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Inhibitory effects of methylglyoxal (MGO) on the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) in model system

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Abstract: Methylglyoxal was produced by the degradation of glucose, and its role in producing 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine **(**PhIP) was studied attempting to clarify the reaction pathways. The study showed that the PhIP yield was decreased when glucose was added to a mixture of phenylacetaldehyde and creatinine or when methylglyoxal was incorporated into a blend of phenylalanine (phenylacetaldehyde), creatinine and glucose in proportions of 1:1:0.5. PhIP yield was firstly increased and then decreased when glucose was incorporated into a blend of phenylalanine and creatinine. When methylglyoxal was added to the creatinine/phenylacetaldehyde/glucose mixture, methylglyoxal achieved around 83.2% inhibition of PhIP. However methylglyoxal achieved around 39% inhibition of PhIP in the creatinine/phenylalanine/glucose mixture. All of these results indicate that the addition of methylglyoxal to the phenylacetaldehyde/creatinine mixture inhibited the formation of PhIP. A pathway which the inhibitory effect of methylglyoxal on PhIP composition is proposed. Meanwhile, this study explained the ways in which glucose are involved in the composition of PhIP, and helped us better study the formation mechanism of PhIP.

Keywords: 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), formation, methyl-glyoxal, pathway, and inhibition

1. Introduction

Heterocyclic aromatic amines (HAAs) created in food when it is heated to a high temperature may be a dangerous factor for certain malignancies in people. Because of their possible mutagenic and carcinogenic qualities, food chemists, nutritionists, and toxicologists are concerned about the formation of HAAs in food [1,2]. According to a recent study, during the high-temperature food processing procedure, nitrogenous bases' nucleosides and nucleotides significantly contributed to the creation of HAA, and different kinds of meat played an indispensable role in HAA formation. HAA formation depended on meat chemical composition, particularly the amount of protein, glucose, nitrogenous compounds, and free amino acids. Furthermore, certain polymers (like proteins) in a food matrix have

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the ability to bind HAAs. Bounded HAAs may be released during digestion in the human digestive tract and contribute to carcinogenesis [3,4]. From food and model systems, over 25 HAAs have been detected and confirmed [5]. Among all HAAs, one of the most prevalent HAA produced is the thermic 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) in foods.

Several investigations have examined the process by which PhIP forms. PhIP is confirmed to be formed through the reaction of phenylalanine and creatinine [6,7]. Furthermore, the reaction is assisted by reactive carbonyls, which change phenylalanine into phenylacetaldehyde; reactive carbonyls are by-products of the Maillard reaction [6]. Traditionally, carbohydrates have been supposed to be the source of these reactive carbonyls [7,8].

Methylglyoxal, a 1,2-dicarbonyl compound, is formed via carbohydrate degradation, i.e., carbohydrate undergoes caramelization and the Maillard reaction [9]. Sugars have been mentioned as methylglyoxal's purported source, amadori arrangement products (ARPs), long-chain dicarbonyls (such as 1-deoxy-glucosone and 3-deoxy-glucosone), and Schiff base [10-12]. Subsequently, Voigt and Glomb [13] outlined the three main processes that result in the production of methylglyoxal as follows: retro-aldol reactions and dicarbonyl cleavages of α and β.

The generation of PhIP in mixtures of creatinine, phenylalanine, or phenylacetaldehyde is the subject of this investigation, and glucose in order to clarify the function of methylglyoxal on PhIP formation. Additionally, the same reaction mixtures were also investigated when methylglyoxal was present. Analyzing the impact of methylglyoxal on PhIP generation has been made possible by a study of these final reactions.

2. Materials and Methods

2.1. Materials

The supplier of 2-Amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) was Toronto Research Chemicals. (North York, Ontario, Canada), solid phase extraction with Oasis MCX cartridges (3 cc/60 mg, 30 mm) of Waters (Milford, Massachusetts, USA). The remaining chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA) and were analytical grade. All solutions used to prepare by double distilled water.

2.2. Formation of PhIP in creatinine/phenylalanine/glucose/methylglyoxal and creatinine/ phenylacetaldehyde/glucose/methylglyoxal reaction mixtures

A model system contained creatinine (0.6 mmol), phenylalanine (0.6 mmol), glucose (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol), and methylglyoxal (0, 0.2, 0.4, 0.6, 0.8, and 1 μmol) in 10 ml water. Another model system contained phenylacetaldehyde (0.6 mmol), creatinine (0.6 mmol), glucose (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol), and methylglyoxal (0, 0.2, 0.4, 0.6, 0.8, and 1 μmol) was in 10 ml water. The mixture were added to PTFE test tubes with an external stainless steel liner, and heated at 200 ℃ for 3 h in a closed hood. Afterward, reactants were immediately cooled in an ice-bath. Reaction mixture (1 ml) was incubated using o-Phenylenediamine (1 ml, 0.5 mg/ml in 0.2 mol/l PBS of pH 7.4) at room temperature for 12 h.

2.3. Purification

SPE was used to accomplish the clean-up for the HPLC analysis. Rinse the cartridges with 2 ml MeOH and 2 ml water. After that, they were filled with 3 ml reaction sample, 2 ml of 0.1 M HCl, 2 ml of 100% methanol, and 2 ml of 40% methanol with 5% ammonium hydroxide (NH4OH) were used for washing. The ultimate elution was carried out using 5% ammonium hydroxide and 95% methanol. For HPLC analysis. The residue was dissolved in 0.5 ml of methanol after the eluent was evaporated.

2.4. Analysis of PhIP and methylglyoxal

20 μl of the extracts was added into an HPLC-DAD-MS system, which was composed of a Waters 2707 autosampler, a Waters 600 pump, 5 μm Waters XBridgeTM Shield RP18 250×4.6 mm column (Waters

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Co., USA), and a Waters 2998 diode array detector (Waters Co., USA), linked with a LCQ-Fleet ion-trap mass spectrometer (Thermo Fisher Scientific, USA), utilizing positive ionization mode (ESI+) electrospray ionization interface. Methanol is the B mobile phase, while water/acetic acid (1000/1, v/v) is the A mobile phase. The program begin with 5% methanol,5% to 15% for 0–16 minutes, followed by linear gradients of 25% to 35% methanol for 16–26 minutes, 35% to 45% methanol for 26–30 minutes,45% to 100% methanol for 30–32 minutes,100% methanol for 32–40 min; and 5% methanol for 41–50 min. The mobile phase was supplied in isocratic mode at 0.5 ml/min. The following were the ESI-MS operating conditions: electrospray voltage 5.0 kV, the heated capillary 275 °C. Nitrogen sheath gas was 35, and nitrogen auxiliary gas was 15 (arbitrary units), respectively. For quantification, the PhIP transition of 225.0→210.1 was employed. Regression formula for the PhIP standard was y=6.4421x-168.09; R^2=0.9992; x= the concentration of PhIP, μg/L; y= PhIP peak area. PhIP was retained for around 26.5 minutes, and in this test, the range was 100–10,000 μg/L, about 95% of the PhIP was recovered in the reaction mixes.

The corresponding quinoxaline 2-MQ was used to assess MGO. (Figure 1) [12]. Filtration was performed on the samples using 0.45μL Millex-HNylon filters. (Millipore, Billerica, USA) after being derivatized with OPD. The same HPLC-DAD equipment was then used for analyzing the percolates. A 1 ml OPD solution (0.5 mg/ml) was combined with the reaction solution (1.0 ml), and the mixture was incubated for 12h at 25 °C in dark. The mobile phase began with 5% methanol for 0–5 minutes, followed by linear gradients of 5% to 30% methanol for 5–10 minutes, and 30% to 40% methanol for 10–45 minutes. Ultimately, an elution time of 45 to 65 minutes was achieved using 100% methanol and flow rate was l ml/min. 20 μl was the injection volume. The column temperature is 25 °C. At 315 nm, chromatograms were obtained, while all peaks spectrum data were gathered in the 200–600 nm range.

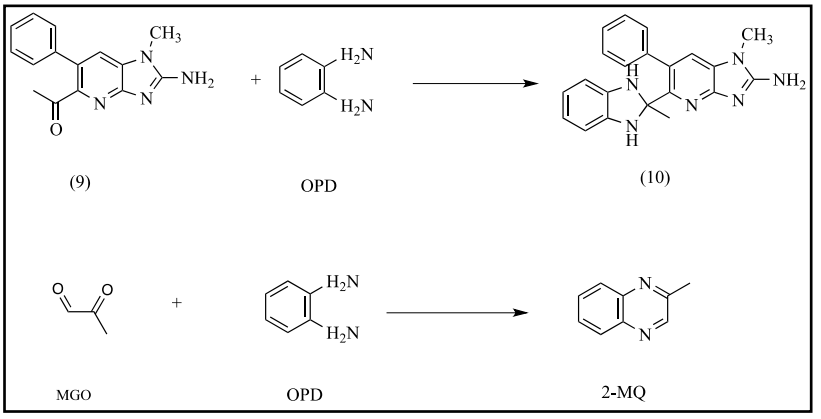

Figure 1. Chemical structure of 2-MQ and compound (10) obtained via derivatization with OPD.

2.5. Analysis of the by-product (9) by HPLC-MS

Because of the instability, the by-product (9) was measured using the corresponding benzimidazoles compound (10) (Figure 1). Filtration was performed on the samples using 0.45μL Millex-HNylon filters (Millipore, Billerica, USA) after sample derivatized with OPD. The same HPLC-DAD-MS equipment was then used for analyzing the percolates. A 1 ml OPD solution (0.5 mg/ml) was combined with the reaction solution (1.0 ml), and the solution was incubated for 12 h at 25 °C in dark. The mobile phase began with 5% methanol for 0–5 minutes, followed by linear gradients of 5% to 30% methanol for 5–10 minutes, and 30% to 40% methanol for 10–45 minutes. Ultimately, an elution time of 45 to 65 minutes was achieved using 100% methanol at a flow rate of l ml/min. The injection volume was 20 μl. It set the column temperature to 25 °C. Chromatograms were recorded at 258 nm, while all peaks spectrum data were gathered in the 200–600 nm range. The operation conditions of ESI-MS are the same as the preceding ones. In this experiment, the mass spectrometer was used to acquire data throughout a mass range of *m/z* 50–500 and *m/z* 357 was detected. The *m/z* 357 was corresponding to the protonated molecular ion of compound (10) ($[M+H]^+$). Figure 2 (B) displays the chromatograms that

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were being monitored in SIM mode at individual m/z values. Chemical structure of the *m/z* 357 ion was analyzed by MS/MS method. From MS/MS fragmentation pattern data, a *m/z* 224 ion was observed, which may be attributed to the loss of $C_6H_4(NH)_2CCH_3$- in compound (10) $[M+H]^+$ in positive ionisation modes. Then the *m/z* 209 ion was observed in the *m/z* 224 ion $[M+H\text{-}C_6H_4(NH)_2CCH_3]^+$ by MS/MS, which may be attributed to loss of a methyl from the precursor ion at m/z 224. So proposed fragmentation pathway of compound (10) (*m/z* 357) are shown in Figure 2(C).

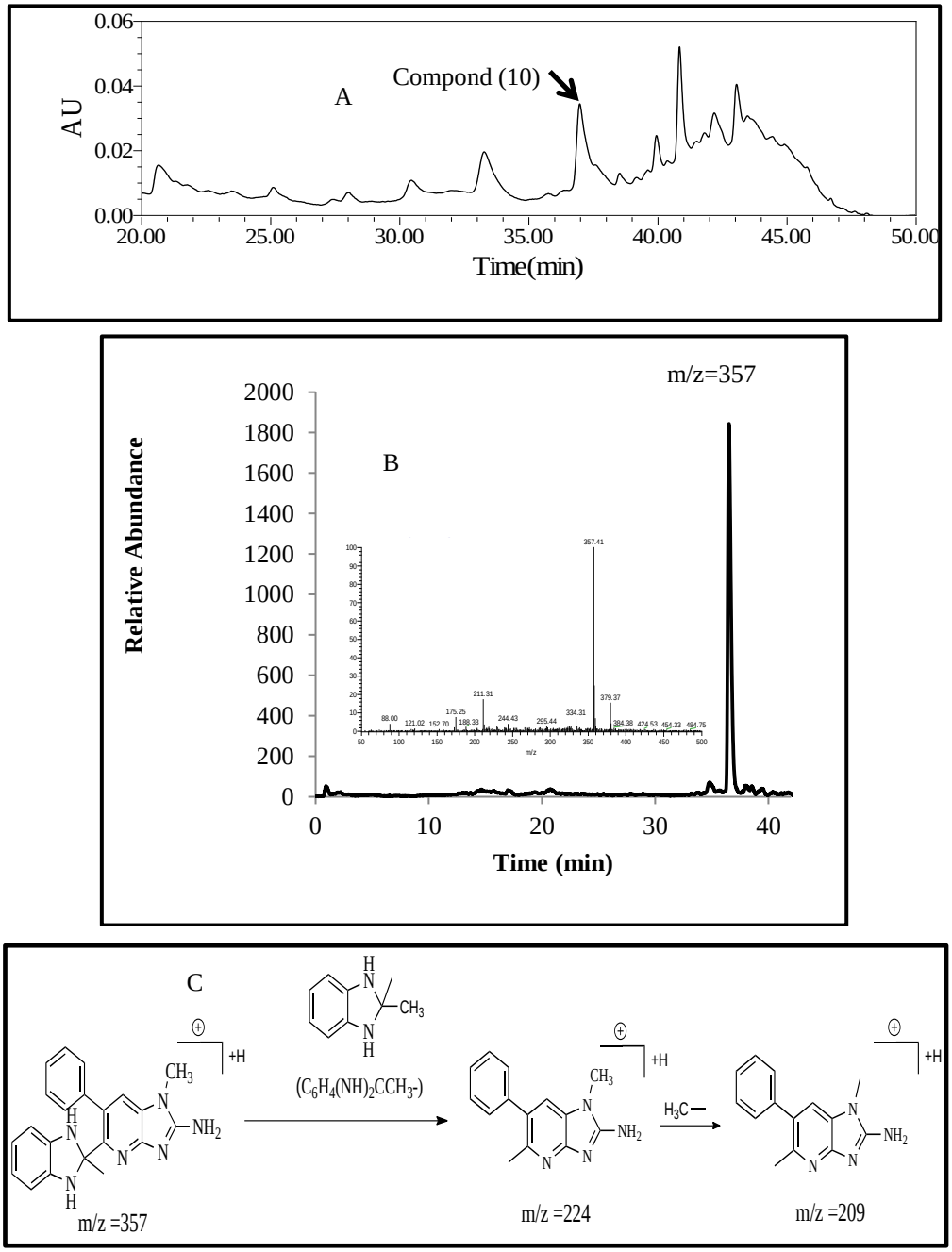

Figure 2. A, DAD chromatogram of compound (10); B, The relative abundance of specific analytes is displayed using MS chromatograms (SIM). (m/z 357); C, Proposed fragmentation pathway of compound (10) (m/z 357).

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2.6. Data analysis

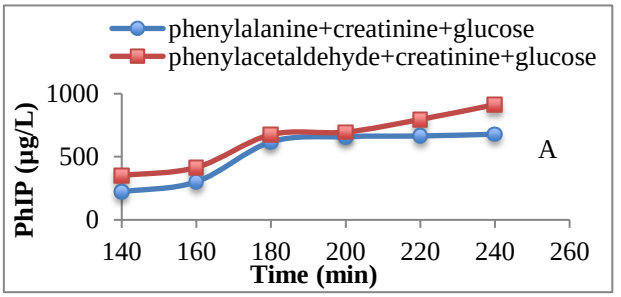
Microsoft excel version 2016 is used for statistical analysis of data. All tests were carried out three times. The average standard deviation is used to express the experimental results.

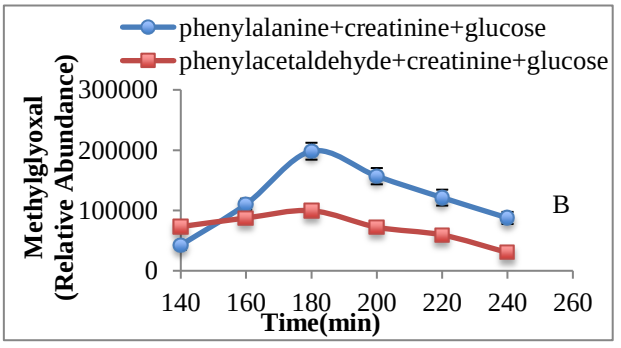
3. Results

3.1. Formation of methylglyoxal, PhIP, and the by-product (9) in creatinine/phenylalanine/glucose/ methylglyoxal reaction mixtures

A solution containing 0.6 mmol of creatinine, 0.6 mmol of phenylalanine, and 0.3 mmol of glucose in 10 ml of water was heated in a closed hood at 200 °C for 140, 160, 180, 200, 220, and 240 min, a small amount of PhIP was formed (Figure 3A). At the same time, methylglyoxal and by-product (9) were detected using HPLC-DAD methods. More details about the change of methylglyoxal and by-product (9) were shown in Figure 3B. The amount of PhIP and by-product (9) formed increased over time, and the amount of methylglyoxal formed initially increased and gradually decreased. Interestingly, the addition of methylglyoxal caused variations in the quantity of PhIP generated in system. Not only did the methylglyoxal assay appear to reduce the amount of PhIP produced, but this reduction was also important. (Figure 3C). Methylglyoxal achieved around 39% inhibition of PhIP in the creatinine/phenylalanine/glucose mixture (Figure 6). In Figure 3C, the amount of by-product (9) formed increased with increasing methylglyoxal concentration.

A solution containing creatinine, phenylalanine, and glucose was heated in a closed hood at 200 °C for 3 h, and the addition of glucose was 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol. The highest amount of PhIP was formed with the addition of glucose (0.3 mmol) (Figure 4). The formation of methylglyoxal and by-product (9) increased with increasing glucose concentration (Figure 4).





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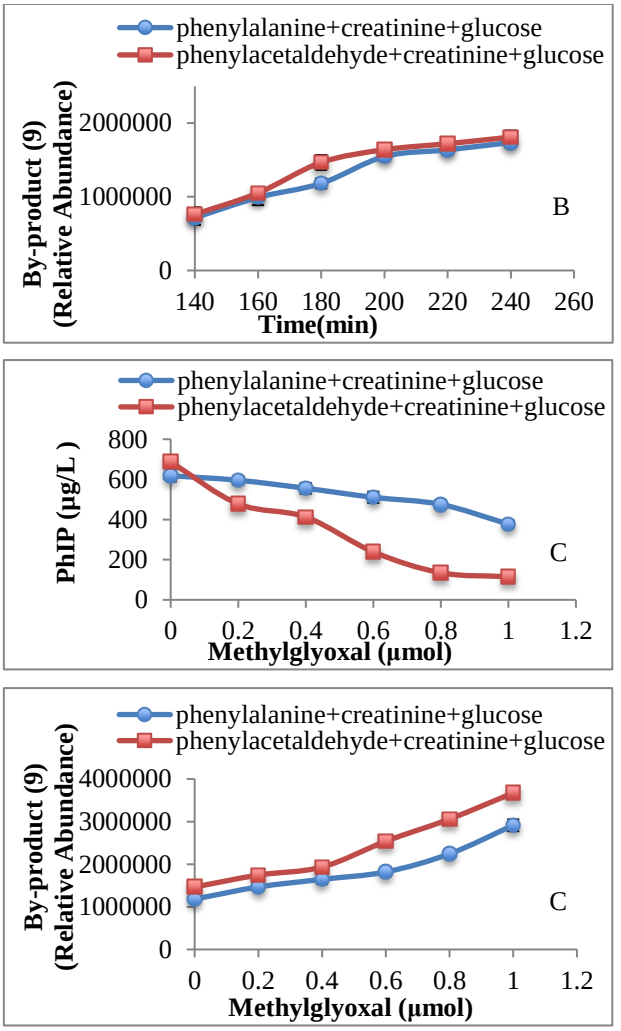

Figure 3. 3A, Impact of temporal variation on PhIP generation in a model system. Samples were heated in 200 °C. Values are mean ± SD (n = 3); 3B, Effect of time on the development of methylglyoxal and by-product (9) in model system. Samples were heated in 200 °C. Values are mean ± SD (n = 3); 3C, Effect of methylglyoxal on the development of PhIP and by-product (9) in model system. Samples were heated for 3 h in 200 °C. Values are mean ± SD (n = 3).

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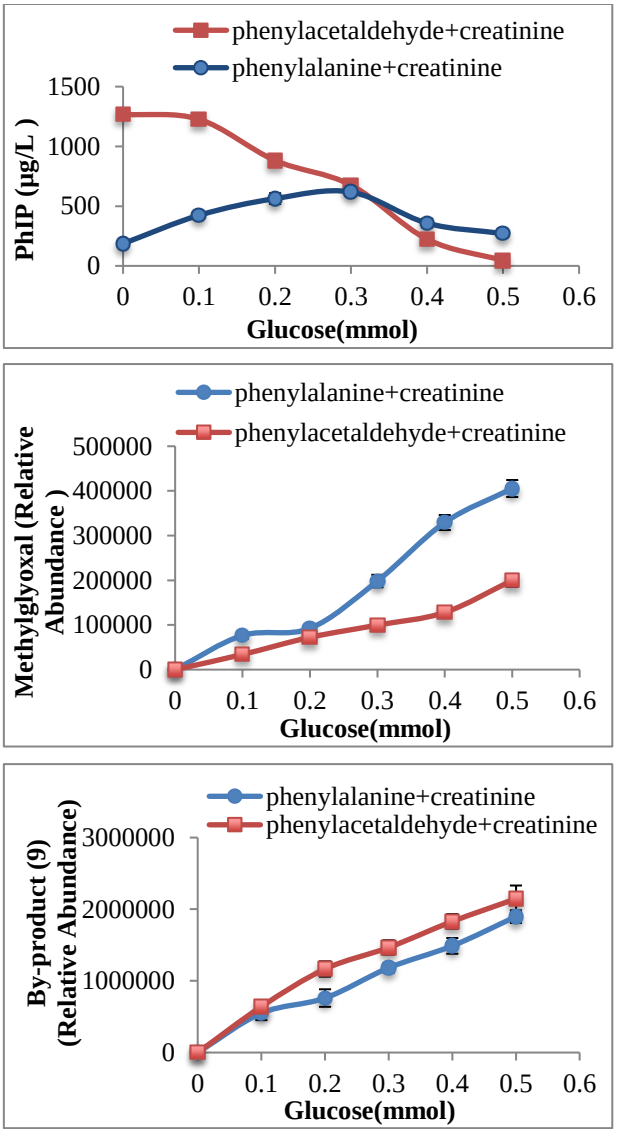

Figure 4. Effect of glucose on the formation of PhIP, methylglyoxal and by-product (9) in model system. Samples were heated for 3 h in 200 °C. Values are mean ± SD (n = 3).

3.2. In creatinine/phenylacetaldehyde/glucose/methylglyoxal reaction combinations, methylglyoxal, PhIP, and the by-product (9) are formed.

After heating a mixture containing glucose, phenylacetaldehyde, and creatinine in a closed hood at 200 °C for 140, 160, 180, 200, 220, and 240 min, PhIP was formed. Unlike the phenomenon that occurred with the addition of phenylalanine, the development of PhIP increased with the addition of phenylacetaldehyde in the same reaction time (Figure 3A). More details about the change of methylglyoxal and by-product (9) are shown in Figure 3B. The trend of methylglyoxal formed was the same as the methylglyoxal formed in creatinine/phenylalanine/glucose reaction system, but when methylglyoxal was added to the creatinine/phenylacetaldehyde/glucose mixture, the amount of produced PhIP decreased much more quickly compared with the PhIP produced in creatinine/phenylalanine/glucose mixtures. Methylglyoxal achieved around 83.2% inhibition of PhIP (Figure 6). The amount of produced by-product (9) increased (Figure 3C).

The solution containing phenylacetaldehyde, creatinine, and glucose was heated in a closed hood at 200 °C for 3 h. Glucose at 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol was added. When the concentration of

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glucose increased, less PhIP was generated. (Figure 4). However, glucose showed dose-dependent effect on the amount of methylglyoxal and by-product (9) (Figure 4).

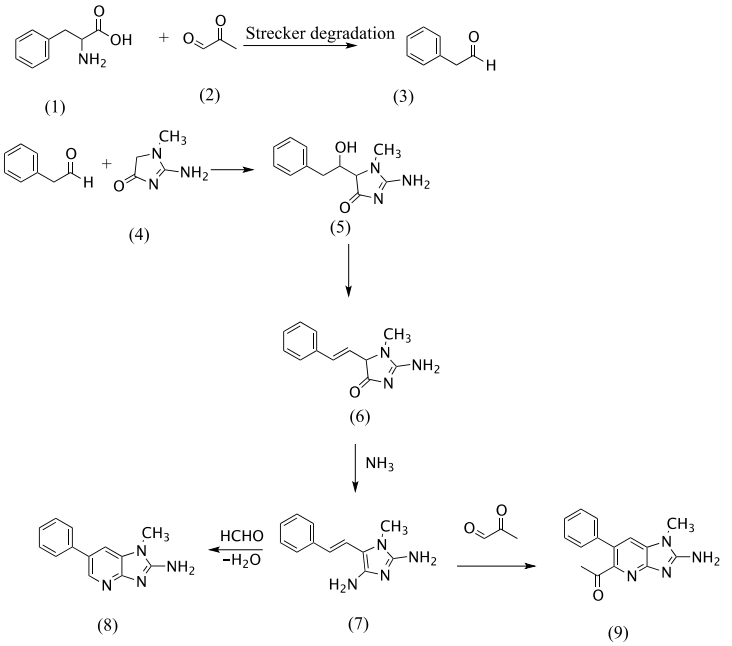

Figure 5. Speculated processes by which methylglyoxal inhibits the synthesis of PhIP.

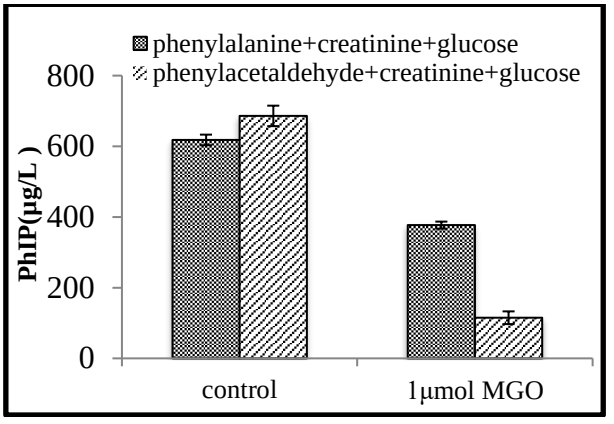

Figure 6. Changes of PhIP in the model system.

4. Discussion

One of the most common HAAs is PhIP. PhIP has been classified by the International Agency for Research on Cancer (IARC), that develops at high temperatures, as potentially carcinogenic to humanity. (Group 2B). A variety of investigations have demonstrated the reaction pathways through which PhIP is formed. Murkovic's group confirmed that phenylacetaldehyde is an intermediate in the reaction [6]. Skog, K., & Jägerstad, M. [14] discovered that creatinine, phenylalanine, and sugar were heated in an aqueous solution to create PhIP, which suggested that reactive carbonyls may have played a role in the reaction. The proportion between creatinine and glucose was close to 1:0.5 and was optimal for the formation of PhIP in modeling studies. However, the reason is also unclear at present.

The present study yielded results that addressed the problem by first identifying other chemicals engaged in the reaction and then clarifying the ways in which the different compounds might react with one other. The most significant ingredient that was absent was methylglyoxal. The amount of

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methylglyoxal formed increased with increasing glucose in Figure 4, and methylglyoxal was absent when glucose was absent. Methylglyoxal is produced from glucose in agreement with the previous studies [11].

When methylglyoxal has been produced, the by-product (9) was detected, and the amount of by-product (9) formed increased with increasing methylglyoxal. However, the amount of PhIP decreased. These data confirm that the by-product (9) is produced during the course of the formation of PhIP in a model reaction. This result is in agreement with the proposed reaction pathways in Figure 5. According to Zöchling and Murkovic, in the reaction system of creatinine, phenylalanine and glucose, and methylglyoxal and phenylalanine would react to produce phenylacetaldehyde

(3) during the strecker degradation. Subsequent reaction with creatinine would occur to yield the corresponding aldol product, after which water loss would occur to produce the corresponding conjugated olefin (6). Compound (6) would react with ammonia to produce the corresponding imine (7). This compound (7) would then react with formaldehyde, according to Rosario Zamora [15], to produce PhIP (8). This imine (7) would react with methylglyoxal as indicated in Figure 5 to produce the by-product (9) and then would suppress the formation of PhIP.

The conclusions of this study and previous studies are explained by this reaction pathway. These chemical pathways concur with the results that were obtained, thereby showing that PhIP yields decreased more with the addition of methylglyoxal to the creatinine/phenylacetaldehyde/glucose reaction mixtures

than to the creatinine/phenylalanine/glucose reaction mixtures (Figures 3C and 6). Because creatinine/phenylacetaldehyde/glucose reaction system did not undergo strecker degradation, methylglyoxal only played a role in suppressing the formation of PhIP. In addition, they also agree with the trend of the formation of PhIP observed with the addition of glucose to the creatinine/phenylacetaldehyde reaction mixtures and to the creatinine/phenylalanine reaction mixtures (Figure 4). In the creatinine/phenylalanine reaction system, when the glucose content reached half the concentration of creatinine, the formation of PhIP was maximum. The balance between the promoting strecker degradation and inhibition of PhIP formation may explain this result by methylglyoxal.

5. Conclusions

When methylglyoxal was introduced to the model system, it inhibited PhIP from establishing and created a byproduct instead. However, methylglyoxal promoted the strecker degradation of phenylalanine to produce phenylacetaldehyde. Meanwhile, this study explained the ways in which glucose are involved in the formation of PhIP.

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