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MACROMOLECULAR CHEMISTRY AND POLYMERIC MATERIALS

Immobilization of α-Galactosidase inside Hybrid Silica Nanocomposites Containing Polysaccharides

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Abstract—α-Galactosidase was encapsulated into sol–gel derived hybrid polysaccharide–silica nanocomposites. The main properties of the immobilized enzyme were studied.

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Immobilization has long been successfully used for storage and prolongation of action of enzymes, as well as for development of artificial biocatalysts [1, 2]. Encapsulation into a matrix or immobilization on a support allows repeated use of enzymes and facilitates isolation of products of enzymatic reaction, which is essential for development of biotechnological processes. An important advantage of immobilization is the stabilization effect, i.e., significant prolongation of the lifetime and enhancement of the stability of enzymes. Therefore, new efficient supports and matrices for encapsulation of enzymes are constantly sought for.

α-Galactosidase (α-*D*-galactoside galactohydrolase, EC 3.2.1.22) is a promising biochemical tool for studying the antigene structure of various biological objects, in particular, blood cells. Immobilization of α-galactosidase was repeatedly attempted. For example, enzyme from coffee beans was covalently bound to dextran activated with cyanogen bromide [3].

α-Galactosidase from *Pycnoporus cinnabarinus* was immobilized on chitin dispersions with glutaric dialdehyde [4]. Enzyme from *Aspergillus oryzae* was immobilized on Eupergit C containing epoxy groups, which provided covalent bonding of the protein by amino, SH, and carboxy groups [5]. α-Galactosidase from the same source was placed into calcium alginate gel, in which it was additionally immobilized with glutaric dialdehyde [6]. Also, this enzyme was immobilized with polyacrylamide gel [7].

It should be noted that, in the cited studies, the enzyme was encapsulated via cross-linking. Immobilization inside a matrix or on a support by covalent bonding can adversely affect the enzyme through disturbing the conformation of the protein macromolecule. In some cases this is the main reason for partial or complete denaturation of not very stable enzymes [8, 9]. In the case of sol–gel technology, immobilization involves no cross-linking agents. The protein macromolecules are encapsulated into the inorganic matrix formed in situ by polycondensation reactions. The macromolecules undergo minimal structural changes, and the enzymes can preserve their activity [1–3]. α-Galactosidase from *Pseudoalteromonas sp*. KMM 701 sea bacterium can eliminate α-1,3-bound galactose residues from the nonreducing end of glucoconjugates, blood group substances, and antigene determinants for erythrocytes in B(III) blood group via suppressing their serologic activity [10]. α-Galactosidase is a thermally labile enzyme. In concentrated solutions at 20°C α-galactosidase remained active for 1 week, and at 30°C lost 50% activity within 10 min [10]. Dilution of concentrated solutions of the enzyme accelerated its inactivation. A low thermal stability and short lifetime of α-galactosidase in solution hinder its biotechnological application.

Our preliminary studies showed that encapsulation of α-galactosidase into polysaccharide–silica matrices obtained by the method we suggested previously [11, 12] significantly prolonged the lifetime of the enzyme. In this study we examined in more detail the conditions for α-galactosidase immobilization inside new hybrid nanocomposites and the properties of immobilized α-galactosidase in order to select candidate nanocomposites for development of biocatalysts suitable for biotechnological and biosensing applications.

EXPERIMENTAL

Tetrakis(2-hydroxyethyl) orthosilicate (THEOS) was synthesized from tetraethoxysilane (ABCR, Germany) as described in [13]. Polysaccharides xanthan and galactomannan (locust bean gum, from here on, LBG) (Fluka) was used without additional purification. Laminaran (β-1,3-glucan) was isolated from *Laminaria cichorioides* brown alga [14], and α-galactosidase, from biomass of *Pseudoalteromonas sp.* KMM 701 sea bacterium, cultivated at the Laboratory of Microbiology, Pacific Ocean Institute of Bioorganic Chemistry, Far-East Division, Russian Academy of Sciences, by the procedure from [10].

α-Galoactosidase was encapsulated into hybrid polysaccharide–silica nanocomposites by the procedure described in our previous paper [12]. First, 1.9 ml of solution containing 10 or 30 wt % precursor, different concentrations (0.25, 0.35, 0.75, and 1.5 wt %) of one of the three polysaccharides, and 0.1 M sodium phosphate buffer solution (to adjust pH) was prepared. It was kept for some time, determined in previous experiments and required for its conversion into a pregel state by partial polymerization of silicic acids formed from hydrolysis of THEOS. Then, 0.1 ml of the enzyme solution with an activity of 10 units ml^{-1} was added. All the solutions were precooled to 3–5°C in a refrigerator. After mixing and thorough stirring, they once again were placed into the refrigerator in order to minimize denaturation of the enzyme during immobilization.

The activity of immobilized α-galactosidase was determined with *p*-nitrophenyl-α-*D*-galactopyranoside (Sigma) as substrate. The reaction mixture was prepared by mixing 0.35 ml of a 3.3×10^{-3} M solution of substrate in the buffer solution (10^{-1} M sodium phosphate, pH 7.2) and 0.05 ml of solution of free α-galactosidase, or 0.05 g of the gel with the enzyme immobilized, and incubated at 20°C. The reaction was terminated by adding 0.6 ml of an aqueous solution of 1 M sodium carbonate, whereupon the gel was separated by centrifugation. The amount of *p*-nitrophenol yielded by the enzymatic reaction was determined spectrophotometrically at 400 nm (ε = 18 300 l mol^{-1} cm^{-1}). The enzyme activity unit *U* was taken equal to the amount of the enzyme in 1 ml of solution (free enzyme) or 1 g of gel (immobilized enzyme) with which 10^{-6} mol of *p*-nitrophenol was formed per minute. The control was a solution of the enzyme (free enzyme) prepared by 20-fold dilution of the initial solution of α-galactosidase with 0.1 M sodium phosphate buffer solution (pH 7.2). It was free from additions of the precursor and polysaccharide used for immobilization.

α-Galactosidase from *Pseudoalteromonas sp.* KMM 701 sea bacterium was immobilized inside hybrid polysaccharide–silica nanocomposites synthesized using a new precursor, THEOS. It is distinguished by complete solubility in water and compatibility with biopolymers. Polysaccharides were used because they catalyze sol–gel processes [15, 16]. In their presence gelation proceeds at any pH, as well as at low temperatures, which is essential for thermolabile enzymes. In particular, to minimize denaturation of α-galactosidase exhibiting a low thermal stability during encapsulation into silica matrix, immobilization was carried out at a temperature close to 0°C and pH 7.2. Commonly used precursors, such as tetramethoxy- and tetraethoxysilanes, do not form gels under such conditions [8, 9, 17, 18].

To elucidate how the structure of the polysaccharide and its charge affect the catalytic properties of α-galactosidase, we tested negatively charged xanthan and neutral LBG and laminaran. Previously we studied the possibility of immobilization of endo-1 → 3-β-*D*-glucanase L_{IV} from *Spisula sacchalinensis* and α-galactosidase in hybrid polysaccharide–silica nanocomposites [12]. The immobilized proteins remained functionally active for 165 days. The activity of the proteins varied with the formulation of the nanocomposite and structure of the polysaccharide, but these aspects were not studied in detail.

Table 1 lists the activities of α-galactosidase immobilized inside hybrid polysaccharide–silica nanocomposites with various formulations, synthesized in this study. It was tested for 16 months after matrix immobilization of the enzyme. It is seen that the enzyme remained active by the end of the test in selected cases only. To elucidate how the catalytic properties of α-galactosidase are influenced by the structure and concentration of the polysaccharide, as well as by the silica concentration in the matrix, we will consider the activities determined just after gel formation.

Table 1 shows that the highest activity was exhibited by sample no. 5 prepared by mixing 0.25 wt % solution of xanthan and 10 wt% solution of THEOS. It was nearly two times that of the free enzyme. Also, it exceeded the activity of galactosidase in a matrix with identical formulation that we examined previously [12], but in that study the content of the enzyme was by an order of magnitude lower.

The enzymatic activity of sample nos. 2 and 3, containing 0.25 and 0.75 wt % LBG, respectively,

Table 1. Activity of α-galactosidase in relation to the immobilization conditions and storage time

Sample no.	SiO_2, wt %	Polysaccharide, wt %	$U \times 10^3$, g^{-1}, at indicated storage time, days						
			0	6	19	30	43	165	417
1	0	Free enzyme*	33.6	21.8	16.5	N.d.**	18.7	0	0
2	10	LBG: 0.25	34.6	50.9	76.8	N.d.	54.1	N.d.	28.9
3	10	0.75	32.8	56.7	81.8	N.d.	63.5	N.d.	N.d.
4	10	1.5	9.2	10.9	12.0	N.d.	8.4	N.d.	9.6
5	10	Xanthan: 0.25	63.9	N.d.	N.d.	21.7	N.d.	N.d.	N.d.
6	30	0.25	14.1	N.d.	N.d.	0.1	N.d.	N.d.	N.d.
7	10	Laminaran, 0.35	20.0	N.d.	N.d.	0	N.d.	N.d.	N.d.

* U, ml^{-1}
** Not determined.

just after immobilization was comparable to that of the enzyme in solution. With increasing amount of LBG in the silica matrix to 1.5 wt % (sample no. 4), the activity decreased almost threefold. This suggests that the activity depends on the concentration of the polysaccharide in the hybrid nanocomposite. As shown in [15, 16], this can be due to the fact that the network structure of the nanocomponent becomes thicker, which can hinder diffusion of the substrate and the enzymatic reaction products.

Most probably, a similar factor also operates when the content of silica in the nanocomposite is increased. Comparison of the data for sample nos. 4 and 5 (Table 1), containing identical amounts of xanthan and different amounts of THEOS (10 and 30 wt %, respectively), shows that the activities of the enzyme immobilized differed for them by a factor of nearly 5. Among the polysaccharides that we studied only laminaran in the gel did not exert a stabilizing effect on the immobilized α-galactosidase. This follows from the fact that the enzymatic activity of control sample no. 1 was 1.5 times that of sample no. 7 (see Table 1).

A 16-month testing of the samples showed that, by the end of this time, α-galactosidase lost its enzymatic activity in most cases (Table 1). The situation with the free enzyme was similar. The activity was preserved by sample nos. 2–4 only: By the end of the test period it was virtually identical to that in the beginning of the experiment. However, the activity of the biocatalysts varied during the tests. For sample nos. 2 and 3 it increased more than twofold within the first 19 days and then tended to decrease. The activity of sample no. 4 significantly varied in the beginning of the experiment, whereupon it remained virtually unchanged throughout the test period.

Tests of xanthan- (nos. 5 and 6) and laminaran-containing (no. 7) samples showed that the activity of the immobilized enzyme decreased with time. Also, the lifetime of α-galactosidase in matrices containing these polysaccharides was shorter than that in the previously discussed samples with LBG.

The retention of the immobilized enzymes in(on) the support is of practical importance. To estimate the strength of immobilization of α-galactosidase in the polysaccharide–silica matrices, we tested sample nos. 2–4 in which the enzyme was best preserved. To 0.1 g of gels, 0.4 ml of 10^{-1} M buffer sodium phosphate solution (pH 7.2) was added. After 2-day keeping in a refrigerator at 4°C with intermittent stirring, the samples were centrifuged at 10000 rpm. The activity was determined in both supernatants and gels by the above-described procedure.

The solutions in which the biocatalysts were kept and separated by centrifugation virtually lack enzymatic activity (Table. 2). This suggests a fairly strong immobilization of the enzyme macromolecules in the gel matrix, when only insignificant number, if any, of them can liberate. However, the activity in gel nos. 2 and 3 proper proved to be significantly lower than the initial activity (Table 2). Probably, centrifugation was responsible for compaction of gels, which do not exhibit high mechanical strength, and

Table 2. Retention of α-galactosidase in the silica nanocomposite (10 wt %) containing different amounts of LBG

Sample no.	LBG, wt %	$U \times 10^3$, g^{-1}	$U \times 10^3$	
			in 1 ml of supernatant	in 1 g of gel
2	0.25	34.6	0.076	3.7
3	0.75	32.8	0.018	12.5
4	1.50	9.2	0.009	12.6

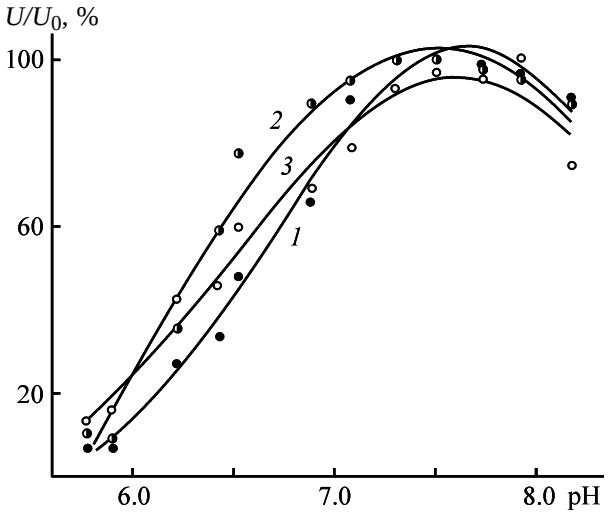

Fig. 1. pH dependence of the residual activity U of (*1*) free and (*2*, *3*) immobilized α-galactosidase in gel nos. 4 (*2*) and (*3*) 5. 0.1 M sodium phosphate buffer solution.

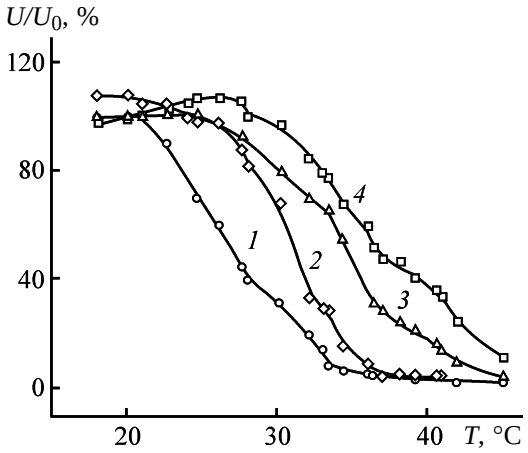

Fig. 2. Temperature dependence of the residual activity U of (*1*, *2*) free and (*3*, *4*) immobilized α-galactosidase in sample nos. (*3*) 4 and (*4*) 5. 0.1 M sodium phosphate buffer solution, pH 7.3. Time of keeping at the required temperature, min: (*1*, *3*) 30 and (*2*, *4*) 10.

this either hindered the accessibility of the enzyme for the substrate, or inactivated the enzyme immobilized. When the LBG concentration in the gel (sample no. 4) was increased, the mechanical exposure affected the enzyme activity to a significantly lesser extent.

We determined the pH corresponding to the optimal activity of immobilized α-galactosidase. To 0.05 ml of free enzyme preparation, or 0.05 g of gel, 0.25 ml of 0.1 M sodium phosphate buffer solution with pH under study and 0.1 ml of 10^{-2} M aqueous solution of the substrate were added. The activity of the enzyme was determined by the above-described procedure. The residual activity was calculated as the ratio, %, of the activity U at various pH values to that of the enzyme at optimal pH values, U_0. Figure 1 presents the pH dependences of the residual activity of the enzyme for free (curve *1*) and immobilized α-galactosidase in gels with LBG (sample no. 2) and xanthan (sample no. 3) (curves *2* and *3*, respectively). It is seen that encapsulation into a silica matrix caused the pH range to broaden to a certain extent, but left the pH corresponding to the optimal activity of the enzyme virtually unaffected.

As shown by analysis of published data, immobilization by cross-linking noticeably shifted the pH corresponding to the highest activity of α-galactosidases. For example, for enzyme isolated from *A. oryzae*, immobilized inside an alginate matrix with glutaric dialdehyde, this pH shifted to the acid region by 0.3 pH unit [6], and for α-galactosidases from *Gibberella fujikuroi* in polyacrylamide gel and from green coffee beans in dextran, by 0.4 pH unit [3, 7]. In the case of sepharose-immobilized enzyme isolated from fig, the pH corresponding to the best activity shifted by 1 pH unit [19]. These published data suggest that the properties of α-galactosidase changed more substantially in the case of immobilization by cross-linking than in the case of the sol–gel technology that we applied here.

Another problem addressed by this study is the thermal stability of α-galactosidase. Gel (0.05 g) with the enzyme immobilized was placed into a thermostat, kept at the required temperature for 10 or 30 min, and cooled to room temperature, whereupon the residual activity was estimated as described above. The control sample was subjected to analogous manipulations.

Figure 2 shows the temperature dependences of the residual activity for free (curves *1* and *2*) and immobilized α-galactosidase in gels with LBG (sample no. 3) and xanthan (sample no. 5) (curves *3* and *4*, respectively). The keeping time was 10 min (curves *2* and *4*) and 30 min (curves *1* and *3*). Analysis of these dependences showed that, after 10-min keeping, free α-galactosidase lost its activity at 37°C (curve *2*), and after 30-min keeping, at 32°C (curve *1*). Under identical conditions, the immobilized enzyme preserved from 50 to 70% of its activity (curves *3* and *4*). Major shift of curves *3* and *4* to higher temperatures relative to curves *1* and *2* suggests that encapsulation into the polysaccharide–silica gel causes the thermal stability of α-galactosidase to increase. This can be due to a stabilizing effect of the inorganic matrix, hindering temperature-induced conformational transitions of the protein molecule. This effect was observed, for example, in the case of proteins immobilized inside

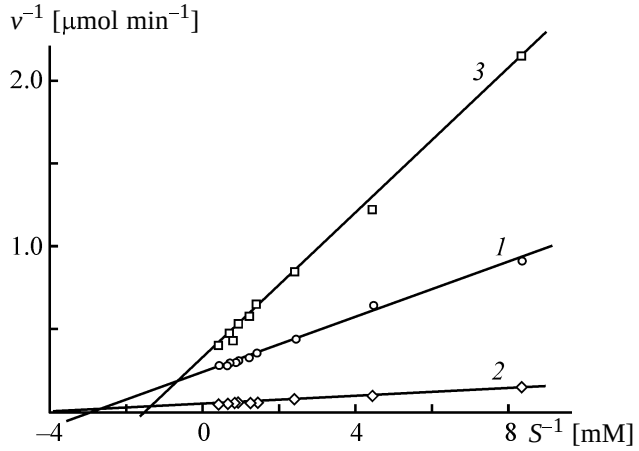

Fig. 3. Reciprocal rate of accumulation of *p*-nitrophenol v^{-1} vs. the reciprocal concentration of *p*-nitrophenyl galactoside S^{-1} for α-galactosidase (*1*) in solution and (*2*, *3*) in immobilized state in sample nos. (*2*) 2 and (*3*) 5.

silica hydrogel [20–23]. Immobilization typically increases the thermal stability of enzymes. In the majority of studies with α-galactosidase it increased by several degrees [3, 5–7, 19]. At the same time, Mitsutomi [4] reported that the thermal stability of α-galactosidase from *P. cinnabarinus*, immobilized in chitin, did not change relative to free α-galactosidase [4].

To compare the catalytic properties of free and gel-immobilized α-galactosidase, we determined the corresponding Michaelis constants K_m. We tested gels containing 0.75 wt % LBG (sample no. 2) and 0.25 wt % xanthan (sample no. 5) at 20°C in 0.1 M sodium phosphate buffer solution, pH 7.7, at a time when the free and immobilized gels still exhibited the maximal activity. The concentration of the substrate, *p*-nitrophenyl galactopyranoside, was varied from 0.12×10^{-3} to 2.49×10^{-3} M. To 0.05 ml of dilute enzyme, or to 0.05 g of gel, 0.350 ml of the buffer solution of the substrate was added. The reaction was terminated by introducing 0.6 ml of aqueous solution of 1 M sodium carbonate. The samples with the gels immobilized were centrifuged at 10000 rpm, whereupon the concentration of *p*-nitrophenol in the supernatant was determined by the above-described procedure. The K_m constant was calculated by the standard Lineweaver–Berk method.

Figure 3 presents the plots of the reciprocal enzymatic reaction rate vs. the reciprocal substrate concentration for α-galactosidase in solution (curve *1*) and in the immobilized state in gel nos. 2 (curve *2*) and 5 (curve *3*), containing LBG and xanthan, respectively. It is seen that immobilization did not substantially affect the catalytic properties of the enzyme. A minor and a twofold increase in K_m, respectively, for the enzyme in gel nos. 2 (0.42×10^{-3} M) and 5 (0.72×10^{-3} M) compared to that for free α-galactosidase (0.36×10^{-3}) can be due to hindered diffusion of the substrate and reaction products in the silica matrix pores. A similar increase in K_m was observed, e.g., for α- and β-galactosidases immobilized in polyacrylamide and alginate gels using cross-linking agents [5, 6, 15].

CONCLUSIONS

(1) α-Galactosidase was immobilized inside sol–gel derived hybrid polysaccharide–silica nanocomposites with different formulations.

(2) The catalytic activity of α-galactosidase varies with the formulation of the silica–polysaccharide matrix. The enzymatic activity tends to decrease with increasing content of the inorganic component. The activity and stability of the enzyme are also influenced by the structure of the polysaccharide and its concentration in the matrix.

(3) α-Galactosidase immobilized inside a tetrakis-(2-hydroxyethyl) orthosilicate (10 wt %) matrix containing galactomannan (0.25 and 0.75%) preserved the catalytic activity for a long time; its thermal stability increased by 10°C. The immobilized α-galactosidase exhibited the maximal activity at the same pH values as the free enzyme; the Michaelis constant K_m did not substantially change.

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