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# Inhibition of chitosan-immobilized urease by slow-binding inhibitors: $Ni^{2+}$, $F^-$ and acetohydroxamic acid

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**Abstract**

The inhibitions by $Ni^{2+}$ and $F^-$ ions and by acetohydroxamic acid of jack bean urease covalently immobilized on chitosan membrane was studied (pH 7.0, 25°C) and compared with those of the native enzyme. The reaction progress curves of the immobilized urease-catalyzed hydrolysis of urea were recorded in the absence and presence of the inhibitors. They revealed that the inhibitions are of the competitive slow-binding type similar to those of native urease. The immobilization weakened the inhibitory effect of the inhibitors on urease as measured by the inhibition constants $K_i^*$. The increase in their values: 17.9-fold for $Ni^{2+}$, 26.5-fold for $F^-$ and 1.7-fold for acetohydroxamic acid, was accounted for by environmental effects generated by heterogeneity of the urease–chitosan system: (1) mass transfer limitations imposed on substrate and reaction product in the external solution, and (2) the increase in local pH on the membrane produced by both the enzymatic reaction and the electric charge of the support. By relating the $K_M/K_i^*$ ratio to the electrostatic potential of chitosan it was found that while the reduced $Ni^{2+}$ inhibition is mainly brought about by the potential, inhibition by acetohydroxamic acid is independent of the potential, and the acid inhibits urease in its non-ionic form. The reduction in $F^-$ inhibition was ascribed to the increased pH in the local environment of the immobilized enzyme. 

*Keywords:* Urease; Chitosan membrane; Immobilization; Inhibition; $Ni^{2+}$ ion; $F^-$ ion; Acetohydroxamic acid

## 1. Introduction

The enzyme urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide: $(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2$ [1–5]. The enzyme is widely distributed in a variety of bacteria, fungi and plants, thus playing an important role in the circulation of nitrogen in nature. The enzyme is also known for its applications [6] in removal of urea from blood or dialysate in the treatment of uraemia, and in analytical determinations of urea. In the above applications free urease can be replaced by its immobilized form, which allows for multiple reuse of the enzyme. In nature and in its applications urease is exposed to inhibitions [2], which depending on circumstances, are advantageous or disadvantageous.

The inhibitions of urease have been extensively studied because of their potential use among others as: (1) therapies for bacterial urease-induced human pathogenic states, such as *Helicobacter pylori*-induced peptic ulcer, urinary stone formation, pyelonephritis and hepatic coma [2,3,5,7,8], (2) control of urea-hydrolysis in soils after use of urea fertilizers in an attempt to protect soils from pH elevation and loss of nitrogen [2,9,10], and (3) as an analytical technique for determination of substances acting as enzyme

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Table 1
Selected urease inhibitors

| Inhibitor | Type of inhibition | Urease (buffer pH) | $K_i$ (mM) | References |
|---|---|---|---|---|
| Boric acid | Competitive | J. bean[a] (buffer-free system pH 7.0) | 0.12 | [16] |
| | | Bact.[b] (25 mM HEPES pH 7.5) | 0.1 | [17] |
| 2-Mercaptoethanol | Competitive | J. bean (HEPES pH 7.1) | 0.72 | [18] |
| | | Bact. (100 mM HEPES pH 7.75) | 0.55 | [19] |
| Phosphate buffer | Competitive at pH <7.6 | J. bean (phosphate pH 7.0) | 19 | [20] |
| | Competitive at pH <7.3 | Bact. (100 mM HEPES pH 7.0) | 40 | [19] |
| Phenylphosphorodiamidate | Competitive slow-binding | Bact. (100 mM HEPES pH 7.75) | $9.4 \times 10^{-8}$ 0.016 | [19] |
| Acetohydroxamic acid | Competitive slow-binding | J. bean (22 mM phosphate pH 7.0) | 0.016 | [c] |
| | | Bact. (100 mM HEPES pH 7.75) | 0.0026 | [19] |
| NaF | Competitive slow-binding | J. bean (22 mM phosphate pH 7.0) | 0.076 | [21] |
| | | Bact. (100 mM HEPES pH 7.0) | 0.17 | [22] |
| $Ni^{2+}$ | Competitive slow-binding | J. bean (20 mM HEPES pH 7.0) | 0.0028 | [23] |

[a] Jack bean urease.
[b] Bacterial urease.
[c] This work.

inhibitors [11]. The latter is of special interest, as it offers fast and simple determinations, and allows very low concentrations to be determined without costly and complex instrumentation and highly trained personnel. From the point of view of economy and ease of handling, procedures based on inhibition of free enzymes can be dramatically improved by using immobilized enzymes, e.g. in bioreactors or biosensors. Numerous biosensors have been produced by integrating immobilized enzymes with different kinds of transducers [11–15]: potentiometric, amperometric, conductometric, thermometric etc. They have found application in various analytical fields including environmental monitoring and screening, bioprocess and food control, and biomedical and pharmaceutical analysis.

The main classes of urease inhibitors are: boron-containing compounds, thiol compounds, phosphate, phosphoroamide compounds, hydroxamic acids, $F^-$ ions, and heavy metal ions. The types of inhibition and the inhibition constants of the representative inhibitors of the above classes are listed in Table 1.

In this study urease was immobilized on gel chitosan membrane. Chitosan (1 → 4)-2-amino-2-deoxy-β-D-glucan, a deacetylated derivative of chitin [24], is a polycationic gel-forming substance, suitable for enzyme/cell immobilization [25]. The preparation and properties of urease covalently immobilized on chitosan membrane [26], as well as its inhibitions by sodium fluoride [21], boric acid [27], and phoshate buffer [28] were described in our previous work. In this work, the inhibition of chitosan membrane-immobilized urease by slow-binding inhibitors, $Ni^{2+}$ and $F^-$ ions and by acetohydroxamic acid were compared to those of native urease determined under the same conditions [21,23], in an attempt to evaluate the changes in the enzyme kinetics brought about by the immobilization.

## 2. Slow-binding inhibition

An enzymatic reaction in the presence of a competitive slow-binding inhibitor proceeds along Scheme 1 [29]:

$$E + S \underset{k_2}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_7} E$$

$$E + I \underset{k_4}{\overset{k_3}{\leftrightarrow}} EI \underset{k_6}{\overset{k_5}{\leftrightarrow}} EI^*$$

slow step

Scheme 1.

In such a reaction, a complex EI is formed together with ES. The EI complex is unstable and undergoes a slow isomerization into a more stable EI* complex. The stability of each of the complexes is characterized by its inhibition constant $K_i$ and $K_i^*$, respectively. The course of the reaction depends on the order of mixing of components of the reaction mixture. If the reaction is initiated by the addition of the enzyme to the substrate-inhibitor mixture (unincubated system),

initially the reaction is weakly inhibited, and its rate $v_o$ is still high as compared to that of the uninhibited reaction. As the reaction proceeds the inhibition grows stronger, and after a certain time the reaction rate falls to a steady-state value $v_s$, much lower than $v_o$. If the inhibitor concentration is much higher than that of the enzyme, the equation describing the slow-binding inhibited reaction progress curve in the unincubated system is

$$P(t) = v_s t + (v_o - v_s)(1 - e^{-k_{app}t})\frac{1}{k_{app}} \quad (1)$$

where $P$ is the concentration of the reaction product, $v_o$ and $v_s$ are the reaction initial and steady-state rates, respectively, $t$ stands for time, and $k_{app}$ denotes the apparent first-order rate constant. The rates $v_o$ and $v_s$ are given by the Michaelis–Menten equation describing competitive inhibition

$$v_o = \frac{v_{max} S}{K_M(1 + I/K_i) + S} \quad (2)$$

$$v_s = \frac{v_{max} S}{K_M(1 + (I/K_i^*) + S} \quad (3)$$

where $S$ and $I$ are substrate and inhibitor concentrations, respectively, $K_M$ is the Michaelis constant and $v_{max}$ the maximum reaction rate of the uninhibited reaction, and $K_i$ and $K_i^*$ are the inhibition constants corresponding to the two steps of the reaction. The reciprocals of $v_o$ and $v_s$ expressed by

$$\frac{1}{v_o} = \frac{K_M}{v_{max} S K_i} I + \frac{1}{v_{max}}\left(1 + \frac{K_M}{S}\right) \quad (4)$$

$$\frac{1}{v_s} = \frac{K_M}{v_{max} S K_i^*} I + \frac{1}{v_{max}}\left(1 + \frac{K_M}{S}\right) \quad (5)$$

help to determine the values of the inhibition constants from the measured $v_o$ and $v_s$ as a function of $I$.

If the enzyme is incubated with the inhibitor prior to the reaction, and the reaction is initiated by adding the substrate (incubated system), reaction progress curves are observed different from those in the unincubated system. If the final concentrations of the enzyme, of the inhibitor and of the substrate are the same as in the unincubated system, the two steady-state rates in both the systems are identical provided that there is no significant enzyme inactivation, substrate depletion, nor other enzyme-product secondary reactions.

## 3. Experimental

### 3.1. Materials

The jack bean urease (type III) of specific activity 33 units/mg protein, HEPES buffer (ultra), urea (molecular biology reagent) and acetohydroxamic acid (analar grade) were from Sigma. The salts $NiCl_2 \cdot 6H_2O$, NaF, sodium phosphates: $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 12H_2O$ and EDTA were from POCh, Gliwice, Poland. HEPES buffer pH 7.0 of concentration 20 mM was prepared by neutralizing potentiometrically the dissolved buffer with NaOH solution. Phosphate buffer pH 7.0 of concentration 22 mM was prepared by mixing sodium phosphates. Chitosan was obtained from the Sea Fisheries Institute in Gdynia, Poland, where it is produced by deacetylation of chitin of Antarctic krill shells. A fraction of grade 0.43–0.75 mm was used whose weight-average molecular weight was $3.3 \times 10^5$ and deacetylation degree was of the order of 70% [30]. Glutaraldehyde was from BDH.

The preparation of chitosan membranes and immobilization of urease were performed as described previously [26]. In brief, membranes supported with glass fabric were cast from 1% solution of chitosan in 0.8% acetic acid, dried and neutralized with NaOH solution. Water-swollen membranes pre-treated with 0.01% glutaraldehyde solution in water for 1.5 h at room temperature were immersed in 0.05% solution of urease in phosphate buffer pH 5.3 for 1 h at room temperature and overnight at 4°C. The membranes thus obtained had the wet state thickness of 0.009–0.01 cm including the glass fabric, and the amount of active enzyme immobilized on both surfaces of the membrane was 0.049 mg/cm$^2$.

### 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 the inhibitors were recorded at 25°C in buffers pH 7.0: $Ni^{2+}$ in 20 mM HEPES, and $F^-$ and acetohydroxamic acid in 22 mM phosphate buffer containing 1 mM EDTA. Two experimental systems were used: in the first (unincubated), the reaction was initiated by dropping the membranes into urea-inhibitor

mixtures, and in the second (incubated), the membranes were incubated with the inhibitor for 20 min prior to the reaction, and the reaction was initiated by adding a small volume of concentrated urea solution. In both the systems, the reaction conditions were the same, i.e. the membrane samples had the surface area 50.2 $cm^2$ (both surfaces), the total volume of the reaction mixture was 100 $cm^3$, the urea concentration was 50 mM, and the concentrations of the inhibitors were chosen according to their inhibitory strength from the ranges: 0.125–5 mM for $Ni^{2+}$, 5–20 mM for $F^-$, and 1–10 mM for acetohydroxamic acid. The reaction mixtures were stirred throughout the period of measurements. The reaction progress curves were recorded by measuring ammonia concentration by the phenol-hypochlorite method [31] in samples removed from the reaction mixtures at time intervals. The BURSTO computer program for fitting product concentration-time experimental data to Eq. (1) was kindly offered by W.W. Cleland [32].

The inhibition of chitosan-immobilized urease by $Ni^{2+}$ ions was investigated in this work in both the unincubated and incubated system. This inhibition is compared with that by $F^-$ and acetohydroxamic acid studied in the unincubated system. The data for $F^-$ inhibition are our previous results [21] extended to longer reaction times, and recomputed for the sake of comparison with the other two inhibitors, with use of the BURSTO program.

## 4. Results and discussion

### 4.1. Uninhibited reaction

The kinetic constants of native and of chitosan membrane-immobilized urease, the Michaelis constant $K_M$ and the maximum reaction rate $v_{max}$ in the absence of the inhibitors, determined in the applied buffers are presented in Table 2.

### 4.2. Inhibited reaction

The reaction progress curves recorded for $Ni^{2+}$, $F^-$ and acetohydroxamic acid inhibitions in the unincubated system are presented in Figs. 1a, 2 and 3, respectively. The solid curves in these figures were computed by non-linear fitting of the experimental data to Eq. (1) with use of the BURSTO program. The shape of the curves in all three cases corresponds to the competitive slow-binding type of inhibition, represented by Scheme 1, i.e. in the initial period of the reaction, the reaction is weakly inhibited, characterized by high reaction rates $v_o$, and in the later period, the inhibition grows stronger, characterized by lower reaction rates $v_s$. The reciprocals of the calculated $v_o$ and $v_s$ values are linear function of inhibitor concentration according to Eqs. (4) and (5) (see inserts to Figs. 1a, 2 and 3). This proves the competitive type of the inhibition in both the initial and steady-state stages of the inhibited reaction. The values of the inhibition constants corresponding to both the initial and steady-state stages of the reaction, $K_i$ and $K_i^*$, obtained from these plots with use of the $K_M$ and $v_{max}$ values in the uninhibited reaction (Table 2) are compiled in Table 3, along with the inhibition constants of native urease determined under the same experimental conditions [21,23].

The reaction progress curves for the inhibition of the immobilized urease by $Ni^{2+}$ ions carried out in the incubated system are presented in Fig. 1b. The reaction progress curves are linear. They exhibit the same reaction rates as the steady-state rates in the unincubated system for the same $Ni^{2+}$ concentrations (see for instance the curves for 1 mM $Ni^{2+}$ in Fig. 1a and b). Therefore, the plot of $1/v_s$ versus $I$ in this system (the insert to Fig. 1b) is a straight line identical with that of $1/v_s$ versus $I$ in the unincubated system (the insert to Fig. 1a). This provides further evidence for the competitive slow-binding inhibition of the chitosan-immobilized urease by $Ni^{2+}$ ions obeying the mechanism presented in Scheme 1.

Table 2
Kinetic constants of native and chitosan membrane-immobilized urease (pH 7.0)

| Buffer | Native urease | | Chitosan-immobilized urease | |
|---|---|---|---|---|
| | $K_M$ (mM) | $v_{max}$ (μmol $NH_3$/min mg protein) | $K_M$ (mM) | $v_{max}$ (μmol $NH_3$/min $cm^2$ membrane) |
| 20 mM HEPES | 3.5 | 72.0 | 10.0 | 1.5 |
| 22 mM phosphate | 7.1 [20] | 47 [20] | 12.0 [28] | 1.5 [28] |

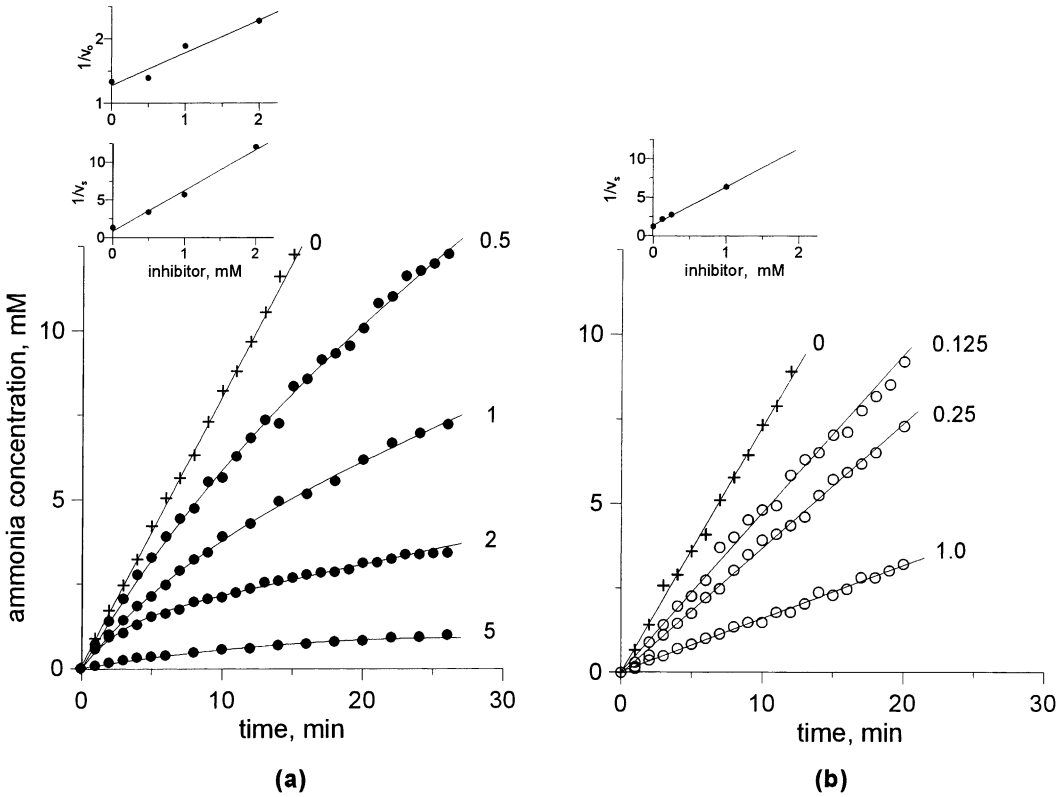


Fig. 1. Progress curves of urea hydrolysis catalyzed by urease immobilized on chitosan membrane carried out in the presence of $Ni^2$ ions: (a) in the unincubated system, (b) in the incubated system. Numbers denote $Ni^{2+}$ concentration (mM). The inserts are reciprocals of reaction rates (initial $v_o$ and steady-state $v_s$ expressed in mM $NH_3$/min) plotted as a function of $Ni^{2+}$ concentration.

As shown in Table 3 the inhibitory strength of the studied inhibitors towards native urease, as judged by the $K_i^*$ values, forms the series: $Ni^{2+}$ > acetohydroxamic acid ≈ $F^-$. The immobilization on chitosan resulted in that: (1) the $K_i^*$ constants of the immobilized urease are higher than those of the native enzyme, the highest increase being for $F^-$ (26.5-fold), then for $Ni^{2+}$ (17.9-fold), and for acetohydroxamic acid (1.7-fold), and (2) that the inhibitory strength series changed into: acetohydroxamic acid > $Ni^{2+}$ > $F^-$.

The changes in kinetic behavior of immobilized enzymes as compared to their native counterparts, such as the one observed here, are commonly accounted for

Table 3
Inhibition constants of $Ni^{2+}$ ions, acetohydroxamic acid and of $F^-$ ions towards native and chitosan membrane-immobilized urease

| | $Ni^{2+}$ | | Acetohydroxamic acid | | $F^-$ | |
|---|---|---|---|---|---|---|
| | Native [23] | Immobilized | Native | Immobilized | Native | Immobilized |
| $K_i$ (mM) | 0.042 | 0.53 | 1.5 | 3.6 | 1.0 | 12.2 |
| $K_i^*$ (mM) | 0.0028 | 0.05 | 0.016 | 0.028 | 0.02 | 0.53 |
| $K^*_{i(\mathrm{imm})}/K^*_{i(\mathrm{nat})}$ | 17.9 | | 1.7 | | 26.5 | |
| $K_M/K_i^*$ | 1286 | 200 | 444 | 429 | 355 | 23 |
| $(K_M/K_i^*)_{\mathrm{imm}}/(K_M/K_i^*)_{\mathrm{nat}}$ | 0.16 | | 0.97 | | 0.065 | |

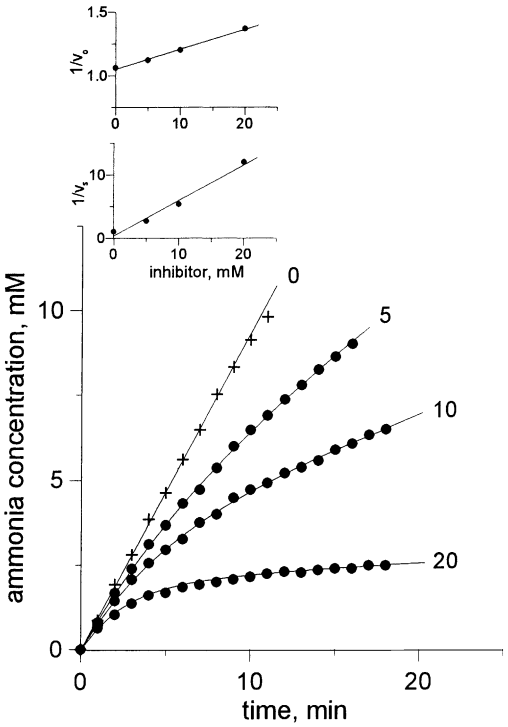


Fig. 2. Progress curves of urea hydrolysis catalyzed by urease immobilized on chitosan membrane carried out in the presence of $F^-$ ions in the unincubated system. Numbers denote $F^-$ concentration (mM). The inserts are reciprocals of reaction rates (initial $v_o$ and steady-state $v_s$ expressed in mM $NH_3$/min) plotted as a function of $F^-$ concentration.

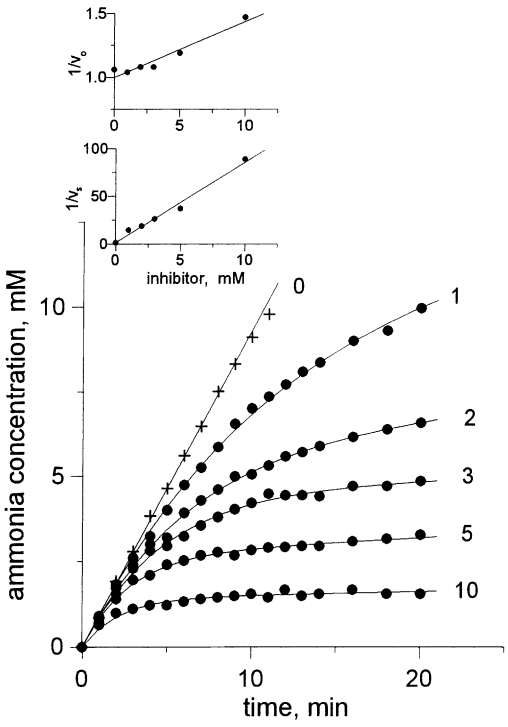


Fig. 3. Progress curves of urea hydrolysis catalyzed by urease immobilized on chitosan membrane carried out in the presence of acetohydroxamic acid in the unincubated system. Numbers denote acetohydroxamic acid concentration (mM). The inserts are reciprocals of reaction rates (initial $v_o$ and steady-state $v_s$ expressed in mM $NH_3$/min) plotted as a function of acetohydroxamic acid concentration.

by structural and environmental effects brought about by enzyme immobilization [33,34]. To the structural effects belong: (1) conformational changes introduced to the enzyme by its binding to a support, and (2) steric effects resulting from limitations on the accessibility of substrate to the enzyme. To the environmental effects belong: (1) 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 (2) modification of the local micro-environment of the bound enzyme by the physico-chemical properties of the support, e.g. electric charge, as well as by the enzymatic reaction itself, e.g. if the reaction releases hydrogen or hydroxyl ions. The environmental effects lead to altered distribution of substrates, products, hydrogen and hydroxyl ions in the vicinity of the immobilized enzyme as compared to the bulk solution.

Since: (1) urease-catalyzed hydrolysis of urea as a reaction liberating ammonia and $CO_2$ generates an increase in pH [20], (2) chitosan as a polycationic substance [24] carrying positive charge leads to accumulation of hydroxyl ions in its vicinity, and (3) the urease–chitosan conjugate as a heterogeneous enzymatic system of high activity is susceptible to mass transfer limitations in solution despite applied stirring [33], the environmental effects in the studied urease–chitosan system can be reckoned as especially pronounced, and therefore, responsible for the observed magnitude of the examined inhibitions. Although the observed behavior is a resultant of all the effects, below we present an analysis of the results aiming at exposing the individual contributions of the environmental effects to the inhibition.

*4.2.1. Mass transfer resistances in the external solution*

The urease–chitosan system was classified in our previous study [28] as moderately controlled by diffusion as judged by the calculated thickness of the unstirred layer adhering to the membrane, through which the substrate has to diffuse before it reaches the enzyme, $\delta \approx 0.0042$ cm, and by the corresponding substrate modulus $\mu = 1.4$ (diffusion control can be neglected when $\mu < 0.1$ [35]). As a rule in diffusion-controlled heterogeneous enzymatic systems, chemical inhibition is suppressed [35,36], i.e. the observed inhibition is weaker than that of the native enzyme in a homogeneous solution. Therefore, the effect of mass transfer resistances in the external solution must be regarded as contributing to the $K_i^*$ values of all three inhibitors studied, which were found to be higher than those of native urease (Table 3), though to different degrees depending on the combination with other effects.

*4.2.2. Modification of the local environment: pH effects*

The positive charge of the chitosan matrix and the enzymatic reaction taking place on the matrix, both lead to the matrix hydrogen ion concentration in the immediate vicinity being lower than that measured in the bulk solution, i.e. local pH is higher than bulk pH. Moreover, the increase in local pH is liable to be enhanced by mass transfer limitations in the external solution. The manifestation of these pH effects was noted previously in the form of the optimum pH shift, ΔpH, of chitosan-immobilized urease towards acidic pH by about 0.25 pH units in our preliminary study of the system [26] and by about 0.85 pH units in our later study on the inhibition of the immobilized urease by phosphate buffer [28], both shifts recorded in 22 mM phosphate buffer. ΔpH is related to the electrostatic potential $\psi$ of the matrix by [33,34]

$$\Delta\mathrm{pH} = \frac{0.434\varepsilon\psi}{kT} \tag{6}$$

where $\varepsilon$ is the electronic charge, $k$ the Boltzman constant and $T$ is the absolute temperature. If we ascribe the observed ΔpH of the urease–chitosan conjugate only to the electrostatic potential of chitosan, then by virtue of Eq. (6) its value falls in the range 0.014–0.045 V.

The effectiveness of competitive inhibitors is commonly measured by the ratio $K_M/K_i$, which is related to the electrostatic potential of the enzyme support by the following expression [34]

$$\frac{(K_M/K_i)_{imm}}{(K_M/K_i)_{nat}} = e^{((z_s - z_i)\varepsilon\psi/kT)} \tag{7}$$

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 $K_M/K_i^*$ ratio and their ratios $(K_M/K_i^*)_{imm}/(K_M/K_i^*)_{nat}$ for the studied competitive slow-binding inhibitors are compiled in Table 3.

For acetohydroxamic acid the ratio $(K_M/K_i^*)_{imm}/(K_M/K_i^*)_{nat}$ is equal to about one (0.97). This proves that the inhibitory action of acetohydroxamic acid on both the ureases is comparable, and that acetohydroxamic acid acts on urease as an inhibitor in its non-ionic form, as having $z_s = 0$ (urea), only with $z_i = 0$ the right-hand side of Eq. (7) is equal to one. The latter conclusion is in agreement with the earlier one by Todd and Hausinger [19] that only neutral molecules of acetohydroxamic acid $CH_3CONHOH$ are an inhibitor of urease, and not $CH_3CONHO^-$ anions. It can be further concluded from Eq. (7) that since for chitosan-immobilized urease $K_M$ and $K_i^*$ of acetohydroxamic acid do not depend on the electrostatic potential of the support, the inhibition of this urease by acetohydroxamic acid is mainly dependent on mass transfer resistances.

For $Ni^{2+}$ ions the ratio $(K_M/K_i^*)_{imm}/(K_M/K_i^*)_{nat}$ is equal to 0.16. This means that the inhibitory effectiveness of $Ni^{2+}$ ions acting on the immobilized urease is considerably lower than that on the native enzyme. This reduction in the inhibition results from repulsion between the support and the inhibitor, both carrying positive charges. The value of the electrostatic potential $\psi$ of the chitosan support calculated from the ratio 0.16 with use of Eq. (7) is equal to 0.022 V, and when recomputed into the shift of the optimum pH according to Eq. (6), gives the value ΔpH = 0.40. This ΔpH value is in a good experimental agreement with the earlier obtained pH shifts for urease between 0.25 and 0.85, thus, confirming the effect of the electrostatic potential on this inhibition. It is also noteworthy that apart from the two aforementioned effects: mass transfer limitations and pH effects, both reducing the strength of the inhibition by $Ni^{2+}$ ions,

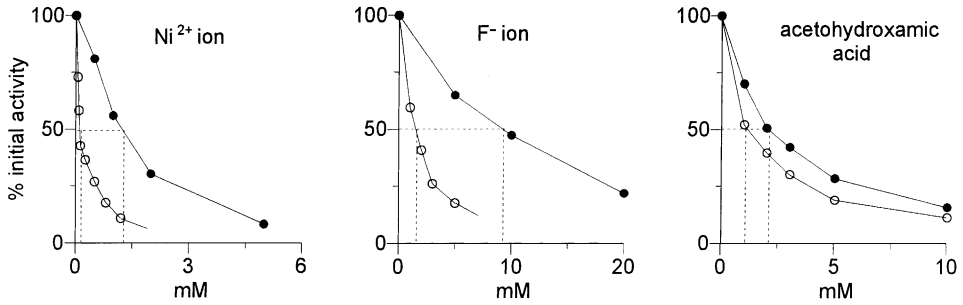


Fig. 4. Percent initial activity plotted as a function of inhibitors concentration for native (○) and chitosan membrane-immobilized urease (●). Dotted lines indicate $I_{50}$, i.e. the inhibitor concentration needed for 50% reduction in enzyme activity.

a chelating effect of chitosan is also possible in this system. Chitosan is known to be a powerful chelating agent, especially towards $Cu^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ [37], which is why in addition to the other two effects, $Ni^{2+}$ ion chelation by chitosan can also participate in the observed reduction of $Ni^{2+}$ inhibition of urease [38].

For $F^-$ ions the ratio of $(K_M/K_i^*)_{imm}/(K_M/K_i^*)_{nat} = 0.065$ shows that the inhibition of chitosan-immobilized urease by these ions does not fall into this direct electrostatic potential-dependent category of behavior. The weaker inhibition of the immobilized urease by these ions than that of native urease can be accounted for by the dependence of this inhibition on pH. This dependence was studied for *Klebsiella aerogenes* urease by Todd and Hausinger [22]. The authors found that with an increase in pH, the fluoride inhibition of the enzyme becomes weaker, i.e. $K_i$ values are higher (the dependence of log $K_i$ versus pH is linear with a slope + 1). As a result of the local pH effects on the membrane, in the examined urease–chitosan system, the inhibitor acts on the enzyme at pH that is higher than the nominal pH in the bulk, at which the inhibition of native urease was measured. Hence the effective inhibition of the immobilized uresae was observed to be weaker (the corresponding $K_i$ higher) than that of free urease in the homogeneous solution.

A practical representation of the inhibition data is that of the percent initial enzyme activity plotted as a function of the inhibitor concentration. In Fig. 4, the data for the inhibition of both native and chitosan-immobilized urease by the three studied inhibitors are presented. The plots confirm the observation that the immobilization of urease on chitosan membrane has a pronounced reducing effect on the inhibition by $Ni^{2+}$ ions and $F^-$ ions as compared to its small effect on acetohydroxamic acid inhibition. In Fig. 4, the dotted lines indicate $I_{50}$ that is the inhibitor concentration needed for 50% reduction in the enzyme activity.

In view of the presented results, it can be summarized that the observed kinetic behavior of chitosan membrane-immobilized urease in the inhibitions by $Ni^{2+}$ and $F^-$ ions and by acetohydroxamic acid results from two effects acting simultaneously, i.e. diffusional limitations in the external solution and the increase in local pH on the membrane.

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## References

[1] R.K. Andrews, R.L. Blakeley, B. Zerner, in: G.L. Eichhorn, L.G. Marzilli (Eds.), Advances in Inorganic Biochemistry, Vol. 6, Elsevier, New York, 1984, p. 245.
[2] H.L.T. Mobley, R. P Hausinger, Microbiol. Rev. 53 (1989) 85.
[3] C.M. Collins, S.E.F. D'Orazio, Mol. Microbiol. 9 (1993) 907.
[4] A.F. Kolodziej, in: K.D. Karlin (Ed.), Progress in Inorganic Chemistry, Vol. 41, Wiley, New York, 1994, p. 493.
[5] H.L.T. Mobley, M.D. Island, R.P. Hausinger, Microbiol. Rev. 59 (1995) 451.
[6] Y. Qin, J.M.S. Cabral, Appl. Biochem. Biotechnol. 49 (1994) 217.
[7] A.J. Pope, C.D.N. Toseland, B. Rushant, S. Richardson, M. McVey, J. Hills, Dig. Dis. Sci. 43 (1998) 109.
[8] J.S. Rodman, Nephron 81 (Suppl.) (1999) 50.

[9] R.L. Mulvaney, J.M. Bremner, in: E.A. Paul, J.N. Ladd (Eds.), Soil Biochemistry, Vol. 5, Marcel Dekker, New York, 1981, p. 153.
[10] K.L. Sahrawat, Plant Soil 57 (1980) 335.
[11] T. Krawczynski vel Krawczyk, Chem. Anal. (Warsaw) 43 (1998) 135.
[12] C. Tran-Minh, J. Mol. Recognit. 9 (1996) 658.
[13] R.T. Andres, R. Narayanaswamy, Analyst 120 (1995) 1549.
[14] K. Jung, G. Bitton, B. Koopman, Water Res. 29 (1995) 1929.
[15] V. Volotovsky, Y.J. Nam, N. Kim, Sens. Actuators B 42 (1997) 233.
[16] B. Krajewska, W. Zaborska, M. Leszko, Z. Brzózka, Polish J. Chem. 73 (1999) 359.
[17] J.M. Breitenbach, R.P. Hausinger, Biochem. J. 250 (1988) 917.
[18] N.E. Dixon, R.L. Blakeley, B. Zerner, Can. J. Biochem. 58 (1980) 481.
[19] M.J. Todd, R.P. Hausinger, J. Biol. Chem. 204 (1989) 15835.
[20] B. Krajewska, W. Zaborska, J. Mol. Catal. B: Enzym. 6 (1999) 75.
[21] M. Leszko, W. Zaborska, J. Chem. Tech. Biotechnol. 57 (1993) 113.
[22] M.J. Todd, R.P. Hausinger, Biochemistry 39 (2000) 5389.
[23] W. Zaborska, B. Krajewska, M. Leszko, Z. Olech, J. Mol. Catal. B: Enzym., in press.
[24] G.A.F. Roberts, Chitin Chemistry, Macmillan, New York, 1992.
[25] B. Krajewska, Acta Biotechnol. 11 (1991) 269.
[26] B. Krajewska, M. Leszko, W. Zaborska, J. Chem. Tech. Biotechnol. 48 (1990) 337.
[27] B. Krajewska, W. Zaborska, M. Leszko, J. Mol. Catal. B: Enzym. 3 (1997) 231.
[28] B. Krajewska, J. Bioact. Compat. Polym. 15 (2000) 155.
[29] J.F. Morrison, C.T. Walsh, Adv. Enzymol. 61 (1988) 201.
[30] B. Krajewska, A. Olech, Polym Gels Networks 4 (1996) 33.
[31] M.W. Weatherburn, Anal. Chem. 39 (1967) 971.
[32] W.W. Cleland, BURSTO computer program.
[33] L. Goldstein, in: K. Mosbach (Ed.), Methods in Enzymology, Vol. 44, Academic Press, New York, 1976, p. 397.
[34] G.P. Royer, in: H.H. Weetall (Ed.), Immobilized Enzymes, Antigens and Antibodies, Marcel Dekker, New York, 1975, p. 49.
[35] C. Horvath, J.M. Engasser, Biotechnol. Bioeng. 16 (1974) 909.
[36] J.M. Engasser, C. Horvath, Biochemistry 13 (1974) 3845.
[37] B. Krajewska, React. Funct. Polym., in press.
[38] B. Krajewska, J. Chem. Tech. Biotechnol. 52 (1991) 157.