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Inhibition of chitosan-immobilized urease by boric acid as determined by integration methods

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Abstract

Jack bean urease was covalently immobilized on glutaraldehyde–pretreated chitosan membranes. Inhibition of the immobilized urease by boric acid was studied. The kinetic integration methods of: Walker and Schmidt, Jennings and Niemann, Booman and Niemann, Yun and Suelter, and Klesov and Berezin were used to determine kinetic constants of the urease-catalyzed urea hydrolysis in phosphate buffer pH 7.0, uninhibited and inhibited. Inhibition of chitosan-immobilized urease by boric acid was found to be competitive similar to that of the free enzyme, with the inhibition constant K_i equal to 0.60 and 0.19 mM, respectively. The effectiveness of the inhibition was evaluated with the $K_M^{(int)}/K_i$ ratio, which was determined to be close in value for both the ureases, 18.3 and 17.9, respectively. This proves that inhibitory potency of boric acid is comparable for both the enzymes. By relating $K_M^{(int)}/K_i$ ratio to the electrostatic potential of chitosan, it was shown that boric acid acts as an inhibitor in its non-ionic form $B(OH)_3$, and not in the $B(OH)_4^-$ form.

Keywords: Immobilized urease; Chitosan membrane; Inhibition; Boric acid; Kinetic integration methods

1. Introduction

The enzyme urease (urea amidohydrolase EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and carbon dioxide, $(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2$. Being widely distributed in a variety of bacteria, fungi and plants, the enzyme plays an important role in the circulation of nitrogen in nature [1–4]. Apart from its natural significance, urease-catalyzed hydrolysis of urea is of practical use in diverse fields [5], e.g. for determination of urea in blood, urine and wastewaters, for removal of urea from blood in the treatment of uraemia [6,7], and for removal of urea from ground- and wastewaters. In all of the above applications free urease can be replaced by its immobilized form, which allows for multiple reuse of the enzyme [8]. Membrane-immobilized urease opened the way for constructing urea sensors [9] and membrane bioreactors [10] applied for urea determinations and urea removal, respectively. In all the above processes the enzyme, free or immobilized, is exposed to inhibition by different substances. Its knowledge, apart from practical importance, provides insight into the urease structure and into urease catalytic mechanism, as well as into

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changes of the above generated by immobilization [11].

The kinetics of inhibition of free urease has been extensively studied [2]. Four major classes of urease inhibitors have been investigated, namely hydroxamic acids [12–14], phosphoroamide compounds [13,15], boric and boronic acids [16,17], and heavy metal ions [18,19]. The first three classes were investigated mainly as potential therapeutic agents against certain bacterial urease-induced human pathogenic states, the fourth class for analytical purposes.

By contrast, studies on kinetics of inhibition of immobilized urease have scarcely been reported.

In this study jack bean urease was immobilized on chitosan membranes. Chitosan, (1 → 4)-2-amino-2-deoxy-β-D-glucan, is a deacetylated product of the alkali treatment of chitin which is obtained from abundant natural resources of crustaceans such as crabs, shrimps, lobsters and krills, whose shells are wastes of seafood processing [20]. Chitosan, as an enzyme immobilization support, offers an attractive set of properties; it is inert, hydrophilic, biocompatible, it shows high affinity to proteins, and the presence of hydroxyl and amino groups facilitates immobilization of enzymes as well as further derivatization of the polymer [21,22]. Due to its solubility in dilute organic acids, chitosan can be processed into different geometrical configurations: membranes, fibers, hollow fibers, capsules and beads [20,21,23].

In a previous report the preparation and properties of urease covalently immobilized on the chitosan membrane were described [24]. The inhibition of urease activity in native and immobilized forms by heavy metal ions [25] and sodium fluoride [26] were evaluated. The urease–chitosan system proved to be stable and thus promising for practical application.

In the present study the kinetics of inhibition of chitosan membrane-immobilized urease by boric acid was investigated. This inhibition is of practical significance e.g. for analysis of urease-based urea determinations in urine, as boric acid is a common urine preservative used prior to analytical tests [17]. Boric acid is known to be a competitive inhibitor for bacterial urease [16]. To estimate the reaction kinetic constants of the studied urease–chitosan system kinetic integration methods were used. The results are compared with those obtained for free urease [27].

2. Kinetic integration methods

Kinetic parameters of an enzyme-catalyzed reaction, Michaelis constant K_M and maximum reaction rate v_{max}, can be obtained either by differential methods based on the differential Michaelis–Menten equation (Eq. (1)) [28] or by integration methods based on the integrated Michaelis–Menten equation (Eqs. (2a) and (2b)). The former require measurements of initial reaction rates at a series of substrate concentrations, whereas the latter require recording of a single total reaction progress curve, product concentration versus time, at a chosen substrate concentration. Compared to differential methods, integration methods are faster, require less enzyme and substrate, which is why they are very useful for fast quality tests in preparing immobilized enzymes.

The Michaelis–Menten equation for an uninhibited enzyme-catalyzed reaction,

$$E + S \rightleftarrows ES \rightarrow E + P,$$

is:

$$-\frac{dS}{dt} = \frac{v_{max} S}{K_M + S} \tag{1}$$

where S is the substrate concentration. Integrating between the limits $S = S_0$ and S_t and $t = 0$ and t gives:

$$t = \frac{S_0 - S_t}{v_{max}} + \frac{K_M}{v_{max}} \ln \frac{S_0}{S_t} \tag{2a}$$

or

$$t = \frac{P_t}{v_{max}} + \frac{K_M}{v_{max}} \ln \frac{S_0}{S_0 - P_t} \quad (2b)$$

where S_0 is the initial substrate concentration, S_t is the concentration of substrate remaining after time t, and P_t is the concentration of product formed in time t.

For a competitively inhibited enzymatic reaction Eq. (2b) becomes:

$$t = \frac{P_t}{v_{max}} + \frac{K_M^I}{v_{max}} \ln \frac{S_0}{S_0 - P_t} \quad (3)$$

where

$$K_M^I = K_M \left(1 + \frac{[I]}{K_i}\right) \quad (3a)$$

is the Michaelis constant for the reaction carried out in the presence of an inhibitor at concentration [I], and K_i is the inhibition constant.

As shown above, the kinetic parameters K_M, v_{max} and K_i can be determined from two successive progress curves monitoring the reactions in the absence and presence of an inhibitor, respectively.

In this study six integration methods were applied. Below are presented equations on which they are based together with the way of obtaining K_M and v_{max}.

1. The method of Walker and Schmidt [29]:

$$\frac{P_t}{t} = v_{max} - \frac{K_M}{t} \ln \frac{S_0}{S_0 - P_t} \quad (4)$$

A plot of P_t/t versus $(1/t)\ln[S_0/(S_0 - P_t)]$ is linear with a slope $-K_M$ and intercept v_{max}.

2. The method of Jennings and Niemann (I) [30]:

$$\frac{t}{P_t} = \frac{1}{v_{max}} + \frac{K_M}{v_{max}} \frac{1}{P_t} \ln \frac{S_0}{S_0 - P_t} \quad (5)$$

A plot of t/P_t versus $(1/P_t)\ln[S_0/(S_0 - P_t)]$ is linear with a slope K_M/v_{max} and intercept $1/v_{max}$.

3. The method of Jennings and Niemann (II) [30]:

$$\frac{t}{\frac{\ln S_0}{(S_0 - P_t)}} = \frac{K_M}{v_{max}} + \frac{1}{v_{max}} \frac{P_t}{\frac{\ln S_0}{(S_0 - P_t)}} \quad (6)$$

A plot of $t/\ln[S_0/(S_0 - P_t)]$ versus $P_t/\ln[S_0/(S_0 - P_t)]$ is linear with a slope $1/v_{max}$ and intercept K_M/v_{max}.

4. The method of Booman and Niemann [31]:

$$\frac{1}{S_0 - S_t} \int_0^t S \, dt = \frac{2K_M + S_0}{2v_{max}} + \frac{S_t}{2v_{max}} \quad (7a)$$

or

$$\frac{S_0 t - \int_0^t P \, dt}{P_t} = \frac{2K_M + S_0}{2v_{max}} + \frac{S_0 - P_t}{2v_{max}} \quad (7b)$$

A plot of $(S_0 t - \int_0^t P \, dt)/P_t$ versus $S_0 - P_t$ is linear with a slope $1/2v_{max}$ and intercept $(2K_M + S_0)/2v_{max}$.

5. The method of Yun and Suelter [32]:

$$\frac{t_j - t_i}{P_j - P_i} = \frac{1}{v_{max}} + \frac{K_M}{v_{max}} \frac{1}{P_j - P_i} \ln \frac{S_0 - P_i}{S_0 - P_j} \quad (8)$$

where P_j and P_i are product concentrations corresponding to time t_j and t_i of the reaction, respectively. A plot of $(t_j - t_i)/(P_j - P_i)$ versus $(1/(P_j - P_i))\ln((S_0 - P_i)/(S_0 - P_j))$ is linear with a slope K_M/v_{max} and intercept $1/v_{max}$.

6. The method of Klesov and Berezin [33]:

$$\frac{P_j - P_i}{t_j - t_i} = v_{max} - \frac{K_M}{t_j - t_i} \ln \frac{S_0 - P_i}{S_0 - P_j} \quad (9)$$

where P_j and P_i denote the same as above.

A plot of $(P_j - P_i)/(t_j - t_i)$ versus $(1/(t_j - t_i))\ln((S_0 - P_i)/(S_0 - P_j))$ is linear with a slope $-K_M$ and intercept v_{max}.

K_M, K_M^I and v_{max} values obtained with use of the above presented methods should be understood in this study as the kinetic constants determined by integration methods, $K_M^{(int)}$, $K_M^{I(int)}$ and $v_{max}^{(int)}$ (this notation will be used throughout, when necessary). Thus obtained values may differ from those obtained by differential methods, because either group of methods makes use of a different period of the reaction: the former monitor the whole duration of the reaction, the latter monitor only the initial short phase of the reaction. As the aim of this study was to investigate the inhibition of the enzyme by an external inhibitor, it was assumed that the intrinsic conditions of the reaction mixture generated by the development of the enzymatic reaction over its whole duration, constituted the same background for the uninhibited and inhibited reaction and had no effect on the studied inhibition, i.e. that K_M and K_M^I, if modified, were modified in the same manner without affecting K_i (see Eq. (3a)).

3. Experimental

3.1. Materials

Chitosan (chitin of Antarctic krill shells) was obtained from the Sea Fisheries Institute in Gdynia, Poland. A fraction of grade 0.43–0.75 mm was used whose weight-average molecular weight was 3.3×10^5 and deacetylation degree was of the order of 70% [23]. Jack bean urease (type III) with specific activity of 33 units/mg protein was obtained from Sigma. One unit of activity is defined as the amount of enzyme that liberates 1.0 μmol NH_3 from urea per minute at pH 7 and 25°C. Glutaraldehyde was from BDH, Poole, England. Urea, boric acid and all other chemicals (analar grade) were from POCh, Gliwice, Poland. Phosphate buffer pH 7.0 of concentration 100 mM containing 2 mM EDTA was prepared from orthophosphoric acid solution titrated potentiometrically with NaOH solution to pH 7.0.

Chitosan membranes with immobilized urease were prepared as described previously [24]. In brief, membranes supported with glass fabric were cast from chitosan solution (1% in 0.8% acetic acid), dried and neutralized with NaOH solution. Water-conditioned membranes were then treated with 0.01% glutaraldehyde solution in water for 1.5 h at room temperature. Urease was immobilized on both surfaces of the membranes by immersing the glutaraldehyde-treated membranes in 0.05% solution of urease in phosphate buffer pH 5.3 for 1 h at room temperature and overnight at 4°C. The wet state thickness of the obtained membranes, including the glass fabric 'backbone', was 0.009–0.01 cm, and the amount of active enzyme immobilized was 0.049 mg/cm^2 membrane.

3.2. Methods

The reaction progress curves, ammonia concentration versus time, for hydrolysis of urea catalyzed by chitosan membrane-immobilized urease in the absence and presence of boric acid were recorded at 25°C in the following way: the membranes (area of two surfaces 2×63.6 cm^2) were dropped into 100 cm^3 of 10 mM urea solution in the phosphate buffer pH 7.0, and into 100 cm^3 of 10 mM urea solution in the phosphate buffer pH 7.0 containing 0.5 mM boric acid, respectively. 0.1 cm^3 samples were removed from the reaction mixtures at 1 and 2 min intervals, respectively, for NH_3 determination by the phenol–hypochlorite method [34]. The reaction mixtures were stirred throughout the period of measurements, and the temperature was controlled to an accuracy of ± 0.5°C. 0.5 mM boric acid was chosen for the experiment according to the suggestion that the inhibitor concentration should be numerically close to the predicted value of K_i [35]. The parameters used in computations based on Eqs. (4)–(6), (7b), (8) and (9) were as follows: $S_0 =$ 10 mM urea, $P_t = [NH_3]/2$, mM, product concentration formed in time t, [I] = 0.5 mM boric acid.

4. Results and discussion

The reaction progress curves, ammonia concentration versus time, recorded for the hydrolysis of urea catalyzed by chitosan membrane-immobilized urease, uninhibited and inhibited by 0.5 mM boric acid are presented in Fig. 1. For further calculations, the experimental progress curves were approximated by polynomials of the third degree.

The linear replots of the polynomial progress curves of Fig. 1 obtained by the integration methods (Eqs. (4)–(6), (7b), (8) and (9) are presented in Fig. 2. The inserts show the sections of the progress curves from which the linear replots were obtained. The points outside these sections, i.e. in the initial and final stages of the reactions deviated from straight lines and were therefore excluded from calculations. For the methods of Yun and Suelter, and Klesov and Berezin the following $(t_j - t_i)$ were chosen: 10 and 15 min for the uninhibited reaction, and 16 and 20 min for the inhibited reaction, respectively.

The values of the kinetic constants obtained by the applied kinetic integration methods for the uninhibited reaction, $K_M^{I(int)}$ and $v_{max}^{(int)}$, and for the inhibited reaction, $K_M^{I(int)}$ and $v_{max}^{(int)}$, are presented with their standard deviations in Table 1. The standard deviations were calculated from the variances and covariances of coefficients of linear regressions applied by the studied methods to the experimental points of the progress curves. The values of the constants determined by the applied methods are consistent. $v_{max}^{(int)}$ was found to have the same value for both the reactions, which provides evidence that the inhibition of chitosan immobilized–urease by boric acid is of competitive type. Accordingly, in Fig. 2 the pairs of replots representing the two studied reactions have either the same intercept (Fig. 2a, b, e, f) or the same slope (Fig. 2c, d). Inhibition constants K_i calculated according to Eq. (3a) are included in Table 1. The standard deviations of K_i were calculated from standard deviations of the corresponding

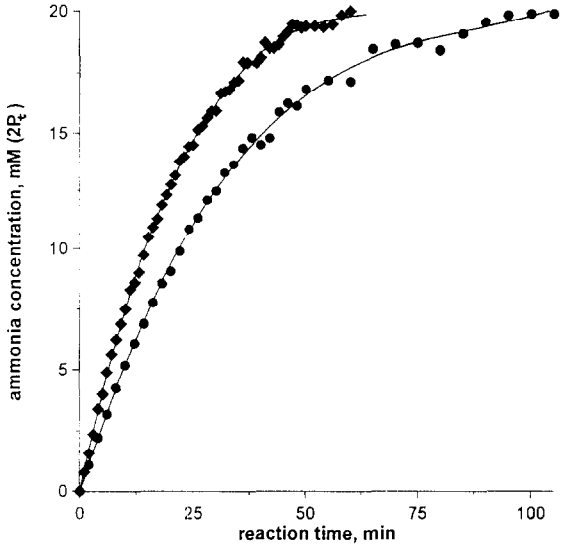

Fig. 1. Reaction progress curves, ammonia concentration versus time, recorded for hydrolysis of urea catalyzed by chitosan membrane-immobilized urease, uninhibited (♦) and inhibited by 0.5 mM boric acid (●), approximated with polynomials of the third degree. Experimental conditions: membrane of surface area 2 × 63.6 cm^2, 100 cm^3 of urea solution in phosphate buffer pH 7.0 (100 mM, 2 mM EDTA) of initial concentration $S_0 = 10$ mM, 25°C. $P_t = [NH_3]/2$ is the concentration of product.

$K_M^{(int)}$ and $K_M^{I(int)}$ values. Table 2 presents the weighted mean values of the kinetic constants of the immobilized and free urease with their standard deviations. The values of free urease were determined by the same methods in the same medium with 0.25 mM boric acid [27]. For the sake of comparison $v_{max}^{(int)}$ of the immobilized urease was recalculated to refer to 1 mg protein.

As commonly observed, the immobilization changed the kinetic properties of urease. It is generally assumed that responsible for the changes following enzyme immobilization are [8,11,36]: (1) conformational and steric effects which result from structural changes introduced to the enzyme by its binding to a support, (2) mass-transfer resistances imposed on diffusion of substrates and products of the reaction to and from the bound enzyme by a stagnant solution layer surrounding the enzyme-support system, and (3) modification of the local microenvironment of the bound enzyme by the physico-

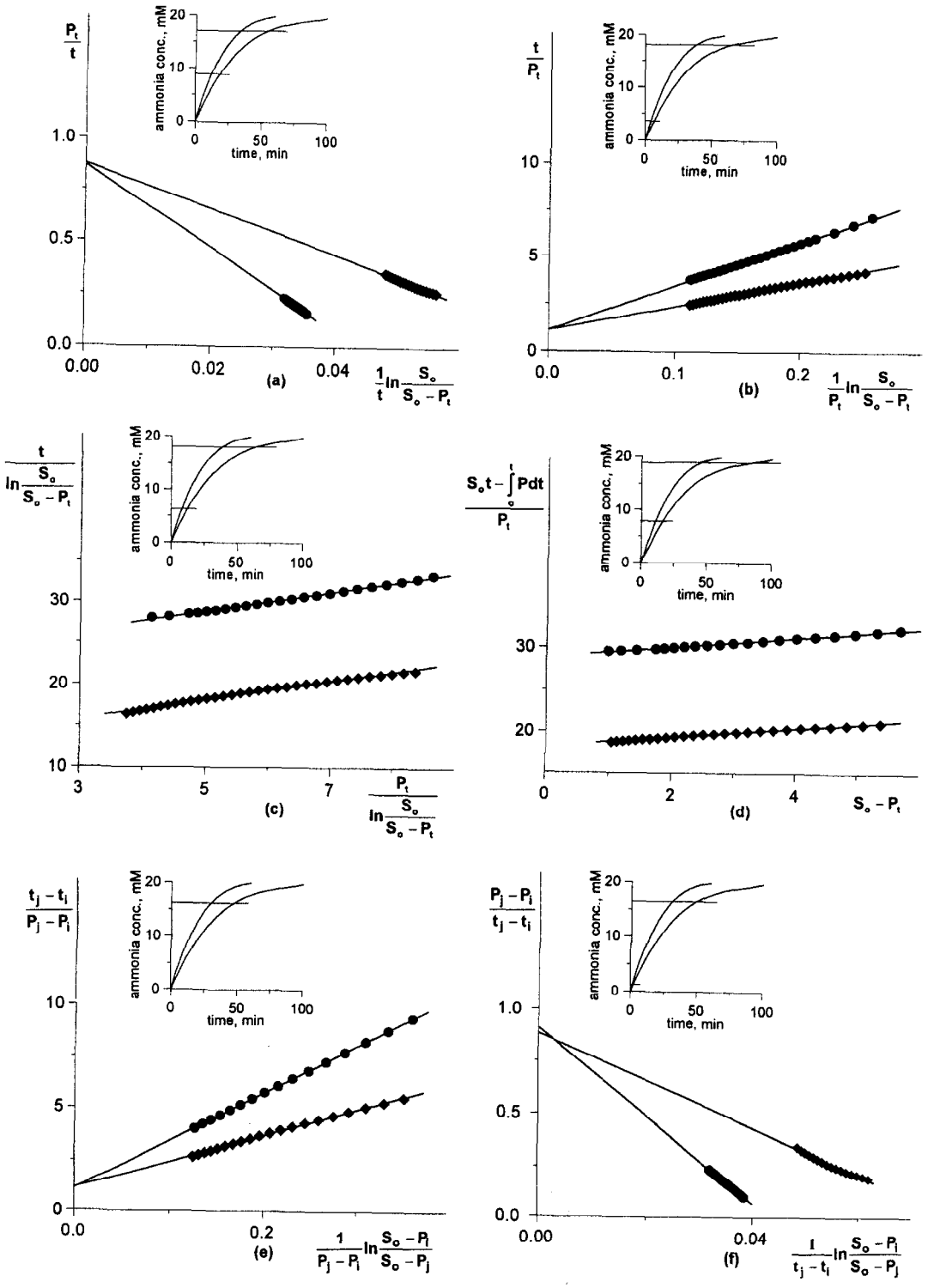

Fig. 2. Replots of the progress curves of hydrolysis of urea catalyzed by chitosan membrane-immobilized urease, uninhibited (♦) and inhibited by 0.5 mM boric acid (●), obtained by the integration methods of: (a) Walker and Schmidt, (b) Jennings and Niemann (I), (c) Jennings and Niemann (II), (d) Booman and Niemann, (e) Yun and Suelter, (f) Klesov and Berezin. S_0 and $P_t = [NH_3]/2$ denote initial concentration of urea and concentration of product formed in time t, respectively.

Table 1
Kinetic constants of urea hydrolysis catalyzed by chitosan membrane–immobilized urease uninhibited and inhibited by boric acid, obtained by integration methods

Method	Uninhibited reaction		Inhibited reaction		K_i (mM)
	$K_M^{(int)}$ (mM)	$v_{max}^{(int)}$ (mM NH_3/min)	$K_M^{I(int)}$ (mM)	$v_{max}^{(int)}$ (mM NH_3/min)	
Walker–Schmidt	11.0 ± 0.7	1.74 ± 0.07	20.0 ± 1.5	1.74 ± 0.10	0.61 ± 0.13
Jennings–Niemann (I)	11.0 ± 0.9	1.76 ± 0.09	20.4 ± 3.9	1.76 ± 0.26	0.58 ± 0.26
Jennings–Niemann (II)	10.8 ± 0.6	1.74 ± 0.06	19.4 ± 1.5	1.70 ± 0.10	0.63 ± 0.13
Booman–Niemann	11.1 ± 0.7	1.76 ± 0.07	19.6 ± 1.0	1.70 ± 0.06	0.65 ± 0.12
Yun–Suelter	11.0 ± 0.7	1.74 ± 0.09	20.5 ± 1.6	1.78 ± 0.13	0.58 ± 0.15
Klesov–Berezin	11.2 ± 0.6	1.76 ± 0.07	21.0 ± 1.5	1.82 ± 0.10	0.56 ± 0.11

chemical nature of the support resulting in altered distribution of substrates, products, hydrogen and hydroxyl ions in the vicinity of the immobilized enzyme as compared to the bulk solution. The determined properties of the immobilized enzyme reflect the resultant of all the above effects, which is why it is difficult to distinguish between them. However, the first two effects are said to be mainly responsible for increased K_M and decreased v_{max} values, whereas the last one for environment-related behaviors of the immobilized enzyme.

In agreement with the above general rule, it was found that chitosan immobilized–urease shows a higher value of $K_M^{(int)}$ and a lower value of $v_{max}^{(int)}$ as compared to the free enzyme (see Table 2).

Microenvironmental effects generated by chitosan help to account for the behavior of the immobilized urease towards inhibition. Chitosan, due to the presence of NH_2 groups in its molecules, is positively charged, as a result of which in its immediate vicinity hydrogen ion concentration is lower than that measured in the bulk solution. The manifestation of this property was noted previously [24] in the form of the optimum pH shift of chitosan immobilized–urease by about 0.25 pH unit toward acidic pH as compared to free urease (optimum pH 7.0). The optimum pH shift is expressed by [11]:

$$\Delta pH = pH_{local} - pH_{bulk} = \frac{0.434\varepsilon\psi}{kT} \qquad (10)$$

where ε is the electronic charge, ψ is the electrostatic potential of the support ($\psi > 0$ for a positively charged support, $\psi < 0$ for a negatively charged support), k the Boltzman constant and T the absolute temperature. From Eq. (10) with the observed ΔpH, the electrostatic potential of the studied chitosan matrix was estimated to be 0.014 V.

The inhibition of chitosan-immobilized urease by boric acid was found to be competitive similar to that of the free enzyme. The effectiveness of competitive inhibitors is commonly measured by the ratio K_M/K_i, which is related

Table 2
Mean values of kinetic constants of urea hydrolysis catalyzed by free [27] and chitosan membrane–immobilized urease, uninhibited and inhibited by boric acid, obtained by integration methods

Enzyme	Uninhibited reaction		Inhibited reaction		K_i (mM)	$K_M^{(int)}/K_i$
	$K_M^{(int)}$ (mM)	$v_{max}^{(int)}$ (μmol NH_3/min · mg protein)	$K_M^{(int)}$ (mM)	$v_{max}^{(int)}$ (μmol NH_3/min · mg protein)		
Free urease	3.41 ± 0.08	33.7 ± 0.3	7.90 ± 0.16	33.3 ± 0.4	0.19 ± 0.01	17.9 ± 1.0
Chitosan membrane–immobilized urease	11.0 ± 0.3	28.1 ± 0.5	20.0 ± 0.6	27.8 ± 0.6	0.60 ± 0.06	18.3 ± 1.9

to the electrostatic potential of the enzyme support by the following expression [11,36]:

$$\frac{(K_M/K_i)_{imm}}{(K_M/K_i)_{free}} = e^{((z_s - z_i)\varepsilon\psi)/(kT)} \quad (11)$$

where $z_s\varepsilon$ and $z_i\varepsilon$ are charges of the substrate and inhibitor, respectively, and other symbols denote the same as above.

The values of $K_M^{(int)}/K_i$ ratio for both the ureases are presented in Table 2. Their ratio (the left-hand side of Eq. (11)) is equal to 1.02 ± 0.12. This proves: (1) that the effectiveness of inhibitory action of boric acid on both the ureases is comparable, and (2) that boric acid acts as an inhibitor in its non-ionic form $B(OH)_3$, as having $z_s = 0$ (urea), only with $z_i = 0$ the right-hand side of Eq. (11) is equal to one. The latter conclusion is in agreement with that of Breitenbach and Hausinger [16] that only trigonal $B(OH)_3$ is an inhibitor of bacterial urease, and not the $B(OH)_4^-$ anion.

It can be further concluded from Eq. (11) that for urease immobilized on chitosan the constant $K_M^{(int)}$ and the constant K_i of boric acid do not depend on the electrostatic potential of the support, i.e. that with sufficient stirring of the reaction mixture minimizing mass transfer resistances, the values of $K_M^{(int)}$ and K_i result only from structural changes introduced to the enzyme by the applied immobilization procedure.

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