Acta Biochimica Polonica

Vol. 42 No. 1/1995

115–118

QUARTERLY

Short Communication

Competitive inhibitors of free and chitosan-immobilized urease

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Received: 30 June, 1994; accepted: 5 December, 1995

Key words: urease inhibitors, chitosan membranes, urease immobilization

The influence of four inhibitors: boric acid, thioglycolic acid, sodium fluoride and acetohydroxamic acid on the activity of urease, both in the native form and immobilized covalently on glutaraldehyde-pretreated chitosan membrane, was studied. Urea hydrolysis was carried out in phosphate buffer, pH 7, at 25°C at urea concentration of 50 mM. The immobilized urease was more resistant than the native one to the action of all the investigated inhibitors, except boric acid. This property of the enzyme offers a possibility of its practical application.

In contrast to the native, the immobilized enzymes are more stable and inhibitor resistant. They can be easily separated from the catalysis products and used several times or applied in flow analysis. Immobilized enzymes find broad application in analytical chemistry, industry, biotechnology and biomedicine [1].

The chitosan membrane-immobilized urease system exhibits high enzymatic activity and can be useful in enzymatic removal of urea from blood in the artificial kidney, for blood detoxication [2] or in the dialysate regeneration system of artificial kidneys [3, 4].

Urease is a nickel-containing enzyme that hydrolyses urea to form ammonia and carbamate, the latter spontaneously decomposing to give carbonic acid and ammonia [5]. Jack bean urease is a homohexameric enzyme (subunit M_r = 90770) containing 2 moles of nickel per one mole of subunit [6]. Zerner and coworkers [5, 7] reported that, in the resting enzyme at neutral pH, one of the Ni(II) ions is coordinated by a water molecule and the other is coordinated by hydroxide ion. Three amino-acid residues are supposed to be also located at the active site: a carboxyl group, a sulfhydryl group, and an unidentified base. The model of the active site proposed by Zerner and coworkers [5, 7] is shown in Fig. 1.

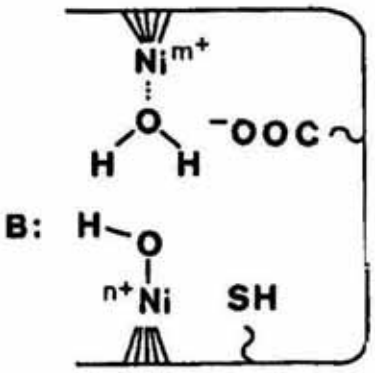

Fig. 1. The model of jack-bean urease active site proposed by Zerner and coworkers [5, 7].

In the course of competitive inhibition the equilibrium between enzyme, inhibitor and the enzyme-inhibitor complex is attained very fast, within milliseconds, and the progress of hydrolysis is linear with time. A particular group of competitive inhibitors, the so called slow-binding inhibitors have been described by Morrison & Walsh [8]. These inhibitors initially form a complex which is slowly transformed to a more stable one. In this case the equilibrium is at-

tained within seconds or minutes, and the progress curves have an asymptotic course. The analysis of the reaction progress curves allows to distinguish the two types of inhibitors.

In previous papers [9–11] the preparation and properties of the urease covalently immobilized on chitosan membrane and the distribution of pore radii was reported. Inhibition of the activity of urease in native and immobilized form by heavy metal ions was also studied [12]. It was found that the toxicity of metal ions correlates well with metal sulfide solubility. The immobilization makes the enzyme more resistant to the inhibitory action of heavy metal ions.

In the present paper the inhibition of free and chitosan membrane-bound urease by four inhibitors: boric acid, thioglycolic acid, sodium fluoride and acetohydroxamic acid has been studied.

EXPERIMENTAL

Materials. Urease was of Sigma type III with specific activity of 32 units/mg of protein. One unit is defined as the amount of enzyme that liberates 1.0 μmole NH_3 from urea per minute at pH 7 and 25°C. The urease was immobilized on chitosan membranes with the use of bifunctional agent, glutaraldehyde, according to the procedure described previously [10]. The amount of immobilized enzyme protein was found to be 0.049 mg • cm^{-2}.

The following compounds were tested as inhibitors of free and chitosan immobilized urease: boric acid and sodium fluoride (POCh, Poland), thioglycolic acid (Fluka), acetohydroxamic acid (Sigma). Urea was purchased from POCh, Poland.

Enzymatic reaction. Urea hydrolysis, catalysed by free and immobilized urease was studied in 22 mM phosphate buffer, pH 7.0, containing 1 mM EDTA at 25°C and at urea concentration of 50 mM. The concentration of inhibitors was varied from 0 to 10 mM. The reaction was initiated by addition of either 1 ml urease solution (concentration 1 mg/ml) or 20 cm^2 chitosan-immobilized urease to 40 ml of assay mixture. The progress of reaction was measured by determination of the concentration of ammonium ions formed during hydrolysis of urea. The reaction was studied over a period of 10 min. Without inhibitor, in both systems studied, the rate of urea hydrolysis was the same.

The concentration of ammonium ions was determined by the phenol-hypochlorite method [13]. Since thioglycolic acid interferes with the phenol-hypochlorite ammonia assay, separate calibration curves for ammonium ions in the presence of thioglycolic acid were made.

RESULTS AND DISCUSSION

Loss of activity of native and chitosan-immobilized urease as a function of concentration of boric acid, thioglycolic acid, sodium fluoride or acetohydroxamic acid is shown in Fig. 2. Progress curves for the rate of urea hydrolysis in the presence of investigated inhibitors are shown in Fig. 3.

Boric acid is the strongest of all the inhibitors studied and affects in the same way both forms of urease, free and bound. Boric acid at 1 mM concentration decreases urease activity to 50% and at 10 mM concentration to 8% of the initial activity (Fig. 2). The rate of urea hydrolysis in the presence of 2 mM boric acid is constant within the investigated range of time (Fig. 3).

Breitenbach & Hausinger [14] have found that boric acid is a competitive inhibitor of *Proteus mirabilis* urease. Basing on the pH dependence of the inhibition constant, it was suggested that the only uncharged trigonal molecule $B(OH)_3$ is the urease inhibitor and it can bind to the metal center.

Thioglycolic acid is rather poor urease inhibitor; an apparent influence on the native urease activity can be observed above the concentration of 4 mM and immobilized urease is almost completely resistant to the inhibitor (Fig. 2). The rates of urea hydrolysis by native and immobilized urease in the presence of 6 mM thioglycolic acid are constant (Fig. 3).

Several thiol compounds were shown to be competitive inhibitors of urease [5, 7, 15]. The pH dependence of inhibition constant and spectroscopic studies demonstrate that –SH group forms a charge transfer complex with nickel ion(s) present on the active site. The protonated thiol is inactive as an inhibitor.

Sodium fluoride is a strong inhibitor of native urease, whereas immobilization protects the enzyme against the inhibition (Fig. 2). Thus,

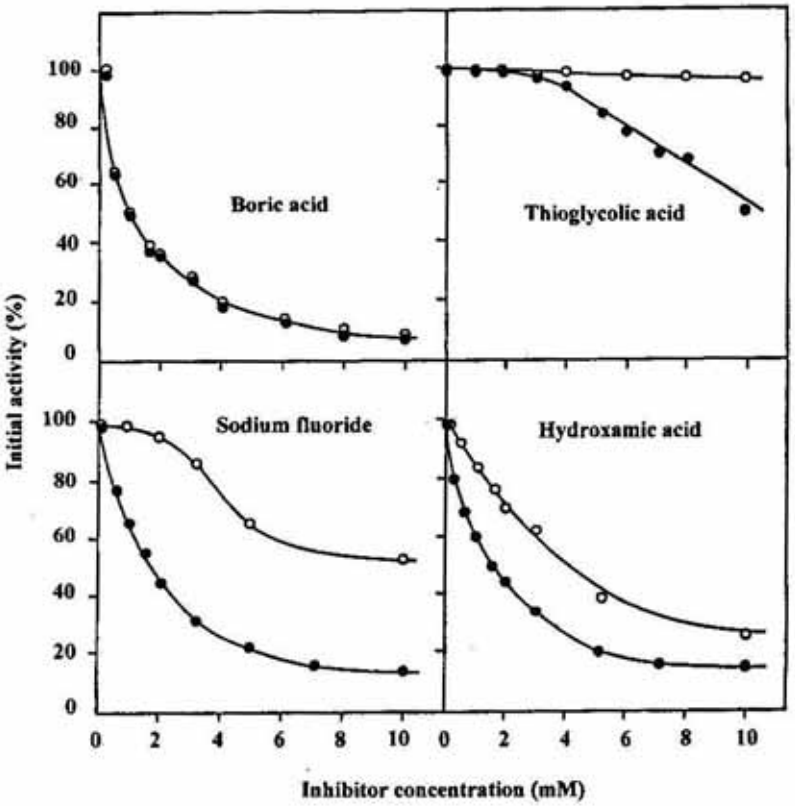

Fig. 2. Loss of activity of urease, free (●) and immobilized on chitosan membrane (○) inhibited by boric acid, thioglycolic acid, sodium fluoride or acetohydroxamic acid.
The measure of activity was the concentration of ammonia after 10 min.

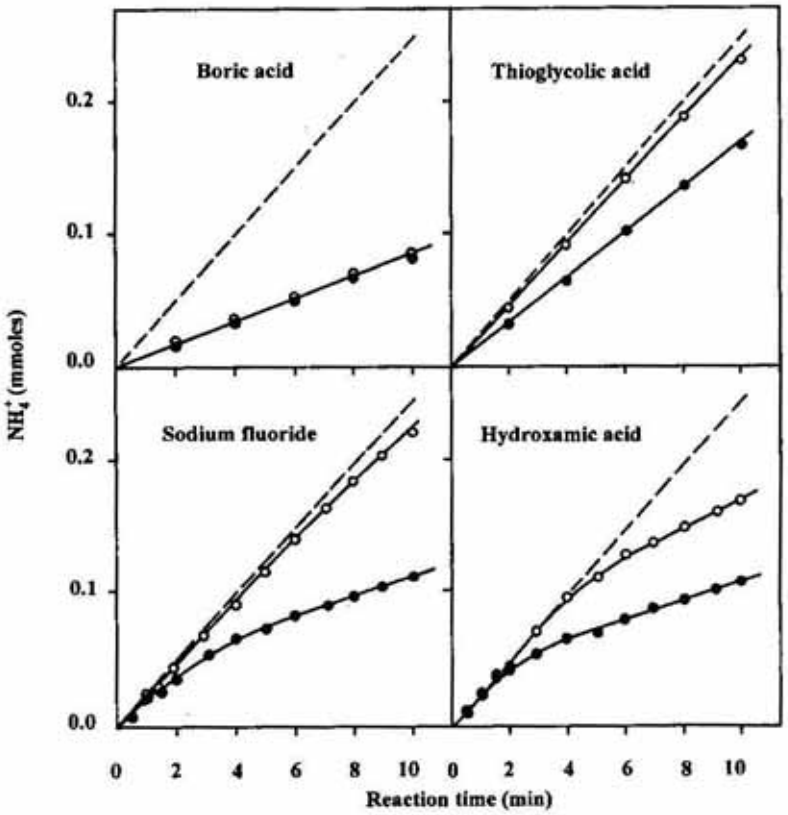

Fig. 3. Progress curves for urease activity in native form (●) and immobilized on chitosan membrane (○) in the presence of boric acid (2 mM), thioglycolic acid (6 mM), sodium fluoride (2 mM) or acetohydroxamic acid (2 mM).
The dotted lines refer to the systems without inhibitor.

for example at 5 mM inhibitor concentration, after 10 min, the immobilized urease maintains 65% and free enzyme only 20% of its initial activity. The hydrolysis of urea by the native enzyme in the presence of fluoride ion proceeds in three steps. The first one, lasting about one minute, can be characterized by the constant velocity, the second step, which occurs between the first and the fifth minute of the reaction, proceeds with decreasing velocity, whereas the

third is stationary and proceeds until the end of the experiment (10 min) at constant velocity (Fig. 3).

The kinetic mechanism of this inhibition was studied previously [16]. It was found that sodium fluoride acted as a competitive slow-binding inhibitor of urease. Spectroscopic studies proved that the fluoride ion binds to nickel ion(s) in the urease molecule [7].

Acetohydroxamic acid is also a strong inhibitor of native urease. The immobilized enzyme undergoes inhibition but it is more inhibitor resistant than native urease (Fig. 2). The reaction rate is high at the beginning, then decreases and reaches a stationary state (Fig. 3).

Acetohydroxamic acid was found to be a competitive slow-binding inhibitor of native urease [15, 17]. According to the model proposed by Blakeley & Zerner [5], hydroxamate anion inhibits the enzyme through bidentate coordination to the nickel ion.

Both simple competitive inhibition of urease (by boric acid and thioglycolic acid) and slow-binding competitive inhibition (by sodium fluoride and acetohydroxamic acid) were studied. The Ni(II) ion(s) in the active site of urease binds the sulfhydryl, the hydroxamate and the fluoride anions as well as the undissociated boric acid.

The native urease is influenced very strongly by boric acid, sodium fluoride and acetohydroxamic acid and less strongly by thioglycolic acid. The resistance of urease to the inhibitors studied, with the exception of boric acid, was considerably improved after enzyme immobilization. This protective effect could have resulted from the zeta potential barrier generated by the chitosan membrane carrier [18]. This potential is not an obstacle for the nonionic substrate and for the weakly dissociated molecules of the product but it repels sulfhydryl, hydroxamate and fluoride anions. The electrical potential of the carrier does not protect the urease against boric acid which is in the nonionic form, and inhibits equally well the native and the immobilized form of urease.

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