Acrylamide Formation Mechanism in Heated Foods

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Recent findings of a potential human carcinogen, acrylamide, in foods have focused research on the possible mechanisms of formation. We present a mechanism for the formation of acrylamide from the reaction of the amino acid asparagine and a carbonyl-containing compound at typical cooking temperatures. The mechanism involves formation of a Schiff base followed by decarboxylation and elimination of either ammonia or a substituted imine under heat to yield acrylamide. Isotope substitution studies and mass spectrometric analysis of heated model systems confirm the presence of key reaction intermediates. Further confirmation of this mechanism is accomplished through selective removal of asparagine with asparaginase that results in a reduced level of acrylamide in a selected heated food.

INTRODUCTION

Great technical interest and rapid research efforts on acrylamide in foods followed an announcement in April 2002 by the Swedish National Food Authority and the University of Stockholm. These researchers found microgram per kilogram to milligram per kilogram levels of acrylamide in foods (*1*). A World Health Organization meeting on this topic provided methods of analysis, potential safety concerns, and mechanistic hypotheses (*2*). The importance of asparagine in the mechanistic formation of acrylamide in foods was reported at the 116th Association of Official Analytical Chemists International Annual Meeting and Exposition (*3*, *4*). A number of potential mechanisms for the formation of acrylamide were published recently. Mottram et al. (*5*) recognized the importance of asparagine as one of the reactants, and speculated that dicarbonyl compounds are necessary co-reactants in a Strecker degradation. Stadler et al. (*6*) reported that the *N*-glycosides (Schiff bases when viewed in their open chain tautomeric form) formed by reaction of reducing sugars with asparagine, when heated, resulted in significant levels of acrylamide, while the *N*-glycosides formed by the reaction of glutamine and methionine only formed minor amounts of acrylamide. Becalski et al. (*7*) recently published their work, also confirming the role of asparagine as a precursor to acrylamide. These previous works by Mottram et al. (*5*), Stadler et al. (*6*), and Becalski et al. (*7*) were performed on relatively dry samples, through pyrolysis, or under pressure in a buffered solution. To understand the interactions that occur in a food system, we developed a model fried potato snack

system to study the role of amino acids and reducing sugars in the formation of acrylamide. This model allowed a more thorough study of key precursors. A second model system allowed identification of intermediates and confirmation of a proposed mechanism for acrylamide formation.

MATERIALS AND METHODS

Chemicals. All solvents used were of High Purity Grade from Burdick and Jackson (Muskegon, MI) and all chemicals were of analytical grade obtained from Sigma-Aldrich (St. Louis, MO) unless specified. The following chemicals were obtained commercially (percentages refer to isotope enrichment): acrylamide-1-^{13}C (99%) from C/D/N Isotopes Inc. (Quebec, Canada); L-asparagine monohydrate (U-$^{13}C_4$, 98%+; U-$^{15}N_2$, 96−99%) from Spectra Stable Isotopes (Columbia, MD); L-asparagine monohydrate (amide-^{15}N, 98%+) from Cambridge Isotopes (Andover, MA); D-glucose-$^{13}C_6$ (99%) and L-asparagine-^{15}N monohydrate (amine-^{15}N, 99%) from Isotec Inc. (Miamisburg, OH); H-$β$-alanine-NH$_2$·HCl (3-aminopropionamide·HCl) from Advanced ChemTech (Louisville, KY); and Asparaginase (A2925) from Sigma-Aldrich. Russet Burbank baking potatoes were obtained from a local grocery store.

Model Fried Potato Snack System. A model food system was developed that resembled a potato chip and provided an inert, "nonacrylamide producing" matrix to study the formation of acrylamide. The optimal system was found to be a combination of Food Grade Potato Starch-ungelatinized (AVEBE, North America) and water (400 g each). Water was initially preheated to 65 °C, and any additives (D-glucose, maltodextrin, amino acids, etc.) were stirred until dissolved. The typical amount of D-glucose added was 5.6 g, while the typical amount of maltodextrin added was 18 g. Amino acid addition was varied between 0 and 10 g. Following additive dissolution, 2.4 g of liquid emulsifier (mono-diglycerides) was added. The last step was the addition of potato starch to the water and preparation of the dough. Once mixed,

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Table 1. Concentrations of Solutions for Aqueous Reaction System Experiments

	amino acid	concn, mM	sugar	concn, mM
1	asparagine	16.8	D-glucose	13.9
2	asparagine	5.6	D-glucose	23.2
3	U-$^{13}C_4$,U-$^{15}N_2$-asparagine	18.2	D-glucose	13.9
4	asparagine	16.8	2-deoxyglucose	15.6
5	asparagine	5.6	2-deoxyglucose	25.9
6	3-aminopropionamide	20.2	none	
7	3-aminopropionamide	13.4	D-glucose	9.3

Table 2. Acrylamide Formation from Amino Acids and D-Glucose in a Model Heated Food System Made from Potato Starch and Water[a]

amino acid	acrylamide (μg/kg)
none (contains only potato starch and water)	<50
asparagine	9270
asparagine[b] (without D-glucose)	117
glutamine	156
alanine, arginine, aspartic acid, cysteine, lysine, methionine, threonine, or valine	<50

[a] The model system contained the following: amino acid (10 g); D-glucose (5.6 g); maltodextrin (18 g); emulsifier (2.4 g); potato starch (400 g); and water (400 g). [b] D-Glucose was omitted from this sample.

the dough was formed by microwaving (Litton Minutemaster microwave oven model 426) the mixture for 1 min at 700 W, stirring for 10 s, and microwaving again for another minute. The resulting material was then milled (twin rolls) to form a sheet with a thickness of 0.50 mm. The sheet was cut into 60 mm × 40 mm ovals and fried in mid oleic sunflower oil at 205 ± 2 °C. The frying time was varied from 3 to 30 s depending on the desired final moisture content. The fried product was drained and allowed to come to room temperature in air. During frying, the oil was exposed to the atmosphere.

Method for Measuring Acrylamide (AA) in Samples and Food Products. Briefly, food products were spiked with 1-^{13}C-acrylamide (^{13}C-AA) and extracted with hot water. The aqueous supernatant was extracted three times with ethyl acetate, and the ethyl acetate extracts were combined and concentrated and analyzed by LC/MS with selected ion monitoring for specific detection of AA and ^{13}C-AA.

Extraction of Sample. Finely crushed sample (6.00 ± 0.01 g) was weighed into a 125-mL Erlenmeyer flask. To this flask was added 120 μL of ^{13}C-AA (100 ng/μL in deionized, distilled water) and 40 mL of deionized, distilled water. The flask was covered with foil, and placed into a 65 °C water bath for 30 min. Ethylene dichloride (10 mL) was added, and the flask contents were homogenized with a Tekmar Tissumizer (SDT-1810) or Ultra-Turrax (T18 Basic) for 30 s, or until uniform. A 25-g portion of the homogenate was placed into an 8-dram vial and centrifuged for 30 min at 2500−5200 rpm. An 8-g aliquot of the aqueous supernatant was transferred to another 8-dram vial (being careful to avoid solid particles) and extracted 3 times with 10, 5, and 5 mL of ethyl acetate, respectively. The extract was dried with addition of anhydrous sodium sulfate, and the solution was concentrated to a final volume of approximately 100−200 μL. The sample was then ready for LC/MS analysis.

Analysis by LC/MS. Samples were analyzed with a Waters 2690 LC (Milford, MA) interfaced to a Micromass Platform LCZ mass spectrometer (Milford, MA) operated in positive electrospray ionization mode. Mobile phase: 100% H_2O, 10 mM ammonium acetate, adjusted to pH 4.6 w/formic acid. Column: 2.0 mm × 150 mm, YMC C18 AQ, 5 μm (Waters; Milford, MA). Flow rate: 0.2 mL/min. LC/MS interface: Direct (no split). Injection volume: 5 μL. Mode: selected ion monitoring (m/z 72, 73). Dwell time: 0.5 s.

Data Analysis. Response ratios (area of AA peak/area of ^{13}C-AA peak) were plotted against the corresponding concentration ratios for a series of five standards in ethyl acetate. All standards contained 4.5 μg/mL of ^{13}C-AA, and AA concentrations ranging from 0 to 5 μg/mL. Linear regression resulted in a calibration curve from which concentration ratios in extracts were determined from measured response ratios.

Aqueous Reaction System for Identifying and Profiling Asparagine/Sugar Reaction Intermediates and Products. Aqueous solutions were prepared with the concentrations shown in **Table 1**. Reproducible heating and agitating of reaction mixtures were achieved by using a Gerstel MPS2, Multi-Purpose Sampler. Aliquots of 2 mL were placed in 20 mL headspace vials (vented with an ∼1/8 in. hole in the Teflon cap), and the vials were heated for 0, 180, 210, 240, and 270 s in a heating block. The heating block maintained a temperature of 190−200 °C while agitating the mixture for the designated heating time. After 180 s, solutions were light brown; after 210 s, solutions were medium brown; after 240 s, free water was gone and samples were moist and dark brown to black; after 270 s, residue was dark brown to black. Solution samples were transferred to LC auto-sampler vials. For analysis of residue samples, 1 mL of deionized water was pipetted onto

samples, samples were swirled, and the contents were transferred to LC auto-sampler vials. The 240- and 270-s samples contained some solid particles that did not go back into solution. Samples were analyzed with the same LC/MS conditions as described in Method for Measuring Acrylamide (AA) in Samples and Food Products, except that the full scan mode was used instead of selected ion monitoring.

Accurate Mass Measurements for Confirmation of LC/MS Peak Identifications. LC/high-resolution MS was carried out on a Hewlett-Packard 1090 LC (Agilent: Foster City, CA) directly coupled to a Finnigan MAT95 Q (ThermoFinnigan: San Jose, CA) operated in positive atmospheric pressure chemical ionization (APCI) mode. APCI was chosen for this work because that mode performed better in the 100% aqueous mobile phase than did electrospray ionization (ESI) on the magnetic sector instrument.

LC Conditions. Column: Phenomenex 150 × 4.6 mm, 4 μ, Synergi Hydro-RP 80+ (Torrance, CA). Mobile phase: 100% H_2O. Flow rate: total flow into MS is 1 mL/min. For accurate mass work, a solution of polypropylene glycol (PPG 425-10 ppm) was added (by tee) to the total flow at a rate of 10 μL/min. The flow from the LC was adjusted to 990 μL/min, resulting in a total of 1 mL/min.

MS Conditions. The resolution was adjusted to ∼2500. Ion source lenses were adjusted for maximum intensity and resolution by using a PPG mass that was near the mass to be measured. The tube lens setting and vaporizer temperature were key variables for this work, and were optimized for a balance of intensities in the PPG spectrum. Ion source temperature: 38 °C. Vaporizer temperature: 400 °C. Capillary temperature: 240 °C. Scan speed: 3 s/decade.

Preparation of Microwaved Potato Product. Unpeeled Russet Burbank baking potatoes were boiled for 1 h, peeled, and then mashed with a fork to break apart the flesh. To 15 g of the mashed potatoes was added 45 g of water and the mixture was mixed until no lumps were present. Asparaginase (50 units) was added to this slurry, which was then shaken every 5 min for a total of 30 min incubation at room temperature. After the 30-min incubation, the product was microwaved (Panasonic microwave, model NN-S5488A) on high for 2-min increments for a total of 10 min until dry (and brown). An identical control sample was prepared the same way except the asparaginase addition step was replaced with only distilled/deionized water.

RESULTS AND DISCUSSION

Model Fried Potato Snack System: Role of Asparagine. Model food system requirements needed for researching acrylamide formation were 2-fold: the finished product needed to be an edible food and the system needed to comprise the simplest starting raw materials that do not produce acrylamide when heated. Potato starch is a particularly good system due to very low amino acid and sugar contents. As shown in **Table 2**, our model system provided an inert matrix to study acrylamide formation as evidenced by the absence of detectable levels of acrylamide when no other ingredients are added. Asparagine and glutamine were the only amino acids tested that formed acrylamide, and the higher yield of acrylamide from asparagine demonstrated that it is the predominant amino acid responsible
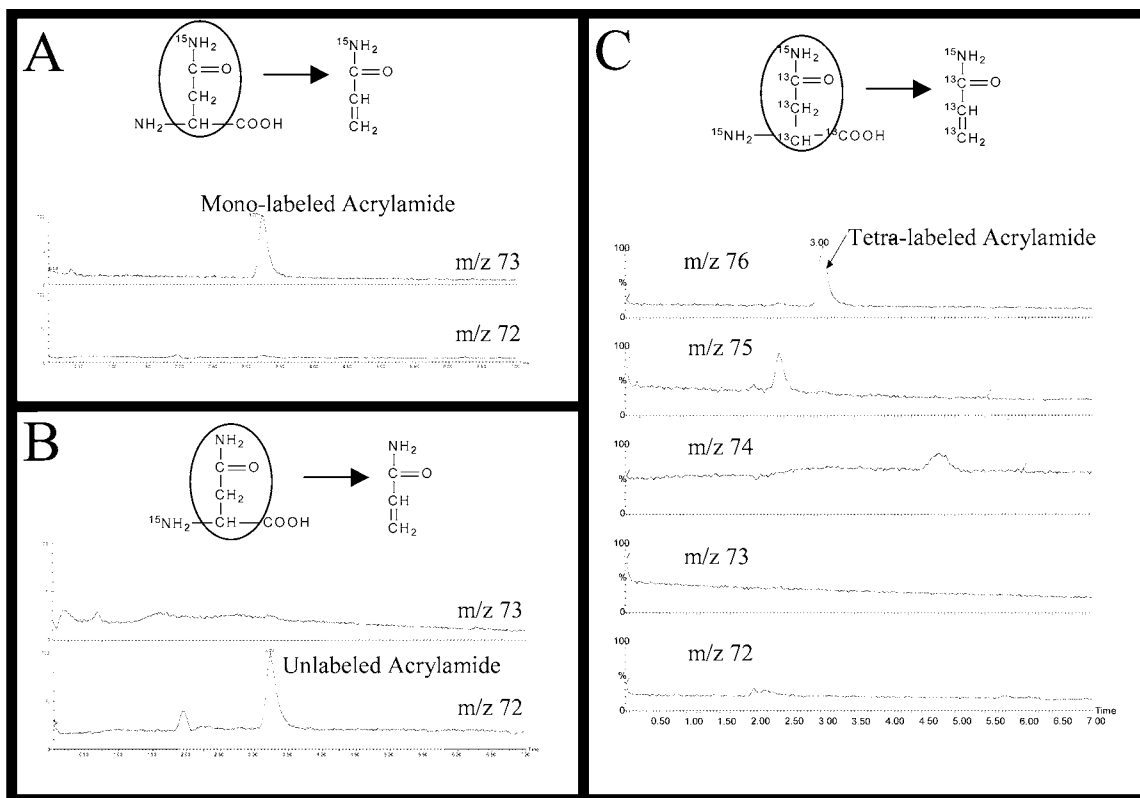
Figure 1. Experiments with isotope-substituted asparagine to determine the source of acrylamide nitrogen and carbon atoms. The ion chromatograms are plotted so that ions in each experiment are viewed with the same sensitivity.

for forming acrylamide. In addition, D-glucose was needed for the efficient formation of acrylamide as shown in the example of asparagine heated without D-glucose.

To elucidate the role of asparagine in generating acrylamide in our model fried potato snack system, we executed a series of stable isotope substitution experiments shown in **Figure 1**. For experiments A and B in which isotope substitutions of the amide and amino nitrogens were separately examined, m/z 72 and 73 were monitored. For experiment C, in which all carbons and nitrogens of asparagine were isotope substituted, m/z 74, 75, and 76 were also monitored to detect all possible combinations of isotope substitutions in the resulting acrylamide. In experiment A, amide ^{15}N-substituted asparagine was heated with D-glucose in the model fried potato snack system. If the amide group is the source of acrylamide nitrogen, ^{15}N-substituted acrylamide should result at m/z 73. Integration of the acrylamide peaks of experiment A revealed that >97% of the acrylamide formed is at m/z 73, positively indicating that the asparagine amide nitrogen was the source of acrylamide nitrogen. In experiment B, α-amino ^{15}N-substituted asparagine was heated with D-glucose. Since the amide group is the source of acrylamide nitrogen, only unsubstituted acrylamide at m/z 72 should result. The only measurable peak in experiment B was the unsubstituted acrylamide peak at m/z 72, indicating that <1% of the acrylamide nitrogen originated from the amino nitrogen of asparagine. In experiment C, uniformly isotope-substituted asparagine (all carbons and nitrogens were isotope substituted) was heated with D-glucose. If the encircled region is the source of acrylamide carbons and nitrogens, then acrylamide with four isotope substitutions at m/z 76 should result. The chromatograms for experiment C reveal that the only measurable peak was the m/z 76 peak, corresponding to incorporation of four isotope substitutions—showing that all three acrylamide carbon atoms, as well as the one nitrogen atom, came from asparagine. An

Table 3. Formation of Acrylamide from Asparagine and Various Carbonyl Compounds in a Model Heated Food System[a]

carbonyl source	acrylamide (μg/kg)
D-glucose	1454[b]
2-deoxyglucose	1036[b]
ribose	2425
glyceraldehyde	2669
glyoxal	3936

[a] The model system contained the following: asparagine (10 g); emulsifier (2.4 g); potato starch (400 g); water (400 g); and carbonyl source (D-glucose, 0.49 g; 2-deoxyglucose, 0.45 g; ribose, 0.37 g; glyceraldehyde, 0.225 g; glyoxal, 0.175 g).
[b] Average of results.

additional experiment with $^{13}C_6$-D-glucose and unsubstituted asparagine in the model snack resulted in only unsubstituted acrylamide. These data conclusively demonstrate that acrylamide is formed from the amide side chain of asparagine.

The Mechanism of Acrylamide Formation. During the typical heating of foods, reducing sugars react with amino acids initiating a cascade of events leading to the browning of foods known as the Maillard reaction. This process is known to generate more reactive monocarbonyl and dicarbonyl compounds that are proposed to be responsible for the browning reaction (*8*). To better understand the mechanism of acrylamide formation from asparagine, we investigated the ability of other carbonyl-containing compounds to generate acrylamide in the potato snack model system (**Table 3**). We found that a variety of carbonyl sources could generate acrylamide from asparagine under heat. As the sugar chain gets shorter, the molecule is strained to form a cyclic hemiacetal structure and subsequently the carbonyl becomes more readily available for nucleophilic attack from the α-amine of asparagine (*9, 10*). Thus, the shorter chain sugars become more reactive, which is shown in **Table 3**. (Approximately equimolar quantities of the carbonyl sources

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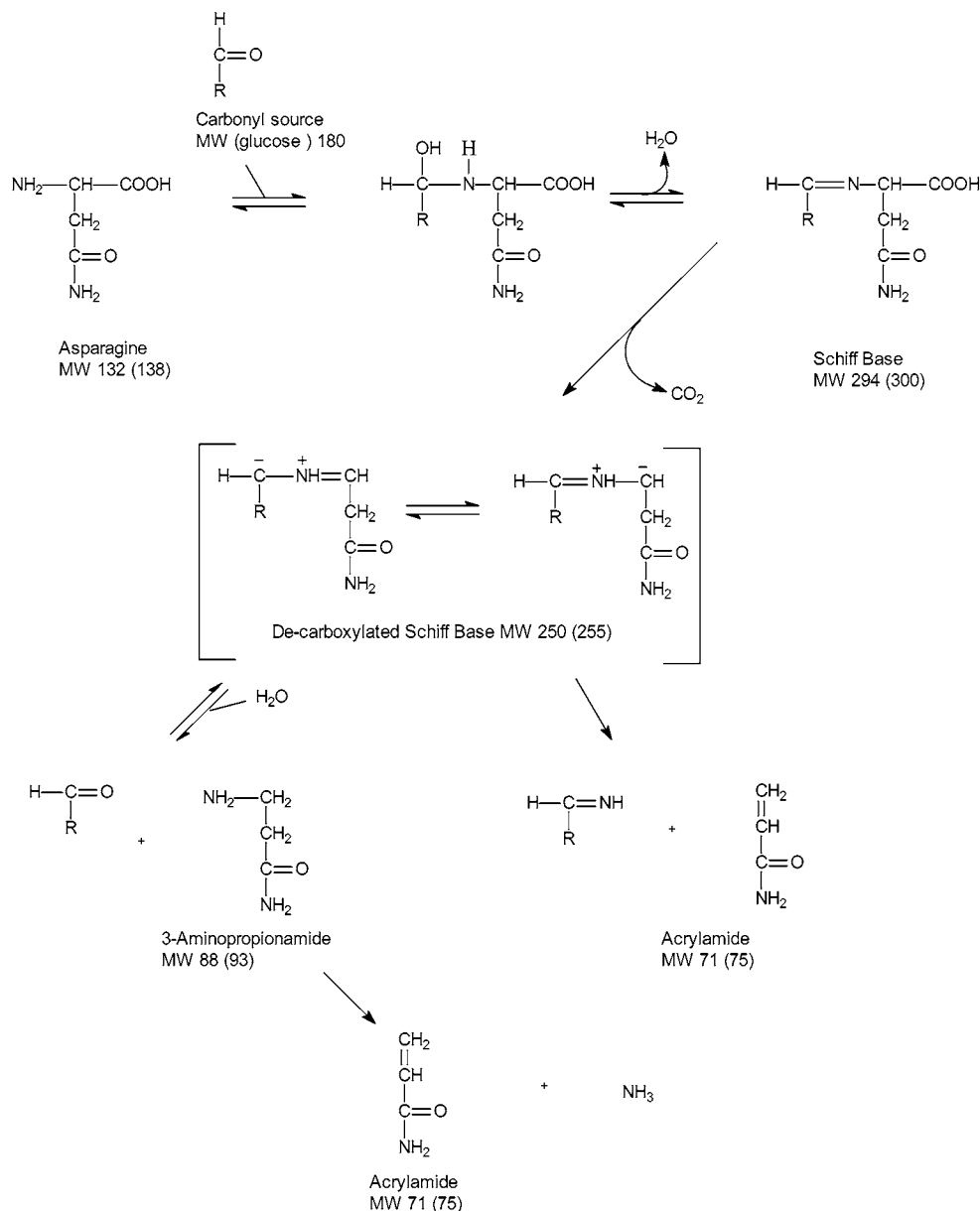
Figure 2. Mechanism of acrylamide formation in heated foods. Numbers in parentheses are the molecular weights observed when uniformly isotope-substituted asparagine was used.

were used, so that acrylamide levels reflect relative reactivity.) The first step in acrylamide production is the Schiff base formation between the carbonyl and the α-amino group of asparagine. In one of our experiments, we used 2-deoxyglucose as the carbonyl source and showed that it too formed comparable levels of acrylamide. Since 2-deoxyglucose does not have a hydroxyl group adjacent to the carbonyl, it can only form the Schiff base adduct and cannot undergo the Amadori rearrangement, which leads to the formation of dicarbonyl compounds, e.g. 3-deoxyglucosone. In addition, Becalski et al. (7) reported that octanal was able to react with asparagine and form acrylamide. Our studies, using decanal, produced similar results (unpublished data). Combined, these observations suggest the necessity of carbonyls in the formation of acrylamide, but that dicarbonyls are not essential for the formation of acrylamide from asparagine.

On the basis of the isotope-substitution results and carbonyl studies described above, we propose the mechanism displayed in **Figure 2**. The α-amino group of free asparagine reacts with a carbonyl source, forming a Schiff base. Under heat, the Schiff base decarboxylates (facilitated by delocalization of negative charge, which Schiff base formation allows), forming a product that can react one of two ways. It can hydrolyze to form 3-aminopropionamide that can further degrade via the elimination of ammonia to form acrylamide when heated. Alternatively, the decarboxylated Schiff base can decompose directly to form acrylamide via elimination of an imine. Additional evidence to support this mechanism was developed by using an aqueous reaction system comprising D-glucose and asparagine as described in the Materials and Methods section. Asparagine and D-glucose were allowed to react in approximately equimolar amounts (row 1 of **Table 1**). **Figure 3A** displays the resulting chromatograms that monitor the disappearance of asparagine and D-glucose, and the formation of Schiff base, 3-aminopropionamide, and acrylamide. Before heating ($t = 0$ s), only asparagine and D-glucose are present. At $t = 180$ s, the Schiff base appears. At $t = 270$ s, D-glucose and Schiff base are depleted, and 3-aminopropionamide and acrylamide appear.

Several techniques were used to identify the intermediates and products indicated in **Figure 3A**. The molecular formulas
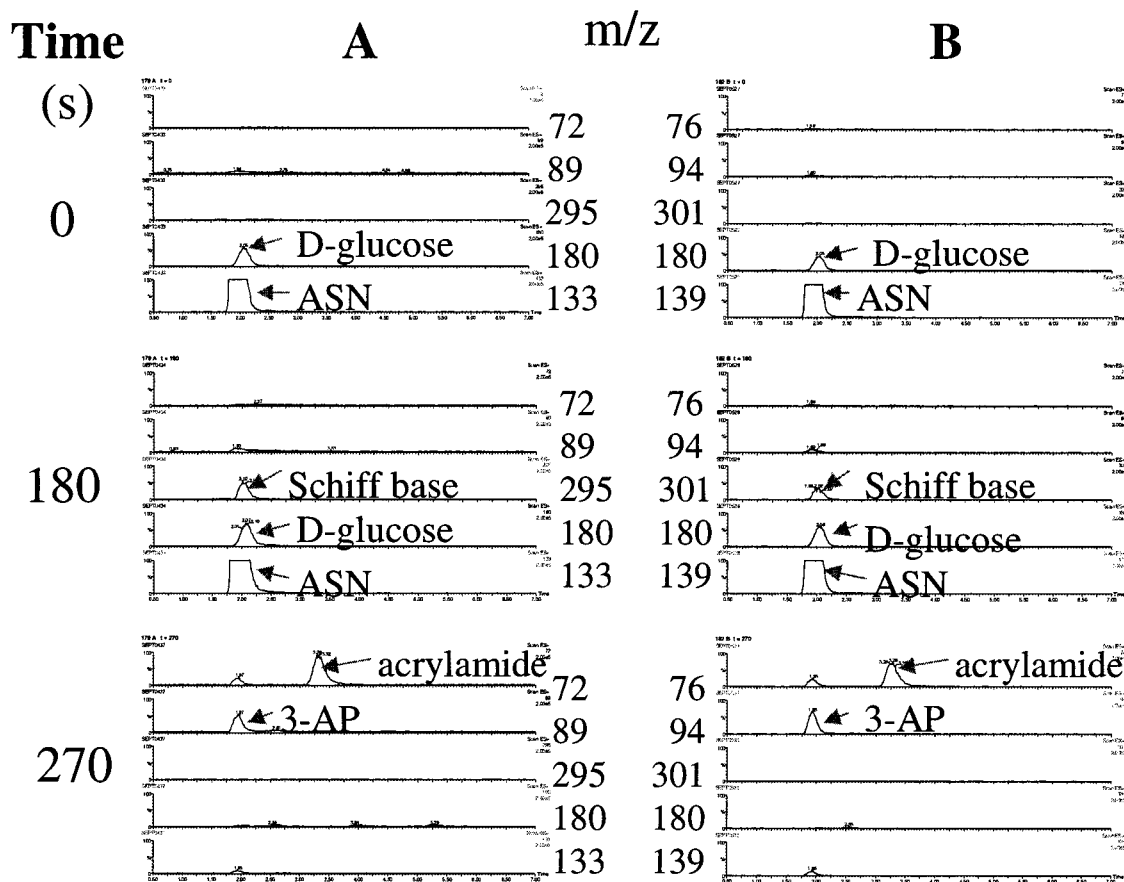
ASN = asparagine and 3-AP = 3-aminopropionamide

Figure 3. Aqueous reaction system. LC/MS profiles for three reaction time samples: A reactions: Unsubstituted asparagine plus D-glucose. B reactions: $^{13}C_4,^{15}N_2$-substituted asparagine plus D-glucose. All components except D-glucose are detected at m/z = MW + 1. For D-glucose, MW + 18(NH_4^+) − 18(H_2O) at m/z 180 is used, because it was the most intense D-glucose ion observed. The ion chromatograms are plotted so that ions in each experiment are viewed with the same sensitivity.

Table 4. High-Resolution MS Investigation of Intermediates in the Formation of Acrylamide[a]

analyte	solution	heat time (s)	measd mass	calcd mass	Δ (ppm)
ASN	ASN/D-glucose	0	133.0613	133.0613	0
D-glucose	ASN/D-glucose	0	180.0877	180.0872	+2.8
Schiff base	ASN/D-glucose	180	295.1116	295.1141	−8.5
acrylamide	ASN/D-glucose	270	72.0457	72.04495	+10
3-AP	3-AP	270	89.0717	89.07153	+2.0
acrylamide	3-AP	270	72.0447	72.04495	−3.5
decarboxylated Schiff base	3-AP/D-glucose	270	251.1240	251.1243	−1.2

[a] ASN = asparagine; 3-AP = 3-aminopropionamide.

of the asparagine, D-glucose, Schiff base, and acrylamide peaks were confirmed by high-resolution mass spectrometry (**Table 4**). Data from the high-resolution system, when plotted as in **Figure 3**, yielded similar profiles, except that 3-aminopropionamide was obscured due to asparagine fragmentation (loss of CO_2 that does not occur in ESI). In the absence of accurate mass data on this component, the 3-aminopropionamide peak was confirmed by injecting a standard and comparing its retention time and +ESI mass spectrum with that of the indicated peak in **Figure 3A**, t = 270 s. Standards were used by Stadler et al. to confirm the Schiff base intermediate in this mechanism (6). Finally, all of these identifications were confirmed in **Figure 3B**: the same experiment as in **Figure 3A**, but with uniformly isotope-substituted asparagine (row 3 of **Table 1**). The ions displayed are for the same compounds

as in the unsubstituted experiment, but adjusted in mass to account for incorporation of the number of isotope substitutions expected from the mechanism in **Figure 2**. For example, 3-aminopropionamide retains five of the six isotope substitutions of the precursor asparagine, and m/z 94 of **Figure 3B** behaves in near-identical fashion to m/z 89 in **Figure 3A**. The appropriate mass shift occurred for each component, confirming the identifications of intermediates and products generated in the mechanism. A remaining issue was whether acrylamide forms in heating 3-aminopropionamide. In our model fried potato snack system, we determined that 3-aminopropionamide heated without D-glucose formed five times the level of acrylamide as compared to an asparagine/D-glucose reaction mixture. Also, acrylamide formation from 3-aminopropionamide was observed in the aqueous reaction system (row 6 of **Table 1**).

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Table 5. Asparaginase-Facilitated Reduction of Acrylamide

potato product	control acrylamide (μg/kg)	asparaginase-treated acrylamide (μg/kg)	% reduction[a]
microwaved potato snack	20500	164	>99

[a] Calculated as (control − asparaginase treated)/control × 100.

The one missing intermediate from **Figure 2** is the decarboxylated Schiff base. Monitoring of *m/z* 251 (in chromatograms similar to **Figure 3**) did not result in the detection of any significant component. Two experiments were carried out to further investigate this intermediate. First, by starting with decarboxylated asparagine (3-aminopropionamide) and D-glucose (row 7, **Table 1**), enough of the **Figure 2** decarboxylated Schiff base was produced to define its retention time, verify its molecular formula (see **Table 4**), and establish its +ESI spectrum. Second, asparagine was again reacted with D-glucose, this time with D-glucose in 4-fold excess (row 2, **Table 1**). In excess D-glucose, decarboxylated Schiff base at *m/z* 251 was readily detected at the correct retention time. In the equimolar D-glucose/asparagine system, the decarboxylated Schiff base is removed as rapidly as it is formed. In excess D-glucose, the 3-aminopropionamide/decarboxylated Schiff base equilibrium is shifted in favor of the decarboxylated Schiff base, allowing its detection. Furthermore, no 3-aminopropionamide was observed in this excess D-glucose experiment, because it was converted in the reaction with D-glucose to form the decarboxylated Schiff base and subsequently eliminated the imine to form acrylamide. These data confirm the mechanism of **Figure 3**, and indicate that decarboxylation is a limiting reaction in the D-glucose/asparagine aqueous reaction system. Similar experiments (rows 4 and 5, **Table 1**) were carried out with 2-deoxyglucose in place of D-glucose as a carbonyl source. The analogous Schiff base was observed (at *m/z* 279), as well as 3-aminopropionamide and acrylamide, with the same kinetic behavior as observed in **Figure 3**.

Verification of Asparagine as the Source of Acrylamide in a Food. Our results indicate that the side chain amide group of asparagine is incorporated into the amide bond of acrylamide. A logical confirmation of this mechanism would be to degrade this amide bond through hydrolysis with either acid or enzyme and measure acrylamide in a food. A way to accomplish this specifically for asparagine is to use the enzyme asparaginase, which catalyzes the hydrolysis of asparagine into aspartic acid and ammonia. We evaluated the effectiveness of asparaginase in reducing acrylamide formation in a microwaved mashed potato product. Asparaginase pretreatment of the snack produced an asparagine reduction of 88% and an acrylamide reduction of greater than 99% when compared to samples prepared by the exact same process but without the enzyme (**Table 5**). This result again shows that the major route to acrylamide formation, in a potato product, is through asparagine.

Thus, we have detailed proof that the major mechanistic pathway in the formation of acrylamide in foods involves asparagine and a reactive carbonyl, proceeding through intermediates that include a Schiff base, decarboxylated Schiff base, and 3-aminopropionamide.

ABBREVIATIONS USED

AA, acrylamide; ASN, asparagine; 3-AP, 3-aminopropionamide; LC/MS, liquid chromatography/mass spectrometry; APCI, atmospheric pressure chemical ionization; PPG, polypropylene glycol; MS, mass spectrometry

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