Amplification of Thermostable Neutral Protease Open Reading Frame from *Geobacillus* sp. DS3 Isolated from Sikidang Crater, Dieng Plateau, Central Java, Indonesia

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ABSTRACT

Neutral protease is a protease that are widely applied in the food industry due to its stability at neutral pH. However, the neutral protease is generally poor in thermal stability which limits its application in some industries that require high temperatures. The thermostable neutral protease-producing bacterium, *Geobacillus* sp. DS3, was isolated from Sikidang Dieng Crater, Central Java, Indonesia. Preliminary research showed the existence of thermostable protease activity from *Geobacillus* sp. DS3 that was grown optimally at 70°C on a minimal synthetic medium agar + 1% skim milk. *Geobacillus* sp. DS3 produces thermostable protease to hydrolyze protein into amino acid. Production of thermozymes requires high temperatures, making the process less efficient. In this work, the open reading frame of NPr gene was amplified and characterized as a starting point to express the gene of interest into mesophilic bacteria. Amplification was carried out by PCR using degenerate primers Pro_F and Pro_R resulted in ~1.6 kbp of NPr sequence. A GenBank accession number of this sequence was ON882245. Analysis using BLASTN confirmed that the amplified ORF had a high similarity with the neutral protease from *Alicyclobacillus acidocaldarius*. Translation of the ORF resulted 546 amino acid residues and theoretical pI/Mw of 5.60 / 59.81 kDa. The NPr had a structure similar to that of PDB 5A3Y or SAD thermolysin with 86.35% sequence identity. The domain and function of NPr were determined using SignalIP and InterPro. SignalIP results showed that the amino acid sequence of NPr consisted of signaling peptides. InterPro results showed that the NPr domain consisted of four major domains, namely, *fungalysin/thermolysin/propeptide* domain from residues 80–124, PepSY regulatory peptide domain from residues 140–21, and peptidase domain consisted of peptidase M4 from residues 235–380 and peptidase M4 C-terminal from residues 383–258. Further study related molecular docking must be performed to further understand the NPr structure.

Keywords: *Amplification, Geobacillus sp. DS3, Thermostable neutral protease (NPr).*

1. INTRODUCTION

Thermozymes from thermophilic or hyperthermophilic microbes are unique in their ability to retain optimal biocatalytic activity at extremely high temperatures (ranging from 60°C to about 120°C) [1]. Proteases are one of the most abundantly demanded thermozymes by various industries and are widely applied in protein hydrolysis and modification and leather, meat, brewing, photographic, dairy, membrane cleansing, and waste treatment industries [2,3]. Proteases can be applied in the food industry to obtain bioactive peptides and process different foods. Most industrial proteases are sourced from genus *Bacillus*. These bacteria can produce high yields of neutral and alkaline proteolytic enzymes with remarkable

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properties, such as high stability toward extreme temperatures, pH, organic solvents, detergents, and oxidizing compounds [4]. Thermostability in industrial enzymes remains a desirable attribute for (1) achieving fast conversion rates, (2) great catalytic efficiencies, and (3) protection from microbial contamination while operating at high temperatures [1].

Several thermophilic bacteria that grow at high temperatures of 60°C–80°C in Sikidang Crater on Dieng Plateau, Central Java, Indonesia have been isolated. One of the potential isolates from Sikidang Crater was characterized as *Geobacillus* sp. DS3 (previously characterized as *Brevibacillus* sp.) that optimally grow at 70°C [6]. *Geobacillus* sp. DS3 can express thermostable enzymes for survival at high temperatures. Phon *et* al. [5] purified and characterized thermostable alkaline serine protease from *Geobacillus* sp. DS3. The other thermostable proteases produced by *Bacillus* are mostly serine proteases from *B. subtilis* [7] and *B. pumilus* [8], cysteine proteases from *B. licheniformis* [9], metalloproteases from *B. stearothermophilus* [10], and aspartic proteases from *B. amyloliquefaciens* [11]. *Bacillus* proteases have a molecular weight of 27–71 kDa and an optimal pH range between 6 and 10 and grow optimally at temperatures between 37°C and 60°C [4]. Zilda *et* al. [12] investigated the protease activity of isolates from Padang Cermin, Lampung and Banyu Wedang, Bali hot springs identified as *Brevibacillus thermoruber* and reported an optimum temperature of 85°C, an optimum pH of 9, and stability of up to 100 min at 75°C. However, thermophilic bacteria require a high temperature to directly produce thermozymes, making the process less efficient. One solution is to express the gene of interest into mesophilic bacteria. To reach this objective, this research focused on amplifying the open reading frame (ORF) of the protease gene using specific primers as a step to establish the cloning process of NPr gene. The 3D structure of the enzyme must also be determined to understand its functions at the molecular level. The availability of DNA sequence from a novel microbial gene and its comparison with homologous enzymes permit us to identify the molecular determinants and amino acid residues involved in the desired features [13].

2. MATERIALS AND METHODS

The method of this research consisted of isolation and growth of *Geobacillus* sp. DS3, DNA genome isolation, primer design and gene target amplification, NPr ORF nucleotide sequence deduction, and amino acid analysis (Figure 1).

2.1. Bacterial growth conditions

Geobacillus sp. DS3 was isolated from the soil sampled from Sikidang Crater Dieng Plateau, Central Java [5] and then grown at 70°C in modified Luria–Bertani (LB) broth containing 1% bacto-tryptone, 0.5% yeast extract, 0.5% mannitol, 0.02% NaCl, 0.01% K2HPO4, 0.02% CaCl2, 0.01% MgSO4.7H2O, and 0.01% FeCl2. *Escherichia coli* strains were grown at 37 °C in LB broth or plated on LB agar containing the appropriate antibiotics as required.

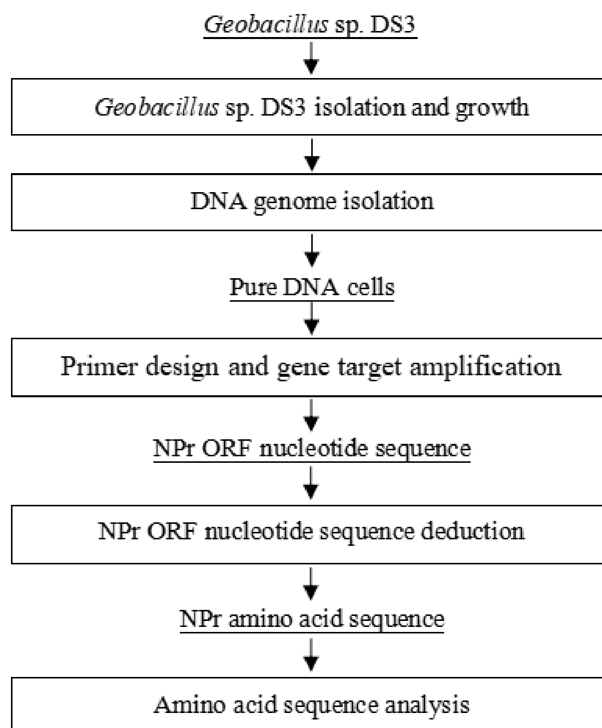

Figure 1 Method flowchart.

2.2. Geobacillus sp. DS3 DNA genome isolation

The cells grown in modified LB broth were harvested using 100 µl of TEN buffer (Tris-Cl 10 mM pH 7,9; EDTA 1 mM; NaCl 1 M). DNA genome was isolated using the method of Witasari *et* al. [6]. The cells were incubated at 37℃ for 10 min, resuspended in 200 µl of lysis solution (Lysozyme 1 mg/mL; EDTA 1 mM; NaCl 1 M; Tris-Cl 10 mM), added with 225 µl of TEN-sarkocyl buffer (Tris-Cl 10 mM pH 7,9; EDTA 1 mM; NaCl 1 M; and 2% sarkocyl), and incubated again at 42°C for 1 h to obtain the cell extract. DNA cells were separated by adding proteinase K (0,1 mg/mL) and conducting overnight incubation at 42℃. Pure DNA cells were obtained after extraction using phenol:chloroform.

2.3. Primer design and preparation and gene target amplification

The primers were Pro-F (5′-ATGRACAAACGGGCGATGCTYG-3′) and Pro-R (5′-

TTAATACACTCCAACCGCATTGAAC -3 ′). PCR was performed at 94°C for 5 min (initial denaturation), followed by 30 cycles of 94°C for 30 s (denaturation), 55 ° C for 30 s (annealing), and 72 ° C for 1 min (extension). After the final cycle, the PCR reaction was terminated at 72°C for 10 min. The mixture contained Go *Taq* Green PCR Mix 2x (Promega), 10 pmol of each primer and 0.5 mg of *Geobacillus* sp. DS3 genomic DNA.

2.4. Amino acid sequence properties

The amino acid sequence of neutral protease (NPr) was deduced from the nucleotide sequence of NPr ORF by using EXPASSY (http://web.expasy.org/translate/) [14]. Theoretical pI/MW was calculated using EXPASSY Compute pI/Mw tool (https://web.expasy.org/compute_pi/) . The amino acid sequences were then analyzed to determine the signal peptide sequence using SignalP-6.0 server [15] and InterPro.

3. RESULTS AND DISCUSSION

3.1. Amplification of DNA NPr gene from Geobacillus sp. DS3 DNA genome

Specific primers were used to amplify the whole sequence of NPr gene from *Geobacillus* sp. DS3. Primers were designed based on the ORF of *Geobacillus stearothermophilus* (M11446.1) using Primer-BLAST program on NCBI. The primers were Pro-F (5′- ATGRACAAACGGGCGATGCTYG-3′) and Pro-R (5′- TTAATACACTCCAACCGCATTGAAC-3′).

The amplification step was carried out using degenerate primers that were designed based on the nucleotide sequences of G. *stearothermophilus* NPr gene. The amplified fragment had a length of approximately ~1.6 kbp. (Figure 2), which was nearly identical to that of the NPr gene of *Brevibacillus brevis* (1607 bp) and the thermostable NPr gene of *Geobacillus stearothermophilus* (1671 bp) [16,17].

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
MULTISPECIES: M4 family metallopeptidase [Geobacillus]	Geobacillus	422	473	99%	1e-142	93.59%	546	WP_014196585.1
M4 family metallopeptidase [Geobacillus sp. GHH01]	Geobacillus sp. ...	421	472	99%	5e-142	93.16%	546	WP_015375654.1
M4 family metallopeptidase [Geobacillus sp. C56-T3]	Geobacillus sp. ...	420	471	99%	2e-141	92.74%	546	WP_013144430.1
bacillolysin [Geobacillus stearothermophilus]	Geobacillus stea...	420	471	99%	2e-141	92.74%	546	KFL16257.1
RecName: Full=Thermolysin; AltName: Full=Thermostable neutral proteinase; Flags: Precursor [[Bacillus] caldol...	[Bacillus] caldoly...	419	470	99%	2e-141	93.16%	546	Q59193.1
alkaline metalloprotease [synthetic construct]	synthetic construct	419	470	99%	3e-141	93.16%	546	AKR05596.1
MULTISPECIES: M4 family metallopeptidase [Geobacillus]	Geobacillus	419	470	99%	3e-141	93.16%	546	WP_047758159.1
MULTISPECIES: M4 family metallopeptidase [Geobacillus]	Geobacillus	419	470	99%	3e-141	93.16%	546	WP_023634539.1
RecName: Full=Thermolysin; AltName: Full=Thermostable neutral proteinase; Flags: Precursor [Bacillus sp. EA1]	Bacillus sp. EA1	419	470	99%	3e-141	93.16%	546	Q59223.1
MULTISPECIES: M4 family metallopeptidase [Geobacillus]	Geobacillus	418	466	99%	1e-140	92.31%	546	WP_063329853.1
M4 family metallopeptidase [Geobacillus sp. A8]	Geobacillus sp. A8	417	468	99%	1e-140	92.74%	546	WP_021322092.1
M4 family metallopeptidase [Geobacillus stearothermophilus]	Geobacillus stea...	417	467	99%	1e-140	92.31%	546	WP_053413931.1
MULTISPECIES: M4 family metallopeptidase [Geobacillus]	Geobacillus	415	464	99%	1e-139	89.74%	546	WP_047752636.1
MULTISPECIES: M4 family metallopeptidase [unclassified Geobacillus]	unclassified Geo...	415	466	99%	2e-139	91.88%	546	WP_013524383.1
M4 family metallopeptidase [Geobacillus thermocatenulatus]	Geobacillus ther...	413	462	99%	7e-139	89.32%	546	WP_025949562.1
M4 family metallopeptidase [Geobacillus sp. LEMMY01]	Geobacillus sp. ...	409	409	77%	3e-137	90.17%	546	WP_079935998.1
M4 family metallopeptidase [Geobacillus proteiniphilus]	Geobacillus prot...	409	409	77%	3e-137	90.17%	546	WP_074044107.1

Figure 3 BLASTX analysis of the amplified DNA sequence.

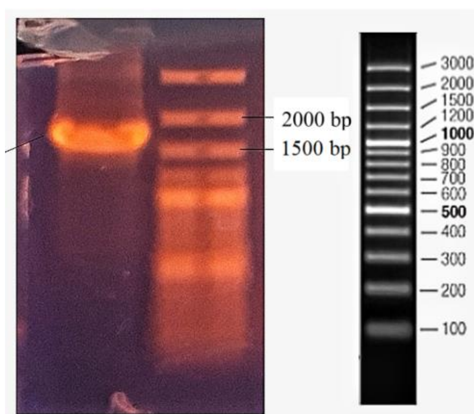

Figure 2 Amplified DNA fragment

Homology analysis using the BLASTX of the DNA sequence showed a 93.59% identity to the metalloprotease gene of *Geobacillus* (Figure 3). BLASTN analysis revealed that the amplified sequence was similar to *Alicyclobacillus acidocaldarius* NPr gene by up to 94.78% (Figure 4). Therefore, the DNA fragment amplified from *Geobacillus* sp. DS3 was homologous to the DNA proteases of other *Geobacillus* and *Bacillus* bacteria [18].

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Geobacillus kaustophilus HTA426 DNA, complete genome	Geobacillus kaustophilus HTA426	409	409	28%	2e-109	95.70%	3544776	BA000043.1
Alicyclobacillus acidocaldarius neutral protease gene, complete cds	Alicyclobacillus acidocaldarius	1399	1399	100%	0.0	94.78%	2231	U07824.1
Geobacillus thermoleovorans strain SGAir0734 chromosome	Geobacillus thermoleovorans	1393	1393	100%	0.0	94.67%	3615919	CP027303.2
Synthetic construct clone BL21 alkaline metalloprotease gene, complete cds	synthetic construct	1393	1393	100%	0.0	94.67%	1644	KP792451.1
Bacillus subtilis strain BSP alkaline metalloprotease gene, partial cds	Bacillus subtilis	1393	1393	100%	0.0	94.67%	1640	KP792450.1
Geobacillus thermoleovorans strain SURF-48B chromosome, complete genome	Geobacillus thermoleovorans	1393	1393	100%	0.0	94.67%	3808577	CP061472.1
Bacillus caldolyticus neutral proteinase (npr) gene, complete cds	[Bacillus] caldolyticus	1393	1393	100%	0.0	94.67%	1641	U25629.1
Geobacillus stearothermophilus strain AD-11 keratinase gene, complete cds	Geobacillus stearothermophilus	1382	1382	99%	0.0	94.64%	1641	KJ783444.1
Geobacillus sp. GHH01, complete genome	Geobacillus sp. GHH01	1387	1387	100%	0.0	94.56%	3583134	CP004008.1
Geobacillus stearothermophilus 10, complete genome	Geobacillus stearothermophilus 10	1382	1382	100%	0.0	94.45%	3654829	CP008934.1
Geobacillus sp. Y412MC52, complete genome	Geobacillus sp. Y412MC52	1382	1382	100%	0.0	94.45%	3628883	CP002442.1
Geobacillus sp. Y412MC61, complete genome	Geobacillus sp. Y412MC61	1382	1382	100%	0.0	94.45%	3622844	CP001794.1
Bacillus sp. neutral proteinase (npr) gene, complete cds	Bacillus sp. (in: Bacteria)	1382	1382	100%	0.0	94.45%	1641	U25630.1

Figure 4 BLASTN analysis of the amplified DNA sequence.

3.2. Sequencing and amino acid sequence properties of the NPr encoding gene

Compute pI/Mw

Theoretical pI/Mw (average) for the user-entered sequence:

```
        10         20         30         40         50         60
MNKRAMLGAI GLAFGLMAWP FGASAKEKSM VWNEQWKTPS FVSGSLLKGE DAPEELVYRY

        70         80         90        100        110        120
LDQEKNTFQL GGQARERLGL IGKQVDELGH TVMRFEQRYH GIPVYGAVLV AHVHNGELSS

       130        140        150        160        170        180
LSGALISNLD KRTLKTEAAI SIQQAERIAK QDAADAVAKE WPAFEEGKPT RLVIYPNGET

       190        200        210        220        230        240
ARLAYEVNVR FLTPVPGNWI YMIDAADGNV LNKWNQMDEA KPGGVQPVAG TSTVGVGRGV

       250        260        270        280        290        300
LGDQKYINTT YSSYYGYYYL QDNTRGSGIF TYDGRNRTVL PGSLWADSDN QFFASYDAAA

       310        320        330        340        350        360
VDAHYYAGVV YDYYKNVHGR LSYDGSNAAI RSTVHYGRGY NNAFWNGSQM VYGDGDGQTF

       370        380        390        400        410        420
LPFSGGIDVV GHELTHAVTD YTAGLVYQNE SGAINEAMSD IFGTLVEFYA NRSPDWEIGE

       430        440        450        460        470        480
DIYTPGIAGD ALRSMSDPAK YGDPDHYSKR YTGTQDNGGV HTNSGIINKA AYLLSQGGVH

       490        500        510        520        530        540
YGVNVTGIGG DKMGKIFYRA LVYYLTPTSN FSQLRAACVQ AAADLYGYRP RSQLGETGVQ

CGWSVL
```

Theoretical pI/Mw: 5.60 / 59813.66

Figure 5 Amino acid residue translated from amplified ORF and its theoretical pI/Mw.

The amino acid sequence was translated from ~1.6 kbp of the NPr sequence from *Geobacillus* sp. DS3. A GenBank accession number of this sequence was ON882245. Amino acid sequence was translated with

ExPASy tool. Frame 1 was chosen to be the NPr domain because it showed the most identical frame as indicated by the longest red highlight consisting of a sequence of 546 amino acid residues. These residues displayed theoretical pI and MW of 5.60 and 59.81 kD, respectively (Figure 5).

The sequence then was compared with other structures in BLASTP and PDB databases to identify other similar structures. As displayed in Figure 6, the 5A3Y structure showed a red line from number 1 to over 500, making it the longest red line among the other structures. Therefore, 5A3Y had the most similar structure to the query with up to 86.35% sequence identity and was identified as the structure of SAD thermolysin. Given that the structure was not 100% similar, it was considered as an incomplete sequence due to undetected or lost residues from residue 232 that might be caused by unknown peptide and propeptide signaling (Figure 7). Therefore, the domain and function of NPr were determined using SignalIP and InterPro.

SignalIP results showed that the amino acid sequence of NPr consisted of signaling peptides (Figure 8a). InterPro results showed that the NPr domain consisted of four major domains, namely, fungalysin/thermolysin/propeptide domain from residues 80–124, PepSY regulatory peptide domain from residues 140–21, and peptidase domain consisted of peptidase M4 from residues 235–380 and peptidase M4 C-terminal from residues 383–258 (Figure 8b) [19].

Figure 6 BLASTP analysis of NPr sequence of *Geobacillus* amino acid structure

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REMARK 465 MISSING RESIDUES
REMARK 465 THE FOLLOWING RESIDUES WERE NOT LOCATED IN THE
REMARK 465 EXPERIMENT. (M=MODEL NUMBER; RES=RESIDUE NAME; C=CHAIN
REMARK 465 IDENTIFIER; SSSEQ=SEQUENCE NUMBER; I=INSERTION CODE.)
REMARK 465
REMARK 465   M RES C SSSEQI
REMARK 465     MET A  -231
REMARK 465     LYS A  -230
REMARK 465     MET A  -229
REMARK 465     LYS A  -228
REMARK 465     MET A  -227
REMARK 465     LYS A  -226
REMARK 465     LEU A  -225
REMARK 465     ALA A  -224
REMARK 465     SER A  -223
REMARK 465     PHE A  -222
REMARK 465     GLY A  -221
REMARK 465     LEU A  -220
REMARK 465     ALA A  -219
REMARK 465     ALA A  -218
REMARK 465     GLY A  -217
REMARK 465     LEU A  -216
REMARK 465     ALA A  -215
REMARK 465     ALA A  -214
REMARK 465     GLN A  -213
REMARK 465     VAL A  -212
REMARK 465     PHE A  -211
REMARK 465     LEU A  -210
REMARK 465     PRO A  -209
REMARK 465     TYR A  -208
REMARK 465     ASN A  -207
REMARK 465     ALA A  -206
REMARK 465     LEU A  -205
REMARK 465     ALA A  -204
REMARK 465     THR A   -34
REMARK 465     PRO A   -33
REMARK 465     GLU A   -32
REMARK 465     PRO A   -31
REMARK 465     GLY A   -30
REMARK 465     ASN A   -29
REMARK 465     TRP A   -28
REMARK 465     LEU A   -27
REMARK 465     TYR A   -26
REMARK 465     ILE A   -25
REMARK 465     ILE A   -24
REMARK 465     ASP A   -23
REMARK 465     ALA A   -22
REMARK 465     VAL A   -21
REMARK 465     ASP A   -20
REMARK 465     GLY A   -19
REMARK 465     LYS A   -18
REMARK 465     ILE A   -17
REMARK 465     LEU A   -16
REMARK 465     ASN A   -15
REMARK 465     LYS A   -14
REMARK 465     PHE A   -13
REMARK 465     ASN A   -12
REMARK 465     GLN A   -11
REMARK 465     LEU A   -10
REMARK 465     ASP A    -9
REMARK 465     ALA A    -8
REMARK 465     ALA A    -7
REMARK 465     LYS A    -6
REMARK 465     PRO A    -5
REMARK 465     GLY A    -4
REMARK 465     ASP A    -3
REMARK 465     VAL A    -2
REMARK 465     LYS A    -1
REMARK 465     SER A     0
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Figure 7 Incomplete sequence of PDB 5A3Y structure

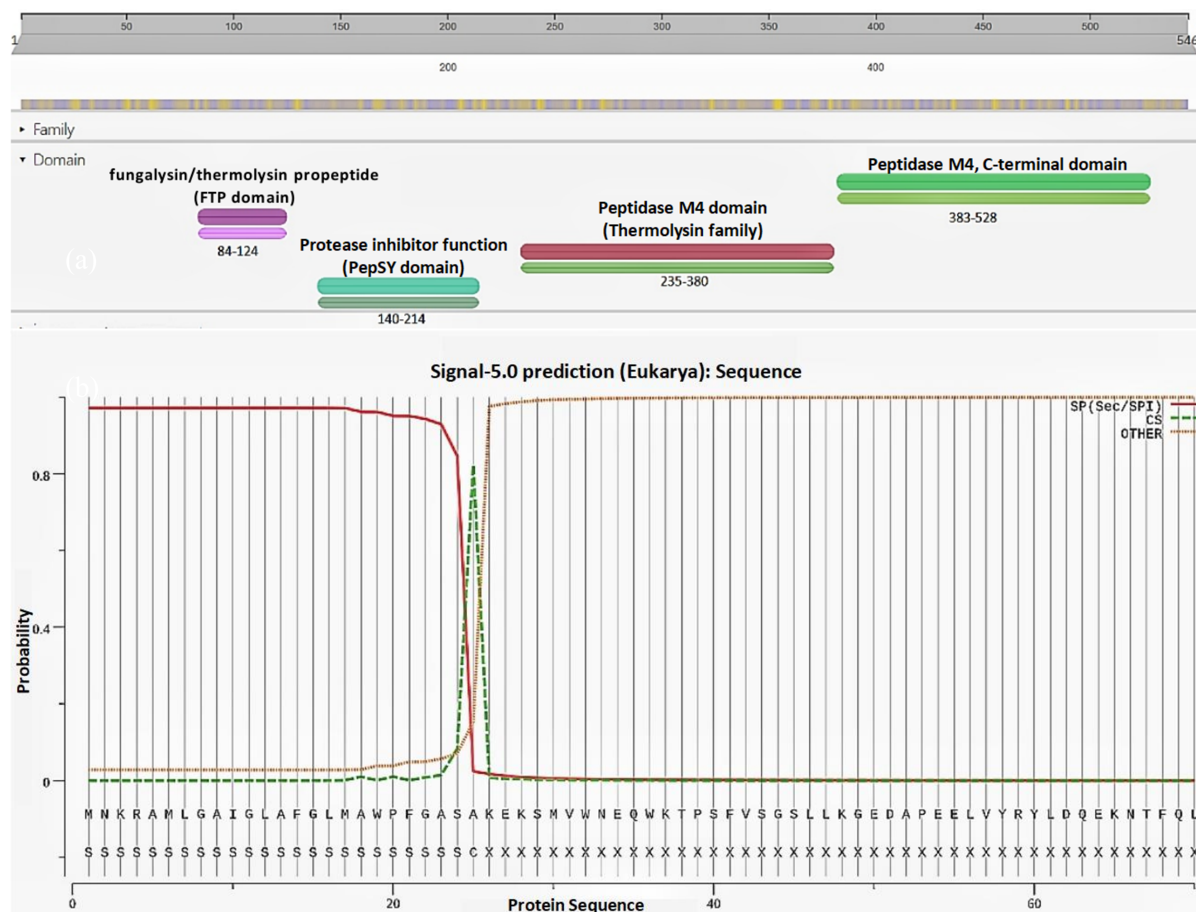

Figure 8 NPr amino acid sequence domain identified by SignalIP (a) and NPr amino acid sequence domain identified by InterPro (b).

4. CONCLUSION

The NPr ORF was successfully amplified from *Geobacillus* sp. DS3 DNA genome using degenerate primers (Pro_F and Pro_R). A sequence of ~1.6 kbp length was obtained and deposited in Genebank database as ON882245. Further cloning process should be conducted to be able to express the NPr gene on mesophilic bacteria. Sequence analysis of the amino acid showed its high similarity to thermolysin with four major domains, namely *fungalysin/thermolysin/propeptide* domain, PepSY regulatory peptide domain, and peptidase domain consisted of peptidase M4 and peptidase M4 C-terminal. Further study related molecular docking must be performed to further understand the NPr structure.

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