Acquisition of Digestive Enzymes by Siricid Woodwasps from Their Fungal Symbiont

Abstract. Larvae of the woodwasp, Sirex cyanus, contain midgut digestive enzymes that enable them to utilize the major fungal and plant polysaccharides found in their food. At least two classes of enzymes, the C₄-cellulases and the xylanases, are not produced by the larvae. Instead, larvae acquire these enzymes while ingesting tissue of Amylostereum chailletii, the fungal symbiont that occurs in the wood on which the larvae feed.

Woodwasps (1, 2) maintain a close association with a fungal symbiont both as larvae and adults. Adult female woodwasps oviposit in dying and dead standing trees. As eggs are laid, the wood is simultaneously inoculated with a mass of fungal oidia which are maintained in special pouches associated with the egg-laying apparatus. The fungus permeates the surrounding wood, and the larval tunnel into this, ingesting both wood and fungal hyphae. Although attempts to study the enzymatic characteristics of the gut fluids of woodwasps have given ambiguous or conflicting results (3, 4), Müller demonstrated significant digestion and assimilation of wood constituents, including cellulose and hemicellulose, by larvae of Sirex gigas and S. phantoma (4).

We have found (Table 1) that the midgut of S. cyanus larvae contains enzymes active against a number of plant polysaccharides, including microcrystalline cellulose (the cellulase complex), carboxymethyl cellulose (C₄-cellulase), xylan (xylanase), pectin (pectinase), and amylase (amylase). The midgut of S. cyanus also contains enzymes active toward laminarin, a β-1,3-glucan representative of a widely distributed class of fungal cell wall polysaccharides. The enzymes are present in larvae that have been reared on balsam fir chips permeated by the mycelium of the fungal symbiont, Amylostereum chailletii (5), as well as larvae collected from their natural galleries in the trunks of standing balsam fir trees. This same suite of carbohydrates is present in both the culture fluid of A. chailletii and in an extract of balsam fir wood permeated by the fungus.

These results suggest that S. cyanus might be acquiring essential digestive carbohydrases when it ingests fungal tissue and fungal secretions along with the wood it consumes. In agreement with this idea is the observation that larvae fare poorly when they are fed a diet of symbiont-free balsam fir chips, extracts of which are virtually enzyme-free (Table 1). Larval mortality is high after only a week, and the level of midgut enzymes decreases dramatically (6).

In order to test the hypothesis that the enzymatic activity of the larval gut fluid is due to ingested fungal enzymes, we have purified the C₄-cellulases and xylanases from larvae feeding on A. chailletii-infested balsam fir chips and from the culture fluid from A. chailletii growing on microcrystalline cellulose in a defined medium, and have compared isoelectric points (pI) of the insect- and fungus-derived enzymes. C₄-cellulase and xylanase were chosen for detailed comparison since Sirex larvae have been shown to assimilate significant portions of cellulose and hemicellulose, which make up nearly 70 percent of the dry matter of balsam fir wood (4, 7). Although the presence of enzymes active against pectin, starch, and laminarin suggests that woodwasp larvae have the capacity to utilize polysaccharides other than cellulose and hemicellulose, the limited quantities of starch and pectin in balsam fir wood (8) and the sparse growth of A. chailletii mycelium in the wood surrounding larval tunnels (9) indicate that these are minor sources of nutrients for the larva.

C₄-Cellulase and xylanase activities were detected in comparable fractions.
when gut extracts and fungus culture fluids were subjected to a purification scheme involving ion exchange chromatography on DEAE-Sepharose CL6B, gel filtration on Sephadex G75, and chromatofocusing on Polybuffer Exchanger 94 (10). Both the larval midgut and the fungus culture extracts yielded two major \( C_r \)-cellulases and three major xylanases. The first cellulase from both sources eluted from the preparative isofocusing column at \( pH \) 4.9, and the second cellulase at \( pH \) below 4.0 (11). The first xylanase eluted at \( pH \) 5.7, the second at \( pH \) 5.3, and the third at \( pH \) 4.5.

A further comparison of insect and fungal enzymes isolated from the preparative isofocusing columns was made by subjecting the cellulase and xylanase fractions to analytical isofocusing on ultrathin polyacrylamide gels (12). This procedure revealed that several proteins were present in each of the cellulase and xylanase fractions (Fig. 1). We did not determine whether the observed protein multiplicity reflected enzyme multiplicity or simply incomplete purification. However, the point of overriding importance is that the patterns seen in each of the cellulase and xylanase fractions from the fungal extract are identical to those seen in the comparable fractions derived from the insect extract. These experiments demonstrate that the \( C_r \)-cellulases and xylanases present in the gut of \( S. \) cyanus larvae are identical to (or indistinguishable from) the enzymes produced by the fungus \( A. \) chailletii and confirm our hypothesis that the woodwasp larvae acquire essential digestive enzymes by the ingestion of their fungal symbiont.

No data are available that allow an estimate of the relative importance to the larvae of polysaccharide digestion products liberated in the gut and those that might have been generated through decay of the wood prior to ingestion. However, Müller's (4) demonstration that \( S. \) gigas and \( S. \) phantoma assimilate 22 and 31 percent, respectively, of the cellulose they ingest indicates that structural polysaccharides digested in the gut are an important source of carbon to these insects.

It has been recognized since the investigations of Buchner (2) and Cleveland (13) that microbial symbionts can play an important role in the digestive processes of insects. It is now well established that permanent populations of hindgut protozoa are responsible for cellulose digestion in wood roaches and in the lower termites (14), and that hindgut bacteria contribute to the digestion of refractile plant polysaccharides in the rhinoceros beetle (15) and American cockroach (16).

The ingestion of active fungal enzymes, first demonstrated by Martin and Martin (17) in their studies of cellulose digestion by the fungus-growing termites, and now shown to occur also in the siricid woodwasps, constitutes an alternative mechanism by which insects exploit the digestive capabilities of microorganisms. Recently, a significant role for acquired fungal cellulases in the digestive processes of the amphibod, Gammaraus fassarum, has been proposed (18). In this system, the fungi which are the source of the enzymes are not obligate symbiotic associates of \( G. \) fassarum, but rather are normal colonizers of the aquatic detritus that constitutes the major food of this invertebrate. We anticipate that additional examples of the acquisition of di-

<table>
<thead>
<tr>
<th>Item</th>
<th>Enzymatic activity [units per milligram (dry weight) of dissolved solids in extract]</th>
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<tbody>
<tr>
<td></td>
<td>Cellulase complex</td>
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<tr>
<td>Larvae</td>
<td></td>
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<tr>
<td>Collected from natural</td>
<td>0.33 ± 0.13</td>
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<tr>
<td>galleries in balsam fir</td>
<td>(isoelectric point)</td>
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<tr>
<td>Cultured 1 week on BFC</td>
<td>0.48 ± 0.01</td>
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<tr>
<td>permeated by ( A. ) chailletii</td>
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<tr>
<td>Cultured 1 week on sterile BFC</td>
<td>0.00</td>
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<tr>
<td>Culture fluid from</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>( A. ) chailletii mycelium</td>
<td></td>
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<tr>
<td>Sterile BFC</td>
<td>0.37 ± 0.00</td>
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Fig. 1. Isofocusing gels of \( C_r \)-cellulases and xylanases of \( A. \) chailletii and \( S. \) cyanus obtained from preparative chromatofocusing. The \( pH \) values are indicated to the left of each figure. (A) \( C_r \)-cellulases. Lanes 1, 4, and 7 contain pl (isoelectric point) marker proteins (12); lanes 2 and 5 are the fungal \( C_r \)-cellulases; lanes 3 and 6 are the insect \( C_r \)-cellulases. The pl values for bands in lanes 2 and 3 are 4.5, 5.1, and 5.3; in lanes 5 and 6, 3.0 and 3.7. (B) Xylanases. Lanes 1, 4, 7, and 10 are the marker proteins; lanes 2, 5, and 8 are the fungal xylanases; lanes 3, 6, and 9 are the insect xylanases. The pl values for bands in lanes 2 and 3 are 4.9, 5.2, 5.4, and 5.5; in lanes 5 and 6, the pl's are 4.5, 4.8, 4.9, 5.1, 5.3, and 5.5; in lanes 8 and 9 the pl's are 4.4, 4.5, and 5.0.

Table 1. Enzymatic activity toward various polysaccharides of extracts of midguts (tissue plus contents) of \( S. \) cyanus larvae and of extracts of cultures of their symbiotic fungus \( A. \) chailletii. Each value is the mean ± standard error of the mean. The number of replicates was five in all cases except that only a single replicate was made for larvae cultured for 1 week on sterile balsam fir chips (BFC) (6). A unit of activity is the amount of enzyme required to liberate 1 \( \mu \)g of maltose equivalents per hour under the conditions of the assay (37°C, \( pH \) 5, incubation volume 1.0 ml). Substrates were microcrystalline cellulose (MC), carboxymethyl cellulose, larchwood xylan, citrus pectin, \( \alpha \)-amylase, and seaweed laminarin (19, 20).
gestive enzymes will be uncovered by further studies of insect-microbial associations and of the utilization of refractory plant materials by arthropods.

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References and Notes
4. W. Müller, Arch. Mikrobiol. 5, 84 (1934).
5. Basidionymetes, Aphyllophorales, Stereaceae; the strain of A. chailletii used in all experiments was a single-cell isolate obtained from the intersegmental sacs of an adult female of S. cyanescens.
6. Most of the larvae died before the end of the first week when fed symbiont-free balsam fir chips. It was necessary to pool the surviving larvae to obtain sufficient material for a single series of enzyme assays.
8. Pectin and starch together make up less than 2 percent of the dry weight of balsam fir wood (7).
10. Larval midguts, in groups of 10 to 20, were homogenized and centrifuged. The extract was desalted on a Pharmacia PD-10 column and soluble proteins were dialyzed against 50 mM acetate, pH 5.0. Cell-free fungal culture fluid was concentrated in a Pellicon Cassette (Millipore, Bedford, MA) and dialyzed against acetate buffer, pH 5.0.
11. Larval and fungal extracts were treated similarly thereafter. Cellulases and xylanases were fractionated on a column (2.6 by 40 cm) of DENC-Sepharose CL-6B equilibrated with 50 mM acetate buffer, pH 5.0, and eluted with a 0 to 500 mM linear NaCl gradient. Four major peaks of xylanase activity were eluted from the column at concentrations of 35 mM, 80 mM, 165 mM, and 345 mM NaCl. Two major cellulases emerged from the column at concentrations of 295 mM and 370 mM NaCl. Active fractions were further fractionated on a column (2.6 by 40 cm) of Sephadex G75 eluted with 50 mM acetate buffer, pH 5.0. Isoelectric focusing on a polyacrylamide gel (chromatofocusing) was performed on a column (1 by 40 cm) of Polybuffer Exchanger 94 (Pharmacia) equilibrated with 25 mM imidazole HCl, pH 7.4, and eluted with 0.0004 M Polybuffer 74 (Pharmacia) per pH unit per milliliter, pH 4.
12. The second cellulase fraction did not elute from the column, since the pH gradient was set to run from pH 7 to 4. This cellulase was eluted with an NaCl solution. That the pH values of the proteins in this fraction are less than pH 4 is confirmed by polyacrylamide gel isofocusing (Fig. 1).
13. Analytical isofocusing was performed on 0.1-mm polyacrylamide gels (Servalyt Precotes, Serva Fine Biochemicals) with a nominal pH range of 3 to 6. Focusing was performed for 2000 volthours (final field strength of 100 V/cm) at 10°C in a column and apparatus. Gels, fixed in 20 percent trichloroacetic acid, were stained with silver nitrate (C. R. Merrill, D. Goldman, S. A. Miller, and T. J. Kollman, Biochem. Biophys. Res. Comm. 211, 1437 [1985]). Amyloglucosidase, glucose oxidase, soybean trypsin inhibitor, β-lactoglobulin A, and bovine carboxy anhydrase B (Serva Fine Biochemicals) were used as pl marker proteins.
20. For an up-to-date discussion of cellulolysis and the cellulases, see P. D. Vangronsveld, J. Insect Physiol. 13, 239 (1967); J. de Ninan, M. Ebert, Science 211, 1437 (1985).
21. We thank A. Sakai, R. VandelKopple, G. Keevil, and E. Weatherbee for field assistance, the University of Michigan Biological Station and the Matthaei Botanical Gardens for use of their facilities, and the National Science Foundation for grants PCM-78-22733 and PCM-82-03537 to M.M.

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Is Thymosin Action Mediated by Prostaglandin Release?

Abstract. Treatment of spleen cells derived from adult thymectomized mice with thymosin fraction 5 resulted in a rapid and dose-dependent stimulation of the release of immunoreactive prostaglandin E2. The release of prostaglandin E2 was associated with induction of theta antigen and was totally inhibited by indomethacin. In contrast, prostaglandin E2 release from spleen cells from intact donors was inhibited by treatment with fraction 5. The data support the concept that prostaglandin E2 mediates the effects of thymosin fraction 5 on lymphocytes.

Prostaglandins have been implicated as possible mediators of the biological activity of thymic factors. Studies in our laboratory showed that an analog of prostaglandin E2 (PGE2) was able to mimic the action of thymic factors in the induction of theta antigen and in the appearance of serum thymic-like activity (STA) when it was given to adult thymectomized mice (1, 2). Moreover, the action of thymosin on theta antigen and on STA appearance was completely abrogated by the administration of indomethacin, a potent inhibitor of prostaglandin synthetase. In the experiments reported here we investigated the mode of action of thymosin on lymphocytes and examined the relation between thymosin and prostaglandins. Our results show that thymosin induces an early and dose-dependent release of high concentrations of PGE2 by lymphocytes collected from thymectomized mice.

For these experiments we used 4- to 8-week-old male C57BL/6J mice obtained from ENEA-Casaccia, Rome, Italy. Spleen cells from thymectomized or intact mice were incubated with 100 µg of thymosin fraction 5 (Fr5) (Hoffmann-La Roche, Nutley, New Jersey) with or without indomethacin. At various time intervals, the amount of PGE released was measured by using the radioimmunoassay described by Jaffe et al. (3). As early as 5 minutes after incubation, spleen cells obtained from thymectomized mice and treated with Fr5 released markedly higher concentrations of PGE2 than untreated spleen cells or spleen cells from thymus-intact mice (Fig. 1A). This difference persisted at least for 1 hour. Conversely, spleen cells from intact donors showed a slight inhibition of the PGE release after exposure to Fr5. Indomethacin inhibited the release both from Fr5-stimulated spleen cells from thymectomized mice and from intact donors.

In a second experiment, spleen cells from thymectomized and intact mice were separated on sodium metrizoate–Ficoll solution (Lymphoprep) and incubated with various concentrations of Fr5. After 15 minutes (that is, at the time of full PGE release) specific PGE2 release was measured by radioimmunoassay. Thymosin Fr5 increased the PGE2 release by lymphocytes from thymectomized mice in a dose-dependent fashion (Fig. 1B). Conversely, in normal splenic lymphocytes, small amounts of Fr5 stimulated a slight PGE2 release, whereas high concentrations of the factor (10 to 100 µg/ml) inhibited the spontaneous release of PGE2. Furthermore, most of the PGE released by splenocytes belonged to the PGE series. Treatment with indomethacin inhibited PGE2 release.

Figure 2 shows the relation between the induction of the theta-antigen in vitro and the release of PGE2 by lymphocytes derived from thymectomized mice and incubated with various amounts of Fr5. The presence of the theta antigen was evaluated by measuring the concentrations of azathioprine (AZ) required for inhibiting splenic, spontaneous rosette-forming cells, according to a modification of Bach’s technique (4). Theta-positive cells were defined as those in which rosetting was inhibited by 1.5 µg of AZ. High concentrations of Fr5 (100 µg/ml) induced the presence of the theta antigen and stimulated the greatest release of PGE2, whereas low levels of the factor (1 to 5 µg/ml) were unable to induce theta antigen and produced only a limited amount of PGE2.