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Properties of *Rhodotorula gracilis* D-Amino Acid Oxidase Immobilized on Magnetic Beads through His-Tag

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D-Amino acid oxidase catalyzes one of the key steps in the production of semisynthetic cephalosporins. We expressed and purified recombinant *Rhodotorula gracilis* D-amino acid oxidase with C-terminal his-tags. This engineered enzyme was immobilized onto Ni^{2+}-chelated nitrilotriacetic acid magnetic beads through the interaction between his-tag and Ni^{2+}. The kinetic constants, storage properties, and the reusability of the immobilized D-amino acid oxidase were determined. The effects of temperature, pH, and hydrogen peroxide on the activity of immobilized D-amino acid oxidase were also studied. The highest activity recovery was 75%. Thermal stability was improved after immobilization; the relative activity of the immobilized enzyme was 56% whereas the free enzyme was completely inactivated after incubation at 50°C for 1 h. In the presence of 10 mM hydrogen peroxide, the immobilized enzyme did not show a rapid loss of activity during the first 2 h of incubation, which was observed in the case of the free enzyme; the residual activity of the immobilized enzyme after 9 h was 72% compared with 22% of the free form. The long-term storage stability was improved; the residual activity of the immobilized enzyme was 74% compared with 20% of the free enzyme when stored at room temperature for 10 d. The immobilized form retained 37% of its initial activity after 20 consecutive reaction cycles.

[**Key words:** D-amino acid oxidase, his-tag, magnetic beads, *Rhodotorula gracilis*, thermal stability]

D-Amino acid oxidase (DAO, EC 1.4.3.3) is an FAD-dependent flavoenzyme, which catalyzes the oxidative deamination of D-amino acids to the corresponding α-keto acids, producing ammonia and hydrogen peroxide during the reaction (1). This enzyme can be found in a wide variety of eukaryotic organisms. For yeasts, the presence of DAO is related to the ability of using D-amino acids for growth (2). Most vertebrates have DAO in the kidney, liver, and brain. However, the physiological function of this enzyme is somewhat unclear for higher organisms because the D-amino acid substrates are considered rare. It has been proposed that the role of DAO in animals is to metabolize both exogenous and endogenous D-amino acids; the latter may have been accumulated during aging (1). In addition to metabolizing D-amino acids, DAO may play a specific role in the brain by regulating the D-serine level (1, 3).

The main industrial application of DAO is the enzymatic deamination of cephalosporin C to 7-(5-oxoadipoamido)cephalosporanic acid, which decarboxylates spontaneously to glutaryl-7-cephalosporanic acid in the presence of hydrogen peroxide. An additional enzymatic step catalyzed by an acylase converts glutaryl-7-cephalosporanic acid to 7-aminocephalosporanic acid, which is an important intermediate in producing semisynthetic cephalosporins. This conversion can also be carried out in the absence of hydrogen peroxide by coimmobilizing DAO with catalase (4). For antibiotics production, DAO from *Rhodotorula gracilis* (*Rg*DAO, *R. gracilis* is also known as *Rhodosporidium toruloides*) and that from *Trigonopsis variabilis* (*Tv*DAO) are the main enzymes used owing to their high turnover rates and stable FAD binding (1, 5). DAO has also been applied to the production of α-keto acids when coupled to catalase (6); these α-keto acids are used as food additives for people with chronic uremia. Other applications of DAO include the enantiomeric separation of amino acids and biosensors for the detection of D-amino acids (7, 8).

One major obstacle using DAO for industrial purposes is its low stability (9). The main mechanism of thermal inactivation of *Rg*DAO is subunit dissociation; the thermal inactivation of *Tv*DAO also involves the dissociation of FAD because *Tv*DAO has a lower affinity towards FAD (K_d between 10^{-6} and 10^{-7} M) than *Rg*DAO ($K_d = 2 \times 10^{-8}$ M) (9, 10). The hydrogen peroxide released during the reaction can inactivate DAO through chemical modification of certain residues (11, 12). Many techniques for immobilizing DAO have been explored owing to its important industrial applications. Mateo *et al.* immobilized *Rg*DAO on polyethylenimine-activated support through ionic adsorption (13). In addition to the physical approach, various chemical methods have been reported and improvements in stability against pH or thermal or hydrogen peroxide inactivation were observed (5, 11, 14, 15).

Compared with physical and chemical immobilization methods, the utilization of affinity tags for DAO immobilization is less developed. Affinity tags such as his-tag, mal-

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tose binding protein, chitin binding domain, and glutathione *S*-transferase are often introduced recombinantly to facilitate protein purification (16). These tags can also be applied to protein immobilization for catalysis, biosensing, expression profiling, and many other purposes. His-tag has been the most widely used affinity tag and the purification or immobilization of his-tagged proteins often requires chelated Ni^{2+} or Co^{2+} as affinity ligand (16). Proteins can also bind to these metal-chelated supports through innate histidine, cysteine or tyrosine residues (17, 18), or through phosphate groups (19). Although his-tag has been applied to the purification of *Rg*DAO and *Tv*DAO, conventional chemical methods were used in subsequent immobilization (5, 20, 21). To the best of our knowledge, Dib *et al.* reported the only successful DAO immobilization using a twelve-amino acid affinity tag called *Strep*-Tag II (22). They fused *Strep*-Tag II to the N-terminus of *Tv*DAO and immobilized this chimeric enzyme on commercial *Strep*-Tactin MacroPrep particles through the bonding between the *Strep*-Tag II and streptavidin.

In this work, we expressed and purified his-tagged *Rg*DAO then immobilized this engineered enzyme onto Ni^{2+}-chelated nitrilotriacetic acid (Ni-NTA) magnetic agarose beads. The main advantage of magnetic beads is the ease of separation. The porous nature of agarose may also prevent enzyme inactivation at gas-liquid interfaces (23). *Rg*DAO is a homodimeric holoenzyme; the subunit is approximately 40 kDa and each contains a noncovalently bound FAD (1). Every his-tagged *Rg*DAO is expected to contain two tags. The kinetic constants, storage properties, and the reusability of the immobilized enzyme were determined. The effects of temperature, pH, and hydrogen peroxide on the activity of the immobilized enzyme were also determined.

MATERIALS AND METHODS

Expression and purification of recombinant *Rg*DAO The recombinant plasmid, pDAO-23, constructed by Liao *et al.* (24), was transformed into *Escherichia coli* BL21(DE3) and used for the expression of *Rg*DAO. Transformed *E. coli* cells were grown in terrific broth (TB) supplemented with 100 μg/ml ampicillin at 37°C with rotary shaking. The DAO expression was induced with the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the cell optical density (OD_{600}) reached 2.0. After further cultivation for 30 h, the culture was harvested by centrifugation at 6000×*g* for 10 min. The bacterial cells were then resuspended and disrupted in BugBuster protein extraction reagent (Novagen, Madison, WI, USA). The expressed recombinant *Rg*DAO was subject to purification by metal chelation chromatography using a His-bind column (Novagen). The purified *Rg*DAO concentration was determined using Bradford's assay (Sigma, St. Louis, MO, USA) and the purity was examined by SDS–PAGE.

DAO activity assay by hydrogen peroxide production DAO activity was determined spectrophotometrically by measuring the absorbance increase accompanying the oxidation of *o*-phenylenediamine. The peroxidase-catalyzed oxidation utilized the hydrogen peroxide released from the DAO reaction, and *o*-phenylenediamine was converted to the orange-brown 2,3-diaminophenazine. A 1 ml assay mixture contained 30 mM D-alanine, 0.03% (w/v) *o*-phenylenediamine, 5 U/ml horseradish peroxidase (Sigma), and an appropriate amount of DAO in 100 mM potassium phosphate buffer, pH 8.0. The reaction was initiated by the addition of DAO and the absorbance increase was monitored at 453 nm for 1 min using a JASCO V-550 spectrophotometer (Jasco, Tokyo). One activity unit was defined as the production of 1 μmole of hydrogen peroxide per min at 25°C.

DAO activity assay by pyruvate production To study the effect of hydrogen peroxide, a different activity assay was used because the previously described method relied on the production of hydrogen peroxide. An appropriate amount of DAO was incubated with 30 mM D-alanine and 4 U/μl catalase (Sigma) for 5 min in 100 μl of 100 mM potassium phosphate buffer, pH 8.0. Forty microliters of 1 mg/ml 2,4-dinitrophenylhydrazine prepared in 2 M HCl was added and incubated for 3 min. The reaction was quenched by incubation with 140 μl of 3 M NaOH and 1 ml of deionized water for an additional 5 min. The production of pyruvate-2,4-dinitrophenylhydrazone was determined by measuring A_{550} with a JASCO V-550 spectrophotometer and the amount of pyruvate produced was determined using a calibration curve. One activity unit was defined as the production of 1 μmole of pyruvate per min at 25°C.

***Rg*DAO immobilization** Thirty microliters of his-tagged *Rg*DAO was incubated with 60 μl of 5% (v/v) suspension of Ni-NTA magnetic beads (Qiagen, Hilden, Germany) for 15 min with rotary mixing at 25°C. The average diameter of the beads was 50 μm, ranging from 20 to 70 μm. The magnetic core was covered with porous 3% cross-linked agarose. The beads were separated from the solution by a magnet separator and the pellet was washed three times with 600 μl of 100 mM potassium phosphate buffer, pH 6.8, followed by resuspension in 60 μl of the same buffer. The residual DAO in the supernatant was determined using a DAO activity standard curve.

RESULTS AND DISCUSSION

The effects of *Rg*DAO concentration on immobilization are shown in Fig. 1. The activity increased as *Rg*DAO concentration increased and started to saturate above 1 mg/ml. However, the amount of immobilized *Rg*DAO did not saturate at the concentration range tested. Such difference could be explained by the porous nature of the support. When the *Rg*DAO concentration increased, the enzyme molecules started to occupy the interior of the beads, where the mass transfer

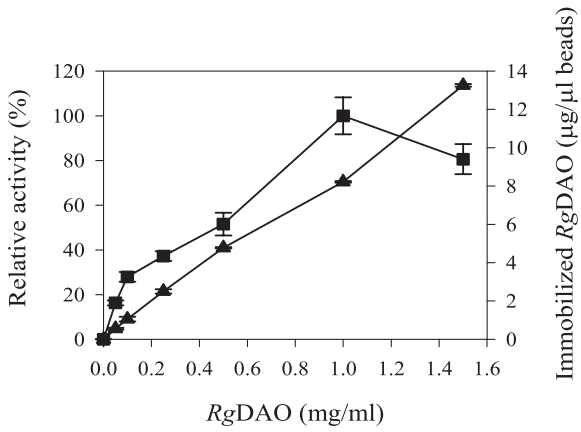

FIG. 1. Effects of his-tagged *Rg*DAO concentration on immobilization. Squares, Relative activity of immobilized *Rg*DAO; triangles, amount of immobilized *Rg*DAO. The amount of immobilized enzyme was determined from the activity change in the supernatant using a DAO standard curve.