Biocatalysis by Penicillin G Acylase Anchored on Beaded Macroreticular Glycidyl methacrylate-divinyl benzene Polymers: Effect of Pore Structure on Catalytic Activity

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Abstract

A series of macroreticular, beaded, glycidyl methacrylate (GMA) and divinylbenzene (DVB) polymers with variance in pore size distribution were synthesised by suspension polymerisation. The internal pore structure of the beads were altered by changing the mole ratio of GMA:DVB, porogen (cyclohexanol, hexanol) or its volume. The porosity was investigated by mercury intrusion porosimetry. The functional group were characterised by IR spectroscopy and wet analysis. The study shows a dependence of surface functional group, surface area and porosity on copolymer composition, porogen type and volume. Immobilisation of penicillin G acylase on the synthesised beads was investigated. The process parameters were optimised to attain good binding and maximum expression of penicillin G acylase. The immobilised enzyme was used in the production of 6-APA, a key intermediate in the production of semi-synthetic penicillins.

Keywords: penicillin G acylase, pore size distribution, immobilised penicillin G acylase, GMA-DVB polymers, 6-amino penicillanic acid, biocatalysis

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1. Introduction

The use of enzymes as biocatalysts for the synthesis of pharmaceutical products, both achiral and chiral, has gained in popularity and the number of important industrial applications of biocatalysis is growing rapidly [1]. The biocatalysis can be carried under very mild conditions and with greater specificity, leading to increased yield by reducing side-products. As regards the preparation of pure enantiomers, the enzymatic catalysis marginalises isomerisation, racemisation, epimeri-ation and rearrangement that tend to occur in equivalent chemical processes. Using soluble enzymes as biocatalyts presents some drawbacks, such as stability under operational conditions, difficult product recovery and the impossibility of repeated reuse, essential to industrial applications. This is overcome by anchoring enzymes on solid supports, termed as enzyme immobilisation. Immobilised enzymes catalysed transformation of one enantiomer in a racemic mixture, allows for an efficient and rapid separation of enantiomers [2-4], presenting the pharmaceutical industry with an attractive approach to enantiomerically pure drugs. In 2002 the world-wide sales

of single-enantiomer drugs topped US$ 159 billion.

Penicillin acylase (PGA) of *Escherichia coli* ATCC 11105 (EC 3.5.1.11) catalyses the hydrolysis of penicillin G to phenylacetic acid (PAA) and the industrially important 6-amino-penicillanic acid (6-APA). This β-lactam nucleus is a building block in the synthesis of semi-synthetic penicillins [5,6]. The reaction is schematically represented in Figure 1. The hydrolysis involves the nucleophilic attack by a serine residue on the acyl carbon of penicillin G, with the formation of a tetrahedral intermediate, resulting in an acyl enzyme and free 6-APA. The acyl enzyme reacts with water to form a second intermediate which further transforms to yield phenylacetic acid [5].

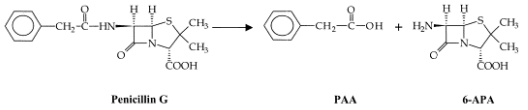

Figure 1. Reaction scheme of penicillin G hydrolysis to yield phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA).

The enzymatic catalysis by PGA of penicillin G to 6-APA is regio- and stereo-specific [7]. It can be carried out in mild conditions and it is more economical than the parallel chemical route. The production of 6-APA constitutes perhaps the largest utilisation of enzymatic catalysis in the production of pharmaceuticals. The enzymatic activity of PGA, associated with the phenacetyl moiety, allows the stereo-specific hydrolysis of a rich variety of phenacetyl derivatives of primary amines as well as alcohols. It even tolerates minor modifications in the phenacetyl moiety [8-11]. The industrial needs has triggered a large production of PGA, and its rather tolerant substrate specificity have led to a wide range of applications.

PGA has been covalently bonded to various supports [12-15] and PGA-immobilised systems have been reported as useful tools for the synthesis of pure optical isomers [11,16-21]. A very large number of immobilisation procedures have been investigated for this industrially important enzyme in order to obtain a heterogeneous catalyst with good stability [22] and catalytic properties [23]. Covalent linking of the enzyme to the polymer support does modify the conformational structure near the active centre of the enzyme resulting in a modulation of the catalytic properties.

Tailored hydrophobic polymer matrices for the immobilisation of PGA are studied because of the commercial implications. Matrices with epoxy groups are preferred over other functional groups because the activation step is avoided, the binding is via formation of a covalent bond and the process of immobilisation is a simple one step procedure [13]. Reaction of epoxy group carrying vinyl monomers (GMA) with a relatively high concentration of divinyl monomer (DVB), as used in the present study, leads to a permanent macroporous (macroreticular) structure, essential to trap and to covalently anchor large sized enzyme molecules. The porous structure is modified by the presence in the polymerisation recipe of inert diluents, termed porogens.

In the present study, a series of hydrophobic epoxy polymers of differing inner pore volume, pore size and its distribution, and concentration of reactive epoxy pendent groups were synthesised by varying porogen volume and crosslink density (GMA:DVB). The effect of these on immobilisation of PGA are reported. The optimally immobilised PGA was evaluated for the biocatalytic activity, in hydrolysis of penicillin G to 6-amino penicillanic acid.

2. Experimental

2.1 Materials

Glycidyl methacrylate (GMA) (Sartomer, USA) and divinylbenzene (DVB) (Merck) were used as received. Cyclohexanol, hexanol were obtained from M/S Aldrich Chemical Co. (USA). Poly(vinyl pyrrolidone) [PVP] (Polysciences, USA) was used as protective colloid. Azobisiso-butyronitrile [AIBN], (SISCO, India) was used as initiator. Penicillin G and Penicillin G acylase (specific activity 7.5 IU/mg) from Escherichia coli were from Hindustan Antibiotics Ltd. (Pimpri, India).

2.2 Synthesis of GMA-DVB Polymers

The suspension polymerisations were conducted in double walled cylindrical reactor at constant agitation set at 300 rpm, at 70 ^{o}C for 3 h. The continuous phase comprised of one weight percent aqueous solution of PVP. The discontinuous organic phase consisted of GMA, crosslinking divinyl monomer (DVB), polymerisation initiator [AIBN] and cyclohexanol or hexanol used as porogen. The discontinuous organic phase was introduced into the aqueous phase, constant stirring (with 6 bladed Rushton turbine) and temperature were set, by circulating thermostated water. The polymer obtained in beaded form was separated by decantation, washed with water and methanol and dried at room temperature under reduced pressure and the yield was noted. The composition of synthesised GMA-DVB polymers are presented in Tables 1 and 2.

2.3 Characterisation

The epoxy functional group/g polymer was determined by titration of the excess reagent present after reaction of the polymer beads with a hydrochloric acid-dioxane solution at 80 ^{o}C for 6 h. Dioxane swells the beads and therefore some of the epoxy groups buried within the bulk of the matrix will be titrated in addition to all present at the surface of the pores. A Shimadzu 8300-Fourier transform infra-red spectrophotometer (FTIR) with a resolution of 1 cm^{-1} in the transmission mode was used to study the infra-red absorption. The GMA-DVB polymers (2 mg) were milled, mixed with potassium bromide (100 mg), and pressed into a solid disk of 1.2 cm diameter prior to the infra-red measurement.

The porous properties were determined by mercury intrusion porosimeter in the pressure range 0 - 4000 kg/cm^2 with an Auto scan 60 mercury porosimeter from Quantachrome, USA.

2.4 Immobilisation of PGA

GMA-EGDM beads (5.0 g) were suspended in 100 cm^3 of 0.05M phosphate buffer pH 7.5 containing approximately 2500 IU of PGA. The flasks were incubated on a rotary shaker (100 rpm) at room temperature for 72 h. After the incubation, the supernatant was assayed for unbound enzyme. The difference between the amount of enzyme loaded and the amount of

enzyme remaining unabsorbed in the supernatant indicates the amount of enzyme bound. The supernatant after the assay was decanted off. The immobilised penicillin G acylase bound beads thus prepared were washed twice with distilled water and stored in 0.05M phosphate buffer pH 7.5 containing 0.02 % sodium azide at 5 ^{o}C. The catalytic activity of immobilised enzyme beads were evaluated using penicillin G as the substrate. The expression of the adsorbed enzyme is defined as the activity of the immobilised enzyme as compared to that of the enzyme bound on the matrix. The activity of soluble and immobilised PGA was determined by measuring 6-APA formed.

Table 1: Porosity of GMA-DVB polymers and immobilisation and expression of PGA: Effect of crosslink density and porogen volume

Polymer No.	CLD[a] (%)	MPR[b] (nm)	PV[c] (mL.g^{-1})	SA[d] (M^2.g^{-1})	EB (IU.g^{-1})	Binding (%)	IEA (IU.g^{-1})	Exp (%)
1.Monomers:cyclohexanol 1:0.5 v/v								
1.1	10	2.8	0.06	42	78.5	16.3	ND	ND
1.2	25	3.5	0.10	58	93.7	19.4	ND	ND
1.3	50	4.4	0.09	44	109	22.6	ND	ND
1.4	75	4.7	0.11	48	83.7	17.3	ND	ND
1.5	100	4.6	0.12	53	65.3	13.5	ND	ND
2. Monomers:cyclohexanol 1:1.0 v/v								
2.1	10	9.4	0.30	64	203	44.7	69.2	15.2
2.2	25	8.7	0.50	112	394	86.9	118	25.9
2.3	50	7.6	0.55	152	442	97.4	101	22.4
2.4	75	8.2	0.64	150	429	95.8	ND	ND
2.5	100	7.8	0.66	160	88.3	18.6	ND	ND
3. Monomers:cyclohexanol 1:1.5 v/v								
3.1	10	14.5	0.41	55	396	74.9	134	33.8
3.2	25	10.8	0.66	120	524	99.1	205	39.0
3.3	50	12.1	0.78	130	526	99.4	167	32.0
3.4	75	13.7	0.85	127	521	98.5	131	25.3
3.5	100	14.9	0.94	127	523	98.9	105	20.1
4. Monomers:cyclohexanol 1:2.0 v/v								
4.1	10	9.2	0.26	57	145	29.6	41.2	28.4
4.2	25	10.0	0.75	148	401	82.0	144	36.0
4.3	50	11.6	1.08	187	471	96.4	150	31.7
4.4	75	13.9	1.24	175	473	96.8	45.6	9.6
4.5	100	13.7	1.34	190	477	97.5	23.3	4.9
5.Monomers:cyclohexanol 1:2.5 v/v								
5.1	10	3.5	0.08	43	131	24.7	19.2	14.7
5.2	25	13.8	0.60	88	440	83.2	134	30.4
5.3	50	13.5	1.3	193	513	97.1	143	27.9
5.4	75	15.0	1.5	201	513	97.1	93.1	18.1
5.5	100	14.9	1.6	220	257	48.5	ND	ND

Combined monomer (GMA+DVB) volume was 32.7 mL in all experiments; AIBN = 0.2 g; CLD[a] = crosslink density = [GMA]/[DVB]; MPR[b], PV[c], SA[d] = mean pore radii, pore volume and surface area, as determined by mercury porosimetry; Polymer sets 1, 2, 3, 4 and 5 were loaded with 483, 454, 529, 488 and 528 IU.g^{-1}; EB = enzyme bound, as determined by enzyme activity in the supernatant; IEA = immobilised enzyme's expressed activity; Exp = expression; ND = not determined.

2.5 Production of 6-APA

The optimal immobilised enzyme was used in the production of 6-APA over exactly 100 cycles, to evaluate activity loss, using immobilised penicillin G acylase based on polymer 3.2 [24].

Table 2: Dependence of porosity on porogen type

Poly. No.	CLD[a] (%)	MPR[b] (nm)	PV[c]	SA[d]
6. Monomers:cyclohexanol 1:1.6 v/v				
6.1	25	4.1	0.15	39.2
6.2	50	3.7	0.16	42.8
6.3	75	4.3	0.19	42.7
6.4	100	4.3	0.21	55.4
6.5	150	13.3	0.40	83.0
7. Monomers:hexanol 1:1.6 v/v				
7.1	25	6.9	1.47	24.5
7.2	50	4.7	1.53	35.4
7.3	75	4.4	1.70	46.1
7.4	100	4.7	1.88	48.0
7.5	150	3.7	1.84	50.8

3. Results and Discussion

GMA-DVB polymers provide reactive epoxy groups which react with functional groups present in the enzyme molecule, mainly primary amino groups, to form permanent covalent linkages between the polymer and the PGA during immobilisation. Besides the surface properties of the polymer beads, the major factor that contributes to the binding and expression of immobilised PGA is pore size and pore size distribution which influences the diffusional phenomenon during the course of catalysis. Pore diffusional limitations are of importance particularly in the preparation of immobilised PGA. Since a pH gradient exists across the porous polymer beads, the rate of hydrolysis of penicillin G is reduced [25,26]. Thus, optimisation of pore size and pore size distribution forms an integrated part of designing polymer matrices for immobilisation of enzymes such as PGA.

Some of the very many factors that contribute to pore size and pore size distribution include porogen type, the volume of porogen relative to the volume of the monomers, the mole ratio between GMA, the reactive monomer and the crosslinker, DVB, termed as crosslink density. The GMA-DVB polymerisation strategies were so planned that the synergistic effect of the above mentioned factors on the immobilisation of PGA could be studied.

The surface tension of GMA, DVB and cyclohexanol in contact with water are approximately 6.9, 16.5 and 34.4 dyne/cm. The surface tension of GMA is lower than DVB and cyclohexanol. Therefore, the monomer with lower surface tension will be enriched on the interface between the continuous phase consisting of a aqueous solution of PVP and the dispersed phase consisting of the porogen (cyclohexanol) and the monomers. As the surface tension of GMA is lower than DVB and cyclohexanol, GMA would be present preferentially at the surface of the polymer beads. It is deduced that the surface state of the prepared polymer beads is controlled by the surface tension between the dispersed phase and the continuous phase.

3.1 Physico-chemical Characterisation

The IR spectra of all GMA-DVB polymers show peaks at 1733 and 1123 cm^{-1} due to stretching vibrations of C=O of ester group and C-O-C of epoxy group, respectively. The peak at 1636

cm^{-1}, a characteristic band of C=C stretching, disappears. This means that epoxy group is present in the polymers, polymerisation proceeds with complete consumption of the vinyl double bond. It is seen that the epoxy content decreases as the relative mole fraction of GMA decreases with increasing crosslink density. This is to be expected. With the increase in crosslink density (decreasing GMA content), the particle size increases and rate of diffusion of the reagent into the pores decreases, resulting in much lower analysable epoxy group in polymers with higher crosslink density. However, the fraction of the epoxy groups which are titratable by the reagent and hence present at or near the surface decreases much more drastically.

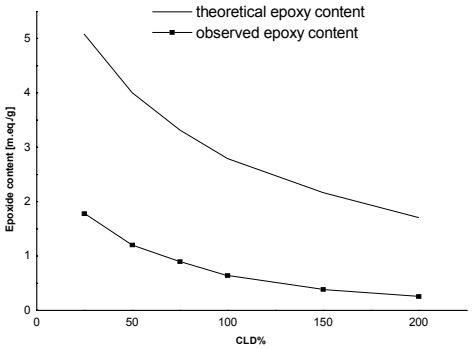

Figure 2: Theoretical and reactive epoxy groups in GMA-DVB polymers vs crosslink density, %CLD.

3.2 Porous structure

Mercury porosimetry provides a good estimate of pore size and pore size distribution in the meso and macroporous region, the range of importance for immobilisation of enzymes. The larger pores, which are responsible for higher pore volume, are located in between agglomerates and arise when larger amount of crosslinker and porogen (cyclohexanol) are used. In beaded polymers macroporous morphology arises during suspension polymerisation due to formation of gel microspheres, agglomeration of these and binding together of the agglomerates. Pore volume is an important property of a polymer that defines its porous structure and mechanical properties. GMA-DVB polymers prepared with lower amount of crosslinker and porogen (cyclohexanol) have low pore volume and surface area. This is because a large number of nuclei are formed which tend to grow through each other. When compositions were repeated using hexanol in exactly the same proportions as porogen, the porosity characteristics are quite different. The pore volume was greater. The surface area measurements reveal just the opposite effect between cyclohexanol and hexanol. For a given polymerisation recipe, the surface area of polymer formed in presence of cyclohexanol were greater than that in presence of hexanol. The increase in surface area with crosslink density is due to decrease in the size of the microsphere. This also points to a bimodel distribution of pore size in the presence of cyclohexanol.

3.3 Immobilisation, biocatalysis

The binding of penicillin G acylase onto GMA-DVB polymers formed at monomer:cyclohexanol v/v ratio of 1:0.8 were very low. The binding increased with increase in the volume of cyclohexanol. Thus, enzyme binding occurs preferentially in the pores. In general, at a fixed volume of cyclohexanol, the amount of enzyme bound initially increased with increase in crosslink density and then remained constant. The catalytic activity of the

immobilised enzyme, however, goes through a maxima. In this polymer series, the crosslinking comonomer, DVB, is very hydrophobic. The increase in hydrophobicity of the polymers at high crosslink density probably disrupts the tertiary structure of the immobilised enzyme, thereby affecting its catalytic activity. The optimal polymer (No. 3.2) showed 99.1 % binding and 39 % expression exhibiting 205 IU g-1. This immobilised enzyme was successfully utilised for 100 cycles in the laboratory for the production of 6-APA, with slightly less than 10% loss in enzyme activity.

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