Effect of pH on Preparation and Performance of Physically Immobilized Amyloglucosidase on DEAE-Cellulose

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This study was to optimize the pH for immobilization as well as for repeated saccharification of dextrinized starch by physically immobilized amyloglucosidase on DEAE-cellulose. Immobilization activity yield for physical immobilization of amyloglucosidase on DEAE-cellulose was high at neutral pH values. Activity of immobilized amyloglucosidase increased as pH decreased and was high at pH 4.0 under our experimental conditions. However, for the repeated batchwise saccharification of dextrinized starch, pH 4.5 was optimal as both degree of saccharification (85%) and enzyme retentivity (72%) of immobilized enzyme were high at the end of third cycle.

Der Einfluß des pH-Wertes auf die Darstellung und Wirkung von physikalisch an DEAE-Cellulose immobilisierter Amyloglucosidase. Diese Untersuchung diente der Optimierung des pH-Wertes zur Immobilisierung als auch zur wiederholten Verzuckerung von dextrinierter Stärke durch an DEAE-Cellulose physikalisch immobilisierter Amyloglucosidase. Die Ausbeute an Immobilisierungsaktivität für die physikalische Immobilisierung von Amyloglucosidase an DEAE-Cellulose war bei neutralen pH-Werten hoch. Die Aktivität von immobilisierter Amyloglucosidase nahm mit abnehmendem pH zu und war unter unseren experimentellen Bedingungen bei pH4 hoch. Für die wiederholte chargenweise Verzuckerung von dextrinierter Stärke war jedoch pH 4,5 optimal, da sowohl der Verzuckerungsgrad (85%) als auch die Haltekraft (72%) des immobilsierten Enzyms am Ende des dritten Cyclus hoch waren.

1 Introduction

In the conversion of starch to glucose, immobilized amyloglucosidase could offer an advantage over the use of soluble enzyme as the immobilized enzyme can be recovered for reuse [1, 2] Amyloglucosidase was immobilized on DEAE-cellulose by many workers [3-6]. Physical immobilization process is more attractive in commercial practice than the covalent process because physical immobilization is a simple and cheap process [2, 5]. Furthermore, physical immobilization conditions are mild and permit easy reloading of carrier [5]. Thus, we have exploited the physical method to immobilize the amyloglucosidase protein to DEAE-cellulose. Chromatogra-

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phic studies have revealed that interactions between a protein and an ionic carrier depend on the pH of the medium. Moreover, the pH has great influence over the activity of enzymes. Hence, this study was focused on the influence of pH both on physical immobilization of amyloglucosidase to DEAE-cellulose and repeated batch saccharification of dextrinized starch by the immobilized enzyme.

2 Experimental

Materials

Amyloglucosidase (Spiritamylase 150 L) and (heat-stable) α-amylase (Termamyl 60 L) were obtained from NOVO Industries. DEAE- cellulose (No.D 8382) was purchased from Sigma Chemical Company, U.S.A., and soluble starch was from B.D.H. Ltd, England.

2.1 Analytical methods

The reducing equivalents were measured in terms of glucose by dinitrosalicylic acid method [7] and protein by micro-*Kjeldahl*-method [8].

2.2 Preparation of dextrinized starch

Dextrinized starch was prepared by incubating 200ml starch suspension (25%w/v) at pH 7.0 and with α-amylase (50μl) at 95°C for 1h and terminating α-amylase activity by lowering the pH to 3.5 and boiling the dextrinized starch solution for 30S [9]. The final volume of the dextrinized starch was made up to 250ml and its concentration and DE values were 20% (w/v) and 36% respectively.

2.3 Assay of amyloglucosidase activity

Activities of soluble and immobilized amyloglucosidase were determined with 20% wv of dextrinized starch DE 36, at pH 4.5, and 57°C. In this experiment, 0-16mg protein of soluble or immobilized enzyme was used. Unit of enzyme activity is defined as the amount of enzyme that releases one mg glucose per min.

2.4 Physical immobilization of amyloglucosidase on DEAE-cellulose at different pH values

Amyloglucosidase (811 units in 65mg protein) in 5.0ml citrate-phosphate buffer of varying pH values (3.0-7.5) was incubated with 4.0ml activated DEAE-cellulose [10] (1.0g) at 30°C for 4h in a reciprocal shaker (120rpm). The immobilized amyloglucosidase was separated by filtration and the activity of amyloglucosidase in the filtrate was determined at pH 4.5 and 57°C. The amount of amyloglucosidase units immobilized on DEAE-cellulose is the difference between the added amyloglucosidase units and amyloglucosidase units in the filtrate that is not immobilized by the resin [11, 12]. The controls were the amyloglucosidase (811 units in 65mg protein) solutions in buffers of respective pH kept under the same conditions.

2.5 Saccharification profile at pH 4.5

Dextrinized starch of DE 36 (16% w/v) was hydrolyzed by soluble and immobilized enzyme (each consisting of 11.3mg protein) at pH 4.5 and at 57°C for 90min.

2.6 Repeated use of immobilized enzyme for the saccharification of dextrinized starch

At different pH (4.0-7.0), same amount (11.3mg protein) of immobilized enzyme preparation was used to saccharify 50ml of 16% w/v dextrinized starch of DE 36 at 57°C. Specific activities of immobilized enzyme were determined at different pH values. At the end of 1h saccharification, DEAE-cellulose immobilized amyloglucosidase was recovered by filtration, washed with 5.0 ml of corresponding buffer and reused in the next saccharification cycles. Degree of saccharification and enzyme retentivity were determined at the respective pH values.

3 Results and Discussion

3.1 Effect of pH on immobilization

The variation in the immobilization activity yield (%) of DEAE-cellulose immobilized amyloglucosidase with pH in the range of 3.0 to 7.5 had a sigmoidal shape (Fig.1). The mid

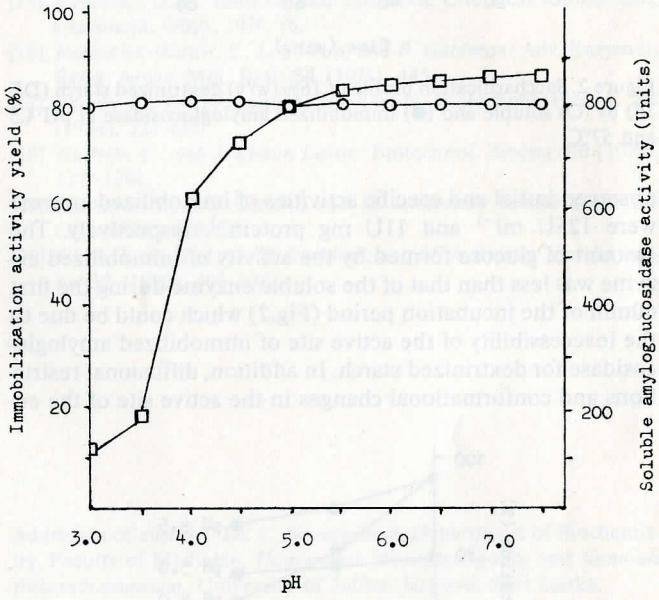


Figure 1. Effect of pH on physical immobilization of amyloglucosidase on DEAE-cellulose at 30°C. (○) Amyloglucosidase units in control after 4h of incubation and (□) immobilization activity yield. Immobilization activity yield = [(amyloglucosidase units added − amyloglucosidase units not immobilized by the resin) / amyloglucosidase units added] x 100.

point of the immobilization activity yield which corresponds to isoelectric point of amyloglucosidase is between pH 3.5 and 4.0. This observation agreed with the report of *Fogarty* (1983) [13]. When immobilizing amyloglucosidase on DEAE-cellulose, immobilization activity yield was high at neutral pH values and it was 86% at pH 7.0. Hence, pH 7.0 was selected as suitable for immobilization.

Since the soluble enzyme was stable for 4h between pH 3.0 and 7.5 (Fig.1) it can be presumed that decrease in activity in the free solution (filtrate) was due to physical binding of enzyme with carrier. Thus, the above observation validates the estimation given by *Woodward* (1985) [11] and *Fradet* et al. (1985) [12].

3.2 Saccharification profile for soluble and immobilized enzyme

The hydrolysis of 16% (w/v) dextrinized starch DE 36 by soluble and immobilized amyloglucosidase is shown in Fig.2. The

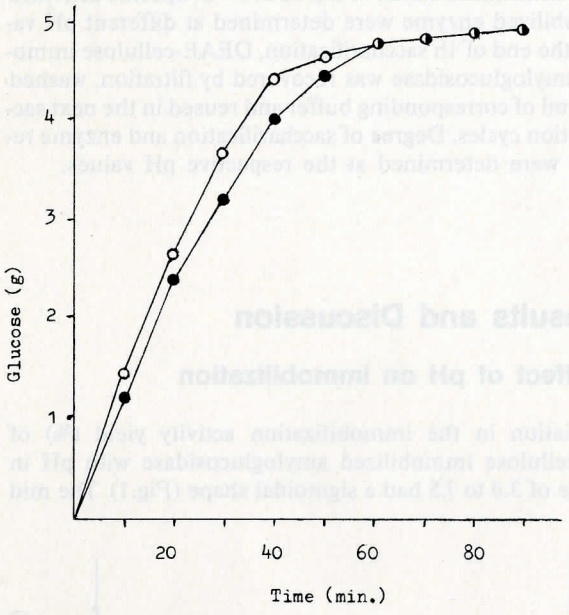


Figure 2. Saccharification profile of 16% (w/v) dextrinized starch (DE 36) by (○) soluble and (●) immobilized amyloglucosidase at pH 4.5 and 57°C.

observed initial and specific activities of immobilized enzyme were 125U ml^{-1} and 11U mg $protein^{-1}$, respectively. The amount of glucose formed by the activity of immobilized enzyme was less than that of the soluble enzyme during the first 60min of the incubation period (Fig.2) which could be due to the inaccessibility of the active site of immobilized amyloglucosidase for dextrinized starch. In addition, diffusional restrictions and conformational changes in the active site of the enzyme due to changed micro-environment can also contribute to the observed low activity of the immobilized amyloglucosidase [11, 14-16]. However, the glucose formation at the end of 1h by both the soluble and immobilized amyloglucosidases was same. This is equivalent to a degree of saccharification of 95.2%. Complete hydrolysis was not achieved although the incubation was prolonged to 90min. This may be due to isomaltose formation by the reverse reaction [13, 17].

3.3 Effect of pH on saccharification performance

The immobilized enzyme showed higher activity at pH 4.0 than at pH 4.5 and the optimum would be well below pH 4.0 (Fig.3). The activity of the immobilized enzyme was not determined below its isoelectric point (pH 4.0) as the net charge on the enzyme is changed leading to its leaching from its carrier.

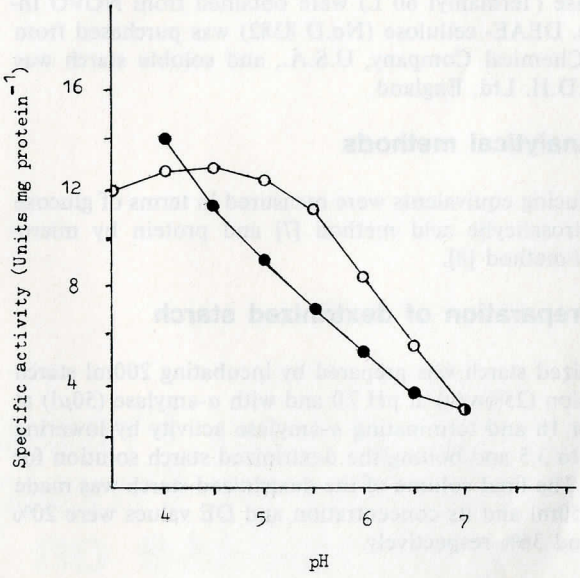
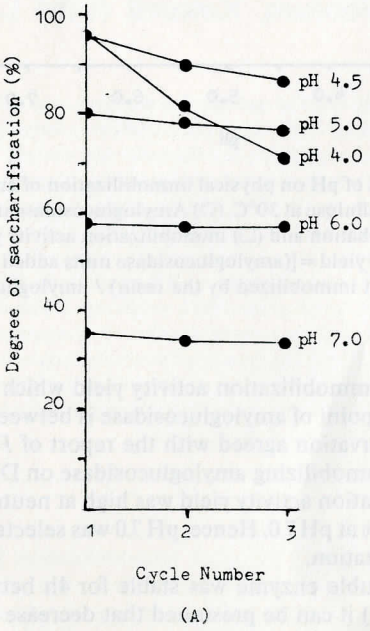


Figure 3. Effect of pH on soluble and immobilized amyloglucosidase activity with dextrinized starch DE 36 at 57°C. Specific activities of (○) soluble and (●) immobilized amyloglucosidase.



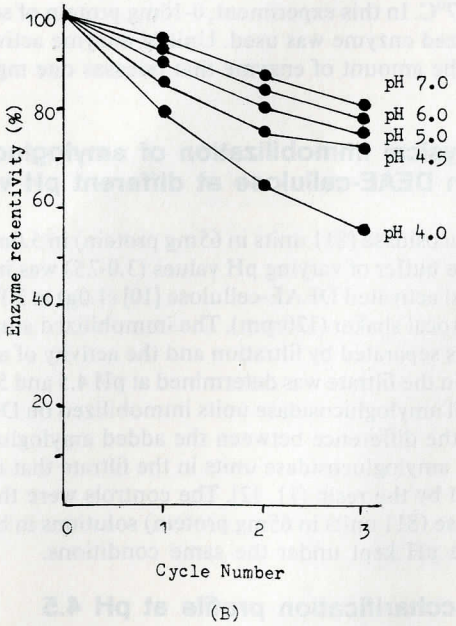


Figure 4. Effect of pH on (A) the degree of saccharification and (B) enzyme retentivity of immobilized enzyme with increasing cycles of saccharification of 16% (w/v) dixtrinized starch (DE 36). Degree of saccharafication = [(reducing equivalents at the end of cycle − reducing equivalents at zero time) / (Total reducing equivalents of the substrate − reducing equivalents at zero time)] x 100; Enzyme retentivity = [(enzyme activity at the end of cycle − enzyme activity at the beginning) / enzyme activity at the beginning] x 100.

Acidic shift on pH optimum had been reported when immobilizing the enzymes on anion- exchange carriers [5, 15, 18]. The repeated batch saccharification experiments carried out with DEAE-cellulose immobilized amyloglucosidase are shown in Fig. 4. With consecutive saccharification cycles, the enzyme activity decreased. This decrease may be due to the influence of many factors such as desorption (or leaching out), heat inactivation and pH inactivation [15, 19]. *Ghali,* et al. (1980) [20] reported that when Amberlite-IR 45 immobilized amyloglucosidase was used in a repeated batch saccharification process carried out at pH 4.5, the residual activity decreased along with the number of reaction cycles. The enzyme retentivity after the completion of 3 repeated batch hydrolysis was high at pH 7.0 (80%). When the hydrolysis was carried at pH of 4.0, the enzyme retentivity after 3 repeated batches was reduced to 56%. As the pH of the hydrolytic medium was brought near to the isoelectric pH of amyloglucosidase, the net negative charge of the enzyme molecule would be progressively reduced and thereby leading to a reduction in the strength of electrostatic interaction between charged enzyme molecule and oppositely charged carrier. Therefore when considering the degree of saccharification, and enzyme retentivity, pH 4.5 could be selected as the optimum working pH for repeated batch hydrolysis of dextrinized starch by the DEAE-cellulose immobilized amyloglucosidase. At this pH, the immobilized enzyme showed 72% of enzyme retentivity and a degree of saccharification of 85% at the end of the 3rd cycle.

4 Conclusion

Our studies have shown that the pH of the medium could greatly influence the immobilization activity yield and the performance of the physically immobilized amyloglucosidase. Furthermore, it could be observed that neutral pH enhances the immobilization activity yield and enzyme retentivity of DEAE-cellulose immobilized amyloglucosidase while weak acidic pH favours the saccharifying activity of immobilized enzyme preparation. To immobilize amyloglucosidase on DEAE-cellulose, pH 7.0 could be selected whereas to use the immobilized enzyme in repeated saccharification process, pH 4.5 was more suitable than other pH values. However, leaching of the physically immobilized amyloglucosidase appears to be a major problem while operating at or near pH 4.5.

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