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COMPARATIVE STUDIES ON IMMOBILIZATION OF HUMAN PROSTATIC ACID PHOSPHATASE

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Summary

Acid phosphatase (othophosphoric monoester phosphohydrolase (acid optimum), EC 3.1.3.2) from the human prostate was immobilized by its protein moiety on cyanogen bromide-activated Sepharose, by carbohydrate moiety on Concanavalin-A-Sepharose, and by Schiff base formation with partially oxidized carbohydrate groups on ethylenediamine-Sepharose.

The highest retention of enzyme activity, 80%, was found for the noncovalent immobilization on Concanavalin-A-Sepharose. It was demonstrated that the optimal pH changes for the Concanavalin-A-Sepharose and CNBr-Sepharose-enzyme complexes are electrostratic in character. In all cases of immobilization the enzyme has higher thermostability than that for the native enzyme under the same conditions.

The effects of the enzyme stabilization were interpreted in terms of the multipoint interaction between the enzyme molecule and the carrier.

Introduction

Acid phosphatase (orthophosphoric monoester phosphohydrolase (acid optimum), EC 3.1.3.2) from the human prostate is a glycoprotein of established chemical composition [1,2]. The theoretical and practical importance of this enzyme attracts attention for elucidation of the role which the protein and carbohydrate components play in the mechanism of the enzyme's catalytic action. For instance, it was demonstrated [3] that the partial modification of the carbohydrate moiety of the enzyme by an action of neuraminidase in prac-

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Abbreviations: Con-A, Concanavalin A; Con-A-Sepharose, Concanavalin-A combined to CNBr-activated Sepharose.

tice does not change its activity towards the low molecular weight substrates.

Immobilization is now a good method for studying some aspects of enzyme behaviour [4]. Acid phosphatases of bacterial and animal origin were immobilized by several adsorption [5,6] and covalent [7,8]methods, but all results are only descriptive. It seemed of interest to explore the existing possibilities for immobilization of prostatic acid phosphatase by various methods involving different parts of its molecule and to compare the preparations obtained in these ways. Thus the enzyme was immobilized by covalent binding with CNBr-activated Sepharose [9], by noncovalent binding with Con-A-Sepharose through the carbohydrate part of the molecule [10], and also by the covalent bond with ethylenediamine-Sepharose after partial oxidative modification of the carbohydrate residues of the enzyme [11].

The preparation and comparison of properties of the immobilized forms of the enzyme is the subject of this paper.

Experimental procedures

Materials

Pure acid phosphatase I from the human prostatic gland was obtained as a solution in 0.05 M phosphate buffer, pH 6.5, containing 0.92 mg/ml and specific activity of 250 I.U. per mg of enzyme protein [12]. Sepharose-4B and CNBr-Sepharose-4B were from Pharmacia, Uppsala (Sweden); *p*-nitrophenylphosphate, disodium salt, was from Merck, Darmstadt (W. Germany); Concanavalin A was obtained from Sigma (USA); methyl-α-D-mannopyranoside was the product of Koch-Light (England). All other reagents were from POCH, Gliwice (Poland).

Methods

Preparation of Con-A-Sepharose. This was prepared according to Porath et al. [10]. The amount of bound Con-A was determined spectrophotometrically at 280 nm from the differences in the amount of protein before and after the reaction. The preparation of Con-A-Sepharose contained 8 mg of the protein/g of carrier.

Preparation of ethylenediamine-Sepharose [11]. The Sepharose 4B gel, 1 g, washed on a sintered glass filter with 200 ml of 0.001 M HCl, was mixed with 50 mg of ethylenediamine in 5 ml of 0.1 M carbonate buffer, pH 8.3, containing 0.5 M NaCl and maintained overnight at 4°C. The preparation was then treated with 1 M ethanolamine at pH 8, then washed several times with 0.1 M acetate buffer pH 4.0 and 0.1 M borate buffer pH 8.0. The product was dried on the filter and stored cold.

Oxidation of carbohydrate groups of acid phosphatase. The enzyme, 50 μg in 5 ml of 0.1 M acetate buffer solution, pH 4.7, was mixed with a solution of 5 mg of $NaIO_4$ in 1 ml of the same buffer and oxidized for 12 h at 4°C. The oxidation reaction was terminated by an addition of 5% thiosulphate solution or a solution of potassium iodide in 0.001 M HCl. (Special precautions should be kept in mind since the free iodine completely inhibits the enzyme activity, [13].) The loss of the enzyme activity in the applied conditions did not exceed 50%. As a result of this reaction, due to the oxidation of the carbohydrate resi-

dues of the enzyme, carbonyl groups are formed which are to react with the amine groups of the carrier [11].

Immobilization of acid phosphatase on CNBr-activated-Sepharose. To the enzyme, 35 μg, in 0.5 ml of 0.1 M $NaHCO_3$ solution containing 0.5 M NaCl, 100 mg of CNBr-Sepharose gel washed with 0.001 M HCl was added and mixed. The immobilization reaction was carried out overnight at 4°C. Then the product was washed on the glass filter with 1 M NaCl, 0.001 M HCl, 0.1 M acetate buffer pH 4.0 and 0.1 M borate buffer pH 8.0, dried on the filter and stored in the cold. Spectrophotometric measurements at 280 nm proved 100% binding of the enzyme by the carrier.

Immobilization of the oxidized acid phosphatase on ethylenediamine-Sepharose. The reaction mixture after the oxidative modification of acid phosphatase was brought up to pH 8.3 and 100 mg of the ethylenediamine-Sepharose were added. Immobilization was carried out for 12 h at 4°C with constant stirring. The preparation was washed, as for the enzyme immobilized on CNBr-Sepharose. 100% binding was found.

Immobilization of acid phosphatase on Con-A-Sepharose. To 35 μg of enzyme protein in 5 ml of 0.1 M acetate buffer pH 6.8, 100 mg of Con-A-Sepharose were added and the reaction was carried out for 12 h at 4°C with constant stirring. The product was washed on the glass filter as above. 100% binding of the enzyme with the carrier was achieved.

Determination of enzymic activity. The activity of insoluble acid phosphatase was determined using 20 mM solution of *p*-nitrophenylphosphate as a substrate in 0.1 M citrate buffer pH 5.2 [14]. Incubation at 37°C was terminated by adding 0.1 M NaOH. The sample was freed of insoluble material by centrifugation and absorbance at 400 nm was measured.

Results and Discussion

Characteristic data for the preparations of the immobilized phosphatase are given in Table I. From these data in follows that in all cases active immobilized acid phosphatase was obtained.

The activity of Con-A-Sepharose bound enzyme is retained to a remarkable extent, 80%, while for the enzyme covalently bound through the protein moiety the loss of activity is 70%. Participation in the immobilization of ε-amino groups of lysine residues of the protein [15] probable leads to deep changes in the conformation of polypeptide chain, which results in loss of enzyme activity. The immobilization of the partially modified enzyme by oxidation with $NaIO_4$ gives almost 90% loss of activity. It was shown [16] that the oxidation itself can lead to some changes in the amino acid residues of the active center and to the distortion of the hydrophylic-hydrophobic equilibrium in the enzyme molecule.

It was further observed that in the case of immobilization on Con-A-Sepharose the enzyme is not bound by the covalent linkages as was previously suggested for other systems, for which Con-A was used as a carrier [15,17]. The noncovalent binding in the complex studied can be unequivocably proved by the complete elution of the enzyme activity after treatment of the complex with an 0.5 M solution of methyl-α-D-glucopyranose in 0.1 M acetate buffer,

TABLE I

PROPERTIES OF PREPARATIONS OF IMMOBILIZED PROSTATIC ACID PHOSPHATASE

Carrier	Reatined enzymic activity in the complex (percent)	pH optimum	K_m
Con-A-Sepharose	80	6.0	$2.0 \cdot 10^{-4}$ **
CNBr-Sepharose	30	4.5	$1.0 \cdot 10^{-3}$
Ethylenediamine-Sepharose	12 *	5.0	$4.5 \cdot 10^{-3}$
Native phosphatase	—	5.0	$2.5 \cdot 10^{-4}$

* Value calculated in respect to the activity of the oxidized phosphatase by $NaIO_4$ (50% of the activity of the native enzyme).

** K_m values were obtained from Lineweaver-Burk plots in 0.1 M citrate buffer solutions at optimal pH for each preparation.

pH 6.0 [18]. Such results were not achieved in the case of acid phosphatase immobilized on CNBr-Sepharose and on ethylenediamine-Sepharose, which proves that covalent binding occurs in these systems.

For the preparations obtained by the immobilization of phosphatase on CNBr-Sepharose and Con-A-Sepharose, changes in the pH optimum profile were observed (cf. Table I). Optimal pH for Con-A-Sepharose bound enzyme is shifted toward the alkaline region (from pH 5.0 to 6.0), whereas for CNBr-Sepharose bound enzyme it is shifted toward the acid side (pH 4.5). This can be explained in terms of electrostatic phenomena, because a local increase of pH near the surface of the carrier should lead to the shift in optimal pH toward the more acidic range [19]. The alkaline shift for Con-A-Sepharose bound enzyme can be only partially explained by the acidic character of the carrier surface, since even at high ionic strength, up to 2 M, of NaCl solutions, one could not regain the pH optimal for native enzyme, which suggests a more complex mechanism for changes in the pH profile of immobilized enzymes [20]. In the case of ethylenediamine-Sepharose bound, iodate-oxidized phosphatase, the pH optimum of the native enzyme is retained but its profile is broader.

The data from Table I for the K_m values of the immobilized preparations of acid phosphatase show that for the non-covalent binding through the carbohydrate moiety this value does not differ in comparison with that for the native enzyme. However, in the case of immobilization through the protein moiety, the K_m value decreased fourfold. This can be explained in terms of steric factors and changes in the conditions of diffusion of the substrate and product molecules, which modulate enzyme kinetic properties [21]. A similar decrease of the K_m value was observed for the immobilized, iodate-modified enzyme.

Investigations of the thermal stability of the products obtained showed enhanced resistance to heating at 55°C of the immobilized acid phosphatase comparing to the native enzyme (Fig. 1). The highest thermal stability shows in the product of non-covalent binding with Con-A-Sepharose. It becomes clear when the process of immobilization is regarded as the strong interaction of the enzyme with the complementary surface of the carrier. In accordance with reports [22,23] on the role of multilateral interactions in the process of stabili-

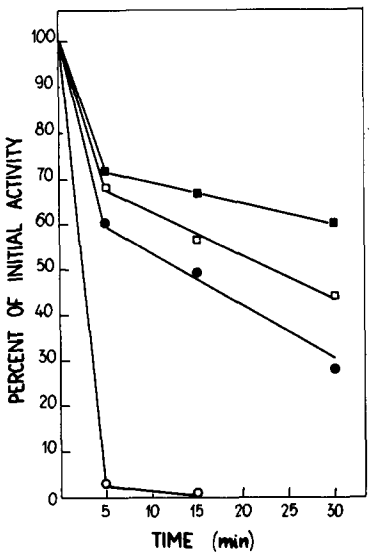

Fig. 1. Rates of thermal inactivation of the native and immobilized prostatic acid phosphatase. Samples in 0.1 M borate buffer, pH 7.1, were heated at 55°C for indicated intervals, then cooled and their activity determined at pH 5.2 (see Methods). ○, Native enzyme; ●, ethylenediamine-Sepharose-phosphatase; □, CNBr-Sepharose-phosphatase; ■, Con-A-Sepharose-phosphatase.

zation of the enzyme conformation, one can conclude that under these circumstances the highest thermal stability should be expected. Similarly, one can predict high thermostability for the covalent immobilization of the enzyme on CNBr-activated Sepharose. In this case a large amount of ϵ-amino groups of the lysine residues in the enzyme [1] provides numerous possibilities for contacts between the enzyme and the carrier matrix. In the case of ethylenediamine-Sepharose-bound enzyme, previously oxidized by iodate, such contacts are limited because the reacting groups are remote and the existence of only a few linkages between the carrier and the enzyme molecule is more plausible. This is partially confirmed by the small thermal stability observed with this preparation (Fig. 1).

It is evident from the results described, that the comparative immobilization of acid phosphatase by various methods and on different carriers enables us not only to obtain the preparation of immobilized enzyme with favorable properties for practical use (for instance, for continuous dephosphorylation of nucleotides) but also to estimate the role of particular components in the catalytic function of the enzyme molecule.

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