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Peptide synthesis catalysed by papain immobilised on polymer supports: Effect of the macromolecular structure and reaction conditions on synthesis

V G JAYAKUMARI and V N RAJASEKHARAN PILLAI*
School of Chemical Sciences, Mahatma Gandhi University, Kottayam 686 631, India

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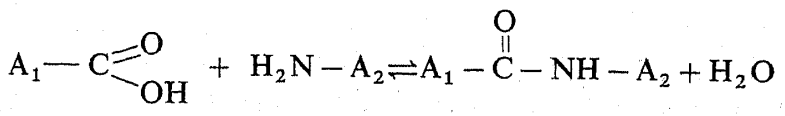
Abstract. Papain immobilised on different types of polymeric supports was used for the synthesis of peptides in aqueous–organic solvent mixtures. The effects of the nature of the polymer support, degree of crosslinking, nature and length of the spacer grouping between the polymer backbone and the point of attachment of the enzyme, and reaction conditions like pH, concentration of nucleophile and the immobilised enzyme content on the course of the synthesis were investigated. Divinylbenzene-crosslinked polystyrene, divinylbenzene-crosslinked polyacrylamide and N,N′-methylene-*bis*-acrylamide-crosslinked polyacrylamide systems immobilised with papain were used for these studies. An increase in the length of the spacer arm and an increase in hydrophilicity invariably resulted in an increase in the yield of the peptide synthesis. Papain immobilised on polystyrene–PEG supports and tetraethyleneglycol-crosslinked polystyrene supports was determined to be more efficient in effecting peptide synthesis when compared to other polystyrene-based supports.

Keywords. Immobilised enzymes; enzymatic peptide synthesis; immobilised papain; functionalised polymeric supports.

1. Introduction

The use of proteases to catalyse peptide bond formation is an interesting alternative to chemical methods (Isowa *et al* 1977). Compared to ordinary chemical methods, enzyme-catalysed reactions offer significant advantages of efficiency, chemoselectivity, regioselectivity, diasterio- and enantioselectivity (Jones and Takemura 1984; Schneider 1986). The first clearcut protease-catalysed synthesis of an amide bond was reported by Bergman and Fraenkel-Conrat (1937). It has been reported that addition of organic cosolvents shifts the peptide formation equilibria towards synthesis (Kuhl *et al* 1980; Jakubke 1987). Thus the use of a biphasic medium consisting of water and a water-immiscible organic solvent is proposed for shifting the chemical equilibrium represented below towards synthesis (Adisson *et al* 1988).



* For correspondence

Thus the synthesis can be considered as a thermodynamically controlled process. This biphasic method has undoubtedly started a novel stage in the development of synthesis of peptides (Konnecke *et al* 1981; Khmelnitski and Martinek 1984).

One major disadvantage in the use of such systems is that high concentrations of organic solvents can denature the free enzyme so that the activity is decreased (Jakubke 1987).

Moreover, the work-up of the reaction mixture is a laborious process in conventional organic synthesis. With the introduction of immobilized enzymes in organic synthesis these problems were solved to a considerable extent. Immobilization offers stability to the attached enzyme so that it is not easily denatured by organic solvents. The work-up procedure is simple and, even on the smallest scale, this route is potentially energy-saving and economical. The long term stability and simple recovery of enzymes guarantee their multiple reuse (Cramer and Horvath 1989; West and Wong 1986). Covalently bound trypsin, chymotrypsin, papain and thermolysin were used for the synthesis of specific peptide bonds (Konnecke and Jakubke 1981; Jukubke and Kuhl 1982; Khmelnitski *et al* 1984; Konnecke *et al* 1985; Jakubke and Konnecke 1987; Babonneau *et al* 1989; Schellenberger *et al* 1989).

This paper describes investigations on the use of immobilized papain for the synthesis of some model peptides. The emphasis is on the study of the effect of the different types of structurally and functionally different polymer supports used for bringing about the peptide synthesis. Ways in which the reaction conditions and the structure of the support affect the yield of peptide synthesis are investigated. Synthesies of a few peptides in water-miscible aqueous-organic solvent systems are also described.

2. Experimental

2.1 *General*

Solvents were reagent grade and were purified according to literature procedures. Microanalyses and amino acid analyses were performed at the Central Drug Research Institute, Lucknow. Melting points were determined on a hot stage melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin–Elmer model 235 B spectrophotometer. ^{1}H NMR measurement was carried out on a Jeol FX 90 Q FT NMR spectrometer using trimethylsilane as internal standard. A Toshniwal model VIS spectrophotometer was used for colorimetric studies. Thin-layer chromatography was performed on pre-coated silica gel plates.

The enzyme papain and the Boc–amino acids used were commercially available (Sigma). Acrylamide, N–N′-methylene-*bis*-acrylamide (NNMBA), divinylbenzene (DVB), tetrathyleneglycol diacrylate (TEGDA), polyethyleneglycol (PEG) and dicyclohexylcarbodimide (DCC) were purchased from Fluka Switzerland. Copolystyrene–divinylbenzene beads (1, 2, 4 and 20% crosslinked, microporous, 200–400 mesh) and Merrifield's chloromethylated polystyrene (2% DVB crosslinked) were also purchased from Fluka.

The carboxymethylpolystyrene resin 1a was prepared starting from chloromethylpolystyrene by treatment with potassium cyanide followed by acid hydrolysis (Ayres and Mann 1965; Frechet *et al* 1979). Aminomethyl polystyrenes were prepared from polystyrene–TEGDA resins and commerically available polystyrene–DVB supports of crosslink densities 1, 2, 4 and 20% by Friedel–Crafts reaction with N-chloromethyl

phthalimide followed by hydrazinolysis (Merrifield 1986). Polystyrene–TEGDA copolymer, acrylamide–DVB and acrylamide–NNMBA resins were prepared by literature procedures (Sandler and Karo 1974). Acrylamide resins were amino-functionalized by treatment with ethylenediamine (Inman and Dintzis 1969).

2.2 *Preparation of carboxyl functional resins 1a–3a, 5a from amino functional polystyrene and polyacrylamide resins: Introduction of the spacer group* $-CH_2NHCO\cdot(CH_2)_3-$*: General procedure*

A mixture of the aminomethyl resin (15 mmol) and a 4-fold molar excess of glutaric anhydride (60 mmol) was suspended in dry DMF (100 ml). The suspension was stirred at 80°C for 10 h on a water bath. The resin was filtered hot, washed successively with dimethylformamide (DMF), CH_2Cl_2 and MeOH (50 ml × 4 times) and dried to constant weight to afford the carboxyl functional resin. IR (KBr): 3500 (–NH, OH), 1720 (–C=O, acid), 1640, 1600 (C=O, amide) cm^{-1}.

2.3 *Conversion of aminofunctional acrylamide–NNMBA resin to carboxyl functional resin (6a)*

Poly (N-2-aminoethylacrylamide) (2 g, 5 mmol) was suspended in 0·1 M NaCl (50 ml). The suspension was stirred, while small portions of glutaric anhydride (20 mmol, 4-fold excess) were added. The pH of the solution was kept near 4 throughout by adding a few drops of 2 N NaOH. The mixture was stirred for 10 h. After completion of reaction, the resin was collected by filtration and washed with water until the filtrate was free from any glutarate. Finally the resin was washed with methanol, drained and dried under vacuum. Yield: 2·7 g, IR (KBr): 3400–3500 (–NH, OH), 1690 (C=O, amide), 1725 (C=O, acid) cm^{-1}.

2.4 *Preparation of carboxyl functionalized polystyrene–polyethyleneglycol graft copolymer (4a)*

Carboxylated PEG_{600} (1·6 g, 4 mmol), DCC (0·42 g, 2 mmol), and CH_2Cl_2 (20 ml) were stirred in an ice-bath at 0°C for 10 minutes. Hydroxymethyl polystyrene (Letsinger and Kalus 1964) (1 g, 2 mmol) was swelled in CH_2Cl_2 (20 ml) and added to the above solution. It was then stirred for one more hour in the ice-bath and then at room temperature for 10 h, filtered, washed with CH_2Cl_2 (20 ml, thrice), $CHCl_3$ (20 ml, twice) and methanol (20 ml, thrice). The polymer was drained and dried. Yield: 2·5 g, capacity of COOH group, 0·78 mmol/g, IR (KBr): 1110 (PEG, C–O–C) and 1740 (>C=O, ester) cm^{-1}.

2.5 *Coupling of carboxyl functional resins with papain: Preparation of immobilized papain derivatives (1b–6b): General procedure*

Carboxyl functional polystyrene resin (3·0 mmol) and DCC (0·62 g, 3 mmol) were taken in dioxane (15 ml) and stirred in an ice-bath at 0°C for 30 minutes. The enzyme, papain (200 mg) was separately dissolved in phosphate buffer (pH 7·8, 10 ml) and the clear solution was added to the pre-activated carboxyl resin in THF. The reaction mixture was stirred at 0°C for 2 h and at room temperature for a further 24 h. The resin was

filtered and washed with buffer till the filtrate was free from any unbound enzyme on testing by the ninhydrin colour reaction. Further washing of the resin was done with tetrahydrofuran (THF), dioxane, CH_3OH, CH_2Cl_2–CH_3OH mixture (1:1) and finally with CH_3OH (20 ml × 2 times) until the resin was free from any dicyclohexylurea (DCU) precipitated during the reaction. The resulting immobilized enzyme was vacuum-dried.

The amount of papain bound on the support was determined by nitrogen estimation by Kjeldahl method (Dumitriu and Popa 1986) and also by Lowry-protein measurements (Lowry *et al* 1951).

2.6 *Synthesis of a model peptide Boc–Gly–Phe–OMe in a water-immiscible organic cosolvent*

Immobilized papain (2b, 1 g, containing 20 mg of bound papain) was pre-activated using $NaBH_4$ (20 mg) after suspending in bicarbonate buffer (10 ml, pH 9) and EDTA solution (0·01 M, 2 ml). A solution of Phe–OMe·HCl (171 mg, 0·8 mmol) neutralised with triethylamine and Boc–Gly (140 mg, 0·8 mmol) in CH_2Cl_2 was added and the mixture stirred at room temperature for 24 h. The polymer was then removed by filtration and washed with CH_2Cl_2. The filtrate along with the washings was collected and washed with saturated $NaHCO_3$, citric acid solution (10%, 10 ml, thrice), saturated sodium chloride solution and finally with water. The organic layer was evaporated and triturated with anhydrous ether to obtain Boc–Gly–Phe–OMe. Yield 75 mg, m.p. 110°C, IR (KBr): 1715 (urethane), 1520, 1645, 3300 cm^{-1} (amide), ^1H NMR δ:1·5 (*s*, Boc), 6·6 (*s*, NH Gly), 4·2 (*s*, $C_\alpha H$ Gly), 3·1 (*d*, $C_\beta H$ Phe), 3·75 (*s*, OCH_3), 4·1 (*q*, $C_\alpha H$ Phe), 7·5 (*s*, aromatic Phe), 6·3 (*d*, NH Phe).

2.7 *Synthesis of Boc–Leu–Met–NH_2 in a water-miscible organic cosolvent*

A solution of methionine amide hydrochloride (185 mg, 1·0 mmol), neutralised with triethylamine, and Boc–Leu (230 mg, 1·0 mmol) in THF (10 ml) was added to the pre-activated enzyme solution and the mixture stirred at room temperature for 24 h. The polymer was removed by filtration and washed with THF (10 ml × 2 min, thrice), CH_2Cl_2 (10 ml × 2 min, thrice) and ethyl acetate (10 ml × 2 min, twice). The filtrate, together with the washings, was collected and evaporated under reduced pressure on a water bath to remove the organic solvents. The peptide was then extracted by repeatedly washing with CH_2Cl_2 and $CHCl_3$. The organic layer was washed using saturated $NaHCO_3$, citric acid (10% solution and saturated NaCl solution (10 ml × 2 times) and finally with water. The organic layer was evaporated under reduced pressure and then triturated with anhydrous ether to obtain Boc–Leu–Met–NH_2. Yield: 90 mg, m.p. 158°C. IR (KBr): 1520, 1650, 3300 (amide), 1710 cm^{-1} (urethane). ^1H NMR, 1·45 (*s*, Boc), 0·95 (*d*, $C_\delta H$ Leu), 1·7 (*m*, $C_\beta H$ and $C_\gamma H$ Leu, $C_\beta H$ Met) 2·5 (*s*, CH_3–S–Met), 3·1 (*t*, $C_\gamma H$ Met) 6·4 (*d*, NH Leu), 6·6 (*d*, NH Met).

2.8 *Synthesis of Boc–Gly–Leu–Met–NH_2 from Boc–Leu–Met–NH_2*

Boc–Leu–Met–NH_2 was suspended in 4N HCl–Dioxane and stirred at room temperature for 1 h for Boc group removal. A solution of hydrochloride of H–Leu–Met–NH_2, neutralised with triethylamine (150 mg, 0·5 mmol) and Boc–Gly (85 mg, 0·5 mmol)

was added to the pre-activated enzyme solution and stirred for 24 h. The immobilized enzyme was then removed by filtration and washed with THF (10 ml × 2 min, thrice), CH_2Cl_2 (10 ml × 3 min, twice) and ethyl acetate (10 ml × 2 min, twice). The filtrate, together with the washings, was collected and evaporated under reduced pressure on a water bath. The peptide was then isolated as described earlier to obtain Boc–Gly–Leu–Met–NH_2; Yield: 125 mg, m.p. 132°. Amino acid analysis: Gly 0·98 (1), Leu 0·09 (1), Met 1·10 (1). ^{1}H NMR 1·45 (*s*, Boc), 6·6 (*d*, NH Gly) 4·3 (*s*, C_αH Gly), 0·95 (*d*, C_δH Leu), 1·7 (*m*, C_γH, C_βH Leu and C_βH Met), 2·45 (*s*, CH_3–S Met), 3·1 (*t*, C_γH Met), 6·6 (*d*, NH Met).

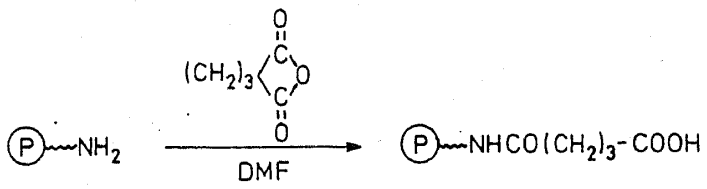
2·9 *Synthesis of Boc–Phe–Gly–Leu–Met–NH_2 from Boc–Gly–Leu–Met–NH_2*

Boc–Gly–Leu–Met–NH_2 was suspended in 4 N HCl-dioxane and stirred at room temperature for 1 h for Boc deprotection. A solution of hydrochloride of H–Gly–Leu–Met–NH_2 neutralised with triethylamine (175 mg, 0·5 mmol) and Boc–Phe (130 mg, 0·5 mmol) in THF was added to the pre-activated enzyme solution, stirred at room temperature for 24 h. The immobilized enzyme was removed by filtration and washed with THF (10 ml × 5 min, thrice), CH_2Cl_2 (10 ml × 3 min, twice) and ethyl acetate (10 ml × 2 min, twice). The filtrate together with the washings were collected and evaporated under reduced pressure on a water bath. The peptide was then isolated as described earlier to obtain Boc–Phe–Leu–Met–NH_2 Yield: 85 mg, m.p. 158°C. R_f: 0·32. Analytical details are given in Table 1. Amino acid analysis: Phe 0·95 (1), Gly 0·92 (1), Leu 0·99 (1), Met 1·02 (1). ^{1}H NMR 1·4 (*s*, Boc), 4·2 (*m*, C_αH Phe, C_αH Gly), 6·2 (*d*, NH Phe), 7·4 (*d*, aromatic Phe), 6·6 (*d*, NH Gly), 0·93 (C_δH Leu), 1·8 (*m*, C_γH and C_βH Leu, C_βH Met), 2·4 (*s*, CH_3– Met), 3·5 (*t*, C_γH Met), 6·4 (*d*, NH Met).

3. Results and discussion

3.1 *Preparation and characterisation of immobilized enzyme derivatives*

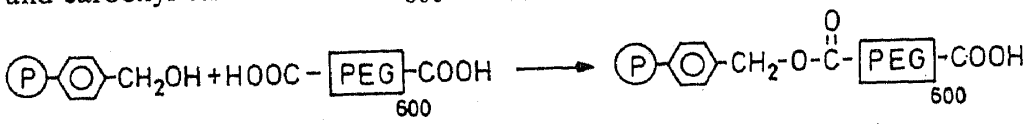
Spacer group in aminofunctional polystyrene and polyacrylamide resins were introduced by the reaction with glutaric anhydride in DMF at 80°C. The resulting carboxyl functional resins possess a $-CH_2NHCO(CH_2)_3-$ spacer in between the functional carboxyl group and the polymer backbone. The reaction is represented in scheme 1.



Scheme 1. Preparation of carboxyl functional resins from amino functional resins.

The purpose of the introduction of the spacer group is to reduce the steric effect imposed by the crosslinked polymer matrix so that the approaching macromolecular enzyme experiences lesser steric hindrance in the process of immobilization. The introduction of a spacer can also minimise the effect of the crosslinked polymer matrix on the activity of the attached enzyme.

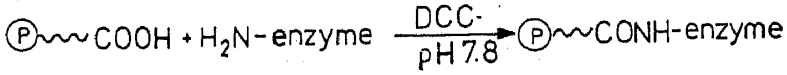
Carboxyl functionalized polystyrene-polyethyleneglycol graft copolymer was prepared by the reaction between hydroxymethylpolystyrene (2% crosslinked with DVB) and carboxyl terminated PEG_{600} as depicted in scheme 2.



Scheme 2. Preparation of carboxyl functional polystyrene–PEG graft copolymer.

The structures of various types of carboxyl functional polystyrene and polyacrylamide-based supports prepared are shown in figure 1. These supports vary in the nature and length of the spacer groups and the nature of the polymeric backbone. Structures 1a, 2a and 4a are divinylbenzene-crosslinked polystyrene supports. But 3a is a polystyrene support with tetraethyleneglycol diacrylate crosslinks, which are flexible and hydrophilic as compared to the hydrophobic and rigid divinylbenzene crosslinks. The structures 5a and 6a are hydrophilic polyacrylamide-based supports. Divinylbenzene is the crosslinking agent in 5a whereas 6a has long, flexible and hydrophilic N,N′-methylene-*bis*-acrylamide as the crosslinking agent.

These carboxyl functional polystyrene and polyacrylamide resins (1a–6a) with differences in molecular character and the extent of crosslinking were reacted with papain in phosphate buffer at pH 7·8 using DCC as the coupling reagent to get immobilized papain derivatives (1b–6b) (scheme 3).



Scheme 3. Immobilization of papain on polymeric supports through DCC-mediated coupling.

After completion of the immobilization process the resin was filtered and washed several times with phosphate buffer to remove all the unbound enzyme. The filtrate, together with the washing, was collected and the enzyme concentration was measured

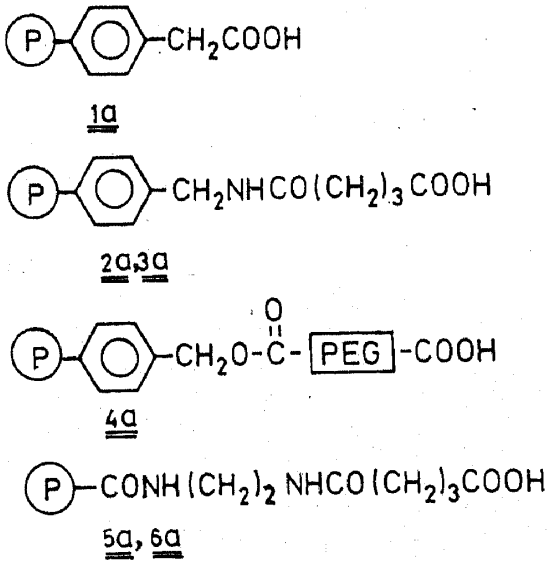


Figure 1. Structures of polymeric supports used for immobilization of papain. 1a, 2a, 4a: polystyrene–DVB support, 3a: polystyrene–TEGDA support, 5a: polyacrylamide–DVB support, 6a: polyacrylamide–NNMBA support.

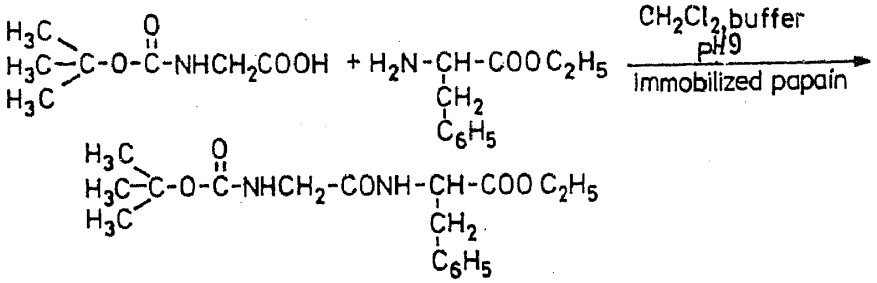
using Folin's Ciocalteu reagent at 750 nm (Lowry-protein measurements) (Allcock and Kwon 1986). The amounts of papain bound on different supports were found to be in the range 10–50 mg of enzyme/g of polymer. This was further confirmed by Kjeldahl's nitrogen estimation procedure (Dumitriu and Popa 1986). The activities of these immobilized enzyme preparations were determined by following spectrophotometrically the hydrolysis of the chromogenic substrate *p*-nitrophenyl acetate at 405 nm. They were found to retain 8–72% of the original free enzyme activity (Bender and Brubacher 1966).

3.2 *Synthesis of peptides using immobilized papain*

Substrate specificity of enzymes and advantages of immobilization were exploited in enzyme-catalysed synthesis of peptide bonds. It is reported that native papain can catalyse the formation of peptide bonds like Gly–Gly, Gly–Phe, Gly–Leu and Phe–Gly (Boyer 1971; Jakubke 1987; Adisson *et al* 1988). Here, immobilized papain was investigated for its use in peptide synthesis. Papain, immobilized on a polystyrene support (2% crosslinked with divinylbenzene) through a $-CH_2NHCO(CH_3)_3-$ spacer arm (2a), was used for the synthetic reactions. The enzyme derivative was found to contain 20 mg of papain per gram of polymer and 31% retention of original free enzyme activity, as determined from the hydrolysis of *p*-nitrophenyl acetate. Synthesis of peptides was carried out in a mixture of organic solvent and bicarbonate buffer (pH 9). The experimental part of the synthetic reaction involves stirring of the amino acid components with preactivated immobilized enzyme at room temperature. Papain, on storage, loses part of its enzymic activity due to the formation of disulphide linkages between the cysteine residues present in it. Therefore the enzyme was pre-activated using a little sodium borohydride or cysteine in the presence of 0·01 M EDTA solution. EDTA removes any metal ions which may inhibit catalytic reactions (Boyer 1971). The formation of the peptide was followed by thin layer chromatography. The procedure for the isolation of the peptide from the reaction mixture involved removal of the insoluble enzyme by filtration followed by washing with organic solvents and the usual work-up of the filtrate to obtain the peptide.

Both water-miscible and water-immiscible organic cosolvents were used for the synthesis to investigate the effect of solvents on the yield. An advantage with water-immiscible organic cosolvents is that in this case product extraction into organic phase is possible. The work-up procedure involves only removal of the resin by filtration and separation of the organic phase. The reaction medium is actually a triphasic system consisting of an immiscible organic solvent, water and the insoluble polymer support. The conditions of peptide formation resemble those of triphasic catalysis involving polymer-supported phase transfer catalysis. Boc–Val–Gly–OEt, Ac–Gly–Gly–OEt, Boc–Leu–Phe–OMe, Boc–Met–Phe–OMe, Boc–Gly–Phe–OMe and Boc–Ala–Phe–OMe were synthesised using this approach. The synthesis of Boc–Gly– Phe–OMe is represented in scheme 4.

Synthesis of the peptide Boc–Leu–Met–NH_2 was carried out in a water-miscible organic solvent. In this case, after the separation of the catalyst by filtration, the solvent was removed from the filtrate by evaporation under reduced pressure and the peptide was extracted with an organic solvent. The yields were less in this approach, but the peptide synthesis was not possible in CH_2Cl_2 or $CHCl_3$ due to solubility problems. The tripeptide Boc–Gly–Leu–Met–NH_2 was prepared starting



Scheme 4. Synthesis of Boc–Gly–Phe–OMe using immobilized papain.

Table 1. Details of preparation of some model peptides using immobilized papain.

Peptide	Solvent	Yield (%)	m.p. (°C)	R_f^a	$(\alpha)D^{30b}$	Elemental analysis (%) Found (required) (Molecular formula) C	H	N
Boc–Val–Gly–OEt 1	CH_2Cl_2	68	65–67	0·558	−8·9	55·10 (55·62) ($C_{14}H_{26}N_2O_5$)	8·30 (8·6)	9·01 (9·27)
Ac–Gly–Gly–OEt 2	CH_2Cl_2	20	118	0·428	—	47·20 (47·52) ($C_8H_{14}N_2O_4$)	6·50 (6·90)	13·70 (13·80)
Boc–Leu–Phe–OMe 3	CH_2Cl_2	30	77	0·389	−23·5	62·50 (64·28) ($C_{21}H_{32}N_2O_5$)	7·80 (8·16)	6·56 (7·14)
Boc–Met–Phe–OMe 4	CH_2Cl_2	30	55–57	0·696	−35·0	58·10 (58·53) ($C_{20}H_{30}N_2O_5S$)	7·20 (7·30)	6·40 (6·82)
Boc–Gly–Phe–OMe 5	CH_2Cl_2	28	110	0·331	+15·1	59·51 (60·71) ($C_{17}H_{24}N_2O_5$)	6·92 (7·14)	7·80 (8·33)
Boc–Ala–Phe–OMe 6	CH_2Cl_2	60	90	0·542	−22·5	59·55 (61·71) ($C_{18}H_{26}N_2O_5$)	6·99 (7·42)	7·52 (8·00)
Boc–Leu–Met–NH_2 7	THF	25	152	0·522	−27·0	52·85 (53·18) ($C_{16}H_{31}N_3O_4$)	8·40 (8·59)	11·28 (11·63)
Boc–Gly–Leu–Met–NH_2 8	THF	60	132	0·421	−26·0	51·30 (51·67) ($C_{18}H_{34}N_4O_5S$)	7·90 (8·13)	12·80 (13·40)
Boc–Phe–Gly–Leu Met–NH_2 9	THF	30	180	0·301	−32·0	56·80 (57·34) ($C_{27}H_{43}N_5O_6S$)	7·60 (7·61)	12·15 (12·38)

[a] in 9:1 Chloroform–methanol mixture

[b] determined as 1% solution in chloroform for (1, 3–6) and as 1% solution in THF for 7–9.

from Boc–Leu–Met–NH_2. The Boc–group was removed by treatment with 4N HCl–dioxane mixture. The hydrochloride of the N-terminal dipeptide was neutralised with triethylamine, Boc–Gly in THF was added and the whole stirred with immobilized papain in bicarbonate buffer (pH 9). The reaction mixture was worked up as described earlier. The tetrapeptide Boc–Phe–Gly–Leu–Met–NH_2 was prepared starting from Boc–Gly–Leu–Met–NH_2. Removal of the Boc group by treatment with HCl–dioxane followed by stirring with Boc–Gly in THF–buffer mixture in the presence of immobilized papain gave the tetrapeptide Boc–Phe–Gly–Leu–Met–NH_2. The analytical details of the peptides synthesized are given in table 1.

3.3 *Role of the support and reaction conditions on peptide synthesis*

The dependence of various parameters characteristic of the polymer support like degree of crosslinking, hydrophobic–hydrophilic nature, nature of the crosslinking agent and nature of spacer grouping on synthetic reactions was investigated. The way in which reaction conditions like pH, duration of reaction, enzyme and nucleophile concentrations affect the synthetic yield was also investigated. The synthetic model selected for these studies was Boc–Gly–Phe–OMe starting from the amino acid components Boc–Gly and Phe–OMe. Boc–Gly is the acyl donor and Phe–OMe takes the role of the nucleophile.

3.3a *Role of the support*: (i) *Effect of crosslink desnity* – In order to investigate the effect of crosslink density on peptide yield, synthesis of Boc–Gly–Phe–OMe was investigated in CH_2Cl_2-buffer mixture at pH 9 using papain supported on different crosslinked polymer supports. Papain immobilized on 1, 2, 4 and 20% crosslinked polystyrene–DVB supports ($\underline{2b_1}$–$\underline{2b_4}$) and 5, 10, 15 and 20% acrylamide–DVB supports ($\underline{5b_1}$–$\underline{5b_4}$) were used for the synthesis of Boc–Gly–Phe–OMe to study the effect of crosslink density on peptide yield.

Equimolar concentrations of amino acid components were taken and stirred with the various immobilized papains containing the same amount of bound papain.

For polystyrene–DVB resins, as the crosslink density increased from 1 to 20%, there was a gradual decrease in peptide yield. For 1% crosslinked resin ($\underline{2b_1}$), the yield was 42% whereas for 20% resin ($\underline{2b_4}$), there was no appreciable synthesis as shown in table 2. But for acrylamide–DVB resins, even though crosslinking influences

Table 2. Yield % of Boc–Gly–Phe–OMe using papain immobilized on different crosslinked polystyrene–DVB supports. (Concentration of Boc–Gly and Phe–OMe, 0·8 mmol each, immobilized papain, polymer containing 20 mg bound papin.)

Resin No.	Crosslink density (%)	Peptide yield[a] (%)
$\underline{2b_1}$	1	42
$\underline{2b_2}$	2	37
$\underline{2b_3}$	4	28
$\underline{2b_4}$	20	—

[a] Duration of reaction: 24 h

the peptide yield, appreciable yields were obtained even for the 20% crosslinked resin (table 3). Here, as crosslinking increases, the rigidity of the support increases but the hydrophobicity of the support, which has a role in catalysis involving water-immiscible organic solvents also increases. For catalysis using acrylamide–DVB resins, a maximum yield of 62% was obtained for 5% crosslinked acrylamide–DVB resin ($\underline{5b_1}$) and for the 20% crosslinked resin ($\underline{5b_4}$), the yield was 22%. A comparison of the yield obtained for various crosslinked polystyrene–DVB and polyacrylamide–DVB immobilized enzymes are presented in figure 2.

Table 3. Yield of Boc–Gly–Phe–OMe using papain immobilized on different crosslinked polyacrylamide–DVB supports. (Concentration of Boc–Gly and Phe–OMe, 0·8 mmol each, immobilized papain, polymer containing 20 mg bound papain.)

Resin No.	Crosslink density (%)	Peptide yield[a] (%)
$\underline{5b_1}$	5	62
$\underline{5b_2}$	10	50
$\underline{2b_3}$	15	33
$\underline{2b_4}$	20	22

[a] Duration of reaction: 24 h

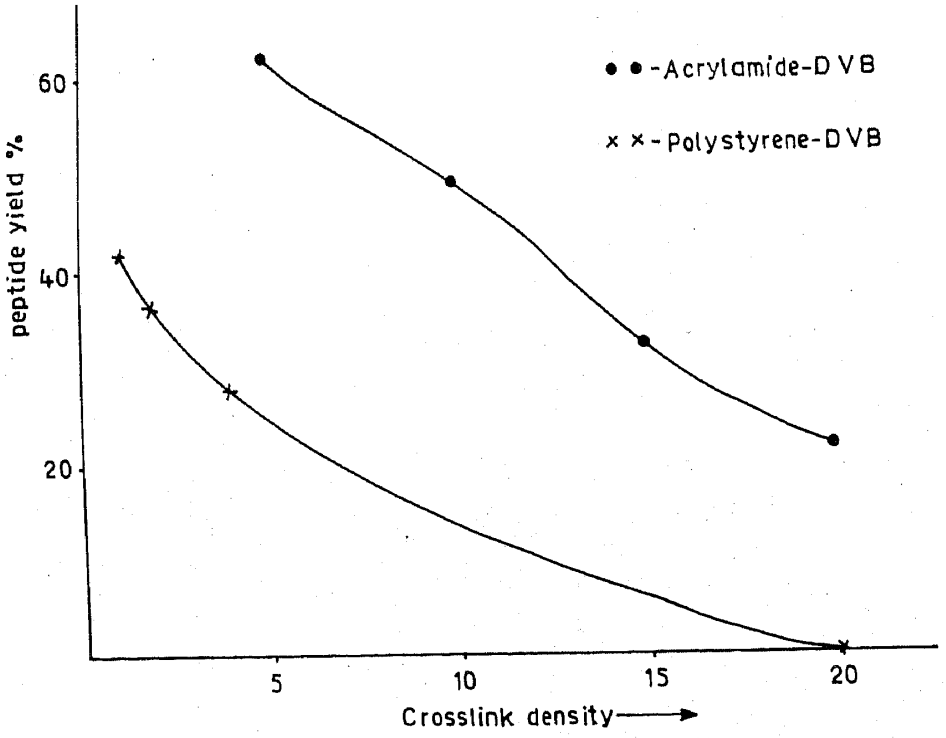


Figure 2. Effect of crosslink density on peptide yield for the synthesis of Boc–Gly–Phe–OMe using papain immobilized on polystyrene–DVB and polyacrylamide–DVB based supports.

(ii) *Influence of the nature of the polymer matrix on peptide yield* – Influence of the nature of the support on peptide synthesis was investigated using resin supports which differ in length of the spacer arm, nature of the crosslinking agent and the hydrophilic/hydrophobic nature. The synthesis of Boc–Gly–Phe-OMe from the amino acid components in CH_2Cl_2–buffer mixture at pH 9 was used as the model reaction. Papain immobilized on DVB–crosslinked polystyrene through spacer groups of varying nature and length (1b, 2b and 4b), TEGDA–crosslinked polystyrene (3b), DVB–crosslinked polyacrylamide ($5b_1$) and NNMBA–crosslinked polyacrylamide (6b) were used for the synthesis of the peptide to investigate the influence of the nature of the support on peptide yield. The crosslink density of the supports used was 5% and different weights of the immobilized enzymes containing the same amount of bound papain were taken.

When the length of the spacer arm between the polystyrene backbone and the functional carboxyl group was increased from CH_2– to $-CH_2NHCO(CH_2)_3$– the peptide yield increased from 6 to 28% (table 4). This effect can be explained on the basis of relief of steric restrictions of the crosslinked polystyrene backbone by the spacer arm. In resin (4b), there is a long hydrophilic polyethyleneoxide spacer between the polystyrene backbone and the macromolecular enzyme. The relatively high yield of peptide (50%) is quite expected as the spacer group orients the hydrophilic enzyme into the aqueous phase of the synthetic reaction where the catalytic reaction is actually taking place.

When the nature of the crosslinking agent was altered by the introduction of a more hydrophilic flexible crosslinking agent such as tetraethyleneglycol diacrylate instead of hydrophobic divinylbenzene, noticeable differences were observed in the yield of synthetic reactions. An yield of 42% was observed here for 5% crosslinked resin (3b) and this can be explained as due to the flexible hydrophilic nature of tetraethyleneglycol chains, which reduces the overall steric effects of the polymer matrix and imparts hydrophilic nature to the support.

Polyacrylamide-based supports, when investigated to study the effect of hydrophilicity of the matrix on peptide yield, also gave interesting results. DVB–crosslinked acrylamide resin (5b) gave a maximum yield of 62% whereas NNMBA–crosslinked polyacrylamide resin (6b) gave 20% yield. The relative inefficiency of polyacrylamide–

Table 4. Yields of Boc–Gly–Phe–OMe obtained using polymer supports of varying nature. (Concentration of Boc–Gly and Phe–OMe, 0·8 mmol each, immobilized papain 50 mg, polymer ≡ 20 mg of bound papain.)

Immobilized enzyme No.	Reaction time (h)	Peptide yield (%)
1b	36	6
2b	24	28
3b	24	42
4b	24	50
5b	24	62
6b	26	20

NNMBA resins in bringing about peptide synthesis reaction is quite interesting and is an example of the importance of hydrophobic–hydrophilic balance in the matrix to bring about synthetic reactions. One plausible explanation for this is offered by the mechanism of the enzyme-catalysed reactions in triphasic medium. As the amino acid components to be reacted are concentrated in the organic phase, the hydrophobic part of the polymer support appears to have some role in bringing about the phase transfer of the components for the production of the peptide in the aqueous medium and its removal into organic phase. In acrylamide–NNMBA resins there is no such hydrophobic part and the yield of the peptide is comparatively less as given in table 4.

3.8b *Effect of reaction conditions*: In order to study the effect of reaction conditions on peptide yield, synthesis of Boc–Gly–Phe–OMe using papain immobilized on a 5% crosslinked polyacrylamide–DVB support 5b was investigated under different conditions. This enzyme derivative had an enzyme content of 40 mg of enzyme/g of polymer and 62% retention of original enzymic activity as determined from the hydrolysis of the chromogenic substrate *p*-nitrophenyl acetate.

(i) *Effect of solvents* – The nature of the solvent plays an important role in enzyme-catalysed synthesis. Various organic solvents such as dichloromethane, chloroform, ethyl acetate, tetrahydrofuran, dimethylformamide and dioxane were used for these studies. The synthesis was done in a 1:1 mixture of an organic solvent and bicarbonate buffer at pH 9.

From table 5 it can be seen that peptide yields are larger with dichloromethane as the cosolvent (55%) as compared to the yield using tetrahydrofuran as cosolvent (28%). However, the effect of using a water-miscible organic solvent is not that prominent as in the case of synthesis using free enzymes. In the case of free enzymes, it is possible that there is less contact with the enzyme and the immiscible organic solvent and hence the reduction in the activity of the enzyme is less. It appears that in the case of an immobilized enzyme, the possibility of contact with organic solvent is greater due to the increased hydrophobic nature of the polymeric supports used. The advantages of using a water-immiscible system in synthetic reactions are described earlier. Here, the reaction medium actually becomes triphasic, consisting of an

Table 5. Effect of solvent on peptide yield for the synthesis of Boc–Gly–Phe–OMe using immobilized papain. (Concentration of Boc–Gly 0·8 mmol, Phe–OMe, 0·8 mmol, immobilized papain, 500 mg polymer ≡ 20 mg of bound papain.)

Solvent	Reaction time (h)	Peptide yield %
CH_2Cl_2	18	55
$CHCl_3$	18	50
Ethylacetate	18	48
THF	24	28
DMF	24	25
Dioxane	24	20

immiscible organic solvent, water and the insoluble polymer support. The conditions of peptide formation resemble those of triphasic catalysis involving polymer supported phase transfer catalysts. Hydrophobic polymer support is concentrated in the organic phase while the hydrophilic enzyme part is oriented in the aqueous medium. The enzyme catalyses the formation of peptide bonds in the aqueous medium which is then transferred to the organic solvent. This increased product concentration in the organic media drives the reaction equilibria towards synthesis, in the presence of a higher concentration of the nucleophile.

As shown in table 5 peptide yields are lower when DMF, THF or dioxane (water-miscible cosolvents) are used (28, 25 and 20% respectively) as compared to dichloromethane, chloroform or ethyl acetate (55, 50 and 48% respectively). Thus the situation arising in a triphasic system, i.e., product extraction into organic layer is more favourable for the formation of a peptide.

(ii) *Effect of pH* – In order to study the effect of pH on peptide synthesis, equimolar amounts of the amino acid components were taken and papain (5b), immobilized on 5% crosslinked polyacrylamide–DVB support was used as the catalyst. The synthesis was carried out in CH_2Cl_2– buffer mixture in pH range 6–11. The yield of the peptide was found to increase with increasing pH up to 9. A maximum yield of 62% was obtained at pH 9 after 24 h as shown in figure 3. On increasing the pH to 11 the yield decreased to 25%. Thus there are appreciable yields even at higher pH values whereas free enzymes are completely inactivated at highly alkaline pH. Thus immobilization protects the enzyme from inactivation.

(iii) *Effect of duration of reaction* – The effect of the extent of duration on the synthesis of the peptide Boc–Gly–Phe–OMe using the immobilized papain (5b) was studied in CH_2Cl_2– buffer mixture at pH 9. The time required for the maximum conversion (62%) was found to be 24 h. The yields of the peptide after definite intervals

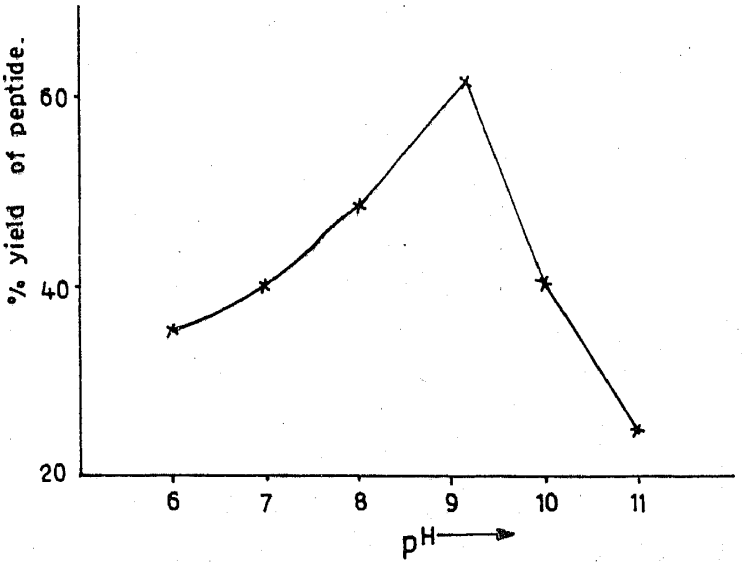


Figure 3. Effect of pH on peptide yield for the synthesis of Boc–Gly–Phe–OMe (concentration of Boc–Gly and Phe–OMe, 0·8 mmol each, immobilized papain 500 mg polymer ≡ 20 mg of bound papain).

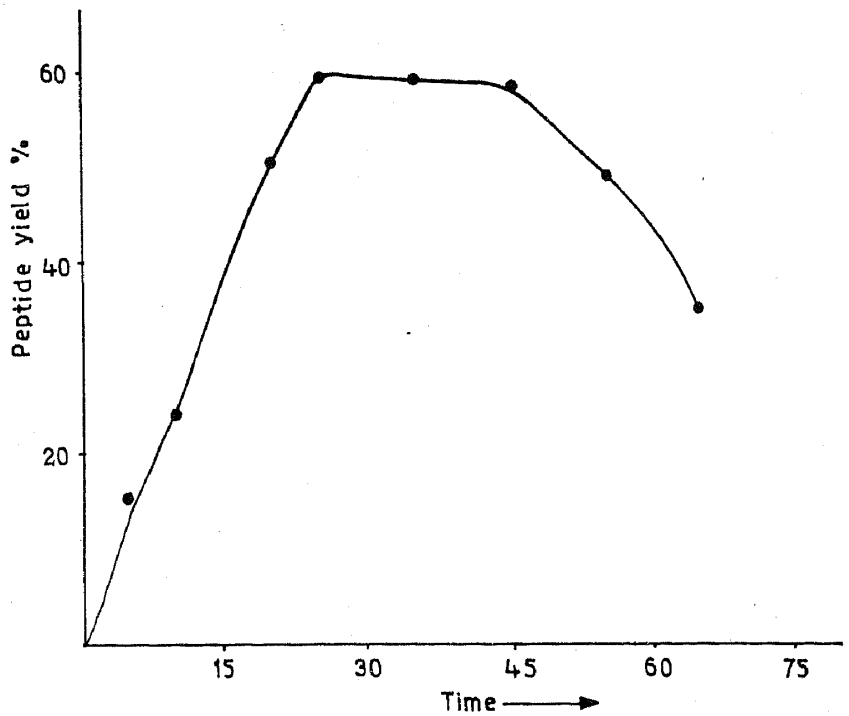


Figure 4. Effect of duration of reaction on peptide yield for the synthesis of Boc–Gly–Phe–OMe (concentration of Boc–Gly and Phe–OMe, 0·8 mmol each, immobilized papain 500 mg polymer ≡ 20 mg of bound papain).

were determined. From figure 4 it can be seen that the yield goes on decreasing after prolonged periods of reaction. From tlc it was clear that concentration of starting compounds increase after 48 h. Thus, on prolonged reaction, there are chances of secondary hydrolysis.

(iv) *Effect of nucleophile concentration* – Concentration of nucleophile plays a role in determining the yield of peptides. High nucleophile concentration is a criterion for driving the reaction equilibria towards synthesis. In the synthesis of Boc–Gly–Phe–OMe, Phe–OMe is the nucleophile. Various concentrations of Phe–OMe were used to study its effect on the reaction. Boc–Gly (0·8 mmol) and varying concentrations of Phe–OMe were used to study its effects on the reaction. The enzyme used was papain immobilized on 5% crosslinked acrylamide–DVB (5b). The reaction was conducted in CH_2Cl_2–buffer mixture at pH 9. As shown in figure 5, the peptide yield goes on increasing upto a nucleophile concentration of 1·6 mmol. No noticeable change was observed after that. Maximum yield was observed with a 4–fold molar concentration of nucleophile with respect to the carboxyl components.

(v) *Effect of concentration of the immobilized enzyme* – Effect of concentration of enzyme on the yield of Boc–Gly–Phe–OMe was studied in CH_2Cl_2–buffer mixture at pH 9. Equimolar concentrations of Boc–Gly and Phe–OMe were used and the enzyme used was papain immobilized on 5% crosslinked polyacrylamide–DVB support (5b). This resin contains 40 mg of bound enzyme/g of polymer. 50, 100, 300, 500, 800 and 1000 mg of the enzyme-immobilized polymers were taken for studies so that it contained 2, 4, 12, 20, 32 and 40 mg of bound enzyme. The yield was only 10%

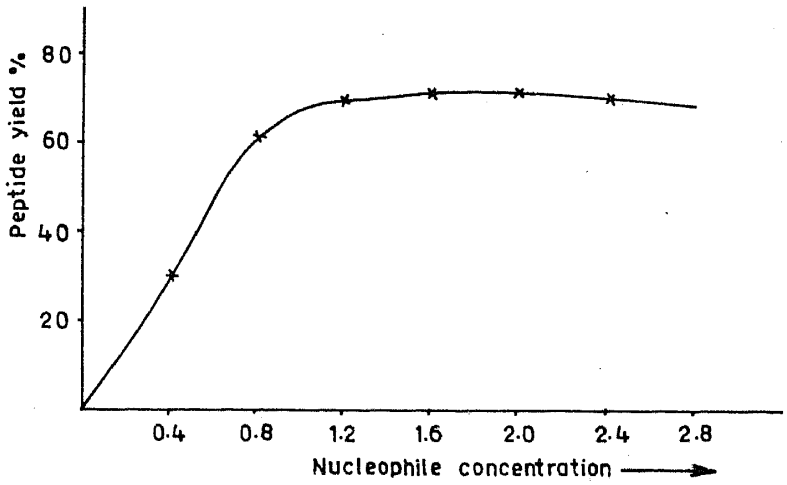


Figure 5. Effect of nucleophile concentration on peptide yield for the synthesis of Boc–Gly–Phe–OMe (concentration of immobilized papain, 500 mg polymer ≡ 20 mg of bound papain).

Table 6. Yield % of Boc–Gly–Phe–OMe using various concentrations of immobilized papain (Concentration of Boc–Gly and Phe–OMe, 0·8 mmol each.)

Wt. of polymer (mg)	Enzyme content (mg)	Peptide[a] yield (%)
50	2	10
100	4	29
300	12	50
500	20	60
800	32	60
1000	42	60

[a] Duration of reaction: 24 h.

with 50 mg of polymer. But as the concentration was increased to 500 mg polymer (= 20 mg of bound enzyme), 60% yield was obtained. Beyond this concentration there was no noticeable change in peptide yield. The yields of peptides obtained for various enzyme concentrations are given in table 6.

Studies on the synthesis of model peptides using papain immobilized on different synthetic functional polymers reveal the effect of the macromolecular support on the synthetic efficiency of the enzyme preparation. The yields were found to be greater when the length of the spacer arm was increased in the case of the polystyrene supports. An increase in hydrophilicity also increased the yield of the peptide. Thus, the yields were larger in the case of peptide synthesis using papain immobilized on acrylamide–DVB supports. Also, papain immobilized on polystyrene–PEG supports and polystyrene–TEGDA supports were found to be more efficient in effecting peptide synthesis when compared to other polystyrene-based supports. Degree of crosslinking and reaction conditions significantly influence the synthetic yield of the peptide.

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