

MYCELIAL GLUCOAMYLASES PRODUCED BY THE THERMOPHILIC FUNGUS *SCYTALIDIUM THERMOPHILUM* STRAINS 15.1 AND 15.8: PURIFICATION AND BIOCHEMICAL CHARACTERIZATION

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ABSTRACT

Two strains (15.1 and 15.8) of the thermophilic fungus *Scytalidium thermophilum* produced high levels of intracellular glucoamylases, with potential for industrial applications. The isoform I of the glucoamylase produced by 15.1 strain was sequentially submitted to DEAE-Cellulose and CM-Cellulose chromatography, and purified 141-fold, with 5.45% recovery. The glucoamylase of strain 15.8 was purified 71-fold by CM-Cellulose and Concanavalin A-Sepharose chromatography, with 7.38% recovery. Temperature and pH optima were in the range of 50-60°C and 5.0-6.0, respectively, using starch and maltose as substrates. The glucoamylase of *S. thermophilum* 15.8 was more stable ($t_{50} > 60$ min) than that of *S. thermophilum* 15.1 ($t_{50} = 11-15$ min), at 60°C. The glucoamylase activities were enhanced by several ions (e.g. Mn^{2+} and Ca^{2+}) and inhibited by β -mercaptoethanol. The glucoamylase from 15.1 strain showed a K_m of 0.094 mg/ml and 0.029 mg/ml and V_{max} of 202 U/mg prot and 109 U/mg prot, for starch and maltose, respectively. The hydrolysis products of starch and maltose, analyzed by TLC, demonstrated glucose as end product and confirming the character of the enzyme as glucoamylase. Differences were observed in relation to the products formed with maltose as substrate between the two strains studied. *S. thermophilum* 15.8 formed maltotriose in contrast with *S. thermophilum* 15.1.

Key-words: glucoamylase, amylase, *Scytalidium thermophilum*, thermostable enzyme, starch hydrolysis

INTRODUCTION

Glucoamylases (GAs) (EC 3.2.1.3) are exo-amylases which hydrolyze α -1,4 glycosidic linkages in raw or soluble starches and related oligosaccharides, producing β -glucose by inversion of the anomeric configuration (26). In addition, they also hydrolyze, but at a lower rate, α -1,6 glycosidic linkages of starch (20).

The conversion of starch into sugar, syrups and dextrins by action of amylases represents the larger part of the starch processing industry (27). The hydrolytes are used as carbon source in fermentation, as well as sources of sweetness in a range of manufactured food products and beverages. Amylases are used in the liquefaction of insoluble starch, manufacture of

oligosaccharides mixtures (substitutes sucrose preventing crystallization in foods and keeping a certain level of hardness of the texture during storage), maltotetraose syrup (to control the freezing points of frozen foods) and of high molecular weight branched dextrins (used as extender and glozing agent for production of powdery foods and rice cakes). Manufacturing starch to maltose is important since that this disaccharide is widely used as sweeter and also as intravenous sugar supplement. In food industries is used due of low tendency to be crystallized and relative nonhygroscopic. To manufacture of high fructose containing syrups, the starch is first converted to glucose by enzymes liquefaction and saccharification.

Several fungal species produce glucoamylases, for instance, *Aspergillus awamori*, *A. terreus* and *Rhizopus oryzae* (21).

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Glucoamylases produced by fungi, generally, occur in multiple forms. They have two domains, a catalytic domain and a starch binding domain. These domains are connected by an *O*-glycosylated polypeptide linker located at the N-terminus (20). Total or partial proteolytic excision of the starch binding domains leads to the formation of glucoamylase capable of hydrolyzing only soluble starch (8).

The fungus *Scytalidium thermophilum* belongs to the thermophilic *Torula-Humicola* complex which has more than thirty isolates with species very variable in macroscopical and microscopical features (24). In our laboratory we previously studied *Scytalidium thermophilum* 15.1 and 15.8, which were excellent producers of trehalase (14) and of conidial and mycelial alkaline phosphatases (13). Regarding amylase activity, substantial differences were detected between *S. thermophilum* 15.1 and 15.8 isolates. The aim of the present study was to compare some biochemical properties of the glucoamylase produced by the mycelial extracts of fungi *S. thermophilum* 15.1 and 15.8. The high amylolytic activity produced by the two strains suggested potential for applications in the saccharification starch process.

MATERIALS AND METHODS

Microorganism and culture

The *S. thermophilum* isolates 15.1 (= CBS 619.91) and 15.8 (= CBS 671.88 = ATCC 669.38) were maintained on slants of solid 4% oatmeal baby food (Quaker) medium, at 45°C. Liquid cultures were prepared in 50 ml of M-5 liquid medium (22), inoculated with of 2.0 ml of a spore suspension (10^5 spores/ml), in 250 ml Erlenmeyer flasks. The culture conditions were standardized according to the exigency of each fungus for maximal enzymatic production, i.g., incubated at 45°C, without agitation, and for 5 or 7 days, respectively, for 15.1 and 15.8 isolates.

Preparation of crude mycelial extract

The cultures were harvested by vacuum filtration on Whatman #1 and the mycelial pads were ground in a porcelain mortar with twice their weight of acid-washed sea sand, at 4°C, extracted in distilled water and centrifuged at 23,700xg for 15 min. The supernatant was the source of crude mycelial extract.

Determination of glucoamylase activity and protein

The glucoamylase activity was determined using 1% starch as substrate in 0.1M sodium acetate buffer, pH 5.5. The reducing sugars formed were quantified according to Miller (18) using dinitrosalicylic acid (DNS). The glucoamylase activity was also determined on maltose, starch, amylopectin and glycogen, as substrates by glucose oxidase method according to Bergmeyer & Bernt (3). One unit of glucoamylase activity was defined as the amount of enzyme that releases one mmol of glucose per minute. The protein was quantified according to Lowry (16)

using bovine serum albumin as standard. Specific activity corresponds to $\mu\text{mol}/\text{min}/\text{mg}$ prot.

Purification and biochemical characterization

Mycelial glucoamylase of *S. thermophilum* 15.1 was purified using a DEAE-Celulose column (21 x 60 mm) equilibrated in 0.01 M sodium acetate buffer, pH 5.5 and two forms (I e II) were separated. The form I did not interact with the resin, and the fractions with amylolytic activity were pooled and applied to a CM-Celulose chromatographic column (21 x 60 mm) equilibrated in the same buffer. The enzyme was eluted as single form, using a linear gradient of NaCl (0-0.5 M), in the same buffer. The fractions with amylase activity were pooled, dialyzed overnight at 4°C, and used for biochemical characterization. The form II was eluted using a linear gradient of NaCl (0-1.0 M) in the same buffer but it has not representative amylolytic levels.

Glucoamylase of *S. thermophilum* 15.8 was purified also by two chromatographic steps. The crude extract was applied to a CM-Celulose column (21 x 60 mm) and eluted as a single form under the same condition mentioned above. The fractions with amylase activity were pooled, dialyzed overnight at 4°C and applied to a Concanavalin A-Sepharose column (10 x 50 mm), equilibrated with 0.020 M Tris-HCl buffer, pH 7.5 added of 0.5 M NaCl, 0.5 mM Mn^{++} and Ca^{++} . The enzyme was eluted as a single form using a linear gradient of methyl α -D-mannopyranoside (0-0.5 M), pooled, dialyzed overnight at 4°C, and used for biochemical characterization.

Electrophoresis

The samples of purified enzymes were run under non-denaturing electrophoresis in polyacrylamide 7% gels PAGE, Davis (9) and stained for protein with AgNO_3 according to Blum (4).

Molecular mass and carbohydrate content determination

The molecular mass of glucoamylase produced by *S. thermophilum* was estimated by gel filtration in a Sephadex G-100 column (20 x 860 mm), equilibrated in 0.05 M Tris-HCl buffer, pH 7.5 added of 0.1 M KCl, using as molecular mass markers alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome-c (14 kDa). The protein carbohydrate content was determined according to Dubois (10).

Kinetic parameters

The kinetic parameters (K_m and V_{max}) were determined using starch, maltose, amylopectin and glycogen as substrate by the Lineweaver-Burk graphic representation (15).

Thin layer chromatography

Chromatographic analysis of the reaction end products of glucoamylase activity produced by *S. thermophilum* 15.1 e 15.8

was carried out using TLC. The reaction mixture was concentrated and applied (10 µl) on silica gel, and two ascendent chromatographic steps were applied, using butanol/ethanol/water (5:3:2) as solvent system. Spots were developed by spraying the air-dried plate with H₂SO₄ and methanol (1:9) containing 0.2% orcinol and heating at 100°C (12).

RESULTS AND DISCUSSION

Enzyme purification

Crude mycelial extract of *S. thermophilum* 15.1 was submitted to chromatography in DEAE-Cellulose (Fig. 1A). Two isoforms (I and II) with amylolytic activities were separated. The form I contained approximately 86% of total amylolytic activity and it did not interact with the resin. Form II, with 14% of total amylolytic activity was eluted as single form with 0.58 M NaCl, using a linear gradient (0-1.0M). The fractions of form I were pooled, dialyzed against buffer and applied to a CM-Cellulose chromatographic column (Figure 1B). The amylolytic activity eluted as single form with 0.16-0.28 M NaCl, with a linear gradient (0-0.5M).

S. thermophilum 15.8 crude mycelial extract was applied to a CM-Cellulose chromatographic column (Fig. 1C), and eluted with a linear NaCl gradient (0-0.5M). The fractions with glucoamylase activity eluted as a single form with 0.18 M NaCl. The activity fraction were pooled, dialyzed, applied to a Concanavalin A-Sepharose column (Fig. 1D) and eluted as single form with 0.05-0.1 M methyl α -D-mannopyranoside using a linear gradient (0-0.5M).

The enzymes of the strains 15.1 and 15.8 were purified 141-fold and 71.23-fold, with recovery of 5.45% and 7.38%, respectively (Table 1). Only a single band on 7% PAGE was observed for the intracellular glucoamylase of strain 15.1, stained for protein by silver and stained for glucoamylase activity (Fig. 2).

Molecular mass determination

Molecular masses of intracellular glucoamylases of *S. thermophilum* 15.1 and 15.8 determined by Sephadex G-100 gel

filtration were, respectively, 54 kDa and 98.7 kDa. These values are in agreement with the literature that reports that the molecular masses of fungal glucoamylases are in the range 48-90 kDa (20), as those of *T. lanuginosus* (26), *Chaetomium thermophilum* (7) and *Sclerotinia sclerotiorum* (17). However, a higher value of 125 kDa was reported for *Aspergillus niger* glucoamylase (25).

Usually, the carbohydrate content of fungal glucoamylase is in the range of 10-20%, as observed for *Rhizopus niveus* (29). But, lower values were observed such as that of the glucoamylase of *Neurospora crassa* (5.1%) (23). Intracellular glucoamylases of *S. thermophilum* 15.1 and 15.8 had carbohydrate contents of 20% and 51%, respectively.

Temperature and pH optima and thermal stability

The values of temperature optima for glucoamylases of the strains 15.1 and 15.8 were compatible with the literature, which reports values among 50°C to 70°C (20). Intracellular glucoamylase of *S. thermophilum* 15.1 exhibited optimum temperature of 50°C and 55°C, with maltose and starch as substrates (Fig. 3A), and 60°C with either substrates for the intracellular glucoamylase of *S. thermophilum* 15.8 (Fig. 3B). A temperature optimum of 50°C is reported for *S. sclerotiorum* glucoamylase (17). Higher activity temperatures were verified also for glucoamylase of *Thermomyces lanuginosus* (26), *Chaetomium thermophilum* (7) and *Trichoderma reesei* (11). Mycelial enzymes of *S. thermophilum* have t_{50} of 11 min and 15 min (strain 15.1) (Fig. 3C), and more than 60 min for strain 15.8 (Fig. 3D), at 60°C, when starch and maltose, respectively, were used as substrates. Glucoamylase of *Chaetomium thermophilum* retained 50% active after 60 min, at 65°C (7). The high percentage of carbohydrate content of the glucoamylase of *S. thermophilum* 15.8, could be responsible by the higher thermal stability compared to *S. thermophilum* 15.1 glucoamylase.

Intracellular glucoamylase of *S. thermophilum* 15.1 had a pH optimum of 5.0 when starch or maltose used as substrates (Fig. 4A), and the mycelial glucoamylase of *S. thermophilum* 15.8 has pH optimum in the range of 5.5-6.0, either with starch or maltose (Fig. 4B). Similar results were obtained for extracellular

Table 1. Purification of glucoamylases produced by *S. thermophilum* 15.1 and 15.8.

Step	<i>S. thermophilum</i> 15.1					<i>S. thermophilum</i> 15.8				
	Activity (Total U)	Protein (Total mg)	Specific Activity (U/mg prot)	Yield (%)	Purification fold (X)	Activity (Total U)	Protein (Total mg)	Specific Activity (U/mg prot)	Yield (%)	Purification fold (X)
Crude extract	72.27	235.06	0.31	100	1	40.92	305.80	0.13	100	1
DEAE-Cellulose	7.39	1.98	3.73	10.22	12.03	-	-	-	-	-
CM-Cellulose	3.94	0.09	43.77	5.45	141.2	4.37	1.32	3.31	10.68	25.46
Con A- Sepharose	-	-	-	-	-	3.02	0.32	9.44	7.38	71.23

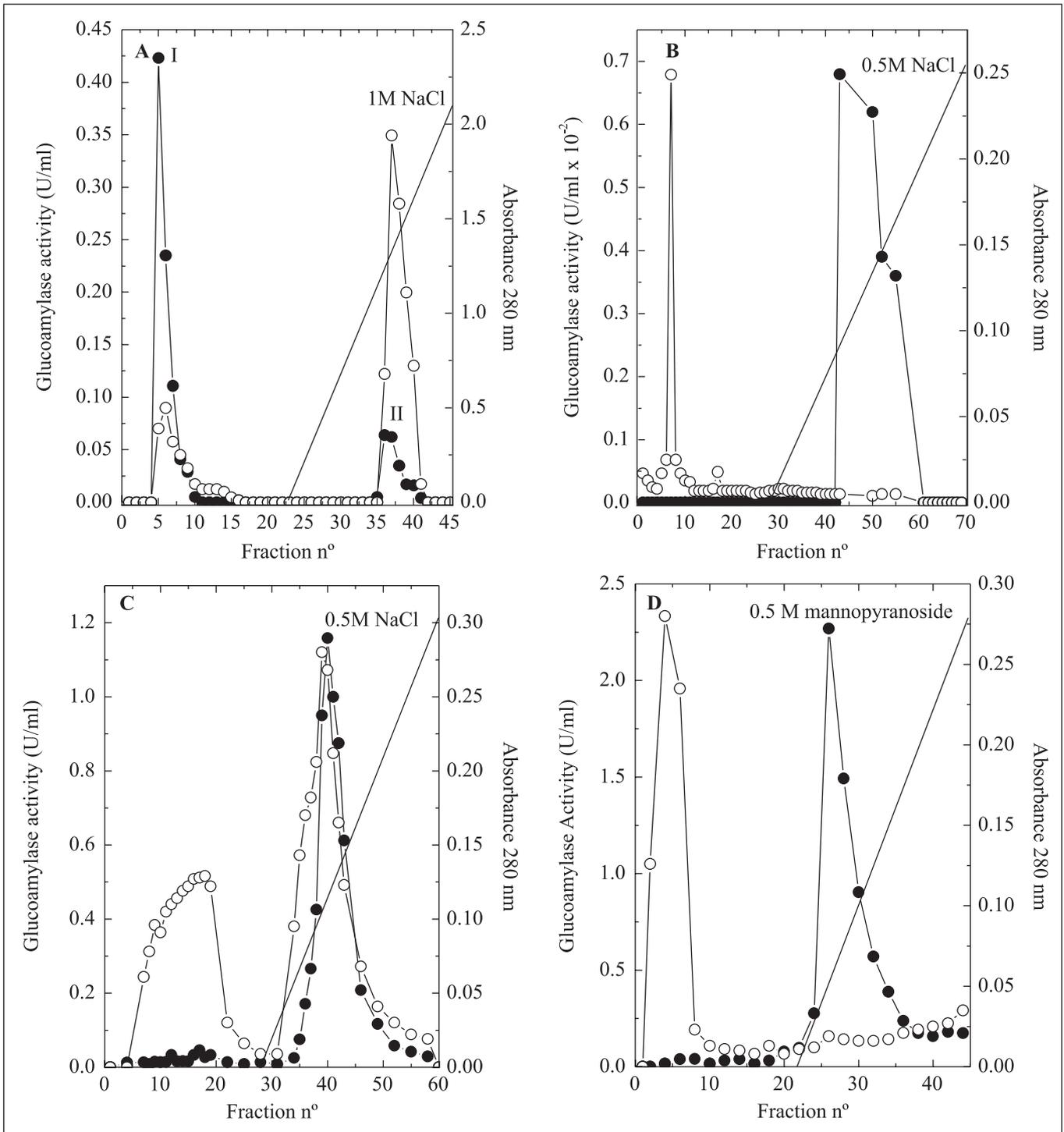


Figure 1. Chromatographic profiles of the glucoamylases produced by fungi *S. thermophilum* 15.1 (A and B) and 15.8 (C and D), on DEAE-Cellulose (A), CM-Cellulose (B and C) and Concanavalin A-Sepharose (D). Symbols: (●) glucoamylase activity; (○) absorbance 280 nm.

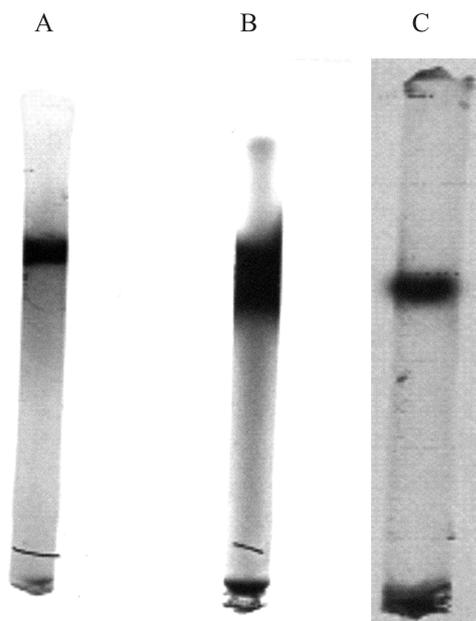


Figure 2. 7% PAGE stained for glucoamylase activity using starch (A) and maltose (B), and stained for protein by silver (C).

glucoamylase of *S. thermophilum* 15.1 (5), *Thermomyces lanuginosus* (26), *Arthrotrichy amerospora* (20) and *Neurospora crassa* (23). The values obtained were higher than those reported for *S. sclerotiorum* (3.5-4.0) (17). Generally, glucoamylases obtained from fungi are active at acidic pH values. However, several forms have variable values of pH optimum.

Influence of ions on the glucoamylase activity

The influence of different ionic compounds on the glucoamylase activities is shown in Table 2. The intracellular glucoamylase activity of *S. thermophilum* 15.1 was enhanced 23-36% by addition of Mn^{2+} , but strongly inhibited by Cu^{2+} , Hg^{2+} and β -mercaptoethanol. These last two metallic ions drastically reduced the 15.8 glucoamylase activity, but in contrast, Cu^{2+} enhanced 23 or 74% the glucoamylase activity, depending on the substrate, starch or maltose, respectively.

The inhibition promoted by β -mercaptoethanol indicates the existence of disulfide bonds in the enzyme molecule. *S. thermophilum* 15.8 glucoamylase was also enhanced by 5mM Ba^{2+} , Mg^{2+} , Na^+ , NH_4^+ , and Ca^{2+} . For both species of *S. thermophilum* were verified considerable differences on glucoamylase activity determined with the substrates starch or maltose in the presence of Mn^{2+} and Zn^{2+} . Glucoamylase of *C. thermophilum* was more active in the presence of Ca^{2+} , Mg^{2+} , Na^+ and K^+ , and inhibited by Fe^{2+} , Ag^+ and Hg^{2+} (7). Mn^{2+} and Zn^{2+} enhanced glucoamylase activity from *Humicola grisea* var. *thermoidea* (28) and lower concentrations of Mn^{2+} and Ca^{2+} enhanced the activity of glucoamylase of *S. sclerotiorum* (17).

Table 2. Effect of ions and other compounds on the glucoamylases produced by *S. thermophilum* 15.1 and 15.8.

Ions/ Compounds	<i>S. thermophilum</i> 15.1		<i>S. thermophilum</i> 15.8	
	Starch	Maltose	Starch	Maltose
Relative activity (%)				
None	100	100	100	100
β -mercaptoethanol	40	24	0	0
$BaCl_2$	70	67	121	98
$CaCl_2$	78	65	109	139
$CuCl_2$	48	38	123	174
$HgCl_2$	7	26	0	0
$MgCl_2$	101	80	140	108
$MgSO_4$	94	86	134	86
$MnCl_2$	123	136	59	203
$NaCl$	95	73	116	76
NH_4Cl	104	76	131	88
$ZnCl_2$	93	12	83	61

Kinetic properties

The kinetic parameters obtained for the glucoamylase of *S. thermophilum* 15.1 are shown in Table 3. The enzyme possesses greater affinity to maltose (K_m 0.029 mg/ml) than for starch (K_m 0.094 mg/ml), which was confirmed by the values of catalytic efficiency. But, probably, the hydrolysis of starch and maltose occurred at the same catalytic site, considering that the rate of hydrolysis of a mixture of starch and maltose (35 U/ml) was intermediary when compared with that of the individual substrates (42 U/ml for starch and 12.5 U/ml for maltose), and lower than the addition of both (54.5 U/ml approximately). The enzyme showed reduced affinity by glycogen.

Both enzymes, GAs 15.1 and 15.8, hydrolyzed starch and maltose as substrate, and glucose was always the main hydrolysis product (Fig. 5), but *S. thermophilum* 15.8 formed maltotriose when maltose was the substrate, in contrast with *S. thermophilum* 15.1. In according to Thorsen (26), di-, tri- and tetrasaccharide can be formed reversibly from high glucose concentrations.

We previously reported the purification of the extracellular amylases produced by *S. thermophilum* 15.1 and 15.8 and significant differences were detected between the enzymes of the two isolates. The first one (15.1) produced an extracellular glucoamylase (1) and an α -amylase (2), which were isolated and purified by chromatographic methods. But, in contrast, under the same conditions, it was no detected α -amylase activity in *S. thermophilum* 15.8, but two isoforms of glucoamylase were separated and characterized (5;6). Here,

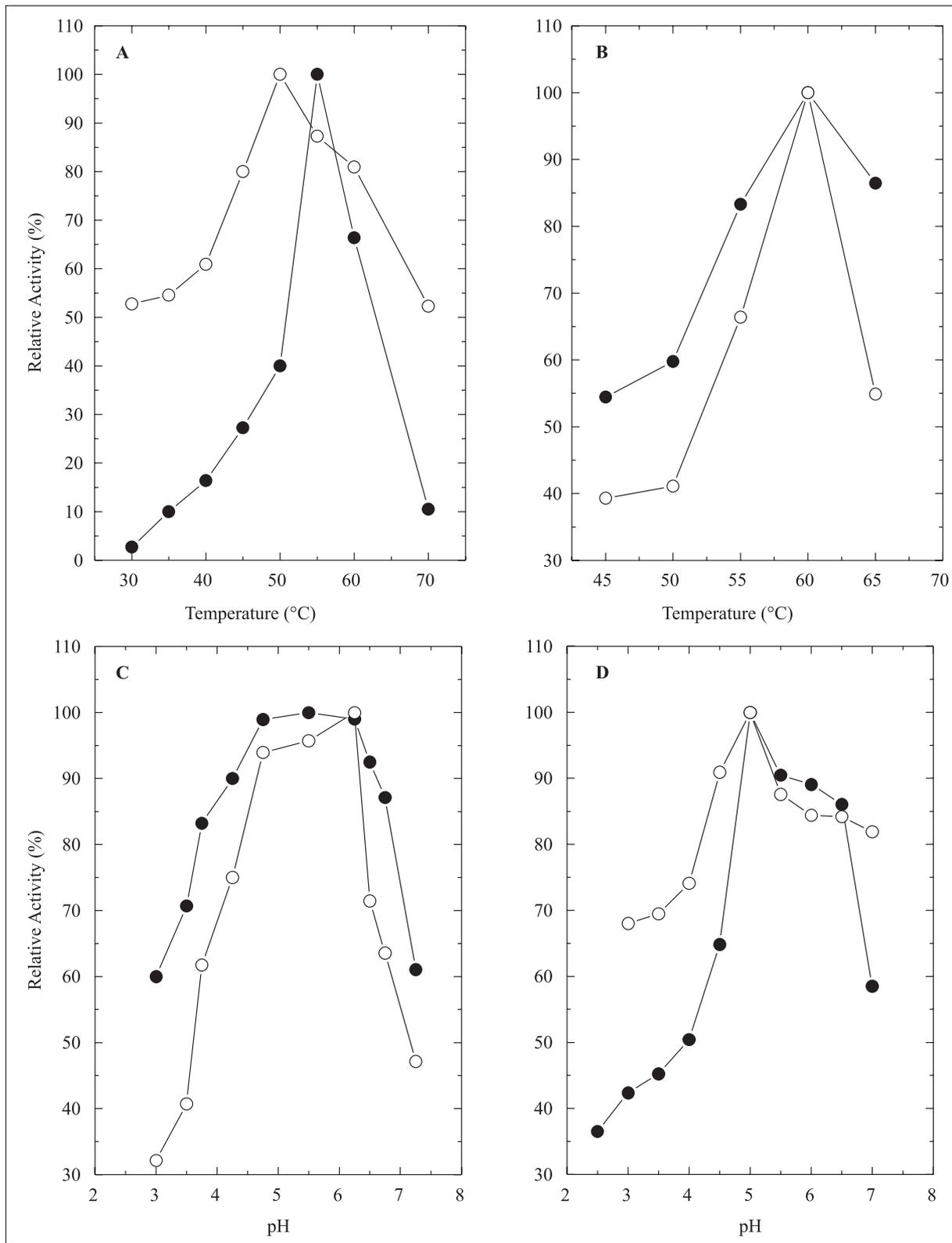


Figure 3. Optima temperature (A and B) and thermal stability on 60°C (C and D) of the glucoamylases produced by *S. thermophilum* 15.1 (A and C) and 15.8 (B and D), using starch (●) and maltose (○) as substrate.

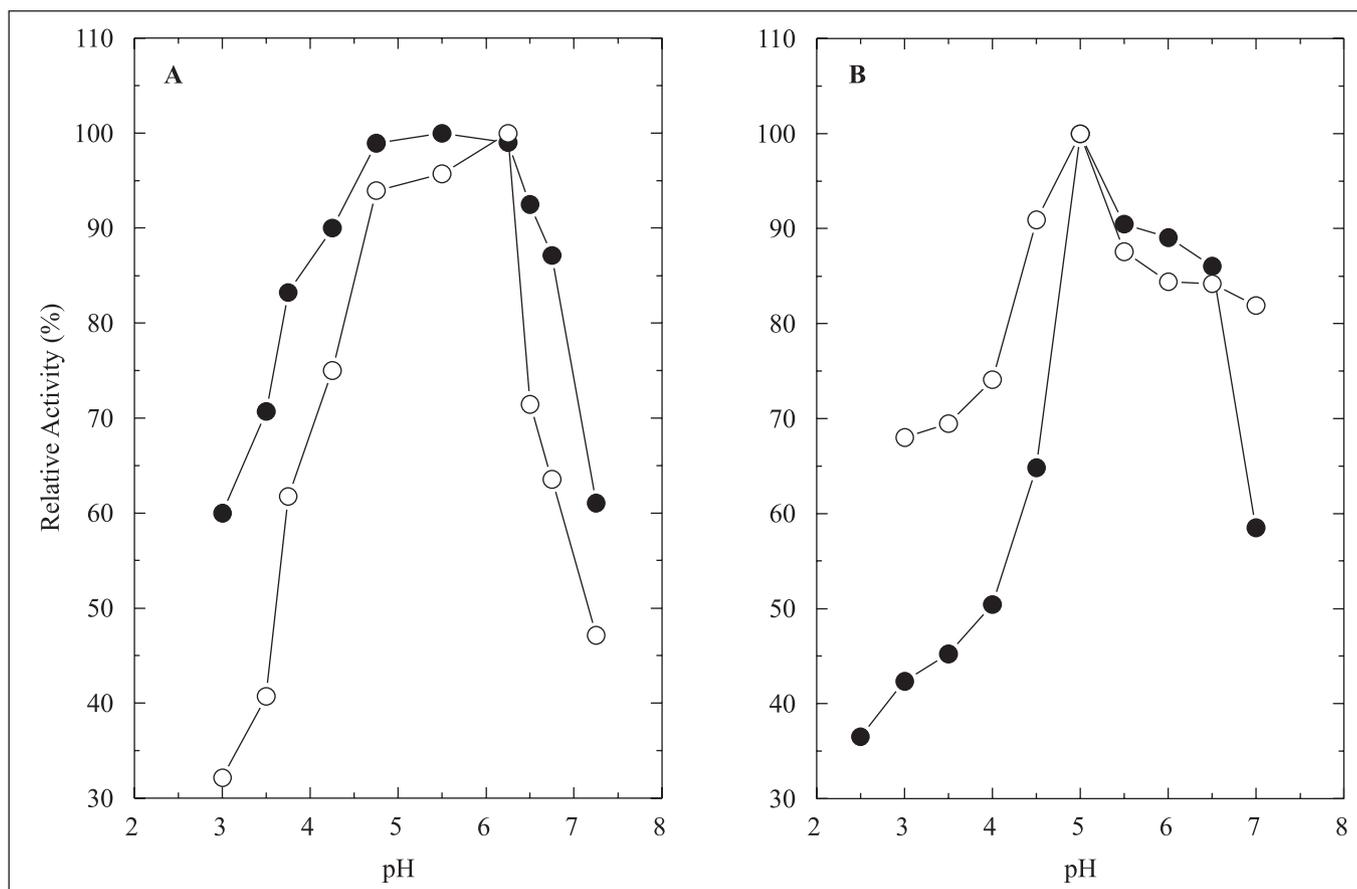


Figure 4. Optimum pH of activity for the glucoamylases produced by *S. thermophilum* 15.1 (A) and 15.8 (B), using starch (●) and maltose (○) as substrate.

Table 3. Determination of kinetic parameters of the purified glucoamylase produced by *S. thermophilum* 15.1.

Substrate	Km (mg/ml)	Vmax (μmol/min/mg prot)	Vmax/Km
Starch	0.094	0.202	2.15
Maltose	0.029	0.109	3.76
Starch + Maltose	0.003	0.070	2.33
Glycogen	0.158	0.185	1.17
Amylopectin	0.045	0.016	0.35

mycelial extracts from two species were purified by two different sequential chromatographic steps. Optimal temperature and pH, for starch and maltose as substrates, were close between the two species (50-60°C and pH 5.0-6.0) but differences were observed as for effect of metallic ions, thermostability, kinetic constants, molecular masses 15.1 and 15.8 and products of hydrolysis. In relation at molecular mass, for example, for *S. thermophilum* 15.1, extra (1) and intracellular glucoamylase

had close values (60 kDa and 54 kDa, respectively), but in contrast, differences were observed to extracellular GAI (68.5 kDa) (6), GAII (83 kDa) (5) and to the intracellular form (98.7 kDa) from *S. thermophilum* 15.8. These results reflect differences structural or in the carbohydrate content since that extracellular glucoamylases of *S. thermophilum* 15.1 and 15.8 had carbohydrate contents of 9.8% and 10-25% (1,5,6), but intracellular glucoamylases of *S. thermophilum* 15.1 and 15.8 had carbohydrate contents of 20% and 51%, respectively. Then, *S. thermophilum* is a potential thermophilic fungus with highest amylolytic levels either extra or intracellular with great possibility industrial application.

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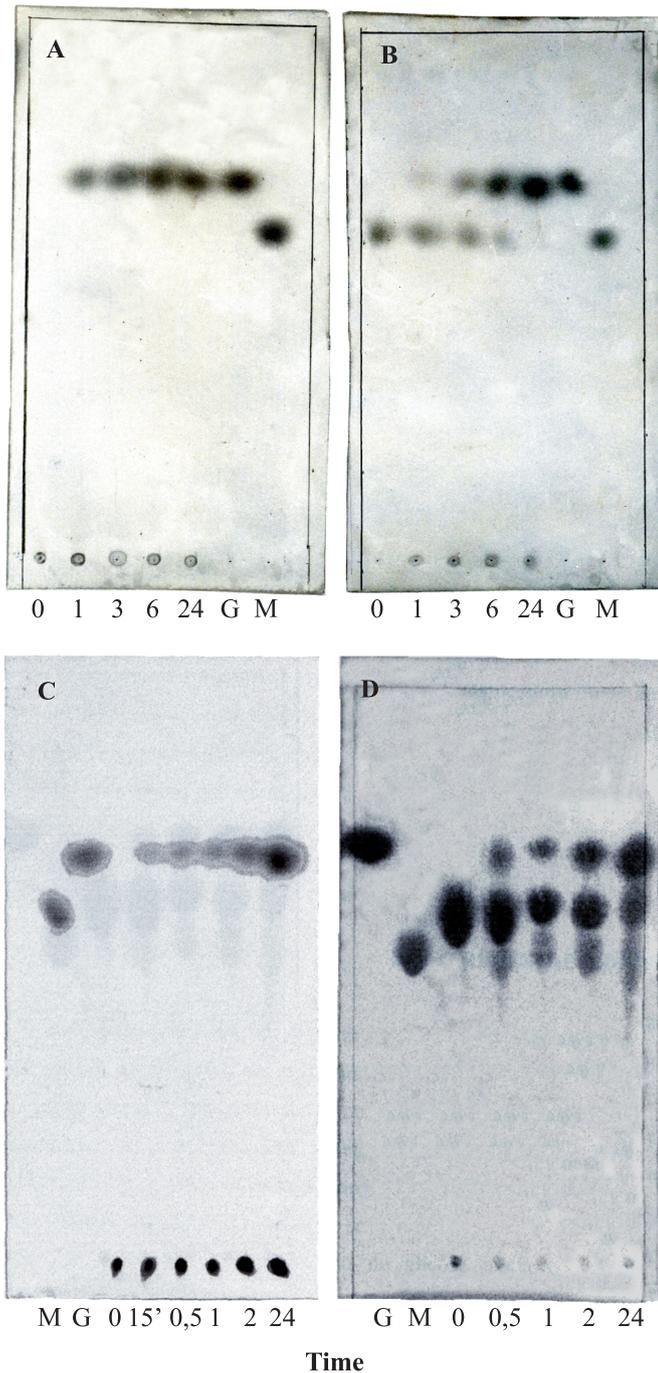


Figure 5. Thin layer chromatography of the hydrolysis products of the glucoamylases of *Scytalidium thermophilum* 15.1 (A,B) and *Scytalidium thermophilum* 15.8 (C,D) using starch (A,C) and maltose (B,D) as substrates. G = glucose; m = maltose. Time is represented in hours, except 15 (minutes).

RESUMO

Glucoamilases miceliais produzidas pelas linhagens 15.1 e 15.8 do fungo termofílico *Scytalidium thermophilum*: Purificação e Caracterização Bioquímica.

Dois linhagens (15.1 e 15.8) do fungo termofílico *Scytalidium thermophilum* se mostraram produtoras de grandes quantidades de glucoamilases, com potencial aplicação industrial. A isoforma I de glucoamilase produzida pela linhagem 15.1 foi submetida seqüencialmente a cromatografia em colunas de DEAE-celulose e CM-celulose, sendo purificada 141 vezes com porcentagem de recuperação de 5,45%. A glucoamilase da linhagem 15.8 foi purificada 71 vezes através do uso de colunas de cromatografia de CM-celulose e Concanavalina A-Sepharose com porcentagem de recuperação de 7,38%. Temperatura e pH ótimo foram de 50-60°C e 5,0-6,0 respectivamente, utilizando-se amido e maltose como substratos. A glucoamilase de *S. thermophilum* 15.8 se mostrou mais estável ($t_{50} > 60$ min) que a de *S. thermophilum* 15.1 ($t_{50} = 11-15$ min) a 60°C. As glucoamilases tiveram suas atividades enzimáticas aumentadas na presença de vários íons (ex: Mn^{2+} , e Ca^{2+}) e inibidas por β -mercaptoetanol. A glucoamilase da linhagem 15.1 apresentou um Km de 0,094 mg/ml e 0,029 mg/ml and Vmax de 202U/mg prot e 109U/mg prot, para amido e maltose respectivamente. A análise do produto da hidrólise de amido e maltose por TLC, demonstrou que o produto final era glucose, confirmando as características da enzima como glucoamilase. Diferenças entre as duas linhagens foram observadas com relação aos produtos formados tendo maltose como substrato, a linhagem 15.8 de *S. thermophilum* produziu maltotriose como produto final em contraste com a linhagem 15.1.

Palavras-chave: glucoamilase, amilase, *Scytalidium thermophilum*, enzima termoestável, hidrólise de amido

REFERENCES

1. Aquino, A.C.; Jorge, J.A.; Terenzi, H.F.; Polizeli, M.L.T.M. (2001). Thermostable glucose-tolerant glucoamylase produced by the thermophilic fungus *Scytalidium thermophilum*. *Folia Microbiol.*, 46 (1), 11-16.
2. Aquino, A.C.M.M.; Jorge, J.A.; Terenzi, H.F.; Polizeli, M.L.T.M. (2003). Studies on a thermostable α -amylase from the thermophilic fungus *Scytalidium thermophilum*. *Appl. Microbiol. Biotechnol.*, 61, 323-328.
3. Bergmeyer, H.U.; Bernt, E. (1974). *Methods of enzymatic analysis*. H.U. Bergmeyer, Verlag-Chimie Academic Press, New York, 1205-1215p.
4. Blum, H.; Beier, H.; Gross, H.J. (1987). Improved silver staining of plant protein, RNA and DNA in polyacrylamide gels. *Electrophoresis*, 81, 93-99.
5. Cereia, M.; Guimarães, L.H.S.; Peixoto-Nogueira, S.C.; Jorge, J.A.; Terenzi, H.F.; Greene, J.L.; Polizeli, M.L.T.M. (2006). Glucoamylase isoform (GAI) purified from a thermophilic fungus *Scytalidium*

- thermophilum* 15.8 with biotechnological potential. *Afr. J. Biotechnol.*, 5 (12), 1239-1245.
6. Cereia, M.; Terenzi, H.F.; Jorge, J.A.; Greene, L.J.; Rosa, J.C.; Polizeli, M.L.T.M. (2000). Glucoamylase activity from the thermophilic fungus *Scytalidium thermophilum*. Biochemical and regulatory properties. *J. Basic Microbiol.*, 2, 83-92.
 7. Chen, J.; Li, D.; Zhang, Y.; Zhou, Q. (2005). Purification and characterization of a thermostable glucoamylase from *Chaetomium thermophilum*. *J. Gen. Appl. Microbiol.*, 55, 175-181.
 8. Coutinho, P.M.; Reilly, P.J. (1997). Glucoamylase, structural, functional and evolutionary relationships. *Proteins*, 29, 334-347.
 9. Davis, B.J. (1964). *Disc electrophoresis II*. Method and application to human serum proteins. *Ann. NY Acad. Sci.*, 121, 407-427.
 10. Dubois, M.; Gilles, K.; Hamilton, J.; Rebers, P.; Smith, F. (1956). Colorimetric methods for determination of sugar and related substances. *Anal. Chem.*, 28, 350-356.
 11. Fagerstrom, R.; Kalkkinen, N. (1995). Characterization, subsite mapping and partial amino acid sequence of glucoamylase from the filamentous fungus *Trichoderma reesei*. *Biotechnol. Appl. Biochem.*, 21, 223-231.
 12. Fontana, J.D.; Gebara, M.; Blumel, M.; Schneider, H.; Mackenzie, C.R.; Johnson, K.G. (1988). α -4-O-methyl-D-glucuronidase component of xylanolytic complexes. *Methods Enzymol.*, 160, 560-571.
 13. Guimarães, L.H.S.; Terenzi, H.F.; Jorge, J.A.; Polizeli, M.L.T.M. (2001). Thermostable conidial and mycelial alkaline phosphatases from the thermophilic fungus *Scytalidium thermophilum*. *J. Ind. Microbiol. Biotechnol.*, 27, 265-270.
 14. Kadowaki, M.K.; Polizeli, M.L.T.M.; Terenzi, H.F.; Jorge, J.A. (1996). Characterization of trehalase activities from the thermophilic fungus *Scytalidium thermophilum*. *Biochim. Biophys. Acta*, 1291, 199-205.
 15. Lineweaver, H.; Burk, D. (1934). The determination of the enzyme dissociation. *J. Am. Chem. Soc.*, 56, 658-666.
 16. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193, 265-275.
 17. Martel, M.B.; Penhoat, C.H.; Létoublon, R.; Fèvre, M. (2002). Purification and characterization of a glucoamylase secreted by plant pathogen *Sclerotinia sclerotiorum*. *Can. J. Microbiol.*, 48, 212-218.
 18. Miller, G.H. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31, 426-429.
 19. Norouzian, D.; Akbarzadeh, A.; Scharer, J.M.; Young, M.M. (2006). Fungal glucoamylases. *Biotechnol. Adv.*, 24, 80-85.
 20. Norouzian, D.; Rostami, K.; Nouri, I.D.; Saleh, M. (2000). Subsite mapping of purified glucoamylases I, II, III produced by *Arthrotrypis amerospora* ATCC 34468. *World J. Microbiol. Biotechnol.*, 16, 155-161.
 21. Pandey, A.; Nigam, P.; Soccol, C.R.; Soccol, V.T.; Singh, D.; Mohan, R. (2000). Advances in microbial amylases. *Biotechnol. Appl. Biochem.*, 31, 135-152.
 22. Peralta, R.M.; Terenzi, H.F.; Jorge, J.A. (1990). β -D glycosidase activities of *Humicola grisea*: biochemical and kinetic characterization of a multifunctional enzyme. *Biochim. Biophys. Acta*, 1033, 243-249.
 23. Spinelli, B.B.L.; Polizeli, M.L.T.M.; Terenzi, H.F.; Jorge, J.A. (1996). Biochemical characterization of glucoamylase from hyper producer exo-1 mutant strain of *Neurospora crassa*. *FEMS Microbiol. Lett.*, 138, 173-177.
 24. Straatsma, G.; Samson, R.A. (1993). Taxonomy of *Scytalidium thermophilum*, an important fungus in mushroom compost. *Mycol. Res.*, 97 (3), 321-328.
 25. Suresh, C.; Dubey, A.K.; Srikanta S.; Kumar, U.S. (1999). Characterization of starch hydrolyzing enzyme of *Aspergillus niger*. *Appl. Microbiol. Biotechnol.*, 51, 673-675.
 26. Thorsen, T.S.; Johnsen, A.H.; Josefsen, K.; Bensen, B. (2006). Identification and characterization of glucoamylase from the fungus *Thermomyces lanuginosus*. *Biochem. Biophys. Acta*, 1764, 671-676.
 27. Torres, R.; Pessela, B.C.; Mateo, C.; Ortiz, C.; Fuentes, M.; Guisan, J.M. (2004). Reversible immobilization of glucoamylase by ionic adsorption on SEPA beads coated with polyethyleneimine. *Biotechnol. Prog.*, 20 (4), 1297-1300.
 28. Tosi, L.R.O.; Terenzi, H.F.; Jorge, J.A. (1993). Purification and characterization of an extracellular glucoamylase from the thermophilic *Humicola grisea* var. *thermoidea*. *Can. J. Microbiol.*, 39, 846-852.
 29. Ueda, S. (1981). Fungal glucoamylase and raw starch digestion. *TIBS* 6, 89-90.