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

## I. THE NATURE OF THE HYDROLASES

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SUMMARY

1. Cell-free extracts prepared from *Euglena gracilis* rapidly hydrolyse a number of β-1,3-glucans as well as show slight, but significant, hydrolytic action on a number of other glucans, glucose oligosaccharides and glucosides.

2. The nature of the enzymes hydrolysing the β-1,3-glucans has been examined. Glucose was the sole product detected during the hydrolysis of paramylon and pachyman and during the early stages of the hydrolysis of laminarin. The hydrolysis of laminarin and paramylon could be substantially blocked by chemical modification of both nonreducing and reducing glucosyl residues but not by modification of reducing residues alone. These observations are consistent with the presence of an exo-hydrolase (β-1,3-glucan glucohydrolase) cleaving terminal, nonreducing glucose residues from these substrates.

3. The extracts also reduced the viscosity of carboxymethylpachyman solutions indicating the presence of an endohydrolase (β-1,3-glucan glucano-hydrolase). Extracts showing little or no endo-hydrolase activity could be obtained by breaking the cells at pH 4.5 rather than pH 5.2, or by heating at 55°. Under both these conditions the exo-hydrolase retained most of its activity.

4. In extracts centrifuged at 9000 × *g* for 30 min, the exohydrolase was present mainly in the supernatant fraction. Variable amounts of the endo-hydrolase (15–39%) were found associated with the washed pellet which consisted largely of paramylon granules.

## INTRODUCTION

Euglenoid flagellates store carbohydrate in characteristic granules, which in all species so far investigated are composed of the essentially linear β-1,3-glucan, para-

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mylon[1]. The enzymes from euglenoids which depolymerise β-1,3-glucans or oligoglucosides may be either hydrolytic[2-4] or phosphorolytic[5-10]. This paper reports the results of a study of the β-1,3-glucan hydrolases from *Euglena gracilis* var. bacillaris.

## EXPERIMENTAL PROCEDURE

### *Organism and culture*

An ultraviolet-bleached strain of *E. gracilis* var. bacillaris, obtained through the courtesy of Dr. J. A. Schiff, Department of Biology, Brandeis University (Waltham, Mass.), was used throughout. Cells were grown in CRAMER–MYERS[11] medium using 1% glucose as carbon source. Cultures were incubated at 26° in the dark in 500-ml conical flasks containing 200 ml of medium and shaken at 130 rev./min. Large-scale cultures were grown in 2 l of medium contained in 10-l vessels which were rotated at 60 rev./min on rollers set at an angle of 30° to the horizontal.

### *Preparation of cell-free extracts*

Cells were harvested from the culture medium by centrifugation at 1000 × *g* for 5 min and the pellet was washed twice with distilled water, twice with ice-cold buffer (0.1 M sodium acetate, pH 4.5 or pH 5.2) and finally suspended in the buffer. Cells were disrupted by passage through a French press (American Instrument Co., Silver Spring, Md.) at an applied pressure of 2 tons (on a 2-inch diameter ram). The whole extract (100% cell breakage) was centrifuged at 9000 × *g* for 30 min, and the supernatant was filtered through glass wool and dialysed overnight against ice-cold buffer. Any precipitate which formed during dialysis was removed by centrifugation. Extracts were either assayed immediately, stored for short periods at 4° or frozen at −20°.

### *Substrates*

*Insoluble laminarin.* The sample used, designated IL 13, was from the Seaweed Research Institute (Midlothian, Scotland) and was a gift of Prof. D. J. Manners, Department of Brewing and Biochemistry, Heriot Watt University (Edinburgh, Scotland). Before use the crude polysaccharide was dissolved in distilled water at 80° and filtered while hot. The solution was allowed to stand for several days at 4° after which time a dark brown precipitate was removed by centrifugation. The supernatant was allowed to stand for a further 5–7 days at 4° when a copious white precipitate was formed. This precipitate was collected by centrifugation and washed several times with distilled water. It was then reprecipitated in the same way from a hot water solution. The precipitate was finally dialysed exhaustively against distilled water, freeze-dried and stored as a powder.

*Pachyman.* Sclerotia of *Poria cocos*, a gift of Prof. K. Nisizawa, Botanical Institute, Tokyo University of Education (Tokyo), were ground to a fine powder, defatted by refluxing for 24 h with chloroform–ethanol (2:1, v/v), and the extracted powder was air-dried.

*β-1,3-Oligoglucosides.* Individual members of the series from laminaribiose to laminaripentaose in a pachyman hydrolysate were separated on a column of Nuchar C unground charcoal (West Virginia Paper and Pulp Co., New York, N.Y.)[12,13]. Final purification of individual oligosaccharides was achieved where necessary by gradient elution from a charcoal column saturated with stearic acid[14,15].

*Paramylon granules.* Granules were isolated[16] from the pooled residues of numerous Euglena extracts which had been stored at −20° until used.

*Dispersed paramylon.* A thick suspension of paramylon granules was chilled in an ice bath and an equal volume of an ice-cold solution of 2 M NaOH, containing 1% sodium borohydride, was added slowly with constant stirring. On neutralising the viscous solution with 2 M HCl a thick gel was formed which was then suspended in water and dialysed until free from chloride. This suspension was stored frozen since after freeze-drying the alkali-treated paramylon could not be readily dispersed in water without retreatment with NaOH.

*Carboxymethylpachyman*[17]. This substrate had a degree of substitution of 0.30 as determined by the acid-wash method[18].

*Polyalcohols derived from laminarin and paramylon.* Laminarin and paramylon polyalcohols were prepared by periodate oxidation, followed by borohydride reduction of the product[19]. Samples which had been treated with periodate previously inactivated with ethylene glycol and then with sodium borohydride served as controls for the effect of borohydride on the substrates. A sample of laminarin polyalcohol was hydrolysed under mild acid conditions (0.005 M $H_2SO_4$, room temperature) to regenerate the nonreducing glucosyl chain ends[19].

*Analyses*

Total reducing sugars in enzymic digests were determined as glucose by the SOMOGYI–NELSON method[20,21]. Glucose was specifically estimated by the glucose oxidase method[22–24]. Protein was determined with the Folin–phenol reagent[25], using bovine serum albumin as standard.

*Enzyme assays*

Enzyme activity was determined by the release of reducing sugars or glucose from insoluble laminarin, paramylon or CM-pachyman as follows: substrate (0.5 ml) in 0.1 M sodium acetate (pH 5.2) was incubated with shaking at 40° with 0.5 ml of extract. The final substrate concentrations were laminarin, 0.8% (w/v), paramylon granules, 3% (w/v) and CM-pachyman, 0.8% (w/v). The incubation time for laminarin was 30 min and for paramylon granules and CM-pachyman was 15 h. Reactions were terminated either by the addition of 1.0 ml of 0.15 M $Ba(OH)_2$ and 1.0 ml of 5% $ZnSO_4$ or by heating the reaction mixture in a sealed tube in a boiling-water bath for 3 min. The $Ba(OH)_2$–$ZnSO_4$ precipitation method could not be used with CM-pachyman hydrolysates due to precipitation of the ionised products of hydrolysis. The inactivated reaction mixtures were assayed for reducing sugars or glucose as described. Results are recorded as the amount of glucose or reducing sugars (as glucose) per incubation period per ml of extract (or mg of protein).

Viscometric assays were made with CM-pachyman as substrate as previously described[17].

*Chromatography*

The products of enzymic hydrolysis were examined by paper chromatography. Incubations were performed in 0.01 M sodium acetate (pH 5.2). 100-μl portions of the reaction mixture were removed at intervals and spotted directly onto Whatman No. 3 chromatography paper. The chromatograms were developed in propan-1-ol–ethyl

TABLE I

SUBSTRATE RANGE OF EUGLENA EXTRACT

Substrate (1.5–3.0 mg) was incubated at 40° and pH 5.2 with the extract in a total volume of 0.5 ml. 0.1-ml samples were removed at intervals, heated on 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; −, no hydrolysis. Abbreviations: Glc, glucose; Lb, laminaribiose; Ltr, laminaritriose; Ltet, laminaritetraose; Lpent, laminaripentaose; oligo, oligosaccharides; Gb, gentiobiose.

| *Substrate* | *Linkage type* | *Degree of hydrolysis* | *Products of hydrolysis* |
|---|---|---|---|
| Laminarin | β-1,3 | +++++ | Glc, Lb, 2 oligo* |
| Laminarin polyalcohol | β-1,3 | ++ | Glc, 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, oligo (48 h) |
| Laminaribiose | β-1,3 | + | Glc |
| Laminaritriose | β-1,3 | +++ | Glc, Lb |
| Laminaripentaose | β-1,3 | ++++ | Glc, Lb, Ltr, Ltet |
| Claviceps glucan | β-1,3; β-1,6 | ++++ | Glc, Gb |
| Yeast glucan | β-1,6; β-1,3 | +++ | Glc |
| Eisenia glucan | β-1,3; β-1,6 | +++ | Glc, oligo |
| Avicell | β-1,4 | − | |
| Swollen cellulose | β-1,4 | + | Glc |
| Cellobiose | β-1,4 | ++ | Glc |
| Cellopentaose | β-1,4 | ++ | 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 |
| Caulerpa xylan | β-1,3 | − | |
| Soluble starch | α-1,4 | ++ | Glc, oligo |
| Maltose | α-1,4 | ++ | Glc |
| Crown gall polysaccharide | β-1,2 | − | |
| Sophorose | β-1,2 | − | |
| Lutean | β-1,6 | ++ | Glc |
| Gentiobiose | β-1,6 | +++ | Glc |
| Dextran | β-1,6 | ++ | Glc |
| Salicin | | + | |
| Sucrose | | − | |
| α,α′-Trehalose | | ++ | Glc |

* Most likely gentiobiose and 1-*O*-β-(glucosyl)-D-mannitol.

acetate–water (6:1:3, v/v/v). The compounds were located by staining with the alkaline $AgNO_3$ reagent[26].

RESULTS

*Action of Euglena extracts on glycosidic substrates*

Table I shows that the most readily hydrolysed substrates were the β-1,3-glucans and the β-1,3-oligoglucosides, although a number of other glycosidic substrates were hydrolysed to a minor extent.

*Action pattern of Euglena extracts on various β-1,3-glucan substrates*

*Insoluble laminarin.* The progress curves for the release of glucose and total

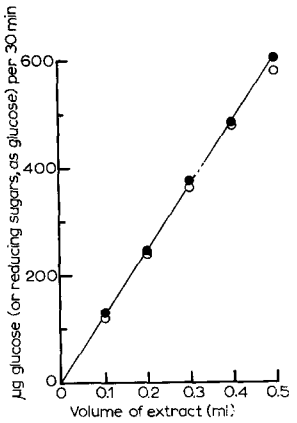


Fig. 1. Hydrolysis of insoluble laminarin by a Euglena extract. Effect of extract dilution on rate of reaction. The cell-free extract was prepared in 0.1 M sodium acetate buffer (pH 5.2) and was incubated with insoluble laminarin at a final concentration of 0.8% (w/v). ○—○, total reducing sugars, as glucose (SOMOGYI–NELSON[20,21]); ●—●, glucose (glucose oxidase).

reducing sugars from laminarin were linear for 4 h under the incubation conditions used (Fig. 4a, upper curves). During this period all the reducing sugars liberated could be accounted for as glucose. The initial rate of hydrolysis was also linear with respect to extract concentration in the range tested (Fig. 1).

*Laminarin polyalcohol.* When laminarin polyalcohol was the substrate, the production of reducing sugars was much lower than from laminarin and a significant proportion of sugars other than glucose was formed (Fig. 4a, lower curves).

*Chromatographic examination of hydrolysis products*

When enzymic digests were examined by paper chromatography, it was found

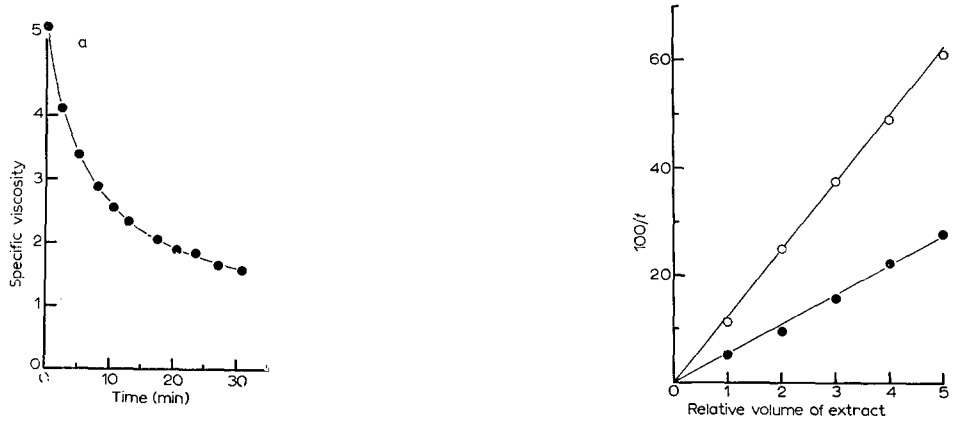


Fig. 2. Action of a Euglena extract on CM-pachyman. The extract was prepared as described for Fig. 1. a. Decrease in specific viscosity of a solution of CM-pachyman (0.8%, w/v) (pH 5.2). b. Plot of $100/t$ against the relative extract volume, where $t$ is the time taken to reduce the specific viscosity of the solution by 20% (○—○), or 30% (●—●).

that glucose was the sole product of the hydrolysis of paramylon and pachyman and also of laminarin in the initial stages of the hydrolysis. In the case of laminarin, products other than glucose were apparent after 30 min and increased in intensity as the hydrolysis was prolonged. The $R_{Glc}$ values of these products did not correspond to those of members of the $\beta$-1,3-oligoglucoside series and they were tentatively identified as gentiobiose and 1-*O*-$\beta$-(glucosyl)-D-mannitol by a comparison with $R_{Glc}$ values given by BULL[27] for this solvent.

*Hydrolysis of CM-pachyman.* Extracts which produced glucose from laminarin also reduced the viscosity of solutions of CM-pachyman (Fig. 2a). For the viscometric assay a linear relationship between $100/t$, where $t$ is the time taken to reduce the specific viscosity by 20% and 30%, and the relative volume of the extract, is shown in Fig. 2b.

*Comment*

The rapid release of glucose from the $\beta$-1,3-glucan substrates and the substantial blocking of this release by modification of the terminal nonreducing residues of laminarin and paramylon suggested that a $\beta$-1,3-glucan exo-hydrolase was present in the extracts. However the slow release of reducing groups from the modified laminarin and paramylon and the ability of the extract to reduce the viscosity of a solution of CM-pachyman indicated that in addition to the exo-glucanase, a $\beta$-1,3-glucan endo-hydrolase was also present. The presence of these two hydrolases was confirmed in a further series of experiments in which the viscometric assay was used to measure the activity of the endohydrolase and the reductometric assay to measure the exo-hydrolase activity.

*Effect of heating on the hydrolases of the Euglena extract*

The effect on the exo- and endo-hydrolase activities of heating a pH 5.2 extract for 1 min at 55° is shown in Table II.

*Effect of the pH of extracting medium on the activity of the exo- and endo-hydrolases*

Extracts were prepared in the usual way at four different pH's and assayed reductometrically against laminarin and viscometrically against CM-pachyman at pH 5.2. The results are shown in Table III and Figs. 3a and b.

Although on a volume basis the pH 5.2 extract showed the highest activity

TABLE II

EFFECT OF HEATING ON THE EXO- AND ENDO-HYDROLASES

The extract was prepared at pH 5.2 and assayed reductometrically against 0.8% (w/v) laminarin and viscometrically against 0.8% CM-pachyman before and after heating for 60 sec on a water bath at 55°.

| *Assay* | *Before heating* | *After heating* | *Inactivation (%)* |
|---|---|---|---|
| Reductometric | 2240 ($\mu$g glucose per 30 min per ml) | 1890 ($\mu$g glucose per 30 min per ml) | 16 |
| Viscometric | 89 ($100/t_{20}$) | 10.9 ($100/t_{20}$) | 88 |

TABLE III

EFFECT OF THE pH OF PREPARATIVE BUFFER ON THE ABILITY OF EUGLENA EXTRACTS TO HYDROLYSE INSOLUBLE LAMINARIN

Four extracts were prepared from suspensions containing equal numbers of cells. The following buffers were used: 0.05 M sodium acetate (pH 4.5 and pH 5.2); 0.05 M sodium maleate (pH 6.2 and pH 6.8). All assays were performed at pH 5.2 at a laminarin concentration of 0.8% (w/v).

| *pH of preparative buffer* | *Protein in extract supernatant (mg/ml)* | *Activity* | |
|---|---|---|---|
| | | *μg Glucose per 30 min per ml extract* | *μg Glucose per 30 min per mg protein* |
| 4.5 | 0.95 | 1300 | 1366 |
| 5.2 | 3.89 | 1600 | 412 |
| 6.2 | 6.16 | 1450 | 235 |
| 6.8 | 6.30 | 1150 | 182 |

towards laminarin, the specific activity was greatest when the extraction pH was 4.5. Furthermore, the pH 5.2 extract rapidly reduced the viscosity of the CM-pachyman solution whereas the pH 4.5 extract, while possessing 81% of the activity of the pH 5.2 extract in the reductometric assay, did not reduce the viscosity of the CM-pachyman solution.

*Action of pH 5.2 and pH 4.5 extracts on laminarin polyalcohol*

The possibility that an endo-hydrolase was present in the pH 5.2 extract was tested in the following way. Extracts were prepared at pH's 4.5 and 5.2 from equal numbers of cells. The dialysed supernatants were assayed for activity towards la-

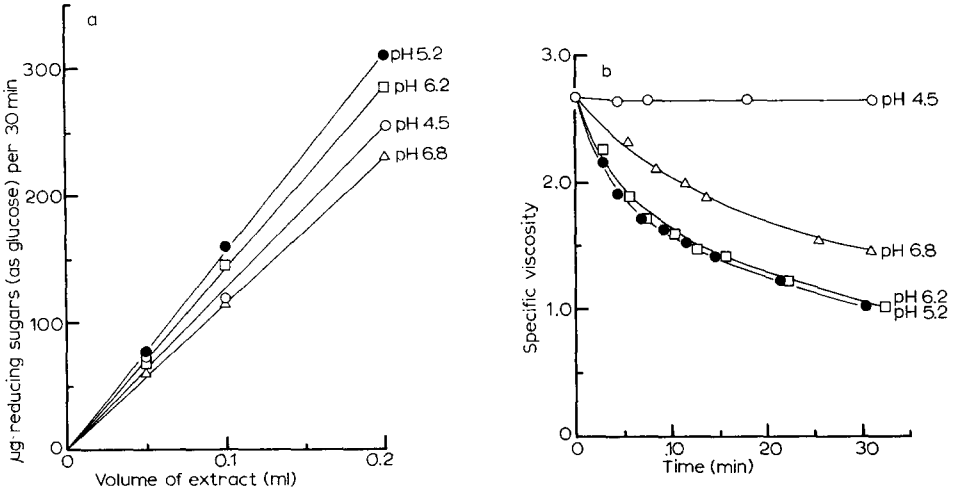


Fig. 3. Effect of the pH of cell extract preparation on the activity of Euglena extracts towards insoluble laminarin and CM-pachyman. For conditions of preparation see Table III. a. Effect on activity towards 0.8% (w/v) laminarin, assayed in 0.1 M sodium acetate buffer (pH 5.2). b. Effect on activity towards 0.8% (w/v) CM-pachyman at pH 5.2.

minarin and the more active preparation diluted so that the two extracts had equal activities on this basis. The pH 5.2 extract actively reduced the viscosity of CM-pachyman but the pH 4.5 preparation did not.

The two extracts were incubated at pH 5.2 with laminarin polyalcohol, reduced laminarin, acid-hydrolysed laminarin polyalcohol and untreated laminarin. Reducing sugars and glucose were measured in samples of the hydrolysates taken at regular intervals. The progress curves for the hydrolysis of laminarin and laminarin poly-

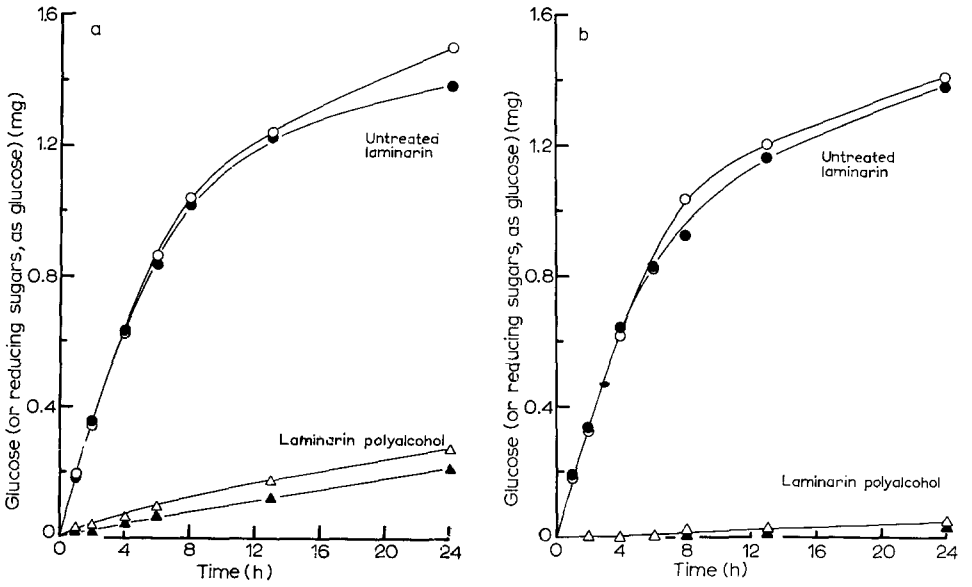


Fig. 4. Action of a Euglena extract prepared at pH 5.2 (a), and pH 4.5 (b) on untreated laminarin and laminarin polyalcohol. The final substrate concentration was 0.2% (w/v) in sodium acetate buffer (pH 5.2). Untreated laminarin: ○—○, total reducing sugars, as glucose; ●—●, glucose. Laminarin polyalcohol: △—△, total reducing sugars, as glucose; ▲—▲, glucose.

alcohol by the pH 5.2 extract are shown in Fig. 4a and for the pH 4.5 extract in Fig. 4b. For both extracts the time-course of hydrolysis of reduced laminarin and acid-hydrolysed laminarin polyalcohol was identical with that of untreated laminarin. The pH 5.2 extract released significant amounts of reducing sugars and glucose from laminarin polyalcohol, whereas the amounts of these products formed by the pH 4.5 extract were very small.

*Action of pH 5.2 and pH 4.5 extracts on paramylon polyalcohol*

The two extracts were tested in a similar manner with dispersed paramylon and dispersed paramylon polyalcohol as substrates. The results are shown in Figs. 5a and 5b, and in general they were the same as for the modified laminarin substrates. It will be noted that the degree of hydrolysis of dispersed paramylon after 24 h by the pH 5.2 extract was higher than by the pH 4.5 extract.

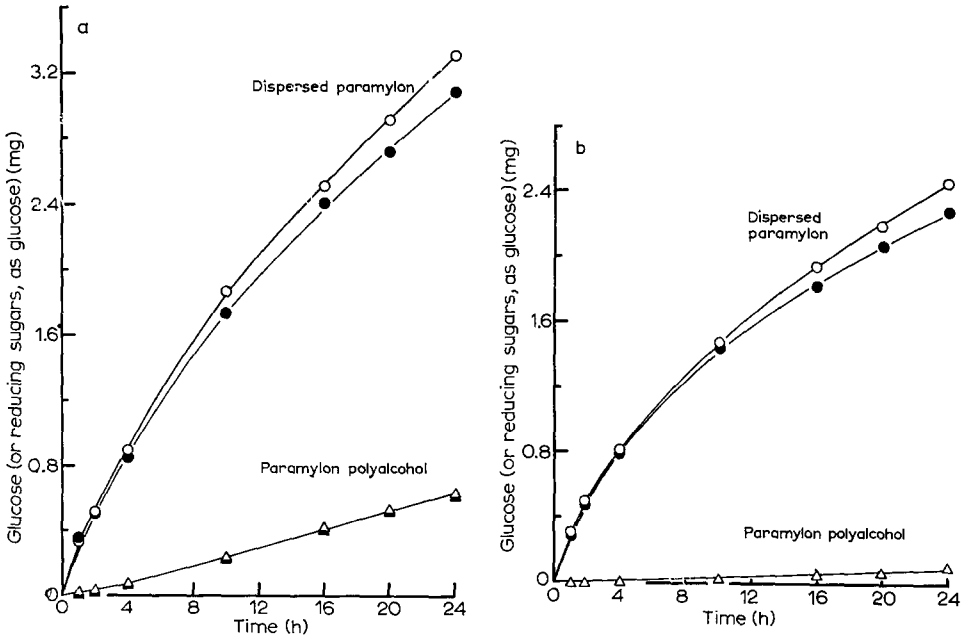


Fig. 5. Action of Euglena extract prepared at pH 5.2 (a), and pH 4.5 (b) on dispersed paramylon and dispersed paramylon polyalcohol. The final concentration of paramylon was 0.09% (w/v). The incubation conditions were similar to those in Fig. 6. The symbols used are the same as for the corresponding laminarin samples in Fig. 4.

*Extent of hydrolysis of laminarin by pH 4.5 and pH 5.2 extracts*

The progress curves for the liberation of glucose from laminarin (0.06%, w/v) by extracts prepared at pH 4.5 and pH 5.2 are shown in Fig. 6. The extracts were adjusted to have the same activity on the basis of assays made with laminarin at a

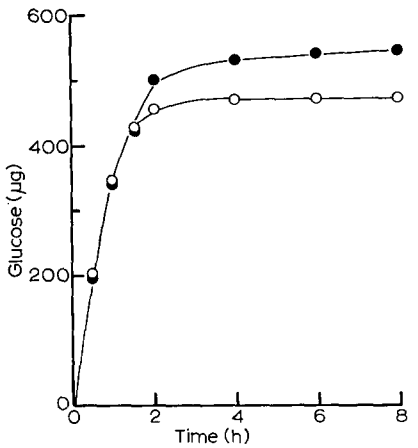


Fig. 6. Effect of pH of extract preparation on the extent of hydrolysis of insoluble laminarin. Extracts were prepared at pH 4.5 and pH 5.2 and their activities adjusted to the same value on the basis of assays with 0.8% (w/v) laminarin at pH 5.2. The figure shows the rate of release of glucose from 0.06% (w/v) laminarin at pH 5.2. ○—○, pH 4.5 extract; ●—●, pH 5.2 extract.

concentration of 0.8% (w/v) *i.e.* well above the substrate saturation concentration which was determined to be 0.1–0.2%.

Only with the pH 5.2 extract did the amount of glucose released approach the theoretical yield, calculated assuming laminarin to be a linear glucan. However, it is possible that the theoretical yield would not be attained because of the deviations in the structure of insoluble laminarin from that of a linear glucan.

*Comparison of the action of the pH 4.5 and pH 5.2 extract on CM-pachyman*

Extracts of Euglena were prepared at pH 4.5 and pH 5.2 as previously described, and their activities matched on the basis of assays on 0.8% laminarin. The rates of decrease in viscosity and release of reducing sugars were measured using 0.8% (w/v) CM-pachyman as substrate and the results are shown in Fig. 7. The maximum

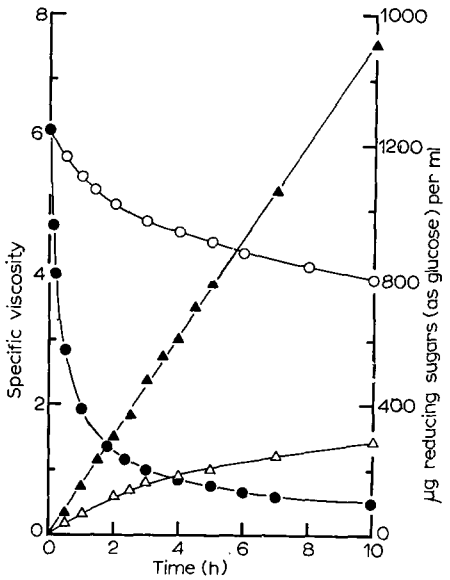


Fig. 7. Reduction in the viscosity and the release of reducing sugars from a solution of CM-pachyman; comparison of extracts prepared at pH 4.5 and pH 5.2. Viscometric assay: 0.8% (w/v) CM-pachyman, pH 5.2. ○—○, pH 4.5 extract; ●—●, pH 5.2 extract. Reductometric assay: substrate conditions as above. △—△, pH 4.5 extract; ▲—▲, pH 5.2 extract. The extracts were matched in activity towards laminarin at pH 5.2 as previously described.

period of incubation reported in Fig. 7 is 10 h, but viscometric and reductometric measurements were continued up to 23 h when the activities measured were as follows:

| | *Specific viscosity* | *Activity (μg reducing sugars per ml)* |
|---|---|---|
| pH 4.5 extract | 3.00 | 385 |
| pH 5.2 extract | 0.29 | 2170 |

TABLE IV

DISTRIBUTION OF THE ENDO-HYDROLASE BETWEEN FRACTIONS OF EUGLENA EXTRACTS

The fractions were assayed against CM-pachyman by the viscometric procedure. Units were expressed as $100/t$ where $t$ is the time taken to reduce the specific viscosity of the solution of CM-pachyman by 20%.

| *Fraction* | *Units/ml of extract* | | | | | *% of units in the whole extract* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Extract No.:* | | | | | *Extract No.:* | | | | |
| | *1* | *2* | *3* | *4* | *5* | *1* | *2* | *3* | *4* | *5* |
| Whole extract | 7.0 | 25.0 | 25.0 | 32.3 | 31.3 | | | | | |
| 9000 × *g* supernatant | 4.1 | 15.5 | 19.0 | 19.6 | 22.9 | 58 | 62 | 76 | 61 | 73 |
| 9000 × *g* pellet | 2.6 | 4.8 | 4.2 | 9.1 | 4.0 | 37 | 19 | 17 | 28 | 13 |
| % Recovery of units in whole extract | | | | | | 95 | 81 | 93 | 89 | 86 |

It is evident that the pH 5.2 extract had a much higher endo-hydrolase activity than the pH 4.5 extract, which in this case was low but measurable in contrast to the endo-hydrolase activity of the pH 4.5 extract used in the experiment in Fig. 3b. Furthermore, there was a continuous linear rate of release of reducing sugars by the pH 5.2 extract up to 23 h, but with the pH 4.5 extract the rate was initially lower and decreased throughout the incubation period to a very low value.

*Subcellular distribution of exo- and endo-hydrolases in Euglena*

Data from experiments with five extracts prepared at pH 5.2 gave an indication of the intracellular distribution of the two β-1,3-glucan hydrolases in Euglena. The activity of the exo- and the endo-enzymes was measured in the whole extract, the supernatant after centrifugation at 9000 × *g* for 30 min and the pellet from this centrifugation after three washings with 0.1 M sodium acetate buffer (pH 5.2). The results for the β-1,3-glucan endo-hydrolase are given in Table IV and for the β-1,3-glucan exo-hydrolase in Table V. The linear response of the viscometric assay system using the whole extract and the pellet suspension was confirmed.

TABLE V

DISTRIBUTION OF THE EXO-HYDROLASE BETWEEN FRACTIONS OF EUGLENA EXTRACTS

The fractions were assayed for the release of glucose from 0.8% solutions of laminarin under the standard incubation conditions. Units are expressed as μg glucose per 30 min.

| *Fraction* | *Units/ml of extract* | | | | | *% of units in the whole extract* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Extract No.:* | | | | | *Extract No.:* | | | | |
| | *1* | *2* | *3* | *4* | *5* | *1* | *2* | *3* | *4* | *5* |
| Whole extract | 150 | 340 | 260 | 210 | 220 | | | | | |
| 9000 × *g* supernatant | 125 | 250 | 200 | 200 | 210 | 83 | 73 | 77 | 95 | 95 |
| 9000 × *g* pellet | 11 | 31 | 34 | — | — | 8 | 9 | 13 | — | — |
| % Recovery of units in whole extract | | | | | | 91 | 82 | 90 | 95 | 95 |

## DISCUSSION

The presence of an active β-1,3-glucan exo-hydrolase (β-1,3-glucan glucohydrolase) in extracts of Euglena was originally suggested by the observations of FELLIG[2], TOCHER[3] and MEEUSE[4], and more recently by VOGEL AND BARBER[28]. The results presented here confirm the presence of this enzyme. Its purification and some of its properties are reported in the accompanying paper[29].

The existence of a second type of β-1,3-glucan hydrolase in Euglena extracts became evident during the examination of the properties of the exo-hydrolase. Extracts prepared at pH 5.2 could rapidly reduce the viscosity of CM-pachyman with the concomitant release of very small amounts of reducing groups (Fig. 7). This behaviour is characteristic of endo-hydrolases, enzymes which cleave internal linkages in polymeric substrates. Using a viscometric assay to measure the endo-hydrolase and a reductometric assay for the exo hydrolase, it was shown that the endo-hydrolase, but not the exo-hydrolase, was inactivated when the pH was held at 4.5 during preparation of the Euglena extract (Fig. 3). The endo-hydrolase was also inactivated by a brief heating at 55°, whereas the exo-hydrolase retained most of its activity (Table II). Subsequent experiments reported in the accompanying paper[29] showed that the exo- and endo-hydrolase could be separated by exclusion and ion exchange chromatography.

The presence of the endo-hydrolase in the pH 5.2 extract explains the appearance of reducing sugars in the hydrolysates of laminarin and paramylon polyalcohol (Figs. 4a and 5a). The endo-hydrolase action on internal glycosidic linkages of these substrates would make unmodified nonreducing chain ends available for attack by the exo-hydrolase. This is also suggested by the finding (Fig. 6) that the pH 5.2 extract containing both enzymes was able to hydrolyse insoluble laminarin more extensively than the preparation containing only the exo-hydrolase.

The dependence of the rate of reducing sugar liberation by the exo-hydrolase on the availability of chain ends is shown by the comparison of the curves in Fig. 7. The zero-order kinetics exhibited by the pH 5.2 preparation may be explained if it is assumed that there is a constant release of hydrolysable nonreducing end groups by the endo-hydrolase which maintains a saturating substrate concentration for the exo-hydrolase over the period of incubation. The low level of endo-hydrolase in the pH 4.5 extract is apparently insufficient to maintain a saturating substrate concentration.

Carboxymethyl substitution of the β-1,3-glucan decreases the hydrolysis by the exo-hydrolase, as shown by the lower initial rate of release of reducing groups from CM-pachyman (Fig. 7) than from paramylon (Fig. 5b), even though the exo-hydrolase activity (based on a reductometric assay with laminarin) of the pH 4.5 preparation used in the CM-pachyman experiment was 6 times higher than for the paramylon hydrolysis. It is probable that only linkages joining unsubstituted glucosyl residues are hydrolysed by the exo-hydrolase, since glucose in small amounts was the only product observed on chromatographic examination of the products of CM-pachyman hydrolysis by the purified exo-hydrolase[29].

The endo-hydrolase proved to be susceptible to heat inactivation and was also denatured during the breakage of the cells in the French press at pH 4.5. In contrast, the exo-hydrolase was stable under both conditions. These differences in stability may be related to the different sub-cellular location of the two enzymes (Tables IV and V). The exo-hydrolase is essentially a soluble enzyme whereas a significant, although

variable, proportion of the endo-hydrolase is sedimentable and appeared, in subfractionation experiments, to be associated with the paramylon granules. There is morphological evidence for a membranous structure associated with the surface of the paramylon granules[30–35] and it seems possible in view of the present findings that the endo-hydrolase is associated with this membrane or with the paramylon granule surface.

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