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Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11001 Belgrade
**IHTM-Department of chemistry, Studentski trg 12-16, 11001 Belgrade*

Penicillin G acylase (PGA, penicillin amidohydrolase EC 3.5.1.11) is an enzyme used commercially for the hydrolysis of penicillin G (Pen G) and cephalosporin G (Cep G) to 6-aminopenicillanic acid (6-APA) and 7-amino-3-deacetoxycephalosporanic acid (7-ADCA), intermediates for the production of semisynthetic penicillins and cephalosporins.[1] Because efficient recovery and reuse of the biocatalyst is a prerequisite for a viable process, much attention has been focused on the immobilization of penicillin G acylase (PGA). Covalent binding of the enzyme to a support is stable and has generally been favored in the case of PGA.[2] However, it has often been difficult to achieve stable binding of high levels of activity because the active site may be blocked from substrate accessibility, multiple point-binding may occur, or the enzyme may be denatured. On the other hand, in a previous article, effectiveness of the chemical method of the *Candida rugosa* lipase immobilization on amino-Eupergit by the enzyme coupling via its carbohydrate moiety has been demonstrated.[3] This seemed to provide a possibility to couple the enzyme without risking reaction at the active site that might cause loss of activity. The method may be applicable for a wide variety of lipases and other glycoproteins. The present article reports that the chemical procedure employed for immobilizing lipase on Eupergit can also be applied for PGA immobilization on amino supports.

Native PGA from *Escherichia coli* is not glycosylated and, hence, the immobilization strategy could not be directly applied for improving the properties of this enzyme. In view of this fact, the alternative proposed in this paper is to chemically modify the enzyme to prepare a synthetic glycoconjugate of PGA with much better possibilities of yielding a more suitable covalent attachment during immobilization. This improved enzyme will then be covalently bound to amino-supports in a similar way that the other glycoenzymes. In this work, PGA molecules are modified by cross-linking with dialdehyde derivatives of starch in order to add them new and useful functions. Starch is a natural polysaccharide with wide biotechnological applications, but to our knowledge, the use of this macromolecule for modifying enzymes has not been reported.

Evidently, it is necessary to use supports that may really yield a very intense multipoint covalent attachment. Thus, to demonstrate the potential of this strategy, the immobilization of starch-PGA on Sepabeads used as a model system. Sepabeads are very stable and have good chemical, mechanical and other properties such as hydrophilic nature, wide pore distribution and almost ideal spherical beads, low swelling tendency in common solvents, high flow rate in column procedures, excellent performance in stirred batch reactors, etc. Although a number of studies have shown that Sepabeads are good carriers for enzyme immobilization, their potential for binding PGA was not fully explored.[4] In this paper, we investigated the optimal condition for covalent immobilization of modified PGA from *E. coli* on two supports, namely Sepabeads EC-EA and Sepabeads EC-HA. The immobilized enzymes were then characterized by evaluating the potential effects of immobilization on their thermal stability, especially in comparison with free non-modified enzyme.

Experimental

Penicillin G acylase from *E. coli* (PGA) was a gift from DSM (The Netherlands). The enzyme was a crude preparation with specific activity of 82.9 U ml^{-1} and 56.13 mg/ml protein based on Lowry`s method for protein assay. Sepabeads EC-EA and EC-HA (particle sizes 150-300 μm, average pore diameter 30-40 nm, water retention 55-65%) were kindly donated by Resindion S.R.L. (Mitsubishi Chemical Corporation, Milan, Italy). 6-Aminopenicillanic acid (6-APA), penicillin G, *p*-dimethylaminobenzaldehyde (PDAB) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent-grade.

Preparation and characterization of starch-PGA conjugate

Prior to conjugation, starch was activated to its 2,3-dialdehyde derivatives by periodate oxidation according to the method described previously.[5] Periodate oxidation of starch molecules was stopped by treating the reaction mixture for 1 h after adding 1 ml ethylene glycol. The starch-dialdehydes were dialysed against water overnight in the cold and dark in order to remove excess of ethylene glycol and sodium periodate.

Four milliliter native PGA solution in 10 mM pH 7.0 phosphate buffer containing phenylacetic acid as active site protector was reacted with 12 ml activated starch solution. The conjugation reaction was preformed as previously described for trypsin.[5] The amount of activated starch in conjugated enzyme solution was measured by the phenol sulphate method of Dubois *et al*.[6]

Immobilization of chemically modified PGA

The immobilization procedure consisted of two main steps: oxidation of starch-PGA by excess sodium periodate, and coupling of oxidized starch-PGA to amino-Sepabeads supports. Therefore, starch-PGA was oxidized by sodium periodate as described previously for lipase by incubating 1 mg/ml of starch-PGA solutions with 5 mM $NaIO_4$ in acetate buffer, pH 5.0, for 6 hours in the dark at 4 °C.[3] The reaction mixture was stirred occasionally and the reaction was quenched with 10 mM ethylene glycol for 30 minutes. To remove the by-products, the oxidized starch-PGA solution was then dialyzed against 50 mM acetate buffer, pH 5.0, for 18 h. Sepabeads® EC-EA or EC-HA were incubated with oxidized starch-PGA in sodium acetate buffer at pH 5.0 and 4 °C for 48 h. After that, the beads were collected by vacuum filtration, washed with 1 M NaCl (3x20 cm^3), afterwards with potassium phosphate buffer, pH 8.0 (3x20 cm^3) and stored in it at 4 °C until use. Samples of the enzyme solution before and after the immobilization, together with the washing solutions, were taken for protein content and enzyme activity assay.

Enzyme activity assay

The activity of free non-modified PGA and imobilized PGA was determined by measuring the penicillin G enzymatic product, 6-APA, spectrophotometrically. One unit of PGA was defined as the amount of the enzyme required to produce 1 μmol of 6-APA per minute under the assay conditions (4% (w/v) penicillin G as substrate solvated in 0.1 M phosphate buffer, pH 7.92 at 37 °C). The amount of 6-APA was determined with the method of PDAB as decribed previously.[7]

Thermal stability assays

The thermal stability assays were performed at 50 °C in an aqueous medium (100 mM phosphate buffer, pH 7.92) by using equivalent number of activity units of biocatalysts (native PGA, immobilized non-modified PGA and immobilized starch-PGA on Sepabeads). Biocatalysts were dissolved in the buffer and incubated in a constant temperature water bath. After different times, samples were taken and the residual activity was determined, taking an unheated control to be 100% active. The model considering first-order enzyme deactivation mechanism has been used to describe the experimental data for biocatalysts:

$$A = A_o e^{-k_1 t} \qquad (1)$$

where k_1 is the first-order deactivation rate constant.

Results and discussion

Preparation and characterization of starch-PGA conjugate

The natural polysaccharide starch, previously activated by periodate treatment, was attached to PGA to generate carbohydrate moiety in enzyme molecule. The prepared neoglycoenzyme starch-PGA contained 47% (w/w) of saccharides. The modified enzyme (starch-PGA complex) retained almost 95% of the original activity.

Immobilization of modified enzyme onto Sepabeads

A simple manner of starch-PGA immobilization is formation of insoluble complexes of such conjugate with amino-supports. The two polymethacrylate particulate polymers containing amino groups with spacers of different lengths used in the study were Sepabeads® EC-EA and EC-HA. The role of the spacer was investigated in terms of amount of protein immobilized and activity of bound enzyme. We studied the influence of the starch-PGA concentration in the attachment solution in the range of 0.14-2.24 mg/ml on the total enzyme loading on Sepabeads as well as enzyme and activity coupling yields. The results are shown in Fig.1. In each experiment, 0.5 g of polymer particles was immersed in a certain volume of modified enzyme solution. The aim was to determine an efficient relationship between the modified enzyme and supports.

It appears that immobilization via the hexamethylendiamino spacer on Sepabeads EC-HA gives a lower loading of protein than that via ethylendiamino on Sepabeads EC-EA. As can been seen, the amount of enzyme bound on Sepabeads EC-EA is slightly higher than that of the other support. However, the difference is small, and the maximum amounts of modified enzyme bound, 123.4 and 96.1 mg/g dry supports for Sepabeads EC-EA and Sepabeads EC-HA, respectively, are highly satisfactory. Despite lower enzyme loadings on Sepabeads EC-HA, the immobilized PGA seems to present better immobilization parameters with an activity in the range of 11.4 to 67.7 U/g and an activity coupling yield of 99.2-32.9%, probably due to the positive effect of the spacer in terms of reduced interaction between modified enzyme and support particles. Note that the activity increases for lower enzyme loadings at first and then decreases, possibly due to close packing of the enzyme on the support surface which could limit the access of substrate needed in the reaction. It is generally acknowledged that the catalytic efficiency of immobilization processes decreased when enzyme loading exceeded a certain value and an

optimum activity should be selected.[8] The loading of 56.6 mg g^{-1} support seems to be most appropriate for use, resulting in a rather high activity yield of 81% with satisfactory degree of enzyme fixed.

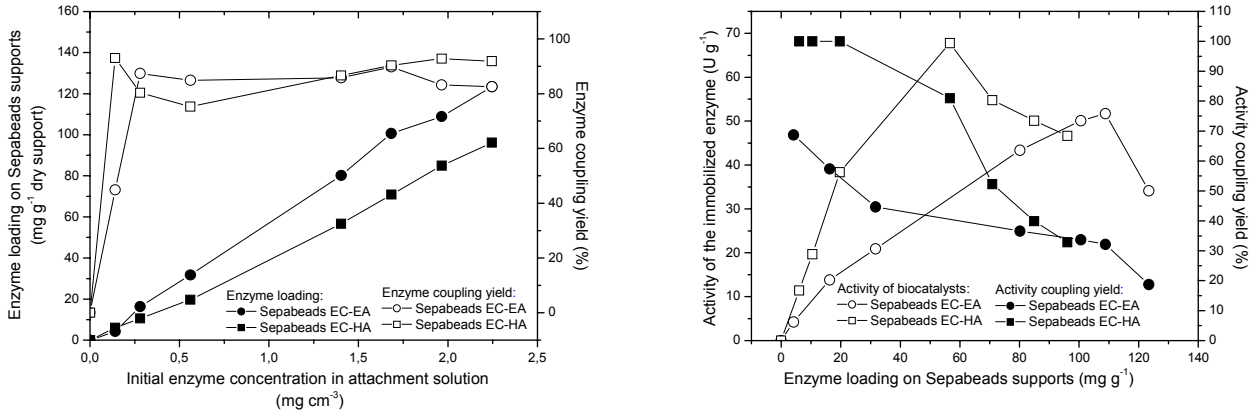

Figure 1. (a) Effects of initial starch-PGA concentration in attachment solution on the enzyme loading and enzyme coupling yield (b) Effects of enzyme loading on the activity of the biocatalysts and the activity coupling yield

Effect of modification and immobilization on enzyme thermal stability

An important consideration when evaluating an immobilized enzyme system for industrial application is reported enzyme inactivation. Moreover, for design and operation of enzyme reactor, proper kinetic deactivation model and kinetic parameters are necessary. Therefore, the thermal stability of the immobilized starch-PGA on Sepabeads EC-EA and EC-HA was studied at 50 ^{o}C in an aqueous medium (100 mM phosphate buffer, pH 7.92) and compared with that of the free non-modified one. As a reference, the thermal study was also carried out with immobilized but non-modified enzyme. The results are presented in Fig. 2.

Results show that the immobilization of the chemically modified enzyme on Sepabeads in both cases offers a high thermoprotection. For example, the immobilized starch-PGA on Sepabeads EC-HA treated at 50 ^{o}C for 2 h still held significant activity of around 78%, whereas the free non-modified enzyme lost its original activity completely at this condition. This fact is of real significance in commercial applications.

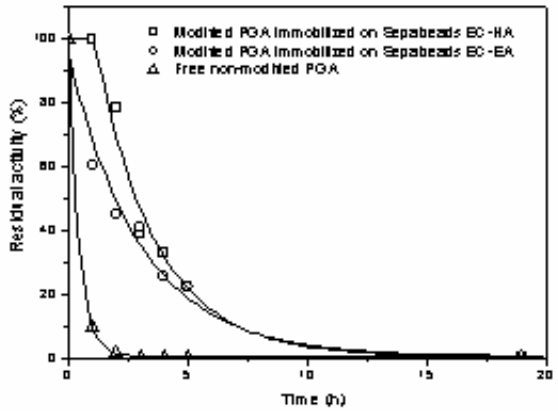

Figure 2. Thermal inactivation of free non-modified PGA and immobilized starch-PGA on Sepabeads at 50 ^{o}C in an aqueous medium (100 mM phosphate buffer, pH 7.92). Symbols are experimental data. The lines represent the best fits of the first-order kinetic deactivation model. Starting activities were taken as 100%.

In order to interpret and analyze the obtained experimental results, the model based on first-order degradation kinetics was fitted to deactivation curves (Eq [1]). Points on the graph are experimental data and the solid lines represent the best fits of the theoretical model predictions. It seems that first order enzyme degradation kinetics fits experimental data well for both free and immobilized starch-PGA, suggesting that the biocatalysts lose their activities in only one step at 50 ^{o}C. The best-fit values of the deactivation rate constant, k_1, are listed in Tab. 1.

Clearly, the immobilized starch-PGA on both Sepabeads EC-HA and EC-EA was superior to the other biocatalysts studied. By comparison of the $t_{1/2}$ values, it can be concluded that starch-PGA immobilized on Sepabeads EC-EA via carbohydrate side chain was almost 4.5-fold more stable than conventionally immobilized one and 7-fold than free non-modified PGA. Similarly, starch-PGA immobilized on Sepabeads EC-HA was around 2-fold more stable than conventionally immobilized one and almost 6-fold than free non-modified enzyme.

Table 1. Best-fit parameters of the model based on first-order degradation kinetics (Eq [1]) for free non-modified PGA, immobilized PGA on Sepabeads and immobilized starch-PGA on Sepabeads at 50 °C

Biocatalyst	k_1, h^{-1}	$t_{1/2}$, h	*F*
Free non-modified PGA	2.32	0.30	1.0
Starch-PGA (modified non-immobilized enzyme)	1.99	0.35	1.2
Starch-PGA immobilized on Sepabeads EC-EA	0.33	2.10	7.0
Starch-PGA immobilized on Sepabeads EC-HA	0.39	1.77	5.9
Non-modified PGA immobilized on Sepabeads EC-EA	1.44	0.48	1.6
Non-modified PGA immobilized on Sepabeads EC-HA	0.71	0.98	3.3

**F is stabilization factor (considered as the ratio between half-lives of immobilized and free non-modified PGA, $t_{1/2}$)*

Generally, the attachment of natural polysaccharides to enzyme molecules has been proposed as a method for improving their stability. However, it seems that starch-PGA is only slightly more stable than the natural one; hence thermal stabilization of starch-PGA immobilized on Sepabeads could not be ascribed by enzyme modification. Therefore, the improved stability should be related to a higher multipoint covalent attachment, involving the new aldehyde groups chemically introduced on the enzyme.

Conclusion

An approach is presented for the stable covalent immobilization of chemically modified PGA from *E. coli* on Sepabeads® carriers with a high retention of hydrolytic activity and thermal stability. The two amino-activated polymethacrylate particulate polymers with spacers of different lengths used in the study were Sepabeads® EC-EA and Sepabeads® EC-HA. The performance of these immobilized catalysts was compared with respect to activity and thermal stability. The thermal stability study shows that starch-PGA immobilized on Sepabeads via the new aldehyde groups chemically introduced on the enzyme is several times more stable than the both native enzyme and conventional immobilized PGA.

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Imobilizacija hemijski glikozilovane penicilin G acilaze na Sepabeads nosače

Penicilin G acilaza je enzim od velikog industrijskog značaja jer katalizuje reakcije hidrolize prirodnih penicilina i sinteze polusintetskih β-laktamskih antibiotika. Kako je efikasno izdvajanje biokatalizatora iz reakcione smeše po završenoj reakciji i njegova reciklacija neophodan uslov za razvoj efikasnog enzimskog procesa, velika pažnja se posvećuje imobilizaciji ovog enzima na različite nosače. U radu je po prvi put ispitana kovalentna imobilizacija hemijski modifikovane PGA iz Escherichia coli *na dva komercijalna nosača i to Sepabeads EC-EA i Sepabeads EC-HA i dobijeni imobilisani enzimi su okarakterisani sa aspekta aktivnosti i termalne stabilnosti u reakciji hidrolize penicilina. Hemijska modifikacija PGA je izvedena pomoću dialdehidnih derivata skroba dobijenih prethodnom oksidacijom perjodatnom metodom. Na ovaj način je omogućeno da se enzim veže za amino-nosače preko uvedenih aldehidnih grupa koje nisu od značaja za njegovu aktivnost. Na osnovu proučavanja kinetike termalne deaktivacije enzima utvrđeno je da predložena metoda ujedno i značajno doprinosi termalnoj stabilnosti enzima.*

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