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A STRUCTURAL-FUNCTIONAL STUDY OF COTTONPLANT GLYCOPROTEINS

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The results are presented of a structural-functional investigation of two groups of cottonplant glycoproteins — lectin-like and extensin-like proteins — using the methods of molecular modeling of various structural fragments of the glycoproteins.

Glycoproteins (GPs) are among the polyfunctional molecules participating directly in various inter- and intracellular interactions linked with processes of biological recognition. This is owing to the high level of structural organization of the oligosaccharide fragments of the GPs and their considerable information capacity, the latter being used in numerous carbohydrate-carbohydrate and carbohydrate-protein contacts taking place at the cell surface (ligand-receptors, adhesion, cell migration, etc.) and inside it (AG-AT, enzyme-substrate, and other interactions) [1, 2]. Carbohydrate-mediated recognition between cells may include several levels of molecular information: the primary sequences of oligosaccharides must be recognizable by a protein receptor; copies of oligosaccharide epitopes (or a multiplicity of different epitopes) may be located along a polypeptide chain, their specific environment creating new functional epitopes that enhance specificity and avidity. The density of their disposition and the comparative orientation of different molecules of glycoconjugates on a cell surface determine the specificity of intercellular interactions [3, 4].

In view of this, a study of membrane and membrane-bound proteins of plant cells and also of cell-wall proteins, a large number of which serve as bearers of information in processes of molecular recognition, is of great interest. Among them, great attention is attracted by lectin- and extensin-like proteins, which possess a broad spectrum of physiological action. Up to the present time, proteins of this group isolated from plants of the Leguminosae, Gramineae, Solanaceae, and other families have been studied in most detail [1, 5, 6].

Among the proteins from cotton seeds that have been investigated, the most studied are the reserve proteins, including the 7S and 11S globulins [7], various enzymes [8], and strongly-bound proteins extractable with 2% sodium dodecyl sulfate [9]; some of them are glycoproteins and exhibit hemagglutinating activity [10]. An analogous activity has also been established for protein fractions isolated from the leaves, stems, and seedlings of cotton plants of varieties Tashkent-1, 108-F and S-4727 [11].

Also known are the participation of glycoproteins, including lectins and lectin-like proteins (LLPs), in the aggregation and adhesion of normal and tumor cells [1] and in the agglutination of blood cells [12], and their mitogenic and cytotoxic properties connected with the action of these proteins at both membrane and intracellular levels [13, 14]. Many lectins and lectin-like proteins affect the process of protein biosynthesis [15].

There is practically no such information on the cell-wall proteins (extensins). it is known that plants contain at least three classes of hydroxyproline-enriched glycoproteins: 1) a group of hemagglutinating lectins the activity of which is specifically inhibited by di- and tri-N-acetylglucosamines [16]; 2) arabinogalactan proteins (AGPs), which are predominantly localized in the intercellular matrix and are found either in the free state or associated with the plasmatic membrane and are

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the main component of plant glue and exudate [17]; and 3) extensins proper — structural proteins participating in the formation of the primary cell wall. The amount of the latter rises sharply on wounding and infectious attack, under the action of low temperatures and of ethylene, and in cell cultures [18—22]. A characteristic feature of extensins is their extremely low solubility, apparently due to inter- and intra-molecular isodityrosine cross-linking [23]. The amount of glycoproteins isolated by salt extraction is very low, and, therefore, solutions of $AlCl_3$ or $CaCl_2$ are most frequently used for their extraction [24].

We have studied two groups of cottonplant proteins — the extensin-like and the lectin-like proteins (ELPs and LLPs).

The extensin-like proteins from cotton seedlings or a suspension culture were isolated by extraction of the water-insoluble material with a 200 mM solution of $CaCl_2$, followed by purification on a column of CM-cellulose [24—26]. The lectin-like proteins were isolated from defatted cotton seeds by salt extraction followed by stepwise salting out with ammonium sulfate. Galactose-specific and Con-A-binding proteins were obtained from the total LLP_{60-80} fraction by affinity chromatography.

All the protein fractions were characterized electrophoretically in the Laemmli system with 0.1% sodium dodecyl sulfate. The presence of carbohydrates in the proteins under investigation was established by treating the electrophoretic gels with concanavalin A—peroxidase followed by staining with diaminobenzidine [26]. The total sugar contents were determined spectrophotometrically, using the anthrone—sulfuric acid reagent. For the LLPs an ELPs, they were 8—11% and 60—65%, respectively.

The presence of hydroxyproline, which is characteristic for proteins of the extensin family, was determined with the aid of PTC derivatives [26].

The LLP_{60-80}s caused agglutination of human erythrocytes, which was most considerable for blood groups I and II; they possessed no activity of the ELP type.

We have previously obtained hybridomas producing monoclonal antibodies (mcABs) to cottonplant membrane proteins isolated from two-day seedlings. Electrophoretic analysis of the membrane proteins showed their highly heterogeneous composition, including polypeptide zones of the glycoproteins isolated from the seeds [27].

The membrane proteins also caused the agglutination of human erythrocytes — most considerably those of blood groups I and II. Immunochemical analysis with the aid of mcABs to the cottonplant membrane proteins showed the reactivity of a number of the mcABs with the LLPs and ELPs [26]. These results, in combination with electrophoretic analysis, permitted the hypothesis that there were common sections of the binding of the LLPs both with the ELPs and with the membrane proteins; i.e., it is possible to speak either of a common nature of the antigenic determinants or of a homology of individual polypeptide sections.

It is known that the mechanism of mitogenic stimulation of lymphoid cells under the action of lectins includes binding with the carbohydrate components of the cell receptors acting as triggers starting the cascade of intracellular processes affecting the nucleus [1]. Therefore, by evaluating the level of synthesis of nucleic acids and proteins in the nucleus it is possible to determined the anti- or proliferative activities of lectins and lectin-like proteins.

In this connection, we have studied the action of cottonplant LLPs and ELPs on the proliferative activity of animal and plant cells from the level of inclusion of ^{3}H-thymidine. It was shown that the ELPs inhibit the inclusion of ^{3}H-thymidine in both plant and animal cells; i.e., they exhibit an antiproliferative activity. The influence of LLPs in various cell systems bears an opposite character. Thus, while in human cells (lymphocytes) and animal cells (mouse myelocytes) these proteins exhibit mitogenic properties, in a homologous system (cottonplant suspension culture) their influence was shown in an inhibition of DNA synthesis. These differences are apparently due to the specific nature of the interaction of ELPs and LLPs with the cell receptors of homologous and heterologous systems [25, 28].

It has been established that plants most frequently contain glycoproteins (GPs) with N-bound oligosaccharide (OSs) of two types: the oligomannose type (for example, the agglutinins of legumes and soybeans) and the xylose-fucose or modified type, specific for plants and found, for example, in *Erythrina* and *Sophora* lectins. Phytohemagglutinins from *Phaseolus vulgaris* contain OSs of both types on a single polypeptide chain but in different glycosylation sites.

The carbohydrate fragments of GPs contain not only *D*-Man but also *D*-Gal, α*D*-GalNAc, *D*-GlcNAc, α-Fuc, and neuraminic acid. Characteristic for the N- and O-glycans is the presence of the core fragments always found in GP molecules and of variable sections differing in composition, size, and degree of branching that determine the specifics of recognition. Plant O-glycans (including extensin) include polypeptide sections having a linear form and resembling a comb in structure — i.e., short carbohydrate chains as teeth project from a rigid proline-rich polypeptide backbone [30].

Lectins of known structure and carbohydrate specificity have been used to analyze and determine the specificity of new

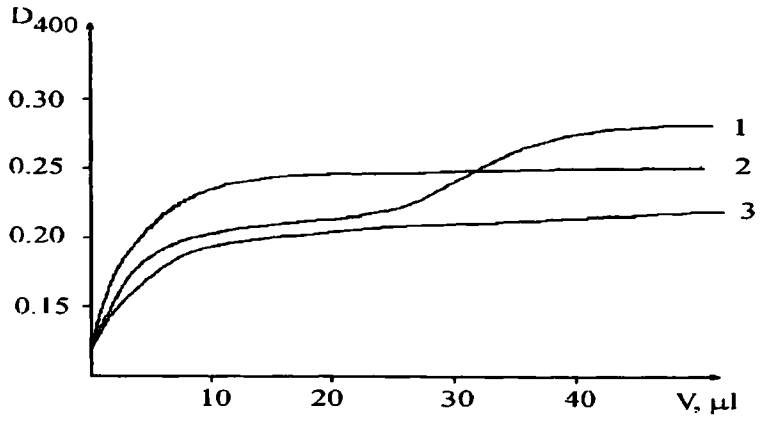


Fig. 1. Turbidimetric study of the precipitation of cottonplant LLPs: *1*) total LLPs + Con A; *2*) total LLPs + peanut lectin; *3*) total LLPs + ricin (RCA_{120}).

glycoproteins [31]. The nature of their interaction in vitro and information obtained from experiments in cell systems have led to the finding of definite relationships and the revelation of structural fragments participating in these interactions. In spite of the similarity, on the whole, of animal and plant GPs, a number of fundamental differences in their structures have been found: thus, plant N-glycans contain the fragments Man-β (core)—1,2-β-*D*-Xyl and GlcNAc-α (core)-1,3-α-*L*-fuc, not appearing in plants [32].

At the present time, some relationships between the structure and specificity of lectins have been established, but none of them are absolute. Thus, the *D*-Man/*D*-Glu-specific lectins belonging to one group from monocotyledonous plants of the Amaryllaceae family can distinguish *D*-Man from *D*-Glu through the axial OH-2 group (in contrast to Con A, the lectin of the peanut (PNA) and others) [33]. Many glycoconjugates (GCs) exhibit affinity not for mono- but for di- and oligosaccharides, while for Asn-bound N-glycans a capacity has been found for distinguishing the degree of branching and the number and composition of the antennae. Thus, a group of 20 *D*-Gal/*D*-GalNAc-specific lectins of animal and plant origin has been divided into six classes according to their specificity for the disaccharides Gal-β-GalNAc of different configurations both in independent form and in the composition of larger determinants [34]. Analysis of the results of known structural-functional investigations in the GP series shows the complex nature of the interaction of GPs with partner molecules ensuring modulation of the biological functions of receptors, enzymes, lectins, antibodies, and other carbohydrate-containing biomolecules [35—37].

A study of the LLPs from cotton seeds has established the presence in them of a set of glycoproteins with different carbohydrate specificities. Competitive hemagglutination has shown the presence in cottonplant LLPs of proteins with *D*-Glu- and D-Gal-specificities. Moreover, in precipitation experiments the turbidimetric method has been used to study the dynamics of the interaction of cottonplant LLPs with lectins of known structure and specificity: Con A, RCA_{120}, and peanut lectin (PNA). The results are presented in Fig. 1.

The total LLPs from cotton seeds reacted most powerfully with Con A, which showed the presence of oligomannosyl glycans in their molecules. However, nonterminal mannose residues substituted at C-2 by a glycosidic bond may also take part in the interaction. The main requirement for strong binding with Con A is the presence of two α-*D*-Man residues attached to a third residue with the formation of a fork-like structure. Thus, the interaction of cottonplant LLPs with Con A witnesses the presence in their molecules of carbohydrate chains of the oligomannosyl type and also of biantennary chains of the complex type bound with Asn. The interaction with RCA_{120} and PNA shows β-*D*-galactosyl specificity, including affinity for the fragment β-*D*-Gal-1,3-β-*D*-Gal.

Together with experimental approaches, a theoretical study of the spatial structures of the carbohydrate fragments of glycoproteins with the aid of molecular modeling by the methods of quantum chemistry and molecular dynamics is making it possible to develop models of the interaction of glycoproteins with ligands in order to discover the mechanisms of recognition at the molecular level.

TABLE 1. Characteristics of the vanderWaals Parameters for Extensin

Fragment	Polar region				Nonpolar region		Total surface	
	I		II					
	S	E	S	E	S	E	S	E
I	200.2	5.0	0.0	0.0	223.0	-16.7	423	-11.7
II	196.1	4.9	0.0	0.0	216.1	-16.2	412	-11.3
III	385.0	9.6	0.0	0.0	368.7	-27.7	753	-18.0
IV	539.2	13.5	0.0	0.0	532.5	-39.9	1071	-26.5
V	358.8	9.0	0.0	0.0	315.4	-23.7	674	-14.7
VI	436.1	10.9	0.0	0.0	342.9	-25.7	778	-14.8
VII	588.6	14.7	69.1	15	463.2	-34.7	1120	-18.5
VIII	694.9	17.4	72.0	1.6	536.9	-40.9	1303	-21.3
IX	811.7	20.3	148.5	3.3	685.3	-51.4	1645	-27.5
X	458.0	11.5	0.0	0.0	413.3	-31.3	875	-19.8
XI	460.9	11.5	0.0	0.0	405.1	-30.4	866	-18.9
XII	451.0	11.3	0.0	0.0	403.8	-30.8	854	-19.0

Note: I) saturated surface; II) unsaturated surface; *S*) surface area, Å; *E* —— energy, kcal.
Here and in Table 2: I —α-*D*-Man-1,3-(α-*D*-Man-1,6)-β-*D*-Man ; II —α-*D*-Man-1,3(α-*D*-Man-1,6)-β-*D*-Man with interMHBs; III — pentasaccharide core fragment of the N-glycans; IV — oligomannosyl fragment of the N-glycans; V —Ser-$(Hyp)_4$-$(Ara)_2$; VI —Ser-$(Hyp)_4$-Val-$(Ara)_2$; VII —Ser-$(Hyp)_4$-Var-IDT-$(Ara)_2$; VIII —Ser-$(Hyp)_4$-Val-IDT-Lys-$(Ara)_2$; IX —Ser-$(Hyp)_4$-Val-IDT-Lys-IDT-$(Ara)_2$; X —Ser-$(Hyp)_4$-$(Ara)_4$; XI —Ser-$(Hyp)_4$-$(Ara)_4$; with hydrogen bonds between Ser and Ara; XII — Ser$(Hyp)_4(Ara)_4$ with hydrogen bonds between Ser—Ara and Ara—Ara.

In this connection, we have created a data base for the structural elements of GPs — mono- and oligosaccharides and amino acids — using the methods of molecular mechanics. Within the framework of the ALCHEMY and PCM—MMX programs we constructed molecular models of mono-, di-, and trimannosides and calculated the geometric and energy parameters of the molecules in the initial state and also with intramolecular hydrogen bonds (interMHBs), which, as is known, play a large role in the organization of the structure and the functional activity of biomolecules.

From the values of ΔH_f obtained we determined the energy of formation of the interMHBs for α-*D*-Man — 2.48 kcal/mole; α,β-1,3-dimannoside — 3.6 kcal/mole; and 1,3(1,6)-trimannoside — 4.49 kcal/mole; these figures agree with known values of hydrogen bond energies [38].The calculation also confirmed the expected gain in energy on the formation of interMHBs, which indicates a stabilization of the structures. The trimannoside of the fork type that we calculated — α-*D*-Man-1,3-(α-*D*-Man-1,6)-β-*D*-Man — (Tables 1 and 2; structures I and II) is the most common fragment of the N-glycans entering into the composition of the pentasaccharide core (Fig. 2, structure III). In this fragment the 1,3 branch is rigidly fixed and in the glycans has a preferred conformation, while the 1,6-branch, in which α-*D*-Man is attached to the extra-ring —CH_2OH group by a glycosidic bond, is labile; the mobility of the 1,6-antenna ensures the possibility of the formation of various conformers.

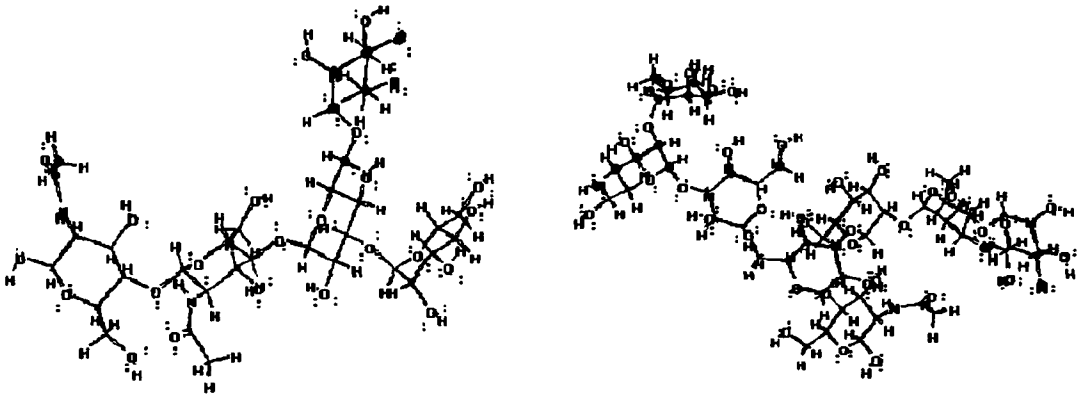
We have carried out the step-by-step molecular modeling of carbohydrate fragments of glycoproteins and have obtained a set of energy and geometric parameters of optimized glycan structures of two types — oligomannosyl antennae forming components of LLPs and tetrahydroxyproline-oligoarabinosides in combination with various peptide fragments (ELPs). Elongation of the antennae of a pentasaccharide in the oligosaccharide chains of the N-glycans change the geometric parameters most favorably in a 1,6-antenna (Fig. 2, structure IV).

In modeling the branched structure of the oligomannosyl fragments of the N-glycosides (Fig. 2, structure IV) it was shown that the torsional angles between the antennae and the core branch changed mainly in the 1—6 antenna.

An evaluation of the electric fields on hydration enabled us to determine the total area and the energies of the polar and nonpolar zones of the molecules, and also the energy of the whole hydration sheath. Thus, a comparison of the parameters of the aqueous solvation sheaths of structures III and IV showed that their hydration is energetically favorable, which reflects the compactization of these molecules in aqueous solutions (Tables 1 and 2, structures I—IV).

TABLE 2. Characteristics of the Aqueous Solvation Sheath of Fragments of Extensin

Fragment	Polar region				Nonpolar region		Total surface	
	I		II					
	S	E	S	E	S	E	S	E
I	272.2	6.8	0.0	0.0	326.8	-24.5	599	-17.7
II	274.2	6.9	0.0	0.0	330.2	-24.8	604	-17.9
III	499.2	12.5	0.0	0.0	504.5	-37.8	1003	-25.4
IV	624.8	15.6	0.0	0.0	721.2	-54.1	1346	-38.5
V	402.7	10.1	0.0	0.0	422.1	-31.7	824	-21.4
VI	485.1	12.1	0.0	0.0	428.6	-32.1	913	-20.0
VII	773.5	19.3	48.0	1.1	638.9	-47.9	1460	-27.5
VIII	876.8	21.9	42.3	1.0	748.6	-56.1	1668	-33.3
IX	1044.0	26.1	99.45	2.2	971.2	-72.8	2115	-44.5
X	488.3	12.2	0.0	0.0	563.4	-42.3	1051	-30.1
XI	509.4	12.7	0.0	0.0	515.2	-38.6	1024	-25.9
XII	503.1	12.6	0.0	0.0	519.1	-38.9	1022	-26.4



III IV

Fig. 2. Pentasaccharide core fragment (III) in the oligomannosyl fragment of N-glycans (IV)

III. α-*D*-Man-1,6 > β-*D*-Man-1,4-β-*D*-Glc-Nac-1,4-β-*D*-Glc-Nac
α-*D*-Man-1,3 >

IV. α-*D*-Man-1,2-α-*D*-Man-1,3-α-*D*-Man-1,6 > β-*D*-Man-1,4-β-*D*-Glc Nac
α-*D*-Man-1,2-α-*D*-Man-1,2-α-*D*-Man-1,3 >

Among O-glycans, the greatest interest is presented by the structure of extensin. As mentioned above, a characteristic feature of the extensins is their high content of hydroxyproline, and also considerable amounts of tyrosine, lysine, serine, histidine, valine, and threonine [22, 39]. Carbohydrates make up about 2/3 of the glycoprotein mass. A study of the fragments obtained by exhaustive acid hydrolysis and also with the aid of polyclonal antibodies obtained to soluble precursors of glycosylated and deglycosylated extensin has shown that hydroxyproline interacts with *L*-arabinose to form an O-glycosidic bond, fragments with mono-, di-, tri-, and tetraarabinosyl-Hyp having been detected [22, 40, 41]. It has been established by the tryptic hydrolysis of deglycosylated extensin that the structure of this molecule is highly conservative, containing frequently repeated sequences of the type of Ser-$(Hyp)_4$, Tyr-Lys-Tyr-Lys, Ser-$(Hyp)_4$-Val-IDT-Lys-IDT-Lys, etc, [24, 39].

We have constructed molecular models of the repeated core fragment of tomato extensin (Fig. 3, structure I) and of one of the most biologically significant fragments obtained by tryptic hydrolysis (Fig. 3, structure II) [22, 24, 42]:

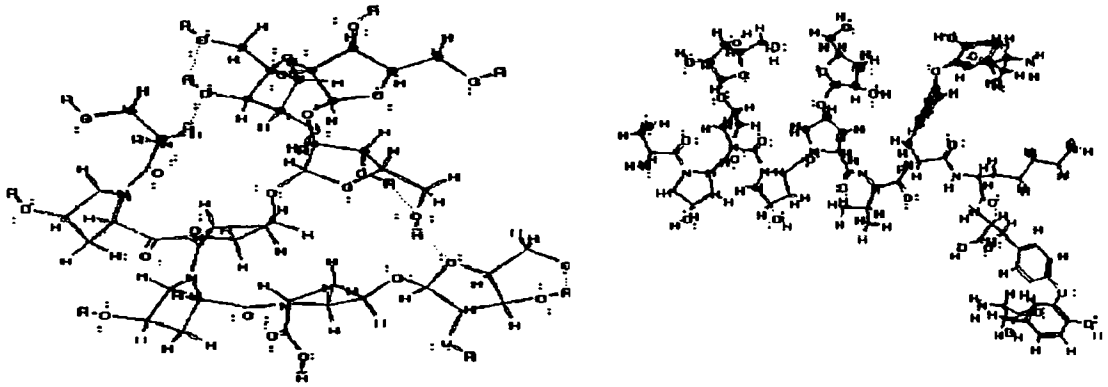


Fig. 3. Molecular models of the extensin fragments:

I. Ser -Hyp-Hyp-Hyp-Hyp
(Ara)$_3$ Ara

II. Ser -Hyp-Hyp-Hyp-Hyp-Val-IDT-Lys-IDT
Ara Ara

As can be seen from the molecular model, the polypeptide chain has a linear structure in which $(Hyr)_4$ forms a second-order β-helix that is stabilized by the formation of hydrogen bonds, both between serine and arabinose and between two arabinoses, which probably imparts rigidity to the hydroxyproline core and to the extensin molecule as a whole [39]. On the other hand, the products of the acid hydrolysis of extensin contain residues of hydroxyproline arabinosylated to various degrees but with the predominant formation of the fragment Hyp-Ara_4 [22]. The molecular modeling of fragments of extensin has shown that the addition of three arabinosyl residues to Hyp forms a kind of cavity (0.5 × 0.6 nm) that is stabilized by hydrogen bonds, although the Ara-Ara hydrogen bond is unstable and exists in only one of the possible transition states, the energy parameters of which are extremely close (Tables 1 and 2, structure VIII; Fig. 3, structure I). On the addition of mono-Ara to Hyp, a comb-like structure arises; such structures play an important role in carbohydrate—carbohydrate and carbohydrate—protein interactions. The introduction of iso-di-tyrosine residues (Fig. 3, structure V) causes rearrangements within the structure due to local change in the torsional angles.

An evaluation of the electrostatic fields of the sheaths of extensin fragments and their van-der-Waals surfaces has permitted a determination of the total area and the energies of the polar and nonpolar zones of these molecules and also of the energy of the whole hydration sheath (Tables 1 and 2). As can be seen from the tables, on the introduction of the aromatic amino acid iso-di-tyrosine, the energy contribution to the nonpolar field changes, which leads to an energetically more favorable state in the whole structure (Table 1 and 2; structures VII—IX).

As can be seen from the three-dimensional model (IX), the iso-di-tyrosine residues are located in the frontal regions of the fragments, which ensures their participation in inter- and intramolecular interactions. In actual fact, as is known, aromatic amino acids can interact with the carbohydrate components of glycoproteins to form charge-transfer complexes of the π—σ and n—π types [42, 43—45]. It is apparently through such interactions that biological recognition with the participation of glycoproteins is effected.

A comparison of calculated and experimental data indicates on perspective using this approach for research of glycoproteins role and their participation in processes of "recognition" on molecular level.

EXPERIMENTAL

The lectin- and extensin-like proteins were isolated and characterized by us as described in [26].

The total membrane proteins were isolated in the following way. Seeds that had been treated with concentrated sulfuric acid were germinated in rolls of moistened filter paper at 28°C for two days. The seedlings were collected and were homogenized in 25 mM tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 3 mM EDTA, 5 mM 2-mercaptoethanol, and 1 mM

PMSF in a Potter homogenizer. All the biochemical work was conducted at 4°C.

The homogenate was filtered through four layers of gauze and was centrifuged at 1500 rpm for 10 min. The deposit was discarded, and the supernatant was centrifuged at 100,000 *g* for 90 min (Beckman L5-75 ultracentrifuge, SW-28 rotor, USA). After centrifugation, the supernatant, containing cytoplasmic proteins, was also discarded, and the deposit that had been obtained was resuspended in 10 mM Na phosphate buffer, pH 7.5, and used for further work. The total membrane proteins were then fractionated in a 10/34/45% stepwise gradient of sucrose in 25 mM tris-HCl buffer, pH 7.4, at 100,000 *g* for 90 min. The interphase layer between 34 and 45% sucrose was carefully extracted and washed with a 10-fold volume of PBS, pH 7.5, and this was also used for further work. The proteins were characterized electrophoretically in 15% PAAG in the presence of 0.5% sodium dodecyl sulfate [26].

The hemagglutinating activity of the membrane proteins was determined with a 2% suspension of human erythrocytes in 96-well polystyrene plates by a standard procedure [46].

The precipitation of the cottonplant LLPs with known lectins was studied turbidimetrically in the following way. In a 1-cm thick optical cell, 0.6 ml (600 μg) of LLPs were mixed with increasing amounts (20—600 μg) of known lectins in 5 mM sodium phosphate buffer, pH 6.8. The mixture was gently stirred and absorption was determined every 10—15 min from the optical density of the solutions at 400 nm until a constant value had been reached. The formation of precipitates was recorded for 24 hours. Optical densities were measured on a Beckman Specord spectrophotometer.

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