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ORIGINAL ARTICLE

Molecular analysis of a fibrin-degrading enzyme from *Bacillus subtilis* K2 isolated from the Indonesian soybean-based fermented food moromi

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Abstract

The screening of proteolytic and fibrinolytic bacteria from moromi (an Indonesian soybean-based fermented food) yielded a number of isolates. Based on morphological and biochemical analyses and sequencing of the 16S rRNA gene, the isolate that exhibited the highest proteolytic and fibrinolytic activity was identified as *Bacillus subtilis* K2. The study was performed to analyze molecular characteristic of a fibrin-degrading enzyme from *B. subtilis* K2. BLASTn analysis of the nucleotide sequence encoding this fibrinolytic protein demonstrated 73.6% homology with the gene encoding the fibrin-degrading enzyme nattokinase of the *B. subtilis* subsp. natto, which was isolated from fermented soybean in Japan. An analysis of the putative amino-acid sequence of this protein indicated that it is a serine protease enzyme with aspartate, histidine, and serine in the catalytic triad. This enzyme was determined to be a 26-kDa molecule, as confirmed with a zymogram assay. Further bioinformatic analysis using Protparam demonstrated that the enzyme has a pI of 6.02, low instability index, high aliphatic index, and low GRAVY value. Molecular docking analysis using HADDOCK indicated that there are favorable interactions between subtilisin K2 and the fibrin substrate, as demonstrated by a high binding affinity (ΔG: − 19.4 kcal/mol) and low Kd value (6.3E-15 M). Overall, the study concluded that subtilisin K2 belong to serine protease enzyme has strong interactions with its fibrin substrate and fibrin can be rapidly degraded by this enzyme, suggesting its application as a treatment for thrombus diseases.

Keywords *Bacillus subtilis* · Bioinformatic · Molecular docking · Moromi · Serine protease

Introduction

Moromi is an intermediate phase in soy sauce fermentation. The high protein content of soybean (35–38%) implies that this product has potential as a growth medium for various proteolytic microorganisms, including fibrin-degrading microorganisms. Wei et al. [1] reported that during spontaneous fermentation of soybean, a number of microorganisms, such as *Zygosaccharomyces rouxii*, *Tetragenococcus halophilus*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens*, which produce hydrolytic enzymes, can be observed.

The alkaline serine protease subtilisin (EC 3.4.21.14) is the dominant extracellular protease of *Bacillus* species [2], and a number of forms of this enzyme have been reported, including nattokinase from *B. subtilis* subsp. natto [3]; subtilisins DJ-4 and DFE from *B. amyloliquefaciens* [4, 5]; subtilisin AprE3-17 from *B. licheniformis* [6]; subtilisin amylosachariticus from *Bacillus amylosachariticus* [7]; thrombinase from *Bacillus spharicus* [8]; and subtilisin CK from *Bacillus* sp. CK [9]. Subtilisin belongs to the second-largest family of serine proteases, which are widely distributed in nature and have been identified as the products of numerous Gram-positive *Bacillus* spp. This occurrence is indicative of narrow functional diversity [10]. Głowacka et al. [11] reported that subtilisins are primarily monomeric extracellular proteases. One of the subtilisin enzymes, nattokinase (EC 3.4.21.62), encoded by the aprN gene, is a product of natto, a traditional Japanese food that is known

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as one of the healthiest foods [3]. Nattokinase exhibits the ability to break down blood clots by directly hydrolyzing the fibrin substrate and activating plasmin [12].

It is possible to employ fibrinolytic bacteria from food-grade microorganisms (generally recognized as safe, or GRAS) for functional food or nutraceutical treatment of cardiovascular diseases [13]. Fibrin-degrading enzymes from food origin have attracted medical interest in recent decades due to their historical record of safety, and these enzymes are therefore considered to be promising medications for thrombolytic therapy. This promise may be observed because this treatment is convenient, can be taken through oral administration, has been proven to be efficacious, exhibits prolonged and preventive effects, is inexpensive, and demonstrates high stability in the digestive tract [14].

Several fibrin-degrading enzymes producing bacteria were screened and isolated from the moromi stage of Indonesian soy sauce fermentation. One particular isolate, identified as *B. subtilis* K2, exhibited strong fibrinolytic activity, as observed in the fibrin plate assay [15]. In this study, we present a molecular analysis of a fibrin-degrading enzyme from *B. subtilis* K2; specifically, we analyze the nucleotide and amino-acid sequences of the enzyme and undertake a structural analysis, bioinformatic study, and molecular docking analysis of enzyme-fibrin substrate interactions.

Materials and methods

Chemicals and bacterial strains

The chemicals employed in this research were analytical-grade and were purchased from Sigma (US) and Oxoid (UK) through local distributors. The *B. subtilis* K2 isolate from moromi (intermediate product from fermented soy sauce) was collected earlier [15].

Amplification of fibrinolytic gene

The reaction mixture (25 µL) contained 3 µL of DNA template (50 ng/µL), 2.5 µL of each primer (10 pmol/µL), 8 µL of PCR Go Taq Master Mix (2x) (Promega, US), and 9 µL of nuclease-free water. The fibrinolytic gene from subtilisin K2 was amplified using genomic DNA from *B. subtilis* K2 as a template for PCR. Primers for the fibrinolytic gene and PCR conditions were previously described in Jeong et al. [16] (forward: 5′-GCG AAT TCG CCG CAT CTG TGT CTT TG-3′, reverse: 5′-GCG AAT TCG AGA ACA GAG AAG CCG CT-3′), with the following PCR conditions being employed: pre-denaturation (94 °C, 5 min), denaturation (94 °C, 15 s), annealing (60 °C, 30 s), polymerization (72 °C, 60 s), amplification (30 cycles), final polymerization (72 °C, 5 min), and cooling (25 °C, 10 min).

DNA electrophoresis, fibrinogen zymography, and protein determination

Agarose gel electrophoresis was performed as described by Sambrook et al. [17]. This method involved the loading of PCR products into a 1% agarose gel containing 0.5 µg/mL EtBr, and electrophoresis was subsequently performed at 70 V for 60 min. The DNA was visualized on a Gel-Doc® System (BioRad). Fibrinogen zymography was conducted following the protocol described by Hwang et al. [18] with several modification. Fibrinogen zymography was performed using 12% polyacrylamide gels containing 0.1% fibrinogen. Electrophoresis was conducted at 70 V and 50 A for 4 h. Protein concentration was analyzed as described by Bradford [19], with bovine serum albumin serving as the standard. Each measurement of protein concentration was performed in triplicate.

DNA sequencing and bioinformatic analysis

The Sanger dideoxy method was adopted to sequence the DNA, which was subsequently analyzed with the ExPASy translate tool (https://web.expasy.org/translate/). In addition, BLAST queries were applied using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch) (RRID:SCR_001598) and BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&BLAST_PROGRAMS=blastx&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthomesoftware) (RRID:SCR_001653) from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) (RRID:SCR_006472) followed by the alignment of nucleotide sequences using the webserver Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (RRID:SCR_001591). The protein characteristics were determined using ProtParam analysis (https://web.expasy.org/protparam/) (RRID:SCR_018087), and prosite (https://prosite.expasy.org/) (RRID:SCR_003457) was employed in the active site analysis.

Deposition of sequence data

The nucleotide sequence of the gene encoding protease subtilisin K2 was deposited in GenBank under accession number MN294987.

Structural modeling and validation of the subtilisin K2 protease model

The 3D structure of subtilisin K2 was constructed using the SWISS-MODEL Workspace (https://swissmodel.expasy.org/) program (RRID:SCR_018123) [20] and nattokinase (PDB ID: 4DWW.1.A) from the Protein Databank (https://www.rcsb.org/pdb/) (RRID:SCR_012820) served as the template structure. Furthermore, the nattokinase (template) and subtilisin K2 sequences were aligned using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/) (RRID:SCR_001591). The predicted structure of subtilisin K2 was analyzed using the PyMOL Molecular Graphics System (Version 2.3.0, Schrödinger, LLC., NY, US) (https://PyMOL.sourceforge.net/) (RRID:SCR_000305) [21], which was subsequently exported as an image. The secondary structure of subtilisin K2 was obtained through the SOPMA Program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) [22]. Then, the structure obtained by SWISS-MODEL (RRID:SCR_018123) was validated by examining the Phi/Psi Ramachandran plot [23] acquired from PROCHECK (https://servicesn.mbi.ucla.edu/PROCHECK/) [24], which improved the quality evaluation of the Ramachandran plot. In addition, the Protein Structure Analysis (PROSA) webserver (https://prosa.services.came.sbg.ac.at/prosa.php) (RRID:SCR_018540) [25] was adopted to evaluate the energy characteristics/specification of the protein model and compared with the X-ray and NMR structures obtained from the database. Afterwards, the plot of PROSA II (RRID:SCR_018540) energy was computed to evaluate all residues' interaction energies in the protein model. Additionally, the model quality was investigated through VERIFY-3D, available at https://servicesn.mbi.ucla.edu/Verify3D/ [26], to measure its compatibility. VERIFY-3D was employed to compare the predicted model with its template structure (based on 3D and sequence score). The SuperPose version 1.0 webserver, available at (https://wishart.biology.ualberta.ca/SuperPose/), was employed to compare the model (subtilisin K2) and template structure (nattokinase) [27], and PyMOL (RRID:SCR_000305) [21] was also utilized.

Molecular docking study

The subtilisin K2 model and fibrin, which served as the substrate, were docked to investigate the interaction between these two proteins to predict the interaction site of subtilisin K2 and fibrin through the CPORT webserver at https://milou.science.uu.nl/services/CPORT/ [28]. The physical and knowledge-based approach for predicting the binding site for protein interaction in the High Ambiguity Driven protein–protein DOCKing (HADDOCK) webserver (https://milou.science.uu.nl/services/HADDOCK2.2/) [29] was employed for protein docking. The 3D docking method for subtilisin K2 was the flexible type with the fibrin molecule from Protein Databank (PDB ID: 2HLO) through HADDOCK. In addition, the energetically important hotspot residues at the interface area of subtilisin K2 and fibrin were identified using the HotRegion webserver (https://prism.ccbb.ku.edu.tr/hotregion/) (RRID:SCR_006022) [30]. Afterwards, the prediction of the binding affinity in protein–protein complexes was achieved with the help of the Prodigy webserver available at https://bianca.science.uu.nl//prodigy/ [31]. Finally, the LigPlot + Version v.2.1 (RRID:SCR_018249) program was utilized to obtain schematic diagrams of the protein–protein interactions (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/install.html) [32].

Results

Analysis of protease subtilisin K2

In this study, primers for a polymerase chain reaction (PCR) to amplify the protease gene, generating a 1700-bp DNA fragment, were designed based on Jeong et al. [16] using the protease gene from *B. subtilis*. The gene sequence of the PCR product was analyzed using BLASTn and BLASTx. The result of the BLASTn analysis of the *B. subtilis* K2 (subtilisin K2) gene demonstrated that the fibrinolytic gene possessed 73.6% homology to that of *B. subtilis* subsp. subtilis str. SRCM101392, and BLASTx analysis of subtilisin K2 indicated that the predicted protein had high (99.6%) homology with nattokinase, an enzyme secreted by *B. subtilis* subsp. natto isolated from Japanese natto, which is known as one of the healthiest foods.

An alignment with a number of homologous proteins demonstrated that the subtilisin K2 enzyme investigated in this study belongs to the subfamily of subtilisin serine proteases. These enzymes possess catalytic residues (red highlighted letter) (Fig. 1) within the catalytic domain between residues 19 and 208 (Fig. 1). The active site residues of the protease subtilisin K2 are characterized by aspartic acid at position 19, histidine at position 51, and serine at position 208, which suggest the existence of a typical serine protease catalytic triad. The open reading frame of the subtilisin K2 gene (measuring 1700 bp) encodes a protein consisting of 262 amino acids with a calculated mass of 26.4 kDa.

The fibrinogenolytic activity of this enzyme was analyzed using the zymography method. Fibrinogen 0.1% was used as the substrate, and the concentration of protein loaded into the gel was 0.99 mg/mL. Clearing bands indicated that the active enzymes were in the range of 60–120 kDa and measured ± 26 kDa (Fig. 2). It is possible that the

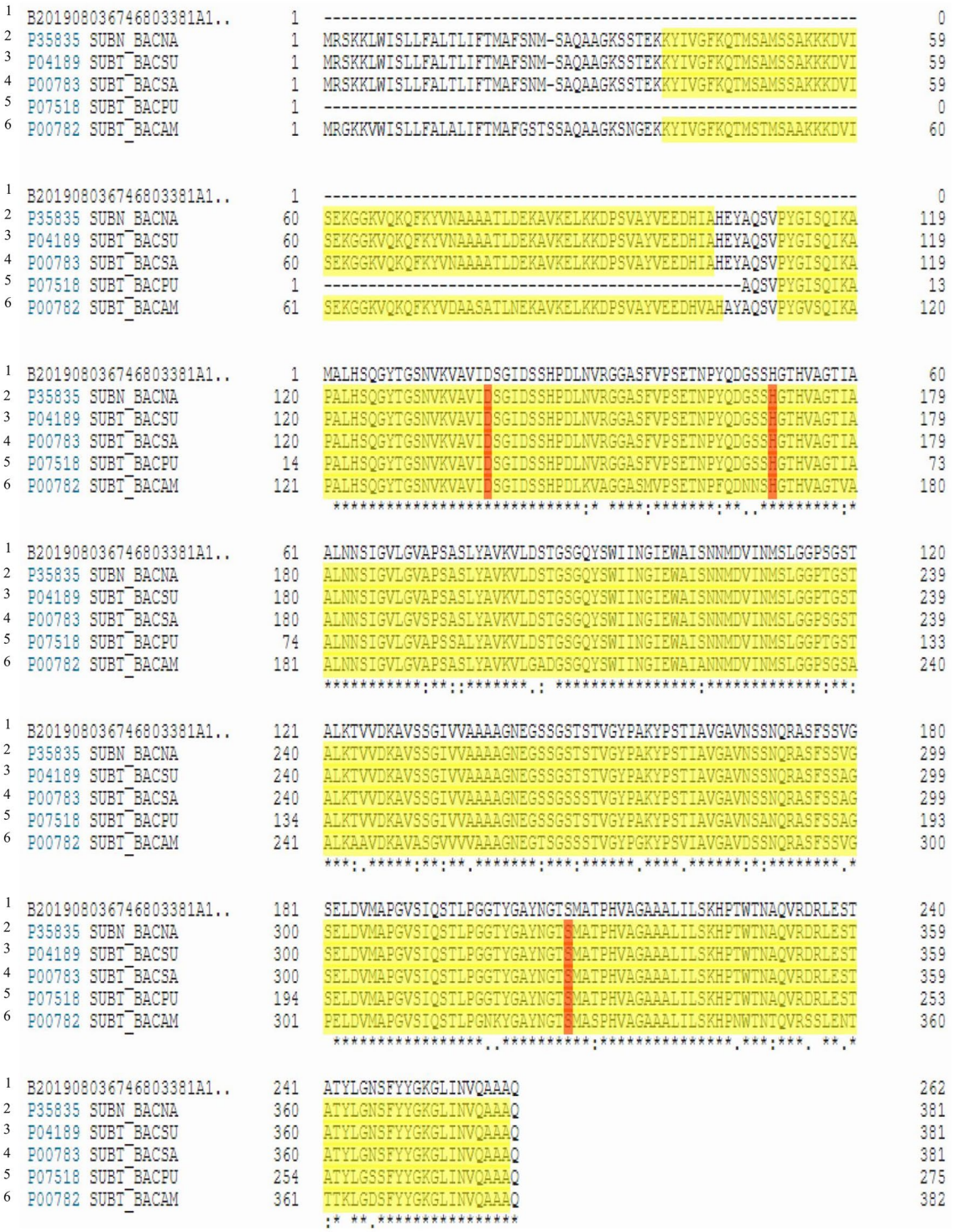

Fig. 1 Sequence alignment of protease from Indonesian *Bacillus* sp. with several homologous proteins. Catalytic triad and domain are indicated by red and yellow highlighted letters, respectively. The proteases used for sequence alignment were as follows: (1) our protease (subtilisin K2), (2) subtilisin nattokinase from *B. subtilis* subsp. *natto,* (3) subtilisin E from *B. subtilis* strain 168, (4) *Subtilisin amylosachariticus* from *B. subtilis* subsp. *amylosachariticus*, (5) subtilisin from *Bacillus pumillus*, and (6) subtilisin BPN' from *B. amyloliquefaciens*

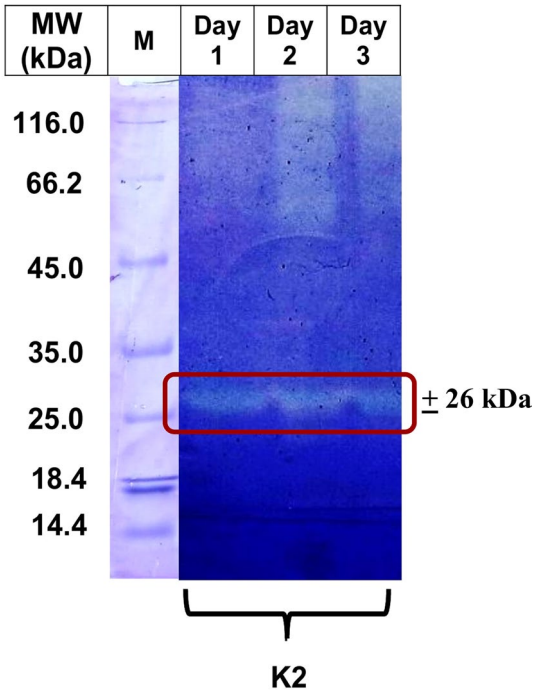

Fig. 2 Fibrinogenolytic activity of subtilisin K2 using zymogram analysis. M: Broad molecular weight standard protein markers (Thermo Fisher Scientific, US); days 1, 2, and 3 are related to different fermentation times to produce the enzyme

fibrinogen-degrading enzyme of *B. subtilis* K2 consists of multiple enzyme fractions, with the smallest fraction measuring 26 kDa. The bioinformatic analysis of subtilisin K2 using ProtParam demonstrated that subtilisin K2 has a calculated mass or molecular weight of 26.4 kDa.

ProtParam was adopted to analyze the physicochemical characteristics of subtilisin K2 and nattokinase (as reference), which demonstrated similarities in various parameters. Subtilisin K2 and nattokinase exhibited a high extinction coefficient (32,890 and 34,380 at 280 nm in water, respectively) and a low instability index (21.43 and 22.91), which indicates that both enzymes are stable, a high aliphatic index (83.05 and 83.75), which suggests that both enzymes are thermostable, and a low grand average hydropathicity (GRAVY) of subtilisin K2 and nattokinase, that is, 0.048 and 0.037, respectively, where a lower value of GRAVY is considered a measure of better interaction of the protein with water. The pI values of subtilisin K2 and nattokinase were determined to be 6.02 and 6.30, respectively. These parameters signify that subtilisin K2 and nattokinase are highly thermostable, as well as soluble in water [33]. In addition, the estimated half-lifes of subtilisin K2 and nattokinase are > 20 h (yeast, in vivo) and > 10 h (*Escherichia coli*, in vivo), and subtilisin K2 and nattokinase exhibited different estimated half-lifes in reticulocyte mammals, that is, 30 h (subtilisin K2), and 4.4 h (nattokinase). This result implies that subtilisin K2 is considerably more stable than nattokinase.

3D model building and validation of subtilisin K2 structure

Analysis of the secondary structure of subtilisin K2 through the SOPMA program indicated that 24.52%, 24.52%, 11.49%, and 39.46% of the amino acid residues are present in alpha helices, beta sheets, beta turns, and random coils, respectively. Based on the results, random coils were accounted for the largest portion of the protein (39.46%) followed by alpha helices, beta sheets, and beta turns, making subtilisin K2 a stable protein. According to Chakraborty et al. [34], a high random coil content is an indication of protein flexibility. The alpha helices and beta sheets were present in equal proportions of 24.52%. In general, thermophilic proteins have a larger fraction of alpha helices conformation [35], while beta sheets induce the protein stable structure [36]. Hence, the alpha helix and beta sheet conformations exhibited by the subtilisin K2 model indicate that the enzyme is stable. Additionally, an analysis performed through the SOPMA program showed that the protein model was useful for homology modeling.

Irajie et al. [37] reported from a secondary structure analysis of nattokinase that it consisted of 23.27% alpha helices, 24% beta sheets, 9.45% beta turns, and 43.27% random coils. The random coil accounted for the largest portion of the nattokinase structure. The beta sheet composition was slightly greater than the alpha helix composition. The alpha helix, beta sheet, and beta turn contents of subtilisin K2 were higher than those of nattokinase, which correlated with the higher stability of subtilisin K2.

The PROCHECK webserver that was used to analyze the subtilisin K2 model built by the SWISS-MODEL Workspace program indicated that 88.9% of amino acid residues were found in the most favored regions, 10.6% were residues in the allowed regions and 0.5% were residues in generously allowed regions. This result indicates that the model had good quality (Fig. 3a). In addition, the results obtained with the PROSA tool employed to evaluate the model with the Z score of the model, specifically – 9.53, support the good quality of the model. Generally, the Z score, which shows the model quality, is utilized to evaluate the score range of the target model observed in native proteins of similar size. The PROSA analysis indicated that the subtilisin K2 model was in the acceptable range of the known X-ray and NMR structures from databases (Fig. 3b). Furthermore, the PROSA analysis of this structure obtained negative interaction energy for all of the residues, as shown in Fig. 3c. Based on the results of the PROCHECK and PROSA analyses, the subtilisin K2 model can be employed for further bioinformatic analysis.

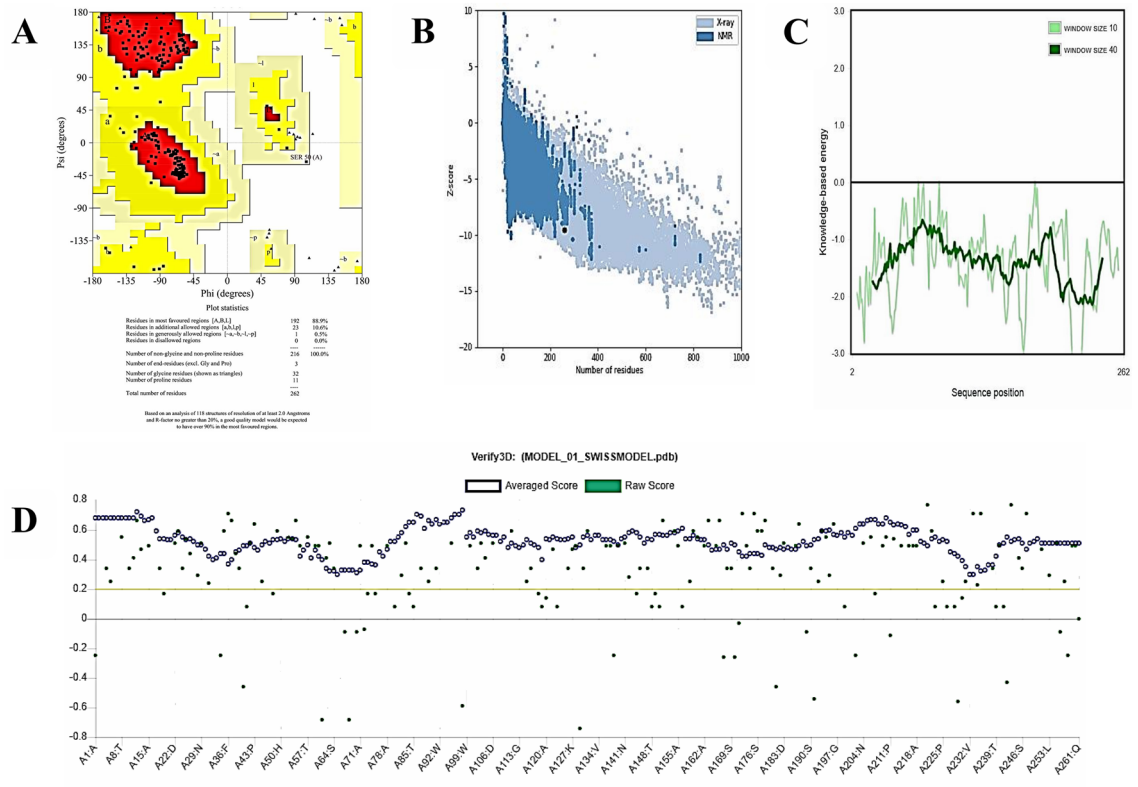

Fig. 3 Ramachandran plot (**a**); validation of the subtilisin K2 model using PROSA. The black dot shows the similarity of the model with X-ray and NMR structures (**b**); interaction energy of the subtilisin K2 model using PROSA (**c**); 3D profile verification results of the subtilisin K2 model (blue) and template (green) (**d**). (Color figure online)

Subtilisin K2 was analyzed by VERIFY-3D to determine the 3D compatibility of the protein structure with its own amino-acid sequence (1D). A compatibility score (3D-1D score) above zero in the VERIFY-3D graph is an indication of acceptable side-chain environments (Fig. 3d). Additionally, 100% of total residues from the subtilisin K2 model built by SWISS-MODEL provided an averaged compatibility score of ≥ 0.2, as shown in Fig. 3d. The vertical axis in VERIFY-3D shows the average 3D–1D protein score for each residue in a 21-residue sliding window, which is necessary for validation of the model.

Furthermore, superimposing the subtilisin K2 molecule model on nattokinase (Accession Number: 4DWW.1. A), as shown in Fig. 4c, was achieved using PyMOL version 2.3.0 (SchrÖdinger, Inc.). This superimposition indicated that good similarity between the two proteins [27] yielded a lower value of root mean square deviation, i.e., 0.12 Å. Figure 4c revealed that the active site residues (Asp19, His51, and Ser208) of subtilisin K2 are slightly different from the nattokinase active site residues (Asp32, His64, and Ser221). Subtilisin K2 from *B. subtilis* K2 (Fig. 4a) contains alpha helix and beta sheet domains that are structurally similar to those of nattokinase (Fig. 4b). Amino acid alignment with subtilisin from *B. subtilis* confirmed the presence of the typical catalytic triad of the serine protease family: histidine, serine, and aspartic acid (blue box) (Fig. 4d).

Molecular docking study of subtilisin K2

Molecular docking between the subtilisin K2 model and fibrin, with fibrin serving as the substrate, was performed to investigate the binding mode and interactions of subtilisin K2. In this research, the interaction site residues of the subtilisin K2 structure were made flexible and then docked with the interaction site of fibrin (PDB ID: 2HLO). The interaction site residues of subtilisin K2 and fibrin were identified using the CPORT server. Subtilisin K2 has 27 residues that are active functional groups and 49 residues that are passive residues, as determined in HADDOCK, while the fibrin substrate has 44 residues that are active functional groups and 41 residues that are passive residues, as determined in HADDOCK. Accordingly, the subtilisin K2 was docked into the binding site of fibrin.

The hotspots of protein interaction were the areas with greater probability of being the interface area or binding domain, and these residues were observed to contribute the most to binding [38]. These hotspots were analyzed using the HotRegion database webserver [30]. The HotRegion

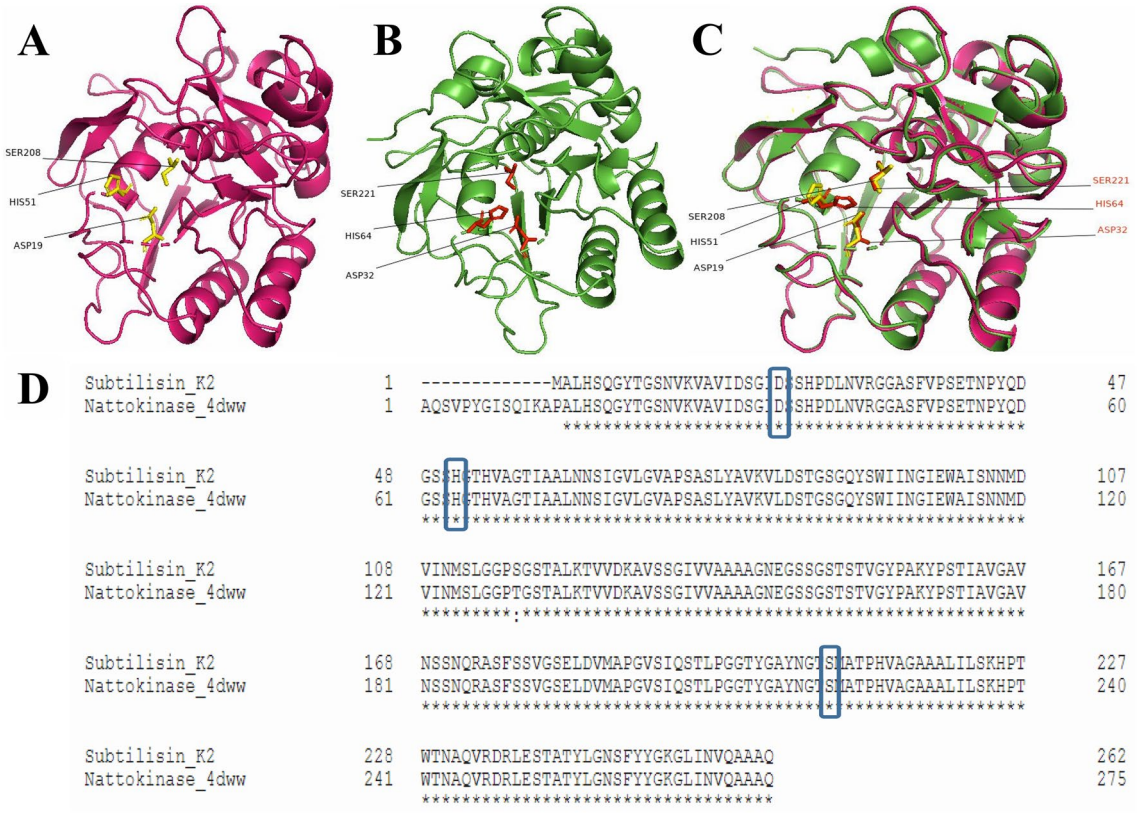

Fig. 4 Modeled structure of protease subtilisin K2 (**a**); structure of nattokinase (reference) (**b**); superposition between subtilisin K2 and reference (magenta = subtilisin K2; green = nattokinase) (**c**); amino acid alignment between protease subtilisin K2 and nattokinase (active site indicated by blue box) (**d**). (Color figure online)

database experimentally shows the derived hotspot and hot region information and protein structural characteristics needed to determine the energetically important hotspot residues in protein complex structures [39]. The hotspots of subtilisin K2 and fibrin are the residues underlined in Table 1. A total of 24 residues of subtilisin K2 and 11 residues of fibrin serve as hotspots of protein interaction.

Subtilisin K2 was observed to recognize fibrin with an extended binding surface through the interface residue between both molecules. Schematic diagrams of protein–protein interactions obtained using the LigPlot + program showed that 40 residues of subtilisin K2 and 32 residues of fibrin belong to the interface area (Table 1, Fig. 5), and these residues are involved in whole binding. Therefore, the residues have the ability to facilitate the formation of the subtilisin K2-fibrin complex and mediate the enzyme–substrate reaction.

Prediction of the binding affinity in the subtilisin K2-fibrin complex was performed with Prodigy. The subtilisin K2-fibrin complex showed a high binding affinity (ΔG) (− 19.4 kcal/mol) and low Kd value (6.3E-15 M) at the active site of the subtilisin K2 model. The binding affinity, also known as the binding free energy (ΔG), between the two proteins was mainly defined by their contact region and interface. This

Table 1 Predicted location of subtilisin K2-fibrin interaction

Interaction	Subtilisin K2*	Fibrin*
Hydrogen	Asn171, Gln193, His213, Leu69, Gly87, Gly89, Ser88, Ser65	Gln421, Met426, Asp425, His429, Arg169, Leu165, Arg166, Arg391, Lys396
Hydrophobic	Gly114, Asn142, Ser191, Ile192, Pro188, Val68, Gly67, Gly70, Leu83, **His51**, Thr58, Ser49, Ser20, **Ser208**, Tyr154, Val71, **Asp19**, Gln258, Ala2, Met1, Tyr91, Ile66, Tyr204, Asn205, Ala203, Phe176, Ser5, Val190	Val167, Leu182, Ser170, Trp418, Gly419, Ala427, Thr431, Gly420, Thr423, Lys428, Trp424, Gly430, Leu168, Ile171, Ser443, Leu172, Trp444, Cys407, Asp432, Ile179, Leu175, Asn174, Lys178, Asn364

*Bold residues are active site of Subtilisin K2 and underlined residues are the hotspots

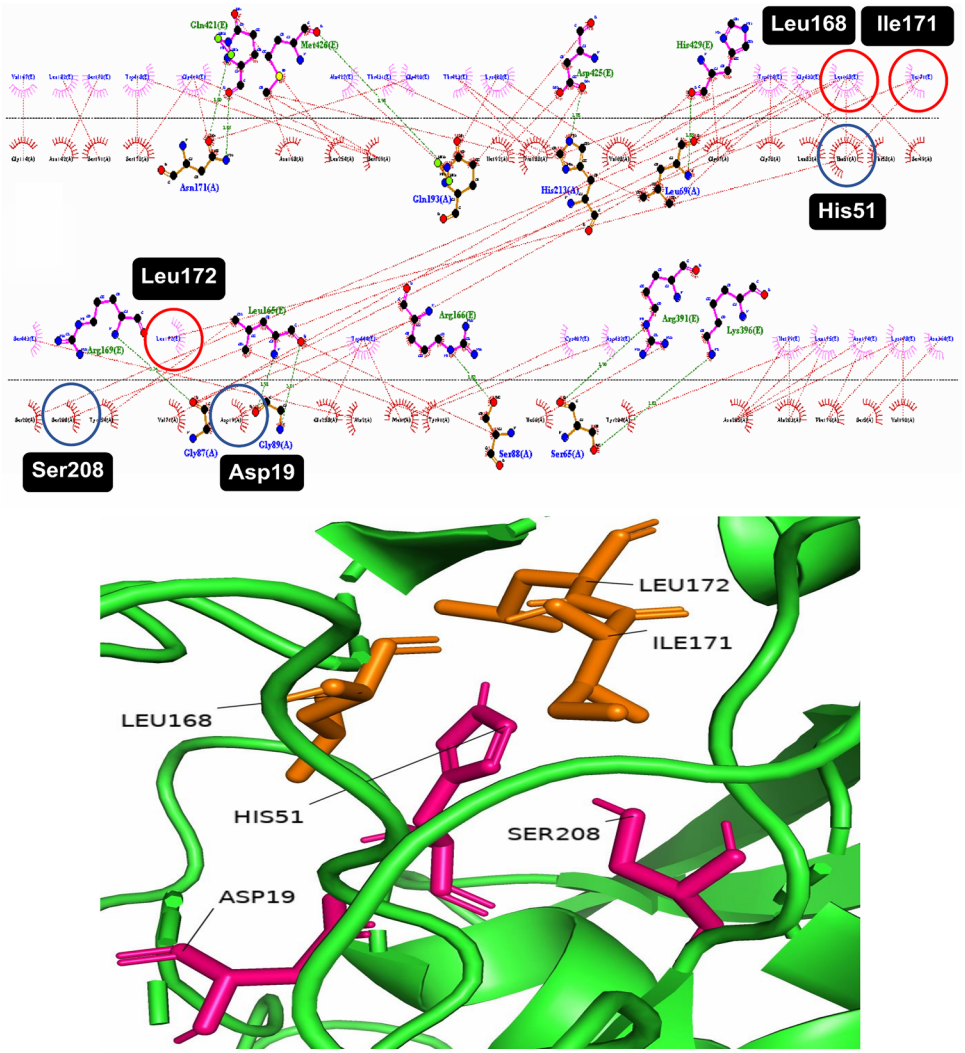

Fig. 5 Subtilisin K2 docked to fibrin at its binding site through the HADDOCK web-server (pink licorice = active site of subtilisin K2, orange licorice = substrate specificity of fibrin). (Color figure online)

binding affinity determines whether the complex formation between both proteins occurs in specific conditions that contribute to their interaction strength [40]. If the binding free energy value is more negative, the interaction occurring between the receptor and ligand is stronger.

Generally, in assessing and determining the order of strengths of bimolecular interactions, binding affinity is measured by the equilibrium dissociation constant (Kd). A higher Kd value indicates a lower binding affinity of the ligand for its target and vice versa. Docking results demonstrated that Asp19, His51, and Ser208, as subtilisin K2's active site, interact with Leu168, Ile171, and Leu172 of the fibrin substrate, which may have potent implications for substrate specificity, as shown in Fig. 5.

Discussion

The serine protease family includes numerous members encompassing digestive enzymes (e.g., trypsin, chymotrypsin, and elastase), fibrinolytic enzymes of mammalian, worm and bacterial origin (nattokinase) and various subtilisin types secreted by *Bacillus* sp., which share the catalytic triad of aspartic acid-histidine-serine. The possibility of varying molecular weights of this fibrinogen-degrading enzymes, among others, (1) Many protease enzymes are synthesized as molecules with various different sizes or multiple forms [41, 42], and (2) this enzyme is also possible that the larger but less active proenzyme, upon

self-hydrolysis, degrades molecules with higher activity. (3) Another possibility is that the 26-kDa fibrinogen-degrading enzyme forms a larger and active enzyme complex with a higher molecular weight after it is synthesized.

The intestinal intake of fibrin-degrading enzymes has been reported [43, 44]. This intake implies that these enzymes can be administered effectively through oral methods. Earlier studies have shown that the oral administration of fibrin-degrading enzymes can enhance fibrinolytic activity in plasma [45]. A bioinformatic analysis performed using ProtParam predicted that subtilisin K2 shows high stability, which supports the ability of the enzyme to remain intact in the gastrointestinal tract [46] and remain active toward thrombi in the bloodstream.

Analysis of the subtilisin K2 model generated by SWISS-MODEL indicated good results, and the model was subsequently employed for molecular docking studies, with fibrin serving as the substrate. This result is important with respect to the different sources and fermentation methods of natto in Japan and moromi in Indonesia. To date, nattokinase-like enzymes with high fibrin-degrading activities have not been reported anywhere except Japan, and this study is the first to show a nattokinase-like enzyme from Indonesia.

At present, there is no report describing a molecular docking analysis between nattokinase and fibrin, with fibrin serving as the substrate. Mohanasrinivasan et al. [47] reported a molecular docking analysis between nattokinase and fibrinogen. Ferrall-Fairbanks et al. [48] also reported a molecular docking analysis between other fibrin-degrading enzymes, namely, cysteine cathepsins (cathepsins K, L, and S) and the fibrinogen α, β, and γ chains, while Zheng et al. [49] docked nattokinase with ligands (substrate model), such as a chromogenic substrate for plasmin (H-D-VLK-pNA) and a streptokinase-activated plasminogen (Suc-AAPF-pNA).

Therefore, this molecular docking study, which demonstrates a strong interaction between a food-derived fibrin-degrading enzyme (subtilisin K2) from Indonesian *B. subtilis* K2 and its fibrin substrate, represents an important addition to the current body of knowledge concerning enzymes that are capable of interacting with and digesting fibrin, and this enzyme may have potential as a treatment for thrombus.

Ezat et al. [50] reported that overall, molecular docking analysis can be employed to probe the interactions between molecules and proteins at the atomic level to characterize the behavior of molecules in the binding sites of target proteins and to explain fundamental biochemical processes. The molecular docking approach between fibrin-degrading enzymes and specific substrates, such as fibrin and fibrinogen, attempts to predict the mechanism of action employed fibrin-degrading enzymes against thrombi. Mohanasrinivasan et al. [47] docked nattokinase and fibrinogen to determine the structural basis of nattokinase and fibrinogen interaction and to identify the best binding mode; Zheng et al. [49] also docked nattokinase structure with other ligands (a chromogenic substrate for plasmin and a streptokinase-activated plasminogen) to elucidate the relation between the structure and function of nattokinase; and Ferrall-Fairbanks et al. [48] performed molecular docking analysis to investigate potential binding interactions and sites of hydrolysis between cathepsins K, L, and S and fibrinogen.

Limitation in our study presently is that purification of the fibrin degrading enzmye from *B. subtilis* K2 of Moromi for physicochemical characterization has not been completed. This aspect would be included in our future work.

Conclusion

Structural analysis demonstrated that the fibrinolytic protease subtilisin K2 is a serine protease with a typical catalytic triad (Asp, His, Ser). The presence of a high extinction coefficient, low instability index, high aliphatic index and low GRAVY value imply that this enzyme is highly thermostable and has excellent solubility in water. Based on the results of the docking studies, it is concluded that subtilisin K2 and fibrin substrate have an extended binding pattern and interact with the crucial residues important for enzyme activity. Molecular docking also indicated a strong interaction between the two compounds, with negative ΔG (high binding affinity) (− 19.4 kcal/mol) and low Kd values (6.3E-15 M) being observed. The strong interactions between this enzyme and its fibrin substrate demonstrate that fibrin can be rapidly degraded by this enzyme, suggesting its application as a treatment for thrombus diseases.

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Compliance with ethical standards

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

Ethical approval The bacteria as source of the gene and enzyme was isolated from local soy fermented food. The data taken for result and discussion were original and came from the experimets 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.

Research involving human and animal participants This research did not use any animal or human subject. All methodology applied were in accordance with the reference cited.

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