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Inactivation of Jack Bean Urease by Allicin

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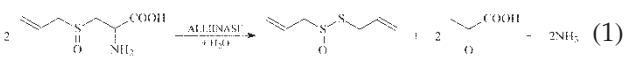
Allicin—diallyl thiosulfinate—is the main biologically active component of freshly crushed garlic. Allicin was synthesized as described elsewhere and was tested for its inhibitory ability against jack bean urease in 20 mM phosphate buffer, pH 7.0 at 22°C. The results indicate that allicin is an enzymatic inactivator. The loss of urease activity was irreversible, time- and concentration dependent and the kinetics of the inactivation was biphasic; each phase, obeyed pseudo-first-order kinetics. The rate constants for inactivation were measured for the fast and slow phases and for several concentrations of allicin. Thiol reagents, and competitive inhibitor (boric acid) protected the enzyme from loss of enzymatic activity. The studies demonstrate that urease inactivation results from the reaction between allicin and the SH-group, situated in the urease active site (Cys^{592}).

Keywords: Garlic; Allicin; Urease; Inactivation; Biphasic kinetics

INTRODUCTION

Since ancient times, garlic (*Allium sativum*) has been used wordwide not only as a spice but also as a medicine. Many beneficial health properties of garlic are attributed to the organosulfur compounds, alk(en)yl thiosulfinates R-S-S(O)-R, of which 70–80% is diallyl thiosulfinate know as allicin.[1–3] It is produced from the nonprotein amino acid allin-in the enzymatic reaction after crushing of garlic:[4]



(1)

Allicin is responsible for garlic's characteristic punget smell and has been shown to posses antimicrobial and antioxidant activity. It inhibits platelet aggregation, effectively reduces serum cholesterol and triglicerydes and lowers ocular pressure.[5,6] The best known and most extensively studied effect of allicin is the strong antibacterial activity. It retards the growth and proliferation of both Gram-positive and Gram-negative bacteria.[6–8] It has been shown that the antibacterial properties of allicin result from the modification of thiol-containing enzymes in the microorganism, by the rapid reaction of allicin with thiol groups. Wills[9] has reported that allicin inhibited nearly all thiol-containing enzymes. The mechanism of the blocking of activity of the following enzymes: SH-protease papain, $NADP^+$-dependent alcohols dehydrogenase, NAD^+-dependent alkohols dehydrogenase, squalene monooxygenase[10,11] has been explained in detail.

The plant urease obtained from jack bean has fifteen free cysteine groups for each subunit, among which Cys^{592} is essential for enzymatic activity and is located on the mobile flap closing the active site of urease.[12] The enzyme urease is present in many pathogenic bacteria including such species as *Proteus, Klebsiella, Staphylococcus* and *Helicobacter pylori*. The first three types of bacteria are responsible for infections of the urinary tract, and the latter is considered to be responsible for the development of gastric and duodenal ulcers as well as gastric cancer.[13] *Helicobacter pylori* produces a large amount of the enzyme urease (10–15% of total protein by weight), which hydolyzes urea present in gastric juice. The ammonia, generated from this reaction, neutralizes the gastric acidity and forms a neutral microenvironment for the survival of the bacteria.[14] The studies carried out *in vitro* and *in vivo* reveal that *Helicobacter pylori*, resistant to many antibiotics, is sensitive to garlic extract in relatively low concentrations.[15,16]

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The use of appropriate inhibitors may result in a change in the level of activity of enzymes; the strongest inhibitors of ureases are phosphoroamides, thiophosphoroamides, hydroxamic acids, heavy metal ions, quinones and their derivatives.[12,13,17–19] Among the above mentioned inhibitors, hydroxamic acids and phosphoroamides have been used to cure diseases caused by bacteria which release urease (e.g. urinary stones, pyelitic, chronic gastric ulcers).[20–22] The results of the therapies were effective but had many side effects.

In the present investigations we studied the kinetics and mechanism of inhibition of jack bean urease by allicin. The obtained results might be used to evaluate the activity of microbiological ureases present in some pathogenic bacteria since it has been found that the structure of the active center and the catalytical mechanism is similar in all known ureases. Allicin is easily available, inexpensive and a nontoxic natural product which may replace synthetic compounds presently used to cure deseases caused by the urease-producing bacteria.

MATERIALS AND METHODS

Materials

Jack bean urease, Sigma type III, with specific activity 22 μmol NH_3/min mg protein, urea (Molecular Biology Reagent), L-cysteine, reduced glutatione, 2-mercaptoethanol, dithiothreitol, 5.5′-dithiobis-(2-nitrobenzoic acid), HEPES buffer, allyl disulfide, were purchased from Sigma. Other chemicals were obtained from POCh, Gliwice, Poland. All reagents used were of analytical grade.

Synthesis of Allicin

Allicin was synthesized through oxidation of diallyl disulfide with perbenzoic acid, essentially following the method reported by Small *et al.*[23] Diallyl disulfide (4.82 g, 33.0 mmol) was dissolved in chloroform (350 ml) and was then oxidized with perbenzoic acid (33.0 mmol) dissolved in 70 ml of chloroform. As the only departure from the published procedure Skellysolve B was substituted with toluene in the extraction step. Allicin (3.25 g) was obtained in 61% yield as a light yellow oil with a characteristic pungent smell.

IR (neat, cm^{-1}): 3084, 3014, 2910, 1635, 1426, 1403, 1230, 1087, 989; ^1H NMR (500 MHz, $CDCl_3$, δ): 3.70–3.90 (m, 4H, CH_2), 5.20–5.50 (m, 4H, CH_2=), 5.86–6.02 (m, 2H, −CH=); ^{13}C NMR (125 MHz, $CDCl_3$, δ): 34.60, 59.40, 118.70, 123.65, 125.50, 132.60.

These data are in agreement with the reported spectra of allicin.[24]

Quantitative Determination of Allicin Concentration

The allicin concentration was estimated using the spectrophotometric method of Han *et al.*[25] This method is based on the quantitative reaction in which one molecule of allicin reacts with two molecules of cysteine to form two molecules of S-allylmercaptocysteine. To the investigated allicine solution, excess cysteine was added and the amount of cysteine which had not been converted into S-allylmercaptocysteine was determined by reaction with 5.5′-dithiobis-(2-nitrobenzoic acid). The amount of the yellow-colored 2-nitro-5-thiobenzoate released in this reaction was measured spectrophotometrically at 412 nm.

Enzymatic Reaction

Urease catalyzes the hydrolysis of urea to yield ammonia and carbon dioxide:[12]

$$CO(NH_2)_2 + H_2O \xrightarrow{UREASE} 2NH_3 + CO_2 \qquad (2)$$

The reaction was monitored by measuring the ammonia concentration by the phenol-hypochlorite spectrophotometric method at 625 nm.[26]

Inactivation Studies

The urease solution was mixed with an equal volume of allicin solution, and the mixture was incubated at 22°C in 20 mM phosphate buffer pH 7.0 and 2 mM EDTA. The urease concentration in incubated mixtures remained constant at 0.5 mg/ml. In order to determine the residual activity of urease at the appropriate time intervals, samples (0.5 cm^3) were transferred into solutions (50 cm^3) containing 100 mM of urea, 20 mM phosphate buffer pH 7.0 and 2 mM EDTA. In a separate experiment the activity of urease was determined in the absence of allicin. The interaction of the phenol-hypochlorite method with allicin was tested. The method did not disturb studies in the allicin concentration (0.006–0.00037 mM) range used.

Protective Effects

Urease was incubated for 10 min with allicin in the presence and absence of thiol-containing compounds: L-cysteine (Cys), 2-mercaptoethanol (2-ME), reduced glutatione (Glu), dithiothreitol (DTT) and residual activity was measured. Reactivation of allicin-modified urease was investigated in the following way: urease was incubated for 10 min with allicin before adding thiol-containing compounds and residual activity was monitored as a function of time. The concentration of allicin and the protective agent in the incubation mixture was 0.5 mM and 5 mM, respectively. The protective effect

of boric acid was determined by incubation of urease in a mixture of allicin (0.25 mM) and boric acid (5 mM). The activity of urease in the presence of allicin or boric acid, alone, was determined in separate experiments.

Stability of Allicin in Water

A solution of allicin (3.3 mM) was divided into two portions: one was stored at 4°C and the other at 22°C. At the appropriate time intervals samples were taken from both portions and were incubated for 10 min with urease solution. Afterwards, residual activity was measured.

Analysis of the Kinetics Data

An enzyme inactivator, in general, is a compound that produces irreversible inhibition of the enzyme. Usually such inactivators form a covalent bond with the enzyme and, therefore, dialysis or gel filtration does not restore enzyme activity. In many cases the inactivation reaction can be characterized by the pseudo-first-order kinetics with respect to time[27,28]:

$$A_t = e^{-kt} \tag{3}$$

where A_t is the percent of the initial enzyme activity at time t (residual activity), and k is the pseudo-first-order constant. The plot of $\ln A_t$ against t is linear with a slope equal to $-k$.

The pseudo-first-order rate constant depends upon the concentration of inactivator, which can be expressed by the following equation:

$$k = a(inactivator)^n \tag{4}$$

where n is a number equal to the average order of the reaction with respect to the concentration of inactivator. Taking the logarithm of both sides leads to

$$\log k = \log a + n \log (inactivator) \tag{5}$$

Therefore, the order of reaction can be experimentally estimated by determing k at different inactivator concentrations.

It was observed in some experiments that the dependence $-\ln A_t$ vs t shows biphasic kinetics.[29–31] A part of the initial activity of the enzyme was lost in the fast phase, while the remaining activity was lost in a slow phase. Each of the phases exhibited pseudo-first-order kinetics. The time course of the reaction can be represented by a rate equation containing two pseudo-first-order terms, corresponding to the fast and slow phases of the reaction:

$$A_t = A_{\text{fast}} e^{-k_{\text{fast}} t} + A_{\text{slow}} e^{-k_{\text{slow}} t} \tag{6}$$

where A_{fast} and A_{slow} are parameters expressed as percent of the initial activity, and k_{fast} and k_{slow} are the pseudo-first-order rate constants of the fast and slow phases, respectively.

RESULTS AND DISCUSSION

Inactivation Kinetics of Urease by Allicin

The plot of the loss of initial urease activity against the allicin incubation time is shown in Figure 1A. Urease was incubated with 0.6, 0.3, 0.15, 0.075 and 0.037 mM of allicin, respectively. The concentration of allicin was much higher as compared to the concentration of urease. The loss of enzyme activity was dependent on the concentration of allicin as well as on the incubation time. A semi-logarithmic plot of the data clearly shows that the loss of urease activity is biphasic. Nearly 30% of urease activity is lost in the fast phase and the rest in the slow phase (Figure 1B).

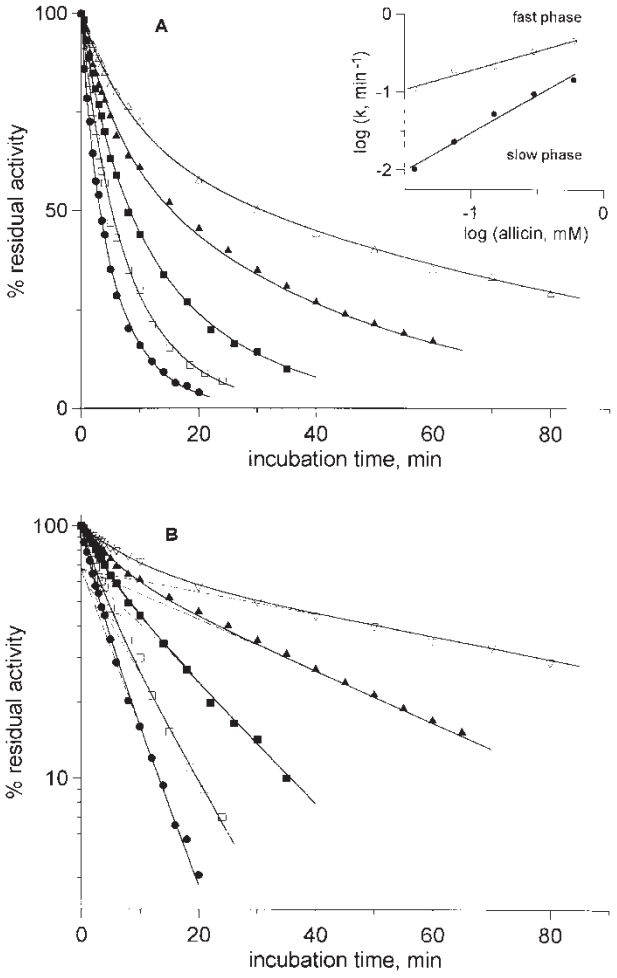


FIGURE 1 (A) Kinetics of the inactivation of urease by allicin. Urease was incubated with the following concentration of allicin: 0.60 mM (●), 0.30 mM (□), 0.15 mM (■), 0.075 mM (▲), 0.037 mM (Δ) and the residual enzymatic activity was monitored as a function of time. (B) Replot on a semi-logarithmic scale. Solid lines were fitted to the experimental points, according to Equation (6), by the least squares method. Inset: double logarithmic plot of pseudo-first-order rate constant (k) of urease inactivation *vs.* allicin concentration.

TABLE I Parameters A_{fast}, A_{slow} and the rate constants k_{fast}, k_{slow} for the inactivation of jack bean urease with various concentration of allicin

Concentration of allicin in the incubation mixture (mM)	Fast phase		Slow phase	
	A_{fast} (%)	k_{fast} (min^{-1})	A_{slow} (%)	k_{slow} (min^{-1})
0.60	32	0.45	68	0.14
0.30	35	0.32	65	0.095
0.15	32	0.24	68	0.052
0.075	30	0.19	70	0.023
0.037	33	0.11	67	0.010

Each phase exhibits the pseudo-first-order kinetics. The overall kinetics of inactivation can be expressed by Equation (6). The values of the pseudo-first-order rate constants (k_{fast}, k_{slow}) and parameters (A_{fast}, A_{slow}) at different concentration of allicin were computed by fitting the experimental data to Equation (6) using a computer program based on nonlinear last squares regression. The resulting values of k_{fast}, k_{slow} and A_{fast}, A_{slow} are compiled in Table I. The pseudo-first-order rate constants k_{fast} and k_{slow} increase with the increasing concentration of allicin both in the fast and slow phases, however, the values of parameters A_{fast} and A_{slow} are nearly the same for all investigated inhibitor concentration: $A_{fast} = 32 \pm 2$ and $A_{slow} = 68 \pm 2$.

The insert to Figure 1A shows a double logarithmic plot of the pseudo-first-rate constant of urease vs. allicin concentration for both phases. The slope of the plot for the slow phase was 0.98, and this is consistent with the inactivation by 1 mol of allicin per enzyme subunit. For the fast phase the slope of the plot is lower and amounts to 0.48. Prakash *et al.*[29] and Srivastawa *et al.*[30,31] have observed similar biphasic kinetics during inhibition of two other plant ureases obtained from *Cajanus cajan* and *Citrullus vulgaris*. As inhibitors they used compounds which can modify the thiol functions such as: 5.5′-dithiobis-(2-nitrobenzoic acid), *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetic acid and also metal ions (Ag^{+}, Hg^{2+}, Cu^{2+}). The biphasic kinetics were clearly seen during thermal inactivation of the enzyme. The above ureases, similar to jack bean urease, have the homohexameric structure. Each subunit possesses two nickel ions and one cysteine residue in the active site, and these are essential for catalytic activity.[12,30] The above-mentioned authors suggested that the characteristic biphasicity is the result of molecular asymmetry in urease; SH-groups can be divided into two categories, those which react faster and those which react slower with the inactivator. In the case of inactivation of jack bean urease with allicin ca. 30% SH-groups react faster while the other 70% reacts slower.

In order to find out whether allicin combines with urease irreversibly, the enzyme was incubated with allicin until total loss of activity had been achieved, and then allicin-modified urease was dialyzed for 24 h in 20 mM phosphate buffer pH 7.0, 2 mM EDTA at 4 °C. Urease did not regain its activity, proving that the inhibitor binds irreversibly to urease.

Protection by Thiol-containing Compounds and Boric Acid

Thiol-containing compounds such as L-cysteine, 2-mercaptoethanol, glutation and dithiothreitol were added before or after 10 minutes incubation of urease with allicin. The addition of each thiol reagent to the incubation mixture indicated significant protection against inactivation by allicin (above 90%), suggesting that allicin reacts with SH-groups in urease (Figure 2A). The addition of dithiothreitol after 10 min of urease incubation with allicin leads to the recovery of urease activity in a time-dependent manner: after 1 h

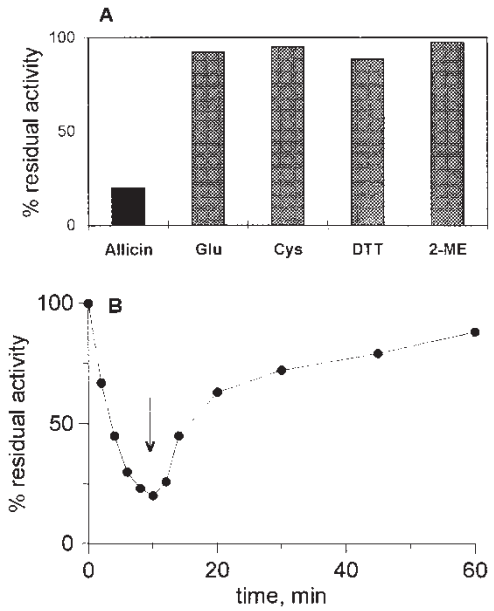


FIGURE 2 The protective effects of thiol-containing compounds on inactivation of urease by allicin. (A) Urease was incubated for 10 min with allicin in the absence or presence of thiol-containing compounds and the residual activity was determined. (B) Slow reactivation of allicin-modified urease in the presence of DTT. Urease was incubated for 10 min with allicin before adding DTT and the residual activity was monitored as a function of time.

the enzyme recovered ca. 90% of its initial activity (Figure 2B). The enzyme did not recover its activity by the addition of monothiols.

Boric acid was tested for its ability to protect against inactivation of urease by allicin. The incubation mixture contained 0.25 mM allicin and 5 mM boric acid. In a separate experiment we investigated the influence of 5 mM boric acid (alone), and 0.25 mM allicin (alone) on urease activity. The equilibrium between boric acid and the enzyme was established rapidly and the enzyme exhibited constant activity with time which amounted to 80% of the initial activity. In a mixture of allicin and boric acid, a decrease of urease activity below 80% in a time-dependent manner was observed (Figure 3). A semi-logarithmic plot of residual activity vs time is shown in the inset of Figure 3. The rate constant for the reaction of urease inactivation with allicin in the presence of boric acid was 0.036 min^{-1}, and this value was nearly 2-fold lower than the rate constant for allicin which was 0.079 min^{-1}. The above data refer to the slow stage of the reaction. For a mixture of allicin and boric acid, the biphasity can hardly be seen. Boric acid is a reversible and competitive inhibitor of urease of medium potency. It has been shown that the active form is the $B(OH)_3$ molecule, which binds with Ni^{2+} ion(s).[13] It has been proved that the small molecules which react with the ligands of the enzyme's active site (e.g. phosphate, boric acid, urea) are effective protectors against sulfhydryl reagents.[32,33] The observed protective ability of boric acid indicates that allicin inactivation of urease takes place at the active site of the enzyme.

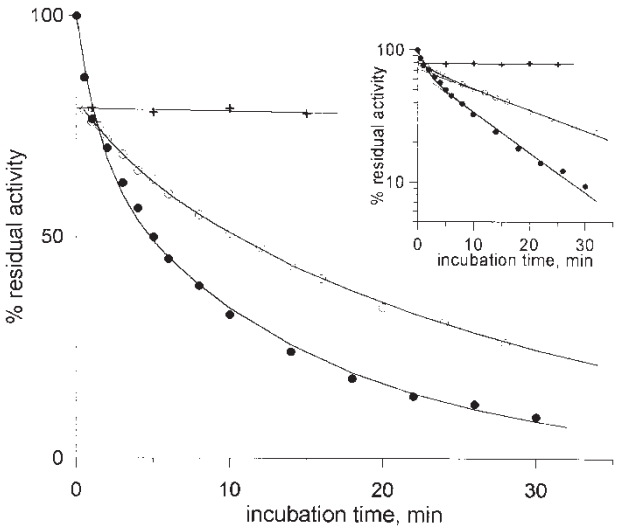


FIGURE 3 The protective effect of boric acid on the inactivation of urease by allicin. Urease was incubated with allicin (●), boric acid (+), and in the mixture of allicin and boric acid (○), and the residual activity was monitored as a function of time. Inset: Replot on a semi-logarithmic scale.

Stability of Allicine in Water

Figure 4A shows how the inhibitory potency of allicin solution in water, stored at 4° and 22°C changes with the storage time, where % inhibition = 100% − % residual activity. Allicin stored at 4°C was found to be relatively stable and even after 260 days still retained over 74% of its initial inhibitory effect while the solution stored at 22°C showed only 32% of its initial inhibitory effect. Figure 4B shows that a decrease in allicin inhibitory potency is correlated with a decrease in the allicin content in solutions stored both at 4°C and 22°C. It has been found that allicin is unstable, and with time is converted into more stable organosulfur compounds, mainly polysulfides.[1] This leads to the loss of inhibitory activity against urease.

Rabinkov *et al.*[10] studied in detail the interaction mechanism of pure allicin with SH- groups in the model system containing L-cysteine. The reaction products are S-allylmercaptocysteine and allyl sulfenic acid, which were detected by NMR and MS studies. Further steps of the reaction are still not clear. It is possible that two molecules of allyl sulfenic acid dimerize to form a molecule of allicin, which then reacts with another molecule of cysteine.

The results obtained herein prove that allicin is an irreversible, time-dependent inhibitor of jack bean urease. Restoration of the activity of allicin-modified urease with dithiothreitol and the protective effect of boric acid indicate that the inactivation of urease by

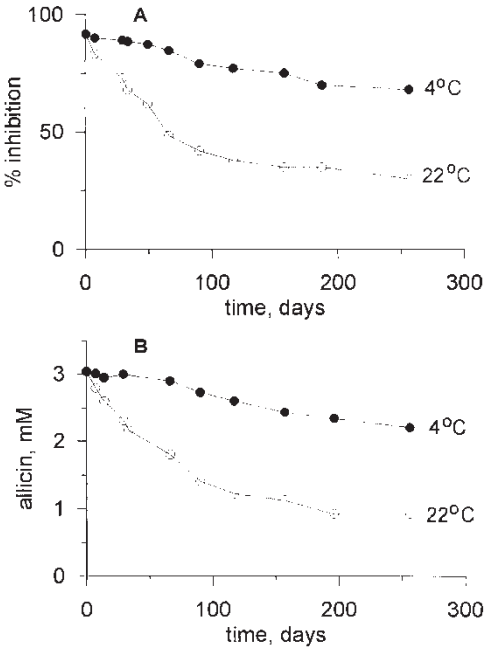
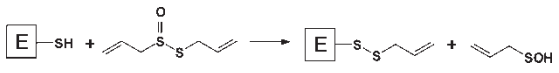


FIGURE 4 The stability of 3.3 mM allicin in water stored at 4° and 22°C. (A) The decrease in inhibitory potency of allicin towards urease. Urease was incubated with the inhibitor for 10 min and its residual activity was measured. The magnitude of inhibition was calculated as: % inhibition = 100% − % residual activity. (B) Decrease of allicin content in the solution.

allicin takes place at the active centre of the enzyme, and this is the result of SH-group modification. These data suggest that the covalent, mixed disulfide is produced according to the equation:



(7)

An analogous mechanism was assumed for allicin inactivation of other thiol-containing enzymes.[10]

The thiol group involved belongs to Cys^{592} and is situated on a mobile flap closing the active center of urease. In the course of urea hydrolysis the flap must be locked as the thiol group participates in the activation of urea. Modification of this simple group leading to the formation of a mixed disulfide results in the deformation of the flap and this, in turn, leads to the loss of urease catalytic activity.

Rabinkov *et al.*[10] and Gupta *et al.*[11] demonstrated that the inhibition of other thiol containing enzymes (papain, dehydrogenases and squalene monooxygenase) by allicin is also irreversible, time- and concentration dependent, and involves binding of the inhibitor to the SH-group at the active site of the enzymes.

The reaction of allicin with SH-groups of the enzymes is one of the major factors responsible for the antibacterial activity of garlic.

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