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Contents

The remainder of this book describes my attempt to take the seventh step in the study of a symbiosis. My laboratory has undertaken a broad comparative study of other insect-fungus associations in which an augmentation of digestive capacity by ingested fungal enzymes seemed possible. Although the number of systems studied is still small, this research has generated several important results. Most important of all, my colleagues and I have demonstrated that the phenomenon discovered in the fungus-growing termites is not unique to that group. Ingested fungal enzymes play an important role in the nutrition of insects from several other orders. Furthermore, because of the diversity of the species included in our survey, we have been able to identify certain phylogenetic, physiological, and ecological factors that favor the evolution of a mutualistic association based upon the use of ingested enzymes.

Chapter 3

Acquired Enzymes in the Siricid Woodwasp *Sirex cyaneus*

Our next research objective was to probe the generality of the phenomenon we had discovered in our investigations of the fungus-growing termites. Was it possible that the acquisition of a digestive capacity by the ingestion of microbial enzymes was actually a widespread mechanism for digesting structural polysaccharides? In searching for another species that might rely upon acquired enzymes for cell wall digestion, we soon directed our attention to the siricid woodwasps. Like the macrotermitine termites, siricid larvae are wood-feeding insects involved in an elaborate obligate symbiotic association with basidiomycetous fungi. Our research on the siricid-fungus symbiosis is described in a single publication (Kukor and Martin 1983).

The Siricid Woodwasps

The Woodwasps and Their Associated Fungi

The siricid woodwasps (Hymenoptera, Siricidae) are insects familiar to foresters because of the damage they do to a variety of commercially important softwoods and hardwoods. The larvae are wood borers (Figure 3.1), and the ovipositing females (Figure 3.2) are vectors of decay fungi. The pest status of the woodwasps is somewhat mitigated by the fact that the females most often invade trees that have already been weakened by fire, disease, or previous insect attack.

Buchner (1928, 1965) first drew attention to the symbiotic association

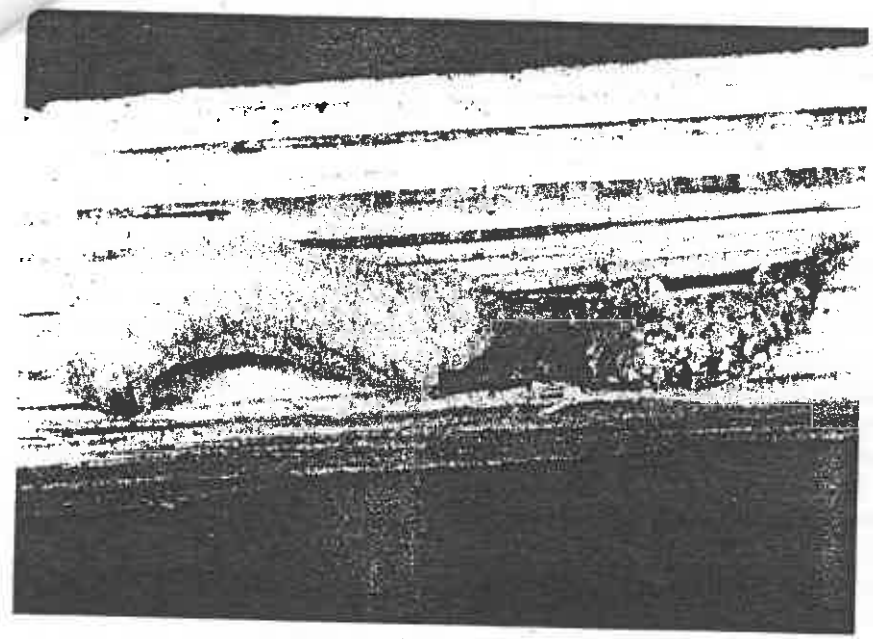

Figure 3.1 *Sirex cyaneus* larva from a gallery in balsam fir. [Photograph by David Bay, Division of Biological Sciences, University of Michigan.]

of the siricid woodwasps with fungi. He observed that two pear-shaped structures at the base of the egg-laying apparatus, structures that he called intersegmental pockets, are filled with fragments of fungal mycelium suspended in a mucous, and he suggested that these mycelial fragments, called oidia or arthrospores, are implanted in the wood with an egg at the time of oviposition. This hypothesis has been verified by careful investigations of oviposition in several species of *Sirex*, *Urocerus*, and *Tremex* (Francke-Grosmann 1939, 1967; Parkin 1942; Stillwell 1964; Coutts and Dolezal 1969; Spradbery 1977; Madden and Coutts 1979).

Following implantation of the arthrospores and the associated mucous into a suitable tree, the fungus produces vegetative hyphae that colonize the wood surrounding the oviposition hole (Francke-Grosmann 1939). The fungus continues to spread in the larval galleries as the larvae grow and bore further into the wood. The fungal hyphae not only grow along the surface of the tunnels but also penetrate into the surrounding wood.

Before the adults emerge from the tree, the intersegmental pouches of the females are inoculated with the fungal symbiont. Francke-Grosmann (1957) established the mechanism by which this inoculation occurs. The

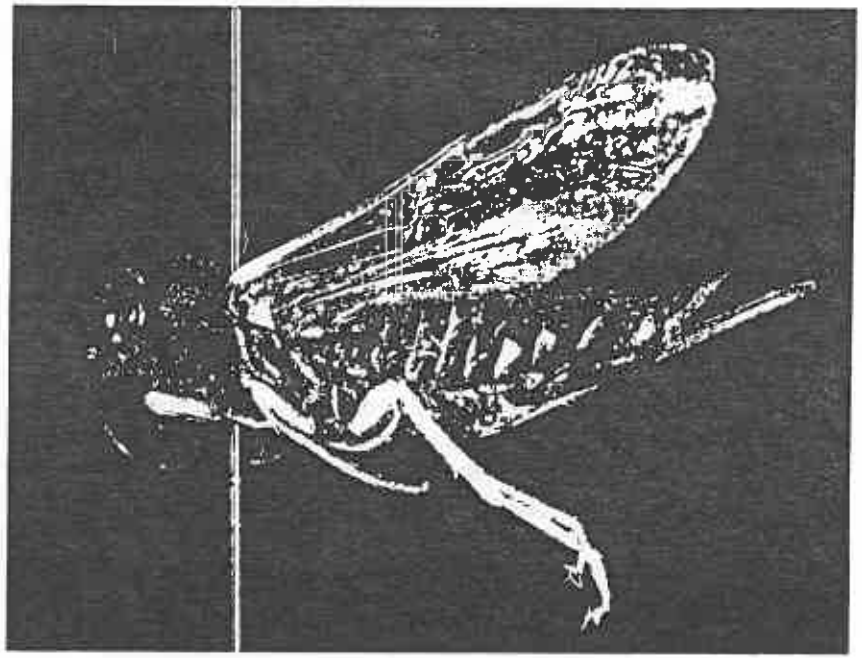

Figure 3.2 *Sirex cyaneus* adult female. [Photograph by David Bay, Division of Biological Sciences, University of Michigan.]

female larvae possess a pair of deep folds between the first and second abdominal segments (Parkin 1942; Rawlings 1951; Stillwell 1965). These structures, called hypopleural organs, are filled with fragments of the symbiotic fungus embedded in a clear secretion that hardens to the consistency of wax before pupation. At the time of eclosion, reflex movements of the emerging adult female cause a mechanical disintegration of the larval exuviae, which remain closely associated with the pupa, and some of the symbiont-containing wax attaches to the ovipositor and is eventually taken up into the intersegmental pouches.

The fungal symbionts of the siricid woodwasps are readily cultured from arthrospores taken from the intersegmental pouches of adult females (Cartwright 1929, 1938; Francke-Grosmann 1939; Parkin 1942). Woodwasps that attack conifers are associated with fungi in the genus *Amylostereum*. *A. chailletii* has been identified as the symbiont of one species of *Sirex* and two of *Urocerus* in North America (Stillwell 1966), whereas *A. aerolatum* has been identified as the symbiont of *S. noctilio* in Australia (Talbot 1964; Gaut 1969). Both *A. chailletii* and *A. sanguinolentum* have been reported from European siricids (Cartwright 1929,

1938; Parkin 1942; Stillwell 1966; Francke-Grosmann 1967). The fungal symbionts of species that attack hardwoods differ markedly from those of species that attack softwoods. The fungal associate of *Tremex columba*, which attacks beech, has been identified as *Daedalea unicolor* (Stillwell 1964).

The fungal associates of the siricid woodwasps are basidiomycetes that can also occur as free-living species. The species of *Amylostereum* associated with *Sirex*, however, seem not to form fruiting bodies readily, and woodwasps are probably major, perhaps exclusive, agents of dispersal of *Amylostereum* in some areas. The fruiting bodies of *A. chailletii*, for example, have rarely been reported in New Brunswick and Nova Scotia, yet in these provinces the fungus is associated with much of the sapwood decay in stands of balsam fir that have been attacked by woodwasps (Stillwell 1964). The propagation of *A. areolatum* in Australia is also believed to be due entirely to *S. noctilio* (Talbot 1964; Gaut 1969).

Stillwell demonstrated the absolute dependence of siricid larvae on their fungal symbionts. By rearing late-instar larvae of *S. juvencus* (Stillwell 1966) and *T. columba* (Stillwell 1967) in horizontal test tubes containing malt agar, a process that apparently prevents the transfer of fungal oidia from the larval exuviae to the intersegmental pouches of the adult, he obtained aposymbiotic adult females. Fungus-free and fungus-infected females were then allowed to oviposit in logs of the natural host wood. Normal larval development occurred only in those logs in which the fungal symbiont had been introduced during oviposition by fungus-infected females. The eggs laid by the fungus-free females hatched, but the larvae failed to survive the first instar.

Several hypotheses have been proposed to account for the dependence of the woodwasp larvae on their fungal symbionts. Fragments of mycelium, dispersed among the more abundant fragments of wood, are always present in the gut contents of siricid larvae, and a nutritional role for the fungus was presumed by most of the early investigators who studied these insects (Buchner 1928, 1965; Cartwright 1929; Francke-Grosmann 1939). Cartwright (1929) demonstrated that the fungal symbiont of *S. cyaneus* had high nutritive quality by maintaining young larvae for 3 weeks on a diet consisting exclusively of a pure culture of the fungus. The quantity of fungal tissue normally consumed, however, is too small for it to serve as a major source of macronutrients for the growing larvae (Francke-Grosmann 1939, 1967), although it does, of course, enrich the larval diet and does contribute important micronutrients (Madden and Coutts 1979). Some researchers have also suggested that the symbiont might predigest the wood surrounding the larval galleries, thereby making it a more nutritious food (Munro 1931; Gilmour 1965) or a softer one that is more easily chewed by the larvae (Francke-Grosmann 1967).

A Possible Role for Acquired Enzymes

All of the observations about the dependence of siricid larvae upon their fungal symbiont are compatible with the hypothesis that the symbiont is a source of enzymes that enable the larvae to digest the structural polysaccharides present in the wood that composes the bulk of the diet. This hypothesis is given further credence by the demonstration that the fungal symbionts of *Sirex* are cellulose digesters (Francke-Grosmann 1939) and that 22% of the cellulose in fir wood and 31% of the cellulose in spruce wood is assimilated during passage through the guts of *S. gigas* and *S. phantoma*, respectively (Müller 1934). Larvae of these two species also assimilate some of the hemicellulose they ingest.

In discussing the siricid woodwasps in his 1928 treatise, *Holznahrung und Symbiose*, Buchner wrote, "One has the impression that the amount of fungal material obtained in this way is not sufficient for the larvae. I would like to suggest that this represents an entirely new variation of ambrosia cultivation, in which the insect utilizes little of the substance of the fungus other than the collection of wood-degrading enzymes. These enzymes are ingested along with mycelial fragments, and together with the enzymes of the animal, act on wood in the gut lumen" (p. 16; my translation).[1] This passage apparently went unnoticed by workers interested in symbiosis. The idea was never tested and was never reiterated by Buchner in any of his later writings. Nonetheless, my colleagues and I are pleased and honored to find that our research conducted more than a half a century later has verified the speculation of this pioneer in the field of insect-microbial interactions.

To test the hypothesis that ingested enzymes contribute to digestion in the siricid woodwasps, we conducted a series of experiments with *S. cyaneus*, a species that is abundant at the University of Michigan Biological Station at Pellston, Michigan, in stands of balsam fir that have been subject to earlier attacks by spruce budworms. The symbiont of *S. cyaneus* is *A. chailletii*. We took an approach similar to the one used in our study of *M. natalensis*, which I described in Chapter 2.

1. "Man hat den Eindruck, dass die auf solche Weise aufgenommene Menge Pilzsubstanz der Larve nicht genügen kann und ich möchte die Vermutung aussprechen, dass hier eine ganz neue Variante der Ambrosiazucht vorliegt, bei der es dem Insekt vielleicht weniger auf die Substanz des Pilzes ankommt, als auf die Bestandteile des Holzes lösenden Nahrungsenzyme, die es sich mit den Mycelstücken zuführt, und die ihre dem Tier abgehende Wirkung auf das Holz auch im Darmlumen geltend machen."

The Enzymes in the Woodwasps' Gut Fluid

Enzymatic Activity of *Sirex cyaneus* Gut Fluid and *Amylostereum chailletii* Culture Fluid

The alimentary tract of *S. cyaneus* is a simple tube with no blind sacs or enlarged regions in the hindgut that might serve as a fermentation chamber. The midgut is by far the largest segment. The midgut fluid of larvae collected from their natural galleries in balsam fir is enzymatically active against a number of plant cell wall polysaccharides, including microcrystalline cellulose, carboxymethylcellulose, larchwood xylan, and citrus pectin (Table 3.1). These results support the conclusion of Müller (1934) that the larvae are capable of digesting both cellulose and hemicellulose, and they identify the midgut as a major site of digestion.

The midgut of *S. cyaneus* also contains enzymes active against potato amylose (amylase) and seaweed laminarin (laminarinase). Amylose is an α-1,4-glucan representative of the storage polysaccharides of both higher plants and fungi, whereas laminarin is a β-1,3-glucan representative of a widely distributed class of polysaccharides present in fungal cell walls as well as in marine algae. β-1,3-Glucans are also present in vascular plants in the form of callose, a polysaccharide that can be deposited in response to injury and may be a significant component of phloem tissue (Eschrich 1975).

The larvae of *S. cyaneus* are well equipped to exploit the energy content of their food. The ability of the larvae to survive on a diet consisting only of their fungal symbiont is readily explained by their ability to digest the storage and structural polysaccharides of fungal tissue. The presence in their midguts of enzymes active against the structural and storage polysaccharides of vascular plants, however, suggests that they should also be able to survive on a fungus-free diet of wood—unless of course they depended upon the fungus for their wood-degrading enzymes.

When *A. chailletii* is grown in a chemically defined liquid medium in which suspended microcrystalline cellulose is the only carbon source, after 2 weeks the cell-free culture fluid contains the same set of enzymatic activities as the gut fluid collected from *S. cyaneus* larvae (Table 3.1). Indeed, the relative ratios of the various activities are strikingly similar in the midgut and culture fluids.

A comparison of the enzymatic activity in gut fluids from larvae fed a fungus-free diet and a fungus-containing diet suggests that enzymes

Table 3.1
Enzymatic activity toward various polysaccharides of extracts of midguts of *Sirex cyaneus* larvae and of a culture medium containing *Amylostereum chailletii*

Source of extract	Units of activity per mg (dry wt) of dissolved solids in extract[a]				
	Cellulase complex	C_x-Cellulase	Xylanase	Pectinase	Amylase	Laminarinase
Larval midgut	0.33 ± 0.13	8.86 ± 6.20	11.59 ± 7.21	8.47 ± 5.30	6.06 ± 2.62	14.23 ± 8.91
Fungus culture medium	0.33 ± 0.03	7.45 ± 0.33	9.24 ± 0.33	9.76 ± 0.46	9.48 ± 0.26	8.47 ± 0.38

Note: Midgut extracts consisted of tissue plus contents of *S. cyaneus* larvae collected from the natural galleries in balsam fir. The fungus culture medium was one in which *A. chailletii* had been growing for 2 weeks on microcrystalline cellulose. Each value is the mean plus or minus the standard error of the mean for five replicates.

[a]A unit of activity is the amount of enzyme required to liberate 1 μmol of maltose equivalents per hour under the conditions of the assay (37°C, pH 5.0, incubation volume 1.0 ml).

Source: J. J. Kukor and M. M. Martin, "Acquisition of Digestive Enzymes by Siricid Woodwasps from Their Fungal Symbiont," *Science* (Washington, D.C.) 220 (1983):1161–1163, © AAAS.

active against cellulose, carboxymethylcellulose, xylan, pectin, and laminarin are all acquired by ingesting fungal tissue. We easily prepared fungus-infected balsam fir wood chips by inoculating sterile wood chips with *A. chailletii* and allowing the culture to grow undisturbed for a month, during which time the fungal mycelium spread throughout the collection of wood chips. The larvae were maintained in the laboratory quite satisfactorily on a diet of such fungus-infected balsam fir wood chips. They fed actively and appeared normal. Their digestive fluids were active against the same polysaccharides as the gut fluids of larvae collected from natural galleries (Table 3.2). When the larvae were put on a diet of sterile balsam fir wood chips, however, they fared badly. Within a week 10 of the 15 larvae originally placed on the diet had died. The gut fluid from the 5 survivors was pooled and concentrated for a single series of enzyme assays. Activity toward cellulose and carboxymethylcellulose was virtually undetectable, whereas activity toward xylan, pectin, and laminarin was very low. Amylose was the only substrate toward which activity was comparable in gut fluids from larvae on fungus-containing and fungus-free wood chips. We also confirmed that an extract of balsam fir wood chips permeated by *A. chailletii* is enzymatically active toward cellulose, carboxymethylcellulose, xylan, pectin, amylose, and laminarin, whereas an extract of sterile balsam fir wood chips is enzymatically inactive. These results clearly support the idea that ingested fungal enzymes are responsible for the ability of the woodwasp larvae to digest the structural polysaccharides of wood.

Midgut and Fungal Enzymes Compared

As a final confirmation of the hypothesis that digestive enzymes in the woodwasp larvae are derived from their fungal symbiont, we carried out direct comparisons of larval and fungal enzymes, using the technique of analytical isoelectric focusing. Because hemicellulose and cellulose together compose nearly 70% of the dry matter of balsam fir wood, whereas pectin and starch together make up less than 2% (Kollman and Côté 1968), cellulases and hemicellulases are the two classes of enzymes that contribute most to the efficient assimilation of wood by these larvae. We therefore limited our comparisons of insect and fungal enzymes to the xylanases and C_x-cellulases.

In comparing the enzymes of *S. cyaneus* and *A. chailletii*, we subjected the enzyme extracts to a much more extensive purification process than in our earlier study of the enzymes of *M. natalensis* and *Termitomyces* sp. Our effort was rewarded with a demonstration of the identity of the insect and fungal enzymes that was based upon three criteria (binding characteristics on an anion exchange resin, molecular

Table 3.2
Enzymatic activity toward various polysaccharides of midgut extracts from *Sirex cyaneus* larvae cultured either on balsam fir wood chips permeated by *Amylostereum chailletii* mycelium or on sterile balsam fir wood chips

Source of extract	Units of activity per mg (dry wt) of dissolved solids in extract[a]					
	Cellulase complex	C_x-Cellulase	Xylanase	Pectinase	Amylase	Laminarinase
Larvae cultured 1 week on BFWC permeated by *A. chailletii* mycelium	0.48 ± 0.01	20.17 ± 0.59	33.41 ± 3.97	11.03 ± 1.11	4.18 ± 1.30	12.91 ± 3.14
Larvae cultured 1 week on sterile BFWC	0.00	0.09	1.39	0.97	2.36	1.62
BFWC permeated by *A. chailletti* mycelium	0.37 ± 0.00	1.58 ± 0.65	6.78 ± 2.53	1.50 ± 0.74	2.54 ± 0.00	5.65 ± 2.12
Sterile BFWC	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01	0.02 ± 0.01

Note: Each value is the mean plus or minus the standard error of the mean. The number of replicants was five in all cases except that only a single determination was possible for larvae cultured on sterile balsam fir wood chips. BFWC = balsam fir wood chips.
[a]Units of activity are as defined in Table 3.1.
Source: J. J. Kukor and M. M. Martin, "Acquisition of Digestive Enzymes by Siricid Woodwasps from Their Fungal Symbiont," *Science* (Washington, D.C.) 220 (1983):1161–1163, © AAAS.

weight, and isoelectric point) rather than just one (isoelectric point) and also with cleaner, better resolved isoelectric focusing gels.

Extracts of larval midguts were centrifuged, desalted on a Sephadex G-25 column, and dialyzed against 50 mM acetate (pH 5.0). The culture fluid in which the fungus had been growing was filtered and concentrated in a Pellicon Cassette, after which proteins were precipitated with ethanol at −20°C and then dialyzed against acetate buffer (pH 5.0). Thereafter larval and fungal extracts were separately subjected to identical separation sequences, the first two steps of which were ion-exchange chromatography on DEAE-Sepharose CL6B and gel filtration on Sephadex G-75. Ion-exchange chromatography separates proteins on the basis of charge, whereas gel filtration chromatography separates them on the basis of molecular size. The separation sequence resulted in the distribution of xylanase and C_x-cellulase activity in identical fractions from the insect and fungal extracts, indicating that the enzymatic activity in the two extracts is due to enzymes with identical distributions of surface charge and identical molecular weights.

The final step in the purification sequence was preparative chromatofocusing on Polybuffer Exchanger 94. This technique, like isoelectric focusing, separates proteins according to their isoelectric points. It yielded two major C_x-cellulases and three major xylanases from both the larval midgut extract and the fungal culture medium. In this step also, activity was distributed identically in the fractions derived from insect and fungal extracts, demonstrating that the enzymes responsible for activity toward carboxymethylcellulose and larchwood xylan in the larval gut fluid and the fungal culture medium have identical isoelectric points. The first cellulase eluted in a fraction with a pH of 4.9. The second cellulase remained bound to the column even when the eluent reached a pH of 4, indicating that it had a pI below 4. This cellulase was eluted with a sodium chloride solution. The three xylanases eluted in fractions with pHs of 5.7, 5.3, and 4.5.

The final test of the identity of the insect and fungal enzymes was a comparison of the protein bands obtained when corresponding active fractions from the insect and fungal extracts, acquired from the chromatofocusing step, were subjected to analytical isoelectric focusing. Initially this comparison was thwarted by the failure of the focused enzymes to elute from sliced segments of a polyacrylamide gel column or to be stained with such standard protein staining agents as Coomassie Blue. Success was finally achieved, however, when isoelectric focusing was carried out on ultrathin polyacrylamide gels, and proteins were stained with silver nitrate (Figure 3.3). Each active fraction contained several proteins. We did not determine whether the observed

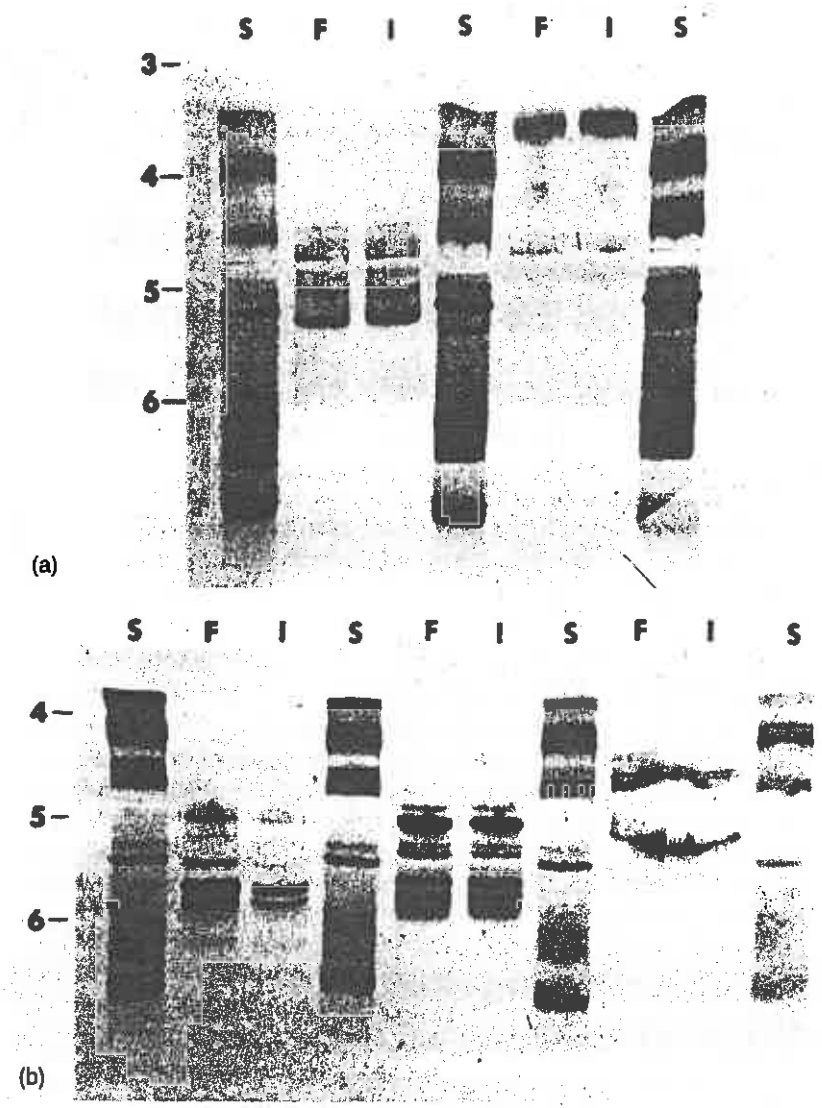

Figure 3.3 Isoelectric focusing gels of C_x-cellulases and xylanases in fractions eluted from a preparative chromatofocusing column. The pH values are indicated to the left of each figure. Lanes 1, 4, and 7 contain pI marker proteins. (a) C_x-Cellulases. Lanes 2 and 3 are the proteins that eluted from the chromatofocusing column at pH 4.9, whereas Lanes 5 and 6 are the proteins that remained bound to the column and had to be eluted with sodium chloride. Lanes 2 and 5 are from *Amylostereum chailletii*, whereas Lanes 3 and 6 are from *Sirex cyaneus*. (b) Xylanases. Lanes 2 and 3 are fractions that eluted at pH 5.7, Lanes 5 and 6 at pH 5.3, and Lanes 8 and 9 at pH 4.5. Lanes 2, 5, and 8 are from *A. chailletii;* Lanes 3, 6, and 9 are from *S. cyaneus*. [From J. J. Kukor and M. M. Martin, "Acquisition of Digestive Enzymes by Siricid Woodwasps from Their Fungal Symbiont," *Science* (Washington, D.C.) 220 (1983): 1161–1163, © AAAS.]

protein multiplicity was a consequence of enzyme multiplicity or only of incomplete purification. The point of overriding importance, however, is that the pattern seen in each of the cellulase and xylanase fractions from the larval gut fluid extract is identical to that seen in the corresponding fraction from the fungal culture fluid. Thus the proteins responsible for activity in the fractions of insect origin are also responsible for activity in the fractions of fungal origin.

Conclusion

The experiments described in this chapter demonstrate that the C_x-cellulases and xylanases in the midgut fluids of *S. cyaneus* larvae are derived from the fungus *A. chailletii*. These experiments confirm our hypothesis that the woodwasp larvae acquire essential digestive enzymes when they ingest their fungal symbiont. The research described in Chapters 2 and 3 has therefore demonstrated the importance of the acquisition of digestive capacity through the ingestion of fungal enzymes in two species of wood-feeding insects from two different orders, Isoptera and Hymenoptera. The presence of this mechanism for digesting plant structural polysaccharides in two such distantly related taxa suggested to us that it might have evolved independently in still other groups as well.

Chapters 4 and 5 describe research designed to probe the role of acquired enzymes in digestive processes in various species of detritus-feeding invertebrates and wood-feeding beetles. These species regularly encounter fungi and consume varying amounts of fungal tissue together with plant tissue, but unlike the macrotermitine termites and the siricid woodwasps, they are not involved in highly coevolved symbiotic associations with specific fungi. In looking for additional examples of invertebrates that rely upon acquired microbial enzymes for the digestion of structural polysaccharides, we were attempting both to establish the generality of the phenomenon and to gain further insights into the conditions that must be met before the phenomenon can evolve.

Chapter 4
Acquired Enzymes in Detritivores

Detritivores appear to be excellent candidates for organisms that could benefit from the ingestion of enzymes produced by members of the decomposer community normally associated with litter. This chapter describes investigations originating from five different laboratories concerned with the digestive biochemistry of detritivores. The work in my laboratory has been with the aquatic larvae of the crane fly *Tipula abdominalis* (Martin et al. 1980) and the terrestrial isopod *Tracheoniscus rathkei* (Kukor and Martin 1986a). In our work with *T. rathkei*, my colleagues and I were finally able to do an experiment that had not been possible with either the siricid woodwasps or the macrotermitine termites. We were able to compare the assimilation efficiency of the isopods' normal food in individuals that had consumed microbial enzymes and in ones that had not. As a result for the first time we could make a quantitative assessment of the contribution of the acquired enzymes to digestive processes occurring in the animal. Another technical improvement in our methodology was the utilization of high-performance liquid chromatography (HPLC) in purifying and comparing enzymes derived from extracts of the gut fluid and detrital food of *T. rathkei*. Several other investigators have addressed the possible significance of ingested enzymes in detritivore nutrition, and their work is also discussed in this chapter. Hassall and Jennings (1975) have studied a different terrestrial isopod, *Philoscia muscorum*, whereas Bärlocher (1982, 1983) and Chamier and Willoughby (1986) have investigated the aquatic amphipods *Gammarus fossarum* and *G. pulex*, respectively. Fi-