney, 1974). However, when this method is used to analyze for deamidation in a complex

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food system that contains carbohydrates, proteins, and lipids, byproducts are formed during the hydrolysis step that interfere with the final ammonia assay (Beutler, 1984). This study incorporates a modification of Kun and Kearney's enzymatic method which eliminates the effects of interfering compounds. The modified method was applied to analyze the effects of temperature, feed moisture, and pH on the levels of deamidation in an extruded wheat flour system.

EXPERIMENTAL PROCEDURES

Materials. High-gluten wheat flour (14% protein, db) was obtained from the Bay State Milling Co. (Clifton, NJ) and is sold under the trade name Bouncer. Adenosine diphosphate (ADP) disodium salt, triethylamine hydrochloride, ammonium chloride, potassium bicarbonate, sodium hydroxide, and reduced nicotinimide adenine dinucleotide (NADH) were purchased from the Sigma Chemical Co. (St. Louis, MO). Glutamate dehydrogenase and 2-oxoglutarate were obtained from Boehringer Mannheim (Indianapolis, IN). Crystalline sodium chloride and hydrochloric acid were purchased from Fisher Scientific Co. (Fair Lawn, NJ).

Extrusion of Wheat Flour. All extrusions were carried out on a Werner Pfliederer ZSK-30 corotating twin-screw extruder (Werner Pfliederer, Ramsey, NJ). The die diameter was 3 mm, and the diameter and length of each screw were 30 and 900 mm, respectively. The extruder barrel was induction heated and contained five independently controlled heating zones. Die temperatures were recorded by a thermocouple inserted at the die plate. Wheat flour was fed into the extruder using a K-Tron Series 7100 volumetric feeding system (K-Tron Corp., Pitman, NJ). A metering pump (U.S. Electric Motors, Milford, CT) was used to add the liquid feed.

Three sets of samples were extruded. In the first set, temperature was varied while all other parameters were held constant. The temperatures extruded were 130, 155, and 170 °C. The samples of this set were extruded at 20% feed moisture, screw speed of 300 rpm, and a mass flow rate of 300 g/min. Feed moisture content of the second set of samples was varied to 20%, 25%, and 30% while a melt temperature of 150 °C, a screw speed of 300 rpm, and a mass flow rate of 300 g/min were held. Finally, the production of extrudates under extremes of pH was accomplished by using multiple reservoirs. First, the proper conditions were met by equilibrating the extruder with a reservoir containing tap water. Once the desired conditions were met (20% feed moisture, screw speed of 300 rpm, mass flow rate of 300 g/min), the tap water reservoir was switched to a smaller reservoir containing water adjusted to the appropriate pH. The pH of the reservoirs was adjusted to the proper values with 1 M HCl and 1 M NaOH. The pH levels examined were 3.0, 7.0, and 12.0. All extrudates generated were stored at 10 °C under nitrogen in sealed 1-qt Mason jars until analysis.

Total Deamidation and Ammonia Isolation. Ten grams of extrudate was ground in the presence of dry ice in a benchtop grinder (Glen Mills, Maywood, NJ) and passed through a 24-mesh sieve to achieve uniform particle size. Dry ice was used to keep the grinder interior from heating up, exposing the extrudates to higher temperatures and resulting in further reactions. The moisture content of the resulting powder was determined according to the AOAC air oven method (AOAC, 1986). To facilitate the release of the residual amides, 10 g (db) of ground extrudate was weighed into a 500-mL Erlenmeyer flask fitted with a ground glass neck to which was added 250 mL of 2 N HCl. The sample was then attached to a condensing unit and refluxed at 100 °C for 3 h while stirring with a Teflon-coated stir bar. This treatment released ammonia from the protein but trapped it in soln. since ammonia is not volatile at strongly acidic pH values. After refluxing, the sample was then cooled for 30 min to 25 °C in an ice bath (0 °C). Upon achieving room temperature, the ammonia liberated from the sample was then volatilized by titrating the hydrolysate to pH 13.0 with saturated NaOH solution. This solution was quickly transferred to a 2000-mL double-neck round-bottom flask and 80 g of NaCl was added to aid in decreasing the solubility of the ammonia. The sample was

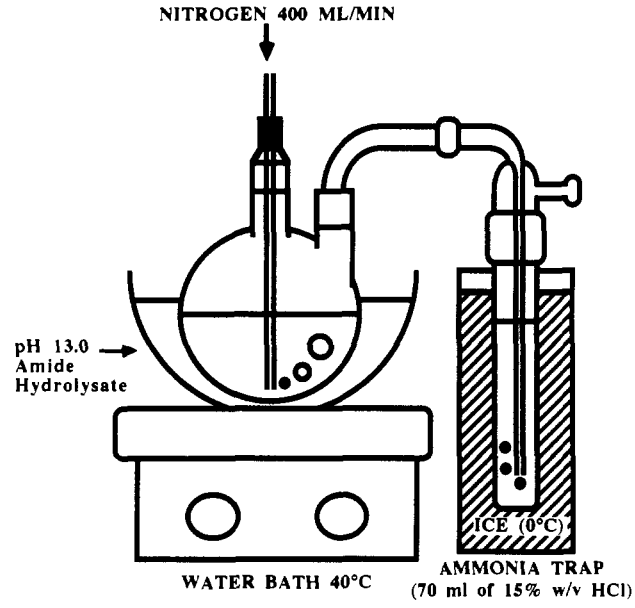

Figure 1. Purge and trap apparatus for ammonia isolation.

then purged with nitrogen (400 mL/min) for a period of 3 h at 40 °C and trapped in 70 mL of a 15% w/v HCl solution cooled to 4 °C in an ice bath (see Figure 1). Since the system analyzed contained carbohydrate, it was important only to warm the sample to 40 °C. Higher temperatures under the alkaline pH values may result in Maillard reactions which could consume ammonia (Izzo and Ho, 1992). After purging, the contents of the trap were stored at 10 °C in sealed amber bottles until their ammonia contents could be analyzed. This purge and trap step was used to isolate the ammonia away from the various compounds in the extract that were found to interfere with the enzymatic ammonia assay described below. A flow chart of the entire ammonia isolation scheme is given in Figure 2.

Ammonia Determination. Ten-milliliter aliquots of each acid trap were pipetted into a 100-mL Erlenmeyer flask and titrated to pH 7.0–7.25 with 25 mL of 2 M $KHCO_3$. Aliquots of the neutralized isolate were immediately assayed for ammonia content using glutamate dehydrogenase [see Kun and Kearney (1974)]. All samples were analyzed on a Hitachi Model U-3110 spectrophotometer (Hitachi Instruments, Danbury, CT).

Determination of the Degree of Deamidation. Deamidation is expressed as a percentage and is based on a determination of the amount of ammonia released from the unextruded sample (SU) vs the extruded sample (SE) with (SU − SE) giving the amount of ammonia lost during the extrusion. The percent deamidation is calculated in the following manner: ((Su − SE)/SU) × 100 = % deamidation. To obtain the most precise value for percent deamidation, a standard curve was produced (r^2 = 0.997) by repeating the purge and trap step several times, spiking the sample side with various concentrations of ammonium chloride. This curve was used to calculate the amount of ammonia liberated from the extrudate. All extrudates were analyzed in triplicate and are reported as a mean ± a standard deviation.

RESULTS AND DISCUSSION

From the results obtained in this study one can see the hypothesis that deamidation does occur in wheat flour during extrusion processing is valid. The unextruded wheat flour was found to exhibit the highest amount of ammonia liberated per gram of flour (5.42 mg of NH_3/g of flour). All samples extruded exhibited a value less then 5.42 mg of NH_3/g of flour, indicating that a loss of amide occurred during the extrusion. Interestingly, some of the reuslts seem to mimic the trends found by other researchers in model protein and peptide systems.

Effect of Temperature. The effect of the degree of temperature treatment on the level of protein deamidation is presented in Table I. As can be seen, there is a positive

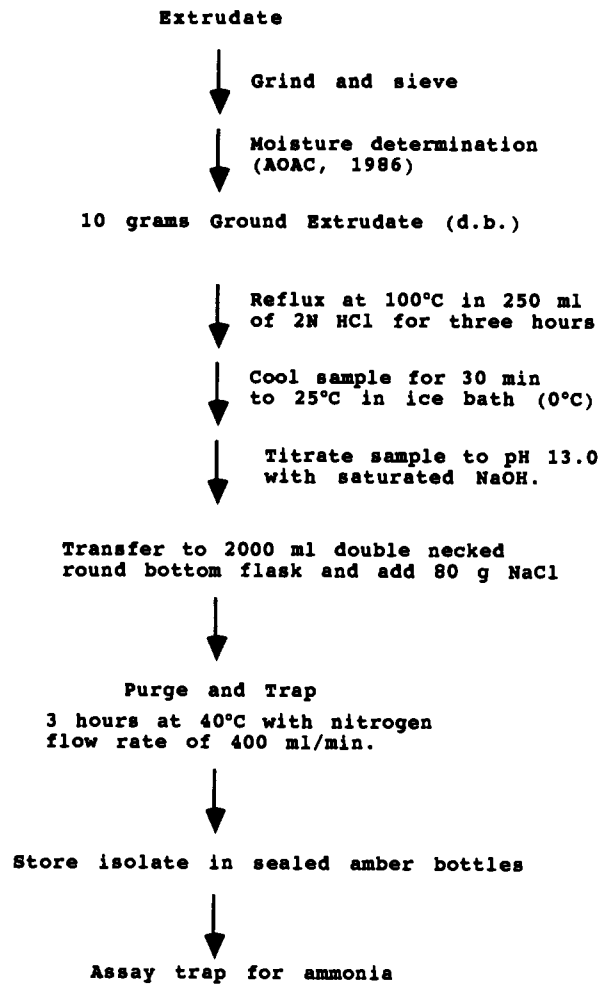

Figure 2. Ammonia isolation scheme.

Table I. Effect of Temperature on Deamidation in Extruded Wheat Flour

melt temp, °C	feed moisture, %	screw speed, rpm	mass flow, g/min	deamidation,[a] %
130	20	300	300	2.80 ± 1.75
155	20	300	300	12.71 ± 1.01
170	20	300	300	21.91 ± 1.97

[a] Results depicted are expressed as the average of three replicates ± their standard deviation.

correlation between increased temperature and greater deamidation. This result seems reasonable since higher temperature, in most cases, dictates higher rates of reaction. This trend has been shown to occur in model systems as well. When studying the effects of increasing temperature on the deamidation of glutamyl residues in pentapeptides, Scotchler and Robinson (1974) found that a 20 °C increase in temperature can cause considerable increases in deamidation rates.

Another factor that could explain the increase in deamidation with temperature is that at higher temperature more bonds and interactions which occur both inter- and intramolecularly between gluten molecules are weakened. This allows for the penetration of water into the structure of the gluten, aiding in the catalysis of deamidation within the core of the protein. As this occurs, repulsive negative charges are produced which can then aid in altering the protein conformation, exposing more potential sites of reaction to the aqueous environment. This effect can be seen when deamidating capabilities of enzymes have been enhanced by heating food proteins

Table II. Effect of Feed Moisture on Deamidation in Extruded Wheat Flour

feed moisture, %	melt temp, °C	screw speed, rpm	mass flow, g/min	deamidation,[a] %
20	150	300	300	15.69 ± 0.26
25	150	300	300	22.62 ± 0.17
30	150	300	300	29.65 ± 0.16

[a] Results depicted are expressed as the average of three replicates ± their standard deviation.

Table III. Effect of pH on Deamidation in Extruded Wheat Flour

pH	feed moisture, %	melt temp, °C	screw speed, rpm	mass flow, g/min	deamidation,[a] %
3.0	20	155	300	300	22.74 ± 0.54
7.0	20	155	300	300	15.29 ± 2.88
12.0	20	155	300	300	25.93 ± 1.72

[a] Results depicted are expressed as the average of three replicates ± their standard deviation.

briefly before the enzyme is added (Hamada, 1992). Water penetration and protein solubilization seem to be key factors in the deamidation process, and both are enhanced by heat.

Effect of Moisture. As mentioned above, moisture plays an important role in the deamidation reaction (Wright, 1991). The effects of increasing moisture content have provided us with some intriguing results. Most chemical reactions that occur during extrusion are enhanced at low moisture content (Izzo, 1989; Wen et al., 1990). Interestingly, the trend here seems to be just the opposite (see Table II). However, when one examines this trend more closely and relates it to the mechanisms introduced in the literature (Lowenson and Clarke, 1988), the trend becomes more understandable. Since it has been shown that deamidation can occur via direct hydrolysis of the amide bond, the presence of water is obviously needed. In this case, increased moisture content may have resulted in increased hydrolysis of the amide bond. It is also possible that the increasing moisture content, coupled with the high temperature, results in a greater degree of protein unfolding and solubilization in the aqueous environment, thus catalyzing deamidation.

Effect of pH. Extremes of pH seem to have a positive effect on deamidation (see Table III). As one can see, at high pH and at low pH the deamidation reaction is favored. These data seem to agree with the data found with model glutamine-containing peptides (Scotchler and Robinson, 1974), where deviations of 2 pH units above and below pH 6.0 resulted in increased deamidation levels. Acidic pH values have been used often to deamidate food proteins via direct hydrolysis of the amide bond. However, it is well established that the deamidation reaction can occur at alkaline pH values by way of intramolecular attack of the peptide nitrogen on the amide carbonyl carbon (Stephenson and Clarke, 1989). This intramolecular attack results in the formation of an unstable succinimide intermediate which can undergo spontaneous hydrolysis, resulting in the formation of normal peptides or isopeptides that redirect the peptide chain through the side-chain carboxyl group (Wright, 1991). Also, the formation of this succinimide intermediate can promote racemization about the α-carbon, resulting in the generation of D-isomers.

Conclusions. The extent of deamidation in a complex system consisting of lipid, protein, and carbohydrate was successfully monitored using the purge and trap technique. Upon analysis of the data, one can see that deamidation

of gluten proteins in an extruded wheat flour system does occur. Increases in temperature and moisture content give rise to an increase in deamidation. Extremes of pH also enhance deamidation but are thought to do so by different mechanisms. Since the loss of amide functions affects charge density, it is possible that different or more macromolecular complexations and interactions are occurring as a result of deamidation in complex systems. Studies in this area may help to relate changes in gluten amide level with other chemical reactions to further explain why under certain conditions of extrusion different physical characteristics are obtained.

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