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Immobilization of glucose oxidase on porous copolymer

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Porous copolymer was prepared by suspension polymerization method of styrene, maleic anhydride and divinylbenzene in a batch type reactor. The characteristics of the copolymer were evaluated using Fourier Transform IR spectroscopy and scanning electron microscopy. The glucose oxidase (GOD) enzyme was selected as a model enzyme to assess its potential applicability for immobilization purpose. Then the glucose oxidase was immobilized on a copolymer under an optimized condition (the incubation time, and pH 6,5, GOD 5 mg/ml). Enzymatic activities of free and immobilized GOD were assessed in terms of the oxidation of D-glucose to D-gluconic acid carried out under stirring by air. The enzyme immobilization yield was 87% with the immobilized GOD activity of 125 U/mg protein. The Michaelis constant for immobilized GOD was disclosed to be 16,3 mmol/l by Lineweaver–Burk plot at 25ºC. The highest enzymatic activity was achieved when the pH of the medium was approximately pH 7.0. Storage stabilities of GOD samples were investigated during 40 days and the retained activity was 70%, higher than the 22% of the free enzyme.

Key words: *maleic anhydride copolymer; glucose oxidase; immobilization*

INTRODUCTION

Glucose-oxidase (GOD, β-D-glucose:oxygen-1-oxidoreductase, EC 1.1.3.4) is a well-characterised enzyme (flavoprotein), that catalyses the oxidation of β-D-glucose to D-gluconolactone which in its turn converts in an aqueous solution into gluconic acid according the following equation:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid}$$

$$\text{gluconic acid} + H_2O_2 \xrightarrow{\text{CAT}} CO_2 + H_2O + \tfrac{1}{2} O_2$$

During the catalytic oxidation of glucose, hydrogen peroxide is generated. Under physiological conditions GOD is exposed to very low concentrations of H_2O_2, but in the industrial applications higher H_2O_2 concentrations are usually obtained, which can attack some residues of the proteins and GOD's enzymatic activity is reduced. The gluconic acid accumulates, reducing pH of the solution. Both gluconic acid and hydrogen peroxide can result in product inhibition of GOD. Therefore, it is inevitable to remove hydrogen peroxide, properly with catalase (CAT).

Industrial use of soluble GOD from *A. niger* is the basis of many applications in the food industry, textile bleaching and as a glucose biosensor. The GOD-catalysed reaction removes oxygen and generates hydrogen peroxide, a trait utilised in food preservation [1, 2]. GOD has also been used in baking, dry egg powder production, wine production, gluconic acid production, etc. Maillard non-enzymatic browning is a result of reaction between the amino group of proteins or amino acid and reducing sugars. In the production of dried egg powder, this reaction causes undesirable browning. Thus, soluble GOD-CAT are mixed into egg products in order to remove traces of glucose from them. The GOD-CAT mixture can be added to the beverage to remove oxygen, and thus to prevent damage by oxidation. This is typically a batch process and contains the enzyme as an impurity in the product.

However, because of the relatively high price of GOD it is no competitive. In order to compensate the high price of enzymes, it was proposed to recover them by immobilisation. Through immobilisation, continuous process is possible and the enzyme can also be retained and recycled. Although immobilized enzymes usually show lower catalytic activity than the free ones, they are more stable and can be reused and therefore they are cheaper and more effective in large scale applications [3]. Many studies were performed involving the immobilization of GOD in different matrices to enhance properties such as reusability, recovery, stability, thermostability and shelf life. GOD has been immobilized on numerous carriers, such as sol–gel matrix, porous silica beads, polymer membrane, polymer microsphere. Besides natural polymers, many synthetic organic polymers have been investigated for that purpose. Among them,

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two common examples are the derivatives of ethylene-maleic anhydride copolymer and those of polystyrene [4,5].

In the present paper, a novel reactive fine powdery copolymer preparation is demonstrated. Its structure and morphology are also investigated. Taking into consideration the economical demands, a simple way of covalently immobilization of GOD is applied. The parameters important for immobilization process were studied. The effect of immobilization on the multiple use of the enzyme was estimated in batch type reactor.

EXPERIMENTAL

Materials

Glucose oxidase isolated from *Aspergillus niger* (Sigma-Aldrich, Product Number GO 49180), its GOD activity was 185 units/mg. Styrene (Lukoil Neftochim Bourgas, Bulgaria, St) was washed successively with a dilute solution of sodium hydroxide and water, dried over anhydrous calcium chloride, and then distilled under reduced nitrogen pressure just before use. Divinylbenzene (DVB), maleic anhydride (MA) and benzoyl peroxide (BPO) used were reagent grade (Sigma-Aldrich). All other chemicals were reagent grade commercial products.

Preparation of copolymer of styrene-maleic anhydride-divinylbenzene (P(St-co-MA-co-DVB)

A glass reaction vessel equipped with a stirrer (l000 rpm), a thermometer, and a reflux condenser was used. 100 ml benzene was put into the reaction vessel and under nitrogen stream a definite weight of the monomers and BPO were added. The copolymerization was carried out at 70°C and stirred for 4 hours. The resultant copolymer was quickly filtered, washed with benzene, and dried up at 70°C under vacuum.

Determination of the maleic anhydride unit content

The total amount of anhydride and free carboxyl groups in copolymer were determined. For this purpose 0,2 g copolymer was hydrolyzed in 25 mL 0,1M NaOH at 90°C for 3 h applying also stirring. After cooling, the mixture was titrated with 0,1 M HC1 using phenolphthalein as indicator. The copolymerized MA quantity was calculated as the MA unit content of the copolymer.

Immobilization procedure

The immobilization of GOD to the active support (P(St-*co*-MA-*co*-DVB) was conducted under defined conditions: 1,0 g of carrier and 100 mg of enzyme were added to 19,0 ml of 0,05 M phosphate buffer (pH 6,5). After incubation at 4°C for 24 hr with occasional stirring, the immobilized enzyme was collected by filtration and washed extensively with several portions of cold buffer to remove the free enzyme.

Determination of protein

Protein determination was performed according to Lowry et al. [6]. The amount of immobilized protein was calculated as the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein found in the filtrate and washing solutions after immobilization.

Assay of GOD activity

The reaction velocity is determined by an increase in absorbance at 460 nm resulting from the oxidation of o-dianisidine through a peroxidase coupled system [7]. One unit causes the oxidation of one micromole of o-dianisidine per minute at 25°C and pH 6,0 under the conditions specified. The mixture of 2,5 ml dianisidine-buffer mixture, 0,3 ml 18% glucose, 0,1 ml peroxidase and 0,1 ml of appropriately diluted enzyme was added into a cuvette and the increases in A_{460} for 4 - 5 minutes were recorded. ΔA_{460} from the initial linear portion of the curve was measured and GOD activities was calculated:

$$Units/mg = \frac{\Delta A_{460}/\min}{11{,}3.mgGOD/ml}$$

Effect of immobilization on the kinetic constants

The activity assays were carried out applying different glucose concentrations (10,0, 20,0, 30,0, 40,0 and 50,0 mM) in order to determine maximum reaction rates (V_{max}) and Michaelis–Menten constants (K_m) of free and immobilized GOD. V_{max} and K_m for free and immobilized GOD were determined from double reciprocal plots. The stability tests were performed at 4°C with 0.5 ml soluble enzyme or 20 mg immobilized enzyme in 10 ml 0,05M acetate buffer (pH 6.0). After appropriate times of incubation the residual activities were assayed.

RESULTS & DISCUSSION

It is known that the copolymerization of MA with St leads to copolymers with nearly equimolar ratio of monomer units (*alt*-copolymers). The incorporation of a third monomer (DVB) in the polymerization process is a prerequisite for the preparation of a porous solid carrier. Styrene and

divinylbenzene were washed with 2 M sodium hydroxide and water to remove inhibitors. Benzene was distiled before use. P(St-*co*-MA-*co*-DVB) was prepared in a batch type reactor. A mixture of 0,04 M MA, 0,1 M St , 0,005M DVB and 0,04 mM benzoyl peroxide were added to a 300 ml of benzene. The mixture was dispersed and the batch temperature was raised to 70ºC. The reaction mixture was continuously stirred for 3 hour. As the benzene is a poor solvent for the crosslinked copolymer, several minutes after the beginning of copolymerization the reaction system turbid. The resulting reactive fine powdery was filtered, washed with benzene and dried.

The chemical structure of the synthesized polymer was confirmed by Fourier transform infrared spectroscopy (FTIR) by using a spectrophotometer (Varian 660-IR). Absorption bands characteristic of stretching vibrations of aromatic and aliphatic C─H bonds were observed at 3100–3000 and 2926 cm^{-1} (Fig.1). Bands were also detected at 1854.6 cm^{-1} (asymmetric C=O stretching vibrations of the maleic anhydride groups), 1771.7 cm^{-1} (symmetric C=O stretching vibrations of the maleic anhydride groups), 1633.2 cm^{-1} (stretching vibrations of the polystyrene aromatic ring), 1495.1 cm^{-1} (bending vibrations of the aromatic ring), 1454.8 cm^{-1} (C─H bending vibrations of the polymer chain), as well as at 763.1 and 702.4 cm^{-1} (C─H stretching vibrations of the mono-substituted aromatic ring of polystyrene). It can be concluded that the synthesized polymer is a copolymer of styrene, maleic anhydride and divinylbenzene.

The microparticles were analyzed with scanning electron microscopy (JEOL JSM-5510, Japan). As seen from Fig. 2 their surface was a highly porous and rough. The copolymerized MA quantity was calculated as the anhydride and free carboxyl groups in copolymer. The MA unit contents of the copolymers were 32%.

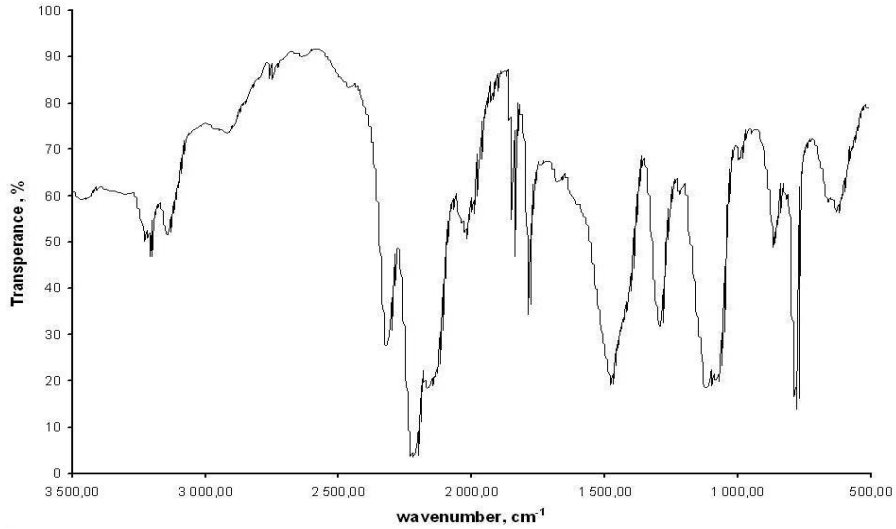


Fig. 1. FTIR spectrum of P(St-co-MA-co-DVB) in KBr pellet.

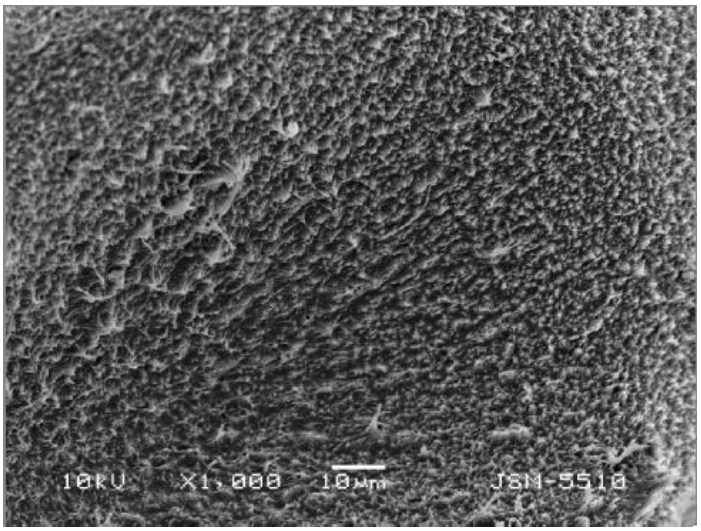


Fig. 2. Scanning electron micrograph of microporous surface of P(St-co-MA-co-DVB).

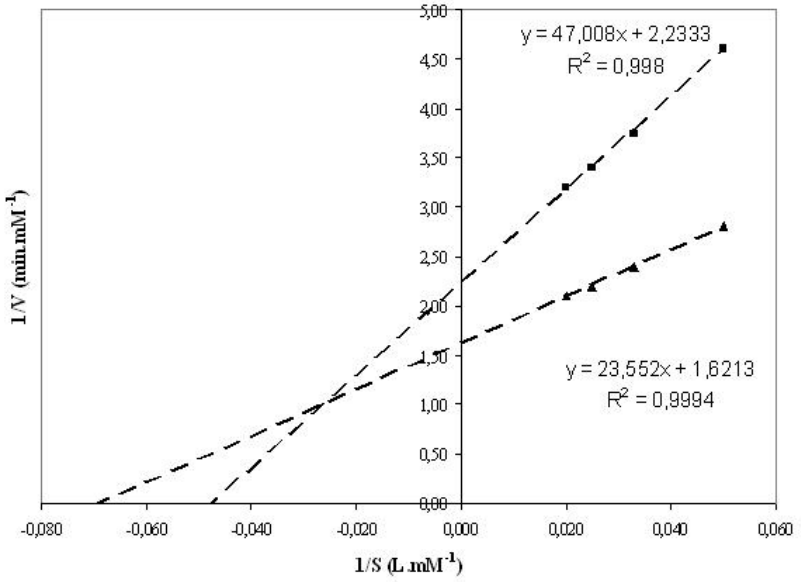
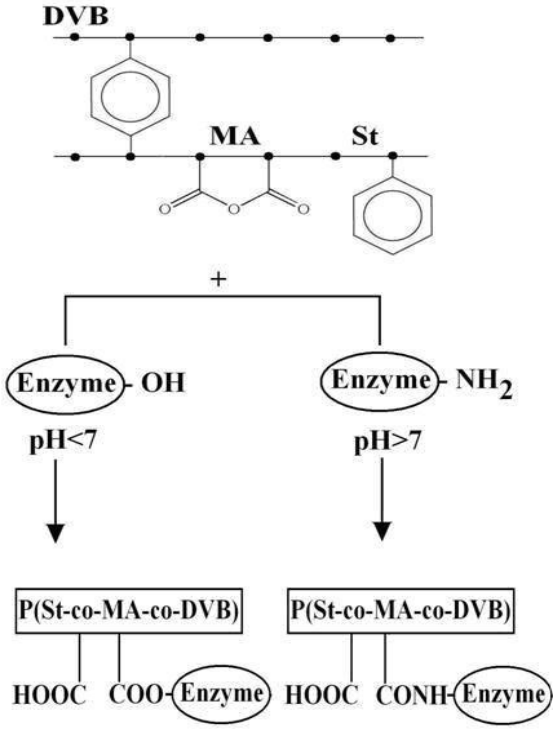


Fig. 3. Lineweaver-Burk plots of free (▲) and immobilized (■) GOD.



Scheme 1.

It must be pointed out that the P(St-*co*-MA-*co*-DVB) undergo the typical reactions of anhydrides with amines and alcohols. The copolymer may also be affected by the water used in the post-treatment of the samples. The proposed method of immobilization of GOD on P(St-*co*-MA-*co*-DVB) includes the reactions represented on Scheme 1. The presence of reactive anhydride groups allows enzymes to bind covalently to the polymer carrier. There are two possibilities for covalent coupling of enzymes depending on the pH of medium. The copolymer binds proteins via its anhydride groups, which may react with different nucleophiles on the protein as a function of pH:

- Through the formation of an amide bonds between the amino groups of lysyl or arginyl residues.

- Through the formation of ester linkages between the hydroxyl groups of seryl and threonyl residues, or the phenols OH groups of tyrosyl residues.

Parameters, characterizing GOD immobilizationare presented in Table 1. The activity of immobilized GOD and protein binding capacity

Table 1. Characteristics of immobilized GOD.

Parameters	Free GOD	Immobilized GOD
Protein content [mg/g of support]	-	87,1
Specific activity [U/mg protein]	180,2	125,0
Relative activity [%]	-	69,4
Immobilization yield [%]	-	87,0
Storage stability after 40 days [%]	22,0	70,5

reached 125,0 U/mg protein and 87,1 mg/g support. The relative activity was 69,40%, compared with the free GOD.

The effects of immobilization on the properties of the immobilized enzymes were studied. It was found that GOD, immobilized onto P(St-*co*-MA-

co-DVB) exhibited typical Lineweaver-Burk behaviour (Fig.3). The linear nature of the Lineweaver-Burk plots proves that in both cases enzyme reactions followed the Michaelis-Menten kinetics, which permitted the determination of the Michaelis constant (K_m) and the maximum rate of the reaction (V_{max}). As the K_m is a characteristic constant for enzyme activity, it was calculated for both immobilized and free enzymes. It was found that V_{max} of the immobilized GOD (0,45 mM/min) decreased with respect to that of the free enzyme (0,62 mM/min), whereas K_m increased from 16,3 mM for the free enzyme to 19,1 mM for the immobilized one. Since the K_m values were of the same magnitude, this means that the catalytic function of the enzyme was not significantly impaired by the coupling process. Probably the increase in the K_m value after the immobilization of GOD is due to changes in the conformation of the enzyme molecules, which impedes the enzyme–substrate interaction or due to hindered access of the substrate to the active sites of the immobilized enzyme (steric and diffusion effects).

The stability of free and immobilized GOD at 4°C was investigated and results obtained are illustrated in the Fig. 4.

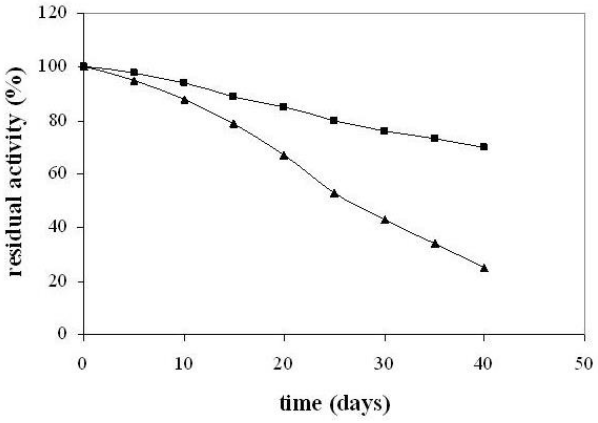


Fig. 4. Storage stability of free (▲) and immobilized (■) GOD at 4 °C. The initial enzyme activity is taken as 100%.

The immobilization procedure stabilized the enzyme structure and the immobilized GOD was much more thermally stable than the free having 70% activity retained compared to 22% for the free GOD after 40 days storage. This phenomenon was also found by Onda *et al*. [8].

The possibility of reuse of the immobilized GOD was studied by measuring its activity after 10 reusing cycles. Covalently bound enzyme shows good operational stability and upon 10 reuses it loses 25% of its activity. This activity reducing is probably due to enzyme deactivation and protein losses from the support upon multiple uses.

CONCLUSIONS

In conclusion, three monomers were used to synthesize porous P(St-*co*-MA-*co*-DVB) with maleic anhydride reactive groups. The immobilized glucose oxidase has better stability upon repeated use than the free enzyme. Probably the covalent immobilization leads to a more stable enzyme conformation in comparison to that of the free enzyme.These properties render the copolymer suitable carrier for immobilization of GOD. The activity values of the immobilized enzyme are suitable for the industrial processing.

REFERENCES

1. W. Gerharrtz, Industrial uses of enzymes. in: Enzymes in industry.Production and applications. New York:VCH Publishers, (1990).
2. C.M. Wong, K. H. Wong, X. D. Chen, *Appl. Microbiol. Biotechnol.*, **78**, 927 (2008).
3. A.Vikartovská, M. Buko, D. Misloviová, V. Pätoprsty, I. Lacík, P. Gemeiner, *Enzyme Microb. Technol.*, **41,** 748 (2007).
4. T. S. Pao, S. Chenga, *Biotechnology and Bioengineering*, **20**, 773 (1978).
5. Y. Mizutani, S. Matsuoka, *J. Appl.Polym. Sci.* **26**, 2113, (1981).
6. O.H. Lowry, N.J. Rosebrough, L. Faer, R.J. Randall, *J. Biol. Chem.*, **193,** 265 (1951).
7. H.U. Bergmeyer, K. Gawehn, M. Grassl, in *Methods of Enzymatic Analysis* Volume I, Second Edition, 457-458, Academic Press, Inc.,NY (1974).
8. M. Onda, K. Ariga, T. Kunitake., *Int. J. Mol. Sci.,***12**, 3052 (2011).

ИМОБИЛИЗИРАНЕ НА ГЛЮКОЗООКСИДАЗА В ПОРЬОЗЕН СЪПОЛИМЕР

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(Резюме)

Синтезиран е носител за имобилизиране на ензими чрез суспензионна съполимеризация на стирен, малеинов анхидрид и дивинилбензен. Съполимерът е охарактеризиран с помощта на FTIR спектроскопия и сканираща електронна микроскопия. Глюкозооксидазата (GOD) е избрана като моделен ензим, за да се изследва възможността за имобилизирането й в полученият съполимер при оптимални условия (време на инкубиране, pH 6,5, GOD 5 mg/ml). Ензимната активност на свободния и имобилизирания GOD са определени чрез окисление на D-глюкозата до D-глюконова киселина при разбъркване и аериране с въздух. Добивът на имобилизиране на ензима е 87%, а активността му е 125 U/mg белтък. Константата на Michaelis за имобилизирания ензим е 16,3 mmol/l, определена с помощтта на графичната зависимост на Lineweaver-Burk. Най-висока ензимна активност е постигната, когато pH на средата е 7.0. Установена е повишена стабилност при съхранение на имобилизирания GOD (остатъчна активност 70%), спрямо свободния ензим (22%).