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CHARACTERIZATION OF THE EXTRACELLULAR ENZYME SYSTEMS OF *TRICHODERMA VIRIDE* AH124*

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*Dedicated to the 70th birthday of Prof. Lajos Ferenczy

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Running title: Extracellular enzyme systems of *T. viride*

A mycoparasitic *Trichoderma viride* strain was investigated for the production of extracellular enzymes important in antagonism by the use of natural, stained-natural and chromogenic substrates. Some of these enzymes, such as β-1,3-glucanases and low levels of proteases were produced constitutively. Under inductive conditions the measurable activities of β-1,3-glucanase, protease and aspecific chitinase enzimes were increased, while in the case of proteases and β-1,3-glucanases the levels were depend both on nitrogen and carbon source. By gel filtration chromatography at least four β-1,3-glucanases, six proteases, two β-glucosidases and one β-1,4-*N*-acetyl-glucosaminidase izoenzyme was detected, under inductive conditions.

Key words: *Trichoderma viride,* extracellular enzymes, regulation

INTRODUCTION

The filamentous fungus *Trichoderma viride* is well known to produce a lot of different extracellular enzymes. Some of these enzymes take part in the decomposition of plant litter and are responsible for the survival of the strains in the soil [13], while others play a role in the degradation of fungal cell walls [14]. The qualitiy and amount of these latter enzymes are important in the biofungicid efficacy of the strains, determining their mycoparasitic abilities - one of the components of their antagonistic properties. The role of β-1,3-glucanases, chitinases and proteases in mycoparasitism was proved in *Trichoderma harzianum* [3].

The aim of this work was to examine the extracellular enzyme system of *T. viride* strain AH124, a strain with the ability to effectively antagonize the filamentous fungus *Fusarium culmorum,* which causes large damages at the germination of maize plants.

MATERIALS AND METHODS

Strains and culture media

The *T. viride* strain AH124 was isolated by Manczinger et al. from the soil of the forest of Ásotthalom, southern Hungary. It was characterized as a strong antagonist against *Fusarium culmorum* (unpublished results). This strain was maintained on minimal medium [11] at 25°**Hiba! A hivatkozási forrás nem található.**C.

The inductive and non-inductive liquid media for the investigation of enzyme production were as follows: T1 (20 g/l glucose, 5 g/l NH_4Cl, 1 g/l KH_2PO_4, 1 g/l $MgSO_4$×**Hiba! A hivatkozási forrás nem található.**7 H_2O); T1F (T1 supplemented with 20 g/l dried *Fusarium culmorum* hyphae); T2 (20 g/l glucose, 5 g/l $NaNO_3$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4$×**Hiba! A hivatkozási forrás nem található.**7 H_2O); T2F (T2 supplemented with 20 g/l dried *F. culmorum* hyphae); T3F (20 g/l dried *Fusarium culmorum* hyphae). Production of *F. culmorum* hyphae: Conidia were inoculated into 100 ml liquid YEGK medium (5 g/l yeast extract, 10 g/l glucose, 5 g/l KH_2PO_4) and the incubation was carried out in 500 ml Erlenmeyer flasks on a rotary shaker at 180 rpm and 25°**Hiba! A hivatkozási forrás nem található.**C. After 3 days the mycelia were collected by filtration, washed with distilled water and dried under vacuum. T4 (5 g/l mannitol, 5 g/l $NaNO_3$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4$×**Hiba! A hivatkozási forrás nem található.**7 H_2O); T5 (5 g/l mannitol, 5 g/l NH_4Cl, 1 g/l KH_2PO_4, 1 g/l $MgSO_4$× **Hiba! A hivatkozási forrás nem található.**7 H_2O); T6 (10 g/l glycerol, 5 g/l $NaNO_3$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4$×**Hiba! A hivatkozási forrás nem található.**7 H_2O); T7 (10 g/l glycerol, 5 g/l NH_4Cl, 1 g/l KH_2PO_4, 1 g/l $MgSO_4$×**Hiba! A hivatkozási forrás nem található.**7 H_2O). For induction, T4, T5, T6 and T7 media were supplemented with 0.5 g/l laminarin, 0.5 g/l colloidal chitin and 0.5 g/l gelatine respectively.

Preparation of supernatants for enzyme assays

Conidia of strain AH124 were inoculated into the appropriate inductive or non-inductive liquid media. The incubation was carried out in 50 ml Erlenmeyer flasks on a rotary shaker at 150 rpm. After culturing at 25°C the mycelial pellets were removed by

centrifugation (3000**Hiba! A hivatkozási forrás nem található.**g for 10 min) and enzyme activities measured in the supernatant.

Spectrophotometrical measurement of extracellular enzyme activities

The weight of hyphae and protein content was determined on the basis of organically bound phosphorus content [7] and by the method of Spector [16] respectively. For measurement of aspecific protease, specific trypsin-like and chymotrypsin-like protease activities, azocasein [9], *N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide [5] and *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide [1] were used respectively. The β**Hiba! A hivatkozási forrás nem található.**-1,3-glucanase activities were detected by using the dinitrosalicylic acid method [18], with laminarin as substrate. Aspecific chitinase **Hiba! A hivatkozási forrás nem található.**activity was assayed by the method of Rodriguez-Kaban et al. [15]. Determination of the β**Hiba! A hivatkozási forrás nem található.**-1,4-*N*-acetyl-glucosaminidase and β**Hiba! A hivatkozási forrás nem található.**-glucosidase activities was carried out with *p*-nitrophenyl-*N*-acetyl-β**Hiba! A hivatkozási forrás nem található.**-D-glucosaminide [20] and *p*-nitrophenyl-β-D-glucopyranoside [8] substrates respectively. The substrates (all derived from Sigma) were dissolved in phosphate buffer (pH 6). All experiments were repeated three times, and the measurements were carried out with a Beckman DU-65 spectrophotometer.

Gel filtration chromatography

For gel filtration experiments strain AH124 was inoculated into 100 ml T3 medium. The incubation was carried out in 500 ml Erlenmeyer flasks on a rotary shaker at 180 rpm and 25°C for 5 days. Amounts of 1.5 ml from the vacuum concentrated supernatant of the centrifuged culture filtrate were fractionated on a 0.9 x 60 cm Sephadex G-100 column (Pharmacia). The column was equilibrated and eluted with 10 mM Tris·HCl buffer (pH 6.8). To each of the 0.2 ml collected fractions 1-1 ml of the same buffer was added. The enzyme activities were determined with spectrophotometer as described above, but substrates were dissolved in 10 mM Tris·HCl buffer (pH 6.8) containing 1mM $MgCl_2$. The mixtures of 0.05 ml diluted fractions and 0.5 ml of substrate

solutions were incubated at 30°**Hiba! A hivatkozási forrás nem található.**C for 2 hours.

RESULTS

Production of extracellular enzymes under non-inductive and inductive conditions in the presence of different carbon and nitrogen sources

For this experiment media T1, T1F, T2, T2F and T3F were used in which the carbon source was changed from glucose to mannitol, alanine or glycerol. Aspecific chitinase was produced only under inductive conditions (Table 1). Aspecific protease and β**Hiba! A hivatkozási forrás nem található.**-1,3-glucanase showed low levels of constitutive activity, which depended in the case of β**Hiba! A hivatkozási forrás nem található.**-1,3-glucanase both on the carbon, and on the nitrogen source used: in the $NaNO_3$ containing media (T2) the level of consitutive β**Hiba! A hivatkozási forrás nem található.**-1,3-glucanase activity proved to be the highest in the case of glycerol as carbon source, while in the presence of NH_4Cl (T1) strong repression was detected. Under inductive conditions the measurable activities of all investigated enzymes were increased in every type of the culture media. In the most cases glycerol as carbon-, and $NaNO_3$ as nitrogen source promoted the secretion of the enzymes at the highest level.

Time course production of extracellular enzymes under inductive and non-inductive conditions

The activities of aspecific protease, β-1,3**Hiba! A hivatkozási forrás nem található.**-glucanase and β**Hiba! A hivatkozási forrás nem található.**-1,4-*N*-acetyl-glucosaminidase were measured for 8 days and referred to hyphae weight. The conidia were inoculated into T4, T5, T6 and T7 culture media and in their derivates containing the appropriate inducers.

The aspecific protease activity was detected in the samples both of the non-inductive and inductive cultures as early as day 1, but much higher level appeared under inductive conditions. From the second day the activities of aspecific proteases were

decreased in all media, possibile due to self-degradation. When $NaNO_3$ was used as sole nitrogen source, the levels of secreted proteases were higher than those produced in the presence of NH_4Cl. The level of enzyme released to the medium was higher when incubated with glycerol than with mannitol.

The β**Hiba! A hivatkozási forrás nem található.**-1,3-glucanase activity appeared on the first day of cultivation, even under non-inductive conditions, but this activity was repressed in the presence of NH_4Cl as sole nitrogen source. From the second day the activity of the initially secreted β**Hiba! A hivatkozási forrás nem található.**-1,3-glucanases under inductive conditions decreased gradually throughout the investigation period, in contrast with the constitutive enzyme level, which showed a weak increasing. During growth on glycerol as sole carbon source, the level of secreted β**Hiba! A hivatkozási forrás nem található.**-1,3-glucanase was higher than that produced on mannitol.

A small level of β-1,4-*N*-acetyl- glucosaminidase activities was detectable just on the first day after inoculation. Surprisingly, higher enzyme activities were measured in the samples derived from under non-inductive conditions than in those derived from under inductive conditions, which is possibly due to the retardation of the growth of hyphae in the presence of chitin. The level of the constitutively secreted enzyme increased to the third day, and than decreased slowly to the end of the investigation period. In the presence of $NaNO_3$ as sole nitrogen source, the levels of secreted β-1,4-*N*-acetyl-glucosaminidases were higher than those produced in the case of NH_4Cl.

Fractionation of enzymes by gel filtration chromatography

Using gel filtration at least six peaks of aspecific protease activity were detected (Fig. 1A), four of them in the high, while the others in the low molecular weight fractions. Six peaks of trypsin-like protease activity (Fig. 1B), and five peaks of chymotrypsin-like protease activity (Fig. 1C) were detected, 1-1 of them in the high molecular weihgt fractions. The similarity of the profile of trypsin-like and chymotrypsin-like activities suggests the possibility of the presence of protein-degrading enzymes with both specific activities. Two peaks of β-glucosidase activity were detected in the high molecular

weight fractions (Fig. 1D). In the case of β-1,3-glucanase enzymes (Fig. 1E) beside the peak in the high molecular weight fractions, further three small peaks in the low molecular weight fractions could be detectable. Based on this experiments, one peak of β-1,4-*N*-acetyl-glucosaminidase activity could be detected (Fig. 1F).

DISCUSSION

The extracellular enzymes important for mycoparasitism were produced by *Trichoderma viride* strain AH124 in a great volume under inductive conditions. In the respect of induction the effects of the presence of another filamentous fungus such as *F. culmorum* hyphae, or laminarin, chitin and gelatine were the same. Except for aspecific chitinase, the investigated enzymes were produced under non-inductive conditions, but constitutive levels were low. The activities of β-1,3-glucanase, aspecific chitinase and aspecific protease were repressable both by carbon and nitrogen sources. In general when $NaNO_3$ was used as sole nitrogen source, the levels of secreted enzymes were higher than those produced in the presence of NH_4Cl, and the same effect was observable in the case of glycerol and mannitol carbon sources (mármint a glycerol szénforráson való növesztés eseténben nagyobb volt az enzimaktivitás mint a manniton valónál). Under inductive conditions, the levels of constitutive enzyme activities were increased except for β-1,4-*N*-acetyl-glucosaminidase, where the presence of chitin repressed the growth of hyphae. By gel filtration chromatography the protease system of *T. viride* was found to be complex: six trypsin-like and five chymotrypsin-like proteases were detected. Geremia et al. [4] reported from the isolation and purification of an extracellular alkaline protease from *T. harzianum* with supposed role in mycoparasitism however, the extracellular proteolytic systems of *Trichoderma* species is rather unrevealed. In comparison the number of extracellular enzymes found in *T. viride* AH124 with the isoenzymes data in literature: two β**Hiba! A hivatkozási forrás nem található.**-glucosidase are described in *Trichoderma pseudokoningii* [2] and in *T. reesei* [17], seven β-1,3-glucanases were detected in *T. harzianum* upon induction with laminarin [19], and two ß-1,4-N-acetyl-glucosaminidases were characterized by Haran et al. in *T. harzianum* [6].

This study examined the extracellular enzymatic systems of *T. viride* AH124, a strain which proved to be a promising candidate for biocontrol based on the earlier examinations. It is also important, that besides protoplast fusion and mutagenesis, transformation possibilities based on hygromycin B resistance are also available for the further breeding of *T. viride* AH124 strain [12].

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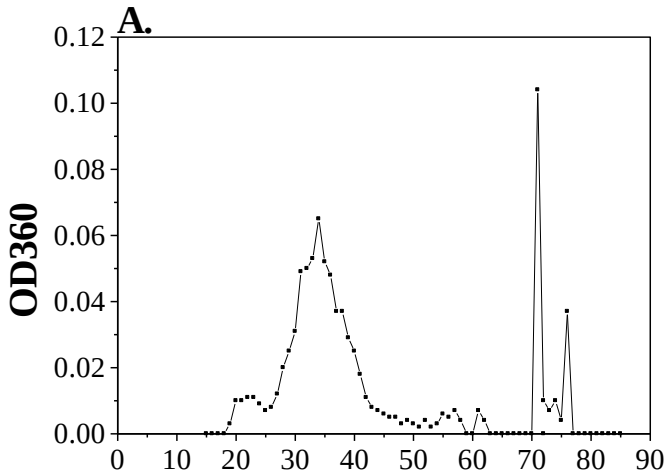
Table 1

Carbon sources		**Aspecific protease (OD_{360})**		**ß-1,3-Glucanase (OD_{540})**		**Aspecific chitinase (OD_{585})**	
		NI	I	NI	I	NI	I
T1	glycerol	0.002	0.473	0.000	0.034	0.000	0.248
	mannitol	0.007	0.092	0.023	0.053	0.000	0.068
	alanine	0.009	0.029	0.000	0.046	0.000	0.134
	glucose	0.013	ND	ND	ND	0.000	ND
T2	glycerol	0.009	0.608	0.063	0.084	0.000	0.168
	mannitol	0.007	0.434	0.015	0.065	0.000	0.195
	alanine	0.010	0.047	0.004	0.036	0.000	0.088
	glucose	0.003	ND	ND	ND	0.000	ND
T-3		ND	0.170	ND	0.032	ND	0.019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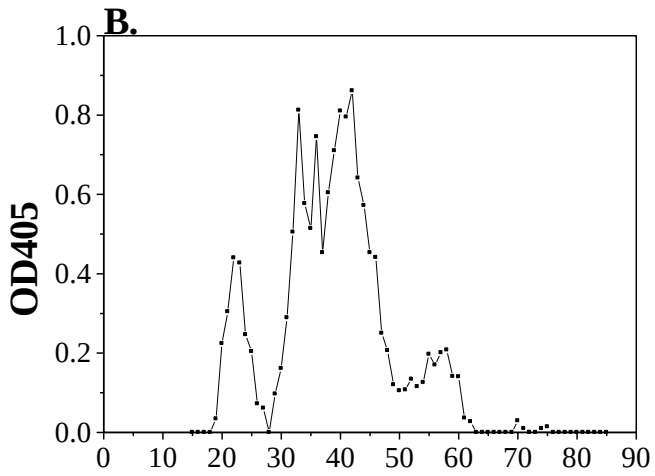
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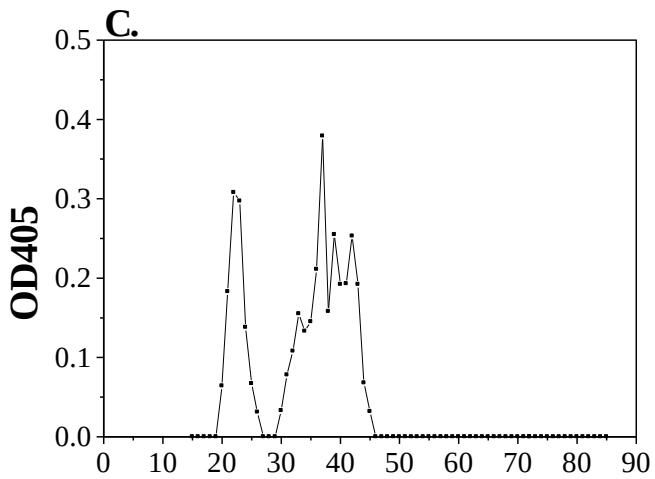
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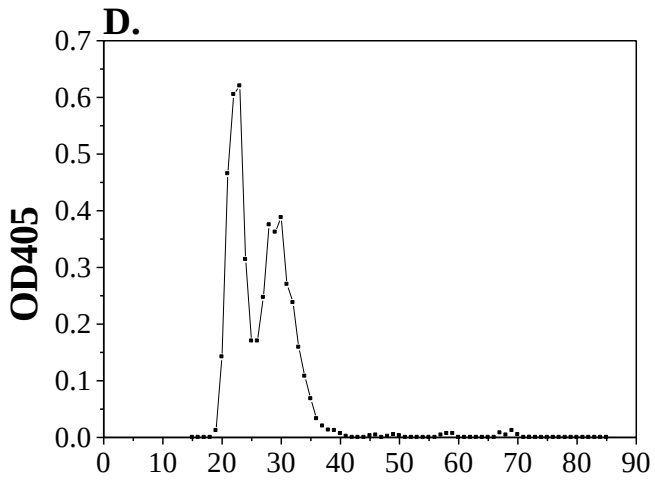
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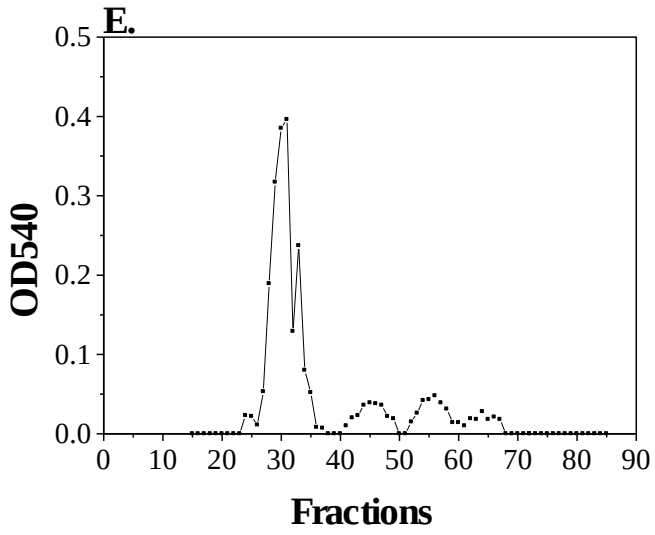

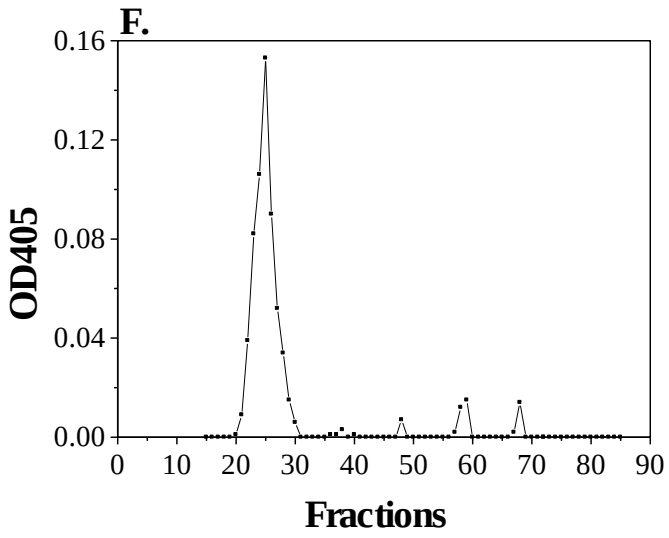

Figure legend:

Table 1 Secretion of extracellular enzymes under inductive (I) and non-inductive(NI) conditions. ND: not determined

Fig. 1. Extracellular enzyme profiles of *Trichoderma viride* T124. A. Aspecific protease activity, B. trypsin-like protease activity, C. chymotrypsin-like protease activity, D. β-glucosidase activity, E. β-1,3-glucanase activity, F. β-1,4-*N*-acetyl-glucosaminidase activity.