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In Silico Studies of Bromelain Protease from MD2 Pineapple

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Abstract
Bromelain is a proteolytic enzyme mainly found in the stem and fruit of Ananas comosus (pineapple). It is known for its therapeutic effects, including anti-inflammatory, fibrinolytic, wound-healing, and cardiovascular benefits. This study focuses on the in silico characterization of bromelain protease (SBT1.7) from MD2 pineapple to evaluate its structural features and fibrinolytic activity for therapeutic potential. The sequence of subtilisin-like protease SBT1.7 was retrieved from the NCBI GenBank database and analyzed for physicochemical characteristics using ProtParam. Multiple sequence alignment revealed conserved catalytic and binding residues. Notably, subtilisin-like protease SBT1.7 contains a catalytic dyad (Ser/His) instead of the typical catalytic triad (Ser-His-Asp) found in classical serine proteases. 3D homology modelling was performed using SWISS-MODEL, with EuRp-61, a known serine protease from *Euphorbia resinifera,* as the reference. The EuRP-61 was selected due to its documented fibrinolytic activity and the highest sequence identity (48.91%) with the target protein. Additionally, AlphaFold prediction was performed to enhance confidence in the model accuracy. The predicted structure comprises 12 alpha helices, 26 beta strands and connecting loops. Structural comparison revealed both differences and similarities between SBT1.7 and EuRP-61, with key variations attributed to the shorter sequence of SBT1.7. Electrostatic mapping and interaction analysis, including salt bridges, disulphide bridges and hydrogen bonds, were performed using PyMOL. The finding suggests that subtilisin-like protease SBT1.7 is an extracellular enzyme with favourable structural stability and potential substrate accessibility. Overall, these results support the potential of subtilisin-like protease SBT1.7 as a fibrinolytic protease and highlight the importance of further experimental validation for therapeutic development.
Keywords: Bromelain; Subtilisin-like protease; Fibrinolytic activity; Catalytic dyad; Serine protease

Introduction

Bromelain is a proteolytic enzyme that can be found in the Bromeliaceae plant family. Pineapple (*Ananas comosus*) is the most well-known plant in this plant family (Varilla et al., 2021). Bromelain exhibits various therapeutic properties, including anti-inflammatory benefits in human inflammatory disorders (Hale et al., 2005). According to Hale et al. (2005), specific cell surface molecules that influence lymphocyte migration are also eliminated by the bromelain enzyme. Furthermore, bromelain is also used to treat cardiovascular diseases such as thrombophlebitis, which is caused by blood clots (Ley et al., 2011). As a result, the bromelain enzyme reduces thrombus formation in patients with cardiovascular disease. Additionally, prior research has found that the bromelain enzyme promotes wound healing by eliminating eschar, which is particularly beneficial for individuals with diabetes and burn injuries.

Prior research has demonstrated that the most commonly used enzyme-based fibrinolytic medications are nattokinase, urokinase, streptokinase, and genetically modified tissue plasminogen activator (r-tPA) (Hazare et al., 2024). For example, papain from papaya has been shown to possess anti-inflammatory and digestive properties, while nattokinase is utilized for its cardiovascular health benefits (Hazare et al., 2024). Furthermore, cucumisin from *Cucumis melo* inhibits platelet aggregation and does not have cytotoxic activity on human erythrocytes. Previous studies have proven.

that serine protease from *Euphorbia resinifera* (EuRP-61), isolated from plant latex, was able to hydrolyze all chains of human fibrin clots (Siritapetawee et al., 2020). Since bromelain is also a fibrinolytic enzyme, it plays a crucial role in preventing the formation of fibrin, a blood-clotting protein (Varilla et al., 2021), by degrading fibrin and thereby reducing blood clot formation. These various therapeutic properties highlight bromelain's potential as a therapeutic enzyme with numerous health benefits.

Bromelain can be extracted from the stem and the fruit of the pineapple plant. Among these, stem bromelain (EC 3.4.22.32) is the most abundant protease in pineapple. Research has demonstrated that bromelains have pharmacological characteristics. The lack of undesirable side effects makes bromelain a safe and suitable option for patients. Bromelain contains various proteinases with different amino acid compositions that lead to differences in their proteolytic specificity and inactivation sensitivity (Hale et al., 2005). Researchers can identify variants with the highest therapeutic potential by analyzing the sequence of bromelain. Bioinformatics analysis plays a crucial role in exploring bromelain's potential medical applications, especially in treating areas of inflammation, wound healing and cardiovascular disease. According to Zatul Iffah et al. (2017), they reported successful recombinant expression of bromelain in hosts such as *Brassica rapa* and *Escherichia coli*, emphasizing the importance of economical, efficient production and downstream processing. These findings suggest that bromelain can be expressed in a range of systems for effective production. Additionally, previous studies have shown that aqueous two-phase extraction (ATPE) has the highest purification factor, although it results in a significant reduction in bromelain activity (Abreu et al., 2019). A more advanced method combining ion exchange and gel filtration chromatography demonstrated a higher purification factor of 16.93 and a high recovery of enzymatic activity at 89% (Costal et al., 2014).

Bromelain is widely utilized in medicine due to its therapeutic effects, which help in the treatment of inflammatory diseases and oxidative stress-related disorders. Research has shown that the genes encoding the enzymes found in commercially available bromelain products have not been identified. Additionally, the origin of these enzymes in terms of evolution remains unknown. Furthermore, prior research has shown a lack of studies on bromelain gene screening for specific applications in applied science. Although bromelain has numerous medicinal qualities, its role and chemical mechanism, particularly at the molecular level, have not yet been thoroughly assessed. Most studies concentrate on the general properties of bromelain rather than its specific enzymatic activity. Finding the bromelain enzyme that can break down fibrin is essential because it may be used to treat cardiovascular conditions where breaking down fibrin is necessary to avoid blood clots and to debride eschar.

This study aims to characterize the bromelain protease from MD2 pineapple using in silico approaches to identify bromelain enzymes that have potential medical applications. This study will also analyze the structure and functional properties of bromelain using bioinformatics tools to assess its suitability for therapeutic use. Analyzing the bromelain sequence can lead to a better understanding of the structure and function of bromelain from MD2 pineapple. According to Abuzinadah et al. (2022), an in silico analysis revealed that bromelain is one of the most effective molecules for controlling the toxicity of foodborne pathogens and COVID-19. Furthermore, past studies have assessed bromelain's pharmacological properties in terms of platelet aggregation inhibition, fibrinolytic activity, anti-inflammatory effects, decreasing SARS-CoV-2 severity, and skin debridement properties. As a result, current knowledge gaps about its biochemical properties and potential medical applications will be filled.

Materials and methods

In this study, various bioinformatics tools and in silico approaches will be utilized to analyze and characterize subtilisin-like protease SBT 1.7. Firstly, the sequence of serine proteases will be retrieved from the GenBank database. GenBank is widely used to retrieve DNA, RNA and protein sequences for various organisms. Subsequently, sequence analysis will be conducted using bioinformatics tools such as ProtParam. The ProtParam tool will be used to analyze basic sequence properties, including amino acid composition, molecular weight, theoretical pI value, and instability index. Additionally, a multiple sequence alignment will be performed using Clustal Omega to identify conserved regions among protease sequences. Additionally, the 3D structure of bromelain protease will be predicted using the

SWISS-MODEL software. The predicted structure will be validated using ERRAT to ensure the accuracy of the 3D model. A comparison of bromelain protease and serine protease (EuRP-61) from *Euphorbia resinifera* will be performed. The structural comparison aims to identify similarities and differences. Finally, structural analysis will be conducted using PyMOL to visualize and analyze the 3D structure of the protein.

The subtilisin-like protease sequence was downloaded from the NCBI GenBank in FASTA format. The retrieved sequence was analyzed using BLAST (Basic Local Alignment Search Tool) and UniProt to identify the corresponding UniProt ID for further sequence analysis. Additionally, the sequence of serine protease (EuRP-61) from *Euphorbia resinifera* was also identified in the PDB database as a homologous sequence to be compared with the bromelain enzyme.

Multiple sequence alignment was performed by using Clustal Omega to align the sequences. Multiple sequence alignment also identified the conserved regions across various sequences. Additionally, conserved regions, which comprise functionally critical amino acid residues, were identified through multiple sequence alignment. Multiple sequence alignment is also performed to study the evolutionary relationships by comparing sequences from different species.

Bioinformatics tools such as SWISS-MODEL were used to predict the 3D structure of bromelain protease. The serine protease (EuRP-61) from *Euphorbia resinifera*, identified in the PDB database and NCBI GenBank, was used as a template for comparative homology modelling. The 3D model of subtilisin-like protease SBT1.7 was built using EuRP-61, a homologous structure, as the template.

The predicted structure was validated using the ERRAT bioinformatics tool to ensure its accuracy and reliability. The validation of the homology model ensures that the structure of subtilisin-like protease SBT1.7 is accurate and error-free. The structure was validated using the ERRAT tool after uploading the predicted structure in PDB format.

AlphaFold-based structure prediction was performed to ensure the accuracy of the homology-modelled structure. The sequence of subtilisin-like protease SBT1.7 was uploaded in the AlphaFold server to generate the predicted structure. The predicted structure from the AlphaFold server was then compared with the homology model to identify structural differences and assess the similarity between the two models.

Structural comparisons were performed using pairwise structure alignment in the Protein Data Bank (PDB). The predicted structure of subtilisin-like protease SBT1.7 was compared with the known structure of EuRP-61. The purpose of comparing homology modelling to known structures is to validate and improve the predicted structure and the enzyme's functional sites. Additionally, PyMol was used to visualize and compare the active and substrate-binding sites of subtilisin-like protease SBT1.7 with those of the template enzyme, EuRP-61. To perform the comparison, the PDB files of the predicted structure and the known structure were uploaded into PyMOL.

The modelled subtilisin-like protease SBT1.7 was uploaded into the PyMol software to analyze the predicted structure. To visualize and analyze the structure, the homology modelling structure from SWISS-MODEL was downloaded and uploaded into PyMol. PyMOL is a molecular visualization system used in this study to investigate the 3D structure of subtilisin-like protease SBT1.7, including secondary structure elements, active site residues, and stabilizing interactions such as hydrogen bonds, salt bridges, and disulphide bonds.

Results and discussion

The sequence of subtilisin-like protease SBT1.7 was retrieved from NCBI GenBank for sequence analysis (Figure 1). This sequence BLAST used UniProt to identify the UniProt ID that shows 100 % similarity. A0A199V3F8 (Uniprot ID), which is an uncharacterized sequence, was identified from the BLAST result. Structural analysis and prediction are anticipated to be based on this sequence. Furthermore, the sequence of serine protease (EuRP-61) from *Euphorbia resinifera* has also been identified in the PDB database (7EOX) to be compared with the sequence of subtilisin-like protease SBT1.7. Additionally, sequences of three different bromelain proteases were retrieved from the UniProt database for multiple sequence alignment (Table 1).

>OAY71431.1 Subtilisin-like protease SBT1.7 [Ananas comosus]
MQPPPPRWKGRCAFNASDCNNKLIGIRAFLHGLDNATMSPYDDGGHGTHVASTAAGMFVDKADVN
GLANGTASGIAPYAHLAVYKVCHQNRCSLSDVLAGMDSAVHDGVDVLSLSLGGLSLPFYDDDLAIGA
LGAVEKGVFVSCATGNSGPSNGTTENEAPWILTVGASTMDRSVRATVELGNNISTFYGESLYQPDNF
SAIPLPLIYPGLRGGLKTPYCVNGSLDGVDVPGKIIVCDAGAVTTVAKGRVVKAAGGLGMIVAYPQAV
GFSTFENPHVLPASEVGYTDGLLIKAYAITASAPTASILFERTIVGTKPAPALVYFSSRGPNQADPNILK
PDIIGPGVNVLAAWPFQVGSSDSGSYFNVISGTSMATPHLSGVAALLKSTHPDWSPAAIKSAIMTTATL
IGNDNKPIVDETRALQISLGSAPAT
Figure 1 FASTA format of Subtilisin-like protease SBT 1.7 from *Ananas comosus*

Table 1: List of bromelain proteases from different organism for multiple sequence alignment

Protein ID	Protein name	Organism
7EOX	Serine protease (EuRP-61)	*Euphorbia resinifera*
Q39547	Cucumisin	Cucumis melo (Muskmelon)
O82777	Subtilisin-like protease SBT3	*Solanum lycopersicum* (Tomato) (Lycopersicon esculentum)
P35835	Subtilisin NAT	Bacillus subtilis subsp. natto

The physicochemical properties of subtilisin-like protease SBT1.7 and serine protease (EuRP-61) from *Euphorbia resinifera* are obtained from ProtParam (Table 2). The information generated by the ProtParam bioinformatics tool includes physicochemical characteristics such as amino acid composition (Table 3), extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY).

Table 2 shows differences in physicochemical properties in subtilisin-like protease SBT1.7 and protease (EuRP-61) from *Euphorbia resinifera*. Subtilisin-like protease SBT1.7 has 431 amino acids, while EuRP-61 has 654 amino acids, which indicates that EuRP-61 is significantly larger. Additionally, the molecular weight of subtilisin-like protease SBT1.7 (~44.4 kDa) is lower than that of EuRP-61 (~140.37 kDa), suggesting a greater size and complexity in EuRP-61. The number of charged residues in EuRP-61 is also higher than that of subtilisin-like protease SBT1.7. The extinction coefficient for EuRP-61 (98375 $M^{-1}cm^{-1}$) is higher than that of subtilisin-like protease SBT1.7, suggesting that the protease from Euphorbia resinifera latex has a greater number of aromatic residues, such as Trp, Tyr, or Cys disulphides (Walker, 2005). Subtilisin-like protease SBT1.7 has a GRAVY score of 0.141, suggesting it is slightly hydrophobic, while EuRP-61 has a GRAVY score of -0.163, suggesting it is more hydrophilic. Despite the observed differences in physicochemical properties, EuRP-61 may be a suitable template due to its structural and functional similarities with the subtilisin-like protease SBT1.7.

Table 2: Physicochemical properties for subtilisin-like protease SBT1.7 and EuRP 61

Physiochemical characteristics	Subtilisin-like protease SBT1.7	Serine protease (EuRP-61) from *Euphorbia resinifera*
Number of amino acids	431	654
Molecular weight (Da)	44405.37	61000.00
Theoretical pI	5.54	4.3
Total number of negatively charged residues (Asp + Glu)	34	70
Total number of positively charged residues (Arg + Lys)	26	43
Extinction coefficient	41745 $M^{-1}cm^{-1}$	98375 $M^{-1}cm^{-1}$
Instability index	27.46	33.99
Aliphatic index	89.68	74.28
Grand average of hydropathicity (GRAVY)	0.141	-0.163

Table 3 shows the amino acid composition of subtilisin-like protease SBT1.7 and serine protease (EuRP-61) from *Euphorbia resinifera.* Both proteases have similar amino acid compositions, with comparable percentages for many residues. This proves that EuRP-61 may be a suitable template for modelling subtilisin-like protease SBT1.7.

The hydrophobic amino acid composition for subtilisin-like protease SBT1.7 includes Val (8.1%), Leu (8.4%), Ile (5.6%), Phe (3.0%), Gly (10.9%), Pro (7.2%) and Ala (11.8%). Meanwhile, its hydrophilic amino acid composition includes Ser (8.6%), Thr (6.5%), Cys (1.6%), Arg (2.6%), Asn (4.9%), Asp (5.8%), Gln (1.6%), Glu (2.1%), His (2.1%), and Lys (3.5%). The higher proportion of hydrophobic residues (55%) indicates that subtilisin-like protease SBT1.7 is more hydrophobic, which could influence its structural stability and activity. In comparison, EuRP-61 contains hydrophobic amino acids, including Val (7.2%), Leu (5.2%), Ile (6.3%), Phe (3.7%), Gly (9.6%), Pro (6.1%), and Ala (8.7%). Its hydrophilic amino acid composition consists of Ser (12.2%), Thr (6.9), Cys (1.5%), Arg (2.8%), Asn (4.3%), Asp (7.3%), Gln (1.8%), Glu (3.4%), His (1.7%) and Lys (3.8%). Although EuRP-61 has a higher proportion of hydrophobic residues (46.8%), the content of hydrophilic amino acids (41.9%) and its negative GRAVY score, -0.105, suggest that it is overall more hydrophilic than subtilisin-like protease SBT1.7. EuRP-61's hydrophilic properties may make it more soluble in aqueous environments. The more compact hydrophobic core of subtilisin-like protease SBT1.7 influences its substrate specificity or structural stability.

Table 3: Amino acid composition of Subtilisin-like protease SBT1.7 and EuRP-61

Amino acid	Subtilisin-like protease SBT 1.7		Serine protease (EuRP-61) from *Euphorbia resinifera*	
	Number of amino acids	Amino-acid composition	Number of amino acids	Amino-acid composition
Ala (A)	51	11.8%	57	8.7%
Arg (R)	11	2.6%	18	2.8%
Asn (N)	21	4.9%	28	4.3%
Asp (D)	25	5.8%	48	7.3%
Cys (C)	7	1.6%	10	1.5%
Gln (Q)	7	1.6%	12	1.8%
Glu (E)	9	2.1%	22	3.4%
Gly (G)	47	10.9%	63	9.6%
His (H)	9	2.1%	11	1.7%
Ile (I)	24	5.6%	41	6.3%
Leu (L)	36	8.4%	34	5.2%
Lys (K)	15	3.5%	25	3.8%
Met (M)	8	1.9%	7	1.1%
Phe (F)	13	3.0%	24	3.7%
Pro (P)	31	7.2%	40	6.1%
Ser (S)	37	8.6%	80	12.2%
Thr (T)	28	6.5%	45	6.9%
Trp (W)	4	0.9%	11	1.7%
Tyr (Y)	13	3.0%	25	3.58%
Val (V)	35	8.1%	47	7.2%

Subtilisin-like protease SBT1.7 and bromelain proteases from different families were retrieved from the UniProt and PDB databases for multiple sequence alignment. Serine protease (EuRP-61) from *Euphorbia resinifera* shares 47.18% identity of its conserved regions with the subtilisin-like protease SBT1.7. Asterisk (*) symbols indicate the fully conserved residues across the aligned serine proteases. Two of the three residues (His 46 and Ser 371) in subtilisin-like protease SBT1.7 share similarities with the active site residues of other bromelain protease sequences. This significant conservation indicates that the subtilisin-like protease SBT1.7 shares functional characteristics with other bromelain proteases, particularly in its catalytic activity. Potential variations in substrate specificity and enzymatic efficiency

could be indicated by the variation in a single active site residue (Asp). Conserved cysteine residues contributing to disulphide bridge formation were observed at positions Cys12-Cys19, Cys87-Cys92 and Cys220-Cys237 in subtilisin-like protease SBT1.7. This suggests that all the aligned serine proteases share a similar thermodynamic stability and exhibit proper protein folding. Additionally, two of the active sites are located in the helix region. This suggests that the helix regions may have a more conserved functional and structural role in subtilisin-like protease SBT1.7 (Robertson et al., 2016).

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sp|P35835|SUBN_BACNA            MRSKKLWISLLFALTLIFTMAFSNMSAQ-AAGKSSTEKKYIVGFKQTMSAMSSAKKK---      56
sp|O82777|SBT3_SOLLC            ---MELLHL------LLFSWALS----AHLFLALAQRSTYIVHLDKSLMPNVFTDHHHWH      47
sp|Q39547|CUCM1_CUCME           MSSSLIFKL------FFFSLFFSNRLASRLDSDDDGKNIYIVYMGRKLEDPDSAH--LHH      52
tr|A0A199V3F8|A0A199V3F8_ANACO  ----------------------------------------------------------      0
7EOX_1|Chains                   ----------------------------------------------------------      0

sp|P35835|SUBN_BACNA            -DVIS--------EKG-----GKVQKQF-KYVNAAAATLDEKAVKELKKDPSVAYVEEDH      101
sp|O82777|SBT3_SOLLC            SSTIDSIKASVPSSVDRFHSAPKLVYSYDNVLHGFSAVLSKDELAALKKLPGFISAYKDR      107
sp|Q39547|CUCM1_CUCME           RAMLE-------QVVGSTFAPESVLHTYKRSFNGFAVKLTEEEAEKIASMEGVVSVFLNE      105
tr|A0A199V3F8|A0A199V3F8_ANACO  ----------------------------------------------------------      0
7EOX_1|Chains                   ----------------------------------------------------------      0

sp|P35835|SUBN_BACNA            IAHEYAQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGAS------      155
sp|O82777|SBT3_SOLLC            TVEPHTTHTSDFLKLNPSSGLWPASGLGQDVIVAVLDSGIWPESASFQDDGMPEIPKRWK      167
sp|Q39547|CUCM1_CUCME           MNELHTTRSWDFLGFPLT--VPRRSQVESNIVVGVLDTGIWPESPSFDDEGFSPPPPKWK      163
tr|A0A199V3F8|A0A199V3F8_ANACO  ----------------------------------------------MQPPPPRWK      9
7EOX_1|Chains                   -----DTHTPEFLGDSSNSGLWPNGNYGEDIIIGVLDTGVWPEHPSFSDSDMSDIPSSWK      55

sp|P35835|SUBN_BACNA            --------F---------------------------VPSETNPYQDGSSHGTHVAGTIA      179
sp|O82777|SBT3_SOLLC            GICKPGTQFNASMCNRKLIGANYFNKGILANDPT---VNITMNSARDTDGHGTHCASITA      224
sp|Q39547|CUCM1_CUCME           GTCETSNNF---RCNRKIIGARSYHIGRPISP-------GDVNGPRDTNGHGTHTASTAA      213
tr|A0A199V3F8|A0A199V3F8_ANACO  GRCA----FNASDCNNKLIGIRAFLHGLDNA----------TMSPYDDGGHGTHVASTAA      55
7EOX_1|Chains                   GTCETSDDFPASSCNKKLIGARAFSKGIVAYQGAPIDKSKDSDSPRDINGHGTHTSTTAG      115
                                       *                                    * ..**** :   .

sp|P35835|SUBN_BACNA            ALNN-SI--------GVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINM      230
sp|O82777|SBT3_SOLLC            GNFAKGVSHFGYAPGTARGVAPRARLAVYKFSFNEGTF-TSDLIAAMDQAVADGVDMISI      283
sp|Q39547|CUCM1_CUCME           GGLVSQANLYGLGLGTARGGVPLARIAAYKVCWNDGCS-DTDILAAYDDAIADGVDIISL      272
tr|A0A199V3F8|A0A199V3F8_ANACO  GMFVDKADVNGLANGTASGIAPYAHLAVYKVCHQNRCS-LSDVLAGMDSAVHDGVDVLSL      114
7EOX_1|Chains                   GSKVQNASFYGYAKGQARGMATKARIAVYKVCWSAGCP-DTDILAAMNQAIEDGVHVISM      174
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sp|P35835|SUBN_BACNA            SLGGPTGST-----ALKTVVDKAVSSGIVVAAAAGNEGSSGSTSTV--------------      271
sp|O82777|SBT3_SOLLC            SYGY--RFIPLYEDAISIASFGAMMKGVLVSASAGNRGPGIGSLNNGSPWILCVASGHTD      341
sp|Q39547|CUCM1_CUCME           SVGGA-NPRHYFVDAIAIGSFHAVERGILTSNSAGNGGPNFFTTASLSPWLLSVAASTMD      331
tr|A0A199V3F8|A0A199V3F8_ANACO  SLG--GLSLPFYDDDLAIGALGAVEKGVFVSCATGNSGPSNGTTENEAPWILTVGASTMD      172
7EOX_1|Chains                   SVGPQGYSPDYYQEASAIGAFNAVKYGIIVSCSAGNSGPKPLTAGNISPWILTVGASTID      234
                                * *                   *:  *:..: ::** *    :

sp|P35835|SUBN_BACNA            ----------------------------GYPAKYPSTIA-------------------      282
sp|O82777|SBT3_SOLLC            RTFAGTLTLGNG-LKIRGWSLFPARAFVRD-SPVIYNKTLSD-----------CSSEELL      388
sp|Q39547|CUCM1_CUCME           RKFVTQVQIGNG-QSFQGVSINT---FDNQYYPLVSGRDIPNTGF-DKSTSRFCTDKSVN      386
tr|A0A199V3F8|A0A199V3F8_ANACO  RSVRATVELGNNISTFYGESLYQPDNFSAIPLPLIYP------GLRGGLKTPYCVNGSLD      226
7EOX_1|Chains                   REFRADVVLGDG-RTFKGSSLYTGEPLQDEFFPLVYA------GYAGS--SRFCTNGSLD      285
                                                                    *
sp|P35835|SUBN_BACNA            ----------------------------------------------------------      282
sp|O82777|SBT3_SOLLC            SQVENPENTIVICDDNGDFSDQMR-IITRARLKAAIFISEDPGVF--RSATFPNPGVVVN      445
sp|Q39547|CUCM1_CUCME           --PNLLKGKIVVCEASFGPHEFFKSL-D--GAAGVLMT----SNTRDYADSYPLPSSVLD      437
tr|A0A199V3F8|A0A199V3F8_ANACO  --GVDVPGKIIVCDAGAVTTVAKGRVVKAAGGLGMIVAYPQAVGFSTFENPHVLPASEVG      284
7EOX_1|Chains                   --SSKVQGKIVICDNGIISREEKGNEVNRAGGAGMIDVTA--EDFLRAGDAYLFPATTVT      341

sp|P35835|SUBN_BACNA            ---------------------------VGAVNSSNQRASFSSVG--------SELDVMAP      307
sp|O82777|SBT3_SOLLC            KKEGKQVINYVKNSVTPTATITFQETYLDTK-PAPVVAASSARGPSRSYLGISKPDILAP      504
sp|Q39547|CUCM1_CUCME           PNDLLATLRYIYSIRSPGATIFKSTTILNA--SAPVVVSFSSRGPNRATKDVIKPDISGP      495
tr|A0A199V3F8|A0A199V3F8_ANACO  YTDGLLIKAYAITASAPTASILFERTIVGTK-PAPALVYFSSRGPNQADPNILKPDIIGP      343
7EOX_1|Chains                   LTDGYEIEYYSVTSQSPTAKIVFLGTVIGNSPPAPKVASFSSRGPNLWTPQILKPDVIAP      401
                                                    :.    :   .  *: *         : *: .*

sp|P35835|SUBN_BACNA            GVSIQSTLPGG--------------TYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVR      353
sp|O82777|SBT3_SOLLC            GVLILAAYPPNVFATSIGTNILLSTDYILESGTSMAAPHAAGIAAMLKAAHPEWSPSAIR      564
sp|Q39547|CUCM1_CUCME           GVEILAAWPSVAPVG----GIRRNTLFNIISGTSMSCPHITGIATYVKTYNPTWSPAAIK      551
tr|A0A199V3F8|A0A199V3F8_ANACO  GVNVLAAWPFQVG------SSDSGSYFNVISGTSMATPHLSGVAALLKSTHPDWSPAAIK      397
7EOX_1|Chains                   GVAILAGWSGAAHPTDLD-NDDRIVQFWLDSGTSMACPHVSGIVALLRKAHPSWSAAAIK      460
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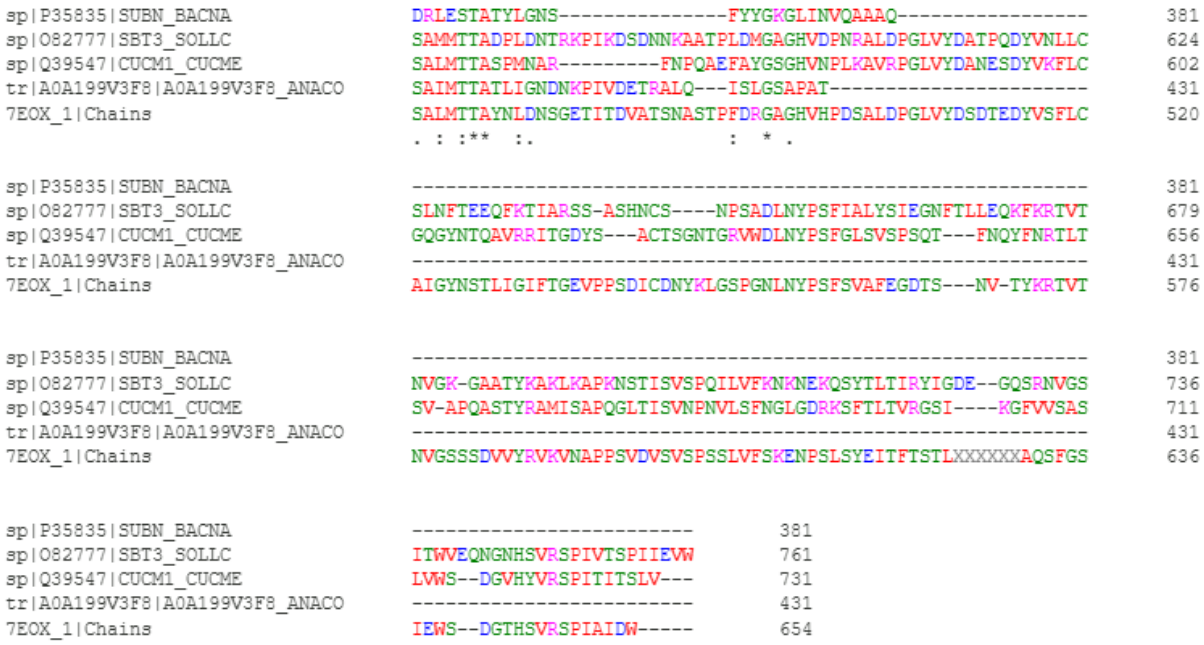

Figure 2 Multiple sequence alignment of subtilisin NAT from bacillus subtilis natto, subtilisin-like protease SBT3 from Solanum lycopersium (tomato), cucumisin from cucumis melo (muskmelon), subtilisin-like protease SBT1.7 from MD2 pineapple and protease from *Euphorbia resinifera* (EuRP-61)

Table 4: Amino acid composition of Subtilisin-like protease SBT1.7 and EuRP-61

	Percentage identity				
	A0A199V3F8	Q39547	O82777	7EOX	P35835
Subtilisin-like protease SBT1.7 (A0A199V3F8)	100	41.48	40.00	47.18	35.55
Cucumisin (Q39547)	41.48	100	35.42	42.79	27.67
Subtilisin-like protease SBT3 (O82777)	40.00	35.42	100	40.92	27.27
Protease from *Euphorbia resinifera* (7EOX)	47.18	42.79	40.92	100	31.48
Subtilisin NAT (P35835)	35.55	27.67	27.27	31.48	100

According to Table 4, when compared to other proteases, the *Euphorbia resinifera* protease has the highest percentage identity (47.18%). Furthermore, cucumisin shares a higher identity (41.48%) than both subtilisin NAT (35.55%) and subtilisin-like protease SBT3 (40.00%). Previous research has shown that *Euphorbia resinifera* protease (EuRP-61) can hydrolyze human fibrin clots. Antithrombin III and α2-macroglobulin, two inhibitors of human blood circulation, did not affect the fibrinolytic activity of this enzyme, resulting in its long-term stability in blood circulation (Siritapetawee et al., 2020). In a dose-dependent manner, cucumisin can also break down human fibrinogen, causing fibrin clot lysis (Silva-López & Gonçalves, 2019). It is hypothesized that subtilisin-like protease SBT1.7 possesses fibrinolytic qualities due to its substantial percentage identity with EuRP-61. Although subtilisin NAT is a fibrinolytic enzyme, its lower percentage identity suggests that sequence similarity may vary depending on the organism's origin. The lower sequence identity between subtilisin NAT and other proteases indicates

that, despite having comparable fibrinolytic activity, the proteases' evolutionary histories have resulted in notable sequence differences.

Figure 3 shows the 3D homology model of the subtilisin-like protease SBT1.7 generated using SWISS-MODEL. The homology modelling is performed using a known structure, serine protease (EuRP-61) from *Euphorbia resinifera* (PDB ID: 7EOX). The EuRP-61 template shared 48.91% similarity with subtilisin-like protease SBT1.7. The structure includes 12 alpha helices, 26 beta strands, and connecting loops. Previous structural homology searches reveal that EuRP-61's loop regions differ slightly from those of subtilisin-like proteases (Jaruwan Siritapetawee et al., 2021). By comparing the structural alignment with the template, the active site was also determined. The subtilisin-like protease SBT1.7 has two active sites, His and Ser, at positions 46 and 371, respectively. Since EuRP-61 is a serine protease that is also distinguished by a catalytic triad of three amino acids, such as Asp32, His106, and Ser434 (Nakagawa et al., 2010).

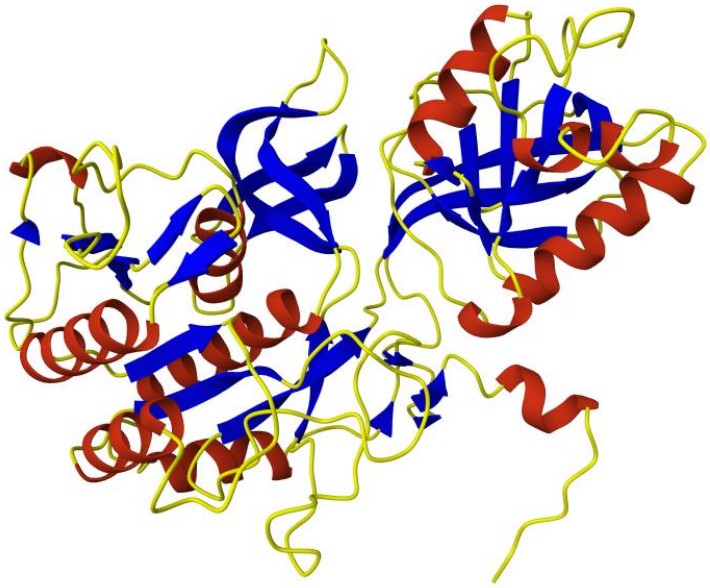

Figure 3 3D homology model of the subtilisin-like protease SBT1.7. Alpha helices, beta strands and coils are colored red, blue and yellow, respectively.

ERRAT tool analyzes the overall quality factor for the homology model of subtilisin-like protease SBT1.7. A protein structure verification algorithm called ERRAT is used to assess the quality of crystallography model construction and refinement (Zaghlool & Ammar, 2016). By utilizing errors that result in random atom distributions, the ERRAT method can identify incorrect regions in protein structures. The overall quality factor of 92.346 was obtained for the modelled subtilisin-like protease SBT1.7. A high-quality model is considered to have a score that is greater than 50 (Omar et al., 2018). Since the score for the model is 92.346, it is proven that the protein structure of subtilisin-like protease SBT1.7 is a high-quality model. As shown in Figure 4, some amino acid residues exceed the error values, indicating that these regions contain significant errors in atomic interactions.

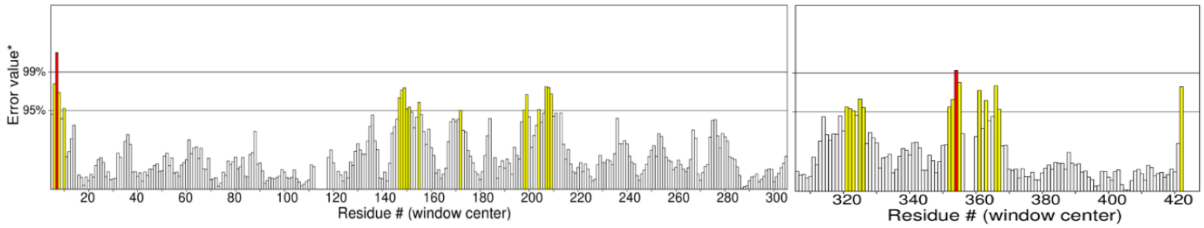

Figure 4 ERRAT plot showing the error values for amino acid residues. The Y axis represents the error value, while the X-axis corresponds to the residue positions in the sequence.

The AlphaFold server predicts protein structure based on the input sequence without requiring a known reference enzyme. The AI-predicted AlphaFold model (Figure 5) exhibited a high pTM score of 0.93, indicating that the overall predicted fold of the model is likely close to the true structure. In addition, a structural comparison between the homology model and the AlphaFold model revealed high similarity, with an RMSD of 1.23 Å. A notable difference was observed at the C-terminal region, where a single residue was modelled in a helical conformation in the homology model but appeared in a loop conformation in the AlphaFold prediction. However, this region is not part of the active site and showed low confidence (pLDDT<70) in the AlphaFold server. This result suggests potential structural flexibility or disorder in that particular region.

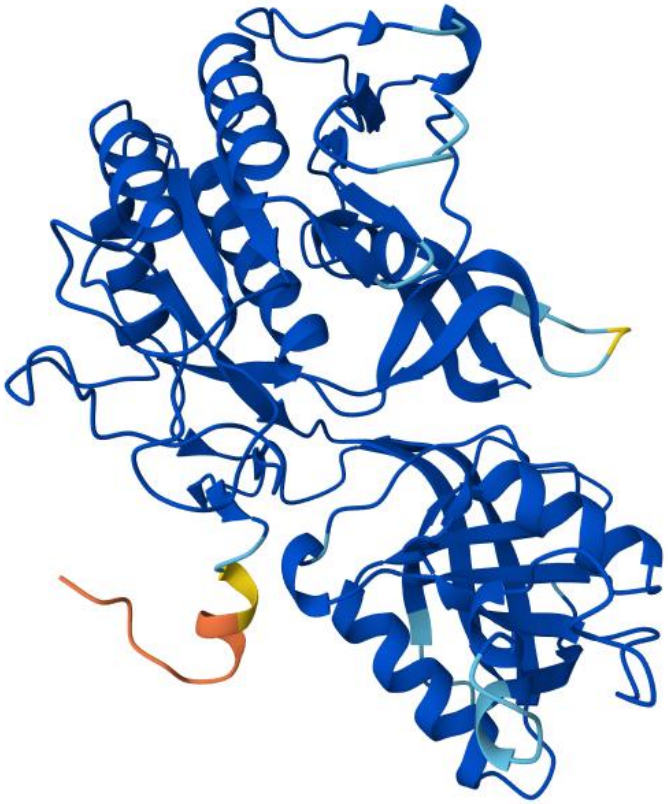

Figure 5 AlphaFold-based structure prediction of subtilisin-like protease SBT1.7. Dark blue indicates very high confidence (pLDDT > 90), light blue indicates confidence (90 > pLDDT > 70), yellow indicates low confidence (70 > pLDDT > 50), and orange indicates very low confidence (pLDDT < 50).

Figure 6 shows that serine protease (EuRP-61) from *Euphorbia resinifera* is larger in terms of its overall size due to its longer amino acid sequence. EuRP-61 consists of 20 helices, 29 beta sheets and

loops that connect them, while the subtilisin-like protease SBT1.7 consists of 12 alpha helices, 26 beta strands and connecting loops. The differences in alpha and beta strands between subtilisin-like protease SBT1.7 and EuRP-61 are caused by sequence length differences. The sequence of the subtilisin-like protease SBT1.7 is shorter than that of EuRP-61. As a consequence, some secondary structures found in EuRP-61 are absent. Furthermore, variations in amino acid composition also result in the absence of certain secondary structural components. Furthermore, the loss of helices and beta sheets can make the subtilisin-like protease SBT1.7 more flexible or unstable, thereby affecting its ability to function under various conditions. Since subtilisin-like protease SBT1.7 has fewer secondary structures that stabilize hydrogen bonds, it is less stable than EuRP-61 at high temperatures.

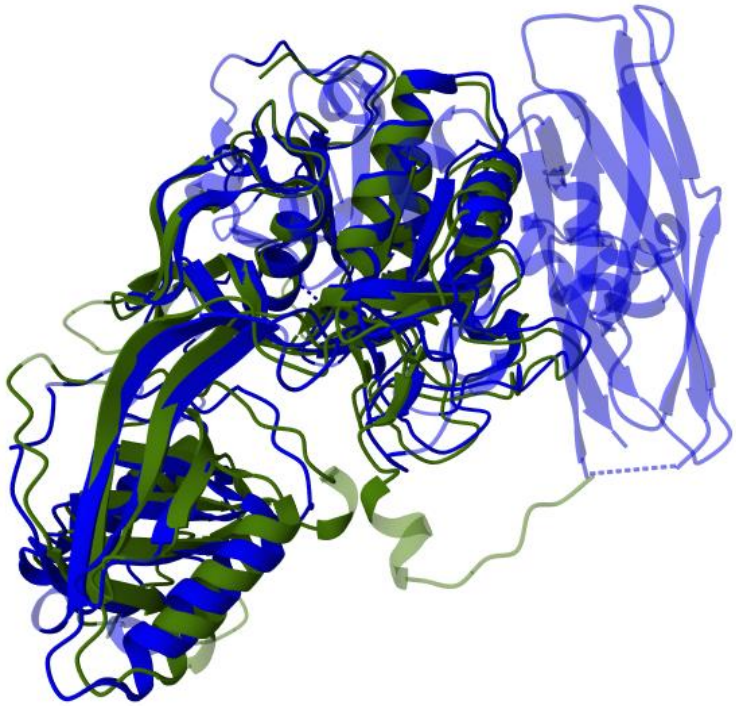

Figure 6 Pairwise structure alignment of subtilisin-like protease SBT1.7 (green) and the protease from *Euphorbia resinifera* (EuRP-61) (blue).

From the result of multiple sequence alignment that is shown in Figure 2, two of the catalytic triads of EuRP-61 and other known proteases are aligned with the His 46 and Ser 371 of the subtilisin-like protease SBT1.7 (Figure 7). The alignment of Asp 32 in EuRP-61 with subtilisin-like protease SBT1.7 is not observed due to the absence of a corresponding sequence in that region. Figure 7 depicts the catalytic triad (His106, Ser434, Asp32) of EuRP-61 and the catalytic dyad (Ser371, His46) of subtilisin-like protease SBT1.7. In rhomboid proteases, which take part in controlled intramembrane proteolysis, the Ser/His catalytic dyad is frequently observed. Because subtilisin-like protease SBT1.7 appears to use a catalytic dyad (Ser/His) rather than the typical Ser-His-Asp catalytic triad found in classical serine proteases, this feature is more similar to rhomboid protease. Although the subtilisin-like protease SBT1.7 is an extracellular enzyme, the observed catalytic dyad suggests structural or mechanistic similarities to rhomboid proteases. This variation may affect the stability, reaction mechanisms, and substrate specificity of the enzyme. To confirm the enzymatic mechanism experimentally, more research is needed.

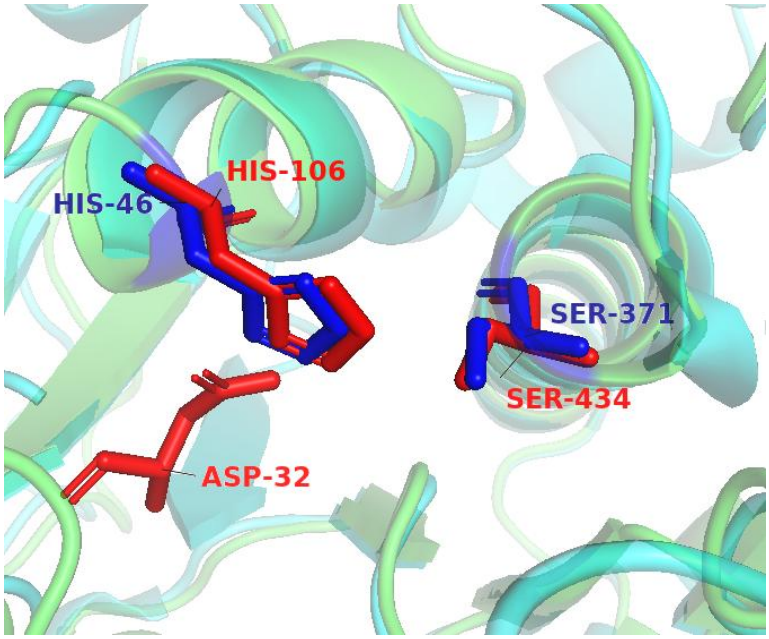

Figure 7 Comparison of active sites between subtilisin-like protease SBT1.7 (catalytic dyad shown in blue) and EuRP-61 (catalytic triad shown in red).

The binding sites of serine proteases for substrates are often referred to as the S1 pocket. The catalytic activity of subtilisin-like proteases is dependent on this binding pocket. Previous research has demonstrated that a nine-residue peptide sequence from the substrate or inhibitor can bind to subtilisin from S6 to S3'. The specificity of serine proteases is mainly determined by the substrate residue located N-terminal to the cleavage site (P1). The multiple sequence alignment of the subtilisin-like protease SBT1.7 reveals its binding sites in Figure 9. The subtilisin-like protease SBT1.7 exhibited sequence alignment with five of the six binding site residues found in the reference enzyme EuRP-61. The variation in one binding site, at position 32 (Asp), indicates that the subtilisin-like protease SBT1.7 lacks a corresponding region. This points to a potential divergence in structure or function at that location. This one change could be a factor in variations in binding affinity or substrate specificity.

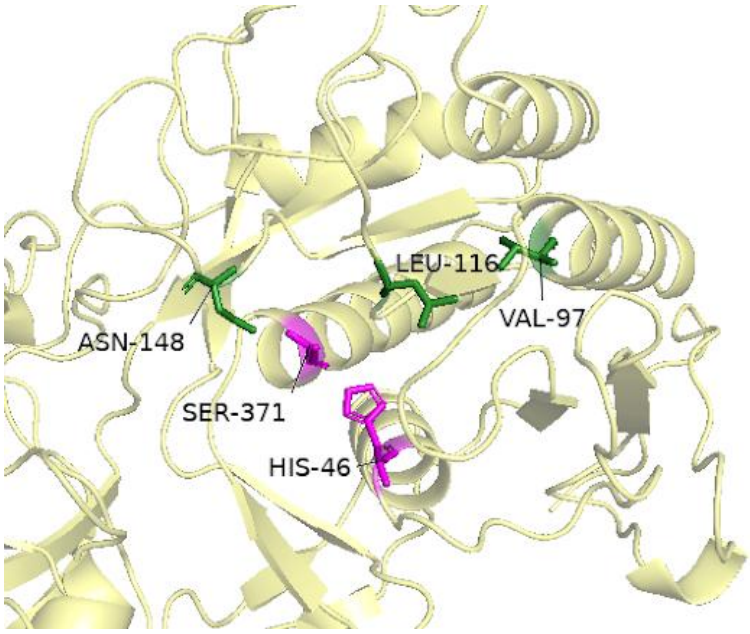

Figure 8 Predicted substrate binding sites of subtilisin-like protease SBT1.7. Active site residues are shown in magenta, while binding site residues are shown in green.

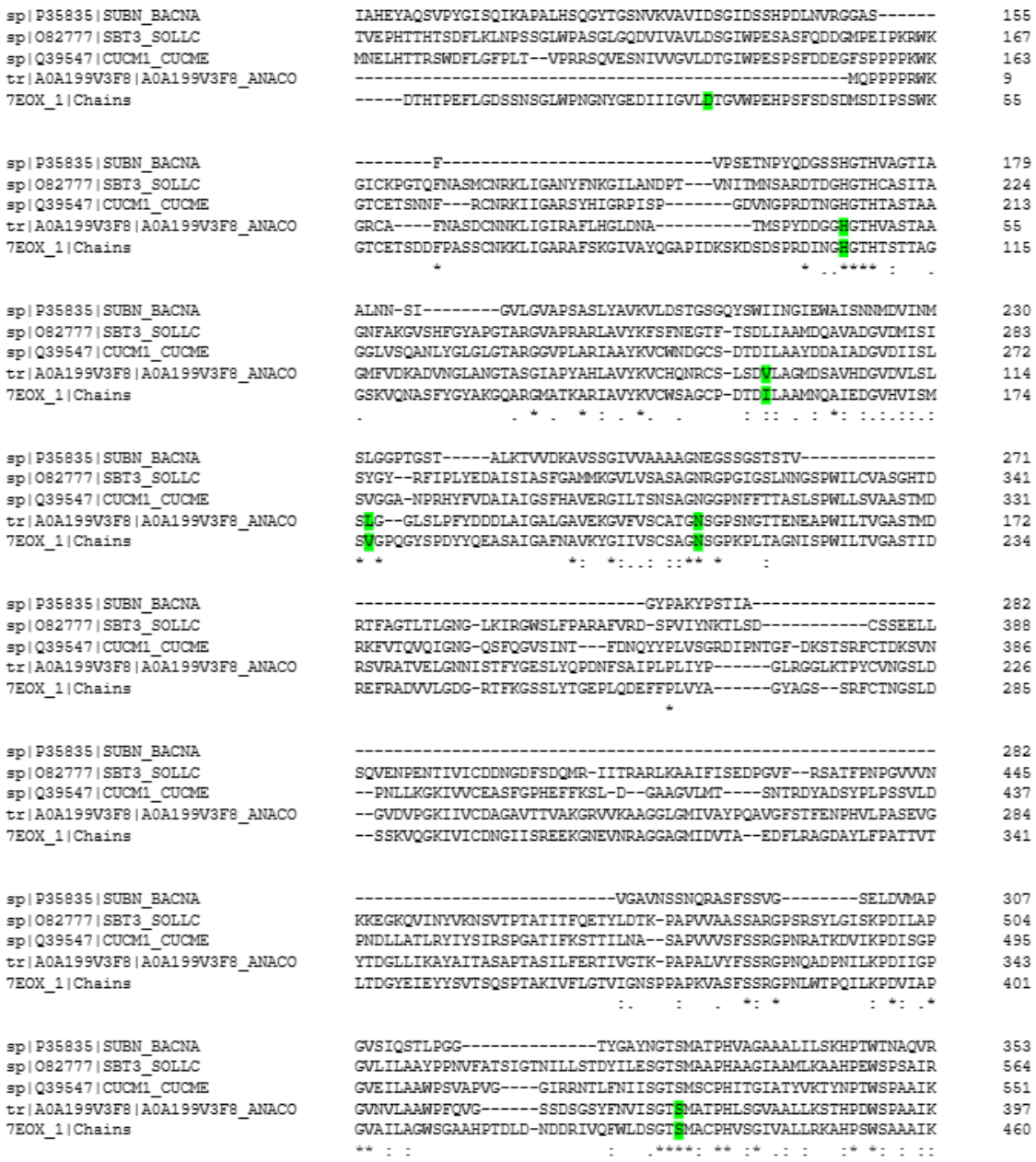

Figure 9 Binding sites (highlighted in green) of subtilisin-like protease SBT1.7 and EuRP-61, as observed from multiple sequence alignment.

Table 5: Amino acid composition of Subtilisin-like protease SBT1.7 and EuRP-61

No	Subtilisin-like protease SBT1.7	EuRP-61
1	-	Asp 32
2	His 46	His 106
3	Val 97	Ile 157
4	Leu 116	Val 176
5	Asn 148	Asn 210
6	Ser 371	Ser 434

Electrostatic analysis of the subtilisin-like protease SBT1.7 reveals a negative electrostatic potential around His36 and a positive electrostatic potential around Ser371. In EuRP-61, however, negative patches were observed around His106 and Asp32, as well as positive patches around Ser434. Previous research has shown that the catalytic triad of subtilisin-like protease is influenced by electrostatic forces. Negatively charged Asp60, located 7.8 Å from the catalytic triad, significantly increases the catalytic activity of serine proteases. According to Zlobin et al. (2025), it stabilizes His64, one of the catalytic triads, in the tetrahedral intermediate states. Similar to the prior study, Asp60 in subtilisin-like protease SBT1.7 carries a negative charge. Further research is needed to confirm the effect of Asp60 on the catalytic dyad using a computational method. The distribution of surface charges is critical to the effectiveness of the catalytic mechanism. Furthermore, electrostatic preorganization is significant because it suggests that the observed surface potential supports the hypothesis that noncatalytic charged residues aid in transition state stabilization in subtilisin-like proteases (Zlobin et al., 2025).

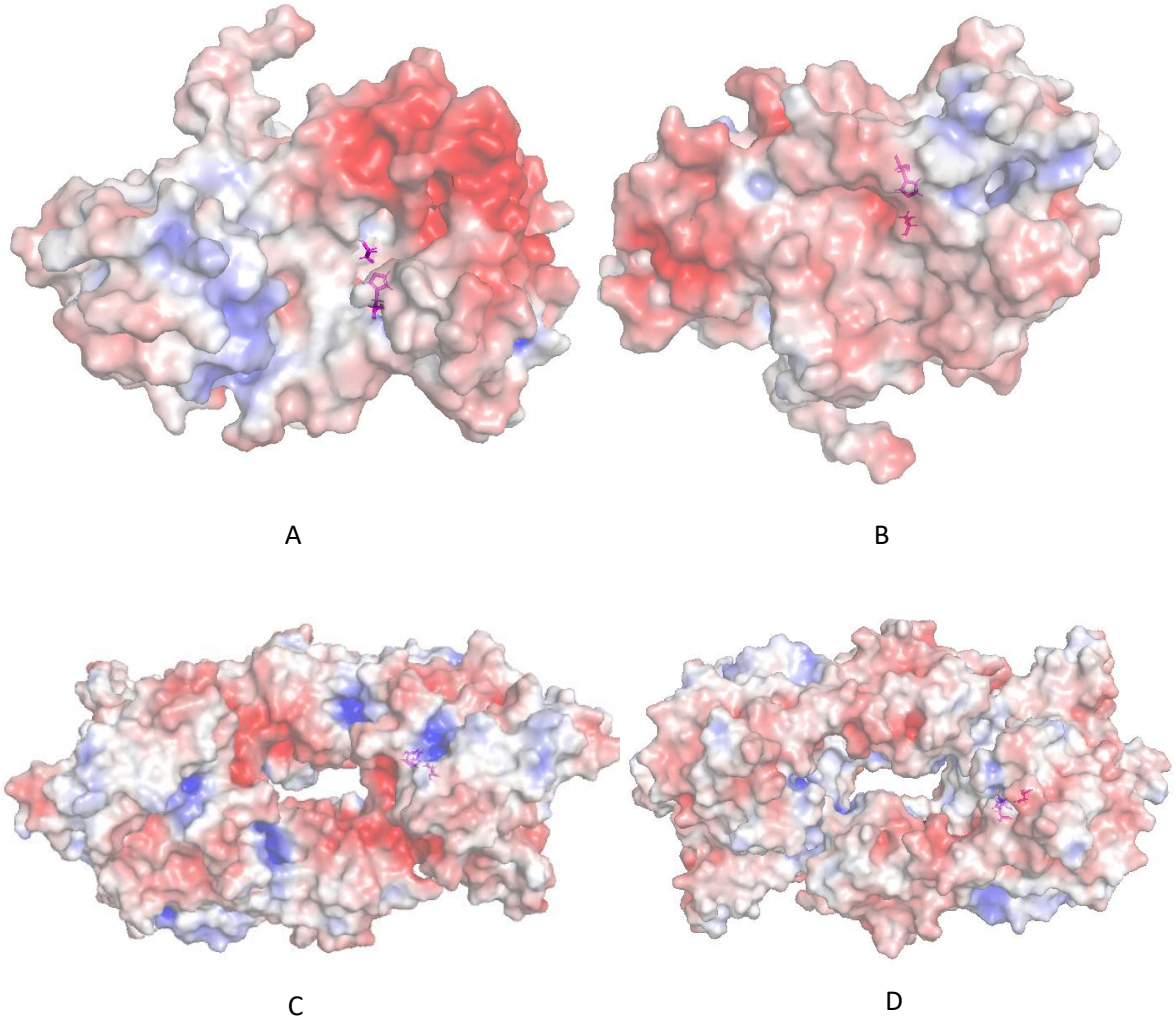

Figure 10 Electrostatic surface representations of subtilisin-like protease SBT1.7 (A, B) and EuRP-61 (C, D). Negative electrostatic potential is shown in red, positive in blue and neutral in white. Panels A and C display the front view of the electrostatic surface, while B and D show the back view. The catalytic dyad and triad residues are represented as sticks.

Enzyme stability is crucial in determining the efficiency of biocatalysis. Under various environmental factors, including temperature, solvent exposure, and pH, stable enzymes maintain their

structural integrity and catalytic activity (Longo & Combes, 1999). Furthermore, a greater percentage of long surface loops is observed in subtilisin-like protease SBT1.7 compared to alpha helices and beta strands. The protein's function is supported by the loop's flexibility rather than its rigidity.

Additionally, disulphide bridges in subtilisin-like protease SBT1.7 are observed between Cys12-Cys19, Cys87-Cys92 and Cys220-Cys237 (Figure 11). Two of these disulphide bridges, Cys87-Cys92 and Cys220-Cys237, are located within the catalytic domain of subtilisin-like protease SBT1.7, corresponding to the peptidase S8/S53 as described in the UniProt database. According to Jaruwan Siritapetawee et al. (2021), the catalytic domain of subtilisin-like protease is stabilized by disulphide bridges.

In subtilisin-like protease SBT1.7, the majority of the salt bridges are visible on the surface (Figure 12). For proteins to retain their structure at high temperatures, salt bridges that are dispersed across their surface are essential (Ban et al., 2019). Moreover, the distribution of salt bridges promotes interactions between proteins and ligands. Protein stability is enhanced by salt bridges, which serve as molecular clips to stabilize large regions in protein-protein interactions (Spassov et al., 2023). Since the subtilisin-like protease SBT1.7 has shown promise as a therapeutic agent in earlier research, understanding its salt bridge interaction may be beneficial in the development of structure-based drugs.

Additionally, a significant number of hydrogen bonds were detected across the subtilisin-like protease SBT1.7. The formation of hydrogen bonds between the peptide backbone atoms of the primary structure stabilizes the secondary structure. On the other hand, surface hydrogen bonds contribute to the protein's flexibility and ligand-protein binding selectivity.

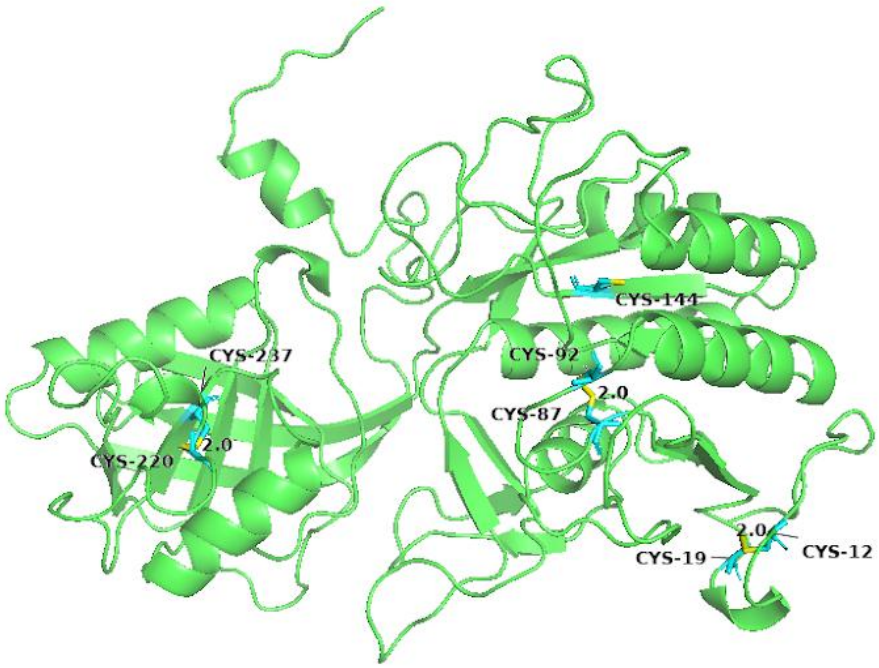

Figure 11 Disulfide bridges in the subtilisin-like protease SBT1.7, observed between Cys12-Cys19, Cys87-Cys92 and Cys220-Cys237. The cysteine residues are shown as cyan-colored sticks and the disulfide bonds are represented by yellow colored sulfur atoms.

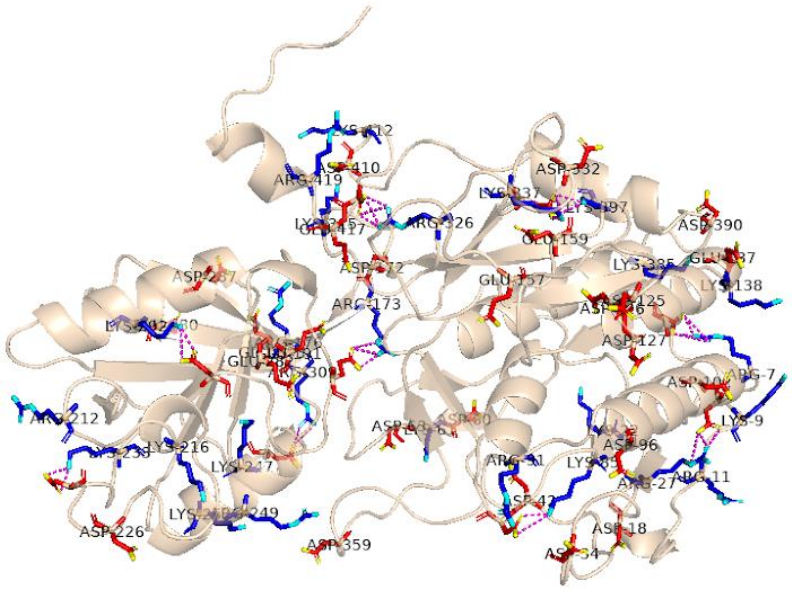

Figure 12 Salt bridges were identified on the surface and core of subtilisin-like protease SBT1.7. Acidic residues are colored red, and basic residues are colored blue. Acidic atoms are shown in yellow, while basic atoms are shown in cyan. The dotted line in magenta represents the salt bridge between acidic and basic atoms.

Conclusion

This study aimed to characterize the subtilisin-like protease SBT1.7 from MD2 pineapple using in silico techniques. Bioinformatic tools were used to explore the structure and function of subtilisin-like protease SBT1.7, particularly its fibrinolytic activity, which may have therapeutic potential in treating inflammation and cardiovascular disease as well as promoting wound healing. The physicochemical properties, including molecular weight and GRAVY, demonstrated several distinctions between EuRP-61 and subtilisin-like protease. According to this analysis, the subtilisin-like protease SBT1.7 is less hydrophilic and slightly more hydrophobic than the template protein. Notably, only two residues corresponding to the usual catalytic triad were conserved, suggesting that subtilisin-like protease SBT1.7 may possess a catalytic dyad rather than the typical triad, which could potentially affect its enzymatic mechanism or efficiency. Homology modeling revealed that subtilisin-like protease SBT1.7 has 48.91% sequence similarity with EuRP-61. The modeled structure demonstrated a 92.346 quality factor, indicating high reliability. Electrostatic analysis revealed a negative surface potential, with charged residues His36 and Ser371 contributing to the active site environment. Disulphide bridges, salt bridges, and hydrogen bonds of subtilisin-like protease SBT1.7 were observed through structural analysis using PyMol. The presence of surface-distributed salt bridges exhibits high thermostability. Overall, subtilisin-like protease SBT1.7 is predicted to be a stable serine protease with potential fibrinolytic activity and structural features favorable for therapeutic applications.

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