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Investigation of the Coupling of Water Soluble Poly(acrylic acid) and Poly(methacrylic acid) to Amines and to β-Galactosidase

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Summary

Acrylic acid and methacrylic acid were polymerized by γ - irradiation to give homogeneous solution of homopolymer. Neither homopolymeric species coupled to benzylamine or phenylpropylamine with water soluble carbodi-imides or Woodward's K Reagent but both homopolymers coupled to β - galactosidase using a water soluble carbodi-imide. In each instance, the activity of the enzyme was unaffected. Evidence for the formation of covalent links between the enzyme and the copolymer was obtained from electrophoresis and by analyzing the products obtained by precipitation of the enzyme-copolymer complexes. Both homopolymer species markedly effected the activity of acid phosphatase without the condensing agent being present.

Introduction

Enzymes attached to water insoluble supports have been widely investigated (ZABORSKY, 1973) but attachment to water soluble polymers has received little attention. Certain industrial substrates such as polysaccharides and proteins are not easy to use because of the physical restraints imposed by the support. However, soluble polysaccharide-bound carbohydrases have been used and have involved ultrafiltration (O'NEILL et al 1971; WYKES et al, 1971; AXEN et al, 1970). Soluble polymer linked enzymes may also be of use with smaller substrates, using semi-permeable membranes. This type of system could be extended towards applications such as affinity chromatography.

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In this initial investigation, we have synthesised poly (acrylic acid) and poly (methacrylic acid) and examined their coupling to β - galactosidase, benzylamine and phenyl propylamine using the water soluble carbodi-imides CMC and EDAC.

Experimental

1 - Ethyl - 3 (3 - dimethyl amino propyl) carbodi-imide (EDAC), 1 - cyclohexyl 1 - 3 (2 - morpholinoethyl) carbodi-imide metho - p - toluene sulphonate (CMC), N - ethyl - 5 - phenyl - isoazolium - 3' - sulphonate (Woodward's Reagent K) (WK), benzylamine phenylpropylamine, acid phosphatase and B - galactosidase (E.Coli) were obtained from Sigma Biochemicals (St. Louis, Missouri). Acrylic acid and methacrylic acid were obtained from Aldrich Chemical Co. and were purified by standard means. Other reagents (analytical grade) were obtained from B.D.H. Ltd., (Poole, Dorset, U.K.).

Substrate Preparation. Solutions of acrylic acid and methacrylic acid (10% (w/w) in water) were irradiated at 2.4 rads per second for 48 mins. The resulting solutions were centrifuged at 5,000 g for 10 minutes to remove heterogeneous species and then they were dialyzed using Visking tubing. The dialysis involved continuous changes of distilled water until no more acidic material was removed. The solids content was determined gravimetrically.

Homopolymeric PMA was isolated from aqueous solution by the addition of 1M $CaCl_2$ until no further precipitation occurred. The precipitate was centrifuged, washed with water, 0.1M phosphate buffer (pH 6.5), distilled water and was re-suspended in distilled water 0.1M EDTA was added dropwise until the precipitate redissolved. The excess calcium ions and EDTA were removed by dialysis, the product is referred to as PMA-P. The supernatant from the precipitation was dialyzed against distilled water and the solids content was determined gravimetrically as previously.

A portion (100 cm^3) of the PMA solution was precipitated, centrifuged and washed as above. The precipitate was redissolved in 0.1M NaOH and dialyzed against 0.1M NaOH solution. After adjustment of the pH, the total dialyzate was titrated with 0.1M

EDTA (BELCHER and NUTTEN (1960)) to determine the calcium content of the precipitate. The poly (acrylic acid) did not precipitate with calcium salts.

Coupling the amines: In a typical reaction, redistilled benzylamine (10 mg/cm^3) in 0.1M citrate buffer (pH = 4.8)(8 cm^3) and PMA-P solution (8.02 mg/cm^3, 10cm^3) were mixed with 0.1M citrate buffer (pH = 4.8)(22 cm^3) and cooled to 4°C. An aliquot (10 cm^3) was removed and EDAC (40 mg) was added to it. The mixture was kept at 40°C overnight before being examined by adding 1 cm^3 to a column (74 cm x 1 cm diameter) of G25 Sephadex gel (Pharmacia Ltd., Uppsala, Sweden). The column was eluted with 0.1M citrate buffer (pH 4.8) at a rate of 2.8 cm^3/minute at ambient temperature. Fractions (3.2 cm^3) of eluent were collected and examined at λ_{252}. In an initial run, the fractions containing PMA were located by adding 1 cm^3 from each fraction to the assay for acid phosphatase and completing the determination. The experiment was repeated in different phosphate buffer solutions (pH = 3.0, 4.0, 4.8 and 6.0) at 4°C and also with a ten fold increase in the benzylamine concentration.

In other experiments, the EDAC was replaced by CMC. To investigate the use of Woodward's 'K' reagent, PMA and PAA (40 mg) in solution, were mixed with either benzylamine or phenylpropylamine (40 mg) in 0.1M citrate buffer at pH 4.8 (20 cm^3) at 4°C. 5 cm^3 were taken and WK (200 mg) was added and kept at 4°C overnight. The solutions were examined using the G25 Sephadex column as before.

Studies of enzyme stability Polymer solution (PMA or PAA) was diluted in buffer solution and 2 cm^3 was added to the acid phosphatase assay (pH 4.8) or the B - galactosidase assay (pH 6.5). The activity, at different concentrations of each polymer solution, was determined.

Condensation of PAA and PMA-P with B - galactosidase PMA-P or PAA solution (6.0 cm^3)(36 mg) was added to portions of β-galactosidase (2.5^3)(5.2 mg) in 0.1M phosphate buffer (pH 6.5). CMC (5 mg) was added and the mixture kept at 4°C overnight. The enzymic activity of the resulting solutions was measured and the values compared with those from copolymer-free and CMC-free enzyme

after redissolving and titrating with EDTA showed that the calcium content was 39% ± 7%.

An attempt was made to couple PMA to benzylamine in the presence of EDAC. The eluent from the G25 column showed that PMA, as shown by its ability to interfere with the acid phosphatase assay, (Table 1) was present in the 20-32 cm^3 fraction. Benzylamine absorbing at λ_{252} was removed on further elution. Coupling of amine to PMA would cause UV absorbing material to appear in the PMA fraction, and a decrease in the amine fraction. No evidence for the coupling was found with either fraction. Variation in the pH (3.0 - 6.0), the temperature (4°C - 20°C) and the benzylamine concentration had no effect. When CMC was used at pH 6.0, no coupling occurred even at a tenfold increase in the CMC concentration.

Tube Number	Optical Density
5	1.017
6	0.460
7	0.196
8	0.142
9	0.147
10	0.686
11	1.052

Table 1 The effect of the addition of 1 cm^3 of column eluent (from the separation of PMA and benzylamine), using a G 25 Sephadex, on the assay of acid phosphatase.

These findings were unexpected since copolymer gels containing poly (acrylic acid) are reported to have coupled with enzymes under identical conditions (MARTENSSON and MOSBACH, 1972; MARTENSSON 1974 (a), and 1974 (b)). Another coupling agent (WK) was investigated but no coupling to benzylamine or phenylpropylamine was obtained.

However, the possibility of coupling to enzymes was investigated. The addition of PMA to acid phosphatase, without coupling agent, had a profound effect on the activity of acid phosphatase (Table 2), which was not seen on the addition of the monomer to the enzyme.

solution. The reaction mixtures were examined further:-
(a) 5.0 cm^3 of the PMA-P enzyme solution and also a solution of PMA-P and enzyme at the same concentrations, were each treated with 0.4 cm^3 of 1% $CaCl_2$. The precipitates were centrifuged, and washed as before but using 0.1M phosphate buffer (pH 7.3). The precipitates were suspended in water and re-dissolved by the dropwise addition of 0.1M EDTA. After dialyzing against water, each solution was made up to 10 cm^3. The supernatent liquors and the redissolved precipitate solutions were assayed for their protein content (LOWRY et al, 1957) and for β - galactosidase activity.
(b) Solutions of β - galactosidase, β -galactosidase with PMA-P added and with PAA added were compared to the reaction mixtures by electrophoresis on cellulose acetate strips in 0.1M barbiturate buffer (pH 8.6) at 200 V for 90 minutes. The strips were then immersed in a 2% solution of Ponceau S for 10 minutes to stain the protein. After removing the excess dyestuff in 5% acetic acid and drying, the strips were examined visually.

Assay of enzyme activity The acid phosphatase activity was determined using p-nitrophenyl phosphate (BESSY et al, (1946)) (1 unit 1 x 10^{-6} mol of p-nitrophenol produced min^{-1} cm^{-3} at pH 4.8 and 20°C). The β- galactosidase activity was assayed using o-nitrophenyl-β, D-galactopyranoside (ONPG) (CRAVEN et al, (1965)) (1 unit 1 x 10^{-6} mol of ONPG hydrolyzed min^{-1} at pH 7.3 and 20°C

Results and Discussion

Solutions of PAA and PMA prepared by γ -irradiation had concentrations of 10.3 and 8.31 mg/cm^3 respectively.

When known volumes of PMA solution were treated with calcium ions, 73% of the PMA remained in solution. This is to be expected since the PMA is highly polydisperse as a consequence of the polymerization initiation procedure. The higher molecular mass chains are of sufficient size to precipitate; the monomer is not precipitated. A calcium precipitate obtained from 100 cm^3 of PMA solution was redissolved and gave a dialyzate that consumed 9.3 cm^3 of EDTA. This is equivalent to 37.2 mg of Ca^{2+} having combined with 216 mg of PMA. It appears that 35% of the carboxyl groups of PMA interacted with the Ca^{2+} ions. Dry ashing of portions of the precipitate

Addition of 0.0165 mg of PMA to 0.45 mg of enzyme caused a marked reduction in the activity. The effect of PMA is 600 times that of the monomer when present in equivalent concentrations of acid groups. The reduction in activity of the enzyme, caused by increased PMA concentration does not totally inhibit the activity. In a related immobilized system, PMA-co-nylon, the inhibition of enzyme activity was not observed (Abdel-Hay et al, 1980).

A possible explanation is that the PMA and the PAA are able to distort the configuration of the enzyme to a significant extent. Because of the inhibition, covalent coupling to this enzyme was not attempted.

Polymer (PMA or PAA) (mg/cm^3)	U.V. absorbance at λ = 410 nm	
	with PAA	with PMA
1.5	0.268	0.219
0.15	0.263	0.271
0.105	0.270	0.266
0.044	0.290	0.271
0.022	0.276	0.311
0.0165	0.530	0.696
0.0033	1.068	1.079
0.00	1.081	1.081

Table 2 Effect of the PAA and PMA concentration on the activity of acid phosphatase. (0.457 mg of acid phosphatase with p-nitrophenyl phosphate (7.8 mg) in a total volume of 6.0 cm^3 at pH 4.8); 2 cm^3 aliquots removed after 10 minutes, added to 2 cm^3 0.1M NaOH and the optical density measured at λ = 410 nm.)

When added to β - galactosidase solution at pH 7.3, neither PMA nor PAA had any effect of the activity, even at concentrations of 2 mg of polymer/cm^3 in the presence of 4 x 10^{-4} mg of enzyme/cm^3. Consequently, the effect of coupling PMA or PAA to β - galactosidase was examined further. The free enzyme control solution had an activity of 107.5 units/cm^3. The PMA-enzyme and PAA-enzyme solutions had activities of 107.3 and 108.3 units/cm^3 respectively,

showing that coupling did not affect the activity.

To prove that coupling had occurred, the PMA-P enzyme mixture was treated with $CaCl_2$ solution and the precipitate was centrifuged and washed. The protein content of the first supernatant liquor was 0.40 mg/cm^3, which showed that 31.5% of the enzyme protein was left in solution and the precipitate should contain 0.964 mg protein which was equivalent to 59.7 mg/g. The enzyme solution control, to which PMA had been added, had a supernatant which contained 1.19 mg/cm^3 which showed that 93.7% of the protein was left in solution. The precipitation procedure could cause some protein to absorb.

When the precipitate was redissolved, the solution contained 0.202 mg of protein/cm^3, which was 66.2% of the total protein and showed that the polymer contained 56.1 mg of protein/g. The protein content of the precipitated protein sample was only a little lower than the value obtained from the supernatant liquor. The PMA-P enzyme complex after precipitating and redissolving, was low with only 9% of the initial enzyme activity being retained. This was improved to 16% when the solution was diayzed free from EDTA and Ca^{2+} ions. Systems of this type would be worthy of further investigation, especially if a good yield of calcium-precipitating PMA could be formed easily. PAA did not give an insoluble calcium salt.

The use of a Sephadex column in investigations of the linkage between PMA or PAA and proteins is likely to be complicated by any heterogeneity within the polymer solutions in that more than one enzyme molecule could be attached to each polymer chain. Consequently, both PMA-P-β - galactosidase and PAA-β - galactosidase were examined by electrophoresis on cellulose acetate strips. Visual examination showed that almost all of the enzyme (mobility 0.027 - 0.028 mm/V/min) had coupled to the PAA (mobility 0.019 - 0.022 mm/V/min) in the PAA-enzyme mixture. The PMA-enzyme had a mobility of 0.022 to 0.024 mm/V/min). This confirms the results obtained with the calcium salt precipitation method.

Conclusions

Despite the lack of covalent attachment to either benzylamine and phenylpropylamine using Woodward's K Reagent, EDAC or CMC the results obtained in coupling to β - galactosidase with full retention of activity suggest that the PMA and PAA systems are

worthy of further attention.

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