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IMMOBILIZATION OF A LIPASE FROM *Pseudomonas aeruginosa* ON A MAGNETIC SUPPORT MODIFIED WITH A POLYAMIDE

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Covalent and adsorption methods for immobilizing an alkaline lipase from Pseudomonas aeruginosa *on magnetite coated with a polyamide have been developed. Highly active preparations of alkaline lipase with magnetic properties have been obtained.*

The immobilization of enzymes permits catalytic properties to be imparted to ordinary materials, while, conversely, the enzymes may acquire the properties of these materials. The immobilization of enzymes on supports with magnetic properties is of interest in this respect. On the one hand, this broadens the possibilities of engineering enzymology in the field of facilitating the separation of the components of a reaction mixture, and, on the other hand, it creates the possibility of the directed transport of substances. Although at the present time it has been proposed to use biomagnetics for immobilizing ligands in the isolation of various biologically active substances in separators with a magnetic field [1], and the practical use of magnetic materials in the purification of waste waters [2], for magnetic radioimmune analysis [3], as sensor elements [4], for the directed transport of drugs [5], etc., is already known, the methods of obtaining such "magnetic" enzymes are small in number and are laborious [3—6]. The magnetic materials used for biotechnical purposes have either a biogenic or a synthetic nature. A source of natural magnetic particles is formed by magnetotactic bacteria [6]. Methods have been developed for the synthesis of artificial magnetic particles based on magnetite Fe_3O_4 [3], although Zn-ferrite, hematite, and other compounds possessing magnetic properties may also be used.

In the present paper we propose a fairly simple method of obtaining immobilized enzymes with magnetic properties, using our experience of the covalent binding of enzymes on polyamide supports. As a polymeric coating of magnetite we have chosen a polyamide used previously for immobilizing a number of proteins and enzymes [7]. We have immobilized an alkaline lipase from *Pseudomonas aeruginosa* on a support with functional amino groups on its surface obtained in this way.

As is known, the activity of an immobilized enzyme and its properties depend to a considerable degree both on the nature of the support and on the method of binding with it. For the immobilization of the *Pseudomonas aeruginosa* lipase on the sorbent that we had synthesized we used two methods — covalent binding and adsorption.

In covalent binding the enzyme was directly attached to the amino groups of the support with the aid of glutaraldehyde, as described in [7], while on the adsorption binding of the enzyme the support was first modified with phosphatidylethanolamine (cephalin). The modification consisted in the covalent addition of cephalin with the aid of glutaraldehyde. Such a sorbent has been used previously for the hydrophobic immobilization of a number of proteins, including a lipase [8].

Sorption of the lipase was carried out from an unpurified preparation of G10X alkaline lipase containing a considerable amount of ballast proteins. Here we pursued the aim of demonstrating the possibility of obtaining highly active lipase preparations with no expensive preliminary purification of the enzyme.

In both variants of immobilization, to select the optimum conditions we varied the amount of enzyme charged onto the support. Table 1 gives indices of the lipase immobilization process. It can be seen that with an increase in the amount of protein charged onto the sorbent (from 25 to 100 mg/g of sorbent) the amount of active enzyme bound with the sorbent rose.

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TABLE 1. Characteristics of the Immobilization of the Alkaline Lipase from *Ps. aeruginosa* on a Magnetic Support

Type of immobilization	Amount		Activity of the immobilized enzyme		Residual activity, %
	adsorbed protein, mg/g of sorbent	bound enzyme, units/g of sorbent	units/g of sorbent	units/mg of protein	
			Covalent (Fe_3O_4—PA—lipase)		
1*	2.33	116	10.2	4.4	8.8
2	1.94	82	27.3	14.1	33.0
3	1.69	60	29.6	17.5	49.0
			Adsorption (Fe_3O_4—PA—PEA—lipase)		
1*	1.62	117	19.8	12.2	16.9
2	1.18	86	33.2	28.1	38.6
3	0.98	43	28.8	67.0	29.4

*The amounts of protein charged onto the sorbent in the immobilization processes were: 1) 100; 2) 50; and 3) 25 mg/g.

TABLE 2. Desorption of Adsorption-Immobilized *Ps. aeruginosa* Lipase

Experiment No.	Eluting solution	Eluted enzyme, %
1	Universal buffer, 0.1 M, pH 7	0
2	pH10	11.3
3	0.2% polyvinyl alcohol	4.6
4	0.5% polyvinyl alcohol	4.4
5	1M NaCl	1.9
6	2M NaCl	6.4
7	5% Triton X-100	7.8
8	60% ethylene glycol	2.9
9	0.1 N EDTA	7.4
10	15% ethanol	6.8
11	15% isopropanol	4.2

However, a simultaneous fall was observed in the specific activity of the immobilized enzyme calculated per milligram of bound protein. Both with covalent and with adsorption immobilization, the maximum specific activity of the enzyme was shown for the smallest amount of protein charged onto the sorbent (25 mg/g), although, thanks to the greater amount of bound protein, the maximum activity of the adsorption-immobilized enzyme calculated to 1 g of sorbent was observed at a protein charge of 50 mg/g of sorbent.

The fall in the specific activity of the immobilized enzyme with an increase in the amount of protein adsorbed is apparently connected with a blocking of the access of the substrate to the active center when the enzyme molecules are close to one another. It can also be seen from Table 1 that the maximum specific activity of the adsorption-immobilized enzyme was higher (29.4 units/mg) than the analogous index for the enzyme immobilized by the covalent method (17.5 units/mg).

On the adsorption immobilization of the enzyme it was possible to achieve the retention of about 67% of the bound enzyme activity on the sorbent. In the case of covalent immobilization, the maximum retention was about 49% of the activity.

Thus, the adsorption method of immobilizing the enzyme enabled a preparation with a higher activity to be obtained. However, together with the evident efficacy of this method of immobilizing the lipase, a fear of instability of the preparation obtained could arise. For this reason, special investigations were made that showed an adequate strength of the binding of the enzyme. As can be seen from Table 2, the bulk of the immobilized lipase was not eluted even under fairly severe conditions.

TABLE 3. Repeatability of the Use of the Adsorption-Immobilized *Ps. aeruginosa* Lipase

Number of times used	Residual activity, % of initial
1	100
2	88
3	76
4	68
5	65
6	63
7	60
8	57
9	48
10	45

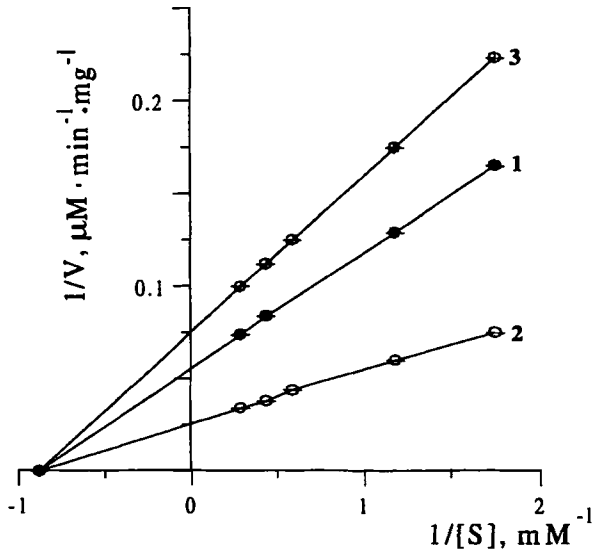

Fig. 1. Substrate dependence of the cleavage of tributyrin by *Ps. aeruginosa* lipase in the Lineweaver—Burk coordinates: *1*) native lipase; *2*) adsorption-immobilized enzyme; *3*) covalently immobilized enzyme.

The strength of the binding of the enzyme was ensured, together with the repeatability of its use, as is shown by the results given in Table 3. Even after ten work cycles, the adsorption-immobilized lipase retained a considerable part of its initial activity.

A comparative analysis of the kinetic parameters of the alkaline lipase immobilized on magnetite-polyamide by the covalent and adsorption methods showed that in neither variant did the affinity of the enzyme for the substrate — tributyrin — change after immobilization, as was shown by identical values of the apparent Michaelis constant (K_M) found graphically in the Lineweaver-Burk coordinates (Fig. 1). After immobilization the maximum rates of reaction had changed. At the same time, the value of V_{max} for the covalently immobilized enzyme was lower than the analogous index for the native lipase, while, conversely, that for the adsorption-bound enzyme was higher. In the second case, such an apparently unexpected fact, when the maximum rate of the reaction of the immobilized enzyme is higher than that for the native enzyme, is obviously due to the selective sorption of the enzyme on the latter sorbent.

A comparative investigation of some properties of the native and the immobilized lipases showed some shift of the pH optimum of he enzyme towards the alkaline region after immobilization by both methods (Fig. 2, *a*), while the temperature optimum (Fig. 2, *b*) did not change in the case of covalent immobilization and shifted slightly in the direction of higher temperatures on adsorption. Unfortunately, in contrast the generally observed rise in the stability of an enzyme after immobilization, the immobilization of the lipase did not lead to stabilization but, on the contrary, when the enzyme was

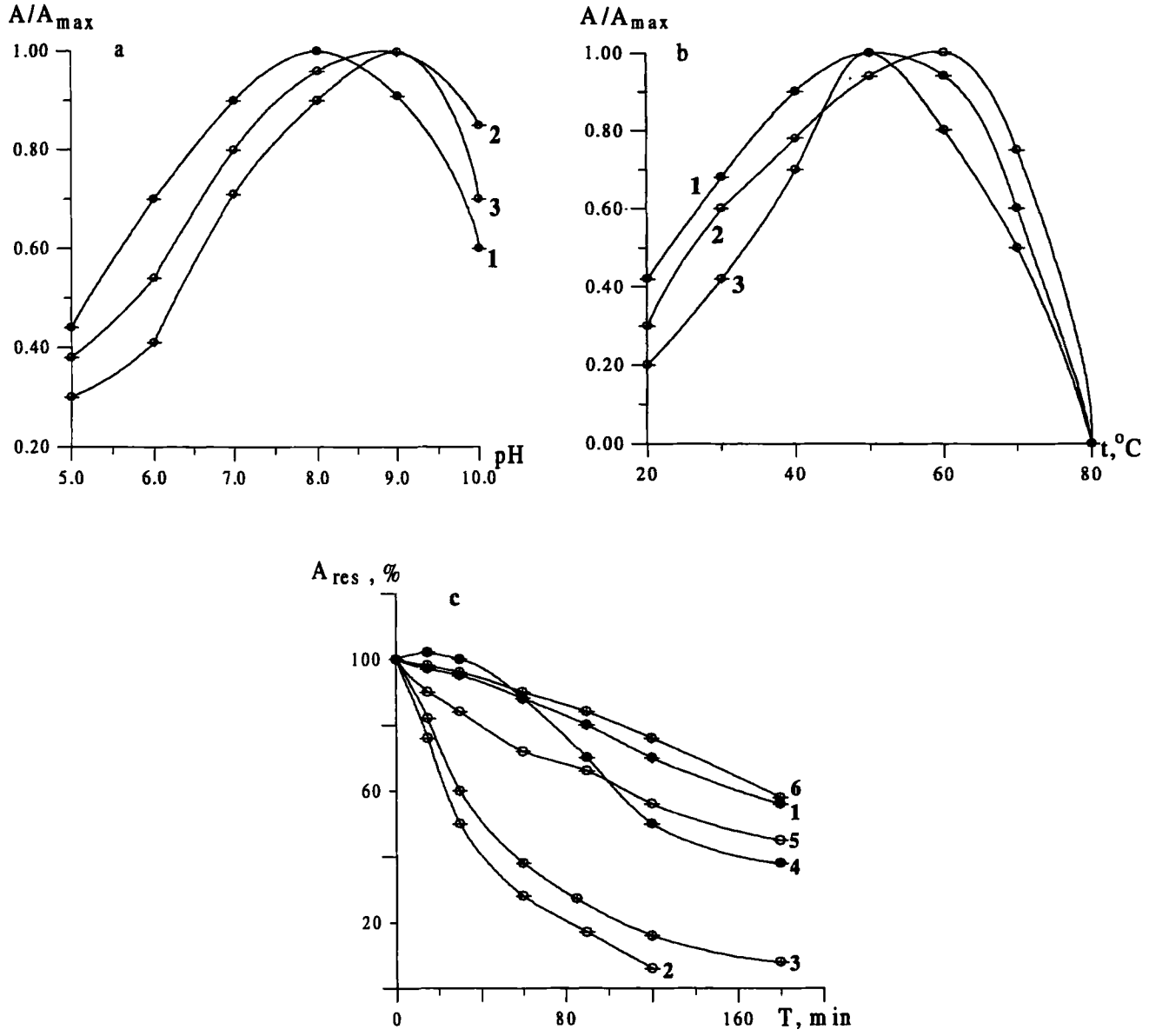

Fig. 2. Some properties of native and immobilized *Ps. aeruginosa* lipase: *a*) pH dependence; *b*) temperature dependence of the activity; *c*) stability of the enzyme at 60°C, pH 9.0; *1*) native enzyme; *2*) adsorption-immobilized lipase; *3*) covalently immobilized lipase; *4*) native enzyme in the presence of the substrate; *5*) adsorption-immobilized enzyme in the presence of the substrate; *6*) covalently immobilized enzyme in the presence of the substrate.

incubated at 60 °C without a substrate there was actually some fall in this index (Fig. 2, *c*; curves *1*—*3*). This is possibly connected with the loss of some kind of stabilizing agents by the enzyme. However, no supplementary investigations elucidating the nature of this phenomenon were performed within the framework of this study. On the other hand, the operative stability of the enzyme, determined in the presence of the substrate tributyrin, rose for the immobilized enzyme after incubation for an hour at 60°C (Fig. 2, *c*; curves *4*—*6*).

Thus, both the methods that have been developed for the immobilization of alkaline lipase on a magnetic support enable highly active preparations for repeated use to be obtained. In the case of adsorption hydrophobic immobilization, thanks to the selectivity of the sorption of the lipase, it was possible to achieve a rise in the maximum rate of the reaction for the immobilized

enzyme and to retain a larger amount of active enzyme in percentage relationship to that theoretically bound. A deficiency of the preparations obtained was the fall in the stability of the immobilized lipase on heating. However, the operative stability of the lipases immobilized by both methods, determined in the presence of the substrate, rose after the preparation had been incubated for an hour at 60°C.

EXPERIMENTAL

We used a preparation of G10X alkaline lipase from the culture liquid of *Ps. aeruginosa* IN-25a provided by the Biotekhnologiya NPO (Scientific Production Combine).

Magnetite Fe_3O_4 was obtained in a similar way to that described in [9], and then the magnetic granules were coated with a layer of polyamide from a solution of the polyamide in formic acid.

Immobilization of the Lipase. 1. Covalent Immobilization. The sorbent (2 g) was equilibrated in 22 ml of 0.1 M borate buffer, pH 8.5, and then 0.3 ml of 25% glutaraldehyde was added and the mixture was incubated with stirring for 2 h, after which it was washed with 0.05 M borate buffer, pH 8.5. Immobilization was carried out in borate buffer, pH 9.0, at 4°C for 24 h. For this, a weighed amount of enzyme (30, 50, or 100 mg, corresponding to the three variants) was dissolved in 22 ml of borate buffer and, with stirring, was incubated with the modified sorbent, after which the support with the immobilized enzyme was washed.

2. Adsorption Immobilization. A suspension of the sorbent (2 g) in 11 ml of 0.1 M borate buffer (pH 9.0) was treated with 11 ml of phosphatidylethanolamine (40 mg/ml) in a mixture of 0.1 M borate buffer (pH 9.0) and ethanol (ratio 1:1, *v/v*) and was stirred at 4°C for 48 h. Then the unbound phosphatidylethanolamine was eliminated by washing the sorbent with a tenfold volume of diethyl ether.

The sorbent so obtained was treated with 21.5 ml of 0.1 M borate buffer, pH 8.5, containing 0.5 ml of ethanolamine and the mixture was stirred at 4°C for another 2 h. The excess of ethanolamine was eliminated by washing the sorbent with a tenfold volume of the initial buffer.

The immobilization of the lipase by method 2 was conducted under the same conditions as on covalent immobilization.

The amount of enzyme bound was determined from the decrease in its activity and the amount of total protein in the supernatant after incubation of the sorbent with a solution of the enzyme.

Lipase activity was determined by potentiometric titration [10]. The standard reaction mixture, with a volume of 10 ml, contained 0.23 mmole of tributyrin, 0.02% of polyvinyl alcohol, and 1.5 mmole of NaCl. Unless otherwise specified, the reaction was conducted at pH 9.0 and 45°C. The specific activity of the enzyme was expressed in micromoles of acid products formed in 1 min calculated to 1 mg of protein or 1 g of sorbent.

Protein was determined by the Lowry method and spectrophotometrically.

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