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Immobilization of glycolate oxidase from *Medicago falcata* on magnetic nanoparticles for application in biosynthesis of glyoxylic acid

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ABSTRACT

Glycolate oxidase was isolated from *Medicago falcata* Linn. after a screening from 13 kinds of C_3 plant leaves, with higher specific activity than the enzyme from spinach. The *M. falcata* glycolate oxidase (MFGO) was partially purified and then immobilized onto hydrothermally synthesized magnetic nanoparticles via physical adsorption. The magnetic nanoparticles were characterized with scanning electron microscope (SEM), transmission electron microscopy (TEM) and Fourier transform infrared (FT-IR) spectroscopy. The maximum load of MFGO was 56 mg/g support and the activity recovery was 45%. Immobilization of MFGO onto magnetic nanoparticles enhanced the enzyme stability, and the optimum temperature was significantly increased from 15 °C to 30 °C. The immobilized biocatalyst was successfully used in a batch reactor for repeated oxidization of glycolic acid to synthesize glyoxylic acid, retaining *ca.* 70% of its initial activity after 4 cycles of reaction at 30 °C for nearly 70 h, and its half-life was calculated to be 117 h.

1. Introduction

Glycolate oxidase (GO, EC. 1.1.3.15) is a peroxisomal enzyme which catalyzes the oxidation of α-hydroxy acid. It widely exists in the green parts of high plants to catalyze the second reaction of the photorespiratory pathway, *i.e.*, the oxidation of glycolate to yield glyoxylate and H_2O_2 [1–3]. GOs also exist in many animals' liver to form oxalate by converting glycolate to glyoxylate, the precursor of oxalate [4,5]. Among various GO sources, the spinach GO has been purified and characterized, and its crystal structure has also been dissolved correctly [6–8].

The application of spinach GO in the biocatalytic production of glyoxylic acid was intensively studied [9–11]. By using soluble spinach glycolate oxidase [9] or engineeried recombinant microbial whole cells [10] as biocatalysts, glycolic acid of 0.25–1.5 M was transformed into glyoxylic acid with high selectivity (>98%) at greater than 99% conversion. The enzymatic oxidation of glycolic acid was accompanied with the formation of by-product H_2O_2. The glyoxylic acid formed would be decomposed by H_2O_2 into formic acid and carbon dioxide or be further oxidized into oxalic acid by GO and oxygen. The by-product H_2O_2 could be decomposed by catalase and the further oxidation of glyoxylic acid could be prevented by the addition of ethylenediamine (EDA), which could react with glyoxylic acid to form *N*-substituted hemiaminal or imine [9]. The application of microbial GO for the production of glyoxylic acid was also reported [11], yielding 0.95 M glyoxylic acid after 15 days of transformation.

Soluble GO was unstable in aqueous medium and could not be reused, therefore the immobilization of GO was investigated. The spinach GO had been immobilized onto various supports, including oxirane acrylic beads [12,13] and silica sol–gel [14]. In the work of Anton et al. [12,13], the spinach GO was coimmobilized with catalase on Eupergit® C, an oxirane acrylic beads, resulting in an increased stability and prolonged operation time of the enzyme, but the activity recovery was only 22%, which was relatively low. The spinach GO was also immobilized on silica sol–gel [14], but a 70% activity loss was observed unless the GO was complexed with poly(ethyleneimine) prior to immobilization. So it was important to find an ideal support for the immobilization of GO with high activity recovery and stability.

Recently the researches on magnetic nanoparticles are of great interest [15–19] and the use of magnetic beads for immobilizing enzymes has been widely investigated [20–24]. Magnetic bead is used as a carrier of biomolecules because of the following advantaged properties [25]: (1) its diameter is much smaller than other supports, such as Eupergit® C, which can cause the perfect dispersal and great delivery of substrate molecules; (2) its high specific surface area can greatly enhance the protein adsorption capacity; and (3) it can be separated easily from the reaction mixture by applying a magnetic field. The advantage in recovery of enzyme from reaction system can also meet the requirements of industrial application. A variety of methods have been employed for immobilizing enzyme onto magnetic beads, such as cross-linking [26] and covalent attachment [27], however none of these reports has ever mentioned the

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immobilization of enzyme onto magnetic beads by simple physical adsorption.

Till now, no information was available regarding the immobilization of GO onto magnetic nanoparticles. In this work, the glycolate oxidation activity was examined among C_3 plants, which had been well known of possessing more GOs than C_4 plants [28], and a facile way was developed to immobilize the GO from *Medicago falcata* Linn. (abbreviated as MFGO) onto magnetic nanoparticles via a simple and mild way of physical adsorption. The reuse of the immobilized MFGO in the biotransformation of glyoxylic acid was investigated.

2. Experimental

2.1. Materials

Fresh plants were bought from market nearby. Bovine serum albumin (BSA) was purchased from Sigma–Aldrich Chemical Co. Ltd. Glycolic acid (70%, w/v), glyoxylic acid (50%, w/v) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) were purchased from Alfa Aesar. Flavin mononucleotide (FMN) was obtained from AppliChem GmbH. Phenylhydrazine hydrochloride and all other chemicals were supplied by Sinopharm Chemical Reagent Co. Ltd. Ethylenediamine (EDA) was obtained from Shanghai Lingfeng Chemical Reagent Co. Ltd.

2.2. Enzyme screening among C_3 plants

Different kinds of C_3 plants were selected as the source of glycolate oxidase. The fresh leaves (5 g) of these plants were triturated with 7 ml of potassium phosphate buffer (100 mM, pH 8.0) at 4 °C, and then centrifuged at 1000 × *g* for 5 min. The cell free extracts were collected, and the total amount of protein and the glycolate oxidation activity were measured.

2.3. Partial purification of glycolate oxidase from M. falcata Linn. and catalase from Baker's yeast

Glycolate oxidase from *M. falcata* Linn. (MFGO) was partially purified using selective ammonium fractionation. Fresh leaves (100 g) were chopped into fine particles using a tissue disruptor in 140 ml phosphate buffer (100 mM, pH 8.0). The liquid fraction was filtrated by 4 layers of gauze, then centrifuged at 6000 × *g* for 8 min, and the supernatant (*approx.* 0.2 l) was collected for ammonium fractionation.

Solid ammonium sulfate (9.35 g) was added into the cell free extract slowly. After all the ammonium sulfate was dissolved, the resulting precipitate was removed by centrifuge at 17,000 × *g* for 8 min, the pellet was discarded and then another 9.35 g solid ammonium sulfate was added into the supernatant (*approx.* 0.17 l) as before. All the steps above were carried out at 4 °C. The pellet was collected by centrifuge, lyophilized and stored at 4 °C until use.

Fresh cells of Baker's yeast were suspended in Tris–HCl buffer (20 mM, pH 7.5) and disrupted by high-pressure homogenizer AH110B (ATS Engineering Inc., Italy). The liquid fraction was centrifuged at 20,000 × *g* for 8 min and then the supernatant was collected for ammonium fractionation. The ammonium sulfate saturation was ranged from 40% to 60%, and the pellet was collected by centrifugation. All the steps above were carried out at 4 °C. The resultant pellet was dissolved in a small quantity of Tris–HCl buffer and dialyzed against the same buffer. After being lyophilized, the catalase was stored at 4 °C until use.

2.4. Preparation of amine-functionalized magnetic nanoparticles

The amine-functionalized magnetic nanoparticles used in the immobilization of enzyme were prepared by hydrothermal synthesis [29]. In brief, a solution consisted of 1,6-hexanediamine (3.6 g), anhydrous sodium acetate (4.0 g) and $FeCl_3{\cdot}6H_2O$ (1.0 g) as a ferric source in glycol (30 ml) was stirred to form a transparent solution, then transferred into a Teflon-lined autoclave and reacted at 200 °C for 6 h. The particles formed were rinsed with hot water and ethanol in turn to remove the solvent and unbound 1,6-hexanediamine, and then dried at 50 °C. The amine-functionalized magnetic nanoparticles were characterized by Fourier transform infrared (FT-IR) spectroscopy; its size and morphology were observed by scanning electron microscopy (SEM).

2.5. Adsorption of glycolate oxidase onto magnetic nanoparticles

The partially purified glycolate oxidase from *M. falcata* Linn. (MFGO) was immobilized onto amine-functioned magnetic nanoparticles via physical adsorption. The magnetic beads (0.5 g) were resuspended in 25 ml Tris–HCl buffer (100 mM, pH 9.0, containing 0.01 mM FMN) by ultrasonication, and then the MFGO was added, with a final concentration ranged from 10 mg/ml to 60 mg/ml. The immobilization process was carried out at 15 °C with a shaking rate of 160 rpm for 24 h. The immobilized enzyme was collected by magnetic field, washed 4 times with fresh buffer and then stored at 4 °C in the same buffer until use. Its morphology was observed by transmission electron microscopy (TEM). The amounts of protein remained in the enzyme solution before and after immobilization were determined by Bradford protein assay method.

2.6. Assay of enzyme activity

Activities of glycolate oxidases in different C_3 plants during screening were assayed spectrophotometrically at 324 nm by monitoring the formation of phenylhydrazone using a literature method [30] with slight modification. The phenylhydrazine can be converted to phenylhydrazone in the presence of glyoxylate at 30 °C and the phenylhydrazone formed has a maximum adsorption at 324 nm. The assay reaction mixture contained 2.5 ml phosphate buffer (100 mM, pH 8.0), 0.3 ml phenylhydrazine hydrochloride solution (100 mM, pH 8.0), 0.1 ml glycolate (100 mM, pH 8.0) and 0.1 ml enzyme solution. The enzymatic reaction was initiated by the addition of glycolate and the increase of absorbance at 324 nm was recorded every 30 s interval. One unit of glycolate oxidase activity is defined as the amount of enzyme that catalyzes the formation of 1.0 μmol glyoxylic acid per minute at pH 8.0 and 30 °C.

Catalase activity was assayed by measuring the change in concentration of discomposed H_2O_2 at 240 nm. The reaction was initiated by the addition of 0.1 ml catalase solution to 2.9 ml H_2O_2 solution (20 mM, pH 7.5), and the increase of absorbance at 240 nm was recorded every 30-s interval. One unit of catalase activity is defined as the amount of enzyme that catalyzes the decomposition of 1.0 μmol H_2O_2 per minute at pH 7.5 and 30 °C.

During the investigation of MFGO immobilization, the activities of the free and immobilized GOs were assayed using MBTH method which was first reported by Paz et al. [31] with slight modification. The free or immobilized GO was added into 2 ml Tris–HCl buffer (100 mM, pH 9.0) containing 50 mM glycolic acid and 0.01 mM FMN, the reaction was carried out at 30 °C for 30 min with a shaking rate of 160 rpm, then stopped with the addition of 0.4 ml HCl solution (1.2 M). After centrifuged, the glyoxylic acid formed in the reaction medium was determined with the addition of 0.2 ml MBTH (1%) and 2.5 ml $FeCl_3$ (0.2%) solution. The amount of tetraazopentamethine cyanine dye formed by glyoxylate, MBTH and Fe^{3+} was measured spectrophotometrically at 610 nm. One unit of glycolate oxidase activity is defined as the amount of enzyme that catalyzes the conversion of 1.0 μmol of glycolic acid to glyoxylic acid per minute at pH 9.0 and 30 °C.