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Immobilization on Chitosan of a Thermophilic β-Glycosidase Expressed in *Saccharomyces Cerevisiae*

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ABSTRACT

A *Sulfolobus solfataricus* β-glycosidase expressed in *Saccharomyces cerevisiae* (Sβgly) was immobilized on chitosan activated with glutaraldehyde. The yield of immobilization was evaluated as 80%. Compared to the free β-glycosidase, the immobilized enzyme showed a similar pH optimum (pH = 7.0), the same increasing activity up to 80°C, improved thermostability, and no inhibition by glucose. Functional studies pointed out that the kinetic constant values for both enzymes were comparable. A bioreactor, assembled with the immobilized Sβgly, was used for glucose production. The values of cellobiose conversion increased on increasing residence time in the bioreactor, following a nonlinear trend. However, the highest glucose production/min was obtained at a flow of 0.5 mL/min.

Index Entries: β-Glycosidase; immobilized; support, chitosan; glutaraldehyde, coupling agent; immobilized β-glycosidase, properties; glucose, production; cellobiose, hydrolysis.

Abbreviations: ONPG, *ortho*-nitrophenyl-β-D-galactopyranoside; Sβgly, *Sulfolobus solfataricus* β-glycosidase.

INTRODUCTION

Cellulose is a linear polymer of D-glucose units linked by 1,4-β-D-glucosidic bonds. The enzymatic system for the cellulose-to-glucose conversion comprises endo-1,4-β-glucanase, cellobiohydrolase, and β-glucosidase.

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Cellulolytic enzymes in conjunction with β-glucosidase sequentially and cooperatively degrade crystalline cellulose into glucose. Endoglucanase acts in a random fashion on the low-crystallinity regions of the cellulosic fiber, whereas cellobiohydrolase removes cellobiose (β-1,4 glucose dimer) units from the nonreducing end of the cellulosic chain.

β-Glucosidases hydrolyze cellobiose and, in some cases cellooligosaccharides, into glucose, and are generally responsible for the regulation of the entire cellulolytic process; moreover, they represent a rate-limiting factor for enzymatic hydrolysis of cellulose, since endoglucanase and cellobiohydrolase activities are often inhibited by cellobiose (*1*). Thus, the β-glucosidase not only produces glucose from cellobiose, but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently. However, like glucanases, almost all β-glucosidases are subject to end product inhibition. Product inhibition and β-glucosidase thermal inactivation are two major barriers to the development of the enzyme hydrolysis of cellulose as a commercial process (*2*).

The use of enzymes in industrial applications is restricted, because most of them are relatively unstable, the cost of enzyme isolation and purification is still high, and it is technically expensive to recover active enzymes from the reaction mixture after completion of the catalytic process. To eliminate some of these deleterious effects, one approach is to attach or entrap enzymes onto/in water-insoluble solid matrixes. Such procedures immobilize enzyme molecules and make them insoluble in aqueous media (*3*).

Chitosan is a polysaccharide containing amino groups. It has been used as a support for enzyme immobilization, in an attempt to develop an inexpensive matrix. It can be easily obtained by partial deacetylation of chitin, one of the most widespread among the naturally occurring polysaccharides. Chitosan, in a soluble form, can be mixed with an enzyme solution, and a gel is formed by adding such a multifunctional crosslinking agent as glutaraldehyde (*4*).

The thermophilic and thermostable β-glycosidase (EC 3.2.1) isolated from the extreme thermophilic archaeon *Sulfolobus solfataricus* (Sβgly) was immobilized on chitosan with glutaraldehyde. This enzyme is of interest because of its broad specificity, its exoglucosidase activity, and its low inhibition by glucose. Since its gene has been cloned and expressed in yeast, the enzyme can be produced in large quantity and used, for example, coupled to cellulose system for the formation of glucose to produce ethanol.

MATERIALS AND METHODS

Reagents

Bacto-yeast nitrogen base, yeast extract, and bacteriological peptone were purchased from Difco; other media components were obtained from

Serva; bovine serum albumin was purchased from Boehringer Mannheim. Protein assay reagent was from Bio-Rad. Chitosan of low mol wt was from Fluka. All chemicals used were of reagent grade.

Strain and Growth Conditions

The engineered yeast (SoKY117 strain), kindly supplied by M. Ciaramella, was grown in defined medium (SD), containing 0.67% yeast nitrogen base supplemented with amino acids (50 μg/mL), or in complete medium (YP) containing 2% peptone and 1% yeast extract. The carbon sources were 2% glucose for SD and 2% galactose for YP media.

Yeast cells were grown in a 10-L bioreactor (New Brunswich); then a pilot experiment in a 100-L bioreactor (Terzano) was performed. To start the 10-L fermenter, 1 L of yeast suspension, grown in SD medium at 30°C in a Dubnoff incubator, was used. The fermenter process in the 10-L bioreactor was performed at 30°C in SD medium plus 50 mL of streptomycin sulfate (40 mg/mL). The fermenter was aerated with air, filtered using a 0.45 μm "on-line" filter (Millipore), at a flow of 20 L/min, and stirred at a speed of 200 rpm. Foam was suppressed by addition of 30 mL of sterilized siliconic antifoamer (2%). After 24 h, the suspension was transferred into a 100-L bioreactor containing YP medium plus 100 mL of sterilized antifoamer. Growth rate was followed by measuring pH and optical density of the suspension at 540 nm. Yeast growth was stopped in the stationary phase, corresponding to the maximum expression of the thermophilic enzyme. The biomass was centrifuged by an Alfa-Laval centrifuge at 12,000 rpm, and stored at −20°C; the yield was 13 g of wet cells/L of culture medium.

Enzyme Assay

The activity of the free enzyme was measured using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The ONPG hydrolysis reaction was followed spectrophotometrically at 75°C measuring the increase in the absorbance at 405 nm owing to the liberation of *o*-nitrophenol in a quartz cuvet of 1.0-cm path length. The standard reaction mixture (final volume 1.0 mL) contained: 50 m*M* sodium phosphate, pH 7.0, 2.8 m*M* ONPG, 5–10 μL of the enzyme solution (0.1 μg of enzyme), and distilled water to 1.0 mL.

Activity toward nonchromogenic substrates was determined as in Nucci et al. (7).

The activity of the chitosan-immobilized enzyme was determined by stirring the enzyme-support complex (0.5 mg) in 2.0 mL of standard reaction mixture under the same assay condition reported for the free enzyme; the reaction was stopped in ice bath after 10 min of incubation, centrifuged, and 1.0 mL of solution was withdrawn and tested as described above.

One enzymatic unit was defined as the amount of enzyme catalyzing the hydrolysis of 1.0 μmol of ONPG under the described conditions, assuming a molar absorption coefficient of 3100 at 405 nm for *o*-nitrophenol (*8*).

Enzyme Purification: Yeast Autolysis and Ultrafiltration

Yeast autolysis was performed suspending the thawed cells (120 g) in 240 mL of distilled water and incubating the resulting suspension in a water bath for 72 h. After autolysis, cell debris was removed by centrifugation in a Beckman centrifuge at 14,000 rpm for 30 min. The opalescent supernatant was clarified by underpressure filtration on a 0.22-μm filter (Millipak-40, Millipore).

The clarified supernatant was decolored and concentrated by using an Amicon ultrafiltration system (model CH2) equipped with a hollow-fiber cartridge (type III P30-20). Sβgly activity was assayed in the concentrate and in the permeate to evaluate the efficiency process.

Matrix Preparation

Low-mol-wt chitosan was solubilized in 1.0% acetic acid to a final concentration of 2.5% w/v. The solution was filtered through Whatman GF/A filters and precipitated by adding NaOH 1.0*M* until the pH 9.0 value was reached. The precipitate was centrifuged in a Sorvall RC 28 centrifuge at 8000 rpm for 5 min, and the pellet was washed several times with distilled water until neutral values of pH were obtained. Neutralized chitosan suspension was finally lyophilized and stored.

Enzyme Immobilization

The immobilization of Sβgly on chitosan was performed by the adsorption-reticulation method. A volume of 30 mL of the enzyme solution (600 U of Sβgly; 0.7 mg/mL protein concentration) in 50 m*M* sodium phosphate buffer, pH 6.0, was adsorbed onto 3.0 g of lyophilized chitosan powder for an enzyme:chitosan ratio of about 1:1000. The bound enzyme was about 80% of the total protein used in the immobilization reaction. The suspension was gently shaken for 2 h at room temperature. A glutaraldehyde solution was then added to the chitosan-enzyme suspension to a 2.5% v/v final concentration, and the mixture was gently stirred on a rotating shaker for 4 h. The resulting matrix was thoroughly rinsed with the same buffer until no absorbance was read at 280 nm; the absence of enzymatic activity in the last washing was checked.

pH Optimum Determination

The pH optimum of the free and immobilized Sβgly for the hydrolysis of ONPG was determined using the following buffers: 50 m*M* glycine-HCl in the pH range 3.0–3.5, 50 m*M* sodium acetate, pH 4.0–5.5,

50 m*M* sodium phosphate, pH 6.0–8.0, and 50 m*M* glycine-NaOH, pH 8.5–10. The buffer pH values were measured at room temperature.

The Sβgly assays for the free and immobilized enzyme were carried out at 75°C in the presence of 2.8 m*M* ONPG using 0.5 or 500 μg of free and immobilized enzymes, respectively.

Optimal Temperature and Thermostability

The temperature dependence of the free and immobilized enzymes was determined by assaying both in the standard mixtures at temperatures ranging from 30–80°C.

The thermal stability of the free and immobilized Sβgly was tested by incubating protein samples in sealed tubes at 75 and 85°C. At intervals, aliquots of the incubated enzymes were withdrawn from the mixture and assayed under standard conditions, following the ONPG hydrolysis reaction.

Kinetic Analysis

The kinetic constants of the free and immobilized enzymes on synthetic and natural substrates were measured at 75°C under standard assay conditions by a spectrophotometer Cary 1E (Varian Ltd.).

The data were analyzed with the Enzifitter program (Data Analysis Program by Leatherbarrow, R. J., Elsevier-Biosoft).

Bioreactor Building Up

A chromatographic column (Pharmacia 1 × 15 cm) was filled with the chitosan-bound enzyme. It was connected to a thermostated bath, a peristaltic pump, and a fraction collector (LKB) following the scheme of Fig. 1.

The bioreactor efficiency was tested at 75°C by a solution of 10 mL 250 m*M* cellobiose in 50 m*M* sodium phosphate buffer, pH 7.0, at a flow speed of 0.1–0.5 mL/min.

RESULTS AND DISCUSSION

The Sβgly expressed in *Saccharomyces cerevisiae* was immobilized on chitosan using the bifunctional agent glutaraldehyde; some properties of the immobilized enzyme were studied and compared with those of the free enzyme.

pH Dependence of Catalytic Activity

The pH optimum for the catalytic activity of the immobilized enzyme was 7.0 in 50 m*M* sodium phosphate buffer, in agreement with the same

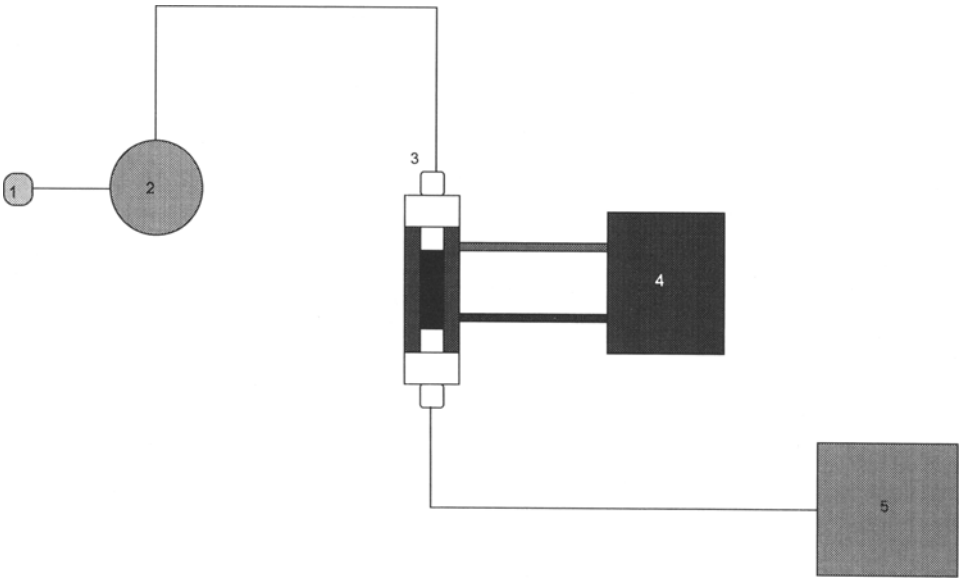

Fig. 1. Scheme of the bioreactor: (1) substrate; (2) peristaltic pump; (3) bioreactor; (4) thermostated bath; (5) fraction collector.

value found for the free enzyme. Enzymatic activity exhibited the typical bell-shaped curve.

Figure 2 shows the pH dependence of immobilized Sβgly activity in the pH range 3.0–10.0.

Temperature Dependence of Catalytic Activity

The temperature dependence of the immobilized Sβgly showed a regular increase in the temperature range 30–80°C in 50 m*M* sodium phosphate buffer, pH 7.0, under standard assay conditions (Fig. 3).

The rate of thermal inactivation of the immobilized Sβgly, in 50 m*M* sodium phosphate buffer, at two temperatures is shown in Fig. 4. After a 5-h incubation at 75 and 85°C, 85 and 75% of the initial activity, respectively, were retained; these results are similar to those previously described for the free enzyme that were 83% at 75°C and 57% at 85°C, thus showing a larger thermostability of the immobilized enzyme at 85°C.

Kinetic Analyses

Kinetic analyses concerned the determination of K_m and k_{cat} values for the chromogenic ONPG and for nonchromogenic cellobiose substrates. The results are summarized in Table 1.

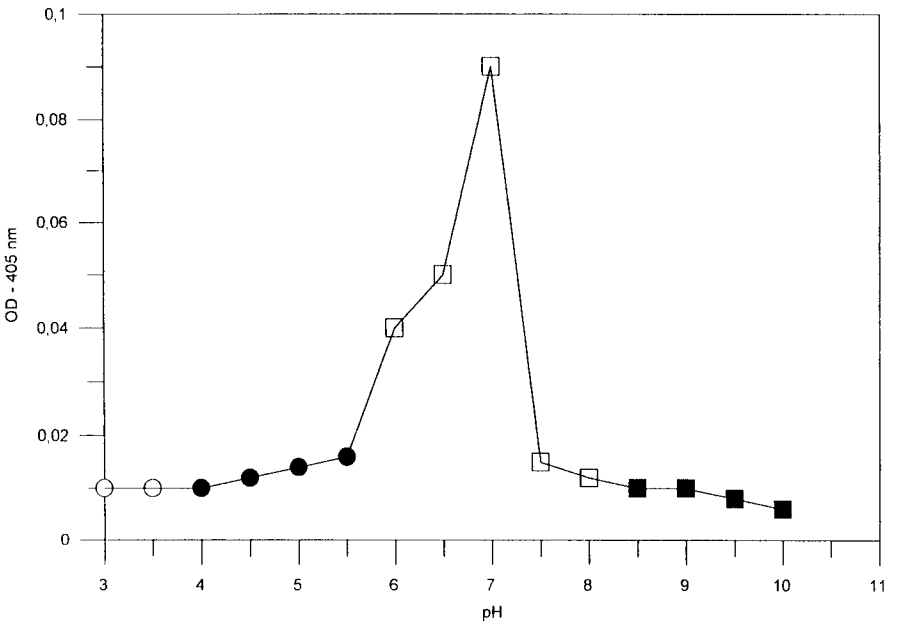

Fig. 2. Effect of pH on the activity of the immobilized Sβgly. Experiments were performed at 75°C, using 2.8 m*M* ONPG as substrate. The buffers used were: ○ Gly-HCl, ● NaAc., □ Na-phosphate, ■ Gly-NaOH.

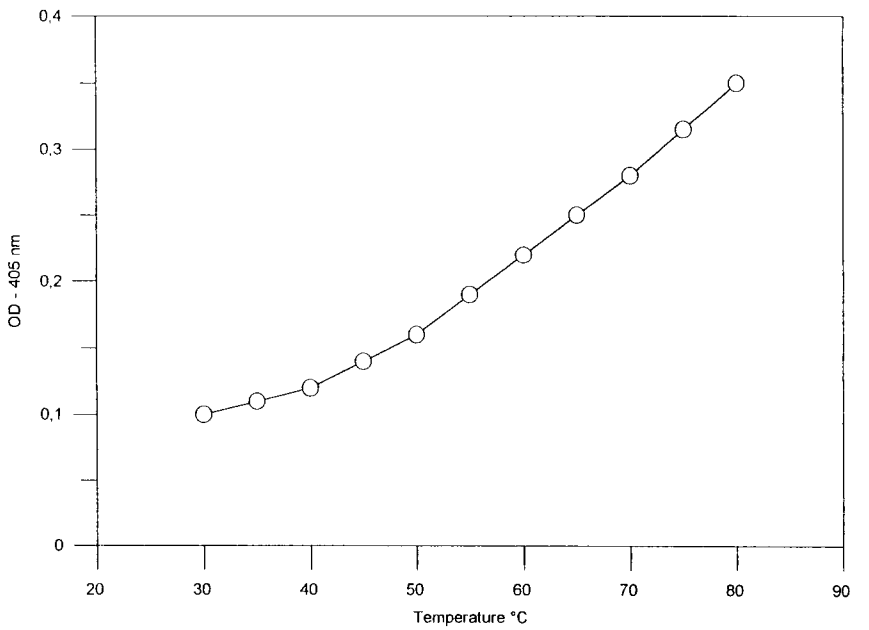

Fig. 3. Effect of temperature on activity of the immobilized Sβgly. Enzymatic assays were performed in 50 m*M* sodium phosphate buffer, pH 7.0, 2.8 m*M* ONPG.

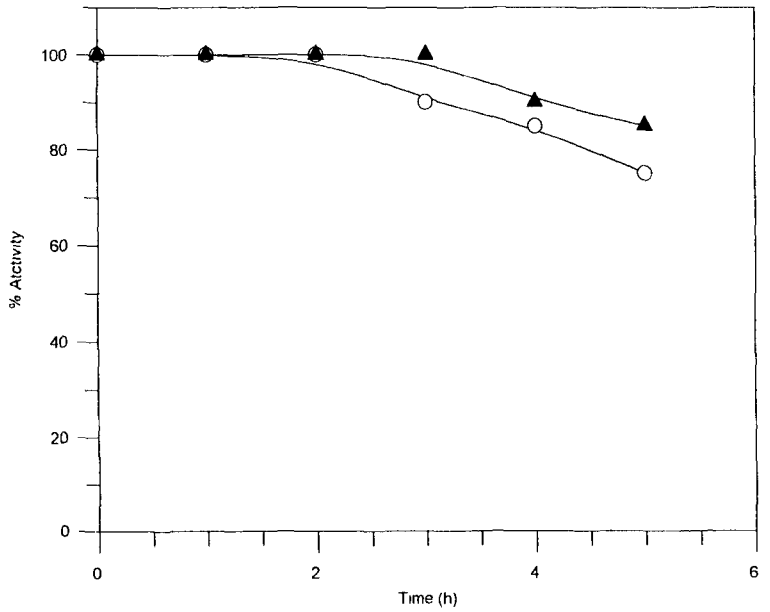

Fig. 4. Thermostability of the immobilized Sβgly. Experiments were carried out at 75°C (—▲—) and 85°C (—○—), using enzymatic solutions at 0.5 mg/mL. Enzymatic assays were carried out as described in Materials and Methods. The starting activity was taken as 100%.

Table 1
Kinetic Constants for the Free and Immobilized Enzymes

	Free enzyme		Immobilized enzyme	
Substrate	K_m, mM	k_{cat}, s^{-1}	K_m, mM	k_{cat}, s^{-1}
Cellobiose	31.5	644	20.0	746
ONPG	3.9	1713	5.0	1686

Enzymatic assays were carried out at 75°C, under the conditions described in Materials and Methods.

The comparison between the kinetic constants of the free and immobilized enzymes, analyzed by the Enzfitter Program, showed similar values at a temperature of 75°C, indicating that the immobilization procedure does not alter these properties of the enzyme. The lower value of K_m observed for the immobilized enzyme tested on cellobiose could be explained in light of a better interaction between a hydrophilic support as chitosan and the hydroxyl groups of the substrate. The kinetic values are the means of two determinations.

Assays on the Bioreactor

The practical use of the immobilized Sβgly was tested in preliminary experiments, filling the bioreactor with the immobilized enzyme for glucose production from cellobiose. The results are summarized in Table 2.

Table 2
Main Distinctive Features
of the Bioreactor as Function of Different Flow Rates

Flow, mL/min	0.1	0.25	0.4	0.5
Transformed cellobiose (mmol/cycle)	0.7	0.35	0.27	0.25
% Yield of transformed cellobiose/cycle	28.0	4.0	11.0	10.0
Time of cycle (min)	100	40	25	20
Transformed cellobiose (μmol/min)	7	8.75	10.8	12.5

A solution, 250 m*M* cellobiose (10 mL), was used for a complete reaction cycle, at 75°C.

Table 3
Bioreactor Yield as Function
of Number Cycles and Flow Speed

No. of cycles	Flow speed 0.5 mL/min	Flow speed 0.4 mL/min
1	9.6%	10.9%
2	19.8%	21.6%
3	36.2%	38.0%

A solution, 250 m*M* cellobiose (10 mL), was used for a complete reaction cycle, at 75°C.

At a flow speed of 0.5 mL/min, the highest production of glucose in μmol/min was observed. In Table 3, we report the final yield (36%) of glucose after three cycles at two different flow speeds.

Interestingly, the bioreactor assayed after 25 cycles of transformation retained 90% of its initial activity.

Storage Stability

The immobilized enzyme was kept at 4°C to test its storage stability. The activity of the immobilized Sβgly did not change for more than 2 mo, and only 20% of the initial activity was lost after a 4-mo storage.

CONCLUDING REMARKS

A continuous cellobiose hydrolysis system for glucose production has been developed with a high degree of conversion by immobilized-Sβgly.

The bioreactor could run at high flow rates with corresponding high productivity and good operational stability.

Chitosan is a suitable biocompatible and inexpensive support for a bioreactor in which the main features of the free enzyme are preserved.

This would allow us to build up a biphase reactor in which a cellulase complex as that present in the thermophilic *Clostridium thermocellum (9)*, that brings together the endo and exo 1,4-β-glucanases required for true cellulase activity is on line with the β-glucosidase and exo-glucosidase activities specific of our Sβgly. The synergistic action of these enzymes will permit the degradation of cellulose to soluble sugars for food industry. The possibility to co-immobilize multiple compatible enzymes into a designed catalyst could drastically change the process economics for saccharification of cellulosic substrates.

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