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Investigation of the Immobilisation of Bovine Serum Albumin, Trypsin, Acid Phosphatase and Alkaline Phosphatase to Poly(Hydroxyethyl Acrylate)-co-Cellulose and Poly(Hydroxyethyl Acrylate)-co-Pectin

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Summary

Poly(hydroxyethyl methacrylate)-co-pectins (107% graft), activated with p-benzoquinone, immobilised 122 mg of bovine serum albumin (BSA) per g of copolymer and 22.4 mg of trypsin per g of copolymer. This latter figure was replicated when p-toluene sulphonyl chloride was used for activating the copolymer. In a separate exercise p-benzoquinone was used to couple diaminohexane to the copolymer. This modified product immobilsed 396 mg of trypsin/g when glutaraldehyde was used in coupling. A 25% grafted, poly(hydroxyethyl methacrylate)-co-cellulose immobilised 39.5 mg of BSA per g of copolymer.

Introduction

Earlier we reported our work on the immobilsation of enzymes and proteins on hydrophobic supports such as Nylon (BEDDOWS et al, 1981) and polyethylene (BEDDOWS et al, 1982). It was shown that significant immobilisation occurred using poly(acrylic acid) grafts. However, the retained activity of the immobilised enzymes was somewhat disappointing. This was deduced to be due to the presence of too rigid a support (ABDEL-HAY et al 1983). Consequently it was decided that immobilisation on more hydrophilic supports be investigated.

Pectin was selected for an initial investigation, though it was recognised that its highly hydrophilic nature would be modified by grafting with hydroxyethyl methacrylate. It was hoped that such modification could be used to advantage. The work ws extended to include regenerated cellulose films. We feel that these systems could offer potential commercial exploitation.

Experimental

Pectin samples (with 30% and 70% methoxy groups) were used as supplied. Regenerated cellulose film was obtained from British Cellophane Ltd., Bridgwater, Somerset, U.K. The hydroxyethyl methacrylate (HEMA) was purifed by standard means. Acid phosphatase (wheat germ), alkaline phosphatase (calf intestine), trypsin (bovine pancreas) and bovine serum albumin (BSA) were used as supplied.

Portions of the pectin (2g) were immersed in a HEMA solution (10% w/v in methanol), 30 cm^3, and irradiated at 18.3 rads/s in a Co(60) source for 72h at 298 K in the presence of air. The irradiated composition was then added to methanol (180 cm^3), filtered and washed thoroughly

with methanol to remove attendant homopolymer. The graft copolymers were dried under reduced pressure at 313 K, to constant weight. In addition, 1g portions of regenerated cellulose film were immersed in a solution of HEMA (10% in methanol), 30 cm^3, and irradiated as above. The post-irradiation treatment was the same as that used in the preparation of the pectin based copolymers. The extent of grafting was determined gravimetrically.

Immobilisation was carried out on activated samples. Four routes to activation were examined. p-Benzoquinone, p-toluene sulphonyl chloride, cyanogen bromide and glutaraldehyde were employed as activating agents. In a typical procedure involving p-benzoquinone, approximately 100 mg of the copolymer were stirred at room temperature with 0.05M p-benzoquinone in 20% ethanol (in 0.1M phosphate buffer, pH = 8.0) for 2 hours. The resulting solids were filtered, washed with 20% ethanol, water, 1M NaCl and water. Aliquots (100 mg) of activated copolymer were shaken at 277K with the protein, dissolved in 0.1M $NaHCO_3$ (4 mg cm^{-3}, 8 cm^3) for 18 hours. The product was filtered, washed with 0.1M $NaHCO_3$ and water. The protein attached was determined by difference (LOWRY et al, 1951).

Activation with p-toluenesulphonyl chloride was achieved using a modification of the method suggested by NILSSON et al (1981). This employed the activating agent (1g) in dry dioxan (6 cm^3). The dry graft copolymer (100 mg) was added. The mixture was stirred and pyridine (1 cm^3) added ovver 60 seconds. After further stirring at room temperature, the product was washed with dioxan and then with water.

Activation with cyanogen bromide was achieved using the method suggested by AXEN et al (1967).

In a separate experiment, the graft copolymer (100 mg) was coupled to 1,6 diaminohexane (40 mg) in dimethyl formamide (5 cm^3) for 18 hours with shaking, at room temperature, after an initial activation with p-benzoquinone had been carried out. The product was treated with glutaraldehyde (12½% (v/v)) in 0.1M borate buffer (pH = 8.5) for 2 hours, filtered and washed with the buffer and then with water. The activated product was added to the relevant protein solution as required.

Immobilised acid phosphatase and alkaline phosphatase were assayed using o-nitrophenol phosphate (BESSAY et al, 1946). Trypsin was assayed using benzoyl-DL-argenine-p-nitroanilide (BAPNA), (ERLANGER et al 1961).

Results and Discussion

Pectin consists of a poly(galacturonic acid) backbone to which is attached esters and glycosides, and the acid groups are usually partially esterified. All these substituents can have a pronounced effect on the physical characteristics of the pectins. Consequently two pectins were selected for the initial investigation. One had 70% of its carboxyl groups esterified (70-pectin), the other had 30% of its carboxyl groups esterified (30-pectin).

Both substrates were grafted with HEMA to give approximately 107% (w/w) add-on. The solubility of these graft copolymers was determined under a variety of conditions. (Table 1).

Table 1

Solubility of the graft copolymers Pectin/HEMA and the pectins under different conditions.

Conditions of treatment	% Methoxy group	Polymer	% Solubility
Water	70	Pectin/HEMA	34
Water	70	Pectin	100
Water	30	Pectin/HEMA	25
Water	30	Pectin	100
BSA in 0.1M $NaHCO_3$	70	Pectin/HEMA	45
	70	Pectin	100
$NaHCO_3$	30	Pectin/HEMA	8.2
$NaHCO_3$	30	Pectin	100
p-benzoquinone	70	Pectin/HEMA	22
p-benzoquinone	70	Pectin	97
p-benzoquinone	30	Pectin/HEMA	0.3
p-benzoquinone	30	Pectin	97

Table 2

Immobilisation of trypsin, in 0.1M $NaHCO_3$, by poly(hydroxyethyl-methacrylate)-co-pectins (30 - pectin and 70 - pectin) after activation.

Copolymer	Activating Agent	Protein Coupled mg g^{-1} copolymer	Activity (Units/g^{-1} copolymer)	'Active'* Enzyme Coupled mg g^{-1}
70-pectin-co-HEMA	p-benzoquinone	72.1	7.7	12.2
	p-toluenesulphonyl chloride	22.4	5.4	8.6
	none	trace	0.1	0.16
30-pectin-co-HEMA	p-benzoquinone	56.9	12.2	19.4
	p-toluenesulphonyl chloride	21.4	9.4	14.9
	none	trace	0.11	0.17

* The free enzyme had an activity of 0.632 BAPNA units min^{-1} mg^{-1}.

Grafting decreased the solubility quite markedly, although the copolymers still have a quite high degree of solubility which is presumably due to ungrafted chains being quite soluble. The presence of BSA protein in 0.1M $NaHCO_3$ decreased the solubility of the graft copolymer prepared from the 70-pectin but not that prepared from the 30-pectin. Treatment with p-benzoquinone generally decreased the level of solubility quite extensively.

The 30-pectin-co-HEMA immobilised 115 mg of protein/g, while the 70-pectin-co-HEMA immobilised 122 mg of protein/g. No protein was adsorbed. The copolymers possessed an appearance somewhat between that of a true solid and a gel. The extent to which a gel has been formed can be inferred from its ability to hold water. The 30-pectin-co-HEMA adosrbed 1.29g of water/g and the 70-pectin-co-HEMA adsorbed 1.24g of water/g. Such values are much lower than would be expected of a true gel.

The amount of p-benzoquinone in each of the activated graft copolymers was determined by adding excess acidified potassium iodide in alcohol. This quantitatively reduced the quinone to hydroquinone and liberated iodine. The iodine produced after 20 minutes was titrated with sodium thiosulphate using starch as indicator. The 70-pectin and the 30-pectin copolymers coupled 108 and 104 mg benzoquinone per gram respectively. The method was used to show that the coupling of benzoquinone reached a constant value after 130 minutes with each copolymer.

The two copolymers were used to immobilised trypsin after activation using p-benzoquinone and also p-toluenesulphonyl chloride. (Table 2). Better results for protein binding were obtained with p-benzoquinone activation, with excellent immobilised enzyme activity. The p-toluene sulphonyl chloride activation gave much better retention of enzyme activity (40-70%). As with BSA, the products were fairly gelatinous and were difficult to filter.

In an attempt to reduce the gel like texture and still obtain a good retention of activity, the graft copolymers were coupled through p-benzoquinone to 1:6 diaminohexane. Glutaraldehyde was used for coupling trypsin. High levels of protein immobilisation occurred. (Table 3). The products had excellent enzymic activities. However, it was found that some of the copolymer composite was soluble in the medium. Soluble fractions were removed and the activation repeated on the truly insoluble fractions. After this treatment, the 70-pectin-co-HEMA coupled 264 mg/g of copolymer with a retention of activity equivalent to 15 mg/g. The 30-pectin-co-HEMA samples immobilised 234 mg/g of copolymer with a retention of activity equivalent to 17 mg of active enzyme/g of copolymer. The products were less gel-like but still proved difficult to filter.

Table 3

Immobilisation of trypsin (0.644 units/min/mg) onto 30-pectin and 70-pectin -co-HEMA linked to the diaminohexane adduct using glutaraldehyde

	Protein coupled mg/g of copolymer	Activity Units/g	Active enzyme coupled mg/g
30-pectin-co-HEMA	394	12.6	19.6
70-pectin-co-HEMA	396	18.6	28.9

As an alternative, cellulose was selected as a less hydrophilic, more internally compact substrate. Thus, cellulose film was grafted with HEMA to provide 25% add-on. The conditions used were similar to those used in preparing the pectin-containing copolymers.

When BSA was coupled to this cellulose-co-HEMA, after activation with p-benzoquinone, ungrafted cellulose was included in the study for comparison purposes. It was found both systems yeilded the same order of coupling (Table 4). When p-toluenesulphonyl chloride was used to activate the copolymer and the cellulose, 34.0 and 6.4 mg of protein/g of substrate were immobilised respectively, indicating a difference in the overall activity of the two substrates towards immobilisation. With cyanogen bromide activation, both the cellulose graft copolymer and the ungrafted cellulose coupled approximately 13 mg of protein/g of substrate.

Table 4

Immobilisation of bovine serum albumen by 25% hydroxyethylmethacrylate-co-cellulose and cellulose using different methods of activation.

Polymer	p-benzoquinone	p-toluene sulphonyl chloride	Cyanogen bromide
'Activated' Cellulose HEMA	39.5	34.0	12.5
Cellulose	14.7	4.2	nd
'Activated' Cellulose	34.2	6.4	13.0
Cellulose	0	0.1	nd

nd denotes not determined

p-Benzoquinone activation was used for immobilising enzymes since this gave the greatest yield with BSA. The amount of enzyme attached was disappointing, and the retention of activity of immobilised trypsin, acid phosphatase and alkaline phosphatase is much less than with the pectin graft copolymer. (Table 5). The integrity of the cellulose film was maintained. A copolymer having a greater extent of grafting could have more hydrophilic properties. However it is possible that such hydrophilic chains can interact with the cellulose surface.

Work is continuing to develop a system with less hydrophilic characteristics than the grafted pectin and yet having sufficient hydrophilic character to retain enzyme activity and stability.

Table 5

Immobilisation of enzymes using p-benzoquinone for activation of cellulose and poly(hydroxyethylmethacrylate-co-cellulose).

	Cellulose-HEMA		Cellulose	
	Activated	Control	Activated	Control
Trypsin*				
Protein coupled mg/g	22.0	6.0	8.0	0.8
Activity U/g	0.63	0.25	0.42	1.0
'Active' enzyme mg/g	1.5	0.6	1.0	0.4
Acid Phosphatase*				
Protein coupled mg/g	16.1	5.0	15.1	3.0
Activity U/g	0.079	0	0.11	0
'Active' enzyme mg/g	0.51	0	0.70	0
Alkaline Phosphatase*				
Protein coupled mg/g	14.0	4.0	7.0	4.1
Activity U/g	0.17	0.04	0.17	0
'Active' enzyme mg/g	0.40	0.1	0.40	0

(* Activity of the pure enzymes, trypsin 0.419 U min^{-1} mg^{-1}, alkaline phosphatase 0.432 U min^{-1} mg^{-1}, acid phosphatase 0.154 U min^{-1} mg^{-1}

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