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Characterization of Antigenic Properties of Horseradish Peroxidase with Monoclonal Antibodies

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Abstract—Monoclonal antibodies (mcAbs) specific to alkaline isoenzymes of horseradish peroxidase were used to characterize the antigenic properties of horseradish peroxidase. The results of a competitive binding assay indicated that monoclonal antibodies can be divided into three groups directed against distinct parts of the protein. The interaction of monoclonal antibodies with native and modified horseradish peroxidase showed also three different patterns of reactivity. Antibodies from groups I and II are directed against epitopes which are conformational and formed by tertiary structure elements. Epitopes recognized by these antibodies are sensitive to heme removal or partial denaturation of peroxidase. Antibodies from group III bind specifically with epitopes consisting of primary or secondary structure elements. The antigenic determinants recognized by antibodies from group III PO_1 and $36F_9$ were shown to be linear (continuous) and formed by amino acid residues 261-267 and 271-277, respectively, as determined by the peptide scanning method (PEPSCAN). The location of revealed linear antigenic determinants in the molecular structure of peroxidase is analyzed.

Key words: antigenic determinants, horseradish peroxidase, epitope mapping, PEPSCAN, monoclonal antibodies

Study of the interaction of protein antigens with specific antibodies is an important field in modern immunochemistry from the point of view of understanding the molecular mechanisms of immunological recognition, definition of kinetic and thermodynamic parameters of "antigen–antibody" reaction, and the application of immunochemical methods of analysis in biotechnology and medicine. Antigenic determinants, or epitopes, are regions of a protein against which specific antibodies develop during the immune response. There are many experimental methods for studies of structure of antigenic determinants [1-5]. Many of them are based on interaction of short peptide fragments with the antibodies against the whole protein. One of the commonly used methods is peptide scanning (PEPSCAN) [6]. The main idea of this method consists in synthesis of short overlapping peptides with amino acid sequence similar to that of the investigated protein and testing of their interaction with specific antibodies by enzyme immunoassay. Based on the strength of binding of peptides with specific antibody, assumptions can be made on the structure of linear antigenic determinants. Using this method, antigenic structures of proteins have been analyzed [6, 7]. But PEPSCAN allows us to determine only linear antigenic determinants, i.e., continuous stretches of the polypeptide chain possessing antigenic activity. However, most observable antigenic determinants are conformational antigenic determinants composed of residues distant from each other in the polypeptide chain but brought together in space by the protein folding [8, 9]. Therefore an investigation of antigenic structure of proteins only by the PEPSCAN method does not provide complete information on antigenic structure of the protein.

It has been assumed that conformational epitopes are formed from several linear ones and that they can be determined by merging some linear epitopes into a single peptide [2, 8]. The structure of the conformational antigenic determinants can be determined by structural method of analysis (X-ray or multidimensional NMR). To localize antigenic determinants one has to perform studies of interaction of antibodies as with the whole protein, and with its fragments, or the modified forms. In the present work we used PEPSCAN in combination with other approaches determining the characteristics of antigenic structure of horseradish peroxidase. This enzyme is the most widespread from a superfamily of plant peroxidases and is widely used in analytical diagnostics. The study of its immunochemical properties is a quite important task. Recently we used PEPSCAN to determine linear antigenic determinants of isoenzyme C of horseradish

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peroxidase with the use of polyclonal antibodies [10]. Polyclonal antibodies contain a set of antibodies of different specificity. Their use for determining the characteristics of antigenic structure allowed us to characterize all possible linear antigenic determinants of peroxidase. Studying the individual antigenic determinants, including conformational ones, by applying polyclonal antibodies is complicated due their great heterogeneity. Thus, here we used monoclonal antibodies that demonstrate high specificity and affinity. The purpose of the present work was to investigate an interaction of eight types of mcAbs specific to horseradish peroxidase with various forms of the enzyme.

MATERIALS AND METHODS

The preparation of horseradish peroxidase (isoenzyme C, RZ = 3.0) was purchased from Biozyme (Great Britain), the chromogenic peroxidase substrates 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and *o*-phenylenediamine hydrochloride (OPD) were from Sigma (USA), polyethylene pins on plates for synthesis of solid phase-linked peptides were from Cambridge Research Biochemicals (Great Britain), and horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin antibodies were from Sigma.

Recombinant horseradish peroxidase expressed in *E. coli* was kindly provided by V. G. Grigorenko [11].

Hybridomas produced mcAbs specific to isoenzyme C of horseradish peroxidase were obtained as described in [12]. Clone $36F_9$ was kindly provided by A. D. Dmitriev (Mental Health Research Center, Russian Academy of Medical Sciences). The monoclonal antibodies were isolated from ascitic fluids by precipitation with 30% ammonium sulfate and purified by ion-exchange chromatography on DEAE-Sephadex.

Spectrophotometric measurements were performed on a Shimadzu UV-1602 spectrophotometer (Japan). Measurements of optical density for 96-well microtiter plates were performed on a microtiter plate reader (Molecular Devices, USA).

Apo-horseradish peroxidase. Apo-HRP was prepared from the holo-HRP by removing heme with methylethylketone at pH 2.5 and 4°C [13]. The aqueous solution containing apo-HRP was neutralized with 1 M ammonium carbonate to pH 7.0 and then purified on a column with Sephadex G-25. Then 0.01 M calcium chloride was added to the final concentration of 0.1 mM; at the final stage the solution was dialyzed against 0.05 M potassium phosphate buffer, pH 8.0, at 4°C.

Chemical modification of peroxidase. The peroxidase was modified by treatment with 10 mM sodium metaperiodate, 0.1 M HCl, or boiling essentially as described in [14]. Briefly, purified horseradish peroxidase at 1 mg/ml was incubated with 10 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.5, at 4°C for 18 h in the dark. At the end of this period, the mixture was neutralized with addition of 10 times concentrated PBS. For acid treatment, the 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) 0.05 M sodium bicarbonate, pH 9.5. Proteins contents were determined by measuring absorption at 230 and 260 nm. Concentration was calculated by the formula:

$$\text{protein (}\mu\text{g/ml)} = 183A_{230\ \text{nm}} - 75.8A_{260\ \text{nm}}\ [15].$$

Enzyme-linked immunosorbent assay (ELISA). Interaction of monoclonal antibodies with samples of native and modified horseradish peroxidase was investigated by means of enzyme immunosorbent assay. Testing was carried out in two ways: in the first the wells of the plate were coated with rabbit-anti mouse antibodies solution diluted 1 : 2000 in 0.01 M sodium carbonate buffer, pH 9.5, overnight at 4°C. After washing the wells with PBS containing 0.05% Tween-20 (PBST) (3× 200 μl per well) the different dilutions of mcAbs in PBST were added, and after 1 h at 37°C the wells were washed 3 times with PBST. Then a solution of horseradish peroxidase (10^{-7} M) in PBST was added to wells, incubated 1 h at 37°C, washed 3 times with PBST, and the activity of the coated enzyme was determined by adding a substrate solution (4 mg/ml *o*-phenylenediamine, 1 mM H_2O_2 in 0.01 M sodium citrate buffer, pH 4.8). The enzymatic reaction was stopped by addition 4 M H_2SO_4. Concentration of formed product was determined by measuring the absorbance at 490 nm. The wells of the plate were coated with 100 μl of HRP at a concentration of 4 μg/μl overnight at 4°C. The wells were washed with PBST (3× 300 μl). Different dilutions of holo- or apo-HRP specific antibodies in PBST were added to coated wells, respectively. The plates were incubated for 1 h at 37°C, then washed with PBST (3× 300 μl). Secondary antibody–alkaline phosphatase conjugate solution (100 μl) diluted 1 : 5000 in PBST were added to the wells followed by another 1 h incubation at 37°C and washing with PBST (4× 300 μl). Freshly prepared substrate solution (100 μl) containing 1 mg/ml *p*-nitrophenylphosphate in buffer solution containing 1 M diethanolamine and 0.5 mM $MgCl_2$, pH 9.8, were added to each well. The optical densities were read at 405 nm. The binding of non-immune rabbit serum was employed as a control.

Competitive binding immunoassay of monoclonal antibodies to horseradish peroxidase. mcAbs of one clone were adsorbed on the surface of the plate at concentration 2 μg/ml and then different dilutions of peroxidase were added from initial concentration of $8 \cdot 10^{-7}$ M with step 2. After 1 h at 37°C and washing 3 times with PBST, enzy-

matic activity of peroxidase coated with antibodies was determined. For performance of the competitive analysis we chose such concentration of peroxidase which binds no more than 50% of the adsorbed mcAbs. Having picked thus the concentration of peroxidase we added it to wells with coated antibodies and different dilutions of mcAbs of another clone. The plates was incubated for 1 h at 37°C and after that the activity of binding enzyme was determined.

Enzyme-linked immunosorbent assay of pin-bound peptides (PEPSCAN). The assay was performed on the pins with bound hexapeptides overlapping the amino acid sequence of HRP as described in [10]. To prevent unspecific protein sorption, the pins with peptides were incubated with a blocking buffer (PBS containing 0.1% Tween 20, 0.1% casein hydrolyzate, 0.02% sodium azide) for 1 h at 37°C. Then the pins were incubated with specific or nonspecific (control) antibodies (175 μl/pin) in a concentration of 5-10 μg/ml diluted with the blocking buffer overnight at 4°C. After that the pins were washed (4 × 10 min) with PBST followed by incubation with peroxidase-labeled secondary antibodies in the blocking buffer without sodium azide for 1 h at room temperature. The pins were then washed again as described above and incubated in a substrate solution (0.5 mM ABTS, 2 mM H_2O_2 in 0.01 M sodium citrate buffer, pH 4.5, 150 μl/pin) for 45 min at room temperature. The reaction was stopped by removing the pins from the wells of the microtiter plate and the optical density of colored solutions in the wells was then measured at 405 nm (A_{405}). After each assay the pins were washed by sonication in a disruption buffer containing 0.1 M sodium phosphate, pH 7.4, 1% sodium dodecyl sulfate, 0.1% mercaptoethanol at 60°C; then the pins were washed twice with water (60°C) and at the end with boiling ethanol.

RESULTS AND DISCUSSION

Determination of specificity of monoclonal antibodies by competitive enzyme immunoassay. Eight types of mcAbs specific to alkaline isoenzymes of horseradish peroxidase were investigated. Using the competitive ELISA method one can clarify whether they are specific to the same or different epitopes on the protein surface [16]. For this purpose mcAbs of one type were coated on the surface of plates. By variation of peroxidase concentration, we found one at which no more than 50% the active centers of antibodies were bound. Then competitive enzyme immunoassay was performed using a mixture of peroxidase at fixed concentration and various concentration of mcAbs of another type. The results of the competitive analysis of monoclonal antibodies PO_2 by antibodies PO_1 and *9D* are shown in Fig. 1. This figure shows that mcAbs PO_1 compete for peroxidase with mcAbs PO_2. mcAbs *9D* bind independently and therefore their addition does not influence the interaction of peroxidase with mcAbs PO_2. The ability of all mcAbs to bind independently was investigated in a similar manner. Results of the competitive analysis (Table 1) allowed us to divide conditionally all the studied mcAbs into at least three groups. Antibodies from group I and III recognize distinct sites of a molecule and do not compete with each other. Antibodies from group II compete with antibodies from group I and III and, probably, bind to overlapped sites on the protein surface. Antibodies *7G* and *2C* compete among themselves and with mcAbs $9F_2$ and *3E* and therefore, most likely, recognize the same epitope. On the basis of the data of the competitive analysis one can suggest a schematic disposition of binding sites of different mcAbs on a molecule of horseradish peroxidase (Fig. 2.)

Reactivity of monoclonal antibodies with recombinant horseradish peroxidase. We attempted to clarify to which part of the molecule (polypeptide chain, heme, or the carbohydrate residues) the mcAbs bodies were developed. It is obvious that the antibodies specific to a carbohydrate part of the enzyme will not bind deglycosylated peroxidase. Therefore binding of monoclonal antibodies with recombinant horseradish peroxidase (lacking carbohydrates) was studied. All tested mcAbs interacted to some extent with the recombinant peroxidase, hence, all of them are specific to a protein part of the enzyme. We observed the differences in binding of the mcAbs of different groups with recombinant peroxidase as compared to native. For antibodies of group III ($36F_9$, PO_1, and PO_2) and antibodies of group II *3E* a considerable increase in binding with recombinant protein was shown, while antibodies from group I ($9F_2$ and *9D*) bind better to native peroxidase (Table 2). One can assume that epitopes

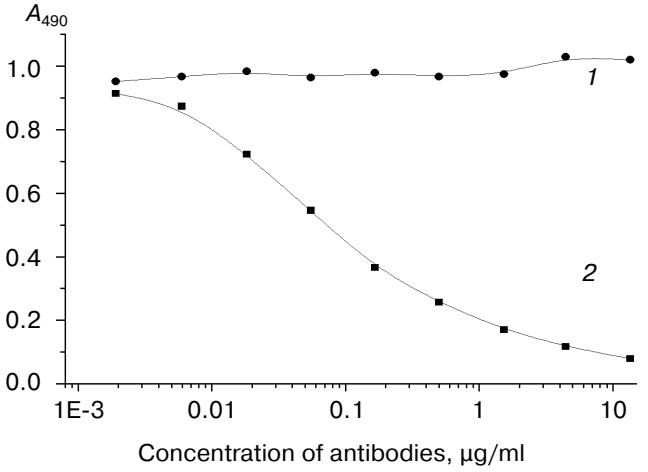

Fig. 1. Binding curves of horseradish peroxidase with coated mcAb PO_2 in the presence of mcAb *9D* (*1*) and PO_1 (*2*). Concentration of peroxidase, 7 nM.

Table 1. Results of competitive enzyme-linked immunosorbent assay of mcAbs to horseradish peroxidase (+, competition; −, no competition)

Group		I		II			III		
		9F$_2$	*9D*	*7G*	*2C*	*3E*	*36F*$_9$	*PO*$_2$	*PO*$_1$
I	*9F*$_2$		+	+	+	−	−	−	−
	9D	+		−	−	+	−	−	−
II	*7G*	+	−		+	+	−	−	−
	2C	+	−	+		+	−	−	−
	3E	−	+	+	+		−	+	−
III	*36F*$_9$	−	−	−	−	−		−	−
	PO$_2$	−	−	−	−	+	−		+
	PO$_1$	−	−	−	−	−	−	+	

for antibodies *9F*$_2$ and *9D* include stretches of polypeptide chain and a carbohydrate fragment.

Reactivity of mcAbs with denatured, chemically modified, and apo-form of peroxidase. Conformational epitopes can be analyzed by investigation a binding of antibodies to protein in different forms—native, chemically modified, and partial denatured. The effects of treatment of the horseradish peroxidase with reagents destroying native structure of protein or the carbohydrate structure on binding abilities of the mcAbs were investigated. Peroxidase was treated with 10 mM $NaIO_4$ which oxidizes the hydroxyl groups of the carbohydrate residues while leaving the polypeptide part intact [17], with 0.1 M HCl, or boiling, which destroys fully or partially the protein tertiary and secondary structure [14]. Reactivity of mcAbs to chemically modified, denatured, and apo-horseradish peroxidase are listed in Table 2. Binding of all mcAbs with exception of clone *3E* did not depend on treatment with sodium periodate. Thus, the investigated mcAbs (except *3E*) recognize epitopes whose structure is not affected by periodate treatment and these epitopes do not contain carbohydrate components, or such treatment does not change their structures.

Boiling and acid treatment affected epitopes binding with antibodies of group I (*9F*$_2$ and *9D*) and especially of group II (*7G*, *2C*, *3E*), which almost completely lost their abilities to react with denatured peroxidase (Table 2). This confirms that antigenic determinants to which antibodies of groups I and II are specific are conformationally dependent, i.e., they are formed by tertiary structure elements which are brought together in space only if the protein is in native conformation. This gives us reason to define at least two non-overlapped conformational antigenic determinants in the peroxidase molecule.

Epitopes for antibodies of group III (*36F*$_9$, *PO*$_1$, and *PO*$_2$) were insensitive to the action of destroying reagents. After boiling and acid treatment binding of peroxidase with antibodies *PO*$_2$ is decreased approximately twofold, and the same treatment has practically no influence on the reactivity of peroxidase toward antibodies *36F*$_9$ and *PO*$_1$. Most likely the epitopes which are recognized by these mcAbs are completely linear, formed by continuous

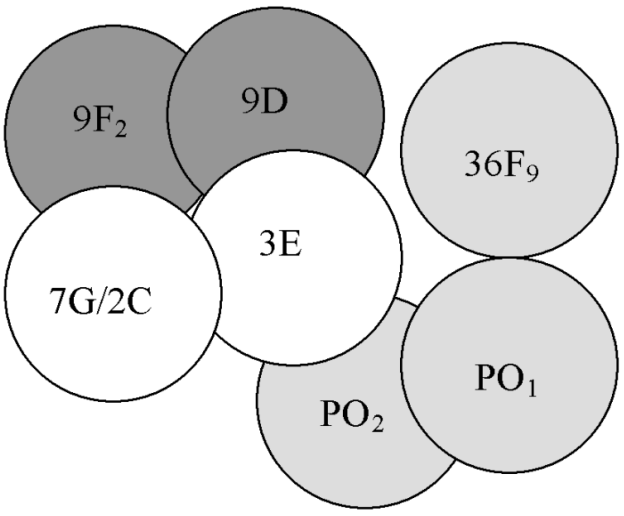

Fig. 2. Scheme for the disposition of binding sites for anti-HRP mcAbs determined with competitive ELISA.

Table 2. Reactivity of mcAbs with chemically modified native HRP, recombinant, and apo HRP (100%, binding with native HRP)

Antibodies		Native HRP after various treatment, %			Apo-HRP, %	Rec-HRP, %
		10 mM $NaIO_4$	0.1 M HCl	boiling		
I	*9F2*	96	10	15	46	32
	9D	89	22	25	42	25
II	*7G*	120	9	9	12	87
	2C	101	16	21	15	94
	3E	155	12	9	16	180
III	$36F_9$	103	86	88	230	200
	PO_2	111	48	43	82	142
	PO_1	96	160	120	170	212

sites of the polypeptide chain, or consist of stable elements of secondary structure [18].

Similar results were obtained from study of interaction of mcAbs with apo-enzyme (Table 2). The structural changes occurring in the molecule of peroxidase upon removing heme influence the ability of some clones to bind apo-peroxidase. For antibodies from group I this ability is reduced to 42-46% as compared with binding to native peroxidase, and antibodies from group II practically do not react with apo-peroxidase (12-16% compared to binding native peroxidase). This confirms that preservation of tertiary structure is an important factor for recognition of protein by the given antibodies, and, on the other hand, specifies that antigenic determinants for these antibodies probably are located in regions which undergo conformation changes upon heme removal. Binding of antibodies of group III with apo-peroxidase practically was not decreased or even increased in some cases.

The binding of antibodies of group III ($36F_9$, PO_1) with apo-peroxidase was markedly increased in comparison with holo-enzyme (230 and 170%, accordingly). Probably, heme removal promotes a partial unfolding of some sites of the molecule that results in exposition of new immunogenic amino acid residues, and, hence, to increase of their availability for antibodies paratopes. This suggests that these amino acid residues are part of the antigenic determinants for the given group of mcAbs.

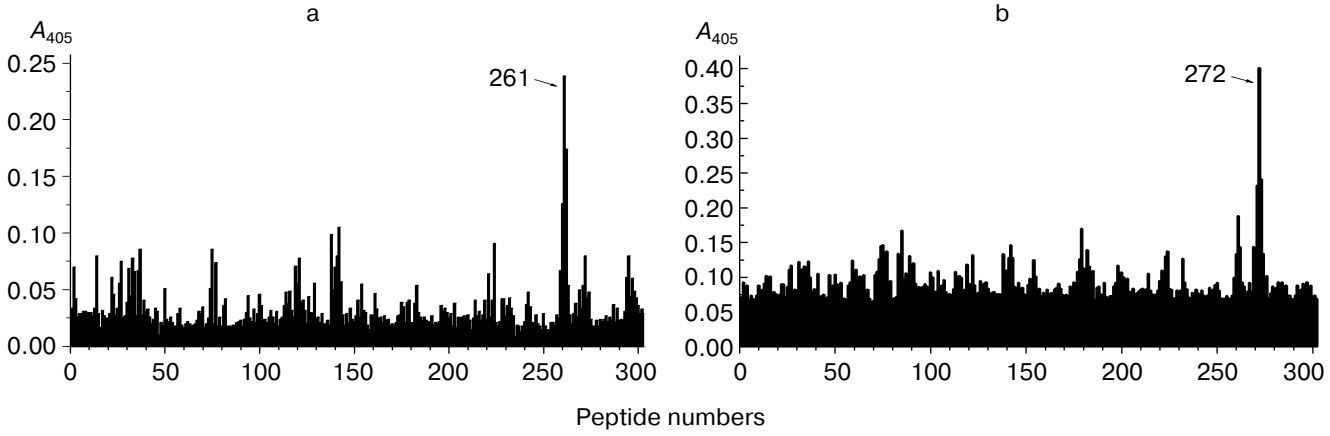

Fig. 3. Interaction of mcAbs PO_1 (a) and $36F_9$ (b) with hexapeptides from the sequence of HRPC.

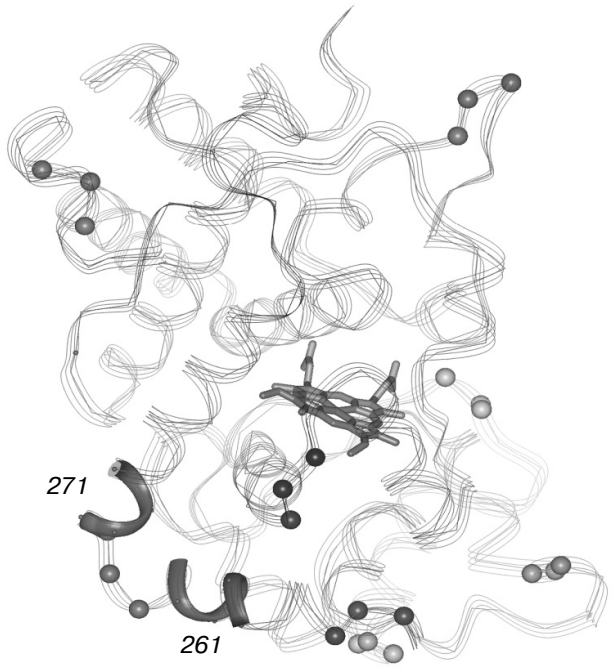

Fig. 4. Structure of horseradish peroxidase molecule with the marked regions of the polypeptide chains corresponding to epitopes for mcAbs PO_1 and $36F_9$. •, sites of glycosylation.

Localization of linear antigenic determinants binding with mcAbs and the analysis of their spatial arrangement in the structure of the horseradish peroxidase molecule. To localize the linear antigenic determinants binding with mcAbs we used peptide scanning (PEPSCAN). The antibodies directed presumably to linear antigenic determinants were tested for their interaction with a panel of hexapeptides overlapping the amino acid sequence of HRP. The binding of antibodies with the hexapeptides can be presented as a characteristic pattern where the abscissa is the peptide number and the ordinate is the optical density obtained in ELISA (that is proportional to the concentration of antibodies bound with that peptide). Figure 3 presents the patterns obtained for antibodies PO_1 and $36F_9$. Both mcAbs interact with linear determinants, which are basically located in the region of the amino acid residues 261-266 for antibodies PO_1 and 271-276 for $36F_9$. Figure 4 shows the model of the spatial structure of HRPC with the marked regions of the polypeptide chains corresponding to epitopes for these antibodies. As follows from the X-rays structure for isoenzyme C horseradish peroxidase [19], these epitopes are linked fragments of adjacent I and J α-helices connected by a loop and are exposed on a surface of the protein. They are located rather far from the active center of the enzyme. Amino acids 268-270 located near the epitope for antibodies $36F_9$ are glycosylated in native peroxidase. Development of antibodies against this region of the polypeptide chain can be considered as evidence that in the course of the immune response there is a partial hydrolysis of the oligocarbohydrates and some parts of the protein shielded earlier by carbohydrates become accessible for immunoglobulins. This argument can also explain the increase in binding of these mcAbs with recombinant (non-glycosylated) peroxidase compared to the native protein. Carbohydrate residues of native peroxidase present steric obstacles for antibodies against corresponding sites on the protein surface.

In summary, eight mcAbs against horseradish peroxidase define several distinct antigenic regions of the protein part of the enzyme molecule. Only two clones of the investigated antibodies (PO_1 and $36F_9$) are directed against linear antigenic determinants, the others are directed against conformational epitopes. Our results of competitive analysis and interaction with modified forms of peroxidase indicated that the mcAbs can be divided into three basic groups. Binding of antibodies from group I and II is reduced upon heme removal and partial denaturation under the influence of boiling and acid treatment, while such treatment has practically no influence on the reactivity of antibodies of group III. These antibodies bind better with recombinant protein than with native.

Based on these results, the protein part of peroxidase can be divided into three immunodominant regions. Two regions (not overlapped) of conformational determinants are located in part of molecule with stable conformation. The third region possesses labile tertiary structure. All antibodies developed on this site are directed to elements of primary or secondary structure.

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