Note

Isolation and Identification of Sodium 2-Propenyl Thiosulfate from Boiled Garlic (Allium sativum) That Oxidizes Canine Erythrocytes

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Sodium 2-propenyl thiosulfate was identified in boiled garlic (Allium sativum). When canine erythrocytes were incubated with sodium 2-propenyl thiosulfate, the methemoglobin concentration and Heinz body percentage in erythrocytes were both increased, indicating that the compound induced oxidative damage in canine erythrocytes. It seems that this compound is one of the causative agents of garlic-induced hemolysis in dogs.

Key words: sodium 2-propenyl thiosulfate; garlic; canine erythrocyte; oxidant

It is well-known in veterinary medicine that onions (Allium cepa) are toxic to erythrocytes, resulting in hemolytic anemia in domestic animals such as dogs, cats, horses, sheep and cattle. Hemolysis is associated with Heinz body formation within erythrocytes, resulting from the precipitation and denaturation of hemoglobin molecules via methemoglobin formation that is oxidatively damaged by certain oxidants in onions. n-Propyl disulfide and three sodium alk(en)yl thiosulfates, n-propyl, trans-1-propenyl and cis-1-propenyl thiosulfates, have been identified as the causative compounds of onion-induced hemolytic anemia. These three non-volatile thiosulfates isolated from a hot water-extract of onions play an important role in the canine disease that often occurs after the ingestion of cooked onions.

The ingestion of garlic (Allium sativum) has also been shown to induce hemolysis in dogs due to the oxidative damage to erythrocytes. We have recently isolated eight organosulfur compounds oxidizing canine erythrocytes in vitro from an aqueous ethanol garlic extract, i.e., bis-2-propenyl trisulfide, bis-2-propenyl tetrasulfide, bis-2-propenyl pentasulfide, bis-2-propenyl thiosulfonate, trans-sulfuric acid allyl ester 3-allylsulfanyl-allyl ester, 2-propene-1-sulfinothioic acid S-methyl ester, 2-propene-1-sulfinothioic acid S-(E)-1-propenyl ester and trans-sulfurous acid allyl ester 3-allylsulfanyl-allyl ester. These compounds are partly responsible for the garlic-induced hemolysis in dogs.

We report in this present paper the novel natural compound oxidizing canine erythrocytes, sodium 2-propenyl thiosulfate, which had been isolated from a hot-water extract of garlic.

To isolate the oxidatively toxic compounds, a bioassay for the oxidation of canine erythrocytes was performed by measuring the methemoglobin formation in vitro. Whole blood from clinically healthy beagle dogs was drawn into a heparinized tube and centrifuged at 1250 × g for 7 min at 4°C. After removing the layer of leukocytes and platelets, the erythrocytes were washed three times with 10 mM PBS (pH 7.4) and resuspended in PBS having a packed cell volume of 25% (v/v). Five hundred μl of this erythrocyte suspension was incubated with each sample of a garlic extract for 2 h at 37°C. The methemoglobin concentration was then measured as described by Hegesh et al., being expressed as a percentage of the total hemoglobin. All procedures

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Abbreviations: PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; FABMS, fast atom bombardment mass spectrometry
using experimental animals were performed in accordance with the guidelines regulating animal use at the Graduate School of Veterinary Medicine, Hokkaido University.

Peeled garlic bulbs (200 g), which had been cultivated in Honam, Korea, were homogenized after the addition of 300 ml of deionized water. The homogenate was boiled for 15 min and filtered through gauze. The resulting filtrate was evaporated in vacuo to approximately 100 ml, and the aqueous concentrate was partitioned three times with 100 ml each of ethyl acetate. Four hundred ml of ethanol was then added to the residual aqueous fraction that showed activity. After removing the precipitate, the filtrate was evaporated to dryness. To the extract (6.1 g), 180 ml 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, 63–200 μm particle size, Merck) with methanol-chloroform (45:55) as the eluent. This column chromatography yielded three main bioactive fractions, and the fraction having the longest elution time and relatively low activity among these main fractions was rechromatographed on silica gel (YMC-gel Sil-60–10–40, 10–40 μm particle size, YMC) with methanol-chloroform (3:7) as the eluent. Finally, an active compound (3.3 mg) was obtained as an amorphous solid which showed a single peak by HPLC in a reversed phase column (Inertsil ODS, 6 × 250 mm, GL Science), using a solvent of 20% aqueous methanol.

The 1H- and 13C-NMR spectra of this purified active compound were recorded in CD3OD with a Jeol EX400 spectrometer (1H: 400 MHz, 13C: 100 MHz). The 1H-NMR spectrum of the compound showed the presence of CH2= (δ 5.07, 1H, dd, J = 10.2, 1.5 Hz; δ 5.22, 1H, dd, J = 14.2, 1.5 Hz), =CH– (δ 6.02, 1H, m) and –CH2– (δ 3.71, 2H, d, J = 7.3 Hz). The 13C-NMR spectrum gave three carbons due to CH2= (δ 118.6), =CH– (δ 135.9) and –CH2– (δ 39.6). These NMR spectral data for the compound showed the partial structure of CH2=CH–CH2–. The FABMS spectrum measured with a Jeol JMS HX-100 mass spectrometer showed a base peak at m/z 153, which was suspected to be C3H5O3S2. A analysis of the electrolytes contained in the compound showed the presence of a sodium ion. These spectral data suggest that the structure of the compound was sodium 2-propenyl thiosulfate, CH2=CH–CH2–S–SO3–Na+.

To confirm the structure of the active compound, sodium 2-propenyl thiosulfate was synthesized as described by Chapelet et al.11) To a stirred solution of sodium thiosulfate (230 mmol) in deionized water (330 ml), a solution of allyl chloride (190 mmol) in toluene (420 ml) was added. To the solution was added benzyltrimethylammonium chloride (8 mmol) as a catalyst, and the reaction mixture was refluxed for 3 h at 105°C. After the reaction, the product was dried in vacuo, and extracted with methanol. The extract was purified by silica gel chromatography (Kieselgel 60, Merck; methanol:chloroform = 45:55, v/v) to afford sodium 2-propenyl thiosulfate (17 g). The NMR and FABMS data for synthetic sodium 2-propenyl thiosulfate completely matched with those of the natural compound, indicating that the oxidant isolated from garlic in the present study was sodium 2-propenyl thiosulfate (Fig. 1).

![Fig. 1. Chemical Structure of Sodium 2-Propenyl Thiosulfate Identified in Hot-water Extract of Garlic.](image-url)

Fig. 2. Methemoglobin Concentration (A), Heinz Body Percentage (B) and Turbidity Index (C) in Canine Erythrocytes after Incubation with 5 mM Sodium 2-Propenyl Thiosulfate at 37°C.

Canine erythrocytes were suspended in 10 mM PBS (pH 7.4) having a packed cell volume of 25%. Each value represents the mean ± standard deviation of the results from five replicates. *P<0.01, t-test compared with the value before incubation.

![Fig. 2. Methemoglobin Concentration (A), Heinz Body Percentage (B) and Turbidity Index (C) in Canine Erythrocytes after Incubation with 5 mM Sodium 2-Propenyl Thiosulfate at 37°C.](image-url)
The oxidizing property of synthetic sodium 2-propenyl thiosulfate was examined in vitro by measuring the methemoglobin concentration, Heinz body percentage and turbidity index, which is another quantitative estimate of Heinz bodies in erythrocytes. When canine erythrocytes suspended in PBS were incubated with 5 mM sodium 2-propenyl thiosulfate at 37°C for 4 h, the methemoglobin concentration, Heinz body percentage and turbidity index were significantly \( P < 0.01 \) increased (Fig. 2), although each parameter hardly changed in the control incubated without sodium 2-propenyl thiosulfate (data not shown).

In the previous study, hemolysis due to oxidative damage to erythrocytes was found to occur in dogs fed with a boiled garlic extract. In the present study, sodium 2-propenyl thiosulfate was isolated from a hot-water extract of garlic and shown to cause oxidative damage to canine erythrocytes, indicating sodium 2-propenyl thiosulfate to be one of the causative compounds in the garlic-induced hemolysis of dogs. However, we have also previously identified eight compounds other than sodium 2-propenyl thiosulfate that oxidized canine erythrocytes. Furthermore, there were other active compounds present in the fractions separated by silica gel chromatography in the present study. The composite effects of many compounds on erythrocytes may be responsible for the garlic-induced hemolysis in dogs.

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References


10) FABMS and NMR data for sodium 2-propenyl thiosulfate. FABMS \( m/z: \) (2M − Na)− and 153 (100) [M − Na]−. \(^1\)H-NMR (CD3OD) \( \delta: \) 3.71 (2H, d, \( J = 7.3 \) Hz, 1-CH2), 5.07 (1H, dd, \( J = 10.2, 1.5 \) Hz, 3-CHa), 5.22 (1H, dd, \( J = 14.2, 1.5 \) Hz, 3-CHb) and 6.02 (1H, m, 2-CH). \(^1\)C-NMR (CD3OD) \( \delta: \) 39.6 (1-CH2), 118.6 (3-CHa) and 135.9 (2-CH).