Purification and properties of an extracellular glucoamylase from a diastatic strain of *Saccharomyces cerevisiae*

Matthew J. KLEINMAN,* Anthony E. WILKINSON,† Ian P. WRIGHT,‡ Ivor H. EVANS§ and E. Alan BEVAN||

*School of Natural Sciences, Hatfield Polytechnic, P.O. Box 109, College Lane, Hatfield, Herts. AL10 9AB, U.K.,

†Department of Biochemistry, University of Manchester, Oxford Road, Manchester M13 9PL, U.K.,

‡Food Group Research Department, Distillers Co., Menstrie, Clacks. FK11 7ES, U.K.,

§School of Biological Sciences and Environmental Health, Thames Polytechnic, Wellington Street, London SE18 6PF, U.K.,

and ||School of Biological Sciences, Queen Mary College, Mile End Road, London E1 4NS, U.K.

The extracellular glucoamylase from certain strains of *Saccharomyces cerevisiae* can be purified from culture medium by a simple chromatographic procedure. The native enzyme is heavily glycosylated and has an M_r of about 250 000, but gel filtration indicates the existence of oligomers of larger size. Dissociation yields a form of M_r about 70 000. The glucoamylase is rich in serine and threonine and in aspartic acid plus asparagine, and has a pI of 4.62 and a pH optimum of 4.5–6.5. The thermostability and resistance to denaturants of the yeast enzyme is compared with those of two other fungal glucoamylases. Kinetic data for the yeast enzyme and a variety of substrates is presented; the enzyme is particularly ineffective in cleaving α -(1→6)-glycosidic bonds.

INTRODUCTION

Diastatic strains of *Saccharomyces cerevisiae* (formerly *Saccharomyces diastaticus*; Barnett *et al.*, 1983) contain one or more polymeric *STA* (or *DEX*) genes and release glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) into the culture medium (Erratt & Nasim, 1987). Laboratory and industrial strains of *S. cerevisiae* lack this capacity and so cannot degrade and utilize exogenous starch as a carbon source. The glucoamylases of these diastatic yeasts are attracting increasing attention because of growing interest in using strains of *S. cerevisiae* for direct production of ethanol or biomass from substrates containing starch as main carbon source (Laluce & Mattoon, 1984; Spencer-Martins & Van Uden, 1977). Additional significance attaches to these glucoamylases as being members of the very small group of proteins that are naturally secreted into the culture medium by *S. cerevisiae* (Schekman & Novick, 1982).

The present paper describes a simple procedure for isolating glucoamylase from the culture medium of *S. cerevisiae* (formerly *S. diastaticus*) N.C.Y.C. 625 and a derivative strain. Some physical and kinetic properties of the enzyme are also reported. Preliminary accounts of this work have appeared elsewhere (Patel *et al.*, 1982, 1984).

EXPERIMENTAL

Materials

Bulk chemicals were from BDH Chemicals, as were the maltodextrins. Dextrin was type I bacteriological grade from Sigma Chemical Co. (catalogue no. D-2006, lot 64C-0250). Standard isoelectric-point markers (cata-

logue no. 44270 2G) were from BDH Chemicals. Fine chemicals were from Sigma Chemical Co. Electrophoretic and gel-filtration standards, DEAE-Sephadex A-50, Sephacryl S300 and polyacrylamide-gradient slab gels were from Pharmacia. Thin-layer polyacrylamide plates for isoelectric focusing (PAG plates) were from LKB Produkter. Bisacrylamide was from Eastman-Kodak Ltd., and *NNN'*-tetramethylethylenediamine from Rose Chemicals Ltd. *Aspergillus oryzae* glucoamylase (A3527) and *Rhizopus* glucoamylase (A7255) were both from Sigma Chemical Co.

Saccharomyces cultures

Strain N.C.Y.C. 625 was obtained from the National Collection of Yeast Cultures, Food Research Institute, Norwich, U.K. Strain lab¹, a prototrophic diploid homozygous for a glucoamylase-determining gene, was derived from an initial cross between a spore culture of strain N.C.Y.C. 625 and a non-diastatic laboratory tester strain (Patel, 1985).

Batch cultures were grown for 5 days at 28 °C in a yeast extract (Difco; 10 g/l)/maltose (20 g/l) medium, with vigorous agitation in an orbital shaker to ensure thorough aeration, starting from a 24 h preculture grown on solid medium.

Enzyme assay

(i) Quantitative determination of glucoamylase activity employed a commercial glucose assay kit (Sigma Chemical Co. catalogue no. 15-UV, containing hexokinase, glucose-6-phosphate dehydrogenase, NADP⁺ and buffer salts) to measure release of glucose from a glucoamylase substrate (routinely maltotriose at 10 mM or soluble starch at 0.01%, w/v, final concentration). A 0.2 ml

§ To whom correspondence should be addressed.

portion of the glucose-assay-kit medium (at 10 times the concentration recommended by Sigma Chemical Co.), distilled water and substrate were added to a 1 ml quartz cuvette, mixed, and the absorption at 340 nm was monitored with a temperature-controlled Pye-Unicam SP.1800 recording spectrophotometer at 30 °C, coupled to a chart recorder. When any small absorbance changes caused by slight amounts of contaminating glucose had ceased, glucoamylase-containing solution at 30 °C was added to adjust the final volume to 1.0 ml, the contents were mixed, the cuvette was immediately returned to the spectrophotometer, and the reaction was allowed to proceed (for approx. 5 min). Enzyme activity was determined from the linear rate of absorbance rise, 1 enzyme unit being defined as the amount of activity releasing 1 μ mol of glucose from substrate/min at 30 °C.

(ii) Semi-quantitative activity determinations (as in preliminary assays of column fractions) employed the chromogenic substrate *p*-nitrophenyl α -D-glucopyranoside. Portions (0.01 ml) of *p*-nitrophenyl α -D-glucopyranoside (1 mg/ml) were pipetted into the wells of a Sterilin micro-titre plate and mixed with an equal volume of solution to be assayed, or water (control). The plate was wrapped in Clingfilm and incubated overnight at 37 °C. Glucoamylase activity caused a visible yellow coloration (*p*-nitrophenol), which could be scored visually or measured, after dilution, in a spectrophotometer, as absorbance at 400 nm.

Protein assay

Protein concentrations were determined either from the absorbance at 280 nm (Warburg & Christian, 1941) or by using the commercial colorimetric assay procedure produced by Bio-Rad Laboratories (catalogue no. 500-0001). The protein standard was bovine serum albumin.

Amino acid analysis

Protein samples (3 mg) were hydrolysed at 110 °C for 18, 24, 36 and 48 h in 5.87 M-HCl. Hydrolysates were analysed on an automatic amino acid analyser (Locarte), with norleucine (25 nm) as an internal standard.

Tryptophan was measured spectrophotometrically in the native protein by the method of Beaven & Holiday (1952).

Carbohydrate assay

Carbohydrate was determined by using the phenol/H₂SO₄ method (Dubois *et al.*, 1956), with glucose standards. All assays were performed in triplicate.

Carbohydrate composition

Alditol acetate derivatives of neutral sugars released from the glycoprotein by acid hydrolysis were prepared essentially as described by Albersheim *et al.* (1967) and analysed by g.l.c. [with a packed column (3.2 mm $\times$ 188 cm) of 5% OV 275 on Chromosorb W with temperature programming from 180 to 225 °C at 2 °C/min], and with a flame ionization detector.

Deglycosylation

Endo- β -N-acetyl-D-glucosaminidase H (Miles Laboratories) was used to attempt to deglycosylate the glucoamylase enzyme, by following the method of Tarentino *et al.* (1974).

Electrophoresis

Non-gradient native polyacrylamide gels (7.5%) in tube or slab form were prepared and run by following standard methods (Hames, 1981).

Pharmacia gradient slab gels (PAA 4/30) were used according to the manufacturer's directions, electrophoresis continuing until proteins had reached their gel-pore limits. A mixture of standard proteins was run in flanking lanes to enable the M_r of the glucoamylase to be determined by plots of $\log M_r$ versus mobility.

For electrophoresis of denatured proteins the running buffer contained either SDS (0.1%, w/v) or SDS (0.1%, w/v) and urea (8 M), the gel having been equilibrated in the same buffer. Glucoamylase samples were denatured by boiling for 30 min in 10 mM-Tris/HCl buffer, pH 8.0, containing 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 1 mM-EDTA, or in the same denaturing buffer supplemented with 8 M-urea. The same protein standards were employed as for the native gels. All gels were stained overnight in a solution of 50% (v/v) methanol/7% (v/v) acetic acid containing 0.2% (w/v) Coomassie Brilliant Blue R. Destaining was effected by repeated washing in 7% (v/v) acetic acid.

Isoelectric focusing

Glucoamylase samples were electrofocused in LKB PAG plates (pH ranges 3.5–9.5 or 4.0–5.0) in an LKB Multiphor unit and were stained and destained, all according to the manufacturer's recommended procedure. Standard proteins were electrofocused simultaneously. The positions of the standard proteins on the PAG gel were determined and yielded a pH profile of the gel from which the glucoamylase isoelectric point was determined.

Gel filtration

A column (2.5 cm $\times$ 40.0 cm) of Sephacryl S300 (super-fine grade) was used to determine the glucoamylase M_r , by following the general method of Andrews (1965). The Sephacryl resin, supplied pre-swollen, was equilibrated with 50 mM-citrate/phosphate buffer, pH 6.0, at 4 °C. The column was calibrated by duplicate gel filtrations of Blue Dextran and standard proteins [thyroglobulin (M_r 669 000), ferritin (M_r 440 000), malate dehydrogenase (M_r 62 000), ovalbumin (M_r 43 000) and cytochrome *c* (M_r 12 300)], eluted with the citrate/phosphate buffer at a constant pressure head of 30 cmH₂O and column effluent being collected in 1 ml or 2 ml fractions. Glucoamylase was run through the column under conditions identical with those used for the standards. The elution volume (V_e) was taken to be the sum of the fractions up to and including the peak fractions for each protein. The void volume (V_0) was assumed to be the elution volume of Blue Dextran. A plot of V_e/V_0 against $\log M_r$ was used to determine glucoamylase M_r .

Analytical velocity centrifugation

Glucoamylase (10 mg of protein/ml) dissolved in 0.1 M-citrate/phosphate buffer, pH 7.0, was centrifuged at 40 000 or 60 000 rev./min in an MSE Centriscan 75 centrifuge at 20 °C. An integral chart recorder was used to trace the schlieren peaks at 8 min intervals. The natural logarithm of the distance between the schlieren peak and the centre of rotation, at different time intervals,

was plotted, and the sedimentation coefficient was calculated by the method of Schachman (1959).

Equilibrium centrifugation

Analytical equilibrium ultracentrifugation of glucoamylase samples was performed on a Beckman model E analytical ultracentrifuge. The protein samples (4.28 and 7.14 mg/ml) were exhaustively dialysed against 0.1 M-potassium phosphate buffer, pH 7.0. Centrifugation, with a 12 mm multichannel centre-piece with sapphire windows in an AnJ rotor, was effected at 4611.5 rev./min for 75 h at 5–6 °C. The solvent density was 1.0008 at 5–6 °C. Observations were made with the use of Raleigh interference optics, and M_r values were calculated by the method of Nazarian (1968).

pH optimum

Samples of glucoamylase were incubated at 23 °C with soluble starch (0.1 %, w/v, starch and 450 µg of protein/ml, final concentrations) in 0.1 M-citrate/phosphate buffer at pH values ranging from 3.0 to 8.0. Concentrations of glucose were determined before and after a 3 min incubation by using enzyme assay (i), and glucoamylase activities (µmol of glucose/min) were calculated.

Thermostability

Samples of fungal glucoamylases were incubated at 41, 51, 60 and 65 °C in 10 mM-citrate/phosphate buffer, pH 6.5. Samples were removed at time intervals, chilled in an ice bath, and later assayed at 30 °C for glucoamylase activity by using enzyme assay (i).

Sensitivity to denaturants

Samples of fungal glucoamylases were incubated with maltotriose (20 mM final concentration) in a range of concentrations of either urea or SDS, at 30 °C for 10 min. Samples (10 µl) of the incubations (total volume 1 ml) were assayed for glucose [by using method (i) and a total assay volume of 1 ml] before and after the incubation in order to determine glucoamylase activity in the presence of the denaturant.

RESULTS AND DISCUSSION

Purification of glucoamylase

The strain mainly used for glucoamylase isolation, lab¹, was a diploid capable of normal sporulation, and it was selected as the enzyme source because it produced substantially higher extracellular glucoamylase activity than the parent diastatic strain, N.C.Y.C. 625, under the standard culture conditions. This difference was also seen at the level of glucoamylase specific activities in the post-culture supernatants, which were about 0.4 µmol of glucose/min per mg of protein for strain lab¹ and 0.03 µmol of glucose/min per mg of protein for strain N.C.Y.C. 625.

For bulk enzyme isolation, cells were separated from the culture medium by low-speed centrifugation (at 4000 g for 10 min at 4 °C). Preliminary experiments showed that no significant concentration of enzyme by precipitation could be effected either by (NH₄)₂SO₄ (up to 76 %, w/v, final concentration) or ethanol (up to 80 %, v/v), so the cell-free supernatant was concentrated by freeze-drying in an FTS Flexi-Dry freeze-drier. The freeze-dried material was dissolved in a minimal volume

Fig. 1. Profile of elution of *S. cerevisiae* lab¹ glucoamylase activity from DEAE-Sephadex A-50

Redissolved freeze-dried medium from the culture of strain lab¹ was chromatographed as described in the text, glucoamylase activity in eluate fractions being assayed by *p*-nitrophenyl α-D-glucopyranoside hydrolysis (method ii). ■, Protein; ●, activity.

of 50 mM-citrate/phosphate buffer, pH 6.0, and applied to a DEAE-Sephadex A-50 column (20 cm × 2 cm) that had been equilibrated in the same buffer. The column was washed with the loading buffer and glucoamylase activity was recovered in the eluate as a broad peak (see Fig. 1). All these operations were conducted at 4 °C.

Table 1 gives an example of the purification. The early fractions from the column had the highest specific activity (e.g. pool 1 in Table 1) and were essentially pure on the basis of several criteria. Polyacrylamide-tube-gel electrophoresis of the purified enzyme yielded only a single band staining with Coomassie Blue, which was coincident with a single peak of glucoamylase activity, as assayed in extracts of gel slices (Kleinman, 1984). Isoelectric focusing of the enzyme preparation also showed only a single Coomassie Blue-staining band (see Fig. 2), and the enzyme sedimented as a single symmetrical peak during velocity ultracentrifugation (Kleinman, 1984).

The specific activity obtained here after purification (1.66 µmol of glucose/min per mg of protein; see Table 1) was similar to the values achieved by Erratt (1980) for five yeast (*S. diastaticus*) glucoamylases (1.43, 0.62, 1.29, 3.4 and 0.465 µmol of glucose/min per mg of protein) by using a four-step procedure. The yield observed here (8%; see Table 1) is similar to the yields (2–7%) reported by Erratt (1980). The purification effected in the present work, in a single step, was assisted by the fact that the glucoamylase specific activities routinely found in the culture supernatants of strain lab¹ were some 10-fold higher than those recorded for other diastatic strains [e.g. 0.03 µmol of glucose/min per mg of protein for strain N.C.Y.C. 625 and around 0.04 µmol/min per mg of protein for the strains used by Erratt (1980)].

Enzyme properties: physical

The M_r of the purified glucoamylase was investigated by polyacrylamide-gradient-gel electrophoresis, equilibrium centrifugation, gel filtration and velocity centrifugation. Glucoamylase electrophoresed to its pore limit in gradient gels occupied a broad band between M_r limits of

Table 1. Purification of *S. cerevisiae* strain 1ab¹ glucoamylase

Freeze-dried yeast (*S. cerevisiae* strain 1ab¹) culture supernatant dissolved in buffer was fractionated on a DEAE-Sephadex A-50 column, and 1 ml fractions were collected and assayed for glucoamylase activity. Further details are given in the text.

	Total enzyme units (μmol of glucose/min)	Concn. of protein (mg/ml)	Total protein (mg)	Specific activity (μmol of glucose/min per mg of protein)	Yield (%)	Purification factor
Cell-free supernatant	464		985	0.47	100	1.0
Pool 1 (fractions 15–21)	38	1.0	23	1.66	8.3	3.5
Pool 2 (22–35)	117	4.1	170	0.69	25	1.5
Pool 3 (36–37)	157	4.5	235	0.67	34	1.4
Pool 4 (48–57)	17	0.4	17	1.00	3.7	2.1
Total (pools)	329		445		71

Fig. 2. Isoelectric focusing of 1ab¹ glucoamylase, commercially prepared yeast α -glucosidase and invertase, and standard proteins

Isoelectric focusing was in an LKB PAG plate, pH range 3.5–9.5, as described in the text, samples being applied at the cathode end of the gel. Tracks 1, 3 and 4 contained respectively yeast α -glucosidase (Sigma, catalogue no. I-4504), 1ab¹ glucoamylase and yeast invertase (Sigma, catalogue no. G-6136). Tracks 2 and 5 contained standard proteins [C-phycocyanin (pI 4.65 and 4.85), azurin (pI 5.65), trifluoroacetylated pig metmyoglobin (pI 5.92), pig metmyoglobin (pI 6.45), sperm-whale metmyoglobin (pI 8.3) and cytochrome *c* (pI 10.6)].

2.4×10^5 and 3.3×10^5 with a mean value of 2.85×10^5 (see Fig. 3; for the standard proteins the plot of $\log M_r$ versus relative mobility was linear). The diffuse banding of the glucoamylase may be attributable to the glycoprotein nature of the enzyme: the phenol/ H_2SO_4 method indicated that 80% of the enzyme is carbohydrate (Kleinman, 1984), other secreted yeast enzymes, such as

Fig. 3. Analysis of 1ab¹ glucoamylase by polyacrylamide-gradient-gel electrophoresis

Protein samples were electrophoresed to their 'pore limits' in Pharmacia gradient slab gels, as described in the text. Tracks 1 and 4, standard proteins [thyroglobulin (M_r 669 000), ferritin (M_r 440 000), catalase (M_r 232 000), lactate dehydrogenase (M_r 140 000) and albumin (M_r 67 000)]; tracks 2 and 3, 1ab¹ glucoamylase.

invertase, being known to contain as much as 50% carbohydrate (Ballou, 1982). The value for the 1ab¹ glucoamylase is exceptionally high, though a similar carbohydrate content (about 80%) has been reported for the glucoamylases of other yeast strains (Erratt, 1980). G.l.c. analysis of derivatives of neutral sugars released from the 1ab¹ glucoamylase by acid hydrolysis yielded two peaks comprising 94% and 6% of the total, on the basis of relative peak areas. The major peak co-ran with an authentic mannose standard and was presumed to be mannose; the minor peak has not been identified. The high (94%, w/w) mannose content disclosed by this analysis indicates that the glucoamylase is, with respect to gross carbohydrate composition, a fairly typical *S. cerevisiae* mannoprotein, mannoproteins being a major

component of the cell wall (see Ballou, 1982). Endo- β -*N*-acetyl-D-glucosaminidase H treatment of the purified glucoamylase altered the behaviour of the enzyme on polyacrylamide-gradient-gel electrophoresis by lowering the M_r to 270 000 and narrowing the zone width, giving limits of 258 000 and 283 000 (Kleinman, 1984). This suggested that at least some of the carbohydrate was linked via chitobiose units to asparagine residues in the protein (Tarentino *et al.*, 1974).

In the equilibrium-centrifugation analyses, plots of $\log \Delta QJ$ against r^2 were linear through most of the cell sector, though slight upward curvature was seen towards the bottom. Fitting straight lines to the plots for the 4.28 mg and 7.14 mg of protein/ml samples, and taking $1 - \bar{\nu}\rho = 0.2694$, yielded M_r values, with standard deviations, of $231\,500 \pm 890$ and $202\,600 \pm 730$ respectively. In the case of gel filtration, purified glucoamylase was eluted from a calibrated Sephacryl S300 column in two

broad overlapping peaks, with mid-points corresponding to M_r values of 200 000 and 570 000 (Kleinman, 1984). The peak of smaller M_r presumably corresponds to the species of mean M_r around 2.5×10^5 seen on gel electrophoresis and equilibrium centrifugation, while the larger peak may represent species generated by self-association, though the anomalous behaviour of glycoproteins on gel filtration (Andrews, 1970) makes interpretation problematical. Sedimentation-velocity experiments also gave anomalous results. Plots of $\ln r$ versus time at 40 000 rev./min and 60 000 rev./min were linear over the 100 min measurement period and gave s values of 3.97 and 3.44 S respectively. Assuming a partial specific volume of 0.74 ml/g, and a diffusion coefficient ($D_{20,w}$) of 6.151 [the mean of the values determined by Tamaki (1980) in his study of three yeast glucoamylases], M_r values of 71 302 and 61 783 were obtained. SDS/urea/polyacrylamide-gradient-gel electrophoresis showed SDS-denatured glucoamylase migrating as a band of mean M_r 72 000 (limits of 66 000 and 78 000) (Kleinman, 1984).

The M_r data collectively suggest that the native glucoamylase is a tetramer of M_r about 250 000. This may be capable of association to higher- M_r forms, and denaturation dissociates the enzyme to a monomer of M_r about 70 000. If the diffusion coefficient taken from Tamaki's (1980) work is indeed similar to that of the lab¹ enzyme, it would seem that centrifugal force can also dissociate the enzyme, as argued by Chu *et al.* (1983) in the case of yeast invertase. The anomalous behaviour of the enzyme and the inconsistencies in M_r determinations are probably consequences of the enzyme's especially large carbohydrate moiety.

The putative tetramer M_r is quite similar to the M_r of 300 000 seen for the five extracellular yeast glucoamylases studied by Erratt (1980). By contrast, the three glucoamylases characterized by Tamaki (1980) have M_r values (68 000, 84 000 and 79 000 for enzymes from strains carrying *STA1*, *STA2* or *STA3* genes respectively) closer to that of the putative lab¹ monomer, whereas in another study the glucoamylase of a *STA1*-gene-bearing strain is reported to be heterogeneous in M_r (ranging from 66 000 to 80 000) and to consist of two non-identical subunits (Yamashita *et al.*, 1984).

The most striking features of the amino acid composition of the lab¹ glucoamylase (see Table 2) are the high content of serine and threonine and of aspartic acid plus asparagine. Some of these residues may occur in the Asn-Xaa-Thr (or Ser) configuration typical of *N*-glycosylation sites (Struck *et al.*, 1978), since endo- β -*N*-acetyl-D-

Table 2. Amino acid composition of yeast (*S. cerevisiae* strain lab¹ and *S. diastaticus* strain 5106-9A) extracellular glucoamylases

The values for strain 5106-9A are derived from the data of Yamashita *et al.* (1985a). Abbreviation: N.D., not determined.

Amino acid	Amino acid composition (mol/100 mol of residues)	
	Strain lab ¹	Strain 5106-9A
Ala	6.76	7.77
Arg	1.59	4.29
Asp + Asn	8.27	16.09
Cys	1.22	N.D.
Glu + Gln	7.42	9.65
Gly	5.69	6.17
His	1.76	3.22
Ile	4.56	6.17
Leu	4.72	11.26
Lys	3.36	2.68
Met	0.00	1.34
Phe	4.10	5.36
Pro	5.95	N.D.
Ser	16.64	9.12
Thr	17.62	7.24
Trp	0.93	N.D.
Tyr	2.54	4.83
Val	6.87	4.83

Table 3. Half-lives at different temperatures of five fungal glucoamylases

The half-lives for the *S. cerevisiae*, *Aspergillus* and *Rhizopus* enzymes were determined as described in the text; those for the *S. diastaticus* strains are from Yamashita *et al.* (1985b).

	Half-life (min)					
	At 40 °C	At 50 °C	At 51 °C	At 55 °C	At 60 °C	At 65 °C
<i>S. cerevisiae</i> strain lab ¹	> 90		> 90		26	6
<i>S. diastaticus</i> strain 5106-9A (<i>STA1</i>)	> 120	> 120		44	12	4
<i>S. diastaticus</i> strain 5301-17B (<i>STA3</i>)	> 120	> 120		65	17	4
<i>Aspergillus oryzae</i>	> 90		> 90		88	32
<i>Rhizopus</i> sp.	> 90		18.5		0.35

Fig. 4. Urea inhibition of fungal glucoamylase activities

The effect of urea on glucoamylase activity was determined as described in the text. The glucoamylases were from *Aspergillus oryzae* (●), *Rhizopus* (▲) and *S. cerevisiae lab1* (■).

glucosaminidase H treatment appeared to remove some carbohydrate, and the glucoamylase of strain 5106-97 (*STA1*-gene-bearing) is extensively *N*-glycosylated (Yamashita *et al.*, 1984). However, in the serine- and threonine-rich extracellular glucoamylases of fungi such as *Rhizopus* (Takahashi *et al.*, 1978), *Aspergillus niger* (Pazur *et al.*, 1970) and *Aspergillus saitoi* (Takahashi *et al.*, 1981), much of the carbohydrate is *O*-linked to

Fig. 5. SDS inhibition of fungal glucoamylase activities

The effect of SDS on glucoamylase activity was determined as described in the text. The glucoamylases were from *Aspergillus oryzae* (●), *Rhizopus* (▲) and *S. cerevisiae lab1* (■).

hydroxy amino acid residues (Pazur *et al.*, 1980), and this might also occur in the *lab1* glucoamylase. The preponderance of acidic over basic residues in this enzyme may be reflected in the isoelectric point, determined by isoelectric focusing as 4.62 (see Fig. 2). The pH-dependency of *lab1* glucoamylase activity showed a broad plateau in the pH range 4.5–6.5 and so is similar in this respect to the *STA1*-gene-specified glucoamylase reported on by Yamashita *et al.* (1985b).

Comparison of the thermostability of the *lab1* glucoamylase to that of commercially prepared glucoamylases from *Aspergillus oryzae* and a *Rhizopus* species (see Table 3) showed that the *lab1* glucoamylase was substantially more thermostable than the *Rhizopus* glucoamylase, but rather less so than the *Aspergillus* enzyme. The *lab1* glucoamylase is marginally more thermostable than the yeast *STA1*-gene- and *STA3*-gene-specified glucoamylase (Yamashita *et al.*, 1985b). The response to urea denatura-

Table 4. Summary of K_m , V_{max} , turnover numbers and relative velocities for the *S. cerevisiae* strain *lab1* glucoamylase

K_m and V_{max} values were mainly derived by using Lineweaver–Burk plots of initial velocities for usually 12–19 (minimum eight) different concentrations of a substrate, as described in the text. The correlation coefficient indicates the goodness of fit to a straight line. Turnover numbers assume an M_r of 2.5×10^5 . Relative velocities relate activities to either 1 mM-maltose or 0.1% (w/v) soluble starch.

Substrate	K_m (mM)	K_m (% w/v)	V_{max} (μ mol/min per mg)	Correlation coefficient	Turnover no. (mol/s per mol of enzyme)	Relative velocity
Maltose	3.54	0.120	0.095	0.996	52	100
Isomaltose	36.47	1.25	0.0101	0.998	6	1.3
Maltotriose	3.79	0.191	0.59	0.989	330	596
Isomaltotriose						0.25
Maltotetraose	4.12	0.275	1.30	0.996	720	1216
Maltopentaose	4.33	0.359	1.44	0.967	800	1299
Maltohexaose	3.65	0.361	1.29	0.995	717	1336
Maltoheptaose	4.15	0.480	1.48	0.998	823	1383
Soluble starch		0.078	0.77	0.983	428	100
Amylopectin		0.090	1.23	0.998	683	155
Glycogen*		0.43	0.59		328	30
Dextrin		10.96	0.74	0.994	413	83
Pullulan						0.63

* K_m and V_{max} values determined from an Eadie–Hofstee plot.

tion of the lab¹, *A. oryzae* and *Rhizopus* enzymes paralleled their thermostability (see Fig. 4), but the lab¹ glucoamylase was more sensitive to SDS than the other two enzymes (see Fig. 5). According to Koffler (1957) enzyme thermostability may be conferred by higher-than-average hydrophobicity. ϕ_{av} and NPS are protein hydrophobicity indices proposed by Bigelow (1967) and are, for the lab¹ enzyme, 4095 J/mol of residue (978 cal/mol of residue) and 0.30 respectively. These values are not above the average of the proteins analysed by Bigelow (1967), so the stability of the lab¹ glucoamylase may be due to other factors, such as the carbohydrate moiety: it is known that deglycosylation lowers the thermostability of *STA1*-gene- and *STA3*-gene-specified yeast glucoamylases (Yamashita *et al.*, 1985b) and the resistance of yeast invertase towards several denaturants, including heat (Chu *et al.*, 1978).

Enzyme properties: kinetic

Table 4 presents K_m and V_{max} values for hydrolysis of a variety of substrates by the lab¹ glucoamylase. Turnover numbers are also given, assuming an enzyme M_r of 250000, and relative velocities at substrate concentrations of either 1 mM (maltose = 100%) or 0.1% (w/v) (soluble starch = 100%). K_m values for the structural series maltose–maltoheptaose are very similar, whereas V_{max} increases some 13-fold between maltose and maltotetraose, increasing little thereafter. Isomaltose is a much poorer substrate than maltose, indicating that α -(1→6)-glycosidic bonds are hydrolysed with difficulty.

Hydrolysis of isomaltotriose was even slower than that of isomaltose (15% of the rate for isomaltose at substrate concentrations of 1 mM) and did not conform to Michaelis–Menten kinetics. This was also the case for pullulan, though measurable hydrolysis did occur (the rate was 0.6% of the rate for soluble starch, at substrate concentrations of 0.1%, w/v). Such data indicate that the α -(1→6) branch points in starch are probably not readily cleaved by this enzyme. Indeed, the V_{max} values for both soluble starch and amylopectin are lower than for maltoheptaose. Lineweaver–Burk analysis of the glycogen data yielded anomalous K_m and V_{max} values, but credible values were obtained with an Eadie–Hofstee plot of the same data. The glycogen V_{max} is the lowest value (0.59) for the four polysaccharides, whereas the K_m (0.43%, w/v) is the highest.

The kinetic behaviour reported here for the lab¹ glucoamylase is similar to that for other fungal glucoamylases. For example, increasing hydrolytic rate with increasing maltodextrin chain length is seen for the *Aspergillus niger* (Lineback *et al.*, 1969) and *Rhizopus niveus* (Tanaka *et al.*, 1983) enzymes, and K_m values are usually high and V_{max} values low for substrates with α -(1→6)-glycosidic linkages, such as isomaltose and pullulan.

The particular ineffectiveness of yeast (*S. cerevisiae*) glucoamylases in debranching may be explicable if they have recently evolved from the internal glucoamylase required during sporulation (Colonna & Magee, 1978), which acts to degrade intracellular glycogen, presumably in concert with an internal debranching enzyme. Southern-blot analysis of genomic DNA from diastatic strains of *S. cerevisiae* with the use of cloned *STA1* and *STA3* genes as probes suggests that these genes arose from the sporulation-specific glucoamylase gene (present in all strains of *S. cerevisiae*) through fusion with

sequences required for secretion (Yamashita *et al.*, 1985b).

We thank Dr. J. F. T. Spencer and Mrs. D. Wright for strains, Dr. D. McCarthy and Mr. P. D. Laverack for help with sedimentation analysis and amino acid analysis, and the Distillers Co. for laboratory facilities and financial assistance. Thanks are also due to Mr. Bob Rastell, Thames Polytechnic School of Biological Sciences, for the carbohydrate analysis. The work was supported by a Science and Engineering Research Council CASE award, and mostly carried out in the School of Biological Sciences, Queen Mary College, London.

REFERENCES

- Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967) Carbohydr. Res. **5**, 340–345
- Andrews, P. (1965) Biochem. J. **96**, 595–606
- Andrews, P. (1970) Methods Biochem. Anal. **18**, 1–53
- Ballou, C. E. (1982) in The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W. & Broach, J. R., eds.), pp. 335–360, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Barnett, J. A., Payne, R. W. & Yarrow, D. (1983) Yeasts: Characterisation and Identification, Cambridge University Press, Cambridge
- Beaven, G. H. & Holiday, E. R. (1952) Adv. Protein Chem. **7**, 319–386
- Bigelow, C. C. (1967) J. Theor. Biol. **16**, 187–211
- Chu, F. K., Trimble, R. B. & Maley, F. (1978) J. Biol. Chem. **253**, 8691–8693
- Chu, F. K., Watorek, W. & Maley, F. (1983) Arch. Biochem. Biophys. **223**, 543–555
- Colonna, W. J. & Magee, P. T. (1978) J. Bacteriol. **134**, 844–853
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) Anal. Chem. **28**, 350–356
- Erratt, J. A. (1980) Ph.D. Thesis, University of Western Ontario
- Erratt, J. A. & Nasim, A. (1987) CRC Crit. Rev. Biotechnol. **5**, 95–104
- Hames, B. D. (1981) in Gel Electrophoresis of Proteins: A Practical Approach (Hames, B. D. & Rickwood, D., eds.), pp. 1–91, IRL Press, Oxford and Washington
- Kleinman, M. J. (1984) Ph.D. Thesis, University of London
- Koffler, H. (1957) Bacteriol. Rev. **21**, 227–240
- Laluce, C. & Mattoon, J. R. (1984) Appl. Environ. Microbiol. **48**, 17–25
- Lineback, D. R., Russell, I. J. & Rasmussen, C. (1969) Arch. Biochem. Biophys. **134**, 539–553
- Nazarian, G. M. (1968) Anal. Chem. **40**, 1766–1769
- Patel, D. (1985) Ph.D. Thesis, University of London
- Patel, D., Kleinman, M. J., Wright, I. P. & Evans, I. H. (1982) Abstr. Int. Conf. Yeast Genetics and Molecular Biology 11th, Montpellier, abstr. no. E57, p. 97
- Patel, D., Kleinman, M. J., Wright, I. P. & Evans, I. H. (1984) Abstr. Int. Conf. Yeast Genetics and Molecular Biology 12th, Edinburgh, abstr. no. P4, p. 280
- Pazur, J. H., Knull, H. R. & Simpson, D. L. (1970) Biochem. Biophys. Res. Commun. **40**, 110–116
- Pazur, J. H., Tominaga, Y., Forsberg, L. S. & Simpson, D. L. (1980) Carbohydr. Res. **84**, 103–114
- Schachman, H. K. (1959) Ultracentrifugation in Biochemistry, Academic Press, London and New York
- Schekman, R. & Novick, P. (1982) in The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W. & Broach, J. R., eds.), pp. 361–393, Cold Spring Harbor Laboratory, Cold Spring Harbor

- Spencer-Martins, I. & Van Uden, N. (1977) *Eur. J. Appl. Microbiol.* **4**, 29–35
- Struck, D. K., Lennarz, W. J. & Brew, K. (1978) *J. Biol. Chem.* **253**, 5786–5794
- Takahashi, T., Tsuchida, Y. & Irie, M. (1978) *J. Biochem. (Tokyo)* **84**, 1183–1194
- Takahashi, T., Inokuchi, N. & Irie, M. (1981) *J. Biochem. (Tokyo)* **89**, 125–134
- Tamaki, H. (1980) *Gakujutsu Kenkyu Nenpo Doshisha Joshi Daigaku* **31**, 270–286
- Tanaka, A., Fukuchi, Y., Ohnishi, M., Hiromi, K., Aibara, S. & Morita, Y. (1983) *Agric. Biol. Chem.* **47**, 573–580
- Tarentino, A. L., Plummer, T. H. & Maley, F. (1974) *J. Biol. Chem.* **249**, 818–824
- Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–421
- Yamashita, I., Hatano, T. & Fukui, S. (1984) *Agric. Biol. Chem.* **48**, 1611–1616
- Yamashita, I., Suzuki, K. & Fukui, S. (1985a) *J. Bacteriol.* **161**, 567–573
- Yamashita, I., Maemura, T., Hatano, T. & Fukui, S. (1985b) *J. Bacteriol.* **161**, 574–582

Received 27 March 1987/22 July 1987; accepted 16 September 1987