Influence of Pentaerythritol Triacrylate Acrylic Carrier Swelling on Immobilization of Enzymes

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SYNOPSIS

Suspension copolymerization and modification of pentaerythritol triacrylate (PENTA)–butyl acrylate (BA) matrices with ethylenediamine (EtDA) are discussed. It is shown that aminolysis in water solution offers gel structure carriers with small pore radii (about 3 nm). These carriers are characterized by the high swelling and sharp correlation between the pH value and the specific volume. The enzymes (acylase, glucoamylase, and peroxydase) are immobilized by means of the glutaraldehyde method. The best matrix seems to be copolymers modified in a 95% solution of ethylenediamine in water. Preparations do not lose activity after 1 month storage at 4°C. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Suspension polymerization and matrice properties of trimethylolpropane trimethacrylate (TMPMA) and its copolymers with either methyl, glycidyl, methacrylate, or acrylamide were investigated by Flodin et al.,[1-6] Reinholdsson et al.,[7,8] Sherrington,[9] and others.[10-12] The TMPMA matrices had small pore diameter when synthesized in the presence of toluene, and were moderately broader when obtained in an ethyl acetate environment[1,6] or in the presence of the linear polymer in solution.[10] The exact investigation of a molecular structure revealed that TMPMA incorporated in extent of 20% to monocyclic and 80% to cyclic chain structures.[13] This molecular organization was mostly responsible for extraordinary swelling properties of the TMPMA copolymers.[6] The small amounts of residual double bounds were used for functionalization of these copolymers.[14-16]

Trimethylolpropane triacrylate (TMPA), its copolymers with acrylonitrile (AN) or methacrylonitrile, and derivatives obtained after aminolysis were successively studied as carriers for immobilization of penicillin acylase, glucoamylase, peroxidase, and lipase.[17-19] The properties of carriers obtained by aminolysis of four kinds of copolymers, TMPMA/AN, TMPMA/BA (BA-butyl acrylate), TMPA/AN, and TMPA/BA, synthesized in the presence of good inert diluents, cyclohexanol and 2-ethylhexanol, were compared.[11] The most suitable for immobilization of acylase were the carriers obtained from TMPMA/BA due to their high porosity and mean size of pores.

To the best of our knowledge, there are no papers dealing with syntheses, properties, and performances in an enzyme immobilization task for either homo- or copolymers of pentaerythritol triacrylate (PENTA). For this reason, the aim of the studies presented was to evaluate the potential usefulness of PENTA and PENTA/BA copolymers as carriers for enzyme immobilization.

EXPERIMENTAL

Materials and Method Synthesis of Matrices

The polymers of PENTA and PENTA/BA were obtained in suspension polymerization initiated by 0.5 wt % benzoil peroxide. The organic phase was suspended in a 10% NaCl water solution containing 1.0 wt % poly(vinyl alcohol) (Gohsenol GH-23) as dispergator. The monomer mixtures of 20 (20P), 40 (40P), 50 (50P), 60 (60P), 80 (80P), and 100% of

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PENTA (100P) were diluted in cyclohexanol and 2-ethylhexanol (1 : 1, v/v). The volume ratio of monomer to diluents was adjusted to 1 : 1. Polymerization was carried out at 70–95°C for 8 h. Finally, the synthesized copolymers were rinsed with hot water and methanol and air dried. The copolymers were extracted with hot toluene in Soxhlet. All reactants were purchased from Aldrich. The properties of the matrix have been described separately.[12]

Aminolysis of Copolymers

Modification of the copolymers was performed in the following way[19]: PENTA or PENTA/BA copolymers (10 g) were placed into a reaction vessel and a solution of ethylenediamine (EtDA) in water or toluene (100 cm^3) was added. After 24 h the reaction mixture was heated and kept boiling under reflux for an appropriate time. The concentration of EtDA and time of aminolysis are juxtaposed in Table I. After modification the carriers were washed with water or toluene and acetone, and then alternatively treated with 1 *M* solution of NaOH and HCl three times successively.

Determination of Carriers Properties

Amine group concentration was calculated from the contents of organic nitrogen. Carboxylic and amine group concentrations were also measured according to our own previously published method.[20]

Water regain was evaluated by centrifugation, and the volume fraction of polymer in swollen gel phase, v_2, was calculated.[21]

Specific volume swelling of carriers was measured in calibrated cylinders by adjusting the pH of the solution with NaOH and HCl, respectively. The swelling was measured in NaCl solution (0.5 *M*) and in phosphate buffer (0.5 *M*).

Inverse steric exclusion chromatography (ISEC) was used to characterize the carriers swollen with water.[19,20]

Immobilization of Enzymes

Enzymes were immobilized by the glutaraldehyde method. The procedure using penicillin acylase attachment and enzyme assay was published elsewhere.[22] For the purpose of this work, the methods were not modified. The amount of enzyme that catalyzed the formation of 1 μmol of 6-aminopenicillanic acid from penicillin G within 1 min under test condition (37°C, pH 7.8) was defined as one (1) activity unit (U). Because of the crude penicillin acylase (0.73 U/mg) preparation was used in immobilization studies, the preferential ability of active enzyme bonding was detected. This parameter was expressed as the ratio of specific activities of bound to native enzyme. The specific volume of preparation was also measured for better characterization of carriers.

Table I Parameters of Aminolysis

Symbol	Amine Concentration (%)	Diluent	Time (h)
A	20	Toluene	1.5
C	50	Toluene	5.0
D	75	Toluene	4.5
E	95	Toluene	5.0
F	95	Water	5.0
G	75	Water	5.0
H	75	Water	2.5
I	75	Water	3.5

The immobilization and activity assays of peroxidase and glucoamylase were performed as described previously.[23]

Enzymes stabilities were evaluated by storing preparations at 4°C for at least 4 weeks.

RESULTS AND DISCUSSION

Aminolysis of PENTA/BA Copolymers

The properties of the polymer networks obtained by suspension polymerization from pentaerythritol triacrylate (PENTA or PENTA/BA) in the presence of the inert diluents cyclohexanol and 2-ethylhexanol have been described in the separate paper.[12] Swelling PENTA/BA copolymers in water is low, in spite of the fact that the crosslinker contains hydroxyl groups (water regain about 0.7 g/g).

To prepare carriers for immobilization of enzyme, the PENTA/BA copolymers were subjected to aminolysis in large excess of ethylenediamine in the parameters presented in Table I. Taking into account the condition of the aminolysis reaction one expects that modification can create on the surface *N*-amidoamines and stoichiometric amounts of hydroxyl groups (Fig. 1). However, at the same aminolysis condition, for PENTA homopolymer (100P), a large number of ester groups hydrolyzed. During this reaction the carboxylic groups and stoichiometric amount of hydroxyl groups appear in the polymer network. Hence, it may be stated that hydrolysis of

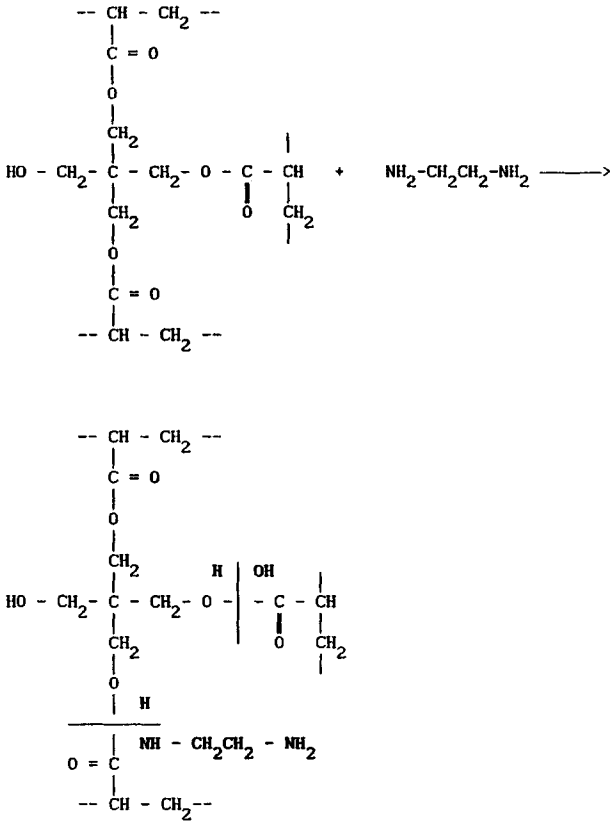

Figure 1 Aminolysis of pentaerythritol triacrylate.

PENTA and/or butyl esters groups appears simultanously (Fig. 1).

Figure 2 presents the influence of aminolysis parameters (F–I, Table I) on amine groups concentration for carriers containing 20, 40, 50, and 60% of PENTA. With the increased time of modification in water–EtDA solution, amine group concentrations also rise and the carriers are more prone to swelling (Fig. 3). Therefore the amine group concentrations in mmol/cm^3 are similar. After 5 h of aminolysis in 75% water–EtDA solution (entry G, Table I) the concentration of both amine and carboxylic groups is highest (see Fig. 4) and swelling of these carriers increases drastically (Fig. 3).

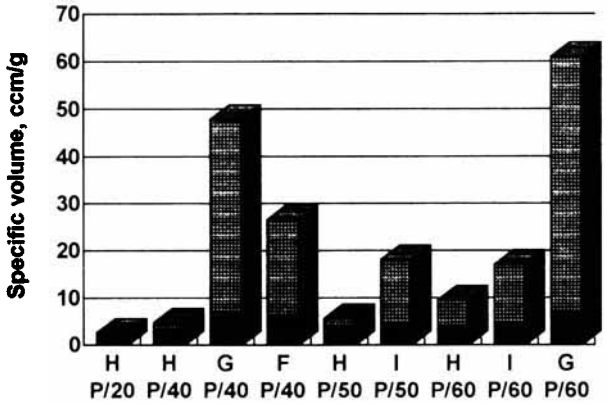

Figure 3 Specific volume for PENTA/BA carriers. (▨) swollen in water; (■) dry state.

Aminolysis performed for a prolong time results in the creation of water-soluble polymers, mostly for 100P (I,G,F), 80P (F), and 20P samples.

The influence of aminolysis parameters on the ionic groups concentration is tested for 40PENTA/BA copolymers. The aminolysis is carried out in solution of EtDA either in water or toluene (Table I). The carriers modified in toluene had about two times fewer ionogenic groups than derivatives prepared with water (Fig. 5). The copolymers modified in 95% EtDA water solution are characterized by a small amount of carboxylic groups, smaller than the

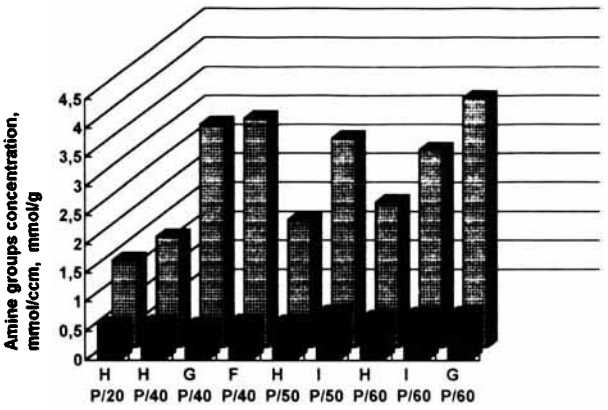

Figure 2 Amine groups concentration for PENTA/BA carriers (parameters F–I see Table I). (▨) mmol/g; (■) mmol/cm^3.

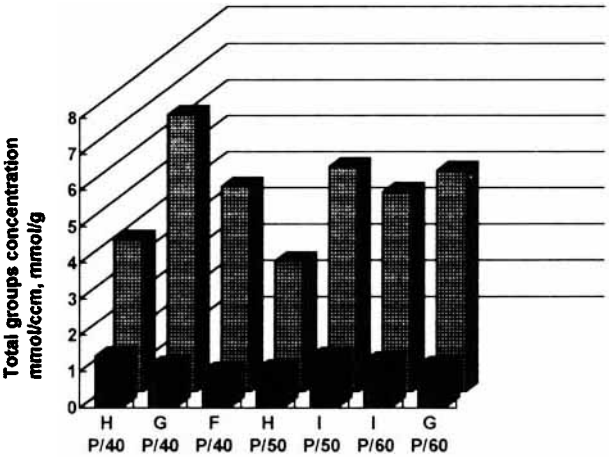

Figure 4 Total groups concentration for PENTA/BA carriers. (▨) mmol/g; (■) mmol/cm^3.

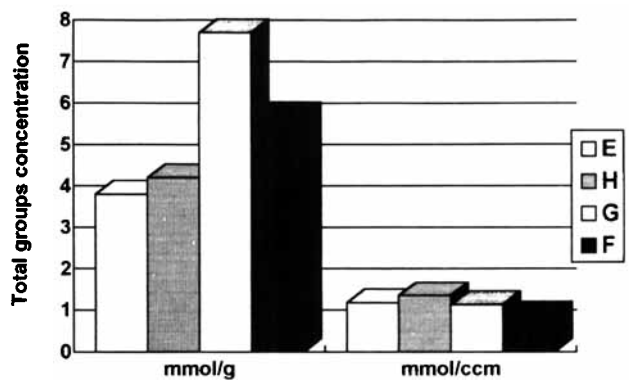

Figure 5 The influence of aminolysis parameters on total groups concentration for 40PENTA/BA carriers (parameters E–F, see Table I).

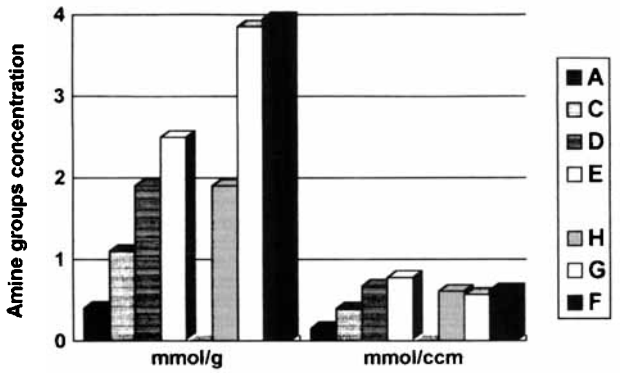

Figure 7 The influence of aminolysis parameters on amine groups concentration for 40PENTA/BA carriers.

concentration of these groups after modification in 75% EtDA–water solution. Comparison between amine and total group concentrations (in mmol/g of dry carriers and mmol/cm^3 of gels) leads to the conclusion that carboxylic groups are responsible for drastic swelling of carriers (Figs. 6 and 7). Thus the concentration of ionic groups in gel (in mmol/cm^3) is less than in the other carriers obtained with the smaller degree of aminolysis (Fig. 5). For this reason the measurement of carriers by the ISEC method is preferred.

The calculated values of gel porosity, surface area, and average pore diameter for selected 40PENTA/BA carriers are presented in Table II. Careful examination of collected data confirms the fact of a vital effect of modification conditions on carrier structures. The 40P copolymer and carriers modified in toluene solution have a porous structure with small average pore radius. With the increasing degree of aminolysis the pores of the derivatives fill up by modified copolymers. As a result, carriers with high hydrophilicity and small avarage pore radius are obtained (carrier E). During aminolysis in toluene the modification takes place only on the surface of agglomerates.

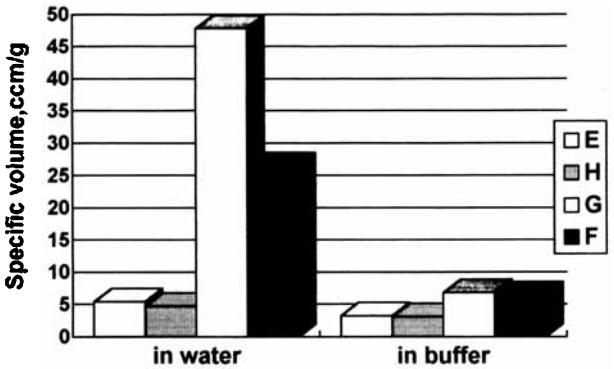

Figure 6 Specific volume for 40PENTA/BA carriers.

Aminolysis in the presance of water, which resulted in the creation of more ionogenic groups, offer an expanded gel structure like xerogel with average pore radius of about 3 nm (Table II). When the extent of hydrolysis is smaller the carriers preserve the matrix superstructure in a similar way to the carriers modified in toluene (carrier H and E) (see Table II, Figs. 6 and 7). This structure of gel type carriers was observed after aminolysis of others crosslinked by triacrylate, butyl acrylate copolymers.[19]

There was another issue to be studied before the matrices were used in some enzyme immobilization tasks. The change of volume has an influence on prepared materials. As a matter of fact the modified PENTA/BA carriers should be considered as polyelectrolytes; hence they should be sensitive to change in pH value. The phenomenon of specific volume alteration of 40P/F carrier vs. pH is shown in Figure 8. The gel has very high specific volume in water and contracts after addition of electrolite (1*N* HCl or 1*N* NaOH). The increase of volume by a factor of 2 or 3 when the pH value is changed from 5 to 7 led to the question of the reasonable standardization of obtained carriers.

In a dilute solution of phosphate buffer (0.05*M*) the change of specific volume vs. pH is comparable, but the contraction of the network under the influence of salt (NaCl 0.5*M*) is significantly higher than under the influence of acid or base (Fig. 8). The influence of phosphate buffer on volume change of carriers is seen also in Figure 8.

Immobilization of Enzymes

Immobilization of enzymes resulted in a drastic decrease in the specific volume of the enzyme–carrier

preparation. The phenomenon may be elucidated by three effects: influence of buffer, crosslinking of swollen polymer chains by glutaraldehyde, and consumption of $-NH_2$ groups. The properties of penicillin acylase–carriers preparation are listed in Table III. The table shows a few important correlations between carrier structure and preparation performances.[24,25] First, the amount of bound protein is higher for copolymers modified in a water environment, because the amine groups in gel are more accessible (v_2 = 0.04) (Table II) and a larger part of aldehyde can react with them. After this reaction the specific volume of carriers decreases but is higher (about 6.4 cm^3/g) than for carriers modified in toluene solution (about 3.2 cm^3/g). On the other hand, the diameter of enzyme molecules was so large that it was difficult for the molecules to enter the pores. Hence, the attachment was not so effective. The fact that the carrier obtained in pure EtDA (95%) (carrier F) is most prone for protein binding is out of the discussion. It has the highest volume concentration of amine groups and the lowest of carboxylic groups.

Also of note is the other relationship. Penicillin acylase is attached to some carriers to a larger extent and presents higher activity. It is assumed that the enzyme active center is not deformed.[19,26,27] The explanation for this effect needs more study. Studies are now in progress and results will be presented soon. Nevertheless, some matrices immobilized active enzyme more preferentially than others. Hence, the specific activity of such materials exceeds the activity of the enzyme used in immobilization. At first glance, there is no correlation between the preferential attachment of active enzymes and carrier structures. Comparison of amounts in systems prepared on carriers modified in water or toluene indicates that the gel structure is more attractive.

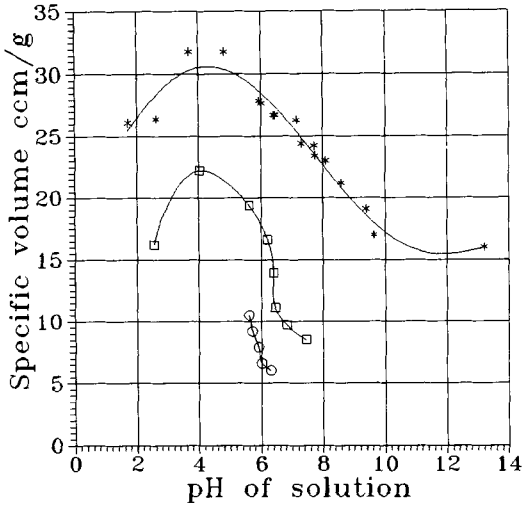

Figure 8 Specific volume for 40PENTA/BA /F carriers as a function of pH. (*) in water; (□) in 0.5*M* NaCl; (○) in 0.05*M* buffer (KH_2PO_4 + Na_2HPO_4) (9:1).

Moreover, the specific volume of gel carriers after immobilization is about twice as high than the others. Thus after immobilization, 1 g of gel carrier has close to twice as much active acylase. However, this relationship should be very carefully checked taking into account the other carrier properties. These studies are in progress.

The success in the selection of a carrier for enzyme immobilization calls for careful consideration of enzyme–carrier preparation activity and stability. Only in 2 of 13 cases did derivatives significantly lose activity after 1 month storage at 4°C (Table III). The rest of the preparations exhibited satisfactory stabilization of penicillin acylase activity.

Taking the properties of the other enzyme preparations into consideration, like peroxidase and glucoamylase (Tables IV and V), it can be concluded

Table II Characteristic of 40 PENTA/BA Carriers in Swollen State (ISEC Method)

Symbol	Amine Groups Concentration (mmol/g)[a]	Water Regain (g/g)	v_2	Porosity	Surface Area, (m^2/cm^3)	Average Pore Radius (nm)
Copolymer	—	0.71	0.96	0.33	376	2.3
C	—	0.93	0.57	0.39	408	2.2
D	—	1.30	0.56	0.45	448	2.0
E	2.41	2.34	0.38	0.60	584	1.5
H	2.97	1.92	0.34	—	—	—
G	3.89	20.9	0.04	0.94	283	3.1
F	4.42	14.6	0.06	0.95	287	3.1

[a] From N analysis.

Table III Activity of Immobilized Acylase on PENTA/BA Carriers

Symbol		Bound Protein (mg/cm³)	Enzyme Activity (U/cm³) Initial	Enzyme Activity (U/cm³) After 30 Days	Active Enzyme (%)	Specific Activity (U/mg)
20P	H	1.6	0.4	0	31.5	0.25
40P	A	3.2	1.7	1.1	66.3	0.52
	C	2.7	3.7	2.0	174.4	1.39
	D	2.9	1.7	2.0	74.9	0.59
	E	6.0	3.4	3.0	72.0	0.57
	H	1.9	2.3	2.3	—	1.21
	G	5.2	4.8	2.0	117.6	0.93
	F	13.4	12.6	8.8	119.4	0.94
50P	H	13.2	5.5	8.7	52.7	0.42
	I	6.5	7.5	6.5	146.3	1.15
60P	H	2.6	3.3	1.0	160.1	1.26
	I	3.5	3.2	—	—	1.90
	G	7.8	4.3	2.5	69.4	0.55

that PENTA/BA offers versatile carriers for immobilization purpose. Both enzyme preparations exhibit successful activity and storage stability. The activity of immobilized glucoamylase is independent of the swelling degree of the carriers and remains nearly the same for 1 month. In comparison with carriers syntethized previously, the bonding of protein to carriers was very high. Studied carriers are the most advisable for immobilization of peroxidase. This is connected with the relatively small mean pore sizes in these gel carriers in comparison with the mean pore sizes in the AN-VA-DVB carriers.[26] The amount of immobilized protein in 1 cm³ of a carrier is large and its activity is very high, the highest whenever it has been received, though the specific activity is higher on carriers crosslinked by TMPA, which bound very little active protein.[19] On some carriers with the highest swelling ratio the activity of peroxidase remains nearly the same over a month, while on porous carriers it diminishes very quickly.

CONCLUSION

The aminolysis of porous copolymers of butyl acrylate and pentaerythritol triacrylate by ethylenediamine leads to the preparation of polymer carriers with various contents of amine and carboxylic groups. The kind and amount of these groups are controlled by the condition of the process and the solvent environment during aminolysis. The prepared carriers appear mostly in the gel structure with small (about 3 nm) pore radii. This morphology imposes a high swelling ratio and sharp correlation between the pH value and specific volume.

The carriers, used in the immobilization of enzymes by the glutaraldehyde method, undergo shrinking of the polymer network, which does not suppress the dominant role of the gel fraction. Copolymers appear in the gel fraction to a large extent. Carriers exhibiting the largest swelling properties are very attractive for immobilization purposes.

Table IV Activity of Immobulized Glukoamylase on PENTA/BA Carriers

Symbol		Bound Protein (mg/cm³)	Enzyme Activity (U/cm³) Initial	Enzyme Activity (U/cm³) After 14 Days	Enzyme Activity (U/cm³) After 30 Days	Specific Activity (U/mg)
40P	F1	5.9	107.3	108.3	110.0	18.1
	F2	6.8	111.5	120.2	112.6	16.4
	F3	5.7	107.8	118.5	111.2	18.9
	G1	5.3	126.7	119.4	110.0	24.0
	G2	5.6	100.8	106.2	120.0	18.1
60P	G	6.2	113.8	118.8	105.4	18.5

Table V Activity of Immobilized Peroxydase on PENTA/BA Carriers

Symbol		Bound Protein (mg/cm^3)	Enzyme (U/cm^3 10^{-5})			Specific Activity (U/mg 10^{-5})
			Initial	After 14 Days	After 30 Days	
40P	F1	8.1	18.5	18.0	15.0	2.28
	F2	14.0	48.0	36.0	4.0	3.44
	F3	12.2	30.0	17.0	12.0	2.45
	G1	13.6	38.0	20.0	7.0	2.80
	G2	15.2	36.6	34.0	24.0	2.40
60P	G	14.3	71.3	45.0	42.0	4.97

They may be considered as versatile supports for enzyme attachment. Moreover, their stabilization of enzyme activity makes PENTA/BA copolymers of great interest to biotechnologists.

REFERENCES

1. J. E. Rosenberg and P. Flodin, *Macromolecules,* **19,** 1543 (1986); **20,** 1518; 1522 (1987); **21,** 2041 (1988); **22,** 155 (1989).
2. A. B. Schmid and P. Flodin, *React. Polym.,* **15,** 49 (1991).
3. M. Walenius and P. Flodin, *Br. Polym. J.,* **23,** 67 (1990).
4. M. Walenius, L. I. Kulin, and P. Flodin, *React. Polym.,* **17,** 309 (1992).
5. A. Schmid, M. Walenius, and P. Flodin, *J. Appl. Polym. Sci.,* **45,** 1995 (1992).
6. A. Schmid, L. I. Kulin, and P. Flodin, *Makromol. Chem.,* **192,** 1223 (1991).
7. P. Reinholdsson, T. Hargitai, R. Isaksson, and B. Tornell, *Angew. Makromol. Chem.,* **192,** 113 (1991).
8. T. Hjertberg, T. Hargitai, and P. Reinholdsson, *Macromolecules,* **23,** 3080 (1990).
9. P. D. Verweij and D. C. Sherrington, *J. Mater. Chem.,* **1,** 371 (1991).
10. M. T. Ratering, J. Meuldijk, P. Piet, and A. L. German, *React. Polym.,* **19,** 233 (1993).
11. B. N. Kolarz, M. Wojaczyńska, J. Bryjak, and B. Pawłów, *Polymer,* to appear.
12. M. Wojaczyńska and B. N. Kolarz, *Polymer,* to appear.
13. A. Matsumoto, H. Ando, and M. Oiwa, *Eur. Polym. J.,* **25,** 385 (1989).
14. T. Hargitai, P. Reinholdsson, B. Tornell, and R. Isaksson, *J. Chromatogr.,* **540,** 145 (1991).
15. P. Reinholdsson, A. Nikitidis, and C. Andersson, *React. Polym.,* **17,** 187 (1992).
16. P. Reinholdsson, T. Hargitai, R. Isaksson, A. Nikitidis, and C. Andersson, *React. Polym.,* **17,** 175 (1992).
17. B. N. Kolarz, M. Wojaczyńska, and A. Trochimczuk, *Makromol. Chem.,* **194,** 1299 (1993).
18. B. N. Kolarz, M. Wojaczyńska, J. Bryjak, and B. Pawłów, *React. Polym.,* **23,** 123 (1994).
19. B. N. Kolarz, M. Wojaczyńska, J. Bryjak, and J. Łobarzewski, *Macromol. Rep.,* **A30,** 201 (1993).
20. B. N. Kolarz, J. Jezierska, and A. Gontarczyk, *React. Polym.,* **23,** 53 (1994).
21. M. Wojaczyńska, B. N. Kolarz, D. Hlavata, J. Liesiene, and A. Gorbunov, *Makromol. Chem.,* **193,** 2259 (1992).
22. J. Bryjak, A. Trochimczuk, and A. Noworyta, *Bioprocess Eng.,* **4,** 159 (1989).
23. J. Łobarzewski, B. N. Kolarz, M. Wojaczyńska, A. Trochimczuk, and T. Błaszczyńska, *Acta Biotechnol.,* **7,** 197 (1988).
24. B. N. Kolarz, A. Trochimczuk, J. Bryjak, M. Wojaczyńska, K. Dzięgielewski, and A. Noworyta, *Angew. Makromol. Chem.,* **179,** 173 (1990).
25. J. Bryjak and A. Noworyta, *J. Chem. Technol. Biotechnol.,* **57,** 79 (1993).
26. B. N. Kolarz, A. Trochimczuk, M. Wojaczyńska, J. Liesienie, J. Łobarzewski, A. Gorbunov, and J. Bryjak, *React. Polym.,* **17,** 51 (1992).
27. B. N. Kolarz, J. Łobarzewski, A. Trochimczuk, and M. Wojaczyńska, *Angew. Makromol. Chem.,* **171,** 201 (1989).

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