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PECTINASE FROM *Phomopsis helianthi* – THE AGENT OF SUNFLOWER STEM CANKER

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SUMMARY

Phomopsis (Diaporthe) helianthi Munt.-Cvet. et al., is an important phytopathogen which causes stem canker of sunflower. When grown in sumberged culture this phytopathogen excretes extracellular pectinase. Production of constitutive exo-pectinease by this fungus grown on glucose was established, while the endo-pectinase and pectinesterase activities could be detected only in the presence of pectin in the medium. Temperature optimum of endo- and exo-pectinases were obtained at 50°C. pH dependence of crude extracellular endo-pectinase activity showed two maxima, at pH 4.5 and 5.7, measured at the optimum temperature (50°C). Maximum activity for exo-pectinase was observed at pH 5.5 (at 50°C), and for pectinesterase at pH 5.0 (20°C).

Key words: ***Phomopsis helianthi*, sunflower, pectinase.**

INTRODUCTION

Phomopsis helianthi (perfect stage, *Diaporthe helianthi*) is a fungal pathogen causing leaf necrosis and stem canker of sunflower (Muntañola-Cvetković et al., 1981). The disease of sunflowers caused by the holomorph *Diaporthe helianthi – Phomopsis helianthi* was first noticed in Yugoslavia in July 1980 (Mihaljčević et al., 1983), Hungary (Vörös et al., 1983) and France (Lamarque and Perny, 1985).

The leaf-petiole-stem route of sunflower invasion by the fungus was established (Muntañola-Cvetković et al., 1991). According to the histological studies by the same authors, after penetration into the sunflower, the infection hyphae invade the intercellular spaces and spread toward the midrib and the petiole. Xylem elements are less affected but the phloem and parenchyma tissues are distintegrated completely. The destructive effect of phytopathogenic plant fungus is often caused by enzymes and phytotoxins (Cervone et al., 1986; Chan and Sackton, 1970a, 1970b, 1972).

Phomopsis helianthi excretes a toxic substance named phomozin, which could be responsible for the disease of plants infected by this fungus (Mazars et al., 1990, 1991). An important role in cell degradation belongs to the group of enzimes named cell wall degrading enzimes, CWDE (Wood, 1967). The main enzymes belonging to this group are pectinolytic enzymes (Bateman et al., 1976), which act first on cell wall polysaccharides, enabling other enzymes to attack other substrates (Karr et al., 1970; Jones et al., 1972).

Phomopsis (Diaporthe) helianthi Munt.-Cvet. et al., is a new causative agent whose mechanism of pathogenesis is insufficiently known. As far as we know, there is no evidence about the capability of this fungus to produce enzymes belonging to CWDE.

The aim of this work was to study the ability of this phytopathogenic fungus to produce extracellular pectinases in submerged culture.

MATERIAL AND METHODS

Microorganism

Phomopsis (Diaporthe) helianthi Munt.-Cvet. et al., was kindly supplied by Dr. M. Mihaljčević. This microorganism was isolated from infected sunflower plants grown on the fields of Vojvodina (Yugoslavia), and maintained on potato-dextrose agar (PDA) in petri dishes.

Media and fermentation procedure

The basal medium containing $(NH_4)_2SO_4$, 2.0 g/l and K_2HPO_4, 2.0 g/l, was supplemented with the chosen carbon source and 0.3% of yeast extract. Apple pectin green band (Obi Pectin, Switzerland), or glucose (Sigma Chemical Co., USA) at 0.5% were used as carbon source. All reagents were of analytical reagent grade. Sterilization was carried out at 121°C and 15 psi for 20 minutes. The initial pH in all experiments was 4.5.

The shake glask inoculum was prepared in three different manners.

In the first procedure (MPDA-disks) carried ont according to Keen and Horton (1966), there disks (0.89 cm^2), were cut from the PDA in petri dishes and transferred to 100 ml of the basal medium supplemented with the appropriate source of carbon.

The second procedure (MS-inoc.) consisted of the preparation of mycelial suspension by swirling the PDA plates with 7 day old mycelia with 10 ml of sterile distilled water. This suspension was used to inoculate 100 ml of basal medium supplemented with the appropriate carbon source.

For the thirid procedure, involving adapted mycelia inoculation (AMI), the mycelial suspension was prepared in the same way as for the second one. The pre-culture flasks containing basal medium with 0.5% green pectin as the sole carbon source were inoculated with the mycelial suspension (10 ml suspension/100 ml medium). The flasks were incubated in a shaker (200 rpm) at 30°C for seven days before 10 ml aliquotos were withdrawn to serve as the inoculum in the fermentation experiments using 100 ml of the basal medium supplemented with the appropriate carbon source.

Fermentation experiments (300 ml flask/100 ml medium) were carried out at 30°C, using a rotatory shaker (200 rpm). In order to avoid difficulties in the withdrawal of samples during the process, a sufficient number of flasks was used and total content of two of them was taken for evalution at the desired time intervals.

The medium containing extracellular enzymes was separated from the fungus cells by centrifugation (17.000xg, 10 min) and stored at 4°C (crude enzymes).

Pectinolytic activity assays

Exo-pectinase activity was measured according to Aguilar and Huitron (1990); one unit of exo-activity was defined as that amount of the enzyme which catalyzed the formation of 1 μmol of galacturonic acid per hour at pH 5.0.

Endo-pectinase activity was determined according to Peričin et al., (1992a), by measuring the decrease of the specific viscosity of the reaction mixture (0.25% apple pectin red ribbon in o.1 mol/l citrate buffer pH 4.5) in a Micro-KPG-Ubbehlohed viscosimeter at 30°C. One unit was defined as the amount of the enzyme which reduced the viscosity of the reaction mixture by 25% in 1 min.

Pectinesterase (PE) activity was determined titrimetrically by measuring the amount of carboxyl groups released from the methyl ester bound in 1% green band pectin at pH 4.5 and 20°C.

pH and temperature optima

Crude enzymes obtained by fermentation on pectin using AMI inoculation were used for determination of pH and temperature optima.

The optimum temperature of endo- and exo-pectinolytic activity was determined by incubating the crude enzymes in a water bath at various temperatures, and measuring the initial velocity of the enzyme catalysis.

The determination of pH optimum was conduced at the optimum temperature of the respective enzymes, except for PE which was measured at 20°C. Buffers (pH 4.0 to 7.0) were prepared using 0.2 mol/l Na_2HPO_4 and 0.1 mol/l citric acid.

Analytical methods

Reducing groups content was determined by the DNS-method (Miller, 1959). Glucose content was measured enzymatically (Bergmayer, 1965).

Biomass production was measured as dry weight. After centrifugation, pellets were washed with 0.1 mol/l NaCl and dried at 100°C to constant weight.

RESULTS

First procedure of inoculation

Secretion of exo-pectinase exhibited the one- and two-phase kinetics, depending on the carbon source used. In the presence of glucose as the sole source of carbon, one-phase secretion was observed and the a maximum activity (6.62 U/ml) was reached the third day after the inoculation. The enzyme secretion in the medium containing pectin exhibited the two-phase kinetics, the first maximum activity (5.12 U/ml) occuring the first day after inoculation, and the second one (4.52 U/ml) three days later.

During fermentation in the presence of glucose, small increases in the glucose content were observed the first and the second day of fermentation (6.1 and 5.9 mg/ml, respectively), and after that, a decrease was noticed at the same time when the maximum of secreted enzyme activity was reached.

The enzyme secretion showed an opposite correlation with the accumulation of the reducing groups in the fermentation experiments with pectin as the sole source of carbon.

During fermentation, pH increased from 4.8 to 5.6 and, when the maximum activity of the enzymes was reached, the pH of the medium in the presence of glucose was 5.3. On the other hand, in the presence of pectin as the carbon source, pH showed a decrease during fermentation reaching a value of 4.5 at the end of the process (Figure 1).

No endo-pectinase and pectinesterase activities could be detected in the fermentation experiments with this type of inoculation.

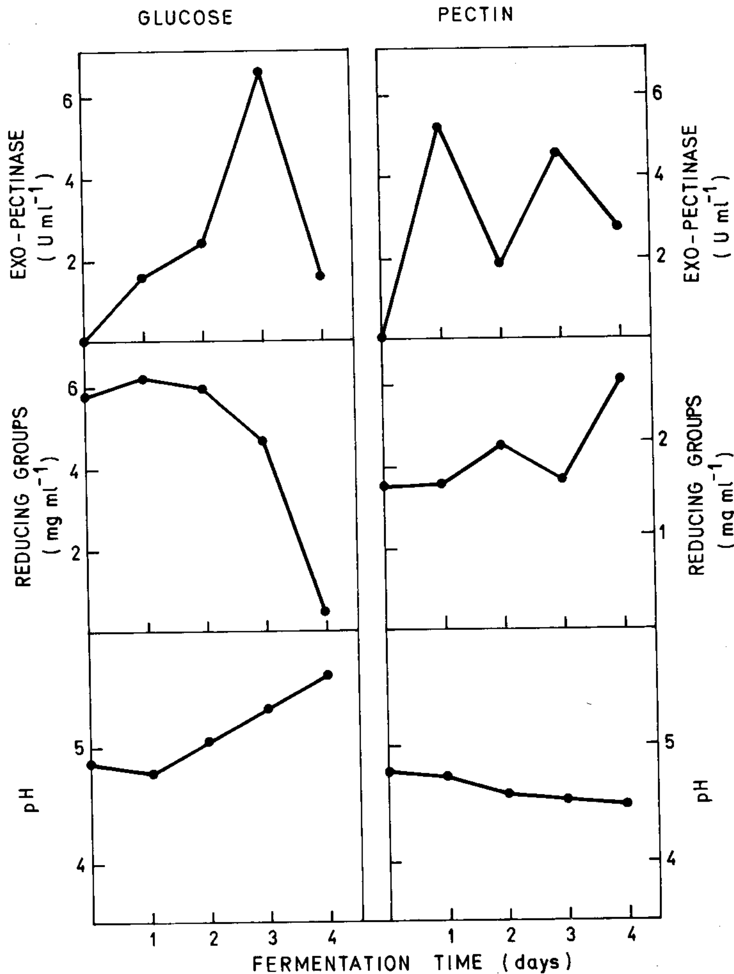

Figure 1. Comparison of exo-pectinase activity, reducing sugar content and pH during submerged fermentation of Phomopsis helianthi in the presence of glusoce or pectin. (MPDA-disk inoculation; carbon source 0.5% glucose or 0.5% pectin, initial pH of the medium: pH 4.7 – 4.8).

Second procedure of inoculation

Inoculation with mycelial suspension only (without PDA discs) ensured conditions for the secretion of a larger number of enzymes of the pectinolytic complex. Under these conditions of inoculation the fungus produced both exo-pectinolytic enzymes (detected also in the preceding inoculation procedure), and endo-pectinolytic enzymes, as well as pectinesterase.

The production of extracellular enzymes reached its maximum on the seventh and eighth day of fermentation. The content of reducing groups (0.10 – 0.16 mg/ml) was lower than in the preceding way of inoculation (Table 1).

Table 1. Enzyme production of *Phomopsis (Diaporthe) helianthi* cultured in the presence of green pectin[1]

Ferment. time	pH	Reduc. group	Pectinase					
			Endo-type		Exo-type		PE	
days		mg/ml	U/ml	U/mg	U/ml	U/mg	U/ml	U/mg
7	6.60	0.16	0.07	0.14	15.0	31.25	0.050	0.104
8	6.97	0.10	0.04	0.08	9.19	19.55	0.035	0.070

[1]The fungus was grown in liquid submerged culture of 0.5% green pectin.
Initial pH of the culturs was 4.5. Inoculation with mycelial suspension.

Third procedure of inoculation

The inoculation conditions employed in this procedure yielded an increased rate of pectinolytic enzymes secretion during fermentation (Figure 2). After two days of fermentation, the activity of endo-pectinase reached its maximum (0.08 U/ml), and this activity level was preserved for three days, after which a slight decrease was observed. The exo-pectinase secretion exibited two-phase kinetics and the maximum activity was reached after three and five days upon inoculation (33 and 25 U/ml, respectively). The maximum pectinesterase activity (0.06 U/ml) was also reached after three days of fermentation.

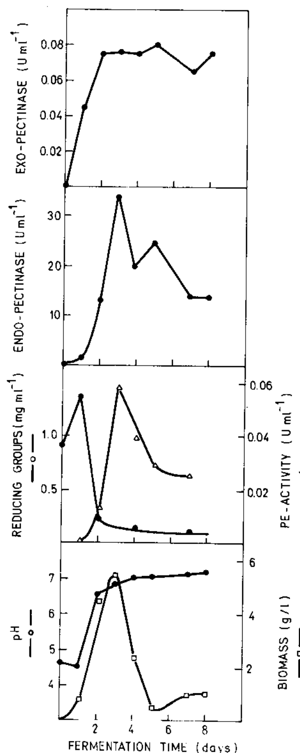

Figure 2.
Time course of mycelar biomass, reducing sugar content, pH and activity of endo-pectinase, exo-pectinaseand PE during submerged fermentation of Phomopsis helianthi (AMI – inoculation, carbon source: 0.5% green pectin).

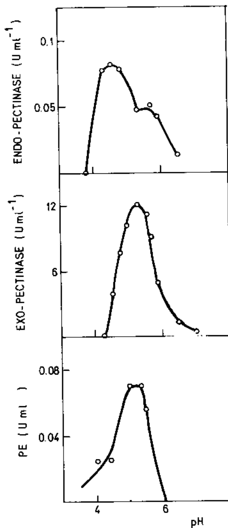

Figure 3.
pH optima of endo- and exo-pectinase, and PE of Phomopsis helianthi. Initial velocity of the enzyme catalyzed reaction was measured. Reaction mixture was buffered with 0.2 mol/l Na_2HPO_4 and 0.1 mol/l citric acid and incubated at 50°C for endo- and exo-pectinase, and at 20°C for PE.

The growing of the fungus in the presence of highly-esterified pectin resulted in a degradation of pectin, which was evident from the accumulation of reducing groups. The accumulation (1.38 mg/ml) was noticed on the first day of fermentation. This accumulation of reducing groups was in good correlation with the beginning of endo-pectinase secretion.

The secretion of exo-pectinase and pectinesterase began one day later than that of endo-pectinase, and after that the content of accumulated reducing groups showed a decrease.

Under these conditions of inoculation and fermentation, the maximum biomass content was reached after three days of fermentation. The secretion of pectinesterase was

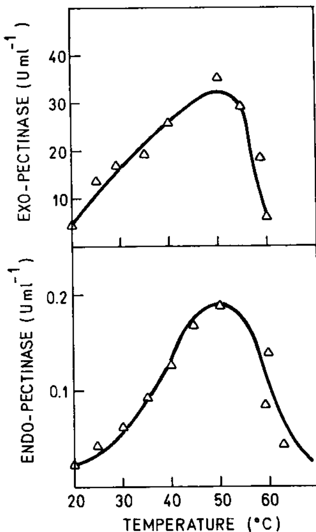

Fig. 4. Dependence of endo- and exo-pectinase activity from Phomopsis helianthi on temperature. Initial velocity of the enzyme catalyzed reaction was measured. Reaction mixture was buffered with 0.1 mol/l citric acid, pH 4.5, for endo-pectinase, and 0.1 mol/l acetic acid buffer, pH 5.0, for exo-pectinase.

associated with the fungus growth, which was not true for the secretion of endo-pectinase and exo-pectinase. During fermentation, pH increased from 4.5 to 7.0, and this value remained constant to the end of fermentation.

Temperature and pH characteristics of extracellular pectinases

The pH dependece of endo-pectinase activity showed two peaks, at pH 4.5 and 5.0. On the other hand, the activity of exo-pectinase and pectinesterase showed only one peak, at pH 5.0 (Figure 3).

The temperature dependence of both exo- and endo-pectinase activity showed a maximum at 50°C (Figure 4).

DISCUSSION

According to the results presented above, the fungus *Phomopsis helianthi* under the submerged conditions of fermentation can secrete extracellular pectinases (Figures 1 and 2 and Table 1), i.e., endo- and exo-pectinases and pectinesterase.

The extracellular pectinase production was dependent of the carbon sources used in the fermentation experiments and the manner of inoculation. The secretion of exo-pectinases by the fungus was detected in all three procedures of inoculation, while the secretion of endo-pectinases and pectinesterase was observed only in the presence of pectin.

In the first procedure of inoculation, which is frequently used for the inoculation of sunflower plants (Tourvielle de Labrouhe et al., 1988), the fungus grew intensively (data not shown) and secreted exo-pectinase in the presence of both carbon sources examined (0.5% glucose and 0.5% highly-esterified pectin). In the beginning, the fungus used primarily carbon from PDA disks. Three days after inoculation, the fungus started to use the added carbon source (glucose or pectin) which was registered as a decrease in glucose content. The exo-pectinase production under these conditions suggests that this enzyme from *Phomopsis helianthi* is a constitutive enzyme. Constitutive exo-pectinases were also found in other fungus species (Aguilar and Huitron, 1990).

The sequence of carbon source utilization, as mentioned above, is probably the cause of the two-phase kinetics of the exo-pectinase secretion. The increased contents of reducing groups observed on the second and fourth day of fermentation suggest accumulation of pectin degradation products caused by the action of exo-pectinase, so that they could not be utilized so fast by the fungus. Similar results were also obtained with another phytopathogenic fungus (Keen and Horton, 1966).

The presence of 0.5% pectin in the medium did not induce secretion of endo-pectinase and pectinesterase by this inoculation procedure during the examined fermentation time.

When the inoculation was carried out by the second procedure (inoculation by suspension of mycelia, without PDA discs), the fungus secreted both endo- and exo-pectinase and pectinesterase (Table 1).

On the other hand, when inoculated by the third procedure, the examined enzymes were present in the medium and they exhibited an approximately maximum activity on the third day after inoculation. The exo- and endo-pectinase secretion was not associated

with the growth of the fungus, whereas the PE secretion did follow its growth. A similar effect of reducing groups accumulation, as for the MPDA-disk inoculation, **was noticed but now on the first day of fermentation.** The accumulated pectin degradation products are utilized by the fungus very fast so that the reducing sugar content rapidly falls to 0.1 mg/ml. This rapid utilization of reducing groups was probably caused by the presence of all enzymes of the pectinolytic complex (Figure 2).

The virulence of some pathogenic microorganisms under natural conditions may be dependent on catabolic repression by glucose and other carbohydrates present in the host plant (Horton and Keen, 1966; Keen and Horth, 1966; Brown et al., 1992). The endo-pectinase repression observed under the experimental conditions of inoculation by PDA disks could be caused by the sugar concentrations in the medium which were higher than the physiological glucose concentration in sunflower. Hence, it could be supposed that the plant inoculation by mycelia on PDA discs can retard infection, especially if the pectinolytic enzymes play a key role in pathogenesis.

Two pH optima for endo-pectinase, at pH 4.5 and 5.7, suggests that this enzyme could occur in two isoenzymatic forms. Multiple forms of endo- and exo-pectinases are frequently present in microorganisms and have been previously reported for other organisms (Peričin et al., 1992b). The exo-type enzyme exibited only one pH optimum (pH 5.5). The obtained pH optimum is almost by one pH unit higher than those usually found for this kind of enzymes. Souch high pH optima were also obtained for pectinases from *Venturia inequalis* (Valssangacomo and Gessler, 1992) and were also found for other pathogenic microorganisms (Wood, 1967).

The pH optimum obtained for pectinesterase (5.0 – 5.5) is only slightly higher than the usual value for this enzyme in the other microorganisms (Wood, 1967).

The temperature dependence of exo- and endo-pectinase showed the optima which are also usual for the other well-known microorganisms (Godfrey and Reichelt, 1986).

The role of microbial pectinases in pathogenesis was studied for a great number of plant diseases caused by different microorganisms as: *Verticilium alboatrum* (Durrands and Cooper, 1988), *Sclerotium bataticola* (Chan and Sackston, 1970a, 1970b, 1972), *Venturia inaqualis* (Valssangacomo and Gessler, 1992), etc. The mechanisms of action of these enzymes in biotrophic pathogenesis are still unknown, especially for diseases caused by *Venturia inaqualis* and *Phomopsis (Diaporthe) helianthi.*

The above information about the capability of the pathogenic fungus *Phomopsis (Diaporthe) helianthi* to produce pectinolytic enzymes and their characterization could contribute to a better understanding of the mechanism of patogenic action of this fungus via CWDE. However, a synergetic action of CWDE and phytotoxins (Mazars et al., 1990,1991) could not be excluded.

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PECTINASA DE *Phomosis helianthi* – EL AGENTE DEL CANCRO DEL TALLO

RESUMEN

Phomopsis (Diaporthe) helianthi Munt-Cvet et al., es un imortante fitopatógeno que causa el cancro del tallo del girasol. Cuando crecen cultivos inundados este fitopatólogeno excreta pectinansas extracelulares. La producción de la expectinasas constitutiva por este hongo crecido sobre glucosa fue establecida mientras las actividades de las endopectinasas y pectinestresas podria ser detectada solo en la presencia de la pectina en el medio. La temperatura optima de endo y ectopectinasas fueron obtenidas a 50°C. La dependencia del pH de la actividad extracelular de endopectinasas mostro dos màximos a pH 4.5 y 5.7 medidos a la temperatura optima de 50°C. La màxima actividad para exopectinasas fue observada a pH 5.5 a 50°C y para pectinerasa a pH 5 y 20°C.

PECTINASE DE *Phomopsis helianthi*, AGENT DU CHANERE DE LA TIGE DU TOURNESOL

RÉSUMÉ

Phomopsis (Diaporthe) helianthi Munt.-Cvet. et al., est un important pathogène responsable la nécrose des tiges de tournesol. Quand il est cultivé en milieu liquide, ce parasite excréte une pectinase extracellulaire. La production d'exo-pectinase par ce champignon a été établie sur milieu glucosé alors que les activités endo-pectinasiques et pectinesterasiques n'ont été mises en évidence qu'en présence de pectine dans le milieu. La température optimale des activités endo- et exo- pectinasiques est de 50°C. Le pH lié à l'activité de l'endopectinase d'extrait brut extracellulaire présente deux maxima à 4.5 et 5.7 mesurés à température optimale (50°C). Le maximum d'activité pour l'exopectinase a été enregistré pour un pH 5.5 (à 50°C) et pour pectinestérase pour un pH 5.0 (20°C).