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Stabilization of α-glucosidase in organic solvents by immobilization on macroporous poly(GMA-co-EGDMA) with different surface characteristics

RADIVOJE M. PRODANOVIĆ[1*#], NENAD B. MILOSAVIĆ[2#], SLOBODAN M. JOVANOVIĆ[3#], TANJA ĆIRKOVIĆ VELIČKOVIĆ[1#], ZORAN M. VUJČIĆ[1#] and RATKO M. JANKOV[1#]

[1]*Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Belgrade,* [2]*Department of Chemistry, ICTM, Belgrade, and* [3]*Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia and Montenegro (e-mail: rprodano@chem.bg.ac.yu)*

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Abstract: *a*-Glucosidase from baker's yeast was immobilized on macroporous copolymers of ethylene glycol dimethacrylate and glycidyl methacrylate, poly(GMA-co-EGDMA), with various surface characteristics and pore sizes ranging from 44 nm to 270 nm. Immobilization was done by glutaraldehyde on the copolymer previously modified with 1,2-diaminoethane. The specific activity of the obtained immobilized enzyme varied from 27 to 81 U/g, depending on the employed copolymer. The half lives of the immobilized enzyme in cosolvents were influenced by the surface characteristics of the copolymer, ranging from 60 to 150 min in 35 % methanol and from 10 to 44 min in 45 % dimethyl sulphoxide (DMSO). The best stabilities were obtained when the enzyme was immobilized onto a copolymer having a pore size of 48 nm in methanol and 270 nm in DMSO.

Keywords: cosolvent, eupergit, maltase, immobilized, stability.

INTRODUCTION

Non-traditional biocatalysis, which includes enzymes in organic solvents for biotransformations, has been investigated extensively over the past two decades. The use of organic solvents has advantages over conventional biocatalysis, for example the biotransformation of substrates which are sparingly soluble in water and increased yield of reverse reactions of hydrolysis due to the decreased water activity. The limiting factor for application is often the low activity and stability of enzymes in non-aqueous environments, which is the result of structural denaturation and diffusional limitations because of the insolubility of native enzymes in organic solvents. Several approaches have been developed to improve the stability and sol-

* Corresponding author.

Serbian Chemical Society active member.

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ubility of proteins in organic solvents: site directed mutagenesis,[1] chemical modification,[2] the use of additives,[3] immobilization,[4] etc.

Immobilization has received a lot of attention as a tool for stabilizing enzymes in organic solvents.[5–7]

Macroporous copolymers of ethylene glycol dimethacrylate and glycidyl methacrylate, poly(GMA-co-EGDMA) (an eupergit-like copolymer) has been previously used for enzyme immobilization.[8–11] They offer a large surface area, a hydrophilic microenvironment, a number of active epoxide groups and the possibility of obtaining targeted porous structures.[12] These varieties of porous structures which can be obtained offer the possibility of finding copolymer with suitable surface characteristics for the stabilization of an investigated enzyme.[7]

α-Glucosidase from *Saccharomyces cerevisiae* (baker's yeast) catalyses glucosylation of menthol[13] and *n*-alkyl alcohols[14] in an aqueous system. Recently it was reported that this enzyme could catalyze glucosylation of various phenolic compounds, such as hydroquinone, catechine and *p*-coumaric acid.[15] The activity of the soluble α-glucosidase was better preserved in the presence of DMSO and methanol than in various organic solvents.[16]

In this study the influence of the surface characteristics of copolymers of poly(GMA-co-EGDMA) on the stability of immobilized α-glucosidase in DMSO and methanol was investigated.

EXPERIMENTAL

Enzyme and chemicals

α-Glucosidase was isolated from baker's yeast by a slightly modified previously published procedure.[17] The obtained α-glucosidase showed a single band on SDS-gel electrophoresis with an approximate molecular weight of 63 kDa.[13] The specific activity of the purified enzyme was 40 U/mg of protein with sucrose as the substrate. Sucrose, pNPG (*p*-nitrophenyl α-D-glucopyranoside), cosolvents and all other chemicals were from Merck, Germany.

Copolymer preparation

The macroporous copolymers were obtained by suspension polymerization as previously described.[12] The particle size distribution was determined by sieve analysis. A commercial mercury porosimeter, Model 2000 Carlo Erba, was used for the determination of the specific pore volume, pore size distribution and specific area. Prior to use, the copolymer was modified with 1 M 1,2-diaminoethane at 60 °C for 4 h at pH 10. The concentration of amino groups was determined by titration with 0.01 M HCl in 0.5 M KCl.

Enzyme immobilization

Dry copolymer (0.30 g) was activated with 2.5 % glutaraldehyde in 50 mM sodium phosphate buffer pH 7.0 for 2 h. Subsequently, the copolymer was rinsed with water and incubated overnight with 75.5 U of enzyme in sodium phosphate buffer pH 7.0 at 4 °C. The immobilized enzyme was then rinsed several times with 1 M NaCl in 50 mM sodium phosphate buffer pH 7.0 (the washings were combined and stored for activity measurements) and stored at 4 °C in 50 mM sodium phosphate buffer pH 7.0 until use.

Enzyme activity

The activity of the soluble enzyme prior to immobilization was measured at a substrate concentration of 10% (w/v) sucrose in 50 mM sodium phosphate buffer pH 7.0. 1 U of enzyme activity was defined as the amount of enzyme that liberates 1 μmol glucose from the substrate in 1 min at 25 °C. The reducing sugars were determined using dinitrosalicylic acid reagent.[18]

Immobilized glucosidase (30–50 mg) was added to 5 ml of 10 % (w/v) sucrose in 50 mM sodium phosphate buffer pH 7.0. The reaction was allowed to proceed for 15 min at 25 °C under constant stirring. The formed reducing sugars were determined using dinitrosalicylic acid reagent. The specific activity of the immobilized enzyme was the amount of glucose in micromoles that was liberated in 1 min at 25 °C from 1 g of dry copolymer with enzyme immobilized on it. The bound activity was the amount of enzyme which was bound per 1 g of dry copolymer. It is calculated from the difference in the activity of the enzyme solution prior to immobilization and the activity of the combined washings after immobilization. The yield was calculated from 100 % x (Specific activity/Bound activity).

Enzyme stability

The enzyme solution (1 mg/ml) was incubated at 25 °C in 100 mM sodium citrate/phosphate buffer pH 5.5 containing various concentrations of cosolvent. After an appropriate time, 2 μL aliquots of the enzyme were taken and the residual activity was measured in 0.6 mL of 1 mM pNPG as the substrate solution in 50 mM sodium phosphate buffer pH 7.0 without cosolvent. The relative activity was determined by the amount of *p*-nitrophenol liberated during the first minute of the reacton. *p*-Nitrophenol was determined spectrophotometrically at 405 nm.

Wet immobilized enzyme (0.1 g) was incubated at 25 °C in 10 mL 100 mM sodium citrate/phosphate buffer pH 5.5 containing various concentrations of cosolvent. After an appropriate time, the immobilized enzyme was rinsed several times with 30 mM sodium phosphate buffer pH 7.0 and the residual activity was measured without cosolvent, as already described for the immobilized enzyme.

RESULTS AND DISCUSSION

The particle diameters of the obtained copolymers were in the range of 150–500 μm. The specific pore volume, specific area and the mean pore diameter of the copolymers were determined prior to modification and the concentration of amino groups was determined after modification with diaminoethane by titration with 0.100 M HCl, Table I.

TABLE I. Surface parameters of the investigated copolymers employed for immobilization[10]

Copolymer type	Pore size $d_{v/2}$ nm	Pore volume V_s ml/g	Surface area $S_{s,Hg}$/m^2 g^{-1}	Amino groups μmol/g
GE-10/4	44	0.580	70	790
GE-10/10	48	0.560	53	1150
A 1-94	60	0.660	56	910
GE-10/14	68	0.740	47	1210
GE-10/16	87	0.755	33	1090
GE-20/12	120	0.960	31	1050
GE-15/16	200	1.020	34	370
GE-20/14	270	1.040	26	1180

It can be seen that the obtained copolymers had different surface characteristics with similar concentrations of amino groups on their surface.

In a previous report, the ability of α-glucosidase from baker's yeast to catalyze glucosylations of various phenolic compounds was described.[15] In order to obtain a more suitable biocatalyst for glucosylation, α-glucosidase was immobilized by the glutaraldehyde method, which had previously been employed for invertase,[8] and the specific activity of the immobilized enzyme was determined (Table II).

TABLE II. Immobilization parameters of the investigated copolymers and enzyme. Immobilization was performed with 252 U of enzyme per 1 g of dry copolymer (previously activated with glutaraldehyde) for one day in 50 mM sodium phosphate buffer pH 7.0 at 4 °C

Copolymer type	Bound activity U/g	Bound activity %	Specific activity U/g	Yield %
GE-10/4	237	94.2	44.8	18.9
GE-10/10	240	95.4	46.8	19.5
A 1-94	227	90.4	56.7	25.0
GE-10/14	241	95.8	56.2	23.3
GE-10/16	227	90.1	53.5	23.6
GE-20/12	244	97.1	27.3	11.2
GE-15/16	246	97.8	43.4	17.7
GE-20/14	227	90.2	80.7	35.5

All the employed copolymers bound almost all of the applied activity, which was probably the consequence of the high concentration of active amino groups on the copolymer surface. The immobilized enzymes showed similar specific activity, around 50 U/g, with a yield of immobilization of around 20 %, except for the copolymer designated GE-20/14, which had a higher specific activity of 80.7 U/g with a 35.5% yield of immobilization. These differences in the activities between the immobilized enzymes could be explained by the different surface characteristics of the employed copolymers.

In a previous study,[16] it was shown that the enzyme activity was better retained an DMSO and methanol than in dioxane, *t*-butanol, tetrahydrofuran and *N,N*-dimethylformamide. In order to determine the influence of the type of copolymer employed for immobilization on the stability of the immobilized α-glucosidase with DMSO and ethanol as organic cosolvents, the activity of the native enzyme, for the synthesis of glucoside at the optimum pH of 5.5[15] was determined as a function of time. The results are shown in Fig. 1.

The half life of the native enzyme was 4 min in 45 % DMSO and 5.5 min in 35 % methanol. With 35% methanol as cosolvent, the half life of the immobilized α-glucosidase was increased and the degree of stabilization depended on the copolymer employed for immobilization, Fig. 2.

This stabilization of the immobilized enzyme could be explained by the regidification of the protein structure which occurs because of multiple bonding of the enzyme to the copolymer surrounding the enzyme molecule.[6] Hence, the mor-

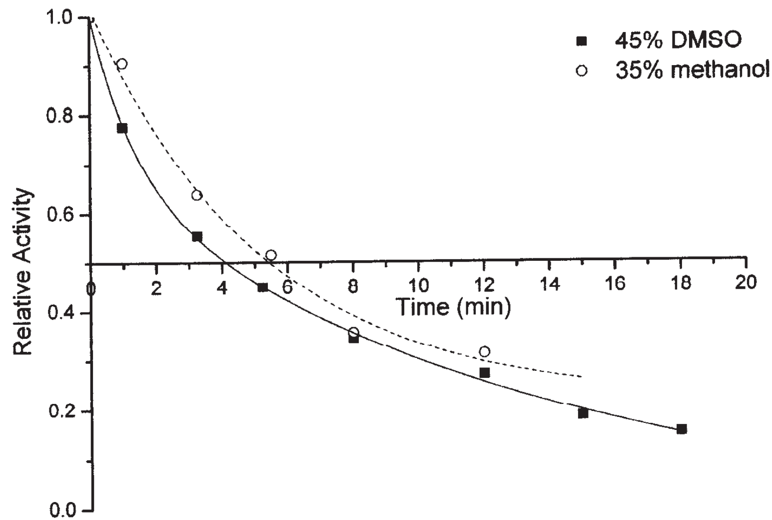

Fig. 1. Dependence of the residual activity on time for the soluble α-glucosidase in DMSO and methanol. The soluble enzyme (1 mg/ml) was incubated at 25 °C in 100 mM sodium citrate/phosphate buffer pH 5.5 containing cosolvent. After appropriate time intervals, aliquots are taken and the residual activity was measured as described in buffer solution without cosolvents.

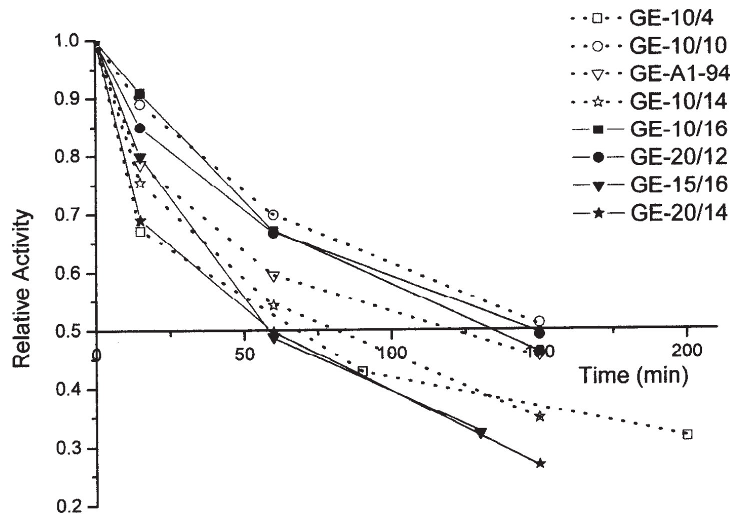

Fig. 2. Dependence of the residual acitivity on time for the immobilized α-glucosidase in 35 % methanol. The wet immobilized enzyme (0.1 g) was incubated at 25 °C in 10 mL 100 mM sodium citrate/phosphate buffer pH 5.5 containing various concentrations of cosolvent. After appropriate time intervals, the immobilized enzyme was rinsed and the residual activity was measured as described in buffer solution without cosolvent.

phology of the copolymer and the diameter of pores in which the immobilized enzyme is situated could be of major importance for determining the stability of the immobilized enzyme.[7]

For the same copolymer used for immobilization, the degree of the stabilization was different in DMSO compared to methanol, Fig. 3.

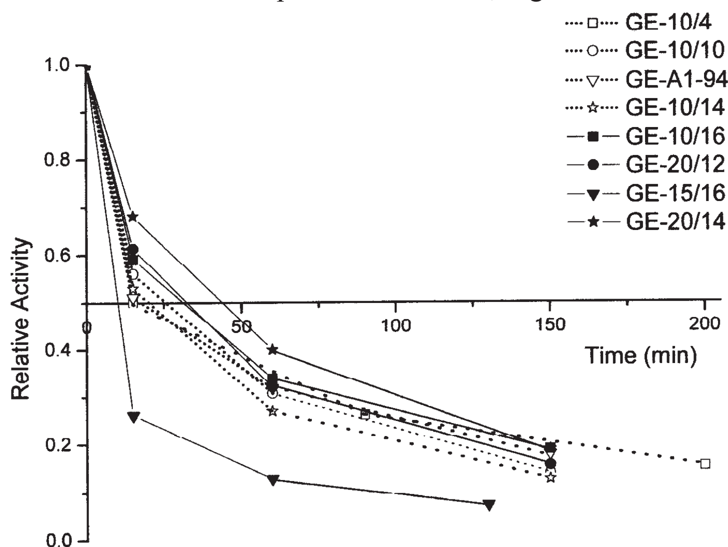

Fig. 3. Dependence of the residual activity on time for immobilized α-glucosidase in 45 % DMSO. The wet immobilized enzyme (0–1 g) was incubated at 25 °C in 10 mL 100 mM sodium citrate/phosphate buffer pH 5.5 containing various concentrations of cosolvent. After appropriate time intervals, the immobilized enzyme was rinsed and the residual activity was measured as described in buffer solution without cosolvent.

These results indicate that the mechanisms of α-glucosidase inactivation by these two cosolvents are different, which was also previously observed for immobilized β-galactosidase.[4]

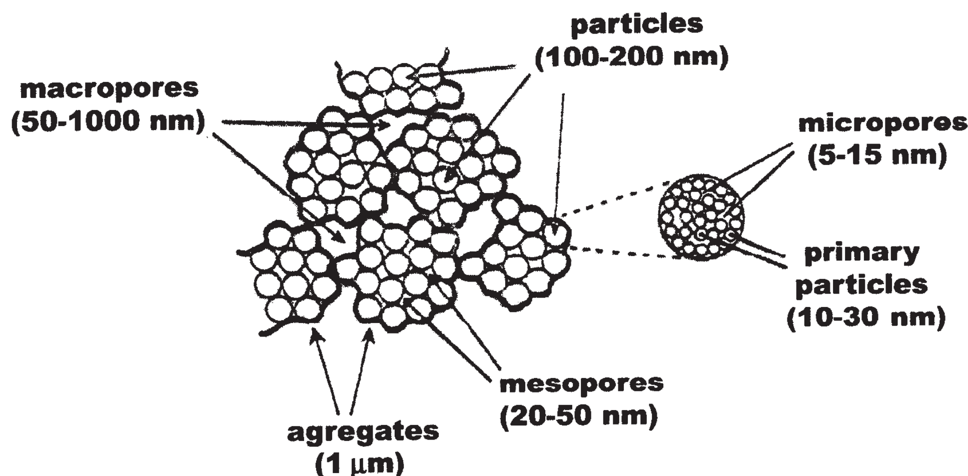

Fig. 4. The Sherrington model of the structure of the macroporous copolymers.

There is also very little correlation between the mean pore size of the copolymer macropores and the degree of stabilization. This observation can be explained by the Sherrington model of the structure of macroporous copolymers, Fig. 4.

According to this model, a macroporous copolymer has macro, meso and micropores of various diameters.[19] Their pattern depends on the type of the copolymer and is called surface morphology. It is evident that the stabilization of an enzyme the diameter of which is around 3 nm depends on all of these pores and not only the macropores, indicating that the overall surface morphology of a macroporous copolymer is the key factor in determining the stability of an immobilized enzyme.

Assumng that the deactivation of the immobilized enzyme follows first order reaction kinetics,[20] the half lives and the first order constants for enzyme deactivation were determined, Table III.

TABLE III. Kinetic parameters for the deactivation of native and immobilized α-glucosidase. The soluble enzyme (1 mg/ml) was incubated at 25 °C in 100 mM sodium citrate/phosphate buffer pH 5.5 containing various concentrations of cosolvent. The wet immobilized enzyme (0.1 g) was incubated at 25 °C in 10 mL 100 mM sodium citrate/phosphate buffer pH 5.5 containing various concentrations of cosolvent. After the appropriate time intervals, the residual activity was measured as described in buffer solution without cosolvents

Enzyme and copolymer	$k_{in(methanol)} \times 10^{-3}$ min^{-1}	$t_{1/2(methanol)}$ min^{-1}	$k_{in(DMSO)} \times 10^{-3}$ min^{-1}	$t_{1/2(DMSO)}$ min^{-1}
Native glucosidase	128	5.40	169	4.10
GE-10/4	10.3	67.6	46.2	15.0
GE-10/10	4.62	150	27.0	25.7
A 1-94	5.78	120	39.4	17.6
GE-10/14	8.54	81.2	34.5	20.1
GE-10/16	5.78	120	22.1	31.4
GE-20/12	4.72	147	21.3	32.5
GE-15/16	11.9	58.3	68.6	10.1
GE-20/14	11.6	60.0	15.8	44.0

The highest degree of stabilization was obtained with one copolymer in DMSO and with another in methanol. Hence, the half life increased on immobilization from 5.4 to 150 min in methanol with the copolymer designated GE-10/14 (pore diameter 48 nm), while in DMSO it increased from 4.0 to 44 min with the copolymer designated GE-20/14 (pore diameter 270 nm). The obtained degrees of stabilization were higher than those obtained for β-galactosidase immobilized on glutaraldehyde–agarose[4] in high concentrations of cosolvents. For example, is was reported that the half life of the enzyme in 36 % (v/v) dimethylformamide increased from 0.3 to 60 min and the half life even decreased in 36 % (v/v) acetone and ethanol.

CONCLUSION

α-Glucosidase from baker's yeast in organic cosolvents was stabilized by an order of magnitude by immobilization onto macroporous poly(GMA-co-EGDMA). This degree of stablization was greatly influenced by the nature of the employed copolymer used, but there was no correlation between the stability of the immobilized enzyme and either the pore size or the volume of the macropores of the copolymer. The reason for this could be the existence of micro and mesopores in the copolymer which could contribute to the stabilization of the enzyme.

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ИЗВОД

СТАБИЛИЗАЦИЈА α-ГЛУКОЗИДАЗЕ У ОРГАНСКИМ РАСТВАРАЧИМА ИМОБИЛИЗАЦИЈОМ НА МАКРОПОРОЗНИМ (ПОЛИ) ГЛИЦИДИЛ МЕТАКРИЛАТИМА РАЗЛИЧИТИХ ПОВРШИНСКИХ КАРАКТЕРИСТИКА

РАДИВОЈЕ М. ПРОДАНОВИЋ[1], НЕНАД Б. МИЛОСАВИЋ[2], СЛОБОДАН М. ЈОВАНОВИЋ[3], ТАЊА ЋИРКОВИЋ ВЕЛИЧКОВИЋ[1], ЗОРАН М. ВУЈЧИЋ[1] и РАТКО ЈАНКОВ[1]

[1]*Катедра за биохемију, Хемијски факултет, Студентски трг 12, Београд,* [2]*Центар за хемију, ИХТМ, Студентски трг 12, Београд и* [3]*Технолошко-металуршки факултет, Карнегијева 4, Београд*

α-Глукозидаза изолована из пекарског квасца је имобилизована на макропорозним глицидил-метакрилатима различитих површинских карактеристика и величина пора од 44 до 270 nm. Имобилизација је изведена глуталдехидом на полимеру претходно модификованом са 1,2-диаминоетаном. Специфична активност добијеног имобилизованог ензима је варирала од 27 до 81 U/g у зависности од врсте коришћеног полимера. Полуживоти имобилизованог ензима у корастварачима су зависили од површинских карактеристика полимера и кретали су се у опсегу од 60 до 150 min у 35 % (v/v) метанолу и од 10 до 44 min у 45 % (v/v) диметилсуфоксиду. Највећа стабилност у метанолу је добијена имобилизацијом ензима на полимеру са величином пора од 48 nm а у диметилсулфоксиду на полимеру са величином пора од 270 nm.

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