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Isolation and Characterization of Alliin From Garlic Bulbs

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마늘중 Alliin의 분리 및 그 특성규명에 관한 연구

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SUMMARY

Alliin was isolated from deep-frozen garlic bulbs and purified into crystalline form. Purity of isolated alliin was assured by melting point determination and thin-layer chromatography. Sulfoxide bond and functional groups of amino acid were confirmed with IR spectrum, and vinyl bond with NMR spectrum. Molecular weight, allyl and other bonds were confirmed with MS spectrum.

INTRODUCTION

The important role of garlic bulbs as a medicinal agent has been recognized from ancient times, however, very little work has been done on the nature of its active principle.

It has been well established that alliin (S-allyl-L-cysteine sulfoxide) is the basic principle present in garlic, and readily degraded by specific enzyme known as alliin lyase, producing allicin which is antibacterial substance.

According to Stoll and Seebeck[1], Rundqvist[2] first attempted to isolate the basic principle from garlic bulbs, and named the crude preparation he obtained "alliin". Since he dealt with considerably contaminated substance, Rundqvist erroneously concluded that the basic principle of garlic bulbs was a glycoside.

Thereafter Stoll and Seebeck[1] succeeded to isolate the basic principle of garlic bulbs in pure crystalline state and undertook the name "alliin". Through an extensive study, they proved that natural alliin was (+)-S-allyl-L-cysteine sulfoxide.

The fact that garlic bulbs exert an antibacterial action has been noticed by a number of ancient workers[1], however, the nature of antibacterial substance has not been clarified until Cavallito and Bailey[3] isolated a watersoluble substance and named it "allicin". Cavallito and Bailey et al.[4] proposed the possible two constitutional formulas for allicin. Stoll and Seebeck[5] confirmed that allicin was allyl ester of allyl thiosulfinic acid agreed with one of constitutional formulas which Cavallito and Bailey[3] presented.

When alliin decomposes into allicin by alliin lyase, pyruvic acid and ammonia[1,6,7] are con-

comitantly produced. Although very little information is available on its physiological action,[8] alliin appears to be an interesting natural product in that it produces allicin which is powerful antibacterial substance and pyruvic acid which is involved in animal metabolism.[1]

Since allicin is quite unstable, isolation of natural alliin was desired as the first step of studies on the active principles of garlic bulbs. Therefore emphasis was placed on the establishment of isolation method for natural alliin.

In order to confirm whether pure alliin was obtained, new instrumental techniques were employed for characterization of alliin isolated from garlic bulbs. Information on instrumental analysis of alliin is extremely rare. Infrared spectrum[9,10] and ultraviolet absorption spectrum[10] are only available with synthetic alliin.

EXPERIMENTAL

Isolation of alliin was based on procedures described by Stoll and Seebeck[1], only with slight modification. The garlic bulbs (*Allium sativum* L.) were deep-frozen for 2 days at-70C, and crumbled to a fine powder while still frozen in a waring blendor with 2 kg of dry ice per 500g bulbs. Powdered sample was extracted with 1.5 liter of absolute ethanol for 1 hour in a shaker bath, and filtered under suction with Toyo No. 5 filter paper. The residue was reextracted with 1 liter of 80% (v/v) ethanol and two extracted solutions were combined and evaporated under reduced pressure till yellowish, syrupy liquid was formed.

Syrupy liquid was again extracted with ether to eliminate the fatty components and impurities, and dried under reduced pressure. Approximately 15g of yellowish powder was produced per 500g garlic bulbs after drying.

Yellowish powders were dissolved in 80ml of distilled water, and mixed with 300ml of absolute ethanol. The resulted turbid solution was stood for 10 hours, and then the liquid was cautiously decanted to from contaminating carbohydrates. The same procedures were repeated two or three times with additional ethanol. Final solution was concentrated under reduced pressure.

As a result of adding cold methanol to concentrated aqueous solution, approximately 0.6g of white precipitate was obtained. After drying them under sulfuric acid under reduced pressure, the white precipitates were dissolved in 6 ml of distilled water. Needlish-bundled crystals were formed when 12 ml of acetone previously warmed at 50C was added to the above solution. They were recrystallized with 75% (v/v) acetone and dried under reduced pressure. All procedures were carried out at 10 C or lower.

Crystals are mounted on slide glass with diluted alcohol and examined under AO spencer type microscope. Melting point was determined with capillary method. Ninhydrin test[11], sodium nitroprussid reaction[12] were performed with approximately 2% (w/v) alliin solution.

The thin-layer chromatography was performed by the method which Lukes[11] developed for cysteine derivative. S-alkyl-L-cysteine sulfoxides which Dr. Mendel Mazelis, Univ. of California donated were concomitantly chromatographed as supplementary standards.

The IR spectrum was obtained on a Beckman IR-4 type spectroscopy. A 1.5mg of sample was finely ground and mixed well with 300mg of potassium bromide. Powders were dried to remove moisture and pressed under high pressure of 20,000 psi into a small disc of 10 mm diameter and 2 mm thickness.

The nuclear magnetic resonance (NMR) spectrum was determined using a Varian HA-100D type NMR spectrometer. This instrument was operated at a fixed frequency of 100 MHz. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was employed as an internal reference standard. Sample was dissolved in D_2O and approximately 0.2M solution was prepared for analysis. NMR spectrum was determined at 24C.

The mass (MS) specctrum was determined with Finnigan 1015 type mass spectrometer, which was equipped with a heated inlet system and operated at 140C. Ionizing energy was 90 ev and approximately 1 mg of sample was employed for determination.

RESULTS AND DISCUSSION

Isolation of Alliin

In the course of establishing isolation method for alliin, frequent failures were encountered at the final step. Intensive garlic odor often developed when syrupy liquid was produced, and then crystallization of alliin was not feasible. This was overcome by the use of dry ice. When alliin was extracted with ethanol and the solution was stored at 4-5C for several days, the same result occurred as no dry ice was added.

It was considered that intensive odor was dependent upon the production of allicin was due to insufficient inactivation of alliin lyase. Evaporation of methanol at elevated temperature resulted in the reduced yield of isolated alliin.

Approximately 200 mg of alliin was obtained per 500g bulbs. Bunched needles of crystallized alliin are shown in Fig. 1.

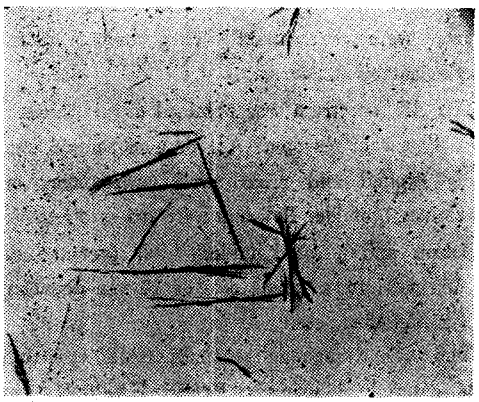


Fig. 1. Bunched needles of crystallized alliin (50×)

Characterization of Isolated Alliin

It was intended to synthesize S-allyl-L-cysteine sulfoxide by the procedured by Theodoropoulos[13] and Stoll and Seebeck[1], and to use it as reference standard for confirmation of isolated alliin. However, it was not feasible since S-allyl-L-cysteine run out of stock in Sigma Chemical Company. Accordingly characterization of purified crystals was desired to confirm whether pure alliin was isolated from garlic bulbs.

Melting point of isolated alliin was shown to be 164 C and it was agreed with range (163-165 C) described by Stoll and Seebeck[5]. Agreement of melting point illustrated that isolated allin was quite pure.

Positive reaction of isolated alliin by ninhydrin test suggested that this compound would be primary amine whose α-carbon possesses a hydrogen atom. Sodium nitroprusside test for identification of sulfhydryl group showed negative reaction with isolated alliin, however, the same test undertaken after alkaline hydrolysis of the sample showed positive reaction. Thus it was assured that isolated alliin had sulfur atom in oxidized form.

The results of thin-layer chromatography (Table 1) showed that isolated alliin was cysteine derivative. It has been also proved that isolated alliin was unique compound, and was not identical with S-methyl-L-cysteine sulfoxide or S-ethyl-L-cysteine sulfoxide.

Table 1. Rf values of isolated alliin and S-alkyl-L-cysteine sulfoxides shown on the thin-layer chromatography plate.

	Solvent type	
	tert-butanol	phenol
Isolated alliin	0.55	0.29
S-methyl-L-cysteine sulfoxide	0.37	0.18
S-ethyl-L-cysteine sulfoxide	0.61	0.32
L-cysteine	0.52	0.21

IR spectrum of isolated alliin is shown in Fig. 2. Characteristically broad and strong absorption resulting from NH^+_3 stretching of free amino acids was easily detected at 3080 cm^{-1} (3.23μ) and NH_3^+ symmetrical bending at 1560 cm^{-1} (6.52μ). The peak of carboxylate ion group ($-C\overset{O}{\underset{O}{\ll}}$) occurred at 1620 cm^{-1} (6.40μ).

It is well established that in IR spectrum, free primary amino acids are characterized by : 1) a broad, strong NH^+_3 stretching band in the 3100—2600cm^{-1} (3.23—3.85μ) region; 2) a weak asymmetric NH_3^+ bending band near 1660—1610 cm^{-1} (6.03—6.21μ) or a fairly strong symmetrical bending band near 1550—1485cm^{-1} (6.45—6.73μ);

and 3) strong absorption of the carboxylate ion group near 1600—1590 cm^{-1} (6.45—6.47μ) or a weaker absorption near 1400 cm^{-1} (7.15μ)[14,15]. Although slight shift occurred in the peak of carboxylate group, the functional groups of amino acids were confirmed by the above absorption bands,

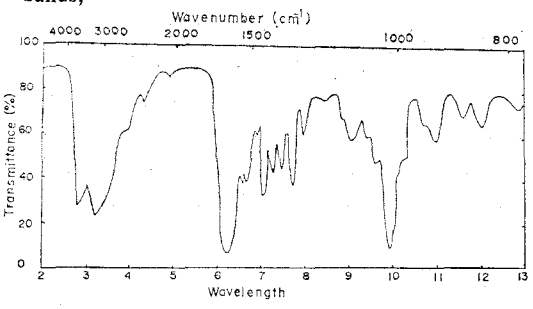


Fig. 2. The infrared spectrum of isolated alliin

Sulfoxide bond showed strong absorption at 1025 cm^{-1} (9.75μ) (Fig. 2). Since sulfoxide groups generally absorb in the region 1070—1030 cm^{-1} (9.35—9.71μ)[15], slight shift appeared to occur in sulfoxide bond. Combining the results of sodium nitroprusside test and IR spectrum, it is almost apparent that isolated alliin has sulfoxide group.

IR spectrum of S-ethyl-L-cysteine sulfoxide was taken as supplementary reference standard to confirm absorptions of functional groups of free amino acid and sulfoxide bond. A broad, strong NH_3^+ stretching band was detected at 3000 cm^{-1} (3.33μ), NH_3^+ symmetrical bending at 1500cm^{-1} (6.66μ), and carboxylate ion group at 1600 cm^{-1} (6.45μ) and sulfoxide bond at 1020 cm^{-1} (9.80μ)

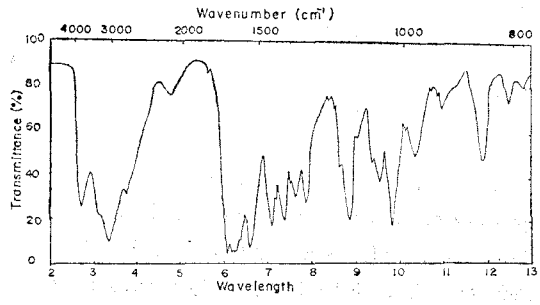


Fig. 3. The infrared spectrum of S-ethyl-L-cysteine sulfooide

in S-ethyl-L-cysteine sulfoxide (Fig. 3).

Thus, in isolated alliin, functional groups of free amino acids and sulfoxide bond in molecule were confirmed by IR absorption spectra, however, it was not feasible to prove carbohydrogen bonds with IR spectrum.

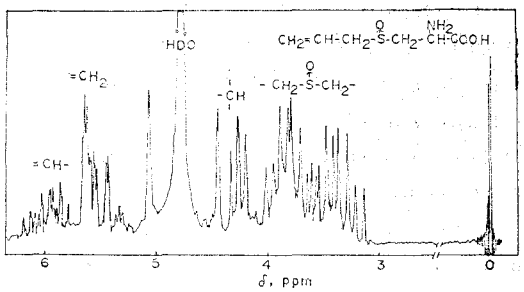


Fig 4. The NMR spectrum of isolated alliin in D_2O

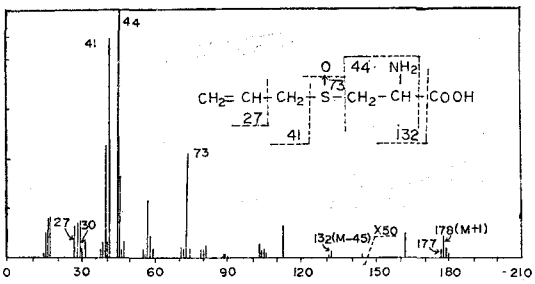
Fig. 4 shows NMR spectrum of isolated alliin. Since general regions of chemical shifts in olefinic structure are known to exist between 4.6—6.4 ppm, signals detected between 5.4 and 6.2 ppm appear to be the ones of vinyl bond, considering number of signals and an area ratio of 1 : 2 in $=CH-$ and $=CH_2$, respectively.

The COOH and NH_2 resonances were not separately detected in isolated alliin which is amino acid derivative. It is probable that the proton in functional groups of amino acid and D_2O were exchanged rapidly, and detected as a single signal[15)16)].

Three signals detected between 4.15 and 4.40 ppm were regarded as the ones for —CH of α-position in isolated alliin, since general regions of chemical shifts in α-disubstituted aliphatic structure are known to exist between 2.4 and 7.2 ppm[15].

Signals of $-CH_2-\overset{O}{\overset{\uparrow}{S}}-CH_2-$ were somewhat ambiguous and difficult to interpret. The only possible signals for $-CH_2-\overset{O}{\overset{\uparrow}{S}}-CH_2-$ were detected between 3.1—4.6 ppm, and this might be possible to interpret as AA'BB' system of $-CH_2-\overset{O}{\overset{\uparrow}{S}}-CH_2-$. However, further study would be needed for definite conclusion.

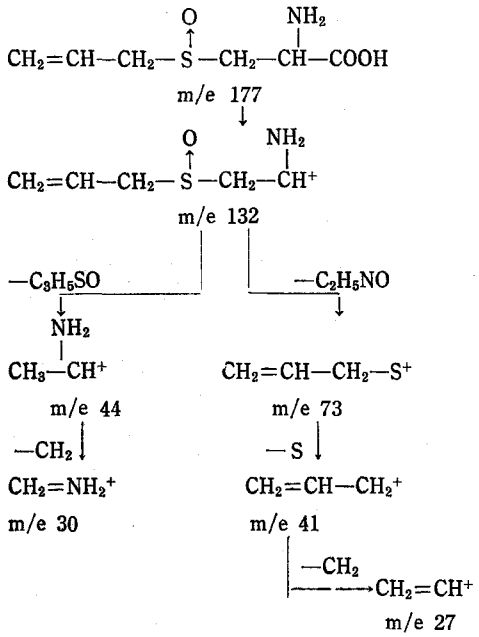
MS spectrum of isolated alliin is shown in Fig. 5. Amino acids are mostly non-volatile, and thus it is common to determine their MS spectrum after undertaking esterification[15,17]. According to Biemann and McClosky[18], it is possible to determine the MS spectra of amino acids without prior conversion to more volatile derivatives. Therefore, MS spectrum of isolated alliin was determined without esterification.



The mass spectrum of isolated alliin.

Fig. 5. The mass spectrum of isolated alliin

Possible fragmentation scheme of isolated alliin is shown below:



The peaks at m/e 73 were regarded due to $CH_2=CH-CH_2-S^+$ bond of isolated alliin. It is presumable that the peak at m/e 41 was formed by the elimination of sulfur atom from $CH_2=CH-CH_2-S^+$ bond and a peak at m/e 27 was formed by the elimination of $-CH_2$ from $CH_2=CH-CH_2^+$ bond. The base peak at m/e 44 was regarded due to $CH_3-CH^+-NH_2$ and a peak at m/e 30 appeared to be formed by the elimination of $-CH_2$ from $CH_3-CH^+-NH_2$. The parent peak of isolated alliin was weakly detected, and M+1 peak was slightly more intense than the parent peak. It is generally recognized that parent peak is always associated with a considerable "M+1" peak and its intensity is somewhat lower[19]. A peak at 132 was regarded due to the elimination of —COOH from isolated alliin.

요 약

냉동시킨 마늘로부터 Alliin을 分離해서 순수한 相態로 結晶化 시켰다. 分離된 Alliin의 純度는 융점측정과 thin-layer chromatography에 의하여 확증되었다. Sulfoxide bond 및 아미노산의 기능기는 IR spectrum에 의하여, vinyl 결합은 NMR spectrum에 의하여 확인되었다. 분자량, allyl 및 기타의 결합은 MS spectrum에 의하여 確認되었다.

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