0273-2289/92/3435—0341$02.00

Cellobiose Hydrolysis by Glutaraldehyde-Treated β-Glucosidase Entrapped in Propylene Glycol Alginate/Bone Gelatin Spheres

Scientific Note

JONATHAN WOODWARD* AND KATHERINE M. CAPPS

Chemical Technology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6194

Index Entries: *Aspergillus niger;* cellobiase; cellobiose; glutaraldehyde crosslinking; gel entrapment.

INTRODUCTION

Aspergillus niger β-glucosidase (EC 3.2.1.21) is a suitable enzyme with which to supplement, and hence enhance, the activity of fungal cellulases because it has high cellobiase specific activity. It will thus hydrolyze cellobiose and prevent end-product inhibition of the cellobiohydrolase component of the cellulase complex mixture of enzymes from fungal sources, such as *Trichoderma reesei* (*1*). Furthermore, it can be added to a reaction mixture containing cellulase in an immobilized form because its substrates are soluble.

Recently, we reported the entrapment of this enzyme adsorbed on concanavalin A-Sepharose (CAS) within propylene glycol alginate/bone gelatin (PGAG) spheres (*2*). In order to impart structural stability to the spheres, it was necessary to store them in sodium hydroxide for several minutes after their formation. However, this resulted in desorption of the enzyme from CAS and its subsequent leakage out of the spheres. In an attempt to overcome this problem, *A. niger* β-glucosidase was treated with

*Author to whom all correspondence and reprint requests should be addressed.

glutaraldehyde, instead of being immobilized on CAS, prior to its entrapment in PGAG spheres. This was done in order to (1) polymerize the enzyme so that it would not leak out of the spheres and (2) enable the spheres to be stored in sodium hydroxide for longer than 4 min, resulting in greater structural stability. Our previous data have shown glutaraldehyde-treated β-glucosidase to be retained within calcium alginate spheres (*3*).

In this article, we present preliminary data suggesting that glutaraldehyde-treated β-glucosidase is almost as active as the native enzyme, possesses enhanced thermal stability, and, when entrapped within PGAG gel spheres, can be used for the continuous hydrolysis of cellobiose in a fixed-bed reactor. As noted previously (*2*), the spheres are maintenance-free in that they do not require exogenously added ions for their structural stability (*4*).

MATERIALS AND METHODS

Enzyme Preparation, Assay, and Activity

One milliliter of a crude preparation of β-glucosidase (Novozyme 188), kindly provided by NOVO Enzymes, Danbury, CT, was diluted to 1/5 of the original concentration with 50 m*M* sodium acetate buffer, pH 5.0, and subjected to gel filtration on a 2.54 × 94-cm BioGel P-100 column equilibrated with the same buffer. The fractions containing cellobiase activity, assayed as described previously using 10 m*M* cellobiose as the substrate (*2,5*), were pooled and used as the enzyme source for all the studies reported. The activity and specific activity of the filtered enzymes were 18.2 U/mL and 14.2 U/mg protein, respectively, when assayed at 40°C and pH 5.0. One unit of activity is defined as the amount of enzyme required to generate 1 μmol of glucose from cellobiose under the specified assay conditions.

Chemical Modification of β-Glucosidase with Glutaraldehyde

β-Glucosidase (1.0 mL of the gel-filtered enzyme, 1.4 mg protein) was incubated overnight (~ 18 h) at 4°C with 8.8 mL 0.1*M* sodium phosphate-buffer, pH 8.0, and 0.2 mL of a 25% aqueous solution of glutaraldehyde (practical grade, purchased from Sigma Chemical Co., St. Louis, MO). The chemical modification was carried out in the presence and absence of 15 m*M* cellobiose. The solution was then subjected to gel filtration using a disposable PD-10 column containing Sephadex G-25 M gel (Pharmacia, Piscataway, NJ) equilibrated with 50 m*M* sodium acetate buffer, pH 5.0, at 23°C. This filtered enzyme was analyzed for cellobiase activity.

Prior to gel entrapment, the enzyme was chemically modified as follows: The enzyme filtered on the BioGel P-100 (~ 100 mL) was concentrated by lyophilization and redissolved in 10 mL of nanopure distilled

Table 1
Effect of Glutaraldehyde on the Activity of β-Glucosidase[a]

	Activity of chemically modified β-glucosidase	
	In absence of cellobiose	In presence of cellobiose
Specific activity, U/mg	0.82	7.6
Relative activity,[b] %	10	96

[a]For details, *see* Materials and Methods.
[b]Refers to the percent of activity relative to the control (i.e., enzyme subjected to the conditions for modification in the absence of glutaraldehyde).

water. The concentrated enzyme was then subjected to gel filtration using a PD-10 column (*see above*) equilibrated with 0.1*M* sodium phosphate buffer, pH 8.0, that yielded 14 mL of enzyme. The latter was then treated with 0.5% (v/v) glutaraldehyde as described above in the presence of 15 m*M* cellobiose.

Entrapment of Glutaraldehyde-Modified β-Glucosidase within PGAG Gel Spheres

The entrapment of the modified enzyme was carried out as described previously (2), and consisted of entrapping 4.2 mL of modified enzyme within 15% gelatin and 2% propylene glycol alginate spheres. Crosslinking and hardening of the spheres were carried out for 15 min in 0.1*N* NaOH. The diameter of the spheres was 3.1 mm.

Cellobiose Hydrolysis by Gel Spheres in a Fixed-Bed Reactor

Forty milliliters of gel spheres were packed into a glass column (2.54×25.4 cm) through which was pumped a 1% (w/v) cellobiose solution containing 0.1% (w/v) sodium azide in 50 m*M* sodium acetate buffer, pH 5.0, at 23°C and a flow rate of 1.0 mL/min. The glucose concentration in the effluent was measured using the hexokinase assay reagent kit (Sigma).

RESULTS AND DISCUSSION

The Effect of Glutaraldehyde on the Catalytic Activity of β-Glucosidase

The chemical modification of β-glucosidase in the presence of its substrate, cellobiose, had a profound effect on the recovery of activity (Table 1). The reason why the modified enzyme is almost as active as the native

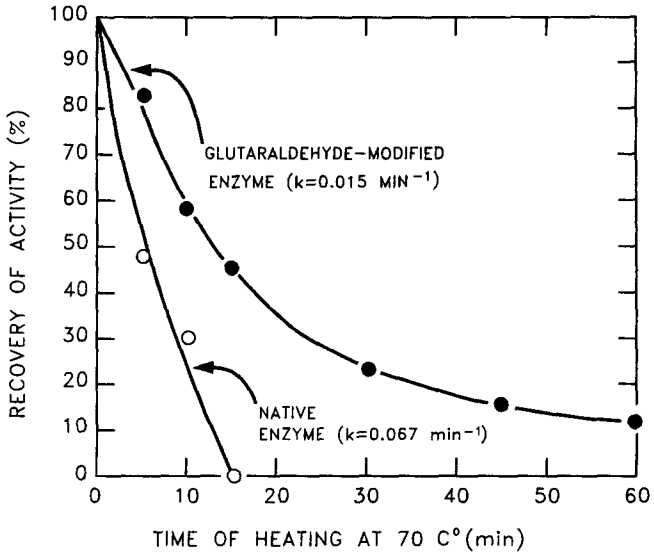

Fig. 1. Thermal stability (70°C) of native and glutaraldehyde-modified β-glucosidase. Native and modified enzyme were heated at a protein concentration of 0.1 mg/mL. At the times indicated, aliquots were removed, cooled to 4°C, and subsequently assayed for activity. The values of k refer to the rate inactivation constants for the native and modified enzyme.

enzyme is not understood. It could be related to either a protection of certain amino acid side chains from modification that otherwise would affect the catalytic site or a protection of the latter from a deleterious conformational change. Previous reports noted that 25% of β-glucosidase activity was lost when it was modified with glutaraldehyde in the absence of cellobiose at room temperatures for 1 h or less (*6,7*). We have also observed that modifying β-glucosidase at 23°C with glutaraldehyde in the presence of 15 m*M* cellobiose for 18 h completely protects the catalytic activity (data not shown).

As expected (*6,7*), the thermal stability of the enzyme was enhanced fivefold by treatment with glutaraldehyde based upon the values of the first-order rate inactivation constant for the native and modified enzyme (Fig. 1). According to Baker et al. (*7*), the reason for the enhanced thermal stability is owing to intramolecular crosslinking. In this study, it was also important that intermolecular crosslinks be formed so that the polymerized enzyme molecules would be retained within the PGAG gel spheres.

Entrapment of Glutaraldehyde-Modified β-Glucosidase within PGAG Gel Spheres

A 0.1-mL volume of gel spheres was assayed for 30 min for cellobiase activity. After this time, the reaction mixture was removed by aspiration leaving the spheres that were washed with 2.5 mL of buffer, pH 5.0, at 23°C, and reassayed. This procedure was repeated six times, consecu-

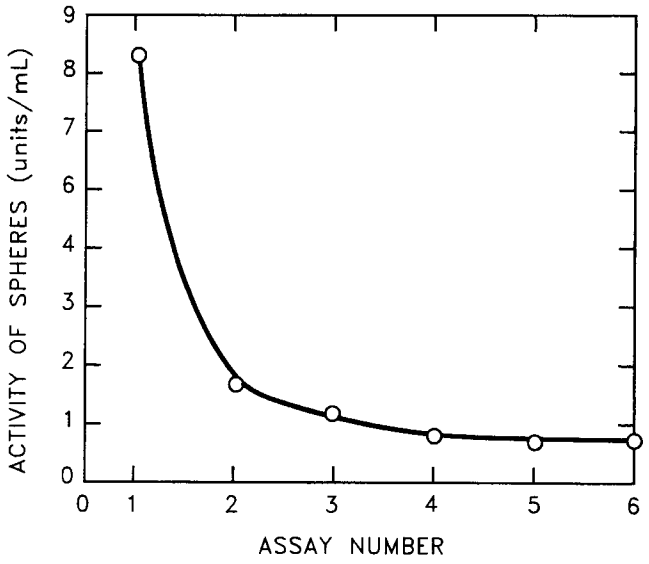

Fig. 2. Stability of glutaraldehyde-modified β-glucosidase entrapped within PGAG gel spheres during repeated batch assays. For details, *see* Results and Discussion.

tively. The data in Fig. 2 show that there was a 90% loss in the initial activity during the repeated batch assays. After the sixth assay, the activity appeared to remain constant at 0.9 U/mL of spheres. There was no apparent physical damage inflicted upon the spheres during these assays.

It can be concluded that most of the enzyme leaked out of the spheres, suggesting that the extent of polymerization was not sufficient to ensure that all the enzyme molecules remained trapped. A comparison of the mol wt of the native and modified enzyme by SDS-gel electrophoresis (*8*) revealed that only a small percentage (~ 10%) of the modified enzyme was large enough to prevent it from penetrating the 10–15% polyacrylamide electrophoresis gel (data not shown). This observation would explain the leakage of most of the enzyme activity from the spheres during the batch assays. In this regard, we previously showed that native β-glucosidase entrapped within calcium alginate gel spheres rapidly leaked out into the external solution (*4*). Activity loss caused by thermal inactivation can be discounted because of the prolonged stability of this enzyme at 40°C (*6,7,9*).

The reason for the leakage may be because of the fact that, after modification of the enzyme with glutaraldehyde, the Schiff-base adducts that formed between glutaraldehyde and the amino groups of the enzyme were not stabilized by reduction (*10*). Consequently, the intermolecular covalent bonds formed would be unstable, resulting in depolymerization and leakage of the enzyme from the spheres.

It should also be noted that in this study the spheres were crosslinked and hardened in sodium hydroxide for 15 min, which is the time necessary to confer maximum structural stability on the spheres (*3*). Storage of

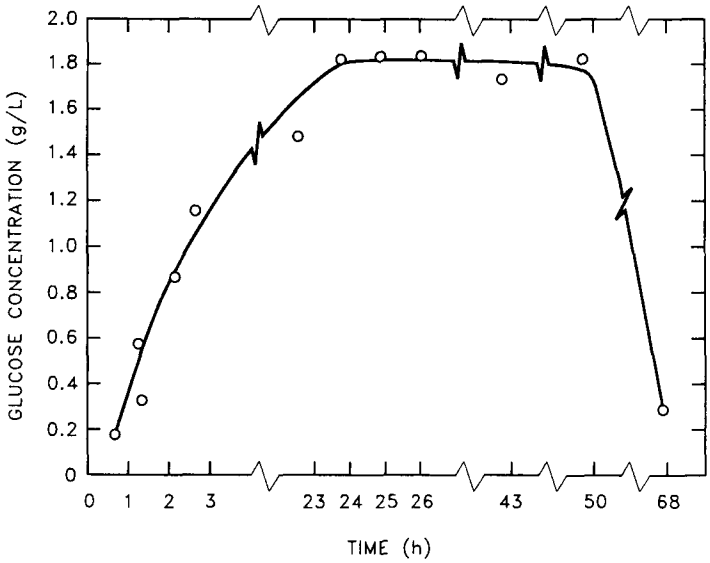

Fig. 3. Continuous hydrolysis of a 1% (w/v) cellobiose solution in a fixed-bed reactor. For details, *see* Materials and Methods.

CAS-β-glucosidase entrapped within PGAG gel spheres in sodium hydroxide for periods longer than 4 min was not possible because all catalytic activity was lost (*2*). Glutaraldehyde-modified β-glucosidase PGAG gel spheres, therefore, possess maximum structural stability and retain catalytic activity.

Cellobiose Hydrolysis by PGAG Gel Spheres in a Fixed-Bed Reactor

The data in Fig. 3 show that the spheres were capable of continuous cellobiose hydrolysis for at least 50 h at 23°C. A maximum of approx 10% conversion of cellobiose to glucose was observed between 24 and 50 h. Presumably, a higher percentage conversion would result if a greater retention of activity within the spheres could be achieved. After 68 h, the extent of conversion had dropped to ~1.6%; although the reason for this is not known, the possibility exists that, ultimately, the polymerized enzyme molecules leaked out for the reason given above. Again, thermal inactivation is an unlikely cause of activity loss because of the known prolonged stability of this enzyme at 23°C (*11*). It is also unlikely that glucose accumulates within the spheres, causing an inhibition of activity (*12*) because of the known rapid diffusion of this sugar from gel beads (*13*).

We have previously noted (*2*) that a major advantage of using PGAG gel spheres, over other kinds of gel spheres used in enzyme and microbial cell immobilization (*14*), is that they do not require exogenously added ions for maintenance of their structural stability. In this regard, the PGAG gel spheres used in the fixed-bed reactor for continuous cellobiose hydrolysis were structurally stable for at least 68 h.

CONCLUSIONS

The use of glutaraldehyde-modified β-glucosidase entrapped within structurally stable PGAG gel spheres for the continuous hydrolysis of cellobiose has been demonstrated. Such spheres may be useful in a bioprocess concerned with the enzymatic conversion of cellulosic materials to fuels and chemicals. It will be important, however, to maintain higher loadings of cellobiase activity within the spheres than those achieved in the present study. This will be possible through the formation of stable covalent bonds between β-glucosidase molecules prior to their entrapment. Finally, if the modification is carried out in the presence of cellobiose, it appears as if there is virtually no loss in catalytic activity.

ACKNOWLEDGMENTS

The authors would like to thank B. D. Faison and M. E. Reeves for reviewing the manuscript, and D. J. Weaver and S. A. 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-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

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