



# **Biochemical Analysis of Type II Metacaspase (mclI-*Pa*)**

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Masters Thesis in Biotechnology

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Biokemiska analys av Typ II Metacaspase (mclI-Pa)

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## **Abstract**

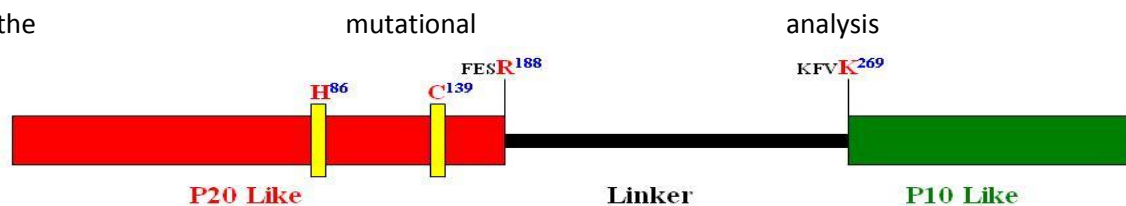
Programmed Cell Death (PCD) is a physiological process of cell death to remove unwanted cells and damaged cells. This physiological process is a cascade of biochemical reactions triggered and completed by Caspase, a cysteine protease, in animals. After discovering PCD in plants scientists tried to find caspase or similar enzyme in plants. However, caspase is not present in plant but a distant homologue metacaspase were found in the end of last decade. Conversely the exact function of metacaspase is not known. The main objective of this study was to assess the biochemistry of type II metacaspase from Norway spruce (mclI-Pa). mclI-Pa is the member of cysteine protease family and contains Cys/His catalytic diad which is conserved in all metacaspases and caspase. In this study we have overexpressed metacaspase in *E. coli* and purified by affinity, ion exchange and gel filtration chromatography. Having several rare codons, mclI-Pa can only express in cells which are engineered with additional tRNAs for translation of rare codons. Reducing agents are required during purification and storage to minimize the chance of formation of disulphide bonds. The activation pH is in the range (7.4) of physiological condition. We found that mclI-Pa is stabilized by Ca ions. Activation of mclI-Pa optimally requires 50mM  $\text{Ca}^{++}$ . In contrast,  $\text{Zn}^{++}$  inhibited mclI-Pa as it was also shown for caspase.  $\text{Mg}^{++}$  had no effect on the activation of mclI-Pa. We found that mutation of Lys269 to Gly inactivate mclI-Pa, suggesting that it may play role in the mclI-Pa activation. mclI-Pa was most active with 300mM NaCl which is in a range of physiological range. We found that mclI-Pa is active at its monomer form, whereas the mclI-Pa dimer is not active. Stabilization experiments of mclI-Pa by CD showed that  $\text{Ca}^{++}$  can stabilize the protein. Understanding the mechanism of the mclI-Pa activation and proteolytic activity requires high resolution structural studies. Our finding that  $\text{Ca}^{++}$  inhibits aggregation of mclI-Pa will help in preparing suitable sample of the protein for crystallization and subsequent X-ray crystallography analysis.

## Introduction:

Programmed cell death (PCD) is the important suicidal machineries for an organism which takes parts in development structures and plays an important role in many diseases. PCD observed in multicellular organisms, including mammals, insects, nematodes, plants. There is no report of PCD in procaryotes. Especially for the eukaryotes, PCD leads to removal of unwanted, damaged or infected cells in an organized way to keep the organism live and healthy (1, 2). In animal, PCD/apoptosis is a cascade of biochemical changes by the activation of Caspase for normal structure development and tissue homeostasis (1, 3, 4). Caspase is a member of cysteine protease family and cleaves specifically after aspartate (4, 5). Caspases are expressed inside the cells as a full length Zymogens. Effectors/initiator caspases (Caspase -2, -8, -9, -10) and Executioner caspases (Caspase -3, -6, -7) are activated through the proteolytic cleavage of zymogens and separated into two subunits, p20 (large) and p10 (small) subunit (5, 6, 7).

Programmed cell death also observed in plants (6, 7, 8). For the homeostasis and development of different organs of plants, PCD plays an important role (9). Chromatin Aggregation, Cell Shrinkage, Cytoplasmic and Nuclear Condensation, DNA Fragmentation are the major characteristics of plant program cell death (8). Although apoptotic characteristics are observed in plant due to responses to Hypersensitive response, Abiotic stress i.e. exposure to  $O_3$ , UV, Chilling & Salt stress (17). For growth and survival, selective cell death is required in plants and these are mostly found in local or large scale (18). The identification of metacaspase gene by Uren et. Al (2000) triggered the assessments of the involvement in PCD of metacaspase. Overproduction of yeast metacaspase YCA1 results the reducing cologenecity (19), *Trypanosoma brucei* metacaspase (TbMCA4) caused cell death (20). The group of P. Bozhkov showed that knockdown of Norway Spruce metacaspase (mclI-Pa) caused cell death during *in vitro* somatic embryogenesis (21). However, the mechanism of this process is not known.

Metacaspase is cytosolic enzyme and cleaves proteins after Arg and Lys (6, 8). It was found in plants fungi and protozoa and it is distantly homologues to caspase (22). Metacaspase contains catalytic diad Cys/His (figure 1) and belongs to the cysteine protease family (6, 8). Biochemical assessment of ATMC 9 (Arabidopsis metacaspase 9) shows the presence of a second catalytic cysteine which was confirmed by the



**Figure 01:** mclI-Pa. Indicating p20 and p10 like subunit and linker. Catalytic diad His86 and Cys139 are present on p20 like subunit. Arg188 and Lys269 are two potential cleavage site of mclI-Pa.

To understand the mechanism of plant cell death and the functional mechanism of metacaspases in death process, we performed a thorough biochemical characterization of mclI-Pa.

## Materials and Methods

### Multiple Alignments:

Multiple alignments of mclI-PA with *Arabidopsis* (ATMC -1, -2, -3, -4, -5, -6, -7, -8, -9), *Scizosaccharomyces*, *Acanthamoeba*, *Leishmania*, *Trypanosoma* metacspases was carried out using Clustalw2 bioinformatics web tool. All protein sequences were obtained from NCBI protein data bank.

### Expression and Purification:

All constructs were made on the basis of pET-11a (amp resistance). mclI-*Pa* cDNA was cloned into vector using NheI/BamHI restriction enzymes. His<sup>6</sup> tag was introduced with primers on N-terminal end of wild Type (WT), C139A mutant, R188G mutant, L269G mutant, R188G-L269G mutants and C-terminal end of C22A mutant and C22A-C139A mutant. All constructs were transformed in *Codonplus* RIL (DE3) competent cells.

*E. coli* (1000 ml) were grown in LB media containing Ampicilin (100 µg/ml) and Chloramphenicol (20 µg/ml) at 37°C till optical density (OD:600) reached 0.4-0.6. IPTG (0.5mM) was induced with half concentration of Ampicilin (50 µg/ml) and Chloramphenicol (10 µg/ml) and cultures were kept at 28°C for two hours (WT, R188G mutant, L269G mutant, R188G-L269G mutant) and for four hours (C139A mutant, C22A Mutant C22A-C139A mutant). Harvested cells were spinned down and resuspended with 40 ml buffer containing 50mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 300mM NaCl, 10mM Imidazole, 5mM DTT. French press was used for lyses the cells. Supernatant were collected for purification.

Affinity (Ni-Chelate) chromatography were used as per companies instruction (GE Healthcare) with loading buffer containing 50mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0 + 300mM NaCl + 10mM Imidazole + 10mM B-Me, and elution buffer containing 50mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0 + 300mM NaCl + 250mM Imidazole + 10 B-Me. Eluted samples were collected and dialyzed against 20mM Tris pH 8.0 + 5mM DTT. Anion Exchange chromatography (MonoQ 8ml Column) and Gel Filtration (120ml Superdex 200 column) were performed with Akta purifier. 20mM Tris pH 8.0, 2mM DTT and 20mM Tris pH 8.0 , 1M NaCl, 5mM DTT were used as loading buffer and elution buffer respectively. Gel filtration buffer contains 50mM HEPES pH 7.4, 150mM NaCl and 5mM DTT.

### Concentration Measurements:

Due to presence of reducing agents, DTT, it is difficult to measure protein concentration with nanodrop. Bradford assay were used to determine the protein concentration. All samples in each steps were measured by this assay.

### Ca<sup>++</sup> Dependence of mclI-Pa:

The Ca<sup>++</sup> dependence of mclI-*Pa* against the substrate FESR-AMC (50 µM) were evaluated in different concentration of CaCl<sub>2</sub> (1mM, 5mM, 10mM, 20mM, 30mM, 50mM, 60mM, 75mM, 100mM and 150mM) with 3ng of mclI-*Pa*. The enzymatic reaction was carried out at 28°C in following buffer: 50mM HEPES pH 7.4, 0.1% CHAPS, 5mM DTT. Activities were measured with BMG-Labtech OMEGA instruments at Genetic Centrum of SLU. Data were evaluated and analyzed with BMG-Labtech Omega Mars software

and the trend lines were made by Origin software. SDS-PAGE also performed separately with the same concentration of  $\text{CaCl}_2$ .

#### pH Dependence of mclI-Pa:

The same substrate and enzyme concentrations were used for the determination of pH dependence with variety of buffers containing different pH. For the pH range 4 to 5.2 50mM NaAc buffer, for 5.4 to 6 50mM Na-Cocadylate, for 6.2 to 6.8 50mM Bis Tris, for 7 to 7.4 50mM HEPES, for 7.6 to 8.8 50mM Tris and for pH 9 50mM TAPS buffer were used. 0.1% CHAPS, 5mM DTT and 50mM  $\text{CaCl}_2$  were present in all buffers. Data were evaluated and analyzed as described above.

#### NaCl dependence of mclI-Pa:

For determination of NaCl dependence different concentration of NaCl (50mM, 75mM, 100mM, 150mM, 200mM, 300mM, 400mM, 500mM, 600mM, 700mM, 800mM, 900mM and 1000mM) were used in the buffer containing 50mM HEPES pH 7.4, 0.1% CHAPS, 5mM DTT, 50mM  $\text{CaCl}_2$ . Same substrates were used for this experiment. Data were evaluated and analyzed as above.

#### Activity of the mutants of mclI-Pa:

Activities of the mutants (C139A, R188G, L269G, R188G-L269G, C22A and C22A-C139A) were measured with same substrate concentration and 3ng of enzymes. Data were evaluated by BMG Labtech Omega Mars software and graphs were prepared by Microsoft Excel. Reaction buffers were 50mM HEPES pH 7.4, 0.1% CHAPS, 5mm DTT containing 50mM  $\text{CaCl}_2$ .

#### Autocatalysis experiment of mclI-Pa:

SDS-PAGE based autocatalysis experiment also performed with two different pH buffer 50mM Bis tris pH 6.0 and 50mM HEPES pH 7.4. 20  $\mu\text{l}$  of Protein was in the reaction buffer. 50mM  $\text{CaCl}_2$  was added to the samples (4 in each pH buffer) at the time point 0, 5, 10 and 15 minutes. Reaction was stopped by adding 50mM EDTA. All experiments were carried out on ice.

Another SDS-PAGE based experiment was carried out with two different concentrations of enzymes (20x dilutions to another) in the buffer containing 50mM HEPES pH 7.4. 20mM  $\text{CaCl}_2$  was added at the time points 0, 5, 10, and 15 minutes. 50mM EDTA was used to stop the reaction. This experiment also carried out on ice.

#### Dimerization Experiments of mclI-Pa

Dimerization Experiments and activity assay of WT mclI-Pa was performed using different concentration (10mM, 20mM, 50mM, 75mM, 100mM, 200mM, 300mM, 400mM 500mM, 600mM, 700mM, 800mM, 900mM and 1000mM) of  $\text{NH}_4$ -Citrate as a dimerization agent. The reaction buffer contained 50mM  $\text{CaCl}_2$ . Activity assay was observed on SDS-PAGE.

#### Determination of the ion sensitivity of mclI-Pa:

The sensitivity towards  $Mg^{++}$  and  $Zn^{++}$  was determined in optimal buffer without EDTA, containing different concentration of  $Mg^{++}$  and  $Zn^{++}$  with 50mM  $CaCl_2$ . Sensitivity towards  $Mg^{++}$  and  $Zn^{++}$  were observed on SDS-PAGE.

#### *In vitro* stability of mclI-Pa:

Circular Dichroism (CD) was used for the determination of *In vitro* stability of the mclI-Pa structure. WT and C139A mclI-Pa was obtained just after Gel Filtration. Temperature range of 20°C to 90°C was used in CD instruments for melting the enzymes with and without  $Ca^{++}$ . The enzyme concentration in the both case was 0.15 mg/ml.

#### Crystallization experiments:

Crystallizations of mclI-Pa were tried several times. C139A mutant were purified till Gel Filtration and placed crystal drops using three different crystallization screens (Crystal screen 1+2, JSCG+ and Cryo 1+2) on 96 well crystallization plates. Proteins were concentrated till 6.12 to 21 mg/ml in different batches of crystallization trials. Plates were kept at 16°C.

#### Oligomerization experiments

To remove the high molecular species, oligomerization experiments were performed just after Gel Filtration of C139A mutant. Sample were divided into six and treated with 50mM  $CaCl_2$ , 0.1% Twin20, 0.5% Octyle  $\beta$  D Glucopyrinoside, 0.1% CHAPS and 0.3% CHAPS added directly to the sample and concentrated 10x. The sixth sample was used as control without adding any compounds. Oligomerization experiments were done by Gel Filtration by using 24 ml Superdex 200 column with Aakta purifier.

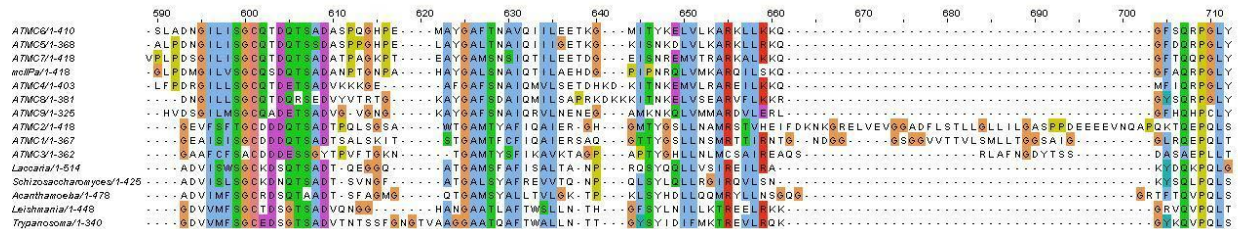
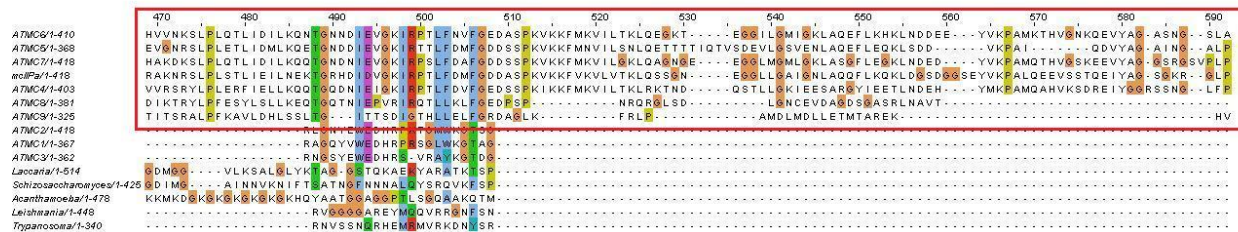
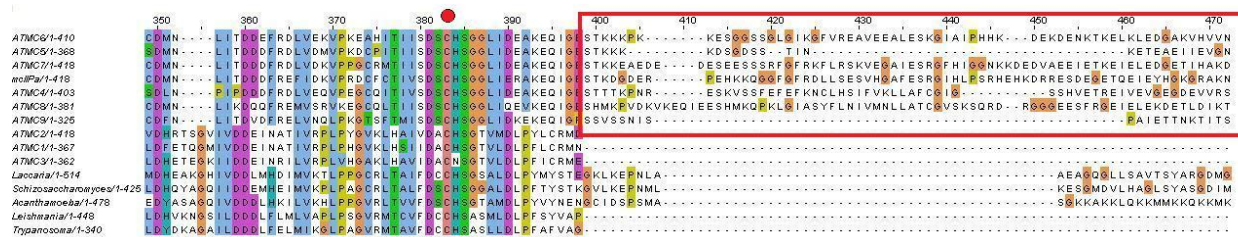
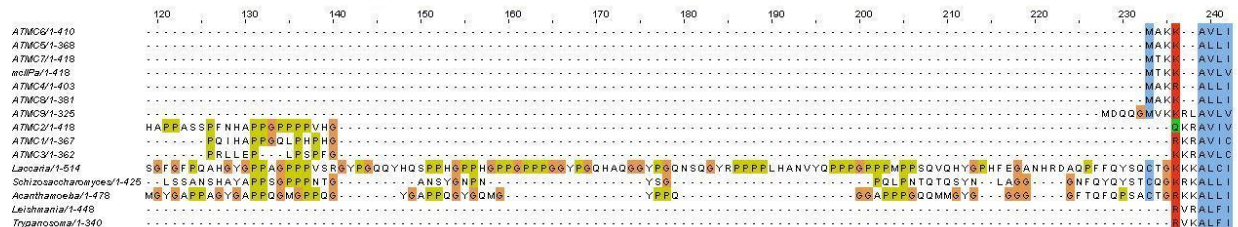
#### Model prediction:

To predict the model of mclI-Pa we used web tool 3D-PSSM. Which is predicted protein structure based on the structure existing in PDB database.

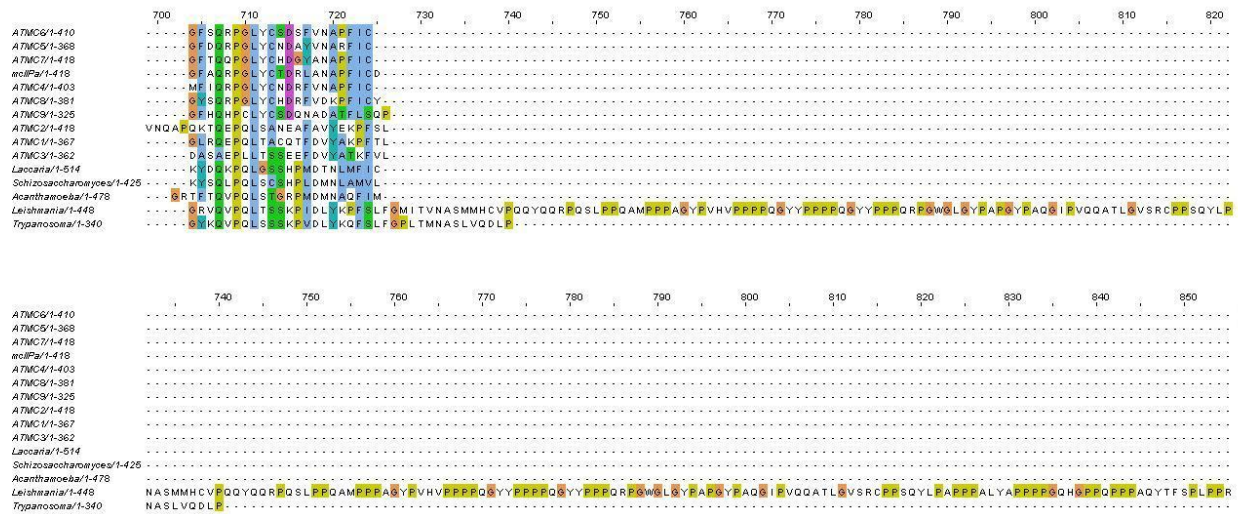
### **Results and Discussion:**

Using Clustalw2 bioinformatics web tool, the multiple alignments of mclI-PA with *Arabidopsis* (ATMC -1, -2, -3, -4, -5, -6, -7, -8, -9), *Scizosaccharomyces*, *Acanthamoeba*, *Leishmania*, *Trypanosoma* metacaspases have been shown in the figure 2. catalytic diad of mclI-Pa (His86 and Cys139) were conserved in all metacaspase (Second and third red circle in figure 2). *Arabidopsis* -1, -2, -3, , *Acanthamoeba*, *Leishmania*, and *Trypanosoma* metacaspases contains N-terminal prodomain which is absent in type two metacaspase (mclI-PA, *Arabidopsis* (ATMC -4, -5, -6, -7, -8, -9). Long linker of type two metacaspases has been observed from the alignment as well, type one contains short linker between two subunits (red rectangular in the figure 1). Vercammen *et.al* (2007) showed that a second cysteine (mclI-PA C22) takes part in catalytic reaction of *Arabidopsis* metacaspase activation. This Cysteine (ATMC -9, Cys29) is also conserved in all metacaspases (first red circle).



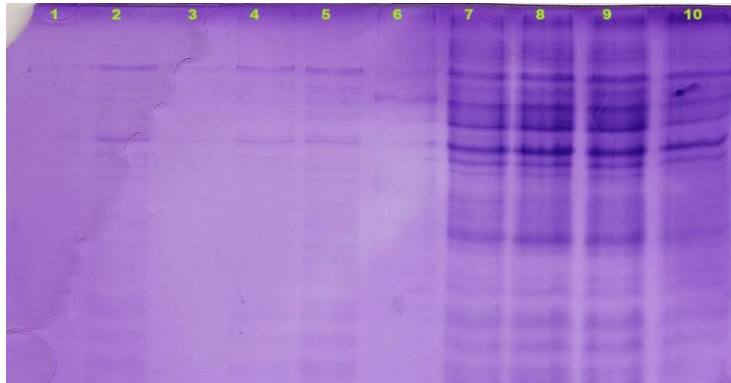




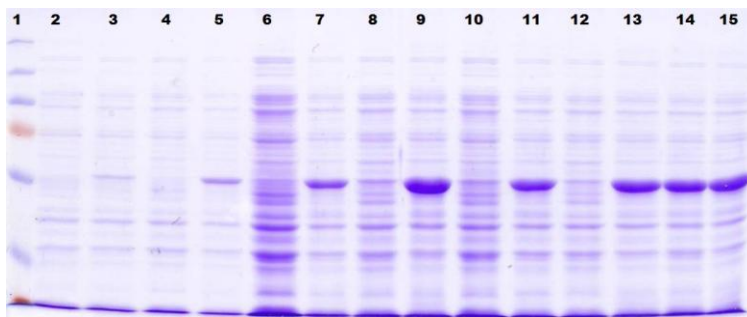


**Figure 02:** Multiple alignment of Metacaspase. Arrows indicated the conserved amino acids among all proteins. Second and third red circles indicating the catalytic dyad (His86 and Cys139). First red circle (Cys22-mcII-Pa) indicating the second Cysteine that could take part in catalytic reaction.

After a long expression optimization, we have managed to express large amount of mcII-*Pa* recombinant protein in *E. coli*, which accumulated in cytoplasm in soluble form. BL 21(DE3) cell can not able to express mcII-*Pa* (figure 3) as mcII-*Pa* contains 22 rare codons. BL 21 (DE3) has not additional tRNAs to translate the protein. Using *Codonplus* RIL (DE3) competent cells solved the problem and gave excellent expression (figure 4).



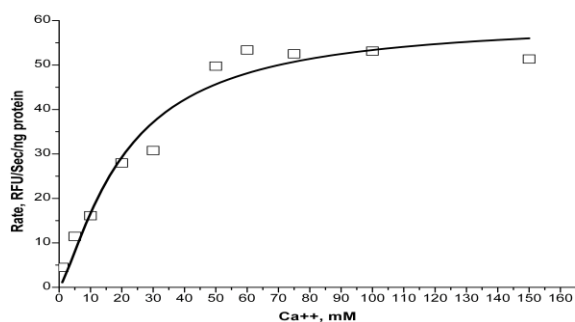
**Figure 3:** SDS-PAGE expression of mcII-*Pa*. in BL 21(DE3) competent cells. Lane 1 to 6 contains soluble fractions after sonication and lane 7 to 10 contains the inclusion bodies.



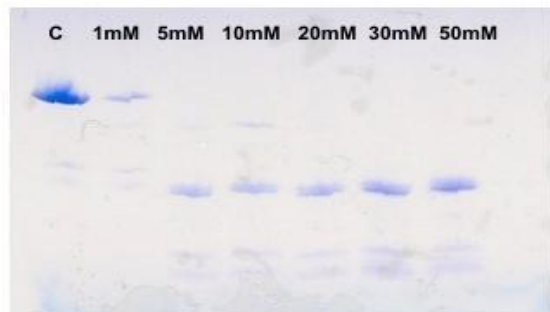
**Figure 04:** SDS-PAGE containing samples after expressing in *Codonplus* RIL (DE3) competent cells with different time points after IPTG induction and temperature. Good Expression of mcll-*Pa* was observed at 28°C after induction (Lane 9).

The next challenge was to purify the protein for further experiments. We tried several times to purify the protein using Ni-chromatography and anion exchange (monoQ). But every time we failed. Protein was aggregated and digested and finally the protein yields were very low compare to the expression. Then we observed that mcll-*Pa* contains 11 cysteins which might create the problem of aggregation by forming disulphide bond between cysteins. This observation led us to use reducing agent to reduce the chance of forming disulphide bonds. Using  $\beta$ -merkaptoethanol (BME) with Ni-chromatography buffer and DDT with other purification buffers, we got a tremendous improvement of protein yield till Gel Filtration. After a couple of optimization trials we used 10mM BME in the both buffers of Ni-chromatography and 2mM and 5mM DTT in Anion exchange chromatography loading and elution buffer respectively. During dialysis and French press we used 5mM and 2mM DTT respectively. Gel filtration buffer contained 5mM DTT.

Bozhkov *et.al.* (2005) showed that  $\text{Ca}^{++}$  requires for activation of mcll-*Pa*. However, their experiments were performed with unpurified protein. We decided to check  $\text{Ca}^{++}$  activity dependence using purified mcll-*Pa*. We observed from the activity assay (figure 5) and SDS-PAGE (figure 6), 50mM  $\text{Ca}^{++}$  are required for the activation of mcll-*Pa*.



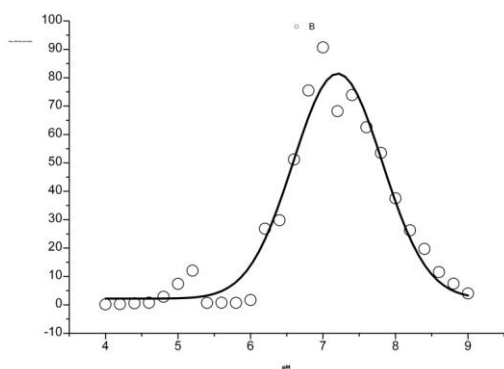
**Figure 05:**  $\text{Ca}^{++}$  dependence of mcll-*Pa*.



**Figure 06:** SDS-PAGE  $\text{Ca}^{++}$  dependence.

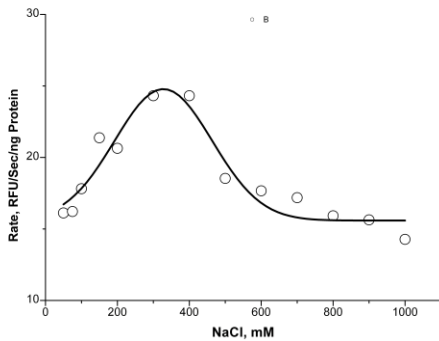
mcll-*Pa* activation requires quite a lot of  $\text{Ca}^{++}$  (50mM) compare to AtMC4, AtMC5 (0.5mM) (10), and *Trypanosoma* metacaspase 2 (1mM) (11).  $\text{Ca}^{++}$  has no effect on mammalian Caspase (12).

Norway Spruce metacaspase was most active at neutral pH (pH 7.4), whereas ATMC 4 and ATMC 8 activated at pH 7.5 to 8.0 (13), while ATMC 9 is active at pH range 5.0 to 5.5 (13). The bell-shaped (figure 7) pH dependence signifies the existence of one active form of the enzyme with increase in activity most likely due to the de-protonation of catalytic Cys residues. the pH dependence also shows that mcll-*Pa* is fully active inside the cell.



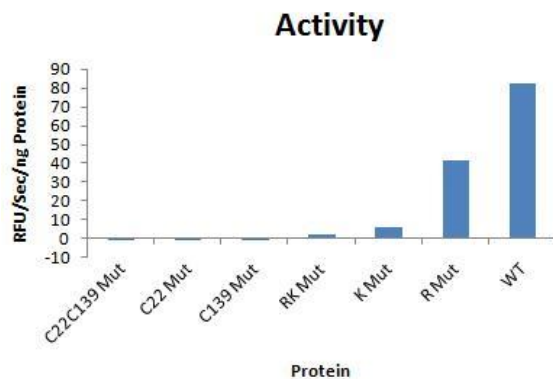
**Figure 07:** pH dependence. Rate, RFU/Sec/ng Protein in Y axis and pH is in X axis.

We used NaCl in the range of 50mM to 1M in the assay buffer to determine the ionic strength of mcll-*Pa* activity. mcll-*Pa* was most active with 300mM NaCl which is in a range of physiological range.



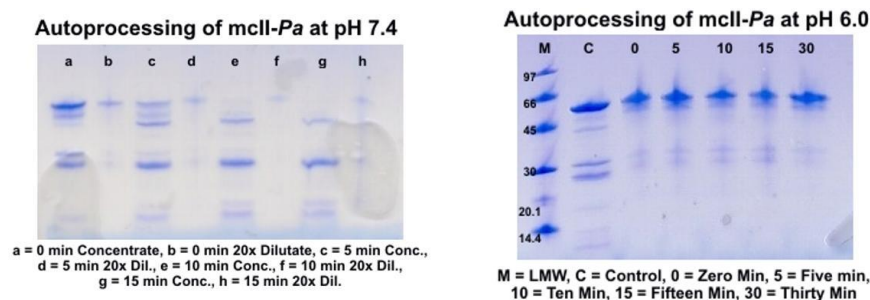
**Figure 08:** NaCl dependence on mclI-*Pa* activity.

Mutation on catalytic site of enzyme makes inactive. For mclI-*Pa* mutation at Cys139 site to Alanine found inactive (figure 9) that ensure the presence of catalytic diad. We have also assessed the activity of C22A mutant enzyme to be sure the presence of second cys which may take part in catalysis as described incase of Arabidopsis (6). The C22A mutant enzyme found inactive.



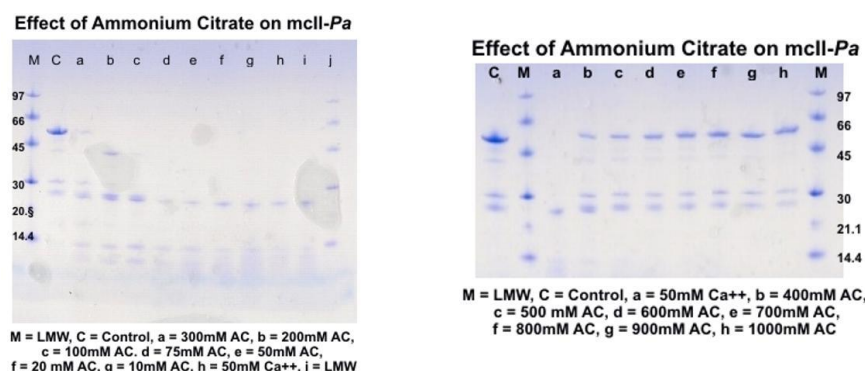
**Figure 10:** Activity assay of WT mclI-*Pa*, C139A mutant, C22A mutant, R188G mutant, K269G mutant, R188GK269G mutant, C22AC139A mutants. R188G mutant shows some activity but K269G nearly inactive. The activity of R188GK269G is almost zero. C22A, C139A and C22AC139A mutants were totally inactive.

*Leishmania* metacaspase (LdMC1 and LdMC2) (14), Arabidopsis metacaspase (6) and mclI-*Pa* (8) acts as trypsin and cleaves substrates after Arg and Lys. Where Caspase cleaves after Asp (12). Bozhkov *et. al.* (2005) and Vercammen *et.al* (2007) shows that to be active, metacaspase requires cleaved themselves. According to their experiments metacaspase cleaved after Arg and Lys to activate itself. To check the cleavage point with activity we assessed the activity of mutant enzyme at the point of Arg188 and Lys269 separately and both (R188GK269G) mutant on same enzyme. We have found Arg188 mutant showed some activity. The activity of Lys269 mutant was nearly zero. But both mutant enzymes showed almost inactivity (figure 10). The autocatalysis pattern was observed on SDS-PAGE. At pH 7.4 enzymes were cleaved itself like a cascade and after 15 minutes mclI-*Pa* were digested almost fully (figure 11). As pH 6 mclI-*Pa* is inactive we haven't found any cleavage pattern on the gel (figure 11). Compare to dilute (20x) enzyme concentrate samples were digested fully.



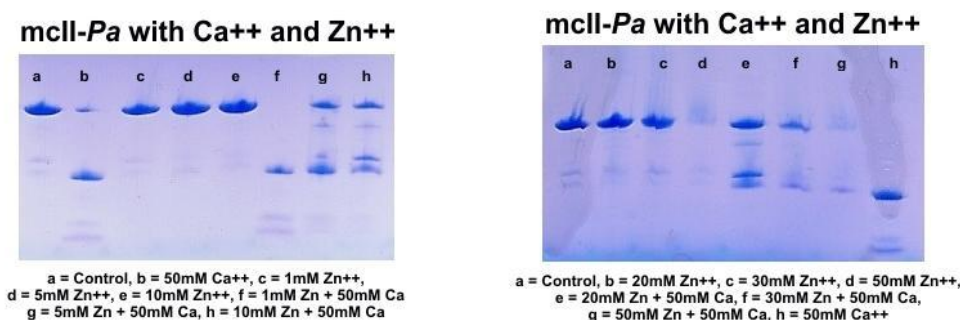
**Figure 11:** Autoprocessing of mcll-Pa at two different pH

Caspases are active when they are formed dimmers (15). We made dimer form of metacaspases with  $\text{NH}_4$ -Citrate and run SDS-PAGE. We found mcll-Pa inactive (figure 12). This experiment proved that metacaspases are active at monomer formed.



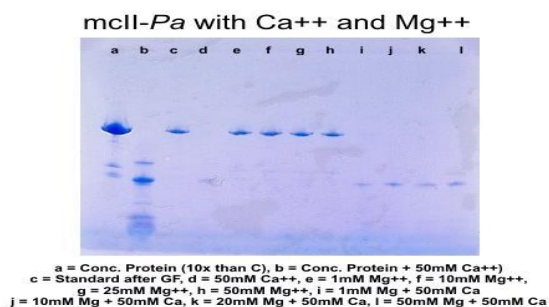
**Figure 12:** Dimerization experiments with  $\text{NH}_4$ -Citrate.

Several studies have been reported that  $\text{Zn}^{++}$  inhibits cell death as well as caspase activity (8, 12, 16). Mostly the inhibition of nuclease by  $\text{Zn}^{++}$  makes the effects. Caspase 3 and Caspase 6 are completely inactivated by 2mM  $\text{Zn}^{++}$  (12). We have found that 5mM  $\text{Zn}^{++}$  is enough for inactivate the metacaspase activity. SDS-PAGE shows the scenario in the figure 13.



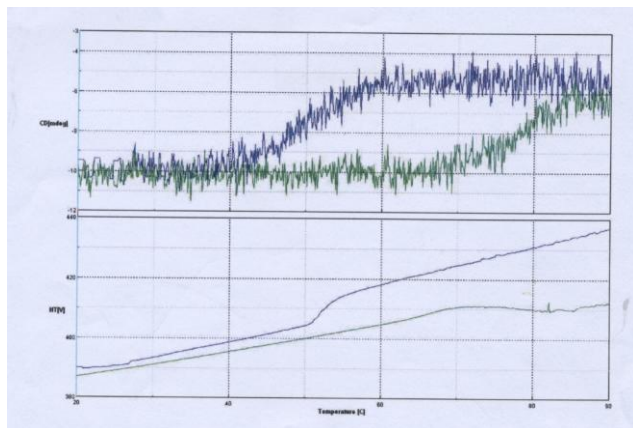
**Figure 13:** Effect of  $\text{Zn}^{++}$  on metacaspase activity.

On the other way  $\text{Mg}^{++}$  has no effect on the activity of metacaspase (figure 14).

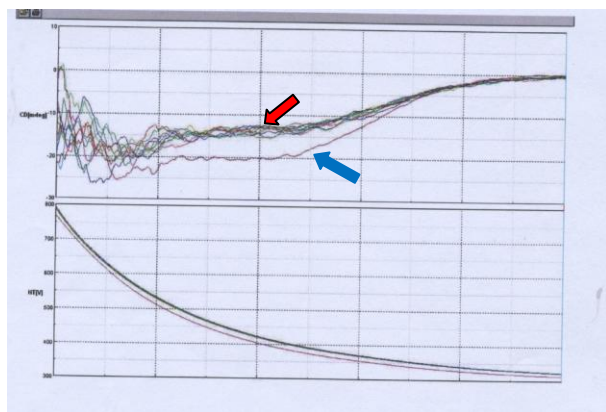


**Figure 14:** Effect of  $\text{Mg}^{++}$  on Metacaspase activity.

$\text{Ca}^{++}$  plays a great role in activation of metacaspase. To be activated metacaspase requires certain amount of  $\text{Ca}^{++}$ . Now the questions are that is  $\text{Ca}^{++}$  made metacaspase stable? To find the answer we carried out the Circular Dichroism (CD) experiments using both WT and C139A mutant metacaspase. We have observed that  $\text{Ca}^{++}$  makes C139A mutant metacaspase stable up to  $70^{\circ}\text{C}$  (figure 15). WT metacaspase also stabilized by  $\text{Ca}^{++}$  (figure 16).



**Figure 15:** Circular Dichroism of Cys139 mutant. Blue graph is for without  $\text{Ca}^{++}$  and the green one contains 50mM  $\text{Ca}^{++}$ .



**Figure 16:** Circular Dichroism of WT mclI-Pa. Blue arrow indicates the WT without  $\text{Ca}^{++}$ . and red arrow indicates with 50mM  $\text{Ca}^{++}$ .



Protein function can be fully known from its structure. We tried several times to crystallize the metacaspase mutant (Cys139). But we failed to get crystals. At highest we got some crystalline precipitation. Every case we got high molecular species on gel filtration chromatogram. Mostly high molecular species has made the differences. To remove the high molecular species, oligomerization experiments were performed just after Gel Filtration of C139A mutant. Sample were divided into six and treated with 50mM  $\text{CaCl}_2$ , 0.1% Twin20, 0.5% Octyle  $\beta$  D Glucopyrinoside, 0.1% CHAPS and 0.3% CHAPS added directly to the sample and concentrated 10x. The sixth sample was used as control without adding any compounds. Oligomerization experiments were done by Gel Filtration by using 24 ml Superdex 200 column with Aakta purifier. We have found that 50mM  $\text{Ca}^{++}$  addition directly to the sample solved the problem (Figure 18). And in all other case no improvement (figure 17, figure 19 to 22).

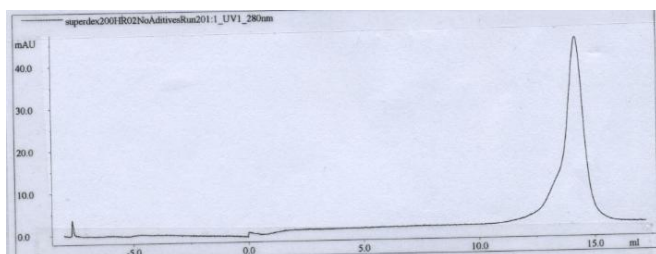


Figure17: GF Chromatogram of Control, without adding any additives.

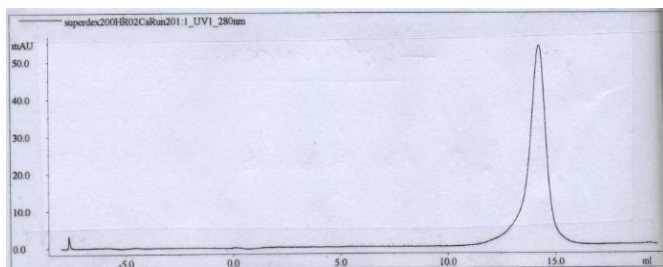


Figure 18: GF Chromatogram of WT containing 50mM  $\text{Ca}^{++}$  in the samples.

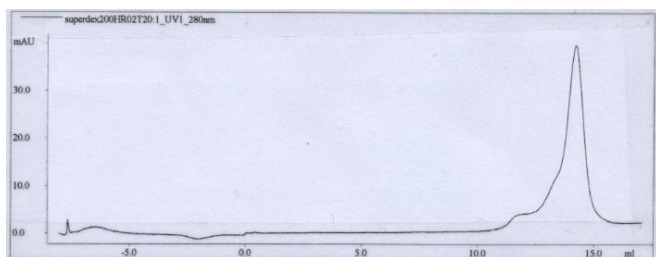


Figure 19: GF Chromatogram of WT containing 0.1% Twin 20 in the sample.



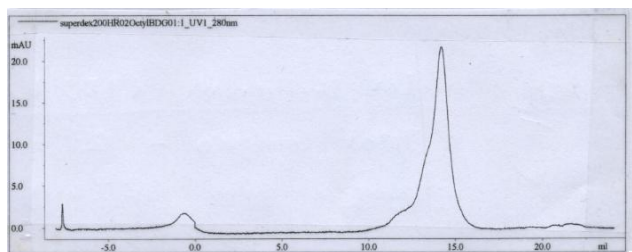


Figure 20: GF Chromatogram of Wt containing 0.5% beta octyl glucopyranoside in the sample

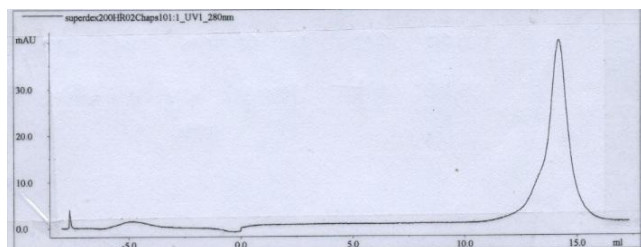


Figure 21: GF chromatogram of WT containing 0.1% CHAPS in the sample.

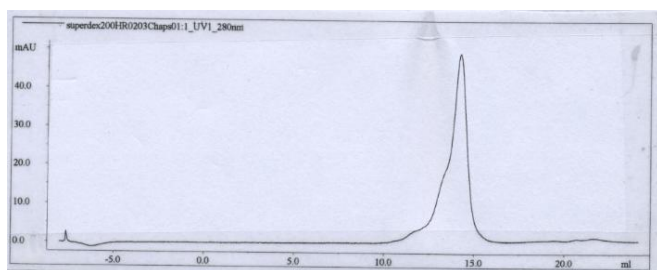
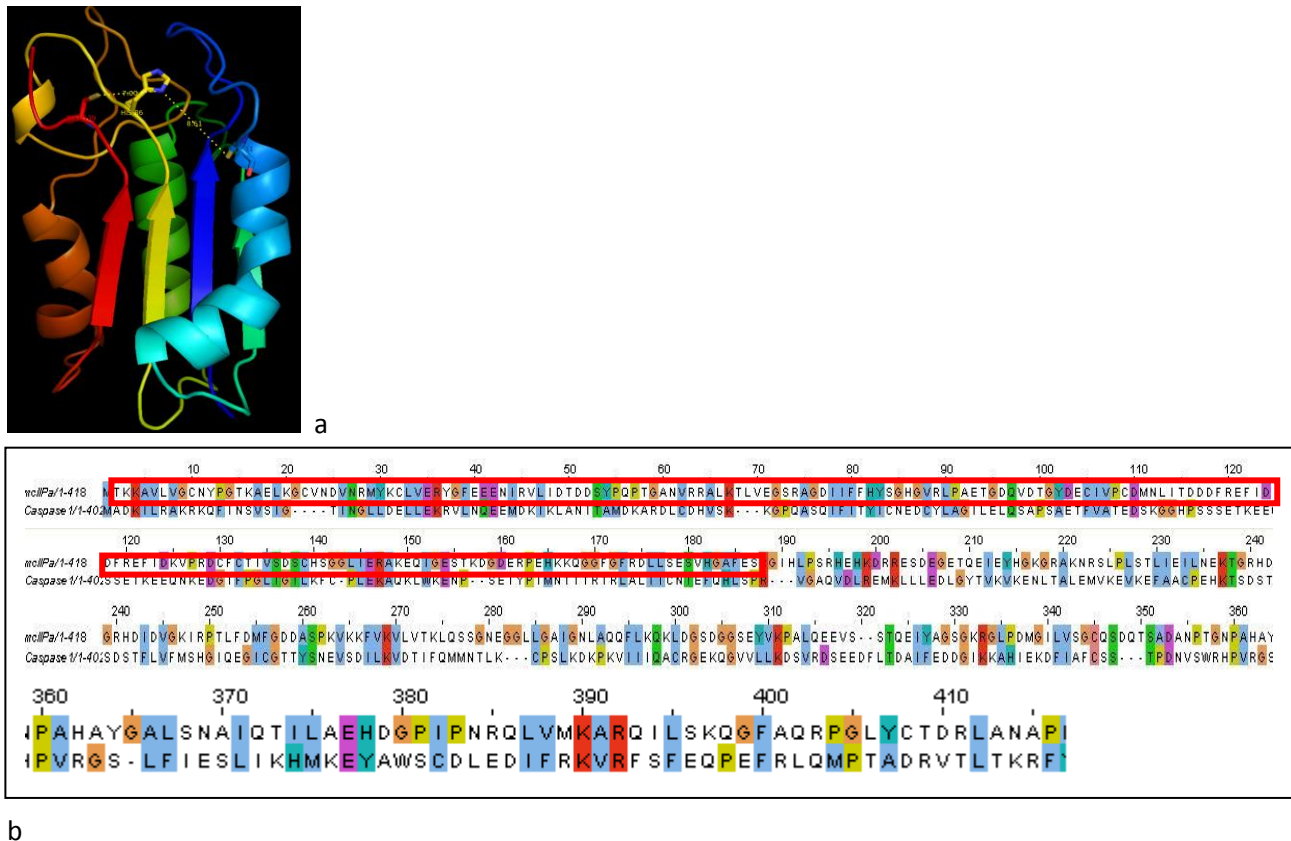


Figure 22: GF Chromatogram of WT containing 0.3% CHAPS in the samples.

Metacaspase model was predicted by web tool 3D PSSM. Only N-terminal part of metacaspase was predicted based on Caspase 1 structure (figure 23). The figure 23 shows the predicted structure with the distance of C22 (8.61 Å) and C139 (7 Å) from H86.



**Figure 23:** a. Predicted structure of metacaspase showing the distance of C22 and C139 from H86. b. Alignment of metacaspase with caspase 1 and also indicating the amino acid sequence of N-terminal part.

## Conclusion

Metacaspase is the prime candidate for PCD in plants. Several reports are indicating that. But the truth is revailing. Comparing to Caspase, metacaspase has basic (Arg and Lys) P1 preferences for substrate where the P1 preference of Caspases is acidic (Asp). The activation pH is in the range (7.4) of physiological condition. For activation and stabilization of metacaspase  $\text{Ca}^{++}$  is necessary.  $\text{Zn}^{++}$  inhibits both metacaspase and caspase. Lys269 is important for activation. Mutation of Lys269 position to Gly made metacaspase inactive. The second catalytic Cys (C22) is not completely understood required further experiments. Structure of mcll-Pa will tell us more about this as well as complete scenario of functions.

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