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Conformational Analysis of Synthesized Ovine Kisspeptin 13 using Circular Dichroism Spectroscopy

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ABSTRACT
Kisspeptin, also called as metastin, is the cognate ligand GPR-54 (G-coupled receptor) which was previously an orphan receptor. Kisspeptin consists of 145 amino acids and its biological activity can be localized to the C-terminal segment which is cleaved into C-10, C-13 and C-14 segment. Kisspeptin-GPR-54 interaction stimulates GnRH secretion and has been shown to be essential for the initiation of the pubertal LH surge. A 13 amino acid derivative of kisspeptin was synthesized by solid phase peptide synthesis using F-moc strategy and it was purified using RP-HPLC. The CD spectra of the peptide (1 mg mL^{-1}) was evaluated in water, 50% and 75% TFE. The CD spectra of kisspeptin 13 in the water mainly exhibited random coil structure with no significant alpha helical or beta sheet content. Increasing concentration of TFE could cause slight induction of both beta sheet and alpha helix structures but still disordered random coil being the major contributor to the conformation. Predominantly disordered structure of kisspeptin 13 as elucidated by the experiment may explain its multifunctional role and non putative receptor binding and may help to develop a few agonists for mimicking the therapeutic action for future drug use.

Key words: Metastin, GPR54, solid phase peptide synthesis, Kiss-1, RP-HPLC

INTRODUCTION
The conformational spectrum of a molecule is determined by its three dimensional structure and its stereospecific orientation in space. Study of conformational stability of a peptide is important with respect to its pharmacological properties as well as intermolecular and intramolecular interactions. Kisspeptins are the peptide products of the Kiss-1 gene which were identified in 2001 as natural ligands of the previously orphan receptors, GPR54 (Kotani *et al*., 2001; Muir *et al*., 2001; Ohtaki *et al*., 2001). Kiss-1 gene was originally reported to be selectively over expressed in metastatis suppressed tumour cell (Lee *et al*., 1996), hence the fragment (68-121) also named as metastin. The Kiss-1 gene in human is localized to chromosome number 1 (West *et al*., 1998) which codes for a 145 amino acid protein which can be proteolytically cleaved into different C-terminal fragments with nearly similar efficacy and affinity. The major C-terminal fragment appears to be metastin (Kisspeptin 54) but other derivatives like kisspeptin 14, kisspeptin 13 and kisspeptin 10 have been identified (Ohtaki *et al*., 2001). All kisspeptin have same C-terminal region as metastin molecule and they have RF-NH_2 motiff (Terao *et al*., 2004). Kisspeptins have been named as

switches to puberty as Kisspeptin-GPR 54 interaction has been shown to mediate the GnRH secretion which is the key event in the onset of puberty (Colledge, 2004). Circular Dichroism (CD) spectroscopy of Kisspeptin 13 can give an account of the bioactive conformation of the kisspeptin which may be critical in understanding the GPR-54/Kisspeptin interaction. Here in the present study, we have synthesized the Kisspeptin 13 by solid phase peptide synthesis followed by its purification through semi preparative RP-HPLC and studied its conformation through CD spectroscopy.

MATERIALS AND METHODS

Present study was conducted from 23rd September 2010 to 10th Jan 2011. Kisspeptin 13 (Val59-Ser60-Ala61-Tyr62-Asn63-Trp64-Asn65-Ser66-Phe67-Gly68-Leu69-Arg70-Tyr71NH_2) was synthesized through solid phase peptide synthesis using HBTU/HOBt coupling method employing Fmoc chemistry. All solvents were analytical grade. 1-(Bis (dimethylamino) methylene)-1H-benzotriazoliumhexafluorophosphate (1-) 3-oxide (HBTU), 1-hydroxy-benzotriazole (HOBt), Trifluoroacetic Acid (TFA) 1, 3-diisopropylcarbodiimide (DIC), Rink Resin (0.72 mmol equiv g^{-1}), piperidine, N,N-dimethylformamide (DMF), diisopropylethylamine (DIPEA) and all Fmoc-amino acids were purchased from Sigma Aldrich, St Louis, MO, USA.

Solid phase peptide synthesis: Kisspeptin-13 was synthesized on the rinkamide resin which is an acid labile resin widely used to prepare amide derivatives of peptides.

Preparation of the resin: Dimethyl Formamide (DMF) was added to solid support rinkamide resin and it was allowed to swell properly for 2 h at room temperature. Proper swelling of resin is critical for the good coupling efficiency.

Coupling of the 1st amino acid using DIPC as coupling agent: In a small glass vial which is containing swollen resin, 6 equivalent of Fmoc tyrosine, 3 equivalent of Diisopropylcarbodimide (DIPC) and 0.1 equivalent of p-dimethyl amino pyridine was added. The contents were mixed and incubated for 4°C for 3 h with constant shaking. Thereafter resin was washed twice with Dimethyl Formamide (DMF) followed by three washing of dichloromethane. Completeness of coupling was determined by use of Kaiser test, a qualitative test sensitive to primary amine. In a small test tube few beads were taken and to it two drops of each ninhydrin, phenol and pyridine reagent was added. This solution was heated for 3 min at 110°C. Development of colourless/yellow solution corresponds to satisfactory coupling efficiency.

Deprotection of Fmoc group: After successful coupling, a-floreny methyloxy carbonyl (Fmoc) groups of the coupled amino acid are to be removed for subsequent coupling of amino acids. To the tube containing coupled amino acid 2 mL of 20% piperidine was added and was kept at room temperature for 20 min with constant shaking. The beads were washed twice with DCM followed by three washing of DMF to get the deprotected tyrosine amino acid attached to rinkamide resin.

Coupling of subsequent amino acids: Subsequent coupling steps were carried out by the treatment of deprotected resin with a threefold molar excess of Fmoc amino acids, HBTU and HOBt for 2 h at room temperature under constant shaking. Between coupling and deprotection, the resin was washed twice with Dimethyl Formamide (DMF) followed by three washing of Dichloromethane (DCM).

Table 1: Constituents composition of the cleavage mixture

Constituents	Percentages
Trifluoroacetic acid (TFA)	82.5
Thioanisole	5.0
Ethanedithiol	5.0
Water	10.0
Phenol	5.0

Final cleavage of peptide from rinkamide resin: Peptide was cleaved out of the resin using the cleavage mixture in following composition given in Table 1.

The tubes containing beads (50 mg) were added with 200 μL of the above cleavage mixture and they were centrifuged at 3000 rpm for 3 min and supernatant from each tube was collected. The peptide was precipitated out of resin with chilled dry ether and washed thrice with diethyl ether. The peptide was finally vacuum dried and stored at -70°C.

Purification and analysis: The peptide was dissolved in HPLC grade water and analyzed for semi preparative RP-HPLC (Shimadzu, Tokyo, Japan) using C18 column employing binary gradient module consisting of 5% acetonitrile (Solvent B) in water and a limiting organic solvent. Absorption was monitored at 214 and 254 nm. Maximum resolution for the synthetic peptide mixture was achieved with a 36 min linear gradient of 0-90% solvent B at a flow rate of 2 mL min^{-1}. The peptide was purified by fine fractionation of the major peak and the elute was vacuum dried to obtain the purified peptide.

CD spectroscopy of the peptide: The spectra of kisspeptin-13 was studied using a CD spectropolarimeter (J-810 Model, JASCO) with 0.2 cm path length cuvettes from 200-320 nm. The routine calibration of the machine is done with d-10 campho sulphonic acid (60 mg 100 mL^{-1} water). The mean residual ellipticity ((θ)-degcm^{2}dmol^{-1}) were calculated using the equation (θ) = 100 θ cnl^{-1}, where θ is the ellipticity (mdeg), c is the peptide concentration (mM), n is the number of residues and l is the path length (cm). Data analyses and acquisition were performed using inbuilt spectra manager software provided with the machine. On an average six scans were taken with scanning rate of 20 nm min^{-1}. In order to ascertain the conformation adopted by the peptide, CD spectra of peptide were obtained in water and trifluoroethanol.

RESULTS AND DISCUSSION

Therapeutic potential of kisspeptin series of peptides have been analyzed for controlling metastatis (Lee *et al.*, 1996). They have been studied in several species as molecular switches for triggering puberty via GPR54/Kisspeptin interaction leading to release of GnRH (Messager *et al.*, 2005; Dhillo *et al.*, 2005; Gottsch *et al.*, 2004; Irwig *et al.*, 2005; Shahab *et al.*, 2005), thus mediating release of LH and FSH from gonadotrophs of the pituitary. Since the kisspetin peptides are important biomolecules having potential to be the vital therapeutic agents for few of the ailments in future, it is necessary to define its bimolecular conformation to further substantiate its role and to develop a few agonists for mimicking the therapeutic action for future drug use. To study the bioactive conformation of peptide, we synthesized kisspeptin 13 by solid phase peptide synthesis on rink resin by Fmoc method. Coupling efficiency was analyzed at every step using

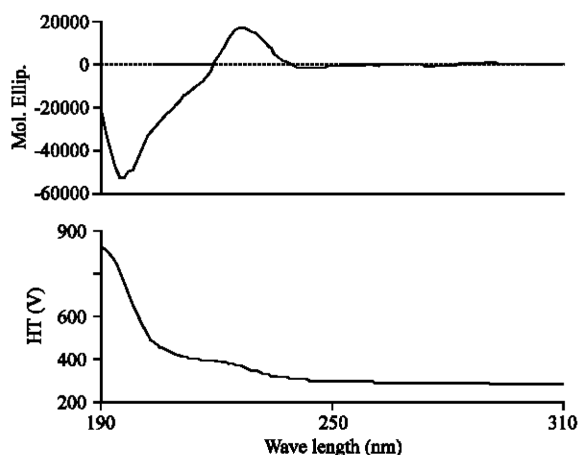

Fig. 1: Spectra of ovine kisspeptin 13 in water as depicted in the above graph in which molar ellipticity is plotted against wavelength

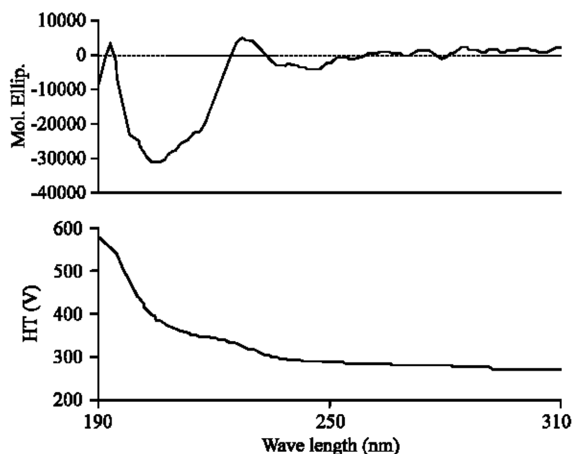

Fig. 2: Spectra of ovine kisspeptin 13 in 50% as depicted in the above graph in which molar ellipticity is plotted against wavelength

Kaiser test and final cleavage of the peptide was performed by using standardized cleavage mixture. The purified peptide was collected by fractionation of major peak corresponding to retention time of 23-24 min in 36 min linear gradient.

The CD spectra of the peptide (1 mg mL^{-1}) was evaluated in water, 50% and 75% Trifluroethanol (TFE). The CD spectra of kisspeptin 13 in the water mainly exhibited random coil structure with no significant alpha helical or beta sheet content which is evident from the negative minimum around 195 nm due to $n \rightarrow \Pi^*$ shift and positive maximum at 226nm due to perpendicular excitons of the $\Pi \rightarrow \Pi^*$ shift of the peptide. Figure 1 describes the CD spectra of peptide in water. Since ovine kisspeptin are rich in aromatic amino acid considerably, it is duly reflected in the CD spectra with positive maxima at 226 nm. The CD spectra of peptide were also recorded in increasing concentration of TFE as it is the structure inducing solvent. In 50% TFE peptide was observed to have slightly increased ordered structure even though still a major contribution to conformation was from disordered random coil structure (Fig. 2). Spectra were analyzed by spectramanager

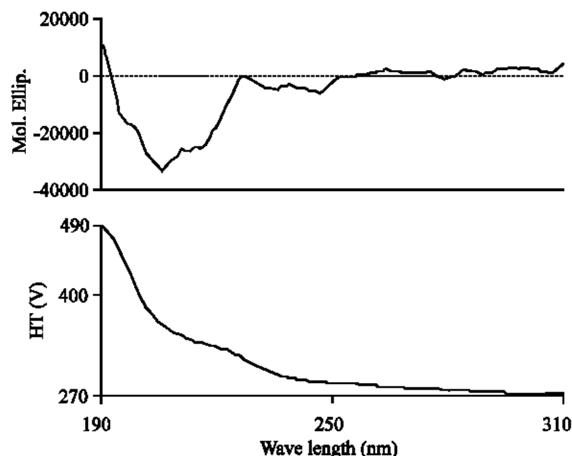

Fig. 3: Spectra recorded by ovine kisspeptin 13 in 75% TFE as depicted in the above graph in which molar ellipticity is plotted against wavelength

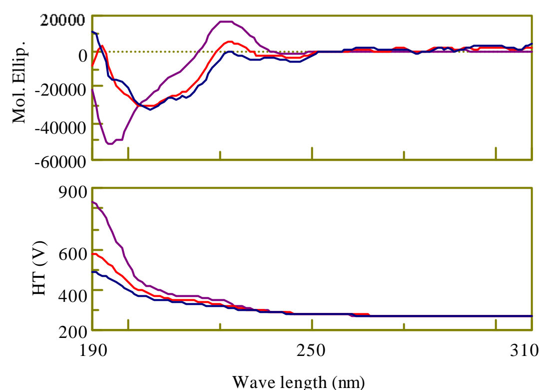

Fig. 4: Overlay of Peptide in water (magenta color), 50% TFE (red color) and 75% TFE (blue color)

software and it predominantly showed the induction of beta sheet like structure (24.1% Beta sheet, 1.6% helix and rest random coil structure). Peptide conformation in 75% TFE showed a typical negative minimum at 208 and 222 nm and increased positive maximum at 190 nm, suggesting the induction of alpha helical structure (Fig. 3). Spectra manager software analysis showed absence of the beta structure, 12.5% helical, 4.3% turn and rest random coil structure. The results of the CD spectra indicated that random coil structure was the major conformation of peptide in solution phase. On being dissolved in increasing concentration of TFE, peptide showed conformational versatility by showing slight induction of both beta sheet and alpha helix structures but still disordered random coil being the major contributor to the conformation (Fig. 4). Primarily disordered state of kisspeptin 13 could explain the mechanism by which it can bind to other non putative receptors and thereby may explain the multifunctional role played by peptide from metastasis suppressor and puberty switch to recently discovered role as insulin inhibitor. Using NMR spectroscopy Orsini *et al.* (2007) revealed kisspeptin 13 in detergent micelles (S.D.S) adopted a stable helix structure by virtue of last seven residues using NMR spectroscopy. In the present

study we could find that the peptide adopted an increasing proportion of stable helix conformation when dissolved in 50% TFE (1.6%) and 75% TFE (12.5%). But surprisingly we are reporting that ovine kisspeptin 13 adopts a sizeable proportion of β sheet content in 50% TFE which may be due to presence of large aromatic (Tyr, Phe and Trp) and β-branched (Val, Ile) amino acid residues taking more regular conformational structure like β sheet in structure inducing solvent. β sheet structure was absent in 75% TFE environment with an increased propensity of helical structure formation by peptide, can be explained by the increased helicogenic environment in 75% TFE.

CONCLUSION

Ovine Kisspeptin 13 adopted a disordered random coil conformation in solution phase and shows conformation versatility in different TFE environment but disordered state always remains the major conformation of peptide in solution. Random coil conformation may favor the multifunctional role of peptide by non-putative receptor binding as well as different binding kinetics for receptor-ligand interaction.

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