Journal of the Serbian Chemical Society

JSCS@tmf.bg.ac.yu • www.shd.org.yu/JSCS

J. Serb. Chem. Soc. 72 (12) 1255–1263 (2007) UDC 547.458.2+54.05:664.642:582.282.23
JSCS–3657 *Original scientific paper*

Preparation and studies on immobilized α-glucosidase from baker's yeast *Saccharomyces cerevisiae*

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(Received 10 July 2007)

Abstract: α-Glucosidase from *S. cerevisiae* was covalently immobilized onto Sepabeads EC-EA by the glutaraldehyde method. An analysis of the variables controlling the immobilization process is first presented and it is shown that the highest coupling of α-glucosidase occurred within 24 h. Also, a loading of 30 mg/g support proved to be effective, resulting in a rather high activity of around 45 U g^{-1} with a satisfactory degree of enzyme fixed. Both free and immobilized enzymes were then characterized by determining the activity profile as a function of pH, temperature and thermal stability. The obtained immobilized preparation showed the same optimum pH, but a higher optimum temperature compared with the soluble one. In addition, the immobilized enzyme treated at 45 ºC for 1 h still retained an activity of around 20 %, whereas the free enzyme completely lost its original activity under this condition. In conclusion, the developed immobilization procedure is quite simple, easily reproducible and provides a promising solution for the application of immobilized α-glucosidase.

Keywords: maltase, Sepabeads EC-EA, immobilization, stabilization.

INTRODUCTION

α-Glucosidase (EC 3.2.1.20) catalyzes not only the hydrolysis of α-glucoside linkages, but also the transglucosylation of α-glucosyl residues to various glucosyl co-substrates, resulting in the synthesis of new oligosaccharides, besides digestion, lysosomal catabolism of glycoconjugates and glycoprotein synthesis. Pompe disease is a genetic fetal muscle disorder caused by a deficiency of acid α-glucosidase, which results in glycogen accumulation in multiple tissues, with cardiac and muscular tissues being most seriously affected. One way of treating the disease is to employ α-glucosidase, *i.e.*, enzyme therapy.[1,2]

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doi: 10.2298/JSC0712255A

The yeast *Saccharomyces cerevisiae* synthesizes a maltose inducible and glucose repressible α-glucosidase or maltase. This intracellular enzyme is a monomer of 63,000 Da and has been purified to homogeneity and characterized.[3]

The concept of immobilizing proteins and enzymes to insoluble supports has been the subject of considerable research for over 30 years and, consequently, many different methodologies and a vast range of applications have been suggested. The aims often included such factors as: the reuse or better use of enzymes, especially if they are scarce or expensive, better quality products as there should be little enzyme in the product requiring inactivation or downstream purification, the production of biosensors, flow-through analytical devices, or the development of continuous manufacturing processes. Although large tonnages of immobilized enzymes are employed industrially, for example in the production of various syrups from starch,[4] and several smaller-scale industrial applications, the introduction of such biocatalysts has been disappointingly slow.

It is well known that multipoint attachment may promote an increased rigidity in immobilized enzymes.[5] This rigidity makes them more resistant to small conformational changes induced by heat, organic solvents, denaturing agents, *etc*. Multipoint attachment was mainly involved to explain the observed stabilization of randomly prepared derivatives and was considered less in developing strategies to obtain optimally stabilized immobilized systems.[6]

For the industrial application of enzymes acting on water-soluble substrates, such as carbohydrates, an effective immobilization method is required to facilitate the continuous processing and reuse of the biocatalyst.[7,8] In this context, the employment of available carriers for covalent immobilization of enzymes is of great interest. Sepabeads EC is a polymethacrylate-based carrier for enzyme immobilization.[9] Sepabeads EC-EA is an aminated support having ethylenediamine groups with a high reactive group density. Compared with other acrylic polymers, Sepabeads EC-EA possess a high mechanical stability and do not swell in water. In addition, the polymer is non-toxic, non-immunogenic and non-antigenic. Furthermore, the raw materials applied for the production of these supports are included in the EU list of resins allowed for the processing of food stuffs.[10]

In this work, α-glucosidase from *Saccharomyces cerevisiae* was immobilized on Sepabeads EC-EA and the conditions for immobilization and characterization of the immobilized enzyme were studied in detail. The first aim was to determine the optimal immobilization conditions, such as enzyme–support contact time and initial enzyme amount in the attachment solution, with respect to enzyme loading, catalytic activity and coupling yields (enzyme and activity). A comparative study between free and immobilized enzymes was then undertaken in terms of pH, temperature and thermal stability.

EXPERIMENTAL

Material

α-Glucosidase, isolated from baker's yeast by a slightly modified, previously published procedure,[11] showed a single band on SDS–gel electrophoresis, with an approx. molecular weight of 63 kDa. Its specific activity was 80 U mg^{-1} proteins and the K_M for 4-nitrophenyl α-D-glucopyranoside was 0.2 mM. All other chemicals were from Merck, Germany. Sepabeads EC-EA was kindly provided by Resindion S. R. L. (Mitsubishi Chemical Corporation, Milan, Italy). *p*-Nitrophenyl α-D-glucopyranoside (α-*p*NpG) was purchased from Fluka Chemie AG. All other reagents and solvents were of the highest available purity and used as purchased from Sigma.

Standard activity assay

The activity of soluble and immobilized α-glucosidase preparations were determined using sucrose as the substrate. The activity of soluble enzyme was measured at a substrate concentration of 10 % (w/v) sucrose in 50 mM sodium phosphate buffer, pH 7.0 at 25 ºC. One unit of enzyme activity was defined as 1 μmol glucose produced per minute under the above reaction conditions. Glucose was determined by the dinitrosalicylic acid reagent.[12]

Immobilization of α-glucosidase

The immobilization procedure consisted of two main steps: glutaraldehyde activation of the polymer and enzyme coupling to the polymer. Glutaraldehyde reacts with the amino groups of Sepabeads EC-EA, and the amino groups of the enzyme are then coupled to the carrier *via* the free carbonyl groups of the dialdehyde bound to the supports. Activation of polymer was performed by incubation of the polymer with 2.5 % (w/v) glutaraldehyde solution buffered at pH 7 (sodium phosphate buffer, 100 mM) for two hours at room temperature in the dark with occasional stirring. Subsequently, the polymer was incubated overnight with different amounts of enzyme (from 5 to 50 mg/g dry polymer) in sodium phosphate buffer, pH 7.0 at 4 ºC. After binding, any unbound enzyme was removed by washing several times with 1 M NaCl in 50 mM sodium phosphate buffer pH 7.0 and with buffer alone (the washing solutions were combined and stored for activity and determination of proteins) and stored at 4 ºC in 50 mM sodium phosphate buffer pH 7.0 until use.

Time-course of immobilization

A total of 0.5 g of support was suspended in 50 ml of enzyme solution (protein concentration 1 mg ml^{-1}) in sodium phosphate pH 7.0, 50 mM at 25 ºC. Periodically, samples of the supernatants were withdrawn and analyzed for enzyme activity.

Determination of proteins

Soluble protein was determined by the Bradford method,[13] using bovine serum albumin as the protein standard.

Effects of pH and temperature on α-glucosidase activity

The effect of temperature on the specific activity of the immobilized and soluble preparations was determined by incubation of the biocatalysts at a temperature ranging from 25 to 90 ºC with 10 % (w/v) sucrose in 50 mM sodium phosphate buffer pH 7.0. The specific activity at 30 ºC for the soluble and 40 ºC for the immobilized form was arbitrarily set as 100 % relative activity.

The optimal pH was determined with 10 % (w/v) sucrose in 50 mM sodium citrate phosphate buffer of pH values varying from 3 to 9 at 25 ºC. The specific activity value obtained at pH 6 for the soluble and 7 for immobilized form was taken as 100 % relative activity.

Enzyme inactivation experiments

Enzyme preparations (soluble and immobilized preparations) were incubated at pH 7.0 and 45 ºC. Periodically, samples were withdrawn and their remaining activities were assayed as des-

cribed above. The residual activity of α-glucosidase was expressed as the percentage of the initial activity for a given incubation time.

Kinetic deactivation model

Thermal deactivation kinetics of free and immobilized enzymes was studied based on a single step, first-order deactivation mechanism, having the final state of the enzyme, E_d, with or without residual activity:

$$\alpha E \xrightarrow{k} E_d \quad (1)$$

where E and E_d are the initial and final state of the enzyme, respectively; k is the first-order deactivation rate coefficient; α is the ratio of the specific activity of the E and E_d enzyme states. This mechanism leads to a model, where the residual enzyme activity at time t can be determined from the following equation:

$$A(t) = A_1 e^{-kt} + A_2 \quad (2)$$

$$A(t) = (100 - \alpha) e^{-kt} + \alpha \quad (3)$$

In the proposed kinetic model, the adjustable parameters are the rate coefficient, k, and the residual activity of the enzyme, α, which were determined by the least-squares fit to the experimental data using MAT-LAB software.

RESULTS AND DISCUSSION

For this immobilization study, a commercially available polymer, Sepabeads EC-EA was employed. The particle diameter, specific pore volumes and other parameters for the employed polymer are presented in Table I.

TABLE I. Physicochemical characteristics of Sepabeads EC-EA

	Active group	Functional group density mmol g^{-1} wet	Particle size range μm	Density g ml^{-1}	Pore diameter peak nm	pH stability range
Sephabeads EC-EA	Ethylendiamino	0.6	150–300	>1.1	>30	0–14

Time-course of α-glucosidase immobilization on Sepabeads EC-EA

The influence of the duration of the coupling was analyzed by measuring the activity of enzyme in the supernatant at various times, as described in the experimental section. The kinetic plot of enzyme coupling is presented in Fig. 1. It seems that during the first 20 h of coupling, around 25 % of the amount of initial enzyme was immobilized. After this initial phase, the rate of enzyme immobilization decreased and gradually ceased after about 24 h. After 20 h, only an addition 10 % of the initial enzyme activity was immobilized. Thus, an enzyme–support contact time of 24 h was considered to be optimal for the immobilization of α-glucosidase on Sepabeads EC-EA under the employed conditions (pH 7.0, 25 °C).

Effects of the amount of enzyme on the enzyme loading and activity coupling yield

An attempt was made to achieve the binding of high levels of enzyme with a high retention of hydrolytic activity. The influence of the amount of α-glucosi-

dase in the attachment solution in the range of 2.5–100 mg of enzyme per gram of dry support was studied. In each experiment, 0.05 g of support particles was immersed in a certain volume of enzyme solution. The aim was to determine an efficient relationship between the enzyme and the support. The results are presented in Fig. 2.

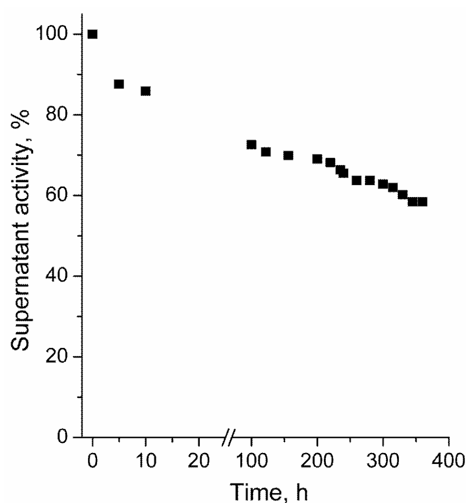

Fig. 1. Kinetics of α-glucosidase immobilization on Sepabeads EC-EA at 25 °C and pH 7.0.

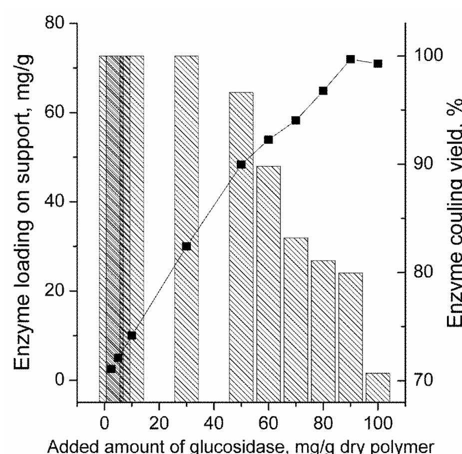

Fig. 2. Effects of the initial amount of α-glucosidase in the attachment solution on the enzyme coupling yield.

It is apparent from Fig. 2 that the enzyme loading on Sephabeads EC-EA initially increased rapidly with increasing amount of α-glucosidase but leveled off at about 70 mg/g. The enzyme coupling yield was in the range of 70–100 %. However, it is apparent from Fig. 3 that the activity yield is inversely related to the amount of enzyme bound. For example, increasing the α-glucosidase loading from 2.5 to 72 mg/g support resulted in a decrease in the activity yield from 96.9 to 13.11 %, possibly due to the close packing of the enzyme on the support surface, which could limit access of the substrate to the enzyme, required for the hydrolysis reaction. It is generally acknowledged that the catalytic efficiency of immobilization processes decreases when the enzyme loading exceeds a certain value and an optimum activity should be selected.[14,15] The loading of 30 mg/g support seems to be most appropriate for use, resulting in an activity yield of 30 % with satisfactory degree of bound enzyme. The present activity of the immobilized enzyme was higher than in a previously published study, where the immobilized α-glucosidase had an activity ranging from 44.8–80.7 U g^{-1}, depending of characteristics of the macroporous support.[16]

Dependence of the catalytic activity on pH and temperature

Both the soluble and immobilized form of α-glucosidase were the most active in the pH range 6–7 (Fig. 4). After immobilization, the optimal pH did not

change when compared with the pH profile of the soluble enzyme; the presented results are very similar to those previously published.[17]

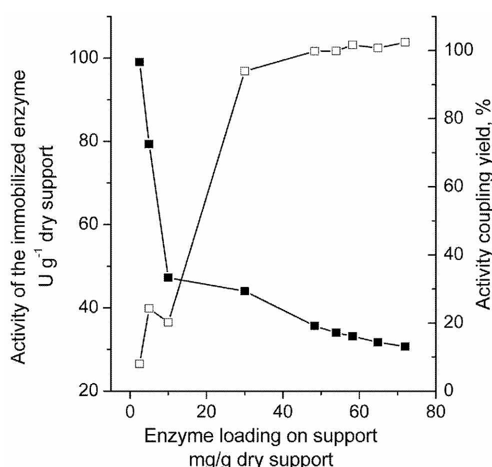

Fig. 3. Effect of enzyme loading on the activity of the biocatalysts and the activity coupling yield. The symbols represent the activity of the immobilized α-glucosidase (□) and the activity coupling yield (■).

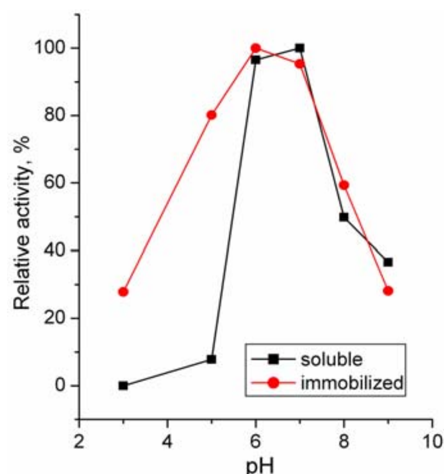

Fig. 4. Dependence of the relative activity of soluble and immobilized on Sephabeads EC-EA α-glucosidase from *S. cerevisiae* on pH (temperature 25 °C, 10 % (w/v) sucrose as substrate).

Temperature dependencies of the activities of the two forms of α-glucosidase, soluble and immobilized, are presented in Fig. 5. It appears that the optimal reaction temperature shifted from 30 °C for soluble α-glucosidase to almost 40 °C for the immobilized enzyme, suggesting a significantly better thermal stability of the immobilized enzyme.

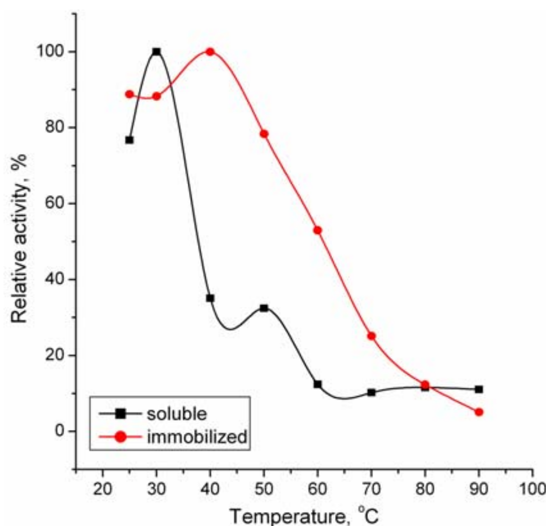

Fig. 5. Dependence of the relative activity of soluble and on Sephabeads EC-EA immobilized α-glucosidase from *S. cerevisiae* on temperature (pH 7.0, 10 % (w/v) sucrose as substrate).

Effect of immobilization on the thermal stability of the enzyme

An important consideration when evaluating an immobilized enzyme system is the reported enzyme deactivation. Moreover, for the design and operation of an enzyme reactor, a proper kinetic deactivation model and kinetic parameters are required. Therefore, the thermal stability of the immobilized enzyme was studied at 45 °C in an aqueous medium (50 mM sodium phosphate buffer, pH 7.0) and compared with that of the free enzyme.

The kinetic deactivation profiles at 45 °C for the free and immobilized enzyme are presented in Fig. 6. In order to interpret and analyze the obtained experimental results, the model based on a single step, first-order degradation kinetics was fitted to the deactivation curves (Eq. 3). The points on the graph are experimental data and the solid lines represent the best fits of the predictions of the theoretical model. The results show that immobilization of the enzyme on Sepabeads EC-EA offers a high degree of thermoprotection. For example, immobilized enzyme treated at 45 °C for 1 h still retained activity of around 20 %, whereas the free enzyme lost its original activity completely under this condition. This fact is of real significance in commercial applications.

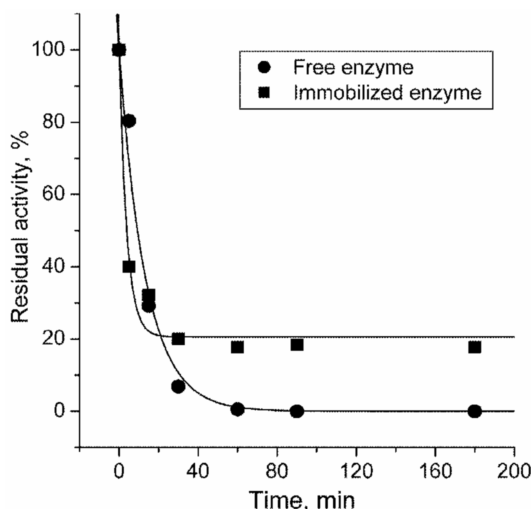

Fig. 6. Thermal deactivation of free (●) and immobilized enzyme (■) at 45 °C. The lines represent the best fits of the single step, first-order kinetic deactivation model. The initial activities were taken as 100 %.

It also seems that first order enzyme degradation kinetics fits the experimental data well for both soluble and immobilized enzymes, suggesting that the biocatalysts lose their activity in only one step at the tested temperature. The best-fit values of the deactivation rate constant, k, half life, $t_{1/2}$, and the residual activity of the final state enzyme, α, are listed in Table II. It appears that the free enzyme is more thermostable than the immobilized one, deduced from the k and $t_{1/2}$ values. However, immobilization enabled the enzyme to attain a final state, E_d, with a significant residual activity, while the final state of the free

enzyme was completely deactivated ($\alpha = 0$). Thus, the shape of the curve obtained for the immobilized enzyme showed that, after a first phase of rapid inactivation, the residual activity of the immobilized enzyme reached a defined plateau, yielding a stable enzyme form with a residual activity of around 20.56 % ($\alpha = 20.56$ %), indicating an improvement in the thermal stability of the enzyme upon immobilization.

TABLE II. Best-fit parameters of the first-order kinetic model (Eq. 3)

Biocatalyst	t / °C	k / min^{-1}	A_1	α (A_2)	$t_{1/2}$ / min
Free enzyme	45	0.075	100	0	9.24
Immobilized enzyme		0.260	79.08	20.56	3.79

CONCLUSION

To summarize, although Sepabeads EC-EA is known to be a good support for enzyme immobilization, its potential for α-glucosidase immobilization has not been fully explored. In the present study, the conditions for immobilization of α-glucosidase, the time course of immobilization and the amount of added enzyme were optimized. To prevent enzyme inactivation, an approach is presented for the stable covalent immobilization of α-glucosidase from *S. cerevisiae* on an amino-containing support (Sepabeads EC-EA) with a high retention of activity. The immobilized enzyme showed greater thermostability than the soluble one; after incubation of the enzymes at 45 °C for 1 h, the soluble form showed no activity but the immobilized enzyme retained a residual activity of around 20.56 %. Further work is in progress on the application of immobilized α-glucosidase for the continuous synthesis of various physiologically active compounds.

Acknowledgments. The authors thank Resindion S. R. L. (Mitsubishi Chemical Corporation, Milan, Italy) for the gift of the Sepabeads samples. Also, the authors are grateful for the financial support of the Ministry of Science of Serbia (Project No. 142020).

И З В О Д

ДОБИЈАЊЕ И ПРОУЧАВАЊЕ ИМОБИЛИЗАЦИЈЕ α-ГЛУКОЗИДАЗЕ ИЗ ПЕКАРСКОГ КВАСЦА *Saccharomyces cerevisiae*

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Малтаза из *S. cerevisiae* је ковалентно имобилизована на Sepabeads EC-EA након активације носача раствором глутаралдехида. Испитивањем кинетике имобилизације утврђено је да се 25 % ензима имобилизује након 24 часа. Имобилизована α-глукозидаза има исти pH оптимум као и растворни ензим, док је оптимална температура за активност имобилизованог ензима увећана за 10 °C у поређењу са растворним ензимом. Када се упореде заостале активности растворне и имобилизоване форме α-глукозидазе, након инкубације од 1 h на

45 °C растворни ензим не показује активност док имобилизована форма задржава око 20 % почетне активности. Имобилизована форма ензима задржава 20 % почетне активности чак и после 3 h инкубације на 45 °C.

(Примљено 10. јула 2007)

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