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IMMOBILIZATION OF A PROTEASE ON GRAFTED POLYETHYLENES

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UDC 577.154

It has been shown that when the protease of *Bacillus mesentericus* is immobilized on polyethylene with grafted-on polyacrylic acid by the carbodiimide method there is a considerable retention of its activity and an increase in its thermal stability, and the immobilized preparations are active on repeated use.

The wide use of proteases makes it necessary to obtain them in the immobilized state [1]. The immobilization of a protease from *Bacillus mesentericus* on Silochromes with the aid of various linking reagents distinguished by a considerable degree of binding of the protein has not given satisfactory results in relation to stability and repeated use [2]. It therefore appeared of interest to consider the possibility of immobilizing the protease on polyethylenes with grafted-on poly(acrylic acid) and poly(allyl alcohol). The advantage of polymeric supports of this type consist in the fact that the functional groups are readily modified; because of the "loose" packing of the grafted-on coating the functional groups are accessible for interaction with cross-linking reagents over the whole depth of the grafted-on layer, i.e., such polymeric supports combine within themselves the advantages of linear homopolymers and of three-dimensional polyligands.

The modification of the grafted polyethylenes was carried out by the following scheme using the carbodiimide, benzoquinone, and azide methods (see scheme, following page.)

As follows from Table 1, the maximum amount of protein is bound to polyethylene with grafted-on polyacrylic acid (PE-gr-PAAc) prepared by the carbodiimide method (ratios of enzyme to support 1:4 and 1:10). In this case, the ratio of linking agent to enzyme also has a considerable influence on the degree of binding of the protein. Thus, at a ratio of 1:2 20.6 mg of protein is bound per 1 g of support, and at a ratio of 1:20 only 1.5 mg. However, the caseinolytic activities of the immobilized preparations depend little on the ratios of linking agent to enzyme and of enzyme to support, amounting to 24.2-33.8%, which corresponds to the results of the immobilization of the *Bacillus mesentericus* protease on Silochromes modified with transition-metal salts [2]. With the azide method of linkage, both the degree of binding and the retention of the caseinolytic activity fall (to 5.5% and 12.2%, respectively). In the case of immobilization on polyethylene with grafted-on poly(allyl alcohol) (PE-gr-PAAl) with the aid of benzoquinone, however, inactive preparations were obtained.

When the protease immobilized on PE-gr-PAAc by the carbodiimide method was stored at 4°C for 4.5 months 94% of the initial activity was retained, while for the preparations immobilized on Silochromes only 22-68% of the activity was retained after a month.

Institute of Physical Chemistry, Academy of Sciences of the Ukrainian SSR, Odessa. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 512-516, July-August, 1983. Original article submitted June 10, 1982.

 0009-3130/83/1904-0482$07.50 © 1984 Plenum Publishing Corporation

TABLE 1. Properties of the *Bacillus mesentericus* Protease Immobilized on Grafted Polyethylenes

Support	Linking reagent	Ratio of linking reagent to enzyme	Ratio of enzyme to support	Amount of immobilized protein, mg/g of support	Degree of binding of the protein, %	Retention of caseinolytic activity, %	Stability of the immobilized proteinase on storage, % of maximum
PE-gr-PAAc	Carbodiimide	1 : 20	1 : 10	1,5	2,0	30,6	94 after storage for 4.5 months
		1 : 10	1 : 10	3,2	4,4	24,2	
		1 : 2	1 : 10	20,6	28,7	30,0	
		1 : 1	1 : 20	10,4	29,0	33,8	
		1 : 5	1 : 4	26,7	15,0	30,2	
PE-gr-PAAc	Azide			8,2	5,5	12,2	
PE-gr-PAA1	Benzoquinone			14,5	14,6	0	

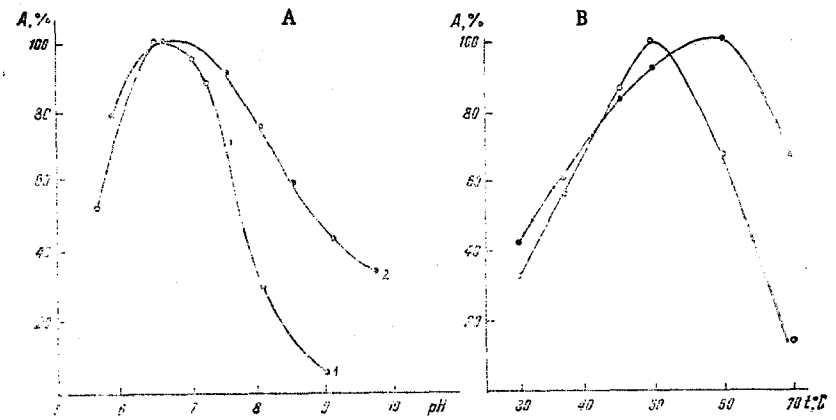
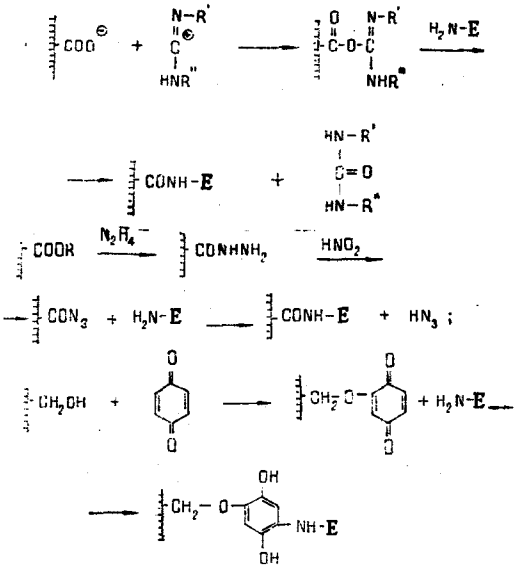


Fig. 1. Dependence of the activity of the *Bacillus mesentericus* protease immobilized on polyethylene with grafted-on poly(acrylic acid) activated by carbodiimide on the pH (A) and the temperature (B) (in % of the maximum): 1) native protease; 2) immobilized protease.



The above-mentioned preparations possess considerable stability (as compared with the preparations immobilized on Silochromes) on repeated use (Table 2). After being used twenty times, the activity had fallen to 17.5%, while the protease immobilized on Silochromes can be used only three to seven times before the caseinolytic activity has disappeared completely.

TABLE 2. Stability of the Immobilized *Bacillus mesentericus* Protease on Repeated Use

Retention of caseinolytic activity (% of the maximum) of the protease immobilized on the following supports	Experiment No.																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
PE-gr-PAAc by the carbodiimide method	100	41	39,8	35,3	35,3	32,5	32,3	29,9	28,4	27,8	23,3	19	19	18,8	18,8	18,5	18,3	18	17,5	17,5
Silochrome ($TiCl_4$ linkage)	100	71,7	44,2	33,2	29,4	24	24	0												
Silochrome ($ZrOCl_2$ linkage)	100	44,7	17,1	5,9	3,3	0														
Silochrome ($HfOCl_2$ linkage)	100	11,0	0,7	0																

The pH optima of the native enzyme and that immobilized on PE-gr-PAAc coincide (Fig. 1A), but at pH 9.0 the activity of the native enzyme is only 5%, and that of the immobilized enzyme 35%, of the maximum at pH 6.8.

A study of temperature inactivation showed that at 70°C 10% of the initial activity of the native protease and 70% of that of the immobilized enzyme were retained (Fig. 1B). The maximum activity of the immobilized enzyme was found at 60°C and that of the native enzyme at 50°C. From the tangent of the angle of slope in the Arrhenius coordinates [3] we determined the activation energies as 5.7 kcal/mole for the native enzyme and 7.5 kcal/mole for the immobilized enzyme. Thermal stability was studied 50°C. For the native and immobilized preparations, the apparent inactivation rate constants were $1.1 \cdot 10^{-2}$ and $3.0 \cdot 10^{-3}$ min^{-1}, respectively.

We investigated the dependence of the rate of hydrolysis of casein on its concentration in solution: For the native protease and that immobilized on PE-gr-PAAc by the carbodiimide method a linear relationship was found in the Lineweaver–Burk coordinates in the range of casein concentrations of from 0.75 to 5.0 mg/ml [4]. The similar values of K_m for the native protease (K_m 1.4 mg/ml) and the immobilized enzyme (K_m 1.65 mg/ml) indicate an invariability of the kinetic properties.

EXPERIMENTAL

We used the *Bacillus mesentericus* protease obtained as described previously [5]. Proteolytic activities were determined by the method of Petrova and Vinugonaite [6]. As the unit of proteolytic activity we took that amount of enzyme that increased the absorption of a solution at 280 nm by 1.0 on incubation with casein for 10 min [7]. The initial preparation contained 71.6% of protein as determined by Lowry's method in Hartree's modification [8].

Polyethylene with poly(acrylic acid) (6-10%) and poly(allyl alcohol) grafted on by the method of gas-phase grafting polymerization induced by ionizing radiation (^{60}Co γ-radiation, the accelerated electrons (ē) of a linear accelerator, or a low-temperature high-frequency discharge plasma) was supplied by A. D. Pomogailo (Institute of Chemical Physics of the Academy of Sciences of the USSR). The 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide p-toluene sulfonate used was a Serva product.

Preparation of the Azide of Poly(acrylic acid) grafted onto Polyethylene and the Immobilization of the *Bacillus mesentericus* Protease. A suspension of 6.7 g of PE-gr-PAAc in 300 g of 1% methanolic HCl was boiled for 40 min. The product was separated off and dried. A suspension of 2 g of the preparation obtained in 330 ml of absolute methanol containing 1.7 ml of hydrazine hydrate was stirred at 38°C for 3 days. The product was centrifuged off, washed with methanol, and dried. With stirring and cooling to 2°C, 6 ml of 3% $NaNO_2$ in water was added to a suspension of 0.62 g of the PE-gr-PAAc hydrazide in 94 ml of 2% HCl. After 20 minutes from the addition of the $NaNO_2$, the azide formed was filtered off and was washed with ice water to neutrality [9] and was suspended in 0.05 M borate buffer, pH 8.55 (10 ml), and then a cooled solution of 100 mg of the enzyme in 15 ml of the same buffer was added. The mixture was stirred at 1-4°C for 1 h 45 min. The product was washed with 100 ml of buffer solution, 100 ml of 1 M KCl, and 200 ml of distilled water.

Immobilization of the Protease on Polyethylene with Grafted-On Poly(allyl alcohol) Activated with Benzoquinone. With stirring, 270 mg of benzoquinone in 10 ml of ethanol was added to a suspension of 500 mg of the support (15% of PAAl) in 40 ml of 0.1 M phosphate buffer, pH 8.0. The mixture was shaken at room temperature for 1 h. Then the support was washed with 200 ml of 20% ethanol, 150 ml of 1 M KCl, and 250 ml of distilled water. To a cooled suspension of the support in 5 ml of 0.1 M phosphate buffer, pH 8.0, was added a cooled solution of 50 mg of the enzyme in 5 ml of the same buffer, and the mixture was shaken at 4°C for a day.

Immobilization of the *Bacillus mesentericus* Protease on Polyethylene With Grafted-On Poly(acrylic acid) with the Aid of Carbodiimide. At 4°C, to 500 ml of the support in 5 ml of Tris-HCl buffer containing 0.005 M $CaCl_2$, pH 7.4, were added solutions of carbodiimide in 2 ml and of the enzyme in 3 ml of the same buffer (see Table 1). The reaction mixture was incubated with shaking at 4°C for 24 h, and the product was washed with 100 ml of cooled 0.05 M Tris-HCl buffer containing 0.005 M $CaCl_2$, with 100 ml of 1 M KCl, and with 200 ml of distilled water. The preparations obtained were stored in distilled water in the refrigerator at 5°C.

Determination of Caseinolytic Activity on Repeated Use. A solution of casein (37°C) was added to the immobilized protease thermostated at 37°C. After 10 minutes' stirring, the immobilized preparation was filtered off, washed with distilled water, and reused. To 1 ml of the filtrate was added 5 ml of a 5% solution of trichloroacetic acid, and the optical density at 280 nm was determined.

Determination of Caseinolytic Activity on Storage. Samples of the immobilized proteinase were kept in distilled water in the refrigerator at 5°C for 2-5 months. Then their caseinolytic activities were determined.

Determination of the Thermal Stability of the Immobilized Proteinase. The immobilized protease in 0.033 M phosphate buffer, pH 7.4, was thermostated at 50°C, and its residual caseinolytic activity was determined after predetermined intervals of time. The apparent inactivation rate constant (K_{in}) of the native and immobilized enzymes were calculated from the formula

$$K_{in} = \frac{2.3}{t} \lg \frac{A}{A_0},$$

where A_0 is the initial activity of the enzyme; and A is the activity at time t.

Determination of the Temperature Optimum. The activities of suspensions of the immobilized and native proteases were determined at 30, 40, 50, 60, and 70°C.

Determination of the pH Optimum. The activities of suspensions or solutions of the enzyme in distilled water with a 2% solution of casein in 0.1 M borate buffer were determined at pH values from 5.5 to 8.5.

SUMMARY

Preparations of *Bacillus mesentericus* protease immobilized on grafted polyethylene preparations have been obtained, and a considerable retention of their activities on immobilization and repeated use and an increase in the thermal stability of the protease immobilized on polyethylene with grafted-on poly(acrylic acid) activated with a solution of carbodiimide have been observed.

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