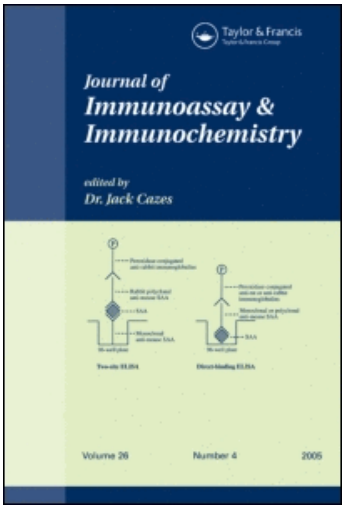

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An Improved Preparation of Antibody-Coated Polystyrene Beads for Sandwich Enzyme Immunoassay

Eiji Ishikawa[a]; Yoshitaka Hamaguchi[a]; Masayoshi Imagawa[a]; Mitsuo Inada[b]; Hiroo Imura[b]; Nobuhiko Nakazawa[c]; Hiroshi Ogawa Daiichi[c]

[a] Department of Biochemistry, Medical College of Miyazaki, Kiyotake, Miyazaki, Japan [b] Second Division, Department of Medicine, Kyoto University School of Medicine, Kyoto, Japan [c] Radioisotope Labs. Ltd., Tokyo, Japan

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AN IMPROVED PREPARATION OF ANTIBODY-COATED POLYSTYRENE BEADS FOR SANDWICH ENZYME IMMUNOASSAY

Eiji Ishikawa, Yoshitaka Hamaguchi and Masayoshi Imagawa
Department of Biochemistry, Medical College of Miyazaki,
Kiyotake, Miyazaki 889-16, Japan

Mitsuo Inada and Hiroo Imura
Second Division, Department of Medicine, Kyoto University
School of Medicine, Kyoto 606, Japan

Nobuhiko Nakazawa and Hiroshi Ogawa
Daiichi Radioisotope Labs., Ltd., 3-10-5,
Nihonbashi, Chuo-ku, Tokyo 103, Japan

ABSTRACT

An improved preparation of antibody-coated polystyrene beads for sandwich enzyme immunoassay of human thyroid-stimulating hormone (TSH) was described. Rabbit anti-TSH IgG was purified by eluting at pH 2.5 from a TSH-Sepharose column, diluted 3 or 9 fold with normal rabbit IgG and used for coating polystyrene beads by physical adsorption. In a sandwich enzyme immunoassay of TSH using rabbit (anti-TSH) Fab'-β-D-galactosidase conjugate, β-D-galactosidase activities specifically bound to thus prepared polystyrene beads in the presence of TSH was 2.8-6.3 fold higher than those bound to polystyrene beads coated with anti-TSH IgG before purification. A similar effect was observed when guinea pig anti-pork insulin IgG, rabbit (anti-human IgE) IgG and goat (anti-human IgE) IgG were treated at pH 2.5. This improvement may be based on a conformational change of Fc in IgG molecule which was caused by the treatment at pH 2.5. Other sandwich immunoassays such as fluoro- and radio-immunoassays may also be improved in the same way. (KEY WORDS: Sandwich enzyme immunoassay, TSH, insulin)

INTRODUCTION

We developed a method for the conjugation of antibodies with enzymes by utilizing the reaction between thiol and maleimide groups (1, 2), and the use of antibody-β-D-galactosidase conjugates prepared by this method allowed development of sandwich enzyme immunoassays with attomole sensitivities for macromolecular antigens such as ornithine δ-aminotransferase from rat liver (3), human thyroglobulin (4), hepatitis B surface antigen (5) and human ferritin (6). However, other antigens including TSH, insulin, IgE and α-fetoprotein have been assayed only at femtomole levels.

The sensitivity of sandwich enzyme immunoassay is limted by several factors (3). One of them is the completeness of antigen binding to antibody-coated solid phase. Another is the binding efficiency of enzyme-antibody conjugates. This paper describes an improved preparation of antibody-coated polystyrene beads to provide efficient binding of antigens and subsequently of enzyme-antibody conjugates.

MATERIALS AND METHODS

Antigens and Antisera

Human TSH was obtained from Calbiochem-Behring Corp., La Jolla. Pork insulin was obtained from Novo Industri A/S, Copenhagen (Actrapid, 40 U/ml). Ornithine δ-aminotransferase was prepared from rat liver as described previously (7).

Anti-TSH was raised in rabbits by giving 3 subcutaneous injections of human TSH (150 μg on the first day and 100 μg each on the 45th and 75th days) in 1 ml of saline emulsified with 1 ml of complete Freund's adjuvant. Blood was collected 10 days after the last injection. Guinea pig anti-pork insulin serum was obtained from Miles Laboratories, Inc., Kankakee. One μl of the anti-insulin serum could bind 0.85 mU of pork insulin. Anti-ornithine δ-aminotransferase was raised in rabbits as described previously (7).

Preparation of IgG, $F(ab')_2$ and Fab'-β-D-Galactosidase Conjugates

IgG and $F(ab')_2$ were prepared by fractionation with Na_2SO_4 and chromatography on a diethylaminoethyl cellulose column (3).

Fab'-β-D-galactosidase conjugates were prepared using N,N'-o-phenylenedimaleimide (1). The amount of conjugates was expressed as units of β-D-galactosidase activity, and one unit of the enzyme activity was defined as that which released 1 μmole of 4-methylumbelliferone per min when assayed with 1×10^{-4} M 4-methylumbelliferyl-β-D-galactoside at 30°C (7).

Purification of Anti-TSH

Rabbit anti-human TSH serum (18 ml) was fractionated by 50 % $(NH_4)_2SO_4$ saturation. The precipitate was washed with 33 % saturated $(NH_4)_2SO_4$ solution and dissolved in 0.01 M sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl. Anti-TSH

adsorbed on a human TSH-Sepharose 4B column (1.0 x 5.0 cm) was eluted with 0.1 M KCl containing 0.008 N HCl, pH 2.3-2.5. The eluate was neutralized with 0.5 M glycine-NaOH buffer, pH 9.0.

Human TSH-Sepharose 4B was prepared by coupling 300 μg of TSH to 1 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala) following Pharmacia's instruction.

Treatment of Antibody IgG at pH 2.5

IgG solutions (1-10 mg/ml) were diluted with 0.1 M glycine-HCl buffer, pH 2.5 containing 1 M NaCl, adjusted to pH 2.5 with 0.1 N HCl, allowed to stand at room temperature for 10 min and finally adjusted to pH 7.3-7.4 with 0.5 M Tris-HCl buffer, pH 8.0.

Preparation of Antibody IgG-Coated Polystyrene Beads

Polystyrene beads (3.2 mm in diameter, Precision Plastic Ball Co., Chicago) were coated with IgG by physical adsorption (3). The concentration of IgG used for coating was about 100 μg/ml.

Sandwich Enzyme Immunoassay

One antibody-coated polystyrene bead per assay tube was incubated with antigen in 0.15 ml of buffer, washed twice with buffer, incubated with Fab'-β-D-galactosidase conjugates in 0.15 ml of buffer, washed twice with buffer and subjected to β-D-galactosidase assay (3). Incubation was performed with

antigens for 4 h at 37°C plus 15 h at 4°C and with conjugates for 4 (for insulin) or 6 (for other antigens) h at 37°C. The amount of conjugate used was 2000 μunits per tube except for ornithine δ-aminotransferase where 1700 μunits per tube was used. The buffer used was 0.01 M sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 % bovine serum albumin and 0.1 % NaN_3.

In most experiments, incubation media which contained antigens were subjected to sandwich enzyme immunoassay (the first assay), and, after removing antibody-coated polystyrene beads used in the first assay, the remaining solution was again subjected to sandwich enzyme immunoassay (the second assay) to define the amount of antigen remaining unbound after exposure to antibody-coated polystyrene beads in the first assay (7).

Calculation of β-D-Galactosidase Activity and the Amount of Conjugates Specifically Bound

β-D-Galactosidase activity specifically bound (μunits) was calculated by subtracting the enzyme activity nonspecifically bound in the absence of antigens (the background) from the enzyme activity bound in the presence of antigens, and divided by 53 μunits to calculate the amount of conjugates specifically bound (fmoles), assuming that the conjugates bound showed the same actvity as their free forms (7).

RESULTS

Effect of Purification of Anti-TSH IgG (Table 1)

When polystyrene beads were coated with rabbit anti-TSH IgG before purification, β-D-galactosidase activities both specifically and nonspecifically bound to the beads were very low. The amount of anti-TSH-β-D-galactosidase conjugate specifically bound in the first assay was calculated to be only 0.028 fmoles when 1 fmole of TSH was added. Comparison of β-D-galactosidase activities bound in the first and second assays indicated that TSH to be assayed was partly bound to the beads even when 1 fmole of TSH was added.

When polystyrene beads were coated with purified anti-TSH IgG, β-D-galactosidase activity specifically bound in the presence of 100 fmoles of TSH in the first assay increased 4.3 fold as compared with that specifically bound before purification. β-D-Galactosidase activity nonspecifically bound in the absence of TSH (the background of assay) also increased 2.2 fold. β-D-Galactosidase activities bound in the second assays indicated that up to 100 fmoles of TSH were almost completely bound to the beads.

Sensitivity with Purified Anti-TSH IgG Diluted with Normal IgG (Table 1)

When purified anti-TSH IgG was diluted 3 fold with normal rabbit IgG and used for coating polystyrene beads, β-D-galacto-

TABLE 1. Sandwich Enzyme Immunoassay of Human TSH

Rabbit anti-TSH for coating beads	Assay	β-D-Galactosidase activity bound					
		TSH added for assay (fmoles / tube)					
		0	0.2	0.5	1	10	100
		μunits					
Not purified							
IgG	1st	1.3	—	—	2.8	6.5	9.6
	2nd	1.0	—	—	2.0	6.1	9.0
IgG treated at pH 2.5	1st	7.0	—	—	11.7	33.2	42.9
	2nd	6.7	—	—	7.5	19.4	37.4
$F(ab')_2$	1st	1.1	—	—	2.3	5.1	6.3
	2nd	1.1	—	—	1.7	5.5	6.5
$F(ab')_2$ treated at pH 2.5	1st	1.1	—	—	2.1	4.9	5.4
	2nd	1.1	—	—	2.0	4.1	5.5
Purified							
IgG not diluted	1st	2.9	—	—	—	7.3	38.8
	2nd	2.9	—	—	—	3.0	4.3
IgG diluted* 3 fold	1st	1.9	—	—	—	18.4	54.0
	2nd	1.8	—	—	—	2.6	13.3
IgG diluted* 9 fold	1st	1.2	2.5	3.4	5.4	23.8	43.5
	2nd	1.3	—	—	1.4	3.4	33.9

*Affinity-purified rabbit anti-TSH IgG was diluted with normal rabbit IgG. Values are means of duplicate assays.

sidase activities specifically bound in the presence of 10 and 100 fmoles of TSH in the first assays increased 3.2 and 6.3 fold, respectively. When diluted 9 fold, β-D-galactosidase activities specifically bound in the presence of 1, 10 and 100 fmoles in the first assays increased 2.8, 4.3 and 5.1 fold, respectively. The background was lowered by dilution. As a result, the sensitivity, that is, the amount of TSH to double the background was enhanced to 0.2 fmoles by using purified and 9 fold-diluted anti-TSH IgG, compared with a sensitivity of 1 fmole with anti-TSH before purification.

β-D-Galactosidase activities bound in the second assays indicated that up to 10 fmoles of TSH were almost completely bound even when diluted 9 fold. When diluted 3 and 9 fold, about 95 and 67 fmoles of TSH, respectively, were bound in the presence of 100 fmoles. These results indicate that the capacity of anti-TSH-coated polystyrene beads to bind TSH increased and that the measurable range of TSH expanded, if a sufficient amount of conjugate is used.

One ml of the purified, 9 fold-diluted anti-TSH IgG solution could be used at least 5 times for coating 45 polystyrene beads with little change in β-D-galactosidase activity bound.

Effect of pH 2.5 Treatment of Anti-TSH (Table 1)

In order to elucidate the mechanism by which bound β-D-galactosidase actvity increased when purified anti-TSH was used to

coat polystyrene beads, rabbit anti-TSH IgG was treated at pH 2.5 without purification and used for coating polystyrene beads, the acid treatment being similar in duration to that received by purified anti-TSH during elution. β-D-Galactosidase activities specifically bound in the presence of 1, 10 and 100 fmoles in the first assays increased 3.1, 5.0 and 4.3 fold, respectively. The background increased 5.4 fold. β-D-Galactosidase activities bound in the second assays indicated that 1 fmole of TSH added was almost completely bound, although not completely bound when more than 1 fmole were added.

When rabbit anti-TSH $F(ab')_2$ was treated at pH 2.5 and used for coating polystyrene beads, β-D-galactosidase activity bound did not change both in the first and second assays.

These results indicate that the presence of Fc is essential for improving the sandwich enzyme immunoassay of TSH by the present method.

Effect of pH 2.5 Treatment of Anti-insulin and Anti-ornithine δ-aminotransferase (Tables 2 and 3)

When guinea pig anti-pork insulin IgG was treated at pH 2.5 and used for coating polystyrene beads, β-D-galactosidase activities specifically bound in the first assays increased 2.1-2.8 fold. β-D-Galactosidase activities specifically bound in the presence of 5 and 10 μU of insulin in the second assays were lowered, indicating that the amount of insulin bound also

TABLE 2. Sandwich Enzyme Immunoassay of Insulin

Guinea pig Anti-insulin IgG for coating beads	Assay	β-D-Galactosidase activity bound				
		Insulin added for assay (μU / tube)				
		0	0.5	1	5	10
		μunits				
Not treated	1st	1.4	3.5	5.7	14.2	19.5
	2nd	0.88	1.1	1.6	7.7	14.8
Treated at pH 2.5						
Not diluted	1st	3.7	9.2	12.9	35.8	54.1
	2nd	3.6	4.3	4.0	6.5	10.5
Diluted 3 fold	1st	2.0	6.7	11.0	32.3	45.9
	2nd	1.9	2.4	2.7	7.2	20.2
Diluted 9 fold	1st	1.2	5.6	9.6	24.3	31.0
	2nd	1.2	1.7	2.1	14.8	24.8

TABLE 3. Sandwich Enzyme Immunoassay of Ornithine δ-Aminotransferase

Rabbit anti-ornithine δ-aminotransferase IgG	Assay	β-D-Galactosidase activity bound			
		Ornithine δ-aminotransferase added (fmoles / tube)			
		0	1	10	100
		μunits			
Not treated	1st	1.1	68.1	234	279
	2nd	0.9	4.3	70.2	256
Treated at pH 2.5	1st	1.7	78.9	313	386
	2nd	1.4	2.4	16.8	278

increased. The background increased 2.6 fold. When anti-insulin IgG was treated at pH 2.5, diluted 9 fold with normal guinea pig IgG and used for coating, the background was lowered to that before the treatment and β-D-galactosidase activities specifically bound in the first assays were 1.8-2.1 fold higher than those before the treatment.

When rabbit anti-ornithine δ-aminotransferase IgG was treated at pH 2.5 and used for coating, β-D-galactosidase activities specifically bound in the first assays increased only 15-38 %.

DISCUSSION

Affinity purification of rabbit anti-TSH at pH 2.5 helped to improve the sandwich enzyme immunoassay of TSH both in the sensitivity and in the measurable range. Treatment of guinea pig anti-insulin IgG at pH 2.5 similarly improved the sandwich enzyme immunoassay of insulin. Treatment at pH 2.5 of (anti-human IgE) IgG from rabbit and goat was similarly effective in preliminary experiments. Therefore, affinity purification or treatment at pH 2.5 may be effective for IgG from rabbit, guinea pig, goat and probably other animals.

When 1 fmole each of TSH, IgE and ornithine δ-aminotransferase and 1 μU (6.7 fmoles) of insulin were subjected to the assay by using antibodies before purification, the numbers of conjugate molecules specifically bound per antigen molecule added were calculated to be 0.028, 0.032 (rabbit anti-human IgE IgG), 1.2 and

0.012, respectively, and those numbers increased to 0.079, 0.17 (rabbit anti-human IgE IgG), 1.4 and 0.026, respectively, by using purified or pH 2.5-treated antibodies. The number increased from 0.058 to 0.15 when goat anti-human IgE IgG was treated at pH 2.5. (Table 4). Therefore, sandwich enzyme immunoassays of other antigens, which are measurable only with a low sensitivity or with

TABLE 4. Number of Antibody-β-D-Galactosidase Conjugate Molecules Specifically Bound per Antigen Molecule Added in Sandwich Enzyme Immunoassay

Antigen	Source of antibody	Number of conjugate molecules specifically bound per antigen molecule added[1]	
		Treatment of antibody IgG for coating polystyrene beads	
		None	pH 2.5-treated
TSH	Rabbit	0.028	0.089
			(0.079)[2]
IgE	Rabbit	0.032	0.17
	Goat	0.058	0.15
Ornithine δ-amino-transferase	Rabbit	1.2	1.4
Insulin	Guinea pig	0.012	0.026

[1]Numbers were calculated as described in METHODS, when 1 μU of insulin or 1 fmole each of other antigens was added.

[2]Affinity-purified anti-TSH IgG was used for coating polystyrene beads.

a low binding efficiency of enzyme actvity, may be improved in this way.

Spectrophotometry for β-D-galactosidase assay requires a longer incubation time than fluorimetry. The enhanced binding efficiency of β-D-galactosidase activity allows performance of the spectrophotometric assay of β-D-galactosidase activity within a short time.

The Fc fragments of IgG molecules undergo a conformational change at pH 2.5 (8) and may not completely restore to their original structure. As a result, pH 2.5 treated IgG may be adsorbed on the surface of polystyrene beads in such a way that antigen may be more efficiently bound and/or the amount of IgG adsorbed may increase.

The enhanced binding efficiency of β-D-galactosidase activity by affinity purification or treatment at pH 2.5 is largely or partly at least due to the efficient binding of antigens. However, it may be partly due to an orientation of antibody IgG molecules on the surface of polystyrene beads that permits the binding of more conjugate molecules, and the possibility that β-D-galactosidase activity is enhanced by binding to pH 2.5 treated antibodies remains to be tested.

Alternative treatments to cause an irreversible conformational change of Fc may be as effective as treatment at pH 2.5, and solid materials other than polystyrene may also be useful for the same purpose. It is possible that the sensitivity of other sandwich immunoassays such as radio- and fluoro-immunoassays may be improved in the same way.

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Correspondence and requests for reprints should be addressed to Eiji Ishikawa, Department of Biochemistry, Medical College of Miyazaki, Kiyotake, Miyazaki 889-16, Japan.

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