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ORIGINAL PAPER

Purification and biochemical characterization of a thermostable extracellular glucoamylase produced by the thermotolerant fungus *Paecilomyces variotii*

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Abstract An extracellular glucoamylase produced by *Paecilomyces variotii* was purified using DEAE-cellulose ion exchange chromatography and Sephadex G-100 gel filtration. The purified protein migrated as a single band in 7% PAGE and 8% SDS-PAGE. The estimated molecular mass was 86.5 kDa (SDS-PAGE). Optima of temperature and pH were 55 °C and 5.0, respectively. In the absence of substrate the purified glucoamylase was stable for 1 h at 50 and 55 °C, with a t_{50} of 45 min at 60 °C. The substrate contributed to protect the enzyme against thermal denaturation. The enzyme was mainly activated by manganese metal ions. The glucoamylase produced by *P. variotii* preferentially hydrolyzed amylopectin, glycogen and starch, and to a lesser extent malto-oligossacarides and amylose. Sucrose, p-nitrophenyl α-D-maltoside, methyl-α-D-glucopyranoside, pullulan, α- and β-cyclodextrin, and trehalose were not hydrolyzed. After 24 h, the products of starch hydrolysis, analyzed by thin layer chromatography, showed only glucose. The circular dichroism spectrum showed a protein rich in α-helix. The sequence of amino acids of the purified enzyme VVTDSFR appears similar to glucoamylases purified from *Talaromyces emersonii* and with the precursor of the glucoamylase from *Aspergillus oryzae*. These results suggested the character of the enzyme studied as a glucoamylase (1,4-α-D-glucan glucohydrolase).

Keywords *Paecilomyces variotii* · Glucoamylase · Purification · Thermostable

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Introduction

Glucoamylases (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.3), also referred to as amyloglucosidases, are exo-acting amylases releasing glucose from the nonreducing end of starch and related oligosaccharides. These enzymes have many applications in industry, being used for dextrose production, in the baking industry, in the brewing of low calorie beer and in whole grain hydrolysis for the alcohol industry, but the most important application of glucoamylase is the production of high-glucose syrups. Others dextrose products formed as a result of the glucoamylase action are high fructose corn syrup and high conversion syrups. The conversion of starch to sugars is one of the most important biotechnological processes [1]. In contrast to α-glucosidases, this enzyme yields glucose in the β-configuration. Glucoamylases are capable of splitting α-1,4-*O*-glucosidic bonds and, at lower rates, α-1,6-*O*-glucosidic bonds in glucans [2–4]. They may be obtained from a large variety of plants, animals and microorganisms, though most of these enzymes occur in fungi. Glucoamylases used commercially for the conversion of malto-oligosaccharides in glucose [5] originate from strains of either *Aspergillus niger* or *Rhizopus* sp. The preference for glucoamylases from these sources in the starch-processing industries is due to their good thermostability and high activity at near neutral pH values [4, 6]. These enzymes are generally regarded as safe (GRAS) by the food and drug administration (FDA).

Preliminary studies of our laboratory showed that a *Paecilomyces variotii* strain of our culture collection abundantly produced an extracellular amylolytic activity, potentially interesting for industrial applications. The present study was aimed to biochemically characterize the enzyme.

Materials and methods

Organism and growth conditions

Paecilomyces variotii strain was isolated in our laboratory from guava (*Psidium guajava*) leaves, in Pereira Barreto (São Paulo State, Brazil), and identified by "Instituto de Pesquisas Tropicais André Tosello" (Brazil). The fungus was maintained at 30 °C, on slants of solid 4% (w/v) PDA and oatmeal baby food (Quaker) media. Conidia from 7-day-old cultures were inoculated into 1,000 ml Erlenmeyer flasks containing 200 ml of the liquid medium described by Rizzatti et al. [7] containing 1.5% oatmeal as carbon source. The cultures were incubated at 30 °C without agitation and after 6 days the mycelial pads were harvested by filtration using Whatman no. 1 filter paper and the filtrates were used as the source of crude extra-cellular amylase.

Purification of glucoamylase

All steps were carried out at 4 °C. The culture filtrate was dialyzed overnight against 10 mM Tris–HCl buffer, pH 7.5, and applied to a DEAE-cellulose column (20 × 140 mm) equilibrated with the same buffer, and eluted with 400 ml of a linear gradient (0–1 M) of sodium chloride in the same buffer. The fractions showing amylolytic activity were pooled, dialyzed against distilled water, lyophilized and suspended in 2 ml of 100 mM sodium acetate buffer, pH 5.0, 150 mM of sodium chloride. This sample was applied to a Sephadex G-100 gel filtration column (15 × 510 mm) equilibrated and eluted with the same buffer. Fractions of 2 ml were collected at a flow rate of 25 ml/h, and the active fractions were pooled and used for enzyme characterization.

Enzymatic assays, determination of protein, sugar content and kinetic constant

Glucoamylase activity was determined by measuring the production of reducing sugar using 3′5-dinitrosalicylic acid (DNS) as described by Miller [8]. The assay was carried out at 55 °C, using a 1.0% starch solution in 0.1 M sodium acetate buffer, pH 5.0. One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of glucose per minute. The amount of residual soluble starch was estimated by the iodine method [9]. Protein concentration was estimated as described by Lowry [10] using bovine serum albumin as a standard. Total neutral carbohydrate was quantified by the phenol-sulfuric acid method of Dubois et al. [11], using D-mannose as a standard. In order to determine which substrate was hydrolyzed most efficiently, the apparent kinetic parameters K_m and K_{cat} of the purified enzyme were determined using starch, amylopectin, amylose and glycogen. The reactions were carried out at 55 °C, and the apparent K_m values were calculated from Hanes plots [12]. The efficiency of substrate utilization was estimated on the basis of K_{cat}/K_m [13].

Polyacrylamide gel electrophoresis analysis

Polyacrylamide gel electrophoresis performed under non-denaturing conditions (7% PAGE) was carried out by the method of Davis [14] and under denaturing conditions (8% SDS-PAGE) according to Laemmli [15]. Molecular weight standards were: myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase b (97.4 kDa); bovine serum albumin (66 kDa); egg albumin (45 kDa); carbonic anhydrase (29 kDa). Protein was stained with silver nitrate as described by Blum et al. [16]. Glucoamylase activity on polyacrylamide gels was detected by the coupled reaction with glucose oxidase by incubating the gels in a buffer containing nitroblue tetrazolium and phenazine methosulphate as electron acceptors, and soluble starch as substrate [17]. Isoelectric focusing was carried out according to O'Farrel et al. [18] using Pharmalite (pH 3.0–10.0).

Chromatography of hydrolysis products

Chromatographic analysis of the reaction end products of glucoamylase activity on soluble starch was carried out using thin layer chromatography (TLC). A volume of 10 µl of the reaction mixture was applied on silica gel plates (DC-Alufolien Kieselgel 60, Merck), and subjected to two sequential ascending chromatography runs using butanol/ethanol/water (5:3:2) as the solvent system. After air-drying the plate, the spots were developed by spraying with H_2SO_4 and methanol (1:9) containing 0.2% orcinol, and heating at 100 °C [19].

Circular dichroism expereriments

All measurements were performed in 20 mM sodium acetate buffer (pH 5.5) at 25 °C. Far UV CD spectra (180–250 nm) were measured with a JASCO 810 spectrometer (JASCO Inc., Tokyo, Japan) using 0.1 mm pathlength cuvettes and 0.76 mg ml^{-1} of native glucoamylase. A total of 12 spectra were collected, averaged and corrected by

subtraction of a blank containing 20 mM sodium acetate buffer pH 5.5.

Peptide sequencing by mass spectrometry (ESI-MS/MS)

Coomassie-stained protein bands was excised from the gel, and digested with trypsin (sequencing grade porcine trypsin, Promega), according to the University of California, San Francisco (UCSF) Mass Spectrometry Facility in-gel digestion procedure (http://donatello.ucsf.edu/ingel.html), and subjected to ESI-MS/MS analysis were performed in a Q-Tof (Micromass) coupled to a CapLC (Waters) chromatographic system. The tryptic peptides were purified using a Waters Opti-Pak C18 trap column. The trapped peptides were eluted using a water/acetonitrile 0.1% (v/v) formic acid gradient and separated by a 75-ml i.d. capillary column home-packed with C18 silica. Data was acquired in data-dependent mode, and multiply charged ions were subjected to MS/MS experiments. The MS/MS spectra were processed using MAXENT3 (Micromass) and manually sequenced using the PEPSEQ program (Micromass).

Results and discussion

Purification of extracellular glucoamylase activity

The culture filtrate was applied to a DEAE-cellulose column, and a single peak containing starch-hydrolyzing activity eluted at approximately 0.55 M NaCl contained starch-hydrolyzing activity (Fig. 1a). After pooling of the fractions containing activity, the concentrated sample was applied to a Sephadex G-100 column equilibrated and eluted as described in "Materials and methods". Two peaks with amylolytic activity were eluted and designated pool I and II, respectively (Fig. 1b). Pool I contained an α-amylase that was not purified until electrophoretic homogeneity by this chromatographic procedure (results not shown). Pool II contained a glucoamylase, and these fractions were used for biochemical studies. Data from glucoamylase purification are summarized in Table 1, which shows that the enzyme was purified approximately 48-fold, with 6.2% recovery.

Properties of the purified glucoamylase

The purified protein migrated as a single polypeptide under 8% SDS-PAGE (Fig. 2—lane A) and 7% PAGE (Fig. 2—lanes B and C). The molecular mass estimated by SDS-PAGE was 86.5 kDa, which is within the range of the majority of molecular masses of other fungal glucoamylases (26–112 kDa) [20]. According to James and Lee [1], the isoeletric point of glucoamylases includes a range pH of

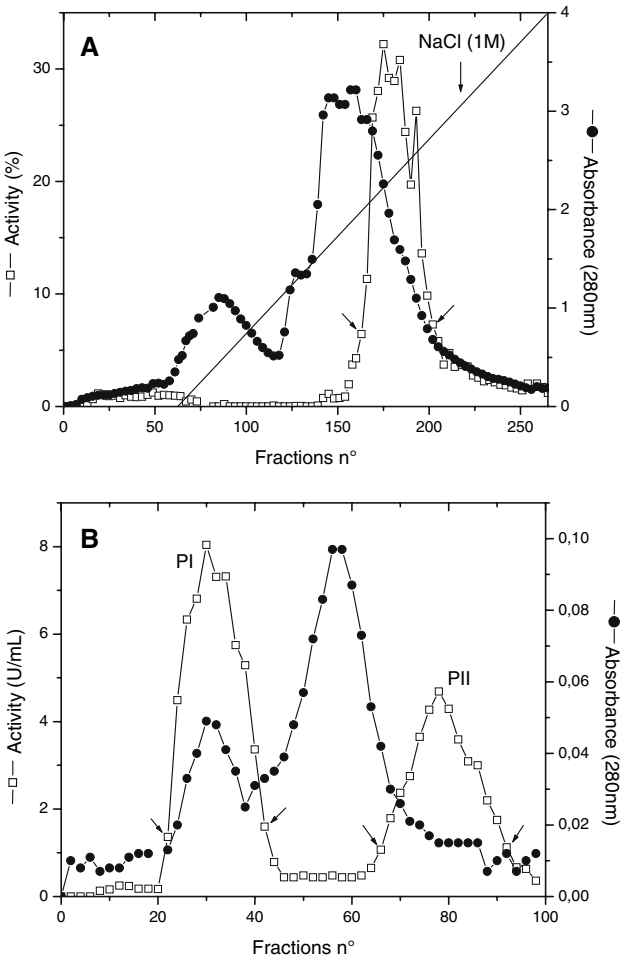


Fig. 1 Profile of the elution of starch hydrolyzing activity from: **a** the DEAE-cellulose column; **b** the Sephadex G-100 gel filtration column. Symbols: *filled circle* absorbance at 280 nm; *open square* amylolytic activity; *forward slash* NaCl gradient; *rightarrow* pool of activity; *PI* peak one; *PII* peak two. Enzymatic activity was quantified based on the amount of reducing sugar (DNS) using starch as substrate

3.7–7.4, and electrofocusing of the purified glucoamylase of *P. variotii* showed a p*I* of about 3.5. Glucoamylases of *A. niger* [21], *A. awamori* [22] and *Thermomyces lanuginosus* [23] presented similar p*I*; however, two varieties of *Scytalidium thermophilum* [17, 24] produce a glucoamylase with a p*I* of approximately 8.0. The *P. variotii* glucoamylase is a glycoprotein that contains 27.5% carbohydrate, which is in agreement with other fungal glucoamylases [24, 25].

Specificity and mechanism of action

The purified glucoamylase was highly specific for the α-anomeric configuration of the glycosidic linkage, catalyzing the hydrolysis of polymeric substrates such as amylopectin, glycogen and starch (around 100%). Maltotriose (21%), maltose (14%), maltopentaose (7%), maltotetraose

Table 1 Summary of the purification steps of the extra-cellular glucoamylase by *P. variotii*

Purification steps	Volume (ml)	Protein total (mg)	Activity total (U)	Spec. act (U/mg prot)	Yield (%)	Purification factor
Filtrate	410	847.7	8212.3	9.7	100	1
Filtrate dialyzed	420	356.7	10004.0	28.0	121.8	2.9
DEAE-cellulose	70	24.5	1703.1	69.5	20.7	7.2
Sephadex G-100	33	1.1	511.5	465.0	6.2	47.9

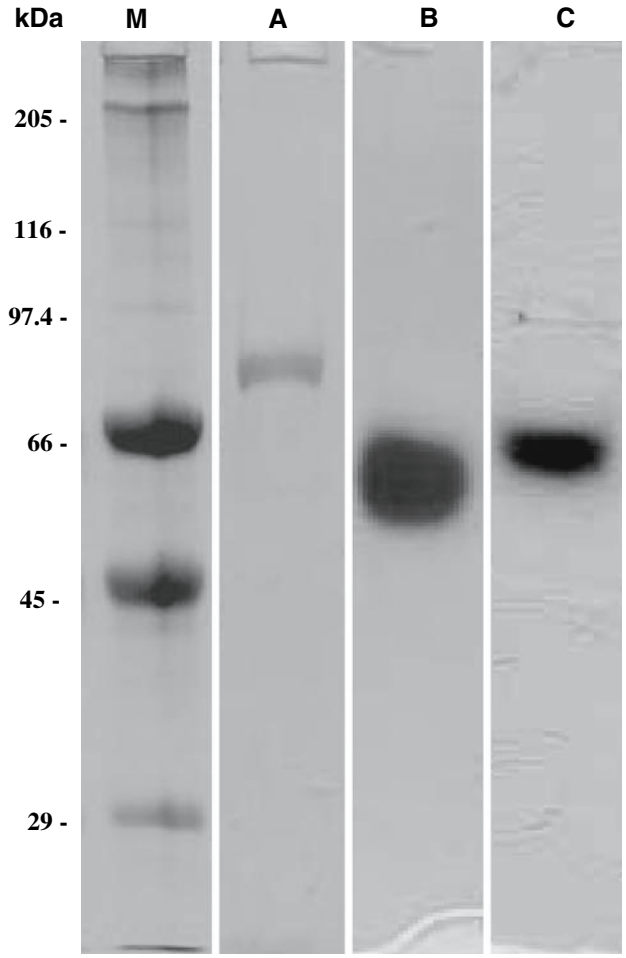


Fig. 2 Electrophoresis analysis of the pool II protein: *M* Molecular weight patterns in 8% SDS-PAGE: myosin (205 kDa), *β*-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa). *A* 8% SDS-PAGE of the purified glucoamylase. *B* 7% PAGE of the purified glucoamylase developed for protein using silver nitrate and *C* developed for glucoamylase activity using glucose oxidase

(5%) and amylose (5%) were hydrolyzed to a lesser degree. The lower affinity of the enzyme for the low molecular weight substrates is consistent with the properties of a true glucoamylase. Furthermore, pullulan, α- and *β*-cyclodextrin, sucrose, *p*-nitrophenyl α-D-maltoside, methyl-α-D-glucopyranoside, and trehalose did not serve as substrates for the purified glucoamylase (data not shown).

The classification of the *P. variotii* enzyme as a glucoamylase was based on the products of starch hydrolysis (Fig. 3). After reaction times varying between 0.5 and 24 h, only glucose was detected as a product of the activity of the pool II enzyme against starch. These results confirm the exo-amylolytic character for the enzyme in pool II, which was therefore classified as a glucoamylase. Besides, the enzyme did not hydrolyze methyl-α-D-glucopyranoside, which is a specific substrate for α-glucosidase [26].

The in vitro influence of glucose is important, considering that its accumulation can inhibit the glucoamylases and α-amylases which take part of the starch saccharification

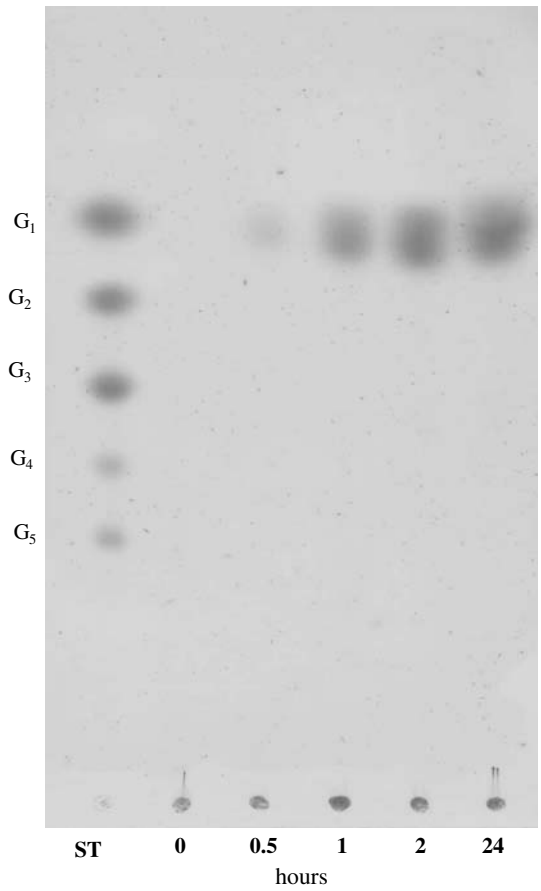


Fig. 3 Thin-layer chromatography of the reaction products of soluble starch hydrolyzed by the purified glucoamylase. Hydrolysis times were 0, 0.5, 1, 2 and 24 h. Standards (*St*) were a mixture of 1 mg ml^{-1} of glucose (G_1), maltose (G_2), maltotriose (G_3), maltotetraose (G_4) and maltopentaose (G_5)

and liquefaction process. The purified glucoamylase was assayed with 1% starch substrate in the absence or presence of glucose at a concentration of 0.5 M, and residual starch was assayed by the iodine method. The rate of starch hydrolysis was around 20–30% lower in the presence of glucose (data not shown), and the low sensitivity to glucose inhibition of the glucoamylase from *P. variotii* is an important property, since the insensibility of the enzyme to the end product is rare among fungal glucoamylases (for example, the glucoamylase of *S. thermophilum*) [17].

Effect of temperature and pH on activity and stability

Studies of the effect of temperature and pH on the enzyme activity were carried out utilizing 100 mM sodium acetate buffer, pH 5.0 over the temperature range of 30–70°C and McIlvaine buffer in the range of pH 2.5–8.0, respectively. Optimum temperature and pH were estimated to be 55 °C and 5.0, respectively (Fig. 4a, b). Similar values of optima temperature and pH were previously reported for the glucoamylase of *Humicola grisea* [27], and the glucoamylases of *Rhizomucor pusillus* [28] and *Schwanniomyces alluvius* [29]. Thermal inactivation was investigated by incubating the enzyme in the presence or absence of starch. In the absence of starch the purified enzyme was stable for 1 h at 50 °C, and decayed with half-lives of approximately 60 min at 55 °C and 45 min at 60 °C (Fig. 5a). Glucoamylases have in general pH optima between 3.5 and 6, and they are thermostable only at temperatures below 60 °C [30]. These results show that the enzyme presented a high level of stability when compared with some glucoamylases of *A. niger* [31–33].

The presence of starch during the heat treatment at 60 °C protected the glucoamylase activity from inactivation, where around 30% of the activity was retained after 2 h of heat treatment (Fig. 5b). Similar results were reported for other fungal glucoamylases [25, 27, 34], and might be due to an increased stability of the enzyme–substrate complex [35]. Another alternative explication, suggested by Fagerstrom et al. [30], is that the high concentration of nonreducing ends of starch will act to protect the enzyme against thermal denaturation. In respect of pH stability, the enzyme was stable over the period of 1 h in the pH range of 2.5–8.0 at 4 °C (Fig. 5c).

Influence of metal ions, β-mercaptoethanol and EDTA

The enzyme activity was significantly activated by metal ions, principally 5 mM Mn^{2+} that increased the activity by 80.7% (Table 2). Similar results have also been reported for glucoamylase from *Neurospora crassa* [34] and *T. lanuginosus* [23]. In contrast, glucoamylase activity was inhibited by several metal ions, such as (1–2.5 mM) Al^{3+}, (5–10 mM)

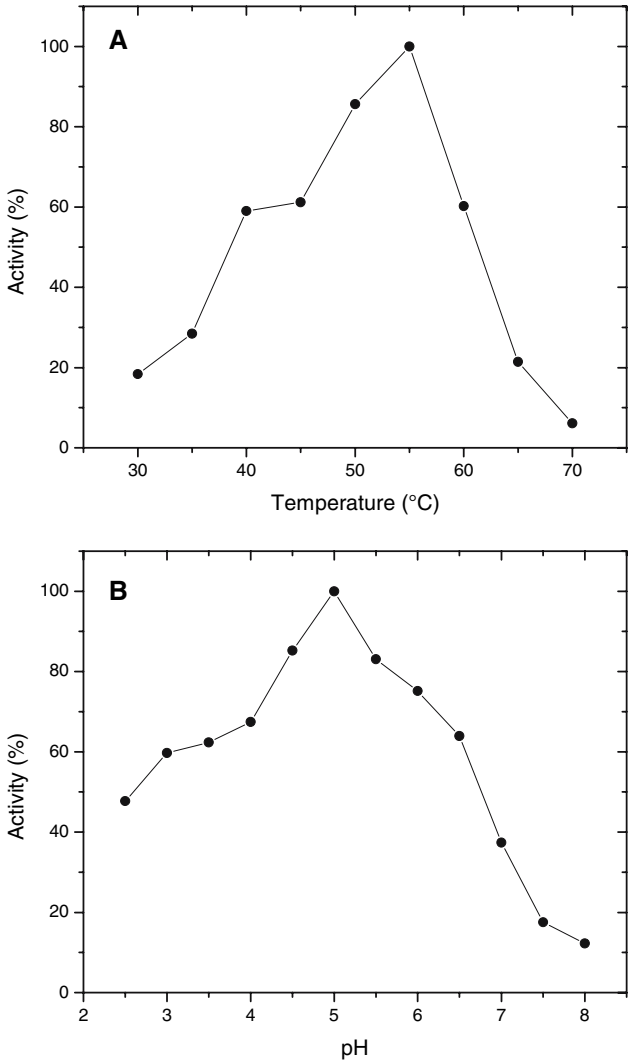
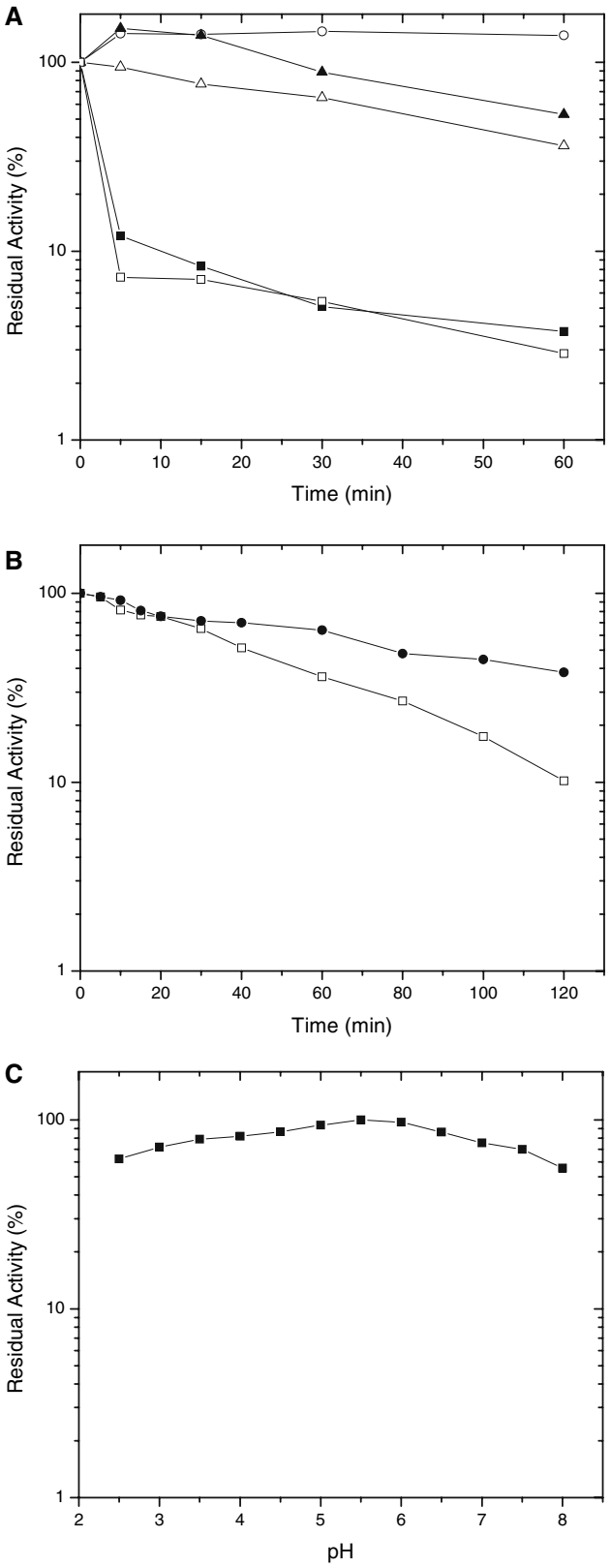


Fig. 4 Influence of temperature (**a**) and pH (**b**) on the glucoamylase activity. The enzyme was assayed at several temperatures and various pH in as described in "Materials and methods". The glucoamylase activity was estimated by the DNS method using starch as substrate

Zn^{2+} and Cu^{2+}, (1–10 mM) Pb^{2+} and Fe^{3+}. This inhibitory effect has also been reported for other glucoamylases [27]. The effect of salts of various cations, such as Co^{2+}, Ca^{2+}, Ba^{2+}, Mg^{2+} and Hg^{2+}, and of other reagents, such as NaCl, NaBr, EDTA, KH_2PO_4, NaH_2PO_4, NH_4F, NH_4Cl, and KCl was examined on the activity of the glucoamylase, but the enzyme was only slightly affected. The fact that Ca^{2+} ions did not inhibit the *P. variotii* glucoamylase is highly significant for the potential industrial use of the enzyme, since calcium is required by the α-amylase used in the liquefaction process, and is detrimental to the activity of the glucoamylase normally used in such process. The results obtained with β-mercaptoethanol suggested that the catalytic activity is unaffected by protein disulphide bond reduction, probably because they are not present in the catalytic site. That is important to emphasize that 10 mM of



◀ **Fig. 5** Thermal inactivation of the glucoamylase activity was carried out at 50 °C (*open circle*), 55 °C (*filled triange*), 60 °C (*open triangle*), 65°C (*filled square*) or at 70 °C (*open square*) in the absence of substrate (**a**) and at 60°C in the presence of 1% starch (*filled circle*) and the absence of starch (*open square*) (**b**). Stability at pH for the glucoamylase (**c**). The glucoamylase activity was estimated by the DNS method using starch as substrate

Table 2 Effect of metal ions, EDTA and β-mercaptoethanol on the activity of the glucoamylase produced from *P. variotii*

Compound	Relative activity (%)			
	1 mM	2.5 mM	5 mM	10 mM
None	100	100	100	100
$AlCl_3$	33.3	68.4	98.1	94.7
$BaCl_2$	118.3	120.1	113.0	101.0
β-mercaptoethanol	120.1	ND	ND	139.8
$CaCl_2$	99.8	114.9	121.5	109.6
$CoCl_2$	122.0	128.8	122.2	116.4
$CuCl_2$	92.8	91.8	85.7	76.5
EDTA	99.0	100.5	115.0	103.8
$FeCl_3$	35.2	31.2	20.8	9.0
$HgCl_2$	107.7	103.8	95.4	79.2
KCl	83.0	91.1	102.4	102.1
KH_2PO_4	104.1	114.9	111.4	98.6
$MgCl_2$	116.9	110.9	108.0	101.0
$MnCl_2$	151.7	157.9	180.7	166.2
NaCl	106.3	114.7	120.1	109.0
NaBr	96.8	104.1	98.6	98.1
NaH_2PO_4	113.3	110.2	103.8	85.3
NH_4Cl	101.5	106.1	98.5	87.7
NH_4F	107.9	108.0	93.7	84.6
$Pb(C_2H_3O_2)_2$	38.2	22.4	11.6	8.5
$ZnCl_2$	98.0	89.1	72.2	63.3

ND not determined

Table 3 Kinetic analyses of the glucoamylase produced by *P. variotii*

Substrate	K_m (mg ml^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mg^{-1} ml^{-1})
Starch (from Reagen)	3.8	41.7	11.0
Starch (from Sigma)	4.1	36.6	8.9
Amylopectin	2.0	34.1	17.1
Amylose	2.5	15.5	6.2
Glycogen	1.7	22.1	13.0

β-mercaptoethanol activated the glucoamylase in 40%. A similar result was previously reported for xylanase with 10 mM β-mercaptoethanol by Sandrim et al. [36].

Kinetic parameter

In order to find out which substrate was hydrolyzed most efficiently, the apparent kinetic parameters K_m and K_{cat} of the purified enzyme were determined using starch, amylopectin, amylose and glycogen. The K_{cat}/K_m values obtained

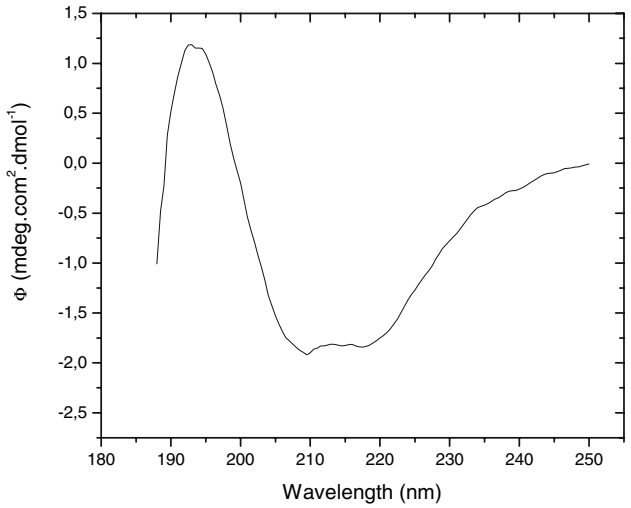


Fig. 6 Far-UV CD spectra of native glucoamylase purified from *P. variotii*. The protein concentration used in the CD was 0.76 mg ml^{-1}

(Table 3) demonstrated that the preferred substrates were amylopectin, glycogen, starch and amylose, respectively. The K_m of glucoamylase from *P. variotti* against starch is lower than that reported to *A. oryzae* [37] and *S. alluvius* [29].

Circular dichroism spectroscopy and amino acid sequence analysis

In order to evaluate possible detrimental effects of the purification procedure on the secondary structure, the quality of the purified glucoamylase was assessed using two complementary spectroscopic techniques. Figure 6 presents the far ultraviolet circular dichroism spectra of the native protein purified from *P. variotti* showing a negative minimum at 209 and 220 nm and a maximum at 193 nm. These features of the spectrum indicate that the purified protein present high α-helix content and indicates that the refolding and purifications protocols yielded protein with native-like secondary structure.

A peptide from the purified enzyme showed the amino acid sequence VVTDSFR, which is identical with glucoamylases purified from *Talaromyces emersonii* and with the precursor of the glucoamylase from *A. oryzae* (Fig. 7), and is highly similar to the VVDSFR sequence from *A. awamori*. The three-dimensional structure of the *A. awamori* glucoamylase has been determined by x-ray crystallography (PDB code 1AGM) [38], and is a protein containing a high proportion (51%) of residues in a α-helical conformation. This observation supports the suggestion that the glucoamylases from *A. awamori* and *P. variotii* are homologous.

Fig. 7 Aminoacids sequence of glucoamylase from *Talaromyces emersonii* and the precursor of the glucoamylase from *Aspergillus oryzae*; showing underlined the points of homology between the same and the glucoamylase from *P. variotii*

[VVTDSFR] glucoamylase of *Paecilomyces variotii*

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T. emersonii  MASLVAGALC ILGLTPAAFA RAPVAARATG SLDSFLATET PIALQGVLNN
A. oryzae     MVSFSS-CLR ALALGSSVLA VQPVLRQATG -LDTWLSTEA NFSRQAILNN

T. emersonii  IGPNGADVAG ASAGIVVASP SRSDPNYFYS WTRDAALTAK YLVDAFIAGN
A. oryzae     IGADGQSAQG ASPGVVIASP SKSDPDYFYT WTRDSGLVMK TLVDLFRGGD

T. emersonii  KDLEQTIQQY ISAQAKVQTI SNPSGDLSTG GLGEPKFNVN ETAFTGPWGR
A. oryzae     ADLLPIIEEF ISSQARIQGI SNPSGALSSG GLGEPKFNVD ETAFTGAWGR

T. emersonii  PQRDGPALRA TALIAYANYL IDNGEASTAD EIIWPIVQND LSYITQYWNS
A. oryzae     PQRDGPALRA TAMISFGEWL VENSHTSIAT DLVWPVVRND LSYVAQYWSQ

T. emersonii  STFDLWEEVE GSSFFTTAVQ HRALVEGNAL ATRLNHTCSN CVSQAPQVLC
A. oryzae     SGFDLWEEVQ GTSFFTVAVS HRALVEGSSF AKTVGSSCPY CDSQAPQVRC

T. emersonii  FLQSYWTGSY VLANFGGSGR SGKDVNSILG SIHTFDPAGG CDDSTFQPCS
A. oryzae     YLQSFWTGSY IQANFGG-GR SGKDINTVLG SIHTFDPQAT CDDATFQPCS

T. emersonii  ARALANHKVV TDSFRSIYAI NSGIAEGSAV AVGRYPEDVY QGGNPWYLAT
A. oryzae     ARALANHKVV TDSFRSIYAI NSGRAENQAV AVGRYPEDSY YNGNPWFLTT

T. emersonii  AAAAEQLYDA IYQWKKIGSI SITDVSLPFF QDIYPSAAVG TYNSGSTTFN
A. oryzae     LAAAEQLYDA LYQWDKIGSL AITDVSLPFF KALYSSAATG TYASSTTVYK

T. emersonii  DIISAVQTYG DGYLSIVEKY TPSDGSLTEQ FSRTDGTPLS ASALTWSYAS
A. oryzae     DIVSAVKAYA DGYVQIVQTY AASTGSMAEQ YTKTDGSQTS ARDLTWSYAA

T. emersonii  LLTASARRQS VVPASWGESS ASSVPAVCSA TSATGPYSTA TNTVWPSSGS
A. oryzae     LLTANNRRNA VVPAPWGETA ATSIPSACST TSASGTYSSV VITSWPTISG

T. emersonii  GSSTTTSSAP CTTPTSVAVT FDEIVSTSYG ETIYLAGSIP ELGNWSTASA
A. oryzae     YPGAPDS--P CQVPTTVSVT FAVKATTVYG ESIKIVGSIS QLGSWNPSSA

T. emersonii  IPLRADAYTN SNPLWYVTVN LPPGTSFEYK FFKNQTDGTI VWEDDPNRSY
A. oryzae     TALNADSYTT DNPLWTGTIN LPAGQSFEYK FIRVQN-GAV TWESDPNRKY

T. emersonii  TVPAYCGQTT AILDDSWQ
A. oryzae     TVPSTCGVKS AVQSDVWR
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Thorsen et al. [3] observed through N-terminal sequences of the purified glucoamylase from *T. lanuginosus*, that it turned out to be closely related to *T. emersonii* glucoamylase (presenting 60% identity according to Nielsen et al. [39]) as they grouped together in the phylogenetic tree with aspergilli group as nearest neighbor. A similar proposal was reported by Coutinho and Reilly [40]. This suggests that *T. lanuginosus*, *T. emersonni* and *P. variotii* are closely related on protein level.

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