Polymer Bulletin 1, 749–753 (1979)

Polymer Bulletin

The Use of Graft Copolymers as Enzyme Supports

I. The Immobilization of β-Galactosidase and Papain onto Nylon-Polyacrylamide Graft Copolymers

C. G. Beddows[1], R. A. Mirauer[1], J. T. Guthrie[2], F. I. Abdel-Hay[2] and C. E. J. Morrish[2]

[1] School of Health and Applied Sciences, Leeds Polytechnic, Calverley Street, Leeds 1, U.K.

[2] Department of Colour Chemistry, University of Leeds LS2 9JK, U.K.

Summary

A new type of polymer support for immobilizing enzymes, a copolymer grafted onto nylon was investigated. Polyacrylamide was used and successfully coupled to β-galactosidase (from yeast and bacteria) using two different methods to give activities of the order of 5 units/g of polymer for the yeast enzyme, and 156 units/g for the E.coli enzyme. Lactose did not affect the yield. The azide method gave better results than the glutaraldehyde method. β-galactosidase coupled enzyme was fairly stable but not as good as the free enzyme. Papain was also coupled to the polyacrylamide grafted nylon using both methods; the glutaraldehyde gave better results with 1.5mg of enzyme being coupled for each gram of polymer.

Introduction

A large variety of natural and synthetic polymers have been used as solid supports for the attachment of enzymes. Immobilized enzymes have advantages over the free enzyme including their ease of recoverability and re-usability (ZABORSKY 1973). The choice of the support is determined by the conditions under which the enzyme can be viably attached, and the use to which the immobilized system is put. The vast majority of supports utilize the reactive groups present in the polymer. With hydrophilic polymers, this often causes the enzyme to become attached throughout the support. Examples of this are the attachment of β-galactosidase (E.C.3.2.1.23) to Sepharose using cyanuric chloride (KAY et al.1968), to DEAE cellulose (LILLY 1971) and AE-cellulose (REGAN et al.1974) and to polyacrylamide gels (DAHLQVIST et al.1973, BUNTING and LAIDLER 1973). Problems can arise in that the substrate and the products have to diffuse through the polymer fairly quickly, if they are to be comparable with the free enzyme. Galactose, for example can inhibit β-galactosidase and considerably reduce its usefulness. The system may also become blocked by other particles in the solution.

Other hydrophilic supports having a more porous structure and with greater control on the number of reactive groups, have

0170-0839/79/0001/0749/$01.00

been prepared (BEDDOWS et al 1979) but have limited value. With hydrophobic supports, coupling generally takes place on the surface. For example, β-galactosidase has been coupled to nylon using glutaraldehyde (NGO et al. 1976, HORNBY and FILIPUSSON 1970), to phenolic resins (STANLEY and PALTER 1973) and even ceramics (WONDOLOWSKI et al. 1974). The enzyme has been coupled to coated porous glass, but the attachment is not wholly covalent in nature. (WEETALL et al. 1974). The effect of the hydrophobic nature of such support systems on the enzyme activity and the relatively small number of reactive groups on the surface tend to make such systems less acceptable. However an unexplored field is that of copolymer grafts. With such systems, the number of reactive groups can be considerably increased, their number controlled and the micro-environment of the enzyme can also be altered. Copolymer grafts are covalent in nature, and are usually prepared by a free radical addition of an unsaturated system onto a relatively large polymer surface. The work of Manecke and colleagues has shown that a number of copolymers derived from unsaturated compounds have a high protein binding capacity (MANECKE and GUNZEL 1968, MANECKE et al. 1970). The grafting of these monomers onto a support, such as nylon, offers a wide range of potentially useful polymer supports with a number of methods available for coupling the enzyme.

In this investigation, polyacrylamide was grafted onto nylon and this support was used to investigate the possibility of immobilizing β-galactosidase and papain. It is hoped that other substrates will be investigated in due course.

Experimental

Analytical grade acrylamide (AM) was supplied by the Aldrich Chemical Company and was purified by standard procedures. 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. 4 sintered crucibles, further washing with methanol and drying to constant weight, under vacuum (313K). The photoinitiator, 2-chlorothioxanthone, was supplied in an analytical grade by Ward-Blenkinsop, Ltd., Widnes. The synergist dimethylaminoethanol (DMAE), was used as the analytical grade supplied by Aldrich Chemical Company Ltd.

Photoinitiation Procedures

The concentrations of the active photoinitiator (2-chlorothioxanthone) and the synergist (DMAE) were 2% and 1% (w/w) of the polyamide/monomer/water/synergist/photoinitiator system, respectively. The acrylamide concentration was initially 20% (w/w) of the bulk system containing 3g aliquots of the polyamide. Polymerizations were carried out in aqueous media after heating (353K) for 1 hour to allow reasonable contact between the polyamide and the monomer/initiator/

synergist composition. Irradiation was carried out for 60 minutes using a PRIMARC medium pressure ultra-violet lamp, care being taken to ensure that overheating of the grafting medium did not take place. The polymerization medium was continuously agitated and provided with a nitrogen gas purge. Sample assembly consisted of a centrally mounted source surrounded by a silica envelope designed to permit cooling from an external water supply. The purged, grafting medium was held in a pyrex photochemical reactor, into which the cooled lamp was assembled. Agitation was provided by a magnetic spiggot. The grafting took place at 313K. After irradiation, the grafted nylon was repeatedly washed with water to remove homopolymer using Soxhlet extraction for 48 hours. It was then washed with methanol, filtered and dried as described above. This entire programme was handled in triplicate.

Coupling Procedures

a) By conversion to the hydrazide. The polyacrylamide-co-nylon (1g) was converted to the hydrazide according to the method of INMAN and DINTZIS (1969) by incubating with hydrazine at 320K for 15 minutes. Portions (100mg) of the washed polymer were used to form the azide. The resin was suspended in $20cm^3$ of 0.25M hydrochloric acid at 277K and $4cm^3$ of cold 0.1M sodium nitrite was added dropwise over 90 secs. After 2 minutes the resin was washed with ice-cold water on a pre-cooled buchner filter, and then transferred to a solution of the enzyme at 277K and stirred for 2 hours. For β-galactosidase from yeast (BDH Ltd. Poole, Dorset) 250mg of enzyme in $25cm^3$ of pH 7.0, 0.1M phosphate buffer was used. For β-galactosidase from E.coli, (purchased from Sigma Biochemicals Ltd.) 200mg of enzyme in $25cm^3$ of 0.1M pH 7.0 phosphate buffer was used. In some experiments with β-galactosidase lactose (1%) was added.

b) Using glutaraldehyde according to the method of WESTON and AVRAMEAS (1971) when the copolymer coated nylon (1g) was initially treated with $25cm^3$ glutaraldehyde for 1 hour in 0.1M pH 7.0 buffer at ambient temperature. After washing free of glutaraldehyde, the number of reducing groups present on the polymer was determined by the method of NELSON (1944). The polymer was then added to enzyme solutions as above, mixed, washed with 0.1N sodium chloride solution and assayed.

Enzyme Assays

The β-galactosidase was assayed using the method of CRAVEN et al (1965) using o-nitrophenol-β-D-galactose (ONPG) as substrate (1 unit ≡ 1×10^{-6} mol of ONPG hydrolysed per min. at 298K) Papain was assayed using α-benzoyl-arginine-ethyl ester at pH 7.8 (BAEE) (SCHWERT and TAKENAKA 1955) (1 unit ≡ change in 0.001 UV units min^{-1} at 298K).

Results and Discussion

The level of graft co-polymerization (Griltex-PAM) was 5% ± 0.2% based on the original weight of polyamide. The extent of homopolymerization, though not quantified was considerable. It is felt that the experimental procedure gave rise to predominantly surface grafts with hydrophilic branches on an essentially hydrophobic substrate. No accurate method of determining the amount of protein coupled to the polymer was available. Some of the results of the experiments with β-galactosidase are given in Table 1. The free enzyme (from yeast) had an activity of 2.5 units/mg; the enzyme from E.coli had an activity of 82.1 units/mg.

TABLE 1

Coupling to β-galactosidase

Source of Enzyme	Coupling Method	Activity (Units/g)	Active Enzyme coupled (mg/g)
Yeast	Azide	5.2	2.1
	Azide (with 1% lactose present)	5.1	2.0
	Glutaraldehyde	1.1	0.4
E.coli	Azide	156	1.9
	Glutaraldehyde	16.4	0.2

All the methods were carried out in triplicate. The azide method gave better results than the glutaraldehyde method, particularly with the yeast enzyme. The presence of lactose which could combine with the active site, had no effect on the extent of coupling. The results are comparable to those obtained using p-amino-carbanilated cellulose (CTC) (BEDDOWS et al.1979), using coated porous glass (WONDOLOWSKY and WOYCHICK 1974) and DEAE-cellulose (KAY et al 1968).

The stability of the coupled E.coli enzyme was comparable to CTC (BEDDOWS et al 1979) with a half life of 15 days, but is less than the free enzyme.

Analysis of the glutaraldehyde - polymer showed that a large excess of reducing groups was present both before and after coupling to the enzyme. This confirms that only a very few groups must have been utilized in coupling to active enzyme. Papain showed an activity of 84.6 BAEE units/mg. Coupling using the azide method gave an activity 107 BAEE units/g so that 1.3mg/g of active enzyme had been coupled. With the

glutaraldehyde method 124 BAEE units/g were coupled (≡ 1.5mg/g of active enzyme coupled to the polymer). The relative activities are the converse of those obtained with the two methods of coupling to β-galactosidase. The amount coupled with the azide method was greater than the amounts coupled using cellulose - carboxymethyl ether (WEETALL 1970).

However the amount of enzyme coupled onto polyacrylamide is less than the amounts of papain that MANECKE et al. (1968) immobilized onto a variety of copolymers having other functional groups. Further work will involve the investigation of the preparation of these copolymers as graft-copolymers and their potential usefulness.

References

BEDDOWS, C.G., MIRAUER, R.A., GUTHRIE, J.T.; Biotech. Bioeng. In Press (1979)
BUNTING, P.S., AND LAIDLER, K.J.; Can. J. Biochem. 51. 1598 (1973)
CRAVEN, G.R., STEERS, E.J. and ANFINSEN, C.B.; J. Biol. Chem. 240, 2468 (1965)
DAHLQVIST, A., MATTIASSON, B., and MOSSBACH, K.; Biotechnol. Bioeng, 15, 395 (1973)
HORNBY, W.E., and FILIPUSSON, H.; Biochem. Biophys. Acta. 220, 343 (1970)
INMAN, J.K., and DINTZIS, H.M.; Biochemistry, 8, 4074 (1969)
KAY, G., LILLY, M.D., SHARP, A.K., and WILSON, R.J.H.; Nature 217, 641 (1968)
LILLY, M.D.; Biotechnol. Bioeng. 13 589 (1971)
MANECKE, G., GUNZEL, G.; Naturwissenschaften, 20, 531 (1968)
NELSON, N.; J. Biol. Chem.; 153,375 (1944)
NGO, T.T., NARINESING, D., and LAIDLER, K.J.; Biotech. Bioeng. 18, 119 (1976)
REGAN, D.L., DUNHILL, P. and LILLY, M.D.; Biotech. Bioeng. 16, 333 (1974)
SCHWERT, G.W., and TAKENAKA, G.; Biochem. Biophys. Acta, 16, 570 (1955)
STANLEY, W.L., and PALTER, R.; Biotech. Bioeng. 16, 1633 (1974)
WEETALL, H.H., HAVEWALA, N.B., PITCHER, W.H., DETAR, C.C., VANN, W.P. and YAVERBAUM, S.; Biotech. Bioeng, 16, 689 (1974)
WEETALL, H.H.; Biochem. Biophys. Acta., 212, 1 (1970)
WESTON, P.D. and AVRAMEAS, S.; Biochem. Biophys. Res. Comm., 45, 1574 (1971)
WONDOLOWSKY, H.V. and WOYCHICK, V.; Biotech. Bioeng. 16 1633 (1974)
ZABORSKY, O.R.; (Review) Immobilized enzymes, Cleveland, Ohio, U.S.A., CRC Press (1973)

Received July 19, 1979