Effect of Microenvironment of Oxirane Groups on the Immobilization of Penicillin G Acylase

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SYNOPSIS

Macroporous beaded terpolymers containing oxirane groups were synthesized for immobilization of penicillin G acylase. The effect of incorporation of various monomers such as hydroxyethyl methacrylate and methyl methacrylate, and the effect of crosslinking agents such as ethylene glycol dimethacrylate and divinyl benzene on binding and expression of penicillin G acylase were studied. The terpolymers were modified to varying extents by treating with polyethyleneimine. The influence of the microenvironment around the oxirane group on the binding and expression of penicillin G acylase was investigated.

INTRODUCTION

Immobilized preparation of penicillin G acylase, an enzyme catalyzing hydrolysis of linear amide bond in the penicillin G molecule, is being used industrially for the production of 6-amino penicillanic acid (6-APA).[1] Immobilization of penicillin G acylase is an important step in the total process for production of 6-APA. Among various matrices explored for the binding of penicillin G acylase, synthetic porous beaded matrices have gained importance because of certain advantages.[1-3] We have earlier reported the role of crosslinking agent, crosslinking density, and pore generation solvent in adsorption and expression of penicillin G acylase onto methacrylate polymers.[2,4]

Immobilization of penicillin G acylase on glycidyl methacrylate-co-ethylene dimethacrylate polymers has been investigated by Drobnik et al.[5] Among various approaches for modifying the oxirane group and introducing spacer arms, modification of oxirane groups with ammonia and activation by glutaraldehyde yields maximum immobilization (39%) of penicillin G acylase.[5]

In the present study, the effect of altering the hydrophilicity in the microenvironment of oxirane groups in the glycidyl methacrylate polymers on the adsorption and expression of penicillin G acylase is described.

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EXPERIMENTAL

Materials

Purified penicillin G acylase preparation (specific activity 7.5 IU/mg) from *Escherichia coli* was obtained from the production unit of Hindustan Antibiotics Ltd., India. The following chemicals were obtained from Fluka AG, Switzerland: hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), methyl methacrylate (MMA), and divinylbenzene (DVB). Azobisisobutyronitrile (AIBN) was from Scientific and Industrial Supplies Corporation, India. Polyethyleneimine (PEI) (MW 2000) in the form of 50% aqueous solution was from BASF, Germany. All other chemicals were of analytical grade from local suppliers.

Support Preparation

Suspension polymerization was conducted as described previously.[2] The monomers, crosslinking agent, polymerization initiator, and pore generating solvent used are as described previously.[2] The beaded polymer obtained was separated by decantation,

then washed with methanol, followed by water, and dried at room temperature under vacuum.

Polyethyleneimine Modification

Polymers containing oxirane groups were modified with polyethyleneimine by placing the beads in contact with polyethyleneimine solution in distilled water for 24 h. Concentrations of polyethyleneimine were adjusted so that the extent of modification varied between 3 and 50% of oxirane groups present in the beads.

Enzyme Immobilization

Polymer beads (5.0 g) were suspended in 100 mL of 0.05 *M* phosphate buffer, pH 7.5 containing 1160 IU of penicillin G acylase. The flasks were incubated on a rotary shaker (100 rpm) at room temperature for 48 h. The immobilized penicillin G acylase beads, thus prepared were washed with distilled water and stored in 0.05 *M* phosphate buffer, pH 7.5 at 5°C. The supernatant was assayed for unbound enzyme. Quantity of enzyme bound indicates the difference between the amount of enzyme loaded and amount of enzyme remaining unadsorbed in the supernatant. Immobilized enzyme beads were processed for activity determination. The expression of the adsorbed enzyme is defined as the activity of the immobilized enzyme as compared to that of the enzyme bound on the matrix.

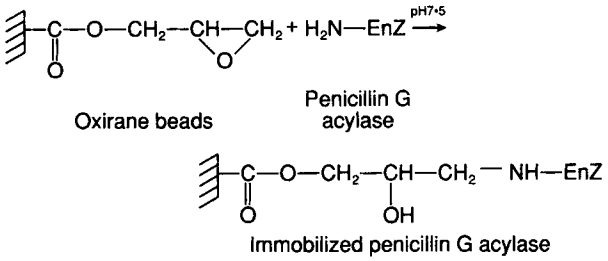
Analytical Methods

The activity of both soluble and immobilized penicillin G acylase preparations was determined as described previously.[2,6] The oxirane groups on the beads were quantitated using the sodium thiosulfate method.[7] Pore size distribution was studied by mercury intrusion porosimetry using a No. 33 mercury porosimeter from Quantachrome (USA).

RESULTS AND DISCUSSION

Synthetic polymer matrices ideally suited to enzyme immobilization should be inert, and provide reactive functional groups to bind enzyme effectively under mild conditions. Oxiranyl polymers offer such facile reactive groups. The covalent binding occurs through the oxirane group and a number of functional groups, mainly primary amino groups, present in the enzyme molecule (Scheme I).

Other structural moieties in the polymer backbone contribute to the enzyme binding by providing suitable topochemical environment and/or by increasing the affinity of the polymer for the enzyme. Optimizing microenvironment and modifying hydrophilic/hydrophobic character form an intergral part of the overall strategy in the identification and design of ideal polymer matrices for a specific enzyme.



Scheme I

Three series of terpolymers were synthesized by the suspension polymerization technique. The polymers were obtained in spherical "beaded" form within each series. Six of the seven process variables were unaltered, to generate terpolymers of differing hydrophilic/hydrophobic character. The variables maintained at constant levels were: relative volumes of the continuous (water) and discontinuous (organic) phases, the amount of protective colloid [poly(vinyl pyrrolidone), K-90], the volumes of pore generating solvent and crosslinking agents (ethyleneglycol dimethacrylate/divinylbenzene), the initiator concentration, and reaction temperature. Thus, the relative performances of the terpolymers could be related to the hydrophilic/hydrophobic balance in the polymers.

The suspension polymerizations were conducted at constant speed (300 rpm) to obtain beads of uniform particle size. The internal pores were generated using a solvent which was freely miscible with the monomers but which did not dissolve the polymer. This pore-generating solvent is dispersed uniformly together with the monomers in the discontinuous phase. During chain growth the pore-generating solvent is expelled as droplets within the discontinuous phase. This generates the pores. The droplet size, which determines the pore size and its distribution, is governed by (i) rate of polymerization and (ii) the rate of aggregation of the pore-generating solvent. Macropores are generated by large differences in the solubility parameters of copolymer and the pore-generating solvent at low rates of polymerization. Micropores would evolve with solvents having a solubility parameter equivalent to the co-

Table I Glycidyl Methacrylate (GMA), Hydroxyethyl Methacrylate (HEMA), and Ethylene Glycol Dimethacrylate (EGDMA) Terpolymers. Composition and Average Pore Radii Values

Polymer No.	GMA (moles)	HEMA (moles)	EGDMA (moles)	Average Pore Radii, (Å)
P1	0.1078	Nil	0.0779	58.0
P2	0.1051	0.0027	0.0779	56.4
P3	0.1024	0.0054	0.0779	60.6
P4	0.0970	0.0108	0.0779	62.3
P5	0.0889	0.0189	0.0779	55.5
P6	0.0809	0.0270	0.0779	53.5
P7	0.0674	0.0404	0.0779	48.6
P8	0.0539	0.0539	0.0779	53.5
P9	0.0270	0.0809	0.0779	53.3

polymer, especially at high rates of polymerization. The divinyl monomer offers supporting bridges to stabilize the pores. Homopolymerization in the presence of pore-generating solvent would initially generate pores. These would collapse in the absence of supporting bridges provided by the crosslinking agent and would result in nonporous beads.

The reactivity ratios for the following binary copolymerizations are reported in literature:[8-10]

1. Glycidyl methacrylate (M_1) : r_1 = 0.98
   Ethylene glycol dimethacrylate (M_2) : r_2 = 1.00
2. Glycidyl methacrylate (M_1) : r_1 = 1.05
   Methyl methacrylate (M_2) : r_2 = 0.80
3. Methyl methacrylate (M_1) : r_1 = 0.41
   Divinylbenzene (M_2) : r_2 = 0.61

In methacrylate terpolymers the composition would be predominantly governed by the feed ratios. Hence, the pore size and its distribution will be independent of conversion. In terpolymers with divinylbenzene as a constituent, the divinyl monomer will be consumed preferentially in the early stages of the polymerization. This system would intrinsically display a wide distribution in pore size. The enzyme molecules would diffuse into only those pores which are larger than its dimensions.

Effect of Hydroxyethyl Methacrylate

In the first series, *P*, one copolymer and eight terpolymers were synthesized by an interplay of the relative mole ratios of hydroxyethyl methacrylate and glycidyl methacrylate at a constant crosslink density, provided by incorporating ethylene glycol dimethacrylate. The data is presented in Table I. Hydrophilicity increases from *P*-1 to *P*-9. The polymers were all macroporous with a distribution of pores in the range 32 to 1000 Å. The average size of the pores, presented in Table I, is between 48.6 and 62.3 Å.

The penicillin G acylase binding characteristics are listed for a few representative polymers. The

Table II Effect of Terpolymer Composition on Binding and Expression of Penicillin G Acylase

Polymer No.	Enzyme Loaded, (IU per g of Polymer)	Enzyme Adsorbed		IME Assay[a]	
		(IU per g)	% Adsorbed	Activity (IU per g)	% Expressed
P1	232	194	83.6	113	58.2
P3	232	146	62.9	64	43.8
P4	232	141	60.7	62	43.9
P6	232	58	25.0	25	43.1
P8	232	44	18.9	20	45.4
P9	232	7	3.0	4	57.1

[a] Immobilized enzyme assay

Table III Glycidyl Methacrylate (GMA), Hydroxyethyl Methacrylate (HEMA), and Divinylbenzene (DVB) Terpolymers. Composition and Average Pore Radii Values

Polymer No.	GMA (Moles)	HEMA (Moles)	DVB (Moles)	Average Pore Radii, (Å)
DVB 1	0.1078	Nil	0.1303	66.2
DVB 2	0.1051	0.0027	0.1303	59.3
DVB 3	0.1024	0.0054	0.1303	62.2
DVB 4	0.0970	0.0108	0.1303	59.0
DVB 5	0.0889	0.0189	0.1303	63.2
DVB 6	0.0809	0.0270	0.1303	55.0
DVB 7	0.0674	0.0404	0.1303	62.2
DVB 8	0.0539	0.0539	0.1303	58.5
DVB 9	0.0270	0.0809	0.1303	67.1

adsorption and expression of bound enzyme are presented in Table II. The binding of penicillin G acylase decreases with increasing HEMA content of the polymer. The percent expression of the bound enzyme was not measurably altered at different levels of HEMA. Thus, increasing the hydrophilicity decreases the adsorption of enzyme on the matrix, a desired prerequisite for binding, and results in less covalent binding.

Effect of Divinylbenzene

In the second series, DVB, one copolymer and eight terpolymers were synthesized. In this series GMA was replaced to varying degrees with HEMA, and divinylbenzene (DVB) was used as the crosslinking agent instead of ethylene glycoldimethacrylate. The degree of crosslinking was held constant. The divinylbenzene used was composed of 60% DVB and 40% 4-ethylstyrene. The composition and crosslink density of the polymers were similar to those in the first series. Incorporation of DVB imparts greater hydrophobicity to these polymers. The composition and average pore radii are presented in Table III. The average pore radii varied between 55.0 and 67.1 Å.

The relative abilities to bind penicillin G acylase are presented in Table IV. A relatively high concentration of the enzyme was bound to the polymer matrix. An increase in hydrophilicity had practically no effect on the binding characteristics. At very high levels of HEMA (Polymer DVB 9) the binding efficiency dropped by 24%. The bound penicillin G acylase does not effectively promote the hydrolysis of penicillin G to 6-aminopenicillanic acid. It appears that the aromatic groups of DVB either disrupt the tertiary structure of the bound penicillin G acylase or hinder the interactions between penicillin G acylase and penicillin G molecules. This decreases the catalytic efficiency.

Table IV Effect of GMA-HEMA-DVB Terpolymer Composition on Binding and Expression of Penicillin G Acylase

Polymer No.	Enzyme Loaded, (IU per g of Polymer)	Enzyme Adsorbed		IME Assay[a]	
		(IU per g)	% Adsorbed	Activity (IU per g)	% Expressed
DVB 1	232	185	79.7	25	13.5
DVB 2	232	166	71.5	21	12.6
DVB 3	232	189	81.4	35	18.5
DVB 4	232	188	81.0	28	14.9
DVB 5	232	188	81.0	21	11.1
DVB 6	232	173	74.5	35	20.2
DVB 7	232	188	81.0	32	17.0
DVB 8	232	190	81.9	29	15.2
DVB 9	232	131	56.4	21	16.0

[a] Immobilized enzyme assay

Table V Glycidyl Methacrylate (GMA), Methyl Methacrylate (MMA), and Ethylene Glycol Dimethacrylate (EGDMA) Terpolymers. Composition and Average Pore Radii Values

Polymer No.	GMA (moles)	MMA (moles)	EGDMA (moles)	Average Pore Radii (Å)
M 1	0.1051	0.0027	0.0779	61.0
M 2	0.1024	0.0054	0.0779	59.6
M 3	0.0970	0.0108	0.0779	54.9
M 4	0.0889	0.0189	0.0779	60.7
M 5	0.0809	0.0270	0.0779	58.1
M 6	0.0674	0.0404	0.0779	54.5
M 7	0.0539	0.0539	0.0779	55.9
M 8	0.0270	0.0809	0.0779	52.6

Effect of Methyl Methacrylate

In this series 2.5 to 75.0 mole percent of glycidyl methacrylate (GMA) was substituted with more hydrophobic but nonreactive methyl methacrylate (MMA). The compositions of the macroporous beaded terpolymers are presented in Table V. The average pore radii in this series varies between 52.6 and 61.0 Å.

The binding efficiencies of polymer supports in this series are presented in Table VI. The binding efficiency decreases initially and goes through a minimum corresponding to 25 mol % of MMA (polymer M 5). The acetate pendant group of MMA is relatively less hydrophobic than the benzene ring in the DVB series. This hydrophobic character of MMA is inadequate to effectively adsorb penicillin G acylase. At higher mole fraction of MMA (polymer M 7 and M 8) the binding efficiency increases. However, the bound enzyme is either inaccessible to penicillin G or its catalytic activity is decreased due to structural reorganization.

Effect of Polyethyleneimine

The oxirane group in the polymer P 1 were partially derivatized with low molecular weight polyethyleneimine (mol wt 2,000), to increase the hydrophilicity without altering the porosity of the beads. The beads have reactive oxirane groups in a more hydrophilic microenvironment. The data is presented in Table VII. The unmodified polymer matrix had an overall immobilization efficiency of 48.2%. The binding efficiency was 82.7% and expression was 58.3%. On modification, the binding and expression of penicillin G acylase are markedly decreased. The binding efficiency dropped by 38% at as low a modification of oxirane groups as 3.13%. Thus, a hydrophilic microenvironment decreases

Table VI Effect of GMA-MMA-DVB Terpolymer Composition on Binding and Expression of Penicillin G Acylase

Polymer No.	Enzyme Loaded, (IU per g of Polymer)	Enzyme Adsorbed		IME Assay[a]	
		(IU per g)	% Adsorbed	Activity (IU per g)	% Expressed
M 1	232	83	35.7	20	24.1
M 2	232	52	22.4	8	15.4
M 3	232	48	20.7	9	18.7
M 4	232	46	19.8	10	21.7
M 5	232	22	9.5	11	50.0
M 6	232	61	26.3	13	21.3
M 7	232	94	40.5	12	12.7
M 8	232	115	49.5	11	9.5

[a] Immobilized enzyme assay

Table VII Effect of Polyethyleneimine (mol wt 2,000) Modification on Binding and Expression of Penicillin G Acylase

Polym. No.	Percent Modification	Enzyme Loaded, (IU per g of Polymer	Enzyme Adsorbed		IME Assay[a]	
			(IU per g)	% Adsorbed	Activity (IU per g)	% Expressed
P 1	Nil	232	190.0	81.80	112.0	57.8
PGMA 1	3.13	232	104.6	45.08	31.7	30.3
PGMA 2	6.25	232	98.2	42.32	26.7	27.1
PGMA 3	12.50	232	82.9	35.73	18.8	22.6
PGMA 4	25.00	232	56.0	24.13	8.7	15.5

[a] Immobilized enzyme assay

immobilization by hindering the interaction of penicillin G acylase with the polymer, and hinders the interactions of penicillin G acylase with penicillin G during the catalytic reaction.

CONCLUSION

A series of hydrophilic and hydrophobic oxiranyl terpolymers with macropores were synthesized. The polymers were tested for binding of penicillin G acylase and the catalytic efficiency of the bound enzyme. Hydrophilic and hydrophobic microenvironments decrease the binding efficiency as well as the expression of the bound enzyme. The binding efficiency decreases less dramatically with increasing hydrophobicity; however, the expression of bound enzyme is drastically reduced. The porosity of the polymer plays a very marginal role in determining the binding efficiency.

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