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BIOINFORMATIC ANALYSIS OF SUBTILISIN-K2 FROM INDONESIAN MOROMI: EVALUATION OF ITS ANTITHROMBOTIC POTENTIAL FOR FUNCTIONAL FOOD APPLICATION

Analisis Bioinformatika Subtilisin-K2 dari Moromi Indonesia: Evaluasi Potensi Antitrombotik Untuk Aplikasi Pangan Fungsional

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ABSTRACT

Microbial fibrinolytic enzymes are commonly found in various fermented foods of both plant and animal origin. While extensive studies have been conducted in Japan, Korea, and China, research in Indonesia remains limited despite its rich diversity of fermented foods. Moromi, an intermediate product of soy sauce fermentation, contains Subtilisin-K2, an enzyme proven in vitro to degrade fibrin and fibrinogen, indicating potential antithrombotic activity. This study investigated the antithrombotic properties of Subtilisin-K2 using bioinformatic approaches, including molecular docking (HADDOCK) and molecular dynamics (GROMACS). Subtilisin-K2 exhibited strong binding affinities with fibrin, fibrinogen, PAI-1, PAI-2, and α-antiplasmin, with Gibbs free energy values of –19.4, –15.6, –15.7, –18.2, and –13.3 kcal/mol, respectively. Molecular dynamics confirmed the stability of these complexes. These findings suggest that Subtilisin-K2 from Indonesian moromi exhibits significant bioactivity, underscoring the potential of Indonesian fermented products as valuable sources of functional enzymes, especially as antithrombotic potential.

Keywords: *Antithrombotic, Bioinformatics, Functional Food, Moromi, Subtilisin-K2*

ABSTRAK

Enzim fibrinolitik mikroba banyak ditemukan pada berbagai pangan fermentasi, baik berbasis nabati maupun hewani. Penelitian mengenai enzim tersebut telah banyak dilakukan di Jepang, Korea, dan Tiongkok. Namun, penelitian di Indonesia terkait enzim fibrinolitik mikroba dari produk fermentasi masih terbatas, padahal Indonesia dikenal sebagai negara yang kaya akan pangan fermentasi. Moromi, produk antara fermentasi kecap di Indonesia, diketahui mengandung Subtilisin-K2, enzim yang terbukti secara in vitro mampu mendegradasi fibrin dan fibrinogen sehingga berpotensi sebagai agen antitrombotik. Penelitian ini bertujuan menginvestigasi sifat antitrombotik Subtilisin-K2 melalui pendekatan bioinformatika, yang berfokus pada kemampuan Subtilisin-K2 dalam menghambat Plasminogen Activator Inhibitor (PAI) dan antiplasmin serta kestabilan ikatannya terhadap fibrin dan fibrinogen. Analisis dilakukan menggunakan penambatan molekul (HADDOCK) dan dinamika molekul (GROMACS) untuk mengevaluasi kestabilan interaksi Subtilisin-K2 dengan fibrin, fibrinogen, PAI-1, PAI-2, dan α-antiplasmin. Hasil penelitian menunjukkan bahwa Subtilisin-K2 memiliki interaksi molekul yang kuat dengan seluruh substrat target dengan nilai energi bebas Gibbs masing-masing sebesar –19,4; –15,6; –15,7; –18,2; dan –13,3 kkal/mol. Interaksi ini menunjukkan bahwa Subtilisin-K2 memiliki po-

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tensi sebagai agen antitrombotik, yang diperkuat dengan hasil dinamika molekul yang menunjukkan interaksi stabil. Temuan ini menunjukkan bahwa Subtilisin-K2 dari moromi Indonesia memiliki bioaktivitas yang signifikan, serta menegaskan potensi produk fermentasi Indonesia sebagai sumber enzim fungsional yang bermanfaat, khususnya sebagai aktivitas antitrombotik.

Kata Kunci: *Antithrombotik, Bioinformatik, Moromi, Pangan Fungsional, Subtilisin-K2*

INTRODUCTION

Indonesia is recognized as one of the countries with a rich diversity of fermented foods (Syahbanu et al. 2020b). These fermented products are not only consumed as staple foods but also possess significant potential as sources of bioactive compounds with health benefits (Egea et al. 2022). Traditional Indonesian fermented products such as tempeh, tape, and soy sauce have long been consumed and are now recognized to contain bioactive compounds with antimicrobial, antioxidant, and potential anti-degenerative benefits (Alfid Kurnianto et al. 2023; Kurnianto et al. 2023b; Kurnianto et al. 2024a). One particularly promising fermented product from Indonesia is moromi, an intermediate product in soy sauce production (Syahbanu et al. 2020b; Syahbanu et al. 2020a; Syahbanu et al. 2022; Kurnianto et al. 2023b; Kurnianto et al. 2023a; Syahbanu and Pawestri 2023; Kurnianto et al. 2024b). Moromi is a soy-based fermented product rich in enzymes and secondary metabolites produced during the fermentation process. Subtilisin-K2 is a key protease produced during moromi fermentation and has demonstrated fibrinolytic activity, including degradation of fibrin, a critical factor in blood clot formation (Syahbanu et al. 2020b; Syahbanu et al. 2020a; Syahbanu et al. 2022a; Kurnianto et al. 2023b; Syahbanu and Pawestri 2023).

Abnormal blood clot formation within blood vessels (thrombosis) is a leading cause of cardiovascular diseases, including stroke, myocardial infarction, and pulmonary embolism (Benjamin et al. 2019). Globally, thrombosis is closely associated with cardiovascular disease (CVD), which remains the foremost cause of mortality worldwide. In 2019, an estimated 18.6 million people died due to CVD, with thrombosis, particularly through ischemic heart disease and stroke, contributing to 85% of all CVD-related deaths (World Health Organization 2018). This condition arises when fibrin, the primary structural component of blood clots, is inadequately degraded by the body's endogenous fibrinolytic system (Cesarman-Maus and Hajjar 2005; Longstaff and Kolev 2015). Therefore, fibrinolytic agents, which can enhance the breakdown of fibrin, are critical in the prevention and treatment of thrombosis (Syahbanu et al. 2020b; Syahbanu et al. 2022).

Other Asian fermented products, such as Japanese natto, have been widely studied for their fibrin-degrading enzyme Nattokinase, which functions as a safe and effective natural antithrombotic agent used in various health products and supplements (Wu et al. 2024). In contrast, the potential of Indonesian fermented food products, such as moromi, to provide similar enzymatic activities has not been thoroughly investigated. Subtilisin-K2, a proteolytic enzyme produced during the fermentation of moromi, has demonstrated in vitro efficacy in degrading fibrin and fibrinogen (Syahbanu et al. 2020b). This enzymatic activity suggests that moromi shows potential as a functional food candidate, particularly in the prevention and management of thrombosis (Syahbanu et al. 2020b; Syahbanu et al. 2020a; Syahbanu et al. 2022).

Beyond its health benefits, developing moromi as a functional food also offers strong economic potential. Global demand for natural, preventive health products creates opportunities for moromi to enter and compete in international markets (Venter de Villiers et al. 2024). Advancing moromi as a functional food product could substantially enhance the understanding of local resources and the potential of traditional Indonesian foods in promoting human health (Sgroi et al. 2024; Surya 2024). This innovation also creates opportunities to investigate other fermented foods that remain underexplored. In a time of rising interest in natural

and holistic nutrition, products like moromi offer timely and impactful health-promoting solutions (Herrero et al. 2023).

This study aimed to investigate the potential of moromi as a natural antithrombotic agent. The focus was to examine the inhibitory effects of Subtilisin-K2 on Plasminogen Activator Inhibitor (PAI) and antiplasmin involved in coagulation and fibrinolysis. The study also evaluated the binding stability of Subtilisin-K2 with fibrin and fibrinogen substrates.

MATERIALS AND METHODS

Time and Place

This study was conducted from August 2024 to September 2025 at the Nutrition Analysis Laboratory, Faculty of Health Sciences, Universitas Singaperbangsa Karawang, Indonesia. The molecular dynamics simulation was performed at the Research Institute Inbio Indonesia, Malang.

Materials

The 3D structure of Subtilisin K2 was constructed using the SWISS-MODEL Workspace program (RRID:SCR_018123) (Arnold et al. 2006; Benkert et al. 2011; Biasini et al. 2014) as reported by (Syahbanu et al. 2020a; Syahbanu et al. 2022), and Nattokinase as reference was retrieved from RCSB PDB (PDB ID: 4DWW). The fibrin, fibrinogen, PAI-1, PAI-2, and α2-Antiplasmin structures were retrieved from RCSB PDB (PDB IDs: 2HLO (fibrin), 3GHG (fibrinogen), PAI-1 (1B3K), PAI-2 (AF_AFP05120F1), and α2-Antiplasmin (AF_AFP08697F1)) as receptors for the molecular docking experiments with Subtilisin K2 and Nattokinase as a ligand. For dynamic molecular simulation only used fibrin (2HLO) and fibrinogen (3GHG) as receptor.

Molecular docking analysis

In this study, molecular docking was performed between the Subtilisin K2 model and Nattokinase as reference. PAI and α2-Antiplasmin models as substrate. The previous study, molecular docking has been conducted between Subtilisin-K2 and Nattokinase as ligand and either fibrin and fibrinogen as substrate (Syahbanu et al. 2022). The High Ambiguity Driven protein-protein DOCKing (HADDOCK) web server (Van Zundert et al. 2016; Vangone et al. 2017) was used to perform protein-protein docking. Prediction of the binding affinity in protein-protein complexes was performed using the Prodigy web server (Vangone and Bonvin 2015; Xue et al. 2016). The LigPlot+ Version v.2.1 (Wallace et al. 1995) was used to generate schematic diagrams of protein-protein interactions.

Molecular dynamic simulation of Subtilisin-K2 and Nattokinase with fibrin and fibrinogen

The computer specifications used are DELL Optiplex 7000 Tower i9-12900K 64GB 2TB+SSD1TB PC and Nvidia GeForce RTX 3070 8GB. The structure of ligand was fixed using MODELLER 10.4 (Eswar et al. 2003) through Chimera1.17.1. Protein and ligand preparation using GROMACS 2023 (Abraham et al. 2015) consisting of protein topology preparation with pdb2gmx. Force field for protein using AMBER99SB (Lindorff-Larsen et al. 2010). For ligand topology, acpype (https://github.com/alanwilter/acpype) was used. Subsequently, protein and ligand topology merging, solvation, ion addition, equilibration, minimization and MD production were performed. The final concentration of the system was set at 0.15 concentration through the addition of Na^+ and Cl^- ions. MD production was performed for 50,000 ps (50 ns). The temperature was set at 310.15 K or equivalent to 37°C. MD interpretation was displayed in the form of root mean square deviation (RMSD) graphs on the backbone, root mean square fluctuation (RMSF) on C-alpha and solvent-accessible surface area on the protein using Grace software (Huang et al. 2021).

RESULTS AND DISCUSSION

Molecular Docking Analysis

Molecular docking between fibrin-degrading enzyme and fibrin as substrate

The mechanism of interaction between Subtilisin-K2 and Nattokinase with various domains of fibrin in its three distinct chains (α, β, and γ) is presented in **Table 1** and **Figure 1**. These enzymes possessed active sites comprising specific residues (Asp19, His51, and Ser208 in Subtilisin-K2

(**Figure 1A, 1C**); Asp32, His64, and Ser221 in Nattokinase (**Figure 1B, 1D**)) that function as the primary regions for interactions with fibrin domains. Each enzyme interacted specifically with domains A through F of the α, β, and γ fibrin chains, exhibiting some overlapping and distinct residues between the two enzymes. For instance, in Domain B of the γ chain, the primary interacting residues for Subtilisin-K2 are Leu168 and Leu172, whereas Nattokinase additionally involved residues such as Leu175, Arg176, and Ile179. These differences indicated that the distinct affinities of the two enzymes for specific regions of fibrin, which play a crucial role in determining their effectiveness and selectivity in fibrin substrate interaction during the fibrinolysis process. These results are in line with the research of Syahbanu et al. 2020b; Syahbanu et al. 2020a; Syahbanu et al. 2022 who reported that each amino acid that interacted between the fibrin-degrading enzyme and their substrates had a certain type of bond that could affect the binding affinity.

Table 1. Molecular interaction between fibrin-degrading enzyme model active site and fibrin domain

Enzyme	Prediction of Fibrin's Interactive Site (2HLO)					
	α Chain		β Chain		γ Chain	
	Domain A	Domain D	Domain B	Domain E	Domain C	Domain F
Subtilisin-K2 **(Active Site: Asp19, His51, Ser208)**	Arg171, Glu151, Ile154, Leu150	Glu151, Arg171, Ile154, Leu150	Leu168, Leu172	**Leu168**, Leu172, **Ile171**	Lys140, Cys139, Leu131, Cys135, Glu132	Lys140, Cys139, Leu131, Cys135, Glu132
Nattokinase (4DWW) **(Active Site: Asp32, His64, Ser221)**	Arg171, Glu151, Ile154, Leu150	Glu151, Arg171, Ile154, Leu150	Leu168, Leu172	**Leu175**, **Arg176,** Leu172, **Ile179**	Lys140, Cys139, Leu131, Cys135, Glu132	Lys140, Cys139, Leu131, Cys135, Glu132

Note: **Bold residues** are the difference of interactive site between Subtilisin-K2 and Nattokinase

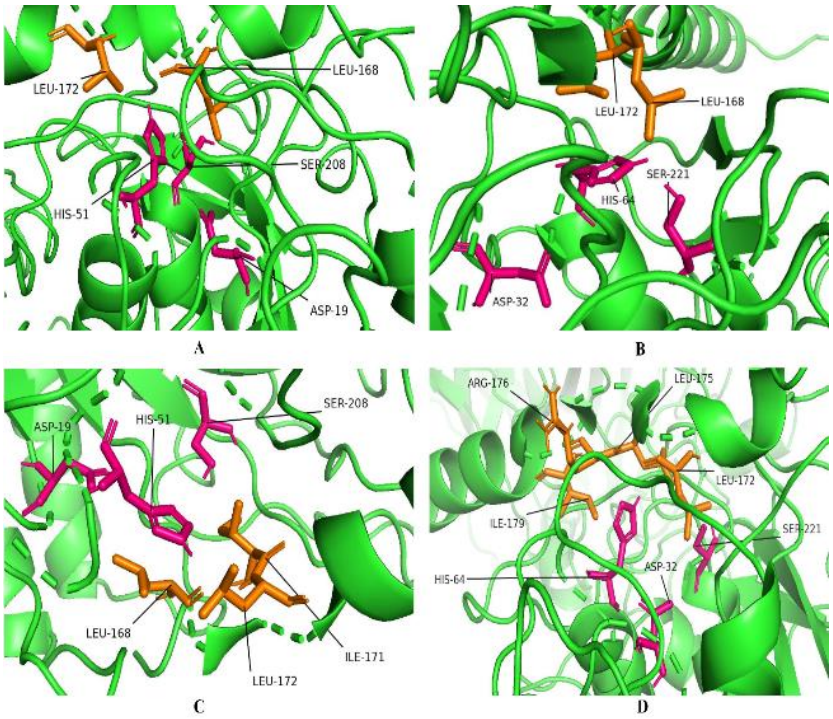


Figure 1. Molecular interaction between Subtilisin-K2 and domain B and E of fibrin (1A, 1C); and also interaction between Nattokinase and domain B and E of fibrin (1B, 1D).

Based on the ligplot results, the hydrogen bonds formed on Subtilisin-K2-fibrin have more hydrogen bonds than nattokinase-fibrin. In addition to hydrogen bonds, the number of hydrophobic interactions on Subtilisin-K2-fibrin was also more than nattokinase-fibrin. This will affect the Gibbs free energy/binding energy (ΔG) and Dissociation constant (Kd) values of the two fibrin-degrading enzymes. These binding affinities determine whether complex formation between the two proteins occurs under certain conditions that contribute to the strength of their interaction. The smaller the Gibbs free energy/binding energy (ΔG) value (more negative energy value), the stronger the interaction formed between the receptor and ligand. The Gibbs free energy/binding energy (ΔG) value, is determined by the equilibrium dissociation constant (Kd). A higher Kd value indicates a lower binding affinity of the ligand for its target and vice versa (Li et al. 2024).

The docking analysis results for Subtilisin-K2 and Nattokinase with fibrin domains are presented using three parameters (**Table 2**): 1) Gibbs free energy/binding energy (ΔG), measured in kcal/mol, which indicates the strength of interaction between the enzyme and the fibrin domain, with lower values signifying more stable binding; 2) Dissociation constant (Kd) at 37°C, which reflects binding affinity, where a smaller Kd indicates a tighter binding of the enzyme to a specific fibrin domain; and 3) RMSD (Root Mean Square Deviation), measuring the positional differences of atoms between the interaction model and the original structure, where a smaller RMSD indicates higher stability of the enzyme-fibrin complex (Xue et al. 2016). For instance, in Domain E of the β chain, Subtilisin-K2 exhibited a ΔG of -19.4 kcal/mol with a Kd of 6.30×10^{-15}, indicating a strong and stable interaction. In contrast, Nattokinase showed a ΔG of -17.1 kcal/mol with a slightly different Kd of 9.10×10^{-13}. Although both enzymes demonstrated comparable binding energies, the slightly higher Kd for Nattokinase suggested that its binding to fibrin in this domain might be slightly less robust than that of Subtilisin-K2.

Nattokinase displayed slightly lower ΔG, Kd, and RMSD values across most domains, except for Domain E, suggesting a stronger tendency for interaction with fibrin strands. However, Subtilisin-K2 exhibited ΔG, Kd, and RMSD values that were not significantly different from those of Nattokinase, indicating that the moromi enzyme possessed fibrinolytic potential comparable to Nattokinase, as both enzymes are capable of degrading pre-formed fibrin. When linked to the interaction mechanisms of Subtilisin-K2 and Nattokinase with various fibrin domains, it was observed that Nattokinase interacted with a greater number of amino acid residues in the fibrin domains compared to Subtilisin-K2. This difference contributed to the variations in ΔG, Kd, and RMSD values observed between the two enzymes.

Molecular docking between fibrin-degrading enzyme and fibrinogen as substrate

The interactions between Subtilisin-K2 and Nattokinase with fibrinogen chains (the precursor of fibrin), which consisted of the α, β, and γ chains, are summarized in **Table 3** and **Figure 2**. Each chain interacted with distinct residues, reflecting the specificity of each enzyme towards fibrinogen. For example, on the β chain of fibrinogen, Subtilisin-K2 bound to residues such as Ser111, Ser114, Met118, Phe115, and Leu121 (**Figure 2A**), while Nattokinase interacted with similar residues but substituted Met118 with Thr110 (**Figure 2B**). This suggested that the interaction profile of Subtilisin-K2 on the β chain of fibrinogen closely resembles that of Nattokinase. Although the differences in residue interactions may appear minor, they could influence the enzymatic efficiency in hydrolyzing fibrinogen. Such variations may affect the rate of fibrinogen conversion to fibrin and consequently impact the reduction of blood clot formation.

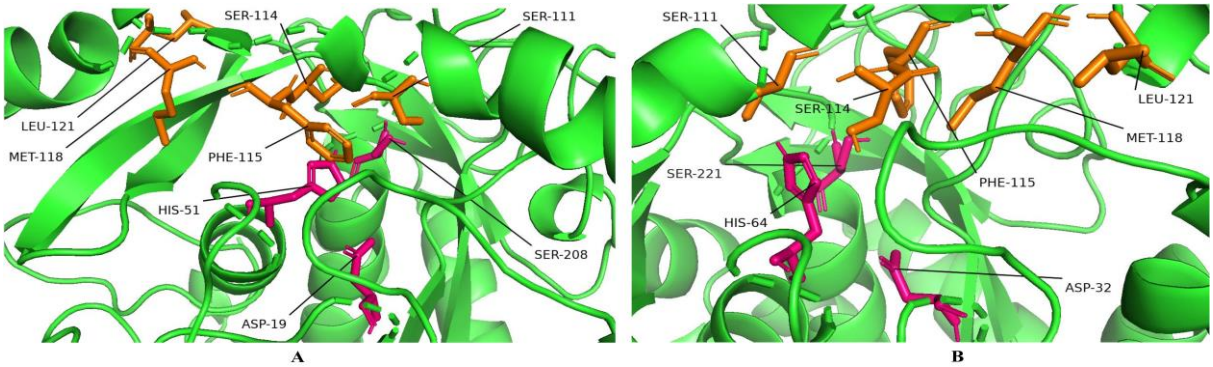


Figure 2. Molecular interaction between Subtilisin-K2 (A) and Nattokinase (B) with β Chain of fibrinogen

Table 2. Docking analysis fibrin-degrading enzyme model with fibrin's domain

Fibrin (2HLO)		Subtilisin-K2 (Syahbanu et al. 2022b)			Nattokinase (4DWW)		
		ΔG (kcal mol-1)	Kd (M) (T= 37°C)	RMSD	ΔG (kcal mol-1)	Kd (M) (T= 37°C)	RMSD
α Chain	Domain A	-14,9	1,10E-11	0,7 ± 0,5	-17,6	3,90E-13	0,4 ± 0,2
	Domain D	-14,8	1,40E-11	0,4 ± 0,2	-17,5	4,50E-13	0,5 ± 0,3
β Chain	**Domain B**	-19	1,10E-14	0,5 ± 0,3	**-19,4**	**2,00E-14**	0,5 ± 0,3
	Domain E	**-19,4**	**6,30E-15**	0,8 ± 0,6	-17,1	9,10E-13	0,9 ± 0,6
γ Chain	Domain C	-13,9	6,50E-11	0,5 ± 0,3	-16,9	1,30E-12	0,5 ± 0,3
	Domain F	-13,5	1,30E-10	1,0 ± 1,0	-14,6	5,00E-11	0,5 ± 0,3

Note: **Bold font** illustrated strong interaction between fibrin-degrading enzymes model (Subtilisin-K2 and Nattokinase) and β chain

Table 3. Molecular interaction between fibrin-degrading enzyme model active site and fibrinogen chain

Enzyme	Prediction of Fibrinogen's Interactive Site (3GHG)		
	α Chain	β Chain	γ Chain
Subtilisin-K2	Phe117, **Asp124**, Asn121	Ser111, Ser114, **Met118**, Phe115, Leu121	Phe54, Thr57, Asp53
Nattokinase (4DWW)	Phe117, **Ala120**, Asn121	Ser111, Ser114, **Thr110**, Phe115, Leu121	Phe54, Thr57, Asp53

Note: **Bold residues** are the difference of interactive site between Subtilisin-K2 and Nattokinase

Table 4. Docking analysis fibrin-degrading enzyme model with fibrinogen's chain

Fibrinogen (3GHG)	Subtilisin-K2 (Syahbanu et al. 2022)			Nattokinase (4DWW)		
	ΔG (kcal mol-1)	Kd (M) (T= 37°C)	RMSD	ΔG (kcal mol-1)	Kd (M) (T= 37°C)	RMSD
α Chain	-14,9	1,10E-11	0,7 ± 0,5	-13,8	2,00E-10	0,5 ± 0,3
β Chain	**-15,6**	**3,90E-12**	0,4 ± 0,3	-14,1	1,10E-10	0,5 ± 0,3
γ Chain	-15,2	6,70E-12	0,4 ± 0,2	**-15,5**	**1,30E-11**	0,4 ± 0,2

Note: **Bold font** illustrated strong interaction between fibrin-degrading enzymes model and β chain

The interactions between Subtilisin-K2 and Nattokinase with fibrinogen are presented in **Table 4**, with the same parameters as in **Table 2** (ΔG, Kd, and RMSD). This analysis aimed to assess the enzyme's affinity for fibrinogen, which is crucial before this protein is converted into fibrin during the coagulation process. Subtilisin-K2 has a ΔG

of -15.6 kcal/mol on the β chain of fibrinogen, with a Kd of 3.90×10^{-12}, indicating a strong binding. In contrast, Nattokinase showed a ΔG of -14.1 kcal/mol with a higher Kd of 1.10×10^{-10}, suggesting that Nattokinase has a slightly weaker affinity for the β chain of fibrinogen compared to Subtilisin-K2. On the γ chain, Subtilisin-K2 exhibited a ΔG of -15.2 kcal/mol with a Kd of 6.70×10^{-12}, indicating a strong interaction between the enzyme and this chain. Nattokinase also interacted strongly with the γ chain, but with a slightly higher ΔG of -15.5 kcal/mol and a Kd of 1.30×10^{-11}. Therefore, Subtilisin-K2 demonstrated more stable and stronger interactions with the β chain of fibrinogen, which may contribute to its role as a more effective fibrinolytic enzyme. The study conducted by (Mohanasrinivasan et al. 2017) reported that Nattokinase and fibrinogen adopted an extended binding pattern and interacted with the important residues to show activity, so it is possible that the interaction pattern affects the binding affinity of nattokinase to fibrinogen.

Binding effectiveness of the fibrin-degrading enzyme to fibrin and fibrinogen

Based on the molecular docking analysis presented in **Table 2** and **Table 4**, the effectiveness of Subtilisin-K2 and Nattokinase in interacting with fibrin and fibrinogen can be evaluated. Some important parameters, such as binding energy (ΔG), dissociation constant (Kd), and interaction stability (RMSD), provide information regarding the strength and stability of each enzyme's bond to its substrate in the fibrin degradation process. From the parameters of binding energy (ΔG), dissociation constant (Kd), and interaction stability (RMSD), an overview of the effectiveness of Subtilisin-K2 and Nattokinase in interacting with fibrin and fibrinogen was obtained. In some fibrin domains, Nattokinase showed lower ΔG values than Subtilisin-K2, indicating a stronger interaction. For example, on Domains A and D of the fibrin α-chain, Nattokinase had ΔG values of -17.6 and -17.5 kcal/mol, lower than Subtilisin-K2 which showed -14.9 and -14.8 kcal/mol, respectively. In contrast, on fibrinogen, Subtilisin-K2 showed lower ΔG values in some interactions, especially in the β and γ chains, as seen in the β chain with a ΔG value of -15.6 kcal/mol for Subtilisin-K2, lower than Nattokinase which has a ΔG of -14.1 kcal/mol. This indicated that Subtilisin-K2 has a stronger bond with fibrinogen, especially on the β-chain, which is an important domain in fibrinogen interaction.

In addition, based on the dissociation constant (Kd) values, Nattokinase generally showed higher affinity to fibrin than Subtilisin-K2 in some domains. In Domains B and E of the β-chain, Nattokinase showed lower Kd values, indicating a stronger binding affinity to fibrin. In contrast, Subtilisin-K2 had lower Kd values on several interactions with fibrinogen, especially on the β and γ chains, indicating a stronger affinity to fibrinogen than than nattokinase's affinity to fibrinogen. In terms of interaction stability, the RMSD values of the two enzymes are relatively similar in various domains of fibrin and fibrinogen, indicating that their binding stability to the substrate is comparable. These small differences in ΔG and Kd values reflect the different affinity and effectiveness of Subtilisin-K2 and Nattokinase in the degradation process of fibrin and fibrinogen, which may affect their potential in fibrinolysis applications. It can be reported that Subtilisin-K2 is effective in preventing the formation of fibrin as well as degrading the already formed fibrin threads, so this moromi enzyme has the potential to be an antithrombosis agent because it has similar advantages to Nattokinase.

Molecular docking between fibrin-degrading enzyme and PAI and α2-Antiplasmin

Subtilisin-K2 interacted with Ala345 and Arg346 residues on PAI-1 through active sites consisting of Asp19, His51, and Ser208 (**Table 5, Figure 3A**). The Gibbs free energy (ΔG) value of -15.7 kcal/mol and the dissociation constant (Kd) of 8.3E-12 M at 37°C indicated that Subtilisin-K2 formed a strong bond with PAI-1. Meanwhile, Nattokinase interacted with residue Arg346 (**Figure 3B**), resulting in a ΔG of -12.1 kcal/mol and a Kd of 3.2E-09 M (**Table 5**), indicating a weaker interaction than Subtilisin-K2. For PAI-2, Subtilisin-K2 interacted with residues Phe81, Gln84, and Ile85, with a ΔG of -18.2 kcal/mol and a Kd of 1.5E-13 M (**Table 6, Figure 3C**). These results indicated that the

interaction between Subtilisin-K2 and PAI-2 was very strong, which is indicated by the low ΔG value and small Kd. In comparison, Nattokinase interacted with five different residues on PAI-2, namely Phe81, Gln84, Ile85, Lys87, and Gly88 (**Figure 3D**), with a ΔG value of -16.6 kcal/mol and a Kd of 4.43E-12 M (**Table 6**). The affinity of Nattokinase to PAI-2 is lower than that of Subtilisin-K2, although Nattokinase interacted with more residues.

Table 5. Molecular interaction and docking analysis between fibrin-degrading enzyme model's active site and plasminogen activator inhibitor-1 (PAI-1)'s chain (1B3K)

Enzyme	Prediction of Plasminogen Activator Inhibitor-1 (PAI-1)'s Interactive Site (1B3K)	ΔG (kcal mol-1)	Kd (M) (T= 37°C)
Subtilisin-K2	**Ala 345**, Arg346	-15.7	8.3E-12
Nattokinase (4DWW)	Arg346	-12.1	3.2E-09

Note: **Bold residues** are the difference of interactive site between Subtilisin-K2 and Nattokinase; Underlined font illustrated strong interaction between fibrin-degrading enzymes model and PAI-1 chain

Table 6. Molecular interaction and docking analysis between fibrin-degrading enzyme model's active site and plasminogen activator inhibitor-2 (PAI-2)'s chain (AF_AFP05120F1)

Enzyme	Prediction of Plasminogen Activator Inhibitor-2 (PAI-2)'s Interactive Site (AF_AFP05120F1)	ΔG (kcal mol-1)	Kd (M) (T= 37°C)
Subtilisin-K2	Phe81, Gln84, Ile85	-18.2	1.5E-13
Nattokinase (4DWW)	Phe81, Gln84, Ile85, **Lys87, Gly88**	-16.6	4.43E-12

Note: **Bold residues** are the difference of interactive site between Subtilisin-K2 and Nattokinase; Underlined font illustrated strong interaction between fibrin-degrading enzymes model and PAI-2 chain

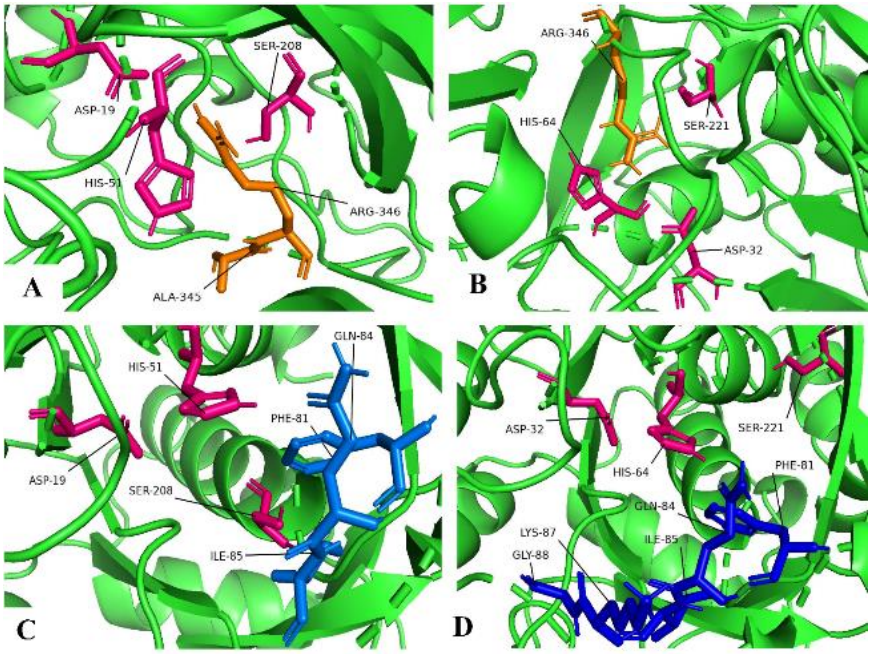


Figure 3. Molecular interaction of Subtilisin-K2 with PAI-1 (orange licorice) (A) and PAI-2 (blue licorice) (C) and also interaction of Nattokinase (B) with PAI-1 (orange licorice) (B) and PAI-2 (blue licorice) (D)

Analysis of the interaction between Subtilisin-K2 and α2-Antiplasmin showed that this enzyme interacted with residues Leu4, Trp5, Leu8, and Val9 (**Figure 4A**). These values indicated high affinity, although lower compared to the interactions with PAI-1 and PAI-2. In contrast, Nattokinase interacted with residues Trp5, Leu8, and Val9 (**Figure 4B**), resulting in a ΔG of -12.4 kcal/mol and a Kd of 3.63E-9 M (**Table 7**). Based on the comparison of these ΔG and Kd values, Subtilisin-K2 showed higher affinity to α2-Antiplasmin than Nattokinase (**Table 7**), although the difference was not as large as that of PAI-1 and PAI-2.

Table 7. Molecular interaction and docking analysis between fibrin-degrading enzyme model's active site and α2-Antiplasmin's chain (AF_AFP08697F1)

Enzyme	Prediction of α2-Antiplasmin 's Interactive Site (AF_AFP08697F1)	ΔG (kcal mol-1)	Kd (M) (T= 37°C)
Subtilisin-K2	**Leu4,** Trp5, Leu8, Val9	-13.3	4.2E-10
Nattokinase (4DWW)	Trp5, Leu8, Val9	-12.4	3.63E-9

Note: **Bold residues** are the difference of interactive site between Subtilisin-K2 and Nattokinase; Underlined font illustrated strong interaction between fibrin-degrading enzymes model and α2-Antiplasmin chain

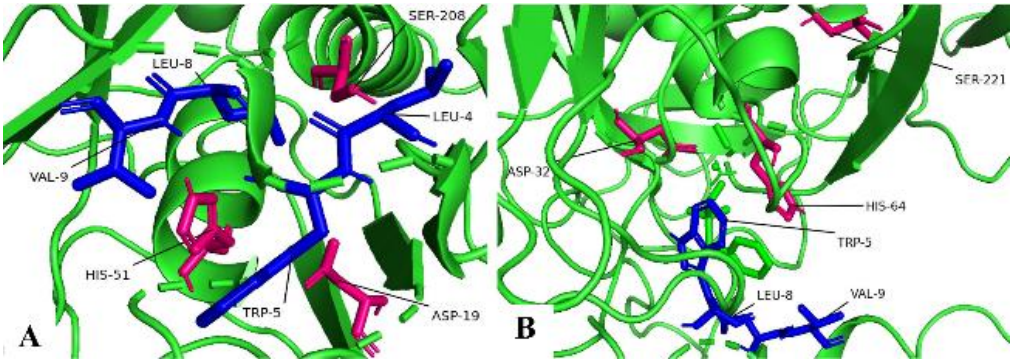


Figure 4. Molecular interaction between Subtilisin-K2 (A) and Nattokinase (B) with α2-Antiplasmin

Implications In Functional Food Development and Fibrinolytic Therapy Applications

Subtilisin-K2 showed stronger interaction affinity with plasminogen inhibitors (Plasminogen Activator Inhibitor-1 (PAI-1), Plasminogen Activator Inhibitor-2 (PAI-2)), and α2-Antiplasmin, compared to Nattokinase; and has similar interaction affinity strength with Nattokinase towards fibrin and fibrinogen substrates. These advantages are important factors in the development of fermentation-based functional food products, such as moromi, where Subtilisin-K2 can act as a bioactive agent that supports cardiovascular health through fibrinolytic activity (**Figure 5**).

These findings are in accordance with studies showing that enzymes with low ΔG values and small dissociation constants (Kd) have greater potential for biomedical and functional food applications (Syahbanu et al. 2022a; Yang et al. 2024). Functional food products containing fibrinolytic enzymes have the potential to reduce the risk of blood clots, improve blood flow, and support overall cardiovascular health. In addition, the high affinity of Subtilisin-K2 to plasminogen inhibitors opens up opportunities for the development of fibrinolytic enzyme-based therapies. Subtilisin-K2 could be a promising candidate for therapies that require enzymes with strong interactions to plasminogen inhibitors, thus more effectively addressing conditions related to blood coagulation or thrombosis. As a natural alternative, the application of Subtilisin-K2 in the form of fermented foods also offers significant health benefits without the need for direct pharmaceutical intervention.

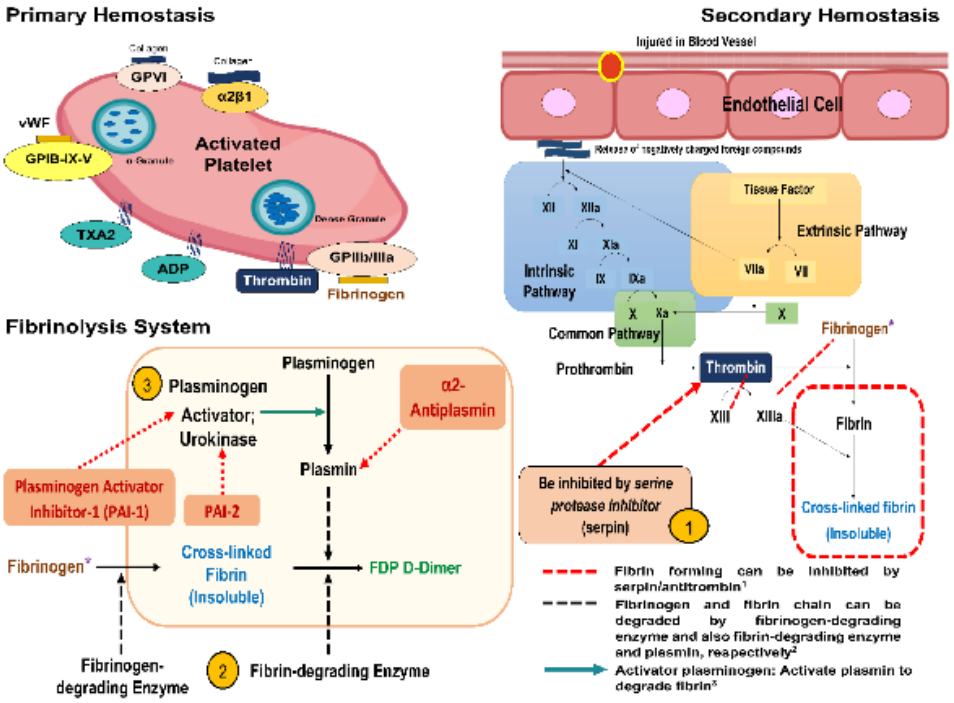


Figure 5. Hemostasis mechanisms (Cesarman-Maus and Hajjar 2005; Kotb 2014; Repetto and De Re 2017)

Molecular Dynamic Simulation

Molecular dynamics simulations using GROMACS software were performed to verify the molecular docking results (Huang et al. 2021). The simulation aimed to understand the behavior and evaluate the stability of the Subtilisin-K2 complex with fibrin and fibrinogen substrates in a simulated environment that mimics biological conditions as well as to compare the stability of the moromi enzyme with Nattokinase. The simulation results showed that the interaction between the enzyme and the substrates was stable throughout the simulation period. This indicated that Subtilisin-K2 not only has affinity for the target substrates, but was also able to maintain a stable interaction under dynamic conditions that resemble the cellular environment.

The results of molecular dynamics simulations on Subtilisin-K2 and Nattokinase showed various levels of interaction and stability in the analyzed system. RMSD, RMSF, and SASA analysis were used to evaluate the stability of the molecular structures and interactions during the 50 ns simulation. These molecular dynamics reinforce previous molecular docking results, where Subtilisin-K2 has strong potential as an antithrombosis agent. During the simulation, no significant shifts in the enzyme or substrate structures were found, indicating that the interaction may be relevant under real biological conditions.

Analysis of Root Mean Square Deviation (RMSD)

Molecular Dynamics Simulation was performed for 50 ns to observe the stability of Subtilisin_K2-Fibrin (SK2-F), Nattokinase-Fibrin (N-F), Subtilisin_K2-Fibrinogen (SK2-Fg), and Nattokinase-Fibrinogen (N-Fg). Simulation results on various protein chains, such as SK2-F, N-F, SK2-Fg, and N-Fg showed variations in RMSD values, indicating the stability of the interaction of each protein (**Figure 6**). SK2-F (Subtilisin_K2-Fibrin) and N-Fg (Nattokinase-Fibrinogen) showed considerable RMSD values reaching about 50 Å and this indicated significant fluctuations in molecular interactions. RMSD values greater than 2 Å generally indicate that the protein structure is not bound to the ligand in a stable manner, so the bond between the protein and the ligand cannot last long.

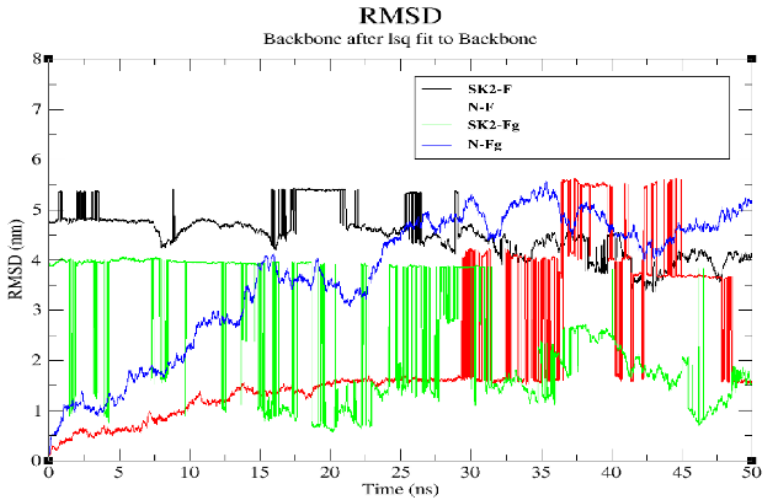


Figure 6. RMSD of fibrin-degrading enzyme and targeted substrate

This may be due to the presence of two weakly interacting or unstable protein chains at certain points during the simulation. Meanwhile, SK2-Fg (Subtilisin_K2-Fibrinogen) displayed an RMSD value of about 20 Å, which is smaller than SK2-F, but still showed some fluctuations. N-F (Nattokinase-Fibrin) had the most stable RMSD value, around 15 Å, indicating stronger and more stable interactions between its chains during the simulation.

The interaction between protein chains

Screening of interactions at 0 ns (initial), 25 ns (midpoint), and 50 ns (final) of the simulation (**Figure 7**) revealed the dynamic behavior between the protein chains. In SK2-F and SK2-Fg, the temporary separation observed throughout the simulation suggests weak or inconsistent interactions. Nevertheless, these complexes may still exhibit antithrombotic potential, as such transient contacts could support fibrinolytic activity under specific conditions or through alternative pathways.

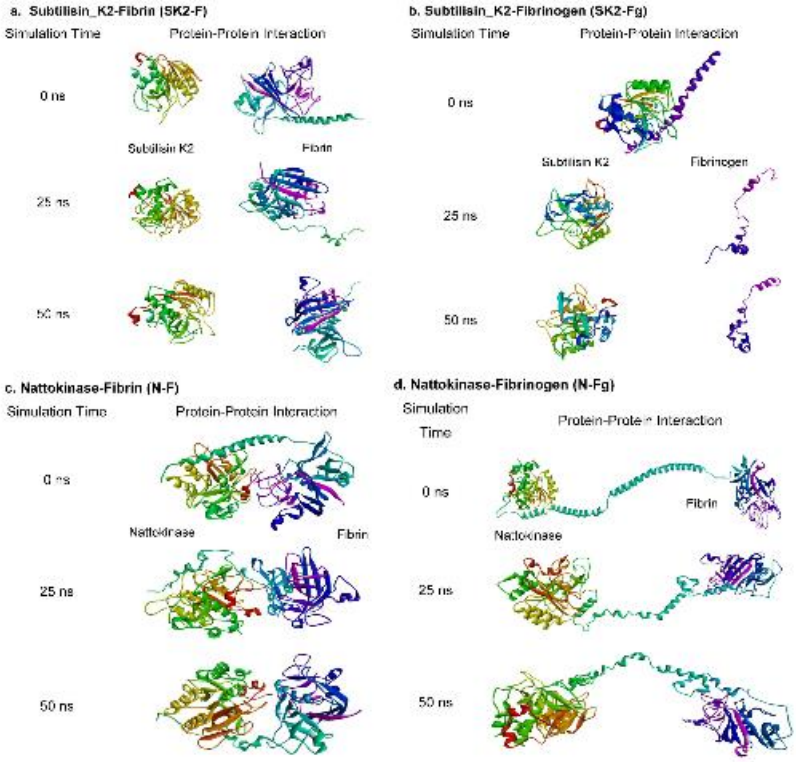


Figure 7. Protein-Protein interaction: a) SK2-F, b) SK2-Fg, c) N-F, and d) N-Fg

In contrast, the N-F and N-Fg complexes maintained persistent interactions throughout the simulation. Although slight separations occurred at several points, the enzymes quickly re-associated in a stable manner, demonstrating strong and continuous binding between Natto–Fibrin and Natto–Fibrinogen. This stability reflects the inherent characteristics of nattokinase and suggests that SK2 may share mechanistic similarities with natto in its antithrombotic action.

CONCLUSION

Subtilisin-K2 was proven to have the ability to inhibit the action of plasminogen inhibitors (PAI-1 and PAI-2) and antiplasmin in silico, and these fibrin-degrading enzymes were also suggested to have stable binding to fibrin and fibrinogen substrates. Although these simulation results provided important insights into the potential of Subtilisin-K2 and Nattokinase as antithrombosis agents, further studies need to be conducted to validate these results through in vitro and in vivo tests.

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