Protease of *Stenotrophomonas* sp. from Indonesian fermented food: gene cloning and analysis

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Abstract

Screening of proteolytic and fibrinolytic bacteria from Indonesian soy bean based fermented food *Oncom* revealed several potential isolates. Based on 16s rDNA gene analysis, one particular isolate with the highest proteolytic and fibrinolytic activity was identified as *Stenotrophomonas* sp. The protease gene was amplified to generate a 1749 bp Polymerase Chain Reaction product and BLAST analysis, revealed 90% homology with gene encoding protease enzyme from *Stenotrophomonas maltophilia*. The putative amino acid sequence indicated a serine protease enzyme with typical amino acid aspartate, histidine and serine in the catalytic triad. The gene was translated into a pre-pro-protein consisted of cleavage site on its N terminal and Pre-Peptidase C-terminal domain. Cloning of the protease gene in pET22b with *Escherichia coli* BL21 DE3 as the host showed that the gene was expressed as insoluble protein fraction. This is the first report for analysis of protease gene from food origin *Stenotrophomonas* sp.

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Contributions: FK: conducting experiment: gene cloning and analysis; RRT: planning methodology and helped discussion on the results, in particular with the protein structure; AY: involved in bioinformatic analysis; MTS: principal investigator, responsible for overall discussion and writing the manuscript.

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Introduction

Stenotrophomonas is a gram-negative bacteria; the genus compromises of eight species, ranging from common soil inhabitants to opportunistic human pathogens. *Stenotrophomonas* can be found throughout the environment, especially on plants. Among these species, full genome of *Stenotrophomonas maltophilia* had been reported.[1] *S. maltophilia* is catalase-positive, oxidase-negative (which distinguishes it from most other members of the genus). *S. maltophilia* is ubiquitous in aqueous environments, soil, and plants. This gram negative bacteria has become important for biotechnological application in agricultural and bioremediation due their ability to produce hydrolytic enzymes such as chitinase, glucanases, lipases, laccases and proteases.[1,2] Extracellular protease from *Stenotrophomonas* sp is already used for biocontrol activity, such as for enhancing suppression of phytopathogenic fungi and virulence factor against giant parasitic nematode.[3]

Protease which catalyze cleavage of peptide bonds in proteins have important role in biochemical investigation, medical and industrial applications. Among the protease enzymes, serine protease is the major protease that has been widely used in technical enzyme applications, such as in leather, food and chemical industries and processes.[4,5] This group of enzyme is excreted by various microorganisms and eukaryote. Some of the commercial serine proteases are subtilisin Carlsberg, subtilisin BPN and Savinase, with their major applications as detergent enzymes.[6] Other serine proteases of medical interest/ applications are urokinase-type from human urine, streptokinase from *Streptococci sp.* and lumbrokinase from earthworms and several microorganisms.[7,8]

Despite numerous commercial proteases available, discovery of novel/unique protease is still being pursued due to demand of novelty, safer, higher activity and less expensive protease for industries and medical applications.[4,5,9] Alkaline extracellular protease potential for detergent aplication from *Stenophomonas maltophilia* isolated from soil and medical samples had been reported.[10,11]

Interesting sources of proteases regarded as safe include some microorganism present in the traditional fermented food. Kim *et al.* screened proteolytic microorganism from *Chungkook-Jang*, a traditional Korean fermented-soybean sauce, with its activity about eight time higher than subtilisin Carlsberg.[12] Protease with higher activity than plasmin known as anti-thrombosis agent was successfully screened by Uesugi *et al.* from the Japanese *natto.*[13]

Screening of proteolytic bacteria from *Oncom* as one of the traditional Indonesian fermented soy bean based product, revealed several potential isolates, namely *Bacillus* sp., *Bacillus cereus* and *Stenotrophomonas* sp. as confirmed by 16srDNA gene analysis *Stenotrophomonas* sp. was selected for further study and characterization due to its highest proteolytic activity among other isolates. This was shown through several quantitative parameters such as protease activity assay, fibrin plate assay and fibrin activity assay, which revealed that at similar protein concentration (40 mg/mL) the enzyme activity was similar to that of the commercial lumbrokinase, a fibrin degrading enzyme of the earthworm.[14] The protease enzyme excreted by *Stenotrophomonas* sp. from fermented food Oncom was found as safe, as shown in the cell culture assay and animal experiments.[15] The enzyme had been purified and found unique.[14] This extracellular protease was the focus of our genomic and bioinformatic studies.

The structural genes encoding extracellular protease from *S. maltophilia* R551-3 (GenBank acession number CP001111) revealed five genes: StmPr1, StmPr2 , StmPr3 and StmPr4 as well as an ATP-dependent metalloprotease FtsH. These serine proteases gene were composed of signal peptide, pro-peptide I, mature protease, and the C-terminal domain The actice site region revealed considerable homology with other serine protease family such as subtilisin, with the typical catalytic triad Asp, His, and Ser in the primary sequence. StmPr1-StmPr3 from *S. maltophilia* strain 19580 have a multi-domain structure including a signal peptide for translocation, a pro-peptide for maturation, the mature peptide and a C-terminal domain. Upon translation, all the domain was translated as proenzyme which later on processed into its mature form.[16]

In this manuscript we reported analysis of the gene encoding protease from Indonesian *Stenotrophomonas* sp. of food origin. The protease structure was predicted using bioinformatic tools.[17] Structural comparison of this protease with similar protease reported earlier is expected to lead us to find best strategy and methods for furthur molecular experiment for increasing enzyme expression, productivity and furthur applications. This is the first report for analysis of protease gene from food origin *Stenotrophomonas* sp.

Materials and methods

Chemicals and reagents

All chemicals used in this study were analytical grade and purchased from Sigma (United States) and Oxoid (United Kingdom) through local distributors.

Bacterial strains and plasmids

Stenotrophomonas sp. isolate originated from *Oncom* was provided from Bogor Agricultural University, Indonesia. Vector pGEM-T Easy (Promega, United States) was used for library construction in *Escherichia coli* DH5α (provided by Atmajaya University, Indonesia). Vector pET22b (Merck, United States) was used for gene expression in *E. coli* BL21 DE3 (Merck, United States).

General recombinant DNA techniques

All DNA manipulations described in this work were performed by standard methods.[18] DNA isolation kit UltraClean®Microbial (MoBio, United States) was used for gDNA isolation. GoTaq Green MasterMix (Promega, United States) was used for Polymerase Chain Reaction (PCR). The PCR was performed in T3000-Thermal cycler (Biometra, Germany). Cloning DNA to pGEM-T Easy (Promega, United States), digestion of DNA with restriction endonuclease (Fermentas, United States), dephosphorylation with alkaline phosphatase (Fermentas, Unites States) and ligation with T4 DNA-ligase (Fermentas, United States) were performed in accordance to the manufacturer's instructions. Plasmids were purified using Miniprep method and DNA fragments were purified by Qiagen DNA purification kits (Qiagen, Germany).

Cloning of protease gene

The protease gene (annotated as *Stenoprotease*) was amplified using genomic DNA from *Stenotrophomonas* sp. as template for PCR. The reaction was performed in a 50 µL volume with 4µM primer StenoF: 5'-CATATGTCCCAGGTAACGC-3' (forward) which has *Nde*I restriction site and StenoR: 5'-CTCGAG-TACTGGGCGTTGA-3' (reverse) which has *Xho*I restriction site, 25 µL of GoTaq Green Master Mix and 100 ngDNA template. DNA amplification was conducted based on the manufacture instruction. The PCR product was gel purified and ligated to pGEM-T Easy vector, yielding plasmid pGEM-T-Steno. The plasmid was transformed into *E. coli* DH5α and the gene was sequenced using M13 primers pair. Plasmid pGEM-T-Steno that have protease gene was digested with *Nde*I and *Xho*I and ligated to pET22b, yielding plasmid pET22b-Steno. The construction of these plasmids can be seen on Figure 1. The plasmid was transformed into *E. coli* BL21 DE3 and used for expression.

Expression of protease Steno in E. coli BL21 DE3

For expression of protease Steno, the plasmid pET22b-Steno was freshly transformed into *E. coli* BL21 DE3 cells. These clones were

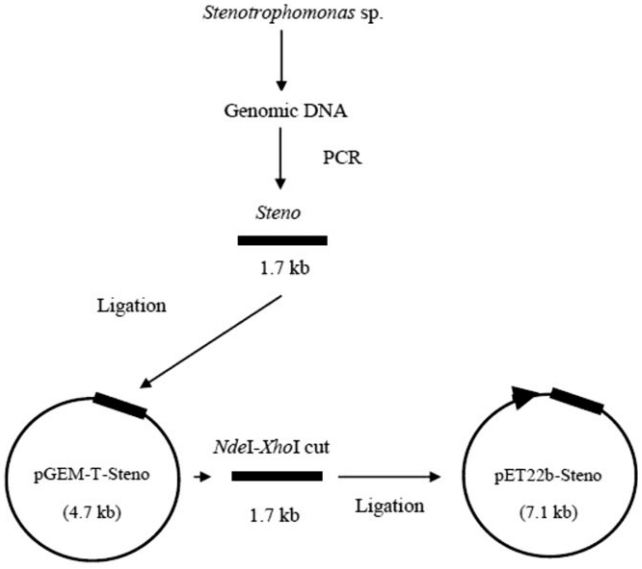


Figure 1. Construction of pGEM-T-Steno and pET22b-Steno recombinant plasmids containing protease gene from Indonesian *Stenotrophomonas* sp.

then inoculated into 5 ml Luria Bertani (LB) broth supplemented with 100 µg/mL ampicillin. The culture was grown overnight at 30°C on a rotary shaker at 150 rpm and utilized to inoculate the main culture, composed of 100 ml freshly prepared LB broth and 100 µg/mL ampicillin. The culture was incubated at 37°C and 150 rpm until an optical density (600 nm) between 0.6-0.8 was reached. The culture was then cooled down to 20°C and induced with IPTG at a final concentration of 0.05 mM. The induced culture was incubated at 20°C with shaking at 150 rpm for approximately 6 hours. Cells were harvested by centrifugation at 5000 g and 4°C for 10 minutes. Cell pellet was resuspended in Phosphate Buffer Saline 1x and sonicated for 5 times with 20 s intervals. The suspension was centrifuged for 30 minutes at 13,000 rpm. The supernatant was collected as soluble fraction and the pellet was the insoluble fraction. These samples were run on SDS PAGE for protein molecular weight analysis.

Agarose gel electrophoresis and SDS PAGE

Agarose gel electrophoresis was carried out based on Sambrook *et al.*;[18] PCR reagents (premixed with loading buffer) were loaded into 1% agarose gel (containing 0.5 µg/mL EtBr) and PCR was performed at 100 V and 45 minutes conditions. DNA was visualized on GelDoc® System (BioRad). SDS PAGE was carried out according to Laemmli;[19] protein-containing samples were denatured with sample buffer (43.5% glycerine, 2% SDS, 5% merchaptoethanol, 0.1% bromphenol blue, 0.06 M Tris Base pH 6.8) and run at 200 V for 40 minutes. Protein bands were stained with Coomassie Briliant Blue R-250 and destained with destaining buffer (10% methanol; 10% acetic glacial acid).

DNA sequencing, database searches and alignments

DNA was sequenced as custom service (1stBASE). Analysis of DNA sequence was performed with Sequence Scanner v1.0, Serial Cloner v2.5, SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and ExPASy translate tool (http://web.expasy.org/translate/). BLAST searches were performed using BLASTn and BLASTx software from National Center for Biotechnology Information (NCBI). Nucleotide sequences were aligned using the webserver Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Structural modeling of protease Steno

3D structure of protease Steno was modeled using the program Modeler v9.11[20] Amino acid sequence of protease Steno was firstly aligned to internal database on Modeller. Several proteases having lowest E value score were selected as templates for modelling the protease. The final templates were decided based on the highest homology, crystallographic resolution and crystallographic R factor.

Deposition of sequence data

The nucleotide sequence of protease Steno was deposited in the GenBank under accession number KJ440091.

Results and discussion

Amplification and analysis of protease Steno

The primers for PCR in this study were designed based on protease gene from *Stenotrophomona smaltophilia*. These primers generated 1749 bp PCR product. The gene sequence was analyzed using BLASTn and BLASTx. BLASTn revealed that the *Stenoprotease* gene showed 90% homology with *Pr2* gene from *S. maltophilia* and BLASTx revealed that the translated nucleotide showed high homology with minor extracellular protease StmPr2 preprotein, peptidase S8 and the secreted serine protease from *S. maltophilia*

Open reading frame of the gene (1740 bp) encodes a protein consisted of 580 amino acids with calculated mass of 58.3 KDa. Sequence alignment with homologous proteins revealed that the enzyme belongs to subfamily of subtilisin like serine proteases from *S. maltophilia*. The protein possess catalytic residue (black highlighted letter) similar to the protease from *S. maltophilia* and other serine protease (Figure 2). Based on the alignment with protein from *S. maltophilia*, we found the catalytic domain located between 177 until 409 residue followed with bacterial Pre-Peptidase C-terminal (PPC) domain until 577 residues. Active site residues of the Steno protease (Indonesian *oncom*) with aspartic acid on position 177, histidine on postion 237 and serine on position 409 pointed to the typical serine protease, such as subtilisin from *Bacillus subtilis* known as enzyme widely used for detergent, or fibrinolytic enzyme or natokinase from *Bacillus subtilis*.[5,6,7] In fact serine protease family which include broader members such as digestive enzyme (trypsin, chymotrypsin, elastase), fibrinolytic enzyme of mammalian and bacterial origin (lumbrokinase, nattokinase) and various subtilisin type of enzyme secreted by *Bacillus* sp share the catalytic triad Asp-Histidine-Serine. Overall sequence analysis shows 94% and 93% similarity with peptidase and minor extracellular protease StmPr2 from *Stenotrophomonas maltophilia* known as an opportunistic bacteria which excretes protease with broad specificity. The alkaline serine enzyme is synthesized as 63-kDa precursor which is processed to the mature protein of 47 kDa.[21]

When comparing our protease Steno with the amino acid sequence of other protease originated from *Bacillus subtilis*, we found only 39% similarity to subtilisin (enzyme used in detergent). Interestingly, eventhough our enzyme was found as fibrinolytic (beside proteolytic), the sequence similarity of 39% was found with nattokinase from *Bacillus nato* (fibrinolytic enzyme used as thrombolytic agent). In our previous study, the *Stenotrophomonas* sp. isolated from fermented food *oncom* was found as producers of strong fibrin and fibrinogen degrading enzymes.[14,15]

3D Modelling of protease gene

Structure modelling of protease Steno of Indonesian *Oncom* was conducted using program Modeller 9.11. There are several proteases served as template for this modelling (residue 170-580). These proteases were subtilisin BPN from *Bacillus amyloliquefaciens* (PDB code 1to2), subtilisin Carlsberg from *Bacillus licheniformis* (PDB code 1r0r), serine protease subtilisin from *Bacillus lentus* (PDB code 1gci), subtilisin from *Bacillus licheniformis* (PDB code 1bh6), Proteinase K from *Engyodontium album* (PDB code 1ic6), AK.1 serine protease from *Bacillus* sp. AK1 (PDB code 1dbi), serine protease from *Lysinibacillus sphaericus* (PDB code 1ea7), mesenterico peptidase from *Bacillus pumilus* (PDB code 1mee), subtilisin like-serine protease from *Fervidobacterium pennivorans* (PDB code 1r6v), subtilisin BPN from *Bacillus amyloliquefaciens* (PDB code 1sbt), extracellular subtilisin-like serine protease from *Vibrio* sp. (PDB code 1sh7) and Thermitase from *Thermoactinomyces vulgaris* (PDB code 1thm). The residues of these templates ranged between 269-671 amino acid residues, some of these templates were in the form of complex with its inhibitor (1to2, 1r0r, 1bh6, and 1mee). The sources of these templates were mostly from *Bacillus* genus (Table 1).

Serine protease subtilisin from *Bacillus lentus* (PDB code 1gci) was selected as template because of better sequence homology (52.26%), better crystallographic resolution (0.8 Å) and better crystallographic R-factor (9.9%). Modelling of protease Steno on *Bacillus lentus* subtilisin (Figure 3A) revealed that the alpha helical and beta sheet domain appeared structurally similar. In comparison to the subtilisin from *Bacillus lentus* (Figure 3B),[22] the beta sheet in pro-

tease Steno appeared to be less accessible (more buried). The protruded random coil chain (indicated by arrow) might be the unprocessed amino acids making up the PPC domain. *In silico* analysis using SignalP,[23] showed that protease Steno possess a signal peptide which is cleaved most likely between Ala32 and Gly33 upon excretion. This finding might indicate that the protein is expressed as precursor molecule. Amino acid alignment with subtilisin from *Bacillus lentus* confirmed the presence of the typical catalytic triad of the serine protease family: histidine, serine and aspartic acid (red letter) (Figure 3C), even though at different positions.

Cloning and expression of protease gene

The Steno protease gene was then subcloned into pET22b with *E. coli* BL21 DE3 as the host. The expressed protein was expected to have larger size due to addition of HisTag on its C terminal. The size was approximately 59.4 KDa (Figure 4) in agreement with the expected protein size based on the previous gene sequence analysis, which revealed open reading frame of 1740 bp encoding a protein of 580 amino acids with calculated mass of 58.3 KDa. Figure 4 showed that the expressed protein was only observed in the total cellular protein and insoluble fraction. This result indicated that the protein was expressed as aggregate (inclusion bodies) form. This might indicate insufficient post translational processing in the *E. coli* host.[24] The protease excreted from *S maltophilla* which share sequence similarity with our protease was reported as about 47 kD mature protein. Even though this protease and our protease belong to serine protease, the molecular weight are somewhat larger than the protease of subtilisin family.

Conclusions

We have succeeded in cloning and analysis of protease Steno from *Stenotrophomonas* sp. originated from *Oncom*, a fermented Indonesian food. This is the first report on protease gene analysis from *Stenotrophomonas* of food origin. Protein modelling and sequence analysis revealed that the protease was a serine protease with typical catalytic triad (Asp, His, Ser) and expressed as an immature protein, mostly as inclusion bodies.

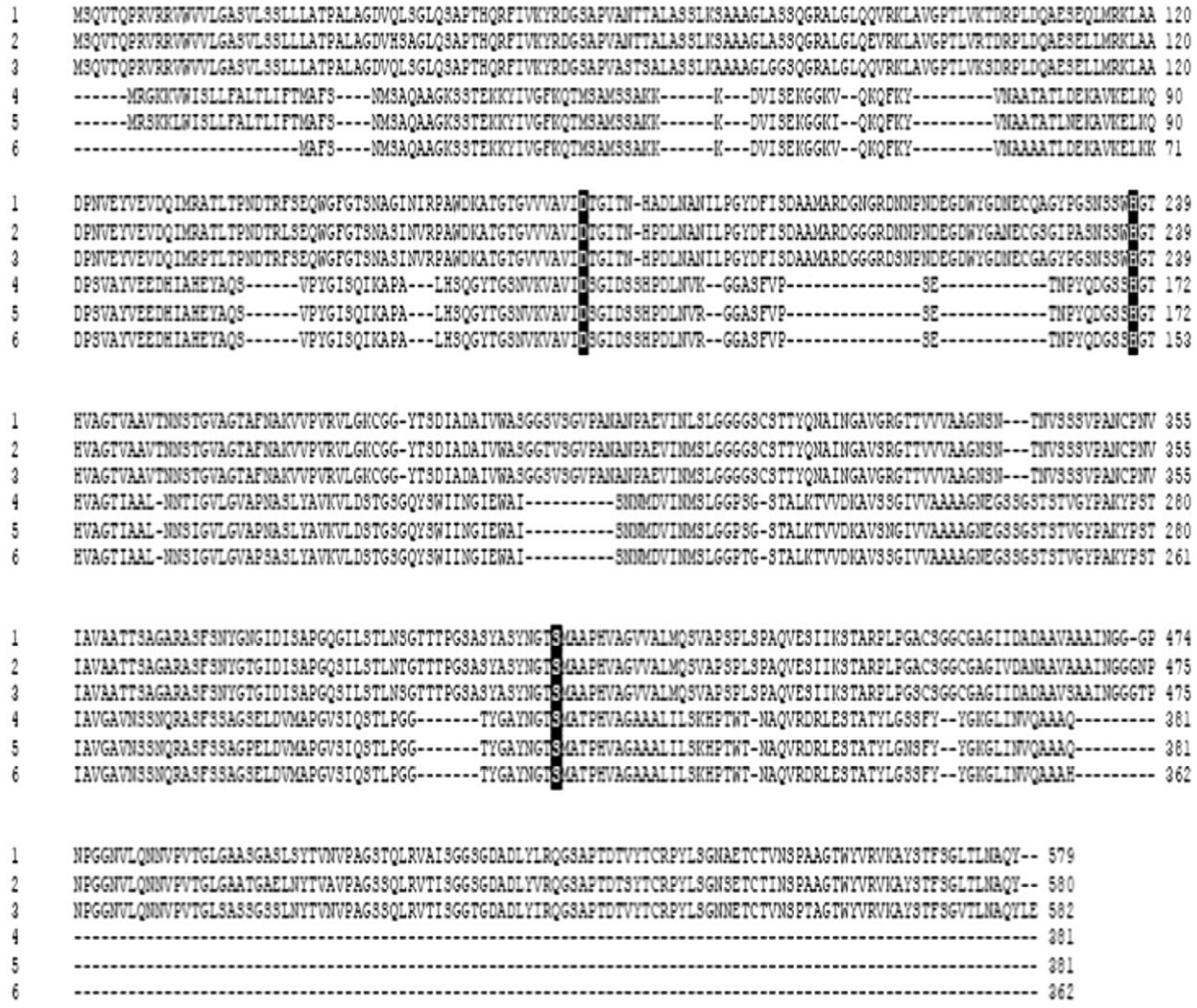
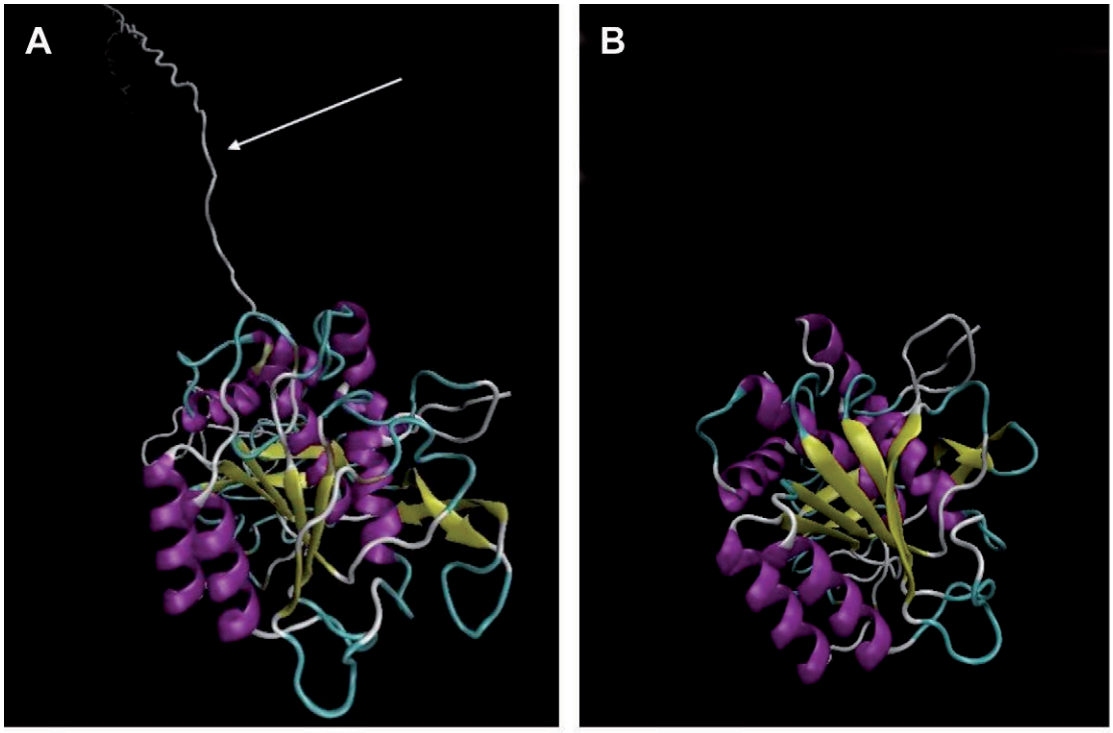


Figure 2. Sequence alignment of protease from Indonesian *Stenotrophomonas* sp with several homologous protein. Catalytic triad is indicated by black highlighted letter. The proteases used for sequence alignment were: (1) peptidase S8 from *Stenotrophomonas maltophilia*, (2) minor extracellular protease StmPr2 (3) our protease (Steno), (4) subtilisin from *Bacillus subtilis*, (5) fibrinolytic enzyme F1 from *Bacillus subtilis*, and (6) nattokinase from *Bacillus subtilis*.



C

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Subtilisin      ------------------------------------------------------------
Protease        MSQVTQPRVRRVWVVLGASVLSSLLLATPALAGDVQLSGLQSAPTHQRFIVKYRDGSAPV

Subtilisin      ------------------------------------------------------------
Protease        ASTSALASSLKAAAAGLGGSQGRALGLQQVRKLAVGPTLVKSDRPLDQAESELLMRKLAA

Subtilisin      ----------------------AQSVPWGISRVQAPAA---HNRGLTGSGVKVAVLDTGI
Protease        DPNVEYVEVDQIMRPTLTPNDTRFSEQWGFGTSNASINVRPAWDKATGTGVVVAVIDTGI

Subtilisin      STHPDLNIR--GGASFVPGEPSTQD----------------------------GNGHGTH
Protease        TNHPDLNANILPGYDFISDAAMARDGGGRDSNPNDEGDWYGDNECGAGYPGSNSSWHGTH

Subtilisin      VAGTIAAL-NNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGM-------
Protease        VAGTVAAVTNNSTGVAGTAFNAKVVPVRVLGKCG-GYTSDIADAIVWASGGSVSGVPANA

Subtilisin      ---HVANLSLGSP-SPSATLEQAVNSATSRGVLVVAASGNSGA-GSISYPARYANAMAVG
Protease        NPAEVINMSLGGGGSCSTTYQNAINGAVGRGTTVVVAAGNSNTNVSSSVPANCPNVIAVA

Subtilisin      ATDQNNNRASFSQYGAGLDIVAPGVNVQSTYP-------GSTYASLNGTSMATPHVAGAA
Protease        ATTSAGARASFSNYGTGIDISAPGQSILSTLNSGTTTPGSASYASYNGTSMAAPHVAGVV

Subtilisin      ALVKQKNPSWS-NVQIRNHLKNTATSLGST--NLYGSGLVNAEAATR-------------
Protease        ALMQSVAPSPLSPAQVESIIKSTARPLPGSCSGGCGAGIIDADAAVSAAINGGGTPNPGG

Subtilisin      ------------------------------------------------------------
Protease        NVLQNNVPVTGLSASSGSSLNYTVNVPAGSSQLRVTISGGTGDADLYIRQGSAPTDTVYT

Subtilisin      ----------------------------------------
Protease        CRPYLSGNNETCTVNSPTAGIWYVRVKAYSTFSGVTLNAQYLE
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Figure 3. Modelled structure of protease Steno (A) with the protruded peptide chain (indicated by arrow); structure of the serine protease subtilisin from *Bacillus lentus* (B) and amino acid alignment (C) between protease Steno from *Stenotrophomonas* sp. and subtilisin from *Bacillus lentus* (active site indicated by red letter).

Table 1. Proteases served as template for modelling protease Steno.

Protein code	Protein name	Source microorganism	Description	Residue
1to2	Subtilisin BPN	*Bacillus amyloliquefaciens*	Crystal structure of the complex of subtilisin BPN' with chymotrypsin inhibitor 2 M59K, in pH 9 cryosoak	Chain E: 281 residue Chain I: 64 residue
1r0r	Subtilisin Carlsberg	*Bacillus licheniformis*	1.1 Angstrom Resolution Structure of the Complex Between the Protein Inhibitor, OMTKY3, and the Serine Protease, Subtilisin Carlsberg	Chain E: 274 residue Chain I: 51 residue
1gci	Serine protease subtilisin	*Bacillus lentus*	0.78 Angstroms Structture of a Serine Protease-Bacillus Lentus Subtilisin	Chain A: 269 residue
1bh6	Subtilisin	*Bacillus licheniformis*	Subtilisin DY in Complex with the Synthetic Inhibitor N-Benzyloxycarbonyl-Ala-Pro-Phe-Chloromethyl Ketone	Chain A: 274 residue
1ic6	Proteinase K	Engyodontium album	Structure of A Serine Protease Proteinase K from Tritirachium Album Limber at 0.98 A resolution	Chain A: 279 residue
1dbi	AK.1 serine protease	*Bacillus sp. AK1*	Crystal Structure of A Thermostable Serine Protease	Chain A: 280 residue
1ea7	Serine protease	*Lysinibacillus sphaericus*	The 0.93A Crystal Structure of Sphericase: A Calcium-Loaded Serine Protease from Bacillus Sphaericus	Chain A: 310 residue
1mee	Mesentericopeptidase	*Bacillus pumilus*	Complex between the subtilisin from a mesophilic bacterium and the leech inhibitor eglin-C	Chain A: 275 residue
1r6v	Subtilisin like-serine protease	*Fervidobacterium pennivorans*	Crystal structure of fervidolysin from Fervidobacterium pennivorans, a keratinolytic enzyme related to subtilisin	Chain A: 671 residue
1sh7	Extracellular subtilisin-like serine protease	*Vibrio sp.*	Crystal structure of a subtilisin-like serine proteinase from a psychrotrophic Vibrio species	Chain A,B: 284 residue
1thm	Thermitase	*Thermoactinomyces vulgaris*	Crystal structure of thermitase at 1.4 A resolution	Chain A: 279 residue

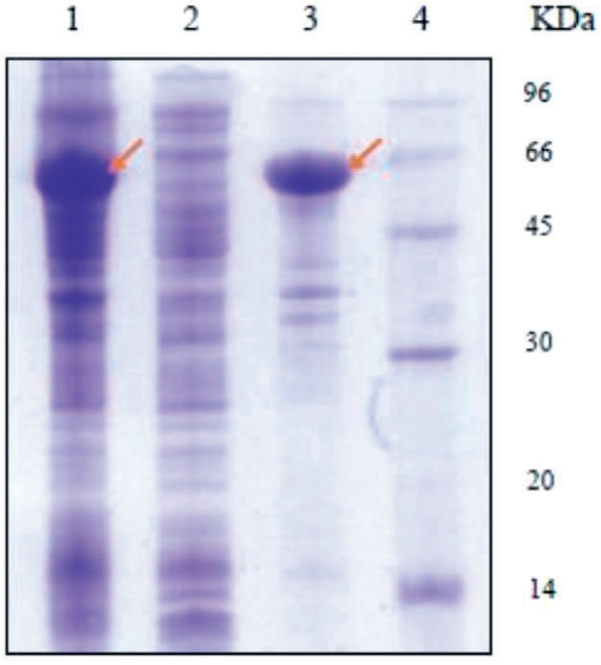

Figure 4. SDS PAGE of expressed protein from BL21 pET22b-Steno. Lane 1: Total cellular protein; Lane 2: Soluble fraction, Lane 3: Insoluble fraction; Lane 4: Low Molecular Weight Marker (GE Healthcare). Expected protein target is shown by the red arrow.

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