Polygalacturonase and xylanase forms produced by *Fusarium avenaceum* in infected lupin roots and their sensitivity to a cell wall glycoprotein

**URSZULA MAŁOLEPSZA, ELŻBIETA KUŹNIAK, JACEK PATYKOWSKI,
HENRYK URBANEK**

Department of Plant Physiology and Biochemistry, University of Łódź
Banacha 12/16, 90-237 Łódź, Poland

(Received: June 06, 1995)

A b s t r a c t

Using CM-Sepharose column chromatography it was shown that *Fusarium avenaceum* produced two forms of endo-polygalacturonase, one exo-polygalacturonase and two forms of endo-xylanase in infected lupin roots. A glycoprotein obtained from lupin seedlings inhibited endo-polygalacturonases, exo-polygalacturonases and xylanases but more the first ones. Two forms of endo-polygalacturonase did not differ in their sensitivity to the glycoprotein.

INTRODUCTION

Glycoproteins inhibiting polygalacturonase of phytopathogenic fungi were isolated from some plants. It was reported that fungi secrete multiple polygalacturonase isoenzymes that could be involved in phytopathogenic processes (D i L e n n a and F i e l d i n g, 1983; M a r c i a n o et al., 1982; S c a l a et al., 1980). A comparison of the sensitivity of particular isoenzymatic forms to the inactivation by the proteinaceous polygalacturonase inhibitors seems to be interesting for explanation of plant-pathogen interactions (A b u - G o u k h and L a b a v i t c h, 1983; B r o w n, 1984; D e L o r e n z o et al., 1990; T u r n e r and H o f f m a n, 1985).

In the preliminary experiments we detected a glycoprotein in cell walls of lupin seedlings, which inhibits not only polygalacturonase but also xylanase activity.

The purpose of this work was to determine whether *Fusarium avenaceum* produced different molecular forms of polygalacturonase and xylanase during infection of lupin and whether they differed from one another in the sensitivity to inhibition by the glycoprotein obtained from lupin seedlings.

MATERIAL AND METHODS

A virulent *Fusarium avenaceum* isolate was cultured from diseased lupin seedlings. The stock culture of this isolate was maintained on potato dextrose agar (PDA).

Infection of lupin roots. From 12-day-old seedlings of lupin cv. Cyd roots were cut and treated with the spore suspension of *F. avenaceum* containing 2×10^7 spores in 1 ml for 24 h. The control was roots treated with the autoclaved spores at the same concentration. After treatment, roots carefully washed with distilled water were transferred on Petri dishes containing filter paper strongly moistened with water and stored at room temperature. After 6, 9, 12, and 15 days samples of diseased roots were homogenized in 0.1 M veronal buffer at pH 5.0, centrifuged out and dialyzed against the same buffer for 24 h. The obtained dialysate was assayed for polygalacturonase and xylanase activities.

To obtain spores necessary to infection of roots, the isolate of *F. avenaceum* was grown on a shaker for 6 days at 30°C on a medium containing in 1 l of water: 30 g saccharose, 3 g of NaNO_3 , 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ and 0.5 g of KCl.

Culture of *F. avenaceum* on homogenized lupin roots. The fungus was grown with shaking at 26°C on a medium containing 80 g of homogenized lupin roots in 1 liter of water. The cultures were made in 1000 ml Erlenmayer flasks containing 95 ml of medium and 5 ml of inoculum which was a spore suspension obtained by addition of sterile water to 10-day-old slant of *F. avenaceum*. After 4 days of growth the flask content was centrifuged out and the culture supernatant dialyzed against 0.01 M veronal buffer, pH 5.0 was used to fractionation of polygalacturonase and xylanase.

Fractionation of xylanase and polygalacturonase. Ammonium sulphate was added to the extract from infected tissue obtained after 12 days of *F. avenaceum* growth on lupin roots and to the 4-day-old culture on homogenized roots. After standing overnight the resulting precipitates were centrifuged, dissolved in 0.01 M NaCl, and the solutions were dialyzed against 0.01 M veronal buffer pH 5.0. The dialysates were applied to a CM-Sephacrose column, which was eluted with a linear gradient of 0-0.8 M NaCl in the above buffer. All operations were carried out at 4°C. The fractions eluted from the column were examined for polygalacturonase and xylanase activities.

Enzyme assays. The activities of polygalacturonase and xylanase were assayed according to Nelson method as described earlier (Zalewska-Sobczak, 1985). The substrate for xylanase was xylan (Koch-Light) and for polygalacturonase – sodium polypectate (Sunkist Growers Inc. USA).

Paper chromatography. Hydrolysis products released by xylanase and polygalacturonase after 12 h of incubation were examined as described earlier (Urbanek et al., 1978).

Isolation of glycoprotein inhibitor from lupin. 10 g of 6-day-old seedlings of lupin were cut into 5 mm segments and homogenized for 5 min with 50 ml of distilled water. The homogenate was centrifuged out, the residue was washed twice with distilled water and then extracted with 0.5 M NaCl for 1 h at room temperature. The extract was centrifuged out and the supernatant was dialyzed against 0.01 M acetate buffer, pH 5.2. The dialysate was applied to a CM-Sephadex C-50 column that was washed with the above buffer and then eluted with the linear gradient of 0-1 M NaCl. The 5 ml fractions were collected and assayed for protein and total carbohydrate contents.

Protein assay. Protein concentrations in the fractions obtained after CM-Sephadex chromatography of glycoprotein inhibitor were determined by the method of Lowry et al. (1951).

Carbohydrate assays. Total carbohydrate in the fractions obtained after CM-Sephadex chromatography of glycoprotein inhibitor was measured according to Dubois et al. (1956).

The effect of lupin glycoprotein fractions on polygalacturonase and xylanase activities. Fractions obtained from CM-Sephadex containing protein and carbohydrate were examined with the respect of being capable of inhibition of polygalacturonase and xylanase activities. Polygalacturonase and xylanase isoenzymatic forms obtained after CM-Sephadex chromatography were pretreated with glycoprotein fractions from CM-Sephadex for 30 min at 30°C and then the remaining activity was assayed.

RESULTS AND DISCUSSION

The first symptoms of root rotting began to be visible on the 9th day after inoculation with the fungus conidia. Only on that day the noticeable increase of xylanase and polygalacturonase activities in the infected tissue was observed (Fig. 1). The activities of these two enzymes rapidly increased until the 12th day and further on they maintained more or less the same level. For the fractionation we used the enzymes from 12-day-old infected roots. To establish host or pathogen origin of the enzymes, those from the infected tissue were fractionated parallelly with the preparation from the culture on homogenized and autoclaved roots.

From the rotten tissue there were obtained: one unadsorbed and three adsorbed on CM-Sephadex peaks of polygalacturonase (Fig 2). These adsorbed peaks

obtained from the culture corresponded to the peaks from the infected tissue. In the case of xylanase, from the diseased tissue there were obtained one unadsorbed and two adsorbed peaks whereas from the culture the number of the adsorbed peaks amounted to three (Fig. 3). Peak II and III from the culture corresponded to peaks II and III from infected tissue.

On the basis of the above results we assumed that fractions II, III and IV of polygalacturonase and fractions II and III of xylanase from diseased tissue were produced by the fungus.

Fig. 1. The secretion of polygalacturonase and xylanase during growth of *Fusarium avenaceum* on lupin roots

Fig. 2. CM-Sepharose column chromatography of polygalacturonase from the infected lupin roots and from the culture on homogenized lupin roots

Fig. 3. CM-Sepharose column chromatography of xylanases from the infected lupin roots and from the culture on homogenized lupin roots

Fig. 4. Chromatography of lupin glycoprotein fractions on CM-Sephadex C-50

Chromatographic analysis of enzymatic hydrolysis of substrates showed that polygalacturonases II and III as well as xylanases II and III were endo-enzymes whereas polygalacturonase IV was an exo-enzyme. These three forms of polygalacturonase and two forms of xylanase were used to study the effect of the glycoprotein fractions obtained from lupin seedlings on their activities.

The pattern of glycoprotein separation on CM-Sephadex column is shown on Fig. 4. Fraction IV of four fractions containing protein and sugar, named glycoprotein, behaved similarly to polygalacturonase inhibitor isolated from bean by A l b e r s h e i m and A n d e r s o n (1971). It inactivated both forms of endopolygalacturonase of *F. avenaceum* equally over 70 % while ratio of enzyme protein to inhibitor protein was 1:3 (Table 1). In the same conditions it also lowered the activities of exo-polygalacturonase and endo-xylanases but only to the extent of 19-28 %. Thus, it was less specific than proteinaceous polygalacturonase inhibitor from bean or pea which inactivated only endo-polygalacturonases (D e L o r e n z o et al., 1990; S c h a r r o c k and L a b a v i t c h, 1994). Recently S h a r r o c k and L a b a v i t c h (1994) have demonstrated that inhibitor from pear showed varied inhibition of the *B. cinerea* polygalacturonase isozymes. The results of our work suggest that proteinaceous inhibitor from lupin may be involved in defence reaction by nonspecific binding and inactivation of different hydrolytic enzymes of pathogen.

Table 1

The effect of lupin glycoprotein fraction IV on polygalacturonase and xylanase activities

Enzyme	Activity (A_{660})		% of inhibition
	Without inhibitor	With inhibitor	
Endo-polygalacturonase (fraction II)	0.718	0.200	72
Endo-polygalacturonase (fraction III)	0.610	0.146	76
Exo-polygalacturonase (fraction IV)	0.737	0.553	25
Endo-xylanase (fraction III)	0.640	0.463	28
Endo-xylanase (fraction III)	0.585	0.474	19

This work was financially supported by University of Łódź (grant No 505/726)

REFERENCES

- A b u - G o u k h A. A., L a b a v i t c h J. M., 1983. The *in vivo* role of pear fruit endopolygalacturonase inhibitor. *Physiol. Plant Pathol.* 23: 123-135.
- A l b e r s h e i m P., A n d e r s o n A. J., 1971. Proteins from plant cells walls inhibit polygalacturonases secreted by plant pathogens. *Proc. Nat. Acad. Sci. USA* 68: 1815-1819.
- B r o w n A. E., 1984. Relationship of endopolygalacturonase inhibitor activity to the rate of fungal rot development in apple fruits. *Phytopath. Z.* 111: 122-132.

- De Lorenzo G., Ito Y., D' Ovidio R., Cervone F., Albersheim P., Darvill A. G., 1990. Host-pathogen interactions. XXXVII. Abilities of the polygalacturonase – inhibiting proteins from four cultivars of *Phaseolus vulgaris* to inhibit the endopolygalacturonases from three races of *Colletotrichum lindemuthianum*. *Physiol. Mol. Plant Pathol.* 36: 421-435.
- Di Lenna P., Fielding A.H., 1983. Multiple forms of polygalacturonase in apple and carrot tissue infected by isolates of *Botrytis cinerea*. *J. Gen. Microbiol.* 129: 3015-3018.
- Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A., Smith F., 1956. Colorimetric method for determination of sugars and related substances. *Analyt. Chem.* 28: 350-356.
- Lowry D. H., Rosenbrough N. I., Farr A. L., Randall R. J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Marciano P., Di Lenna P., Magro P., 1982. Polygalacturonase isoenzymes produced by *Sclerotinia sclerotiorum* *in vivo* and *in vitro*. *Physiol. Plant Pathol.* 20:201-212.
- Scala A., Camardella L., Scala F., Cervone F., 1980. Multiple forms of polygalacturonase in two strains of *Rhizoctonia solani*. *J. Gen. Microbiol.* 116: 207-211.
- Sharrock K. R., Labavitch J. M., 1994. Polygalacturonase inhibitors of Bartlett pear fruits: differential effects on *Botrytis cinerea* polygalacturonase isozymes, and influence on products of fungal hydrolysis of pear cell walls and on ethylene induction in cell culture. *Physiol. Mol. Plant Pathol.* 45: 305-319.
- Turner J. S., Hoffman R. M., 1985. Effect of polygalacturonase inhibitor from pea on the hydrolysis of pea cell walls by the endopolygalacturonase from *Ascochyta pisi*. *Plant Pathol.* 34: 54-60.
- Urbanek H., Zalewska-Sobczak J., Borowińska A., 1978. Isolation and properties of extracellular cellulase-hemicellulase complex of *Phoma hibernica*. *Arch. Microbiol.* 118: 265-269.
- Zalewska-Sobczak J., 1985. Sequential secretion of cell wall degrading enzymes by *Botrytis fabae* and *Fusarium avenaceum* during growth on host and non-host plants. *Biochem. Physiol. Pflanzen* 180: 169-175.

Formy molekularne poligalakturonazy i ksylanazy wytwarzane przez *Fusarium avenaceum* w zainfekowanych korzeniach łubinu i ich wrażliwość na glikoproteid ścian komórkowych

Streszczenie

Wykazano, że *Fusarium avenaceum* wytwarza dwie formy endo-poligalakturonazy, jedną egzo-poligalakturonazę oraz dwie formy endo-ksylanazy w zainfekowanych korzeniach łubinu.

Z siewek łubinu wyizolowano glikoproteid, który hamował endo-poligalakturonazy i w mniejszym stopniu egzo-poligalakturonazę i ksylanazy. Otrzymane formy endo-poligalakturonazy nie różniły się w ich wrażliwości na glikoproteid.

Wydaje się, że otrzymany glikoproteid może brać udział w reakcjach obronnych łubinu na *F. avenaceum* przez niespecyficzne hamowanie i inaktywację enzymów tego patogena.