Anthraquinones isolated from the browned Chinese chestnut kernels (*Castanea mollissima blume*)

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Abstract. Anthraquinones (AQS) represent a group of secondary metallic products in plants. AQS are often naturally occurring in plants and microorganisms. In a previous study, we found that AQS were produced by enzymatic browning reaction in Chinese chestnut kernels. To find out whether non-enzymatic browning reaction in the kernels could produce AQS too, AQS were extracted from three groups of chestnut kernels: fresh kernels, non-enzymatic browned kernels, and browned kernels, and the contents of AQS were determined. High performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) methods were used to identify two compounds of AQS, rhein(1) and emodin(2). AQS were barely exists in the fresh kernels, while both browned kernel groups sample contained a high amount of AQS. Thus, we confirmed that AQS could be produced during both enzymatic and non-enzymatic browning process. Rhein and emodin were the main components of AQS in the browned kernels.

1. Introduction

The Chinese chestnut, *Castanea Mollissima Blume*, has been utilized as food since ancient times in northern China, and are now widely distributed and consumed around the world [1]. In the processing and storage of chestnuts, browning is a major problem as it brings undesirable sensory properties changes and may even produce some harmful products [2]. It is very important to get farther
understanding of the browning reaction. Currently, the main substrates and enzymes in the enzymatically browned kernels were well studied [3-9]. Phenolic compounds in chestnuts are mediated by polyphenol oxidase (PPO) and peroxidase (POD) initially producing quinones, which rapidly condense to produce relatively insoluble brown polymers [10]. But there were few studies referring to the components and the change of components in browned kernels.

Anthraquinones (AQS) represent a group of secondary metallic products in plants. AQS derivatives and AQS-containing plants are widely used as mild laxatives [11]. AQS are also characterized by their antibacterial, antiviral [12-13], antifungal [14-15], antioxidant [16], and anticancer [17] properties. AQS are often naturally occurring in plants and microorganisms.

In our previous study, we investigated the components in the enzymatic browned Chinese chestnut kernels and found that rhein and emodin were the main components of AQS in the browned kernels [18]. It was the first time that AQS were found in the enzymatic browned chestnut kernels.

In the present study, to find out whether AQS could be produced by non-enzymatic browning reaction, AQS were extracted from three groups of Chinese chestnut:

a. fresh kernels group, which was set as control group to confirm that AQS were produced by browning rather than already exited in the fresh chestnut kernels;

b. non-enzymatic browning kernels group, which was set to find out whether this type of browning could produce AQS too;

c. browned kernels, which were browned by two pathways: enzymatic and non-enzymatic browning.

Then the AQS contents of these three groups were determined. HPLC and NMR were used to identify AQS in group c.

The result of this study might be helpful to understand the safety of the browned products in Chinese chestnuts kernels and the formation of AQS in browning reactions.

2. Materials and methods

2.1. Plant materials
Chinese chestnuts (Castanea mollissima), called Yanshan Red Chestnuts, were purchased from the Miyun District, Beijing.

2.2. Reagents
All solvents used for extraction of AQS were of analytical grade. Rhein (>99%) and emodin (>99%) used for high-performance liquid chromatography (HPLC) analysis were of HPLC grade and were purchased from the National Institutes for Food and Drug Control (BJ, China). Water used for HPLC analysis was purified by a Milliporeultra-pure water system (0.4-mm filter; Watford, UK).

2.3. Extraction of AQS
Fresh kernels of Chinese chestnuts were powderized (~20 mesh). The powder was separated into 3 groups: fresh kernels, non-enzymatic browned kernels and browned kernels as discribed in the instruction part. The powder for non-enzymatic browned kernels group was processed in a microwave
After heating at 45°C for 60 min. The alcoholic solution was recycled with a rotary vacuum evaporator at 70°C under 31kpa. The sediments were hydrolyzed with 50mL 2.3 M sulfuric acid in a 250mL flask followed by heating at 100°C for 60 min. After cooling down, the solution was extracted three times with trichloromethane. The combined extracts were evaporated at 45°C under 12kpa. The residue in browned paste state was resuspended in methanol (total volume: 50mL) for AQS analyses.

2.4. AQS contents
The AQS content of the extracts were determined by the modified method of Yuan Y [19]. Briefly, 10mL extract solution was mixed with 0.5g/100mL acetic acid-magnesium-methanol (2mL) and diluted with methanol to a total volume of 50mL. The absorbance was measured against methanol at 510nm by spectrophotometry (TU-1810, Beijing, China). AQS contents were calculated from the calibration curve of 1,8-hydroxyl AQS and expressed as 1,8-hydroxyl AQS equivalents.

2.5 Separation and purification of AQS
The browned kernels sample was used to separate and purify AQS according to the method of Yang XY [20]. AQS was sequentially extracted with 5g/100mL aqueous sodium bicarbonate. The extraction was completed in three cycles with a 3-h static period. The extracts were collected and acidified with 2.3 M hydrochloric acid. The yellow sediments were rinsed with distilled water. These extracts were crystallized three times with acetic acid, and crystals of Compound 1 (purity of 92%) were collected. Compound 2 was extracted with 5% aqueous sodium carbonate from the AQS after extracted with 5% aqueous sodium bicarbonate. Acidification and crystallization were the same as Compound 1. The resulting crystals were Compound 2 (purity of 92.5%).

2.6 HPLC Analysis
Compound 1 and 2 were analyzed by HPLC (Shimadzu SCL-10AVP, Japan) equipped with a diode array detector (UV at 254nm); the column was a Keystone (Bellefonte, PA) ODS/3v 250 mm, 4.0 mm i.d., 5 µm; the oven was set to 30°C; the mobile phase was 75% methanol/25% water with an isocratic flow rate of 1.0mL/min; the injection volume was 10µL, and the run length was 6 min. The elution time of rhein was approximately 2.8 min, and the elution time of emodin was approximately 3.5 min.

2.7 NMR spectrometer Analysis
The structures of the two compounds were determined using an ECA-600 spectrometer (JEOL, Tokyo, Japan). Both $^1$H- and $^{13}$C-NMR spectra were determined in DMSO-d6 solvent, using TMS as an internal standard. The chemical shifts are expressed in ppm values, referenced to TMS ($\delta_H = 2.5$ ppm) for the $^1$H-NMR spectra and the residual solvent signal ($\delta_c = 39.5$ ppm) for the $^{13}$C-NMR spectra. All spectra were recorded at ambient temperature. $^1$H-NMR was measured using a frequency of 600 MHz, a 90° pulse supplied from the decoupler, a 1.455-s acquisition time, and a 1-s relaxation delay.
$^{13}$C-NMR was measured using a frequency of 150 MHz, a 90° pulse supplied from the decoupler, a 0.692-s acquisition time, and a 2-s relaxation delay.

2.8 Statistical analysis
The experiments for determination of AQS contents were performed in triplicate and the results were expressed as the mean ± standard deviation. Statistical comparisons were made using the Student’s t-test. Statistical analyses were carried out by the Statistical Analysis System (SAS, 1985) software package. Analysis of variance was performed by analysis of variance procedures.

3. Results and discussion
The contents of AQS in browned Chinese chestnut kernels were summarized in Table 1. The total amount of AQS was significantly greater in browned kernels (11.18 μg/mL) than in fresh kernels (0.08 μg/mL) and in non-enzymatic browned kernels (0.53 μg/mL). These results illustrated that AQS could be produced during both enzymatic and non-enzymatic browning reaction in the kernels of Chinese chestnuts.

Table 1. AQS contents in three samples (μg/mL).

| Samples                              | AQS             |
|--------------------------------------|-----------------|
| Extracts in fresh kernels            | 0.08±0.02$^A$   |
| Extracts in non-enzymatic browned kernels | 0.53±0.12$^B$ |
| Extracts in browned kernels          | 11.18±3.78$^C$ |

$^A-C$ Differences within columns (samples connected by the different capital letter are statistically different at p < 0.05). Values are means ± standard deviation (n = 3).

The HPLC/UV analysis showed that the AQS profile of browned kernels was composed of emodin and rhein. The two compounds isolated from the browned kernels were rhein (Compound 1, Figure 1) and emodin (Compound 2, Figure 2). A robust baseline separation was achieved in 6 min. The retention time of Compound 1 was 2.8 (±0.54) min and the retention time of Compound 2 which was 3.5 (±0.24) min.

Figure 1. HPLC chromatogram of standard rhein (A) and Compound 1 in 5% sodium bicarbonate solution (B).

Figure 2. HPLC chromatogram of standard emodin (A) and Compound 2 in 5 g/100 mL sodium carbonate solution (B).
The spectrum of Compound 1 (Table 2) confirmed that Compound 1 was rhein and the molecular formula of Compound 1 was assigned as $\text{C}_{15}\text{H}_8\text{O}_6$.

There were two aromatic hydroxyl signals at $\delta_{\text{H}}$ 11.89 (with two hydrogen atoms), a carboxyl signal at $\delta_{\text{H}}$ 13.59, and five methyl signals in this spectrum: signals at $\delta_{\text{H}}$ 7.41, $\delta_{\text{H}}$ 7.72, $\delta_{\text{H}}$ 7.82, $\delta_{\text{H}}$ 7.75, and $\delta_{\text{H}}$ 8.16. The structure of -CH-CH-CH- was present in Compound 1. Proton signals at $\delta_{\text{H}}$ 7.41 and $\delta_{\text{H}}$ 7.75 respectively coupled with the adjacent protons. The signal at $\delta_{\text{H}}$ 7.82 coupled with two protons. The signals at $\delta_{\text{H}}$ 7.75 and $\delta_{\text{H}}$ 8.16 respectively indicated only one proton without coupling [21]. The $^1\text{C}-\text{NMR}$ of Compound 1 showed that there were four types of carbons ($\alpha$, $\beta$, carbonyl, and quaternary) in the skeleton of AQS. The signal at $\delta 182.5$ was the carbonyl carbon and the signal at $\delta 178$ was the $\alpha$ carbon substituted by a hydroxyl. Two signals at $\delta 190.82$ and $\delta 182.13$ belonged to

\begin{table}[h]
\centering
\begin{tabular}{ccc}
\hline
No. & $\delta_{\text{H}}$ & $\delta_{\text{C}}$ \\
\hline
1  & / & 161.52 \\
2  & 7.75 ($^1\text{H}, \text{d}, J=2.5$) & 124.80 \\
3  & / & 148.20 \\
4  & 8.16 ($^1\text{H}, \text{d}, J=2.5$) & 125.70 \\
5  & 7.72 ($^1\text{H}, \text{dd}, J=7.5,11$) & 118.40 \\
6  & 7.82 ($^1\text{H}, \text{dd}, J=7.5,11$) & 134.50 \\
7  & 7.41 ($^1\text{H}, \text{dd}, J=8.22,11$) & 125.70 \\
8  & / & 161.12 \\
9  & / & 190.82 \\
10 & / & 182.31 \\
4a & / & 126.60 \\
8a & / & 126.30 \\
9a & / & 126.10 \\
10a & / & 126.40 \\
1-OH & 11.89 & / \\
8-OH & 11.89 & / \\
3-OOH & 13.59 & / \\
\hline
\end{tabular}
\caption{\textsuperscript{1}H and \textsuperscript{13}C-NMR data for Compound 1 (600 and 150MHz, DMSO-d6, $\text{J}$ in Hz and $\delta$ in ppm).}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{ccc}
\hline
No. & $\delta_{\text{H}}$ & $\delta_{\text{C}}$ \\
\hline
1  & / & 162.1 \\
2  & 6.52 ($^1\text{H}, \text{d}, J=2.1$) & 126.7 \\
3  & / & 165.02 \\
4  & 7.06 ($^1\text{H}, \text{d}, J=2.1$) & 126.4 \\
5  & 7.43 ($^1\text{H}, \text{d}, J=1$) & 134.8 \\
6  & / & 132.6 \\
7  & 7.02 ($^1\text{H}, \text{d}, J=2.5$) & 161.6 \\
8  & / & 166.5 \\
9  & / & 190.4 \\
10 & / & 181.31 \\
4a & / & 126.1 \\
8a & / & 126.6 \\
9a & / & 121.5 \\
10a & / & 121.3 \\
1-OH & 11.38 & / \\
3-OH & 11.97 & / \\
8-OH & 12.01 & / \\
6-Me & 2.36 & / \\
\hline
\end{tabular}
\caption{\textsuperscript{1}H and \textsuperscript{13}C-NMR data for Compound 2 (600 and 150MHz, DMSO-d6, $\text{J}$ in Hz and $\delta$ in ppm).}
\end{table}
carbonyl carbons. A signal at $\delta 190.82$ was an $\alpha$ carbon connected to two hydroxyl groups, and the signal at $\delta 182.13$ was not connected with any hydroxyl group. The chemical shift of the $\alpha$ carbon was 126.6 without any substituent group. Connected with a hydroxyl group, the chemical shift increased to 162. Signals at $\delta 161.52$ and $\delta 161.62$ indicated two hydroxyl groups were connected with the $\alpha$ carbon. Two hydroxyl groups were respectively located on either side of the carbonyl carbon.$^{21}$

In conclusion, H-7 ($\delta 7.41$), H-5 ($\delta 7.72$), H-6 ($\delta 7.82$), H-2 ($\delta 7.75$), and H-4 ($\delta 8.16$) were found in the $^1$H-NMR of Compound 1. H-7 was connected with an aromatic hydroxyl group at position 1, and H-2 was connected with an aromatic hydroxyl group at position 7. The signal at $\delta 13.59$ suggested that a carboxyl group existed at H-3. The signal at $\delta 11.89$ indicated that two aromatic hydroxyl groups were presented. The $^{13}$C-NMR of Compound 1 suggested that there were two carbonyl carbons in the structure of Fraction 1 and two hydroxyl groups were substituted at H-1 and H-8.

The structure of Compound 1 (Figure 3) corresponded to the structure of rhein (1,8-2-hydroxyl-3-carbonyl-AQS).

The spectrum results of Compound 2 were listed in Table 3.

There was a methyl signal at $\delta 2.36$ and a aromatic hydroxyl signal at $\delta 11.38$. Two aromatic hydroxyl signals were also observed at $\delta 11.97$ and $\delta 12.01$ in the $^1$H-NMR of Compound 2. There were four methyl groups. Signals at $\delta 6.52$ and at $\delta 7.02$ were coupled with meta-position protons. The other two signals at $\delta 7.06$ and $\delta 7.43$ were split. These signals indicated that four methyl groups were not adjacent and the signal of the proton at $\delta 6.52$ was surrounded by strong electronic groups, such as aromatic hydroxyl and aromatic methyl groups, and the other signals at $\delta 7.02$, $\delta 7.06$, and $\delta 7.43$. Two carbonyl carbons were found at $\delta 190.4$ and $\delta 181.31$ in the $^{13}$C-NMR of Compound 2. Two positions of the $\alpha$ carbon were connected with hydroxyl groups at $\delta 190.4$, but the carbonyl carbon signal at $\delta 181.31$ was not coupled. The chemical shift was 126.6 without substituent groups for the $\alpha$ carbon, but the chemical shift was approximately 162 with hydroxyl groups on the $\alpha$ carbon. For the $\beta$ carbon, the chemical shift was 134.3 without substituent groups, and the chemical shift was approximately 163 with hydroxyl groups. There were three carbons at $\delta 162.1$, $\delta 165.02$, and $\delta 166.5$ with hydroxyl groups, and the positions of the substituent groups could not be determined. Two hydroxyl groups were connected to the $\alpha$ position of the carbonyl carbon at $\delta 190.4$, and no hydroxyl groups were found with another carbonyl carbon at $\delta 181.31$. The carbon with the last hydroxyl group was at the $\beta$ position.

Signals of protons at $\delta 6.52$ (s, $^3$H), $\delta 7.02$ (s, $^3$H), $\delta 7.06$ (s, $^4$H), and $\delta 7.43$ (s, $^4$H) were found in the $^1$H-NMR of Compound 2. The correlation from the proton ($\delta 2.37$) to H-6 indicated the location of the methyl group. Two hydroxyl groups were substituted with two protons at $\delta 11.38$ (s, $^1$H), $\delta 11.97$ (s, $^3$H) and $\delta 12.01$ (s, $^3$H). Two carbonyl carbons were observed in the $^{13}$C-NMR of Compound 2. The locations of H-1 and H-8 at the $\alpha$-position of the carbonyl carbon were occupied by two hydroxyl groups. Another hydroxyl group was located at H-3. The $^1$H-NMR analysis showed that it was coincident with the $^{13}$C-NMR analysis.

The configuration of emodin was identified as 1, 3, 8-trihydroxy-6-methyl-AQS. Two aromatic hydroxyl groups were found at H-2, and one aromatic hydroxyl group and one aromatic methyl group were found at H-7. Additionally, an aromatic hydroxyl group was observed at H-4 and an aromatic methyl group was found at H-5. According to the strength of the electronic groups, the sequence of the four protons in the magnetic field was H-2>H-7>H-4>H-5.
The structure of Compound 2 (Figure 4) corresponded to the structure of emodin.

4. Conclusions

AQS belongs to a large group of substances that includes substitutions at the β position and the 1 and 2 positions, but most have three or four substitutions, such as emodin, rhein, and chrysophanol [22]. In this study, we found that contents of AQS increased after browning reaction in Chinese chestnut kernels indicating AQS could be produced during browning process. Rhein and emodin were the main components of AQS in the browned kernels. Additional information about other components of AQS in browned kernels should be researched in the future.

This was the first time to find AQS can be produced during both enzymatic and non-enzymatic browning reaction of Chinese chestnut kernels. In the future, other browned materials should be used to confirm this result. The formation and safety of AQS in browning reactions should be studied in detail.

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