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Chemical characterization of α hormones of *Phytophthora parasitica*

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Abstract. A method for the large scale hormone α1 and α2 extraction from agar cultures of A1 and A2 mating types of *Phytophthora parasitica* has been developed. Using a total of 240 L of organic solvents to extract 40 L of the agar medium, sufficient amounts of α hormones were obtained to permit characterization of their physical and chemical properties. Activities of both hormones were in the neutral fractions in acid-base partition. These hormones were not precipitated by cold acetone or digitonin, nor were their activities destroyed by saponification. Fractionation on an aminopropyl column showed that both hormones were neutral lipids with polarity similar to that of monoglycerides and that α2 had a polarity greater than that of α1. Both hormones were stable at 200°C under nitrogen, and α1 was more volatile than α2. After acetylation, the hormonal activity of α1 was retained, while that of α2 was greatly reduced. Base hydrolysis of the acetylated α2 led to the restoration of its hormonal activity. Using semi-preparative gas chromatography, α1 activity was eluted between 230 and 278°C while α2 could not be eluted under the same conditions. Results from this study suggest that α hormones are not phospholipids, glycolipids, glycerides, or steroids, but that they are probably neutral lipids with hydroxyl functional group(s). Structural elucidation of α1 and α2, however, would require an even greater scale of operation than that provided by the present experiment.

Keywords: *Phytophthora parasitica*; α hormones; Sexual reproduction of fungi.

Introduction

Hormonal regulation of sexual reproduction in both heterothallic and homothallic species of *Phytophthora* has been demonstrated with a polycarbonate membrane technique by Ko (1978, 1980). For heterothallic species of *Phytophthora*, it has been postulated that the initiation of oospore formation by A2 mating type depends on the presence of an active receptor to the hormone α1 secreted by A1 mating type of the same or different *Phytophthora* species, and *vice versa* for the oospore formation by A1 mating type. These species have therefore been termed cross-inducing *Phytophthora* (Ko, 1980). According to this hypothesis, homothallic species of *Phytophthora* are those capable of producing oospores in single cultures due to the presence of receptors to hormones produced by themselves, and these have therefore been termed self-inducing *Phytophthora* (Ko, 1980).

Previous attempts to isolate α hormones directly from cultures of *P. parasitica* were unsuccessful, and the physical and chemical characteristics of these hormones remained unclear (Gooday, 1974). An indirect adsorption method developed by Ko (1983) showed that when a culture block of *P. parasitica* was incubated with a piece of Millipore filter separated by a polycarbonate membrane, the Millipore filter was able to absorb hormone produced by the culture and could stimulate oospore formation of the opposite type. It was also found that α hormones could be extracted from the Millipore filters by ethyl ether. The extracts were active when they were readsorbed on a piece of Millipore filter, but not when they were added directly to an agar culture.

The Millipore adsorption method is tedious and unsuitable for collecting α hormones for purification and characterization. A project was initiated to isolate α hormones directly from cultures of *P. parasitica*. Various lipid extraction methods were tested since α hormones were found to have some characteristics of lipids (Chern, 1995). Development of a direct isolation method eventually succeeded. Using a total of 240 L of organic solvents to extract 40 L of agar the medium, sufficient amounts of α hormones were obtained for the characterization of their physical and chemical properties. However, due to their extremely low concentration in the culture medium, structural elucidation of these important fungal hormones would require an even greater scale of operation than available in the present experiment.

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Materials and Methods

Microorganisms

The A1 mating type (isolate P991) of *P. parasitica* was supplied by Dr. G. A. Zentmyer, while the A2 type (isolate 6134, ATCC 62654) of the same species was isolated from an egg plant (Ann and Ko, 1988). These fungi were maintained on 10% V-8 agar (10% V-8 juice, 0.02% $CaCO_3$, and 2% agar) by subculture every two to three weeks.

Bioassay of Hormone Activity

The A2 isolates of *P. parasitica* grown on discs (6 mm diam., 3 mm thick) of 5% V-8 agar (5% V-8 juice, 0.02% $CaCO_3$ and 2% agar adjusted to pH 8 with 1 N KOH before autoclaving) for 6–8 days were used as receptors of hormones α2 and α1, respectively (Ko, 1983). Three µl of the organic solvent containing the hormone was pipetted onto a disc (6 mm diam.) of Millipore filter (GSWP, 0.22 µm, Millipore, Bedford, MA, USA). After evaporation of the solvent, the disc was inverted on the same size of disc of receptor culture for 6 days to induce oospore formation (Figure 1). The number of oospores produced on the contact surface of the receptor culture was counted under a microscope and used to evaluate hormone activity. One unit was defined as the amount of hormone α1 or α2 capable of inducing formation of 10 oospores by A2 or A1 isolate, respectively.

Isolation of α Hormones

Twelve culture blocks (1 × 1 × 3 mm) of A1 or A2 mating type of *P. parasitica* were evenly distributed on a 40% V-8 (40% V-8 juice, 0.02% $CaCO_3$, pH adjusted to 8 with 6 N KOH and 2% agar) agar plate. After incubation in darkness at 24°C for 4 to 6 days, the culture in a plate was homogenized twice each with 30 ml of organic solvent with a Brinkmann Homogenizer (Brinkmann Instruments, Westbury, NY 11590) at the speed setting of 4.5 for 5–10 min. The agar and mycelial fragments were removed by filtration through 2–3 layers of Kaydry wipers (Kimberly-Clark Co., Roswell, GA 30076). Chloroform-methanol (2:1) mixture was used to extract α1. After homogenization and filtration, 0.88% KCl solution was added amounting to one-fourth of the final volume to facilitate separation of organic and aqueous phases. The organic phase was collected and removed with a rotary evaporator under reduced pressure. The residue was redissolved in chloroform for bioassay. For extraction of hormone α2, 95% ethanol was used. After most of the ethanol was evaporated under reduced pressure in a rotary evaporator, the aqueous solution was partitioned 3 times with an equal volume of ethyl acetate. The organic extracts were combined and evaporated, and the residue was redissolved in 95% ethanol for bioassay and characterization.

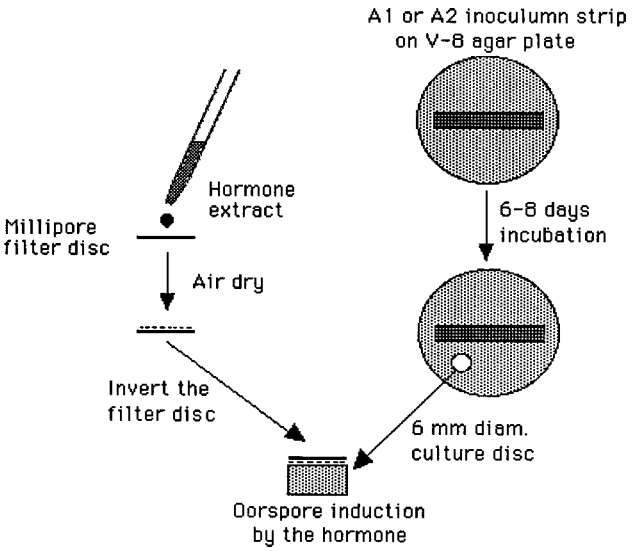

Figure 1. Diagrammatic description of bioassay method for determining hormone activity in solvent extracts.

Acid-Base Partition

To determine if the hormones were basic, acidic or neutral lipids (Harwood and Moody, 1989), the α1 or α2 preparation was dissolved in 100 ml of ethyl ether. The ether solution was extracted twice with 100 ml of 2 N KOH. The organic phase was then extracted twice with 100 ml of 2 N HCl. This ether phase after the acid and base extraction was the neutral fraction. The aqueous KOH extracts were combined and adjusted to pH 2 with 6 N HCl, and then extracted twice with 100 ml of ethyl ether. The ether extracts were combined as the acidic fraction. The aqueous HCl extracts were combined and adjusted to pH 12 with 6 N KOH and then extracted twice with 100 ml of ethyl ether. The ether extracts were collected as the basic fraction. All fractions were evaporated and the residues redissolved in 1 ml of chloroform for α1 or in 1 ml of 95% ethanol for α2 for bioassay.

Acetone Precipitation

To separate polar lipids from neutral lipids, the hormone extract was dissolved in acetone, chilled in ice for 6 h, and centrifuged at 2,500 rpm for 10 min in a refrigerated centrifuge (Kates, 1986). This procedure was repeated, and each time the supernatant was separated from precipitates by centrifugation. Acetone in the supernatant was removed by evaporation under a stream of nitrogen, and the residue was dissolved in chloroform. The combined precipitates were also dissolved in chloroform for α1 and 95% ethanol for α2 for bioassay.

Separation of Hormone Extract Into Different Lipid Classes

The method of lipid classification using aminopropyl bonded phase column (Bond Elute, Varian, Harbor City, CA) developed by Kaluzny et al. (1985) was adopted. The hormone sample was dissolved in a small amount of chloroform, loaded on the first aminopropyl column, and separated into neutral lipids, fatty acids, and phospholip-

ids by eluting with 4 ml of chloroform-isopropanol (2:1), 4 ml of acetic acid in ether, and 4 ml of methanol, respectively. The neutral lipid fraction (i.e., eluate of chloroform-isopropanol) was evaporated to dryness under a stream of nitrogen and redissolved in hexane for further fractionation on a new aminopropyl column. The eluants sequentially were 6 ml of hexane, 6 ml of ethyl ether-methylene chloride-hexane (1:10:89), 12 ml of ethyl acetate-hexane (15:85), and 4 ml of chloroform-methanol (2:1). The lipid classes separated, respectively, were cholesterol esters, triglycerides, cholesterol, diglycerides, and monoglycerides. All of the fractions were evaporated, and chloroform or 95% ethanol was used to redissolve the residues of α1 or α2, respectively, for bioassay.

Saponification

The analytical procedure published by the American Oil Chemist's Society (1971) was used. Five ml of hormone extract was evaporated, and the residue was dissolved in 30 ml of 95% ethanol and 5 ml of 50% KOH. The solution was refluxed for 2 h. After the solution cooled down to room temperature, 40 ml of distilled water was added. The solution was then extracted three times with 50 ml of petroleum ether. The organic extracts were combined as the non-saponifiable fraction. The saponifiable fraction in the aqueous phase was adjusted to pH 2 with 6 N HCl and extracted with petroleum ether or ethyl ether. After removal of solvents, residues of both of the saponifiable and non-saponifiable fraction were redissolved in 5 ml of chloroform for α1 or 5 ml of 95% ethanol for α2 for bioassay.

Acetylation

To determine the effects of acetylation on hormone activity, 0.2 g of the hormone sample was dissolved in 2 ml of acetic anhydride in pyrimidine (5:1, V/V) and left at 24°C overnight (Christie, 1982). The reagents were removed in a stream of nitrogen at 70°C. The residues of α1 and α2 were redissolved in chloroform and 95% ethanol, respectively, for bioassay. If the hormone activity disappeared after acetylation, the acetylated sample was hydrolyzed by saponification as described above to determine if the activity could be restored.

Digitonin Precipitation

To test if α hormones were digitonin preciptible sterols, *ca*. 5 mg of each sample was dissolved in 1 ml of acetone and 95% ethanol (1:1 V/V), mixed with 1 ml of 1% digitonin solution. The digitonin solution was prepared by dissolving 1 g of digitonin in 50 ml of 95% ethanol and adjusting the solution to 100 ml with deionized water (Kates, 1986). After incubation at 24°C for 10 min, the mixture was centrifuged at 3,000 rpm for 5 min. The supernatant was saved for bioassay, and the precipitates were washed with acetone and centrifuged again. The digitonides were dissolved in 2 ml of pyridine, and ethyl ether was added until precipitates started to form. The precipitated digitonin was removed by centrifugation. The supernatant was evaporated, and the residue was dissolved in chloroform for α1 or 95 % ethanol for α2 for bioassay.

Volatility of α Hormones

Volatility of the hormones was determined by a solvent sweep co-distiller (K-5050 Sweep Co-distiller, Kontes Glass Co., Vineland, NJ). A sample tube (22 × 0.6 cm i.d.) fitted with a silicon septum was loosely packed with glass wool and preconditioned by heating at 150°C for 24 h before use (Storherr and Watts, 1965). The hormone extract, dissolved in 250 µl of ethyl acetate, was injected into the heated sample tube, followed by four consecutive injections of 250 µl of ethyl acetate. The conditions for the co-distillation were a constant nitrogen flow rate of 300 ml/min and a sample tube temperature of 200°C. Only volatile compounds in the sample could co-distill with the solvent and pass through the sample tube filled with glass wool. These compounds were subsequently condensed in a Teflon tubing coil immersed in an ice bath. The condensate was collected and concentrated for bioassay. The glass wool was then removed from the sample tube and extracted with ethyl acetate. The extract was collected and evaporated, and the residue was reconstituted with chloroform for α1 or 95% ethanol for α2 for bioassay.

Gas Chromatography

Hormone α1 in chloroform or hormone α2 in ethyl acetate was injected into an HP 5830A gas chromatograph equipped with a thermocoductivity detector (TCD). The glass column (1830 × 2 mm id.) was packed with 10% SE-30 on Chromosorb W (100–120 mesh). The oven temperature was 150°C for 5 min, increased to 280°C at 8 °C/min and finally maintained at 280°C for 9 min. A glass capillary tube buried in ice was attached to the gas outlet to collect fractions. Chloroform was passed through the capillary tube three times for a total of 600 µl to wash the condensate out for bioassay and further analysis on an analytical GC (HP 5890) equipped with a flame ion detector (FID) and a capillary column (DB-5, 0.25 µm × 30 m, J&W Science, Folsom, CA). Conditions for the FID-GC were: detector temp., 300°C; injector temp., 250°C; carrier gas (helium) flow rate, 1 ml/min; hydrogen flow rate, 20 ml/min; and air flow rate, 280 ml/min. The oven temperature was programmed from 90 to 220°C at 10 °C/min, to 290°C at 7 °C/min, and maintained at 290°C for 15 min. Cholesterol was used as an external standard.

Results

Isolation of α Hormones

For α1, approximately 120 L of the mixture of chloroform-methanol was used to extract 1,354 plates (20 L of V-8 agar medium) of A1 culture. Each plate contained *ca*. 5,900 units of α1 and a total of 8×10^6 units was obtained. For α2, about 120 L of 95% ethanol were used

to extract 1,296 plates (20 L of V-8 agar medium) of A2 culture. About 4,500 units of α2 were extracted from each plate and a total of 5.8×10^6 units was obtained.

Acid-Base Extraction

For both α1 and α2, most of the activity remained in the neutral fraction (Table 1). No activity or only trace activity was detected in the acidic and basic fractions. These results suggest that α hormones are compounds without acidic or basic functional groups.

Acetone Precipitation

According to bioassay results, α hormones did not precipitate in cold acetone (Table 2). This indicates that a hormones are not likely to be polar lipids such as phospholipids or glycolipids, which are generally insoluble in cold acetone (Kates, 1986).

Table 1. Activity of α hormones in fractions from Acid-Base-Neutral extraction.

Fraction	Hormone activity (unit)[a]	
	α1	α2
Neutral	65.1	28.3
Acidic	0	0.7
Basic	1.3	1.0
Original sample	62.9	27.8

[a]One unit = The amount of hormone α1 or α2 capable of inducing formation of 10 oospores by A2 or A1 isolate, respectively.

Table 2. Effect of acetone (0°C) precipitation on the activity of α hormones.

Fraction	Hormone activity (unit)	
	α1	α2
Acetone insoluble	0	0
Acetone soluble	58.0	59.3
Original sample	69.4	27.8

Separation of Hormone Extract to Different Lipid Classes

When the hormone preparations were separated into fatty acids, phospholipids, and neutral lipids on an aminopropyl column by sequential elution with different solvent systems, only the fraction containing neutral lipid was active (Table 3). When the neutral lipids were further fractionated on new aminopropyl columns, the fraction containing substances with polarity similar to that of monoglycerides was most active for both α1 and α2 (Table 3). Based on these results, α1 and α2 have polarity similar to that of monoglycerides. However, since some α1, but not α2 activity, was detected in the fraction with polarity similar to that of cholesterol and diglycerides, α1 should be less polar than α2.

Saponification of the Hormone Extracts

The activity of both α1 and α2 survived saponification. To recover hormone activity from the aqueous phase, petroleum ether served as a good solvent for α1, but for α2, a relatively polar solvent, ethyl ether was required for complete extraction (Table 4).

Acetylation

Acetylation did not affect α1 but destroyed the activity of α2. However, hydrolysis of the acetylated α2 restored its activity (Table 5).

Table 4. Activity of α hormones subjected to saponification and extraction with petroleum ether or ethyl ether.

Fraction	Hormone activity (unit)	
	α1	α2
Extraction with petroleum ether		
Non-saponifiable	17.6	28.0
Saponifiable	0	47.5
Original sample	36.1	39.3
Extraction with ethyl ether		
Non-saponifiable		41.5
Saponifiable		0
Original sample		34.5

Table 3. Activity of α hormones in fractions eluted from aminopropyl column.

Solvent[a]	Possible lipid classes eluted	Hormone activity (unit)	
		α1	α2
1	Neutral lipids	+[b]	+
2	Fatty acids	–	–
3	Phospholipids	–	–
4	Cholesterol esters	0	0
5	Triglycerides	0	0
6	Cholesterol	5.1	0
7	Diglycerides	6.0	0
8	Monoglycerides	36.9	50.2
Original sample		98.3	20.2

[a]Solvent systems used: 1, Chloroform-isopropanol 2:1, 4 ml; 2, 2% Acetic acid in ethyl ether, 4 ml; 3, Methanol, 4 ml; 4, hexane, 4 ml; 5, 1% Ethyl ether, 10% methylene chloride in hexane, 6 ml; 6, 5% Ethyl acetate in hexane, 12 ml; 7, 15% Ethyl acetate in hexane, 4 ml; 8, Chloroform-methanol 2:1, 4 ml.

[b]Symbols: + = active; – = inactive.

Table 5. Effect of acetylation on the activity of α hormones.

Treatment	Hormone activity (unit)	
	α1	α2
None	70.8	201.0
Acetylation	80.0	4.9
Hydrolysis[a]	NA[b]	180.0

[a]Hydrolysis by soponification of the acetylated hormone α2.
[b]Not applicable.

Digitonin Precipitation

Digitonin precipitation removed 81% of the weight of α1 extract without significantly reducing the hormone activity in the supernatant (Table 6). Although only a trace amount of precipitate was formed when hormone α2 extract was treated with digitonin, the treatment doubled the hormone activity in the supernatant. Results indicate that neither α hormone is a digitonin precipitable sterol.

Volatility of α Hormones

After solvent co-distillation, *ca*. 50% α1 activity was recovered in the distillate, indicating that α1 is a volatile compound (Table 7). In contrast, no α2 activity was detected in the distillate, suggesting that α2 did not volatilize under the solvent sweep co-distillation conditions. Residues recovered from the glass wool packing for both hormones were active, indicating that they were stable at 200°C under nitrogen gas.

Gas Chromatography

Standard cholesterol showed a retention time of 270 to 280 °C on the semi-preparative GC equipped with a SE-30 packed column. When the α1 extract was fractionated under the same conditions, hormone activity was eluted between 230 to 278 °C. However, no detectable peaks corresponded to the hormonal activity. The active fraction was collected and re-injected on an analytical FID-GC, but again the chromatogram did not show any significant peak(s), suggesting that the sample size was below the detection limit of the analytical CG system. All efforts to collect active α2 fraction(s) from the semi-preparative GC failed, suggesting α2 was too polar to be analyzed by gas chromatography.

Discussion

Hormones α1 and α2 were extracted directly from A1 and A2 cultures of *P. parasitica*, respectively, with organic solvents for the first time with the method developed in this present study. Although the crude extracts contained high levels of impurity, the unprecedented quantities of hormones obtained from a total of 40 L of agar cultures enabled us to determine the chemical and physical properties of α1 and α2.

Results summarized in Table 8 indicate that both hormones are non-saponifiable neutral lipids and are, therefore, not esters or carboxylic acids. Acid-base fractionation showed that they are not organic base either. Our results

Table 6. Effect of digitonin precipitation on the activity of α hormones.

Fraction	Hormone activity (unit)	
	α1	α2
Supernatant	4.4 ± 1.3	22.6 ± 3.8
Precipitate	0	0
Original sample	6.7 ± 0.3	11.5 ± 3.7

Table 7. Activity of α hormones following solvent sweep co-distillation.

Fraction	Hormone activity (unit)	
	α1	α2
Distillate	28.2	0
Residue	26.7	27.5
Original sample	61.6	28.6

Table 8. Comparison of characteristics of hormone α1 and hormone α2.

Treatment	Characteristics of α1	Characteristics of α2
1. Molecular weight[a]	100–500	100–500
2. Effective solvents for extraction	Petroleum ether, methylene chloride	Ethyl ether, ethanol
3. Stability at 200°C	Stable	Stable
4. Co-distillation at 200°C	Volatile	Non-volatile
5. Saponification	Non-saponifiable	Non-saponifiable
6. Acetone precipitation	Non-precipitable	Non-precipitable
7. Digitonin test	Not a 3-ß-hydroxyl sterol	Not a 3-ß-hydroxyl sterol
8. Acid-base-neutral extraction	Neutral lipid	Neutral lipid
9. Aminopropyl column chromatography	Polarity similar to that of monoglycerides	Polarity similar but greater than monoglycerides
10. Thin layer chromatogra[b]	Polarity similar to that of monoglycerides	Polarity similar but greater than monoglycerides
11. Acetylation	No effect on activity	Activity destroyed but regained by hydrolysis

[a]Data from Ko (1983).
[b]Data from Chern (1995).

do not support the suggestion that these hormones are steroids similar to antheridiol (Gooday, 1974). Digitonin precipitation ruled out the possibility of α hormones being 3-β hydroxyl steroids which include most sterols found in plants and fungi (Goodwin and Mercer, 1983). Based on lipid classification (Kaluzny et al., 1985), α1 and α2 belong to the class of monoglycerides. Therefore, it is possible that both hormones contain two hydroxyl functional groups. On the other hand, the fact that only α1 passed through the solvent sweep co-distillation and an SE-30 GC column with a retention time less than that of cholesterol suggests that α1 may only have one hydroxyl group instead. Acetylation further provided evidence that only the hydroxyl group(s) on α2 are essential to its hormonal activity, and the blocking of activity is reversed by the removal of the acetyl group(s) through base hydrolysis. The hydroxyl group(s) on α1 is not essential to its activity.

Because of the minute quantities of hormones in the culture, we did not observe any identifiable peaks in TCD-GC or by re-injecting the collected peak onto a more sensitive FID-GC with a capillary column. Useful reactions for functional group determination, such as periodate oxidation of 1,2-diols, were also prohibited by the paucity of sample sizes. Obviously, despite our efforts in preparing large quantities of the hormones, the amounts were still insufficient for their chemical identification. Further work on improving the collection techniques and increasing the operation size should make their structural elucidation possible.

Two sex hormones produced by other members of Oomycetes have been isolated and chemically identified. They are antheridiol (Arsenault et al., 1968; Barksdale and Losure, 1974) and oogoniols (McMorris, 1978a; 1978b). The molecular weight of antheridiol is 470, and that of oogoniol-1 is 546 (McMorris, 1978a). Both hormones are steroids. Antheridiol produced by female strains of *Achlya* sp. can initiate formation of antheridial branches and attract male gametes (Kochert, 1978; McMorris, 1978a; 1978b). Oogoniol produced by male strains of *Achlya* sp. can induce differentiation of oogonia. Hormones α1 and α2 can induce A2 and A1 isolates of *P. parasitica*, respectively, to form both antheridia and oogonia (Ko, 1983; Chern, 1995). Therefore, α hormones have the functions of both male and female hormones.

Zentmyer (1979) reported the observation of oospore formation by *Phytophthora cinnamomi* in aqueous extracts of avocado roots. The stimulatory factors in the avocado root extracts were subsequently identified as oleic acid and its triglyceride (Zaki et al., 1983). Although these substances are not α hormones, they are capable of mimicking the activity of hormone α1 by inducing the formation of antheridia and oogonia of A2 isolates of *P. cinnamomi*.

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