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STUDY OF THE STRUCTURE OF THE LECTINS OF *Datura innoxia* SEEDS. III. RESISTANCE OF THE LECTINS TO THE ACTION OF VARIOUS PROTEASES

S. V. Levitskaya and T. S. Yunusov

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The capacity of the lectins of Datura innoxia *for being digested by proteases with various specificities and pH optima has been studied. Under the conditions of limited proteolysis the hemagglutinating activity did not decrease, and the degree of hydrolysis determined from the reaction with trinitrobenzenesulfonic acid was close to zero.*

In a study of the interrelationship of the structure and activity of lectins, as of other proteins, it is important to determine the conditions under which the molecule retains its native structure and also the limits of the modification permissible for the retention of biological activity.

The lectins from Hindu datura (*Datura innoxia)* seeds (DIL_1 and DIL_2), highly active hemagglutinins, possess a considerable resistance to the action of high temperatures and to denaturing and chelating agents, and are active within wide pH limits [1]. One of the criteria for evaluating the stability of protein molecules is their capacity for undergoing digestion by various proteases.

From an analysis of the composition of the lectins (high levels of carbohydrates and Cys) [2], it was possible to assume that the molecules of these glycoproteins would be resistant to proteolysis. In view of this, it was of interest to ascertain to what extent the molecules of the *Datura innoxia* lectins were resistant to the action of proteases under physiological conditions, what is the influence of hydrolysis on the activity of the lectins, and, if hydrolysis does take place, how the activity depends on its extent. In order to answer these questions, we chose enzymes and enzyme preparations with different specificities and pH optima. As a measure of activity we used the titer of the hemagglutinating activity (HA). The degree of hydrolysis was determined from the reaction of the newly formed amino groups with trinitrobenzenesulfonic acid TNBS by Adler-Nissen's method [3]. The degree of hydrolysis was expressed in mM/eq. Leu. For this purpose we first plotted the calibration curve $D_{340} = f(C_{Leu})$ (Fig. 1). In addition, we chose buffer systems in which a suspension of erythrocytes was stable. Hydrolysis by trypsin was carried out in 0.05 M PBS at a ratio of protein to enzyme of 50:1, 37°C, with constant stirring. Aliquots were taken after 15, 30, and 45 min, and 1, 2, and 20 h. Hydrolysis was stopped by freezing with liquid nitrogen. The lectin was treated with pepsin, pronase, and pectofoetidin in the way described in the Experimental part.

It followed from the results of analysis of the HAs of the lectin samples under all types of hydrolysis (Table 1) that either the lectin did not undergo hydrolysis under the conditions described or hydrolysis, to whatever degree it took place, did not affect the interaction of the lectin with erythrocytes. In determining the degree of hydrolysis in pronase cleavage, the absorption at 340 nm of a solution of the lectin with a concentration of 10 mg/ml corresponded to 0.7 mM Leu eq. At the same point, the HA titer amounted to 100% of the control. In all the other experiments the value of D_{340} was outside the range of determination. At the same time, the hemagglutinating activity did not decrease.

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (3712) 89 14 75. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 93-95, January-February, 1996. Original article submitted July 3, 1995.

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TABLE 1. Treatment of the Hindu Datura Lectin with Proteinases

Time of hydrolysis	Trypsin		Pepsin		Pronase		Pectofoetidin	
	T_{HA}	D_{340}	T_{HA}	D_{340}	T_{HA}	D_{340}	T_{HA}	D_{340}
0 min	2^{-7}	0	2^{-5}	0.05	2^{-11}	0.21	2^{-8}	0
15 min	2^{-7}	0	2^{-5}	0.05	2^{-11}	0.21	2^{-8}	0
30 min	2^{-7}	0	2^{-6}	0.05	2^{-11}	0.21	2^{-8}	0
45 min	2^{-7}	0	2^{-6}	0.05	2^{-11}	–	2^{-8}	0
60 min	2^{-8}	0	2^{-6}	0.05	2^{-11}	0.21	2^{-8}	0
2 h	2^{-8}	0	2^{-7}	0.05	2^{-11}	2.0	2^{-8}	0
4 h	2^{-9}	0	2^{-7}	0.05	2^{-11}	2.0	2^{-8}	0
24 h	2^{-10}	0	2^{-8}	0.05	2^{-11}	–	2^{-8}	0

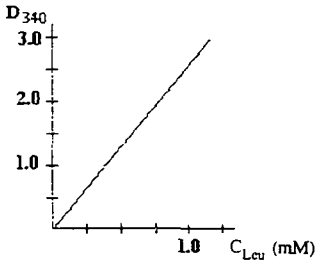

Fig. 1. Calibration curve $D_{340} = f(C_{Leu})$.

The results obtained showed the resistance of the Hindu datura lectin to limited proteolysis. As is known, limited proteolysis takes place under conditions in which the molecules of the proteins are in the native state, at peptide bonds accessible on the surface of the molecule. The resistance of the datura lectin to proteolysis is apparently explained by the fact that carbohydrates occupy a large part of the surface of the molecules and screen the peptide bonds from the action of proteases. Furthermore, the lectin molecules are stabilized by numerous disulfide bonds, thanks to which the native structure is retained even at some slight degree of hydrolysis (as in the case of pronase cleavage). In actual fact, after deglycosylation, reduced and carboxymethylated lectin derivatives are readily digested by trypsin (Fig. 2).

Thus, the lectins of Hindu datura proved to be resistant to the action of various proteases: trypsin, pepsin, pronase, and pectofoetidin; i.e., proteases with different specificities and pH optima. Under the conditions of limited hydrolysis, the HAs did not decrease and the degree of hydrolysis determined from the reaction with trinitrobenzenesulfonic acid was close to zero. The results obtained may be a consequence of the inhibitory activity of the lectin preparation, as in the case of lupin lectin [4]; however, in a study of the hydrolysis of hemoglobin in the presence of the lectin this hypothesis was not confirmed.

EXPERIMENTAL

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

The hemagglutination reaction was conducted in micro-test-tubes using a series of twofold dilutions of the samples of pectin in PBS, pH 7.4, and a 2% solution of erythrocytes (rabbit or human) as described in [6]. Hemagglutinating activity was expressed as a titer. As the titer we took the mximum dilution at which agglutination was still observed.

Proteolysis was conducted under the following conditions:

a) Hydrolysis by pepsin. A solution of the lectin (1 mg/ml) in PBS, pH 2.5 [sic], was incubated at 37°C for 15 min. Then aliquots of the lectin (1 ml) were each treated with 0.1 ml of pepsin (1 mg/10 ml of water). Successive aliquots were taken every 15 min for 1 h, and after 2, 4, and 20 h. Digestion was conducted at 37°C with constant stirring. Hydrolysis was stopped by freezing.

b) Hydrolysis by trypsin. Solutions of the lectin with concentrations of 1 and 10 mg/ml in PBS, pH 7.5, were hydrolyzed at 37°C with constant stirring. The enzyme was added in an enzyme:substrate ratio of 1:50. Aliquots were taken and analyzed as described above.

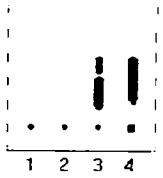

Fig. 2. Thin-layer chromatography of tryptic hydrolysates on 6 × 9 cm cellulose-coated plates in the *n*-BuOH—CH_3COOH—Py—H_2O system: 1) DIL_1; 2) DIL_2; 3) deglycosylated DIL_1; 4) deglycosylated DIL_2.

c) Hydrolysis by pectofoetidin was conducted in 0.05 M Na acetate buffer, pH 4.5, the pectofoetidin being added in a ratio of enzyme to protein of 1:50. Hydrolysis was conducted in a thermostat at 50°C with constant stirring. Analysis was performed as in the case of hydrolysis by pectin.

d) Hydrolysis by pronase. Each of a series of dilutions of solutions of the lectin (0.5 mg/ml) in PBS was treated with 50 μl of a 0.4% solution of pronase and incubation was carried out at 37°C for 2 h. After this, the samples were titrated with a 2% solution of erythrocytes. The results were expressed in percentages of the HA of a control.

e) Hydrolysis of hemoglobin by trypsin in the presence of the lectin. Trypsin was added in a ratio of 1:100 (e/p) to the following samples: 1) a solution of hemoglobin (Hb) (1 mg/ml); 2) a solution of the lectin (1 mg/ml); 3) a solution containing 1 mg/ml of Hb and 1 mg/ml of the lectin. The samples were incubated at 37°C. Aliquots were taken after 1.5, 2.5, 3.5, and 4 h for determining hemagglutinating activity and for analyzing proteolysis by TLC on plates coated with cellulose.

The degree of proteolysis was determined with the aid of trinitrobenzenesulfonic acid [3].

Thin-layer chromatography was conducted on plates (6 × 9 cm) coated with cellulose in the butan-1-ol—acetic acid—water—pyridine (15:3:12:10) system. The spots were revealed with a 0.5% solution of ninhydrin in acetone.

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