0273-2289/01/95/0083/$12.50

Simultaneous Purification and Reversible Immobilization of D-Amino Acid Oxidase from *Trigonopsis variabilis* on a Hydrophobic Support

Stanislaus F. D'Souza* and Ashwini Deshpande

Nuclear Agriculture and Biotechnology Division,
Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India,
E-mail: enztech@magnum.barc.ernet.in

Received September 8, 1998; Revised December 14, 1999;
Accepted December 16, 1999

Abstract

Purification and reversible immobilization of D-amino acid oxidase from *Trigonopsis variabilis* could be simultaneously accomplished by hydrophobic interaction on Phenyl Sepharose CL-4B in the presence of 50 m*M* pyrophosphate buffer (pH 8.5). The presence of a high salt concentration of 2 *M*, which is generally required for the hydrophobic interactions, was not essential for the hydrophobic immobilization. The enzyme in free as well as immobilized form was optimally active between pH 7.0 and 9.0. The immobilized preparation could be reused in a batch process for the conversion of D-amino acids to α-keto acids. When the activity of the preparation dropped below practical limits, the gel could be regenerated by water wash and recharged with fresh crude extract from yeast.

Index Entries: Immobilization; *Trigonopsis variabilis*; hydrophobic support; Phenyl Sepharose CL-4B; reversible immobilization; α-keto acids; D-amino acid oxidase.

Introduction

The preparation of α-keto acids is gaining importance in view of their therapeutic applications in the treatment of chronic uremia *(1)*. Enzymatic methods investigated for the preparation of α-keto acids include the use of

*Author to whom all correspondence and reprint requests should be addressed.

either L- or D-amino acid oxidases *(2,3)*. The use of D-amino acid oxidase (D-amino acid:oxygen oxidoreductase [deaminating] EC 1.4.3.3.) has the advantage of simultaneous separation of the natural L-isomer from DL-racemates along with the formation of keto acids. Various D-amino acid oxidases have been investigated for this purpose. Of the several microorganisms screened, the yeast *Trigonopsis variabilis* has been found to be one of the potent sources of D-amino acid oxidase, with a broad substrate specificity to deaminate most of the D-amino acids *(4–8)*. Earlier studies from our laboratory and others have shown the feasibility of immobilizing the enzyme by entrapment of whole cells in Ca-alginate *(5)*, polyacrylamide *(9)*, and radiation-polymerized gelatin *(10)*. Large-scale application of immobilized D-amino acid oxidase has been hampered, mainly owing to its very short operational half-life, which ranges from a few hours to about 5 to 9 d *(5,9,11)*, thus making the classical immobilization techniques not too attractive for its continuous use. In view of this, attempts were made toward developing a reversible immobilization technique.

D-Amino acid oxidase was found to be adsorbed on hydrophobic supports such as Phenyl Sepharose, and this property was used earlier in our laboratory in developing a rapid method for the purification of the enzyme from crude extracts of *T. variabilis (12)*. This observation could be exploited to yield an immobilized preparation by operation under noneluting conditions favoring the hydrophobic interaction. On the loss of activity, the enzyme immobilized by this method could be removed and the system could be recharged as desired for full activity. Such attempts on simultaneous purification and reversible immobilization of D-amino acid oxidase on hydrophobic Phenyl Sepharose are discussed in this article.

Materials and Methods

Materials

Phenyl Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden). DL-Methionine was obtained from Sigma (St. Louis, MO). The strain of *T. variabilis* NCIM 3344 was obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory (Pune, India). Other chemicals of analytical reagent grade were obtained from standard sources.

Methods

Culture Conditions and Induction of Enzyme

T. variabilis was maintained by a biweekly transfer on agar slants containing 0.2% malt extract, 1% glucose, 0.3% yeast extract, and 0.5% peptone (pH 6.4–6.8), in 2% agar. For the induction of D-amino acid oxidase, they were grown aerobically for 3 d in a liquid medium containing 0.3% DL-methionine *(13)*. The cells were harvested by centrifugation at 8000*g* for 5 min and washed twice with cold isotonic saline.

Preparation of Cell-Free Extract

Yeast cells (20 g) were suspended in 10 mL of cold distilled water and mixed with 80 g of glass beads (150–200 µ, Type III; Sigma), in a mortar. Liquid nitrogen was added to the suspension and the frozen cell paste was thawed by thoroughly grinding with a pestle. The process was repeated twice, the cell lysate was centrifuged, and the precipitate was extracted twice with 50 mL of cold 0.2% Tween-80. The cell lysate and the washings were pooled.

Simultaneous Purification and Immobilization of D-Amino Acid Oxidase on Phenyl Sepharose CL-4B

The crude extract was fractionated using solid ammonium sulfate. The fractions obtained between 0 and 60% saturation were dissolved in 50 m*M* pyrophosphate buffer (pH 8.5) with or without 2 *M* KCl, and 5 mL of this was added to 20 mL of Phenyl Sepharose (wet gel volume). The mixture was stirred gently on a "test tube rocker" at room temperature for 60 min. The supernatant was decanted and the gel was washed with 50 m*M* pyrophosphate buffer (pH 8.5) with or without 2 *M* KCl until the supernatant was free from proteins.

Desorption of Immobilized Enzyme

The bound D-amino acid oxidase was desorbed when required by washing the gel with distilled water until the supernatant was free of proteins. The gel could then be reloaded with fresh sample as described above for immobilization.

Regeneration of the Gel

The gel could be regenerated by washing with distilled water, followed by an equal volume of distilled ethanol, followed by two washes with equal volumes of *n*-butanol and ethanol, and finally with excess distilled water until free of alcohol.

Analytical Methods

D-Amino acid oxidase activity was determined using an oxygen electrode (Gilson). The reaction mixture (2 mL) contained 20 m*M* DL-methionine and 0.002% sodium azide (to inhibit catalase without affecting D-amino acid oxidase) in 50 m*M* sodium pyrophosphate buffer (pH 8.5) *(12)*. One unit of enzyme activity has been defined as micromoles of oxygen consumed per minute at 25°C. α-Ketoacids were estimated using dinitrophenylhydrazine reagent *(5)*. Protein was estimated by the method of Lowry et al. *(14)* using bovine serum albumin as the standard.

Results

Optimal Conditions for Immobilization of D-Amino Acid Oxidase on Phenyl Sepharose CL-4B

Effect of KCl on Enzyme Activity and Binding Stability

D-Amino acid oxidase from crude extracts of *T. variabilis* was found to bind to hydrophobic matrix in the presence of high salt concentration (2 *M* KCl),

Table 1
Immobilization of D-Amino Acid Oxidase
on Phenyl Sepharose

Sample	2 *M* KCl	Buffer
Total units added to gel	250	289
Units recovered		
Gel	137	126
Supernatant	17	11
Wash	28	39
Total units recovered	182	176

Immobilization and washing of the gels were done in the presence of 2 *M* KCl and in buffer (50 mm pyrophosphate buffer, pH 8.5), respectively. Different preparations were assayed in the absence of KCl. Other details are as stated in the text.

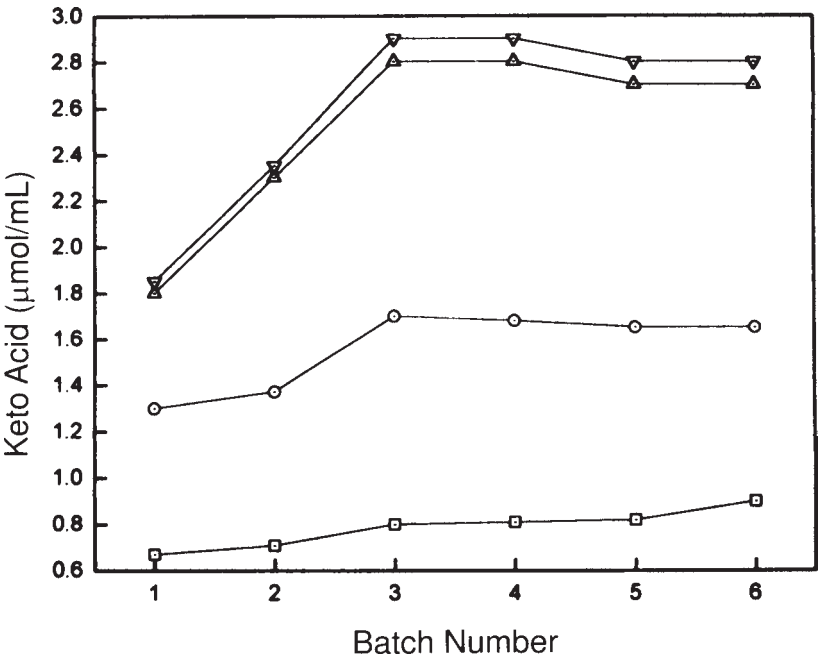

Fig. 1. Effect of KCl on the activity and reuse of D-amino acid oxidase immobilized on Phenyl Sepharose. (▽), 0 *M* KCl; (△), 0.2 *M* KCl; (○), 1.0 *M* KCl; (□), 2.0 *M* KCl.

and this property was used in the earlier studies for the purification of enzyme using Phenyl Sepharose CL-4B *(12)*. When bound enzyme was assayed in the presence of 2 *M* KCl, only 30% of the original activity could be detected. Also, the free soluble enzyme, when assayed in the presence of 2 *M* KCl, showed a retention of 50% of its original activity. Hence, the immobilization of D-amino acid oxidase was attempted in the presence as well as in the absence of 2 *M* KCl in order to ascertain the requirement of

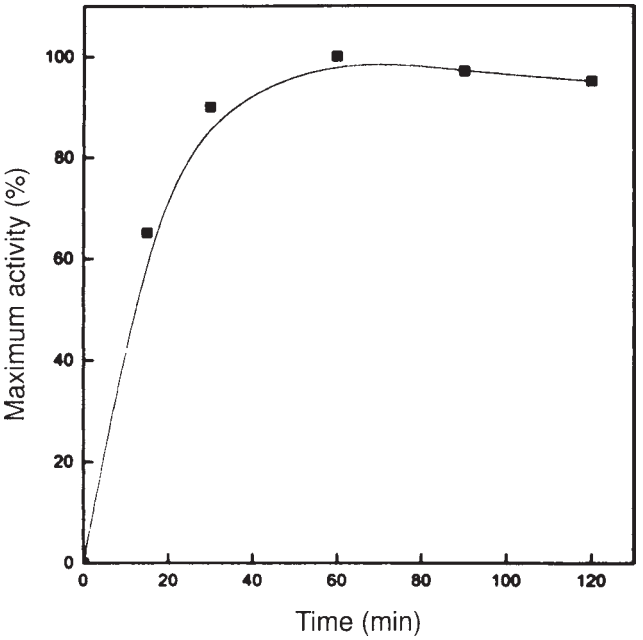

Fig. 2. Optimal contact time for hydrophobic immobilization of D-amino acid oxidase. Maximum activity obtained was taken as 100%.

salt for the hydrophobic immobilization. Ammonium sulfate precipitate (60%) in 50 m*M* pyrophosphate buffer with and without 2 *M* KCl was stirred with Phenyl Sepharose CL-4B at room temperature for 60 min. The gels were washed with buffer and assayed for D-amino acid oxidase activity using a substrate-buffer mixture without KCl. The results are presented in Table 1. It is apparent that 2 *M* KCl was not obligatory for binding of the enzyme. Sepharose CL-4B, which was used as a control, did not show any binding of the enzyme either in the presence of KCl or buffer, indicating the need for hydrophobic ligand. Also, the enzyme was not found to bind the Phenyl Sepharose CL-4B in the water in the absence of 50 m*M* buffer. Moreover, the bound enzyme could be eluted from the column by washing with a very low concentration (5 m*M*) of the buffer or only distilled water. The immobilized enzyme preparations were used for the conversion of amino acids to keto acids in repeated batches in the presence and absence of KCl. The enzyme was not found to leak in the absence of 2 *M* KCl (Fig. 1). Therefore, in all the further studies the immobilization was carried out in 50 m*M* pyrophosphate buffer using 60% ammonium sulfate precipitate of the crude extract. This process also resulted in a 10-fold purification of the enzyme.

Effect of Contact Time

The effect of contact time between enzyme and Phenyl Sepharose is shown in Fig. 2. A considerable amount of binding took place immediately

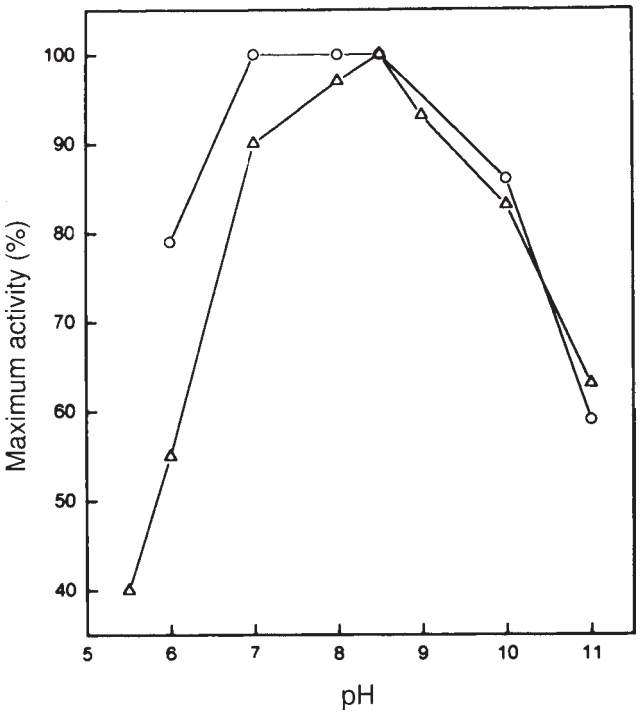

Fig. 3. The pH activity profile of Phenyl Sepharose immobilized D-amino acid oxidase. The buffers used were 50 m*M* phosphate in the range of 5.5–8.0 and 50 m*M* Tris-HCl in the range of 8.5–11. The activity obtained at optimum pH was taken as 100%. (△), soluble enzyme; (○), immobilized enzyme.

after contacting the enzyme with the hydrophobic gel. A contact time of 60 min was found to be optimal for maximal enzyme binding.

Properties of Immobilized D-Amino Acid Oxidase

The pH activity profile of Phenyl Sepharose immobilized D-amino acid oxidase is shown in Fig. 3. Both the preparations showed a pH optima of about 8.5. However, the pH activity profile of immobilized enzyme was broader toward the acidic side. The reason for this is not known. However, such changes in pH profile on immobilization have often been reported *(15)*. Optimum temperature and thermostability characteristics were not significantly altered on immobilization.

Operational Stability of Phenyl Sepharose Immobilized D-Amino Acid Oxidase

The enzyme-bound gel was stirred with 50 mL of 50 m*M* pyrophosphate buffer (pH 8.5) containing 20 m*M* DL-methionine for 5 h at room temperature. The keto acids formed in the supernatants were estimated after sedimenting the gel. The gel was then washed with the buffer and resuspended in a fresh batch of substrate-buffer mixture. One batch per day

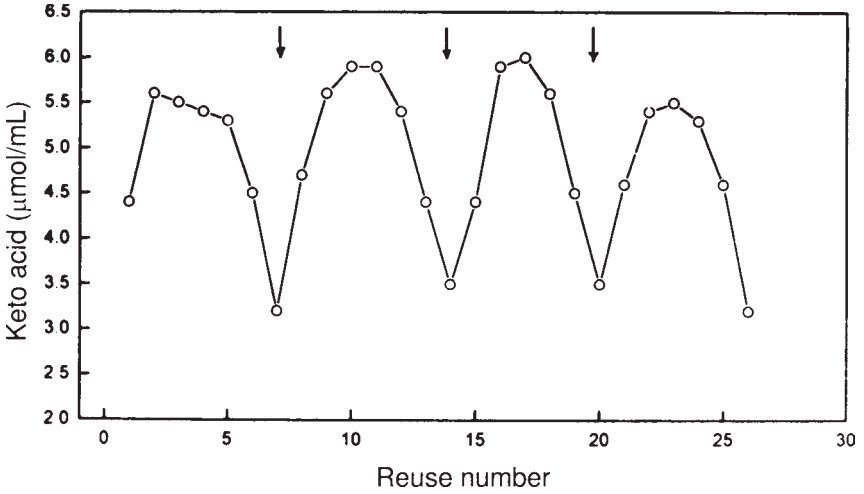

Fig. 4. Operational stability of Phenyl Sepharose immobilized D-amino acid oxidase. Experimental details are as stated in the text. Arrows indicate the recovery of the support by water wash and reloading with a fresh extract of yeast cells.

was carried out. When not in use, the enzyme preparations were stored at 0–4°C in buffer in the cold. The immobilized D-amino acid oxidase preparation could be reused in several batches for the preparation of keto acids. When the activity of the enzyme fell beyond practical limits, the support was recovered by elution of the bound protein by washing with distilled water. The gel was then loaded with a fresh batch of the crude enzyme (0–60% ammonium sulfate precipitate). This cycle was repeated several times, as shown in Fig. 4. After every six to seven cycles the support was regenerated using organic solvents as described in Materials and Methods.

Discussion

Enzymes and cells immobilized through adsorption offer several advantages over covalent binding *(15–18)*. The ease with which the reaction is carried out and the possibility of achieving its reversal for the reuse of the support are some of the appealing features. In the case of enzymes immobilized through ionic interactions, a dynamic equilibrium is normally observed between the adsorbed enzyme, which is often affected by pH, as well as the ionic strength of the surrounding medium. Enzymes have also been immobilized by biospecific affinity adsorption on metal-chelate supports *(19)*, antibody bound supports *(20)*, or immobilized lectins *(21)*. Even though all of these methods are amenable for reversible immobilization of enzymes, the versatility of the method is often limited by the high cost, and by the problems of immobilizing them without impairment of its catalytic activity or desorption during operational conditions.

Reversible immobilization of the enzymes is achieved by operation under noneluting conditions and in principle is reversible only on an

appropriate change in adsorbate environment. Immobilization by adsorption through hydrophobic interaction has gained considerable importance *(22–27)*. Depending on their biological location and function, proteins are known to contain nonpolar regions on the surface, which, when brought in contact with nonpolar residues attached to hydrophilic gels, form an adsorbate with good stability so that the protein can be considered to be practically immobilized. One of the important features of this technique, which is of great significance, is that unlike ionic binding, hydrophobic interactions are usually stabilized by high ionic concentrations. This enables the use of high substrate concentrations without desorption of the enzyme.

A number of enzymes have been immobilized on hydrophobic supports *(22–27)*. One of the main limitations observed so far has been the use of a high concentration of salt during immobilization and subsequent operation. High salt concentration is known to be detrimental to enzymes, including D-amino acid oxidase as observed in the present study, thus allowing for utilization of the enzyme at suboptimal activity. Moreover, the presence of salt in the final product enhances the problems of downstream processing and product recovery. The practical application of the hydrophobic immobilization technique will depend on the ability to operate in a salt-free or near salt-free solution. The present studies show that D-amino acid oxidase can be successfully immobilized on Phenyl Sepharose in 50 m*M* buffer solution in the absence of salt. The ability of this enzyme to bind to hydrophobic supports under low ionic concentration indicates that the enzyme is strongly hydrophobic. Our earlier studies have shown that a considerable amount of the enzyme activity in *T. variabilis* is membrane associated and could be solubilized only by extraction with Tween-80 *(12)*. In addition, the method gives a simple technique for the simultaneous purification and immobilization of the enzyme, thus obviating the cost- and labor-intensive process of purification normally involved in covalent binding of cell-free enzymes. Such an approach for the simultaneous isolation and immobilization of streptavidin-β-galactosidase has been reported recently by Huang et al. *(28)*.

Immobilization of enzymes on hydrophobic supports, even though used in view of regeneration of the support, is of special significance for enzymes which are otherwise very labile and require frequent regeneration of the support. Studies reported by Brodelius et al. *(5)*, and from our own laboratory *(9)*, have indicated that one of the major limitations in the practical application of D-amino acid oxidase for keto acid production has been the low operational stability which has been attributed to its low *in situ* stability. The technique developed in this paper may be a useful demonstration of a practical way of utilizing D-amino acid oxidase for keto acid production. Reversible immobilization can be useful in the application of labile enzymes *(29)*.

The exact reason for the enhancement of activity initially on repeated use is not clear. Similar observations have been made in the case of glucose isomerase immobilized on controlled pore alumina *(30)*, and glucose oxi-

dase immobilized on polyethylenimine-coated cotton cloth *(31)*, or in hen egg white foam *(32)*. It was explained as follows: initially, some of the enzyme active sites may be bound to the carrier or to adjacent enzyme molecules. Since the affinity of the enzyme is more for substrate than for either the carrier, or the adjacent enzyme molecules, those sites become available as the substrate diffuses in the vicinity. This was referred to as reversible activity loss *(30,31)*.

The high cost of the agarose gels constitutes a drawback for the technological utilization of enzymatically active agarose conjugates. However, preparation of cheaper hydrophobic supports, such as hydrophobic cloth have been reported *(33,34)*, which could be useful for commercial scale hydrophobic enzyme immobilization. Thus, the reversible immobilization of D-amino acid oxidase by hydrophobic interaction offers an attractive, simple, and reusable system with a potential for large-scale application.

Acknowledgment

A. Deshpande was a recipient of a DAE Senior Research Fellowship. We thank Dr. G. B. Nadkarni for his keen interest in this work and for useful discussions.

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