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ANALYSIS OF STRUCTURE OF HIRUDIN AND ITS MECHANISM OF INTERACTION WITH THROMBIN

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Abstract:
Hirudin is a naturally occurring peptide in the salivary glands of medicinal leeches . It has a blood anticoagulant property. Hirudin is the main chemical compound in the secretion of leeches that allows them to suck out blood freely from the body after they attach to the skin. The leech saliva contains substances that anesthetize the wound area, dilate the blood vessels to increase blood flow, and prevent the blood from clotting. The search for the development of hirudin from leech extract to genetically engineered products as an alternative anticoagulant has been resumed. The pharmacological profiling of the isolated thrombin inhibitor has shown that native hirudin is an antithrombotic agent of high quality. However, its clinical use has remained limited, because the substance has not been available in adequate amounts. The progress in molecular biology has stimulated the interest in the structure and function of hirudin. This development resulted in the production of recombinant hirudins (r-hirudins) through gene technology. The crystallographic structure of a recombinant hirudin-thrombin complex has been solved at 2.3 angstrom (A) resolution. Hirudin consists of an NH2-terminal globular domain and a long (39 A) COOH-terminal extended domain. Residues Ile1 to Tyr3 of hirudin form a parallel beta-strand with Ser214 to Glu217 of thrombin with the nitrogen atom of Ile1 making a hydrogen bond with Ser195 O gamma atom of the catalytic site, but the specificity pocket of thrombin is not involved in the interaction. The COOH-terminal segment makes numerous electrostatic interactions with an anion-binding exosite of thrombin, whereas the last five residues are in a helical loop that forms many hydrophobic contacts. In all, 27 of the 65 residues of hirudin have contacts less than 4.0 A with thrombin (10 ion pairs and 23 hydrogen bonds). Such abundant interactions may account for the high affinity and specificity of hirudin.

Keyword: Hirudin-thrombin-platelet-diseases

Introduction:

Hirudin was used for the first parenteral anticoagulation in humans in 1909. (Engelmann ***et*** *al.,* 1909) and as the anticoagulant for the first hemodialysis in humans. (Haas1926) Soon after, heparin became available and has since become the most widely used drug for parenteral anticoagulation. Heparin, however, can induce a life-threatening adverse immune reaction, heparin-induced thrombocytopenia (HIT), (Warkentin ***et*** *al.,* 1995). The thrombin inhibitory mechanism of hirudin has been extensively investigated . (Chang, 1983a; Chang, 1989; Dennis ***et*** *al.,* 1990).Hirudin consists of an N-terminal globular domain and an extended C-terminal domain. Residues 1-3 form a parallel beta- strand with residues 214-217 of thrombin, the nitrogen atom of residue 1 making a hydrogen bond with the Ser195 O gamma atom of the catalytic site. The C-terminal domain makes numerous electrostatic interactions with an anion-binding exosite of thrombin, while the last five residues are in a helical loop that forms many hydrophobic contacts. Stone and Hofsteenge,1986 Recombinant Hirudin is a potent thrombin inhibitor originally derived from the medicinal leech unlike heparin. Hirudin acts directly on thrombin rather than through other clotting factors. The mechanism of Hirudin-thrombin appears to be unique. The conversion of fibrinogen into fibrin by the serine protease enzyme thrombin is a major event in the final stages of blood coagulation. In the final stages of coagulation prothrombinase converts prothrombin into thrombin. Fibrin is subsequently cross linked by factor XIII to form a blood clot. The primary inhibitor of thrombin in normal blood circulation is antithrombin III. The anticoagulatant activity of hirudin is derived from its ability to inhibit the pro-coagulant activity of thrombin (similar to antithrombin III activity). Hirudin is the strongest natural inhibitor of thrombin. Hirudin binds to and inhibits only the activity of thrombin forms with a specific activity on fibrinogen contrasting to antithrombin III activity.(Dodt ***et*** *al.,* 1985)Therefore, hirudin has a thrombolytic activity since it prevents or dissolves the formation of clots and thrombi. Hirudin also has therapeutic significance in blood coagulation disorders, 5in the treatment of skin hematomas and of superficial varicose veins. Hirudin does not hinder with the biological activity of other serum proteins and can also act on complexed thrombin, thus having an advantage over more common anticoagulants and thrombolytics like heparin for example. (Rydel ***et*** *al.,* 1990;Gruetter ***et*** *al.,* 1990) It is complicated to extract large quantities of hirudin from natural sources; therefore a method for producing and purifying hirudin using recombinant biotechnology has been developed.

Materials and Methods:

The present study was carried out on starved *Poecilobdella viridis* weighing about 2.5 to 3 gms. Leeches were collected locally.

Experimental Setup

Blood samples from volunteers before and immediately after leech application were collected in small eppendorf tubes. Serum was prepared from venous blood within 30 min of collection by centrifugation at – 20^0 C for 10 minutes. In the third set of experiment r-Hirudin procurred from Dr. Jurgen Hofirger NAMOS (Nanotechnology of Biomimeties on surfaces), Germany. It was used as antigen to test its immune reaction *in vitro*. For this 5 volunteers were applied leeches (one each) for different duration's and antibodies against the leech saliva were allowed to form in volunteers for 96 hr. and then venous blood from the volunteers was removed after 96 hrs of leeching. Serum was separated, which contain antibodies against natural hirudin and other ingradients in leech saliva. The serum was used for immunoelectrophoresis.

Five volunteers were applied leeches (one each) for 1 hr and antibodies against the leech saliva were allowed to form in the volunteers for 96 hours. and then venous blood from the volunteers was taken after 96 hr of leeching, serum was separated, which contained antibodies against natural hirudin and other ingradients in leech saliva. This serum was used for immunoelectrophoresis, by radial immunodiffusion assay. Antibodies produced after leeching were present in serum of all the volunteers. They reacted with the r- hirudin to form a lattice that precipitate to form a precipitin ring. The serum samples containing antibodies were mixed with the gel and the 4 wells in the gel were filled up with different concentrations of r – Hirudin (5 □l, 10 □l, 15 □l and 20 □l). The diameters of precipitin rings obtained after incubation of 48 hours at 37 ^{0}C were measured .(diameter of precipitin rings against r-hirudin concentration) was prepared

Then another group of 5 healthy volunteers were applied with leech (one each) for 1 hour and immediately after leeching the venous blood was removed from the nearby site of leech bite. Plasma was separated which contained the injected leech saliva (hirudin + other biochemical secretions). This hirudin is treated as the natural antigen. 20□l of plasma was poured into a well of freshly prepared immunoelectrophoresis gel mixed with antibodies as above. The plate was then incubated at 37 ^{0}C for 48 hours and then the diameter of precipitin ring was measured.

Observation and Discussion:

Thrombin is a allosteric serine protease. Hirudin N- terminal sequence is known to interact with the catalytic site of thrombin. The crystal structures of the complexes of human alpha– thrombin with recombinant hirudin variants 1 (Grutter *et al.*, 1990) and 2 (Rydel *et al.*,1990,1991) have been determined. These studies reveal a mode of binding that has not been previously observed for a protease inhibitor. The unique findings of their studies can be summariesed as below to justify the present results. The contact area between hirudin and thrombin in the complex is large Of the 65 residues of hirudin, 27 actually contact with thrombin which probably accounts for the high affinity selectivity of hirudin for thrombin resulting in increased clotting and bleeding time. The first 3 residues of hirudin bind at the active site of thrombin, but the primary specificity site of thrombin is not occupied by hirudin. The last 16 residues of hirudin are in an extended conformation and bind at an anion binding exosite on the surface of thrombin that extends from the active site and is probably the secondary fibrinogen binding site. These binding characteristics may be the cause of increased clotting time when more than two leeches were used simultaneously resulting into entry of more hirudin molecules in the venous blood circulation of volunteers in the present investigation.

Radial immunodiffusin assay of antihirudin raised against hirudin (Table 1).

Before predicting the epitope of hirudin let us briefly see the molecular characterization of hirudin. There are three disulfide linkages located with-in the first 39 N- terminal residues of naïve hirudin. Hirudin contains a highly acidic N- terminal segment. There are 5 acidic amino acids, 4 glutamin and one Tyrosine SO_3H , within the last 9C-terminal residues. One of the likely reactive sites of hirudin, is a lysin residue flanked by two prolins. The part of molecule of hirudin shows hydrophilicity on kyte Doolittle scale and hydrophobicity on Hopp-Wood scale and These scales show 3 hydrophobic domains in a particular region. Removal of the acidic c-terminal amino acids of naïve hirudin results into loss of hirudin inhibition activity. Hirudin contains 3 molecules of lysine and 6 molecules of cysteine per molecule.

The fragment between 14 to 22 (clcegsnvc) forms 4 motifs and they share the beta sheets fig1 .

1. The epitope region is in beta sheet region.
2. The motif map (Fig 5) shows 4 motifs in epitope region of which three motifs are MHC class I related and one motifs is B-cell related.
3. The molecule shows high beta hydrophobic moment (Fig3)
4. The ratio of hydrophilicity to hydrophobicity is 1.54298
5. Percentage of hydrophilic amino acids is 53.0612 and that of hydrophobic amino acids is 46.9388.
6. The estimated molecular weight of hirudin is 6969.57
7. Atomic composition is carbon (287) hydrogen (446) , Nitrogen (80), Oxygen (110) and sulfur (6) thus total number of atoms is 929
8. The details of amino acid composition is indicates that hirudin is glycerin rich with a frequency of 0.138..
9. The isoelectric point is 3.82623.
10. The hirudin N-terminus is globular and very tight because of the presence of the three disulfide bridges and its C-terminus is rather light with numerous amino acid residues.
11. The hirudin N-terminal sequence is known to interact with the catalytic site of thrombin. In this context amino acid residues 52 to 56 are predicted to be extremely important for the link between hirudin and thrombin).

Table 1

S.No.	Quantity of r-hirudin (micro g)	Diameter of precipitin ring (mm)
1	0.844	6.0±0.26
2	1.688	8.0±0.15
3	2.532	11.4±0.12
4	3.376	12.8±0.18
5	4.250*	134.0±0.20

Values are ±SE of 5 observations.

Figure 1 Hirudin thrombin complex showing β sheet

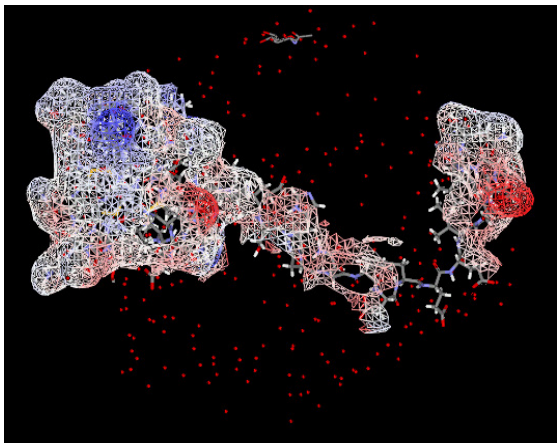

Figure 2 Hirudin-thrombin complex showing Electron Density Map of Hirudin Molecule

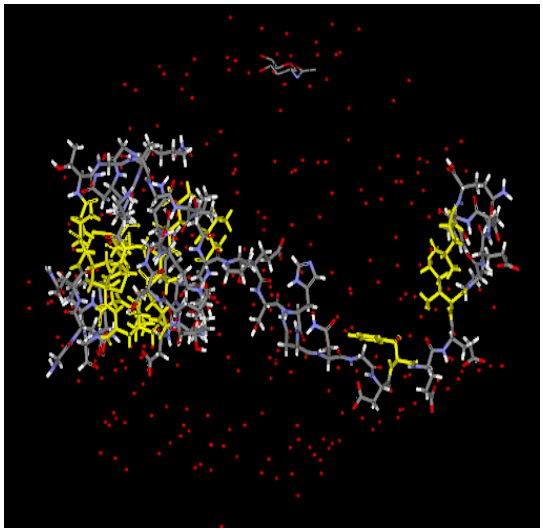

Figure 3. Hirudin-thrombin complex showing hydrophobic amino acids of Hirudin

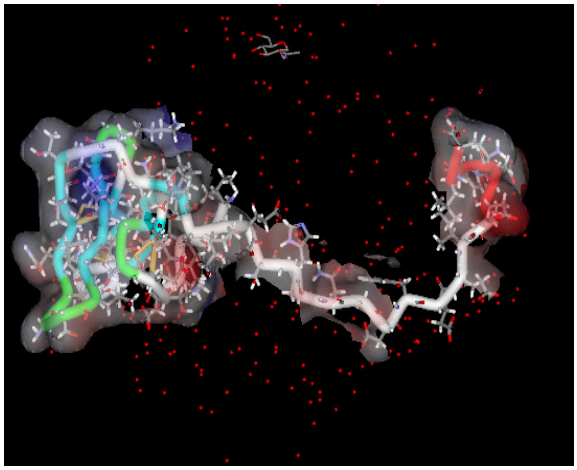

Figure 4 Hirudin-thrombin complex showing Surface showing Activity of Hirudin Molecule

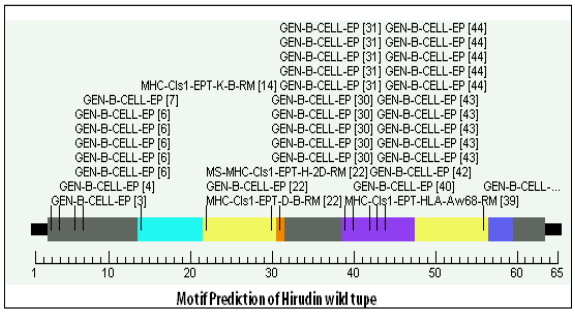

Figure 5. Motif-prediction of Hirudin

Conclusion:

As a polypeptide natural recombinant hirudin may elicit an immunological response in humans, this may also be due to the long phylogenetic distance from invertebrates (Song *et al.*, 1999). In the present study, generation of antihirudin antibodies was observed using immunodiffusion test. The r - hirudin radial immunodiffusion demonstrated binding of the human anti-hirudin, antibody to r – hirudin. The production of anti hirudin antibodies raise the question as to whether the generation of anti-hirudin antibodies interferes with the anticoagulant activities of r hirudin. The radial immunodiffusion assay in the present investigation demonstrated *in vitro* binding of the human anti hirudin antibody to r hirudin. The above characterization is taken from DS gene protein sequence site after running the hirudin sequence. The purpose was to give the proof for our results of hirudin strong antegenicity of hirudin observed in radial immunodiffusion assay in the present investigation. Using various tools of bioinformatics, characterization of hirudin was investigated. It is found that a segment of hirudin between amino acid number 10 to 30 is antigenic in nature and this epitope contains a sequence, " GQNLCLCEGSNVCGQGNKCIL". The average antigenic prospensity for hirudin is found to be 1.0245.

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