Chemistry of Natural Compounds, Vol. 37, No. 2, 2001

ISOLATION, PURIFICATION, AND CERTAIN PROPERTIES OF β-1,3-GLUCANASE FROM THE FUNGUS *Verticillium dahliae*

R. S. Sattarova

UDC 577.154

Two homogeneous molecular forms of β-1,3-glucanase are isolated and purified from the culture fluid of Verticillium dahliae *Kleb. Isolation and purification methods including ion exchange on DEAE-TSK 650M with subsequent gel filtration on TSK HW 50, preparative electrophoresis, and adsorption chromatography on HA-Ultrogel were compared. Certain kinetic properties of the molecular forms of β-1,3-glucanase for laminarin were studied. The effect of amino acids on the activity of both forms of β-1,3-glucanase was investigated.*

Key words: *Verticillium dahliae*, β-1,3-glucanase, purification, activity, molecular forms.

Protective reactions in plant tissues are initiated when receptors recognize elicitors of pathogenic microorganisms and when enzymes of a fungal pathogen decompose hydrolytically plant tissue and liberate from it endogenous elicitors. In addition to others, these include β-bound glycosides [1-3]. β-1,3-Glucanase plays a very important role in this process.

We previously isolated β-1,3-glucanase from the fungus *Verticillium dahliae*, which causes cotton wilt, by preparative electrophoresis in polyacrylamide gel by determining the β-1,3-glucanase activity in gel segments and preparing zymograms [4]. However, preparative electrophoresis is difficult to use. Special reagents that color the enzymolysis products in the gel are not always available, i.e., enzyme in the gel cannot be identified, zymograms cannot be obtained, extraction of enzyme from the gel is complicated, etc.

We developed a more effective, simple, and quick method for isolating and purifying β-1,3-glucanase from *V. dahliae* and determined certain kinetic characteristics of the isolated enzyme.

β-1,3-Glucanase was isolated using ion-exchange chromatography of the enzyme preparation on DEAE-TSK 650M anion exchanger with a linear concentration gradient of ammonium-bicarbonate buffer from 0.05 to 1.0 M at pH 7.8. Seven fractions were produced. The fraction eluted with 0.24 M ammonium bicarbonate has β-1,3-glucanase activity. The active fraction was purified by gel chromatography on TSK-gel HW 50 in a column equilibrated with 0.05 M bicarbonate buffer at pH 7.8 (Fig. 1**a**). This separated proteins with β-1,3-glucanase activity into two fractions that were molecular forms of the enzyme.

Table 1 gives results of purifying the molecular forms of β-1,3-glucanase. It can be seen that the specific activity increased by three times in the initial purification stages. Thus, whereas the specific activity increased by 12 times after ion-exchange chromatography on DEAE-TSK 650M, gel chromatography on TSK HW 50 produced a 32-fold purification of form I; 19-fold, of form II.

Results from the purification described above show that the purification level and yield of active molecular forms approach those obtained by isolating them by preparative electrophoresis, which we have previously reported [4].

Thus, the present experiments and previous work [5, 6] suggest that *V. dahliae* produces two molecular forms of extracellular β-1,3-glucanase. According to N-terminal analysis and electrophoresis in sodium-dodecylsulfate polyacrylamide gel, these are homogeneous. The N-terminal amino acid of form I is glycine; of form II, alanine.

Institute of Microbiology, Academy of Sciences, Republic of Uzbekistan, 700128, Tashkent, fax (998712) 41 71 29, e-mail: sregina@janus.silk.org. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 154-157, March-April, 2001. Original article submitted July 24, 2000.

0009-3130/01/3702-0177$25.00

TABLE 1. Purification of β-1,3-Glucanase from *V. dahliae* by Gel and Ion-Exchange Chromatography

Purification stage	Protein, mg	Total activity, E	Specific activity, E/mg	Activity yield, %	Purification level
Raw material	2217.6	3326.4	1.5	100.0	1.0
Gel chromatography on Sephadex G-50	554.4	2328.5	4.2	70.0	3.0
Ion-exchange chromatography on DEAE-TSK 650M	110.8	1933.8	17.4	58.1	12.0
Gel chromatography on TSK-gel HW 50					
form I	22.1	1054.8	47.7	31.8	32.0
form II	24.5	703.2	28.7	21.1	19.0

TABLE 2. Isolation and Purification of β-1,3-Glucanase from *V. dahliae* on HA-Ultrogel

Purification stage	Protein, mg	Total activity, E	Specific activity, E/mg	Activity yield, %	Purification level
Raw material	408.8	555.5	1.4	100.0	1.0
Adsorption chromatography on HA-Ultrogel					
form I	0.2	6.41	32.1	1.2	23.0
form II	9.4	241.1	25.6	43.4	18.0

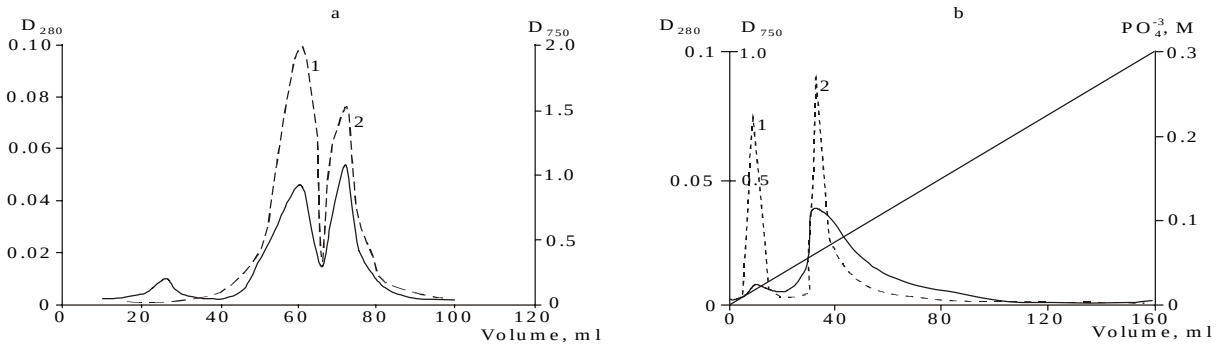

Fig. 1. Gel chromatography of the fraction with β-1,3-glucanase activity on TSK HW 50 (*a*) and adsorption chromatography of culture liquid of *V. dahliae* on HA-Ultrogel (**b**). β-1,3-glucanase form I (1) and II (2).

Hydrated calcium-phosphate gels have been used successfully to adsorb selectively proteins with elution by highly concentrated salt solutions. The best results are obtained if crystalline hydroxyapatite is used. This method is simply and quick. Therefore, we attempted to isolate β-1,3-glucanase by adsorption chromatography on HA-Ultrogel sorbent, which is a spherical gel coated with hydroxyapatite. The properties of this sorbent are clearly superior to those of other forms of hydroxyapatite, in particular, it has a relatively high adsorption capacity.

Cultural liquid of *V. dahliae* was passed through a column of HA-Ultrogel with elution by a linear gradient of phosphate buffer from 0.01 to 0.3 M at pH 6.8 (Fig. 1**b**). Two fractions with β-1,3-glucanase activity were obtained. This also confirmed that two forms of β-1,3-glucanase exist. The results of this purification of β-1,3-glucanase are listed in Table 2.

Therefore, it can be concluded that the isolation methods that include precipitation, ion-exchange and gel-filtration chromatography in addition to preparative electrophoresis are the most effective compared with adsorption chromatogrpahy.

Enzyme kinetics typically study the effect of the chemical nature of the reactants and the reaction conditions on the rate of the reaction. The rate, in turn, depends on the chemical nature of the reactants, enzyme concentration, the reactants, various physical factors, and the presence of various activators and inhibitors.

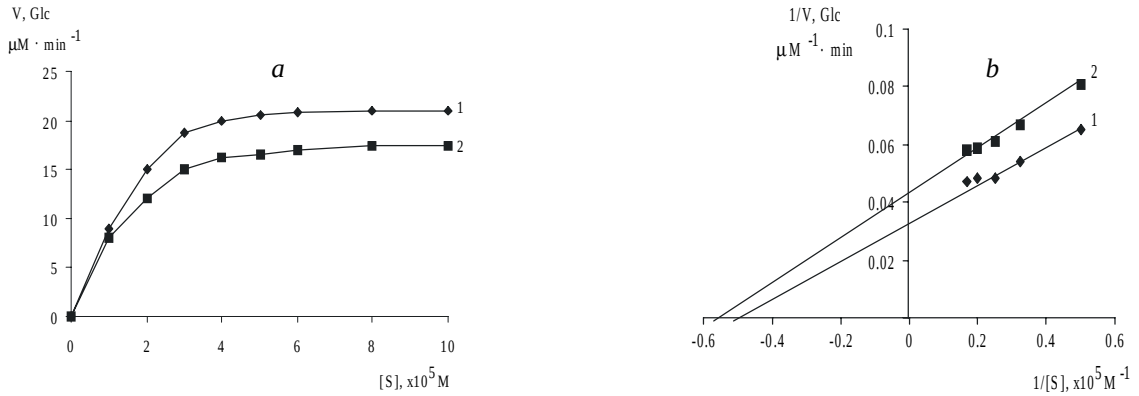

Fig. 2. β-1,3-Glucanase activity as a function of laminarin concentration in Michaelis—Menten (*a*) and Lineweaver—Burke (*b*) coordinates; β-1,3-glucanase form I (*a*) and II (*b*).

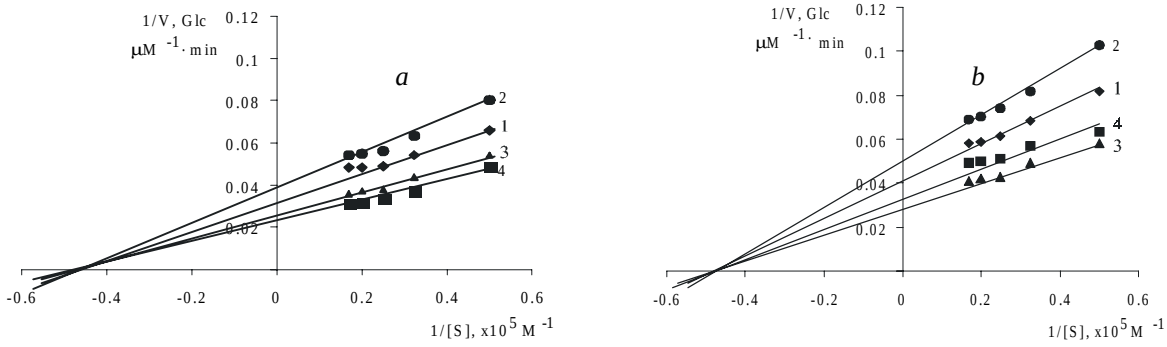

Fig. 3. Effect of amino acids on rate of laminarin decomposition by β-1,3-glucanase form I (*a*) and II (*b*); without effector (1), valine (2), threonine (3), lysine (4).

We first studied the concentration of reaction products as a function of time of reaction of the molecular forms of β-1,3-glucanase and laminarin (polymerization degree n = 30). It was found that the glucose (reaction product) accumulation curve for both forms has two portions. In the first of these, the rate of glucose accumulation for a given substrate polymerization degree is independent of n. In the second, the rate begins to decrease smoothly as n V_{Glc} decreases. This is observed after 10 min of hydrolysis for form I; after 20, for form II.

We then studied the rate of the enzymatic reaction as a function of substrate concentration. Figure 2 shows that the curves of enzymatic reaction rate as functions of laminarin concentration are close to hyperbolic, i.e., hydrolysis of substrate by β-1,3-glucanase (both forms) follows the Michaelis—Menten equation. The quantities K_m and V for laminarin that are calculated by Lineweaver—Burke double inverses are 2.4×10^{-5} M and 33.5 μM/min for form I; 2.0×10^{-5} M and 24.5 μM/min, for form II.

A study of the regulation of the activity of the molecular forms of β-1,3-glucanase by amino acids found that both forms are activated by lysine and threonine. Valine was negative or inhibiting. The nature of the activation or inhibition of the rate of the enzymatic reactions in the presence of the amino acids mentioned above showed that they are noncompeting effectors (Fig. 3), i.e., they bind to the enzyme not at the active center where substrate binds but at a different part of the molecule. For lysine and threonine, the conformation of the enzyme apparently changes so that the catalytic center is reversibly activated; for valine, inactivated.

EXPERIMENTAL

Isolation and Cultivation. Strain 2 of *V. dahliae* was obtained from the laboratory collection of microorganisms at the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan and was grown under steady-state conditions in Czapek Dox sterile medium (200 mL) at 28°C for 15 d in 1-L flasks.

Determination of β-1,3-Glucanase Activity. Enzyme activity was determined from the increase of reducing sugars by the Somogyi—Nelson method [7] using glucose as a standard. The substrate was laminarin from the brown alga *Laminaria cycharioides* [8]. One unit of enzyme activity was taken as the amount of enzyme required to form 1 μmole of reducing sugars (calculated as glucose) in 1 min under optimal conditions of enzyme activity. The specific activity was determined from the number of units per mg of protein.

Isolation and Purification of β-1,3-Glucanase. Gel and Ion-Exchange Chromatography. The active fraction of β-1,3-glucanase preparation from gel charomatography on Sephadex G-50 [9] was placed on a column (1.5×20 cm) packed with DEAE-TSK 650M equilibrated with ammonium bicarbonate buffer (0.05 M) at pH 7.8. A linear concentration gradient of ammonium bicarbonate buffer (0.05-1.0 M) at pH 7.8 was used. The active fraction isolated from ion-exchange chromatography on DEAE-TSK 650M was dried by lyophilization and placed on a column (1.6×65 cm) packed with TSK HW 50 gel equilibrated with ammonium bicarbonate buffer (0.05 M) at pH 7.8.

Adsorption Chromatography. Culture liquid of *V. dahliae* was passed through a column (1.3×17 cm) of HA-Ultrogel equilibrated with phosphate buffer (0.01 M). A linear gradient of phosphate buffer (0.01-0.3 M) at pH 6.8 was used.

Analytical Electrophoresis in Sodium Dodecylsulfate Polyacrylamide Gel. Electrophoresis was performed by the Weber and Osborn method in 10% polyacrylamide gel [10]. Gels were colored by coumassie R-250 solution and silver [11].

N-Terminal amino acids of β-1,3-glucanase molecular forms were determined according to Gray [12].

REFERENCES

1. R. L. Wijesundera, J. A. Bailey, R. J. Byrde, and A. H. Fielding, *Physiol. Mol. Plant Pathol.*, **34**, 403 (1989).
2. A. Darvill, C. Augur, C. Bergmann, R. W. Carlson, J. Cheong, S. Eberhard, G. Hahn, V. Lo, V. Marfa, B. Meyer, D. Mohnen, M. A. O'Neill, M. D. Spiro, H. van Halbeek, W. S. York, and P. Albersheim, *Glycobiology*, **2**, No. 3, 181 (1992).
3. Y. Okinaka, K. Minori, and K. Takeo, *Plant Physiol.*, **109**, No. 3, 839 (1995).
4. A. G. Khalmuradov, R. S. Sattarova, and E. V. Konnova, *Biokhimiya*, **61**, No. 4, 467 (1996).
5. I. V. Zaikina, N. A. Tiunova, N. Ya. Kobzeva, and A. M. Bezborodov, *Prikl. Biokhim. Microbiol.*, **21**, No. 4, 461 (1985).
6. T. Yukui, H. Tezuka, and S. Yabuuchi, *Agric. Biol. Chem.*, **53**, 2341 (1989).
7. S. D. Varfolomeeva, *Progress in Science and Technology, Series Biotechnology* [in Russian], VINITI, Moscow (1990), Vol. 25, p. 30.
8. L. A. Elyakova and T. N. Zwyagintseva, *Carbohydr. Res.*, **34**, 241 (1974).
9. R. S. Sattarova, M. T. Adylbekov, and A. G. Khalmuradov, *Dokl. Akad. Nauk Uzb. SSR*, No. 9, 51 (1991).
10. K. Weber and M. Osborn, *J. Biol. Chem.*, **24**, 4406 (1969).
11. B. R. Oakley, D. R. Kirsch, and N. R. Morris, *Anal. Biochem.*, **105**, 361 (1980).
12. W. R. Gray, *Methods Enzymol.*, **25**, 121 (1972).