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Insolubilized D-Amino Acid Oxidase: Properties and Potential Use

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Abstract

D-Amino acid oxidase has been amide-linked to ω-aminoalkyl agarose particles. The insolubilized enzyme has characteristics similar to the soluble form with respect to stability, pH optimum, affinity for flavin adenine dinucleotide, and general substrate specificity, although smaller Michaelis constants were found for some substrates. The potential application for the separation of the natural L-amino acid isomers from racemates has been demonstrated.

INTRODUCTION

The effective use of biochemically specific absorbents is rapidly increasing. The basic principles of using insolubilized substrates, competitive inhibitors, and coenzymes were illustrated in the purification of flavokinase (*1*), flavin mononucleotide (FMN) dependent enzymes (*2*), and some other cases (*3*). More recently, the use of longer bridges between ligands and the solid matrices to which they are attached has allowed more frequent and effective binding of compounds to be isolated (*4*). Successful applications in the isolation of antigens, antibodies, nucleic acids, and other complex biological structures have also been reported (*5*). As a major extension of such solid-state biochemistry,

insoluble carriers have been used especially to bind enzymes, both for theoretical (*6*) and practical (*7*) purposes. In this report we describe the potential application of a carrier-bound enzyme, D-amino acid oxidase, which can specifically exhaust the unnatural, and usually undesired, D-isomer of amino acid racemates for the purpose of isolating the natural, desired, L-form.

The flavin adenine dinucleotide (FAD) dependent D-amino acid oxidase catalyzes the oxidation of D-amino acid with the formation of H_2O_2 and imino acid, which latter hydrolyzes spontaneously to the respective keto acid (*8*). Several important features of this enzyme make it excellent for the purpose intended. For example, this enzyme can be obtained commercially or through straightforward purification with good yield and stability (*9*). The high content of aspartyl and glutamyl residues (*10*) suggests the probability of coupling to solid matrices through amide linkages. Most importantly, this enzyme has a broad and absolute specificity for D-amino acids, with the exception of glycine, and is insensitive to most L-amino acids as inhibitors (*11*).

EXPERIMENTAL PROCEDURE

Electrophoretically-purified D-amino acid oxidase from Worthington, with a specific activity of 13 units* per mg protein, and Affinose 102A (agarose-$NH(CH_2)_3NH(CH_2)_3NH_2$) from Bio·Rad Laboratories, with approximately 10 μmoles of amino groups per ml of packed volume, were used. Prior to coupling, the Affinose was washed with 20 bed volumes each of 0.1 *M* NaCl (pH 10) and distilled water. Generally, 40 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 0.5 ml of water was slowly added to a 9-ml, screw-capped test tube containing a 3-ml water suspension of 20 mg (266 units) of oxidase and 2 ml bed volume of Affinose. The capped tube was rotated slowly at 2 to 4° for 2 or 4 days in the dark. The reaction mixture was then transferred to a centrifuge tube, washed 7 times by stirring for a few minutes with 10 ml of 0.05 *M* sodium pyrophosphate buffer (pH 8.3) at 0°, and decanting the supernatant after low-speed centrifugation. Finally, the washed agarose-bound enzyme was suspended in the same buffer containing 0.1 m*M* FAD and stored at 0°. The Lowry method (*12*) was used for protein quantitation and, with modification (*13*), for carrier-bound samples. Un-

* One unit of activity is defined as the uptake of 1 μmole of O_2, or the oxidation of 1 μmole of D-alanine, per minute at 38° and pH 8.3.

less designated otherwise, enzymic activity was determined at 38°, pH 8.3, in buffer containing 37 mM D-alanine, 2×10^{-5} M FAD, and an appropriate amount of free or bound enzyme, employing a Gilson oxygraph for the measurements of oxygen uptake. A final yield of 4.0 to 4.4 mg of bound enzyme, with specific activity of 2.0 to 2.5 units per mg protein, was obtained after either 2 or 4 days of treatment. No activity could be detected in the buffer washes after 4 washings. The agarose-bound enzyme was shown to be free from soluble oxidase, since the latter can be completely separated from the agarose particles by washing 3 times after the same treatment in the absence of carbodiimide.

RESULTS AND DISCUSSION

Some physical and catalytic properties of both free and bound enzymes were examined, and the results are summarized in Table 1. Similar stabilities were found when the enzymes were stored in 0.05 M buffer containing 0.1 mM FAD. Affinity for FAD, pH optimum, and specificity for amino acids as substrate or inhibitor were also found to be about the same. The bound enzyme has smaller apparent Michaelis constants (K_m) for the indicated substrates.

To examine the FAD-binding affinities, apoenzymes were prepared by

TABLE 1

Comparison of Some Physical and Catalytic Properties of Soluble and Agarose-Bound D-Amino Acid Oxidase[a]

Properties	Enzyme		°C	pH
	Soluble	Bound		
Half-life (days)	14	11	23	8.3
K_{dissoc} for FAD ($10^7 \times M$)	1	1	38	8.3
pH Optimum	9.3	9.3	23	7–11
Specificity for D-amino acids	+	+	23	8.5
Inhibition by L-leucine	+	+	23	8.5
K_m ($10^3 \times M$) for				
D-Alanine	1.5	0.8	38	8.3
D-Phenylglycine	3.0	0.9		

[a] Details of experimental conditions are described in the text.

the method of Massey and Curti (*14*) for the soluble enzyme and, with the following modification, for the bound form. The particles were washed 6 times in 30 bed volumes of 0.1 *M* pyrophosphate buffer (pH 8.5), containing 1 *M* KBr and 3 m*M* EDTA, by stirring gently for 30 to 60 min at 0° and decanting the supernatant after centrifugation. Another 6 washes were done with 0.05 *M* buffer (pH 8.3). The resultant preparation was catalytically inactive, but full recovery of activity could be obtained after the addition of excess FAD. Apparent dissociation constants for FAD were calculated (*15*) from the titration curves for the restoration of activity by FAD.

For the comparison of pH optima, 0.08 *M* buffers of phosphate-citrate, pyrophosphate, and triethylamine were used to maintain the pH at 7 to 8, 8 to 10, and 10 to 11, respectively, while maximal enzymic activity was detected (near pH 9.3 with both enzyme samples) using 37 m*M* D-alanine as substrate. Five D-amino acids (Ala, Val, Ile, Met, and Phenylgly) and 18 L-amino acids (Ala, Val, Leu, Ile, Ser, Thr, Asp, Glu, Asn, Met, Cys, His, Arg, Lys, Phe, Try, Trp, and Pro) were tested for substrate activity; the latter were also tested as inhibitors. Absolute specificity for D-amino acids was obtained with enzymes of both forms, and only L-leucine (10 m*M*) was found to inhibit, partially, both preparations using D-alanine (5 m*M*) as substrate. The apparent K_m for D-alanine and D-phenylglycine with the soluble and the bound enzyme were determined; smaller values were found with the latter. This interesting effect may be attributed either to a change in enzyme structure resultant from insolubilization or to a higher local concentration of substrate around the bound enzyme molecules created by the solid matrices.

Since the bound enzyme retains complete specificity for D-amino acid, is resistant to inhibition by most L-amino acids, and is suitable for fast separation from the reaction solution, it seems reasonable that the isolation of L-amino acids from their racemic mixtures can be easily achieved by using such an enzyme preparation. As a small-scale demonstration of this, 40 min incubation at 23° of a 1.7-ml suspension containing 10 μmoles of D-alanine, 3.4×10^{-8} moles of FAD, and 1.3 mg of agarose-bound enzyme in 0.05 *M* pyrophosphate buffer (pH 8.5) resulted in a complete loss of amino acid (by ninhydrin test) in the supernatant after centrifugation. When an additional 10 μmoles of L-alanine was also included, only half of the total amino acid was exhausted after a similar period of incubation. The formation of trace amounts of pyruvate from the D-alanine was detected with 2,4-di-

nitrophenylhydrazine in both cases, although H_2O_2 simultaneously generated further degradation of the keto acid product.

In conclusion, the use of bound D-amino acid oxidase for obtaining the natural L-amino acid isomers from chemical racemates has been demonstrated. The present use of agarose particles has some obvious advantageous features for quick laboratory-scale operations. In large-scale use, however, enzymes attached to inorganic materials have many advantages over those coupled to organic supports (*7*). The use of inorganic carriers, such as alkylamine glass particles (*7*), for coupling D-amino acid oxidase in the same manner, viz., using carbodiimides, also deserves elaboration.

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