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The Use of Graft Copolymers as Enzyme Supports

IV. The Immobilization of Invertase, Pepsin, Acid and Alkaline Phosphatases and Bovine Serum Albumin to Hydrolysed and Reduced Nylon-Co-Acrylonitrile Graft Copolymers

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Summary

The grafting of acrylonitrile on to nylon powder was carried out in DMF solution after vacuum impregnation. Initiation was radiation induced. A sample of graft copolymer was reduced and coupled to enzyme using a water soluble carbodi-imide, or using glutaraldehyde.

Some samples of the graft copolymer were selectively hydrolysed to the corresponding acrylic acid form and used for coupling to bovine serum albumin, invertase, pepsin, acid and alkaline phosphate, using water soluble carbodi-imides.

Introduction

The unique value of using graft copolymers as enzyme supports has been reported, (ABDEL-HAY et al. 1979; BEDDOWS et al. 1979.). This work concentrated on grafting polyacrylamide onto nylon and cellulose and using the amide to couple to enzymes through the azide or through glutaraldehyde. The reasoning behind using this type of copolymer system with acrylic type grafts is that a polymer having limited hydrophilic properties could be obtained, whereas homopolymeric acrylic systems invariably form hydrophilic gels by crosslinking.

Both enzyme inclusion (BUNTING and LAIDLER 1973) and covalent coupling with such gels have been utilised (LLOYD and BURNS 1979) with some success, but there are inherent problems.

The ease of handling copolymer systems has encouraged us to investigate the system further. Acrylonitrile was selected as the graft comonomer, as previous experience has shown that it is relatively easy to obtain a suitably grafted copolymer, with little or no formation of homopolymeric gel.

Acrylonitrile is also particularly convenient in this context since its polymer can be readily converted to the corresponding carboxylic acid by hydrolysis or the amine by reduction. Both of these functional groups allow for coupling to enzymes with peptide forming reagents such as the carbodi-imines as well as using

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glutaraldehyde for the amine.

Materials

Pepsin was obtained from Fisons Ltd. Loughborough, U.K., Acid phosphate from Sigma Biochemicals Ltd., Missouri, U.S.A.; Invertase, alkaline, phosphatase, 1.6 diaminohexane and other reagents (analytical grades) from BDH Ltd., Poole, Dorset. The polyamide substrate, Griltex, was supplied by Grilon Plastics Ltd., Dover, U.K., as a very finely divided powder. This was washed with water, then methanol before filtering on No.6 sintered crucibles, further washing and drying to constant weight, under vacuum (313K). The acrylonitrile (AN), obtained from the Aldrich Chemical Company, was freed from inhibitor by standard means and purified by distillation. The dimethylformamide (DMF) was purified by the method of Batty (1975).

Experimental

Polymerisation Procedure: Tared, dry polyamide powder (5g) was dispersed in DMF/AN mixtures of known molar ratio of solvent and monomer.

Ampoules, containing the polyamide, monomer and solvent (30 cm^3) were subjected to a series of freeze/thaw cycles before being sealed at 101.3×10^{-2} Nm^{-2} at room temperature (291 K). On thawing of the contents, the ampoules were placed in a constant temperature environment of 338 K for 30 minutes. After cooling to room temperature, the ampoules were subjected to irradiation under controlled conditions. Variables examined included the monomer, intensity and total dose dependence of the grafting process. On cessation of irradiation, the ampoule contents were dispersed in DMF and the whole poured into a 4 times excess of water, filtered on No.4 porosity, sintered crucibles before drying to constant weight under vacuum at 313 K. The nylon-AN copolymers were then Soxhlet extracted in DMF for 48 hours to remove any available polyacrylonitrile. The extracted copolymeric samples were thoroughly washed with water, then filtered and dried as described above.

Hydrolysis: Portions (3 g) of acrylonitrile-nylon of known composition were hydrolysed by refluxing with 30 cm^3 of 5 M HCl for specified periods up to three hours at 373 K. After cooling, the products were filtered, thoroughly washed with water, then methanol, before drying at 313 K under vacuum. The copolymer with 12.8% graft was fully hydrolysed, as shown by the disappearance of the -C≡N absorption band in the IR spectra, after 5 hours refluxing and was used for enzyme coupling (Copolymer I).

An attempt was made to hydrolyse with 5 M NaOH. The -C≡N peak was considerably reduced but was not fully absent (Copolymer II).

Reduction: Reduction of selected Nylon-co-AN grafts was achieved by dispersion of 2 g through gradual addition, with constant stirring, to 30 cm^3 of absolute alcohol containing 2 g of finely divided sodium. The system was refluxed for two hours and allowed to cool at room temperature. The product (copolymer III) was isolated and dried as above in each case. Evidence of reduction was seen in the white to orange colour change and the loss of absorption in the infra red region arising from the -C≡N group.

Coupling to Enzymes: Carbodi-imide condensation; In a typical preparation, 100 mg of the copolymer was added to 5 cm^3 of 0.1 M phosphate buffer solution at 277 K. To this was added 15 cm^3 of cooled enzyme solution (4 mg/cm^3) and 40 mg of 1-ethyl-3(3-dimethylaminopropyl) carbodi-imide (EDAC) or 1-cyclohexyl-3(-2-morpholinoethyl) cabodi-imide metho-p-toluene sulphonate (CMC) and the mixture was shaken at 277 K for 16 hours. The copolymer was filtered and washed well with buffer, water and 0.1 M NaCl. The supernatant liquor and washings were made up to 100 cm^3 and assayed for protein by the method of LOWRY et al (1951). A portion of the washed polymer was assayed for enzymic activity.

Glutaraldehyde condensation; 150 mg of copolymer III in 10 cm^3 0.1 M borate buffer (pH = 8.5), was treated with 10 cm^3 of 25% glutaraldehyde at 277 K for 1 hour. The polymer was filtered and washed thoroughly with borate buffer (4 x 100 cm^3), 0.1 M saline (2 x 50 cm^3) and water (3 x 100 cm^3). A portion of the solid was removed for assay of reducing groups by the method of NELSON (1944). The bulk of the solid was added to 10 cm^3 0.1 M, pH 8.5 borate buffer and 10 cm^3 of alkaline phosphatase (4 mg/cm^3) at 277 K and shaken at this temperature overnight. The solid was filtered and washed. A portion of the copolymer was used to determine its enzymic activity and the filtrate and washings were used to determine the amount of protein present (LOWRY 1951).

Assay of invertase activity was carried out by incubating 0.2 cm^3 of enzyme solution with 1% sucrose in 0.05 M acetate buffer, pH 4.7, at 290 K and removing 1.0 cm^3 after 10 minutes, heating rapidly to destroy the enzyme. After cooling, the glucose was determined using glucose oxidase (HUGGET and NIXON 1957) (1 unit = 1 mg glucose produced min^{-1} at 290 K). Pepsin activity was measured using 2% haemoglobin according to the method of ANSON (1939) (1 unit = formation of 0.001 UV unit at 750 nm at pH 2.0). Acid and alkaline phosphatase activities were measured using p-nitrophenol phosphate (BESSY et al 1946) (1 unit = 1 x 10^{-6} M p-nitrophenol produced cm^{-3} at 310K).

Results and Discussion

The rate of grafting may be expressed as mol of AN converted to grafted polymer per litre of bulk solution per second, thus

$$R_G = [((w_f - w_i^c)/w_i^c) \times (1000/30) \times 1/63] \text{ per second, where}$$

w_i^c is the initial weight of nylon powder, after correction for any change in weight of ungrafted nylon as acquired from blanks.

w_f is the weight of extracted, dry, grafted nylon. The rates of grafting for four different bulk monomer concentrations of 0.79, 1.58, 2.28 and 3.17 mol of AN per litre of bulk solution were determined. These were 0.49×10^{-5}, 1.00×10^{-5}, 1.9×10^{-5} and 5.75×10^{-5} mol of AN converted to grafted polymer l^{-1} s^{-1} respectively. The dependence of the grafting reaction on the radiation intensity is shown in figure 1. The non-linear nature of the relationship indicates the complexity of the grafting process. This observation can be interpreted in terms of Langmuir adsorption behaviour on the surface of the finely divided nylon powder. Thus, with increasing intensity, an increasing number of surface radical sites is created. These sites compete for available monomer. With increasing propagation, access to the newly created surface sites becomes restricted and initiation of grafting becomes diffusion controlled. However, such diffusion control of initiation of grafting does not prevent the occurrence of pseudo "Norrish-Trommsdorff" effects as may be seen from the non-linear dependence of the grafting rates on the bulk monomer concentration.

Enzyme coupling. Portions of a polyacrylonitrile-nylon (12.8% w/w) graft were hydrolysed with NaOH or HCl.

When both acid and alkali hydrolysed co-polymers were condensed with bovine serum albumin using the water-soluble carbodi-imide, CMC, the acid hydrolysed product gave a yield of 250 mg of protein per gram of polymer whereas virtually no protein was coupled to the alkali hydrolysed material.

The acid hydrolysed co-polymer (Polymer I), which can be considered to be virtually poly (acrylic acid)-co-nylon was coupled to a variety of enzymes (Table 1) using either EDAC or CMC. In each instance, a control, without coupling agent present, but containing co-polymer and enzyme, was also prepared. The protein content, if any, was subtracted from the protein present in the coupled enzyme-copolymer. The activity of the control was also determined. The carbodi-imide condensed products had high protein contents. However the activities of the coupled enzymes were disappointingly low in comparison. Nevertheless, the acid phosphatase-copolymer system showed significant activity. The CMC condensing agent gave better results than EDAC with invertase.

The yields of protein coupled are far in excess of those we obtained with fluorinated polymer-nylon copolymers (ABDEL- HAY et al 1980); with polyacrylamide-nylon copolymer (where coupling was either by the acid azide route or through glutaraldehyde) - (ABDEL-HAY et al 1979); with cellulose-polyacrylamide copolymers (where coupling was either by the acid azide route or through glutaraldehyde)- (BEDDOWS et al 1979) and with diazotised p-amino carbonilated cellulose derivatives (BEDDOWS et al 1980).

The coupling to acid phosphatase was tried at range of pH values 2.0 - 5.0, but no improvement in activity of the enzyme polymer was obtained.

The reduced product (copolymer III) was used to couple to acid phosphatase at pH 4.8 using CMC. The reduced form was compared to the product of the acid copolymer I coupled to 1,6 diaminohexane with CMC at pH 4.8 (copolymer Ia). When acid phosphatase was coupled to copolymer Ia, a yield of 58 mg/g of protein was obtained with 0.881 U/g of activity and 3.67 mg of active enzyme/g. However,it is possible that both amino groups of the diaminohexane and the carboxyl groups of uncoupled polyacryiic acid could be involved in the attachment of the enzyme. The reduced copolymer III coupled very little protein and showed negligible activity with acid phosphatase. This copolymer absorbed 10 mg/g acid phosphatase. Consequently copolymer III was coupled, using glutaraldehyde ,to alkaline phosphatase. The intermediate aldehyde complex contained 4.6 mM reducing groups/g which is in great excess. Although 40 mg/g of alkaline phosphatase protein was coupled to the polymer, only 0.25 mg/g of active enzyme was present.

TABLE 1

Activity and Binding Yield of Enzymes with Copolymer I

Enzyme	Condensing Agent	pH	Yield [mg/g Polymer]	Activity [U/g]	Yield Active Enzyme [mg/g]
Invertase	EDAC	4.8	13	30.03	0.13
Invertase	CMC	4.8	72	315	1.4
Pepsin	CMC	4.0	210	321	0.16
Acid Phosphatase	CMC	4.8	176	0.89	4.67

Conclusions

The reduced graft copolymer showed relatively little prospect as a possible enzyme immobilising substrate; however the hydrolysed product showed great promise with large yields of coupled protein being obtained. This is being investigated further.

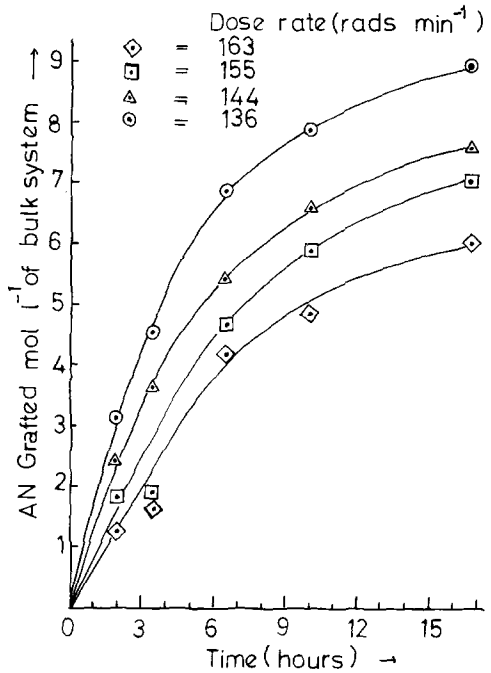

Fig. 1. Variation in the extent of grafting of acrylonitrile (AN), on nylon powder, with time of irradiation for different dose rates

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