Comparison of two glucoamylases from *Hormoconis resinae*

RICHARD FAGERSTRÖM,^{1*} ARJA VAINIO,¹ KARI SUORANTA,¹ TIINA PAKULA,¹ NISSE KALKKINEN² and HELENA TORKKELI¹

¹Research Laboratories, Alko Ltd, POB 350, SF-00101, Helsinki, Finland

²Institute of Biotechnology, Helsinki University, Valimotie 7, SF-00380, Helsinki, Finland

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Two extracellular glucoamylases (EC 3.2.1.3), glucoamylase P and glucoamylase S, were purified to homogeneity from the culture medium of *Hormoconis resinae* (ATCC 20495; formerly *Cladosporium resinae*) by a new method. Their apparent molecular masses (71 kDa glucoamylase P; 78 kDa glucoamylase S) and catalytic properties agreed well with those previously reported in the literature. Heat inactivation studies suggested that the high debranching (1,6-glycosidic) activity of glucoamylase P preparations (measured with pullulan) may reside in the same protein molecule as its 1,4-glycosidic activity (measured with soluble starch). Although glucoamylase S had virtually no debranching activity, it cross-reacted with polyclonal antibodies raised against glucoamylase P, and the two enzymes had very similar amino acid compositions. However, peptide mapping and amino-terminal sequencing studies of the peptides showed that the two enzymes have different sequences and must be encoded by different genes.

Introduction

Enzymes that degrade poly- and oligosaccharides are commercially important for the food and fermentation industries. Glucoamylases (1,4-glucan glucohydrolases; EC 3.2.1.3) release glucose units sequentially from the nonreducing end of polymeric carbohydrates. The ability of glucoamylases to hydrolyse 1,6-glycosidic bonds is called debranching activity and is of great importance in industrial processes requiring complete degradation of starch to glucose.

Glucoamylases are produced by a wide variety of micro-organisms (Manjunath *et al.*, 1983). Most of the known glucoamylases have very low activity towards 1,6-glycosidic bonds. The analytical measurement of debranching activity is not straightforward, since the activity of glucoamylases on substrates containing only 1,6-glycosidic bonds is usually quite low. To avoid this difficulty, substrates like pullulan (every third bond 1,6-glycosidic) are often used to estimate 1,6-hydrolysing activity. Many fungal glucoamylases have been reported to exist in multiple forms (Lineback & Bauman, 1970; Tsuboi *et al.*, 1974; Yamasaki *et al.*, 1977; Takahashi *et al.*, 1978, 1981). In order to clone the appropriate gene it is essential to know if these multiple forms are coded by different genes, or if they are results of post-translational

modification and/or different mRNA splicing (Haya-shida *et al.*, 1988; Ono *et al.*, 1988). For example, the two glucoamylases G1 and G2 produced by *Aspergillus niger* have identical amino-terminal sequences and peptide profiles but different carboxy-terminal sequences. According to Boel *et al.* (1984) these two forms of the enzyme are products of two differently spliced mRNA molecules coded by the same gene, whereas Svensson *et al.* (1986) have pointed out that proteolytic modification also affects the multiplicity of this enzyme.

The fungus *Hormoconis resinae* (ATCC 20495; formerly *Cladosporium resinae*) has been reported to produce two forms of glucoamylase which exhibit different substrate specificities and have different molecular masses and pI values (McCleary & Anderson, 1980). The smaller glucoamylase P has a very high debranching activity, while the larger glucoamylase S has virtually no debranching activity. In the present work, we examined whether the 1,4- and 1,6-glycosidic activities of glucoamylase P are both functions of a single protein, and, secondly, whether glucoamylase P and S are products of a single gene or of two distinct genes.

Methods

Culture conditions. *H. resinae* (ATCC 20495) was stored as a suspension of spores in 0.1% SDS and 10% (v/v) glycerol at –80 °C. A 1 ml sample of this suspension was incubated at 30 °C for 2 d in 50 ml of

Abbreviations: FMOC, 9-fluorenylmethoxycarbonyl; OPA, o-phthalaldehyde; PTH, phenylthiohydantoin.

a medium containing 2% (w/v) proteose peptone, 1% (w/v) yeast extract and 2% (w/v) glucose. The cells were collected by centrifugation at 1000 g for 15 min, washed once with 5 ml sterile water and suspended in sterile water. The suspension was inoculated into a medium containing 1% (w/v) proteose peptone and 0.2% NH_4Cl as nitrogen sources, 0.1% glucose and 2% (w/v) starch as carbon sources and 0.2 M-potassium phosphate, pH 5.5. One litre of medium was supplemented with 100 μg CaCl_2 , 20 μg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 50 μg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μg MnCl_2 , 10 μg ZnSO_4 , 100 μg CuSO_4 , 10 μg CoCl_2 , 100 μg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 12.5 mg tetracycline and 8.0 mg chloramphenicol. The inoculated medium was incubated at 30 °C in Erlenmeyer flasks filled to 10–15% of capacity and aerated by shaking (270 r.p.m.) at 30 °C. Samples of 2 ml were taken from the cultures every 4–7 d to assay their ability to degrade starch and pullulan. Every 7 d, starch (8 g l^{-1}) was added to the culture. Growth was prolonged to between 24 and 27 d, depending on the level of glucoamylase activity.

Activity measurements. The activity measurements were done as initial velocity measurements. Samples of 50 μl were incubated at 30 °C with 0.5% (w/v) substrate in 0.1 M-sodium acetate pH 4.3 containing 200 μg bovine serum albumin ml^{-1} (Sigma; essentially fatty acid and globulin free) in a final volume of 1.0 ml. Samples were withdrawn at suitable time intervals (usually 0–30 min), and the reaction was stopped by the addition of 10 vols 2% (v/v) perchloric acid. After centrifugation at 15000 g for 5 min, the glucose content of the supernatant was measured using a glucose dehydrogenase kit (Merck). One unit is defined as 1 μmol glucose released in 1 min.

Purification of glucoamylase. All steps were performed at 5 °C. Cells were sedimented by centrifugation at 10000 g for 30 min, and the supernatant was diluted with an equal volume of 0.2 M-potassium phosphate, pH 7.5, containing 0.2 mM-EDTA and 2 mM- MgCl_2 . To this solution was added 250 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$. The supernatant obtained by centrifugation for 20 min at 10000 g was diluted with 0.02 M-potassium phosphate, pH 7.5, containing 0.1 mM-EDTA and 1 mM- MgCl_2 (buffer A) to give a final concentration of 200 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$, and then applied at 200 ml h^{-1} to an Octyl-Sepharose CL-4B (Pharmacia) column (5 $\times$ 26 cm) equilibrated with buffer A containing 200 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$. Elution was performed with a linear gradient (1000 ml) from 200 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$ in buffer A to buffer A alone at 160 ml h^{-1} . Fractions of 11 ml were collected. The fractions degrading starch or starch and pullulan were pooled separately and $(\text{NH}_4)_2\text{SO}_4$ added at 450 g l^{-1} . The precipitate containing the enzyme was collected by centrifugation for 20 min at 10000 g.

The pullulan-degrading fraction was purified as follows. The $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved in 10 ml 20 mM-potassium phosphate, pH 7.5 (buffer B), then loaded onto and eluted at 180 ml h^{-1} from a Biogel P-30 (Bio-Rad) column (3 $\times$ 24 cm) equilibrated with buffer B. The fractions (4.8 ml) containing activity were pooled and then applied to an Accell QMA (Waters Associates) FPLC anion-exchange column (1 $\times$ 10 cm) equilibrated with buffer B. Elution was performed with a linear gradient of 0 to 0.3 M-NaCl in 80 ml buffer B. Fractions capable of degrading both pullulan and starch were pooled, diluted with water to less than 30 mM-NaCl, and applied to a Mono-P column (0.5 $\times$ 20 cm; Pharmacia). The column was equilibrated with 25 mM-Bistris/HCl, pH 6.3, and eluted at 30 ml h^{-1} with a mixture of 5 ml PB-74, 1 ml Pharmalyte pH 2.5–5 (Pharmacia) and 100 ml 25 mM-Bistris/HCl, pH 6.3, adjusted to pH 3.0 with HCl. To elevate the pH of the eluant immediately fractions of 0.5 ml were collected into 0.2 ml 0.5 M-Tris/HCl, pH 6.8, except for analytical runs where the pH was monitored. Fractions containing the pullulan/starch-degrading activity were pooled, concentrated fivefold with Centricon 30 microconcentrators (Amicon), and 500 μl portions chromatographed on a Superose-12 column (1 $\times$ 30 cm; Pharmacia) equilibrated with 0.05 M-potassium phosphate, pH 6.5, containing 0.15 M-NaCl, and eluted at 25 ml h^{-1} .

The Octyl-Sepharose fraction that degraded only starch was purified

as follows. The $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved in 11 ml buffer B and chromatographed on a Biogel P-30 column as above. Fractions containing activity were pooled and chromatographed on an Accell QMA anion-exchange column as above. Fractions containing starch-degrading activity were pooled, diluted with water to 30 mM-NaCl and applied to an FPLC Mono-Q column (0.5 $\times$ 5 cm; Pharmacia) equilibrated with 20 mM-ammonium acetate, pH 6.3. Elution was performed at 60 ml h^{-1} with a 0–0.3 M-NaCl gradient in 30 ml 20 mM-ammonium acetate, pH 6.3. Fractions of 0.7 ml were collected. Fractions containing starch-degrading activity were diluted to 30 mM-NaCl, chromatographed on Mono-P, concentrated, and run on Superose 12 as above.

Purified preparations were stored at –20 °C.

Amino acid composition. Protein samples in 50 mM-potassium phosphate, pH 6.5, containing 0.15 M-NaCl were hydrolysed with either 6 M-HCl (110 °C, 24 h and 72 h) or 3 M-mercaptoethanesulphonic acid (110 °C, 24 h) (Penke *et al.*, 1974; Reeck, 1987).

Amino acids were separated either by HPLC (Varian Vista 5000 LL and AminoTag column) and quantified as FMOC derivatives (Varian Fluorichrom detector) (Einarsson *et al.*, 1983), or by using a Komtron Liquimat III amino acid analyser equipped with an Interaction AA-511 column and Pierce Buffelute buffers and quantified as OPA derivatives.

Peptide mapping by HPLC. Protein samples (50–100 μg) containing glucoamylase P or S activity were digested with lysylendopeptidase C (Wako Chemicals) in 70 mM-Tris/HCl, pH 9.0, at 37 °C with 4% (w/w) of the protease for 20 h. Trypsin (TPCK-treated, Sigma) digestions were performed by incubating similar samples at 37 °C for 2 h with 3% (w/w) trypsin in the presence of 1% (w/v) ammonium bicarbonate and then with a further 2% (w/w) trypsin for about 18 h.

The peptides were separated on a Vydac 218 TP B5 reverse-phase column (0.46 $\times$ 25 cm; The Sep/ara/tions Group, Hesperia, California, USA) using a Waters HPLC apparatus. Elution was performed at 1 ml min^{-1} with a linear gradient from 0.1% trifluoroacetic acid (TFA) to 60% (v/v) acetonitrile containing 0.05% TFA in 60 min at 30 °C. Absorbance at 218 nm was measured.

Amino acid sequencing. Amino-terminal sequencing of the proteins and peptides was done by degrading them in a gas-pulsed-liquid-phase sequencer (Kalkinen & Tilgmann, 1988). The released PTH-amino acids were analysed on-line by using narrow-bore reverse-phase HPLC.

Production and purification of glucoamylase P antibodies. Antibodies against purified glucoamylase P were raised in rabbits. Samples of 50 μg protein in 0.2 M-NaCl were emulsified with equal volumes of Freund's complete adjuvant and injected intradermally. Four additional doses of 50 μg protein emulsified with Freud's incomplete adjuvant were given at 2 week intervals. The rabbits were bled by cardiac puncture 7–12 d after the last booster injection.

The antisera were purified by affinity chromatography on a column of Sepharose 4B (Pharmacia) that contained covalently linked purified glucoamylase P. The protein was coupled to Sepharose 4B by cyanogen bromide according to Axén *et al.* (1967). Low-affinity antibodies were eluted with 0.1 M-sodium acetate, pH 4.5. High-affinity antibodies were eluted with 0.1 M-glycine, pH 3.0. Fractions (1 ml) were collected in tubes containing 35 μl 1 M-Tris base. The purified antibody solution was adjusted to pH 7.5 and stored at –20 °C.

Endoglycosidase treatment. SDS-treated samples (diluted to contain less than 0.2% w/v, SDS) were deglycosylated with Endo H of *Streptomyces plicatus* produced in *S. lividans* (Boehringer) in 0.1 M-sodium acetate, pH 5.7, containing 1% (w/v) phenylmethylsulphonyl fluoride and 10 μM -pepstatin A (Sigma). The samples were incubated at 37 °C for 20 h with 30 mU of Endo H per mg of protein.

Fig. 1. Separation of glucoamylase P and glucoamylase S by Octyl-Sepharose 4B chromatography. The column was run as described in Methods. Δ , A_{280} ; $\bullet$, relative activity measured with pullulan as substrate; $\square$, relative activity measured with starch as substrate; —, $(\text{NH}_4)_2\text{SO}_4$ gradient. S-pool and P-pool show fractions combined to purify glucoamylases S and P, respectively. A relative activity of 100% corresponds to 1.32 U ml^{-1} .

Table 1. Purification of glucoamylase P and glucoamylase S

Activities were measured as described in Methods. The penultimate column shows the activity towards pullulan as a percentage of that towards starch. The recovery of glucoamylase P was calculated from the activity towards pullulan. That of glucoamylase S is based on the activity towards starch and the calculation that, for the example shown, glucoamylase P is responsible for 28% of this activity in the growth medium (see text).

Step	Protein (mg ml ⁻¹)	Pullulan ($\mu\text{mol min}^{-1} \text{ mg}^{-1}$)	Starch ($\mu\text{mol min}^{-1} \text{ mg}^{-1}$)	Debranching (%)	Recovery (%)
Growth medium	0.45	0.7	3.6	20	100
<i>Glucoamylase P</i>					
Octyl-Sepharose	10.3	1.2	0.9	67	38
Accell QMA	0.94	10.4	15.0	70	30
Mono P	0.50	14.1	20.3	70	22
Superose 12	0.38	18.3	26.1	70	22
<i>Glucoamylase S</i>					
Octyl-Sepharose	12.9	0.2	2.8	14	30
Accell QMA	3.4	0.7	6.7	10	19
Mono Q	4.0	0.6	9.5	6	17
Mono P	0.9	0.4	19.0	1	15
Superose 12	0.71	0.3	24.2	1	14

Immunodiffusion. Glass plates covered with 0.8% (w/v) low-melting-point agarose (Bethesda Research Laboratories) in 50 mM-potassium phosphate, pH 7.0, containing 150 mM-NaCl were used. After 4 d of diffusion at room temperature, the plates were soaked in buffer for 10 min and dried under a stack of filter papers for 10 min. This was repeated three times. Dry plates were stained with 1% (w/v) amido black in 2% (v/v) acetic acid for 30 min. The stained plates were destained with 2% (v/v) acetic acid.

Electrophoresis. Polyacrylamide slab gels (6–13%) containing 0.1% SDS were used and stained with Coomassie Brilliant Blue as described by Laemmli (1970).

Protein concentrations. The method of Bradford (1976) was used with γ -globulin as standard. Turbid protein samples, such as the growth medium, were first precipitated with perchloric acid. Proteins being eluted from the chromatographic columns were monitored at 280 nm.

Chemicals. Pullulan was from Sigma and Zulkowsky starch from Merck. Partially purified *Aspergillus niger* and *Rhizopus delemar* glucoamylases were from Sigma.

Results

The purification procedures are summarized in Table 1. The Octyl-Sepharose column separated the starch- (glucoamylase S) and pullulan/starch- (glucoamylase P) degrading activities (Fig. 1). The Accell QMA step gave a relatively large purification of both enzymes. A small polypeptide (about 32 kDa) still present in glucoamylase P preparations after the Mono P column was removed by

Fig. 2. SDS-PAGE analysis of the glucoamylases. Growth medium (Medium), purified glucoamylases P and S before (GA-P, GA-S) and after (GA-P/Endo H, GA-S/Endo H) treatment with endoglycosidase H and molecular mass standards [St: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), lactate dehydrogenase (35 kDa), triosephosphate isomerase (26.5 kDa), myoglobin (17.2 kDa) and cytochrome *c* (11.7 kDa)] were run on a 6–13% polyacrylamide slab gel and stained with Coomassie Brilliant Blue as described in the text.

gel exclusion chromatography on Superose 12. The ratio of activities towards pullulan and starch changed by only a few percent during the 15-fold purification of glucoamylase P from the Octyl-Sepharose P-pool, and was constant through the last two purification steps. Residual debranching activity in the S-pool from Octyl-Sepharose was mostly removed by the Accell QMA, Mono Q and Mono P columns. The final step (Superose 12) caused no further change in the proportion of debranching activity (1%) but caused a 25% increase in specific activity and

Fig. 3. Temperature dependence of glucoamylase P activity. Samples of 50 μ l enzyme protein solution were incubated at different temperatures with 0.5% substrate in the presence of 100 mM-sodium acetate, pH 4.3, containing 0.2 mg bovine serum albumin ml^{-1} . At suitable times (the same for starch as for pullulan at each temperature) samples were withdrawn and assayed for released glucose as described in Methods. The activity of starch degradation at 60 °C (11.2 U ml^{-1}) was taken as 100% relative activity. The activities with pullulan (●) or starch (□) as substrate, and their ratio (pullulan $\times$ 100/starch) (▲) are shown.

removed the polybuffer components from the preparation. The purified preparations were both stable at -20 °C for at least a year.

The recovery of glucoamylase P was 22% calculated from the total pullulan-degrading activity, and the purification was 27-fold (Table 1). The recovery of glucoamylase S could not be calculated from the total starch-degrading activity of the growth medium, because glucoamylase P also degrades starch. The contribution of glucoamylase P was calculated on the basis that the activity of purified glucoamylase P towards starch was 1.43 times its activity towards pullulan, whereas purified glucoamylase S had negligible activity towards pullulan. On this basis, recovery of glucoamylase S was 14% with a purification of ninefold (Table 1). Both enzymes appeared homogeneous on SDS-PAGE, and had apparent molecular masses of 71 kDa (glucoamylase P) and 78 kDa (glucoamylase S) (Fig. 2). The deglycosylated proteins showed apparent molecular masses of 63 kDa and 71 kDa respectively, giving an apparent glycosylation of 11% for glucoamylase P and 9% for glucoamylase S. Analytical runs on Superose 12 gave apparent molecular masses of 72 kDa (glucoamylase P) and 81 kDa (glucoamylase S) for the native proteins (results not shown). The isoelectric points obtained from the chromatofocusing experiments (not shown) were 3.8 for glucoamylase P and 4.1 for glucoamylase S.

The pH optimum for both enzymes was broad at 30 °C with maximum activity between pH 3.5 and 4.6 (results not shown). The temperature dependence of the activities of glucoamylase P is shown in Fig. 3.

Fig. 4. Thermal inactivation of glucoamylase P. Three samples of 1080 μ l of purified enzyme protein were incubated at 30 °C, 56 °C and 60 °C, respectively, in 50 mM-potassium phosphate, pH 6.5. At suitable times, samples of 50 μ l were withdrawn and assayed at 37 °C with pullulan or starch as described in Methods. A relative activity of 100% corresponds to 0.9 U ml⁻¹ for starch-degrading activity and 0.5 U ml⁻¹ for pullulan-degrading activity. ●, Pullulan as substrate; □, starch as substrate.

Fig. 4 shows the thermal inactivation of purified glucoamylase P at 30 °C, 56 °C and 60 °C (pH 6.5) with both pullulan and starch as substrates. Identical thermal inactivation profiles were obtained for the two substrates.

The amino acid compositions of the two enzymes were very similar (Table 2). Antibodies raised in rabbits against glucoamylase P and purified by binding to immobilized glucoamylase P gave a precipitation band with glucoamylase P, but also with glucoamylase S when the latter was used in at least three times greater amounts than those of the glucoamylase P (results not shown). No precipitation bands were found when the *H. resinae* glucoamylases were substituted by commercial preparations of glucoamylases from *Aspergillus niger* and *Rhizopus delemar* even in excess.

Many attempts were made to digest glucoamylases P and S with yeast carboxypeptidase Y, but no amino acids were released. Both nonalkylated samples (in 5 M-urea) and alkylated (Friedman *et al.*, 1970; Fullmer, 1984) samples were treated with carboxypeptidase for various times and then derivatized with Dabs-Cl (Chang *et al.*, 1983) before amino acid analysis. Under identical conditions, the correct carboxy-terminal sequence (-Leu/Gly-Phe-Gln-Gly-OH) was obtained for equine skeletal muscle myoglobin.

Different peptide maps were obtained from glucoamylase P and S, respectively, after digestion with either lysylendopeptidase C (Fig. 5) or trypsin (results not shown).

The amino-terminal sequences of the native proteins were distinct, but showed 59% identity in the first 22 residues (Table 3). Several pairs of peptides (one member

Fig. 5. Peptide profiles of (a) glucoamylase P and (b) glucoamylase S digested with lysylendopeptidase C. Protein samples were digested with lysylendopeptidase C and the resulting peptides were separated by reverse-phase HPLC as described in Methods. Peptides were monitored at 218 nm.

from each enzyme) were also found that had distinct but similar amino-terminal sequences: S lys-C 28.1 and P trypsin 21.4 (78% identity over 27 amino acids); S lys-C 32.2 and P trypsin 35.0 (50% identity over 20 amino acids); S trypsin 26.0 and P lys-C 39.8 (63% identity over 8 amino acids); S trypsin 23.9 and P trypsin 20.1 (80%

Table 2. *Amino acid composition of glucoamylase P and S*

The amino acid contents of the glucoamylases were analysed as described in Methods. For residues marked with asterisks, results from mercaptoethanesulphonic acid hydrolysates were used; other results are from 6 M-HCl hydrolysates. For cysteine the average value from several glucoamylases (Manjunath *et al.*, 1983) was used.

Amino acid	Glucoamylase P		Glucoamylase S	
	Content (mol per 100 mol)	No. of residues (per 63 000 Da)	Content (mol per 100 mol)	No. of residues (per 71 000 Da)
Asx	11.0	63.3	10.8	69.7
Thr*	8.5	48.9	10.0	64.6
Ser*	9.5	54.5	11.7	75.4
Glx	8.2	46.8	8.8	56.8
Pro	5.9	33.9	3.8	24.8
Gly	7.9	45.3	6.8	43.9
Ala	11.0	63.3	7.8	50.6
Cys. SH	1.4	8	1.2	8
Val	5.6	31.9	5.2	33.6
Met*	0.9	5.1	1.0	6.7
Ile	5.4	30.9	4.7	30.5
Leu	6.8	39.1	7.4	47.5
Tyr*	5.8	33.4	6.2	40.3
Phe	3.6	20.6	4.4	28.4
His	1.2	6.7	1.4	9.3
Lys	2.0	11.3	2.6	17.0
Trp*	2.1	11.8	2.3	15.0
Arg	3.1	18.0	3.6	23.2

Table 3. *Amino acid sequence of the N-terminus and N-terminal peptide sequences from glucoamylase P and glucoamylase S*

Amino-terminal sequences of native glucoamylases P and S as well as peptides isolated by HPLC from trypsin and lysylendopeptidase digests were performed with a gas-phase sequencer as described in Methods. Peptides are numbered according to their appearance time in the peptide maps. Lys-C, lysylendopeptidase C; GA-P, glucoamylase P; GA-S, glucoamylase S; x, not determined.

Source	Digest	Peptide	Sequence
GA-S	None	N-term.	SVDSFIESEYAIADLLxNIG
GA-P	None	N-term.	DLSSFIASERAIALOGALNNIGPD
GA-S	Lys-C	28.1	TAFTGSWGRPQDGPALRATALIAYSK
GA-P	Trypsin	21.4	FNGPWGRPQR
GA-S	Lys-C	32.2	DANTLEFSIOTFDPDAGSDATTFQP
GA-P	Trypsin	35.0	TGLDGSILGAISTFDIDAYCDS
GA-S	Trypsin	26.0	ILANINEN
GA-P	Lys-C	39.8	YIVSNINVNNGRTG
GA-S	Trypsin	23.9	QsxGSLSEQFS
GA-P	Trypsin	20.1	YIPsxGSLSEQFNR
GA-S	Trypsin	42.6	VNPDYFY
GA-P	Lys-C	30.5	ANPDYFYTWSRDSALTLK
GA-S	Trypsin	16.7	TLAAOHR
GA-P	Trypsin	11.0	TIQNOHR

identity over 10 amino acids); S trypsin 42.6 and P lys-C 30.5 (86% identity over 7 amino acids) and S trypsin 16.7 and P trypsin 11.0 (57% identity over 7 amino acids) (Table 3).

Discussion

The separation of glucoamylase P and S by hydrophobic interaction chromatography is effective, and also convenient because it can easily be scaled up for commercial-scale purification. The optimal ratio of 1,6- to 1,4-glucosidic activity (as well as other amylolytic activities) in starch degradation depends greatly on the process and substrate used (Labout, 1985; Kennedy *et al.*, 1988). Hence, large-scale purification of glucoamylase P may be important, if the debranching activity is to be used efficiently in industrial processes.

The apparent molecular masses (glucoamylase P, 71 kDa; glucoamylase S, 78 kDa) estimated by SDS-PAGE were only slightly different from the values reported by McCleary & Anderson (1980) (70 kDa and 82 kDa, respectively), and the specific activities were also similar. The molecular masses of the native proteins estimated by gel exclusion chromatography indicate that both enzymes are monomeric in their native states.

The activity ratio of glucoamylase P towards pullulan compared to starch decreased when the temperature was increased (Fig. 3). This behaviour could result from a stabilization of glucoamylase P by the higher concentration of non-reducing end-groups in starch compared to pullulan, as proposed by McCleary & Anderson (1980).

The identical thermal inactivations of the two activities in glucoamylase P (Fig. 4) makes the presence of two enzymes in the preparation very unlikely.

A covalently blocked carboxy-terminus could be responsible for the failure of carboxypeptidase Y to release amino acids from glucoamylase P and glucoamylase S. Another possibility could be that the carboxy-terminal sequence is inaccessible to carboxypeptidase Y even in the presence of 5 M-urea.

The peptide maps of glucoamylases P and S showed large differences, but amino acid sequences of some of the corresponding peptides had high identity. Whereas the high percentage identity seems to indicate a homology between the two enzymes, the differences between the highly similar sequences, especially between the amino-terminal sequences of the proteins, shows that they must be coded by different mRNA species. A combination of intron splicing and post-translational modification is very unlikely, because so many splicing events would be required to generate the widely scattered sequence differences observed. This is also supported by the larger amount of proline and alanine in the smaller

glucoamylase P compared to the larger glucoamylase S. Thus, the two enzymes must be encoded by distinct genes.

The cross-reactivity of the glucoamylase P polyclonal antibodies with glucoamylase S indicates some similar antigenic determinants in either the carbohydrate or protein portions of the two enzymes.

Putative homologies between various sequenced glucoamylases have recently been described (Tanaka *et al.*, 1986; Itoh *et al.*, 1987). There seem to be five distinct regions (S1 to S5) of high homology in glucoamylases. Sequences similar to parts of the S1 consensus sequence (G---AS-S---PDY-Y-W-RD---) are found (Table 3) in the peptides GA-S trypsin 42.6 (PDYFY) and GA-P lys-C 30.5 (PDYFYTWSRD). Sequences similar to parts of the S2 consensus sequence (LG-PK-N-D---T--WGRPQ-DGPA-R---) are found in the peptides GA-S lys-C 28.1 (TGSWGRPQRDGPALR) and GA-P trypsin 21.4 (WGRPQ).

Further sequencing is necessary in order to find the overall homology to other glucoamylases, as well as the homology of glucoamylases P and S.

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