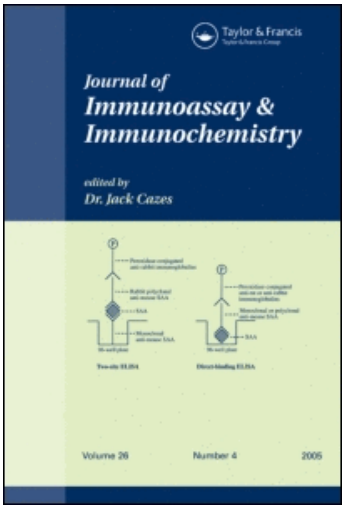

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Hashida, Seiichi , Imagawa, Masayoshi , Ishikawa, Eiji and Freytag, J. William(1985) 'A Simple Method for the Conjugation of Affinity-Purified Fab to Horseradish Peroxidase and β-D-Galactosidase from *Escherichia Coli*', Journal of Immunoassay and Immunochemistry, 6: 1, 111 — 123
To link to this Article: DOI: 10.1080/01971528508063024
URL: http://dx.doi.org/10.1080/01971528508063024

JOURNAL OF IMMUNOASSAY, 6(1&2), 111-123 (1985)

A SIMPLE METHOD FOR THE CONJUGATION OF AFFINITY-PURIFIED FAB' TO HORSERADISH PEROXIDASE AND ß-D-GALACTOSIDASE FROM ESCHERICHIA COLI

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ABSTRACT

A simple method was described for the conjugation of affinity-purified Fab' to horseradish peroxidase and ß-D-galactosidase from Escherichia coli. IgG was subjected to successive processes of pepsin digestion, reduction with 2-mercaptoethylamine, affinity-purification and reaction with maleimide groups introduced into the enzymes. In the present method, gel filtration was required only once to separate the conjugate from unconjugated components in the final step, while gel filtration had to be repeated 3-4 times in the previous methods. The conjugate preparations obtained by the present method contained less nonspecific conjugate and gave a lower background by immunoenzymometric assay technique than those obtained by the previous method.

KEY WORDS: Peroxidase, ß-D-Galactosidase, Fab', Affinity-Purification, Enzyme-Labeling.

INTRODUCTION

We developed methods for the conjugation of Fab' to enzymes through thiol groups in the hinge of Fab' by the reaction between

 0197-1522/85/0601-0111$3.50/0

thiol and maleimide groups (the hinge methods) (1, 2). The use of affinity-purified Fab'-enzyme conjugates prepared by the hinge methods made it possible to measure macromolecular antigens at attomole levels by sandwich enzyme immunoassay technique (3, 4). However, the hinge methods for the conjugation of affinity-purified Fab' to enzymes were time-consuming with repeated processes of gel filtration and concentration. This paper describes a simple method, in which gel filtration was used only once.

MATERIALS AND METHODS

Buffers

The frequently used buffers were 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1 % bovine serum albumin (buffer A) and 0.01 M sodium phosphate buffer, pH 6.5 or 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 % bovine serum albumin and 0.1 % NaN_3 (buffer B, pH 6.5 or 7.0) (1).

Enzymes and Their Assay

Pepsin from porcine gastric mucosa, horseradish peroxidase (grade I, lyophilized, RZ = 3.0) and ß-D-galactosidase from Escherichia coli (lyophilized for enzyme immunoassay) were obtained from Boehringer Mannheim GmbH, Mannheim, West Germany.

Peroxidase activity was assayed by fluorimetry using 3-(p-hydroxyphenyl)propionic acid as a substrate (2). ß-D-Galacto-

sidase activity was assayed by fluorimetry using 4-methylumbelliferyl ß-D-galactoside as a substrate (1).

Antigens and Antibodies

Human chorionic gonadotropin (hCG) was obtained from Calbiochem-Behring Corporation, San Diego, California. IgG was prepared by fractionation of serum with Na_2SO_4 followed by passage through a column of diethylaminoethyl cellulose (1). Fluorescein-labeled rabbit (anti-human IgG) IgG and goat (anti-rabbit IgG) IgG were obtained from Cappel Laboratories Inc., Cochranville, Pennsylvania. Rabbit anti-hCG IgG was obtained from Dakopatts, Copenhagen, Denmark. Rabbit (anti-fluorescein) serum was a generous gift from Dr. Edward W. Voss, Jr. (Department of Microbiology, University of Illinois, Urbana-Champaign, Illinois), who prepared it as described previously (5).

Preparation of Protein-Sepharose 4B

IgG (10 mg), hCG (30,000 U) or bovine serum albumin (10 mg) was coupled to CNBr-activated Sepharose 4B (1 g, Pharmacia Fine Chemicals AB, Uppsala, Sweden) according to the instructions of Pharmacia.

Present Method for the Preparation of Fab'-Peroxidase Conjugates

(Anti-human IgG) IgG (20 mg) in 1.55 ml of 0.1 M sodium acetate buffer, pH 4.5, containing 0.1 M NaCl was digested to $F(ab')_2$ by incubation with 0.4 mg of pepsin in 0.05 ml of the same buffer at 37°C for 24 h. After adjusting pH to 6.0 by

adding 0.2 ml of 0.5 M Na_2HPO_4, the reaction mixture was incubated with 0.2 ml of 0.1 M 2-mercaptoethylamine in 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA at 37°C for 90 min to reduce the $F(ab')_2$ to Fab'. The reaction mixture was diluted with 8 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl and 5 mM EDTA and applied to a column (0.55 x 1.0 cm) of human IgG-Sepharose 4B at a flow rate of 5 ml/h. After washing the column with the same buffer, the (anti-human IgG) Fab' was eluted with 3.2 mM HCl, pH 2.5, containing 0.1 M NaCl. pH of each eluate fraction (1 ml) was adjusted to 6.0 by adding 0.02 ml of 0.2 M sodium phosphate buffer, pH 8.0, and each fraction was mixed with 0.1 ml of 0.25 M sodium phosphate buffer, pH 6.0, and 0.05 ml of 100 mM EDTA. Fractions containing the affinity-purified Fab' were pooled and concentrated with dialysis against 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA. The amount of affinity-purified (anti-human IgG) Fab' was 1.2 mg. In the same way, affinity-purified anti-hCG Fab' (1.1 mg) was prepared from anti-hCG IgG (40 mg) using a hCG-Sepharose 4B column (0.55 x 2.5 cm).

The affinity-purified Fab' was conjugated to the maleimide-peroxidase which was prepared using N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (1, 2).

Present Method for the Preparation of Fab'-ß-D-Galactosidase Conjugates

The affinity-purified Fab' prepared as described above was conjugated to the maleimide-ß-D-galactosidase which was prepared

using N,N'-o-phenylenedimaleimide (1). When the conjugate was prepared by incubation of 2 nmol/ml of the maleimide-ß-D-galactosidase and excess (10 nmol/ml) of Fab', the maleimide-ß-D-galactosidase was completely converted to the conjugate, which consisted of one ß-D-galactosidase molecule and 3.2-3.9 Fab' molecules (1). When prepared by incubation of 2 nmol of the maleimide-ß-D-galactosidase and 1-2 nmol/ml of fluorescein-labeled Fab', the maleimide-ß-D-galactosidase remained partially unconjugated, and the monomeric conjugate formed was separated from unconjugated ß-D-galactosidase by affinity chromatography. Fluorescein-labeled Fab'-ß-D-galactosidase conjugate preparation (19-24 U) in 2.5 ml of buffer B, pH 6.5, was applied to a column (0.55 x 0.9 cm) of rabbit (anti-fluorescein) IgG-Sepharose 4B at a flow rate of 1 ml/h. After washing the column with buffer B, pH 6.5, the conjugate was eluted with 4 ml of 0.1 mM fluorescein sodium in the same buffer. The eluate (4.0 ml) was subjected to gel filtration on a column (2.0 x 45 cm) of Sephadex G-25 using the same buffer.

Previous Method for the Preparation of Fab'-Enzyme Conjugates

Fab'-enzyme conjugates were prepared by successive processes of pepsin digestion of IgG, gel filtration, affinity-purification, reduction to Fab', gel filtration, reaction with the maleimide-enzymes and gel filtration (1-4). Affinity-purification of $F(ab')_2$ was performed as in the present method.

Separation of Fab'-Peroxidase Conjugate from Unconjugated $F(ab')_2$

Fab'-peroxidase conjugate (14-20 μg) was applied to a column (0.35 x 3.0 cm) of concanavalin A-Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and the conjugate was eluted with 0.1 M α-methyl-D-mannoside as described previously (1) except that buffer A was used throughout. Peroxidase activity applied was almost completely (99-100 %) adsorbed to the column, and 98-103 % of the activity adsorbed was found in the eluate.

Calculation and Expression of the Amount of Proteins

The amount of IgG, its fragments and β-D-galactosidase was calculated from the measured absorbance at 280 nm (1). The amount of peroxidase and Fab'-peroxidase conjugate was calculated from the measured absorbance at 403 nm (1). The amount of Fab'-β-D-galactosidase conjugate was expressed in units of β-D-galactosidase activity (1).

Test for the Proportion of Nonspecific Conjugates

The preparation of (anti-human IgG) or anti-hCG Fab'-enzyme conjugates was passed through a column of human IgG- or hCG-Sepharose 4B, and the enzyme activity in the effluent was compared with that applied (1).

Test for the Presence of Unconjugated Enzymes

The preparation of rabbit Fab'-enzyme conjugates was passed through a column of (anti-rabbit IgG) IgG-Sepharose 4B, and the

enzyme activity in the effluent was compared with that applied (1).

Sandwich Enzyme Immunoassay Technique for hCG

An anti-hCG IgG-coated polystyrene ball was incubated with hCG at 37°C for 4 h and at 4°C overnight and then with 20 ng of affinity-purified anti-hCG Fab'-peroxidase conjugate or 0.2 mU of affinity-purified anti-hCG Fab'-ß-D-galactosidase conjugate at 20°C for 4 h (2, 3). Peroxidase or ß-D-galactosidase activity bound was assayed by incubation at 30°C for 60 min or 30 min as described above.

Immunoenzymometric Assay Technique for hCG

HCG in 0.05 ml of buffer A was mixed with affinity-purified anti-hCG Fab'-peroxidase conjugate (10 ng) in 0.05 ml of buffer A, and incubated at 37°C for 6 h with continuous shaking and at 4°C overnight without shaking. After the incubation, 0.05 ml of the reaction mixture was passed through a column of hCG-Sepharose 4B at a flow rate of 1 ml/h, and washed with 1 ml of buffer A. Peroxidase activity in the effluent was assayed by incubation at 30°C for 30 min as described above. The column consisted of a mixture of 0.02 ml in wet packed volume of hCG-Sepharose 4B and 0.03 ml in wet packed volume of bovine serum albumin-Sepharose 4B in GILSON C-200 Blue Tip.

RESULTS

Proportion of Nonspecific Conjugate

Affinity-purified (anti-human IgG) Fab'- and affinity-purified anti-hCG Fab'-peroxidase conjugates were prepared by the

present method and applied to columns of human IgG- and hCG-Sepharose 4B, respectively. The adsorption of peroxidase activity in the conjugate preparations to the column was 89-93 % both before and after purification with concanavalin A-Sepharose 4B. By contrast, the corresponding conjugates prepared by the previous method was less (80-85 %) adsorbed (Table 1). Similar results were obtained with affinity-purified fluorescein-labeled (anti-human IgG) Fab'-ß-D-galactosidase conjugates which were prepared by incubation of 2 nmol/ml of the maleimide-ß-D-galactosidase and 1-2 nmol/ml of Fab' and affinity-purified with (anti-fluorescein) IgG-Sepharose 4B (Table 1).

Thus, the present method, which was simpler, could significantly reduce the proportion of nonspecific conjugate in the conjugate preparations.

Absence of Unconjugated Enzymes

Rabbit Fab'-enzyme conjugates were prepared by both the present and previous methods and applied to a column of goat (anti-rabbit IgG) IgG-Sepharose 4B. The adsorption to the column of the enzymes activities in the conjugate preparations was almost complete, indicating that there was little unconjugated enzymes in the conjugate preparations (Table 1).

Proportion of Unconjugated Antibodies

By gel filtration, Fab'-ß-D-galactosidase conjugate was separated from unconjugated Fab', $F(ab')_2$ and IgG, and Fab'-peroxidase conjugate was separated from unconjugated Fab' and IgG,

TABLE 1

Purity and antigen-binding activity of affinity-purified fluorescein-labeled rabbit (anti-human IgG) Fab' conjugates and affinity-purified rabbit anti-hCG Fab' conjugates with peroxidase and ß-D-galactosidase

Enzyme	Conjugation method	Adsorption of enzyme activity (%) to (Anti-rabbit IgG) IgG-Sepharose 4B	Human IgG-Sepharose 4B	hCG-Sepharose 4B
Peroxidase	Present method	99	93	—
		99	—	89
		98[a]	91[a]	—
		99[a]	—	89[a]
	Previous method	99	85	—
		98[a]	84[a]	—
		99	—	80
ß-D-Galactosidase	Present method	99[b]	94[b]	—
		98[c]	97[c]	—
		99[c]	—	98[c]
	Previous method	99[b]	86[b]	—
		99[c]	98[c]	—
		100[c]	—	98[c]

[a]The conjugates were purified using concanavalin A-Sepharose 4B.

[b]The concentrations of the maleimide-ß-D-galactosidase and fluorescein-labeled Fab' in the reaction mixture for conjugation were 2 nmol/ml, and the conjugate was affinity-purified using an (anti-fluorescein) IgG-Sepharose 4B column.

[c]The concentrations of the maleimide-ß-D-galactosidase and Fab' in the reaction mixture for conjugation were 2 nmol/ml and 10 nmol/ml, respectively, and the average number of Fab' molecules conjugated per ß-D-galactosidase molecule was 3.2-3.9.

but not from $F(ab')_2$, if any (1). When the preparation of affinity-purified fluorescein-labeled (anti-human IgG) Fab'-peroxidase conjugate obtained by the present method was applied to a concanavalin A-Sepharose 4B column, 79 % of fluorescence intensity applied was adsorbed, and 76 % of fluorescence intensity in the effluent was adsorbed to human IgG-Sepharose 4B. This indicated that 16 % of fluorescence intensity in the conjugate preparation was associated with anti-human IgG but unconjugated. In the corresponding conjugate prepared by the previous method, 85 % was adsorbed to concanavalin A-Sepharose 4B, and 45 % was adsorbed to human IgG-Sepharose 4B.

Recovery of Peroxidase, ß-D-Galactosidase and Fab'

The recovery in the conjugate of peroxidase incubated for conjugation was calculated from absorbance at 403 nm in the elution profile by gel filtration (1). It was 58-64 % in the present method and 68-70 % in the previous method. The recovery of Fab' must have been similar (1).

The recovery in the ß-D-galactosidase conjugate of Fab' incubated for conjugation was calculated from fluorescence intensity of fluorescein-labeled Fab' in the elution profile by gel filtration (1). It was 60-78 % in the present method and 64-81 % in the previous method.

The recovery of ß-D-galactosidase in the conjugate varied under different conditions for conjugation. When the conjugate was prepared by incubation of 2 nmol/ml of the maleimide-ß-D-galactosidase with excess (10 nmol/ml) of Fab', the maleimide-ß-D-

galactosidase was almost completely converted to the conjugate (Table 1). However, when prepared using 2 nmol/ml of the maleimide-ß-D-galactosidase and 1-2 nmol/ml of fluorescein-labeled Fab', only 14-17 % of ß-D-galactosidase activity in the conjugate preparation in the peak fraction from a Sepharose 6B column was adsorbed to a column of (anti-fluorescein) IgG-Sepharose 4B, and 49-59 % of the activity adsorbed was eluted with fluorescein. This low recovery was caused largely by the fact that the average number of fluorescein molecules coupled per Fab' molecule was only 0.3.

Usefulness of the Conjugates

The affinity-purified anti-hCG Fab'-enzyme conjugates prepared by the present method, whether they had been purified on a column of concanavalin A-Sepharose 4B or not, gave the same sensitivity of hCG dose response curves by sandwich enzyme immunoassay technique as those prepared by the previous methods. And a lower background was obtained for hCG dose response curves by immunoenzymometric assay technique using the conjugates prepared by the present method, which contained less nonspecific conjugates.

DISCUSSION

In the present method, gel filtration was required only once, while it was repeated 3-4 times in the previous method (1). In addition, the affinity-purified Fab'-enzyme conjugate preparations

obtained by the present method contained less nonspecific conjugate and gave a lower background in immunoenzymometric assay than those obtained by the previous method. This may have been effected by prior reduction of $F(ab')_2$ and subsequent affinity-purification, for affinity-purification must have eliminated useless Fab' which lost the antigen-binding activity by reduction.

In the present method, thiol groups of Fab' may have been partially oxidized during affinity-purification. Probably as a result of this, the yield of the conjugate tended to be lower in the present method than in the previous one, and the proportion of unconjugated $F(ab')_2$ in the peroxidase conjugate preparation slightly increased. However, the sensitivity of immunoassays was not affected, and the ß-D-galactosidase conjugate preparation contained no unconjugated $F(ab')_2$, since it was separated by gel filtration. Unconjugated $F(ab')_2$ in the peroxidase conjugate preparation could be removed using concanavalin A-Sepharose 4B, if necessary.

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