Short Communication

Novel Heinz Body Hemolysis Factors in Onion (Allium cepa)

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Heinz body hemolysis factors were isolated from boiled onion extracts and characterized as three novel sulfur-containing compounds: sodium trans-1-propenylthiosulfate, sodium cis-1-propenylthiosulfate, sodium n-propylthiosulfate.

Onions (Allium cepa) are known to cause Heinz body hemolytic anemia in many kinds of domestic animals.1-4) This disease has been called “onion poisoning” and is often found in veterinary clinics for small animals. Small animals such as dogs and cats which eat boiled onions or other Allium plants develop hemolytic anemia. In our previous study,5) we have experimentally demonstrated that the oral administration of boiled onions to dogs caused Heinz body hemolytic anemia, and speculated about certain properties of the compounds responsible for this disease. The factors causing this disease have been thought for a long time to be some disulfides6) contained in the onion oil. In this study, we report the isolation and structural elucidation of the Heinz body hemolysis factors (1-3) from boiled onions, using a canine erythrocyte oxidation test.

The bioassay for the oxidation of canine erythrocytes was performed by the methemoglobin formation in vitro. Whole blood from normal mongrel dogs was drawn into a heparinized tube and centrifuged at 1250 x g for 5 min at 4°C. After removing the Buffy coat, the canine erythrocytes were washed three times with 154 mM NaCl and 10 mM phosphate-buffered saline (pH 7.4, 4°C, PBS), and resuspended in PBS having a packed cell volume of 15%. The suspension was incubated with each sample of the onion extract for one or two hours at 37°C, and the methemoglobin concentration was then measured as described by Hegesh et al.7)

Peeled onion bulbs (6 kg) were chopped and homogenized. After making up to 13 liters with deionized water, the homogenate was boiled for 15 min and filtered through gauze. The filtrate was evaporated to one liter, and the aqueous concentrate was partitioned with ethyl acetate. Four liters of ethanol was added to the aqueous fraction which showed activity. After removing the precipitate, the filtrate was evaporated to dryness. To the extract (279.1 g), 2.25 liters of methanol–chloroform (1:2) was added, and the insoluble materials were removed by filtration. The filtrate was evaporated to dryness, and then chromatographed on silica gel (Kieselgel 60, Merck) with methanol–chloroform (1:1) as the eluent. The bioactive fraction was further purified by HPLC in a µBondapak C18 column (19 x 300 mm, Waters), using a solvent of 30% aqueous methanol. Finally, an active mixture (2.9 mg) was obtained, which showed a single peak by HPLC in an Inertsil ODS column (6 x 250 mm, Gasukuro Kogyo), using a solvent of 80% aqueous methanol.

The 1H-NMR spectrum showed that the peak factor consisted of three compounds: 1 (70%), 2 (20%), and 3 (10%). The spectrum of major compound 1 showed the presence of CH3 (δ 1.83, dd, J = 1.7, 6.6 Hz) and of H=C–C=CH (δ 6.05, dq, J = 6.6, 14.8 Hz and δ 6.41, dq, J = 1.7, 14.8 Hz). The 13C-NMR spectrum gave three carbons due to CH3 (δ 18.7) and –CH=–CH– (δ 137.0 and 121.7). These NMR spectral data for 1 showed the partial structure CH3–C–C–

Similarly the partial structures of 2 and 3 were determined to be CH3–C–C–C– and CH3CH2CH2–C–C–, respectively. Assignments of the chemical shifts were confirmed by 1H-1H COSY and HMBC experiments (Table).

The active compounds gave a positive reaction to iodine-azide reagent, showing an organic sulfur-containing compound. The IR spectrum of the mixture exhibited the presence of thiosulfates (1190 and 1010 cm⁻¹), and flame photometry showed the presence of Na⁺. These data led to the residual inorganic partial structure being assigned to sodium thiosulfate.

In the FD-MS spectrum of the active mixture, no molecular ion peak was observed, but it showed two base peaks at m/z 146 and 148, and two fragment peaks at m/z 178 and 180. By high resolution EI-MS, m/z 146, 148, 178, 180 and 64 (base peak) were analyzed for C6H10S2, C6H9S2, C6H9O2S2, C6H12O2S2, and O2S2, respectively. Consequently, the active compounds in the mixture were determined to be sodium trans-1-propenylthiosulfate (1), sodium cis-1-propenylthiosulfate (2), and sodium n-propylthiosulfate (3). Fragment peaks at m/z 146 and 148 were assigned to CH3CH=CHSSCH=CHCH3 and CH3CH=CHSSCH2CH2CH3, respectively. The peak at

m/z 178 was assigned to CH3CH=CHSSCH=CHCH3,

and at m/z 180 to CH3CH=CHSSCH2CH2CH3 and/or

CH3CH=CHSSCH2CH2CH3. These fragment peaks seem to have resulted from the combination of C3 units after the decomposition.

One of these active compounds, 3, was synthesized as described by Chapelet et al.9) Sodium thiosulfate (30 mmol) was dissolved in distilled water (60 ml), and propane chloride (25 mmol) was dissolved in benzene (80 ml). These two solutions were mixed and refluxed for 24 h, after trimethylbenzlammonium chloride (1 mmol) had been
added to the mixed solution. The mixed solution was evaporated to dryness and extracted with methanol, the extract being chromatographed on silica gel (Kieselgel 60) with methanol–chloroform (1:1) as the eluent. Compound 3 as synthesized was purified by HPLC in an Inertsil ODS column, using a solvent of 80% aqueous methanol. The retention time by HPLC and the analytical data for the synthetic compound (3) completely agreed with those of the natural product. A mixture of the natural active compounds (1–3) resulted in 13% of methemoglobin formation at a concentration of 50 ppm, whereas synthetic compound 3 showed a little weaker activity (11%) at 500 ppm. The mixture of natural compounds showed approximately 10-fold the activity of synthetic compound 3 with respect of concentration, this probably resulting from the higher activity of compounds 1 and 2 than of compound 3.

The Heinz body hemolysis factors in boiled onion were revealed to be three novel thiosulfates. These novel active compounds (1–3) with thiosulfate groups would probably contribute to the oxidation mechanism of blood. We have not yet been able to completely purify the mixture by HPLC, using various columns and solvent systems. Syntheses of 1 and 2 are in progress.

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References

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<th>Compound</th>
<th>H (ppm)</th>
<th>C (13C (ppm))</th>
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<tr>
<td>O</td>
<td>1 6.41 (dq, J = 1.7, 14.8 Hz)</td>
<td>1 121.7</td>
</tr>
<tr>
<td>O</td>
<td>2 6.05 (dq, J = 6.6, 14.8 Hz)</td>
<td>2 137.0</td>
</tr>
<tr>
<td>O</td>
<td>3 1.83 (dd, J = 1.7, 6.6 Hz)</td>
<td>3 18.7</td>
</tr>
<tr>
<td>O</td>
<td>1 6.46 (dq, J = 1.4, 9.3 Hz)</td>
<td>1 123.1</td>
</tr>
<tr>
<td>O</td>
<td>2 5.90 (dq, J = 6.8, 9.3 Hz)</td>
<td>2 129.4</td>
</tr>
<tr>
<td>O</td>
<td>3 1.73 (dd, J = 1.4, 6.8 Hz)</td>
<td>3 14.9</td>
</tr>
<tr>
<td>O</td>
<td>1 3.04 (t, J = 7.2 Hz)</td>
<td>1 38.0</td>
</tr>
<tr>
<td>O</td>
<td>2 1.79 (m)</td>
<td>2 24.0</td>
</tr>
<tr>
<td>O</td>
<td>3 1.01 (t, J = 7.4 Hz)</td>
<td>3 13.7</td>
</tr>
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1H-NMR (500 MHz) and 13C-NMR (125.8 MHz) chemical shifts were recorded in CD3OD with a Bruker AM-500 spectrometer.