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Food Chemistry 85 (2004) 553–558

Food Chemistry

www.elsevier.com/locate/foodchem

A study of the inhibition of jack bean urease by garlic extract

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Received 17 January 2003; received in revised form 3 July 2003; accepted 17 July 2003

Abstract

An inhibiting effect of fresh garlic extract (GE) on jack bean urease has been investigated in order to elucidate the kinetics and mechanism of inhibition. The system: jack bean urease-garlic extract-urea, can be treated as a model system in the studies on the utility of garlic in the therapy of diseases caused by *Helicobacter pylori* and other bacteria producing urease. The influence of fresh aqueous garlic extract on the activity of jack bean urease at 22 °C in 20 mM phosphate buffer, pH 7 was investigated. The loss of urease activity was found to be directly proportional to alk(en)yl thiosulfinates (TS) content in the garlic extract. The inhibitory activity of GE was compared with the activity of synthetic allicin. The results indicate that the inhibition of urease by GE is irreversible and incubation time-dependent. The kinetics of the inhibition was found to be biphasic; each phase obeyed first-order kinetics. Thiol reagents (L-cysteine, 2-mercaptoethanol, glutathione, dithiothreithol) strongly protect the enzyme from the loss of enzymatic activity, while urea and boric acid show weaker protection. GE-modified urease could be reactivated with dithiothreitol. This study demonstrates, that the inhibition of urease by GE should be atrributed to the reaction of TS with the SH-group (Cys^{592}) found in the active site of urease.

Keywords: *Allium sativum*; Garlic extract; Inhibition; Alk(en)yl thiosulfinates; Jack bean urease; Biphasic kinetics

1. Introduction

Garlic has been used in herbal medicine for thousands of years. At present garlic preparations such as fresh garlic juice, aqueous and alcoholic extracts, lyophilized powders and steam distilled oil are a subject of numerous chemical, pharmacological and clinical studies. Garlic is known for its strong antibacterial, antifungal and antioxidant activity. Moreover, it inhibits platelet aggregation, effectively reduces serum cholesterol and triglicerydes and lowers ocular pressure (Ankri & Mirelman, 1999; Reuter, 1995). Responsible for these beneficial health properties of garlic preparations are biologically active sulphur compounds (Block, 1992; Ferary & Auger 1996; Lawson, Wood, & Hughes, 1991). The primary component of fresh aqueous garlic extract are alk(en)yl thiosulfinates, which are produced from alk(en)ylcysteine sulfoxides in the enzymatic reaction after the crushing of garlic [Eq. (1)]:

$$\begin{aligned} &\mathrm{R{-}S(O){-}CH_2{-}CH\ (NH_2)\ CO_2H + H_2O} \xrightarrow{\text{alliinase}} \\ &\mathrm{R{-}SOH + CH_3{-}C(O){-}CO_2H + NH_3} \\ &\mathrm{2R{-}SOH \rightarrow R{-}S(O)S{-}R + H_2O} \end{aligned} \quad (1)$$

At the initial period of this reaction pyruvate, ammonia and alk(en)yl sulfenic acid RS(O)H are formed; the latter undergoes rapid condensation to form thiosulfinates R–S(O)S–R, wherein R represents one of the following groups: methyl, 1-propenyl or 2-propenyl.

Garlic is a strong antibacterial agent and acts as an inhibitor on both Gram-positive and Gram-negative bacteria including such species as *Escherichia, Salmonella, Streptococcus, Staphylococcus, Klebsiella, Proteus* and *Helicobacter pylori* (Ankri & Mirelman, 1999; Reuter, 1995; Small, Bailey, & Cavallito, 1947). The latter is considered to be one of the main reasons of gastric and duodenal ulcers as well as gastric cancer. Recent in vitro and in vivo research has proved that *Helicobacter pylori*, resistant to many antibiotics, is sensitive to garlic extract in relatively low concentrations. Sivam, Lampe, Ulness, Swanzy, and Potter (1997)

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doi:10.1016/j.foodchem.2003.07.033

investigated the antimicrobial activity of aqueous extract of garlic against *H. pylori* and found that the minimum inhibitory concentration of the extract was 40 μg thiosulfinates/ml. Cellini, Di Campli, Masulli, Di Bartolomeo, and Allocati (1996) tested 16 clinical isolates of *H. pylori* and showed the concentration of garlic extract required to inhibit the bacterial growth was between 2 and 5 mg/ml. The inhibitory concentration of garlic reported in the above studies is achievable in the stomach by consuming a medium size clove of garlic. *H. pylori* is a rare bacterium known to colonize the human stomach.

Interestingly, it can survive and grow in the acidic environment of the stomach by producing abundant amounts of the enzyme urease (10–15% of total proteins by weight), which hydrolyzes urea present in gastric juice. The ammonia, generated from this reaction, produces a local alkaline microenvironment, thereby protecting the bacterium against the hostile acidic conditions (Ha, Oh, Sung, Cha, Lee, & Oh, 2001). The enzyme urease is also present in many other pathogenic bacteria, for example in the above mentioned, garlic extract sensitive strains such as *Proteus, Klebsiella, Staphylococcus*. Those bacteria are responsible for infections of urinary tracts. Independently on their origin, Ni^{2+} ions and the –SH group of the active site of the enzyme are essential for the catalytic activity of all ureases. The strongest inhibitors of ureases are phosphoroamides, acetohydroxamic acid and heavy metal ions while thiols were found to be weaker inhibitors (Blakeley & Zerner, 1984; Ciurli, Benini, Rypniewski, Wilson, Miletti, & Mangani, 1999; Kot, Zaborska, & Juszkiewicz, 2000; Mobley, Island, & Hausinger, 1995; Zaborska, Krajewska, Leszko, & Olech, 2001). Among the above mentioned inhibitors, acetohydroxamic acid, because of its effectiveness and non-toxicity, is most frequently studied inhibitor of urease, used in the therapy of diseases caused by bacteria producing urease. It has been found out that the inhibitors mechanism of action and the kinetics of inhibition for bacteria urease and jack bean urease are similar (Ciurli et al., 1999).

In the present study the inhibiting effect of fresh garlic extract on jack bean (*Canavalia ensiformis*) urease has bean investigated in order to elucidate the kinetics and mechanism of inhibition.

2. Materials and methods

2.1. Materials

Jack bean urease, Sigma type III, with specific activity 22 μmol NH_3/min mg protein, urea (Molecular Biology Reagent), L-cysteine (CYS), reduced glutathione (GLU), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), 5.5′-dithiobis-(2-nitrobenzoic acid), HEPES buffer, allyl disulfide, were purchased from Sigma. All the remaining chemicals were of analytical grade.

Allicin was syntesized from allyl disulfide after oxidation by perbenzoic acid by the procedure of Small et al. (1947). The garlic (*Allium sativum*) used was from China.

2.2. Preparation of garlic extract

Garlic cloves were crushed and extracted with 5 ml of distilled water per gram of garlic. Next, the homogenate was shaken for 20 minutes, filtered first through gauze and afterwards through a 0.2 μm filter. The obtained solution was then divided into two parts. One part was stored in the fridge (at 4 °C) and the other one at room temperature (22 °C). The aqueous garlic extract was standardized for its thiosulfinate concentration, using spectrophotometric method of Han, Lawson, Han, and Han (1995). The total thiosulfinate concentration was determined to be 4.4±0.3 mM (22±2 μmol TS/g wet mass). For kinetic measurements the extract was diluted 2, 5 and 10 times.

2.3. Enzymatic reaction

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

$$CO(NH_2)_2 + H_2O \overset{\text{urease}}{\rightarrow} 2NH_3 + CO_2 \quad (2)$$

The reaction was monitored by measuring ammonia concentration with the phenol-hypochlorite method (Weatherburn, 1967). Prior to this reaction, ammonia concentration in the garlic extract produced in the reaction of thiosulfinate formation [Eq. (1)] was determined. This amount was subtracted from the total amount of ammonia in the system.

2.4. Inhibition studies

Urease solution was mixed with equal volume of GE or allicin, and the mixture was incubated at 22 °C. The mixture always contained 0.5 mg/ml of urease, 20 mM phosphate buffer of pH 7.0 and 2 mM EDTA. Aliquots were taken at different time intervals and were immediately transferred to assay mixtures containing 100 mM urea, 20 mM phosphate buffer, pH 7.0 and 2mM EDTA in order to determine the residual activity.

2.5. Inhibition of urease in the presence of protective agents

Urease was incubated for 15 min with garlic extract in the presence of thiol-containing compounds (L-cysteine, 2-ME, glutathione, DTT), urea or boric acid. The concentration of TS and the protective agent in the

incubating mixture was 1.1 mM and 5 mM, respectively. Reactivation of the inhibited urease by TS was done with 5 mM thiols.

2.6. Stability of garlic extract

Samples of garlic extract stored at 4 and 22 °C were taken at particular time intervals and incubated for 10 min with urease solution. Afterwards, the residual activity was measured.

3. Results and discussion

3.1. Interaction of garlic extract and allicin with urease

GE was tested for its ability to inhibit urease. The main components of the fresh, aqueous garlic extract are alk(en)yl thiosulfinates, which are formed from alk(en)ylcysteine sulfoxides in the enzymatic reaction [Eq. (1)] after the crushing of garlic. The analysis of the aqueous garlic extract revealed the presence of all possible combinations of 2-propenyl (allyl), methyl, and 1-propenyl groups except of di-1-propenyl thiosulfinates; where 60–80% of thiosulfinates stands for diallyl thiosulfinate known as allicin.

Fig. 1A presents the decrease of urease activity after 10 min of an incubation with garlic extract of growing content of garlic. Half of the inhibitory concentration was achieved with 5.6 g/l GE and the activity was completely inhibited with 100 g/l GE. Total TS concentration in the former solution was 0.20 mM, while in the latter was 2.2 mM.

The inhibitory properties of GE thiosulfinates and synthetic diallyl thiosulfinate are compared in Fig. 1B. Urease was incubated with 1.6 mM allicin or GE containing 1.6 mM of TS. Samples were taken from incubating mixtures at particular time intervals to measure the residual activity of urease. The results show that the urease activity decreases with the increase of incubation time equally in both investigated systems. This means that the thiosulfinates are active inhibitors in the garlic extract and that the inhibition of urease does not depend on the kind of alk(en)yl groups of thiosulfinates but depends on TS concentration and incubation time with urease. The 18-min incubation of the enzyme with the inhibitor causes total loss of urease activity, i.e. inactivation.

3.2. Stability of fresh aqueous garlic extract

Fig. 2A shows how the inhibitory strength of GE stored at 4 and 22 °C changes with the storage time. While the extract stored at 4 °C was found to be relatively stable and even after 200 days still retained over 40% of its original inhibitory potential, the solution

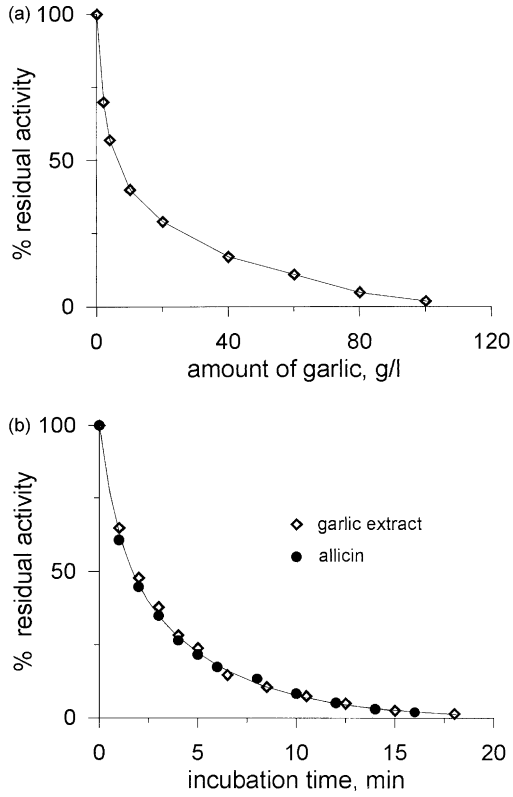

Fig. 1. A—Effect of garlic extract on urease activity. Urease was incubated for 10 min with different amounts of garlic extract. B—Time-dependent inhibition of urease by 1.6 mM allicin and garlic extract in which total TS concentration was also 1.6 mM.

stored at 22 °C showed only 3% of its inhibitory potential after 57 days. Fig. 2B shows that a decrease in the GE inhibition strength is correlated with the decrease in the TS content in the extract stored both at 4 and 22 °C. The data provide evidence that the actual inhibiting agents in the GE are thiosulfinates. TS are not stable and transform with time into more stable components: polysulfides and thiosulphonates (Block, 1992). This explains why the extract looses its inhibitory properties towards urease.

3.3. Protection by thiol-containing compounds, urea and boric acid

Thiol-containing compounds were tested for their ability to reverse and protect against the inactivation of urease by garlic. Monothiols such as cysteine, 2-ME, glutathione and dithiol: DTT were added before or after 15 min incubation. The addition of each thiol reagent to the incubation mixture indicates the significant protection against inactivation by garlic, suggesting that TS react with SH-groups in urease—Fig. 3A. The –SH groups present in the protein are much less reactive than externally added –SH in the form of 2-ME, cysteine, glutathione or DTT (Goodno, Swaisgood, & Catignani, 1981). The addition of DTT after 10 min incubation of

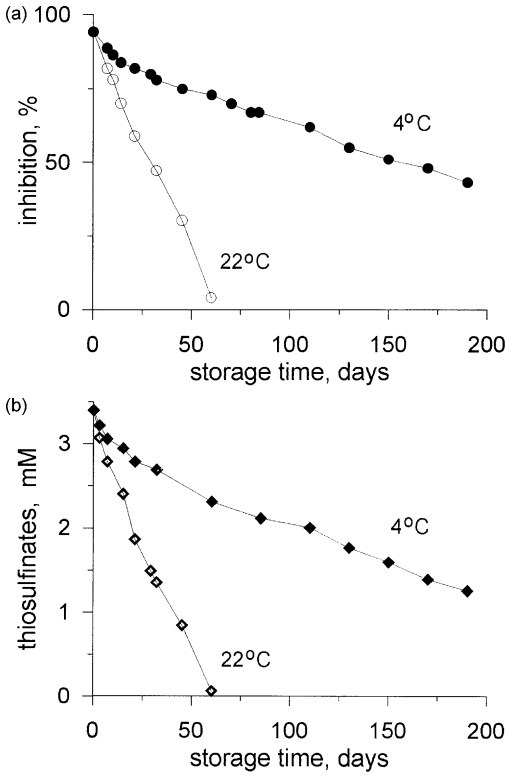

Fig. 2. The stability of garlic extract on storage at 4 and 22 °C. A—The decrease in inhibitory properties of the extract towards urease. Urease was incubated with the inhibitor for 10 min and its residual activity was measured. The magnitude of inhibition was calculated from:% inhibition = 100% − % residual activity. B—Decrease in TS content in the extract.

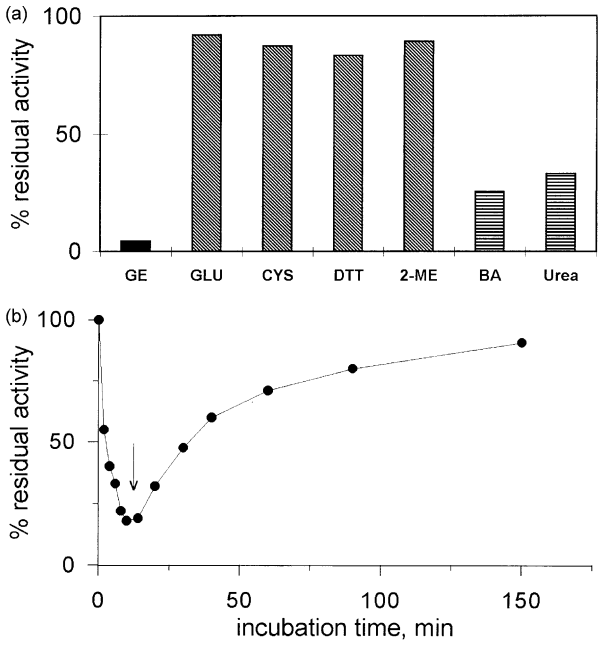

Fig. 3. A—The effects of thiol-containing compounds, urea and boric acid (BA) on the inhibition of urease by garlic extract. Urease was incubated for 15 min with garlic extract in which TS concentration was equal to 1.1 mM in the absence or presence of 5 mM protection agent. B—Slow reactivation of TS-modified urease in the presence of DTT. Urease was incubated for 10 min with garlic extract before added DTT and residual activity was monitored as a function of time.

urease with GE leads to the recovery of urease activity in the time-dependent way: after 2.5 h the enzyme recovers ca. 95% of its initial activity–Fig. 3B. DDT recovers the enzyme activity also when the enzyme is incubated with GE for 60 min. The enzyme does not recover the activity by the addition of monothiols. The substrate and boric acid provide weaker protection against GE. The boric acid is simple, reversible and competitive inhibitor of medium strength: $K_i = 0.12$ mM (Kot, Krajewska, Leszko, Olech, & Zaborska, 1998). It was shown that the active form is $B(OH)_3$ molecule. Being of small size, this molecule binds Ni^{2+} ions which allows for blocking the active site of the enzyme, protecting it from TS inactivation. The obtained results indicate that the inactivation of urease by TS takes place in the active centre of the enzyme.

3.4. Irreversibility of the inhibition

In order to determine whether TS interacts with urease irreversibly, the enzyme was incubated with GE for 1 h and then TS modified-urease was dialyzed for 24 h in 20 mM phosphate buffer of pH 7.0, 2 mM EDTA at 4 °C. Urease did not regain its activity, proving that the inhibitor binds irreversibly to urease.

3.5. Determination of inactivation kinetics

The kinetic study performed in this paper showed that the inhibition of urease by TS was dependent on the concentration of the TS as well as on the incubation time. A semi-log plot of the residual activity shows biphasic kinetics in which ca. 30% of the initial activity was lost in the fast phase and the remainder in a slow phase. Each phase exhibited first-order-kinetics—Fig. 4. The time course of the reaction can be represented by a rate equation containing of two first-order terms, corresponding to the fast and slow phases of the reaction (Prakash & Bhushan, 1998):

$$A_t = A_{fast} e^{-k_{fast} \cdot t} + A_{slow} e^{-k_{slow} \cdot t} \quad (3)$$

where A_t is the percent of the residual activity at time t, A_{fast} and A_{slow} are amplitudes (expressed as percent of the starting activity), and k_{fast} and k_{slow} are the first-order rate constants of the fast and slow phases, respectively. The value of A_{fast}, A_{slow}, k_{fast} and k_{slow} with different concentration TS in GE were computed from the fit of the residual activity versus time, according to Eq. (3), by least square method (Table 1). The first-order rate constants k_{fast} and k_{slow} increase with the increasing amount of TS in garlic extract both in the fast and slow phases, however, the values of amplitudes A_{fast} and A_{slow} are nearly the same for all investigated inhibitor concentration.

Table 1
Amplitudes and the rate constants for the inhibition of jack bean urease by thiosulfinates (TS) present in the aqueous garlic extract

Concentration of TS in the incubation mixture (mM)	Fast phase		Slow phase	
	A_{fast} (%)	k_{fast} (min^{-1})	A_{slow} (%)	k_{slow} (min^{-1})
1.1	30	1.26	70	0.18
0.44	33	0.36	67	0.084
0.22	30	0.20	70	0.047

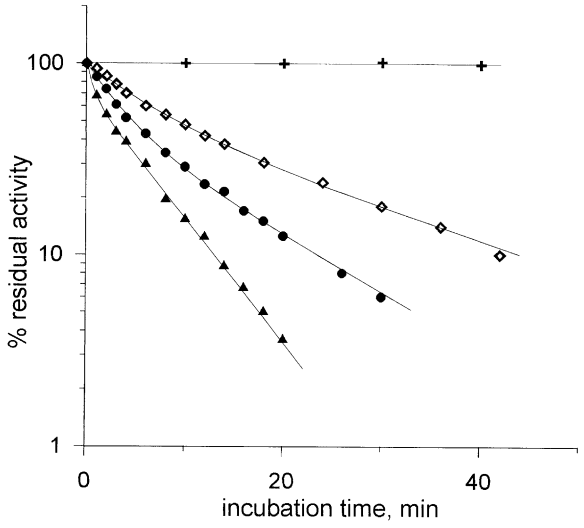

Fig. 4. Kinetics of the inhibition of urease by garlic extract. Urease was incubated with garlic extract containing TS in the following concentration: 1.1 mM (▲), 0.44 mM (●), 0.22 mM (□), 0 mM (+) and the residual enzymatic activity was monitored as a function of time. Solid lines were fited to the experimental points, according to Eq. (3), by the least square method.

Similar biphasic kinetics was observed during inhibition of two other plant ureases (*Cajanus cajan* and *Citrullus vulgaris*) using thiol reagents (5.5′-dithiobis-(2-nitrobenzoic acid), p-chloromercuribenzoate, N-ethylmaleimide), acetohydroxamic acid and metal ions (Prakash & Bhushan, 1998; Prakash & Vishwakarma, 2001; Srivastawa & Kayastha, 2000; Srivastawa, Kayastha, & Jagannadham, 2002). The above ureases, similarly to jack bean urease, are homohexameric proteins, which contain in the active site one cysteine residue, essential for the catalytic activity of urease, per subunit. The authors suggest, that the characteristic biphasicity is the result of different reactivity of these cysteine residues. Six –SH groups can be divided into two categories: those which react faster and those which react slower. In the jack bean urease, the –SH group belongs to Cys^{592} located on the mobile flap closing the active site (Ciurli et al., 1999).

The results obtained in this paper prove that the irreversible, time-dependent inhibition of jack bean urease by aqueous garlic extract is the result of the reaction of the –SH group, situated in the active site of urease, with thiosulfinates. Rabinkov, Miron, Konstatinovski, Wilchek, Mirelman, and Weiner (1998) studied in details the interaction mechanism of diallyl thiosulfinate with –SH groups in the model system containing L-cysteine. S-allylmercaptocysteine (Cys-S-S-All) is produced in the course of this reaction, which was proved with NMR as well as SM studies. Taking all the above into consideration, the inhibition of jack bean urease by TS can be considered as the reaction between the –SH group (Cys^{592}), present in the active site of urease, and the sulfur of the -S(O)-S-in thiosulfinates, leading to the formation of the covalent disulphur compound:

$$\mathbf{E}\text{-SH} + \text{R-S(O)S-R} \rightarrow \mathbf{E}\text{-S-S-R} + \text{R-SOH}$$

where R represents the groups: methyl, 1-propenyl or 2-propenyl.

The above reaction leads to deformation of the flap and in consequence to the loss of catalytic activity of urease.

The results for jack bean urease presented in this paper are in accordance with those reported for other thiol containing enzymes. Rabinkov et al. (1998) studied the effect of allicin on the activity thiol containing proteins: papain and dehydrogenases and found that the inhibition was irreversible and time-dependent, and was caused by modification of –SH group(s) of the enzymes. Gupta and Porter (2001) demonstrated that inhibition of squalene monooxygenase by fresh water garlic extract is also irreversible and time-dependent, and involves binding of the inhibitor to the –SH groups in the active site of the enzyme.

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