IMMOBILIZATION OF THE PROTEASE OF *Bacillus mesentericus* ON SILOCHROMES

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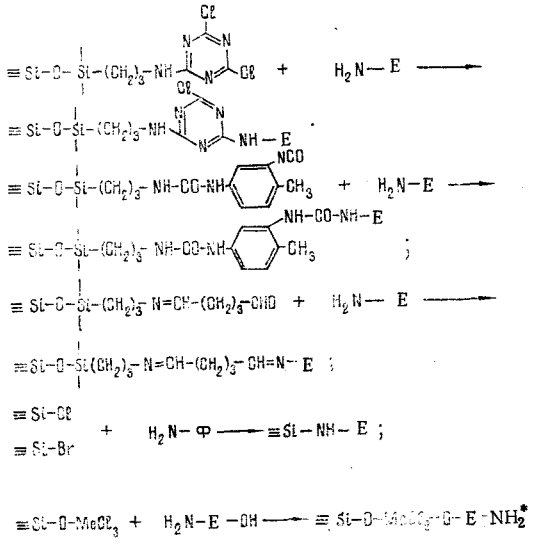
The immobilization of the protease of *Bacillus mesentericus* on Silochrome using cyanuryl chloride, 2,4-toluylene diisocyanate, glutaraldehyde, thionyl chloride, phosphorus tribromide, titanium tetrachloride, and zirconium and hafnium oxychlorides as cross-linking reagents has been studied. The greatest binding and retention of activity were observed with the use of transition-metal salts. A comparison of the properties of the native and immobilized preparations has been made. The stability of the immobilized protease on storage and repeated use has been investigated.

At the present time, proteases are finding use in domestic chemistry, the foodstuffs and leather industries, for the purification of effluents, and in medicine [1]. The production of immobilized enzymes of this class would possibly, in addition to increasing their stability to repeated use, cheapen them and expand their applications.

The general principles of the immobilization of proteases have been described previously [2, 3], but the concrete elucidation of the physicochemical properties of immobilized enzymes, their catalytic activity, and the cost of the supports requires new experimental investigations. In the present paper we consider the immobilization of the protease of *Bacillus mesentericus* on Silochromes with the aid of various cross-linking reagents (cyanuryl chloride, 2,4-toluylene diisocyanate, glutaraldehyde, thionyl chloride, phosphorus tribromide, titanium tetrachloride, and zirconium and hafnium oxychlorides).

In the choice of supports we were guided by such factors as availability, low cost, possibility of obtaining supports with various reactive groups (chlorotriazine, diisothiocyanate, aldehyde groups) both remote from the surface of the Silochrome (through γ-aminopropyltriethoxysilane) and also directly bound to the supports (chlorine- and bromine-containing, Silochromes modified with transition-metal salts) formed according to the following assumed reactions:



* The nature of the $-Me-O-E-$ bond has not been definitely established.

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TABLE 1. Properties of the Protease from Bacillus mesentericus Immobilized on Silochrome

Method of immobilization (or reagent)	Amount of immobilized protein, mg/g of support	Degree of binding of the protein, %	Retention of caseinolytic activity, %
Cyanuryl chloride	98.7	71.8	7.7
2,4-Toluylene diisocyanate*	125.1	79.9	5.3
Glutaraldehyde	71.5	52.6	13.4
Thionyl chloride	94.7	69.6	11.5
Phosphorus tribromide	107.0	78.7	6.5
Titanium tetrachloride	111.0	81.6	21.5
Zirconium oxychloride	124.7	91.7	29.0
Hafnium oxychloride	94.4	69.4	33.8

*The support and enzyme were taken in a ratio of 156 mg of enzyme per 1 g of support.

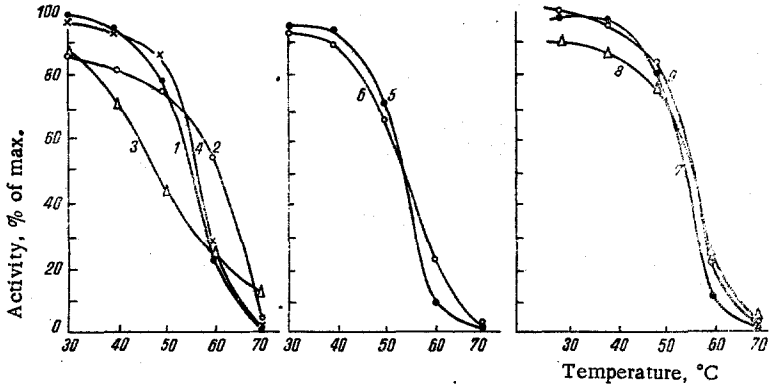

Fig. 1. Dependence of the activity of the protease of Bacillus mesentericus on the temperature (A – activity of the immobilized enzyme, % of maximum). 1) Native protease; Bacillus mesentericus protease immobilized with the aid of 2) cyanuryl chloride; 3) 2,4-toluylene diisocyanate; 4) glutaraldehyde; 5) thionyl chloride; 6) phosphorus tribromide; 7) titanium tetrachloride; 8) zirconyl oxychloride; 9) hafnium oxychloride.

As follows from Table 1, the specific amount of enzyme per gram of support depends on the method of immobilization and ranges from 71.5 mg of protein (glutaraldehyde) to 125.1 (in the case of 2,4-toluylene diisocyanate) and 124.7 mg of protein (for zirconium oxychloride). The method of immobilization affects the activity of the preparations obtained to a still greater extent: the greatest percentage retention of caseinolytic activity was observed with the use of glutaraldehyde, thionyl chloride, and transition-metal salts. As has been reported previously [4], for an amino-substituted silica gel the activity of Bacillus mesentericus depends little on the method of immobilization on aminoorgano-Silochromes, except for the fact that the protease immobilized with the aid of glutaraldehyde was more active. It is interesting to note the different degrees of retention of activity for the enzyme immobilized on the chlorine- and bromine-containing supports. This effect is apparently due to the inhibition of the enzyme by the HBr liberated, as we have also observed in the immobilization of pectawamorin G.

When immobilized preparations of the protease were stored at 4°C for 10, 30, and 38 days the activity of the enzyme changed differently for the different cross-linking reagents (Table 2). The most stable were prepa-

TABLE 2. Stability of the Immobilized Proteases of Bacillus mesentericus

Storage at 5°C for, days	Retention of caseinolytic activity, % of initial							
	cyanuryl chloride	2,4-toluylene diisocyanate	glutaraldehyde	thionyl chloride	phosphorus tribromide	titanium tetrachloride	zirconium oxychloride	hafnium oxychloride
10	77.1	61.4	—	66.3	79.3	—	—	—
30	65.3	59.5	77.0	35.8	70.6	22	52	68
38	62.6	33.7	63.7	21.1	59.4	—	—	—

rations obtained by the use of cyanuryl chloride, glutaraldehyde, and phosphorus tribromide (~60% of the initial activity was retained after 38 days) and of hafnium oxychloride on storage for 30 days (68% of the initial activity).

However, in a study of the possibility of the repeated use of a preparation immobilized with the aid of hafnium oxychloride, after the first experiment only 11% of the activity was retained, and inactivation was complete when it had been used three times. Apparently, the hydrolysis products are competitive ligands and decompose the complex of metal ions with the enzyme. Such a phenomenon has been reported by Slobodyanikova [5], who considered the immobilization of the proteases of Bacillus subtilis on a copolymer of styrene and maleic anhydride. In actual fact, after the enzyme immobilized with the aid of hafnium oxychloride had been kept for 10 min in a 5% solution of a mixture of 18 amino acids (equimolar amounts of alanine, glycine, serine, threonine, glutamic and aspartic acids, proline, valine, methionine, cystine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, histidine, lysine, and arginine) the caseinolytic activity was only 26% of the initial value. The protease preparations immobilized with the aid of zirconium oxychloride and titanium tetrachloride were more stable, losing 33% and 14% of their initial activity after treatment with amino acids.

Where a chlorine-containing support was used, again, the initial activity fell considerably. However, when the immobilized preparation was stored at 4°C in distilled water for a day the activity increased by a factor of 1.5-2 after use three and four times.

A study of temperature inactivation showed (Fig. 1) that at 70°C in the majority of cases the immobilized and native forms of the protease underwent complete inactivition. For all the immobilized enzymes we obtained results of the dependence of their activity similar to that for the native protease, with the exception of immobilization with the aid of cyanuryl chloride and 2,4-toluylene diisocyanate. Thus, in the last case at 50°C the preparation lost 55% of its initial activity, while the native protease lost 22%; however, at 70°C the immobilized preparation retained 13.4% of its activity.

EXPERIMENTAL

We used Bacillus mesentericus protease obtained as described previously [6]. The caseinolytic activities of the native and immobilized preparations were determined by a modification of Anson's method [7]. In the method used, as the unit of activity of the enzyme we took that amount that forms, during one minute, proteolysis products incapable of being precipitated by trichloroacetic acid containing one microequivalent of tyrosine. The initial preparation contained 64% of protein determined by the Lowry method [8]. Its activity was 1.39 units/mg. The amounts of protein in the immobilized preparations were estimated from the difference between the amount taken for immobilization and that found in the wash-waters.

Amino-Silochrome was obtained by the method of Tertykh et al. [9] and the activation of the aminoorgano-Silochrome with cyanuryl chloride, 2,4-toluylene diisocyanate, and glutaraldehyde was carried out as described in the literature [9, 10]. The capacity of the initial Silochrome C-120 was determined from the concentration of OH groups measured according to a known method [11].

Preparation of Silochrome Activated with Thionyl Chloride. To 10 g of Silochrome C-120 was added 20 ml of thionyl chloride. With constant stirring, the mixture was heated under reflux at 90 °C for 8 h. The support obtained was washed with 450 ml of chloroform and 200 ml of absolute benzene and was dried in vacuum at 120 °C to constant weight.

Preparation of Silochrome Activated with Phosphorus Tribromide. To 10 g of Silochrome C-120 was added 10 ml of chloroform and then, with stirring, 20 ml of phosphorus tribromide. With constant stirring, the mixture was heated under reflux at 90°C for 8 h. Then the chloroform and the excess of PBr_3 were distilled off. The support obtained was washed with 450 ml of chloroform and 200 ml of absolute benzene and was dried in vacuum at 120 °C to constant weight.

Preparation of Silochrome Activated with Transition-Metal Salts. A suspension of 3 g of Silochrome C-120 in 15 ml of distilled water was stirred vigorously for 1 h and boiled for 15 min. After cooling to 5-7°C, a solution of 5.59 g of hafnium oxychloride in 10 ml of water was added, the pH of the reaction mixture being kept between 3.0 and 4.0 by the addition of 25% ammonia solution. The solution was stirred for 30 min with cooling and 30 min at room temperature. The product was washed with 150 ml of 0.05 N HCl by decantation, and then with distilled water to neutrality.

Preparation of the Immobilized Bacillus mesentericus Protease. The activated support, in a proportion of 1 g of support to 136 mg of enzyme, was added to 15-20 mg of enzyme in 2 ml of tris-HCl buffer containing 0.005 M $CaCl_2$ (pH 7.4). The reaction mixture was incubated at 25°C with shaking for 30 min and was then washed

with cooled tris-HCl buffer (pH 7.4) until the wash-waters no longer showed absorption at 280 nm. The resulting preparation of immobilized enzyme was stored in tris-HCl buffer at 5°C in the refrigerator.

Dependence of the Activity of the Immobilized Protease on the Temperature. The immobilized protease was thermostated in tris-HCl buffer at 30-70°C for 15 min and was then cooled in a beaker with ice, and its caseinolytic activity was determined. In a control experiment, the activity was determined without heating.

Change in Caseinolytic Activity on Repeated Use. A solution of casein (37°C) was added to the immobilized protease thermostated at 37°C. After ten minutes' stirring, the immobilized preparation was filtered off, washed with 100 ml of distilled water, suspended, and again added to the reaction vessel for re-use. To 3 ml of the filtrate was added 5 ml of 5% trichloroacetic acid solution, and the optical density at 280 nm was determined.

Determination of Caseinolytic Activity on Storage. Samples of immobilized protease were kept in tris-HCl buffer (pH 7.4) in the refrigerator at 5°C for 10, 30 , and 38 days. Then their caseinolytic activities were determined.

SUMMARY

Preparations of Bacillus mesentericus protease immobilized on Silochromes with the aid of various cross-linking reagents (cyanaryl chloride, 2,4-toluylene diisocyanate, glutaraldehyde, thionyl chloride, phosphorus tribromide, and transition-metal salts) have been obtained.

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