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β-1,3-GLUCAN HYDROLASES FROM *EUGLENA GRACILIS*

II. PURIFICATION AND PROPERTIES OF THE β-1,3-GLUCAN EXO-HYDROLASE

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SUMMARY

1. The β-1,3-glucan exo- and endo-hydrolases from extracts of *Euglena gracilis* may be partially resolved by chromatography on Biogel P-200 and P-300. Both the exo- and endo-hydrolases are adsorbed by CM-cellulose but only the exo-hydrolase is eluted by 150 mM sodium acetate.

2. The exo-hydrolase in extracts prepared at pH 4.5 and free of endo-hydrolase was chromatographed on CM-cellulose, yielding two active components.

3. The first of these components was further investigated and shown to be highly specific for β-1,3-glucan substrates. Its relative rate of reaction decreased as the chain length of the substrate decreased. Laminaribiose was not hydrolysed. The K_m for laminaripentaose was $11.1 \cdot 10^{-5}$ M and for insoluble laminarin 0.008% (w/v). The pH–activity optimum was in the range 4.7–5.2 and the enzyme was inactive below pH 3 and above pH 7. No transglycosylation from laminarin to glucose or reversion reaction with glucose alone was observed. Glucose was released in the α-configuration during hydrolysis. The enzyme was not stimulated by Mn^{2+} or inhibited by 1,5-gluconolactone.

INTRODUCTION

It has been established[1] that extracts of *Euglena gracilis* contain both β-1,3-glucan exo- and endo-hydrolases. This paper describes the purification of the β-1,3-glucan exo-hydrolase (β-1,3-glucan gluco-hydrolase) and some of its properties.

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EXPERIMENTAL PROCEDURE

Crude enzyme extracts

Cells of an ultraviolet-bleached strain of *E. gracilis* var. bacillaris were grown, and cell-free extracts were prepared as described previously[1].

Substrates and enzyme assays

The substrates were the same as described in the accompanying paper[1]. The exo-hydrolase activity was measured by a reductometric assay and the endo-hydrolase by a viscometric assay[1].

Gel filtration

Polyacrylamide beads, Biogel P-200 and P-300, 50-150 mesh (Bio-Rad Laboratories, Calif.), were prepared for use by gently stirring the gels in 0.01 M sodium acetate (pH 5.2) made 6.25 mM with respect to sodium azide and containing a dilute Euglena extract. Sodium azide was shown not to have any inhibitory effect on the exo- and endo-enzymes. The swelling was allowed to proceed at 4° for at least 2 weeks before the gels were used. The dilute Euglena extract was included in the buffer to ensure the maximum swelling possible, following the observation[2] that a culture filtrate of *Trichoderma viride* caused an irreversible swelling of Sephadex G-75. After the swelling period the gels were washed several times with buffer. Biogel P-200 was poured into a 1.5 cm × 60 cm column onto a sintered polythene disc. The column was thoroughly washed with 0.01 M sodium acetate (pH 5.2) (6.25 mM NaN_3) before use. Biogel P-300 was poured into a 2.5 cm × 60 cm column onto a small bed of Biogel P-10. The surfaces of the columns were stabilised by discs of nylon mesh mounted in cylindrical plastic inserts.

CM-cellulose chromatography

CM-cellulose for use in column chromatography was precycled by suspending the powder in 0.5 M NaOH, washing to pH 7 with distilled water and resuspending in 0.5 M HCl. The acid CM-cellulose was washed with distilled water until the washings were above pH 4.5, then equilibrated with 0.2 M sodium acetate (buffered at the desired pH, either 4.5 or 5.2) and finally washed with 0.01 M buffer. The slurry was poured into a column and washed with the equilibrating buffer before use. The form of the elution gradients of sodium acetate at pH 5.2 were followed by determination of the Na^+ concentration by flame photometry.

Paper chromatography

Reaction products in enzymic digests were analysed by paper chromatography on Whatman No. 3 paper using the solvent propan-1-ol–ethyl acetate–water (6:1:3, v/v/v). The time of development was 24 h. After drying, the products were detected with the alkaline $AgNO_3$ reagent[3].

Buffers

The buffer systems used in the determination of the pH–activity relationships of extracts in the hydrolysis of several substrates were as follows:

pH 3.1–4.0, formic acid–sodium formate; pH 4.5–5.3, acetic acid–sodium ace-

tate; pH 5.7–6.6, sodium acid maleate–NaOH; pH 6.9–7.8, imidazole or Tris–HCl; pH 8.4–9.4, glycine–HCl.

[^{14}C]-D-Glucose

The uniformly ^{14}C-labelled-D-glucose used in transglycosylation experiments was obtained from The Radiochemical Centre, (Amersham, Great Britain) and had a specific activity of 0.157 μC/mg.

Gas–liquid chromatography

Trimethylsilyl derivatives of D-glucose released by the action of the β-1,3-glucan exo-hydrolase were prepared by the addition of dry pyridine, 0.1 ml, hexamethyldisilazane 0.05 ml, and trimethylchlorosilane 0.05 ml, to freeze-dried samples of the enzymic hydrolysate[4]. The reaction mixture was allowed to stand at room temperature for 30 min. The solvent and excess reagents were removed by blowing in a stream of nitrogen, the sample was dissolved in CS_2 and 1–10 μl of this solution were used for chromatography. An F and M 5780 gas chromatography unit with a dual flame hydrogen ionisation detector was used in conjunction with a coiled stainless-steel column, 180 cm × 0.25 cm (internal diameter), packed with 3% SE-30 on Gaschrom Q. The oven temperature was 180°.

RESULTS

Fractionation of the β-1,3-glucan hydrolase complex of Euglena

Gel filtration. Crude Euglena extracts were freeze-dried and dissolved in a minimum volume of buffer for application to the Biogel columns. Enzyme activities against insoluble laminarin (reductometric assay), pachyman (reductometric assay) and CM-pachyman (reductometric and viscometric assay) in fractions from a Biogel P-200 column are shown in Fig. 1. Similar results were obtained with Biogel P-200 columns. The total recovery of the exo-hydrolase from the Biogel columns was between 93 and 98% based on reductometric assays. As shown in Fig. 1 the exo-hydrolase appeared to be partially resolved into two components. However in view of the overlap of the elution profiles of the exo- and endo-hydrolases, it was considered possible that the leading shoulder of the exo-hydrolase elution pattern might have resulted from the combined action of the two enzymes in the region of maximum overlap. In order to test this possibility Fractions 60–130 from the Biogel P-300 column (Fig. 1) were assayed against insoluble laminarin and CM-pachyman before and after heating at 55° for 5 min, the endo-hydrolase having previously been shown[1] to be more labile than the exo-hydrolase under these conditions. Both exohydrolase peaks were still evident, in the same proportions, after this treatment, while the endo-hydrolase was no longer detectable. It would thus appear that the exo-hydrolase was partially resolved into two components by passage through the column of Biogel P-300.

CM-cellulose column chromatography. Preliminary experiments indicated that under the particular conditions tested both the exo- and the endo-hydrolases were adsorbed by CM-cellulose, whereas only the exo-hydrolase could be detected in sodium acetate eluates. In view of this observation and the fact that the exo- and endo-enzymes were not completely resolved by a single passage through the Biogel P-300 column, it

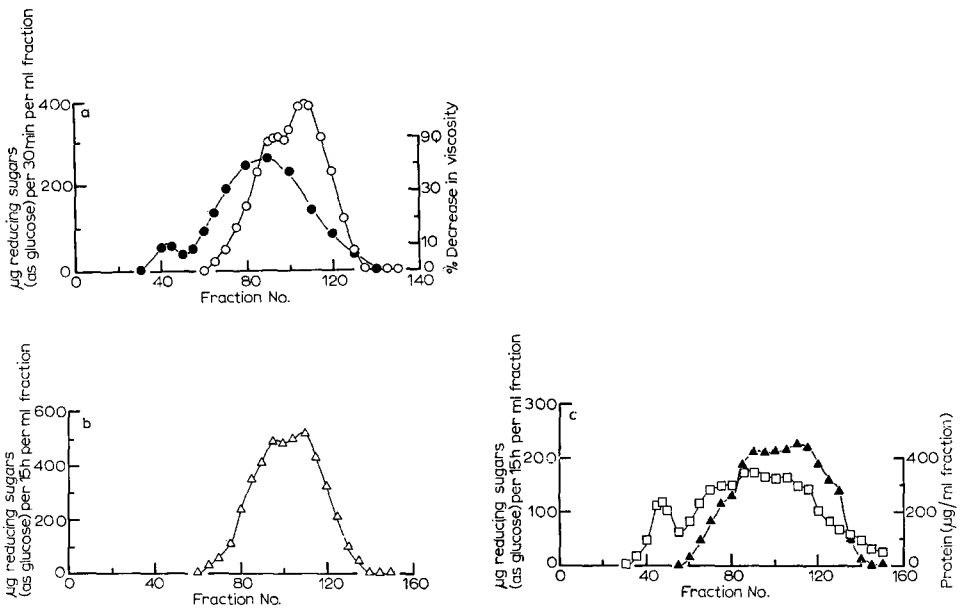

Fig. 1. Chromatography on Biogel P-300. The extract was prepared at pH 5.2 in acetate buffer and dissolved, after freeze-drying, in a minimum volume of 0.01 M sodium acetate (pH 5.2) (6.25 mM NaN_3). 2.0 ml was applied to the column and elution was carried out with the above buffer. Fractions were assayed for protein content (□—□) and for reductometric activity towards laminarin (○—○), paramylon granules (△—△), and CM-pachyman (▲—▲). Selected fractions were assayed viscometrically against CM-pachyman (●—●).

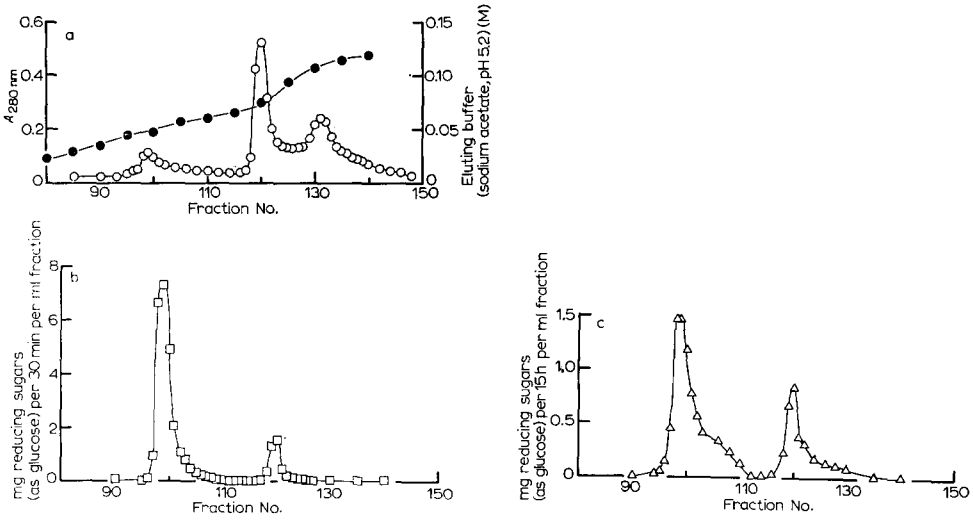

Fig. 2. Chromatography on CM-cellulose. Euglena extracts were prepared in sodium acetate buffe r (pH 4.5) and adjusted to pH 5.2 15 h before addition to the column previously equilibrated a t pH 5.2. The total protein added to the column was 273 mg. The column was eluted with a gradien t of sodium acetate (pH 5.2) ranging in concentration from 0.01 M to 0.15 M (●—●). The protein elution pattern was followed by measuring $A_{280\,nm}$ (○—○). The fractions were assayed reductometrically against laminarin (□—□) and paramylon granules (△—△).

TABLE I

ENZYME PURIFICATION ON CM-CELLULOSE

The conditions of chromatography are described in Fig. 1. Assay conditions as described[1].

Sample assayed	*Protein (mg/ml)*	*Activity*		*Purification factor*	*Recovery (%)*
		(μg Glucose per 30 min per ml)	*(μg Glucose per 30 min per mg protein)*		
Initial extract	5.35	1600	300	—	100
Extract added to column*	1.27	1080	850	2.8	67
PI	0.188	3000	16 000	53	26
PII	0.712	1400	1960	6.5	8

* This extract was prepared from a crude extract by dialysis followed by removal of the precipitated protein by centrifugation and was stored at −20°. Attempted fractionation by $(NH_4)_2SO_4$ precipitation resulted in a rapid inactivation of the β-1,3-glucan hydrolase.

was decided to attempt the purification of the exo-hydrolase by ion-exchange chromatography on CM-cellulose.

All extracts were prepared in 0.1 M sodium acetate buffer (pH 4.5) and showed no endo-hydrolase activity in the viscometric assay with CM-pachyman[1]. The results of a preparative scale CM-cellulose column are shown in Fig. 2. The exo-hydrolase was eluted from the CM-cellulose column in two peaks, the major peak (PI) at a buffer concentration of 0.05 M and the minor peak (PII) at 0.073 M. Fractions from the first

TABLE II

SUBSTRATE SPECIFICITY

Substrate (1.5–3.0 mg) was incubated at 40° and pH 5.2 with the enzyme in a total volume of 0.5 ml. 0.1-ml samples were removed at intervals, heated in a boiling-water bath for 3 min, dried *in vacuo* and analysed by paper chromatography as described in the text. Key: +++++, very strong hydrolysis; ++++, strong hydrolysis; +++, moderate hydrolysis; ++, weak hydrolysis; +, very weak hydrolysis. Abbreviations: Glc, glucose; Lb, laminaribiose; Ltr, laminaritriose; Ltet, laminaritetraose, Gb, gentiobiose. Negative results were obtained with the following: laminarin polyalcohol, laminaribiose, avicell, swollen cellulose, cellobiose, cellopentaose, Caulerpa xylan, soluble starch, maltose, crown gall polysaccharide, sophorose, lutean, gentiobiose, dextran, salicin, sucrose, α,α′-trehalose, β,β′ trehalose.

Substrate	*Linkage type*	*Degree of hydrolysis*	*Products of hydrolysis*
Laminarin	β-1,3	+++++	Glc, Lb, 2 oligo
Paramylon (dispersed)	β-1,3	+++++	Glc
Pachyman	β-1,3	+++++	Glc
Oligoglucosides from pachyman	β-1,3	++++	Glc
CM-pachyman	β-1,3	+	Glc
Laminaritriose	β-1,3	+++	Glc, Lb
Laminaritetraose	β-1,3	+++	Glc, Ltr
Laminaripentaose	β-1,3	++++	Glc, Lb, Ltr, Ltet
Claviceps glucan	β-1,3; β-1,6	++++	Glc, Gb
Yeast glucan	β-1,3; β-1,3	+++	Glc
Lichenin	β-1,3; β-1,4	++	Glc
Barley β-glucan	β-1,3; β-1,4	++	Glc
Oat β-glucan	β-1,3; β-1,4	++	Glc

peak were pooled to a volume of 30 ml and those of the second peak to a volume of 20 ml. The recovery of the exo-hydrolase in the pooled fractions was 34%. The purification data for the preparative column (Fig. 1) is summarised in Table I.

Properties of Euglena β-1,3-glucan exo-hydrolase

The preparation used in these investigations was Peak I from the CM-cellulose column (see Fig. 2 and Table I).

Stability on storage

After storage at −20° for 5 months the preparation was found to have an activity of 12 000 μg glucose per 30 min per mg protein when assayed against 0.8% laminarin. This value represented 75% of the original activity (Table I).

Substrate specificity

The preparation was incubated with the same range of substrates used to test

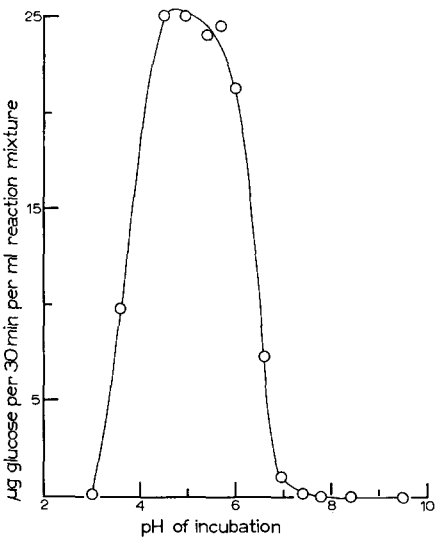

Fig. 3. pH–activity relationships for the exo-hydrolase. The laminarin concentration was 0.8% (w/v) and the temperature of incubation was 40°. Glucose was measured by the glucose oxidase method.

the hydrolytic activity of the crude extract[1]. The results are recorded in Table II. The purified preparation shows a high specificity for substrates with β-1,3-glucosidic linkages, although no hydrolysis of laminaribiose was detected.

pH optimum

The effect of the pH on reaction rate was tested using insoluble laminarin as substrate and the buffer systems listed under *Buffers*. Appropriate substrate blank determinations were made at each pH and the pH of each incubation was checked at 40°. The results are shown in Fig. 3.

Transglycosylation reaction

The possibility of enzymic transglycosylation from a substrate to glucose or a reversion reaction from glucose alone was tested by incubating the preparation with uniformly ^{14}C-labelled D-glucose and carrier glucose at concentrations of 2.5, 25 and 50% (w/v) with or without the addition of 0.25% (w/v) dispersed paramylon. Samples were removed at 8, 24, 48 and 72 h and chromatographed under the conditions described. The developed chromatograms were auto-radiographed on Ilford X-ray film using a 10-day exposure. The chromatograms were then treated with the alkaline $AgNO_3$ reagent.

The control glucose sample showed several slow-moving radioactive areas which were also detected by the $AgNO_3$ reagent. Apart from these areas and those corresponding to the original glucose no other areas of radioactive material were found on any of the chromatograms.

Anomeric form of glucose released in the hydrolysis of laminaripentaose and laminarin

Trimethylsilyl derivatives of the glucose released from the two substrates by the action of the enzyme were prepared and analysed by gas–liquid chromatography

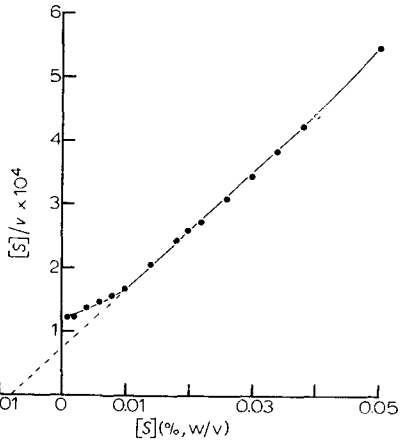

Fig. 4. Reaction velocity data for the action of Euglena exo-hydrolase on insoluble laminarin. The enzyme preparation (0.05 ml) was incubated at 40°, pH 5.2 for 30 min with a series of concentrations of laminarin from 0.001% (w/v) to 0.1% (w/v), in a total volume of 1.0 ml. The reaction was terminated by heating in a boiling-water bath for 3 min. The glucose content was assayed by the glucose oxidase method.

(see EXPERIMENTAL PROCEDURE). The results of the analyses are shown in Table III. It is evident from the results that the D-glucose is released from the β-linked substrates as the α-anomer.

Estimation of the K_m (laminarin) and the K_m (laminaripentaose)

The reaction velocity data for the incubation of the enzyme with laminarin is presented in Fig. 4 as a plot of $[S]/v$ against $[S]$ (ref. 5). The K_m (laminarin) found was 0.008% (w/v).

Similar incubations were set up with laminaripentaose as substrate. The results

TABLE III

ANOMERIC FORM OF D-GLUCOSE RELEASED

Substrate (7.5 mg) was dispersed in water (0.05 ml) at 40°. The enzyme preparation (0.35 ml) was added and 0.1-ml samples were removed at 0, 10, 20 and 30 min. The samples were immediately frozen in liquid air and freeze-dried. Trimethylsilyl derivatives were prepared as described. The peak areas were calculated by the triangulation method.

Time of incubation (min)	*Anomer ratio (β/α)**	
	Laminaripentaose	*Laminarin*
10	0.59	—
20	0.75	0.63
30	0.98	0.94

* The equilibrium ratio β/α = 1.35–1.51 (refs. 4, 16). Equilibrium ratios determined in the present study were ($\alpha \rightarrow \beta$) 1.54; ($\beta \rightarrow \alpha$) 1.63.

are given in Table IV. A plot of $[S]/v$ against $[S]$ gave a K_m (laminaripentaose) of 0.009% (w/v) or $11.1 \cdot 10^{-5}$ M.

Relative rates of hydrolysis of β-1,3-oligoglucosides

The data for the relative rates of hydrolysis of laminaribiose, -triose, -tetraose, -pentaose and laminarin are given in Table V.

Effect of Mn^{2+} on exo-hydrolase activity

In view of an earlier report[6] of the stimulatory effect of Mn^{2+} on the activity of Euglena β-1,3-glucan hydrolase, the effect of Mn^{2+} on the purified exo-hydrolase was examined. The enzyme was assayed both before and after dialysis against an ice-cold solution of 2 mM EDTA in the presence and absence of 2 mM Mn^{2+}. The results are shown in Table VI. It is evident that when the high substrate blanks in the presence of Mn^{2+} are taken into account no activation of the enzyme by Mn^{2+} could be detected.

TABLE IV

DATA FOR K_m DETERMINATION FOR LAMINARIPENTAOSE

0.05 ml of the enzyme preparation was incubated at 40° and pH 5.2 for 10 min with a series of concentrations of laminaripentaose from 0.005% (w/v) to 0.1% (w/v), in a total volume of 1.0 ml. The reaction was terminated by heating in a boiling-water bath for 3 min and the glucose released was assayed by the glucose oxidase method.

[S]		*v*	*[S]* / *v* × 10^{-4}
% (w/v)	*mM*	*(μg Glucose per 10 min per 0.05 ml)*	
0.005	0.062	21	2.38
0.010	0.124	39	2.56
0.020	0.248	56	3.57
0.030	0.372	65	4.61
0.040	0.496	69	5.80
0.050	0.620	70	7.15
0.100	1.240	71	10.40

TABLE V

RELATIVE RATES OF HYDROLYSIS OF β-1,3-OLIGOGLUCOSIDES

A sample (0.05 ml) of the purified extract was incubated with 0.118 mM solutions of laminaribiose, laminaritriose, laminaritetraose, laminaripentaose and insoluble laminarin for 10 min at 40°, pH 5.2. The reaction was stopped by heating in a boiling-water bath for 3 min and the glucose in the hydrolysate was assayed by the glucose oxidase method. Corrections for substrate blanks were made.

Substrate	*Relative reaction rate (µg glucose per 10 min per 0.05 ml extract)*	*% Rate**
Laminarin	42	100
Laminaripentaose	34	81
Laminaritetraose	21	50
Laminaritriose	9	22
Laminaribiose	0	0

* Laminarin = 100.

TABLE VI

EFFECT OF Mn^{2+} ON EXO-HYDROLASE ACTIVITY

The incubation conditions are described in the text. Mn^{2+} concentration was 2 mM.

Extract	*Activity (µg glucose per 30 min per ml)*	
	− Mn^{2+}	*+ Mn^{2+}*
Before dialysis	25*	18*
After dialysis against 2 mM EDTA	25*	24*
Substrate blank	7	35

* These values have been corrected for the substrate blank.

TABLE VII

EFFECT OF 1,5-GLUCONOLACTONE

The incubation mixture contained purified enzyme extract, insoluble laminarin (0.8% final concentration), acetate buffer 0.05 M (pH 5.2) and 1,5-gluconolactone at the concentrations shown. The stock lactone solution was prepared immediately before use. The mixtures were incubated for 30 min at 40° and the reactions stopped by heating. Glucose in the hydrolysates was measured by the glucose oxidase method. Control experiments showed that the inclusion of up to 25 mM gluconolactone did not interfere with the determination of glucose in the concentration range used.

Gluconolactone concn. (final) (mM)	*Activity (µg glucose per 30 min per ml)*
—	264
1	272
2.5	280
10	296
25	292

Effect of 1,5-gluconolactone

Table VII shows the effect of 1,5-gluconolactone on exo-hydrolase activity towards insoluble laminarin.

DISCUSSION

The two exo-glucanase components detected in the Euglena extracts by gel filtration and ion-exchange chromatography on CM-cellulose were not tested for differences in substrate specificities or kinetic parameters. The β-1,3-glucan exo-hydrolase from Basidiomycete QM806 (ref. 7) apparently exists as two isozymes and the two components observed here may be similarly explained. However, unpublished experiments in which the conditions of pH and azide concentration in the enzyme preparation varied were shown to affect the relative amounts of the two exo-glucanase components from the CM-cellulose columns. This suggests the possibility of their inter-conversion and that they may have arisen during the preparative procedures[8].

The Euglena exo-hydrolase showed a marked specificity for substrates containing the β-1,3-glucosidic linkage, in contrast to the crude extract[1] which also hydrolysed maltose, starch, sophorose, lutean, gentiobiose, dextran and α,α'-trehalose. The relative rate of hydrolysis of β-1,3-oligoglucosides increases with increasing degree of polymerisation of the substrate, but the maximum rate did not appear to have been reached even with laminarin. There was no detectable hydrolysis of laminaribiose. A low but significant hydrolysis of this disaccharide by the Basidiomycete QM806 β-1,3-glucan exo-hydrolase has been reported[7]. The hydrolysis of the Claviceps glucan[9,10] by the Euglena enzyme with the production of glucose and gentiobiose, indicated that the action of the enzyme was not prevented by glucosyl substitution at the C-6 position of every fourth glucosyl residue along the β-1,3-linked backbone of the molecule. The Claviceps glucan[9,10] and a similar glucan from Sclerotium[11] are also hydrolysed, by the Basidiomycete QM806 enzyme, to glucose and gentiobiose. EBATA AND SATOMURA[12] have described a β-1,3-glucan exo-hydrolase from *Sclerotinia libertiana* which hydrolyses sclerotan[13], a glucan very similar in structure to the Sclerotium glucan[11], producing glucose and gentiobiose. The low degree of hydrolysis of yeast glucan, lichenin and the barley and oat β-glucans with the production of glucose alone was probably due to the hydrolysis of the terminal β-1,3-linked glucosyl residues in these substrates.

β-1,3-Glucan hydrolases from a wide variety of sources[14,15] show pH optima in the region of 5. The Euglena exo-hydrolase is no exception, being maximally active between pH 4.5 and 5.5 and inactive below pH 3 and above 7. The pH–activity curve of the Basidomycete exo-hydrolase[7], in contrast to the Euglena enzyme, showed a much broader pH optimum between 4.0 and 6.0 and was still active below pH 3 and above 7.

In studies aimed at establishing criteria for the differentiation of glucoside hydrolases and glucan exo-hydrolases, it was shown[16,17] that, of the enzymes examined, the glucoside hydrolases retained the configuration of the glucose released in the form in which it occurred in the substrate, while the glucan exo-hydrolases caused an inversion of the configuration during hydrolysis. The Euglena enzyme fulfils this particular criterion of an exo-hydrolase in that it causes an inversion of the anomeric configuration of the glucose unit from the β-form in the substrate to the α-form in the

product of hydrolysis. REESE *et al.*[17] also showed that while α- and β-glucoside hydrolases were both inhibited by very low concentrations of 1,5-gluconolactone relative to substrate concentration, very much higher concentrations were needed to inhibit the α- and β-glucan exo-hydrolases. The results in Table VII show that the Euglena enzyme is not inhibited at lactone/substrate ratios which are effective with glucoside hydrolases, thus giving further support to its classification as a β-glucan exo-hydrolase.

β-Glucoside hydrolases are known to catalyse the transfer of glucosyl groups[18,19], whereas a number of glucan exo-hydrolases have been found to show little or no tendency to catalyse transfer reactions[17]. However, under forcing conditions, with high concentrations of glucose, glucoamylases from *Aspergillus niger* and *Rhizopus delamar*, which were apparently α-glucoside hydrolase free, catalyse the formation of nigerose, isomaltose and isomaltotriose by reversion reactions[20]. The Euglena enzyme showed no transfer reaction under similar conditions.

FELLIG[6] reported the activation by Mn^{2+} of a crude Euglena β-1,3-glucan hydrolase and CHESTERS AND BULL[21] found that both β-1,3-glucan exo- and endo-hydrolases from a variety of microfungi were activated by Mn^{2+}, with laminarin as substrate. Such an activation was not detected with the purified Euglena exo-hydrolase, although it was noted that Mn^{2+} greatly increased the laminarin substrate blank after incubation at 40°.

The Euglena enzyme belongs to a group of highly specific exo-hydrolases of which the Badisiomycete QM806 (refs. 7 and 14), Sclerotinia[12] and *Sporotrichum pruinosum*[14] enzymes are the most studied, although such enzymes are common fungal products[15]. This group is distinct from other less specific β-glucan exo-hydrolases which have been found in yeast and have been studied extensively from the point of view of their substrate range[22].

The presence in Euglena of both hydrolytic[1] and phosphorolytic[23–26] systems for the depolymerisation of paramylon clearly provides an enzymological basis for the rapid removal of this β-1,3-glucan which is observed under certain physiological conditions[27]. Whether both systems can function simultaneously, how their activity is controlled and the nature of their sub-cellular location remain to be defined. There is, however, a superficial resemblance between the systems for the depolymerisation of paramylon in Euglena and the phosphorolytic and hydrolytic pathways existing in mammalian liver and other tissues for the breakdown of glycogen[28], where the two systems apparently act independently and have different roles in the cellular economy.

REFERENCES

1 D. R. BARRAS AND B. A. STONE, *Biochim. Biophys. Acta*, 191 (1969) 329.
2 K. SELBY AND C. C. MAITLAND, *Biochem. J.*, 104 (1965) 716.
3 W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444.
4 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497.
5 G. N. WILKINSON, *Biochem. J.*, 80 (1961) 324.
6 J. FELLIG, *Science*, 131 (1960) 832.
7 F. I. HUOTARI, T. E. NELSON, F. SMITH AND S. KIRKWOOD, *J. Biol. Chem.*, 243 (1968) 952.
8 E. S. VESELL, *Ann. N.Y. Acad. Sci.*, 151 (1968) 5.
9 A. S. PERLIN AND W. A. TABER, *Can. J. Chem.*, 41 (1963) 2278.
10 K. W. BUCK, A. W. CHEN, A. G. DICKERSON AND E. B. CHAIN, *J. Gen. Microbiol.*, 51 (1968) 337.
11 J. JOHNSON, S. KIRKWOOD, A. MISAKI, T. E. NELSON, J. V. SCALETTI AND F. SMITH, *Chem. Ind. London*, (1963) 820.

12 J. EBATA AND Y. SATOMURA, *Agr. Biol. Chem. Tokyo*, 27 (1963) 478.
13 Y. TAKEUCHI AND M. KITAHARA, *Agr. Biol. Chem. Tokyo*, 30 (1966) 523.
14 E. T. REESE AND M. MANDELS, *Can. J. Microbiol.*, 5 (1959) 173.
15 A. T. BULL AND C. G. C. CHESTERS, *Advan. Enzymol.*, 28 (1966) 325.
16 F. W. PARRISH AND E. T. REESE, *Carbohydrate Res.*, 3 (1967) 424.
17 E. T. REESE, A. H. MAGUIRE AND F. W. PARRISH, *Can. J. Biochem.*, 46 (1968) 25.
18 E. M. CROOK AND B. A. STONE, *Biochem. J.*, 65 (1957) 1.
19 M. A. JERMYN, *Rev. Pure Appl. Chem.*, 11 (1961) 92.
20 J. H. PAZUR AND S. OKADA, *Carbohydrate Res.*, 4 (1967) 371.
21 C. G. C. CHESTERS AND A. T. BULL, *Biochem. J.*, 86 (1963) 38.
22 A. T. H. ABD-EL-AL AND H. J. PHAFF, *Biochem. J.*, 109 (1968) 347.
23 L. R. MARECHAL AND S. H. GOLDEMBERG, *Biochem. Biophys. Res. Commun.*, 13 (1963) 106.
24 S. H. GOLDEMBERG, L. R. MARECHAL AND B. C. DE SOUZA, *J. Biol. Chem.*, 241 (1966) 45.
25 L. R. MARECHAL, *Biochim. Biophys. Acta*, 146 (1967) 417.
26 L. R. MARECHAL, *Biochim. Biophys. Acta*, 146 (1967) 431.
27 D. R. BARRAS AND B. A. STONE, in D. E. BUETOW, *The Biology of Euglena*, Vol. 2, Academic Press, New York, 1968, p. 149.
28 E. E. SMITH, P. M. TAYLOR AND W. J. WHELAN, in F. DICKENS, P. J. RANDLE AND W. J. WHELAN, *Carbohydrate Metabolism and its Disorders*, Vol. 1, Academic Press, London, 1968, p. 89.