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THE APPLICATION OF THIN-LAYER GEL FILTRATION CHROMATOGRAPHY IN THE RAPID ASSESSMENT OF PROTEIN–PROTEIN COUPLING REACTIONS

J. H. KENNEDY, L. J. KRICKA* and P. WILDING

Department of Clinical Chemistry, Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Birmingham, B15 2TH (Great Britain)

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SUMMARY

The use of enzymes as markers when covalently coupled to various antigens or antibodies has wide application in medical science. A variety of bifunctional reagents has been used to produce the conjugates, and there have been some attempts to investigate the conditions of reaction necessary to obtain good coupling, whilst preserving the biological function of the molecules.

The present study describes the influence of coupling conditions on the coupling of a α-amylase and albumin by a range of commercially available bifunctional coupling reagents, and the application of thin-layer gel filtration chromatography for the rapid qualitative/semi-quantitative assessment of coupling reactions.

INTRODUCTION

Protein–protein conjugates have been used as reagents in a variety of analytical techniques including immunoelectrophoresis, immunodiffusion, haemagglutination and enzyme immunoassay[1–4]. Enzyme- or ferritin-labelled antibodies have also found use as immunohistochemical markers. Such conjugates have been prepared using a range of coupling agents, including dialdehydes, diisocyanates, carbodiimides, bis-maleimides, nitrogen mustards, *s*-triazines, bisdiazobenzidines and fluoronitrobenzenes. These studies, however, have not always demonstrated that optimum conditions were employed for the coupling reaction, and the relative merits of each coupling agent in different systems have not been fully explored.

The criteria for the assessment of coupling agents are related to the efficiency of coupling, and the subsequent maintenance of the integrity of biological activity of the conjugated proteins. Impairment of biological function will reduce the sensitivity of analytical procedures utilising these coupled proteins. Various techniques have been employed for assessing the results of protein–protein coupling reactions, and purifying the conjugated species. These have included ultracentrifugation, electro-

* To whom correspondence should be addressed.

phoresis, precipitin studies, chemical analysis and gel filtration in columns. These methods do not readily lend themselves to the rapid assessment of the large numbers of conjugation reactions required for deciding optimum coupling conditions. We present here an account of the use of thin-layer gel filtration (TLG) for rapid qualitative and semi-quantitative assessment of conjugation in model protein–protein coupling reactions between human albumin and α-amylase.

EXPERIMENTAL

Materials

Human albumin and α-amylase (Grade II bacterial) were purchased from Sigma (London) (London, Great Britain) and used without further purification. Amylase substrate was prepared essentially by the method of Rinderknecht *et al.*[5]. Remazol Brilliant Blue R salt (Calbiochem, Los Angeles, Calif., U.S.A.) was covalently linked to corn starch (Sigma) and the insoluble product was obtained in quantitative yield as a waxy solid (RBB starch).

3,6-Bis(acetoxymercurimethyl)dioxane was prepared from mercury(II) acetate and allyl alcohol[6]. 4,4′-Diaminobibenzyl, m.p. 129–131° (ref. 7: m.p. 132°) was obtained by reduction of the corresponding 4,4′-dinitro compound available via nitration of bibenzyl[7].

Methods

Sephadex® G-200 Superfine (Pharmacia, Uppsala, Sweden) (21.0 g) was equilibrated for three days at room temperature in 500 ml of phosphate buffer saline pH 7.2–7.4 (phosphate 0.02 *M*, NaCl 0.5 *M*), prior to use. The gel was spread 0.6 mm thick on 20 × 40 cm glass plates. Following overnight equilibration in a Pharmacia TLG apparatus, the plates were placed flat, and 20-μl samples (8–10 per plate) were applied using an Eppendorf automatic pipette. The plate was inclined at an angle of 10° and developed for 8–10 h. A replication technique was then used to detect the chromatographed substances, by soaking up the excess buffer with a sheet of filter paper (Whatman 3MM, 180 g/m²).

The protein stain used was Coomassie Brilliant Blue R250, 0.25% in methanol–glacial acetic acid (90:10, v/v). Paper replicates were stained for 15 min and excess stain was removed by washing three times with water, then twice with methanol–glacial acetic acid–water (50:10:50, v/v), and finally twice more with water.

α-Amylase activity was localised by a modification of the method described by Rosalki[8] for use with cellulose acetate electrophoresis strips. Agarose (1.5%) was dissolved in phosphate buffer saline pH 7.2 (phosphate 0.015 *M*, NaCl 0.15 *M*). Agarose gel (20 ml) was melted in a boiling water bath and RBB starch (1 g) was added. The mixture was then poured on a 20 × 20 cm glass plate, giving a gel of 0.5 mm thickness. The wet filter-paper replica was placed face-down on top of the gel and left in the open at room temperature for up to one hour. The areas of amylase activity showed up as blue spots on the paper replica.

Immunoelectrophoresis was performed essentially by the method of Scheidegger[9]. Agarose gel (1%) was made up in barbitone buffer pH 8.6 (ionic strength 0.025) containing polyethylene glycol 6000 (4%) and sodium azide (0.02%). The melted gel was poured 0.8 mm thick on to 10 × 10 cm glass plates, and 1-μl samples

were pipetted into each well. Electrophoresis, in barbitone buffer pH 8.6 (ionic strength 0.05), was performed at constant voltage of 2–3 V/cm for 55 min. Double diffusion, using 50 μl of antiserum in each trough, was allowed to proceed at room temperature for 20–24 h. The gels were then press-dried[10] to remove soluble proteins and stained as for the TLG replicates.

Typical coupling reaction

Solutions of human albumin (500 μl, 20 mg/ml) and α-amylase (700 μl, 20 mg/ml) in phosphate buffer (0.1 *M*, pH 7.8) were treated with 40 μl of a solution of hexamethylene diisocyanate (100 mg/ml in acetone), and the mixture was agitated gently at 0–4° for 24 h. The reaction mixture was then dialysed against 2 l of ammonium carbonate solution (0.1 *M*) at 0–4° for 16 h, and finally centrifuged at 4000 *g* for 15 min. The supernatant was then subjected to TLG, and filter paper replicates were stained for protein and enzyme.

RESULTS AND DISCUSSION

Mixtures of human albumin and α-amylase have been reacted under varying conditions of protein concentration, (2, 20 g/l), pH (2–10.8), and reagent concentration (10^{-4}–10^{-2} *M*) with a range of commercially available bifunctional protein–protein coupling reagents (Table I). α-Amylase was chosen as the enzyme for this model study by virtue of its stability and low cost, both highly desirable features of an enzyme label.

The general approach to assessing each of the bifunctional, protein–protein coupling reagents is outlined in the scheme[11] below.

Reagent	→	Coupling conditions	→	Qualitative/semi-quantative assessment	→	Characterisation of coupled products
		Temperature, pH, reagent and protein concentrations		Thin-layer gel filtration chromatography		Immunoelectrophoresis

Reaction mixtures were subjected to TLG and a paper replicate of the chromatogram stained for either protein or α-amylase activity. In these studies TLG has proved an extremely valuable analytical tool for the simultaneous and rapid assessment of large numbers of reaction mixtures. Inspection of protein stained replicates quickly reveals the extent of protein coupling and any polymerisation of the reactant proteins. A typical protein stained replicate is shown in Fig. 1.

A comparison of results obtained by TLG and conventional gel filtration on a column of Sephadex G-200, for a mixture of albumin and α-amylase which had been treated with hexamethylene diisocyanate at 38° for 2 h, is shown in Fig. 2. The enzyme stained replica (b) indicated that the high-molecular-weight products have very little amylase activity. Initial attempts to visualise amylase activity after replication utilised the starch–iodine reaction. However, the reaction also detects the presence of albumin[12], and the use of an insolubilised dyed starch, previously reported for the localisation of α-amylase activity on cellulose acetate electrophoresis strips[8], was adopted instead.

TABLE I

PROTEIN–PROTEIN COUPLING REAGENTS

Suppliers: a, Aldrich, Milwaukee, Wisc., U.S.A.; b, BDH, Poole, Great Britain: c, Cambrian Chemicals, Croydon, Great Britain; d, Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; e, kindly supplied by I.C.I. Organics Division, Manchester, Great Britain; f, Sigma (London), London, Great Britain; g, Pierce & Warriner, Chester, Great Britain.

Type	*Product*	*Supplier*
Dialdehydes	Malondialdehyde	a
	Glutaraldehyde	b
	Terephthal-dicarboxaldehyde	a
Diisocyanates	Hexamethylene diisocyanate	a
	p-Phenylene diisocyanate	c
	m-Phenylene diisocyanate	c
	Toluene-2,4-diisocyanate	b
	3,3′-Dimethoxy-4,4′-biphenyl diisocyanate	d
	4,4′-Diphenylmethane diisocyanate	e
	4,4′-Dicyclohexylmethane diisocyanate	e
s-Triazines	Cyanuric chloride	a
	Procion yellow MX-4R	e
Bismaleimides	N,N′-*o*-Phenylene dimaleimide	a
	N,N′-*p*-Phenylene dimaleimide	a
Carbodiimides	1-Ethyl-3-(dimethylaminopropyl)-carbodiimide	f
Nitrogen mustards	1,1-Bis(2-chloroethyl)-4,4′-bipiperidine	a
Isoxazoles	N-Ethyl-5-phenyl isoxazolium-3′-sulphonate	f
Sulphonyl chlorides	*m*-Benzene disulphonyl chloride	a
Mercurials	3,6-Bis(acetoxymercurimethyl)dioxane	
Bisdiazonium salts	*o*-Dianisidine	f
	p,p′-Diaminodiphenyl ethane	
Fluoronitrobenzenes	1,5-Difluoro-2,4-dinitrobenzene	a
	Bis(4-fluoro-3-nitrophenyl)sulphone	f
Imidoesters	Dimethyl suberimidate	g
	Dimethyl adipimidate	g

High-molecular-weight products of coupling reactions, selected on the basis of the TLG analysis, were characterised by immuno electrophoresis. Samples were run in duplicate and stained for protein and α-amylase activity, in order to detect conjugates of α-amylase and albumin. These analyses revealed that the extent of coupling of α-amylase to albumin was very low since little enzyme activity could be demonstrated for products showing immunological activity with anti-albumin. This finding was in agreement with the results from TLG which suggested that with many of the coupling reagents, especially those which effected coupling via amino groups (diisocyanates) or thiol groups (mercurials), albumin reacted preferentially to form albumin polymers.

In general protein–protein coupling was insignificant at low protein concentrations (2 g/l) as compared with corresponding reactions at protein concentrations of 20 g/l. The effect of reagent concentration and pH on a typical series of coupling reactions is illustrated by results obtained with several diisocyanates (Table II).

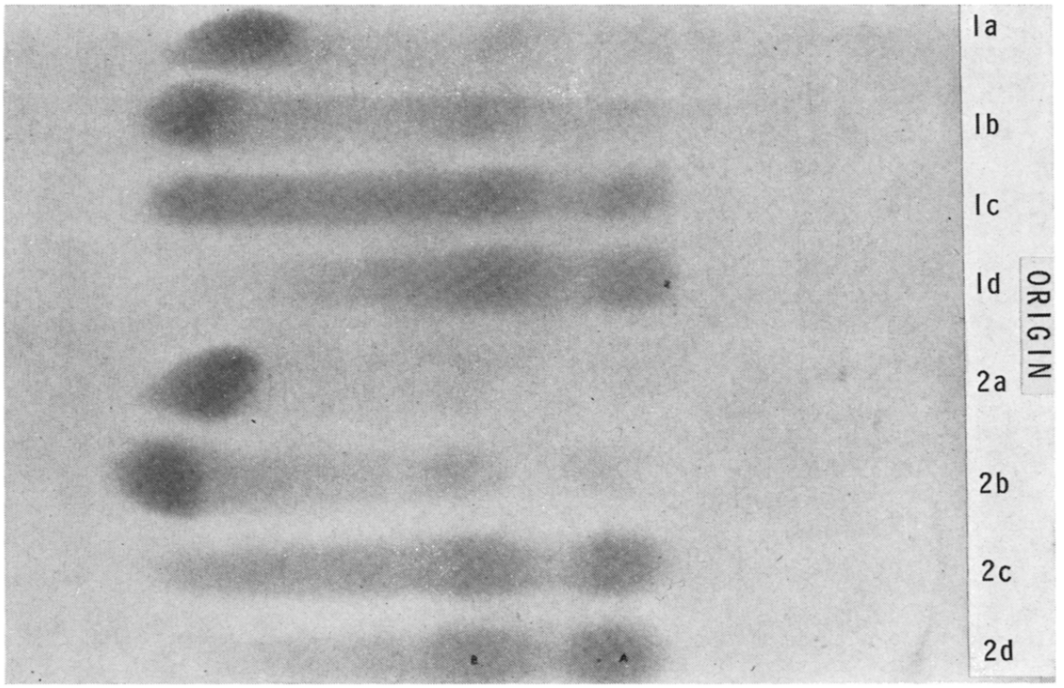

Fig. 1. Replicate of thin-layer gel filtration chromatogram of mixtures of α-amylase (A) and albumin (B) following treatment with glutaraldehyde. Protein concentration 20 g/l; pH: 1, 7.4; 2, 7.0; concentration of glutaraldehyde: a, 3.2×10^{-2} *M*; b, 1.6×10^{-2} *M*; c, 8.0×10^{-3} *M*; d, 4.0×10^{-3} *M*.

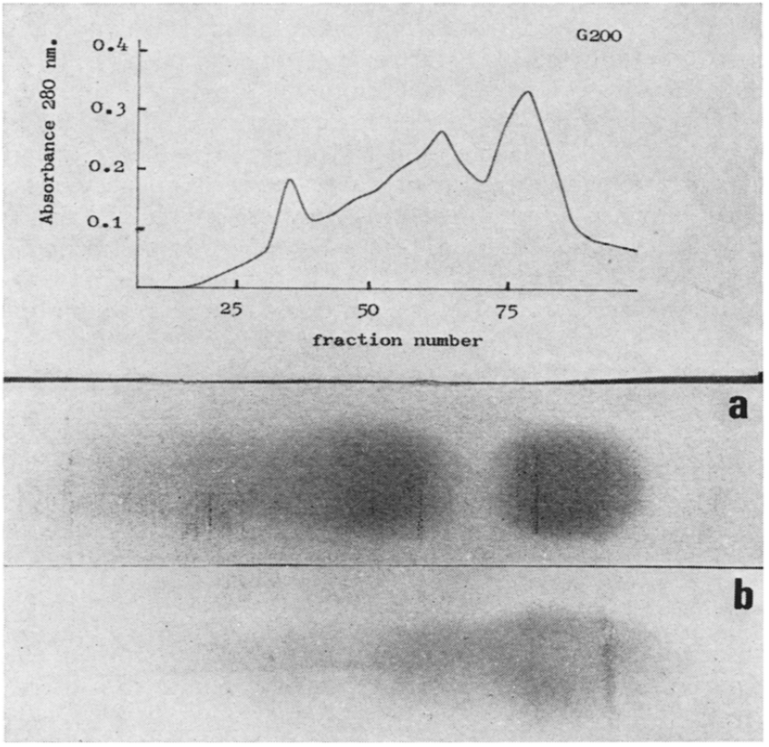

Fig. 2. Comparison of TLG and gel-filtration on a column. Top: reaction of albumin and α-amylase with hexamethylene diisocyanate at 38° for 2 h. Bottom: paper replicates stained for (a) protein and (b) α-amylase.

TABLE II

SUMMARY OF RESULTS FOR THE COUPLING OF α-AMYLASE AND ALBUMIN BY THE BIFUNCTIONAL COUPLING REAGENTS, *m*- AND *p*-PHENYLENE DIISOCYANATE, TOLUENE-2,4-DIISOCYANATE, AND HEXAMETHYLENE DIISOCYANATE

c Denotes the formation of high molecular weight products. Protein concentration, 20 g/l. Reaction mixtures contained equimolar proportions of α-amylase and albumin.

Coupling reagent	*Concentration (M)*	*pH*				
		6.6	*7.0*	*7.4*	*7.8*	*8.2*
p-Phenylene diisocyanate	5×10^{-3}				c	c
	1×10^{-2}			c	c	c
	2×10^{-2}	c	c	c	c	c
	4×10^{-2}	c	c	c	c	c
m-Phenylene diisocyanate	5×10^{-3}					c
	1×10^{-2}				c	c
	2×10^{-2}	c	c	c	c	c
	4×10^{-2}	c	c	c	c	c
Toluene-2,4-diisocyanate	5×10^{-3}				c	c
	1×10^{-2}	c	c	c	c	c
	2×10^{-2}	c	c	c	c	c
	4×10^{-2}	c	c	c	c	c
Hexamethylene diisocyanate	5×10^{-3}				c	c
	1×10^{-2}			c	c	c
	2×10^{-2}			c	c	c
	4×10^{-2}			c	c	c

ncreasing reagent concentration led to increased formation of high-molecular-weight products, whilst the combination of high pH and high reagent concentration produced insoluble products. The trend towards increased reaction at high pH can be understood from the mechanism by which diisocyanates couple proteins. Diisocyanates effect coupling principally via reaction with free amino groups on proteins. At low pH values such groups are protonated and therefore unreactive towards an isocyanate.

In the model system α-amylase–albumin the most reactive coupling reagents were found to be glutaraldehyde, cyanuric chloride, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (ECDI), toluene-2,4-diisocyanate (TDIC), and N-ethyl-5-phenyl isoxazolium-3′-sulphonate (Woodwards Reagent K). In contrast to the other coupling reagents listed in Table I, these five coupling reagents produced good yields of soluble high-molecular-weight products.

The low yield of conjugates which was observed with many of the bifunctional coupling reagents may be attributed first to the poor solubility of the reagents in aqueous media, although this was overcome in part by using the reagents dissolved in a water-miscible solvent, *e.g.* ethanol or dioxane, and secondly to the relatively unreactive nature of α-amylase as compared with albumin.

CONCLUSIONS

Design of successful coupling experiments requires an appreciation of (a) the specificity of the coupling reagent, and (b) the relative reactivity of the two proteins

to be coupled. The latter is related to the nature and number of functional groups on the protein available for reaction with the coupling reagent. TLG offers a method whereby the influence on a protein–protein coupling reaction of coupling conditions, type of coupling agent, and reactivity of the two proteins, may be rapidly assessed, thus guiding the selection of an appropriate coupling reagent and conditions for a particular pair of proteins.

For the system α-amylase–albumin the most reactive coupling reagents were found to be glutaraldehyde, cyanuric chloride, ECDI, TDIC, and Woodwards Reagent K.

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