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EXPERIMENTAL ARTICLES

Proteolytic Activity of Lectins from the Nitrogen-Fixing Bacterium *Bacillus polymyxa*

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Abstract—Two lectins (LI and LII) stripped from the surface of *Bacillus polymyxa* 1460 cells were found to possess proteolytic activity, which was associated with their hemagglutinating activity. The inhibition of the hemagglutinating activity of lectins by specific carbohydrate haptens changed their enzyme activities. The inhibition of hemagglutinating activity by glucuronic acid or fructose 1,6-diphosphate decreased the proteolytic activities of both lectins, whereas the blocking of this activity with D-glucosamine or D-galactosamine increased the proteolytic activity of LII. The molecules of the *B. polymyxa* lectins are suggested to contain two active centers responsible for hemagglutinating and proteolytic activities.

Key words: *Bacillus polymyxa*, lectins, carbohydrate haptens, hemagglutination, proteolytic activity.

In recent years, lectins, as polyfunctional proteins, have attracted the increased interest of researchers. Lectins are now isolated from various microorganisms, however, only few of them possess both hemagglutinating and enzymatic activities.These are the toxins of some enteropathogenic bacteria [1–4] and the proteolytic enzymes of *Rhizobium* [5]. The functional role of lectins, including those of soil nitrogen-fixing bacteria, is not studied in depth. The ability of *Rhizobium* and *Azospirillum* cells to penetrate into the plant roots is explained by the presence of various hydrolytic enzymes in these cells [6, 7]. However, no information is available on the presence of hydrolytic enzymes in *B. polymyxa* cells, which are active nitrogen fixers and can associate with the wheat, winter rye [8], and sugar cane [9] plants. At the same time, other species of this genus are known as the producers of proteolytic enzymes [10, 11]. In view of this, it was of interest to elucidate whether the lectins of *B. polymyxa* possess enzymatic activity. By now, we have established that the lectins of *B. polymyxa* are involved in the attachment of bacterial cells to the wheat seedling roots, serving as adhesins [12, 13].

To gain closer insight into the role of lectins in the interactions of *B. polymyxa* cells with higher plants, in this work we studied their proteolytic activity.

MATERIALS AND METHODS

The strain *Bacillus polymyxa* 1460 was obtained from the Czechoslovak Collection of Microorganisms, J.E. Purkyne University, Brno. The strain was grown in synthetic Moore medium [14] at 28°C for 72 h; the biomass was harvested by centrifugation at 6000 *g*. To isolate agglutinins, cell suspension in 1% NaCl was repeatedly passed through a syringe needle 0.1 mm in diameter and then centrifuged. Agglutinins were precipitated from the supernatant with 2.5 volumes of acetone, dialyzed against distilled water for 15–20 h, and purified by gel filtration on a column with Toyopearl HW-55. Proteins were eluted from the column with 0.05 M glycine buffer (pH 3). The optical density of the eluate was recorded on an Uvicord SII monitor (LKB) at 278 nm [12]. Protein concentration was determined by the Bradford method [15].

The hemagglutinating activity of lectins was assayed by agglutinating a 2% suspension of trypsin-treated rabbit erythrocytes. To determine the proteolytic activity of lectins [16], 0.05 ml of a freshly prepared solution of hemoglobin (10 μg/ml) and 0.1 ml of 0.2 M acetate buffer (pH 4.5) were added to 0.1 ml of lectin solution and the mixture was incubated at 30°C for 2 h. The reaction was stopped by adding 0.25 ml of 20% trichloroacetic acid and the mixture was centri-

Table 1. Proteolytic activity of the *B. polymyxa* 1460 lectins at different stages of their purification

Purification step	Proteolytic activity, μg alanine/(min μg protein)
Cells	0.0002
Crude extract	0.0013
Lectin LI	0.0800
Lectin LII	0.0180

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Table 2. Proteolytic activity of lectin LI, whose hemagglutinating activity was inhibited by specific carbohydrate haptens

Carbohydrate	Hemagglutination titer	Proteolytic activity, μg alanine/(min μg protein)	*P*
Glucuronic acid	–	0.004 ± 0.0015	<0.001
Fructose-1,6-diphosphate	–	0.006 ± 0.0001	<0.001
Mixture of the carbohydrates (1 : 1)	–	0.003 ± 0.0006	<0.001
Control	1 : 4	0.080 ± 0.015	–

Table 3. Proteolytic activity of lectin LII, whose hemagglutinating activity was inhibited by specific carbohydrate haptens

Carbohydrate	Hemagglutination titer	Proteolytic activity, μg alanine/(min μg protein)	*P*
Glucuronic acid	–	0.008 ± 0.002	<0.05
Fructose-1,6-diphosphate	–	0.007 ± 0.001	<0.05
D- Galactosamine	–	0.036 ± 0.007	<0.05
D-Glucosamine	–	0.180 ± 0.003	<0.001
Mixture of the carbohydrates (1 : 1 : 1 : 1)	–	0.049 ± 0.006	<0.001
Control	1 : 4	0.018 ± 0.004	–

fuged at 1400 *g* for 10 min. The content of free amino acids in the supernatant was determined using the ninhydrin reagent containing 1.72 g of ninhydrin and 0.24 g of $SnCl_2$ in 16.8 ml of 0.2 M acetate buffer (pH 4.5). The calibration curve was constructed with alanine concentrations ranging from 0.005 to 0.1%. The absorbance of the ninhydrin complex with amino acids was measured with an SF-26 spectrophotometer at 570 nm. Enzyme activity was expressed as the amount of alanine (μg) formed per minute by 1 μg of protein under the given experimental conditions.

Data were statistically processed as described by Oivin [17].

RESULTS AND DISCUSSION

Our preliminary studies showed that *B. polymyxa* 1460 cells have a total proteolytic activity of 0.0002 μg alanine/(min μg protein). It was found that the proteolytic activities of lectins LI and LII isolated from the cell surface of *B. polymyxa* increased as they were purified. As can be seen from the data presented in Table 1, the crude preparation of agglutinins exhibited higher proteolytic activity than the whole cells. Further purification of lectin preparations increased their proteolytic activities to 0.080 and 0.018 μg alanine/(min μg protein) for LI and LII, respectively. According to the data available in the literature, lectins of some bacteria, including the non-nitrogen-fixing species of the genus *Bacillus*, possess both proteolytic and hemagglutinating activities. For example, the lectins isolated from *Bacillus subtilis* 316M cells exhibited a proteolytic activity of 0.2–0.3 units/μg protein [18].

To elucidate the relationship between the proteolytic and hemagglutinating activities of lectins, we inhibited hemagglutination by the lectin-specific carbohydrate haptens. For this purpose, a carbohydrate solution (0.3 M) was added to the lectin solution with a hemagglutination titer of 1 : 4 in a ratio of 1 : 1, the mixture was incubated at 28°C for 30 min, and then its proteolytic activity was measured. According to our earlier data, lectin LI is specific for glucuronic acid and fructose-1,6-diphosphate, whereas lectin LII is additionally specific for D-galactosamine and D-glucosamine [12].

Further experiments showed that the inhibition of hemagglutination by carbohydrates may increase or decrease the proteolytic activities of lectins, depending on the carbohydrate.

As can be seen from Table 2, the proteolytic activity of lectin LI decreased, as compared with the control, when hemagglutination was inhibited by glucuronic acid or fructose-1,6-diphosphate. The decline in the proteolytic activity was more pronounced when these carbohydrates were added together. The incubation of the other lectin, LII, with glucuronic acid or fructose 1,6-diphosphate decreased its proteolytic activity by 2.2 and 2.6 times, respectively (Table 3). Thus, the inhibition of hemagglutination by the two carbohydrates decreased the proteolytic activities of both *B. polymyxa* 1460 lectins.

The inhibition of the hemagglutination of lectin LII by two other carbohydrate haptens, D-galactosamine and D-glucosamine, produced an entirely different effect on its proteolytic activity, which increased under the action of these carbohydrates two- and tenfold, respectively (Table 3). The inhibition of hemagglutination by a mixture of the four carbohydrates increased

the proteolytic activity of LII, possibly due to the overwhelming effect of D-glucosamine and D-galactosamine.

Analysis of the results obtained allowed us to suggest that the lectin molecule contains two active centers, one of which is responsible for enzymatic activity and the other for hemagglutination. The latter center presumably contains two to four carbohydrate-binding sites. The blocking of the hemagglutination center by carbohydrates obviously causes conformational changes in the lectin molecule and thus also affects the center responsible for enzymatic activity. The existence of the two, enzymatic and agglutinating, centers in the α-galactosidase molecule was also suggested by other researchers [19].

The processes of the soil bacterial interactions with the plant root cells are now known to involve bacterial lectins [13, 20, 21], which play the role of adhesins. The specific contacts between the carbohydrate components of plants and the receptors of microorganisms are believed to occur in mucin [22, 23]. The mucin secreted by wheat roots contains various monosaccharide residues, including the carbohydrate haptens specific for the lectins of *B. polymyxa*, such as glucuronic acid, galactosamine, and glucosamine [23]. These residues may function as receptors for bacterial lectins, inhibiting their hemagglutinating activity. As a result, the lectins of *B. polymyxa* may play, to a greater or lesser extent, the role of proteolytic enzymes and promote the penetration of bacterial cells into the plant root cells. This suggestion is confirmed by the data available in the literature that the lectins of pathogenic bacteria (such as clostridial and cholera toxins) exhibit both lectin and enzymatic properties during their interactions with the macroorganism [1–4]. The enzymatic activity of bacillary lectins together with their known adhesive properties allow us to suggest a more active role of these proteins in the formation of nitrogen-fixing associations.

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