COUPLING Fab' FRAGMENT OF RABBIT ANTI-HUMAN IgG ANTIBODY TO β-D-GALACTOSIDASE AND A HIGHLY SENSITIVE IMMUNOASSAY OF HUMAN IgG

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1. Introduction

Techniques for coupling antigens or antibodies to enzymes are essential in enzyme-linked immunoassay and immunohistochemistry.

We have recently developed a novel method for synthesis of the insulin-β-D-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23) complex [1]. Insulin is first mercaptosuccinylated and then coupled to the enzyme, which contains sulfhydryl groups, using *N,N'-o*-phenylenedimaleimide. The complex is shown to be useful for insulin immunoassay [1]. In a similar manner, rabbit immunoglobulin G (IgG) is coupled to β-D-galactosidase [2]. Rabbit anti-human IgG antibody is mildly reduced by 2-mercaptoethylamine and coupled to the enzyme. The amount of human IgG determined with the complex is as little as 5 fmol.

The present paper describes the conjugation of Fab' fragment of rabbit anti-human IgG antibody with β-D-galactosidase and a highly sensitive immunoassay of human IgG.

2. Materials and methods

Rabbit anti-human IgG antibody was purified by affinity chromatography using human IgG-coupled Sepharose. $F(ab')_2$ fragment of the purified antibody was mildly reduced by 10 mM 2-mercaptoethylamine and then coupled to β-D-galactosidase from *Escherichia coli* with *N,N'-o*-phenylenedimaleimide. ($F(ab')_2$: pepsin-treated antibody, a dimer of two identical units, the Fab' fragments, each with one combining site, linked by one disulfide bond [3,4]). The complex proved to be useful for measuring human IgG as little as 0.3 fmol (50 pg) by sandwich method.

2.1. *Preparation of rabbit (anti-human IgG) Fab'-β-D-galactosidase complex*

The (anti-human IgG) antibody was purified about 10-fold from the Na_2SO_4 fraction [5] of the rabbit anti-serum (Medical and Biological Laboratories LTD., Nagoya). The Na_2SO_4 fraction (30 to 90 mg) was applied on an affinity column (human IgG (Miles Laboratories, Inc., Illinois) coupled to CNBr-activated Sepharose 4B, 0.7 × 10 cm), which was equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, and the antibody was eluted with 0.1 M glycine-HCl buffer, pH 2.5 containing 1 M NaCl.

About 3 mg of the purified (anti-human IgG) antibody was incubated with 60 μg of pepsin (from swine gastric mucosa, Boehringer Mannheim, Mannheim) at pH 4.5 at 37°C for 16 hr in a final vol of 1.5 ml. After incubation, the pH of the reaction mixture was adjusted to 8 with 1 N NaOH, and $F(ab')_2$ fragment of the purified antibody was separated from other components on a Sephadex G-150 column (1.5 × 40 cm), which had been washed with 0.1 M sodium borate buffer pH 8.0. The $F(ab')_2$ fragment (1.5 mg) was concentrated in a Collodion-Bag (Sartorius-Membrane-filter GmbH, Göttingen) under a mild vacuum of about 20 Torr, dialyzed against 0.1 M sodium acetate buffer, pH 5.0, and reduced with 10 mM 2-mercaptoethylamine as described by Nisonoff and Rivers [6]. The Fab' fragment thus prepared was separated from compounds of lower molecular weight on a Sephadex G-25 column, equilibrated with 0.1 M sodium acetate buffer, pH 5.0. The Fab' fragment in a volume of 1.6 ml with an absorption of 0.57 at 280 nm (in a cell of 1 cm light path) was incubated with 0.25 ml of saturated *N,N'-o*-phenylenedimaleimide (Aldrich Chemical Company, Inc., Milwaukee) solution (in 0.1 M sodium acetate

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buffer, pH 5.0) at 30°C for 20 min. Unreacted *N,N'-o*-phenylenedimaleimide was removed on a Sephadex G-25 column, similar to that described above. The maleimide-treated Fab' fragment obtained was shown to contain maleimide residues which reacted with 2-mercaptoethylamine. (The quantitative characterization of the reactive maleimide residues will be described elsewhere.) Amounts of IgG, and $F(ab')_2$ or Fab' fragment were estimated by the absorbance at 280 nm, being based on $E^{1\%}_{280} = 15$ [7] and 14.8 [8], respectively. Sulfhydryl groups were determined by the method of Grassetti and Murray [9].

One ml of the dimaleimide-treated Fab' fragment, with an absorption of 0.35 at 280 nm (1 cm light path), was incubated with 5 μl of β-D-galactosidase (5 mg/ml, from *Escherichia coli*, Boehringer Mannheim, Mannheim) at 30°C for 20 min. No decrease in enzyme activity was detected during the incubation. After neutralization with 1 N NaOH, 20 μl of 5% bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Chicago) and 2 μl of 1 M $MgCl_2$ were added to stabilize the enzyme activity. The mixture was kept at 4°C for 24 hr to 72 hr and then applied on a Sepharose 6B column (1.5 × 40 cm), which had been washed with 0.01 M sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% NaN_3 and 0.1% bovine serum albumin (Buffer A), to separate the (anti-human IgG) Fab'-β-D-galactosidase complex from the antibody which was not coupled to the enzyme. Amounts of the Fab' coupled to the enzyme remains to be determined. The fractions in the peak of the enzyme activity were used for the assay of human IgG. The Fab'-β-D-galactosidase complex thus obtained was stable at 4°C for at least 1 month.

2.2. *Assay of human IgG with the purified rabbit (anti-human IgG) Fab'-β-D-galactosidase complex*

Human IgG was assayed with the sandwich technique [10]. The anti-human IgG antibody-coupled Sepharose for a solid phase was prepared as described previously [2]. (10 mg of IgG fraction of rabbit (anti-human IgG) serum were coupled to 1 g of CNBr-activated Sepharose 4B, and the antibody-coupled Sepharose was suspendend in 10 ml of 0.1 M sodium phosphate buffer, pH 7.0.)

The antibody-coupled Sepharose, corresponding to 10 μl of the suspension, was incubated with various amounts of human IgG (Miles Laboratories, Inc., Illinois) with shaking at 37°C in a final vol of 0.15 ml of Buffer A. After 4 hr of incubation, the antibody-coupled Sepharose was washed once with 1 ml of Buffer A by centrifugation, and then incubated with the Fab'-β-D-galactosidase complex (5 μl of the Sepharose 6B eluate) with shaking at 37°C for 6 hr in a final vol of 0.15 ml of Buffer A. The antibody-coupled Sepharose was then washed twice with 1 ml of Buffer A by centrifugation. The activity of β-D-galactosidase bound to the antibody-coupled Sepharose was determined with 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co., St. Louis) as described previously [1]. Amounts of the antibody-coupled Sepharose and the Fab'-β-D-galactosidase complex were adjusted to obtain the maximum ratio of the enzyme activity bound to the antibody-coupled Sepharose in the presence of 10 fmol of human IgG to that in the absence of human IgG.

3. Results and discussion

A typical result of human IgG assay is shown in fig.1, indicating that small quantities of human IgG such as 0.3 fmol (50 pg) can be determined. The sensitivity attained by the present assay markedly

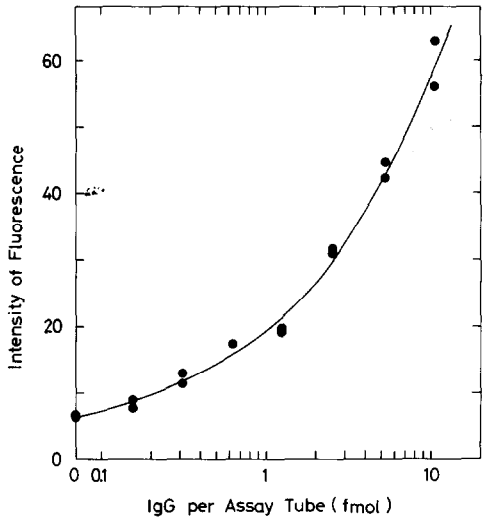

Fig.1. Assay of human IgG with the purified rabbit (anti-human IgG) Fab'-β-D-galactosidase complex. One unit of fluorescence intensity corresponds to the intensity of 1×10^{-8} M 4-methylumbelliferone.

exceeds those achieved by Belanger et al. [10] and Kato et al. [2] who have reported sensitive enzyme-linked immunoassays of rat α-fetoprotein and human IgG respectively. The enzyme activity of the Fab′-β-D-galactosidase complex bound to the antibody-coupled Sepharose in the absence of human IgG was about 2.3% of the total activity incubated. A similar percentage of the enzyme activity was also bound to the antibody-coupled Sepharose, when β-D-galactosidase itself in place of the Fab′-β-D-galactosidase complex was incubated in the same manner. When the free enzyme was incubated, however, no increase in the enzyme activity bound to the antibody-coupled Sepharose was observed with increasing amounts of human IgG.

The present experiments have demonstrated that rabbit Fab′ can easily be coupled to β-D-galactosidase under mild conditions. Sulfhydryl groups can readily be introduced into proteins under mild condition [11]. So, rabbit Fab′ fragment may be coupled to other enzymes in a similar manner to obtain Fab′–enzyme complexes which are useful not only in immunoassay but also in immunohistochemistry [12].

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