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In Silico Analysis of Protein of Milk, Soybean, and Kefir as Anti-Thrombotic Bioactive Peptide

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ABSTRACT

Currently, Cardiovascular Diseases (CVDs) cases are increasing significantly in Indonesia and the world. WHO reported that in 2019-related CVD patients accounted for 85%, which increased to 87% in 2021. The cases increased significantly by 2%, which is a significant number as it covered the world population. Stroke is a disease affected by platelets undergoing thrombolysis by the α-thrombin (IIa) receptor with coagulation factors on the heart blood vessel. Chemical or synthesis drugs to treat thrombolytics such as heparin are already available, but they would be harmful if misused. In this study, we focused on the activity, interaction, and prediction of the physicochemical of anti-thrombotic peptides. We found that k-casein from kefir has the highest activity of 0.037 A based on BIOPEP analysis with 17 peptide libraries and molecular docking visualization analysis using PyMol, PyRx, and Discovery Studio for screening the best peptide. ToxinPred and AlgPred showed there are no potentially harmful peptides, but the protein variant of β-conglycinin subunit can be an allergen known to the immune system IgE. In conclusion, the GPR peptide has the highest binding affinity, with a total energy of -6.8 kcal/mol.

1. Introduction

Cardiovascular Diseases (CVDs) are diseases with a high mortality rate on a global scale. Based on the World Health Organization (WHO) in 2019, it was recorded that 85% of the population had a stroke or heart attack and had killed 32% of the world population and an increase of 2% stroke patients in 2021. CVDs are caused by embolism or a collection of diseases of the heart and blockage of blood vessels, such as stroke, coronary heart disease, and heart infections (Cheng *et al.* 2019). Based on the Ministry of Health Republic of Indonesia, the data on stroke cases had a percentage of 10.9%, with the elderly (55-64 years) with a percentage of 33.3% in 2018 in Indonesia. Poor diet or excessive food consumption is a factor that causes embolism in patients with CVDs to occur. Thrombosis is the main process of producing thrombin as a receptor and will interact with blood platelets so that platelets will attach and clog tissues or cause blood clots in the body (Tu *et al.* 2019). Therefore, anti-thrombotic-based treatment is needed as an agent to prevent the thrombosis process from occurring abnormally.

Anti-thrombotic is currently obtained from animal protein synthesis, which is formulated into medical drugs to inhibit the action of α-thrombin (IIa) receptors, such as heparin, aspirin, hirudin, fondaparinux, and others (Eikelboom and Weitz 2010). The interaction of α-thrombin (IIa) and thrombotic factor occurs precisely at the receptor's active site at residues His57, Asp102/109, and Ser195 as well as in the exosite I or exosite II receptor (Cheng *et al.* 2019). This active site has fibrinogen which is convent soluble. It can convert fibrinogen into fibrin filaments when interacting with thrombotic factors. Furthermore, when factors V, XII, and XI are active, they will cause negative feedback and form a thrombus (Tu *et al.* 2017). Therefore, the food-derived bioactive peptide can be used as a good alternative as an anti-thrombotic bioactive peptide agent in preventing blood clot and avoiding the negative impact of conventional medicine. The purpose of this study was to conduct an *in silico* analysis of the activity of bioactive peptides, the potential for toxicity and allergies to sources of milk casein

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protein, soy protein, kefir protein, and to understand the molecular docking interaction as a representative of animal protein, vegetable protein, and protein produced from fermentation. *In silico* approach could be used as a guideline or supporting analysis during the *in vitro* or *in vivo* validation stage.

2. Materials and Methods

2.1. Protein Sequences Availability

The α-thrombin receptor can be accessed via the Protein Data Bank (PDB: https://www.rcsb.org/structure/2BVS) with code 2BVS. The 2BVS is selected because its most favored region of Ramachandran plot value almost reached 90% in which there were no other proteins with the value as close as 2BVS (PDBsum: http://www.ebi.ac.uk/thornton- srv/databases/cgi-bin/pdbsum/GetPage.pl). The constituent proteins to be analyzed were found from the Uniprot platform with the codes listed in Table 1. while the anti-thrombotic peptide sequences were predicted using BIOPEP and built via PyMOL 2.5. The results of visualization from PyMOL were modified using Microsoft PowerPoint.

2.2. BIOPEP Analysis

The presence of anti-thrombotic peptide in protein is predicted by inputting the sequence into BIOPEP server (https://biochemia.uwm.edu.pl/metachemibio/). Bioactive peptides will be measured using the tool calculations and select activity with anti- thrombotic to be detected, then enter the amino acid sequence of each protein constituent. BIOPEP will display the results of the bioactive activity (A) and provide peptide sequences with the anti-thrombotic potential. The activity of bioactive peptides (A) contained in the amino acid sequence could be measured by the equation of A = a/N (Zulvana *et al.* 2019).

2.3. Bioactive Peptide Selection by Peptide Ranker

The bioactive score of anti-thrombotic peptides will be calculated to analyze which peptide has the best bioactive through the Peptide Ranker server (http://distilldeep.ucd.ie/PeptideRanker/). The peptide sequences were calculated using computational prediction based on the characteristics that responded to the anti-thrombotic biological activity. The probability rank of a peptide to have bioactive activity at a score close to 1.0 while a score of 0.0 means the peptide is weak activity. PeptideRanker was used at a threshold of 0.5, meaning any peptide predicted over the threshold is labeled as bioactive (Tu *et al.* 2019).

2.4. Allergen and Toxicity Analysis

Allergy and peptide toxicity need to be evaluated for product safety purposes. Allergies are analyzed using AlgPred server (https://webs.iiitd.edu.in/raghava/algpred/submission.html) by inputting amino acids and submitting the sequences. The AlgPred uses an IgE epitope indicator as an antibody to be recognized by amino acids that can potentially cause allergic

Table 1. Anti-thrombotic bioactive peptides and prediction of protein source allergy

Protein sources	Constituent protein	Uniprot code	Anti-thrombotic peptide	Allergen prediction
Milk casein	β-casein	P02666	√	-
	k-casein	P02668	√	-
	α-S1-casein	P02662	-	-
	α-S2-casein	P02663	√	-
	7s globulin	P13917	√	-
	11s glycin	P04347	√	-
Soybean	β-conglycinin, β-chain	P25974	√	√
	β-conglycinin, α-chain	A0A445F555	√	√
	β-conglycinin, α'-chain	P11827	√	√
	α-lactalbumin	A5JSS8	-	-
	α-S1-casein	P18626	-	-
	α-S2-casein	P33049	√	-
	Bac7.5 protein	Q9XSQ9	√	-
Kefir	β-1,4-galactosiltransferase I	E9NRZ3	√	-
	β-casein	P33048	√	-
	β-lactoglobulin	P02756	-	-
	Fibrinogen, α-chain	P02672	√	-
	k-casein	Q7YRX5	√	-

reactions. Meanwhile, the toxicity levels are analyzed by using the ToxinPred server (https://webs.iiitd.edu.in/raghava/toxinpred/design.php), which is based on the support vector machine (SVM). If a peptide with a threshold >0.0 is found, the peptide could be indicated as toxic (Gupta *et al.* 2013).

2.5. Evaluation of Molecular Docking Interaction

Peptides with the best rank will be evaluated specifically by molecular docking using PyRx to obtain the lowest binding affinity for each peptide within the α-thrombin (PDB: 2BVS) receptor and control complex comparison. The peptide was built by assembling residue using the PyMol tool. PyRx works in measuring the driving force of α-thrombin receptors and anti-thrombotic peptide ligands. Autodock Vina was performed to calculate the RMSD value between α-thrombin receptors and anti-thrombotic peptide ligands. The GridBox size of center X: 12.1777, Y: -15.2274, Z: 16.7219 and dimensions (Angstrom) X: 14.5448, Y: 15.6320, Z: 11.7720. Then, the docking results are visualized to see the interacting residues and the types of interactions that occur through the BIOVIA Discovery Studio software.

3. Results

3.1. Activity of Anti-Thrombotic Bioactive Peptide

The protein sources presented in Table 1 illustrate that not all of the protein constituents were found to have anti-thrombotic bioactive peptide activity. Based on the results of the BIOPEP analysis, proteins that did not have anti-thrombotic activity including α-S1-casein in milk casein and α-lactalbumin, α-S1-casein, and β-lactoglobulin in kefir were not found. Meanwhile, soy protein is predicted to have anti-thrombotic activity in all its constituent proteins.

The bioactive anti-thrombotic peptide fragment that occurs in the three analysis sources can be seen in Figure 1, k-casein in kefir showed the highest bioactive frequency value of 0.037 A and followed by the fibrinogen alpha chain of 0.0358. Soybean protein showed a low bioactive activity value compared to other proteins. There were 17 anti-thrombotic peptides found with a peptide score of PeptideRanker above the threshold, which is above 0.5 as shown in Table 2. These peptides are suspected of having a better bioactive ability, where a value close to 1.0 is the best peptide sequence as a bioactive agent. Peptide sequences GP = 0.90;

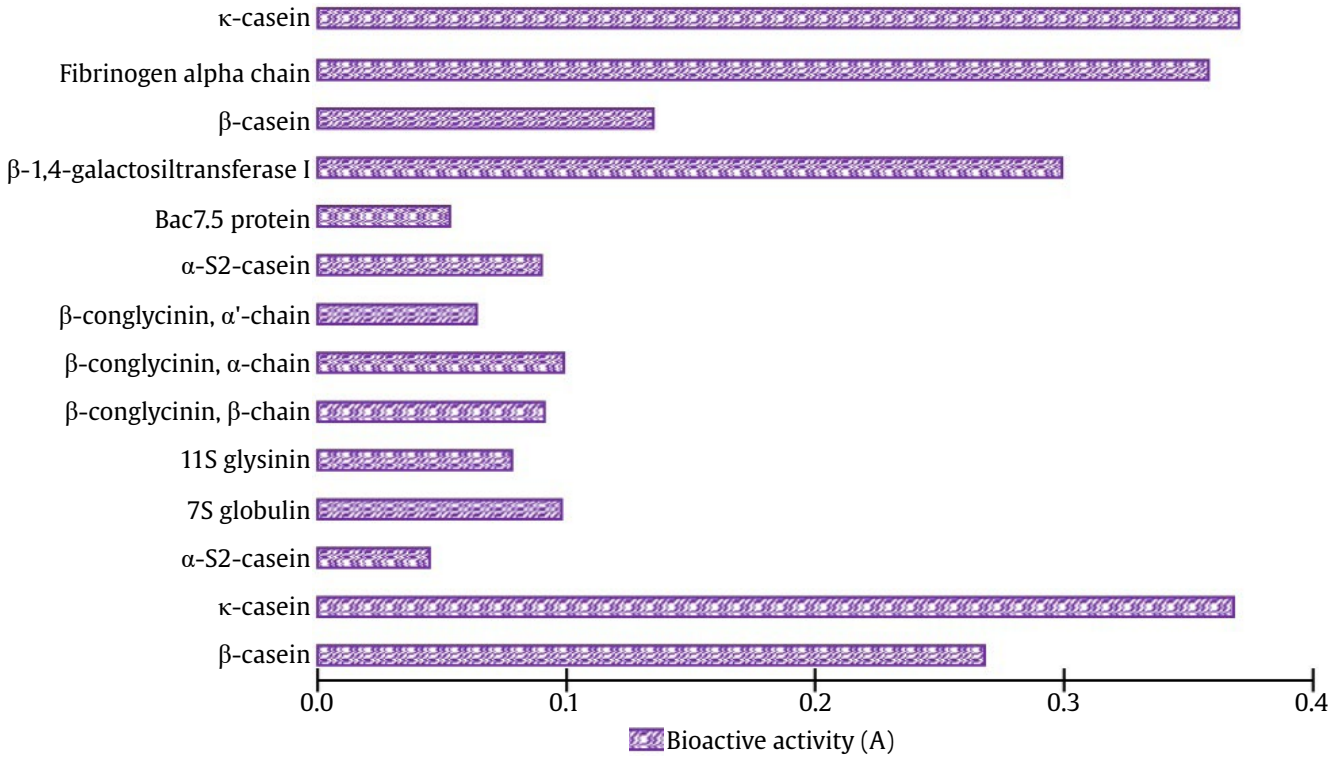

Figure 1. Graph of the anti-thrombotic bioactive peptide value (A) on the X-axis and the constituent proteins on the Y-axis

Table 2. Anti-thrombotic bioactive peptide prediction analysis

Peptide sequence	Peptide ranker	Toxicity prediction	SVM score	Physicochemical characteristics		
				Charge	pI	MW
GP	0.905487	Non	-0.79	0.00	5.88	172.20
PGP	0.908686	Non	-0.75	0.00	5.88	269.33
PG	0.877086	Non	-0.80	0.00	5.88	172.20
DEE	0.0306847	Non	-0.82	-3.00	3.58	391.36
GPR	0.865974	Non	-0.71	1.00	10.11	328.40
RGDS	0.191523	Non	-0.83	0.00	6.19	433.46
RGDF	0.85532	Non	-0.89	0.00	6.19	493.56
RGD	0.367398	Non	-0.81	0.00	6.19	346.37
TAQVTSTEV	0.0300416	Non	-1.30	-1.00	4.00	935.12
QVTSTEV	0.031806	Non	-1.27	-1.00	4.00	762.91
KDQDK	0.0764868	Non	-0.93	0.00	6.31	632.73
MAIPPKKNQDK	0.184142	Non	-0.85	2.00	9.72	1269.68
NQDK	0.0741907	Non	-0.90	0.00	6.19	503.56
MAIPPK	0.594919	Non	-0.28	1.00	9.11	655.93
MAIPPKK	0.506137	Non	-0.44	2.00	10.02	784.12
KNQDK	0.0677995	Non	-0.95	1.00	8.94	631.75
PPK	0.189236	Non	-0.81	2.00	10.02	317.51

SVM score = support vector machine, pI = isoelectric point, and MW = molecular weight

PGP = 0.90; PG = 0.87; GPR = 0.86; and RGDF = 0.85 were five peptides with PeptideRanker exceeding the threshold of >0.5 and the highest compared to the other 12 peptide sequences. Therefore, these five peptides will be used as further references for molecular docking analysis.

3.2. Anti-Thrombotic Bioactive Peptide Prediction and Toxin Allergy

Allergy analysis with AlgPred in Table 1 and Figure 2 found potential allergies in soybeans. The potential allergies were β-conglycinin, β-chain; β-conglycinin, α-chain; and β-conglycinin, α'-chain against IgE antibody interactions, while milk casein and kefir were not predicted to have the potential for such interactions. Based on Table 2, no toxic peptide potential of the 17 anti-thrombotic peptides was found. This could be seen from the SVM value, which was negative or did not exceed the limit (threshold score >0.0) for peptides that are indicated to be toxic. This SVM value was developed from a machine-learning calculation from various computations such as amino acid composition, dipeptide composition, and terminal residue composition. Therefore, 17 peptides that are predicted to be anti-thrombotic could be tested to find their safety usage through a clinical trial and then further study for their drug development.

3.3. Molecular Docking and Thrombin-Peptide Antithrombotic Interaction

The results shown after molecular docking can be analyzed based on Figure 3 and Table 3. Control compounds and 5 peptides (GP, PGP, PG, GPR, and RGDF) were successfully placed at specific locations with control compounds. The control compound 2CE binds to the S1 pocket α-thrombin residues of Gly216, Gly219, and Glu192 by hydrogen bond and hydrophobic bonds with Trp60D, Cys191, Trp215, Ala190, Val190, Val213, Typ228, and Cyc220.

The GPR ligand binds to both catalytic sites of α-thrombin, precisely at the active site gap and pocket S1 α-thrombin, where hydrogen interacts with Gly216, Gly219, Glu192, Ala190, and Ser195 and hydrophobically binds to His57 and Trp60D. Even though the binding affinity of the GPR ligand = -6.8 kcal/mol was still low compared with control 2CE = -6.9 kcal/mol, GPR ligand became an alternative to FDBP as a bioactive peptide.

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B-conglycinin_A-chain       MMRARFPLLLLG-LVFLASVSVSFGIAYWEKENPKHNKCLQSCNSERDSY
B-conglycinin_A'-chain      MMRARFPLLLLG-VVFLASVSVSFGIAYWEKQNPSHNKCLRSCNSEKDSY
B-conglycinin_B-chain       MMRVRFPLLVLLGTVFLASVCVSLKVREDE--------------------
                            ***.*****:*   ******.**: :   *

B-conglycinin_A-chain       RNQACHARCNLLKVEKEECEEGEIPRPRPRPQHPEREPQQPGEKEEDEDE
B-conglycinin_A'-chain      RNQACHARCNLLKVEEEEECE-EGQIPRPRPQHPERERQQHGEKEEDEGE
B-conglycinin_B-chain       --------------------------------------------------

B-conglycinin_A-chain       QPRPIPFPRP-QPRQEEEHEQREEQEWPRKEEKRGEKGSEEEDED-----
B-conglycinin_A'-chain      QPRPFPFPRPRQPHQEEEHEQKEEHEWHRKEEKHGGKGSEEEQDEREHPR
B-conglycinin_B-chain       --------------------------------------------------

B-conglycinin_A-chain       -------EDEEQDERQFPFPRPPHQKEERKQEEDEDEEQQQESEESEDSE
B-conglycinin_A'-chain      PHQPHQKEEEKHEWQHKQEKHQGKESEEEEDQDEDEEQDKESQESEGSE
B-conglycinin_B-chain       --------------------------------------------------

B-conglycinin_A-chain       ----LRRHKNKNPFLFGSNRFETLFKNQYGRIRVLQRFNQRSPQLQNLRD
B-conglycinin_A'-chain      SQREPRRHKNKNPFHFNSKRFQTLFKNQYGHVRVLQRFNKRSQQLQNLRD
B-conglycinin_B-chain       ---------NNPFYLRSSNSFQTLFENQNGRIRLLQRFNKRSPQLENLRD
                                  *:  :  .*: *:***:** *::*:*****:** **:****

B-conglycinin_A-chain       YRILEFNSKPNTLLLPNHADADYLIVILNGTAILSLVNNDDRDSYRLQSG
B-conglycinin_A'-chain      YRILEFNSKPNTLLLPHHADADYLIVILNGTAILTLVNNDDRDSYNLQSG
B-conglycinin_B-chain       YRIVQFQSKPNTILLPHHADADFLLFVLSGRAILTLVNNDDRDSYNLHPG
                            ***::*:*****:***:*****:*:.:*.* ***:**********.*:.*

B-conglycinin_A-chain       DALRVPSGTTYYVVNPDNNENLRLITLAIPVNKPGRFESFFLSSTEAQQS
B-conglycinin_A'-chain      DALRVPAGTTYYVVNPDNDENLRMITLAIPVNKPGRFESFFLSSTQAQQS
B-conglycinin_B-chain       DAQRIPAGTTYYLVNPHDHQNLKIIKLAIPVNKPGRYDDFFLSSTQAQQS
                            ** *:*:*****:***.:.:**::*.**********::.******:****

B-conglycinin_A-chain       YLQGFSRNILEASYDTKFEEINKVLFSREEGQQQGEQRLQESVIVEISKE
B-conglycinin_A'-chain      YLQGFSKNILEASYDTKFEEINKVLFGREEGQQQGEERLQESVIVEISKK
B-conglycinin_B-chain       YLQGFSHNILETSFHSEFEEINRVLFGEEE-----EQRQQEGVIVELSKE
                            ******:****:*:.::*****:***..**     *:* **.****:**:

B-conglycinin_A-chain       QIRALSKRAKSSSRKTISSEDKPFNLRSRDPIYSNKLGKFFEITPEKNPQ
B-conglycinin_A'-chain      QIRELSKHAKSSSRKTISSEDKPFNLRSRDPIYSNKLGKLFEITPEKNPQ
B-conglycinin_B-chain       QIRQLSRRAKSSSRKTISSEDEPFNLRSRNPIYSNNFGKFFEITPEKNPQ
                            *** **::**************:*******:*****::**:**********

B-conglycinin_A-chain       LRDLDIFLSIVDMNEGALLLPHFNSKAIVILVINEGDANIELVGLKEQQQ
B-conglycinin_A'-chain      LRDLDVFLSVVDMNEGALFLPHFNSKAIVVLVINEGEANIELVGIKEQQQ
B-conglycinin_B-chain       LRDLDIFLSSVDINEGALLLPHFNSKAIVILVINEGDANIELVGIKEQQQ
                            *****:*** **:*****:**********:******:*******:*****

B-conglycinin_A-chain       EQQQEEQPLEVRKYRAELSEQDIFVIPAGYPVVVNATSNLNFFAIGINAE
B-conglycinin_A'-chain      RQQQEEQPLEVRKYRAELSEQDIFVIPAGYPVVVNATSDLNFFAFGINAE
B-conglycinin_B-chain       KQKQEEEPLEVQRYRAELSEDDVFVIPAAYPFVVNATSNLNFLAFGINAE
                            .*:***:****::*******:*:*****.**.******:***:*:*****

B-conglycinin_A-chain       NNQRNFLAGSQDNVISQIPSQVQELAFPGSAQAVEKLLKNQRESYFVDAQ
B-conglycinin_A'-chain      NNQRNFLAGSKDNVISQIPSQVQELAFPGSAKDIENLIKSQSESYFVDAQ
B-conglycinin_B-chain       NNQRNFLAGEKDNVVRQIERQVQELAFPGSAQDVERLLKKQRESYFVDAQ
                            *********.:***: **  ***********: :*.*:*.* ********

B-conglycinin_A-chain       PKKKEEGNKGRKGPLSSILRAFY
B-conglycinin_A'-chain      PQQKEEGNKGRKGPLSSILRAFY
B-conglycinin_B-chain       PQQKEEGSKGRKGPFPSILGALY
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Figure 2. The sequences highlighted in blue are peptides, while the sequences highlighted in yellow are sequences recognized by the IgE epitope

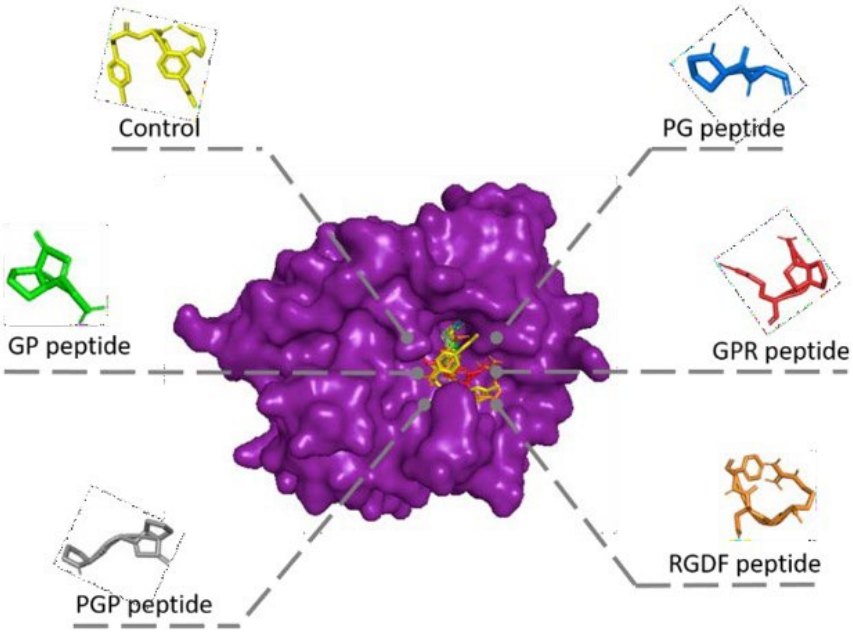

Figure 3. Interaction visualization of α-thrombin (purple) with anti-thrombotic ligands. (A) 2CE control: yellow, (B) GP peptide: green, (C) PGP peptide: gray, (D) PG peptide: blue, (E) GPR peptide: red, (F) RGDF peptide: orange

Table 3. Molecular Docking Analysis and Molecular Interactions

Ligand	Binding affinity (kcal/mol)	Type of interactions	
		Hydrogen bond	Hydrophobic bond
Control (2CE)	-6.9	GLY216 GLY219 GLU192	TRP60D CYS191 TRP215 ALA190 VAL213 TYR228
GP	-4.7	TRP215 SER195 GLY219	CYS220
PGP	-6.6	GLU192 SER195 SER214	LEU99 ALA190 HIS57 TYR60A TRP60D
PG	-5.1	GLY219 SER195 GLY216 ASP189 ALA190 SER214	ALA190
GPR	-6.8	ASP189 GLY216 SER195 GLY219 ALA190 GLY193 GLU192	LEU99 HIS57 TYR60A TRP60D
RGDF	-7.4	TYR60A GLY216 GLY219	CYS191 TRP215 LEU99 ALA190

4. Discussion

The greater the A value means the higher bioactive activity which correlates with the amount of peptides exist. The frequency could be calculated by several fragments with activity from the protein sequence, and N as the number of protein amino acid residues (Zulvana *et al.* 2019). PeptideRanker consideration in determining the threshold in this ranking is based on the two main components of the location and composition of amino acids in the peptide sequence (Baghban *et al.* 2021). A protein with bioactivity could still have an allergic reaction with the predicted sequences that matched the IgE epitope. It means that the sequences has no anti-thrombotic potential. This explains what caused allergies which were the three subunits of the beta-conglycinin protein. However, the anti-thrombotic peptide found in the β-conglycinin protein was predicted to have no allergic potential because its

sequences were not recognized by the IgE immune system (Sharma *et al.* 2020). Allergies will trigger hypersensitivity in the body and will mediate the immune system to produce immunoglobulin E (IgE) as the first type of defense against allergens in the body. The allergen-specific IgE epitope will bind to mast cells and basophils which play a role in sensitization if there is an allergen that has been recognized by IgE. In addition, mast cells degranulated and release inflammatory mediators such as histamine (Kumar *et al.* 2012). Based on Rishnan *et al.* (2009), 25% of the population is sensitive to the β-conglycinin subunit with a high reaction as an allergen in hypersensitivity of the immune system.

The SVM value was developed from a machine-learning calculation from various computations such as amino acid composition, dipeptide composition, and terminal residue composition (Gupta *et al.* 2013). Therefore, 17 peptides predicted to be anti- thrombotic were safe to be used as therapeutic agents for drug development or food-derived bioactive peptides. The presented physicochemical characteristics, including charge, isoelectric point (pI), and molecular weight (g/mol), will help demonstrate the optimization of peptide performance in interacting with the target protein, namely α-thrombin. The charge on the protein at pH conditions equal to pI is 0. The charge on the protein at pH conditions greater than pI is negative. The protein charge at pH conditions less than pI is positive (Fahri *et al.* 2021). The solubility of α-thrombin is predominantly hydrophobic precisely at the catalytic active site which is divided into 4 specific pockets: S_1, S_2, S_3, and S_4 (Neumann *et al.* 2005; Cheng *et al.* 2019). Meanwhile, other α-thrombin residues such as Ser195 and His57 are residues for ligand binding in the α-thrombin active site gap according to the literature (Figueiredo *et al.* 2012). The inhibition potential of this bioactive peptide as an anti-thrombotic agent as an IIa (thrombin) inhibitor is shown in Figure 4 The bioactive will bind to the active site of α-thrombin which will affect the work of thrombin in converting soluble fibrinogen into

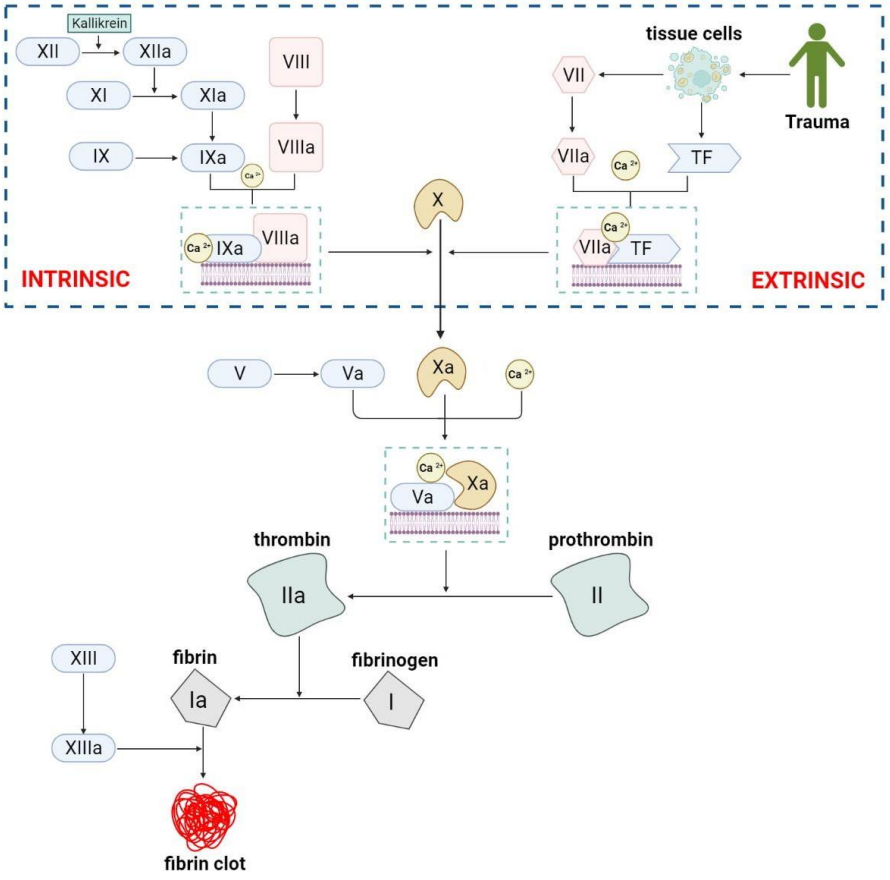

Figure 4. Schematic of the coagulation cascade formation. Intrinsic complex (IXa/VIIIa/Ca^{2+}/PL) and extrinsic complex (VIIa/TF/Ca^{2+}/PL) will convert factor X into factor Xa, then factor Xa will bind to factor Va in PL3 with the help of Ca2+ (Xa/Va/Ca^{2+}/PL) to catalyze prothrombin (II) into thrombin (IIa). Thrombin (IIa) will be activated, convert fibrinogen into fibrin, and then form cross-links between fibrin (fibrin clot) to become a platelet plug. Schematic diagram was adapted from (Cheng *et al.* 2019). Figure created with Biorender.com

fibrin to activate factors V, VII, XI, and XIII to form a thrombus. Factor V, which cannot be converted to Va due to IIa inhibition, will affect factor Xa as it could be disrupted in forming a complex (Xa/Va/Ca^{2+}/PL) in the regulation of thrombolysis (Cheng *et al.* 2019). Therefore, the role of bioactive anti- thrombotic peptides can be a solution to prevent or treat blood coagulation and reduce stroke cases.

In conclusion, food protein sources could provide health benefits, such as being an anti-thrombotic agent found in milk, soybean, and kefir. Based on the database, the best protein for bioactive activity was k-casein in kefir with no toxic potential. However, the β-conglycinin subunit is a source of protein recognized by the IgG epitope which could cause hypersensitive reaction immune system. Molecular docking explains that anti-thrombotic peptides have a positive interaction in inhibiting the action of α-thrombin through the active catalytic site and S1 pocket by GPR peptides with a binding affinity of -6.8 kcal/mol. This research requires further study of molecular dynamics and still needs to be proven *in vitro* and *in vivo*.

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