Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 500-505

CHARACTERIZATION OF EPITOPES ON THE CATIONIC PEANUT PEROXIDASE BY FOUR MONOCLONAL ANTIBODIES

Chunfang Hu and Robert B. van Huystee

Department of Plant Sciences, The University of Western Ontario, London, Ontario N6A 5B7, Canada

Received August 15, 1988

The epitope sites on the cationic peanut peroxidase were characterized by four monoclonal antibodies raised against this isozyme. Evidence is presented showing that the epitope for monoclonal antibody 1B is located on the polypeptide. Sensitivity of the epitopes recognized by 1M and 2F to 0.1M HCl, boiling, 10 mM periodate and trifluoromethane sulfonic acid treatment indicate that they occur at regions where oligosaccharides are linked to the polypeptide backbone. The antigenic specificity of 2A is, in addition, dependent on the conformation of the epitope site which is destroyed after partial proteolysis of the peroxidase.

Proteins are multideterminant antigens. They elicit in the immunized animals a variety of heterogeneous antibody families, each family being comprised of members able to recognized one particular epitope on the surface of the protein (1,2,3). The advent of hybridoma technology (4) to produce monoclonal antibodies (McAb), each of which recognizes a specific antigenic site or epitope, has provided a powerful tool for elucidating the complex antigenic structures of proteins (2).

The cationic peanut peroxidase is a glycohemoprotein (5) containing about 20% N-linked oligosaccharides (manuscript submitted). Ten McAbs were previously produced against this peroxidase and were divided into four groups according to their binding specifities to the antigen (6). In the present study, the molecular nature of the epitopes recognized by McAbs 1M, 1B, 2A and 2F, each representing an antibody group, was determined by the reactivities of the antibodies towards chemically modified or

0006-291X/88 $1.50

partially fragmented cationic peroxidase in enzyme-linked immunosorbent assay (ELISA) or Western blotting.

Materials and Methods

Isolation of the cationic peroxidase and production of McAbs. The peroxidase was isolated from the spent medium of peanut cell suspension culture according to Chibbar and van Huystee (7). Fusion of spleen cells from mice immunized with purified cationic peroxidase and myeloma cells, cloning of hybridomas for McAb production and preliminary characterization of the specific McAbs were reported (6).

Chemical modification of the cationic peroxidase. The peroxidase was deglycosylated by trifluoromethane sulfonic acid (TFMS, Aldrich Chemical Company) according to Edge *et al* (8) or modified by treatment with 10 mM Na metaperiodate, 0.1 M HCl or boiling essentially as described by Homan *et al* (9). Briefly, purified cationic peanut peroxidase at 1 mg/ml was incubated with 10 mM $NaIO_4$ in 50 mM Na acetate buffer pH 4.5 at 4°C for 1 or 18 h in the dark. At the end of this period, the mixture was neutralized with addition of ethylene glycol to a final concentration of 16% and/or 10 times concentrated PBS (phosphate buffered saline). For acid treatment, the cationic peroxidase (1 mg/ml) was incubated with 0.1 M HCl for 24 h at room temperature and neutralized by addition of 3 N NaOH. Alternatively, the peroxidase was kept at 100°C over a water bath for 5 h. The samples were then dialyzed against (for samples treated with periodate or acid)or resuspended in (for the boiled sample) the coating buffer for ELISA (0.05 M Na bicarbonate pH 9.6) for latter assay of antibody activities. The controls were not boiled or were kept under the same conditions with omission of the chemicals.

Partial proteolysis. The purified cationic peroxidase was digested under denaturing conditions by incubation with *S. aureus* V_8 protease (Miles Laboratory), trypsin or chymotrypsin (Boehringer Mannheim Biochemica) at a substrate:enzyme ratio of 15:1 at 37°C for 15 minutes according to Cleveland *et al* (10).

SDS polyacryamide electrophoresis (PAGE) and Western blotting. The TFMS deglycosylated cationic peroxidase or partially proteolytically cleaved peroxidase peptides were separated on SDS-PAGE and blotted to nitrocellulose membranes as described (6).

Others. ELISA was performed as described (6). Protein contents were measured according to Lowry *et al* (11).

Results

To elucidate the molecular nature of the epitope sites recognized by these antibodies, the effects of treatments of the cationic peroxidase with reagents destroying protein or carbohydrate structure on binding abilities of the McAbs were studied. The peroxidase was treated with 10 mM periodate, which oxidizes the vicinyl hydroxyl groups of carbohydrate residues while leaving the polypeptide intact (12,13), with 0.1 M HCl or boiling, which destroys the protein structure (9). Or it was deglycosylated by TFMS. Of the four McAbs tested, only 1B clearly

Table 1. Reactivities of monoclonal antibodies to chemically modified peanut cationic peroxidase as determined by ELISA. The hybridoma culture supernatants (1B 10^{-2}, 1M 10^{-1}, 2F 10^{-1}, 2A 10^{-0}) were assayed against control and treated cationic peanut peroxidase. Antibody binding was detected by the enzyme reaction of alkaline phosphatase conjugated to goat anti-mouse IgGs secondary antibodies. Each datum represents the mean±s.d. (n=3). The absorbance obtained with non-immune mouse IgGs has been substracted.

Treatment	A_{405}			
	1B	1M	2F	2A
10 mM $NaIO_4$				
Control	1.281±0.160	1.538±0.024	0.763±0.070	1.219±0.122
Treatment	1.303±0.149	0.703±0.065	0.153±0.022	0.653±0.055
% of Control	102	46	20	54
0.1 M HCl				
Control	1.201±0.098	1.456±0.103	0.831±0.075	1.266±0.250
Treatment	0.998±0.092	1.002±0.064	0.190±0.030	0.680±0.065
% of Control	83	69	23	54
Boiling				
Control	1.314±0.341	1.610±0.335	0.919±0.103	1.005±0.152
Treatment	0.641±0.231	0.605±0.025	0.005±0.001	0.153±0.054
% of Control	49	38	1	15

recognized a protein-defined epitope. This epitope was insensitive to either periodate oxidation (Table 1) or to TFMS-deglycosylation (Fig 1). On the other hand, the antibody activity of 1B was reduced to about half of the control when the peroxidase was boiled, and also slightly affected by acid treatment of the antigen, suggesting that 1B-epitope is located on the protein moiety of the peroxidase (Table 1).

The results obtained with McAbs 1M, 2F and 2H indicated that their epitopes seemed to be composed of both protein and carbohydrate groups. Pre-treatment of the cationic peroxidase with 10 mM periodate reduced the binding of these three antibodies. Results after 18 h incubation with periodate were presented in Table 1. Essentially the same effects were achieved with 1 h incubation (data not shown). Similarly, the antibody activities were abolished when the carbohydrate moiety was removed from the peroxidase by TFMS (Fig 1). Moreover, the epitopes recognized by these antibodies were sensitive to acid treatment and boiling (Table 1). However, the degree of involvement of carbohydrate seemed to vary among different epitopes. The antibody activity of 2F was decreased to approximately 20% that of control after periodation of the antigen, a two times greater reduction than that achieved when 2A

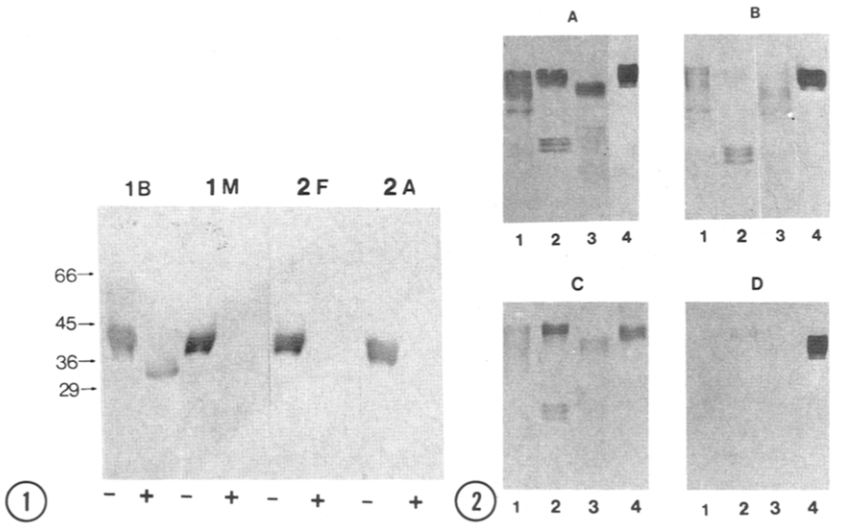

Fig 1. Western blotting of the deglycosylated cationic peanut peroxidase. The cationic peroxidase was incubated with TFMS for 3 h at 0°C. The protein recovered form the mixture and the control was electrophoresed and transferred to nitrocellulose membranes. The membranes were probed with McAbs 1B, 1M, 2F and 2A, respectively. -, control; +, deglycosylated peroxidass.

Fig 2. Western blotting of peptide fragments of the cationic peroxidase after partial proteolysis. Lane 1, digested with trypsin; 2, with V_8 protease; 3, with chymotrypsin and 4, control. Panel A, probed with 1B; B, 1M; C, 2F; D, 2A.

or 1M was used. In addition, the 2A-epitope was sensitive to all protease treatments suggesting that it is conformation- as well as protein-dependent (Fig 2D). On the other hand, some of the fragmented peptides were recognized by 1B, 1M and 2F (Fig 2A, B and C).

Discussion

We have investigated the molecular nature of the epitopes recognized by anti-cationic peanut peroxidase McAbs with the intention of determining the involvement, if any, of the carbohydrate side chains. Of the four McAbs studied, 1B recognized a protein- defined epitope. The results for McAb 1M was of particular interest. The 1M-epitope was affected by periodate oxidation, TFMS-deglycosylation as well as by boiling and acid treatment (Table 1, Fig 1). Thus, it appeared that 1M recognized a region on the cationic peroxidase that comprised of both protein and carbohydrate. This region must be in the vicinity of the enzyme active site, since 1M was previously shown to be inhibitory of peroxidase activity (6). This is not

impossible considering that there are two carbohydrate chains close or in the substrate access channel in the structural model suggested for plant peroxidase (14). Besides, carbohydrates were shown to be essential for the expression of peroxidase activity (manuscript submitted).

Similar to 1M, 2A and 2F seemed to recognized carbohydrate-containing epitopes. The binding of these antibodies were sensitive to treatments that modified protein or carbohydrate structure (Table 1, Fig 1). These results suggest that carbohydrate residues, in combination with protein sequences, form the reactive epitopes. Alternatively, removal of carbohydrate might induce considerable conformational changes such that protein-defined epitopes could no longer be recognized by these McAbs, since carbohydrates may interact with domains of the protein and thus modulate the conformation of a glycoprotein (15). To test these possibilities, the cationic peroxidase was partially digested with V_8 protease, trypsin or chymotrypsin. The fragmented peptides were subjected to SDS-PAGE and Western blotting (Fig 2). No staining of the peptides were observed by using 2A, suggesting that 2A-epitope is conformation- and protein-dependent. The peptide patterns recognized by 1B, 1M and 2F were similar. Because only relatively large fragments (>19 kD) were detected, no distinctions could be made between the binding specificities of 1M, 1B and 2F from Western blotting. At this time, it is uncertain whether the sensitivity of the 2A-epitope to carbohydrate-modifying treatments was due to a direct involvement of carbohydrate in the epitope or to a conformational change caused by the treatments. Nevertheless, it is clear from above that the four McAbs studied here belong to four antibody groups, which is in accordance with previous classification by competition experiment (6). In conclusion, both protein and carbohydrate moieties are antigenic in peanut peroxidase, as was true for many other glycoproteins (9,16,17,18). Monoclonal antibodies are well suited as probes for the surface structures of the enzyme.

Acknowledgment:This work was supported by a grant from Natural Sciences and Engineering Research Council of Canada.

References

1. Atassi, M.Z. (1984) Eur. J. Biochem. 145, 1-20

2. Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G.,

Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E. and Wilson, A.C. (1984) Ann. Rev. Immunol. 2, 67-101

3. Geysen, H.M., Tainer, J.A., Rodda, S.J., Mason, T.J., Alexandeer, H., Getzoff, E.D. and Lerner, R.A. (1987) Science 235, 1184-1190

4. Köhler, G. and Milstein, C. (1975) Nature 256, 495-497

5. van Huystee, R.B. (1987) Ann. Rev. Plant Physiol. 38, 205-219

6. Hu, C., Carbonera, D. and van Huystee, R.B. (1987) Plant Physiol. 85, 299-3036.

7. Chibbar, R.N. and van Huystee, R.B. (1984) Plant Physol. 75, 294-298

8. Edge, A.S., Faltynak, C.R., Hof,L., Reichert, L.E.,Jr. and Weber, P. (1981) Anal. Biochem. 118, 131-137

9. Homan, W.L., van Kalshoven, H., Kolk, A.H.J., Musgrave, A., Schuring, F. and van den Ende, H. (1987) Planta 170, 328-335

10. Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102-1106

11. Lowry, O.H., Rosebrough, N.L., Farr, L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

12. Woodward, M.P., Young, W.W. and Bloodgood, R.A. (1985) J. Immunol. Methods 78, 143-153

13. Dyer, J.T. (1956) Methods Biochem. Anal. 3, 112-152

14. Welinder, K.G. (1985) Eur. J. Biochem. 57, 497-504

15. Schwarz, R.T. and Datema (1982) In The Glycoconjugates (M.I. Horowitz,Ed), Vol. III,pp.47-79. Academic Press, New York London Toronto

16. Roberts, K., Phillipa, J., Shaw, P. and Smith, E. (1985) In Biochemistry of Plant Cell Walls (C.T. Brett & R. Hillman,Eds), pp.125-154. Cambridge University Press, Cambridge London New York

17. Stafstorm, J.P. and Staehelin, L.A. (1988) Planta 174, 321-332

18. Frelinger, A.L. and Rutishauser, U. (1986) J. Cell Biol. 103, 1729-1737