Pak. J. Sci. Ind. Res. 2004 ***47****(4) 292-297*

PARTIAL PURIFICATION AND CHARACTERIZATION OF GLYCOLIPID ISOLATED FROM *COLLETOTRICHUM LINDEMUTHIANUM*

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(Received May 5, 2003; accepted June 11, 2004)

Colletotrichum lindemuthianum, the causative agent of anthracnose in beans (*Phaseolus vulgaris)* was successfully cultured in a complex medium of glucose/neopeptone. The extracellular High Molecular Weight Culture Filtrate Elicitor (HMWCFE) was isolated by simultaneous dialysis and ultrafiltration of nominal cutoff 30,000 dalton cartridge systems. Chromatographic separation provided two fractions Partially Purified Fraction, PPF-I (Mr 2000,000) and PPF-II (Mr < 40,000). Extraction with organic solvents showed that lipid was found in crude preparations. Methanolysis of PPF-I and II analyzed by GC, provided a series of fatty acids characterized by GC-MS viz: behenic (C_{22}), arachidic (C_{20}), isostearic (C_{18}), oleic ($C^{9}_{18:1}$), palmitic (C_{16}), myristic (C_{14}) and capric (C_{10}) acids. Not all the lipid material was released under mild basic hydrolyzing conditions indicating the presence of some stable linkages between lipid and sugar moieties.

Key words**:** *Colletotrichum lindemuthianum*, Elicitor, Fatty acids, Carbohydrate.

Introduction

Substances obtained from culture filtrate and cell wall of plant pathogen i.e. fungi elicit hypersensitive defence responses when applied to tissues or cell cultures of incompatible plants (Knogge 1996; Paiva 2000). These substances are chemically diverse in nature such as polysaccharides (Clarence and Edward 1991), proteins and glycoproteins (Voglsang *et al* 1994), glycolipid/lipids (Castoria *et al* 1995). Elicitor active material from *Colletotrichum lindemuthianum*, the causal agent of anthracnose disease in beans is first known from culture filtrate of hot water extracts of fungal cell wall (α race) (Anderson and Albersheim 1975), the active material shown to contain a high molecular weight β-1, 3 and 1,4-linked glucose polysaccharides. Subsequently, elicitor active preparations were studied from culture filtrate of the α (Anderson 1978; Anderson 1980a), β (Anderson 1980b, Tepper and Anderson 1986) and IMI 112166 (Hamdan and Dixon 1986, 1987) races. The accumulation of isoflavonoid phytoalexins was studied in several Colombian bean cultivars resistant and susceptible to *C. lindemuthianum* (Dieg *et al* 2002).

In these studies, methanolysis and hexane extraction of partially purified glycoconjugate obtained from culture filtrate of *C. lindemuthianum* IMI 112166 provided a series of fatty acids on separation and characterization by GC and GC-MS.

Materials and Methods

Elicitor preparation and purification. Shake cultures of *C. lindemuthianum* race IMI 112166 (International Mycological Institute Virginia water, Surrey UK) were grown in a complex medium of glucose/neopeptone (Mathur *et al* 1949) as modified by Anderson and Albersheim (1975), by the use of 15 g glucose/l. Crude High Molecular Weight Culture Filtrate Elicitor (HMWCFE) was isolated by simultaneous dialysis with distilled water and ultrafiltration cartridge system of pore size 30,000 dalton.

Extraction of HMWCFE with organic solvent. An aqueous solution (2 mg/ml) of HMWCFE with a total sugar content of 30% was extracted with 4 ml of chloroform: acetone 1:1 v/v an emulsion was formed at the interface. The aqueous layer was separated and the organic layer was washed twice with water. The combined aqueous layer was freeze dried and analysed for sugar content. The crude HMWCFE were fractionated by size exclusion chromatography on Fractogel HW-65S and Partially Purified Fractions PPF-I and PPF-II were collected.

Methanolysis of PPF-I & II and GLC of methyl esters. Reagent A: 0.45 ml of acetyl chloride added to 10 ml of dry MeOH (reagent should be prepared fresh before use).

PPF-I and II (0.25 ml), at a concentration of 1mg/ml were completely dried under N_2 in PTFE-lined screw capped 5 ml vials. 0.4 ml of reagent A and 1 ml of methyl acetate were added, flushed with N_2 and heated for 16h at 70°C. Methanol was removed by blowing nitrogen and the residue was extracted with hexane (3x1.5 ml). The hexane fractions were dried on anhydrous Na_2SO_4, concentrated and applied for GLC separation.

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Table 1
Fatty acids liberated from HMWCFE, PPF-I of *C. lindemuthianum*

Peak #	Scan #	Abbreviated symbol	Common name	Systematic name	Structural formula
1	788	C_{16}	Palmitic acid	14-methylated-decanoic acid	CH_3-CH-$(CH_2)_{12}$-CO_2H | CH_3
3	926	$C^{9}_{18:1}$	Oleic acid	Octadecanoic acid	CH_3-$(CH_2)_7$-CH=CH$(CH_2)_7$-CO_2H
4	935	C_{18}	Isostearic acid	16-methyl heptadecanoic acid	CH_3-CH- $(CH_2)_{14}$-CO_2H | CH_3
5	1068	C_{20}	Arachidic acid	Eicosanoic acid	$CH_3(CH_2)_{18}$-CO_2H

GLC analysis was carried out using a Perkin Elmer Model 8410 chromatograph fitted with a flame ionization detector. Separation was achieved on a 25 m x 0.35 mm of BP 10 polyphenyl cyanopropylmethyl siloxane column, operated in the splitless mode. Helium at 15 ml/min was used as a carrier gas and the oven temperature was programmed from 90˚C (1 min hold) to 230˚C with an increment of 2˚C/min. Standard fatty acids were derivatized and run under similar conditions on GC. The peaks separated by capillary gas chromatography were further characterized by GC-MS.

Acid and base/borohydride hydrolysis of PPF- I & II. Acid hydrolysis. PPF-I (400µl) and PPF-II (200 µl) of concentration 1 mg/ml were dried under N_2 in vials and 0.5 ml of 2M trifluro acetic acid was added to each sample, flushed with N_2 and heated at 120˚C for 90 min. Hydrolyzed material was extracted with 3 ml hexane twice the combined hexane fractions were dried and esterified.

Base/borohydride hydrolysis. PPF-I & II 1 mg/ml were stirred at room temperature for 10h into 0.5 ml of 0.05M NaOH containing 0.5 ml of 1M $NaBH_4$. The hydrolysates were acidified with acetic acid (glacial) to pH 4 and extracted twice with 3 ml of hexane. An emulsion was formed during the extraction at the interface of the organic and aqueous layers. This emulsion was shown to contain carbohydrate when tested by phenol/H_2SO_4.

Esterification of liberated free fatty acids. To each acid and base hexane fraction 0.1 ml of BF_3 (etherate) and 500 µl of dry MeOH was added and heated for 10 min at 60-65˚C, cooled to room temperature, extracted with hexane (2 x 2 ml) concentrated and applied for GLC analysis. The gas chromatographic conditions were the same with a modified temperature programme such that initial temperature 150˚C (1 min hold) to 240˚C with an increment of 4˚C per minute.

Results and Discussion

Crude High Molecular Weight Preparations from culture filtrate of *C.lindemuthianum* were partially purified on Fractogel HW-65S. Total solid recovered in Partially Purified Fractions, PPF-1 and II were in the range of 84% w/w. PPF-I showed 27% recovery with a sugar content of 14%, whereas 57% material was recovered in PPF-II with a carbohydrate content of 11.5% (Fatima and Finch 1998). Extraction of HMW crude preparations of *C.lindemuthianum* with organic solvents aimed to remove any organic material which is non polar leaving behind elicitor preparations enriched with sugar components. Extraction showed that an emulsion was formed at the interface, this emulsion showed a positive test for sugar with the phenol/H_2SO_4. The recovery in aqueous phase was about 21% with a sugar content of 13%.

Low column recoveries and a remarkable loss of carbohydrate in the interfacial emulsion during organic extraction suggested that a major portion of sugar was trapped on the column or lost in the interfacial emulsion. These results gave a strong indication that sugar moieties in these preparations were attached to some hydrocarbon or lipid material.

To resolve this finding methanolysis of PPF-I and II were carried out by acid catalyzed degradation of these glycolipids. Samples were refluxed with a large excess of anhydrous methanol in the presence of an acid catalyst (Woung *et al* 1980). The polysaccharides were converted to methyl glycosides of constituent monosaccharide and methylated esters of librated fatty acids. After methanolysis of 16h the methyl esters of PPF-I and II were partitioned and extracted into hexane. During extraction a band of gel like emulsion was formed at the interface of the aqueous and organic hexane layer, which swalled after standing for some time exhibited

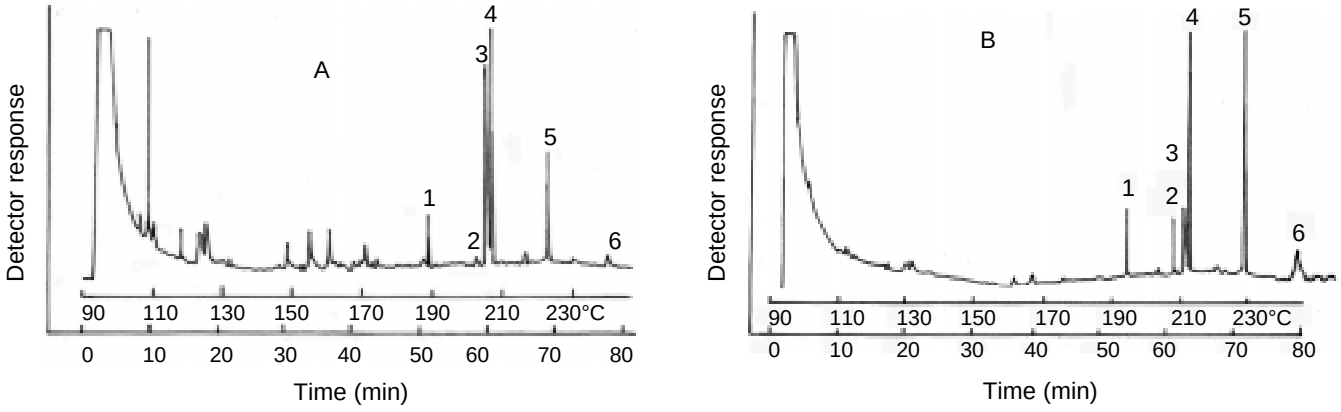

Fig 1a & 1b. GLC separation of methyl esters of *C. lindemuthianum* (A) Partially purified HMWCFE fraction I, (B) HMWCFE fraction II, oven temperature was increased from 90˚C to 230˚ at 2˚/min, peak numbers correspond to the methylester listed in Tables 1 and 2.

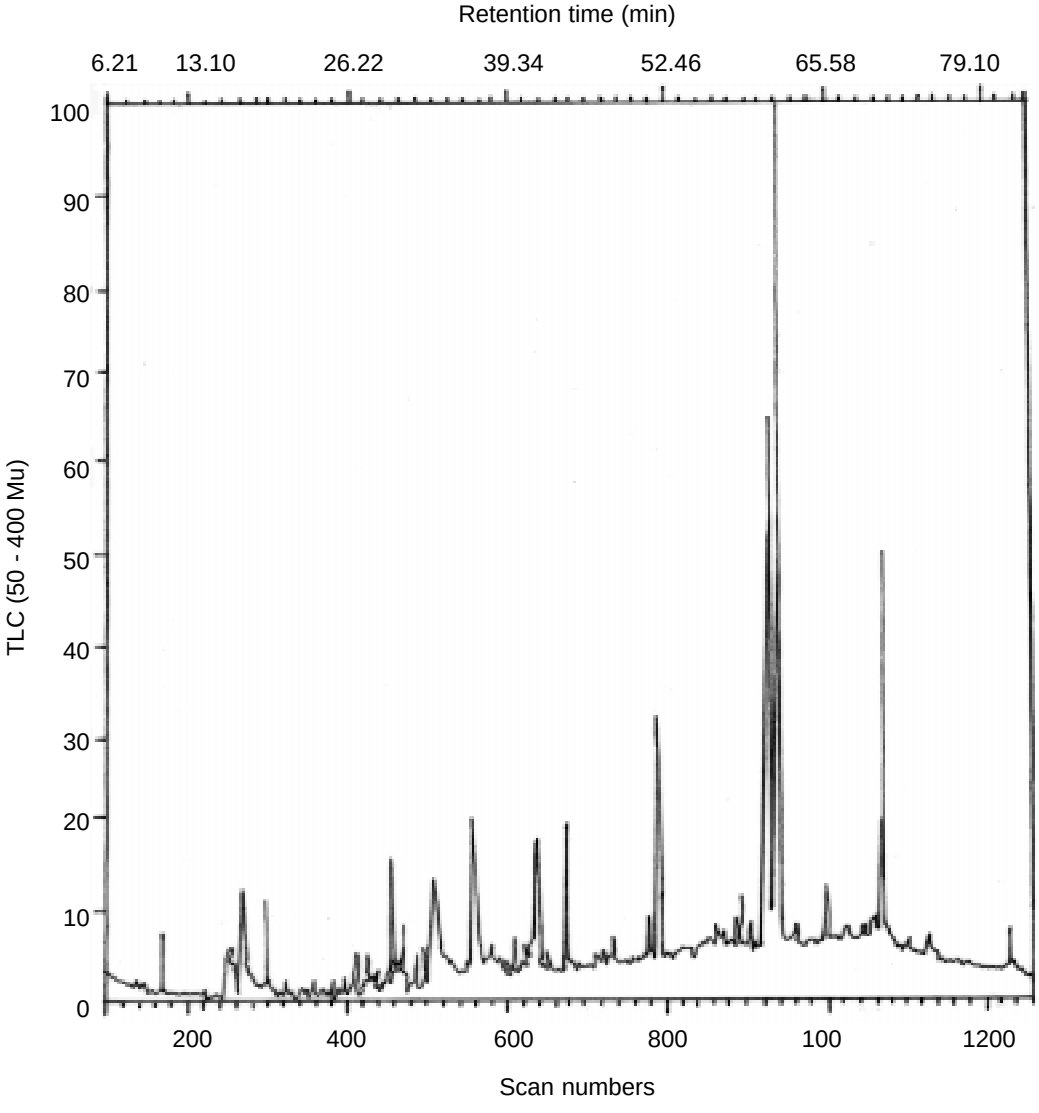

Fig 2. Total ion current chromatogram of methyl esters of fatty acids of HMWCFE of fraction I.

Table 2
Fatty acids liberated from HMWCFE, PPF-II of *C. lindemuthianum*

Peak #	Scan #	Abbreviated symbol	Common name	Systematic name	Structural formula
	498	C_{10}	Capric acid	Decanoic acid	CH_3-$(CH_2)_8$-CO_2H
	669	C_{14}	Myristic acid	Tetradecanoic acid	CH_3-$(CH_2)_{12}$-CO_2H
1	827	C_{16}	Palmitic acid	14-methylated decanoic acid	CH_3-CH(CH_3)-$(CH_2)_{12}$-CO_2H
2			Unidentified		
3	961	$C_{18:2}$	Linoleic acid	Octodeca-dienoic acid	$CH_3(CH_2)_4$-CH=CH-CH_2-CH=CH$(CH_2)_7CO_2H$
3a	979	C_{18}	Stearic acid	Octadecanoic acid	CH_3-$(CH_2)_{16}$-CO_2H
4	1034	C_{18}	Isostearic acid	16-methylhepta-decanoic acid	CH_3-CH(CH_3)-$(CH_2)_{14}$-CO_2H
5	1106	C_{20}	Arachidic acid	Eicosanoic acid	CH_3-$(CH_2)_{18}$-CO_2H
6	1272	C_{22}	Behenic acid	Docosanoic acid	CH_3-$(CH_2)_{20}$-CO_2H

positive sugar test. This gel like material was more pronounced in Fraction-I and suggested that there may be some unhydrolysed material still present which may require either more vigorous or different hydrolyzing conditions.

Hexane extracts were analysed by GLC and fatty acid esters eluted in the range of 90-230˚C were usually between 18-34 carbon chain lengths (Fig 1a & b). The characterization of these peaks has been done by co-chromatography of standard samples. The peaks separated by GLC were further characterized by electron impact mass spectra and library search to fit the best matches of the major eight peaks to reference spectra. Total ion current chromatogram of HMWCFE of PPF-I and II are presented in Fig 2-3.

Four major peaks separated by GLC with retention times identical to palmitic acid (1), oleic acid (3), isostearic acid (4) and arachidic acid (5) were detected in the hexane extract of PPF-I (Fig 1a and Table 1). The integrated area of major peak was considered as 100% (Fig 2) and the relative proportions of each fatty acid was concluded as follows, palmitic acid 8.7%, oleic acid 30%, isostearic acid 43% and arachidic acid 17%.

Similarly five major fatty acids of PPF-II (Fig 1b and Table 2) were identical viz: palmitic acid, 2.0% (1), isostearic acid, 52% (4), arachidic acid, 25% (5) and behenic acid 5.1% (6). Other smaller peaks were identified as capric acid and myristic acid. It was observed that palmitic, isostearic and arachidic acids are common to both the fractions. Arachidic and other fatty acids were identified from *Pythium infestans* and elicited fungitoxic sesquiterpenes in potato (Bostock *et al* 1981).

Various possible linkages have been shown to exist between lipids and polysaccharides. We know that HMWCFE polysaccharides in this study contain phosphate as well as amino sugars and structures are likely to be complex (Fatima and Finch 1998). Hence to establish the linkages of the fatty acids to the polysaccharides, PPF-I and II were hydrolysed under acidic and very mild basic conditions in the presence of $NaBH_4$ to prevent the peeling effect of NaOH. The liberated fatty acids were extracted by hexane and esterified with boron trifluoride etherate in anhydrous methanol (Anon 1989). GLC of the methyl ester showed that PPF-I and II gave similar set of peaks although the peaks provided by acid hydrolysis were slightly different from those produced by base hydrolysis. A typical chromatogram for acid and base hydrolysed material in Fig 4 showed that a peak 3 (arachidic acid) with a retention time of 25.93 min was absent from the sample produced by basic hydrolysis. This apparent stability of a small portion of elicitor fatty acids to a mild base may indicate that this fraction is linked to other compounds via amide bonding, which is labile to acids but stable to base hydrolysis. PPF-I has been shown to release a significant proportion of glucosamine on acid hydrolysis, this sugar could act as the site for amide bonding. Few examples of this type of glycolipid exist in the literature (Kennedy and White 1983).

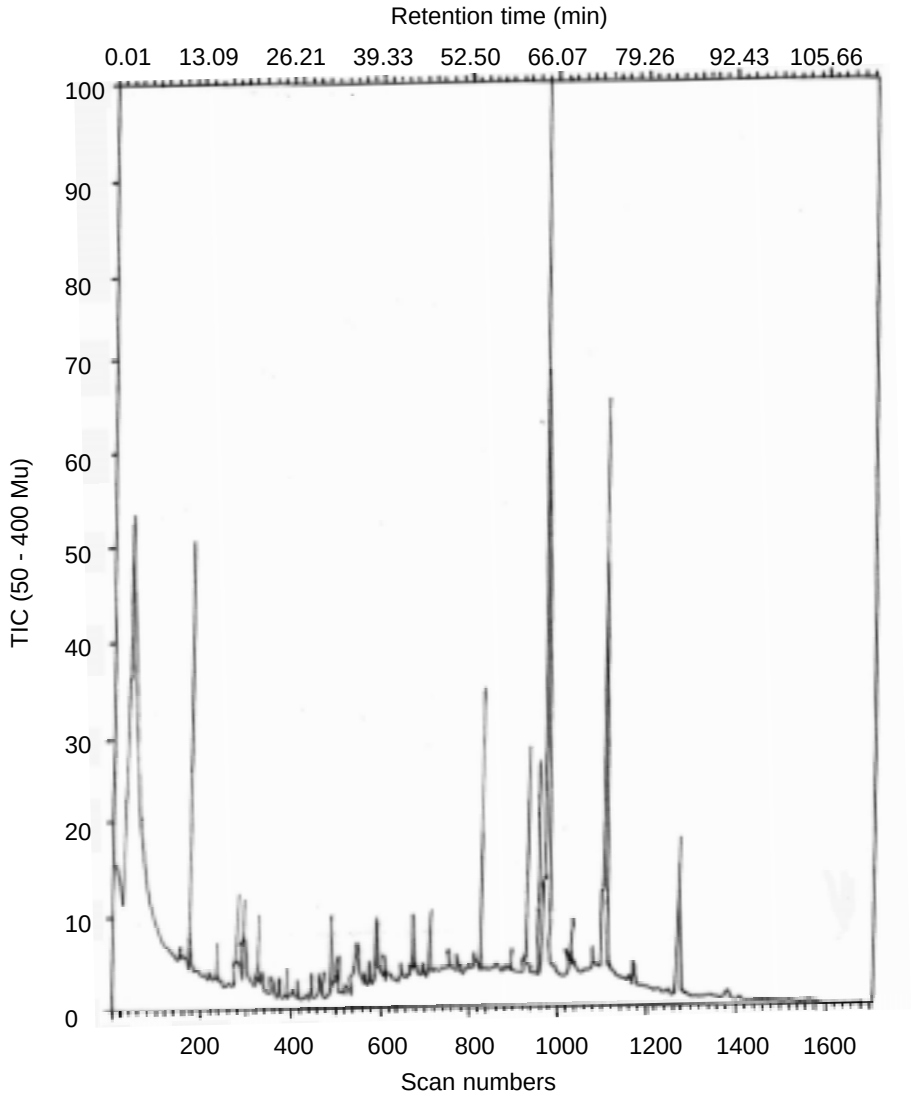

Fig 3. Total ion current chromatogram of methyl esters of fatty acids HMWCFE of fraction II.

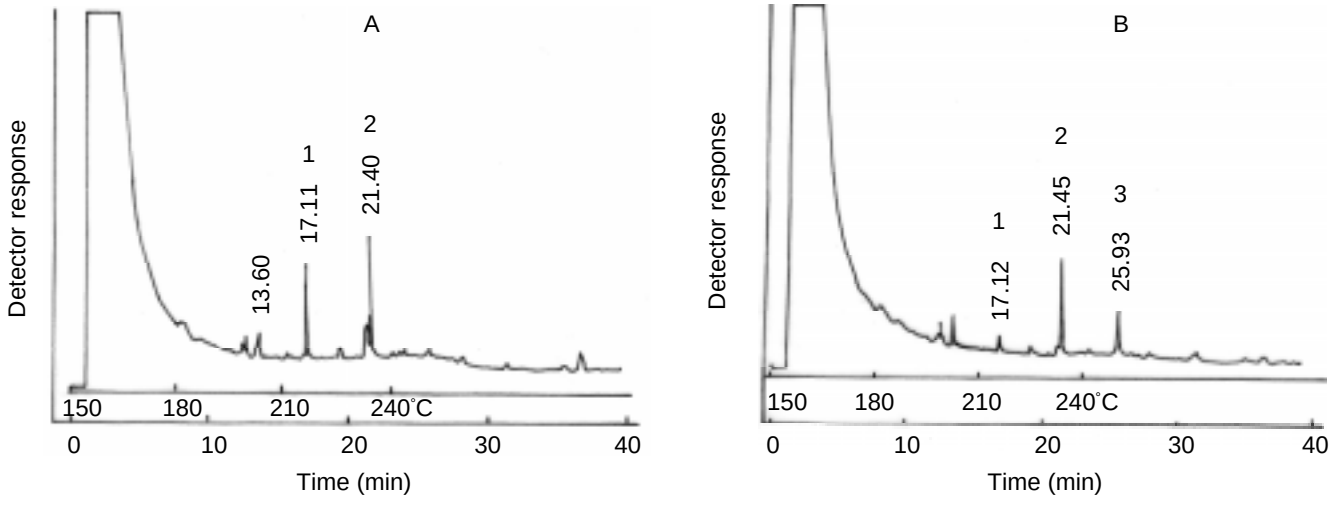

Fig 4a & 4b. Gas Liquid chromatograms of fatty acid methyl esters from *C. lindemuthianum* (A) Base hydrolysed and esterified sample of HMWCFE fraction I, (B) Acid hydrolysed and esterified sample of HMWCFE fraction 1.

All these evidence suggest that extracellular culture filtrate product of *C.lindemuthianum* in this case are not a pure polysaccharide but a glyco-conjugate consisting of simple or complex glycolipid. Lipid material was not reported previously in culture filtrate preparation of *C. lindemuthianum* and significance of the lipid in this system is not established.

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