Cysteine proteases of hookworm *Necator Americanus* as virulence factors and implications for future drug design: A bioinformatics-based study.

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Abstract:

Human hookworm *Necator Americanus* causes iron deficiency anemia, as the parasite ingests blood from the gastrointestinal tract of its human host. The virulence factors of this blood feeding nematode have not been researched extensively. This bioinformatics based study focuses on the cathepsin B like cysteine proteases (CPs) of the worm, which could have immense pathogenic potential. The role of the individual CPs remain vaguely determined except for CP3 which has been shown to act as globinase in the hemoglobin degradation pathway. In this study, the cysteine proteases were subjected to predictive molecular characterizations viz: probability of extracellular secretion to the interface between pathogen and host, ability for hemoglobin degradation, and/or interaction with host plasma proteins. CP1- CP6, which harbored the active site cysteine were also observed to have N terminal signal peptide for extracellular localization, and were relevantly predicted to be secretory. Amongst these, CP2 and CP3 showed the presence of hemoglobinase motif derived in this study that could be a prerequisite for globin or hemoglobin degradation. Active site correlation of the secretory CPs with human pKal that cleaves high molecular weight kininogen (HMWK) to prevent platelet activation is suggestive of the involvement of hookworm CPs in preventing the formation of blood clots via this pathway. *NA* CP1, CP2, CP3, CP5 and CP6 were predicted to bind heparin, which is the glycosaminoglycan molecule that has been demonstrated to aid the functionality of other cysteine proteases like human cathepsin B and cruzain. Heparin docked onto the *NA* CPs at the C terminal domain, away from the active site, similar to what has been shown for heparin binding to cathepsin B, and cruzain that cleaves HMWK. These observations therefore lead to the hypothesis that the functions of the hookworm CPs, which would probably include blood clot prevention, could be assisted by heparin. This study underscores the potential of synthetic heparin analogs as molecular treatment for hookworm infection, which could have implications for future drug design.

Introduction:

Hookworm infection in humans is a neglected tropical disease that affects over 700 million people worldwide, mostly in the developing countries of the tropical and subtropical regions. (Hotez *et al*, 2004; Hotez *et al*, 2010) *Necator Americanus (NA)* species of hookworm constitutes the majority of these infections (~85%) (Loukas *et al*, 2011).

The clinical manifestation of the disease includes anemia, malnutrition in pregnant women, and cognitive and/or physical development impairment in children (Diemert *et al*, 2008). These helminth blood feeders on reaching maturity can feed up to 9ml of blood per day in an infected individual by attaching themselves to the intestinal mucosa of the host, through cutting plates as in *NA* (Pearson *et al* 2012). Iron deficiency anemia is the direct effect of the hookworm's blood feeding (Kassebaum *et al*, 2014), resulting in other subsidiary consequences of the hookworm disease (Sakti *et al* 1999; Hotez *et al*, 2008).

The infective larval stage (L3) worm penetrates into the host skin from soil (Vetter *et al*, 1977; Vetter *et al*, 1977) and then invades the circulatory system to reach heart and lungs, wherefrom it migrates to alveoli and then to trachea. The parasite eventually reaches gastrointestinal tract as fourth stage larvae (L4) to develop into blood feeding adult stage hookworms (Hotez *et al*, 2004).

An array of diverse enzymes and molecules in *NA*'s biomolecule repertoire facilitate the pathogen's survival in the host for up to seven years or longer during the different stages of its lifecycle (Pearson *et al* 2012). The most important therapeutic targets are the enzymes involved in interaction with host and in nutrient acquisition. These are often found in the excretory-secretory (ES) products of the worm. The ES proteins have been shown to engage in crucial functions like tissue degradation for host invasion (Pearson *et al* 2012, Brooker *et al*, 2004), fibrinogen degradation (Brown *et al*, 1995) for preventing blood clots, hemoglobin degradation (Brown *et al*, 1995) for nutrient acquisition, and evasion of the host immune system (Bungiro *et al*, 2011). The enzymes in the ES potpourri of *NA* are not yet completely characterized. However, Brown *et al*, had reported cysteine and serine proteases from ES products to degrade hemoglobin and fibrinogen, and detected the presence of at least two cysteine proteases operating at different pH optima (Brown *et al*, 1995).

The cysteine proteases (CPs) in *NA* form the ninth most gut-expressed abundant gene family (Ranjit *et al* 2006) These are most similar to *H.Contortus* (a blood feeding ruminant) CPs that dominate (~16%) intestinal transcriptome of the barber pole worm (Jasmer *et al*, 2001), highlighting the importance of pathogenic CPs in host blood degradation. The CPs are synthesized as precursor molecules, where in their folded proenzyme form, a self-inhibitory peptide blocks their mature catalytic domain. The removal of the inhibitory peptide upon

proteolytic action of other peptidases releases the functional active cysteine proteases. The active sites of the *NA*-CPs contain the catalytic triad residues: Cysteine, Histidine and Asparagine. Classification of cysteine proteases relies on the sequence homology spanning the catalytic residues (Sajid *et al*, 2002). CPs of parasitic organisms are divided into clans CA and CD. The clans are further classified into families in which Cathepsin B-like proteases belong to C1. The *NA*-CPs which are cathepsin B-like, therefore belong to the clan CA C1 family of cysteine proteases, which have an amino-terminal domain that is mostly α-helical, and the carboxy-terminal is β sheet dominated. Another clan CA gut-localized and hemoglobin-degrading cathepsin B C1 peptidase - SmCB1 enzyme from *S.mansoni* (Caffrey *et al*, 2004) – is a close structural homologue of the *NA* CPs. The antiparallel β strand rich C-terminal domain shared by these proteases has been implicated to act as hemolysin in lysine-dependent gingipain (Li *et al*, 2010) which is a clan CD cysteine protease having similar mechanisms to clan CA proteases. The entire cascade of hemoglobin degradation in hookworm has not been elucidated. However, certain key enzymes have been shown to participate in the degradation pathway. Aspartic protease *NA*-APR1 acts on hemoglobin, whereas metalloprotease MEP1 and cysteine protease CP3 degrade globin fragments (Ranjit *et al* 2009). While ingestion and digestion of blood, anticoagulant proteins are secreted to prevent clot formation (Stanssens *et al*, 1996; Harrison *et al*, 2002; Furmidge *et al*, 1995). Cysteine proteases from *NA* (Brown *et al*, 1995), and from phylogenetically close *H. contortus* (Cox *et al*, 1990) have been implicated to have anticoagulation properties (Brown *et al*, 1995). *NA* adopts a number of complementary strategies to evade host procoagulation system (Furmidge *et al*, 1995), very few of which have been elucidated so far.

Taken together, cysteine proteases from different pathogenic organisms perform diverse functions pertaining to blood feeding. This study focuses on eight cysteine proteases viz: CP1, CP2, CP3, CP4, CP4b, CP5, CP6, CP7 encoded by *NA* genome; of which CP2, CP3, CP4 and CP5 genes are reportedly expressed in abundance in the gut tissue of the adult worm (Ranjit *et al* 2008). Only CP3 amongst these has been characterized as a globinase (Ranjit *et al* 2009). The expression of the other CP genes in the *NA* gut is suggestive of their involvement in digestive or other assistive functions. Despite *NA*-CPs' importance in the parasite's physiology, they are under-researched and not much is known about the individual proteases and which of these constitute the ES products of *NA*. This bioinformatics based study on the molecular characterization of the CPs probes into those aspects of the proteases, which could have pathogenic potential such as cell invasion, hemoglobin degradation and blood clot prevention. Several bioinformatics methodologies have been applied ranging from sequence-based predictive methods, homology modeling, docking, motif derivation from sequence patterns, and mapping of molecular interactions to elucidate the role of the CPs as possible virulence factors and hence target for therapeutics. The approaches to some of the methods adopted here and some discussions pertaining to the physiology of the hookworm are in the context of

other relevant cysteine proteases. Implications for the usage of heparin-analogs as inhibitors of the worm have been outlined based on docking of heparin in the *NA*-CPs.

Materials and Methods:

1. Sequence alignments and pattern detection:

The following *NA* cysteine protease sequences were retrieved for analyses from Uniprot protein sequence database (The UniProt Consortium, 2015)(Uniprot ID in parentheses): Necpain or CP1 (Q9U938), CP2 (A1YUM4), CP3 (A1YUM5), CP4 (A1YUM6), CP4b (W2TRZ7), CP5 (A1YUM7), CP6 (W2T0C4) and CP7 (W2SQD9) (the organism code part of the ID is omitted for brevity). The alignments were done in ICM (Abagyan *et al*, 1994) by BLOSUM62 scoring matrix, with gap opening penalty of 2.40 and gap extension penalty of 0.15. Patterns of relevance and predicted and/or deciphered sites of functional importance were mapped onto the aligned CP sequences to denote their positions in the protein sequence.

1.1 N-terminal:

The N-terminal pre-sequences of *NA*-CP1 - CP6 were used to derive PRATT (Jonassen *et al*, 1995) patterns within them. The lack of these patterns was looked for in CP7 by ScanProsite (Sigrist *et al*, 2002) to determine CP1-6 specific N-terminal signature, as such signals often holds clue to protein sorting (Blobel, 1980).

1.2 Signal peptide cleavage:

SignalP (Petersen *et al*, 2011) was used to predict cleavage sites in the proteases, where the signal peptide would be cleaved off to generate the proenzymes.

1.3. Subcellular localization:

The *NA*-CP sequences were submitted for subcellular location prediction to TargetP (Emanuelsson *et al*, 2007), iPSORT (Bannai *et al*, 2002), TMHMM (Krogh *et al*, 2001), LocSigDB (King *et al*, 2007), Bacello (Pierleoni *et al*, 2006), Protein Prowler (Bodén *et al*, 2005), Cello (Yu *et al*, 2006) and PrediSi (Hiller *et al*, 2004) webservers to determine which of the proteases would be prone to secretion. While the algorithms for most of these programs take N-terminal signals into account, Bacello predicts localization on the basis of the information contained in the folded protein and LocSigDB is a signature pattern database derived from proteins whose localization has been confirmed by experiments.

1.4 Hemoglobinase motif:

The incompletely elucidated hemoglobin degradation pathway in *NA* describes the role of only CP3 as a globinase, amongst other CPs in the cysteine protease repertoire. In an effort to investigate the involvement of the rest of the *NA* CPs in hemoglobin degradation, cysteine protease sequences from other organisms - known to degrade hemoglobin - were taken along with *NA*-CP3 to derive conserved patterns unique to these proteins. Those organisms, the proteins, and their genbank (Benson *et al*, 2013) accession numbers (in parenthesis) are: *Necator Americanus* CP3 (ABL85237.1), *A. Canine* CP1 (Q11006), *A. Canine* CP2 (AIG62903.1), *S.mansoni* CB1 (3QSD_A), *P.falciparum* falcipain2 (AAK06665.1), *P.falciparum* falcipain 3(KOB61544.1), *S.japonicum* Cathepsin B (P43157.1), *H. Contortus* AC3 (Q25032), *H.Contortus* AC4 (Q25031), *P.Westermani* CP1 (AAF21461.1) and *O. ostertagi* CP1 (P25802.3) and *A. Suum* CP (AAB40605.1) Conserved patterns from the aforementioned proteins were derived in PRATT (Jonassen *et al*, 1995) and the motifs were scanned against some other non-hemoglobin degrading proteins in ScanProsite (Sigrist *et al*, 2002) to pinpoint patterns specific to the hemoglobin degrading enzymes. The organisms' proteins in the non-hemoglobin degrading set were: C. elegans_CPR3 (AAA98789.1), C.elegans_CPR4 (AAA98785.1) and L. major cathepsin B (AAB48119.1). Such patterns specific to the hemoglobin degrading enzymes (when found) were scanned in the rest of the *NA* CP sequences.

1.5 Emulation of human plasma kallekrein activity:

Human plasma kallekrein (pKal), a component of the anticoagulation pathway cleaves high molecular weight kininogen (HMWK) to eventually block platelet activation and degranulation (Da'dara *et al*, 2011; Maurer *et al,* 2011). Emulation of human plasma kallekrein activity has been reported for cruzain - cysteine protease of *T.Cruzi* (Del Nery *et al*, 1997; Lima *et al, 2002*). Such function has been suggested also for SmSP1 - serine protease of *S.mansoni* (Mebius *et al*, 2013), as kallikrein-like activity had been reported for a serine protease from *S.mansoni* (Carvalho *et al*, 1998). The pathogens secreting these proteases traverse host bloodstream and therefore have a survival need to prevent clot formation around them, which explains their HMWK-cleaving activity. As *NA* larvae too traces migratory route through blood capillaries of human hosts, the *NA*-CPs were scrutinized for molecular features, which could carry out such functions as cleaving HMWK. Homology of the *NA*-CPs with human plasma kallekrein's (pKal's) active site was studied with the help of PDB structure 4OGX at 2.4Å resolution, which is a co-crystal of pKal and IgG1 monoclonal antibody Fab DX-2930, where the antibody blocks the active site of pKal (Kenniston *et al*, 2014). The antibody-interacting active site residues from kallekrein were determined in Chimera (Pettersen *et al*, 2004). Fab Chain H residues from Ser25 to His31 close to pKal were selected, and then the residues within 4Å of the selection were listed to figure the antibody-interacting pKal residues. The process was repeated with Fab Chain H for the residue stretch Gly102-Glu108, and for Fab Chain L residues Ser52-Val58. The pKal active site residues thus listed from

Chimera were mapped onto the pKal – *NA*- CP sequence alignment by clustalW (Thompson *et al*, 1994). The alignment was generated in PBIL server (Perriere *et al*, 2003), where the scoring matrix was set to BLOSUM, with gap opening penalty of 2.40 and gap extension penalty of 0.15.

2. Heparin binding:

2.1 Heparin binding domain prediction:

The sequences were subjected to query by ProDom (Bru *et al*, 2005), a protein domain family database derived from Uniprot knowledgebase. The search was carried out for the purpose of finding any domains from other organisms, with known heparin binding functionality.

2.2. Heparin binding docking simulation:

Heparin was included in the study to explore the feasibility of its interaction with the *NA*-CPs, as heparin-like glycosaminoglycan (GAGs) displayed on host proteoglycans (Bartlett *et al,* 2010*)*, are most probably encountered by the worm ES products at the host-pathogen interface.

Lack of experimental three-dimensional structure of the cysteine proteases prompted BLAST search for homology model templates, against Protein Data Bank (PDB) (Berman *et al,* 2000). 3QSD - mature CathepsinB1 enzyme from *Schistosoma Mansoni* - was chosen from the search results for building models as it aligned well at the active site and had a resolution of 1.3 Å. Also, this structure had co-ordinates for the two occluding loop residues near the active site, which have been designated crucial for the exopeptidase activity for this class of cathepsin B like proteases (Illy *et al,* 1997) Homology models were built within the internal co-ordinates mechanics protocol of ICM software (Abagyan *et al,* 1994). The sequence alignment between the template and the model sequence was generated by using BLOSUM62 matrix, with gap opening penalty of 2.40 and gap extension penalty of 0.15. Further, for generating reliable models, the alignment around the active site was edited wherever necessary, according to conservation propensity of residues and for modeling the occluding loop residues. Loops were sampled for the alignment gaps where the template did not have co-ordinates for the model sequence. The loop refinement parameters were used according to the default settings of the procedure. Acceptance ratio during the simulation was 1.25. The *NA*-CP model structures were then built within the full refinement module of the software. The quality of the homology models were checked using PROCHECK (Laskowski *et al*, 1993) which showed 100% of the residues from most of the CPs to lie within the allowed regions of the Ramachandran plot. CP2 and CP5 were the exceptions, which had 99.5% residues in the allowed regions.

The co-ordinates for heparin were taken from its complex deposited in PDB (ID: 5D65) and saved as SDF formatted ligand. The CP homology models were

converted to ICM formatted receptors for docking the heparin molecule. The sequence stretch of the CPs encompassing the predicted fibronectin domain, which can putatively bind heparin like molecules (Pankov *et al,* 2002) was selected for docking the heparin tetrasachharide which had alternating units of N, O6-disulfo-glucosamine (PDB ID: SGN) and 2-O-sulfo-alpha-L-idopyranuronic acid (PDB ID: IDS). The receptor maps were generated with grid step of 0.50. The dockings were performed with a thoroughness level of 3, with the generation of three initial ligand conformations for each simulation.

The heparin-bound *NA* CP models were rendered in electrostatic surface representation by ICM (Abagyan *et al,* 1994), where the potential scale was set to 5.0 along with the assignment of simple charges, for the purpose of viewing the electrostatics of the protein sites occupied by the negatively charged heparin.

Results:

1. Sequence alignments and pattern detection:

1.1 N-terminal:

The *NA*-CP alignment showed that the CP7 lacked the N-terminal signal pre-sequence present in the other proteases (**Figure1**). The PRATT derived motif unique to CP1-6's pre-sequence was M-x(4,5)-L (**Figure 2**). CP7's N-terminal however had a lysosomal targeting pattern [DE]x{3}L[LI], according to LocSigDB. The specific patterns in the pre-sequences are summarized in **Table1**, along with the lysosome targeting peptide in CP7.

1.2 Signal peptide cleavage:

SignalP derived cleavage sites predicted the length and the peptide sequence for the signals contained in the pre-sequences of CP1-6. **Figure 2** shows the positioning of the cleavage sites where the signal peptide would be cleaved off to generate the proenzymes. The lengths of these signal peptides across the *NA* CPs were approximately the same.

1.3 Subcellular localization:

The consensus from the subcellular localization prediction methods deemed CP1-6 to be secretory proteins, with the aforementioned presence of pre-sequences, which signal for the proteases' extracellular localization. CP7 was predicted to be a lysosome directed protease (**Table 1**).

1.4 Hemoglobinase motif:

The hemoglobinase motif Y-[WY]-[IL]-[IV]-x-N-S-W-x-[DEGNQST]-[DGQ]-W-G-E-x(1,2)-G-x-[FI]-[NR]-[FILM]-x(2)-[DG]-x-[DGNS] was derived from the hemoglobin degrading cysteine proteases of the following blood feeders viz: *NA* (CP3), *A. Canine*, *S.mansoni*, *S.japonicum*, *H. Contortus*, *O. ostertagi* and *A. Suum*. The

pattern detected here is longer than the previously reported Y-W-[IL]-[IV]–A-N-SW-X–X–D-W-G-E motif by Baig *et al*, (Baig *et al*, 2002). See comparison in **Figure 3**. The training data set for deriving this new motif additionally included falcipain2 and falcipain3 of *P.falciparum* and CP1 of *P.westermani* and the derived motif was absent in the cysteine proteases of the non-blood feeders viz: *C. elegans* and *L. major*. The observations from this study are similar to the previous study in the context of the presence of the motif in blood feeding proteases and its absence in the non-blood feeding proteases. The derived motif when searched in the *NA*-CPs (excluding CP3 of the training dataset) was detected only in CP2.

1.5 Emulation of human plasma kallekrein activity:

Scanning of the *NA*-CPs for their possible emulation of human pKal's function of cleaving HMWK to prevent platelet activation and degranulation was motivated by such cleaving functions reported for cruzain from *T.Cruzi* which survive in host bloodstream as trypomastigotes (Del Nery *et al*, 1997; Lima *et al, 2002*). The scan or sequence homology search was additionally prompted by mouse pKal-homologous SmSP1 from *S.mansoni* which is an obligatory blood fluke and whose serine protease had been reported to exhibit kallikrein-like activity (Carvalho et al, 1998). SmSP1 therefore has been proposed to perform HMWK cleaving functions to prevent blood clots for the survival of *S.mansoni* in blood (Mebius et al, 2013). In case of bloodstream navigating *NA* (larvae stage), though there was minimal identity/similarity between its secretory CPs and human pKal, the structures shared similar domains with antiparallel β strands (**Figure 4**). The overall sequence identity between pKal and CP1-6 were within 15.12% to 19.37%, and the sequence similarity ranged from 20.70% to 24.56%. The antibody-blocked pKal active site corresponded with the Cysteine and Asparagine residues of the *NA* CP catalytic triad in the sequence alignment (**Figure 5A**, **5C**). Conservation with the CPs was noted along the LCG (**Figure 5A**) and WGEG (**Figure 5C**) sequence stretches of the pKal active site.

2. Heparin binding:

2.1 Heparin binding domain prediction:

CP2 and CP5 sequences were predicted by ProDom to have type III fibronectin domain, which is capable of binding glycosaminoglycan such as heparin or heparan sulfate of extracellular proteoglycans (Pankov *et al*, 2002). With CP2 as reference, the fibronectin domain region in the rest of the secretory CPs showed sequence identity within 52.72% to 60.37% and the sequence similarity ranged from 55.21% to 60.26%. The high sequence identity and similarity implied that all the CPs would be predisposed to heparin binding, and so the entire stretch – dominated by antiparallel β strands and loops at the C-terminal (**Figure 4**) – encompassing the predicted fibronectin domain (**Figure 6**) was considered for docking heparin.

2.2. Heparin binding docking simulation:

Heparin on being docked at the putative *heparin-binding* fibronectin domain of the cathepsin B-like CPs showed the best scored conformation to bind surface loops away from the enzymatic cleft (**Figure 7**), at a site similar to what has been reported in an earlier docking/MD simulation study on human cathepsin B - heparin interaction (*Costa et al, 2010*). The crucial interactions made there by the human cathepsin B basic residues K154 and R235 for binding the negatively charged heparin, mapped close to this study's heparin-interacting K and R residues in most of the *NA*-CPs (derived from cathepsin B - *NA* CP: alignment not shown). This is the closest comparison, which could be drawn, with no structures of cathepsin B – heparin complex available in PDB (**Figure 6**). The binding site residues within 4Å of the ligand are underscored in the sequence alignment (**Figure 6**). Barring CP6, heparin occupied similar region in all the CP homology models. The positioning of heparin was slightly shifted in CP6, albeit away from the active site (**Figure 7**). The overall electrostatics at the heparin-bound sites of the CPs varied from predominantly neutral to positive (except CP2, CP4 and CP4b), depending on the site-residues and long-range electrostatic effects from residues beyond. The negative sulfate groups in the glycosaminoglycan molecule mostly interacted with basic/neutral residues at the binding site. Some of the heparin-contacts of the CPs showed the patterns: BBX, XBBX, BXB, and BXXBB, which were entirely or partially in conformity with previously reported heparin-binding motifs (Forster *et al,* 2006; Proudfoot *et al,* 2001*;* Mann *et al,* 1994*;* Fromm *et al,* 1997), where B is a basic residue and X is any amino acid. The highly negative binding-site electrostatics of CP4 and CP4b contributed to unfavorable docking scores for these proteases. The rest of the CPs showed scores for good binding of heparin. **Table 2** summarizes the scores, contact residues, sequence patterns, and H-bonding interactions of the highest scored conformations of heparin.

Discussion:

NA infection and survival in human hosts requires a repertoire of proteolytic enzymes. The parasite's physiology involves cysteine proteases for digestive purposes and evading potentially damaging host hemostatic events, only some of which have been characterized – that this study attempts to decode.

1. Sequence alignments and pattern detection:

1.1 N-terminal:

CP1-6 proteins' hydrophobic N-terminal pre-sequence are presumably signal peptides for extracellular localization. N-terminal signals are extremely degenerate across various proteins. The conserved hydrophobic M-x(4,5)-L sequence pattern in the *NA*-CPs therefore forms part of the unique signal peptide for these proteins.

1.2 Signal peptide cleavage:

CP7, which lacked the N-terminal pre-sequence, was not predicted to have any signal peptide cleavage site. The observation implies and re-emphasizes that this protease gets synthesized without the signal for extracellular localization, unlike the other CPs.

1.3 Subcellular localization:

CP1-6 are secreted out as per the subcellular localization predictions from this study, suggesting their presence in the ES products for host-pathogen interactions. CP3, amongst these, has been implicated to be present in the gut of an adult worm (Ranjit *et al*, 2008) and has been shown to be involved in the hemoglobin degradation pathway (Ranjit *et al* 2009). Therefore, CP3 being predicted to be secretory; CP1-6 - as per their predicted localization - could be expected to be in the ES products of *NA* for similar or other supportive functions pertaining to blood feeding. Whereas, CP7 that lacks the active site cysteine residue is predicted to reside in lysosome for unknown purposes.

1.4 Hemoglobinase motif:

The cysteine proteases from the *NA* ES products of the parasite had been demonstrated to cleave hemoglobin (Brown *et al*, 1995). However, only CP3 in the worm has been characterized to have such role. The molecular features pertaining to such function in *NA* CPs has not been researched. Hemoglobin degrading activity in Cathepsin B like proteases from blood feeding helminths were attributed to Y-W-[IL]-[IV]–A-N-SW-X–X–D-W-G-E sequence motif by Baig *et al*. In this study, a specific sequence pattern generic to hemoglobin degrading enzymes was sought, without emphasis on a particular family of cysteine proteases. This study therefore included hemoglobin-cleaving non-cathepsin B enzymes from *P.falciparum* and *P.Westermani*, which were not taken into account by Baig *et al*. Despite adopting a different methodology (mentioned in materials and methods) from the previous study for deriving the motif, a pattern unique to the blood degrading enzymes emerged. The hemoglobinase motif Y-[WY]-[IL]-[IV]-x-N-S-W-x-[DEGNQST]-[DGQ]-W-G-E-x(1,2)-G-x-[FI]-[NR]-[FILM]-x(2)-[DG]-x-[DGNS] which is being reported here is a longer pattern and working with such motif is advantageous in terms of avoiding false positives. The derived motif could be investigated in different enzymes across blood feeding pathogens. CP2 was the only protein in the repertoire of the *NA* cysteine proteases to have the motif, other than the already established globinase CP3. This observation is suggestive of CP2's involvement in hemoglobin degradation, along with CP3.

1.5 Emulation of human plasma kallekrein activity:

Human plasma Kallekrein (pKal) is a serine protease that cleaves high molecular weight kininogen (HMWK) to produce bradykinin (Da'dara *et al*, 2011; Maurer *et al*, 2011). This molecule stimulates prostacyclin (PGI_2) release from endothelial cells, and PGI_2 in turn is an inhibitor of platelet activation and degranulation pathway (Maurer *et al*, 2011). Cruzain from *T.Cruzi* has been shown, and

SmSP1 from *S. mansoni* has been proposed, to play roles in the production of bradykinin by acting on HMWK, the way plasma Kallekrein does (Del Nery *et al*, 1997; Lima *et al,* 2002, Mebius *et al*, 2013), and thereby evading host's primary hemostasis. The cysteine proteases of *NA,* could possibly mimic the active site of pKal, for the purpose of blood thinning to survive in the bloodstream like *T.Cruzi* and *S.mansoni*. The HMWK degradation function could be especially possible with the active sites of pKal and the *NA* CPs corresponding in alignment, encompassing the Asparagine and Cysteine residues of the catalytic triad of the CPs (**Figure 5A**, **5C**). Blood coagulation prevention by thwarting primary hemostasis via this pathway could be employed by cysteine proteases of *NA* larvae, which migrate through blood circulation.

2. Heparin binding:

2.1 Heparin binding domain prediction:

The β sheet and loop dominated C-terminal domains of the *NA*-CPs encompassing the predicted segments of fibronectin domain; structurally resembled the C-terminal domains of cruzain and human cathepsin B (**Figure 4**), which are known to bind heparin (Lima *et al, 2002,* Almeida *et al* 2001, Costa *et al*, 2010). The negatively charged heparin occupied neutral-positive electrostatics patches in most of the different *NA* CPs (**Figure 7**) at about the same location as the low-energy heparin-docked region of human cathepsin B (Costa *et al*, 2010). Similar results were obtained in this study despite adopting a different methodology of pattern-based prediction method to specify the region for docking heparin, as compared to the Costa *et al* study, where they relied on protein patches having positive electrostatic potential. Heparin bound to the *NA*-CPs, away from the active site, making the K and R contacts as made by human cathepsin B's K154 and R235 (**Figure 6**).

2. 2 Heparin binding docking simulation:

Heparin-like glycosaminoglycan present in animal plasma membrane and ECM, form components of the host-pathogen interface. The GAGs serve as recognition factors for molecular interactions, controlling activities like cell adhesion and parasitic infection (Bartlett *et al,* 2010*;* Lima *et al,* 2002*;* Judice *et al,* 2013*).* These negatively charged molecules are covalently attached to syndecans and glypicans proteoglycans of host cells, harboring important binding sites for parasitic cysteine proteases (Bartlett *et al,* 2010*).* The simultaneous binding of these proteases with GAG and their protein substrate results in the formation of ternary complexes (Lima *et al,* 2002*;* Judice *et al,* 2013), which facilitates the enzymatic action of the proteases. Soluble proteoglycans or GAG chains released upon proteolytic cleavage of ECM or cell surface components can function in similar ways as their immobilized counterpart (Bartlett *et al,* 2010). Furthermore, this type of heparin binding modulates the activity of the cysteine proteases, specifically cathepsin B - which otherwise tends towards alkaline pH

induced inactivation. (Almeida *et al* 2001*,* Costa *et al,* 2010). The implications of heparin binding to *NA* CP1, CP2, CP3, CP5 and CP6, as indicated by the docking scores, (**Table 2**) are discussed in the light of heparin binding to other cysteine proteases as follows.

2.2.1 Modulation of catalytic activity:

Human Cathepsin B bound by heparin/heparin-sulfate does not undergo alkaline pH induced loss of catalytic activity. Inactivation of cathepsin B under alkaline conditions occurs due to disruption of electrostatic interactions like the thiolate-imidazolium ion pair of the active site, and breakage of crucial salt bridge interactions. Heparin binding prevents the loss of such interactions and helps stabilize the protease's structure at alkaline pH by maintaining the helical content of the active enzyme (Almeida *et al* 2001). This allosteric mechanism mediated by heparin's binding away from the active site has been corroborated by computational studies (Costa *et al,* 2010*). NA*-CPs being cathepsin B-like are likely to be affected by heparin binding in the same way. The molecule docked on to the *NA*-CPs, at a region similar to what has been reported by Costa *et al* in their cathepsin B–heparin dockings (**Figure 6**). This is suggestive of possible allosteric control that could be exercised by heparin over the enzymatic action of the *NA*-CPs to make them functional even at alkaline pH. Such controls could be crucial for the survival of hookworm, which encounters different pH conditions within the host, and the cysteine proteases of which of have varying degrees of overall surface electrostatics (**Figure 7**).

2.2.2 RBC lysis and hemoglobin degradation:

Heparan sulfate proteoglycans have been identified on the cell surfaces of Red Blood Cells (Vogt *et al*, 2004). These possibly serve as receptors for parasitic cysteine proteases like Lysine-dependent gingipain with antiparallel β strand rich domain that has been implicated to be a hemolysin (Li *et al,* 2010). *NA* hookworm CPs which share the antiparallel β sheet fold and many of which are believed to be present in the gut of the adult worm to assist blood feeding (Ranjit *et al,* 2008), could be utilizing such proteoglycans as co-receptors (by forming ternary complexes with heparin) to gain access to the red blood cells for hemoglobin degrading purposes.

2.2.3 Activation of anticoagulation pathway:

2.2.3.1 Kininogenonase activity:

Parasitic cysteine protease cruzain from *T.Cruzi* (which traverses capillary vessels and bloodstream in human hosts as trypomastigotes) is known to activate the kinin pathway to produce Lys-bradykinin (Del Nery *et al*, 1997; Lima *et al,* 2002), which is a potent vasodilator (Carvalho *et al,* 1998) and is also capable of stimulating prostacyclin (PGI_2) production from endothelial cells (Maurer *et al,* 2011). PGI_2, which is another vasodilator is also an inhibitor of

platelet activation and degranulation pathway and in effect could trigger the anticoagulation pathway (Mebius *et al,* 2013). Cruzain with its cathepsin B-like substrate specificity has been implicated in the cleavage of high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK), via its carboxyl-terminal domain, to produce Lys-bradykinin (Del Nery *et al,* 1997*)*. Heparin induces the kininogenasic activity in cruzain by forming a complex with kininogen and cruzipain, for assisting the proteolytic cleavage of kininogen to release Lys-bradykinin (Lima *et al,* 2002). The docking results from this study suggest high likelihood of heparin binding to most of the cathepsin B-like *NA* CPs, which in turn can bind kininogen as its substrate to form ternary complex. The implications of such interactions underscore hookworm CPs role in possible kininogen cleaving activity for generating vasodilators to aid hookworm larvae's migration through small blood vessels.

2.2.3.2 Antithrombin activity:

The disruption in flow (stasis) of the ingested blood at the adult hookworm's gut would tend to trigger coagulation (Lowe 2003*;* Bagot *et al,* 2008). Inhibiting such coagulation would be a prerequisite for blood degradation. Heparin is known to serve as co-factors to antithrombin - an inhibitor of thrombin and other coagulation factors in the blood plasma. The molecule by increasing the thrombin binding efficiency of antithrombin by 2,000 to 10,000 fold (Beck *et al,* 1985) becomes extremely effective in preventing blood clots. Soluble free heparin-like GAG chains could be possibly released by *NA* CPs from the proteolytic cleavage of host proteoglycans. The free heparin chains thus released could bind plasma antithrombin to enhance anticoagulation activity of hookworm.

2.2.4 Cell invasion and migration:

Fibronectin is a ubiquitous protein of ECM that forms the scaffolding material for maintaining tissue organization and composition (To *et al,* 2011). Parasitic cysteine protease B secreted from *Leishmania sp* has been shown to degrade ECM fibronectin (Kulkarni *et al,* 2008), aided by heparin (Judice *et al,* 2013) Similarly, heparin-bound cysteine proteases of *NA* can potentially participate in fibronectin degradation, where the ECM proteoglycans could possibly facilitate the proteases' interaction with fibronectin. Though aspartic proteases have been identified as the key players in fibronectin degradation for skin penetration (Brown *et al,* 1999), the implication of parasitic cysteine protease in local migration of *Leishmania* (Kulkarni *et al,* 2008*)*, leaves the scope for hypothesizing that heparin-bound *NA*-CPs could degrade fibronection possibly for tissue invasion (along with their reported fibrinogenolytic activity, possibly for blood clot degradation), during the migratory course of the hookworm larvae from heart-lungs-gastrointestinal tract (Pearson *et al,* 2012).

2.2.5 Therapy:

NA-CPs' suggestive role in RBC lysis, hemoglobin degradation, anticoagulation and cell invasion/migration - all aided by heparin-like molecules as mechanistically described before, probably requires abolishment of GAG binding

to the *NA*-CPs for preventing the proteases' pathogenic activities. Blocking the putative GAG binding site on the parasite's CPs, (see residues in **Table 2**) could help thwart hookworm infection. Soluble heparin/heparin sulfate usage as competitive inhibitors have the chances of causing adverse physiological effects due to their anticoagulant/immunogenic properties (Bartlett *et al,* 2010). However, synthetic GAG mimetics with limited biological activity (minimum active structure) or polysaccharides (Vann *et al*, 1981*;* Copeland *et al,* 2008) targeted at GAG binding site, could be used for inhibiting hookworm infection in conjunction with active-site inhibitors.

This study was initiated to explore the role of *NA* CPs in human hookworm pathogenesis*,* as the proteases remain uncharacterized despite their demonstrated pathogenic potential (Brown *et al*, 1995). Bioinformatics based analyses of the *NA*-CPs is indicative of the presence of CP1, CP2, CP3, CP4, CP4b, CP5 and CP6 in the ES content of the hookworm, localized strategically for important host-pathogen interactions*.* The hemoglobinase motif derived here is harbored by CP2 and CP3, which suggests hemoglobinase activity for these two proteins, of which only CP3 has been confirmed to degrade globin (Ranjit *et al*, 2009). The *NA* CPs' active site similarity with pKal, presence of fibronectin domain signature and capability to bind heparin hint towards the CPs' role in evading the host hemostatic system for preventing blood clots in order to facilitate feeding and survival. The heparin docking results from this study points towards *NA* CPs' heparin-assisted engagement in HMWK-cleaving activity for blood thinning. Such function, verified for the proteases of other parasites like *T.Cruzi* (Del Nery *et al*, 1997; Lima *et al,* 2002) and *S.mansoni* (Carvalho *et al*, 1998) that navigate blood capillaries, calls for the hypothesis of similar survival strategy adoption by larvae-stage *NA* for migrating through blood capillaries. The predicted extracellular localizations of CP1-CP6 and their predicted pathogenic roles render these CPs to be possibly multi-targeted for heparin-analog binding, which could inhibit hookworm infection. This study attempts to provide molecular level information based on computational predictions, decoding previously unreported possible functions of the *NA* cysteine proteases, which could be subjected to further experimental verification.

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NA Cysteine Protease	Localization	Signal motif	Motif specific to proteas
CP1	Secretory	M-x(4,5)-L	MLLFLTL
CP2	Secretory	M-x(4,5)-L	MLTLAAL
CP3	Secretory	M-x(4,5)-L	LILIAL
CP4	Secretory	M-x(4,5)-L	MKANFAL
CP4b	Secretory	M-x(4,5)-L	MKANIAL
CP5	Secretory	M-x(4,5)-L	MITIITL
CP6	Secretory	M-x(4,5)-L	MLITLAL
CP7	Non-secretory (Lysosomal)	[DE]x{3}L[LI]	DDKDLL

Table1: The subcellular localization of the *NA* cysteine proteases, and the motifs pertaining to the signals for the localization.

NA-CP	Score	Contact Residues	Motif pattern	H-bonding residues
CP1	-21.75	**K270, R271, G272**, I273, Y274, K275, K277, D295, N296, T298, Y300, E313, R318	BBX	R271, K275, N296, R318
CP2	-24.22	Y276, Y277, K278, N279, G280, I281, Y282, M283, E303, N304, V306, R326.		Y276, K278, N279, M283, E303, N304, R326
CP3	-20.33	D266, F269, Y270, E271, K272, G273, V274, Y275, K297, V298, N299, G300, T301, L303		E271, K272, V274, N299, G300
CP4	-3.809	**H268, Y269, K270**, E271, G272, I273, Y274, K275, T277, Y278.	BXB	K270, I273, K275, T277
CP4b	-8.224	E90, R91, R95, K270, E271, G272, G293, T294, E295, N296, G297, Y300, L302, Y309, G312, E313, N314, G315, T316, R318		R91, E271, T294, E295, N296, G297, Y300, E313, N314
CP5	-15.4	**Y272, K273, K274, G275,** V276, Y277, V278, Q298, D299, L301, Y303, L305, G315, D316, E317, R321	XBBX	K273, K274, Q298
CP6	-27.69	F256, Y258, V270, Q271, **K272, A273, G274, K275, R276**, T318, N319, N320, C321, S322, E325	BXXBB	Q271, G274, R276, N319, N320, S322

Table 2: Docking scores, contact residues, sequence motif patterns, and H-bonding interactions for the highest scored conformations of heparin molecule docked onto *NA* cysteine proteases.

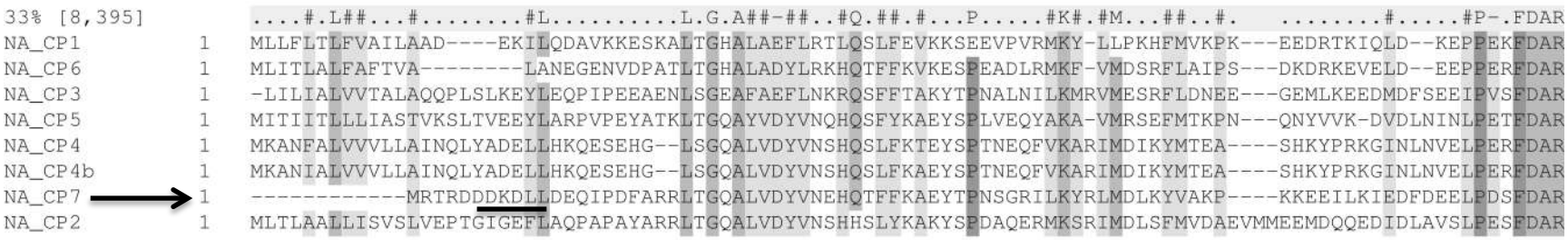

Figure 1: N-terminal alignment of the *NA* CP sequences showing the lack the signal sequence in CP7 (denoted by arrow). The predicted lysosome targeting sequence for the protease is underscored.

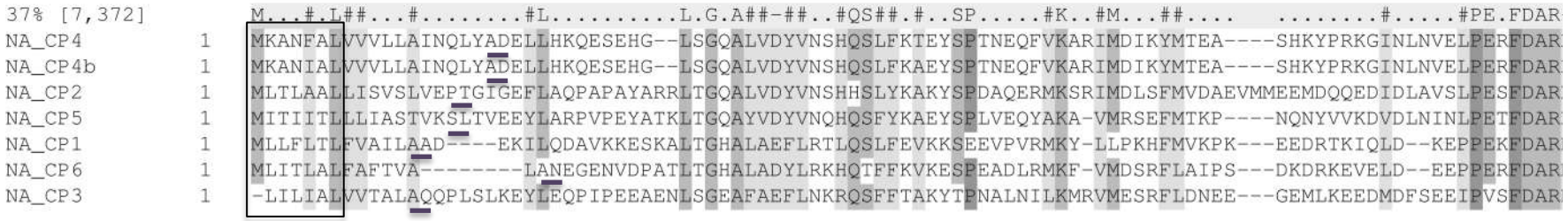

Figure 2: N-terminal alignment of the *NA* CP1-CP6 sequences. The unique pattern M-x(4,5)-L derived from these pre-sequences is boxed. The residues encompassing the cleavage sites for each of the proteases are underscored.

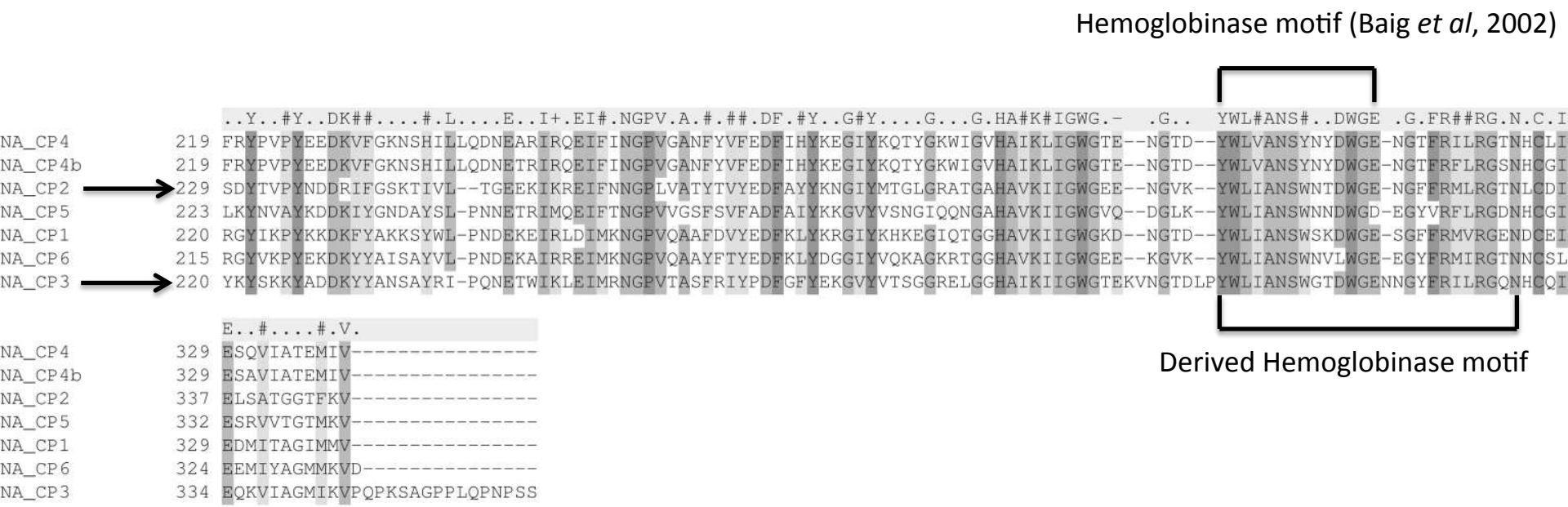

Figure 3: Partial sequence alignment of the *NA*-CPs mapping the region of the derived longer hemoglobinase motif Y-[WY]-[IL]-[IV]-x-N-S-W-x-[DEGNQST]-[DGQ]-W-G-E-x(1,2)-G-x-[FI]-[NR]-[FILM]-x(2)-[DG]-x-[DGNS], as compared to previously determined hemoglobinase motif by Baig *et al*. *NA* CP2 and CP3 which show the presence of the motif are denoted by arrows.

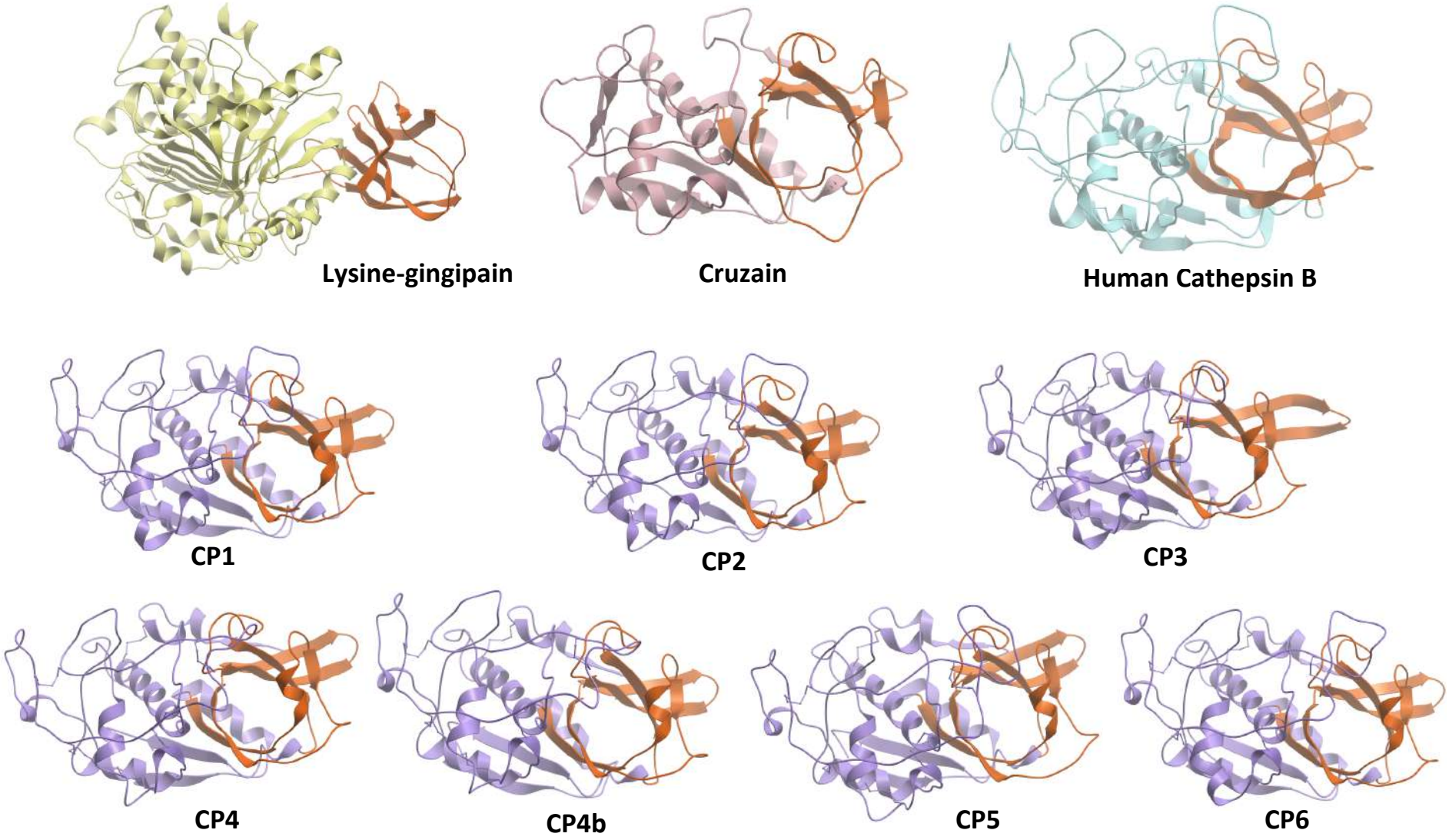

Figure 4: The C-terminal domains (orange) of hemolysis-causing Lysine-gingipain (PDB ID:4RBM), HMWK-cleaving Cruzain (PDB ID: 2OZ2), and human Cathepsin B (PDB ID: 1HUC) compared to: structurally similar C-terminal domains (orange) of the *NA*-CPs dominated by antiparallel beta strands and loops, and harboring the enzymatic residues **H** (Histidine) and **N** (Asparagine). The outer fringes of the *NA* CPs' C-terminal domains (loop regions) make contacts with the docked heparin molecule.

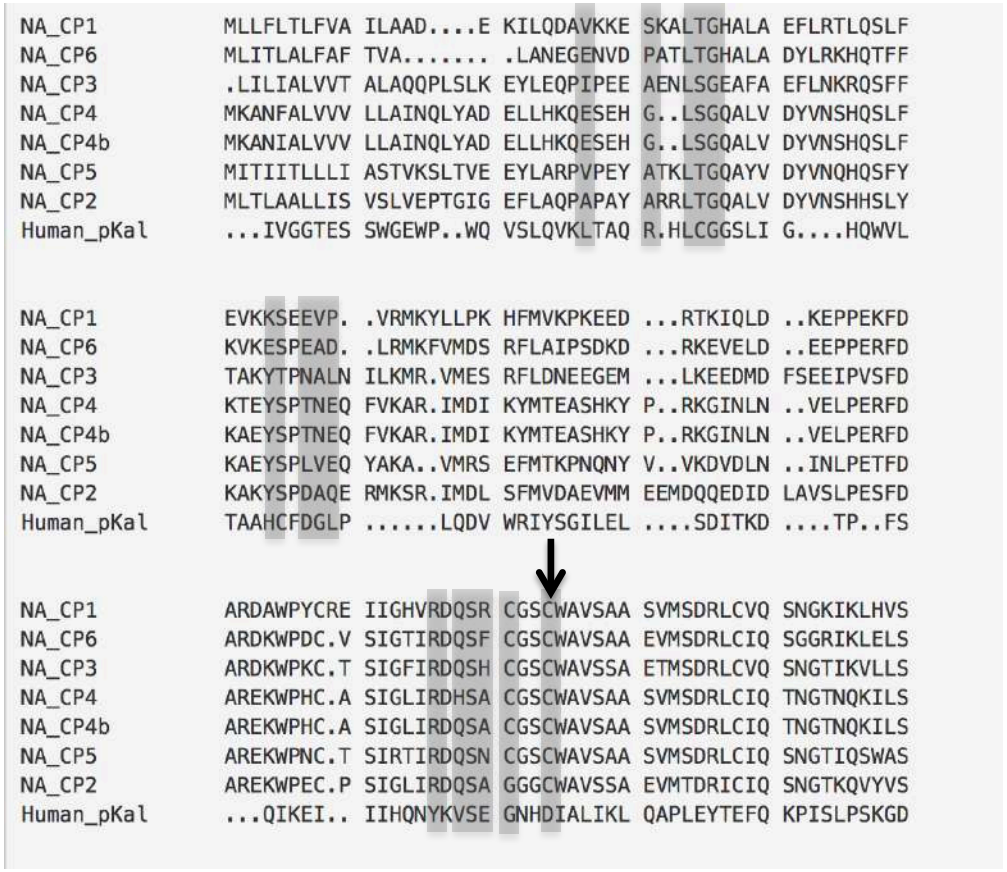

Figure 5A: Sequence alignment of human pKal with the *NA* cysteine proteases, where catalytic triad **C** (Cysteine) of the hookworm protease is shown by arrow. The active site residues of the pKal and the corresponding residues of *NA*-CPs in the alignment are shaded in grey.

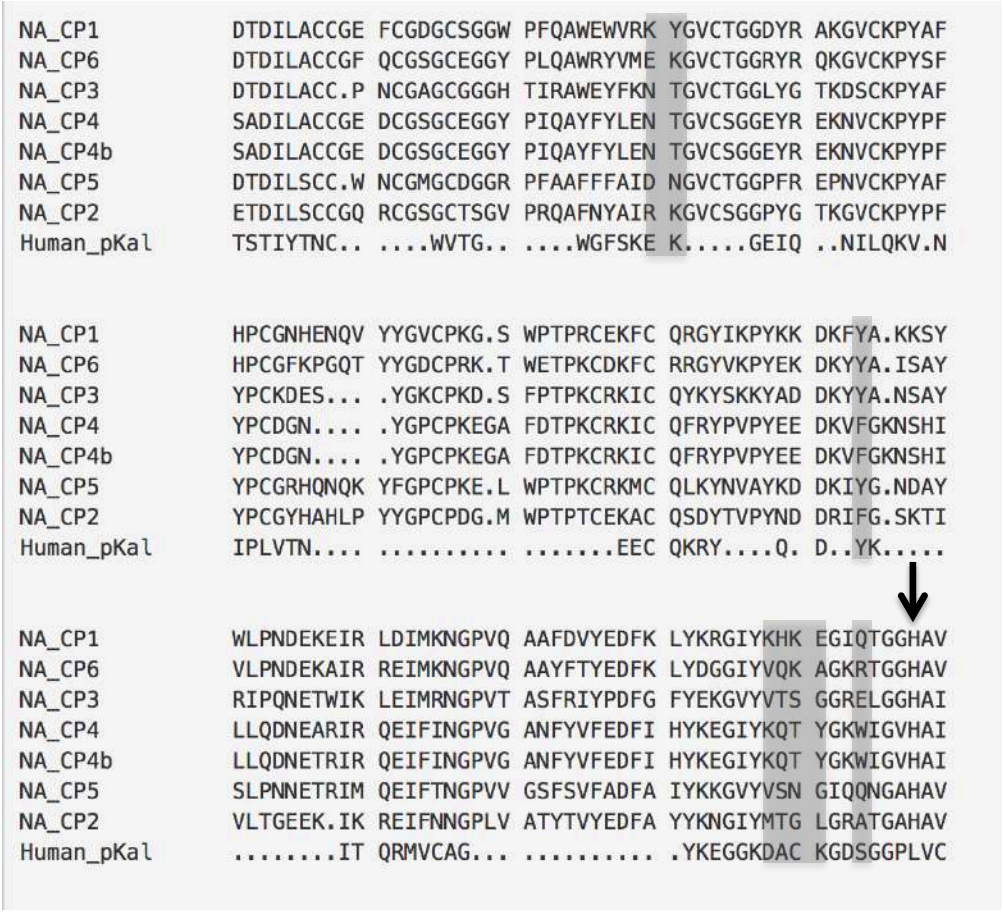

Figure 5B: Sequence alignment of human pKal with the *NA* cysteine proteases, where the catalytic triad **H** (Histidine) of the hookworm protease is shown by arrow. The active site residues of the pKal and the corresponding residues of *NA*-CPs in the alignment are shaded in grey.

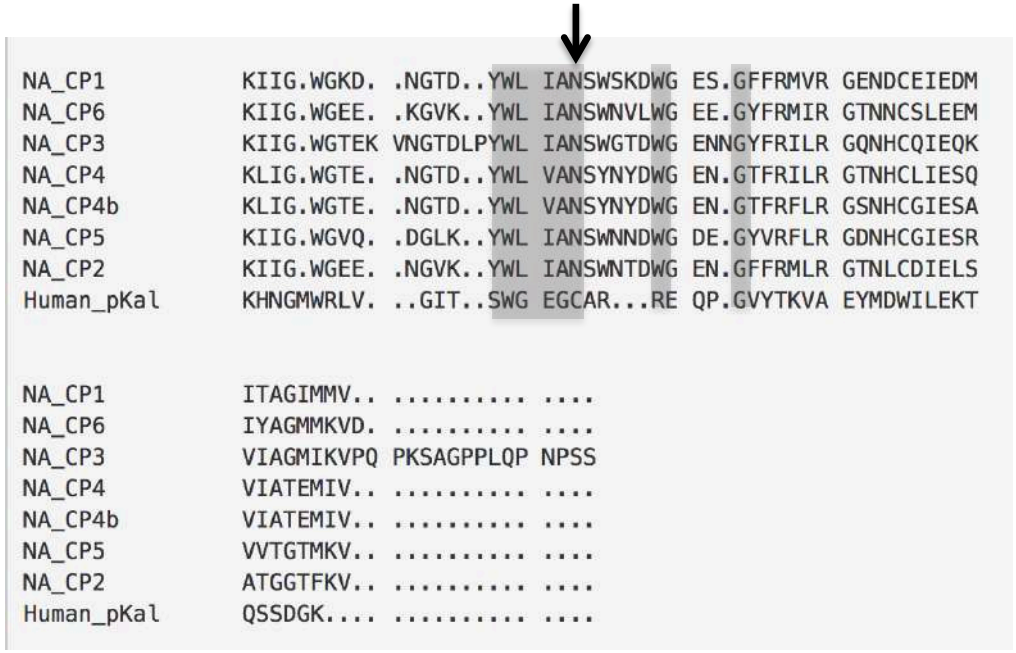

Figure 5C: Sequence alignment of human pKal with the *NA* cysteine proteases, where the catalytic triad **N** (Asparagine) of the hookworm protease is shown by arrow. The active site residues of the pKal and the corresponding residues of *NA*-CPs in the alignment are shaded in grey.

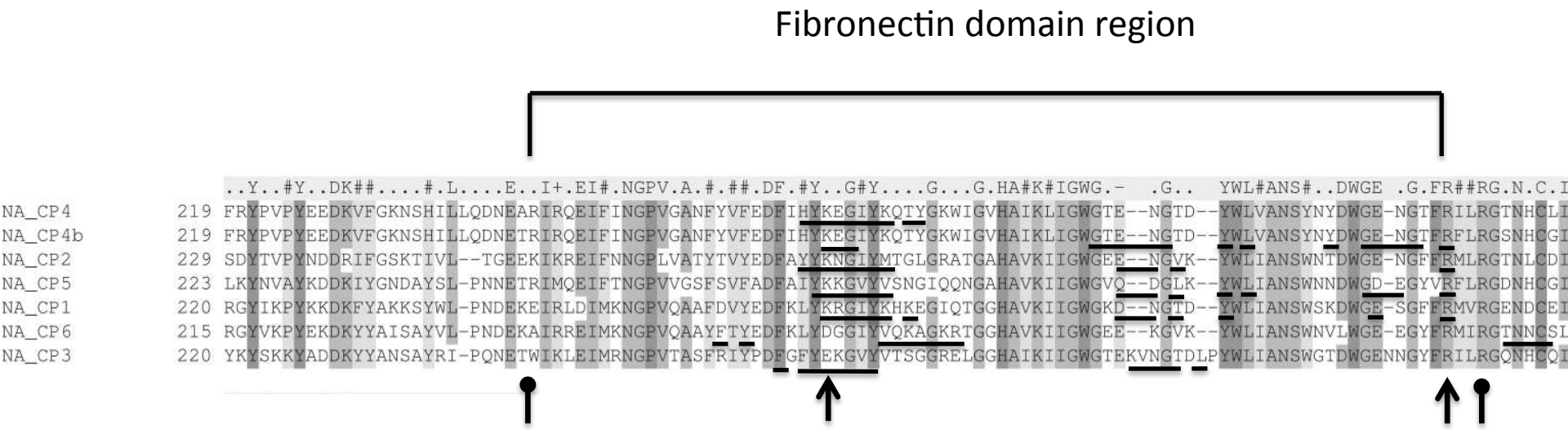

Figure 6: Partial sequence alignment of *NA* CP1-CP6 : region encompassing all the patches of the predicted fibronectin domain (for CP2 and CP5) has been denoted. The residue contacts made by heparin molecule in the proteases are underscored. Some of the N-terminal contacts (few) are not shown for brevity. The K and R residues in contact with heparin from most of the *NA* CP-heparin dockings are shown by arrow. The residues corresponding to human cathepsin B's K154 and R235 (derived from cathepsin B - *NA* CP alignment), which bind heparin according to human Cathepsin B-heparin docking/MD (Costa *et al*, 2010), are shown by spherical arrowheads.

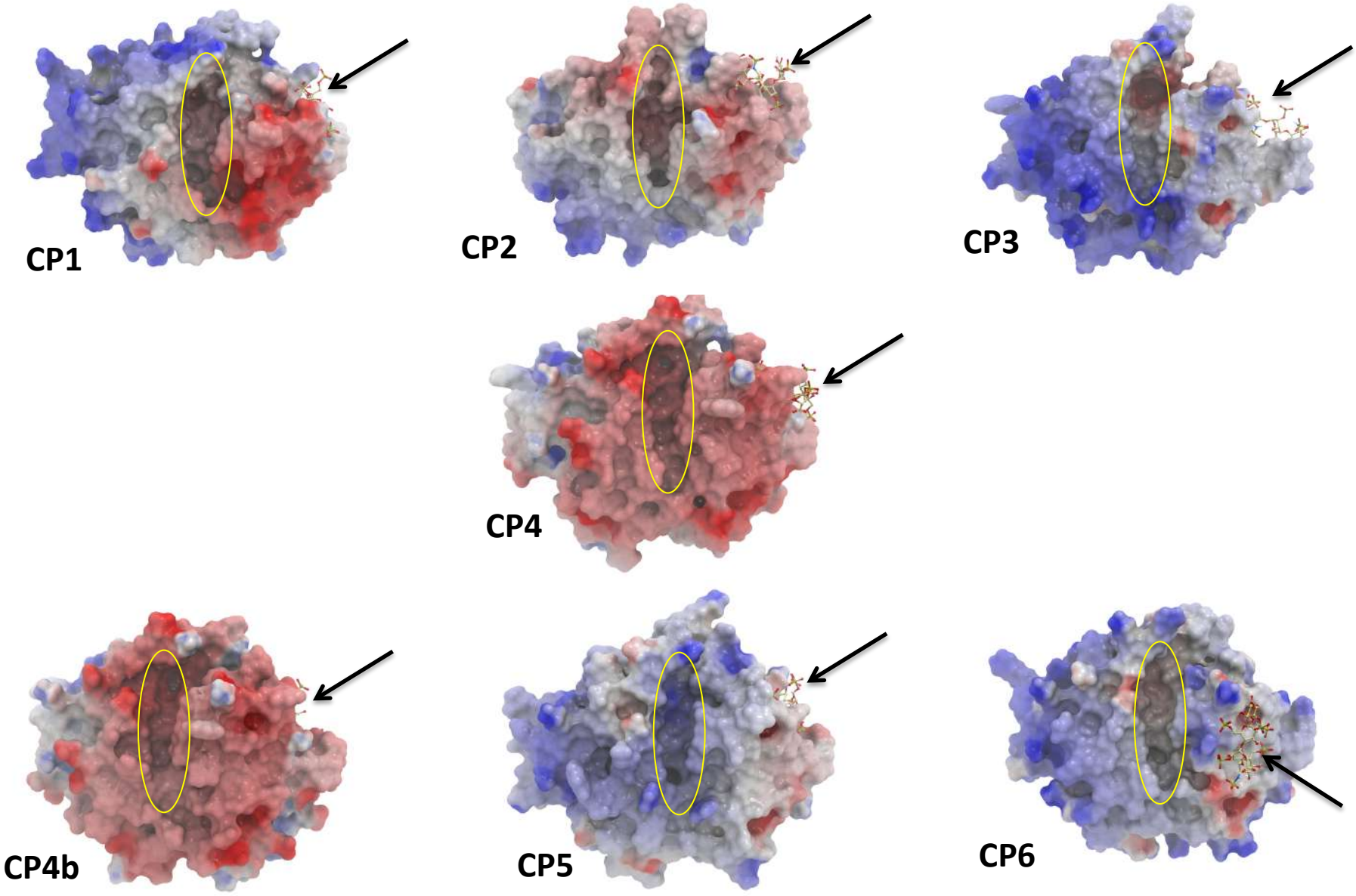

Figure 7: Heparin-bound three dimensional structures of the *NA*-CPs in electrostatic surface represention . The active site of the cysteine proteases which appears as clefts are facing the front (ovaled in yellow) and the location of the heparin (represented in stick form) which bind away from the clefts are indicated by arrows.