JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Why Asparagine Needs Carbohydrates To Generate Acrylamide

VAROUJAN A. YAYLAYAN,* ANDRZEJ WNOROWSKI, AND CAROLINA PEREZ LOCAS

Department of Food Science and Agricultural Chemistry, McGill University, 21,111 Lakeshore, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

Structural considerations dictate that asparagine alone may be converted thermally into acrylamide through decarboxylation and deamination reactions. However, the main product of the thermal decomposition of asparagine was maleimide, mainly due to the fast intramolecular cyclization reaction that prevents the formation of acrylamide. On the other hand, asparagine, in the presence of reducing sugars, was able to generate acrylamide in addition to maleimide. Model reactions were performed using FTIR analysis, and labeling studies were carried out using pyrolysis-GC/MS as an integrated reaction, separation, and identification system to investigate the role of reducing sugars. The data have indicated that a decarboxylated Amadori product of asparagine with reducing sugars is the key precursor of acrylamide. Furthermore, the decarboxylated Amadori product can be formed under mild conditions through the intramolecular cyclization of the initial Schiff base and formation of oxazolidin-5-one. The low-energy decarboxylation of this intermediate makes it possible to bypass the cyclization reaction, which is in competition with thermally induced decarboxylation, and hence promote the formation of acrylamide in carbohydrate/asparagine mixtures. Although the decarboxylated Amadori compound can be formed under mild conditions, it requires elevated temperatures to cleave the carbon−nitrogen covalent bond and produce acrylamide.

KEYWORDS: Asparagine; acrylamide; mechanism; decarboxylated Amadori product; FTIR; Py-GC/MS; ^{13}C-labeled glucose

INTRODUCTION

Recent findings correlating acrylamide concentrations in animal subjects to the intake of fried feed (*1*) have led to the investigation of its presence in food (*2*). These studies have indicated that acrylamide formation in laboratory-heated foods is temperature dependent and that only moderate levels were detected in protein-rich foods, whereas carbohydrate-rich foods exhibited much higher levels of acrylamide. Recently, two studies (*3*, *4*) confirmed asparagine to be the amino acid precursor of acrylamide. Furthermore, Stadler et al. (*3*) have provided evidence that the sugar asparagine adduct, *N*-glycosylasparagine, is a direct precursor of acrylamide, indicating the involvement of the Maillard reaction. To control the formation of this potential carcinogen in food, detailed knowledge of its mechanism of formation is of critical importance. In this paper, using pyrolysis−gas chromatography/mass spectrometry (Py-GC/MS) (*5*) and Fourier transform infrared (FTIR) analysis, we provide evidence that the ability of the open-chain form of *N*-glycosylasparagine (the Schiff base) to undergo intramolecular cyclization and formation of oxazolidin-5-one is the key step that allows decarboxylation of asparagine and subsequent formation of acrylamide. Py-GC/MS is also a cost-effective and convenient method to perform experiments with labeled reactants (*5*). Although under pyrolytic conditions a higher number of products are formed compared with aqueous reactions, most of the products identified in aqueous systems are also formed under pyrolytic conditions, albeit in different amounts. In addition, experimental evidence was provided that the position and label distribution in the common products observed in the same model systems, between aqueous and pyrolytic reactions, are identical (*6*). This indicates the similarity of mechanisms of formation of the common products under both conditions. Consequently, mechanistic conclusions derived from label incorporation in the products observed under pyrolytic conditions, which are common to both systems, have relevance to the aqueous reactions. Using variously ^{13}C-labeled glucoses we have also provided evidence for the origin of niacinamide—an indicator for the formation of decarboxylated Amadori compound in the glucose/asparagine model system.

MATERIALS AND METHODS

All reagents and chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. The labeled sugars [1-^{13}C]glucose (99%), [3-^{13}C]glucose (99%), [4-^{13}C]glucose (98%), and [6-^{13}C]glucose (98%) were purchased from Cambridge Isotope Laboratories (Andover, MA).

Py-GC/MS Analysis. A Varian CP-3800 gas chromatograph coupled to a Saturn 2000 ion trap detector interfaced to a CDS Pyroprobe 2000 unit, through a valved interface (CDS 1500), was used for Py-GC/MS

* Corresponding author [telephone (514) 398-7918; fax (514) 398-7977; e-mail varoujan.yaylayan@mcgill.ca].

10.1021/jf0261506 CCC: $25.00
Published on Web 02/11/2003

Table 1. Formation Efficiency of Acrylamide Expressed as Gas Chromatographic Peak Area per Mole of Starting Asparagine Generated at 350 °C from Different Model Systems

model system	efficiency (area/mol)
asparagine alone	0
asparagine + sorbitol	0
asparagine + 2,3-pentanedione	trace
asparagine + glucose	4.9×10^{11}
asparagine + glucose[a]	1.4×10^{11}
asparagine + glycolaldehyde[a]	2.8×10^{11}
asparagine + fructose	6.6×10^{11}
asparagine + glyceraldehyde	8.6×10^{11}
asparagine + sucrose	18×10^{11}

[a] At 250 °C.

analysis. In all experiments, asparagine model mixtures (2.0 mg, equimolar with sugars) were introduced inside the quartz tube (0.3 mm thickness), plugged with quartz wool, and inserted into the coil probe. The pyroprobe was set at the desired temperature at a heating rate of 50 °C ms^{-1} with a total heating time of 20 s. The pyroprobe interface temperature was set at 250 °C. The initial temperature of the column was set at −5 °C for 12 min and then increased to 50 °C at a rate of 50 °C min^{-1}; immediately the temperature was further increased to 250 °C at a rate of 8 °C min^{-1} and kept at 250 °C for 5 min. A constant flow of 1.5 mL min^{-1} was used during analysis. Capillary direct MS interface temperature was 250 °C; ion source temperature was 180 °C. The ionization voltage was 70 eV, and the electron multiplier was 2047 V. The mass range analyzed was 29−300 amu. The column was a fused silica DB-5 MS column (50 m length × 0.2 mm i.d. × 0.33 μm film thickness; J&W Scientific). The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1 software. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

FTIR Analysis. Saturated solutions were prepared by mixing equimolar amounts of asparagine and glyceraldehyde in methanol. Samples were analyzed after evaporation of methanol and formation of a thin film on the surface of the cell window. Infrared spectra were recorded on a MIDAC Prospect Fourier transform infrared spectrometer purged with dry air and equipped with a deuterated triglycine sulfate (DTGS) detector. The spectra were acquired on a CaF_2 cell without spacer at room temperature unless otherwise specified. A total of 128 scans at 4 cm^{-1} resolution were co-added. Processing of the FTIR data were performed using Galactic GRAMS 32/AI.

FTIR Temperature Studies. Samples were placed in a CaF_2 cell without spacer. The temperature of the sample was regulated by placing the IR cell in a temperature-controlled cell holder. Infrared spectra were recorded as described above.

RESULTS AND DISCUSSION

Asparagine model systems containing either glucose, fructose, sucrose, sorbitol, glyceraldehyde, glycolaldehyde, or 2,3-pentanedione were reacted using Py-GC/MS as detailed under Materials and Methods. All model systems generated acrylamide at 250 °C; however, the intensity of the acrylamide increased at higher temperatures. Interestingly, at 250 °C, the asparagine/glycolaldehyde model system was more efficient than the asparagine/glucose system in generating acrylamide; however, at 350 °C asparagine/sucrose was more efficient than the asparagine/glucose model system. The least efficient system in generating acrylamide was that of asparagine/2,3-pentanedione, which generated acrylamide in trace amounts at both temperatures (see **Table 1**).

A cursory look at the structures of naturally occurring amino acids in food would immediately pinpoint asparagine as a possible source of acrylamide, because decarboxylation and

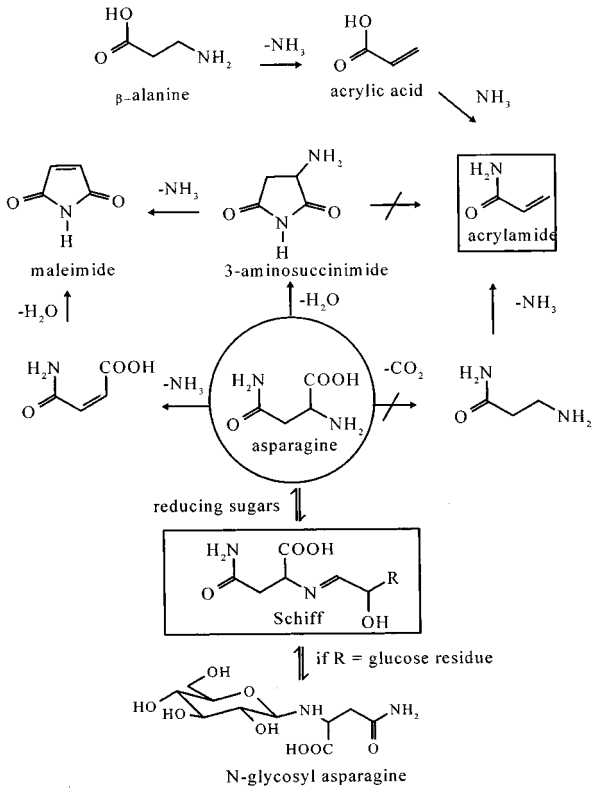

Figure 1. Thermal decomposition products of asparagine and its reaction with reducing sugars based on products identified by Py-GC/MS.

deamination of this amino acid, in principle, can produce acrylamide and because such reactions are common thermally allowed reactions in food. However, the main degradation product of asparagine, identified during Py-GC/MS analysis, was maleimide (**Figure 1** and **Table 2**). Evidently, intramolecular cyclization to form an imide is much faster compared with the decarboxylation reaction due to entropy factor (*7*). In theory, this reaction should reduce the toxicity of asparagine in food by preventing its conversion into acrylamide. The intermediate 3-aminosuccinimide (**Figure 1**) was detected in the pyrogram of asparagine model systems along with maleimide. On the other hand, a fast deamination reaction can also prevent intact asparagine from its conversion into acrylamide, because decarboxylation of this intermediate is not favored due to the destabilization of the resulting negative charge at the proximity of the π electrons of the double bond. When β-alanine was pyrolyzed under the same conditions as asparagine, only two products were detected: acrylic acid and the product of its subsequent reaction with ammonia, acrylamide, indicating the propensity of such moieties to deaminate rather than to decarboxylate.

The role of reducing sugars in enabling the conversion of asparagine into acrylamide should be considered, therefore, in the light of the above findings. The first intermediate formed when reducing sugars react with asparagine is the so-called Schiff base (in equilibrium with *N*-glycosylasparagine). This intermediate stabilizes itself through an Amadori rearrangement process (pathway A in **Figure 2**), which leaves the carboxylic acid free to undergo intramolecular cyclization at elevated temperatures, similar to the free asparagine, and form an Amadori product with N-substituted succinimide. The main residue generated from the amino acid moiety identified in the pyrolysates of asparagine model systems was succinimide (**Figure 3b** and **Table 2**). This process, similar to that occurring

Table 2. Mass Spectrometric Data (10 Highest Peaks) of Compounds Identified in the Model Systems

compound	*m/z* (relative intensity)
maleimide	98 (4.6), 97 (100), 70 (4.3), 69 (31.2), 54 (48.1), 53 (18.0), 52 (5.2), 43 (9.4), 42 (5.0), 40 (4.3)
maleimide[a]	98 (5.1), 97 (100), 70 (4.3), 69 (39.3), 54 (58.1), 53 (29.5), 52 (2.9), 43 (16.5), 42 (9.4), 40 (6.1)
succinimide	100 (4.7), 99 (100), 70 (9.0), 57 (2.0), 56 (11.0), 55 (10.1), 54 (4.0), 44 (3.9), 43 (9.8), 42 (10.3)
succinimide[a]	100 (4.8), 99 (100), 70 (5.0), 57 (2.2), 56 (8.0), 55 (7.3), 54 (2.1), 44 (3.8), 43 (5.8), 42 (7.3)
3-aminosuccinimide	114 (9.2), 86 (37.8), 70 (7.8), 59 (5.2), 57 (3.2), 56 (1.1), 55 (6.3), 44 (6.2), 43 (100), 42 (66.8)
3-aminosuccinimide[a]	114 (7.4), 86 (32.0), 70 (6.2), 59 (4.1), 57 (1.1), 56 (1.5), 55 (2.3), 44 (5.8), 43 (100), 42 (77.1)
niacinamide	122 (100), 106 (54.9), 94 (6.3), 79 (8.9), 78 (78.4), 77 (8.0), 52 (14.4), 51 (33.8), 50 (17.8), 44 (11.6)
niacinamide[a]	122 (100), 106 (57.6), 94 (4.3), 79 (7.9), 78 (75.6), 77 (7.2), 52 (17.4), 51 (45.4), 50 (19.9), 44 (12.3)

[a] NIST library.

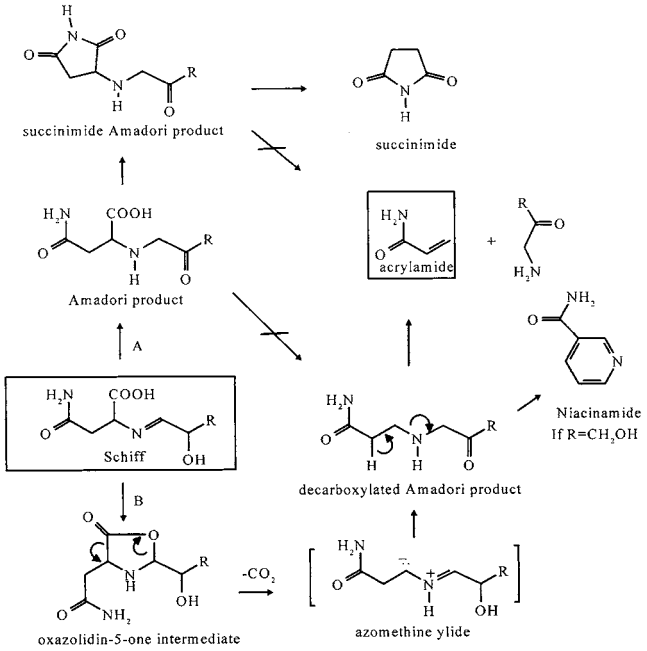

Figure 2. Mechanism of acrylamide formation from a decarboxylated Amadori product of asparagine. R = H, $[CH(OH)]_nCH_2(OH)$; n = 0–3.

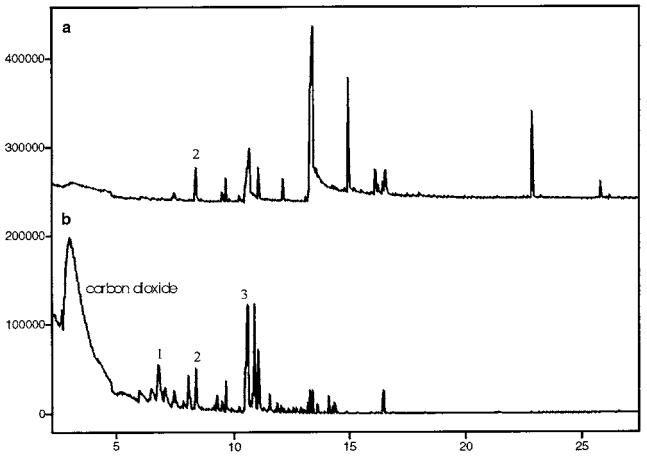

Figure 3. Pyrograms of (a) sorbitol/asparagine and (b) glucose/asparagine model systems. Peaks: 1, acrylamide; 2, maleimide; 3 succinimide.

in asparagine alone (**Figure 1**), prevents the formation of acrylamide. A competing mechanism, therefore, should exist that allows the Amadori product to decarboxylate before it can be trapped as the inactive succinimide moiety by intramolecular cyclization.

In addition to undergoing Amadori rearrangement, the Schiff form of *N*-glycosylasparagine can also stabilize itself through intramolecular cyclization (pathway B in **Figure 2**) initiated by

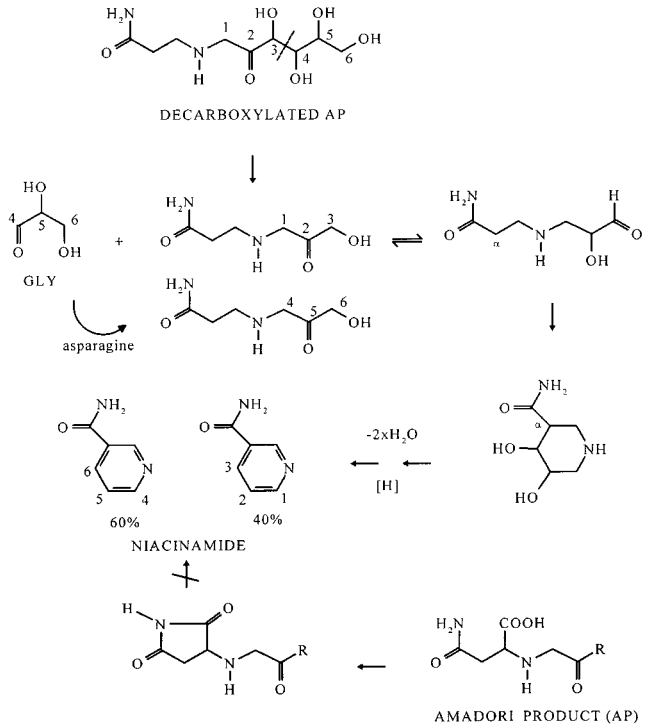

Figure 4. Mechanism of niacinamide formation in asparagine/glucose model system and percent label incorporation. Carbon numbers represent original glucose carbon atom locations. AP, Amadori product; Gly, glyceraldehyde.

the carboxylate anion (*8*, *9*) and formation of oxazolidin-5-one (*8*). Manini et al. (*8*) have observed the formation of such an intermediate and its facile decarboxylation at room temperature in a D-glucose/L-DOPA model system. This facile decarboxylation of the oxazolidin-5-one intermediate at room temperature affords a stable azomethine ylide (*8*), which after tautomerization and protonation can generate decarboxylated Amadori product (see **Figure 2**). The existence of such a pathway will allow some of the Amadori rearrangement product to decarboxylate, thus preventing the formation of succinimide moiety, and eventually allow the formation of acrylamide. Although the decarboxylated intermediate can be formed under mild conditions, it requires elevated temperatures to cleave the strong carbon−nitrogen covalent bond and produce acrylamide as shown in **Figure 2**.

Py-GC/MS Experiments with ^{13}C-Labeled Glucose. Evidence in favor of the formation of the decarboxylated Amadori intermediate comes from identification of niacinamide (see **Table 2** and **Figures 2** and **4**) in the reaction mixture of the asparagine/glucose model system. Niacinamide can be formed only from the decarboxylated Amadori product of asparagine with glyceraldehyde through cyclization and dehydration reac-

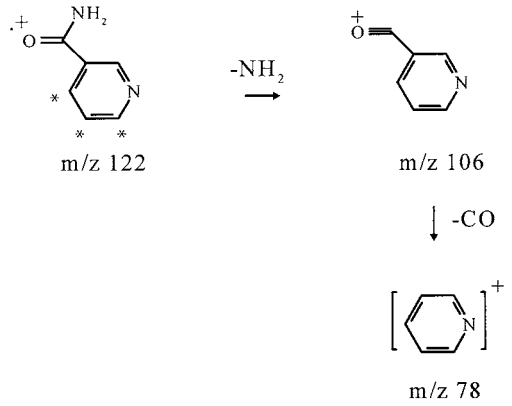

Figure 5. Mass spectral fragmentation pattern of niacinamide. * represents glucose carbon atoms.

Table 3. Percent Label Distribution in Selected Mass Spectral Fragments[a] of Niacinamide Formed in Different Model Systems

model system	*m/z* 122 M	*m/z* 123 M + 1	*m/z* 106 M	*m/z* 107 M + 1	*m/z* 78 M	*m/z* 79 M + 1
D-glu/L-asn	100	0	100	0	100	0
D-[1-^{13}C]glu/L-asn	60	40	60	40	60	40
D-[3-^{13}C]glu/L-asn	60	40	60	40	60	40
D-[4-^{13}C]glu/L-asn	40	60	40	60	40	60
D-[6-^{13}C]glu/L-asn	40	60	40	60	40	60

[a] See **Figure 5** for the structures of ions at *m/z* 122, 106, and 78; glu, glucose; asn, asparagine.

tions (see **Figure 4**). The Amadori product, on the other hand, will quickly undergo intramolecular cyclization and form a succinimide intermediate, which is unable to form niacinamide due to its altered structure, as shown in **Figure 4**. Furthermore, Maillard model systems containing glucose are known to produce free glyceraldehyde and glyceraldehyde Amadori product (*10*) through retro-aldol reactions (**Figure 4**). In fact, when glyceraldehyde was pyrolyzed in the presence of asparagine, both acrylamide and niacinamide were detected in higher amounts relative to the glucose model system (see **Table 1** for acrylamide values). In addition, as illustrated in **Figure 4**, when the glucose Amadori product undergoes retro-aldol cleavage, it produces a glyceraldehyde Amadori product that retains C-1, C-2, and C-3 atoms of glucose and a free glyceraldehyde molecule with the C-4, C-5, and C-6 remaining atoms of glucose. Theoretically, the niacinamide molecule should be formed containing either C-1−C-2−C-3 or C-4−C-5−C-6 unscrambled fragments as part of the aromatic ring carbon chain as shown in **Figure 4**. The percent incorporation of these two fragments into the niacinamide structure will depend on the concentration of these two fragments in the reaction mixture. Furthermore, **Figure 5** indicates that these sugar carbon atoms should be retained in the ions *m/z* 122 (molecular ion), *m/z* 106, and *m/z* 78 during the electron impact induced fragmentation of niacinamide (see **Table 2**). To confirm the above prediction, asparagine was reacted with variously labeled glucoses (see **Table 3**) and the percent label incorporation in niacinamide was calculated and is shown in **Table 3**. According to this table 40% of niacinamide was formed with the C-1−C-2−C-3 fragment and 60% with the C-4−C-5−C-6 fragment, confirming the proposed mechanism of niacinamide formation.

Monitoring the Decarboxylation by FTIR Spectroscopy and Py-GC/MS. To confirm that the ease of decarboxylation of the asparagine moiety was due to the formation of a Schiff base adduct, asparagine was reacted with glucose and sorbitol; the latter is unable to form a Schiff base with asparagine, and the resulting pyrograms were compared. **Figure 3** clearly indicates the presence of a significant peak due to CO_2 release in the case of the glucose model system and its complete absence in the case of the sorbitol model system.

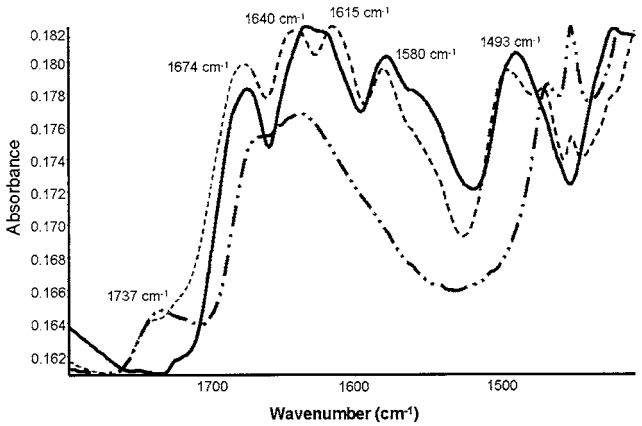

Figure 6. Overlaid infrared spectra (1400–1800 cm^{-1}) of (a) asparagine (solid line), (b) asparagine/glyceraldehyde after 15 min of incubation at room temperature (dotted line), and (c) asparagine/glyceraldehyde after 6 days of incubation at 40 °C (dash-dot line).

Additional evidence for the facile formation of a decarboxylated asparagine Amadori product was obtained from infrared spectral analysis of a mixture of asparagine and glyceraldehyde in methanol. These studies have indicated that within 15 min of incubation at room temperature, the Amadori product has already started to form (pathway A, **Figure 1**) as indicated by the appearance of the absorption band at 1737 cm^{-1}, which was assigned to the carbonyl band (*11*, *12*) of the Amadori product (see **Figure 6b**). This assignment was based on the fact that the Amadori product is a derivative of dihydroxyacetone (*11*). Further incubation of the sample at 40 °C for 6 days caused the disappearance of both symmetrical and antisymmetrical stretching frequencies of the carboxylate bands at 1493 and 1580 cm^{-1} (see **Figure 6c**), indicating decarboxylation reaction and formation of a decarboxylated Amadori product (pathway B, **Figure 2**). The spectral properties of this mixture did not change upon further incubation at 40 °C; however, when the temperature of the mixture was raised to 180 °C and the spectrum was acquired, the data showed the disappearance of the amide I (1674 cm^{-1}) and amide II (1615 cm^{-1}) absorption bands of the amino acid, indicating cleavage of the asparagine moiety as shown in **Figure 2**.

Furthermore, the cyclic form of the Schiff base, *N*-glycosylasparagine, has already been confirmed (*3*) to be an intermediate in the asparagine/glucose reaction, and the acrylamide yield from the pyrolysis of synthetic *N*-glycosylasparagine was significant and comparable to the yield from the asparagine/glucose model system under the same conditions (*3*), providing further evidence for the intermediacy of a decarboxylated Amadori product as the precursor of acrylamide.

Similar to asparagine, a high-temperature-induced cyclization reaction to form succinimide Amadori product (see **Figure 2**) is highly favored over the competing thermal decarboxylation reaction required for the formation of acrylamide. However, the low-energy pathway (pathway B, **Figure 2**) provided for the decarboxylation of the *N*-glycosylasparagine through the formation of oxazolidin-5-one makes it possible to bypass the cyclization reaction and promote the formation of acrylamide in carbohydrate/asparagine mixtures.

LITERATURE CITED

(1) Tareke, E.; Rydberg, P Karlsson, P.; Eriksson, S.; Törnqvist, M. Acrylamide: A cooking carcinogen? *Chem. Res. Toxicol.* **2000**, *13*, 517−522.

(2) Tareke, E.; Rydberg, P.; Karlsson, P.; Eriksson, S.; Törnqvist, M. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J. Agric. Food Chem*. **2002**, *50*, 4998−5006.

(3) Stadler, R. H.; Blank, I.; Varga, N.; Robert, F.; Hau, J.; Guy, P.; Robert, M.-C.; Riediker, S. Acrylamide from Maillard reaction products. *Nature* **2002**, *419*, 449−450.

(4) Motram, D. S.; Wedzicha, B. L.; Dodson, A. T. Acrylamide is formed in the Maillard reaction. *Nature* **2002**, *419*, 448−449.

(5) Yaylayan, V. A. Analysis of complex reaction mixtures: Novel applications of Py-GC/MS and microwave assisted synthesis (MAS). *Am. Lab*. **1999**, *31*, 30−31.

(6) Yaylayan, V. A.; Wronowski, A. The influence of pyrolytic and aqueous phase reactions on the mechanism of formation of Maillard products. *J. Agric. Food Chem.* **2000**, *48*, 3549−3554.

(7) Sykes, P. *A Guidebook to Mechanism in Organic Chemistry*, 4th ed.; Longman Group: London, U.K., 1975; pp 33−36.

(8) Manini, P.; d'Ischia, M.; Prota, G. An unusual decarboxylative Maillard reaction between L-DOPA and D-glucose under biomimetic conditions: Factors governing competition with Pictet−Spengler condensation. *J. Org. Chem*. **2001**, *66*, 5048−5053.

(9) Yaylayan, V.; Wnorowski, A. The role of *β*-hydroxyamino acids in the Maillard reaction−transamination route to Amadori products. In *Maillard Reaction in Medicine and Food Chemistry and Medical Science*: *Update for the Postgenomic Eva*; Horiuchi, S., Taniguchi, N., Hayase, F., Kurata, T., Osawa, T., Eds; International Congress Series 1245; Elsevier Science: Amsterdam, The Netherlands, 2002, 195−200.

(10) Keyhani, A.; Yaylayan, V. Elucidation of the mechanism of pyrazinone formation in glycine model systems using labeled sugars and amino acids. *J. Agric. Food Chem.* **1996**, *44*, 2511−2516.

(11) Yaylayan, V.; Harty-Majors, S.; Ismail, A. Investigation of DL-glyceraldehyde−dihydroxyacetone interconversion by FTIR spectroscopy. *Carbohydr. Res.* **1999**, *318*, 34−39.

(12) Yaylayan, V.; Harty-Majors, S.; Ismail, A. Monitoring carbonylamine reaction and enolization of 1-hydroxy-2-propanone by FTIR spectroscopy. *J. Agric. Food Chem.* **1999**, *47*, 2335−2340.

Received for review November 21, 2002. Revised manuscript received January 7, 2003. Accepted January 13, 2003. We acknowledge funding for this research by the Natural Sciences and Engineering Research Council of Canada (NSERC).

JF0261506