

Properties of Two Enzymes Involved in the Biosynthesis of the Fungal Pathogenicity Factor HC-Toxin

Jonathan D. Walton and Frank R. Holden

Department of Energy-Plant Research Laboratory, Michigan State University, E. Lansing, MI 48824 U.S.A.
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Host-selective toxins are chemical mediators of the plant-pathogen interactions in which they occur. In the fungal maize pathogen *Cochliobolus carbonum* race 1, a single genetic locus controls production of its host-selective toxin, HC-toxin. This genetic locus is associated with two separable enzymatic activities from *C. carbonum*, which have the properties expected of enzymes involved in the biosynthesis of HC-toxin (Walton, Proc. Natl. Acad. Sci. USA 84:8444, 1987). These enzymes have been purified and characterized. One enzyme, called HTS-1, activates L-proline by adenosine triphosphate/inorganic pyrophosphate exchange, binds L-proline as the thioester, and epimerizes L-proline to D-proline, the isomer of proline found in HC-toxin. The second enzyme, called HTS-2, activates both L-alanine and D-alanine, forms the thioesters of each amino acid, and epimerizes L-alanine to D-alanine, but not D-alanine to L-alanine. The two enzymes were

purified by precipitation with ammonium sulfate and anion exchange, gel filtration, and hydroxyapatite chromatography. By HPLC gel filtration HTS-1 and HTS-2 both have apparent molecular weights of 310,000, but by sodium dodecyl sulfate-PAGE the apparent molecular weights are 220,000 and 160,000, respectively. HTS-1 has a Michaelis constant for L-proline of 17.6 mM. HTS-2 has Michaelis constants of 3.4 and 101 mM for D-alanine and L-alanine, respectively, but the maximum velocity for L-alanine is three times that for D-alanine and approximately equal to that of HTS-1 for L-proline. The results in this paper demonstrate the involvement of HTS-1 and HTS-2 in the biosynthesis of HC-toxin and describe methods to purify the enzymes, opening an avenue to elucidate one aspect of the molecular biology of the interaction between *C. carbonum* race 1 and maize.

Additional keywords: cyclic peptide biosynthesis, maize

HC-toxin is a phytotoxin produced by race 1 of *Cochliobolus carbonum* Nelson (anamorph *Helminthosporium carbonum* Ullstrup or *Bipolaris zeicola* (Nisak. and Miyake) Shoem.), a pathogen of certain genotypes of maize, *Zea mays* L. Several studies have demonstrated that HC-toxin is a primary determinant of disease. Only maize lines that are homozygous recessive at the nuclear *Hm* locus (genotype *hmhm*) are susceptible to *C. carbonum* race 1 and sensitive to HC-toxin (Ullstrup and Brunson 1947; Scheffer and Ullstrup 1965). Races of *C. carbonum* that cannot make HC-toxin are not pathogenic to maize with genotype *hmhm* (Scheffer *et al.* 1967). In genetic crosses between pathogenic and nonpathogenic isolates of *C. carbonum*, or between *C. carbonum* race 1 and *C. victoriae*, a pathogen of oats, pathogenicity on *hmhm* maize is controlled by a single genetic locus that segregates with HC-toxin production (Nelson and Ullstrup 1961; Scheffer *et al.* 1967). A nonpathogenic isolate of *C. carbonum* is able to infect *hmhm* maize if HC-toxin is added to the infection court (Comstock and Scheffer 1973).

HC-toxin is a cyclic tetrapeptide of structure cyclo(D-propyl-L-alanyl-D-alanyl-L-AOE). AOE stands for 2-amino-8-oxo-9,10-epoxidecanoic acid (Liesch *et al.* 1982; Walton *et al.* 1982; Pope *et al.* 1983; Kawai *et al.* 1983; Kawai and Rich 1983).

Research on the molecular biology of the genes, known as the *Tox* loci, which control the biosynthesis of host-selective toxins by *Cochliobolus* spp., would allow a better understanding of the processes by which this group of plant pathogens evolves. One approach to identifying the *Tox*

genes is to start by identifying the products of those genes, which are likely to be the toxin biosynthetic enzymes. Toward this end, we have begun a study of the enzymes that biosynthesize HC-toxin, based on the knowledge of how other cyclic peptides such as gramicidin-S and tyrocidine are synthesized (Kleinkauf and von Döhren 1981).

C. carbonum race 1 has two enzymes, one that catalyzes adenosine triphosphate/inorganic pyrophosphate (ATP/PP_i) exchange in the presence of L-proline (and that we now call HC-toxin synthetase 1, or HTS-1), and one that catalyzes ATP/PP_i exchange in the presence of L-alanine or D-alanine (called HTS-2) (Walton 1987; Wessel *et al.* 1987). HTS-1 and HTS-2 are present only in isolates of *C. carbonum* that make HC-toxin, and in a genetic cross they segregate with the *Tox* locus, which controls HC-toxin production (Walton 1987). These data indicated that these two enzymatic activities were involved in the biosynthesis of HC-toxin. They therefore can be considered crucial biochemical entities in the pathogenic relationship between *C. carbonum* race 1 and its host.

This paper reports on the characterization and purification of these two enzymes. The results demonstrate their similarity to other cyclic peptide synthetases and further support their role in the biosynthesis of HC-toxin.

MATERIALS AND METHODS

Fungal growth. All experiments were done with *C. carbonum* race 1 isolate SB111 (obtained from S. Briggs, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The fungus was maintained on V-8 juice agar and for enzyme production grown in 1-l flasks on modified Fries' medium (Walton *et al.* 1982), 125 ml per flask, for 4 days on

an open laboratory bench. At harvest the mycelial mats were rinsed in 50 mM KCl plus 2 mM EDTA, and freeze-dried.

Enzyme purification. The initial stages of purification were based on those used by Zocher *et al.* (1982) in the purification of enniatin synthetase. All buffers contained 4 mM dithiothreitol and 10% (v/v) glycerol. All manipulations were done either on ice or at 4°C. Freeze-dried mycelial mats were ground in a mortar and pestle in liquid nitrogen and then again in 50 mM KH₂PO₄, pH 7, 0.2 M KCl, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). In some experiments the protease inhibitors leupeptin and pepstatin were also added, but without any noticeable effect. The cell-free homogenate was stirred for 30 min at 4°C and then centrifuged at 36,000 × *g* for 20 min. Neutralized polyethylenimine was added to the supernatant to a final concentration of 0.2% (v/v), and the precipitate collected by centrifugation (21,000 × *g*) and discarded. Ammonium sulfate was added to the supernatant to 40% saturation (22.6 g per 100 ml), and after stirring for 20 min the precipitate was collected by centrifugation (21,000 × *g*, 15 min).

After resuspension in 25 mM KH₂PO₄, pH 7, and desalting on a G-25 Sephadex column, the protein preparation (typically 100–200 mg of total protein) was loaded onto a column of Fast-Flow DEAE-Sepharose (total column volume 100 ml) and eluted with a gradient of 25 mM KH₂PO₄, pH 7, to 300 mM KH₂PO₄, pH 7, in a total volume of 600 ml. Anion exchange chromatography separates HTS-1 from HTS-2; unresolved activity was rerun on DEAE-Sepharose. All further purification of the two enzymes was done separately, in parallel.

High-pressure liquid chromatography. All HPLC was done on a Beckman instrument equipped with two model 114M pumps, a model 163 UV detector set at 280 nm, and a model 421A controller. All gradients were linear. The buffer for gel filtration chromatography contained 1 mM EDTA.

After the initial ion exchange separation of HTS-1 and HTS-2, they were chromatographed by preparative HPLC gel filtration (TSK 3000SW, 21.5 × 600 mm) in 100 mM KH₂PO₄, pH 7, flow rate 4 ml/min. Gel filtration molecular weight standards (Bio-Rad) were thyroglobulin (*M_r* 670,000), gamma-globulin (*M_r* 158,000), ovalbumin (*M_r* 44,000), and myoglobin (*M_r* 17,000). Active fractions were pooled, concentrated by precipitation with ammonium sulfate, and run on HPLC HCA-hydroxyapatite (Rainin Instrument Co.; dimensions 7.6 × 100 mm) with a gradient of 10 mM KH₂PO₄, pH 7, to 500 mM KH₂PO₄, pH 7, in 100 min, flow rate 0.8 ml/min. Finally, active fractions from this step were pooled, concentrated, and chromatographed by HPLC anion exchange (TSK IEX-DEAE-5PW, 7.5 mm × 7.5 cm) in 50 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid with a gradient of 0–0.6 M NaCl in 40 min, flow rate 1 ml/min.

ATP/PP_i exchange assay. ATP/PP_i exchange was assayed as previously described (Lee and Lipmann 1975; Walton 1987). The standard amino acid concentration was 4 mM and the PP_i concentration was 1 mM.

Thioester assay and analysis of enzyme-bound amino acids. All radiolabeled amino acids were purchased from Amersham. Reactions were run with 100 μl of enzyme fraction in 20 mM MgCl₂ and 15 mM ATP, plus 0.25 μCi of [¹⁴C]L-proline (specific activity 250 mCi/mmol), [¹⁴C]L-alanine (specific activity 168 mCi/mmol), or [¹⁴C]D-alanine (specific activity 46 mCi/mmol) (Keller 1987). Incubations were for 30 min at 30°C. Reactions were terminated by addition of an equal volume of 15% (w/v) trichloroacetic

acid (TCA) and the precipitates collected on GF/C (Whatman) glass fiber filters. The filters were washed five times with 7% (w/v) TCA, once with ethanol, and counted (Keller 1987). Alternatively, the reaction mixtures were made 2% (w/v) in sodium dodecyl sulfate (SDS) and after heating to 90°C for 3 min analyzed by SDS-PAGE.

To determine the nature of the protein-bound amino acid, the reaction mixtures were centrifuged instead of being filtered, and washed four times with TCA. The protein pellets then were washed twice with ethanol, dried, and resuspended in 0.5 ml of formic acid or performic acid. After incubation at room temperature for 1 hr, the samples were dried, the protein again precipitated with TCA, and centrifuged. The supernatants were assayed for radioactive amino acids released by the acid treatments.

To analyze the chiralities of the amino acids released from HTS-1 and HTS-2 by the treatment with performic acid, the amino acids were derivatized with 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide (FDAA; Marfey's reagent; Pierce Chemical Co.) according to the manufacturer's instructions and Marfey (1984). Derivatized amino acids were separated by HPLC using a Beckman 5μ ODS Ultrasphere column, 4.6 mm × 25 cm, and a gradient from 15% solvent A to 65% solvent B in 25 min. Solvents A and B were water and acetonitrile, respectively, each containing 0.1% trifluoroacetic acid. For the controls, radioactivity was detected with a Radiomatic Flo-1 radioactive flow detector equipped with a solid cell. For analysis of the unknowns, fractions were collected and the radioactivity measured in a scintillation counter. As internal standards, after performic acid treatment but before derivatization with FDAA, each unknown sample was spiked with unlabeled amino acid of the same chirality as originally incubated with the enzyme. The amount of internal standard used was sufficient to be detected by absorption at 340 nm (Marfey 1984). Hydrolyzed FDAA served as an additional internal standard. This technique was also used to confirm the optical purity of the radiolabeled amino acids used in these experiments.

Protein was measured by the method of Bradford (1976). SDS-PAGE was on a Pharmacia PhastSystem, following the manufacturer's protocols for running and staining the gels with silver. Gels contained 7.5% acrylamide. Molecular weight standards (Bio-Rad) were myosin (*M_r* 200,000), β-galactosidase (*M_r* 115,600), phosphorylase b (*M_r* 97,000), bovine serum albumin (*M_r* 66,000), and ovalbumin (*M_r* 43,000). Additional molecular weight standards (Pharmacia) were used in some experiments to estimate the molecular weights of HTS-1 and HTS-2. These included thyroglobulin (*M_r* 330,000) and ferritin (*M_r* 220,000). SDS-PAGE to show proteins radiolabeled with [¹⁴C]amino acids was on a 5% stacking and 7% resolving gel in a Hoefer "Mighty-Small" apparatus as described by Hames and Rickwood (1981), but in the absence of any thiol reagent (Keller 1987). Dried gels containing radiolabeled proteins were analyzed with a Bioscan (Washington, DC) System 200 gas-flow imaging scanner.

RESULTS

Purification of HC-toxin synthetases 1 and 2. On most chromatography media, HTS-1 and HTS-2 were poorly resolved from each other. This was observed even though, on the basis of their behavior when run separately on the same medium, they should have been well resolved. This apparent tendency of HTS-1 and HTS-2 to form a complex

could be reliably overcome only by anion exchange chromatography, which was therefore used as the first purification step after ammonium sulfate precipitation.

The two HTSs free of significant contamination by the other were further purified in parallel by gel filtration HPLC, hydroxyapatite HPLC, and then finally anion exchange HPLC. When material purified in this way was analyzed by SDS-PAGE, a band of molecular weight of 220,000 was consistently associated with HTS-1 activity (Fig. 1). This is in reasonable agreement with its molecular weight, 310,000, estimated by gel filtration (Walton 1987; Fig. 2). HTS-2 activity in several different preparations, however, was consistently associated with a band of M_r 160,000 (Fig. 1), although it, too, has an apparent molecular weight of 310,000 by gel filtration (Walton 1987; Fig. 3).

One possible explanation for the discrepancy in apparent molecular weight of HTS-2 as deduced from gel filtration and from SDS-PAGE is that HTS-2 is a homodimer, but there is no precedent for this among cyclic peptide synthetases. Perhaps dimerization of HTS-2 occurs in the absence of HTS-1. This result is probably not due to proteolysis because it was not influenced by protease inhibitors such as EDTA, PMSF, leupeptin, or pepstatin, nor by altering purification parameters such as temperature and time that would be expected to promote or inhibit proteolytic activity. We have seen apparent proteolysis of HTS-2 (but never HTS-1) in some enzyme preparations

when EDTA was omitted from the grinding buffer. In this case, by gel filtration chromatography there was either instead of or in addition to the peak of M_r 310,000 a distinct

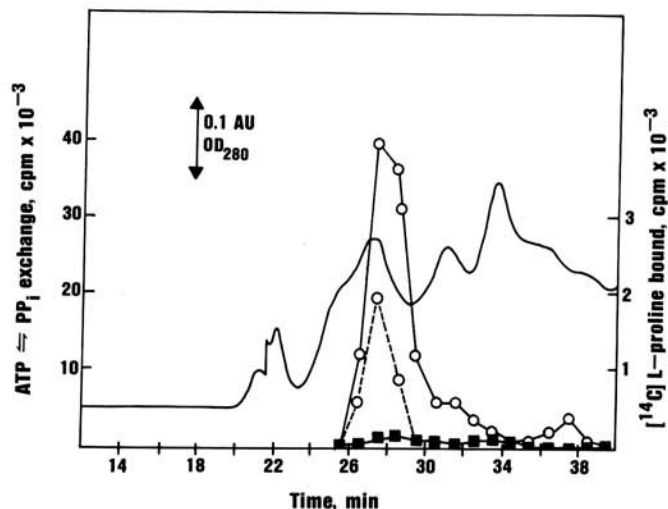


Fig. 2. Gel filtration of HTS-1 and binding of [14 C]L-proline. The enzyme preparation had been purified previously by ammonium sulfate precipitation and DEAE-ion exchange and was largely free of HTS-2. The gel filtration column was a TSK 3000SW (Beckman), 21.5 \times 600 mm. A total of 14.3 mg of protein was injected. One minute (4-ml) fractions were collected and assayed for adenosine triphosphate/inorganic pyrophosphate (ATP/PP_i) exchange dependent on L-proline (o—o) and D-alanine (■—■), and for binding of [14 C]L-proline (o—o). The reaction buffers for ATP/PP_i exchange contained 425,000–455,000 cpm of [32 P]PP_i. For exchange activity, 25 μ l of enzyme was assayed; for amino acid binding, 100 μ l. Solid line—absorption at 280 nm. Molecular weights and elution times of standard proteins were: 760,000, 22.5 min; 158,000, 32 min; 44,000, 39 min; 17,000, 45 min.

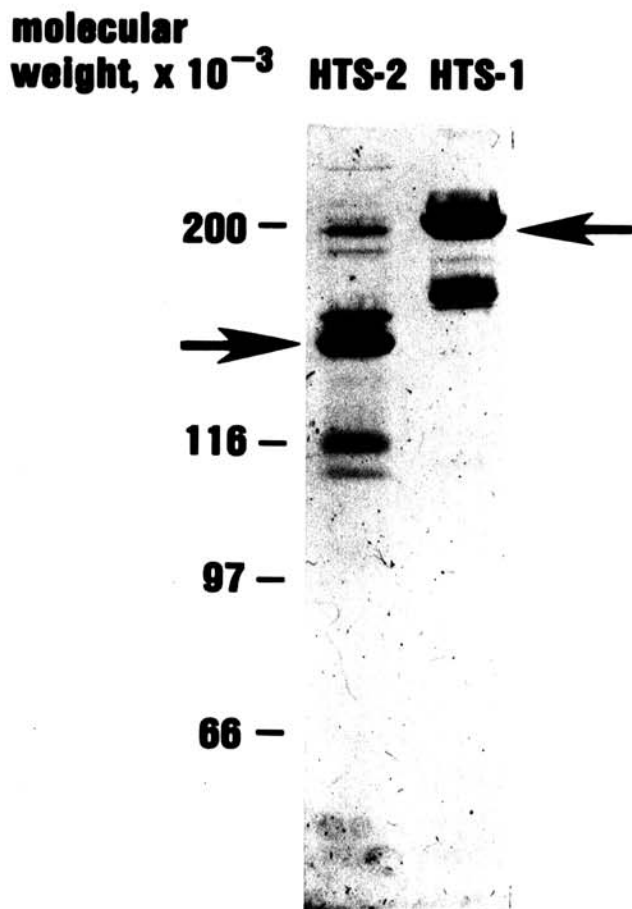


Fig. 1. SDS-PAGE of highly enriched preparations of HTS-1 and HTS-2. Following purification by ion exchange, gel filtration, and hydroxyapatite chromatography, the two enzyme preparations were further purified by analytical anion exchange HPLC before analysis by SDS-PAGE on a Pharmacia PhastSystem. The gel was stained with silver. Molecular weights of standard proteins are shown on the left (values in kD). HTS-1 and HTS-2 are indicated by arrows.

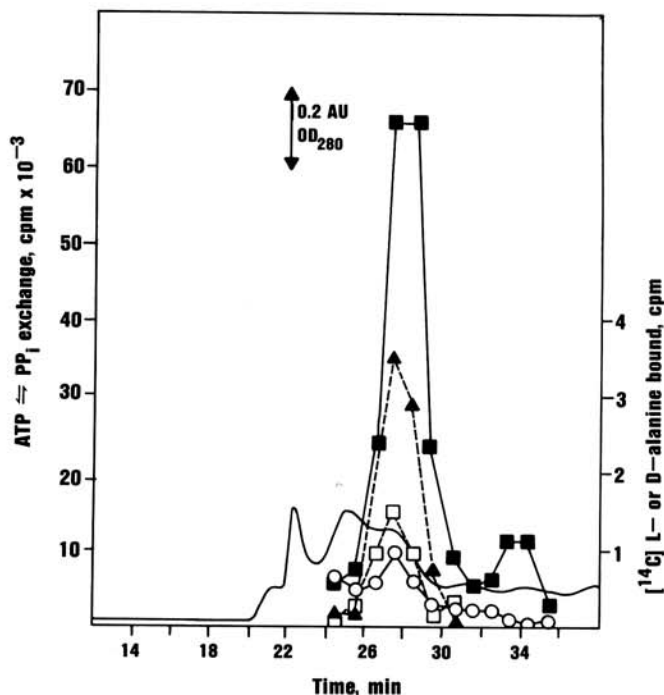


Fig. 3. Gel filtration of HTS-2 and binding of [14 C]L-alanine and [14 C]D-alanine. The enzyme preparation, previously purified by ammonium sulfate precipitation and DEAE-ion exchange, was largely free of HTS-1. Protocols were identical to the experiment shown in Figure 2. A total of 10.2 mg of protein was loaded. Fractions were assayed for ATP/PP_i exchange activity dependent on D-alanine (■—■) and L-proline (o—o), and binding of [14 C]L-alanine (▲—▲) and [14 C]D-alanine (□—□). Solid line—absorbance at 280 nm.

peak of HTS-1 activity of M_r 110,000.

Binding of amino acids as thioesters by HC-toxin synthetase. Before initiating peptide bond formation, enzymes that synthesize cyclic peptides activate the amino acids as aminoacyl thioesters (Gevers *et al.* 1969; Zocher *et al.* 1982; Zocher *et al.* 1986; Keller 1987). Amino acid-binding activities were co-eluted with amino acid-dependent ATP/PP_i exchange activities from a gel filtration column (Figs. 2 and 3). HTS-1 bound [¹⁴C]L-proline (Fig. 2), and HTS-2 bound [¹⁴C]L-alanine and [¹⁴C]D-alanine (Fig. 3). This binding was dependent on ATP. These experiments also demonstrate that proline and alanine amino acid activation are separable activities and that independently the enzymes have the same apparent molecular weight by gel filtration of 310,000 (Walton 1987).

The radiolabeled amino acids that were bound to HTS-1 and HTS-2 could be released by treatment with performic acid but not formic acid (Table 1). This demonstrates that the amino acids had been bound as thioesters (Keller 1987).

Aliquots of highly purified HTS-1 and HTS-2 were incubated with [¹⁴C]L-proline and [¹⁴C]L-alanine as for the thioester assay and analyzed by PAGE in the presence of SDS but without a thiol reagent. Radiolabeled HTS-1 gave a single peak of radioactivity in the resolving gel, with an estimated molecular weight of 210,000 (Fig. 4). The substantial amount of radioactivity remaining in the stacking gel in Figure 4 is due to overloading, which was necessary to get enough radioactivity to enter the gel. There was a single peak of radiolabeled HTS-2 with an estimated molecular weight of 154,000 (Fig. 5). These data support the conclusion that the major protein bands seen with silver staining in highly purified preparations (Fig. 1) represent HTS-1 and HTS-2.

HC-toxin synthetase epimerizes L-proline and L-alanine. The chiralities of the amino acids that had bound to HTS-1 and HTS-2 as thioesters were determined by derivatization with a chiral reagent, FDAA, and separation by reverse phase HPLC (Marfey 1984). Figure 6 shows the ability of this technique to resolve standard mixtures of D- and L-alanine completely. The peak of radioactivity eluting within 2 min is probably underivatized alanine, indicating that under our conditions the reaction with FDAA was not complete. Derivatized L-proline and D-proline were also completely resolved (data not shown). Derivatized L-alanine and L-proline (and D-alanine and D-proline) had similar retention times. The D isomers of both alanine and proline were eluted after hydrolyzed FDAA and their peak shapes were less symmetrical (Marfey 1984; Fig. 6). Marfey (1984) has demonstrated that FDAA reacts equally well with D- and with L-amino acids.

Table 1. Aminoacyl thioester formation by HC-toxin synthetase

[¹⁴ C]-Labeled amino acid ^a	Enzyme	Acid treatment	Cpm released
L-proline	HTS-1	formic	60
		performic	5,780
D-alanine	HTS-2	formic	71
		performic	4,490
L-alanine	HTS-2	formic	92
		performic	12,030

^a[¹⁴C]-labeled amino acids (0.25 μ Ci) were incubated with ATP and either HTS-1, which catalyzes proline-dependent ATP/PP_i exchange, or HTS-2, which catalyzes alanine-dependent exchange. The samples were treated with either formic acid or performic acid and the proteins then precipitated with trichloroacetic acid. The results show the radioactivity remaining in the supernatant. Amino acids bound as aminoacyl thioesters are released by treatment with performic acid but not formic acid (Keller 1987).

When HTS-2 was incubated with [¹⁴C]D-alanine and then treated with performic acid, only D-alanine was released (Fig. 7A). However, after HTS-2 was incubated with [¹⁴C]L-alanine, a mixture of L- and D-alanine was released (Fig. 7B), indicating that HTS-2 had converted some of the L-alanine to D-alanine. Likewise, HTS-1 partially converted [¹⁴C]L-proline to D-proline (Fig. 7C).

Kinetic constants. Table 2 shows the kinetic constants for HTS-1 and HTS-2 for their respective amino acid substrates

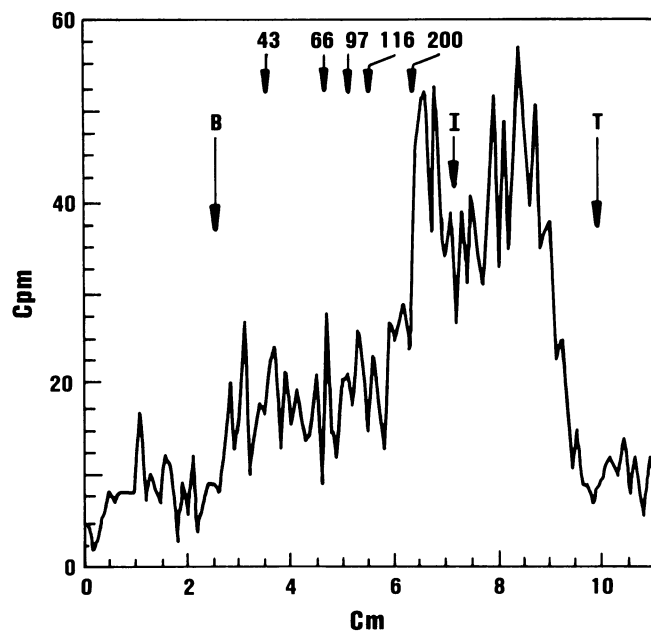


Fig. 4. Radioactivity scan of separation by sodium dodecyl sulfate (SDS)-PAGE of HTS-1 labeled with [¹⁴C]L-proline. After incubation, the assay mixture was made 2% in SDS and loaded on a 7% acrylamide gel. A total of 1800 cpm of aminoacylated HTS-1 was loaded. After electrophoresis the gel was dried and radioactivity in each lane measured with a gas-flow radioactivity scanner. The position of the running front is indicated with a "B," the interface between the stacking and the resolving gel with an "I," and the top of the stacking gel with a "T." The abscissa shows migration distance and is calibrated in centimeters. Positions of molecular weight markers (values in kilodaltons) are indicated across the top.

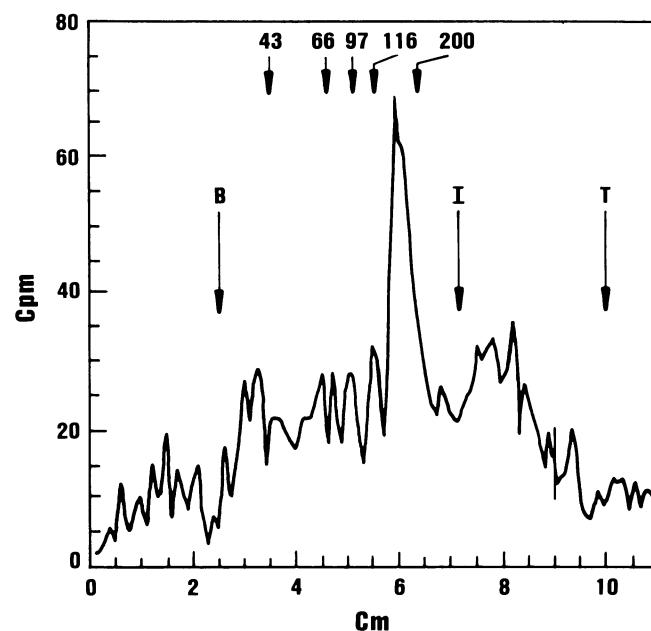


Fig. 5. Radioactivity scan of separation by SDS-PAGE of HTS-2 labeled with [¹⁴C]L-alanine. Protocols were identical to the experiment shown in Figure 4. A total of 4,800 cpm of aminoacylated HTS-2 was loaded.

in the ATP/PP_i exchange assay. Substrate consumption was linear over the course of the experiment. The kinetics gave normal, linear curves when the reciprocal of the velocity was plotted against the reciprocal of the substrate concentration. The values in Table 2 were measured in an enzyme preparation that had been purified only as far as precipitation with ammonium sulfate, in order to be able to compare the V_{\max} s for HTS-1 and HTS-2. Similar K_m s were found for highly purified preparations (data not shown).

Because the K_m s for the amino acid substrates, especially

L-alanine, were so high, we made some attempts to exclude the possibility of an artifact due to sub- or supra-optimal concentrations of the other substrates. We determined the K_m of HTS-2 for PP_i to be 0.2 mM and for ATP to be also 0.2 mM. This K_m for ATP is similar to that of gramicidin-S synthetase (Kittelberger *et al.* 1982). A tenfold higher

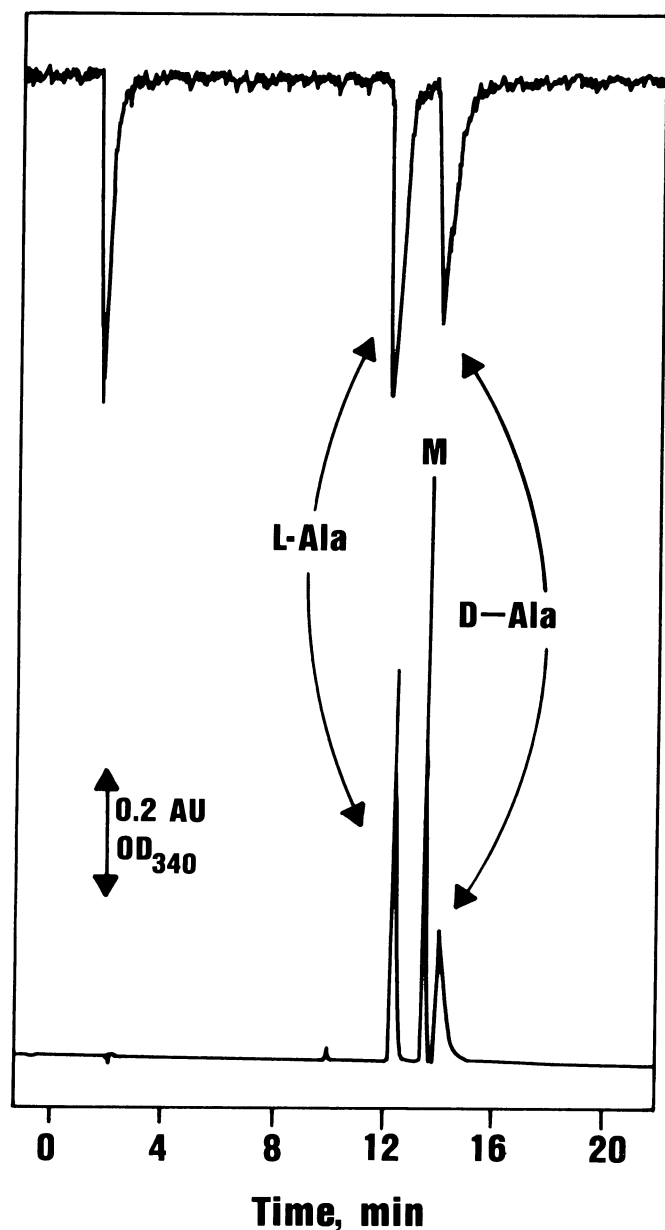


Fig. 6. Separation of the 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide (FDAA) derivatives of L-alanine and D-alanine by reverse phase HPLC (Marfey 1984). The preparation contained an equimolar mixture of L-alanine and D-alanine to which 10,000 cpm each of [¹⁴C]L-alanine and [¹⁴C]D-alanine were added before derivatization. A total of 7 nmol of derivatized D-alanine and L-alanine was injected. Optical absorbance was monitored at 340 nm, 2.0 AU full scale. Radioactivity was monitored with an in-line radioactive flow detector (Radiomatic model CT) equipped with a solid cell, 3,000 cpm full scale. Lower trace—absorbance at 340 nm. Upper trace—radioactivity (chart recorder polarity reversed). Due to the void volume between the absorbance and radioactivity detectors, and to chart pen offset, the two traces do not correspond exactly. “M” indicates unreacted FDAA. The peak of radioactivity with an elution time of 1.8 min is unreacted alanine.

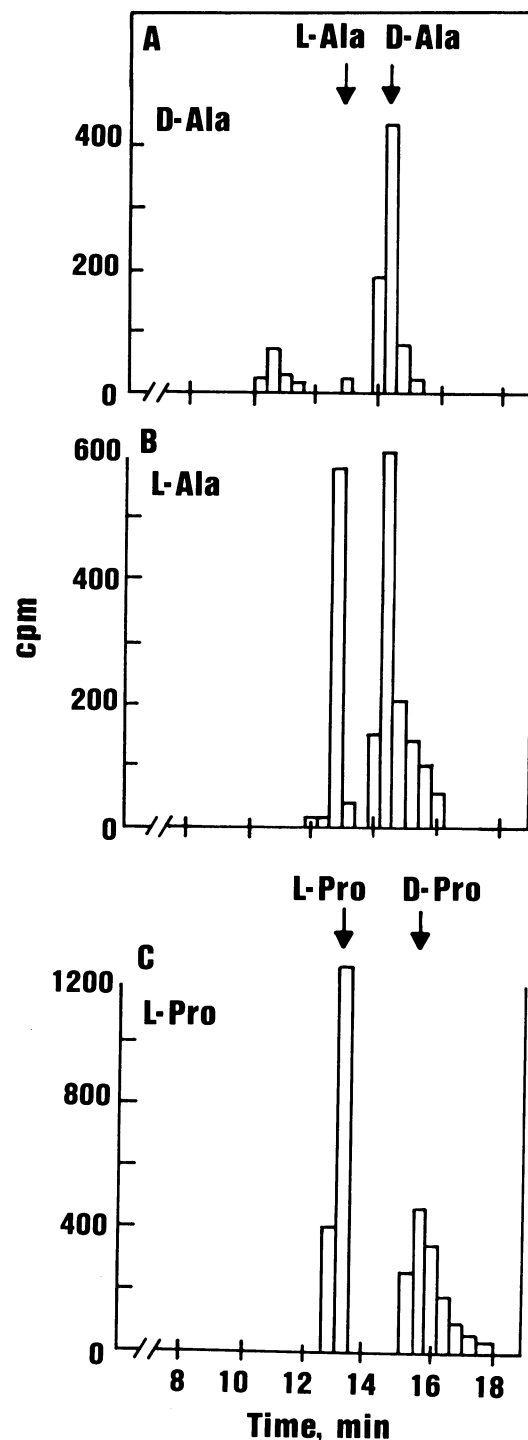


Fig. 7. Analysis of the stereochemistry of [¹⁴C]amino acids thioesterified to HTS-1 and HTS-2. Radiolabeled amino acids bound to the enzymes were released by performic acid treatment (see Table 1), derivatized with FDAA, and analyzed by reverse phase HPLC as in Figure 6. Fractions were collected and the radioactivity measured by scintillation counting. Unlabeled amino acid was added to each preparation as an internal standard that could be monitored by absorbance at 340 nm (not shown); elution positions of these standards are indicated by arrows in (A) and (C). Incubation of: (A) HTS-2 with [¹⁴C]D-alanine, (B) HTS-2 with [¹⁴C]L-alanine, and (C) HTS-1 with [¹⁴C]L-proline.

concentration of either of these substrates did not inhibit the enzyme. The optimum Mg^{+2} concentration for HTS-2 was between 4 and 6 mM, with slight inhibition at our standard concentration of 10 mM, but the K_m for L-alanine was the same at 5 mM Mg^{+2} . We conclude that the K_m values in Table 2 are a reasonable estimate of the affinities of HTS-1 and HTS-2 for their amino acid substrates.

The possibility of competition between L-alanine and D-alanine was tested by assaying HTS-2 with and without 30 mM D-alanine and varying concentrations of L-alanine. At all concentrations of L-alanine tested (0–500 mM), the ATP/PP_i exchange activity was the sum of the exchange activity catalyzed by each amino acid alone, indicating that activation of L-alanine and D-alanine occurs at different, independent sites (data not shown).

DISCUSSION

The data presented in this paper substantiate a role for two enzymes that catalyze ATP/PP_i exchange dependent on L-proline, L-alanine, and D-alanine in the biosynthesis of the pathogenicity factor HC-toxin. This conclusion is especially strengthened by the demonstration (Fig. 7C) that one of these enzymes, HTS-1, can convert L-proline to D-proline, which is the isomer of proline found in HC-toxin (Walton *et al.* 1982).

HTS-2, the enzyme that catalyzes ATP/PP_i exchange in the presence of D-alanine and L-alanine, both of which are found in HC-toxin, can also epimerize L-alanine to D-alanine. HTS-2 cannot convert D-alanine to L-alanine, which is consistent with the fact that when [¹⁴C]D-alanine was fed to *C. carbonum* race 1 *in vivo*, it was incorporated only into the D-alanine moiety of HC-toxin and not into the L-alanine moiety (unpublished data). This means that both the L-alanine and the D-alanine in HC-toxin could come from free cytoplasmic L-alanine, but that the L-alanine in HC-toxin could not come from cytoplasmic D-alanine. Considering that HTS-2 activates D-alanine, and in fact has a much higher affinity for it than for L-alanine (Table 2), there is the possibility that D-alanine, in addition to L-alanine, is a natural substrate for the enzyme *in vivo*. Free D-alanine could come from either an unknown D-alanine biosynthetic pathway in the fungus or from the infected plant. It is not known if there is free D-alanine in *C. carbonum*. Free D-alanine does occur at low levels in some higher plants (Fukuda *et al.* 1973; Frahn and Illman 1975), including, perhaps, the host of *C. carbonum*, maize (Aldag *et al.* 1971).

Neither HTS-1 nor HTS-2 catalyzes complete conversion of L-proline and L-alanine, respectively, to the corresponding D isomers (Figs. 7B and 7C). The HTS-2 activity is not a racemase because it does not convert D-alanine to L-alanine

(Fig. 7A). Since HC-toxin contains both D-alanine and L-alanine, one would expect there to be an aminoacyl thioester binding site for each isomer. However, it is not known if L-alanine is epimerized after it binds to the D-alanine site, or whether the epimerization occurs while it is being transferred from the L-alanine site to the D-alanine site. A study of competition between D-alanine and L-alanine in the [¹⁴C]amino acid binding assay (Figs. 2 and 3) might clarify this.

In the case of HTS-1, the data suggest that there are two thioester sites, one for L-proline and one for D-proline. An alternate explanation is that there is only a single proline thioester site but that the epimerization reaction had not gone to completion in the course of our experiments. Regardless of the number of aminoacyl thioester sites on HTS-1 and HTS-2, all of them are probably "peripheral" thioesters, following the nomenclature of Lynen (Lipmann 1982), because we have not been able to find enzyme-linked pantetheine in either HTS-1 or HTS-2 (unpublished data).

The cyclic decapeptide gramicidin-S contains D-phenylalanine, and gramicidin synthetase activates both L- and D-phenylalanine (Vater and Kleinkauf 1976). In contrast, bacitracin contains D-ornithine, but bacitracin synthetase activates only L-ornithine (Frøyshov and Laland 1974). Actinomycin synthetase is similar to bacitracin synthetase in this regard; although actinomycin contains D-valine, only L-valine is recognized by the enzyme (Keller 1987). In its ability to use either L-alanine or D-alanine as substrate for the D-alanine in HC-toxin, HTS-2 is similar to gramicidin-S synthetase. In its inability to activate D-proline, HTS-1 is similar to bacitracin and actinomycin synthetases. HTS-1 differs from actinomycin synthetase in that L-alanine remains in the L-configuration after it is thioesterified to actinomycin synthetase (Keller 1987), but L-proline becomes partially converted to D-proline after (or in the process of) being thioesterified to HTS-1 (Fig. 7C).

The affinities of HTS-1 and HTS-2 for all three amino acid substrates are very low (Table 2). The large difference in the K_m s for D-alanine and L-alanine explains why, at the amino acid concentration, 4 mM, used in our standard ATP/PP_i exchange assays, the D-alanine-dependent exchange activity appeared to be much stronger than the L-alanine-dependent activity (Walton 1987).

Published K_m s for amino acid substrates of other cyclic peptide synthetases are usually less than 1 mM (e.g., Kittelberger *et al.* 1982). However, other enzymes are known that have low affinities for L-alanine. Alanine racemases from various bacteria have K_m s for L-alanine of 8.5 (Wood and Gunsalus 1951), 30 (Julius *et al.* 1970), and 54 mM (Marr and Wilson 1954).

Why do HTS-1 and HTS-2 have such low affinities for their amino acid substrates? One speculative answer is that it is in order to prevent HC-toxin production from interfering with primary metabolism. Bacterial cyclic peptides are synthesized only after cell growth and division are slowing down or have stopped, in this respect being typical secondary metabolites (Katz *et al.* 1965; Frøyshov and Laland 1974; Lee and Lipmann 1975). Therefore, for bacterial cyclic peptides there is little or no competition between primary and secondary metabolism for the substrates. In contrast, HC-toxin, like other host-selective toxins, is produced constitutively (Nishimura and Scheffer 1965; Comstock and Scheffer 1973; Walton, unpublished data), and therefore HC-toxin biosynthesis occurs even while the fungus is rapidly growing and the rate of primary metabolism is high. The low affinity of HTS-1 and HTS-2

Table 2. Kinetic constants for HTS-1, which catalyzes L-proline-dependent ATP/PP_i exchange, and HTS-2, which catalyzes L- and D-alanine-dependent exchange

Substrate ^a	Enzyme	K_m (mM)	V_{max} (relative to L-proline)
L-proline	HTS-1	17.6 ± 3.8	1.00
L-alanine	HTS-2	101 ± 15	1.12
D-alanine	HTS-2	3.4 ± 0.8	0.36

^aThe enzyme preparation used in this experiment was a 40%-saturation ammonium sulfate cut. Each reaction, done in triplicate, contained 9 μg of protein. The concentration of ATP was 2 mM; of PP_i, 1 mM; and of Mg^{+2} , 10 mM. Reactions were run for 30 min at 30°C. Total [³²P]exchanged was always less than 10%. Values were calculated from Lineweaver-Burk (reciprocal) plots. Michaelis constant values are ± one standard deviation.

for proline and alanine might prevent an excessive flux of substrates away from primary metabolism. Whether HC-toxin synthesis is sequestered from primary metabolism by such a "passive kinetic" mechanism or not, some sort of control would seem to be necessary, especially to regulate the consumption of alanine, a compound only one enzymatic step away from the central primary metabolite pyruvate.

A thorough analysis of the biosynthesis of HC-toxin and hence of the molecular genetics of this pathogenicity factor still requires identification of the enzyme or enzymes that synthesize and activate the fourth amino acid in HC-toxin, L-2-amino-8-oxo-9,10-epoxidecanoic acid (AOE). In a step toward clarifying AOE's biosynthetic origins, Wessel *et al.* (1988) showed acetate to be incorporated into AOE *in vivo*.

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LITERATURE CITED

- Aldag, R. W., Young, J. L., and Yamamoto, M. 1971. An enzymatic chromatographic procedure for the determination of D-amino acids in plant and soil extracts. *Phytochemistry* 10:267-274.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Comstock, J. C., and Scheffer, R. P. 1973. Role of host-selective toxin in colonization of corn leaves by *Helminthosporium carbonum*. *Phytopathology* 63:24-29.
- Frahn, J. L., and Illman, R. J. 1975. The occurrence of D-alanine and D-alanyl-D-alanine in *Phalaris tuberosa*. *Phytochemistry* 14:1464-1465.
- Frøyshov, Ø., and Laland, S. G. 1974. On the biosynthesis of bacitracin by a soluble enzyme complex from *Bacillus licheniformis*. *Eur. J. Biochem.* 46:235-242.
- Fukuda, M., Tokumura, A., and Ogawa, T. 1973. D-alanine in germinating *Pisum sativum* seedlings. *Phytochemistry* 12:2593-2595.
- Gevers, W., Kleinkauf, H., and Lipmann, F. 1969. Peptidyl transfers in gramicidin S biosynthesis from enzyme-bound thioester intermediates. *Proc. Natl. Acad. Sci. USA* 63:1335-1342.
- Hames, B. D., and Rickwood, D., eds. 1981. *Gel Electrophoresis of Proteins*. IRL Press, Oxford. 290 pp.
- Julius, M., Free, C. A., and Barry, G. T. 1970. Alanine racemase (*Pseudomonas*) *Meth. Enzymol.* 17A:171-176.
- Katz, E., Wise, M., and Weissbach, H. 1965. Actinomycin biosynthesis: Differential effect of chloramphenicol on protein and peptide antibiotic synthesis. *J. Biol. Chem.* 240:3071-3078.
- Kawai, M., and Rich, D. H. 1983. Total synthesis of the cyclic tetrapeptide, HC-toxin. *Tetrahedron Lett.* 24:5309-5312.
- Kawai, M., Rich, D. H., and Walton, J. D. 1983. The structure and conformation of HC-toxin. *Biochem. Biophys. Res. Comm.* 111:398-403.
- Keller, U. 1987. Actinomycin synthetases. *J. Biol. Chem.* 262:5852-5856.
- Kittelberger, R., Altmann, M., and von Döhren, H. 1982. Kinetics of amino acid activation in gramicidin S synthesis. In: *Peptide Antibiotics, Biosynthesis and Functions*. H. Kleinkauf and H. von Döhren, eds., pp. 209-218. Walter de Gruyter, Berlin.
- Kleinkauf, H., and von Döhren, H. 1981. Nucleic acid independent synthesis of peptides. *Curr. Top. Microbiol. Immunol.* 91:129-177.
- Lee, S. G., and Lipmann, F. 1975. Tyrocidine synthetase system. *Meth. Enzymol.* 43:585-602.
- Liesch, J. M., Sweeley, C. C., Staffeld, G. D., Anderson, M. S., Weber, D. J., and Scheffer, R. P. 1982. Structure of HC-toxin, a cyclic tetrapeptide from *Helminthosporium carbonum*. *Tetrahedron* 38:45-48.
- Lipmann, F. 1982. On biosynthesis of the cyclic antibiotics gramicidin S and tyrocidine, and of linear gramicidin. In: *Peptide Antibiotics, Biosynthesis and Functions*. H. Kleinkauf and H. von Döhren, eds., pp. 23-45. Walter de Gruyter, Berlin.
- Marfey, P. 1984. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Res. Commun.* 49:591-596.
- Marr, A. G., and Wilson, P. W. 1954. The alanine racemase of *Brucella abortus*. *Arch. Biochem. Biophys.* 49:424-433.
- Nelson, R. R., and Ullstrup, A. J. 1961. The inheritance of pathogenicity in *Cochliobolus carbonum*. *Phytopathology* 51:1-2.
- Nishimura, S., and Scheffer, R. P. 1965. Interactions between *Helminthosporium victoriae* spores and oat tissue. *Phytopathology* 55:629-634.
- Pope, M. R., Ciuffetti, L. M., Knoche, H. W., McCrery, D., Daly, J. M., and Dunkle, L. D. 1983. Structure of the host-specific toxin produced by *Helminthosporium carbonum*. *Biochemistry* 22:3502-3506.
- Scheffer, R. P., and Ullstrup, A. J. 1965. A host-specific toxic metabolite from *Helminthosporium carbonum*. *Phytopathology* 55:1037-1038.
- Scheffer, R. P., Nelson, R. R., and Ullstrup, A. J. 1967. Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae*. *Phytopathology* 57:1288-1291.
- Ullstrup, A. J., and Brunson, A. M. 1947. Linkage relationships of a gene in corn determining susceptibility to a *Helminthosporium* leaf spot. *J. Am. Soc. Agron.* 39:606-609.
- Vater, J., and Kleinkauf, H. 1976. Gramicidin S-synthetase. A further characterization of phenylalanine racemase, the light enzyme of gramicidin S-synthetase. *Biochim. Biophys. Acta* 429:1062-1072.
- Walton, J. D. 1987. Two enzymes involved in biosynthesis of the host-selective phytotoxin HC-toxin. *Proc. Natl. Acad. Sci. USA* 84:8444-8447.
- Walton, J. D., Earle, E. D., and Gibson, B. W. 1982. Purification and structure of the host-specific toxin from *Helminthosporium carbonum* race I. *Biochem. Biophys. Res. Comm.* 107:785-794.
- Wessel, W. L., Clare, K. A., and Gibbons, W. A. 1987. Enzymatic biosynthesis of peptide toxins by plant-specific fungi. *Biochem. Soc. Trans.* 15:917-918.
- Wessel, W. L., Clare, K. A., and Gibbons, W. A. 1988. Biosynthesis of L-AOE, the toxic determinant of the phytotoxin produced by *H. carbonum*. *Biochem. Soc. Trans.* In press.
- Wood, W. A., and Gunsalus, I. C. 1951. D-alanine formation: A racemase in *Streptococcus faecalis*. *J. Biol. Chem.* 190:403-416.
- Zocher, R., Keller, U., and Kleinkauf, H. 1982. Enniatin synthetase, a novel type of multifunctional enzyme catalyzing depsipeptide synthesis in *Fusarium oxysporum*. *Biochemistry* 21:43-48.
- Zocher, R., Nihira, T., Paul, E., Madry, N., Peeters, H., Kleinkauf, H., and Keller, U. 1986. Biosynthesis of cyclosporin A: Partial purification and properties of a multifunctional enzyme from *Tolypocladium inflatum*. *Biochemistry* 25:550-553.