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Specific inhibition by *N*-acetyl-D-galactosamine of the interaction between soybean agglutinin and animal cell surfaces

Certain plants contain proteins, known as phytohemagglutinins or lectins[1-3], which agglutinate the erythrocytes of various animals, including man. Many of these hemagglutinins have been shown by hapten inhibition studies to react with specific saccharides on the erythrocyte surface. Two phytohemagglutinins have been found to react also with specific sites on the surface membranes of other types of somatic cells: a glycoprotein from wheat germ with *N*-acetyl-D-glucosamine-like sites[4,5] and the Jack bean protein concanavalin A with α-D-glucopyranoside-like sites[6,7]. Here we present data showing that the agglutination of rabbit and human erythrocytes using soybean hemagglutinin, a highly purified glycoprotein isolated from soybean oil meal[8,9] which will be referred to as soybean agglutinin, is inhibited specifically by low concentrations of *N*-acetyl-D-galactosamine (D-GalNAc) or of disaccharides with D-GalNAc at their nonreducing end. The same compounds were also found by us to inhibit the agglutination of other types of somatic cells by soybean agglutinin[10]. We conclude therefore that the saccharide-binding site of soybean agglutinin is specific for D-GalNAc or a closely related structure.

Soybean agglutinin was isolated and purified as previously described[9]. The disaccharides β-D-GalNAc-(1→3)-D-Gal, β-D-GalNAc-(1→4)-D-Gal and β-D-GalNAc-(1→6)-D-Gal were a gift of Professor D. Shapiro and Dr. A. Acher, and α-D-GalNAc-(1→3)-D-Gal was a gift of Professor E. A. Kabat. All other saccharides were commercially available preparations of the highest purity.

Agglutination experiments were performed with rabbit erythrocytes and human (A, B and O type) erythrocytes, essentially as described by LIENER[11]. All erythrocytes were washed 4 times with saline. For trypsinization, a 4 % suspension of erythrocytes in phosphate buffered (0.05 M, pH 7.4) saline was treated with trypsin (Bacto-trypsin, Difco, 1 mg/ml) for 1 h at 37°, and the trypsinized erythrocytes were washed 5 times again with saline. The erythrocytes (trypsinized or untreated) were suspended in saline (about 2 % suspension, $1 \cdot 10^8$ cells/ml) to give an absorbance of 1.0 at 620 nm. The absorbance was measured in a Coleman Junior Spectrophotometer equipped with a special adaptor[11] using 10 mm × 75 mm round couvettes. Serial 2-fold dilutions of soybean agglutinin in saline were made in a final volume of 1 ml. To each tube was added 1 ml of the erythrocyte suspension and the absorbance of the mixture was read after it had been left standing for 2.5 h at room temperature. The amount of soybean agglutinin required to decrease the absorbance from the initial value of 0.50 to 0.25, was defined as a unit of agglutinating activity. In the absence of soybean agglutinin, there was no decrease in absorbance after 2.5 h. For inhibition studies, the serial dilutions of soybean agglutinin were made with saline containing varying concentrations of the inhibitors. From the specific activity of soybean agglutinin in the absence and presence of the inhibitor, the percentage of inhibition was calculated.

The results of the experiments on the agglutination with soybean agglutinin of trypsinized and untreated erythrocytes of different origin are given in Table I. It can be seen that soybean agglutinin interacts only weakly with all untreated erythrocytes tested, and that the agglutinability of rabbit blood cells is higher than that of human

blood cells of any type. With human erythrocytes, the agglutination was highest with type A, as expected from the fact that D-GalNAc is part of this blood group determinant[12]. The much lower agglutination of types B and O may be due to the presence of some D-GalNAc-like sites on the surface of the erythrocytes which cannot be detected by anti-A serum. In all cases, there was a marked increase in the agglutination of the erythrocytes following their treatment with trypsin. This shows that the sites with which soybean agglutinin can interact are predominantly in a cryptic form on the erythrocytes.

TABLE 1

ACTIVITY OF SOYBEAN AGGLUTININ TESTED WITH DIFFERENT TYPES OF ERYTHROCYTES

For experimental details, see text.

	Agglutinating units/mg soybean agglutinin			
	Rabbit	*Human, type*		
		A	*B*	*O*
Untreated	20	10	0.5	2
Trypsinized	4000	320	70	150

Agglutination using soybean agglutinin in the absence of trypsin treatment has also been found with various transformed cell lines[10]. Thus mouse cells transformed by polyoma virus and Simian virus 40 were agglutinated with soybean agglutinin at a concentration of 10–1000 μg/ml, under the assay conditions previously described[6].

The results of the inhibition studies with soybean agglutinin and trypsinized rabbit erythrocytes are shown in Fig. 1. It is clear that D-GalNAc and its disaccharides, whether α- or β-linked, are strong specific inhibitors of the agglutination reaction in the system tested. The amount of D-GalNAc or its derivatives required to give 50 % inhibition (0.03–0.04 μmole/ml) is about one twentieth that of D-galactose and its derivatives. Other saccharides tested (D-glucose, methyl α- and β-D-glucosides, *N*-acetyl-D-glucosamine, D-mannose, and D- and L-fucose) did not inhibit the agglutination reaction to any significant extent at concentrations as high as 50 μmoles/ml. Similar results were obtained when the effect of various saccharides on the agglutination of trypsinized human erythrocytes types A, B and O was tested.

D-GalNAc was also found to inhibit specifically the agglutination of transformed cells using soybean agglutinin. The concentration of D-GalNAc (or of its two disaccharides tested, β-D-GalNAc-(1→3)-D-Gal and β-D-GalNAc-(1→6)-D-Gal), necessary to cause 50 % inhibition of this agglutination reaction was 0.1 μmole/ml, close to the concentration needed for the inhibition of the agglutination of erythrocytes. D-Galactose inhibited the agglutination of transformed cells at 1 μmole/ml, whereas D-glucose, methyl α-D-glucoside, *N*-acetyl-D-glucosamine, D-mannose and D- and L-fucose did not affect the reaction at 100 μmoles/ml.

Further evidence for the specificity of soybean agglutinin for D-GalNAc was obtained in experiments in which the effect of various saccharides on agglutinated aggregates was tested. In these experiments, the aggregates of transformed cells, formed after incubation with 100 μg/ml of soybean agglutinin were washed 3 times

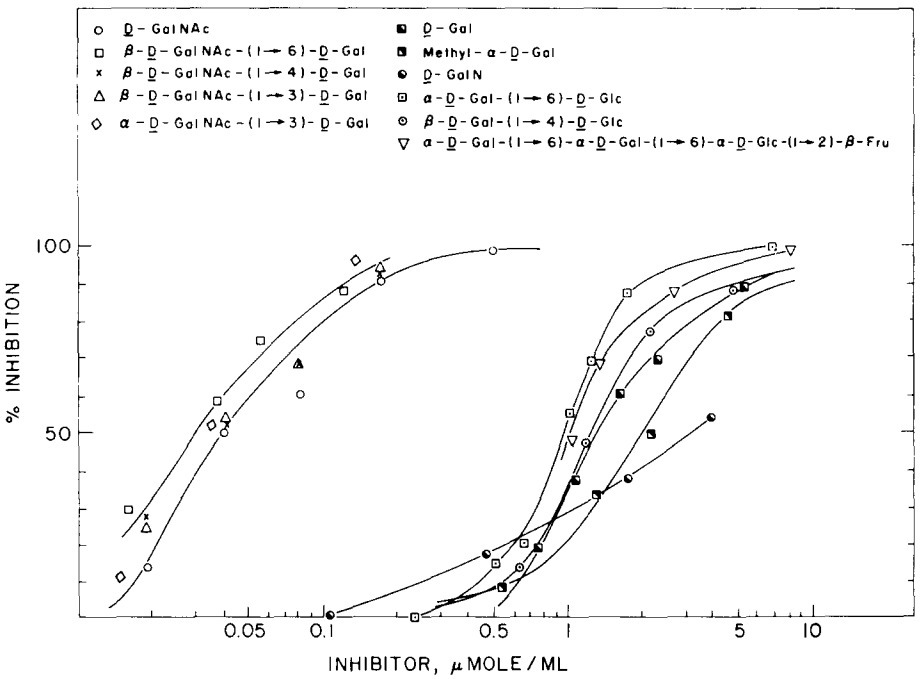

Fig. 1. Inhibition by various saccharides of the agglutination of trypsinized rabbit erythrocytes using soybean agglutinin.

with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline[6] and suspended in this buffer containing varying amounts of different saccharides. Ca^{2+}- and Mg^{2+}-free buffer was used to prevent nonspecific clumping of cells. It was found that D-GalNAc at a concentration of 0.2 μmole/ml caused complete dissociation of the aggregates within 20 min; D-Gal caused dissociation at a concentration of 4 μmoles/ml, while the noninhibitory saccharides tested (see above) were without effect even at a concentration of 1 mmole/ml. After dissociation of the aggregates, the cells were collected by centrifugation, washed 3 times with Ca^{2+}- and Mg^{2+}-free buffer and again incubated with soybean agglutinin (100 μg/ml in Ca^{2+}- and Mg^{2+}-free buffer) causing once again agglutination of the cells. Aggregates of untrypsinized rabbit erythrocytes could also be dispersed by the addition of D-GalNAc (2 μmoles/ml) or D-galactose (50 μmoles/ml). Treatment of the erythrocytes thus obtained with soybean agglutinin again caused agglutination. These results demonstrate that agglutination by soybean agglutinin is reversible.

Since *N*-acetyl-D-glucosamine and D-galactose or its derivatives are much poorer inhibitors of the agglutination reaction than D-GalNAc, it seems that soybean agglutinin requires for the binding of saccharides an equatorial 2-acetamido group and an axial 4-OH group. Furthermore, the fact that D-fucose was not an inhibitor under the experimental conditions used, shows that a 6-CH_2OH group is necessary for interaction with soybean agglutinin. Disaccharides of D-GalNAc and of D-galactose were not significantly better inhibitors than the corresponding monosaccharides (Fig. 1). This finding suggests that the combining region of the soybean agglutinin

may be no larger than the size of a monosaccharide, as found for example for concanavalin A (ref. 13) and for the agglutinin of *Helix pomatia*[14].

The specific inhibition of soybean agglutinin agglutination by D-GalNAc and its disaccharides indicates that a D-GalNAc-like saccharide is most probably part of the receptor sites of the cell surface membrane. Soybean agglutinin can therefore be used for the detection of D-GalNAc-like residues of cell membranes.

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