0273-2289/91/2829—0277$02.00

Hydrolysis of Cellobiose by Immobilized β-Glucosidase Entrapped in Maintenance-Free Gel Spheres

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ABSTRACT

A crude preparation of *Aspergillus niger* β-glucosidase (27.5 cellobiase U/mg protein at 40°C, pH 5.0) was immobilized on concanavalin A-Sepharose (CAS). The cellobiase activity of the immobilized enzyme was 1334 U/mg dried CAS or 108 U/mL CAS gel. The β-glucosidase-CAS complex was entrapped within crosslinked propylene glycol alginate/bone-geletin gel spheres that possessed between 0.67 and 2.35 cellobiase U/mL spheres, depending on their size. The effect of cellobiose concentration (10–300 m*M*) on the activity of native, immobilized, and gel-entrapped enzyme was determined. It was shown that concentrations of cellobiose between 10 and 180 m*M* were not inhibitory to the entrapped enzyme, although inhibition was found to occur with the native and immobilized enzyme. Exogenous ion addition was not necessary to maintain the structural integrity of the spheres, which were stable for 4 d at 40°C.

Index Entries: Immobilization; β-glucosidase; propylene glycol alginate/bone gelatin; cellobiose hydrolysis.

INTRODUCTION

The enzyme β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of alkyl and aryl β-D-glucosides, as well as glycosides containing only carbohydrate residues (e.g., cellobiose) (*1*).

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It is a key component of fungal cellulase enzyme complexes, but, unlike the cellobiohydrolase and endoglucanase components (*2*), does not require adsorption to the insoluble cellulosic surface in order to perform its catalytic function. This, taken together with the soluble nature of the substrates of β-glucosidase (e.g., cellobiose, cellotriose), allows its utilization in immobilized form (*3*).

Calcium alginate gel spheres have been used to entrap glutaraldehyde-modified and CAS-immobilized *Aspergillus niger* β-glucosidase (*4,5*), but their use in continuous-flow systems requires the addition of calcium ions for maintenance of their structural stability. In contrast, propylene glycol alginate–bone gelatin (PGAG) gel spheres developed for the entrapment of microorganisms (*6*) are maintenance-free in that, once the spheres are formed, the addition of exogenously added ions is not necessary for maintaining their structural integrity and long-term stability.

We now report the entrapment of β-glucosidase adsorbed on CAS within PGAG gel spheres and describe the kinetic properties of such spheres with respect to their ability to hydrolyze cellobiose. Such spheres have not been used previously for the entrapment of enzymes and may have general utility in biocatalyst immobilization.

MATERIALS AND METHODS

Chemicals

A commercial preparation of *Aspergillus niger* β-glucosidase (Cellobiase 250L) was obtained from NOVO Laboratories, Inc., Wilton, CT. Propylene glycol alginate (Kelcoloid S) was purchased from Kelco, Clark, NJ, and deionized bone gelatin was obtained from Rousselot, Paris, France. Cellobiose and the hexokinase reagent for glucose assay were purchased from Sigma Chemical Co., St. Louis, MO. CAS was purchased from Pharmacia, Piscataway, NJ. Protein determinations were made using the Coomassie blue reagent (Bio Rad, Richmond, CA).

Immobilization and Assay of β-Glucosidase

Adsorption of β-glucosidase on CAS was carried out as described previously (*7*). In this procedure, 5.0 mL of undiluted enzyme was stirred with 5.0 mL of CAS for 15 min at 23°C, after which the CAS-enzyme complex was filtered and washed with 50-mL portions of 50 m*M* sodium acetate buffer, pH 5.0, until negligible protein and enzyme activity were detectable in the washings. The CAS–enzyme complex was suspended in 10 mL of buffer, pH 5.0. It should be noted that washing the CAS–enzyme complex in the presence of exogenously added $MnCl_2$ and $CaCl_2$ required to preserve the binding activity of concanavalin A below pH 5.0, was not necessary, because, once bound to CAS, β-glucosidase is extremely difficult to elute under the usual conditions employed

for the elution of glycoproteins (*8*). β-Glucosidase, in soluble form, adsorbed on CAS and entrapped within PGAG gel spheres, was routinely assayed by incubating, at 40°C, a given volume of enzyme in 10 mL total volume of 50 m*M* sodium acetate buffer, pH 5.0, containing 10 m*M* cellobiose, and monitoring the rate of glucose production using the hexokinase reagent. One unit of enzyme activity is defined as the amount of enzyme required to generate 1 μmol of glucose from cellobiose under the specified assay conditions.

Entrapment of CAS-Immobilized β-Glucosidase Within PGAG Gel Spheres

Bone gelation (15 g) was added to ~80 mL of distilled water and dissolved by stirring for 30 min at 48°C. The solution was maintained at this temperature by use of a jacketed beaker, and 2 g of propylene glycol alginate and 4.2 mL of the CAS-enzyme gel were added. After stirring manually, the alginate-gelatin-enzyme mixture (100 mL) was allowed to stand for 60 min to allow the air bubbles to dissipate. Gel spheres (3.1 mm in diameter) were formed by adding the mixture dropwise (by syringe) into a 2-L beaker containing ~700 mL mineral oil at 23°C on the surface of 500 mL of ice-cold 0.1*N* NaOH. The gel spheres (liquid phase) formed in the mineral oil through which they fell, and subsequently crosslinked and hardened (solid phase) upon reaching the alkali.

A variation of this procedure was used to generate different sizes of spheres, averaging 1.85 mm and 3.1 mm in diam. It consisted of adding 10 mL of the alginate-gelatin-enzyme mixture to 20 mL of mineral oil warmed to 45°C and shaking the mixture by hand. The mixture was then poured into the beaker containing the mineral oil and ice-cold sodium hydroxide (*see above*). Beads formed at the oil/alkali interface, and the mixture was then stirred for 1–2 min to allow crosslinking and hardening of the spheres to occur. The beads were washed with distilled water (4×500 mL) and 50 m*M* sodium acetate buffer, pH 5.0 (3×200 mL), separated according to size, and stored in the acetate buffer at 4°C.

Cellobiose Hydrolysis by Gel Spheres

Cellobiose hydrolysis by the immobilized β-glucosidase entrapped within the PGAG spheres was carried out in jacketed reactors in a 10-mL volume containing 0.5 mL of spheres and a given concentration of cellobiose in 50 m*M* sodium acetate buffer, pH 5.0. After 15 min of incubation at 40°C, a 100-μL aliquot of the reactor mixture was assayed for glucose.

RESULTS AND DISCUSSION

The activity and specific activity of the β-glucosidase preparation used in this study was calculated to be 921 U/mL and 27.5 U/mg protein, re-

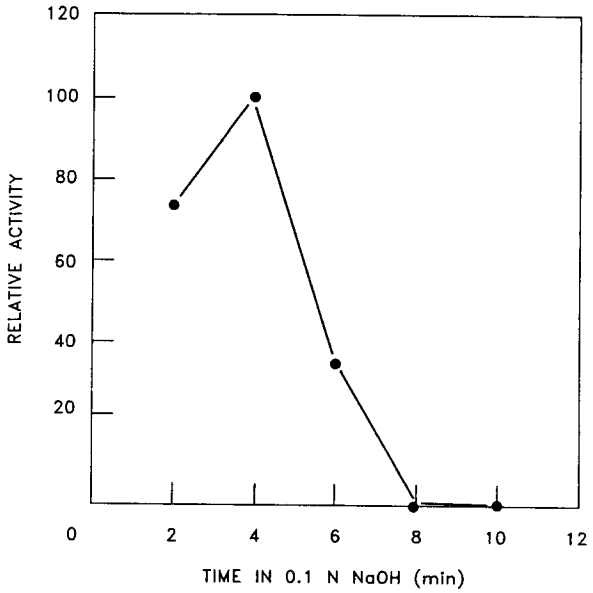

Fig. 1. Dependence of the retention of β-glucosidase activity entrapped in gel spheres on the time spent in 0.1*N* sodium hydroxide. Gel spheres (2.0 mm in diameter) containing immobilized β-glucosidase were made as described in the Materials and Methods section. Several spheres were removed from the alkaline solution at 2-min intervals; washed thoroughly with distilled water followed by buffer, pH 5.0; and assayed for their ability to hydrolyze 10 m*M* cellobiose. Relative activity of 100% ≡ 2.35 U/mL spheres.

spectively. The rationale for immobilizing β-glucosidase prior to its gel entrapment was to prevent leakage of enzyme from the gel spheres, which would have occurred otherwise (*5*). Adsorbed on CAS, the activity of β-glucosidase was 1334 U/g dry CAS or 108 U/mL gel slurry.

It was found that immobilized β-glucosidase was easily entrapped within PGAG gel spheres using the methods described, but that the time that the spheres spent in sodium hydroxide was critical to the retention of enzyme activity, which was maximal at 4 min (Fig. 1). The reason for the loss in activity that occurred in sodium hydroxide appeared to be mainly the desorption of enzyme from CAS under these conditions, effecting its leakage from the spheres (data not shown). However, pH inactivation of β-glucosidase was also possible. Previous studies have shown that cross-linking of β-glucosidase with glutaraldehyde prior to entrapment in 2% calcium alginate gel spheres prevents leakage of the enzyme, as well as increasing its thermal stability (*4,5,9*). It is possible, therefore, that PGAG gel spheres containing glutaraldehyde-modified β-glucosidase will retain enzymatic activity for longer periods when incubated in sodium hydroxide. The longer the spheres are stored in the alkaline solution, the greater their structural stability (*6*). Consideration must be given, therefore, to retention of enzyme activity during the storage time. The gel spheres con-

Table 1
Entrapment of CAS-Immobilized β-Glucosidase in PGAG Gel Spheres

Initial CAS-immobilized β-glucosidase activity (units)[a]	Entrapped activity		Effectiveness factor (B/A) (%)
	Theoretical (A) (units)[a]	Actual (B) (units)[b]	
151.2	151.2	63.5	42

[a]Units refer to the amount of total activity used in the initial entrapment.
[b]Units refer to the activity found in the total number of gel spheres, (3.1 mm in dia).

taining β-glucosidase activity stored for 4 min in sodium hydroxide appeared structurally stable, since they remained intact when placed in distilled water at 80°C and incubated for 15 min. They were also structurally stable when stirred in water at 23°C for 60 h or in buffer, pH 5.0, at 40°C for 96 h. However, they were unstable when stirred in buffer at 50°C for 60 h. In order for the spheres to be structurally stable for extended periods of time at elevated temperatures, it will probably be necessary to crosslink the spheres in sodium hydroxide for periods longer than 4 min, during which time the enzyme, ideally, should be stable, or at least recover its activity upon being returned to the solution at the pH for optimum activity.

The data in Table 1 show that there is a 42% retention of total activity upon entrapment, which is similar to that observed for CAS-immobilized β-glucosidase entrapped in calcium alginate gel spheres (*5,7*). Our previous data showed that substrate inhibition of *A. niger* β-glucosidase, which occurs with cellobiose above 10 m*M* in the reaction mixture, did not occur with cellobiose up to 10 m*M* when β-glucosidase was entrapped within calcium alginate gel spheres (*7*). The effect of cellobiose concentrations (up to 300 m*M*) on the initial rate of hydrolysis by native, CAS-immobilized PGAG gel spheres of two sizes containing β-glucosidase was determined (Figs. 2 and 3). Above 10 m*M*, the initial rate of cellobiose hydrolysis by either native or CAS-immobilized β-glucosidase was reduced, but it should be noted that significant hydrolysis occurred even with 300 m*M* cellobiose in the reaction mixture. The rate of cellobiose hydrolysis by both sizes of spheres increased above 10 m*M* cellobiose and was maximal between 40 and 180 m*M* cellobiose. Above 180–200 m*M* cellobiose, the activity tended to decline, but, as with the native and CAS-immobilized enzyme, there was significant hydrolysis with 300 m*M* cellobiose. Substrate diffusion is the major cause of the apparent lack of substrate inhibition of β-glucosidase entrapped within the spheres, since the concentration of cellobiose within the spheres will be less than that in the surrounding solution (*7*). The K_m of the immobilized entrapped enzyme was increased two- and fourfold over that of the native enzyme (2.7 m*M*) for the small and large spheres, respectively.

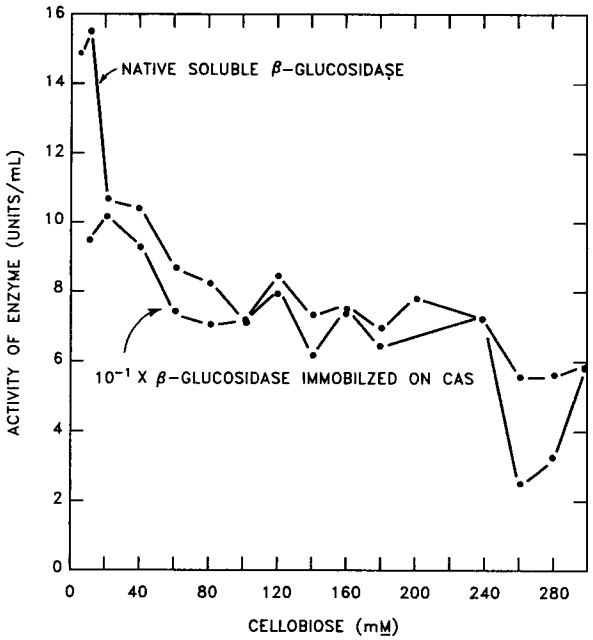

Fig. 2. The effect of cellobiose concentration on the activity of native and CAS-immobilized β-glucosidase. Native enzyme (50 μL of original solution diluted×50) and immobilized enzyme (50 μL of slurry; *see* Materials and Methods section) were incubated in a 10-mL volume of 50 m*M* sodium acetate buffer, pH 5.0, containing a given concentration of cellobiose, for 15 min at 40°C. Glucose concentration was measured.

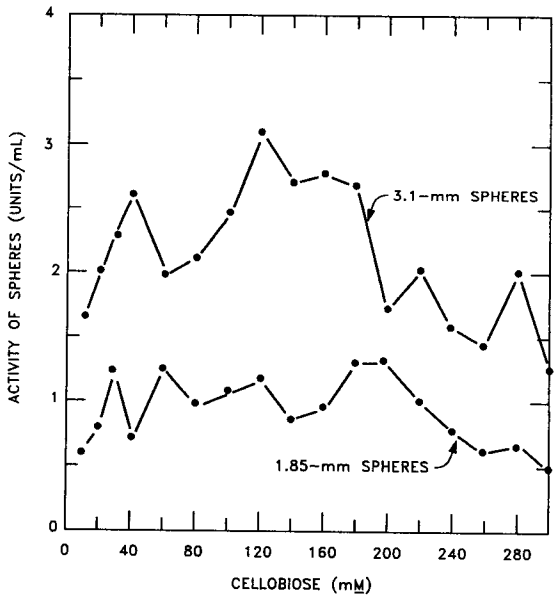

Fig. 3. The effect of cellobiose concentration on the activity of two sizes of gel-entrapped CAS-immobilized β-glucosidase. For details, *see* legend to Fig. 2. The volume of spheres used in the assay was 500 μL.

The reason for the activity of the smaller spheres (1.85 mm in diameter) being lower than that of the larger spheres is not known, but could be the effect of storing the spheres in sodium hydroxide. The smaller spheres would not retain their activity as long as the larger ones, because diffusion of the alkali through them would be faster and lead to rapid desorption of enzyme from CAS. Finally, there was ~40% retention of β-glucosidase activity after the spheres had been stirred for 4 d at 40°C.

CONCLUSIONS

Propylene glycol alginate/bone-gelatin gel spheres can be produced that contain immobilized β-glucosidase and are capable of cellobiose hydrolysis. The strutural integrity of the spheres depends on the time spent in sodium hydroxide, which is necessary to crosslink the alginate and gelatin. Once this has been achieved, there is no requirement for exogenous-ion addition to maintain structural stability. Such spheres may have general utility for the entrapment of biocatalysts and in continuous-flow bioreactor systems.

ACKNOWLEDGMENTS

The authors would like to thank C. A. Woodward for technical advice, G. W. Strandberg and B. D. Faison for reviewing the manuscript, and D. Weaver and S. Hoglund for secretarial assistance. This work was supported by the Chemical Sciences Division, Office of Basic Energy Sciences, US Department of Energy under contract DE-AC05-85OR21400 with Martin Marietta Energy Systems, Inc., and by the Solar Energy Research Institute.

REFERENCES

1. Woodward, J. and Wiseman, A. (1982), *Enzyme Microb. Technol.* **4,** 73–79.
2. Woodward, J. (1987), *Carbon Substrates in Biotechnology,* vol. 21, Chapter 4, Stowell, J. D., Beardsmore, A. J., Keevil, C. W., and Woodward, J. R., eds., IRL, Oxford, pp. 45–65.
3. Woodward, J. (1989), *J. Biotechnol.* **11,** 299–312.
4. Woodward, J., Whaley, K. S., Zachry, G. S., and Wohlpart, D. L. (1981), *Biotechnol. Bioeng. Symp.* **11,** 619–629.
5. Woodward, J., Krasniak, S. R., Smith, R. D., Spielberg, F., and Zachry, G. S. (1982), *Biotechnol. Bioeng. Symp.* **12,** 485–489.
6. Scott, C. D., Woodward, C. A., and Byers, C. H. (1989), US patent application.
7. Lee, J. M., and Woodward, J. (1983), *Biotechnol. Bioeng.* **25,** 2441–2451.
8. Woodward, J., Marquess, H. J., and Picker, C. S. (1986), *Prep. Biochem.* **16,** 337–352.
9. Baker, J. O., Oh, K. K., Grohmann, K., and Himmel, M. E. (1988), *Biotechnol. Lett.* **10,** 325–330.