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Immobilization of formate dehydrogenase from *Candida boidinii* through cross-linked enzyme aggregates

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ABSTRACT

We employed a cross-linked enzyme aggregate (CLEA) method to immobilize formate dehydrogenase (FDH) from *Candida boidinii*. The optimal conditions for the preparation of CLEAs were determined by examining effects of various parameters: the nature and amount of cross-linking reagent, additive concentration, cross-linking time, and pH during CLEA preparation. The recovered activities of CLEAs were significantly dependent on the concentration of glutaraldehyde; however, the recovered activity was not severely influenced by the content of dextran polyaldehyde as a mild cross-linker. Bovine serum albumin (BSA) was also used as a proteic feeder and enhanced the activity recovery by 130%. The highest recovered activity of CLEA was 18% for formate oxidation reaction and 25% for CO_2 reduction reaction. The residual activity of CLEA prepared with dextran polyaldehyde (Dex-CLEA) was over 95% after 10 cycles of reuse. The thermal stability of Dex-CLEA was increased by a factor of 3.6 more than that of the free enzyme. CLEAs of FDH could be utilized efficiently for both NADH regeneration and CO_2 reduction.

1. Introduction

Formate dehydrogenase (FDH) catalyzes both the oxidation of formate to CO_2 in the presence of NAD^+ and the reduction of CO_2 to formate with an excess concentration of NADH. This enzyme has considerable potential for applications in biotechnology fields, such as the regeneration of NADH during enzymatic reduction of chiral compounds [1–3], the determination of formic acid in solution [4], and the production of methanol from CO_2 [5–7]. Commercially available FDH from *Candida boidinii* has a quaternary structure of a homodimer when it is catalytically active [8,9]. In order to apply FDH to various reactions, strategies to stabilize the enzyme are needed.

Immobilization has been effectively used as a method for increasing stability and reusability of enzymes. Previous studies have immobilized FDH with various types of carriers, such as polystyrene [6], dextran sulphate [9], agarose derivatives [10], and magnetic nanoparticles [11]. However, the association of enzymes with solid supports results in diluted enzymatic activities [12]. Enzymes can also be immobilized without extra carriers by cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) [12,13]. To prepare CLEAs, the enzyme is precipitated from an aqueous solution by adding precipitants such as salts, water-miscible organic solvents, or polyethylene glycol. In a subsequent step, the physical aggregates of the enzymes are cross-linked with glutaraldehyde or polymeric cross-linkers. The methodology of CLEA as a carrier-free preparation is simple and robust, and does not require highly purified enzymes unlike CLECs. CLEAs have many economic and environmental benefits, such as improved storage and operational stability toward denaturation by heat, organic solvents, and autoproteolysis [13]. CLEA technology can also stabilize the quaternary structures of multimeric enzymes. Thus far, CLEAs of various enzymes, such as tyrosinases, alcohol dehydrogenases, penicillin acylases, lipases, and nitrilases, have been prepared [12–17]. However, the recovered activities of CLEAs were highly dependent on the kinds of enzymes and the preparation conditions. Therefore, the CLEA preparation process should be specifically optimized for the target enzyme. Interestingly, very few results have been reported for the CLEA preparation of dehydrogenases, which may be attributable to the very low recovery of dehydrogenase activity. For example, Mateo et al. [16] reported the CLEA of alcohol dehydrogenase, but the recovered activity was less than 10% under optimized conditions. Schoevaart et al. [13] prepared the CLEA of FDH by an optimization process; however, the recovered activity was only 7%.

In this work, we investigated if the CLEA of FDH could be prepared to enhance the activity recovery by changing various influencing factors such as the type of cross-linker, cross-linking

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time, pH, and additives such as a proteic feeder. The activity and stability of prepared FDH CLEAs were also studied by monitoring formate oxidation and CO_2 reduction reactions.

2. Materials and methods

2.1. Materials

Formate dehydrogenase from *C. boidinii* (liquid, 50 U/mL), reduced disodium salt hydrate of nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide hydrate (NAD^+), albumin from bovine serum (BSA), dextran from *Leuconostoc* sp. (MW 100,000), and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium formate was obtained from Junsei Chemical (Tokyo, Japan). All other chemicals used in this study were of analytical grade and used without further purification.

2.2. Preparation of dextran polyaldehyde

Dextran (1.65 g) was dissolved in 50 mL of water. Sodium metaperiodate (3.85 g) was added to the dextran solution, and the mixture was stirred at room temperature for 90 min. The resulting solution was dialyzed five times with a MW cutoff of 3 kDa [16]. The dextran polyaldehyde solution was frozen overnight at −70 °C and freeze-dried.

2.3. CLEA preparation

A volume of 1 mL protein solution containing 0.1 mg/mL FDH and 0–0.5 mg/mL BSA in 0.1 M buffer (citrate buffer for pH 5, phosphate buffer for pH ranging from 6 to 8, and glycine–NaOH buffer for pH 9 and 10) was mixed with 9 mL of a saturated ammonium sulfate solution. After 20 min of mixing at 4 °C, glutaraldehyde or dextran polyaldehyde was slowly added to the mixture to a final concentration of 2–30 mM or 6–150 μg/mL, respectively. After cross-linking at 4 °C, the suspension was centrifuged at 12,000 rpm for 15 min. The pellet was then resuspended and washed twice with 0.1 M phosphate buffer (pH 7.0) by centrifugation. The recovered pellet was stored in phosphate buffer at 4 °C before use.

2.4. Activity assay for FDH

To perform FDH-catalyzed formate oxidation, free FDH or the prepared CLEAs were placed in a conical tube together with 10 mL of a 55 mM sodium formate solution in 0.1 M phosphate buffer (pH 7.0). The reaction was started by adding 1 mL of NAD^+ solution (3.3 mM) and carried out at 25 °C in a water bath with shaking at 100 rpm. Periodically 0.2 mL aliquots were taken and diluted with 0.8 mL buffer. The activity was determined by measuring the increase in absorbance at 340 nm by the NADH produced during the formate oxidation. In order to measure the CO_2 reduction activity of FDH, free FDH or the prepared CLEAs were mixed with 10 mL of 55 mM sodium bicarbonate. The reaction was started by adding 1 mL of a 3.3 mM NADH solution. The activity was determined by measuring the decreased NADH concentration. The reaction conditions for CO_2 reduction were the same as those for formate oxidation. The recovered activities in the CLEAs were calculated by the following equation:

$$\text{Recovered activity}(\%) = \frac{\text{total activity of CLEA}}{\text{total free enzyme activity used for CLEA preparation}} \quad (1)$$

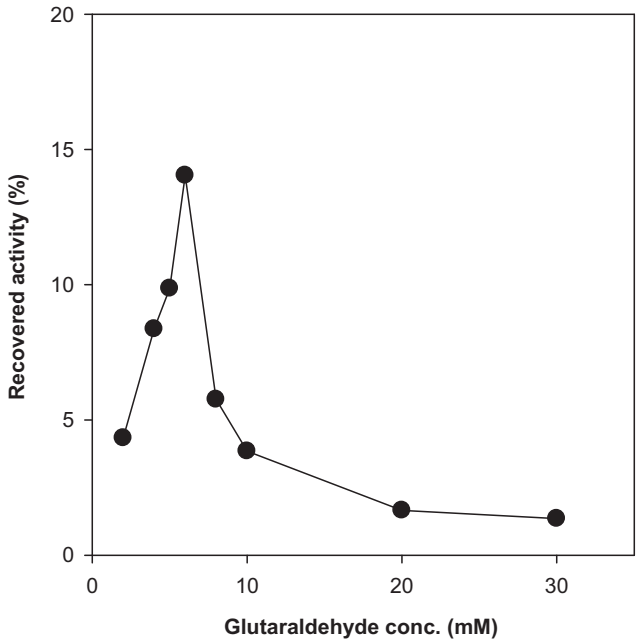

Fig. 1. The effect of glutaraldehyde concentration as a cross-linking reagent on the recovered activity of prepared CLEAs. Cross-linking time was 150 min.

2.5. Measuring the thermal stability of CLEAs

In order to measure the thermal stability of the CLEAs, free FDH, or FDH CLEAs were incubated in 1 mL of a 0.1 M phosphate buffer (pH 7.0) at 50 °C. After incubation for the determined period of time, the residual activity was measured by adding 9 mL of a sodium formate solution (61 mM) in 0.1 M phosphate buffer (pH 7.0) and 1 mL of a NAD^+ solution (3.3 mM) to the enzyme solution, as described above.

2.6. Measuring the operational stability of CLEAs

After the FDH CLEAs were used for formate oxidation, as described above, the CLEAs were washed and recovered by centrifugation at 12,000 rpm for 20 min. Recovered CLEAs were 10 times reused for the formate oxidation and the residual activity of CLEAs was measured.

2.7. Scanning electron microscopy (SEM) analysis

The CLEAs were freeze-dried and sputter-coated with gold prior to observation. SEM images were recorded with the AURIGA Field-Emission Scanning Electronic Microscope (Carl Zeiss, Germany).

3. Results and discussion

3.1. Optimization of CLEA preparation

3.1.1. Effect of the cross-linker

Glutaraldehyde has been traditionally used as the cross-linking agent to prepare CLEAs of various enzymes such as lipase, tyrosinase, glucose oxidase, and penicillin acylase [12–17]. Generally, the recovered activity of CLEAs prepared using glutaraldehyde is highly dependent on the kind of enzyme and the concentration of glutaraldehyde because of high reactivity and small size of glutaraldehyde. The recovered activity of CLEAs will be very low if the catalytically important residues of the enzyme react with glutaraldehyde. Fig. 1 shows the influence of glutaraldehyde