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EXPERIMENTAL ARTICLES

Effect of the *Rhizobium leguminosarum* 252 Agglutinins on the Activity of Certain Enzymes in Plant Cells

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Abstract—The incubation of pea seedling roots with the surface agglutinins R_1 and R_2 of *Rhizobium leguminosarum* 252 brought about an increase in the activity of proteases, β-glucosidase, and, especially, succinate dehydrogenase in the roots. The data presented show that rhizobial agglutinins play an important part in the formation and functioning of legume–rhizobial associations.

Key words: *Rhizobium leguminosarum*, agglutinins, enzymes, pea seedling roots, symbiosis.

The formation of nitrogen-fixing plant–bacterial associations is known to be mediated by plant agglutinins (lectins). There is increasing evidence that bacterial lectins may also play a role in this process, providing, like the agglutinins of pathogenic bacteria, for the attachment of bacterial cells to the host plant roots [1–4]. The findings that the lectins of pseudomonads, azospirilla, and rhizobia can influence the activity of lytic enzymes, including their own [5, 6], suggest that the lectins of soil bacteria may also perform some other functions.

The aim of the present work was to study the effect of the *Rhizobium leguminosarum* 252 agglutinins on the activity of some hydrolytic and respiratory enzymes of plant cells.

MATERIALS AND METHODS

Rhizobium leguminosarum bv. *viciae* strain 252 was obtained from the collection of rhizobia at the All-Russia Research Institute of Agricultural Microbiology in Pushkin, St. Petersburg.

Agglutinins were stripped from the surface of *R. leguminosarum* 252 cells as described earlier [7].

For enzyme assay, the cut roots (0.5 g) of four-day-old seedlings of the pea cultivar Uladovskii yubileinyi were placed in a 5 mM solution of $CaCl_2$ and incubated at 18–20°C for 1 h to repair root damage. After being washed with distilled water, the roots were incubated in a solution of rhizobial agglutinins for 1 h (the optimum values of agglutinin concentration and incubation time were chosen experimentally). Then the roots were again washed with distilled water and used for the preparation of root extracts to assay enzymatic activities.

Root extract for the determination of proteolytic and β-glucosidase activities was prepared as follows: washed roots were ground in cold 0.2 M acetate buffer (pH 5.0) and centrifuged at 1400 *g* for 10 min.

Proteolytic activity was measured by the method of Preston and Kruger [8]. Root extract (0.1 ml) was mixed with the equal volume of acetate buffer and 0.05 ml of a freshly prepared solution of hemoglobin (10 mg/ml) and incubated at 30°C for 2 h. The reaction was stopped by the addition of 0.25 ml 20% trichloroacetic acid (TCA). The same amount of TCA was added to the control reaction mixture before its incubation. The concentration of amino acids and peptides nonprecipitated with 20% TCA was determined with the ninhydrin reagent. A calibration curve was constructed using alanine as the standard. Specific proteolytic activity was defined as the amount of amino acids and nonprecipitable peptides (expressed in mg of alanine) formed in min per mg protein.

β-Glucosidase (EC 3.2.1.21) was assayed by the method of Ki-Sun Kwon *et al.* [9]. Root extract (0.1 ml) was mixed with 0.4 ml of a substrate solution (2 mM *p*-nitrophenyl-β-D-glucopyranoside in 50 mM acetate buffer with pH 5.0) and incubated at 55°C for 30 min. The reaction was stopped by adding 1 ml of 1 M Na_2CO_3. One unit of enzymatic activity was defined as the amount of enzyme producing 1 mmol of *p*-nitrophenol in min per mg protein. The absorbance of *p*-nitrophenol was measured at 420 nm with an SF-26 spectrophotometer (LOMO, Russia).

Root extract for the assay of malate dehydrogenase (MDH, EC 1.1.1.37) and NADH dehydrogenase (EC 1.6.1.1) was prepared as follows. Cut and washed pea seedling roots (0.5 g) were homogenized in 1 ml of a mixture containing equal volumes of 2% K_2CO_3 and 0.2% $Na_2S_2O_5$. The homogenate was centrifuged at 5000 *g* for 10 min and the resultant supernatant was concentrated by adding an amount of dry Sephadex

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G-250 (fine). After 10-min incubation, the swollen Sephadex was removed by centrifugation.

MDH was assayed by a modified method of Sal'kova [10]. The measuring cuvette contained 1 ml of 0.1 M Tris–HCl buffer (pH 7.2), 0.2 ml of 0.27% $MnSO_4$, 0.6 ml 0.2% malate, 0.2 ml of root extract, 0.2 ml of 0.089% $NADP^+$, and 0.3 ml of H_2O. The reference cuvette contained an additional 0.2 ml of water instead of the root extract. The concentration of NADPH was determined by measuring the absorbance of the reaction mixture at 340 nm 1, 2, and 3 min after the initiation of the reaction. Enzymatic activity was calculated by the formula:

$$A = \frac{(D_2 - D_1)V}{\Delta tH},$$

where A is the activity of MDH; D_1 is the initial absorbance; D_2 is the final absorbance; Δt is the time interval between the absorbance measurements; V is the volume of the root extract in the reaction mixture measured in ml; and H is the amount of the added protein measured in mg. The specific activity of MDH was expressed in OD units/(min mg protein).

The activity of NADH dehydrogenase was determined from the reduction rate of the artificial electron acceptor 2,6-dichlorophenolindophenol (DPIP) [11]. The measuring cuvette contained 2 ml of 50 mM phosphate buffer (pH 7.5), 0.5 ml of 0.04 mM DPIP, 0.2 ml of root extract, and 0.1 ml of 0.1 M NADH. The reference cuvette contained 0.2 ml of distilled water instead of the root extract. The absorbance of DPIP was measured at 600 nm. Enzymatic activity was calculated by the formula:

$$A = 3\frac{\Delta E_{600}}{\Delta tH21},$$

where ΔE_{600} is the absorbance of DPIP measured at 600 nm; 21 is the coefficient of the molar extinction of DPIP measured in μM^{-1}; Δt is the time interval measured in min; 3 is the total volume of the reaction mixture in ml; and H is the amount of the added protein measured in mg. The specific activity of NADH dehydrogenase was expressed in OD units/(min mg protein).

The activities of succinate dehydrogenase (SDH, EC 1.3.99.1) and lactate dehydrogenase (LDH, EC 1.1.1.27) were determined from the reduction of almost colorless 2,3,5-triphenyltetrazolium chloride (TPT) to red-colored triphenylformazan [12]. Cut and washed pea seedling roots (0.5 g) were thoroughly ground in 2.5 ml of cold 0.1 M phosphate buffer (pH 7.8), and the root homogenate was placed in experimental and control test tubes in 1-ml amounts. Enzymatic substrate (either succinate or lactate) was added to the experimental tube in the form of a mixture (0.2 ml) of equal volumes of 0.2 M substrate and 1% TPT. The equivalent volume (0.2 ml) of phosphate buffer (pH 7.8) was added to the control tube. The tubes were evacuated with a vacuum pump and incubated at 20–22°C in the dark for 1.5 h. Then the triphenylformazan formed was dissolved by adding an ethanol–acetic acid mixture (19 : 1, v/v). The absorbance of triphenylformazan was measured at 496 nm. The specific activities of SDH and LDH were expressed as mmol formazan/(min mg protein).

Protein was quantified by the Bradford method [13]. The results obtained were statistically processed using Student's *t*-test [14].

RESULTS AND DISCUSSION

R. leguminosarum 252 cells contain two surface agglutinins, R_1 and R_2 [7]. The involvement of these agglutinins in the attachment of rhizobial cells to pea roots and the existence of agglutinin-specific receptors on the root surface [3, 7] allowed us to assume that rhizobial agglutinins play a role in the formation of legume–rhizobial associations.

The close symbiosis of rhizobia with plant roots suggests that rhizobial cells may penetrate into plant cells. This process is associated with the partial lysis of the cell wall of the legume root hairs due to the action of the hydrolytic enzymes of the plant [15] and, probably, of the rhizobia. Electron microscopic studies showed that the infected root hairs have zones of the cell wall hydrolysis with attached rhizobial cells [16]. Many rhizobial strains were shown to exhibit activities of a number of hydrolytic enzymes [17, 18]. As was shown earlier [6], *R. leguminosarum* 252 cells possess proteolytic and pectinolytic activities, as well as the activities of acid and alkaline phosphatases and β-glucosidase. Of interest is the fact that the rhizobial agglutinins R_1 and R_2 partially inhibit the activity of rhizobial β-glucosidase, pectinase, and acid and alkaline phosphatases and completely suppress the proteolytic activity of rhizobia.

We assumed that rhizobial agglutinins may also affect the hydrolytic enzymes of host plants and attempted to verify this assumption by investigating the effect of these agglutinins on the β-glucosidase and proteolytic activities of pea seedling roots.

Experiments showed that the incubation of the rhizobial agglutinins R_1 and R_2 (2.5 μg/ml) with the pea seedling roots for 1 h led to an increase in the β-glucosidase activity of the roots by 1.7–2 times (Table 1). The incubation of the pea seedling roots with the higher concentration of these agglutinins (5 μg/ml) increased the proteolytic activity of the roots by 1.7–1.8 times (Table 1).

At present, there is no generally accepted mechanism of the action of lectins on enzymes. Taking into account the hypothesis that bacterial lectins cofunction with lytic enzymes [5], we may assume that, due to their carbohydrate-binding capacity, the *R. leguminosarum* 252 agglutinins bind to the active center of such enzymes, thus causing conformational changes leading to an increase in the affinity of these enzymes for their substrates and, hence, in the catalytic activity.

Table 1. Effect of the *R. leguminosarum* 252 agglutinins R_1 and R_2 on the activity of hydrolytic enzymes in the pea seedling root extract*

Enzymes	Control	Agglutinin			
		R_1		R_2	
	$M \pm m$	$M \pm m$	P	$M \pm m$	P
Proteases	0.012 ± 0.0018	0.022 ± 0.0023	<0.01	0.020 ± 0.0024	<0.05
β-Glucosidase	0.003 ± 0.0004	0.006 ± 0.0005	<0.001	0.005 ± 0.0003	<0.001

*See MATERIALS AND METHODS for enzyme activity units.

Table 2. Effect of the *R. leguminosarum* 252 agglutinins R_1 and R_2 on the activity of some dehydrogenases in the pea seedling root extract*

Enzymes	Control	Agglutinin			
		R_1		R_2	
	$M \pm m$	$M \pm m$	P	$M \pm m$	P
Malate dehydrogenase	0.022 ± 0.004	0.020 ± 0.004	>0.1	0.030 ± 0.007	>0.1
Lactate dehydrogenase	0.007 ± 0.0007	0.007 ± 0.0004	>0.1	0.006 ± 0.0005	>0.1
NADH dehydrogenase	0.002 ± 0.0003	0.002 ± 0.0004	>0.1	0.003 ± 0.0005	>0.1
Succinate dehydrogenase	0.006 ± 0.0008	0.020 ± 0.005	<0.02	0.010 ± 0.001	<0.01

*See MATERIALS AND METHODS for enzyme activity units.

Alternatively, bacterial agglutinins may influence the synthesis of hydrolytic enzymes.

Increasing the activity of the hydrolytic enzymes of root cells, rhizobial agglutinins may enhance the degradation of the main structural components of the cell wall of the root cells and thus promote the penetration of rhizobial cells to the root tissues and, hence, the formation of nitrogen-fixing bacterial–plant associations.

The legume–rhizobial symbiosis suggests the functional interaction of symbionts. Earlier, we found that rhizobial agglutinins interact with the membrane proteins of the pea seedling roots [7] and assumed that the agglutinins may affect the functional state of the cytoplasmic membrane of the host plants. This state can be characterized by the activity of some primary membrane-bound respiratory enzymes, such as the dehydrogenases of malate, lactate, succinate, and NADH [19].

The measurement of these activities in the pea seedling root extracts showed that both rhizobial agglutinins, R_1 and R_2, influenced little the activity of malate, lactate, and NADH dehydrogenases but increased the activity of the succinate dehydrogenase of the roots by 3.3 times (agglutinin R_1) and 1.7 times (agglutinin R_2) (Table 2). These data suggest that the functional activity of plant mitochondria may also be enhanced, since succinate dehydrogenase is the only enzyme of the tricarboxylic acid cycle that is built in the internal mitochondrial membrane [20].

The data presented imply that the surface agglutinins of *R. leguminosarum* 252 may function not only as adhesins, but also as modifiers of various enzymes in bacterial and plant cells.

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REFERENCES

1. Smit, G., Kijne, J.W., and Lugtenberg, B.J.J., Involvement of Both Cellulose Fibrils and a Ca^{2+}-Dependent Adhesin in the Attachment of *Rhizobium leguminosarum* to Pea Root Hair Tips, *J. Bacteriol.*, 1987, vol. 169, no. 9, pp. 4294–4301.
2. Wisniewski, J.P. and Delmontte, F.M., Modulation of Carbohydrate-Binding Capacities and Attachment Ability of *Bradyrhizobium* sp. (Lupinus) to White Lupin Roots, *Microbiology*, 1996, vol. 42, pp. 234–242.
3. Karpunina, L.V., Savenkova, N.N., Vishnevetskaya, O.A., Mashkina, A.V., and Nikitina, V.E., Role of the Surface Agglutinins of *Rhizobium leguminosarum* Cells in Their Attachment to Pea Roots, *Mikrobiologiya*, 1996, vol. 65, pp. 512–516.
4. Karpunina, L.V., Mel'nikova, U.Yu., Soboleva, E.F., Vishnevetskaya, O.A., and Nikitina, V.E., Investigation of the Adhesive Properties of *Bacillus polymyxa* Cells and the Role of Bacillary Lectins in Adhesion, *Mikrobiologiya*, 1999, vol. 68, no. 4, pp. 445–448.
5. Gilboa-Garber, N. and Garber, N., Microbial Lectin Cofunction with Lytic Activities as a Model for a Gen-

eral Basic Lectin Role, *FEMS Microbiol. Rev.*, 1989, vol. 63, pp. 221–222.

6. Karpunina, L.V., Soboleva, E.F., and Pronina, O.A., Study of the Effect of *Rhizobium leguminosarum* 252 Agglutinins on the Activities of Certain Hydrolytic Enzymes, *17th Int. Lectin Meeting*, Wurzburg (Germany), September 24–27, 1997, p. 16.
7. Karpunina, L.V., Savenkova, N.N., Vladimirova, M.V., Koltunova, E.F., and Nikitina, V.E., Agglutinins of *Rhizobium leguminosarum* and Their Role in Interactions with Plants, *Izv. Akad. Nauk, Ser. Biol.*, 1996, no. 6, pp. 698–704.
8. Preston, K.R. and Kruger, J.E., Physiological Control of Exo- and Endoproteolytic Activities in Germinating Wheat and Their Relationship to Storage Protein Hydrolysis, *Plant Physiol.*, 1979, vol. 64, pp. 450–454.
9. Ki-Sun Kwon, Hyung Cyeo Kang, and Yung Chil Hak, Purification and Characterization of Two Extracellular β-Glucosidases from *Aspergillus nidulans*, *FEMS Microbiol. Lett.*, 1992, vol. 97, pp. 149–154.
10. *Metody biokhimicheskogo issledovaniya rastenii* (Biochemical Methods for Investigation of Plants), Ermakova, A.I., Ed., Leningrad: Agropromizdat, 1987, p. 49.
11. *Metody izucheniya membran rastitel'nykh kletok. Uchebnoe posobie* (Methods for Investigation of Plant Cell Membranes: A Handbook), Polevoi, V.V. *et al.*, Eds., Leningrad: Leningrad Univ., 1986, p. 54.
12. Pleshkov, B.P., *Praktikum po biokhimii rastenii* (Laboratory Manual on Plant Biochemistry), Moscow: Kolos, 1985, pp. 212–215.
13. Bradford, M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein–Dye Binding, *Anal. Biochem.*, 1976, vol. 72, pp. 248–254.
14. Oivin, I.A., Statistical Processing of Experimental Results, *Patol. Fiziol. Eksperim. Terap.*, 1960, no. 4, pp. 76–89.
15. Ljunggren, H. and Fahraens, C., The Role of Polygalacturonase in Root-Hair Invasion by Nodule Bacteria, *J. Gen. Microbiol.*, 1961, vol. 25, pp. 521–528.
16. Callaham, D.A. and Torrey, J.G., The Structural Basis for Infection of Root Hairs of *Trifolium repens* by *Rhizobium*, *Can. J. Bot.*, 1981, vol. 59, no. 9, pp. 1647–1664.
17. Martinez-Molina, E., Morales, V.M., and Hubbell, D.H., Hydrolytic Enzyme Production by *Rhizobium*, *Appl. Environ. Microbiol.*, 1979, vol. 38, pp. 1186–1189.
18. Skochinskaya, N.N., Aizenberg, V.A., Antipchuk, A.F., and Tantsyurenko, E.F., About the Pectinolytic Activity of Nodule Bacteria, *Mikrobiol. Zh.* (Kiev), 1990, vol. 52, no. 1, pp. 22–23.
19. Ivanishchev, V.V. and Kurganov, B.I., Enzymes of Malate Metabolism: Characterization, Activity, and Biological Role, *Biokhimiya*, 1992, vol. 57, no. 5, pp. 653–662.
20. Igamberdiev, A.U. and Falaleeva, M.I., Isolation and Characterization of the Succinate Dehydrogenase Complex of Plant Mitochondria, *Biokhimiya*, 1994, vol. 59, no. 8, pp. 1198–1206.