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HYDROLYSIS OF α-D-GLUCANS AND α-D-GLUCO-OLIGOSACCHARIDES BY *Cladosporium resinae* GLUCOAMYLASES

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ABSTRACT

Culture filtrates of *Cladosporium resinae* ATCC 20495 contain a mixture of enzymes able to convert starch and pullulan efficiently into D-glucose. Culture conditions for optimal production of the pullulan-degrading activity have been established. The amylolytic enzyme preparation was fractionated by ion-exchange and molecular-sieve chromatography, and shown to contain α-D-glucosidase, alpha-amylase, and two glucoamylases. The glucoamylases have been purified to homogeneity and their substrate specificities investigated. One of the glucoamylases (termed P) readily hydrolyses the (1→6)-α-D linkages in pullulan, amylopectin, isomaltose, panose, and 6^3-α-D-glucosylmaltotriose. Each of the glucoamylases cleaves the (1→6)-α-D linkage in panose much more readily than that in isomaltose.

INTRODUCTION

Glucoamylases (EC 3.2.1.3) are important commercially for the conversion of malto-oligosaccharides into D-glucose[1]. Consequently, the properties of these enzymes from numerous microbial sources[2–6] have been studied in detail. All glucoamylases readily cleave the (1→4)-α-D linkages in starch, glycogen, and gluco-oligosaccharides, but (1→6)-α-D linkages appear to be more resistant to hydrolysis[7–14]. Maltose is hydrolysed by most glucoamylases at ~40 times the rate for isomaltose[13]. Also, the linear polysaccharide pullulan, which consists mainly of repeating maltotriosyl units joined by (1→6)-α-D linkages, is apparently quite resistant to hydrolysis[7–9]. At substrate concentrations of ~10 mg/ml, it is hydrolysed at less than one fortieth the rate for starch.

Most glucoamylases almost completely convert starch into D-glucose, but only when they are used in a large excess and over extended incubation times[11,15–17].

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The typical hydrolysis curves of starch or amylopectin by glucoamylase show a rapid, initial conversion into D-glucose followed by a continuously decreasing rate, thought to be due to the resistance to hydrolysis of the (1→6)-α-D linkages in the malto-oligosaccharides. The use of large excesses of enzyme, long incubation times, and high concentrations of malto-oligosaccharides (~30% w/v) under industrial conditions may cause reversion, especially the resynthesis of isomaltose from D-glucose[1].

There are two reports of glucoamylases capable of readily cleaving (1→6)-linked α-D-glucosyl residues in pullulan and branched amylaceous polysaccharides. Both of these glucoamylases were produced extracellularly, one by *Cladosporium resinae*[18] and one by *Aspergillus oryzae*[19].

Detailed studies of the physicochemical and kinetic properties of the novel glucoamylase from *C. resinae* (termed glucoamylase P) are now reported.

MATERIALS AND METHODS

Chromatography. — Paper chromatography was performed at 22° on Whatman 3MM paper with ethyl acetate–pyridine–water (10:4:3), and detection with silver nitrate[20]. T.l.c. was performed on Eastman Chromatogram sheets (silica gel No. 6061), which were developed twice with 1-propanol–ethanol–water (7:1:2). Spots were developed by spraying the plates with 5% sulphuric acid in ethanol and heating to 110°.

Culture of Cladosporium resinae. — *C. resinae* ATCC 20495 was originally provided by Dr. E. T. Reese (Quartermaster and Engineering Center, Natick, Massachusetts). The fungus was grown in a culture medium containing 1.0% of KH_2PO_4, 0.14% of $(NH_4)_2SO_4$, 0.03% of urea, 0.03% of $MgSO_4 \cdot 7\ H_2O$, 0.03% of $CaCl_2$, 0.10% of Proteose Peptone, and 1 ml per litre of trace metal solution containing 500 mg of $FeSO_4 \cdot 7\ H_2O$, 150 mg of $MnSO_4 \cdot H_2O$, 160 mg of $ZnCl_2$, 200 mg of $CoCl_2$, and 1 ml of 2M HCl per 100 ml of water. To this mineral solution was added carbohydrate to a final concentration of 0.5 to 0.8% and, unless otherwise stated, the pH was adjusted to 5.3.

Cultures were stored on agar slants containing 0.1% of NH_4NO_3, 0.03% of $MgSO_4 \cdot 7\ H_2O$, 0.12% of KH_2PO_4, 0.001% of yeast extract, 0.001% of Proteose Peptone, 3% of D-glucose, and 3% of Bacto agar at 4°.

The fungus could be readily grown in solution or on wheat-bran solid. The procedure generally used involved transferring the entire culture from a single slant to 200 ml of culture solution containing 0.5% of D-glucose as the carbon source. After growth for 3 days, aliquots (10 ml) of this culture were added to 250-ml flasks containing 50 ml of culture solution either at various pH values or containing various carbon sources or various mineral-element levels. These cultures were grown in a controlled-environment incubator-shaker at 30° for up to 21 days. Aliquots were removed at ~3-day intervals, centrifuged to remove mycelium, dialysed overnight against distilled water, and then assayed for activity against starch and pullulan.

Alternatively, aliquots (5 ml) of the 3-day D-glucose culture were used to inoculate a bran medium prepared by kneading wheat bran (15 g) and distilled water (10 ml) in a 250-ml Erlenmeyer flask. All media were autoclaved at 15 p.s.i. and 121° for 15 min before use. After inoculation, the wheat-bran cultures were incubated at 30° for up to 20 days. The bran was extracted with 1.5% aqueous NaCl (60 ml), which was then filtered through cheesecloth. The bran was then re-extracted with another aliquot (40 ml) of NaCl solution. The combined extracts were centrifuged, and dialysed against distilled water for 18 h.

Large-scale preparation of glucoamylases involved the inoculation of culture solution (1 litre in a 3-litre flask) with 50 ml of a 3-day D-glucose culture of the organism. D-Glucose (0.5%) was the carbon source. Large-scale solid-cultures were prepared by kneading wheat bran (150 g) and distilled water (50 ml) in a beaker and then transferring to a 4-litre flask which was autoclaved at 15 p.s.i. and 121° for 1 h. After cooling, the bran was inoculated with a 3-day D-glucose liquid-culture (50 ml) of the organism. The bran was extracted with 1.5% aqueous sodium chloride after incubation of the culture at 30° for 13 days.

Enzyme assays. — Glucoamylase was assayed on the basis of the rate of release of D-glucose from soluble starch (J. T. Baker, Lintner), pullulan, or maltose. Enzyme solution (0.1 ml) was incubated with substrate solution (0.5 ml, 5 mg/ml) in 0.1M sodium acetate buffer (pH 4.3). The reaction was stopped and D-glucose was determined by the addition of D-glucose oxidase reagent[21] (1 ml). After incubation at 37° for 1 h, 5M HCl (2 ml) was added to terminate the reaction and generate colour. Alpha-amylase was assayed by the method of Marshall *et al.*[22]. Maltase (α-D-glucosidase) was assayed as for glucoamylase; the substrate was maltose (5 mg/ml) in 0.1M sodium acetate buffer (pH 4.3). Pullulanase and beta-amylase were assayed by incubating enzyme solution (0.1 ml) with substrate solution (0.5 ml) at 37°, the reaction was terminated by addition of Nelson–Somogyi solution A (1 ml), and reducing sugar was determined by the Nelson–Somogyi method[23,24] with D-glucose as the standard. Pullulan and soluble starch (5 mg/ml) in 0.1M sodium acetate buffer (pH 5) were used as substrates.

Purification of C. resinae *glucoamylases.* — All steps were performed at 4°. Culture solution (1 litre) was centrifuged (8,000*g*, 20 min), and solid ammonium sulphate (to 80% saturation) and Celite (1 g) were added. The precipitate was allowed to settle overnight and the bulk of the supernatant solution was removed by suction. The remaining solution was centrifuged (8,000*g*, 20 min), and the recovered pellet was suspended in H_2O (50 ml). Celite was removed by centrifugation, and the supernatant solution was dialysed for 18 h at 4° against citrate–phosphate buffer (0.01M, pH 8). The pH was adjusted to 5.2, and the solution was centrifuged and then applied to a column (2.5 × 10 cm) of pre-equilibrated DEAE-cellulose which was washed with sodium acetate buffer (0.01M, pH 5.2). Fractions were assayed for activity against starch, pullulan, maltose, and sodium starch glycollate (alpha-amylase). The glucoamylase fraction was concentrated by ammonium sulphate precipitation and dialysis, applied to a column (2.5 × 80 cm) of Ultrogel AcA 34, and eluted with

citrate–phosphate buffer (0.1M, pH 8). The active fraction was concentrated, and applied to a column (1.7 × 83 cm) of Sephadex G-100 in 0.1M citrate–phosphate buffer (pH 8). This step separated glucoamylase P (eluted first) from glucoamylase S. Glucoamylase P has a high affinity for pullulan. Glucoamylase S, as recovered from Sephadex G-100, was homogeneous by the criterion of SDS-polyacrylamide and conventional gel-electrophoresis and gel isoelectric focusing, and, apparently, was completely devoid of alpha-amylase. Glucoamylase P was further purified by chromatography on a column (1.6 × 5.5 cm) of DEAE-Sepharose which was pre-equilibrated with citrate–phosphate buffer (0.01M, pH 8). Enzyme activity was eluted with a linear salt-gradient (0→0.4M KCl). This step removed last traces of non-enzyme protein and alpha-amylase.

The purification of these enzymes from wheat bran required the inclusion of two further steps. After the initial extraction and filtration, Bentonite clay (25 g/litre) was added with stirring, and then removed by centrifugation. The Bentonite adsorbed most of the coloured material from the culture solution and gave a less than 10% decrease in glucoamylase activity. To remove the last traces of non-enzyme protein, the glucoamylase P preparation was chromatographed on CM-Sepharose in 20mM sodium acetate buffer (pH 4); the enzyme was eluted with a linear salt-gradient (0→0.4M KCl).

A. niger *glucoamylases I and II.* — Details of the purification of these enzymes will be presented elsewhere[25]. The purified enzymes were devoid of alpha-amylase and had final specific activities of ~434 nkat/mg.

Beta-amylase. — This enzyme was prepared from North Carolina Jumbo sweet-potatoes (3.5 kg after washing and peeling) essentially according to the procedure of Nakayama *et al.*[26]. The purified preparation had a final specific activity of 16.7 μkat/mg and was completely devoid of glucoamylase and α-D-glucosidase. Contamination with alpha-amylase was ~1 unit per 10 million beta-amylase units.

Pullulanase. — This enzyme was prepared essentially according to the procedure of Enevoldsen *et al.*[27] by chromatography on immobilised cycloamylose. Crude "pullulanase K2000" (ABM Chemicals Ltd., Woodley, Stockport, Great Britain) was used. The enzyme (from 100 g of crude preparation) was purified by chromatography on DEAE-cellulose and DEAE-Sepharose, both equilibrated to pH 8 with 10mM citrate–phosphate buffer. Activity was eluted by using a linear salt-gradient (0→400mM KCl). The enzyme was then applied to a column (1.5 × 5 cm) of cyclohepta-amylose–epoxySepharose which was washed with M sodium acetate (pH 5) in M KCl (100 ml). Pullulanase was eluted with the same solution (100 ml) containing 1% of cyclohepta-amylose. The recovered enzyme solution was concentrated and washed exhaustively in a Diaflo cell with a PM-10 membrane. The purified pullulanase had a specific activity of 668 nkat/mg (pH 5), contained traces of alpha-amylase, but was completely devoid of glucoamylase.

Polysaccharide substrates. — Waxy maize starch was prepared by the method of Schoch[28] and was defatted by refluxing in 80% methanol for 8 h. The product was washed with methanol and acetone and dried. For use as substrate, waxy maize

starch (1 g) was dissolved in 0.5M sodium hydroxide (50 ml) by heating to 80°. Conc. acetic acid (1.5 ml) was added to the cooled solution and the pH adjusted to 4.3 by dropwise addition of 5M HCl. Pullulan (1 g) or soluble starch (1 g) was dissolved in 0.1M sodium acetate buffer (pH 4.3, 100 ml) by vigorous stirring and heating to 100°. Commercial glycogen (Sigma Chemical Co.) was purified by chromatography through DEAE-Sepharose at pH 8, to remove protein. Glycogen beta-limit dextrin was prepared by incubating purified, commercial oyster glycogen with beta-amylase, essentially according to the procedure of Whelan[29]. Glycogen (10 g) dissolved in 0.1M sodium acetate buffer (100 ml, pH 4.3) was incubated in a dialysis sac with beta-amylase (16.7 μkat) at 30°, with frequent changes of the external solution during 2 days.

O-(*Carboxymethyl*)*pullulan*[30]. — To a solution of pullulan (10 g) in water (200 ml) at 70° was added sodium sulphate (66.6 g) followed, after dissolution, by chloroacetic acid (1.1 g) in H_2O (5 ml) and the slow addition of NaOH (1.3 g) in H_2O (10 ml). The solution was stirred at 70° for 1.5 h, the *O*-(carboxymethyl)pullulan was recovered by filtration and dissolved in hot water (200 ml), and the solution was dialysed against flowing tap-water for 24 h and then freeze-dried. The d.s. (carboxymethylation), determined by the procedure of Thewlis[30], was 0.2.

Oligosaccharide substrates. — Members of the series maltose to maltohexaose were isolated from a mixture of linear oligosaccharides (Corn Products Co) by thick-paper chromatography (Whatman 3MM); marker strips were treated with silver nitrate–sodium hydroxide[20]. Each oligosaccharide was eluted from the paper with water, the extract was concentrated to dryness on a rotary evaporator, and a solution of the residue in 60% ethanol was centrifuged at 20,000*g* for 20 min and filtered through glass-fibre paper to remove cellulosic material derived from the chromatography paper. Each oligosaccharide solution was concentrated to dryness, the residue dissolved in a minimum volume (~2 ml) of water, and the solution checked for purity by paper chromatography. Oligosaccharide concentration was determined by incubating a sample (0.1 ml) with a large excess of *C. resinae* glucoamylase (17 nkat), to ensure complete hydrolysis, and quantification of the released D-glucose by the D-glucose oxidase method[21].

Panose was prepared essentially by the procedure of Smith and Whelan[31]: a partially purified sample of panose was a kind gift from Dr. E. E. Smith.

6^3-α-D-Glucosylmaltotriose was prepared as described elsewhere[32]. 6^3-α-D-Glucosyl-6^3-α-maltotriosylmaltotriose was prepared from 6^3-α-maltotriosyl-6^3-α-maltotriosylmaltotriose obtained on partial hydrolysis of pullulan by pullulanase. Pullulan (10 g) in 0.1M acetate buffer (pH 5) was hydrolysed to 50% of the theoretical maximum by incubation with pullulanase (2 μkat) for 1 h at 37°. Pullulanase was inactivated by incubating the solution at 100° for 10 min. The mixture was then incubated with beta-amylase (33 μkat) at 37° and pH 5 until all of the 6^3-α-maltotriosyl-6^3-α-maltotriosylmaltotriose had been converted into the desired heptasaccharide (~24 h). Beta-amylase was inactivated by incubating the solution at 100° for 10 min, the solution was deionised and concentrated (rotary evaporation) to

~10% (w/v), and the oligosaccharides were separated by thick-paper chromatography as described for the maltosaccharides.

Glycogen alpha-limit dextrin was prepared by incubating purified, commercial oyster glycogen (50 g), dissolved in 5mM sodium glycerophosphate buffer (pH 6.9, 800 ml) containing 5mM calcium chloride, with dialysed, salivary alpha-amylase (100 μkat). The solution was overlaid with a few drops of toluene and incubated at 37°. After 2 days, the solution was incubated at 100° to denature the alpha-amylase, and washed, dry yeast (*Saccharomyces cerevisiae*, 60 g dry weight before washing with distilled water) was added to the cooled solution. After 2 days, the yeast was removed by centrifugation, and the supernatant solution was deionised with Bio Rad mixed-bed resin (AG 501-X8, 20–50 mesh) in the carbonate form and then freeze-dried.

Degrees of hydrolysis of polysaccharide substrates by glucoamylases. — Enzyme solution (0.1 ml, 25 nkat) was incubated with polysaccharide solution (2 ml, 10 mg/ml) in 0.1M sodium acetate buffer (pH 4.3) at 37°. Aliquots (0.1 ml) were removed from the reaction mixture at 7.5, 15, and 30 min, and 1, 2, 4, and 18 h, and incubated at 100° for 2 min to inactivate the enzyme. Each sample was diluted with water (5 ml), and aliquots (0.1 ml) were removed for the determination of total carbohydrate by the phenol–sulphuric acid method[33], and D-glucose by the D-glucose oxidase procedure[21]. Total carbohydrate in starch hydrolysates was also measured by incubating aliquots (0.1 ml) with an aliquot (0.1 ml) of a mixture of glucoamylase P (10 nkat) and *Aspergillus oryzae* alpha-amylase (2 nkat; Sigma Chemical Co., A6630) for 30 min, and then determining D-glucose by the D-glucose oxidase procedure[21].

Relative, initial rates of hydrolysis. — Enzyme preparation (0.1 ml) was incubated with substrate solution (0.5 ml) in sodium acetate buffer (0.1M, pH 4.3) at 37° and the reaction terminated after incubation for 0, 5, and 10 min. Substrate concentrations were 10 mg/ml for polysaccharides and 5 mg/ml for oligosaccharides.

Kinetic constants. — The kinetic constants were determined by incubating enzyme preparation (0.1 ml) with substrate solution (0.5 ml, 0.01–30 mg/ml) in sodium acetate buffer (0.1M, pH 4.3) at 37°, the reaction was terminated after incubation for 0, 5, and 10 min, by the addition of D-glucose oxidase reagent[21].

Properties of glucoamylases. — The carbohydrate content of the purified enzymes was determined by the phenol–sulphuric acid method[33] and protein levels[34] by the Folin–Lowry method. Values are reported as the weight of carbohydrate as a percentage of the total weight of carbohydrate plus protein. Isoelectric focusing was performed essentially as previously described[35], and isoelectric points were determined by slicing the gels into 5-mm segments; the segments were crushed and extracted with water (2 ml), and the enzyme activity and pH of the extracts measured. Temperature activities of the glucoamylases were determined by incubating enzyme preparation (0.1 ml) with substrate solution (0.5 ml, 5 mg/ml) for 10 min at 25–65°. Reaction was terminated and the released D-glucose measured by adding D-glucose oxidase reagent[21]. Temperature stability was measured by incubating aliquots (0.5 ml) of the enzyme at 25–65° for 10 min; aliquots (0.1 ml) were assayed for

activity. The optimal pH for activity was determined by incubating enzyme preparation (0.1 ml) with substrate solution (5 mg/ml, 0.5 ml) in citrate–phosphate buffer (pH 2.5–7.5). The stability of the enzymes at different pH values was determined by adding enzyme preparation (0.025 ml) to citrate–phosphate buffer solutions (0.1M, 0.2 ml; pH 2.5–7) and storing at 4° or 37° for 20 h. The solutions were then diluted 10-fold with 0.2M sodium acetate buffer (pH 4.3), and aliquots (0.1 ml) were assayed for enzyme activity. The anomeric configuration of the D-glucose released on glucoamylase hydrolysis of starch was determined by the procedure of Semenza *et al.*[36]. Molecular weights were determined by the procedure of Fehrnstrom and Moberg in 5% polyacrylamide gels, using the phosphate and the imidazole buffer systems[37]. To determine the degree of inhibition by metal ions, enzyme preparation (0.1 ml) was preincubated with metal-ion solution (0.1 ml, 2 mg/ml) for 10 min at 37°. Activity was then assayed by adding starch-substrate solution (0.5 ml, 5 mg/ml) and incubating for 5 min. Released D-glucose was measured by the Nelson–Somogyi method[23,24].

RESULTS AND DISCUSSION

Production of glucoamylase by C. resinae. — In liquid culture with D-glucose as the source of carbon, *C. resinae* grew vigorously during the first 3 days, but with little production of enzyme. Release of starch- and pullulan-degrading enzyme activities increased significantly up to 20 days, even though most of the carbohydrate carbon-source was utilised in the first 2 days of growth.

The level of glucoamylase produced was greatly influenced by the nature of the carbon source used in the culture medium. Significantly more enzyme was produced

TABLE I

PRODUCTION[a] OF EXO-STARCH AND EXO-PULLULAN HYDROLYTIC ACTIVITIES BY *Cladosporium resinae*

Culture conditions	*Carbon source*	*Total activity (nkat)*	
		Exo-starch	*Exo-pullulan*
Liquid culture	D-Glucose (0.5%)	1340	200
	Starch (0.5%)	918	117
	Pullulan (0.5%)	750	150
	Pullulan (0.5%) plus D-glucose (0.5%)	1250	200
	Maltose (0.5%)	1169	167
	Nigeran (0.5%)	418	84
Solid culture	Wheat bran	22545	3507

[a]Liquid cultures were prepared by inoculating the culture solution (50 ml) with an aliquot (10 ml) of a 3-day D-glucose culture. The solution was then grown in a rotational incubator for 17 days at 30°. Solid cultures were prepared by inoculating wheat bran (15 g) plus water (10 ml) with a 3-day D-glucose culture (5 ml) of the organism; the flasks (250 ml) were then incubated for 13 days at 30°.

when the carbon source was starch or pullulan than when D-glucose or maltose was employed. However, on continued sub-culturing of the organism over several months, the production of glucoamylase became independent of the carbohydrate used as the carbon source (Table I). The best production of enzyme was attained at phosphate levels of 1.0%; lower levels of phosphate resulted in lower production of enzyme and more sporadic results. Urea could be replaced by equivalent amounts of ammonium sulphate (nitrogen) with little effect on enzyme production. The optimal, initial pH in the culture solution was 4.0–5.5.

On a wheat-bran solid-culture, *C. resinae* produced considerably higher levels of glucoamylase (Table I), with the maximum after 11 days of growth if fresh D-glucose cultures (*i.e.*, a 3-day culture) were used to inoculate the bran.

Purification of glucoamylases P and S. — Although much higher levels of glucoamylase were produced by *C. resinae* grown on solid culture, it was more practical to purify the enzymes from liquid culture. Soluble proteins extracted from the wheat bran used in the solid cultures made purification of the enzymes more difficult.

A typical scheme used for the purification of the two glucoamylases from solution cultures of *C. resinae* is shown in Table II. These purified enzymes appeared as single-protein bands on SDS-polyacrylamide gel-electrophoresis and on gel-slab isoelectric focusing. The two enzymes were produced in almost equal quantities and the overall recovery was ~57% of the total glucoamylase activity present in the culture solutions.

Alpha-amylase contamination of the glucoamylase preparation was almost completely removed by chromatography on DEAE-cellulose (Fig. 1). The two glucoamylases could not be separated by chromatography on Ultrogel AcA 34, but were completely separated on Sephadex G100 (Fig. 2). Glucoamylase P showed similar behaviour on both these materials, but glucoamylase S was highly retarded

TABLE II

PURIFICATION OF *C. resinae* GLUCOAMYLASES S AND P

Step	*Protein (mg)*	*Activity (μkat)*	*Specific activity (nkat/mg)*	*Recovery (%)*	*Purification (-fold)*
Crude culture filtrate (1 litre)	5856	18.37	3.1	100	1
Ammonium sulphate precipitate (80%)	2684	18.20	6.8	99	2.2
DEAE-cellulose	129	17.03	132	93	42.6
Ultrogel AcA 34	43	14.40	334	78	107.7
Sephadex G-100					
P	19	5.76	303	31	97.7
S	12	5.84	487	32	157.1
DEAE-Sepharose					
P	10	4.68	468	25	151.0

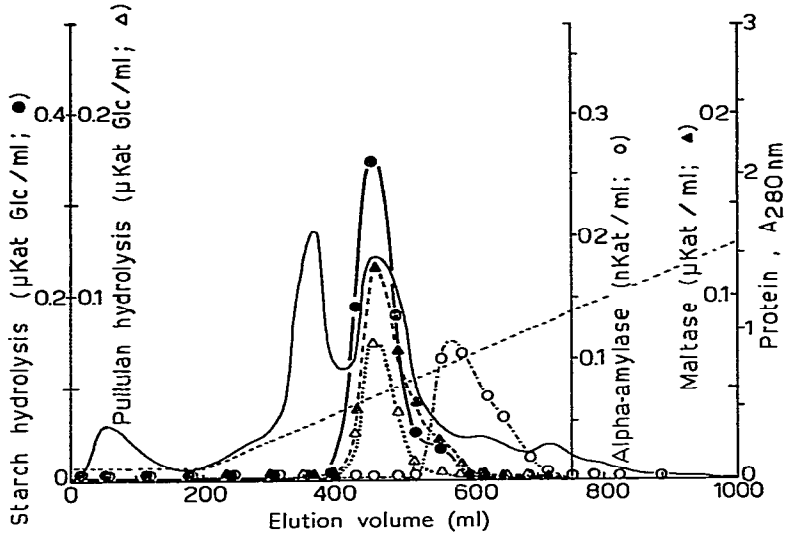

Fig. 1. Chromatography of *C. resinae* glucoamylases on a column (2.5 × 10 cm) of DEAE-cellulose with 20mM acetate buffer (pH 5.2) at 4° and a 0→400mM KCl gradient. The material chromatographed was obtained by ammonium sulphate preparation of a crude culture filtrate.

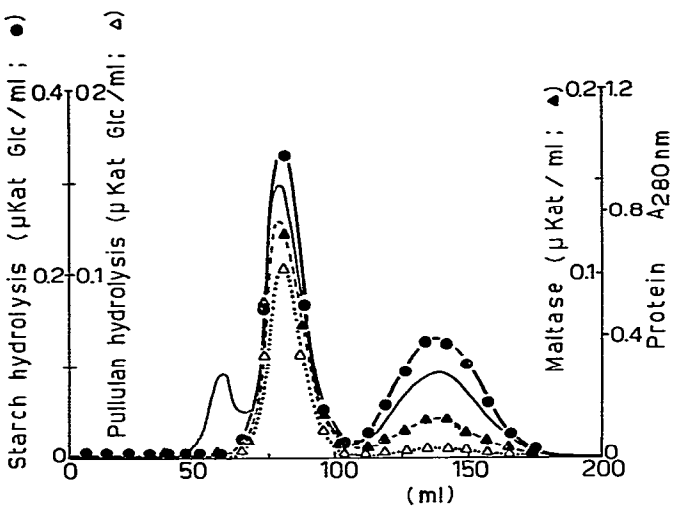

Fig. 2. Chromatography of *C. resinae* glucoamylases on a column (1.5 × 83 cm) of Sephadex G100 with 0.1M citrate–phosphate buffer (pH 8.0) at 4°. The sample chromatographed was obtained by chromatography on Ultrogel AcA34.

on Sephadex G100 and was eluted as a broad peak. It seems that glucoamylase S has some affinity for Sephadex G100; the eluted enzyme was homogeneous by the criteria of SDS-polyacrylamide and conventional gel-electrophoresis and by isoelectric focusing. Glucoamylase P was freed of traces of alpha-amylase and other protein by chromatography on DEAE-Sepharose.

TABLE III

PROPERTIES OF *C. resinae* GLUCOAMYLASES

Property	*Glucoamylase P*	*Glucoamylase S*
Molecular weight	70,000	82,000
pI	4.6	4.5
Carbohydrate (%)	4.6	8.2
pH Optimum	3.5–4.0	3.5
pH Stability (37°, 20 h)	4.0–7.0	4.0–6.0
Temperature optimum	60°	60°
Temperature stability (pH 4.3, 10 min)	up to 55°	up to 48°
Anomeric configuration of the released D-glucose	β	β

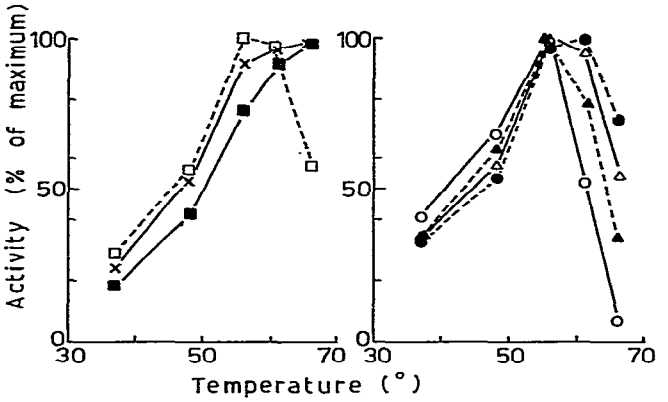

Fig. 3. Temperature activity of glucoamylases. (*a*) Starch substrate: *A. niger* I (■), *A. niger* II (×), and *C. resinae* S (□). (*b*) *C. resinae* P on pullulan (○), starch (●), maltose (▲), and pullulan hydrolysed to 58% of maximum by pullulanase (△).

Properties of glucoamylases P and S. — Some properties of the glucoamylases are shown in Table III. The enzymes have similar temperature stabilities and activities, pH optima and stabilities, and isoelectric points. However, they differ in their carbohydrate contents and molecular weights (SDS-gel electrophoresis).

Glucoamylase P hydrolysed starch, maltose, and pullulan, but the temperature activity for each of these substrates was slightly different (Fig. 3). The results indicated that the enzyme is stabilised at high temperatures by substrates having a high concentration of non-reducing end-groups. Thus, it was stabilised by starch (which has a high concentration of non-reducing end-groups) but not by pullulan (which is a linear molecule of high $\overline{\text{d.p.}}$). Pullulan that had been partially hydrolysed by pullulanase was much more effective in stabilising the enzyme at high temperatures than was native pullulan.

Of the metal ions tested, only Hg^{2+}, Fe^{3+}, and Mn^{2+} caused any appreciable

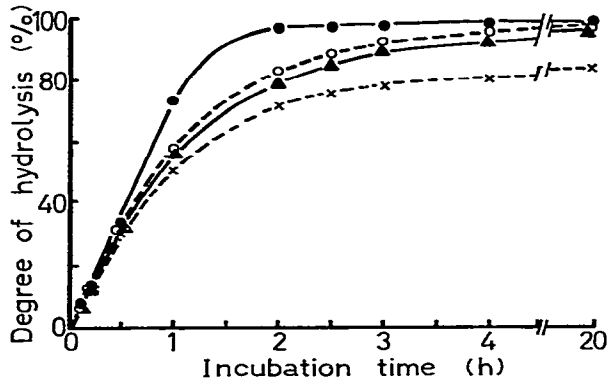

Fig. 4. Hydrolysis of waxy maize starch (8.3 mg/ml) by glucoamylases (12 nkat/ml) at 37°: *C. resinae* P (●), *C. resinae* S (▲), *A. niger* I (○), and *A. niger* II (×).

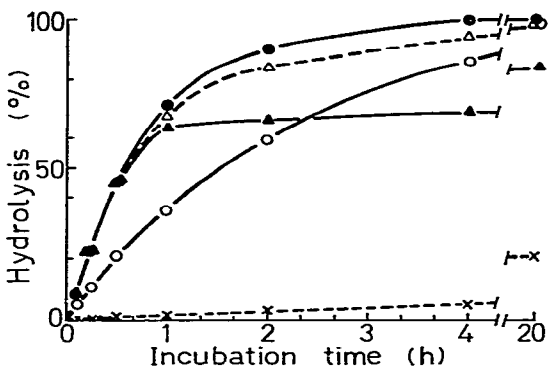

Fig. 5. Hydrolysis of α-D-glucans (8.3 mg/ml) by *C. resinae* glucoamylase S (17 nkat/ml) at 37°: waxy maize starch (●), glycogen (△), potato amylopectin (▲), glycogen beta-limit dextrin (○), and pullulan (×).

inactivation at mM concentration. Each of these ions inhibited both glucoamylase S and P to a similar degree: Hg^{2+}, 83–90%; Fe^{3+}, 35–40%; and Mn^{2+}, 20–30%. Other metal ions tested, namely Cd^{2+}, Ba^{2+}, Cu^{2+}, Ca^{2+}, Pb^{+}, Zn^{2+}, and Ag^{+}, as well as sodium arsenate and sodium tungstate, gave no detectable inhibition at mM concentrations. Both of the glucoamylases released D-glucose from starch in the β-anomeric configuration[36].

Hydrolysis of α-D-glucans and α-D-gluco-oligosaccharides by C. resinae *and* A. niger *glucoamylases.* — Hydrolysis of waxy maize starch by *C. resinae* glucoamylases S and P and by *A. niger* glucoamylases I and II is shown in Fig. 4. The initial rates of hydrolysis of waxy maize starch by each of the glucoamylases were the same; but, after ~60% hydrolysis, there were marked differences in the rates of conversion into D-glucose. None of the glucoamylases effected 100% conversion of waxy maize starch into D-glucose. The extents of conversion after extended incubation

with glucoamylases S, P, I, and II were 97, 98, 96, and 80%, respectively. The results in Fig. 4 show that *C. resinae* glucoamylase P is much more effective than the other three glucoamylases in the conversion of starch into D-glucose. In the presence of alpha-amylase (2 nkat), glucoamylases P, S, and I gave 100% conversion of starch into D-glucose within 20 hours, under the incubation conditions described in Fig. 4.

Hydrolysis of various α-D-glucans by *C. resinae* glucoamylase S is shown in Fig. 5. At substrate concentrations of ~10 mg/ml, waxy maize starch, glycogen, and potato amylopectin were hydrolysed at approximately the same initial rate. However, after incubation for 2 h, waxy maize starch was hydrolysed ~8% more than glycogen. This may be due to the greater concentration of (1→6)-linked α-D-glucosyl residues in glycogen than in waxy maize starch. The initial rate of hydrolysis of glycogen beta-limit dextrin by this enzyme is considerably less than the initial rate of hydrolysis of waxy maize starch, demonstrating the resistance to hydrolysis of (1→6)-linked compared to (1→4)-linked α-D-glucosyl residues. The rate of hydrolysis of pullulan by glucoamylase S is extremely low compared to that of waxy maize starch. Similar results have been reported for most other glucoamylases where the action on pullulan has been studied[7–9].

The action of *C. resinae* glucoamylase P on pullulan and other α-D-glucans is shown in Fig. 6. As previously reported, the initial rates of hydrolysis by this enzyme of pullulan and of glycogen beta-limit dextrin, compared to waxy maize starch, are much higher than the comparative rates of hydrolysis of these substrates by glucoamylase S. A second glucoamylase that also readily hydrolyses pullulan was reported by Saha *et al.*[19] in culture solutions of *A. oryzae*.

Glucoamylase S and P both gave a rapid, initial rate of hydrolysis of potato amylopectin, but hydrolysis essentially ceased after 65% conversion of the polysaccharide into D-glucose, probably because of the presence of phosphate groups in this polymer[38]. Since it is known that the introduction of a chromophore group or hydroxyethyl group into amylose blocks the action of such exo-enzymes as gluco-

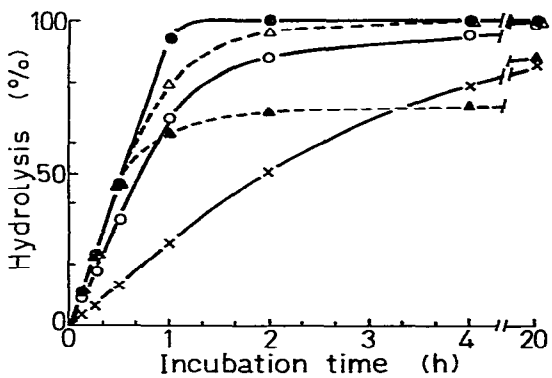

Fig. 6. Hydrolysis of α-D-glucans (8.3 mg/ml) by *C. resinae* glucoamylase P (17 nkat/ml) at 37°: waxy maize starch (●), glycogen (△), potato amylopectin (▲), glycogen beta-limit dextrin (○), and pullulan (×).

amylase, beta-amylase, and phosphorylase, it would not be surprising if the presence of phosphate groups had a similar effect. The fact that there was a slight, but significant, further hydrolysis of potato amylopectin on greatly extended incubation with glucoamylases S or P (or with *A. niger* glucoamylases I and II purified in this laboratory) may indicate that the glucoamylases still contained traces of alpha-amylase. However, attempts to detect such traces by using the highly sensitive starch glycollate assay[22] were unsuccessful, even when incubation periods of up to 48 h were employed. Thus, the alpha-amylase activity, if present, was at levels of <1 unit per 5 million units of glucoamylase.

Glucoamylases P and S from *C. resinae* were much more effective than *A. niger* glucoamylase II in the nearly complete conversion of waxy maize starch, glycogen, and glycogen beta-limit dextrin into D-glucose. This is shown in Figs. 7–9. The

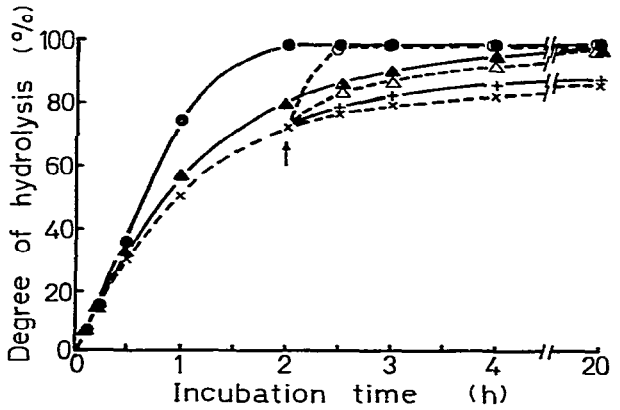

Fig. 7. Hydrolysis of waxy maize starch (8.3 mg/ml) by glucoamylases at 37°: *C. resinae* P, 12.5 nkat/ml (●); *C. resinae* S, 12.5 nkat/ml (▲); *A. niger* II, 12.5 nkat/ml (×); II, 12.5 nkat/ml, followed by II, 12.5 nkat/ml (+); II, 12.5 nkat/ml, followed by S, 12.5 nkat/ml (△); II, 12.5 nkat/ml, followed by P, 12.5 nkat/ml (○).

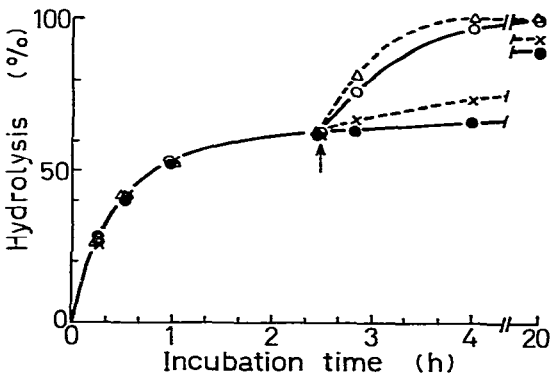

Fig. 8. Hydrolysis of glycogen (8.3 mg/ml) by glucoamylases at 37°: *A. niger* II, 25 nkat/ml (●); II, 25 nkat/ml, followed by II, 25 nkat/ml (×); II, 25 nkat/ml, followed by *C. resinae* S, 25 nkat/ml (○); II, 25 nkat/ml, followed by *C. resinae* P, 25 nkat/ml (△).

effect of adding glucoamylases S or P to solutions of waxy maize starch partially degraded by glucoamylase II is shown in Fig. 7. Addition of glucoamylase P caused a rapid conversion of the remaining polysaccharide into D-glucose. Addition of extra glucoamylase II gave only a slight increase in the rate of hydrolysis of this material.

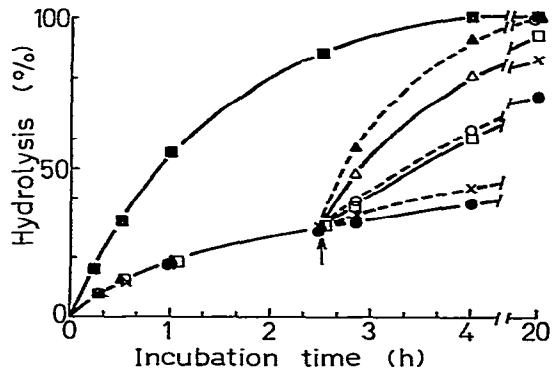

Fig. 9. Hydrolysis of glycogen beta-limit dextrin (8.3 mg/ml) by glucoamylases at 37°: *C. resinae* P, 12.5 nkat/ml (■); *A. niger* II, 12.5 nkat/ml (●); II, 25 nkat/ml, followed by II, 25 nkat/ml (×); II, 25 nkat/ml, followed by P, 2.5 nkat/ml (○); II, 25 nkat/ml, followed by P, 5.0 nkat/ml (△); II, 25 nkat/ml, followed by P, 12.5 nkat/ml (▲); II, 25 nkat/ml, followed by *C. resinae* S, 12.5 nkat/ml (□).

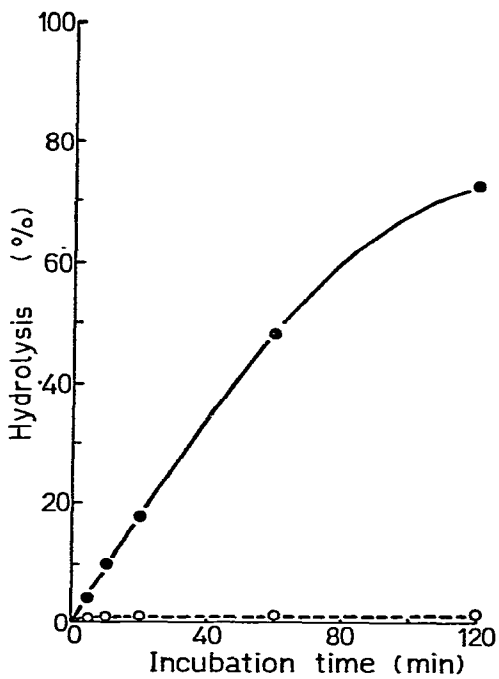

Fig. 10. Hydrolysis of pullulans (4.2 mg/ml) by *C. resinae* glucoamylase P (25 nkat/ml): pullulan (●) and CM-pullulan (○).

Glycogen beta-limit dextrin was very resistant to hydrolysis by glucoamylase II, but glucoamylase P rapidly hydrolysed it to D-glucose (Fig. 9).

C. resinae glucoamylase P, unlike other glucoamylases, readily hydrolysed the (1→6)-α-D linkages in pullulan, even at low concentrations of substrate. To confirm that this enzyme was not contaminated with endo-pullulanase, its activity on pullulan and *O*-(carboxymethyl)pullulan (CM-pullulan; d.s. 0.2) was studied. The results in Fig. 10 show that this low d.s. with carboxymethyl groups greatly limited the degree of hydrolysis by glucoamylase P. This confirmed that glucoamylase P acted as a typical glucoamylase, removing single D-glucosyl groups from the non-reducing end of the polysaccharide. Highly purified pullulanase (see Materials and Methods) had the same activity on pullulan and CM-pullulan, indicating that the d.s. was not sufficient to hinder endo-pullulanase activity.

The relative rates of hydrolysis of several gluco-oligosaccharides and polysaccharides by the various glucoamylases are shown in Table IV. Each glucoamylase readily hydrolysed starch, amylopectin, and glycogen, but *C. resinae* glucoamylase P was the most effective in hydrolysing glycogen beta-limit dextrin, pullulan, and isomaltose, demonstrating its greater ability to cleave (1→6)-linked α-D-glucosyl residues in oligo- and poly-saccharides. Since the hydrolysis of maltose and isomaltose releases 2 D-glucose molecules, for comparative purposes the D-glucose

TABLE IV

RELATIVE RATES OF HYDROLYSIS OF POLY- AND OLIGO-SACCHARIDES BY *A. niger* AND *C. resinae* GLUCOAMYLASES[a]

Substrate	C. resinae *glucoamylases*		A. niger *glucoamylases*	
	P	*S*	*I*	*II*
Soluble starch	100	100	100	100
Waxy maize starch	100	100	100	100
Potato amylopectin	100	100	100	100
Glycogen	100	100	100	96
Glycogen beta-limit dextrin	78	44	42	41
Glycogen alpha-limit dextrin	52	35	37	38
Pullulan	39	3	2	1.5
Dextran	2	0.03	—	—
Maltose	42(21)[b]	20(10)[b]	26(13)[b]	24(12)[b]
Maltotriose	79	47	52	59
Maltotetraose	95	95	100	96
Maltopentaose	95	95	100	96
Maltohexaose	100	100	100	100
Isomaltose	7(3.5)[b]	0.2(0.1)[b]	0.4(0.2)[b]	0.4(0.2)[b]
Panose	33(11)[c]	9(3)[c]	14(4.7)[c]	11(3.7)[c]
6^3-α-D-Glucosylmaltotriose	100(25)[d]	24(6)[d]	28(7)[d]	18(4.5)[d]

[a]Activities were determined in solutions containing substrate at a concentration of 8.3 mg/ml (polysaccharide) and 4.2 mg/ml (oligosaccharide). [b]D-Glucose values divided by 2. [c]D-Glucose values divided by 3. [d]D-Glucose values divided by 4.

TABLE V

KINETIC CONSTANTS OF *C. resinae* GLUCOAMYLASES

Substrate	*Glucoamylase P*		*Glucoamylase S*	
	K_m[a]	V_{max}[b]	K_m[a]	V_{max}[b]
Soluble starch	0.007	470	0.007	483
Waxy maize starch	0.007	470	0.007	483
Potato amylopectin	0.007	470	0.007	483
Glycogen	0.03	470	0.03	483
Glycogen beta-limit dextrin	0.03	367	0.05	250
Pullulan	0.58	180	~150	250
Maltose	0.80(2.2)	123[c]	0.6(1.8)	48.3[c]
Maltotriose	0.32(0.6)	370	0.3(0.6)	227
Maltotetraose	0.32(0.5)	447	0.2(0.3)	460
Maltopentaose	0.30(0.4)	450	0.2(0.2)	452
Maltohexaose	0.08(0.08)	467	0.2(0.2)	483
Isomaltose	5.4(16)	50[c]	9.9(29)	3.3[c]
Panose	6.3(13)	297	11.0(22)	35
6^3-α-D-Glucosylmaltotriose	1.1(1.6)	297	2.0(3)	58
6^3-α-D-Glucosyl-6^3-α-maltotriosylmaltotriose	0.2(0.2)	202	3.3(3)	82

[a]Values are expressed as mg/ml, except those in brackets which are expressed as mM. [b]Nkat of D-glucose released/min/mg of protein at 37°, pH 4.3. [c]D-Glucose values divided by 2; all other D-glucose values are unaltered.

TABLE VI

KINETIC CONSTANTS OF *A. niger* GLUCOAMYLASES

Substrate	*Glucoamylase I*		*Glucoamylase II*	
	K_m[a]	V_{max}[b]	K_m[a]	V_{max}[b]
Waxy maize starch	0.007	433	0.5	433
Pullulan	~150	292	~150	222
Maltose	—	—	0.5(1.1)	40[c]
Maltotriose	—	—	0.2(0.4)	255
Maltotetraose	—	—	0.2(0.2)	417
Isomaltose	8.9(26)	1.2[c]	8.3(24)	1.2[c]
Panose	11.1(22)	37	11.1(22)	38
6^3-α-D-Glucosylmaltotriose	1.4(2.1)	30	1.3(2.0)	32
6^3-α-D-Glucosyl-6^3-α-maltotriosylmaltotriose	2.1(1.8)	33	2.1(1.8)	43

[a]Values are expressed as mg/ml, except those in brackets which are expressed as mM. [b]Nkat of D-glucose released/min/mg of protein at 37°. [c]D-Glucose values divided by 2; all other D-glucose values are unaltered.

oxidase values have been divided by 2 (Table IV, values in brackets). With panose, hydrolysis of the (1→6)-α-D linkage is more difficult than that of the subsequent (1→4)-α-D linkage, and consequently values for panose have been divided by 3; using the same logic, values for 6^3-α-D-glucosylmaltotriose have been divided by 4.

Kinetic studies. — In order to examine further the relative abilities of the glucoamylases to hydrolyse (1→6)- and (1→4)-α-D-glucosidic linkages, a range of α-D-glucans and α-D-gluco-oligosaccharides was employed in detailed kinetic studies. The results obtained are shown in Tables V and VI; each of the glucoamylases was widely effective. However, glucoamylase P was unique in its ability to readily hydrolyse (1→6)-linked α-D-glucosyl residues. This enzyme had a much higher affinity (lower K_m) for pullulan than did the other glucoamylases currently studied (Fig. 11), but the relative V_{max} of each of the glucoamylases on pullulan compared to starch is similar (*cf.* Ref. 18). The pronounced difference in the K_m values of glucoamylases S and P for pullulan is contrasted by their similar affinities for starch, amylopectin, and glycogen. The K_m values of these enzymes for starch and amylopectin are much lower than those reported for most other glucoamylases, with the exception of *Trichoderma viride* glucoamylase[39] which has K_m values for amylopectin and starch that are similar to those now reported for *C. resinae* glucoamylases and *A. niger* glucoamylase I. The K_m values currently reported for *A. niger* glucoamylases are considerably lower than those reported by Smiley *et al.*[40].

Detailed studies of the hydrolysis of maltosaccharides by *Rh. delemar* glucoamylase have been reported[41]; as the chain length of maltosaccharides increases from 2 to 5, the K_m values decrease about 10-fold and V_{max} increases ~4-fold. Very similar results were obtained in the current study using the two glucoamylases from *C. resinae* as well as glucoamylase II from *A. niger*. For maximum rate of hydrolysis, the minimum d.p. of the maltosaccharide is 4, but K_m values continue to decrease

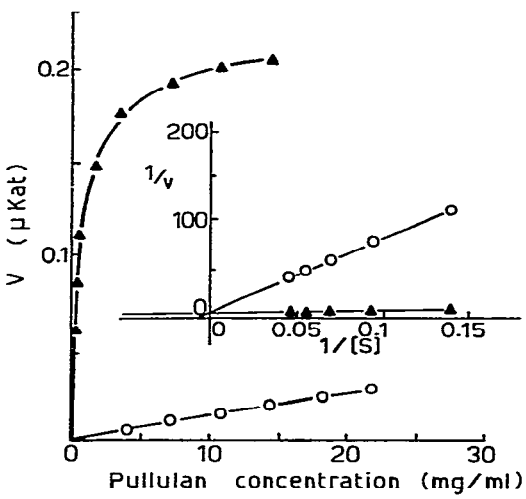

Fig. 11. Kinetic plots for *C. resinae* glucoamylases on pullulan: S (○); P (▲).

as the d.p. increases to 6. Ono *et al.*[41] suggested that the improved binding to the enzyme surface of maltosaccharides of increasing d.p. facilitates the hydrolysis of the first glycosidic linkage to an extent depending on the binding strength, even though these additional residues are not directly attached to the linkage being cleaved.

C. resinae glucoamylase P has a much greater ability than the other glucoamylases to hydrolyse (1→6)-α-D linkages in gluco-oligosaccharides. The relative rate of hydrolysis of isomaltose by this enzyme is almost half that of maltose, whereas *C. resinae* glucoamylase S and the *A. niger* glucoamylase II hydrolyse isomaltose at approximately one twentieth the rate (V_{max}) for maltose. The V_{max} for the hydrolysis of panose by each of the glucoamylases is much higher than that for isomaltose, even though the K_m values are essentially unchanged. These results agree with those of Abdullah *et al.*[12] who, from studies of the relative rates of hydrolysis of these oligosaccharides by *A. niger* glucoamylase, concluded that the environment of the linkage undergoing hydrolysis is an important factor in determining the rate of hydrolysis. After isomaltose and panose, the next oligosaccharide in this series is 6^3-α-D-glucosylmaltotriose, which has two (1→4)-linked α-D-glucosyl residues at the reducing end of the molecule. Each of the glucoamylases had a K_m value for this oligosaccharide which was 8 times lower than that for panose, but the V_{max} values for the two oligosaccharides were similar. The rates of hydrolysis of panose and 6^3-α-D-glucosylmaltotriose by *C. resinae* glucoamylase P are only slightly lower than those for maltotriose and maltotetraose, thus demonstrating the ease with which the enzyme cleaves (1→6)-linked α-D-glucosyl residues. In contrast, the maximum rate of hydrolysis of panose and 6^3-α-D-glucosylmaltotriose by *C. resinae* glucoamylase S and *A. niger* glucoamylase II is approximately one eighth that of the malto-oligosaccharides. This is undoubtedly why *C. resinae* glucoamylase P is so effective in the rapid and almost complete conversion of amylopectin and glycogen into D-glucose, *i.e.*, (1→6)-linked α-D-glucosyl residues in these polysaccharides pose essentially no resistance to hydrolysis.

As discussed by Ono *et al.*[41], caution is required in the interpretation of the observed, initial rate of reactions catalysed by exo-enzymes, especially if the first linkage of the substrate is attacked less rapidly than the second. In this case, the observed rate would be enlarged to a certain degree depending on the relative rate of reaction for the first and second linkage. This factor is important when the rates of hydrolysis of panose, 6^3-α-D-glucosylmaltotriose, and 6^3-α-D-glucosyl-6^3-α-D-maltotriosylmaltotriose are compared with those of the linear oligosaccharides.

Chromatographic examination of the products of hydrolysis of panose and 6^3-α-D-glucosylmaltotriose by *C. resinae* glucoamylase S and *A. niger* glucoamylases I and II revealed only glucose. This finding indicates that the (1→4)-α-D linkages in maltose (released on removal of the (1→6)-linked α-D-glucosyl group at the non-reducing end of panose) and in maltotriose (released on removal of the (1→6)-linked D-glucosyl group at the non-reducing end of 6^3-α-D-glucosylmaltotriose) are hydrolysed more rapidly than the (1→6)-linked α-D-glucosyl group at the non-reducing end of these substrates. In contrast, examination of the partial hydrolysates of panose

and 6^3-α-D-glucosylmaltotriose by *C. resinae* glucoamylase P revealed the presence of maltose in panose hydrolysates, and maltose plus maltotriose in 6^3-α-D-glucosylmaltotriose hydrolysates. This finding suggests that the ease of hydrolysis of the (1→6)-α-D linkage in these substrates by glucoamylase P is comparable to that of the (1→4)-α-D linkages.

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