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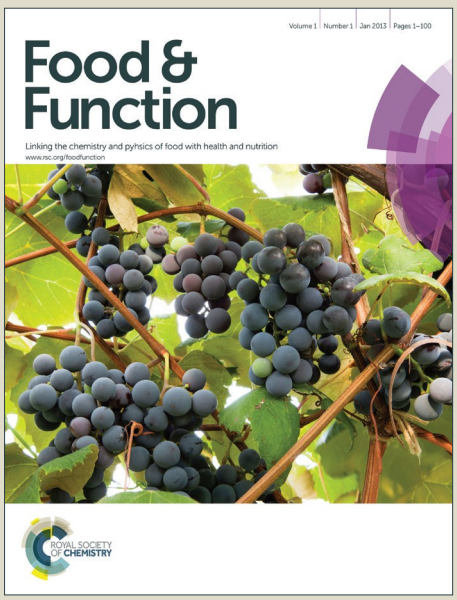

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Effect of waxy rice starch and short chain amylose (SCA) on the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in model system

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Abstract: Starch is a glucose polymer of vast importance to mankind. It forms the major component of all our staple foods. Starch is often used as an important material in the cooking meat. In this study, the effects of waxy rice starch and short chain amylose (SCA) from debranched waxy rice starch on the formation of PhIP in model system were investigated and compared. The results showed that addition of waxy rice starch and SCA significantly decreased PhIP, and the effect of SCA was more pronounced than that of waxy rice starch. This decrease may be attributed to the fact that the glucose residues of starch condense with amino group of creatinine formed N-Glycosyl conjugate. This reaction path could disturb the reaction of creatinine with phenylacetaldehyde, subsequently influence the aldol condensation product formation, and finally suppress the formation of PhIP. Furthermore, the complex spatial structure of waxy rice starch disturb the reaction of glucosyl hydroxyl groups of glucose with amino group of creatinine in model reaction. So the effect of SCA was more pronounced than that of waxy rice starch on suppressing PhIP formation. A possible mechanism of waxy rice starch and SCA during inhibiting PhIP formation in the model system is also proposed.

Keywords: waxy rice starch, short chain amylose (SCA), aldol condensation product, creatinine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

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 [1-6]. On 26 October 2015 in Lyon, France The International Agency for Research on Cancer (IARC), the cancer agency of the World Health Organization, has evaluated the carcinogenicity of the consumption of red meat and processed meat. In recent work was published that nitrogenous base their nucleosides and nucleotides play important role in HAA formation during high temperature processing of food, and the different kind of meat play very important role in HAA formation too, it is dependent on chemical composition of meat (especially free amino acid, protein, nitrogenous compounds and glucose content). Moreover we have known that some polymers in food matrix for example proteins can bind heterocyclic aromatic amines. During digestion in human digestive track bounded HAA may be released and play important role in carcinogenesis [7-13].

Over 25 HAAs have been isolated and identified from food and model systems [14]. Given their potent mutagenic activity and the fact that they can be formed even during ordinary household cooking [15], 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 [16]. Reducing HAA formation is one of the most widely

recognized approaches to attenuate HAA-associated health risks. The thermic 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 [4, 17, 18]. 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 [17]. According to Zamora, Alcón, and Hidalgo (2014), PhIP originates from the reaction of this aldol condensation product, formaldehyde, and ammonia; hence, several factors may influence the formation of PhIP.

Starch is a glucose polymer of vast importance to mankind. It forms the major component of all our staple foods. Starch is a biopolymer and consists of two major components: amylose and amylopectin. Amylose that builds up to 15–35% of the granules in most plants, is a primarily linear polysaccharide with α[1→4]-linked D-glucose units. Some amylose molecules, particularly those of large molecular weight may have up to ten or more branches. Amylopectin, is a highly branched molecule, with α[1→4]-linked D-glucose backbones and exhibits about 5% of α[1→6]-linked branches, which have a profound effect on the physical and biological properties.

Recently, substantial efforts have been made to modify the starch to reduce its digestibility, including temperature-cycled crystallization, enzymatic modification by debranching, hydrothermal treatment, retrogradation treatment, chemical modification, acid modification, and functional starch resources [19, 20]. Among the enzymatic methods, pullulanase debranching and crystallization treatment are cost-effective, safe and more suitable for commercial use[21-23]. Enzymatic modification by debranching generates short linear α[1→4]-linked glucans, resulting from reforming of double helix structure by low temperature crystallization. Waxy rice starch is known as composed of highly polymerized amylopectin [22]. A process for making short chain amylose (SCA) by using debranching waxy rice starch has been reported [21, 24].

The objective of this work was to investigate the effects of SCA and waxy rice starch on the formation of PhIP in model system. A possible mechanism to explain the changes to PhIP and its precursors in the presence of SCA and waxy rice starch is proposed. This information will be useful for better designing starch-based food ingredients with improved health benefits. The results of this study will be useful in producing an effective additive to minimize PhIP formation in cooked meats, the SCA suggest a great potential for practical application in daily cuisine.

Experimental

Materials

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine(PhIP) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Waxy rice starch (0%

amylose) was obtained from Jiangsu Baobao Group (Nantong, China). Pullulanase (EC 3.2.1.41, 2800 ASPU/g) from Bacillus licheniformis was attained from Guangzhou Yulibao Biotechnology Co., Ltd. (Guangzhou, China).

HPLC grade methanol and acetic acid were purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), and were analytical grade. All solutions were prepared by double distilled water.

Experiments

Preparation of short chain amylose (SCA). Waxy rice starch (15 g, dry basis) slurry (10% w/v in diluted pH 5.2 buffer solution containing 0.2 M sodium acetate and 0.2 M acetic acid) was boiled with continuous stirred for 30 min. The temperature of cooked starch was adjusted to 58°C and debranched by pullulanase at 60 ASPU/g of starch for 12 h. The debranched starch gels were proved to be linear [19, 22]. Immediately after the reaction, the starch gel was exposed to a deactivated enzyme reaction at 100 °C for 30 min and cooled to room temperature. The starch gel was stored at 4 °C for 4 days. After that, the precipitated specimen was centrifuged, washed 3 times with distilled water and air-dried then milled to pass through a 100-mesh sieve for the further experiment. The pullulanase debranched starch was designated as 1 time of gelatinization–crystallization. Debranching and crystallization resulted in the formation of the short chain amylose (SCA) crystals.

High-performancesize-exclusionchromatography (HPSEC) and multi-angle laser-light scattering (MALLS) with refractive index (RI) detector. Starch sample (12.5 mg) was stirred in 25 mL of dimethyl sulfoxide (DMSO) which contain 50 mM LiBr and heated it in a boiling water bath for 30min. After that, the liquid system was stirred for 24h at room temperature. The solutions were then filtered through 0.22 μm Millex-HNnylon filters (Millipore, Billerica, USA) before injection into the MALLS system (Wyatt Technology, Santa Babara, CA, USA) consisting of a pump (P2000, Spectra System, San Hose, CA, USA), an injector valve with a 1mL loop, SEC column (P8514-806, Showa Denko, Tokyo, Japan), a MALLS fitted with an argon laser (488 nm), and an Optilab 903 refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The sample (1 mL) was injected into the system and ran at a flow rate of 0.3 mL/min. The mobile phase was DMSO and degassed under vacuum. The column oven temperature was controlled at 40 °C. The molecular weights were calculated using the ASTRA 6.1 software program (Wyatt Technology).

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 waxy rice starch and short chain amylose (SCA). Creatinine (0.6 mmol) and phenylacetaldehyde (0.6 mmol) were combined in 25 mL PTFE test tubes with a stainless steel exterior liner. The containers were placed in a forced air oven at 200 °C for 3 h. Varying amounts (0, 0.01, 0.025, 0.05, 0.075, and 0.1 g) of waxy rice starch and SCA were respectively added to the reaction mixture. The total reaction volume

was adjusted to 10 mL with water in each case. The pH of the reaction mixture was measured as 6.36 prior to addition of waxy rice starch and SCA. Adding up to 0.1 g of waxy rice starch and SCA 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 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.

Analysis of glucose and maltose in model reaction. The analysis of glucose and maltose were performed in an HPLC- refractive index detector (RID) system, which consisted of a Sugar-Pak^{TM}1 6.5×300 mm column (Waters Co., USA), a Waters 600 pump and a Waters 2414 refractive index detector (Waters Co., USA). The injection volume of the reaction mixtures was 20 μL (sterilized through 0.22 μm filters), and the mobile phase was 0.0001M Ethylenediaminetetraacetic acid calcium disodium salt hydrate solution at a flow rate of 0.5 mL/min. Column temperature was set at 90 °C. The retention time of glucose was about 10 min. The retention time of maltose was about 8 min. HPLC-RID analysis showed glucose and maltose were not present in the reaction mixtures.

Analysis of PhIP, creatinine, and aldol condensation product. For HPLC-MS analysis of PhIP, creatinine, and the aldol condensation product, 20 μL (sterilized through 0.22 μm filters) of the reaction mixtures was injected into an

144 HPLC-DAD-MS system, which consisted of a 5 μm Waters XBridgeTM Shield RP18
145 250×4.6 mm column (Waters Co., USA), a Waters 600 pump, a Waters 2707
146 autosampler, and a Waters 2998 diode array detector (Waters Co., USA), connected to
147 a LCQ-Fleet ion-trap mass spectrometer (Thermo Fisher Scientific, USA) using an
148 electrospray ionization interface in positive ionization mode (ESI+). Mobile phase A
149 was composed of water/acetic acid (1000/1, *v/v*) at pH 3.6 (adjusted with 30% of
150 ammonium hydroxide), and mobile phase B was composed of methanol. The gradient
151 program was as follows: 5% B, 0 min; 5%–15% B, 0–15 min; 25%–35% B, 15–
152 25 min; 35%–45% B, 25–30 min; 45%–100% B, 30–31 min; 100%B, 31–40 min; and
153 5%B, 41–50 min. The mobile phase was delivered at 0.5 mL/min in isocratic mode.
154 The ESI-MS operating conditions included an electrospray voltage of 5.0 kV and
155 nitrogen sheath and auxiliary gases set to 35 and 15 (arbitrary units), respectively. The
156 temperature of the heated capillary was set to 275 °C. The 225.0→210.1 transition for
157 PhIP was used for quantification purposes in this study, and the retention time of PhIP
158 was about 26.5 min. The 114→86 transition for creatinine was used for quantification
159 purposes in this study, and the retention time of creatinine was about 3.36 min.

160 According to Zöchling and Murkovic (2002), the first steps of the reaction involve
161 formation of the aldol condensation product between creatinine and
162 phenylacetaldehyde. The aldol condensation product is a substance with an expected
163 molecular mass of 215 dominating the chromatogram. For data acquisition, the mass
164 spectrometer was operated over the mass range of m/z 50–500 and for special

identification the SIM (single ion monitoring) mode was used m/z 216 for the aldol condensation product of phenylacetaldehyde and creatinine in positive ionisation mode (ESI+).

HPLC analysis of benzimidazole formed from phenylacetaldehyde with *o*-phenylenediamine (OPD). Given that phenylacetaldehyde cannot be measured directly, it was measured using the corresponding benzimidazole 2-PB (Figure 1). After derivatization with OPD, the samples were filtered through 0.22 μm Millex-HNnylon filters (Millipore, Billerica, USA). The percolates were then analyzed by an HPLC-DAD system consisting of a 5 μm Waters XBridgeTM Shield RP18 250×4.6 mm column (Waters Co., USA), a Waters 600 pump, and a Waters 2998 diode array detector (Waters Co., USA). 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 (sterilized 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 min to 10 min, 30%–40% methanol from 10 min to 45 min, and 100% methanol from 45 min 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.

Statistical analysis. Means and standard deviations of the data were calculated for each treatment. Analysis of variance was performed to determine any significant

differences ($p < 0.05$) among the treatments, using SPSS 17.0 for Windows.

Results and discussion

Weight-average molar mass, gyration radius and molecular density. The weight-average molar mass (M_W), gyration radius (R_Z) and molecular density (ρ) from the starch samples are shown in Table 1. The M_W of native waxy rice starch was larger than the debrabched starch. This indicated that native waxy rice starch was composed of highly polymerized amylopectin. The M_W of native waxy rice starch obtained in our study was similar to that reported in [25].

The lower M_W of the debrabched starch resulted from the degradation of α-(1→6) glycosidic by pululanase debrabching. The radius of gyration (R_Z) is related to the theoretical probability of finding the molecule at a given distance from the center. The larger proportion of long branch-chains of amylopectin of the waxy rice starch may result in higher R_Z, since it is dependent on the volume occupied by the molecule in solution [26]. A higher R_Z and a lower dispersed molecular density ($\rho=M_W/R_Z^3$) was found in native starch than the debrabched starch. These results indicated that the structure of the debrabched starch molecules has been changed on the process of pululanase debrabching. Debranching and crystallization resulted in the formation of the short chain amylose (SCA) crystals.

Identification of the aldol condensation product. The identification of the aldol condensation product in model system was done by comparing the molecular mass and UV spectra with the paper reported [17]. The MS chromatogram (SIM) and DAD

chromatogram of the reaction product of creatinine and phenylacetaldehyde in the model system are shown in Figure 2. The results corresponded to the data of the paper reported [17].

Study of waxy rice starch and SCA in the model reaction. When creatinine, phenylacetaldehyde, and waxy rice starch/SCA were heated at 200 °C for 3 h, Figure 3 and Figure 4 shows residual creatinine and phenylacetaldehyde contents, respectively. Addition of waxy rice starch and SCA significantly decreased the residual creatinine, and the effect of SCA was more pronounced than that of waxy rice starch. However, the amount of the residual phenylacetaldehyde increased with increasing waxy rice starch/SCA amount, and the effect of SCA was more pronounced than that of waxy rice starch. In previous studies [4, 17, 18], the aldol condensation product have been shown to be intermediate products in the reaction of phenylacetaldehyde with creatinine. In this study Figure 5 showed that the amount of the aldol condensation product formation decreased with increasing waxy rice starch/SCA amount. Meanwhile, the amount of PhIP also decreased Figure 6. Interestingly, the effect of SCA was more pronounced than that of waxy rice starch. This phenomenon may be explained as follows: Waxy rice starch and SCA were consisted of α-D-glucose polymers [27]. Mechanistic studies have proposed that glycoconjugates, such as N-glycosides and related compounds formed in the early phase of Maillard reaction [28-30]. Based on these studies, the glucose residues might condense with amino group of creatinine formed N-Glycosyl conjugate. This reaction

path could disturb the reaction of creatinine with phenylacetaldehyde, subsequently influence the aldol condensation product formation, and finally suppress the formation of PhIP. Figure 7 presented the proposed pathway of inhibition of PhIP formation in the presence of waxy rice starch and SCA. But the main components of waxy rice starch and SCA are different. Waxy rice starch is known as composed of highly polymerized amylopectin [22]. In this study SCA is composed of the short chain amylose. Amylose has an essentially unbranched α[1→4] linked glucan, and amylose chains can form double helices. The glucosyl hydroxyl groups of α[1→4] glucose chains are located on the outer surface of the helix [27]. This characteristic structure of amylose may easily produce to reaction of SCA with creatinine. However, the structure of amylopectin is very complex, and amylopectin has chains of α[1→4] linked glucoses arranged in a highly branched structure with α[1→6] branching links [23]. The amylopectin branches may be classified according to their pattern of substitution: A-chains are defined as unsubstituted, B-chains are substituted by other chains, and there is a single C-chain that caries the reducing glucose. Meanwhile, the complex spatial structure of amylopectin disturb the reaction of glucosyl hydroxyl groups of glucose with amino group of creatinine in model reaction. So the effect of SCA was more pronounced than that of waxy rice starch on suppressing PhIP formation.

Conclusion

The SCA showed a high level of ability of inhibition of PhIP in model reaction. Starch is often used as an important material in the cooking meat. Therefore, the SCA may be applicable to various meat systems. The results of the current study would be useful in producing an effective additive to minimize PhIP formation in cooked meats, the SCA suggest a great potential for practical application in daily cuisine.

Acknowledgements

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Figure Legends:

Figure 1. Chemical structure of corresponding benzimidazole of phenylacetaldehyde obtained via derivatization with OPD.

Figure 2. A, UV spectra of the aldol condensation product in model system; B, DAD chromatogram of reaction product of creatinine and phenylacetaldehyde; C, MS chromatograms (SIM) showing relative abundance of selected analytes (*m/z 216*) in model system. Samples were heated for 180 min in 200 °C.

Figure 3. Contents of the residual creatinine in the model system containing phenylacetaldehyde, creatinine, and waxy rice starch/SCA. Samples were heated at 200 °C for 180 min.

Figure 4. Contents of the residual phenylacetaldehyde in the model system containing phenylacetaldehyde, creatinine, and waxy rice starch/SCA. Samples were heated at 200 °C for 180 min.

Figure 5. Effect of waxy rice starch and SCA on the formation of the aldol condensation product in the model system containing phenylacetaldehyde and creatinine. Samples were heated at 200 °C for 180 min.

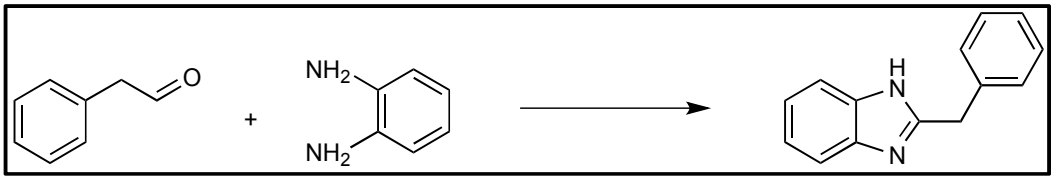
Figure 6. Effect of waxy rice starch and SCA on the formation of PhIP in the model system containing phenylacetaldehyde and creatinine. Samples were heated at 200 °C for 180 min.

Figure 7. Proposed pathways for the inhibitory activity of waxy rice starch and SCA against PhIP formation.

Table 1. Weight-average molar mass, radius of gyration and molecular density of starches

Sample	$M_w \times 10^5$ (g/mol)	R_z (nm)	ρ (g/mol/nm^3)
Native waxy rice starch	1037.75 ± 116.32^a	337.35 ± 32.74^a	2.83 ± 1.11^b
SCA	3.69 ± 0.18^b	25.95 ± 0.49^b	21.10 ± 0.18^a

M_w: weight-average molar mass; R_z: z-average radius of gyration; ρ: molecular density (M_w/R_z^3). Values with different superscripts within a column are significantly different ($P<0.05$).Experimental data are the means of duplicates.



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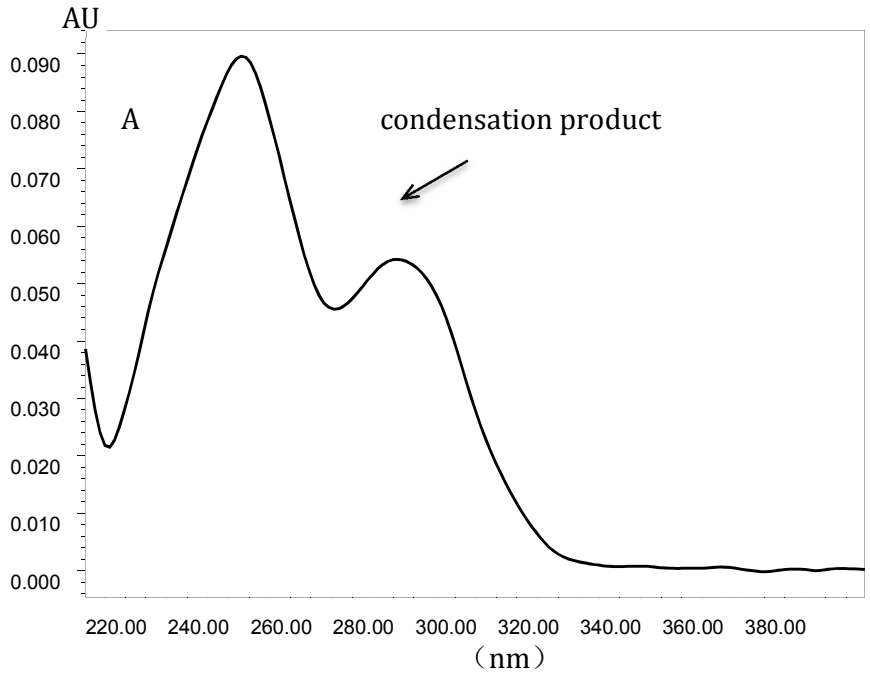
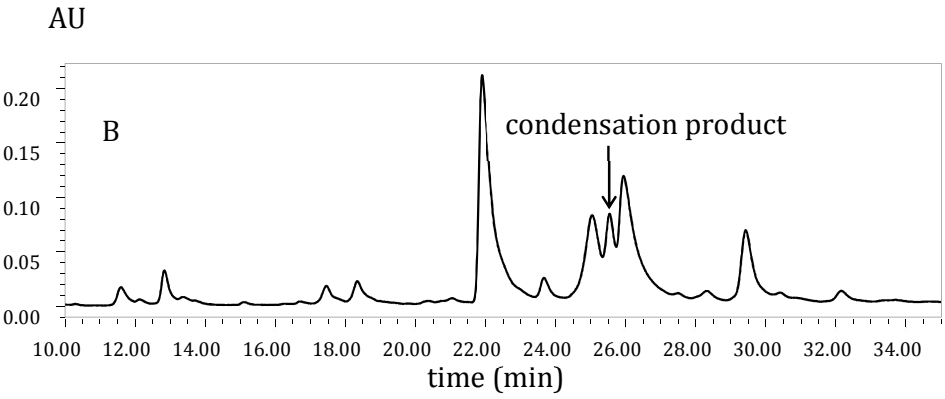




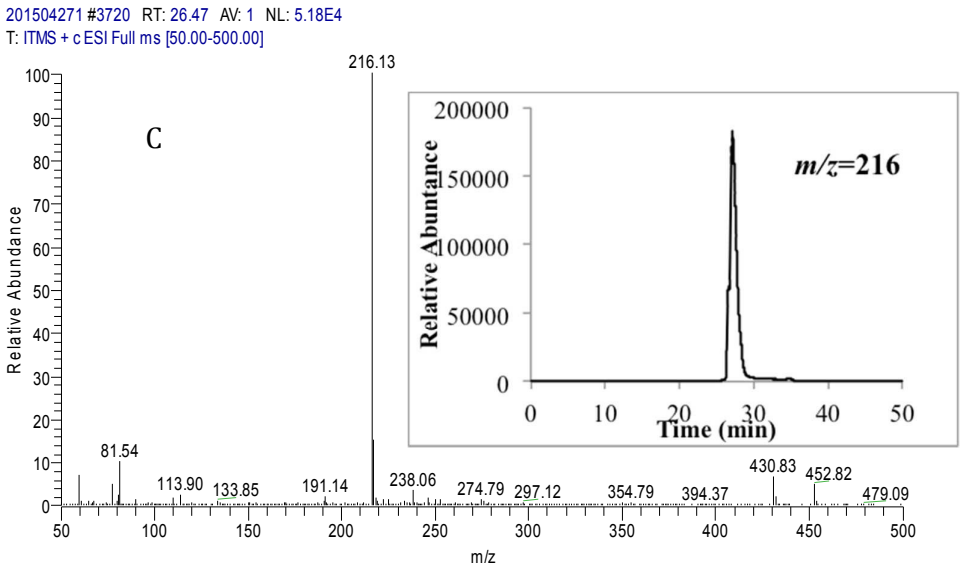
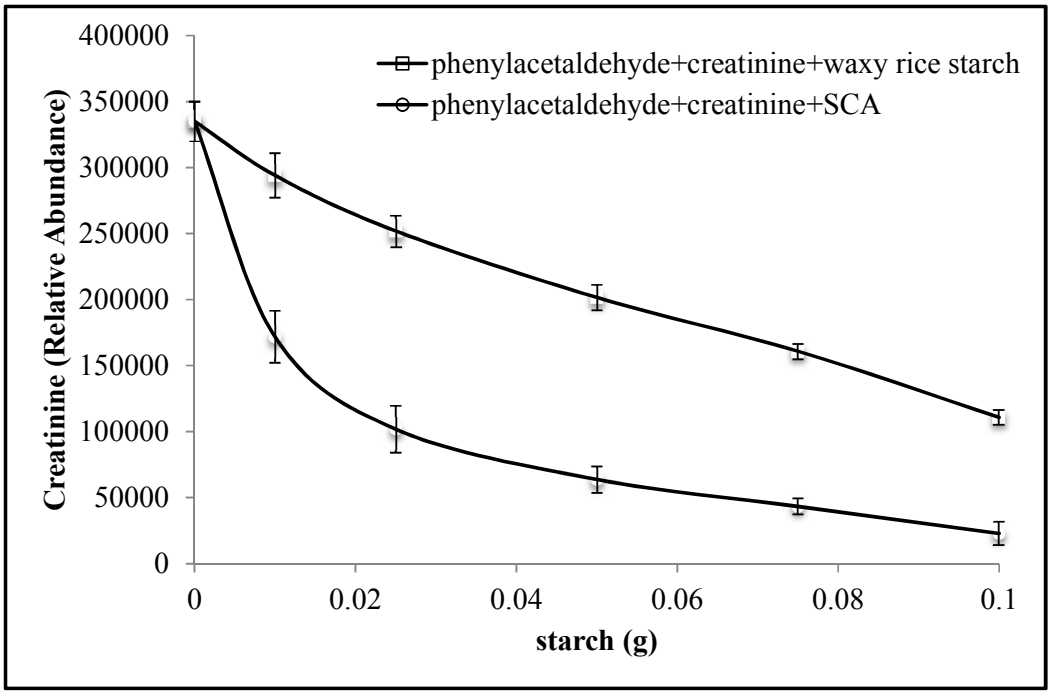


Figure 2



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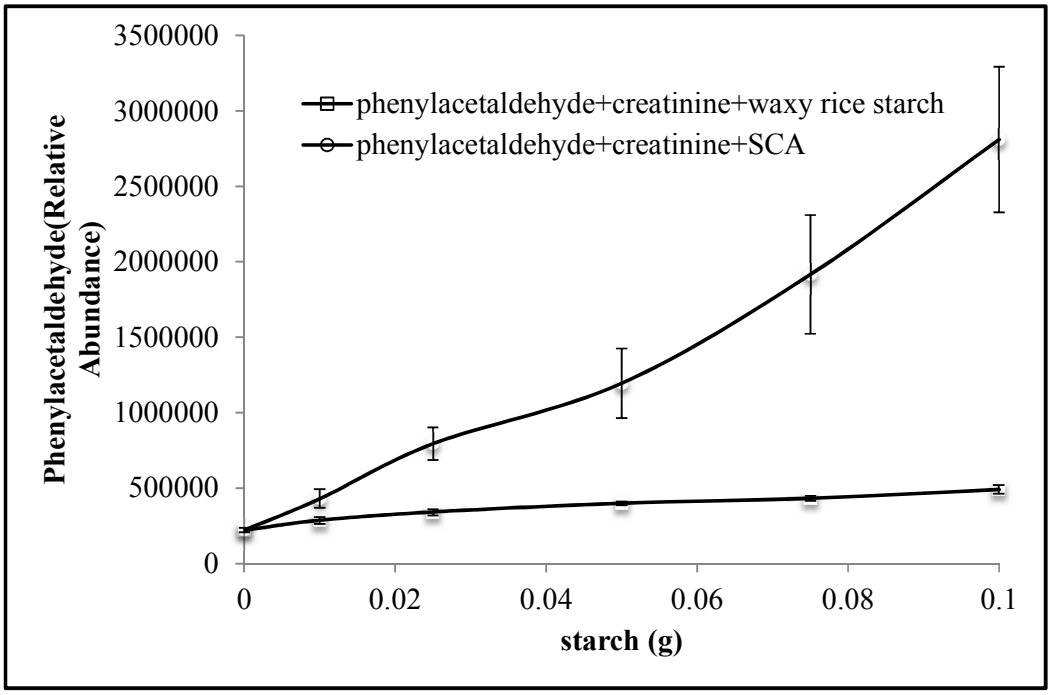

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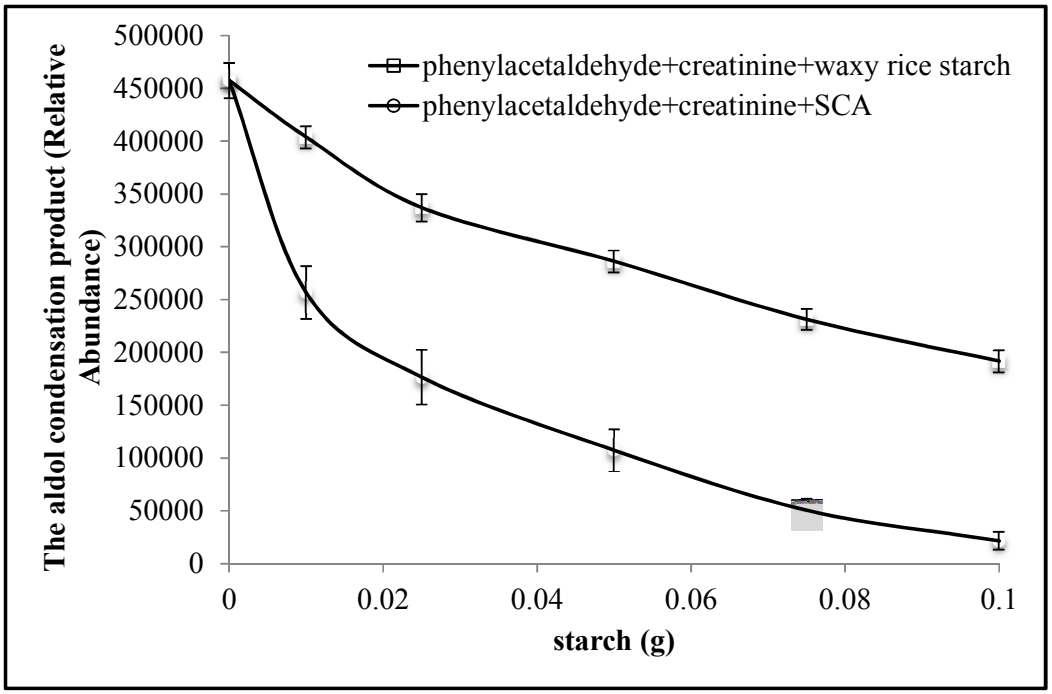


Figure 5

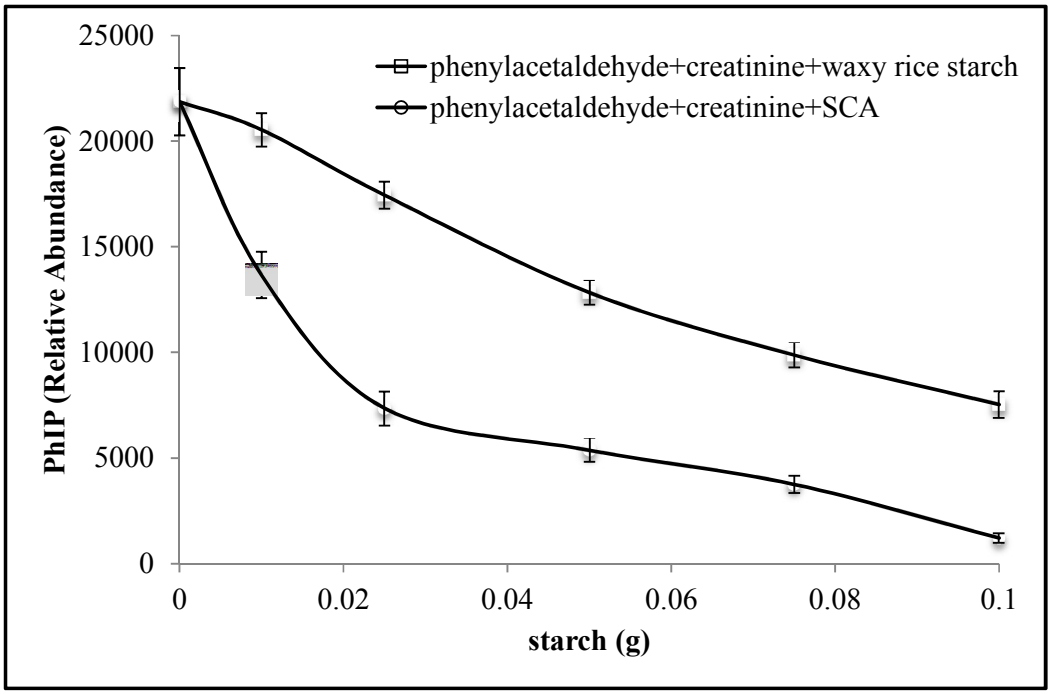


Figure 6

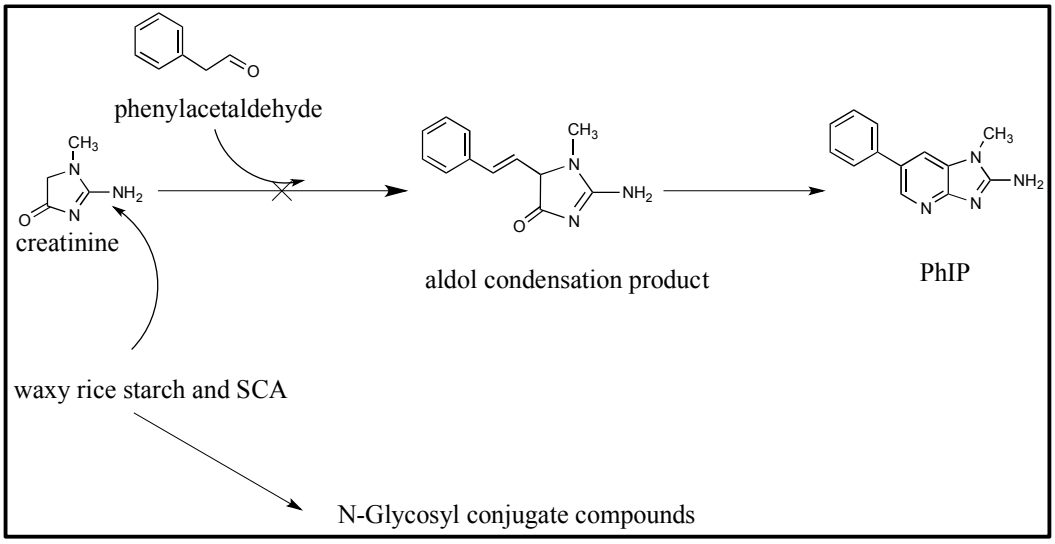
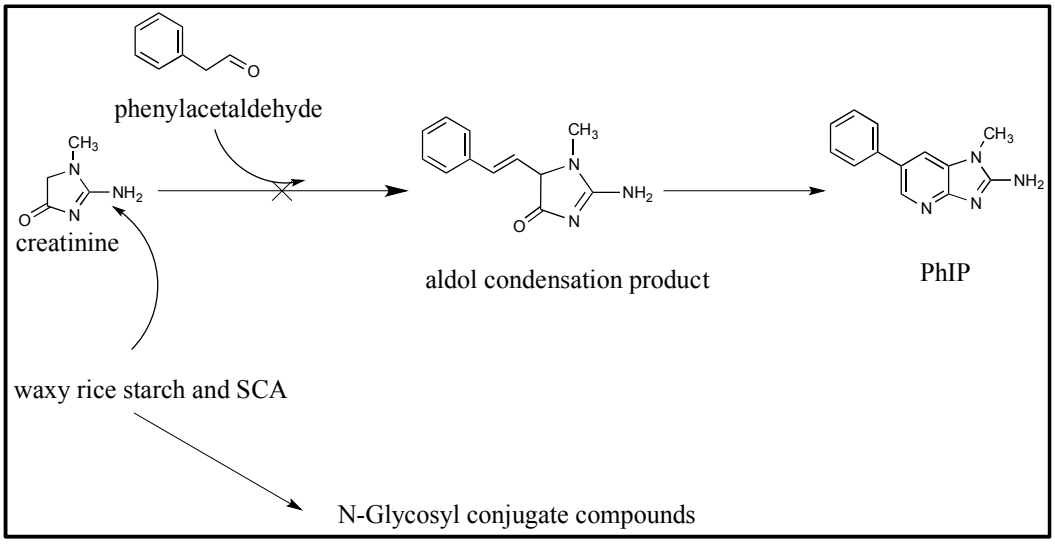


Figure 7



A possible mechanism of waxy rice starch and SCA during inhibiting PhIP formation in the model system is also proposed.