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Effects of some cations on the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in a model system

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ABSTRACT

The present study aimed to investigate in detail the changes to 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and its precursors in the presence of some cations (i.e., K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+}) in a creatinine/phenylacetaldehyde model system. Results showed that PhIP yields decreased when $Fe^{2+}$and Fe^{3+} were added to a mixture of phenylacetaldehyde and creatinine. This decrease may be attributed to the fact that Fe^{3+} can form complexes with various properties with creatinine and accelerate creatinine degradation. This pathway can disturb the reaction with phenylacetaldehyde, influence aldol condensation product formation, and suppress PhIP formation. Furthermore, $Ca^{2+}$and Mg^{2+} enhanced PhIP content. Such enhancement may be attributed to the fact that $CaCl_2$ and $MgCl_2$ promote aldol and aldol condensation product reactions with ammonia and formaldehyde. A possible mechanism for the action of cations during PhIP formation in a model system is also proposed.

1. Introduction

Heterocyclic aromatic amines (HAAs) produced in food during heating at high temperatures are a risk factor for certain human cancers. The formation of HAAs in food has been an ongoing concern among food chemists, nutritionists, and toxicologists because of the potential mutagenic/carcinogenic properties of these compounds (Damašius, Venskutonis, Ferracane, & Fogliano, 2011; Gibis & Weiss, 2012; Nakai & Nonomura, 2013; Turesky & Le Marchand, 2011; Yu, Chen, & Yu, 2016; Zamora, Alcón, & Hidalgo, 2013; Zur Hausen, 2012). Over 25 HAAs have been isolated and identified from food and model systems (Moon & Shin, 2013). Given their potent mutagenic activity and the fact that they can be formed even during ordinary household cooking (Warzecha et al., 2004), a great deal of research effort has focused on developing strategies that can effectively lower HAA contents in food and reduce human exposure to HAAs (Cheng, Chen, & Wang, 2006).

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is one of the most abundant HAA produced in foods. Several researchers have studied the formation mechanism of PhIP, and a number of hypothetical formation pathways of PhIP in various reaction models have been proposed in recent years. PhIP is produced through the reaction of phenylacetaldehyde with creati(ni)ne (Zamora, Alcón, & Hidalgo, 2014; Zamora et al., 2013; Zöchling & Murkovic, 2002). An aldol condensation product with a molecular mass of 215 was found to be an intermediate in this reaction, and the product was later identified as 2-amino-1-methyl-5-(2′-phenylethenyl)-imidazol-4-one (Zöchling & Murkovic, 2002). According to Zamora et al. (2014), PhIP originates from the reaction of this aldol condensation product, formaldehyde, and ammonia; hence, several factors may influence the formation of PhIP.

Recent reports have indicated that metal cations can reduce acrylamide formation in thermally processed foods (Gökmen &

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Şenyuva, 2006, 2007). However, the effects of metal cations on PhIP formation have been ignored in recent literature. In the present paper, we investigated the effects of several cations (K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+}) at different concentrations on the formation of PhIP and its precursors in a creatinine/phenylacetaldehyde model system. A possible mechanism to explain the changes to PhIP and its precursors in the presence of these cations is proposed.

2. Materials and methods

2.1. Materials

PhIP was purchased from Toronto Research Chemicals (North York, ON, Canada). HPLC-grade methanol and acetic acid were purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). The NaCl, KI, $CaCl_2$, $MgCl_2{\cdot}6H_2O$, $FeCl_2{\cdot}4H_2O$, and $FeCl_3{\cdot}6H_2O$ used in this work were of reagent grade and purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals were purchased from Sigma–Aldrich and of analytical-reagent grade. All solutions were prepared with double-distilled water.

2.2. Preparation of model reaction mixtures

A model system composed of creatinine and phenylacetaldehyde was used to study the formation of PhIP in the presence of certain cations (K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}). Creatinine (0.6 mmol) and phenylacetaldehyde (0.6 mmol) were combined in 25-mL PTFE test tubes with a stainless steel exterior liner. Varying amounts of each cation were added to the reaction mixture. The total reaction volume was adjusted to 10 mL with water in each case. The final concentrations of each cation were 0, 0.5, 1, 2, 4, or 8 mM. The pH of the reaction mixture was measured as 6.36 prior to addition of the cations. Adding up to 8 mM of cations to the reaction mixture did not change the pH significantly. The reactants were heated in a closed hood. After heating, the samples were taken and immediately cooled in iced water. Then, 1.0 mL of the reaction solution was mixed with 1 mL of (*o*-phenylenediamine) OPD solution (1 mg/mL in 1 M PBS of pH 7.4) and then reacted at 25 °C for 12 h in the dark. The remainder of the sample was stored at 4 °C for further analyses.

2.3. Analysis of PhIP, creatinine, and aldol condensation product

For HPLC–MS analysis of PhIP, creatinine, and the aldol condensation product, 10 μL of the reaction mixtures were injected into an HPLC–DAD–MS system, which consisted of a 5 μm Waters XBridgeTM Shield RP18 250 × 4.6 mm column (Waters Corp., Milford, MA), a Waters 600 pump, a Waters 2707 autosampler, and a Waters 2998 diode array detector, connected to a LCQ-Fleet ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) using an electrospray ionisation interface in positive ionisation mode (ESI+). Mobile phase **A** was composed of water/acetic acid (1000/1, *v*/*v*) at pH 3.6 (adjusted with 35% of ammonium hydroxide), and mobile phase **B** was composed of methanol. The gradient program was as follows: 5% **B**, 0 min; 5–15% **B**, 0–15 min; 25–35% **B**, 15–25 min; 35–45% **B**, 25–30 min; 45–100% **B**, 30–31 min; 100% **B**, 31–40 min; and 5% **B**, 41–50 min. The mobile phase was delivered at 0.5 mL/min in isocratic mode. The ESI-MS operating conditions included an electrospray voltage of 5.0 kV and nitrogen sheath and auxiliary gases set to 35 and 15 (arbitrary units), respectively. The temperature of the heated capillary was set to 275 °C. The 225.0 → 210.1 transition for PhIP was used for quantification purposes in this study. The regression equation for PhIP standard was $y = 36.986x + 106.7$; $r^2 = 0.9994$; where x = PhIP concentration, μg/L; y = peak area of PhIP. The retention time of PhIP was about 26.5 min. The 114 → 86 transition for creatinine was used for quantification purposes in this study. The regression equation for creatinine standard was $y = 18.225x + 5984.4$; $r^2 = 0.9968$; where x = creatinine concentration, μg/L; y = peak area of creatinine. The retention time of creatinine was about 3.36 min.

According to Zöchling and Murkovic (2002), the first steps of the reaction involve formation of the aldol condensation product between creatinine and phenylacetaldehyde. The aldol condensation product is a substance with an expected molecular mass of 215 dominating the chromatogram. For data acquisition, the mass spectrometer was operated over the mass range of m/z 50–500 and for special identification the SIM (selected ion monitoring) mode was used (m/z 216) for the aldol condensation product of phenylacetaldehyde and creatinine in positive ionisation mode (ESI+).

2.4. HPLC analysis of benzimidazole formed from phenylacetaldehyde with o-phenylenediamine

Given that phenylacetaldehyde cannot be measured directly, it was measured using the corresponding benzimidazole 2-PB (Fig. 1). After derivatisation with OPD, the samples were filtered through 0.45-μm Millex-HNnylon filters (Millipore, Billerica, MA). The percolates were then analysed by an HPLC–DAD system consisting of a 5 μm Waters XBridgeTM Shield RP18 250 × 4.6 mm column, a Waters 600 pump, and a Waters 2998 diode array detector. Approximately 1.0 mL of reaction solution was mixed with 1 mL of OPD solution (0.5 mg/mL) and reacted at 25 °C for 12 h in the dark. The injection volume was 10 μL (sterilised through 0.45-μm filters). The mobile phase was 5% methanol, 0–5 min, and the linear gradient program was as follows: 5–30% methanol from 5 to 10 min, 30–40% methanol from 10 to 45 min, and 100% methanol from 45 to 65 min at a flow rate of l mL/min. The column temperature was set to 25 °C. Spectral data from all peaks were accumulated in the range 200–600 nm, and chromatograms were recorded at 280 nm. The retention time of 2-PB was about 42 min.

3. Results

3.1. Changes of phenylacetaldehyde in model reaction

In this study, the sample was derivatised using OPD before HPLC analysis. Table 1 shows the contents of phenylacetaldehyde in creatinine, phenylacetaldehyde, and some cation reaction mixtures. Addition of K^+ and Na^+ did not significantly influence the residual phenylacetaldehyde. By contrast, addition of Ca^{2+} and Mg^{2+} decreased the residual phenylacetaldehyde content. Interestingly, addition of Fe^{2+} and Fe^{3+} increased the residual phenylacetaldehyde content, and the effect of Fe^{3+} was more pronounced than that of Fe^{2+}.

3.2. Changes of creatinine in model reaction

Table 2 shows residual creatinine contents when creatinine, phenylacetaldehyde, and some cations were heated at 200 °C for 2 h. Addition of K^+ and Na^+ did not significantly influence the residual creatinine. By contrast, addition of Ca^{2+}, Mg^{2+}, Fe^{2+}, and Fe^{3+}

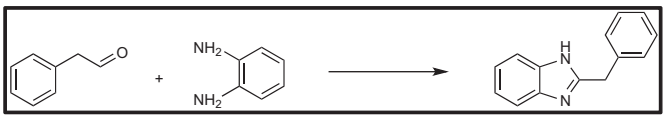

Fig. 1. Chemical structure of the corresponding benzimidazole of phenylacetaldehyde obtained by derivatisation with OPD.

Table 1
Contents of phenylacetaldehyde in a model system containing phenylacetaldehyde, creatinine, and some cations. Samples were heated at 200 °C for 120 min.

Cation amount (μmol)	Phenylacetaldehyde mean ± SD (relative abundance)					
	K^+	Na^+	Mg^{2+}	Ca^{2+}	Fe^{2+}	Fe^{3+}
0	1,094,230 ± 56169[a]	1,094,230 ± 56,169[a]	1,094,230 ± 56,169[a]	1,094,230 ± 56,169[a]	1,094,230 ± 56,169[a]	1,094,230 ± 56,169[a]
5	1,084,725 ± 386,816[a]	1,074,419 ± 125,378[a]	874,607 ± 105,269[b]	435,380 ± 307,373[b]	1,402,617 ± 252,236[b]	3,015,514 ± 114,382[b]
10	1,031,311 ± 53,197[a]	1,084,061 ± 211,973[a]	490,066 ± 133,403[c]	170,871 ± 119,759[b,c]	1,861,692 ± 99,237[c]	6,981,448 ± 196,410[c]
20	1,059,455 ± 169,790[a]	1,175,432 ± 104,001[a]	143,171 ± 71,132[d]	60,603 ± 41,672[c]	2,662,325 ± 131,947[d]	7,946,186 ± 234,042[d]
40	1,090,811 ± 100,417[a]	1,161,456 ± 215,071[a]	59,839 ± 14,551[e]	7580 ± 1009[d]	3,343,453 ± 96,496[e]	10,400,516 ± 874,144[e]
80	1,081,783 ± 337,045[a]	1,075,814 ± 202,019[a]	16,747 ± 7529[f]	6256 ± 742[e]	4,803,449 ± 160,756[f]	12,576,443 ± 143,3905[f]

Means with different letters are significantly different $p < 0.05$.

Table 2
Contents of creatinine in a model system containing phenylacetaldehyde, creatinine, and some cations. Samples were heated at 200 °C for 120 min.

Cation amount (μmol)	Creatinine mean ± SD (μg/L)					
	K^+	Na^+	Mg^{2+}	Ca^{2+}	Fe^{2+}	Fe^{3+}
0	51,542 ± 1308[a]	51,542 ± 1308[a]	51,542 ± 1308[a]	51,542 ± 1308[a]	51,542 ± 1308[a]	51,542 ± 1308[a]
5	60,350 ± 2129[b]	55,777 ± 628[b]	32,553 ± 776[b]	46,351 ± 1147[b]	43,737 ± 829[b]	36,636 ± 736[b]
10	48,766 ± 1234[c]	54,149 ± 1798[a,b]	31,483 ± 785[b]	40,310 ± 1838[c]	37,126 ± 385[c]	21,940 ± 790[c]
20	51,708 ± 630[a]	56,459 ± 885[b]	15,064 ± 274[c]	29,131 ± 1141[d]	27,950 ± 1371[d]	21,480 ± 1334[c]
40	59,197 ± 274[b]	60,568 ± 491[c]	8874 ± 327[d]	10,105 ± 685[e]	19,169 ± 775[e]	8152 ± 242[d]
80	53,704 ± 2355[a]	50,209 ± 759[a]	6705 ± 228[e]	8281 ± 480[f]	11,490 ± 987[f]	4593 ± 91[e]

Means with different letters are significantly different $p < 0.05$.

decreased residual creatinine contents, and the effect of Fe^{3+} was more pronounced than that of Fe^{2+}.

3.3. Identification of the aldol condensation product

Identification of the aldol condensation product in a model system was conducted by comparing molecular masses and UV spectra with the values reported in a previous paper (Zöchling & Murkovic, 2002). The MS (SIM) and DAD chromatograms of the reaction product of creatinine and phenylacetaldehyde in the model system are shown in Fig. 2. The results corresponded to the previously reported data (Zöchling & Murkovic, 2002).

3.4. Changes in the aldol condensation product in the reaction model

Certain amounts of the aldol condensation product were formed after heating the creatinine and phenylacetaldehyde mixture without including any cation in the reaction mixture. Addition of K^+ and Na^+ did not significantly influence the aldol condensation product. By contrast, the amounts of aldol condensation product formed during the reaction significantly decreased with increasing amounts of Ca^{2+} and Mg^{2+} in the reaction mixture. Increasing the amounts of Fe^{2+} and Fe^{3+} also decreased the amount of the aldol condensation product formed, and the inhibition of Fe^{3+} was stronger than that of Fe^{2+} in the reaction model (Table 3).

3.5. Changes of PhIP in model reaction

Table 3 shows the results of PhIP analysis in the model reaction when a solution mixture of creatinine, phenylacetaldehyde, and metal cations was heated at 200 °C for 2 h, and then added with 0, 5, 10, 20, 40, or 80 μmol of metal cations. The amount of PhIP formation did not change significantly with increasing K^+ and Na^+ amounts. The amounts of PhIP formed during the reaction also increased with increasing amounts of Ca^{2+} and Mg^{2+} in the reaction mixture. PhIP content was significantly reduced with increasing Fe^{2+} and Fe^{3+} concentrations, and the effect of Fe^{3+} was more pronounced than that of Fe^{2+}.

4. Discussion

The present literature indicates that Fe^{3+} can induce oxidative decarboxylation of amino acids and formation of Strecker aldehydes because of its high oxidation potential (Bodiga, Eda, & Bodiga, 2014; Nashalian & Yaylayan, 2014; Zhuang et al., 2012). Fe^{3+} can form complexes with various properties with carbonyl and promote further oxidative degradation of these complexes (Nashalian & Yaylayan, 2014). The amount of residual creatinine decreased with increasing Fe^{3+}, as shown in Table 2. By contrast, phenylacetaldehyde contents increased, as shown in Table 1. The amount of the aldol condensation product and PhIP formed also decreased. This phenomenon may be explained as follows: Fe^{3+} can form complexes of various properties with creatinine and accelerate creatinine degradation. This pathway can disturb the reaction with phenylacetaldehyde, influence aldol condensation product formation, and finally suppress PhIP formation. The results obtained in the present study show that the effects of Fe^{3+} and Fe^{2+} are very similar in inhibiting PhIP. Given that Fe^{2+} is oxidised to Fe^{3+} in the reaction, the effect of Fe^{3+} was stronger than that of Fe^{2+} in inhibiting PhIP in the reaction model.

Several studies have shown that $CaCl_2$ and $MgCl_2$ are very good catalysts for promoting formation of the aldol product and the aldol condensation product reactions with ammonia and formaldehyde (Asadullah, Kitamura, & Fujiwara, 2000; Gangadasu, Narender, ChinaRaju, & JayathirthaRao, 2006; Kossev, Koseva, & Troev, 2003; Miura, Nakagawa, & Hosomi, 2002). Thus, the amount of residual phenylacetaldehyde and creatinine decreased with increasing Ca^{2+} and Mg^{2+} contents in Tables 1 and 2, respectively. Meanwhile, because $CaCl_2$ and $MgCl_2$ promote PhIP formation of the aldol condensation product reactions with ammonia and formaldehyde, the amounts of the aldol condensation product formed decreased with increasing amounts of Ca^{2+} and Mg^{2+} in the reaction mixture (Table 3). As a result, the amounts of PhIP formed during the reaction increased with increasing amounts of Ca^{2+} and Mg^{2+} in the reaction mixture (Table 3). Based on previous studies and the present results, the mechanism of some cations on the formation of PhIP in a model system is proposed in Fig. 3. The mechanism contributes significantly to our understanding of the PhIP-inhibitory activity of metal cations, providing useful informa-

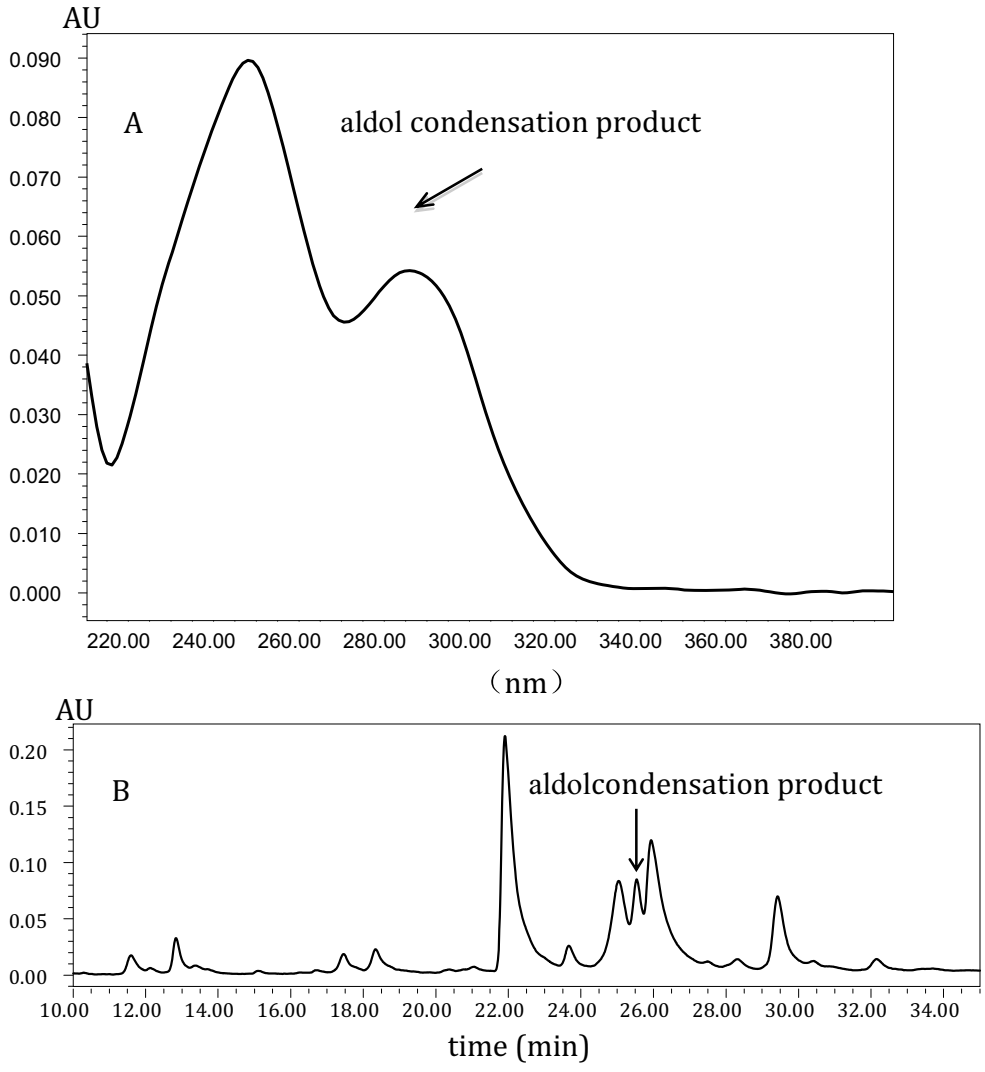

Fig. 2. (A) UV spectrum of the aldol condensation product in the model system; (B) DAD chromatogram of the reaction product of creatinine and phenylacetaldehyde; (C) MS chromatograms (SIM) showing the relative abundance of the selected analytes (*m*/*z* 216) in the model system. Samples were heated at 200 °C for 120 min.

Table 3
Effect of some cations on the formation of the aldol condensation product and PhIP in a model system containing phenylacetaldehyde and creatinine. Samples were heated at 200 °C for 120 min.

Cation amount (μmol)	Aldol condensation product mean ± SD (relative abundance)					
	K^+	Na^+	Mg^{2+}	Ca^{2+}	Fe^{2+}	Fe^{3+}
0	1,383,817 ± 83,929[a]	1,383,817 ± 83,929[a]	1,383,817 ± 83,929[a]	1,383,817 ± 83,929[a]	1,383,817 ± 83,929[a]	1,383,817 ± 83,929[a]
5	1,541,864 ± 76,748[a,b]	1,315,529 ± 97,364[a,b]	1,232,183 ± 67,839[a,b]	531,596 ± 33,784[b]	1,006,154 ± 87,466[b]	566,621 ± 17,278[b]
10	1,472,886 ± 64,778[a,b]	1,212,028 ± 87,177[a,b]	1,117,545 ± 110,938[b,c]	405,443 ± 44,029[c]	824,141 ± 54,747[c]	379,666 ± 26,698[c]
20	1,517,897 ± 54,748[a,b]	1,443,396 ± 99,827[a,b]	422,209 ± 50,297[d]	108,895 ± 13,409[d]	600,029 ± 36,828[d]	201,576 ± 9029[d]
40	1,246,310 ± 89,773[a,c]	1,340,741 ± 89,871[a,b,c]	75,597 ± 6932[e]	35,806 ± 6847[e]	464,263 ± 33,647[e]	134,263 ± 8889[e]
80	1,441,812 ± 98,377[a,b,c]	1,206,419 ± 38,177[b,c]	68,559 ± 5874[e]	24,800 ± 5847[e]	214,244 ± 23,788[f]	61,092 ± 6289[f]
	PhIP Mean ± SD (μg/L)					
	K^+	Na^+	Mg^{2+}	Ca^{2+}	Fe^{2+}	Fe^{3+}
0	634 ± 92.1[a]	633 ± 92.1[a]	633 ± 92.1[a]	633 ± 92.1[a]	633 ± 92.1[a]	633 ± 92.1[a]
5	607 ± 129[a]	673 ± 181[a]	1031 ± 98.3[b]	1481 ± 104[b]	484 ± 30.5[b]	256 ± 44.0[b]
10	659 ± 91.3[a]	621 ± 121[a]	1896 ± 104[c]	2294 ± 61.7[c]	413 ± 32.2[c]	129 ± 12.4[c]
20	569 ± 61.6[a]	613 ± 84.0[a]	2211 ± 76.4[d]	2759 ± 102[d]	317 ± 26.8[d]	81 ± 10.0[d]
40	571 ± 50.8[a]	661 ± 129[a]	2884 ± 36.4[e]	3963 ± 51.3[e]	232 ± 24.4[e]	69 ± 8.9[d]
80	613 ± 132[a]	618 ± 159[a]	2975 ± 62.9[e]	4043 ± 159[e]	204 ± 24.0[e]	33 ± 3.0[e]

Means with different letters are significantly different $p < 0.05$.

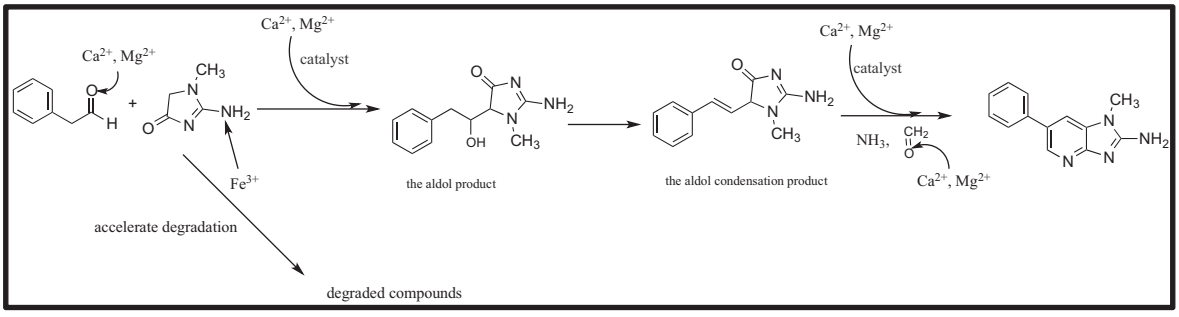

Fig. 3. Proposed mechanism of some cations during PhIP formation in a model system.

tion for further studies in the field of food chemistry and toxicology.

5. Conclusion

Minimisation of the PhIP formed during thermal processing of food is important from the viewpoint of food safety. Metal ions play an important role in the human diet. This study found that Ca^{2+} and Mg^{2+} addition to the model system increased the formation of PhIP, whereas Fe^{3+} and Fe^{2+} addition decreased the formation of PhIP. This study also explained the mechanism of metal cations during PhIP formation in a model system. Drinking water contains various metal ions, and the hardness of water in different places varies despite the assurance offered by numerous regulatory agencies that the domestic drinking water in China is safe. The national standard hardness of drinking water in China is below 4.5 μmol/mL of $CaCO_3$. The concentration range of cations is 0–8 μmol/mL in this study. So this study provides a theoretical basis on whether the use of water with different hardness levels in cooking food can reduce the formation of PhIP. It also provides answers on whether to use the iron wok in cooking.

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