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Immobilization of glucose oxidase by acrylonitrile copolymer coated silica supports

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Abstract

New polymer/silica gel hybrid supports were prepared by coating high surface area of silica gel with eight different copolymers of acrylonitrile: acrylonitrile-vinyl pyridine, acrylonitrile-vinylimidazole, acrylonitrile-*N*,*N*-dimethylaminoethylmethacrylate, acrylonitrile-acrylic acid, acrylonitrile-2,6-dichlorphenilmaleimide, acrylonitrile-maleic anhydride, acrylonitrile-hydroxyethylmethacrylate and acrylonitrile-methylmethacrylate and Na-vinylsulfonate. Glucose oxidase was covalently immobilized on six types of acrylonitrile copolymer/silica gel hybrid supports. Glutaraldehyde was used as coupling agent between the support treated with hexamethylenediamine (spacer) and the enzyme in the case of $S_{AN\text{-}MA}$, $S_{AN\text{-}HEMA}$, $S_{AN\text{-}AAc(HMDA)}$ and S_{PAN}. The carboxylic groups of $S_{AN\text{-}AAc}$ support were activated with *N*,*N*′-dicyclohexylcarbodiimide – $S_{AN\text{-}AAc(CDI)}$, for covalent immobilization of glucose oxidase. Direct covalent immobilization was achieved on $S_{AN\text{-}DCPMI}$ via the reactive Cl-ions of the support and the ε-amino group of lysine residue of the enzyme. Glucose oxidase was immobilized on $S_{AN\text{-}VP}$, $S_{AN\text{-}VI}$ and $S_{AN\text{-}DMAEM}$ by adsorption via ionic bonding. The greatest amount of bound protein and relative enzyme activity was presented among the covalently immobilized glucose oxidase on $S_{AN\text{-}HEMA}$, S_{PAN} and $S_{AN\text{-}MA}$. The relative activity of the immobilized glucose oxidase via ionic bonding is also high since no conformation changes in enzyme molecules could be noticed. S_{PAN} immobilized system showed the highest thermal stability at 60 °C (78% residual activity at the fifth hour), followed by GOD immobilized on $S_{AN\text{-}HEMA}$ and $S_{AN\text{-}DCPMI}$ (72 and 70%). The most stable for 50 days storage time is GOD covalently immobilized on S_{PAN} (80% residual activity) followed by GOD immobilized on $S_{AN\text{-}HEMA}$ (73%).

Keywords: Glucose oxidase; Immobilization; Silica gel; Coatings; Acrylonitrile copolymers

1. Introduction

Inorganic materials have been successfully used as carriers for enzyme immobilization [1–5]. They show good mechanical properties, thermal and pH stability and resistance against microbial attack and organic solvents. Silica gel is an amorphous inorganic polymer composed of siloxane groups (Si–O–Si) in the inward region and silanol groups (Si–OH) distributed on the surface.

The modification of silica gel has been a subject of considerable interest due to many possible applications. They are usually achieved by coating silica surface with appropriate organosilane agents [6–9] or different kind of polymers. The most commonly used polymers for surface modification of silica particles are polyethylenimine (PEI) [10,11], poly(4-vinylpyridine) (P4VP) [10,12], poly-*N*,*N*-dimethylaminoethylmethacrylate, copolymers of 1-vinyl-2-pyrrolidone and maleic anhydride [13] in order to produce derivatives with suitable functional groups for further utilization in the immobilization of bioactive compounds.

Polyacrylonitrile (PAN) has a good film forming property, but it has not active groups for enzyme immobilization [14]. The chemically modified PAN in the shape of beads [15] or membranes [16–18] is very suitable for that purpose. Only a few copolymers of acrylonitrile (AN) with glycidyl methacrylate and vinylimidazole monomers containing suitable active groups for enzyme immobilization also find an application [19,20]. The latter has not good mechanical properties, pH stability. The combination of high surface area of silica gel with AN-copolymer coating would give support very suitable for enzyme immobilization.

The aim of this study was to obtain new polymer/silica gel hybrid supports for glucose oxidase immobilization using

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different copolymers of acrylonitrile. The basic characteristics of immobilized glucose oxidase were investigated and the most effective carrier and method for immobilization was found.

2. Materials and methods

2.1. Materials

Silica particles (Silica gel 100) were coated with eight different copolymers of acrylonitrile – AN-VP, AN-VI, AN-DMAEM, AN-AAc, AN-DCPMI, AN-MA, AN-HEMA, and AN-MMA-Na-VS (PAN). The first seven copolymers were laboratory prepared in Shumen University "Episkop Konstantin Preslavski". The last one is a commercial product of LukOil-Neftochim"-Bourgas. PAN was modified with NaOH supplied by Merck. Silica gel 100 with mean particle size 0.2–0.5 mm was also supplied by Merck (Germany). Dimethylformamide as a solvent for the copolymers of acrylonitrile was supplied by Merck (Germany). The covalent immobilization of glucose oxidase from Aspergillus Niger with specific activity 119.3 U/mg was carried out with glutaraldehyde (GA), hexamethylenediamine (HMDA) and *N*,*N*′-dicyclohexylcarbodiimide (CDI) all supplied by Merck (Germany). All other reagents used for analysis of enzyme activity and bound protein were reagent grade, Fluka (Buchs, Switzerland).

2.2. Preparation of AN-copolymers

The seven copolymers of AN mentioned above were prepared by radical copolymerization. The copolymerization reactions of AN with VP, VI, DMAEM, AAc and HEMA were conducted in dimethylformamide at 60 °C, using AIBN as initiator. The copolymer reactions of AN with MA and DCFMI were conducted in dioxane at 60 °C and the same initiator. All the AN copolymers were precipitated in alcohol solution and then twice precipitated in acetone solution after the polymerization.

2.3. Modification of silica gel beads

Silica particles (Silica gel 100) were suspended in 2% solution of different acrylonitrile copolymers in dimethylformamide for 24 h. The optimal conditions for polymer coating of silica gel beads were determined in our previous work [21]. PAN was beforehand partially hydrolyzed with 10% NaOH at 50 °C for 60 min. The polymer coated silica gel beads ($S_{AN\text{-}VP}$, $S_{AN\text{-}VI}$, $S_{AN\text{-}DMAEM}$, $S_{AN\text{-}AAc}$, $S_{AN\text{-}DCPMI}$, $S_{AN\text{-}MA}$, $S_{AN\text{-}HEMA}$ and S_{PAN}) were removed by filtration, washed with methanol and then dried till constant weight. $S_{AN\text{-}AAc}$, $S_{AN\text{-}MA}$, $S_{AN\text{-}HEMA}$ and S_{PAN} were treated with 10% solution of HMDA (pH 11) for 1 h at 20 °C. The latter ones were washed thoroughly with distilled water. Then they were suspended in 0.5% solution of GA (pH 7) for 1 h at 4 °C [21]. $S_{AN\text{-}HEMA}$, before treatment with HMDA and GA, was treated with $NaIO_4$ (pH 4.5) at 20 °C for 1h. For $S_{AN\text{-}AAc}$ was also used CDI (1% solution in ethylacetate, pH 4.6) as activating coupling agent. The modification reaction was carried out at 20° for 1.5 h. Then treated with CDI silica gel particles were washed with cold ice water.

2.4. Immobilization of glucose oxidase

The nine types of surface-modified silica gel beads were immersed in 0.1% solution of glucose oxidase in 0.1 M sodium phosphate buffer (pH 5.8) for 24 h at 4 °C. Then the particles were washed with distilled water, 1 M solution of NaCl and 0.1 M buffer solution with pH corresponding to the pH optimum of every immobilized system till no traces of enzyme activity was found in the rinse solutions.

2.5. Assay of GOD activity

The enzyme activity measurement of the immobilized GOD is based on the spectrophotometric determination of the amount of the hydrogen peroxide formed. The assay mixture (2.8 ml) is containing 2.5 ml of dye buffer solution (0.1 ml of 1% solution *o*-dianizidine in ethyl alcohol and 12 ml 0.1 M buffer solution with pH corresponding to the pH optimum of every immobilization system) and 0.3 ml of 18% β-D-glucose. That assay mixture was added to 0.1 g immobilized with GOD silica gel beads for 5 min at 30 °C. Then GOD-silica particles were removed and 0.1 ml peroxidase (0.2% solution) was added to the mixture. The absorption of solution was measured after 10 min at 460 nm with Specol 11, Carl Zeiss Jena [22]. The effect of pH on enzyme activity was studied in 0.1 M buffer solution in pH range from 2.0 to 9.0 at temperature optimum of every immobilized system. The effect of temperature on enzyme activity was studied by varying the temperature in the range of 25–35 °C at pH optimum of every immobilized system. The thermal stability of the enzyme was studied by measuring the residual activity after incubation of the immobilized enzyme in buffer with pH optimum at 60 °C for 5 h. The enzyme activity was measured at every 1 h. The storage stability of the immobilized enzyme was evaluated by incubating them in 0.1 M buffer solution with pH corresponding to the pH optimum of each immobilized system, at 4 °C for 50 days. The enzyme activity was measured every 10 days.

2.6. Analyses

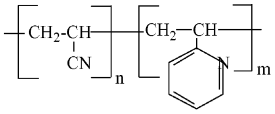
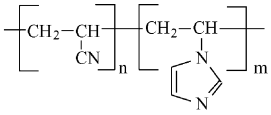
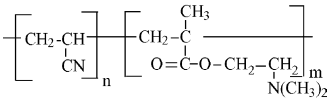
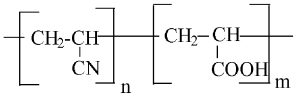
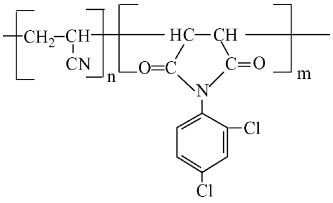
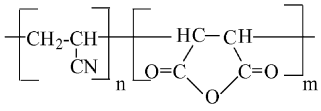
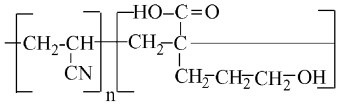
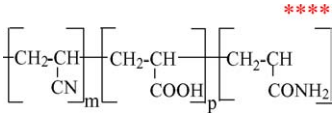
The composition of the copolymers was determined by elemental automatic analyzer (Carlo Erba, Italy). The amount Cl-ion in AN-DCPMI was determined by mercurimertric titration with sodium rhodanide indicator in "oxygen flask" [23]. The amount of bound protein was determined by the method of the Lowry based on spectrophotometric measurement at 750 nm (Specol 11, Carl Zeiss Jena) [24].

3. Results and discussion

3.1. Screening of polymer/silica gel hybrid supports and method for enzyme immobilization

Silica particles were coated with eight different copolymers of acrylonitrile. The chemical structures and the composition of the copolymers are presented in Table 1. For preparation of AN-copolymer membrane the amount of the second monomer unit (M_2) usually has to be less than 8%. This limited the

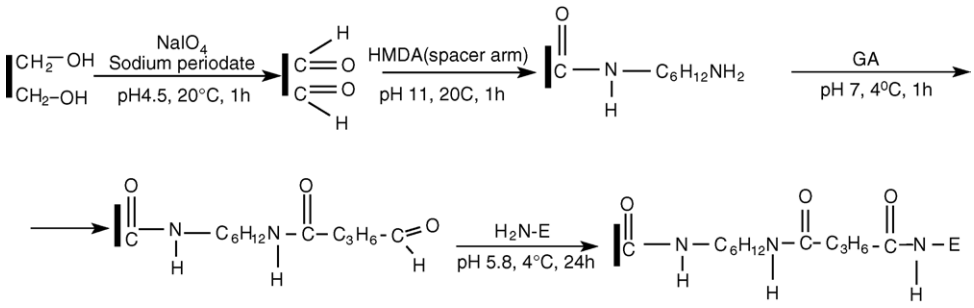
Table 1
Basic characteristics of acrylonitrile copolymers

Support	Chemical structure of AN-copolymer	Composition of copolymer		Active groups-NH_2	Protein content (mg protein/g dry carrier)	Relative activity[a] (%)
		M_1 (AN) (mol%)	M_2 (mol%)			
S_{AN-VP}		78.07	21.93	$-\overset{+}{N}=$	2.3	71
S_{AN-VI}		75.6	24.4	$-\overset{+}{N}=$	2.0	97
$S_{AN-DMAEM}$		62.40	37.60	$-\overset{+}{N}R_2=$	3.4	87
S_{AN-AAc}		78.90	21.10	–COOH[b] $-NH_2$[c]	3.1[b] 7.4[c]	35[b] 35[c]
$S_{AN-DCPMI}$		84.10	15.90	–Cl	2.7	32
S_{AN-MA}		87.57	12.43	$-NH_2$	6.0	63
$S_{AN-HEMA}$		65.00	35.0	$-NH_2$	3.8	98
S_{PAN}		92.30	7.3[d]; 1.4[e]	$-NH_2$	2.9	70

(****) The chemical structure of AN-MMA-Na-VS copolymer (PAN) is presented only with AN monomer units and their modified derivatives.
[a] The relative activity was calculated from the ratio: (specific activity for immobilized enzyme/specific activity for soluble enzyme) × 100.
[b] S_{AN-AAc} modified with CDI.
[c] S_{AN-AAc} modified with HMDA and GA.
[d] The content of MMA in PAN.
[e] The content of Na-Vs in PAN.

amount of the active groups on the membrane surface for enzyme immobilization. This disadvantage could be avoided when using polymer/silica gel hybrid support where the amount of M_2 unit in the copolymer composition is higher hence the percentage of active groups is also higher.

Polymer/silica gel hybrid supports were used as enzyme carrier for immobilization of glucose oxidase. GOD was covalently immobilized on six AN-copolymer/silica gel hybrid supports. GA was used as coupling agent between the support and the enzyme in the case of S_{AN-MA}, $S_{AN-HEMA}$, $S_{AN-AAc(HMDA)}$ and S_{PAN}. These four supports are initially treated with HMDA. The covalent bonding was achieved via the amino group of the activated carrier and the ε-amino group of lysine residue of the enzyme by GA. The mechanism of the reaction is well known and described in details in many publications [25,26]. It was aimed using HMDA as spacer-arm to move the enzyme

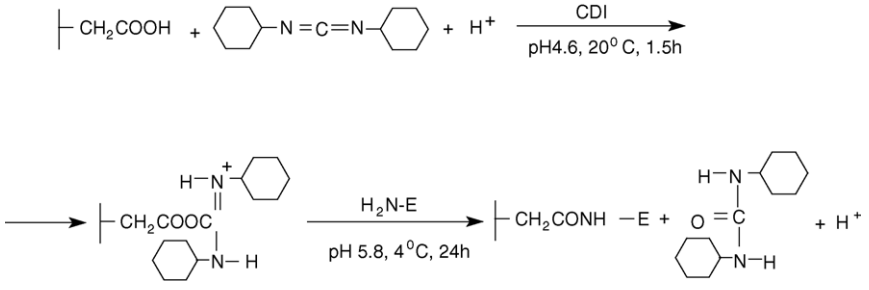


Scheme 1. Immobilization of GOD on $S_{AN\text{-}HEMA}$ using HMDA and GA as activating agents.

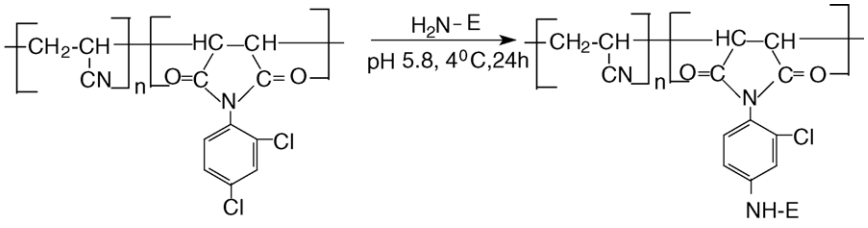
away from the surface area of the support. As a result of that the diffusion limitations and the negative influence of the support were reduced. $S_{AN\text{-}HEMA}$ was firstly treated with $NaIO_4$ to activate the OH groups of the support and then treated with HMDA and GA. The mechanism is presented in Scheme 1. The carboxylic groups of $S_{AN\text{-}AAc}$ support were activated with *N*,*N*′-dicyclohexylcarbodiimide for covalent immobilization of glucose oxidase. The mechanism of coupling the enzyme with this support surface is presented in Scheme 2. GOD was directly covalently immobilized on $S_{AN\text{-}DCPMI}$ via the reactive Cl-ions of the support and the *ε*-amino group of lysine residue of the enzyme. The mechanism is presented in Scheme 3. It is well known that Cl-ion in some Cl-derivatives is very active and that allows direct immobilization of enzyme [26]. Considering the copolymer structure, interaction could be expected with the Cl-ion in *p*-position.

GOD was immobilized on $S_{AN\text{-}VP}$, $S_{AN\text{-}VI}$ and $S_{AN\text{-}DMAEM}$ by adsorption via ionic bonding. The immobilization was achieved at the expense of the positively charged N atom in co-VP, co-VI and co-DMAEM units and negatively charged carboxylic groups of the enzyme. These immobilized systems were kept in buffer solution with pH 3–4 (pH optimum of these immobilized systems), because the higher values of pH cause desorption of enzyme molecules from the support's surface. This is evidence for the ionic character of that bonding.

The amount of bound protein and the relative enzyme activity were determined. From the results presented in Table 1 could be seen that the amount of bound protein in the case of GOD immobilization by adsorption is comparatively low. This is related to the week interactions between the enzyme and the support and the washing out of the enzyme from silica surface. The relative activity of the immobilized GOD on these supports is very high at the same time. This is typically for enzyme immobilization by ionic bonding where no conformation changes in enzyme molecules could be noticed [26]. The greatest amount of bound protein among the covalently immobilized GOD is presented in immobilized systems $S_{AN\text{-}MA}$ and $S_{AN\text{-}AAc(HMDA)}$. The highest activity among the covalently immobilized systems is presented in $S_{AN\text{-}HEMA}$, followed by $S_{AN\text{-}MA}$ and S_{PAN}. Combining the high activity with great protein concentration makes $S_{AN\text{-}MA}$ immobilized systems very effective one. Regardless of the low amount of bound protein in $S_{AN\text{-}HEMA}$ and S_{PAN}, these immobilized systems are effective ones. It could make an impression that the enzyme activity was the highest ($S_{AN\text{-}MA}$,



Scheme 2. Immobilization of GOD on $S_{AN\text{-}AAc(CDI)}$ using CDI as coupling agent.



Scheme 3. Direct covalent immobilization of GOD on $S_{AN\text{-}DCPMI}$.

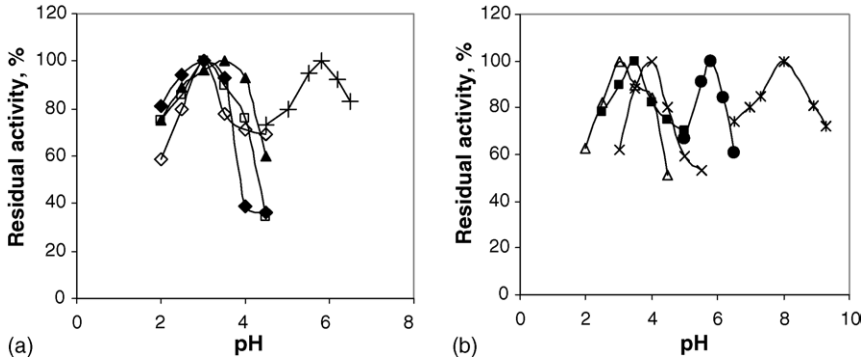


Fig. 1. The activity of free (●) and immobilized GOD on S_{AN-VP} (♦), S_{AN-VI} (□), $S_{AN-DMAEM}$ (▲), $S_{AN-AAc(CDI)}$ (×), $S_{AN-DCPMI}$ (∗), S_{AN-MA} (Δ), $S_{AN-HEMA}$ (◊), $S_{AN-AAc(HMDA)}$ (■),S_{PAN} (+) at different pH values.

$S_{AN-HEMA}$ and S_{PAN}) when HMDA was used as a spacer-arm in covalent immobilization. This is quite logically because HMDA reduced the influence of the charge of the support's surface on the enzyme molecule and decrease the substrate and product diffusion resistance. $S_{AN-AAc(HMDA)}$ immobilized system is an exclusion. Despite of the spacer HMDA the relative activity is low. This is related to steric impediments, which prevent the substrate molecules from penetrating to the active centers of the enzyme molecules as a result of local concentration of the immobilized enzyme (7.4 mg/g). In the case of $S_{AN-DCPMI}$ the amount of bound protein is very low and the activity is also low. This may be due to the low concentration of the Cl-ions in the co-DCPMI unit (Cl concentration-8.43%, measured by mercurimertric titration) and the direct bonding between the enzyme and the support without spacer.

3.2. Effect of pH and temperature on enzyme activity

The pH optimum of immobilized GOD on different type of hybrid supports was studied. From the results presented in Fig. 1 could be seen that the immobilized systems with residual positively charged groups of the initial carriers (S_{AN-VP}, S_{AN-VI}, $S_{AN-DMAEM}$) or modified carriers with HMDA ($S_{AN-HEMA}$, S_{AN-MA}, S_{AN-AAc}) and with CDI (S_{AN-AAc}) have pH optimum in the acidic area (3.0–4.0). The other two immobilized systems with $S_{AN-DCPMI}$ and S_{PAN} carriers have pH optimum 7.0 and 5.8, respectively. Only GOD immobilized on S_{PAN} has the same pH optimum as the free enzyme. Obviously all the amino groups on this support are bound with GOD. pH optimum of GOD immobilized on $S_{AN-DCPMI}$ is shifted to the alkali pH, because of the residual negatively charged Cl-ions on the support's surface.

The temperature optimum of all immobilized systems was also studied and compared to the temperature optimum of the free enzyme. There is no significant difference between the profiles of the temperature optimum for free and immobilized GOD It was found out that the temperature optimum of all the immobilized systems was 2–3 °C higher than the free enzyme (28 °C).

It is of great interest to investigate the thermal stability of immobilized systems and to study the influence of the support and the method of immobilization on the enzyme stability. GOD was immobilized on all type of hybrid supports and incubated at temperature 60 °C (Fig. 2) for 5 h. From the results presented in Fig. 2 could be seen that S_{PAN} immobilized system showed the highest thermal stability at that temperature (78% residual activity at the fifth hour). The second place of the thermal stability of immobilized GOD was for the system with $S_{AN-HEMA}$ (72%) and the third place for GOD immobilized on $S_{AN-DCPMI}$ with 70% residual activity. GOD was covalently immobilized on $S_{AN-HEMA}$, S_{PAN}, $S_{AN-DCPMI}$ and it is likely the main reason for the good thermal stability of the enzyme. Only the covalently immobilized systems with $S_{AN-AAc(CDI)}$ presented the lowest thermal stability, even worse than GOD immobilized on hybrid supports by adsorption (S_{AN-VP}, S_{AN-VI} and $S_{AN-DMAEM}$), residual activity 40–50% at the fifth hour. The thermal stability of all

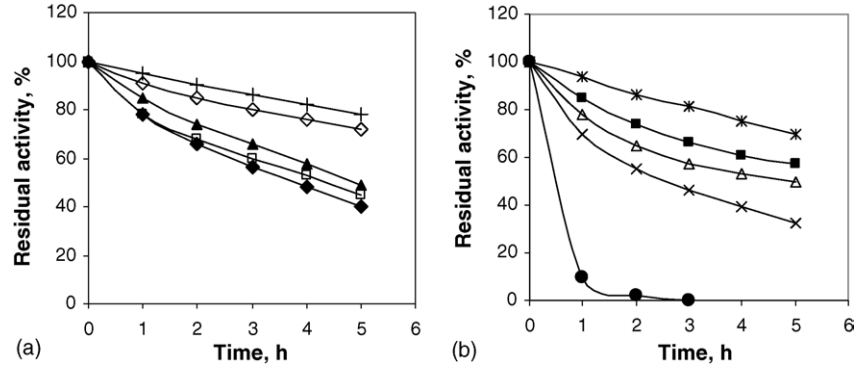


Fig. 2. Thermal stability of GOD immobilized on S_{AN-VP} (♦), S_{AN-VI} (□), $S_{AN-DMAEM}$ (▲), $S_{AN-AAc(CDI)}$ (×), $S_{AN-DCPMI}$ (∗), S_{AN-MA} (Δ), $S_{AN-HEMA}$ (◊), $S_{AN-AAc(HMDA)}$ (■),S_{PAN} (+) and the free GOD (●) for 5 h at 60 °C.

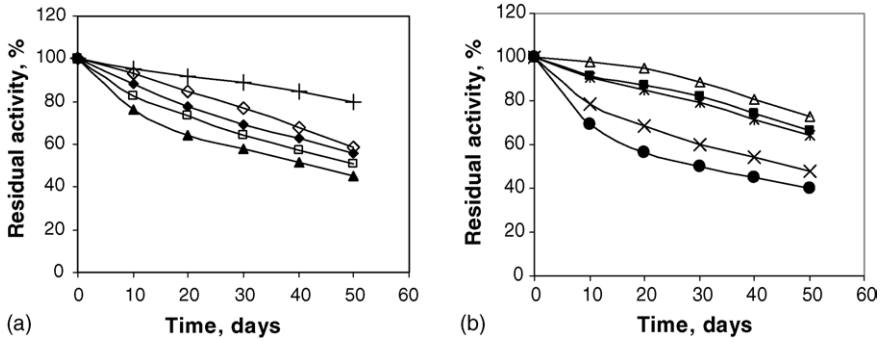


Fig. 3. Storage stability of GOD immobilized on $S_{AN\text{-}VP}$ (♦), $S_{AN\text{-}VI}$ (□), $S_{AN\text{-}DMAEM}$ (▲), $S_{AN\text{-}AAc(CDI)}$ (×), $S_{AN\text{-}DCPMI}$ (∗), $S_{AN\text{-}MA}$ (Δ), $S_{AN\text{-}HEMA}$ (◊), $S_{AN\text{-}AAc(HMDA)}$ (■), S_{PAN} (+) and the free GOD (●) for 50 days at 4 °C.

the immobilized systems was compared to the thermal stability of the free enzyme at same conditions. Free GOD completely lost its activity on the third hour.

3.3. Storage stability studies

One of the most important characteristics of the immobilized enzyme is the storage stability. The results for storage stability are presented in Fig. 3. The most stable is GOD covalently immobilized on S_{PAN} (80% residual activity for 50 days) followed by GOD immobilized on $S_{AN\text{-}HEMA}$ (73%). With respect to the other hybrid supports the storage stability of GOD did not distinguish from one to another (residual activity is about 60% after 50 days).All the immobilized GOD showed better residual activity than the free enzyme (40% at the 50th day).

4. Conclusion

New hybrid supports for GOD immobilization were obtained on the base of different acrylonitrile copolymer coatings of silica gel beads. GOD was immobilized covalently or by adsorption depending on the type of the carrier. From the results of the protein content and the enzyme activity could be concluded that the most effective immobilized systems are GOD immobilized on S_{PAN}, $S_{AN\text{-}HEMA}$ and $S_{AN\text{-}MA}$. Hence the most suitable method for enzyme immobilization is the covalent bonding with spacer-arm HMDA and GA. Best results according to the thermal and the storage stability presented GOD covalently immobilized on S_{PAN} and $S_{AN\text{-}HEMA}$.

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