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ISOLATION AND PARTIAL PURIFICATION OF ELICITOR MOLECULES FROM *COLLETOTRICHUM LINDEMUTHIANUM*

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Abstract

Colletotrichum lindemuthianum grown in glucose/neopeptone natural liquid medium showed that yield of crude 'High Molecular Weight Culture Filtrate Elicitor' (HMWCFE) obtained by simultaneous ultrafiltration and dialysis were little high 0.8 g/L in 6th day culture. A range of browning was observed in response to various elicitor preparations of each day culture of *C.lindemuthianum*. Maximum browning was produced by the elicitor preparation in 6th day culture. Total carbohydrate and protein contents were found to be 18.4 -33.4% and about 1.6–2.7% respectively. The presence of phosphate (4-12%) in high molecular weight culture filtrate elicitor is reported for the first time from *C. lindemuthianum*. Prelininary elution profile and apparent range of molecular weight of HMWCFE was >60 000 dalton determined by gel permeation chromatography.

Introduction

Polysaccharides and glycoproteins obtained from culture filtrate and cell wall of plant pathogenic fungi elicit hypersensitive defense response when applied to tissues or cell cultures of incompatible plant (Knogge, 1996). These molecules are called elicitors (Keen *et al.*, 1972). Many elicitors have been described including various polysaccharides (Hughes & Dickerson, 1991), oligosaccharides (Clarence & Edward, 1991), protein/enzymes (Vogelsang *et al.*, 1994) and fatty acids (Castoria *et al.*, 1995). Isolation and partial purification of elicitor active substances from *Colletotrichum lindemuthianum*, the causal agent of anthracnose disease in beans has been described by gel permeation and ion exchange chromatography of α, β and λ races (Anderson & Albersheim, 1975; Tepper & Anderson 1986). Elicitor active molecule were also obtained and purified from cell wall of IMI 112166, β races and culture filtrate of *C.lindemuthianum* (Dixon *et al.*, 1981, Hamdan & Dixon 1986).

In the present study shake cultures of *C.lindemuthianum* were grown in a complex medium of glucose/neopeptone to measure the elicitor production at different growth stages and to examine the elicitation behavior of these crude preparations. Purification and fractionation of HMWCFE on Biogel P-60 was aimed to produce a homogenous fraction rich in sugar content for further characterization and elicitor activity. Elicitor activity was measured in terms of induced browning in cotyledons of French beans (*Phaseolus vulgaris*).

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Material and Method

Stock cultures of *C.lindemuthianum* (Commanwealth Mycological Institute Isolate IMI 112166) were maintained on semisolid glucose neopeptone complex medium (Mathur *et al.*, 1949), as modified by Anderson & Albersheim (1975) by the use of 15 g glucose/L. Suspension cultures of *C. lindemuthianum* were grown on a complex medium containing 15 g glucose, 2.7 g KH_2PO_4 1.23 g $MgSO_4.7H_2O$, 2 g peptone per litre.

Initiation of suspension culture: Shake cultures of *C. lindemuthianum* were initiated by sterile transfer of 1 cm, conditioned friable clumps of mycelium, cut from the stock cultures to the 500 mL of the Mathur modified liquid medium in Erlenmeyer flask. Cultures were grown in an orbital incubator shaker at 25°C with low agitation rate of 100-110 rpm. The fungus took 10-15 days to form a dense actively growing mycelial suspension, which was used as stock liquid culture for further inoculation of fresh liquid medium.

Growth curve and elicitor preparation: Further inoculation of liquid media was carried out by sterile transfer of 10 mL of growing inoculum into twelve 1-L flasks, each containing 250 mL of autoclaved medium, inoculated under similar condition as mentioned above, with an agitation rate of 100-110 rpm.

Two flasks were removed at 4 days incubation and mycelium was separated by filtration through Whatman filter paper no-1. The culture filtrate was heated on boiling water for 20 min., to deactivate enzymes and was deproteinized by shaking with chloroform:butanol 5:1 v/v. Duplicate samples of 250 mL each day culture filtrate were ultrafiltered and dialyzed simultaneously using an Amicon hollow fiber ultrafiltration cartridge system of pore size 3000 dalton Mr cutoff to remove salts and low mol. wt. sugars. Concentrate of high molecular weight from the reservoir were collected, lyophilized and stored at –20 °C. Three batches of fungus were grown.

HMWCFE collected by ethanol precipitation: In a parallel sample (250 mL each) of a second batch of culture, 750 mL of, ethanol was added and the culture filtrate was stored at 4°C for 4 days. A brown precipitate was deposited and the supernatant liquid was siphoned off. The precipitate was collected and redissolved in 5-10 mL water, dialysed against distilled water, freeze dried and stored at –20 °C.

Fractionation of HMWCFE on Biogel P-60: Ten mL (2 mg/mL) solution of crude HMWCFE from each days culture of batch 11 was fractionated on a Biogel P-60 column (44x 2 cm) with a fractionation range of 3000-60 000 dalton for Dextran. The column was washed and sample was eluted with water and calibrated with Dextran T-70 and glucose. Fractions were monitored by phenol/H_2SO_4.

Chemical Composition and Elicitor Activity of HMWCFE: Crude elicitor preparations were chmically analysed. Total carbohydrate was determined by phenol/H_2SO_4 in terms of glucose (Dubois *et al.*, 1956). Total protein was determined by the method of Bradford (1976). Total phosphate was determined by the method of Chen *et al.*, (1956).

Elicitor activity of HMWCFE was determined in terms of induced browning by the method previously described by Whitehead *et al.*, (1982). Cut surfaces of cotyledons of French bean variety "The Prince" were inoculated with the dilution 20, 40 and 100 µg glu eq/mL of each day HMWCFE preparations and control samples were prepared by treatment of sterile water or simply by wounding, incubated at 25 ^{0}C for 24 h in the dark.

Results

Elicitor preparation: Three separate batches of *C.lindemuthianum* cultures were grown and the lag phase of about 3 days was observed followed by a period of exponential growth which was confined to 5th and 6th day of active growth (Table 1). Dry weight of mycelium gradually increased and peaked at maximum (1.0-1.6 g / 250 mL) on 6th day of incubation.

Table 1. Yield of HMW culture filtrate elicitor of *Colletotrichum lindemuthianum* in complex medium[a] by ultra filtration.

Day of culture	Dry wt. (g) Mycelium[b]	Dry wt. (g) HMWCFE[b]	Carbohydrate content HMWCFE (% W/W)	Protein content HMWCFE (% W/W)	PO_4^{-2}
4th	1.04	0.076	18.4	2.5	4
5th	1.4	0.068	26.8	2.7	4
6th	1.64	0.2	33.4	1.6	4.5
7th	1.50	0.072	28.3	1.8	6
8th	1.56	0.14	31.3	1.7	12

[a]Data are mean of 2 replicates and findings of three determinations.
[b]Gram per 250 ml

Deactivated and deprotenized dry weight of crude HMWCF products obtained by simultaneous dialysis and ultrafiltration were higher (0.8 g/L) in 6th day of culture than reported (0.19 g/L) by Hamdan & Dixon (1986), obtained by ethanol precipitation. Total carbohydrate content of HMWCFE in terms of glucose increased (18 - 33.4%) steadily throughout the growth. Protein content was found very low (1.6 - 2.7%) and decreased slightly in later stages of growth. Phosphate (4-12%) was associated with each sample, not previously reported from this organism, but has been found in polysaccharide obtained from CFE of various races of liquid cultures of same fungal isolates grown in similar medium of *Cladosporium fulvum* (Dow & Callow, 1979).

HMWCFE obtained by ethanol precipitation: The brown precipitate was collected and analyzed for its carbohydrate and protein contents (Table 2), showed that dry weight of HMWCFE (0.6 g/L in 6th day of culture) were generally lower than those obtained by ultrafiltration and still higher than reported by Hamdan & Dixon (1986). The carbohydrate content of HMWCFE was found in the range of (16-36%), considerably high 22 and 36% in 5th and 6th days sample, respectively. Increased amount of protein ranging 15-2% was found in each day's culture. Sugar and protein ratio 1:1 was found in the 4th day sample. Protein and carbohydrate contents of CFE obtained by ethanol precipitation from α and β races and IM I 112166 isolates of *C. lindemuthianum* were in the ratio of 1:1 and 1:43 respectively (Anderson & Albersheim 1975; Tepper & Anderson 1986; Hamdan & Dixon 1986).

Table 2. Yield of HMWCFE of *Colletotrichum lindemuthianum* obtained by ethanol precipitation from complex medium.

Day of culture	Dry wt. (g) Mycelium[a]	Dry wt. (g) HMWCFE[a]	Carbohydrate content HMWCFE (% w/w)	Protein content HMWCFE (% w/w)
4th	1.55	0.04	16	15
5th	1.6	0.048	22	14
6th	2.1	0.15	36	7
7th	1.8	0.082	19	5
8th	1.84	0.072	17	2

[a]Gram per 250 ml

Table 3. Carbohydrate recovered from Biogel P-60 chromatography of crude HMWCFE of *Colletotrichum lindemuthianum*.

Day of culture	Total sugar recovered (%) by wt.	Sugar recovered in fraction a + h (%) by wt.
4th	54	68
5th	56	73
6th	66	88
7th	33	65
8th	19	45

Separation of HMWCFE on Biogel P-60: Crude HMWCFE of *C.lindemuthianum* was fractionated on Biogel P-60. A typical chromatogram (Fig. 1) showed a disperse peak of carbohydrate eluted in or near the void volume of Mr >60 000 dalton. The polysaccharide of each day's culture produced an identical elution profile with similar molecular size distribution pattern. On the basis of carbohydrate, fractions a, b, and c were pooled and collected. It was noticed that maximum sugar 56 and 66% were recovered in the 5th and 6th day sample (Table 3) and 73-88% respectively of this sugar was eluted in the fraction a+b of Biogel P-60.

Presence of salts and other charged material in crude and P-60 purified fractions were further investigated by conductance measurement (results are not shown here). Results showed lowest conductance in 5th and 6th days sample and these samples were also rich in carbohydrates.

Elicitor activity: Elicitor activity determined in terms of induced browning, by the elicior treatment of cotyledons of French bean variety 'The Prince' showed a distinct and definite increase in browning by the elicitor treated samples as compared to the control samples, although the extent of surface browning was different for each sample (Table 4). Maximum browning was produced by the elicitor preparation of 6th day culture; this preparation has high sugar content 33.4%. Intermediate dilution of 40 µg glc eq/mL was less active than two other dilutions of 20 and 100 µg glc eq/mL. Fraction 'b' from Biogel P-60 of each days culture preparation showed various degree of browning (results are not included here).

Table 4. Elicitor activity in terms of induced browning[a]

	Crude HMWCFE μg glc eq/ml				
Day of culture	**20**	**40**	**100**	**Control H_2O**	**Control Wound**
4^{th}	++	+++	+	++/+	0/+
5^{th}	+	++	++		
6^{th}	+++	+	++++		
7^{th}	++	++	+++		
8^{th}	++	++	++		

[a]Result are average of duplicate sample and replicate of three experiments. Degree of browning = Least 0/+, Maximum ++++

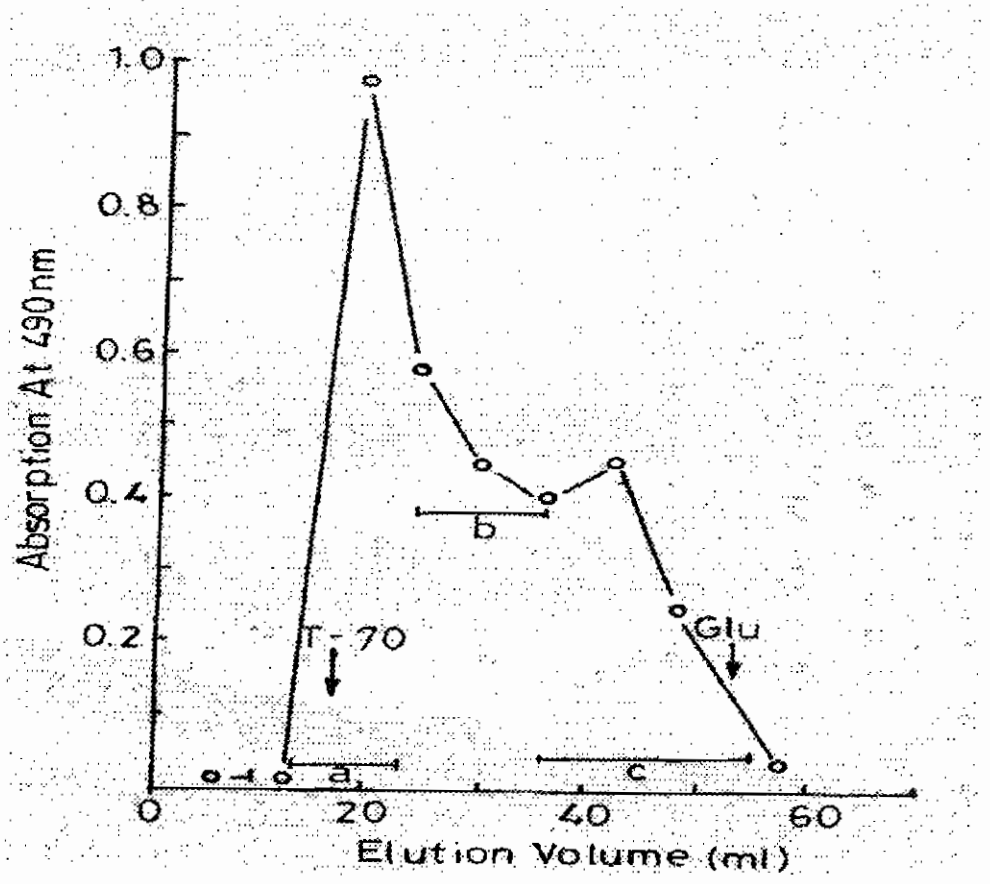

Fig. 1. Gel filteration of HMWCFE (SS) crude preparation of *C. lindemuthianum* on Biogel P-60. Fractions A, B and C were collected on the basis of carbohydrate. Arrow indicate the elution volume of glucose and dextran T-70.

Discussion

Colletotrichum lindemuthianum was successfully cultured in a complex medium, an improved yield of crude HMWCFE 0.8 g/L and 0.6 g/L were obtained by ultrafiltration and ethanol precipitation respectively. In both cases values were higher than reported by

Hamdan & Dixon (1986). Chemical analysis of HMWCFE showed that amount of carbohydrate was found in similar range in crude elicitor preparations obtained by either method, whereas increased amount of protein (15%) was found in HMWCFE obtained by ethanol precipitation. High content of protein in early days of culture may arise from added peptone and precipitated by addition of ethanol. At initial stages of growth the sugar and protein ratio was 1:1 in the 4th day sample, this finding confirmed that protein and sugar contents of CFE obtained by ethanol precipitation from α and β races and IMI 112166 isolate of *C. lindemuthianum* were in the ratio of 1:1 and 1:43 (Anderson, 1978; Hamdan & Dixon 1986). Phosphate was associated with each sample and is reported for the first time from this organism. Preliminary results showed that despite making allowance for counter ion to phosphate the aggregate analytical data fall below 100% and suggested that some other constituents are present, previously unrecognized in this preparation. Purification and fractionation of HMWCFE on Biogel P-60 was aimed at providing a homogenous pure fraction for characterization and elicitor activity studies. The elution profile indicated that material was highly polymeric and quite heterogenous in construction. Maximum sugar was recovered in 5th and 6th days sample whereas 7th and 8th days cultures contained lower amounts of sugar. High conductivity exhibited by the sample of 1st day, may be arised from some undialysed salt of the medium and the high conductance of 7th and 8th days culture preparations suggest incorporation of charged groups (phosphates) at later stages of growth. It has been reported that preparation on strong anion exchange resin followed molecular size separation provided a polydisperse high molecular weight fraction with an apparent Mr of 5,000,000 dalton (Anderson & Albersheim, 1975). Anion exchange, size exclusion and finally affinity chromatography of CFE of IMI 112166 isolate provided a mannose rich elicitor active fraction (Hamdan & Dixon, 1987).

Differential response of induced browning was observed by the tissues of *P. vulgaris* (French bean) on treatment of crude and partially purified HMWCFE preparation of each days culture of *C.lindemuthianum*. It was observed that intensity of the browning was dependent on the type of elicitor preparations and its dilution. Maximum browning was produced by the 6th day culture preparation with high sugar content. This suggests that amount of sugar has some direct effect on elicitor activity. Carbohydrate is the important constituent for manifestation of elicitor activity (Hamdan & Dixon, 1986, Anderson *et al.*, 1991). Various dilutions of each days culture used and elicitor of 20 and 100 μg glc eq/mL were found as the more effective dilutions (Table 4).

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