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Thermostability of Allicin Determined by Chemical and Biological Assays

Hiroyuki FUJISAWA,[1,2] Kaoru SUMA,[3] Kana ORIGUCHI,[3] Taiichiro SEKI,[1,3] and Toyohiko ARIGA[1,3,†]

[1]*Nihon University Graduate School of Applied Life Sciences, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan*
[2]*Nagaoka Perfumery Co., Ltd., 2-2-6 Kitakyuhoujimachi, Chuo-ku, Osaka 541-0057, Japan*
[3]*Department of Agricultural and Biological Chemistry, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan*

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The garlic-derived antibacterial principle, alk(en)yl sulfinate compounds, has long been considered as very short-lived substance. However, there are some data showing a rather more stable nature of allicin. We determined here the thermostability of allicin by a systematic analyses employing chemical quantification and an antibacterial activity assay. Allicin in an aqueous extract of garlic was degraded stoichiometrically in proportion to the temperature; we estimated the half-life of allicin to be about a year at 4 °C (from 1.8 mg/ml to 0.9 mg/ml) and 32 d at 15 °C, but only 1 d at 37 °C (from 2.0 mg/ml to 1.0 mg/ml). The half-life values for antibacterial activity showed a similar trend in results: 63 d or more at 4 °C for both antibacterial activities, 14 d for anti-staphylococcal activity, and 26 d for anti-escherichia activity at 15 °C, but only 1.2 d and 1.9 d for the respective activities at 37 °C. Such antibacterial activities were attributable to the major allicin, allyl 2-propenylthiosulfinate. Surprisingly, the decline in the quantity of allicin was not accompanied by its degradation; instead, allicin became a larger molecule, ajoene, which was 3-times larger than allicin.

Key words: allicin; garlic; thermostability; ajoene; antibacterial activity

Garlic (*Allium sativum* L.) has an extraordinary amount of sulfur, mainly in the form of organosulfur compounds called alliin,[1,2] which is a general name for a class of alk(en)yl-L-cysteine sulfoxides containing allyl (2-propenyl), 1-propenyl, propyl or methyl groups as their alk(en)yl moieties.[3,4] When crushed, garlic produces sulfinyl compounds, known as allicin, from alliin *via* the enzymatic action of alliinase (alliin lyase, EC 4.4.1.4).[5] Garlic contains most abundantly allyl-L-cysteine sulfoxide as alliin, and thus yields allicin, which has the diallyl-*S*-sulfinyl oxide structure, and is chemically known as allyl 2-propenylthiosulfinate.[6] Since the garlic plant utilizes allicin as its most effective protector against microbial and fungal attacks, or from animal bites and other damage, they have survived only with the vegetative propagation in many places on the earth, even after losing the ability for seed propagation.[7,8] Garlic bulbs store as much as 6 mg/g of alliin, which correspond to 1.7% of their dry weight, and they also contain as much as 2.8% of alliinase protein on a dry weight basis.[4,9] Mankind is thought to be the only creature who eats garlic. Garlic has also been one of the most useful vegetables for preserving meat and fish, disinfecting skin scratches, seasoning dishes, and providing nourishing meals.[7] The antimicrobial potential of garlic has been recognized since ancient Egypt. In particular, before the advent of penicillin and sulfanilamides, which became available in the 1940s and 1930s, respectively, garlic was one of the most effective antibiotic substances.

In respect of the antibacterial activity of allicin, it has been reported that this activity is bacteriocidal, whereas that of allicin-derived sulfides is bacteriostatic.[10] Allicin is effective toward most Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* and multidrug-resistant *Shigella dysenteriae*.[11] However, such mucoid strains as *Pseudomonas aeruginosa*, S*treptococcus β hemolyticus*, and *Enterococcus faecium* are known to be resistant to allicin.[12] Although the reason is not yet clear, it has been assumed that the hydrophilic capsular or mucoid layers prevent the penetration of allicin into the bacteria. Characteristically, most bacteria cannot develop resistance against allicin, the reason being that allicin directly modifies thiol enzymes or certain proteins involved in cell division. It is also known that allicin inhibits RNA synthesis more strongly than DNA and protein synthesis in *Salmonella typhimurium*.[13]

We have little evidence at present for the medicinal use of allicin, especially for humans. Allicin has recently

† To whom correspondence should be addressed. Tel: +81-466-84-3948; Fax: +81-466-84-3949; E-mail: ariga@brs.nihon-u.ac.jp

Abbreviations: HPLC, high-performance liquid chromatography; *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; NB, nutrient broth; LC, liquid chromatography; MS, mass spectrometry

been utilized by Israeli scientists as an anticancer agent inducing apoptosis in cancer cells.[14,15] Their method is based on the fact that allicin can be produced from alliin by the plant enzyme alliinase only at the cancer cell surface, where the enzyme has initially been delivered as a conjugate with a specific antibody.

Considering these valuable effects of allicin, we should use it even now as an antibiotic agent that is easily obtainable from garlic. More efficient use of garlic-derived allicin has involved modern technology for its identification, quantification, and determination of stability. Although a few reports have described allicin as being relatively stable in aqueous and ethanolic solutions,[4] little work has been done to determine the thermostability of allicin in an ordinary garlic extract by systematic analyses employing chemical and biological assays at the same time. Since we have recently established the analytical high-performance liquid chromatography (HPLC) for the quantification of garlic-derived allicin, a comparative study on the thermostability of allicin has now become possible. This is the first report describing the thermostability of allicin, which can be prepared by a generally acceptable way of extraction, studied by chromatographic quantification and in terms of its antibacterial activities against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*.

Materials and Methods

Quantitative analysis of allicin. Allicin was separated by HPLC in a C18 column of MG-II (5 μm, 4.6 mm × 250 mm; Shiseido, Tokyo, Japan). A pump (LC-10AT, Shimadzu, Kyoto, Japan) and Chromato-PRO data integrator (Run Time Co., Kanagawa, Japan) equipped with a 655A UV monitor (Hitachi, Tokyo, Japan) constituted the HPLC system. The solvent for operating the column was a 0.02 M phosphate buffer (pH 6.5) containing acetonitrile and 1,4-dioxane in the ratio of 7:1:2. Twenty μl of allicin or an allicin-containing garlic extract were applied to the column and eluted by isocratic application of the solvent at a flow rate of 0.5 ml/min. Allicin in eluate was detected by measuring its optical absorption at 220 nm, and quantified by comparing the peak area (V·s of the electric signal) produced by authentic allicin with that of the garlic extract. Authentic allicin was a reagent-grade preparation with 99.39% purity (LKT Laboratories, MN, USA), and was kept at −70 °C until being used.

Preparation of the garlic aqueous extract and its incubation. Garlic (*Allium sativum* L., 'Fukuchi White') grown in Aomori, Japan, was obtained at a market, stored at 4 °C, and used for analysis within 30 d. The allicin-containing garlic extract was prepared from 10 g of garlic cloves. The cloves were crushed with an electric vegetable crusher (TK-102, Tescom Co., Tokyo, Japan), and the juice and debris were collected in a centrifuge tube by flushing the crusher with 10 ml of water. After having been shaken 10 times, the tube was allowed to stand for 10 min at room temperature and then centrifuged twice at 5,500 rpm for 5 min at 4 °C. The supernatants were combined and then analyzed for antibacterial activity and the amount of allicin, as described next. Except for the incubation at various temperatures, the experiments were all performed at room temperature (23–25 °C). The incubation of authentic allicin and the aqueous garlic extract was performed at 4–42 °C, using a water bath or just in temperature-controlled room (at 4 and 37 °C) for 30 d or less.

Assay of antibacterial activity. The bacteria used were Gram-positive *Staphylococcus aureus* (Rosenback 1884 NBRC 12732) and Gram-negative *Escherichia coli* C600, kindly provided by Dr. R. Takahashi of Nihon University. These bacteria were cultured on a nutrient broth (NB) agar plate for 24 h or more at room temperature, and the colonies that formed were picked up twice with a platinum loop and uniformly inoculated onto the surface of NB agar plates (20 ml of agar/9-cm Petri dish) with a sterilized cotton stick. A 50 μl amount of the garlic extract was put on an ethanol-sterilized paper disk (8 mm diam., EB-101; Advantech, Tokyo, Japan) and the disk was placed on the plate. After incubating at 37 °C for 24 h, the antibacterial activity was determined as the square of the diameter (mm^2) of the bacteria-free zone.

Liquid chromatography (LC)/MS/MS. Mass spectrometry was performed by using a Waters Acquity Ultra Performance LC system and Quattro Premier XE MS system (Waters, MS, USA) with a 50 mm × 2.1 mm column of Acquity BEH C18 (1.7 μm). A sample volume of 5 μl was injected with an autosampler. The mobile phase was composed of solvent A (0.1% formic acid in water) and solvent B (acetonitrile/1,4-dioxane = 1:2) and introduced at a constant flow rate of 0.2 ml/min. The graded mobile phase was programmed to increase the amount of B from an initial 30% to a final 100% in 7 min. The MS/MS analysis was performed with a positive ion mode electrospray interface, loading cone voltage of 20 V, and capillary voltage of 2.5 kV.

Results and Discussion

Thermostability of allicin generated in the aqueous extract from freshly crushed garlic

The amount of allicin contained in an ordinary aqueous garlic extract (10 g of cloves with 10 ml of water in this study) was determined to be approximately 2 mg/ml, and it decreased with time and temperature after the extraction. As shown in Fig. 1, the quantity of allicin decreased linearly depending either on the incubation time (days) or temperature. Although, in this experiment, we used two different extracts of garlic containing allicin at 1.84 mg/ml and 2.04 mg/ml, and

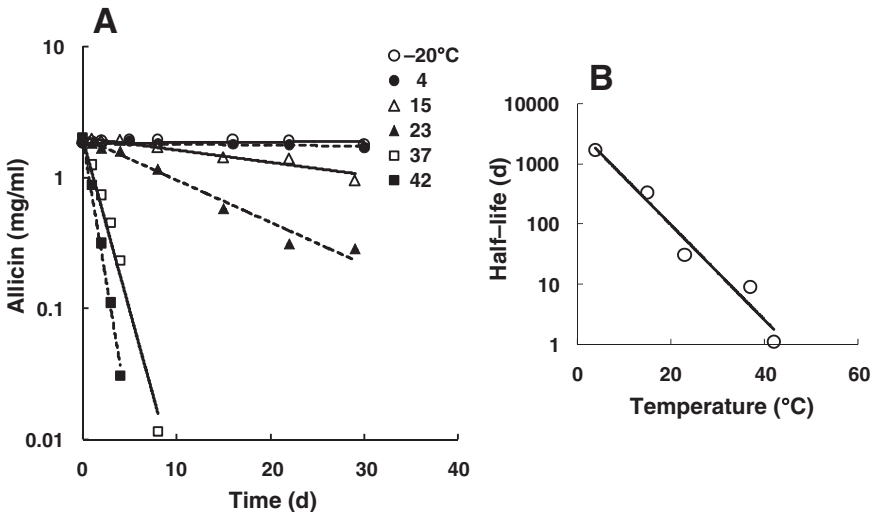

Fig. 1. Thermostability of Allicin in an Aqueous Garlic Extract.

A, Decay curves for allicin in the extracts incubated for 30 d at various temperatures from −20 to 42 °C. The content of allicin (allyl 2-propenylthiosulfinate) was assayed by HPLC during the time plotted in the figure. B, Arrhenius plots showing that allicin decreased exponentially as the temperature increased. The half-life of allicin was calculated from the curves shown in Fig. 1A (see also Table 1), and is presented on the ordinate with a logarithmic scale.

incubated the former at −20–4 °C and the latter at 15–42 °C, both for up to 30 d, we were able to obtain precise characteristics for the decrease of allicin as a function of temperature (see the Arrhenius plots, Fig. 1B). At temperatures lower than 15 °C, allicin was stable, and even at 23 °C, it remained at about 50% of the initial quantity 10 d after the preparation. Since allicin decreased with first–order kinetics at a given temperature, the equation for the exponential decay curve (a) could be adopted to calculate the amount of allicin remaining in the extract:

$$\text{(a)} \quad N_t = N_o e^{-kt}$$

where N_t and N_o are the current (at the time t day) and initial quantities of allicin in its aqueous solution, respectively. The *k*-value is the rate constant for allicin's thermal degradation. In practice, the *k*-value was obtained as the slope of semi-logarithmic plots of the amounts of allicin determined several times by HPLC during the incubation for 30 d. The half-life, $t_{1/2}$, can be defined as the t-value at the moment giving $N_t/N_0 = 1/2$. In other words, it is the time when $N_0 = 1$ (100%) becomes $N_t = 1/2$ (50%), and can be obtained from equation (b) as follows:

$$\text{(b)} \quad t_{1/2} = \ln 1/2 / -k = -0.693/-k$$

The *k*-values thus obtained at various temperatures from 4 to 42 °C gave the half-life of allicin as shown in Table 1. The half-life of allicin thus calculated was changed greatly by increasing the temperature; *e.g.*, it changed from 1 d at 37 °C to 346 d at 4 °C, although the latter life, 346 d, is an extrapolated figure. It was difficult to determine such a long life for allicin from only a 30-d experiment. Nevertheless, we were able to identify the relationship between the temperature of the extract and allicin's half-life, as shown in Fig. 1B; the half-life become shorter by about one-order of magnitude by each 10 °C increase in the temperature. According to Canizares *et al.*,[16] allicin in ethanolic and acetone extracts of garlic remained at about 50% of the initial amount when it was stored at 6 °C for 5 months. As allicin is known to be more stable in an aqueous solution than in an organic solvent,[4] we consider as plausible the half-life at 4 °C which we estimated.

Table 1. Rate Constant for Thermal Degradation and Half-Life of Allicin

Temperature (°C)	*k*	$t_{1/2}$ (d)
−20	—	—
4	0.002	346.6
15	0.022	31.5
23	0.074	9.37
37	0.604	1.15
42	0.997	0.70

Lawson *et al.* have reported that the half-life of allicin in an aqueous garlic extract was 30–40 d at 23 °C.[4] This value is 4-times longer than ours, 9.4 d, determined for a similar garlic extract (Table 1). The reason for such a big difference is not yet clear; but as Lawson *et al.* reported,[4] the stability of allicin increased in a highly diluted aqueous solution. Therefore, the extracts that we prepared, 50% (garlic/water = 1:1, 10 g:10 ml), would not have been as dilute as theirs, which was 10% or less.

Thermostability of allicin as determined by the antibacterial activity assay

The biological activity of allicin decreased in proportion to the incubation time (Fig. 2). As shown in Fig. 2A, the anti-staphylococcal activity of the garlic

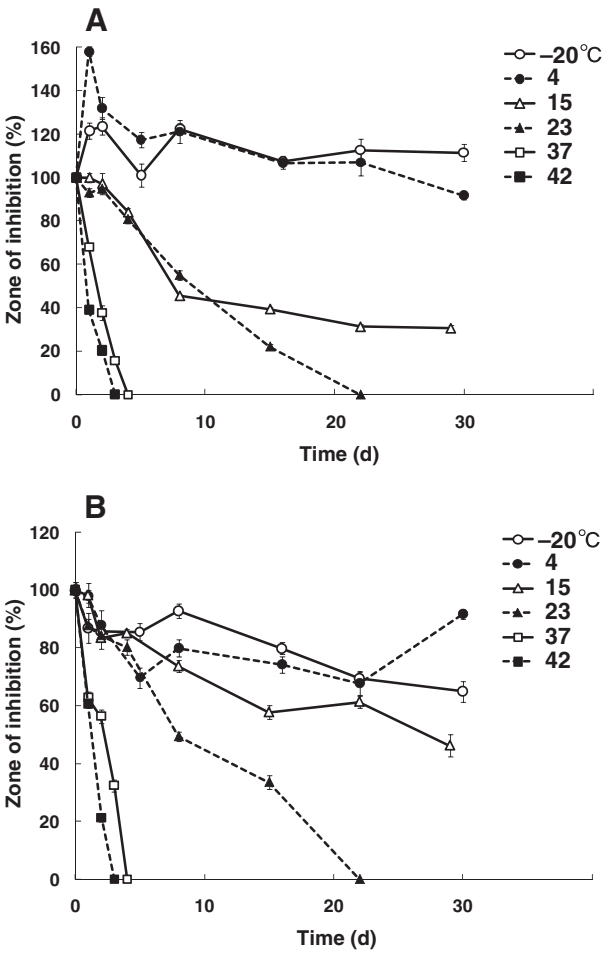

Fig. 2. Thermostability of Allicin in the Garlic Extract Determined by Its Antibacterial Activity.

Curves showing the decrease in anti-staphylococcal (A) and anti-escherichia (B) activity at various temperatures. The aqueous extract of garlic was incubated for up to 30 d at the temperatures indicated in the figure, and the antibacterial activity toward each bacterium was measured. The activity (zone of inhibition) is expressed by its percentage remaining in each extract during the incubation period. Each plot is the mean ± S.E. obtained with 6 disks from a single extract.

extract decreased quickly at temperatures higher than 15 °C, but more slowly at those lower than 4 °C, these rates being quite similar to those for the degradation of allicin determined by HPLC (see Fig. 1). During the incubation period, there was some fluctuation in the activities measured for the extracts incubated at temperatures lower than 4 °C. This would have been due to the low solubility of allicin in such an aqueous extract at low temperatures.[4] The large fluctuation at −20 °C would have resulted from freezing and thawing of the extract, in addition to the lower solubility of allicin in this extract.[17]

The anti-escherichia activity of the garlic extract also decreased temperature and time dependently in a similar fashion to that of the anti-staphylococcal activity (Fig. 2B). Although there were some difference in the pattern of decrease between the antibacterial activities

Table 2. Half-Life of Allicin in the Garlic Extract as Determined by Biological Assays Using *S. aureus* and *E. coli*

Temperature (°C)	*S. aureus*		*E. coli*		Allicin*
	k	$t_{1/2}$ (d)	k	$t_{1/2}$ (d)	$t_{1/2}$ (d)
−20	—	—	—	—	—
4	—	—	0.011	63.0	346.6
15	0.05	13.9	0.027	25.7	31.5
23	0.092	7.5	0.075	9.2	9.37
37	0.565	1.2	0.357	1.9	1.15
42	0.838	0.8	0.719	1.0	0.70

*The half-life of authentic allicin is presented again here for comparison (see Table 1).

toward *S. aureus* (Fig. 2A) and *E. coli* (Fig. 2B), we could not find any difference between the two at temperatures higher than 23 °C. These antibacterial activity curves were used to obtain the rate constant (k value); therefore, the biological half-life of allicin could be calculated according to equation (b). As shown in Table 2, the half-life of allicin at 4 and −20 °C was not correctly determined, because the 30-d incubation period that we performed in this experiment was too short to determine a half-life longer than a few months. However, in respect of the half-lives shorter than 1 month at 15 °C or higher, both bacteria gave values close to each other at a given temperature. The reason why the half-life of allicin determined from the anti-escherichia activity was a little longer than that from the anti-staphylococcal activity is not completely clear, but would undoubtedly involve the difference in susceptibility of the two bacteria to allicin; Gram-positive bacteria (*e.g.*, *S. aureus*) are more sensitive to allicin than are Gram-negative bacteria (*e.g.*, *E. coli*).[11,18]

Relationships for the antibacterial activity and amount of allicin in garlic extracts incubated at various temperatures for up to 30 d

As shown by the solid lines in Fig. 3A and B, all the extracts, which were treated under several thermal conditions for up to 30 d, exhibited a good correlation between their antibacterial activity and amount of allicin (assayed as allyl 2-propenylthiosulfinate). As represented by the plots encircled in the Figure, there were some departures from the averaged activity line. Those plots lower than the average were derived from the extracts incubated at temperatures lower than 15 °C (see Fig. 3A and B, encircled as "b"), and those higher than the average were from the extracts incubated at temperatures higher than 23 °C (Fig. 3A and B, encircled as "a"). Apart from experimental error, these results could be explained by the presence of a component other than allicin (allyl 2-propenylthiosulfinate) which may have been produced or become active at the higher temperatures, but not so at the lower temperatures in exhibiting certain antibacterial activity.

Compared with authentic allicin, the garlic extract containing an equal amount of allicin exhibited stronger

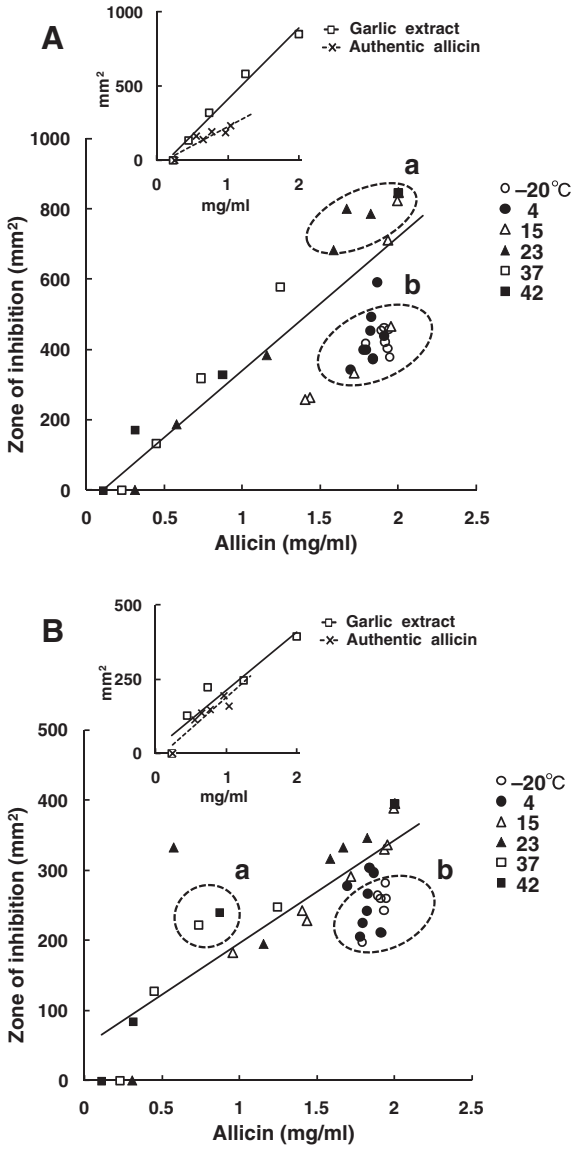

Fig. 3. Relationships between the Antibacterial Activity and Quantity of Allicin Determined for the Garlic Extracts and Authentic Allicin.

A, Anti-staphylococcal activity and allicin in the garlic extract. The solid lines show a correlation between the anti-streptococcal activity and quantity of allicin measured for the garlic extracts which were incubated for up to 30 d at various temperatures as noted inside the figure. The encircled plots are those for activities higher (a) and lower (b) than those on the average line of the correlation. The inset in A shows different slopes in the antibacterial activities between the garlic extract and authentic allicin when incubated at 37 °C for up to 8 d. B, Anti-escherichia activity and allicin in the garlic extract. The solid line and the encircled plots were obtained from *E. coli* in a similar ways to that for *S. aureus*. The inset in B shows the same slopes for the antibacterial activities between the garlic extract and authentic allicin incubated at 37 °C for up to 8 d.

anti-staphylococcal activity (see the inset in Fig. 3A, showing 2 lines with different slopes). Since, as already described, we quantified allyl 2-propenylthiosulfinate, the main component representing approximately 70% of the thiosulfinyl compounds in the garlic extract,[4] the unquantified compounds in the extract would have had anti-staphylococcal activity. These compounds would have been sulfinates containing methyl or 1-propenyl groups as reported by Lawson *et al.*[4] and Canizares *et al.*[16] Although Canizares *et al.* have reported that, among the eight sulfinates, only allicin (allyl 2-propenylthiosulfinate) had antibacterial activity toward *Helicobacter pylori*,[16] we cannot exclude the participation of sulfinates other than allicin in inhibiting the growth of *S. aureus*.

The antibacterial activity toward *E. coli* and the quantity of allicin in the aqueous garlic extract were correlated with $r^2 = 0.6689$ (Fig. 3B). Different from the case of *S. aureus*, the anti-escherichia activity of the garlic extract was similar to that of authentic allicin (see the inset in Fig. 3B). Therefore, the most abundant allicin would apparently have been the only substance to determine the antibacterial power toward the less sensitive bacterium, *E. coli*.

Identification of the breakdown products of allicin

As described above, once allicin is dissolved in water or generated from garlic, its quantity as well as its biological activity decrease hourly or daily depending on the temperature. It has been reported that allicin degrades into small fragments by releasing oxygen, and then the fragments form stable sulfides binding with each other.[19] However, there have been few reports describing the fate of pure allicin in water. Pure allicin dissolved in water (1 mg/ml) showed a 50% decrease in amount when incubated at 37 °C for 4 d (data not shown, but similar to allicin in the extract as shown in Fig. 1). We analyzed such an allicin-containing solution immediately, and 4, and 10 d after the preparation. The LC pattern for a freshly prepared solution formed a single peak at 7.61 min corresponding to allicin (allyl 2-propenylthiosulfinate) and decreased in proportion to the number of days of incubation, producing new peaks at 6.2 and 10.02 min (Fig. 4A). Although we could not obtain any MS fragment from the 6.20 min peak, the 10.02 min peak gave MS/MS fragment patterns making it obvious that the newly produced substance on the LC (10.02 min peak) was a 3-times larger molecule ($m/z = 491$) than that of allicin ($MH^+ = 163$; Fig. 4B and C). This mother molecule is likely to have been composed of two ajoene molecules and one sodium ion bound together. The fragment with m/z 235, which is assumed to have been that of protonated ajoene, could be fragmented further on the second MS, by which allicin ($M^+ = 162.8$) was detected as a mother molecule (see the MS pattern in Fig. 4C). The degradation of allicin in terms of its chemical and biological natures would thus appear to involve binding among the molecules of allicin, by which large molecules would be formed through an ionized form or a short-lived radical. It is noteworthy that the antibiotic activity of an aqueous extract of garlic is determined primarily by the amount of allicin. Hence ajoene that we identified from the incubated aqueous solution of allicin would not have

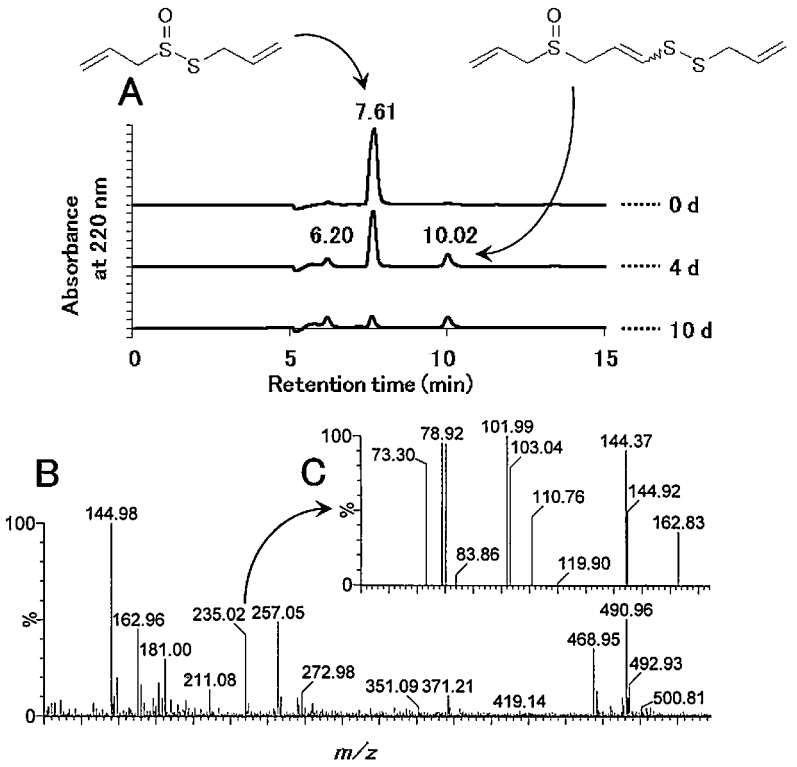

Fig. 4. Identification of the Breakdown Products of Allicin in Water by an LC/MS/MS Analysis.

A, LC patterns for allicin (7.61 min peak) and its breakdown products in the aqueous solutions incubated at 37 °C for 0, 4 and 10 d. B, MS spectrum for the newly appearing substance by LC having a retention time of 10.02 min. C, MS data for the fragment $M^+ = 235$, which is assumed to have been a protonated ajoene.

had antibacterial potential comparable to that of allicin. Since we failed to identify any deoxygenated products from allicin in the aqueous solution, another solvent should be employed for analyzing such hydrophobic compounds.

We conclude that the antibiotic activity of the garlic extracts that we routinely prepared by crushing the cloves was due to allicin (allyl 2-propenylthiosulfinate). Although allicin in the extract was extremely thermosensitive, it was relatively stable at a temperature lower than 4 °C. This may be useful to prevent bacterial growth in putrefactive foods in a refrigerator. The loss of allicin activity could equally be verified by its chemical change and decrease in antibacterial potential against either Gram-positive *S. aureus* or Gram-negative *E. coli*. The change of allicin directly led to the formation of a molecule larger than its own, which we determined to be ajoene; however, we could not observe any intermediate derived from allicin as described by Block *et al.*[19]

Acknowledgments

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