RECOGNITION BETWEEN THE N_2-FIXING *ANABAENA* AND THE WATER FERN *AZOLLA*

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1. Introduction

The symbiosis between the nitrogen fixing blue–green alga (cyanobacterium) *Anabaena azollae* and the water fern *Azolla* is of highly agronomical importance because of its value as a green manure [1,2]. The *Anabaena* occupies a specific cavity in the leaf of the *Azolla* and forms close association with the host cells [3]. The molecular mechanisms, leading to the recognition and association between the two cell types, have not been yet clarified.

The hypothesis, underlying the research reported here, is that sugar-binding proteins (lectins) localized on the cell surface of the alga mediate the adherence of the *Anabaena* to the *Azolla*. Such lectins and their glycoconjugate receptors have been implicated in cellular recognition and adhesion in several systems, such as interactions between Rhizobia and legumes, slime mould differentiation and lichens symbiosis [4–6]. This work describes a lectin activity in cell-extracts of *Anabaena* and the possible presence of a glycoconjugate receptor for this lectin in the leaf cavities of the fern, in which the algae are accommodated during symbiosis.

2. Materials and methods

2.1. *Cultures*

A culture of *Anabaena azollae* Stras. which has been isolated from *Azolla caroliniana* Wild. was obtained from Dr J. W. Newton (USDA, Peoria IL). Algae were grown in a BG-11 medium [7] at 25°C, under constant illumination of cool fluorescent light (10 W . cm^{-2}).

Anabaena 7120 was obtained from Dr R. Pippka (Pasteur Institute, Paris) and *Nostoc muscorum* 7119 was obtained from Professor D. I. Arnon (Univ. of Calif., Berkeley CA). Cultures were grown in Allen and Arnon medium [8] as above.

Azolla filiculoides was collected at the botanical gardens of the Tel Aviv University and maintained in a 1/8 strength Hoagland medium [9], under a light–dark regime of 16:8 h at temperatures of 27°C and 22°C, respectively.

2.2. *Algal extracts*

Cells were collected by centrifugation, suspended in growth medium and ruptured by sonication at 20 W for 60 s (Branson sonifier, model W140). The homogenate was then centrifuged at 10 000 × *g* for 2 min.

2.3. *Isolation of leaf cavity envelopes*

The algae-containing leaf cavity envelopes (LCE) were prepared essentially as in [10]. The fronds were incubated in a medium containing 1% Cellulysin (Calbiochem) (without Triton X-100) after vacuum infiltration at 25°C with moderate shaking for 18 h. The homogenate was diluted 3-fold with distilled water and was filtered through a 20 mesh net. The filtrate was allowed to settle for 30 min, and was then centrifuged at 1000 × *g* for 30 min. The supernatant containing the LCE, was centrifuged at 35 000 × *g* for 10 min. The LCE pellet was suspended in saline containing 0.25% Zwittergen (Ciba), sonicated, and centrifuged at 10 000 × *g* for 2 min. The supernatant obtained, 'LCE extract', was used for the inhibition studies.

2.4. *Preparation of fixed erythrocytes*

Outdated human blood of types A, B and O were received from the local blood bank. The cells were collected by centrifugation (1000 × *g* for 5 min), washed

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4 times with 5 vol. saline/packed cells, and incubated in 5 vol. phosphate-buffered saline (PBS, pH 7.2) containing 1% glutaraldehyde for 1 h at room temperature with moderate shaking. The cells were collected and washed twice with 5 vol. 0.1 M glycine in PBS and twice with saline. The cells were stored at 4°C as a 10% suspension in saline. Before assay, the cells were diluted with saline to produce a 4% suspension.

2.5. *Hemagglutination assay*

This assay was performed by using serial 2-fold dilutions of extract in V-shaped microtiter plates (Cooke Engineering Co.). Each well contained 25 μl algal extract diluted in saline, 25 μl bovine serum albumin (1%) in saline, 25 μl saline, and 25 μl of the 4% erythrocytes suspension. To study the effects of different saccharide haptens on the hemagglutinating activity, appropriate concentrations of the inhibitors in saline were added to the well in 25 μl portions, replacing the equivalent volume of saline. Agglutination titers were determined after 1 h at room temperature. The titer was defined as the reciprocal of the highest dilution of extracts that agglutinates the cells. Specific activity is defined as the titer divided by the number of milligrams of protein per millimeter of algal extract. Protein concentrations were determined by the Lowry procedure [11]. All saccharides used were purchased from Pfanstiehl or Sigma.

3. Results

Preliminary tests have confirmed the presence of an agglutinin in extracts of *Anabaena azollae*. No difference in the agglutinating activity of the sonicates was detected when tested with erythrocytes before or after their fixation with glutaraldehyde.

In order to locate a possible biological role of this hemagglutinin, we compared the agglutinating activity in extracts from several algal species. As shown in table 1, the hemagglutinating activity was high in preparations from the symbiotic *A. azollae* and was absent in preparations from the free-living algae, *Nostoc muscorum* 7119 and *Anabaena* 7120. Residual activity found in sonicates of *Azolla* fronds was attributed to the algal symbiont.

Table 1
Comparison between the hemagglutinating activity of free-living and symbiotic N_2-fixing blue–green algae

Species	Spec. act.
Anabaena azollae	640
Anabaena 7120	0
Nostoc muscorum 7119	0
(*Azolla filiculoides*)	40

Assay was done with extracts from the different species containing 100 μg protein/sample

Table 2
Subcellular distribution of hemagglutinating activity in extracts of *Anabaena azollae*

Fraction	Titer
Complete extract (sonicate)	32
Supernatant (20 000 × *g* for 10 min)	128
Pellet (20 000 × *g* for 10 min)	4

Experiment was done with *Anabaena azollae* at mid-log phase of growth (2 weeks). Complete extract contained 87.5 μg protein/sample

Table 2 describes the distribution of the hemagglutinating activity in cell fractions of *A. azollae*. The hemagglutinin was solubilized during the sonication process and was completely recovered in the supernatant (20 000 × *g* for 10 min) of the sonicated cells, while the residual activity combined with cell walls and membranes in the pellet was very low.

Lectins are operationally defined as carbohydrate-binding proteins that can agglutinate a variety of cells. The protein nature of the hemagglutinin is shown in table 3. The activity was heat-labile and was destroyed by trypsin treatment. In order to clarify the sugar specificity of the algal agglutinin, the inhibitory potential of a large number of saccharide haptens on the hemagglutinating activity was tested. Two monosaccharides, D-fucose and L-rhamnose were found to be potent inhibitors of hemagglutinating activity as shown in fig.1. The specificity of the algal

Table 3
Effect of several treatments on the hemagglutinating activity of the algal extract

Treatment	Titer
None	64
Heating (100°C for 10 min)	0
Trypsin (0.1%; 1 h at 37°C)	0
Autoproteolysis (1 h at 37°C)	64

Assay was done as in section 2. Each sample contained 50 μg protein

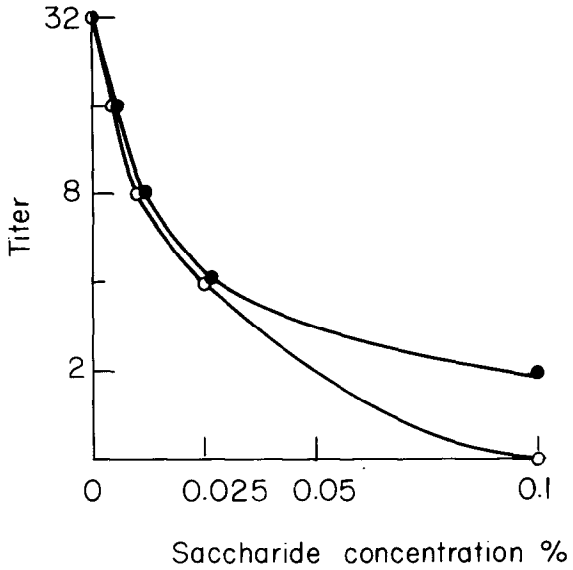

Fig.1. The effect of L-rhamnose (○) and D-fucose (●) on the hemagglutinating activity of *Anabaena azollae*. The assay was done as in section 2. Each sample contained 26.6 μg protein of algal extract.

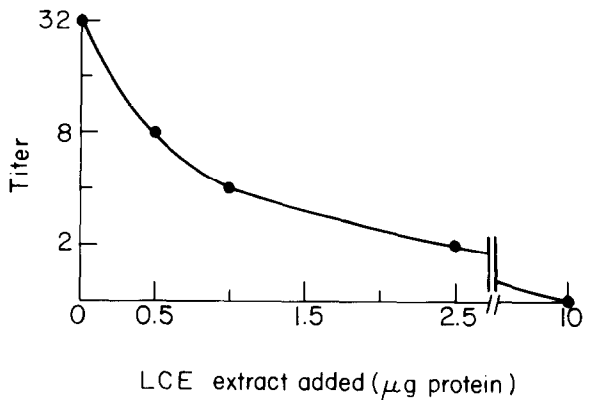

Fig.2. Inhibition of the algal hemagglutinating activity by leaf cavity envelopes of *Azolla*. Each sample contained 53.3 mg protein of algal extract. Leaf cavity envelope extract was prepared as in section 2.

lectin was emphasized by the saccharides which did not show any inhibition of hemagglutination. The monosaccharides glucose, galactose, mannose, xylose, and ribose, the disaccharides sucrose, lactose, and maltose and the aminosugars *N*-acetylglucosamine and *N*-acetylgalactosamine did not inhibit the agglutination mediated by the algal lectin (up to 0.06% final conc.).

The possibility of a specific receptor for the lectin, which would be localized within the fern's cavity, was tested. Leaf cavity envelopes were prepared and the detergent-treated supernatant showed inhibitory activity when tested in the agglutination assay (fig.2). This result indicates that the leaf cavity envelope extract contains an algal hemagglutinin inhibitor, which may serve as an endogenous receptor.

4. Discussion

The major goal of this research is to elucidate the molecular mechanisms underlying the early processes in *Anabaena–Azolla* interaction. Descriptions of an algal lectin and its possible receptor in the fern leaves, two components whose interaction may mediate the formation of the symbiotic association, are reported here. The lectin–receptor interaction may play an essential role in the penetration and settlement of the endosymbiont *Anabaena* into the cavity of a premature differentiated leaf of the fern. However, temporary activity of at least 1 of the 2 components requires developmental or environmental regulation of its appearance. Evidence for such regulation was found in preliminary studies (not shown) which indicate quantitative changes in lectin content during growth phase of *Anabaena azollae* and in different growth regimes.

We intend to quantitate the amount of the saccharide hapten in the leaf cavity of the *Azolla*, and to follow the relationship between maturation of the leaf cavity, its sugar content, and its binding capacity as well as its effect on the efficiency of N_2-fixation.

The lectin was shown to be soluble or loosely bound to the membrane of *Anabaena*. However, in order to enable the lectin to mediate the adherence of algae to other cells, it must be present on the cell surface of the algae. Several mechanisms of adherence involving soluble lectin may be postulated, but the question of cell surface localization of the lectin deserves a thorough investigation.

It is also unclear yet, whether all 6 species of *Azolla* accommodate the same species of *Anabaena*. These results may be of value for a systematic definition of *Anabaena azollae* strains vs *Azolla* species.

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