Biochemistry (Moscow), Vol. 62, No. 8, 1997, pp. 870-871. Translated from Biokhimiya, Vol. 62, No. 8, pp. 1015-1017.

Assessment of Dot-Blot ELISA Sensitivity on Membrane Sorbent Using Various Peroxidase Substrates

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Submitted February 18, 1997; revision submitted April 17, 1997.

The sensitivity of the peroxidase reaction in dot-blot ELISA significantly depends on the substrate. The highest sensitivity is observed using benzidine and diamine–phenol combinations as the substrates due to the reaction of the coupled oxidation (NADI).

KEY WORDS: ELISA, phenols, naphthols, aromatic amines.

Peroxidase is the most often used label among the great variety of labels used in ELISA (enzymes, lanthanoids, colloidal gold and silver, ferritin, etc. [1, 2]). This is due to the relative stability of the enzyme, its high sensitivity, and a great number of soluble and complex-forming substrates. The latter is of importance for construction of ELISA test systems on various carriers: polystyrene plates when the reaction is registered in the fluid volume using spectrophotometry, or membrane sorbents when the reaction is registered visually by staining of the carrier [3]. Since peroxidase is a relatively nonspecific reducer of two molecules of hydrogen peroxide, various chemical substances can be used as electron donors. The best known soluble substrates are ortho-phenylenediamine, 5-aminosalicylic acid, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) [4], while the complex-forming substrates are 4-chloro-1-naphthol, benzidine, and 3,3′-diaminobenzidine [5]. The sensitivity of ELISA can be increased based on the choice of substrates or their combinations [6, 7].

The purpose of this work was to assess the sensitivity of dot-blot ELISA on the membrane sorbent in the determination of paramyxovirus antigens depending on peroxidase substrate.

MATERIALS AND METHODS

Sendai virus (strain 960) was grown in the allantoid cavity of 9-10-day-old chick embryos. The virus was purified and concentrated by ultracentrifugation.

Protein was determined by the method of Bradford [8].

The reaction of dot-immunobinding on a membrane sorbent was carried out as described earlier [9]. The antigen in serial dilutions was sorbed onto a Vladipor (Russia) acetate-cellulose membrane. The membrane with the sorbed material was blocked by treatment with a mixture of 10% normal horse serum (Immunopreparat, Ufa, Russia) and 0.05% Tween 20 (Serva, Germany) for 60 min at 37°C. The antigen was detected directly using a conjugate of specific antibodies to this antigen with horseradish peroxidase. After incubation for 1 h at 37°C, the unbound conjugate was removed by washing the immunosorbent with buffer (0.05 M Tris-HCl, 0.9% NaCl, 0.05% Tween 20). The staining was carried out using a chromogenic complex-forming substrate. All substrate solutions contained 1 μg of the compound being tested and 0.03% hydrogen peroxide. The results were registered visually by clearly stained spots on the membrane. The antigen titer was determined as the greatest antigen dilution producing the spots. Concentrated allantoid fluid without virus antigens was used for the control.

Some phenols (hydroquinone, resorcinol (Reakhim, Russia)); guaiacol, vanillin (Ferak, Germany)), naphthols (4-chloro-1-naphthol, α-naphthol), aromatic amines (benzidine, diaminobenzidine, *o*-phenylenediamine (Serva, Germany)), diethyl *p*-phenylenediamine sulfate, and ethylhydroxyethyl *p*-phenylenediamine sulfate (Reakhim) were used.

RESULTS AND DISCUSSION

The rate of formation of colored products of the substrate mixture oxidation is one of the factors which

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0006-2979/97/6208-0870$18.00 © 1997 МАИК Наука/Interperiodica Publishing

TABLE 1. Sensitivity of Dot-Blot ELISA on Membrane Sorbent Depending on Peroxidase Substrate

Substrate	Detection limit, ng
Ortho-phenylenediamine	2.000 ± 0.600
Guaiacol	0.500 ± 0.150
Hydroquinone	64.000 ± 5.000
Resorcinol	64.000 ± 5.000
Vanillin	1.000 ± 0.300
α-Naphthol	0.500 ± 0.150
4-Chloro-1-naphthol	0.500 ± 0.150
Diethyl *p*-phenylenediamine sulfate	0.500 ± 0.150
Ethylhydroxyethyl *p*-phenylenediamine sulfate	0.500 ± 0.150
3,3'-Diaminobenzidine	0.500 ± 0.150
3,3'-Diaminobenzidine + α-naphthol	0.063 ± 0.010
Diethyl *p*-phenylenediamine sulfate + α-naphthol	0.125 ± 0.040
Ortho-phenylenediamine + α-naphthol	0.125 ± 0.040
Ortho-phenylenediamine + guaiacol	0.125 ± 0.040
Ortho-phenylenediamine + vanillin	0.500 ± 0.150
Diethyl *p*-phenylenediamine sulfate + guaiacol	0.500 ± 0.150
Diethyl *p*-phenylenediamine sulfate + vanillin	0.500 ± 0.150
Benzidine	0.125 ± 0.040

limit the sensitivity of dot-immunobinding. The resulting complexes should be insoluble, and this was a criterion for selection among chemical compounds under study which were phenols, naphthols, and aromatic amines.

The sensitivity of dot-blot ELISA on a membrane sorbent was rather high when various chemical compounds were used (Table 1). Benzidine was found to be the most sensitive, and this seems to be due to specific features of this substrate oxidation which resulted in the formation of a large multimolecular complex [5]. Moreover, the stepwise mechanism of generation of the reaction product permitted modification not only of the reaction rate but also of staining intensity by varying the reaction conditions, e.g., changing the pH of the solution, the contents of some metal ions, etc, [10, 11].

In other words, the sensitivity of the dot-immunobinding reaction significantly depended on generation of large substrate complexes of limited diffusion ability. Based on this suggestion, in subsequent experiments substrate mixtures were used which could be involved in reactions of oxidative combination (the so-called NADI and ADI reactions) [7, 12].

Oxidative combinations of some compounds with each other were found to increase the sensitivity of dot-blot ELISA on membrane sorbent 4-8 times compared to either of the compounds without addition of the other ingredients (Table 1).

Thus, the sensitivity of the dot-immunobinding reaction significantly depended on the substrate used. The highest sensitivity was found for substrates which generated water-insoluble large multimolecular complexes during the oxidation. Benzidine and various combinations of diamines and phenols or amines and diamines are such substrates.

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