Food Chemistry 112 (2009) 42–45

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Modification of jack bean urease thiols by thiosulphinates contained in garlic extract: DTNB titration studies

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ARTICLE INFO

Article history:
Received 12 November 2007
Accepted 8 May 2008

Keywords:
Garlic extract
Inhibition
Alk(en)yl thiosulphinates
Urease
5,5′-Dithiobis(2-nitrobenzoic acid)

ABSTRACT

The reactivity of the thiol groups in urease with thiosulphinate contained in garlic extract was spectroscopically characterised. The enzyme was incubated with the garlic extract and the reaction progress curves were recorded in the presence of thiol-selective reagent 5,5′-dithiobis(2-nitrobenzoic acid). Simultaneously the enzyme residual activity was also determined. The process was studied in 50 mM phosphate buffer, at pH 7.8 and ambient temperature. It was found that thiosulphinates act as time- and concentration-dependent inactivators of urease. The observed decrease of the enzyme activity corresponds to the number of the urease thiols modified by thiosulphinates. The modification of half of all the urease thiols (18 of 36) causes a slight (only 8–10%) decrease of the catalytic activity. The modification of the remaining 18 thiols results in significant disturbance of urease action until complete loss of the catalytic function occurs. This provides the evidence that Cys^{592}, the critical residue for urease activity, belongs to the enzyme thiols, which are less reactive and more resistant to chemical modification than the other thiols.

1. Introduction

Urease (urea amidohydrolase (EC 3.5.1.5) catalyses the hydrolysis of urea to ammonia and carbon dioxide; it is synthesised by plants, algae, fungi and bacteria (Blakeley & Zerner, 1984; Mobley & Hausinger, 1989; Sirko & Brodzik, 2000). Many bacteria producing urease have a negative effect on human health. Strains such as *Proteus*, *Klebsiella* and *Staphylococcus* are responsible for urinary tract infections (Burne & Chen, 2000). *Helicobacter pylori* is believed to be responsible for developing gastric and duodenal ulcers as well as gastric cancer. Interestingly, it can survive and grow in the acidic environment of the stomach by releasing large amounts of urease (10–15% of total proteins by weight), which hydrolyses urea present in the gastric juice. The ammonia, produced from this reaction, alkalises the microenvironment, thereby protecting the bacteria from the hostile acidic conditions (Ha et al., 2001).

The structure, number and type of subunits, molecular weight and amino acid sequence of urease depend on its origin. The bacterial ureases contain three subunits α, β and γ. Their molecular weights are in the range 68–73 kDa (α), 8–17 kDa (β) and 8–12 kDa (γ). The molecules of bacterial urease are mostly trimers containing three subunits: $(\alpha\beta\gamma)_3$. In contrast, *Helicobacter pylori* urease possesses only two types of subunits: α (60–66 kDa) and β (26–31 kDa). However, the basic structural elements are trimers: $(\alpha\beta)_3$ which form a tetrahedral complex in the $((\alpha\beta)_3)_4$ stoichiometry, the molecular weight of which equals 1100 kDa. This unique supramolecular assembly may be important for the protection of the microorganism against acidic environment (Benini et al., 1999; Jabri, Carr, Hausinger, & Karplus, 1995; Mobley, Island, & Hausinger, 1995).

The plant urease from jack bean is a homohexameric molecule (α_6). The subunit contains 840 amino acid residues and its molecular weight is equal to 90.77 ± 0.05 kDa (Mamiya et al., 1985).

Despite the differences in the molecular structures, the amino acid sequences of the active sites are principally similar in all known ureases. The consequence of this fact is the same catalytic mechanism. The active sites are always located in α subunits and contain the binuclear nickel centre, in which the Ni–Ni distances range from 3.5 to 3.7 Å (Ciurli et al., 1999; Dixon, Blakeley, & Zerner, 1980).

Ureases are thiol-rich enzymes. The total thiol content in jack bean urease has been determined by DTNB titration in the presence of 6 M guanidinum chloride. It was proved that urease contains 15 thiol groups per subunit. However, only six out of 15 cysteines are accessible to the thiol-selective reagent (without denaturation of the enzyme). One of those six cysteines, Cys^{592}, is located on the mobile flap closing the active site of urease. This cysteine plays a critical role in catalysis. The chemical modification of Cys^{592} results in inactivation of the enzyme. However, the covalent modification of five other cysteine residues, which are more

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doi:10.1016/j.foodchem.2008.05.029

reactive than Cys^{592}, does not affect the enzyme activity (Norris & Brocklehurst, 1976; Takishima, Suga, & Mamiya, 1988).

The study of urease inhibition is of medical significance. Urease inhibitors such as hydroxamic acids and phosphoroamides (Burne & Chen, 2000) have been applied in medicine but have many side effects; therefore, a safe and efficient inhibitor of urease is still being sought.

In our previous paper we presented the results of urease inhibition by a natural plant agent such as garlic extract (Juszkiewicz, Zaborska, Łaptaś, & Olech, 2004). Garlic is known for its strong antibacterial and antifungal activity (Ankri & Mirelman, 1999; Reuter, 1995). The primary components of fresh aqueous garlic extract are alk(en)yl thiosulphinates, which are produced from alk(en)ylcysteine sulphoxides in the enzymatic reaction after crushing of garlic (Block, 1992; Lawson, Wood, & Hughes, 1991):

$$2R-S(O)-CH_2-CH(NH_2)CO_2H \xrightarrow[+H_2O]{\text{allinase}} R-S(O)S-R+2CH_3-C(O)-CO_2H+2NH_3$$

where R represents groups: methyl, 1-propenyl and 2-propenyl. The products of the enzymatic reaction are alk(en)yl thiosulphinates, pyruvate and ammonia.

Our studies showed that the urease inhibition by thiosulphinates is irreversible. The kinetics of the inhibition was found to be biphasic. The loss of urease activity was directly proportional to alk(en)yl thiosulphinates content in garlic extract. The test, based on the reaction with thiol reagents (L-cysteine, 2-mercaptoethanol, glutathione, dithiothreitol) confirmed the essential role of the sulphydryl groups in the inhibition of urease by garlic extract.

The aim of this paper is to study the thiol groups modification in the system urease/urease–thiosulphinate complexes by the use of the thiol-selective reagent, DTNB. The influence of the incubation time and garlic extract concentration on accessibility of the urease –SH groups to DTNB is also investigated.

2. Materials and methods

2.1. Materials

Jack bean urease (Sigma type III, with specific activity 16 U/mg solid) urea (Molecular Biology Reagent), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (St. Louis, MO). Other chemicals were obtained from POCh, Gliwice, Poland. All reagents used were of analytical grade. Phosphate buffer (50 mM, pH 7.80) was prepared by adjusting the pH of phosphoric(V) acid with NaOH. Two millimolar EDTA was added to all enzyme-containing solutions.

2.2. Preparation of garlic extract

Garlic cloves were crushed and extracted with 10 ml of distilled water per gram of garlic. Next, the homogenate was shaken for 20 min and filtered through gauze. Undissolved material was removed by centrifugation at 300*g* for 4 min. The aqueous garlic extract was standardised for its thiosulphinate (TS) concentration, using the spectrophotometric method of Han, Lawson, Han, and Han (1995). The total thiosulphinate concentration was determined to be: 3.0 ± 0.3 mM. For measurements, the stock garlic extract was diluted 10–1000 times.

2.3. Standard urease activity assay

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

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

The standard assay mixture (25 ml) consisted of 50 mM urea in 50 mM phosphate buffer (pH 7.8) and 2 mM EDTA. The reactions were initiated by the addition of small aliquots of the enzyme-containing (0.5 mg urease) solution, and the urease activity was determined by measuring ammonia concentration after 5 min reaction time. Ammonia was determined by the spectrophotometric, phenol–hypochlorite method. The absorbance was registered at 630 nm (Weatherburn, 1967). Prior to the enzymatic reaction, ammonia concentration in the garlic extract produced during thiosulphinate formation was measured.

The measurements were performed at ambient temperature. The activity of uninhibited urease was designated as the control activity of 100%.

2.4. Inactivation of urease by thiosulphinates (TS)

The urease solutions (2.0 mg solid/ml) and the garlic extract were mixed in the ratio 1:1. The concentrations of TS used in the incubation mixtures were in the range 1.5–1000 μM. During the incubations, 0.5 ml aliquots of the incubation mixture at different time intervals were transferred into the standard assay mixtures (25 ml) for urease residual activity determination.

2.5. Spectroscopic assays of –SH groups with DTNB in thiosulphinates-modified urease

Urease (2 mg/ml in 50 mM phosphate buffer, pH 7.8, 2 mM EDTA) and the inhibitor (garlic extract) solutions were mixed at the ratio 1:1. Upon incubation, the urease activity was assayed under standard conditions, and 2.5 ml of incubation mixture was

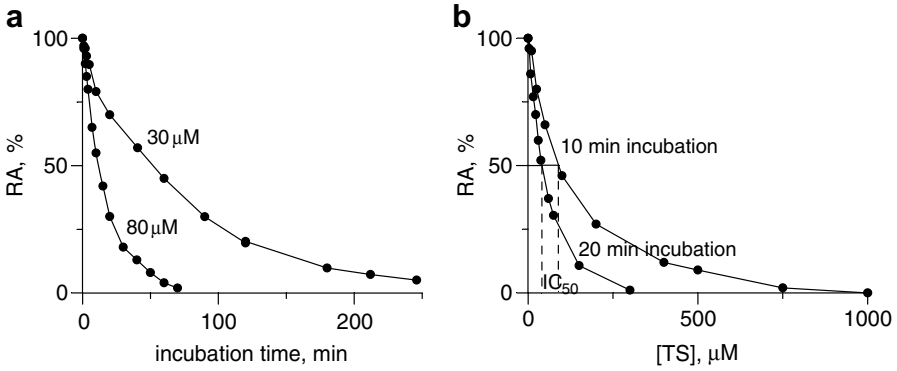

Fig. 1. Inactivation of urease by garlic extract: (a) Time progress of inactivation, urease residual activity (RA) *vs* incubation time. TS concentration is shown. (b) Urease residual activity (RA) as a function of TS concentration in the incubation mixture corresponding to 10 and 20 min incubation.

transferred to a cuvette (light path 5 cm) and mixed with 2.5 ml 0.15 mM DTNB (prepared in 50 mM phosphate buffer, pH 7.8). The absorbance of mixtures was measured at 412 nm in continuous mode for 15 min (Ellman, 1959). Prior to these measurements, the control measurements of absorbance of the mixtures used were performed: urease and DTNB–garlic extract in the proportions corresponding to the final reaction mixture at 412 nm into continuous mode for 15 min. The recorded control absorbances were subtracted from the absorbances recorded for the reaction mixtures, i.e., urease–DTNB–garlic extract.

3. Results and discussion

3.1. Inactivation of urease by TS

The inhibition effect of thiosulphinates contained in the garlic extract on urease action is presented in Fig. 1a and b. Fig. 1a shows the decrease of urease activity with increase of incubation time. The incubation time increases until complete loss of urease catalytic functions occurs. The experiment was performed at 30 and 80 μM TS concentration. The observed half-life time was equal to 60 min at 30 μM and 15 min at 80 μM TS. In Fig. 1b the urease residual activity corresponding to the 10 and 20 min incubation time is plotted against inhibitor concentration. The resulting IC_{50} values, which specify an inhibitor concentration causing 50% of enzyme inactivation are 80 and 50 μM, respectively for the 10 and 20 min incubation time. The obtained results show that urease is inhibited by garlic extract thiosulphinates in time- and concentration-dependent manners, which qualifies thiosulphinates as the inactivators of urease.

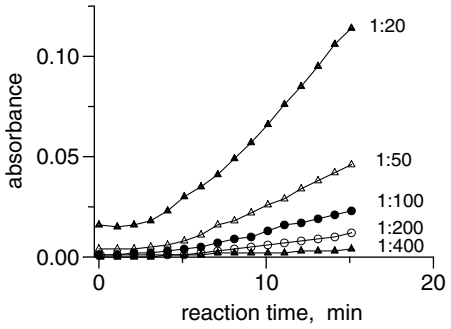

Fig. 2. Absorbance curves of DTNB–garlic extract reaction. Curves correspond to 400, 200, 100, 50 and 20 times dilution of the stock garlic extract, which relates to a TS concentration equal to 7.5, 15, 30, 60 and 150 μM, respectively.

3.2. Spectroscopic assays of –SH groups with DTNB in thiosulphinates-modified urease

The mechanism of urease inactivation was studied by the spectroscopic titration of thiol groups in thiosulphinate-modified urease with the use of DTNB. The procedure was proposed in our previous study on urease-Hg(II) ion system (Krajewska, Zaborska, & Chudy, 2004) and was also successfully applied in our study of quinones effects on the enzyme (Krajewska & Zaborska, 2007). The change of absorbance as a function of time corresponds to the reaction progress. The registered absorbance curves were corrected for the absorbance changes resulting from the DTNB–garlic extract interaction. Fig. 2 presents the absorbance curves recorded after mixing of the garlic extract with DTNB. The observed increase of absorbance was caused by compounds containing free thiol groups, e.g., proteins and allylmercaptan, which are contained in garlic extract, and are DTNB reactive (Bianchini & Vainio, 2001).

The absorbance curves of DTNB reaction with urease and TS-modified urease are showed in Fig. 3a. Urease was incubated with

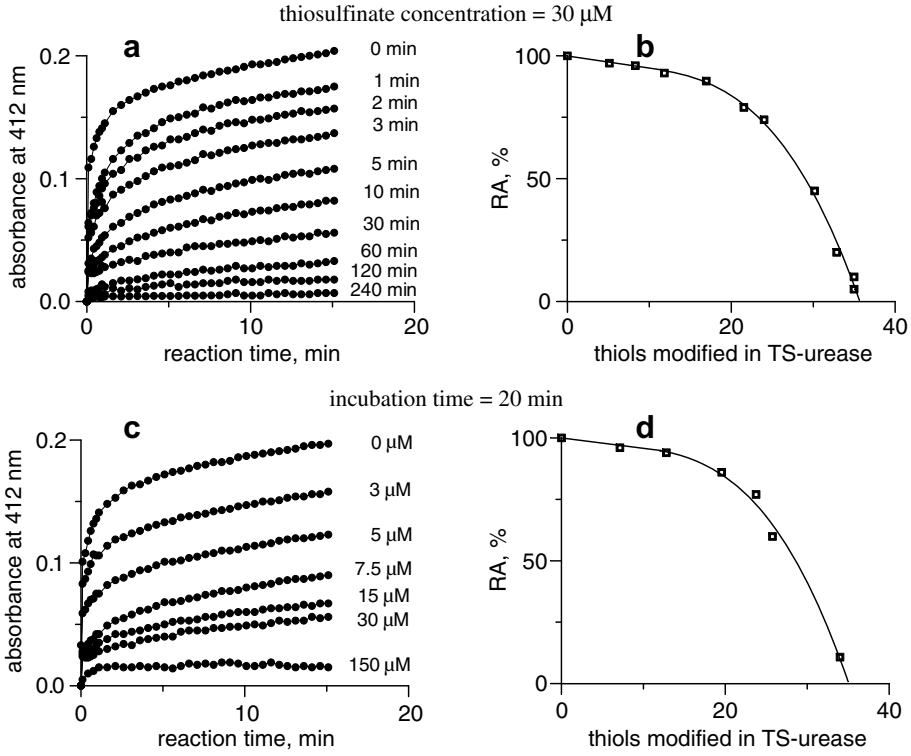

Fig. 3. DTNB titration of thiol groups in urease modified by TS: (a) absorbance curves of DTNB reaction with urease and urease modified by TS. TS concentration was equal to 30 μM, the incubation time is shown. (b) Urease residual activity as a function of number of enzyme thiols modified by TS. Points relate to the respective absorbance curves of reactions presented in Fig. 3a. (c) Absorbance curves of DTNB reaction with urease and urease modified by TS after a 20 min incubation. TS concentrations are shown. (d) Urease residual activity as a function of number of enzyme thiols modified by TS. Points relate to the respective absorbance curves presented in Fig. 3c.

the garlic extract containing a constant TS concentration equal to 30 μM (related to 1:100 dilution of the stock garlic extract). The incubation time was in the range 1–240 min. The obtained absorbance curves provide evidence that the reaction between urease and the garlic extract is time-dependent. For each curve depicted in Fig. 3a the amount of thiols modified by TS in urease during different incubation times was calculated. Assuming that the absorbance recorded for uninhibited urease (at non-denaturating conditions) corresponded to 36 thiols per enzyme molecule (Takishima et al., 1988), the 15-min absorbance of TS-modified urease was converted, by direct proportion between absorbance and the number of thiol groups, into the number of TS-modified thiols per urease molecule. Fig. 3b shows the relation between the urease residual activity and the calculated number of modified enzyme thiols. At the beginning of inhibition (up to 5 min) a half of all urease thiols (18 of 36) are blocked by TS. Interestingly, that causes a weak inhibition effect. The urease activity decreases insignificantly to ca. 92% of the total activity. The modification of the remaining 18 urease thiols results in a sharp activity decrease until complete activity loss. This indicates that Cys^{592}, a critical residue for urease catalytical function, remains unmodified by TS, at the beginning of the incubation period and reacts at a later incubation time. This confirms previous observation that Cys^{592}, situated in the urease active site, is less reactive than other urease thiols (Norris & Brocklehurst, 1976).

Fig. 3c presents the time-courses of the reaction of DTNB with urease modified by different concentrations of TS (3–150 μM) at a constant incubation time equal to 20 min. Fig. 3d shows that the number of urease thiol groups available for DTNB after 20 min incubation decreases with an increase in the TS concentration used in the incubation mixtures. The low TS concentrations up to 5 μM modify about 13 –SH groups without considerably altering the enzyme activity. Only higher inhibitor concentrations give rise to the modification of further –SH groups, up to 36 –SHs, inducing a quick drop in the enzyme activity. It appears that Cys^{592} is among the 23 thiols responsible for the rapid activity decrease. The modification of all 36 –SH groups (including the active site flap Cys^{592}) accessible in urease under non-denaturating conditions causes the complete inactivation of urease. This observation confirms that modification of thiol groups by TS, as well as thiols in other thiol-containing enzymes (Gupta & Porter, 2001; Rabinkov et al., 1998; Wills, 1956), results from the reaction between the –SH groups and thiosulphinates. This leads to the formation of the covalent disulphide compound:

$$E-SH+R-S(O)S-R \rightarrow E-S-S-R+R-SOH$$

where R represents the groups methyl, 1-propenyl or 2-propenyl, and E represents urease.

The structural investigations shows that Cys^{592} situated on the mobile flap closing the entrance to the active site, plays an essential role in the mechanism of urea hydrolysis (Takishima et al., 1988). If this residue is chemically modified, the flap loses its mobility and the reaction is disturbed. Our investigations prove that thiosulphinates contained in garlic are able to react with urease thiols, completely blocking the urease action. This ability makes garlic a natural antibiotic against the urease pathogenic bacteria.

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