A SIMPLE METHOD FOR THE PREPARATION OF ENZYME–ANTIBODY CONJUGATES

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1. Introduction

Enzyme–antibody conjugates are valuable tools in immunohistochemistry [1,2]. More recently they have been used to detect and quantitate antigens and antibodies [3,4]. Unfortunately, there are very few generally applicable methods for the production of such conjugates. Glutaraldehyde is the most widely used coupling reagent. Typically the conjugates are produced in low yield with considerable loss of both enzyme and antibody activity. Extensive polymerisation resulting in high molecular weight conjugates often occurs [5].

We describe here a simple but efficient method for the preparation of antibody conjugates using the hetero-bifunctional reagent *meta*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). This reagent has been used to prepare an insulin–β-D-galactosidase conjugate [6].

2. Materials and methods

2.1. *Reagents*

Mercaptoethanol, papain, 5,5′-dithio-2-nitrobenzoic acid (DTNB), *O*-nitrophenyl-β-D-galactopyranoside, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, triiodothyronine (T3) and Sepharose 4B were purchased from Sigma Chemical Co. (St Louis), Sephadex G-25 from Pharmacia Ltd. (Uppsala), DEAE-Biogel A from Bio-Rad Labs (Richmond), *m*-nitrobenzyloxymethyl pyridinium chloride and polyoxyethylene (20) sorbitan mono-oleate (Tween 80) from BDH Chemicals Ltd. (Poole), cellulose powder CC41 from Whatman Ltd. (Maidstone), 1,4-butanediol diglycidyl ether (bisoxirane) from Aldrich Chemical Co. (Milwaukee), bovine albumin (BSA) from Armour Pharmaceutical Co. Ltd. (Eastbourne), β-D-galactosidase from Boehringer-Mannheim and MBS from Pierce Chemical Co. (Rockford).

2.2. *Preparation of MBS-acylated antibody*

Purified donkey antibodies raised against sheep Fc fragments were tested for the presence of free thiols using DTNB [7]. (The preparation and purification of these antibodies will be described in a later paper.) The antibodies were used to prepare the conjugates.

Dioxan (50 μl) containing 0.73 mg MBS was added to 3.5 mg antibody dissolved in 7.0 ml 0.1 M phosphate buffer (pH 7.0) containing 0.09 M NaCl. The solution was kept at 30°C for 1 h, then chromatographed on a Sephadex G-25 column (60 × 0.9 cm) using 0.01 M phosphate buffer pH 7.0 as eluent. Fractions containing protein were pooled and the number of maleimide residues introduced per antibody molecule determined by measuring the decrease of thiol content [7] on addition to excess mercaptoethanol.

2.3. *Preparation of β-D-galactosidase–MBS antibody conjugates*

β-D-Galactosidase, 1.4 mg, and 1.4 mg MBS-acylated antibody were dissolved in 5.5 ml 0.01 M phosphate buffer (pH 7.0) containing 0.01 M $MgCl_2$ and held at 30°C for 1 h. Mercaptoethanol (2 M) was

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then added to final conc. 0.01 M. The solution was applied to a DEAE-agarose column (15 × 1 cm) equilibrated with 0.01 M Tris–HCl buffer (pH 7.0) containing 0.01 M $MgCl_2$, 0.01 M mercaptoethanol and 0.05 M NaCl. The column was eluted with Tris buffer (30 ml) before applying a linear gradient consisting of Tris–HCl buffer containing 250 ml 0.05 M NaCl in the mixing chamber and 250 ml 0.2 M NaCl in the reservoir. Fractions (2.7 ml) were collected into Tris–HCl buffer (0.3 ml) containing 1% w/v BSA and assayed for total and bound enzyme activity and antibody activity.

2.4. *Determination of antibody, total and bound enzyme activity*

Antibody activity was measured using a sandwich-type assay. Antibody solution was mixed with a cellulose–sheep Ig solid phase [8] maintained at room temperature for 3 h, and washed twice with 2% w/v saline. Iodinated sheep IgG [9] was added to the solid phase, left overnight at room temperature, washed as above and the radioisotope content of the solid phase counted in an LKB Wallac 8000 gamma counter. The amount of radioactivity bound to the solid phase was found to be dependent on the quantity of specific antibody present.

Total enzyme activity was measured using *O*-nitrophenyl-β-galactopyranoside as substrate [10]. A_{420} was measured on a Bausch and Lomb Spectronic 710. Formation of the enzyme–antibody conjugate was determined by measuring the binding of treated enzyme to a cellulose–sheep IgG solid phase. Enzyme conjugate (0.1 ml) was incubated for 4 h with 0.1 mg solid phase suspended in 0.1 ml 0.05 M barbitone buffer (pH 8.6) containing 0.1% w/v BSA. The solid phase was washed twice with barbitone buffer, enzyme activity was then measured on the solid phase.

2.5. *Analysis of enzyme–antibody conjugates by gel filtration*

The fractions from the ion-exchange column containing bound enzyme were pooled. A 2 ml aliquot was applied to a Sepharose 4B column (100 × 1 cm), eluted with Tris buffer and assayed for total and bound enzyme activity.

2.6. *Detection of T_3 antibodies*

T_3 antibodies were raised by immunising sheep with a T_3–BSA conjugate prepared using EDC [11]. Aliquots (0.1 ml) of the T_3 antisera diluted in 0.05 M barbitone buffer (pH 8.6) containing 0.1% w/v BSA were mixed with a T_3–cellulose (0.1 mg, 0.1 ml) solid phase [12] suspended in barbitone buffer containing 2% w/v Tween 80. The solid phase was left overnight at 4°C before washing twice with barbitone buffer containing 5% w/v NaCl. Enzyme–antibody conjugate was added to the solid phase, left overnight and washed as before. Enzyme activity attached to the solid phase was measured. A comparison was made with the binding of label to the solid phase in the presence of normal sheep serum.

3. Results

No free thiols could be detected in the purified antibody preparations. Following modification of these antibodies with MBS, an average of 3 maleimide residues were introduced per antibody molecule. No loss of enzyme activity occurred during conjugation to antibody. Following the conjugation reaction 55% of the total enzyme activity was bound by a sheep IgG solid phase. Non-specific binding of untreated enzyme to the same solid phase was 5%, indicating that 50% of the enzyme was conjugated to immunologically-active antibody. When the concentration of antibody and enzyme was increased 3-fold a 20% decrease in enzyme and antibody activity occurred on conjugation. However, 90% of the enzyme was bound to immunologically-active antibody.

Unreacted antibody was effectively removed from the conjugate by ion-exchange chromatography. However, the purification step did not effectively remove all the free enzyme, although the proportion of bound enzyme activity was greater in the leading edge of the enzyme peak. The pooled conjugate contained ~40% free enzyme. When the partially purified label was analysed by gel filtration most of the bound enzyme was present in low molecular weight conjugates, although some high molecular weight conjugates were formed.

At an initial dilution of T_3 antiserum of 1:25 600, 10% of the total enzyme activity was bound to the T_3 solid phase. With normal sheep serum at an initial dilution of 1:400 only 5% of the total enzyme activity was bound to the solid phase. These results indicate

that the label can be used to detect low levels of specific antibodies.

When stored for 4 months in solution at 4°C in the presence of 0.05% w/v sodium azide the label retained 85% of its enzyme and antibody activity.

4. Discussion

This paper describes the use of MBS in the preparation of enzyme–antibody conjugates. This reagent contains an active ester which can selectively react with amino residues and so introduce reactive maleimide residues into the antibody molecule. As antibodies contain no free thiols, these residues will be free to react with thiol-containing enzymes. The method is in theory widely applicable as thiols can be introduced into many enzymes using 4-methyl mercaptobutyrimidate [13]. The coupling of β-D-galactosidase to purified donkey anti-sheep Fc antibodies by MBS was efficient with little loss in enzyme or antibody activity and minimal conjugate polymerisation.

It is advisable to test for the presence of free thiol residues in the enzyme samples used for coupling. Although studies [14] have indicated that β-galactosidase contained ~40 reactive cysteine residues, the number of free thiol groups present in commercial enzyme preparations was generally much lower. In some batches no free thiols could be detected. The enzyme batch used to prepare the label contained an average of 3 free thiol residues/enzyme molecule. The use of enzyme–antibody labels for the detection and quantitation of antibodies and antigens will be described in a later paper.

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