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# Synthesis and conformation of a hexapeptide fragment (3-8) of SPF peptide by NMR and restrained molecular dynamics

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Seminalplasmin (SPLN) is a 47-residue peptide from bovine seminal plasma which shows broad spectrum antimicrobial activity but no hemolytic activity and it lyses dividing mammalian cells, but not resting cells. It is reported that the 28-40 segment of SPLN, designated as SPF, is the most hydrophobic stretch of SPLN and primarily responsible for the membrane-perturbing activity of SPLN. The SPF peptide has a helical structure as shown by CD analysis. Molecular dynamics studies show that SPF peptide has two helical segments, regions Leu4-Phe7 and Lys10-Ile12. The helical structure makes it biologically active. The hexapeptide studied presently, Leu-Leu-Glu-Thr-Phe-Leu, forms the 3-8 region of SPF. NMR analysis shows that the hexapeptide Leu-Leu-Glu-Thr-Phe-Leu has an extended backbone conformation. Molecular dynamics studies show that the electrostatic attraction between opposite charges at 2nd and 5th positions stabilises the helical structure of SPF peptide. The lack of 2nd residue (Lys) in the experimental 3-8 peptide fragment of SPF explains its extended structure. This information has led to the conclusion that the regions other than this hexapeptide motif are essential to maintain the helical structure of SPF peptide.

**Keywords**: Seminalplasmin, SPF peptide, peptide conformation, NMR, restrained molecular dynamics

Seminalplasmin (SPLN) is a 47-residue peptide from bovine seminal plasma and has broad spectrum antibacterial and antifungal activities, without any hemolytic activity[1-3]. SPLN might exert its anti-bacterial activity by rapidly permeabilizing the outer and cytoplasmic membranes of bacteria[4]. The protein is also capable of lysing dividing mammalian cells, but not resting cells[5]. Nuclear magnetic resonance (NMR) studies showed that SPLN has α-helical conformations for the residues 21-27 and 31-47 with an interruption at 34, in the presence of detergents[6].

The 28-40 segment of SPLN (Pro-Lys-Leu-Leu-Glu-Thr-Phe-Leu-Ser-Lys-Trp-Ile-Gly) designated as SPF is the most hydrophobic stretch of SPLN[7]. This 13-mer peptide shows antimicrobial and hemolytic activities[8-10]. This peptide segment is primarily responsible for the membrane-perturbing activity of SPLN[11]. Circular dichroism studies have indicated that SPF adopts a helical conformation, the active conformation, in a hydrophobic environment[10]. Molecular dynamics studies showed that SPF peptide has two helical segments, namely in regions Leu4-Phe7 and Lys10-Ile12 (Ref. 12). The hexapeptide, Leu-Leu-Glu-Thr-Phe-Leu, studied in the present report forms the 3-8 fragment of SPF peptide. Based on molecular dynamics studies, this hexapeptide is seen to form the first helix of SPF peptide. This paper describes the synthesis and conformation of this hexapeptide fragment by two dimensional 2D-NMR and restrained molecular dynamics in dimethyl sulphoxide (DMSO). This study highlights the significance of the regions other than this hexapeptide region in maintaining the helical structure of SPF peptide.

## Materials and Methods

### Synthesis of Leu-Leu-Glu-Thr-Phe-Leu

Chloromethylated 2% cross-linked butanediol-dimethacrylate-polystyrene (BDDMA-PS, 2.0 mM –Cl/g) was pre-swollen for 2 hr in dichloromethane (DCM) by shaking in a reaction vessel equipped with a sintered glass bottom. The attachment of the first amino acid was done by caesium salt method[13]. The extent of first amino acid attachment was estimated by picric acid method[13]. The butyloxycarbonyl (Boc)

protecting group was removed from the N-terminus of the attached amino acid by reaction with 30% trifluoroacetic acid (TFA) in DCM (2×15 min) followed by neutralization with 5% diisopropylethylamine (DIEA) in DCM (5 min) and 5% DIEA in N-methylpyrrolidone (NMP)-DCM mixture (1:1 v/v, 5 min) to free the amino group. 1-Hydroxybenzotriazole (HOBt) active ester method[13] was used for the formation of the peptide bond. Three equivalents of reagents were used in each step. Completion of reaction was monitored by ninhydrin test[13]. The peptide was cleaved from the resin and deprotected by treating with the cleavage mixture consisting of 82.5% TFA, 5% water, 5% phenol, 5% thioanisole and 2.5% ethanedithiol. The crude peptide was purified by reverse-phased FPLC (AKTA, Amersham Biosciences) using Superdex peptide HR 10/30 column and solvent system consisting of 0.1% aqueous trifluoroacetic acid (TFA, solvent A) and 0.1% TFA in 80% acetonitrile-water (solvent B). The peptides were characterized by amino acid analysis and sequence specific resonance assignment using 2D NMR techniques.

## NMR analysis

The NMR experiments were carried out in DMSO-$d_6$. DMSO has been used as a membrane mimicking solvent. The structure determinations of peptides by NMR in DMSO-$d_6$, as in the cases of transmembrane helices of rhodopsin[14-16] and bacteriorhodopsin[17], have enabled the visualization of the secondary structure of native protein with as much fidelity as seen in the X-ray crystal structures. Furthermore, DMSO is not known to induce particular secondary structure and thus is preferable to solvents such as trifluoroethanol, which would strongly stabilize α-helix. The sample was prepared by dissolving 5 mg of the peptide in 0.5 mL DMSO-$d_6$. NMR experiments were performed on a Bruker AMX 400 MHz spectrometer at 298 K. Double quantum filtered COSY (DQF-COSY) spectra were acquired with the pulse sequence of Derome *et al.*[18] The TOCSY spectra were obtained using the MLEV-17 spin lock sequence[18] with mixing time of 75 ms. 2D rotating-frame Overhauser enhancement spectra (ROESY)[18], with a mixing time of 500 ms, were acquired to obtain the distance constraints. In 2D-NMR experiments, time domain data points were 512 and 2048 along $t_1$ and $t_2$ dimensions respectively. The data multiplied with sine bell window functions shifted by $\pi/4$ and $\pi/8$ along $t_1$ and $t_2$ axes respectively, was zero-filled to 1024 data points along $t_1$ dimensions prior to 2D Fourier transformation (FT). $^1$H NMR chemical shift calibrations were carried out with respect to the methyl signal (at 0.0 ppm) of 3-(trimethylsilyl) [3, 3, 2, 2-$^2$H] propionate-$d_4$ (TSP), which was used as the external reference.

## Molecular modeling

Molecular modeling by restrained molecular dynamics was performed by using the software package CYANA[19]. Ten thousand torsional angle dynamics (TAD) steps were performed with a simulated annealing procedure on each of the 1000 initially generated structures. The structures were visualized and analyzed by means of the software package MOLMOL[20].

## Results and Discussion

### Synthesis of Leu-Leu-Glu-Thr-Phe-Leu

Attachment of the C-terminal amino acid Leu was found to be quantitative. All the couplings, except Leu2-Leu1 coupling, required single coupling with three molar excess of the reagents for completion. The Leu2-Leu1 coupling required double coupling for completion. This might be due to steric or secondary structural factors. 2% Cross-linked butanedioldimethacrylate-polystyrene (BDDMA-PS) was found to be an efficient polymer matrix for the synthesis of oligopeptides.

### NMR analysis

*Sequence specific resonance assignment and secondary structure of peptides*

Sequence specific resonance assignments of the peptides were done by following standard procedures developed by Wüthrich *et al.*[21] Combined use of 2D DQF-COSY, TOCSY and ROESY spectra helped the complete resonance assignments. The $H^N$-$H^\alpha$ regions of TOCSY (75 ms) and ROESY (500 ms) of Leu-Leu-Glu-Thr-Phe-Leu are shown in **Figure 1** and **Figure 2** respectively. The observed chemical shift values are given in **Table I**. $H^N$- $H^N$ region of the ROESY spectrum is shown in **Figure 3**. The chemical shift index (CSI) developed by Wishart *et al.*[22] is very useful to identify secondary structures. A stretch of four or more consecutive +ve indices indicates a β-strand and a stretch of four or more consecutive -ve indices indicates an α-helix. Plot of CSI versus residue of Leu-Leu-Glu-Thr-Phe-Leu is shown in **Figure 4**. CSI plot

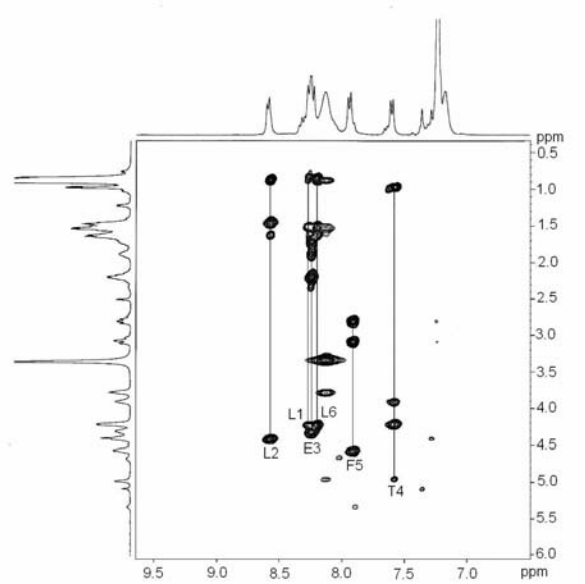


**Figure 1** — $H^N$-aliphatic region of TOCSY of Leu-Leu-Glu-Thr-Phe-Leu in DMSO-$d_6$ at 25°C.

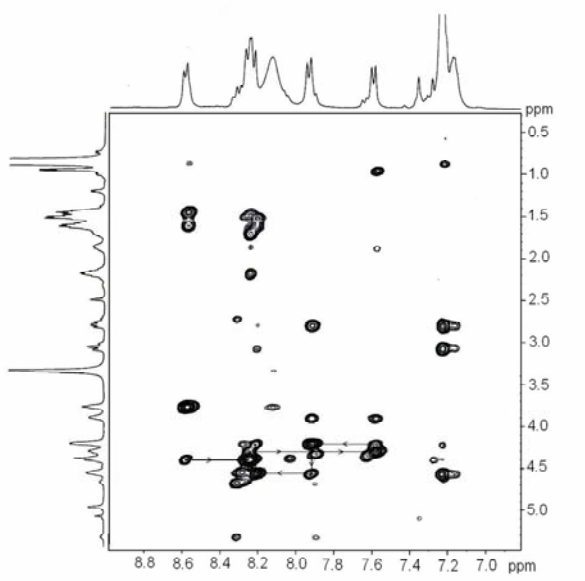


**Figure 2** — $H^N$-aliphatic region of ROESY of Leu-Leu-Glu-Thr-Phe-Leu with mixing time 500 ms in DMSO-$d_6$ at 25°C.

showed that Leu-Leu-Glu-Thr-Phe-Leu formed an extended backbone conformation.

Diagnostic NOE connectivities of Leu-Leu-Glu-Thr-Phe-Leu are shown in **Figure 5**. The $^3J_{NH\alpha}$ values can be correlated to ϕ-torsional angles. For a β-strand, the $^3J_{NH\alpha}$ values are greater than 8.5 Hz. For an extended backbone conformation, the $^3J_{NH\alpha}$ values are between 7 and 8 Hz. The $^3J_{NH\alpha}$ and ϕ values of Leu-Leu-Glu-Thr-Phe-Leu are shown in **Table II**. The NOE connectivities and the $^3J_{NH\alpha}$ values also supported the results from CSI

**Table I** — The observed chemical shift values of Leu-Leu-Glu-Thr-Phe-Leu

| Residue | $H^N$ | $H^\alpha$ | $H^\beta$ | Others |
|---|---|---|---|---|
| L1 | 8.28 | 4.22 | 1.65, 1.65 | $H^\gamma$ 1.50, $H^\delta$ 0.86 |
| L2 | 8.58 | 4.45 | 1.62, 1.62 | $H^\gamma$ 1.45 $H^\delta$ 0.84 |
| E3 | 8.26 | 4.32 | 1.70, 1.90 | $H^\gamma$ 2.20, 2.21 |
| T4 | 7.59 | 4.23 | 3.91 | $H^\gamma$ 0.91, $OH^\gamma$ 4.99 |
| F5 | 7.94 | 4.58 | 2.80, 3.11 | Aromatic 2,6 H 7.23 3,5 H 7.35 4H 7.18 |
| L6 | 8.23 | 4.22 | 1.65, 1.65 | $H^\gamma$1.50 $H^\delta$ 0.89 |

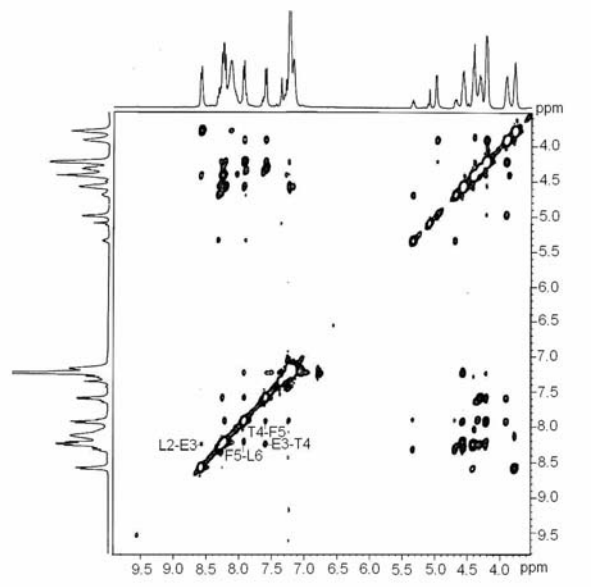


**Figure 3** — $H^N$-$H^N$ region of ROESY with mixing time 500 ms of Leu-Leu-Glu-Thr-Phe-Leu in DMSO-$d_6$ at 25°C

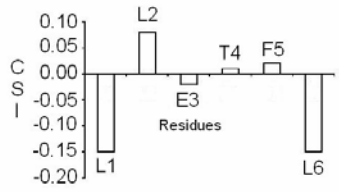


**Figure 4** — CSI versus residue plot of Leu-Leu-Glu-Thr-Phe-Leu


**Figure 5** — Diagnostic NOE connectivities of Leu-Leu-Glu-Thr-Phe-Leu in DMSO-$d_6$ at 25°C.

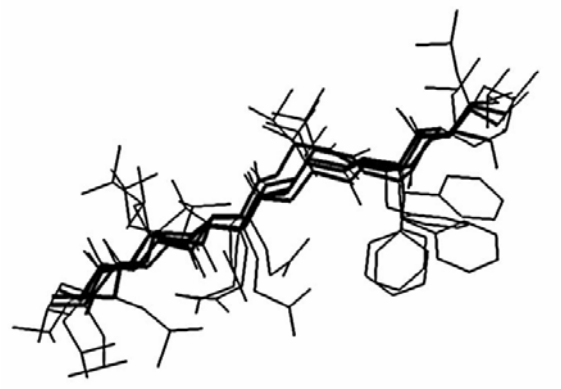

**Figure 6** — Best fit backbone superposition of the 5 conformers of Leu-Leu-Glu-Thr-Phe-Leu in DMSO-$d_6$ at 25°C.

**Table II** — The observed $^3J_{HN\alpha}$, $\phi$ and CSI ($H^\alpha$) values of Leu-Leu-Glu-Thr-Phe-Leu

| Residue | $^3J_{HN\alpha}$ (Hz) | $\phi$ (°) | CSI ($H^\alpha$) |
|---|---|---|---|
| L1 | Undetermined | - | -0.15 |
| L2 | 7.40 | -155° | 0.08 |
| E3 | 8.19 | -145° | -0.02 |
| T4 | 7.87 | -147° | 0.01 |
| F5 | 8.23 | -143° | 0.02 |
| L6 | Undetermined | - | -0.15 |

values that the peptide is in an extended backbone conformation

*Structure calculation*

Restrained torsion angle dynamics using CYANA package was used to obtain the 3D structure from NMR data. CSI values, inter-proton distances and $\phi$ values were given as input restraints. The NOE cross peak intensities obtained in the ROESY spectrum were translated into distance constraints. The NOE cross-peak intensities were classified as strong (<2.5 Å), medium (2.5 Å< x < 3.5 Å) and weak (3.5 Å < x < 5.0 Å). Thirty-two upper limit distance restraints (both inter- and intra-residue) were obtained. These distance restraints were complemented with four $\phi$-torsion angles and chemical shift restraints. Out of the 1000 random conformers subjected to TAD, the 20 structures with the lowest global deviation (minimum target function) from the input restraints were sorted out and submitted to statistical analysis. Best fit backbone superposition of the 5 conformers of Leu-Leu-Glu-Thr-Phe-Leu peptide is shown in **Figure 6**. The mean global backbone rmsd of the best fit structures is 0.53 Å. Overall, the hexapeptide adopts an unfolded extended backbone conformation. CD analysis has shown that the 28-40 fragment of seminalplasmin (SPF), Pro-Lys-Leu-Leu-Glu-Thr-Phe-Leu-Ser-Lys-Trp-Ile-Gly, formed a helical conformation, which forms the active conformation[10]. Molecular dynamics studies showed that SPF peptide has two $3_{10}$-helical segments, regions Leu4-Phe7 and Lys10-Ile12 (Ref. 12). The peptide examined in the present study, Leu-Leu-Glu-Thr-Phe-Leu, belongs to the first helical segment of SPF. NMR studies show that the peptide adopts an extended backbone conformation.

Molecular dynamics studies showed that the electrostatic attraction between opposite charges at 2nd and 5th positions stabilizes the helical structure of SPF peptide. The lack of 2nd residue (Lys) in the experimental 3-8 peptide fragment of SPF explains its extended structure. This information led to the conclusion that the regions other than this hexapeptide motif are essential to maintain the helical structure of SPF peptide.

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