MOLECULAR DOCKING OF SUBTILISIN K2, FIBRIN DEGRADING ENZYME FROM INDONESIAN MOROMI WITH FIBRIN SUBSTRATE

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Abstract

Fibrinogen supplies the primary building block of the blood clot or thrombus after α-thrombin converts this fibrinogen to fibrin during the final phases of coagulation. When the homeostasis system is disrupted, blood clots that aggregate in the blood vessels can lead to thrombosis. Fibrin degrading enzyme from Bacillus subtilis K2 (Subtilisin K2) has many excellent characteristics with strong fibrinolytic activity. Bioinformatic analysis indicated that the enzyme molecule appeared to share conserved domain, with peptidase s8 super family also known as the subtilase family with its motif: Asp subtilase (motif: VAVIDSGIDSsH), His subtilase (motif: HGTHVAGTIAA) and Ser subtilase (motif: GTSMATPHVAG). Study on molecular docking between this fibrin degrading enzymes and the specific substrates, fibrin and fibrinogen was aimed to predict the mechanism of action. This analysis, revealed no productive interaction between Subtilisin K2 and fibrinogen. However, hydrolysis reaction is indicated strongly between Subtilisin K2 and fibrin substrate. Amino acid Asp19, His51, and Ser208 in the Subtilisin K2's active site interacted with Leu168, Ile171, and Leu172 of the fibrin substrate with [?]G of –19.4 kcal/mol that showed suitable substrate specificity. The fibrin degrading enzyme Subtilisin K2 tend to act more as fibrin degrading enzyme than as fibrinogen degrading enzyme.

INTRODUCTION

Fibrinogen and fibrin show overlapping important roles in wound healing, blood clotting, fibrinolysis, neoplasia, interactions of cellular and matrix, and also inflammatory response. Interactive sites on fibrinogen and fibrin adjusted these functions, some of which are concealed or otherwise not available on fibrinogen, and also they frequently evolve as a consequence of fibrin formation or fibrinogen–surface interactions[1]. Fibrinogen is a plentiful plasma protein. Fibrinogen provides the primary building blocks of thrombus during the final phases of coagulation, when α-thrombin converts it into fibrin clot. Increased concentrations of fibrinogen in the blood have been related with risk for thrombosis. In recent decades, many related studies on the abnormal fibrin structure as one of the causes of thrombosis. The high fiber density of fibrin clots and increased resistance to fibrinolysis have been consistently related with risk for thrombosis[2–5].

Fibrinogen comprise some structural domains with different functions. Domain that contains the cleavage sites for thrombin, the central E domain, is connected to two D domains consisting binding pockets for polymerization by two coiled coil domains that provide elasticity to this molecule[6]. The Aα-chain protrudes from the D domain and it forms the flexible αC domain, whereas the Bβ- and γ-chains end in the D domain[7]. Thrombin converts fibrinogen into fibrin through the cleavage of two fibrinopeptides A from the Aα chain (16 residues) and two fibrinopeptide B from the Bβ-chain (14 residues)[1,6]. The fibrinopeptide A is cleaved first and exposes GPR (Gly-Pro-Arg) binding site on the E domain of fibrin for attaching to the binding

pocket in the D domain. This interaction causes to the binding of several fibrin molecules, which result to the formation of fibrin polymer, also called the protofibril that consists of half-staggered, overlapping fibrin molecules (Figure 1).

The present bioinformatic work was carried out to elucidate the mechanism of bacterial fibrinolytic enzyme Subtilisin K2 to interact before degrade fibrin or fibrinogen. The information will be useful and used as the basic for further strategy in drug development for CVD. The three-dimensional model of the Subtilisin K2 protease from*Bacillus subtilis* K2 was developed by using SWISS-MODEL Workspace[8–11]. Our previous study revealed Subtilisin K2 posessed typical active site residue Asp19, His51, and Ser208 which interacts with Leu168, Ile171, and Leu172 of the fibrin with high binding affinity (ΔG: –19.4 kcal/mol)[12]. In this study, we observe the interactions that occur between the enzyme and several fibrin domains (domains A, B, C, D, E, F), and also interactions with several fibrinogen chains (α, β, γ chains).

MATERIALS AND METHODS

Materials

The protein sequence of fibrin degrading enzyme, Subtilisin K2 from*Bacillus subtilis* K2 (this isolate originated from moromi and was collected earlier (Syahbanu et al. 2020)) was retrieved from GenBank (*accession number* MN294987). It comprised of 262 amino acids and was saved in FASTA format. The 3D structure of Subtilisin K2 that was constructed using SWISS-MODEL Workspace (https://swissmodel.expasy.org/) program **(RRID:SCR_018123)** [8–11] was obtained by Syahbanu et al.[12]. Fibrin and fibrinogen structure was retrieved from RCSB PDB (PDB ID: 2HLO (fibrin) and 3GHG (fibrinogen)) as a receptor for the molecular docking with Subtilisin K2 as ligand.

Prediction of Motifs and Conserve Domain.

The presence of motif seequence in Subtilisin K2 protease was assessed by Myhits https://myhits.isb-sib.ch/cgi-bin/motif_scan[13], conserve domain was predicted using Conserved Domain Architecture Retrieval Tool (CDART) https://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi[14].

Molecular Docking Study

Molecular docking was performed between Subtilisin K2 model and fibrin, fibrinogen substrates. The docking analysis predicted the interaction site of Subtilisin K2 and fibrin, fibrinogen using CPORT (Consensus Prediction Of interface Residues in Transient complexes) webserver (https://milou.science.uu.nl/services/CPORT/)[15]. It was a physical and knowledge-based approach to predict interactive or binding site for protein interaction. High Ambiguity Driven protein–protein Docking (HADDOCK) webserver[16,17] was used to perform protein docking. HADDOCK (High Ambiguity Driven protein–protein Docking) webserver[16,17](https://milou.science.uu.nl/services/HADDOCK2.2/) use of chemical shift perturbation data resulting from NMR titration experiments, mutagenesis data, and bioinformatics predictions. The 3D Subtilisin K2 was docked by flexible docking method with the fibrin and fibrinogen molecule retrieved from Research Collaboratory for Structural Bioinformatics (RCSB) Protein Databank (https://www.rcsb.org/) using the HADDOCK webserver[16,17]. Prediction of the binding affinity in protein-protein complexes using Prodigy (Protein Binding Energy Prediction) webserver[18,19](https://bianca.science.uu.nl//prodigy/). LigPlot+ Version v.2.1 (https://www.ebi.ac.uk/thorntonsrv/software/LigPlus/install.html)[20]was used to generate schematic diagrams of protein-protein interactions.

RESULTS AND DISCUSSION

Motif and Conserved Domain Prediction

The prediction of conserved domain in Subtilisin K2 was performed through Myhits tool[13]. In this study, analysis of the enzyme encoded by fibrinolytic gene revealed conserved domain, shared with the peptidase s8 super family also known as the subtilase family, the second largest family of serine peptidases, both in terms of number of sequences and the characterized peptidases. Determination of the protein motif was carried out

using https://myhits.isb-sib.ch/cgi-bin/motif_scan[13]. Based on the results of the search for protein motifs, this enzyme has typical motifs, namely, Asp subtilase (motif: VAVIDSGIDSsH) (Figure 2), His subtilase (motif: HGTHVAGTIAA) (Figure 2), and Ser subtilase (motif: GTSMATPHVAG) (Figure 2). This motif indicated that the enzyme belongs to peptidase S8 family. In addition, based on the results of the motif search, several short motifs were found consisting of: ASN_GLYCOSYLATION motif, CK2_PHOSPHO_-SITE motif, and MYRISTYL motif.

Molecular Docking Analysis

The fibrin structure consists of three chains, namely α chains (domain A and domain D), β chains (domain B and domain E), and γ chains (domain C and domain F). Using ligplots, gave information on the interactions occuring between enzymes and substrates through hydrogen bonds and hydrophobic interactions. The more formation of hydrogen bonds and hydrophobic interactions, the better the interactions between enzymes and substrates. This bioinformatic study show that domains A and D of fibrin have favourable interactions with enzymes but no hydrolysis reaction occurs in the two domains (Figure 3). The results from using Prodigy[18,19] shows high binding affinity energy (ΔG) and dissociation constant (Kd) in A domain (ΔG = -14.9 kcal/mol; Kd = 1.1E-11 M) and D domain (ΔG = -14.8 kcal/mol; Kd = 1.4E-11 M). Binding affinity energy of A and D domain were higher than C and F domain of fibrin, and also dissociation constant of A and D domain were lower than C and F domain of fibrin (Table 1).

The binding affinity (or binding free energy) (ΔG), represents whether complex formation takes place favourably or not in a specific conditions[21]. Binding affinity is the strength of the binding interaction between a single biological macromolecules (such as protein or DNA) to its ligand/binding partner (such as protein, drug, or inhibitor). The more negative of binding free energy (ΔG) value, the stronger interaction/binding between receptor and ligand, and vice versa. Binding affinity is used to assess and rank order strengths of binding partners interactions and measured by the equilibrium dissociation constant (Kd). Therefore, the higher the Kd value, the lower the binding affinity of the ligand to its target site and vice versa. Binding affinity between the two molecules is affected by non-covalent intermolecular interactions such as hydrogen bonding, electrostatic interactions, hydrophobic interaction, and Van der Waals forces, as well as the presence of other molecules.

The highest negative value of ΔG with the lowest Kd value occurs in the beta chain binding (domain B and E) of fibrin (Figure 4) with the Subtilisin K2 enzyme (Table 1). However, the hydrolysis reaction occurs in the E domain as there were catalytic triad residues (Asp19, His51, and Ser208) of the enzyme model interacting with the E domain (Figure 4). The three amino acid residues that act as catalytic triads were not located in other domains. Interaction of C and F domain of the gamma chain on fibrin and the enzyme (Figure 5) show the highest values of ΔG (low affinity energy) and Kd (Table 1), which means that the hydrophobic interactions and hydrogen bonds occuring between the enzyme and these two domains are much less when compared with other domains.

Hydrogen interactions can take place between the -OH group with O, the -NH group with O, the -NH group with N, and the -OH group with N. Hydrophobic interactions occur between the aromatic ring with the -CH group in amino acid residues[22]. Docking results demonstrated that Asp19, His51. and Ser208 as Subtilisin K2's the active sites interact with Leu168, Ile171, and Leu172 of E domain in the fibrin substrate that might act as the substrate specificity.

Hydrophobic interactions take place more frequently between enzyme (fibrin degrading enzyme) and substrates (fibrin and fibrinogen) than hydrogen bonds. This might be caused by the fact that the enzyme and its substrate are large molecules, and the interactions which occur between those two large molecules are interfaces interactions. The Ligplot+ program has four numeric parameters related to the HBPLUS program and were used in calculating the potential hydrogen bonds and non-bonded contacts (such as hydrophobic interactions).

The two parameters define the hydrogen bonding and denote to the maximum hydrogen-acceptor (H-A) and donor-acceptor (D-A) distances and the default range of maximum H-A and D-A distance value were 2.70 Å

and 3.35 Å, respectively. Increasing these values cause more additional interactions. The next two parameters define the distances range of non-bonded contacts (such as contacts between atoms that are neither covalently bonded, nor interacting via hydrogen bonds) and the default range of this contact distance value were 2.9-3.9 Å.

Based on ligplot analysis, amino acid residues which are the active site of fibrin degrading enzyme (Ser208 and His51) interacted with amino acid residues of domains A (Figure 3) and D (Figure 3) on the fibrin substrate (Ile154, Glu151, Arg171, and Leu150). The enzyme's active site also interacted with Leu168 of domain B (Figure 4), where Leu168 of domain E also interacted with active site of the enzyme (Figure 4). Residues from the E domain on the fibrin substrate that interacted with active site of the enzyme (Asp19, His51, dan Ser208) were Leu168, Ile171, and Leu172. Whereas, Leu131, Glu132, and Val128 are residues from the C (Figure 5) and F (Figure 5) domains that interacted with active site of the enzyme.

Fibrinogen is composed of two symmetric half molecules (dimer), each dimer is composed of one assembly of three different polypeptide chains Aα, Bβ and γ (trimer) (Figure 1). The six peptide chains are held together by 29 disulfide bonds and sulfhydryl groups is not contained in this fibrinogen chain. The half-cystine residues are more concentrated in the three clusters along each chain (one N-terminal, one intermediate, and one C-terminal cluster) in the fibrinogen primary structure[23,24]. Based on analysis using Prodigy[18,19], interaction between Bβ chain and Subtilisin K2 showed more negative values of ΔG (high binding affinity) (-15.6 kcal/mol) and low Kd (3.9E-12 M) followed by γ and Aα chain.

Catalytic triad residues were not indicated in the docking result between Subtilisin K2 and the three chains of fibrinogen (Figure 6, Figure 7). Interactions between residues of active site of the enzyme with residues of the fibrinogen chain (Aα, Bβ, and γ chains) also occur. However, only His51 and Ser208 residues of enzyme's active site residues were found to have interactions with residues from the Aα, Bβ, and γ chains. Phe117 of the α chain, Trp125 and Leu121 of the β chain, as well as Phe54, Asp53, and Thr57 of the γ chain showed interactions with His51 and Ser208.

Based on bioinformatic study, interaction between Subtilisin K2 enzyme and E domain of the fibrin substrate showed value of ΔG and Kd 1.2 times greater and 0.0016 times lower than value of ΔG and Kd of interaction between this enzyme and Bβ fibrinogen chain. It showed that Subtilisin K2 enzyme tend to interact much more with fibrin, and thus this enzyme more act as fibrin degrading enzyme than as fibrinogen degrading enzyme because might be catalytic reaction occur on domain E of the fibrin. Fujita et al.[25] reported that the cleavage fibrin chains by nattokinase was 6 times more efficient than plasmin, but the cleavage fibrinogen chains by nattokinase was 3 times more efficient compared to plasmin and measured with kcat Km^{-1}. It can be concluded that the enzyme nattokinase was less sensitive to cleavage fibrinogen chains, but more sensitive to cleavage fibrin chains.

CONCLUSION

Molecular docking analysis showed that Subtilisin K2 and fibrinogen and also fibrin use an extended binding pattern in their interactions with the important residues to run the enzyme activity. Furthur analysis demonstrated that hydrolysis reaction may occur between Subtilisin K2 and E domain of fibrin. Asp19, His51, and Ser208 as Subtilisin K2's active site interacted with Leu168, Ile171, and Leu172 of the fibrin substrate that might be potent as substrate specificity. There were no indication of interaction between catalytic triad residues of Subtilisn K2 and the three chains of fibrinogen. Therefore, it can be concluded that Subtilisin K2 tend to act more as fibrin degrading enzyme than fibrinogen degrading enzyme.

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DECLARATIONS

Disclosure of Potential Conflicts Of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Approval/Ethical Disclosure

- This manuscript is original, has not been published elsewhere and has never been submitted to any journal other than *PROTEINS: Structure, Function, and Bioinformatics*
- The bacteria as source of the gene and enzyme was isolated from local soy fermented food.
- This research did not use any animal or human subject. All methodology applied were in accordance with the reference cited.
- The data taken for Result and Discussion were original and came from the experiments stated in the methodology.
- This research did not involve the type of work that could be a threat to public health, does not produce any biological agents or toxins.
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- Puspo E Giriwono, a member of PhD supervision is a lecturer and researcher at Departement of Food Science and Technology, Bogor Agricultural University.
- Raymond Tjandrawinata, a member of PhD supervision is associated with Dexa Laboratory for Biomolecular Science, Dexa Medica Pharmaceutical Company.

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Figure Caption

Figure 1 Fibrinogen and fibrin structure

Figure 2 Motif sequences of fibrin degrading enzyme from *Bacillus Subtilis* K2

Figure 3 Interaction between enzyme model with alpha chain of fibrin (A and D domain) (blue circle and pink licorice = active site of enzyme; red circle and orange licorice = substrate specificity of fibrin).

Figure 4 Interaction between enzyme model with beta chain of fibrin (E and B domain) (blue circle and pink licorice = active site of enzyme; red circle and orange licorice = substrate specificity of fibrin).

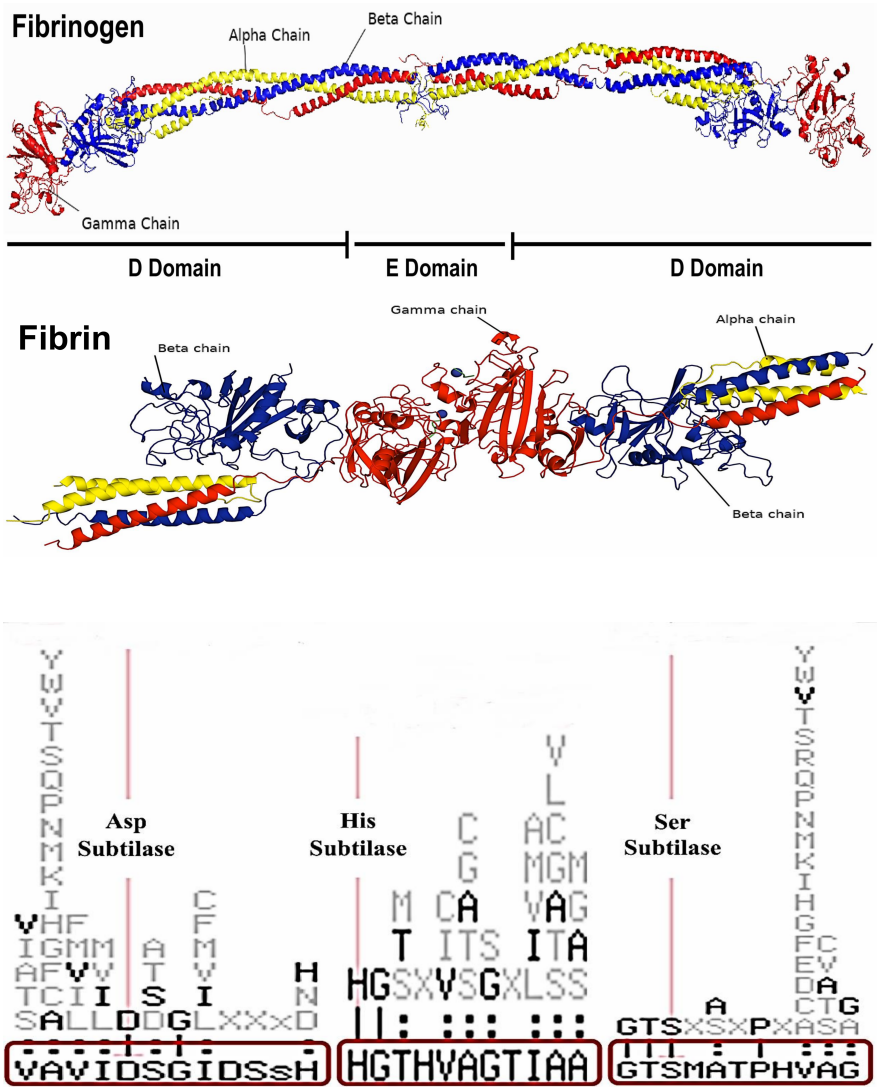
Figure 5 Interaction between enzyme model with gamma chain of fibrin (C and F domain) (blue circle and pink licorice = active site of enzyme; red circle and orange licorice = substrate specificity of fibrin).

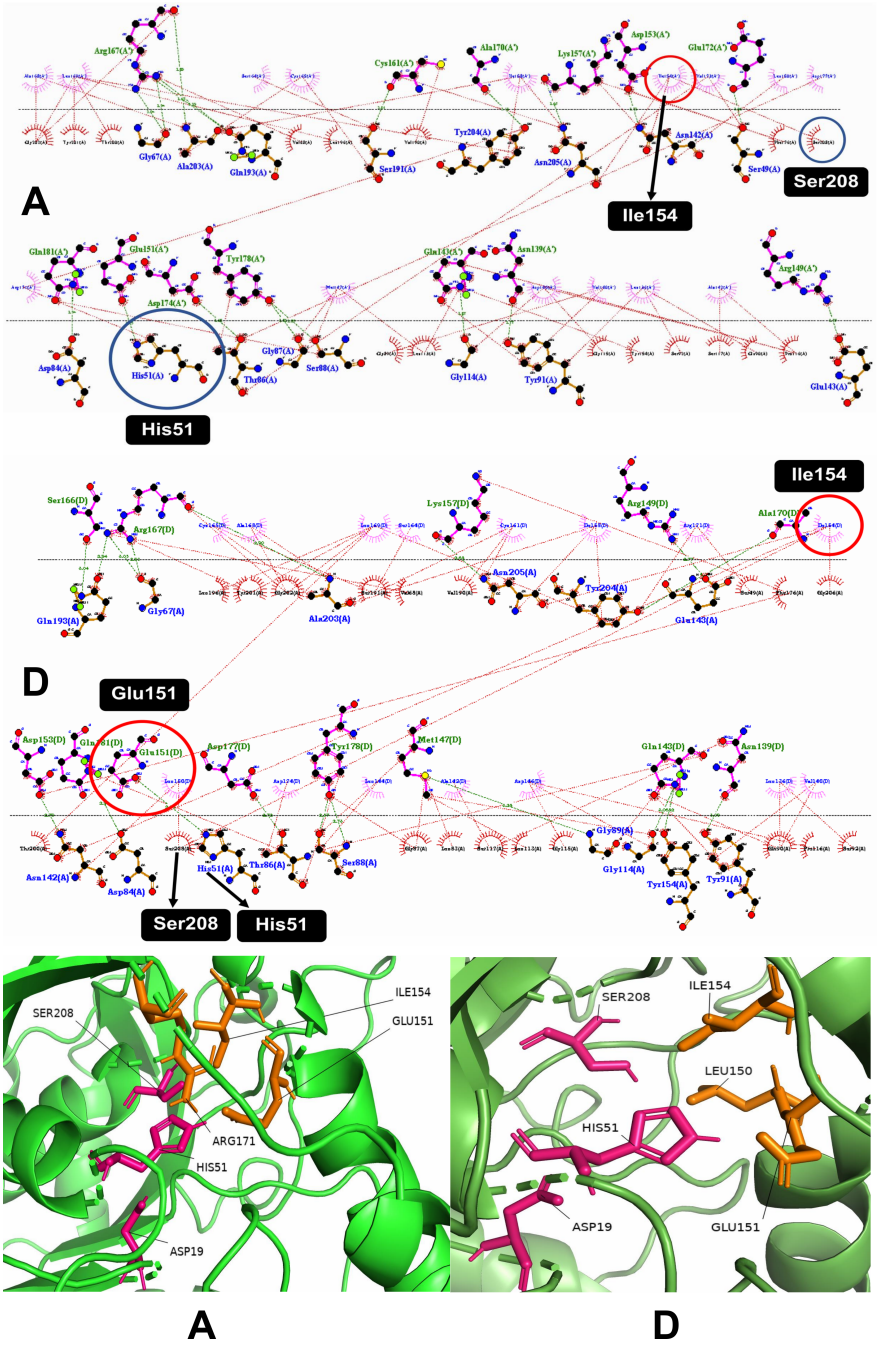
Figure 6 Interaction between enzyme model with α, β, and γ chain of fibrinogen (blue circle = enzyme's active site; red circle = substrate residue that interacted with enzyme's active site).

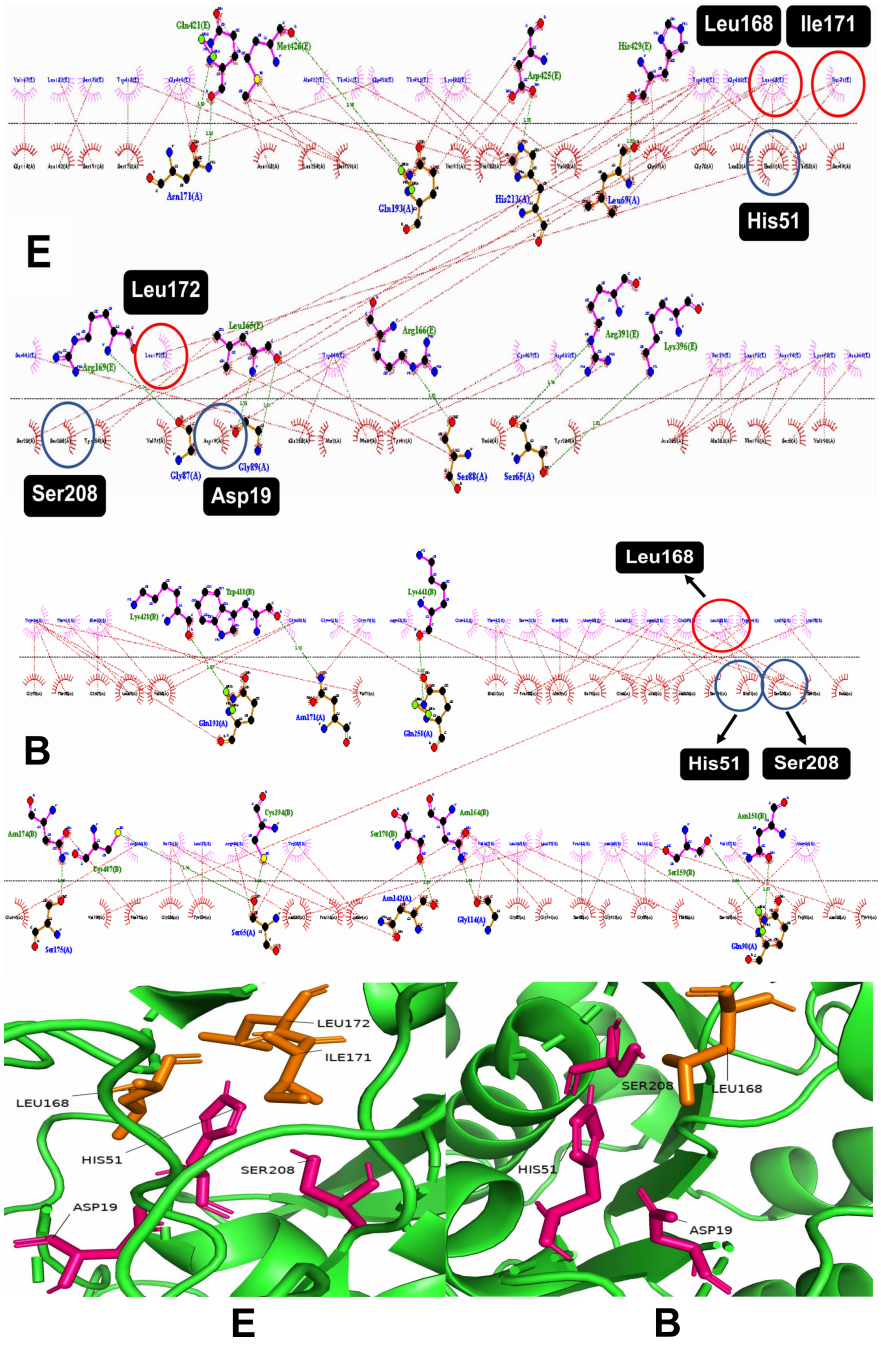
Figure 7 Interaction between enzyme model with α, β, and γ chain of fibrinogen (pink licorice = enzyme's active site; orange licorice = substrate residue that interacted with enzyme's active site).

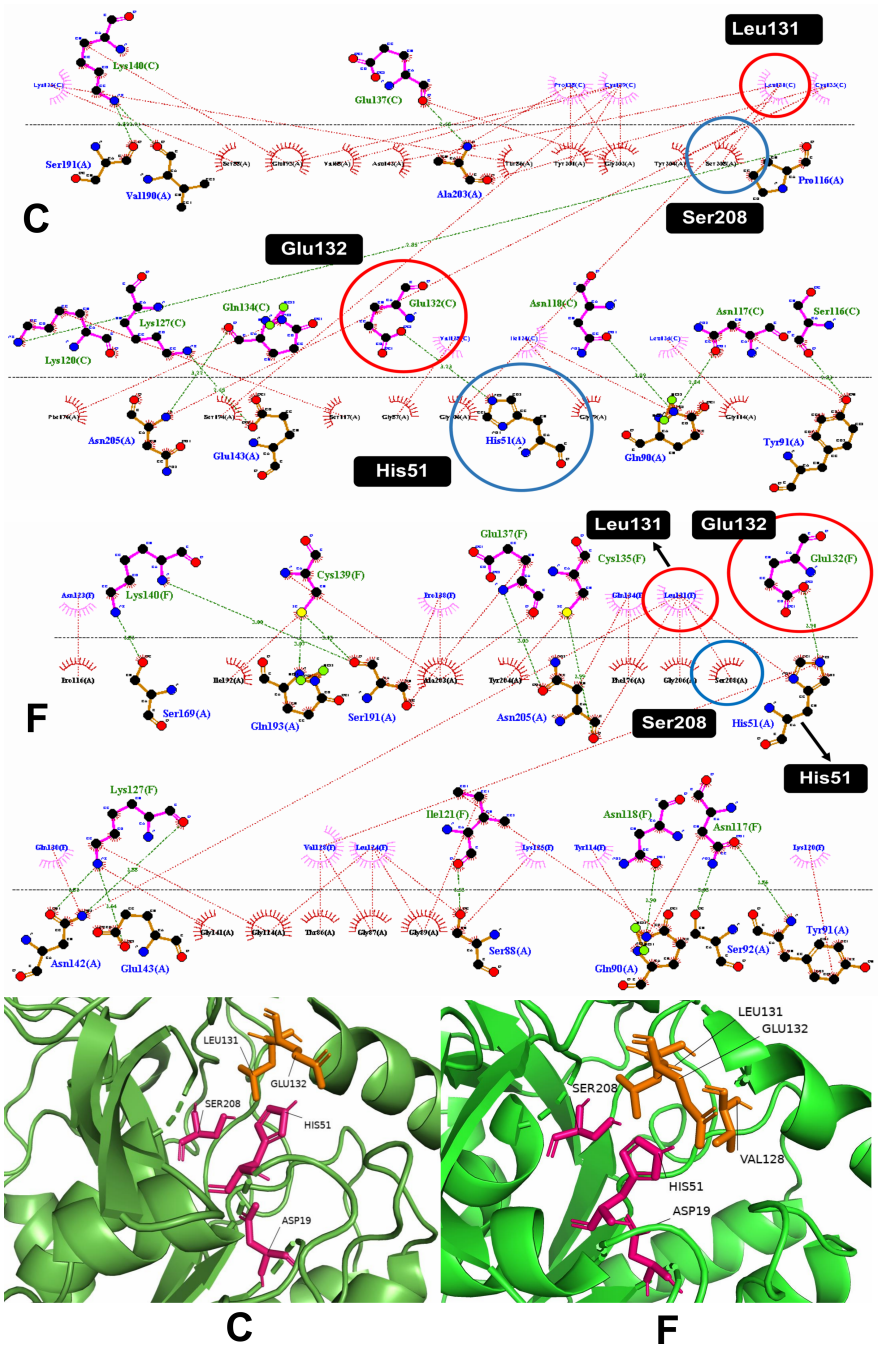
Table Caption

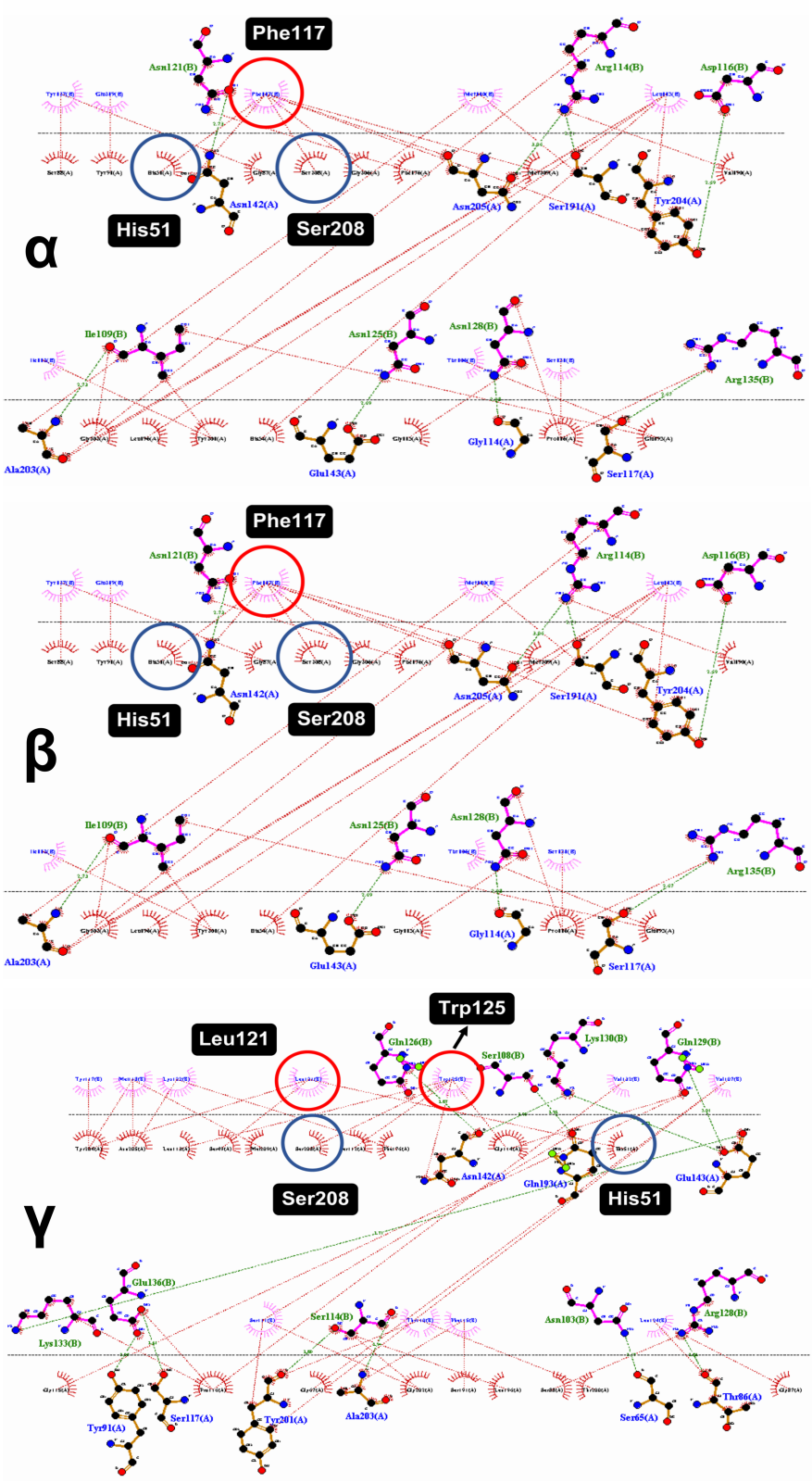
Table 1 Result of Docking of of enzyme model with the substrate

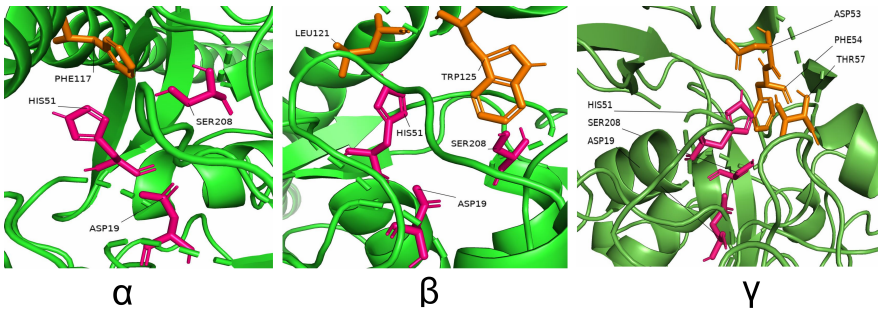












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