PROTEIN-PROTEIN CONJUGATION ON A LECTIN MATRIX

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SUMMARY An efficient method of protein-protein conjugation yielding primarily monoconjugates is described.A glycoprotein enzyme, invertase,was 'spaced-out' on a succinyl concanavalin A sepharose matrix and reacted with 1% glutaraldehyde.The excess glutaraldehyde was washed out and a second,non-glycoprotein,enzyme,urease, was reacted with the'activated' invertase.The column was washed till the washings were free of enzymatic activity.On elution with α-methyl glucoside both enzymes were detected in the eluate.Resolution on Sepharose 6B revealed that the eluted invertase was completely conjugated to urease.The molecular size of the conjugate suggested that it was a monoconjugate.The glutaraldehyde treated enzyme retained its immunological reactivity in the conjugate.This method of protein-protein conjugation is applicable if one of the two involved proteins is a glycoprotein.

INTRODUCTION Covalent conjugates between two dissimilar proteins have many applications in biology and medicine.Ideally such coupled proteins should not contain any conjugates resulting from coupling between molecules of the same protein species,and both proteins should be in stoichiometric equivalence,preferably in the form of monoconjugates.

Most methods of protein-protein conjugation employing bifunctional reagents are of low efficiency and result in mixed,varying, and often large conjugates of indeterminate stoichiometry (1-3), unsuitable for quantitative purposes such as enzyme-linked immunoassay.Moreover such conjugates are likely to be more immunogenic,and to possess new antigenic determinants,factors which render them less desirable in the realm of clinical application.

We considered that the spacing out of one of the proteins on a matrix,(from which it is capable of being eluted under mild conditions),would hinder coupling between the molecules of this protein species.Subsequently,selective 'activation' of the former protein with a bifunctional reagent was considered a means of preventing the second protein species from coupling to itself. These considerations led to the development of an efficient

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conjugation procedure yielding primarily monoconjugates.This procedure is applicable if at least one of the two involved proteins is a glycoprotein,and utilises succinyl concanavalin A sepharose as a matrix.

METHODS Protein was estimated according to the method of Lowry et al using crystalline bovine serum albumin (Sigma) as standard (4).

Preparation of succinyl concanavalin A sepharose Concanavalin A was prepared according to the method of Surolia et al (5) and coupled to sepharose 6B according to the cyanogen bromide method of Porath et al (6).To 10 ml of this gel (4 mg concanavalin A/ml of gel) suspended in saturated sodium acetate,was added 30 mg of succinic anhydride.After stirring for 2 hours at 4°C,the gel was washed in a sintered glass funnel,resuspended in saturated sodium acetate and mixed with 30 mg of succinic anhydride for a further 12 hours at 4°C.The gel was then washed with water and suspended overnight in 0.05M sodium acetate buffer pH 5.6 containing 0.15M NaCl and 0.001M each of $MgCl_2$,$MnCl_2$ and $CaCl_2$.The gel was washed with 0.05M sodium acetate buffer pH 5.6 containing 0.15M NaCl before use.

The absence of reactive amino groups in the matrix after succinylation was confirmed by the trinitrobenzenesulphonic acid method of Failla and Santi (7) using plain sepharose 6B and concanavalin A sepharose as controls.

Conjugation 8 ml of succinyl concanavalin A sepharose suspended in 10 ml of 0.05M sodium acetate buffer pH 5.6 containing 0.15M NaCl was mixed overnight at 4°C with 1 ml of a 2.3 mg/ml solution of yeast invertase (Type VI Sigma).The gel was then washed in a sintered glass funnel with the above buffer till the washings showed no invertase activity.The gel was then mixed with 8 ml of plain sepharose 6B for 2 hours at 4°C,packed into a column and equilibrated with 0.1M sodium phosphate buffer pH 7.2 containing 0.15M NaCl. 25% glutaraldehyde (Koch-Light) was diluted in the same buffer to give a 1% solution,16 ml of which were introduced into the column.The column was closed for 18 hours at 4°C.It was then washed out with 160 ml of the same buffer.16 ml of a 1 mg/ml solution of urease (City Chemical Co.New York) were introduced into the column which was closed for a further 18 hours at 4°C. The column was then washed extensively with the same buffer until no urease activity was detectable in the washings.16 ml of a 0.01 % ethanolamine solution were introduced into the column for 4 hours at 4°C,following which the column was once again washed extensively with the above buffer.The column was eluted with 10 % α-methyl glucoside in 0.1M sodium phosphate buffer pH 7.2 containing 0.15M NaCl.One ml fractions were collected and assayed for invertase and urease activities.Active fractions were pooled and concentrated using Aquacide II (Calbiochem).

Enzyme Assay Invertase was assayed using a coupled glucose oxidase -peroxidase system described elsewhere (8).One unit of invertase activity was defined as that quantity of enzyme that liberates one micromole of glucose per 10 minutes of assay time at 37°C. Urease was assayed using an adaptation of the method of Chaney and Marbach (9).One unit of urease activity was defined as the quantity of enzyme that liberates one nanomole of ammonia per 30 minutes of assay time at 37°C.

Gel Filtration Gel filtration was performed on a Sepharose 6B column (50 x 1.1 cm).The flow rate was 15 ml/hour and the fraction volume was 1.1 ml.The column was equilibrated with 0.1M sodium phosphate buffer pH 7.2 containing 0.15M NaCl for studies involving the α-methyl glucoside eluate and free invertase.It was equilibrated with 0.02M sodium veronal buffer pH 8.3 containing 0.15M NaCl for studies involving keyhole limpet haemocyanin (Schwarz Mann).

Gel filtration was performed succesively with the following:

a) 0.5 ml of a concentrate of the α-methyl glucoside eluate containing 74.4 enzyme units of invertase.Each fraction was assayed for invertase and urease.
b) 0.5 ml of a 0.23 mg/ml solution of free invertase and
c) 0.5 ml of a 2.64 mg/ml solution of keyhole limpet haemocyanin.

Antiserum Liposomes containing invertase were prepared as described elsewhere (8).Liposome preparations,each with a total of 2.25 mg surface invertase were injected as single doses (by multiple subcutaneous puncture) to rabbits.The rabbits were bled at 10 and 22 days post injection and the sera were pooled.Invertase was coupled to Sepharose 4B by the cyanogen bromide method of Porath et al (6).A 15 ml invertase-sepharose column(0.52 mg protein/ml of gel) was equilibrated with 0.05M sodium phosphate buffer pH 7.2 containing 0.15M NaCl,and was loaded with 12 ml of the pooled antiserum.It was washed with the above buffer till no protein was detectable in the washings and then eluted with 3M KSCN in the above buffer.Following concentration and dialysis,the eluted protein was loaded,in the central well of an agarose plate for immunodiffusion studies.

Immunodiffusion This was performed using the Ouchterloney technique (10),with wells of 0.02 ml capacity in a veronal(0.02M,pH8.3) buffered 1% agarose gel.The central well contained antiinvertase. Active fractions following gel filtration of the α-methyl glucoside eluate were pooled and concentrated and loaded in the outer wells of the immunodiffusion plate.The plate was photographed after 36 hours of incubation at 4°C.

RESULTS Each of the active fractions obtained from the α-methyl glucoside elution of the lectin matrix contained both invertase and urease activities.These fractions were pooled and concentrated to a volume of 2.5 ml containing 148.8 enzyme units of invertase per ml.0.5 ml of this sample were used in the gel filtration studies described under 'methods'.

It was expected that the invertase activity would appear in two peaks,one representing conjugated invertase (and which would coincide with the urease peak) and the other,later, peak representing unconjugated invertase.It was considered that the ratio of the peak sizes would be an index of the efficiency of the conjugation.

A solitary invertase peak,however,was obtained which coincided with the peak for urease obtained on assaying the same fractions. The invertase activity recovered in this peak (76.4 enzyme units)

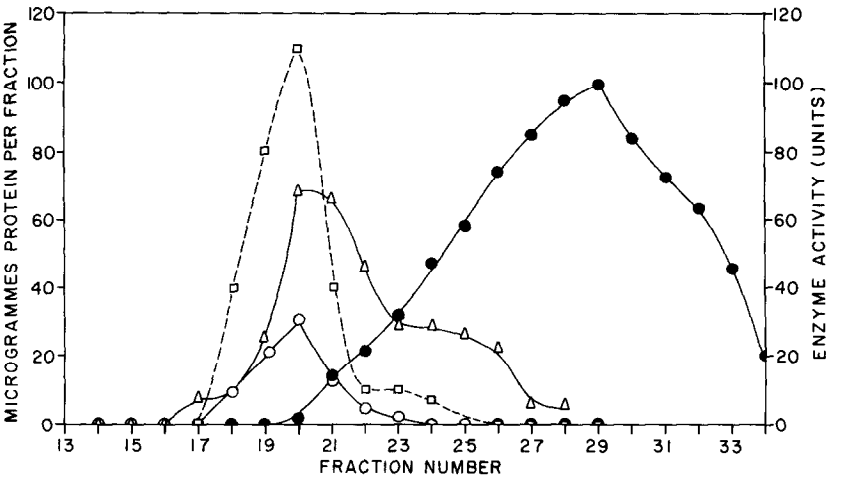

Fig.1. **Gel filtration studies of the conjugate and marker proteins on a Sepharose 6B column**

□---□ **urease activity in the conjugate/fraction**
○——○ **invertase activity in the conjugate/fraction**
Δ——Δ **keyhole limpet haemocyanin microgrammes protein/fraction**
●——● **free invertase activity/0.1 ml**

wholly accounted for the amount of invertase loaded (Fig.1). Free invertase, separately chromatographed on the same column, gave a distinct peak, very well separated from that of the conjugate (Fig.1).

Although the gel filtration experiments revealed that the conjugation was virtually complete and that the conjugate was uniform, the stoichiometry of the conjugate remained to be confirmed. The molecular weight of urease is approximately 483000 daltons (11) and that of invertase around 270000 daltons (12). On an addition basis, it was reasoned that if the conjugate was a monoconjugate, on gel filtration its molecular size would roughly correspond to that of keyhole limpet haemocyanin at pH 8.3 . On chromatography, haemocyanin at this pH was found to peak at virtually the same volume as the conjugate, indicating that the latter is a monoconjugate.

On immunodiffusion, it was found that the glutaraldehyde-treated enzyme, invertase, retained its immunological reactivity in the conjugate.

DISCUSSION For accuracy and reproducibility in quantitative enzyme-linked immunoassays it is essential that enzyme and antibody be linked in a uniform stoichiometric fashion. Quantification of receptors for hormones, lectins or antibodies on cell surfaces would ideally require monoconjugates. In histochemical use small conjugates are preferred since they more readily penetrate cells. The linking of one protein to a carrier protein, to increase its immunogenicity, would be less likely to give rise to new antigenic determinants if monoconjugates were used. The use of antibodies to target deficient enzymes or drugs to specific tissues has been suggested. When the targeting involves proteins, a monoconjugate would be a more effective vehicle than a larger, mixed conjugate. Many potential uses of protein-protein monoconjugates may be postulated. Indeed, the ability to efficiently and reproducibly synthesize such conjugates may well lead to newer applications. Periodate oxidation of a fluorodinitrobenzene treated glycoprotein enzyme, peroxidase, and the subsequent Schiff base coupling of the carbohydrate side chains of the enzyme to immunoglobulin has been recently reported (13). This represents a method of conjugation in which coupling within the same protein species is prevented. The stoichiometry of the conjugate was however not reported. Theoretically, such a procedure carried out in solution might be expected to yield only a limited proportion of monoconjugates. Moreover, the conditions of blocking and reduction may prove too harsh for certain enzymes. A diminution in the activity of peroxidase itself following periodate oxidation (14) has been reported.

The gel filtration studies outlined above demonstrated the formation of monoconjugates between invertase and urease. The large molecular size of urease relative to the matrix attached invertase may have been an important factor in the production of monoconjugates.

Established methods exist for the introduction of sugar side chains into proteins which lack them (15-17). We would like to suggest that matrix methods, similar to the one outlined above, could be developed to facilitate the formation of monoconjugates from any two proteins. An alternative to a lectin matrix, applicable to proteins containing -SH groups, could be a dilute thiol matrix.

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