SUMMARY

It has been shown that adsorption on AE-cellulose partially protected by the palmitoyl residue is an effective method of immobilizing the lipases of *Rhizopus microsporus*. The kinetics of the lipases are characterized by substrate inhibition. Immobilization considerably increases the heat stability of the lipases.

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PREPARATION AND PROPERTIES OF ENZYMES IMMOBILIZED ON SUPPORTS ACTIVATED BY METAL IONS

V. A. Laurinavichyus and Yu. Yu. Kulis

UDC 577.15.02

The adsorption of enzymes forms the simplest method of immobilizing them [1, 2]. Recently, to improve the parameters of supports they have been modified by coating the surface with hydrophobic compounds [3], by the "grafting on" of hydrophobic radicals [4], or by treating the surface with transition-metal ions [4-15]. Such treatment of the sorbents leads to an increase in the amount of bound enzyme and to an improvement in the stability of the enzyme. The mechanism of the activation of supports by hydrophobic compounds has been studied in detail by Poltorak et al. [3], and the hydrophobic nature of the fixation of the enzymes has been shown. The nature of the activation of supports by metal ions has been little investigated, in spite of the fact that biocatalysts obtained in this way are of great practical interest [6, 11]. Moreover, this method of immobilizing enzyems is used mainly in the region of low pH values. The immobilization and properties of enzymes obtained at neutral pH values have not been studied.

Our aim was to investigate the immobilization of enzymes at neutral pH values on supports activated by transition-metal ions, and also to study the properties of the heterogeneous biocatalysts obtained.

On considering the immobilizaiton of enzymes at neutral pH values on supports activated by metal ions, as enzymes we selected the well-studied proteolytic enzymes α-chymotrypsin

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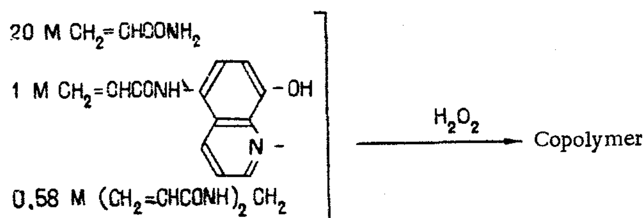
TABLE 1. Properties of the Immobilized Enzymes

Support	Enzyme	Activator	Amount of bound enzyme, mg/g of support	Activity		Ratio of activity (%) to the native enzyme
				U/g of support	U/mg of enzyme	
I. Acrylamide–hydroxyquinoline	α-Chymotrypsin	$SnCl_4$	—	82,2	—	—
I. Acrylamide–hydroxyquinoline	α-Chymotrypsin	$TiCl_4$	—	105,4	—	—
II. Sephadex–lysine	α-Chymotrypsin	$SnCl_4$	—	291,1	—	—
II. Sephadex–lysine	α-Chymotrypsin	$TiCl_4$	—	101,3	—	—
III. Sephadex	α-Chymotrypsin	$TiCl_4$	54,4	9402,2	99,3	60,1
III. Sephadex	α-Chymotrypsin	$SnCl_4$	22,7	2013,7	88,8	53,8
III. Sephadex	α-Chymotrypsin	$FeCl_3$	4,6	237,6	51,4	31,1
III. Sephadex	α-Chymotrypsin	$Ni(NO_3)_2$	5,6	84,0	15,1	9,1
IV. Microcrystalline cellulose	Glucose oxidase	$SnCl_4$	3,56	57,7	16,2	90,0
V. Tin hydroxide	Glucose oxidase	$SnCl_4$	6,4	631,5	99,2	53,9
V. Tin hydroxide	α-Chymotrypsin	$SnCl_4$	21,6	2738,0	126,8	76,8
V. Tin hydroxide	Trypsin	$SnCl_4$	64,2	599,4	9,3	49,9
VI. Titanium hydroxide	α-Chymotrypsin	$TiCl_4$	26,3	92,2	3,5	2,2
VI. Titanium hydroxide	Trypsin	$TiCl_4$	15,0	—	—	—
VII. Chromium hydroxide	Glucose oxidase	$Cr_2(SO_4)_3$	13,3	9,4	1,5	11,5

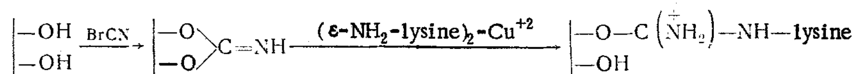
and trypsin, which possess an action convenient for investigating the pH optimum. As an oxidizing enzyme we used glucose oxidase.

To immobilize enzymes with the use of metal ions, we synthesized a ligand-containing polymer having an 8-hydroxyquinoline or lysine grouping (supports I and II in Table 1) [16], and we also used Sephadex (III) and microcrystalline cellulose (IV), Table 1. In the synthesis of the support (I), we obtained a copolymer of acrylamide and 5-acryloylamino-8-hydroquinoline (acrylamide–hydroxyquinoline):



The copolymer obtained contained one hydroxyquinoline grouping to 21.2 vinyl residues. The charging of support (I) with tin or titanium ions followed by treatment with a solution of α-chymotrypsin gave an immobilized enzyme with an activity of 82-105 U/g of support.

The synthesis of the lysine-containing support (Sephadex-lysine) was carried out by the following scheme:



The synthesis of this support led to the formation of a sorbent containing 2.52 mmole of lysine per 1 g of support, which corresponds to one lysine residue per 1.41 carbohydrate residues. Such a support when treated with titanium or tin ions strongly adsorbes α-chymotrypsin with the formation of an immobilized enzyme having an activity of 101-291 U/g of support (Table 1).

The treatment of Sephadex with titanium, tin, iron, or nickel ions led to strong activation of the support, so that as the result of immobilization enzymes were obtained which possessed specific activities of 9400-84 U/g of support at 60-9% retention of the initial activity. The immobilization of glucose oxidase on microcrystalline cellulose (IV) treated with tin ions led to the formation of an enzyme having an activity of 58 U/g. The activity yield amounted to 90%.

Using a method proposed previously for immobilizing enzymes on metal hydroxides [17], we obtained insoluble α-chymotrypsin, trypsin, and glucose oxidase sorbed on tin hydroxide (V), titanium hydroxide (VI), and chromium hydroxide (VII) and each containing 64 mg of protien per 1 g of support with an activity yield of 77-2.2% (Table 1).

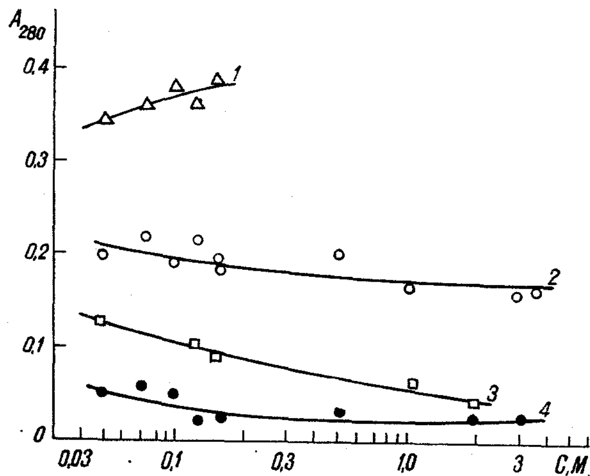


Fig. 1

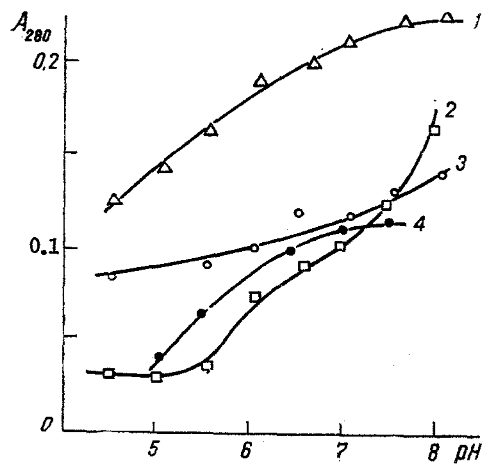


Fig. 2

Fig. 1. Dependence of the equilibrium concentration of enzyme in solution on the ionic strength: 1, 2, 4) (tin hydroxide)—α-chymotrypsin; 3) acrylamide—hydroxyquinoline—Sr—(glucose oxidase); 1, 3) pH 8.0, 2) pH 7.0, 4) pH 6.0; 50 mg of immobilized enzyme was incubated in 3 ml of 0.01 M phosphate buffer containing KCl at 25°C for 12 h.

Fig. 2. Dependence of the equilibrium concentration of enzyme on the pH of the solution: 1) acrylamide—hydroxyquinoline—Sn—(glucose oxidase); 2) tin hydroxide; 3) acrylamide—hydroxyquinoline—Sn—α-chymotrypsin; 4) (tin hydroxide)—α- chymotrypsin; incubation at 25°C in 0.25 M phosphate buffer containing 1 M KCl for 12 h.

No desorption of the immobilized α-chymotrypsin from the synthesized support (I) or from the hydrated tin oxidase (V) took place even at a high ionic strength of the solution (Fig. 1). However, as Figs. 1 and 2 show, the equilibrium concentration of protein in the solutions above the immobilized enzyme depends on the pH. Both in the case of the enzymes adsorbed on the ligand-containing sorbent (I) and for enzymes sorbed on hydrated metal oxides, with an increase in the pH of the solution the equilibrium concentration of the proteins in the solution rises. The strongest pH dependence of the desorption of enzymes was observed in the case of glucose oxidase immobilized on hydrated tin oxides and of α-chymotrypsin adsorbed on the support (I).

At an ionic strength of the solution of 10-20 mM, the pH dependence of the activity of the immobilized enzymes was shifted into the alkaline region as compared with the native enzyme. The dependence of the activity on the pH is described by the equation of a one-proton transfer (Fig. 3). The calculated $pK_{a(app)}$ values of the ionogenic groups of the enzymes are given in Table 2. It can be seen that the magnitude of the shift ($\Delta pK_{a(app)}$) is between 0.26 and 1.19 units for α-chymotrypsin and between 0.75 and 0.93 unit for trypsin and depends on the method of immobilization. In the case of immobilization on hydrated titanium oxides, the magnitude of the shift also depends on the conditions of formation of the hydroxides (enzymes 5-7). The value of ΔpK_b for glucose oxidase was 0.61 and 0.94 unit, respectively, for the enzymes (12 and 13).

To study the influence of the support on the thermodynamic parameters of the immobilized enzymes we investigated the Michaelis constants, and also the inhibition of the enzymes (1-7) by the negatively charged competitive inhibitor N-acetyl-L-tryptophan. The results obtained show that the Michaelis constant for immobilized α-chymotrypsin (3, 4, 6, 7) scarcely changes. In the case of enzymes (2 and 5), $K_{M(app)}$ increased 2.1- to 2.2-fold. In contrast to this, inhibition by N-acetyl-L-tryptophan of the immobilized α-chymotrypsins became worse (Table 2 and Fig. 4). In the case of enzymes 2-5 and 7, K_I increased 1.5- to 4.5-fold in comparison with the native enzyme. For enzyme (6) immobilized on hydrated titanium oxides precipitated from ammonia, the increase in the inhibitor constant amounted to a factor of almost 22.

In the case of immobilized trypsin (9, 10), to determine the activity of which we used a positively charged derivative of arginine, the Michaelis constants were 5- and 1.13-fold smaller than for the native enzyme.

TABLE 2. Parameters of the Immobilized Enzymes and Properties of the Supports at 25°C

Immobilized enzyme	pK values of the ionogenic groups of the enzyme	Charge of the support from the pK value, mV	$K_{M(app)}$ of the enzyme, mM	K_I of the enzyme, mM	Charge of the support calculated from the K_I value, mV
1. Native α-chymotrypsin	6,78	—	0,72	4,7	—
2. Acrylamide–hydroxyquinoline–Sn–α-chymotrypsin	7,46	—40,5	1,50	6,5	— 8.7
3. Sephadex–Sn–α-chymotrypsin	7,04	—15,3	0,73	15,0	—30,1
4. (Tin hydroxide)–α-chymotrypsin	7,06	—16,7	1.08	21,3	—39.3
5. (Titanium hydroxide)–α-chymotrypsin*	7,97	—70.9	1,58	11,0	—22.1
6. (Titanium hydroxide)–α-chymotrypsin†	7,58	—47.8	0,63	101,6	—79,9
7. (Titanium hydroxide)–α-chymotrypsin‡	7,70	—55.0	0,87	11,4	—23,4
8. Native trypsin	5,79	—	0.53	—	—
9. (Tin hydroxide)–trypsin	6,54	—44,7	0,11	—	—40,7
10. Sephadex–lysine–Sn–trypsin	6,72	—56,0	0,47	—	— 2,9
11. Native glucose oxidase	7,49	—	—	—	—
12. (Tin hydroxides)–(glucose oxidase)	8,10	—36,5	—	—	—
13. (Chromium hydroxides)–(glucose oxidase	8,45	—57.4	—	—	—

*The hydroxides were obtained in phosphate buffer.
†The hydroxides were obtained by precipitation with ammonia solution.
‡The hydroxides were obtained in carbonate buffer.

The absence of the desorption of the proteins under the action of ionic strength on the immobilized enzymes shows that the protein globules are not fixed to the supports with the aid of hydrophobic, hydrogen, or dispersion forces. The capacity of the transition-metal ions for forming coordination bonds shows that the enzymes are fixed to the supports by the formation of coordination bonds. This nature of the support–enzyme bonds is also confirmed by the influence of the Ph on the degree of binding of the protein. Just such a pH dependence is observed in the case of the binding of metal ions by chelating polymers [18-20].

A similar conclusion was arrived at by Kennedy and Kay [21], who investigated the immobilization of enzymes in the acid pH range. However, attention is attracted by the decrease in the binding capacity of the supports on the introduction of additional ligand-forming residues, as in the case of the copolymer (I) and the support (II). This is explained by the fact that in the synthesis of a support (II) using cyanogen bromide, positively charged isourea groupings are formed. The presence of these residues weakens the complex-formation of the transition-metal ions with the modified supports, in spite of the fact that these ions form extremely stable complexes with polyhydroxy compounds. The result of this is a decrease in the capacity of the supports and a weakening of their binding capacity. These are grounds for assuming that the low binding capacity of the support (I) is due to the poorly developed three-dimensional structure of the copolymer.

The mechanism of the fixation of enzymes on hydrated oxides is complex. On the adsorption of enzymes on these supports, coordination bonds are also realized, since the influences of the ionic strength and the pH are similar, as for the ligand-containing supports. However, on the fixation of enzymes by this method mechanical fixation of the protein globules in the structure of the polynuclear complexes formed from the hydrated oxides also takes place [21]. The fact that such a mechanism exists is shown by the increase in the stability of the immobilized enzymes on standing during the period of the formation of the structure of the polynuclear complexes, which takes place for 12-24 h.

The immobilized enzymes studied show a shift of the pH optimum in the alkaline direction. the magnitude of this shift falls with an increase in the ionic strength of the solution. Consequently, this phenomenon can be explained by local acidification [22]. On the basis of the Boltzmann distribution of hydrogen ions, following [22, 23], we estimated the potential of the matrix due to this acidification and the shift of the enzymatic activity in the alkaline direction. The calculated values of these potentials are in the range from —15 to —71 mV (Table 2), reaching the greatest value for α-chymotrypsin immobilized on hydrated titanium oxides.

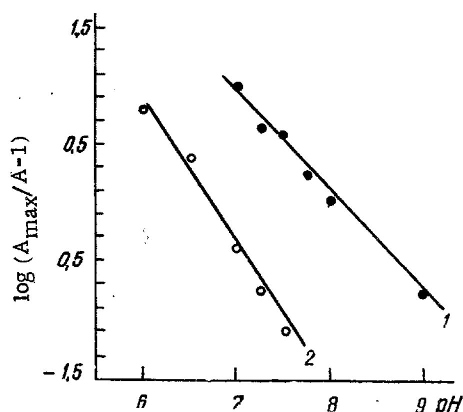


Fig. 3

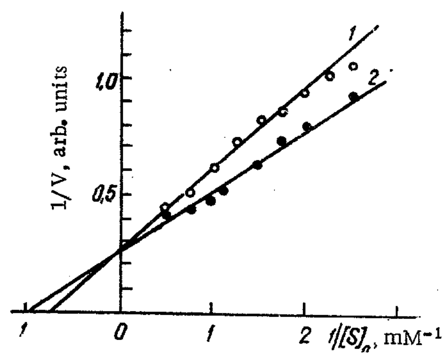


Fig. 4

Fig. 3. Dependence of the activity of (titanium hydroxide) — α-chymotrypsin (1) and native α-chymotrypsin (2) on the pH of the solutions. The immobilized enzyme was obtained in phosphate buffer.

Fig. 4. Dependence of the initial rate of hydrolysis of the ethyl ester of N-acetyl-L-trypsine by (tin hydroxides) — α-chymotrypsin on the concentration of the substrate in the presence of 5 mM N-acetyl-L-tryptophan (1) and in its absence (2). Experimental conditions: 1 mM $CaCl_2$, 10 mM KCl, pH 8.0, 25°C.

If the pH optimum of the action of the enzyme is due to the charge of the matrix, it should also be reflected in the values of K_M and K_I for charged compounds. In actual fact, for a positively charged substrate of trypsin $K_{M(app)}$ decreased, and in the case of a negatively charged inhibitor of α-chymotrypsin the inhibition constant increased. On the assumption of a local concentration of these substances, the action of the charge of the support, it is also possible to take the surface charge into account [23]. The calculated values of the potentials are also negative (Table 2). However, their absolute magnitudes differ from those obtained on the basis of the pH shift of the action optimum. The greatest discrepancy of the calculated potentials is observed for enzyme (2) obtained from a support possessing hydrophobic groupings. In this case, the increase found in the value of the potentials is due to the formation of a hydrophobic bond between the support and the hydrophobic inhibitor, leading to a decrease in the repulsive action of the charge of the support. In the case of other enzymes the discrepancy in the calculated potentials can be explained by diffusion hindrance of the substrate or the inhibitor, and also by its specific interaction with the matrix. Diffusion hindrances may appear, in particular, in the case of the comparatively large particles of Sephadex (enzymes 2 and 10).

The values of the potential of the matrix obtained relate to the zone of action of the active center of the enzyme, i.e., at a distance approximately 2 nm from the surface of the support. Extrapolation of the potential to the surface of the support by means of the Poisson — Boltzmann equation [24] shows that the potential at the surface of the matrix, particularly at an ionic strength of 0.05 M may exceed the calculated value by a factor of 2 and more. The calculated charge density for hydrated tin oxides then amounts to one charge per 0.25 nm^2.

The values of the surface charge of the supports that have been obtained agree with the known values of the isoelectric points of hydrated oxides, which are between 4 and 6.6 [21]. Since the charge is negative, it is not due to the metal ions. It is assumed that the charge of the matrix is determined by the dissociation of the acidic groups and (or) by the adsorption of anions [25]. The acid groups may be represented by the hydroxy groupings forming ligand complex, since the hydrated oxides of the transition metals possess amphoteric properties.

EXPERIMENTAL

The work was carried out with the enxymes trypsin (E.C. 3.4.4.4.), type A, and α-chymotrypsin (E.C. 3.4.4.5.), type A, from the Olaine chemical reagents factory. Crystalline

glucose oxidase (E.C. 1.1.3.4.) from *Penicillium vitale* was obtained as described elsewhere [26]. Sephadex G-200 Fine was from Pharmacia (Sweden). N-Benzoyl-L-arginine ethyl ester and N-acetyl-L-tryptophan were from Reanal (Hungary). N-Benzoyl-D,L-arginine p-nitroanilide was a reagent of the Voikovo chemical reagents factory. N-Acetyl-L-tyrosine ethyl ester was synthesized as described in the literature [27]. Sucrose (kh.ch. ["chemically pure"]), β-D-glucose (kh.ch.), tin tetrachloride (ch.d.a. ["pure for analysis"]), titanium tetrachloride (ch. ["pure"]), iron trichloride (ch.), nickel nitrate (ch.d.a.), and chromium sulfate (ch. d.a.) were used without additional purification.

The synthesis of the ligand-containing support Sephadex—lysine was carried out by adding the copper complex of lysine through the ε-amino group to cyanogen-bromide-activated Sephadex G-200. The acrylamide—hydroxyquinoline was synthesized by the addition of 5-amino-8-hydroxyquinoline to acryloyl chloride followed by the polymerization of the product obtained with acrylamide and N,N'-methylenebisacrylamide [16].

The charging of the supports with metal ions was carried out by stirring 0.1 g of a support in a 5% solution of a metal salt at room temperature for 4 h. The activated support was washed with 100 ml of water and 100 ml of the buffer in which the immobilization of the enzyme was to be performed.

Immobilization of the Enzymes. The support charged with metal ions was placed in a buffer solution of the enzyme the concentration of which was 5 mg/ml, and the mixture was stirred at 3°C for 12-14 h. To immobilize trypsin and α-chymotrypsin we used 0.1 M phosphate buffer, pH 7.2, and for glucose oxidase 0.2 M acetate buffer, pH 5.8. The immobilization of the enzymes on the hydrated metal oxides was carried out at pH 7.2 as described previously [7].

The determination of the amount of bound protein was carried out by a modification of the Lowry—Hartree method [17]. Since the matrix itself gives a strong color reaction with the Folin reagent, the amount of protein was not determined in the case of the synthetic ligand-containing supports.

The determination of the activities of the native and immobilized glucose oxidases was carried out with the aid of an oxygen electrode in 0.2 M acetate buffer, pH 5.8, t 25°C, with 0.17 M glucose [28]. The enzymatic esterase activity of the proteases was determined on a Radiometer TTT-2 pH-stat (Denmark), t 25°C, 0.1 M KCl, pH 8.0, with 5 mM N-acetyl-L-tyrosine ethyl ester for α-chymotrypsin and with 5 mM N-benzoyl-L-arginine ethyl ester in the case of trypsin.

The determination of the $K_{M(app)}$ values of the native and immobilized trypsins was performed by using N-benzoyl-D,L-arginine p-nitroanilide. The initial rate of hydrolysis of this substrate by native trypsin was determined on a Specord UV-VIS spectrophotometer (GDR) at 388 nm (pH 8.2, 0.05 M Tris-HCl, 1 mM $CaCl_2$). In the case of the immobilized trypsin, it was performed in the following manner: 1.9 ml of 0.9-0.13 mM substrate solution was thermostated at 25°C. To this solution was added 0.1 ml of a suspension of the immobilized enzyme in 0.05 M Tris-HCl buffer thermostated at the same temperature. The mixture was stirred for 10 min and the reaction was stopped by boiling it for 2 min. Then the mixture was centrifuged and the optical density of the centrifugates was determined at 388 nm.

The determination of the competitive inhibition constant of α-chymotrypsin under the action of N-acetyl-L-tryptophan was carried out 0.01 M KCl solution at concentrations of the substrate of 0.4-2 mM, of the inhibitor of 5 mM, and of $CaCl_2$ of 1 mM. The calculations of the constants were carried out by the method usually adopted [29].

The determination of the pH dependence of the activities of the immobilized protease was carried out in 0.01 M KCl solution and of the glucose oxidase in 0.01 M phosphate buffer solutions at pH 6-8.5.

The influence of the ionic strength on the immobilized enzymes was investigated in 3 ml of buffer solution by keeping 40-50 mg of immobilized enzymes at 25°C for 12 h and determining the optical densities of these solutions at 280 nm.

SUMMARY

1. Preparations of trypsin, α-chymotrypsin, and glucose oxidase immobilized on synthetic and polysaccharide supports charged with transition-metal ions contain 3-64 mg of protein per 1 g of support.

2. The activity of immobilized enzymes amounts to 100-10,000 U/g. The activity yield is 2.2-90%.

3. The pH dependence of the enzymes is shifted in the alkaline direction by 0.26-1.19 units. The Michaelis constants and inhibitor constants have decreased by factors of 1.5-21.

4. The mechanism of the fixation of enzymes is determined by the formation of coordination bonds and by inclusion in inorganic gels. The properties of the enzymes are due to the surface charge of the activated supports.

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