MECHANISTIC PATHWAYS OF FORMATION OF ACRYLAMIDE FROM DIFFERENT AMINO ACIDS

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Abstract: Studies on model systems of amino acids and sugars have indicated that acrylamide can be generated from asparagine or from amino acids that can produce acrylic acid either directly such as β-alanine, aspartic acid and carnosine or indirectly such as cysteine and serine. The main pathway specifically involves asparagine and produces acrylamide directly after a sugar-assisted decarboxylation and 1,2-elimination steps and the second non-specific pathway involves the initial formation of acrylic acid from different sources and its subsequent interaction with ammonia to produce acrylamide. Aspartic acid, β-alanine and carnosine were found to follow acrylic acid pathway. Labeling studies with [^{13}C-4]aspartic acid have confirmed the occurrence in aspartic acid model system, of a previously proposed sugar-assisted decarboxylation mechanism identified in asparagine model systems. In addition, creatine was found to be a good source of methylamine and was responsible for the formation of N-methylacrylamide in model systems through acrylic acid pathway. Furthermore, certain amino acids such as serine and cysteine were found to generate pyruvic acid that can be converted into acrylic acid and generate acrylamide when reacted with ammonia.

Key words: asparagine, acrylic acid, pyruvic acid, aspartic acid, creatine, carnosine, acrylamide, N-methylacrylamide, mechanisms of acrylamide formation.

1. INTRODUCTION

Preliminary studies (Stadler et al., 2002; Mottram et al., 2002) that followed the initial discovery of acrylamide in cooked food have lead not

Chemistry and Safety of Acrylamid in Food, edited by
Friedman and Mottram, Springer Science+Business Media, Inc., 2005

only to the unambiguous identification of asparagine as the main amino acid precursor of acrylamide, but also confirmed the origin of its carbon atoms and the amide nitrogen through labeling studies. Although thermal decarboxylation and deamination reactions (Yaylayan et al., 2003) of asparagine alone, in principle, can produce acrylamide, the presence of sugars was necessary to effect the conversion of asparagine into acrylamide. Subsequent studies (Becalski et al., 2003; Zyzak et al, 2003) have indicated that any carbonyl containing moiety can perform a similar transformation and that asparagine alone prefers to undergo intramolecular cyclization and form an imide rather than decarboxylate to form acrylamide. Studies related to the detailed mechanism (Yaylayan et al. 2003) of this transformation in model systems have indicated that decarboxylated Amadori product of asparagine is the key precursor of acrylamide. Furthermore, the decarboxylated Amadori product was shown to be formed under relatively mild conditions through the intramolecular cyclization of the initial Schiff base and formation of oxazolidin-5-one intermediate (Manini et al., 2001) and subsequent generation of a stable azomethine ylide which is prone to undergo an irreversible 1,2-prototropic shift (Grigg 1989) to produce decarboxylated Schiff base and eventually decarboxylated Amadori product. Similar conclusions, using model food systems, were drawn by Zyzak et al. (2003) depicting direct decarboxylation of the Schiff base, but without invoking, oxazolidin-5-one as an intermediate. As part of our investigation of other sources of acrylamide in food and using Py-GC/MS as in integrated reaction, separation and identification system (Yaylayan, 1999) 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.

2. MATERIALS AND METHODS

All reagents, chemicals and $^{15}NH_4Cl$ were purchased from Aldrich Chemical company (Milwaukee, WI) and used without further purification. The labeled [^{13}C-4]aspartic acid was purchased from Cambridge Isotope Laboratories (Andover, MA).

2.1 Pyrolysis-GC/MS analysis

A Hewlett-Packard GC with Mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit was used for the Py-GC/MS analysis. One mg samples of pure reactants was introduced inside a quartz tube (0.3mm thickness), plugged with quartz wool, and inserted inside the coil probe with a total heating time of 20s. The column was a fused silica

DB-5 column (50m length x 0.2mm i.d. x 0.33 μm film thickness; J&W Scientific). The pyroprobe interface temperature was set at 250°C. Capillary direct MS interface temperature was 280°C; ion source temperature was 180°C. The ionization voltage was 70 eV, and the electron multiplier was 2471 V. All samples were injected in splitless mode. Three methods of analysis were used.

- **Method 1** had a delayed pulse of 65 psi followed by constant flow of 0.775mL/min, and a septum purge of 2 mL/min. The initial temperature of the column was set at 40°C for 2 minutes 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 constant flow of 1 mL/min, and a septum purge of 2 mL/min. The initial temperature of the column was set at -5°C for 2 minutes 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 followed by constant flow of 1mL/min, and a septum purge of 2 mL/min. The initial temperature of the column was set at -5°C for 2 minutes 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.

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 % enrichment) are the average of duplicate analyses and are rounded to the nearest multiple of 5%. Fig. 4, shows examples of the three methods.

3. RESULTS AND DISCUSSION

Studies with model systems containing selected amino acids and glucose (see Table 1) have indicated that there are two general pathways of acrylamide formation; a major pathway that generates acrylamide directly from asparagine and the second minor pathway that generates acrylamide though reaction of ammonia with acrylic acid (see Fig. 1). Furthermore, studies have also indicated that acrylic acid itself can be generated either directly from certain amino acids or dipeptides such as carnosine, β-alanine and aspartic acid or indirectly from amino acids such as serine and cysteine, through reduction of pyruvic acid into lactic acid and its subsequent dehydration into acrylic acid.

Table 1. Efficiency (area/mole of amino acid) of acrylic acid & acrylamide generation from 1 mg samples of either amino acid or amino acid/glucose (3:1) mixtures pyrolyzed at 350° C (data generated using method 1)

Model System	Acrylic acid x 10^{12}	Acrylamide x 10^{12}
Asparagine	not detected	trace
Asparagine/glucose	1.20 ± 0.18	5.18 ± 0.6=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

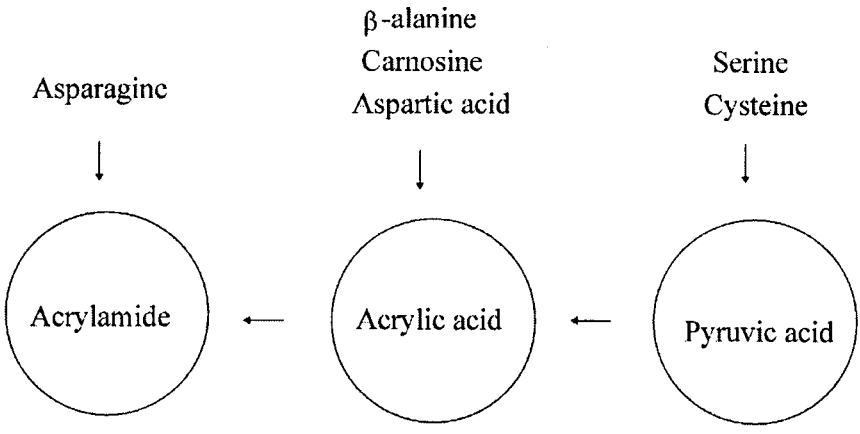

Figure 1. Precursors of acrylamide.

3.1 Direct formation of acrylamide from asparagine

Asparagine is the only amino acid capable of directly generating acrylamide. Consequently it is considered the main source of acrylamide in food. The studies related to the detailed mechanism of this transformation have indicated that sugars and other carbonyl compounds play a specific role in the decarboxylation process of asparagine – a necessary step in the generation of acrylamide. It has been proposed (Yaylayan et al., 2003) that

Schiff base intermediate formed between asparagine and the sugar provides a low energy alternative to the decarboxylation from the intact Amadori product through generation and decomposition of oxazolidin-5-one intermediate (Manini et al., 2001) leading to the formation of a relatively stable azomethine ylide (see Fig. 2).

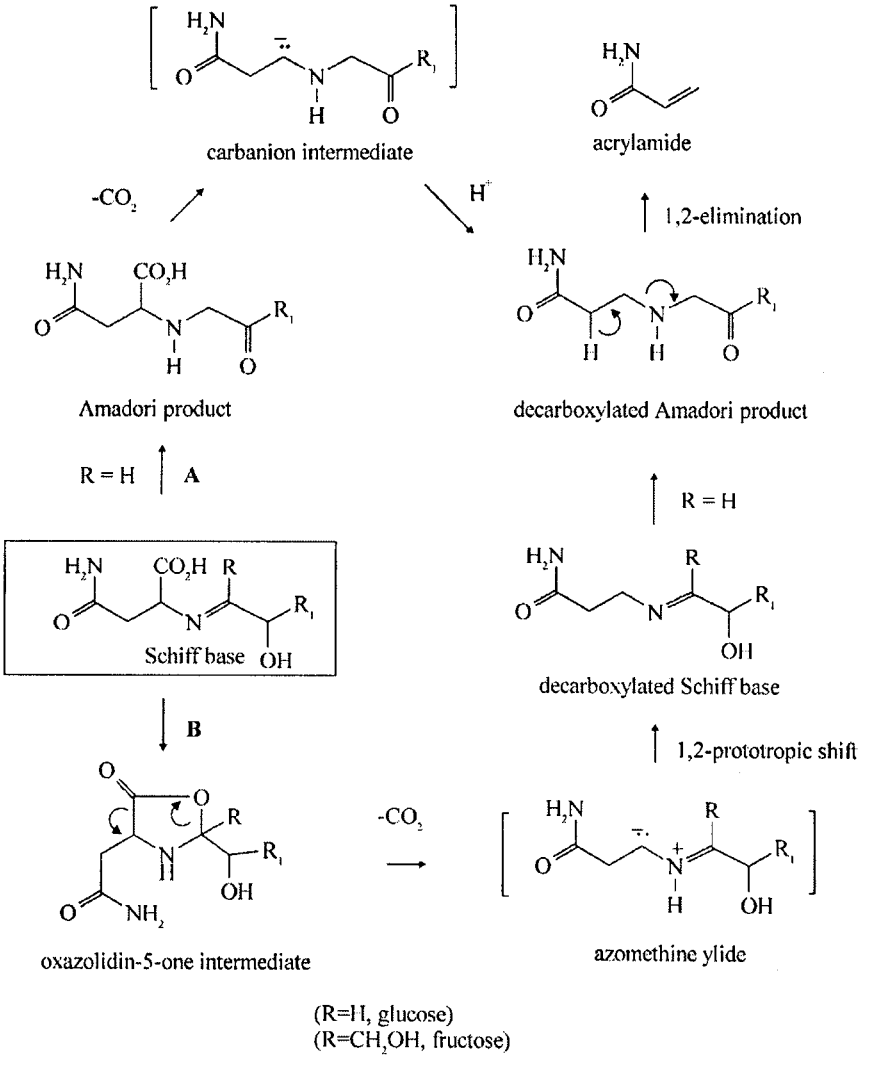

Figure 2. Mechanism of formation of acrylamide through thermally-induced decarboxylation of intact Amadori products (pathway A) and through sugar-assisted pre-Amadori decarboxylation (pathway B).

Literature data indicates the propensity of such protonated ylides to undergo irreversible 1,2-prototropic shift (Grigg et al., 1989) and produce, in this case, decarboxylated Schiff base which can easily rearrange into corresponding Amadori product. Decarboxylated Amadori products can either undergo the well known β-elimination process initiated by the sugar moiety to produce 3-aminopropanamide and 1-deoxyglucosone or undergo 1,2-elimination initiated by the amino acid moiety to directly generate acrylamide. On the other hand the decarboxylated Schiff intermediate can either hydrolyze and release 3-aminopropanamide or similarly undergo amino acid initiated 1,2-elimination to directly form acrylamide (Yaylayan and Stadler, 2004). However, their relative contribution to acrylamide formation is still under investigation.

3.2 Direct formation of acrylic acid from β-alanine, carnosine and aspartic acid

Some amino acids can generate acrylic acid directly during their thermal decomposition. Such amino acids require the presence of ammonia to convert acrylic acid into acrylamide. One of the main sources of ammonia in food is the free amino acids. Sohn and Ho (1995) have identified asparagine, glutamine, cysteine and aspartic acid as the most efficient ammonia generating amino acids under thermal treatment.

3.2.1 Formation of acrylamide from β-alanine

The mechanism of decomposition of β-alanine to generate both reactants required for the formation of acrylamide, ammonia and acrylic acid, is shown in Fig. 3. 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 (Fig. 4b). When β-alanine was pyrolyzed in the presence of excess $^{15}NH_4Cl$ the resulting acrylamide incorporated both the labeled (added) and 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 (Fig. 4a). Comparison of figures 4a and 4b 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).

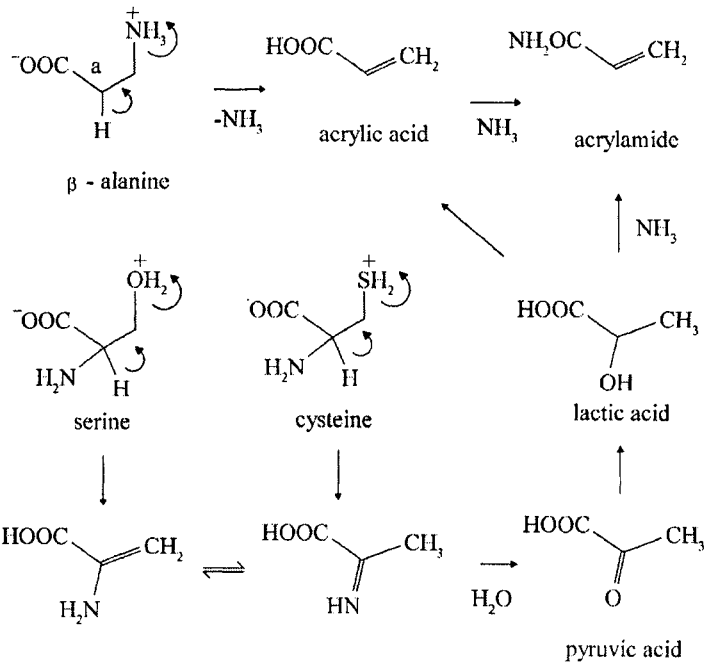

Figure 3. Proposed mechanisms of formation of acrylamide from β-alanine, serine and cysteine.

3.2.2 Formation of acrylamide from aspartic acid

Aspartic acid, on the other hand, can also form acrylic acid and subsequently acrylamide (Stadler et al., 2003; Yaylayan et al., 2004; Becalski et al., 2003) (Fig. 4c), but unlike β-alanine and similar to asparagine, it produces more acrylic acid in the presence of glucose (see Table 1). In order to identify the mechanism of acrylic acid formation from aspartic acid, [^{13}C-4]-aspartic acid was pyrolyzed alone and in the presence of glucose. According to Fig. 5, 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 Fig. 5. Unlike α-alanine, β-alanine is known to produce acrylic acid and 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% unlabelled product (Fig. 6c) indicating existence of a third pathway capable of formation of acrylamide with C-4 decarboxylation.

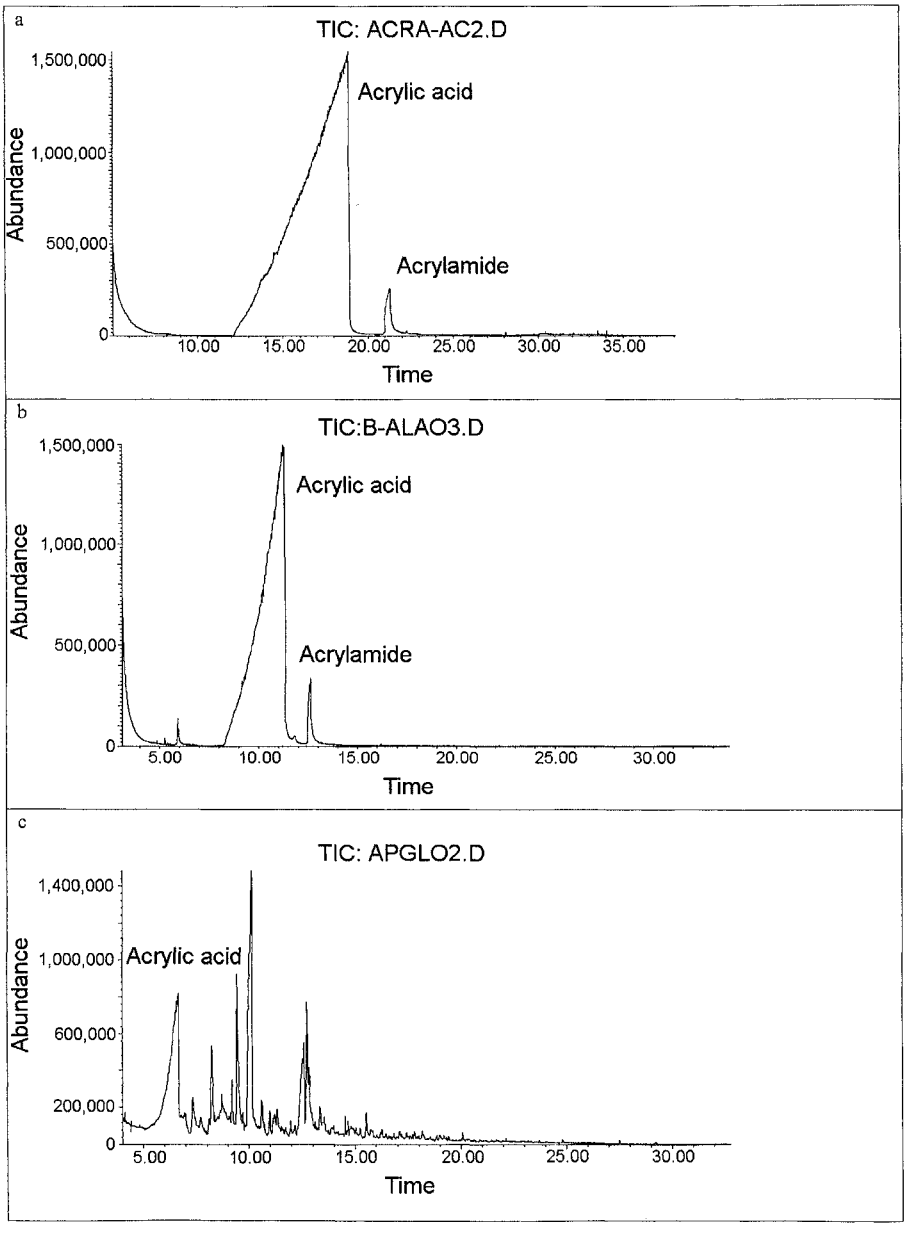

Figure 4. Pyrograms of model systems consisting of equimolar mixtures of (a) acrylic acid/ammonium carbonate (using method 3) (b) β-alanine alone (using method 2) and (c) aspartic acid/glucose (using method 1).

A concerted mechanism where decarboxylation occurs simultaneously with deamination can explain the formation of unlabelled acrylic acid as

shown in Fig. 5. Interestingly, when [^{13}C-4]-aspartic acid was pyrolyzed in the presence of glucose only 100% labeled acrylic acid was observed (Fig. 6b), indicating preferential decarboxylation of C-1 carboxylate moiety consistent with the mechanism of sugar-assisted decarboxylation shown in Fig. 2. This observation, along with increased ability of aspartic acid to generate acrylamide in the presence of glucose (see Table 1), provides evidence for the ability of the Schiff base to provide a low energy pathway for decarboxylation of amino acids relative to decraboxylation from intact Amadori products that passes through a carbanion intermediate rather than the more stable azomethine ylide as shown in Fig. 2. Furthermore, similar to asparagine, reaction with sugar and formation of oxazolidine intermediate can prevent cyclization to form maleic anhydride (equivalent to succinimide in the case of asparagine) and enhance acrylic acid generation as observed.

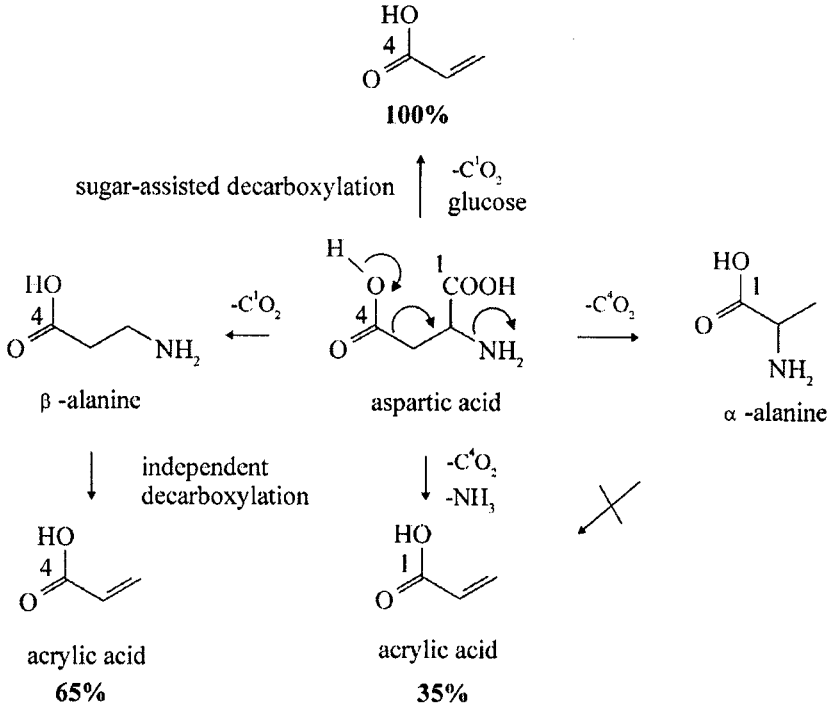

Figure 5. Decarboxylation pathways of aspartic acid based on labeling studies.

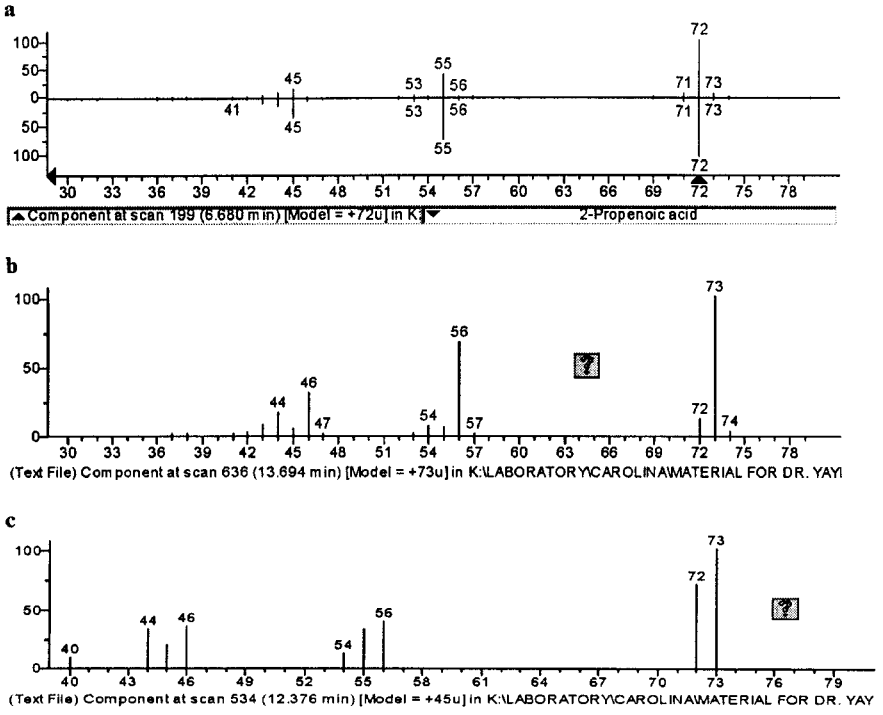

Figure 6. Mass spectrum of (a) acrylic acid generated from unlabeled aspartic acid compared with authentic NIST library spectrum in head to tail fashion. (b) acrylic acid generated from [^{13}C-4]aspartic acid/glucose mixture. (c) acrylic acid generated from [^{13}C-4]aspartic acid alone.

3.2.3 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 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 (Chen and Ho, 2002). Fig. 7 depicts two possible pathways of formation of acrylamide from carnosine, one through hydrolysis of the peptide bond and release of β-alanine and its subsequent deamination, the second through release of 3-aminopropanamdie and its deamination. However, the conspicuous absence of acrylamide in meat products at the scale expected to that of potatoes (Friedman, 2003) has lead 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 effect on the

amounts of acrylamide and its precursor acrylic acid was calculated. 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 potentially toxic (Hashimoto et al., 1981 & WHO, 1985) acrylamide derivatives; N-methylacrylamide and N,N-dimethylacrylamide. The decrease in acrylic acid formation can be explained by its accelerated conversion into acrylamide derivatives due to the efficient generation of ammonia and methylamines from added creatine (Yaylayan et al., 2004).

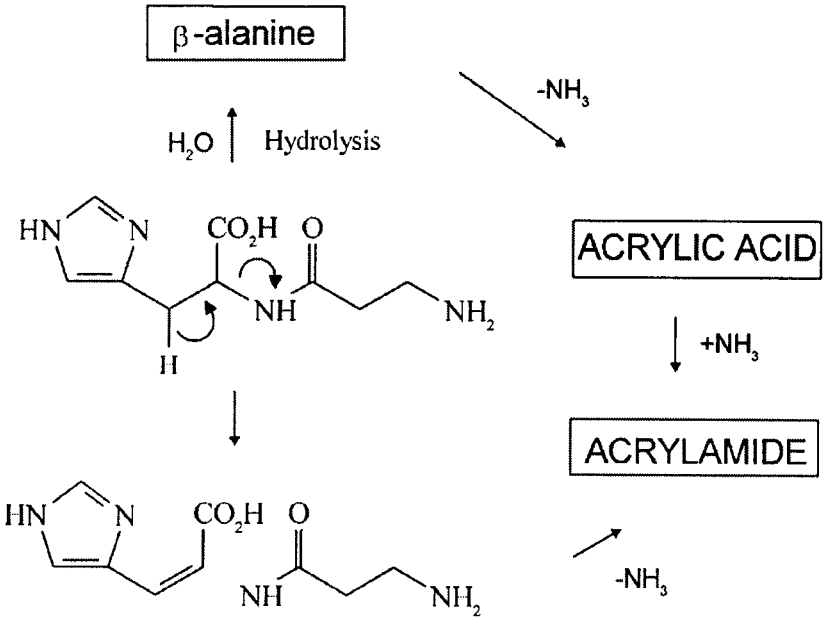

Figure 7. Proposed mechanism of acrylamide formation from carnosine.

3.3 Indirect formation of acrylic acid from serine & cysteine

Dehydration of serine alone (see Fig. 3) and in the presence of sugars has been shown to generate pyruvic acid (Wnorowski and Yaylayan, 2003). Conversion of β-alanine into acrylic acid and release of acrylamide from

decarboxylated Amadori product follow a similar mechanism of 1,2-elimination (see Figs 2 & 3). Cysteine can also lose a hydrogen sulfide molecule to generate acrylic acid as shown in Fig. 3. Acrylic acid was detected along with pyruvic acid when serine was pyrolyzed at 350°C. This observation can be justified by proposing the reduction of pyruvic acid into lactic acid and its subsequent dehydration into acrylic acid. Model studies with lactic acid have indicated that such transformations are possible in the presence of ammonia; mixtures of lactic acid and ammonium salts produced lactamide, acrylic acid and acrylamide when pyrolyzed at 350°C.

4. CONCLUSION

Although, in theory, there are more than one amino acid that can generate acrylamide, however, the efficiency of the conversion of acrylic acid into acrylamide is limited by the availability of free ammonia in the vicinity of its production in the food matrix, in addition, this limitation is further compounded by the extreme volatility of ammonia at temperatures that are conducive to acrylamide formation. Recent studies (Stadler et al., 2003) have indicated that aspartic acid/fructose mixtures generated acrylamide at levels 1000-fold below the levels measured for asparagines/fructose mixtures.

ACKNOWLEDGMENTS

The authors acknowledge funding for this research by the Natural Sciences and Engineering Research Council of Canada (NSERC) and by Danone (France).

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