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Purification and Characterization of an Aspecific Glycoside Hydrolase from the Anaerobic Ruminal Fungus *Neocallimastix frontalis*

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A glycoside hydrolase characterized by β-fucosidase (EC 3.2.1.38) and β-glucosidase (EC 3.2.1.21) activities was purified from the culture medium of the anaerobic ruminal phycomycete *Neocallimastix frontalis* grown on 0.5% Avicel. The enzyme had a molecular mass of 120 kilodaltons and a pI of 3.85. Optimal activity against *p*-nitrophenyl-β-D-fucoside and *p*-nitrophenyl-β-D-glucoside occurred at pH 6.0 and 50°C. The β-fucosidase and β-glucosidase activities were stable from pH 6.0 to pH 7.8 and up to 40°C. They were both inhibited by gluconolactone, sodium dodecyl sulfate, *p*-chloromercuribenzoate, and Hg^{2+} cation. The enzyme had K_ms of 0.26 mg/ml for *p*-nitrophenyl-β-D-fucoside and 0.08 mg/ml for *p*-nitrophenyl-β-D-glucoside. The purified protein also had low β-galactosidase activity.

Anaerobic ruminal phycomycetes synthesize a large variety of hydrolytic enzymes, comprising glycoside and polysaccharide hydrolases, able to degrade cellulosic and hemicellulosic materials from plant cell walls to simple sugars (8, 10, 14, 18, 24, 25). Their capacity to invade and penetrate fibrous plant tissues and to secrete cellulolytic, hemicellulolytic, and proteolytic (23) enzymes indicates that they play an important role in cell wall degradation and fiber digestion in the rumen (1, 7, 17).

The extracellular enzyme system produced by the anaerobic ruminal fungus *Neocallimastix frontalis*, when grown in a coculture with the methanogenic bacterium *Methanobrevibacter smithii*, is able to solubilize cotton fiber more efficiently than that of mutant strain C30 of *Trichoderma reesei* (27). This ability to degrade crystalline cellulose demonstrates that this fungus has a complete cellulolytic system.

In *T. reesei*, this complex consists of three principal types of enzyme, namely, endo-β-1,4-glycanase, exo-β-1,4-glucanase or cellobiohydrolase, and β-glucosidase. These enzymes have little or no action on highly hydrogen bond-ordered cellulose when they act separately. However, when they act synergistically, they are able to degrade the most highly ordered crystalline cellulose. In the cellulolytic system, the β-glucosidase enzyme is obviously an important component because it provides glucose necessary for the metabolism and growth of the fungus and degrades cellobiose, an inhibitory product of the action of the other cellulolytic enzymes.

To appreciate the role and the efficiency of the various components of the hydrolytic complexes secreted by anaerobic fungi, protocols for the purification of enzymes must be set.

In this report, we describe the first purification of a glycoside hydrolase from an anaerobic ruminal fungus. We characterize some properties of the enzyme, which has a high β-glucosidase activity combined with a very high β-fucosidase activity and a weak β-galactosidase activity.

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MATERIALS AND METHODS

Organism and culture conditions. *N. frontalis* MCH3 was isolated from sheep rumen as previously described (8) and identified at the Laboratoire de Microbiologie, INRA-CRZV, Theix, France. The strain was maintained in semisynthetic liquid medium B described by Lowe et al. (11) and modified in our laboratory (6). The fungus was grown anaerobically at 39°C in 5-ml culture tubes with a gas phase of 100% CO_2.

Enzyme preparation. β-Fucosidase and β-glucosidase were produced in flasks of 500 ml of medium containing 0.5% Avicel (microcrystalline cellulose; Merck & Co., Inc.) as the carbon source and inoculated with 2 ml of a zoospore suspension from a 3-day-old culture. After 5 to 7 days of growth, the medium was centrifuged for 15 min at 3,000 × *g* to pellet mycelia. The supernatants were pooled and dialyzed for 24 h at 4°C against distilled water. This preparation was freeze-dried, and the powder was used for enzyme purification.

Enzyme assays. β-Fucosidase (β-D-fucoside fucohydrolase; EC 3.2.1.38) and β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) activities were determined by measuring the amount of *p*-nitrophenol (pNP) released from the substrates *p*-nitrophenyl-β-D-fucopyranoside (pNPF; Sigma Chemical Co.) and *p*-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma), respectively. The standard reaction mixture (1 ml) contained samples of enzyme solution incubated at 50°C with 0.7 mg of pNPF or 0.5 mg of pNPG dissolved in 0.1 M citrate-phosphate buffer (CPB) (pH 6.0). The reaction was stopped after 5 to 20 min by the addition of 0.1 M Na_2CO_3. The pNP liberated was measured spectrophotometrically at 400 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol of pNP per min under the above-described conditions, and the specific activity was expressed as units per milligram of protein. All enzyme reactions were linear over the assay period.

TABLE 1. Summary of purification of β-glucosidase and β-fucosidase from *N. frontalis*

Step	Total protein (mg)	Total activity (U)		β-Glucosidase			β-Fucosidase		
		β-Glucosidase	β-Fucosidase	Sp act (U/mg)	Yield (%)	Purification (fold)	Sp act (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	110	74.3	132.6	0.675	100	1	1.21	100	1
Ammonium sulfate precipitation	55	63.1	119.3	1.15	85	1.7	2.17	90	1.8
DEAE-Bio-Gel A	5	47.3	96.4	9.45	63.7	14	19.3	72.7	16
Ultrogel AcA 34	2.3	26.4	63.8	11.48	35.5	17	27.7	48.1	23
Preparative IEF	0.2	3.78	15.9	18.9	5.1	28	79.5	12	66

Protein was measured by the method of Bradford (3) with bovine serum albumin as a standard.

Enzyme purification. All the purification procedures were done at 4°C unless otherwise stated. The powder from the freeze-dried medium was solubilized in 0.01 M CPB (pH 6.0). The preparation was precipitated to 40% saturation with ammonium sulfate and centrifuged at 8,000 × *g* for 10 min. The supernatant was brought to 85% saturation with ammonium sulfate, and the pellet was dissolved in the smallest possible volume of 0.01 M CPB (pH 6.0). The concentrated preparation was first dialyzed against distilled water and then was dialyzed against 0.01 M CPB (pH 6.0).

DEAE-Bio-Gel A (Bio-Rad Laboratories) ion-exchange chromatography was done in a column (2 by 30 cm) equilibrated with 0.01 M CPB (pH 6.0) containing 0.05 M NaCl. The proteins were eluted with a linear NaCl gradient (0.05 to 0.4 M) in CPB at a flow rate of 25 ml/h. Fractions of 2.3 ml were collected. The fractions containing the enzyme activities were pooled and concentrated by successive centrifugations (700 × *g*, 10 min) with CF 25 membrane cones (Amicon Corp.).

Gel filtration was done in a column (2 by 45 cm) of Ultrogel AcA 34 (IBF) equilibrated with 0.01 M CPB (pH 6.0). Elution was performed at a flow rate of 12 ml/h with CPB containing 0.1 M NaCl. The fractions (2.3 ml) were screened for β-glucosidase and β-fucosidase activities. The elution peak was concentrated as described before.

The last step of purification was performed by preparative isoelectric focusing (IEF) in a Sephadex gel (Serva) for 16 h. The pH gradient, formed with carrier ampholytes (pH range, 3.0 to 6.0; Serva), was measured with a surface pH electrode (LKB Instruments, Inc.). Thirty fractions were collected in hemolysis tubes, and the proteins were removed by successive treatments with 0.1 M CPB (pH 6.0).

Analytical determinations. Nondenaturing analytical polyacrylamide gel electrophoresis (PAGE) and analytical IEF were used to ascertain the degree of protein purity derived from preparative IEF. Electrophoresis was done at 4°C in 7.5% (wt/vol) polyacrylamide gels (pH 8.9) by the method of Ornstein and Davis (16). Protein bands were visualized by staining with Coomassie brilliant blue R-250.

Analytical IEF-PAGE was performed with Servalyt Precotes containing 5% ampholytes (pH range, 3.0 to 10.0; Serva). The gel (5% T; 3% C; 0.15 mm thick) was prefocused up to 500 V before samples were applied. The samples were focused at a constant power for 2 h up to a final voltage of 1,700 V. A part of the slab gel was stained with Coomassie brilliant blue for protein determination; the other part was used to detect the enzyme activities by hydrolysis of methylumbelliferyl-β-D-glucoside (MUG) or methylumbelliferyl-β-D-fucoside (MUF) solubilized in 0.1 M CPB (pH 6.0). The activities were revealed by UV exposure at 365 nm.

pH optimum and stability. The optimum pH for activity was determined by measuring enzyme activity in 0.1 M CPB at pHs ranging from 3.0 to 7.8. The effect of pH on enzyme stability was tested by incubating purified enzyme adjusted to pH 3.0 to pH 7.8 in 0.1 M CPB. After incubation of the enzyme for 1 h at 20°C, residual activity was measured under standard assay conditions.

Temperature optimum and stability. The temperature optimum for activity was determined by measuring enzyme activity at temperatures ranging from 25 to 70°C. Thermal stability was measured by incubating purified enzyme without substrate in 0.1 M CPB (pH 6.0) for 1 h at temperatures ranging from 18 to 70°C. Following incubation, the enzyme was cooled and residual activity was determined under standard assay conditions.

Kinetics. The K_ms were determined from Lineweaver-Burk plots with pNPG and pNPF as substrates in 0.1 M CPB. The concentration range of the substrates was 0.05 to 4 mg/ml. Assays were performed at 50°C.

Inhibition by gluconolactone at concentrations of 0.05 to 1,000 μM was studied in culture filtrates with pNPG and pNPF as substrates, and the apparent k_is were determined from Dixon plots.

Effect of metallic ions and reagents. The effect of several metallic ions and reagents on the purified enzyme was tested at an ion or reagent concentration of 2 mM in 0.1 M CPB (pH 6.0). Residual β-glucosidase and β-fucosidase activities were measured at 50°C, and the activities assayed in the absence of any addition were taken as 100%.

Substrate specificity. The purified enzyme was incubated with a variety of pNP glycosides and polysaccharides at concentrations of 5 mM and 2 mg/ml, respectively. The enzyme assay was conducted in the standard manner with 0.1 M CPB (pH 6.0).

RESULTS

Enzyme purification. The first step in purification consisted of two successive precipitations to recover the fraction that was 40 to 85% saturated in ammonium sulfate. This fraction contained, respectively, 85 and 90% of the β-glucosidase and β-fucosidase activities and eliminated 50% of the total proteins (Table 1).

The precipitate, suspended in a small volume of 0.01 M CPB (pH 6.0), was dialyzed overnight and applied to a DEAE-Bio-Gel A Column.

The β-glucosidase and β-fucosidase activities were eluted as a single peak at 0.165 M NaCl (Fig. 1). A low β-galactosidase activity was also detected in these fractions. All these enzyme activities were eluted at that molarity, as no activity was detected before the application of the salt gradient or in the rinsing volume with 0.01 M CPB (pH 6.0) containing 1 M NaCl.

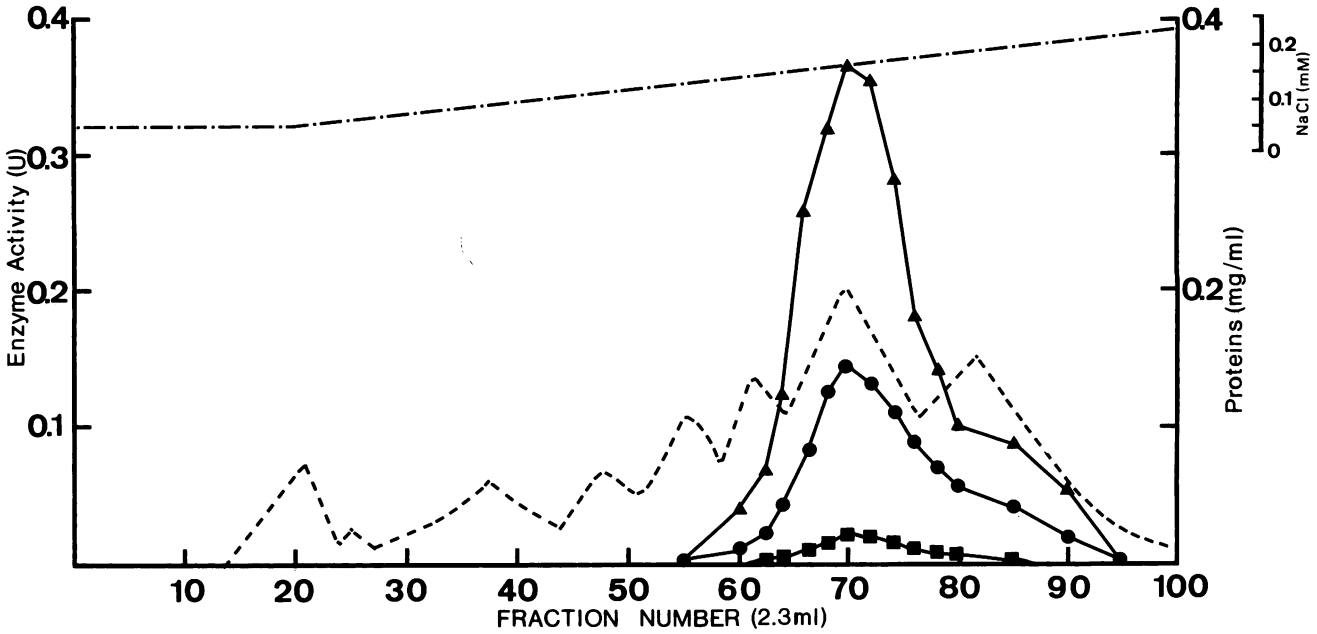

FIG. 1. DEAE–Bio-Gel A chromatography of the *N. frontalis* fraction that was 40 to 85% saturated in ammonium sulfate. Elution was performed with a linear gradient of NaCl (0.05 to 0.4 M) in 0.01 M CPB (pH 6.0). Symbols: ●, β-glucosidase; ▲, β-fucosidase; ■, β-galactosidase; – – – –, proteins; —.—, NaCl gradient.

Fractions of higher activity were pooled, concentrated, and filtered in an Ultrogel AcA 34 column equilibrated and eluted with 0.01 M CPB (pH 6.0) containing 0.1 M NaCl. The β-glucosidase and β-fucosidase activities were collected in the same fractions. The molecular mass of the enzyme was estimated by calibrating the column with standard proteins of known molecular masses. A linear relationship was obtained when the elution volumes (V_e/V_o) of the standard proteins were plotted against the log of the molecular mass. The molecular mass of the enzyme was estimated to be 120 kilodaltons (kDa).

The active fractions concentrated by centrifugation were further purified by preparative IEF in a Sephadex gel. The pH gradient was achieved with carrier ampholytes (pH range, 3.0 to 6.0). After migration, proteins were removed from the different gel fractions with 0.1 M CPB (pH 6.0) and the enzyme activities were measured in supernatants of each fraction. β-Glucosidase and β-fucosidase activities were revealed at the same pI, 3.85.

At this stage, a 28-fold-purified β-glucosidase and a 66-fold-purified β-fucosidase were recovered, with specific activities of 18.9 and 79.5 U/mg of protein, respectively (Table 1).

Analysis of the purified enzyme. Analysis of the protein purified after preparative IEF and containing the two enzyme activities was done by analytical IEF and nondenaturing PAGE. Analytical IEF, with staining with Coomassie brilliant blue R-250, revealed a single protein band at a pI of 3.85 (Fig. 2, lane 3). Analysis of the enzyme activities by hydrolysis of MUG and MUF revealed a single active band at a pI of 3.85 for the β-glucosidase and β-fucosidase activities (Fig. 2, lanes 4 and 5, respectively). Native electrophoresis of the purified protein confirmed the presence of a single band with a mobility near the front of the gel, confirming that the protein had a negative global charge (Fig. 2, lane 6).

Effect of pH on activity and stability. The β-glucosidase and β-fucosidase activities were measured at various pHs ranging from 3.0 to 7.8 in 0.1 M CPB. The optimum pH (pH 6.0) was similar for the two activities. Slight deviations from this optimum resulted in a rapid decrease in enzyme activities; approximately 70% of maximal activities were found at pH 5.5 or 6.5, and the enzyme was inactive at pH 4.0 or 7.8.

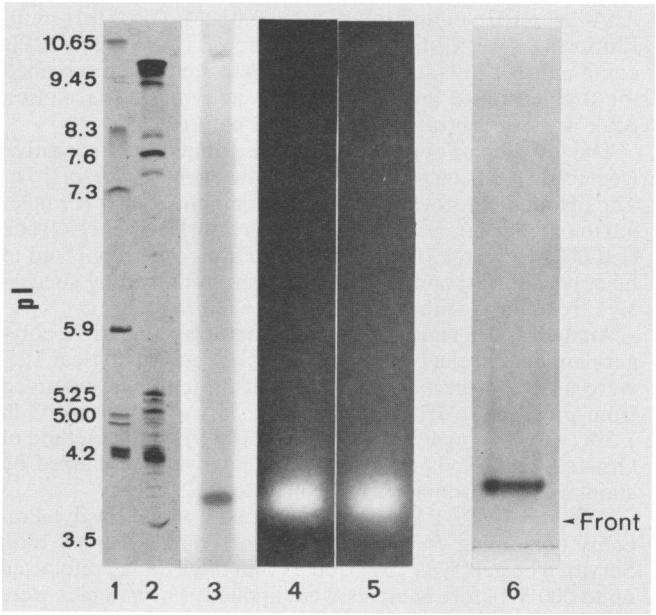

FIG. 2. Analysis of the purified glycosidase by analytical IEF-PAGE at pHs 3.0 to 10.0 (lanes 1 to 5) and by nondenaturing PAGE (lane 6). Lanes: 1, protein standards (Protein Test Mix 9; Serva); 2, *Ancistrodon* venom standard (Serva); 3, purified protein stained by Coomassie brilliant blue R-250; 4 and 5, enzyme activities revealed by hydrolysis of MUG and MUF, respectively, under UV irradiation at 365 nm.

Enzyme stability was tested by measuring the residual activities after 1 h of incubation at various pHs. Similar curves were observed for the β-glucosidase and β-fucosidase activities, which were stable from the optimum pH (pH 6.0) to pH 7.8. Enzyme stability was less affected than enzyme activity below pH 6.0; approximately 50% of both activities were recovered after incubation at pH 4.0.

Effect of temperature on activity and stability. The purified protein was tested for β-glucosidase and β-fucosidase activities at various temperatures ranging from 25 to 70°C in 0.1 M CPB (pH 6.0). The optimum temperature for the two enzyme activities was 50°C. The enzyme was weakly active at 25°C, corresponding to about 20% of maximal β-glucosidase and β-fucosidase activities. A sharp decrease took place above 55°C, and the enzyme was inactivated at 70°C.

The thermostabilities were investigated by measuring the residual activities after 1 h of incubation of the enzyme at temperatures ranging from 18 to 70°C. A significant loss of activity appeared above 40°C, and complete inactivation was obtained at 60°C. The behavior of the two enzyme activities was similar.

Kinetic properties. The affinity of the purified enzyme for the substrates pNPG and pNPF was examined with a Lineweaver-Burk plot. The K_ms against pNPG and pNPF were 0.08 and 0.26 mg of substrate per ml, i.e., 0.266 and 0.91 mM, respectively.

Gluconolactone inhibition of β-glucosidase and β-fucosidase activities was tested with a crude enzyme preparation and pNPG and pNPF as substrates. The two enzyme activities were inhibited by gluconolactone, and the apparent K_is, determined from Dixon plots, were 95 and 70 μM for β-glucosidase and β-fucosidase, respectively.

Effect of metallic ions and inhibitors on the activities. The effects of various reagents at 2 mM concentrations on both enzyme activities were investigated. The two activities were similarly affected, with a weak inhibition by Fe^{3+} (25%) and a strong inactivation (more than 90%) in the presence of Hg^{2+}. The other metallic ions tested, Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}, and Cu^{2+}, and the chelating agent disodium EDTA had no significant effect. On the other hand, sodium dodecyl sulfate and *p*-chloromercuribenzoate inhibited both enzyme activities by 40% and 66%, respectively.

Substrate specificity. Hydrolysis of several pNP-glycosides and polysaccharides at concentrations of 5 mM and 2 mg/ml, respectively, was tested to examine the substrate specificity of the enzyme. The enzyme was active against pNPG and pNPF, with specific activities of 2.3 and 4.1 U/mg of protein, respectively. The enzyme also was weakly active against *p*-nitrophenyl-β-D-galactoside (0.07 U/mg) but was not active against carboxymethyl cellulose, xylan, pNP-β-D-lactoside, or pNP-β-D-xyloside.

DISCUSSION

Since the first description of a sheep anaerobic ruminal flagellate, *N. frontalis*, identified as an aquatic phycomycete (8), many fungal species have been isolated from the rumens of a large ruminant population.

Studies carried out on several species of anaerobic phycomycetes have suggested that these species are directly involved in the degradation processes of the plant cell wall polysaccharides and glycoproteins. A large hydrolytic complex seems to be common to these species (8, 24, 25) and is characterized by very close physicochemical properties (8). Among the glycoside- and polysaccharide-degrading enzymes of *N. frontalis*, β-glucosidase and β-fucosidase are particularly active (8). They also play a key role in providing simple sugars from degraded cell wall polysaccharides. Whatever the carbon source used in the growth medium, the β-glucosidase activity always represents 40 to 60% of the β-fucosidase activity (unpublished observation). We have purified the β-glucosidase and β-fucosidase activities from a culture medium containing 0.5% Avicel, the best carbon source for their production (70 and 170 mU/ml for β-glucosidase and β-fucosidase, respectively, after 7 days of growth).

At each step of purification, the β-glucosidase and β-fucosidase activities were always recovered in the same fractions. Analysis by native electrophoresis and analytical IEF of the purified fraction obtained by preparative IEF revealed a single band. This protein band hydrolyzed the fluorogenic substrates MUG and MUF. The β-glucosidase and β-fucosidase activities are therefore carried by the same protein, which is aspecific and also exhibits a weak β-galactosidase activity.

The aspecificity of glycoside hydrolases has already been observed for several fungi. In particular, this is the case for the *T. longibrachiatum* β-glucosidase, which has a β-galactosidase activity (20), and the *T. viride* β-glucosidase, which is active against xylan, carboxymethyl cellulose, pNP-β-D-xyloside, and pNP-α-L-arabinoside (2). However, to our knowledge, this is the first time that a β-glucosidase activity associated with a β-fucosidase activity has been observed in a fungus. Such aspecific glycoside hydrolases with very broad specificities are known in marine organisms as well as in mammalian and human organs like the kidneys or liver (4).

The microbial β-glucosidases are generally highly specific and hydrolyze only β-D-glucosides; examples include the β-glucosidases from *Geotrichum candidum* and *Aspergillus terreus* (20), *A. niger* (12), *Sclerotium rolfsii* (21), and the ruminal bacterium and *Ruminococcus albus* (15). For the last, the specificity of the β-glucosidase is limited to the β-1,4-glucoside linkages; in contrast, positional isomery appears less critical for the fungal β-glucosidases, which hydrolyze disaccharides having β-1,6-glucoside, β-1,3-glucoside, and β-1,2-glucoside linkages. On the other hand, these enzymes have a strict requirement for a β-D-glucopyranosyl configuration for activity and do not hydrolyze glucosides with an α configuration or the glycans such as galactosides, fucosides, mannosides, xylosides, and arabinosides.

In the crude extract and during the first four stages of purification, the ratio between β-fucosidase and β-glucosidase specific activities was always the same (approximately 2). However, after preparative IEF, this ratio increased to 4 and, consequently, the fold purification values became very different. The electrophoretic analysis of the enzyme activities during the different steps of purification did not reveal the presence of another β-glucosidase (data not shown) which could be separated by the preparative IEF and may explain the change in the ratio between the two activities. These results suggest that the β-glucosidase activity was partially denatured and that the catalytic center of the protein for the β-glucosidase activity was probably less stable than that for the β-fucosidase activity.

The molecular mass of the aspecific glycoside hydrolase from *N. frontalis* was estimated to be 120 kDa by gel filtration in Ultrogel AcA 34. This value distinguishes the enzyme from other extracellular β-glucosidases from *T. longibrachiatum* (20), *T. viride* (2), *T. reesei* (22), and *T. koningii* (26), which were reported to have molecular masses of 350, 76, 70, and 39.8 kDa, respectively. On the other hand, the *N. frontalis* glycosidase has a molecular mass

similar to those of the β-glucosidases from *A. niger* (118 kDa; 12) and *R. albus* (116 kDa; 15) and in the same range as those of *Talaromyces emersonii* β-glucosidase I (135 kDa; 13) and the four β-glucosidases from *S. rolfsii* (90 to 107 kDa; 21). The β-glucosidase–β-fucosidase of *N. frontalis* has a molecular mass similar to those of the basic unit of the *G. candidum* (115 kDa; 20) and *A. fumigatus* (95 kDa; 9) β-glucosidases.

The pI of the *N. frontalis* enzyme (3.85) is similar to those of the β-glucosidases from *T. viride* (2) and *A. niger* (12), which were found to be 3.9 and 4.0, respectively. However, the pIs of the extracellular β-glucosidases are generally more basic, being 4.2 for *T. longibrachiatum* (20) and 8.4 to 8.5 for *T. reesei* (5, 22).

The optimum pH for the β-glucosidase and β-fucosidase activities from the purified protein was 6.0, while the optimum pHs for the activities of many fungal β-glucosidases are found to lie between 3.0 and 5.5. The optimum pHs of the glycoside and polysaccharide hydrolases from ruminal fungi (8, 10, 14) and bacteria (15, 19) are correlated with the rumen pH ($6.0 < pH < 7.0$).

The maximal activity of the purified protein on both substrates was reached at 50°C. The enzyme activities were stable up to 35 to 40°C, decreased rapidly above 40°C, and were completely inactivated at 60°C.

The Michaelis constants of the purified protein against pNPG and pNPF were 0.266 and 0.91 mM, respectively, and were similar to the values of 0.31 and 1.3 mM found for *N. patriciarum* β-glucosidase and β-fucosidase, respectively (25). Gluconolactone, a specific inhibitor of β-glucosidase, also inhibited β-fucosidase activity. The apparent inhibition constants, determined in the crude culture filtrate with pNPG and pNPF as substrates, were 95 and 70 μM for β-glucosidase and β-fucosidase, respectively.

Various metallic ions and reagents produced similar effects on β-glucosidase and β-fucosidase activities, confirming the aspecificity of the purified protein. Hg^{2+} and *p*-chloromercuribenzoate, a sulfhydryl-reacting reagent, strongly inhibited both activities, suggesting that sulfhydryl groups are involved in the catalytic active centers of the protein, as observed for the β-glucosidases from *A. fumigatus* (9) and *R. albus* (15).

The results presented here show that *N. frontalis* MCH3 synthesizes and secretes an aspecific glycoside hydrolase characterized by β-glucosidase, β-fucosidase, and weak β-galatosidase activities and able to hydrolyze disaccharides derived from either cellulosic and/or hemicellulosic polymers. The production of this enzyme and its high level of activity on pNPG and pNPF substrates allow us to postulate that this fungus plays an important role in the degradation processes of plant cell walls, releasing simple sugars useful for metabolism and growth of the microorganisms and for animal nutrition. Such an enzyme may represent a very interesting tool in the area of biotechnology and particularly in the fermentative processes of residual hemicellulosic materials.

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