Isolation and Identification of Causal Constituents of Green Discoloration in Garlic Puree

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The green discoloration of garlic (Allium sativum L.) puree, a serious problem in garlic processing, was investigated. Two thiosulfinates, 2-propenyl E-1-propenethiosulfinate (2P1PTS) and (E/Z)-1-propenyl 2-propenethiosulfinate (1P2PTS), were isolated from unheated garlic puree (homogenate), and were confirmed to be the direct causal constituents ('color developer') of green discoloration based on a color developing assay. 2P1PTS and/or 1P2PTS were reacted with glycine to develop a blue-green color similar to the discoloration of garlic purees. 2P1PTS and 1P2PTS showed synergy for greening; the color developing activities of three solutions, 2P1PTS, 1P2PTS and an equimolar mixture of them (0.5-2P1PTS + 0.5-1P2PTS; similar to the natural abundance ratio) were in the rank order: (0.5-2P1PTS + 0.5-1P2PTS) > 2P1PTS > 1P2PTS.

Keywords: alliin, alliinase, discoloration, isoalliin, garlic, greening, thiosulfinate

Introduction

A serious problem in garlic (Allium sativum L.) processing is the green discoloration (greening) of the product, such as that of puree and pickles (Joslyn and Sano, 1956; Rejano et al., 1997; Bai et al., 2005; Block, 2010). Greening decreases product quality, resulting in serious economic losses. Previously, Lukes (1986) reported that the greening of garlic puree is governed by isoalliin in garlic bulbs. Elucidation of the greening phenomenon in puree remains uncertain at the compound level (Lee et al., 2007), although some methods for decreasing greening have been reported (Bae and Lee, 1990; Kim et al., 1999).

Recently, Kubec et al. (2004) reported that a reaction mixture consisting of S-(E-1-propenyl)-L-cysteine sulfoxide (isoalliin), S-(2-propenyl)-L-cysteine sulfoxide (alliin), alliinase (EC 4.4.1.4) and glycine developed a blue color. They also reported in the same paper that synthesized 2-propenyl 1-propenethiosulfinate (2P1PTS) or 1-propenyl 2-propenethiosulfinate (1P2PTS) reacts with glycine to produce a blue color. Additionally, Imai et al. (2006a, b) reported on the discoloration (greening) of a mixture of onion (Allium cepa L.) and garlic, also referring to a plausible mechanism. Subsequently, Kubec and Velíšek (2007) reported differences in reactivity and color development using combinations of thiosulfinates and amino acids, and Cho et al. (2009) reported on the relationships between color development and kinds of amino acids.

These model studies, using purified specific compounds, have provided important clues to the clarification of the greening phenomenon, which has remained unsolved for more than fifty years. However, the greening mechanism in garlic puree has not been sufficiently elucidated, as isolation and structure determination of the causal substances of greening in unheated purees has not been completed. For example, whether the thiosulfinates used in model studies are principal causal substances in puree has not been confirmed.

In this study, we isolated two naturally occurring causal constituents (‘color developer’) of greening from garlic homogenate (puree), using color development screening with glycine. The structures of the causal constituents were identical to synthetic thiosulfinates, 2P1PTS and 1P2PTS, used in a model study by Kubec et al. (2004).
the precipitate. The combined supernatants were loaded on a Lichroprep RP-18 (75 g, 40 – 63 mesh, Merck KG, Germany) using a Buchner funnel (75 mm i.d.). Fractionation was performed by eluting with 500 mL each of MeOH-0.1%HCOOH (0:100, 10:90, 50:50, and 100:0, v/v). Fractions (1 mL) were heated with 5 mL of 0.1 M aqueous glycine in test tubes, at 80°C for 60 min in a block heater, and cooled to room temperature. Then, absorption spectra of the cooled solutions were measured at 350 – 750 nm. For these assays, the active fraction obtained by eluting with MeOH-0.1%HCOOH (50:50, v/v) was extracted with CH$_2$Cl$_2$, after the removal of MeOH in vacuo (<30°C). The CH$_2$Cl$_2$ layer was concentrated (<18°C) to a residue as crude active material (970 mg). The purity of the extracts and active fractions described below were assessed with HPLC (column: µ-Bondasphere C18 (3.9 mm i.d. × 150 mm, Waters Corp.), mobile phase (flow rate: 1 mL/min): MeOH-0.1%HCOOH (40:60, v/v)).

**Separation of causal substances from the crude active material** The crude active material thus obtained (970 mg) was subjected to preparative HPLC (columns: two serially connected µ-Bondasphere C18 (19 mm i.d. × 150 mm), mobile phase (flow rate: 5 mL/min): MeOH-0.1% HCOOH (10:90 (v/v) (100 mL), 10:90 to 50:50 (400 mL, linear gradient for 80 min), 50:50 (250 mL). The eluent was fractionated (5 mL fractions), and an aliquot (0.1 mL) of each fraction was heated with 5 mL of 0.1 M aqueous glycine in test tubes at 70°C for 90 min in a water bath, and cooled to room temperature. Then, the absorbance of the solutions was...
measured at 585 nm. From the assay result, the active fractions No. 105 through No. 114 were combined. The solvent of the combined aqueous MeOH solution was replaced with CH₂Cl₂ using a Sep-pak C18 (10 g, Waters Corp.) cartridge as described in Fig. 2. The acquired CH₂Cl₂ solution was concentrated (< 18°C) to yield a crude oily causal substance (211 mg).

Isolation of the two compounds from the crude causal substance The crude causal substance (211 mg) was subjected to recycling HPLC (column: µ-Bondasphere C18 (19 mm i.d. × 150 mm), mobile phase (flow rate: 5 mL/min, MeOH-0.1%HCOOH (40:60, v/v), 10 rounds recycle) to separate into two fractions; the major two fractions, the front and the rear ones, were separately collected. The solvent of each fraction was replaced with CH₂Cl₂ using Sep-pak C18 cartridges as described above (Fig. 2), and concentrated (< 18°C) to yield two colorless oily compounds, 36 mg (the front fraction) and 51 mg (the rear fraction).

The NMR and MS spectral data of the two compounds were as follows: the front fraction (2P1PTS), HRESI-MS m/z: (MH⁺), calcd. for C₆H₁₁OS₂: 163.0251, found: 163.0279; ¹H-NMR (CDCl₃) δ: 6.60 (1H, dq, J = 14.8, 6.8 Hz, H-2'), 6.47 (1H, dd, J = 14.8, 1.5 Hz, H-1'), 5.96 (1H, m, H-2), 5.33 (1H, d, J = 15.6 Hz, H-3), 5.23 (H, d, J = 9.9 Hz, H-3), 3.75 (2H, dq, J = 14.0, 7.6 Hz, H-1), 1.97 (3H, dd, J = 6.8, 1.5 Hz, H-3') ppm; the rear fraction (1P2PTS), HRESI-MS m/z: (MH⁺), calcd. for C₆H₁₁OS₂: 163.0251, found: 163.0279; ¹H-NMR (CDCl₃) δ: 6.25-6.43 (2H, m, H-1 & H-2), 5.95 (1H, m, H-2'), 5.46 (2H, m, H-3'), 3.83 (2H, m, H-1'), 1.89 (2H, d, J = 5.1 Hz, H-3 (E-isomer)), 1.84 (1H, d, J = 6.8 Hz, H-3 (Z-isomer)) ppm; E/Z ratio = ca. 2/1. The two isolated compounds (2P1PTS, 1P2PTS) were dissolved in MeOH-0.1%HCOOH (10:90, v/v) to a concentration of 10 mM, and stored at −75°C until use.

Color developing activities of the two isolated compounds Color developing activities of the two compounds were evaluated using the stored solutions described above.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Procedure</th>
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<tbody>
<tr>
<td>1</td>
<td>Dilute the 50% MeOH fraction to 10% MeOH with 0.1% aq. HCOOH (v/v)</td>
</tr>
<tr>
<td>2</td>
<td>Load the diluted solution on a Sep-pak C18 cartridge (10 g)</td>
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<tr>
<td>3</td>
<td>Wash the cartridge with 100 mL of H₂O</td>
</tr>
<tr>
<td>4</td>
<td>Elute the cartridge with 100 mL of CH₂Cl₂</td>
</tr>
<tr>
<td>5</td>
<td>Remove the H₂O layer with a separatory funnel</td>
</tr>
<tr>
<td>6</td>
<td>Wash the CH₂Cl₂ layer with brine</td>
</tr>
<tr>
<td>7</td>
<td>Dry the CH₂Cl₂ layer with Na₂SO₄ (anhydrous), followed by filtration</td>
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</tbody>
</table>

Fig. 2. Solvent exchange procedure with Sep-pak C18 cartridge chromatography.
the above two reaction products was almost the same as that of garlic puree homogenates in our estimation.

In reference to the above-mentioned pre-examination results, we carried out preparative scale separation and isolation of causal agents using the color developing assay.

**Isolation of causal compounds from garlic puree**

The constituents of garlic homogenate retained on the Lichroprep RP-18 (ODS) column were eluted stepwise with MeOH-0.1%HCOOH (0:100, 10:90, 50:50 and 100:0, v/v) and four fractions collected. In the color developing assay, the first two fractions were colorless and the last two were blue-green (λmax: 585 nm) and pale pink (λmax: 525 nm), respectively (Fig. 3). It was thought that the causal agents were present in only the third fraction eluted with MeOH-0.1%HCOOH (50:50, v/v), because of its similar blue-green color with that of heated garlic puree.

The crude active material was further purified using a reverse phase HPLC. In the gradient C18-HPLC of the crude active material, fractions No.105 through No.114 showed blue color developing activity (Fig. 4). Although fraction No.83 had color developing activity, it was not collected because it showed pink not blue.

On the other hand, when the eluted aqueous methanol solution was treated in vacuo for sufficient time, diminution of color developing activity was observed. This indicates that the causal substances might be volatile compounds. Next, we attempted to replace the causal substances solvent, from aqueous MeOH to the more volatile CH₂Cl₂, using a C18 cartridge as an absorbent (Fig. 2). As a result, sufficient amounts of causal compounds were obtained for both structure determinations by spectroscopic analysis and the color developing test.

The crude causal substances obtained by gradient HPLC were fractionated into two pure compounds, which showed only one peak each, using recycling C18-HPLC (Fig. 5, D and E). The two isolated fractions each showed color developing activity.

The structures of the two compounds were determined to be (E)-1-propene-1-sulfinothioic acid S-2-propenyl ester.
(2-propenyl 1-propenethiosulfinate; 2P1PTS) and 2-propene-1-sulfinothioic acid S-(E,Z)-1-propenyl ester ((E,Z)-1-propenyl 2-propanethiosulfinate; 1P2PTS) by comparison of the obtained NMR and MS data with that previously reported (Lawson et al., 1991; Block et al., 1996; Calvey et al., 1997; Yoshida et al., 1999). Both compounds are known to be present in garlic homogenates (Lawson et al., 1991; Block et al., 1992).

The two isolated compounds, 2P1PTS and 1P2PTS, were each dissolved in MeOH-0.1% HCOOH (10:90, v/v) to give 10 mM solutions, and stored at −75°C until color developing experiments were carried out, due to the instability of concentrated oily thiosulfimates. This storage condition is comparable to that for allicin (2P2PTS) (Lawson, 1996).

**Color developing activities of the two isolated thiosulfimates and their mixture** The three test solutions, 2P1PTS solution, 1P2PTS solution and a mixture of 2P1PTS and 1P2PTS (0.5-2P1PTS + 0.5-1P2PTS), developed a blue-green color (λmax (abs): 586 (0.184), 577 (0.108) and 584 (0.193) nm, respectively) by heating with glycine solution (Fig. 6). The observed discoloration in these solutions is similar to that of greening in puree.

Interestingly, the equimolar solution of 2P1PTS and 1P2PTS developed a more intense color than that of 2P1PTS or 1P2PTS solution alone. Reportedly, in unheated garlic puree the concentrations of 2P1PTS and 1P2PTS are almost equal (Lawson et al., 1991). The reason for the synergistic effect observed in color developing activity, (0.5-2P1PTS + 0.5-1P2PTS) > 2P1PTS > 1P2PTS, has not yet been elucidated. Further study is necessary to explain this result.

**Principal causal agents of garlic bulb greening** From the results of the examinations herein, we concluded that the principal causal constituents of the green discoloration in garlic puree were the thiosulfimates 2P1PTS and 1P2PTS, in addition to ubiquitous amino acids. When allicin (2P2PTS) was removed during purification, a decrease in coloration was observed (data not shown). Therefore, although allicin is involved in discoloration, it is not the predominant compound, as it does not develop color when reacted with glycine. This conclusion is supported by a previous report using synthetic thiosulfimates (Kubec et al., 2004). These results indicate that the main causal agents involved originally in garlic bulbs are alliin, isoalliin and alliinase (Yamazaki et al., 2011).

Consequently, to prevent greening of garlic puree, generation of causal thiosulfimates should be reduced. The principal means to achieve this are control of alliin, isoalliin or alliinase in garlic bulbs before processing (Fig. 1). We have reported effective methods for decreasing isoalliin in garlic bulbs (Yamazaki et al., 2012).

**References**


