# THE PREPARATION AND PURIFICATION OF A β-D-GALACTOSIDASE–OESTRADIOL-17β CONJUGATE FOR ENZYME IMMUNOASSAY

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## 1. Introduction

Recent studies have indicated that both enzyme and enzyme immunoassay may overcome some serious drawbacks inherent in the radioimmunoassay technique [1–3]. The sensitivity and precision of any immunoassay system however depends in part on the purity of the label used and unfortunately previous workers in the enzyme immunoassay field have not extensively purified their enzyme-conjugate labels. Affinity chromatography procedures for the purification of enzyme conjugates which enables pure conjugates to be obtained are therefore described.

The development of any sensitive enzyme immunoassay or enzyme inhibition technique demands a critical choice of high specific activity enzyme [1], which has to be stable on chemical modification, and the measurement of its enzyme activity must be simple and sensitive. The properties of *E. coli*, (β-D-galactoside galactohydrolase EC 3.2.1.23) appear to satisfy most of these requirements, and a pure oestradiol-17β conjugate of this enzyme has been prepared.

Purification of this conjugate must be such that it does not contain any unconjugated (active or denatured) enzyme, or any unconjugated steroid. Consideration of the antibody–steroid interaction involved in enzyme immunoassay shows that maximum sensitivity is achieved by a 1:1 enzyme/steroid ratio, and that maximum specificity is obtained when the homologous hapten (identical to that used for the assay antiserum) is conjugated to the enzyme. The preparation of an *E. coli* β-D-galactosidase–oestradiol-17β conjugate which takes all the above considerations into account is described.

## 2. Materials and methods

### 2.1. *Reagents*

[2.4.6.7-$^3$H]Oestradiol-17β (spec. act. 85 Ci/mM) was obtained from the Radiochemical Centre, Amersham, England and non-radioactive steroids from Koch-Light, Colnbrook, Bucks, England. 1 Cyclohexyl-3-(2-morphinoethyl) carbodiimide metho-*p*-toluene sulphonate (CMC) and 1-ethyl-3-(3)-dimethyl-aminopropyl carbodiimide–HCl (EDC) were obtained from Sigma, St Louis, USA. Sepharose 4B and 6B, CH–Sepharose 4B, DEAE–Sephadex A 50 and Sephadex G–10 were obtained from Pharmacia, Uppsala, Sweden. Norit A charcoal was purchased from Sigma, St Louis, USA and the synthetic substrate 4-methylumbelliferyl-galactoside from Koch-Light, Colnbrook, Bucks, England.

The hapten used for enzyme conjugation, oestradiol-17β–6-(*O*-carboxymethyl)-oxime ($E_2$6CMO) was synthesised according to Dean, Exley and Johnson [4]. Oestrone-6-(*O*-carboxymethyl) oxime ($E_1$6CMO) was prepared according to Dean, Rowe and Exley [5]. Antiserum to oestradiol-17β raised by $E_2$6CMO–BSA was prepared according to Exley, Johnson and Dean [6] and to oestrone raised by $E_1$6CMO–BSA by the method of Dean, Rowe and Exley [5]. Antiserum to oestradiol-17β–3-glucuronide was a gift from Professor A. E. Kellie of the Middlesex Hospital, London, England. The enzyme activity of β-D-galactosidase was measured using the fluorimetric technique of Robinson [7]. Galactosylamine was coupled to CH–Sepharose 4B by EDC using a modification of the method of Cuatrecasas and Anfinsen [8]. The product was stored at 4°C until use. Phosphate–azid–saline (PAS)

buffer, pH 7.0, was prepared according to Abrahams [9].

2.2. *Preparation of the conjugate*

Figure 1 shows the steps involved in the preparation and purification of the conjugate. The first step was to prepare the extensively purified enzyme from *E. coli*. Mutant K12 3300 cells were cultured and harvested in the normal way and then the cells ruptured in a French press machine. The resultant suspension was centrifuged to remove insoluble material before subjection to 40% w/v ammonium sulphate precipitation. The crude enzyme was extensively purified by gel filtration on Sepharose 6B (Abuknesha and Brown to be published).

The conjugate was prepared using $E_2$6CMO. Condensation of this compound with lysyl groups of the enzyme to form a peptide link was effected using the carbodiimide CMC. An intermediate of $E_2$6CMO and CMC was first formed in weak (0.01 M) acetate buffer, pH 4.7, by reacting 20 mg enzyme with 0.4 mg of $E_2$6CMO at 10–15°C for 30 min. This intermediate was added slowly over 10 min to a solution of the enzyme in (0.1 M) acetate buffer, pH 5.8. The stoichiometry of the reaction was arranged to give a minimal steroid/enzyme ratio (usually a 20–50 m ratio of steroid to enzyme was required to give about 30% yield of such conjugate). The mixture was then stirred at 4°C overnight and the reaction stopped by adding phosphate buffer, pH 7.2.

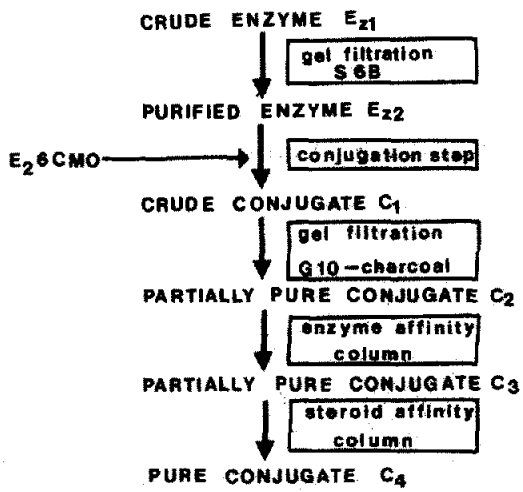


Fig.1. Flow chart of preparation and purification of enzyme–steroid conjugate.

2.3. *Purification of the conjugate*

As shown in fig.1, 3 steps are involved in the purification of the crude conjugate ($C_1$). First, the majority of the unconjugated steroid, and carbodiimide by-products were removed by dialysis, and then last traces by use of a Sephadex G-10 column in series with a small charcoal column. After this treatment fractions containing the enzyme were collected and concentrated. This was the partially purified conjugate ($C_2$). The next purification step involved affinity chromatography to remove non-enzymically active molecules (whether conjugated to steroid or not) from $C_2$. This conjugate was poured down a column of CH–Sepharose 4B–galactosylamine which was washed with PAS buffer until no further protein was detected in the eluate. The affinity bound active enzyme was then eluted with 100 mM galactose. Fractions containing the active enzyme were concentrated and dialysed and provided the partially purified conjugate ($C_3$).

The last step in the purification, one that removes all unconjugated enzyme, is a focal one. This again involved affinity chromatography. Antiserum raised against oestrone–6-(*O*-carboxymethyl) oxime ($E_1$6CMO–BSA which is heterologous to the hapten ($E_2$6CMO) conjugated to the enzyme was coupled to activated Sepharose 4B. In this heterologous affinity chromatography technique the affinity of the bound antiserum for the $E_2$6CMO moiety of the conjugates was only 10% of that for its homologous hapten ($E_1$6CMO), thus excess antibodies were bound to ensure efficient immunoadsorption. This affinity column attracted the enzyme–$E_2$6CMO conjugate only. After immunoadsorption and elution of the free enzyme by PAS buffer, the bound conjugate was eluted from the column by the stronger affinity homologous hapten $E_1$6CMO. The column fractions containing the conjugate were then concentrated and dialysed to remove most of the $E_1$6CMO eluant, then subjected to Sephadex G-10 and charcoal treatment before finally concentrating with polyethylene glycol. This was the final purified conjugate $C_4$.

## 3. Results

Results showed the final enzyme specific activity of conjugate $C_4$ to be 90% or more of that of the original enzyme before conjugation ($E_{z2}$) (fig.1) suggesting

that little enzymatic activity had been destroyed in the process. The immunogenicity of the conjugate $C_4$ to the homologous anti $E_2$6CMO–BSA serum was tested before and after the final affinity chromatography purification step, by comparing the inhibition of binding of [2,4,6,7-$^3$H]oestradiol-17$\beta$ produced by different amounts of the conjugate with that of known quantities of non-radioactive oestradiol-17$\beta$. The radioimmunoassay technique of Hotchkiss, Atkinson and Knobil [10] was used for this test, result showed that the immunogenicity of $C_4$ was greatly increased by the last affinity chromatography step and that it was highly immunogenic. Methods of determining the number of steroids bound per enzyme molecule are now being developed.

A typical enzyme immunoassay standard curve for the determination of oestradiol-17$\beta$ is shown in fig.2. Curves were obtained using the $\beta$-D-galactosidase–oestradiol-17$\beta$ conjugate and using either the homologous (anti $E_2$6CMO–BSA) serum or an heterologous antiserum to oestradiol-17$\beta$–3 glucuronide–BSA. The heterologous antiserum gives better sensitivity but this is at the expense of specificity. The

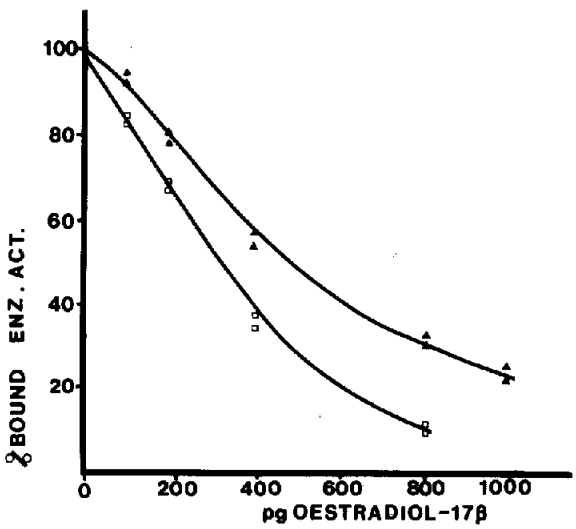


Fig.2. Standard curves showing % bound enzyme activity against pg oestradiol-17$\beta$ added, obtained for enzyme-immunoassay using the $E_2$6CMO–$\beta$-D-galactosidase conjugate with homologous antiserum in $E_2$6CMO–BSA (▲ - ▲) and with heterologous antiserum, i.e., $E_2$–3 glucuronide–BSA (□ - □). Enzyme was assayed using 4-methylumbelliferyl-galactoside.

method of separating bound from free conjugate was by the double antibody solid phase (DASP) method in which the second antibody (goat anti-rabbit $\gamma$-globulin) was bound to Sepharose 4B.

## 4. Discussion

The present enzyme-conjugate purification procedure is more comprehensive than those previously described by other workers who invariably have not included any step to remove non-enzymically active molecules. Despite the necessity of all the purification steps, once prepared, a gram of enzyme conjugate will provide enough label for millions of enzyme immunoassays. Present sensitivity of the enzyme immunoassay of oestradiol-17$\beta$ using the purified conjugate (fig.2) approaches that of radioimmunoassay for this hormone. However the double antibody solid phase (DASP) system used for the assay separation stage did not enable the maximum potential sensitivity to be obtained, thus other separation steps are being studied.

The present preparation and purification procedure can be used for enzyme-conjugates used in enzyme inhibition immunoassays. It is this technique in particular [2] which could in the future supersede radioimmunoassay, on the counts of cost, simplicity, speed and ease of automation.

## Acknowledgement

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