Enzyme and Microbial Technology 43 (2008) 495–499

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Thermodynamic, kinetic, and operational stabilities of yeast alcohol dehydrogenase in sugar and compatible osmolyte solutions

Osato Miyawaki[a,*], Ge-Li Ma[b], Tomokazu Horie[c], Ayumi Hibi[c], Toru Ishikawa[c], Shoji Kimura[c]

[a] Department of Food Science, Ishikawa Prefectural University, Suematu 1-308, Nonoichi, Ishikawa-gun, Ishikawa 921-8836, Japan
[b] Department of Food and Biology Engineering, Zhengzhou University of Light Industry, Zhengzhou, Henan 450002, PR China
[c] Department of Environmental Chemical Engineering, Kogakuin University, Nakano-machi 2665-1, Hachioji, Tokyo 192-0015, Japan

ARTICLE INFO

Article history:
Received 1 March 2008
Received in revised form 25 August 2008
Accepted 9 September 2008

Keywords:
Stability of enzyme
Thermodynamic stability
Kinetic stability
Operational stability
Yeast alcohol dehydrogenase
Compatible osmolyte
Sugar
Ultrafiltration membrane reactor

ABSTRACT

Thermodynamic, kinetic, and operational stabilities of yeast alcohol dehydrogenase (YADH) were measured and compared in aqueous solutions containing various sugars (sucrose, glucose, and ribose) and compatible osmolytes (betaine and sarcosine). In the measurement of operational stability, native YADH was entrapped and physically immobilized in an ultrafiltration hollow fiber tube to retain the native characteristics of the enzyme. All the additives tested increased thermodynamic stability and kinetic stability of YADH. The order of the magnitude of stabilization effect among additives was different between thermodynamic and kinetic stabilities. Compared to the thermodynamic and kinetic stabilities, the effects of additives were much different in operational stability. Sucrose, glucose, and betaine stabilized YADH substantially while ribose and sarcosine destabilized the enzyme. These results show that the thermodynamic and kinetic stabilities do not necessarily guarantee the operational stability of YADH. The coexistence of stabilizing solute was proved effective to increase the productivity of the bioreactor with immobilized YADH.

1. Introduction

In spite of their high catalytic activity, enzymes are inherently labile. Therefore, stability of enzyme is very important in their practical applications. In the literature, various types of stabilities have been discussed for enzyme stability [1–3]. Among those, thermodynamic stability of protein unfolding [3], kinetic stability in catalytic activity [3], and operational stability in continuous usage of enzyme as a biocatalyst [1] are important practically. These three stabilities, however, have not been compared directly because thermodynamic and kinetic stabilities are measured for native enzyme while operational stability is measured for immobilized enzyme in most cases.

There are various methods for immobilization of enzymes such as chemical modification, crosslinkage, gel entrapment, etc [4]. Immobilization, however, introduces additional variables to enzyme characteristics so that operational stability of immobilized enzyme cannot be compared directly with thermodynamic and kinetic stability of native enzymes. In a bioreactor with an immobilized enzyme, however, operational stability of enzyme in the continuous operation is ultimately important in practice [4–10] and thermodynamic and/or kinetic stabilities of enzyme is expected to be prerequisite to long term operational stability of the bioreactor.

In the present paper, thermodynamic and kinetic stabilities for yeast alcohol dehydrogenase (YADH) are measured in solutions with various stabilizing additives such as sugars [11,12] and compatible osmolytes [13]. Then, native YADH is entrapped and physically immobilized in an ultrafiltration hollow fiber tube [14,15] so that the immobilized YADH can retain its native characteristics. Operational stability is measured for this immobilized enzyme in the continuous operation with continuous feed of solutions containing substrates and stabilizing additives. Thus measured operational stability is compared with thermodynamic and kinetic stabilities to explore the difference among the three stabilities of YADH.

2. Materials and methods

2.1. Materials

Yeast alcohol dehydrogenase (YADH, lyophilized powder with 30% sucrose and 10% phosphate), bovine heart L-lactate dehydrogenase (LDH, crystallized suspension in 3.3 M ammonium sulfate), and β-NAD^+ were purchased from Roche Applied Science (Tokyo, Japan). D(−)Ribose was from Sigma–Aldrich (Tokyo, Japan). Sarcosine and betaine monohydrate were from Nacalai Tesque (Kyoto, Japan). All other reagents used were of reagent grade.

* Corresponding author. Tel.: +81 76 227 7465; fax: +81 76 227 7410.
E-mail address: osato@ishikawa-pu.ac.jp (O. Miyawaki).

0141-0229/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2008.09.002

2.2. Measurement of thermodynamic stability

YADH was dissolved at 1 mg-protein/ml in 0.05 M Tris buffer, pH 8.0 at room temperature. Then the temperature of enzyme solution was raised at 1°/min by a program controller (Jasco, ETC-505S, Tokyo, Japan) and the optical absorbance was monitored at λ = 250 nm by a spectrophotometer (Jasco, V-560, Tokyo, Japan). When the thermal denaturation of protein begins, a clear turn was observed in the temperature scan curve from which thermal denaturation temperature, T_d was determined. With the presence of additives such as sugars and compatible osmolytes, T_d changed depending on the type of additives and their concentration. The deviation of T_d from that without additive, ΔT_d was measured and compared.

2.3. Measurement of kinetic stability

YADH dissolved at 0.6 mg-protein/ml (240 U/ml) in 0.05 M Tris buffer, pH 8.0 was treated at 50, 55, or 60 °C for 2–25 min. The residual enzyme activity was measured at 25 °C with a substrate solution containing 3 mM NAD and 5 vol% ethanol by measuring the optical absorbance increase at 340 nm. From the decrease in activity with an increase in thermal treatment time, thermal deactivation rate constant, k_d was determined.

2.4. Ultrafiltration hollow fiber membrane reactor and measurement of operational stability of enzyme

Before the measurement of operational stability, YADH (1200 U, 3 mg-protein/ml) was entrapped and coimmobilzed with LDH (2750 U, 5 mg-protein/ml) in an ultrafiltration hollow fiber membrane tube (Asahi Kasei HC, Tokyo, Japan; nominal cut-off molecular weight = 13,000, inner diameter = 0.8 mm, outer diameter = 1.4 mm, 60 cm in length). The hollow fiber tube with the immobilized enzymes was clogged by stoppers at the both ends and inserted in a silicon tube (inner diameter = 2.5 mm) to form a membrane bioreactor as shown in Fig. 1 [14]. The reactor was immersed in a water bath controlled at 50 °C. Substrate solution in 0.05 M Tris buffer, pH 8.0 contained 5 vol% ethanol, 0.05 M pyruvate, 50 μM NAD and additives for enzyme stabilization. The substrate solution was continuously supplied into the space between the hollow fiber and the silicon tube. Substrates and cofactors for enzyme reactions permeate in through the hollow fiber membrane and products permeate out while enzymes cannot permeate through the membrane because of their high molecular weight (YADH, 141,000; LDH, 150,000) [15]. The mean residence time of the feed solution was adjusted at 1.5 h. In the reactor, following conjugated reactions proceed with NAD regeneration.

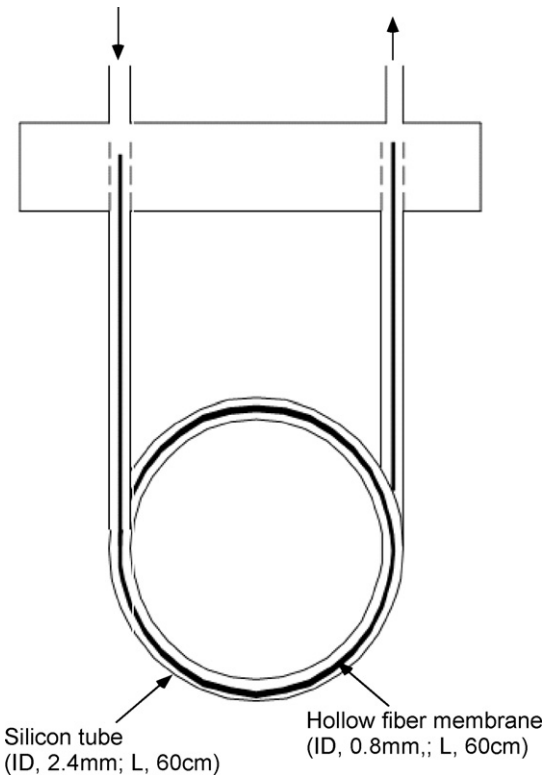

Fig. 1. Ultrafiltration hollow fiber membrane bioreactor for coimmobilization of conjugated enzyme system with YADH and LDH. Enzymes were physically immobilized in the hollow fiber tube and substrate solution was supplied into the space between hollow fiber and silicon tube.

$$\text{YADHethanol} + \text{NAD}^+ \rightarrow \text{acetaldehyde} + \text{NADH} + \text{H}^+ \quad (1)$$

$$\text{LDHpyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{L-lactate} + \text{NAD}^+ \quad (2)$$

Because of this conjugation, the concentration of the expensive cofactor, NAD, could be kept at a low level. The performance of this system was established before [15].

2.5. Assay of lactate

The final product of the membrane bioreactor, L-lactate was analyzed as reported before [15]. 1.0 ml of 0.4 M hydrazine in 1.0 M glycine, 0.2 ml of 0.05 M NAD, 0.01–0.1 ml of sample and deionized water were mixed to adjust the total volume to be 2.2 ml. To this mixture, 0.01 ml (55 U) of LDH was added to measure the increase in optical absorbance at 340 nm by the formation of NADH, the concentration of which corresponds to that of L-lactate.

2.6. Determination of water activity

The effect of different additives on the enzyme stability should be compared at the same water activity [16]. Water activity (a_w) of a solution with a low molecular solute is theoretically described by the following equation [17].

$$a_w = (1 - X_S)\exp(\alpha X_S^2) \quad (3)$$

where X_S is molar fraction of solute. The experimental parameter, α have been reported in the literature [18] for sucrose, glucose, and ribose to be −7.405, −2.734, and −1.099, respectively. No data were available for sarcosine and betaine so that water activity was measured by water activity meter (Ainex, CX-3, Tokyo, Japan) in these cases. As a result, α's for sarcosine and betaine were 0.0356 and −7.835, respectively. The very low α for sarcosine means that sarcosine solution is very close to an ideal solution in terms of water activity.

3. Results and discussion

3.1. Thermodynamic stability of YADH

Thermal denaturation temperature T_d for YADH in the simple Tris buffer (0.05 M, pH 8.0) without additives was 61.3 °C, which seemed to be the beginning of the irreversible precipitation of the protein. In the thermal unfolding process of monomeric proteins, sigmoidal curves are observed in UV absorption or fluorescence analysis [11,12,19,20]. YADH, however, is known to be a tetrameric protein [21] so that the thermal denaturation process was not simple as that of monomeric protein. Therefore, the present method seemed appropriate to compare the different effect of additives on thermal stability of YADH although the process was irreversible.

Fig. 2 shows the effect of water activity on change in thermal denaturation temperature, ΔT_d, of YADH in various solutions. All the additives used here increased thermodynamic stability of YADH but the effects were different among those. Sucrose, a sugar also known as a compatible osmolyte, showed the largest increase in ΔT_d of more than 12 °C at a_w = 0.97. When compared at the same water activity, the stabilization effect was the largest for sucrose followed by sarcosine, glucose, betaine, and ribose.

In the preceding paper [20], we measured and compared thermodynamic stabilities of ribonuclease A, lysozyme, and α-chymotrypsinogen A in various sugar solutions with water activity varied. Among sugars, sucrose showed the highest stabilizing effect, ribose showed the lowest, and glucose intermediate, as was observed here for YADH. The stabilizing effects of sugars on proteins are the balance between the positive effect of dehydration through the reduction in water activity to prevent protein unfolding and the negative effect of direct binding on the protein to facilitate protein unfolding [20]. As sucrose is nonreducing sugar and is preferentially excluded from the protein surface [11], this solute has the only stabilizing effect while glucose and ribose are reducing sugar so that these have a chance to form a Shiff-base at the aldehyde terminal with an amino group of proteins at a high temperature. Ribose,