https://doi.org/10.15218/zjms.2025.009

Inhibitory effect of various recombinant corn trypsin inhibitor variants against trypsin

Received: 27/02/2023 Accepted: 07/05/2023

Badraldin Kareem Hamad [1*]

Abstract

Background and objective: Trypsin is best known for playing a role in enteric digestion, among the most commonly examined serine proteases. Trypsin is expressed extra-pancreatically by a number of cancer types (for instance, Ovarian cancer), and it is thought that both *in vivo* and *in vitro* carcinogenesis are facilitated by trypsin. A new study suggests that trypsin overexpression may promote Coronavirus transmission. In this investigation, several corn trypsin inhibitor (CTI) variants will be expressed and purified, and their trypsin inhibitory action will be assessed.

Methods: CTI was cloned and expressed, and then different variants of rCTI were expressed and assessed for their ability to inhibit trypsin enzyme activity.

Results: The Arg34Ala amino acid substitution negated all inhibitory activity, whereas Gly32Trp, Trp22Ala reduced inhibitory activity by considerable degrees, respectively six-fold and seven-fold; Arg27Ala, Glu39Ala, Arg42Ala, Arg27Ala-Arg42Ala didn't affect inhibitory activity either.

Conclusion: CTI's central inhibition loop is critical for binding trypsin, and the data confirm that Arg34, Trp22, and Gly32 of the loop are involved. It is possible to design new, specific, and safe drug candidates based on the residues for the treatment of ovarian cancer using this framework. This study can also serve as a starting point for designing drugs that reduce the symptoms of COVID-19 caused by trypsin-induced cytokinestorm.

Keywords: CTI; Mutation, Inhibition; Trypsin; Ovarian cancer; COVID-19.

Introduction

One of the serine proteases that is most often examined and is well known for its function in enteric digestion is trypsin. After being created by the acinar cells of the pancreas,[1-4] enterokinase is in charge of activating trypsinogen, the proform of trypsin, in the duodenum. It uses the same enzymatic mechanism as other serine proteases. The catalytic triad of these enzymes is composed of serine 195, aspartate 102, and histidine 57.[5]

Trypsin has been shown to be expressed extra-pancreatically in a number of cancer types and to have tumor-promoting properties both in vivo and in vitro.[2,5-7] In ovarian cancer, trypsinogen expression is linked to tumor aggressivity.[8,9]

Women with ovarian cancer have trypsin-like activity or trypsin (ogen) in their cyst fluid, serum, and ascites.[8,10-12] Trypsin promotes the invasion of ovarian cancers by activating matrix metalloproteinases (MMP) and urokinases-plasminogen activators in addition to destroying extracellular matrix (ECM) components.[10,13-16]

Seven transmembrane G protein-coupled receptors known as protease-activated receptors (PARs) are activated by serine proteases. There are four different isoforms of PARs, each of which is activated by thrombin, whereas PAR2 is activated by trypsin.[16-19] Through a proteolytic process, the PAR2 agonist (such as trypsin) attaches to and cleaves

[1] Department of Pharmacology, College of Pharmacy, Hawler Medical University, Erbil, Iraq.
Correspondence: badraldin.hamad@hmu.edu.krd

https://doi.org/10.15218/zjms.2025.009

the amino-terminus of the receptor, activating it. Through this method, a tethered ligand sequence—like SLIGKV—is produced and after receptor cleavage, binds to and activates the core receptor.[16,17,20]

In many different forms of cancer, including ovarian cancer, there is a correlation between the expression of PAR2 and the tumor's aggressiveness.[21,22] Particularly in gynecologic malignancies, PAR2 encourages cancer cell proliferation, invasion, migration, and metastasis.

Serine proteases activate seven transmembrane G protein-coupled receptors known as protease-activated receptors (PARs). In PARs, there are four isoforms: PAR2 is activated by trypsin, and PAR1,3, and 4 are all activated by thrombin.[16-19] PAR2 is activated through a proteolytic mechanism, in which the PAR2 agonist (e.g. trypsin) binds to and cleaves the receptor's amino-terminus. Through this process, a tethered ligand sequence, such as SLIGKV, is generated that bind to and activates the core receptor following receptor cleavage.[16,17,20]

There is an association between PAR2 expression and tumor aggressiveness in several types of cancer, including ovarian cancer.[21,22] Specifically, PAR2 promotes the proliferation, invasion, migration, and metastasis of cancer cells in gynecologic cancers. Despite the lack of clarity concerning the role of trypsin-PAR2 signaling in ovarian cancer, PAR2 has been linked to increased levels of IL-8, VEGF, and MMP.[7,22] Trypsin upregulation might facilitate the transmission of Coronavirus into patients, according to a recent study.[23]

Corn trypsin inhibitor (CTI) is a bifunctional inhibitor of serine proteases and amylases with a molecular weight of 13.6 kDa and 127 residues.[24-29] It was discovered and isolated from corn and shown to have inhibitory activity against trypsin.[27,29-31]

CTI was first expressed in BL21 (DE3) cells of Escherichia coli . It was, however, insoluble due to the presence of 7 amino acids from the vector linker. In a refolding procedure, the refolded protein was indistinguishable from the commercially available native type CHFI when inhibiting FXIIa.[24-29] As observed in a 1984 study, Arg34 and Leu35 form a scissile bond in the exposed region of CTI. The bond is believed to contribute to interactions with trypsin and possibly activated coagulation factor XIIa (FXIIa). In 1998, the crystal structure of CTI suggested that its specific, atypical conformation of its protease inhibitory site is not responsible for CTI's selective inhibition of factor XIIa.[32,33] With reference to a study Substitution of Arg34 with Ala completely abolished the inhibition of FXIIa. Trp22 at the N-terminus and Arg43 at the C-terminus of the central inhibition loop are two key residues for interaction of CTI with FXIIa.[27-34]

The aim of this study is firstly to perform molecular cloning of CTI, and then, to develop an efficient expression and purification system for producing soluble and functional recombinant wild type CTI, using BL21 (DE3), origami™2 (DE3) cells (Escherichia coli) and pCOLD I-GST vector. Lastly, to characterize the construct viainhibitory activity against FXIIa. Another objective is to investigate the interaction between CTI different variants and trypsin, which was essential to elucidate inhibitory activity of the former against the latter. This study can be considered as an early, necessary approach to design novel, specific and safe drug like candidates for the treatment of ovarian cancer. Using this study as a starting point, drugs can also be designed that reduce cytokine storms caused by trypsin in COVID-19 patients.

Methods

A codon-optimized CTI cDNA inserted into the cloning vector MCS-PUC57, was obtained from Gens-cript (Piscataway, NJ, USA). The sequence codon was optimized for bacterial expression. NdeI (CATATG) and XbaI (TCTAGA) restriction sites were put at two ends of the sequence

https://doi.org/10.15218/zjms.2025.009

respectively. The expression vector, pCOLD I-GST, and the host strain E. coli Rosetta-Gami 2 DE3 were obtained from (EMD Millipore Corporation, Billerica, MA, USA). QuikChange XL Site-Directed Mutagenesis Kit was obtained from Agilent Technologies Stratagene Products Division. Commercial CTI was obtained from Enzyme Research Laboratories (Swansea, UK) and trypsin from Sigma-Aldrich® UK:Trypsin 10x (Catalogue No.: T1763).Phosphate buffered saline (PBS) tablet (Catalogue No.: P4417). S2302 (a chromogenic substrate peptide mimic) was obtained from Chromogenix (UK). The primers were ordered from Eurofins MWG (Ebersberg, Germany) as shown in (Table 1).

Cloning and site-directed mutagenesis

The study was conducted at Nottingham University's center for biomolecular science. The vector, pCOLD I-GST, was used to clone the CTI cDNA.[6, 35] A recombinant fusion protein made up of a His6 tag, an FXa site, a glutathione-S-transferase (GST) tag, an HRV3C protease site, and recombinant CTI (rCTI) was the result of the construct. The www.agilent.com/genomics/qcpd tool, MWG Eurofins site/unmodified DNA oligos, was used to create a forward and reverse primer pair for each of the mutations, and a site-directed mutagenesis kit was used to carry out the mutagenesis (Table 1). Restriction analysis and DNA sequencing provided proof of the mutagenesis.

Table 1 List of the mutagenic primers used in the PCR site directed mutagenesis. The red codons encoding the point sites of interest

Point mutations	Forward Primers (5' to 3')	Reverse Primers (5' to 3')
Trp22Ala	ctgccgtcctgtcgcgcgtatgtcacctcacg	cgtgaggtgacatacgcgcgacaggacggcag
Arg27Ala	ctggtatgtcacctcagccacctgtggcattggt	accaatgccacaggtggctgaggtgacataccag
Gly32Trp	ctcacgcacctgtggcatttggccgcgtctgc	gcagacgcggccaaatgccacaggtgcgtgag
Arg34Ala	tggcattggtccggctctgccgtggccg	cggccacggcagagccggaccaatgcca
Glu39Ala	ctgccgtggccggcactgaaacgtcgc	gcgacgtttcagtgccggccacggcag
Arg43Ala	gccggaactgaaagctgcctgctgtcgtgaactg	cagttcacgacagcaggcagctttcagttccggc
Arg27Ala Arg42Ala	ctggtatgtcacctcagccacctgtggcattggt	accaatgccacaggtggctgaggtgacataccag

https://doi.org/10.15218/zjms.2025.009

Expression and purification:

An OD of 0.9 was attained after the CTI construct was transfected into the expression strain Origami 2 (DE3) and cultured at 37 °C under antibiotic selection. After that, 1M iso-propylthiogalactoside was added to the culture, and it was incubated at 10 °C for 16 hours. Sonication was used to lyse bacterial pellets in a solution of 300 mM NaCl, 100 mM sodium phosphate, and 10% glycerol. Ni2+ -nitrilotriacetic acid agarose column affinity chromatography was used for purification. In order to elute the beads, binding buffer containing 500 mM imidazole was used after the beads had been washed in 20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, and 20 mM imidazole. The protein concentration was measured Via nanodrop and the equal loading on an sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel served as confirmation of protein quantification.(34)

The relevant CTI construct was transfected into expression strain Origami 2 (DE3) and grown under antibiotic selection at 37° C until an OD of 0.9 was reached. The culture was then induced with 500 1M iso-propylthiogalactoside, and incubated for 16 h at 10 °C. Bacterial pellets were collected and lysed by sonication in 100 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, and 10% glycerol. Purification was performed with Ni2+–nitrilotriacetic acid agarose column affinity chromatography. Beads were washed in binding buffer (20 mM sodium phosphate buffer [pH 7.4], 500 mM NaCl, and 20 mM imidazole), and eluted with binding buffer containing 500 mM imidazole. The protein concentration was quantified and verified by equal loading on an SDS-PAGE gel. Where indicated, protein was purified further by gel filtration chromatography in 50 mM Tris-Cland 150 mM NaCl (pH 7.4) on a HiLoad 16/600 Superdex 200 preparative grade column with a fast protein liquid chromatography system (GE Healthcare, Little Chalfont, UK).[34]

Trypsin enzymatic assay

To determine whether recombinant CTI inhibits the enzymatic activity of trypsin as compared to commercial CTI, the release of pNA chromophore from substrate H-D-Pro-Phe-Arg-pNA (S-23002) was monitored. Perkin Elmer Envision plate readers were used to measure pNA release at 405 nm at 33 °C in 96-well plates. Absorbance values were converted to pNA concentrations by comparison with a standard curve obtained under exactly the same instrument conditions.

PBS was added to each test well in duplicate. Afterwards, a fixed substrate concentration (0.2 mM S-2302) was used, along with different concentrations (0, 10, 30, 60, 100, 300, 600, 1000 nM) of commercial and rCTIs; a fixed trypsin con-centration (20 nM) was added last. The following controls were used: S-2302, CTI+PBS, S-2302+PBS, trypsin+PBS, and PBS alone. The reaction volume was 100 µl.

Upon completion, the plate was transferred to the plate reader for monitoring amidolytic activity of the enzyme at 33°C and 405nm wave length. This was done for 30 minutes at 5-minute intervals. Using wild type rCTI as a reference, the mutant proteins of CTI were tested for inhibitory assay using the same methods mentioned above.

Results

Cloning, expression and purification of different CTI variants

Following molecular cloning and expression of rCTI, site-directed mutagenesis was successfully used to produce seven CTI mutants. CTI recombinant variants, such as rCTI-Trp 22Ala, rCTI-Arg27Ala, rCTI-Gly32Trp, rCTI -Arg34Ala, rCTI-Glu39Ala, rCTI-Arg42Ala, and rCTI-Arg27Arg42Ala, have all been expressed and purified successfully in soluble form using the established, effective expression and purification system for rCTI (Figure 1)

https://doi.org/10.15218/zjms.2025.009

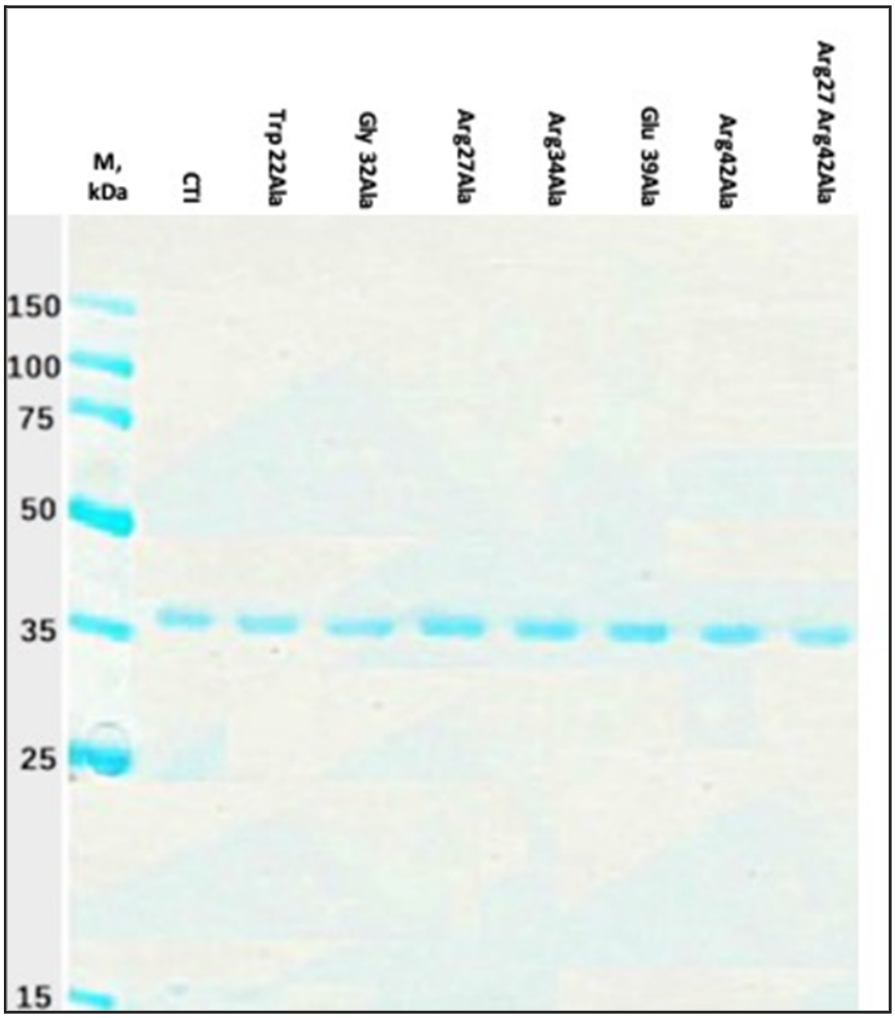

Figure 1 Expression and purification of different CTI variants. recombinant CTI (rCTI) and seven mutant proteins with desired point mutations were successfully produced through site-directed mutagenesis. Using the established, effective expression and purification system for rCTI, the different recombinant variants of CTI, rCTI-Trp22Ala, rCTI-Arg27Ala, rCTI-Gly32Trp, rCTI-Arg34Ala, rCTI-Glu39Ala, rCTI-Arg42Ala, and rCTI-Arg27Arg42Ala, were all successfully expressed and purified in soluble form.

https://doi.org/10.15218/zjms.2025.009

Inhibition assay of rCTI

Both commercial CTI and rCTI exhibit similar behaviors and have comparable inhibitory potency using trypsin-S2302 enzymatic assay (Figure 2).

Effect of replacement of Arg34 by Ala:

Replacement of Arg34 with Ala completely eliminated inhibition of trypsin as shown in (Figure 3).

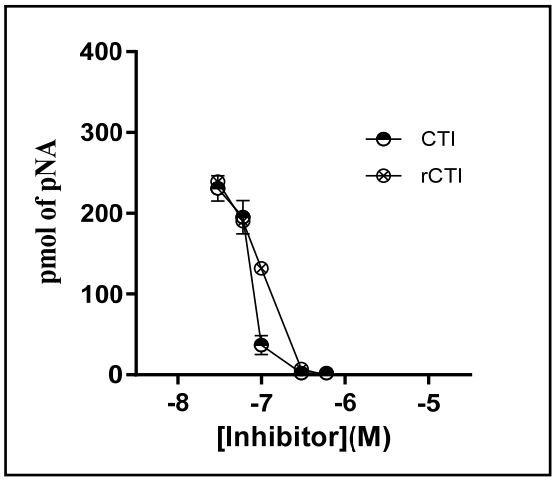

Figure 2 Commercial CTI versus rCTI inhibition assay. Using the calibration equation, the amount of the yellow product (chromophore) produced was converted from the absorbance unit. Trypsin activity after CTI treatment is shown as (pmol of product/pmol of enzyme/sec). The assay was carried out using various concentrations (10-600 nM) of commercial CTI and rCTI co-incubated with 0.2 mM S-2302, and the mixture was then added with 10 nM Trypsin; the substrate cleavage was observed at 405 nm wave length at 33 °C for 30 min (5 minutes time interval). Commercial CTI and rCTI exhibit similar behaviors and have comparable inhibitory potencies. The outcome is the average of three separate experiments. The error bar is the average error across all separate experiments.

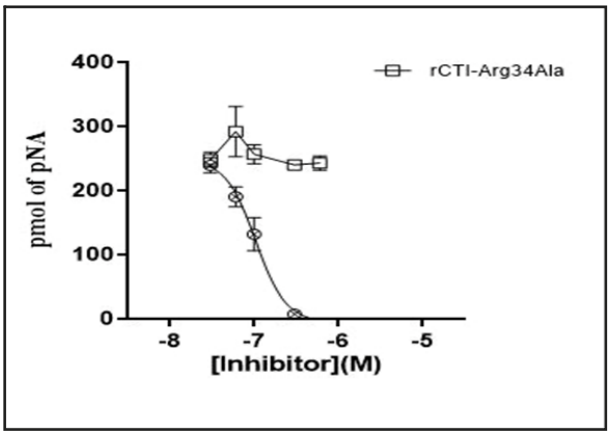

Figure 3 Experimental verification of the central role of Arg34. Inhibition of trypsin was fully removed by replacing this residue with Ala. The positive control, as a reference, showed significant inhibition. Three independent experiments were used to calculate the error bar. Each recombinant variant of CTI was tested in duplicate in each experiment. Duplicate results were averaged.

https://doi.org/10.15218/zjms.2025.009

Effect of replacement of Gly32 byTrp
According to (Figure 4), the mutant protein rCTI Gly32Trp partially inhibits trypsin.
Effect of replacement of Trp22 byAla.
Trypsin is partially inhibited by the recombinant variant, rCTI-Trp22Ala. The reduction in inhibitory activity when Ala is substituted for Trp 22 as shown in (Figure 5).

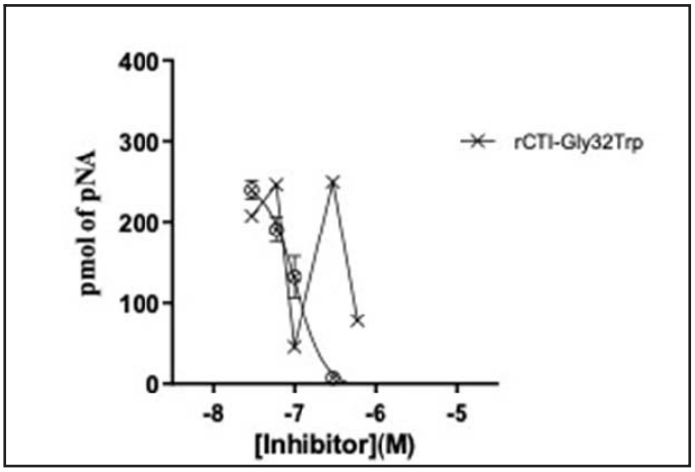

Figure 4 Trypsin is partially inhibited by the rCTI Gly32Trp mutant protein. By substituting TRP for Gly32, the inhibitory activity was reduced as compared with the wild-type inhibition effect. Trypsin's amidolytic activity is reported in terms of pmol of S-2302 converted per pmol of enzyme per second. A standard error of the mean of three independent experiments is displayed in the error bar. A duplicate of each experiment was carried out for each concentration of the inhibitor. The average of the duplicate results was calculated.

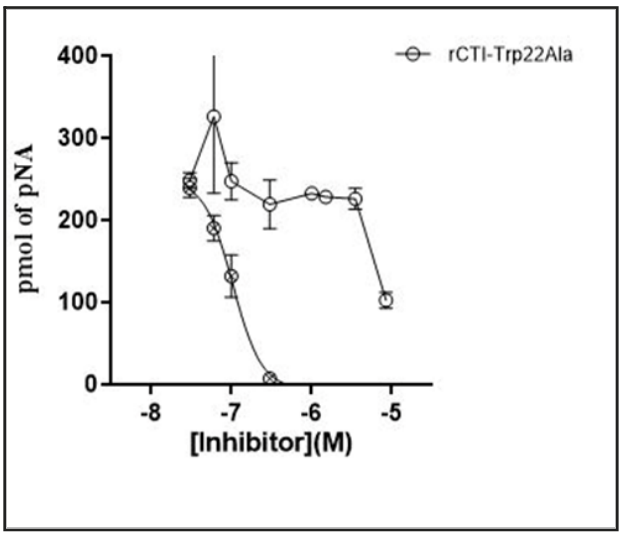

Figure 5 rCTI-Trp22Ala partially inhibits trypsin. Ala was substituted for Trp 22 to reduce the inhibitory activity. The amidolytic activity of trypsin is reported in terms of pmol of S-2302 converted per pmol of enzyme per second. Error bars show the standard error of three independent experiments. For each concentration of the inhibitor, duplicate experiments were conducted. Using duplicate results, the average was calculated.

https://doi.org/10.15218/zjms.2025.009

Effect of Ala substitutions for Arg27, Glu39, and Arg42, and double point mutations

There was no difference in the behaviour of rCTI-Arg27Ala, rCTI-Glu39Ala, rCTI-Arg42Ala, or rCTI-Arg27Ala-Arg42Ala compared to wild-type rCTI as exhibited in (Figure 6)

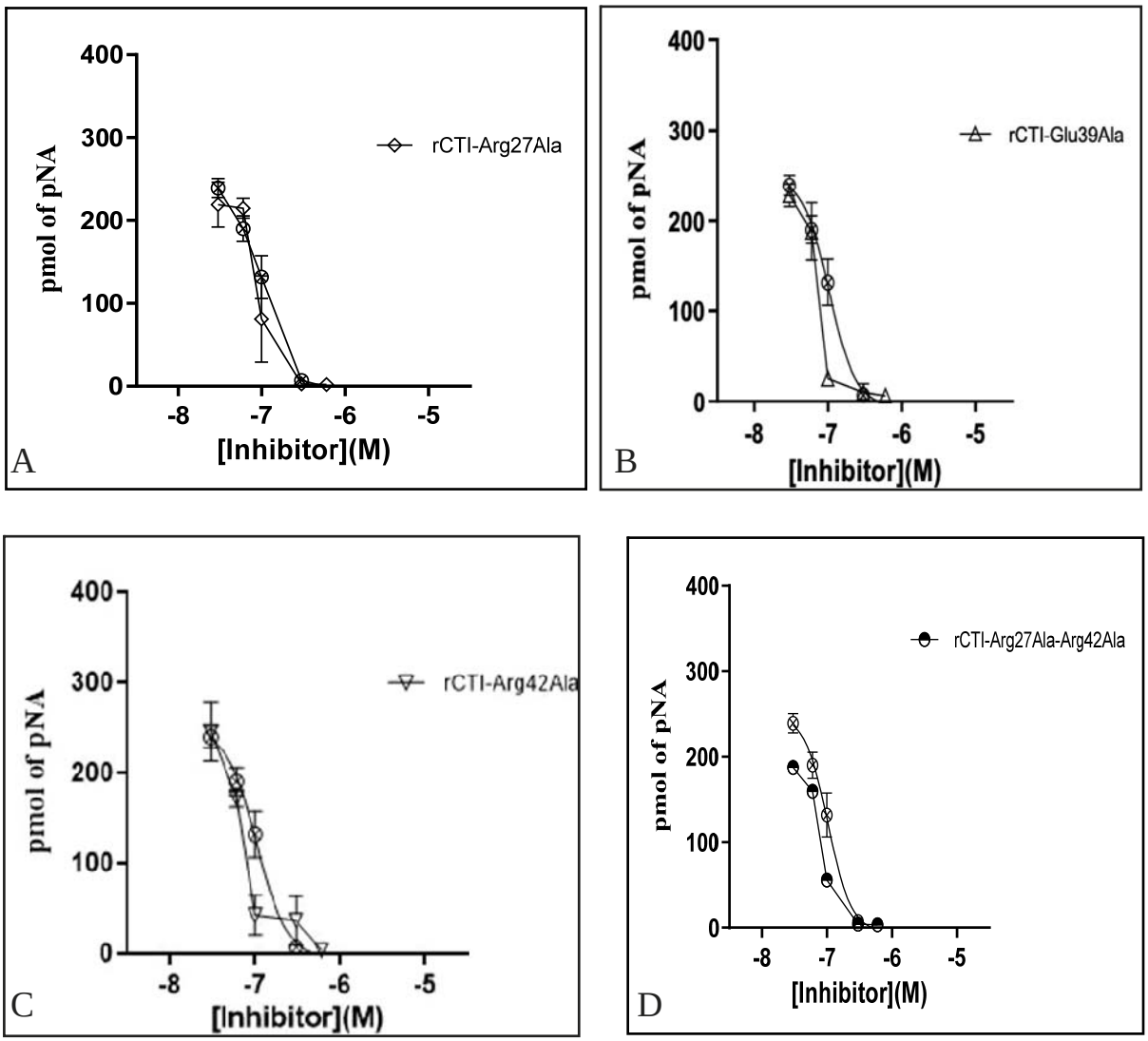

Figure 6 Inhibitory activity of the mutant proteins: **A**. rCTI-Arg27Ala, **B**. rCTI-Glu39Ala, **C**. rCTI-Arg42Ala, and **D**. rCTI-Arg27Ala-Arg42Ala.
They exhibit the same behavior as rCTI wild type, which was employed as a positive control. The hydrolytic activity of trypsin after treatment with various rCTI variants is indicative of the amount of S-2302 that is converted to enzyme per second. The standard error of the mean from three separate experiments is represented by the error bar. For each concentration of rCTI in various forms, a duplicate was utilized in each experiment. The duplicate results' average was calculated.

https://doi.org/10.15218/zjms.2025.009

Inhibition potency of different mutant proteins versus wild type rCTI

Wild type like mutant proteins, as well as non wild type like mutant proteins were compared with the native recombinant protein (rCTI) for their inhibitory effectiveness or IC50 (nM) (Table 2).

Discussion

To assess the inhibition effectiveness of different CTI constructs towards trypsin, the wild type CTI was firstly constructed. The gene of interest (CTI gene) was cloned into an expression vector, pCOLD I-GST vector, successfully. As a result, a fusion gene encoded a fusion protein containing the 6xHis tag, the factor Xa site, the GST tag, and the HRV3C and CTI are situated from the N-terminus to the C-terminus of the protein was generated. An effective protocol for soluble expression and single step purification of recombinant CTI was established using pCOLD I-GST vector and the bacterial expression systems, BL21 DE3 and origami™ 2 (DE3).[34]

the molar absorptivity of the pNA chromogen is found. The inhibitory activity, meaning inhibition effect of the CTI different forms on the catalytic activity of trypsin enzyme, was measured by the influence of rCTI, and commercial CTI on the amount of pNA generated from amidolytic breakdown of the chromogenic substrate by the enzyme. The recombinant wild type CTI displayed tremendous and complete inhibitory activity against trypsin; this establishes the following points. First, the recombinant protein is as active inhibitor as commercial CTI and is properly folded after expression, purification and storage. Inhibition concentration fifty (IC_{50}) of the commercial and the recombinant form is 100±20 and 102 ±16 respectively. This assay can be used as a principal assay and a reference guide for evaluation of the inhibitory activity of the mutant proteins against trypsin in the characterization study of CTI-Trypsin interaction.

Using the recombinant wild type, rCTI, as a reference, substitution of Alanine 34 for Arginine 34 completely abolishes trypsin inhibition. As a result, the central Arg34 at the top of the fully exposed region of the CTI inhibition loop plays an important role in inhibiting human trypsin. Additionally, Arg34 importance is also described in studies characterizing CTI structure and assessing its interaction with FXII.[32-34]

With the purpose of evaluating the

Table 2 Inhibition effectiveness of the different recombinant constructs in comparison with the commercial wild type CTI. The error is the standard error of the mean of three independent experiments. In every experiment, a duplicate was used for testing each concentration of the different forms. The average of the duplicate results was taken.

Different recombinant variants	IC_{50}(nM)	Comparative inhibitory activity (folds)
CTI	100±20	1
rCTI	102 ±16	1
rCTI-Arg27Ala	95 ±18	0.95
rCTI-Arg27Ala-Arg42Ala	90 ±16	0.9
rCTI-Gly32Ala	600 ±60	6
rCTI-Arg34Ala	-	-
rCTI-Trp22Ala	740 ±80	7.4
rCTI-Glu37Ala	90 ±21	0.9
rCTI-Arg42Ala	94 ±10	0.94

https://doi.org/10.15218/zjms.2025.009

contribution of Gly32 and Try22 to the function of CTI, substitutions with Trp32 and Ala22 were introduced and the inhibitory activity of rHIS-GST- -rCTI-Gly32Try and rCTI-Try22Ala was conducted. None of these were able to inhibit FXIIa at low concentrations, but they did at higher concentration. They would be described as partial inhibitors. The inhibition effectiveness of rCTI-Gly32Try was approximately 6 times less potent than that of the wild type, rCTI .

The minimum concentration value of rCTI-Gly32Trp at which 50% inhibition is achieved is 600±60, meaning that its inhibition effect is 6 times less than recombinant wild type. There may be the following reasons behind this observation. replacement of Gly with Trp results in the addition of a new bulky side chain, as glycine possesses only hydrogen as its 'side chain'. In addition, glycine is generally often found at the surface of proteins, often within loops, providing high conformational flexibility to these regions. Due to its location nearer the top of the convex-extended protease binding loop and the central Arg34, Gly32 could have a greater impact on inhibition effects. Furthermore, by replacing Gly32 with Trp, Pro33 would become less flexible because its side chains are connected twice to the protein backbone, forming a five-membered ring (proline, on the other hand, provides rigidity to the protein structure). Thus, Arg34's function would be affected by the rigidity of the loop. A study also observed that mutation of glycine 32 can cause a substantial decline in inhibition effect towards FXII.[34]

This assay was designed to validate Trp22's relevance experimentally. rCTI-Trp22Ala was tested for inhibitory activity. The minimum concentration of the construct to achieve 50% inhibition is 740 nM resulting in 7.5 times less inhibition than the wild type. Trp 22 was also demonstrated to be relevant to the inhibition effect in another study, which used FXII as a protease.[34]

Combined findings from the trypsin study and FXII find Trp22 to be a key residue in explaining CTI's specificity against FXII and trypsin.

As part of this experiment, the point mutations Arg27Ala, Glu39Ala, and Arg42Ala were evaluated for their effects on trypsin function. CTI-Arg27Ala, CTI-Glu39Ala, and CTI-Arg42Ala mutant proteins exhibited no effect on inhibitory activity and behaved like the wild type CTI. The potency of their inhibition was comparable to that of rCTI. The values of IC50 for rCTI-Arg27Ala, rCTI-Glu39Ala, and rCTI-Arg42Ala are 95 ±18, 90±21, 94 ±10 nM respectively. Thus, Replacing Arg27, Glu39, and Arg42 with Ala left the central inhibition loop's affinity unaffected, suggesting they were not essential to inhibiting trypsin with CTI. A mutant, rCTI-Arg27Arg42Ala, with two-point mutations was generated and tested against trypsin to further investigate their potential effect in combination. CTI binding to trypsin was not affected by the mutant proteins. The value of IC50 of rCTI-Arg27Arg42Ala is 90±16 nM.

Conclusion

The data confirm that Arg34, Trp22, and Gly32 of the CTI's central inhibition loop are required for binding trypsin. Using a residue-based framework, it is possible to design safe, specific, and effective drug candidates for ovarian cancer treatment. In addition, this study may provide a basis for developing drugs that reduce the symptoms of COVID-19 caused by an overproduction of cytokines induced by trypsin.

Competing interests

The author declares that he has no competing interests.

References

1. Navarro-Guillen C, Yufera M, Perera E. Biochemical features and modulation of digestive enzymes by environmental temperature in the greater amberjack, Seriola dumerili. Front Mar Sci. 2022; 1391. doi: 10.3389/fmars.2022.960746.

2. Patel S. A critical review on serine protease: key immune manipulator and pathology mediator. Allergol Immunopathol (Madr.). 2017; 45(6):579-91. doi: 10.1016/j.aller.2016.10.011.
3. Forrest CM, McNair K, Vincenten MC, Darlington LG, Stone TW. Selective depletion of tumour suppressors Deleted in Colorectal Cancer (DCC) and neogenin by environmental and endogenous serine proteases: linking diet and cancer. BMC Cancer. 2016; 16(1):1-19. doi: 10.1186/s12885-016-2795.
4. Rashid MY, Noor A, Patel V, Henin S, Cuello-Ramírez A, Gnawali A, et al. Role of SCO-792, a novel enteropeptidase inhibitor, in the prevention of post-endoscopic retrograde cholangiopancreatography pancreatitis. Cureus. 2021; 13(3):1324. doi: 10.7759/cureus.
5. Malthouse JPG. Kinetic Studies of the Effect of pH on the Trypsin-Catalyzed Hydrolysis of N-α-benzyloxycarbonyl-l-lysine-p-nitroanilide: Mechanism of Trypsin Catalysis. ACS omega. 2020; 5(10):4915-23. doi: 10.1021/acsomega.9b03750.
6. Wojtukiewicz MZ, Hempel D, Sierko E, Tucker SC, Honn KV. Protease-activated receptors (PARs)—biology and role in cancer invasion and metastasis. Cancer Metastasis Rev. 2015; 34:775-96. doi: 10.1007/s10555-015-9599-4.
7. Martin CE, List K. Cell surface–anchored serine proteases in cancer progression and metastasis. Cancer Metastasis Rev. 2019; 38:357-87. doi: 10.1007/s10555-019-09811-7.
8. Dong Y, Loessner D, Irving-Rodgers H, Obermair A, Nicklin JL, Clements JA. Metastasis of ovarian cancer is mediated by kallikrein related peptidases. Clin. Exp. Metastasis. 2014; 31:135-47. doi: 10.1007/s10585-013-9615-4.
9. Goettig P, Brandstetter H, Magdolen V. Surface loops of trypsin-like serine proteases as determinants of function. Biochimie. 2019; 166:52-76. doi: 10.1021/acs.jproteome.7b00541.
10. Kim KK, Turner R, Khazan N, Kodza A, Jones A, Singh RK et al. Role of trypsin and protease-activated receptor-2 in ovarian cancer. PLoS One. 2020; 15(5):e0232253. doi: 10.1371/journal.pone.0232253.
11. O'Connell C, VandenHeuvel S, Kamat A, Raghavan S, Godin B. The Proteolytic Landscape of Ovarian Cancer: Applications in Nanomedicine. Int J Mol Sci. 2022; 23(17):9981. doi: 10.3390/ijms23179981.
12. Peregrina-Sandoval J, del Toro-Arreola S, Oceguera-Villanueva A, Cerda-Camacho F, del Toro-Arreola A, Gonzalez-Ramella O, et al. Trypsin proteolytic activity in cervical cancer and precursor lesions. Int J Clin Exp Pathol. 2017; 10(5):5587-93. www.ijcep.com/ISSN:1936-2625/IJCEP0051629.
13. Kenny HA, Leonhardt P, Ladanyi A, Yamada SD, Montag A, Im HK, et al. Targeting the Urokinase Plasminogen Activator Receptor Inhibits Ovarian Cancer MetastasisTargeting of u-PAR Inhibits Ovarian Cancer Invasion and Metastasis. Clin. Cancer Res. 2011; 17(3):459-71. doi: 10.1158/1078-0432.CCR-10-2258.
14. Mahmood N, Mihalcioiu C, Rabbani SA. Multifaceted role of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR): diagnostic, prognostic, and therapeutic applications. Front Oncol. 2018; 8:24. https://doi.org/10.3389/fonc.2018.00024. PMID:29484286; PMCID:PMC5816037.
15. Zhang Y, Kenny HA, Swindell EP, Mitra AK, Hankins PL, Ahn RW, et al. Urokinase Plasminogen Activator System–Targeted Delivery of Nanobins as a Novel Ovarian Cancer Therapy uPA-Targeted Nanobin Therapy in Ovarian Cancer. Mol. Cancer Ther. 2013; 12(12):2628-39. doi: 10.1158/1535-7163.MCT-13-0204.
16. Elste AP, Petersen I. Expression of proteinase-activated receptor 1-4 (PAR 1-4) in human cancer. J Mol Histol. 2010; 41:89-99. doi: 110.1007/s10735-010-9274-6.
17. Gratio V, Beaufort N, Seiz L, Maier J, Virca GD, Debela M, et al. Kallikrein-related peptidase 4: a new activator of the aberrantly expressed protease-activated receptor 1 in colon cancer cells. Am J Pathol. 2010; 176(3):1452-61. doi: 10.2353/ajpath.2010.090523.
18. Han N, Jin K, He K, Cao J, Teng L. Protease-activated receptors in cancer: A systematic review. Oncol Lett. 2011; 2(4):599-608. doi: 10.3892/ol.2011.291.
19. Sébert M, Sola-Tapias N, Mas E, Barreau F, Ferrand A. Protease-activated receptors in the intestine: focus on inflammation and cancer. Front Endocrinol (Lausanne). 2019; 10:717. doi: 10.3389/fendo.2019.00717.
20. Falconer AM, Chan CM, Gray J, Nagashima I, Holland RA, Shimizu H, et al. Collagenolytic matrix metalloproteinases antagonize proteinase-activated receptor-2 activation, providing insights into extracellular matrix turnover. J Biol Chem. 2019; 294(26):10266-77. doi: 10.1074/jbc.RA119.006974.
21. Barry GD, Suen JY, Le GT, Cotterell A, Reid RC, Fairlie DP. Novel agonists and antagonists for human protease activated receptor 2. J Med-Chem. 2010; 53(20):7428-40. doi: 10.1021/jm100984y. PMID: 20873792.
22. Chanakira A, Westmark PR, Ong IM, Sheehan JP. Tissue factor-factor VIIa complex triggers protease activated receptor 2-dependent growth factor release and migration in ovarian cancer. Gynecol Oncol. 2017; 145(1):167-75. doi: 10.1016/j.ygyno.2017.01.022.
23. Chen H, Huang S, Chen Q, Liu Q, Lv X. Trypsin may induce cytokine storm in M1 macrophages, resulting in critical coronavirus disease. Respir Physiol Neurobiol. 2022; 303:103920. doi: 10.1016/j.resp.2022.103920.

24. Touw CE, de Jong Y, van Hylckama Vlieg A. The influence of corn trypsin inhibitor on the contribution of coagulation determinants to the Technoclone Thrombin Generation Assay (TGA) and the Calibrated Automated Thrombogram (CAT). PLoS One. 2022; 17(2):e0263960. doi: 10.1371/journal.pone.0263960. PMID: 35213588; PMCID: PMC8880747.
25. Parunov L, Surov S, Tucker E, Ovanesov M. The effect of corn trypsin inhibitor and inhibiting antibodies for FXIa and FXIIa on coagulation of plasma and whole blood: comment. J Thromb Haemost. 2015; 13(8):1527-30. doi: 10.1111/jth.12707. Epub 2014 Sep 30. PMID: 25142753.
26. Alibeik S, Zhu S, Yau JW, Weitz JI, Brash JL. Modification of polyurethane with polyethylene glycol–corn trypsin inhibitor for inhibition of factor XIIa in blood contact. J Biomater Sci Polym Ed. 2012; 23(15):1981-93. doi: 10.1163/092050611X603250.
27. Hellum M, Franco-Lie I, Øvstebø R, Hauge T, Henriksson CE. The effect of corn trypsin inhibitor, anti-tissue factor pathway inhibitor antibodies and phospholipids on microvesicle-associated thrombin generation in patients with pancreatic cancer and healthy controls. PLoS One. 2017; 12(9):e0184579. doi: 10.1371/journal.pone.0184579. PMID: 28910348; PMCID:PMC5598995.
28. Mohammed B, Martin E, Salinas V, Carmona R, Young G, Brophy D. Failure of corn trypsin inhibitor to affect the thrombin generation assay in plasma from severe hemophiliacs. J Thromb Haemost. 2014; 12(9):1558-61. doi: 10.1111/jth.12659.
29. Hansson K, Nielsen S, Elg M, Deinum J. The effect of corn trypsin inhibitor and inhibiting antibodies for FXIa and FXIIa on coagulation of plasma and whole blood. J Thromb Haemost. 2014; 12(10):1678-86. doi: 10.1111/jth.12707.
30. Butenas S, Mann K. The effect of corn trypsin inhibitor and inhibiting antibodies for FXIa and FXIIa on coagulation of plasma and whole blood: comment'. J Thromb Haemost. 2015; 13(3):487. doi: 10..1111/jth.12812.
31. Alharbi A, Iyer N, Al Qaryoute A, Raman R, Burks DJ, Azad RK, et al. Role of ribosomal RNA released from red cells in blood coagulation in zebrafish and humans. Blood Advances. 2021; 5(22):4634-47. doi: 10.1182/bloodadvances.2020003325.
32. Baeriswyl V, Calzavarini S, Chen S, Zorzi A, Bologna L, Angelillo-Scherrer A, et al. A synthetic factor XIIa inhibitor blocks selectively intrinsic coagulation initiation. ACS Chem Biol. 2015; 10 (8):1861-70. doi: 10./.1021/acschembio.5b00103.
33. Meng D, Andre P, Bateman TJ, Berger R, Chen Y-H, Desai K, et al. Development of a novel tricyclic class of potent and selective FIXa inhibitors. Bioorg Med Chem Lett. 2015; 25(22):5437-43. doi: 10.1016/j.bmcl.2015.07.078.
34. Hamad BK, Pathak M, Manna R, Fischer PM, Emsley J, Dekker LV. Assessment of the protein interaction between coagulation factor XII and corn trypsin inhibitor by molecular docking and biochemical validation. J Thromb Haemost. 2017; 15(9):1818-28. doi: 10.1111/jth.13773.
35. Liu T, Zhao H, Jian S, Gong S, Li S, Ma Y, et al. Functional expression, purification and identification of interaction partners of PACRG. Molecules. 2021; 26(8):2308. doi: 10.3390/molecules26082308. PMID: 33923444; PMCID: PMC8074078.