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Full Length Research Paper

Glucoamylase isoform (GAII) purified from a thermophilic fungus *Scytalidium thermophilum* 15.8 with biotechnological potential

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***Scytalidium thermophilum* 15.8 produced two extracellular glucoamylases. Using a DEAE-Cellulose chromatographic column glucoamylases form II (GAII) was separated and purified from glucoamylases form I (GAI) that was previously purified and characterised (Cereia et al., 2000) when the filtrate of the culture medium was applied to a DEAE-Cellulose chromatographic column. GAII bound to the DEAE-Cellulose and was eluted with a NaCl gradient, while GAI did not bind to the resin. GAII presented electrophoretic homogeneity in 6% denaturing and non-denaturing PAGE, separately, with a molecular mass of 83 kDa, after the second round DEAE-Cellulose purification step. The enzyme pI was 7.2. Optima pH and activity temperature were 5.5 and 55ºC respectively for starch and maltose as substrates, with a termostability of 2.5 min at 60ºC. Enzymatic activities were activated by 1 mM Na^{+}, Mn^{2+} and Mg^{2+} or 10 mM NH^{4+}, Ba^{2+} and Mg^{2+}. The carbohydrate content was 10%. The kinetic parameters Km and Vmax with starch and maltose as substrate were 0.2 and 1.5 mg/ml, and 22.3 and 4.39 U/mg of protein, respectively. The amino acid sequence of GAII had 92% homology with the glucoamylase of *Humicola grisea* var. *thermoidea* after 13 cycles. Generally, GAII had different properties compared with GAI (Cereia et al., 2000).**

Key words: Glucoamylase, *Scytalidium thermophilum*, starch hydrolysis, amylase, fungus.

INTRODUCTION

Enzymes responsible for degradation of starch and related saccharides are produced either by prokaryotic or eukaryotic organisms. Hydrolyses of starch involves four groups of enzymes: endo- and exo-amylases, debranching enzymes attacking mainly the α-1, 6 linkages and cyclodextrin glycosyltransferase (Harváthova et al., 2000; Aiyer, 2005). α-Amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3) are the endo- and exo-amylases most studied respectively. These enzymes represent about 25-33% of the world enzyme market, second after proteases. Their main applications are in the production of high glucose syrup (HGS) from starch and in the production of high fructose corn syrup (HFCS) (Nguyen et al., 2002). Glucose from starch is used in the production of citric acid, glutamic acid, lactic acid, lysine, glucitol, ethanol and crystalline glucose (Pazur et al., 1990). Glucoamylase is an exo-enzyme that liberates β-D-glucose from the non-reducing chain ends of amylose, amylopectin and glycogen by consecutively hydrolyzing α-1,4; α-1,6 and rare α-1,3 linkages. Usually these enzymes are produced from several microorganisms, but mainly *Aspergillus niger* and *Rhizopus oryzae* are used in biotechnology applications (Coutinho and Reilly, 1997). Thermostable enzymes from microorganisms have advantages in industrial biotechnological applications (Pandey et al., 2000) and thermostable glucoamylases have been studied in the

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latter regard (Chen et al., 2005). In this context, we have been studying amylases produced by thermophilic filamentous fungi such as *Humicola grisea* (Tosi et al., 1993) and *Scytalidium thermophilum* (Cereia et al., 2000; Aquino et al., 2001). According to Straatsma and Sanson (1993), there are different varieties of *S. thermophilum*. In this work an extracellular glucoamylase (GAII) from *S. thermophilum* 15.8 was purified and characterised. Characteristics of GAII were compared with the extracellular glucoamylase (GAI) that was previously purified and characterised from the same fungus (Cereia et al., 2000).

MATERIALS AND METHODS

Microorganism and culture conditions

The fungus *S. thermophilum* (15.8) [= CBS 672.88 = ATCC 66938] was kept at 45ºC, in slants of solid 4% oatmeal baby food (Quaker) medium. Conidia from 10 day-old cultures were inoculated into 500 ml Erlenmeyer flasks containing 100 ml liquid medium according to Cereia et al. (2000). The cultures were incubated at 45ºC without agitation. After seven days, the cultures were harvested by filtration and the filtrate was used as a source of extracellular glucoamylase.

Determination of glucoamylase activity

The activity of glucoamylase was determined at 60ºC, in a reaction mixture containing 0.2 ml of diluted enzyme and 0.2 ml of 1% starch in 100 mM sodium acetate buffer, pH 5.5. The amount of reducing sugar released was estimated by glucose-oxidase procedure according to Bergmayer and Bernt (1974). Maltase activity was assayed as described above using 1.7% maltose as substrate. Glucose was used as a standard. An enzyme unit is the amount of enzyme that produces 1 μmol of glucose/min.

Quantification of protein and carbohydrate

Protein was determined by methodology described by Lowry et al. (1951) using bovine serum albumin as a standard. The carbohydrate content of the purified protein was determined by the phenol sulfuric method as described by Dubois et al. (1956) using D-mannose as a standard.

Purification on DEAE-Cellulose

The crude filtrate with glucoamylase activity was dialysed overnight against 10 mM Tris-HCl buffer, pH 7.5 and applied to a DEAE-Cellulose (1.9 x 20 cm) chromatographic column, equilibrated with the same buffer and eluted with a linear gradient of NaCl (0-500 mM). Fractions of 10 ml were collected, at a flow rate of 28 ml/h, at 4ºC. The fractions which adsorved to DEAE-Cellulose were pooled and dialysed in the same buffer and subjected to the same procedure described above. The fractions with glucoamylase activity collected after the latter purification step were pooled and termed GAII.

Electrophoresis

Non-denaturating PAGE (6%) electrophoresis was carried out at pH 4.5 according to Reisfield et al. (1962) method for alkaline proteins, and SDS-PAGE (6%) according to Laemmli (1970). Molecular markers were: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase-b (97 kDa), bovine serum albumin (66 kDa), ovoalbumin (45 kDa), and carbonic anydrase (29 kDa). Protein was stained with silver (Blum et al., 1987). Isoelectric focusing was carried out according to the O'Farrel et al. (1977) method using Pharmalite pH 3-10.

Amino acid sequencing and western blotting

For the amino acid sequencing, the protein was electroblotted onto a PVDF membrane after SDS-PAGE. The protein band was subjected to automatic Edman degradation. The sequencing was carried out in a procise 491 protein sequencer (PE-Applied Biosystems, Foster City) using gas phase chemistry with on-line identification of phenylthiohydantoin derivatives. A 10 picomol standard was used to quantify PTH-amino acids. The derived amino acid sequence was compared with the amino acid sequence of *Humicola grisea* var. *thermoidea* glucoamylase, access number M89475 from the GenBank for homology.

Antibody anti-glucoamylase was produced from New Zealand rabbits (Hames and Jurd, 1985) and quantified using ELISA (Enzyme linked immunosorbent assay) at 490 nm (Wilson and Nakane, 1978). This antibody was used for immunoassays and Western blotting using the procedure as described by Towbin et al. (1979).

Chromatography of hydrolysis product

The hydrolysis products of glucoamylase activity from soluble starch were studied using thin-layer chromatography on silica gel (DC-Alufolien Kieselgel 60, Merk). Buthanol/ethanol/water (5:3:2, V/V) mix was used as a mobile phase and the sugars were detected with orcinol (Fontana et al., 1988).

RESULTS AND DISCUSSION

Purification of glucoamylase GAII

Glucoamylase activity was obtained as two fractions, form I (did not adsorved to the resin) (GAI) and form II (GAII). GAII was retained in the resin and was eluted with a linear gradient of NaCl (52 mM) prepared in the same buffer (Figure 1A). GAII corresponded to approximately 30% of total glucoamylase produced by the fungus. However, there were some protein contaminants and another round of dialysation and chromatography with a new DEAE-Cellulose column successfully purified GAII (Figure 1B). Curiously, GAII did not interact with the resin as previously but the contaminant proteins were eliminated and GAII purified (Table 1).

Physicochemical properties

Glucoamylase GAII was purified to apparent homogeneity on SDS-PAGE (Figure 2A) and PAGE (Figure 2B). The molecular mass was estimated at approximately 83 kDa and differed with only 3 kDa from

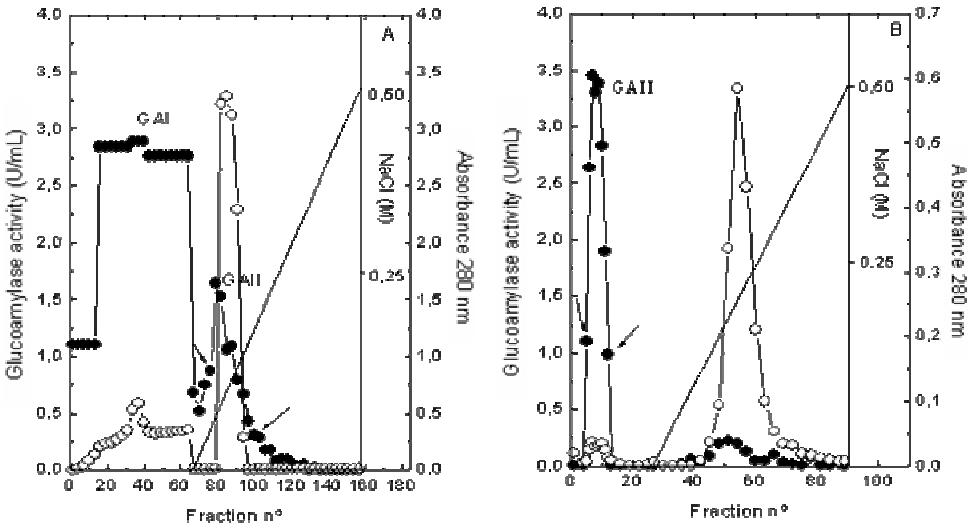

Figure 1. Chromatographic profile on DEAE-Cellulose of extracellular crude extract (A) and GAII (B). The column (1.9 x 20.0 cm) was equilibrated with 10 mM Tris-HCl buffer, pH 7.5 and eluted with a linear gradient of NaCl (0-500 mM), in the same buffer, at a flow rate of 28.0 ml/h. Ten ml fractions were collected. Symbols: (•) glucoamylase activity; (o) absorbance 280 nm. The arrows indicate the fractions pooled.

Table 1. Purification of GAII produced by *S. thermophilum* 15.8.

Steps	Volume (mL)	Protein (Total mg)	Activity (Total U)	Specific activity (U/mg)	Yield (%)	Purification (x)
Crude Extract	780	303.4	2828.3	9.3	100	1
DEAE-Cellulose I	164	56.6	682.2	12.1	24.1	1.3
DEAE-Cellulose II	72	8.13	284.8	35	10	3.8

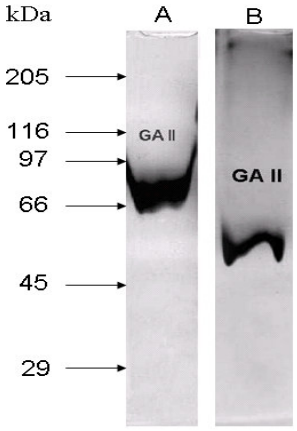

Figure 2. Denaturing gel electrophoresis SDS-PAGE 6% (A) and non-denaturing PAGE 6% (B) of purified glucoamylase (GAII) stained with silver. Molecular markers used: myosin (205 kDa), β-galactosidase (116 kDa), phosphorilase b (97 kDa), BSA (66 kDa), albumin egg (45 kDa), carbonic anhydrase (29 kDa).

GAI (Cereia et al., 2000). Moreover, GAII, a glycoprotein, had a 10% carbohydrate content compared with the 25.5% carbohydrate content of GAI (Cereia et al., 2000). The carbohydrate content for GAII was approximately similar to that reported for *Chaetomium thermophilum* glucoamylase (11.4%) (Chen et al., 2005). The molecular mass of both GAI and GAII falls within the glucoamylase molecular mass range of 26 kDa to 112 kDa (James and Lee, 1997; Aquino et al., 2001; Chen et al., 2005).

GAII hydrolysis of starch produced only glucose after 30 min as shown by thin-layer chromatography (TLC) (Figure 3), indicative of glucoamylase (EC 3.2.1.3) activity and consistent with results reported for the extracellular glucoamylase of *C. thermophilum* (Chen et al., 2005).

Influence of temperature and pH on glucoamylase activity

Optimum pH of GAII activity was 5.5 for both starch and maltose substrates (Figure 4A), while for GAI it was 6.5 and 5.0 on the same substrates, respectively (Cereia et al., 2000). *T. lanuginosus* glucoamylase was effectiveness in an optimum pH range from 4.4 to 5.6 (Nguyen et al., 2002); *Humicola lanuginosa* glucoamylase at pH 4.9

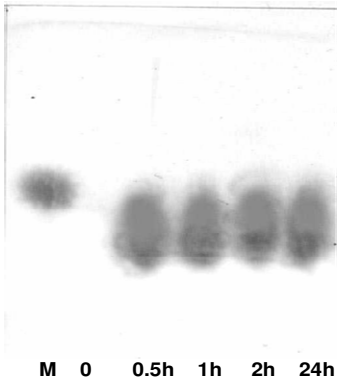

Figure 3. Analysis of hydrolysis products obtained using purified glucoamylase activity of *S. thermophilum* (GAII) on soluble starch as substrate in thin layer chromatography (TLC). M: markers.

Table 2. Influence of several compounds on GAII activity.

Compounds	Starch		Maltose	
	1mM	10mM	1mM	10mM
Control	100	100	100	100
$BaCl_2$	97.8	98.6	85.2	106.8
β -mercaptoethanol	0	Nd	0	Nd
$CaCl_2$	79	93.9	81.4	96.6
$CuCl_2$	108.2	71.7	56	31.8
EDTA	102	93.4	77.8	91.4
$HgCl_2$	80.1	9	53.4	10.2
$MgCl_2$	108.9	98.1	74	100.7
$MnCl_2$	104.4	89.1	73.1	93.4
NaCl	103.6	93.3	79.6	90.5
NH_4Cl	91.7	95.9	75.8	106.4
$ZnCl_2$	99.9	98.7	58.9	79.5

nd – Not determined; control is assay without addition of metallic ion.

Table 3. Kinetic parameters of GAII.

Parameters	Starch	Maltose
Km (mg/ml)	0.2	1.5
Vmax (µmol/min/mg protein)	22.3	4.39
Vmax/Km	111.5	2.93

These parameters were determined using 0.05-5 mg/ml of starch and 0.2-68 mg/ml of maltose.

and 6.6 (Taylor et al., 1978) and *C. thermophilum* glucoamylase at 4.0 (Chen et al., 2005). The optimum pH range of glucoamylase varies from 4.5 to 5.5 with stability at pH 7.0 (Taylor et al., 1978). The pI for GAII was 7.2 compared with 8.4 for GAI and 8.4 for *H. grisea* glucoamylase (Campos and Félix, 1995).

According to James and Lee (1997), the range of glucoamylases pI is between 3.7 and 7.4; however the results from this study as well as results from others (Cereia et al., 2000; Campos and Félix, 1995) indicate that the upper limit of the pI range for glucoamylases needs to be revised to 8.4.

Optimum temperature of glucoamylase activity was 60ºC and 55ºC on starch and maltose substrates (Figure 4B), respectively, with a half-time life (t_{50}) of 3.2 min at 60ºC. At higher temperatures than 60ºC and 55ºC, the activities decreased. The GAII t_{50} was enhanced to approximately 25 min when the enzyme was incubated at 60ºC in the presence of several substances as possible protectors against thermoinactivation, and starch (Figure 4C) or maltose (Figure 4D), as substrates. As can be seen, sorbitol, starch, and to some extent pectin and glycerol protected GAII activity against thermoinactivation. These observations are in agreement with those of Fargeström et al. (1990) and Tosi et al. (1993). At room temperature the enzyme was stable for 92 h for both substrates. Similar optimum temperature was observed by Campos and Félix (1995) for glucoamylase of *Humicola grisea* and for two glucoamylases from *Lipomyces kononenkoae* CBS 5608 (Chun et al., 1995). GAI showed the same optima temperature of activity with t_{50} of 2.5 min (Cereia et al., 2000) and *T. lanuginosus* with optima temperature of 70ºC (Nguyen et al., 2002).

Influence of metal ions on enzyme activity

According to Table 2, glucoamylase activity was enhanced by 1 mM Mg^{2+} (8.9%), Cu^{2+} (8.2%), Mn^{2+} (4.4%), Na^{+} (3.6%) and EDTA (2%), and inhibited by 21% by Ca^{2+} and 20% by Hg^{2+}, when starch was used as a substrate. At higher concentrations (10 mM) the activities were inhibited, especially by Hg^{2+} with an activity inhibition of 91%. These results are generally consistent with results reported for other glucoamylases (Cereia et al., 2000; Nguyen et al., 2002; Chen et al., 2005). When metal ions effect on activity of GAII was conducted using maltose as substrate, no increment of activity was observed with 1 mM ions and the activity levels decreased by 15-46%. 10 mM Ba^{2+} and NH_4^{+} enhanced the glucoamylase activity by 6% and Hg^{2+} inhibited 90% of activity. β-Mercaptoethanol, a reducing agent that disrupts disulfide bonds, inhibited glucoamylase activity completely on both substrates, thus indicating the presence of such bonds in the enzyme molecule. EDTA, an ion chelater, was not effective on GAII activity suggesting that this enzyme does not require metallic ions in it active site for its activity.

Kinetic parameters

Table 3 summarizes the data of the kinetic parameters of

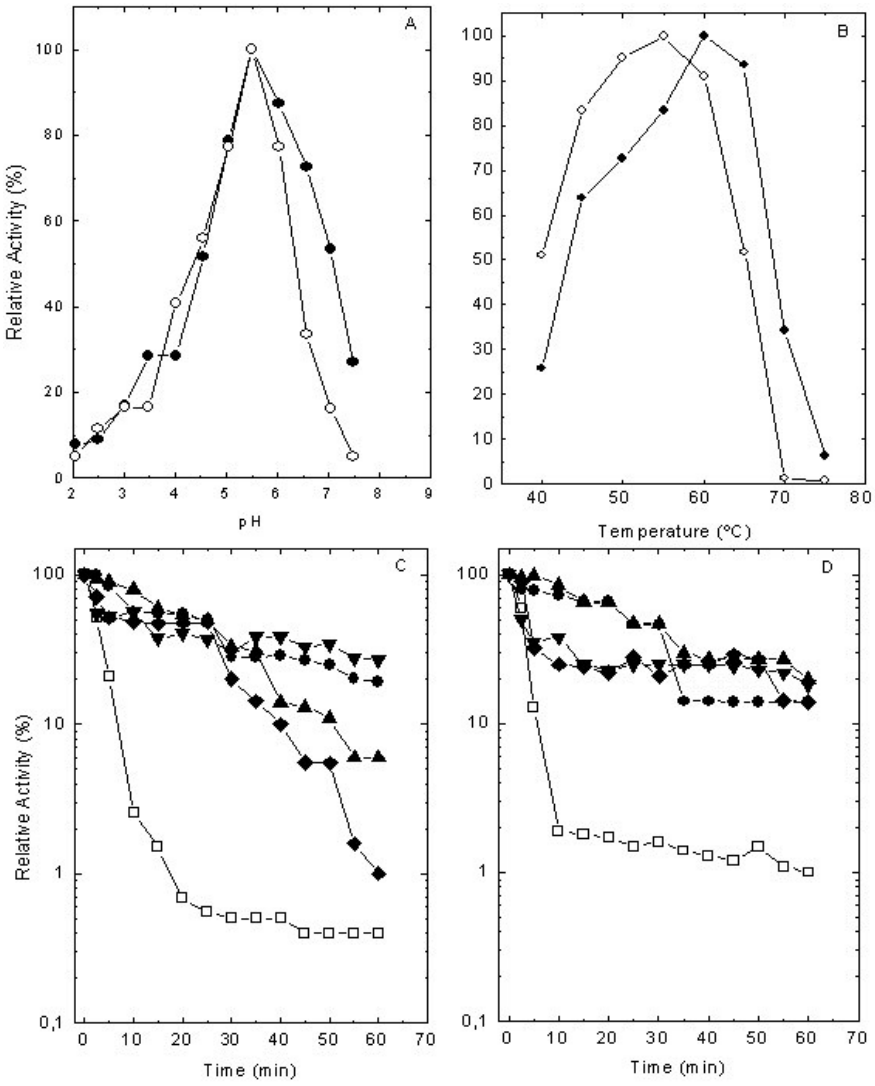

Figure 4. Optimum pH (A) and optimal temperature (B) of GAII activity on starch (•) and maltose (o). Thermal stability of GAII on starch (C) and maltose (D) as substrates in the presence of several protector agents: (•) 0.5% starch; (▲) 0.5% pectin; (▼) 25% sorbitol; (▲) 5% glycerol; (▼) without protector.

glucoamylase GAII using starch and maltose as substrates. This enzyme showed Michaelian behavior with major affinity for starch (0.2 mg/ml), Vmax of 22.3 µmol/min/mg protein, and a catalytic efficiency of 111.5 for starch and 2.93 for maltose. These kinetic values for GAII were similar to the other glucoamylases reported in literature. For example, *Humicola grisea* glucoamylase had a Km of 0.14 mg/ml and Vmax of 0.21 µmol glucose/min/µg (Campos and Félix, 1995), and GAI had a Km and Vmax of 0.28 mg/ml, 67.2 µmol/min/mg, respectively (Cereia et al., 2000). The Km values on maltose and starch of *T. lanuginosus* glucoamylase were 6.5 mM and 0.8 mg/ml, respectively (Nguyen et al., 2002).

Amino acid sequencing and western blotting

The sequence data of the amino terminal residues of GAII (Table 4) showed 92% homology with glucoamylase of thermophilic fungus *Humicola grisea* var. *thermoidea* (“GenBank” access number M89475). When compared to the sequence of GAI, only one amino acid was different at position 42 (serine in GAI and glutamine in GAII). No signal peptide was found. 13-14 amino acids

Table 4. Amino acid sequence data of GAII.

Glucoamylase	Sequence
Humicola grisea	37 intek**p**iawnkllan 51
GAI *S. thermophilum*	37 intek**s**iawnkllan 51
GAII *S. thermophilum*	39 tek**q**iawnkllan 51

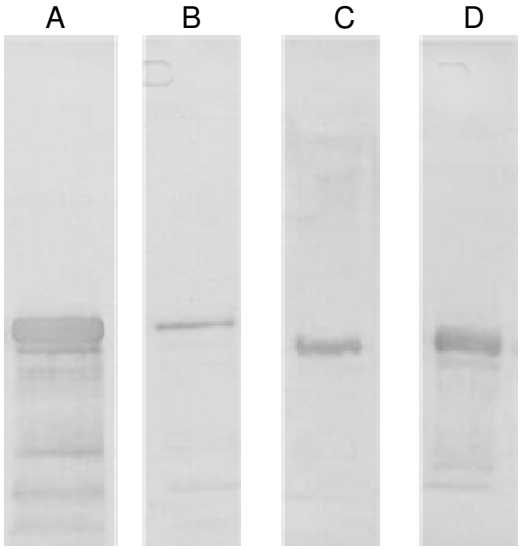

Figure 5. Western blotting with antibodies anti-GAI and anti-GAII is showing homology between the two enzymes. (A) anti-GAI x antigen GAI; (B) anti-GAII x antigen GAII; (C) anti-GAII x antigen GAI; (D) anti-GAI x antigen GAII.

were sequenced, and only one residue was different between GAI and GAII (Table 4). According to Chen et al. (2005) fungal glucoamylases are not conserved among species, but the sequenced data obtained for GAI and GAII of *S. thermophilum* 15.8, and glucoamylase of *H. grisea*, showed conservation.

Antigen-antibody complex were observed in the immunodiffusion experiments for specific and cross reactions between GAI/anti-GAII and GAII/anti-GAI (data not shown) demonstrating structural homology between GAI and GAII. This homology was confirmed by Western blotting (Figure 5). This fact is according to what was reported by Coutinho and Reilly (1997), who proposed that glucoamylases showed homology because derived from the same ancestral bacterial gene.

The sequence-based classification of glycoside hydrolases show that α-amylases, β-amylases and glucoamylases are found in families 13, 14 and 15, respectively. Glucoamylase are found in only family 15 whereas α-amylases are found not only in family 13, but in the related families 70 and 77, and in the family 57.

This implies that there is a rather long evolutionary distance among these enzymes (Horváthová et al., 2000).

In conclusion, in spite of some minor differences, GAII and GAI are indeed very similar enzymes, including in their immunological properties, thus it seems unlikely that they are products of different genes. There are some possible explanations to account for the observed differences. For instance, the two forms (GAI and GAII) could be proteolytic products from a single protein, as is demonstrated for a glucoamylase from *Aspergillus niger* (MacKenzie et al., 2000). Second, these two isoforms could represent alternative structural products which arose during the purification process, where part of the starch-binding domain was lost, as was described by Coutinho and Reilly (1997). Third, these isoforms could be consequence of different post-translational processing, for instance different degree of glycosylation (Iwashita et al., 1998). At the present time we cannot offer a conclusive explanation for the origin of these glucoamylase isoforms. However, since there were no significant molecular mass differences the two first possibilities appear unlikely. On the other hand, these two isoforms contained strikingly different amount of carbohydrate, which may be the actual cause of the observed biochemical differences.

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