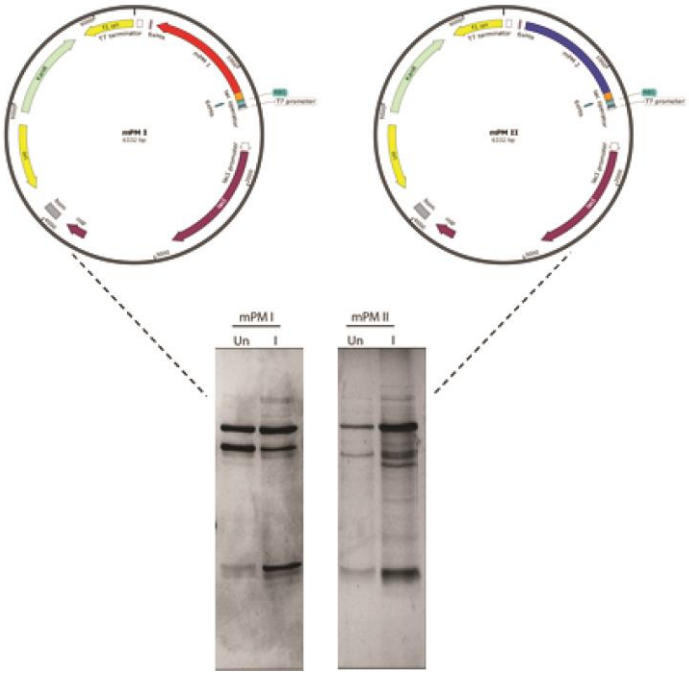
Chapter 3

Cloning and expression of Plasmepsin I and Plasmepsin II from *Plasmodium falciparum* and search for their inhibitors



3.1 Introduction

PM I and PM II are the enzymes known to perform initial cleavage of the hemoglobin molecule, which makes them potential targets for developing novel drugs against malaria. Studies for the development of enzyme inhibitors would require the enzymes in significant quantities. Considering only trivial amounts of material that can be isolated from the parasite, the production of the enzymes as recombinant proteins becomes essential to permit such studies. Recombinant expression of PMs and attaining their active forms have generally been challenging. Attempts made initially to express the full-length proPMs in *E. coli* resulted in very low expression levels, possibly due to the toxic effects of the hydrophobic segment, and the products expressed lacked ability to activate into mature forms (Moon et al., 1997). Subsequently, expression of the recombinant proforms of PM II, PM IV and HAP, which lacked hydrophobic transmembrane domain and which were capable of auto-activation to generate mature forms was achieved (Hill et al., 1994; Wyatt and Berry, 2002; Xiao et al., 2006). However, the active expression of PM I presented some major difficulties which were overcome slightly by introducing a Lys110P to Val (P indicates a propart residue) mutation into the propart of the zymogen which auto-activated at 12 and 7 residues upstream of the native cleavage site, but the overall efficiency of refolding and auto-activation still remained low (Moon et al., 1997). Later three to ten-fold diminution in the k_{cat} values for substrate hydrolysis as compared with the native PM I was reported by this mutant (Tyas et al., 1999). Then Xiao et al. (2007) came up with a simpler protocol for activation of PM I which activated at 7 residues upstream of the native cleavage site and the yields were again limited.

Various postulations prevail regarding the role of the prosegment. The mature form of PM II without prosegment was expressed and it was demonstrated that the enzyme could achieve its active conformation without undergoing the activation process (Parr-Vasquez and Yada, 2010). A study with PM II suggested that prosegment is required to catalyze the folding of the enzyme (Xiao et al., 2011). Conversely, in the case of expression as inclusion bodies in *Escherichia coli* followed by denaturation and refolding, it was found that expressing the mature form of PM II resulted in a three-fold greater recovery of the active enzyme as compared to PM II with partial prosegment (Istvan and Goldberg, 2005), indicating that truncated prosegment actually hindered folding (Xiao et al., 2011).

Considering all the above mentioned, efforts were done to establish an expression system that can express the mature PMs and while bypassing the auto-activation process they can be converted to active enzymes. A convenient method to achieve the properly refolded active PMs was also sought. Since inhibitors of PM I and PM II can be good antimalarial weapons and there is an abundance of compounds with protease inhibitory activity including those against aspartic proteases in plants, the plants already found to have promising activity against the *P. falciparum* parasite which are mentioned in chapter 2 were explored as a source of inhibitors against the parasite's PMs, PM I and PM II.

3.2 Materials and methods

3.2.1 Cloning and preparation of expression constructs

Genomic DNA was extracted from the *P. falciparum* CQ-sensitive 3D7 strain procured from ICMR-NIMR, New Delhi, India, using the protocol proposed by Beck (2002). The genomic DNA was used for the isolation of PM genes. Amplification of the full-length PM I gene was done using the forward primer PM I FW (ATG GCT TTA TCA ATT AAA GAA G) and reverse primer PM I RV (TTA CAA TTT TTT TTT GGC AAG G). The prosegment region of the gene was truncated by using the nested primers mPM I FW (CTA GCT AGC AAT GCT GGT GAT AGT GTA AC) and mPM II RV (CGC GGA TCC TTA CAA TTT TTT TTT GGC AAG G) to amplify only the segment coding for the mature PM I enzyme (mPM I). The restriction site in the primer sequences is underlined. In a similar manner, to amplify the full-length PM II gene the forward primer PM II FW (ATG GAT ATT ACA GTA AGA GAA CAT G) and the reverse primer PM II RV (TTA TAA ATT CTT TTT AGC AAG AGC AAT ACC) were used. The region coding for the prosegment was deleted using the nested primers mPM II FW (CTA GCT AGC AGT TCA AAT GAT AAT ATC G) and mPM II RV (CGC GGA TCC TTA TAA ATT CTT TTT AGC) and only the segment coding for the mature PM II enzyme (mPM II) was amplified. The restriction site in the primer sequences is underlined. The final amplicons produced from the full-length PM I and PM II genes were then digested with NheI and BamHI and were cloned into pET-28a(+) vector to give rise to constructs mPM I and mPM II. The resulting constructs mPM I and mPM II, encoded polyhistidine tag followed by enterokinase cleavage site and the mature regions of the enzymes coded by amino acids 124-452 in proPM I and 125-453 in proPM II respectively. The constructs were transformed to *E. coli* DH5α using the $CaCl_2$ method (Sambrook and Russel, 2001). The transformants harboring the constructs were confirmed by colony PCR

using the gene-specific primers (mPM I FW and mPM I RV; mPM II FW and mPM II RV in the case of mPM I and mPM II harboring transformants respectively) and by checking for insert release after performing restriction digestion of the constructs using NheI and BamHI. The amplicons generated from the plasmids of the confirmed transformants using the gene-specific primers were verified by bidirectional sequencing (Barcode Biosciences, Bangalore, India).

3.2.2 Expression of the recombinant enzymes

E.coli BL21(DE3)pLysS were transformed with the constructs mPM I and mPM II for the expression of the enzymes mPM I and mPM II respectively. Transformants found were grown overnight at 37°C in terrific broth [tryptone 12 g/l, yeast extract 24 g/l, glycerol 4 ml/l, 100 ml/l potassium phosphate buffer (0.17 M monobasic, 0.72 M dibasic), pH 7.2 ± 0.2] supplemented with 50 µg/ml of kanamycin and the inoculum thus obtained was inoculated at final 1% concentration to 50 ml of the same medium. The culture was grown until OD_{600} reached 2.0 at 37°C and then it was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37°C. Cells were harvested by centrifugation (10000 g for 10 min).

3.2.3 Purification of the recombinant enzymes

The purification procedure followed for both mPM I and mPM II enzymes was the same. The cells harvested were resuspended by adding 10 ml of NPI-10 buffer (50 mM NaH2PO4, 0.3 M NaCl, 10 mM imidazole, pH 7.5) to a pellet of approximately 0.35 g and the cells were lysed by sonication. The suspension was centrifuged at 12000 g for 10 min. The supernatant was collected and analyzed by 12% SDS-PAGE. The supernatant was subjected to purification by Ni-affinity chromatography. Briefly, 250 µl of the supernatant was applied to the 5ml His-tag Ni affinity column (HiMedia, Maharashtra, India), the amount applied not exceeding 5% of that of the column volume. The column was first equilibrated with NPI-10 buffer, then following 20 min incubation, the unbound proteins were washed using five column volumes of the wash buffer NPI-20 (50 mM NaH_2PO_4, 0.3 M NaCl, 20 mM imidazole, pH 7.5). The protein bound was then eluted using NPI-250 buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 250 mM imidazole, pH 7.5). Further, the eluent collected was purified and desalted by gel filtration chromatography with SuperoseTM 12 10/300 GL (GE Healthcare, Uppsala, Sweden) column equilibrated with 20 mM Tris pH 8.0 and 100 mM

NaCl. The fractions containing the protein were pooled and concentrated using Microsep advance centrifugal device, 1K Omega. The protein was then digested with thrombin (Sigma-Aldrich, Bangalore, India) (50 000:1, protein:thrombin, w:w) for 1 h at 37°C to remove the His-tag from the protein. After digestion, the sample was applied to a p-aminobenzamidine agarose (Sigma-Aldrich, Bangalore, India) column and following the manufacturer's instructions the mature enzyme was recovered in Tris 50 mM and 0.5 M NaCl, pH 8.0 buffer and the eluted fraction was concentrated.

The refolding of the proteins was accomplished using the thermal-assisted refolding technique. The heat-induced unfolding of each of the samples was initiated by diluting 2 µl of the protein sample (110 µg/ml of mPM I or mPM II) into198 µl of refolding buffer (20 mM Tris-HCl, 1 mM GSH, 0.1 mM GSSG, 10% glycerol; pH 7.0) preheated at 70°C. Thoroughly mixed samples were incubated for 6 min at 70°C and then left in ice for refolding at 0°C for 24 h. Subsequently, the enzyme mixture was concentrated. The protein samples were monitored at each step of purification and after refolding using 12% SDS-PAGE.

3.2.4 Activity assay

The activity of the expressed PMs was determined spectrophotometrically by detecting hemoglobin degradation. This was performed by addition of 100 µl (20 ng/µl) of enzyme to 1900 µl of 100 mM sodium citrate buffer, pH 5.0 and incubating the enzyme for preactivation at 37°C for 5 min. Subsequently, 50 µl of human hemoglobin (2 mg/ml) was added to the mixture and the thoroughly mixed reaction was read at room temperature at 406 nm, which is the wavelength of maximum absorbance (λ_{max}) for hemoglobin (Rane and Datta, 2001), against a blank where the enzyme was substituted with the refolding buffer. The change in absorbance was recorded for 5 min. One enzyme unit was defined as the amount of enzyme required to cleave 1 µmole of hemoglobin in 1 min. The enzyme activity was determined using the following equation:

Enzyme activity (µM/min) = [Δ Absorbance of hemoglobin per min × Total reaction volume (ml) × 10^6]/[ε × Enzyme volume (ml) × Path length (cm)]

Where ε is the molar extinction coefficient of human hemoglobin at 406 nm which is reported as 276069.66 $M^{-1}cm^{-1}$ (Rane and Datta, 2001) and path length is 1 cm.

A time-course analysis of the activities of enzymes was also performed. The reaction system for assessing the activity spectrophotometrically was scaled down to the final volume of 500 µl for this purpose. After setting the reaction at 37°C, 50 µl aliquots were withdrawn after 0, 10, 30, 60 min incubation. The reaction was stopped in every aliquot by immediately adding 6× Laemlli SDS sample buffer and boiling for 10 min. Until the completion of the experiment, the stopped reactions were stored at 0°C, to prevent further denaturation of hemoglobin. The reactions were analyzed by loading onto 12% SDS-PAGE.

3.2.5 pH optimum determination

The pH optimum for the activity of the mature expressed enzymes was determined by scaling down the reaction system used for assessing the enzyme activity spectrophotometrically to 500 µl and carrying out the reactions at various pH values using one of the following 100 mM buffers: sodium acetate pH 4.0; sodium acetate pH 4.5; sodium citrate pH 5.0; sodium citrate pH 5.5 and sodium phosphate pH 6.0. After incubation at 37°C for 1h, the reactions were stopped and stored as mentioned previously for time-course analysis of the activities of enzymes. The reactions were analyzed by 12% SDS-PAGE.

3.2.6 Protein concentration determination

Protein concentration/enzyme concentrations were determined by the Lowry's assay using bovine serum albumin as a standard (Lowry et al., 1951).

3.2.7 PM inhibition assay

The plant extracts having > 70% antiplasmodial activity mentioned in chapter 2 were screened for their ability to inhibit PMs. The search was conducted using the expressed recombinant *P. falciparum* 3D7 PMs, mPM I and mPM II. The ability of the extracts to inhibit the activity of mPM I and mPM II to degrade hemoglobin was assessed by spectrophotometric microtiter plate based assay performed in triplicate. Briefly, the mPM I or mPM II solution in 100 mM sodium citrate buffer at pH 5.0 was prepared freshly and incubated at 37°C for 5 min for enzyme preactivation. The assay consisted of four different types of reactions namely, 100% reaction control, pepstatin A control, extract control and test sample reaction. Then 135 µl of the enzyme solution (containing 10 µl of 20 ng/µl enzyme) was added to all the reactions but to the extract controls where the amount was substituted with the sodium citrate buffer. Then 5 µl of the extracts (50 µg/ml) were added

to the test sample reactions and to the extract controls. Whereas 100% reaction controls received 5 µl of the sodium citrate buffer and pepstatin A controls received 1 µM of the aspartic protease inhibitor pepstatin A in 5 µl volume, instead of a candidate extract. Subsequently, 10 µl of hemoglobin (2 mg/ml) was added to all the wells. Following proper mixing, the reactions were read at 406 nm for the initial reading. The reaction systems were then incubated at 37°C for 40 min. After the end of incubation, the reactions were read again at 406 nm for the final reading. The reading from the respective extract control was used for blank correction. The percentage inhibition of the enzyme was calculated as % Inhibition = $[1 - (\Delta A_S/\Delta A_{100\%})] \times 100$, where ΔA_S is change in absorbance of test sample reaction control or pepstatin A control and $\Delta A_{100\%}$ is change in absorbance of 100% reaction control, which were obtained by subtracting the corresponding initial absorbance reading from their final absorbance reading.

3.3 Results and discussion

3.3.1 Cloning and preparation of constructs

The primers designed successfully amplified the PM genes under the decided PCR conditions. The full-length PM I gene consisting of 1359 bp was amplified (Figure 3.1A). Further using the amplified gene as a template and the nested primers designed, an amplicon of 1008 bp that included the sequence coding for the mature part of PM I was amplified. Similarly, in the case of PM II, the specific primer pairs designed based on the full-length gene and the mature region of the enzyme amplified 1362 bp and 1008 bp amplicons respectively (Figure 3.1B).

The constructs prepared after ligation of the amplicons with encoded mature regions of the enzymes were transferred to *E.coli* DH5α. Transformation with the construct carrying the region for mature PM I gene named mPM I construct (Figure 3.2A) yielded numerous transformants, out of which ten randomly selected transformants were screened. Upon performing colony PCR with primers mPM I FW1 and mPM I RV2, all the screened clones resulted in an amplicon of 1008 bp (Figure 3.1C), the size similar to that of the enzyme's mature region gene part i.e. 990 bp, indicating the amplicon carrying the desired segment. Restriction digestion of the isolated plasmids from the selected clones using NheI and BamHI released an insert corresponding to 996 bp from the vector backbone of 5336 bp (Figure 3.1C). Thus, the screenings confirmed the presence of the desired construct in the

selected clones. Further, insert sequence analysis was performed using a clone which was named mPM I DH5α and BLAST analysis confirmed the presence of the gene sequence coding for the mature region of PM I in the cloned insert.

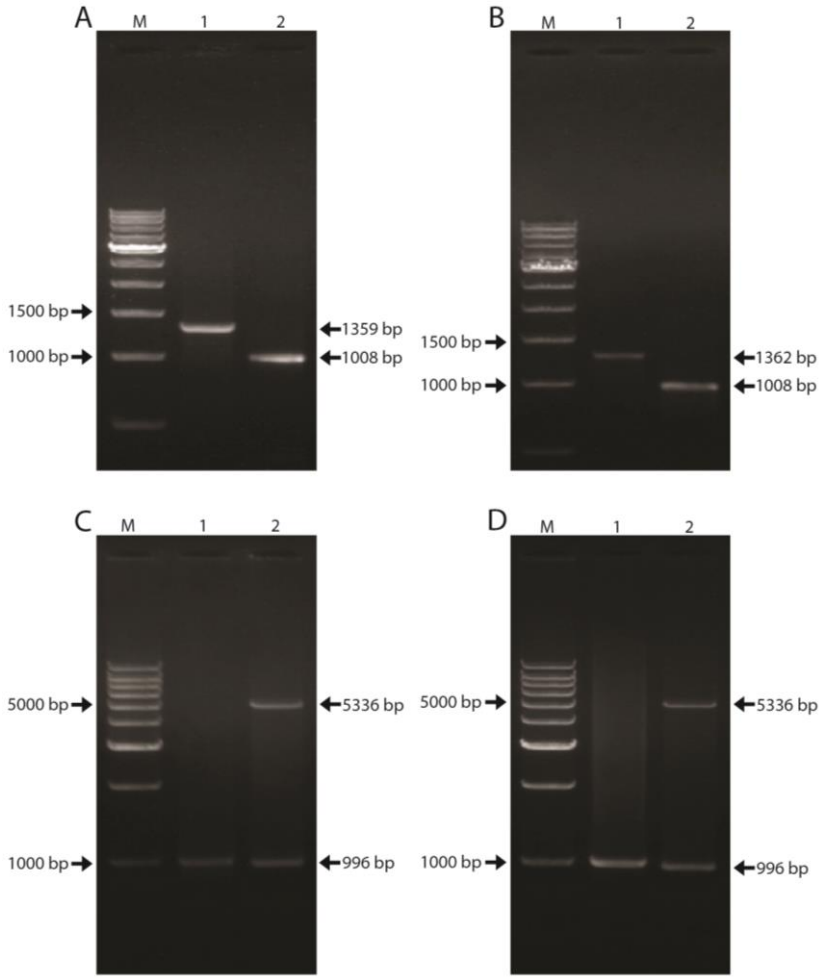


Figure 3.1: Amplification and cloning of *P. falciparum* 3D7 PM I and PM II.

(A) Agarose gel showing amplification of the full-length PM I gene and the segment of the gene coding for the mature enzyme, mPM I. Lane M: molecular size marker; Lane 1: 1359 bp PM I gene PCR product; Lane 2: 1008 bp mPM I PCR product.
(B) Agarose gel showing amplification of the full-length PM II gene and the segment of the gene coding for the mature enzyme, mPM II. Lane M: molecular size marker; Lane 1: 1362 bp PM II gene PCR product; Lane 2: 1008 bp mPM II PCR product.
(C) Agarose gel showing confirmation of the mPM I insert in a representative clone by colony PCR and restriction digestion. Lane M: molecular size marker; Lane 1: 1008 bp mPM I amplified from the clone; Lane 2: 996 bp mPM I insert released from the clone vector backbone of 5336 bp on digestion with NheI and BamHI restriction enzymes.
(D) Agarose gel showing confirmation of mPM II insert in a representative clone by colony PCR and restriction digestion. Lane M: molecular size marker; Lane 1: 1008 bp mPM II amplified from the clone; Lane 2: 996 bp mPM II insert released from the clone vector backbone of 5336 bp on digestion with NheI and BamHI restriction enzymes.

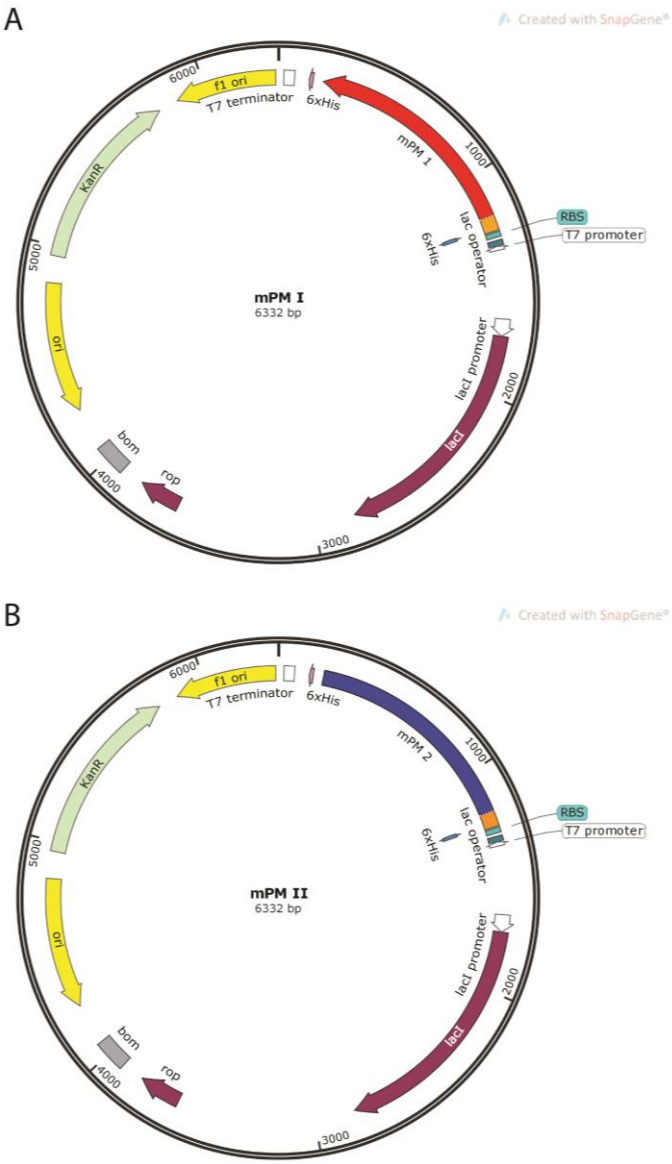


Figure 3.2: Plasmid map of mPM I and mPM II expression vectors.

(A) The segment encoding the mature region of PM I, shown in red was cloned in the NheI and BamHI sites of pET-28a(+) vector.
(B) The segment encoding the mature region of PM II, shown in blue was cloned in the NheI and BamHI sites of pET-28a(+) vector.

In a similar manner, the transformation of mPM II construct (Figure 3.2B) bearing the region coding the mature part of PM II yielded numerous clones, out of which ten randomly selected clones were analyzed. The colony PCR of the transformants resulted in an amplicon length of 1008 bp (Figure 3.1D), the size similar to that of the mature region of PM II i.e. 990 bp. The restriction digestion of the plasmids obtained from the clones with NheI and BamHI released an insert of 996 bp and the size of the vector backbone corresponded to 5336 bp (Figure 3.1D). The colony PCR and restriction digestion results confirmed that the

screened clones had constructs with an insert in the correct orientation. For sequence verification, a clone was selected from the screened set and was named mPM II DH5α. The insert of the construct isolated from the clone was sequenced and BLAST analysis using the sequence generated confirmed the presence of the gene sequence coding for the mature part of PM II in the insert.

3.3.2 Expression of the recombinant enzymes

E. coli BL21(DE3)pLysS was used to express the mature enzymes. The expression system, was chosen considering the pET-28a(+) vector backbone used to prepare the constructs, which uses T7 promoter for gene expression. Leaky expression or expression without induction, associated with these IPTG inducible promoters is a problem while dealing with a protein that can be toxic to the cells, a probable situation when the mature enzymes are possibly active during the expression. This issue was overcome by using *E. coli* BL21(DE3)pLysS strain for expression in which plasmid pLysS produces T7 lysozyme, a natural repressor of T7 RNA polymerase to reduce the basal level expression of the cloned enzymes.

So, the amplified constructs, mPM I and mPM II, in mPM I DH5α and mPM II DH5α respectively were isolated and transformed into *E. coli* BL21(DE3)pLysS competent cells. Out of the various transformants obtained after both the transformations, ten clones from each set were randomly selected and screened for the presence of the inserted DNA in the constructs coding for the mature enzymes by colony PCR, using the respective specific primers pair. After colony PCR, a band of 1008 bp observed in the case of both mPM I and mPM II putative clones, confirmed that the clones were harboring the desired constructs. The expression of the enzymes was performed using 1 clone for each enzyme. So, for recombinant PM I expression the clone chosen was named mPM I pLysS and that for the expression of recombinant PM II was named mPM II pLysS.

Recombinant mature PM I enzyme was expressed in mPM I pLysS under specified expression conditions, as a 6×His-tagged fusion protein of 352 amino acids, in which 329 amino acids belonged to the mature enzyme (Figure 3.3). Similarly, expression of recombinant mature PM II carried out using mPM II pLysS, under given conditions, produced a 6×His-tagged fusion protein of 352 amino acids, where 329 amino acids coded for the mature enzyme (Figure 3.4). The expression of both the proteins was found to be low, so the confirmation of the expression could be achieved after further purification.

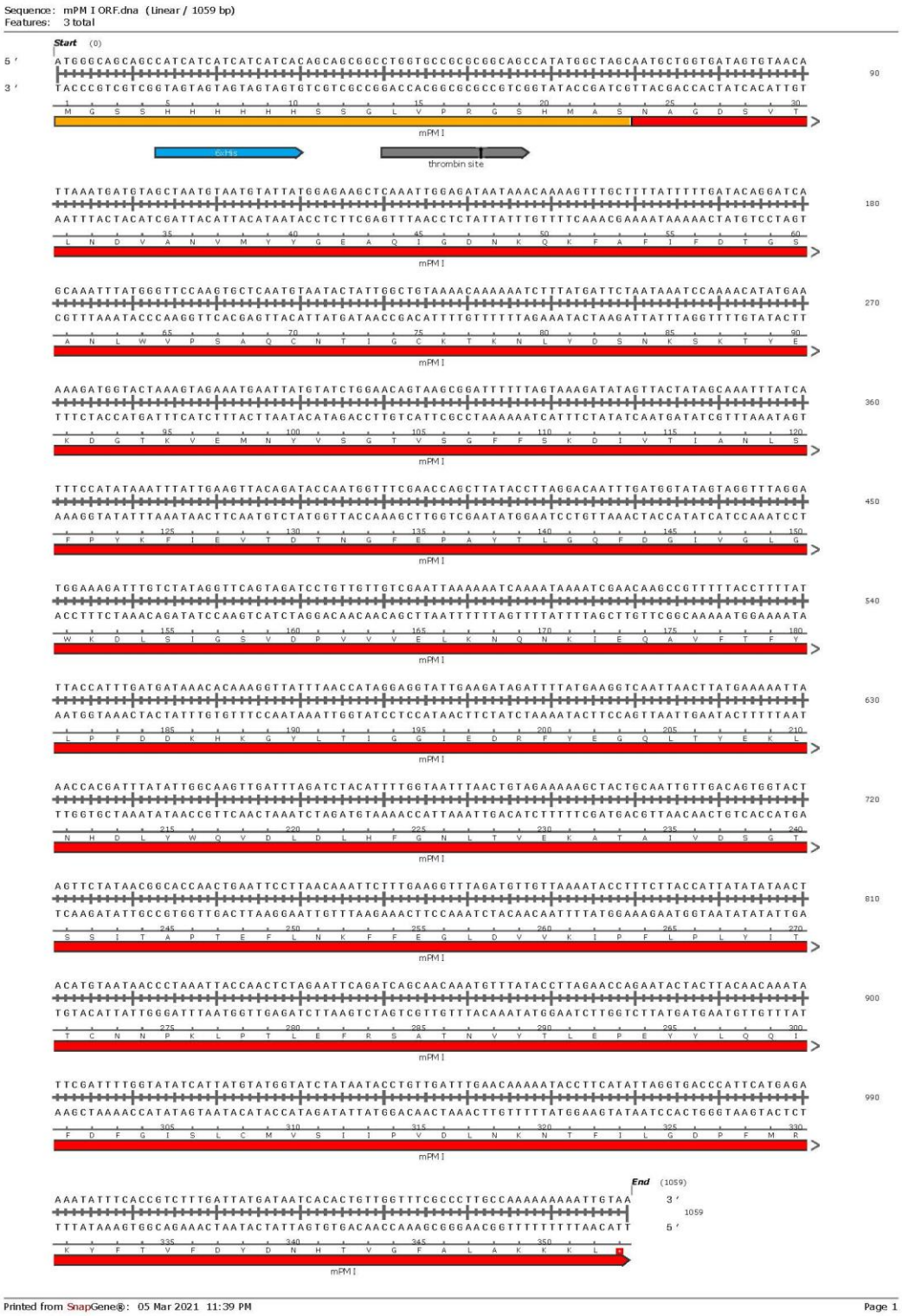


Figure 3.3: Linear map showing key features of mPM I ORF.

The plasmid derived section is shown in yellow and the mature region of PM I is shown in red. The His-tag site and the thrombin cleavage site are depicted by azure blue and grey bars respectively. The arrow in the thrombin cleavage site denotes the cleavage site. The active enzyme mPM I consisting of the amino acid residues of the mature region had the first 6 amino acids (GSHMAS) derived from pET-28a(+) vector.

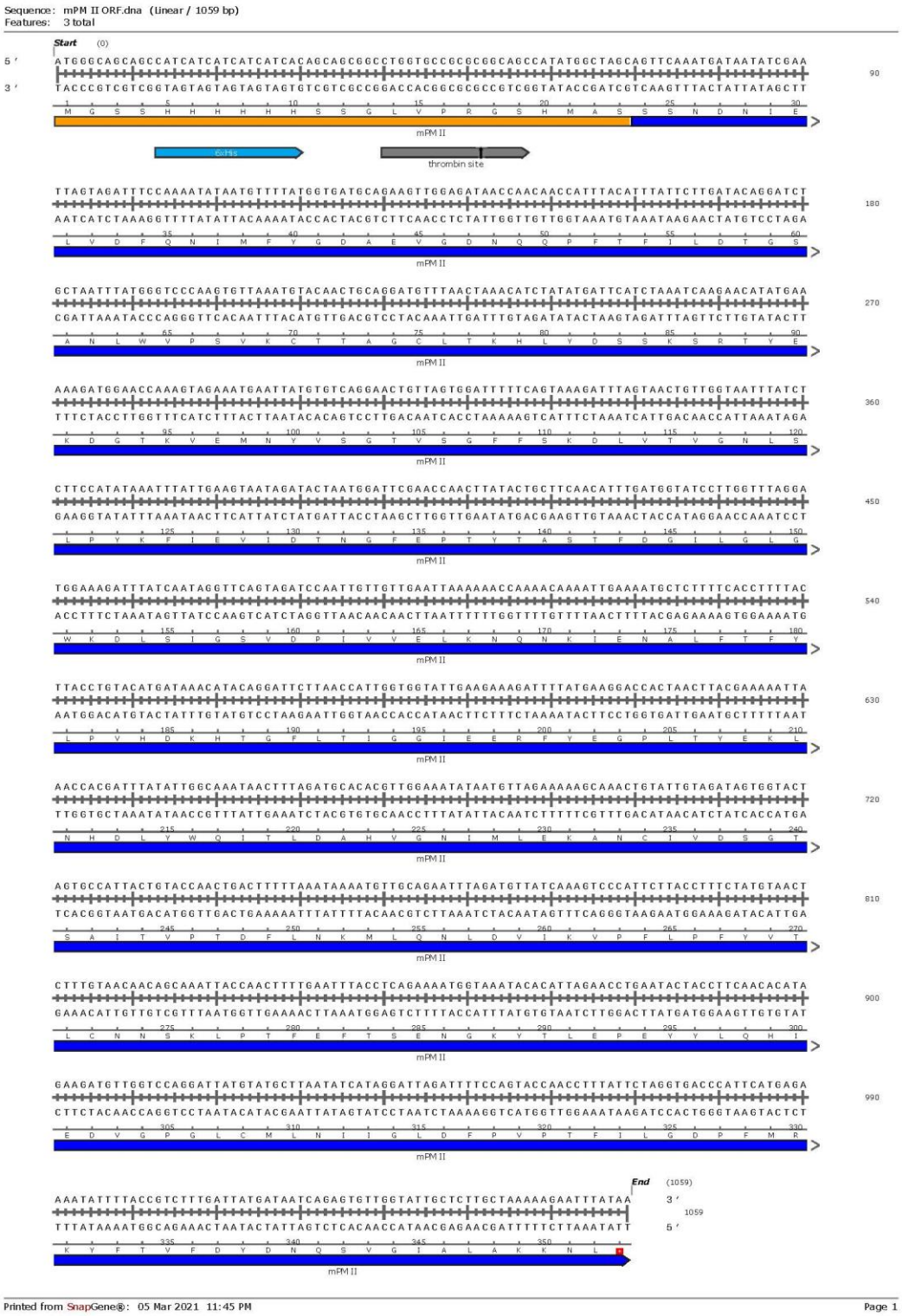


Figure 3.4: Linear map showing key features of mPM II ORF.

The plasmid derived section is shown in yellow and the mature region of PM II is shown in blue. The His-tag site and the thrombin cleavage site are depicted by azure blue and grey bars respectively. The arrow in the thrombin cleavage site denotes the cleavage site. The active enzyme mPM II consisting of the amino acid residues of the mature region had the first 6 amino acids (GSHMAS) derived from pET-28a(+) vector.

The purification of cell lysate supernatant using Ni-affinity chromatography efficiently purified the His-tagged recombinant enzymes, excluding out majority of the protein contaminants in the first step of purification (Figure 3.5A, B). The analysis of the eluted proteins on SDS-PAGE, showed prominent bands below 43 kDa in the case of both mPM I pLysS and mPM II pLysS, which were found to correspond to their molecular weights 39.51 kDa and 39.37 kDa respectively, determined theoretically using their amino acid sequences. Hence, confirmation of the expression of the enzymes was achieved and it was also established that the enzymes were expressed specifically upon induction with IPTG. SDS- PAGE analysis revealed that further purification of the enzyme fractions along with desalting by size exclusion gel filtration chromatography resulted in highly purified mPM I and mPM II enzymes, devoid of non-specific proteins (Figure 3.5C, D). The resulting solutions were concentrated and removal of the His-tag from the proteins was accomplished by the treatment of the enzymes with thrombin. Following thrombin removal and concentration, the resulting solution in the case of both mPM I and mPM II showed prominent bands below 43 kDa which corresponded to theoretically determined molecular weights 37.63 kDa and 37.49 kDa respectively (Figure 3.5C, D), suggesting the presence of mature enzymes in their intact forms. The mature enzymes were then refolded following the described procedure and the enzyme concentrates were subsequently analyzed for the activity by performing hemoglobin degradation assays. Both PM I and PM II perform an initial cleavage between Phe33 and Leu34 of the α-chain of hemoglobin (Gupta et al., 2010). This results in disruption of the native tetramer structure of hemoglobin which is observed as a decrease in absorbance by native hemoglobin in the solution. In the spectrophotometric assay, both the mature enzymes, mPM I and mPM II were able to degrade hemoglobin efficiently (Figure 3.6), which suggested that the refolding of the enzymes into their biologically active conformation was successfully achieved. The activity of mPM I was found to be 1.37 ± 0.06 IU/ml and for mPM II it was calculated as 1.91 ± 0.07 IU/ml, which was significantly higher than that of mPM I ($p < 0.05$).

The molecular weight of the α-chain monomer of human hemoglobin is 15.13 kDa and that of the β-chain monomer is 15.87 kDa. So, when resolved on SDS-PAGE gel the undegraded human hemoglobin is observed as a doublet around 16 kDa. The time-course analysis of hemoglobin degradation showed a decrease in the intensity of the band containing both the hemoglobin monomers over time in the reactions containing either mPM I or mPM II (Figure 3.7A), while the decrease was marginal in the control reactions. This indicated the

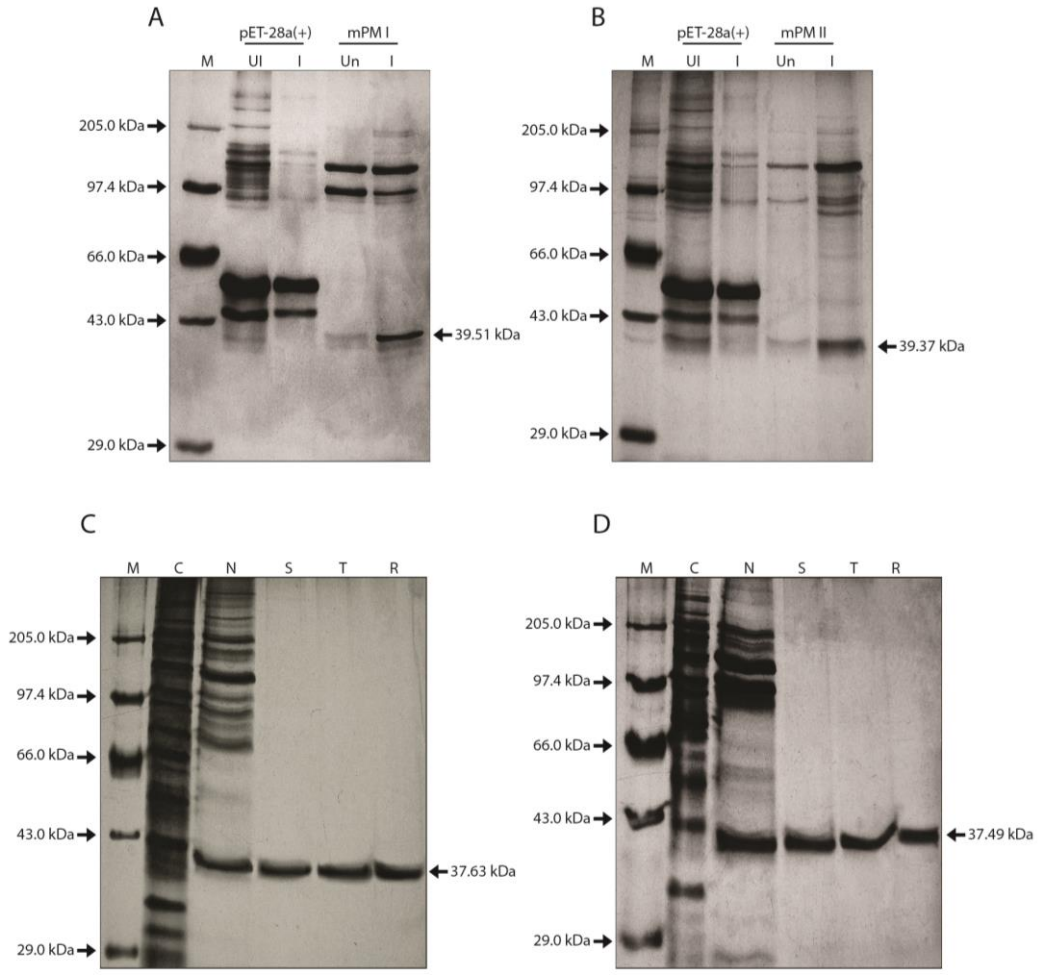


Figure 3.5: Silver stained SDS-PAGE gel analysis showing expression and purification of mPM I and mPM II.

(A) Expression of mPM I observed after Ni- affinity chromatography. Expression from *E. coli* BL21(DE3)pLysS clone transformed with pET-28a(+) vector and mPM I pLysS clone is compared under uninduced and induced conditions. mPM I clone expressed mPM I enzyme with an estimated molecular weight of 39.51 kDa under induced conditions. Lane M: molecular weight marker; Lane UI: uninduced; Lane I: induced with 1mM IPTG at 37°C for 4 h.

(B) Expression of mPM II observed after Ni- affinity chromatography. Expression from *E. coli* BL21(DE3)pLysS clone transformed with pET-28a(+) vector and mPM II pLysS clone is compared under uninduced and induced conditions. mPM II clone expressed mPM II enzyme with an estimated molecular weight of 39.37 kDa under induced conditions. Lane M: molecular weight marker; Lane UI: uninduced; Lane I: induced with 1mM IPTG at 37°C for 4 h.

(C) Sample obtained during purification of mPM I. Lane M: molecular weight marker; Lane C: cell lysate; Lane N: post Ni- affinity chromatography; Lane S: post size exclusion chromatography; Lane T: mPM I post thrombin digestion with an estimated molecular weight of 37.63 kDa; Lane R: post refolding.

(D) Sample obtained during purification of mPM II. Lane M: molecular weight marker; lane C: cell lysate; Lane N: post Ni- affinity chromatography; Lane S: post size exclusion chromatography; Lane T: mPM I post thrombin digestion with an estimated molecular weight of 37.49 kDa; Lane R: post refolding.

Table 3.1: Summary of mPM I and mPM II purification.

Fraction	Average yield (mg)[b]	
	mPM I	mPM II
Cell lysate[a]	12.97	16.46
Ni-affinity chromatography	0.48	0.45
Size exclusion + Ultrafiltration	0.19	0.20
Thrombin digestion + Ultrafiltration	0.15	0.17
Refolding + Ultrafiltration	0.11	0.12

a. From pellet obtained from 1 l cell culture.
b. Protein concentration determined by Lowry assay using BSA as a standard protein.

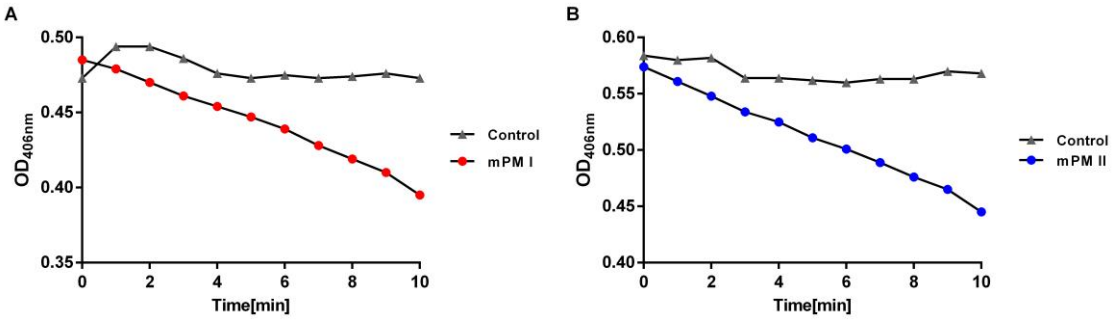


Figure 3.6: Hemoglobin degradation by mPM I and mPM II.

Hemoglobin was incubated without enzyme (Control), with mPM I (A) and with mPM II (B) at pH 5.0 at room temperature and optical density was recorded at 406 nm for 10 min.

action of the enzymes on the α-chain of hemoglobin. In the case of both the enzymes the concentration of the hemoglobin was reduced to approximately half of its initial concentration after 60 min of reaction, suggesting the cleavage of all the α-chain monomers present in the system. The degradation of the hemoglobin by the enzymes observed on SDS-PAGE after loading the reaction systems set for spectrophotometric assay, supported the usage of the spectrophotometric assay for detecting hemoglobin degradation and also confirmed that the enzymes were refolded into their catalytically active forms.

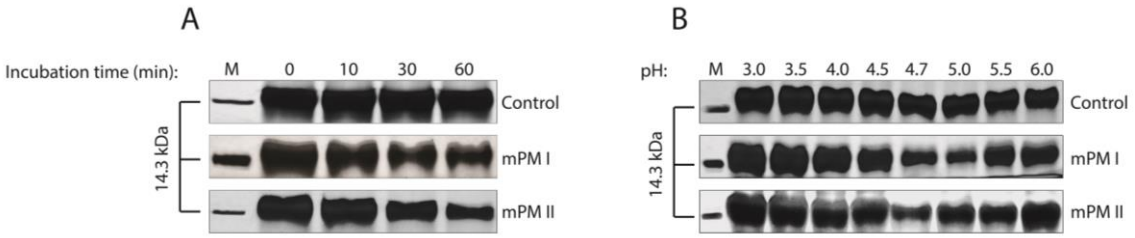


Figure 3.7: Silver stained SDS-PAGE gel showing hemoglobin degradation.

(A) Time course of hemoglobin degradation. Hemoglobin was incubated without enzyme (Control), with mPM I and with mPM II at pH 5.0 at 37°C for 0, 10, 30 and 60 min.
(B) pH optimum analysis for hemoglobin degradation. Hemoglobin was incubated without enzyme (Control), with mPM I and with mPM II at various pH at 37°C for 1 h. Optimum hemoglobin degradation was observed at pH 5.0 with mPM I and pH 4.7 with mPM II.

Previous strategies to produce active PM I included expression of truncated proenzymes after the introduction of mutation into the propart of the zymogen which auto-activated at 12 and 7 residues upstream of the native cleavage site (Moon et al., 1997) and as a thioredoxin fusion which activated at 7 residues upstream of the native cleavage site with yields that were still limited (Xiao et al., 2007). Further, it was found that when refolded from an unfolded form, the mature PMs have the propensity to rapidly acquire misfolded inactive conformation (Xiao et al., 2011). Similarly, in the case of reduced lysozyme, it has been suggested that very early during the folding procedure unproductive transition states intermediates are formed which are trapped into local energy minima, producing aggregates (Sakamoto et al., 2004). To overcome this, the thermal-assisted refolding approach was used, which successfully converted the enzymes into their native forms which were active also. Previously, this approach has been used successfully to increase the refolding yields of reduced lysozyme (Sakamoto et al., 2004). In the current study, the expressed mature enzymes mPM I and mPM II underwent heat induced unfolding which produces the unfolded states that persistently lack a tertiary structure. The formation of misfolded proteins is a common problem associated with refolding carried out at room temperatures. During refolding of the enzymes performed at high temperature, the heat induced molecular fluctuations destabilising the misfolded forms allowed the correct rearrangements of disulfide bonds and also prevented the formation of misfolded forms (Sakamoto et al., 2004).

The prosegment has been suggested to prevent misfolding of the PMs during the transition from a zymogen to a mature enzyme, ultimately enabling them to achieve their active

conformation (Parr-Vasquez and Yada, 2010). Also, it has been hypothesized that prosegment is required by the enzymes to catalyze the folding to practical time scales of seconds (Xiao et al., 2011). It is suggested that once the folding is complete, the prosegment is removed and the native is maintained as a kinetically trapped state, separated from all other denatured states by a large unfolding activation barrier (Xiao et al., 2011). Upon refolding an unfolded mature enzyme there is an equally large folding barrier. It indicates the possibility that the truncated prosegments expressed with the mature parts in earlier efforts were unable to properly catalyze the folding and the expressed recombinant enzyme could not cross the energy barrier and was present predominantly in misfolded states. So, the expression of prosegments may have been another factor in addition to the refolding conditions that governed the formation of active enzymes. However, in the current study, the high temperature used during the refolding procedure provided activation energy to the mature PMs in unfolded state or misfolded states to convert them into their folded native forms.

The yield of the final purified mPM I enzyme capable of hemoglobin degradation was found to be 110 µg/l culture (Table 3.1). A similar yield was obtained for the final purified active mPM II enzyme, which was found to be 120 µg/l culture (Table 3.1). Earlier for the expression of soluble PM I along with truncated prosegment that could be activated to mature form, a comparatively low production yield of 62 µg/l culture was obtained (Xiao et al., 2007). Considering the large amounts of pure PM I and PM II required by the studies including the crystallization experiments the yields obtained in the current work were also scanty. The absence of any effect on the growth of the *E. coli* host following induction and the lack of activity right after expression in the case of both the enzymes mPM I and mPM II, reduces the possibility of the enzymes being toxic towards the host that might have resulted in low expression levels. Further exploration of this matter can certainly lead to improvement in the production yield of the enzymes.

3.3.3 Optimization of the pH

The catalytic activity of the enzymes was assessed at various pH levels (Figure 3.7B). mPM I showed efficient hemoglobin degradation at pH 5.0. At other acidic conditions (3.0-4.7) and (5.5-6.0) the hemoglobin degradation and thus the catalytic activity was found to be low. While mPM II performed its optimum substrate cleavage at pH 4.7, and when tested at either side of this range from pH 3.0-4.5 and pH 5.0-6.0, the cleavage efficiency, hence

the catalytic activity was found to be low. Overall, the optimum pH for both mPM I and mPM II was found to be between pH 4.7-5.0, which is in agreement with the previously reported optimum pH for PMs which was between 4.5-5.0 (Coombs et al., 2001) and to the pH of the food vacuole which is 5.0. In addition, the same optimum pH range has been suggested for the native PM I (Goldberg 1991).

3.3.4 Plasmepsin inhibition studies

The putative antiplasmodial plant extracts demonstrating *Plasmodium* parasite inhibition above 70% showed varying degrees of inhibition of the expressed recombinant PMs, mPM I and mPM II (Figure 3.8). The inhibition of the enzymes above 10% was considered as significant. Of all the extracts tested, the *A. paniculata* EtOH, *C. wightii* AQ, *C. zedoaria* DCM and *P. amarus* DCM extracts significantly inhibited both the enzymes with % inhibition of more than 50%. Thus, these four extracts were selected for further studies. In the case of most of the extracts, no significant difference was observed in the inhibition extents of both the enzymes. Also, the Pearson correlation coefficient r of 0.996, between the % inhibition of mPM I and that of mPM II suggested a strong positive correlation between them.

There is a lacuna in the literature on PM inhibitors of natural or biological origin. Inhibitors found against PMs earlier are chemically synthesized molecules, which are either peptidyl compounds based on statine core found in pepstatin A (Dell'Agli et al., 2006; Gupta et al., 2010) or are nonpeptidyl compounds containing a diphenylurea moiety, which mimics the core statin residue region (Jiang et al., 2001). Three peptidomimetic analogs have been reported to produce potent irreversible inactivation of recombinant PM II with the IC_{50} values in the low nanomolar range (Gupta et al., 2010). But the cost involved in their synthesis is a major drawback. So, in light of the need to find economical and better alternatives, the study was the first of its kind to explore the plants for PM inhibitors. Inhibition of mPM I and mPM II by the four extracts suggests the inhibition of the parasite's PM I and PM II to be one of the mechanisms or the only mechanism of the antiplasmodial action presented by these extracts. The high correlation observed between the inhibition extent of the enzymes by the tested extracts can be backed by the fact that the four food vacuole PMs are redundant (Bhaumik et al., 2011), which implicates that they have similar active sites.

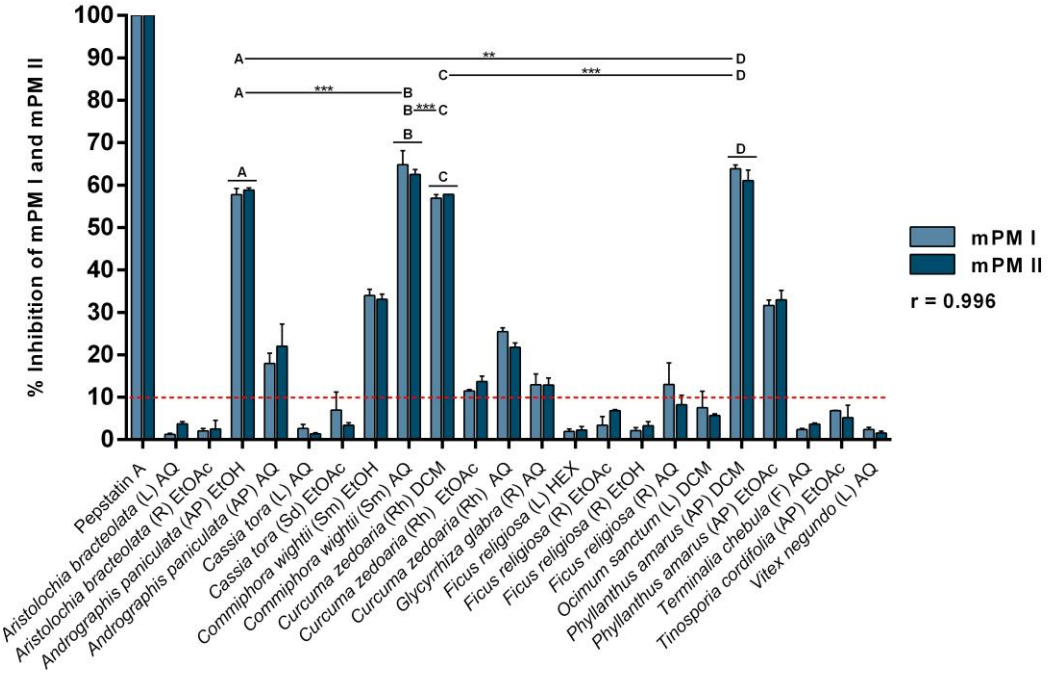


Figure 3.8: Inhibition of mPM I and mPM II activity by plant extracts.

The enzymes mPM I and mPM II were incubated with the positive control pepstatin A and the antiplasmodial plant extracts. Four plant extracts viz., the *A. paniculata* EtOH, *C. wightii* AQ, *C. zedoaria* DCM and *P. amarus* DCM extracts resulted in more than 50% inhibition of activity of both the enzymes. High correlation was observed between the inhibition extent of the enzymes by the extracts. Data are represented as mean ± standard deviation from an experiment performed in triplicate. AP: Aerial parts; F: Fruits; L: Leaves; Rh: Rhizome; R: Roots; Sd: Seeds; Sm: Stem.

In conclusion, the introduction of the thermal-assisted refolding technique in the study allowed the successful conversion of expressed recombinant PMs, mPM I and mPM II into their catalytic active forms which were capable of hemoglobin degradation. Improvement in the expression level of both the enzymes can further increase the overall yields of the active enzymes. The current work introduces an expression system to achieve PM I and PM II in their active forms, which is an approach simpler than those reported earlier and which can certainly be extended to the expression of other PMs. Furthermore, the study demonstrates the PM inhibition activity from the four antiplasmodial plant extracts namely, *A. paniculata* EtOH, *C. wightii* AQ, *C. zedoaria* DCM and *P. amarus* DCM that can be studied ahead to find out the PM inhibitors of natural origin.