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# PREPARATION OF AFFINITY SORBENTS BASED ON MONOCLONAL ANTIBODIES TO COTTONPLANT MEMBRANE PROTEINS

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*An immunosorbent has been obtained by conjugating mcABs 2C8.C7 to membrane proteins from cotton seedlings with BrCN-Sepharose 4B. As a result of the affinity chromatography of the total lectin-like proteins of the cotton plant on this sorbent, two fractions of polypeptides have been isolated, and these have been subjected to electrophoretic analysis.*

Interest in the study of the plasmatic membranes of plant cells is due to their polyfunctionality and participation in various inter- and intracellular processes. The majority of these proteins are glycoproteins, which determines the specifics of their functioning in such most important processes as differentiation, protective reactions, and protein—receptor and AG—AB interactions. The key role in them is played by protein—carbohydrate and carbohydrate—carbohydrate contacts, which form the chemical basis of the physiological action of hormone-receptor complexes, lectins, stress proteins, enzymes, cell wall proteins, etc. [1, 2].

However, the revelation, identification, and structural-functional study of the proteins of the cell wall and of membrane and membrane-bound proteins is greatly hindered by their small amount and high degree of aggregation. The discovery of the method of monoclonal antibodies (mcABs) has opened up a new and extremely promising biotechnological approach to the solution of these problems [3, 4].

We have previously obtained a hybridoma producing mcABs to membrane proteins isolated from two-day cottonplant seedlings [5, 6]. Among them, a number of antibodies exhibit affinity for lectin-like proteins (LLPs) isolated from cotton seeds. Interest in LLPs is due to the fact that these proteins fulfil a protective function in plants and cause the aggregation and adhesion of cells, the agglutination of blood cells, etc. [1, 7]. In view of this, the isolation, purification, and identification of these proteins with the use of biospecific sorbents is of great interest.

In the present paper we consider the preparation of a biospecific sorbent based on mcABs for fractionating lectin-like and membrane proteins isolated from the cotton plant. From among the mcABs obtained, for the creation of an affinity column we chose mcABs of the 2C8.C7 clone, since these hybridomas are more stable and give a higher antibody titer. The antibodies were isolated preparatively from the ascitic liquid of a 2C8.C7 hybdridoma by stepwise precipitation with ammonium sulfate, followed by purification on a column of DEAE-cellulose. The details are given in Fig. 1, *a*. The working concentration of the 2C8.C7 mcABs was determined with the aid of solid-phase enzyme immunoassay as 12 μg/ml. Using a Mouse Monoclonal Typing Kit and various antisera, we also determined the type and isotype of the antibodies obtained. It was found that the mcABs of the clone 2C8.C7 belonged to the subclass IgC2a.

Lectin-like proteins were isolated from cotton seeds ($LLPs_{60-80}$) by a method described previously [8]. Electrophoretic analysis showed that the total lectin-like proteins from cotton seeds consisted of a complex multicomponent protein system including glycoproteins (Fig. 1, *b*); its separation and the isolation of individual proteins required repeated precipitation with ammonium sulfate, further purification on various sorbents, and so on. However, the preparation of mcABs with a high affinity for these proteins permitted the use of a modern methodological approach.

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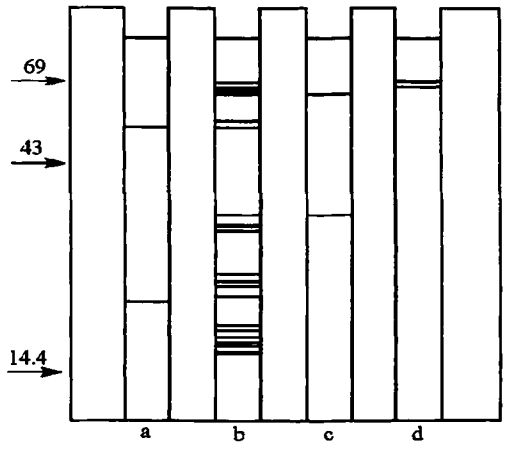


Fig. 1

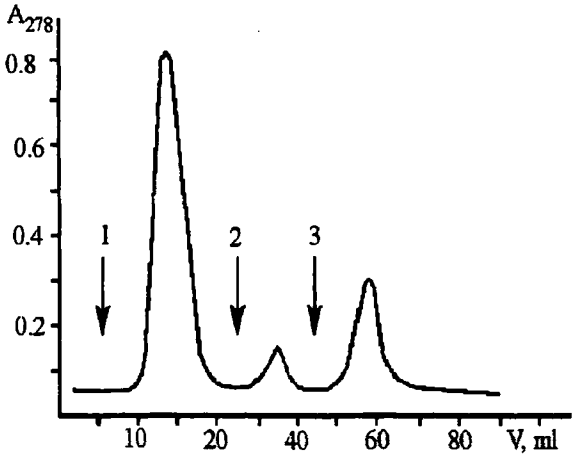


Fig. 2

Fig. 1. Electrophoregrams of proteins in Na-DDS PAAG: *a*) mcABs 2C8.C7; *b*) total LLPs; *c*) the $LLP_{60-80}$ fraction eluted from the column at peak 2; *d*) the $LLP_{60-80}$ fraction eluted from the column at peak 3.

Fig. 2. Elution profile in the affinity chromatography of the $LLPs_{60-80}$ on a column with 2C8.C7 mcABs—BrCN—Sepharose.

To synthesize the immunosorbent we conjugated the 2C8.C7 mcABs with BrCN-Sepharose 4B, as described in the Experimental section.

The proteins $LLPs_{60-80}$ were deposited in 0.1 M Na borate buffer, pH 8.5—0.5 M NaCl on a column and were incubated in the gel for maximum binding. Then the column was washed with the same buffer to eliminate unbound proteins; elution was performed stepwise in two stages with O.1 M Na acetate buffer at different pH values and ionic strengths, and also in the presence of salts of bivalent metals.

The affinity chromatography of the LLPs on the immunosorbent obtained is illustrated in Fig. 2. When 10 mg of $LLPs_{60-80}$ was deposited, 6.85 mg of unbound proteins was found in peak 1; when the bound components were eluted, 0.6 mg of protein was collected in each of peaks 2 and 3.

Thus, of the total mass of $LLPs_{60-80}$, about 70% showed no affinity for the 2C8.C7 mcABs used as ligand; about 12% of proteins out of the whole set of $LLPs_{60-80}$ bound to the sorbent, and these were eluted from the column with an increase in the acidity of the eluent and a change in its ionic strength. It must be mentioned that the presence of ions of bivalent metals is a necessary condition for the specific binding of many lectins and lectin-like proteins, including enzymes, receptors, etc. [10]. The chromatographic profile shows the stepwise nature of the elution of the bound LLPs.

Electrophoretic analysis of the chromatographic fractions obtained (peaks 2 and 3) showed the presence in peak 2 of two polypeptides with molecular masses of approximately 60 kDa for the major component and 30 kDa for the minor zone (Fig. 1, *c*). In peak 3, two closely adjacent zones with a molecular mass of about 70 kDa were revealed (Fig. 1, *d*). A comparison with electrophoregrams of membrane proteins and $LLPs_{60-80}$, showed that these protein zones were present in them, which is apparently the reason for the immunochemical cross-reactions of the membrane proteins and $LLPs_{60-80}$ with the 2C8.C7 mcABs that we have found previously [5]. Also of interest in this connection is the fact that the lectin Con-A, the molecule of which is known to lack carbohydrate fragments, likewise interacts with the bound mcABs; this may show a polypeptide nature of the determinant sections in the LLP molecules that are responsible for binding with the 2C8.C7 mcABs.

The results obtained show that from the heterogeneous mass of $LLPs_{60-80}$ it is possible to isolate narrow fractions with a specific affinity for mcABs used as ligands. The electrophoretic map gives grounds for assuming that the fractions obtained in the second and third peaks corresponded to peptides or glycopeptides with a high degree of homology and, possibly, a modification of individual positions in the determinant sections. The latter apparently explains the stepwise nature of elution, which depends on the ionic conditions of the medium.

Thus, we have shown the possibility of fractionating a complex multicomponent mixture of plant proteins with the aid of immobilized mcABs, which will permit the creation of a basis for the development of new strategies in the investigation of the structure and functions of biopolymers.

## EXPERIMENTAL

Hybridomas producing mcABs to cottonplant membrane proteins were obtained as described in [5].

**Ascitic fluid** containing mcABs was obtained in the following way. 2C8.C7 hybrid cells were sown in 50 ml flasks at a concentration of $10^6$ cells/ml in a volume of 5 ml. After some days, the biomass of cells was collected by centrifuging at 1000 rpm for 10 min on a K-23 centrifuge. The deposit was resuspended in RPMI-1640 medium, and, at a concentration of $10^6$ cells/ml in a volume of 0.5 ml, was administered intraperitoneally to mice of the BALB/c line that had been injected with 0.5 ml of adjuvant (Sigma, USA) one day before. Ascitic fluid was extracted from the abdominal cavity and was centrifuged at 10 thousand rpm for 10 min on a K-24 centrifuge, after which the deposit was discarded.

**The 2C8.C7 mcABs** were isolated from the supernatant by two precipitations with 50% ammonium sulfate; after centrifugation, the deposit was resuspended in 1 ml of 5 mM sodium phosphate buffer (SPB), pH 7.8, and the proteins obtained were desalted on a 1 × 50 cm column of Sephadex G-25 (Pharmacia, Sweden).

**The ion-exchange chromatography** of the desalted proteins was conducted on a 2 × 26 cm column of DEAE-cellulose (Whatman, United Kingdom), using for elution a molarity gradient of NaCl from 0 to 0.25 M in SPB. The antibodies obtained from the DEAE-cellulose column were characterized electrophoretically in 10% PAAG with 0.1% sodium dodecyl sulfate [10] followed by staining with Coomassie Blue. After each column, the affinity of the antibodies was checked by the ELISA (enzyme-linked immunosorbent assay) method as described in [5]. The antibodies collected from the column were stored in 50% glycerol.

**The isotype of the antibodies** was determined with the aid of an ICN Immunobiologicals Mouse Monoclonal Typing Kit and of individual antibodies of various isotypes — IgG1, IgG2a, IgG2b, and IGM (Sigma, USA). In the latter case, we took Schleicher & Schuell nitrocellulose filters. These were wetted in distilled water and dried at room temperature overnight or at 60°C for 1 h. Then the mcABs were deposited spotwise (0.5 μl) with the aid of a capillary, and the filters were incubated at 60°C for 1 h to fix the antibodies. After this, they were placed in a buffer containing 50 mM Tris-HCl, 200 mM NaCl (buffer 1) and 0.1% Tween-20, pH 7.4 (TBS-T) and were incubated at room temperature with constant shaking for 40 min. The filters were placed in individual test-tubes containing various antisera, and were incubated at room temperature with constant shaking for 1 h. They were washed several times with TBS-T buffer and were treated with a IgG—horseradish peroxidase conjugate (Sigma, USA) in TBS-T buffer at room temperature for 1 h. The filters were washed as described above, and the peroxidase substrate — 0.56 ml of 2-bromo-1-naphthol (conc. 2 mg/ml in methanol)—7.5 ml of 10 mM Tris-HCl, pH 7.4—0.26 ml of 0.3% $H_2O_2$ — was added to the wells. In the case of a positive response a deep blue color developed.

**The immunosorbent** was prepared by conjugating 2C8.C7 mcABs with BrCN-Sepharose 4B (Sigma, USA) according to the recommendation of the Pharmacia firm. For this, dialyzed antibodies (three changes against 0.1 M Na carbonate buffer, pH 8.3—0.5 M NaCl) were mixed with BrCN-Sepharose that had been swollen in 1 mM HCl and washed free from acid, and the mixture was left on a shaking machine at 4°C for conjugation overnight. The excess of unbound protein was eliminated by washing with 0.1 M carbonate buffer, pH 8.0. The suspension was treated with a 1 M solution of ethanolamine, pH 9.0, over 2 hours, after which the gel was carefully washed with 0.1 M Na carbonate buffer, pH 8.0, and then with 0.1 M Na acetate, pH 4.0, and 0.1 M Na borate, pH 8.5, buffers containing 1 M NaCl. The last buffer was also used for storing the sorbent.

**Affinity chromatography** was conducted on a 2.6 × 8 cm column charged with 6 ml of the immunosorbent gel containing 30 mg of 2C8.C7 antibodies. Before chromatography, the $LLPs_{60-80}$ were dissolved and dialyzed against 0.1 M Na borate buffer, pH 8.5—0.5 M NaCl. After being deposited on the column, the proteins were incubated in the gel for 2 h by recycling with the aid of a peristaltic pump. Elution was conducted with 0.1 N Na borate buffer, pH 8.5—0.5 M NaCl (solution 1), then with 0.1 M Na acetate buffer, pH 3.2—0.5 M NaCl (solution 2), and with 0.1 M acetate buffer, pH 6.0, containing 0.15 M NaCl and 1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$ (solution 3). An elution profile of the proteins was recorded with the aid of a Uvicord having a light filter with $\lambda = 278$ nm. The protein fractions collected were combined, dialyzed, and lyophilized.

**The electrophoresis of the proteins** was conducted in 15% PAAG with 0.1% Na DDS in the Laemmli system, followed

by staining the gel with silver nitrate [11]. As markers we used bovine serum albumin (69 kDa), ovalbumin (43 kDa), and lysozyme (14.4 kDa).

Protein contents were determined by the Lowry method [12].

All the reagents used were of chda [pure for analysis] or khch [chemically pure] grade.

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