Allixin Accumulation with Long-term Storage of Garlic

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Extremely high accumulation of allixin, a phytoalexin derived from garlic, was observed in necrotic tissue areas after long-term storage. The allixin produced recrystallized on the surface of the garlic clove. The amount of allixin produced in raw garlic with necrotic tissue areas was 1400 ng/mg wet garlic, which exceeds the minimum exhibitory concentration of allixin. After approximately 2 years of storage, amount of allixin accumulated reached slightly less than 1% of the dry weight of garlic cloves.

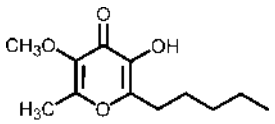
Key words allixin; accumulation; long-term storage; phytoalexin; recrystallization

Plants have several defense systems against the invasion of microorganisms, one of which is systems the accumulation of newly produced chemicals called phytoalexins.[1,2] Allixin was the first compound isolated as a phytoalexin from garlic and it has several unique biological properties.[3—6] Although the antimicrobial activities of phytoalexins are weak, they are accumulated to repel the invasion of microbes on the local surface of plants. Garlic may accumulate allixin in response to microbial invasion because allixin also has weak antimicrobial activities, although the amount of allixin accumulated in previous induction studies was lower than the minimum inhibitory concentration (MIC).[3] Therefore it is assumed that induction of allixin might not be sufficient to combat the invasion of microorganisms. On the other hand, we often observed higher levels of allixin in garlic stored than those induced by chemical, physical, and biological methods. The present paper reports evidence of antimicrobial activity by extremely high amounts of allixin accumulated and crystallized during long-term storage.

Result and Discussion

An extremely high accumulation of allixin was found in garlic after about 9 months of storage in a drafty room without air-conditioning after harvesting and curing at midsummer (Fig. 1a). Although the outside appearance of the garlic bulbs was the same as at the beginning of storage, a crystallized substance was observed to adhere to the surface of some raw garlic cloves with necrotic tissue areas. The substance was not observed on the skin surface of garlic or on nonnecrotic areas, but only on necrotic areas (Fig. 1a). Figure 1c shows a garlic clove with the adhering substance after storage for about 2 years. No such substance was observed on the skins of garlic cloves. All of the adhering crystalline substance found on the raw garlic with necrotic tissue areas and dehydrated cloves was identified as allixin by ^{1}H- and ^{13}C-NMR spectroscopy and comparison with data in previous reports.[3,7]

^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=6.96), 1.34 (4H, m), 1.61 (2H, m), 2.34 (3H, s, –Me), 2.67 (2H, t, J=8.06), 3.89 (3H, s, –OMe), 6.1—6.35 (1H, br, –OH), ^{13}C-NMR ($CDCl_3$) δ: 13.94 (C-5′), 15.06 (–Me), 22.33 (C-4′), 26.39 (C-1′), 28.31 (C-2′), 31.26 (C-3′), 60.14 (–OMe), 141.84 (C-3, C-5), 150.15 (C-2), 158.09 (C-6), 169.48 (C=O).



Structure of allixin

Extensive surface area necrosis was observed on the raw garlic with necrotic tissue areas (Fig. 1a), and the necrosis occurred within a few millimeters from the surface (yellowish-brown tissue area, Fig. 1b). Crystalline allixin was only observed to adhere to the surface of the necrotic area. Figure 2 shows the relationship between the amount of allixin accumulated and the distance from the surface of the raw garlic with necrotic tissue areas with adhering allixin shown in Fig. 1a and b. Most allixin was produced and accumulated around the necrotic surface region. This may indicate that the plant resists the invasion of microorganisms by accumulating phytoalexins on necrotic surface areas.[1] Although the antimicrobial effects of phytoalexins are generally quite weak, the amount of allixin accumulated on the surface was sufficient to protect against the invasion of microorganisms, *i.e.*, almost ten times the antimicrobial activity level and higher than the MIC of allixin (MIC: >160 μg/ml; *Aspergillus niger*, >100 μg/ml; *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, 80 μg/ml; *Candida albicans*, MIC data not shown previously).[3] Therefore based on the allixin accumulation described above, it is assumed that allixin is produced in a site-specific manner.[8,9] Furthermore, the resistance against microorganisms on the surface might be very strong in this case because the amount of allixin adhering to the necrotic garlic surface may significantly exceed the MIC of allixin.

Although many garlic cloves stored for approximately 2 years became fragmented by the invasion of microorganisms, the cloves with allixin crystalline adhesions retained their original shape well but with slight shrinkage (Fig. 1c). The allixin produced in these dried garlic cloves after about 2-year storage was analyzed, and Table 1 compares the results with those in previous reports.[3,10] Allixin was not observed in fresh garlic under stress-free conditions. However, garlic produced allixin in response to physical, chemical, or biological stress, as in previously reported induction experiments.

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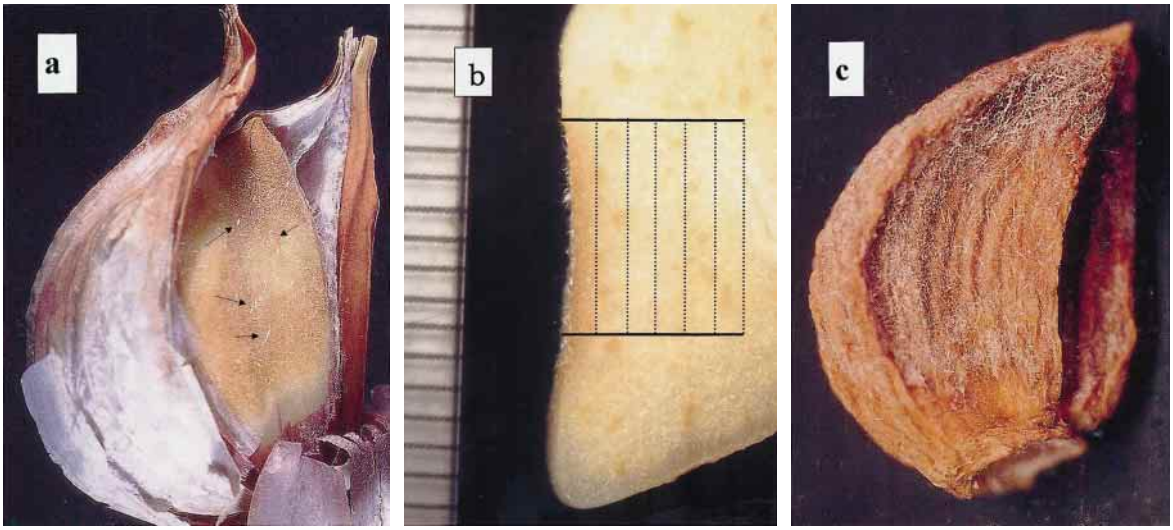

Fig. 1. (a) Appearance of Living Garlic with Adhering Allixin (about 9 Months of Storage)

The crystalline substance on the brown area of necrosis is allixin. The area without necrosis did not have allixin adhesion. Arrows indicate the crystalline of allixin adherences.

(b) Magnification of Allixin Content in Part of Fig. 1a Which was Cut Horizontally before Removing the Adhered Allixin

The line in the figure illustrates the method of tissue sample preparation for analysis of allixin content. Each rectangular part was used as a sample (for results on allixin content, see Fig. 2). Graduation on scale the is 1 mm.

(c) Appearance of Dried Garlic with Adhered Allixin (about 2 Years of Storage)

The adhering needle-like substance on the dried garlic surface is crystalline allixin.

Table 1. Allixin Content in Garlic Samples

Sample			Content (μg/g)
Intact garlic[a,c,f]			Trace
Induction[b,c,f]		UV light	3
		$HgCl_2$	20
		Pectinase	130
Long-term storage	9 months[d,f]		Not more than 290
	2 years[e]	1	3270
		2	1940
		3	7160
		4	3730
		5	7400

a) Raw garlic clove; *b*) previously reported[3,10]; *c*) content in wet weight; *d*) calculated amount based on results of Fig. 2 (mean number, content in surface to 5 mm from surface); *e*) content in dry weight; *f*) water content in raw garlic is 60—70%.

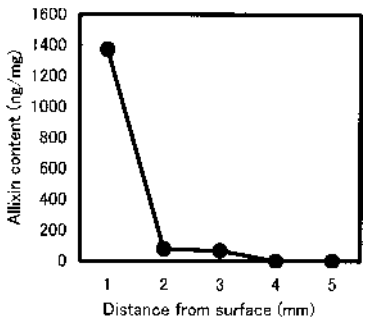

Fig. 2. Relationship between Accumulation of Allixin on Local Necrotic Surface and Distance from Surface

Allixin adhering to surface was removed with a brush and rinsed with methanol, and then garlic was cut into small pieces (8 mm in cross section, 4 mm in vertical section, 1 mm in thick), and the allixin content in each piece was analyzed.

The amount of allixin produced in induction experiments did not exceed 130 μg/g of raw garlic. The amount of allixin accumulated in the whole clove shown in Figs. 1a—c not be more than 290 μg/g based on the results in Fig. 2, although the amount accumulated in the surface region was nearly 1400 μg/g (content in piece of 1 mm from surface in Fig. 2). However, some specimens after long-term storage contained more than 10 times the amount of allixin obtained in induction experiments (Fig. 1c, Table 1). Additionally, the accumulation of nearly 1% of the dry weight of the clove was the highest observed.

The hypothesis that allixin might not be the final secondary metabolite in garlic was proposed in a previous report because the amount of allixin accumulated decreased with time after C_{max}, and allixin disappeared after the stress ceased.[3,10] However, the amount of allixin accumulated in garlic cloves after 2 years of storage was more than 5 times that in garlic after 9 months of storage (calculated based on the water content in fresh garlic). This remarkable accumulation of allixin after a further 15 months of storage might be due to production in response to continuous stress, migration of allixin produced to the surface region, recrystallization of allixin on the surface, and protection of allixin by tightly fitting skin. Thus accumulated allixin might protect the garlic clove against fragmentation by invasion of microorganisms.

The stored garlic cloves were completely dehydrated, light brown in color, and the adhesion of crystalline allixin was also observed to varying degrees. Adhering allixin was seen in both raw garlic with necrotic tissue areas and dehydrated garlic only in cloves protected by tightly fitting and thickened hard-gloss skins. This type of skin might have prevented the disappearance of allixin during the storage period. Thus the phenomenon of recrystallization on the surface may indicate that: i) allixin is capable of sublimation; ii) the storage temperature is appropriate suitable; and/or iii) the garlic cloves

were tightly wrapped in thickened hard-gloss skin to prevent the loss of allixin.

To our knowledge, the adhesion of phytoalexin or crystallized phytoalexin on the surface of living plants has not yet been observed, so far. Allixin, a *de novo* synthesized substance classified as phytoalexin, might play a prohibitory, inhibitory, or postinhibitory antimicrobial role in garlic.[1,2]

Experimental

Structural analysis was performed using NMR (JNM-ECP500, Jeol, Tokyo, Japan), and quantity analyses were performed using a Shimadzu LC 10A HPLC system (Shimadzu, Kyoto, Japan). All chemicals for experiments were of analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

Plant Materials and Storage Conditions Garlic (*Allium sativum* L.) bulbs were cultivated in a green house in a Wakunaga Pharmaceutical experimental field. Garlic bulbs were harvested in July and then were cured in a drafty, shaded area for several weeks until the stems had dried completely. The resulting garlic bulbs were placed in nets and stored in a drafty room without any air-conditioning for more than 9 months.

Quantity Analyses The following method was used for the preparation of sample solution for allixin analysis in raw garlic with necrotic tissue areas after 9-month storage. The skin was carefully removed from the cloves. Garlic cloves with adhering crystalline substance were used as samples to quantify allixin. The a adhering crystalline substance on garlic samples was carefully brushed from the necrotic surface and the samples were cut into round slices 4 mm thick with the necrotic part included. A rectangular parallele piece 8 mm wide and about 6 mm long from the necrotic surface to the central part was cut from the round slice of garlic (see Fig. 1b). This rectangular parallele piece was cut into further small rectangular parallele pieces (8 mm in cross section, 4 mm in the vertical section, 1 mm thick) in an orderly sequence from the surface using a stereomicroscope. Each piece was used as sample for analysis of the allixin contained. Each piece was immediately placed into a previously weighed sample cup, the cup and sample were weighed, and then each piece was homogenized with about 300 μl of methanol. The resulting mixture was transferred to a tube marked with a measuring scale and cap, and the volume was adjusted to 5 ml with methanol. About 1 ml of this mixture was transferred to capped test tubes and then centrifuged at 15000 rpm for 10 min. The supernatant obtained was used for analysis.

Preparation of Sample Solution on Dehydrated Garlic: Garlic skin was carefully removed from cloves dehydrated as a result of long-term storage. Garlic cloves with adhering crystalline substance were used for allixin quantification. Dehydrated garlic was pulverized in a Waring blender and the resulting substance was extracted with 50 ml of methanol. Part of the methanol extract was centrifuged at 15000 rpm for 10 min and supernatant obtained was used for analysis.

HPLC Conditions: HPLC analysis was performed according to the method in a previous report.[3]

NMR Analysis of Adhering Crystalline Substance Adhering crystalline substance was carefully brushed from the garlic surface, dissolved in chloroform-*d*, and subjected to NMR analysis. The observed NMR spectrum was compared with that of authentic allixin and in previous reports.[3,7]

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