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The Use of Graft Copolymers as Enzyme Supports

II. The Immobilization of β-Galactosidase and Glucose Oxidase on Cellulose – Polyacrylamide Graft Copolymers

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Summary

β-Galactosidase and glucose oxidase have been immobilized on cellulose-polyacrylamide (C-PAM) graft copolymers, using the azide method or through glutaraldehyde. The original (C-PAM) copolymers were prepared via radiation-induced grafting under controlled conditions by both post-irradiation and simultaneous procedures. The optimum conditions for coupling of the copolymers to the enzymes were established as were the levels of activity of the immobilized enzymes. Grafting by the simultaneous route was seen to be more efficient than post-irradiation grafting. Indirect evidence of grafting was found from elemental and thermal analysis. Binding of β-galactoside was found to be more successful than that of glucose oxidase. A relationship between the level of immobilized enzyme activity and the extent of grafting in the copolymer was established for the β-galactosidase system.

Introduction

A large number of different supports have been used for immobilizing enzymes. Immobilized enzymes offer advantages over free enzymes in many systems. Their ease of handling and recovery is considerably increased due to the much larger bulk of the immobilized enzyme system. Secondly, the chemical and physical properties, notably solubility, stability and reactivity may be beneficially altered by immobilization. The choice of support is governed by the conditions under which the enzyme is to be used and the method of coupling.

We are particularly interested in the use of selected graft copolymeric supports as substrates for enzyme attachment by means of covalent links, in the relationships between the substrate/monomer combination, the extent of grafting, the efficiency of enzyme binding and the resultant enzymic activity (Beddows et al. 1979 (a) Guthrie et al 1972, Guthrie and Percival 1977). This report deals with the preparation of cellulose-co-polyacrylamide graft copolymers. (An initial investigation into the potential use of graft copolymers as enzyme supports has been carried out (Beddows et al, 1979 (b)).

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The enzymes β-galactosidase and glucose oxidase were bound either using the azide procedure or through glutaraldehyde. Aspects of the kinetics of the of the grafting process are covered in some detail as are certain facets of the thermal stability of the graft copolymers. Such details provide important information relevant to an understanding of the behaviour of the bound enzyme system.

Materials

β-Galactoside (from E. coli) was obtained from Sigma Biochemicals Ltd., Missouri, U.S.A. and glucose oxidase was obtained from B.D.H. Ltd., Poole, Dorset, U.K. High grade wood pulp was supplied by British Cellophane Ltd., Bridgwater, Somerset, U.K. All other reagents used in this work were of analytical quality and were purified when necessary.

Grafting Procedures

The finely divided wood pulp was thoroughly washed with boiling, distilled water, then methanol and dried to constant weight in vacuo at 313K.

Post-irradiation:- The wood pulp cellulose, contained in sealable polyethylene bags was irradiated at 700 rads/min to a total dose of 0.67 M rads using the Co(60) facility, located in the Department of Physical Chemistry at the University of Leeds. Within two minutes of removal from the radiation source the tared cellulose samples were immersed into solutions of known concentrations of acrylamide in water . After standing for one hour, the cellulose/acrylamide copolymer was washed thoroughly with water, then methanol, before drying in vacuo at 313K.

Simultaneous irradiation:- The cellulose was immersed in aqueous solutions of acrylamide at various known concentrations and liquor/cellulose ratios, in sealable glass vessels. The monomer dependence and the total dose dependence of grafting were investigated. Homopolymer removal was achieved by continuous extraction with water. The products were then washed and dried as described previously.

Dosimetry was carried out using the Fe (II) sulphate secondary dosimeter. The samples were subjected to elemental analysis and differential thermal analysis.

Enzyme coupling was carried out either by converting to the hydrazide and treating with nitrous acid to give the azide according to the method of Inman and Dintzis (1969) or by treating with glutaraldehyde (Western and Avrameas, 1971) and adding each of the derivatives to the particular enzyme in solution. The addition to β-galactosidase was at pH 7.2 in 0.1M phosphate buffer and to glucose oxidase in pH 7.0, 0.1M phosphate buffer.

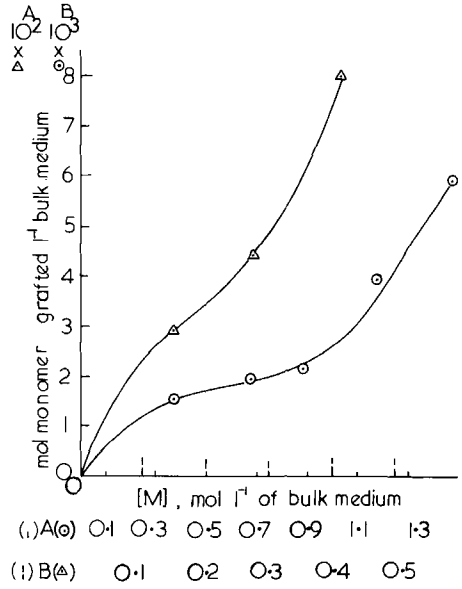

Fig. 1

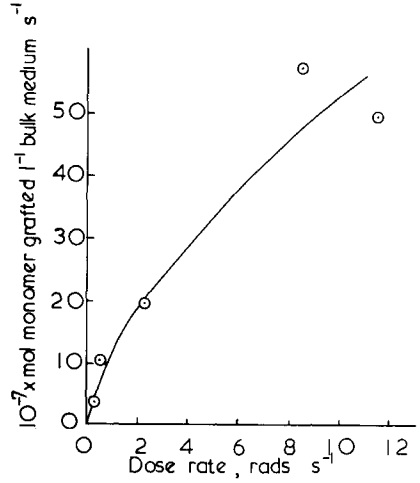

Fig. 2

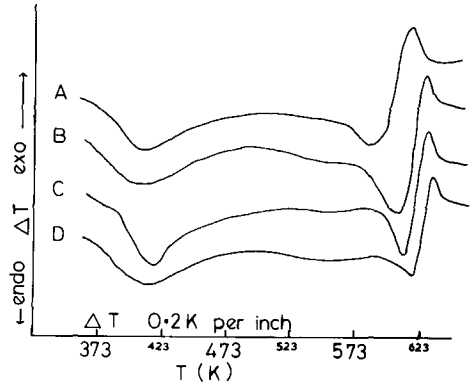

Fig. 3

Assay of β-galactosidase activity was carried out using o-nitrophenol-β, D-galactopyranoside as substrate (Craven et al. 1965) (1 unit ≡ 1 x 10^{-6} mole of ONPG hydrolysed per min. at pH7.2 and 298K) and the assay of glucose oxidase activity according to the method of Huggett and Nixon (1957) (1 unit = oxidation of 10^{-6} mol of glucose per min. at 298K.

Results and Discussion

For post-irridation, figure 1A shows the relationship between the number of mol. of acrylamide grafted per litre of bulk grafting medium, (the denominator expression being calculated using a value of 1.47 g cm^{-3} for the density of cellulose (13), and the concentration of acrylamide in the bulk medium. A non-linear profile is followed though the extent of grafting is low. The corresponding grafting rates for monomer concentrations of 0.37, 0.68, 0.87, 1.17 and 1.58 mol of acrylamide of bulk medium are 4.16, 5.28, 5.83, 10.8 and 16.1 (all x 10^{-7}) mol of acrylamide grafted l^{-1} sec^{-1} respectively.

With simultaneous irradiation, severe problems with gelation arose at even moderate total doses. Hence for this route much lower total doses were used (84 krad). Figure 1 B gives the relationship between the extent of grafting and the bulk monomer concentration, using the relationships previously described. The rates of grafting using the simultaneous route, for bulk monomer concentrations of 0.14, 0.28 and 0.42 mol of acrylamide l^{-1} of bulk solution are 4.02, 6.10 and 11.1 respectively (all x 10^{-6}) mol of acrylamide grafted l^{-1} of bulk solution sec^{-1}. Thus the use of the simultaneous procedure gives grafting of approximately 10 times the rate seen on post-irradiation grafting. In view of the complexity of the traces shown in figure 1 the monomer dependence of the grafting reaction could not be determined. For both systems there is significant evidence of the fact that diffusion dependent processes operate. This is supported by the grafting rates quoted above. It appears that, on grafting, the cellulose is either rendered more accessible to monomer or that Trommsdorff effects are operating as a result of reduced termination possibilities.

Figure 2 gives the dependence of the grafting reaction under simultaneous conditions on the dose rate for a constant monomer concentration. Samples irradiated beyond 97 krad had the appearance of heavily crosslinked jelly-like masses surrounding the cellulose particles and proved impossible to process to a homopolymer-free state.

In assembling the data given in Figure 2, the monomer concentration was maintained at 0.42 mol l^{-1} of bulk medium and the dose rate varied in the range from 20 to 700 rads min^{-1} and the total dose from 2.76 x 10^{3} rads to 9.66 x 10^{4} rads. The rate of grafting shows a dependence on the dose rate to the power 0.68, indicating a deviation away from steady state kinetics. This deviation arises from the complexity of the grafting system.

Figure 3 gives the differential thermograms of the wood pulp cellulose (A) and a series of cellulose-polyacrylamide copolymers (B), (C), (D). The extents of grafting (%) are as follows: A, 0.0; B, 3.7; C, 5.6; and D 10.2. In all the traces, an endotherm is seen at 418 to 424K which may correspond with the removal of bound water. A second endotherm is seen at A, 594K; B, 611K; C, 612K and D, 614K. These are followed by a decomposition exotherm at A, 623K; B, 632K; C, 633K; D, 635K. In the endotherms minimizing at approximately 611K and the exotherms maximizing at approximately 630K, we have an indication of the change in structure of the cellulose which arises from copolymerization. Thus both features of the thermal profile show an upward trend with increasing temperature on increasing the level of grafting.

The above thermal analysis data relate to samples prepared by the simultaneous route. These samples were subsequently used in studies of enzyme binding.

The cellulose-co-polyacrylamide grafts containing different levels of grafting were then evaluated for their potential as supporting media for enzyme immobilization. The substrates prepared, as described, by varying the bulk monomer concentration and irradiating to a constant dose at a constant dose rate (Table 1) or by varying the total dose at a constant monomer concentration (Table 2).

The data in Tables 1 and 2 relate to β-galactosidase and glucose oxidase bound to the cellulose-co-polyacrylamide copolymers via the azide coupling procedure. Since the copolymers referred to in Tables 1 and 2 have been prepared under different physical/chemical conditions the results ought not to be thought of as being directly comparable.

TABLE 1

Activity of bound enzymes on cell-PAM (azide-binding). Grafting via variation in (acrylamide) in bulk medium.

	Units of Activity per g.	
% grafted acrylamide	β-galactosidase	glucose oxidase
3.7	19.3	213
5.6	23.1	7
10.2	48.8	80
free enzyme	10,500	--

TABLE 2

Activity of bound enzymes on cell-PAM (azide binding). Grafting via variation in total dose and intensity, constant (acrylamide) in bulk medium.

	Units of Activity per g.	
% Grafted Acrylamide	β- galactosidase	glucose oxidase
1.86	44.8	173
5.59	37.2	173
8.07	29.0	-
16.8	26.6	168

Glutaraldehyde coupling of β-galactosidase and glucose oxidase onto the various cell-PAM supports was largely unsuccessful in that irrespective of the conditions employed in preparing the grafts a limiting activity of 3.0 units of activity were recorded per gram of bound substrate. In Table 1 it can be seen that the activity of the bound β-galactosidase increases regularly with increases in the level of grafting while the bound glucose oxidase levels are inconsistant. The substrates used in binding have received a uniform radiation dose under standard conditions with the variation being in the quantity of acrylamide available to take advantage of the grafting sites. Table 2 shows that an inverse relationship exists between the extent of grafting and the enzyme activity when β-galactosidase is the bound enzyme. The activity of the glucose oxidase - (cell-PAM) system is seen to be independent of the level of grafting. Here we see the effect of an increase in the number of grafting sites through radiation exposure and competition for these sites by a constant monomer concentration. Ideally the situation described in Table 1 is one which is likely to produce a constant number of grafted branches whose length will be dependent on the monomer concentration. The number of sites would be expected to be low relative to the situation represented in Table 2. Here an increasing number of sites will compete for the restricted monomer. Hence the grafts obtained under the conditions described in producing the data in Table 1 would be longer and thus much more accessible to enzyme binding than would the shorter, more hindered branches outlined in Table 2.

The possibility of crosslinking reactions having occurred also arises. These will be more likely at higher dose rates and monomer concentrations, especially at extended reaction times. This factor will need to be examined further.

The levels of activity obtained are generally disappointingly low, though the bound substrates are easy to handle and recover. In view of the amount of acrylamide present in the graft copolymers, it appears that modifications are needed, perhaps involving the coupling procedure, the substrate and the monomer. This has been clearly shown by the fact that use of a β-galactosidase enzyme preparation having 3 times the activity of the β-galactosidase sample used in the major part of this study, gave no increase in the overall activity of bound substrate. This type of system shows promise because of the ease of handling. Work is being carried out on other substrates and monomers, (substituted styrenes), and will be reported in due course.

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