IMMOBILIZATION OF UREASE ON MODIFIED STYRENE/POLYVINYLBENZENE MATRICES

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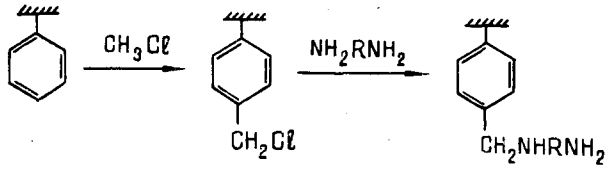
With copolymers of styrene and divinylbenzene containing various modification groups and the use of glutaric dialdehyde for their activation, immobilized forms of urease for watermelon seeds and from Staphylococcus saprophyticus have been obtained. The properties of the immobilized preparations have been studied: the pH optimum, the temperature optimum and thermal stability, the influence of stabilizing components, kinetic features of the hydrolysis of urea, and work under column and batch conditions. The high stability of the immobilized preparations has been shown.

Recently, work has been performed on obtaining a stable preparation of immobilized urease for the analytial determination of urea and heavy metals in the dialysate regeneration systems of "artificial kidney" apparatuses, and for the decomposition of urea in biological liquids and effluents. The promising nature of the use for these purposes of macroporous copolymers of styrene and divinylbenzene containing in their structure chemically active aldehyde groups has been shown [1-3]. In view of the high ion-exchange properties of the supports and also the possibility of modifying the polystyrene by the introduction of amino groups into it by the following scheme



with a change in the length of the alkyl radical within wide limits [4], we have studied the immobilization of urease from watermelon seeds and from Staphylococcus saprophyticus (samples given to us by S. I. Dikhtyarev and V. T. Chernobai, Khar'kov Institute of Pharmaceutical Chemistry, and D. Yu. Yuodval;kite and A. A. Glezhma, "Ferment" Scientific Production Amalgamation, Vilnius) on styrene-divinylbenzene matrices of various structures.

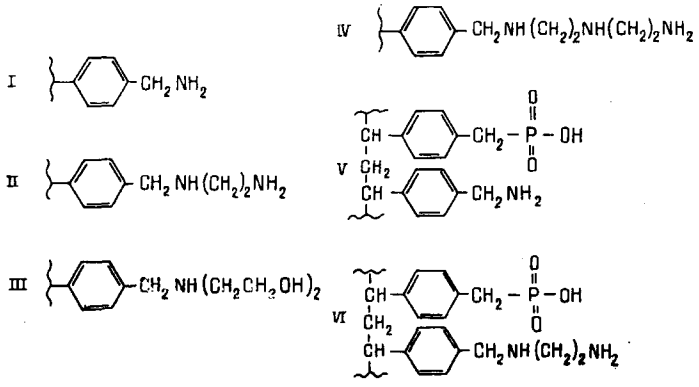


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TABLE 1. Properties of Urease Immobilized on Modified Styrene-Divinylbenzene Matrices

Support	Urease	Amt. of enzyme, mg of protein/g of support	Binding of protein on immobilization, %	Activity of immobilized urease, units/g	Retention of the activity of the immobolized urease, %
I	From watermelon seed	0	0	0	0
II		13.4	44	170	33
III		6	18	18	3,5
IV		7	23	203	39
V		13.7	45	108	21
VI		22	72	382	74
VII	Fr.Staphylococcus saprophyticus	7,5	10	90	6

TABLE 2. Influence of the Ratio of Enzyme Protein to Support and of the Time of Immobilization on the Activity of the Immobilized Urease

Protein/support ratio, mg/g	Activity, % of maximum	Time of immobilization, h	Activity, % of maximum
50	75	6(A)	64
100	99	6(B)	87
200	100	18(A)	86
300	87	18(B)	100
400	49	24(A)	86
		24(B)	100

A – protein/support ratio 100 mg/g; B – protein/support ratio 200 mg/g.

Table 1 gives some properties of urease immobilized on the styrene-divinylbenzene matrices with structures (I-VI) using for their activation the bifunctional reagent glutaric dialdehyde. For the samples obtained we determined the amount of protein bond to the support and their urease activities. As follows from Table 1, the binding of the protein and the retention of urease activity was affected both by the nature of the support and by the length of the modification "spacer" of the copolymers. Thus, urease was not bound to support (I), while for (VI) there was a 74% retention of activity with 72% binding of the protein. The dependence of the activity of the immobilized forms of urease on the length of the modification "spacer" of the polymers can be clearly seen: for (I) (R = NH_2) no urease activity was observed, while for (II) (R = $NH(CH_2)_2NH(CH_2)_2NH_2$) the urease activity was 203 units/g of support.

The samples of immobilized urease from watermelon seeds and from Staphylococcus saprophyticus possessed different activities, and while the retention of the initial activity on the support (IV) for the urease from watermelon seed was 39% for the urease from Staphylococcus saprophyticus it was only 6%.

The modification of support (V) by glutaric dialdehyde was performed at pH 7.0 and 3.5-4.0. The amounts of aldehyde groups in the supports obtained were approximately the same: 0.93% (pH 3.5-4.0) and 0.87% (pH 7.0), and, on immobilization, preparations with similar activities were obtained. In view of the favorable results of the stabilization of trypsin on treatment with glutaric dialdehyde [5], we considered a similar treatment of urease. However, the immobilized preparations obtained had activities 40-50% lower than on the immobilization of the native urease. The use of albumin here protected the urease from the inhibiting influence of the glutaric dialdehyde, and on the immobilization of the microbiological urease in the presence of albumin a preparation six times more active was obtained.

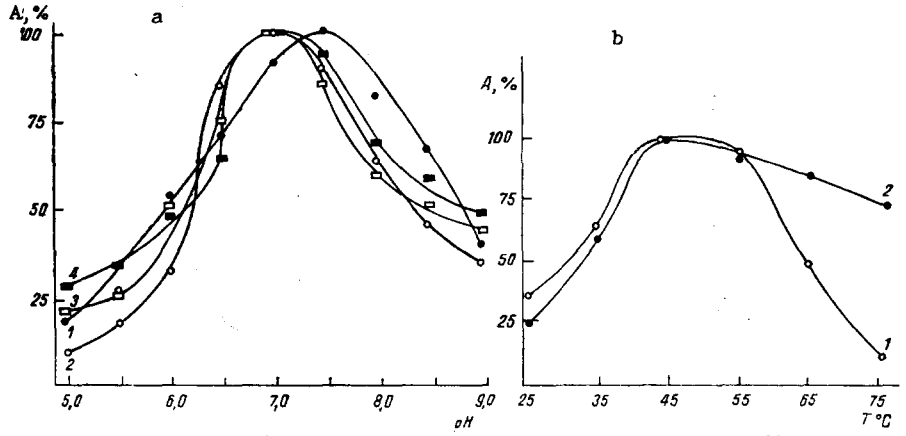

Fig. 1a Fig. 1b

Fig. 1a. Dependence of the activity of watermelon seed urease (% of the maximum) on the pH of the incubation medium: 1) native urease; 2) immobilized on support (IV); 3) on support (II); 4) on support (VI).

Fig. 1b. Temperature dependence of the activity of native urease from watermelon seeds (1) and of that immobilized on support (IV) (2).

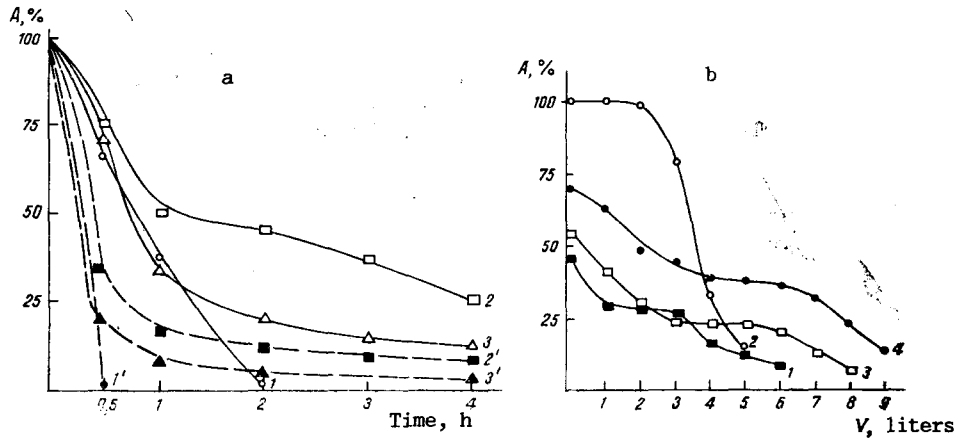

Fig. 2a Fig. 2b

Fig. 2a. Dependence of the activity of watermelon seed urease on the time of incubation at 50°C (1-3) and 70°C (1'-3'): 1) native urease; 2) immobilized on support (IV); 3) on support (II).

Fig. 2b. Dependence of the activity of immobilized watermelon seed urease when working under column conditions on the amount of urea solution passed through: 1) on support (II); 2) (IV); 3) (V); 4) (VI).

TABLE 3. Kinetic Parametrs of Native and Immobilized Ureases

Urease from watermelon seeds	Urea concentration, mM	K_M (mM)	$V_M \cdot 10^4$ (M/min)	ρ
Native	1—1000	5,29±0,6	2,8±0,04	0,999
Immobilized on support (IV)	1—1000	5,58±0,7	5,65±0,16	0,999
Immobilized on support (V)	1—200	11,5±0,8	5,94±0,33	0,9878
	100—1000	117±4	6,13±0,03	0,999

In addition to albumin we studied the influence of sorbitol on the activity and degree of binding of the urease. In spite of literature information on its stabilizing influence [6], no favorable effect was observed in our case.

A study of the dependence of the activity of the preparations obtained on the time of immobilization showed (Table 2) that the process was basically complete in 6 h, but up to 16-18 h there was an increase in activity and in the degree of binding, after which these parameters remained unchanged.

The figures of Table 2 show that the activity of the preparations immobilized on support (V) was a maximum at a protein/support ratio of 100-200 mg/g.

Figure 1, a shows the influence of a change in the pH of the incubation medium on the activities of native and immobilized ureases. The pH optimum of the native preparation was 7.2-7.5 and the pH optima of the immobilized preparations shifted into acid region for all the supports: for 0.06 M phosphate buffer the shift amounted to 0.2-0.4 unit, for phosphate buffers at concentrations less than 0.06 M the shift increased, while at >1 M no shift was observed. A possible explanation may be an accumulation of NH_4^+ ions in the pores of the support, which will lead to an increase in the pH of the medium close to the active sites of the enzyme because of diffusional hindrance in the pores of the supports.

The temperature optimum (Fig. 1b) of urease immobilized on support (IV) (45-50°C) did not differ from that of the native enzyme. With a further rise in the temperature the native preparation was rapidly inactivated: at 65°C 20% of the maximum activity remained, and at 75°C only 11%. The immobilized urease retained 76% of its maximum activity at 65°C and 70% at 75°C. The thermal stability of the immobilized urease had also increased (Fig. 2a). At 50°C after 4 h up to 30% of the initial activity was retained, while the native preparation was completely inactivated after 2 h, while at 70°C the immobilized enzyme retained 10% of its initial activity and the native enzyme was inactivated after half an hour.

On repeated use, the most stable preparation was that immobilized on support (IV): after this preparation had been used 40 times its activity remained unchanged, and then a uniform fall in activity was observed and after 100-fold use 40% of the initial activity remained.

The results on the hydrolysis of urea (concentration 350 mg %) in a reactor of the column type are shown in Fig. 2b. The urease preparation immobilized on support (VI) are the most stable. After 9 liters of solution had been passed through a column containing 0.5 g of this preparation its activity amounted to 20% of the initial activity.

When the immobilized urease from watermelon seeds was stored (3-4°C) in 0.06 M phosphate buffer with the addition of 50% of glycerol and in the dry state, 93 and 21% of the initial activity remained after 6 months, and 78 and 7%, respectively, after 1 year 7 months.

The enzymatic hydrolysis of urea by urease is described by the Michaelis—Menten scheme [7]. According to an investigation of the kinetics of urease immobilized on Silochromes the K_M values at low (5-100 mM) concentrations of urea were similar [8, 9]. In the case of urease immobilized on copolymers of styrene and divinylbenzene direct dependences of the rate of hydrolysis of urea on the concentration in the coordinates (S, S/V) were not observed in all cases. When the graphical method of analysis [10] was used, in the case of support (V) two different values of K_M were observed, for low and high concentrations of urea. The parameters of the two straight lines corresponding to the dependence of the rate of hydrolysis on the concentration of urea within the ranges 10-200 mM and 200-1000 mM were found mathematically (Table 3). It is possible that in this case, as in [9], diffusional inhibition with respect to the substrate is being observed for the immobilized urease. But it is also

impossible to exclude the competitive inhibition of the urease by the product of the hydrolysis of urea – ammonia – since at high concentrations of urea this contribution may be substantial. As follows from Table 3, the values of K_M for the native preparation and that immobilized on support (IV) were close, whole for the enzyme immobilized on support (V) a second value, K_{M_2} was found at high concentrations of the substrate.

EXPERIMENTAL

Preparations of watermelon seed urease supplied by the Khar'kov Institute of Pharmaceutical Chemistry that contained 30% of protein, as determined by Lowry's method [11], and had an activity of 27.5 units/mg, and Staphylococcus saprophyticus urease from the "Ferment" Scientific Production Amalgamation with 40% of protein and an activity of 200 units/mg, were used together with styrene-divinylbenzene copolymers (I-VI) produced by ITEA [All-Union Scientific-Research Institute of Chemical Reagents and Ultrapure Chemical Substances) and "Serva" glutaric dialdehyde.

Urease activities were determined at 37°C by the incubation for 30 min with shaking of 10 mg of the immobilized urease with 2 ml of a 1.5% solution of urea in 0.06 M phosphate buffer, pH 6.8 [12]. As the unit of activity we took the amount of enzyme protein that catalyzed the formation of 1 μmole of ammonia in 1 min at 37°C. The amount of protein in an immobilized urease preparation was determined from the difference between the concentrations of protein in solution before and after immobilization.

The immobilization of the urease was performed through its covalent addition to the surface of supports I-VI via glutaric dialdehyde. For this purpose, a weighed sample of support was added to a 2.5% solution of glutaric dialdehyde in 0.06 M phosphate buffer, pH 6.8, in a proportion of 20 ml of solution to 1 g of support. After stirring at 20°C for 4 hours, the support obtained was washed free from unbound glutaric dialdehyde on a Schott filter and was brought into reaction with the urease.

Immobilization was carried out at 3-4°C for 16-18 h with shaking in 0.06 M phosphate buffer, pH 6.8, containing 20% of glycerol and 1 mmole of dithiothreitol and EDTA. It was shown by special experiments that the immobilization process was completed during this time. The preparation of immobilized urease obtained was separated off by decantation and was then washed free from unbound protein with double-distilled water and 0.06 M phosphate buffer, pH 6.8.

The pH dependence of the urease activity was determined by changing the pH of the medium from 4.5 to 9. The temperature dependence of the activity was estimated by varying the temperature of incubation from 25 to 70°C.

To investigate the thermal stability of the immobilzed and native preparations, weighed amounts of them in 1 ml of 0.06 M phosphate buffer, pH 6.8, were thermostated for a predetermined time (0-4 h) at 50 and 70°C, and then 1 ml of a 3% solution of urea was added and the urease activity was determined as described above [12].

The repeatability of the action of the immobilized urease was checked in the following way: 0.1 g of the immobilized urease was placed in a reactor and the enzymatic hydrolysis of 10 ml of a 0.17% solution of urea in 0.06 M phosphate buffer, pH 6.8, was performed with stirring for 10 min. After this, the immobilized enzyme preparation was separated from the reaction mixture and was washed with double-distilled water, and hydrolysis was repeated with a new portion of urea. In each experiment the concentration of NH_4^+ ions was determined with the aid of the Nessler reagent.

To study the hydrolysis of urea by immobilized urease under column conditions, 1 g of immobilized enzyme was placed in a thermostated (37°C) column with an internal diameter of 20 mm, and then a solution of urea (350 mg % in 0.06 M phosphate buffer, pH 6.8) was passed through the column with the aid of a peristaltic pump at the rate of 4-10 ml/min.

The kinetics of the hydrolysis of urea by the immobilized and native ureases were studied on the basis of the initial rates of hydrolysis by the pH-stating method in the Institute of Physical Chemistry of the UkrSSR Academy of Sciences. The calculations of the rates of hydrolysis of urea on the initial sections of the kinetic curve were carried out by the method of [13].

Assistance in the performance of the kinetic studies was provided by V. A. Tertykh and G. V. Lyubinskii of the Institute of Physical Chemistry of the UkrSSR Academy of Sciences.

SUMMARY

Immobilized forms of the ureases from watermelon seeds and from Staphylococcus saprophyticus have been obtained from copolymers of styrene and divinylbenzene containing various modification groups using glutaric dialdehyde for activation. The properties of the samples of immobilized enzymes have been studied and their high stability has been shown.

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