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New hydrophilic acrylate monomers suitable for the gel bead entrapment of enzymes

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INTRODUCTION

In recent years, there have been many reports of enzyme immobilization by gel entrapment methods[1–4]. However, relatively few different types of enzyme entrapment matrices have been described. These include starch gel[5], polyacrylamide[6], silastic resins[7], poly(vinyl alcohol)[8], poly(2-hydroxyethylmethacrylate)[9], poly(2-hydroxyethylacrylate)[10], nylon capsules[11], cellulose triacetate[12] and poly(vinyl chloride)[13]. Of these matrices, starch gel exhibits poor mechanical stability and enzyme retention properties[5] and silastic resin conjugates exhibit poor permeability to substrates[7]. Only crosslinked polyacrylamide[14–21], and, to a lesser extent, poly(2-hydroxyethylmethacrylate)[22–24] gels have found widespread use.

A feature common to many enzyme entrapment conjugates is that they are prepared by methods involving bulk solution polymerization. This gives blocks of conjugate. These have to be broken up mechanically resulting in irregular-shaped particles. A few workers have prepared enzyme entrapment conjugates in bead form. These bead conjugates are derived usually from crosslinked polyacrylamide[25–28]. Compared to the irregular particulate conjugates, bead conjugates exhibit superior flow rates when applied in packed beds, are less susceptible to enzyme 'leakage', and exhibit much superior mechanical stability to fragmentation when used in stirred tank reactors[14].

In the present study we have attempted to extend the range of bead entrapment systems available for enzyme immobilization. New acrylate monomers, possessing hydrophilic properties arising from ketal, ketone and ether residues have been prepared. These monomers have been used for the gel bead entrapment of β-D-glucosidase.

EXPERIMENTAL

Synthesis of 1-acryloylpiperidine-4-spiro-2′-(1′,3′-dioxacyclopentane)

Method (*a*). Piperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) (5.00 g, 0.035 mol) (Aldrich Ltd., U.S.A.) was dissolved in peroxide free, dry ether (20 cm^3) and the solution was maintained at 0°C. Acryloyl chloride (1.58 g, 0.018 mol) was added slowly over 20 min with efficient mechanical stirring. Stirring was continued for 10 min after which the precipitate of piperidine-4-spiro-2′-(1′-3′-dioxacyclopentan) hydrochloride was removed by filtration. The filtrate was washed with 2 M sodium hydroxide (10 cm^3), dried over anhydrous magnesium sulphate and the solvent removed under reduced pressure to give 1-acryloylpiperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) (Monomer A) (2.64 g, 76%) as a white crystalline solid; m.p. 37–42°C (from diethylether-light petroleum b.p. 40–60°); ν_{max} (KBr disc) 1650 (amide $C=O$ str.), 1620($C=C$ str.) and 1100 cm^{-1} (ketal C O str.); δ(CCl_4; 60 MHz) 1.5–1.7 (4H, m, $C\underline{H}_2CC\underline{H}_2$), 3.5–3.8 (4H, m, $C\underline{H}_2N\underline{CH}_2$), 3.9 (4H, s, $O(C\underline{H}_2)_2O$), 5.4–6.3 (2H, m, $C\underline{H}_2=CHCO$) and 6.5–6.9 ppm (1H, m, $CH_2=C\underline{H}CO$). (Found: C, 61.02%; H, 7.72%; N, 7.08%; M$^+$, 197. $C_{10}H_{15}NO_3$ requires: C, 60.96%; H, 7.68%; N, 7.11%; M, 197).

Method (*b*). Piperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) (10.00 g, 0.070 mol) was dissolved in a slurry of sodium carbonate 11.11 g, 0.11 mol) and water (50 cm^3) and the solution was maintained at 0°C. Acryloyl chloride (7.00 g, 0.077 mol) was added slowly over 30 min with efficient mechanical stirring. Stirring was continued for 20 min after which excess sodium carbonate was removed by filtration. The filtrate was extracted with ether (4 x 100 cm^3) and the combined ether extracts were dried over magnesium sulphate and evaporated under reduced pressure to give 1-acryloylpiperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) (Monomer A) (9.00 g, 65%) [Product identical to that prepared by Method (a)].

Synthesis of 1-acryloyl-4-piperidone

Method (*a*). 1-Acryloylpiperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) (1.44 g, 0.007 mol) was dissolved in 2M hydrochloric acid (5 cm^3). The solution was stirred for 1h, extracted with ether (4 × 10 cm^3), and the ether extracts dried over magnesium sulphate. The ether was evaporated under reduced pressure to give 1-acryloyl-4-piperidone (Monomer B) (0.79 g, 73%) as a colourless oil, ν_{max} (liquid film) 1730 (cyclic $C=O$ str.), 1650 (amide $C=O$ str.) and 1620 cm^{-1} ($C=C$ str.); δ($CDCl_3$; 60 MHz) 2.4–2.6 (4H, m, $C\underline{H}_2COCH_2$), 3.8–4.0 (4H, m, $CH_2C\underline{H}_2NC\underline{H}_2CH_2$), 5.6–6.4 (2H, m, $C\underline{H}_2=CHCO$) and 6.6–7.0 ppm (1H, m, $CH_2=C\underline{H}CO$). (Found: M$^+$, 153; $C_8H_{11}NO_2$ requires M, 153).

Semicarbazone. m.p. 152–154°C (from chloroform-diethyl ether). (Found: C, 51.22%; H, 6.84%; N, 26.48%. $C_9H_{14}N_4O_2$ requires: C, 51.41%; H, 6.72%; N, 26.65%); *2,4-dinotrophenylhydrazone*, m.p. 234–237°C (from benzene). (Found: C, 50.55%; H, 4.54%; N, 20.59%. $C_{14}H_{15}N_5O_5$ requires C, 50.44%; H, 4.54%; N, 21.01%).

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Table 1 Experimental details for the preparation of unadulterated copolymers

Copolymer	Weight of primary monomer (g)	Weight of crosslinker (g)	Volume of 3% $K_2S_2O_8$ (cm^3)	Volume of polymerization mixture (cm^3)
Copolymer A	9.03 g (0.046 mol)	0.72 g (0.0046 mol)	2	40
Copolymer B	10.01 g (0.065 mol)	0.99 g (0.0065 mol)	1	38
Copolymer C	2.05 g (0.020 mol)	0.32 g (0.0021 mol)	2	7

Table 2 Experimental details for the preparation of copolymer entrapped β-D-glucosidase

β-D-glucosidase conjugate	Weight of primary monomer (g)	Weight of crosslinker (g)	Weight of enzyme (g)	Volume of 3% $K_2S_2O_8$ (cm^3)	Volume of polymerization mixture (cm^3)	Enzyme units (×10^{-3}) in polymerization mixture	Active enzyme (×10^{-3}) entrapped	Practical activity retention
Conjugate A	8.05 (Monomer A)	0.63	0.0094	2.5	32	8.7	2.27	26%
Conjugate B	10.00 (Monomer B)	1.00	0.0127	2.0	40	12.7	1.91	15%
Conjugate C	7.17 (Monomer C)	1.13	0.0156	2.5	31	15.6	0.93	6%

Method (*b*). 4-Piperidone monohydrate hydrochloride (15.00 g, 0.098 mol) (Aldrich Ltd., U.S.A.) was dissolved in a slurry of sodium hydrogen carbonate (24.66 g, 0.29 mol) and water (75 cm^3) and the solution was maintained at 0°C. Acryloyl chloride (10.43 g, 0.12 mol) was added slowly over 30 min with efficient mechanical stirring. Stirring was continued for 30 min and excess sodium hydrogen carbonate was removed by filtration. The filtrate was extracted with chloroform (3 × 100 cm^3), the combined extracts were washed with 2M hydrochloric acid (2 × 20 cm^3) and dried over magnesium sulphate. The solvent was removed under reduced pressure to give 1-acryloyl-4-piperidone (Monomer B) (8.62 g, 57%) as a colourless oil. [Product identical to that prepared by Method (a)].

Synthesis of acryloylmethoxyamine

Methoxyamine hydrochloride (10.00 g, 0.12 mol) was dissolved in a solution of sodium hydroxide (4.80 g, 0.12 mol) in water (50 cm^3) at 0°C and sodium hydrogen carbonate (13.10 g, 0.16 mol) was added to the stirred solution. Acryloyl chloride (14.46 g, 0.16 mol) was added slowly over 30 min. Stirring was continued for 30 min and excess sodium hydrogen carbonate was removed by filtration. The filtrate was extracted with chloroform (5 × 50 cm^3), the combined chloroform extracts were washed with M hydrochloric acid (2 × 10 cm^3), dried over magnesium sulphate and evaporated under reduced pressure to give acryloylmethoxyamine (Monomer C) (7.10 g, 58%) as a white crystalline solid; m.p. 48°C (from light petroleum b.p. 40–60°) ν_{max} (KBr disc) 1660 (amide C=O str.), 1630 (C=C str.) and 1060 cm^{-1} (O-CH$_3$ str.); δ(CDCl$_3$; 60 MHz) 3.8 (3H, s, OC$\underline{H}_3$), 5.6–6.4 (2H, m, C$\underline{H}_2$=CHCO), 6.6–7.1 (1H, m, CH$_2$=C$\underline{H}$CO) and 10.6 ppm (1H, s, CON$\underline{H}$O). (Found: C, 47.29%; H, 6.95%; N, 13.76%; M$^+$, 101. $C_4N_7NO_2$ requires: C, 47.56%, H, 6.99%; N, 13.87%; M, 101).

Suspension polymerization

*Bead copolymer of 1-acryloylpiperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) and N,N′-methylene*bis*acrylamide.* Liquid paraffin (ρ^{20} 0.85 g cm^{-3}, η^{20} 3.5–4.0 N sec m^{-2}) (500 cm^3) and sorbitan trioleate (10 cm^3), were placed in a 1 dm^3 glass polymerization vessel fitted with a semi-circular paddle stirrer that had horizontal slats. The paddle just swept the wall of the flask. Nitrogen was bubbled vigorously into the solution for 3 h via a fine glass jet at such a rate as to give an aerosol mist above the solution. A solution of 1-acryloyl-piperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) (Monomer A) (9.03 g, 0.046 mol) and *N,N′*-methylene*bis*acrylamide (0.72 g, 0.0046 mol) in water (38 cm^3) and an aqueous solution of 3% w/v potassium persulphate were deoxygenated in a similar fashion for 2 h. Polymerization was initiated by the addition, with rapid stirring, of an aliquot (2 cm^3) of the potassium persulphate solution to the deoxygenated aqueous monomer solution. The initiated solution was dispersed immediately in the paraffin mixture. A short period of rapid stirring was required to disperse the aqueous phase into droplets of 40–200 μm in diameter before the stirring rate was reduced to 45 r.p.m. A nitrogen atmosphere was maintained in the flask during polymerization. Polymerization was evident after 1 h and assumed to be complete after 5 h.

The beads of gel copolymer (Copolymer A) were left to settle and most of the paraffin was removed by decantation. The beads of Copolymer A were washed finally with light petroleum ether (b.p. 40–60°) (3 × 250 cm^3), water (3 × 250 cm^3) and stored suspended in water.

Polymerization Times. To obtain reproducible polymerization times it was necessary to predetermine the amount of initiator for each bead polymerization in a preliminary, small-scale, block copolymerization experiment. An initiator concentration that would produce a firm block copolymer in 10–20 min was found convenient.

*Bead copolymers of (i) 1-acryloyl-4-piperidone and N,N′-methylene*bis*acrylamide and (ii) acryloylmethoxyamine and N,N′-methylene*bis*acrylamide.* Beaded copolymers of poly[1-acryloyl-4-piperidone (Monomer B)/ *N*,*N*′-methylene*bis*acrylamide] (Copolymer B) and of poly[acryloylmethoxyamine (Monomer C)/*N*,*N*′-methylene*bis*acrylamide] (Copolymer C) were prepared following the method described above. The quantities of reagents used are given in *Table 1*.

*Immobilization of β-D-glucosidase by entrapment within bead copolymerized poly[1-acryloylpiperidine-4-spiro-2′-(1′,3′-dioxacyclopentane)/N,N′-methylene*bis*acrylamide].* The continuous phase used for this suspension polymerization was identical to that used for the preparation of the unadulterated bead copolymers. 1-Acryloylpiperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) (Monomer A) (8.05 g, 0.041 mol) and *N*,*N*′-methylene*bis*acrylamide (0.63 g, 0.0041 mol) were dissolved in water (28 cm^3) and the solution was deoxygenated. Polymerization was initiated by the addition, with rapid stirring, of an aliquot (2.5 cm^3) of deoxygenated 3% w/v aqueous potassium persulphate solution, followed by the immediate addition of a solution of β-D-glucosidase (E.C. 3.2.1.21) (Koch-Light Laboratories) (9.5 mg) in deoxygenated aqueous 0.2M sodium acetate (1.5 cm^3) at pH 5.0. The solution was then dispersed immediately in the continuous phase. When polymerization was complete, the beads of the β-D-glucosidase entrapment conjugate (Conjugate A) were allowed to settle and most of the paraffin was removed by decantation. The beads of Conjugate A were washed with light petroleum ether (b.p. 40°–60°C) (3 × 250 cm^3), water (3 × 250 cm^3) and aqueous 0.2 M sodium acetate (3 × 250 cm^3) at pH 5.0.

Polymerization times: Small-scale block copolymerizations in the presence of the enzyme were carried out prior to the bead polymerization to predetermine optimum initiator concentration.

*Immobilization of β-D-glucosidase by entrapment within bead copolymerized poly(1-acryloyl-4-piperidone/N,N′-methylene*bis*acrylamide) and within bead copolymerized poly(acryloylmethoxyamine/N,N′-methylene*bis*acrylamide).* β-D-Glucosidase was incorporated within beads of poly[1-acryloyl-4-piperidone (Monomer B)/*N*,*N*′-methylene*bis*acrylamide] and within beads of poly[acryloylmethoxyamine (Monomer C)/*N*,*N*′-methylene*bis*acrylamide] to give β-D-glucosidase entrapment conjugates (Conjugate B and Conjugate C respectively), using the method described above. The quantities of reagents used are given in *Table 2*.

Determination of β-D-glucosidase activity

β-D-Glucosidase activity was determined by the method of Agrawal and Bahl[29]. The substrate *p*-nitrophenyl-β-D-glucopyranoside (200 mg) was dissolved in 0.2M sodium acetate buffer (100 cm^3) at pH 5.0. Solvent external to the beads of gel–enzyme conjugate was drawn off by suction under reduced pressure and an accurately weighed sample (0.1–0.3 g) of beads was suspended in aqueous 0.2M sodium acetate (0.5 cm^3) at pH 5.0. An aliquot (3 cm^3) of substrate solution was added to the suspension which was stirred for 30 min. After centrifugation, the supernatant (0.5 cm^3) was added to an aqueous solution (2.5 cm^3) of 0.2M sodium carbonate in 0.2M sodium acetate. The absorbance of the supernatant at 420 nm was determined. One β-D-glucosidase unit was taken to be that which caused the hydrolysis of one μmole of *p*-nitrophenyl-β-D-glucopyranoside per min at pH 5.0 at 25°C.

Heat stability of immobilised β-D-glucosidase

The heat stability of the immobilized enzyme was determined by incubation of the gels at 50°C in 0.2M sodium acetate buffer pH 5.0. Enzyme activity was determined at regular intervals (*Figure 3*). A control experiment on the native enzyme in a similar buffer solution was carried out.

Storage stability of each enzyme conjugate was evaluated by redetermining the enzymic activity of each conjugate after storage for four weeks at 0°C suspended at pH 5.0 in 0.2M sodium acetate buffer.

RESULTS AND DISCUSSION

1-Acryloylpiperidine-4-spiro-2′-(1′,3′-dioxacyclopentane) (Monomer A) (*Figure 1*) was prepared by the addition of acryloylchloride to two molar equivalents of the amine, piperidine-4-spiro-2′-(1′,3′-dioxacyclopentane), in ether. Monomer A was also prepared in water by the addition of acryloyl chloride to the amine in the presence of sodium carbonate. The amine was not used as its own base in the latter method and this route was used to prepare 1-acryloyl-4-piperidone (Monomer B) and acryloylmethoxyamine (Monomer C) (*Figure 1*). Controlled acid hydrolysis of Monomer A also gave Monomer B in good yield.

Monomer A, Monomer B and Monomer C were each copolymerized with *N*,*N*′-methylene*bis*acrylamide using a suspension polymerization technique[30] to give bead copolymers (Copolymer A, Copolymer B and Copolymer C) (*Figure 2*). Copolymer C [crosslinked poly(acryloylmethoxyamine] was obtained as a homo-

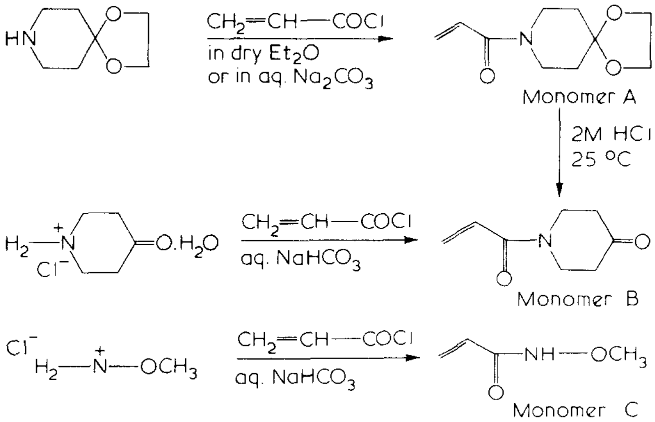

Figure 1 Reaction schemes for the preparation of 1-acryloylpiperidine-4-spiro-2′-(1′, 3′-dioxacyclopentane) (Monomer A), 1-acryloyl-4-piperidone (Monomer B) and acryloylmethoxyamine (Monomer C)

Table 3a Swelling properties of crosslinked poly[acryloylpiperidine-4-spiro-2'-(1',3'-dioxacyclopentane)] (Copolymer A)

Solvent	Gel bed volume (V_B) (cm^3 g^{-1})	Solvent regain of gel (S_R) (cm^3 g^{-1})	Imbibition ratio (I)*
n-Hexane	1.79	0.00	0.00
Diethyl ether	1.88	0.00	0.00
Acetone	4.28	1.63	0.95
Methanol	4.33	1.73	1.01
Ethanol	4.48	1.90	1.11
Water	4.27	1.71	1.00
Dichloromethane	5.13	2.42	1.42
Chloroform	5.82	2.82	1.65
Carbon tetrachloride	2.18	0.60	0.35
1,4-Dioxan	5.02	2.29	1.34
Dimethylformamide	3.83	1.42	0.83
Dimethylsulphoxide	3.93	2.94	1.72
Acetic acid	7.10	2.42	1.42

$$\text{* Imbibition ratio} = \frac{S_R \text{ of the polymer in the given solvent}}{S_R \text{ of the polymer in water}}$$

Table 3b Swelling properties of crosslinked poly(acryloyl-4-piperidone) (Copolymer B)

Solvent	Gel bed volume (V_B) (cm^3 g^{-1})	Solvent regain of gel (S_R) (cm^3 g^{-1})	Imbibition ratio (I)*
n-Hexane	1.27	0.00	0.00
Diethyl ether	1.42	0.00	0.00
Acetone	1.96	0.98	0.24
Methanol	3.95	2.98	0.72
Ethanol	2.18	0.94	0.23
Water	5.21	4.03	1.00
Dichloromethane	3.85	2.76	0.68
Chloroform	4.61	3.31	0.82
Carbon tetrachloride	2.08	0.90	0.22
1,4-Dioxan	1.70	0.25	0.06
Dimethylformamide	5.04	2.58	0.64
Dimethylsulphoxide	5.34	2.67	0.66
Acetic acid	4.87	1.13	0.28

$$\text{* Imbibition ratio} = \frac{S_R \text{ of the polymer in given solvent}}{S_R \text{ of the polymer in water}}$$

genous preparation of discrete beads whereas Copolymer A (crosslinked poly[acryloylpiperidine-4-spiro-2'-(1',3'-dioxacyclopentane)]) and Copolymer B (crosslinked-poly(1-acryloyl-4-piperidone) gave mainly discrete beads but contained some agglomerates. Solvent imbibition measurements on Copolymer A, Copolymer B and Copolymer C showed that these matrices swelled appreciably in polar organic solvents and particularly in water (*Tables 3a, b* and *c*). The latter observation suggested that the three matrices could be suitable for enzyme entrapment studies.

Bulk copolymerization of the acrylate monomers with *N.N'*-methylene*bis*acrylamide were carried out in buffered solution in the presence of *β*-D-glucosidase. Rather long gel times were obtained indicating that the enzyme inhibited polymerization. In subsequent *β*-D-glucosidase bead entrapment studies, it was necessary to adjust the gel times by changing the initiator concentrations (*Table 2*). The *β*-D-glucosidase conjugates derived from Monomer A, Monomer B and Monomer C are designated Conjugate A, Conjugate B and Conjugate C respectively.

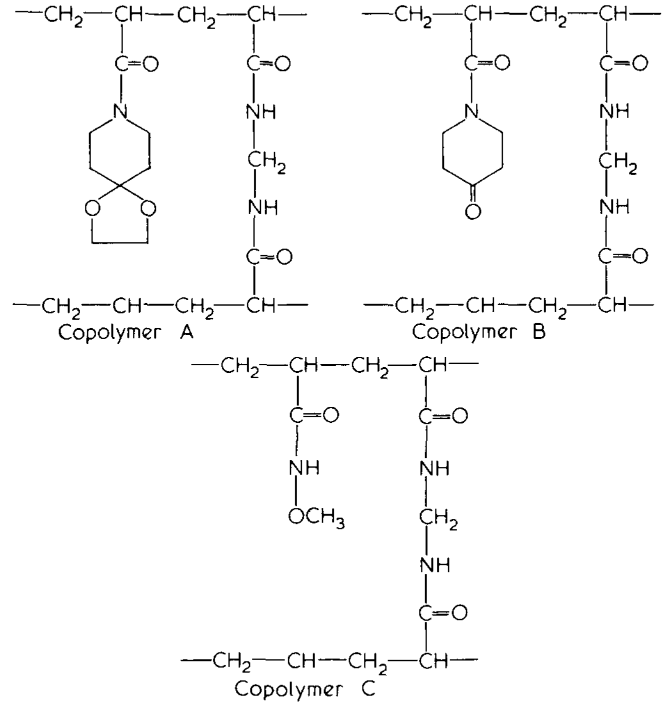

Figure 2 Crosslinked poly(1-acryloylpiperidine-4-spiro-2'-(1',3'-dioxacyclopentane) (Copolymer A); crosslinked poly(1-acryloyl-4-piperidone) (Copolymer B); crosslinked poly(acryloylmethoxyamine) (Copolymer C)

Table 3c Swelling properties of crosslinked poly(acryloylmethoxyamine) (Copolymer C)

Solvent	Gel bed volume (V_B) (cm^3 g^{-1})	Solvent regain of gel (S_R) (cm^3 g^{-1})	Imbibition ratio (I)*
n-Hexane	1.38	0.00	0.00
Diethyl ether	1.31	0.00	0.00
Acetone	1.73	0.00	0.00
Methanol	5.43	2.66	0.29
Ethanol	3.96	2.65	0.29
Water	15.56	9.31	1.00
Dichloromethane	2.13	0.71	0.08
Chloroform	2.28	0.38	0.04
Carbon tetrachloride	1.98	0.33	0.04
1,4-Dioxan	1.86	0.18	0.02
Dimethylformamide	12.64	11.30	1.21
Dimethylsulphoxide	16.13	9.63	1.03
Acetic acid	12.82	3.42	0.31
Ethane-1,2-diol	11.33	8.09	0.87
Acetonitrile	2.60	1.30	0.14
Benzyl alcohol	4.83	4.83	0.52
Benzene	1.89	0.24	0.03

$$\text{* Imbibition ratio} = \frac{S_R \text{ of the polymer in the given solvent}}{S_R \text{ of the polymer in water}}$$

On completion of each *β*-D-glucosidase bead entrapment copolymerization, the polymer beads were washed with light petroleum (b.p. 40°–60°C), water and 0.2M aqueous sodium acetate buffer, pH 5.0. No enzyme activity was detected in the water, or aqueous buffer washings, indicating that in all cases the enzyme had been wholly entrapped within the copolymer beads.

The enzyme activity of each bead entrapped enzyme was determined and compared with a comparable amount of the native enzyme in free solution. Reasonable

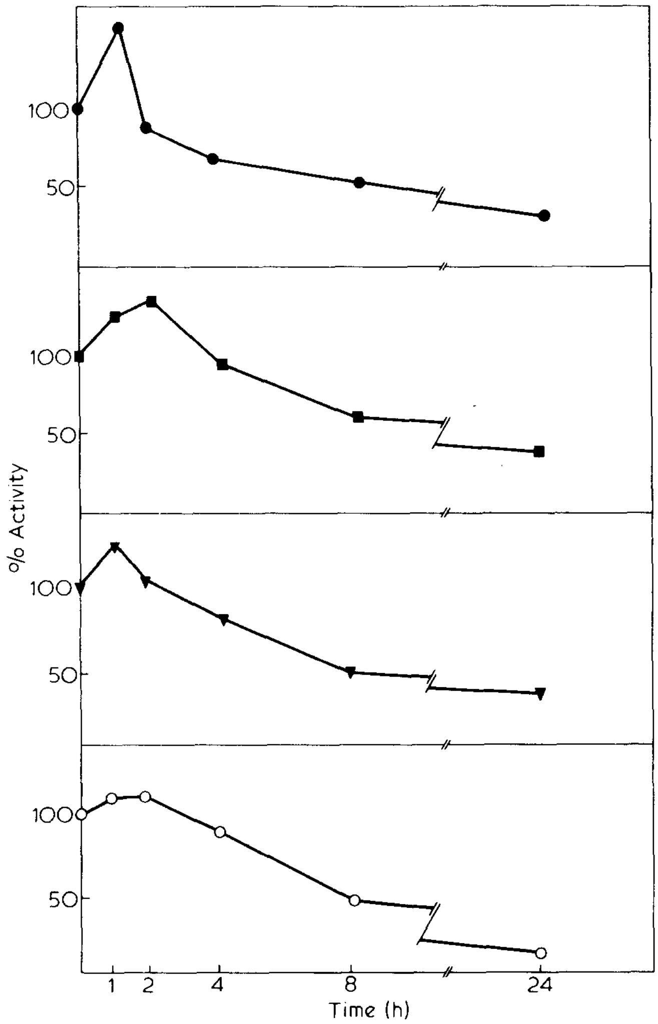

Figure 3 Heat denaturation at 50°C of β-D-glucosidase Conjugate A (crosslinked poly[1-acryloylpiperidine-4-spiro-2'-(1',3'-dioxacyclopentane)]) (●); Conjugate B [crosslinked poly(1-acryloyl-4-piperidone)] (■); Conjugate C [crosslinked poly(methoxyamine)] (▲) and of native β-D-glucosidase in solution (○)

practical enzyme activity retentions were obtained in the case of all three conjugates (*Table 2*).

The three β-D-glucosidase conjugates and the native enzyme in solution had similar stability to heat denaturation at 50°C over incubation periods of up to a few hours (*Figure 3*). However, the native enzyme was substantially denaturated over 24 h whereas the conjugates all retained appreciable enzyme activity. This indicated that the tertiary structure of the enzyme was being preserved by the constraining gel matrix. Conjugate A, Conjugate B and Conjugate C retained 68%, 57% and 44% of their original enzyme activities on storage for 4 weeks in suspension in acetate buffer at 0°C.

All three bead copolymers would seem viable matrices for the gel bead entrapment of enzymes. Copolymer C, derived from acryloylmethoxyamine, is the most stable chemically and could be applicable as a useful base matrix for bio-organic reagent applications. Further work is in progress to evaluate this possibility.

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