Physico-chemical and *In silico* analysis of a phytocystatin purified from *Brassica juncea* cultivar RoAgro 5444

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1 **Physico-chemical and *In silico* analysis of a phytocystatin purified from *Brassica juncea***
2 **cultivar RoAgro 5444**

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Draft

1 **Abstract**

2 This study describes the isolation and purification of a phytocystatin from seeds of *Brassica juncea* (*B.juncea*),
 3 (cultivar RoAgro 5444), an important oilseed crop with pervasive agricultural and economic ramifications. The
 4 protein was purified by gel filtration chromatography with 24.3% yield and 204 fold purification and visualised
 5 by 2D gel electrophoresis. The 18.1kDa mustard cystatin was highly specific for cysteine proteinases. The plant
 6 cystatin inhibited cathepsin B, confirming its role in conferring pest resistance. The inhibitor was highly stable
 7 over a pH range of 3-10 and retained significant inhibitory potential till 70°C. The stoichiometry of its
 8 interaction with papain, determined by Isothermal Calorimetry suggests a 1:1 complex. Secondary structural
 9 elements calculated by far UV circular dichroism (CD) spectroscopy show 18.8% α helical and 21% β sheet
 10 structure. The protein was a non-competitive inhibitor of thiol proteinases. The stoke's radius and frictional co-
 11 efficient were used to describe the shape and size of the protein.. Homology modelling and docking studies
 12 proposed a prototype illustrating the Brassica phytocystatin mediated papain inhibition. Molecular dynamics
 13 (MD) study revealed prominent stability of papain-phytocystatin complex during 100 nanoseconds (ns) long
 14 simulation. Detailed results identify the mustard cystatin as an important member of the phytocystatin family.

15 **Key words:** *Brassica juncea*, Phytocystatin, Circular dichroism, Isothermal calorimetry, Protein-Protein
 16 Docking

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1. Introduction

Cystatins comprise a class of cysteine proteinase inhibitors that form equimolar, tight and reversible complexes with the thiol proteinases. Animal cystatins are well characterised and are classified into three main families: Stefins, Cystatins and Kininogens (Abrahamson et al. 2003; Turk et al. 1997). The plant cystatins, commonly called Phytocystatins, form an independent subfamily on the cystatin evolutionary tree (Brown and Dziegielewska 1997) with molecular weight in the range of 8-25kDa (García Olmedo et al. 1987; Ryan 1990). These are basically classified into three groups - Group I: low molecular weight phytocystatins (~12-16kDa), Group II: 23kDa phytocystatins that are capable of inhibiting the Legumain (C13) family of proteinases (Lim et al. 1996; Shyu et al. 2004) and Group III: high molecular weight plant cystatins (Walsh and Strickland 1993; Wu and Haard 2000). These cysteine proteinase inhibitors are devoid of free thiol groups, disulphide bridges and putative glycosylation sites (Martinez and Diaz 2010). Phytocystatins bear the highly conserved QXVXG sequence, characteristic of the cystatin superfamily. A Proline-Tryptophan dipeptide sequence in the C-terminal region and a conserved Glycine residue in the N-terminal region are also present. A consensus LARFAV-like sequence, present upstream of the reactive QXVXG site is unique to phytocystatins.

Phytocystatins are instrumental in the regulatory and defence response of plants to both biotic and abiotic stimuli. They regulate intracellular protein turnover during seed development and germination (Arai et al. 2002; Corre-Menguy et al. 2002; Martinez et al. 2005) and during programmed cell death (Belenghi et al. 2003; Solomon et al. 1999). A defence role has also been conferred on them due to their ability to inhibit the exogenous cysteine proteinases such as the gut proteinases of invading insects (Haq et al. 2004; Kuroda et al. 1996; Pernas et al. 1998). Enhanced pest resistance has been observed in transgenic plants overexpressing cystatin genes (Álvarez-Alfageme et al. 2007; Gutierrez-Campos et al. 1999; Walker et al. 1999). Phytocystatins are instrumental in inhibition of fungal growth (Martinez et al. 2005). Their expression is induced by wounding and also by exposure to methyl jasmonate, thereby further establishing the role of the inhibitors in defence response (Bolter 1993; Pernas et al. 2000; Zhao et al. 1996).

Rapeseed mustard (*Brassica* spp.) is the third important oilseed crop grown worldwide with the Indian subcontinent, Central Africa and parts of Russia being the principle growing regions. In India, *Brassica* spp constitute 28.6% of the total oilseed production (Shekhawat et al. 2012). Of the various mustard species, oil yielding varieties are *Brassica juncea* (*B. juncea*), and *Brassica nigra* (*B. nigra*) with *Brassica alba* (*B. alba*) being cultivated primarily as a catch crop in India (Panda 2010) and other countries like Poland, Canada etc.

1 *B. juncea* commonly known as Indian mustard is the major edible brassica oilseed crop in India and across the
2 world. The crop, however, is highly susceptible to diseases like seedling blight and damping off caused by
3 *Rhizoctonia solani*, *Sclerotium rolfsii* and/or *Fusarium* species. *Brassica juncea* cultivation is most affected by
4 salinity (Gill et al. 2012) and drought (Zhang et al. 2014). Literature reports that abiotic stress conditions like
5 salinity (Shabala 2009), heavy metal toxicity, temperature extremes (Kratsch and Wise 2000; Tian et al. 2000;
6 Zhang et al. 2014), water stress (Claeys and Inze 2013) and hypoxia (Chang-jie et al. 2004) cause oxidative
7 stress and activation of apoptotic pathways. The mustard plants are also attacked by a number of pests like flea
8 beetles (*Phyllotreta* species), weevils (*Ceutorhynchus assimilis*), pollen beetles (*Meligethus aeneus*) etc. In India,
9 *Bagrada hilaris*, *Pieris brassicae* and *Lipaphis erysimi* (mustard aphid) cause serious damage to the *B. juncea*
10 crop.

11 Previously reported studies on phytocystatins provide ample evidence of their inherent anti-fungal activity
12 (Wang et al. 2008) and efficacy in modifying programmed cell death (Santana et al. 2014). Their ability to
13 confer enhanced pest resistance and stability under extreme conditions is also well documented. Indian mustard
14 (*B. juncea*) is an important crop plant with widespread economic ramifications on the Indian agricultural industry
15 and this knowledge prompted an investigation of its innate cysteine proteinase inhibitor. The present
16 communication provides detailed purification and characterization of a phytocystatin from seeds of *B. juncea* in
17 terms of its physicochemical properties, kinetic parameters, immunological properties and secondary structural
18 content. The papain-phytocystatin interaction has also been elucidated by molecular docking using a *Brassica*
19 *rapa* ssp *pekinensis* phytocystatin model as a prototype for Brassica phytocystatins. The stability of the complex
20 was evaluated via 100 ns long MD simulation studies.

21 2. Experimental

22 2.1. Materials

23 The enzymes (papain, ficin, bromelain, trypsin, chymotrypsin and cathepsin B), substrates (casein, azocasein
24 and p-nitrophenyl phosphate), anti-rabbit IgG and Sephacryl S100-HR were obtained from Sigma Aldrich (St.
25 Louis, MO). Electrophoresis reagents (acrylamide, bisacrylamide, ammonium persulphate) ethylene diamine
26 tetra acetate (EDTA) and L-cysteine were obtained from SRL Pvt. Ltd., India and SERVA Electrophoresis
27 GmbH Medium molecular weight markers, Freund's complete and incomplete adjuvants were obtained from
28 Genei, India Limited. All other chemicals used were of highest purity grade available commercially.

29 2.2. Methods

2.2.1. Purification of a thiol proteinase inhibitor (TPI) from Indian mustard seeds

The mustard TPI was purified by modifying the method of Sharma et al. 2006. 100gm of RoAgro 5444 mustard seeds were homogenized in homogenization buffer: 50mM sodium phosphate buffer, pH 7.5 containing 0.15M sodium chloride (NaCl), 3mM EDTA and 2% n-butanol. The crude extract was centrifuged at 8000rpm for 15 minutes in a REMI cooling centrifuge. Particulate matter was discarded. The supernatant was collected and fractionated by 40-60% ammonium sulphate saturation. The precipitate collected by centrifugation at 10,000 rpm for 30 minutes at 4°C was dissolved in minimum amount of 50mM sodium phosphate buffer pH 7.5 and dialyzed thrice against the same buffer. The dialyzed sample was chromatographed on the Sephacryl S-100 column (55×1.9), equilibrated with 50mM sodium phosphate buffer pH 7.5. Fractions of 5 ml were collected and assayed for protein and TPI activity.

11

2.2.2. Assay of thiol proteinase inhibitory activity

The specificity of the purified cystatin towards thiol proteinases was assessed by its ability to inhibit the caseinolytic activity of papain by the method of Kunitz (1947). Aliquots of papain solution (0.2mg/mL) were activated with 0.14 M cysteine and 0.047 M EDTA at 37°C for 10 minutes in 50mM sodium phosphate buffer, pH 7.5. the activated papain was then incubated with 0.2mL of the purified inhibitor for 30 minutes at 37°C. the reaction volume was 1mL. the reaction was initiated by addition of 1mL of 2% casein solution to the reaction mixture and incubated for 30 minutes at 37°C. the reaction was stopped by adding 1mL of 10% trichloroacetic acid solution . the particulate matter was removed by centrifugation at 2500rpm for 10 minutes. Colour was developed in the supernatant by the method of Lowry et al. (1951). A blank was prepared that was devoid of the purified phytocystatin. The inhibitory activity was defined as the decrease in casein hydrolysing activity per mL of inhibitor solution per minute reaction time. One unit of inhibitor activity of the purified cystatin was defined as the amount of inhibitor bringing about 0.001 change in absorbance per minute per mL.

The ability of the purified inhibitor to inhibit ficin and bromelain was also determined using casein as substrate. Inhibition of cathepsin B by the inhibitor was assayed using the protocol described by Michaud et al. (1993). The protein content of each sample was estimated by the method of Lowry et al. (1951).

27

2.2.3. Electrophoresis

The homogeneity of the purified protein preparation was checked by 2D gel electrophoresis. 200µg of the chromatographed protein was focused on IPG strips, pI 3-10, according to the protocol of Khan et al. (2014), using a IEF100 First-dimension Isoelectric Focusing Unit, Hoefer, Inc. USA. After focussing, the IPG strip was subjected to the same treatment as that described by the aforementioned group. The IPG strip was then placed on a 12.5% SDS-polyacrylamide gel and electrophoresis was performed at a constant voltage of 100V. The gels were stained with a 0.1% Coomassie brilliant blue R-250 preparation for 1 hour. Destaining was performed using a methanol (20%), glacial acetic acid (10%) and distilled water solution.

To determine the molecular mass of the purified TPI, the samples were electrophoresed on 12.5% SDS polyacrylamide gels in the presence and absence of reducing agent, β mercapto ethanol (Laemmli 1970). The molecular weight of the mustard cystatin in the native state was also determined by passing several marker proteins along with the inhibitor on Sephacryl S100-HR column.

13

2.2.4. Thiol group estimation

The presence of thiol groups in the purified mustard cystatin was determined by the method of Ellman (1959). L-cysteine was used to prepare the standard plot.

17

2.2.5. Carbohydrate estimation

The carbohydrate content of the purified protein was measured by the method of Dubois et al. (1956) where D-Glucose was used as standard.

21

2.2.6. Thermal stability of the inhibitor

a. 50µg of the inhibitor was incubated in 50mM sodium phosphate buffer (pH 7.5) at various temperatures ranging from 30°C- 90°C for 30 minutes. The samples were rapidly cooled and the remaining inhibitory activity against 50µg papain was determined (Kunitz 1947).

- 1 b. The purified inhibitor was incubated at 80°C. 50µg aliquots of the protein were drawn from the
2 incubated protein at different time intervals of 30, 60, 90 and 120 minutes and assayed for thiol
3 proteinase inhibitory activity.
- 4 c. The purified inhibitor was stored at -20°C and 4°C. 50µg aliquots of the protein were drawn at intervals
5 of 7, 14, 30, 45, 60 and 90 days and assayed for papain inhibition.

6

7 **2.2.7. pH stability of the inhibitor**

8 50µg of the inhibitor was incubated with buffers of different pH in the range of 3-12: 50mM sodium acetate
9 buffer (pH 3.0, 4.0 and 5.0) 50mM sodium phosphate buffers (pH 6.0, 7.0 and 8.0), 50mM Tris-HCl buffer (pH
10 9.0 and 10.0) and 50mM NaOH-Glycine buffer (pH 11.0 and 12.0) for 30 minutes. The potency of papain
11 inhibition by the treated mustard cystatin was determined by the procedure reported by Kunitz (1947).

12

13 **2.2.8. Hydrodynamic Properties**

14 The stokes radius (r) of the purified inhibitor was determined by running the inhibitor on a Sephacryl S100-HR
15 column, calibrated with proteins of known stokes radii (Andrews 1964; Laurent and Killander 1964). The
16 frictional co-efficient (f) and diffusion constant (D) were then calculated (Erickson 2009).

17

18 **2.2.9. Immunological Properties**

19 The immunological purity of the purified protein was determined by raising antisera in male albino rabbits in
20 response to subcutaneous injections of 300µg inhibitor emulsified in Freund's complete adjuvant. Following this
21 injection, the purified cystatin was administered weekly in Freund's incomplete adjuvant. The rabbit was bled
22 every 14 days. The collected blood was allowed to stand and coagulate at room temperature for 3hr. De-
23 complementation of the anti-sera was performed by incubation at 56°C for 30 minutes. The de-complemented
24 sera was then stored at -20° C. Immuno-diffusion was performed according to the protocol described by
25 Ouchterlony and Nillson (1978).

26 Direct binding ELISA was performed to measure the antigen specific antibodies generated in mustard TPI
27 immunized rabbits (Voller et al. 1976).

2.2.10. Kinetic Analysis

Stoichiometry of papain inhibition by mustard TPI

The stoichiometry of papain inhibition by mustard cystatin was determined by Isothermal Calorimetry (ITC) using MicroCal VP-ITC (Malvern Instruments). Both papain and mustard cystatin solutions were prepared in 50mM sodium phosphate buffer (pH 7.5). Prior to experiment, the papain (100 μ M), cystatin (10 μ M) and buffer solutions were filtered and then degassed for 10 minutes at 37°C using the Microcal Thermovac attachment of the instrument. The reference and sample cells were filled with the buffer and inhibitor solutions respectively. The protein solution in the sample cell was stirred at 309rpm and titrated with 29 consecutive 10 μ L injections of papain. Control experiments involved titration of papain with buffer alone and the integrated data was subtracted from the data obtained from the papain-cystatin titration to eliminate the heat of dilution. The integrated heat data of the titration were then analysed by the MicroCal analyser.

K_i determination

Varying concentrations of the purified TPI (0.6 μ M-0.30 μ M) were made to react with papain, ficin and bromelain fixed at 0.6 μ M. Residual activity was measured using casein as substrate (Kunitz 1947). Four different substrate concentrations were used: 0.5 K_m , 1 K_m , 2 K_m and 3 K_m . The results were analysed by constructing double reciprocal plots (Dixon and Webb 1979).

Determination of dissociation rate constant (K_{-1})

Maximal association between the purified inhibitor and enzyme was achieved by incubating the TPI and enzyme at 37°C for 30 minutes. The reaction was then shifted towards dissociation by adding excess substrate (6% casein) which also binds the free enzyme. The substrate was allowed to interact with the TPI-enzyme complex for different time periods before the reaction was stopped by the addition of TCA and the enzyme activity was then determined.

The association rate constant (K_{+1}) was determined using the equation (Abrahamson et al. 1986)

$$K_{+1} = K_{-1}/K_i$$

The half-life ($t_{1/2}$) of the inhibitor-enzyme complex and the dissociation rate constant are related by the equation

$$t_{1/2} = 0.693/K_{-1}$$

2.2.11. Spectral analysis

Ultra violet spectrum of purified mustard TPI (2 μ M) was measured on UV mini spectrophotometer. The wavelength range was 190-300nm and path length was 1.0 cm. Each spectrum was the average of three scans.

Far UV (190-240nm) CD spectrum of the purified inhibitor were recorded on a JascoJ-815 spectropolarimeter at room temperature using 1nm bandwidth, 100nm min⁻¹ scanning speed and 1mm slit width. The protein concentration was 0.2mg/mL and the ellipticity values were expressed in millidegrees. The fractional percentages of the secondary structure of the protein were calculated by the online server, BestSel (Micsonai et al. 2015).

9

2.2.12. Homology Modeling

A 101 amino acid long phytocystatin sequence of *Brassica rapa ssp pekinensis* was retrieved from the universal protein resource (UniProt) database (ID: Q71QE1) in FASTA format (UniProt 2015). The protein-protein BLAST (blastp) program (Altschul et al. 1990) was used against Protein Data Bank to identify the homologues sequences with their structures. The top hit was considered as a template to construct the desired three dimensional (3D) model of phytocystatin. This template was also subjected to Clustal Omega (V.1.2.1) (Sievers et al. 2011) for multiple sequence alignment to identify the conserved residues in the phytocystatin sequence and the template. The 3D model of phytocystatin was constructed using the *automodel* routine of Modeller (V.9.12) (Šali and Blundell 1993). 100 theoretical model were constructed which needed further evaluation to select the best one for future experiments in this study. Starting with the inbuilt routine of Modeller called DOPE score, top 10 models were selected. These models were subjected to various evaluation programs like PROCHECK (Laskowski et al. 1993), ProQ (Wallner and Elofsson 2003) and QMEAN (Benkert et al. 2009).

22

2.2.13. Docking studies

Docking of modelled phytocystatin with papain was performed using the docking program ZDOCK (Chen et al. 2003) included in Discovery Studio which is a software suite developed and distributed by Accelrys. ZDOCK is rigid-body docking program and requires minimal information about the binding site. Crystal structure coordinates of papain (PDB code: 3E1Z, chain B) from the plant, *Carica papaya* was obtained from Protein

Data Bank. It is a complex of parasite protease inhibitor, chagasin (chain A) and papain (Redzynia et al. 2009). From this protein structure, chain A and all hetero atoms including water were removed. The resulting structure was subjected to protein preparation protocol ‘Prepare Protein’ of Discovery Studio. Default parameters were used in this step. Similarly the modelled structure of phytocystatin was also subjected to protein preparation protocol. Docking was performed using default parameters. For instance, angular step size set to 6 degrees, maximum of 2000 top poses allowed and number of cluster set to maximum of 100. Euler angle sampling sizes can be adjusted from 6 degrees to 15 degrees. Higher the value of angular step size, faster is the search. But maximum accurate search can be performed keeping the angular step size to 6 degrees. Molecular visualisation was aided by a visualisation tool, UCSF-Chimera (V1.8.1) (Pettersen et al. 2004).

2.2.14. Molecular Dynamics (MD) simulations

To evaluate the stability of the papain and phytocystatin complex, MD studies were performed. GROningen MAchine for Chemical Simulations (GROMACS) v5.0.7 package (Abraham et al., 2015) was considered for this study. Protein topology was prepared considering ‘GROMOS96 43a1’ force field (Scott et al., 1999) using the module ‘gmx pdb2gmx’ of GROMACS. The solvated system had a net charge of +2.0. Therefore, 2 Chloride ions were added to neutralize the system. The system was then minimized using the steepest descent method. Minimized system was subjected to equilibration run in two consecutive steps, NVT (Number of particles, Volume and Temperature) equilibration run and NPT (Number of particles, Pressure and Temperature) equilibration run. Equilibrated system was subjected to a 100 ns long MD run at 300K to confirm stability of the given system. The hydrogen bonds between papain-phytocystatin and the root mean square deviation (RMSD) of the proteins, its complex were calculated using the module ‘gmx hbond’ and ‘gmx rms’ modules of GROMACS respectively. Similarly, ‘gmx energy’ was considered to elucidate the interaction energy pattern during 100 ns MD simulation.

Statistical Analyses

Each experiment was performed three times to check the reproducibility of the results. The data was expressed as the Mean $\pm$ standard error mean (SEM) for three independent experiments. The statistical significance was evaluated by one-way ANOVA software. The probability of occurrence was determined at $p \leq 0.05$.

1 **3. Results**

2 **3.1. Purification of the inhibitor**

3 In the present work, the TPI purification procedure involved two steps after homogenization: ammonium
4 sulphate precipitation (40-60%) and gel filtration chromatography on Sephacryl S-100 HR column. Table 1
5 summarises the progress of purification. The inhibitor eluted as a single peak giving significant papain
6 inhibition (figure 1). The fractions corresponding to the peak were pooled and used for further analysis. The
7 inhibitor was purified with a fold purification of 204 and a per cent yield of 24.3.

8

9 **3.2. Homogeneity of the purified mustard TPI**

10 The inhibitor eluted as a single symmetric peak with significant papain inhibitory activity (figure 1). Moreover,
11 the purity and homogeneity of the preparation was envisioned by 2D gel electrophoresis (figure 2).

12

13 **3.3. Properties of the purified TPI**

14 **3.3.1. Molecular weight determination**

15 A single band was obtained in SDS-PAGE under both reducing and non-reducing conditions indicating that the
16 purified inhibitor is a monomeric protein with no sub-units (figure 3). The molecular weight of the purified TPI
17 as determined by gel filtration on Sephacryl S100-HR column was 18.6kDa. It was further confirmed by SDS-
18 PAGE under reducing and non-reducing conditions. The molecular weight obtained under reducing conditions
19 was 18.3kDa while that under non-reducing conditions was 18.1kDa.

20

21 **3.3.2. Sulfhydryl group estimation**

22 Neither free thiol groups nor disulphide linkages were observed in the purified mustard cystatin.

23

24 **3.3.3. Carbohydrate group estimation**

25 The TPI was devoid of carbohydrate groups as estimated spectrophotometrically.

3.3.4. Stability Studies

Temperature profile

The purified TPI was found to be quite stable and active in the temperature range of 30-60°C (figure 4a). It retained about 75% of its anti-papain activity at 70°C. A progressive decrease in its inhibitory activity was noted beyond 60°C, with significant loss of activity at 90°C. When incubated at 90°C, the purified protein retained 40% of its anti-papain activity till 90 minutes (figure 4b). The inhibitor also retained 60% of its inhibitory potential for upto 120 days at -20°C and 55% of its inhibitory potential for upto 45 days at 4°C (data not shown).

pH stability profile

Stability of the purified TPI was investigated as a function of pH between pH 3-12. Figure 5 shows that the inhibitor is stable in the pH range of 3.0-9.0 and exhibits maximal activity at pH 7.5. Above pH 9.0, the TPI retained minimal anti-papain activity with complete loss of activity at pH 12.0.

3.3.5. Hydrodynamic properties

Hydrodynamic parameters as determined by the gel filtration behaviour of the purified inhibitor suggested a stokes radius of 2.07 nm. Using the Stokes radius, the frictional coefficient was calculated to be 3.903×10^{-8} g/s. The Diffusion co-efficient was found to be 1.05×10^{-10} m²/s.

3.3.6. Immunological properties

Immunodiffusion results proved the immunogenic purity of the inhibitor as a single precipitin line was observed (supplementary data figure 1). Direct binding ELISA gave a titer value of 23442.29 hinting at a high antigenicity of the protein (supplementary data figure 2).

3.3.7. Kinetic properties

The energetics of the papain-mustard cystatin interaction were monitored by ITC. Figure 6 represents the isotherm for the reaction. These calorimetric titrations yield a stoichiometry of $\sim 1(\pm 0.0306)$ indicating that a

specific 1:1 complex is formed. The mustard cystatin exhibited significant affinity for papain with a binding constant (K) value of $1.2 \times 10^6 \pm 4.23 \times 10^4 \text{ M}^{-1}$.

The dissociation equilibrium constant (measured as K_i), the dissociation rate constant, the association rate constant and the half-life of the inhibitor-enzyme complex for papain, ficin and bromelain have been mentioned in Table 2. The K_i values obtained for papain, ficin and bromelain are 0.102, 0.306, 0.468 μM respectively, implying the highest affinity of inhibitor for papain. The K_{-1} values for the enzyme-inhibitor complex were determined by the displacement procedure, in which the inhibitor released from the complex with increase in time was trapped by excess substrate. The K_{-1} values obtained for papain, ficin and bromelain are 6.6×10^{-4} , 9.23×10^{-4} , $10.25 \times 10^{-4} \text{ s}^{-1}$ respectively. Association rate constant and hence the affinity of the inhibitor for proteinases is in the following order: papain ($6.47 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) > ficin ($3.01 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) > bromelain ($2.19 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). The half-life of the enzyme inhibitor complex calculated with papain was observed to be $1.05 \times 10^3 \text{ s}$ while with ficin it was $0.75 \times 10^3 \text{ s}$ and for bromelain, the half-life was calculated to be $0.67 \times 10^3 \text{ s}$.

3.3.8. Spectral studies

The purified mustard cystatin yields typical UV absorption spectra with a maximum at 279 nm and a minimum at 253 nm. The molar extinction co-efficient calculated for the protein at 280 nm was $13,233.3 \text{ M}^{-1} \text{ cm}^{-1}$ while the 280/260 absorbance ratio is 1.3. The secondary structural contents of the inhibitor were determined from the far UV CD data (figure 7). The α helical, β sheet and β turn content of the protein, determined by the BestSel server was 18.8%, 21% and 15% respectively.

3.4. In silico analysis

The protein-protein BLAST resulted into eight hits from protein data bank. The top hit, 3IMA was considered as a template model. 3IMA shows 90% query coverage and 59% sequence identity with the phytocystatin sequence. The template model, 3IMA has four chains. Only chain B and D constitutes the tarocystatin from the plant *Colocasia esculenta* (taro). The chain D of 3IMA was considered for modelling the structure of queried phytocystatin. Multiple sequence alignment of 3IMA using Clustal Omega with the query sequence revealed the conservation of several regions in both the proteins (figure 8).

1 The 3D models constructed through modeller were subjected to several programs for evaluation. Top ten models
2 on the basis of DOPE score, were picked from all the 100 models constructed. Theoretical Model 07 (figure 9),
3 was found to be the best model among ten shortlisted. Complete evaluation details of the top ten models are
4 summarised (Table 3). According to PROCHECK evaluation of Model 07, 92.3% residues of this model fall in
5 the most favoured region, 7.7% residues fall in the additionally allowed region. And none residues were found
6 in the generously allowed and disallowed region (figure 10). QMEAN (Z-score) is a comparison with non-
7 redundant set of PDB structures. The score for this evaluation comes out to be -0.43 (figure 11). Protein quality
8 estimation was also performed using ProQ web server, which evaluates the model on the basis of LG score and
9 MaxSub score. LG score and MaxSub score of Model 07 were found to be 2.096 and 0.307 respectively. LG
10 score >1.5 and MaxSub score >0.1 corresponds to correct model.

11 Molecular visualisation of phytocystatin model provided secondary structure information, which illustrated six
12 beta sheets and one alpha helix. From the docking conformations, all 2000 poses were browsed. This enabled
13 the listing of all 2000 poses on the basis of Z Rank score. Best docked conformation (pose 1 from cluster 13)
14 had Z-Rank Score: -111.95 (Z Dock Score: 17.16). This conformation (figure 12) was saved and visualised in
15 detail for possible interactions in between the two proteins. As evident from the image (figure 13), modelled
16 phytocystatin interacts with papain via seven hydrogen bonds. Details of the hydrogen bond interactions are
17 summarised in Table 4.

18 MD study revealed detailed information of the papain-phytocystatin interaction and its stability. Higher number
19 of hydrogen bond formation represents higher number of protein to protein/ligand interactions and hence higher
20 stability. On an average, 13.242 hydrogen bonds were formed between the two proteins during 100 ns long
21 simulation. In final stages of simulation (i.e. from 65 ns onwards), >20 hydrogen bonds were also seen at some
22 instance (figure 14). Protein complex was saved at 20 ns, 40 ns, 60 ns, 80 ns and 100 ns for detailed analysis of
23 residue pairs involved in formation of papain-phytocystatin complex. Corresponding changes were noticed in
24 the interaction energy pattern evolved due to protein-protein interaction (figure 15). Energy was found least for
25 all the instances where hydrogen bond were maximum and vice versa. Falling energy levels clearly indicates
26 the increased stability of the complex with the passage of time. Table 5 lists the residue pairs involved in the
27 formation of hydrogen bonds during the MD simulation. RMSD of the proteins from their initial structure was
28 also evaluated. Proteins were found coming to stabilized state by the end of simulation on the basis of the
29 RMSD plot (supplementary data figure 3). RMSD of papain was comparatively lower than phytocystatin or its

complex. The possible reason for this drastic variation in RMSD could be the homology modelled structure of phytocystatin. Phytocystatin alone had more structural variation from 40 ns onwards. However, the RMSD of the papain-phytocystatin complex is found stabilized by the end of simulation supporting that papain and the phytocystatin have adapted themselves in due course and bind strongly with each other.

4. Discussion

Phytocystatins have important regulatory roles in plant physiology owing to their cysteine proteinase inhibitory activity. Several other roles have been attributed to cystatins in plants: from the control of various physiological and cellular processes in plants (organogenesis, seed development, programmed cell death and storage protein turnover) to the inhibition of exogenous cysteine proteases secreted by herbivorous arthropods and pathogens to digest or colonize plant tissues. The isolation of a thiol proteinase inhibitor from oil yielding mustard seeds had not been previously attempted. Thus, it was envisaged that a systematic study of a thiol proteinase inhibitor from the new source i.e. *Brassica juncea* will be useful in adding to the existing data and knowledge on phytocystatins.

The present report describes the purification and characterization of a thiol proteinase inhibitor from Indian mustard which was found to be highly specific for cysteine proteinases namely papain, ficin, bromelain and cathepsin B. Amongst these, the TPI was found to be maximally effective towards papain followed by cathepsin B, ficin and then bromelain. Practically no inhibitory action was observed against serine (trypsin and chymotrypsin) proteinases (supplementary data figure 4). The ability of the purified protein to inhibit cathepsin B makes it a key player in conferring resistance against pests and predators. Phytocystatins inhibiting animal cathepsins have been reported in strawberry (Martinez et al. 2005), rice (Ohtsubo et al. 2005) and kiwifruit (Rassam and Laing 2004). A simple two step purification procedure involving 40-60% ammonium sulphate fractionation and gel filtration on Sephacryl S100-HR column provided a 204 fold purification and a 24.3 per cent yield. The purified TPI, when passed through a Sephacryl S100-HR column indicated a molecular mass of 18.6kDa. This was further corroborated by electrophoresis wherein the molecular mass was calculated to be 18.1kDa. A 26kDa phytocystatin has been observed in yellow mustard seeds (*Brassica alba*) while a 23kDa protein was reported in *B.rapa* flower buds (Hong et al. 2008). The inhibitor migrated as a single band on SDS-PAGE indicating the single subunit structure of the protein and absence of disulphide bonds. The TPI was found to be devoid of thiol groups and carbohydrate moieties. These physical properties of the purified inhibitor

1 are consistent with the intrinsic properties that identify a protein as a group II Phytocystatin (Benchabane et al.
2 2010).

3 Most of the purified phytocystatins are stable to extremes of temperature and pH (Pernas et al. 1998). The
4 ability of the purified *B.juncea* seed cystatin to remain stable over a wide pH range of 3-9 and temperature range
5 of 30-60°C holds it in good stead as compared to other Brassica phytocystatins like yellow mustard (*B.alba*)
6 cystatin (Ahmed et al., 2016) and chinese cabbage (*B.rapa*) cystatin (Hong et al., 2012) that show stability in the
7 pH ranges of 6-8 and up to pH 7 respectively. The storage stability of the Indian mustard cystatin shows that it is
8 viable up to 4 months when stored at -20°C and 45 days when stored at 4°C. Storage stability of up to 4 months
9 has been reported for goat pancreas cystatin when stored at 0°C (Priyadarshini and Bano, 2005).

10 An investigation of the hydrodynamic parameters revealed a stokes radius of 2.07nm and a frictional co-
11 efficient of 3.903×10^{-8} g/s. This is in accordance with the hydrodynamic parameters reported for *Phaseolus*
12 *mungo* cystatin II wherein the reported stokes radius was 2.05nm (Sharma et al. 2006). A comparison of the
13 frictional co-efficient (f) with the minimal frictional co-efficient (f_{min}) which is the frictional co-efficient
14 obtained if the protein was a smooth sphere of radius r_{min} without the bound water molecules suggests the shape
15 of the protein. In our case, this comparison suggests a slight deviation from the globular structure of the purified
16 protein. The purified cystatin invokes a good immune response as is evident from the antibody titer value
17 obtained. A similar value has been reported for α glycinin and other soy proteins that function as potent
18 allergens (Lalles et al. 1995). Interestingly, a titer value of 22000 was observed for *B.alba* cystatin that has a
19 molecular weight of 26kDa while the ~ 18kDa *B.juncea* cystatin mounted an immune response with an antibody
20 titer value of 23442.29, suggesting higher antigenicity of the *B.juncea* cystatin.

21 A 280/260 absorbance ratio of 1.3 hints at a sufficiently pure protein preparation devoid of any significant
22 nucleic acid contamination (Zabriskie and DiPaolo 1988). The secondary structure of the purified inhibitor was
23 elucidated by the far UV CD spectrum which shows negative peaks at 208 and 223nm, corresponding to the
24 alpha-helix conformational elements. Beta sheet shows a negative band at 218 nm and a positive one at 196 nm.
25 The α -helical content of the inhibitor calculated using the BestSel online program is 18.8% while the β sheet and
26 β turn conformations are 21% and 15% respectively. Reports have shown comparable secondary structural
27 contents for phytocystatins from pineapple (Irene et al., 2012) , yellow mustard (Ahmed et al. 2016), *Phaseolus*
28 *mungo* (Sharma et al. 2006). The calculated secondary structure for mustard cystatin is similar to animal

1 cystatins like goat pancreatic (17%) and human placental (21%) cystatins previously described in our lab
2 (Priyadarshini and Bano 2010; Rashid et al. 2006).

3 Titrations of papain with mustard cystatin consistently indicate that one mole of papain is inhibited by one mole
4 of the purified TPI suggesting that one papain binding site is present per mustard cystatin polypeptide.
5 Stoichiometric ratios other than 1:1 have also been reported. An inhibitor-papain binding ratio of 1:1.1 was
6 observed in *Clitocylin* (Brzin et al. 2000), 1:2 for a synthetic peptide (Björck et al. 1989) and 1:8 in tomato (Wu
7 and Haard 2000). The papain-mustard cystatin interaction is highly endothermic and the purified inhibitor
8 showed significant affinity towards papain as is evident by its K_d value. These binding affinity values are
9 comparable to those determined by ITC experiments for the potato multicystatin 5 ($K_d = 7.2 \mu\text{M}$) (Green et al.
10 2013).

11 The purified protein is a potent inhibitor of thiol proteinases. Of the plant cysteine proteinases studied, the
12 mustard TPI binds most tightly to papain, followed by ficin and then bromelain (supplementary data figure 4).
13 The ability of the plant cystatin to inhibit an animal cysteine proteinase like cathepsin B makes it a key player in
14 conferring resistance against predators and insects. The K_i value towards papain is $1.02 \times 10^{-7} \text{M}$ are comparable
15 to those reported in yellow mustard ($K_i: 3 \times 10^{-7} \text{M}$), the dimeric form of chinese cabbage ($K_i: 1.01 \times 10^{-7} \text{M}$) (Hong
16 et al. 2012) and soya ($K_i: 1.9 \times 10^{-7} \text{M}$) but are one order of magnitude lower than the values for strawberry
17 (Martinez et al. 2005), barley (Martinez et al. 2003) and sesame (Shyu et al. 2004). Amongst the mustard
18 cystatins, the *B.juncea* cystatin is a more potent inhibitor than *B.alba* (yellow mustard) cystatins. The K_i values
19 obtained are in good comparison with thiol proteinase inhibitors from both the plant (Sharma et al. 2006) and
20 the mammalian system (Rashid et al. 2006; Sumbul and Bano 2006). Tight binding inhibitors have high
21 association rate constants. Among the thiol proteinases studied, papain has the highest K_{+1} . The weaker binding
22 of mustard TPI to bromelain is largely due to a low dissociation rate constant, K_{-1} . The Line-weaver Burk plots
23 for the mustard TPI-enzyme interactions suggest a non-competitive mode of inhibition (supplementary data
24 figure 5). Non-competitive mode of inhibition by phytocystatins has previously been reported in soyabean
25 cystatins (Zhao et al. 1996). Additionally, the non-competitive inhibition of cysteine proteinases by
26 phytocystatins is further supported by the docking model of cysteine proteinase inhibitor interaction with
27 cysteine proteinases proposed from crystallographic data (Bode et al. 1988; Stubbs et al. 1990). The $t_{1/2}$ values
28 for the enzyme inhibitor complex for papain, ficin and bromelain are 1.05×10^3 , 7.5×10^2 6.7×10^2 s
29 respectively indicating that the papain binary complex is more stable than the either the ficin or bromelain

1 binary complexes. Similar trends have been observed for the goat pancreas and lung cystatins (Khan and Bano
2 2009; Priyadarshini and Bano 2010).

3 Papain is a prominent member of the cysteine proteinase family. The specificity and potency of papain
4 inhibition by mustard cystatin prompted the investigation of its papain inhibitory nature at an atomic level. In
5 lieu of the availability of the amino acid sequence of the phytocystatins purified from *B.juncea*, the available
6 phytocystatin sequence from *B.rapa ssp pekinensis* (UniProt ID: Q71QE1) was used as a Brassica cystatin
7 prototype. A pairwise sequence alignment between phytocystatins from *B.rapa ssp pekinensis* (UniProt ID:
8 Q71QE1) and *B.campestris* (Q39270) using the EMBOSS Water tool gave 64% identity and 79% similarity.
9 Values of 33% identity and 51% similarity were observed for an alignment between *B.rapa ssp pekinensis*
10 (UniProt ID: Q71QE1) and *B. oleracea* (UniProt ID: Q2A9P9) cystatins. Based on these alignments, it was safe
11 to assume that the purified mustard cystatin will show comparable sequence homology with the above
12 mentioned members of the Brassica family and the docked papain-*B.rapa ssp pekinensis* cystatin model may
13 serve as an archetype and provide vital information of the papain-*B.juncea* inhibitor interaction.

14 Papain has four active site residues (Redzynia et al. 2009) namely, Gln19, Cys25, His159 and Asn175. The
15 Gln19 residue of papain has been implicated in catalysis since it stabilises the tetrahedral catalytic intermediate
16 by forming an oxyanion hole (Menard et al. 1991). After inspecting the interactions of the docked complex, it
17 was evident that the Val56 of the modelled phytocystatin binds with the papain's active site residue Gln19
18 which may check the proteinase activity of the protein. This interaction was also viable during the last phases of
19 the simulation (Table 5). At 80 ns, the Val56 of cystatin was found interacting with papain's Gln19, but Val57
20 takes the place of Val56 at 100 ns. Moreover, several other important interactions leading to hydrogen bond
21 formation were also discernible from visual inspection of docked complex. Gly11 of modelled phytocystatin
22 was found interacting with Asp158 of papain. This interaction remained persistent throughout the 100 ns
23 simulation. The docking study was validated with the help of known complex structure of tarocystatin with
24 papain (PDB id: 3IMA) (Chu et al. 2011). The hydrogen bond interactions of this complex reveal similar
25 interactions between the conserved Gly5 of tarocystatin and Asp158 of papain (Chu et al. 2011). This
26 interaction is also found to be conserved in the papain-pineapple cystatin complex (Irene et al. 2012). Modelled
27 phytocystatin have also been found interacting with the Gly20, Gly66 of papain similar to tarocystatin-papain
28 complex (3IMA) (supplementary data Table 1). Additionally, some interactions of phytocystatin were found
29 near S₂ pocket of papain. S₂ pocket residues bestow major specificity to the P₂ substrate residues (Zafar et al.

2015). The residues lining the S₂ pocket of papain are (Redzynia et al. 2009) Tyr67, Pro68, Val133, Val157, Ala160 and Ser205.

Based on the docking cum MD simulation studies, we propose that conserved residues Val56 or Val57 (of the QVVAG sequence) of the modelled cystatin makes the first point of contact with Gln19 of papain. Gly11 of the N-terminal conserved region then interacts with Asp158 of papain in the catalytic cleft. The complex formed is stabilised by interactions between the residues preceding the conserved glycine residue, like Leu10 and the S₂ subsite (Machleidt et al. 1989). Since all residues involved in the inhibition are conserved across cystatins, it would be prudent to say that *B.juncea* cystatin's mechanism of papain inhibition would be the same. This report provides the first elucidation of the papain-Brassica phytocystatin interaction mechanism

On the basis of its physical and chemical properties, the *B.juncea* seed cystatin is a good cohort of other seed phytocystatins. Its ability to withstand a wide range of temperature and pH and successfully inhibit both plant and animal cysteine proteinases (cathepsin B), holds it in good stead as a putative target for genetically modifying the expression and activity of endogenous proteinase inhibitors. These genetically modified plants may be better equipped to overcome both biotic and abiotic stress conditions, thereby increasing the gross productivity of the plant and consequent yield of mustard oil.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Table 1: Progress of purification of mustard cystatin

Procedure	Volume (mL)	Total Protein (mg)	Total Units†	Specific Activity (Units/mg)	Fold Purification	%Yield
Crude homogenate	300	6600	201	0.030	1	100
40-60% ammonium sulphate precipitation	20	452	70	0.15	5	34
Gel filtration S-100	10	8	49	6.125	204	24.3

† 1 unit of enzyme inhibitory activity is defined as the amount of inhibitor bringing about 0.001 change in OD ml⁻¹ min⁻¹ under the described assay conditions

Table 2. Kinetic constants obtained upon interaction of mustard cystatin with papain, ficin and bromelain

Proteinase	K_i (μM)	K_{+1} ($\text{M}^{-1}\text{s}^{-1}$)	K_{-1} (s^{-1})	Half-life of complex (s)
Papain	0.102 ± 0.005	$6.47 \pm 0.2 \times 10^3$	$6.6 \pm 0.25 \times 10^{-4}$	1.05×10^3
Ficin	0.306 ± 0.003	$3.01 \pm 0.1 \times 10^3$	$9.23 \pm 0.1 \times 10^{-4}$	7.5×10^2
Bromelain	0.468 ± 0.003	$2.19 \pm 0.5 \times 10^3$	$10.25 \pm 0.2 \times 10^{-4}$	6.7×10^2

Results represent the $\pm\text{SEM}$ calculated from three independent experiments.

Table 3.Validation scores from PROCHECK, Qmean and ProQ for top ten theoretical models

Model	PROCHECK*				Qmean (Z-score)	ProQ (LGscore)	ProQ (MaxSub)
	A	b	c	d			
Model_07	92.3	7.7	0	0	-0.43	2.096	0.307
Model_13	90.1	8.8	0	1.1	-0.3	1.485	0.275
Model_32	86.8	12.1	1.1	0	-0.68	1.81	0.292
Model_34	91.2	8.8	0	0	-1.61	1.527	0.252
Model_35	85.7	14.3	0	0	-1.03	1.415	0.248
Model_40	93.4	6.6	0	0	-1.41	1.915	0.306
Model_47	87.9	12.1	0	0	-1.12	1.418	0.261
Model_83	90.1	9.9	0	0	-1.04	1.53	0.282
Model_86	91.2	7.7	1.1	0	-1.61	1.559	0.26
Model_89	89	8.8	1.1	1.1	-1.03	1.465	0.265

*a, b, c, d, values correspond to the percentages of residues that are located in the most favourable, additionally allowed, generously allowed and disallowed regions, respectively

Table 4. Summary of hydrogen bond interactions between papain and modelled phytocystatin.

<i>Papain</i>	<i>Phytocystatin</i>	<i>Bond length (Å)</i>
Asp158	Gly11	3.21
Gln19	Val56	3.41
Gly20	Ala58	2.05
Ser21	Glu93	3.48
Gly66	Leu10	3.17
Lys139	Asp22	2.63
Lys139	Asp22	2.79

Table 5. Residue pairs involved in hydrogen bonding at various intervals during the simulation.

Time Period	H-bonds	Papain and Cystatin complex: pair residues involved*
20 ns	14	Gly20-Ala58, Gly62-Asp3, Cys63-Gln4, Cys63-Gln55, Asn64-Gly11 , Gln92-Asp3, Gln92-Gln4, Gln142-Glu83 , Gln142-Lys84, Arg145-Ala85, Arg145-Asn88, Lys156-Arg14, Lys156-Arg14, Asp158-Gly11
40 ns	16	Gly20-Ala58, Asn64-Gln4, Asn64-Gln5, Asn64-Gly6, Asn64-Gly11 , Asn64-Val56, Gln92-Gln4, Gln142-Thr60, Gln142-Glu83 , Gln142-Ala85, Arg145-Trp86, Arg145-Trp86, Lys156-Arg14, Lys156-Arg14, Asp158-Gly11 , Trp177-Val57
60 ns	11	Gly20-Ala58, Asn64-Gln4, Asn64-Gly6, Asn64-Gly11 , Asn64-Val56, Gln92-Gln4, Gln142-Thr60, Gln142-Glu83 , Gln142-Ala85, Asp158-Gly11 , Asp158-Arg14
80 ns	18	Gln19-Val56, Ser21-Met1, Ser21-Ala2, Cys25-Gly11, Cys25-Val56, Cys63-Ala2, Cys63-Gln4, Asn64-Gly6, Asn64-Gly11 , Asn64-Gln55, Asn64-Val56, Gln142-Ala58, Gln142-Glu83 , Lys156-Arg14, Lys156-Asp15, Asp158-Gly11 , Asp158-Arg14, Trp177-Val57
100 ns	17	Gln19-Val57, Gln19-Ala58, Asn64-Gln4, Asn64-Gly6, Asn64-Gly11 , Asn64-Gly12, Asn64-Gln55, Asn64-Val56, Gln92-Gln4, Gln142-Glu83 , Gln142-Lys84, Gln142-Trp86, Lys156-Arg14, Asp158-Gly11 , Asp158-Arg14, Asp158-Arg14, Trp177-Val57

Constantly appearing residue pairs are highlighted.
*from the residue pairs, the first belongs to papain and second belongs to cystatin model.

Figure captions

Figure 1. Purification profile of a mustard cystatin. The crude extract was subjected to ammonium sulphate fractionation followed by Sephacryl S-100 HR gel filtration chromatography. The fractions collected were assayed for inhibitory activity and protein concentration (O.D at 660nm).

Figure 2. 2D gel electrophoresis of the purified mustard cystatin. 200µg of the purified mustard cystatin was focussed on an IPG strip and then subjected to SDS-PAGE on a 12.5% gel preparation. A single spot observed on the gel suggested the purity of the sample.

Figure 3. Molecular weight determination of cystatin by SDS-PAGE electrophoresis. Lane a contained the molecular mass standards: A- phosphorylase b (97.4 kDa), B- bovine serum albumin (68 kDa), C- ovalbumin (45 kDa), D- carbonic anhydrase (29.1 kDa), E- soyabean trypsin inhibitor (20 kDa), F- lysozyme (14.3 kDa). Lane b contained 60µg of the purified inhibitor and lane c contained 60µg of 2-mercaptoethanol treated purified inhibitor.

Figure 4. Effect of temperature on the purified TPI:(a) 50µg of the inhibitor was incubated in 50mM sodium phosphate buffer, pH 7.5, at various temperatures for 30 min. Remaining % inhibitory activity was analyzed against 50µg of papain at 37°C. (b) The purified *B.juncea* cystatin, when incubated at 90°C for varying time intervals, retained its anti-papain activity up to 90 minutes. The results are the average of three independent experiments.

Figure 5.pH stability profile of purified TPI. 50µg of the inhibitor was incubated in buffers in the pH range of 3-12 for 30 mins. Remaining % inhibitory activity was analysed against 50µg of papain at 37°C. The results are the average of three independent experiments.

Figure 6. Stoichiometry of papain binding by ITC. The heat released on injecting papain at regular intervals into the mustard cystatin was monitored to study the energetics of binding.

Figure 7.Far UV CD spectrum of the purified inhibitor. Mustard cystatin at a concentration of 0.2mg/mL was subjected to CD spectroscopy. The fractional percentages of secondary structural elements were then calculated. The spectrum is the average of multiple runs.

Figure 8.Multiple sequence alignment to identify the conserved residues. The sequences of phytocystatins and considered template(PDB: 3IMA) were aligned using clustal omega. The conserved residues are marked with *.

Figure 9.Modelled Brassica phytocystatin.The secondary structure of the modelled phytocystatin was found to be composed of one α helix and six β sheets.

- 1 **Figure 10. Ramachandran plot of the homology modelled structure of phytocystatin.** The most favoured,
2 additionally allowed, generously allowed and disallowed regions respectively are defined in the figure.
- 3 **Figure 11. Model validation through QMean Z-score.** Absolute quality of modelled structure as evaluated.
4 Good models are generally located in the dark (black) region.
- 5 **Figure 12. Binding mode of phytocystatin with papain.** Surface view of the docked complex- Brassica
6 phytocystatin (magenta) and papain (green).
- 7 **Figure 13. Papain-inhibitor interaction.** Comprehensive details of the interaction between the Brassica
8 phytocystatins (magenta) and papain (green). Black dashes represent hydrogen bonds.
- 9 **Figure 14. Analysis of protein-protein interaction through hydrogen bonds.** Hydrogen bonding pattern as a
10 function of time reveals increased stability of the protein-protein complex with passage of time.
- 11 **Figure 15. Energy pattern for protein-protein interaction.** The pattern of energy corresponds to the hydrogen
12 bond formation. Decline in energy on the time scale suggests the strong binding between the proteins.
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