A computational structural analysis of functional attributes of hypodermin A and B proteins: A way forward for vaccine development

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Abstract: Hypodermosis is a parasitic disease of cattle. The pathogenicity of the disease is attributed to Hypodermin proteins (Hypodermin A, Hypodermin B and Hypodermin C). Studies suggest that Hypodermin proteins may be defined as Serine proteases and collagenases. The structure of both proteases Hypodermin A and Hypodermin B were modeled using the Swiss-model server followed by its validation using Procheck, Errat and Verify-3D. Afterwards, both Hypodermin A and Hypodermin B were docked against collagen in order to study its interaction with respective Hypodermin proteins. The structure of both Hypodermin A and Hypodermin B showed more bent towards hydrophobic nature as more beta sheets were present in them. Both structures were also superimposed to check out similarities and differences present between them. Serine, Aspartic acid, Histidine, Glutamic acid and Lysine are found as interacting residues that are involved in hydrogen bonding with collagen. The interactions are found in the active domain region of Hypodermin proteins. The interacting residues were present in the active region of the hypodermin proteins thus validating the docking studies. This study may help in the drug development against hypodermosis with least side effects.

Keywords: Hypodermosis, hypodermin A, hypodermin B, collagen, modeling, docking.

INTRODUCTION

Hypodermosis is a subcutaneous myiasis and larvae of the order Diptera are responsible for causing this disease and has worldwide geographical distribution (Ahmed *et al.*, 2012; 2013; 2016; 2017). The defined hosts for the parasite of this disease are the domestic and wild animals. Cattle Hypodermosis caused by the warble flies: *Hypoderma lineatum* (HL) and *Hypoderma bovis* (HB). The infection results in low performance of the animal and is also responsible for the economic damage (Hassan *et al*., 2002). Adult *Hypoderma* flies have a period of free aerial activity from May to August Eggs of around 1 mm in length are deposited by the female *Hypoderma* on the hairs of the host. The selected sites for the attachment of the eggs are the legs or lower regions of the cattle. The incubation of eggs is aided by the body temperature of the host which gives rise to L_1 larvae, which bore into the skin through enzymatic activity of Hypodermin proteins with host's collagen, the Hypodermins secreted by the blind mid gut of the larvae. The incubation period of eggs is normally 3-7 days. Then the L_1 migrate through the body to the submucosa of the oesophagus (HL) or to the spinal canal (HB) again with the help of enzymatic activity of Hypodermin proteins, now this time secretion is from the anterior part of the larvae. They remain there until the following spring travel through tissues of body and finally reaching the subcutaneous layer of the dorsal lumbar region (Scholl, 1993). After their migration, they form skin nodules which are called warbles ultimately burrowing through the skin to develop mature adult warble flies.

The ability of *Hypoderma* larvae to infect to the host is chiefly dependent on the serine proteolytic activity of Hypodermin against host's skin collagen tissues. There are three types of Hypodermin proteins that are found to be responsible for causing hypodermosis in cattle i.e. Hypodermin A, B and C (Lecroisey *et al*., 1979). These proteins are secreted by the blind mid gut and anterior part of the larvae Hypodermin A (HyA) and Hypodermin B (HyB) belong to a Trypsin family while Hypodermin C (HyC) is a member of Chymotrypsin family. The amino acid composition and N-terminal of HyA, HyB and HyC suggest their structural homology with Trypsin family (Lecroisey *et al*., 1983).

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The major objectives of this study include modelling the structures of HyA and HyB proteins, undertake their structural and functional comparison. We also studied how Hypodermins bind with collagen which may help devise strategies to develop vaccine against hypodermosis.

MATERIALS AND METHODS

The protein sequences of HyA and HyB proteins of warble flies were retrieved from the National Centre for Biotechnology Information (NCBI) server. The sequences were subjected to NCBI BLAST which retrieved structures of Salmon trypsin and human Mannan-binding lectin serine protease, 1UTJ and 4IGD,as the most homologous templates for HyA and HyB modelling respectively based on the maximum score, sequence identity and query coverage. The models were predicted at Swiss Modeller followed by their validation using different tools such as ProCheck, Errat and Verify 3D. The generated models were energy minimized and refined at Yasara. The non-aligned residues were modeled as loops. The structures of HyA and HyB were modified after truncating the non-aligned part that was creating extra loops in the beginning and end of the structure.

Once the structures have been minimized, the Hypodermin proteins HyA and HyB were docked against Collagen protein to analyze their binding conformations. The model of crystal structure of bovine collagen was downloaded from RCSB-Protein Data Bank. The purpose of docking was to get information about the residues that are involved in the interaction of Hypodermin proteins with Collagen. For the docking of Hypodermin proteins HyA and HyB, Cluspro Server was used. The selected models were analyzed in DimPlot, the protein-protein interaction analyzing tool of LigPlot+ Analysis software. The DimPlot showed the protein-protein interaction between the different chains of both docked complexes.

RESULTS

PDB structure of salmon trypsin, 1UTJ, was selected to model of HyA, with maximum query coverage 95%, residue range covered was from 9-253 and 39.39% identity. To model HBPDB structure of human Mannan-binding lectin serine protease, 4IGD, was selected with maximum query coverage of about 98% and sequence identity of 36.78%.The predicted 3D structure of HyA was visualized on Chimera. The structures of hypodermin proteins are predominantly composed of β strandsα helices. The HyA structure is typical of a serine protease family made up of three α helices and eleven β sheets were 11 as shown in fig. 1, showing that core of the protein is largely hydrophobic in nature. HyB structure also adopts a similar fold as observed in case of HyA. Threeα helices and 12β sheets were found in the structure of HyB as shown in fig 2. Addition of an extra strand in HyB, explains its differential expression and functional specificity.

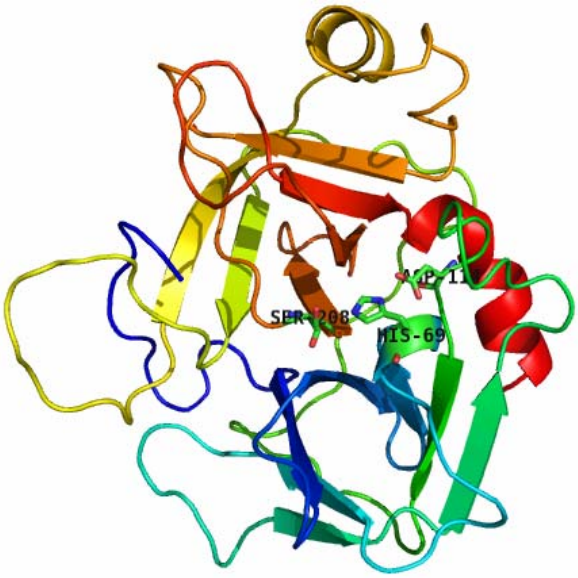

Fig. 1: Structure of Hypodermin A protein. Blue lines show the hydrogen bonding in the structure

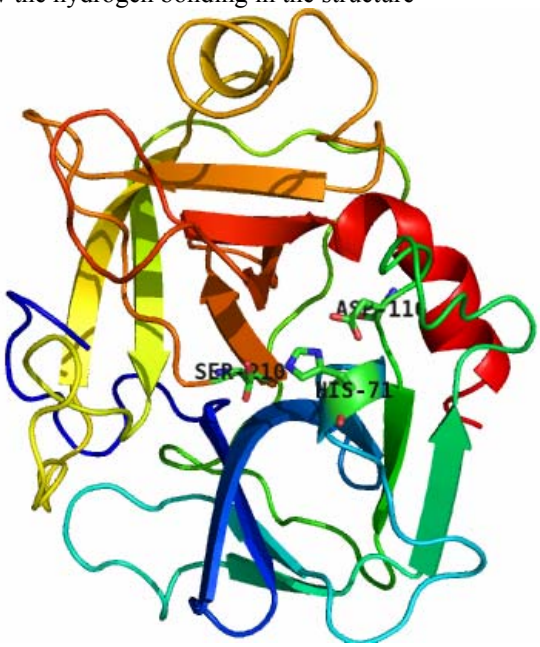

Fig. 2: Predicted structure of Hypodermin B, visualized in Chimera. Blue color lines show the hydrogen bonds present in the structure.

The predicted structures of both proteins were validated for two times, first validation was done before minimizing the energy of protein structure and the other was done after structure minimization step. When the energy of both protein structures was minimized, the overall quality factors improved, stabilizing the structures for docking. The structure of HyA has been validated as shown by Ramachandran plot, 99% of its residues were lying in allowed region fig. 3, structure was acceptable and could be used for further *in-silico* assays (Laskowski *et al.*

1993). The QMEAN6 score was 0.66, much above the preferred threshold. The Z-score value of the predicted structure of HyA is shown in fig 4. The overall quality factor evaluated by Errat for the structure of HyA was 76.498 as shown in fig 5. The Ramachandran plot for HyB was showing that 80.6% residues were in core allowed region and 14.4% was in allowed region. 4% of residues were lying in generously allowed area while only 1% was in disallowed region. Overall the structure of HyB was also very good that 99% of its residues after summing up were present in the allowed region of Ramachandran plot. The QMEAN6 score was 0.658 which is really good score. The Z-score value of the predicted structure of HyB protein is shown in fig 6. The overall quality factor evaluated by Errat for HyB was 71.064 as shown in fig 7 and 8. Both the protein structures, when superimposed, illustrate a conserved structure the residues HIS, ASP and SER occupying the same position thereby conserving the functional integrity, fig 9.

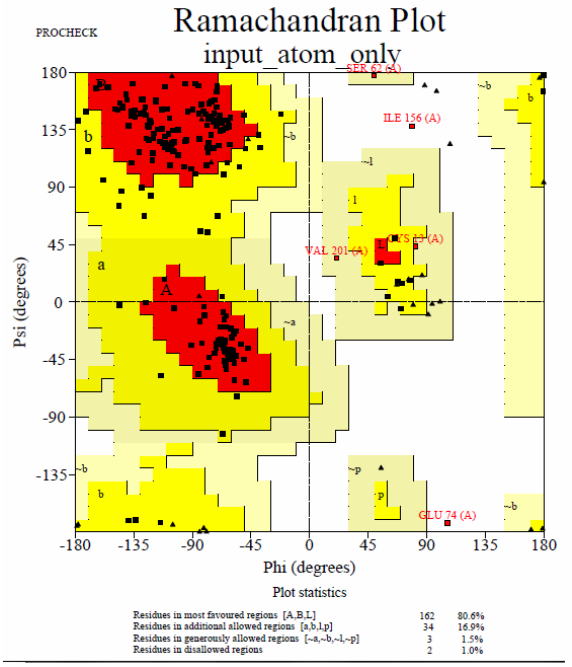

Fig. 3: ProCheck Ramachandran Plot. Red colour shows the 80.6% core allowed area of structure residues of Hypodermin A. Bright yellow shows the 16.9% allowed region. Light yellow shows the 1.5% generously allowed region of residues while the dimmest yellow colour shows 1% disallowed region of structure residues.

The docked complex of HyA had 147 residues involved in docking with free energy of -10.234 kcal/mol. The hydrogen bonds and hydrophobic interactions were observed via LigPlot's Dimplot add-on. Serine 182 of chain A of HyA protein was found interacting with the Arginine 44 of chain B of Collagen. When examined the docked complexes at Dimplot Serine 182 formed hydrogen bond with Arginine 44, their bond length was 2.64 Å. Aspartate 183 of HyA was also showing hydrophobic interaction with the Arginine 44 of chain B of collagen as shown. Aspartate of HyA also formed hydrogen bond with Alanine 73 of collagen and Serine 182 of HyA showed hydrophobic relation with the same residue of collagen, the bond length was 2.94Å. Histidine 54 and Glutamate 228 of chain of HyA showed hydrophobic relation with Proline 34 of collagen. Glutamic acid 184 of HA was also hydrophobically interacting with Iso-leucine 70 of collagen, fig 10. Besides, fig. 11 shows the interaction between the residues of HyB protein and collagen protein. Serine of HyB was not found interacting with collagen. But Aspartate 183 of HyB protein was found interacting with the Arginine 44 of chain B of Collagen. It has formed hydrogen bond with Arginine 44 and the bond length was 2.68Å. Lysine 203 formed hydrogen bond with Glycine 39 with 2.48Å bond length. Glutamate 228 could also be seen in hydrogen bonding with another Glycine 36 residue of collagen and their bond length was 2.93Å as shown in fig 11. Serine 182 and Aspartate 183 of HyB also showed hydrophobic relation with Alanine 73 of collagen. Glutamate 184 and Aspartate 70 of HyB were found hydrophobically interacting with Iso-leucine 70 of chain C of collagen. The strong interaction between HIS 69, ASP 114 and SER 208 of both HyA and HyB with positively charged residues of collagen such as arginine and lysine.

Scoring function term	Raw score	Z-score
C_beta interaction energy	-42.78	-1.94
All-atom pairwise energy	-2274.74	-2.72
Solvation energy	-0.27	-2.87
Torsion angle energy	-52.92	-0.92
Secondary structure agreement	79.5%	-0.49
Solvent accessibility agreement	75.4%	-0.83
QMEAN6 score	0.662	-1.15

Fig. 4: ProCheck- QMEAN6 Score and Z score for the predicted structure of Hypodermin A protein.

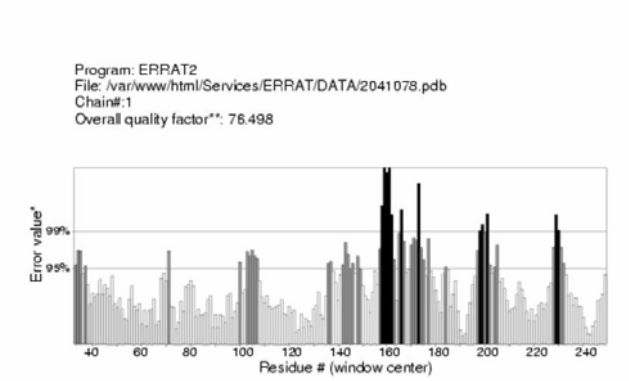

Fig. 5: Errat Protein Structure Validation Tool. This figure shows the overall quality factor of predicted structure of Hypodermin A.

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Scoring function term	Raw score	Z-score
C_beta interaction energy	-29.75	-2.29
All-atom pairwise energy	-2030.42	-2.85
Solvation energy	2.00	-3.13
Torsion angle energy	-33.00	-2.10
Secondary structure agreement	81.1%	-0.20
Solvent accessibility agreement	77.0%	-0.53
QMEAN6 score	0.658	-1.20

Fig. 6: ProCheck-QMEAN6 Score and Z score for the predicted structure of Hypodermin B protein.

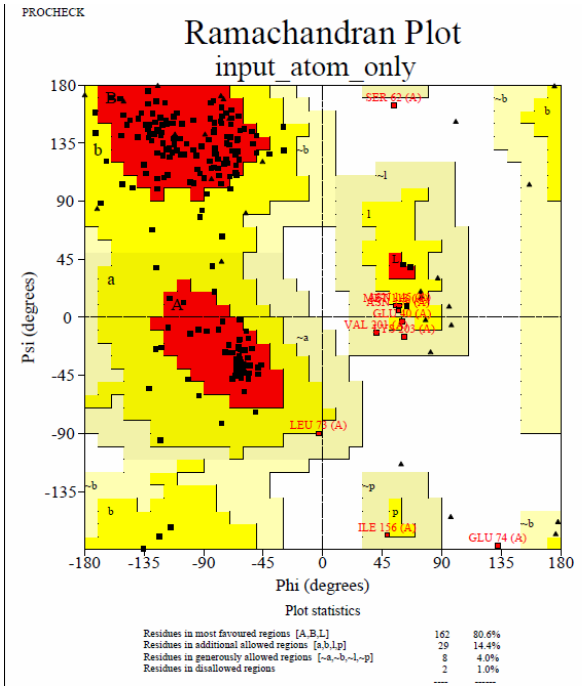

Fig. 7: ProCheck-Ramachandran Plot. Red color shows the 80.6% core allowed area of structure residues of Hypodermin B. Bright yellow shows the 14.4% allowed region. Light yellow shows the 4% generously allowed region of residues while the dimmest yellow color

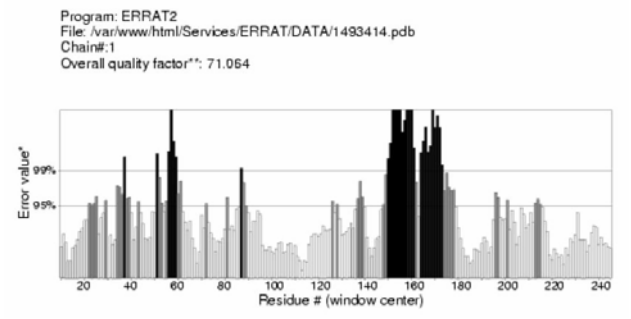

Fig. 8: Errat Protein Structure Validation Tool. This figure shows the overall quality factor of predicted structure of Hypodermin B.

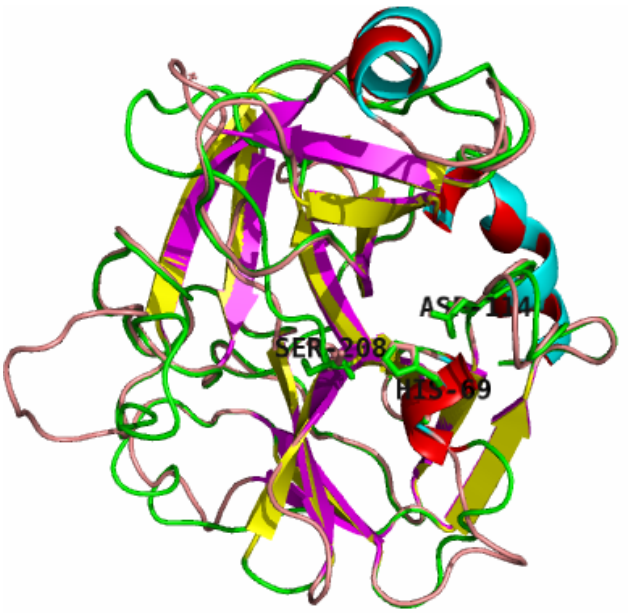

Fig. 9: HA and HB superimposed, notice the residues HIS 69, ASP 114 and SER 208 all occupying adopting similar spatial arrangement whereas some of the secondary structure elements do adopt variable conformations.

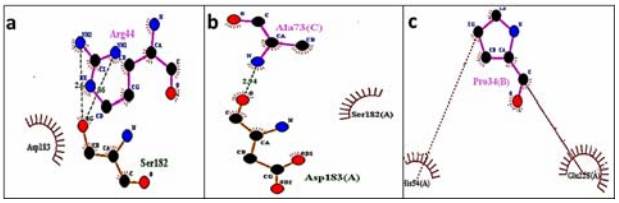

Fig. 10: Interaction between various residues of collagen and Hypodermin A, from left to right a: Ser 182 of HA formed Hydrogen bond with Arg 44 of Collagen. Asp 183 of chain A of HA shows Hydrophobic relation with Arg 44. b: Asp 183 of chain A of HA formed Hydrogen bond with Ala 73 of chain B of Collagen. Ser 182 of chain A of HA shows Hydrophobic relation with same residue chain C of Collagen.c: Hydrophobic interaction between His 54 and Glu 228 of HA with chain B of Collagen.

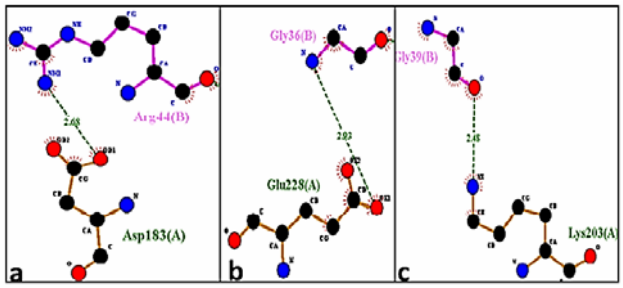

Fig. 11: Interaction between various residues of Collagen and Hypodermin B, from left to right, a, and c: Asp 183,

Glu 228 and Lys 203 of chain of HB formed Hydrogen bond with Arg 44, Gly 36 and Gly 39 of collagen respectively.

Table 1: Hydrogen bond details between different residues of chain A of HA and chain B and C of Collagen.

Residue of Chain A of HA	Residue of Chain B of Collagen	Bond Length	Residue of Chain C of Collagen	Bond Length
Ala 231	Pro 37	3.06	Pro 67	3.11
Gly 9	Pro 37	3.02	-	-
Gly 27	Pro 55	2.91	Gly 84	2.98
Gly 30	Pro 58	2.96	Gly 90	2.95
Tyr 53	Gly 33	2.87	-	-
Gly 6	Gly 33	2.91	-	-
Thr 155	Pro 34	2.99	-	-
Lys 203	Gly 39	2.57	-	-
Gly 24	Pro 52	2.97	-	-
Ile 10	Ile 40	2.75	-	-
Gly 12	Ile 40	2.85	-	-
Val 179	Arg 44	2.73	-	-
Ser 182	Arg 44	2.64	-	-
Arg 14	Arg 44	2.67	-	-
Arg 14	Ala 43	2.82	-	-
Arg 14	Gly 45	2.82	-	-
Ala 13	Gly 42	3.23	-	-
Gly 18	Leu 46	2.88	Arg 74	2.92
Gly 21	Pro 49	2.86	-	-
Gly 15	Leu 46	3.20	-	-
Gly 15	Gly 42	3.07	-	-
Tyr 232	-	-	Pro 67	3.03
Pro 28	-	-	Gly 87	2.90
Asp 183	-	-	Ala 73	2.94
Pro 22	-	-	Gly 81	2.93
Pro 19	-	-	Gly 78	3.03
Leu 16	-	-	Gly 75	2.91
Ala 17	-	-	Arg 74	2.70

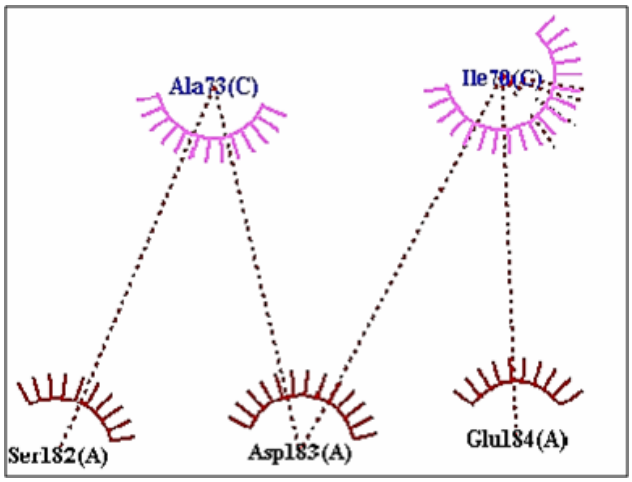

Fig. 12: Hydrophobic interactions between Ser 182 and Asp 183 of HB with Ala 73 of Collagen. Asp 183 and Glu 184 of HB with Ile 70 of Collagen

DISCUSSION

As HyA and HyB have been found to be serine proteases, the main focus of evaluation was to check and analyze how serine is involved in interaction leading to the collagenase activity of the enzymes as in these proteases serine, histidine and aspartate are known to adopt a specific spatial arrangement critical to proteolyse the collagen. They cleave positively charged amino acids like arginine and lysine. The cleavage is always done by the residues that are driven by the histidine, aspartate and serine. Serine plays major role in breaking bonds of the molecule (Shoulders *et al.*, 2009) it attacks. It can be directly or indirectly involved in the proteolysis of other molecule.

Hypodermin proteins are termed as serine proteases orcollagenases belonging to Trypsin family (Polgar, 2005). These proteases are capable to hydrolyze the peptide bond present in the amino acids of a protein. They are also known as endoproteases as they cleave bonds within a protein. Their active site contains serine, aspartate and histidine, also known as catalytic triad, but recent studies have discovered (Khaznadji *et al*., 2003) that the glutamate and lysine residues are also essential for the endo-proteolysis. The catalytic triad comprising of Asp-Ser-His is present in the active site of the Serine protease. This triad plays an important role in the dissolution of peptide bond of the substrate. Substrate binds to the surface of the Hypodermin, serine acts as a nucleophile as it has hydroxyl group (OH). The hydroxyl group of Serine attacks the carbonyl carbon of the scissile peptide bond of the substrate with the assistance of Histidine. The hydrogen of OH of Serine is accepted by

Table 2: Hydrophobic interaction between different residues of chain A of HA and chain B and C of Collagen

Residue of Chain A of HA	Residue of Chain B of Collagen	Residue of Chain C of Collagen
Pro 4, Gly 3	Pro 31, Gly 33	-
Tyr 53	Pro 31,Gly 33, Pro 34	-
Gly 6	Gly 33, Pro 37	-
His 54, Phe 55, Gly 154,Thr 155	Pro 34	-
Glu 228	Pro 34, Gly 36, pro 37	-
Gly 229	Gly 36	-
Cys 206, Pro 7, Cys 230	Pro 37	-
Gly 9	Pro 37, Gly 39	-
Pro 28	Pro 58, Gly 57	Pro 85, Gly 84, Pro 88
Gly 27	Gly 57	
Pro 25	Pro 55	Gly 84, Pro 82
Gly 24	Gly 54, Pro 52, Pro 55	Gly 81, Pro 82
Tyr 157,	Gly 39	-
Ile 10	Gly 39, Ile 40	-
Thr 11, Val 201	Ile 40	-
Val 201, Gly 234, Phe 235	Thr 41	-
Asp 183, Ser 182, Arg 14	Arg44	-
Arg 14, Phe 235	Ala 43	-
Gly 15, Arg 14	Gly 45	-
Pro 19	Pro 49, Gly 48	-
Gly 18	Pro 49, Leu 46, Gly 48, Ala 47	Leu 76, Gly 75, Arg 74
Ala 17	Leu 46, Gly 48, Ala 47	Arg 74
Ile 10, Gly 12, Ala 231	-	Gly 60, Gly 66
Ala 231	-	Pro 67, Ile 70
Gly 229	-	Pro 67
Tyr 232, Glu 184, Tyr 187	-	Ile 70
Ala 13	-	Thr 71
Gly 30	-	Pro 88, Gly 90, Gly 87
Gly 186, Gly 15	-	Gly 72, Ala 73
Asp 182, Ser 182	-	Ala 73
Pro 22	-	Gly 81, Pro 79, Gly 78
Gly 21	-	Gly 78
Pro 19	-	Ala 77, Leu 76, Gly 75
Leu 16	-	Gly 75, Arg 74

the nitrogen of Histidine and the pair of electron moves from double bond of carbonyl oxygen of substrate to the oxygen of Serine forming a tetrahedral intermediate. The peptide bond present in the substrate is now broken. The electrons making this bond now move to attack the hydrogen of Histidine. The connection breaks and the previously moved electrons from carbonyl oxygen move back from negative oxygen to recreate the bond. Water formed during the reaction, replaces the N terminus of the cleaved substrate and attacks carbonyl carbon. Again the electrons move to the oxygen and make it negative oxygen. The bond between the oxygen of water and the carbon is formed. This reaction is again assisted by Histidine as it takes a proton from water forming another tetrahedral intermediate. The electrons that make the bond between the serine and carbonyl carbon move to attack this hydrogen that Histidine just acquired from water. Now carbonyl carbon is electron deficient (Khaznadji *et al*., 2003) and it recreates a double bond with the oxygen and C terminus of substrate is also ejected.

HyA is associated with inflammatory and specific immune responses in cattle hosts. Association between recombinant HA and guinea-pig complement component 3 (C3) through a co-immunoprecipitation assay was explored by Chen and colleagues. Cos7 cells stably expressing HyA were generated, and were found to be more resistant to lysis by guinea-pig C3 than the controls. They DNA binding site of HA with guinea-pig C3 was detected by an electrophoretic mobility shift assay (EMSA). In contrast, after stable transfection, mHA was unable to reduce the amount of C3 or to inhibit its cytotoxicity, while HA could degrade guinea-pig C3 and inhibit the complement pathway. So it can be suggested that recombinant HyA could serve as an immunosuppressive agent against organ rejection after

Table 3: Hydrogen bond details between different residues of chain A of HB and chain B and C of Collagen.

Residue of Chain A of HB	Residue of Chain B of Collagen	Bond Length	Residue of chain C of Collagen	Bond Length
Gly 6	Pro 34	2.89	-	-
Glu 228	Gly 36	2.93	-	-
Cys 230	Gly 36	3.33	-	-
Gly 9	Pro 37	2.92	-	-
Lys 203	Gly 39	2.48	-	-
Ile 10	Ile 40	2.96	Gly 69	2.78
Gly 12	Ile 40	2.88	-	-
Gly 234	Thr 41	2.90	-	-
Asp 183	Arg 44	2.68	-	-
Arg 14	Arg 44	2.79	-	-
Gly 15	Ala 43	3.00	-	-
Arg 14	Gly 45	2.72	-	-
Gly 18	Leu 46	2.91	Arg 74	2.87
Pro 19	Gly 48	3.26	Gly 78	2.99
Gly 21	Pro 49	2.87	-	-
Gly 24	Pro 52	3.00	-	-
Gly 27	Pro 55	2.91	Gly 84	3.00
Gly 30	Pro 58	2.96	Gly 90	2.95
Ala 13	-	-	Gly 72	2.91
Pro 7	-	-	Gly 66	2.91
Ala 17	-	-	Arg 74	2.73
Leu 16	-	-	Gly 75	2.94
Pro 22	-	-	Gly 81	2.93
Pro 28	-	-	Gly 27	2.90

Table 4: Hydrophobic interactions between different residues of chain A of HB and chain B and C of Collagen

Residue of chain A of HB	Residue of chain B of Collagen	Residue of chain C of Collagen
Tyr 53, Pro 4	Pro 31, Gly 33, Pro 34	-
Gly 3	Pro 31	-
Glu 228, Gly 6	Gly 36	-
Thr 155, Gly 6, Cys 206, Ile 156	Pro 37	-
Gly 12, Ala 13, Phe 235	Gly 42	-
Gly 27, Pro 28	Gly 57	-
Arg 14	Arg 44, Ala 43, Gly 45, Leu 46	-
Asp 183	Arg 44	-
Val 201, Phe 235	Thr 41	-
Ser 183, Asp 182	-	Ala 73
Glu 184, Asp 182, Phe 235, Tyr 232, Tyr 187	-	Ile 70
Ala 13, Tyr 187	-	Thr 71
Thr 11, Ala 231, Gly 12	-	Gly 69
Glu 228, Gly 6	-	Pro 64
Leu 16, Gly 18	-	Arg 74
Gly 21	-	Gly 78, Pro 79

xenotransplantation (Chen *et al.*, 2014). Panadero *et al*. (2009) reported the immuno-modulatory impact of three serine proteinases from first instars larvae of *H. lineatum*. Colwell (2011) reported the immunization efficiency of soluble fractions of *H. lineatum* 3rd *Hypoderma* instar fat body mixed with fraction Quil A. The resulting vaccine was used to immunize three different calve groups while 4th and 5th groups were adjuvant treated or untreated controls. In vaccinated cattle the mortality was 100%. A significant increase in the mortality rate of first instar larvae migration was noted as well as increase in mortality rate of 2nd and 3rd instars in vaccinated animals compared to untreated animals controls and adjuvant treated animals was observed. The candidate proteins with protein scores of >400 are the hexamerins/arylphorins (also known as larval serum proteins), which are the storage proteins of the haemocyanin family. Glutathione-S-transferase belongs to a multifunctional family of enzymes. It protects the cells by preventing the damaging effects of oxygen and other free radicals. That's why they

are commonly used in antiparasite vaccines (Parizi *et al.,* 2011). Arginine kinase is another important enzyme that catalyses the transfer of phosphoryl groups from ATP to arginine in insects. That is why it could be an excellent candidate for the development of a drug or vaccine. It has also been implicated as a major human allergen (Ilg and Werr, 2012).

There are different key challenges in current vaccine technologies of myiasis. They are reviewed mainly in primary research genera of *Hypoderma* spp. These flies are one of the causes of morbidity and mortality in livestock sector and so far no control strategies exist. From the last decade the researchers are trying to develop vaccines against myiasis. Although there is a dire need of the rapid development in genomic and proteomic analysis, the alternative controls strategies are constantly evolving due to the drug resistance that has a strong impact on animal welfare (Colwell, 2013). In this study, the structure of HyA and HyB was predicted on the basis of homology modelling and its interaction with the collagen protein was studied via molecular docking technique. We are reporting the 3D structure of HyA and HyB for the first time and have shown how the catalytic triad asspatially arranged in the Hypodermin to bring about the proteolytic activity. The models of HyA and HyB were docked against their substrate collagen which showed for the first time how the nucleophilic triad of the HyA and HyB hydrolyze the peptide bond of the host's collagenase, responsible for puncturing the skin of cattle by breaking down the collagen protein which is the integral part of epidermis in order to penetrate into the body of cattle (Khaznadji *et al*., 2003). Our results have illustrated how the characteristic structure of the HyA and HyB of warble fly larvae has been functionally evolved to to proteolyse the skin collagen of its host, essential for parasitic activity. These studies also highlight the possibility of developing vaccines against HyA and HyB antigens as their structure is known.

CONCLUSION

This study has modeled the structure of HyA and HyB for the first time. We have also highlighted for the first time the active site topology of Hypodermins. We have also shown how collagenase, the substrate of HyA and HyB, binds at the active site. The specificity of collagen binding with HyA and HyB has been studied through understanding the binding mechanism of catalytic triad with collagen electrophiles. These results may be helpful for designing various therapeutic strategies against hypodermosis.

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