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Production of β-Galactosidase by *Trichoderma reesei* FTKO-39 in Wheat Bran

Partial Purification of Two Isozymes

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Abstract

Trichoderma reesei FTKO-39 grown at 35°C for 5 d on wheat bran supplemented with $MgCl_2$ and lactose as the carbon source produced two isozymes of β-galactosidase: BGT I and BGT II. These isozymes were partially purified on a DEAE-Trisacryl column. Both BGT I and BGT II fractions exhibited optimum activity at 65°C, but the pH optima were 4.0 and 6.5, respectively. The isozymes also showed similar thermal stability. However, BGT I was more stable than BGT II in a pH range of 3.0–10.0. At least two different β-galactosidases are produced by *T. reesei*, as revealed by the two bands seen on a 6% polyacrylamide gel stained for activity.

Index Entries: β-Galactosidase; isozymes; partial purification; semisolid fermentation; *Trichoderma reesei*.

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Introduction

Because of their industrial applications in lactose hydrolysis and their uses in nutritional and clinical diets, β-galactosidases (EC 3.2.1.23) from diverse sources have been isolated and characterized and their properties (stability, activation and inhibition effects, transgalactosylation activity, and immobilization on various supports) have been studied *(1–3)*. Various isozymes are produced by a number of organisms and are widely distributed in nature. This fact suggests that β-galactosidases play an important physiologic role *(1,4–13).* Their major application so far has been in the dairy industries *(14)* in the removal of lactose. For this purpose, hydrolysis at elevated temperatures has several advantages, such as improved lactose solubility and rate of hydrolysis, reduction in crystallization and viscosity, and prevention of contamination *(11,15)*. On the other hand, high temperatures are limited by the stability of the enzyme and, thus, factors or compounds that increase β-galactosidase stability are of interest *(3)*.

The aim of the present work was to study the partial purification and characterization of two isozymes of β-galactosidase produced by *Trichoderma reesei* strain FTKO-39 in semisolid fermentation on wheat bran.

Materials and Methods

Strain, Growth, and Enzyme Extraction

T. reesei strain FTKO-39 was donated by the Department of Agro-Industry, Food and Nutrition of ESALQ (University of São Paulo at Piracicaba, SP, Brazil). The strain was grown on semisolid medium containing 5.0 g of wheat bran mixed with 10 mL of 0.010% $MgCl_2$ and 2% lactose. Cultures were grown at 35°C for 5 d and then stirred for 15 min at 4°C with 30 mL of 0.050 mol/L sodium phosphate buffer, pH 7.5. The crude extract was filtered on paper and centrifuged at 9000*g* at 4°C for 20 min. The sediment was discharged, and the supernatant was dialyzed against distilled water and used as the source of extracellular β-galactosidase.

Enzyme Assays

Extracts and fractions were assayed for β-galactosidase by Lederberg's *(16)* method. The reaction mixture contained 0.015 mol/L of *o*-nitrophenyl β-D-galactopyranoside (*o*NPG), 0.050 mol/L of sodium phosphate buffer at pH 7.5, 0.001 mol/L of $MgCl_2$, and the enzyme in a final volume of 0.50 mL and was incubated at 30°C. The amount of *o*-nitrophenol released after the enzymatic hydrolysis of the substrate was determined from the absorbance at 405 nm in an Ultrospec 1000 UV/VIS spectrophotometer (Amersham Biosciences). For the eluted proteins from chromatographs, 0.10 mL of each fraction was assayed with lactose as substrate. After incubation at 35°C for 30 min, the reaction was stopped by immersing the flasks in boiling water for 5 min. The glucose released was determined by the glucose oxidase/peroxidase system in an Ultrospec 1000 UV/VIS

spectrophotometer at 505 nm. One enzyme unit is defined as the amount that releases glucose at an initial rate of 1 μmol/min. Protein was determined by Lowry's *(17)* method using bovine serum albumin as the standard. Proteins in the eluted fractions were detected in an Ultrospec 1000 UV/VIS spectrophotometer at 280 nm.

Anion Exchange and Gel Filtration Chromatography

The pH of the crude extract was adjusted to 7.5 with 0.200 mol/L of sodium phosphate solution and was then clarified on a DEAE-cellulose column (100 H 35 mm) equilibrated with standard buffer (0.050 mol/L of sodium phosphate buffer at pH 7.5, 0.001 mol/L of $MgCl_2$). Fractions with β-galactosidase activity were mixed, and the proteins were concentrated by adding ammonium sulfate slowly to 30% saturation and centrifuging at 9000*g* and 4°C for 20 min. The precipitate was discarded, and the proteins of the supernatant were precipitated with 70% ammonium sulfate. This precipitate was dissolved in buffer and dialyzed against distilled water, the pH was adjusted to 7.5, and the sample was applied to a DEAE-Trisacryl column (150 H 25 mm) equilibrated with the standard buffer to which 0.02% Triton X-100 and 0.0001 mol/L of EDTA had been added. After 500 mL of washout, a linear gradient of NaCl (0–0.500 mol/L) in the same buffer was applied to the column. The fractions with β-galactosidase activity were mixed, dialyzed against the same buffer, and lyophilized. The sample obtained was applied to a Sephacryl S-300 HR column (600 H 15 mm).

Characterization of β*-Galactosidase Isozymes*

Optimal temperatures of the BGT I and BGT II isozymes were determined in the range 20–90°C using *o*NPG as the substrate in the standard buffer. Optimal pH was determined using McIlvaine buffer with a pH ranging from 3.0 to 8.5 at 65°C. For thermal stability, the isozymes were incubated at 65°C for 3 h in sodium phosphate buffer (pH 4.0 for BGT I and pH 6.5 for BGT II) plus 0.001 mol/L of $MgCl_2$. At 30–min intervals, aliquots of the solutions were placed in an ice bath for 10 min and the remaining activity was determined. To determine pH stability, the enzymes were incubated in McIlvaine buffer containing 0.001 mol/L of $MgCl_2$ with the pH ranging from 3.0 to 10.0 at 30°C. After 30 min, the remaining activities were assayed at 65°C. The apparent values of *Km* and V_{max} for each isozyme were determined for the substrates *o*NPG from 0 to 0.020 mol/L and lactose from 0 to 0.200 mol/L at the optimal temperature and pH.

Results and Discussion

Partial Purification of β*-Galactosidase*

T. reesei strain FTKO-39 was grown at 35°C for 5 d on wheat bran supplemented with 0.01% $MgCl_2$ and 2% lactose, with the purpose of producing β-galactosidase. The crude extract was clarified on a DEAE-cellulose column, and proteins were concentrated with 30–70% ammonium

Table 1
Purification of Extracellular β-Galactosidases From *T. reesei* FTKO-39

Step	Total protein (mg/mL)	Total activity (μmol/[min·mL])[a]	Specific activity (μmol/[min·mg])[a]	Purification (fold)	Yield (%)
Crude extract	10.54	25.43	2.41	1.00	100.00
DEAE-Cellulose[b]	9.89	24.54	2.48	1.03	96.50
30–70% $(NH_4)_2SO_4$	1.97	12.25	6.22	2.58	48.17
DEAE-Trisacryl[b]					
BGT-I	0.12	2.53	21.08	8.75	9.95
BGT-II	0.09	1.83	20.33	8.44	7.20
S-300 HR[b]					
BGT I	0.04	1.25	31.25	12.97	4.92

[a]Assayed in 0.015 mol/L of *o*NPG, 0.05 mol/L of sodium phosphate, 0.001 mol/L of $MgCl_2$ (pH 7.5, 30°C).
[b]Eluted with standard buffer of 0.010 mol/L of sodium phosphate, 0.02% Triton X-100, 0.0001 mol/L of EDTA, and 0.001 mol/L of $MgCl_2$ (4°C, pH 7.5).

sulfate. After dialysis, the sample was applied to a DEAE-Trisacryl column. The chromatographic profile of the sample revealed two fractions with β-galactosidase activity, named BGT I and BGT II, eluted with solutions of NaCl at 0.15 and 0.25 mol/L, respectively. Table 1 summarizes the results of partial purification of both enzymes. This separation of β-galactosidases from *T. reesei* strain FTKO-39 into more than one form is similar to the pattern observed in other sources.

Characterization of the β*-Galactosidase Isozymes*

The optimum temperature was found to be 65°C for both fractions, BGT I and BGT II (Fig. 1). A chimeric β-galactosidase of *Thermus* sp. had an optimum temperature of 90°C *(15)*, while that of an immobilized thermostable β-galactosidase was 78°C *(18)*. Li et al. *(7)* characterized in mung bean seedling five β-galactosidase isozymes with temperature optima varying from 50 to 53°C. Hoyoux et al. *(19)* found that a cold-adapted β-galactosidase from an Antarctic psychrophile exhibited an optimum at 10°C. The great variation in optimal temperatures of β-galactosidase from different sources reflects its wide distribution in nature. The energy of activation (*EA*) was determined as 32.5 and 35.0 kcal/mol for BGT I and BGT II, respectively (Fig. 2). When incubated at 65°C, the half-lives were 128 and 118 min for BGT I and BGT II, respectively. However, BGT II showed a rise of 40% in its activity at 60 min (Fig. 3). Lau *(6)* determined 50% thermal deactivation of the maximum of a human β-galactosidase after 20 min at 45°C. Thermophilic bacteria *Thermus* sp. produced a β-galactosidase that retained 50% of its initial activity after 60 min at 70°C *(15)*, and *Saccharopolyspora rectivirgula* produced an enzyme stable for 22 h at 70°C *(11)*. Coordinated metal, substrate, and pH dependence are cited as thermal

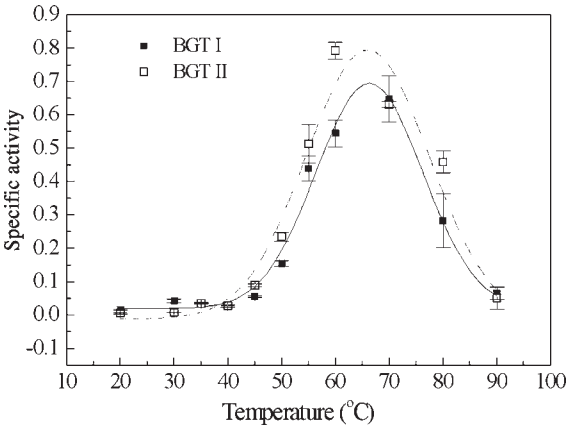

Fig. 1. Effect of temperature on BGT I and BGT II fractions.

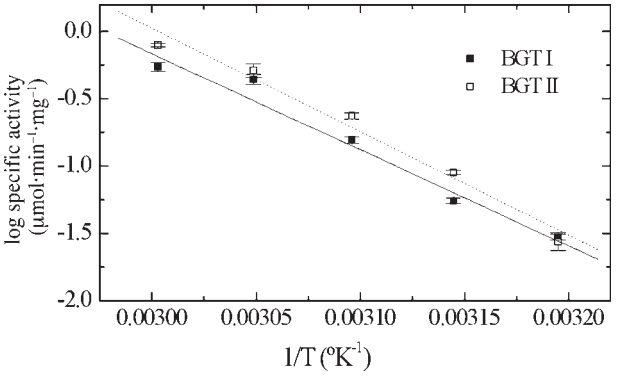

Fig. 2. Arrhenius plot for BGT I and BGT II fractions using *o*NPG as substrate.

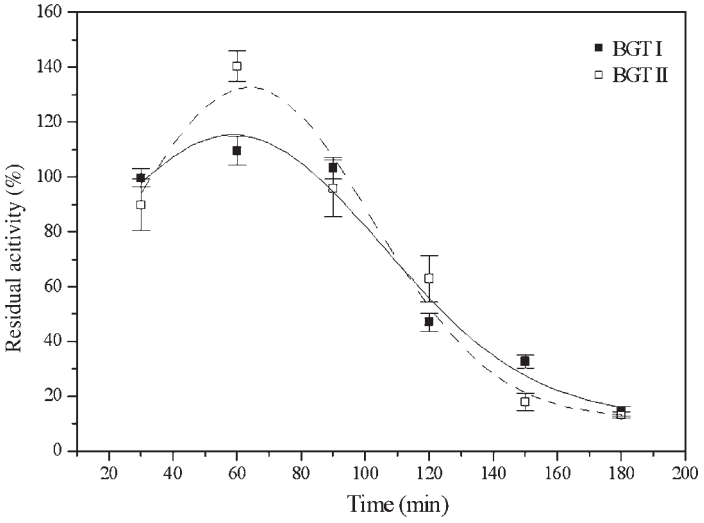

Fig. 3. Stability of β-galactosidase activity at 65°C using *o*NPG as substrate at pH 4.5 for BGT I and pH 6.5 for BGT II fraction.

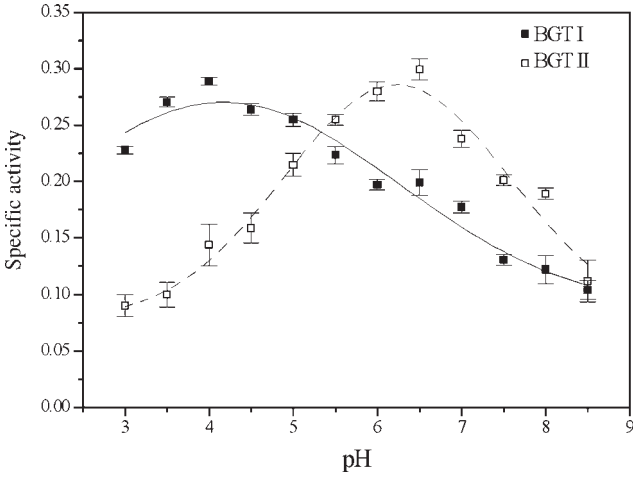

Fig. 4. Influence of pH on BGT I and BGT II activity using *o*NPG as substrate at 65°C.

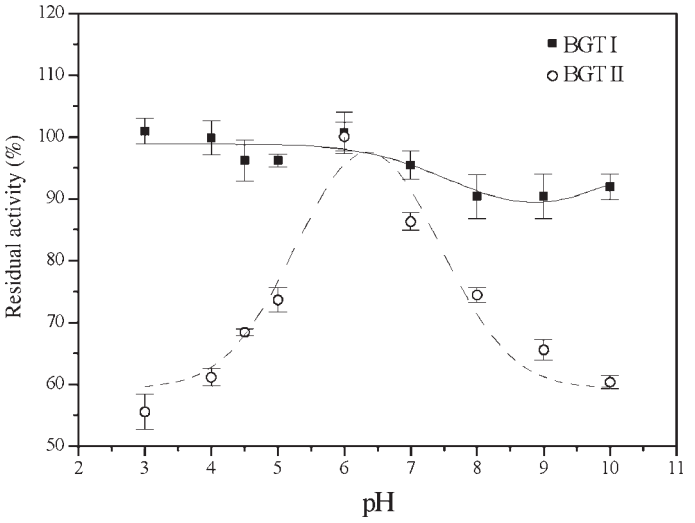

Fig. 5. pH stability of BGT I and BGT II after 30 min at 65°C using *o*NPG as substrate.

stability effectors of some β-galactosidases *(1,3,13,20)*. The initial rise in the BGT II activity could indicate metal coordination and/or pH dependence.

The enzymes clearly exhibit distinct pH optima, despite their similar thermal stability and optimal temperature. Variation of optimal pH occurs frequently among isozymes from a given source. The optimum for BGT I was 4.0 whereas that for BGT II was 6.5 (Fig. 4). Mbuyi-Kabala et al. *(9)* isolated four isozymes of β-galactosidase from *Saccharomyces lactis* that exhibited maximum activity around pH 7.0 and differed in their physical properties and kinetic characteristics. Van Laere et al. *(21)* purified

Table 2
Kinetic Parameters of *T. reesei* FTKO-39 β-Galactosidases Determined at 65°C and pH 4.5 for BGT I or 6.5 for BGT II With *o*NPG and Lactose as Substrates

	*o*NPG		Lactose	
Isozymes	*Km* (m*M*)	V_{max} (μmol/[min·mg])	*Km* (m*M*)	V_{max} (μmol/[min·mg])
BGT I	1.65	0.55	2.66	0.41
BGT II	21.68	0.26	19.01	0.18

a β-galactosidase from *Bifidobacterium adolescentis* with maximum activity at pH 6.0. A β-galactosidase from *Actinomyces viscosus* had an optimal pH between 6.0 and 6.5 *(22)*, and a β-galactosidase from the human intestine had a pH optimum at 6.0 *(6)*. Li et al. *(7)* described five β-galactosidase isozymes from mung bean with optimal pH between 3.6 and 4.0. BGT I activity was more stable than BGT II over a pH range of 3.0–10.0 for a period of 30 min, exhibiting a residual activity above 90% over the tested pH range and time. On the other hand, BGT II had lower residual activity than BGT I, being above 50% in these conditions (Fig. 5).

Kinetic Parameters

Km and V_{max} were determined for partially purified BGT I and BGT II using *o*NPG and lactose as substrates (Table 2). Isozyme BGT I, and other β-galactosidases, exhibited a higher affinity for *o*NPG than for lactose. However, BGT II exhibited more affinity for lactose than *o*NPG. Cavaille and Combes *(1)* purified a β-galactosidase from a yeast that presented a *Km* and V_{max} of 1.7 m*M* and 77.6 μmol/(min·mg) for *o*NPG and 17.0 m*M* and 123.7 μmol/(min·mg) for lactose, respectively. Nakao et al. *(11)* purified a β-galactosidase from thermophilic *Saccharopolyspora rectivirgula* with a *Km* of 0.030 m*M* for *o*NPG and 0.75 m*M* for lactose as substrates, and a V_{max} of 19.7 and 26.1 μmol/(min·mg) for the same substrates, respectively.

The differences found in thermal stabilities, optimal pH, pH stabilities, and kinetic constants suggest that the enzymes studied in the present work are quite distinct from each other. In addition, a 6% polyacrylamide gel (PAGE) *(23)* stained for activity revealed two proteic bands in the crude extract, with an *Rm* of 0.63 and 0.52. BGT I and BGT II fractions analyzed separately by the same methodology presented an *Rm* of 0.65 and 0.51, respectively (results not shown). However, BGT I and BGT II fractions submitted to 6% sodium dodecyl sulfate-PAGE *(24)* did not show migration, even when high temperatures, variation of ionic strength, chelating agents, surfactants, and detergents were used. Hence, the molecular masses of the fractions obtained in the present work will need other techniques for their elucidation.

Conclusion

The results presented showed that *T. reesei* strain FTKO-39 produced at least two β-galactosidase isozymes, separable by DEAE-Trisacryl, identified as BGT I and BGT II. Differences in physical and kinetic properties confirmed the presence of these enzymes. The thermotolerance exhibited by both *T. reesei* β-galactosidases implies that these enzymes, which can be produced in wheat bran, may be of use in the food industry.

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