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Evidence of Multiple Extracellular Phospholipase Activities of *Aspergillus fumigatus*

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Extracellular phospholipase activity has been implicated in the pathogenesis of several bacterial infections. Recently, extracellular phospholipase activity has been proposed as a virulence factor in the opportunistic yeast *Candida albicans*. *Aspergillus fumigatus* is the most pathogenic member of its genus, responsible for >90% of infections. Previously, no specific virulence factors have been determined. We investigated the ability of *A. fumigatus* to produce extracellular phospholipases at 37°C. Fast atom bombardment was used to compare lipid-containing media before and at 5-h intervals during shaking culture of *A. fumigatus*. Lipids were extracted and analyzed. Many anions corresponding to phospholipid breakdown products were identified. Specific anion species identified indicated phospholipase A, B, C (PLC), and D activities. PLC activity was further investigated by using the synthetic substrate *p*-nitrophenylphosphorylcholine. PLC activity was initially observed after 30 h of growth and accumulated in broth cultures up to 50 h. At 55 h, there was a sharp increase in PLC activity which coincided with cultures reaching the stationary phase. Activity of the PLC was measured at different temperatures, with greater activity occurring at 37°C than at lower temperatures. Phospholipases could represent a virulence determinant in *A. fumigatus*.

The genus *Aspergillus* is composed of over 150 species and contains members that are of considerable economic importance. Certain species are of increasing importance in nosocomial infections. *Aspergillus fumigatus* is the most common invasive pathogen mold worldwide and accounts for the vast majority of infections (>90%). Several pathogenicity determinants for the seemingly increased virulence of *A. fumigatus* over other *Aspergillus* species have been suggested. These include small spore size (15), elastase production (8), ribotoxin production (9), and production of other extracellular toxins (7, 12). To date, there is no conclusive evidence that any of these factors are critical to the virulence of *Aspergillus fumigatus*.

Phospholipases are a ubiquitous class of enzymes which degrade phospholipids. Phospholipases A_1 and A_2 cleave the fatty acyl chains of phospholipids at positions sn1 and sn2, respectively, to form lysophospholipids. Phospholipase B removes both fatty acyl chains simultaneously to produce a glycerophosphoryl compound. Diacylglycerides are formed by the removal of both the phosphate moiety and polar head group by the action of phospholipase C (PLC), which cleaves the phosphodiester bond between the glycerol and phosphate groups. Similarly, phospholipase D cleaves the phosphodiester bond between the polar head group and phosphate moiety to form phosphoglycerides.

Phospholipases are frequently found in association with membranes and may play a role in intracellular signalling pathways (4, 17).

The production of extracellular phospholipases has been shown to be important in the pathogenesis of several bacterial infections, including those caused by *Clostridium*, *Listeria*, and *Pseudomonas* spp. (5, 11, 16). Increased phospholipase activity has also been correlated to increased mucosal pathogenicity in the opportunistic yeast *Candida albicans* (2). Recently, blood isolates of *C. albicans* have been shown to produce increased extracellular phospholipases compared with mucosal isolates (6). Exogenous PLC has been shown to cause transient permeability in mammalian cells sufficient to allow entry of the protein toxin alpha-sarcin (13).

Fast atom bombardment-mass spectrometry (FAB-MS) has been applied to the study of bacterial phospholipids for chemotaxonomic purposes (3) and is reviewed elsewhere (10). The technique provides useful information about phospholipids and fatty acids and can be applied to complex mixtures of these compounds.

In this study, we used negative-ion FAB-MS to monitor the breakdown of soybean phospholipids by *A. fumigatus* extracellular enzymes. We also assayed for the presence of extracellular PLC activity and investigated the temperature sensitivity of this activity.

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MATERIALS AND METHODS

Materials. Crude phosphatidylcholine and *p*-nitrophenylphosphorylcholine (pNPPC) were purchased from Sigma Chemical Co., Poole, Dorset, United Kingdom. Chloroform and methanol were of high-performance liquid chromatography grade.

Growth media and cultivation of fungi. *A. fumigatus* ATCC 90240 was cultured from frozen stocks in 75-cm^2 tissue culture flasks containing potato dextrose agar. The flasks were incubated at 35°C aerobically for 7 days. Conidia were harvested in phosphate-buffered saline–Tween (0.1%) and counted with an improved Neubauer hemocytometer. Adjustments were made so that the final conidial concentration was approximately 10^8/ml.

One milliliter of conidial suspension was inoculated into an Erlenmeyer flask containing 100 ml of Vogel's medium, except that sucrose was replaced by either 1% (wt/vol) glucose or 0.5% (wt/vol) soybean phospholipid. Cultures were incubated at 37°C for up to 60 h with constant shaking (200 rpm).

Control cultures were set up and incubated as described above but were not

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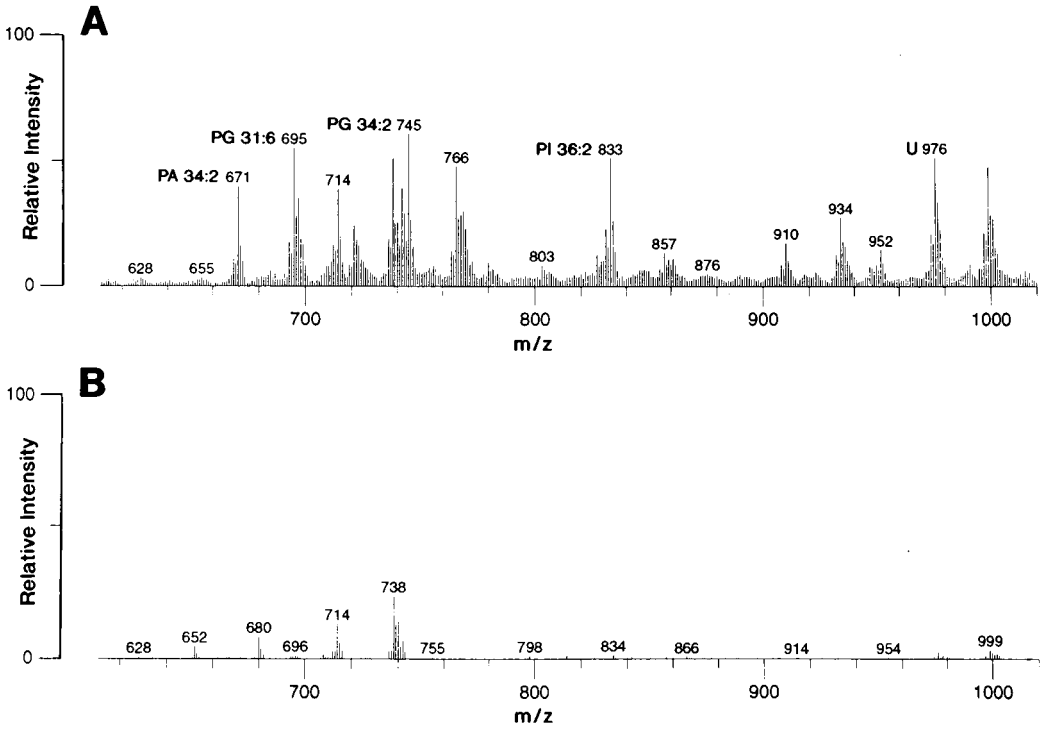

FIG. 1. FAB-MS spectra (*m/z* 500 to 1,000) of culture supernatants containing soybean phospholipids. (A) Phospholipids present in supernatants prior to the addition of *A. fumigatus* conidia and subsequent incubation at 37°C; (B) phospholipids present in supernatants after 45 h of incubation with *A. fumigatus* conidia at 37°C. Specific phospholipid anions are indicated in panel A; chemical identities are given in Table 1. U, unassigned.

inoculated with *A. fumigatus* conidia. This was to determine the role of lipid oxidation and lipid degradation under the experimental conditions.

An additional control containing 1% (wt/vol) glucose instead of soybean phospholipid, inoculated and incubated as described above, was also included.

FAB-MS. At 5-h intervals after inoculation, 5-ml aliquots of culture were removed and filtered through a 0.45-μm-pore-size membrane (Gelman Sciences, Ann Arbor, Mich.) filter. The filtrate was extracted with an equal volume of methanol-chloroform (2:1, vol/vol), and the lower solvent phase was removed and taken to dryness under a stream of nitrogen. Dried samples were stored in a vacuum desiccator at 4°C until FAB-MS was performed.

Samples were dissolved in 1 μl of *meta*-nitrobenzyl alcohol and subjected to negative-ion FAB-MS with a Concept mass spectrometer (Kratos Instruments, Manchester, United Kingdom). Most phospholipid classes are detected with much higher sensitivity in negative-mode FAB-MS. The exception to this is phosphatidylcholine, which, as a result of the presence of a quaternary nitrogen atom, is detected with greater intensity in the positive mode. Neutral lipids give weak signals in FAB-MS spectra.

Because the soybean phospholipid contained all major classes of phospholipid, negative-ion FAB-MS only was undertaken.

Significant anions were identified as having a relative intensity of 5% or greater and were normalized to allow comparisons at different time intervals.

PLC assay. Culture filtrates were tested for PLC activity by using pNPPC as the substrate. A 20 mM solution of pNPPC was prepared in 0.25 M Tris-HCl buffer (pH 7.4) containing 60% (wt/vol) sorbitol. Reactions were carried out in cuvettes containing 1.9 ml of pNPPC solution and 100 μl of culture filtrate. Cuvettes were incubated at 37°C, and the A_{410} was read periodically.

The activity of *A. fumigatus* extracellular PLC from a 48-h culture supernatant was assayed at different incubation temperatures. Cuvettes containing pNPPC (as described above) were incubated at 10, 20, 25, 30, and 37°C, and the A_{410} was measured. The effect of boiling 55-h culture supernatant for 5 min at 100°C on the rate of hydrolysis of pNPPC was determined by the method described above. The presence of PLC in control cultures was also determined as described above.

RESULTS

FAB-MS analysis of soybean phospholipid. FAB-MS analysis of soybean phospholipid revealed anions at mass-to-charge ratios (*m/z*) which corresponded to several major classes of phospholipid. These included phosphatidic acid, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol (Fig. 1). Peaks corresponding to fatty acid anions were also observed in the *m/z* region from 150 to 300. The major fatty acid anions were at *m/z* 279 ($C_{18:2}$), 255 ($C_{16:0}$), 277 ($C_{18:1}$), and 281 ($C_{18:3}$).

FAB-MS spectra from uninoculated controls incubated for 55 h showed no significant difference in phospholipid and fatty acid profiles from FAB-MS spectra obtained prior to incubation at 37°C.

Individual phospholipids together with their fatty acyl substituents could be identified (Table 1). Common fatty acyl substituent combinations were 34:2 and 36:4. This corresponded to 18:2-16:0 and 18:2-18:2 fatty acid combinations, which were the most intense fatty acid peaks observed.

FAB-MS analysis of soybean phospholipid breakdown products. During the first 25 h of incubation, the normalized relative intensity of several major anions initially decreased (Table 1) up to 25 h of incubation. Anions at *m/z* 671, 696, 833, 835, 976, 977, 999, and 1,000 were no longer detected in spectra after 30 h of incubation (Fig. 1), indicating complete degradation of these phospholipids. After 25 h of incubation, the normalized relative intensity of several anions began to increase up to 50 h, the greatest rate of increase being between 45 and 50 h of incubation (Table 1). At this point, cultures were approaching the stationary phase as determined by the A_{600} of the supernatant (see Fig. 3).

The number of anions present in the intermediate *m/z* range (*m/z* 300 to 600) increased steadily from 3 anions initially to 44 anions after 50 h of incubation (data not shown). Anions present in this region of FAB-MS spectra correspond to the expected mass-to-charge ratio of lysophospholipids produced by phospholipase A activity or glycerophosphoryl anions produced by phospholipase B activity on high-molecular-weight phospholipids.

TABLE 1. Mean relative normalized intensities for most abundant soybean phospholipid anions over a 55-h incubation period after inoculation with *A. fumigatus* conidia

m/z	Chemical identity[a]	Mean relative normalized intensity at:									
		0 h[b]	5 h	10 h	20 h	25 h	30 h	40 h	45 h	50 h	55 h
1,001	DGDG (40:1)		6.2	5.3	4.5	2.2	2.1	1.9	<1.0	<1.0	<1.0
1,000	U	5.7	5.4	4.1	2.8	2.1	2.0	<1.0	<1.0	<1.0	<1.0
999	DGDG (40:2)		8.4	7.8	7.3	3.7	3.7	2.5	<1.0	<1.0	<1.0
977	DGDG (39:6)		4.8	3.0	2.9	2.0	1.9	1.2	<1.0	<1.0	<1.0
976	U	10.8	7.4	6.6	3.0	1.7	1.3	<1.0	<1.0	<1.0	<1.0
835	PI (36:1)	3.8	3.8	9.5	8.6	7.6	<1.0	<1.0	<1.0	<1.0	<1.0
833	PI (36:2)	7.4	19.1	7.6	6.5	2.7	<1.0	<1.0	<1.0	<1.0	<1.0
745	PG (34:2)	8.8	5.6	3.2	2.1	1.5	1.1	<1.0	<1.0	<1.0	<1.0
743	PG (34:3)	4.2	4.0	3.8	1.6	1.0	2.2	3.4	5.5	6.5	<1.0
742	PE (36:2)	5.6	4.2	3.7	2.9	2.8	2.0	3.6	4.6	26.5	11.2
741	PG (34:2)	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	6.2	21.7	9.1
740	PG (34:3)	3.8	3.7	3.5	2.6	2.1	1.8	4.3	6.9	<1.0	<1.0
739	PI (27:0)	3.9	3.7	2.9	4.0	4.0	6.0	6.8	8.9	25.3	9.7
738	PE (36:4)	8.0	7.6	7.3	6.2	5.3	7.8	12.5	15.9	42.1	11.4
714	PE (34:2)	6.9	5.5	4.6	6.6	9.0	11.1	11.5	12.5	38.2	18.4
697	PG (31:5)	7.4	5.0	4.6	4.4	3.8	3.4	<1.0	<1.0	<1.0	<1.0
696	PA (36:4)	6.1	7.2	7.9	10.6	10.9	11.2	<1.0	<1.0	<1.0	<1.0
695	PG (31:6)	5.1	4.7	4.2	3.8	3.5	<1.0	<1.0	<1.0	3.2	4.2
671	PA (34:2)	5.7	6.4	7.4	10.5	13.8	<1.0	<1.0	<1.0	<1.0	<1.0

[a] Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; DGDG, digalactosyldiacylglycerol; U, unassigned. Total fatty acyl substituents (carbon atoms:unsaturations [e.g., 40:1]) are given in parentheses.
[b] Incubation time.

An increase in the anion at *m/z* 671 (phosphatidic acid with 34:2 fatty acyl substituents) was observed in spectra up to 25 h of incubation, after which the anion was no longer observed. The increase in the *m/z* 671 anion is consistent with a phospholipase D activity on 34:2 fatty acyl substituent phospholipids. Anions possessing this fatty acyl substituent combination decreased in normalized relative intensity as the *m/z* 671 anion increased, indicating degradation of these species.

Evidence was also found for phospholipase A activity on specific phospholipids. The anion at *m/z* 714 corresponds to phosphatidylethanolamine with 34:2 fatty acyl substituents. Lysophospholipid fragment anions were observed in FAB-MS spectra which were consistent with the removal of both 16:0 and 18:2 fatty acyl moieties from the parent anion (Table 2). The presence of both predicted lysophospholipid anions is indicative of phospholipase A_1 and A_2 activities.

An anion at *m/z* 214 (Table 2) appeared in spectra after 40 h of incubation of cultures. This anion corresponds to the predicted *m/z* of glycerophosphatidylethanolamine and would be formed by the removal of both fatty acid residues from phosphatidylethanolamines. Complete deacylation of phospholipids could be brought about by the action of phospholipase B or by a combination of phospholipase A and lysophospholipase.

Anions which corresponded to the expected degradation products of the phosphoinositols initially present at the start of incubation were also observed. Three anions at *m/z* 259, 241, and 223 (Table 2) were transiently seen in spectra after 20 h of incubation and reappeared in spectra after 50 and 55 h of incubation. These anions correspond to monophosphorylinositol (*m/z* 259) and dehydrated derivatives (*m/z* 241 and 223). The occurrence of these anions indicates PLC activity on the phosphatidylinositol anions seen in the initial spectra.

A dehydrated glycerophosphorylinositol anion (*m/z* 297) was seen in spectra after 20 and 25 h of incubation. Again, the presence of this phosphatidylinositol degradative product is consistent with phospholipase B or a combination of phospholipase A and lysophospholipase activities.

Activity of PLC. Specific anions were observed in FAB-MS spectra during the growth of *A. fumigatus* that indicated the presence of PLC activity (see above). However, because diacylglycerides are poorly represented in anionic spectra, PLC activity was assayed directly with pNPPC as the substrate.

PLC activity was first detected in culture supernatants 30 h after inoculation and increased steadily in supernatants up to 50 h of incubation. A large increase in activity was observed after 50 h of incubation which corresponded to cultures reaching the stationary phase (Fig. 2). No PLC activity was detected in uninoculated controls or controls containing 1% glucose. Similarly, no PLC activity was detected in boiled 55-h culture supernatant.

The activity of *A. fumigatus* extracellular PLC was assessed at differing temperatures (Fig. 3). The rate of release of nitrophenol from pNPPC was found to increase with increasing temperature, with the greatest rate occurring at 37°C (Fig. 3).

TABLE 2. Major soybean phospholipid anions seen in FAB-MS spectra together with diagnostic anions indicating specific phospholipase activities

Parent anion (*m/z*)	Diagnostic anion (*m/z*)	Fatty acyl anion (*m/z*)	Phospholipid fragment anion (*m/z*)	Phospholipase activity indicated
671	153, 79	255, 279	433, 409	A
714	214	255, 279	435, 459	A
			671	D
			595	C
738	214	279, 279	459	A
740		255, 279	671	D
			595	C
745		255, 279	671	C
			595	D
742		279, 279	695	D
833	223, 241, 259, 297	255, 279	415, 391	A or B
835	223, 241, 259, 297	255, 281	415, 391	A or B

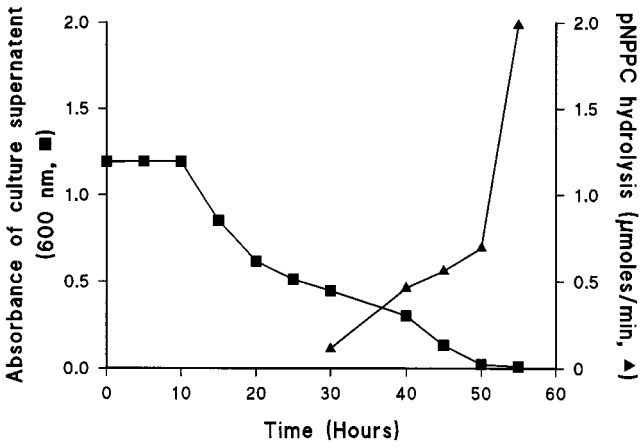

FIG. 2. Decrease in A_{600} of culture supernatant and increase in PLC activity over a 55-h incubation period.

DISCUSSION

The data generated by FAB-MS analysis of phospholipid breakdown products strongly indicate the presence of multiple phospholipase and lysophospholipase activities. The increase in the number of intermediate-range anions (*m/z* 300 to 600) from 3 initially to 44 after 50 h of incubation strongly indicates phospholipase A activities. Indeed lysophospholipid anions corresponding to phospholipase A activity on specific parent anions can be seen in spectra (Table 2). The increase in high-range anions (*m/z* 600 to 1,000) after an initial decrease can be explained by phospholipase activity on phospholipids outside the scan range.

The changes in the phospholipid composition of the culture supernatant cannot be due to chemical breakdown or lipid oxidation since spectra from uninoculated controls did not differ significantly in phospholipid composition after incubation for 55 h at 37°C.

The presence of glycerophosphoryl anions is indicative of phospholipase B activity or a lysophospholipase activity. FAB-MS analysis alone does not differentiate between these two activities; however, it is possible that both exist. Phospholipase A and B, PLC, and lysophospholipase activities have been described previously for *C. albicans* (1, 2, 14).

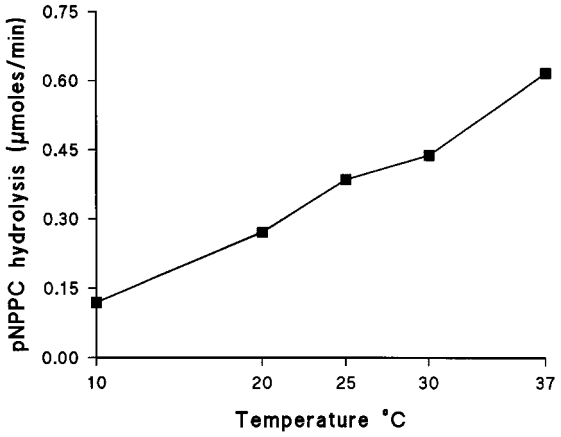

FIG. 3. Increase in activity of *A. fumigatus* PLC with increasing temperature.

Considerable evidence exists for the presence of phospholipase D activity. The increase in the phosphatidic acid anion at *m/z* 671 and the subsequent drop in the relative intensities of 34:2 fatty acid substituent phospholipids are wholly consistent with this conclusion. The products of phospholipase D activity, i.e., ethanolamine, serine, inositol, and glycerol, are potentially extremely useful metabolites for emerging germlings. Therefore, it is not surprising that this activity seems to appear first.

Both PLC and phospholipase D act on the hydrophilic residues of phospholipids which reside on the outer surface of cell membranes. Removal of these residues by phospholipase activity would alter membrane stability and possibly lead to cell lysis and altered permeability (13) and may even alter host cell surface characteristics to increase adherence of pathogens (6).

PLC activity is conclusively demonstrated with diagnostic anions present in FAB-MS spectra and by cleavage of nitrophenol from pNPPC. The enhanced activity of this enzyme at 37°C poses important questions as to the role of this and other phospholipases in the pathogenesis of aspergillosis. Further work is needed to better characterize the described phospholipase activities and to confirm phospholipase B activity.

There are considerable similarities between the pathological appearances of necrotizing fasciitis or gangrene caused by *Clostridium* spp., ecthyma gangrenosum caused by *Pseudomonas* spp., and invasive aspergillosis in that blood vessel occlusion, tissue infarction, and necrosis are characteristic. Both *Clostridium* spp. (16) and *Pseudomonas aeruginosa* (11) produce extracellular PLC. It is therefore possible that the extracellular phospholipase activities that we have observed in *A. fumigatus* and, in particular, that of PLC may account, at least in part, for some of the pathological consequences of invasive aspergillosis. This is to our knowledge the first description of extracellular phospholipase activities in *A. fumigatus*. Clearly, further characterization work is necessary to clarify the role of phospholipases in the pathogenesis of *Aspergillus* infections.

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