Proteolytic truncation of human transthyretin linked to amyloidosis is mediated by a trypsin like enzyme: *In vitro* demonstration using model peptides

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Abstract

**Background**: A number of human conditions collectively known as amyloidosis were found to be associated with self-aggregation of a specific group of proteins. This fibril like protein aggregates deposit in different tissues including the central nervous system leading to amyloid polyneuropathies (AP), systemic senile amyloidosis (SSA), amyloidotic cardiomyopathy (AC) and carpal tunnel syndrome (CTS). The most common protein in this group is human transthyretin (hTTR). Similar to beta amyloid peptide of Alzheimer’s Disease, hTTR forms amyloid type fibrils following its proteolytic cleavage at the C-(carboxy) terminus leading to aggregation. hTTR is normally present in blood where it binds with thyroid hormone thyroxine T4 and Retinol Binding Protein-Vitamin A complex and mediates their transport. It is also present in lower amount in cerebrospinal fluid as a major carrier of thyroxine hormone. Deposition of hTTR fibril aggregates is responsible for SSA usually observed in older population with age >60 years which is regulated by its mutant forms. The exact mechanism of this disorder is still unclear. Studies confirmed that C-terminally truncated hTTR undergoes rapid self-aggregation leading to amyloidosis. The site of this cleavage and the protease involved has not been fully characterized although it is proposed to be at Lys48↓Thr49 residues.

**Objective**: The major goal of this study is to confirm that hTTR is cleaved by a trypsin like enzyme at the position indicated by vertical arrow Ala-Ser-Gly-Lys 48↓Thr49-Ser-Glu-Ser and that this cleavage can be blocked by a trypsin inhibitor.

**Methods**: A 20 mer peptide comprising the wild type sequence from residue (41-60) of hTTR was synthesized, purified by RP-HPLC and fully characterized by mass spectrometry. Three additional mutant peptides namely Lys48/Ala, Ser52/Pro and Thr49/Ala were also prepared. These mutant peptides were designed because of their pathological implications in SSA syndrome in varying degrees. All peptides were digested in vitro with trypsin under identical condition to examine and compare their cleavage efficiencies.

**Results**: Our results showed that while the control wild type peptide was fully cleaved at Lys48↓Thr49 by trypsin within an hour the corresponding Lys48/Ala mutant remained completely uncleaved under identical condition. In contrast the Ser52/Pro and Thr49/Ala mutant peptides were cleaved with varying efficiency. These results are consistent with those reported for the physiological full length hTTR proteins. Moreover the above cleavage can be blocked efficiently by a trypsin inhibitor (dimethyl formamide), reported for the first time in this study. Energy minimised 3D modeling study revealed that Lys48/Ala mutant exhibits a unique structural conformation compared to the corresponding wild type control peptide suggesting its potential role in regulating proteolytic cleavage. Overall our data confirmed the key role of a trypsin like enzyme in hTTR proteolysis leading to its truncation which facilitates its self-aggregation leading to amyloidosis. The study also highlighted a potential therapeutic strategy for hTTR associated amyloidosis by targeting this enzyme with specific inhibitors.

**Keywords**: Human transthyretin (hTTR), hTTR proteolysis, hTTR mutation, trypsin, reverse phase high performance layer chromatography (RP-HPLC), mass spectrum, trypsin inhibitor, trypsin activation, protein aggregation
Introduction
Misfolding and self aggregation of a specific group of proteins in physiological systems have been linked to various human disorders and diseases [1,2]. Of particular interest and importance is the protein human transthyretin (hTTR) (also known as prealbumin) which has major implication in Familial Amyloidotic Polynecropathy (FAP) and associated conditions [3,5]. hTTR is mainly synthesized in the liver and brain’s choroid plexus region. It is then secreted and circulated in the plasma as a soluble protein [6,7]. FAP comprises a family of inherited amyloidosis affecting the peripheral nerves and this is caused by deposition of highly neurotoxic insoluble self-aggregated hTTR and this can often be fatal [8,9]. Our understanding of this disease and associated hTTR aggregation is now slowly beginning to emerge following extensive research in the field [10,11]. hTTR is known to exist in various genetic mutant forms. In fact accumulated research has established that mutations in hTTR can be linked to aggregation state and pathogenesis of FAP [12-16]. For example among others hTTR variants with Val10Met and Ser52Pro mutations became of high interest because of their potential implications in FAP [15,16]. Pathogenic mutated TTR proteins form fibril like aggregates more efficiently than the nonpathogenic and usually non-mutated TTR. The deposition of these aggregates in different tissues including central nervous system leads to FAP as well as Systemic Senile Amyloidosis (SSA) [17], Amyloidotic Cardiomyopathy (AC) [18] and Carpal Tunnel Syndrome [19].

It was further established that calcium influx in neurons mediated by TTR via specific calcium and voltage-gated sodium channels can be regulated depending on the nature of mutation in TTR and this remains an important contributing factor in pathogenic stage of FAP [20]. Interestingly this type of calcium dysregulation is also associated with other neurodegenerative diseases involving amyloidosis that include Alzheimer’s disease [21]. Recently various biochemical and histological studies in cells and tissues suggested that the aggregation and fibril formation of hTTR are dependent not only on the types of mutation but also more importantly on its proteolytic cleavage. This cleavage leads to a C-terminally truncated hTTR fragment which has been found to aggregate far more efficiently than the full length or uncleaved physiological form [22,23]. Such molecular events involving proteolytic truncation favouring subsequent aggregation have been noted for human tau and alpha synuclein proteins which have been linked to Alzheimer’s and Parkinson’s diseases respectively [24,25]. It is important to point out that these and other amyloid proteins do not bear any amino acid sequence homology. However they all possess a high content of beta sheet structure and propensity to form fibrillar structures. The overall secondary structure of the protein promotes the formation of aggregated protein that is responsible for neurotoxicity [26].

hTTR is a 60 kDa extracellular protein which is composed of 4 identical subunits of 15 kDa size. It is synthesized in the liver and brain and its gene is located in chromosome number 18. As a soluble protein it circulates in the blood where it binds with thyroid hormone thyroxine T4 and Retinol Binding Protein-Vitamin A complex and mediates their transport. It is also present in lower amount in cerebrospinal fluid as a major carrier of thyroxine hormone [27,28]. hTTR mediated FAP is an inherited autosomal dominant disease which has no global boundary. It mostly affects older population age higher than 60 years. The disease exhibits variable clinical symptoms the most common of which are acute heat sensation, painful paresthesias leading to autonomic dysfunction [17]. In recent years hTTR has also been described as an enzyme with matrix metalloprotease activity [29,30]. Several substrates of hTTR have been reported among which the most important is Apo-1A protein [31].

Despite many functional activities, the aggregation of hTTR and its pathogenesis associated with amyloidosis remain as the major focus of hTTR research. So far the detailed molecular mechanism leading to hTTR aggregation is still not completely understood although significant progress has been made in this regard. Thus recent studies confirmed that C-terminally truncated hTTR produced by proteolysis undergoes self-aggregation much more rapidly and efficiently than the full length mature hTTR and this occurs in a fibrillary and amyloidogenic pathway leading to neurotoxicity. The site for this cleavage has recently been proposed as between Lys48 and Thr49 but the protease responsible has not yet been identified although it was proposed to be a member of trypsin family. Moreover structural studies revealed that recombinant hTTR protein lacking the first 50 amino acids (from residue 21-71) exhibit an unfolded sheet-like structure unlike the full length mature protein which may explain its rapid self-aggregation compared to the full length protein [32]. Interestingly recombinant hTTR protein lacking the first 37 amino acids (residue 21-57) exhibits a folded structure like the full length protein and showed much less propensity for self-aggregation [32].

A more recent study revealed that for both wild type and variant hTTR, a significantly high level of hTTR (49-127) fragment was found to be present in ex vivo fibril aggregates along with only a small quantity of full length proteins [33]. Moreover the above hTTR sequence domain has also been reported to be the major constituent of wild type and hTTR variants derived amyloid fibrils linked to cardiomyopathy [34]. Based on enzyme digestion study of recombinant and variant hTTR proteins it is further proposed that proteolytic cleavage of hTTR and its variants at Lys48 and Thr49 leading to the formation of C-terminal fragment (49-127) may be a crucial event in hTTR aggregation and associated pathogenesis. The presence of high levels of this fragment aggregate in the amyloid deposit supports this conviction. So far the enzyme responsible for this cleavage has not yet been identified although it is predicted to be a trypsin like enzyme. The proposed biosynthesis of mature hTTR protein from its precursor including its proteolysis leading to enhanced aggregation is shown schematically in Figure 1.

The objective of this study is to confirm using model peptides that hTTR is cleaved by a trypsin-like enzyme at the carboxy
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The study on the role of hTTR mutation is challenging and the goal is to study the functional consequence of those key hTTR mutations that are located in close proximity of the cleavage site and are associated with severe pathogenic amyloidosis. The study of the role of hTTR mutation is challenging and complex due the discovery of at least 100 different point mutations in hTTR with severe to mild or no implications to amyloidosis [http://amyloidosismutations.com/atrr.html]. A significant number of these mutations have effects on hTTR aggregation and associated pathogenesis [34]. However, only a selected number of mutations in hTTR have been strongly linked to high pathogenic severity of amyloidosis. It is suggested that such mutations in hTTR lead to instability of its tetrameric structure which ultimately fold apart as monomer that then self-aggregates into fibril structures promoted by its tetrameric structure which ultimately fold apart as monomer to cause rapid aggregation of hTTR and high degree of pathogenic amyloidosis. The solid thick line mutations are known to cause less aggregation.

Figure 1. Schematic diagram showing the biosynthesis of mature hTTR protein and its various structural domains. The figure also highlights some of the key natural mutation sites with either pathological or non-pathological consequence as well as the proposed proteolytic cleavage site indicated by a vertical arrow with the amino acid sequence. NT: N-terminal; CT: C-terminal. The mutations are indicated in vertical solid and dotted lines. The dotted line mutation is known to cause rapid aggregation of hTTR and high degree of pathogenic amyloidosis. The solid thick line mutations are known to cause less aggregation.

Materials and methods
All Fmoc amino terminal protected amino acids (L-configuration) with an additional side chain protection as needed, peptide coupling agents like N,N,N’,N’-Tetramethyl-O-[(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), Diisopropyl ethyl amine (DIEA), Poly Amino Linke Polyethylene Glycol Polystyrene (PAL-PEG-PS) resin for peptide synthesis, organic solvents such as Acetonitrile (ACN, HPLC grade); Dimethyl sulphoxide (DMSO, analytical grade) and Dime-thyl formamide (DMF, analytical grade) were obtained from Bachem Inc (Torrance, Can, USA), Calbiochem Novabiochem Inc. (San Diego, CA, USA), Sigma-Aldrich Chemical Company (Milwaukee, WI, USA), Neosystems Inc. (Strasbourg, France) and PE-Biosystems (Foster City, CA, USA). Trifluoroacetic acid (TFA, analytical grade) and all reagents such as Phenol, Triisopropyl Silane (TIS) and Ethane Dithiol (EDT) constituting Reagent B [36] for peptide deprotection and its cleavage from bound resin were purchased from Aldrich-Sigma Chemical (Milwaukee, WI, USA). Matrix Assisted Laser Desorption Ionization (MALDI) and Surface Enhanced Laser Desorption Ionization (SELDI) time of flight (tof) mass spectra (MS) were recorded using Voyager (Model: DE-pro, Bench top, Applied (previously PerSeptive) Biosystems, Framingham, Ma, USA) and Ciphergen Protein Chips (Fremont, Ca, USA) instruments respectively. The mass spectra plates namely the re-usable gold plates for SELDI and stainless steel plates for MALDI were obtained from respective companies. 1-Cyano 4-Hydroxy Cinnamic acid (CHCA) or 2,5 Di-Hydroxy Benzoic acid (DHB) (Sigma-Aldrich Chemical Company) were used as energy absorbing matrices. The enzyme recombinant bovine trypsin (Mol Wt 23.3 kDa) and its fluorogenic substrate Boc-Glu-Ala-Arg-MCA, and Boc-Arg-Val-Arg-Arg-MCA (MCA: 4 Methyl 7 Coumarinamide) required for in vitro trypsin assay were bought from Sigma-Aldrich Company.

Peptide synthesis
All peptides derived from hTTR sequence (Table 1), each 20 amino acids long were synthesized on an automated solid-phase peptide synthesizer instrument (Intavis, Multipep model, Germany) using Fmoc (Fluorenyl methoxy carbonyl) mediated chemistry and HBTU in presence of DIEA as coupling reagent [21]. The following amino acid side chain protecting groups were used, tBu (tertiary butyl) for Ser, Thr and Glu residues; Trityl (triphenyl methyl) for His and Boc (t-butyloxycarbonyl) for Lys. The synthesis started from carboxy (C-) to amino terminus (N-) end on an unloaded Fmoc-protected tentagel (a PAL-PEG [poly amino linker poly ethylene glycol] cross linked to PS [polystyrene]) resin. Following completion of
synthesis, peptides were cleaved off from the resin and fully deprotected at the same time by treatment with Reagent B consisting of 88% TFA, 5% phenol, 5% water and 2% TIS [36,37] for 3h at ambient temperature. The crude peptides thus obtained were purified by reverse Phase High Performance Layer Chromatography (RP-HPLC) as described later and fully characterized by SELDI and MALDI Mass Spectrometer using CHCA or DHB as an energy absorbing matrix [38].

Peptide purification by RP-HPLC All crude peptides were purified by RP-HPLC using C18 Silica gel analytical column (Varian, 10x25 cm column size). During RP-HPLC purification, peptides were separated using a linear gradient of Solvent B from 10% to 90% in Solvent A [Solvent B=0.1% TFA in ACN and Solvent A=0.1% TFA in water]. Fractions were collected and analyzed as the elution was monitored on-line by photodiode array (PDA) detector with wavelength fixed at 214, 230, 260 and 280 nm. Peaks were collected and lyophilized twice to a white fluffy powder. It was then subjected to analysis by mass spectrometry for their identifications as described in [39].

SELDI-tof mass spectrometry SELDI-tof mass spectrum was performed on re-usable clean gold plate chips with 2 μl of sample and 2 μl of CHCA or DHB matrix solution as described earlier [38,39]. Each mass spectrum was calibrated against pure commercial ACTH (1-17) peptide (MW 2,093.71 Da), purchased from Sigma-Aldrich Company both as an internal and external standard.

Trypsin assay The protease activity of commercial trypsin was measured using the fluorogenic substrate Boc-Arg-Val-Arg-MCA or Boc-Glu-Ala-Arg-MCA (100 μM final concentration). Typically 5 μl of freshly prepared trypsin solution (0.1 mg/ml in water) was incubated with peptidyl fluorogenic substrate in an aqueous buffer consisting of 25 mM Tris + 25 mM Mes, pH 7.4 and the fluorescence released at various times was measured with a fluorometer instrument with excitation and emission wavelengths fixed at 370 and 460 nm respectively. The experiment was conducted in a 96-microtitre well plate (black color, 50 μl volume capacity) using fluorescence spectrophotometer (Molecular Devices Co, USA) [38,39].

Trypsin inhibition by DMF: K\textsubscript{i} determination This was performed by initial rate and stop-time assays at 37°C in 50 μl final volume in a 96-well plate (Dynetac) in a buffer solution of 25M Tris + 25M Mes, pH 7.4. The experiment was performed with three substrate (Boc-RVRR-MCA) concentrations of 25 μM, 50 μM, and 100 μM, 1 mg/ml trypsin and varying amounts of DMF ranging from 0-30 μl and buffer. The release of fluorescence was monitored by a spectrofluorometer (Spectramax, Gemini XS) at excitation and emission wavelengths of 360 nm and 470 nm respectively. The K\textsubscript{i} (inhibition constant) was derived from Cornish-Bowden plot using the protocol published earlier [39,40].

Peptide digestion study hTTR peptide digestion with trypsin Absence of trypsin inhibitor hTTR(41-60) peptide (10 μg) was dissolved in water (1μl), then diluted with buffer 25M Tris + 25M MES, pH 7.4 (30 μl), and digested with trypsin (10μl, 1 mg/mL) with shaking (60rpm) at 37°C. The reaction was monitored at various times ranging from 0 min - 3 hour, depending on the nature of the peptide used, by extracting 10μl of the reaction aliquot and terminating the reaction by adding 2μl of glacial acetic acid. This procedure was also followed for the digestion of other peptides, namely the mutants: S52/P, K48/A, and T49/A (Table 1). Each aliquot was analyzed by RP-HPLC for the cleavage efficiency. Peaks were collected, lyophilized, and characterized by SELD-tof mass spectroscopy.

Presence of trypsin inhibitor (DMF) Each peptide digestion with trypsin was monitored in the absence and presence of a trypsin-specific inhibitor, namely DMF, reported for the first time in this paper. The protocol used is the same as indicated above, except that along with the control digestion, we have included two additional digestion experiments using the inhibitor at two different concentrations, one considered as low and the other as high. The blockage of the cleavage in the presence of the inhibitor was assessed by RP-HPLC chromatograms as discussed earlier.

3-D molecular modeling study 3D molecular model structures of all synthetic hTTR peptides as well as the full length protein molecule including the mutants were generated by using algorithms based on Hyperchem v 11.0 software program (Hypercube Inc). Theoretical energy minimised geometry in vacuo was used following Polak-Ribiere energy minimisation algorithm [39,40]. The presence

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Da: Dalton; MW: Molecular weight; ↓: Proposed cleavage site. The mutated amino acid residues are indicated in bold underlined characters.

Table 1. List of various peptides derived from hTTR protein sequence encompassing the proposed protease cleavage site and a few key mutations. Each peptide is 20 amino acids long.

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of potential H-bonds were assessed and were displayed in dotted lines in the model figure.

Statistical analysis
Unless otherwise indicated, results were compared using Student’s t test. A p value of less than 0.05 was considered significant. Each experiment was performed in triplicates and the data were used for statistical purposes.

Results
Peptide design
hTTR monomeric protein bio-synthesised in the physiological system as a 147 amino acid long precursor protein (~20 to 127) (Calculated MW: 15,887 Da) which following cleavage by signal peptidase generates the mature monomeric chain of 127 amino acids long (1-127 residues) (Calculated MW: 13,761 Da). For numbering of amino acid sequence in all hTTR protein and its peptides we follow the above common system based on 127 amino acids long mature hTTR protein. The biosynthesis, the various structural domains, cleavage sites and a few selected key mutations in hTTR are shown schematically in Figure 1. The above single monomeric chain forms a strong tetrameric complex (Calculated MW ~55,044 Da) that exhibits its physiological and biological activity [33-35]. A recent study with hTTR wild type as well as its key physiological mutant variants suggested that each monomeric form of hTTR is proteolytically cleaved likely by a trypsin like enzyme and this site may be located not far from 50 amino acids from the N-terminal end. This conclusion is derived from the findings that (i) an N-terminally 50 amino acids truncated hTTR recombinant protein aggregates at a much faster kinetic rate than the full length hTTR protein and (ii) in contrast an N-terminally 37 amino acids truncated recombinant hTTR protein aggregates almost at the same rate as the recombinant full length hTTR [27]. A more recent study identified this cleavage site as between Lys48 and Thr49 [33].

In order to confirm this we designed a peptide encompassing this cleavage site covering 20 amino acids (from residue 41-60 of mature hTTR sequence) with 10 amino acids on either side of the proposed cleavage site. Along with this wild type peptide we also designed two additional peptides which contain the physiologically relevant mutations Ser52/Pro and Thr49/Ala. The rationale for this is based on the published observation that implicated the first mutation (Ser52/Pro) to most pathogenic state of amyloidosis followed by the second mutation (Thr49/Ala) [33,35]. A fourth peptide containing Lys48/Ala mutation has also been designed in order to examine the importance of basic Lys48 residue in hTTR proteolysis. All peptides with their molecular weights and amino acid sequences are listed in Table 1.

Peptide synthesis and characterization
All hTTR peptides with carboxy terminal in amide form, were successfully synthesized by Fmoc based solid phase chemistry, purified by RP-HPLC and fully characterized by SELDI tof mass spectrometry [39-41].

Thus the SELDI tof mass spectra of purified peptides P1, P2, P3 and P4 as displayed in Figure 2 exhibited intense peaks at m/z 2,133.4, 2,143.6, 2,075.9 and 2,104.1 Da respectively which are in excellent agreement with the corresponding calculated values (Table 1). As expected each peptide also exhibited in its mass spectrum an additional peak at [M+Na (23 Da)] for example the peak at m/z 2,098 for peptide P3 (Figure 2). The mass spectral data thus confirmed the identity of each peptide and its purity since there was only one major molecular ion peak with a few minor additional peaks as impurities in their mass spectra.

Trypsin assay and its inhibition
Trypsin activity was determined by in vitro assay using Boc-RVRR-MCA substrate by both progress curve or on line and stop time or end point reading methods. While working with various low-volatile water miscible organic solvents we surprisingly noted that the solvent N,N’-Dimethyl formamide (DMF) exhibits a modest protease inhibitory activity towards trypsin. This is demonstrated in Figures 3A and 3B which showed a gradual decrease in trypsin activity (both on line progress curve and stop time data) following incubation with increasing amounts of DMF. To our knowledge this is the first clear demonstration of trypsin inhibition by DMF which is well known for its high polarity and dissolving property for materials that are not soluble in aqueous system. Previously there were reports of protease activity stabilisation of subtilisin enzyme by a mixture of aqueous organic solvent containing DMF as much as 70% [42]. Regulatory effect of organic solvents that includes DMF on the activity of another enzyme thermolysin has also been reported [43]. Based on our current observation we
decided to use DMF as trypsin inhibitor in the present study instead of more commonly used trypsin inhibitors such as SBTI (Soybean Trypsin Inhibitor) [44] or PMSF (Phenyl Methyl Sulfonyl Fluoride) [45].

**K\text{\textsubscript{I}} determination**

The inhibitory action of DMF towards trypsin was further investigated by Cornish-Bowden plot using three different concentrations (1000, 500 and 250 μM) of the fluorogenic substrate Boc-RVRR-MCA [39,40]. The data is presented in **Figure 4**. As shown in the figure the three lines pass through a common intersection point suggesting a competitive inhibition of trypsin by DMF. The K\text{\textsubscript{I}} value based on the intersection of curves 1 & 2, curves 2 & 3 and curves 1 & 3 are respectively 129.75, 127.08 and 123.67 μM. Thus based on these the measured average K\text{\textsubscript{I}} value for inhibition of trypsin-mediated cleavage of Boc-RVRR-MCA by DMF is 126.8±6.08 μM.

**Peptide digestion by trypsin**

Each hTRT peptide was examined for its cleavage by commercial trypsin and the rates of their cleavages were determined by RP-HPLC chromatograms and compared under identical conditions. **Figure 5** shows the RP-HPLC chromatograms of crude digest of wild type hTRT peptide P1 [\textsuperscript{41}WEPFASGK↓TSESGELHGLTT\textsuperscript{60}] following incubation with trypsin for 7 min. Besides the peak for the undigested peptide eluting at Rt: 31 min as confirmed by its mass spectrum (calculated MW=2,134.29 Da, observed 2,133.4 Da), the chromatogram shows two additional peaks eluting at Rt: 23.8 min and 28.7 min which were found to be more intense in 25 min digest RP-HPLC chromatogram (Not shown). The SELDI-tof mass spectra of materials corresponding to these HPLC peaks showed strong peaks at m/z 1,230.5 Da and 921.0 Da respectively which are consistent with the calculated masses of the C-terminal fragment (TSESGELHGLTT, calculated MW=1,231.58 Da) and N-terminal fragment (WEPFASGK, calculated MW=921.44 Da). These results confirmed cleavage of peptide P1 by trypsin at K↓T site which became more significant (>90%) after 25 minutes of digestion (See later). The cleavage of peptide P1 bytrypsin was further investigated in a time dependent manner ranging from 30 sec to 60 min. The results are shown in **Figure 6** where the RP-HPLC chromatograms of crude digest at various times were overlaid in a stacking format for better visualization purpose. The data showed the formation of in-
increasing amounts of fragment peaks at Rt 23.8 and 28.7 mins with the time of digestion. Additionally our data also revealed that both mutant peptides P2 and P4 were also cleaved by trypsin at the same K↓T site in a differential manner. Thus we noted that peptide P2 was cleaved far more efficiently than control wild type peptide P1 while peptide P4 was cleaved a lot less efficiently compared to that of control wild type peptide P1. A similar time dependent cleavage study of peptide P2 by trypsin was also performed and the results are displayed in Figure 7. The measured half life time or time for 50% cleavage of each peptide under identical condition is measured and the results are shown in Table 2. The data revealed that under identical digestion condition, S52/P mutant peptide P2 [↓TSEPFASGK][TSEPFASGK] peptide at various times as indicated here, I: 30 seconds, II: 45 seconds, III: 1 min, IV: 5 min, V: 10 min CT: C-terminal, NT: N-terminal, UP: Undigested peptide, mAUFS: Milli Absorbance Units Full Scale.

**Table 2.** List of measured half cleavage times of various hTTR peptides following digestion with trypsin in the absence and presence of trypsin inhibitor (only for peptide P1) (For details see Materials Methods section), DMF, an inhibitor of trypsin mediated peptide cleavage

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$T_{1/2}$ (min)</th>
<th>Relative fold $T_{1/2}$</th>
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<tr>
<td>P1</td>
<td>7.4 ± 0.99</td>
<td>7.5</td>
</tr>
<tr>
<td>P2</td>
<td>0.985 ± 0.098</td>
<td>1</td>
</tr>
<tr>
<td>P3</td>
<td>NC</td>
<td>ND</td>
</tr>
<tr>
<td>P4</td>
<td>~10% Cleavage in 60 min</td>
<td>ND</td>
</tr>
<tr>
<td>P1 + Inhibitor</td>
<td>76.6 + 0.97</td>
<td>77.8</td>
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NA: Not Applicable; NC: No cleavage; ND: Not determined, $T_{1/2}$: Half or 50% cleavage time
cleavage of peptidyl fluorogenic substrate Boc-RVRR-MCA can also block almost completely the trypsin mediated cleavage of peptide P1 at post Lys residue. Similarly we also observed that DMF can block trypsin mediated cleavage of mutant peptides P2 and P4 under similar condition (data not shown). These results support the previous observation that hTTR and its mutants are proteolytically cleaved by trypsin like enzyme and therefore this cleavage may likely be regulated by inhibitor of trypsin [33].

Regulator of trypsin activity and effect on hTTR cleavage

Various studies in the past have reported that polyhydroxy organic solvents such as ethylene glycol, isopropanol or glycerine can stabilize the protease activity of various enzymes in an effective manner [43]. As a result such solvents mixed with aqueous buffer have been routinely used in the past to store proteases in active form at low temperature where it does not freeze. Upto 50% of such organic solvents in aqueous medium have been used for this purpose. Based on this observation we became interested to examine whether trypsin activity can be regulated by the presence higher molecular weight polyhydroxy compounds such as glucose, heparin and glycerine. trypsin (10 μl, concentration 0.1 mg/ml or ~0.043 nmol) was incubated separately for 10 min at room temperature with aqueous solution of each of these substances at various molar ratios (1:1, 1:10 and 1:100) and the solutions were then lyophilised. Each lyophilized powder was then re-dissolved in water and immediately tested for its activity in comparison to a control sample of trypsin which was not incubated with any hydroxyl compound. Trypsin activity was then measured using peptidyl fluorogenic substrate (done in triplicates). A representative data is shown in Figures 9A and 9B displaying trypsin regulatory activity of heparin at various molar ratios.

Our data so far indicated that glycerine helps to maintain the enzyme activity and act as a stabilizer and not a regulator. Glucose on the other hand down regulates trypsin activity almost in a dose-dependent manner. A maximum of 20% inhibition was noted with 1:100 molar ratio treated sample. In contrast heparin treated trypsin exhibited a modest increase in
activity compared to control under similar conditions, displaying a maximum of 30% enhanced protease activity against the fluorogenic peptide. This is demonstrated by both stop time and on line or progress curve assays as shown in Figures 9A and 9B. Similar observations were also noted when hTTR peptides P1 and P2 were incubated in parallel with glucose and heparin treated trypsin samples and the results were compared to that with control untreated trypsin. Thus heparin treated trypsin cleaved both peptides P1 and P2 nearly 25-30% more efficiently than regular trypsin (Data not shown).

Previous studies reported enzyme regulatory activity of both heparin and glucose towards several proteolytic enzymes mostly of serine protease family which includes trypsin [46-48].

3-D model study of hTTR peptides
All synthetic hTTR41-60 peptides namely the wild type P1, along with the mutant peptides S52/P (P2), K48/A (P3) and T49/A (P4) (Table 1) were analysed based on their 3-D in vacuo generated energy minimised model structures. These structures were obtained by Hyperchem software professional program (v 11.0. Hypercube Company, USA) using the Polak-Ribiere geometry minimisation algorithm and all possible H-bonds were computed [39,41]. Similarly, a much longer hTTR10-90 polypeptide as well as its S52/P, K48/A, and T49/A variants were also subjected to structural analysis using their energy minimised 3D structures generated in a similar manner. The size of this polypeptide was chosen such that it is relevant as much as possible to the actual conformation of the full length mature hTTR protein.

The comparative analyses of structures of these polypeptides are conducted in the context of similar structure analyses on the corresponding shorter peptides. Theoretically derived 3D model structure of two key peptides namely peptide P1 and peptide P2 are shown in Figure 10 where all the potential H-bonds are indicated with white dotted lines. The secondary structural conformations for the backbone of the above two peptides are displayed in Figure 11.

Peptide model analysis
Presence of H-bonds has been detected in 3 out of 4 peptides studied. The wild type P1 peptide hTTR41-60 exhibited a distinct beta-turn structure exposing the Lys↓Thr cleavage site as shown by arrow in Figure 10. Its 3D model structure also showed the presence of a turn sheet structure followed by short helix like structure in the backbone (Figure 11). In contrast the S52/P mutant peptide P2 exhibited a significantly different backbone secondary structure compared to that of peptide P1. Thus P2 peptide possesses mostly an extended linear helical structure stabilized by two strong H-bonds involving Pro, with Ser and Gly, with Ser. These H-bonds may be involved in stabilizing the observed helical structure. Interestingly there is only one observed H (hydrogen)-bond in P1 peptide (His is H-bonded to Thr). Despite this P1 peptide still assumes a non-extended backbone structure as indicated above. Thus significant structural differences have been noted between peptides P1 and P2. This may allow more exposure of the trypsin-cleavage site to the environment leading to an increased proteolysis. Interestingly K48/A mutant hTTR peptide P3 which is not cleaved by trypsin showed one strong H-bond in its structure involving Trp, and Ala residues. Moreover the remaining peptide hTTR T49/A mutant which is cleaved by trypsin with least efficiency did not exhibit any H-bond in its 3D structure. Like the short peptides, the longer hTTR polypeptide and its mutant variants also exhibited differences in their overall 3D structures, backbone secondary structures and H-bond profiles. Thus 20 H-bonds are observed in hTTR10-90 whereas 19 such bonds are noted in K48/A mutant variant. Of these, 16 were common to both. The 4 remaining
H-bonds that differ in hTTR46-90 peptide are those which involve Ala48 with Thr41; Asp49 with Glu52; Phe75 with Glu77 and Gly78 with Tyr80. Instead of these the corresponding K48/A mutant exhibited the following remaining 3 H-bonds involving Asp49 with Thr51; Ser52 with Glu53 and Phe75 with Ile79. These differences may explain the structural diversity between the two. This may likely be unique to the mutation and may account for the differences in kinetics of proteolysis by trypsin. The differences in the pattern of H-bonds were also observed between the short peptide and the corresponding long peptide. For example, His16 is H-bonded to Thr20 in hTTR10-90 peptide but not in the long hTTR46-90 peptide. Also the H-bond between Trp1 to Ala6 observed in short K48/A mutant peptide is not observed in the long K48/A peptide (Model figures not shown).

Discussion

Peptide cleavage

Our in vitro trypsin digestion data using designed hTTR peptides clearly confirmed that trypsin cleaves wild type peptide sequence at Lys48Thr site as proposed in a recent study with full length mature recombinant wild type and variant hTTR proteins [33]. The result also demonstrated that Ser52Pro variant peptide P2 is processed in a significantly faster kinetics compared to the corresponding wild type peptide P1. In fact under identical in vitro condition P2 is cleaved nearly 7.5-fold faster than P1 as reflected by the measured T1/2 or 50% cleavage times (Table 2). This is also in agreement with the previous study demonstrating by gel electrophoresis that recombinant hTTR Ser52/Pro variant protein is cleaved more efficiently in vitro by trypsin compared to the corresponding wild type hTTR protein [33]. However this study did not provide any comparative analysis of this cleavage between the two proteins. This mutation first found in British nationals is responsible for rapid progression of amyloidosis and cardiomyopathy [49]. However both studies provide rationale for the observed high pathogenenicity associated with this hTTR mutation in amyloidosisis since such mutation is expected to generate an increased level of hTTR C-terminal (49-127) fragment that undergoes self-aggregation much more rapidly than the wild type full length mature hTTR protein [33].

Furthermore our data also suggested that physiologically relevant Thr48/Ala mutant hTTR peptide P4 is processed by trypsin at an extremely slow kinetic rate under identical condition compared to wild type peptide P1, thereby protecting hTTR from proteolysis which is considered as a key factor for promoting aggregation leading to pathogenic form of amyloidosis [32,33]. In fact this mutation commonly observed in Italian and French nationals led to neuropathy and cardiomyopathy but with other different clinical features [50]. An important finding of our study is that the mutation of Lys48 by Ala in hTTR completely abrogates the cleavage by trypsin. Although this mutation has not yet been reported in physiological condition, it has a great implication in hTTR associated amyloidosis, polynephropathy and its pathogenesis.

Such a mutation may be considered as a protector against hTTR proteolysis and possibly it’s associated amyloidosis.

Our study reinforces the previous findings that the proteolysis of hTTR is mediated by a trypsin like enzyme. However the precise enzyme responsible for this cleavage is yet to be fully identified. Our study with peptides showed this enzyme is a Lys specific cleaving enzyme like endoproteinase Lys-C enzyme [51] and not necessarily a trypsin like enzyme. Although endoproteinase Lys-C enzyme has been detected largely in bacteria [52,53], a mammalian homolog of such an enzyme is yet to be characterized. However existence of such an enzyme in mammalians including human is currently hypothetical but is still a possibility. If identified such an enzyme could have enormous implication in hTTR associated pathogenesis.

Our study has established that trypsin mediated cleavage in hTTR peptide at Lys48Thr can be efficiently blocked by NN′-dimethyl formamide (DMF) which is described for the first time in this study as an inhibitor of trypsin. Inhibition of trypsin by DMF has been confirmed in vitro by both on line and stop time assay against peptidyl fluorogenic substrate such as Boc-RVRR-MCA as shown in Figures 3A and 3B. This is further reinforced by the determination of inhibition constant K for DMF against trypsin using Boc-RVRR-MCA. This is described as a moderate inhibitor with measured K ~126 µM (Figure 4). Although our finding of DMF as an inhibitor of trypsin is quite unexpected and accidental but it is not completely unrealistic since compounds containing similar functional moiety such as benzamidines, guanidines and even amides have been reported to inhibit in vitro protease activity of serine proteases like thrombin, trypsin etc [54,55].

Regulation of trypsin activity

In addition to above findings our study also revealed that proteolytic activity of trypsin can not only be stabilized by adding di or tri-hydroxyl organic solvents such as glycercine but can also be up- or down-regulated particularly by adding polyhydroxy compounds such as glucose or heparin. In fact our data indicated that while addition of glucose led to a slight inhibitory effect on trypsin, in contrast addition heparin induced a modest upregulation of activity for trypsin. Such regulatory effects have been demonstrated against the cleavages of fluoroergic peptide as well as the hTTR peptides P1 and P2. Protease stabilizing activity of glycercine and similar solvents has been reported in the past in literature [42]. In fact such solvents are routinely used for preservation and storage of enzymes at low temperature for a long period of time [36,37,42]. In terms of sugars a few of them such as sucrose, glucose, lactose, sorbitol and heparin have been reported as enzyme and protein stabilizers. These have been shown to prevent degradation of both enzymes and proteins [46,47]. Recently glycerol has also been used as a stabilizer for antibodies [56]. Our demonstration of trypsin regulatory activity in vitro by the addition of glucose and heparin is interesting although such compounds have been shown to

regulate activity of other protease family such as MMPs [57].

Lately studies revealed that hTTR itself also behaves as protease most likely of MMP type [58]. A few substrates such as Apo-E for hTTR have been identified. However the exact functional role of such proteolytic activity of hTTR in its aggregation is not yet clear. Further studies will be warranted to investigate this aspect [31].

3D model
In this study we have also theoretically performed 3D structural studies of a 20 mer hTTR peptide and its 3 mutant variant peptides in vacuo. Comparison of these structures revealed that the pathologically aggressive Ser52/Pro mutant hTTR peptide exhibited a widely different backbone secondary structure compared to the corresponding wild type peptide. In fact it showed a predominantly helix structure that is likely stabilized by two H-bonds leading to more exposure and access to the Lys1-Thr cleavage site. On the contrary the wild type peptide showed a turn structure that is likely to be flexible as it did not exhibit any H-bonds. These findings may provide rationale for observed rapid cleavage of Ser52/Pro mutant peptide or protein by trypsin. Another interesting observation was that Lys48/Ala variant peptide also exhibited a specific backbone structure of mixed type that is characterized by two different types of H-bonds. This made the cleavage site completely inaccessible that may explain its resistance to proteolytic cleavage by trypsin.

Conclusions
In summary our in vitro study using designed model peptides based on hTTR sequence and its key mutant variants demonstrated that wild type hTTR peptide is indeed cleaved by trypsin at Lys48-Thr49 site quite efficiently and cleanly and that this cleavage is significantly increased for Ser52/Pro mutant peptide. This provides rationale for the observed high pathogenic amyloidosis outcome for the carrier of this particular mutation since such cleavage has been linked to rapid aggregation of hTTR fragment form. This study also showed that the above cleavage in hTTR may be mediated by a trypsin like enzyme which can be inhibited by a trypsin inhibitor or by mutation of Lys48 to Ala. However there is a high possibility that a more specific enzyme that belongs to bacterial Endoproteinase Lys-C family may play a role in this cleavage. So far such an enzyme has not yet been elucidated in mammalian systems including humans although a few have been predicted or proposed in the past. Though highly theoretical our energy minimised 3D molecular modeling study in vacuo revealed that both Lys48/Ala and Ser52/Pro mutant peptides exhibited significantly different backbone structural conformations compared to the corresponding wild type peptide and this provided necessary support for the observed differences in their proteolytic processing rates by trypsin. This study also highlighted a potential therapeutic strategy for hTTR associated amyloidosis by targeting the Lys48-Thr49 cleaving enzyme with specific inhibitors.

List of abbreviations
Aa: Amino acid
ACN: Acetonitrile
CHCA: α-Cyano 4-hydroxy cinnamic acid
da: Dalton
DHB: 2, 5 dihydroxy benzoic acid
DMF: N, N’-Dimethyl formamide
EDT: Ethane dithiol
h: Human
HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate
HBTU: N,N,N’,N’-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate
HOBOT: 1-Hydroxy benzotriazole
kDa: Kilodalton
mAUF: Milli Absorbance Units Full Scale
MS: Mass Spectrum
MW: Molecular weight
PAL PEG PS: Poly amino linker poly ethylene glycol poly styrene
RP-HPLC: Reverse Phase High Performance Liquid/Layer Chromatography
RPM: Revolution per minute
Rt: Retention time
SERDI tof MS: Surface Enhanced Laser Desorption Ionization Time of Flight Mass Spectrometry
TFA: Trifluoro acetic acid
TCEP: Tris (2-carboxy ethyl) phosphine
TIS: Tri isopropyl silane
TTR: Transthyretin

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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