Effect of Residual Amide Content on Aroma Generation and Browning in Heated Gluten-Glucose Model Systems

Henry V. Izzo and Chi-Tang Ho*

Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08903

The effect of residual amide levels in wheat gluten on the thermal generation of aromas and the formation of brown color was studied. This was accomplished by deamidating native wheat gluten to different levels and heating each fraction with glucose in a closed system at 150 °C for 60 min. The volatiles from the resulting reactant mixtures were then isolated and identified by GC/MS. Brown color development was measured spectrophotometrically. It was found that the Maillard-derived aromas decreased as amide levels decreased, as did the formation of brown color. Explanations for these trends are discussed.

INTRODUCTION

The Maillard reaction is responsible for the thermal generation of roasted, toasted, or caramel-like aromas as well as the development of brown colors in protein and carbohydrate-rich foods (Nursten, 1986). Due to the inherent complexity of food systems, particularly their protein components, the majority of the work done by studying the Maillard reaction has been accomplished in reference to simple model systems of amino acids and reducing sugars (Tressl et al., 1985; Shu and Ho, 1988). However, some work has been done on the participation of intact proteins in thermal aroma generation. These studies focus for the most part on glycosylation of the ϵ-amino group of lysine which activates the sugar, initiating the Maillard reaction (Alaimo et al., 1992; Wu et al., 1990).

Another reaction that proteins have been shown to undergo when heated is the spontaneous deamidation of endogenous asparagine and glutamine residues (Robinson and Rudd, 1974; Wright, 1991). Deamidation is described as the loss of the amide function of a glutamine or asparagine side chain resulting in the formation of glutamic and aspartic acid residues. The reaction affects protein structure and function by increasing charge density, which leads to protein unfolding and thus enhanced solubility (Matsudomi et al., 1981). Also, as a consequence of this reaction, a molecule of free ammonia is liberated from the protein.

Interestingly, in recent studies, we have shown that ammonia can contribute to the Maillard browning reactions in complex systems (Izzo and Ho, 1992). In the past, ammonia has been speculated by some researchers to be a key intermediate in the formation of pyrazine compounds, a very important class of Maillard-generated aromas (Van Praag et al., 1968). Ammonia has also been found as a volatile constituent of roasted barley (Wang et al., 1969). Finally, both glutamine and asparagine have been shown to produce considerably more pyrazines than do their corresponding acids when heated with reducing sugars (Koehler et al., 1969). This seems to suggest that the amide nitrogen, possibly through deamidation, is available to contribute to amino/carbonyl interactions leading to aroma generation via the Maillard reaction.

One very important class of food proteins which are significantly high in amide content is wheat proteins. These proteins, known collectively as gluten proteins, exhibit a very high glutamine content. About 30% of the overall amino acid content of gluten is glutamine. Wheat flour, which contains these proteins, is widely used to produce a variety of leavened breads and ready-to-eat breakfast cereals, as well as a wide array of snack foods (Hoseney and Rogers, 1990). Perhaps amide nitrogen liberated from the production of these food products may play an important role in ensuing Maillard reactions.

The purpose of this study was to determine the contribution of amide levels in gluten protein to the formation of thermally generated aroma compounds. This was accomplished by generating gluten protein fractions exhibiting different levels of residual amide nitrogen. These fractions were then heated in the presence of reducing sugars, and the subsequent volatiles formed were isolated and identified and quantitated using GC/MS techniques.

MATERIALS AND METHODS

Materials. Wheat gluten was purchased from the Sigma Chemical Co. (St. Louis, MO). Hydrochloric acid (82%), D-glucose, hexane (HPLC grade), trichloroacetic acid (TCA), and ethanol (200 proof) were purchased from the Fisher Scientific Co. (Fair Lawn, NJ). The reagents used for ammonia analysis were purchased from the Sigma and are adenosine diphosphate (ADP) disodium salt, triethylamine hydrochloride (TEA), ammonium chloride, potassium bicarbonate, and reduced nicotinamide adenine dinucleotide (NADH). The enzymes, glutamate dehydrogenase and 2-oxoglutarate, were obtained from Boehringer Mannheim (Indianapolis, IN). The carbon standard (C_5-C_{26} *n*-paraffin) for volatile analysis was purchased from Alltech Associates Inc. (Deerfield, IL).

Preparation of Deamidated Gluten Fractions. The wheat gluten used for this experiment was purchased from Sigma and, according to the manufacturer, contained approximately 7% lipid. Since our goal was only to work mainly with the proteins of gluten, the loosely bound lipid needed to be removed with as little damage as possible to the native protein. Therefore, 600 g (db) of wheat gluten was weighed into a 2000-mL Erlenmeyer flask to which was added 1200 mL of hexane. The flask was sealed and placed in an incubator/shaker (New Brunswick Scientific Co., Edison, NJ) equilibrated to a temperature of 50 °C. The gluten was extracted at this temperature for 12 hr while shaking at a speed of 180 rpm. Upon completion of the extraction, the gluten suspension was vacuum filtered through Whatman No. 2 filter paper to obtain the defatted residue. This residue was then reextracted under conditions identical to the first extraction with the exception of extraction time which was only 6 hr. After completion of the second extraction, the gluten suspension was filtered as before and the residue was placed in a shallow aluminum pan (14 in. × 20 in. × 3 in.). The defatted gluten powder was spread out in a thin layer on the bottom of the pan and placed in the vacuum hood for 36 hr to remove any excess hexane.

0021-8561/93/1441-2364$04.00/0

After defatting, 100 g (db) of the gluten powder was weighed into each of five 200-mL Erlenmeyer flasks. To produce protein fractions that exhibited different levels of deamidation, the samples were suspended in increasingly acidic solutions of hydrochloric acid (150 mL) to facilitate hydrolysis of amide side chains. The solution strengths used were 0.00 (blank), 0.075, 0.150, 0.225, and 0.300 N. The flasks containing the suspensions were then sealed with aluminum foil and placed in an incubator/shaker set at 70 °C and 150 rpm for a period of 3 hr. The resulting protein suspensions were then dialyzed exhaustively against deionized water for 48 hr to remove the HCl, and the final fractions were freeze-dried.

Measurement of Degree of Deamidation. To facilitate the measurement of residual amides in each deamidated fraction, 1 g (db) of each sample was weighed into sealable polycarbonate centrifuge tubes (Fisher Scientific) to which was added 20 mL of 2 N HCl. The tubes were capped and placed in a glycerol bath at 110 °C for a period of 3 h. This was done to convert the residual amides to ammonia. Upon completion of the hydrolysis, soluble protein was precipitated by the rapid addition of 20 mL of 10% (w/v) TCA. All five samples were then centrifuged in a Sorvall superspeed automatic refrigerated centrifuge at 10 °C and 12 000 rpm to remove the precipitate. Aliquots of the supernatant were then adjusted to pH 7.0–7.5 with 2 M potassium bicarbonate, and the resultant solutions were assayed for ammonia content using glutamate dehydrogenase (Kun and Kearney, 1974). All measurements were performed in triplicate.

Calculation of Percent of Deamidation. To calculate the percent of deamidation, the value obtained for the blank (gluten in 0.00 N HCl) was treated as the value of 100% amide level. The values obtained for the acid-treated samples were subtracted from this value and subsequently expressed as a percentage of the value obtained for the blank. This percentage can be expressed by the equation $[(A - B)/A] \times 100$, where A is the value obtained for the blank undeamidated sample and B is the value obtained for each acid-treated or deamidated sample.

Reaction of Gluten Samples with Glucose. Ten grams (db) of each gluten sample was blended with 2 g of glucose, and 40 mL of distilled water was added. The mixture was swirled vigorously, then frozen, and subsequently freeze-dried. This step was to facilitate a coating of the proteins with the reducing sugar. This ensures that the system is well mixed and brings the reactants into close proximity to one another. After freeze-drying, the resultant sugar–protein systems were sealed in glass jars and heated at 150 °C in a laboratory air oven (Fisher Isotemp Oven 400 Series) for 1 h. The final reacted systems were then ground into a fine powder with a mortar and pestle and stored at −10 °C in sealed glass jars until analysis.

Volatile Isolation and Analysis. A purge and trap-direct thermal desorption/gas chromatography/mass spectrometry method recently described by Hartman et al. (1993) was used to isolate, analyze, and quantify the volatile compounds in the reacted samples. This method serves as a useful means of quantifying the levels of components present in the samples analyzed. The analysis, however, can be only considered as semiquantitative.

Two grams (db) of each reacted system was weighed into a glass cylinder which was connected to a thermal desorption sample collecting system (Scientific Instrument Services, Ringoes, NJ). At this time an internal standard (1 mL of 1 mg of D-toluene in methanol) was spiked directly into the matrix of the solid sample to facilitate quantitation later on in the analysis. Purge-and-trap isolation was then performed on the sample, and the volatiles were trapped onto a polymer cartridge consisting of Tenax TA (Alltech) and Carbotrap (Supelco). The conditions for trapping were as follows: 80 °C heating block temperature, 40 mL/min nitrogen gas flow, and 1-h purge time. After trapping was completed, the polymer cartridges were dried by purging for an extra 30 min with nitrogen (40 mL/min) at ambient temperatures.

The trapped volatiles were desorbed directly into the GC column (220 °C, 5 min, helium flow 1 mL/min) using a Model TD-1 short path thermal desorption apparatus (Scientific Instrument Services) (Hartman et al., 1993). Separation of the volatiles was accomplished using a Varian 3400 gas chromatograph equipped with a nonpolar fused silica capillary column (DB-1, J&W Scientific; 60 m × 0.32 mm i.d., 0.25-μm film thickness). The GC was operated with an injector temperature of 250 °C, a helium flow rate of 1 mL/min, and a split ratio of 10:1. The program for volatile separation was as follows: initial column temperature of −20 °C with a 5-min hold during thermal desorption and a temperature increase of 10 °C/min from −20 to 280 °C with a 20-min isothermal hold. The separated volatiles were then detected and identified with a Finnigan MAT 8320 high-resolution mass spectrometer. The ionization was set at 70 eV, and the source temperature was 250 °C with a filament emission current of 1 mA. Spectra obtained were identified by utilizing an on-line computer library (NBS) and the Eight Peak Mass Spectra Data (MSDC, 1984). The volatile compounds identified were semiquantified by relating the integrated peak areas of the identified compounds to that of the internal standard (D-toluene). Linear retention indices were determined through the use of a C_5–C_{26} *n*-paraffin standard. Retention indices for the volatiles identified were calculated as described by Majlat et al. (1974).

Browning Measurement. One gram (db) of each heated system was weighed into a glass test tube. To this was added 20 mL of ethanol. The tube was then sealed and the brown color extracted by vigorously shaking for a period of 60 s. The capped tubes were then centrifuged for 20 min in a Sorvall superspeed automatic refrigerated centrifuge at 10 °C and 15 000 rpm. Aliquots of the supernatant were then taken and their absorbances were determined against a water blank at 420 nm on a Milton Roy Spectronic Model 301 spectrophotometer. Each reaction system was done in triplicate.

RESULTS AND DISCUSSION

A total of five gluten fractions exhibiting different levels of residual amide were generated. The levels of deamidation (expressed as five deamidations) measured for each acid-treated sample were as follows: 18.59 ± 0.37% (0.075 N), 22.43 ± 0.67% (0.150 N), 26.54 ± 0.66% (0.225 N), 32.97 ± 0.86% (0.300 N). It was found that a total of 27 volatile compounds comprising several different chemical classes was generated during the heating of various partially deamidated gluten fractions with glucose. Most volatile compounds were common to all systems; however, variations in quantity were apparent and in some cases quite interesting. The approximate quantity for each compound identified, along with its corresponding retention index, is listed in Table I.

Lipid-Derived Volatiles. Even though a defatting step was incorporated into the procedure, it seems that not all of the lipid was removed from the protein. Volatiles suspected to be derived from lipid sources were the alcohols and aldehydes. The alcohols identified in the gluten–glucose reaction systems were pentanol, hexanol, heptanol, 1-hepten-3-ol, octanol, and 2-decen-1-ol. The evidence of residual lipid is also apparent when one examines the aldehyde content of the samples. Hexanal, heptanal, nonanal, and nonenal were all identified in the reaction systems. They, too, are indicators of lipid oxidation. Also, the ketone, 2-heptanone, as well as the hydrocarbons, methylcyclopentane and benzene, could have originated from lipid as well. Examination of the quantitative data obtained for the lipid-derived volatiles shows that in every case the highest level of deamidation (32.97%) gave the lowest amount of volatiles when compared to the volatile quantity of the least deamidated sample (0.00%). It is possible that the acid hydrolysis used to produce the different levels of amide content freed the lipid due to changes in protein structure brought about by deamidation or hydrolysis. This lipid was then washed away in the dialysis step and thus unavailable for further reaction, resulting in lower levels of lipid-derived volatile compounds at higher acid treatment levels.

Maillard-Derived Volatiles. The Maillard compounds identified in the heated gluten–glucose samples

Table I. Volatiles Generated during Heating of the Partially Deamidated Gluten Samples with Glucose (150 °C, 60 min)

compound	I_K[a] (DB-1) 0%	concentration (ppm) at the following extents of deamidation				
		0%	18.59%	22.43%	26.54%	32.97%
alcohols						
1-pentanol	700	–[b]	0.062	–	tr[c]	–
3-methyl-1-butanol	719	1.254	0.773	0.587	0.891	0.246
1-hexanol	811	tr	0.147	0.095	0.101	0.094
1-heptanol	900	0.489	0.094	–	0.073	tr
1-hepten-3-ol	905	0.801	0.404	0.222	0.303	0.031
1-octanol	991	0.383	0.169	0.069	0.138	tr
2-decen-1-ol	1101	0.084	0.172	0.080	0.075	0.076
aldehydes						
hexanal	732	2.352	tr	0.870	0.999	0.677
heptanal	833	tr	tr	0.146	0.150	–
nonanal	1017	0.665	0.419	0.372	0.235	0.208
nonenal	1069	0.200	0.205	0.118	0.082	0.115
furan compounds						
2-propylfuran	642	0.460	0.344	0.370	0.229	0.227
2-furfural	759	17.164	5.455	6.369	7.906	7.458
furfuryl alcohol	790	5.642	0.752	–	0.075	0.448
2-acetylfuran	835	1.804	0.489	0.156	0.065	0.868
5-methyl-2-furfural	872	3.513	1.282	1.569	1.473	1.945
2-pentylfuran	917	0.875	0.510	0.381	0.392	0.386
2,4-dimethyltetrahydrofuran	961	tr	0.497	0.025	0.096	–
hydrocarbons						
methylcyclopentane	596	0.163	0.171	–	0.073	0.036
benzene	617	3.318	0.537	–	0.419	0.032
ketones						
3-methyl-2-pentanone	651	0.889	–	–	0.557	0.297
1-hydroxy-2-propanone	681	–	0.096	–	–	–
2-heptanone	814	1.259	0.263	0.194	0.133	0.351
nitrogen compounds						
dimethylpyrazine	837	0.405	0.220	–	–	tr
sulfur compounds						
dimethyl disulfide	683	0.675	0.138	–	0.115	0.103
dimethylthiophene	799	1.574	tr	–	0.111	0.609
dimethyl trisulfide	885	tr	0.081	–	0.028	0.122

[a] Retention indices measured according to the method of Majlat et al. (1974). [b] Not found. [c] Present, but in trace amounts (<0.0001 ppm).

were of most interest. It is these compounds to which the ammonia liberated from amides could have contributed. One key class of Maillard-derived aroma compounds which were found in the samples was furans. These compounds are quite important to the palatability of heated foods as they provide warm sugary flavor notes. Furans can be the result of a cyclization of the sugar moiety after sugar activation and Amadori rearrangement via interaction with amine compounds. It is possible that the ammonia liberated from amides could have played a role as an activator for sugar degradation and not a contributor to the final structure of the aroma compounds. Quantitatively, furfural was the major peak in all of the samples and seemed to decrease and then increase as the extent of deamidation increased. This trend was found to be true with several other furan compounds as well (furfuryl alcohol, 2-acetylfuran, 5-methyl-2-furfural). It is possible that at the lowest and highest levels of deamidation sugar activation was enhanced. This could be due to the fact that at lower levels of deamidation more ammonia is released (due to the presence of more amide), resulting in more sugar being activated. This leads to the formation of higher quantities of these compounds. The fact that the concentration of these compounds diminishes and then increases as deamidation increases may be due to changes in protein structure brought about by deamidation or acid hydrolysis of peptide bonds. As the protein was intentionally deamidated in the preparation steps, slight hydrolysis of peptide bonds, as well as changes in protein structure due to the manifestation of changes, could have occurred. As proteolysis occurred, more free amino groups from amino acids could have been available to activate sugars. At the same time, more amide groups could have been exposed to the surface of the protein due to charge-induced conformational changes. The deamidation of these newly exposed amides could have occurred during the heating of the protein with glucose. This again would result in the liberation of more ammonia and the subsequently enhanced activation of glucose leading to the formation of more Maillard-specific volatile compounds.

The only nitrogen-containing heterocyclic compound identified in the model system which is characteristic of the Maillard reaction was dimethylpyrazine. Interestingly, one of the ketones identified, 1-hydroxy-2-propanone, is a precursor of dimethylpyrazine. Upon examination of the quantitative data for the generation of dimethylpyrazine, we saw a trend similar to that observed with the furans. Once again, as the amide level of the protein decreased, a subsequent decrease in dimethylpyrazine content was observed, to the point where it was no longer even detected, and then the compound appeared once again in trace amounts. As was mentioned above, deamidation and subsequent unfolding and exposure of more reactive amides could be the causative factor, or the liberation of free amino acids from the gluten protein during acid hydrolysis could have contributed as well. In any case, the quantity of dimethylpyrazine definitely decreases with the amide level in each system. This again indicates the possibility that the ammonia produced from the deamidation of amides in proteins can contribute to aroma generation via the Maillard reaction.

Brown Color Development. As examination of the development of brown color in the heated gluten–glucose samples showed some interesting results (Figure 1), it was found that the brown color formation was most favored in samples which had undergone the least levels of deamidation. Between 18.59% and 26.54% deamidation, the browning significantly decreased and then seemed to

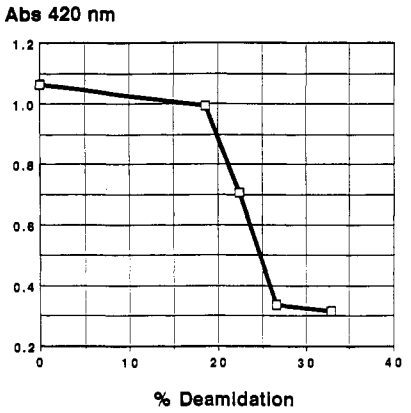

Figure 1. Brown color development of partially deamidated gluten samples heated with glucose (150 °C, 1 h, closed system).

level off. The high levels of brown color in the less deamidated samples can be attributed to the increased levels of residual amides. Again, as with the volatiles generated, these amides were most likely converted to ammonia via deamidation, and the ammonia liberated subsequently interacted with glucose. It is most likely that this interaction led to a polymerization of sugars followed by brown pigment formation.

Conclusion. The effect of amide levels on the extent of browning in the heated gluten–glucose systems provided rather clear trends. It seems that the extent of browning polymer formation was favored in systems having more amide present. This trend is clear-cut for browning but not so for aroma generation. Even though the furans and dimethylpyrazine were generated and seemed to be enhanced in reaction systems which exhibited higher amide levels, it was nonetheless surprising that at the lowest amide levels these volatiles become prevalent once again. It is possible that this phenomenon is related to structural changes induced by deamidation. As more deamidation occurs, the protein unfolds due to increased charge density. Perhaps amides that were protected within the interior of the protein are now exposed to the surface and become more labile. Thus, they can liberate ammonia, contributing to the Maillard reaction. In any case, these results seem to indicate that more available amide results in more Maillard reaction.

ACKNOWLEDGMENT

New Jersey Agricultural Experiment Station Publication D-10544-2-93 supported by State Funds and the Center for Advanced Food Technology. The Center for Advanced Food Technology is a member of the New Jersey Commission for Science and Technology. We thank Mrs. Joan B. Shumsky for secretarial aid and Dr. Thomas G. Hartman for help with the mass spectrometry analysis.

LITERATURE CITED

Alaimo, L. H.; Ho, C.-T.; Rosen, J. D. Effect of protein glycation on subsequent volatile formation. *J. Agric. Food chem.* **1992**, *40*, 280–283.

Hartman, T. G.; Lech, J.; Karmas, K.; Salinas, J.; Rosen, R. T.; Ho, C-T. Flavor characterization using adsorbent trapping-thermal desorption or direct thermal desorption-gas chromatography and gas chromatography-mass spectrometry. In *Flavor Measurement*; Ho, C.-T., Manley, C. H., Eds.; Dekker: New York, 1993; pp 37–60.

Hoseney, R. C.; Rogers, D. E. The formation and properties of wheat flour doughs. *CRC Crit. Rev. Food Sci. Nutr.* **1990**, *29*, 73–93.

Izzo, H. V.; Ho, C.-T. Ammonia affects Maillard chemistry of an extruded autolyzed yeast extract: Pyrazine aroma generation and brown color formation. *J. Food Sci.* **1992**, *57*, 657–659.

Koehler, P. E.; Mason, M. E.; Newell, J. A. Formation of pyrazine compounds in sugar-amino acid model systems. *J. Agric. Food Chem.* **1969**, *17*, 393–396.

Kun, E.; Kearney, E. B. Ammonia. In *Methods of Enzymatic Analysis;* Bergmeyer, H.U., Ed.; Academic Press: New York, 1974; Vol. 4., pp 1802–1806.

Majlat, P.; Erdos, Z.; Takacs, J. Calculation and application of reaction indices in programmed temperature gas chromatography. *J. Chromatogr.* **1974**, *91*, 89–103.

Matsudomi, N.; Keneko, S.; Kato, A.; Kobayashi, K. Functional properties of deamidated gluten. *Nippon Nogeikagu* **1981**, *55*, 983–989.

MSDC. *1984, Eight Peak Index of Mass Spectra*; Royal Society of Chemistry: London, U.K., 1984.

Nursten, H. E. Aroma compounds from the Maillard reaction. In *Development in Food Flavours;* Birch, G. G., Lindley, M. G., Eds.; Elsevier: New York, 1986; pp 173–190.

Robinson, A. B.; Rudd, C. J. Deamidation of glutaminyl and asparaginyl residues in peptides and proteins. *Curr. Top. Cell. Regul.* **1974**, *8*, 247–295.

Shu, C. K.; Ho, C.-T. Effect of pH on the volatile formation from the reaction between cysteine and 2,5-dimethyl-4-hydroxy-3(2H)-furanone. *J. Agric. Food Chem.* **1988**, *36*, 801–803.

Tressl, R.; Grünewald, G. K.; Krersten, E.; Rewicki, D. Formation of pyrroles and tetrahydroindolizin-6-ones as hydroxyproline-specific Maillard products from glucose and rhamnose. *J. Agric. Food Chem.* **1985**, *33*, 1137–1142.

Van Praag, M.; Stein, H. S.; Tibbetts, M. S. Steam volatile aroma constituents of roasted cocoa beans. *J. Agric. Food Chem.* **1968**, *16*, 1005–1008.

Wang, P. S.; Kato, H.; Fujimaki, M. Studies on the flavor components of roasted barley. Part III. The major volatile basic compounds. *Agric. Biol. Chem.* **1969**, *33*, 1775–1781.

Wright, H. T. Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Crit. Rev. Biochem. Mol. Biol.* **1991**, *26*, 1–51.

Wu, H.; Govindarajan, S.; Smith, T.; Rosen, J. D.; Ho, C.-T. Glucose-lysozyme reactions in a restricted water environment. In *The Maillard Reaction in Food Processing, Human Nutrition, and Physiology;* Finot, P. A., Aeschbacher, H. U., Hurrell, R. F., Liardon, R., Eds.; Birkhauser Verlag: Basel, Switzerland, 1990; pp 85–90.

Received for review May 3, 1993. Revised manuscript received September 16, 1993. Accepted September 27, 1993.[⊛]

[⊛] Abstract published in *Advance ACS Abstracts,* November 1, 1993.