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Immobilization of Glucoamylase Onto Novel Porous Polymer Supports of Vinylene Carbonate and 2-Hydroxyethyl Methacrylate

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Abstract

Glucoamylase was immobilized onto novel porous polymer supports. The properties of immobilized glucoamylase and the relationship between the activity of immobilized enzyme and the properties of porous polymer supports were investigated. Compared with the native enzyme, the temperature profile of immobilized glucoamylase was widened, and the optimum pH was also changed. The optimum substrate concentration of immobilized glucoamylase was higher than that of native enzyme. After storage for 23 d, the immobilized glucoamylase still maintained about 84% of its initial activity, whereas the native enzyme only maintained about 58% of the initial activity. Moreover, after using repeatedly seven times, the immobilized enzyme maintained about 85% of its initial activity. Furthermore, the properties of porous polymer supports had an effect on the activity of the immobilized glucoamylase.

Index Entries: Glucoamylase; immobilization; porous supports; vinylene carbonate; 2-hydroxyethyl methacrylate.

Introduction

Glucoamylase (EC 3.2.1.3), an exosplitting enzyme that catalyzes the stepwise hydrolysis of α-(l,4) linkages and, to a lesser extent, α-(l,6) linkages

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from the nonreducing ends of starch and glycogen, is widely used in industry. Because of its valuable application in the industrial starch conversion process, the immobilization of glucoamylase is important. Therefore, much attention has been paid to immobilization methods, through which the enzyme can be immobilized onto a suitable support, and the resulting immobilized enzyme could possess much better properties, such as higher activity and better operational, thermal, and storage stabilities than the native enzyme. Among all of the methods used for immobilizing glucoamylase, covalent bonding was widely investigated because of its obvious advantages (e.g., stable, wide range of choices, etc.) compared with the other methods *(1–10)*. To effectively immobilize glucoamylase onto supports through covalent bonding, many quite different support materials, such as porous glass fibers and beads *(1)*, chitin particles *(2)*, hen egg white *(3)*, natural polymer materials *(4)*, artificial polymer materials, and magnetic microparticles *(5–10)*, were employed. Compared with other supports, artificial polymer materials and correspondingly derived magnetic supports have relatively good properties owing to the possibility of designing the supports' structure through polymerization or chemical and physical modification. Recently, Arica and colleagues *(11–14)* prepared magnetic poly (methylmethacrylate) microspheres, poly (2-hydroxyethylmethacrylate) membranes, and poly (2-hydroxyethylmethacrylate-ethylene glycol dimethacrylate) microspheres that were covalently attached to a six-carbon spacer arm (i.e., 3 hexamethylene diamine) before enzyme immobilization. After the enzyme was immobilized onto these supports, its operational, thermal, and storage stabilities were increased. Although these artificial polymer materials possess many unique advantages, before they are employed to immobilize enzymes, they must undergo an additional activation processing by using some functional coupling reagents.

Bahar and Celebi *(15–17)* investigated the properties of glucoamylase immobilized directly onto low-cost magnetic poly (styrene) particles including active groups in one step without any additional activation process. The maximum activity of the immobilized glucoamylase was approx 70% of the free one, and the activity half-life of immobilized enzyme was improved to 190 h, much longer than the 33.8 h of the free one. Recently, a series of nonporous beadlike and hydrophilic polymer materials containing reactive cyclic carbonate groups was employed in trypsin immobilization as an initial evaluation *(18–22)*. Since the cyclic carbonate group on the support materials can react with the amino groups under mild conditions, these supports could be used to immobilize enzymes without any additional activation process. It was found that the amount of enzymes coupled to the supports and the specific activity of the immobilized trypsin were related to the content of cyclic carbonate structure units and the reaction time.

In the present study, glucoamylase was immobilized on a series of porous beaded hydrophilic polymer materials containing reactive cyclic carbonate groups. These porous polymer matrices were synthesized via

reverse-phase suspension copolymerization of vinylene carbonate (VCA) and 2-hydroxyethyl methacrylate (HEMA) in paraffin oil *(23)*. We studied a variety of kinetic properties and stability parameters of glucoamylase in both free and 4 immobilized states by using liquefied starch as substrates.

Materials and Methods

Materials

Glucoamylase (Novozym AG) was purchased from Novo Nordisk (Tianjin, China). Liquefied starch (D.E. 15-20) was obtained from Funing Glucose Factory (Qinhuangdao, China). Porous polymer materials were synthesized by antiphase suspension polymerization of VCA and HEMA, with different organic solvents as porogenic reagent. The detailed synthesis and properties of porous polymer materials are provided in ref. *23*. Before use, these polymer supports were swelled with acetic acid–sodium acetate buffer (pH 6.26, 0.2 mol/L).

Immobilization of Glucoamylase

Glucoamylase (100,000 U/mL, liquid) was used. The standard amount of glucoamylase for all experiments, unless stated otherwise, was 28 U/mL, for which U/mL is defined as the amount of glucose (mg) liberated from liquefied starch under the catalysis of 1 mL of glucoamylase of 60-min duration at pH 4.6 and 40°C. Immobilizing glucoamylase to supports was implemented by adding enzyme to a tube containing 30 mg of polymer particles, followed by mixing at 100 rpm for 20 h at 10°C. After immobilization, excess solution was poured out of the tube, and the residue was successively washed by using corresponding acetic acid–sodium acetate buffer (0.2 mol/L), whose pH was the same as that of the enzyme solution, to the extent that no glucoamylase could be detected any more from the buffer.

Determination of Amount of Immobilized Glucoamylase

The amount of glucoamylase immobilized onto the polymer supports was estimated from the difference between the amount of glucoamylase in the enzyme-buffer solution before and after immobilization. The glucoamylase was measured using a spectrophotometer 3010 (Hitachi Model U-3010 type) at 225 nm.

Glucoamylase Assay

Glucoamylase activity was determined by measuring the glucose liberated from liquefied starch. Standard reactions were carried out at 40°C using 4% (v/v) liquefied starch. Substrate solution (1 mL) was added to either 0.5 mL of native glucoamylase or 30 mg of the immobilized glucoamylase (dry weight) in a tube. The duration of enzyme catalysis was 30 min. After catalysis, the reaction solution was boiled for 10 min to inactivate the residual glucoamylase. The amount of reducing sugar produced

was determined colorimetrically by using 3,5-dinitrosalicylic acid as color-developing reagent *(24)*. Absorbance was measured at 520 nm with a double-beam spectrophotometer (Hitachi Model U-3010 type) using glucose solution as the standard. The enzyme activity of the immobilized glucoamylase was assessed on the basis of 1 mg of dried polymer particles.

Determination of Optimum Binding Conditions

Glucoamylase solution with different pH values (3.40–6.26) was added to the polymer 6 supports to test the optimum pH of the enzyme to be immobilized. Glucoamylase with different concentrations (16–40 U/mL) was added to the supports to determine the maximum amount of enzyme that could be immobilized. Furthermore, glucoamylase was immobilized on polymer supports for up to 48 h to determine the optimum binding time.

Enzymatic Properties of Immobilized Glucoamylase Compared With Native Glucoamylase

Temperature of Enzyme Reaction

The effect of temperature on the activity of immobilized enzyme was investigated by tuning the temperature of the reaction within the range of 30–70°C at pH 4.58 for 30 min.

pH of Substrate Solution

The effect of the pH of the substrate solution on the activity of immobilized enzyme was investigated by adjusting the pH of the 4% liquefied starch acetic acid–sodium acetate solution 3.40 to 6.20 at 55°C for 30 min.

Concentration of Substrate Solution

The influence of the concentration of the substrate solution was investigated within the range of 1–8% liquefied starch at pH 5.78 and 55°C for 30 min.

Storage Stability

Native and immobilized glucoamylase (without substrate) were stored at 0–5°C for a certain time before their activities were measured. The immobilized glucoamylase was washed using acetic acid–sodium acetate buffer (pH 5.78, 0.2 mol/L) before storage.

Operational Stability

Immobilized glucoamylase that had been used in an enzyme reaction was washed with acetic acid–sodium acetate buffer (pH 5.78, 0.2 mol/L), filtered, and then employed in a catalytic reaction at 55°C for 30 min. This process was repeated, and the corresponding activity was measured.

Effect of Properties of Supports

Glucoamylase was immobilized onto a variety of porous polymer supports with different porogenic reagents and various molar ratios of VCA and HEMA. The activity was measured and then the relationship

Table 1
Effect of pH of Enzyme Solution on Immobilization of Enzyme onto Support[a]

pH	Coupling yield (%)	Activity of immobilized enzyme (U/g)	Retention of activity (%)	Relative activity (%)
3.40	40.5	40.1	7.52	12.4
3.78	51.4	27.5	5.15	9.58
4.19	37.8	30.5	5.71	14.0
5.38	41.9	41.5	7.79	17.4
5.78	27.0	27.8	5.22	17.1
6.26	44.6	34.9	6.54	19.8

[a]Immobilization conditions: enzyme concentration = 28 U/mL; temperature = 10°C; duration time = 16 h.

Table 2
Effect of Time on Immobilization of Enzyme onto Support[a]

Time (h)	Coupling yield (%)	Activity of immobilized enzyme (U/g)	Retention of activity (%)	Relative activity (%)
16	35.1	68.8	12.9	33.9
20	58.8	61.9	11.6	19.1
24	53.4	29.0	5.43	9.76
35	49.3	47.5	8.91	17.2
48	53.4	37.1	6.96	12.5

[a]Immobilization conditions: enzyme concentration = 28 U/mL; temperature = 10°C; pH of enzyme solution = 5.40.

between immobilized glucoamylase activity and properties of the supports was obtained.

Results and Discussion

Immobilization of Glucoamylase Onto Porous Polymer Supports

To find the optimum binding condition, we measured the immobilized glucoamylase activity under the conditions of pH value, glucoamylase concentration, and binding time. It can be seen from Table 1 that the immobilized enzyme presented a relatively high activity when the pH value of the enzyme solution was 5.38. Prolonging the immobilization time more than 20 h did not improve the activity of immobilized glucoamylase, as shown in Table 2. In this case, a possible interpretation is that the reactive functional groups on the support were saturated by the enzyme molecules after a duration of 20 h, and lengthening the duration time further could not improve immobilized enzyme activity. Instead, the activity may be reduced owing to the spatial repulsion of overcrowded enzyme molecules immobilized onto supports.

Table 3
Effect of Glucoamylase Concentration
on Immobilization of Enzyme onto Support[a]

Concentration of glucoamylase (U/mL)	Coupling yield (%)	Activity of immobilized enzyme (U/g)	Retention of activity (%)	Relative activity (%)
16	28.0	26.7	5.01	15.9
20	20.0	37.6	5.64	23.7
24	18.6	50.6	6.33	28.1
28	21.2	90.3	9.67	38.7
32	32.2	107	10.0	28.2
40	51.3	94.7	7.10	13.2

[a]Immobilization conditions: pH of enzyme solution = 5.40; temperature = 10°C; duration time = 24 h.

Table 3 shows the effect of glucoamylase concentration (16–40 U/mL) on the activity of enzyme immobilized onto the supports. The relative activity attained the optimum value when the concentration of glucoamylase was 28 U/mL. When the concentration of enzyme was increased further, the relative activity decreased. This was likely caused by the jostling of too many enzymes on the supports. Therefore, these immobilization conditions were fixed in the following immobilization experiments: pH of enzyme solution = 5.38; binding time = 20 h; concentration of glucoamylase = 28 U/mL.

Effect of Temperature on Relative Activity

The effect of reaction temperature on the relative activity of native and immobilized glucoamylase is shown in Fig. 1. The glucoamylase itself is a kind of heat-resisting enzyme and exhibits relatively higher activity under high temperature than low temperature within a certain range of temperature. However, when the glucoamylase was immobilized onto supports, it presented relatively high activity under both high and low temperature. This indicates that the catalytic reaction temperature profile was widened when the glucoamylase was immobilized onto support.

Effect of pH on Relative Activity

Figure 2 shows the effect of the pH of substrate solution on the relative activity of native and immobilized glucoamylase. The optimum pH of the native enzyme was within the range of 4.0–4.5, whereas for the immobilized enzyme it was 5.78, which is higher than that of the native enzyme, indicating that immobilization shifts the optimum pH of the glucoamylase to right direction. Actually, for the immobilized enzyme, the optimum pH value depends on the properties of supports. For instance, the maximum activity of immobilized glucoamylase was also obtained at pH 4.5 when the enzyme was immobilized on the magnetic poly (styrene) particles *(15)*.

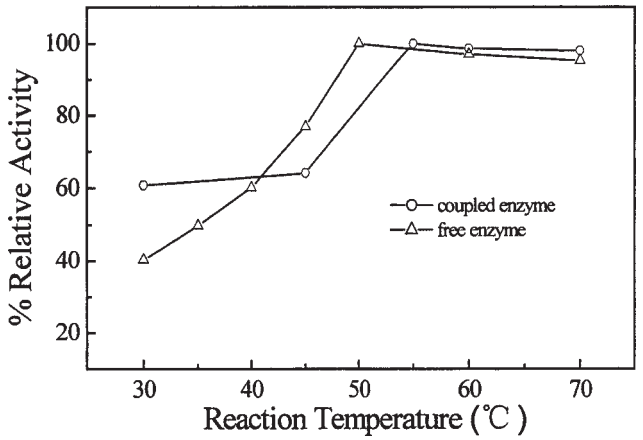

Fig. 1. Effect of reaction temperature on activity of free and immobilized glucoamylase (pH = 4.58; reaction time = 30 min).

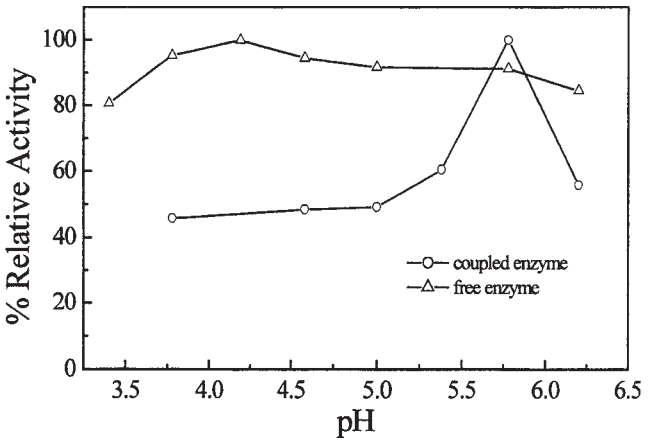

Fig. 2. Effect of pH of substrate solution on activity of free and immobilized glucoamylase (T = 55°C; reaction time = 30 min).

Effect of Concentration of Substrate Solution on Relative Activity

The effect of concentration of substrate (liquefied starch) solution on the relative activity of native and immobilized glucoamylase is shown in Fig. 3. The optimum concentration of substrate solution for native glucoamylase was 2%, whereas for the immobilized enzyme it was 6%. For both cases, a common characteristic was that after the extremum, the activities of both native and immobilized enzymes reduced with augmentation of substrate concentration. This indicates that substrate restraint exists for both native and immobilized glucoamylase. However, for the immobilized enzyme, the effect of substrate restraint occurred at a larger substrate concentration owing to the diffusion limitation of the substrate to the immobilized glucoamylase.

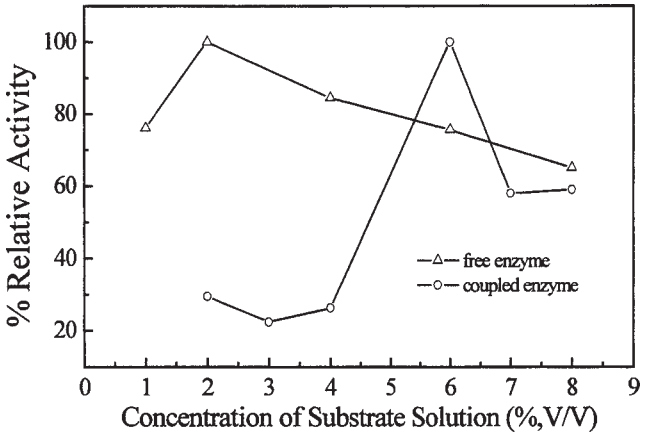

Fig. 3. Effect of concentration of substrate solution on activity of free and immobilized glucoamylase (pH = 5.78; T = 55°C; reaction time = 30 min).

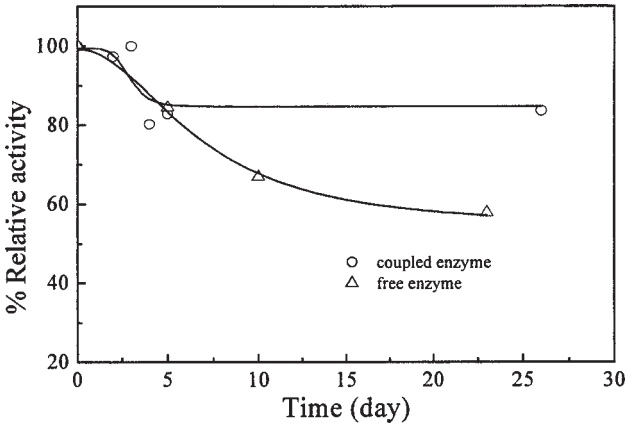

Fig. 4. Storage stability of free and immobilized glucoamylase (pH = 5.78; T = 55°C; reaction time = 30 min).

Storage Stability

Immobilized glucoamylase was stored at 0–5°C, and its catalytic activity was measured after storage for 1, 2, 3, 4, 5, and 26 d. For comparison, the native glucoamylase was also stored under the same condition, and the catalytic activity was measured. The results, shown in Fig. 4, demonstrate that the immobilized glucoamylase still maintained about 84% of its initial activity after storage for 26 d. Compared with immobilized glucoamylase, the native one only maintained 58% of the initial activity after storage for 23 d under the same condition.

Operational Stability

In general, native enzyme can only be employed once. After catalytic reactions, the residual enzyme cannot be easily separated with the prod-

Table 4
Effect of Porogenic Reagent and Ratio of VCA:HEMA (molar ratio) on Retention of Activity of Immobilized Glucoamylase

Support	Porogenic reagent (% [v/v])	Ratio of VCA:HEMA (mol)	Retention of activity (%)
S1	Ethanol (100%)	3:2	6.33
S2	Ethylene glycol (100%)	3:2	9.81
S3	Glycerol (17%)	3:2	7.93
S4	Glycerol (35%)	3:2	9.05
S5	Glycerol (87%)	3:2	11.83
S6	Ethanol (100%)	2:1	5.15
S7	Ethanol (100%)	4:3	6.61
S8	Ethanol (100%)	2:3	6.19

ucts, so it has to be deactivated with high temperature or other methods to avoid polluting the products. However, the immobilized enzyme can be easily separated with the products and can be used repeatedly if its activity does not decrease strongly. We investigated the operational stability of the immobilized glucoamylase and found that after use repeatedly seven times, the-immobilized glucoamylase still maintained about 85% of its original activity.

Effect of Properties of Porous Polymer Supports on Relative Activity

To investigate the effect of the properties of porous polymer supports on the activity of immobilized glucoamylase and to study the relationship between them, we immobilized glucoamylase onto different porous polymer supports and measured the activity of the resulting immobilized enzyme. The activity of glucoamylase immobilized onto porous polymer supports with different porogenic reagents is shown in Table 4. When glycerol (87%) was used as porogenic reagent, immobilized glucoamylase showed the highest catalytic activity among the three kinds of porogenic reagents. The difference in activity was likely caused by the different pore structure of the polymer particle. When glycerol was used as porogenic reagent, the pore structure was more uniform and the specific surface area was higher *(23)*. From Table 4 it can be seen that the activity of immobilized glucoamylase increased with an increase in the amount of glycerol.

The activity of glucoamylase immobilized onto porous polymer supports with different molar ratios of two kinds of monomers, VCA and HEMA, is also shown in Table 4. The activity of immobilized glucoamylase attained an optimum when the molar ratio of the two monomers was 4:3, indicating that the content of VCA and HEMA of the polymer support had an effect on the activity of immobilized glucoamylase. Of the two monomers, VCA has the functional group, cyclic carbonate, which can react with the free $-NH_2$ group on glucoamylase under moderate conditions.

HEMA is a type of hydrophilic monomer, and the purpose of using it as comonomer is to increase the hydrophilicity of the porous polymer supports, thus encourage the immobilization of glucoamylase onto support materials. Therefore, only when the content ratio of the two monomers is suitable can the activity of immobilized glucoamylase attain the optimum value.

Conclusion

The porous polymer supports containing cyclic carbonate could be used to immobilize glucoamylase. The operation could be carried out in one step and without any additional activation, which presents a very simple, mild, and time-efficient process. The immobilized glucoamylase had a wider catalytic reaction temperature profile than native enzyme. Compared with the native enzyme, the immobilized enzyme had a different optimum pH. The optimum substrate concentration of immobilized enzyme was higher than that used for native enzyme. After storage for 26 d and repeated use seven times, the immobilized glucoamylase still maintained about 84 and 85% of its initial activity, respectively. Furthermore, the properties of porous polymer supports had an effect on the activity of the immobilized glucoamylase. The application of porous polymer supports containing cyclic carbonate groups shows promise for becoming one of the preferred methods for enzyme immobilization.

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