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STUDY OF THE STRUCTURE OF THE LECTINS OF *Daturia innoxia* SEEDS. I. INFLUENCE OF VARIOUS FACTORS ON THE ACTIVITY OF THE LECTINS

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Lectins I_1 and I_2 of Datura innoxia *– highly active hemagglutinins – possess a high resistance to the effects of heat, of the pH of the medium, and of pronase. On reduction, the lectins lose their activity.*

A high hemagglutinating activity has been found in an extract of the seeds of *Datura innoxia*, fam. Solanaceae, growing in Central Asia [1]. Responsible for its manifestation are two lectins – I_1 and I_2 – which agglutinate erythrocytes, regardless of their group affinity, and leucocytes at a minimum concentration of 0.1 μg/ml [2]. With the absence of toxicity the lectins exhibit immunosuppressive properties of the humoral type [3]. In order to understand the mechanism of the manifestation of physiological activity by the lectins it is apparently necessary to discover the target molecules. In this connection, an important characteristic of the lectins is their carbohydrate specificity.

To determine carbohydrate specificity, we broadened the set of carbohydrates proposed by Lutsik et al. [4]. Oligo- and polysaccharides, glycopeptides, and glycoproteins were included in the inhibitory analysis. The hemagglutination reaction was inhibited only on the addition of a mixture of chitooligosaccharides and ovalbumin. Incubation with tetrachitobioside at a molar ratio of carbohydrate and lectin of 300:1 lowered the titer of hemagglutinating activity (HA) by a factor of only 2-4.

In the study of the structure-activity relationships of lectins it is important to find conditions in which the molecule retains its native structure — i.e., is not denatured. As is known, the generally accepted approach for the study of protein denaturation is the action of high temperatures, the pH, high salt concentrations, and such agents as urea, guanidine chloride, and organic solvents.

The *Datura innoxia* lectins proved to be extremely heat-resistant (Table 1). On thermostating at 70°C for 15 min, the hemagglutinating activity of the lectins remained at the level of the control. At 100°C, 25% of the initial activity was retained after 1 min, and it fell to zero only after boiling for 15 min.

A study of the influence of the pH of the medium on the hemagglutinating activity showed that both lectins were stable in the range $1.9 < pH < 9.0$. The titer of hemagglutinating activity remained at the level of the control.

pH	1.9	3.0	4.0	5.0	5.8	7.15	8.0	8.9
T_{HA}	2^{-6}	2^{-6}	2^{-7}	2^{-7}	2^{-7}	2^{-7}	2^{-7}	2^{-7}

The *Datura innoxia* lectins proved to be resistant to the action of pronase.

In the manifestation of binding properties by lectins I_1 and I_2 the following structural features may be important. The presence of carbohydrates in the lectin molecules may be significant and so also may be high levels of hydroxyproline and cysteine [5]. The preceding communication was devoted to the study of the first question. So far as concerns cysteine, the existence both of disulfide bonds and of sulfhydryl groups may be assumed. The role of SH groups in the binding of carbohydrates has been well studied only in the case of a lectin from the lima bean (*Phaseolus lunatus*) [6-8]. It was established that

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TABLE 1. Influence of Various Factors on the Hemagglutinating Activity (HA) of the *Datura innoxia* Lectins in PBS, pH 7.4

Conditions of treating lectins I_1 and I_2	% of $HA_{contr.}$
50°C, 30 min	100
60°C, 30 min	100
70°C, 15 min	100
75°C, 5 min	50
80°C, 5 min	50
100°C, 1 min	25
100°C, 5 min	0
2<pH<9	100
Pronase, 0.2%, 37°C, 2 h	100
2 M urea	100
Na_2EDTA	100
—S–S—	0

the reactivity of SH groups is connected with the presence of Ca^{2+} and Mn^{2+} ions. Elimination of the metals by dialysis against EDTA inactivated the lectin and increased the activity of the SH groups 60-fold [7].

Since zinc has been detected in the *Datura innoxia* lectins [5], it was of interest to elucidate its role in the hemagglutinating activity the lectins under study. We dialyzed the lectins against EDTA and, in addition, against salts of bivalent metals. However, in not one of the experiments were any changes in the titer of the hemagglutinating activity in one direction or the other observed. Incubation with cysteine (a thiol reagent) likewise did not change the HA; consequently free SH groups, if they are present, are not involved in the activity. Derivatives of the lectins obtained by reductive carboxymethylation possessed no hemagglutinating activity.

Thus, it may be assumed that the most essential factor in the stability–activity of the *Datura innoxia* lectins is the presence of disulfide bonds. There is no doubt that the number of –S–S– bonds and their localization need to be determined.

EXPERIMENTAL

The lectins were extracted from *Datura innoxia* seeds and purified as described in [2].

The hemagglutination reaction was performed in micro test tubes using a series of twofold dilutions of samples of the lectins in PBS, pH 7.4, and a 2% suspension of erythrocytes (rabbit or human) by the method of [4]. Hemagglutinating activities were expressed as titers. As the titer we took the maximum dilution at which agglutination was still observed.

Carbohydrate specificity was determined from inhibition of the hemagglutination reaction [4]. In the determination of carbohydrate specificity we used the following carbohydrates and carbohydrate-containing compounds: *D*-glucose, *D*-mannose, methyl *D*-gluco- and -mannopyranosides, *D*-galactose, lactose, N-acetyl-*D*-galactosamine, N-acetyl-*D*-glucosamine, chitooligosaccharides, *L*-fucose, xylose, *i*-inositol, *D*-galacturonic acid, rhamnose, *D*-fructose, *L*-arabinose, maltose, raffinose, N-acetylneuraminic acid, methyl 6-O-tosyl-*D*-glucopyranoside, glucogalactan, mannan, xylogalactan, xyloglucogalactan, vancomycin (containing a $GlcNH_2$ residue), ristomycin, kanamycin, and ovalbumin.

Protein was determined by Lowry's method, using BSA as standard.

Incubation with Urea. Urea to final concentrations of 2, 4, 6, and 8 M was added to solutions of the lectins in PBS, pH 7.4 (0.5 mg/ml). The mixtures were incubated at 25°C for a day and were then intensively dialyzed against water and PBS for two days, and hemagglutinating activities were determined.

Study of Heat Stability. Initial solutions of the lectins in PBS, pH 7.4, with a concentration of 0.5 mg/ml were thermostated at 50, 60, 70, 75, 80, 85, and 100°C (see Table 1). Then they were cooled to room temperature and analyzed.

Study of the Influence of the pH of the Medium. The lectins were incubated in buffers with various pH values for 24 h. Then they were dialyzed against PBS and their HAs were determined.

Study of the Influence of Metal Ions on the Hemagglutinating Activities of the Lectins [8]. Initial solutions of the lectins in PBS (0.5 mg/ml) were dialyzed against 0.1 M EDTA and then against 1 mM NaCl or PBS. After this, the lectin

samples obtained were dialyzed against solutions containing 1 mM $MgCl_2$ and, to eliminate unbound Mg^{2+} ions, against PBS. This was repeated with Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} salts.

Protein contents and HAs were determined in the initial solutions, taken as controls, in the solutions that had been dialyzed against EDTA, and in the solutions that had been dialyzed against metal salts. In addition, HAs were determined after the direct addition of an EDTA solution to solutions of the lectins in PBS. Two controls were used, in which the EDTA or the lectin was replaced by PBS.

Study of the Influence of a Thiol Reagent. A series of dilutions was prepared from 50 μl of a solution of a lectin in PBS (0.5 mg/ml). To each test tube was added 50 μl of a solution of cysteine (13 mg/ml of PBS). After incubation for 20 and 60 min, 50 μl of a 2% suspension of erythrocytes was added to each tube and hemagglutinating activities were determined after another 15 min. A lectin solution without cysteine was titrated in a control experiment. In addition, erythrocytes were incubated with a cysteine solution.

Reduction and Carboxymethylation. To 10 mg of protein in a dark glass bottle with a tightly closing lid were added successively: 3.16 g of dry deionized urea, 0.30 ml of EDTA solution (50 mg/ml), and 3.0 ml of Tris/HCl buffer, pH 8.6 (5.23 g of Tris and 9 ml of 1.0 N HCl); then water was added to a volume of 7.5 ml, the bottle was flushed with nitrogen, and 0.1 ml of β-mercaptoethanol was added. The bottle was tightly closed and was incubated at 60°C for 30 min and at 100°C for 3 min.

After reduction, 0.268 g of freshly crystallized iodoacetic acid in 1.0 ml of 1.0 N NaOH was added. Alkylation was conducted at room temperature for 15-20 min. The reduced and carboxymethylated smple was desalted on a column equibrated with 5% CH_3COOH.

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