Original Article

Structural and Biophysical Characterization of *Cajanus cajan* Protease Inhibitor

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Abstract

Context: A large number of studies have proven that Protease inhibitors (PIs), specifically serine protease inhibitors, show immense divergence in regulation of proteolysis by targeting their specific proteases and hence, they play a key role in healthcare. **Objective:** We aimed to access *in-vitro* anticancer potential of PI from *Cajanus cajan* (CCPI). Also, crystallization of CCPI was targetted alongwith structure determination and its structure-function relationship. **Materials and Methods:** CCPI was purified from *Cajanus cajan* seeds by chromatographic techniques. The purity and molecular mass was determined by SDS-PAGE. Anticancer potential of CCPI was determined by MTT assay in normal HEK and cancerous A549 cells. The crystallization screening of CCPI was performed by commercially available screens. CCPI sequence was subject to BLASTp with homologous PIs. Progressive multiple alignment was performed using clustalw2 and was modelled using *ab initio* protocol of I-TASSER. **Results:** The results showed ~14kDa CCPI was purified in homogeneity. Also, CCPI showed low cytotoxic effects of in HEK i.e., 27% as compared with 51% cytotoxicity in A549 cells. CCPI crystallized at 16°C using 15% PEG 6000 in 0.1M potassium phosphate buffer (pH 6.0) in 2-3weeks as rod or needles visualized as clusters under the microscope. The molecular modelling revealed that it contains 3 beta sheets, 3 beta hairpins, 2 β-bulges, 6 strands, 3 helices, 1helix-helix interaction, 41 β-turns and 27 γ-turns. **Discussion and Conclusion:** The results indicate that CCPI may help to treat cancer *in vivo* aswell. Also, this is the first report on preliminary crystallization and structural studies of CCPI.

Keywords: Anticancer, *Cajanus cajan*, crystallization, homology modeling, protease inhibitor, sequence analysis

Introduction

Protein protease inhibitors (PPIs) are proteins that diminish the proteolytic activity of proteases. They form a stable complex with target proteinase by either altering, blocking, or preventing access to the active site of enzyme and hence play important role in regulation of proteolysis.[1] PPIs are omnipresent and versatile and hence are used in a wide range of field including their role as therapeutic agents in diseases, specifically cellular transformation, osteoporosis, blood clotting disorders, retroviral disease, cancer, etc., Currently, PPIs are keenly investigated for their role as anticancer agents, i.e., inhibition of transformed cell growth.[2-5] Metastasis of cancer cells requires action of the matrix metalloproteinases and serine proteases that constitute a complex interacting protease cascade system. Hence, inhibition of such processes and proteins is most likely to be the molecular targets for cancer prevention.[6] Furthermore, studies are being performed to investigate protease inhibitors (PIs) as novel drugs in highly active antiretroviral combination therapy, which aim to increase life expectancy of an HIV-positive patient.[7,8]

In field of agriculture, to counterbalance the loss caused by chemical pesticides, plant PPIs have gained remarkable attention as natural defense agents in plants.[9] Along with the role of growth inhibition of insects and pests, PPIs also show inhibitory activity of pathogenic nematodes such as *Globodera tabaccum*[10] and pathogenic fungi such as *Trichoderma reesei*[11] and *Alternaria alternata*.[12] With the wide range of applications in the field of medicine and agriculture, researchers have gained a keen interest in searching novel PPIs and their therapeutic

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importance.[13] Therefore, a number of PPIs have been isolated and characterized from various plant sources.[14-20]

Plants have ability to produce certain biologically active compounds that are believed to be involved in the defense mechanism against pests, insects, and microbial attacks. This system includes use of defense proteins such as PIs, lectins, amylase inhibitors, and few pathogenesis-related proteins.[21-24] To be specific, pigeon pea (*Cajanus cajan* L.) seeds contain PIs of trypsin, chymotrypsin, and amylases,[25,26] as well as secondary metabolites and phytolectins,[27,28] as the defense machinery against pest and microbial infection. Pichare and Kachole have reported seven isoforms of trypsin-chymotrypsin inhibitors and two isoforms of trypsin inhibitors (TIs) from *C. cajan* seeds.[29] In addition, Godbole *et al*. and Haq and Khan depicted *C. cajan* PI (CCPI) as Kunitz-type PI having inhibitory activity against trypsin and chymotrypsin.[17,30] Our study aimed to purify CCPI from the seeds of *C. cajan* and its role in cellular cytotoxicity in normal and cancer cell line was assessed. For the first time, crystallization study of CCPI was performed and its *in-silico* analysis was done to support the structure-function relationship.

Materials and Methods

Chemicals, reagents, and materials

C. cajan (PUSA-992 variety), commonly known as "*arhar*," was received from IARI, New Delhi. Chemicals: trypsin (bovine pancreatic trypsin), Nα-benzoyl-DL-argi nine-4-nitroanilide hydrochloride (BAPNA), acrylamide, tetramethylethylenediamine, bis-acrylamide, and ammonium persulfate were obtained from Sigma-Aldrich. For an initial screen, commercially available crystallization screens were purchased from Molecular Dimensions. All other reagents and chemicals used were of analytical grade.

Cell lines and cell culture

Human embryonic kidney (HEK) and adenocarcinomic human alveolar basal epithelial cells (A549) were maintained in RPMI-1640 grown in 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The cells were cultured at 37°C, 5% CO_2 humid condition in CO_2 incubator (Thermo). All the other chemicals were procured from Sigma-Aldrich and Merck.

Purification of *Cajanus cajan* protease inhibitor

CCPI was purified as per the protocol mentioned by Haq and Khan[17] Fractions showing inhibitory activity against trypsin were pooled together, dialyzed against Tris buffer (pH 8.2) for desalting, and loaded onto fast protein liquid chromatography (FPLC) gel filtration Superdex-75 column preequilibrated with Tris buffer (pH 8.2). CCPI was eluted at the rate of 0.5 ml/min. The purified and active fractions of CCPI were taken as sample for further research.

Determination of purity and molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The purity and molecular mass of CCPI were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on 12% polyacrylamide slab gel under reducing conditions using method of Laemmli.[31] CCPI sample was mixed with a sample buffer (0.125-M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, and 10% β-mercaptoethanol) in equal ratio. The mixture was then brought to boiling for 5 min. Fifteen microliters of protein sample was loaded onto gel composed of 5% stacking gel and 12% resolving gel. The electrophoresis was carried out at constant 100V current using Mini-Protean apparatus (Bio-Rad). After electrophoresis, gel was stained with 0.1% Coomassie Brilliant blue R-250 of water:methanol:acetic acid in 50:40:10 for 1 h and destained with solution of water: methanol:acetic acid in 50:40:10 overnight. The molecular weight of CCPI was estimated by comparing with Puregene prestained Protein Ladder, Broad Range (10–250 kDa).

Evaluation of trypsin-inhibitory activity

The enzymatic activity of CCPI against trypsin was checked as per the protocol of Erlanger *et al*. with minor modifications.[32] The residual enzymatic activity was checked using BAPNA-HCl as substrate. Twenty microliters of trypsin (1 mg/m1) was incubated with 100 µl of CCPI sample and 80 µl of Tris buffer (pH 8.2) for 10 min at room temperature. The reaction was initiated by addition 500 µl BAPNA solution (1.5 mM) and was incubated at room temperature. Three hundred microliters of 30% acetic acid solution was added after 10 min to terminate the reaction. The total reaction mixture volume was 1 ml. The decrease in intensity of yellow color due to enzymatic hydrolysis of the BAPNA was visualized at 410 nm, which corresponded to release of p-nitroaniline and hence the TI activity.

Assessment of cytotoxic activity against cancer cell line

Anti-proliferative activity of CCPI was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the protocol of Verma *et al*.[33] Anti-proliferative studies of CCPI were performed using MTT assay on adenocarcinomic human alveolar basal epithelial cells (A549) and nontumor HEK cell lines, respectively. Cells were seeded at the density of 5×10^3 cells/well in a 96-well plate supplemented with 2.5% FBS. After treatment with the PPIs, cells were incubated at 37°C with two different concentrations, i.e., 10 µg/ml and 5 µg/ml for 24 and 48 h. After the necessary time period, 20 µl of MTT solution (5 mg/ml in PBS buffer, pH 7.4) was added to wells and incubated for 4 h. After adding 150 µl of dimethyl sulfoxide that dissolves formazan crystals formed from cellular reduction of MTT in well, the plate was read at the optical density of 540 nm wavelengths on the ELISA-reader (Synergy HT, Biotek, USA).

All measurements were done in triplicates. The percent cytotoxicity values were determined by:

$$\% \text{ cytotoxicity} = ([A]_{control} - [A]_{test})/(A)_{control} \times 100$$

Where $(A)_{control}$ is the absorbance of control sample and $(A)_{test}$ is absorbance of test sample.

Screening and developing *Cajanus cajan* protease inhibitor crystals

For an initial screen, commercially available screens were used that exploit the trial conditions. These screens included (1) PACT premier; (2) JCSG plus; (3) Morpheus; (4) Proplex; (5) Three-dimensional (3D) structure; (6) MacroSol; (7) PGA screen; (8) Structural screen. CCPI sample was spun at 15 min/18,000 ×g/4°C to settle down dust and aggregated proteins (if any). The sample was concentrated to the final concentration 5 mg/ml using an Amicon filter. The 96-well hanging drop tray was filled with 100 µl of reservoir buffers according to the tray setup scheme as per screen kits. Ten microliters of CCPI was loaded for each row of well on the cover slide which was then distributed in all wells using mosquito pipetting robot (TTP Labtech). The cover slide was flipped gently and laid down on the grease ring on top of the well. The slide was pressed gently to allow the air entrapped to escape and keep the well sealed. The trays were incubated at 16°C undisturbed and were observed regularly under microscope to visualize the crystal formation.

Primary X-ray diffraction analysis

For initial characterization, CCPI crystals were observed under an R-AXIS IV++ image-plate detector and Rigaku rotating-anode X-ray generator at room temperature using Cu K-radiation.

Sequence analysis and annotations

CCPI sequence retrieved from the UniProt database (Uniprot: Q5U9N0) (http://www.uniprot.org/). This sequence information was analyzed to determine regulatory sequences, structural motifs, and repetitive sequences. A comparison of genes within a species or between different species can show either similarities between protein functions or relations between species. The CCPI sequence was subject to BLASTp with homologous PIs from different species. Progressive multiple alignment was performed using clustalw2.

Homology modeling

CCPI (Uniprot: Q5U9N0) was modeled using *ab initio* protocol of the I-TASSER.[34] Subsequently, five models were generated and assessed on the basis of RMSD and TM-score. This online server theoretically measures various physicochemical parameters such as molecular mass. The overall quality factor score of CCPI was predicted by ERRAT (http://nihserver.mbi.ucla.edu/ERRAT/). The refined structure was validated using SAVES (http://services.mbi.ucla.edu/SAVES/). The topological analysis of the given CCPI structure was done using PDBsum, for understanding the structural features of CCPI structure in detail.

Results and Discussion

Importance of *Cajanus cajan* protease inhibitor

CCPI has been found to be of importance in plant defense. Various studies performed *in-vitro* and *in-vivo* have suggested that the CCPI are potentially active against proteases of larval guts, which lead to impaired digestion and amino acid absorption, cause retarded growth and development of larvae, and lead to loss of fertility and productivity of adult moths. For instance, CCPI shows moderate inhibition potential toward insect *Helicoverpa armigera* gut proteinases.[35] It also diminished the activity of proteinases of larval midgut showing trypsin-like nature in *Manduca sexta*.[36] CCPI being smaller in size can be expressed in castor plants to protect them against their lethal pest *Achaea janata* by inhibiting its midgut trypsin-like proteases.[37]

Purification and characterization for activity, molecular weight, and purity

CCPI was isolated and purified in homogeneity from *C. cajan* seeds. It involved three-step chromatography method which included double ion-exchange (from previous study) and FPLC on Superdex 75 column where an elution profile was obtained [Figure 1a]. The CCPI obtained was subjected to protein concentration determination and TI activity assay at each step [Table 1]. Although PPIs from *C. cajan* have earlier been purified, the family or type of inhibitor to which they belong does not depict a clear picture. Godbole *et al*. purified two PIs kDa from *C. cajan* cv. TAT-10 showing molecular weight of ~15 and ~10.5 and proposed that the PPI belonged to Bowman–Birk inhibitor (BBI) family.[30] However, as we know, PIs belonging to BBI family have lower molecular weight of around 6–9 kDa.[38] Furthermore, Haq and Khan purified CCPI of molecular weight ~14 kDa and concluded on basis of its N-terminal sequence that CCPI belonged to Kunitz family.[17] Further, Osowole *et al*. isolated PPI from *C. cajan* weighing ~18.2 kDa.[39] Norioka *et al*. showed that CCPIs are only BBI on the basis of gel filtration peaks.[40] Further, Prasad *et al*. purified BBI-type PI from *C. cajan* naming it Red Gram PI.[36] Our protocol resulted in purification of CCPI

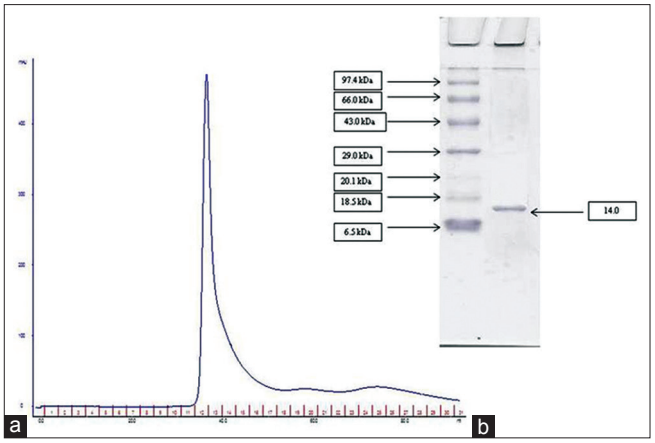


Figure 1: (a) Fast protein liquid chromatography chromatogram of *Cajanus cajan* protease inhibitor eluted on Superdex 75 column. The graph represents concentration of *Cajanus cajan* protease inhibitor eluted on Y axis with eluted fraction on Y axis. (b) Electrophoretogram of purified *Cajanus cajan* protease inhibitor on sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) shows molecular-weight markers (Lane 1) and a single band of molecular mass 14 kDa eluted by fast protein liquid chromatography on Superdex 75 column (Lane 2)

with homogeneity which was depicted as a thick single band at 14 kDa investigated on 12% SDS-PAGE [Figure 1b].

Assessment of cytotoxic activity against cancer cell line

Our results clearly proved that the CCPI was found active against tumor cells when compared to nontumor cells in time- and concentration-dependent manner. CCPI was added in two different concentrations and the cells were incubated for two different time intervals and cytotoxic effect of CCPI on cells was accessed. MTT end-points suggested that the IC_{50} value of CCPI for A549 cells was ~9.84 μg/ml which was lower as compared to IC_{50} value for HEK cells, i.e., ~18.18 μg/ml. CCPI showed low cytotoxic effects in HEK (27%) than A549 cells (51%) at 48 h, which were higher to the values obtained at 24 h interval. Hence, we can conclude that CCPI shows higher cytotoxicity against A549 cells as compared to HEK cells in time- and concentration-dependent manner [Figure 2]. Rakashanda *et al*. reported the IC_{50} values of *Lavatera cashmeriana* PIs to be 36 ± 2 μg/ml in human lung cancer cell line (NCIH322), which was quite higher than the results obtained in our studies.[6] Hence, CCPI demonstrates more inhibitory effect on cancer cell lines and therefore can be depicted as an antitumor drug in near future.

Screening and crystallization

CCPI was screened preliminarily with seven different screens. Morpheus and 3D structural screen were repeated.

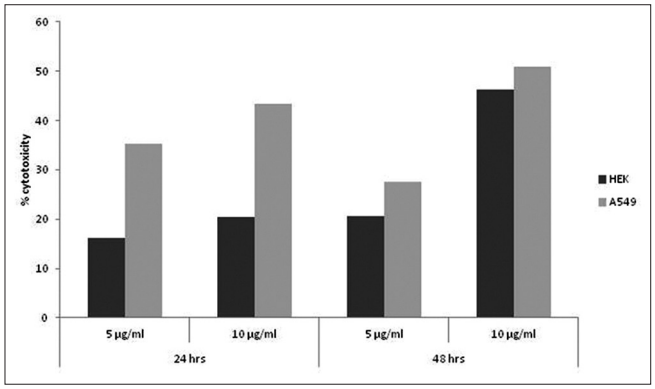


Figure 2: Cytotoxic effect of *Cajanus cajan* protease inhibitor on A549 and human embryonic kidney cell lines. Results are depicted as bar diagrams. The cells were incubated with *Cajanus cajan* protease inhibitor at 5 μg/ml and 10 μg/ml in a 96-well plate. Optical density at 540 nm was measured after 24 and 48 h and percentage cytotoxicity was determined

The conditions showing sign of crystal growth were repeated manually on a 24-well plate. Crystals of the CCPI were obtained in 2–3 weeks using 15% polyethylene glycol (PEG) 6000 in 0.1M potassium phosphate buffer, pH 6.0 [Figure 3]. The crystals obtained were either rectangular- or rod-like structure grouped in the form of clusters which was quite similar to the orthorhombic crystals of PI from *Tamarindus indica*.[20] Due to lack of symmetry and homogeneity, the crystals of CCPI could not diffracted by X-ray. Therefore, the size of crystals could not be determined, but this was the first study in the context of crystallization of CCPI till date.

Sequence and structure determination

The CCPI sequence obtained from http://www.uniprot.org/ (Uniprot: Q5U9N0) has 176 amino acid sequences; first 1–19 are the signal peptide shown in red and 20–176 are chain [Figure 4a]. The molecular mass of *C. cajan* was 19.97 kDa with isoelectric point 9.54.[41] Procheck showed that 77.6% of the residues were in the allowed region of Ramachandran plot. The overall quality factor score predicted by ERRAT was 69.04 for PI. PDBsum showed that initial CCPI contained strands 7.4% (13 aa), alpha helix 11.4% (20 aa), and other 81.2% (143 aa). The structure showed the presence of 3 beta-sheets, 3 beta-hairpins, 2 β-bulges, 6 strands, 3 helices, 1 helix–helix interaction, 41 β-turns, and 27 γ-turns. Moreover, there was no disulfide bonds were found in the structure of *C. cajan* [Figure 4b].[42]

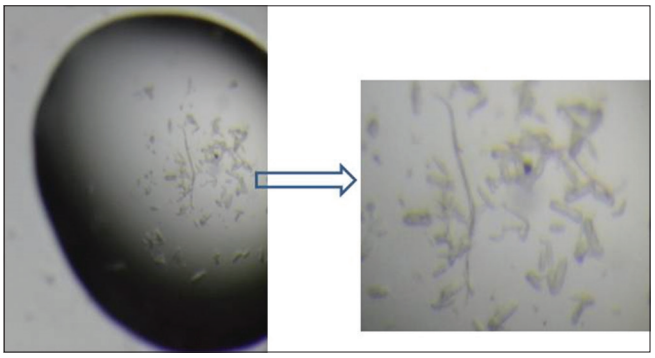
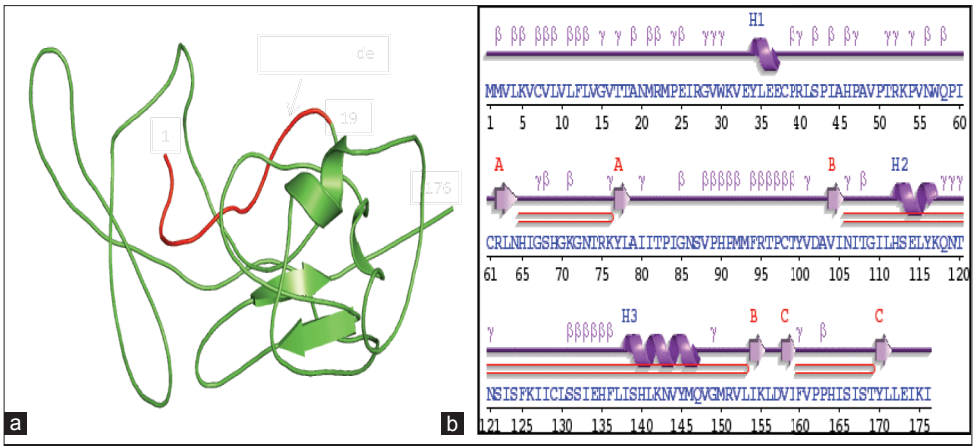


Figure 3: The purified *Cajanus cajan* protease inhibitor was crystallized by hanging drop technique using 0.1M potassium phosphate buffer pH 6.0 and 15% (w/v) polyethylene glycol 6000 within 2–3 weeks. The crystals were visualized under high resolution microscope

Table 1: Purification profile of *Cajanus cajan* protease inhibitors and its activity at each step

Step	Protein concentration (mg/ml)	Amount (ml)	Total protein (mg)	Yield (%)	Activity (U)	Specific activity (U/mg)	Purification (fold)
Homogenate	3.2	120	384	100	2,539,298.66	6612.756927	1
30%-50% $(NH_4)_2SO_4$ precipitate	3.027	60	181.62	37.69	1,176,541.72	6478.04	1.2
First eluent	1.8	40	72	14.94	530,108.83	7362.62	1.4
Second eluent	1.2	30	36	7.47	313,108.83	8697.46	1.66
FPLC eluent	0.8	30	24	6.25	350,120.91	14,588.37	2.21

FPLC: Fast protein liquid chromatography



Sequence	Position	Description	Cell Compartment	Pattern
TYVDA	99-103[A]	Caspase3 And caspase7 Cleavage site.	Cytosol, nucleus	[DSTE][^P] [^DEWHFYC]D[GSAN]
TRK	51-53[A]	NArg dibasic	Extracellular,golgi apparatus, cell surface	(.RK)|(RR[^KR])
TRK	74-76[A]	Convertase (NRD/ Nardilysin) cleavage site (X|RKor R|RX).	Golgi apparatus, cell surface	(.RK)|(RR[^KR])
TRKYLAIIT	74-82[A]	An RxxL based motif that binds to the Cdh1 and Cdc20 components of APC/C thereby targeting the protein for destruction in a cell cycle dependent manner	Nucleus, cytosol	.R..L..[LIVM].
TRKPVNWQ	51-58[A]	Protein phosphatase1 catalytic subunit (PP1c) interacting motif binds targeting proteins that dock to the substrate for dephosphorylationThe motif defined is [RK]0,1}[VI][^P] [FW].	Nucleus, protein phosphatase type 1 complex, cytosol	..[RK].{0,1}[VIL][^P][FW].
NSISF	121-125[A]	Phosphopeptide motif which directly interacts with the BRCT (carboxyterminal) domain of the Breast Cancer Gene BRCA1 with low affinity	Nucleus, BRCA1BARD1 complex	.(S)..F
NITGILH	106-112[A]	Phosphothreonine	Nucleus	..(T)..[ILV].
PCTYVDA	97-103[A]	Phosphothreonine motif binding a subset of FHA domains that have a preference for an acidic amino acid at the pT+3 position.	Nucleus, Replication fork	..(T)..[DE].
SFKII	124128[A]	Canonical LIR motif that binds to Atg8 protein family members to mediate processes involved inautophagy	Cytosol, cytoplasmic side of late endosome membrane	[EDST].{0,2}[WFY]..[ILV]
LLEIKI	171176[A]	The Cterminal class 2 PDZbinding motif is classically represented by a pattern such as (VYF)X(VIL)*	Cytosol, internal side of plasma membrane	...[VLIFY].[ACVILF]$

c

Figure 4: (a) Three-dimensional structure of *Cajanus cajan* protease inhibitor with 3 α-helices, and 3 β-sheets. (b) The topology map of *Cajanus cajan* protease inhibitor generated by PDBsum showing secondary structure elements in the framework. (c) Representation of functional motifs present in *Cajanus cajan* protease inhibitor with patterns and position found in the query sequence

Homology modeling

Multiple Sequence Alignment of CCPI is shown in Figure 5 where less conserved and highly conserved residues are highlighted in light and dark gray, respectively, while the conserved cysteine residue as highlighted in yellow.[43,44] The motif presents in CCPI by Eukaryotic Linear Motifs resource for the functional sites in proteins. In a protein, the motifs are key signatures of protein families and can be preferably used to define the protein function [Figure 4c].[45]

Conclusions

We have purified ~10 mg CCPI from 100 g seeds of *C. cajan*. The results suggested that CCPI showed low cytotoxic effects of in HEK, i.e., 27% as compared in adenocarcinomic human alveolar basal epithelial A549 cells with 51% cytotoxicity. The CCPI protein was crystallized in 0.1M potassium phosphate buffer, pH 6.0, and 15% (w/v) PEG 6000 conditions in the interval of 2–3 weeks. The crystals developed were rod-shaped but could not be diffracted due to some reasons. The CCPI

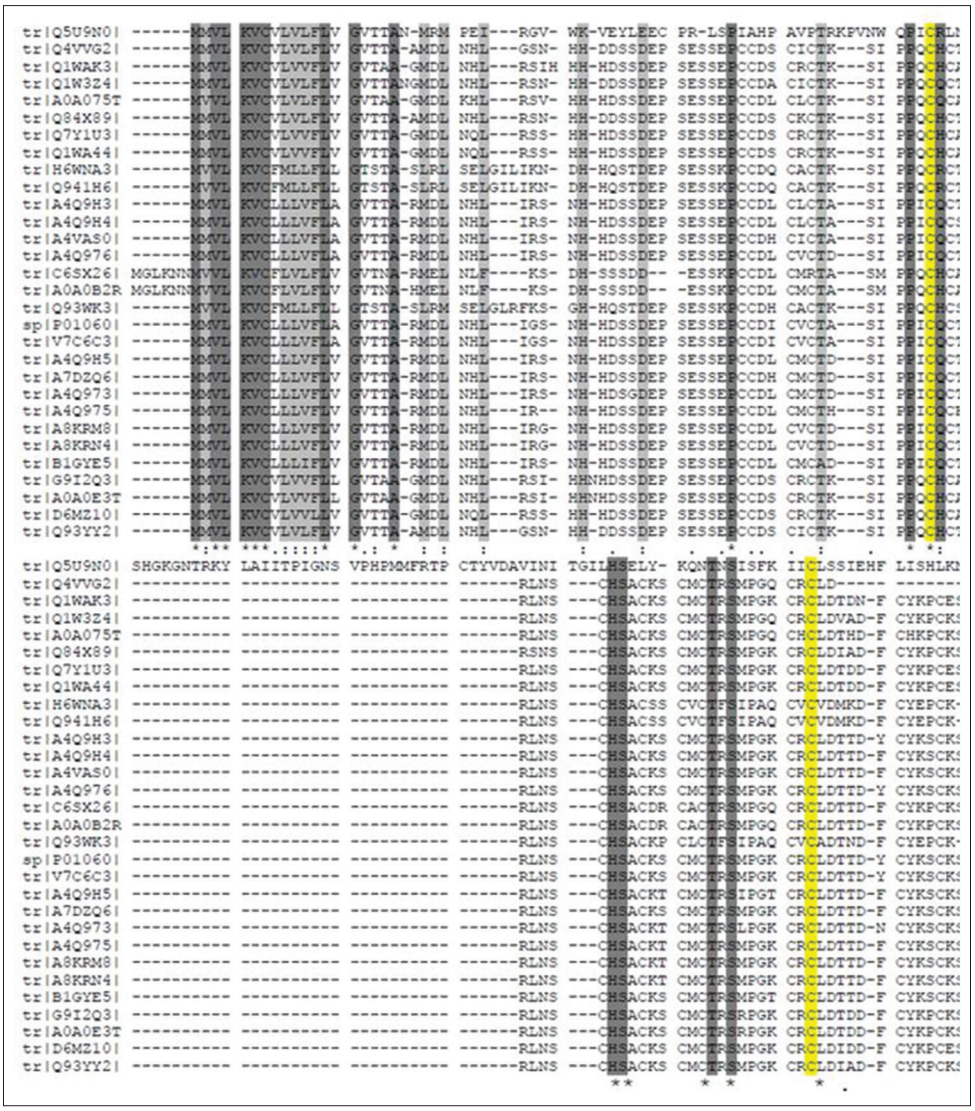


Figure 5: Multiple Sequence Alignment of *Cajanus cajan* protease inhibitor (Q5U9N0) with different species. The highly conserved and less conserved residues are highlighted in dark and light grey, respectively. While the conserved cysteine residue as highlighted in yellow

sequence (Uniprot: Q5U9N0) was analyzed and showed that it had 176 amino acid sequences; first 1–19 were signal peptides and rest were chain. The 3D structure created elucidated the presence of 3 beta-sheets, 3 beta-hairpins, 2 β-bulges, 6 strands, 3 helices, 1 helix–helix interaction, 41 β-turns, and 27 γ-turns. To conclude, CCPI crystal can further be refined so that it can be used as a lead molecule in the drug discovery pipeline against tumor cells. With this lead in hand, we intend to exploit it further to study the exact mode of action and elaborated studies in the near future.

Acknowledgments

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Nil.

Conflicts of interest

There are no conflicts of interest.

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