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Action of Chitinase on Spines of the Diatom *Thalassiosira fluviatilis*

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SUMMARY

An electron microscopic study of the degradation of the chitin spines of the diatom Thalassiosira fluviatilis *by* Streptomyces *chitinase revealed that only the apices of fibrils are broken down. The result suggests that the enzymolysis of crystalline chitinous structures may be rate-limited by the area of microfibril apices available, which varies with chitin source. A suspension of spines can be used as an assay for chitinase activity by monitoring the rate of loss of turbidity, and although this is not a sensitive assay, it does allow assessment of activity on a crystalline substrate.*

INTRODUCTION

Studies on the action of chitinolytic enzymes have revealed that the complete hydrolysis of chitin to *N*-acetylglucosamine (GlcNAc) monomers is typically brought about by two enzymes acting consecutively. Chitinase (poly-β(1 → 4) (2-acetamido-2-deoxy)-D-glucoside glycanohydrolase, E.C.3.2.1.14) hydrolyses tetramers and higher oligomers to dimers which are cleaved by chitobiase (chitobiose acetamidodeoxyglucohydrolase, E.C.3.2.1.30) to produce monomers (Jeuniaux, 1966; Mommsen, 1980). The enzymes are distributed widely throughout the living world (Muzzarelli, 1977), but the exact process by which the

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chitin microfibrils of naturally occurring chitinous structures are broken down remains unclear.

A study by Berger & Reynolds (1958) revealed that whereas the *Streptomyces griseus* chitinase system attacked soluble chitin oligosaccharides randomly (indicated by the occurrence of intermediate oligomers as well as dimers in the reaction mixture after incubation), the same enzyme appeared to act only on terminal disaccharides when colloidal chitin purified from crab shells was used as substrate (indicated by the presence of only dimers in the reaction mixture after incubation). This suggests that only the ends of chitin microfibrils are attacked by this chitinase. This notion, however, has not been investigated in detail in spite of the abundance of chitin. Arguably, the annual production of chitin exceeds that of cellulose (Berkeley, 1979) and its degradation in the environment, which is probably exclusively enzyme-mediated, must play a significant role in carbon and nitrogen cycles, especially in aquatic systems.

The chitin substrate used by Berger & Reynolds (1958) had been altered from its native state by a harsh purification procedure, involving acid/alkali extraction, which would alter characteristics such as solubility, molecular weight, optical rotation and acetyl values (Brine & Austin, 1981), which in turn would affect its specificity as a chitinase substrate. It would be desirable, therefore, to examine the action of chitinase on pure, unmodified, crystalline chitin. The extracellular β-chitin spines of the centric diatoms such as *Thalassiosira fluviatilis* are the only idealised chitin known in nature, being completely acetylated and not associated with other substances (McLachlan *et al.*, 1965). The strap-like spines are up to 80 μm long, about 150 nm wide and 50 nm thick although exact dimensions vary with culture conditions. Three microfibrils constitute a spine, running continuously along its length, and each microfibril is composed of GlcNAc chains arranged parallel both to each other and to the longitudinal axis of the structure (Dweltz *et al.*, 1968). The potential of centric diatoms for the production of pure chitin has been assessed by Allan *et al.* (1978).

In this report we describe results of a study of the action of *Streptomyces* chitinase on the chitin spines of *Thalassiosira fluviatilis*. There were three aims: primarily to investigate the mode of action of the chitinase preparation on a defined substrate, but also to assess the suitability of a suspension of spines as a turbidometric substrate for chitinase, and to investigate further the structure of the spines.

MATERIALS AND METHODS

Preparation of spines

Culture medium was prepared with 80% seawater collected from Cove Bay near Aberdeen, filtered through GF/C glass microfibre filters and aged for at least 4 weeks at 4°C prior to use. The diatom was grown in the enriched seawater medium of Laing (1979) modified by the addition of filtered (0·22 μm) penicillin 0·1% (w/v) (sodium salt) and streptomycin 0·05% (w/v).

A unialgal culture of *Thalassiosira fluviatilis* Hustedt was supplied by Dr J. McLachlan, Atlantic Regional Laboratory, Halifax, Nova Scotia. Approximately 10 ml of a stock culture (maintained at 4°C and transferred to fresh medium at 6-week intervals) was used to inoculate 100 ml of medium in 150 ml Erlenmeyer flasks and the algae grown under cool white fluorescent light (intensity 50 $\mu E\ m^{-2}\ s^{-1}$) at 20°C for 7 days. These cultures were used to inoculate either 1·6 litres of medium in 2-litre Erlenmeyer flasks or, for larger-scale preparations, 18-litre of medium in 20-litre Pyrex vessels. The cultures were supplied with a 1·5% CO_2/98·5% air mixture, fed through a filter series terminating in a 0·15 μm membrane filter at a rate of 100 ml min^{-1} per flask or 1 litre min^{-1} per larger vessel and grown under cool white fluorescent light (intensity 130 $\mu E\ m^{-2}\ s^{-1}$) at 20°C for 72 h.

For electron microscopy, suspensions of spines were prepared by centrifuging a 1·6-litre culture at 4000 *g* for 15 min, and discarding the pellet of cells. The suspended spines were concentrated into a small volume by filtration using a 3-μm membrane filter. They were then washed three times with 50 ml of distilled water, reconcentrating the spines on each occasion. The amount of chitin present was estimated (turbidometrically) as 0·025 mg/ml. Light microscopic examination (× 1000, dark field) revealed that the preparation consisted of spines and spine fragments free of cellular debris.

For other studies, spines were prepared from 72 litres of culture by a modification of the method of McLachlan *et al.* (1965). The culture was processed in a Waring blender for 2–3 s to dislodge the spines with minimum cell disruption, and allowed to sediment for 24 h at 20°C. The supernatant was decanted for processing and the sediment was vigorously shaken and centrifuged (5000 *g*, 5 min). The supernatants were combined and centrifuged in a continuous flow rotor at 12 000 *g*,

500 ml min^{-1}. Spines were collected from the supernatant by membrane filtration (3 μm filter, 142 mm diameter), and washed twice by resuspension in 1 litre distilled water and centrifugation (23 000 *g*, 90 min), and then successively with 1 litre each of 95% ethanol, methanol and acetone, centrifugation in each case being 20 000 *g*, 20 min. The final pellet was freeze-dried to yield a white leathery mass (approximately 350 mg from 72 litres of culture).

The chemical nature of the spines as pure chitin was confirmed by (a) i.r. spectroscopy in a KBr disc, giving a very similar spectrum to those of Falk *et al.* (1966) and McLachlan & Craigie (1966); (b) X-ray diffraction, giving a very similar pattern to those of Falk *et al.* (1966) and Herth & Zugenmaier (1977); (c) acid hydrolysis (6 N HCl, 10 h, 100°C) giving glucosamine as product (with the merest trace of amino acids).

Electron microscopy of spine enzymolysis

Suspended spines (0·5 ml) were mixed with 0·5 ml of a 5 mg/ml solution of *Streptomyces* chitinase (Calbiochem-Behring, USA; purified by the method of Jeuniaux, 1966) prepared in 0·025 M citric acid : NaOH buffer, pH 5·4, in a 1 ml glass reaction vial and incubated at 37°C in a shaker operating at 100 motions per minute. In the controls, heat-treated enzyme solution (100°C for 10 min) was substituted.

Samples from the reaction mixtures were taken initially and at intervals over 6 h and examined as follows. A drop of reaction mixture and a drop of negative stain (potassium phosphotungstate, made by adjusting phosphotungstic acid, 2%, to pH 6·9 with KOH) were mixed and dried at room temperature on a carbon/formvar-coated copper grid and examined in a JEOL CX electron microscope operating at 80 kV.

Measurements of length were made from micrographs printed with a magnification of × 5000. Width of spines was measured on micrographs printed with a magnification of × 60 000, as mean distance across the axis of spines at different points along their length at least 1 μm from their apices. (It was usually not possible to distinguish width from height in these negatively stained preparations, but the low standard errors of measurements suggested that most were truly of width.)

Attempts at comminuting diatom chitin

In order to produce uniform suspensions of fragmented spines, the following homogenising instruments were tried: a Potter-Elvehjem

homogeniser for up to 30 min, a Polytron homogeniser (Northern Media Supply Ltd, Brough, UK) for up to 2 min, an MSE top drive homogeniser (MSE Ltd, Crawley, UK) for up to 20 min, a Braun MSK mill with glass beads for 1 min, frozen samples in an X-press (Jarfalla, Sweden), a French press, frozen samples in an agate pestle and mortar, and an agate multiple micromill (Agate and General Stone Cutters Ltd, London, UK) for up to 20 min.

Turbidometric assay of spine enzymolysis

The diatom chitin, when resuspended, had a gel-like characteristic that lent itself to a turbidometric assay procedure. The spines were suspended at 1 mg ml^{-1} in 0·1 M-citrate : NaOH buffer, pH 6·25, and homogenised at high speed for 10 min at 0°C in a Potter–Elvehjem homogeniser. To 1 ml of this suspension was added 0·5 ml enzyme preparation, both having been pre-warmed to 37°C, and absorbance at 450 nm was monitored.

RESULTS

Electron microscopy showed that most spines as prepared here were pointed at both ends (Fig. 1a–c) but it was not considered feasible to recognise proximal and distal ends unequivocally. In zero time and heat-treated enzyme controls, most spines were long (> 16 μm), but incubation with the *Streptomyces* enzyme preparation resulted in a considerable shortening, so that after 6 h most fragments were 2–4 μm long (Figs 1f, 2). During this shortening, the ends of the spines remained pointed (Figs 1e, f, 3). The width of the spines showed no change on incubating with the chitinase, with measurements of 72 ± 2·5 nm (± S.E., $n = 36$) and 75 ± 4·2 nm ($n = 32$) respectively for 6 h enzyme treated and 6 h control samples (cf. Fig. 3). A similar pattern of chitinolytic attack on the spines was observed when they were treated with a cod gastric chitinase preparation (results not shown).

About half of the spines in all reaction mixtures had some degree of splitting (e.g. Fig. 1d). Categorisation of these splits into large (> 2 μm) and small (< 2 μm), terminal or central, revealed no differences in splitting that could be related to enzyme activity. Thus the splits are probably a consequence of the preparation procedures.

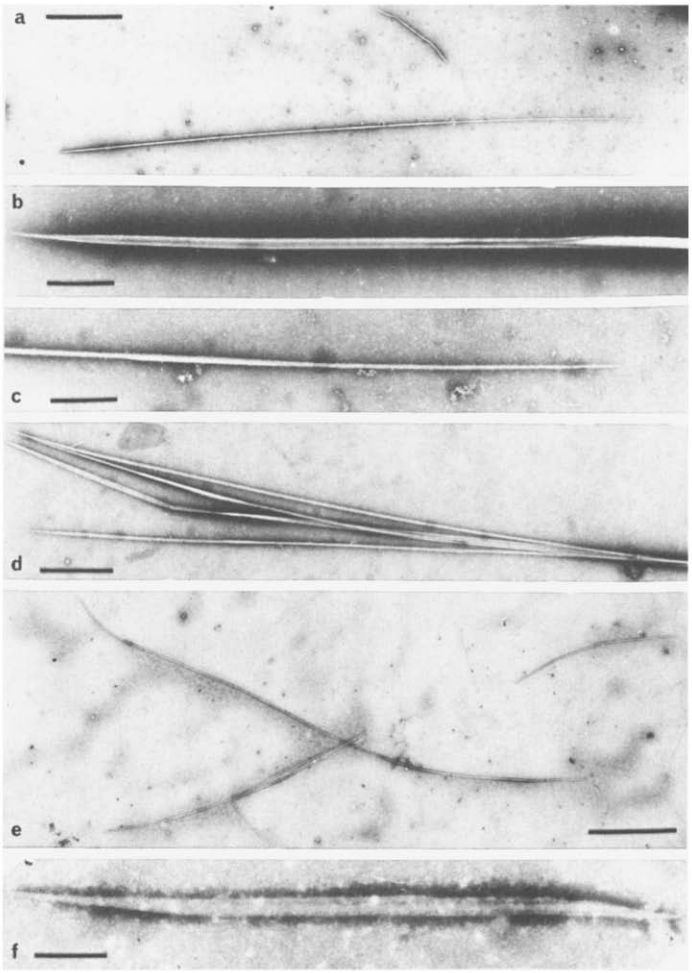

Fig. 1. Electron micrographs of spine preparations incubated with chitinase. a–d, Zero time controls; e, 3 h; f, 6 h. a, One long spine, one short kinked fragment, Mic. no. 3463, scale bar 5 μm; b, c, the two pointed ends of one long spine, Mic. nos. 3469, 3470, scale bars 1 μm; d, split end of a spine, Mic. no. 3471, scale bar 2 μm; e, range of sizes of fragments, Mic. no. 3458, scale bar 10 μm; f, short fragment showing both ends pointed, Mic. no. 3489, scale bar 0·5 μm.

As the electron microscopy showed that chitinase attacked the spines at their ends, short fragments of spines should provide a better substrate for development of a chitinase assay than the long pieces in our pre-

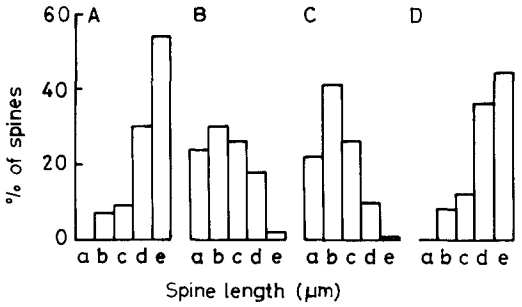

Fig. 2. The effect of chitinase on diatom spine length. A, Zero time control; B, 3 h incubation; C, 6 h incubation; D, 6 h control incubation with boiled enzyme. Spine lengths: a, 0-2 μm; b, 2-4 μm; c, 4-8 μm; d, 8-16 μm; e, > 16 μm.

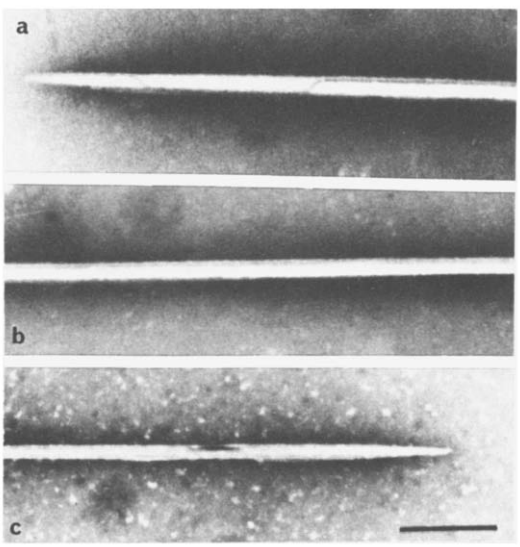

Fig. 3. Electron micrographs of the two ends (a, c) and a mid-section (b) of a spine incubated for 6 h with chitinase. Mic. nos. 3828, 3829, 3844. Scale bar 1 μm.

parations. However, the spines proved to be remarkably resilient, and diverse attempts (outlined in Materials and Methods) to produce broken spine preparations all failed, except for the agate multiple mill which after 20 min at full speed produced some fragmentation, but unfortunately with contamination from the agate balls and chamber.

Thus for the turbidometric assay, the spines were homogenised with a Potter-Elvehjem homogeniser as described earlier, which gave a uniformly turbid suspension, the absorbance of which at 450 nm was proportional to the amount of chitin (Fig. 4). Treatment of these suspensions with chitinase gave a lessening of their turbidity in a non-linear fashion (Fig. 5). Initial rates of reaction could be calculated by the interpolation method of Algranati (1962), using the calibration curve to determine amounts of chitin hydrolysed at regular time intervals after the start of the reaction. Thus the activity shown in Fig. 5 represents an initial rate of hydrolysis of 2·67 μg diatom chitin min^{-1} (mg enzyme preparation)$^{-1}$.

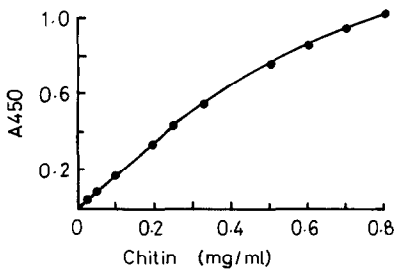

Fig. 4. Absorbance at 450 nm of homogenised suspensions of diatom spines.

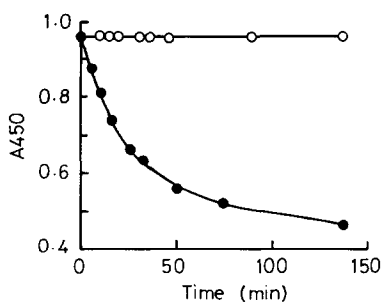

Fig. 5. Turbidity of diatom spine suspensions treated with chitinase (●) or boiled chitinase (○), recorded against a blank of enzyme in buffer.

DISCUSSION

The electron microscopic observation that chitinase hydrolysed the spines only at their apices supports the suggestion that attack on a crystalline chitin is not random, but is confined to the ends of the microfibrils (Berger & Reynolds, 1958). This finding has relevance to the significance of results of assays using non-crystalline chitin as substrate, as they will not allow accurate prediction of breakdown rates of natural chitinous substrates. As the physico-chemical characteristics of chitin microfibrils vary significantly between and within organisms it is to be expected that rates of hydrolysis will vary likewise.

There are many assays available for chitinases (reviewed by Stirling *et al.*, 1979) but nearly all employ soluble or colloidal substrates. The use of diatom spines as an assay for chitinase, although not as sensitive as some other assays, may be a useful adjunct in elucidating enzymic mechanisms, as the spines are physically and chemically well defined, and so provide a highly reproducible substrate.

The non-linear course of reaction (Fig. 4) was also observed by Berger & Reynolds (1958) with colloidal chitin as a turbidometric substrate. We have not investigated the reason for this.

The spines stay in uniform suspension very well during the course of assays (cf. control readings in Fig. 5). This is consistent with their role *in vivo* as form-resistant flotation structures (Walsby & Xypolyta, 1977).

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