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Preparation and characterization of amyloglucosidase adsorbed on activated charcoal

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Abstract

Amyloglucosidase (AMG) [α-1, 4-D-glucan glucohydrolase (E.C.3.2.1.3)] is an exo-enzyme, which is used in the hydrolysis of starch to glucose in industries. To increase the efficiency and profitability of this process, AMG was immobilized on activated charcoal by physical adsorption without the aid of any cross-linking agent, characterized by hydrolysis of dextrin and compared with the native enzyme. The immobilized enzyme has 90% catalytic activity of the native enzyme. Optimum pH of the immobilized enzyme was six, which shifted to basic side by one unit when compared to the optimum pH of the native enzyme (5.0). Optimum temperature of the immobilized enzyme was 60°C, decreased by 10°C when compared to optimum temperature (70°C) of the native enzyme. $K_{m(app)}$ and $V_{max\ (app)}$ values of immobilized enzyme were found to be 1.0×10^{-3} g/l and 3.8×10^{-4} g/min/unit of enzyme, respectively which were less than the values of the native enzyme. The immobilized system can be used repeatedly and continuously for a longer period of time.

Keywords: Amyloglucosidase; Activated charcoal; Preparation; Characterization; Sanzyme; Starch; Dextrin; Glucose

1. Introduction

Amyloglucosidase (AMG) from *Aspergillus niger* is one of the most economically important industrial enzymes. At present, glucose is mainly produced by enzymatic hydrolysis of starch using α-amylase and AMG since it is economical and efficient, and pure product is obtained in the final stages of the process. In industrial conventional enzymatic reactions, a mixture of the substrate and soluble enzyme is incubated. After completion of each batch of reaction, the amylases are inactivated. Naturally, the process would be more economical if the enzyme could be reused, for example, by immobilization. The production of glucose from starch by AMG alone is a slow and inefficient process. Since it is an exo-enzyme, it degrades the starch molecules to glucose acting on α-1, 4 and α-1, 6 links. During hydrolysis, the glucose units are removed sequentially starting from the non-reducing end of the substrate molecules [1].

To eliminate the disadvantages present in the conventional process, AMG was immobilized on various insoluble carriers with the retention of its catalytic properties which can be used repeatedly and continuously. Although much

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research has been carried out to develop immobilized AMG systems, there has been little success in satisfying industrial requirements. Activated charcoal may be formulated with very high surface areas (600–1000 m^2/g) and a significant fraction (10–30%) of its pore volume in the 300–1000 Å range was suitable for the enzyme immobilization. However, very few reports are available on the immobilization of AMG on the activated charcoal. The immobilization of enzymes by adsorption on activated charcoal and the immobilization of lactase using glutaraldehyde as cross-linking agent were carried out [2–4]. Usami et al. [5] immobilized the AMG from *Rhizopus niveus* on the activated charcoal with the retention of 80% of the activity even after 8 h. Adsorption complex of the AMG on acid clay or activated charcoal was prepared with the retention of 30–40% of the native enzyme activity [6]. In our previous studies, we have immobilized the AMG on chitin without the aid of any cross-linking agent with the retention of 98% of native enzyme activity [7]. AMG has been immobilized on a number of other insoluble carriers [8–13]. In this study, an effort was made to immobilize the AMG on activated charcoal, since it is a cheaper carrier economically.

In this study, we have used dextrin as a substrate for the native and immobilized enzymes instead of starch itself. Dextrin was prepared by adding Sanzyme (commercially available α-amylase complex) to gelatinized cornstarch. As conformational changes of enzyme protein may occur upon immobilization, and the affinity between enzyme and the substrate may be changed, the investigation of optimum pH, optimum temperature, and kinetic constants is very important.

2. Materials and methods

Activated charcoal (LR) prepared from wood, CH_3COONa, CH_3COOH, Folin–phenol, 3, 5-dinitro salicylic acid, NaH_2PO_4, NaOH, etc., were purchased from S.D. Fine Chemicals, India. Sanzyme was obtained from Uni-Sankyo, Hyderabad, India and AMG was obtained from Novo-Nordisk Bio Medical group, Bangalore, India. Locally available cornstarch was used as a source of carbohydrate polymer. Reducing values were determined by 3, 5-dinitro salicylic acid method [14,15]. Protein content in AMG was estimated by Folin–phenol method [16].

The activities of AMG and Sanzyme were calculated as 3428 and 26 units, respectively. One unit is defined as 1 μ mole of glucose produced/min/ml of enzyme. Protein content per 1 ml of AMG was estimated as 21.25 mg and protein content of Sanzyme was 20 mg.

2.1. Preparation of activated charcoal immobilized AMG

Twenty grams of activated charcoal was suspended in 50 ml of distilled water, filtered, washed and transferred into a beaker containing 2742.4 units (17 mg protein) of AMG in a total volume of 100 ml (0.8 ml enzyme dissolved in 100 ml distilled water) and soaked at room temperature for 90 min. It was filtered and washed with 2.5 l of distilled water until the washings showed no enzyme activity. The above preparation was pressed to remove free water, weighed without drying and kept in a sealed container at 4°C. Washings were determined for protein content by the Folin–phenol method and subtracted from the initial protein content present in the enzyme taken for immobilization.

2.2. Assay of AMG

One gram of corn starch, gradually suspended in 200 ml of 0.02 M phosphate buffer (pH 5) was slowly heated on a water bath. Liquefying fungal Sanzyme (α-amylase complex) was added at the ratio of 60 mg per 1 g of starch at an initial temperature of 60°C. After liquefaction, the resulting hydrolysate was stirred for 1 h and kept overnight and was used as substrate, dextrin. By doing so, it was ob-

served that all the starch molecules were broken down into dextrins.

The activity of free as well as immobilized AMG was determined by estimating the amount of glucose produced by 3,5–dinitro salicylic acid method using dextrin as a substrate. The hydrolysis reactions were carried out in 0.02-M phosphate buffer of pH 5 and the mixture was incubated at 30°C for 60 min. The liberated glucose was measured spectrophotometrically at 540 μm for the activity of AMG. Before spectrophotometrical measurement, in the experiments involved activated charcoal immobilized enzyme, the charcoal was filtered off and readsorbed glucose on charcoal was eluted with 50% ethanol.

2.3. Stability measurements

Comparison of pH, temperature, and kinetic constants (K_m and V_{max}) of immobilized AMG with free AMG was carried out through appropriate experiments. The kinetics of the enzymatic reactions was studied through simple graphs because of high and inaccurate molecular weight of dextrin.

3. Results and discussions

To increase the efficiency of the AMG in the industrial production of glucose, another enzyme α-amylase is added for liquefaction. The α-amylase is an endo-enzyme, which increases the number of non-reducing ends by its random action on starch molecules and enhances the rate of AMG reaction [17].

During immobilization of the enzyme, the amount of enzyme protein that came down into filtrate was estimated as 619.6 units (3.84 mg). Thus, the amount of the enzyme that was bound to the activated charcoal was 2122.8 units (13.16 mg). The AMG was tightly bound on charcoal by physical adsorption method. The morphology of the activated charcoal was suitable to large loadings of enzymes for immobilization due to its very high surface area and its pore volume. No leakage of enzyme takes place during repeated uses. The preparation of this immobilized system is cheaper when compared to other systems because the activated charcoal is an inexpensive carrier. The charcoal immobilized AMG contains 36.6 units of enzyme activity per 1 g of immobilized system.

3.1. Activity

After immobilization on charcoal, the activity of AMG was calculated as 3111 units/min/ml (Fig. 1). It is 90% when compared to the activity of native enzyme (3428 units). The decrease in the activity of the immobilized enzyme when compared to the native enzyme was probably due to change of the enzyme itself, physical and chemical properties of carriers, and steric hindrance caused by the carrier at the active site.

3.2. Effect of pH

The changes in optimum pH and pH activity curve depend on the charge of the enzyme protein and/or of the water insoluble carrier. This change is useful in understanding the structure–function relationship of enzyme protein and to compare the activity of free and immobilized enzymes as a function of pH. The optimum pH of the activated charcoal immobilized AMG

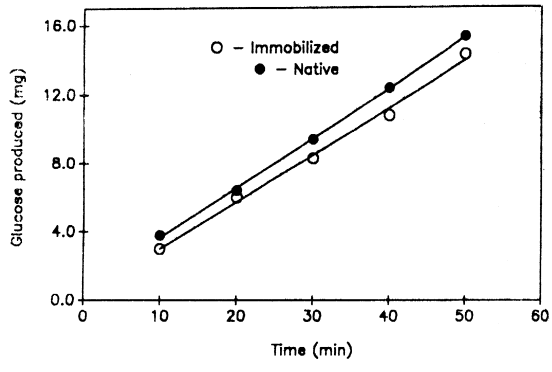

Fig. 1. Comparison of activities of immobilized and native AMG on dextrin hydrolysis.

Table 1
Comparison of characters of native and charcoal immobilized AMG

Enzyme	Optimum pH	Optimum temperature (°C)	K_m (g/l)	V_{max} (g/min/unit)
Free AMG	5.0	70	1.12×10^{-3}	4.49×10^{-4}
Charcoal immobilized AMG	6.0	60	1.0×10^{-3}	3.8×10^{-4}

was six and the optimum pH of native enzyme was five (Table 1). The optimum pH of immobilized enzyme shifted to basic side by one unit (Fig. 2), when compared to the optimum pH of native enzyme. In this figure, the activity of the enzyme was depicted as milligrams of glucose produced at 20 min of hydrolysis reaction. This increase of optimum pH may be attributed to the microenvironment of the charcoal immobilized enzyme which becomes more acidic than the external solution so that the optimum pH of immobilized enzyme shifts to acidic side.

3.3. Effect of temperature

The effect of temperature on the activity of free and immobilized AMG was studied over 30–70°C. The optimum temperature of charcoal immobilized enzyme was 60°C, whereas the optimum temperature of native enzyme was 70°C (Table 1). The optimum temperature of charcoal immobilized enzyme was decreased by 10°C although it was stable at a wide range of temperatures (Fig. 3). Also, in this figure, the activity of enzyme was depicted as milligrams of glucose produced at 20 min of hydrolysis reaction. This decrease in optimum temperature was caused by the process undertaken for immobilization, which decreases the activation energy for immobilized AMG. Most of the enzymes immobilized by physical adsorption showed a decrease in heat stability [18,19]. As charcoal immobilized enzyme shows maximum activity at lower temperature when compared to native enzyme, it leads to decrease in the cost of production of glucose because the amount of energy that is required will be minimal.

3.4. Kinetic constants

Kinetic constants measured with immobilized enzymes are not true kinetic constants equivalent to those obtained in homogeneous reactions, but are apparent values because of the effect of diffusion and other physical factors. Hence, maximum velocity and Michaeli's constant should be referred to as apparent V_{max} ($V_{max(app)}$) and apparent K_m ($K_{m(app)}$). These kinetic constants are related to the effect of substrate concentration on the activity of en-

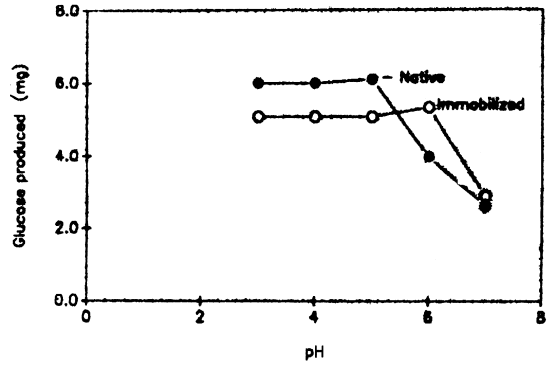

Fig. 2. Effect of pH on the activity of native and immobilized AMG.

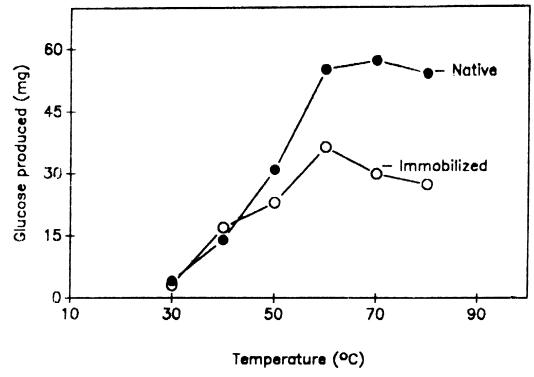

Fig. 3. Effect of temperature on the activity of native and immobilized AMG.

zyme when the concentration of enzyme was kept constant. Kinetic constants were calculated by plotting the rates of enzyme hydrolysis reactions against the concentration of substrate by plotting a simple graph. From the graphs, the $K_{m(app)}$ and $V_{max(app)}$ values were calculated.

$K_{m(app)}$ and $V_{max(app)}$ values of the activated charcoal immobilized AMG were 1.0×10^{-3} g/l and 3.8×10^{-4} g/min/unit, respectively, whereas the values for native enzyme were 1.12×10^{-3} g/l and 4.4×10^{-4} g/min/unit, respectively (Table 1). Michaeli's constant is related to the rate of reaction where the K_m value is inversely proportional to the affinity between enzyme and substrate, and also to the stability of the enzyme substrate complex. The decrease in $K_{m(app)}$ value of the charcoal-immobilized enzyme was not due to high affinity between the enzyme and substrate or higher stability of [E–S] complex. This decrease in $K_{m(app)}$ value is attributed to the electrostatic interaction between the substrate and carrier [20,21]. In carrier binding method, the electrostatic interaction between the carrier and substrate is considered to be one of the reasons for changes in K_m value upon immobilization. The $V_{max(app)}$ value of the immobilized enzyme is less than that of the native enzyme V_{max} value, which is in agreement with the activity of this immobilized system.

3.5. Continuous production of glucose in laboratory scale

A column of 2.4 cm in diameter and 57 cm in length was loaded with the charcoal immobilized AMG giving a total bed volume of 240 ml. The column was equilibrated with 0.02 M acetate buffer at pH 3.8 and 30°C (room temperature). A 10% dextrin solution was fed continuously in the column with inflow and outflow rate of 8 ml/h. To prevent microbial contamination, a small amount of toluene was added periodically. Glucose solution was checked for other carbohydrates [22] and the amount of glucose was estimated by DNS method. Glucose was the only product obtained in the solution in good yields, i.e., 390 mg per 5 ml. This immobilized enzyme was stable for 2 weeks. After 2 weeks, the activity of immobilized column gradually decreased which was recharged and recycled.

4. Conclusions

Activated charcoal is a good supporting material for the immobilization of the AMG and cheaper when compared to other carriers used for immobilization. Immobilized system should not be dried which leads to a decrease in activity. This immobilized system produces more glucose at 60°C and pH 6. The charcoal immobilized AMG can be employed for the continuous production of glucose from dextrin in industries which can be recycled and recharged after prolonged use.

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