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# A Highly Active Fungal β-Glucosidase

## Purification and Properties

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## ABSTRACT

A highly active thermostable β-glucosidase was purified to homogeneity from a strain of *Trichoderma sp.* The enzyme was an extracellular glycoprotein and showed hydrolytic activity toward several β-glucosides.

Cellobiose was found to be the substrate of choice for this enzyme. This finding could suggest future technological applications of the purified protein.

**Index Entries:** β-Glucosidase; purification; β-glucosides; cellobiose.

## INTRODUCTION

Much worldwide attention is devoted to the hydrolysis of cellulose because it is the most abundant renewable carbon source (*1*). However, cellulose hydrolysis to glucose is not as easy as starch hydrolysis, and therefore high temperatures and strong mineral acids are usually required to obtain satisfactory degrees of hydrolysis. Moreover, the resulting glucose could be partially altered if the conditions of reaction are too drastic.

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An alternative route from cellulose to glucose could be represented by enzymic hydrolysis; however, industrial processes based on the use of cellulolytic enzymes have several limitations:

1. Large amounts of enzyme are required;
2. Crystalline regions within the cellulose fibers are not easily attacked by cellulases because of strong steric hindrances; and
3. Some components of the cellulase complex (endoglucanase, EC 3.2.1.4 and exoglucanase; EC 3.2.1.91) are inhibited by cellobiose, an intermediate product of hydrolysis.

In the enzymic hydrolysis of cellulose the β-glucosidase activity of the enzyme complex is generally the factor limiting the kinetics of the process (*2,3*). Consistently, this enzyme is inhibited by its reaction product, glucose, thus leading to accumulation of cellobiose, which inturn inhibits the exo-1,4-β-glucanase and endo-1,4-β-glucanase activity of the complex. On the other hand, glucose solutions of high concentrations are needed for an efficient and economic fermentation of the sugar to alcohol (*15*).

In order to overcome these problems, the enzyme was made reusable by immobilization on solid supports (*5*) and efforts were made to select strains (wild or mutant), producing greater amounts of β-glucosidase. Preliminary work has shown that inhibition by glucose was substantially reduced for immobilized β-glucosidase in comparison with its soluble counterpart.

This work describes the purification and some properties of a highly active β-glucosidase from a mutant of the fungus *Trichoderma sp.* strain M7.

## MATERIALS AND METHODS

### Strain

β-Glucosidase was obtained from a strain of *Trichoderma sp.* isolated in the Institute of Microbiology of the University of Genoa by G. Satta. This strain is quite different from the well known *Trichoderma reesei* on the basis of both morphological and physiological evidences. The fungus was characterized by a complete pattern of cellulolytic enzymes and a good hemicellulose degrading activity. It was grown as described by Pompei (*6*).

### Growth Conditions

The fermentation was performed in a bioreactor LKB 1601 with 12 L of the medium M1, added with 0.2% antifoam fluid and 1% wheat bran. Parameters of culture were as follow: agitation, 300 rpm; temperature, 28°C; air flux, 6 L/min; pH 5.5.

At various times during fermentation, samples were taken to check enzyme production.

## β-Glucosidase Activity

The activity of β-glucosidase was tested using p-nitrophenyl-β-D-glucopyranoside (β-pNPG) as the substrate. Experimental conditions are described elsewhere (*7*).

In a typical experiment, 0.1 mL of enzyme solution was added to 1.9 mL of 0.1*M* sodium acetate buffer, pH 5, containing 10 μmol of β-pNPG. The reaction mixture was incubated for 10 min at optimal temperature under continuous stirring; after incubation, 1 mL of the solution was transferred to a test tube containing 1 mL of 1*M* $Na_2CO_3$. The quantity of *p*-nitrophenol produced was measured by the OD change at 400 nm. One unit of enzyme activity is defined as the amount of protein required to produce 1 μmole of *p*-nitrophenol/minute, at 55°C of temperature.

## Cellobiase Activity

This was determined by the method of Sternberg (*8*). The reaction mixture contained 0.1 mL of enzyme solution and 0.1 mL of 20 m*M* cellobiose solution. After incubating the mixture at 55°C, the glucose produced was estimated using glucose oxidase, following the method of MacEwen (*9*). One unit of enzyme activity is defined as the amount of protein required to produce 2 μmoles of glucose/min, at 55°C.

## Determination of Protein

Protein concentration were determined by the method of Bradford (*10*), using a commercial concentrated reagent. BSA was used as the standard protein.

## Sugar Determination

Neutral sugars were determined following the method of Dubois (*11*).

## Electrophoresis

Slab gel electrophoresis was performed following the method of Gabriel (*12*) with minor modification. The gels were suitable for qualitative enzyme determination using an appropriate solution of β-pNPG.

## SDS-PAGE

This was performed following the method of Weber (*13*) with minor modification; as the standard was used "Molecular Weight Standards, High," obtained from Bio-Rad (Richmond, CA).

## The Isoelectric Point

This was determined following the method of Righetti (*14*); "Isoelectric Focusing Calibration Kit" by Pharmacia (Uppsala, Sweden) was utilized as standard, using ampholine (3.5–10 and 4–6) obtained by LKB.

### The Aminoacids Composition of the Enzyme

This was determined using DABS Aminoacids Kit, obtained from Beckman (Fullerton, CA), with a Beckman System Gold HPLC.

Tryptophan was determined by the method of Edelhoch (*15*).

HPLC size exclusion chromatography was performed by a BIO-SIL SEC-250 300 × 7.5 mm column obtained from Bio-Rad (mobile phase: 0.1*M* $Na_2SO_4$, 0.1*M* $NaH_2PO_4$, pH 6.8; flowrate 1 mL/min; $T$ = 25°C).

## RESULTS AND DISCUSSION

### Purification of β-Glucosidase

1. Twelve liters of the crude culture filtrate was centrifuged at 4°C and 14,300*g* for 45 min. The supernatant was passed through no. 1 Whatman filter paper (Maidstone, England) and the resulting solution was concentrated and dialyzed by means of a Minitan apparatus equipped with polysulfone membranes (cutoff 10,000 kDa).
2. The solution obtained from the step 1 was passed through a DEAE-cellulose column (5.0 × 15 cm) previously equilibrated with 10 m*M* potassium phosphate buffer, pH 6.5. The column was then eluted in a stepwise manner with the following buffer:
   a. the same buffer as above;
   b. 50 m*M* phosphate buffer, pH 6.5; and
   c. 75 m*M* sodium acetate buffer, pH 5.
   The enzyme was eluted with the last buffer and the β-glucosidase was again concentrated and dialyzed against 10 m*M* potassium phosphate buffer, pH 6.5.
3. The concentrated enzyme solution was subjected to chromatography on a column (2.5 × 15 cm) of DEAE Sepharose, which had been equilibrated with 10 m*M* potassium phosphate buffer pH 6.5.
   The elution was carried out as described in step 2 above.
4. The concentrated enzyme solution (55 mL) was loaded on a column (3 × 100 cm) of Sephacryl S-300 previously equilibrated with 50 m*M* sodium acetate buffer, pH 5. The elution was carried out with the same buffer. Two peaks were obtained with β-glucosidase activity recovered in the first peak. The active fractions were pooled and concentrated to obtain an enzyme solution suitable for further experiments.

The results of β-glucosidase purification are reported in Table 1.

The purified enzyme showed a single sharp band on SDS-PAGE.

Table 1
Steps of Purification of Trichoderma M7 β-Glucosidase and Activity with p-NPG as Substrate[a]

| Step | Total volume, mL | Protein, mg/mL | Specific activity | Total units | Purification, fold | Yield, % |
|---|---|---|---|---|---|---|
| Crude culture filtrate | 12,000 | — | — | — | — | — |
| Centrifugation 9000 rpm Minitan S Ultrafiltration | 800 | 3.36 | 11.24 | 30,300 | 1 | 100 |
| DEAE cellulose column chromatography | 800 | 0.42 | 77.3 | 26,000 | 6.8 | 87 |
| DEAE Sepharose column chromatography | 55 | 3.0 | 96.5 | 15,900 | 8.6 | 51.5 |
| Sephacryl S-300 column chromatography | 132 | 0.47 | 254 | 15,270 | 22 | 50.4 |

[a]Experimental details are given in the text.

## Properties of the Enzyme

### *Molecular Weight*

The enzyme preparation was homogeneous by the criteria of SDS-PAGE. In fact only one band was found when the preparation was subjected to PAGE under denaturing conditions, which corresponded to a single activity band.

Determination of the mol wt, performed under denaturing conditions by SDS-PAGE, in the presence and absence of mercaptoethanol, gave values of 109,000 ± 2000 kDa.

A mol wt of 178,000 kDa was estimated for the native enzyme by gel filtration under nondenaturing conditions.

The noticeable difference seen in the mol-wt determinations could be attributable to the high sugar content of the enzyme, with could deeply change the SDS/protein ratio.

### *Carbohydrate Concentration*

Carbohydrate analysis showed the presence of neutral sugars in amount of about 75%. Acid sugars were not determined.

### *Isoelectric Point*

In the crude extract and during the initial step of the purification procedure, two forms of the enzyme with different electrophoretic mobilities were evidentiated. Similar results were obtained by electrofocusing. These two forms showed an isoelectric point of 4.2 and 4.8, respectively. The predominant form was that with a pI of 4.8. The other form was present in a low quantity and was lost at the third step of purification. No attempt was made to purify this form of the enzyme.

Table 2
Aminoacid Composition
M7 β-Glucosidase[a]

| Amino acid | Mol% |
|---|---|
| Gly | 10.40 |
| Ala | 9.46 |
| Val | 8.64 |
| Leu | 3.97 |
| Ile | 7.00 |
| Ser | 5.72 |
| Thr | 6.54 |
| Cys | 0.23 |
| Met | 2.45 |
| Asp | 11.68 |
| Glu | 7.83 |
| Arg | 3.27 |
| Lys | 3.15 |
| His | 1.87 |
| Phe | 3.50 |
| Tyr | 8.06 |
| Trp | 1.11 |
| Pro | 7.19 |

[a]Tryptophan was determined by the method of Edelhoch. Ammonia was not determined.

The presence of multiple forms of β-glucosidase has been reported for the enzyme extracted from several fungi (*16*), but their origin and function are not clear.

### *Amino Acid Composition*

The amino acid composition (Table 2) showed that the total number of acidic amino acids was greater than that of the basic ones. This finding is in keeping with the pI of the protein and suggests the presence of a relatively low quantity of amide groups.

### *Optimum pH and Temperature*

The pH optimum for the activity was found to be 4.0.

The activity was also tested at different temperatures, ranging from 30 to 90°C with 5°C intervals; the activity was unaffected up to 50°C for 1 h. After 1 h incubation at 60°C, 80% activity could still be observed. The thermostability of protein could be attributed to the high content of nonpolar amino acids (*17*).

### *Substrate Specificity and Kinetic Properties*

β-Glucosidase activity was tested on various substrates. Table 3 shows that the enzyme is properly described as a β-glucosidase because its activity

Table 3
β-Glucosidase Activity on Various Substrates[a]

| | |
|---|---|
| Cellobiose | 100 |
| β-pNPG | 100 |
| Cellotriose | 78 |
| Cellotetraose | 69 |
| Crystalline cellulose | — |
| Soluble CMC | — |
| Insoluble CMC | — |
| Esculin | 25 |
| 4-MUG | 83 |

[a]Relative activities expressed as percentage of the activity on cellobiose.

Table 4
Main Properties of β-Glucosidase from Trichoderma M7

| | $K_M$, mM | $V_{max}$[a] | Optimal pH | Optimal temperature |
|---|---|---|---|---|
| β-pNPG | 0.096 | 351 | 4 | 75°C |
| Cellobiose | 0.8 | 331 | 3.5–5 | 65°C |

[a]$V_{max}$: Glycosidic bonds cleaved/min/mg of protein. These were determined by checking glucose or *p*-nitrophenol concentrations in the assay mixtures. Experimental details are given in the text.

toward β-pNPG is almost identical to that on cellobiose, which is the substrate of choice in typical experiments on cellulolytic enzymes.

It is worth noting that whereas esculin (6,7-dihydroxycoumarin-6-β-D-glucopyranoside) is only slowly attached and β-oNPG is a poor substrate, 4-methyl-umbelliferyl-β-glucopyranoside (4-MUG) is rapidly hydrolyzed by the enzyme. These results suggest that M7 β-glucosidase activity could be inhibited by sterical hindrance.

The main kinetic properties of the enzyme are given in Table 4. The kinetic data show that the enzyme has a very high affinity for its main substrates cellobiose and β-pNPG, comparable with that of some known β-glucosidase (*5,7,17*); the value for $V_{max}$ was also very interesting in comparison with this value for similar enzymes (*5,7,17*).

The enzyme was competitively inhibited by glucose ($K_I$ = 0.65 m*M*) and by D-gluconolactone ($K_I = 9 \times 10^{-4}$ m*M*).

## CONCLUSION

The new strain of *Trichoderma sp.* used for β-glucosidase production and purification grew easily in a simple medium.

The purification of the enzyme was rather rapid in that relatively low levels of other protein were found in the crude culture medium.

The enzyme was very active towards cellobiose whereas routine activity measurements could be performed by using β-pNPG as a chromogenic substrate.

Other β-glucosides are comparatively poor substrates, as expected for an enzyme that is part of a cellulolytic complex and could be more properly named cellobiase.

In conclusion, the enzyme shows a good tolerance against inhibition by glucose and is thermoresistant enough to allow it to be successfully used in technological applications involving cellulose hydrolysis.

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