Studies on the Digestion of Cellulose by the Larvae of the Eucalyptus Borer, *Phoracantha semipunctata* (Coleoptera: Cerambycidae)

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**Abstract**

Larvae of the native Australian xylophagous insect *P. semipunctata*, the adults of which lay eggs only on *Eucalyptus* species, penetrate the bark, then the phloem and feed at the boundary between the phloem and the xylem, causing deep furrows. As the phloem dries, they penetrate the xylem, which they damage by the formation of galleries. Digestion of wood is facilitated by glycosidases acting not only on oligosaccharides but also on many polysaccharides, notably starch, various hemicelluloses and cellulose. The role of a cellulase produced by the midgut epithelium of the larvae was examined and the characteristics of a partially purified preparation of this enzyme from the midgut epithelium studied. The enzyme was shown to have a molecular weight of less than 34500 and to hydrolyse carboxymethylcellulose and Cellulose–Azure. It was identified as being an endo-β-1,4-glucanase.

**Introduction**

*Phoracantha semipunctata* F. (Coleoptera: Cerambycidae) is a xylophagous insect native to Australia, specific for *Eucalyptus*. The insect has been introduced into several countries, notably New Zealand and the Union of South Africa as well as Israel, Tunisia, Algeria, Morocco, Spain and Italy. Bytinski-Salz and Neumark (1952) referred to very significant damage to the *Eucalyptus* plantations in Israel. More recently, the insect has been introduced into Tunisia: more than three million *Eucalyptus* trees, notably *E. camaldulensis*, *E. diversicolor*, *E. ovata*, *E. tereticornis*, *E. viminalis*, *E. maculata* and *E. gomphocephala*, decayed between 1966 and 1972 in all bioclimatic areas of Tunisia, from the middle and the south to the north of the country (Chararas 1969).

Adults (Fig. 1a) have nocturnal habits. The females insert their ovipositors into cracks in the bark and lay 20–60 eggs, occasionally more (Fig. 1b). They can lay eggs successively on several trees. Immediately after hatching, larvae penetrate the bark and then the phloem. They feed at the boundary between the phloem and the xylem, causing many furrows (Figs 1c and 1d). As the phloem dries, they penetrate the xylem in order to escape bad surface conditions, causing further damage by the formation of galleries 40 cm long and occasionally reaching a depth of 10 cm. They change into pupae, then into imagos, and mature females make their way towards the trunks of other *Eucalyptus* in order to lay eggs. In Tunisia, Chararas et al. (1971) observed two generations in a year. A first generation begins in the second half of
April, the larvae feeding from May to August, and a second generation commences at the beginning of September.

*Eucalyptus* species exposed to adverse ecological conditions (high temperature, low humidity, drying up of soil) are unable to resist vigorously such infestation by

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**Fig. 1.** *Phoracantha semipunctata.* (a) Imago. (b) Eggs. (c) Larval galleries furrowing the xylem. (d) Larvae.
secretion of sap and kino, whereas healthy trees in favourable environments and with phloem osmotic pressure of 1–1.2 kPa (1–12 atm) can defend themselves against the penetration of young larvae, turgescence providing effective resistance to this penetration (Chararas et al. 1971).

Larvae feed in the phloem and then in the xylem (Chararas et al. 1972). Phloem is rich in soluble carbohydrates, notably in glucose, fructose, sucrose and raffinose; arabinose and xylose are also present, as also are starch, cellulose, hemicelluloses (notably araban, galactan, xylan and mannan) and polygalacturonic acid (pectin). Activities of enzymes of the larval digestive tract are very high against sucrose, maltose, arbutin, salicin, α- and β-methyl-D-glucopyranoside, but very low against lactose; significant activities were reported against starch, pectin, xylan, carboxymethylcellulose and even swollen cellulose. A very complex system of enzymes acting on oligosaccharides, heterosides and polysaccharides is thus exhibited (Chararas et al. 1971, 1972).

In the present paper, the origin of the cellulases of the gut of P. semipunctata larvae is examined. It is well known that some animals can produce cellulase. The presence of cellulase in the gut contents of certain insects, such as the thysanuran Ctenolepisma lineata (cf. Lasker and Giese 1956), various cockroaches, termites and cerambycids (cf. Mansour and Mansour-Beck 1934), has already been demonstrated and their origin investigated. Schlottke and Becker (1942) have shown that the larvae of the cerambycid Hylotrupes bajulus have no symbionts and yet can digest cellulose, which indicates that they produce cellulase. According to Chararas (1981a), larvae of the cerambycids Ergates faber and Criocephalus syriacus, feeding on a medium containing both antibiotics and fungicides, could still digest cellulose: enzymatic hydrolytic activity on swollen cellulose was the same after 8 days. Moreover, Chararas (1981a) demonstrated that cultures of yeasts isolated from the whole gut of larvae of the cerambycids C. syriacus, C. rusticus, Rhagium inquisitor and E. faber did not act on swollen cellulose; notably the cultures of the yeast Picha stipitis isolated from E. faber larvae and of another yeast, Hansenula capsulata, from those of C. syriacus exhibited no hydrolytic activity towards cellulose (Chararas 1981b). This indicates that the larvae of the former four cerambycids produce a cellulase.

Following the purification by chromatography, as described elsewhere (cf. Chararas and Chipoulet 1982), of a β-glucosidase (EC 3.2.1.21) from the larvae of Phoracantha semipunctata, the partial purification of a cellulase from the midgut epithelium of the larvae and the determination of some of its properties are now described.

Materials and Methods

Extraction and Purification of Enzyme

Actively feeding larvae were collected from the phloem of Eucalyptus spp. trees and then homogenized in ice-cold 0.9% (w/v) NaCl. After the homogenate was centrifuged, the very fatty supernatant was filtered twice through glass wool, in order to remove the fat. The filtrate (crude extract) was used in purifying the enzyme.

The crude extract [containing 90 mg of protein, and strongly solubilizing Cellulose–Azure* (Calbiochem)] was applied to a column (2.5 cm i.d. by 65 cm) of coarse Sephadex G 200. The proteins

* Cellulose–Azure is an insoluble, substituted and dyed derivative of cellulose, which is obtained from pure cotton cellulose by acid treatment and dying with Remazol brilliant blue R.
were eluted at 4°C with distilled water and 1·5-ml fractions were collected. Fractions were assayed for release of dye from Cellulose–Azure in 3 days and the results of this assay are given in the following tabulation (+ + + high release; + + medium release; + low release; — no release):

Fraction No.  10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210
Release of dye — — — — — — — — — — — — — — + + + + ++ + + + + + + + + + + + + + — — — — — — — — — — Combined fractions

Fractions 110-180, which contained all of the enzyme activity, were combined, the pool was dialysed against 5 mM phosphate buffer (pH 6·0), then applied to a hydroxyapatite column (1·3 cm i.d. by 7 cm) equilibrated with 5 mM phosphate buffer (pH 6·0). The proteins were eluted at 4°C with 39 ml of 5 mM and 81 ml of 40 mM phosphate buffer (pH 6·0), 3-ml fractions being collected. Certain fractions were tested for release of dye from Cellulose–Azure in 24 h. Fractions 46–50, corresponding to 40 mM buffer, were combined, the pool was dialysed against 5 mM phosphate buffer (pH 6·0), then applied to another hydroxyapatite column (1 cm i.d. by 3·5 cm) equilibrated with 5 mM phosphate buffer (pH 6·0). The proteins were eluted with 15 ml of 5 mM, 18 ml of 10 mM and 72 ml of 15 mM phosphate buffer (pH 6·0), 3-ml fractions being collected. Certain fractions were assayed for release of dye from Cellulose–Azure in 6 days. Fractions 19-41, corresponding to 15 mM buffer, were combined, then the pool was concentrated 12-fold with a dialysis membrane (Naturin) using sucrose as a dehydrant. The protein concentration in the fractions from the first and second hydroxyapatite columns was determined by the absorption at 280 nm. Total protein was measured by the biuret photometric method using bovine serum albumin as a standard (Zamenhof 1957).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Ornstein and Davis (1962), the concentration of acrylamide in the gel being 8·2%(w/v) and current intensity 5 mA per tube. Electrophoresis was stopped when the tracking dye, bromophenol blue, was at the bottom of the gel. After the run, the gel was stained with Coomassie brilliant blue R to reveal the proteins; the protein bands were scanned at 580 nm using an Isco model 1310 gel-scanner. In certain cases, the gel was sliced into 1·6-mm wide pieces, which were assayed for release of the dye Remazol brilliant blue R from Cellulose–Azure. The reaction mixture for the assays contained 2 mg Cellulose–Azure particles, 50 µl of distilled water, ground slice, 25 µl of 200 mM phosphate buffer (pH 6·0) and 5 µl of toluene to inhibit microbial growth. After incubation at 37°C for 1-4 days, the concentration of Remazol brilliant blue R of the liquid zone was estimated.

Continuous gradient polyacrylamide gel electrophoresis (Uniscil/Gradiapore) was also used in Tris–borate–EDTA buffer (pH 8·3). Acrylamide concentrations ranged from 2·5 to 28% (w/v). The molecular weight of the cellulase was estimated using this technique, with bovine serum albumin monomer (66000), dimer (132000), trimer (198000) and tetramer (264000) (Nutritional Biochemicals Corp.) and pepsin A (34500) (BDH Chemicals Ltd) as protein markers.

Assay of Enzymatic Activity

The effect of enzymatic solutions on the solubilization of Cellulose–Azure was determined. The reaction mixture contained 2 mg of Cellulose–Azure particles, 50 µl of distilled water, 50 µl of enzymatic solution, 25 µl of 200 mM phosphate buffer (pH 6·0) and 5 µl of toluene. After incubation at 37°C, lasting from some hours to several days without shaking, the amount of dye released in the liquid zone was determined. It is known that there is a linear correlation between the solubilization of dyed substrates, such as Cellulose–Azure, and the release of dye into the liquid zone (cf. Leisola and Linko 1976a, 1976b; Enari and Nybergh 1979; Ng and Zeikus 1980). Because of the small volume (125 µl) of the liquid zone, the absorbance at 595 nm due to the dye could not be measured spectrophotometrically by the usual method. An aliquot of this zone (10 µl for example) was therefore deposited on white paper (Schleicher and Schüll) and the amount of dye present in the liquid zone estimated by reference to the colour intensity of the spot. The effect of various cations (Hg²⁺, Fe³⁺, Cu²⁺, Ca²⁺, Mn²⁺, Zn²⁺ and Mg²⁺) on the amount of dye released from Cellulose–Azure in presence of the cellulase for 4 days was determined by adding 25 µl of 60 mM
solution of the tested cation to the reaction mixture buffered with 10 mM acetate buffer (pH 4.0) (final concentration of the cation in the reaction mixture, 10 mM).

In addition, enzymatic hydrolytic activity was determined for various substrates: celllobiose (Sigma), carboxymethylcellulose (Pronova), swollen cellulose (Halliwell) and microcrystalline cellulose (Sigma). The reaction mixture contained 100 μl of substrate solution (50 mM for celllobiose, 1 g per 100 ml for CMC) or substrate suspension (1 g per 100 ml for cellulose), 100 μl of enzymatic solution, 50 μl of 200 mM phosphate buffer (pH 6.0) and 10 μl of toluene. After incubation at 37°C, the amount of reducing groups liberated by enzymatic hydrolysis, expressed as micrograms of glucose, was determined by the method of Nelson (1944) and Somogyi (1945) and the final carbohydrate composition of the reaction mixture was tested by paper chromatography. In certain cases, the amount of glucose liberated by enzymatic hydrolysis was determined by the glucose oxidase method.

Enzymatic hydrolysis of p-nitrophenyl-β-D-glucopyranoside (PNPG; Sigma) was determined spectrophotometrically by the amount of p-nitrophenol liberated. The reaction mixture contained 50 μl of 20 mM PNPG, 50 μl of enzymatic solution, 25 μl of 200 mM phosphate buffer (pH 6.0). After incubation at 30°C, the reaction was stopped by addition of 3 ml of Na2CO3 2% (w/v) and the absorbance was then measured at 400 nm.

**Paper Chromatography**

The oligosaccharides were separated by descending paper (Schleicher and Schüll) chromatography with isopropanol:n-butanol:water, 7:1:2 (by vol.). After the front had run for 24 h, the paper was dried, sprayed with a mixture of an alcoholic solution of 0.66% (w/v) o-phenylenediamine and an aqueous solution of 5% (w/v) oxalic acid (3:2 by vol.) and then developed at 90°C for 20 min.

**Results**

**Origin of the Cellulases in the Gut of the Larvae**

When larvae were fed on moistened *Eucalyptus* spp. phloem sawdust containing about 45% cellulose (dry wt) as determined using the method of Cross *et al.* (1918), 25% cellulose, with respect to dry weight, was found in the excreta, thus showing a partial utilization of the cellulose by the larvae. In addition, the guts of larvae showed enzymatic activities against swollen cellulose and Cellulose-Azure.

The larvae reared on compact discs made either from Whatman cellulose powder, aqueous agar (standard agar medium, of which the moisture content was 45% with respect to dry weight) or from moistened sawdust of *Eucalyptus* spp. phloem, each of which was impregnated with a mixture of different kinds of antibiotics (e.g. ampicillin and seporexin, which act against a wide range of Gram-positive and Gram-negative bacteria) and wide-spectrum fungicides such as nystatin and hamycin, were shown by electron microscopy to lack yeasts and bacteria in the gut.

When larvae were placed on moistened cellulose powder impregnated with antibiotics and fungicides, they started feeding on this artificial medium and 8 days later fresh excreta were collected and suspended in distilled water. The midgut wall was isolated. Paper chromatography of excreta revealed the presence of celllobiose and large quantities of glucose. Moreover, the midgut wall showed enzymatic activities against swollen cellulose, which indicates the presence of cellulases of epithelial origin in the gut contents of the larvae.

The guts of larvae feeding on a medium containing both antibiotics and fungicides showed no decrease in enzymatic activity against swollen cellulose compared with larvae fed medium without antibiotics and fungicides. This would tend to show that the cellulases in the gut contents are of exclusively epithelial origin.
Purification of the Enzyme

The results of polyacrylamide disc gel electrophoresis of the crude extract, followed by gel slicing and determination of the amount of dye released by ground slices from Cellulose–Azure in 4 days are given in the following tabulation (+ + + high release; + + medium release; + low release; – no release).

Slice No.                  Release of dye
1-23                      24-25  26-28  29  30-39  40-44  45-47  48-52  53-55  56-64
                                 –       +      ++     –     +      ++   +++   ++    –

Dye was released in two groups of slices, indicating the presence of two cellulases in the gel. The faster one, corresponding to the major amount of dye release, was partially purified. The steps in the purification procedure are shown in Figs 2 and 3.

Figs 2 and 3. Chromatography on hydroxyapatite columns of pooled fractions 110–180 from Sephadex G 200 column (Fig. 2) and of pooled fractions 46–50 from the first hydroxyapatite column (Fig. 3). Elutions with phosphate buffer (see Materials and Methods) and extent of release of dye from Cellulose–Azure are indicated.

100 μl of fraction 20 from the second hydroxyapatite column, concentrated 10-fold, was analysed using polyacrylamide disc gel electrophoresis. One of the two parallel gels was stained for proteins with Coomassie brilliant blue R, the other was cut into slices, and their solubilizing activity was determined after grinding (Fig. 4). Fraction 20 showed two protein peaks, one of which coincided with an area of dye release. Next, polyacrylamide gel electrophoresis of pooled fractions 19–41 from the second hydroxyapatite column, concentrated 12-fold, was carried out on gradient plates. After
electrophoresis, a vertical gel band was horizontally sliced, with application of reference marks along an adjacent gel band, which was then stained for proteins. Release of dye from Cellulose-Azure by the gel slices was determined. With 200 V

![Graph](image_url)

**Fig. 4.** Polyacrylamide disc gel electrophoresis of fraction 20 from the second hydroxyapatite column (Fig. 3). Release of dye from Cellulose-Azure is shown.

![Graph](image_url)

**Fig. 5.** Gradient polyacrylamide gel electrophoresis of pooled fractions 19–41 from the second hydroxyapatite column. Gel I was run for 600 Vh, and gel II for 200 Vh. Release of dye from Cellulose-Azure is shown. Arrow indicates the position of the cellulase.
and after 3 h, two consecutive active slices were obtained, corresponding to two very close protein bands on the gel band stained for proteins. With 200 V and after 1 h, only one active slice was obtained, coinciding on the stained gel band with a narrow protein band, which was a cellulase. The preparation contained some proteins without cellulolytic activity (Fig. 5). No further enzyme purification was attempted.

**Characterization of the Enzyme**

The properties of the enzyme were studied using pooled fractions 19–41 from the second hydroxyapatite column, concentrated 12-fold.

**Molecular weight.** Gradient polyacrylamide gel electrophoresis showed that the enzyme had a molecular weight lower than that of pepsin A (34 500) (Fig. 6).

**Optimum pH.** 200 mM acetate buffers (pH 3·0, 4·0 and 5·0) and 200 mM phosphate buffers (pH 6·0, 7·0 and 8·0) were used. Release of dye from Cellulose–Azure in the presence of the enzyme in 4 days was about threefold higher at pH 3·0 and 4·0 than at pH 7·0 and 8·0, which indicates a pH optimum of between 3·0 and 4·0.

**Effect of various cations.** The amount of dye released in 4 days from Cellulose–Azure in the presence of Cu²⁺, Ca²⁺, Mn²⁺, Zn²⁺ and Mg²⁺ differed little from the amount released in the absence of the cations. The release of dye was reduced to half by the presence of Hg²⁺ and to almost zero by the presence of Fe³⁺.

**Substrate Specificity of the Enzyme**

Various substrates were incubated with fraction 20 from the second hydroxyapatite column. No activity was detected on p-nitrophenyl-β-D-glucopyranoside for 1 h (no p-nitrophenol liberated) or on cellobiose after 18 h (no glucose liberated). Hence, the cellulase present in fraction 20 does not attack p-nitrophenyl-β-D-glucopyranoside or cellobiose and therefore is not a β-glucosidase. No hydrolytic activity was observed with microcrystalline cellulose after 6 days (no liberation of reducing groups). On the other hand, hydrolytic activity was significant with carboxymethyl-cellulose after 6 days (decrease in the viscosity of the reaction mixture and liberation.
of reducing groups) (Fig. 7). Among the enzymatic hydrolysis products, identified by paper chromatography, were various cellobioigosaccharides including cellobiose, but no glucose (Fig. 7). The paper chromatogram of the enzymatic hydrolysis products showed that the cellulase is an endo-β-1,4 glucanase (EC 3.2.1.4).

![Graph showing glucose production over time](image)

**Fig. 7.** Hydrolysis of carboxymethylcellulose by fraction 20 from the second hydroxyapatite column. Left, quantity of reducing groups liberated, expressed as micrograms of glucose. Right, paper chromatogram of the hydrolysis products present in the reaction mixture (M) after incubation for 6 days. s, Sugar mixture (glu, glucose; cel, cellobiose).

**Discussion**

Crude extracts of the guts of *P. semipunctata* larvae hydrolysed no more than 25% of a sample of swollen cellulose during an incubation period of 24 h at 37°C. Under the same conditions, sucrose and starch were broken down by 90 and 80% respectively (Courtois and Chararas 1966). The larvae are, therefore, adapted to phloem, which is rich in soluble carbohydrates and starch. Cellulose acts only as a secondary component in the nutrition, accounting for the 60% mortality after 3 months, of larvae feeding on discs made from standard medium.

The experiments described above showed an exclusively epithelial origin for the cellulases of the larvae of *P. semipunctata*. It should be noted that Chararas and Pignal (1981) demonstrated that the cultures of two yeasts, isolated from the whole gut (wall + contents) of *P. semipunctata* larvae and identified as *Candida guillermondii* and *C. tenuis*, were able to degrade neither carboxymethylcellulose nor swollen cellulose. Moreover, cultures of other isolated yeasts (*C. intermedia*, *C. diddensii*, *Torulopsis molischiana*) did not exhibit any activity against swollen cellulose (Chararas et al. 1983).

The larvae of *P. semipunctata* produce at least two cellulases, as demonstrated by polyacrylamide disc gel electrophoresis of the crude extract. The cellulase that was partially purified is a small protein, with a molecular weight less than 34 500.

A very low pH optimum was observed for the solubilization of Cellulose-Azure in the presence of the enzyme. The midgut contents contain food particles suspended in a liquid, the pH of which is particularly low, about 5·0 (tested with pH paper). The enzyme seems to be adapted to the acidity of the midgut contents. The situation is the same for the β-glucosidase that we have purified from larvae and which has a
pH optimum for hydrolysis of p-nitrophenyl-β-D-glucopyranoside between 5 and 5.5 (cf. Chararas and Chipoulet 1982). The solubilization of Cellulose-Azure in the presence of the cellulase is reduced to half by Hg²⁺ and to zero by Fe³⁺. It was shown that cellulase is an endo-β-1,4-glucanase, which randomly cuts the linkages in the cellulose chains. Microcrystalline cellulose is not attacked.

_Eucalyptus_ spp. phloem is chewed by the larva’s mandibles in the presence of saliva. The pectocellulosic cell walls enter the midgut space where they meet with enzymes. The polygalacturonans are degraded by the action of polygalacturonases. Cellulases such as the endo-β-1,4-glucanase purified in this study play a role in the degradation of cellulose. The resulting cellobioisaccharides can be hydrolysed by β-glucosidase with liberation of glucose and a lower celloisaccharide such as cellobiose, which is hydrolysed by cellobiase (cf. Chararas and Chipoulet 1982).

References


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Manuscript received 10 August 1982, accepted 22 February 1983