Pak J Sci Ind Res 2000 43 (1) 19-22

ENZYMIC DEGRADATION OF HIGH MOLECULAR WEIGHT CULTURE FILTRATE ELICITOR (POLYSACCHARIDE) OF *COLLETOTRICHUM LINDEMUTHIANUM**

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(Received 11 May 1998; accepted 10 May 1999)

Attempts were made to establish enzymatic procedure which may selectively degrade polysaccharide obtained from culture filtrate of *Colletotrichum lindemuthianum* to produce elicitor active fragments.

Key words: Elicitor, *Colletotrichum lindemuthianum*, Enzymic degradation.

Introduction

It is reported that polysaccharides and oligosacchrides from plant or microbial cell wall and culture filtrate can regulate both defensive and development processes in plants (Darvill and Albersheim 1984; Wilhelm 1994). Fungal cell wall and culture filtrate polysaccharides of *Colletotrichum lindemuthianum* have been found to be potent inducers of plant defense responses (Anderson and Albersheim 1975; Hamdan and Dixon 1986; Anderson *et al* 1991).

A partially hydrolysed highly branched β glucan elicitor from *Phytophthora megasperma* has been reported to induce viral resistance in tissues of several tobacco species when sprayed, injected or inoculated before or within 8 h after inoculation of tobacco leaves with virus (Kopp *et al* 1989). The inducing activity derived from fungal cell wall β-glucan generated by hydrolytic enzymes of plants has been shown to be a hepta-β-glucosyl fragment (Sharp *et al* 1986, Wijesundera *et al* 1989; Sepai 1993). In this study attempts are made to establish enzymatic procedure which may selectively degrade polysaccharide obtained from culture filtrate of *C lindemuthianum*, to produce elicitor active fragments.

Experimental

Elicitor preparation and purification. Shake cultures of *C lindemuthianum* were grown in a complex medium of glucose/neopeptone (Mathur *et al* 1949), as modified by Anderson and Albersheim (1975), by the use of 15g glucose l^{-1}. Originally crude High Molecular Weight Culture Filtrate Elicitor (HMWCFE) was obtained by ultrafiltration and dialysis with distilled water simultaneously, employing Amicon hollow fiber ultrafiltration cartridge system of pore size 30,000 dalton.

* This paper is based in part on the Ph.D thesis of Fatima Bi.
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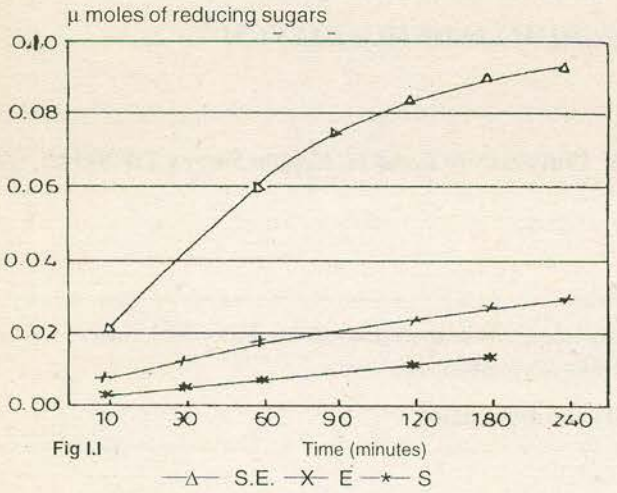
Enzymes used. The following enzymes were applied in the attempts to degrade polysaccharide. (1) Finizyme (Novo enzyme), obtained from *Aspergillus niger* with 1,3 and 1,4-β-endo-glucanase activity. (2) Cereflo (Novo Enzyme), obtained from selected strains of *Bacillus subtilis* and the enzyme is an endo-glucanase. (3) Driselase (Sigma Chemicals Company Ltd. D 9515), obtained from *Basidiomycetes* with a protein content approximately 15% and contained Laminarase, xylanase and cellulase activity.

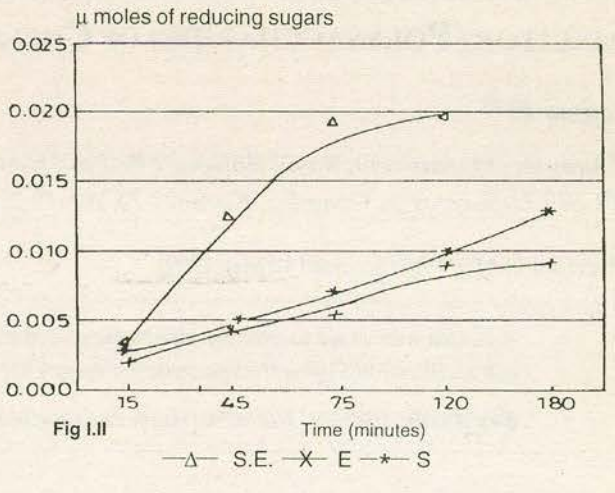
Enzymic degradation of HMWCFE Polysaccharide of C lindemuthianum. 10mg (1 mg ml^{-1}) aqueous solution of HMWCFE, standard barley β-glucan was separately placed in Amicon ultrafiltration apparatus and ultrafiltered using a polysulphone-based membrane of a nominal cut off value 10,000 dalton in acqueous medium, pH = 7 at room temperature 21 + 1°C, with a pressure head keeping a steady flow rate and a constant cell volume. Fractions were collected over a period of time and hydrolysis was monitored by measuring the reducing power of effluent using a colorimetric assay (Lever 1972). Total carbohydrate was determined by phenol/H_2SO_4 method (Dubois *et al* 1956).

Gel permeation chromatography of hydrolysed material. After enzymatic hydrolysis, fractions were pooled and applied on column of Fractogel HW 50S (90 x 3 cm) eluted with 10 mM ammonium formate at a flow rate of 30 ml h^{-1} and monitored by RI detector. 200 fractions, each of 3 ml were collected. Sugar content of each fraction was determined by phenol/H_2SO_4. Column was calibrated by chromatography of Glucose and Dextran T-40.

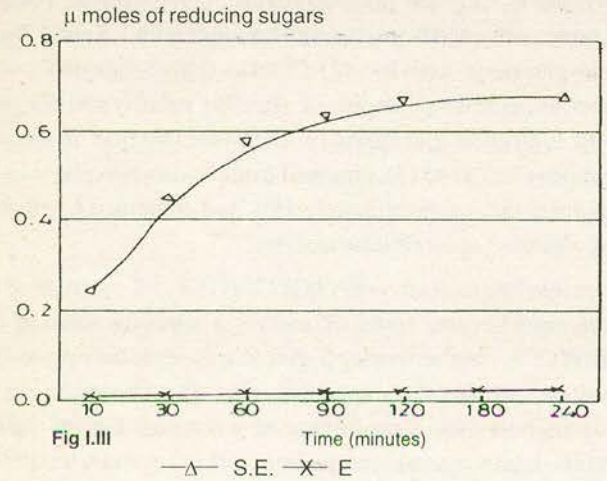
Seed germination and elicitor treatment. A general method of elicitor application was employed in all experiments as previously described by Whitehead (1982). Seeds of French bean (*Phaseolus vulgaris*) were germinated on autoclaved, moist vermiculite at 25°C in the dark. The excised cotyledons (4-5



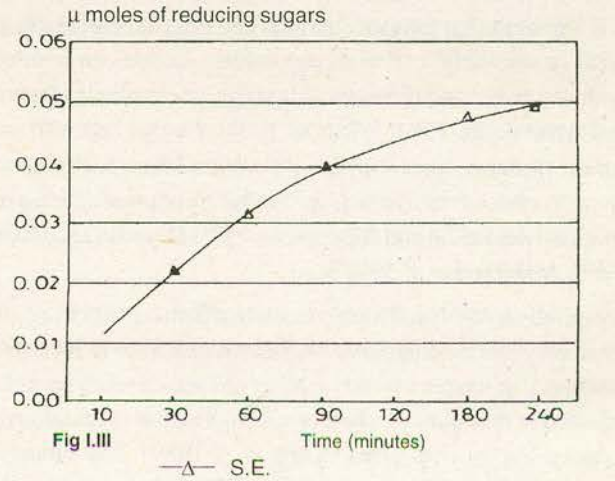
S, Substrate HMWCFE; E, Enzyme (*A.niger* 1,3 and 1,4-B-gluconase)



S, Substrate HMWCFE; E, Enzyme (*B.subtilis* 1,3 and 1,4-B-gluconase)



S,Substrate (Barley B-glucan); E, Enzyme (*A. niger* 1,3 and 1,4-B-gluconase)



S, Substrate (HMWCFE); E, Enzyme (Driselase from *Basidiomycetes* with cell Laminarase and Xylanase activity)

Fig 1. Enzymic degradation and ultrafiltration of polysaccharides obtained from *Colletotrichum lindemuthianum.*

Table 1

Enzymic degradation and ultrafiltration of HMWCFE (crude) obtained from *Colletotrichum lindemuthianum*

Enzyme source (µg protein)	Activity units	Substrate sugar (mg 10 ml^{-1})	Flow rate ($ml\ min^{-1}$)	Total carbohydrate degraded (% $time^{-1}$)
Aspergillus niger 115 µg	1.7	Barley β-glucan 10	0.28	92/30 min
Aspergillus niger 115 µg	0.017	Crude HMWCFE 3.5	0.18	9/3.6 h
Bacillus subtilis 55 µg	0.04	Crude HMWCFE 3.5	0.1	10/3.6h
Basidiomycetes 500 µg	1×10^{-3}	Crude HMWCFE 3.5	0.21	20/3.6h
Basidiomycetes 3000 µg	1.8×10^{-3}	Crude HMWCFE 1.5	n/a	6/4 h

days) were surface sterilised by immersion in 1% sodium hypochlorite for 5 min and then washed extensively with distilled water, finally rinsed with sterile water. Elicitor preparation at a concentration of 10, 40 and 100 µg glu eq ml^{-1} of crude and hydrolysed substance were used. Treated and control samples were prepared by application of 50 µl of droplets of test solution and sterile H_2O (as control) to the cut surfaces of cotyledons and incubated in a petri dish on moist filter paper at 25°C in the dark for a specified period of time.

Results and Discussion

Simultaneous enzymic hydrolysis and ultrafiltration of the degraded products of HMWCFE of *C. lindemuthianum* through a membrane of 10,000 nominal Mr cut off was carried out under the conditions specified in Experimental Section. The maximum degradation was observed by measuring the levels of reducing sugar of the effluent as soon as 10 min after addition of enzymes; µ moles of reducing sugar released was plotted against time (Fig 1, I-IV) for each combination of enzyme and substrate.

The results showed that enzymes acted quickly and the maximum degradation was observed within 30 min followed by a gradual and slow hydrolysis of the material (Figs I.I & I.II). *A niger, B subtilis*, endo-glucanases degraded specifically 1,3 and 1,4-β-glucan and showed a degradation of 9-10% (Table 1) in total 3-4 h time, which is slightly higher than the percentage of glucose determined by GLC and HPLC. The activity of *A niger* 1,3 and 1,4-β-endo-glucanase was assessed against the recommended substrate of barley-β-glucan Fig I.III and showed breakdown greater than 85% within 30 min. The hydrolysis of HMWCFE (crude) was also attempted by using an endoglucanase 'Driselase' obtained from *Basidiomycetes* (Fig I.IV). This enzyme contained laminarase, xylanase and cellulase activities. Enzymic treatment and ultrafiltration of the degraded product exhibited a higher degradation of HMWCFE, upto 20%, indicating the existence of more than one type of linkage and a complex monosaccharide composition.

Table 2

Browning produced by cotyledons of *P vulgaris* treated with culture filtrate elicitor of *C lindemuthianum*

HMWCFE (Crude)	Activity
10	+++/+++
40	++ / +++
100	+++/++++
Partially purified fraction (HW 50S, Peak-A)	
100	++ / +++
Control H_2O (Sterile)	0 / +

Data is mean of two replicates and finding of two determinations. 0 / + shows least browning.

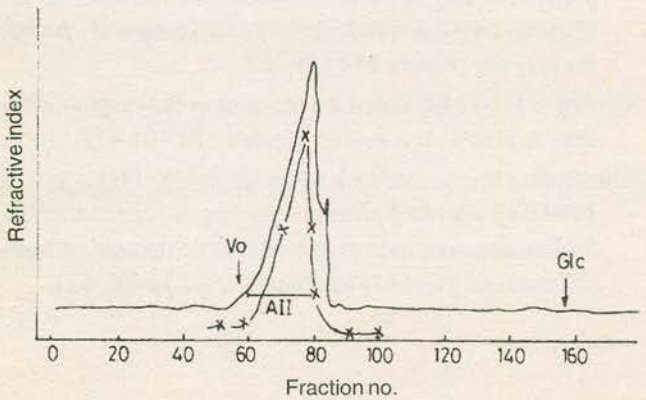


Fig 2. Size separation of enzymically degraded and ultrafiltered products of HMWCEP polysaccharides of *C. lindemuthianum* on Fractogel HW-50S column eluted with water elution profile monitored by refractive index—— and for sugars x—x—x. Arrows indicate the elution volume V_o and glucose Glc.

The enzymic degraded products initially collected through 10,000 Mr cut off membrane were lyophilised and redissolved in 10mM ammonium formate and passed through Fractogel HW-50S column and eluted in the same buffer, an attempt to resolve oligomers of characterisable fragment of approximate size of 10-20 sugar units. The elution profile in Fig 2 showed a highly asymetric partially seperated peak, eluted near the void volume of the column, with apparent Mr 40,000 dalton, suggesting that either some aggregation of the material or the substance was highly charged and eluted directly without being retained on the column as the column packing possessed some residual negative charge. It was previously reported, that the purified glucan elicitors from culture filtrate and cell wall of the **B** race of *C lindemuthianum* were not degraded by prolonged incubation with a purified endo-1, 3-glucanase that was isolated from bean tissues (Abeles *et al* 1970). However Bauer *et al* (1973) showed that degradation was achieved upon incubation of elicitor with a partially purified enzyme preparation from *Trichoderma viride* which possessed both 1,4 and 1, 3- β-glucanase activity.

The elicitor activity of crude and hydrolysed products was determined by the classical method of recording the induced browning (Table 2). Repeated experiments revealed that crude elicitor preparation were active especially at a concentration of 10 and 100 µg glu eq ml^{-1} concentration. Intermediate con-

centration 40 µg glu eq ml^{-1} was less active. Induced browning profile of hydrolysed product at a concentration of 100 µg glu eq ml^{-1} was low. This suggests that active fragment of crude HMWCEP were eliminated during enzymic hydrolysis and passing through membrane of 10,000 cut off. Second possibility is that fragment eluted through 10,000 membrane aggregated in different manner and lost the activity to the similar extent of browning as exhibited by the crude samples. In literature, the elicitor activity of crude and hydrolysed products in various plant pathogen interaction were determined by the classical methods of recording the induced browning (Theodorou and Smith 1979) and phytoalexin production (Johal and Rahe 1990; Anderson *et al* 1991).

Conclusion

These results suggest that polysaccharide under investigation were found to be quite heterogenous and may be composed of some glucan chains with 1, 3 and 1, 4-β-linkages. On the other hand driselase from *Basidiomycetes* showed a higher degradation (about 20%), indicating the existence of more than one type of linkages and complex monosaccharide composition.

Acknowledgement. Thanks to Paul Finch, my Supervisor for his invaluable guidance, and Ministry of Science and Technology of the Government of Pakistan for their financial grant.

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