Soluble polymer–protein conjugates: 1. Reactive *N*-(sym-trinitroaryl) polyacrylamide/acrylhydrazide copolymers and derived carbonic anhydrase conjugates

R. Epton, G. Marr and G. J. Morgan
Department of Physical Sciences, Wolverhampton Polytechnic, Wolverhampton WV1 1LY, UK
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Two copolymers of acrylamide and *N*-acryl-*N'*-t-butoxycarbonyl hydrazine were prepared and some of the t-butoxycarbonyl groups removed by acid hydrolysis to give acyl hydrazide residues pendant on the hydrocarbon backbone. The modified copolymers were dyed by reaction with sodium trinitrobenzene sulphonate and more acyl hydrazide residues generated by removal of the remaining t-butoxycarbonyl groups. The intense red polymers so produced were activated by treatment with nitrous acid and coupled with carbonic anhydrase to give coloured, water soluble enzyme conjugates. Compared to the native carbonic anhydrase the soluble conjugates were more stable to heat denaturation and exhibited much reduced values of K_m when *p*-nitrophenylacetate was used as substrate.

INTRODUCTION

Numerous methods have been reported for the modification of biologically active proteins by conjugation with synthetic and semisynthetic polymers[1–3]. Most of these procedures were devised for the preparation of water insoluble or gelatinous materials which could be applied as heterogeneous catalysts or adsorbents. Usually, the reason for studying these materials was either their ease of recovery from aqueous suspension or their potential for application in packed beds and chromatographic columns.

During the course of studies on immobilized enzymes it has emerged that conjugation can lead to important changes in biocatalytic properties such as K_m, V, pH-activity profile and heat stability[4]. Changes in K_m and V may arise as a result of diffusional and steric limitations as well as more profound effects, such as active site modification[5], microenvironmental changes[4], allosteric phenomena[6] and general perturbation of enzyme structure[7]. Often it is difficult to determine which of these effects is responsible for a given change in K_m or V. The biocatalytic properties of water soluble polymer–enzyme conjugates are, in contrast, subject to minimal interference from diffusional and steric effects. Consequently, these conjugates are much more readily studied and maximum biocatalytic activity is easier to realize.

Water soluble polymer–enzyme conjugates which have been prepared to date include those of chymotrypsin [by coupling to dextran, CM–cellulose, poly(acrylic acid) and poly(*L*-glutamic acid)][8–9], trypsin (by coupling to dextran[10]) and α-amylase (by coupling to dextran, CM- and DEAE-cellulose)[10,11]. In several instances improved stability to heat denaturation and storage was reported. It is surprising that water soluble polymer–enzyme conjugates have not been studied more widely. This is probably due to the difficulties encountered in the preparation and purification of these materials.

In this report we describe the synthesis of coloured, intensely hydrophilic polyacrylamides and the preparation of conjugates of these polymers with the enzyme, carbonic anhydrase. Experimental procedures such as chromatographic purification, electrophoresis and dispensing have been simplified considerably by the use of these coloured derivatives.

EXPERIMENTAL

Synthesis of copolymers

*Poly(acrylamide/*N*-acryl-*N'*-t-butoxycarbonylhydrazine) (molar ratio 10:1).* Acrylamide (7.14 g, 0.1 mol) and *N*-acryl-*N'*-t-butoxycarbonylhydrazine (1.84 g, 0.01 mol)[12] were dissolved in ethylene glycol (50 cm^3). The solution was purged continually with nitrogen while irradiating from above using a Hanovia 80 W u.v. lamp placed at a distance of 20 cm. After 60 h the viscous solution was dialysed exhaustively against distilled water and reconcentrated to 50 cm^3 by rotary evaporation at 50°C. On pouring this solution into methanol the copolymer was obtained as a white, brittle solid (4.6 g, 51%), ν_{max} (film): 3300–3400 (N–H str), 2910 (C–H str) and 1665 cm^{-1} (C=O str).

*Poly(acrylamide/*N*-acryl-*N'*-t-butoxycarbonylhydrazine) (molar ratio 20:1).* The procedure outlined above was repeated using acrylamide (7.14 g, 0.1 mol) and *N*-acryl-*N'*-t-butoxycarbonylhydrazine (0.92 g, 0.005 mol) and resulted in a similar polymer (4.8 g, 59%).

Controlled hydrolysis and dyeing of copolymers

Samples (0.18 g) of each copolymer were dissolved in distilled water (10 cm^3) and each solution was mixed well with aqueous 2 M HCl (10 cm^3). The solutions were incubated at 30°C. Aliquots (1 cm^3) were withdrawn at 15 min intervals and added immediately to a 1% w/v solution (5 cm^3) of trinitrobenzene sulphonic acid (TNBS) in saturated aqueous disodium tetraborate[13]. After 10 min the assay solution was diluted 15 fold with distilled water and the ex-

tinction (500 nm) measured against a control prepared by treating non-hydrolysed copolymer with TNBS reagent. The change in extinction was related to acrylhydrazide concentration by means of a standard graph prepared by TNBS treatment of acetyl hydrazide solutions of known concentration. Acetyl hydrazide solutions were prepared immediately prior to assay by controlled acid cleavage of t-butoxycarbonyl groups from known amounts of *N*-acetyl-*N'*-t-butoxycarbonyl hydrazine in suspension.

Larger scale preparation of dyed copolymers

The conditions described in the previous experiment were adapted. Samples (1.6 g) of each copolymer were dissolved in aliquots (30 cm^3) of distilled water. Aliquots (30 cm^3) of aqueous 2 M HCl were added and the solutions incubated at 30°C. Hydrolysis of the poly(acrylamide/acryl-*N'*-t-butoxycarbonyl hydrazine) copolymers, molar ratios 10:1 and 20:1, was stopped after 20 min and 7 min, respectively, by pouring into an excess of 1% w/v TNBS solution in saturated disodium tetraborate. The solutions were dialysed exhaustively against distilled water, concentrated to approximately 20 cm^3 by rotary evaporation at 50°C, and the bright red copolymers, 'chromopolymers A and B' (0.8–1.0 g), were recovered by methanol precipitation.

Preparation of enzyme conjugates

Samples (0.8 g) of the 'chromopolymers A and B' were each dissolved in distilled water (3 cm^3) and diluted with an equal volume of aqueous 2 M HCl. The solutions were incubated at 30°C for 2 h and chilled to 0°C. An aqueous M $NaNO_2$ solution (2 cm^3), also at 0°C, was added to each with magnetic stirring. After 2 min the solutions were adjusted to pH 9 by dropwise addition of triethylamine. The activated copolymers were precipitated by addition of ice-cold ethanol (50 cm^3), collected by centrifugation, and redissolved in a 3% w/v solution of carbonic anhydrase in 50 mM phosphate buffer (pH 8.0, 2 cm^3). Coupling was allowed to proceed with magnetic stirring at 0°C over 2 h after which an aliquot (0.1 cm^3) of aqueous 25% w/v methylamine solution was added to each reaction mixture. The resulting conjugates, 'chromo-enzyme A' and 'chromo-enzyme B' were recovered by lyophilization and stored at 0°C.

Assay of carbonic anhydrase activity

The method of Armstrong *et al.*[14] was adapted. The substrate, *p*-nitrophenylacetate (20 mg), was dissolved in acetone (1 cm^3) and diluted to (25 cm^3) with distilled water immediately prior to each determination. Aliquots (0.1 cm^3) of the solution of enzyme or enzyme conjugate were mixed with 50 mM Tris HCl buffer (pH 8.0, 1.9 cm^3) and substrate solutions (1.0 cm^3) were added. The solution was incubated at 25°C and the change in extinction (348 nm) related to *p*-nitrophenol concentration with the aid of a standard graph. One unit of carbonic anhydrase activity was taken to be that which released 1 μmol of *p*-nitrophenol in 1 min at 25°C.

Protein content of enzyme conjugates

Each enzyme conjugate was hydrolysed by treatment with 6 M HCl at 110°C for 18 h. The amino-acids produced were separated into groups[6] by paper chromatography and estimated by quantitative ninhydrin assay[15]. The amount of protein originally present in the conjugates was estimated by reference to a standard graph obtained on chromatographic assay of a hydrolysate of native carbonic anhydrase.

Confirmation of conjugate homogeneity

Gel filtration. An aliquot (2 cm^3) of a 5% w/v aqueous solution of each conjugate was chromatographed on a column (32 x 2.5 cm) packed with Sephadex G200. A 500 mM solution of NaCl in 20 mM phosphate buffer (pH 8.0) was used as eluent. Fractions (5 cm^3) were collected and assayed for carbonic anhydrase activity. Aliquots (2 cm^3) of aqueous solutions containing 1% w/v Blue Dextran 2000 (Pharmacia Ltd, UK) and 2% w/v native carbonic anhydrase were eluted under similar conditions. The coloured polymers and Blue Dextran were monitored spectrophotometrically at 500 nm. The soluble enzyme and coloured conjugates were monitored at 280 nm.

Polyacrylamide gel electrophoresis. Electrophoresis was conducted at 7 mA over 3 h in glass columns (9.0 x 0.75 cm) containing 10% polyacrylamide gel with 10 mM phosphate buffer (pH 8.1) as the solvent component. Samples (0.1 cm^3) of 2% w/v solutions of the 'chromopolymer', 'chromo-enzyme' and native enzyme in the electrophoresis buffer were layered on the cathode (top) end of separate gel columns and a 1% w/v solution of bromophenol blue in the same buffer (0.05 cm^3) added to each as front marker. After electrophoresis protein bands within the gel were located by staining with Coomassie Brilliant Blue following the method of Weber *et al.*[16].

Demonstration of reuse potential

'Chromo-enzyme A' (17 mg) was dissolved in 100 mM phosphate buffer (pH 6.75, 5 cm^3) and added to *p*-nitrophenylacetate (0.13 g) in 4% v/v acetone/water (160 cm^3). The solution was incubated for 2 h at 35°C and assayed spectrophotometrically in the usual way. After incubation the volume of the solution was reduced to 5 cm^3 by lyophilization. The solution was then eluted through a Sephadex G-50 column (30 x 1.5 cm) with distilled water. The excluded, coloured band was collected, lyophilized and assayed for carbonic anhydrase activity. The above procedure was repeated for four reuse cycles.

Conjugate stability

Heat stability. Solutions of the coloured and native carbonic anhydrase were incubated in 50 mM phosphate buffer (pH 8.0) at 60°C and the enzyme activity redetermined at intervals.

Storage stability. The activity of the soluble conjugates and native carbonic anydrase were redetermined after storage for 6 weeks at 0°C in 50 mM phosphate buffer solution (pH 8.0).

Determination of K_m *and* V

The activities of the chromo-enzymes and native carbonic anhydrase were determined at a series of *p*-nitrophenylacetate concentrations between 0.2 mM and 3.6 mM at 25°C using the standard assay procedure described above. The Eadie–Hofstee[17,18] method was used to calculate K_m and V.

RESULTS AND DISCUSSION

With the exception of some highly ionic chymotrypsin conjugates of poly(acrylic acid) and poly(*L*-glutamic acid)[9]

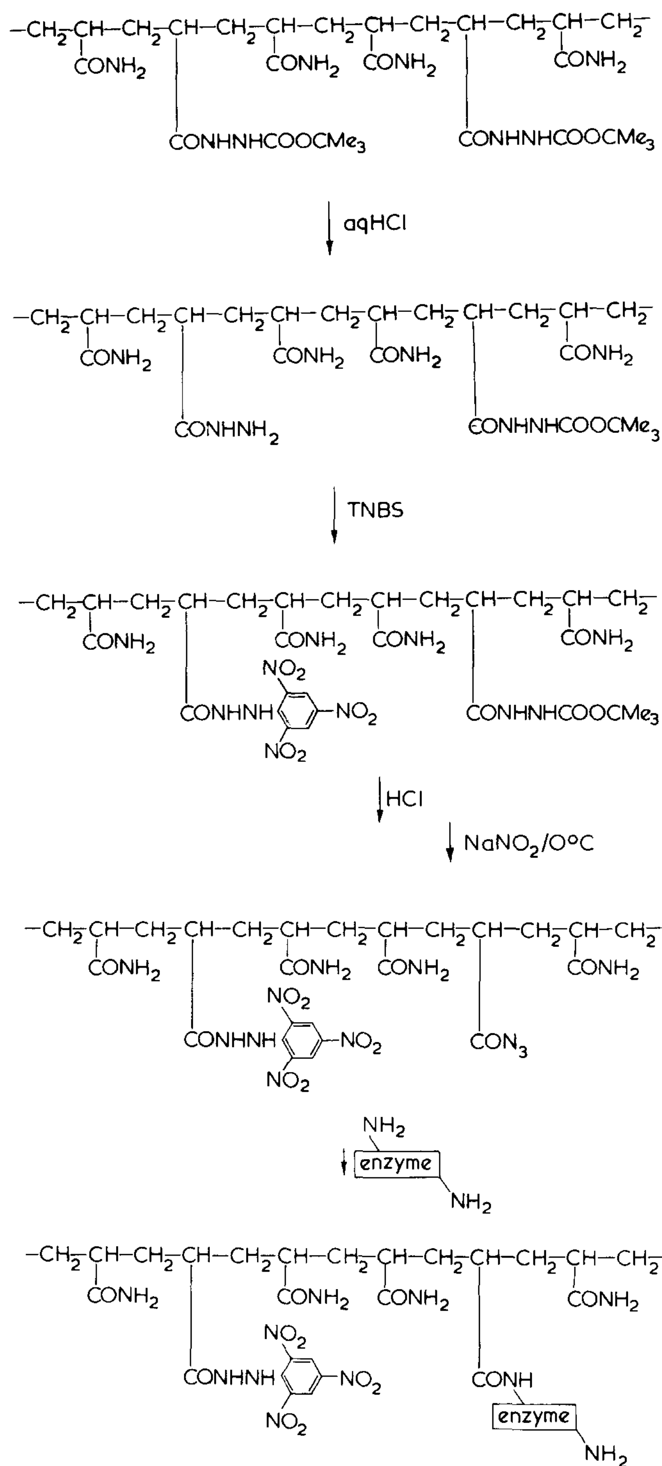

Figure 1 Dyeing of poly(acrylamide/*N*-acryl-*N'*-t-butoxycarbonyl hydrazine) and subsequent preparation of coloured carbonic anhydrase conjugates

there has been little systematic effort to prepare water soluble conjugates of enzymes with wholly synthetic polymers. In the present studies we have synthesised essentially non-ionic, reactive polyacrylamides for use in conjugate preparation. It is known from studies on immobilized enzymes that such polymers confer enhanced stability on a number of covalently attached enzymes[12]. Our reaction scheme (*Figure 1*) involved initial copolymerization of acrylamide and *N*-acryl-*N'*-t-butoxycarbonylhydrazine to give acrylamide/acrylhydrazide copolymers in which the acylhydrazide residues were protected by the acid labile *N*-t-butoxycarbonyl groups. Two copolymers, molar ratio of acrylamide/*N*-acryl-*N'*-t-butoxycarbonylhydrazine 10/1 and 20/1, were studied. We were able to effect controlled acid hydrolysis of the *N*-t-butoxycarbonyl groups to give derivatives of varying acyl hydrazide content. The acyl hydrazide groups were dyed by treatment with sodium trinitrobenzene sulphonate and the degree of substitution estimated spectrophotometrically (*Figure 2*).

Dyed polymers in which the degree of arylation was approximately 20% maximum were subjected to further acid hydrolysis to remove the remaining *N*-t-butoxycarbonyl groups. The reactive 'chromopolymers' so produced were used to prepare coloured conjugates of carbonic anhydrase by acid azide coupling (*Figure 1*). The conjugates, 'chromo-enzymes A and B', derived from the poly(acrylamide/*N*-acryl-*N'*-t-butoxycarbonylhydrazine) copolymers had protein contents of 34.4 and 33.7 mg/g.

Gel permeation chromatography on Sephadex G200 was employed to verify that the 'chromo-enzymes' were genuine covalent conjugates (*Figure 3*). Buffered 0.5 M NaCl was used as the eluent in order to dissociate any ionically bound enzyme. Chromatography of either conjugate gave an elution profile in which enzyme activity was proportional to colour yield (i.e. to 'chromopolymer'

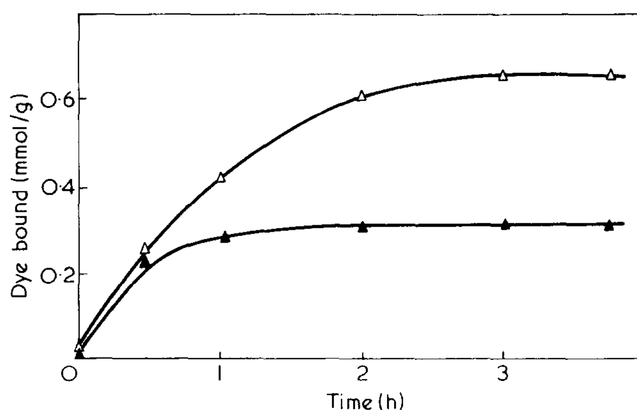

Figure 2 Spectrophotometric determination of dye bound to poly(acrylamide/acryl-*N'*-t-butoxycarbonyl hydrazine) following controlled hydrolysis with 2 M HCl at 30°C and treatment with TNBS. △, 10:1 copolymer; ▲, 20:1 copolymer

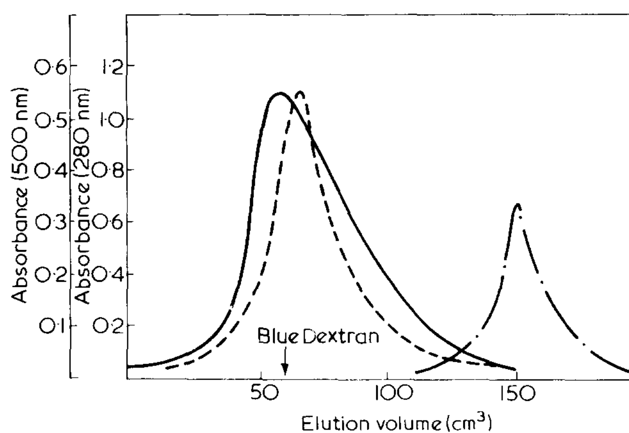

Figure 3 Confirmation of conjugate homogeneity by permeation chromatography on Sephadex G200. Dyed poly(acrylamide/*N*-acryl-*N'*-t-butoxycarbonyl hydrazine) (10:1 copolymer) (— — — —); 'chromo-enzyme A' (———); carbonic anhydrase (—·—·). Corresponding elution profiles were obtained for the 20:1 dyed copolymer and for 'chromo-enzyme B'

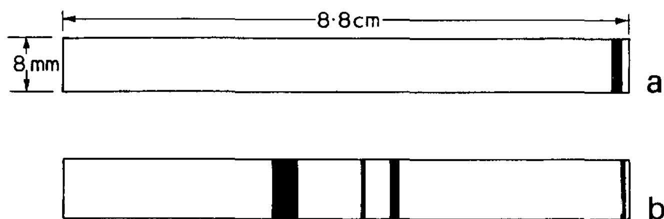

Figure 4 Polyacrylamide gel electrophoresis of (a) coloured conjugate; (b) native carbonic anhydrase

Table 1 Protein content, enzyme activity and kinetic parameters for coloured polyacrylamide–carbonic anhydrase conjugates

Preparation	Bound protein (mg/g carrier)	Enzyme units/ mg protein	Practical activity retention (%)	K_m* (mM)	V* (mmol /min/ mg)
Native enzyme	–	0.78	–	48.7	217
'Chromo-enzyme A'	34.4	1.02	130	24.7	180
'Chromo-enzyme B'	33.7	1.50	190	9.4	90

* Calculated by the Eadie–Hofstee[17,18] method

concentration). Significantly, little activity was detected at the point along the elution profile where the native enzyme emerged when chromatographed alone.

Further evidence for the covalent character of the conjugates was obtained from electrophoretic experiments (*Figure 4*). The enzyme conjugates migrated slowly as a single band whereas the native enzyme migrated rapidly splitting into a number of characteristic bands distributed over the gel column.

At the concentration of the *p*-nitrophenylacetate substrate used to estimate carbonic anhydrase activity the native enzyme, 'chromo-enzyme A' and 'chromo-enzyme B' exhibited activities of 0.78, 1.02 and 1.50 enzyme units/ mg protein. This corresponds to practical activity retentions of 130 and 190%, respectively, for the 'chromo-enzymes A and B. These high activities were observed partly because substantial decreases in K_m occurred on conjugate formation (*Table 1*) and partly because saturation kinetics are not obtained in the *p*-nitrophenylacetate assay.

The enzyme conjugates exhibited excellent reuse potential (*Figure 5*) and could be stored at 0°–2°C in buffer solution for several weeks without loss of activity. Furthermore, the stability of covalently bound carbonic anhydrase to heat denaturation was improved dramatically (*Figure 6*). Major increases in heat stability have been observed by others[10] for soluble dextran–enzyme conjugates and it is of interest that stabilization may be obtained also using wholly synthetic polymers. 'Chromo-enzymes' are much easier to handle and recover than native enzymes and could be used with similar facility in conventional, industrial biochemical reactors. Potentially, the coloured conjugates are applicable to solid, semisolid and macromolecular substrates and could be synthesized from crude enzyme preparations. In the above respects they differ significantly from the more familiar (water insoluble) polymer–enzyme conjugates.

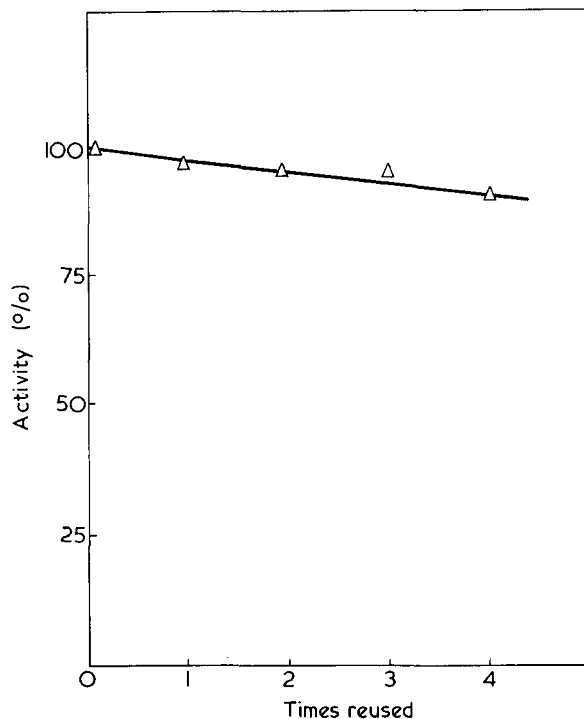

Figure 5 Recovery and reuse potential of 'chromo-enzyme A'

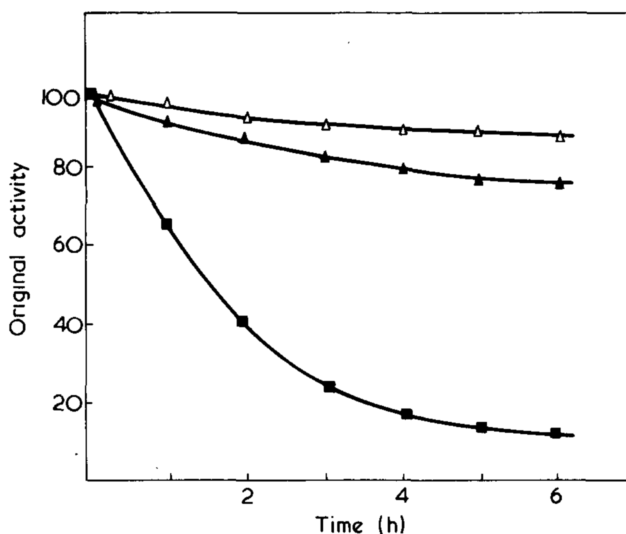

Figure 6 Heat denaturation of carbonic anhydrase and coloured derivatives in phosphate buffer at 60°C: △, 'chrome-enzyme A'; ▲, 'chromo-enzyme B'; ■, native enzyme

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