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(Departamento de Biologia – Faculdade de Filosofia Ciências e Letras de Ribeirão Preto – Universidade de São Paulo; [1]Centro de Química de Proteínas and Departamento de Ginecologia e Obstetrícia – Faculdade de Medicina de Ribeirão Preto – Universidade de São Paulo)

Glucoamylase activity from the thermophilic fungus *Scytalidium thermophilum.* Biochemical and regulatory properties

MARIANA CEREIA, HÉCTOR F. TERENZI, JOÃO A. JORGE, LEWIS J. GREENE[1], JOSE C. ROSA[1] and MARIA DE LOURDES T. M. POLIZELI

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A glucoamylase activity produced by the thermophilic fungus *Scytalidium thermophilum* was purified 6.8-fold by ion exchange chromatography. The protein exhibited a molecular mass of about 86 kDa (7% PAGE and SDS-PAGE), or 68.5 kDa (Bio Sil SEC-400 FPLC). The pI of the enzyme was 8.4. Optima of pH and temperature, with starch or maltose as substrates were, 6.5/60 °C and 5.0/55 °C, respectively. The enzyme had a half-life of 22 min at 55 °C with starch as substrate, and it was fully stable with maltose. Maltase activity was activated by 10 mM Ba^{++} (7.8%); Mn^{++} (12.4%) or Mg^{++} (28%). The enzyme contained approximately 25.5% carbohydrate. K_m and V_{max} values for starch and maltose were 0.28 mg/ml, 67.2 U/mg protein and 1.40 mg/ml, 5.61 U/mg protein, respectively. The products of hydrolysis of starch, detected by thin layer chromatography, showed only glucose after 30 min, indicating a glucoamylase activity. The amino terminal sequence of the purified protein showed 93% homology with a glucoamylase activity purified from *Humicola grisea* var. *thermoidea*.

Biotechnological industries show increasing interest in the use of microbial sources of amylolytic activity (VIHINEN and MÄNTSÄLÄ 1989, GUZMÁN-MALDONADO and PAREDEZ-LÓPEZ 1995, CRABB and MITCHINSON 1997, JAMES and LEE 1997). For instance, glucoamylase [α(1,4)-D-glucan glucohydrolase, EC 3.2.1.3] activity, which attacks α-1,4 linkages from the nonreducing end of starch, releasing D-glucose in the β-configuration, is widely used for the production of high-glucose (96 to 98% glucose) and high-fructose (55% fructose) syrups.

Thermophilic microorganisms are regarded as an attractive source of thermostable amylolytic enzymes, for industrial use (TOSI *et al.* 1993, ALMEIDA *et al.* 1995, KADOWAKI *et al.* 1996, MISHRA and MAHESHWARI 1996, POLIZELI *et al.* 1996, BUSCH *et al.* 1997, BUAINAIN *et al.* 1998, VAN DEN BURG *et al.* 1998). In the present study we describe an extracellular glucoamylase activity produced by *Scytalidium thermophilum*, a fungus isolated from phase II of mushroom compost soil (STRAATSMA and SAMSON 1993). We describe an optimised medium for the enzyme production, and some of its biochemical properties. This enzyme was partially sequenced at the amino terminus, which showed homology with a glucoamylase produced by the thermophilic fungus *Humicola grisea* var. *thermoidea*.

Materials and methods

Organism and growth conditions: *Scytalidium thermophilum* (15.8) [= CBS 671.88 = ATCC 66938] was maintained at 45 °C, in slants of solid 4% oatmeal baby food (QUAKER) medium. Conidia from 10-day-old cultures were inoculated into 125 ml ERLENMEYER flasks containing 25 ml liquid medium: 0.1% calcium carbonate; 0.6% yeast extract; 0.1% peptone; 1.0% sodium chloride; 0.6% ammonium

acetate and 1.0% cassava flour, pH 6.0. The cultures were incubated at 45 °C without agitation. After seven days of incubation, when glucoamylase activity reached a maximum, the cultures were harvested by filtration and the filtrate was saved as a source of crude extracellular glucoamylase. Mycelial pads were ground with sea sand, at 0 °C with ten vol. of cold 100 mM sodium acetate buffer, pH 5.5 (buffer A). After centrifugation (20,000 × g, 20 min, 4 °C) the supernatant fraction was the source of intracellular enzyme.

Enzymatic assays, determination of protein and neutral carbohydrate: Glucoamylase activity was assayed at 60 °C, in a reaction mixture containing 0.2 ml of diluted enzyme and 0.2 ml of 1.0% starch in 100 mM sodium acetate buffer, pH 5.5. The amount of glucose released was estimated by the glucose oxidase procedure (BERGMEYER and BERNT 1974). Maltase activity was assayed as described above, except that 1.7% maltose was used as substrate. An enzyme unit is the amount that produces 1 μmol of glucose per minute. Protein was determined by the method of LOWRY *et al.* (1951). Total neutral carbohydrate was quantified by the phenol sulphuric acid method of DUBOIS *et al.* (1956), using D-mannose as standard.

Gel filtration: The molecular weight of the purified enzyme was estimated by FPLC using a Bio Sil-SEC-400 filtration column (0.78 × 30.0 cm) equilibrated and eluted with 50 mM Tris-HCl, pH 7.5 plus 100 mM KCl. The flow rate was 1 ml/min and 1.0 ml fractions were collected and assayed. Void volume *(Vo)* was determined with Blue Dextran, using as MW markers β-amylase (200 kDa); alcohol dehydrogenase (150 kDa); bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

Electrophoresis and amino acid sequencing: Non-denaturating PAGE (7.0%) electrophoresis was carried out by the method of REISFIELD *et al.* (1962) and SDS-PAGE (7.0%) according to LAEMMLI (1970). MW markers were: β-galactosidase (116 kDa); phosphorylase-b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa), and carbonic anydrase (29 kDa). Protein was stained with silver (BLUM *et al.* 1987). Glucoamylase activity was visualised on polyacrylamide gels as described by BASAVESWARA RAO *et al.* (1981). Isoelectric focusing was carried out according to O'FARRELL *et al.* (1977) using Pharmalite pH 8.0–10.5. For amino acid sequencing the protein was electroblotted onto a PVDF membrane after SDS PAGE. The blotted protein band was submitted to automatic EDMAN degradation. The sequencing was carried out in a Procise model 491 protein sequencer (PE-Applied Biosystem, Foster city) using gas-phase chemistry with on-line identification of phenylthiohydantoin derivative. A 10 picomol standard was used to quantify PTH-amino acids.

Chromatography of hydrolysis products: The hydrolysis products of glucoamylase activity on soluble starch or maltose as substrates were analysed by thin-layer chromatography on silica gel (DC-Alufolien Kieselgel 60, MERCK). The mobile phase was: butanol/ethanol/water (5:3:2, by vol.). Sugars were detected with orcinol (FONTANA *et al.* 1988).

Chemicals: DEAE-cellulose, CM-cellulose, acrylamide and molecular weight standards were purchased from SIGMA Chemicals Co. Maltose was purchased from MERCK. Cassava flour was manufactured in Maceio, State of Alagoas, Brazil. All other reagents were of analytical grade.

Results

The composition of the culture medium (M-5; TOSI *et al.* 1993) was optimised for maximal production and secretion of glucoamylase activity, by modifying the original concentration, or substituting some of its constituents. The enzyme levels were not significantly affected by altering the concentrations of calcium carbonate, peptone or yeast extract (not shown). However, glucoamylase production, and principally its secretion, were markedly stimulated by increasing the concentration of sodium chloride above 0.4% (Table 1). In the absence of this salt, the amount of glucoamylase released into the medium was approximately one-fourth of total activity, in contrast with cultures grown with 1% of sodium chloride, which secreted about 80% of the enzyme. Higher concentrations of sodium chloride diminished growth and did not improve further the enzyme production.

Table 1
Effect of NaCl on the production of glucoamylase activity and on the growth of *Scytalidium thermophilum*

NaCl* %	Extra (Total U)	Intra (Total U)	Protein (Total mg)
0.0	1.78	5.42	7.75
0.2	2.66	6.76	11.47
0.4	3.39	6.34	11.32
0.5	6.51	5.74	10.98
0.8	10.25	2.71	10.05
1.0	10.88	2.40	10.01

* NaCl was added to 25 ml of M-5 medium containing 0.1% $CaCO_3$; 0.1% peptone; 0.6% yeast extract; 0.2% gelatine and 1.0% starch. The pH was adjusted to 6.0.

Other nitrogen-containing compounds were tested as substitutes for gelatine (Table 2). Ammonium acetate, ammonium sulphate and ammonium nitrate increased glucoamylase secretion from about 87% to 93%–96%. A very high stimulus of glucoamylase production was elicited by ammonium acetate, which increased five-fold specific activity. When other carbohydrate sources were used as substitutes for starch, the best result was obtained with cassava flour, which doubled the specific enzyme production. Altogether, the modifications introduced in the composition of the M-5 medium increased by 36-fold (from about 1.8 to 65 U/mg cell protein) the specific activity of amylase, and the enzyme was almost totally (96%) released into the culture medium.

In order to purify the extracellular glucoamylase, a dialysed crude filtrate was applied to a DEAE-cellulose column (1.9 × 20.0 cm) equilibrated and eluted with 10 mM Tris-HCl pH 7.5, at the flow rate of 28 ml/h. Ten ml fractions were collected and assayed for enzyme activity. Glucoamylase activity was eluted into two fractions. Form I (76% of total activity) did not bind to the resin and eluted with a bulk of protein in the void volume, whereas form II (24% of total activity) was retained and eluted at 52 mM concentration of a linear (0–500 mM) NaCl gradient, prepared in the same buffer (Fig. 1). We concentrated in purifying and characterising glucoamylase form I only, considering that it represented the larger enzymatic component, in comparison with form II. Nevertheless, preliminary results (not shown) had revealed that glucoamylase form I and form II exhibited quite similar enzymatic properties.

Table 2
Effect of nitrogen sources on glucoamylase production

Compounds added *	Extracellular (total U)	Intracellular (total U)	Protein (total U)
none	2.66	0.50	1.71
gelatine	9.19	1.33	2.71
CH_3COONH_4	83.88	3.05	4.13
$(NH_4)H_2PO_3$	1.85	1.27	2.49
NH_4NO_3	9.67	0.30	1.56
$(NH_4)_2SO_4$	19.80	1.53	3.35
Urea	8.59	1.57	3.16

* The compounds were added (1% w/v) to 25 ml of M-5 medium containing by 0.1% $CaCO_3$; 0.1% peptone; 0.5% NaCl; 0.6% yeast extract and 1.0% starch. The pH was adjusted to 6.0.

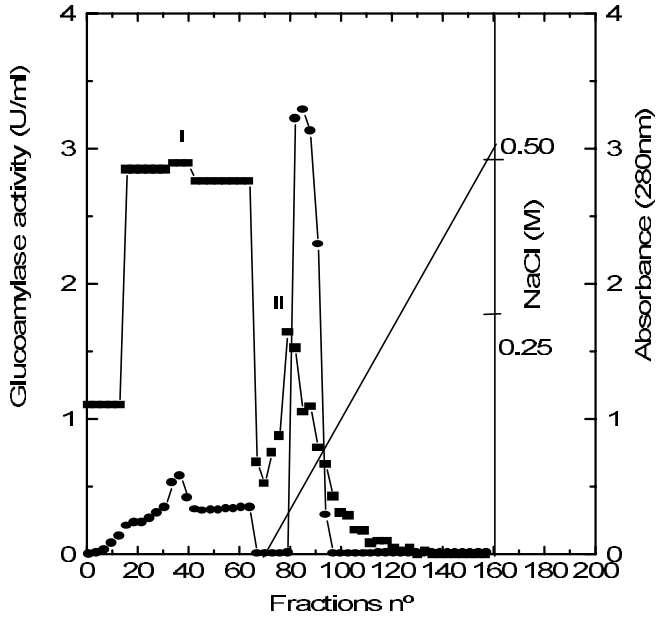

Fig. 1
Separation of extracellular glucoamylase activities by DEAE-cellulose chromatography. The column (1.9 × 20.0 cm) was equilibrated with 10 mM Tris-HCl pH 7.5 and eluted with a linear (0–500 mM) gradient of NaCl, in the same buffer, at a flow rate of 28.0 ml/h. Ten millilitre fractions were collected and assayed. Symbols: (■) glucoamylase activities, Form I and Form II; (●) absorbance 280 nm; (—) NaCl gradient

In order to purify Form I, fractions with this activity were pooled, dialysed, and applied to a CM-cellulose (2.0 × 6.0 cm) column equilibrated with 10 mM sodium acetate buffer, pH 5.5, and eluted with a linear gradient of NaCl (0–500 mM) in the same buffer. Five ml fractions were collected at a flow rate of 24.4 ml/h (Fig. 2). All steps were carried out at 4 °C. Glucoamylase activity was eluted as a single peak with 125 mM of salt with a recovery of 41.6% and purification of 6.8-fold (Table 3).

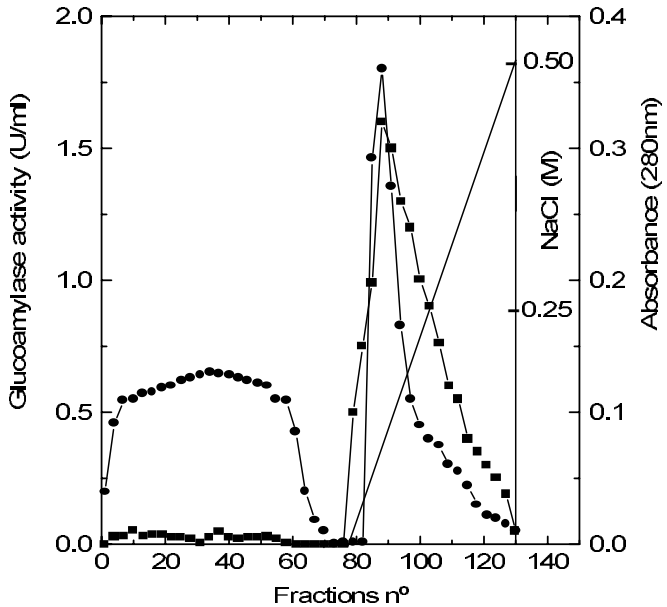

Fig. 2
Separation of extracellular glucoamylase (form I) by CM-cellulose chromatography. The column (2.0 × 6.0 cm) was equilibrated with 10 mM sodium acetate buffer, pH 5.5 and eluted with a linear gradient of NaCl (0–500 mM) in the same buffer at a flow rate of 24.4 ml/h. Five millilitre fractions were collected and assayed. Symbols: (■) glucoamylase activities; (●) absorbance 280 nm; (—) NaCl gradient. Other details as described in the text

Table 3
Summary of the purification steps of extracellular glucoamylase form I

Step	Total volume (ml)	Total protein (mg)	Total activity	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Filtrate	780	303.4	2828	9.3	100	–
DEAE-cellulose						
Form I	710	50.4	2146	42.6	75.9	4.6
Form II	164	56.6	682	12.1	24.1	1.3
CM-cellulose	175	18.7	1176	62.9	41.6	6.8

The purified enzyme showed a single protein band in PAGE (Fig. 3A) and SDS-PAGE (Fig. 3B) coincident with glucoamylase activity against starch (Fig. 3C) or maltose (Fig. 3D), as substrates. The molecular mass of the enzyme was estimated to be 86.0 kDa (SDS-PAGE), or 68.5 kDa (Bio Sil-SEC-400 filtration). This difference in apparent molecular mass could be due to the fact that the enzyme was a glycoprotein (25.5% carbohydrate content). According to HAMES and RICKWOOD (1985) many glycoproteins migrate anomalously, even in the presence of excess SDS and thiol reagents, probably because they bind SDS only to the protein part of the molecule. The reduced net charge resulting from reduced SDS binding lowers the polypeptide mobility during electrophoresis, yielding artifactually high molecular weight estimates.

Thin layer chromatography (TLC) of the products of hydrolysis of starch (Fig. 4A) or maltose (Fig. 4B) revealed only glucose as the hydrolysis product, after 30 min of reaction, indicating that the purified enzyme was a glucoamylase [α(1,4)-D-glucan glucohydrolase, EC 3.2.1.3].

Table 4 summarises some biochemical properties of *S. thermophilum* glucoamylase activity using either starch or maltose as substrates. Activation energy values (Ea) for the hydrolysis of starch and maltose, calculated from ARRHENIUS plots were, 2.64 and 1.97 Kcal mol^{-1} respectively. The isoelectric point was 8.4. Optima of pH and temperature, using starch or maltose as substrates, were 6.5/60 °C and 5.0/55 °C, respectively. Glucoamylase had a half-life of 22 minutes at 55 °C using starch as substrate, and it was completely stable with maltose. The thermostability of the enzyme was improved (100% after 60 min at 60 °C) when it was incubated at 60 °C in the presence of either 1% (w/v) starch, or 10% sorbitol, and then assayed for glucoamylase activity using starch or maltose as substrates. Similar results were reported by others (TOSI *et al.* 1993, SILVA and PERALTA 1998).

The preferred substrates for *S. thermophilum* glucoamylase activity were starch, maltose, glycogen, amylopectin, amylose, and isomaltose, in that order (data not shown). These data are compatible with the values of K_m and V_{max} calculated from HANES plots (HANES 1932), determined at the optimum temperature for glucoamylase and maltase activities, using starch or maltose as substrates at concentrations from 0.05 to 4.5 mg/ml and 0.2 to 10.0 mg/ml, respectively (Table 4). Glucoamylase activity was inhibited by several metal ions used at 1 mM concentration, such as Ba^{++} (20%), Ca^{++} (22.7%), Cu^{++} (17.2%), EDTA (23%), Hg^{+} (24.4%), Mg^{++} (20.8%), Mn^{++} (21.1%), Na^{+} (22.8%), NH_4^{+} (13.7%), Zn^{++} (24.1%). On the other hand, maltase activity was activated by 10 mM Ba^{++} (7.8%), 10 mM Mn^{++} (12.4%), and 10 mM Mg^{++} (28%). Other reagents inhibited 20 to 87% the enzyme. β-mercaptoethanol completely inhibited the hydrolysis of both substrates. This result might suggest the participation of protein disulphide bonds in the catalytic activity.

Sequence data of the first fifteen amino terminal residues of the purified protein (Table 5) revealed that *S. thermophilum* glucoamylase shared 93% homology with that of the thermophilic fungus *Humicola grisea* var. *thermoidea.* No signal peptide was found.

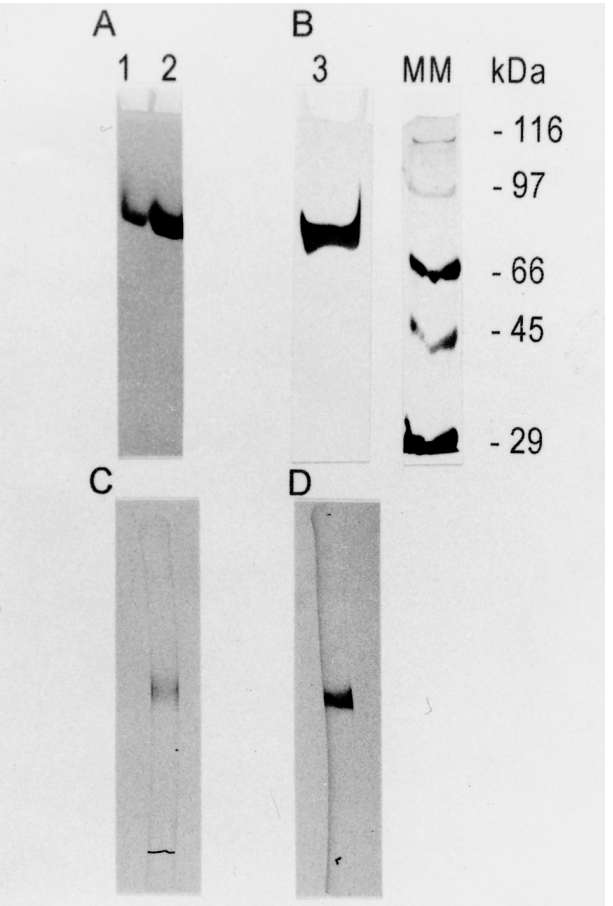

Fig. 3
Gel electrophoresis of purified glucoamylase activity. (A) silver-stained 7% PAGE. Lanes 1 and 2, 0.62 μg and 1.0 μg, respectively; (B) 7% SDS-PAGE. Lane 3, purified glucoamylase (2.5 μg). MM, molecular mass markers. (C) glucoamylase activity gels of samples from the purification steps using starch and (D) maltose as substrate

Discussion

The production of glucoamylase by *S. thermophilum* was largely increased by replacing starch by cassava flour, and gelatine by ammonium acetate, in the original M5 culture medium. Production of microbial glucoamylase in cassava flour was reported by others (KILIKIAN 1996, REDDY and BASAPPA 1996). Glucoamylase activity from *Aspergillus flavus* cultivated on cassava peel was 170-times higher than that obtained with soluble starch (SANI *et al.* 1992).

An interesting result was observed when *S. thermophilum* was cultivated in the presence of different concentrations of sodium chloride (Table 1). This salt increased the secretion glucoamylase activity. The influence of osmotic potential on enzyme levels has been studied in only a few microbial systems, and different effects have been reported. Osmotic stress favours the production and secretion of a periplasmic β-galactosidase from *Penicillium notatum* (FIEDUREK 1998). Similar results were obtained for cellulase activity in *Trichoderma reesei* and *Sporotrichum cellulophilum* growing on wheat bran (KIM *et al.* 1985). However, the possibility that the increased glucoamylase secretion in *S. thermophilum* is due to changes in external osmolarity, is still a speculation.

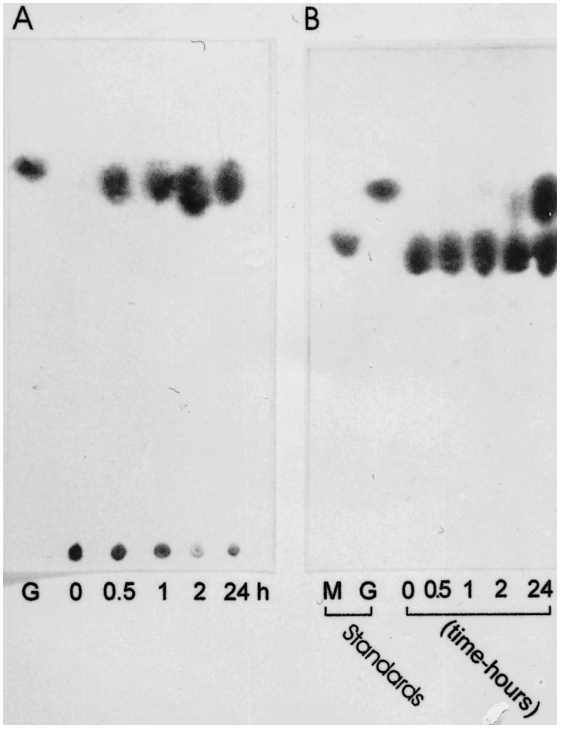

Fig. 4
Analysis of the hydrolysis products released by the purified *S. thermophilum* glucoamylase acting on starch (A) or maltose (B). Glucoamylase (3.6 mU) was incubated with the substrates (10 mg/ml) in 100 mM sodium acetate buffer, pH 5.5, at 60 °C, for different times (0; 0.5; 1; 2; 24 hours). Abbreviations: G, glucose; M, maltose

Table 4
Kinetic constants and some intrinsic properties of *S. thermophilum* glucoamylase activities using starch and maltose as substrate

Parameters	Starch	Maltose
Activation energy (Ea Kcal mol^{-1})	2.64	1.97
Carbohydrate content (%)	25.50	
Isoelectric point (pI)	8.40	
Molecular mass (Filtration Gel, kDa)	68.50	
Molecular mass (SDS-PAGE, kDa)	86.00	
Optimum pH	6.50	5.00
Optimum temperature (°C)	60.00	55.00
Stability 55 °C (T_{50} – min)	22.00	60.00
K_m (mg ml^{-1})	0.28	1.40
V_{max} (U mg^{-1} protein)	67.20	5.61

S. thermophilum glucoamylase activity was resolved into two components by ion exchange chromatography. Form I (76% of total activity) was purified 6.8-fold. Some properties of this glucoamylase, such as glycoproteic nature, molecular mass, specific activity and ability to hydrolyse starch and maltose, resembled those of other fungal glucoamylases (reviewed by VIHINEN and MÄNTSÄLÄ 1989, GUZMÁN-MALDONADO and PAREDEZ-LÓPEZ 1995, JAMES and LEE 1997). This enzyme hydrolysed preferentially starch, but also exhibited a significant maltase activity. Maltase and glucoamylase activities co-migrated in PAGE (Fig. 3 A–D). These activities differed in optimum pH, effect of temperature and also activation energy. Apparently, the activity against starch was protected at higher pH and tempe-

Table 5
N-terminal amino acid sequence of the protein produced by the thermophilic fungi *Scytalidium thermophilum*

Edman cycle	Amino acid	pmol	*H. grisea*
01	Ile	2.27	Ile
02	Asn	1.81	Asn
03	Thr	1.28	Thr
04	Glu	1.58	Glu
05	Lys	1.10	Lys
06	Ser	0.11	Pro
07	Ile	0.98	Ile
08	Ala	1.09	Ala
09	Trp	0.45	Trp
10	Asn	0.78	Asn
11	Lys	0.53	Lys
12	Leu	0.81	Leu
13	Leu	0.99	Leu
14	Ala	1.03	Ala
15	Asn	0.72	Asn

Sequence data of *Humicola grisea* var. *thermoidea* homologous enzyme was take from Gen Bank database, (accession number M89475). The recovery of PTH – amino acids is given in picomol, which corresponds 80% of the product and is reported without any corrections

ratures. This observation agrees with *H. grisea* glucoamylase (TOSI *et al.* 1993) and suggested a common catalytic site for starch and maltose hydrolysis, but specialised subsites for each of these substrates (SIVAKAMI and RADHAKRISHNAN 1976, KISS *et al.* 1981).

The purified glucoamylase presented 93% homology with 15 amino terminal residues of the same enzyme from the thermophilic fungus *Humicola grisea* var. *thermoidea.* Glucoamylase structural, functional, and evolutionary relationships had been revised recently (COUTINHO and REILLY 1997).

The present study may contribute to the knowledge of the evolutionary tree of glucoamylase sequences, and to the basic knowledge of a glucoamylase that could be used industrially.

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Mailing address: MARIA DE LOURDES TEIXERA DE MORAES POLIZELI, Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, 14.040-901 – Ribeirão Preto – SP, Brazil
e-mail: polizeli@ffclrp.usp.br