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Original scientific paper

IMMOBILIZATION OF PENICILLIN ACYLASE FROM *Escherichia coli* ON COMMERCIAL SEPABEADS EC-EP CARRIER

Milena G. Žuža, Slavica S. Šiler-Marinković and Zorica D. Knežević

This paper describes the covalent immobilization of penicillin G acylase from Escherichia coli on sepabeads EC-EP, an epoxy-activated polymethacrylic carrier and kinetic properties of the immobilized enzyme. The selected enzyme belongs to a class of biocatalysts whose industrial interest is due to their versatility to mediate hydrolysis of penicillins and semi-synthetic β-lactam antibiotics synthesis reactions.

About 2.7 mg of the pure enzyme was immobilized onto each gram of sepabeads with an enzyme coupling yield of 96.9%. However, it seems that the activity coupling yield is not correlated with the amount of enzyme bound and the maximum yield of 89.4% can be achieved working at low enzyme loading (0.14 mg g^{-1}). Immobilization of the penicillin acylase resulted in slightly different pH activity profile and temperature optima, indicating that the immobilization by this method imparted structural and conformational stability of this enzyme. It appears that both free and immobilized penicillin acylase followed simple Michaelis-Menten kinetics, implying the same reaction mechanism in both systems.

KEYWORDS: Penicillin acylase; covalent immobilization; sepabeads carrier, Michaelis-Menten kinetics

INTRODUCTION

Penicillin acylases (penicillin amidohydrolase; EC 3.5.1.11) catalyze the hydrolysis of penicillins into 6-aminopenicillanic acid (6-APA) and another organic acid whose structure depends on the type of penicillin hydrolyzed (1). Biotechnological applications of penicillin acylases (PA) have emerged as a serious alternative to traditional chemical procedures for the manufacture of β-lactam antibiotics, small peptides and pure isomers from racemic mixtures. However, penicillin acylases are involved mainly in industrial production of semi-synthetic penicillins (2).

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They are classified in three sub-groups, according to their preferential substrate: penicillin V acylase, penicillin G acylase and ampicillin amidase (3). Penicillin G acylase (PGA), catalyzing the hydrolysis of penicillin G, is the most important acylase because penicillin G is the cheapest raw material for the production of 6-APA, the nucleus from which a range of semi-synthetic penicillins are made. The standard industrial practice to produce semi-synthetic β-lactam antibiotics employs a chemical route, with protection/de-protection of reactive groups, low temperatures (-30 ^{o}C or less), and organochloride solvents. Hence, their enzymatic synthesis has received great attention as possible „green chemistry" alternative (4).

To fully exploit the technical and economical advantages of highly cost penicillin acylase, it is recommended to use them in an immobilized form (5). This is because free enzyme, as a biocatalyst, is lacking long-term stability under the process conditions and is difficult to recover and recycle from the reaction mixture, making the reuse of the enzyme impossible. Hence, the idea of immobilizing the enzyme on a rigid solid support, enabling easy separation and the possibility of operation in a packed-bed or fluidized-bed reactor, has been of great industrial interest for many years (6). Among the immobilization methods, covalent attachment is more advantageous than the other methods since diffusional restrictions to substrate or products are decreased considerably. Moreover, the formation of the rigid enzyme-support linkage provides both kinetic and thermodynamic stabilization of the three-dimensional structure of the active catalytic site and often improves the enzyme thermal stability.

The technology of PGA immobilization has been improved in the last decades, and PGA has been covalently immobilized onto various supports such as microparticulate and monolithic silica supports (2), poly(vinyl acetate–co-diviyl benzene) beads (7), activated agarose (8), grafted nylon membranes (9), etc. However, various problems associated with these supports were also reported. One common problem is the lack of active sites on the polymer. Consequently, most previous studies used glutaraldehyde as a non-specific cross-linking agent to fix the enzyme on the polymeric matrix, but the results were often unsatisfactory, with low immobilization yield and low final enzyme activity. Therefore, the development of new techniques for PGA immobilization on inexpensive and industrially applicable carriers is of economical significance.

Epoxy-activated supports are almost ideal to perform very easy immobilization of enzymes at both laboratory and industrial scale. Epoxide groups are convenient for the covalent binding of enzymes since they are able to directly react with amino, hydroxyl, or sulfhydryl groups of enzymes, depending on pH of the buffer used. Thus, it is not necessary to activate the carrier or enzyme to achieve covalent immobilization. Moreover, the N-C, O-C or S-C bonds formed by the epoxide groups are extremely stable, so that the epoxide-containing commercial polymers such as Eupergit® or Sepabeads can be successfully used for the immobilization of enzymes and proteins (10, 11).

Although a number of studies have shown that epoxide-containing polymers are good carriers for enzyme immobilization, their potential for binding penicillin acylases has not been fully explored. In this paper, we investigated the covalent immobilization of penicillin G acylase from *E. coli* on Sepabeads EC-EP, a commercial polymethacrylic carrier that has introduced functional epoxy groups. The support is very stable and has good chemical, mechanical and other properties such as hydrophilicity, wide pore distribution, and almost ideal spherical beads that allow simple immobilization procedure, high pro-

tein binding capacity, low swelling tendency in high molar solution and in common solvents, high flow rate in column procedures, excellent performance in stirred batch reactors, etc. The first aim of the study was to investigate the effects of immobilization conditions such as initial enzyme concentration on enzyme and activity coupling yields. Having obtained the highly efficient immobilized PGA, it was then characterized its pH and thermal stability, as well as the reaction kinetics.

EXPERIMENTAL

Materials

Penicillin G acylase (E.C. 3.5.1.11) from *E. coli* (PGA) was a gift from DSM (The Netherlands). The enzyme was a crude preparation with specific activity of 167 U mg^{-1} protein and 90% (w/v) protein based on Lowry`s method for protein assay (12). Sepabeads EC-EP (particle sizes 200-240 μm, average pore diameter 30-40 nm, water retention 55-65%, specific volume 2.8-3.3 cm^3/dry g) was kindly donated by Resindion S.R.L. (Mitsubishi Chemical Corporation, Milan, Italy). 6-Aminopenicillanic acid (6-APA), penicillin G, *p*-dimethylaminobenzaldehyde (PDAB), Folin Ciocalteu's Phenol Reagent were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent-grade.

Immobilization of penicillin G acylase

The method for enzyme immobilization on Sepabeads EC-EP support involves the direct enzyme binding on polymers via epoxide groups (Fig. 1). Unmodified Sepabeads EC-EP (500 mg of wet carrier) was incubated with 10 cm^3 of native enzyme attachment solutions in a shaking water bath (130 strokes per min) at 25 °C. Enzyme attachment solutions containing 7.5-120 U/g wet support were prepared in a 1.25 M potassium phosphate buffer at pH 8.0. After incubation for 48 h, the beads were collected by vacuum filtration using a glass filter (Whatman), washed with 1 M NaCl (3x20 cm^3), then with potassium phosphate buffer pH 8.0 (3x20 cm^3), and stored in it at 4 °C until use. Samples of the filtrate and enzyme solution before immobilization, together with the washing solutions, were taken for protein content and enzyme activity assay.

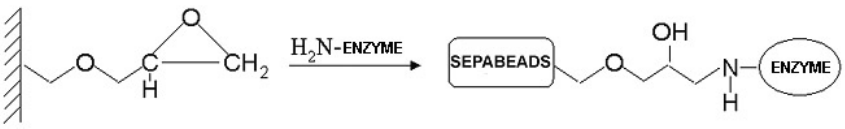


Fig. 1. Schematic illustration of the covalent method for the enzyme immobilization on Sepabeads EC-EP support

The efficiency of immobilization was evaluated in terms of enzyme and activity coupling yields. The enzyme coupling yield, Y_E (%) and activity coupling yield, Y_A (%) were calculated as follows:

$$Y_E(\%) = \frac{P_1}{P_0} \times 100 \quad [1]$$

$$Y_A(\%) = \frac{SA_2}{SA_1} \times 100 \quad [2]$$

where P_1 is the amount of immobilized enzyme; P_0 the initial amount of enzyme; SA_2 the specific activity of immobilized penicillin acylase; and SA_1 is the specific activity of free penicillin acylase.

Enzyme activity assay

The enzyme activity of penicillin acylase was determined by measuring the penicillin G enzymatic product 6-APA spectrophotometrically (13). One unit of penicillin acylase 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 ^{o}C). The amount of 6-APA was determined with the method of PDAB. The experimental procedures were as follows: firstly, the solutions of 20% (v/v) of acetic acid (A), 0.5% (w/v) of NaOH (B) and 0.5% (w/v) of PDAB in methanol (C) were prepared; secondly, A,B and C were added to 0.5 ml of the sample solution in sequence to the volume ratio 2:1:0.5; finally, after reaction for 20 min at room temperature, the absorbance value of solution was determined at 415 nm.

Determination of penicillin acylase concentration

Penicillin acylase concentration was determined spectrophotometrically at 210 nm by using bovine serum albumin as a standard. The penicillin acylase concentration was determined from a standard curve which was constructed for each measurement. The amount of bound enzyme was determined indirectly from the difference between the amount of enzyme introduced into the coupling reaction mixture and the amount of enzyme in the filtrate and in the washing solutions.

Kinetics evaluation

Kinetic parameters of free and immobilized enzyme were determined by measurements of enzyme activity with various concentrations of substrates (0.25 to 4% of penicillin G in 0.1 M phosphate buffer, pH 7.9). The enzyme concentration was constant (0.1 g of immobilized enzyme or corresponding amount of free enzyme). All data are the averages of duplicate samples and were reproducible within ±5%. Mean and standard deviation of the results from at least two independent experiments were calculate using Microsoft Excel (Redmond, WA, USA) software.

Assuming Michaelis-Menten type reaction kinetics, the apparent Michaelis-Menten constant, K_m, and the maximum apparent initial rate, V_{max} of the free and immobilized enzyme were calculated directly from the model by nonlinear regression analysis using the MATLAB software (version 6.5, Release 13, The MathWorks, Juc, Matick, MA, USA). The extent of mass-transfer control could be expressed by the effectiveness factor, *EF* (considered as the ratio between maximum rates of the reaction catalyzed by immobilized and free penicillin acylase under otherwise identical conditions).

pH and temperature profile

The effect of pH on free and immobilized enzyme was studied by assaying the preparations at different pH values in the range from 4.5 to 9.3 at 37 ^{o}C. The effect of temperature on activities of free and immobilized penicillin acylase was also determined at temperatures from 8 to 75 ^{o}C under assay conditions (4% (w/v) penicillin G as substrate solvated in 0.1 M phosphate buffer, pH 7.9).

RESULTS AND DISCUSSION

The basic characteristics of an immobilized enzyme include the amount of immobilized protein, its activity and specific activity, thermal and pH profile as well as kinetic properties. The first aim of this study was to determine the relationship between penicillin acylase and support in respect to enzyme loading, specific activity of the immobilized enzyme and enzyme and activity coupling yields. Then, a comparative study involving free and immobilized penicillin acylase is performed in terms of reaction kinetics and activity profile as a function of pH and temperature.

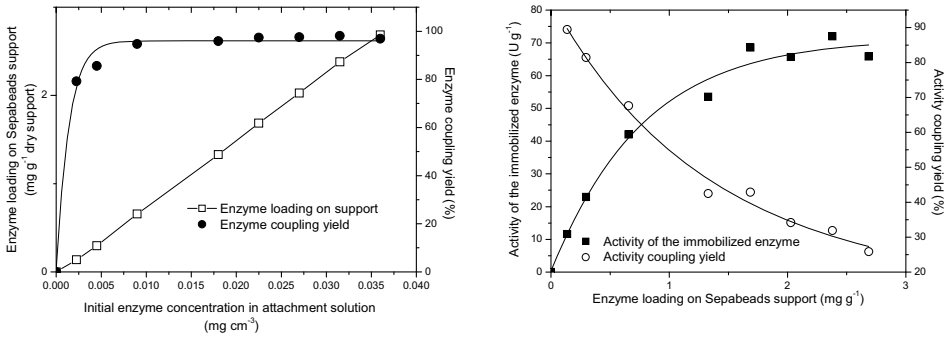
Optimization of immobilization conditions

An attempt was made to achieve binding of high levels of enzyme with a high retention of its initial activity. Thus, the effect of varying penicillin acylase concentration in the attachment solution in the range of $2.25\text{-}36\cdot10^{-3}$ mg/cm^3 (7.5-120 U/g wet support) on the total protein loading on the sepabeads support and enzyme and activity coupling yields is investigated. The results are shown in Fig. 2. In each experiment, 0.5 g of wet polymer beads were immersed in 10 cm^3 of enzyme attachment solution.

It seems that the increase of the initial enzyme concentration in the attachment solution results in almost linear increase of the enzyme loading on support (Fig. 1a). It is interesting to notice that the enzyme coupling yield also increases as the initial enzyme concentration is increased to $9\cdot10^{-3}$ mg/cm^3 and levels off at about 97.5 ±0.5%. Thus, the maximal obtained enzyme loading under this immobilization condition of approximately 2.7 mg of the protein/g of the dry support was equivalent to 96.9% enzyme coupling yield.

The activity of the immobilized penicillin acylase increases rapidly with enzyme loading on supports up to about 1.7 mg g^{-1} and then becomes essentially constant (Fig. 2.b). However, it seems that the activity coupling yield is not correlated with the amount of enzyme bound. For example, as the enzyme concentration in the attachment solution increased from 2.25 to $36\cdot10^{-3}$ mg/cm^3, the enzyme coupling yield increased slightly (by 22.4%), while the activity coupling yield decreased from 89.4 to 25.9%. The maximum activity coupling yield of 89.4% can be achieved working at low enzyme loading (0.14 mg g^{-1}). The possible reason for the lower activity yields at high enzyme loadings may be close packing of the enzyme on the support surface, which could limit the access of substrates needed in the hydrolysis reaction. Nevertheless, the immobilized enzyme showed good performance in considering that generally, covalent binding leads to significant inactivation of enzymes. Especially, the activity coupling yield achieved in this study

(89.4%) was much higher than the values reported in the literature for covalently immobilized penicillin acylase (7-9).



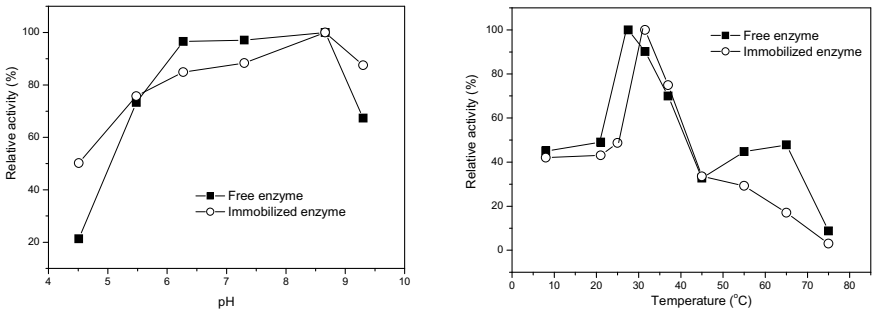
(a) (b)

Fig. 2. (a) Effects of initial enzyme concentration in attachment solution on the enzyme loading and enzyme coupling yield (b) Effects of enzyme loading on the activity of the biocatalyst and activity coupling yield

Effect of immobilization on optimum pH and temperature

The activity of the immobilized penicillin acylase was investigated in the pH range 4.5-9.3 at 37 ^{o}C and compared with that of free enzyme. Results are presented in Fig. 3a.

The pH activity profile of the immobilized enzyme was slightly narrower than that of the free enzyme having no essential effect on the pH optimum. The trends of the curves are similar in both cases. This similar behavior is related to the electrically neutral character of the support, which did not alter the pH of the microenvironment of the immobilized enzyme.



(a) (b)

Fig. 3. Effects of pH (a) and temperature (b) on activities of free and immobilized penicillin acylase. The enzyme activity was determined under assay conditions (4% (w/v) penicillin G as substrate solvated in 0.1 M buffer at indicated pH and temperature).

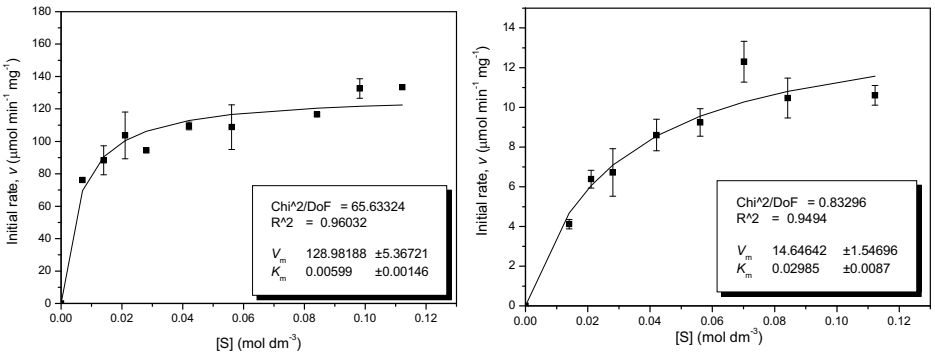
However, the immobilized enzyme shows a higher activity at lower (<5.5) and higher (>8.7) pH values than the soluble one, indicating that the immobilization of penicillin acylase imparted structural and conformational stability to this enzyme.

The effect of temperature on activities of free and immobilized enzyme was also studied and the results are shown in Fig. 3b. It appears that the optimal reaction temperature shifted from 27.5 ^{o}C for the free penicillin acylase to 31.5 ^{o}C for the immobilized enzyme, suggesting a marginally better thermal stability of the immobilized enzyme.

Kinetics properties

The reaction kinetics of immobilized penicillin acylase was compared with that of the free enzyme using penicillin G hydrolysis as a model reaction. The initial reaction rates for free and immobilized penicillin acylase were determined at different concentrations of substrate ranging from 0.25 to 4%, and the results are shown in Fig. 4. It seems that both free and immobilized penicillin acylase followed simple Michaelis-Menten kinetics. The values of kinetic parameters of free and immobilized enzyme are obtained directly by fitting the experimental data to the kinetic model.

The nonlinear regression analysis indicated that the quality of the fit was quite good, with r^2 value of 0.960 and 0.949 for free and immobilized enzyme, respectively. The K_m value for the free enzyme was determined to be 5.99±1.46 mmol dm^{-3}, whereas V_{max} value was 128.98±5.37 μmol min^{-1} mg^{-1}. The apparent value of K_m for the immobilized enzyme was 29.85±8.7 mmol dm^{-3}, approximately 5-fold higher than that of the free enzyme, suggesting that enzyme immobilization by this method caused a decrease in the enzyme-substrate affinity.



(a) (b)

Fig. 4. Effect of penicillin G concentration on initial rates of free (a) and immobilized penicillin acylase (b). The lines represent the best fits of the Michaelis-Menten kinetic model. Assay conditions: t=37 ^{o}C; 0.1 M phosphate buffer, pH 7.9; 0.1 g immobilized enzyme or the corresponding amount of free penicillin acylase.

Similarly, the value of V_m for the immobilized enzyme appeared to be approximately 9-fold lower than that of the free enzyme (V_m=14.65±1.55 μmol min^{-1} mg^{-1}; *EF*=0.11), indicating that the catalytic property of the enzyme was significantly modified by the

immobilization process. Nevertheless, the immobilized penicillin acylase showed favorable kinetic properties in considering that the value of V_m was generally considerably lower upon covalent immobilization (7, 9, 14). The increase in K_m values for immobilized enzymes has also been reported by various authors (7, 14). Wang et al. (14) reported that K_m value of immmobilized penicillin acylase was 7 times higher than that of the free enzyme owing to the influence of the support. These changes in kinetic parameters may be a consequence of either structural change in the enzyme occurring upon immobilization or lower accessibility of substrate to the active sites of the immobilized enzyme.

CONCLUSION

This work provides preliminary results on the use of Sepabeads EC-EP as a support matrix for covalent immobilization of penicilllin G acylase and application of the immobilized enzyme for penicillin G hydrolysis as a study model reaction. The immobilization procedure developed is quite simple, and easily reproduced, and involves the direct enzyme binding on polymers via epoxide groups.

Penicillin G acylase from *E. coli* has been successfully immobilized on Sepabeads EC-EP using the covalent binding method. The highest activity coupling yield of 89.4% was achieved working at low enzyme loading (0.14 mg g^{-1}). Both free and immobilized enzyme were characterized by determining the activity profile as a function of pH, temperature and reaction kinetics. The optimal pH for the immobilized enzyme activity was found to be 8.7. A slightly higher value for optimum temperature (31.5 ^{o}C) was found for the immobilized enzyme in comparison with that displayed by the free one (27.5 ^{o}C). The immobilized enzyme appears to have acceptable kinetic properties in the industrially feasible reaction system of penicillin G hydrolysis. The method is simple, inexpensive, and scaleable for industrial applications.

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ИМОБИЛИЗАЦИЈА ПЕНИЦИЛИН-АЦИЛАЗЕ ИЗ *Escherichia coli* НА КОМЕРЦИЈАЛНОМ SEPABEADS НОСАЧУ

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У раду је испитана ковалентна имобилизација пеницилин-ацилазе из *Escherichia coli* на комерцијалном полиметакрилатном носачу са епоксидним функционалним групама (Sepabeads EC-EP) и добијени имобилисани ензим је окарактерисан у реакцији хидролизе пеницилина. Изабрани ензим је од великог индустријског значаја јер катализује реакције хидролизе природних пеницилина и синтезе полусинтетских β-лактамских антибиотика.

Испитан је утицај почетне концентрације ензима на масу и активност имобилисаног ензима, као и на масени принос имобилизације и принос активности. Највећа маса имобилисаног ензима је износила 2,5 mg по јединици масе носача, што одговара масеном приносу од 96,9%. Међутим, принос активности је обрнуто пропорционалан маси имобилисаног ензима тако да се максимални принос од 89,4% постиже при имобилизацији најмање масе ензима (0,14 mg/g носача). Имобилизација ензима је проузроковала мање промене у pH профилу активности и оптималним вредностима температуре биокатализатора, што указује на незнатну стабилизацију ензима услед имобилизације. Кинетика реакције хидролизе природног пеницилина слободним и имобилисаним ензимом може се описати Михаелис-Ментеновом једначином и одређене су вредности кинетичких константи за оба система.

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