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The Role of Creatine in the Generation of N-Methylacrylamide: A New Toxicant in Cooked Meat

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Investigations of different sources of acrylamide formation in model systems consisting of amino acids and sugars have indicated the presence of two pathways of acrylamide generation; the main pathway specifically involves asparagine to directly produce acrylamide after a sugar-assisted decarboxylation step, and the second, nonspecific pathway involves the initial formation of acrylic acid from different sources and its subsequent interaction with ammonia and/or amines to produce acrylamide or its N-alkylated derivatives. Aspartic acid, β-alanine, and carnosine were found to follow the acrylic acid pathway. Labeling studies using [^{13}C-4]aspartic acid have confirmed the occurrence in this amino acid of a previously proposed sugar-assisted decarboxylation mechanism identified in the asparagine/glucose model system. In addition, creatine was found to be a good source of methylamine in model systems and was responsible for the formation of N-methylacrylamide through the acrylic acid pathway. Labeling studies using creatine (methyl-d_3) and $^{15}NH_4Cl$ have indicated that both the nitrogen and the methyl groups of methylamine had originated from creatine. Furthermore, analysis of cooked meat samples has also confirmed the formation of N-methylacrylamide during cooking.

KEYWORDS: Asparagine; aspartic acid; creatine; carnosine; acrylamide; N-methylacrylamide; N,N-dimethylacrylamide; beef; mechanism of methylamine and N-methylacrylamide formation; Py-GC/MS analysis

INTRODUCTION

Asparagine has emerged as the major amino acid responsible for the formation of acrylamide (*1*) in food products heated at high temperatures in the presence of reducing sugars or carbonyl compounds (*2*). Studies on the detailed mechanism (*3*) of this transformation in model systems have indicated that the decarboxylated Amadori product of asparagine is the key precursor of acrylamide (**Figure 1**). Furthermore, the decarboxylated Amadori product was shown to be formed under relatively mild conditions through the intramolecular cyclization of the initial Schiff base (**Figure 1**, pathway C) and formation of the oxazolidinone intermediate (*4*). The low energy sugar-assisted decarboxylation of this intermediate makes it possible to bypass the thermally induced cyclization reaction (**Figure 1**, pathway B), which is in competition with the high energy decarboxylation of intact Amadori product (**Figure 1**, pathway A), and hence promote the formation of acrylamide in carbohydrate/asparagine mixtures. Similar conclusions were drawn by other researchers (*5*, *6*) using food systems and liquid chromatography/tandem mass spectrometry (LC/MS/MS) analyses. As part of our investigation of other sources of acrylamide in food and using pyrolysis (Py)-gas chromatography/mass spectrometry (GC/MS) as an integrated reaction, separation, and identification system (*7*, *8*), we have studied, in addition to selected α-amino acids, β-alanine and the dipeptide carnosine (N-β-alanyl-L-histidine) as potential sources of acrylamide. **Table 1** summarizes the efficiency of each amino acid alone and in the presence of glucose, to produce acrylamide and acrylic acid under pyrolytic conditions, expressed as chromatographic peak areas per mole of amino acid. According to this table, carnosine had a similar acrylamide-generating efficiency to asparagine in model systems containing reducing sugars. In consideration of the fact that the carnosine content (*9*) of beef (21 μmol/g of fresh beef) is also comparable, if not more, than that of asparagine (*10*) in potatoes (15 μmol/g of fresh potato), it is reasonable to assume that acrylamide should be formed in cooked meat as abundantly as in potatoes. However, many studies have detected relatively low levels of acrylamide in meat products (~20 ppb) relative to cooked potatoes (up to 4000 ppb), and this was attributed to the ability of certain amino acids, such as cysteine and lysine, to undergo Michael type addition reactions to the double bond of acrylamide and form noncarcinogenic adducts. However, such interactions have not been

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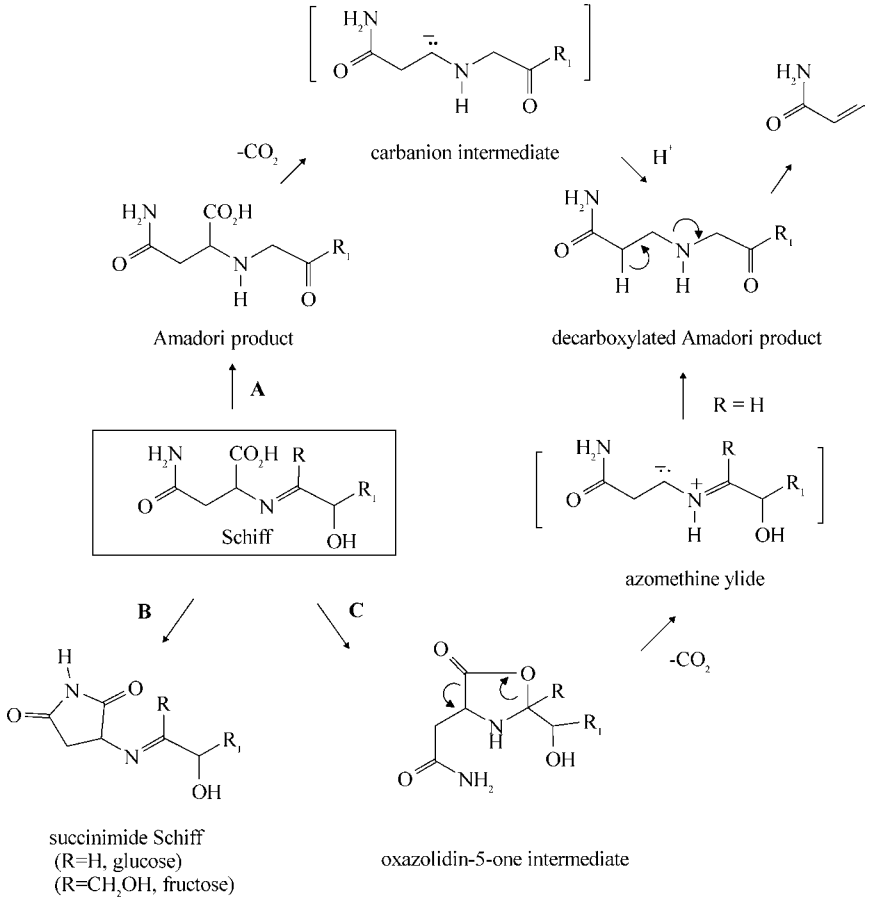

Figure 1. Mechanism of formation of acrylamide through sugar-assisted decarboxylation (pathway C) and thermally induced decarboxylation of intact Amadori product (pathway A).

Table 1. Efficiency (Area/mol of Amino Acid) of Acrylic Acid and Acrylamide Generation from 1 mg Samples of Either Amino Acid or Amino Acid/Glucose (3:1) Mixtures Pyrolyzed at 350 °C[a]

model system	acrylic acid × 10^{12}	acrylamide × 10^{12}
asparagine	not detected	trace
asparagine/glucose	1.20 ± 0.18	5.18 ± 0.57
β-alanine	98.30 ± 0.35	14.4 ± 0.5
β-alanine/glucose	108.00 ± 7.10	13.9 ± 0.7
carnosine	27.5 ± 6.9	12.86 ± 4.15
carnosine/glucose	18.46 ± 1.08	5.11 ± 0.55
aspartic acid	2.0 ± 0.08	0.18 ± 0.08
aspartic acid/glucose	22.53 ± 2.95	0.53 ± 0.04
cysteine	1.7 ± 0.1	trace
cysteine/glucose	1.5 ± 0.1	trace
serine	0.38 ± 0.01	not detected
serine/glucose	0.68 ± 0.01	not detected

[a] Average of duplicate analyses using method 1.

studied at high temperature conditions of cooking and the fate of acrylamide formed in meat products is not fully understood.

MATERIALS AND METHODS

All reagents and chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification. The labeled [^{13}C-4]aspartic acid was purchased from Cambridge Isotope Laboratories (Andover, MA), and creatine (methyl-d_3) was purchased from CDN Isotopes (Point Clair, Quebec, Canada).

Py-GC/MS Analysis. A Helwett-Packard GC with a mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit was used for the Py-GC/MS analysis. One milligram samples of pure reactants or 6.8 mg of beef extract was introduced inside a quartz tube (0.3 mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at 350 °C with a total heating time of 20 s. The column was a fused silica DB-5 column (50 m length × 0.2 mm i.d. × 0.33 μm film thickness; J&W Scientific). The pyroprobe interface temperature was set at 250 °C. The capillary direct MS interface temperature was 280 °C; the ion source temperature was 180 °C. The ionization voltage was 70 eV, and the electron multiplier was 2471 V. All injections were in the splitless mode. Three methods of analysis were optimized for specific model systems listed in **Figure 3**. Method 1 had a delayed pulse of 65 psi followed by a constant flow of 0.775 mL/min and a septum purge of 2 mL/min. Two modes of analysis were used, the scan and selected ion monitoring (SIM) modes. In the scan mode, the mass range analyzed was 33−650 amu, whereas in the SIM mode, only ions of masses 99, 98, 87, 85, 84, 72, 71, 61, 58, 55, 45, and 44 amu were monitored. The initial temperature of the column was set at 40 °C for 2 min and was increased to 100 °C at a rate of 30 °C/min; immediately, the temperature was further increased to 250 °C at a rate of 8 °C/min and kept at 250 °C for 5 min. Method 2 had a delayed pulse of 65 psi followed by a constant flow of 1 mL/min and a septum purge of 2 mL/min. The mass range analyzed was 33−650 amu. The initial temperature of the column was set at −5 °C for 2 min and was increased to 50 °C at a rate of 30 °C/min; immediately, the temperature was further increased to 250 °C at a rate of 8 °C/min and kept at 250 °C for 5 min. Method 3 had a delayed pulse of 65 psi

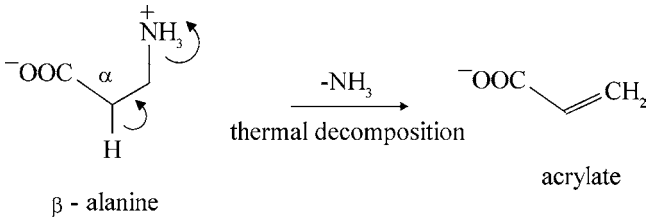

Figure 2. Proposed mechanism of deamination of β-alanine.

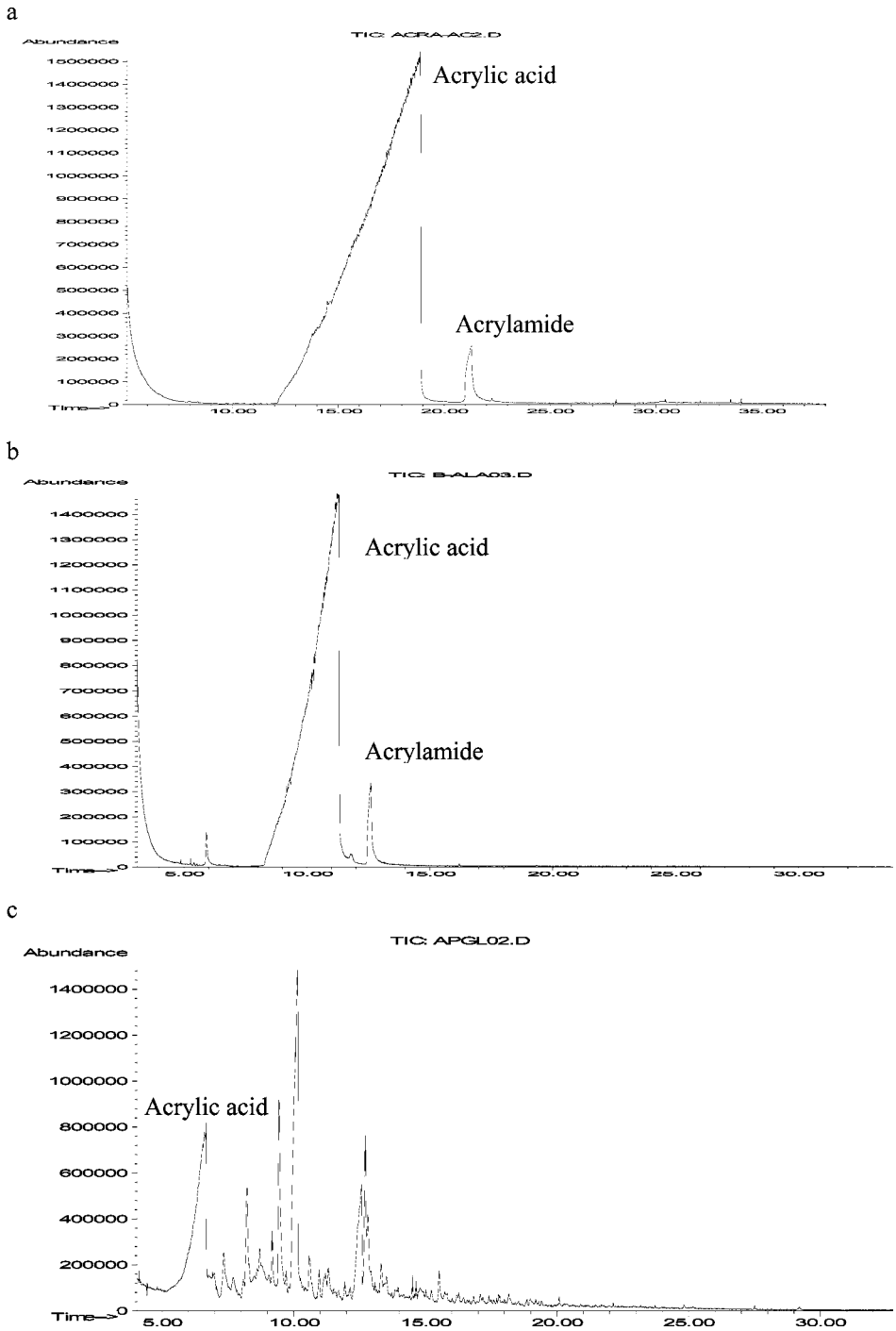

Figure 3. Pyrograms of model systems consisting of equimolar mixtures of (**a**) acrylic acid/ammonium carbonate (using method 3), (**b**) β-alanine alone (using method 2), and (**c**) aspartic acid/glucose (using method 1).

followed by a constant flow of 1 mL/min and a septum purge of 2 mL/min. The mass range analyzed was 33−650 amu. The initial temperature of the column was set at −5 °C for 2 min and was increased to 50 °C at a rate of 8 °C/min; immediately, the temperature was further increased to 100 °C at a rate of 3 °C/min followed by a increase to 250 °C at a rate of 20 °C/min and kept at 250 °C for 5 min (**Figure 3** shows examples of the three methods). The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1. The reported percent label incorporation values (corrected for natural abundance and for % enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

Extraction of N-Methylacrylamide from Beef. Beef cubes (obtained from a local store) were roasted in a domestic oven at 250 °C for 30 min. A portion (28 g) of the roasted meat was homogenized and extracted by stirring for 2 h with 40% methanol/water (100 mL). After filtration, the filtrate was evaporated for 48 h under the fume hood and analyzed (after mixing 3.8 mg of the dry extract with 3 mg of neutral alumina) with and without the addition of labeled creatine (methyl-d_3) by Py-GC/MS using method 1 in the SIM mode.

RESULTS AND DISCUSSION

Studies on model systems containing selected amino acids and glucose (see **Table 1**) have indicated that there are in general two pathways of acrylamide generation from amino acids. The main pathway specifically involves asparagine (**Figure 1**, pathway C) to directly produce acrylamide after a sugar-assisted decarboxylation step (*3*). The second, nonspecific pathway involves the initial formation of acrylic acid from different

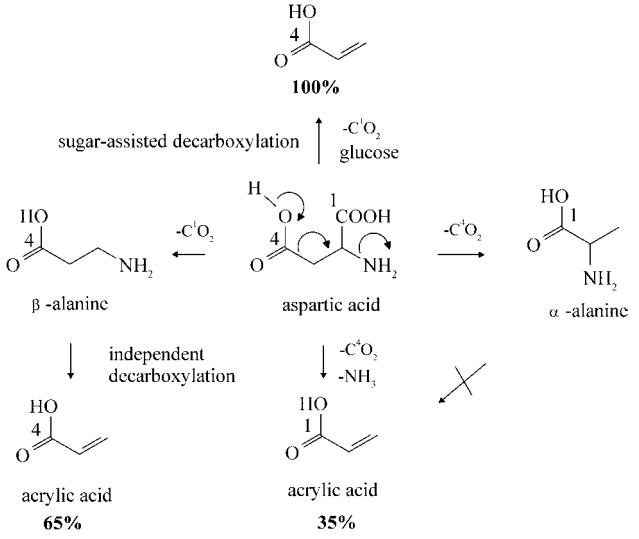

Figure 4. Decarboxylation pathways of aspartic acid.

Table 2. Percent Label Distribution in Acrylic Acid Generated from Different Model Systems

model system	M (*m/z* 72)	M + 1 (*m/z* 73)
aspartic acid	100	0
[^{13}C-4]aspartic acid	35	65
[^{13}C-4]aspartic acid/glucose	0	100

sources and its subsequent interaction with ammonia to produce acrylamide. Aspartic acid, β-alanine, and carnosine follow the acrylic acid pathway.

Formation of Acrylamide from *β*-Alanine. The mechanism of decomposition of β-alanine generates both reactants required for the formation of acrylamide; ammonia and acrylic acid are shown in **Figure 2**. The pyrolysis of β-alanine alone generated mainly acrylic acid and acrylamide, indicating deamination as a major pathway of thermal decomposition of β-alanine. The resulting acid can then interact with the available ammonia to form acrylamide (**Figure 3b**). When β-alanine was pyrolyzed in the presence of excess $^{15}NH_4Cl$, the resulting acrylamide incorporated both the labeled (added) and the unlabeled (generated from β-alanine) ammonia. Similarly, pyrolysis of commercial acrylic acid in the presence of an ammonia source [NH_4Cl, $(NH_4)_2CO_3$, etc.] also generated acrylamide (**Figure 3a**). A comparison of **Figure 3a**,**b** indicates the efficiency of conversion of β-alanine into acrylic acid and ammonia. No significant change in the efficiency of β-alanine conversion into acrylamide was observed in the presence of glucose (see **Table 1**).

Formation of Acrylamide from Aspartic Acid. Aspartic acid on the other hand can also form acrylic acid and subsequently acrylamide (**Figure 3c**), but unlike β-alanine and similar to asparagine, aspartic acid produces more acrylamide in the presence of glucose (**Table 1**). To identify the mechanism of acrylic acid formation from aspartic acid, [^{13}C-4]aspartic acid

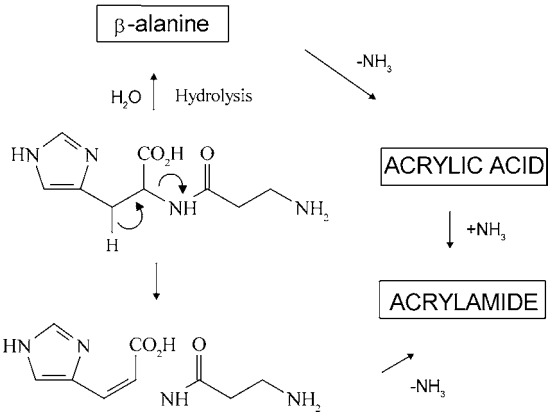

Figure 5. Different pathways of acrylamide formation from carnosine.

was pyrolyzed alone and in the presence of glucose. According to **Figure 4**, aspartic acid can undergo decarboxylation of either C-1 or C-4 carboxylate moieties. C-1 decarboxylation can generate β-alanine, and C-4 decarboxylation can generate α-alanine as shown in **Figure 4**. Unlike α-alanine, β-alanine is known to produce acrylic acid; consequently, it was expected to observe 100% label retention in the acrylic acid mass spectrum when [^{13}C-4]aspartic acid was pyrolyzed alone. However, analysis of the data showed the formation of 65% of labeled acrylamide and 35% unlabeled product (**Table 2**) indicating the existence of a third pathway capable of formation of acrylic acid with C-4 decarboxylation. A concerted mechanism where decarboxylation occurs simultaneously with deamination can explain the formation of unlabeled acrylic acid as shown in **Figure 4**. Interestingly, when [^{13}C-4]aspartic acid was pyrolyzed in the presence of glucose, only 100% labeled acrylic acid was observed (see **Table 2**), indicating the preferential decarboxylation of the C-1 carboxylate moiety consistent with the mechanism of sugar-assisted decarboxylation shown in **Figure 1**. This observation along with an increased ability to generate acrylamide in the presence of glucose (see **Table 1**) provide conclusive evidence for the ability of the Schiff base to provide a low energy pathway for decarboxylation of amino acids relative to decarboxylation from an intact Amadori product that passes through a carbanion intermediate rather than the more stable azomethine ylide as shown in **Figure 1**. Furthermore, similar to asparagine, reaction with sugar and formation of the oxazolidinone intermediate can prevent cyclization to form maleic anhydride (equivalent to succinimide in the case of asparagine) and enhance acrylic acid generation as shown in **Table 1**.

Formation of Acrylamide from Carnosine. The dipeptide carnosine (N-β-alanyl-L-histidine) when pyrolyzed alone produced acrylic acid and acrylamide in amounts higher than the asparagine/glucose model system. However, in the presence of glucose, the amounts became comparable (see **Table 1**) due to the interaction of carnosine with reducing sugars (*11*). **Figure 5** depicts two possible pathways of formation of acrylamide from carnosine, one through hydrolysis of the peptide bond and

Table 3. Efficiency (Peak Area/mol of Carnosine) of Acrylic Acid and Acrylamide Generation from 1 mg Samples of Carnosine Alone and in the Presence of Creatine or Lysine Pyrolyzed at 350 °C[a]

model system	acrylic acid $\times 10^{12}$	acrylamide (AA) $\times 10^{12}$	N-methyl-AA $\times 10^{12}$	N,N-dimethyl-AA $\times 10^{5}$
carnosine alone	13.8 ± 4.6	4.31 ± 1.3	not detected	not detected
carnosine + creatine	5.50 ± 0.6	6.42 ± 0.10	7.05 ± 0.10	6.6 ± 0.10
carnosine + lysine	16.9 ± 5.5	6.16 ± 2.2	not detected	not detected

[a] Average of duplicate analyses using method 2.

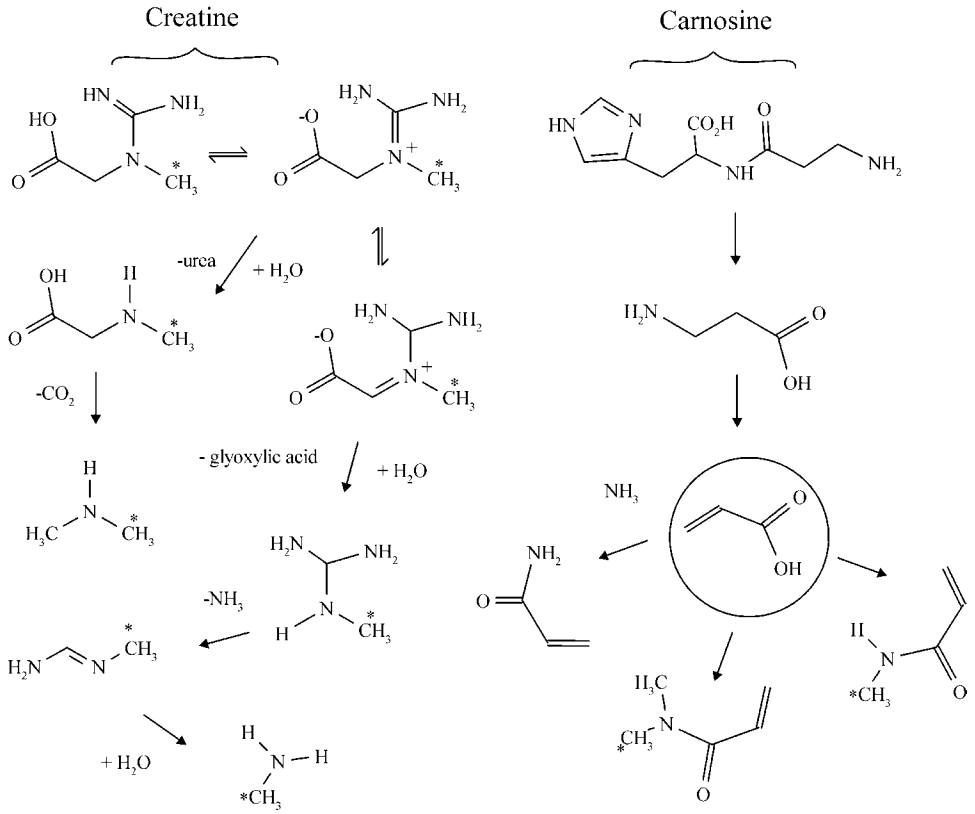

Figure 6. Proposed mechanism of generation of methyl and dimethylamines from creatine and formation of N-methylated acrylamides.

release of β-alanine and its subsequent deamination and the second through elimination of β-alanine amide and its deamination. The conspicuous absence of acrylamide in meat products (*10*) at the scale expected to that of potatoes has led us to investigate its possible fate in meat products using carnosine-containing model systems. Carnosine was reacted in the presence of lysine (a reactive amino acid) and creatine (a major constituent of meat), and their effects on the amounts of acrylamide and its precursor acrylic acid were calculated as shown in **Table 3**. Lysine did not exert any significant effect on the formation efficiencies of acrylamide and acrylic acid. Creatine, on the other hand, not only significantly reduced the acrylic acid content but also gave rise to two new acrylamide derivatives: N-methylacrylamide and N,N-dimethylacrylamide. Both acrylamide derivatives appear to exhibit similar toxicological profiles to acrylamide, a known animal carcinogen and neurotoxin (*12*, *13*). The decrease in acrylic acid formation can be explained by its accelerated conversion into acrylamide derivatives due to the efficient generation of methylamines from added creatine (**Figure 6**).

Mechanism of Formation of N-Methylated Acrylamides in the Carnosine/Creatine Model System. To identify the detailed mechanism of formation of N-methylated derivatives of acrylamide in the carnosine/creatine model system, carnosine was reacted with labeled creatine (methyl-d_3) under the same conditions in the presence and absence of excess labeled $^{15}NH_4Cl$ and label incorporation patterns were calculated for N-methylacrylamide (see **Table 4**). According to these data, N-methylacrylamide incorporated 100% the N-methyl group of creatine and N,N-dimethyl acrylamide incorporated 100% one labeled N-methyl group and one unlabeled methyl group; both acrylamide derivatives did not incorporate any nitrogen atoms from the free ammonium chloride, indicating formation of methyl and dimethylamines directly from creatine. **Figure 7a**,**b** shows the mass spectra of N-methylacrylamide generated from

Table 4. Percent Label Distribution in N-Substituted Acrylic Acids Generated from Isotopically Enriched Model Systems

model system	compd	M	M + 1	M + 3	M + 4
carnosine/creatine (methyl-d_3)	MA[a]	0	0	100	0
carnosine/creatine (methyl-d_3)	DMA[b]	0	0	100	0
carnosine/creatine/$^{15}NH_4Cl$	MA	0	0	100	0
carnosine/creatine/$^{15}NH_4Cl$	DMA	0	0	100	0

[a] MA = N-methylacrylamide. [b] DMA = N,N-dimethylacrylamide.

labeled and unlabeled model systems. The label incorporation patterns can be explained by proposing a reaction between acrylic acid (generated from carnosine) and methylamine or dimethylamine, both generated from creatine (see **Figure 6**). The consumption of creatine by human subjects also significantly increased their urine levels of methylamine, indicating the existence of a metabolic equivalent of this transformation in human biochemistry (*14*). The proposed pathway of generation of methyl and dimethylamines from creatine is shown in **Figure 6**. According to this figure, creatine in its imminium zwitterionic form can undergo hydrolysis to release urea and N-methylglycine, which after decarboxylation can generate dimethylamine. Alternatively, the zwitterionic form can undergo isomerization followed by hydrolysis to generate glyoxylic acid and N-methyl-triaminomethane. This unstable intermediate can undergo deamination followed by hydrolysis to produce methylamine as shown in **Figure 6**. Alternatively, it can undergo hydrolysis to produce N-methylformamide, which after oxidation and decarboxylation steps can also generate methylamine. This proposed pathway was verified by reacting creatine with acrylic acid and acrylamide separately. The reaction of acrylic acid with creatine generated acrylamide, N-methylacrylamide (major product), and N,N-dimethylacrylamide, whereas the acrylamide reaction with creatine generated none of the methylated products, confirming the proposed pathway.

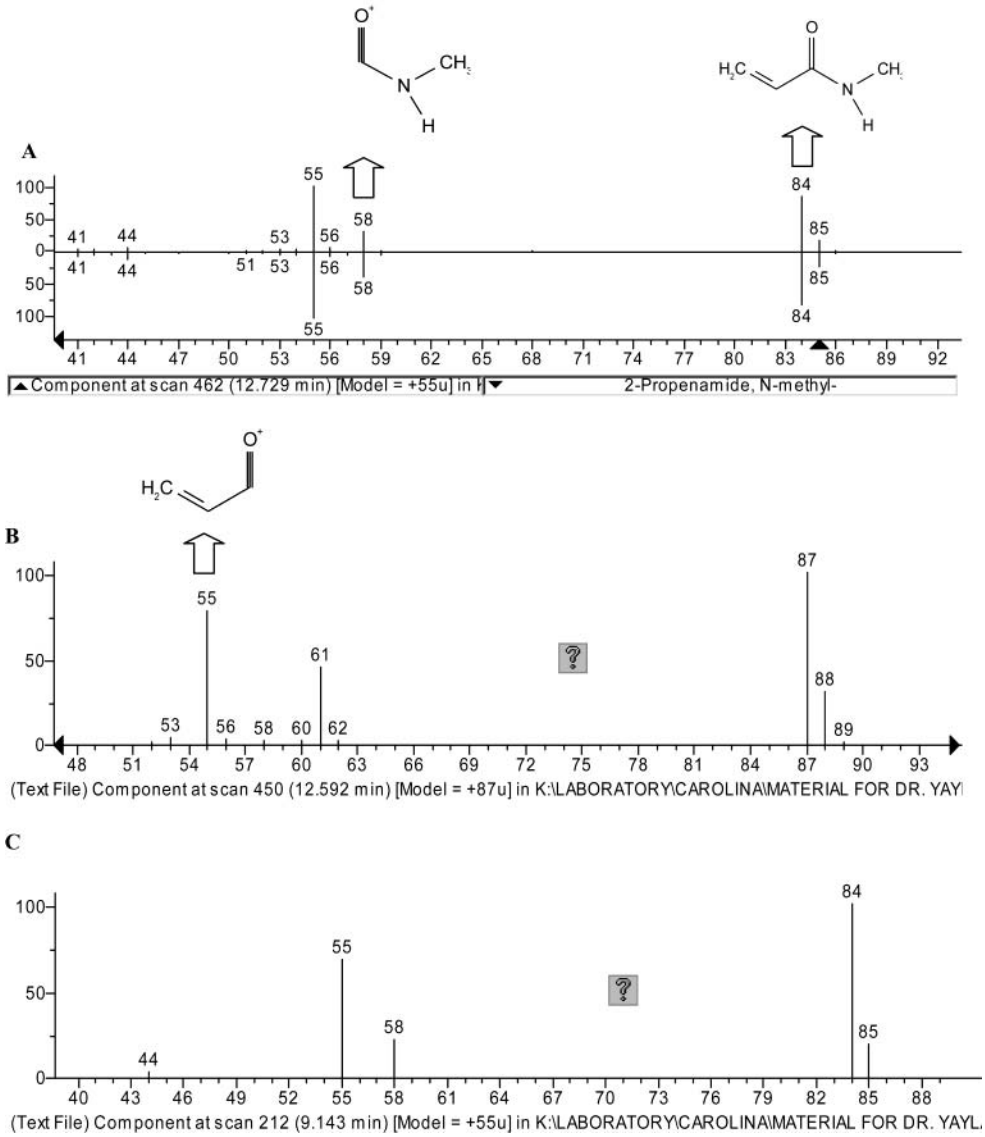

Figure 7. Mass spectrum of (**a**) N-methylacrylamide generated form unlabeled creatine/carnosine model system as compared with authentic NIST library spectrum in head to tail fashion, (**b**) N-methylacrylamide generated from creatine methyl-d_3/carnosine mixture, and (**c**) N-methylacrylamide generated from meat extract.

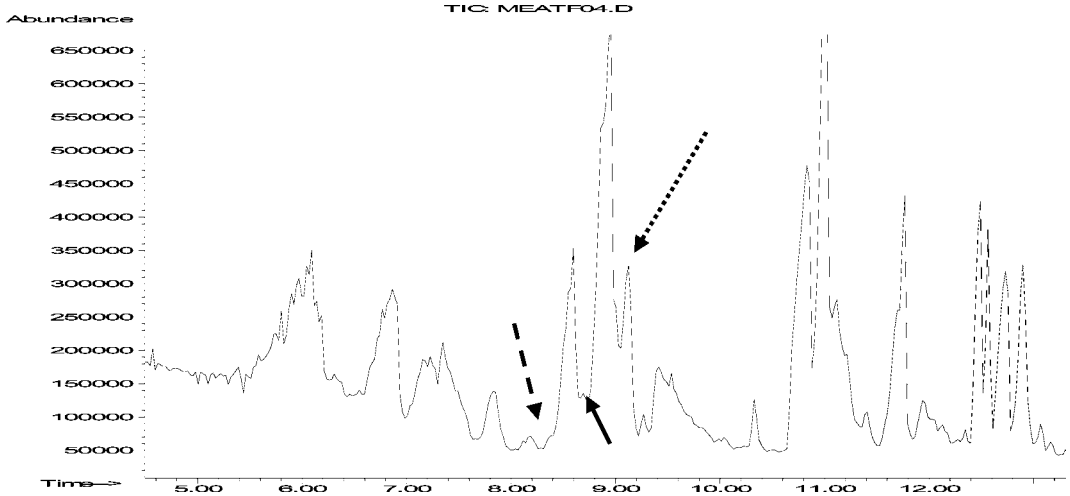

Figure 8. Partial pyrogram of meat extract. The dotted arrow (t = 9.14 min) indicates the N-methylacrylamide peak (mass spectrum shown in **Figure 7c**), the dashed arrow indicates the acrylic acid peak, and the block arrow indicates the acrylamide peak.

Detection of N-Methylacrylamide in Heated Beef Samples. Py-GC/SIM-MS analysis of extracts prepared from heated beef at 250 °C have indicated the presence of trace amounts of acrylic acid and acrylamide but have exhibited a significant intensity

of the peak identified as N-methylacrylamide (see **Figures 7c** and **8**). When the same extract was also analyzed after the addition of excess creatine-d_3, the peak identified as N-methylacrylamide showed 60% label incorporation. When sodium acrylate was pyrolyzed in the presence of methylamine hydrochloride, a peak was generated at the same retention time as the proposed N-methylacrylamide peak and had an identical mass spectrum. In addition, the following acrylamide derivatives N,N-dimethylacrylamide, N,N′-methylene-bis-2-propenamide, and tetrahydrofurfuryl acrylate were also detected in trace amounts.

In conclusion, considering the relatively high detection limit of the method employed in measuring acrylamide derivatives in meat samples, the results obtained have provided enough evidence to speculate that levels of N-methylacrylamide in cooked meat could be as high as acrylamide levels in potato products. Further studies are needed to quantify the levels of N-methylacrylamide in different meat-related consumer products to assess the risk factors associated with its consumption.

LITERATURE CITED

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

(2) 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.

(3) Yaylayan, V. A.; Wnorowski, A.; Perez, L. C. Why asparagine needs carbohydrates to generate acrylamide. *J. Agric. Food Chem.* **2003**, *51*, 1753−1757.

(4) 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.

(5) Zyzak, D.; Sanders, R. A.; Stojanovic, M.; Tallmade, D. H.; Eberhart, B. L.; Ewald, D. K.; Gruber, D. C.; Morsch, T. R.; Strothers, M. A.; Rizzi, G. P.; Villagran, M. D. Acrylamide formation mechanism in heated foods. *J. Agric. Food Chem.* **2003**, *51*, 4782−4787.

(6) Stadler, R. H.; Verzegnassi, L.; Varga, N.; Grigorov, M.; Studer, A.; Reidiker, S.; Schilter, B. *Chem. Res. Toxicol*. **2003**, *16*, 1242−1250.

(7) 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.

(8) Yaylayan, V. A.; Wnorowski, 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.

(9) Arvidsson, P.; Van Boekel, M. A. J. S.; Skog, K.; Jagerstad, M. Kinetics of formation of polar heterocyclic amines in a meat model system. *J. Food Sci*. **1997**, *62*, 911−916.

(10) Friedman, M. Chemistry, biochemistry, and safety of acrylamide. A review. *J. Agric. Food Chem.* **2003**, *51*, 4504−4526.

(11) Chen, Y.; Ho, C.-T. Effects of carnosine on volatile generation from maillard reaction of ribose and cysteine. *J. Agric. Food Chem.* **2002**, *50*, 2372−2376.

(12) World Health Organization. *International Programme on Chemical Safety*; Environmental Health Criteria No. 49; WHO: Geneva, 1985.

(13) Hashimoto, K.; Sahamoto, J.; Tanii, H. Neurotoxicity of acrylamide and related compounds and their effect on male gonads in mice. *Arch. Toxicol*. **1981**, *47*, 179−189.

(14) Mitchell, S. C.; Zhang, A. Q. Methylamine in human urine. *Clin. Chem. Acta* **2001**, *312*, 107−114.

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