CHINESE JOURNAL OF BIOTECHNOLOGY
Volume 24, Issue 4, April 2008
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chin J Biotech, 2008, 24(4), 617−621.

RESEARCH PAPER

Research on Orientedly Immobilized Urease via Concanavalin A

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Abstract: In this study, urease was immobilized on chitosan beads via a saccharide-concanavalin A binding. Concanavalin A (ConA) was immobilized on a pre-activated chitosan microsphere, and then oriented immobilization of urease was carried out based on the strong interaction between ConA and glycoprotein. The optimum immobilization conditions were as follows: glutaraldehyde concentration 3.5%, ConA concentration 1 mg/mL, ConA pH 7.0, and urease concentration 0.4 mg/mL. For orientedly immobilized urease, the highest activity was allowed at pH 5.0–6.0 and temperature 77°C, and the Michaelis constant (*K*m) was disclosed to be 11.76 mmol/L by Lineweaver-Burk plot. Compared with the free urease and the randomly immobilized urease, the optimum pH of the orientedly immobilized urease was smaller and the pH domain wider. Orientedly immobilized urease presents higher temperature resistance, higher affinity to the substrate, and higher stability of operation. Hence, oriented immobilization of urease via ConA can be a versatile tool for immobilization of proteins and offers great promise in clinical as well as industrial use.

Keywords: urease; concanavalin A; oriented immobilization

Introduction

The oriented immobilization of enzyme occurs when a precise site of the enzyme that is usually far away from the active site is bound on the support. Oriented immobilization of enzyme ensures that enzymes are well ordered on the support leading to a high number of properly oriented active sites being available for catalytic action and an increase in catalytic efficiencies. Several different site-directed immobilization strategies have been employed[1−7], such as immobilization based on antibody-antigen interactions, immobilization based on avidin-biotin interactions, immobilization of the glycoprotein through a carbohydrate chain, immobilization of enzyme on metal chelating carriers, immobilization via a fusion affinity tag genetically added to enzymes.

As a result of the immobilization of the glycoprotein via a saccharide-concanavalin A binding, the access to the active sites is ensured and the amino acid residues can be protected from chemical denaturation. Concanavalin A (Con A), a sugar-binding protein from lectin class, recognizes D-mannose and D-glucose residues. Thus, Con A is being used extensively as affinity ligand for the purification of glycoproteins, immune globulins, serum proteins and cells. At pH 7.0, ConA is a tetrameric protein and every subunit contains a saccharide-binding site[8]. Urease (urea amidohydrolase, EC 3.5.1.5) is a glycoprotein and its carbohydrate chains are rich in glucose. Thus, these chains form stable complexes with Con A by self-assembly.

In the present study, the oriented immobilization method was used to immobilize urease. The Con A was first immobilized on the support by glutaraldehyde, and then, urease was immobilized on the "activated" support using the strong affinity links between the Con A and the glucose residues of the enzyme. The enzymatic kinetics of the immobilized urease via a saccharide-concanavalin A binding

Received: July 30, 2007; Accepted: September 29, 2007
Supported by:Natural Science Foundation of Jiangsu Province (No. BK2007051)
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(ConA-urease) such as optimum pH, optimum temperature, and Michaelis constant, etc. are discussed in this article. In addition, the enzymatic kinetics of the free enzyme and the immobilized urease using glutaraldehyde (GA-urease) have also been studied for comparison.

1 Materials and methods

1.1 Materials

Urease was purchased from Worthington, and was dissolved in the buffer. Chitosan was obtained from Sinopharm Chemical Reagent Co., LtdS (Shanghai, China), ConA extracted from Jack Bean (Canavalia ensiformis) was supplied by Medicago AB (Sweden). All other reagents used in the study were of analytical grade.

1.2 Immobilization procedure

1.2.1 Preparation of chitosan microspheres: A chitosan solution (2.5% *W/V*) in water was afforded by stirring a given amount of chitosan with 1% acetic acid in water to form a yellow solution, which was then extruded using 5# needle into a solution of NaOH (20% *W/V*) and CH_3OH (30% *V/V*) to form microspheres. The obtained microspheres were washed with distilled water to neutrality, and then stored at 4°C for use.

1.2.2 Urease immobilization via concanavalin A (ConA-urease): Working solution of ConA was prepared in PBS (phosphate buffer saline, pH 7.0, 0.1 M KCl, 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$), 6 h before utilization to allow the reactivation of the denatured lectin by Ca^{2+} and Mn^{2+}.

The enzyme immobilization via concanavalin A was performed by the following steps: 0.5 g of chitosan microspheres was put in glutaraldehyde solution and stirred for 2.5 h, followed by rinsing with distilled water to get rid of glutaraldehyde. Then, ConA was immobilized on the activated support, which was achieved by shaking the activated chitosan microspheres with the ConA solution for 17.5 h. Finally, urease (prepared in PBS) was allowed make contact with the resulted functionalized support for 3 h. The microspheres, taken out from the solution, were again rinsed with distilled water to remove urease.

1.2.3 Urease immobilization using glutaraldehyde (GA-urease): The enzyme immobilization using glutaraldehyde was performed by the following steps: 0.5 g of chitosan microspheres was put in glutaraldehyde solution and stirred for 2.5 h, followed by rinsing with distilled water to get rid of glutaraldehyde. Then, urease (prepared in PBS) was allowed to make contact with the support. The microspheres, taken out from solution, were again rinsed three times with distilled water to remove urease.

1.3 Protein determination

Protein concentration was determined following the method of Bradford using bovine serum albumin as the standard.

Percentage of ConA-immobilization=(total weight of protein-residual weight of protein)/ total weight of protein

1.4 Activity assays of urease

The activity of urease was determined using Nessler's reagent. One unit of urease activity is defined as the amount of enzyme, which liberates 1 μmoL NH_3 from urea per min at the desired pH and 37°C.

A 0.6 mL of phosphate buffer (pH 7.0) was added to 0.35 mL of urea solution (0.2 M) and by adding 0.05 mL of enzyme solution (1.0 mg urease per mL), the reaction was started. At 37°C, the enzymic reaction was maintained for 10 min and then quenched using 0.5ml of $ZnSO_4$ solution (10%). After adding 0.5 mL of NaOH solution (0.5 M) and 3 mL of distilled water into the solution, the mixture was filtered and 1 mL of filtrate was taken out to mix with 4.5 mL of distilled water, 0.5 mL of tartrate (10% *W/V*), 0.5 mL NaOH (0.5 M), and 1.0 mL Nessler's reagent. 10 min later, the yellow-orange color produced was measured spectrophotometrically at 460 nm (Shimadzu UV-2401PC). The amount of NH_3 liberated in the test solution was calculated by calibrating the Nessler's reagent with standard $(NH_4)_2SO_4$ solution.

The immobilized urease activity was determined as described above except that 0.25 g immobilized urease microspheres were used instead of 0.05 mL enzyme solution.

To investigate the effects of parameters on the activity of the orientedly immobilized urease, including glutaraldehyde concentration, ConA concentration, pH value of ConA solution, and urease concentration, the data were normalized for each parameter concerned, and the outcomes show the relative enzyme activity.

2 Results and Discussion

2.1 Optimal conditions for immobilizing urease

2.1.1 Effect of glutaraldehyde concentration on urease immobilization: The effect of glutaraldehyde concentration on urease immobilization was investigated in the range of 2%-5%. As seen in Fig. 1, with the increase in glutaraldehyde concentration, the percentage of ConA-immobilization onto microspheres increased continuously, and reached the maximum value of 75%, but this leveled off at the glutaraldehyde concentration of 3.5%. The activity of the orientedly immobilized urease increased when the glutaraldehyde concentration increased and reached the maximum activity when the concentration of glutaraldehyde was 3.5% and then decreased. ConA was covalently immobilized onto chitosan microspheres in the presence of glutaraldehyde, a bifunctional reagent. Therefore, the percentage of ConA-immobilization onto microspheres depended on the glutaraldehyde concentration. On one hand, by increasing the glutaraldehyde concentration, more ConA was linked on the support, so the immobilized urease