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Preparation and characterization of two types of covalently immobilized amyloglucosidase

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Abstract: Amyloglucosidase from *A. niger* was covalently immobilized onto poly(GMA-co-EGDMA) by the glutaraldehyde and periodate method. The immobilization of amyloglucosidase after periodate oxidation gave a preparate with the highest specific activity reported so far on similar polymers. The obtained immobilized preparates show the same pH optimum, but a higher temperature optimum compared with the soluble enzyme. The kinetic parameters for the hydrolysis of soluble starch by free and both immobilized enzymes were determined.

Keywords: glucoamylase, poly(GMA-co-EGDMA), immobilization, periodate, starch.

INTRODUCTION

Amyloglucosidase (AMG) from *Aspergillus niger* EC 3.2.1.3 is one of the most economocal industrial enzymes. At present, for economic reasons, glucose is mainly produced by the enzymatic hydrolysis of starch using α-amylase and AMG. Naturally, the process would be even more economical if the enzyme could be reused, for example by immobilization.

Immobilization of enzymes onto insoluble polymeric matrices is a very effective way to stabilize them. Immobilization leads to a heterogeneous system which enables enzyme separation from the reaction media. Biocatalysts have been immobilized using a variety of supports and techniques. The method of immobilization should be gentle, in order not to inactivate the enzyme, and bind as much enzyme as possible to the support. The morphology of the support plays an important role in continuous bioprocesses using immobilized biocatalysts. The levels of activity

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decrease and the diffusional limitation resulting from immobilization are mainly dependent on the properties of the support and the immobilization conditions.

Macroporous copolymers of glycidyl methacrylate and ethylene glycol dimethacrylate (poly(GMA-co-EGDMA)) are hydrophilic and have good mechanical and chemical properties. There is only one report concerning amyloglucosidase immobilized on glycidyl methacrylate.[1] Biocatalysts immobilized on granular or particulate supports are often employed in packed beds or strirred tank reactors.

Binding of an enzyme to a support may be achieved by chemical or physical means. Adsorption of amyloglucosidase[2,3,4] and covalent immobilization,[5,6] especially immobilization with glutarldehyde[7] are the most often used methods. The first one is mild but enzyme leakage can occur, while the other one can bind enzyme tightly to the support but very often denaturation of enzyme is observed. Covalent immobilization of enzyme *via* its carbohydrate moiety after periodate oxidation is mild and the enzyme is bound very tightly. In spite of this, very few reports have been published about amyloglucosidase immobilization via its carbohydrate moiety.[8] The present study compared glutaraldehyde immobilization of AMG with a procedure for the immobilization of enzymes after oxidation with periodate, which was published for invertase.[9] The support for the immobilization of amyloglucosidase was macroporus poly(GMA-co-EGDMA) obtained by a new procedure.[10]

EXPERIMENTAL

Material

An industrial amyloglucosidase (amyloglucosidase; *exo*-1,4-α-glucosidase; EC 3.2.1.3, from *Aspergillus niger*) was supplied by Mapol, Warszawa. The lyophilized enzyme had a specific activity of 134 U/mg. Glycidyl methacrylate and ethylene glycol dimethacrylate were from Rohm-Darmstadt, Germany. All other chemicals were from Merck, Germany.

Polymer

The macroporous copolymer was prepared by suspension polymerization as previously described.[10] The particle size distribution was determined by sieve analysis. A commercial mercury porosimeter, Model 2000 Carlo Erba, was used for the determination of the specific pore volume, pore size distribution and specific area.

Polymer activation

Polymer with particle size of 150-500 μm was modified with 1M 1,2-diaminoethane at 60 °C for 4 h at pH 10. After modification, the polymer was washed several times with water and the concentration of amino groups was determined by titration with 0.01 M HCl in 0.5 M KCl.

Amyloglucosidase immobilization

Glutaraldehyde method

Activated polymer was incubated in 2 % (w/v) glutaraldehyde in 50 mM sodium phosphate buffer at pH 8 for 2h. The polymer was then washed several times with the same buffer and incubated with native (unmodified) amyloglucosidase in sodium phosphate buffer at pH 7 and 4 °C for 48 h. After incubation with enzyme, the polymer was washed 3 times with 1M NaCl in 50 mM acetate buffer pH 4.5 and 3 times with acetate buffer alone. The washings were collected and the insoluble enzyme preparate was stored in acetate buffer pH 4.5 at 4 °C prior to use.

Periodate method

Amyloglucosidase was oxidized by periodate in the same way as previously described for invertase.[11] The activated polymer was incubated with oxidized amyloglucosidase in 50 mM sodium acetate buffer pH 5, at 4 °C for 48 h. Subsequently, the polymer was washed 3 times with 1M NaCl in 50 mM acetate buffer pH 4.5 and 3 times with the buffer. The immobilized enzyme was stored prior to use as above.

Determination of proteins

The amount of immobilized protein was estimated as the difference between the amount of protein applied to the support and the amount of protein recovered in the supernatants and washings. The content of protein in the solutions was determined by the Lowry method[12] using BSA as a standard.

Determination of enzyme activity

The activity of the soluble amyloglucosidase was determined according to the Bernfeld method[13] at 60 °C using 0.05 M sodium acetate buffer, pH 4.5.

Immobilized amyloglucosidase (25 mg) was added to 25 mL of 4 % (w/v) soluble starch in 50 mM acetate buffer pH 4.5. The reaction proceeded under constant stirring and a sample of the reaction mixture (0.5 mL) was withdrawn every 5 min for a period of 20 min. The reducing sugars formed in this way then quantified, again by the Bernfeld method.

One amyloglucosidase unit was defined as the amount of enzyme which releases reducing carbohydrates equivalent to 1 μmol glucose from soluble starch in 1 min at pH 4.5 and at 60 °C. The specific activity of the immobilized enzyme was defined as the number of micromoles of glucose which were liberated in one minute by one gram of dried immobilizate at 60 °C.

Effects of pH and temperature on amyloglucosidase activity

The effect of temperature on the enzyme activity was studied in the temperature range 40 to 80 °C with a soluble starch concentration of 1% (w/v) in 50 mM acetate buffer pH 4.5. The effect of pH on the enzyme activity was determined with a soluble starch concentration of 1% (w/v) in 50 mM sodium citrate-phosphate buffer of an appropriate pH at 25 °C.

Determination of K_m *and* V_{max}

K_m and V_{max} were determined by measuring the initial production rate from soluble starch [0.1-4% (w/v)] in 50 mM acetate buffer pH 4.5 at 60 °C.

RESULTS AND DISCUSSION

Immobilization of amyloglucosidase on macroporous polymer

For the binding of amyloglucosidase, the particle diameters of the obtained polymer were in the range of 150-500 μm. The specific pore volume was 0.6 mL/g, and the specific area 50 m^2/g. The mean pore diameter of the support was 51 nm and the concentration of amino group after modification was 1.2 mmol/g.

With increasing amount of added protein per gram of polymer, the specific activity initially increased, and reached a plateau of 750 U/g for glutaraldehyde immobilization, Fig. 1. The plateau is the result of diffusional limitation caused by the polymer which reduces the access of the substrate to the active sites of the bound enzyme.

The obtained specific activity was slightly higher than in previously published papers.[10–14] However, when the amyloglucosidase was immobilized *via* its carbo-

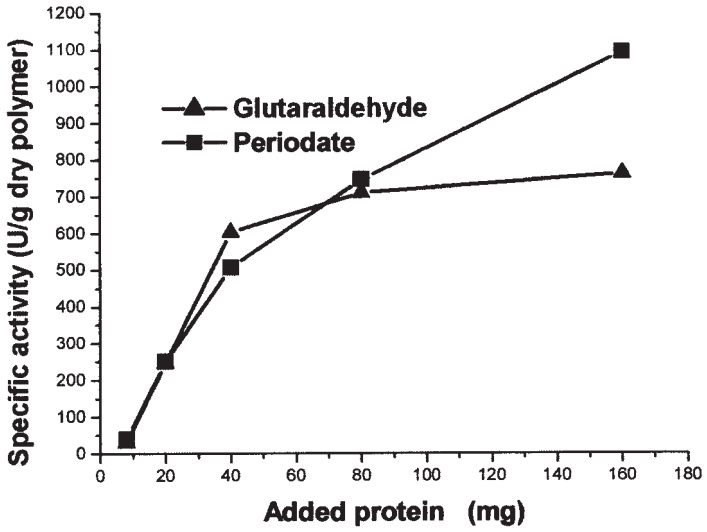

Fig. 1. Dependence of the specific activity of immobilized amyloglucosidase on the amount of added enzyme.

hydrate moiety, after oxidation of the enzyme with periodate, the specific activity of the immobilized preparate was about 50% higher than with glutaraldehyde immobilization. With the addition of 160 mg of enzyme per 1 g of polymer, the specific activity was 1100 U/g, Fig. 1.

This specific activity of immobilized amyloglucosidase was higher than the previously highest one reported for a macroporous polymer.[15]

The yield of immobilization for both preparation methods were in accordance with literature for the immobilization of amyloglucosidase on a macroporous polymer.[1]

Effect of pH and temperature on immobilized amyloglucosidase activity

The effect of pH on the enzyme activity was examined and the results are shown in Fig.2. The enzymatic activity is presented as the relative activity with respect to the maximum value at pH 4.5.

After immobilization, the optimal pH did not change for either the glutaraldehyde or the periodate preparates. The temperature–activity profiles for the free and both the immobilized amyloglucosidases are shown in Fig. 3.

Both the immobilized amyloglucosidases have highest activity at 70 °C while the native enzyme reaches maximum activity at 65 °C. The increase in optimum temperature was caused by the physical and chemical properties of the enzyme changing. This could be explained by the creation of conformational limitations on the movement of the enzyme as a consequence of the formation of covalent bonds between the enzyme and the support.

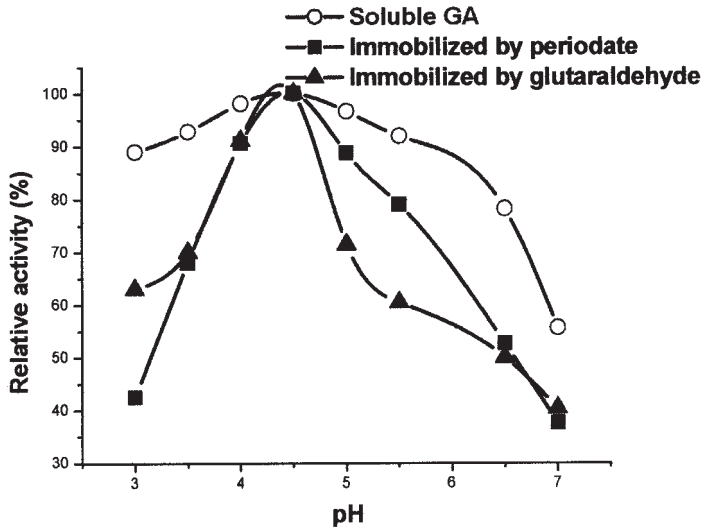

Fig. 2. Effect of pH on free and the two forms of immobilized amyloglucosidase, 50 mM sodium citrate-phosphate buffer 25 °C.

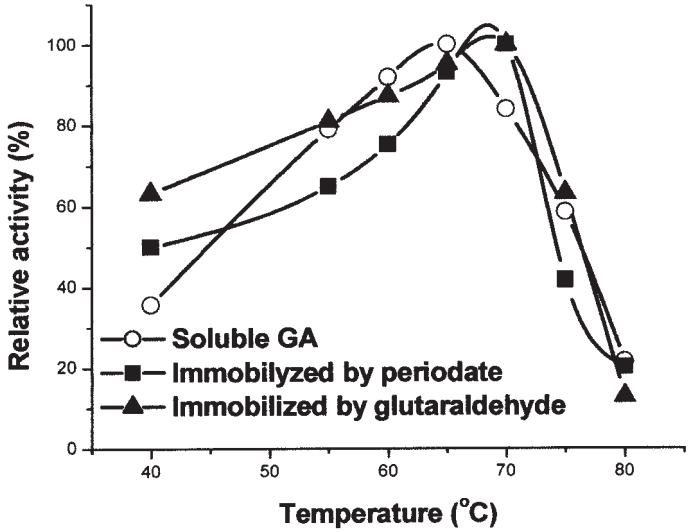

Fig. 3. Effect of temperature on free and the two forms of immobilized amyloglucosidase, pH 4.5, substrate concentration 1 % (w/v).

Kinetic constants

The kinetic parameters for the free and immobilized amyloglucosidases were detrermined using soluble starch as the substrate (Table I).

The values of the apparent K_m for the covalently bonded amyloglucosidase were approximately five times higher than that of the free enzyme. The obtained results are similar to those previously published.[16]

TABLE I. Characteristics of native and two forms of immobilized amyloglucosidase

Amyloglucosidase	K_m/%	Bound proteins mg/g support	Specific activity Ug^{-1} support
Free	0.22	-	-
Immobilized with glutaraldehyde	1.28	35	750
Immobilized with periodate	1.22	97	1100

CONCLUSIONS

– The presented method of periodate immobilization resulted in a 1.5 time higher specific activity than the most used one, glutaraldehyde method.

– The immobilized enzymes were more thermostable than the native enzyme.

– Both immobilized enzymes had similar K_m values.

– The method of periodate immobilization of amyloglucosidase is better than the one most often used, because immobilized enzyme exhibited the highest specific activity reported so far for immobilization on a macroporous polymer.

ИЗВОД

ДОБИЈАЊЕ И КАРАКТЕРИЗАЦИЈА ДВА ТИПА ИМОБИЛИЗОВАНЕ АМИЛОГЛУКОЗИДАЗЕ

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Амилоглукозидаза из *A.niger* је имобилизована на poly(GMA-co-EGDMA) глутаралдехидном и перјодатном методом. Имобилизација амилоглукозидазе након перјодатне оксидације даје препарат са највећом до сада објављеном специфичном активности на сличним полимерима. Добијени имобилизовани препарат има исти pH оптимум али повећани термооптимум у поређењу са растворним ензимом. Одређени су и кинетички параметри за хидролизу растворног скроба имобилизованим као и растворним ензимом.

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