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EQUILIBRATION SHIFT FROM HOMOSERINE LACTONE TO HOMOSERINE IN MULTIMER CLONED RECOMBINANT T-20 PEPTIDE FORMED BY CNBr CLEAVAGE

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ABSTRACT

The CNBr molecule cleaves the protein molecule at the C-terminal end of the methionine leaves an extra amino acid homoserine lactone and homoserine which are in equilibration state. In analytical HPLC the homoserine form of the molecule eluted first followed by homoserine lactone form which creates the purity related consequences, 40-60%, having each form of the T-20 molecule. In this present work we cloned the T-20 peptide as Pentamer for high yield requirements with CNBr cleavage site having the histidine tag. In purification process, after CNBr cleavage of the Pentamer, the cleaved monomer was separated from other related impurities by reverse phase chromatography in which the purified monomer having an extra amino acid was showed two forms in HPLC. So the equilibration shift was needed to one form which was done by keeping the peptide at 37 C for 16 h with an unknown mechanism. The physical and chemical parameters of the two forms of the peptides in comparison with standard T-20 peptide were characterized by HPLC, Biological activity, Mass spectroscophy, Amino acid sequencing by MS.

KEYWORDS

T-20, Homoserine and Homoserine lactone.

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INTRODUCTION[1-3]

HIV type 1 (HIV-1) virus is a major causative agent for AIDS. Throughout the world over 33 million people affected with HIV and 20 million were died. So far 31 anti HIV drugs and combinations have been approved for clinical use. The current antiviral drugs directed at the reverse transcriptase and protease was first introduced in clinical practice. However, the presence of mutant forms of HIV

within a single infected individual means that no single drug is able to successfully suppress the virus. The therapy with potent combinations of three or more antiviral drugs called highly activated antiviral therapy (HAART) has been shown to rapidly reduce the viral count below detectable range for periods of several years or more. The mechanism of viral entry into target cell requires two functional envelope glyco proteins gp41 and gp120. The gp41 is a transmembrane glycoprotein and the molecular sequence includes heptad-repeat regions (HR1 and HR2), reflecting the presence of periodic hydrophobic regions found in α-helical coiled-coil structures. The gp120 is a surface glycoprotein and some of the portions of the gp120 bind to the CD4 receptors as well as chemokine coreceptors on target T-cells. After gp120-CD4-coreceptor binding the gp41 subunit undergoes a conformational change that promotes the fusion of viral and cellular membranes, resulting the entry of the viral core into the cell, entered into nucleus and finally integrated into viral genome. T- 20 also known as Enfuvirtide or Fusion is the first in a new class of anti retroviral drugs targeting the entry stage of the virus life cycle. It is a 36 amino acid peptide that preventing the gp41-gp120 mediated fusion with the host cell membrane (2-7). In this present strategy, we transformed the T-20 Pentamer gene present in the expression vector pET 28a having histide tag on both N and C terminal sides of the fusion protein and having CNBr cleavage site between each monomer into E.coli. The expressed fusion protein was primarily purified by Ni-Affinity chromatography and the fusion protein was cleaved by CNBr. Finally the cleaved monomer was purified by reverse phase chromatography. The purified monomer having two forms which are in equilibration state, homoserine T-20 and homoserine lactone T-20 are converted into single form by equilibration shift study which was done by using different parameters like temperature, time and various peptide concentrations.

MATERIAL AND METHODS[4,5]

Chemicals, Kits and tools

Restriction enzymes (MBA Fermentas), Primers, DNA sequencing (MWG Bengalore), LB Media (Hi Meadia), Gel extraction, plasmid purification, protein estimation kits (Genei, Bengalore), S-30 Matrix, Ni Sepharose, FPLC (GE healthcare, Sweden), Analytical C-18 column (Vydac), HPLC (Shimadzu, Japan), FPLC (USA), Lyophilizer (Virtis).

Expression

The recombinant expression vector having T-20 pentamer gene was transformed into E.coli (BL21) and plated on kanamysin positive plates. The single positive colony was picked and inoculated into 5 ml LB madia, having 10µg/ml kanamycin, cultured overnight at 370C.The overnight culture was sub cultured in the ratio of 1:25 and grown the cells upto the OD600 reached to 0.9-1.0. The cells were induced with 1mM IPTG and continued upto 4 h. The samples were collected every hour lysed and loaded on the SDS-PAGE for expression analysis.

Fermentation

The glycerol stock which is having E.coli Rosetta T20-pentamer was grown for 6h in 100ml LB medium containing 10 µg/ml kanamycin at 37oC and 200 rpm. Then the culture was inoculated into 5l fermentor (B. Braun, Germany). The cells were grown upto the OD600 reached to 10-12, and then the fermentation batch was induced with 1mM IPTG. After completion of 4hrs post induction the culture was collected and centrifuged at 4000rpm for 20min at 4oC, the cell pellet was stored at -20oC for further processing.

Purification methods

The cell pellet was mechanically lysed by using bead beater in Tris buffer pH 8.0. The lysate was solubulized by 1% Triton X-100, centrifuged at 12000rpm, and collected the supernatant. The supernatant was loaded on to Ni-Affinity column and eluted with 100mM Imidazole. The eluted fusion protein was precipitated by adjusting the pH from 8.0 to 5.0 by 0.1N HCl. The precipitated fusion protein was collected by centrifugation.

The fusion protein was dissolved in 70% formic acid and the CNBr was added in the molar ratio of 1:150mM, protein to CNBr and the final concentration of fusion protein was adjusted to 10mg/ml. The reaction was done at room temperature in dark room for 16h and the reaction was terminated by the dilution of reaction mixture with water in the ration of 1:5. The diluted cleavage sample was loaded on to the reverse phase S-30 column and the unbound sample was neutralized with sodium hypo chlorite. The bounded protein was eluted with the linear gradient of acetonitrile having 0.1% TFA. The pure fractions were pooled and lyophilized.

Standard T-20 preparation

The standard T-20 which is having 36 amino acids like native peptide was produced by cloned into pGEX vector having Glutathione sulphonyl transferase (GST) with Fxa cleavage site. The fusion protein was purified by GST-Affinity chromatography and the fusion protein was dialyzed against Factor Xa activation buffer (50mm Tris, pH 8.0, 2mM CaCl2). The fusion protein was cleaved by FXa in the ratio of enzyme to substrate is 1:100. The cleaved solution was loaded on to GST column collected the unbound and loaded on to reverse phase column. Finally the pure fractions were lyophilized and used as standard peptide.

Equilibration shift from Homoserine lactone to Homoserine

The lyophilized peptide fractions were dissolved in carbonate buffer, pH >8.0. The separate experiments were designed for equilibration shift studies by setting different parameters which are followed by peptide concentrations (1mg, 2mg and 5mg/ml), different temperatures (4^0C, 25^0C and 37^0C) and time factor (24, 48 and 72 hrs). After completion of each reaction the test sample was loaded on to analytical HPLC by using C-18 column and analyzed the results by overlapping the all tested peaks.

Biological activity by T-20 phenotypic sensitivity assay

The biological activity was carried out by using p24 antigen capture assay kit in which the p24 is a viral capsid protein represents the viral content in the test sample. The T-20 peptides having Homoserine lactone form , Homoserine form and standard T-20 were preincubated with virus in 100µl RPMI-1640 plus 10% fetal calf serum (FCS) for 1h. Following this 5 X 10^5 SUP-T1 cells in 100ul was added to give final concentrations of 1, 0.5, 0.2 and 0.1µg/ml T-20. Levels of p24 antigen in supernatants were measured on day 5 by p24 antigen assay kit[6-10].

RESULTS

The transformed T20-pentamer plasmid expression was 23% from total E. coli proteins. After harvesting, the cell pellet was lysed and the fusion protein was observed that partially came in lysis supernatant. So for the complete recovery of the fusion protein the lysate was solubulized in the non ionic detergent, Triton X-100 and to the Ni-Affinity column, the eluted fusion protein purity was said to be greater than 80%. The cleavage efficiency and further purification steps were affected by the purity of the fusion protein. After the CNBr cleavage slight other related contaminants (dimer, trimers) were separated by reverse phase chromatography with greater than 95% purity. The pure acetonitrile fractions were analyzed on SDS-PAGE and lyophilized. The lyophized powder was negligible solubility in water, low solubility in neutral buffers and more at the pH greater than 8.0 buffers. The standard T-20 peptide having 36 amino acids was purified by GST-Affinity and reverse phase chromatography was having 98% purity and Comercial protein from Roche and are used as standard for structural and functional studies in the present study in Figure No.1-10.

T-20 peptide showing two forms was kept at different conditions like temperature, peptide concentration in various time points were studied by using analytical RP-HPLC. The study results were tabulated in the Table No.1 and 2.

DISCUSSION

The therapeutic peptides drugs were gaining importance regularly for most of the life threatening diseases. AIDS is most privilege disease in the

world wide. The new class of drug T-20 used as fusion inhibitor in the treatment of HIV-1 infected patients. The high therapeutic dose was needed for this drug (90mg twice daily). For increasing the production of T-20 peptide we followed the recombinant methods by multimer cloning approach. The CNBr cleavage is the method of choice for high efficient cleavage with less cost. But in the final stages of peptide purification by the above described methods leads to the conversion of 37nth amino acid, CNBr cleavage site methionine into two forms of T-20, HSL and HL. The methods developed earlier for the conversion HSL to HL which is slow conversion reaction (8) in which the protein solution was treated with pyridine acetate buffer, pH 6.5 but in this case the solubility of the peptide was negligible at this pH. Solubility of the T-20 is more than 100mg/ml at pH is >8.0. Buffer of choice is sodium bicarbonate buffer pH9.2. RP HPLC is the method of choice to access the purity. Due to presence of HS and HSL the main peak is split into two peaks in RP HPLC. With time the HS and HSL ratios show variations. Peptide with high concentrations HSL to HS shift is less with increasing temperature and time. But the peptide is less stable at higher temperature. Whereas with lower concentrations of peptide HSL to HS shift is more. This also confirmed by MS[9]. Shifting of HSL to HS does not affecting the potency (biological activity), structure and stability of the peptide. This will help in the analysis of peptide with host derived impurities and degraded or peptide related impurities.

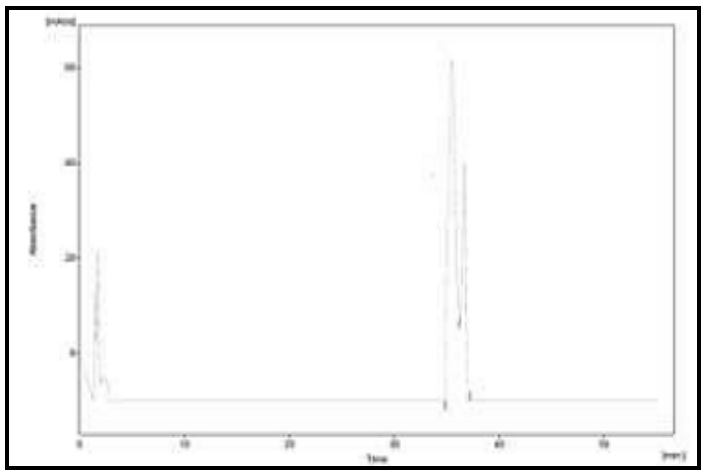
Table No.1: Percentage of two T-20 forms at different temperatures

Initial	4°C (1mg/ml)			25°C (1mg/ml)			37°C (1mg/ml)		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
HS – form (40%)	40%	45%	47%	45%	55%	60%	>95%	The HS form of T-20 undergone different modified forms	The HS form of T-20 purity was decreased
HSL-form (60%)	60%	55%	53%	55%	45%	40%	<5%		

Table No.2: Percentage of two T-20 forms at different peptide concentrations

Initial	1mg/ml (37°C)			2mg/ml (37°C)			5mg/ml (37°C)		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
HS – form (40%)	>95%	The HS form of T-20 undergone different modified forms	The HS form of T20 purity was decreased	55%	65%	80%	40%	The both forms of T-20 undergone different modified forms	The both forms of T-20 purity was decreased
HSL-form (60%)	<5%			45%	35% (purity was decreased)	20% (purity was decreased)	60%		

HS= Homoserine; HSL= Homoserine lactone



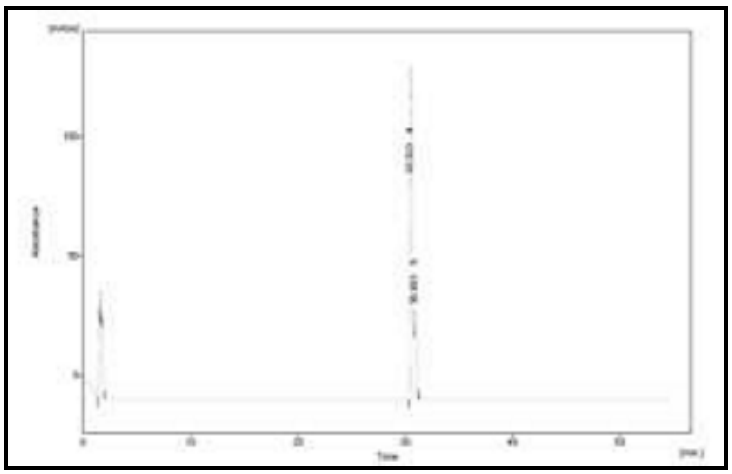
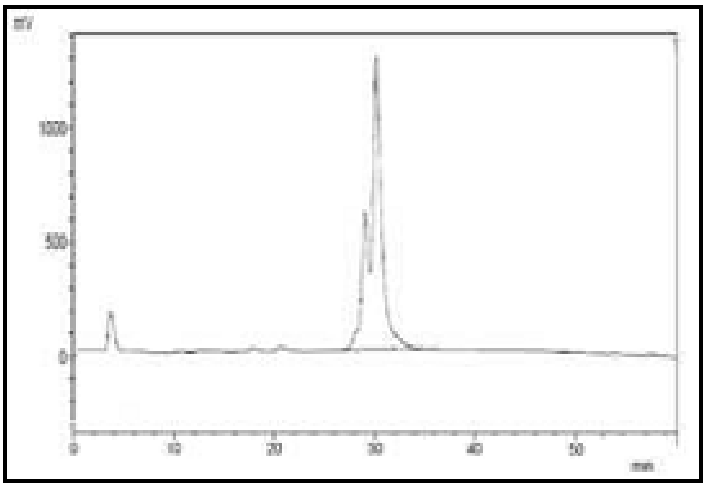


Figure No.1: Homoserine 60% Homoserinelactone 40%: Figure No.2: homoserine 80% Homoserine lactone 20%



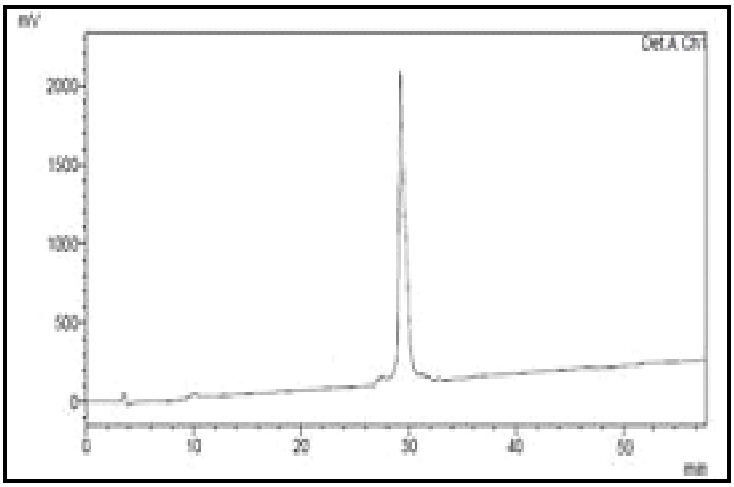
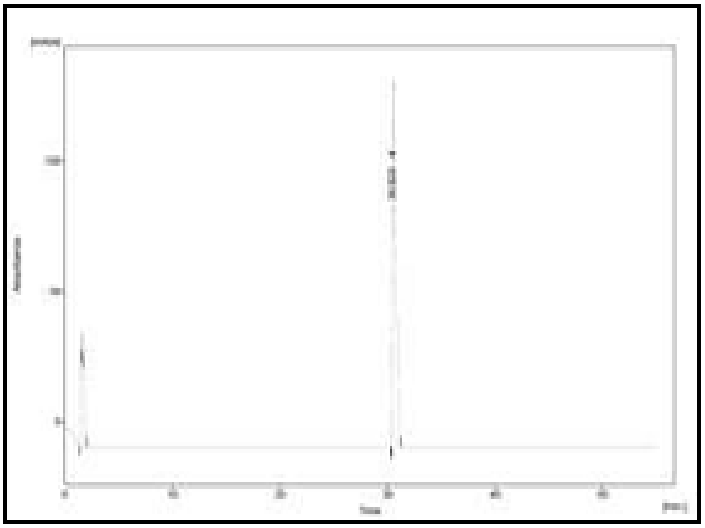


Figure No.3: homoserine 40% Homoserine lactone 60%: Figure No.4: Homoserine 5% Homoserine lactone 95%



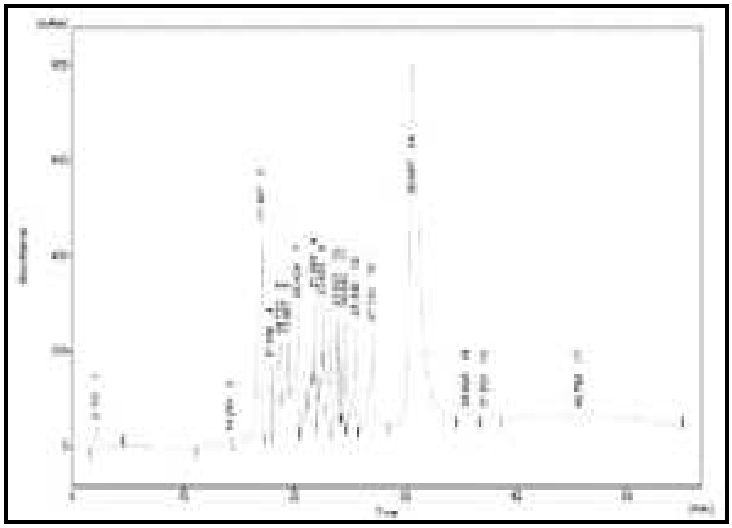


Figure No.5: Standard peptide (From GST clone) **Figure No.6: Degraded**

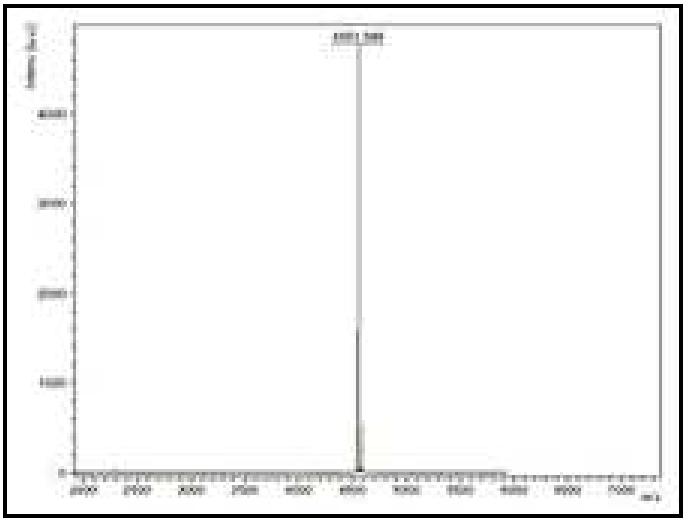


Figure No.7: HS form of T-20

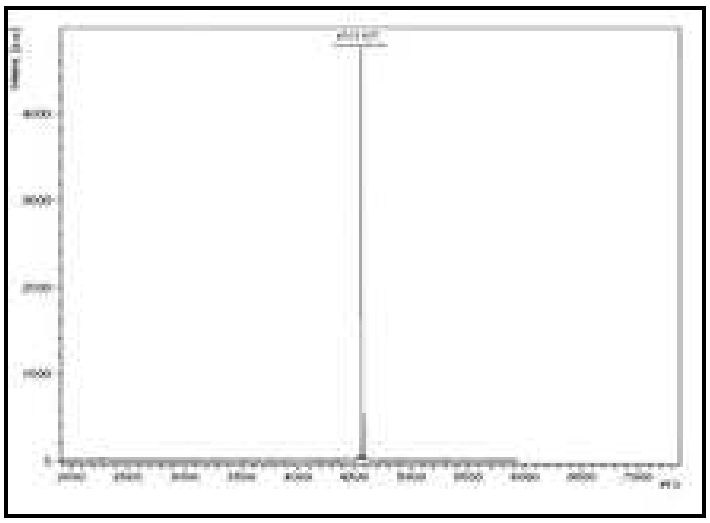


Figure No.8: HL form of T-20

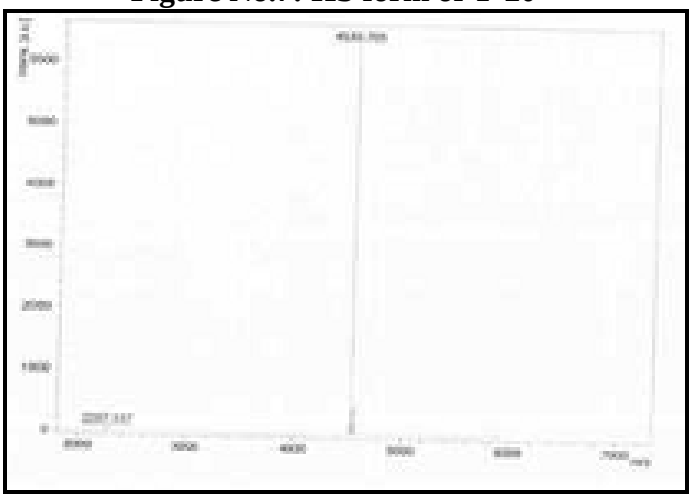


Figure No.9: Standard T-20

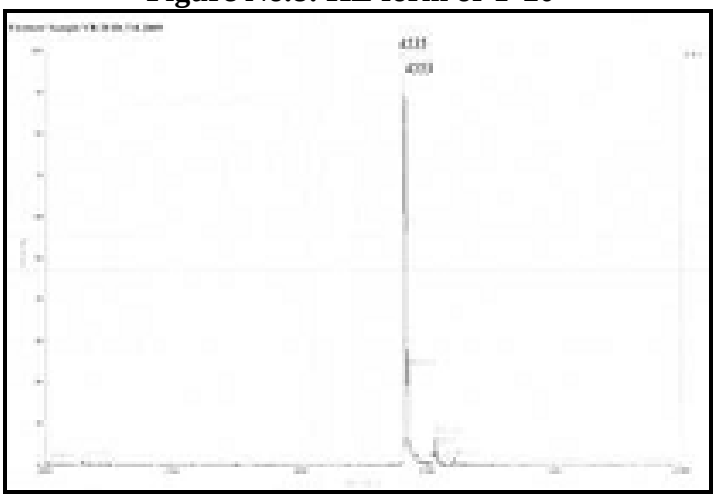


Figure No. 10: Both HL and HS forms of T-20

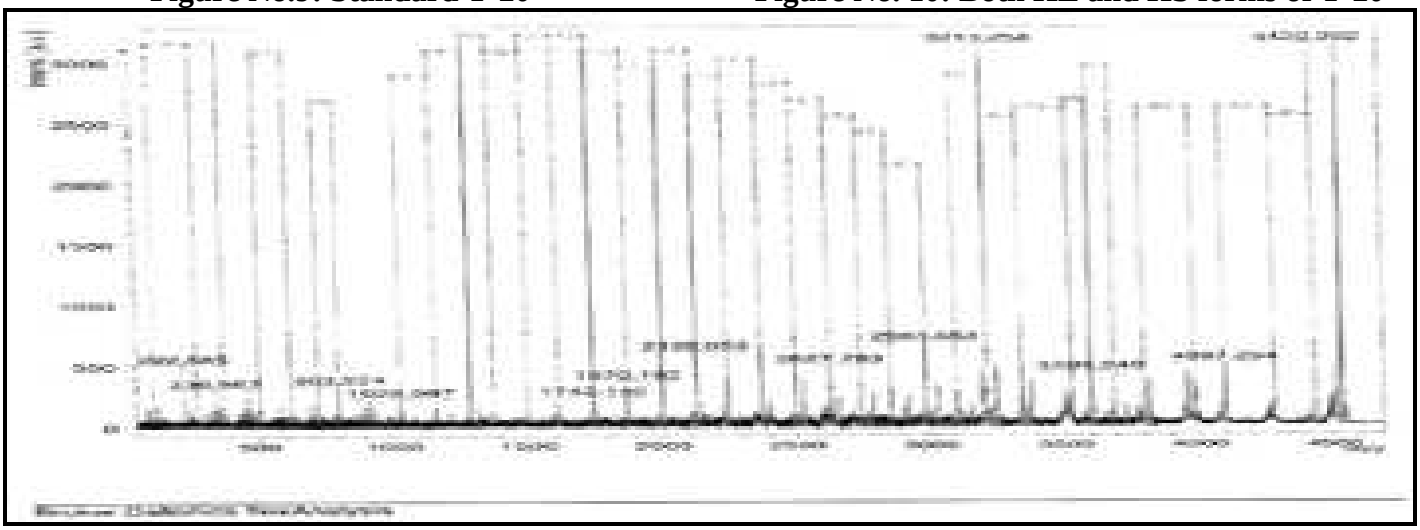


Figure No.11: MS of T-20

CONCLUSION

The chemical cleavage reaction resulted in two forms of peptide molecules i.e. Home serine and Homoserine lactone. The current study has been examined the chemical conversion of Homoserine lactone to Homo serine without any other chemical contribution. Also, the study has demonstrated that the target monomer purity was achieved post conversion into single form

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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