ScienceAsia 28 (2002) : **247-251**

Immobilization of a thermostable alpha-amylase

Saiyavit Varavinit[a], *, Narisa Chaokasem[a] and Sujin Shobsngob[b]
[a] Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.
[b] Department of Chemistry, Faculty of Science, Mahidol University.
* Corresponding author, E-mail: scsva@mahidol.ac.th

Received 15 May 2001
Accepted 14 Dec 2001

ABSTRACT Cellulose fibers from bagasse were oxidized by periodic acid at positions 2 and 3 of the anhydroglucose unit to obtain dialdehyde cellulose. The aldehyde groups of the dialdehyde cellulose were able to react with amino groups of a thermostable alpha-amylase to form covalent bonds and resulted in a dialdehyde cellulose immobilized enzyme. The optimum pH of this immobilized enzyme was pH 7-9 while that of the free enzyme was pH 7.0. The optimum temperature for free and immobilized enzymes was 90 °C and 95 °C, respectively. The activity yield of the immobilized enzyme was 44% . Thermostable alpha- amylase is normally used as starch liquefying enzyme in the production of dextrose. The stability of immobilized enzyme was tested by studying its ability to liquefy 5% gelatinized tapioca starch over 10 reused cycles. The viscosity of 5% gelatinized tapioca starch solution was 2330 cP, while viscosity of the liquefied starch solution produced by the reused immobilized enzyme after more than 10 reused cycles was only 50 cP. Thus, it was very effective in reducing starch viscosity and the immobilized enzyme was very stable.

KEYWORDS: cellulose, covalent binding, dialdehyde, immobilization, thermostable alpha- amylase.

INTRODUCTION

Immobilized enzymes are used in food technology, biotechnology, biomedicine and analytical chemistry. They have various advantages over free enzymes including easy separation of the reactants, products and reaction media, easy recovery of the enzyme, and repeated or continuous reuse. Enzymes can be immobilized to a multitude of different carriers by entrapment, adsorption, ionic binding and covalent binding. Covalent binding is very effective in retaining the enzyme and can achieve high activity after immobilization, if amino acid residues that are covalently bound with the support material are not involved in the active site or substrate-binding site.

Enzyme immobilization by covalent binding has the following advantages: (1) enzymes do not leak or detach from the support during utilization because of tight binding; (2) the immobilized enzyme can easily contact with the substrate because it is localized on the support surface; and (3) an increase in heat stability is often observed because of the strong interaction between the enzyme and support material.[1]

Cellulosic materials have been widely used as carriers for immobilized enzymes. Their advantages are accessibility, cheapness, hydrophilic character and a great number of hydroxyl groups on the surface capable of chemical reaction. Disadvantages as a carrier are low mechanical resistance and facility for biodegradation.[2] Enzymes can be immobilized on cellulose and its derivatives in a variety of ways. These include adsorption on cellulose ion exchangers (eg DEAE-cellulose[3]), binding via chelate ring formation on the surface of cellulose activated by the salts of transition metals and covalent binding[2]. Among the latter may be mentioned binding with cellulose activated by cyanogen bromide[4], binding by means of glutaraldehyde with aminethyl cellulose[5], binding with the use of triazine[6], and binding by the azide method to carboxymethyl cellulose.[7] The aim of the present work was to examine the possibility of immobilization of thermostable alpha-amylase on bagasse (ie, a natural cellulosic material) by activation with periodic acid.

MATERIALS AND METHODS

Materials

Thermostable alpha-amylase of *Bacillus licheneformis* (Termamyl 60 L) was purchased from Novo industries, Denmark. Bagasse used as carrier and tapioca starch were purchased from the local market. Sodium meta periodate, sulphuric acid and soluble potato starch were obtained from Merck Co Ltd.

METHODS

Bagasse purification and proximate analysis

A 1 kg of bagasse was boiled 3 times in 6 L of distilled water (3x2000 mL) for 1 hour each time and dried in hot air oven and then cut into small pieces (range between 180 μm and 355 mm). After that, 15 g of bagasse was purified by boiling in 2000 mL of 1.25% sulphuric acid and 2000 mL of 1.25 % sodium hydroxide for 1 hour each time, followed by washing with 1000 mL of distilled water and 500 mL of 95% ethanol. Then it was dried in hot air oven at 100 °C for 2 hours.

Protein[8], fat and crude fiber[9], ash and moisture[10] contents were analyzed by AOAC (1990) method. Cellulose content was analyzed by the method described in Food and Chemical Codex.[11]

Bagasse oxidation[12]

A 5 g of purified bagasse obtained from the above step was presoaked in 375 mL of 0.03 M periodic acid. The solution was adjusted to pH 3.0, followed by heating in water bath at 90 °C with constant shaking at 200 rpm for 15 hours. The bagasse was then filtered and washed 3 times with distilled water (3x1000 mL) before drying. The number of aldehyde groups obtained were analyzed by the method described elsewhere.[13]

Immobilization of thermostable alpha-amylase

A 1.0 g sample of dried oxidized bagasse was immersed in 30 mL of thermostable alpha-amylase solution and incubated in water bath with constant shaking at 50 °C for 30 minutes. Then the bagasse was filtered to remove excess enzyme and followed by washing with 800 mL of distilled water. Next, the immobilized enzyme was washed again with 100 mL of distilled water. The washed water was tested for the enzyme activity which was found to be absent.

Determination of activity yield

The activity yield was defined here as the yield for enzyme which was immobilized on the bagasse and expressed by the following equation.

$$\text{Activity yield (\%)} = 100 \times \frac{\text{Activity of immobilized enzyme}}{A - B} \quad \ldots (1)$$

Where A is the activity of free enzyme added, and B is the activity of unimmobilized enzyme (remaining enzyme and unimmobilized enzyme in washed water)

Assay of free and immobilized enzyme activity

The activity of free enzyme was determined by taking 0.50 mL of free enzyme into 1.5 mL of 2% (w/v) soluble potato starch solution containing 500 ppm of Ca^{2+} (cofactor) and 1.0 ml of tris (hydroxymethyl aminomethane/HCl) buffer (pH 7.0). The mixture was incubated in a water bath with constant shaking at 40 °C for 15 minutes. Then the reaction mixture was taken for measuring reducing sugar released by the DNS (dinitrosalicylic acid) method.[14]

The activity of immobilized enzyme was determined by adding 0.25 g of immobilized enzyme (dry weight) into 20 mL of 2% soluble potato starch solution containing 500 ppm of Ca^{+2} and 13 mL of tris (hydroxymethyl aminomethane/HCl) buffer (pH 7.0). The mixture was incubated in water bath with constant shaking at 40 °C for 15 minutes. The reaction was stopped by removing the bagasse from solution.

One unit of thermostable alpha-amylase activity was expressed in terms of reducing sugar released (mmole/ml min) as measured by the DNS method. The immobilized enzyme had to be washed with distilled water before re-measuring its activity in successive used cycles.

Liquefaction process

A 5 g sample (dry weight) of immobilized enzyme was added and stirred in 500 mL of 5% cooked tapioca starch containing 500 ppm of Ca^{+2} at 95 °C for 5 minutes. The immobilized enzyme was filtered from solution and then the viscosity of the solution was measured at 30 °C by viscometer (Brookfield Digital Viscometer Model DV-II + Version 3.0). After each cycle, the immobilized enzyme was washed with distilled water before reused.

RESULTS

Purified bagasse was analyzed for moisture, protein, fat, ash and crude fiber contents as shown in Table 1. Cellulose in bagasse was purified by leaching of the impurities from bagasse with diluted sodium hydroxide and sulphuric acid. The purified bagasse was analyzed for cellulose content and it was found to be 71.22% of dried weight. Immobilization of enzyme through covalent binding to bagasse was based on the fact that cellulose is the main component in bagasse and that it can be reacted with periodic acid to form dialdehyde cellulose[12] (Figure1). The coupling reaction between enzyme and dialdehyde groups is shown in Figure 1. The

number of aldehyde groups obtained at various oxidation times is shown in Figure 2.

The data for enzyme immobilization is presented in Table 2. The activity yield calculated from equation (1) and Table 2 was 44%. The optimum pH at 40 °C of free and immobilized thermostable alpha-amylase on oxidized bagasse were somewhat different (Figure 3). At 40 °C, the optimum pH for the free enzyme was in the range of 6 to 7, while that for the immobilized enzyme was over a broader range of 7 to 9. The immobilized enzyme had higher activity than that of the free enzyme over the pH range 8 to12. At 90 °C, activity for both the free and the immobilized enzyme was optimum at pH 7.0 (Figure 4).

The data for relative enzyme activity versus temperature at pH 7 is shown in Figure 5. It was

Table 1. Proximate analysis[a] of purified bagasse.

Analytical items	%
protein (Nx6.25)	0.42
fat	0
ash	0.56
crude fiber	79.58
moisture	8.38

[a] Values represent are the mean of two determinations.

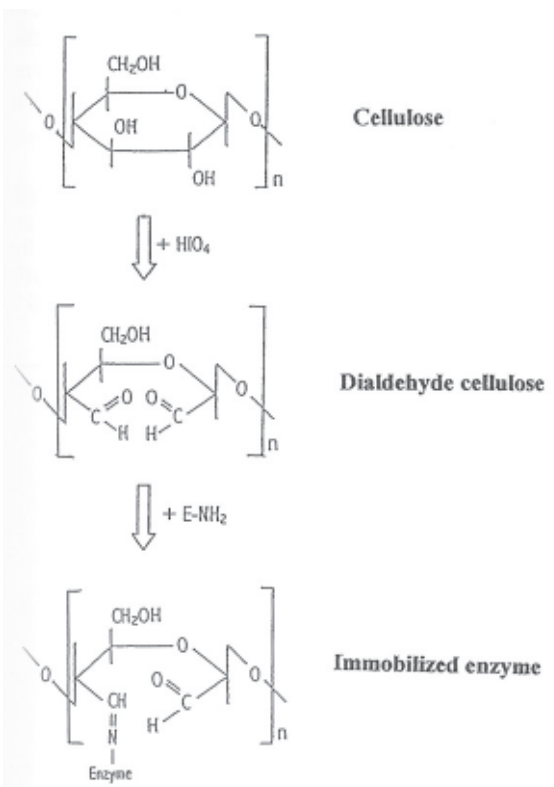

Fig 1. Scheme of the enzyme immobilization.

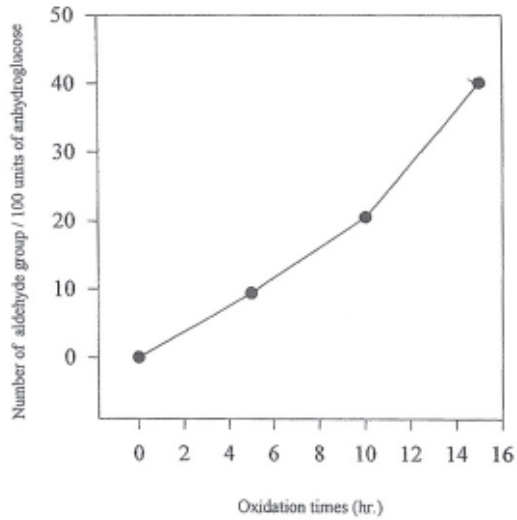

Fig 2. The quantity of aldehyde groups in oxidized bagasse obtained at various oxidation times.

Table 2. Thermostable alpha-amylase activities in various fractions during the immobilization process.

Step	Total thermostable alpha-amylase activity (units)[a]
Soluble enzyme before immobilization 30 ml)	666,400 ± 371
Remaining enzyme removed after immersion process (23ml)	510,907 ± 708
Unimmobilized enzyme in washed water (800 mL)	148,706 ±1324
Immobilized enzyme (0.85 g)	2,967 ± 14

[a] Values represent are the mean of three determinations.

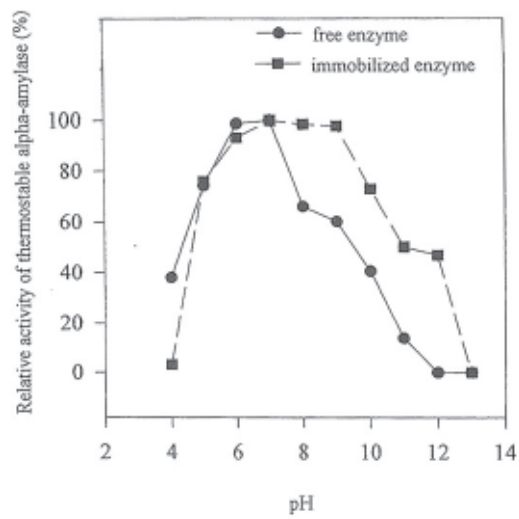

Fig 3. Effect of pH on enzyme activity of free and immobilized thermostable alpha-amylase. The activity was determined at 40 °C at various pH with 2% soluble potato starch containing 500 ppm of Ca^{2+} as substrate.

found that the optimum temperature for free and immobilized enzyme was 90 °C and 95 °C, respectively. However, the immobilized enzyme gave higher activity than that of the free enzyme over the temperature range 30 °C to 80 °C. The activity decreased sharply at temperatures higher than 90°C for free enzyme and 95 °C for immobilized enzyme.

The stability of the immobilized enzyme was also tested by studying its ability to liquefy starch in 10 cycles of reuse (Table 3). The immobilized enzyme was filtered and washed before reuse in each cycle . Viscosity of the liquefied starch was very low for the first cycle and it increased continuously thereafter until the tenth cycle.

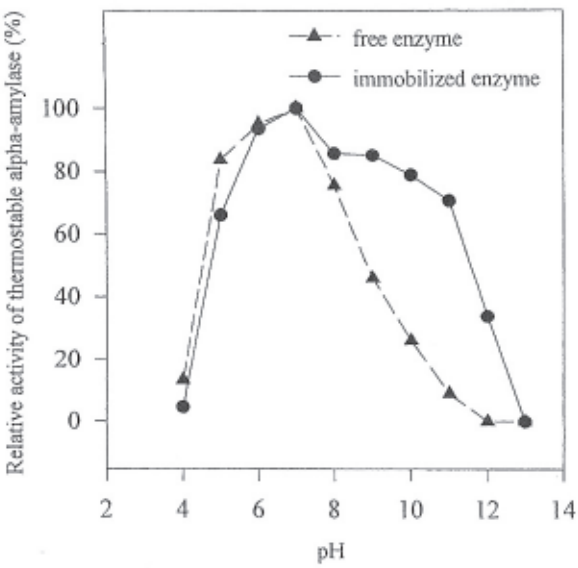

Fig 4. Effect of pH on enzyme activity of free and immobilized thermostable alpha-amylase. The activity was determined at 90 °C at various pH with 2% soluble potato starch containing 500 ppm of Ca^{2+} as substrate.

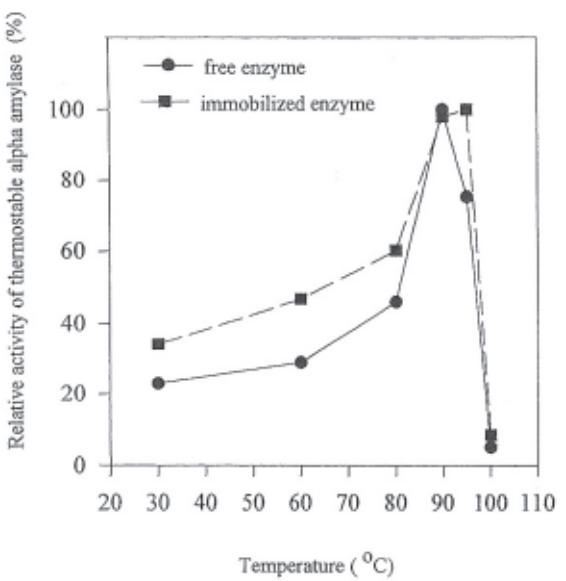

Fig 5. Effect of temperature on enzyme activity of free and immobilized thermostable alpha-amylase. The activity was determined at pH 7 at various temperature with 2% soluble potato starch containing 500 ppm of Ca^{2+} as substrate.

Discussion

The number of aldehyde groups increased with the increase of reaction time. Longer oxidation time (after 16 hours) caused total destruction of cellulose fiber to produce a very fine cellulose powder, and is not suitable for the recovery of immobilized enzyme after the enzymatic reaction. In the immobilization process, the activity yield should be as high as possible. Very low activity yield indicated a worthless immobilization process. If 1% activity yield is obtained, it is necessary to operate 100 cycles of immobilized enzyme to be equivalent to the use of free enzyme. The 44 % activity yield for this study should be acceptable, since only 3 cycles of operation of this immobilized enzyme is worth better than the use of free enzyme. For the optimization of pH at 40 °C and 90 °C, these results demonstrated that the immobilized enzyme provided higher relative activity than that of free enzyme, especially at the pH range 8 to 12 . For optimization of the temperature of immobilized enzyme, the relative activity was higher at the temperature range of 30 °C to 80 °C than that of free enzyme. The stability of immobilized enzyme was tested by studying its ability to liquefy starch in 10 cycles of reuse (Table 3).

Table 3. Effect of number of reused cycles of immobilized enzyme on the starch viscosity. The viscosity of 5% of tapioca starch paste containing 500 ppm of Ca^{2+} after liquefying at 95 °C.

Number of cycles	Viscosity(cP)[a]
0	2,330±15
1	12±0.7
2	13±0.8
3	16±0.9
4	19±0.6
5	21±0.9
6	31±0.7
7	51±2
8	64±4
9	65±2
10	66±2

[a] Values represent are the mean of three determinations.

The viscosity of a 5% gelatinized tapioca starch solution was 2330 cP, while viscosity of the liquefied starch solution produced by the immobilized enzyme after more than ten cycles of reuse was only 66 cP. Thus, it was very effective in reducing starch viscosity. This would confirm the good stability of the immobilized thermostable alpha -amylase.

This investigation has developed a technique to prepare long life immobilized enzyme using bagasse as a cheap immobilization support. The iodic acid or iodate ion resulting from the reaction of periodic acid and bagasse can be reoxidized to periodic acid by reacting with calcium hypochlorite or other stronger oxidizing agents than periodic acid[12,15-17]. The iodic acid can also be oxidized electrochemically to periodic acid.[18]

Conclusion

Cellulose in bagasse can be oxidized by periodic acid at position 2, 3 of anhydroglucose unit to form dialdehyde cellulose (bagasse), which can further be reacted with the amino groups of thermostable alpha-amylase to form covalently bound immobilized enzyme. This immobilized enzyme can be used to liquefy starch for further use in the production of dextrose.

Acknowledgement

The authors would like to thank to the Mahidol University for the financial support.

References

1. Gerhartz W (1990) General Production Methods In: *Enzymes in Industry*: VCH Publishers, New York (USA) , p 67.
2. Przybyt M and Sugier H (1988) Immobilization of glucoamylase. *Starch/Staerke* **40**, 275-9.
3. Jach M and Sugier H (1983) Adsorption of glucoamylase. EC-3.21.3. *Starch/Staerke* **35**, 427.
4. Porath J (1967) Chemical coupling of peptides and proteins to polysacchrides. *Nature* **214**, 1302.
5. Lilly MD (1970) Immobilized Enzymes In: *Method in Enzymology*. Ed K Mosbach: Academic Press. New York **44**, 46.
6. Kay G and Crook EM (1967) Coupling of enzymes to cellulose using chloro-δ-triazines. *Nature* **216**, 514.
7. Crook EM et al (1970) Cellulose-Insolubilized Enzymes In: *Methods in Enzymology* Ed GE Perlmann. L Lovand: Academic Press, New York **19**, 963.
8. AOAC, Official Method of Analysis , protein, 15th edition, Association of Official Analytical Chemistry, Arlington, USA, 1990, p 781.
9. AOAC , Official Method of Analysis , fat and crude fiber 15th edition, Association of Official Analytical Chemistry, Arlington, USA, 1990, p 780.
10. AOAC , Official Method of Analysis , ash and moisture content, 15th edition, Association of Official Analytical Chemistry, Arlington, USA, 1990, p 777.
11. Committee on CODEX specification. Food chemical codex. 3rd edition. Washington, DC: National Academy Press; (1981).
12. Mehltretter CL (1967) Production and use of dialdehyde starch, In *Starch: Chemistry and Technology*. Whistler, RL and Paschall, EF, Eds, Academic Press. New York 2, chap 18.
13. Shriner RL (1980) The detection of functional groups. *Complete structure determination*. John Wiley & Sons, New York, 170.
14. Bernfeld P (1995) Amylase and Enzymes of Carbohydrate Metabolism. In: *Methods in Enzymology*. 1 p 149-58.
15. Mehltretter CL (1966) Recent progress in dialdehyde starch technology. *Die Staerke* **18**, 208.
16. Mehltretter CL(1963) Some landmarks in the chemical technology of carbohydrate oxidation. *Die Staerke* **15**, 313.
17. Pfeifer VF (1960) Dialdehyde starch using electrolytic regeneration of periodic acid. *Ind Eng Chem* **52**, 201.
18. McGuire TA and Mehltretter CL (1971) Chemical process for making dialdehyde starch. *Die Staerke* **23**, 42.