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Changes in Organosulfur Compounds in Garlic Cloves during Storage

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We determined the changes in the contents of three *γ*-glutamyl peptides and four sulfoxides in garlic cloves during storage at −3, 4, and 23 °C for 150 days using a validated high-performance liquid chromatography method that we reported recently. When garlic was stored at 4 °C for 150 days, marked conversion of the *γ*-glutamyl peptides, *γ*-L-glutamyl-*S*-allyl-L-cysteine and *γ*-L-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine (GSPC), to sulfoxides, alliin and isoalliin, was observed. Interestingly, however, when garlic was stored at 23 °C, a decrease in GSPC and a marked increase in cycloalliin, rather than isoalliin, occurred. To elucidate in detail the mechanism involved, the conversion of isoalliin to cycloalliin in both buffer solutions (pH 4.6, 5.5, and 6.5) and garlic cloves at 25 and 35 °C was examined. Decreases in the concentration of isoalliin in both the solutions and the garlic cloves during storage followed first-order kinetics and coincided with the conversion of cycloalliin. Our data indicated that isoalliin produced enzymatically from GSPC is chemically converted to cycloalliin and that the cycloalliin content of garlic cloves increases during storage at higher temperature. These data may be useful for controlling the quality and biological activities of garlic and its preparations.

KEYWORDS: ***Allium sativum*; garlic; organosulfur compound; storage temperature; cycloalliin**

INTRODUCTION

Garlic (*Allium sativum* L.) has been used for culinary and medicinal purposes since ancient times. Numerous beneficial effects of garlic have been reported, including a reduction in the risk of cancer and cardiovascular and age-related diseases (*1*). It has been strongly suggested that these beneficial properties of garlic are attributable to specific organosulfur compounds including sulfoxides and *γ*-glutamyl peptides that are present in raw garlic (*2*−*6*; **Figure 1**). Among these biologically active compounds, it has been reported that (+)-*S*-allyl-L-cysteine sulfoxide (alliin) and (1*S*,3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid 1-oxide (cycloalliin) have anticancer (*2*, *3*) and lipid-lowering effects (*4*) and that *γ*-glutamyl peptides lower systolic blood pressure (*5*) and blood cholesterol levels (*6*).

The contents of organosulfur compounds in garlic bulbs change during cultivation (*7*, *8*) and storage (*9*). Lancaster and Shaw (*10*) and Lawson (*11*) have reported that *γ*-glutamyl peptides are converted into the corresponding sulfoxides by *γ*-glutamyl transpeptidase and oxidase in garlic (**Figure 1**). However, the biosynthetic pathway of cycloalliin is still not sufficiently understood.

As garlic bulbs are stored for several months after harvest to ensure year-round supplies for customers, the storage conditions are important to prevent loss of shelf life and quality, such as surface discoloration, moisture loss, sprouting, and rooting. In particular, storage temperature affects the breaking of dormancy and sprout and root development (*12*). Takagi (*13*) has reported that sprouting and rooting in garlic bulbs were suppressed during storage at −2 °C and that quality characteristics such as firmness and taste were retained when they were stored at −3 °C for 9 months (*14*). Also, it has been reported previously that the optimum temperature for induction of sprouting and rooting is about 4 °C (*13*−*16*). However, all of the above studies were merely morphological observations and not combined with chemical analytical data. Only Lawson et al. (*9*) have reported changes in the contents of two major compounds, *γ*-L-glutamyl-*S*-allyl-L-cysteine (GSAC) and *γ*-L-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine (GSPC), when garlic cloves were stored at 4 °C. These data suggest that the contents of major organosulfur compounds in garlic change under different storage conditions and that this may be one of the important factors controlling the quality and biological activities of garlic.

In the present study, we investigated changes in the contents of three *γ*-glutamyl peptides and four sulfoxides in garlic cloves during storage at −3, 4, and 23 °C using a validated high-performance liquid chromatography (HPLC) method that we reported recently (*17*). Our data indicated that isoalliin produced enzymatically from GSPC is chemically converted to cycloalliin and that the cycloalliin content of garlic cloves increases during storage at higher temperatures. These data may be useful for

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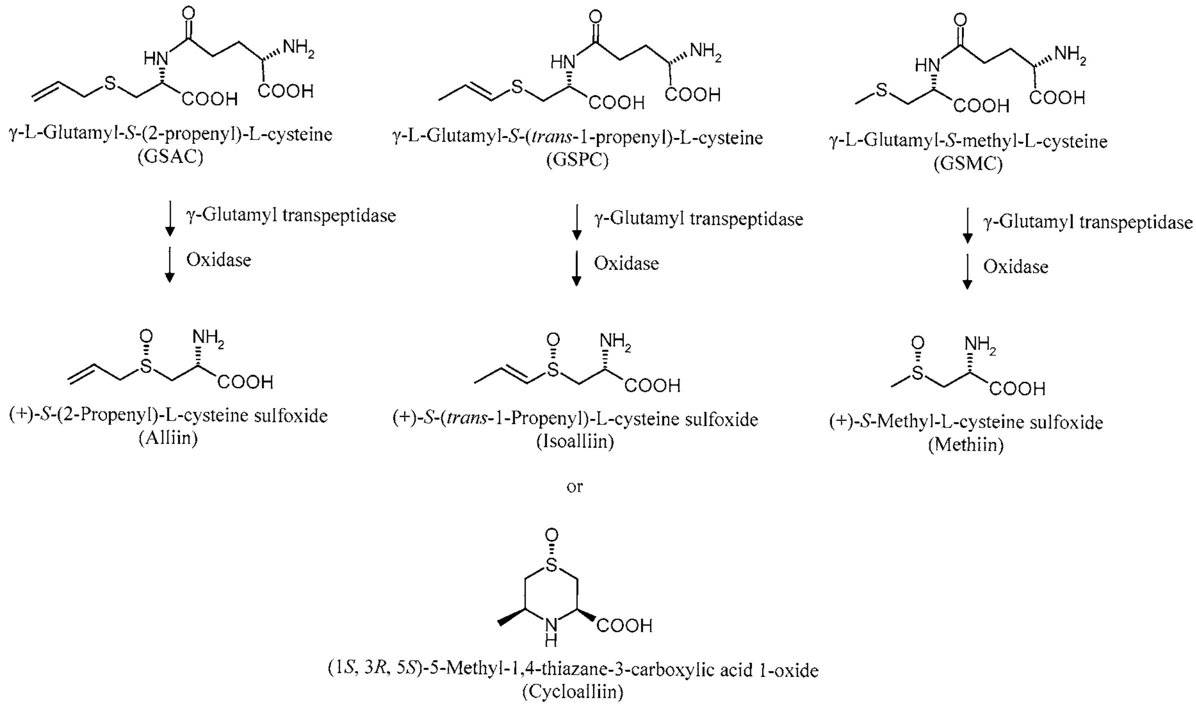

Figure 1. Biosynthetic pathway of organosulfur compounds in garlic.

controlling the quality and biological activities of garlic and its preparations.

MATERIALS AND METHODS

Chemicals. Alliin, (+)-*S*-methyl-L-cysteine sulfoxide (methiin), cycloalliin, GSAC, and γ-L-glutamyl-*S*-methyl-L-cysteine (GSMC) were synthesized as described previously (*9*, *18*−*20*). GSPC and (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide (isoalliin) were isolated from garlic and onion bulbs, respectively, according to the methods of Lawson et al. (*9*) and Shen et al. (*21*) with slight modifications. Acetonitrile and methanol for HPLC were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals of reagent grade were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd., or Yoneyama Yakuhin Kogyo Co., Ltd. (Osaka, Japan). Water purified through a Milli-Q Labo (Millipore, Bedford, MA) water system was used for all sample preparations and mobile phases.

Garlic Samples. Two kinds of the garlic cultivar Fukuchi-howaito, sample A and sample B, were purchased from different farms in Hokkaido, Japan. They were harvested in July 2004, dried naturally to remove excess moisture at room temperature, and then stored in boxes.

Sample Preparation for Determination of Organosulfur Compounds in Garlic Cloves. Sample preparation for HPLC analysis was performed according to the method described previously (*17*). Simply, a peeled garlic clove (4−10 g) in a 250 mL homogenizing cup was added to 150 mL of 90% methanol solution containing 0.01 M HCl and then homogenized at the highest speed for 1 min using a 7012 laboratory blender (Waring Products, Inc., Torrington, CT). The homogenate was decanted into a 250 mL flask. The homogenizing cup was washed with 90% methanol solution containing 0.01 M HCl, and the washing solution was combined with the homogenate to make exactly 250 mL. The mixture was centrifuged at 11000*g* for 5 min, and the supernatant was analyzed by HPLC.

HPLC Instrument and Analytical Conditions. HPLC analysis was performed as described previously (*17*). The assay was performed on two Shimadzu LC-10AVP systems (Shimadzu, Kyoto, Japan). The HPLC conditions for alliin, isoalliin, methiin, cycloalliin, and GSMC were as follows: column, Shodex Asahipak NH2P-50 2D (5 μm, 150 mm × 2 mm, Showa Denko, Tokyo, Japan); column temperature, 25 °C; flow rate, 0.2 mL/min; mobile phase, acetonitrile/water (84:16, v/v) containing 0.2% phosphoric acid; wavelength, UV 210 nm; and injection volume, 1 μL. The HPLC conditions for GSAC and GSPC were as follows: column, Symmetry C18 (5 μm, 150 mm × 3.9 mm, Waters, Milford, MA); column temperature, 25 °C; flow rate, 0.8 mL/min; mobile phase, 50 mM phosphate buffer (pH 2.6)/methanol (85:15, v/v); wavelength, UV 205 nm; and injection volume, 10 μL. The analytical method for determination of three γ-glutamyl peptides and four sulfoxides in garlic cloves has good specificity, linearity ($r > 0.999$), detection limit (0.2−2.4 μg/mL), recovery (97.1−102.3%), intraday precision (RSD < 2.6%), and interday precision (RSD < 4.6%) (*17*).

Moisture Content. The moisture content of garlic cloves was determined by measuring weight loss after the cloves had been sliced and dried using an FD-230 infrared moisture determination balance (80 °C, Kett Electric Laboratory, Tokyo, Japan).

Changes of Organosulfur Compounds in Garlic Cloves during Storage. The garlic cloves (sample A) were stored at −3, 4, and 23 °C for 0, 10, 20, 30, 45, 60, 90, 120, or 150 days. The organosulfur compound contents in garlic cloves were determined according to the quantitative method described above, and at least quintuplicate analyses were performed with the garlic samples for each storage condition.

Changes of Isoalliin in Aqueous Solution and Garlic Cloves. Isoalliin (42 μM) was dissolved in 20 mM potassium phosphate solution (pH 4.6, 5.5, or 6.5), and this solution was transferred to a 1 mL glass vial fitted with a polyethylene cap. Garlic samples (sample B) that had been stored at 4 °C for 120 days and contained a low content of GSPC (0.7 ± 0.7 μmol/g dry weight) were used, to minimize the conversion of GSPC to isoalliin or cycloalliin during storage experiments. The vials and the garlic samples were stored at 25 and 35 °C for 0, 1, 2, 4, 7, 10, 15, 20, or 30 days. To determine the concentrations of isoalliin and cycloalliin, the solution in the vial was directly injected into the HPLC apparatus, while the stored garlic samples were determined according to the quantitative method described above at least three times for each storage condition. The degradation of isoalliin was analyzed in accordance with first-order kinetics, and the rate constants (k) were calculated according to the equation:

$$k = 1/t \cdot \log C_0/C$$

where t is the storage time (day), C_0 is the initial concentration (or content) of isoalliin, and C is the concentration (or content) of isoalliin remaining after storage.

Statistical Analyses. The results are expressed as means ± standard deviations (SDs) of at least three different garlic cloves. The statistical

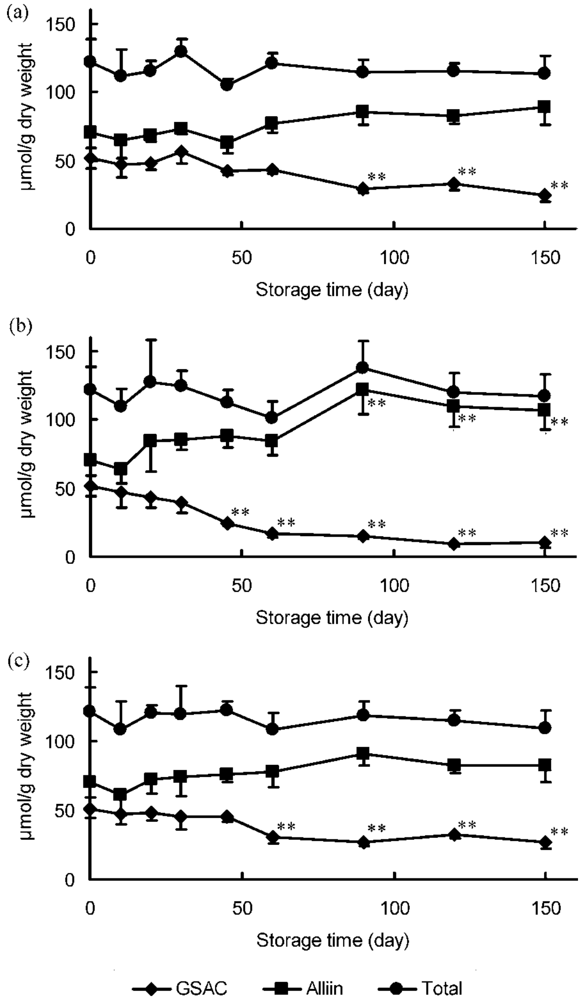

Figure 2. Effects of storage temperature on the contents of GSAC and alliin in garlic cloves stored at –3 (**a**), 4 (**b**), or 23 °C (**c**). The total group consists of the compounds GSAC and alliin. Each point presents the mean ± SD of at least five garlic cloves. Symbols indicate significance of differences as compared with day 0 (Scheffe's test): $**P < 0.01$.

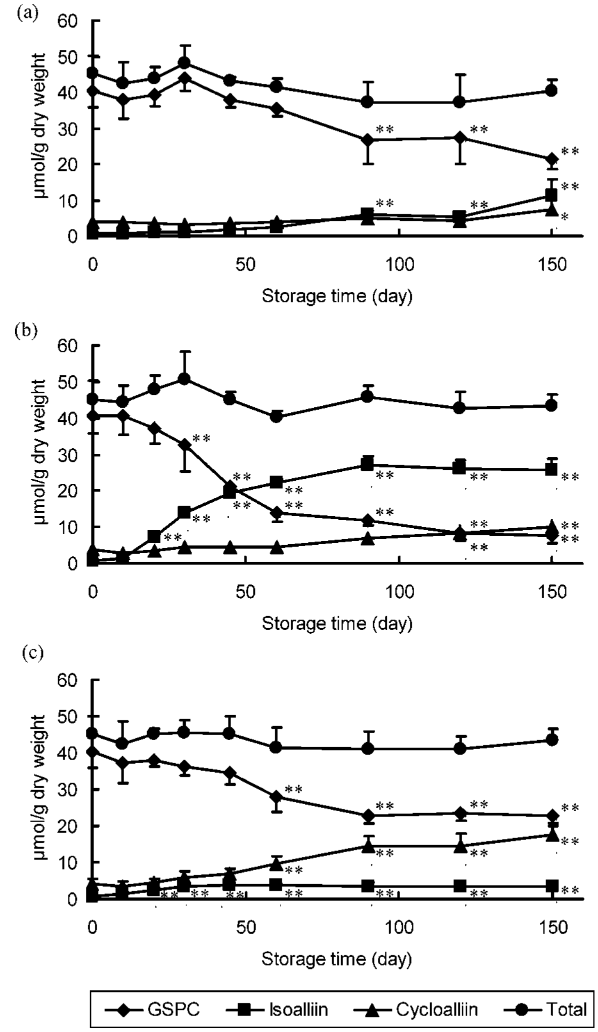

Figure 3. Effects of storage temperature on the contents of GSPC, isoalliin, and cycloalliin in garlic cloves stored at –3 (**a**), 4 (**b**), or 23 °C (**c**). The total group consists of the compounds GSPC, isoalliin, and cycloalliin. Each point presents the mean ± SD of at least five garlic cloves. Symbols indicate significance of differences as compared with day 0 (Scheffe's test): $*P < 0.05$; $**P < 0.01$.

significance of differences between means was determined using one-way analysis of variance followed by Scheffe's multiple comparison test.

RESULTS AND DISCUSSION

Changes of Organosulfur Compounds in Garlic Cloves during Storage. The changes of organosulfur compounds in garlic cloves during storage at −3, 4, or 23 °C for 150 days are shown in **Figures 2−4**. Decreases in GSAC and increases in alliin were observed at all storage temperatures (**Figure 2**). Especially at 4 °C, the GSAC content changed markedly and was reduced by 66% during the first 60 days of storage (51.2 ± 7.3 to 17.2 ± 2.7 μmol/g dry weight), thereafter showing a further decrease during the next 90 days. The total content of GSAC and alliin showed no significant changes at any temperature, suggesting that GSAC was converted to alliin during storage. **Figure 3** shows the changes in GSPC, isoalliin, and cycloalliin in garlic cloves during storage at −3, 4, and 23 °C. At −3 °C, the GSPC content did not change until 60 days but then decreased gradually thereafter and finally declined by 53% in comparison with that on day 0 (40.4 ± 4.6 to 21.6 ± 2.9 μmol/g dry weight). During storage at 4 °C, the GSPC content changed markedly between 10 and 60 days and decreased by 66 and 81% after 60 and 150 days of storage (40.4 ± 4.6 to 13.8 ± 2.4 and 7.7 ± 2.1 μmol/g dry weight), respectively. The GSPC content diminished slowly during the first 90 days at 23 °C and reached a plateau at about 23 μmol/g dry weight (57% to 0 day). The increases in isoalliin almost coincided with the decreases in GSPC at −3 and 4 °C, and the isoalliin content showed 14- and 32-fold increases at 150 days, respectively (0.8 ± 0.1 to 11.2 ± 4.5 and 25.7 ± 2.9 μmol/g dry weight). Although only a 4-fold increase was observed after 150 days of storage at 23 °C (0.8 ± 0.1 to 3.4 ± 1.1 μmol/g dry weight), the reduction of the GSPC content was very similar to that at −3 °C. On the other hand, the content of cycloalliin increased 1.9-, 2.5-, and 4.4-fold at −3, 4, and 23 °C, respectively, during 150 days (4.0 ± 1.5 to 7.5 ± 3.5, 10.0 ± 2.0, and 17.5 ± 3.1 μmol/g dry weight). The total contents of GSPC, isoalliin, and cycloalliin showed no significant changes during storage, suggesting that GSPC was converted to isoalliin or cycloalliin. **Figure 4** shows that the GSMC content decreased by 39% during storage at 4 °C for 150 days, whereas only slight decreases were observed at −3 and 23 °C. However, no clear changes in the methiin content, or the total content of GSMC and methiin, were observed under any storage conditions.

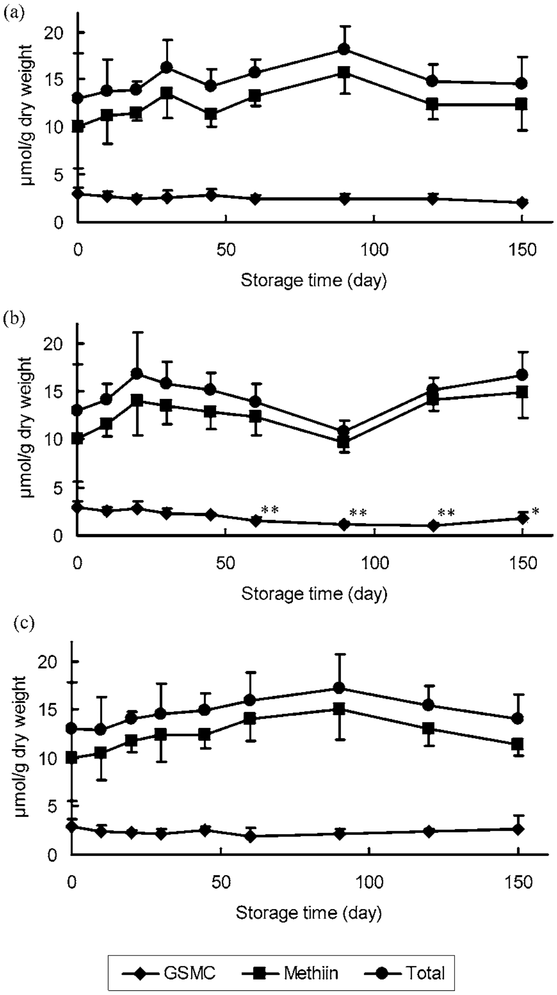

Figure 4. Effects of storage temperature on the contents of GSMC and methiin in garlic cloves stored at –3 (**a**), 4 (**b**), or 23 °C (**c**). The total group consists of the compounds GSMC and methiin. Each point presents the mean ± SD of at least five garlic cloves. Symbols indicate significance of differences as compared with day 0 (Scheffe's test): *$P < 0.05$; **$P < 0.01$.

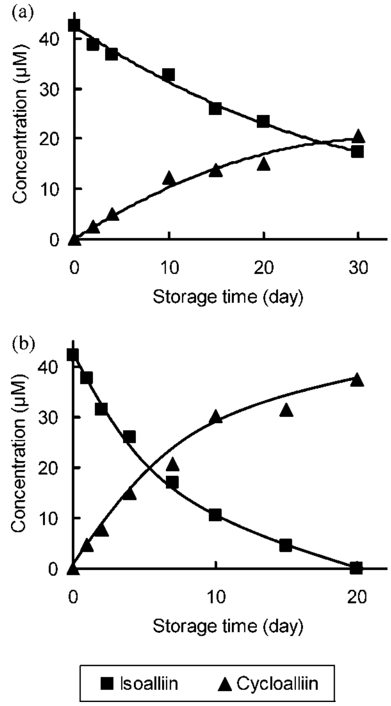

Figure 5. Changes of isoalliin and cycloalliin in isoalliin solution (pH 5.5) stored at 25 (**a**) or 35 °C (**b**).

It has been reported previously that sprouting garlic and onion show higher γ-glutamyl transpeptidase activity than dormant bulbs (*22*, *23*). Our data indicated that the GSAC and GSPC contents of garlic cloves changed markedly at 4 °C, suggesting that these γ-glutamyl peptides, GSAC and GSPC, might be converted to sulfoxides such as alliin, isoalliin, and/or cycloalliin, due to the activation of γ-glutamyl peptidase in garlic that accompanies the breaking of dormancy during storage at 4 °C. This change of γ-glutamyl peptides to sulfoxides may have a close relationship with storage at 4 °C as an optimum temperature for induction of sprouting and rooting, as reported previously (*13*−*16*). It has been reported that garlic bulbs stored at −3 °C for 9 months retained the quality characteristic, firmness, and taste (*14*). As shown in **Figures 2a**, **3a**, and **4a**, no changes of the γ-glutamyl peptides (GSAC, GSPC, and GSMC) in garlic cloves stored at −3 °C were observed until 60 days, indicating that storage at this temperature may be useful for maintaining the dormant phase.

Changes of Isoalliin in Aqueous Solution and Garlic Cloves. Granroth and Virtanen (*24*, *25*) have proposed the biosynthetic pathway of cycloalliin in onion bulbs, where the precursor, *S*-(*trans*-1-propenyl) cysteine, is converted by cyclization and oxidation of the thioether. However, the biosynthetic pathway of cycloalliin in garlic blubs is not yet sufficiently understood. Also, it has been reported that isoalliin is chemically converted to cycloalliin under alkaline conditions or in boiled water at 98 °C (*26*−*28*). Therefore, to clarify the biosynthetic pathway of cycloalliin in garlic, we compared the changes of isoalliin and cycloalliin in garlic cloves with those in isoalliin solution during storage at 25 and 35 °C. **Figure 5** shows the changes of isoalliin and cycloalliin in solution (pH 5.5) during storage at 25 and 35 °C. Decreases in isoalliin and simultaneous increases in cycloalliin were observed under both conditions, and marked changes were observed at 35 °C. **Figure 6** shows the contents of isoalliin and cycloalliin in garlic cloves during storage at 25 and 35 °C. Our data indicated that conversion of isoalliin to cycloalliin occurred under both conditions and that this change was remarkable at 35 °C. Ueda et al. (*28*) have reported that the degradation of isoalliin in solution followed first-order kinetics. The first-order degradation rate constants (k, day^{-1}) of isoalliin and the correlation coefficients (r^2) in three different pH buffer solutions (pH 4.6, 5.5, and 6.5) and in garlic cloves at 25 and 35 °C are given in **Table 1**. The r^2 values ranged from 0.984 to 0.999 in the solutions and from 0.939 to 0.970 in the garlic cloves. These data indicated that the conversion of isoalliin to cycloalliin during storage followed first-order kinetics in both buffer solutions and garlic cloves. The degradation of isoalliin in the solutions was shown to accelerate at higher pH, in agreement with the data reported previously by Ueda et al. (*28*), and the k value obtained from garlic cloves was similar to that from the pH 5.5 solution.

Taken together, our data indicate that GSPC shows marked conversion to isoalliin at 4 °C, followed by a chemical change to cycloalliin when garlic cloves are stored at a higher temperature. The mechanism by which cycloalliin is formed in garlic cloves differs from that in onion, where the precursor, *S*-(*trans*-1-propenyl) cysteine, is converted by cyclization and oxidation of the thioether (*24*, *25*).

Garlic has been reported to have numerous beneficial properties including anticancer effects and prevention of cardiovascular diseases (*2*−*6*, *29*, *30*). The organosulfur compounds are thought to be responsible for its biological activities. Sulfoxides such

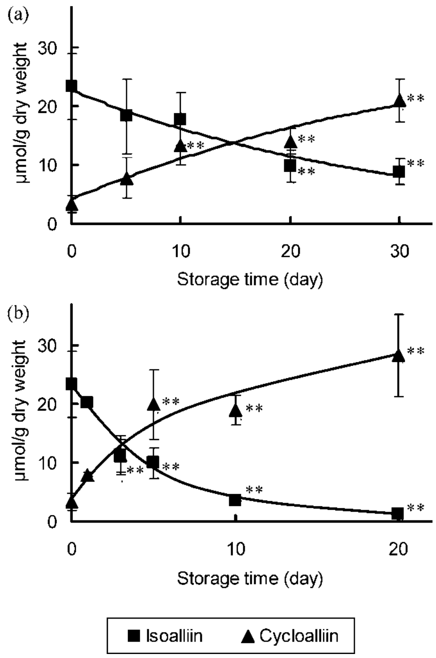

Figure 6. Changes of isoalliin and cycloalliin in garlic cloves stored at 25 (**a**) or 35 °C (**b**). Each point presents the mean ± SD of at least three garlic cloves. Symbols indicate significance of differences as compared with day 0 (Scheffe's test): $^{**}P < 0.01$.

Table 1. First-Order Rate Constants (k) for the Degradation of Isoalliin in Buffer Solution at Different pH Values and in Garlic Cloves

	pH 4.6		pH 5.5		pH 6.5		garlic clove[a]	
temperature	k (day^{-1})	r^2	k (day^{-1})	r^2	k (day^{-1})	r^2	k (day^{-1})	r^2
25 °C	0.027	0.995	0.029	0.993	0.150	0.998	0.034	0.939
35 °C	0.095	0.984	0.148	0.995	0.522	0.999	0.144	0.970

[a] Value calculated from the average of at least three replicate experiments.

as alliin and cycloalliin have anticancer (*2*, *3*) and lipid-lowering effects (*4*), whereas γ-glutamyl peptides lower systolic blood pressure (*5*) and blood cholesterol levels (*6*). Our data suggest that decreases in γ-glutamyl peptides and increases in sulfoxides during storage of garlic cloves might give different biological activities although it appears the same in garlic cloves. Furthermore, various processing of garlic can yield quite different preparations. For example, sulfoxides except cycloalliin are transformed into thiosulfinates such that alliin is transformed into allicin by alliinase when raw garlic is cut or crushed (*11*, *31*). During the extraction by aqueous ethanol solution, GSAC is converted to *S*-allyl-L-cysteine, which shows a variety of biological activities, including prevention of cardiovascular diseases (*32*−*34*) and cancer (*35*), and antioxidant properties (*36*−*38*). On the other hand, cycloalliin revealed the biosynthetic pathway in this study is stable during the processing of garlic (*39*) and has biological activities (*4*, *29*, *30*). This suggests that cycloalliin might be useful as a chemical marker for quality control of garlic and its preparations.

In summary, we have studied the changes in organosulfur compounds in garlic cloves during storage at different temperatures. Our data indicate that γ-glutamyl peptides undergo marked conversion to sulfoxides when garlic cloves are stored at 4 °C. We also demonstrated that isoalliin produced enzymatically from GSPC is chemically converted to cycloalliin and that the cycloalliin content increases when garlic cloves are stored at higher temperature. These data may be useful for controlling the quality and biological activities of garlic and its preparations.

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