IMMOBILIZATION OF PEKTAVAMORIN G10x ON POLYVINYL ALCOHOL DERIVATIVES

T. I. Davidenko and A. V. Moiseev

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The immobilization of Pektavamorin G10x on polyvinyl alcohol modified with glutaraldehyde and with titanium, zirconium, and hafnium salts has been investigated and the properties (stability on storage, pH optimum, temperature optimum, and diversity of action) of the immobilized preparations have been studied.

Recently, polyvinyl alcohol derivatives have been successfully used for the immobilization of enzymes [1-3]. Their cheapness, low toxicity, and good mechanical properties have permitted them to be used for the immobilization of enzymes for medicinal and food purposes. However, the possibilities of this type of supports have been studied inadequately, and therefore it appeared of interest to determine the desirability of using for the immobilization of Pektavamorin G10x suppports obtained from polyvinyl alcohol and the influence of various cross-linking reagents (glutaraldehyde, titanium, zirconium, and hafnium salts) on the properties of the immobilized preparations.

Table 1 gives some properties of Pektavamorin G10x immobilized on polyvinyl alcohol using various cross-linking reagents. As follows from the table, the nature and number of the cross-linkages and the conditions of synthesizing the supports have an effect on the binding of the protein and the retention of the pectin esterase activity. Thus, when Pektavamorin G10x was immobilized in polyvinyl alcohol the retention of the activity was 26% when 4.9 mg of protein/g of support was bound, while on cross-linkage with glutaraldehyde the retention of activity was 70% with the binding of 2.4 g of protein/g of support. In [3], for the immobilization of penicillin amidohydrolase on polyvinyl alcohol using various cross-linking reagents the best results were obtained for glutaraldehyde, for which binding amounted to 40 mg of protein/g of support, but the retention of activity was only 28%. However, the ratio of the components (moles of glutaraldehyde/moles of polyvinyl alcohol (0.5:1) in the formation of the support also differed considerably from that which we used (50:1). In fact the use of the ratio mentioned in their paper [3] did not only lead to a low (28%) retention of the initial activity but also to the formation of immobilized enzyme preparation the multiple use of which was difficult because of the inadequate hydrodynamic indices of the gel formed. At a ratio of the components of 50:1, immobilized preparations were formed which not only had 70% retention of the initial activity but also possessed the necessary mechanical strength.

It is interesting to note on the use of support (II) obtained from polyvinyl alcohol dissolved in different amounts of water (10 and 20 ml) immobilized preparations with different degrees of binding and retentions of activity were obtained. For example, for (II) immobilized preparations with a higher degree of binding and a lower retention of activity were formed with $TiCl_4$ and $ZrOCl_2$ in the case of the support with the higher dilution.

An increase in the content of metal ions (Ti, from 1.46 to 3.6 mmole; Zr from 1.3 to 6.2 mmole per 1 g of support) led to a rise in the degree of binding of the protein (from 6.2 to 8.5%) and in the retention of activity (from 56 to 74%) for support (II) treated with $TiCl_4$, while for support (II) modified with $ZrOCl_2$ only an increase in binding (from 5.6 to 11.9%) was observed, the pectin esterase activity remaining constant (64-65%). In the latter case, the activity of the sample was less "sensitive" to the content of metal ions on the support than the binding of protein. At the same time, the nature of the complex-forming metal ion (Tr, Zr, or Hf) when using supports based on polyvinyl alcohol and, as has been shown previously [4], on Silichromes has little effect on the pectin esterase activity of the immobilized Pektavamorin.

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TABLE 1. Properties of Pektavamorin G10x Immobilized on Polyvinyl Alcohol Derivatives

Support	Content of metal ions, mmole/g of support	Content of enzyme, mg of protein/g of support	Binding of the protein on immobilization, %	Pectin esterase activity of the immobilized Pektavamorin G10x, units/g	Retention of the activity of the bound protein, %	Pectin esterase activity after storage for 30 days (% of max.)
Polyvinyl alcohol	—	4,9	26	26	32	13
I	—	2,4	13	57	70	24
	1,46	2,2	6,2	45	56	—
II. $TiCl_4$	3,6	3,5	8,5	60	74	—
	2,8*	6,6	14,7	17	21	78†
	1,3	2,9	5,6	52	64	—
II. $ZiOCl_2$	2,7	3,0	6,1	52	64	—
	6,2	5,4	11,8	53	65	—
	3,75*	2,2	11,5	33	41	30
III. $TiCl_4$	0,05	2,5	11	60	73	70
III. $HfOCl_2$	0,003	1,1	2,5	73	89	69

*Support obtained by dissolving 1 g of polyvinyl alcohol in 20 ml of water.

†Storage for 15 days.

After the storage of the immobilized Pektavamorin preparations at 4°C in Na acetate—acetic buffer for a month, the activity of the enzyme changed differently for different cross-linking reagents (Table 1). The most stable were preparations obtained in immobilization on support (II) treated both with $TiCl_4$ and $HfOCl_2$, and the least stable was Pektavamorin in polyvinyl alcohol (13% of the initial activity was retained for 30 days). On storage under the same conditions the native preparation lost 96.1% of its pectin esterase activity in 30 h and had become inactive after 2 days.

On repeated use (Table 2), the most stable preparation was that immobilized on support (I); after being used seven times this preparation retained 54% of its initial activity, the corresponding figures for supports (II) and (III) being 17-33%. However, on storage for a month the preparation immobilized on support (I) retained 24% of its initial activity.

Figure 1a shows the influence of a change in the pH of the incubation medium on the activity of the native and immobilized Pektavamorin G10x. The optimum pH of the native preparation was 4.3-4.5 and that of the immobilized preparations shifted in the alkaline direction for all the supports apart from (II) ($ZrOCl_2$) by 0.5-1 unit, in contrast to the pH optimum of Pektavamorin immobilized on modified Silochromes [4], for which a shift in the acid direction of up to 2 pH units was observed.

The temperature optimum (Fig. 1b) of the immobilized enzymes shifted by 10-15°C (to 30°C) as compared with the native enzyme (40°C) with the exception of the Pektavamorin G10x immobilized on polyvinyl alcohol modified with glutaraldehyde, for which the maximum activity was observed at 40°C.

Thus, a comparison of the results given above with those for the immobilization of Pektavamorin G10x on Silichromes using different cross-linking reagents [4] has permitted us to conclude that the use of supports based on polyvinyl alcohols (particularly those modified with glutaraldehyde) for obtaining modified enzyme preparations distinguished by a high level of pectin esterase activity and stability is promising.

EXPERIMENTAL

As the initial material for obtaining supports we used polyvinyl alcohol (ch. ["pure"]) with a molecular weight of 20,000 (produced by the Erevan reagents factory). To create a three-dimensional gel lattice the following reagents were used: glutaraldehyde (Reanal), titanium tetrachloride (ch.) from the Leningrad "Krasnyi khimik" factory, and zirconyl and hafnium oxychlorides (o.s.ch. ["ultrapure"]). For immobilization we used Pektavamorin G10x from *Aspergillus awamori*, grade C (Biokhimreaktiv NPO [Scientific-Production Amalgamation]).

The supports were obtained by the following methods:

I. At 25°C, 0.5 ml of concentrated HCl was added to a solution of 1 g of polyvinyl alcohol in 10 ml of water that had been obtained at 80°C, the mixture was stirred for 10 min,

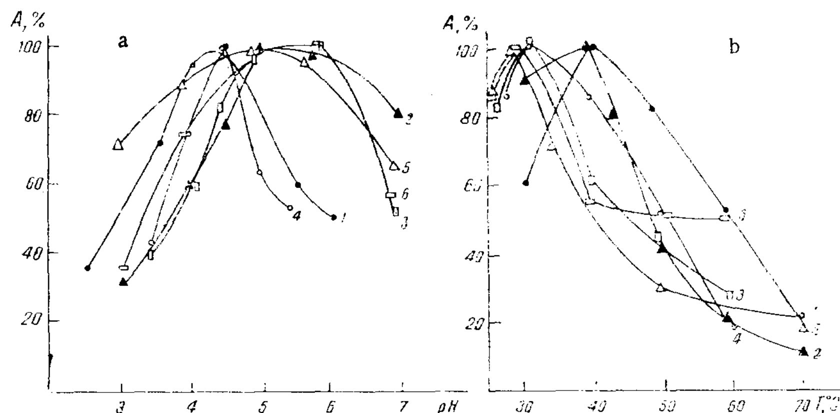

Fig. 1. Activity of the Pektavamorin G10x preparations at various pH values of the incubation medium at 30°C (a) and at various temperatures of the incubation medium (b): 1) initial Pektavamorin G10x; immobilized on polyvinyl alcohol modified with: 2) glutaraldehyde; 3) titanium tetrachloride; zirconium oxychloride; 5) glutaraldehyde and titanium tetrachloride; 6) glutaraldehyde and hafnium oxychloride.

1 ml of 25% aqueous glutaraldehyde was added, and stirring was continued until gel-formation began, after which the stirrer was stopped and the block of gel was left for 1 h; it was then comminuted through a sieve (0.5 mm) and was washed with water to pH 7.0 and with ethanol.

II. At 25°C with stirring, 1 ml of titanium tetrachloride or 3.3 ml of a solution containing 1.86 g of zirconium oxychloride (or 2.6 g of hafnium oxychloride) was added dropwise to a solution of 1 g of polyvinyl alcohol in 10 ml of water that had been obtained at 80°C. The pH of the medium was kept within the interval of 3-4 with the aid of a 25% solution of ammonia. The stirrer was stopped, the block of gel was kept at 25°C for 1 h, and then it was comminuted by passage through a sieve (0.5 mm) and was washed with water and ethanol.

III. At 25°C, 0.5 ml of titanium tetrachloride or 3.3 ml of a solution containing 0.2 g of hafnium oxychloride was added to 1 g of glutaraldehyde-cross-linked polyvinyl alcohol in 10 ml of water, the pH of the medium being kept in the range of 3-4 with the aid of a 25% solution of ammonia. The support obtained was filtered off and washed with water, and dried.

The titanium contents were determined with the aid of diantipyrylmethane [5] after the support had been decomposed with a mixture of concentrated nitric and hydrochloric acids (1:3), and zirconium with the aid of arsenazo III [5], hafnium being determined in the same way as zirconium with the only difference that in this case 1 N hydrochloric acid was used [5].

The pectin esterase activities of the preparations were evaluated by the titrimetric method from the capacity of the enzyme preparation for catalyzing the hydrolysis of the ester bonds in pectin. A 1% solution of highly methoxylated sugar beet pectin (ch) produced by the Biokhimreaktiv NPO was used as the substrate. The amount of protein in the immobilized preparations was estimated from the difference between the protein taken for the reaction and that found in the mother solution and the wash-waters by Lowry's method [6] or directly on the support by Hartree's method [7].

The immobilized forms of the enzymes were obtained by the following procedure: 0.3-0.5 g of modified support was added to a solution of Pektavamorin G10x (55.7 mg of protein in 10 ml of 0.1 M Na acetate—citric acid buffer, pH 4.2) and the mixture was stirred at 25°C for 4 h. After the end of the reaction, the immobilized enzyme was filtered off and it was washed repeatedly with cooled buffer (a control was washed simultaneously) and was stored in the same buffer at 4°C.

The dependence of the pectin esterase activity on the pH was determined by 30°C by the addition to 0.16-0.22 g of immobilized enzyme of 25 mg of Na acetate—citric acid buffer, pH 4.2, and 2 ml of pectin. Then the pectin esterase activities were determined at the given pH and at pH values changed by 0.5 or 1 unit.

The dependence of the pectin esterase activity on the temperature was evaluated at 30-70°C after the charges had been thermostated at the appropriate temperatures for 30 min.

TABLE 2. Stability of Immobilized Pektavamorin G10x on Being Used for Seven Experiments

Support	Pectin esterase activity, % of the maximum on use for the following numbers of experiments						
	1	2	3	4	5	6	7
I	100	92	83	75	72	63	54
II $TiCl_4$	100	42	40	25	25	22	17
II $ZrOCl_2$	100	95	90	84	47	43	32
III $TiCl_4$	100	87	84	79	65	39	18
III $HfOCl_2$	100	62	44	44	44	36	33

The effect of the repeated use of the immobilized Pektavamorin G10x was checked in the following way: 0.2 g of the immobilized Pektavamorin G10x was placed in a reactor and the enzymatic hydrolysis of a 1% solution of highly methoxylated sugar beet pectin was carried out for 20 min. After this, the immobilized enzyme preparation was separated from the reaction mixture and was washed with distilled water, and the hydrolysis was repeated with a new portion of pectin. The pectin esterase activity was determined in each experiment.

SUMMARY

A number of supports for immobilization have been synthesized from polyvinyl alcohol. Pektavamorin G10x has been immobilized on the modified supports and the properties of the samples of immobilized enzyme have been studied.

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