ADSORPTION AND CHEMISORPTION OF GALACTOSE OXIDASE ON SILICA SURFACE

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Abstract

Experience accumulated over a number of years in developing of methods of immobilization of galactose oxidase from *Fusarium graminearum* on parent and modified silica matrices is analyzed. Sturdy adsorption of galactose oxidase on silica surface was observed, such heterogeneous specimens possessed by enhanced biocatalyst stability and activity as compared with enzyme solutions. Covalent immobilization of galactose oxidase was carried out on the amine-containing silicas activated by 2,4-tolylene diisocyanate and cyanuric chloride. It was also shown that in the presence of the substrate (galactose) enzyme chemisorption takes place on the surface on amine-containing silica matrices. Immobilized preparations were successfully applied for analytical determination of galactose-containing carbohydrates (galactose, lactose, raffinose) in complex mixtures.

Introduction

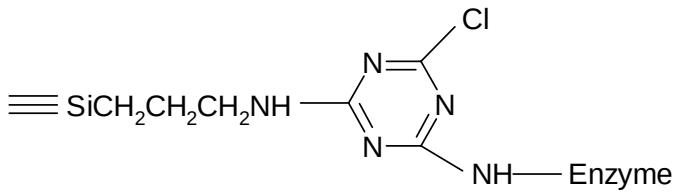
Galactose oxidase (*D*-galactose: EC 1139) came to the attention in the connection with possibility to use for analytical determination of *D*-galactose and galactose-containing sugars. In this case oligo- and polysugars, which contain *D*-galactose, display a high affinity to the enzyme, and they are oxidized fast by galactose oxidase (GalO) [1-4]. An ability of GalO to stereospecific oxidation of the tribasic alcohols may be used for enzymatic synthesis of some optically pure isomers. Many of the difficulties, connected with use of the soluble GalO in analysis and synthesis, can be successfully solved by application of immobilized enzyme preparations. As known, in the majority of cases a bound enzyme becomes more stable, the immobilized biocatalyst may be easily separated from the reaction products and used repeatedly. At the same time the data, devoted to methods of obtaining and studies of properties of the immobilized GalO preparations, are limited in number [5-12]. In the present work, experience, which has been accumulated over a number of years in the field of using of active and stable preparations of GalO for analytical purposes and in organic synthesis, is discussed. Such preparations can be obtained both as a result of the enzyme adsorption on the silica and by covalent anchoring on the modified surface.

Experimental

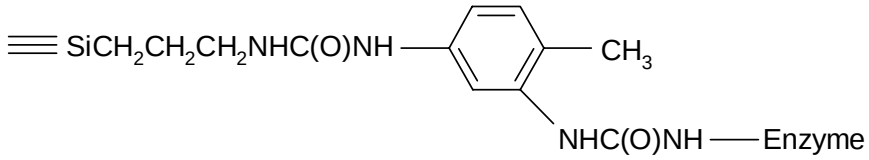
Silochrom S-80, macroporous silica (prepared from fumed silica, the Stavropol plant of pure chemical reagents and luminophores) with specific surface area 80 m^2/g, the total pore volume 1.2 cm^3/g, and the particle size 0.25-0.50 mm was applied as a support for the GalO adsorption and immobilization. GalO preparations were obtained from *Fusarium graminearum IMV-1060*. They have been used in solutions and in the form of liophilic-dried powder [13]. For the determination of activity of soluble and immobilized GalO preparations *D*-galactose (purified grade, may contain 1-2% glucose) was used as a substrate. *D*-galactose solutions were prepared in the form of 100 mM solution in 0.05 M phosphate buffer (pH=7.0), containing 100 μM potassium ferricyanide and 1M Na-EDTA [11].

The adsorption of galactose oxidase was carried out in the following way: the previously prepared buffer enzyme solution was mixed with a weighted specimen of the carrier and incubated at 25°C for 2 h. Then, for removing of an unbound enzyme the carrier was washed by the buffer solution [14]. In this case about 10 ml of the buffer solution on 1g of a carrier was usually applied.

Covalent attachment of GalO was carried out on the modified amine-containing silicas, activated by cyanuric chloride (*a*) and 2,4-tolylene diisocyanate (*b*) [11, 15, 16]. In these cases enzyme anchorage are achieved by the nitrogen-carbon (*a*) and urine bonds (*b*) formation in accordance with the following schemes:



(*a*)



(*b*)

Syntheses of the activated silica carriers were executed by methods described in our previous works [15, 16]. The immobilization process was carried out at room temperature by proceeding as follows: to 100 mg of the appropriate activated support was added the enzyme solution in 0.05 M phosphate buffer (pH=7.0) and mixture was stirred for 2 h. Thereafter the carrier with immobilized biocatalyst was washed by the buffer solution.

As is shown in [17] and this work, when a solution contains galactose oxidase and its substrate (galactose), a peculiar process of autoimmobilization of the enzyme on the amine-containing silica (aminoorganosilica) surface takes place. In this situation, under the action of a biocatalyst galactose is transformed into 2,4-galactohexodialdose that serves as a cross-linking agent and provides bonding of the enzyme with the amine groups of the modified matrix surface with formation of azomethine bonds. Thus, in this case the immobilization may be carried out through the mixing of the buffer enzyme solution, galactose and aminoorganosilica in the various ratios. Usually 50 ml of the galactose solution per 1g of a carrier is used. After incubation of the mixture for 2 h at room temperature, for removing of an unbound enzyme the immobilized preparations were washed by the buffer. In the control experiments the GalO adsorption was performed onto the same aminoorganosilica carrier in the absence of the substrate.

The activity of the soluble GalO was evaluated per a rate of oxygen consumption under the certain conditions employing the Clark oxygen electrode. The activity of the immobilized GalO was determined in the flow-type reactor. Measurements were done both in the impulsive and continuous regimes of a substrate flowing through a column with the immobilized biocatalyst. In the former case oxygen consumption is registered as a peak and in the latter case as a stationary O_2 concentration at the output of the reactor.

Results and discussion

GalO adsorption on the silica surface. The very firm GalO adsorption has been observed on the initial silica surface. We could not completely remove enzyme from the carrier surface by buffer solutions with the various pH (4.5, 5.5, 8.2) and molarity. This is due to the fact that

in water the silica particles go negative charge and the enzyme macromolecules have clearly defined positive charge [18]. It should be particularly emphasized that in the most of cases at various enzyme/carrier ratios, taking for immobilization, it has been possible to obtain the heterogeneous specimens with an enhanced enzyme activity (in the some of cases enough essential) as compared with the enzyme activity in the starting solution (Table 1). It should be also noted that the pH-optimum of the soluble GalO was found at pH=7.0 and the deviations from this value reduce the enzyme activity. It is significant that preparations of adsorbed GalO possess sufficiently more high stability as compared with enzyme solutions. The activities of immobilized specimens are still retained for several months of storage. It is suggested [6] that enzyme attachment to surface of the support, which is unaffected by temperature or pH alteration, provides a biocatalyst by the protective frame and preserves enzyme from the conformational changes. It is quite possible that in the case of GalO adsorption on the silica surface high conformational stability of the biocatalyst and thus its high activity are available. Similar, but more significant effect was observed [19] in time of an incubation of the enzyme by phosphate “bridges”, which have also stabilized the conformation of the protein molecules.

Table 1. Dependence of activity of galactose oxidase adsorbed by silica on concentration of soluble enzyme in initial solution (optimum pH=7.0).

Example	Enzyme activity in initial solution, Int. units*/ml	Enzyme/Silica ratio taken for adsorption, Int.units/g	Activity of adsorbed preparation, Int. units/g
1	0.13	1.3	28.6
2	0.01	0.1	2.4
3	0.25	2.5	13.0
4	0.002	0.02	0.1
5	0.27	2.7	2.6

*1 international unit of activity is equal to enzyme quantity, which catalyzes an oxidation
of 1 μmol of the substrate for 1 min.

As follows from obtained data, of great importance for obtaining of the active preparations is an enzyme/carrier ratio taken for adsorption. This can best be done in the buffer solutions where the enzyme/carrier ratio is equal to 1.3 units/g (international units of an enzyme activity per 1 g of a carrier). The deviation from this value causes decrease in the specimen activity. Nevertheless, in these cases relatively active heterogeneous preparations were obtained. At increasing of an enzyme/carrier ratio up to 2.7 (Table 1, sample 5) a growth of activity of the enzyme was not detected at all. The observed trend can result from an enzyme inactivation because of protein/protein interactions at high surface loadings. Less active specimens were prepared at low surface loadings. It is conceivable that the observed results may be explained by strong electrostatic interactions of adsorbed enzyme molecules with the surface sites. In a case of such interaction the considerable conformational changes in the GalO molecule may occur resulting in a lowering of the immobilised enzyme activity.

Variations in the pH values and buffer type (pH=4.5, 5.8, 6.1, 8.2, and 9.1; Na-acetate and Tris-HCl buffer) have a small effect on a degree of the enzyme activation at sorption on the silica surface. However, the enzyme stability is pH-dependent, and it may be connected with distribution of protons and presence of the ionized groups on a matrix surface. Therefore the

obtained preparations of adsorbed GalO are usually characterized by more low stability as compared with the specimens prepared at the optimal pH-value.

Covalent GalO immobilization. As known, stable preparations of immobilized enzymes, which are not removed from the carrier surface at changing of pH and ionic strength of a solution, may be obtained at covalent binding. In our previous works [10, 11] we proposed methods for GalO chemical binding to surface of the silica matrix. For this purpose amine-containing silicas, activated by cyanuric chloride and 2,4-tolylene diisocyanate, were applied. The use of these activating reagents, whose groups of the same composition differ substantially in their chemical activity, permits one to carry out a process of bonding in two steps. First, a reaction is brought about for amine groups of the modified silica matrix, and then functional groups of an enzyme subjected to immobilization enter into the reaction. In particular, isocyanate groups of 2,4-tolylene diisocyanate bound to benzene rings in position *4* are more active than NCO group in position *2*. Therefore, when amine-containing silica comes into contact with solutions of 2,4-tolylene diisocyanate in carbon tetrachloride, it is isocyanate groups in position *4* that predominantly enter into the surface reaction. The modified silica matrices activated in this way contain grafted isocyanate groups in position *2* of benzene rings. Then the grafted isocyanate groups interact with amine groups of enzyme macromolecule. In a six-member triazine ring of cyanuric chloride in position *2,4,6* there are ≡CCl groups with a highly labile chlorine atom. Interaction between one of these bonds (C–Cl) of the activating agent and a grafted aminopropyl group can result in formation of chemical compounds attached to the surface by hydrolytically stable C–NH–C bonds. It is significant that if one of the ≡CCl groups of cyanuric chloride molecule enters into the reaction, the activity of the rest of the C–Cl bonds in the surface chemical compounds formed slightly decreases. At the same time, the mobility of chlorine atoms in such C-Cl bonds remained sufficiently high to allow bonding of enzyme molecules under mild conditions.

The data represented in the Table 2 show that in a case of using for immobilization of the GalO solutions with the activity 12-80 units the obtained immobilized preparations preserve 75-100% of initial activity. It should be noted that a growth of the enzyme activity at chemical binding with surface do not occur, as it is in a case of the GalO adsorption on silica surface. If the enzyme solutions, having more high activity (115-140 units), were used for covalent attachment, the preparations obtained conserved only 54-59% of an initial activity. It is conceivable that at more high concentrations of protein the role of protein-protein interactions increase resulting in a loss of activity. It is significant that the influence of activation method of amine-containing silica surface (by cyanuric chloride or 2,4-tolylene diisocyanate) on the activity of immobilized preparations obtained be not detected (Table 2).

Immobilization by product of the enzymatic reaction. As known, GalO catalyzes an oxidation of *D*-galactose in the C-6 position by molecular oxygen. The enzymatic reaction product is dialdehyde - 2,4-galactohexodialdose. We know from experience that dialdehydes (glutaraldehyde, gossypol) are effective cross-linking reagents, which provide covalent binding of enzymes and other biologically active compounds to the surface of the amine-containing silicas. It has been found that 2,4-galactohexodialdose at the neutral pH-values can react with amine groups both the enzyme and matrix surface. From the data, represented in the Table 3, one can see that an activity of immobilized preparations depends on the ratio of the taken components. In the result of interaction of amine-containing carrier, enzyme and substrate the immobilized enzyme specimens were obtained. These preparations preserved their activity for 2 months in the condition of flow-injection analyzer (passing through 50 L of a buffer and 1000-fold volume of a substrate). The stability of the obtained preparations is no different from stability enzyme, immobilized by covalent binding to modified silica surfaces activated by cyanuric chloride or 2,4-tolylene diisocyanate. Without addition of the substrate (galactose) we

have observed the enzyme desorption after washing by two volumes of 0.05 M phosphate buffer. It is obvious that positive charge of amine-containing silica surface ($\equiv SiCH_2CH_2CH_2NH_3^+$) is not favoured to binding of the GalO molecules also having positive charge [18].

Table 2. Dependence of activity of galactose oxidase immobilized on amine-containing silicas activated by cyanuric chloride (AsilCC-sample) or 2,4-tolylene diisocyanate (AsilTDI-sample) on activity of enzyme in initial solution.

N/N	Enzyme activity in initial solution, Int. units	AsilCC-sample		AsilTDI-sample	
		Activity of immobilized preparation, Int. units	Degree of activity conservation, %	Activity of immobilized preparation, Int. units	Degree of activity conservation, %
1	23	21	90	20.5	89
2	115	69	60	68	59
3	12	12	100	12	100
4	140	75	54	76	54
5	80	59	74	60	75

Table 3. Immobilization of galactose oxidase on the surface of amine-containing silica matrices in the presence of various amounts of substrate (galactose).

N/N	Galactose/Silica ratio	Initial galactose oxidase activity, Int. units	Activity of immobilized galactose oxidase, Int. units	Degree of activity conservation, %
1	0.36	46	9	20
2	2.88	46	45	98
3	0.18	46	4.5	10
4	3.24	46	45	98
5	1.44	46	38	83
6	0.90	23	20	87
7	0.90	115	89	77
8	0.90	140	89	64
9	0.90	12	12	100
10	0.90	80	75	93
11	–	23	1	4
12	–	69	4	6
13	–	115	5	4

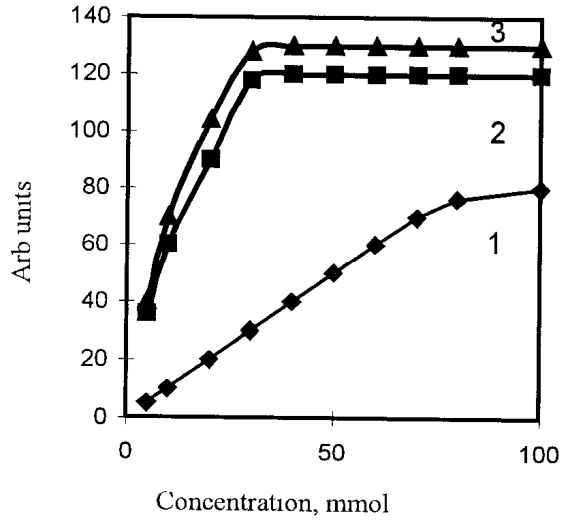


Fig. 1. Oxygen expenditure at determination of lactose (*1*), galactose (*2*), and raffinose (*3*) by immobilized galactose oxidase on addition of $3 \cdot 10^{-6}$ M $CuCl_2$ to 0.05 M (pH=7.0) phosphate buffer.

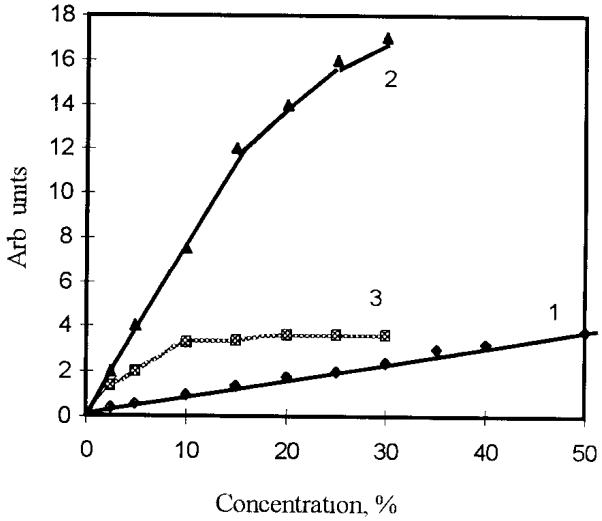


Fig. 2. Dependence of O_2 expenditure by immobilized galactose oxidase on glycerol concentration: *1* - without activator; *2* - on addition of 100 μmol $K_3[Fe(CN)_6]$; *3* - on addition of Na-EDTA and $CuCl_2$ (10^{-5} mol/L).

From the data in the Table 3, one can see that a high enzymatic activity of heterogeneous preparations was observed at an addition 0.90-2.88 g of galactose per 1g of the carrier. However further enhancement of galactose amount up to 3.24 g does not lead to a growth of immobilized enzyme activity. In a similar manner a lowering of galactose amount to 0.18 and 0.36 g results in a lowering of activity of preparations immobilized on the amine-containing silicas. At the designated galactose concentration (0.90 g/g) a high activities of immobilized GalO preparations were detected at using of solutions containing 80-115 units of enzyme activity.

As can be readily appreciated, the proposed method allows one to exclude some stages, in particular a carrier surface activation, and obtain the active preparations of immobilized enzyme. Besides, at using of activating agents it is not inconceivable that these reagents at the interaction with enzyme can deactivate the active centres of biocatalyst [6]. It is hoped that this is not the case when the product of enzymatic reaction is used for enzyme immobilization.

The obtained specimens of GalO, immobilized on the initial and modified silica matrices, are stable and active preparations, which have considerable promise for the determination of galactose-containing sugars and in the fine organic synthesis [20]. These preparations were successfully used (Figs. 1, 2) for analytical determination of galactose-containing carbohydrates in a complex mixture (the linear region for analysis: 0.1-20 mmol/L for raffinose; 2-20 mmol/L for galactose; and 5-100 mmol/L for lactose).

Conclusions

Method of adsorption immobilization of galactose oxidase on the silica surface at the optimal pH-value makes possible obtaining of active heterogeneous enzyme preparations. In addition the resulting specimens possess many a time by more high activity as compared with an enzyme activity in solution taken for adsorption. As this takes place, the carrier has no need of a surface preliminary activation. Growth of an enzyme activity was not detected at covalent

binding of galactose oxidase on amine-containing silicas activated by cyanuric chloride or 2,4-tolylene diisocyanate. It must be indicated that method of activation has no noticeable effect on activity and stability of immobilized preparations. It has been found that in the presence of galactose covalent immobilization of galactose oxidase is rendered possible on amine-containing silicas without a stage of activation of the carrier surface. It is suggested that binding with surface amine groups is executed with the use of 2,4-galactohexodialdose, forming at enzymatic oxidation of galactose. The adsorbed and chemically bound preparations of galactose oxidase have considerable promise in analyses of its substrates and in some organic syntheses.

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References

1. Lukomskaya I.S., Ellanskaya I.A., and Gorodetskii V.K. Experimental Micology. – Kyiv: Naukova Dumka. – 1968 (in Russian).
2. Gansedo J.M., Gansedo C., and Asensio C. Widespread occurrence of galactose oxidase and glucose oxidase in fungi // Archives Biochem. Bioph.- 1967.-V.119, N1-3.- P.588-590.
3. Avigad G., Amoral D., Asensio C., and Horecker B.U. The galactose oxidase of Polyporus circinatus // J. Biol. Chem.-1962.-V.237, N9.-P.2736-2743.
4. Klibanov A.M., Albert B.N., and Marletta M.A. Stereospecific oxidation of aliphatic alcohols catalyzed by galactose oxidase // Biochem. Biophys. Res. Commun.- 1982.- V.108, N2.- P.804-808.
5. Tertykh V.A. and Yanishpolskii V.V. Chemical aspects of enzyme immobilization on inorganic matrices // Adsorption and Adsorbents. – 1980, N8. – P.3-27 (in Russian).
6. Triven M. Immobilized Enzymes. Moscow: Mir. –1983 (in Russian).
7. Hamilton G.A., De Jersey J., and Adolf P.K. Galactose oxidase: the complexities of a simple enzyme. In: Oxidases and Related Redox Systems.- Baltimore- London: Univ., Park Press., 1973.- P.103-119.
8. Pat. USA 4220503. - Jonson J., Stabilization of activated galactose oxidase enzyme, Appl. 28.04.78, Publ. 2.09.80.
9. Taylor R.J., Kmetec E., and Jonson J.M. Design, construction and application of a galactose selective electrodes // Anal. Chem.- 1977.-V. 49, N6.-P.789-794.
10. Kondakova L.V., Yanishpolskii V.V., Tertykh V.A., Buglova T.T., and Koroleva O.V. Some properties of galactose oxidase from *Fusarium graminearum IMV-F-N1060* immobilized on aminoorganosilicas // Ukr. Biokhim. Zhurn. – 1984. – V.56, N4. – P.394-398.
11. Kondakova L.V., Yanishpolskii V.V., Tertykh V.A., and Buglova T.T., Effect of potassium ferricyanide and Cu(II) ions on properties of immobilized galactose oxidase // Ukr. Biokhim. Zhurn. – 1988. – V.60, N6. – P.34-38.
12. Tertykh V.A. and Yanishpolskii V.V. Adsorption and chemisorption of enzymes and other natural macromolecules on silicas // In: Adsorption on Silicas, Ed. by E. Papirer, Marcel Dekker, New York, 2000, P.523-564.
13. Pat. USSR 889705. – Buglova T.T., Kirilenko T.S., and Zakharova I.Ya. Method of preparation of galactose oxidase. – Byull. Izobret., 1981, N46.

14. Pat. USSR 1723124. – Kondakova L.V., Yanishpolskii V.V., Tertykh V.A., and Buglova T.T., Method of galactose oxidase immobilization. – Byull. Izobret., 1992, N2.
15. Tertykh V.A., Yanishpolskii V.V., Chuiko A.A., Galich I.P., Tsyperovich A.S., and Koval'chuk T.A. Immobilization of α-amilase on surface of highly dispersed silica // Doklady AN UkrSSR, ser. B. – 1977. – N7. –P.654-657.
16. Yanishpolskii V.V., Tertykh V.A., and Lyubinskii G.V. Trypsin immobilization on organosilica surfaces // Ukr. Biokhim. Zhurn. – 1979. – V.51, N4. – P.324-329.
17. Pat. USSR 1521775. – Kondakova L.V., Yanishpolskii V.V., Tertykh V.A., Buglova T.T. and Skorobogat'ko O.V. Method of galactose oxidase immobilization. – Byull. Izobret., 1989, N42.
18. Buglova T.T., Skorobogat'ko O.V., and Zakharova I.Ya. Characterization of purified preparations of galactose oxidase from *Fusarium graminearum IMV-F-N1060* // Mikrobiol. Zhurn. – 1983. – V.45, N6. – P.47-52.
19. Rippa M., Signorini M., and Bellini T. The effect of inorganic phosphate on the stability of some enzymes // Biochem. J.- 1982.-V.197, N3.-P.747-749.
20. Kondakova L.V., Yanishpolskii V.V., and Tertykh V.A. Immobilization of galactose oxidase on parent and modified silica matrices // Abstr. 3th Int. Conf. Chemistry of highly organized substances and scientific bases of nanotechnology. – St.-Petersburg, 2001. – P.303-306.