

The Synthesis of UV-Sensitized Polymeric Enzyme Mimic

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UV-sensitized polymeric enzyme mimic based on vinyl monomers having cavity for β -phenyl propionic acid based esters of *p*-nitrophenol is synthesized. It involves synthesis and uv-sensitization of polymeric enzyme mimic bound to a microporous support. The activity of enzyme mimic is found to be inhibited by acylating the active site with *cis*-cinnamoyl imidazole. On irradiation it restores inhibited enzyme mimic's activity which is monitored by studying the hydrolysis profile. On irradiation *cis*-cinnamoyl is isomerized to *trans*-isomer generating free polymeric enzyme mimic. This switching off and on mechanism may be used to light-regulate catalytic activity which has potential application in photoimaging.

Introduction

Enzyme proteins have inherently associated problems such as incompatibility with organic solvents, inactivation at elevated temperature, and pH variations. Although the catalytic activity is characterized by high reaction rates and high selectivity, however, its inherent drawbacks limits its reuse. Even immobilisation on a support did not overcome the problems. Use of highly crosslinked polymeric chemical catalysts with some desirable features of enzymes, having functional groups involved in enzymatic catalysis and exhibiting selectivity due to geometric control, can overcome the inherent associated problems with bio-catalysts^{1,2}. It is formed by bringing the functional groups present in close proximity by complex formation in the presence of divalent metal ion and immobilising on microporous support². Molecular imprinting is used for creating cavities specific to molecule of which imprint has been created.

Focus is on creating artificial cavities with desirable functional groups by molecular imprinting^{3,4} as they are stable and can be reused. Optimization of geometries led to rate acceleration. Breslow¹ has reviewed polymers, which in solution act as catalysts for hydrolysis of esters and amides, however, they were difficult to recover from reaction system¹. On the other hand, highly crosslinked polymers exhibiting high catalytic activity were also synthesized. Leonhardt and Mosbach² have synthesized reactive polymers immobilized on solid

phase using molecular imprinting to create sites specific for substrates to be hydrolyzed². The cavities mimic substrate-binding properties of an enzyme, exhibited substrate specificity and increased hydrolysis rate. 4-(5)-vinylimidazole was copolymerised with divinylbenzene along with an imprint template (*N*-protected amino acids) in the presence of transition metal ions (Co^{2+}). Following elution of the template the polymeric mimic was used for hydrolysing *p*-nitrophenyl esters of the corresponding amino acids. Synthesis of highly crosslinked polymer composition adsorbed on microporous support has been reported⁵ earlier. It consisted of vinyl based polymers with appropriate functional groups which were brought in close proximity by complexation in the presence of the Co^{2+} ion and using *N*-isobutyl-6-aminocaproyl-L-phenylalanyl aminopyridine to create an active site for hydrolysis of corresponding nitrophenol substrate to corresponding alcohols and amines⁵. Polymeric enzyme mimics based on *N*-methacrylate-L-serine, *N*-methacrylate-L-histidine, and *N*-methacrylate-L-aspartic were also synthesized.

Van Deynse *et al.*⁶ have modified α -chymotrypsin to a uv-sensitive enzyme derivative by acylating active-195 residue with a cinnamoyl group or analogue. The uv-sensitized enzyme derivative was used for photographic applications. The interaction of *trans*-cinnamoyl imidazole with chymotrypsin's active site was instantaneous⁷. When exposed to uv-radiation the *trans*-cinnamoyl imidazole gave a less

reactive *cis*-isomer which formed a stable *cis*-cinnamoyl- α -chymotrypsin⁶⁻¹⁰. However, residual activity of 0.5-2 per cent remained which was blocked by using diisopropyl fluorophosphate⁶. On irradiation *cis*-isomer isomerized to *trans*-isomer which deacylated restoring enzymic activity. Substrates having close resemblance to β -phenylpropionic acid esters of *p*-nitrophenol were selected because of good interaction with the active site, higher selectivity and structural resemblance to cinnamoyl derivatives^{6,8}. Such systems were used for photographic and photoimaging applications^{6,11-12}.

The present study deals with the synthesis of uv-sensitized polymeric vinyl based enzyme mimic having imidazole group inside a cavity, which catalyse hydrolysis of a specific substrate which can fit in the imprinted cavity. It involved synthesis of uv-sensitized polymeric enzyme mimic from vinyl based monomers bound to a microporous support and having a cavity suitable for β -phenylpropionic acid based esters. This was achieved by using an imprint molecule similar to substrate and a complexing agent to obtain enzyme mimic for specific applications. This was followed by acylation of the hydroxy group in the active site with *cis*-cinnamoyl group or analogue. On irradiation *cis*-cinnamoyl isomerized to *trans*-isomer which deacylated, generating free polymeric enzyme mimic in spite of its insolubility in aqueous media, thus making it suitable alternative for practical applications. Insolubility of enzymic mimic in aqueous system does not appear to lower inhibition and reactivation levels desired for uv-sensitization.

Experimental Procedure

Materials and Methods

2-Hydroxyethylmethacrylate, ethyleneglycol dimethacrylate, glycidyl methacrylate, and 1,3-dicyclohexylcarbodiimide were obtained from Aldrich Chemical Company, USA. Methacrylic acid was obtained from Fluka Chemie, Switzerland. L-tyrosine, L-histidine, and nicotinic acid were obtained from LOBA, India. *N*-CBZ-L-tyrosine (*N*-carboxybenzyloxy-L-tyrosine) was obtained from Sigma, USA. Benzyl alcohol and polyvinylpyrrolidone were obtained from Aldrich Chemical Company, USA. The chemicals were used directly without further purification. Solvents were distilled before use. *p*-nitrophenol was recrystallized from water. Cinnamoyl imidazole was synthesized, as reported earlier¹³. *N*-

nicotinoyl-L-tyrosine benzyl ester was synthesized by esterification of L-tyrosine with benzyl alcohol using *p*-toluene sulphonic acid monohydrate followed by reacting L-tyrosinebenzyl ester with nicotinic acid using dicyclohexylcarbodiimide. *N*-CBZ-L-tyrosine-*p*-nitrophenyl ester was formed by reacting *N*-CBZ-L-tyrosine with *p*-nitrophenol using dicyclohexylcarbodiimide. *N*-methacryloyl-L-histidine was obtained by reacting L-histidine with *N*-methacryloyl chloride. Poly(glycidylmethacrylate-ethyleneglycol dimethacrylate) (3:7 w/w) copolymer was synthesized by free radical polymerization. Enzymatic reactions were carried out in buffer solution. Phosphate buffer (0.05 M) adjusted to pH 7.8 was prepared by dissolving di-sodium hydrogen orthophosphate (1.7745 g) in 250 mL distilled water and adding sodium dihydrogen orthophosphate to maintain pH at 7.8, followed by addition of sodium azide (62.5 mg).

Synthesis

Synthesis of Uninhibited Polymeric Enzyme Mimic

2-Hydroxyethyl methacrylate (HEMA) (1) (0.285 g, 2.18×10^{-3} mole), methacrylic acid (MAA) (2) (0.188g, 2.18×10^{-3} mole), *N*-methacryloyl-L-histidine (MAH) (3) (0.488g, 2.18×10^{-3} mole), *N*-nicotinoyl-L-tyrosinebenzyl ester (4) (0.825 g, 2.18×10^{-3} mole) and Co²⁺ chloride hexahydrate (0.521g, 2.18×10^{-3} mole) were taken in equimolar quantities and mixed in 10 mL methanol at room temperature for 1h to give a blue coloured Co²⁺ complex. Free radical initiator azobisisobutyronitrile (9.61 mg, 1 per cent w/w) was added, followed by addition of poly(glycidylmethacrylate-ethyleneglycol dimethacrylate) (3:7 w/w) microporous spheres (45-75 μ) in equiproportion by weight to monomers. Stirring was continued for 24 h and a blue coloured solid mass was obtained. After filtering the adsorbed mass was polymerized at 75 °C for 48 h followed by elution of Co²⁺ and the imprint molecule with methanol and dil. HCl to obtain a polymeric crosslinked enzyme mimic. The concentration of active sites based on imidazole concentration was 3.8×10^{-4} mole/g of polymeric enzyme mimic.

Inhibition of the Polymeric Enzyme Mimic

Polymeric enzyme mimic (0.100 g, 3.8×10^{-5} mole) was taken in a 100 mL conical flask. To this 24 mL 0.05 M phosphate buffer (pH = 7.8) and 24 mL photostationary mixture of *trans*-cinnamoyl imidazole (5) were added. The photostationary mixture of *cis*- and *trans*-isomer was obtained by

irradiating *trans*-cinnamoyl imidazole solution (0.02g , 1×10^{-4} mole) in acetonitrile (6 mL) and water (25 mL) with *uv* radiation (170 to 400 nm) using 400 W medium pressure Hanovia lamp (300 nm) [Photostationary mixture contained 4.55×10^{-5} mole *cis*-isomer of cinnamoyl imidazole]. The mixture was stirred in dark for 14 h and kept at 4°C overnight. The enzyme mimic was washed with acetonitrile/buffer solution, filtered, and vacuum dried. The inhibition of enzyme mimic was determined by assaying the enzyme mimic for *p*-nitrophenol released, using corresponding substrate as mentioned subsequently.

Hydrolysis Profile of Enzyme Mimic

The enzyme mimic was characterized by hydrolysing *N*-carbobenzyloxy-L-tyrosine-*p*-nitrophenyl ester (**6**) in acetonitrile/buffer ($\text{pH}=7.8$) system. Polymeric enzyme mimic (0.025g , 0.95×10^{-5} mole) was taken in a double walled reactor. To this acetonitrile (4 mL) and 0.05 M phosphate buffer (6 mL , $\text{pH} = 7.8$) were added. Further *N*-carbobenzyloxy-L-tyrosine-*p*-nitrophenyl ester dissolved in 1 mL acetonitrile was added. The contents were stirred slowly at 37°C . After every 10 min , $10\text{ }\mu\text{L}$ of the solution was withdrawn and diluted to 1 mL with acetonitrile/phosphate buffer solution. *p*-nitrophenol released was monitored by measuring absorbance at 400 nm .

Reactivation of Inhibited Enzyme Mimic

Inhibited enzyme mimic was taken in acetonitrile/phosphate buffer ($\text{pH}=7.8$) and irradiated with *uv* radiation using 400 W medium pressure Hanovia lamp (300 nm) along with stirring for 3 h . The reactivation of the enzyme mimic was determined by assaying the enzyme mimic for *p*-nitrophenol released in enzyme hydrolysis using *N*-carbobenzyloxy-L-tyrosine-*p*-nitrophenyl ester as substrate.

Result and Discussion

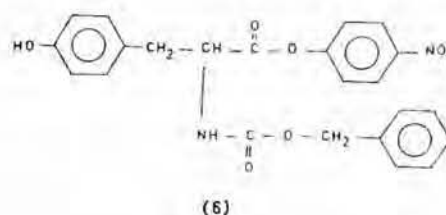
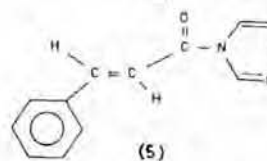
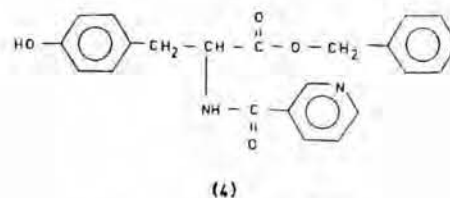
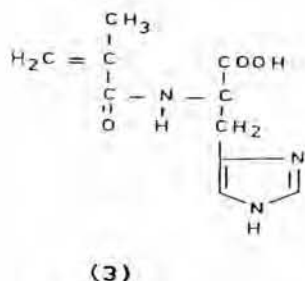
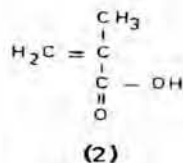
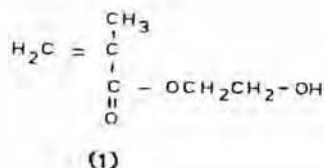
Simple chemical catalysts have been designed to achieve some desirable features of enzymes. These novel catalysts are not proteins, but they may incorporate typical enzyme catalytic groups and achieve selectivity due to geometric control of the cavity. α -Chymotrypsin used in photoimaging has many limitations. Being water soluble, it might dissolve and diffuse in processing bath, resulting in loss of sharpness of image⁶. Another drawback of α -chymotrypsin in photoimaging is overlapping of *uv*-

absorption bands of the protein and *cis*-cinnamoyl group. The sensitivity of the system can be altered by using 4-nitrocinnamoyl derivative which possesses suitable spectral properties^{10,13}. However, it is less suitable because of lack of solubility in buffer⁶.

The present work is related to the synthesis of *uv*-sensitized polymeric vinyl based enzyme mimic immobilized on a microporous support and having imidazole group inside imprinted cavity which catalyzed hydrolysis of corresponding *p*-nitrophenyl ester which can fit in the imprinted cavity. It was done by bringing the functional groups present in close proximity by complex formation in the presence of transition metal ion (Co^{2+}) and then polymerising into a crosslinked polymeric enzyme mimic. Molecular imprinting was used to create a site suitable for hydrolysis of β -phenylpropionic acid esters of *p*-nitrophenol because of close structural resemblance to cinnamic acid derivatives. The imprint molecule was eluted out to create a cavity. The resulting reactive polymer showed enzymic properties such as substrate specificity and catalytic cavity. Such a system is stable and can be reused thus offering advantage over enzyme system.

The polymeric enzyme mimic was used for obtaining a *uv*-sensitized chemical system which could be used for photoimaging applications and for controlling enzymic reactions. 2-Hydroxyethylmethacrylate (HEMA) (**1**) monomer of the polymeric mimic was acylated with *cis*-cinnamoyl imidazole to give a *uv*-sensitive enzyme mimic which when irradiated with *uv*- radiation gets deacylated because of isomerization of *cis*- to *trans*-isomer and the process could be used for switching off and on enzymatic process. The polymeric enzyme mimic was stable and could be reused several times without losing reactivity and selectivity thus offering advantage over natural enzyme systems. Moreover, the catalysis action of the enzyme mimic for the β -phenoxypionic acid based substrate was higher than other similar enzymic systems because of higher selectivity and higher reactivity.

2-Hydroxyethylmethacrylate (HEMA) (**1**), methacrylic acid (MAA) (**2**), *N*-methacryloyl-L-histidine (MAH) (**3**), and *N*-nicotinoyl-L-tyrosine benzyl ester (**4**) were mixed in equimolar quantities in methanol ($10\text{--}15\text{ mL}$) with stirring at room temperature followed by addition of equimolar Co^{2+} chloride hexahydrate, resulting in the formation of a blue complex. After 1 h stirring, free radical initiator



azobisisobutyronitrile (1 per cent w/w) was added and stirred. Polymeric microporous spheres of poly(glycidylmethacrylate-ethyleneglycoldimethacrylate) (3:7 w/w), in the range 45-75 μ and in equivalent proportion by weight to the monomers, was also added with stirring. The adsorbed mass was polymerised at 75°C for 48 h to obtain enzyme mimic, followed by eluting imprint molecule (4) and Co^{2+} with methanol and dil HCl. Active site concentration was determined by Ninhydrin test and Michaelis-Menten constants were determined by hydrolysis profile of the enzyme mimic based on *N*-carbobenzyloxy-L-tyrosine-*p*-nitrophenol (6). The active site concentration of the enzyme mimic was found to be 3.8×10^{-4} mole/g. The active site was inhibited with *cis*-cinnamoyl imidazole (slightly more than the active site concentration) in 0.05 M phosphate buffer with stirring for 3h, followed by storage at 4°C overnight. Photostationary mixture of *trans*-cinnamoyl imidazole having composition *cis*/*trans*-cinnamoyl derivative (1.4:1) was obtained by uv-irradiation of *trans*-cinnamoyl imidazole in acetonitrile/water for 3h⁶. The inhibited enzyme mimic was reactivated in 0.05 M phosphate buffer by uv irradiation for 15 min to 3 h.

The transition metal ion Co^{2+} formed a complex with pyridine, imidazole, and hydroxy groups. Following elution of template (4) after polymeri-

zation hydrolysis of *p*-nitrophenyl esters of corresponding amino acid (6) was carried out. Hydrolysis was catalyzed by imidazole group. Template (4) was used because *p*-nitrophenyl group does not complex with the transition metal.

In order to establish that combination of molecular imprinting and introduction of catalytic groups resulted in acceleration of reaction controlled reactions were carried out. Blank reactions were done by polymerization in the absence of template (4). It gave far slower rate of hydrolysis. This was because imidazole was randomly distributed. Further, with free imidazole, rate of hydrolysis was still lower. Change of amino acid also leads to lowering of catalytic activity. This concludes that mimics show specificity and catalytic activity.

Inhibition of polymeric enzyme mimic with *cis*-cinnamoyl imidazole at pH=7.8 was carried out, as mentioned earlier. The inhibition of the enzyme mimic was determined by assaying the enzyme mimic for *p*-nitrophenol released in enzymic hydrolysis of *N*-carbobenzyloxy-L-tyrosine-*p*-nitrophenol ester. Based on hydrolysis profiles of active and inhibited enzyme mimic, inhibition was found to be >96 per cent as shown in Figure1. Inhibited enzyme mimic was stable for a considerably longer duration at 4°C. Inhibited enzyme was stirred in acetonitrile/phosphate buffer (pH=7.8) and irradiated with uv radiation using

400 W medium pressure Hanovia lamp (300 nm). *Cis*-cinnamoyl imidazole was isomerized to *trans*-isomer which deacylated, thus generating free enzyme mimic. Reactivation was determined by assaying the enzyme mimic for *p*-nitrophenol released using *N*-carbobenzyloxy-L-tyrosine-*p*-nitrophenol ester. Reactivation of the inhibited enzyme mimic was found to be > 83 per cent, as shown in Figure 1.

Hydrolysis profile of the active enzyme mimic is shown in Table 1. Michaelis-Menten constants of uninhibited enzyme mimic system were found to be $K_m = 1.34 \times 10^{-3} M$ and $K_{cat} = 1.465 \times 10^3 / \text{min}$. K_{cat} for chymotrypsin is in the order of $10^2 - 10^3 / \text{s}$. The substrate *N*-carbobenzyloxy-L-tyrosine-*p*-nitrophenyl ester was taken five-times more than the active site concentration, as shown in Table 1. The concentration of the substrate was increased to 8, 10, 12 and 15-times the enzyme mimic active site concentration. The effect of increased substrate concentration on hydrolysis of the *p*-nitrophenol based substrate was studied. Figure 2, showed *p*-nitrophenol (mmole) released against time (min) at different concentrations. Increase in hydrolysis rate with increased substrate concentration as many fold, as shown in Figure 2 confirmed hydrolysis taking place inside cavities. In spite of being insoluble in aqueous solution the artificial enzyme mimic was inhibited and reactivated effectively.

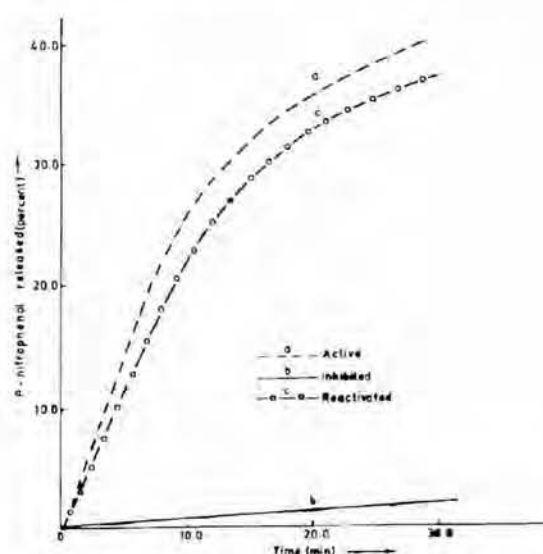


Figure 1 — Hydrolysis profile of polymeric enzyme mimic, (a) active, (b) inhibited, and (c) reactivated using *N*-CBZ-L-tyrosine-*p*-nitrophenol as substrate

Inhibition and reactivation of the enzyme mimic reported was compared with the enzyme mimic based

Table 1— Hydrolysis profile of active HEMA-MAA-MAH based enzyme mimic

Sl No	Substrate* mmole	Time, min	Absorbance at 400 nm	<i>p</i> -Nitrophenol mmol
1	0.0475	00.0	—	0.0000
		10.0	0.425	0.0117
		20.0	0.699	0.0192
2	0.0760	00.0	—	0.0000
		10.0	0.759	0.0209
		20.0	1.041	0.0287
3	0.0950	00.0	—	0.0000
		10.0	1.035	0.0286
		20.0	1.250	0.0346
4	0.1140	00.0	—	0.00000
		10.0	1.249	0.03455
		20.0	1.660	0.04593
5	0.1425	00.0	—	0.00000
		10.0	1.688	0.04679
		20.0	2.020	0.05598

*Enzyme mimic = 0.0095 mmole

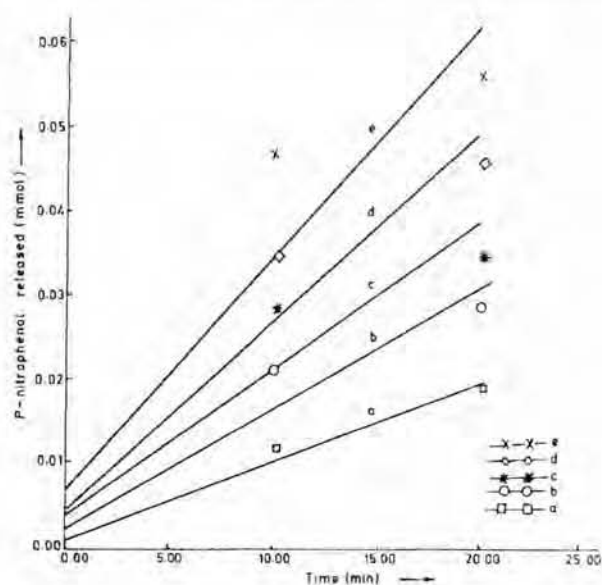


Figure 2 — Hydrolysis profile of polymeric enzyme mimic using *N*-CBZ-L-tyrosine-*p*-nitrophenol as substrate, (a) 5-times, (b) 8-times, (c) 10-times, (d) 12-times, and (e) 15-times the concentration of active site of polymeric enzyme mimic

on *N*-methacryloyl-L-serine, *N*-methacryloyl-L-histidine, *N*-methacryloyl-L-aspartic prepared under similar conditions. It had active site concentration based on imidazole concentration of 2.32×10^{-5} mole/g. The hydrolysis of the enzyme mimic using same substrate as used earlier, showed that enzymic catalysis is much lower than the system reported earlier, because of poor binding and lower reactivity in spite of high substrate concentration. To obtain reasonable amount of catalytic action substrate concentration used was 50-times and higher than the active site concentration (Table 2) Michaelis-Menten constants were found to be $K_m = 5.307 \times 10^{-4}$ M, and $K_{cat} = 10.58 \times 10^3/\text{min}$. Inhibition at pH = 7.8 was found to be >95.8 percent and reactivation was found to be >82.4 per cent. On reactivation the enzyme mimic showed $K_m = 2.487 \times 10^{-4}$ M, and $K_{cat} = 5.054 \times 10^3/\text{min}$.

The reported polymeric mimic based on monomers **1**, **2**, and **3** in spite of its insolubility, proved to be a suitable alternative for practical applications. Thus, switching off and on mechanism could be used to regulate catalytic activity. It has potential application in photoimaging, in particular, and for other enzymic reactions involving chemical catalysis.

Conclusions

It relates to the synthesis of uv-sensitized polymeric enzyme mimic bound to a microporous support by acylating the active site of the enzyme mimic having hydroxy group with *cis*-cinnamoyl derivative which on irradiation restored inhibited enzyme' mimic's activity monitored by studying the hydrolysis profile. This switching off and on mechanism could be used to regulate catalytic activity. Use of β -phenoxypropionic acid esters of *p*-nitrophenol resulted in better selectivity and reactivity because of resemblance to cinnamoyl imidazole, used for inhibition of active site. Other enzymic systems were not found satisfactory because of poor catalysis. Photoreceptive enzymatic systems using light-sensitive substrates could be employed for light-regulating the enzymatic process and for photographic applications.

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Table 2— Hydrolysis profile of methacryloyl based active and reactivated enzyme mimic

Sl No	Substrate*, mmole	Time, min	Absorbance at 400 nm		<i>p</i> -Nitrophenol, mmole	
			Active	Reactivated	Active	Reactivated
1	0.0275	0.0	—	—	—	—
		10.0	0.336	0.267	0.0092	0.0074
		20.0	0.457	0.404	0.0125	0.0113
2	0.044	0.0	—	—	—	—
		10.0	0.504	0.414	0.0140	0.0111
		20.0	0.755	0.550	0.0211	0.0151
3	0.055	0.0	—	—	—	—
		10.0	0.650	0.556	0.0179	0.0153
		20.0	0.876	0.750	0.0241	0.0206
4	0.066	0.0	—	—	—	—
		10.0	0.785	0.689	0.0216	0.0190
		20.0	0.975	0.906	0.0269	0.0249

*Enzyme mimic = 0.00058 mmole

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References

- Breslow R, *Science*, **218** (1982) 532.
- Leonhardt A & Mosbach K, *React Polym*, **6** (1987) 285.
- Andersson L I, Selligren B & Mosbach K, *Tetrahedron Lett.* (1984) 5211.
- Mosbach K & Ramstrom O, *Biotechnology*, **14** (1996) 163.
- Mashelkar R A, Kulkarni M G & Karmar R N, *US Pat. 5, 780, 578* (to Council of Scientific & Industrial Research) 14 July 1998; *Chem Abstr*, **129** (1998) 136666.
- Van Deynse D, Desie G, DeSchryver F C & Leenders L, *Biotechnol Bioeng*, **29** (1987) 403.
- Martinek K, Varfolomeyev S D & Berezin I V, *Eur J Biochem*, **19** (1971) 242.
- Bender M L, Schonbaun G R & Zerner B, *J Am Chem Soc*, **84** (1962) 2540.
- Berezin I V, Varfolomeyev S D & Martinek K, *FEBS Lett*, **8**(4) (1970) 173.
- Varfolomeyev S D, Klibanov A M, Martinek K & Berezin I V, *FEBS Lett*, **15**(2) (1971) 118.
- Kuan K N, Lee Y Y & Melius P, *Biotechnol Bioeng*, **22** (1980) 1725.
- Kuan K N, Lee Y Y, Tebetts L & Melius P, *Biotechnol Bioeng*, **21** (1979) 443.
- Schonbaum G R, Zerner B & Bender M L, *J Biol Chem*, **236** (1961) 2930.