CHAPTER IV

SYNTHESIS OF MODEL PEPTIDES AND BIOACTIVE PEPTIDES USING THE NEW SUPPORTS

In order to assess the suitability of the new resin supports in peptide synthesis, model peptides were synthesised following standard solid phase methodology. From the reactivity studies based on model aminolysis reaction, it was found that 20% DVB-crosslinked poly(acrylamide) was the most suitable in terms of percentage coupling. Three model syntheses were carried out with the DVB-crosslinked amino resins. The major objective of these syntheses was to observe the extent of attachment of the anchoring group, the first amino acid, coupling of individual Boc-amino acids and subsequent deprotection as well as final cleavage of the peptide from the resin support.

IV. 1. Preparation of 4-bromomethyl benzoyl aminoethyl poly(acrylamide) resin

4-bromomethyl benzoic acid was prepared from 4-methyl benzoic acid following literature procedure. This was attached to amino resin derived from resin D4 in dichloromethane using DCC as the condensing agent (Scheme IV. 1). The extent of coupling was ascertained by the semiquantitative ninhydrin test.
Scheme IV. 1. Attachment of bromomethyl benzoic acid anchoring group to N-2-\textsuperscript{2}aminoethyl poly-(acrylamide) resin

The bromine content of the resulting bromo resin was estimated by pyridine fusion followed by the modified Volhard's method of bromine estimation\textsuperscript{199}. The bromine substitution was found to be very low and therefore coupling with the anchoring group was repeated thrice to improve the substitution level. A drop of pyridine was added to the reaction mixture as a basic catalyst which was found to have a positive effect. The final substitution level was 0.40mmoles/g. However, the resin still contained free amino groups detected by the ninhydrin test. These residual amino groups were capped\textsuperscript{135} by acetylation using a mixture of acetic anhydride and triethylamine (TEA).

V. 2. Synthesis of Gly-Ala

The first amino acid Boc-Ala was esterified to the blocked resin in ethyl acetate in presence of TEA for 44h under nitrogen. The Boc-protecting group was removed by
treatment with HCl/dioxane (4N) followed by neutralisation and subsequent washing. Positive ninhydrin test showed removal of Boc groups. The second amino acid Boc-Gly was then coupled to the amino acyl resin in dichloromethane at room temperature for 4h. The anhydride of Boc-Gly was prepared in presence of dicyclohexylcarbodiimide (DCC): dicyclohexyl urea (DCU) was removed by filtration and the filtrate was added to the resin for coupling. Boc group was deprotected using HCl/dioxane (4N) followed by neutralisation with TEA. The dipeptide was cleaved from the resin support using neat trifluoroacetic acid (TFA) at $37^\circ$C for 36h. The peptide was precipitated by ice-cold ether and isolated. The synthetic sequence is represented in Scheme IV. 2.

Scheme IV. 2. Synthesis of model peptide Gly-Ala (G A)

The model synthesis of a tetrapeptide was done using the aminoethylacrylamide resin crosslinked with 20% DVB. The bromine capacity of the resin was estimated after anchoring the amino resins with 4-bromomethyl benzoic acid. Bromo resin of capacity 0.15mmole /g was used for the synthesis. The residual amino groups were blocked by acetylation. Boc-Gly was esterified to the bromo resin through the triethyl ammonium salt. Boc group was deprotected using 4N HCl/dioxane mixture. The second Boc amino acid, Boc-Gly was coupled to the aminoacyl resin through the formation of its anhydride using DCC in DCM. The coupling was done for 1h under nitrogen. The efficiency of coupling was monitored by the ninhydrin test. To ensure completion of coupling, a second coupling was also performed. However, the coupling was sluggish and the free amino groups were capped by acetylation. The Boc group was deblocked and the next amino acid Boc-Ala was coupled by the same method. After Boc-deprotection, the fourth amino acid Boc-Ala was coupled. At the end of the assembly of the amino acids, the peptide resin was cleaved using neat TFA at 40°C for 36h. The crude peptide was precipitated using ice-cold ether. The crude peptide was characterised by TLC using butanol/acetic acid/water (4: 1: 1: V/V) mixture. The entire synthetic procedure is outlined in Scheme IV. 3.
Scheme IV. 3. Synthesis of Ala-Ala-Gly-Gly by solid phase method

IV. 4. Synthesis of Phe-Leu-Leu

The suitability of 4% TEGDMA-crosslinked polystyrene resin was tested by a model synthesis of a tripeptide Phe-Leu-Leu. The chloromethylated resin of chlorine capacity of 1.06mmol/g was used for the synthesis. The
first amino acid Boc-Leu was esterified to the chloromethyl resin by the cesium salt method of Gisin\textsuperscript{299}. The loading level of Boc-Leu was found to be 0.4mmol/g by the picric acid method\textsuperscript{300}. The next amino acid Boc-Leu was coupled to the aminoacyl resin using DCC in DCM. The extent of coupling was monitored by ninhydrin test. Two couplings were necessary for completion of the reaction. Leu-Leu, coupling however, was slow and was therefore done thrice. The synthetic protocol is depicted in Scheme IV. 4.

\begin{scheme}
\begin{align*}
\text{Boc-} & \text{NH-} \text{CH-} \text{C-} \text{O-} \text{Cs} + \text{Cl-CH}_2 \text{CH}_2 \text{O} \\
\text{DMF, 50}^\circ \text{C, 40 h} & \\
\text{Boc-} & \text{NH-} \text{CH-} \text{C-} \text{O-CH}_2 \text{CH}_2 \text{O} \\
\text{30\% TFA/DCM, 5\% DIEA/DCM} & \\
\text{Boc-Leu-} & \text{OH} + \text{H}_2\text{N-CH-C-O-CH}_2 \text{CH}_2 \text{O} \\
\text{33\% EtOH/DCM, DCC/DCM} \quad & \text{1 h} \\
\text{Couple with Boc-Phe} & \\
\text{Boc-Phe-Leu-} & \text{CO-NH-CH-C-O-CH}_2 \text{CH}_2 \text{O} \\
\text{TFA, 40}^\circ \text{C, 36 h} & \\
\text{Phe-Leu-Leu} & \\
\text{R=CH}_2 \text{-CH-} \{(\text{CH}_3)_2 \}
\end{align*}
\end{scheme}

Scheme IV. 4. Solid phase synthesis of Phe-Leu-Leu on 4% PS-TEGDMA chloromethyl resin
The amphiphilic PS-TEGDMA resin support provided excellent coupling conditions in the swollen state. The gel-like swollen resin permitted smooth permeation of solvents and reagents into the resin core and brought about near-quantitative coupling. The flexibility of the crosslinks and the rigidity of the matrix are factors responsible for the increased efficiency of the synthesis. When the assembly of the amino acids was over, the peptide was cleaved from the support using neat TFA. The solvent was evaporated off and the peptide precipitated by the addition of ice-cold ether. The crude peptide was characterised by TLC and amino acid analysis. The solvent system used was butanol/ acetic acid/ water/ pyridine (4: 1: 1: 1).

Synthesis of biologically relevant sequences

The feasibility of the application of the DVB-crosslinked poly(acrylamide) resins in the synthesis of longer peptide sequences was tested by the synthesis of biologically relevant sequences. The impetus for the synthesis was the fact that the model synthesis worked although the yield was poor. An attempt was made to synthesise a 13-residue bioactive peptide on 20% DVB-crosslinked poly(acrylamide).
IV. 5. Synthesis of Crabrolin - a peptide toxin -  
Phe-Leu-Pro-Leu-Ile-Leu-Arg-Lys-Ile-Val-Thr-Ala-Leu  
(F L P L I L R K I V T A L)

Crabrolin is a 13-residue toxic peptide isolated from European hornets. The primary sequence of crabrolin is Phe-Leu-Pro-Leu-Ile-Leu-Arg-Lys-Ile-Val-Thr-Ala-Leu. It shares a variety of properties with mellitin. It has the ability to lyse erythrocytes and elicit release of histamin from mast cells. The native tridecapeptide is capable of forming amphiphilic alpha-helix. This lytic peptide like seminalplasmin forms pores in membranes allowing the passage of aqueous ions through the hydrophobic membrane bilayers.

The synthesis of crabrolin was attempted on 20% DVB-crosslinked poly(acrylamide) support. N-2-aminoethyl functionalised poly(acrylamide) was anchored with 4-bromo methyl benzoic acid and the bromine capacity determined. The resin with 0.4mmol/g bromine capacity was employed for the synthesis. The first amino acid Boc Leu was attached to the bromo resin by Gisin's method. The level of substitution was estimated by Gisin's picric acid method and was found to be 0.4mmol/g.

Boc benzyl strategy of peptide synthesis was followed. The algorithm of the synthesis of crabrolin is represented in Scheme. IV. 5. A 3-fold molar excess of each protected amino acid was used for each coupling. DCM was used throughout as the solvent for the synthesis. The side
DCC/DCM

---CH₂-CH₂-CH₂-CH₂-CH₂-CH---

C=O  C=O
NH₂  NH₂
NH₂  NH₂
C=O  C=O

---CH₂-CH₂-CH₂-CH₂-CH---

Scheme IV. 5. Solid phase synthesis of crabrolin on a DVB-crosslinked poly(acrylamide) support

Boc-Leu-OH+Br-CH₂-OH → CO·NH·(CH₂)₂CO

Cs CO₃, DMF → 42h, 50°C

Boc-Leu-O-CH₂-OH → CO·NH·(CH₂)₂CO

30% TEA/DCM → 5% DIEA/DCM, 30min

H₂N-Leu-O-CH₂-OH → CO·NH·(CH₂)₂CO

DCC/DCM → Boc-Ala/DCM, 1h

Boc-Ala-Leu-O-CH₂-OH → CO·NH·(CH₂)₂CO

11 Cycles of deprotection, neutralisation and coupling

Ile-Val-Thr (OBz)₆-Ala-Leu-O-CH₂-OH → CO·NH·(CH₂)₂CO

Mts C1z₈

Boc-Phe-Leu-Phe-Leu-Ile-Leu Arg-Lys-

TFA, 50°C, 24h

Phe-Leu-Phe-Leu-Ile-Leu-Arg-Lys-Ile-Val-Thr-Ala-Leu

Prepared by BeeHive Digital Concepts for Mahatma Gandhi University
chains of the following amino acids were protected: Boc(Z)Thr, Boc(ClZ)Lys and Boc(Mts)Arg. Boc-deprotection was achieved by 30% trifluoroacetic acid in DCM for 30min and neutralisation by 5% diisopropylethylamine (DIEA) in DCM. Couplings were effected by DCC in DCM. Whenever coupleings were incomplete in the first stage, they were repeated twice or thrice to ensure the completion of the reaction. Three coupleings were given for valine, isoleucine, threonine and arginine. The usual coupling time was 30 minutes and the progress of coupling was checked at every step by semiquantitative ninhydrin test. When the assembly of the peptide was over an aliquot of the peptidyl resin was hydrolysed and subjected to amino acid analysis to find out whether all the amino acids have been incorporated into the chain. All residues were found to be present in the chain. Another portion was cleaved using neat TFA at 50°C for 24h in the presence of thioanisole and m-cresol. The peptide was precipitated by ice-cold ether, washed free of scavenging reagents and dried. The peptide was identified by TLC using butanol/acetic acid/water mixture (4: 1: 5, V/V).

A methanolic solution of the crude peptide was checked by FPLC for its purity. Solvent A consisted of water containing 0.1% TFA and solvent B consisted of acetonitrile containing 0.1% TFA. The FPLC profile is given in Fig. IV. 1.
Fig. IV. 1. FPLC profile of crude crabrolin

The peaks were not collected and analysed as the concentration of the peptide was very low even when 100 microlitres of the methanolic solution was injected.
IV. 6. Problems encountered in the synthesis

Incomplete coupling: The very low yield of the peptide was the major problem of the synthesis, which can be due to poor and incomplete coupling. But the FPLC profile gave only 2 peaks and the amino acid analysis of the peptidyl resin showed the incorporation of all the residues. However, the amino acid ratios obtained from the analysis of the peptidyl resin are not agreeable with the expected values. Generally low values were obtained. This may be due to the improper or incomplete coupling of those amino acids which are repeated in the sequence.

Low substitution levels of anchoring groups: The low yield can also be attributed to the low substitution levels of the resin. The amino resin on anchoring with 4-bromomethyl benzoic acid gave very low substitution as revealed by the bromine capacity values. Attempts were also made to attach the anchoring groups as its active acid chloride, 4-bromomethyl benzoylchloride. But there was no improvement of the earlier substitution levels.

Low first amino acid incorporation: The bromine capacity got further reduced when the first amino acid was incorporated. A comparison of the Gisin's method and the triethylamine salt method of first amino acid incorporation clearly suggests that the substitution is enhanced in the latter method. An esterification catalyst dimethylaminopyridine (DMAP) was also tried in the esterification
steps with slight enhancement of the substitution level. DMAP has been used as a catalyst for the esterification of the first amino acid on to the resin in Fmoc-based solid phase peptide synthesis.\(^\text{303}\)

The above observations suggest that the relatively low reaction rates of the resin was associated with some inherent problem of the acrylamide-based supports. It has been reported that the support structure influences the reaction rates tremendously.\(^\text{218}\) A second possibility is the incompatibility of the anchoring group with the polar support. Another anchoring group capable of forming an acid cleavable resin-peptide linkage could perhaps improve the substitution level considerably. This requires further investigations. The relative difficulty in establishing an ester bond compared to an amide bond has been reported by Merrifield et al.

IV. 7. Synthetic attempts with other acrylamide-based supports

Attempts were made to check the feasibility of other acrylamide-based supports described earlier as possible supports by incorporating Boc-Gly as the first amino acid with no C-alpha side chains. NNMBA-, TTEGDA- and other DVB-crosslinked supports were tried. These studies indicate the strong influence of the support on the reactivity of the functional groups towards the coupling of the anchoring
group as well as the attachment of the first amino acid. The results of these studies are summarised in Table IV. 1.

Table IV. 1. Comparative study of the loading capacities of the crosslinked poly(acrylamide)s

<table>
<thead>
<tr>
<th>Amino cross cross amino</th>
<th>bromine method</th>
<th>time (h)</th>
<th>Gly substitution (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>resin links link from</td>
<td>capacity (%)(mmol/g)</td>
<td>capacity used (mmol/g)</td>
<td></td>
</tr>
<tr>
<td>D1 DVB 5 2.8 0.85 TEA*</td>
<td>60</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>D2 DVB 10 3.8 0.76 TEA*</td>
<td>70</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>D3 DVB 15 2.8 0.6 TEA*</td>
<td>60</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>A1 NNMBA 15 3.4 0.98 TEA*</td>
<td>75</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>B2 TTEGDA 20 5.4 0.30 Gisin</td>
<td>70</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>C4 TEGDMA 20 4.1 0.91 Gisin</td>
<td>70</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

* DMAB was added as a catalyst in trace amounts in all TEA methods

All DVB-crosslinked poly(acrylamide)s were put to synthetic tests because of their higher coupling yields in the model aminolysis reaction. Other resins tested were also those that gave higher coupling efficiencies. However, all the resins were found to be unsuitable for the actual synthesis because of very poor loading levels of the anchoring group as well as that of the first amino acid even after prolonged esterification for 70-75h.

IV. 8. Gisin’s method vs. triethylammonium salt method for the first amino acid attachment

In order to study the influence of the esterification conditions and to optimise them attempt was made to attach
the first amino acid under different esterification conditions. The poly(acrylamide) resins showed better amino acid substitution level in the triethylammonium salt method of esterification. A comparative study of the rates of esterification of Boc-Gly to 20% DVB-crosslinked poly-(acrylamide) by the two methods are given in Fig. IV. 2.

Fig. IV. 2. Loading levels of Boc-Gly on poly (N-2-aminoethylacrylamide) resin derived from resin D4 under different esterification conditions. (a) 50 °C  (b) 70 °C

The above observations suggest that Boc-Gly esterification onto amino resin from D4 proceeds best with triethylamine in presence of a trace of DMAP catalyst in ethyl acetate solvent. Esterification in the absence of the catalyst also gave higher loading levels than the cesium salt method of Gisin. However in the case of polystyrene resins, it was found that Gisin's method often gave better substitution. Stahl et al.\textsuperscript{179} have determined the substitution levels of various Boc-amino acids to the
4-(oxymethyl)phenyl acetamido poly(acrylylpyrrolidine)-[OMPA-PAP]-resin. Barlos et al.\(^4\) determined the loading of various Fmoc amino acids on 2-chlorotrityl chloride resin.

The compatibility of the polar poly(acrylamide) support with the ethyl acetate may be responsible for the increased loading level in the triethylamine method.

**IV. 9. Attempt to attach the anchoring group as its acid chloride**

Since the extent of coupling of 4-bromomethyl benzoic acid to the amino poly(acrylamide) resin was sluggish, an attempt was made to attach the anchoring group as its more reactive acid chloride derivative - 4-bromomethyl benzoyl chloride. 4-bromomethyl benzoyl derivative of poly(ethylene glycol) [PEG] has earlier been used as a soluble support for peptide synthesis\(^3\). 4-Bromomethyl benzoyl chloride was prepared from the corresponding acid by treatment with thionyl chloride. The acid chloride formed was attached to the amino resin and the bromine capacity estimated. The substitution level was not very much improved.

These attempts to increase the substitution levels by increasing the duration of the reaction, repeating the coupling reaction 2-3 times, and modifying the reagent function to improve its reactivity characteristics did not bring about any substantial improvement.
IV. 10. Inherent problems with the poly(acrylamide)-based supports

The lack of reactivity of the poly(acrylamide) resins may be due to the nonavailability of the functional carboxamide group which could be engaged in the formation of intramolecular hydrogen bonding. It has been reported that the monomer acrylamide has a strong tendency to undergo intermolecular H-bonding. In the polymer chain the pendant carboxamide groups are in close proximity such that the carbonyl oxygen can enter into intramolecular H-bonding with the amide nitrogen. A hypothetical situation is represented in Fig. IV. 3.

**Fig. IV. 3. Intramolecular H-bonding in crosslinked poly(acrylamide) [--- crosslinks]**

Further support to establish the existence of H-bonds in crosslinked poly(acrylamide)s is obtained from the IR spectra of the copolymers. IR (KBr): 1680 cm\(^{-1}\) (N-H bend); 1720 cm\(^{-1}\) (C=O str); 3400 cm\(^{-1}\) (N-H str); (Fig III. 1, 2, and
3). These absorptions are characteristic of the H-bonded CO and NH groups which will deprive the primary amide group of its expected reactivity.

IV. 11. Crosslinked terpolymers of acrylamide and N, N-dimethylacrylamide

These terpolymers were prepared with a view to preventing the H-bonding tendency of poly(acrylamide)s by introducing dimethylacrylamide residues at alternate positions. The separation between the carboxamide groups would be less favourable for such H-bonding. Dimethylacrylamide contains no amide hydrogens. It was anticipated that this terpolymer would be more reactive. However capacity and reactivity determinations showed that they are not very much different from the binary copolymers. The desired effect of dimethylacrylamide acting as a spacer separating the carboxamide groups of the chain was not observed. This was particularly due to the fact that during the random copolymerisation process, the distribution of the chains in the polymer may not be uniform. In the 3-dimensional network polymer, the space occupied by the pendant groups is also decisive. If the dimethylamino groups are oriented in a plane opposite to the carboxamide groups due to the main chain flexibility, they become closer permitting easy H-bond formation. The situation is depicted in Fig. IV. 4. However since the capacity and reactivity did not improve, no further synthesis was attempted on these poly(acrylamide)s.

The concept of drug targeting was described by Paul Ehrlich about 80 years ago. It started from the observation that most of the pharmacological agents and drugs are not very selective with regard to their cellular sites of action and present various side-effects which are neither expected nor needed. Targeting involves the concept that the selectivity of drugs can be enhanced and their toxic side-effects reduced by associating them with carriers which pharmacologically inactive themselves will convey the drug selectively to the target cells. An efficient carrier should be able to transport the drug from the site of administration to its target cell and interact with this later as selectively as possible. The drug should remain inactive as long as it is carried in the blood stream. The link between the carrier and the drug should be stable in
the blood stream but be reversible after interaction with the target cell. Several classes of carriers have been advocated and used experimentally to transport a large variety of antitumour and antiinfectious drugs. The most promising carriers seem to be proteins such as antibodies, peptide hormones or glycoproteins because they possess a wide variety of specificities, are degradable and can be non-immunogenic. Trouet et al. developed a general method of the linking the antitumour drug daunorubicin (DNR) to proteins which would fulfill the criteria required by the targeting concept. Trouet's group incorporated a spacer arm of 1-4 amino acids between DNR and protein to overcome the steric hindrance experienced in the case of smaller peptides. When a tetrapeptide with leucyl amino acid adjacent to DNR was introduced, 60-80% release of DNR was intercalated. Among the various amino acid derivatives tested, leucyl DNR was the most extensively and rapidly hydrolysed one into DNR by liposomal enzymes. Thus a tetrapeptide Ala-Leu-Ala-Leu can very well act as a carrier and therefore its synthesis was attempted on the new flexible support.

a. The polymer support for peptide synthesis

The polymer support used was 4% TEGDMA-crosslinked poly(styrene). The maximal swelling and solvation ensured completion of reaction at every stage of the multistep synthesis. Functionalisation of the resin with chloromethyl
groups proceeded smoothly giving chloromethyl resin of chlorine capacity 1.04 mmol/g. The conditions of controlled chloromethylation afforded resins of medium capacity. The efficiency of multistep synthesis depends on several parameters such as the nature of monomers, nature and extent of crosslinks, the balance between hydrophilicity and hydrophobicity of the support, its topographical nature, porosity of the resin beads, swelling and solvation and stability under the synthetic environments. Attempts to optimise such parameters would help design tailor-made macromolecules for specific purposes. In copoly (PS-2%-TEGDM) some of the above characteristics were found and the synthesis of the tetrapeptide is an illustration of that. Couplings proceeded almost completely in the first stage itself and whenever it was incomplete, a second coupling ensured completion which was monitored by the ninhydrin test. The entire synthetic sequence is depicted in Scheme IV. 6. (p.178).

b. Deprotection and cleavage

The peptide was cleaved from the peptidyl resin (200mg) by treatment with neat TFA at room temperature for 15h. The resin was removed by filtration and TFA removed by rotary evaporation. The residual oil was cooled well and the crude peptide precipitated from ice-cold ether. The crude peptide was filtered through a sintered funnel, washed free of TFA, drained and dried in a vacuum desiccator to give 88mg peptide.
A: coupling in DCC/DCM. B: deprotection; 30% TFA/DCM
C: cleavage; TFA

Scheme IV. 6. Solid phase synthesis of a drug targeting tetrapeptide on a 4% PS-TEGDMA support

c. Purification by FPLC

The crude peptide was purified by FPLC using a reverse phase semi-preparative column. The solvent system consisted of A: 0.1% TFA in double distilled water and B: 0.1% TFA in
acetonitrile. The FPLC profile (Fig. IV. 5) shows only one major peak which was separated and analysed.

![FPLC profile](image)

**Fig. IV. 5. FPLC profile of crude drug targeting tetra-peptide: Ala-Leu-Ala-Leu (A L A L)**

d. Amino acid analysis

The amino acid analysis of the purified peptide gave the following results. Ala, 1.89 (2); Leu, 2.2 (2).

The synthesis of the tetrapeptide on a 4% TEGDMA-crosslinked polystyrene resin was accomplished successfully in fairly good yield and purity. The success of the synthesis also points to the positive role of polymer supports with flexible crosslinks in solid phase peptide synthesis.

Growth hormone releasing factor (GRF) stimulates release of pituitary growth hormone (GH)\textsuperscript{311}. Some structurally unrelated short peptides have also been reported to elicit growth hormone secretion by a different mechanism\textsuperscript{312}. Human growth hormone releasing factor is a 44-residue peptide amide with an amidated C-terminus and its primary structure has been established\textsuperscript{313}. Biological activity is located in the N-terminus of the molecule and hGRF segment (1-29) retains the full biological activity of the native molecule\textsuperscript{314}. Total synthesis of hGRF was achieved by Ling et al\textsuperscript{(1980)} on a p-methoxy benzhydramine (pMBHA) resin using a peptide synthesiser\textsuperscript{315} which was purified by ion exchange and partition chromatography. Chou-Fasman method\textsuperscript{316} of secondary structure prediction suggests that positions 6-11 may assume beta-turn conformation. More recently Sato et al\textsuperscript{317} reported the solid phase synthesis of hGRF analogs containing a bicyclic beta-turn dipeptide at 7-8, 8-9, and 9-10 positions using a MBHA support. Synthesis of hGRF(1-29) was accomplished using urethane protected amino acids and N-carboxy anhydrides by Fuller et al\textsuperscript{318}. The present synthesis was undertaken to ascertain whether the newly developed flexible support would be compatible with the short hydrophobic stretch of hGRF (12-15).
a. Peptide Synthesis

The synthesis was accomplished on a 1% PS-TEGDMA resin. The first amino acid Boc-Gly was esterified to the chloromethyl resin following the Gisin's method$^{299}$. The incorporation of the first amino acid proceeded smoothly in DMF and the level of Boc-Gly substitution in the Boc amino acyl resin was estimated by the picric acid method$^{300}$.

b. Assembly of Lys-Val-Leu-Gly (K V L G)

The amino acyl resin of Gly substitution 1.00mmol/g was used for further synthesis. The standard solid phase assembly technique$^{14}$ was followed for incorporating the remaining amino acids. Only Boc-amino acids were used except for lysine where a chloro benzyloxy carbonyl side-chain protection was employed besides the Boc group. Couplings of Boc amino acids were achieved through dicyclohexylcarbodiimide (DCC). A three-fold molar excess of the Boc amino acids was used for the couplings. Two couplings were essential for the completion of the reaction which was monitored in each step by the semiquantitative ninhydrin test$^{298}$. The peptidyl resin assumed a gel nature during the coupling and deprotection steps which facilitated smooth and facile permeation of the low molecular weight reagents into the resin core. This ensured completion of the coupling and deprotection procedure in every step of the synthesis, which was evidenced by the absence of any
deletion sequences. Incomplete deprotection and coupling has been shown to be the major factors responsible for deletion peptides. Boc deprotection was achieved by 30% TFA in dichloromethane and neutralisation by 5% diisopropylethylamine in DCM. Dicyclohexylurea precipitated during the couplings was removed by washing with 33% ethanol in DCM. The synthetic outline is depicted in Scheme IV. 7.

Scheme IV. 7. Solid phase synthesis of Lys-Val-Leu-Gly (KVLG) on 1% PS-TEGDMA resin.
The PS-2%-TEGDM resin due to its hydrophilic/hydrophobic balance and molecular character swelled well in dichloromethane and therefore the solvation problems attached to the peptide bearing resin in the initial stages of the synthesis in the case of the Merrifield resin were totally absent in this synthesis. The scanning electron micrograph of the chloromethylated resin and the peptide bearing resin are shown in Fig. IV. 6. (a and b), [p.184].

The micrographs reveal the significant changes in the surface characteristics brought about by the attachment of the peptide chains. The smooth resin surface became rough after the synthesis.

c. Deprotection and cleavage

Boc and side chain protecting groups as well as the peptide to resin linkage were cleaved in a single step by treatment with neat trifluoroacetic acid.

d. Purification of the crude peptide

A methanol solution of the crude peptide was purified by FPLC. Fig. IV. 7 shows the FPLC profile of the crude and purified peptides. The major peak was separately collected and analysed.
Fig. IV. 6. Scanning Electron Micrographs of chloromethyl copoly(PS-2%-TEGDMA) resin
(a) before synthesis  (b) after synthesis.
Conditions: solvent A: water containing 0.1% TFA; solvent B: Acetonitrile containing 0.1% TFA; flow rate: 0.5ml/min; detection: 214nm

<table>
<thead>
<tr>
<th>Time</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>35</td>
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<tr>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>55</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. IV. 7. FPLC profiles of the crude and purified (inset) peptide Lys-Val-Leu-Gly

e. Amino acid analysis

Amino acid analysis of the hydrolysed sample gave the following results: Lys. 1.06 (1); Val. 0.67 (1); Leu. 1.14 (1); Gly. 0.93 (1). The results are in good agreement with the expected values showing the purity of the peptide.
IV. 14. Synthesis of a contraceptive tetrapeptide:
Thr-Pro-Arg-Lys (TPRK)

With the advance of electronic theory and structural chemical concepts, much attention has been focussed on the study of conformation of peptides and to correlate the specific conformations with biological activity. Understanding the structure-activity correlation has become more feasible with the increased capacity of nuclear magnetic resonance spectroscopy. Kent Jr. isolated a tetrapeptide contraceptive from the two cell zygotes in the golden hamster by repeated passages through a sephadex G-10 column. The tetrapeptide was found to inhibit ovulation in non-bred hamsters. A year later Kent Jr. established the amino acid sequence of the tetrapeptide by Edman degradation and subsequent dansylation. The solid-phase synthesis of the contraceptive tetrapeptide was attempted on Merrifield resin by Chang et al. The solid-phase synthesis of the tetrapeptide on a 6% hexanediol diacrylate (HDODA)-crosslinked polystyrene (PS) support was accomplished following the Boc benzyl strategy. The crude peptide was obtained in 30% yield upon cleavage from the resin by TFA. It was purified by reverse phase HPLC and amino acid analysis showed agreeable ratios. The major objective of this tetrapeptide synthesis was to establish the use of high capacity resins with flexible crosslinks in solid phase peptide synthesis.
a. Attachment of Boc(C1Z)-Lysine to chloromethyl resin

The first amino acid Boc(C1Z)-Lysine was esterified to the chloromethyl resin in presence of a base DIEA and an esterification catalyst dimethylaminopyridine (DMAP). The extent of amino acid incorporation was assessed by the picric acid method and was found to be 0.53 mmol/g.

b. Synthesis of Thr-Pro-Arg-Lys (T P R K)

The remaining amino acids of the sequence were assembled one after the other following the normal solid phase technique (Scheme IV. 8.). Dichloromethane was used as the solvent medium for the reaction. A 3-fold molar excess of the Boc amino acids was used for each coupling. Boc-Arg(Mts) was coupled by the active ester method using HOBt. Other Boc-amino acids were coupled in DCM solution by DCC activation. 30% TFA in DCM was used for Boc-deprotection and 33% ethanol in DCM for removing the precipitated DCU. The efficiency of each coupling was monitored by semiquantitative ninhydrin test. Couplings were found to be complete in two stages showing the efficiency of the couplings. The synthesis is outlined in Scheme IV. 8. (p. 188)
Boc-Lys(C1Z) + Cl-CH₂-C₆H₄-Resin
EtOAc ↓ DIEA. DMAP: 65°C, 45h

Boc-Lys(C1Z) -O- CH₂-C₆H₄-Resin

1. 30% TFA/DCM ↓ 2. 5% TEA/DCM

Boc-Arg(Mts) + H₂N-Lys(C1Z) -O- CH₂-C₆H₄-Resin

DMF, DCC/HOBt ↓ 3h

Boc-Arg(Mts)-Lys(C1Z) -O- CH₂-C₆H₄-Resin

Deprotection Neutralisation ↓ Coupling of Boc-Pro and Boc-Thr(Bzl)
in DCM (2 cycles)

Boc-Thr(Bzl)-Pro-Arg(Mts)-Lys(C1Z) -O- CH₂-C₆H₄-Resin

Cleavage

40°C, 36h ↓ TFA, thioanisole.
m-cresol

Thr-Pro-Arg-Lys

Scheme IV. 8. Solid phase synthesis of Thr-Pro-Arg-Lys

d. Cleavage and purification

The protecting groups were removed and the peptide was cleaved from the support using neat TFA following the method of Bodanszky. Complete deprotection of Arg(Mts) was monitored by the Sakaguchi test. 200mg of the peptidyl resin gave 60mg of crude peptide corresponding to an yield of 30%. The crude peptide was dissolved in methanol and was purified by HPLC. The solvent system consisted of water (A) and acetonitrile 60% (B) both containing 0.1% TFA. The HPLC profile is given in Fig. IV. 8. The peaks were separately collected and analysed.
Fig. IV. 8. HPLC Profile of crude contraceptive tetrapeptide
e. Amino acid analysis

Samples were analysed after hydrolysis with 6N HCl at 110°C for 22 h. The amino acid ratios are as follows:
Thr. 1.12 (1); Pro. 1.07 (1); Arg. 0.91 (1); Lys. 1.58 (1). The ratios are in good agreement with the expected values.

The synthesis of T P R K on a 6% hexanediol diacrylate-crosslinked polystyrene support suggests that peptide synthesis can be done on tightly crosslinked polystyrene
resins also, provided the crosslinks are flexible. On the conventional DVB-crosslinked polystyrene supports, peptide synthesis can be carried out efficiently when the crosslink percentage is in the range 1-2. In a number of cases it has been shown that increasing the rigidity of the support by increasing the crosslink density brought about solvation problems and consequent retardation of coupling efficiency. The new support with amphiphilic and flexible characteristics can therefore overcome certain limitations of the conventional PS-DVB support.


Fujimaki et al.\textsuperscript{326} reported on delicious di and tri peptides obtained from fish protein hydrolysate mixture. The relation between taste and physicochemical properties was investigated by Arai et al.\textsuperscript{326} from a dipeptide containing L-glutamyl residues. Yamasaki and Mackawa\textsuperscript{327} isolated a delicious peptide from beef meat extracts treated with enzyme papain and elucidated its primary structure. The solid phase synthesis of a delicious octapeptide on a flexible polymer support was achieved in good yield. The TEGDMA crosslinked polystyrene support facilitates the optimal conditions for solid phase synthesis ensuring near-quantitative conversions at every stage of the synthesis.
a. Assembly of the peptide Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala (KGDEESLA)

The C-terminal amino acid Boc-Ala was esterified to the chloromethyl resin by the Gisin's method. The level of Boc-Ala substitution in the Boc-Ala resin was determined to be 0.53 mmol/g by picric acid method. Boc-Ala resin (400:0.21 mmol) was used for further synthesis. The next amino acid in the sequence, Boc-Leu, was incorporated into the aminoacyl resin through dicyclohexylcarbodiimide (DCC)-mediated coupling. The remaining Boc-amino acids were assembled on the Boc-amino acyl resin following standard solid phase assembly technique\textsuperscript{14}. The entire synthetic sequence is depicted in Scheme IV.9. (p.192).

b. Cleavage of the peptide, purification and amino acid analysis

The finished peptide was obtained from the peptidyl resin by treatment with TFA in the absence of scavenging reagents for 30h. The crude peptide was precipitated by addition of ice-cold ether and isolated after repeated washings with fresh ether. 200mg of peptidyl resin gave 122mg crude peptide corresponding to an yield of 61%. The crude sample was purified by HPLC. The HPLC profile of the crude delicious peptide is given in Fig. IV.9.
Boc-Ala-OH + Cl-CH$_2$-C$_6$H$_4$- Resin

Cs$_2$CO$_3$: DMF $\xrightarrow{60^\circ C, 72h}$ Boc-Ala -O- CH$_2$-C$_6$H$_4$- Resin

1. 30% TFA/DCM
2. 10% TEA/DCM

Boc-Leu, DCC/DCM

1. 30% TFA/DCM
2. 10% TEA/DCM

Boc-Leu-Ala -O- CH$_2$-C$_6$H$_4$- Resin

Boc-Ser(OBz1), Boc-Glu(OBz1), Boc-Asp(OBz1), Boc-Gly, Boc-Lys(C1Z) (in 6 cycles)

TFA cleavage $\xrightarrow{40^\circ C, 30h}$

Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala

Scheme IV. 9. Solid-phase synthesis of Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala (KGDEESLA)
Fig. IV. 9. HPLC profile of crude delicious peptide:
Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala

Conditions: Solvent A consisted of water with 0.1% TFA and solvent B consisted of 60% acetonitrile containing 0.1% TFA. Detection wavelength 214 nm.

The amino acid analysis gave the following results:
Lys, 1 (1); Gly, 0.8 (1); Asp, 1 (1); Glu, 2.4 (2); Ser 1.2 (1); Leu, 1 (1); Ala, 1.3 (1).
IV. 16. Synthesis of Seminalplasmin segment (14-26)

Ser-Leu-Ser-Arg-Tyr-Ala-Lys-Leu-Ala-Asn-Arg-Leu-Ala

(SLSRYAKLANRLA)

Seminalplasmin (SPLN) is a 47-residue peptide isolated from bovine seminal plasma in India. It is one of the few peptides isolated from mammalian sources and its primary structure has been elucidated.

H-Ser\(^1\)-Asp-Glu-Lys-Ala\(^5\)-Ser-Pro-Asp-Lys-His\(^{10}\)-His-Arg-Phe-Ser-Leu\(^{15}\)-Ser-Arg-Tyr-Ala-Lys\(^{20}\)-Leu-Ala-Asn-Arg-Leu\(^{25}\)-Ala-Asn-Pro-Lys-Leu\(^{30}\)-Leu-Glu-Thr-Phe-Leu\(^{35}\)-Ser-Lys-Trp-Ile-Gly\(^{40}\)-Asp-Arg-Gly-Asn-Arg\(^{45}\)-Ser-Val-OH

(SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV)

It possesses potent antibacterial activity and inhibits RNA synthesis. Seminalplasmin increases the fluidity of sperm acrosomal membrane and sperm plasma membrane and causes lysis of dividing eukaryotic cells. It binds readily to spermatozoa and inhibits sperm mobility and thereby its fertilising ability. In order to study the biological activity of this protein and to delineate the structure-activity correlation, the present study was undertaken.
The solid phase synthesis of seminalplasmin segment (14-26) was achieved on a 2% TEGDMA-crosslinked polystyrene support in 61% overall yield and in high purity. The resin used for the synthesis exhibits good swelling and solvation behaviour in the solvents employed for the synthesis facilitating almost quantitative stepwise peptide coupling. The physicochemical and mechanical properties of the resin and the favourable conditions of synthesis and the final cleavage of the peptide points to potential use of the resin in the synthesis of model peptides and bioactive protein sequences.

a. Peptide Synthesis

The first amino acid Boc-Ala was esterified to the chloromethyl resin and its loading level was determined to be 0.53 mmol/g. All couplings were done in DCM (except Boc-Arg(Mts) and Boc-Asn), using 3-fold molar excess of Boc-amino acids, following the symmetrical anhydride method. Boc-Arg(Mts) and Boc-Asn were coupled in DMF in the presence of 1-hydroxybenzotriazole (HOBt) for 2.5h. The dehydration problem of these amino acids were essentially avoided by changing the carbodiimide method to HOBt active ester method using DMF for longer duration\textsuperscript{364}. Whenever couplings were incomplete even after two subsequent trials, a third one also was performed. Final cleavage of the finished peptide from the resin was effected by TFA in the presence of scavengers. The entire synthetic sequence is represented in Scheme IV. 10.
Scheme IV. 10. Solid phase synthesis of seminalplasmin segment (14-26)
b. Purification of the peptide

Crude peptide was purified by reverse phase FPLC. The FPLC profile of the purified peptide is shown in Fig. IV. 10.

Fig. IV. 10. FPLC profile of purified seminalplasmin segment (14-26). Buffer A: water containing 0.1% TFA; Buffer B: acetonitrile containing 0.1% TFA. Detection wavelength: 214nm; column used: PepRPC HR5/5 (C18); flow rate: 0.5ml/min; sensitivity: 0.5; chart speed: 0.5 cm/min.

The following gradient was used.

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>50</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>51</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>52</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>53</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
Purification of biologically active protein segments and peptides can be very well accomplished by reverse phase FPLC using C18 semiprep columns. The column consists of C18 covalently bonded silica beads packed in the column. In this technique, separation is effected based on the hydrophilic/hydrophobic nature of the peptide. It involves partition between an aqueous and an organic phase. The beaded support represents the organic phase while the eluent represents the aqueous phase. A hydrophilic component of the peptide would prefer to stay in the aqueous environment and would therefore pass through the column rapidly while the hydrophobic component would remain attached to the hydrophobic hydrocarbon coating of the beads and would be eluted through the column relatively slowly. The following conditions affect the behaviour of the components in the sample.

a. Change in the hydrophobicity of the elution buffer. This is achieved by adding acetonitrile to the aqueous phase.

b. Variation in the pH of the buffer

c. Alteration of the nature of the hydrophobic chains attached to the silica beads. Solvents used in FPLC includes acetonitrile and water both containing small quantities of TFA to enhance the peak sharpening ability. Nanomoles of the crude peptide was dissolved in a suitable solvent like water, methanol or a mixture of the two and injected into the column and the peptide detected at 214nm.

The FPLC profile of the crude peptide is given in Fig. IV. 11.
Six different peaks were seen in the profile labelled A-F. These peaks were separately collected, hydrolysed and subjected to amino acid analysis to ascertain their composition and hence to estimate the extent of deletion sequences.
c. Analysis of the peaks

Peak A. The eluate collected from peak A was hydrolysed with 6N HCl and TFA (2:1; v/v) after rotavaporating the solvent, heated at 110°C for 22h and analysed. The results obtained by the analysis of peak A are given in Table IV. 2.

Table IV. 2. Amino acid ratios of peak A from the synthesis of seminalplasmin segment (14-26)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Expected</th>
<th>found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp[^a]</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
<td>0.5[^b]</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>2.94</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>0.3[^c]</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>2.71</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>1.80</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>1.14</td>
</tr>
</tbody>
</table>

\[^a\] Asp gets converted to Asp during hydrolysis
\[^b\] Ser gave low values due to loss during hydrolysis
\[^c\] Tyr undergoes degradation under hydrolytic conditions

Peak B. The amino acid analysis of peak B gave the following results (Table IV. 3)
Table IV. 3. Amino acid ratios of peak B from the synthesis of seminalplasmin (14-26)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Expected</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
<td>0.8³</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>2.98</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>0.13*</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>2.70</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>1.60</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>1.10</td>
</tr>
</tbody>
</table>

a. Ser value is low due to loss during hydrolysis.

* Tyr value is less due to hydrolytic degradation.

Both peaks A and B gave the correct analysis with no deletion of the amino acids. Peak A conforms to the most agreeable analysis and therefore peak A is regarded as the correct FPLC profile of the pure peptide.

Peak C. The results of the analysis of peak C is depicted in Table IV. 4.
Table IV. 4. Amino acid analysis of peak C of the synthesis of seminalplasmin segment (14-26)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Expected</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
<td>0.5^a</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>2.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>0.14*</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>2.3</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>1.06</td>
</tr>
</tbody>
</table>

^a. Ser lost during hydrolysis
^*. Tyr undergoes degradation during hydrolysis

Leu content is slightly less in this peak. However all the amino acids are present in this peak also.

Peak D. Peak D gave the following analysis (Table IV. 5)

Table IV. 5. Analysis of peak D of the FPLC profile of the synthesis of seminalplasmin segment (14-26)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Expected</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
<td>0.56^a</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>4.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>0.21*</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>6.00</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>1.83</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>1.58</td>
</tr>
</tbody>
</table>

^a. Ser lost during hydrolysis
^*. Tyr undergoes degradation during hydrolysis
The contents of alanine and leucine is very high in this peak. However, all residues are present showing the absence of any deletion.

**Peak E**

Amino acid ratios in peak E is given in Table IV. 6.

### Table IV. 6. Amino acid ratios of peak E of the synthesis of seminalplasmin segment (14-26)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Expected</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1</td>
<td>0.94</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
<td>0.44</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>2.52</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>0.10</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>2.28</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>1.44</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* a. serine undergoes degradation.
* * Tyr undergoes hydrolytic degradation

In this peak, the ratios of Ala, Leu and Arg are found to be less.

**Peak F.** The analysis of peak F gave the following amino acid ratios (Table IV. 7).
Table IV. 7. Amino acid analysis of peak F of the synthesis of seminalplasmin segment (14-26)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Expected</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>2.74</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>not found</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>2.17</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Peak F gave no tyrosine which shows that it has been deleted from the chain during the synthesis. Therefore Peak F corresponds to a deletion sequence of the synthesis. Arg and Lys were found in excess.

Among the 6 peaks of the profile, only one corresponds to a deletion sequence. This points out the success of the synthesis. By careful control of the coupling conditions and systematic monitoring of the progress of couplings, the efficiency of the synthesis can be enhanced.

FPLC can be successfully employed for the purification of crude synthetic peptides as well as for estimation of the extent of deletions involved in the synthesis. This in turn is an indirect assessment of the efficiency of the synthesis.
d. Manual microsequencing of seminalplasmin segment (14-26)

The manual microsequencing of the peptide segment was done by the method of Tarr. The sequencing result is given in Table IV. 8.

Table IV. 8. Sequencing data of seminalplasmin segment (14-26)

<table>
<thead>
<tr>
<th>Call</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
<th>Match</th>
<th>Known</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>V</td>
<td>G</td>
<td>++</td>
<td>S</td>
</tr>
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<td>2</td>
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<td>L</td>
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<td>K</td>
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<td>A</td>
<td>+++</td>
<td>L</td>
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<td>A</td>
<td>N</td>
<td>D</td>
<td>+++</td>
<td>A</td>
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<td>10</td>
<td>N</td>
<td>D</td>
<td>P</td>
<td>+++</td>
<td>N</td>
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<td>11</td>
<td>L</td>
<td>R</td>
<td>M</td>
<td>+++</td>
<td>R</td>
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<tr>
<td>14</td>
<td>T</td>
<td>K</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results agree with the correct sequence of amino acids in the peptide synthesised.

e. Circular dichroism spectra of Ser-Leu-Ser-Arg-Tyr-Ala-Lys-Leu-Ala-Asn-Arg-Leu-Ala (S L S R Y A K L A N R L A)

The circular dichroism (CD) spectrum of the peptide segment was recorded by CD measurement in a helicogenic solvent, methanol (Fig. IV. 12). The spectrum suggests that the peptide segment has a random coil structure in methanol.
The solid-phase synthesis of seminalplasmin segment (14-26) was achieved on a 2% TEGDMA-crosslinked polystyrene support in 61% overall yield. The resin used for the synthesis exhibits good swelling and solvation behaviour in the solvents employed for the synthesis facilitating almost quantitative stepwise peptide coupling. The physicochemical and mechanical properties of the resin and the favourable conditions of synthesis and the final cleavage of the peptide point to extensive use of the resin in the synthesis of model peptides and bioactive protein sequences.
IV. 17. Synthesis of Seminalplasmin segment (28-40):

Pro-Lys-Leu-Leu-Lys-Thr-Phe-Leu-Ser-Lys-Trp-Ile-Gly

(P K L L K T F L S K W I G)

Seminalplasmin (SPLN) is a 47-residue peptide. In order to study the biological activity of this protein and to delineate the structure-activity correlation, the present study was undertaken. Earlier, the 28-40 fragment was synthesised by the solid phase technique on a 1% crosslinked Merrifield resin\(^{333}\). This fragment exhibited antibacterial and cell lytic activity. The present synthesis was carried out in order to investigate whether a selective replacement of residue 32- Glutamic acid - by Lysine would improve the antibacterial properties of the protein. The standard stepwise manual solid phase assembly technique was followed.


The application of the new support for solid phase peptide synthesis of biologically active peptide analogues was illustrated by the stepwise assembly of Seminalplasmin segment (28-40) in high yields and very high purity. The first amino acid, Boc-Gly was esterified to the chloromethylated support by the cesium salt method of Gisin and the Boc-Gly substitution in the resin was found to be 1.00 mmol/g. The remaining Boc-amino acids were assembled on the support resin by standard solid phase methodology\(^{14}\). N-t-Boc protection was used throughout the synthesis along
with suitable side-chain protecting groups. Boc-amino acids were coupled by the symmetric anhydride method in DCM/dimethyl formamide (DMF). The progress of the couplings was monitored by the semiquantitative ninhydrin test. Dicyclohexyl urea (DCU) was washed off in 33% ethanol in DCM. Boc groups were deprotected by 30% TFA in DCM. The synthetic sequence is depicted in Scheme IV. 10.

\[
\text{Boc.Gly.Cs+Cl.CH}_2\text{C}_6\text{H}_4\text{-Resin} \\
\text{DMF} \quad 50^\circ\text{C, 60h} \\
\text{Boc.Gly.O.CH}_2\text{C}_6\text{H}_4\text{-Resin} \\
30\%\text{TFA/DCM} \quad 5\%\text{DIEA/DCM} \\
\text{Boc.NH.Ile.OH+H}_2\text{N.Gly.O.CH}_2\text{C}_6\text{H}_4\text{-Resin} \\
\text{DCC/DCM} \\
\text{Boc.NH.Ile.CO.H.Gly.O.CH}_2\text{C}_6\text{H}_4\text{-Resin} \\
11\text{ cycles of coupling} \\
\text{Leu.Ser(OBzl).Lys(CIZ).Trp(CHO).Ile.CO.NH.Gly.O.CH}_2\text{C}_6\text{H}_4\text{-Resin} \\
\text{TFA, Thioanisole} \\
m.\text{Cresol, 1,2-ethanediol} \\
\text{40} \\
\text{Scheme IV. 10. Solid phase synthesis of seminalplasmin segment (28-40)}
\]
b. Cleavage, purification and amino acid analysis

The finished peptide was cleaved from the support by treatment with neat TFA in the presence of scavenging reagents like 1,2-ethane dithiol, m-cresol and thioanisole. The crude peptide was precipitated from cold ether and the scavenging reagents were washed off by repeated washings with fresh ether. The crude peptide, obtained in 80% yield was purified by FPLC using a PepRPC C18 column. The FPLC profile showed a major peak (Fig. IV. 13) which was analysed.

![FPLC profiles of the crude and purified peptide (inset). Conditions- Solvent A: water containing 0.25% TFA; solvent B: acetonitrile containing 0.25% TFA; detection: 214nm; flow rate: 0.5 ml/min; chart speed: 0.5 cm/min; sensitivity 0.5; column employed: PepRPC HR 5/5 (C18)]
The following gradient was used:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>140</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>145</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>160</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

The FPLC profiles of the crude and purified peptides showed only one major peak. The crude peptide exhibited the same biological activity as the purified sample pointing out that the crude peptide was as good as the purified sample in terms of biological activity. This in turn highlights the significance of the synthesis and the polymer support employed for the synthesis.

The results of the amino acid analysis of the purified peptide (Table IV. 9; p.211) agree with the theoretical amino acid composition of the target sequence.

c. Sequencing of the peptide

The sequencing of the purified peptide was done by Edmann degradation technique which revealed the homogeneity of the peptide.
Table IV. 9. **Amino acid analysis of seminalplasmin (28-40)**

(P K L L K T F L S K W I G)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Expected</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro</td>
<td>1</td>
<td>1.20</td>
</tr>
<tr>
<td>Thr</td>
<td>1</td>
<td>0.88</td>
</tr>
<tr>
<td>Ser</td>
<td>1</td>
<td>0.85</td>
</tr>
<tr>
<td>Phe</td>
<td>1</td>
<td>1.09</td>
</tr>
<tr>
<td>Ile</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Gly</td>
<td>1</td>
<td>1.12</td>
</tr>
<tr>
<td>Lys</td>
<td>3</td>
<td>2.83</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>2.97</td>
</tr>
</tbody>
</table>

### d. CD studies

The conformation of the peptide segment was investigated by Circular Dichroism (CD) in helicogenic solvents like trifluoroethanol (TFE) and methanol as well as in a biological buffer HEPES. The CD spectra are given separately in Fig. IV. 14. (a, b) and Fig. IV. 15. (p. 212)

The peptides assumed an alpha helical conformation in both the helicogenic solvents while in a biological buffer N-(2-hydroxyethylpiperazine N'-(2-ethanesulphonic acid)) (HEPES) it assumed a random-coil conformation.
Fig. IV. 14. Circular dichroism spectra of seminalplasmin segment (28-40) in (a) TFE and (b) methanol.

Fig. IV. 15. CD spectra of the 13-peptide in HEPES.
e. Biological activity

The biological activity of the peptide segment was studied by Sitaram et al. It was observed that the peptide possessed enhanced antibacterial activity than the original segment with Glu at position 31. However, the hemolytic activity was not altered.

f. Stability of the PS-TEGDMA support

![Graph showing IR spectra of 1% PS-TEGDMA (a) resin, (b) chloro- methyl resin, and (c) TFA-treated resin.]

Fig. IV. 16. IR spectra of 1% PS-TEGDMA (a) resin, (b) chloro- methyl resin, and (c) TFA-treated resin.
The stability of the TEGDMA-crosslinked poly(styrene) support under the peptide synthetic conditions was tested by treating aliquots of the resin separately with neat TFA at 50°C, with 30% TFA in DCM, DIEA (5%) in DCM and 33% ethanol in DCM for 72h. The resin was recovered and analysed. It was found that the resin remained intact under the above conditions. The chloromethylated resin also was similarly analysed and found that there was no degradation or rupture of the crosslinks during chloromethylation. The IR spectra of the PS-TEGDMA resin, chloromethyl resin and TFA treated resin are given for comparison in Fig. IV. 16(P.213).

The synthesis of seminalplasmin fragment (28-40) illustrates the applicability of the PS-TEGDMA resin as a new solid support for the efficient synthesis of peptides. The added advantage of the support is that the attachment of the first amino acid was quantitative and the subsequent couplings of Boc-amino acids were driven to completion in a single step, showing the efficient solvation and optimal swelling of the support under the peptide synthetic conditions. The new support can be economically and efficiently used for the synthesis of biologically active peptides and protein sequences.