Recombinant human Transmembrane Protease-serine4, extra cellular domain and catalytic domain expressed as soluble secreted protein in Mammalian cells.

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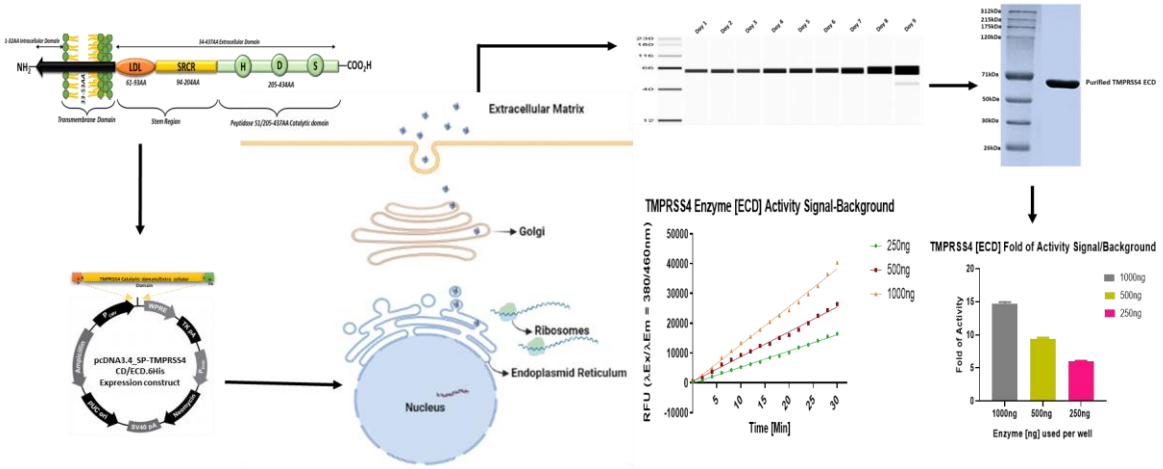
Abstract

Overexpression of the transmembrane protease, serine 4 (TMPRSS4), belonging to type II family of serine proteases in carcinomas, and malignancies of epithelial tissue, signify as a potential therapeutic target in cancer. Its membrane-bound characteristic feature is identified as a suitable target in the development of blocking antibodies and its soluble active protease domain as a non-invasive diagnostic marker. In this scenario, researching and generating active TMPRSS4 protein for the functional studies and moving forward for the elucidation of the structural properties come across as essential. In this study, we aimed to express the extracellular and catalytic domains eliminating the membrane anchoring region from the full-length TMPRSS4 protein in soluble secretory form in mammalian cells. Strategies were designed to express the catalytic domain and extracellular domain as secretory expressions using alternate secretory peptide signals. In this study reported expression and purification of TMPRSS4 extracellular domain (ECD) and catalytic domain(CD) from suspension Chinese Hamster Ovarian cell line (CHO) and Human Embryonic Kindly cell line (HEK) as soluble secretory protein using alternative signal peptide and purified TMPRSS4 ECD and CD protein by affinity column chromatography and confirmed the biological activity of the enzyme through fluorescence assay and this is the first report on secretory expression and biologically active TMPRSS4 ECD production.

Graphical abstract:



Keywords
TMPRSS4, secretory protein expression, serine protease, Expi CHO, HEK Freestyle, protease assay

Introduction

Proteases are factors playing a key role in the pathophysiology of tumorous diseases [1]. Several hallmarks of aggressive cancer are a direct result of proteolytic activity, including, but not limited to, tumour cell invasion, angiogenesis, and metastasis [2]. The advancement to malignancy is mostly connected with irregular proteolysis mechanisms resulting in altered activity of a large number of proteases, with many proteases becoming potential therapeutic targets. Among these, serine proteases are the most abundant proteases, and this functionally varied group occupies one-third of all known proteolytic enzymes [3,4].

From this important class of proteases, at the turn of the millennium, genome analysis and expression sequence data unveiled an emerging class of cell surface proteolytic enzymes [5,6,7] named as type II transmembrane serine proteases (TTSPs). Subsequently, the number of known TTSPs doubled, and the need to understand the physiological functions of each TTSP and its role in human disease increased greatly. From the day of TTSPs, the cell-surface-associated proteases identification, much of the focus is/was on the role of type II transmembrane serine proteases, which commonly have a proteolytic domain, transmembrane domain, a short cytoplasmic domain, and variable-length stem region containing modular structure domains [8,6,7,9].

Recent studies have reported the biological functions of TMPRSS4 during tumorigenesis, demonstrating TMPRSS4 as an important mediator during invasion, metastasis, migration, adhesion, and the epithelial & mesenchymal transition (EMT) in human epithelial cancer cells, presenting itself as a new therapeutic target for cancer [10,11,12,19]. In this study attempted to express and purify the active CD and the ECD of TMPRSS4 using mammalian expression systems.

TMPRSS4 is made up of 437 amino acids, with a predictive molecular weight of 48kDa, encompass a serine protease domain at the C-terminus (234-437AA), followed by a scavenger receptor cysteine-rich domain (SRDR) and a low-density lipoprotein receptor class a domain (Figure 1). Total TMPRSS4 activity depends on the catalytic triad, His, Asp and Ser, which is highly conserved among different TTSPs [13]. The enzymatic activity is modulated by a substrate-binding pocket that determines the enzyme specificity. In this study mainly communicates the strategy adapted in expressing soluble TMPRSS4 CD and ECD as a secretory expression in CHO and HEK cells using alternative secretory signal peptides.

Alternative signal peptides are non-native signal sequences fused with the Protein of Interest (POI) at its N terminus to express it as secretory protein. It has been demonstrated [14] that alternative signal peptides or signal sequences can lead to increased protein secretion when fused with protein of interest [15,16]. Hesketh et al. proved [17] that a native signal peptide is not necessarily the most effective signal peptide in aiding the protein into secretory pathway, and fusion of potent alternative signal peptides with the target of interest can improve the secretion efficiency [18]. Signal peptide denoted as SP in this article, is a hydrophobic string of amino acids, which would be recognized as it transpires from the ribosome by the signal recognition particle (SRP) eventually secreting the protein of interest into extra cellular matrix. With this contextual research, investigated to identify the potent signal peptides to fuse with TMPRSS4 ECD and CD domains at the N terminus. This study describes successful expression of TMPRSS4 catalytic domain and extracellular domain as soluble secretory form demonstrating biological activity.

Materials and methods:

Cell Culture

Expi CHO S cells (Life Technologies, A29127) were grown in suspension in a S41i CO_2 Shaker incubator (Eppendorf) at 120RPM, 8% CO2 and 37^0C temperature in Expi CHO expression media (Life Technologies, A29100). Similarly Free style 293 F cells (Life Technologies, R79007) were grown in S41i CO2 shaker incubator at 120 RPM, 5% CO2 and 37^0C temperature in Freestyle F17 culture media (Life technologies A1383503) in suspension conditions. Corning polycarbonate Erlenmeyer flasks with vent cap were used for culturing the cells.

Plasmids and Cloning

TMPRSS4 (207-437AA) catalytic domain expressing constructs were generated using the pcDNA3.4 expression vector (Thermo, A14308)) to express in the ExpiCHO and HEK F Expression systems. Five alternative signal peptides chosen for these studies were ligated upstream to the TMPRSS4 CD and ECD gene sequences which were having a C terminus 6 His tag. Following Standard RE cloning, the gene inserts were ligated into pcDNA3.4 vector. Totally 5 different secretory signal peptides containing TMPRSS4 CD expression constructs and 1 secretory signal peptide containing TMPRSS4 ECD expression constructs were generated and confirmed the insertion by restriction digestion. The confirmed positive constructs were amplified further and purified by using PurLink HiPure plasmid midi prep kit (Life technologies K2100-14) to generate enough DNA for transfection.

Transfection

Transfected Expi CHO Cells with respective Plasmid DNA constructs at the cell density of $6X10^6$ cells/ml using Expifectamine CHO transfection reagent from the kit (Life technologies, A29129). We have followed standard protein expression protocol to express TMPRSS4 ECD and CD domains. Added Feed and Enhancers (Life Technologies, A29129) a day after transfection and incubated the culture at 120RPM, 8% CO2 and 37^0C temperature. HEK F cells were transfected at the cell density of $2X10^6$cells/ml using PEI max-transfection grade linear polyethyleneimine Hydrochloride MW 40,000 (Poly biosciences 24765) and incubated the cells at 120RPM, 5% CO2 and 37^0C temperature.

The expression was monitored for nine days for Expi CHO Cells, and for 7 days for HEK F cells from the day of transfection in the media supernatant. Cell viability was determined by microscopic observation by staining the cells with trypan blue (Life Technologies, 15250-161). Collected 0.5ml of the culture media and separated the cells centrifuging at 5,000RPM for 15 minutes. Culture media supernatant from the control ExpiCHO cells without the POI constructs and the expression control transfected with some other proteins of interest were analysed for protein expression by western blot on the Protein Simple WES platform. We estimated the total protein in the clarified media with BCA, and the expression profile of TMPRSS4 catalytic domain was evaluated by western blot using anti TMPRSS4 antibody (Sigma, SAB2501048).

For large scale protein production, transfected 500ml sized cultures and harvested the batches when the cell viability dropped down below 85-90%, which typically was 9 days for Expi CHO cells and 5-7 days for HEK F cells.

Purification by affinity chromatography

Following transient transfection and expression the conditioned media was centrifuged at 5000RPM for 20minutes, recovering the media supernatant. Media supernatant was filtered using 0.22µm sterile PES rapid flow bottle filters. The clarified media supernatant was subjected for NiNTA column purification using Pierce High-capacity Ni IMAC resin, EDTA compatible beads (Thermo, A50585). Preconditioned beads with 20mM Tris pH8.0 and 150mM NaCl were added to the media supernatant and allowed TMPRSS4 CD and ECD to bind for ON at 4^0C. Next day centrifuged the beads at 3000RPM, for 30minutes, carefully collecting the unbound, leaving the beads with ~50ml of the sample. Packed XK16/20 column with the beads at 40C, collecting the remaining 50ml sample as flow through. Washed the beads with 5 column volumes of Equilibration buffer (20mM Tris, pH8.0, 150mM NaCl) collecting 5m sized fractions. Eluted the proteins with Equilibration buffer containing 500mM Imidazole in a linear gradient of 0-500mM Imidazole while collecting the eluate as 5ml fractions. Analysed the fractions by SDS PAGE and stained the proteins with Coomassie brilliant blue R250. Pooled the fractions having ECD of ~50kDa, and CD~26kDa and concentrated while exchanging the buffer to 50mM Tris pH8.0, 100mM NaCl. Added glycerol to 20% and protein aliquots were stored in -80^0C.

Peptidase assay For Functional assessment.

The functional activity of the TMPRSS4 ECD enzyme was evaluated by fluorescence assay using fluorogenic peptide Boc.Gln-Ala-Arg-7amido4-methyl coumarin hydrochloride (Sigma,

B4153). The protein activity was optimized in three different buffers that were 100mM Tris pH8.0, 10mM CaCl2; 30mMTris pH8.0, 30mM Imidazole and 200mM NaCl; 20mM Tris pH8.0, 200mM NaCl and two different concentrations of the substrate. Assayed the activity under optimal buffer conditions in 100mM Tris pH8.0, 10mM CaCl2 and 1µM ZnCl2. Fluorogenic substrate prepared as 2mM Stock in methanol was diluted to 200uM in the assay buffer. Proteolysis was initiated by adding 1µg, 0.5µg and 0.25µg of TMPRSS4 ECD per well in 100µl of assay buffer in flat bottom black well plates and adding 100µl of a substrate prepared in assay buffer. The fluorescence was monitored using Tcan Spark multimode reader at λEx_{380} and λEm_{460}nm at 30 °C, and the rate of substrate hydrolysis was analysed with the Spark Control Magellan software. Enzyme unaccompanied with substrate and only substrate without enzyme in the assay buffer is used a negative assay control.

Statistical analysis: Substrate background signal is subtracted from the enzyme activity signal and plotted the enzyme activity using graph pad Prism software.

SignalP 4.0 : This tool used for proteomic analysis of cellular compartments it gives the subcellular locations of a specific protein, or protein complex, which in turn can be a valuable step towards understanding protein function [19].

Results

Mammalian expression construct generation :

TMPRSS4 (207-437AA) catalytic domain (Uniprot ID: Q9NRS4) expressed constructs were generated using the pcDNA3.4 expression vector to express in the ExpiCHO and HEK F Expression systems. Five alternative signal peptides were ligated upstream to the TMPRSS4 CD gene sequences which were having a C terminus 6 His Tag, thus generating five different pcDNA3.4 expression constructs. The confirmed positive constructs using restriction digestion (HindIII, XhoI) were amplified further.

All the five SP(x)-TMPRSS4.CD-6His_pcDNA3.4 expression constructs were purified by midi prep for transfection. ExpiCHO cells grown in ExpiCHO culture media at 37 °C with 8% CO_2 were transfected with the TMPRSS4 CD expression constructs using Expi CHO Fectamine in suspension conditions. Where HEK F cells were grown in Freestyle culture media, at 37^0C, 5% CO_2 were transfected with the TMPRSS4 CD expression constructs using PEI 40Kda in suspensions conditions. The expression was monitored for nine days for Expi CHO Cells, and for 7 days for HEK F cells, from the day of transfection in the media supernatant by collecting 0.5ml of the cells. Culture media supernatant from the control ExpiCHO cells without the POI constructs and the expression control transfected with some other proteins of interest were analysed for protein expression by western blot on the Protein Simple WES platform.

Further, a single expression construct to express the extracellular domain of the TMPRSS4 was generated by fusing it with the SP1 at its N-terminus region. The generated expression construct was used to transfect the ExpiCHO Cells, and we monitored the expression profile until the 9^{th} day of transfection. Samples withdrawn from the date of transfection subjected to total protein estimation, and the expression levels were evaluated by western blot analysis using the anti TMPRSS4 antibody.

TMPRSS4 catalytic domain and extracellular domain expression in mammalian cells as soluble secretory protein :

Five expression constructs by fusing each signal peptide, as listed in Table 1, with the catalytic domain of TMPRSS4 at its N-terminus in pcDNA3.4 expression vector backbone. The performance of all the five fused alternative signal peptides was evaluated whilst expressing the catalytic domain of TMPRSS4 in HEK F and Expi CHO cells. Transfected Expi CHO Cells at $6X10^6$cells/ml density, and HEK F cells at $2X10^6$cells/ml density cells were grown in 50ml culture and monitored every day for the cell density and viability. The culture was harvested on noted viability drop from day 5-7 for HEK and Day 9-10 for Expi CHO. Expression of catalytic domain fused with all five alternative peptides in culture media, confirming the secretory mode of expression releasing the POI into the culture media (Figure 2 and 3). Increased expression levels were observed with alternative SP1, SP2 and alternative SP3 confirmed and judged based on the chemiluminescence intensity of the bands from the western blot analysis carried out (Figure 4 and 5). Same SP1 alternative signal peptide was used to generate ECD expressing construct. Significant expression levels of ECD achieved from day one until the 9^{th} day of transfection as stable and soluble form (Figure 3). Expression levels compared in Expi CHO and in Freestyle HEK F cells, and observed significant higher yields in Expi CHO cells (Figure 4 and 5).

Protein Purification Process:

TMPRSS4 CD and ECD were purified using NiNTA column chromatography followed by gel filtration chromatography from 500ml of Expi CHO and HEK F cultures (Figure 6) and achieved ~90% purity. Fractions were analysed by SDS PAGE and pooled the fractions having pure ECD and CD which were observed as ~50kDa and ~26kDa respectively.

Functional activity assay

Protease assay using the flourogenic substrate was performed to assess the biological activity of the expressed protein. TMPRSS4 CD and ECD proteins expressed and purified from CHO cells observed to have greater biological activity on HEK F expressed and purified proteins. We have observed 18 folds for CD and 14 folds for ECD biological activity corresponding to the protein concentrations used (Figure 7).

Titrated the flourogenic substrate peptide Boc.Gln-Ala-Arg-7amido4-methyl coumarin hydrochloride was performed for CD and ECD proteins purified from CHO cells. Using 500ng of each protein per well, titrated 5µM, 10µM, 25µM, 50µM, 100µM and 200µM substrate concentrations to calculate the substrate velocity. The resulted data fit with nonlinear regression using GraphPad Prism to calculate Vmax and Km (Figure 8)

Aprotinin (Sigma, A6106), a well-known serine protease inhibitor is used to validate the assay. 100µM, 50µM, 25µM and 10µM of Aprotinin was incubated with 500ng of enzyme for 20-30minutes and substrate was added as per the determined Km concentration (ECD 17µM and CD 12µM). Further incubated the reaction for 30minutes and measured the enzyme activity using Tcan Spark multimode reader at λEx_{380} and λEm_{460}nm at 30 °C.

Discussions

Achieving a fully functional protein supporting the biophysical and biochemical studies is very much essential while studying the recombinant proteins. The efficacious production of recombinant protein often necessitates the optimization of several factors, different host systems, expression conditions, gene sequence manipulation and fusion of appropriate purification tags. We chose mammalian cells to express TMPRSS4 proteins, as these cells are well known to simulate the properties of human proteins. Being a membrane protein in nature and its significant role in cancer [20], TMPRSS4 is an attractive target for the development of biological inhibitory tools. The role of TMPRSS4 in cancer development and metastasis suggest that this membrane-anchored serine protease merits further consideration as a novel potential therapeutic target in solid tumours [18]. Ever since the TMPRSS4 gene discovered, this is the first report on successful expression and purification of TMPRSS4 protein (ECD and CD) in soluble secretory form. With this in the background, we have investigated the potential to express TMPRSS4 as a soluble protein in mammalian cells and researched to express the target as secretory form into the culture media using alternative signal peptides.

TMPRSS4 Expressed as soluble secretory form and considered the nature of the POI, commonly utilized, and reported alternative signal peptides for heterologous protein expression and a compatible signal peptide with the host system aimed to use. In this process we have selected five probable signal sequences as alternative SPs to fuse with TMPRSS4 CD after extensive literature search [14,15, 16, 18]. The pool of five signal sequences encompasses, 1 signal peptide belonging to the serine protease family (Thrombin), 2 signal peptides which were utilized frequently and proven as potential alternative signals peptides to express heterologous proteins as secretory (Human Serum Albumin and Azuricidin) and 2 signal peptides of CHO endogenous proteins into the culture media (Vascular endothelial growth factor C (VEGF C) and Fibroblast growth factor (FGF)). Amino acid sequences of the all the five signal peptides are as in table 1.

All the five signal sequences fused at N terminus of the CD and ECD of TMPRSS4 were subjected to predict the discrimination scores (D-scores) with the aid of a secretome computation tool, Signal P 4.1, all are found to be potent secretory signal peptides with significant discrimination score (D-score) [19]. Generated expression construct in pcDNA3.4 vector backbone by fusing each signal peptide coding gene with catalytic domain TMPRSS4 expressing gene. Expressed the protein by transient transfection in Expi CHO and HEK F cells for 7-9 days. Expression was confirmed by Western blot using TMPRSS4 antibody in the culture media withdrawn from Day 1 of transfection.

Though the prediction algorithm scores of all the five alternative signal peptides in the background of the mature TMPRSS4 catalytic domain sequence did correlate with the secretory protein expression profiles observed, secretion levels of POI differed significantly with each signal peptides. Signal peptide Human Serum albumin, Thrombin and Azuricidin showed the best performance as heterologous secretory signal peptides and are in accord with the reported studies. Signal peptide Vascular endothelial growth factor and fibroblast growth factor mediated weak secretion levels of POI although the secretome prediction scores are significant.

Classically a newly synthesized protein, comprising signal peptide at its N terminus is recognized by the signal recognition particle (SRP) in the cytosol while the protein is still being synthesized on the ribosome. SRP, a ribonucleoprotein complex containing three domains, the N domain, the G-domain, and the M-domain play a very important role in recognizing the signal peptide for secretory pathway. The hydrophobic groove from N terminal helix of the M-domain interacts with the hydrophobic H-domain of the signal peptide, which typically contains various numbers of hydrophobic residues. In addition to hydrophobic core and its interaction with SRP, conservation of residues in specific positions is also very vital like harbouring neutral amino acids at positions -1 (last amino acid of the signal peptide) and -3 of signal peptides is important. This sequence in combination with the amino acid sequence downstream to the signal peptide (first amino acid of the protein of interest) can also affect the secretory efficiency of signal peptide.

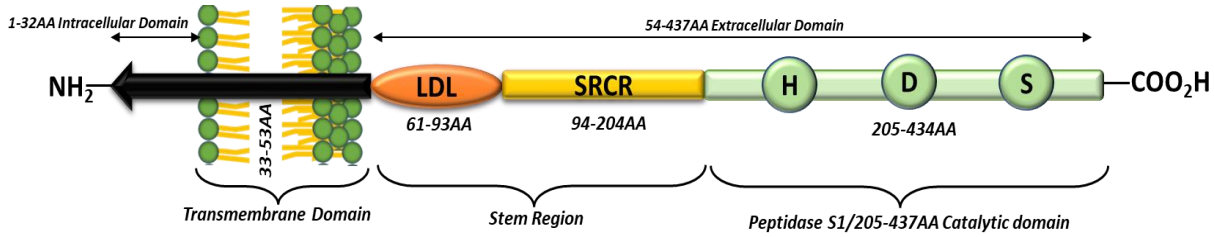
The data generated by western blot analysis to confirm the TMPRSS4 catalytic domain and extracellular domain expression into culture media helped to identify appropriate alternative signal peptide which can be used for the target protein expression in secretory form. As demonstrated in this studies, Expi CHO observed to produce better expression yields in comparison with HEK F cells.

Conclusion

In this studies, we have reported the expression and purification of biologically active TMPRSS4 extra cellular domain and catalytic domain for the first time from mammalian cells. The described process provides an effective means for making TMPRSS4 ECD and CD that can be used for functional and structural elucidations. From these observations, it is predicted that the used approach could be used to successfully generate the difficult to express proteins, that are of significant biological value and careful design/selection of the signal sequences can help in improving the expression levels of desired target proteins. Also, it should be noted that further optimizations can lead significant yields of POI in secretory form.

Table 1: List of Alternative signal peptides identified and used to express truncated TMPRSS4 protein

S.No	Signal peptide (No)	Secretory signal	Secretory signal Amino acid Sequence	Discrimination score
1	SP1	Human Thrombin	MNPLLILTFVAAALA	0.758
2	SP2	Human Serum Albumin	KWVTFLSLLFLFSSAYS	0.892
3	SP3	Human Azuricidin	MTRLTVLALLAGLLASSRA	0.876
4	SP4	Rat VEGF C	MHLLCFLSLACSLLAAALIPGPREAPATVAA	0.825
5	SP5	Chinese hamster FGF	MLGTCLRLLVGVLCSACSLGTVRA	0.848



***LDL receptor**: Low Density Lipoprotein receptor class A domain*
***SRCR Domain**: Scavenger Receptor Cysteine Rich Domain*
***S1 Peptidase**: H, D & S Protease domain indicating the position of the three catalytic residues*

Figure 1: Schematic diagram of the TMPRSS4 structure: TMPRSS4 is a single-pass type II membrane protein compassing a serine protease domain/catalytic domain (Peptidase S1), followed by a scavenger receptor cysteine-rich domain (SRCR) and a low-density lipoprotein domain.

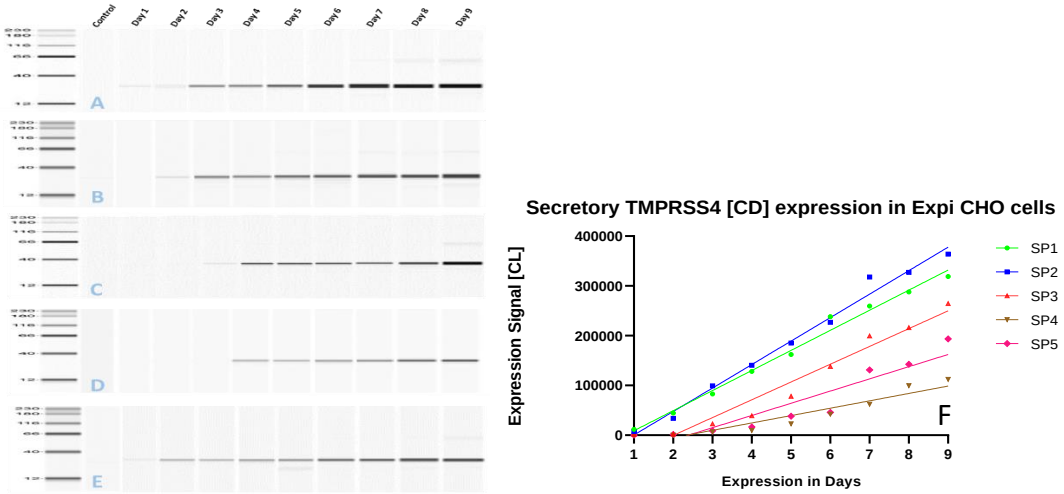


Figure 2: Western blot analysis confirming the expression of TMPRSS4 catalytic domain as soluble secretory protein in Expi CHO Cells. Figure 2 represents the expression of TMPRSS4 CD fused with 2(A)Signal peptide -Thrombin, 2(B)Signal peptide 2-Human Serum Albumin, 2(C)Signal peptide 3-Azuricidn, 2(D) Signal peptide 4-VEGF and 2(D) Signal peptide 5-FGF, from day 1 to the 9th day of transfection in culture media. Protein expression analysis was carried out on the protein simple WES platform and used anti TMPRSS4 antibody (Sigma) for the analysis. Expression levels from Day 1 of transfection were measured in chemiluminescence counts as denoted in Fig 2(F). The product yields are plotted with the corresponding chemiluminescence signal obtained from Western blot analysis.

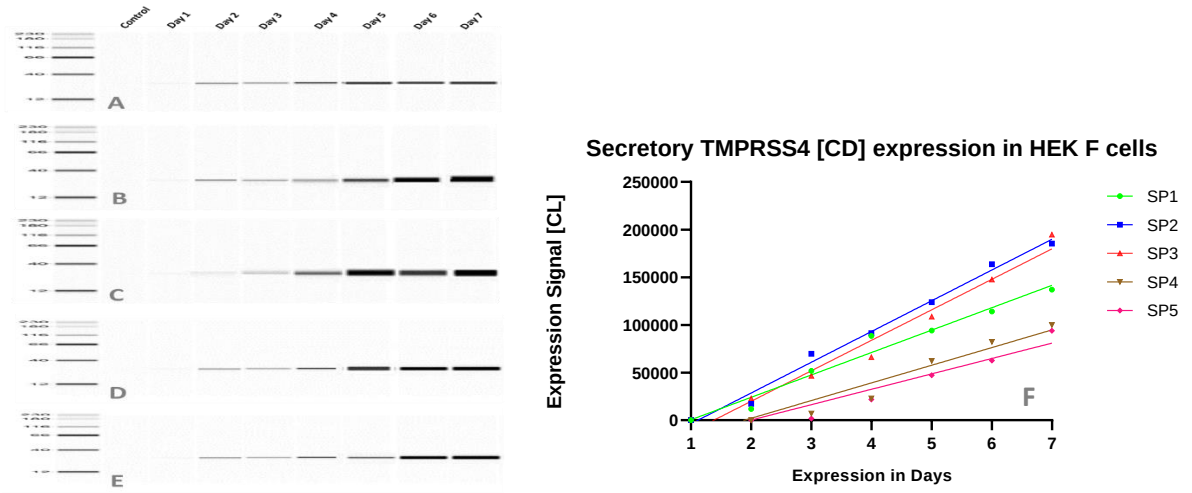


Figure 3: Western blot analysis confirming the expression of TMPRSS4 catalytic domain as soluble secretory protein in HEK F cells. Fig 3 represents the expression of TMPRSS4 CD fused with 3(A), signal peptide 1, 3(B) signal peptide 2, 3(C) signal peptide 3, 3(D) Signal peptide 4 and 3(5) signal peptide 5 from day 1 to 7th day of transfection in culture media. Protein analysis was carried on Protein simple WES platform using anti TMPRSS4 antibody (SIGMA). Expression levels from Day 1 of transfection were measured in chemiluminescence counts as denoted in Fig 3(F). The product yields are plotted with the corresponding chemiluminescence signal obtained from Western blot analysis.

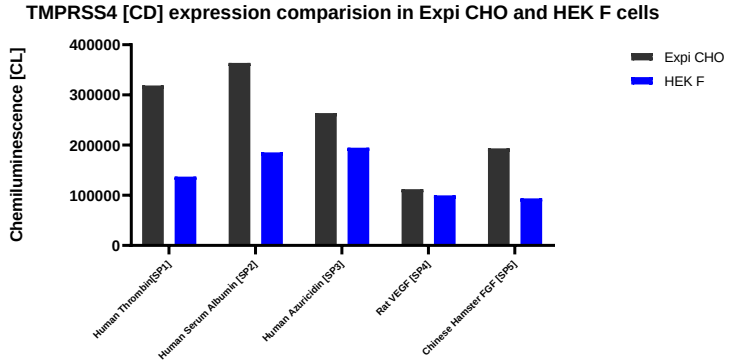
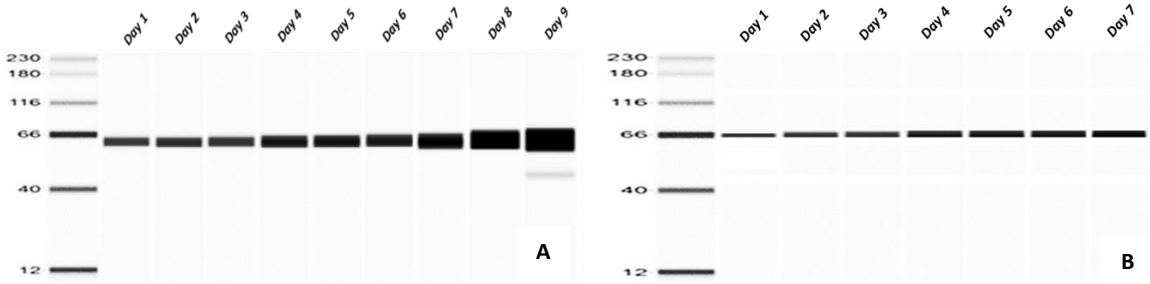


Figure 4: TMPRSS4 [CD] expression yields comparison in Expi CHO and HEK F cells. Expression yields as indicated in this graph are determined with the corresponding chemiluminescence signal obtained from Western blot analysis on 9th day of transfection from Expi CHO and 7th day of transfection from HEK F cells.



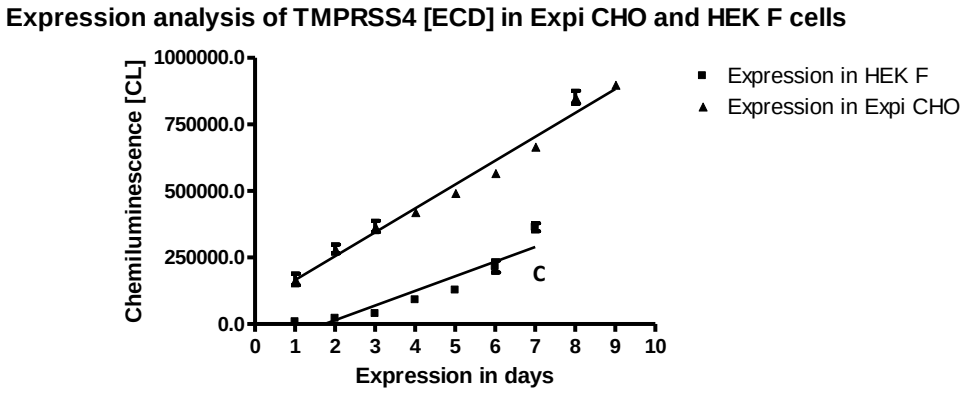


Figure 5: Fig 5(A and C) Western blot analysis of TMPRSS4 extracellular domain expressed as soluble secretory protein fused with Thrombin secretory signal peptide (signal sequence is as in table 1). The figure represents the expression of TMPRSS4 ECD in Expi CHO Cells from day 1 to the 9th day of transfection in culture media and in HEK F cells from day 1 to 7th day of transfection in the culture media. Expression analysis was carried out on the protein simple WES platform using anti TMPRSS4 antibody (Sigma). The product yields are plotted as in Fig 5(B and C) with the corresponding chemiluminescence signal obtained from Western blot analysis.

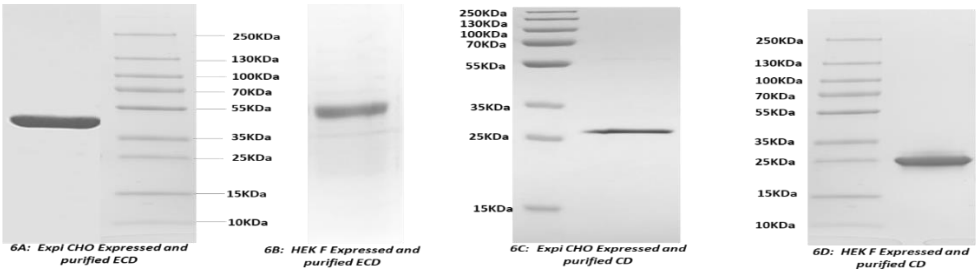


Figure 6 SDS PAGE Analysis of purified TMPRSS4 extracellular domain and catalytic domain from Expi CHO and HEK F cells. Fractions having pure TMPRSS4 ECD and CD were pooled and concentrated using Amicon Centricons into storage buffer.

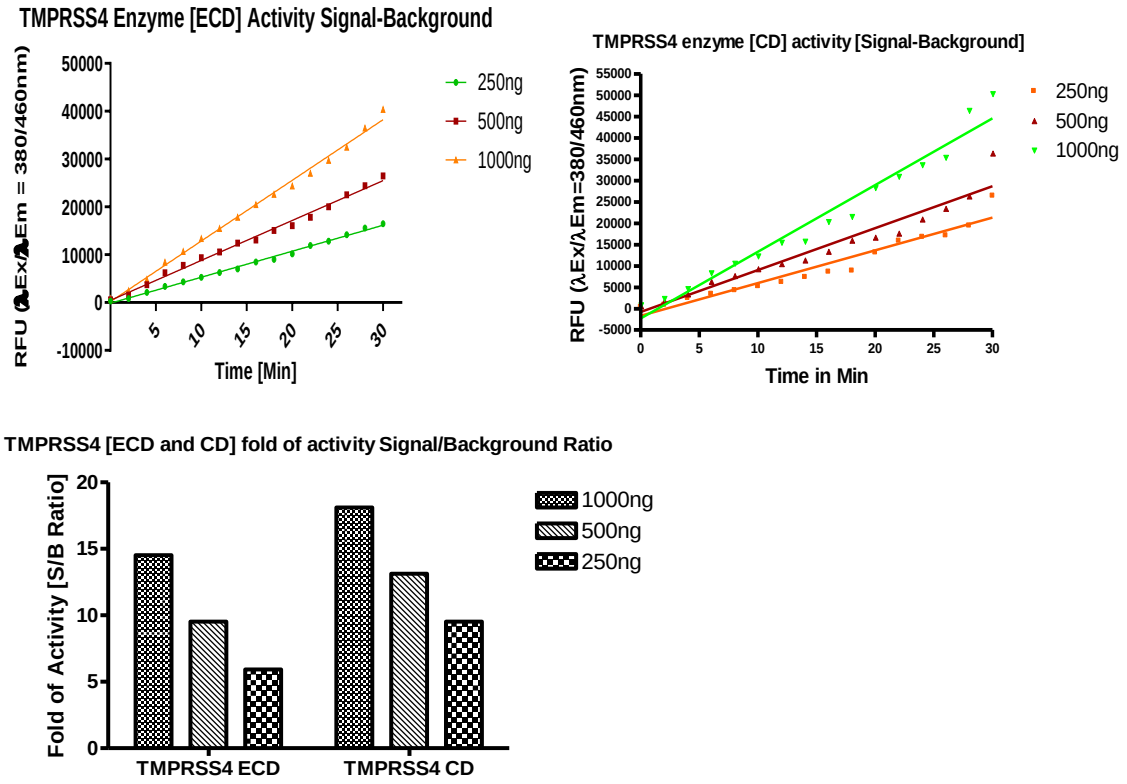


Figure 7: Enzymatic activity of purified TMPRSS4 ECD and CD was measured by fluorescence assay using a fluorogenic peptide as a substrate. TMPRSS4 ECD and CD enzymes prepared, observed to show proteolytic activity until 30minutes in the tested assay conditions and used concentrations of the enzyme.

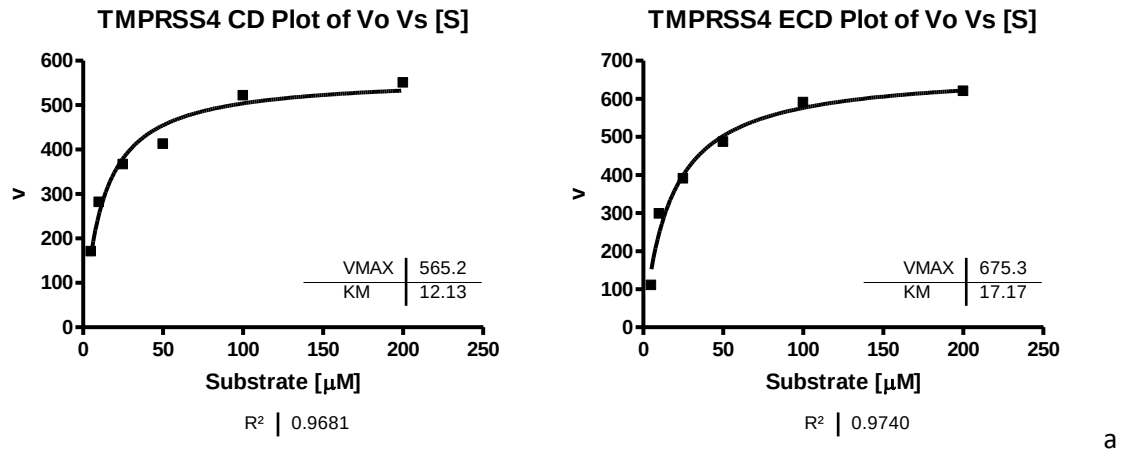


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Figure 8: Michealis – Menten curve representing effect of concentration of substrate (Boc.Gln-Ala-Arg-7AMC.HCl) on the activity of TMPRSS4 CD and ECD.

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