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# Monosaccharide Composition of High Molecular Weight Culture Filtrate Elicitor of *Colletotrichum lindemuthianum*, Determined by High Performance Anion-Exchange/Pulsed Amperometric Detection (HPAE-PAD)

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Monosaccharide composition of crude and partially purified high molecular weight culture filtrate elicitor (HMWCFE) fractions-I and II isolated by molecular sieve chromatography from *Colletotrichum lindemuthianum* IMI (112166) isolate were acid hydrolysed for 0.5, 1.5, 3 and 5 h. Liberated monosaccharides were analysed directly using high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) Mannose galactose were found as major sugars of each sample whereas glucosamine, arabinose and rhamnose were additional sugars. Glucosamine was found as a major sugar (26% w/w) of Fraction-I and first time reported from preparations of this organism.

***Key words:*** *Colletotrichum lindemuthianum*, Elicitor, Monosaccharide composition.

## Introduction

Polysaccharides and glycoproteins obtained from culture filtrate and cell walls of plant pathogenic fungi elicit hypersensitive defence responses when applied to tissues or cell cultures of incompatible plants (Knogge 1996). Elicitor active substances from *Colletotrichum lindemuthianum,* the causal agent of anthracnose disease in bean, were first knewn from culture filtrates or hot water extracts of fungal cell wall (α race) (Anderson and Albersheim 1975); the active material was 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 1978a; Anderson 1980a; Tepper and Anderson 1986) and β (Anderson 1980b, Tepper and Anderson 1986), (Anderson 1980b) and IMI 112166 (Hamdan and Dixon 1986, 1987) races.

In contrast to the original work, neutral sugar analysis of crude and fractionated culture filtrate elicitors revealed significant proportions of galactose and mannose as well as glucose, the β and IMI 112166 races also elaborated rhamnose. In this study crude and partially purified glycoconjugates obtained from culture filtrate of *C. lindemuthianum* IMI 112166 isolate were analysed for monosaccharide composition using high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Towsend *et al* 1988; Hardy 1989). The development of pulsed electrochemical method made possible a sensitive detection (10-100 p mol) of underivatised carbohydrates of biological importance. The technique has been applied to the detection of neutral, acidic, basic monosaccharides and oligosaccharides (Sullivan and Maurice 1994; Kerherve *et al* 1995). Glucosamine was quantitatively determined and reported first time from these preparations.

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## Experimental

*Elicitor preparations and purifications.* Shake cultures of *Colletotrichum lindemithianum* 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 $l^{-1}$. Crude high molecular weight culture filtrate elicitors (HMWCFE) were obtained by ultrafiltration and dialysis with distilled water simultaneously using an Amicon hollow fibre ultrafiltration cartridge system of pore size 30,000 dalton. Partially purified fraction-I of Mr 2000,000 dalton and fraction-II Mr

40,000 were obtained by Fractogel HW-65S, size exclusion chromatography of column size (90x3cm) and eluted with 10 mM ammonium formate with a flow rate 0.5ml $min^{-1}$.

*Acid hydrolysis.* 2 mg of crude HMWCFE and partially purified fractions I and II were dissolved in 1 ml of 2M trifluoro acetic acid (TFA) in reaction vials with screw caps and PTFE dics (Pierce), flushed with $N_2$ and heated at 120°C for hydrolysis. 200 µl aliquots were withdrawn at 0.5, 1.5, 3. and 5 h intervals and dried by $N_2$ blowing. The residue was extracted with hexane twice to remove liberated fatty acids and lipid materials. Hydrolysed polysaccharide was dissolved in 0.8 ml of water, filtered by centrifugation through 10,000 Mr cutoff membrane centrifuge tubes and applied for HPLC separation.

*Chromatographic separation and detection of monosaccharides by HPAE -PAD.* Chromatography was performed on Dionex HPAE-PAD system using a Bio-LC gradient pump module and model 2 detector and the Dionex eluent with helium. In these experiments eluent 1 was 150 mM NaoH and eluent 2 was 18 Moh high purity deionised water. Sample injection was via a Rheodyne 7125 valve equipped with a 25 µl sample loop and Tefzel rotor seal to withstand the alkalinity of the eluent.

Monosaccharides were separated on a column (4.6x250 mm) of Dionex Carbo pac PA-I with a guard column, using a flow rate of 1ml $min^{-1}$ at room temperature. The analysis was carried out at an isocratic NaOH concentration of 15 mM i-e (10% eluent 1 and 90% eluent 2) followed by 15 min washing in 150 mM NaOH and then flushing with starting eluent for 10 min. In order to optimise the detector response 300 mM NaOH was added to the post column effluent via a mixing tee at a flow rate 1 ml $min^{-1}$. The resulting chromatographic data were plotted and integrated by interfacing to Dell system 200 computer and Epson FX-850 printer-printer plotter using Dionex Aution 450 software.

## Results and Discusssion

Partial purification of HMWCFE was carried out on Fractogel HW-65S eluted with 10 mM ammonium formate. The separation achieved on this column resulted in two well resolved fractions. Fraction-1 of Mr $2x10^6$ or greater, Fraction-II of Mr 40,000 dalton. Pattern of the elution curves, Fig-1 suggested that species of different molecular weights and possibly of quite different compositions were present within these fractions. Column recoveries and the chemical composition of these fractions are given in Table-1.

In this study monosaccharide composition of a glycoconjugate (HMWCFE) *Colletotrichum lindemuthianum* was determined by HPAE-PAD system of sugar analysis. Previous analysis of culture filtrate and cell wall elicitor of *C. lindemuthianum* were rather selective and restricted to neutral sugars. Earlier work reported that elicitor active molecule obtained after sizing and ion-exchange chromatography were predominantly glucan (Anderson and Albersheim 1975) impure and partially purified preparations contained considerable amount of galactose and mannose (Anderson 1980b; Kerherve *et al* 1995). In this study mannose and galactose were found as dominating sugar of all three samples glucosamine arabinose and rhamnose were the additional sugars of these preparations. One unidentified sugar was also present.

Results in Table 2 showed that after 30 min, glucose, galactose and mannose liberated were in the following proportions crude HMWCFE (1:4:1), fraction I (1:2:4:2) and fraction II (1:11:5.5).Arabinose was found in significant amount only in the crude preparations and liberated at 30 min and 3hr of hydrolysis. It is interesting to note that glucosamine was first time reported from HMWCFE preparations of this organism, released througout the hydrolysis period and became the major sugar component (16-26% w/w) of fraction-I; a very small quantity was associated with fraction-II. The glucosamine detected is most likely derived from the hydrolysis of Chitin (poly-β-1,4 linked N-acetylglucosamine) which was detected as minor (5-9% w/w) constituent of the cell walls and IMI 112166 races. (O'Connell and Ride 1990) However the chitin was excluded from fungal infection structures (O' Connell and Ride 1990) and its absence may be important in the infection process despite the reported (Miller *et al* 1992) accumulation of a

**Table-1**

Column recoveries and chemical composition of HMWCFE fraction-1 and II obtained from fractogel HW-65S chromatography

| HMWCFE | Solid recovered % w/w | Sugar cont. % | Protein sugar % | Phosphate % | Acidic sugar % |
|---|---|---|---|---|---|
| Fraction-1 | 27.1 | 14 | 2.5 | NF | 1.5 |
| Fraction-II | 57.0 | 11.5 | 0.03 | 6.1 | 1.0 |

NF= Not found

**Table 2**

Monosaccharide composition of crude and partially purified fraction of HMWCF of *C.lindemuthianum* determined by HPAE-PAD

| Sample | Hydrolysis time (h) | Monosaccharide content % w/w | | | | | |
|---|---|---|---|---|---|---|---|
| | | Glc | Gal | Man | Rha | Ara | Glc NH2 |
| HMWCFE | 0.5 | 6.2 | 25 | 6.0 | 0.26 | 9.5 | 3.1 |
| Crude | 1.5 | 0.75 | 1.1 | 0.75 | 0.2 | 0.45 | 4.1 |
| | 3.0 | 0.18 | 0.2 | 3.15 | 1.2 | 6.9 | 1.3 |
| | 5.0 | 0.2 | 0.3 | 0.4 | 0.07 | 0.03 | 1.5 |
| HMWCFE | 0.5 | 6.0 | 14.3 | 12.4 | 0.45 | 0.1 | 16.2 |
| Fraction -I | 1.5 | 4.2 | 15.5 | 14.7 | 0.03 | 0.16 | 16.3 |
| | 3.0 | 3.75 | 13.7 | 12.0 | 0.03 | 0.16 | 25.0 |
| | 5.0 | 3.5 | 12.0 | 11.0 | ND | ND | 26.0 |
| HMWCFE | 0.5 | 2.4 | 27.0 | 11.0 | 0.05 | 1.6 | 0.8 |
| Fraction -II | 1.5 | 2.5 | 27.0 | 15.0 | 0.03 | 2.1 | 1.5 |
| | 3.0 | 2.4 | 24.0 | 13.0 | 1.1 | ND | 1.4 |
| | 5.0 | 2.2 | 21.0 | 11.0 | 1.1 | ND | 1.5 |

ND=Not Determined.

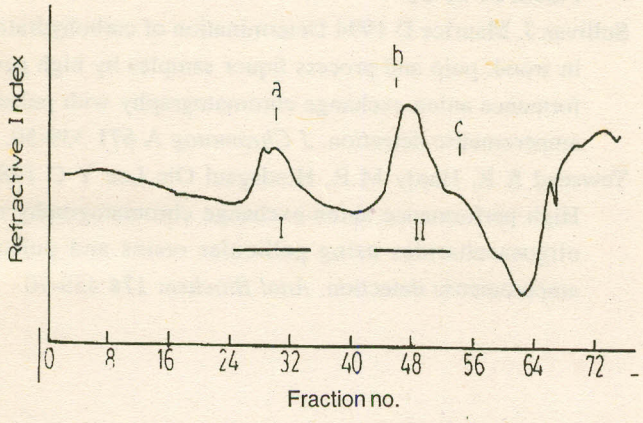


**Fig 1.** Fractogel HW-65S separation of crude HMWCFE and fractions I and II pooled as indicated by the bars. Arrows are the marker for (a) Dextran T-2000, (b) Dextran T-40, and (c) glucose.

plant derived chitin-binding protein at growing hyphal tips. During later stages of hydrolysis the amount of sugar liberated either remained unchanged or significant decrease was observed. In general the amount and the pattern of sugars released in fractions I and II clearly suggest that two distinct class of polysaccharides well resolved by fractogel HW-65S were present in the crude HMWCFE of *C. lindemuthianum.*

TLC or GLC of alditol acetate are not well suited to analyse acidic and basic sugars; advantage of HPAE-PAD is the ability to resolve the free amino sugars and neutral monosaccharides using a single chromatographic condition. In this case separation mixtures of neutral and amino sugar (standards) and HMWCFE samples were achieved in 15 min into well resolved symmetrical peaks Fig-2.

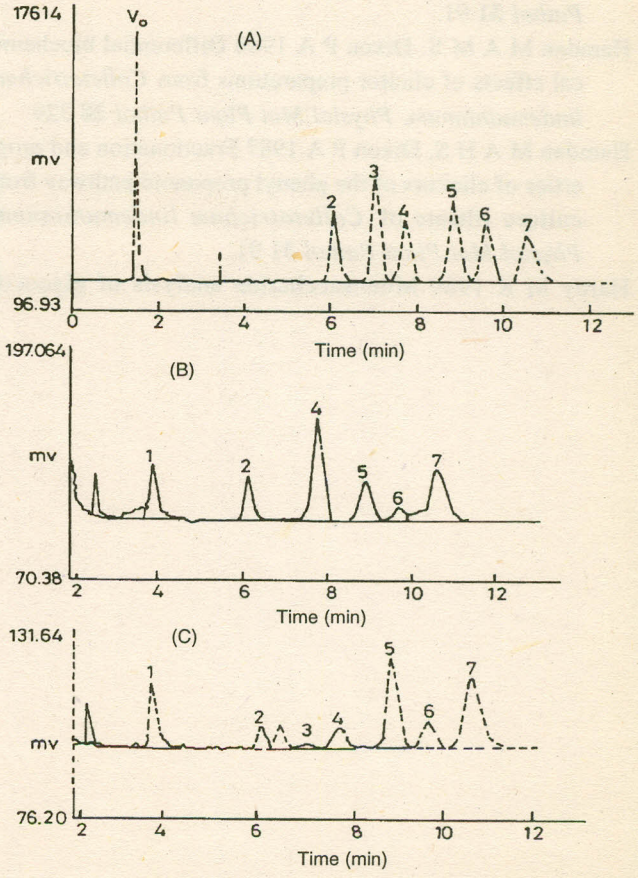


**Fig 2.** Chromatography of monosaccharides using a high performance an-ion-exchange (HPAE) carbopac PAI column with Pulsed Amperometric Detector (PAD) eluted with 15 mM NaOH with flow rate 1mlmin$^{-1}$. (A) separation of standard mixture of neutral and amino sugar. Peak: Vo = myoinositol, 1 = fucose, 2 = arabinose, 3 = rhamnose, 4 = glucosamine, 5 = galactose, 6 = glucose, 7 = mannose. (B) HMWCFE I. (C) HMWCFE II.

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