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Effect of *Azospirillum* lectins on the activities of wheat-root hydrolytic enzymes

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Abstract

This work studied the effect of two cell-surface lectins isolated from the nitrogen-fixing soil bacterium *Azospirillum brasilense* Sp7 and from its mutant defective in hemagglutinating activity, *A. brasilense* Sp7.2.3, on the activities of α-glucosidase, β-glucosidase and β-galactosidase in the exocomponent, membrane and apoplast fractions of wheat-seedling roots. Lectin (40 μg mL^{-1}) incubation for 1 h of the plant fractions increased the enzymes' activities; both wild-type and mutant lectins were most stimulatory to the activities of all the exocomponent-fraction enzymes studied and to the apoplast-fraction β-glucosidase. Pretreatment of the lectins with their carbohydrate hapten, L-fucose, lowered the effect. The observed differences in the lectins' ability to influence enzyme catalytic activity are explained by change in the antigenic properties of the mutant lectin.

Introduction

Azospirilla are widely distributed plant-growth-promoting bacteria, which enhance the development of many important crop and noncrop plants (Döbereiner, 1992). There are known *Azospirillum* factors involved in plant-growth promotion (e.g. Steenhoudt and Vanderleyden, 2000; Bashan et al., 2004) but there is almost no way to firmly establish what factor of *Azospirillum* activity is key to bacterial stimulation of the host plant's growth and development. An important element of recognition between plant and microbe is the direct *Azospirillum*–plant-cell contact mediated by specific molecular structures. Bacterial-surface carbohydrates and plant lectins have traditionally been considered to be such structures. Wheat germ agglutinin has been long thought the only lectin involved in the interactions occurring in the wheat–*Azospirillum* system (Iosipenko et al., 1996). Interestingly, wheat germ agglutinin not only fixes the spatial relations between *Azospirillum* and the plant cell but also can influence the bacterial partner's metabolism (Antonyuk et al., 1997).

Earlier we isolated a cell-surface lectin from the nitrogen-fixing bacterium *A. brasilense* Sp7 (Italians kaya et al., 1989). The lectin is a glycoprotein, has a molecular mass of 36 kDa and is specific for L--fucose (1.87 mM) and D-galactose (20 mM). We also generated a mutant of *A. brasilense* Sp7 (*A. brasilense* Sp7.2.3; Alen'kina et al., 1998) whose cells did not agglutinate erythrocytes but had a surface lectin of the same molecular mass and carbohydrate specificity as the wild-type lectin (Figure 1). However, the mutant lectin did not react with antibodies raised against the wild-type lectin, suggesting that its molecule had undergone changes.

Previous work from this laboratory (Nikitina et al., 1996) showed that the *A. brasilense* Sp7 lectin is involved in the plant–bacterial interaction and that bacterial adhesion to roots is inhibited by

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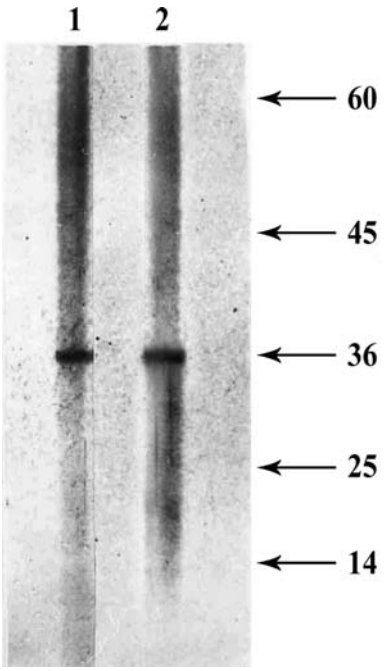

Figure 1. SDS-PAGE patterns of the cell-surface lectins from (1) *A. brasilense* Sp7 and (2) *A. brasilense* Sp7.2.3. Arrows indicate the position of marker proteins: catalase, 50 kDa; ovalbumin, 45 kDa; lactate dehydrogenase, 36 kDa; chymotrypsinogen, 25 kDa; and egg lysozyme, 14 kDa.

receptor analogues and by lectin-specific antibodies. Comparison of the adhesion of parent and mutant cells showed a severe decrease in the mutant's root-adhesion capacity (Alen'kina et al., 1998).

Data on the ability of bacterial lectins to bind to hydrolytic enzymes and to modify the enzymes' activity are fragmentary. In particular, *Pseudomonas* lectins can interact with lytic enzymes (Gilboa-Garber and Garber, 1989), and *Rhizobium* agglutinins can have an effect on hydrolytic enzyme activity (Karpunina and Soboleva, 2001). Lakhtin (1989) showed that lectins may be regulatory to enzyme activities.

In this work we aimed to describe the effect of *Azospirillum* wild-type and mutant lectins on wheat-root hydrolytic enzymes.

Materials and methods

Organisms and growth conditions

The microorganisms used were wild-type *Azospirillum brasilense* Sp7 (Russian Academy of Sciences' Institute of Microbiology, Moscow) and *A. brasilense* Sp7.2.3. (a mutant defective in hemagglutinating activity), derived from strain Sp7 by transposon mutagenesis (Alen'kina et al., 1998). The cultures were grown in the synthetic medium of Sadasivan and Neyra (1985) at 37 °C for 18 h.

Isolation of lectins

Lectins were isolated from the cell surface of the *A. brasilense* wild-type and mutant strains by the method of Eshdat (1978) and were purified by gel filtration on a 30×2.2-cm column of Sephadex G-75 (40–120 μm particle diameter). The emergence of protein fractions was followed at 278 nm with a Uvicord SII apparatus (LKB, Sweden). The eluents were 0.1 M CH_3COOH (pH 4.8) and 0.05 M phosphate buffer (pH 7.0) containing 0.15 M NaCl. The flow rate was 1.5 mL min^{-1}. The working solution contained 40 μg mL^{-1} protein.

Preparation of wheat-seedling-root fractions

The roots of 4-day-old wheat (*Triticum aestivum* L. ,Saratovskaya 29') seedlings, grown aseptically from surface-sterilized seeds, were used to obtain three fractions. The exocomponent fraction was prepared by mild agitation of whole roots in phosphate-buffered saline (pH 7.2). The working solution contained 5 μg mL^{-1} protein. The membrane fraction was prepared as follows. After isolation of exocomponents, the whole roots were subjected to maceration (0.1 N HCl for 0.5 h) and to osmotic shock (treatment with glycerol for 2 h). The roots were extensively washed with distilled water until no protein remained in the washings and then were treated overnight with a 1% solution of Triton X-100 at 4 °C. The Triton X-100 was removed by gel centrifugation by using Toyopearl HW 40F (Polevoi and Maksimova, 1978). The working solution of the membrane fraction contained 3 mg mL^{-1} protein. The apoplast fraction was prepared as follows. The roots were excised, washed three times in distilled water, placed in 0.1 M Na-phosphate buffer (pH 6) and infiltrated for 15 min by using a vacuum pump. For apoplast isolation, the roots were centrifuged at $2000 \times g$ for 10 min (Rohringer et al., 1983). The working solution contained 40 μg mL^{-1} protein.

Enzyme assays

The fractions were incubated with lectin solutions for 1 h. (The lectin concentration and the incubation time had been chosen experimentally.) The activities of α-glucosidase, β-glucosidase and β-galactosidase were determined by the amount of nitrophenol formed from the substrates (4-nitrophenyl-α-D-glucopyranoside, 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl-β-D-galactopyranoside, respectively; Chaplin and Kennedy, 1986). The amount of nitrophenol formed was measured spectrophotometrically at 425 nm. One unit of enzyme activity was defined as the amount required to convert 1 nM substrate per min.

Treatment of lectins with a specific hapten

For hapten treatment, 0.1 mL of lectin solutions (40 μg mL^{-1}) was incubated with 0.1 mL of L-fucose (0.2 mM), and the lectins' effect on enzyme catalytic activity was determined.

Statistics

The data were statistically processed by using the programs SigmaPlot for Windows 4.01 and SPSS for Windows 8.0.0 (SPSS Inc., USA).

Results and discussion

We decided to test whether the azospirillar lectins located on the bacterial outer membrane could function as effectors of the plant-cell hydrolytic enzymes. A 1-h incubation of the *A. brasilense* Sp7 lectin (40 μg mL^{-1}) with the exocomponent, membrane and apoplast fractions increased the activities of α-glucosidase, β-glucosidase and β-galactosidase, as compared with controls (root-fraction enzyme activities in the absence of the lectin). The mutant lectin (40 μg mL^{-1}) also increased the enzymes' activities but to a much lesser degree (Table 1). Possibly, the decrease in the mutant's adhesion capacity and the impaired effect of the lectin are a pleiotropic effect of the mutation.

Both lectins were most stimulatory to the activities of all the exocomponent-fraction enzymes studied and to the activity of the apoplast-fraction β-glucosidase. According to Umali-Garcia (1980), Rougier and Chaboud (1985), and others, the most probable receptors for *Azospirillum* surface lectins are the proteins and carbohydrates of the wheat-seedling-root exocomponent fraction. This opinion was corroborated by the results of our earlier immunodot analysis showing that, indeed, the *A. brasilense* Sp7 lectin reacts more intensely with the exocomponent fraction than it does with other fractions (Nikitina et al., 1996).

There are few data on the endophytic colonization of wheat roots by *Azospirillum*. The bacteria may be located in root hairs, intercellular spaces and cells of the root vascular system. Such location ensures close interaction between micro- and macropartner and makes possible an xchange of metabolites (Assmus et al., 1995; Schloter and Hartmann, 1998). The considerable increase in the apoplast-fraction β-glucosidase activity for both strains (Table 1) might suggest that the *Azospirillum* lectins, by enhancing apoplastic-cellulose degradation, can (i) alter the metabolite concentration and consequently the milieu in which the cell functions and (ii) influence metabolite exchange, the preferred compartment for which is the apoplast.

To prove that the carbohydrate-binding sites are involved in the lectins' effect, we attempted to treat the lectins with a specific hapten. Because both lectins showed marked specificity for L-fucose (1.87 mM), this hapten was chosen for preincubation. When the lectins were treated with L-fucose before being incubated with the root fractions, their effect on all the enzymes' activities decreased (Table 1). Incomplete inhibition of the effects as a result of cell pretreatment with L-fucose was found previously in studies on *Azospirillum* adhesion to wheat roots (Nikitina et al., 1996) and on bacterial aggregation (Nikitina et al., 2001), indicating that carbohydrate-binding sites are not solely responsible for the lectin-induced phenomena. No doubt the lectins' carbohydrate-binding properties have a role to play, since pretreatment with L-fucose changes the pattern of the observed effect. The differences in the effects caused by the antigenically distinct wild-type and mutant lectins suggest that the lectins' effect on enzyme

Table 1. The root-fraction enzyme activities induced by the lectins of *A. brasilense* Sp7 and Sp7.2.3

Root fractions	*A. brasilense* Sp7 lectin[a]			*A. brasilense* Sp7.2.3 lectin[b]		
	α-glucosidase[c]	β-glucosidase[d]	β-galactosidase[e]	α-aglucosidase	β-glucosidase	β-galactosidase
F_e	4 ± 0.2	2 ± 0.2	2 ± 0.5	4 ± 0.2	2 ± 0.2	2 ± 0.5
F_e + L	50 ± 0.8	57 ± 0.8	51 ± 1.2	34 ± 2.6	29 ± 1.1	22 ± 2.5
F_e + (L + F)	6 ± 0.2	4 ± 0.1	10 ± 0.4	20 ± 1.4	17 ± 0.9	6 ± 1.0
F_m	15 ± 0.5	13 ± 0.4	6 ± 0.6	15 ± 0.5	13 ± 0.4	6 ± 0.6
F_m + L	47 ± 1.2	76 ± 2.0	43 ± 1.2	20 ± 1.2	20 ± 0.5	16 ± 1.6
F_m + (L + F)	19 ± 0.6	21 ± 0.3	30 ± 1.2	17 ± 1.8	19 ± 1.5[f]	12 ± 0.9
F_a	29 ± 0.8	2 ± 0.2	4 ± 0.2	29 ± 0.8	2 ± 0.2	4 ± 0.2
F_a + L	61 ± 0.8	31 ± 0.3	24 ± 1.8	30 ± 0.5[f]	19 ± 1.4	13 ± 1.2
F_a + (L + F)	32 ± 1.2	15 ± 0.9	6 ± 0.4	27 ± 1.4[f]	16 ± 1.4[f]	12 ± 1.0[f]

F_e – The exocomponent fraction; F_m – The membrane fraction; F_a – The apoplast fraction; F + L – lectin-incubated fractions; F + (L + F) – fractions incubated with L-fucose-pretreated lectins.
Results are means ± SE ($n = 5$). Differences were considered significant at the $P < 0.05$ level.
[a]α-Glucosidase, β-glucosidase and β-galactosidase activities of the Sp7 lectin preparation: not found.
[b]α-Glucosidase, β-glucosidase and β-galactosidase activities of the Sp7.2.3. lectin preparation: not found.
[c,d,e]One unit of activity was defined as the amount required to catalyze the decomposition of 1 nM substrate per min under specified conditions. Specific activity was calculated per μg of protein.
[f]Not significant at $P > 0.05$.

catalytic activity is associated not with carbohydrate specificity but with the structural changes of the lectin molecule that have occurred during mutagenesis.

Currently there is no clear idea of the mechanism underlying the influence of lectins on enzymes. There have been many examples of microbial lectins and lysins sticking together in heterologous as well as endogenous systems (Gilboa-Garber and Garber, 1989). The enhanced enzyme activities might be due to possible lectin-induced conformational changes of the enzyme molecules. Other mechanisms are also possible.

Conclusions

We showed that the cell-surface lectins isolated from *A. brasilense* Sp7 and its mutant defective in hemagglutinating activity, *A. brasilense* Sp7.2.3, could enhance, to a variable degree, the activities of α-glucosidase, β-glucosidase and β-galactosidase in wheat-seedling roots. The activity of the mutant lectin was weaker than that of the wild-type lectin. Possibly, the decrease in the mutant's adhesion capacity and the impaired effect of the lectin are a pleiotropic effect of the mutation. The inactivation of the lectins' carbohydrate-binding centers by L-fucose relieved the stimulatory effect, suggesting a role for these centers in stimulation. The differences in the lectins' ability to influence enzyme catalytic activity could be explained by change in the antigenic properties of the mutant lectin. The findings obtained in this study and their similarity to the results presented earlier for the agglutinative proteins of other soil bacteria (Karpunina and Soboleva, 2001) are of interest for the understanding of the biological role of lectins in plant relationships during the establishment of a plant–bacterial nitrogen-fixing association.

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