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A STUDY OF THE LECTINS OF *Datura innoxia* SEEDS II. DEGLYCOSYLATION WITH TRIFLUOROMETHANE SULFONIC ACID

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Lectins I_1 and I_2 of Datura innoxia, *which are glycoproteins glycosylated to a very high degree, have been deglycosylated completely by the use of trifluoromethanesulfonic acid. The deglycosylated lectins retained their hemagglutinating activity.*

Various hydroxyproline-rich glycoproteins (HRGPs) are widely distributed in plants. They include the HRGPs of cell walls — extensin and its precursors, the HRGPs of extracellular secretions of plant tissue cultures, and the glycoside-binding mucopolysaccharides of seeds [1].

Lectins I_1 and I_2 from the seeds of *Datura innoxia* (fam. Solanaceae) contain more than 50% of carbohydrates and a considerable amount of hydroxyproline [3] and are highly active hem- and leukoagglutinins.

To elucidate the role of the carbohydrate component in binding activity and to study their structures it is necessary to obtain lectin derivatives completely free from carbohydrates but at the same time retaining the native nature of the peptide component. On the basis of their amino acid and carbohydrate compositions [3] we may assume the presence in lectins of HyP-Ara, Ser-Gal, and Ser-Xyl O-glycosidic bonds between the monosaccharide residues. Their stabilities are different, and, therefore, to obtain deglycosylated derivatives we varied the conditions of cleavage.

It was not possible by alkaline hydrolysis or hydrolysis with oxalic and sulfuric acids to achieve complete deglycosylation of the lectins with retention of the peptide bonds. When hydrolysis was carried out with 3 N or 1 N hydrochloric acid at 60°C and below, peptide bonds were retained but, at the same time, the glycosidic bonds were hydrolyzed to different degrees, and mainly arabinose was split out. Glycosidic bonds were cleaved to the maximum extent, with retention of intact peptide bonds, in 1 N HCl at 60°C for 1 h. This gave lectin derivatives ~50% deglycosylated (35% of carbohydrates remained in I_1 and 38% in I_2)

Edge et al. [4] have shown that, in glycoproteins, trifluoromethanesulfonic (TFMS) acid quantitatively splits the bonds of serine and threonine with N-acetylgalactosamine and of serine with xylose, and also O-glycosidic bonds between sugars. In a study of a potato lectin, Desai et al. [5] showed that Hyp-Ara and Ser-Gal bonds were cleaved completely, with no addition cleavage of peptide bonds, and the resulting lectin derivative retained binding activity.

We deglycosylated the lectins of *Datura innoxia* with TMFS at room temperature for 1 h and at 0°C for 3 h. The products were purified by the extraction of the reagents and dialysis. The completeness of the reaction was monitored by disk electrophoresis, from the absence of staining for carbohydrates, and by TLC. The absence of cleavage of the peptide bonds was judged from the results of disk electrophoresis, TLC on cellulose, and the determination of N-terminal amino acids. Since in the native lectins the N-terminal amino acids are blocked [3], the absence in TLC of spots after dansylation showed that the polypeptide chain had remained intact on treatment with TMFS. As in the case of the native lectins, after methanolysis of the deglycosylated derivatives under conditions leading to the opening of the proline ring, Glu was identified. Analysis of the amino acid composition showed no losses on treatemt with TFMS as compared with the initial protein [3].

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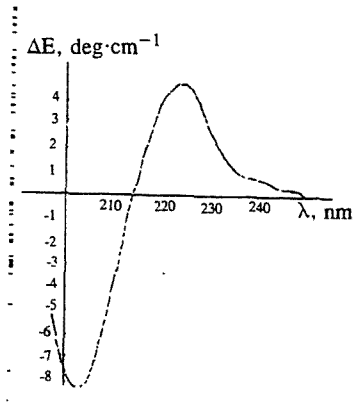

Fig. 1

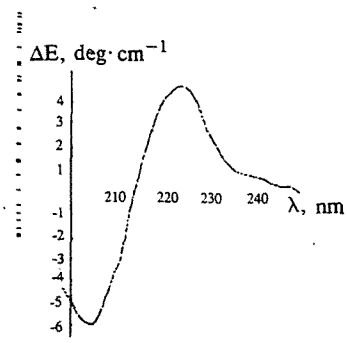

Fig. 2

Fig. 1. CD spectrum of lectin I_1 from *Datura innoxia*. This and the other figures give the spectra of solutions of the lectins in FBS at a concentration of 0.5 mg/ml.

Fig. 2. CD spectrum of the native lectin I_2 of *Datura innoxia*.

Thus, TFMS effectively cleaves glycosidic bonds in the lectins of *Datura innoxia*. The yield of deglycosylation products was 17%, which is satisfactory in the light of the carbohydrate content of the initial protein (64%).

The lectins deglycosylated at 0°C possessed hemagglutinating activity with a titer of 2^{-11}. When the reaction was performed at room temperature the lectins lost their activity completely, apparently as a result of denaturation. The results obtained permit the assumption that the carbohydrate moieties of the lectin molecules are not responsible for the manifestation of binding activity *in vitro*.

It has been reported previously that the lectins of *Datura innoxia* have unusual CD spectra, with λ_{min} at 203 nm and λ_{max} at 222 nm [3] and they lack the maxima in the 200 nm region that are characteristic for α-helices and β-structures [6]. The CD spectra of lectins I_1 and I_2 (Figs. 1 and 2) were qualitatively similar to the CD spectra of polyhydroxyproline, showing the existence of the conformation of polyproline II (PP II — a levorotatory helix with three residues per complete turn and a pitch height of 0.94 nm) [7]. As is known, a negative Cotton effect at 200 nm also shows a random-coil conformation [8]. However, these structures can be distinguished by the size of the negative maximum [7, 9]. Thus, in spite of the indeterminacy of the assumptions in a comparison with spectra described in the literature, it must be assumed that the probability of the existence of the *Datura innoxia* lectins in the PP II conformation is very high.

The presence of carbohydrates imposed limitations on the calculation of elements of the secondary structure. It therefore remained to compare the CD spectra of specimens of the lectins subjected to various modifications only qualitatively.

The CD spectrum of a deglycosylated lectin had the same bands as the CD spectrum of the native lectin, but with a lower intensity (Fig. 3). The absence of degradation on deglycosylation was shown by gel electrophoresis, TLC, and N-terminal amino acid determination. Consequently, the difference in the spectra of the native and the deglycosylated lectins reflected differences in the conformation of the polypeptide, possibly as a consequence of the partial denaturation of the deglycosylated material. This may mean that the carbohydrates are important for the native conformation of the protein component.

Figures 4 and 5 show CD spectra of lectins thermostated at high temperatures. At a denaturation temperature of 75°C, there was only a slight increase in the minimum of the Cotton effect and a decrease in the maximum at the same wavelengths as for the initial proteins. Boiling for five minutes led to a considerable decrease in the maximum in the 220 nm region, and this was accompanied by complete loss of activity. The resistance of the polyproline-II conformation to complete denaturation at 75°C has also been reported for a glycoprotein of the carrot cell wall and for synthetic poly(HyP) [9].

EXPERIMENTAL

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

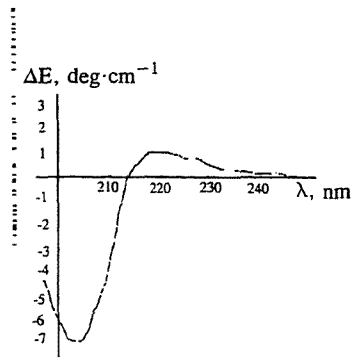

Fig. 3. CD spectrum of deglycosylated lectin I_1.

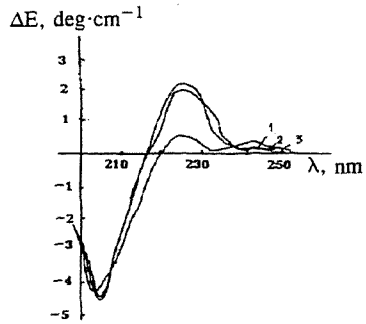

Fig. 4. CD spectra of the *Datura innoxia* lectin I_1 thermostated at: *1*) 55°C; *2*) 75°C (30 min); *3*) 100°C, 5 min.

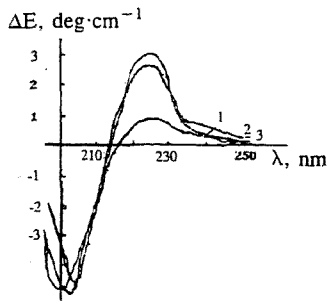

Fig. 5. CD spectrum of lectin I_2 thermostated at: *1*) 55°C; *2*) 75°C (30 min); *3*) 100°C, 5 min.

The hemagglutination reaction was conducted 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 a standard method [10]. Hemagglutinating activity is expressed in the form of a titer. As the titer we took the maximum dilution at which hemagglutination was observed.

Total nitrogen was determined by the micro-Kjeldahl method [11] and protein by Lowry's method [12], using BSA as standard.

Disk electrophoresis in 7.5 and 15% PAAG was conducted by Laemmli's method [13] at pH 8.3. The gel was fixed with 25% *n*-propanol containing 10% CH_3COOH and was stained with 2.5% Coomassie Bright Blue R-25O. Carbohydrates were revealed with the thymol/H_2SO_4 reagent [14].

Amino acid compositions were determined with the aid of a T-339 amino acid analyzer (Czechoslovakia) after hydrolysis in 5.7 N HCl at 110°C in evacuated tubes for 24, 48, and 72 h. The Ile and Val contents were determined from the results of the 72-hour hydrolysis. The Ser and Thr contents were extrapolated to zero time.

Thin-layer chromatography was conducted on plates (6 × 9 cm) coated with cellulose in the *n*-butanol—acetic acid—water—pyridine (15:3:12:10) system. The substances were revealed with a 0.5% solution of ninhydrin in acetone. The thin-layer chromatography of carbohydrates was conducted on Silufol plates in water-saturated phenol containing 1% NH_3. The revealing agent was a 0.5% solution of diphenylamine in acetone containing 0.5% H_3PO_4.

Amino sugars were determined on the amino acid analyzer after the hydrolysis of the lectins in 4 N HCl at 100°C for 4 and 16 h.

N-Terminal amino acids were found by the dansyl method [15], the dansyl derivatives being analyzed by TLC [16].

Pyroglutamic acid was determined by TLC in the form of DNS-Glu after dansylation of the product of eight-hour methanolysis according to Kawasaki [17].

Electronic absorption spectra were recorded on a Hitachi UV spectrometer (Japan) for aqueous solutions of the lectins (0.5-1.0 mg/ml) in cells with a thickness of 1.0 cm. Circular dichroism spectra were taken with the aid of a JASCO J2O dichrograph for solutions with a concentration of 0.5-1.0 mg/ml in water and in PBS in 0.5-cm cells at a sensitivity of 0.002°/cm with a time constant of 16 sec.

The lectins were deglycosylated in the following ways.

1. A lectin (2 mg) was incubated in 1 ml of 5 M NaOH in a sealed tube at 100°C for 24 h. Then the mixture was cooled to room temperature, neutralized with 5 M HCl, and analyzed.

2. A lectin was hydrolyzed in 0.5 M NaOH at 4°C for two days, followed by dialysis against water.

3. Hydrolysis with oxalic acid (12 mM) was conducted in sealed tubes at 100°C for 5 h. After cooling to room temperature the reaction mixture was desalted on a column of Sephadex G-25.

4. The lectins were hydrolyzed with hydrochloric acid under the following conditions: 1 N HCl at 23°C for 2 h, 6 h, and 20 h; 1 N HCl at 37°C for 1 h, 6 h, and 24 h; 1 N HCl at 60°C for 1 h and 20 h; 1 N HCl at 100°C for 30 min, 1 h, and 2 h; 3 N HCl at 20°C for 24 h, at 37°C for 2 h and 24 h, and at 100°C for 30 min, 1 h, and 3 h.

5. Deglycosylation with trifluoromethanesulfonic acid was carried out by Edge's method [4]. The freeze-dried lectin (97 mg) was additionally dried in vacuum over P_2O_5 for a day. A mixture of 6 ml of TFMS and 3 ml of anisole was cooled to 0°C. The lectin was dissolved in the resulting mixture, and nitrogen was passed through the solution for 30 sec. The reaction was performed in an ice bath with constant stirring in a current of nitrogen for 3 h and at room temperature for 1 h.

The deglycosylated samples were freed from reagents and monosaccharides by extraction followed by dialysis. For this, the reaction mixture was dissolved in a twofold excess of diethyl ether cooled to −40°C. To the cooled solution was added an equal volume of 50% v/v aqueous pyridine cooled to 0°C. The precipitate of pyridine salts that formed was redissolved, and the ether phase was discarded. The aqueous phase was washed with ether again. The reaction product was dialyzed against 2 mM pyridine acetate buffer, pH 5.5, and was freeze-dried.

Study of Thermal Stability. The initial solutions of the lectins in PBS, pH 7.4, with a concentration of 0.5 mg/ml, were thermostated at 50°C for 30 min, at 60°C for 30 min, at 70°C for 15 and 30 min, at 75°C for 5, 15, and 30 min, at 80°C for 5 and 30 min, at 85°C for 15 min, and at 100°C for 1 and 5 min. Then they were cooled to room temperature and analyzed.

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