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Development and Validation of an Enzymatic Assay for TMPRSS4: Evaluation of Molecular Inhibitors

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Abstract

TMPRSS4, a member of the transmembrane serine protease (TTSP) family, has earned significant attention due to its pronounced overexpression in various carcinoma types, involvement in disease processes, and viral entry. However, despite its pivotal role in disease biology, little is known about its structural characteristics and potential drug targets. Accurate measurement of TMPRSS4 activity is crucial for understanding its role in these disease processes and for developing potential therapeutic interventions. In this context, we have expressed and purified TMPRSS4 protein and report the development of an enzymatic assay for the quantitative measurement of TMPRSS4 activity. The assay is based on a synthetic fluorogenic peptide substrate that mimics the natural cleavage site of TMPRSS4/serine proteases substrates. The Assay development workflow includes enzyme preparation, optimization of assay materials, reagents, and conditions. The assay is further validated using a known serine protease inhibitor, Aprotinin. Our results demonstrate that the developed enzymatic assay exhibits a wide dynamic range and successfully applied the assay to assess molecular inhibitors predicted by our computational studies. In conclusion, our study presents a robust enzymatic assay for TMPRSS4 activity that provides researchers and clinicians with a valuable tool for studying this protease's function and potential clinical applications.

Keywords: *Fluorogenic biochemical assay, Peptidase activity, TMPRSS4 inhibitors, Drug discovery, Assay development*

1. Introduction

TMPRSS4, also known as Transmembrane Protease, Serine 4, is a type II transmembrane serine protease enzyme encoded by the TMPRSS4 gene in humans (Aberasturi[1]). This enzyme belongs to the serine protease family and is involved in various biological processes, including protein processing and activation, cell signalling, and tissue remodelling (Joyce[2]). One of the well-studied functions of TMPRSS4 is its involvement in the activation of other proteins, particularly protease-activated receptors (PARs) and viral glycoproteins. This serine protease has been extensively studied in the context of cancer, as an upregulated enzyme in various cancer types including pancreatic cancer, colorectal cancer, lung cancer and breast cancer (Choi[3], Min[4], Netzel[5]). It is reported that it is associated with tumour progression, invasion, and metastasis. It cleaves (Figure 1) and activates the proteins involved in cell adhesion, and invasion thus promoting the spread of cancer cells to distant organs, and it also reported that overexpression of TMPRSS4 is linked to poor prognosis in several cancer types (*Netzel*[5], *Chaipan*[6])

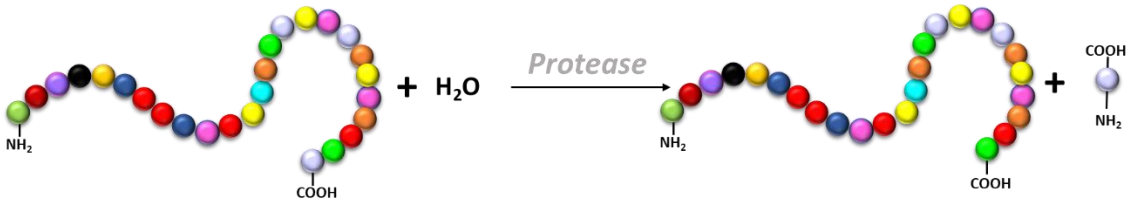
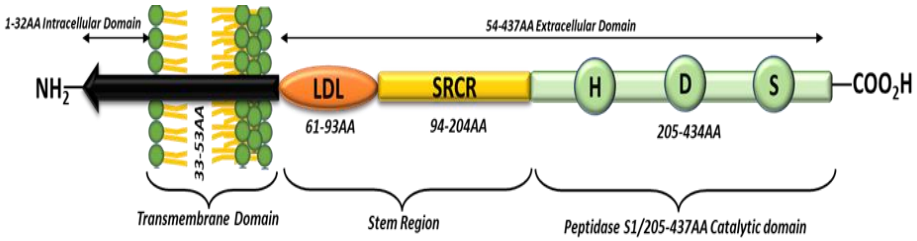


Figure 1: *Illustrates the general process of polypeptide hydrolysis by proteases in the presence of water, resulting in the release of individual amino acids.*

Source: Author

TMPRSS4 role has been reported in the activation of the influenza A virus hemagglutinin (HA) protein, a crucial step in viral entry into host cells (Zang[7]) Its involvement in the activation of SARS-CoV-2 a viral pathogen caused COVID 19, is also reported during the global pandemic (Puente[8]). Its role in facilitating viral entry highlights its significance in respiratory infections. Some research suggests that TMPRSS4 may play a role in cardiovascular diseases. It has been detected in vascular endothelial cells, and its expression has been linked to endothelial dysfunction, which is associated with conditions like atherosclerosis and hypertension. However, the precise mechanisms and implications of TMPRSS4 in these diseases are not fully understood and require further investigation.

TMPRSS4 by structure is a multi-domain protein consisting of 437 amino acids with specific domains located along its length (Figure 2). The multi-domain nature of TMPRSS4 indicates its potential involvement in various cellular processes, including proteolysis, protein binding, and interaction with other molecules (Bugg [9]). The N terminus of the protein encompasses the membrane signal peptide followed by a transmembrane domain, which functions as an anchor. The transmembrane domain is followed by the low-density lipoprotein receptor class A (LDLa: 61-93AA) domain which is known for its involvement in mediating protein-protein interactions and ligand binding. The presence of this domain in TMPRSS4 suggests that it may interact with specific ligands or proteins, potentially influencing its function and localization. From 93AA to 204AA, Scavenger Receptor Cysteine-Rich domain spans, which is typically involved in protein-protein interactions and may play a role in substrate recognition and binding. From 234AA-427AA spans the catalytic domain of the TMPRSS4, also called the serine protease domain. This is responsible for its protease activity, which typically contains the charge relay system or catalytic triad (Hooper[10]) of amino acids namely Serine, Histidine, and Aspartate which are required for enzymatic activity, and this protease domain is responsible for cleaving peptide bonds in target proteins (Figure 3).



LDL receptor*: Low Density Lipoprotein receptor (of class A domain)*
SRCR Domain*: Scavenger Receptor Cysteine Rich Domain*
S1 Peptidase*: H, D & S Protease domain indicating the site 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.*

Source: Author made adapting the concept from Antalis TM

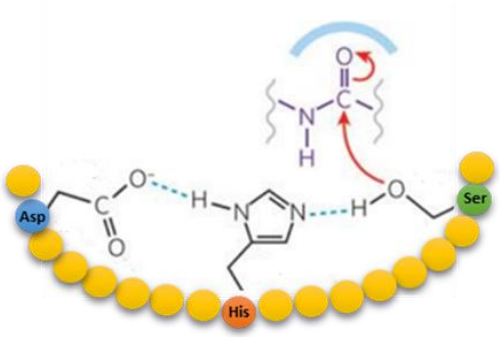


Figure 2: *Describes the catalytic triad or charge relay system found in serine proteases, typically composed of Aspartate (Asp), Histidine (His), and Serine (Ser). This triad plays a crucial role in the catalytic mechanism of serine proteases, including TMPRSS4. These three amino acids, are located far from one another, a proper folding of the protein allows the proximity of all three, and this particular geometry of the triad is highly characteristic of their biological activity.*

Source: The author adapted the concept from N. K. Poddar et. Al

While the exact enzymatic mechanism of TMPRSS4 may not be extensively characterized as some of the other serine proteases, serine proteas enzymatic action principles can be relatable for TMPRSS4 as well, as typically they share a common mechanism of action as illustrated in Figure 4.

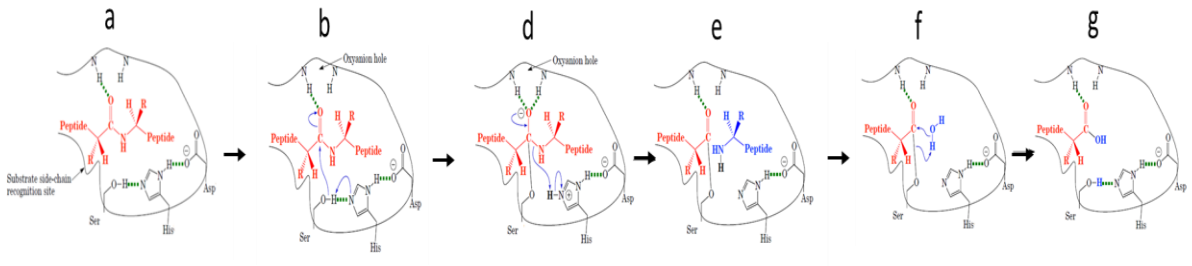


Figure 4: *[a-g] In the catalytic mechanism of serine protease/TMPRSS4: The serine residue within the catalytic triad is a key participant. It becomes activated through deprotonation; a process facilitated by histidine. Once activated, serine becomes nucleophilic and is poised to initiate a nucleophilic attack on a peptide bond. The substrate, which can be a peptide or protein to be cleaved, binds to the active site of TMPRSS4. Specific amino acid residues in the substrate interact with complementary residues in the enzyme through hydrogen bonds and other non-covalent interactions. The activated serine hydroxyl group (ser-OH) performs a nucleophilic attack on the peptide bond, leading to the formation of a covalent acyl-enzyme intermediate. This cleavage step results in the separation of the substrate into two parts. The acyl-enzyme intermediate is rapidly hydrolyzed, with water (H_2O) serving as a nucleophile. Water attaches to the acyl-enzyme intermediate, breaking the covalent bond between the enzyme and the substrate. This hydrolysis process releases the cleaved products and regenerates the active enzyme. The cleaved products, which may consist of fragments from the original substrate, are liberated from the enzyme's active site. The catalytic triad of the enzyme is then regenerated and can participate in additional rounds of catalysis.*

Acknowledgment: Special thanks to Mark Brandit, Ph.D, these Illustrations are used from his chapter Enzyme mechanics.

Proteases, as enzymes, play a pivotal role in catalyzing the degradation of biological molecules into smaller polypeptide chains through a process known as hydrolysis They exhibit varying degrees of substrate specificity, where the presence of basic amino acids such as Lysine and Arginine at the P1 site emerges as a prominent and well-fitting feature for enzyme-substrate interaction and subsequent hydrolysis by TMPRSS4 (Table1 and Figure 5)

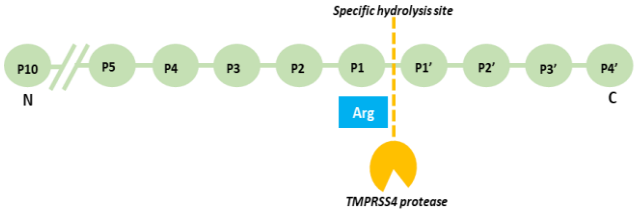


Figure 5*: Illustrates serine protease/ TMPRSS4 reaction (General) in which it cleaves a scissile amide bond from the substrate, which can be either a protein or a synthetic peptide. The amino acid at the P1 position is crucial or specific for the respective proteases to act on, and for TMPRSS4, a basic amino acid Arginine is the best reported AA. Substrate amino acids that are on the amino (N) terminus of the scissile bond are numbered P2, P3, P4, Pn after P1 and similarly the residues that are on the carboxyl (C) terminus are numbered as P1', P2', P3', P4' and ...Pn'*

Source: Author made adapting the concept from Irvine Lian Hao Ong et. Al

Amino acid	P4	P3	P2	P1	P1'	P2'	P3'	P4'
Gly	0	0	0	0	0	1	1	1
Pro	0	1	0	0	0	0	0	1
Ala	0	0	1	0	0	1	0	0
Val	0	0	0	0	0	1	0	0
Leu	2	1	0	0	1	1	2	0
Ile	0	0	1	0	1	2	0	1
Met	0	0	0	0	0	0	1	0
Phe	1	0	0	0	0	0	1	0
Tyr	0	0	3	0	1	0	0	0
Trp	0	0	0	0	0	0	0	0
Ser	0	2	1	0	2	0	2	2
Thr	1	1	0	0	2	2	0	0
Cys	0	0	0	0	0	0	0	0
Asn	2	3	0	0	0	1	0	0
Gln	0	0	1	0	0	0	0	1
Asp	0	0	2	0	1	0	0	0
Glu	1	1	0	0	2	1	3	4
Lys-Potential Basic AA for cleavage	0	1	0	3	0	0	0	0
Arg-Potential Basic AA for cleavage	3	0	1	7	0	0	0	0
His	0	0	0	0	0	0	0	0

Table 1: *Table showing the potential AA at P1 position of a peptide or protein substrate for serine protease activity, where Arg, followed by Lys have the highest potential to be attacked by deprotonated serine nucleophile.*

In the context of protease assays, a commonly utilized method for readout involves fluorescence measurement. This fluorescence assay employs a specific peptide substrate labelled with a fluorescent dye, such as AMC, BFC, MFC, among others. The selection of these substrates is tailored to the specific protease being studied. In the course of hydrolysis reactions, a water molecule is introduced into the peptide substrate, resulting in the liberation of the attached fluorophore, as depicted in Figure 6. Notably, this released fluorophore emits fluorescence within a distinct range of excitation and emission wavelengths. In recent years, the exploration of type II transmembrane proteases derived from mammals has seen remarkable advancements. Researchers have made substantial efforts to clone and report numerous members of the TTSP family, shedding light on their diverse roles and functions in various physiological and pathological processes.

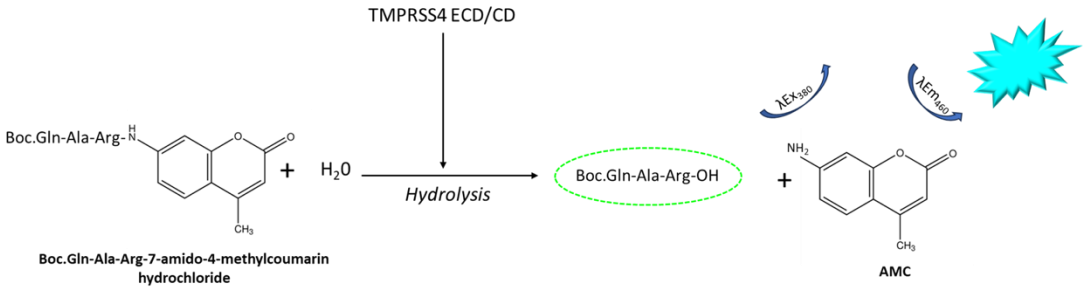


Figure 6: Depicts the hydrolysis reaction of fluorescent dye MCA, linked with Boc.Gln.Ala.Arg at C terminus through peptidase activity of TMPRSS4/serine protease enzymes. The freed AMC, on excitation at a specific wavelength 380nm, emits energy which can be detected at 460nm wavelength.
Source: Author made

A significant milestone in this ongoing research journey was achieved by Nozomi Yamaguchi et al. (Yamaguchi[11]), whose pioneering work contributed significantly to our understanding of the fluorophore labelled substrates. In their diligent pursuit, Yamaguchi et al conducted an extensive screening process, aimed at assessing the suitability of various fluorescent peptides as substrates (Table 2) for the TMPRSS5 enzyme, a member of the transmembrane protease family. This research endeavour led to the identification of Boc-Gln-Ala-Arg-MCA as an exceptionally suitable substrate for evaluating Transmembrane serine protease enzyme activities.

No	***Substrate***
1	Boc-Gln-Ala-Arg-MCA,
2	Boc-Phe-Ser-Arg-MCA,
3	Bz-Arg-MCA,
4	Boc-Val-Leu-Lys-MCA,
5	Pyr-Gly-Arg-MCA,
6	Pro-Phe-Arg-MCA,
7	Boc-Val-Pro-Arg-MCA,
8	Z-Arg, Arg-MCA,
9	Arg-MCA, or Z-Phe-Arg-MCA

Table 2: *List of the substrates evaluated by Nozomi Yamaguchi et al., while conducting TMPRSS5 enzymatic assays.*

Source: Yamaguchi et. Al

In the context of our own investigation into TMPRSS4, the insights gleaned from Yamaguchi et al.'s work on TMPRSS5 and its substrates provide a valuable reference point, and we employed Boc. Gln-Ala-Arg-MCA as TMPRSS4 substrate in our research work. To facilitate our study, we embarked on the development of a robust assay methodology for recombinant TMPRSS4, expressed and purified as ECD and CD variants. This assay methodology served as a cornerstone for the comprehensive examination of TMPRSS4 enzymatic activity. Using the synthetic peptide substrate Boc-Gln-Ala-Arg-MCA as our investigative tool, initially employed a 96-well format for these enzymatic assays. Subsequently, we miniaturized the assay to a higher-density 384-well format, enhancing our throughput capabilities for systematic experimentation.

Through this investigative process, we thoroughly assessed various assay factors, for both versions of TMPRSS4 enzymes. Our optimization efforts encompassed critical parameters, including the selection of buffers, determination of optimal time intervals, precise enzyme concentrations, and the calculation of substrate Km values. This rigorous optimization phase ensured the reliability and accuracy of our enzymatic assays, laying a solid foundation for subsequent analyses.

Furthermore, to validate the efficacy of our assay system, we employed a well-established serine protease inhibitor, aprotinin, as a benchmark. This validation step allowed us to gauge the assay's sensitivity and specificity, providing an additional layer of confidence in our experimental results. In summary, our study represents a comprehensive exploration of TMPRSS4 enzymatic activity through the development and validation of assay methodology.

2. Materials And Methods

Recombinant protein production

We have expressed and purified the catalytic domain (CD) and extracellular domain (ECD) of TMPRSS4 in Expi CHO S cells (Life Technologies, A29127). The extracellular and catalytic domains of TMPRSS4 were fused with secretory signal peptides and expressed the proteins as secretory into the extracellular environment i.e., culture media.

All the details of the protein generation are described in our previous publication. Purified, the protein by affinity chromatography, pooled the fractions having the purest form and stored the protein in 50mM Tris pH 8.0, containing 100mM NaCl until use.

Substrate Boc.QAR.AMC preparation

The fluorogenic substrate was prepared as 10mM stock in 100% methanol and stored as aliquots in -80^{0}C for future use.

Test CPDs and Aprotinin

Inhouse compounds tested with our assay developed were designed and synthesized at Gitam University, by Dr. Rambabu Gundla and team. Aprotinin was purchased commercially (Sigma, catalogue#A6103)

Peptidase assay For Functional assessment procedure.

The functional activity of the extracellular domain (ECD) and catalytic domain (CD) of TMPRSS4 were evaluated using a fluorescence-based assay employing the fluorogenic peptide Boc.Gln-Ala-Arg-7amido4-methyl coumarin hydrochloride (Sigma, Catalogu#B4153) as a substrate. Prepared 10X stock solutions of Trizma base (Sigma, Catalogue#T6791), $CaCl_2$(Sigma, Catalogue#C5670), Imidazole (Sigma, Catalogue#I5513). To optimize the enzyme's activity primarily, three distinct buffer conditions Buffer A (100mM Tris pH 8.0, 10mM CaCl2), Buffer B (30mM Tris pH 8.0, 30mM Imidazole, 200mM NaCl) and Buffer C (20mM Tris pH 8.0, 200mM NaCl), and two substrate concentrations (100µM and 200µM) were examined.

The assay was done using optiplate-96 black micro plates (Perkin, catalogue#6005270). Reactions volumes of 100µl were used in the assay protocol, where substrate, tool compound and enzyme were added as per the plate maps prepared for the respective activities. Readings were taken at 30°C using a Tcan Spark multimode reader in kinetic mode, for 60minutes with excitation and emission wavelengths set at λEx_{380} and λEm_{460}nm, respectively, and the rate of substrate hydrolysis was analysed using the Spark Control Magellan software. Negative assay controls comprising wells devoid of enzyme and wells containing substrate but lacking the enzyme in the assay buffer were included to establish baseline measurement. We have attempted to further miniature the assay in 384 well format and achieved similar profile.

DMSO tolerance

A range of DMSO concentrations (1%, 2%, 4%, 6%, 8%, and 10%) were prepared by dilution with the respective assay buffers. These DMSO concentrations were selected to cover a broad spectrum of typical DMSO concentrations used in biological assays. TMPRSS4 CD and ECD were separately incubated with the substrates at their optimal reaction conditions in the presence of above mentioned DMSO concentrations, and read the reaction at λEx_{380} and λEm_{460}nm.

Test CPD preparation and screening

Prepared all the test CPDs in 100% DMSO as 30mM Stocks. Preliminary screening was done using 3 concentrations of the test compounds at 100µM, 10µM and 1µM in duplicates in a 384 well format. DMSO, Buffer, Substrate and full reaction controls were added in Top and bottom rows in duplicates. Purified TMPRSS4 ECD and CD enzymes were incubated with respective concentrations of the test compounds in 100mM Tris, pH 8.0, 10mM CaCl2 for 30minutes at room temperature. Fluorogenic peptide was added as per the determined Km for ECD and CD domains of TMPRSS4. Incubated the reaction at room temperature, the fluorescence was monitored using Tcan Spark multimode reader at λEx_{380} and λEm_{460}nm at 30°C. Compounds tested positive with > 80% inhibition at top concentration used were further subjected to determine the half maximal inhibitory concentrations (IC_{50}) values.

Statistical Analysis:
Statistical analysis was performed to ensure the accuracy of the enzymatic activity measurements. To achieve this, the background signal from the substrate was subtracted from the enzyme activity signal. The resulting data were then analysed and plotted using GraphPad Prism software.

***Detection Method*:** Fluorescence based readout is used for the assay.

3. Results and Discussion
To screen potential inhibitors of TMPRSS4 identified through molecular docking (12), we developed a fluorescent biochemical assay using the extracellular domain (ECD) and catalytic domain (CD) of TMPRSS4. Successful expression and purification of TMPRSS4 ECD and CD as secretory proteins were achieved from Expi CHO as well HEK F cells, followed by Ni-NTA protein purification, resulting in high-quality proteins (**Figure 7).**

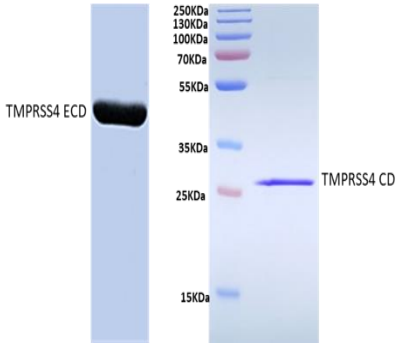


Figure 7*: SDS PAGE Image, showing the purified TMPRSS4 catalytic domain and extra cellular domain by NiNTA column chromatography.*

To assess the biological activity of the expressed protein, we conducted a protease assay using the fluorogenic substrate Boc.Gln-Ala-Arg-7-amido-4-methylcoumarin hydrochloride. This substrate is known to be cleaved by many serine proteases as reported in previous studies. The peptide contains a 7-amino-4-methylcoumarin fluorophore, which is released upon enzymatic activity of TMPRSS4 under optimal conditions and fluoresce at specified excitation, emission wavelengths.

Biological activity evaluations were performed using three different buffer compositions: Buffer A (100mM Tris pH 8.0, 10mM CaCl2), Buffer B (30mM Tris pH 8.0, 30mM Imidazole, 200mM NaCl), and Buffer C (20mM Tris pH 8.0, 200mM NaCl). Buffer A was found to be the most effective buffering composition, resulting in a higher signal compared to the other buffers (Figure 8). Comparing proteins expressed and purified from CHO cells with those from HEK F cells, we observed significantly greater biological activity for both TMPRSS4 CD and ECD proteins expressed and purified from CHO S cells (Figure 8).

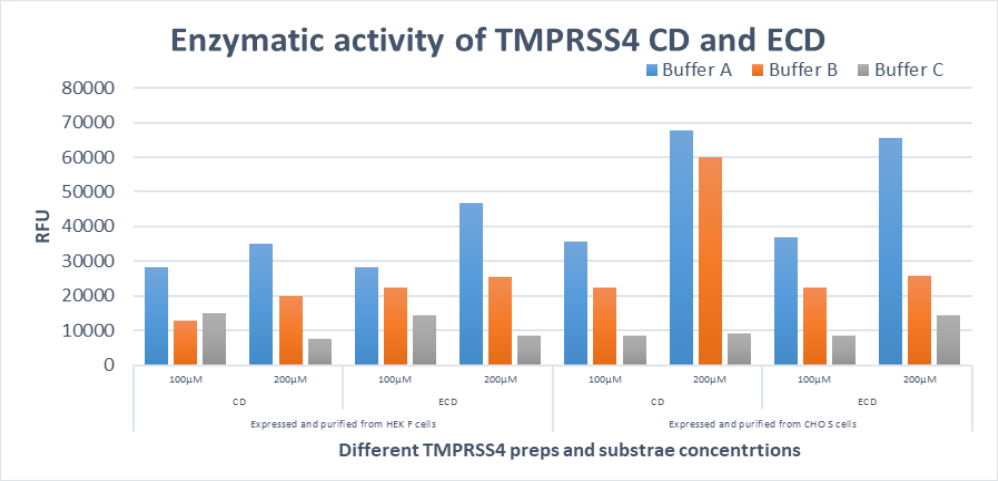


Figure 8: Assessment of TMPRSS4 CD And ECD produced from HEK F and Expi CHO S cells using different assay buffer compositions, and two concentrations of the fluorescent substrate.

For further assay development, we employed Buffer A and a fixed substrate concentration of 100µM, conducting a TMPRSS4 titration. We used enzyme quantities of 0.2µg, 0.5µg, and 1µg per well to determine the optimal enzyme quantity for achieving linearity and a resilient signal. A significant signal was obtained with 0.5µg of the protein. The CD protein exhibited an 18-fold increase in biological activity, while the ECD protein displayed a 14-fold increase, correlating with the protein quantities used (Figure 9C). To gain deeper insights, we conducted further analysis by considering the molar concentration of both TMPRSS4 CD and ECD proteins, allowing us to evaluate enzymatic activity on an equimolar basis. Remarkably the enzymatic activity based on molar concentrations remained similar (Figure 9D)

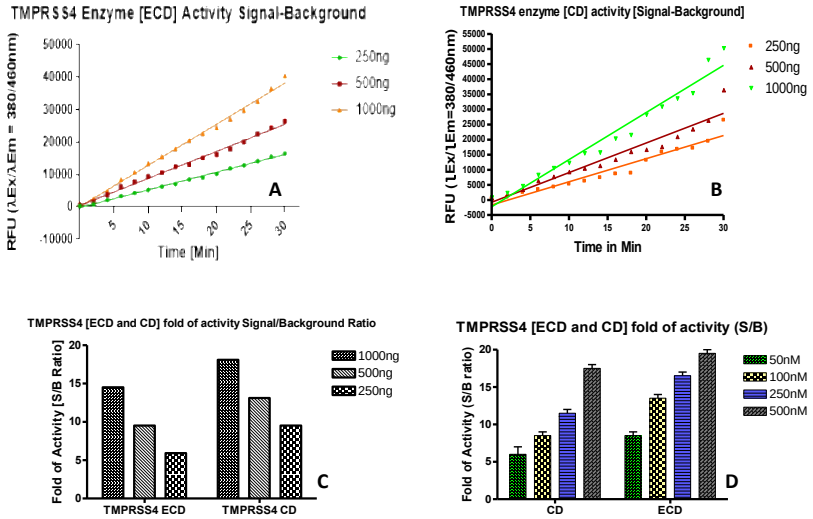


Figure 9: TMPRSS4 peptidase activity (in-vitro). *Figure 9A and 9B illustrates the enzymatic reaction linearity of TMPRSS4 Catalytic Domain (CD) and Extracellular Domain (ECD) using a fixed substrate concentration of 100µM. Three different enzyme quantities (0.25µg, 0.5µg, and 1µg) were tested in kinetic mode. Figure 9C and 9D illustrates the enzymatic activity window achieved considering protein quantities in ug as well in molar concentrations. The reaction rates (measured in RFU) are plotted on the y-axis against the reaction time. The data points show the relationship between enzyme quantity and reaction rate, indicating the linearity of the enzymatic reactions.*

Subsequently, we utilized 200nM (~0.4-0.8ug) of the proteins per well and varied the substrate concentration (5µM, 10µM, 25µM, 50µM, 100µM, and 200µM) to calculate substrate velocity. The resulting data were fitted with nonlinear regression using GraphPad Prism to determine Vmax and Km values (Figure 10). Using our optimized assay protocol, we report Km values of 17µM for the ECD enzyme and 12µM for the CD enzyme.

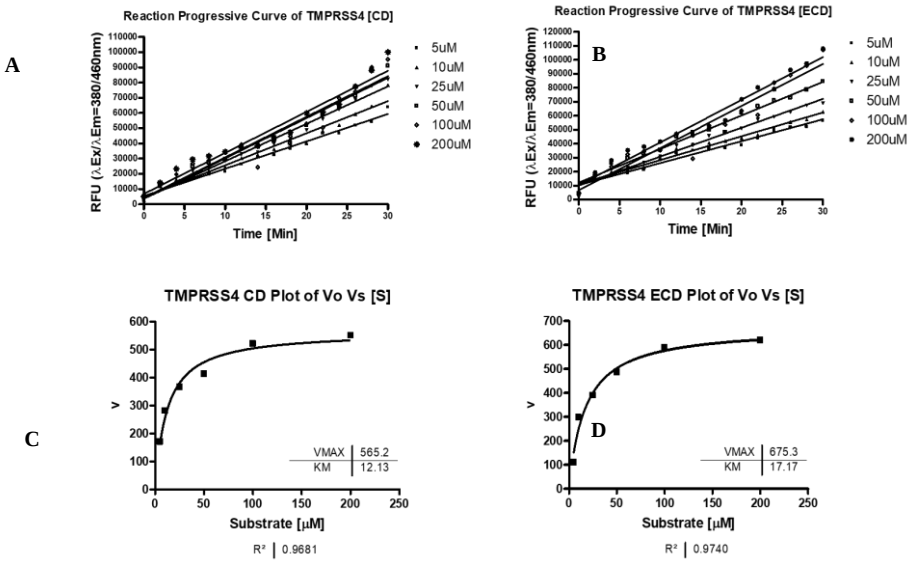


Figure 10: *Figure A and B displays the progressive curves for TMPRSS4 CD (Catalytic Domain) and ECD (Extracellular domain) enzyme kinetics. The graphs illustrate the temporal progression of enzymatic reactions, providing insights into substrate conversion rates over time. C & D illustrates Michaelis-Menten plots for TMPRSS4 CD and ECD measuring the reaction velocities and curve fitting in GraphPad.*

While testing the DMSO tolerance, both TMPRSS4 CD and ECD proteins exhibited a robust enzymatic activity profile up to a DMSO concentration of 4%, at this point enzymatic activity began to decline with increasing DMSO concentrations (Figure 11).

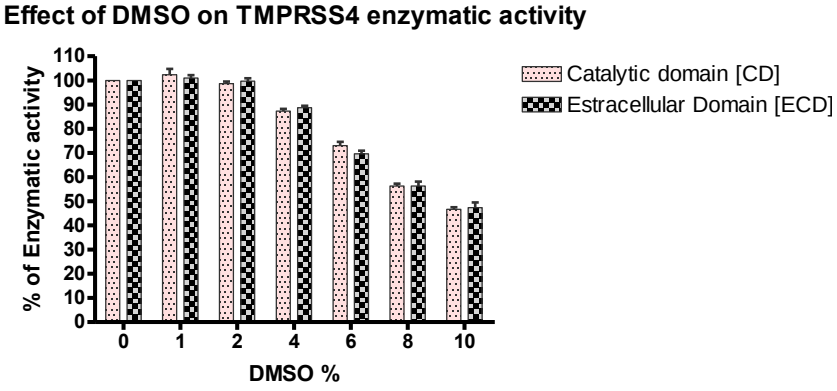
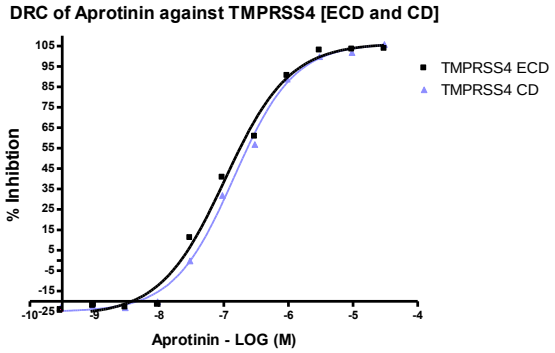


Figure 11: *DMSO tolerance profile of TMPRSS4 ECD and CD.*

To validate the assay, we used Aprotinin (Sigma, A6106), a well-known serine protease inhibitor. Aprotinin diluted in half log concentrations were incubated with enzymes at for 5-10 minutes. Subsequently, the substrate was added at concentrations corresponding to the determined Km values and the reaction was incubated for an additional 30 minutes. Enzyme activity was measured using a Tecan Spark multimode reader at λEx_{380} and λEm_{460nm} at 30 °C. We have observed signals with substrate not having enzyme and used this signal for the signal-background calculations. We have observed enzyme activity inhibition and IC_{50} of aprotinin in the range of 104µM-140µM (Figure 12). These results demonstrate the successful development of a robust and optimized assay for evaluating

TMPRSS4 enzymatic activity, providing important insights into the kinetic parameters of the enzyme and validating the assay using a known inhibitor, Aprotinin.



	TMPRSS4 ECD	TMPRSS4 CD
HILLSLOPE	0.8714	0.9305
EC50	1.046e-007	1.436e-007

Figure 11: *Activity and DRC of Aprotinin, a well-known serine protease inhibitor on inhibiting the enzymatic activity of CD as well ECD of TMPRSS4 (Expressed and purified from Expi CHO S Cells). Half maximal inhibitory concentration of Aprotinin against TMPRSS4* Catalytic domain [CD] and Extracellular domain [ECD] is determined as 143nM and 104nM respectively.

Following the optimized conditions, we have screened ~80 potential anti-TMPRSS4 compounds discovered in our previous studies through molecular docking (13).

Our investigation into the inhibitory potential of scaffold analogues against TMPRSS4 encompassed a meticulous DRC analysis and discovered potent TMPRSS4 inhibitors. This analysis aimed to provide a comprehensive understanding of the concentration-dependent inhibitory effects of the analogues on both the extracellular domain (ECD) and the catalytic domain (CD) of TMPRSS4. These findings underscore the diverse inhibitory profiles exhibited by the scaffold analogues against TMPRSS4 ECD and CD (Table 3).

	Compund IDs	IC50 in µM against TMPRSS4	
		Catalytic domain [CD]	Extracellular domain [ECD]
Scafold 1 analogues	1a	>100	>100
	1b	>100	>100
	1c	>100	>100
	1d	>100	>100
	1e	>100	>100
	1f	>100	>100
	1g	1.1	7.5
	1h	>50	>50
	1i	>50	>50
	1j	2.3	5.5
	1k	>100	>100
	1l	>100	>100
	1m	>100	>100
	1n	>100	>100
	1o	>100	>100
	1p	>100	>100
	1q	3.27	0.99
	1r	>50	>50
	1s	>100	>100
	1t	>100	>100

	Compund IDs	IC50 in µM against TMPRSS4	
		Catalytic domain [CD]	Extracellular domain [ECD]
Scafold 2 analogues	2a	0.36	0.98
	2b	1	1.24
	2c	3.5	4.8
	2d	>100	>100
	2e	3.4	3.8
	2f	1	1.24
	2g	>100	>100
	2j	4.1	3.23

	Compund IDs	IC50 in µM against TMPRSS4	
		Catalytic domain [CD]	Extracellular domain [ECD]
Scafold 5 analogues	5a	>100	>100
	5b	>100	>100
	5c	>100	>100
	5d	>100	>100
	5e	0.19	0.81
	5f	0.99	0.62
	5g	>100	>100
	5h	>100	>100
	5i	1.32	0.96
	5j	>100	>100
	5k	>100	>100
	5l	>100	>100
	5m	4.2	1.8
	5n	>100	>100
	5o	0.96	0.56

	Compund IDs	IC50 in µM against TMPRSS4	
		Catalytic domain [CD]	Extracellular domain [ECD]
Scafold 4 analogues	4a	>100	>100
	4b	>100	>100
	4c	>100	>100
	4d	>100	>100
	4e	>100	>100
	4f	>100 7	>100
	4g	>100	>100
	4h	>100	>100
	4i	0.13	0.85
	4j	1.06	1.4
	4k	0.91	0.98
	4l	8	11.4
	4m	>100	>100
	4n	>100	>100
	4o	0.92	0.85

	Compund IDs	IC50 in µM against TMPRSS4	
		Catalytic domain [CD]	Extracellular domain [ECD]
Scafold 3 analogues	3a	0.36	0.7
	3b	1.1	1.6
	3c	>100	>100
	3s	>100	>100
	3e	0.97	1.45
	3f	3.5	1.1
	3g	1	0.79
	3h	>100	>100

Table 3: *Scaffold analogues subjected to preliminary screening followed by 11point DRC analysis to calculate the half maximal inhibitory concentrations against TMPRSS4 CD and ECD.*

Obtaining a fully functional protein that supports both biophysical and biochemical studies is paramount when investigating recombinant proteins for structural and functional insights. In our study, we opted for mammalian cells as the expression system for TMPRSS4 proteins.

Our study has yielded noteworthy results pertaining to the biological activity of TMPRSS4, specifically catalytic and extracellular domains expressed and purified from CHO S Cells. Additionally, the well-established biological activity of these domains positions them as valuable tools for future studies.

The hydrolysis of peptide chains by proteases is a mechanistic process guided by intricate interactions between the catalytic sites of proteases and complementary amino acid sequences. These interactions encompass the creation of covalent bonds, electrostatic attractions, hydrogen bonds, and van der Waals forces (Irvine Lian Hao Ong et. Al,[12]). Consequently, protease activities exhibit a remarkable level of specificity, resulting in the cleavage of peptide chains that possess distinct amino acid sequences. In light of this understanding, customized peptide substrates are frequently utilized in protease assays to evaluate the biological activity of the target protease.

The mechanistic underpinning of this assay lies in the recognition and binding of the substrate's specific amino acid sequence, Gln-Ala-Arg, by the active serine protease TMPRSS4. The enzyme then cleaves the peptide bond between Arginine (Arg) and MCA, catalyzing a hydrolysis reaction that involves the addition of a water molecule (H_2O) to the peptide bond. This hydrolysis event leads to the separation of the substrate, liberating MCA into the surrounding solution. The released MCA molecule possesses the ability to absorb light at a well-defined wavelength range, typically around 380-400 nanometres, contingent upon the specific substrate utilized. Consequently, it emits fluorescence at a distinct wavelength range, typically around 440-460 nanometres, when excited. The intensity of this emitted fluorescence directly correlates with the protease activity.

The selection of the Boc-Gln-Ala-Arg-MCA substrate in our assay has been pivotal in our assessment of the enzymatic activity of recombinant TMPRSS4 ECD and CD versions. This synthetic peptide substrate, comprising four amino acids (Gln-Ala-Arg) followed by the fluorophore 7-amino-4-methylcoumarin (MCA), represents a well-established choice for such protease assays. MCA, a known fluorophore, exhibits fluorescence upon excitation at specific wavelengths of light, thus facilitating the quantification of enzymatic activity.

In enzymatic assays targeting serine proteases, the choice of buffer pH is a crucial consideration, as it profoundly impacts assay performance and the stability of the enzyme, substrate as well product formed. The existing body of research in this field has consistently highlighted the importance of maintaining buffer pH within the range of 7 to 9 for optimal enzymatic activity. In our experimental design, we made a deliberate choice to fix the pH of the buffer components at 8.0 as it is well reported in many serine protease assays (Yamaguchi et. Al[11]).

In the tested conditions, our enzymatic assay revealed a noteworthy pattern of product formation. Specifically, the enzyme displayed a linear progression of product formation over 60 minutes. This observation suggests that under the experimental parameters employed, the enzymatic reaction proceeded in a controlled and predictable manner, indicative of steady-state kinetics.

The DMSO tolerance testing results during the hydrolysis of the fluorogenic substrate indicate that the enzymatic activity of TMPRSS4 CD and ECD is well-preserved up to 4% DMSO, after which a decrease in activity is observed. These findings underscore the importance of considering DMSO concentrations in experimental design and highlight the need for optimization to maintain optimal enzymatic activity in the presence of co-solvents.

This highly sensitive and quantitative assay provides a robust means of measuring protease activity in our biological samples. It is a versatile tool that has found extensive utility in enzyme kinetics studies, drug discovery efforts, and diverse biochemical research applications. By monitoring the change in fluorescence over time, we were able to precisely quantify the rate of substrate cleavage. This assay

has enabled us to assess the enzymatic activity of TMPRSS4 under various conditions and has positioned us to effectively screen and identify potential TMPRSS4 inhibitors.

4. Conclusion

Overall, our choice of assay methodology and substrate has been instrumental in advancing our understanding of TMPRSS4 and its potential implications in both physiological and pathological processes. Our research attempts led to the creation of a novel and highly specific fluorescent biochemical assay designed to assess the biological activity of human TMPRSS4. This assay not only serves as a valuable tool for understanding the functional aspects of TMPRSS4 but is also strategically compatible with a 96-well format. This compatibility enhances its utility for large-scale applications, particularly in the context of screening potential inhibitors or compound libraries. The successful development of such an assay not only underscores the robustness of our methodology but also presents a versatile platform for future investigations into TMPRSS4-related research, drug discovery, and therapeutic interventions.

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