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RESEARCH ARTICLE

In vitro and *in silico* studies on fibrinolytic activity of nattokinase: A clot buster from *Bacillus* sp.

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BACKGROUND: Nattokinase (NK) is a serine protease enzyme of the subtilisin family. It exhibits a strong fibrinolytic activity. The fibrinolytic enzymes from *Bacillus* sp. have attracted interest as thrombolytic agents because of their efficiency in the fibrinolytic process including plasmin activation.
METHODS: In the present study, VIT garden soil was collected and subjected to isolation process in order to screen for the NK production. Screening for NK enzyme was performed by radial caseinolytic assay. The production of NK enzyme was done in two different production medium for comparative studies. The NK enzyme was purified by gel permeation chromatography. The activity of the purified NK was checked by clot lysis and casein digestion assay. To investigate the structural basis of NK and fibrinogen interaction and also to identify the best binding mode, molecular dynamics and docking studies were performed.
RESULTS: Based on the morphological and biochemical characterization, the isolate was identified as *Bacillus* sp. The overall purification fold of NK was about 3 with the specific activity of 664U/mg and 9.9% yield. Homogeneity of the purified enzyme was analyzed and confirmed by the single band obtained in SDS-PAGE. Molecular weight of the purified protease was estimated as 25 kDa. Purified NK enzyme exhibited 97% of effective clot lysis activity. The NK was docked in to the knob region of the fibrinogen at its binding site using Dock server. A total of 26 residues of fibrinogen and 29 residues of NK constitute the interface region. However, 9 residues of fibrinogen (THR238, MET264, LYS266, ARG275, THR277, ALA279, ASN308, MET310, and LYS321) and 8 residues of NK (GLY61, SER63, THR99, PHE189, LEU209, TYR217, ASN218, and MET222) are involved in intact binding.
CONCLUSIONS: A significant amount of NK enzyme was obtained from *Bacillus* sp. The docking analysis revealed that the NK and fibrinogen adopt an extended binding pattern and interacts with the crucial residues to exhibit their activity.

Keywords nattokinase (NK), *Bacillus* sp., clot busters, docking

Introduction

Nattokinase(NK, EC 3.4.21.62) was first extracted and purified from natto, a Japanese food, which is a fermented soybean. NK is a serine protease: its molecular weight is 27-32 kDa. The enzyme has a dual function of hydrolyzing blood thrombi in vivo and also involved in the conversion of plasminogen to plasmin (Matta and Punj, 1998). Myocardial infarction is a very common death cause in current scenario. World Health Organization reports state that, nearly 8 million people suffer from myocardial infarction. Over the past decades, enzyme therapy has been widely used to treat various forms of cardiovascular diseases. To stop any form of bleeding in the vascular system, circulating platelets respond very quickly and seal the leak by forming a blood clot. Not all forms of blood clots are desirable. Thrombosis is a blood clot in the blood vessel. The clot gets detached from the vascular wall and it travels and blocks any part of the circulatory system such as the lungs, brain, etc. It is the fibrin which is involved in many heart attacks, since cardiac arrest usually occurs after plaque's cap fractures, causing blood to clot over the rupture thus blocking the blood flow. The process of forming a clot is complex and involves several enzymes. However, the body mainly produces one central enzyme for dissolving a clot, plasmin. Scientists have found out that the properties of NK are similar to plasmin. NK is particularly

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effective because it enhances the body's natural ability to fight blood clots in several different ways. It dissolves fibrin directly and appears to enhance the body's natural production of both plasmin and other clot-dissolving enzymes like urokinase. An in vivo study was undertaken to demonstrate the thrombolytic activity of NK, plasmin and elastase on an induced clot in the common carotid artery of laboratory rats. The results indicate that the thrombolytic activity of NK is stronger than that of plasmin or elastasein vivo conditions. NK also enhances the production of tissue plasminogen activator which promotes the transformation of plasminogen to plasmin, thus fibrin is degraded. As of now, research has been focused on isolation and screening of microbes for production of enzyme with high fibrinolytic activity (Chang et al., 2000) also purification and characterization of newly found enzyme. Hence NK has the capability to cleave active recombinant prokaryotic plasminogen activator inhibitor into low molecular weight fragments (Venkataraman et al., 2009). So far, many researchers had focused their efforts on the isolating and screening of microorganisms for enzyme production with high fibrinolytic activity. However, most results showed that *Bacillus* spp. produce a variety of extracellular and intracellular fibrinolytic enzymes/NK. The fibrinolytic enzymes from *Bacillus* sp. have attracted interest as thrombolytic agents because of their efficiency in the fibrinolytic process including plasmin activation. In this study, we had screened a bacterium to produce NK and investigated the structural basis of NK and fibrinogen interaction by molecular dynamics and docking studies.

Materials and methods

Chemicals

Chemicals used were nutrient agar, nutrient broth, agarose, magnesium sulfate, orthophosphate, phosphate buffer solution, tris base, trisHCl and peptone, casein, ammonium sulfate, Bovine serum albumin, sodium potassium tartarate, copper sulfate, skimmed milk powder, shrimp shell. All the chemicals used were of HI Media Chemicals, Mumbai.

Isolation of NK producing *Bacillus* sp.

Soil sample was collected from VIT nursery and serial dilution was done up to 10^{-10}. Two sets were maintained, one was subjected to heat shock, the other non-heat shock. Spread plate technique was performed on nutrient agar to obtain isolated colonies. The isolated colonies were selected based on morphology and streaked on *Bacillus* differential media. Based on morphological and biochemical characterization *Bacillus* sp. was confirmed.

Caseinolytic assay

Nutrient agar was supplemented with 2% casein (w/v) was poured. Primary streaking was done on the plate and it was incubated at 37°C for 24 h. The organism which showed zone of hydrolysis was selected for further studies (Dubey et al., 2011).

Nattokinase production

The production of NK enzyme was done in two different production medium for comparative studies. 100 mL of production medium was prepared with shrimp shell and without shrimp shell separately which contains peptone 0.5%, potassium orthophosphate 0.1%, magnesium sulfate 0.05% and sodium chloride 0.5%. To the production medium broth 1% of inoculum was added. The production medium with shrimp shell was incubated at 37°C, 120 r/min for 48 h. The other medium without shrimp shell was incubated at 37°C, 120 r/min for 7 days .

Radial caseinolytic assay

Screening for NK enzyme was performed by supplementing nutrient agar with 2% casein, 0.2% plasma. Culture supernatants were acquired by centrifuging the production medium. On the medium, wells were made with the help of a sterile borer including positive and negative control. 100 µL of culture supernatant was added and plated were kept at 4°C for 30 min, so that radial diffusion occurs (Dubey et al., 2011). The plates were then incubated at 37°C for 24 h. The organism showing maximum zone of lysis was used for further assay.

Purification

For the separation of enzyme, the production medium was centrifuged at 8000 r/min for 20 min at 4°C. The crude enzyme obtained was subjected to ammonium sulfate precipitation. Initially 30%, then 60%, and finally 80% ammonium sulfate was added in cold conditions (at 4°C), while the beakers were mechanically stirred for several minutes after which it was kept in the refrigerator overnight for settling of the fractionated proteins. After overnight incubation the crude enzyme was centrifuged at 10000 r/min for 30 min. Pellet obtained was dissolved in phosphate buffer (pH 7.2) (Mohanasrinivasan et al., 2013).

Ultra filtration

The saturated protein was further subjected to ultra-filtration. The molecular cut off (MWCO) of 50 kDa was used and the sample was loaded and centrifuged at 1000 r/min for 20 min at 4°C. The filtrate was collected and stored in refrigerated

conditions for further purification (Mohanasrinivasan et al., 2013).

Gel permeation chromatography

The filtrate obtained was then analyzed by gel permeation chromatography. The enzyme was introduced into a column containing sephadex G gel. It was run by continuous addition of phosphate buffer and the aliquots were collected in fractions and its OD was measured in UV spectrophotometer (Mohanasrinivasan et al., 2013).

Holmstorm method

To determine the enzyme activity, modified Holmstorm method (Holmstrom, 1965). In 2.0 mL eppendorff tubes, 200 μL of human blood and equal amount of 25 mM of $CaCl_2$ was added and placed in a water bath at 37°C for 30 min. When the blood clots, the filtered enzyme was added in various concentration of 20 μL–100 μL. The tubes were incubated for 4 h at 37°C. After incubation, the tubes were inverted and analyzed for the extent of clot lysis and the percentage of clot lysis activity was determined.

Casein digestion assay

Tubes were prepared containing 0.1 mL of 2% casein, 0.7 mL of 0.1 M sodium phosphate buffer and 0.1 mL enzyme from each of crude extract, ammonium sulfate saturated fractions, ultra-filtrated and gel permeated solutions and incubated for 5min in room temperature. The reaction was terminated by adding 0.1 mL of 1.5 M trichloroacetic acid. Each tube was allowed to stand at 4°C for 30min, after which the samples were centrifuged at 8000 r/min for 10min and absorbance of the supernatant was measured at 560nm. The readings were converted into their tyrosine equivalents and amount of amount proteins were determined.

Molecular weight determination by SDS-PAGE

The isolated protein was run on SDS-PAGE and its molecular weight was determined by comparing it with a standard marker (Laemmli, 1970; San-Lang et al., 2009)

Molecular docking

To investigate the structural basis of NK and fibrinogen interaction and also to identify the best binding mode, molecular dynamics and docking studies were performed. The structural model of NK used for docking was retrieved from PDB (4DWW: A: R- 1.74Å) The benefit of using molecular dynamics simulation was to partially fix conformational problems and solvent errors associated with X-ray structures. The molecular dynamics simulation was performed in water (simple point charge model) at 300 K for 50 ns by using GROMACS 4.5.4 (GROningenMAchine for Chemical Simulations). The structure was energy minimized by steepest descent method. This refinement would improve the relative stability of the structure (Van Der Spoel et al., 2005). The docking studies predicted the interaction site of NK sites using KFC (Knowledge-based FADE and Contacts) server 2. It was a physical and knowledge-based approach to predict binding hot spots for protein interaction. Haddock (High Ambiguity Driven protein–protein Docking) server was used to perform protein docking. Haddock, use of chemical shift perturbation data resulting from NMR titration experiments, mutagenesis data or bioinformatics predictions. The 3D NK was docked by flexible docking method with the fibrinogen molecule (PDB ID: 2HLO) using the HadDock server (Dominguez et al., 2003)

Results and discussion

A total of 15 different colonies were isolated from VIT garden soil, eight isolates were selected on the basis of its appearance and features. The preliminary screening was carried out for all the 8 isolates on casein agar of which one strain B2 showed prominent translucent zone.The results were compared according to the description in Bergey's *Manual of Determinative Bacteriology.* The Gram's staining results showed gram positive with rod shaped morphology. An individual colony of white coloration was observed with irregular, creamy, fast growing colonies, spore forming and non- motile. (Fig. 1 A,B) . A clear distinct zone was observed on the radial caseinolytic assay agar plate with 12 mm and surrounding was appeared to be transparent. (Fig. 2 A, B) The extracted NK was purified to homogeneity. The 30%-60% $(NH_4)_2SO_4$ precipitate was subjected to Sephadex G-150 gel filtration chromatography, which furnished an elution profile with several peaks. Fractions showing highest NK activity from the respective fraction numbers were pooled (Fig. 3). The overall purification fold of NK was about 3 with the specific activity of 664 U/mg and 9.9% yield (Table 1). Clot lysis activity was observed with highest activity at 100μL concentration showing 97% of clot lysis (Fig. 4). The enzyme was subjected to SDS-PAGE on a 10% polyacrylamide slab

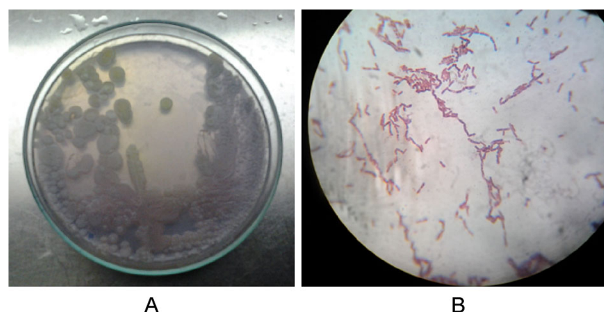

Figure 1 *Bacillus* sp. (A) Pure culture (B) Microscopy 40×.

gel where the purified protein exhibited a single band (Fig. 5) indicating homogeneity of the preparation. Molecular weight of the protein was determined to be 25 kDa corresponding to the BSA marker protein. The NK structure showed energy minimized at 302 steps with potential energy –4.4e + 05 and with the maximum force 9e + 02 on atom 2111 (Fig. 6). On molecular dynamics simulation for 50ns, RMSD and RMSF plot were generated to study the stable conformation of the protein. The plot showed that the root mean square deviation was below 0.2 nm and most of the residue fluctuations were below 0.2 nm. To study the hydrogen bonds, the hydrogen bond existence map was generated. Consistency and abundance of hydrogen bonds indicated that structure was compact and very stable (Fig. 7). Number of hydrogen bonds ranges from 180 to 210 during the molecular dynamic simulation for time period 50 ns (Fig. 8). Gromacs energies (potential, kinetic and total energy plots) of the model were also generated (Fig. 9).This has produced energetically more favorable hydrogen bond geometries and interactions. Hot spots of protein interaction were the regions that have greater probability of being the interface region and hence the binding domain. This hotspot was predicted using KFC server. Accordingly, the NK was docked in to the knob region of the fibrinogen at its binding site using HadDock server

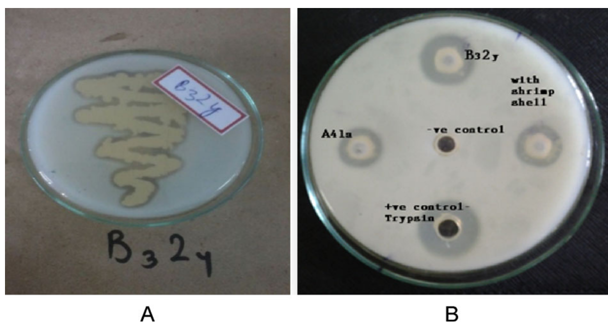

Figure 2 (A) *Bacillus* sp. showing zone of hydrolysis on casein plate. (B) Hydrolysis of casein by *Bacillus* sp. expressed in mm.

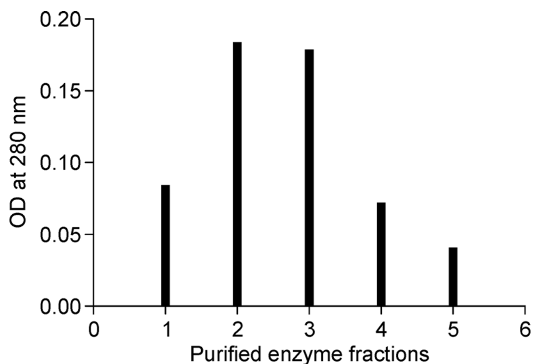

Figure 3 Elution profile of NK enzyme extracted from *Bacillus* sp.

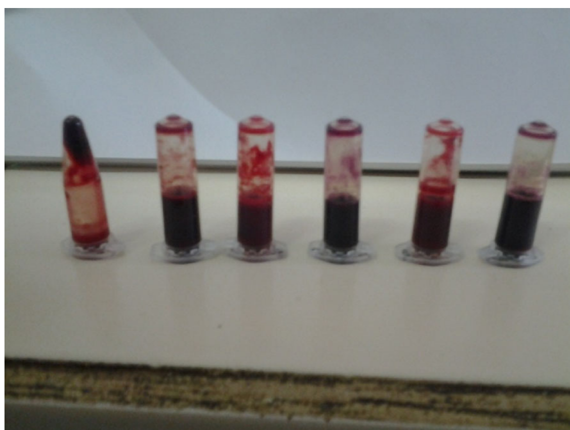

Figure 4 Clot lysis activity.

Figure 5 SDS-PAGE of NK enzyme.

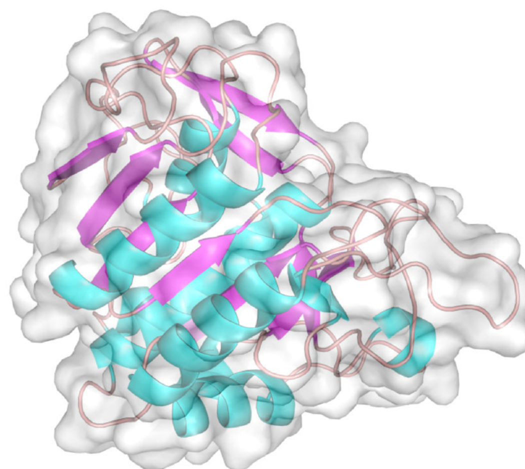

Figure 6 Structure of NK (4DWW) visualized using Pymol.

Table 1 NK enzyme activity

Sample	OD	µ mol	Activity (µmol /mL)	Activity *dilution factor	Total activity (U*mL)	Total protein (mg*mL)	Specific activity (U/mg)	Fold purification	% Yield
Crude	0.08	192	128	256	12800	63.5	202	1	100
Ammonium sulphate precipitate	0.1	242	161	323	1613	47.1	34	0	13
Ultra filtration	0.11	267	178	356	1424	13.2	108	1	11
Gel permeation	0.13	317	211	423	1268	1.9	664	3	9.9

(Table 2). NK recognized fibrinogen with an extended binding surface. The interface residue between NK and fibrinogen was found. A total of 29 residues of NK and 26 residues of fibrinogen constitute the interface region (Table 3). However 8 residues of NK (GLY61, SER63, THR99, PHE189, LEU209, TYR217, ASN218, and MET222) and 9 residues of fibrinogen (THR238, MET264, LYS266, ARG275, THR277, ALA279, ASN308, MET310, and LYS321) were involved in intact binding. Hence these residues may favor the formation of functionally efficient NK-fibrinogen complex. This study suggesting that these fibrinolytic enzymes were the members of the subtilisin family of serine proteases (Bryan, 2000) .Studies have already reported to suggest using recombinant technology inorder to increase the productivity .When the nucleotide sequence of recombinant natto-1.3 kb and wild natto strain obtained from fermented Kombucha were comparatively studied. The nucleotide sequence of recombinant had an open reading frame of 1332 base pairs encoding 106 amino acids for signal peptide and 275 amino acids for mature subtilisin. It showed 100, 99.74% and 98.69% identities with subtilisin NAT, subtilisin E and subtilisin J from *Bacillus subtilis*, respectively. While sequence of the other one has 1088 base pairs encoding only 87 amino acids for signal peptide and 275 amino acids for mature subtilisin (Motaal et al., 2015). A shuttle vector constructed by fusion of *Staphylococcus aureus* plasmid pUB110 with suicidal R6K plasmid origin of *Escherichia coli* was used for the expression of nattokinase in *Bacillus subtilis.* The resultant hybrid plasmid pUB110/R6K displayed full structural stability, leading to a high-level production of recombinant nattokinase (Chen et al., 2007). Altering the NK gene, wild strain 10 element region of PaprN showed increased production of nattokinase by 136% which is far greater than those reported in literatures. (Wu et al., 2011). Thus further recombinant technology can be used to improve the strain potency and to proceed for scale-up process.

Table 2 The Haddock score and energy values of NK-fibrinogen complex

Parameter	Values
HADDOCK score	−114.3 ± 4.7
Cluster size	117
RMSD from the overall lowest-energy structure	0.7 ± 0.5
Van der Waals energy	−66.6 ± 4.0
Electrostatic energy	−316.7 ± 32.1
Desolvation energy	14.8 ± 11.2
Restraints violation energy	8.4 ± 11.47
Buried surface area	2040.2 ± 58.1
Z-score	−1.0

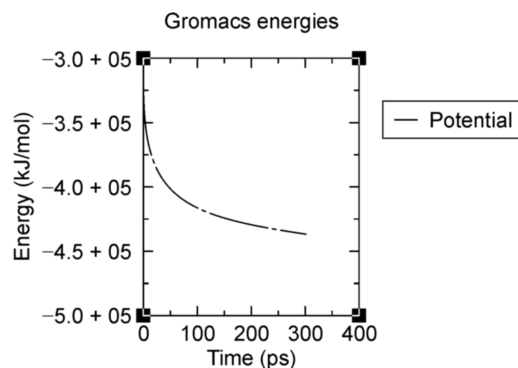

Figure 7 Potential energy plot showing energy minimization (using GROMACS).

Table 3 Interaction analysis of NK-fibrinogen complex representing the interface and interacting residues

Macromolecule	Interface residues	Interacting residues
NK	Ser53, Glu54, Gln59, Asp60, Ser62, His64, Asp97, Ser98, Gly100, Ser101, Pro129, Asn155, Glu156, Asn181, Gln185, Arg186, Ala187, Ser188, Val203, Ser204, Gln206, Pro210, Gly211, Thr213, Tyr214, Gly215, Ala216, Gly219, Ser221	Gly61, Ser63, Thr99, Phe189, Leu209, Tyr217, Asn218, Met222
Fibrinogen	Lys173, His234, Leu235, Gln239, Ser240, Ala241, Pro243, Ala263, Val267, Gly268, Pro269, Ala271, Asp272, Tyr278, Tyr280, Asp291, Phe303, Phe304, Ser306, Gly309, Gln311, Asn319, Asp320, Asn337, Lys338, Phe389	Thr238, Met264, Lys266, Arg275, Thr277, Ala279, Asn308, Met310, Lys321

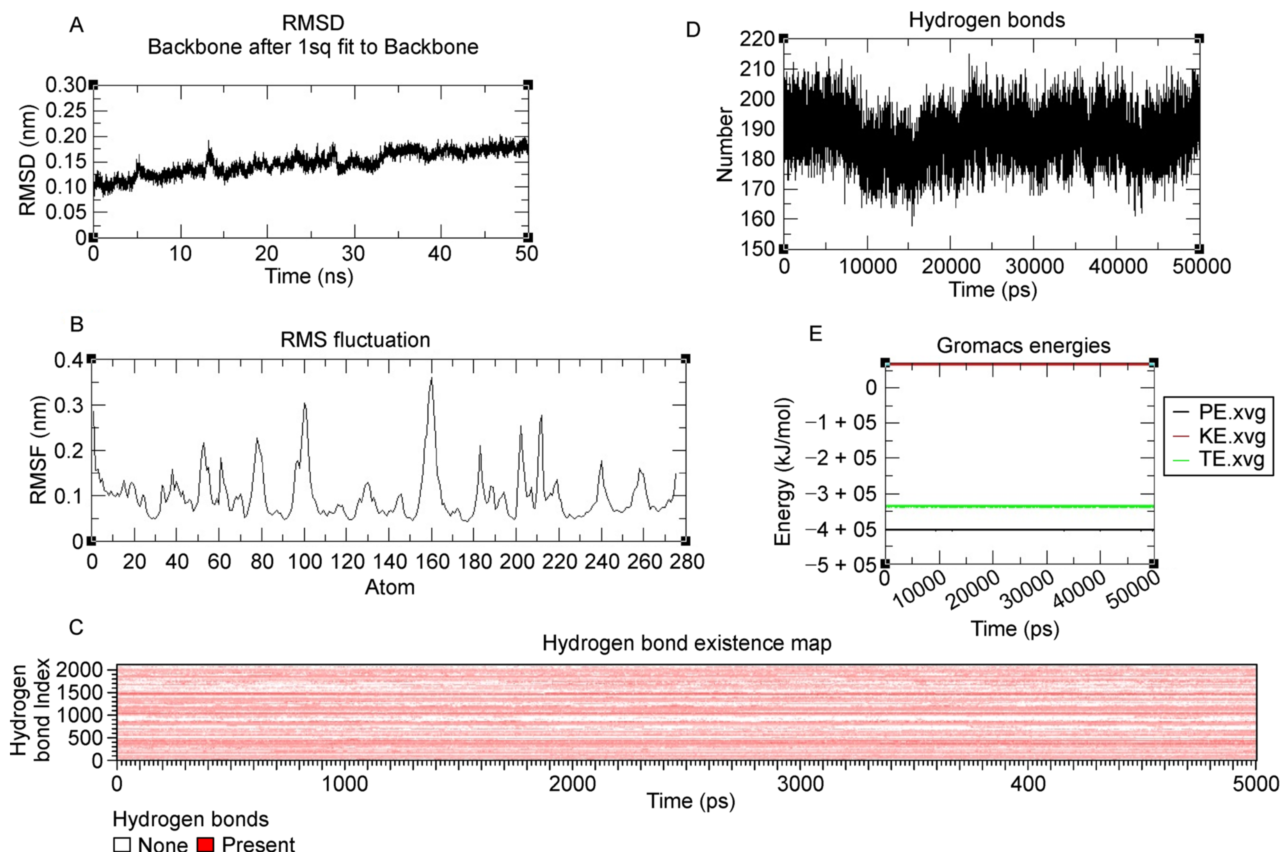

Figure 8 Molecular dynamics simulation results. (A) RMSD graph – RMSD of structure is within 0.2 nm suggesting the stability of NK structure. Structure reaches stable conformation after 30 ns during simulation. (B) RMSF plot – Most of the residues (other than residues at position 53, 79, 100, 160, 205, 212) have fluctuaion less than 0.2 nm which confirms the stable conformation of the NK structure. (C) Presence of hydrogen bond in each atom of NK is shown in hydrogen bond existence map. Here red and white color represents presence and absence of hydrogen bond respectively. (D) Number of hydrogen bonds within protein structure is shown in the graph. (D) Negligible variation in total energy was observed during the simulation study. Constant energy graph represents the stabilty of the structure.

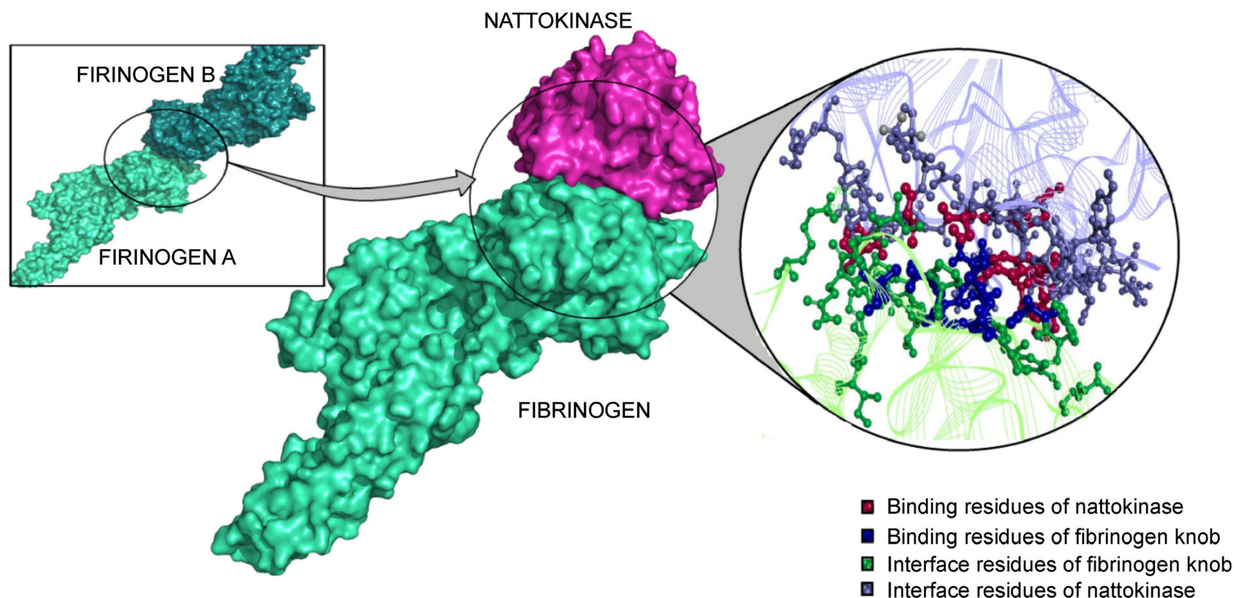

Figure 9 NK docked to fibrinogen knob at its binding site using Haddock server.

Conclusion

Hence, this study concludes, the protein of interest exhibits immense potential to lyse blood clots. A significant amount of NK enzyme was obtained from *Bacillus* sp. The docking analysis revealed that the NK and fibrinogen adopt an extended binding pattern and interacts with the crucial residues to exhibit their activity. Further optimization will help us in understanding the optimum level of enzyme activity.

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Compliance with ethics guidelines

Mohan, Priya, Swaroop, Sourav, Srinath, Sweta., Merlyn & Subathra declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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