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Mechanism and kinetics of synthesis of allicin

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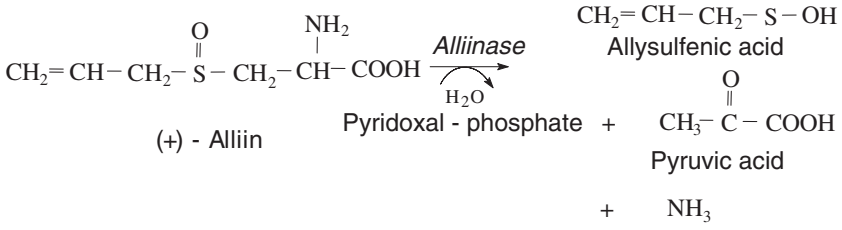
Allicin, allyl-thiosulfinate, a pharmacologically active compound with considerable fungicidal, bactericidal, antioxidant and anticarcinogenic effects, was obtained by oxidizing allyl disulfide with acid hydrogen peroxide. The synthesis mechanism was studied by the ESR spin trap method. The kinetics of allicin synthesis was ascertained by determination of the concentration of the limiting reactant during the synthesis using HPLC and it was found that the allicin synthesis reaction was of zero order. The allicin obtained was determined using UV, FT-IR, MS, ^{1}H and ^{13}C NMR analysis.

1. Introduction

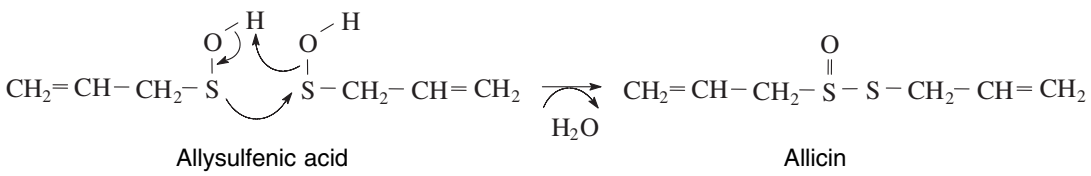
Allicin is a thioester of sulfenic acid, i.e. allyl thiosulfinate. The liquid is oily, and slightly yellowish in colour, with the characteristic garlic (*Allium sativum* L.) odor (Sticher 1991). Allicin is pharmacologically the most important and the most active substance in the raw aqueous extract of garlic (Koch et al. 1989, Lawson et al. 1991a, Lawson et al. 1991b). It has a wide range of antimicrobial effects. It acts upon a great number of bacteria, viruses, fungi, and on some parasites (Adetumbi and Lau 1983, Hughes and Lavson 1991, Koch 1993). It inhibits the growth of *Staphylococcus*, *Streptococcus*, *Bacillus*, *Brucella, Vibrio* and *Candida* species in low concentrations (Cavallito and Bailey 1944, Huddleson 1944). Its antimycotic effect is stronger than that of nystatin and other antimycotics (Adetumbi and Lau 1983, Moore and Atkins 1977, Prasad and Sharma 1980. Sandhu et al. 1980). It shows virucidal effects (Nagai 1973, Weber 1992) *in vitro* on *Herpes simplex* type 1 and 2, *Parainfluenza* virus type 3, *Vaccinia* virus, *Vesicular stomatitis* virus and *Human rhinovirus* type 2.

Besides its antimicrobial effects allicin has an important clinical application in the prevention of cancer and cardiovascular diseases, as well as antioxidant activity as a free radicals scavenger (Criss 1982, Ernst 1987, Kroning 1964, Lau et al. 1983, Meng and Shyu 1990, Phelps and Haris 1993). It is produced by the transformation of alliin [(+)-S-allyl-L-cysteine sulfoxide] in the garlic bulb under the influence of alliinase from plant tissue (Sticher 1991, Stool and Seebeck 1949, Scheme 1).

Scheme 1



Scheme 2



At room temperature, the allyl sulfenic acid is very unstable and very reactive. Two molecules of allyl sulfenic acid condense spontaneously into allicin with the elimination of water (Stricher 1991, Scheme 2).

Due to its high instability and volatility, the isolation, determination and standardization of allicin based products are very difficult. It is hard to obtain commercially pure allicin, and in recent decades there has been considerable interst in the synthesis of allicin. Most of the procedures for the synthesis of allicin involve the oxidation of allyl disulfide with hydrogen peroxide in acid medium (Cavallito et al. 1944, Freeman and Kodera 1995, Iberl et al. 1990, Vedejs et al. 1982), the oxidation of allyl disulfide with m-chloroperbenzoic acid in chloroform (Jansen et al. 1987) or the treatment of dichloromethane solution of allyl disulfide with magnesium monoperoxyhydrate in ammonium-butyl-sulfate (Cruz-Villalon 2001). It is characteristic of all these procedure that they take place at low temperatures (from zero to room temperature) and that, depending on the purification method used, various purity degrees of allicins are obtained.

In this paper investigations of the mechanism and kinetics of the synthesis of allicin from allyl disulfide with hydrogen peroxide in acidic medium are reporter.

2. Investigations, results and discussion

2.1. Mechanism of synthesis of allicin with acid hydrogen peroxide

During the allicin synthesis (Scheme 3) the ESR trap method was used to determine the mechanism of synthesis and the antioxidant activity, and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was used as the spin-trap compound. Fenton reaction was used as a reference.

DMPO reacts directly with hydroxy radicals and produces the DMPO-OH spin adduct, which is a more stable oxygen radical with longer life and easier to detect by ESR methods (Brunet 1998, Scheme 4).

To determine the mechanism of producing reactive free hydroxy radicals in the allicin synthesis reaction mixture, the following model system was used: 1 ml of 0.01 mol/l $FeCl_2 \cdot 4\,H_2O$, 1 ml of 0.01 mol/l H_2O_2, 0.089 mol/l of DMPO and 1 ml of sodium phosphate buffer, pH = 7.2, the ESR spectrum of which is given in Fig. 1a.

In the spectrum, a hyperfine structure is observed (Fig. 1a) showing four lines of relative intensities 1 : 2 : 2 : 1 and

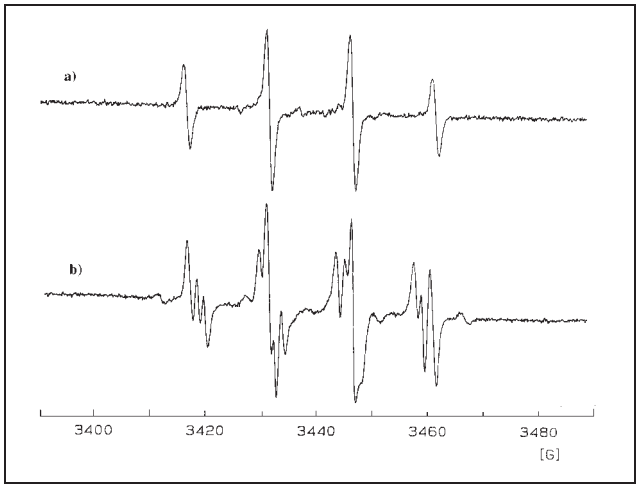


Fig. 1: Electron spin resonance spectrum of a) DMPO-OH spin adduct in the model system (Fenton reaction), b) free hydroxy radical produced in acid medium by decomposition of hydrogen peroxide without adding DMPO

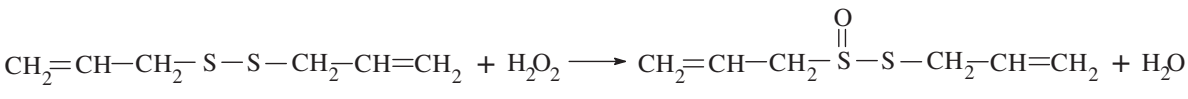
splitting constants for one ^{14}N atom (I = 1) $a_N = 14.9$ G, and one ^{1}H atom (I = 1/2) $a_H = 14.9$ G. The spectroscopic splitting factor (g-value) determining the position of the absorption line of the ESR spectrum for the nitroxide radical obtained (DMPO-OH spin-adduct) was 2.0060.

Since the dissociation energy of the O−O bond in hydrogen peroxide is about 200 kJ/mol (Oaэ 1975), it is concluded that the bond readily breaks and gives hydroxy radicals in the reaction system of allicin synthesis. This assumption is confirmed by the ESR analysis results (Fig. 1b) of the partially formed reaction mixture (1.5 ml of 30% hydrogen peroxide, made up to 10 ml with glacial acetic acid).

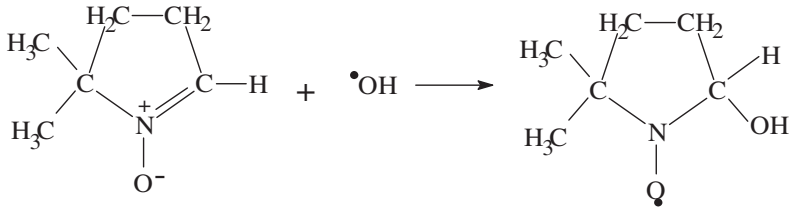
The hyperfine structure of this spectrum is complex and consists of a number of fine spectral lines because no DMPO was added and there is no stable radical.

The presence of hydroxy radicals during the allicin synthesis was confirmed by ESR spectroscopy of the complete reaction mixture with the spin-trap DMPO added. The spectrum obtained retained the hyperfine structure from Fig. 1a, while the intensity of the signal was decreased to 67%. This indicates that allicin reacts with the free hydroxy radicals. If the allicin concentration is increased to 10 mg/ml there is no signal in the ESR spectrum, which

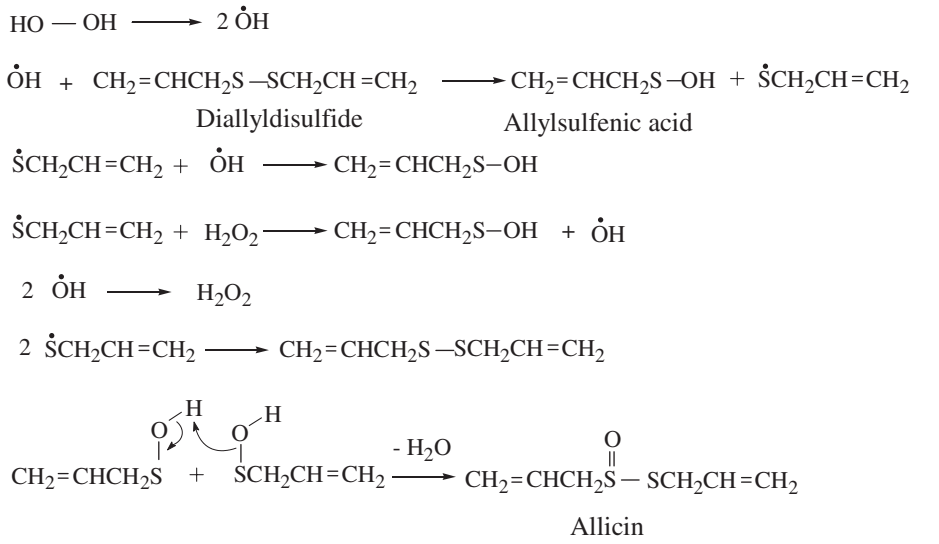
Scheme 3



Scheme 4



Scheme 5



shows that allicin has a strong antioxidant activity. The antioxidant activity of allicin is related to its antimutagenic and anticarcinogenic effect, since the hydroxy radicals contribute to these diseases (Gutteridge et al. 1990, Halliwell 1978, Halliwell 1994, Halliwell and Gutteridge 1984).

Based on the ESR analysis confirming the existence of hydroxy radicals, and the mechanism of condensation of allyl sulfenic acid to allicin (Sticher 1991), the mechanism of the synthesis in Schemen 5 can be given.

Since the dissociation energy of the O–O bond in the peroxide (200.9 kJ/mol) is weaker than the dissociation energy of the S–S bond (301.39 kJ/mol) (Oaэ 1975), it favors the formation of hydroxy radicals, which attack the allyl disulfide molecule and produce a thiil radical and allyl sulfenic acid. The thiil radical can be further combined with a hydroxy radical to produce unstable allyl sulfenic acid, or react with non-decomposed hydrogen peroxide and produce another hydroxy radical and allyl sulfenic acid. It is also possible to recombine two thiil radicals to obtain allyl disulfide, the starting substrate for allicin synthesis. Two molecules of allyl sulfenic acid, which is a very unstable compound, react and produce allicin isolating a water molecule. The slowest phase in the synthesis of allicin is the decomposition of the compound into radicals, whose concentration is constant in the stationary state, which indicates that the synthesis is a zero order reaction, which can be confirmed by observing kinetics of allicin synthesis.

2.2. The kinetics of allicin synthesis

The allicin synthesis reaction can be presented as: $A + B = C + D$ where A is allyl disulfide, B is hydrogen peroxide, C is allicin and D is water. Allyl disulfide is a limiting reactant because hydrogen peroxide was given with a small surplus of about 10%. The kinetics of allicin synthesis was examined by HPLC. The test results for syntheses at 13 °C are given in Fig. 2.

The peak with a retention time of 2.19 min corresponds to acetic acid, whil an allyl disulfide peak is seen at $R_t = 20.44$ min, and with the progress of the reaction its area decreases. The hydrogen peroxide peak at $R_t = 2.01$ min

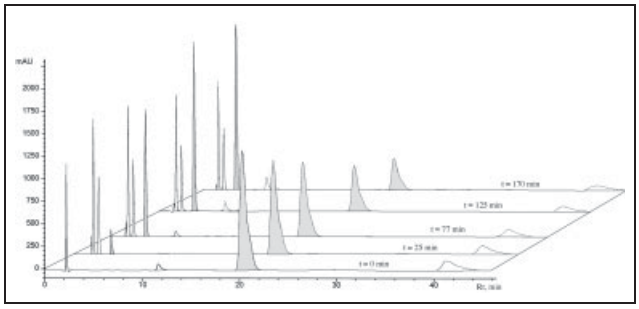


Fig. 2: HPLC chromatograms of the samples taken from the reaction mixture for allicin synthesis at various time intervals (t = 0, 25, 77, 125, 170 min)

shows a decrease in area due to consumption during the synthesis. Allicin, as the synthesis product, has the peak at $R_t = 4.00$ min and its area increases continually. The peak areas are proportional to the concentrations of the corresponding compounds. HPLC shows another peak at about $R_t = 41$ min, coming from allyl trisulfide, present in the initial reactant, allyl disulfide. During the reaction its concentration changes slightly, which shows that within the temperature range of the reaction process it does not take part in the reaction. On the other hand, HPLC diagrams show almost no peaks that could indicate more important secondary products.

The concentrations of allyl disulfide and hydrogen peroxide decrease lineally with reaction time (Fig. 3), indicating that the allicin synthesis reaction is of zero order. Application of the differential equation for the zero order reaction rate:

$$-\frac{dC_A}{dt} = k \qquad (1)$$

for the initial conditions where $C_A = C_{A0}$ for $t = 0$ the calculation gives:

$$C_A = C_{A0} - k \cdot t \qquad (2)$$

Using eq. (2) the rate constant for the allicin synthesis reaction, i.e. the consumption of allyl disulfide at 13 °C, was determined as $k = 0.00126$ mol/l min. Similary, the synthesis was carried out at 26 °C and the reaction rate constant of allyl disulfide depletion was obtained at

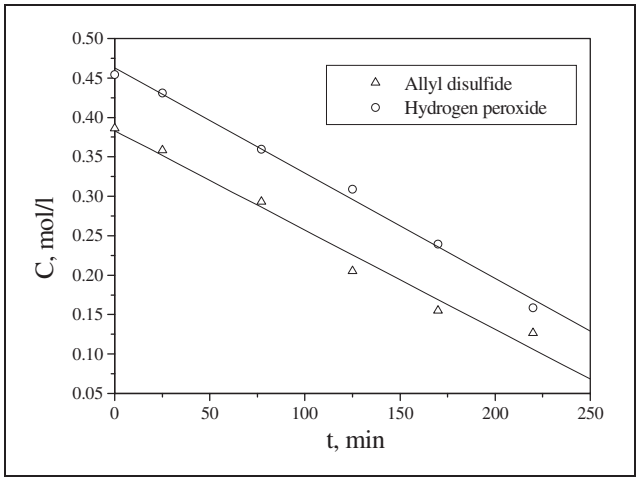


Fig. 3: Variations of concentrations of allyl disulfide and hydrogen peroxide with respect to the reaction time

$k = 0.00236$ mol/l min. With the two values of the rate constant at two different temperatures we obtain the Arrhenius's equation:

$$k = 2.338 \cdot 10^3 \cdot e^{-\frac{34320}{R \cdot T}} \quad (3)$$

The synthesis reaction is very selective, and no relevant secondary products are produced during the synthesis, as seen in Fig. 2.

The synthesized allicin structure was characterized by UV, FT-IR (Fig.), ^{1}HNMR, ^{13}CNMR and MS.

In the UV-spectrum of the aqueous allicin solution two bands are observed with peaks at 198 nm ($\pi \rightarrow \pi^*$ transition from isolated C=C bond and $n \rightarrow \sigma^*$ from S=O) and 254 nm ($n(p_y) \rightarrow \pi^*$ from C=C).

In the IR spectrum of the synthesized allicin, the sharp band of medium intensity at 1635 cm^{-1} is indicative of two terminal C=C bonds and a high intensity band at 1087 cm^{-1} is indicative of valence vibrations of S=O group.

3. Experimental

3.1. Reagents

Allyl disulfide (80%, $\varrho = 1.008$ g/ml) and 5,5-dimethyl-1-pyrroline N-oxide (97%, $\varrho = 1.015$ g/ml) were purchased from Aldrich Chemical Co. Other reagents used in the work were of analytical quality.

3.2. Synthesis and purification

Dissolve allyl disulfide 80% (3.5 ml) in glacial acetic acid, make up with glacial acetic acid to 25 ml, and leave the solution in a cold bath (0 °C). Add to the allyl disulfide solution the acid solution of hydrogen peroxide dropwise for 25 min with intensive stirring. The acid solution of hydrogen peroxide is prepared by dissolving 30% hydrogen peroxide (3.5 ml) in glacial acetic acid and the volume is adjusted to 25 ml. After the whole volume of hydrogen peroxide has been added, continue the synthesis reaction with intensive stirring for several hours at constant temperature between 10 and 30 °C. Neutralize the reaction mixture by adding potassium hydroxide solution (43 g potassium hydroxide was dissolved in distilled water and volume adjusted to 50 ml) dropwise with intensive stirring and cooling. If potassium acetate should form and separate during the neutralization, add distilled water (5–10 ml) to the reaction mixture to dissolve the separated potassium acetate. During the neutralization phase, an oily liquid is separated with the characteristic intensive garlic odor.

The allicin is isolated from the raw reaction mixture by extraction with diethyl ether (4×50 ml). Ether is evaporated from the coalesced ether fractions and two oily fractions are obtained which are not miscible, a colorless heavier fraction and a light yellow lighter fraction, allicin.

3.3. ESR investigations

Electron spin resonance spectral determinations were carried out on a Bruker 300 E spectrometer, with microwave radiation with nominal frequency 9.5 GHz (x-rays), and the following operating characteristics: modulation frequency 100 kH, modulation amplitude 0.204 G, time constant 327.68 ms, measuring time range 1310.72 ms, field center 3440 G, total measuring range 100 G, microwave frequency 9.64 GHz, microwave area power 20.0 mW, and measurement temperature 20 °C. The reaction mixture samples were contained in a standard Bruker ER-160 FC quartz cuvette for aqueous solutions.

3.4. HPLC analysis

Allicin determination and content were carried out during the synthesis process by HPLC under the following conditions: Apparatus: Hewlett Packard 1100 (pump, detector, software). Column: LiChrosorb®; RP-18, 5 µm. Eluent: methanol : water : formic acid = 60 : 40 : 0.1. Flow rate: 1.2 ml/min. Task volume: 20 µl. Detection: UV detector 205 nm.

3.5. UV spectroscopy

The UV spectrum was obtained in water on a Perkin-Elmer lambda 15 UV/VIS spectrophotometer in quartz cuvettes 1 cm thick. UV, λ_{max} (H_2O): 198 and 254 nm.

3.6. FT-IR spectroscopy

IR spectrum was recorded on Bomem Hartmann 7 Braunm MB series FT-IR spectrophotometer between KBr plates with 0.1 µm film thickness, wavelength region between 4000 and 400 cm^{-1}. IR, ν_{max} (KBr), cm^{-1}: 3083, 2978, 1635, 989, 926, 1043, 1087.

3.7. ^{1}H NMR and ^{13}C NMR spectrometry

^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker AC 250 E spectrometer with operating frequency 250 and 62.5 MHz, respectively, in 5 mm dia glass cuvettes at room temperature by the impulse method with multiple repetitions of scans to obtain ^{13}C NMR spectra. ^{1}H NMR (250 MHz, $CDCl_3$) at δ: 3.25–3.5 (2 H, $C\underline{CH_2}SO$), 3.5–3.6 (2 H, $S\underline{CH_2}C$), 5.16–5.3 (2 H, $\underline{CH_2}C$), 5.8–5.96 (1 H, $CH_2\underline{CH}CH_2$). ^{13}C NMR (62.5 MHz, CDCl) at δ: 33.17 (3b, $S\underline{CH_2}CH$), 53.6 (3a, $CH\underline{CH_2}SO$), 118.55 (1b, $CH\underline{CH_2}$), 123.96 (1a, $\underline{CH_2}CH$), 125.3 (2b, $CH_2\underline{CH}CH_2$), 132.6 (2a, $CH_2\underline{CH}CH_2$).

3.8. MS spectrometry

MS spectra were obtained on a GC-MS Voyager spectrometer with electron ionization 70 eV. EI MS (70 eV) m/z (rel. intensity) 162 (M^+, 10), 144 (24), 73 (M-C_3H_5OS, 28), 72 (40), 45 (48), 41 (M-$C_3H_5OS_2$, 100).

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