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Research Article

In silico analysis of Nattokinase from *Bacillus subtilis* sp natto

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ABSTRACT

Nattokinase or subtilisin NAT (EC 3.4.21.62) is one of the most remarkable enzymes produced by *Bacillus subtilis* sp. Natto, which posses direct fibrinolytic activity. The aim of this study is *in silico* analysis of Nattokinase structure and function. The three-dimensional structure of serine protease Nattokinase from *Bacillus subtilis sp. natto* was determined using homology modeling performed by Geno3D2 Web Server and refined by ModRefiner. The obtained models were validated via programs such as RAMPAGE, ERRAT, 3D Match and verify 3D for consistency; moreover, functional analysis performed by PFP from Kihara Bioinformatics laboratory. RAMPAGE analysis showed that 96.7% of the residues are located in the favored region, 3.0% in allowed region and 0.4% in outlier region of the Ramachandran plot. The verify 3D value of 0.73 indicates that the environmental sketch of the model is fine. SOPMA and PSIPRED were exploited for computation of the secondary structural properties of serine protease Nattokinase. Active site determination via AADS suggested that this enzyme can be applied as a potent enzyme for cardiovascular therapy. However, these results should be more confirmed by wet lab researches for designing the more active enzyme for better functions on its fibrinolysis activity.

Keywords: *In silico* analysis, Bioinformatics, Nattokinase, *Bacillus subtilis* natto.

INTRODUCTION

Nattokinase (EC 3.4.21.62), which is encoded by *aprN* gene, is a serine protease enzyme that mainly extracted and purified from natto, a traditional Japanese food[1]. The enzyme has no disulfide bond in its structure, and belongs to cysteine-free protease family. Nattokinase was inhibited by phenylmethylsulfonyl fluoride (PMSF) indicating that is a membership of serine protease family enzymes[2-4]. This enzyme could be also found in some different sources, such as Korean Chungkook-jang soy sauce, Korean Doen-jang, and various microorganisms, in which the genus *Bacillus* strain natto is the most important Nattokinase producer from traditional fermented foods[1,5-7]. Nattokinase is considered to be a promising remedy for thrombosis healing due to its potent fibrinolytic activity[1]. Based on its food source and relatively robust fibrinolytic activity, nattokinase has benefits over other available commercially used drug in prophylactic effects, stability in the GI tract and comfortable oral administration[1]. Several reports showed that oral administration of nattokinase could diminish plasma levels of fibrinogen, factor VIII, and factor VII, which may be useful as a nutraceutical for cardiovascular disease[8]. It was also reported that this enzyme had the beneficial effect on treating heart disease and Alzheimer's disease[9,10]. In adults suffering hypercholesterolemia, nattokinase had a positive effect on the reduction of serum cholesterol level together with low-cholesterol diet. Intravenous administration of fibrinolytic drugs such as tissue plasminogen activator (tPA) and urokinase has been extensively used in clinical platform for thrombolytic treatment[11]. In this regard, microbial fibrinolytic enzymes such as nattokinase have now pulled toward much more consideration than current thrombolytic drugs due to the costly prices and the detrimental side effects[10].

Nattokinase resembles direct fibrinolytic activity on fibrin clot in comparison to tPA and urokinase[12-14]. It directly degrades fibrin as well as increases the liberation of tissue plasminogen activator from cells to break down fibrin[15]. Furthermore, nattokinase also enhances plasmin and pro-urokinase production, which act as clot-dissolving agents. In comparison to plasmin, Nattokinase possesses less susceptible on fibrinogen cleavage but more susceptible on the breakdown of cross-linked fibrin[16]. The fibrinolytic activity of nattokinase remains in blood circulation more than three hours[17]. Diminishing the plasma levels of factor VII, factor VIII and fibrinogen, is another aspect, which offers Nattokinase as a nutraceutical application in cardiovascular disorders[8].

The in silico characterization of important proteases has been reported recently; however, few studies are available for fibrinolytic enzymes[18,19]. This study reports in silico characterization of Nattokinase that has medically important in cardiovascular diseases. The biochemical properties, predicting 3D structure of Nattokinase as well as forecasting active sites of enzyme was performed for

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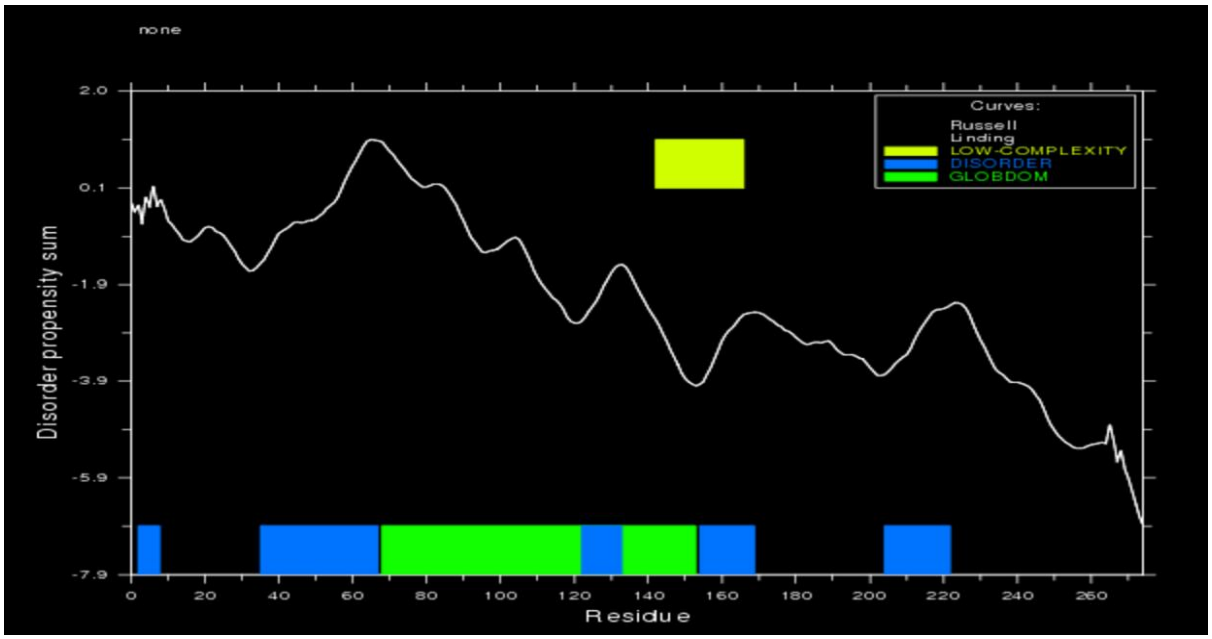

Figure 1: Globplot result displays the disease causing regions of Nattokinase.

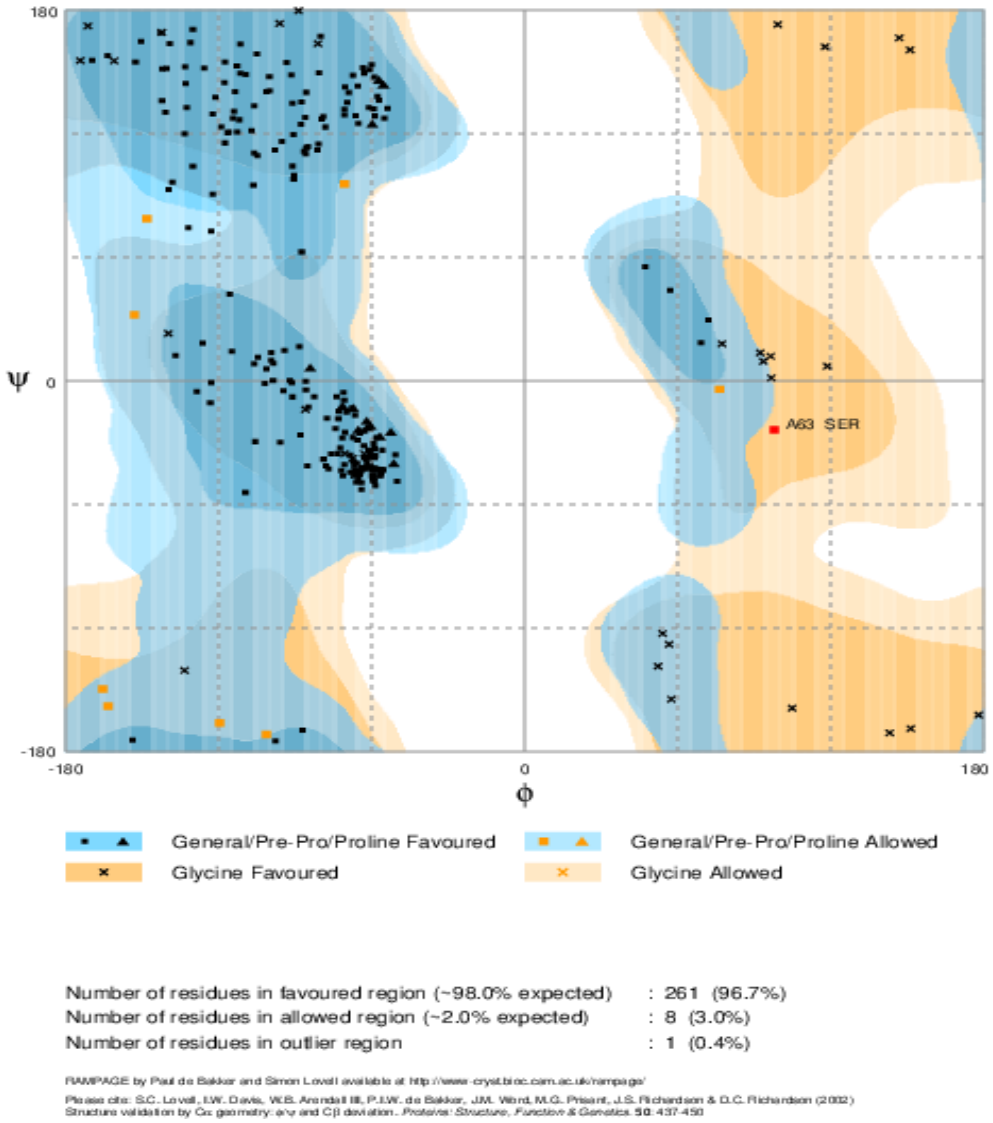

Figure 2: Ramachandran plot of Nattokinase from Bacillus subtilis natto achieved via the modeling tool.

possible drug formulation.

Methodology

The amino acid sequence of Nattokinase was retrieved from UniProt database. The physiochemical properties was obtained using ExPASy ProtParam tool (http://us.expasy.org/tools/protparam.html). The putative amino acid sequence of Nattokinase was evaluated for a signal sequence by the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The AlgPred web server available at http://www.imtech.res.in/raghava/algpred/ was used to predict enzyme allergenicity. The accuracy of Hybrid prediction approach (SVMc + ARPs BLAST + IgEepitope + MAST) is about 85 %. Crystal structure of available Nattokinase was selected as template to create the three dimensional model for Nattokinase. The PSIPRED and SOPMA tools were used for forecasting of secondary structure of proteins[20-22]. The most reputed online homology modeling tool, Geno3D2 Web Server was exploited to generate a total of most suitable ten models of target protein and refined by ModRefiner. The tool uses a distance geometry approach in homology modeling are no a priori choice in loops construction and easy identification of well-defined regions[23]. The stereochemical quality and accuracy of the predicted models were assessed with RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) by Ramachandran plot analysis (Laskowski et al. 1993). The best predicted model was appointed on the basis of overall G-factor, number of residues in core, allowed, generously allowed and disallowed regions. The selected model was then confirmed by 3D Match, verify 3D[24] and ERRAT[25] tool. Finally, the obtained models were visualized using UCSF Chimera version 1.7 (CA.USA). Active site analysis was performed by means of AADS (http://www.scfbio-iitd.res.in/dock/ActiveSite_new.jsp). Functional analysis of Nattokinase was carried out by exploiting a specialized tool PFP from Kihara Bioinformatics laboratory (http://kiharalab.org/web/pfp.php). GlobPlot 2.322 was applied for the forecasting of disease causing regions. Proteolytic cleavage sites were recognized by employing a web-based tool peptide cutter (http://web.expasy.org/peptide_cutter/), which predicts the proteolytic cleavage sites in a given protein sequence.

RESULTS AND DISCUSSION

A sequence of Nattokinase AprN or Subtilisin NAT was retrieved from the Knowledge Base (UniProtKB) UniProt with the accession ID of A2TJV0 that contains 275 amino acids. Physicochemical features of Nattokinase in this research were analyzed via ProtParam with regard to various parameters. The results presented this enzyme has an extremely high extinction coefficient (34380 at 280 nm in water), low instability index (22.91) that shows the enzyme is stable, high aliphatic index (83.05) of enzyme indicates that the enzyme is thermostable, grand average hydropathicity (GRAVY) of enzyme is 0.028, a lower value of GRAVY is considered as a measure of better interaction of a protein with water, molecular weight and pI of enzyme are 27.96 kDa and of 6.30 respectively. The significances of all parameters signify that this enzyme is highly thermostable as well as good solubility in water[22]. Moreover, the estimated half-life of enzyme is: 4.4 hours (mammalian reticulocytes, in vitro), > 20 hours (yeast, in vivo) and > 10 hours (Escherichia coli, in vivo). The most important obstacle to annotating function of various organisms' genomes is an absence of consistency in functional annotation[26]. In this regard, an innovative algo-rithm, PFP from Kihara Bioinformatics Laboratory provides an extended PSI-BLAST search tool that excerpts and scores gene ontology (GO) annotations on the basis of the frequency of their incidence in retrieved sequences, was applied to forecast the functions of desired protein[27]. Function prognosis was carried out with respect to cellular components; molecular function and biological process (Table 1). The results disclosed that function of the enzyme is restricted to proteolysis activity indicating that it is safe to be applied for human used.

Absence of regular secondary structure can be explained as protein disorder[28]. GlobPlot 2.3 was employed to recognize disordered region inside protein based on a performing sum of the tendency for amino acids to be in a disordered or ordered situation. This tool is able to recognize a particular kind of regions by seeking known disordered proteins and domains in databases[29]. Nattokinase includes five disordered regions from sequence 1 to 9, 35 to 68, 121 to 132, 153 to 169 and 204 to 222, respectively with down warding tendency sum (Figure 1). On the other hand, the hybrid approach showed that enzyme is a non-allergen (data not shown). Although the current analysis provided some disorder regions within the Nattokinase sequences, recently reports have not revealed any complication or side effect for this valuable therapeutic enzyme[30,31].

Prediction of protein digestion with protease can be helpful for performing trials on a segment of a protein, segregating the domains in a protein, removing a tag protein when expressing a fusion protein, or ensuring that the desired protein is not susceptible to endogenous proteases. Proteolytic analysis of Nattokinase was performed, which revealed that this enzyme has various cleavage sites for known proteases, including pepsin, trypsin, ec, ta (Table

Table 1: Predicted functions of Nattokinase

Molecular function	Biological process	Cellular component
Serine hydrolase activity	Proteolysis	Extracellular region
Serine type endopeptidase activity	Regulation of catalytic activity	Intrinsic to membrane
Peptidase activity, acting on L amino acid peptides	Regulation of molecular function	Membrane part
Endopeptidase activity	Protein metabolic process	Cell part

Table 2: The predicted cleavage sites from various enzymes for Nattokinase

Name of enzyme	No. of cleavages	Positions of cleavage site
Asp-N endopeptidase	9	31 35 40 59 96 119 139 196 247
Chymotrypsin-high specificity (C-term to [FYW], not before P)	16	6 21 50 58 91 104 106 113 189 214 217 241 256 261 262 263
Chymotrypsin-low specificity (C-term to [FYWML], not before P)	38	6 16 17 21 42 50 58 64 67 75 82 90 91 96 104 106 113 119 124 126 135 189 196 199 214 217 222 226 233 235 241 250 256 257 261 262 263 267
Pepsin (pH1.3)	28	16 42 49 74 75 81 82 89 90 95 125 126 134 135 189 195 196 209 232 233 234 235 250 256 257 260 261 266
Pepsin (pH>2)	49	5 16 20 21 42 49 57 74 75 81 82 89 90 91 95 103 104 105 106 112 113 125 126 134 135 167 171 189 195 196 209 213 214 216 217 232 233 234 235 241 250 255 256 257 260 261 262 263 266
Trypsin	12	12 27 45 94 136 141 170 186 237 247 249 265
Arg-C proteinase	4	45 186 247 249
Asp-N endopeptidase + N-terminal Glu	14	31 35 40 53 59 96 111 119 139 155 194 196 247 250

Table 3: Ramachandran plot statistics of serine protease Nattokinase from *Bacillus subtilis* natto.

Ramachandran PlotStatistics	No. of Residue	%
Residues in the favored regions	261	96.7
Residues in the allowed regions	8	3
Residues in the outlier regions	1	0.4
Number of non-glycine and non-proline residues	227	100
Number of end-residues [excl. Gly and Pro]	2	
Number of glycine residues [shown in triangles]	33	
Number of proline residues	13	
Total number of residues	275	

Table 4: The predicted secondary structure of Nattokinase enzymes

Structure	Number of residues	Percentage (%)
Alpha helix [Hh]	64	23.27
3_{10} helix [Gg]	0	0
Pi helix [Ii]	0	0
Beta bridge [Bb]	0	0
Extended strand [Ee]	66	24
Beta turn [Tt]	26	9.45
Bend region [Ss]	0	0
Random coil [Cc]	119	43.27
Ambiguous states [?]	0	0
Other states	0	0

2). Although Nattokinase is sensitive to gastric and intestinal enzymes, the intestinal intake has been reported successfully[11,32]. However, by performing such formulations, including compression into a tablet, coating with Eudragit L100-55 and hydroxypropyl cellulose as enteric materials can improve the gastrointestinal intake[33]. One of the most important parts of proteomics is determination of Three-dimensional (3D). The 3D structure of proteins offer valuable intuitions into the molecular basis of protein function, allowing an efficient design of trials, such as studies of disease-associated mutations, site-directed mutagenesis or the structure based design of specific inhibitors[34]. Thus, the key to perceive and manipulate of protein cellular and biochemical functions are the high resolution 3D structure of the desired protein[23]. The theoretical structure of Nattokinase from *Bacillus subtilis* sp. natto is generated using Geno3D2Web Server. The generated models were validated by RAMPAGE, verify 3D and ERRAT tool. The Ramachandran plot analysis from RAMPAGE server showed that 96.7% of the residues are located in the favored region, 3.0% in allowed region and 0.4% in outlier region and all non-proline and non-glycine residues are in the allowed region of plot (Figure 2 and Table 3). Moreover, validation of the model was performed with ERRAT and verify3D programs from softberry. Data analysis of ERRAT and verify 3D programs and Ramachandran plot from RAMPAGE disclosed that all residues are inside the limits of the Ramachandran plot, and consequently, it can be regarded as a valid model. The huge score of 0.73 in the verify 3D graph points out that the surrounding sketch of the model is fine and the total quality of the model in ERRAT analysis was 93.258, described as the percentage of the protein for which the calculated error value falls below 95% rejection limit. Finally, the modeled target protein visualized via UCSF Chimera version 1.7 (CA.USA) is shown in Figure 3.

Figure 3: Final model of Nattokinase from *Bacillus subtilis* natto.

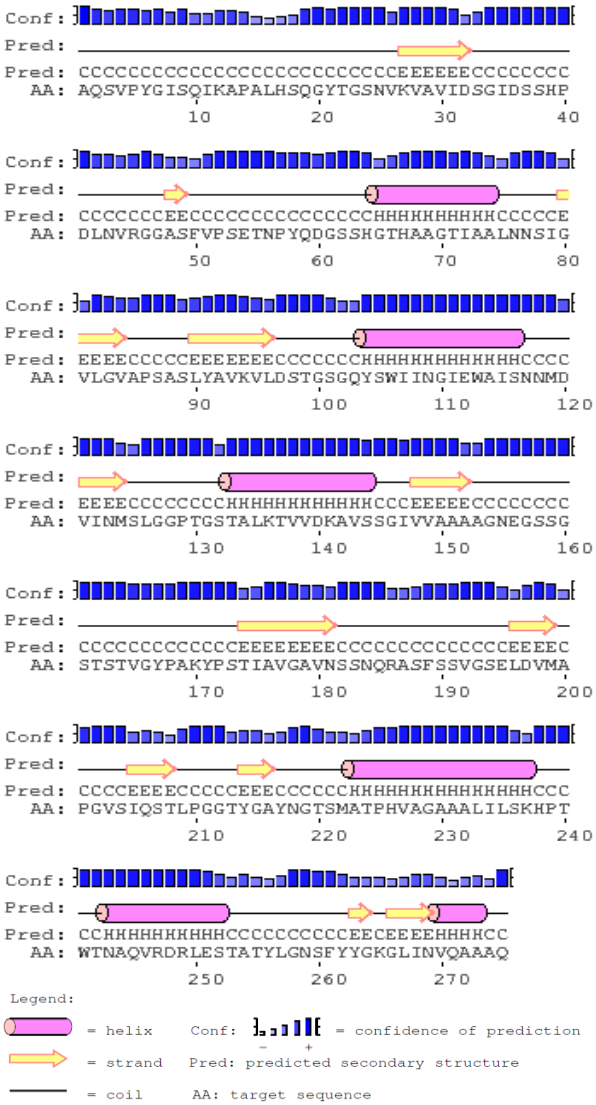

Figure 4: secondary structure profile of Nattokinase obtained from PSIPRED

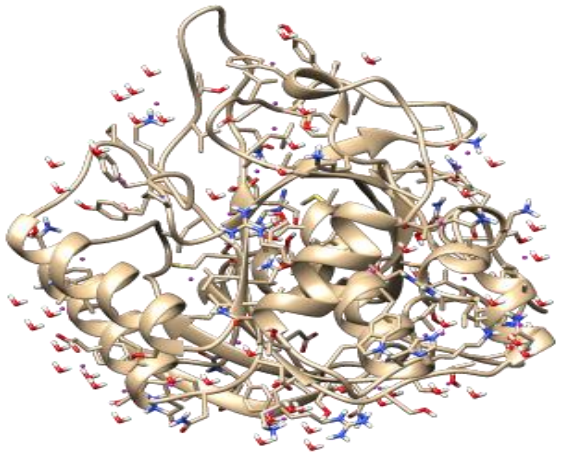

Figure 5: The 3D structure of best active site of Nattokinase obtained from AADS tools that amino acids involving in active site are illustrated in backbone state

The secondary structure analysis of the modeled Nattokinase obtained from SOPMA and PSIPRED revealed that it includes 23.27% alpha helix, 24% extended strand and 43.27% random coil (Table 4 and Figure 4). The characterization and identification of active sites on proteins has progressively become a field of interest. Analysis of the active site residues for the binding of ligands, offers acumen toward to designing of inhibitors for enzymes. In this work, we have also reported the best active site area of the Nattokinase in addition to the number of amino acids involved in the corresponding active region. Figure 5 illustrated the area and the volume for various active sites of Nattokinase and the best active site places in a cavity point of 16.339 and a cavity volume of 1388, which positions from 32 to 224 that contain Beta-sheet and Alpha-helix among them. In addition, it is desirable to recognize which amino acid residues are needed for catalytic activity in order to design enzymatic variants with a specific type of or enhanced activity[35].

CONCLUSION

In this research, the 3D structure of serine protease Nattokinase from *B. subtilis* sp. natto was forecasted and evaluated using different bioinformatics tools. The present of extremely high extinction coefficient, low instability index and high aliphatic index as well as negative GRAVY implies that this enzyme is highly thermostable as well as having excellent solubility in water. Three-dimensional structure of Nattokinase was predicted using Nattokinase PBD ID of 4dww as a template. Structure validation by ERRAT, RAMPAGE, Verify 3D and 3D Match confirmed the reliability of the model. The predicted features of Nattokinase may assist in better application of enzyme for various medicinal applications, including oral thrombolytic therapy.

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