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Production and separation of formate dehydrogenase from *Candida boidinii*

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Abstract

The production of FDH in a 40-l bioreactor was carried out with methanol as the inducer to reach the final intracellular FDH activity of 35 U/g cell. Among different permeabilization methods studied, treatment with toluene at a relatively small amount resulted in the cells with the highest FDH activity. Crude FDH cell extract obtained by ultrasonically breaking down the cell wall was treated with polyethyleneimine (PEI) to separate FDH from other proteins. By adding PEI at a low concentration of 0.04 mg/ml to the cell extract, ~50% of the proteins formed aggregates with PEI and precipitated; however, FDH was not in these aggregates and remained in the solution. After the PEI treatment, the specific FDH activity increased by 1.6-fold. SDS-PAGE analysis showed that PEI precipitation removed some impurity protein molecules that cannot be separated by affinity chromatography with Sepharose-Procion Blue HERB as the separation ligand, and thus improved the separation efficiency. The adsorbed FDH in the affinity column was eluted with KCl solution. Adding 5 mM NAD^+ in 0.2 M KCl improved the FDH elution and increased the specific FDH activity by 1.38-fold as compared to elution with 1 M KCl. Overall, the PEI precipitation and dye affinity chromatographic process obtained a high recovery yield of 56% with a 5.5-fold increase in the specific FDH activity from the crude cell extract.

Keywords: Formate dehydrogenase (FDH); Dye affinity chromatograph; Polyethyleneimine (PEI); *Candida boidinii*

1. Introduction

NAD^+-dependent formate dehydrogenase (FDH, EC 1.2.1.2) catalyzes the oxidation of formate anion with concomitant reduction of NAD^+ to NADH [1]. It catalyzes the final step in the methanol oxidation pathway found in many methylotrophic bacteria and yeasts. The production of FDH in these cells is induced by the addition of methanol in the growth media. FDH, as one of the most promising NADH regenerating enzymes, has many important applications in diagnostics, such as for the determination of oxalic acid [2], and in biotransformation, such as for the production of chiral drug intermediates [3–5]. As an intracellular enzyme associated with cell membrane, FDH biocatalysis requires to use either permeabilized whole cells or cell extract containing partially purified FDH enzyme. The latter is desirable because of its better mass transfer characteristics. However, industrial production of FDH for large-scale biocatalysis is difficult and currently not available. The goal of this study was to evaluate the feasibility of developing a simple, economical process for FDH production for industrial biocatalysis applications.

Although many enzyme purification processes have been reported, most of them contained multi-chromatographic steps or are laborious [1,6]. Affinity chromatography is a well-established method for protein purification. Various molecules, including cofactors, coenzymes, enzyme substrates, antibodies, and protein A and G can be used as the affinity ligands. However, the high cost and difficulty in their immobilization on the solid matrix limit their applications. Commercially available triazine reactive dyes are inexpensive, easy to immobilize, and have high binding capacity for a wide variety of proteins. They are generally considered as the most promising pseudo-affinity ligands that can overcome the drawbacks of the affinity ligands mentioned above. However, the interaction between the dye ligand and protein molecules is not specific enough and more than one types of protein molecules can bind to the dye ligand. One approach to improve the specific interaction between the affinity ligand and protein molecules is to modify and redesign the dye molecules to form biomimetic ligand, which has been used in a single-step dye affinity chromatograph for FDH purification [7]. However, the biomimetic dye-ligand requires tedious synthesis

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steps, that add the cost for the whole process, and is not as robust as the commercially available dyes, which can be screened for specific interactions between the ligand and its target protein for various industrial applications [8].

In this study, several commercial dyes were screened for their affinity for use to isolate and purify FDH from cell extract of *Candida boidinii*. Procion Blue HERB was found as a good dye-ligand for FDH and then used in the dye affinity chromatograph. In addition, polyethyleneimine (PEI), a highly branched cationic polymer that has been widely used in nucleotide, protein, and cell immobilization due to its ability to adsorb negatively charged species via electrostatic interaction [9–13], was investigated for its possible application in the fractionation and purification of FDH from the crude cell extract produced in a two-stage fed-batch fermentation. The effect of NAD^+ on the elution of FDH by KCl from the dye-affinity column was also studied.

2. Materials and methods

2.1. Production of FDH in C. boidinii

2.1.1. Culture

The stock culture of *C. boidinii* ATCC 32195 was maintained in a 50% glycerol solution at −80 °C. The inoculum was prepared in 250-ml flasks each containing 50 ml of the medium (composition, per liter: 20 g glucose, 10 g yeast extract, and 20 g peptone). After incubation for 24 h at 30 °C and 150 rpm agitation rate, a total of 200 ml of the cell suspension from four 250-ml flasks were used to inoculate the fermentor.

2.1.2. Fermentation

Fed-batch fermentation was carried out in a 40-l fermentor (B. Braun Biostat C bioreactor) containing 20 l of the medium operated under the following conditions: aeration 0.5–1.0 vvm; agitation 300–500 rpm; DO > 20%, 30 °C, pH 6.0 (controlled by adding 12.5% NH_4OH, which also served as the nitrogen source). The fermentation was carried out in two phases: cell growth phase and enzyme induction phase. During the growth phase, the initial fermentation medium contained (per liter): 20 g glucose, 10 g yeast extract, 2 g $MgSO_4{\cdot}7H_2O$, 2 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, and 1 ml antifoam A. Foaming was controlled by periodically adding antifoam A to the fermentor as needed. After ∼10 h of fermentation, a concentrated medium containing 50% (w/v) glucose, 0.8% (w/v) $MgSO_4{\cdot}7H_2O$, 1.5% (w/v) KH_2PO_4, 3% (v/v) trace metal solution, and 3% (v/v) vitamin solution were fed into the fermentor to continue cell growth for another 5 h to reach a cell density of 35 g/l (OD = 70). Then, the cells were induced to produce FDH by adding 300 ml of the induction medium (composition: 50% (v/v) methanol, 25% glucose, 10% yeast extract, 0.8% $MgSO_4{\cdot}7H_2O$, 1.5% KH_2PO_4, 3% trace metal solution and 3% vitamin solution) three times at 2–3 h interval. After that, 200 ml of methanol were added and the fermentation was allowed for another 2 h before cells were harvested by centrifugation at 10,000 rpm for 15 min. The harvested cells were stored in a freezer at −20 °C.

The trace metal solution contained (per liter): 2.7 g $FeCl_3{\cdot}6H_2O$, 2 g $ZnCl_2{\cdot}4H_2O$, 2 g $CaCl_2{\cdot}2H_2O$, 2 g NaMoO4·2H2O, 2 g $CuSO_4{\cdot}5H_2O$, 0.5 g H_3BO_3, 2 g $MnSO_4$, pH 7.0. The vitamin solution contained (per liter): 5.4 g pantothenic acid, 1.4 g pyridoxine, 6.1 g niacin, 0.04 g folic acid, 0.06 g biotin. These two solutions were sterilized by microfiltration (membrane pore: 0.2 μm).

2.2. Permeabilization of C. boidinii

FDH is an intracellular enzyme associated with cell membrane. In order to assay the intracellular FDH activity from cells produced in the fermentation process, four cell permeabilization methods were tested for their ability to obtain the highest FDH activity in the permeabilized *C. boidinii*. Cells obtained from the culture broth were washed three times with potassium phosphate buffer (50 mM, pH 7.4), and then resuspended to a final concentration of OD = 1 in an appropriate buffer solution according to the permeabilization method used. The first method was the osmotic shock (OS) method [14], in which cells were treated with 33% glycerol in the ice bath for 10 min, centrifuged at 13,200 rpm for 5 min, washed three times with potassium phosphate buffer (50 mM, pH 7.4), and then resuspended in the assay solution. The second method used the surfactant, Triton X-100, at 0.1% in the reaction solution to change the permeability of cell membrane. The third and fourth methods used a solvent, 2.5% (v/v) toluene:ethanol (1:4) solution [15] and 30–35% (v/v) acetone, respectively, to treat the cells for 3–5 min at room temperature, followed by centrifugation at 13,200 rpm for 5 min and washing three times with potassium phosphate buffer (50 mM, pH 7.4).

2.3. Isolation and purification of FDH

To obtain cell extract containing the FDH enzyme, the cells harvested from fermentation were washed with potassium phosphate buffer (50 mM pH 7.4) three times, broken down by using a cell disruptor (Sonifier Cell disruptor 350, Danbury, CT), and then centrifuged at 13,200 rpm for 5 min to remove the cell debris. To isolate and purify the FDH enzyme, the supernatant containing the FDH enzyme was treated with PEI to remove some impurity proteins and then further purified by the affinity dye chromatography described below.

2.3.1. PEI pretreatment of crude cell extract

One milliliter of crude cell extract was mixed with various amounts of PEI (MW 75,000, Sigma–Aldrich, St. Louis, MO) solution (2 g/l, pH 7.2) in a 1.5 ml Eppendorff tube and incubated at room temperature for 5 min to allow PEI to form aggregates with some proteins. The mixture was then centrifuged at 4 °C, 13,500 rpm for 5 min to remove the aggregates. The supernatant, which contained FDH, was collected for further purification by affinity dye chromatography.

2.3.2. Synthesis of dye-conjugated Sepharose for affinity chromatography

The immobilization of dye-ligands followed a published protocol with minor modifications [16]. Briefly, 0.25 g of a selected dye (Procion Red HE3B, Procion Red HE7B, Procion Blue HERB, and Remazol Black B; Dystar Inc., Charlotte, NC) was dissolved in 8 ml of distilled water in a 50-ml flask. Then, 10 g of Sepharose (CL-6B, sucked-dry gel, Amersham Biosciences, Piscataway, NJ) were added into the dye solution, shaken for 10 min, followed by adding 2 ml of NaCl (20%, w/v) and shaking for another 30 min. Finally, solid sodium carbonate (0.2 g) was added to a final concentration of 0.1 M, and the mixture was incubated at 27 °C with shaking for 5 days. The Sepharose gels with immobilized dye were then washed with sufficient water until the washing solution was colorless, and were stored at 4 °C until use.

2.3.3. Screening of dye affinity

Four different dyes (Procion Red HE3B, Procion Red HE7B, Procion Blue HERB, and Remazol Black B) were compared in their abilities for adsorption and desorption of FDH. One milliliter of crude FDH with various concentrations was mixed with 0.1 g of dye-Sepharose in a 1.5-ml Eppendorff tube. After 15 min, samples were taken and FDH activity and protein concentration were determined. The amount of enzyme bound to the adsorbent was estimated from the total initial FDH activity minus the remaining FDH activity in the supernatant at equilibrium. The resins with adsorbed FDH were washed with 1 ml of 50 mM potassium phosphate buffer for three times, and then resuspended in 1 M KCl solution to desorb FDH from the resins. After 15 min, the desorbed FDH activity and protein concentration in the supernatant were determined. Procion Blue HERB was found to be the best one among the dyes studied and was thus used in the affinity dye chromatograph for FDH separation.

2.3.4. Desorption of FDH from affinity dye

The desorption of FDH from Procion Blue HERB was studied to find the optimal KCl concentration needed to effectively recover the adsorbed FDH. The effect of adding NAD^+ at a low concentration of 5 mM on FDH desorption was also studied.

2.3.5. Dye affinity chromatography

Separation of FDH by affinity chromatography in a column (diameter: 1.47 cm; height: 4.8 cm) packed with 5 ml Procion Blue HERB-Sepharose CL-