Formation of Pyrazines from the Maillard Reaction of Glucose and Glutamine-*amide*-^{15}N

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The contribution of amino and amide nitrogen atoms to pyrazine formation in both dry and aqueous systems was investigated. The ^{15}N isotope labeled at the amide side chain of glutamine was chosen to react with glucose at 180 °C in the studies. Pyrazine, methylpyrazine, ethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3-dimethylpyrazine, vinylpyrazine, and 2-ethyl-5-methylpyrazine were identified from heating the isotope-labeled glutamine with glucose in a dry system. Similar types of pyrazines were also found in an aqueous system. The exception was 2-vinyl-5-methylpyrazine, which was produced instead of 2-ethyl-5-methylpyrazine. These results demonstrated that deamidation did participate in pyrazine production and more than half of the nitrogen sources of pyrazines came from the amide side chains of glutamine. The yields of pyrazines from the dry system were higher than that from the aqueous system.

INTRODUCTION

The nonenzymatic deamidation of proteins has been recognized by biochemists for many years (Wright, 1991). Deamidation is described as the loss of the amide function of a glutamine (Gln) or asparagine (Asn) side chain, resulting in the formation of glutamic and aspartic acids. As a consequence of this reaction, free ammonia is liberated from the protein.

Pyrazines are nitrogen-containing heterocyclics that are potent characteristic flavorants found in a wide range of raw and processed foods (Maga, 1992). Pyrazines are usually associated with the generation of roast and burnt flavor notes. These unique and desirable sensory properties make pyrazines essential to the food industry (Maga, 1982). The most direct route for pyrazine formation results from the interaction of reducing sugars and α-amino acids through Strecker degradation. However, at higher temperatures the amino acids can undergo reactions other than traditional Strecker degradation (de Rijke et al., 1981). The degradation involves decarboxylation and/or deamination to generate a series of compounds including ammonia, amines, carbon dioxide, and the Strecker aldehyde. These compounds can serve as reactants to form pyrazines. Wang and Odell (1973) as well as Baltes and Bochmann (1987) reported that pyrazines could be obtained by heating individual hydroxyamino acids such as serine and threonine at high temperature in the absence of sugars.

In a recent study, Izzo and Ho (1992) have shown that ammonia can contribute to the Maillard reaction in complex systems. In the past, 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 pyrazine generation. To clarify the participation of the deamidation reaction in pyrazine formation, we used a glutamine with a labeled ^{15}N isotope at the amide side chain and a ^{14}N at the α-amino group to investigate the relative contribution of the α-amino nitrogen and the amide nitrogen to pyrazine formation.

EXPERIMENTAL PROCEDURES

Materials. L-Glutamine and wheat starch were purchased from Sigma Chemical Co. (St. Louis, MO). Glucose and deuterated toluene (toluene-d_8), the internal standard, were obtained from Aldrich Chemical Co. (Milwaukee, WI). L-Glutamine-*amide*-^{15}N was purchased from Isotec, Inc. (Miamisburg, OH) and has a stated purity of 99%. Tenax-TA (2,6-diphenyl-*p*-phenylene oxide) adsorbent (60–80 mesh) was obtained from Alltech Associates (Deerfield, IL). Carbotrap (activated graphitized carbon) adsorbent (20–40 mesh), C_5–C_{25} *n*-paraffin standard, and silanized glass wool were purchased from Supelco Inc. (Bellefonte, PA).

Volatile Generation and Isolation from the Dry System. Fifty grams of wheat starch, 500 mg of glucose, and 100 mg of L-glutamine/or L-glutamine-*amide*-^{15}N were mixed with 500 mL of deionized water and then freeze-dried. The solid mixture was further placed in the upper level of a desiccator; a Pyrex dish containing 20 mL of deionized water was placed in the lower level to adjust moisture content of the samples back to 12–14%. The moisture content of the samples was measured according to the AOAC air over method (AOAC, 1986). The samples were then transferred into a reaction vessel and heated at 180 °C for 1 h.

The heated sample (1 g) was packed in the center of a glass tube, and the silanized glass wool was placed on the two ends of the tube. Deuterated toluene (1 μL of a 1.001 mg/mL solution) was spiked into the tube as the internal standard. The tube was further sealed in a Scientific Instrument Services (SIS) solid-sample purge-and-trap apparatus (Ringoes, NJ), and the volatiles were purged with nitrogen at a flow rate of 40 mL/min to a silanized glass-lined stainless steel desorption tube (4.0 mm i.d. × 10 cm length). This desorption tube was from Scientific Instrument Services, Inc. (Ringoes, NJ) and consisted of 3-cm bed volume of Tenax-TA adsorbent and 3-cm bed volume of Carbotrap adsorbent. This volatile isolation was carried out at 80 °C for 1 h.

Volatile Generation and Isolation from the Aqueous System. Glucose (500 mg) and 100 mg of L-glutamine/or L-glutamine-*amide*-^{15}N were dissolved in 100 mL of deionized water, and the pH was adjusted to 10 by 1 N NaOH. The samples were transferred to a reaction vessel and heated at 180 °C for 1 h. The pH of the heated mixtures dropped to 3–4 and was titrated back to pH = 12 to increase the extraction efficiency of pyrazines.

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Table I. Relative Ion Abundance (Percent) of Alkylpyrazines Generated from Glutamine with Glucose in the Dry and Aqueous System[a]

compound	$(M-1)_n$[b]	M_n[b]	$(M+1)_n$[b]	M_{exp}[c]	$(M+1)_{exp}$[c]	$(M+2)_{exp}$[c]	M_{exp}[d]	$(M+1)_{exp}$[d]	$(M+2)_{exp}$[d]
pyrazine	0.4	100	6.6	7.6	62.8	100	6.9	37.3	100
methylpyrazine	1.9	86.8	5.6	4.6	60.4	100	2.6	85.6	100
2,5- and 2,6-dimethylpyrazine	3.1	100	17.6	30.8	84.9	85.1	19.4	100	40.2
ethylpyrazine	49.2	42	0	57.1	100	46.7	37.1	100	42.8
2,3-dimethylpyrazine	6.7	70.8	4.8	9.4	47.1	61.7	15.9	100	83.2
vinylpyrazine	20	100	6.8	10.2	42.1	65.8	3.4	31.2	100
2-ethyl-5-methylpyrazine	100	63.3	6.6	41.4	60.1	27.7	–[e]	–	–
2-vinyl-6-methylpyrazine	20.4	51.6	12.7	–	–	–	14.9	43.3	21.4

[a] Numbers are the average of five separate determinations. [b] $(M-1)_n$, M_n, and $(M+1)_n$ are the relative ion abundances from the reaction of nonlabeled glutamine with glucose. [c] M_{exp}, $(M+1)_{exp}$, and $(M+2)_{exp}$ are the relative ion abundances from the reaction of glutamine-*amide*-^{15}N with glucose in the dry system. [d] M_{exp}, $(M+1)_{exp}$, and $(M+2)_{exp}$ are the relative ion abundances from the reaction of glutamine-*amide*-^{15}N with glucose in the aqueous system. [e] Not observed.

The heated reaction solution was introduced into a 100-mL Wheaton liquid-sample purge-and-trap apparatus obtained from Fisher Scientific Co. (Fair Lawn, NJ). Volatiles were isolated by using nitrogen at a flow rate of 40 mL/min into a desorption tube that consisted of Tenax-TA and Carbotrap adsorbents (1:1) at 50 °C for 1 h.

Volatile Analysis for the Dry System by Gas Chromatography–Mass Spectrometry (GC–MS). The desorption tube prepared from the isolation procedure was connected to a SIS Model TD-1 short-path thermal desorption unit. This desorption unit was interfaced into a Varian 3400 gas chromatograph coupled with a Finnigan MAT 8320 high-resolution, double-focusing magnetic sector mass spectrometer (TD–GC–MS). The volatiles trapped in the desorption tube were then thermally desorbed into the GC at 220 °C for 5 min. However, the temperature of the GC chamber was maintained at −20 °C by dry ice to cryofocus the volatiles as a narrow band at the head of the capillary column during the desorption process. After the desorption tube was removed from the injection port of the GC, the volatiles were analyzed by GC–MS. The GC was operated with an injector temperature of 250 °C with a split ratio of 10:1, and a helium carrier flow rate of 1.0 mL/min. The GC column was a nonpolar fused-silica capillary column [60 m × 0.32 mm i.d., 0.25-μm thickness, DB-1 (J&W Scientific Co.)] and was temperature-programmed from −20 to 280 °C at a rate of 5 °C/min with a 20-min hold at the upper limit. Volatiles were quantified via peak area ratio calculation to that of the internal standard deutrated toluene. Linear retention indices for the volatiles were determined through the use of a C_5–C_{25} *n*-paraffin standard, according to the method of Majlat et al. (1974). The mass spectrometer electron ionization was set at 70 eV, and the source temperature was 250 °C with a filament emission current of 1 mA, scanning masses 35–350, and a 0.8-s interscan time. All mass spectra obtained were identified by utilizing an on-line computer library (NIST).

Volatile Analysis for the Aqueous System by Gas Chromatography–Mass Spectrometry (GC–MS). The GC–MS operating conditions for the aqueous system studies were conducted the same as described for the dry system studies, except for the program for GC separation. The temperature program of the GC was from −20 to 40 °C at a rate of 5 °C/min, from 40 to 150 °C at a rate of 2 °C/min, and then to 280 °C at a rate of 10 °C/min, and the final hold time was 10 min.

Calculations for the Relative Contribution of ^{14}N Nitrogen and ^{15}N Nitrogen to Pyrazine Formation. When ^{15}N nitrogen atoms are incorporated in a pyrazine ring, the molecular weight of the pyrazine will increase one or two mass units depending on the number of ^{15}N nitrogen atoms in the ring. Therefore, each pyrazine may have three different molecular weights, denoted W_1, W_2, and W_3. W_1, W_2, and W_3 represent two ^{14}N nitrogen atoms in the pyrazine ring, one ^{14}N and one ^{15}N nitrogen atoms in the pyrazine ring, and two ^{15}N nitrogen atoms in the pyrazine ring, respectively.

The pyrazines containing one ^{15}N atom in the ring shift the $M-1$ (loss of one hydrogen atom from molecular ion), M (molecular ion), and $M+1$ (natural isotope) triplet one mass higher. The pyrazines containing two ^{15}N atoms shift the cluster two mass units higher. The following equations are used to solve for the contributions of each component (W_1, W_2, and W_3) present in a mixture.

$$M_{exp} = M_n W_1 + (M-1)_n W_2$$

$$(M+1)_{exp} = (M+1)_n W_1 + M_n W_2 + (M-1)_n W_3$$

$$(M+2)_{exp} = (M+1)_n W_2 + M_n W_3$$

Here $(M-1)_n$, M_n, and $(M+1)_n$ are the experimental relative abundances of the ion peaks of the pyrazines from the reaction of nonlabeled glutamine with glucose. M_{exp}, $(M+1)_{exp}$, and $(M+2)_{exp}$ are the experimental abundances of the ion peaks of the pyrazines generated from the reaction of glutamine-^{15}N (labeled at the amide nitrogen only) with glucose. The experimental data are shown in Table I.

After the relative contributions of the three different compounds (W_1, W_2, and W_3) were calculated, the percent contribution from amide nitrogen (from a starting material containing labeled amide and unlabeled amine nitrogens) could be determined by using the equation

$$\% \text{ contribution of amide nitrogen} = [(W_3 + {}^1/_2 W_2)/(W_1 + W_2 + W_3)] \times 100$$

As mentioned above, W_2 contains one ^{14}N nitrogen atom and one ^{15}N nitrogen atom in the pyrazine ring; therefore, half of the nitrogen of the component W_2 is from the amide side chains. The component W_3 contains two ^{15}N nitrogen atoms, both coming from the amide nitrogens.

RESULTS AND DISCUSSION

The pyrazines that were identified from heating isotope-labeled glutamine with glucose in the dry system included pyrazine, methylpyrazine, ethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3-dimethylpyrazine, vinylpyrazine, and 2-ethyl-5-methylpyrazine. Similar types of pyrazines were also found in the aqueous system, except that 2-vinyl-5-methylpyrazine was found instead of 2-ethyl-5-methylpyrazine.

The relative contributions of amide nitrogens to pyrazine formation are shown in Figures 1 and 2. From these figures it is obviously revealed that more than half of the pyrazines consisted of ^{15}N nitrogen atoms that came from the amide chains of glutamine. These data demonstrated that deamidation did happen and could participate in the pyrazine formation. The occurrence of deamidation in food systems was also proved by Izzo et al. (1993). In addition, the ammonia from deamidation had a greater contribution than the α-amino groups during thermal generation of pyrazines in both systems. Similar results were also investigated by Bohnenstengel and Baltes (1992). They found that, in asparagine and glucose mixtures, predominantly nitrogen-containing heterocycles (pyrazines, pyridines, pyrroles) were formed, whereas mixtures of aspartic acid/glucose yielded furans and aliphatic

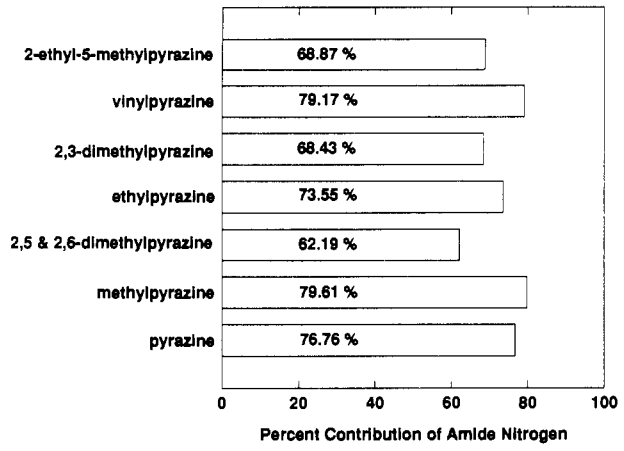

Figure 1. Relative contributions of amide nitrogens to pyrazine formation in the reaction of labeled glutamine with glucose from the dry system.

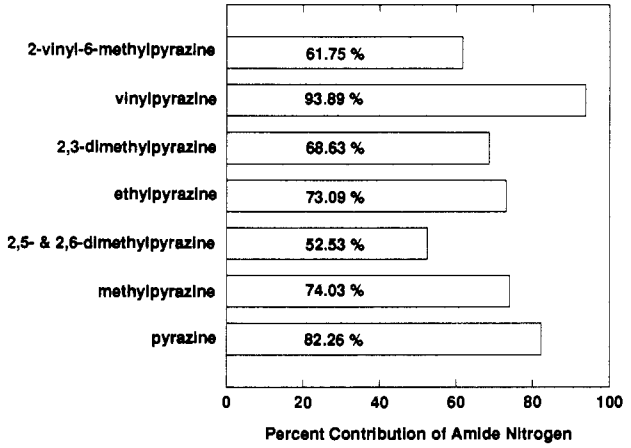

Figure 2. Relative contributions of amide nitrogens to pyrazine formation in the reaction of labeled glutamine with glucose from the aqueous system.

Table II. Alkylpyrazines Identified from Heating Glutamine-*amide*-^{15}N with Glucose in the Dry and Aqueous System

compound	yield (μg/g glucose)		
	I_k[a]	D[b]	A[b]
pyrazine	699	74.9	1.1
methylpyrazine	794	89.1	3.8
2,5- and 2,6-dimethylpyrazine	880	38.1	1.3
ethylpyrazine	884	38.1	0.4
2,3-dimethylpyrazine	886	25.3	0.9
vinylpyrazine	894	28.8	0.8
2-ethyl-5-methylpyrazine	979	6.3	–[c]
2-vinyl-6-methylpyrazine	980	–	0.7

[a] I_k = calculated retention indices with n-paraffins (C_5–C_{25}) as references. [b] D = amounts in the dry system; A = amounts in the aqueous system. [c] Not observed.

carbonyls. These results further implied that ammonia from deamidating the side chains of glutamine more easily reacted with dicarbonyl than the α-amino groups of glutamine. This was reasonable because the α-amino groups could not directly react with dicarbonyl like ammonia and had to proceed through Strecker degradation to generate pyrazines. These results supported that deamidation has a profound effect on flavor generation.

Table II summarizes the quantitation data and retention indices for all pyrazines that were identified in the dry and aqueous systems. In this study, we found that the yields of pyrazines from the dry system were 20–100 times higher than those from the aqueous system. Since deamidation is a hydrolytic reaction requiring water to form products (Wright, 1991), it is assumed that this reaction is more favored in the aqueous system than in the dry system. However, the Maillard reaction and Strecker degradation have a maximum reaction rate at the intermediate water level. Overall, reactions seemed to be controlled by the Maillard type of reactions rather than the deamidation reaction. If the reaction condition was undesirable for pyrazine formation, the ammonia that was liberated from deamidation may have participated in other reactions such as color formation or simply loss to air or water.

The pH of the reaction mixture can also affect both the deamidation reaction and pyrazine production. We observed that pyrazines will disappear if the pH of the solution changed from alkaline to neutral in the aqueous system. It is well-known that the base can enhance the yields of pyrazines by increasing the reactivity of the amino groups toward the carbonyl of glucose and increasing the rearrangement and fragmentation of sugars (Koehler and Odell, 1970). The deamidation reaction can be also catalyzed by either acid or base (Scotchler and Robinson, 1974; Patel and Borchardt, 1990).

We found that the yields of unsubstituted pyrazine and methylpyrazine were the highest two in the dry system (Table II). This suggested that the large amounts of unsubstituted pyrazine and methylpyrazine resulted from the relative abundance of two- and three-carbon fragments. These two- and three-carbon fragments, which were the principal ones involved in pyrazine formation, could be formed from retro-aldol condensation. Shibamoto and Bernhard (1977) indicated that the retro-aldol reaction was catalyzed by amine. Therefore, the ammonia from deamidation of glutamine may be not only a reactant but also a catalyst to accelerate the fragmentation of the sugar moiety during the reaction. Koehler et al. (1969) also indicated that ammonia tended to yield mostly pyrazine and only traces of alkylated pyrazines. From their results, it was further confirmed that ammonia assisted the fragmentation of glucose. The yield of methylpyrazine was greatest in the aqueous system; however, the relative distribution for unsubstituted pyrazine in the aqueous system was not as plentiful as in the dry system (Table II). This might be a consequence of lower amounts of the fragments that were obtained from Amadori rearrangements and retro-aldol reactions. The fragments that required dehydration of a sugar moiety for their formation might be more difficult to form in aqueous solutions.

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