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Single-Step Purification and Immobilization of Penicillin Acylase Using Hydrophobic Ligands

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Abstract

Five different hydrophobic ligands immobilized on 4% (4XL) and 6% (6XL) crosslinked agarose were used to study the single-step purification of penicillin acylase from cell lysate. The 4XL gels showed relatively higher specific activity and recovery than the 6XL gels. In single-step purification, highly active enzyme (42 U/mg) was obtained using moderately hydrophobic ligand (octyl). The crude enzyme immobilized on octyl gel by adsorption showed significant operational stability over a period of 30 d at room temperature. Reactor studies demonstrated the feasibility of hydrophobic ligands as a medium for immobilization.

Index Entries: Penicillin acylase; 6-aminopenicillanic acid; hydrophobic ligand; purification; immobilization; conversion.

Introduction

Annually, about 10–30 t of immobilized penicillin acylase (EC 3.5.1.11) is consumed for the production of 6-aminopenicillanic acid (6-APA) *(1)*. High specific activity of immobilized enzyme is important at the industrial level, and this requires highly purified enzyme. The methods developed so far for the purification of penicillin acylase are mainly based on multistep operations *(2,3)*. Recently, some studies have reported the single-step purification of penicillin acylase *(4–6)*; however, most achieved partial purification or low specific activity. Conventionally, penicillin acylase is immobilized using covalent bonding or copolymerization techniques.

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Disposal of inactivated immobilized enzyme preparation creates significant environmental as well as economical problems. We have selected hydrophobic ligands to study single-step purification and immobilization of penicillin acylase because of their peculiar characteristics and economical feasibility. It has been reported that the purification of proteins using hydrophobic ligand was more advantageous than the other purification methods *(7)*. In addition, the enzyme adsorbed on the hydrophobic ligands can be directly used as an immobilized preparation *(8)*. Because there is no irreversible binding, the inactivated adsorbed enzyme can be replaced easily with fresh enzyme, and, thus, the adsorbent can be used repeatedly. Although the hydrophobic ligands are being used routinely for enzyme purification *(9)*, no information is available regarding the role of different hydrophobic ligands in single-step purification and immobilization of penicillin acylase. The purpose of this study was to examine the possibility of using hydrophobic ligands as a medium for single-step purification and immobilization of penicillin acylase.

Materials and Methods

Growth of Organism and Preparation of Enzyme

Escherichia coli NCIM 2350 was obtained from the National Collection of Industrial Microorganisms, Pune, and maintained routinely on nutrient agar slants. Cells were grown in a medium containing the following 3.0 g/L of peptone, 3.0 g/L of yeast extract, 3.5 g/L of NaCl, 3.0 g/L of K_2HPO_4, 0.3 g/L of KH_2PO_4, 40.0 g/L of corn steep liquor (Anil Starch, Ahamdabad, India), 1.0 g/L of phenylacetic acid, 0.1 g/L of polypropylene glycol (antifoam). The pH of the medium was adjusted to 7.2 with NaOH. The inoculum was developed in a 500-mL conical flask containing 100 mL of medium incubated at 23°C for 24 h on a rotary shaker (300 rpm). The inoculum was transferred aseptically into a 14-L LKB fermentor containing 10 L of medium. The fermentation was carried out at 23°C under agitation at 300 rpm and 0.5 vvm aeration for 24 h. After fermentation, the cells were collected by centrifugation using a tubular Sharples centrifuge (10,000*g*). Because the penicillin acylase is an intracellular enzyme, cell disruptions was carried out in a bead mill (Dynomill) to collect the enzyme. The harvested cells were suspended in 50 m*M* sodium phosphate buffer, pH 7.0 (harvesting buffer), to make a 6% slurry. The slurry was then introduced in a continuous mode into the 0.6-L chamber of bead meal under cryogenic conditions (–18°C) to disrupt the cells. The disrupted material was centrifuged at 10,000*g* for 15 min, and the supernatant was concentrated (5X) by ultrafiltration using an M_r 10,000 cutoff membrane (Millipore, Bedford, MA) and treated as crude enzyme.

Enzyme Assay

Penicillin acylase assay was carried out using 3.72% penicillin G K^+ salt (Hindustan Antibiotics, Pimpri, India) as the substrate in 0.1 *M* phosphate

buffer, pH 7.0. To the mixture of the 0.2-mL sample containing enzyme and 0.8 mL of 0.1 *M* phosphate buffer, pH 7.0, 1 mL of substrate was added and then incubated at 40°C for 1 h. The reaction was terminated by pipetting 0.2 mL of the reaction mixture into 3.8 mL of citrate-phosphate buffer, pH 2.5. The enzymatic activity was estimated by determining the amount of 6-APA formed using *p*-dimethylaminobenzaldehyde reagent *(10)*. The concentration of protein was estimated using Folin phenol reagent *(11)*. The specific activity of the enzyme was expressed as micromoles of 6-APA formed per minute per milligram of protein at pH 7.0 and 40°C (units/milligram).

Adsorption Study

The hydrophobic ligands comprised of a butyl, hexyl, octyl, decyl, and phenyl immobilized on 4% (4XL) and 6% (6XL) crosslinked agarose by a neutral and highly stable epoxide linkage were obtained from Affinity Chromatography (Freeport, Ballasalla, Isle of Man, British Isles [UK]). The concentration of ligand for 4XL and 6XL gel was about 35–40 and 50–60 μmol/g of moist gel, respectively. Experiments were conducted at 22 ± 2°C. Each column (20 × 8 mm) was equilibrated with an appropriate $(NH_4)_2SO_4$ (salt) concentration (1, 1.5, and 2 *M*) prepared in harvesting buffer. Adsorption was carried out by applying onto each column 2 mL of crude enzyme solution (6.0 mg/mL) with the appropriate salt concentration. Prior to the application, the enzyme solution was diluted by mixing with an equal volume of 2, 3, and 4 *M* salt prepared in harvesting buffer to achieve the appropriate salt concentration. After washing with 5 mL of harvesting buffer containing the appropriate salt concentration, the elution was carried out with 5 mL of harvesting buffer containing no salt. Prior to the next run, the columns were washed with 1 *N* NaOH and equilibrated with harvesting buffer containing appropriate salt concentrations.

Stepwise Elution Study

All the 4XL gels were equilibrated with 2 *M* salt prepared in harvesting buffer. Adsorption was carried out as described in the previous section using a 2 *M* salt concentration. After adsorption, all the columns were washed with 5 mL of harvesting buffer containing a 2 *M* salt concentration. After washing, the elution was carried out sequentially with 5 mL of harvesting buffer containing 1.5, 1, 0.5, and 0 *M* salt concentration. The enzyme activity and protein were estimated in collected samples. The gradient elution study was carried out using octyl 4XL gel. Elution of the adsorbed enzyme was carried out at a flow rate of 0.3 mL/min using a linearly decreasing salt gradient ($2 \rightarrow 0$ *M*) prepared in harvesting buffer. The 31 fractions (2.0 mL each) were collected and the enzyme activity and protein were estimated.

Reactor Study

The reactor was prepared by adsorbing crude enzyme on octyl gel as reported earlier *(12)*. The crude enzyme solution was diluted 1:1 with a 3 *M* salt concentration prepared in harvesting buffer, and 2 mL (6.0 mg/mL) was applied on octyl 4XL gel (20 × 8 mm) and treated as a column reactor. Before the application of enzyme, the reactor was equilibrated with harvesting buffer containing 1.5 *M* salt. The operational stability of the reactor was studied over a period of 30 d. The reactor was used in a batch mode on the randomly selected days. After washing with 10 mL of harvesting buffer containing 1.5 *M* salt, the substrate (feed) was fed at a flow rate of 0.2 mL/min. Feed was prepared by dissolving penicillin G K^+ salt (3.72%) in harvesting buffer containing 1.5 *M* salt. The reaction was carried out at room temperature (28± 2°C). Every day, a total of nine samples of 2.0 mL volume were collected. The first sample was rejected, and the concentration of 6-APA was estimated in the remaining eight samples using feed as a blank. The average value of the eight samples was taken for the calculation of substrate conversion. After collection of the nine samples, the reactor was washed with 10 mL of harvesting buffer containing 1.5 *M* salt and stored at room temperature (28± 2°C) until the next run. Prior to the next study, immobilized enzyme was removed by washing the reactor with harvesting buffer containing no salt. The enzyme reactor was prepared as already described to carry out the residence time study. The feed was applied at a flow rate of 0.2 and 0.1 mL/min. Samples were collected as already described and the concentration of 6-APA was estimated. The 30 and 40°C temperature experiments were carried out by keeping the feed in a fixed-temperature water bath.

Results and Discussion

The variables such as ligand hydrophobicity and salt concentration were used to select the most suitable ligand for single-step purification. Table 1 shows the adsorption and purification pattern of penicillin acylase. For a 1 *M* salt concentration, only phenyl 4XL and 6XL showed 100% adsorption. The 100% adsorption on both the phenyl gels at a relatively lower salt concentration suggested the presence of other adsorption effects. Generally, aromatic ligands behave differently owing to the presence of double bonds *(13)*. For a 1.5 *M* salt concentration, all the gels showed 100% adsorption except octyl and decyl 6XL gels. For a 2.0 *M* salt concentration, all the gels showed 100% adsorption. The overall increase in specific activity and recovery was observed with the increase in salt concentration. However, an overall increase in specific activity and recovery was relatively higher for 4XL gels than 6XL gels. This indicated the significant role of ligand concentration, because 6XL gels have ~45% higher ligand concentration than the 4XL gels. The higher ligand concentration of 6XL gels may have led to the irreversible binding of the enzyme, which resulted in low specific activity and recovery. In conclusion, the salt and ligand concentrations are impor-

Table 1
Adsorption and Purification Pattern
of Enzyme for Different Salt Concentrations and Ligands

	Salt concentration[a]								
	1.0 *M*			1.5 *M*			2.0 *M*		
Name of gel	AD	SA	RE	AD	SA	RE	AD	SA	RE
Butyl (4XL)	91.0	29.2	44.0	100	34.4	52.0	100	42.4	64.2
Hexyl (4XL)	66.3	30.2	45.7	100	34.2	51.8	100	47.8	72.3
Octyl (4XL)	74.5	31.0	46.9	100	34.0	51.5	100	46.9	71.0
Decyl (4XL)	83.3	31.2	47.2	100	34.0	51.3	100	47.6	72.0
Phenyl (4XL)	100	27.2	41.2	100	28.0	42.3	100	41.6	63.0
Butyl (6XL)	96.7	16.2	24.4	100	20.0	30.3	100	30.7	46.5
Hexyl (6XL)	97.3	28.5	43.0	100	28.8	43.6	100	41.2	62.4
Octyl (6XL)	81.2	19.2	29.0	87.0	28.7	43.4	100	38.0	57.4
Decyl (6XL)	73.7	19.0	29.0	78.0	28.3	42.8	100	39.34	59.5
Phenyl (6XL)	100	26.6	40.3	100	26.3	39.7	100	38.59	58.4

[a]AD, % adsorption (feed – wash × 100/feed); SA, specific activity (U/mg); RE, % recovery.

Table 2
Stepwise Elution Pattern of Enzyme at Different Salt Concentrations

	Salt concentration[a]								
	1.0 *M*			0.5 *M*			0.0 *M*		
Name of gel	SA	PF	RE	SA	PF	RE	SA	PF	RE
Butyl (4XL)	NA	NA	NA	38.1	2.6	73	7.5	0.51	2.0
Hexyl (4XL)	91.6	6.3	23	40.8	2.8	56	9.3	0.63	1.7
Octyl (4XL)	166.5	11.4	30	44.0	3.0	53	11.4	0.78	3.7
Decyl (4XL)	157.5	10.8	14	38.8	2.6	57	19.5	1.34	7.0
Phenyl (4XL)	NA	NA	NA	15.1	1.0	2.7	28.0	1.92	63.0

[a]SA, specific activity (U/mg); PF, purification fold; RE, % recovery; NA, no penicillin acylase activity.

tant factors for purification of penicillin acylase using hydrophobic ligands. Because the overall specific activity and recovery was higher for the 4XL gels, they were selected for further study.

Table 2 shows the stepwise elution pattern of penicillin acylase for different salt concentrations. No elution of penicillin acylase was obtained for 2 and 1.5 *M* salt concentrations. Splitting of the elution pattern of penicillin acylase was observed for 1, 0.5, and 0 *M* salt concentrations. This indicated the variations in adsorption pattern of the enzyme. The elution pattern of penicillin acylase obtained for a 1 *M* concentration suggested that the enzyme loosely adsorbed on the surface of the gel was eluted first. Since the concentration of the eluted protein was less, the specific activity was higher. The variations in the specific activity obtained from all the 4XL

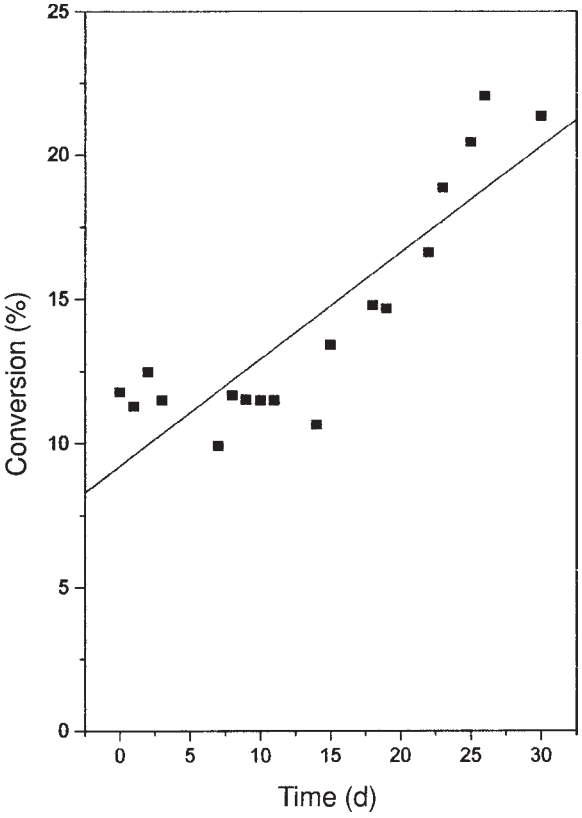

Fig. 1. Operational stability profile of reactor.

gels indicated the significant role of salt concentration in elution. In conclusion, the higher purification of the enzyme can be obtained by optimizing salt and ligand concentration as well as ligand hydrophobicity. Because higher specific activity of penicillin acylase was obtained from the octyl gel, it was selected for further study. It appears that some hydrophobic groups are likely to be present on the surface of the enzyme because the higher purification of penicillin acylase was obtained from a moderately hydrophobic ligand such as octyl.

The gradient elution study was carried out to improve the overall recovery of the enzyme. Higher penicillin acylase activity was obtained in three fractions, which were pooled and the activity was estimated. The pooled sample showed 62% recovery, 42.3 U/mg of specific activity, and 2.9-fold purification. By contrast, 36.8 U/mg of specific activity was the highest value reported so far for the enzyme purified from *E. coli* in which three purification steps were used *(14)*. Although there was an increase in the recovery as compared to the stepwise elution study, the specific activity was not significantly improved. However, the overall results indicated that the octyl 4XL gel can be used efficiently for a single-step purification of penicillin acylase.

Table 3
Effect of Residence Time on Substrate Conversion

Temperature (°C)[a]	Flow rate (mL/min)	Residence time (h)[b]	Conversion (%)
30	0.2	0.08	14
40	0.1	0.16	21

[a]Feed was maintained at respective temperature.
[b]Reactor volume/flow rate.

The possibility of using adsorbed enzyme on hydrophobic gel as an immobilized enzyme was studied. The column in which penicillin acylase was adsorbed on the octyl (4XL) gel was treated as an enzyme reactor. The study of operational stability was carried out because it is an important parameter to check the practical utility of the reactor. Significant operational stability of the reactor was observed over a period of 30 d at room temperature (Fig. 1). This suggests that the enzyme immobilized by adsorption on hydrophobic gel can be used efficiently in an enzyme reactor. However, the upward trend observed in percentage conversion appears to be an unusual behavior (Fig. 1). Generally, the immobilized enzyme loses activity as the number of operations increases. This may be attributed to the gradual removal of inhibitors present in the reactor as the crude enzyme was used for immobilization, because after each run the reactor was washed prior to the storage. It was observed that when the reactor stored without washing, it loses ~70% activity within a 24-h period. In general, the operational parameters such as residence time and temperature can significantly influence the reactor performance. To check this, the substrate conversion was studied at a higher residence time and temperature. Table 3 shows that the reactor responded significantly to the change in residence time and temperature. However, the overall substrate conversion was low. This can be improved by optimizing operational parameters and using highly active enzyme for immobilization. In conclusion, hydrophobic ligands can be used significantly for single-step purification as well as immobilization of enzyme.

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