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Improving of Stability of Formate Dehydrogenase from *Candida methylica* by Immobilization onto Eupergit C 250 L

Formate dehydrogenase (FDH) from *Candida methylica* has covalently been immobilized onto Eupergit C 250 L to improve its stability and reusability. The immobilization and activity yields were obtained as 80% and 42%, respectively. The optimum pH values were 7.0 and the optimum temperature values were 35 °C for either free or immobilized FDH preparations. The apparent Km—k_{cat} values were 4.18±0.22 mM—0.182±0.008 s^{-1} for the free FDH and 2.93±0.11 mM—0.077±0.004 s^{-1} for the immobilized FDH. The free FDH completely lost its initial activity at 35°C and 50 °C after 24 h of preincubation, whereas the immobilized FDH retained 84% and 77% of its initial activity under these conditions, respectively. The immobilized FDH retained 62% of its initial activity after 10 reuses in a batch type reactor.

Key words: formate dehydrogenase, Eupergit C 250 L, immobilization, stabilization.

Formate dehydrogenase (FDH, EC 1.2.1.2) catalyzes oxidation of formate to carbon dioxide (CO_2) using NAD^+ as coenzyme [1—3]. This enzyme can also catalyze the reverse reaction, the reduction of CO_2 to formate, in the presence of excessive NADH concentration [4]. FDH is industrially used in combination with other dehydrogenases, such as alcohol dehydrogenase and lactate dehydrogenase, to regenerate NADH [5]. The end product of the FDH activity, formic acid, is widely used as preservatives in foodstuffs, vinegar, wine and honey [6], and in the methanol production from CO_2 [7].

A few free FDH preparations from *Candida boidinii* and *Escherichia coli* are commercially available; however, the free FDH forms have a limited long-term operational stability and relatively high cost. Enzyme immobilization has been recognized as a convenient approach to improving enzymes stability and reusability. FDH has been immobilized onto various types of carriers, such as polystyrene, dextran sulphate, agarose derivatives [8], and magnetic nanoparticles [9].

Epoxy group-bearing commercial supports of Eupergit C and Eupergit C 250 L are most frequently

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Abbreviations: NAD, nicotinamide adenine dinucleotide; NADH, NAD^+, NADP, $NADP^+$, NAD reduced, oxidized, phosphorylated and phosphorylated oxidized, respectively; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecul sulfate.

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used matrices for the immobilization of numerous enzymes, such as lipase [10], catalase [11], hydroxynitrile lyase [12], glucose isomerase [13] in both laboratory and industrial scales. These supports consist of a copolymer of methacrylamide, glycidyl methacrylate and allyl glycidyl ether, cross-linked with N,N-methylene-bis(methacrylamide) [14]. Eupergit C and Eupergit C 250 L are ready-to-use supports, and their modification is not necessary for the enzyme immobilization. The procedure of enzyme immobilization on these supports is very simple owing to the capacity of epoxy groups to easily react with various nucleophiles highly abundant on the protein surface, such as primary amines, thiols or aromatic hydroxyl groups. Therefore, the stabilization of enzymes can be improved due to the multipoint covalent attachment and also enzyme—support interactions [15].

In this study, we aimed at covalent immobilization of NAD^+-dependent FDH from *Candida methylica* onto Eupergit C 250 L (which was performed for the first time) and establishing of optimum conditions (thermal and operational stability in a batch type reactor) for the formate oxidation by the free and immobilized FDH preparations.

EXPERIMENTAL

Materials. FDH was purified from *Candida methylica* up to more than 90% purity (based on SDS-PAGE) and activity 2.4 U/mL (see below). NAD^+ was purchased from Acros Organics (USA). Sodium formate and Eupergit C 250 L (particle size 250 μm, oxirane content ≥ 200 μmol/g dry support) were supplied by Sigma-Aldrich (St. Louis, USA). All other chemicals used in this study were of analytical grade and used without further purification.

Methods

Purification of *C. methylica* FDH. The purification of FDH was performed according to Demir *et al*. [16]. Briefly, 7 g of wet *E. coli* (a derivative of the Rosetta (DE3) host strain) cell paste containing the expressed FDH protein was suspended in 10 ml of a buffer solution (20 mM Tris-HCl, pH 7.8, 0.5 M NaCl, 5 mM imidazole) at 4°C. The cells were then disrupted by sonication and the cell debris was harvested by centrifugation (28,000 g, 30 min) at 4°C. The pellet was resuspended in an ice-cold buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 30 mM imidazole, pH 7.4), and the mixture was additionally lysed by 25 ml of a freshly prepared lysozyme solution (10 mg/ml in 10 mM Tris-HCl, pH 8.0). The lysate was then passed through a 0.45-μm cellulose membrane. The filtered samples were loaded on a His-trap column (GE Healthcare) equilibrated with 5 ml of the ice-cold buffer (see above), and the column was washed with 5 ml of the same buffer. FDH was eluted with a series of elution buffers: 3 ml of 20 mM NaH_2PO_4, 0.5 M NaCl with 0.1 M imidazole, pH 7.4; 5 ml of 20 mM NaH_2PO_4, 0.5 M NaCl with 0.2 M imidazole, pH 7.4; and finally 3 ml 20 mM NaH_2PO_4, 0.5 M NaCl with 0.4 M imidazole pH 7.4. The collected fractions were analyzed by SDS-PAGE.

Immobilization of FDH. The immobilization procedure was performed according to Alagöz *et al.* [17]. The FDH solution (9.0 ml containing 1.0 mg/ml protein in 1.0 M phosphate buffer, pH 7.0) was added to 1 g of Eupergit C 250 L support (Fig. 1). The mixture was gently shaken at 25°C in a water bath for 24 h. The immobilized FDH preparations were collected by filtration on paper (Whatman Grade 1) and washed with distilled water. The protein content in the filtrate was assessed from the absorbance at 280

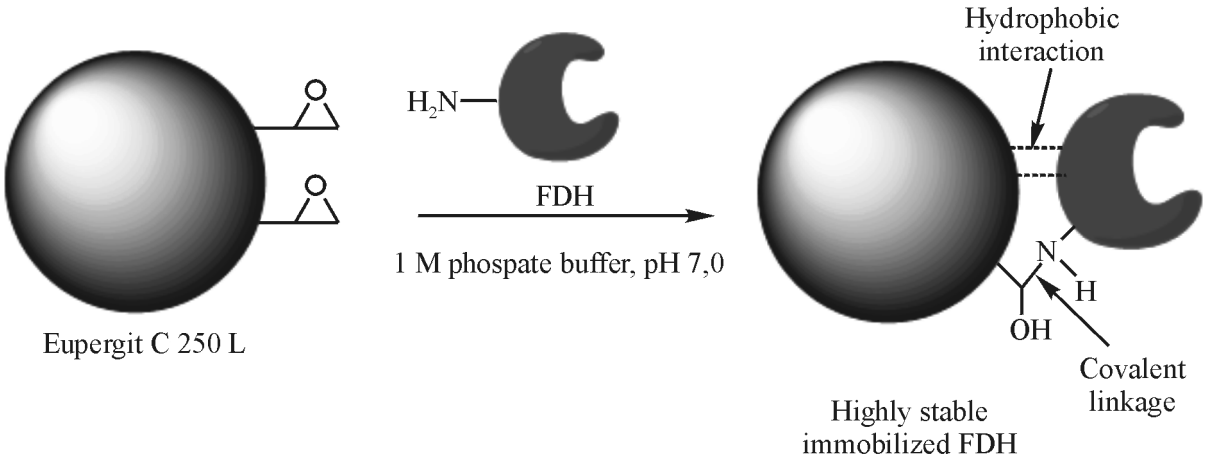

Fig. 1. Scheme of *Candida methylica* FDH immobilization onto Eupergit C 250 L

nm and the washing procedure was continued until no optical density was detected in the filtrate. After that, the immobilized FDH preparations were stored at 5°C until use. The amount of immobilized protein onto the support was determined using the Bradford protein assay [18].

FDH activity assay. The FDH activity was measured spectrophotometrically at 340 nm according to Özgün *et al*. [19]. Five mg of the immobilized FDH, 2.6 ml of phosphate buffer (0.1 M, pH 7.0) and 0.5 ml of the 0.1 M sodium formate solution (in the 0.1 M pH 7.0 phosphate buffer) were sequentially added in a test tube. The reaction was started by the addition of 0.1 ml of the β-NAD^+ solution (10 mM in water) at 25°C in a water bath. After 10 min of the reaction time, a 3-ml aliquot was taken from the reaction mixture and its absorbance was measured at 340 nm. The same procedure was applied to blank tube containing neither free nor immobilized FDH. A unit of the FDH activity was defined as the amount of enzyme producing 1.0 μmole of CO_2 from formate in the presence of NAD^+ under the assay conditions.

FDH characteristics. The effect of pH on the activities of the free and immobilized FDH at pH values ranging from 5.0 to 8.0 was investigated. The optimal temperatures for the free and immobilized FDH preparations were determined within the temperature range of 25—50°C. The apparent *Km* of free and immobilized FDH preparations were determined by performing the FDH assay with various sodium formate concentrations (5—100 mM). The activities of the free and immobilized preparations were measured under their optimum conditions. Enzyme Kinetics Module software (Sigma-Plot 12.0) was used to determine the apparent *Km* values for the FDH preparations.

The thermal stability of the free and immobilized FDH preparations was tested by incubating them at 35°C and 50°C and measuring the activities of the samples with certain time intervals. The first-order inactivation rate constant (k_i) and half-life ($t_{1/2}$) of the free and immobilized FDH preparations were determined when observing the residual catalytic activity during 24 h.

The stabilization factor (SF) at a specific temperature was calculated by dividing a half-life of each immobilized FDH to that of the free FDH.

Operational stability of immobilized FDH. The operational stability of the immobilized enzyme was investigated in a batch type column reactor (7 cm length, 1 cm internal diameter, 5 ml volume capacity, GE Healthcare). 100 mg of the immobilized FDH preparations were loaded to the reactor, and 2.6 ml of phosphate buffer (0.1 M, pH 7.0) and 0.5 ml of 0.2 M sodium formate solution (0.1 M in pH 7.0 phosphate buffer) were added. The reaction was started by the addition of 0.1 ml of the NAD^+ solution (10 mM in water) at 25°C in a water bath. After 10 min, a 3-ml aliquot was taken from the reaction mixture and its absorbance was measured at 340 nm. The immobilized FDH was rinsed with phosphate buffer (0.1 M, pH 7.0), the fresh reaction mixture was then added and the next cycle of the enzymatic reaction was performed.

RESULTS AND DISCUSSION

In this study, *C. methylica* FDH was covalently immobilized onto Eupergit C 250 L. The amount of the bound protein was determined as 80% of the initial loaded value (9 mg per 1 g of the Eupergit C 250 L support). The enzyme retained 42% activity of the free FDH upon immobilization.

The free and immobilized FDH preparations showed 2% and 57%, respectively, of their maximum activities at pH 4.0 (Fig. 2). The activities of both free and immobilized FDH preparations increased with pH enhancing, and their maximum activities were observed at pH 7.0. The similar optimum pH value was earlier reported by Gao *et al*. concerning a mutant FDH immobilized onto polydopamine-coated iron oxide nanoparticles (PD-IONPs) [20] and by Bolivar *et al*. concerning free and immobilized onto glyoxyl-agarose *Pseudomonas sp*. 101 FDH [21]. In that study, the determined activities of free and immobilized FDH preparations were 95 and 66% of their maximum activities, respectively, when pH was further increased up to 8.0.

In our study, the measured activities were 67% and 69%, respectively, for the free and immobilized FDH preparations at 25°C (Fig. 3). The activities of free and immobilized FDH increased with the temperature growing from 25°C to 35°C and the free and immobilized FDH both showed their maximum activities at 35°C. The activities of free and immobilized FDH preparations decreased at the temperatures above 35 °C. The optimum temperature values were found as 37°C for either free mutant FDH or mutant FDH immobilized onto PD-IONPs [21]. Netto *et al*. [9] reported that the optimum temperature for the free *Candida boidinii* FDH was 37°C. When the enzyme was immobilized onto magnetite nanoparticles silanized with (3-aminopropyl)triethoxysilane, the optimum temperature for the free FDH was increased up to 42°C. If this support was further coated with glyoxyl-agarose, the optimum temperature for the free FDH activity decreased up to 27°C.

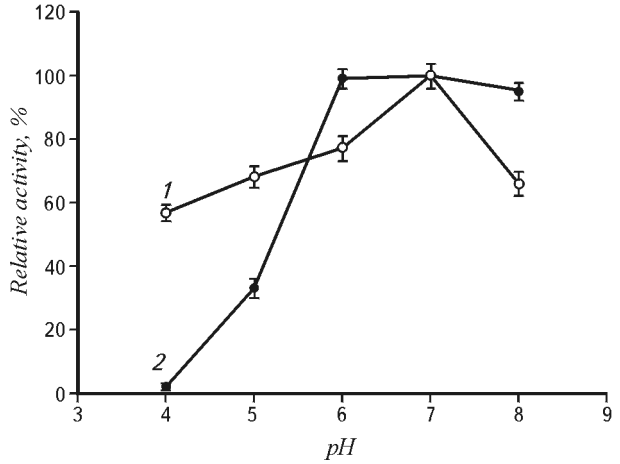

Fig. 2. Effect of pH on activities of free (*2*) and immobilized (*1*) FDH preparations. The FDH activity at pH 7.0 was taken as 100% for either of the enzyme forms. The experiments were run in triplicate

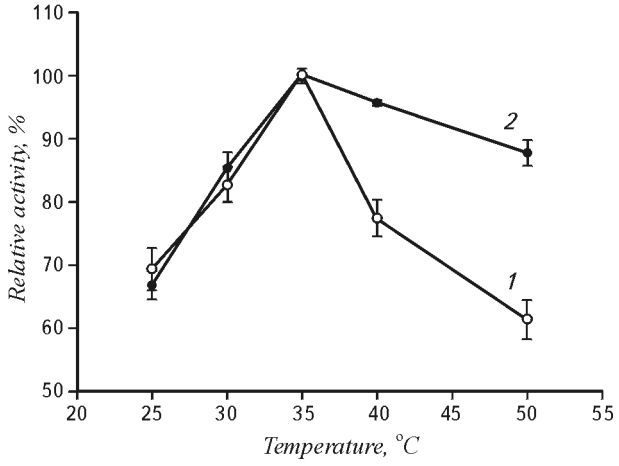

Fig. 3. Effect of temperature on activities of free (*2*) and immobilized (*1*) FDH preparations. Enzyme activity at 35°C is taken as 100% for either of the enzyme forms. The experiments were run in triplicate

The kinetic parameters for free and immobilized FDH preparations were given in Table 1. *Km* values of free and immobilized *C. methylica* FDH for formate were determined as 4.18±0.22 mM and 2.93±0.11 mM, respectively. The immobilization procedure can cause some positive changes in the substrate affinity of the enzyme, and therefore, the *Km* value of the immobilized FDH was reduced. The k_{cat} and k_{cat}/Km values were 0.182±0.008 s^{-1} and 0.044 s^{-1} mM^{-1}, respectively, for the free enzyme. For the immobilized FDH, the corresponding values were 0.077±0.004 s^{-1} and 0.026 s^{-1} mM^{-1}, respectively. Ordu *et al*. [22] reported that the *Km* value for formate for the free *C. methylica* FDH preparation was 4.75±0.3 mM at pH 8.0 and 25°C. Avilova *et al*. [23] observed that the *Km* value for the free *C. methylica* enzyme was 13 mM. Ordu *et al*. [22] reported that k_{cat} and k_{cat}/Km values were 1.13± 0.1 s^{-1} and 0.24 s^{-1} mM^{-1}, respectively for the free *C. methylica* FDH.

It is an advantageous trend in industrial applications to protect by immobilization of sensitive enzyme regions from environment. In general, the activity of an immobilized enzyme, especially in a covalently bound system, is more resistant than that of the soluble form to heat and denaturing agents [24]. The thermal stability experiments were performed by incubating the free and immobilized forms of FDH at 35°C and 50°C and determining their residual activities at several time points during 24 h. The half-life times ($t_{1/2}$) of free and immobilized FDH preparations were calculated as 10.6 h and 69.2 h, respectively, at 35°C (Table 2). The $t_{1/2}$ values at 50°C were 8.1 h and 49.6 h. These results show that the FDH was stabilized upon the immobilization by 6.5 and 6.1 times, respectively, at 35°C and 50°C. Kim *et al*. [25] reported that cross-linked enzyme aggregates of *C. boidinii* FDH prepared with dextrane polyaldehyde and glutaraldehyde showed the 3.6- and 4.0-fold higher stability, respectively, than the free enzyme at 50°C.

The operational stability of an immobilized enzyme is one of the most important characteristics in enzyme industrial applications. The successful reusability of immobilized enzymes could lower operational costs and contribute to the advantage of immobilized enzymes over their free forms [26]. The operational stability of the immobilized FDH was tested in the batch type reactor during 10 reuses (Fig. 4).

Table 1

Kinetic parameters of free and immobilized FDH preparations for formate

Enzyme form	*Km*, mM	k_{cat}, s^{-1}	k_{cat}/Km, s^{-1} mM^{-1}
Free	4.18±0.22	0.182±0.008	0.044
Immobilized	2.93±0.11	0.077±0.004	0.026

Table 2

Thermal stability assays of free and immobilized FDH at 35°C and 50°C

Enzyme form	Temperature, °C	$t_{1/2}$, h	k_i, h^{-1}	Stabilization factor
Free	35	10.6	$6.5 \cdot 10^{-2}$	–
	50	8.1	$8.5 \cdot 10^{-2}$	–
Immobilized	35	69.2	$1.0 \cdot 10^{-2}$	6.5
	50	49.6	$1.4 \cdot 10^{-2}$	6.1

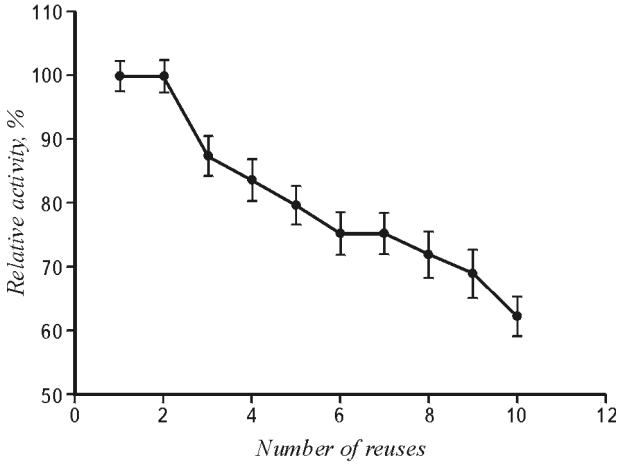

Fig. 4. Reusability of immobilized FDH

As is seen, the immobilized enzyme retained its initial activity up to 2 reuses. After this point, the activity of immobilized FDH slightly decreased, and the residual value after 10 reuses was equal to 62 % of its initial activity. Gao *et al*. [20] reported that the mutant FDH immobilized onto PD-IONPs possessed 60% of its initial activity after 17 cycles. Kim *et al.* [25] reported that *C. boidinii* FDH immobilized as cross-linked enzyme aggregates prepared with dextrane polyaldehyde or glutaraldehyde retained 96% and 89% of their initial activities, respectively, after 10 cycles.

Thus, the immobilization of *C. methylica* FDH onto Eupergit C 250 L was performed. As a result, the enzyme activity was stabilized by 6.5 and 6.1 times. The immobilized FDH retained 62% of its initial activity after 10 reuses. In conclusion, the immobilized FDH may be used in combination with other dehydrogenases to regenerate NADH and thereby making bioprocess economically viable.

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