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Urease Immobilized on an Aminated Polysulphone Membrane – Inhibition by Boric Acid

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Summary

An enzymatic membrane for application in the processes of decomposition and removal of urea from aqueous solutions was prepared: jack bean urease was immobilized on an aminated polysulphone membrane by adsorption. The inhibition of the system by boric acid was studied using procedures based on the MICHAELIS-MENTEN integrated equation (non-linear regression, and the linear transformations of WALKER and SCHMIDT, JENNINGS and NIEMANN, and BOOMAN and NIEMANN). The reaction was carried out in a 100 mM phosphate buffer of pH 7.0, containing 2 mM EDTA, obtained by neutralization of orthophosphoric acid with NaOH, at an initial urea concentration of 10 mM, and a temperature of 25 °C. The reaction was initiated by the addition of the enzyme to the urea solution, and was monitored by removing samples of the reaction mixture for NH_3 determinations by the phenol-hypochlorite method until the urea was exhausted. The results were compared with those obtained earlier under the same reaction conditions for free urease and urease covalently immobilized on chitosan. The inhibition was found to be competitive, similar to that of the free enzyme and urease immobilized on chitosan, with inhibition constants K_i equal to 0.36, 0.19 and 0.60 mM. The results show that adsorption of the enzyme on a polysulphone membrane changed the enzyme to a lesser degree than covalent immobilization of the enzyme on a chitosan membrane.

Introduction

Hydrolysis of urea catalyzed by the urease enzyme in the native or immobilized form: $(NH_2)_2CO + H_2O \rightarrow 2\,NH_3 + CO_2$, is used in the processes of decomposition and removal of urea in medical therapies (the treatment of uraemia), analytical determinations (biosensors) and in environmental protection (the treatment of waste- and groundwaters containing urea) [1]. Urease immobilized on / in membranes offers the potential of performing the processes in membrane bioreactors, whose advantage is the separation of the enzyme from substrates and products of the reaction. The support and technique chosen for immobilization determine the kinetic properties of the immobilized enzyme [2], among others its resistance to being inhibited. Inhibition of urease, depending on the application, is an advantageous or disadvantageous phenomenon. Nevertheless, the knowledge of its kinetics is necessary. The study of inhibition also

provides insight into the enzyme structure and its changes generated by immobilization.

In this study, urease was immobilized on an aminated polysulphone membrane by adsorption. Polysulphone is a useful membranous enzyme immobilization support because of ease of chemical modification, ease of membrane preparation and because of its chemical and biological stability [3]. In a previous report [4], the preparation and properties of urease immobilized on an aminated polysulphone membrane by adsorption were described. The immobilization improved the pH and temperature stability of the enzyme. The immobilized urease exhibited good storage and operational stability and good reusability, properties that prove the applicability of the obtained system in enzyme-membrane reactors.

In the present study, the kinetics of inhibition of urease, by boric acid, immobilized on a polysulphone membrane was investigated. This inhibition is of practical importance e.g. for an analysis of urease-based urea determinations in urine. This is because boric acid is a common urine preservative used prior to analytical tests [5]. Boric acid was found to be a competitive inhibitor of bacterial ureases [6], as well as of jack bean urease in the native form [7] and covalently immobilized on chitosan [8]. In order to estimate the kinetic constants of the reaction of the urease-polysulphone membrane system studied, the kinetic integration methods based on the MICHAELIS-MENTEN integrated equations were used.

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 MICHAELIS-MENTEN differential equation (Eq. 1) or by integration methods based on the MICHAELIS-MENTEN integrated equations (Eqs. 2 and 3) [9].

The MICHAELIS-MENTEN equation for an uninhibited enzyme-reaction ($E + S \Leftrightarrow ES \rightarrow E + P$) is:

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

where S is the substrate concentration. Eq. 1 can be integrated in two ways; one leads to Eq. 2, the other to Eq.3:

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

$$K_M (S_t - S_o) + \frac{1}{2}(S_t^2 - S_o^2) = -v_{max} \int_o^t S_t dt \tag{3}$$

where S_o is the initial substrate concentration and S_t the concentration of the substrate remaining after time t, ($P_t + S_t = S_0$).

A competitively inhibited enzymatic reaction is described by Eqs. 1, 2 and 3, in which K_M is replaced by K_M^I, the MICHAELIS constant in the presence of an inhibitor, expressed by:

$$K_M^I = K_M\left(1+\frac{I}{K_i}\right) \tag{4}$$

where I is the inhibitor concentration and K_i is the inhibition constant.
The integrated forms of the MICHAELIS-MENTEN equation (Eqs. 2 and 3) together with Eq. 4 allow the determination of the kinetic parameters K_M, K_M^I, v_{max} and K_i from two successive progress curves monitoring the reactions in the absence and presence of an inhibitor.
In this study, the kinetic constants were calculated from the progress curves using non-linear regression applied to Eq. 2, and using linear transformations of Eq. 2 (the method of WALKER and SCHMIDT [10], the methods of JENNINGS and NIEMANN (I) and (II) [11]) and of Eq. 3 (the method of BOOMAN and NIEMANN [12]). In non-linear regression, the minimizing procedure MINUIT from the CERN Computer Library was used. The procedure searches for the minimum of the sum

$$\sum_i [S_{t,theor} - S_{t,exp}]_i^2$$

as a function of the fitted parameters K_M, v_{max} and K_M^I, v_{max}. $S_{t,theor}$ stands for S_t values for given times t calculated from Eq. 2 by a separate subroutine. The summary of the linear transformations is presented in [8].

Experiments and Methods

The aminated polysulphone membrane was prepared by G. POŹNIAK *et al.* at the Technical University of Wrocław, Poland [4]. Polysulphone was modified by chlorosulphonation followed by amination with ethylenediamine. Porous asymmetric membranes were formed by phase inversion. The membranes obtained had a finger-like structure and their properties were as follows: concentration of $-NH_2$ groups: 0.15 mmole/g dry membrane; permeability of water: 0.6 $cm^3/cm^2 \times s$ MPa; total porosity: 80.4%; mean pore diameter: 33.8 nm.
The jack bean urease was SIGMA type III of a specific activity of 33 units/mg protein. Urea, boric acid and all other chemicals were from POCh, Gliwice, Poland. Phosphate buffer of pH 7.0 and a concentration of 100 mM containing 2 mM EDTA was prepared from orthophosphoric acid solution titrated potentiometrically with NaOH solution to a pH of 7.0 and diluted as necessary.
Urease was immobilized on both sides of the polysulphone membrane by adsorption [4]: The membrane was immersed in the 0.125% (w/v) solution of urease in phosphate buffer of pH 5.3 for 1 h at room temperature and overnight at 4 °C. The membrane was next washed with the buffer until the washings were free of urease.
The reaction progress curves, ammonia concentration vs. time, for the hydrolysis of urea catalyzed by a polysulphone membrane-immobilized urease in the absence and presence of boric acid were recorded at 25 °C as follows: the membranes (area of two surfaces 2×100 cm^2) were dropped into 100 cm^3 of 10 mM urea solution in a phosphate buffer of pH 7.0 or into 100 cm^3 of 10 mM urea solution in a phosphate buffer of pH 7.0 containing 0.25 mM boric acid. 0.1 cm^3 samples were removed from the reaction mixtures at 0.5 and 1-minute intervals, respectively, in order to determine the NH_3 by the phenol-hypochlorite method [13]. The reactions were carried out until the substrate was exhausted. The reaction mixtures were stirred throughout the period of measurements, and the temperature was controlled to an accuracy of ± 0.5 °C. 0.25 mM boric acid was chosen for the experiment according to the principle that the inhibitor concentration should be numerically close to the predicted value of K_i [14]. The parameters used in the estimations based on Eqs. 2 and 3 were: $S_o = 10$ mM urea, $P_t = [NH_3]/2$, product concentration formed in time t, $I = 0.25$ mM boric acid.

To check the accuracy of the determinations and also a possible interference of product (ammonia) inhibition with the results obtained, the constants K_M and v_{max} for the uninhibited urea hydrolysis catalyzed by polysulphone membrane-immobilized urease were determined using the MICHAELIS-MENTEN differential equation (Eq. 1). For that purpose, the initial rates of the reaction were measured for the membranes of a surface area of 2×6.25 cm^2 in 50 cm^3 of urea solutions of a concentration range of 1.6–133.3 mM in the same 100 mM phosphate buffer.
The values of the kinetic constants were obtained by non-linear regression, applied to Eq. 1, calculated by finding the minimum of the sum

$$\sum_i [v_{theor} - v_{exp}]_i^2$$

as a function of the fitted parameters K_M and v_{max}.

Results and Discussion

The reaction progress curves, ammonia concentration vs. time, recorded for the hydrolysis of urea catalyzed by polysulphone membrane-immobilized urease, uninhibited and inhibited by 0.25 mM boric acid are presented in Fig 1.

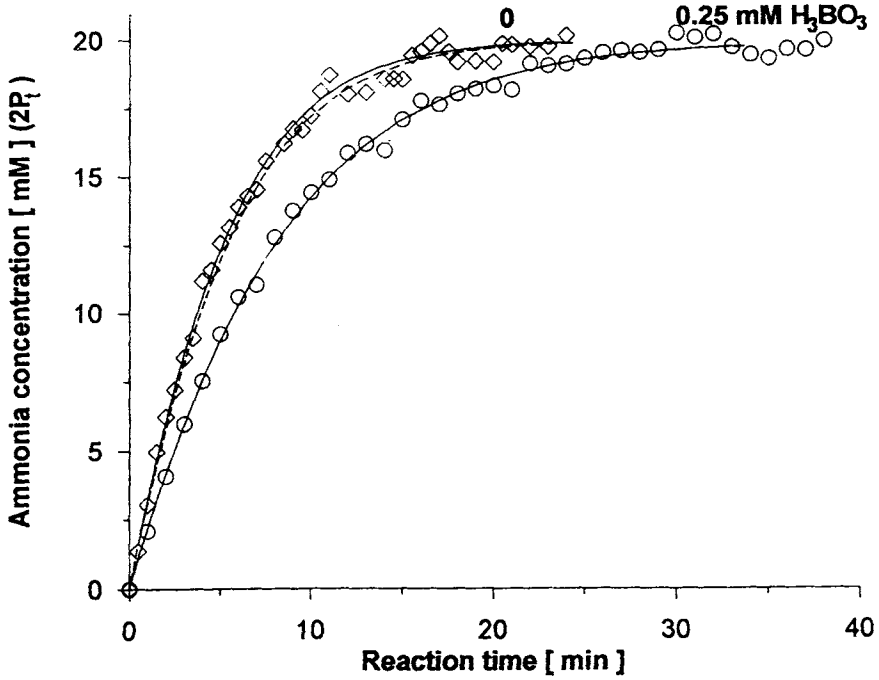

Fig. 1. Reaction progress curves, ammonia concentration vs. time, recorded for the hydrolysis of urea catalyzed by polysulphone membrane-immobilized urease, uninhibited (◊) and inhibited by 0.25 mM boric acid (o), approximated with polynomials of the fourth degree (solid lines)
$P_t = [NH_3]/2$ is the concentration of product formed in time t [mM]; the dotted line represents a "theoretical" progress curve for the uninhibited reaction calculated with values of K_M = 23.3 mM and v_{max} = 5.19 µmoles NH_3 /min × cm^2 of membrane obtained from the MICHAELIS-MENTEN differential equation by non-linear regression.

For further calculations, the experimental progress curves were approximated by polynomials of the fourth degree. To Fig. 1 is added a "theoretical" progress curve of the uninhibited reaction calculated according to Eq. 2 with the values of K_M = 23.3 mM and v_{max} = 5.19 μmoles NH_3/min × cm^2 membrane (5.19 mM urea/min) obtained from the MICHAELIS-MENTEN differential equation by non-linear regression. In Fig. 1, the experimental and "theoretical" progress curves obtained for the uninhibited reaction are in agreement within the limits of experimental error. This provides evidence that inhibition by a product (ammonia), reported to be of a noncompetitive type for free urease in a phosphate buffer [1], does not take place in the reaction catalyzed by polysulphone membrane-immobilized urease in 10 mM urea in a 100 mM phosphate buffer at pH 7.0. The above observation justified the elimination of product inhibition from the determinations of the kinetic constants by the integration methods.

The linear replots of the polynomial progress curves of Fig. 1 obtained by the integration methods are presented in Figs. 2 a–2 d. The progress curves were transformed into the linear replots within the fraction conversion range of 0.1÷0.95. The linear replots representing the two reactions studied have either the same intercept (Figs. 2 a and 2 b) or the same slope (Figs. 2 c and 2 d), which provides evidence that boric acid is a competitive inhibitor of polysulphone membrane-immobilized urease, similar to native urease [7] and urease covalently immobilized on chitosan membrane [8].

The values of the kinetic constants obtained by the applied methods are presented in Tab. 1. As a group, they are consistent, and the K_M and v_{max} values for the uninhibited reaction are consistent with the corresponding values obtained by the differential method. This proves that product/ NH_4^+ inhibition of polysulphone membrane-immobilized urease does not occur. This can be accounted for by the fact that the polysulphone matrix with its NH_2 groups is positively charged. As a result of this, the pH is higher in its direct vicinity than in the bulk solution. The manifestation of this property was noted previously [4] in the form of an optimum pH shift of polysulphone membrane-immobilized urease towards acidic pH values compared to free urease, which is the behaviour commonly observed for positively charged supports [15, 16]. The repulsion of NH_4^+ ions by the polysulphone matrix decreases the concentration of the ions at the reaction site, thus weakening their inhibitory action.

Tab 1. Kinetic constants of urea hydrolysis catalyzed by polysulphone membrane-immobilized urease uninhibited and inhibited by 0.25 mM boric acid, obtained by integration methods

Method	Uninhibited reaction		Inhibited reaction		
	K_M [mM]	$v_{max}\left[\frac{\text{mMurea}}{\text{min}}\right]$	K_M^I [mM]	$v_{max}\left[\frac{\text{mMurea}}{\text{min}}\right]$	K_i [mM]
Non-linear regression	20.8	5.23	35.2	5.11	0.36
WALKER-SCHMIDT	20.5	5.17	35.4	5.14	0.34
JENNINGS-NIEMANN (I)	20.9	5.25	35.4	5.13	0.36
JENNINGS-NIEMANN (II)	20.8	5.24	35.4	5.13	0.36
BOOMAN-NIEMANN	21.3	5.32	35.2	5.11	0.38
Mean values	20.9	5.24	35.2	5.12	0.36

The mean value of K_i of boric acid of polysulphone membrane-immobilized urease was found to be 0.36 mM. This value was confirmed by the DIXON relation: the recip-

rocal of the initial rate of the reaction in 10 mM urea solution measured for three inhibitor concentrations: 0, 0.25 and 0.5 mM H_3BO_3 (K_i = 0.36 mM).

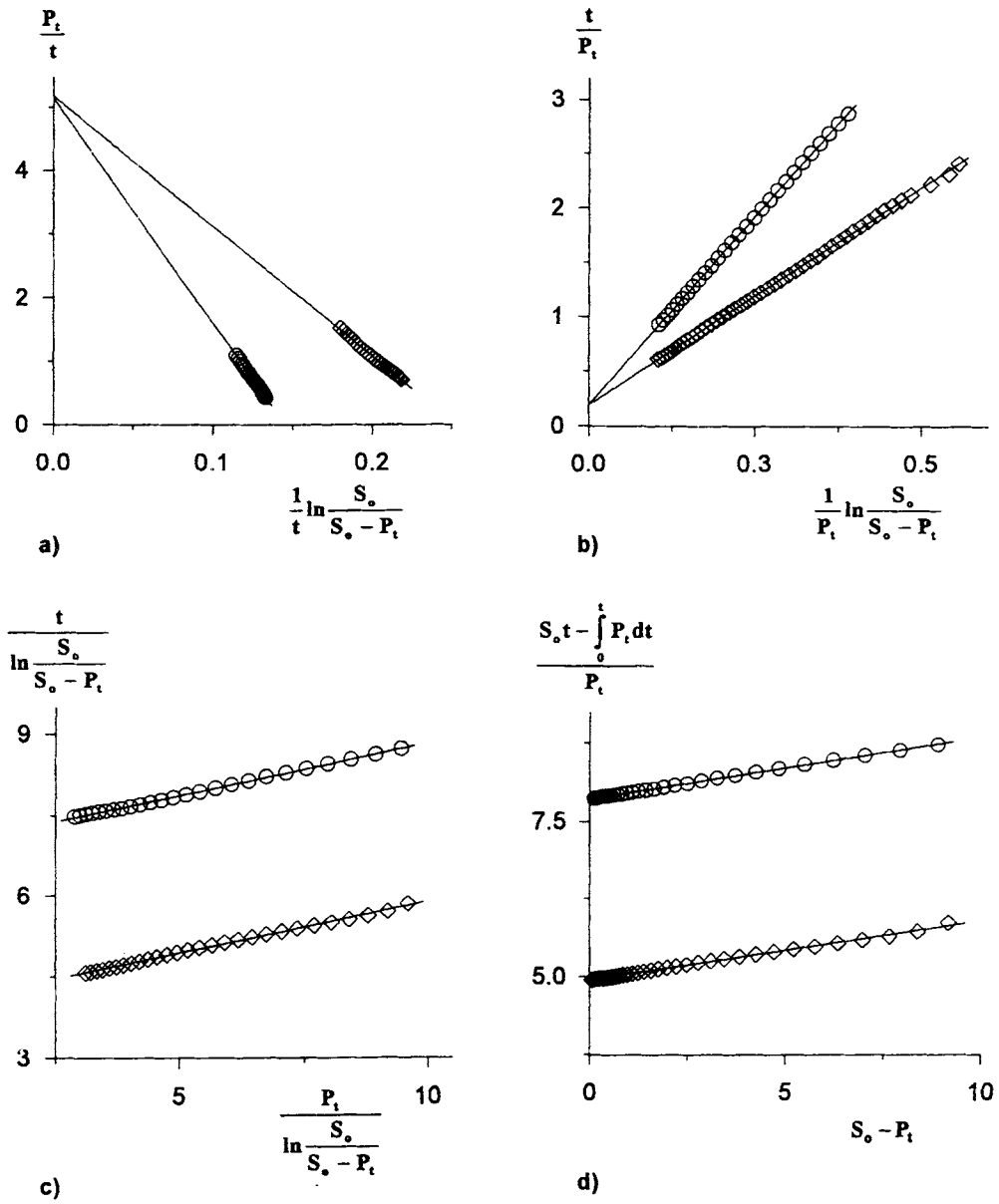

Fig. 2. Replots of the progress curves of hydrolysis of urea catalyzed by polysulphone membrane-immobilized urease (Fig. 1) uninhibited (◊) and inhibited by 0.25 mM boric acid (o), obtained by the integration methods of:
(a) WALKER and SCHMIDT; (b) JENNINGS and NIEMANN (I); (c) JENNINGS and NIEMANN (II) and (d) BOOMAN and NIEMANN.

The inhibition constants K_i of different ureases by boric acid are compared in Tab. 2. The K_i values of jack bean ureases: in their native form and immobilized on a polysulphone membrane by adsorption, and immobilized covalently on a chitosan membrane were determined in the same medium, i.e. 100 mM phosphate buffer, 2 mM EDTA, pH 7.0. The phosphate buffer, being also a competitive inhibitor of urease [17], constitutes here a background which allows the studied ureases to be compared. The obtained K_i constants reflect the degree of changes introduced to the native enzyme by the immobilization techniques applied. Adsorption of the enzyme on a polysulphone membrane changed the enzyme to a lesser degree than covalent immobilization of the enzyme on a chitosan membrane. The studied property, together with the properties presented previously [4], give a fuller picture of polysulphone membrane-immobilized urease useful in the possible practical application of the system.

Tab. 2. Constants of the inhibition of ureases by boric acid

Urease	K_i [mM]	Reference
Native bacterial urease *Proteus mirabilis*	0.099	[6]
Native plant jack bean urease	0.19	[7]
Jack bean urease immobilized by adsorption on a polysulphone membrane	0.36	this paper
Jack bean urease immobilized covalently on a chitosan membrane	0.60	[8]

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