Chemistry of Natural Compounds, Vol. 30, No. 1, 1994

STUDY OF THE MEMBRANE PROTEINS OF THE COTTON PLANT USING MONOCLONAL ANTIBODIES

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UDC 577.112

The total membranes from cottonplant shoots have been used as the antigenic material for obtaining monoclonal antibodies (mcABs). Eleven stable hybrids producing mcABs to components of the membrane proteins have been selected. Antibodies to various membrane fractions and to fractions of lectin-like proteins have been revealed. Structural characteristics of the carbohydrate components of cottonplant glycoproteins have been determined by cross-immunochemical analysis.

Proteins of the plasmatic membranes of plant cells fulfill extremely important functions. They are endo- and exogenous factors of differentiation and intercellular contacts, enzymes, receptors, lectins, and stress proteins. Chemically, the majority of them are glycoproteins.

Experimental difficulties in the revelation and purification of membrane proteins and also the problem of the absence of clear biochemical markers can be considerably simplified by the use of mcABs to functionally important antigens of cytoplasmic membranes.

The greatest attention is being attracted by extensin and by lectin-like proteins (LLPs), since the chemical aspects of their biological action have been inadequately studied. Only for some lectins such as those of the potato and of maize has information been obtained on the specific nature of the protein–carbohydrate interactions that are responsible for the participation of lectins in the regulation of cell processes and in the recognition of a pathogen by the plant [1, 2].

The hydroxyproline-enriched protein called extensin is also a glycoprotein. It participates in the formation of the primary cell wall. The expression of extensin is sharply intensified after wound injury or an attack of pathogens, and also under the action of low temperatures on the plant, which shows its role in the protective strategy of plants [3, 4].

We have developed a hybridoma technology of obtaining monoclonal antibodies to various epitopes of membrane proteins, using as the immunogen the total proteins of the plasmatic membranes of the cotton plant (MPs). Hybridomas were obtained from myeloma X63Ag8.653 cells and splenocytes of immunized mice. Hybridization was achieved by Milstein's method with some modification [5]. The hybridomas were screened by the ELISA solid-phase immunoenzyme method. Because of the lack of individual plasmatic membrane proteins, the total proteins were used both as the immunogen and as the antigen for the primary screening of the hybridomas. More than a hundred hybridomas were obtained. The producing hybridomas were then cloned by the limiting dilution method. Eleven populations of stable producing clones were selected and these were then grown in Balb/c mice to obtain ascitic fluid. Individual mcABs were obtained from the ascitic fluid by precipitation with ammonium sulfate and were purified on CM-cellulose.

Table 1 shows the 11 stable populations of mcABs selected from their affinity for the total proteins of the plasmatic membranes. The membrane proteins are extremely heterogeneous in composition [6], as was confirmed by electrophoretic analysis (Fig. 1, *A*). In view of this, we fractionated the MPs in a sucrose density gradient (34-45%) ($MP_{gr}s$) and also characterized them electrophoretically (Fig. 1, *B*). It was found that clone 2C82 exhibited the greatest affinity for this fraction; slight interaction was detected with clones 2C83 and 4G7A9. It must be mentioned that the $MP_{gr}s$ were less heterogeneous in composition (Fig. 1, *B*). It is known that a fraction enriched with ATPase activity is found in this interval of a sucrose density gradient [7]. Specific reactivity with these mcABs in combination with subsequent biochemical analysis can apparently be used for marking membrane epitopes.

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0009-3130/94/3001-0073$12.50

TABLE 1. Immunochemical Analysis of mcABs with Various Antigens

Clones	MPs	MP_{gr}	Extensin	$LLP_{60—80}$	LLP-1	LLP-2	Con-A	RCA_{120}
2C82	+++	+	+++	+++	–	+++	+	–
2C83	+	+	–	–				
4G7A9	+	+	–	+				
2C88	+	–	–	+			+	
2C8C7	+	–	–	+	–	–	+	
2C85	+	–	–	+			+	
4G7B3	+	–		–				
3A10	+	–	+	+	–			
3G5D9	+	–	–	+	–	–	–	+
1C6	+	–	–	+	+	–	–	+
1C81	+	–	–	–				

*Antigen–antibody (AG–AB) affinity was determined by ELISA solid-phase immunoenzyme analysis and is denoted as: (–) — no binding; +, ++, +++ – degrees of binding; in the other cases, affinity was not determined

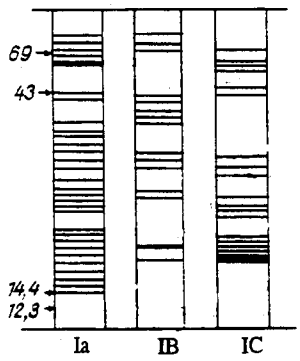

Fig. 1. Electrophoresis in slab polyacrylamide gel in the Laemmli system - 15% PAAG with 0.1% SDS: IA) total membrane proteins; IB) fractions of membrane proteins obtained in a 33-45% sucrose density gradient (MP_{gr}s); lectin-like proteins. The arrows show the positions of markers: BSA (69 kDa), ovalbumin (43 kDa), lysozyme (14.4 kDa), and Cc (12.3 kDa).

For a cross-immunochemical study of these populations relative to various fractions of cottonplant proteins we used extensin-like proteins that we had isolated from a suspension cottonplant culture. Chromatography of Pth derivatives of a hydrolysate of this fraction showed the presence of hydroxyproline (Hyp) (Fig. 2, *A*, *B*). In addition, we isolated the total lectin-like proteins and narrower fractions of them obtained on the basis of their affinity with various biosorbents. The latter included the *D*-galactosyl-bindable lectin-like proteins isolated by chromatography on Sepharose 4B (LLP-1) and the Con-A-

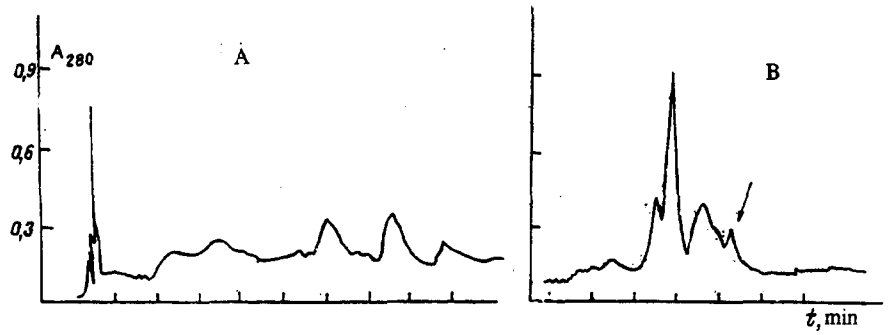

Fig. 2. Chromatograms of Pth derivatives from a hydrolysate of an extensin-like protein (150°C for 1.5 h with a mixture of 5.8 M HCl and TFA (2:1) containing 2.5% β-ME). Chromatograph Gold system (Beckman), 2.4 × 125 cm Spherisorb ODS column (Pharmacia), rate of flow 1.2 ml/min; mobile phases: *A*) 14 mM NaOAc, pH 6.7; *B*) A + 50% CH_3CN. Pump program: 0-2 min, 2% B; 2-25 min, gradient of B from 2 to 50%. The arrow shows the Hyp peak; the other peaks are of derivatives of aminosugars. *A*) Total pattern of the Pth derivatives of the hydrolysate; *B*) region of elution of hydroxyproline (Hyp).

bindable proteins from the usual $LLP_{60\text{-}80}s$ isolated by affinity chromatography on Con-A–Sepharose 4B (LLP-2). The elctrophoretic spectrum of the $LLP_{60\text{-}80}s$ is given in Fig. 1, *C*. In the hemagglutination of human and murine blood erythrocytes the $LLP_{60\text{-}80}s$ and narrower fractions isolated from them exhibited specific activities of 50-70 μg/ml.

We also used in cross-analysis lectins with a known specificity — concanavalin A (Con-A) and *Ricinus communis* agglutinin (RCA_{120}) (Sigma, USA). A number of clones of the 2C8 population — 2C82, 2C87, and 2C88 — reacted unambiguously both with the total membrane proteins and with the lectin-like proteins $LLP_{60\text{-}80}s$, which confirmed the common nature of their antigenic determinants that we had found previously [6]. Furthermore, the clones 2C82 and 3A10 also exhibited affinity for the extensin-like proteins. A comparative electrophoretic analysis of the membrane proteins showed,the presence among them of polypeptide zones of the $LLP_{60\text{-}80}s$ and an extensin-like protein.

It is known that extensins are coded by a small multigenic family and various transcripts are activated in response to different signals. It may be assumed that clones 2C82 and 3A10 have affinity for different polypeptides belonging to the extensin family or for different epitopes, possibly including the carbohydrate fragments.

The results that we have obtained show the localization of the listed proteins in the plasmatic membranes, which is important for the study of the mechanism of their action in the process of recognition and the role of the glyco sections in this. Clone 3G5D9 exhibited affinity for the $LLP_{60\text{-}80}s$ and for RCA_{120}. The overlapping of the immunochemical reactions of clone 1C6 with the $LLP_{60\text{-}80}s$ and also with the galactosyl-bindable fractions isolated from them with the reactions of the lectins (Con-A and RCA_{120}) themselves indicate the possible presence in the antigenic determinants of protein structures with β-*D*-galactosyl terminals. In addition, the fact is also interesting that the antibodies of clone 2C82 bind selectively with Con-A and with the Con-A-specific proteins LLP-2. These results permit the assumption that the lectin-like membrane-bound proteins have molecules with carbohydrate chains of the oligomannoside type and also bi-antigenic chains of the complex type bound with asparagine [8].

Thus, we have obtained panels of mcABs to the membrane proteins of the cotton plant. Using two stages of screening, we have selected producing clones and have studied the antigenic properties of various fractions of the cottonplant proteins. The reactivity of the protein antigens with a set of mcABs can be used as markers for membrane epitopes associated with specific functions and also for the purposeful fractionation of heterogeneous membrane proteins.

EXPERIMENTAL

Isolation of Membrane Proteins from Cottonplant Shoots. Two-day shoots were suspended in 25 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 3 mM EDTA, and 5 mM β-mercaptoethanol. The homogenate was filtered through four

layers of gauze and was centrifuged at 1500 rpm for 10 min. Then the supernatant was centrifuged at 100,000 *g* for 1 h, and the resulting deposit was resuspended in 0.1 M sodium phosphate buffer, pH 7.4, and was used to immunize mice. The protein concentration was determined by Lowry's method [9]. The proteins obtained were characterized electrophoretically [10].

The total membrane proteins were fractionated by centrifugation in a 35-45% sucrose density gradient in 25 mM Tris-HCl buffer, pH 7. 4, at 100,000 *g* for 1 h. The interphase layer was washed with a tenfold volme of PBS, and the aliquot obtained was was used as the antigenic material.

Isolation of Lectin-like Proteins. The lectin-like proteins from the seeds of a cotton plant of variety 108-F were isolated by extraction with salt solutions from the seed flour that had been treated for defatting and the elimination of pigments and were then subjected to stepwise salting-out with ammonium sulfate at from 30 to 80% saturation [11]. The extracting solutions contained a protease inhibitor — 0.1 mM PMSF — and also 1 mM ascorbic acid to prevent the oxidation of the numerous phenols of the cotton plant, which, in the quinoid form, denature proteins. The galactose-specific proteins were isolated by affinity chromatography on a 1.5 × 24 cm column of Sepharose 4B (Pharmacia) in PBS with desorption by 0.1 M *D*-galactose or *D*-lactose, To isolate the Con-A-bindable proteins we used a column with Con-A−Sepharose 4B in 0.15 M NaCl−0.1 M sodium acetate buffer, pH 6; desorption with 50 mM methyl α-*D*-glucoside.

Isolation of the Extensin-like Proteins. The extensin-like proteins were isolated from a cottonplant suspension culture by Lamport's method with some modification [4]. The suspension culture was kept at 4°C for 24 h. Then the cells were gathered by centrifugation and were washed 5-6 times with a tenfold volume of water. The proteins were extracted from the deposit by three extractions with 0.2 M $CaCl_2$. The resulting extract was concentrated to 1/3 volume and was treated with TCA to a final concentration of 10%. The acid-soluble fraction was collected, dialyzed against water, and freeze-dried.

Determination of Hemagglutinating Activity. The hemagglutinating activities of the LLPs were determined with the use of a 2% suspension of human or murine blood in 96-U-shaped-well polystyrene plates without trypsin by a standard procedure [11].

Immunization of Mice and Production of Hybridomas. Mice of the Balb/c line were immunized with the total membrane proteins in an amont of 2 mg of protein in 0.5 ml of PBS intraperitoneally three times at one-week intervals, and, three days before hybridization, with 1 mg of protein in 0.25 ml of PBS intravenously.

For hybridization, splenocytes of the immunized mice were fused with myeloma X63Ag8. 653 cells in the presence of PEG by a modification of Milstein's method [5]. After hybridization, the cell suspension was transferred to 96-well plates. The hybridomas were cultivated in HAT selective medium in the presence of 20% of fetal serum. Clones of hybrid cells appeared after 2 weeks. The colonies secreting antibodies against the immunogen were then cloned by the method of limiting dilution to 1, 2, and 10 cells per well in the presence of a feeder layer. As the feeder layer we took macrophages from the mouse abdominal cavity. The producing hybridomas were cultivated in a medium with 10% of serum in order to obtain a stable line of cells and were injected in the presence of Freund's adjuvant into mice of the Balb/c line for the production of ascitic fluid.

The Screening of the Producing Hybridomas and the Study of Antigenic Properties. To each well of 96-well plates was added 100 μl of antigen solution with a concentration of 20 μg/ml, and they were kept at 4°C for 12 h. The unbound antigen was eliminated, and the plates were treated with a 0.1% solution of Tween-20 or with 5% BSA (bovine serum albumin). Either the culture liquid or the ascitic fluid or the pure mcABs were added to the washed plates, and these were then kept at 37°C for 1 h and were washed with PBS containing 0.1% of Tween-20. The antigen−antibody reaction was revealed with the aid of a conjugate of mouse anti-IgG with horseradish peroxidase. The conjugate was diluted 1:1000, 30 μl was added to each well, and the plate was kept at 37°C or 1 h. Binding was determined from the AG−AB color reaction in the prresence of the dye *o*-phenylenediamine (Sigma) in 0.1 M sodium citrate.

Production and Purification of the mcABs. The ascitic fluid was extracted from the abdominal cavities of mice and was centrifuged. The supernatant was diluted twofold and an equal volume of saturated ammonium sulfate solution was added with uniform stirring over 1 h, followed by centrifugation at 10,000 rpm. The deposit was dissolved in the initial volume of PBS, and a 2/3-saturated solution of ammonium sulfate was added. The resulting precipitate was dissolved in 5 mM sodium phosphate buffer, pH 8, and was desalted on a column of Sephadex G-25. Further purification was achieved on a column of DEAE-cellulose.

REFERENCES

1. J. M. Lord, Plant Physiol., **85**, 1 (1987).
2. J. M. Daly, Annu. Rev. Phytopathol., **22**, 273 (1984).
3. D. J. Meyer, C. L. Afonso, and D. W. Galbraith, J. Cell Biol., **107**, 163 (1988).
4. J. P. Stafstrom and L. A. Staenelin, Plant. Physiol., **81**, 234 (1986).
5. G. Köhler and C. Milstein, Nature (London), **256**, 495 (1975).
6. Yu. S. Mangutova, Z. S. Khashimova, D. M. Katalaeva, M. É. Suslo, and V. B. Leont'ev, Dokl. Akad. Nauk UzSSR, No. 3, 49 (1989).
7. S. R. Gallagher and R. T. Leonard, Plant Physiol., **83**, 265 (1987).
8. R. Colin Hughes, Glycoproteins, Methuen, New York (1983).
9. O. H. Lowry et al., J. Biol. Chem., **193**, 265 (1951).
10. U. K. Laemmli, Nature (London), **227**, 680 (1970).
11. J. Roth, Exp. Pathol. (Jena), suppl. 3, 196 (1978).
12. J. J. Smith, E. P. Muldoon, J. J. Willard,and D. T. A. Lamport, Phytochemistry, **25**, 1021 (1986).