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ARTICLE

Acrylamide Formed at Physiological Temperature as a Result of Asparagine Oxidation

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Acrylamide is a probable human carcinogen that is neurotoxic to both humans and animals. It is known to be formed during cooking of foods at temperatures higher than 120 °C. The present study demonstrates that acrylamide can also be formed at physiological conditions (37 °C, pH 7.4) when asparagine is incubated in the presence of hydrogen peroxide (H_2O_2). The formation of acrylamide under these conditions is dependent on the incubation time and the concentration of H_2O_2. Thus, the results raise the question of the possible endogenous formation of acrylamide in pathological conditions that are associated with long-term oxidative stress. Further studies are therefore warranted to clarify the possible endogenous formation of acrylamide and its significance in chronic conditions that are known to be associated with oxidative stress.

KEYWORDS: Acrylamide; asparagine; hydrogen peroxide

INTRODUCTION

Acrylamide is formed when foods rich in carbohydrates are heated. The formation of acrylamide in many staple foods was first reported in 2002 (*1*). The Maillard reaction with the main precursors glucose and asparagine was later proposed as a possible mechanism for the heat-induced formation of acrylamide (*2*, *3*). Similar reactions between glucose and amino acids are also known to take place endogenously under physiological conditions (*4*). Therefore, it was hypothesized that acrylamide may also be formed at physiological temperature. Recently, it was reported that acrylamide and glycidamide hemoglobin adduct levels were significantly increased in mice treated with chemicals that induce oxidative stress (*5*), suggesting that acrylamide may be formed as a result of oxidation.

In the present study, we investigated the possible formation of acrylamide at 37 °C using hydrogen peroxide (H_2O_2) to induce oxidation. Because asparagine and glucose are known precursors in the heat-induced formation of acrylamide, our initial hypothesis was that acrylamide could be formed by similar reactions under oxidative conditions. To test this hypothesis, we incubated asparagine with glucose in phosphate-buffered saline (PBS) in the presence of H_2O_2 at 37 °C. The production of acrylamide over time was monitored by liquid chromatography−electrospray ionization−tandem mass spectrometry (LC-ESI-MS/MS) in multiple reaction monitoring (MRM) mode.

EXPERIMENTAL PROCEDURES

Chemicals. D-(+)-Glucose (ultra-anhydrous), L-asparagine (anhydrous), ethylenediaminetetraacetic acid (EDTA), and 30% stabilized and nonstabilized H_2O_2 were purchased from Sigma-Aldrich, St. Louis, MO; L-asparagine:H_2O (amide-^{15}N, 98%+) and $^{13}C_3$-acrylamide (1,2,3-$^{13}C_3$, 99%, 1 mg/mL in methanol with 100 ppm hydroquinone) were purchased from Cambridge Isotope Laboratories, Inc., Andover, MA; and Invitrogen Dulbecco's phosphate-buffered saline (PBS) was purchased from GIBCO, Carlsbad, CA. All solvents used for LC-MS were of HPLC grade or higher.

Reaction Mixtures. A PBS solution of 6.8 mM asparagine with or without 4.1 mM glucose was incubated at 37 °C either with 120 mM H_2O_2 for the time course study or with different levels (5, 10, and 25 mM) of H_2O_2. Prior to the addition of H_2O_2, 10 ng of $^{13}C_3$-acrylamide internal standard was added to each 1.0 mL of reaction mixture for quantification purposes. Additionally, 1 mM EDTA was added to some of the reaction mixtures to investigate the role of contaminating metal ions.

LC-ESI-MS/MS Analysis. The samples were analyzed using a TSQ Quantum Ultra mass spectrometer (ThermoFinnigan, San Jose, CA) coupled with an 1100 Agilent HPLC system (Agilent, Palo Alto, CA). The instrument was operated in the positive-ion electrospray mode with argon collision gas set at 1.2 mTorr. Other ESI conditions were as follows: temperature, 350 °C; capillary voltage, 4.0 kV; CID offset, 14 V; sheath gas, 50; auxiliary gas, 20; sweep gas, 0. MRM transitions m/z 72→55 at 11 eV and m/z 72→44 at 30 eV were monitored to assess and confirm acrylamide formation. MRM transitions m/z 73→55 at 11 eV and m/z 73→45 at 30 eV were monitored to assess and confirm ^{15}N-acrylamide formation. The $^{13}C_3$-acrylamide internal standard was monitored with the transition m/z 75→58 at 11 eV. Each transition was 0.2 s for a total cycle time of 1.0 s. This method for monitoring acrylamide formation was used in all analyses. A Synergi HydroRP column 4 μm 2 × 250 mm (Phenomenex, Torrance, CA) was maintained at 30 °C with a column heater. The mobile phase, delivered at 200 μL/min, was isocratic aqueous 0.1% formic acid, and acrylamide eluted at 7.8 min (**Figure 1**). During the first 6 min the column eluate was diverted to waste, and data were only collected from 6 to 10 min. The samples were incubated in the thermostated autosampler at 37 °C. In the time course study, the formation of acrylamide was monitored by LC-ESI-MS/MS once every 2 h for 72 h. To check the purity of the ^{15}N-asparagine, full scans were acquired from m/z 90 to 200 and the same HPLC column and conditions were used.

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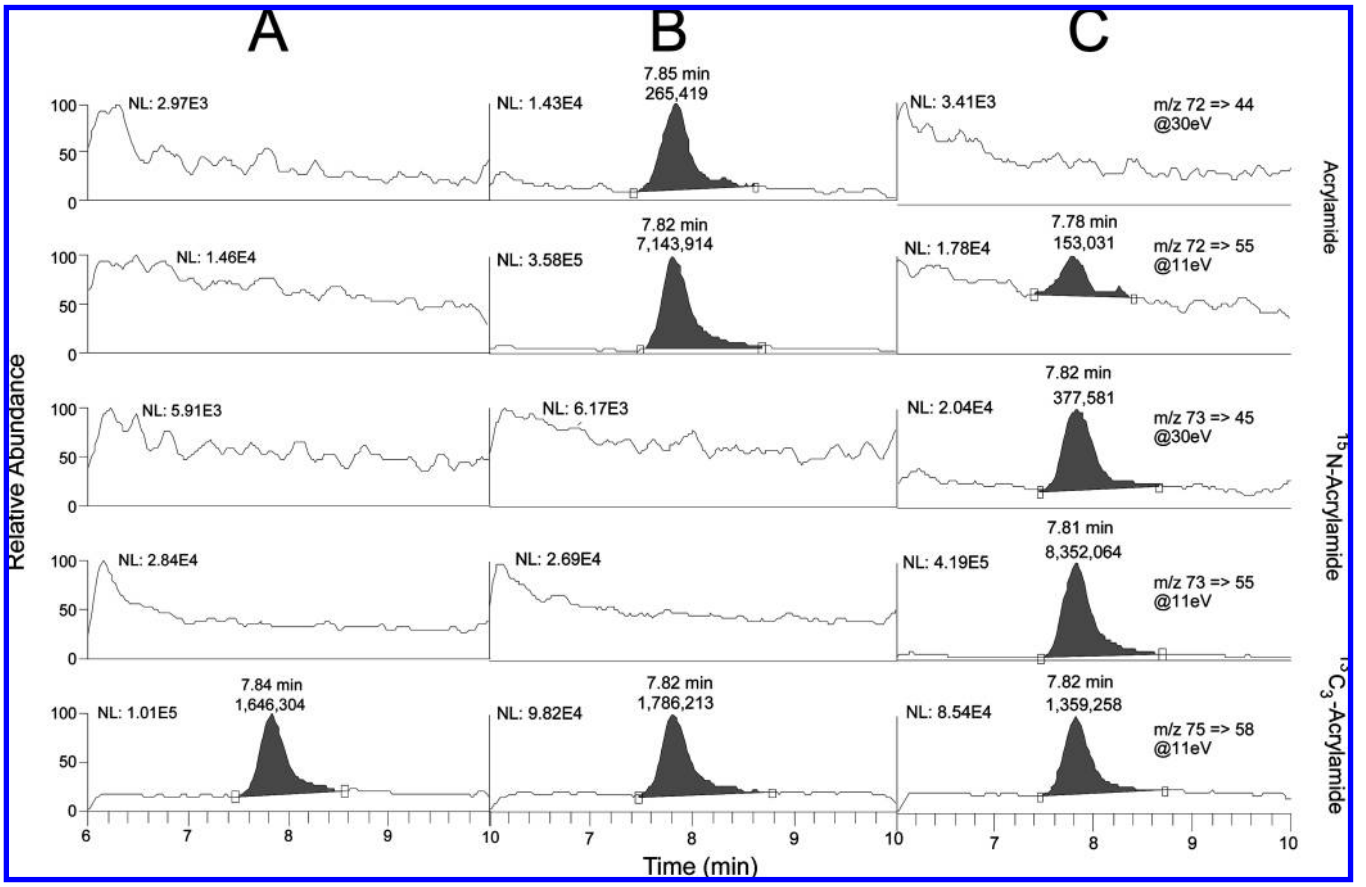

Figure 1. LC-ESI-MS/MS chromatograms of acrylamide or ^{15}N-acrylamide in solutions of 6.8 mM asparagine or ^{15}N-asparagine and 120 mM H_2O_2 in PBS incubated at 37 °C: (**A**) asparagine solution at 5 h; (**B**) asparagine solution after 50 h; (**C**) ^{15}N-asparagine solution at 51 h. MRM conditions were as noted.

RESULTS AND DISCUSSION

Our results reveal that acrylamide is formed in a solution of asparagine and H_2O_2 in PBS (pH 7.4) incubated at physiological temperature (37 °C). **Figure 1** depicts MRM chromatograms of acrylamide formed in 6.8 mM solutions of asparagine and 120 mM H_2O_2 in PBS following 5 h (**Figure 1A**) and 50 h (**Figure 1B**) of incubation at 37 °C. Detectable levels of acrylamide were formed after approximately 6 h of incubation at 37 °C. The formation of acrylamide in the reaction mixture was monitored once every 2 h for 72 h and was time-dependent (**Figure 2**); no acrylamide was formed in the absence of H_2O_2 or asparagine. Water, TRIS and ammonium acetate pH 7.4 buffers, also led to the formation of acrylamide (results not shown). To investigate the involvement of Maillard-like reactions in the formation of acrylamide at 37 °C, we incubated asparagine with H_2O_2 in the presence or absence of glucose and determined the generation of acrylamide over time. Our results showed that glucose was not required for the formation of acrylamide under these oxidative conditions (**Figure 2**).

To clarify the origin of acrylamide, asparagine was replaced by [^{15}N-amide]-labeled asparagine (^{15}N-asparagine). A time course experiment was performed in a solution of 6.8 mM ^{15}N-asparagine with or without 4.1 mM glucose, incubated at 37 °C in the presence of 120 mM H_2O_2. The formation of ^{15}N-acrylamide in the solution was determined by monitoring the MRM transition m/z 73→55 and confirmed using the transition m/z 73→45 (**Figure 1C**). A time-dependent formation of ^{15}N-acrylamide was observed (**Figure 2**). Our results demonstrated that the ^{15}N from the amide moiety of ^{15}N-asparagine was incorporated in the ^{15}N-acrylamide structure [cf. confirmatory transition 73→45

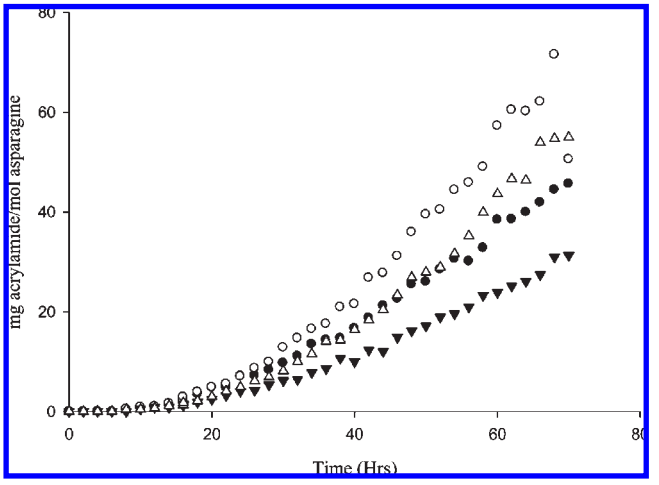

Figure 2. Time-dependent formation of acrylamide or ^{15}N-acrylamide in PBS solutions of 6.8 mM asparagine or ^{15}N-asparagine, respectively, with or without 4.1 mM glucose, in PBS incubated at 37 °C in the presence of 120 mM of H_2O_2.: (●) acrylamide formed in PBS solution of asparagine; (▼) acrylamide formed in PBS solution of asparagine and glucose; (○) ^{15}N-acrylamide formed in PBS solution of ^{15}N-asparagine; (△) ^{15}N-acrylamide formed in PBS solution of ^{15}N-asparagine and glucose. The results are presented in milligrams of acrylamide per mole of asparagine or milligrams of ^{15}N-acrylamide per mole of ^{15}N-asparagine.

$(CO^{15}NH_2)^+$, **Figure 1C**]. These results further support that the origin of the detected acrylamide is asparagine and provide insight into the reaction mechanism. The experiment with

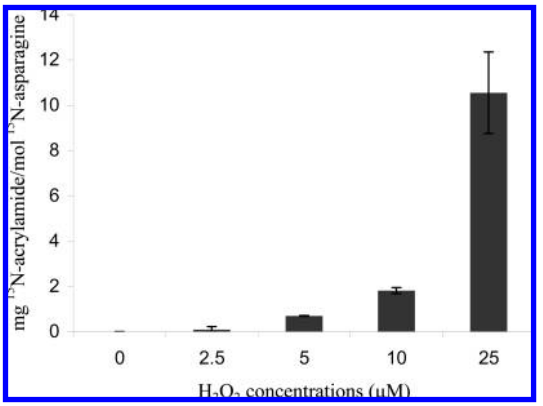

Figure 3. Formation of ^{15}N-acrylamide (mean ± SD) in a solution of 6.8 mM ^{15}N-asparagine in PBS incubated at 37 °C with different levels of H_2O_2 for 120 h.

^{15}N-asparagine confirmed that glucose was not required for the formation of ^{15}N-acrylamide (**Figure 2**). The addition of 1 mM EDTA totally inhibited the formation of acrylamide in both cases, suggesting that the reaction is catalyzed by traces of metal ions present in the reaction mixtures.

Reaction mixtures with asparagine and ^{15}N-asparagine led, respectively, to the formation of acrylamide and ^{15}N-acrylamide (**Figure 1B**,**C**). As demonstrated in **Figure 1C**, a peak for unlabeled acrylamide, integrating for approximately 1.8% of the ^{15}N-acrylamide, was detected in the ^{15}N-asparagine samples incubated for >50 h. To clarify the origin of the unlabeled acrylamide, the isotopic purity of the ^{15}N-asparagine was determined using LC-MS by acquiring full scans from m/z 90 to 200 using the same HPLC column and conditions and was found to contain approximately 1.8% of unlabeled asparagine. The observed 1.8% of unlabeled acrylamide was therefore derived from this unlabeled asparagine isotopic impurity that was found in the ^{15}N-asparagine.

To investigate the minimum levels of H_2O_2 that under our reaction conditions led to the formation of quantifiable levels of acrylamide, ^{15}N-asparagine was incubated with different concentrations (2.5, 5, 10, and 25 mM) of H_2O_2 for 24, 48, or 120 h. The experiment was done in triplicate. Quantifiable levels of acrylamide were formed following incubation with 25 mM H_2O_2 for 24 h, 10 mM H_2O_2 for 48 h, and as low as 5 mM of H_2O_2 for 120 h. The formation of acrylamide in solutions with different levels of H_2O_2 after incubation for 120 h is presented in **Figure 3**.

Our results show that acrylamide is formed at physiological temperature (37 °C) under oxidative conditions. Although the reaction mechanism leading to the formation of acrylamide is still under investigation, our results demonstrate the incorporation of the ^{15}N from the amide moiety of ^{15}N-asparagine in the ^{15}N-acrylamide structure, thus providing further support that the origin of the detected acrylamide is asparagine and insight into the reaction mechanism. Moreover, our results demonstrate that a reducing sugar is not required. The fact that the reaction was totally inhibited by the addition of EDTA, a chemical that chelates metal ions, also suggests that traces of these ions present in the reaction media may play a catalytic role in the mechanism. Interestingly, phytate, a naturally occurring chelator, was also observed to reduce acrylamide formation in heat-induced reactions (*6*).

Under the conditions used in this study 5 mM H_2O_2 was the lowest concentration that led to the formation of measurable levels of acrylamide. H_2O_2 levels reaching 0.577 mM (*7*) have been measured in human blood under normal physiological conditions. Considering that H_2O_2 levels are known to increase in pathological conditions with local inflammation and in long-term chronic conditions (*8*, *9*); it is reasonable to propose the possible endogenous formation of acrylamide under pathological conditions. Moreover, it cannot be excluded that a number of other species such as $O_2^{\bullet -}$, $OH^{\bullet}$, and $ONOO^-$ (*10*, *11*) formed in oxidative environments may be involved in similar reactions capable of leading to the formation of acrylamide. The total free amino acid level in plasma is about 3.2 mM, of which asparagine is 50 μM. The aspragine level used in the study was 6.8 mM, which is higher than the normal free asparagine levels expected endogenously. The possible role of other amino acids in the formation of acrylamide needs to be investigated (*12*).

Our results clearly demonstrate that acrylamide can be formed in vitro from asparagine by the action of H_2O_2 at physiological temperature, pH, and ionic strength, raising the question of a possible formation of acrylamide in vivo. The hypothesis that acrylamide may be formed endogenously in pathological conditions that are linked to oxidative stress is supported by the observation that acrylamide adduct levels were increased in mice treated with chemicals that induce oxidative stress (*5*). Although the levels of H_2O_2 used in this experiment are higher than what is expected under normal physiological conditions, chronic conditions are known to lead to increased oxidative stress, which may facilitate the endogenous formation of acrylamide. Therefore, more studies are warranted to investigate the possible endogenous formation of acrylamide and its consequence in the pathological conditions—such as diabetes and Alzheimer's disease—that are associated with chronic oxidative stress.

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