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Three-dimensional structure modeling of a protease from lactic acid bacteria *Leuconostoc mesenteroides* K7 using automated protein homology analysis

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Abstract. *Hidayat H, Haryadi W, Raharjo TJ. 2020. Three-dimensional structure modeling of a protease from lactic acid bacteria* Leuconostoc mesenteroides *K7 using automated protein homology analysis. Biodiversitas 21: 3156-3162.* This study aimed to characterize the protease encoding gene of *Leuconostoc mesenteroides* K7 isolated from Kelengkeng (*Dimocarpus longan*) fruit as well as to predict the structure of the protein using in silico approach. Gene characterization was performed using PCR employs primers designed based on protease gene of other *Leuconostoc* species, followed by cloning and sequencing of the PCR product. Protein structural modeling was targeted to the deduced amino acid sequence of the gene employ multiple sequence alignment and SWISS-Model online software. As a result, the sequence of the PCR product contains an open reading frame with a size of 1,140 bp, which can be translated into 379 amino acids. The amino acid sequence shares 98.60% identity with protease from *Leuconostoc suionicum* (AP017935.1). Three conserved sequences of QTDA, INPGNSGGPL, and FAIP are known as the signature from the Serine endoprotease DegS family are detected. The three-dimensional modeling structure application shows that the protein share similarity of 37.62% to Protease Do-like I chloroplastic that belong to serine protease.

Keywords: In silico modeling, kelengkeng, *Leuconostoc mesenteroides* K7, PCR, protease

INTRODUCTION

Protease enzyme produced by microbes is more beneficial and stable than proteases obtained from plants and animals and it is easy to be produced on a large scale. Protease enzymes derived from bacteria account for nearly 60% of the world enzyme production. Microbial proteases can be provided at a relatively short time and low cost (Anbu 2013; Ali et al. 2016). Furthermore, microbial proteases have a high yield potential and diverse properties available and can be applied in various fields of industries (Banerjee et al. 2017; Asgher et al. 2018; Razzaq et al. 2019). Lactic acid bacteria (LAB) play an essential role in food processing using the fermentation technique. During the fermentation process, the food taste, texture, and aroma will change, and its nutritional value will increase. The process is catalyzed by enzymes produced by LAB and involves the hydrolysis of macromolecules, such as carbohydrate, protein, and fat, into simple sugar, peptides, amino acid, and fatty acid. Thus, LAB can be considered a good source of hydrolysis enzyme, such as protease (Toledano et al. 2011; Afriani et al. 2018; Lim et al. 2019; Sun et al. 2019; Sun et al. 2020). Protease from *Lueconostoc* bacterial such as *Leuconostoc mesenteroides* (Argüello-Morales et al. 2005) and *Leuconostoc sp.* (Frantzen et al. 2017) have been reported to have high activity.

Leuconostoc mesenteroides strain K7 has been isolated from Kelengkeng fruit. This LAB can survive in high levels of sugar, salt, and alcohol and can ferment monosaccharides and disaccharides (Hidayat et al. 2019). As a LAB, Leuconostoc has the potential to be used as a bacterium for food fermentation. *L. mesenteroides* is able to produce different types of extracellular enzymes, including protease enzyme. *L. mesenteroides* K7 exhibits protease enzyme activity as indicated by the formation of a clear zone around the bacterium colony as a result of the loss of particles in the skim milk medium. The protease enzyme activity and enzyme-specific activity have been determined to be 6.18 unit/mL and 2.81 unit/mg, respectively, with a clear zone of 17 mm (Hidayat et al. 2019).

In order to take advantage of *L. mesenteroides* K7 protease that has been characterized for its activity (Hidayat et al. 2019), further investigation of the structure and catalytic mechanism of the enzyme is essential. Information regarding genes that encode the protease of *L. mesenteroides* K7 is vital for protease structural investigation. Gene characterization of bacterial such as *L. mesenteroides* K7 can be carried out using PCR gene amplification followed sequencing of the PCR product and further analysis of the sequence such as homology analysis as well as modeling of the deductive protein sequence encoded by gene (Biasini et al. 2014; Yan and Fong 2018; Nataningtyas et al. 2019). The protease-coding gene has a diverse sequence between organisms, which can be traced in vitro through polymerase chain reaction (PCR)

technique using primers that are designed based on known gene sequences from other species that have a close taxonomy relationship. In the present investigation, we conducted the sequence analysis of protease encoding gene of *L. mesenteroides* K7 isolated from Kelengkeng fruit amplified using PCR. The deductive amino acid sequence of the protease generated from gene sequence was furtherly analyzed for its secondary and tertiary structures of protease using the SWISS-MODEL.

MATERIALS AND METHODS

Materials

Leuconostoc mesenteroides strain K7 isolated from Kelengkeng fruit was used as the genomic DNA source. All of the analytical and molecular biology grade chemicals used in this study, namely, nutrient agar and nutrient broth (Sigma Aldrich), DNA ladder (Vivantis), DNA loading dye, Illustra™ Ready-To-Go PCR beads (GE Healthcare), nuclease-free water (Gibco), PureLink™ Quick Gel Extraction Kit (Life Technologies), and BigDye XTerminator Kit (Applied Biosystems), were commercially available.

Procedures

Primer design of protease-encode gene

The forward and reverse primers were designed using the gene sequence encoding the LAB protease enzyme obtained from GenBank (http://www.ncbi.nlm.nih.gov) using the online Primer-BLAST software targeted whole open reading frame (ORF) of the gene. The primary parameters used for primer designing were as follows: melting temperature (Tm) of the forward and reverse primers is above 52 °C, with the GC content is set to more than 50%. The selected designed PCR primers used were synthesized at IDT DNA, Singapore.

Amplification of the protease gene

The bacterium was grown in liquid nutrient broth at 37 °C for 24 h. Then, the bacterium cell was harvested by centrifugation at 12,000 rpm for 3 min. The *L. mesenteroides* K7 bacterium genomic DNA was isolated using the modified Sambrook's phenol-chloroform-isoamyl alcohol isolation method (Raharjo et al. 2018). The isolated DNA used as the template for the PCR amplification process using the synthesized primers. The total 25 µL PCR mix consisted of 2 µL Illustra™ Ready-To-Go PCR beads, 2 µL (50 ng) bacterium DNA, 1 µL (10 pmol) each forward and reversed primers and 19 µL nuclease-free water was prepared in PCR tube. Subsequently, the PCR reaction was carried out under the following conditions. In essence, initial denaturation at 95 °C for 5 min, followed by 35 cycles amplification consisted of denaturation step at 95 °C for 1 min, annealing step at 50 °C for 1.5 min, elongation step at 72 °C for 1 min, and final elongation for 10 min at 72 °C. PCR product fragment from the amplification was analyzed on the electrophoresis gel agarose. The PCR product was sequenced at IDT DNA Singapore. The fragment was initially cloned into a pGEM-T vector and the positive clone was sequenced using BigDye XTerminator Kit and Applied Biosystems 3500 Genetic Analyzer equipment. The sequence of the PCR fragment was identified using the BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Once the sequence revealed an open reading frame (ORF) was searched, and the encoded amino acid sequence was deducted.

Three-dimensional (3D) structure modeling of protease

Homology analysis is conducted to identify the conserved areas of the amino acid sequence of the protease that was performed using the ConSeq online server (http://consurf.tau.ac.il/). Meanwhile, the phylogenetic tree of the *L. mesenteroides* K7 protease with other protease was performed using Molecular Evolutionary Genetic Analysis (MEGA-X). Afterward, the UniRef90 database was analyzed to determine the similarity of a homologous amino acid sequence from the alignment. Besides, the default parameter score of conservation was calculated with the Bayesian method using Multiple Alignment using Fast Fourier Transform (https://www.ebi.ac.uk/Tools/msa/mafft/) via Multiple Sequence Alignment (MSA). Finally, to determine the 3D secondary and tertiary structures, the structure of amino acid protease was aligned using the SWISS-MODEL online server (https://swissmodel.expasy.org) (Biasini et al. 2014).

RESULTS AND DISCUSSION

Characterization of the protease-encoding gene

The primer for PCR aimed to amplify protease gen of *L. mesenteroides* strain K7, were designed using the sequence of protease genes reported from other LAB, which are available at the GenBank database Accession number: CP003101.3 region: 500207-500941; CP013016.1 region: 500559-501293; CP015442.1 region: 532157-532891) (https://www.ncbi.nlm.nih.gov/nuccore/CP003101.3;https://www.ncbi.nlm.nih.gov/nuccore/CP013016.1; https://www.ncbi.nlm.nih.gov/nuccore/CP015442.1). Base on the series of homology analysis including BLAST and Clustering, the selected primers, ProLK7F (5′-ATGGTACAATCAGCATTAAC-3′) and ProLK7R (5′-TTAGCTTGCTAATTTCGTTG-3′) were synthesized. This primer covers a complete open reading frame of the gene; therefore whole part of the protease of the gen was expected as a product of amplification. The PCR amplification has successfully amplified a single fragment with a size of approximately 1,200 bp (Figure 1).

The results of the sequencing of PCR fragments, from pGEMT clon, showed that PCR fragments contained 1140 bp, confirm with the size resulted by electrophoresis. The PCR fragment proved to be the expected fragment as the results of a query on PCR fragments revealed the existence of an ORF gene sequence, as shown in Figure 2.A. To ascertain whether the gene encodes the protease gene, a deduction of the amino acid sequence encoded by that gene. The amino acid sequence that was revealed (Figure 2.B) then analyzed by Blasting resulted in high similarity to

the protease of *Leuconostoc suionicum* (98.60%) (AP017935.1), *Leuconostoc gasicomitatum* (74.21%) (FN822744.1), *Leuconostoc gelidum* (74.11%) (CP017197.1), and *Leuconostoc lactis* (88.24%) (CP004884.1). Figure 3 showed the alignment between amino acid sequence of the amplified gene to the amino acid sequence of the protease of *Leuconostoc suionicum*, which shares the highest similarity. This result confirms that the PCR successfully amplify the targeted gene.

The approach of protease gene characterization employing PCR using gene-specific primers is common for a prokaryotic organism such as bacterial (Bach et al. 1999; Kaur et al. 2012; Suberu et al. 2019; Hou et al. 2020). The characterization of the protease gene of *L. pseudomesenteroides* K7 strain was helped by the fact that several genome of other *L. pseudomesenteroides* strain as well other species of Leuconostoc have been reported. Traditionally characterization of complete gene sequences employs several PCRs step using gene-specific primers. However since the similarity among protease sequence of Leuconostoc are relatively high in both starting position of ORF and sequence at stop codon, therefore both sequence can be used as gene-specific primer leading to amplification of whole gene.

Modeling of *L. mesenteroides* K7 protease

The microbial protease gene family has different capabilities and mechanisms of action as hydrolysis enzymes in different families leading to different properties and giving a benefit on a variety of application possibilities. To obtain structural insights of the *L. mesenteroides* protease into the alignment of many amino acid sequences in biologically important protein residues, multiple sequence alignment (MSA) was conducted using the ConSeq online server (http://consurf.tau.ac.il/2016/).

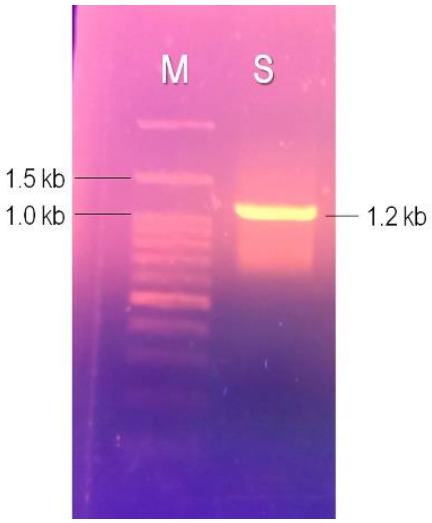

Figure 1. Gel agarose electrophoresis result of PCR amplification of *Leuconostoc mesenteroides* strain K7 using ProLK7F and ProLK7R primer. M: DNA ladder (marker); S = sample of PCR product.

ATGGTACAATCAGCATTAACAAAATTATGGTTAACTGGCGTCATTGCTGGTGTTGTTGGTGGAGGAGCAATTTTGTATGGGCAACAAGGTG
TACAATTGTTACAAAATCAAAATCAAAAAGTAAGTACGACAGCAACAAGTACAAAAACGATTGCCAAGAATGCAACCGCTACATCAGCTTA
TAATAAGGTATCAGATGCAGTTGTATCTGTATTAAACTTTACTAAATCTAGTCAAGGTAGTTATCAAGAATCGTCAGAAGGTTCTGGCGTG
ATCTATAAAAAAACAGATGGATCGGCATTTATTGTAACGAATAATCACGTCATCACTGGAGCGGCTAAAATTCAAGTTATGCTGCACAGTG
GGAAGAAAGTGACCGCTACACTAGTCGGCAAAGATGCGATGACTGACTTAGCTGTTCTGAAAATCGACGGCACAGACGTTACGACAACTGC
ACAATTTGGCGATTCAAGTAAAATAACTGTTGGTGAAAACGTCTTGGCAATTGGTTCGCCGCTGGGATCTGAGTATGCTTCGTCAGTGACG
CAAGGTATCATTTCTGCCAAAAAAAGGTTAGTTGAGACGACATCTGAAAATGGGCAGAATTATGGTGGATCCACGGTTATTCAGACGGATG
CTGCCATTAACCCTGGTAACTCAGGGGGACCATTAATTAATTTTGCAGGACAAGTTATTGGTATTAACTCAATGAAGTTATCAACATCTTC
TTCGGGAACGAGCGTTGAGGGGATGGGTTTTGCAATTCCTTCTGATCAAGTCGTTGACATTGTTAACAAATTAGTCAAAAATGGTAAGGTC
ACACGACCAGCAATCGGTATTAGCTTGATTAATTTGTCGGAAGTAACAGCTTCTGAGCAAAAATCAACTTTGAAAATTCCAGATAGTGTTA
CTGGTGGTGTTGTAGTAATGAGTTTGACTAACAATGGCCCAGCGGATAAAGCTGGATTGAAAAAGTACGATGTTATCGTTGGCATTAATGG
TAAAAAGGTAAGTTCACAGGCTGATCTGCGAGAAGAACGGTACAAGAATTCGCTTGGGGACCACATAACATTGACTTATTATCATCGGGAT
ACTAAGAAGACAGTCCAGGGTAAATTAACACCGAATTTACCAAGCTAA

(a)

MVQSALTKLWLTGVIAGVVGGGAILYGQQGVQLLQNQNQKVSTTATSTKTIAKNATATSAYNKVSDAVVSVLNFTKSSQGSYQESSEGSGV
IYKKTDGSAFIVTNNHVITGAAKIQVMLHSGKKVTATLVGKDAMTDLAVLKIDGTDVTTTAQFGDSSKITVGENVLAIGSPLGSEYASSVT
QGIISAKKRLVETTSENGQNYGGSTVIQTDAAINPGNSGGPLINFAGQVIGINSMKLSTSSSGTSVEGMGFAIPSDQVVDIVNKLVKNGKV
TRPAIGISLINLSEVTASEQKSTLKIPDSVTGGVVVMSLTNNGPADKAGLKKYDVIVGINGKKVSSQADLREERYKNSLGDHITLTYYHRD
TKKTVQGKLTPNLPS

(b)

Figure 2. The sequence information on the DNA fragment resulted from protease targeted PCR amplification of *L. mesenteroides* K7. (A) nucleotide sequence (B) deducted amino acid sequence.

```
Score: 747.1 bits (747.1),                    Expect: 1.1e-221
Identities: 367/378 (97%)                     Positives: 369/378 (97%)

Query : 1      Mvqsaltklwltgviagvvgggailygqqgvqllqnqnqkvsttatstktiaknatatsa  60
Sbjct : 1      MVQSALTKLWLTGVIAGVVGGGAILYGQQGVQLLQNQNQKVSTTATSTKTIAKNATATSA  60
Query : 61     ynkvsdavvsvlnftkssqgsyqessegsgviykktdgsafivtnnhvitgaakiqvmlh 120
Sbjct : 61     YNKVSDAVVSVLNFTKSSQGSYQESSEGSGVIYKKTDGSAFIVTNNHVITGAAKIQVMLH 120
Query : 121    sgkkvtatlvgkdamtdlavlkidgtdvtttaqfgdsskitvgenvlaigsplgseyass 180
Sbjct : 121    SGKKVTATLVGKDAMTDLAVLKIDGTDVTTTAQFGDSSKITVGENVLAIGSPLGSEYASS 180
Query : 181    vtqgiisakkrlvettsengqnyggstviqtdaainpgnsggplinfagqviginsmkls 240
Sbjct : 181    VTQGIISAKKRLVETTSENGQNYGGSTVIQTDAAINPGNSGGPLINFAGQVIGINSMKLS 240
Query : 241    tsssgtsvegmgfaipsdqvvdivnklvkngkvtrpaigislinlsevtaseqkstlkip 300
Sbjct : 241    TSSSGTSVEGMGFAIPSDQVVDIVNKLVKNGKVTRPAIGISLINLSEVTASEQKSTLKIP 300
Query : 301    dsvtggvvvmsltnngpadkaglkkydvivgingkkvssqadlreeryknslgdhitlty 360
Sbjct : 301    DSVTGGVVVMSLTNNGPADKAGLKKYDVIVGINGKKVSSQADLREERYKNSLGDHITLTY 360
Query : 361    yhrdtkktvqgkltpnlp   378
Sbjct : 361    YHRDTKKTVQGKLTPNLP   378
```

Figure 3. The top ranks of the sequence with the highest similarity to the amino acid sequence are reduced from the sequence of DNA fragment nucleotides from PCR amplification targeting the *L. mesenteroides* K7 protease gene. Query corresponds to the sequence obtained from *L. Mesenteroides* K7. Subject corresponds to the sequence found in the database.

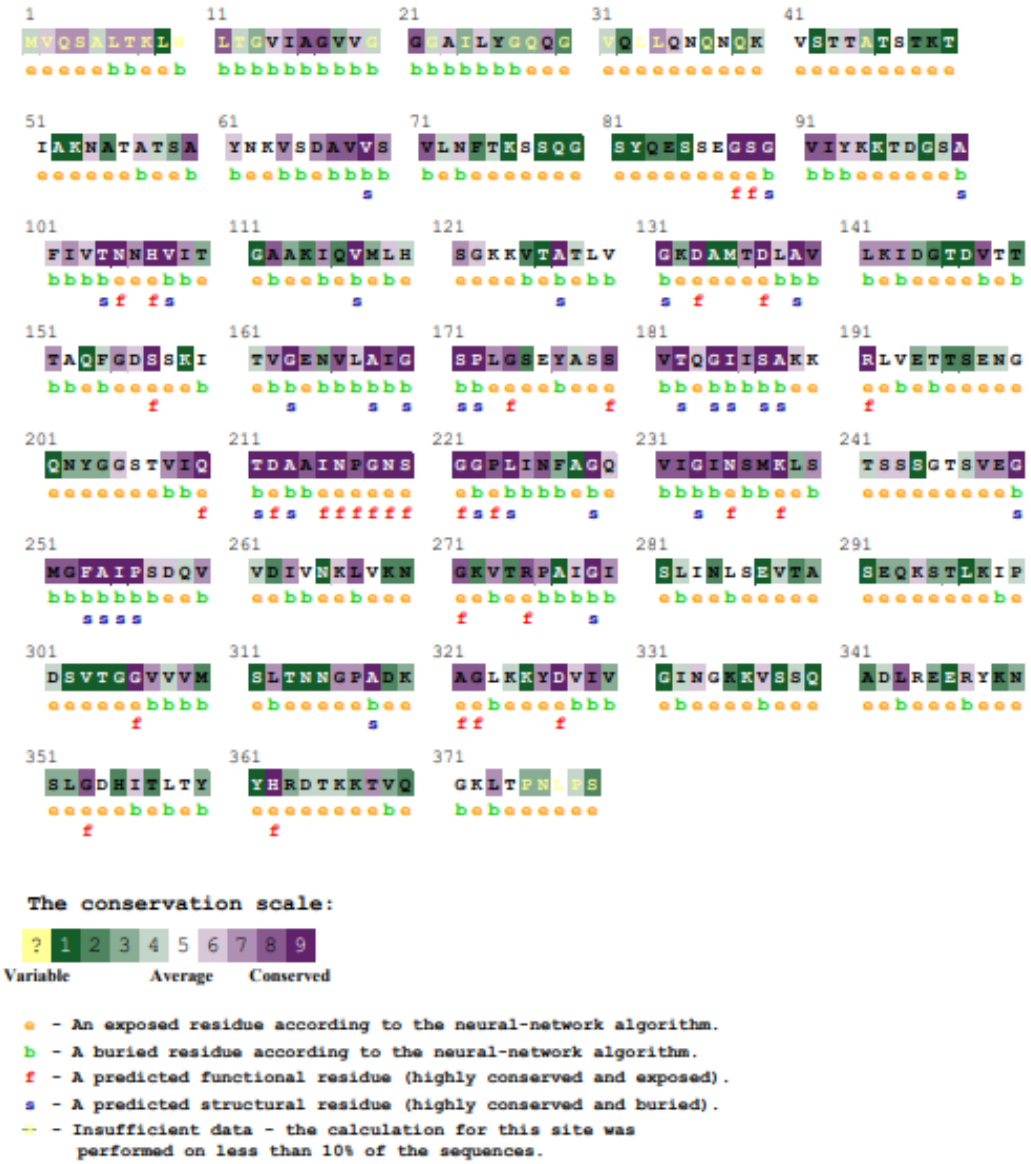

Figure 4. The output of the ConSeq MSA online server of the *L. mesenteroides* K7 protease

The MSA output shows that a well-known conserved region exists in every protease gene sequence. Figure 4 shows that a total of 379 amino acids can be obtained from the protease *L. mesenteroides* K2 protease gene. Among these amino acids, 30 amino acids which are denoted by the purple color, are the predicted functional residues, and that of amino acid with f code has highly conserved and exposed properties with the active side outside when it reacts with enzyme. By contrast, 32 amino acids are the predicted structural residues, which mean that the s code has highly conserved and buried properties with the active side inside when it reacts with enzyme. If the environment is water, then f is hydrophilic and forms hydrogen bonding networks, which contribute to the stabilization of the 3D structure, whereas s is hydrophobic. Three sustainable areas consisting of QTDA, INPGNSGGPL, and FAIP are detected were predicted to be responsible for the hydrolytic activity (Watanabe and Hayano 1994; Poliana and MacCabe 2007). Moreover, the sequence of INPGNSGGPL contains amino acid serine at position 220. Many reports suggested that hydrolase activity of protease determine by serine residue (Jeong et al. 2018; Matkawala et al. 2019; Nazari & Mehrabi 2019; Ji et al. 2020). This conclusion supported by the homology-based on the phylogenetic tree (Figure 5) that shows protease of *L. mesenteroides* K7, named as "sample" at Fig. 5, closely related to serine protease.

Prediction and 3D protein structure modeling are conducted after the active side identification on the basis of the MSA results using the ConSeq online server. During prediction and 3D protein structure modeling, the amino acid sequence data are inputted in the same manner as the results of modeling predictions into a new modeling project table (https://swiss model.expasy.org/interactive) obtained from the SWISS-MODEL online server (https://swissmodel.expasy.org/). The output is shown in Figure 6 as the secondary structure of each model generated by the software. Stabilization of the 3D structure can be achieved using the MSA output, which showed that the residues present in the protein N and C ends are highly exposed and can interact with the aqueous environment to form hydrogen bonding networks.

The 3D modeling predictions and sequential identification results are obtained using the SWISS-MODEL template library (SMTL) for target protein whose evolution-related structures match the target sequence of the 3qo6.1.A template (Table 1) because this template has the highest quality of identification and prediction from the alignment process template-target. A total of 656 templates are identified from the search using the SMTL server. Identification and prediction between protease of *L. mesenteroides* K7 (target protein) with the templates Protease Do-like 1 chloroplastic, which is a serine protease, show highest similarity with a level of similarity of 37.62%. This finding can be attributed to the fact that each of the identified templates that has the best prediction quality can be used to determine the target protein sequence.

The 3D structure of the target protein model transferred by homology to the SWISS-MODEL template library (SMTL) server (Figure 7) includes oligo-state properties, namely homo hexatrimer with target protein structure model resolution of 2.50 Å meaning the distance between protons in target proteins are very closed and always interact with each other because the resolution limits for each protein are around 1-2 Å. Moreover, the atomic structures of 289 proteins have been revealed. The detailed molecular structure illustrates how proteins are recognized and bind with other molecules and how they function as enzymes. Our understanding of this phenomenon will continue to grow with the discovery of a new protease gene from *L. mesenteroides* K7 bacteria isolated from Kelengkeng fruit.

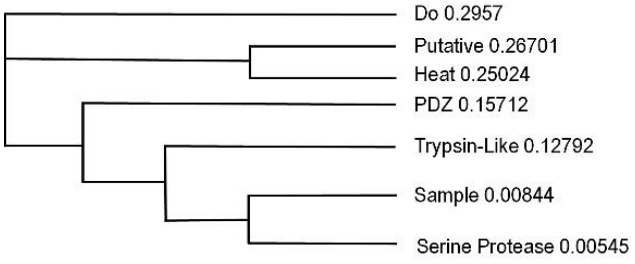

Figure 5. Phylogenetic tree of *L. mesenteroides* K7 protease to the other proteases

Table 1. Identification and prediction from the alignment process template-target

Template	Sequence identity	Oligomeric state	QSQE	Found by	Method	Resolution	Sequence similarity	Coverage	Description
3qo6.1.A	37.62	Homohexamer	0.24	HHblits	X-ray	2.50 Å	0.37	0.80	Protease Do-like 1 chloroplastic
4yo1.1.A	35.29	Homotrimer	0.53	HHblits	X-ray	2.80 Å	0.36	0.81	DegQ
5jd8.1.A	33.98	Homotrimer	0.55	HHblits	X-ray	1.85 Å	0.36	0.82	Periplasmic serine protease DegS
5tny.1.A	31.13	Homotrimer	0.44	HHblits	X-ray	1.70 Å	0.35	0.80	Serine protease HTRA2, mitochondrial

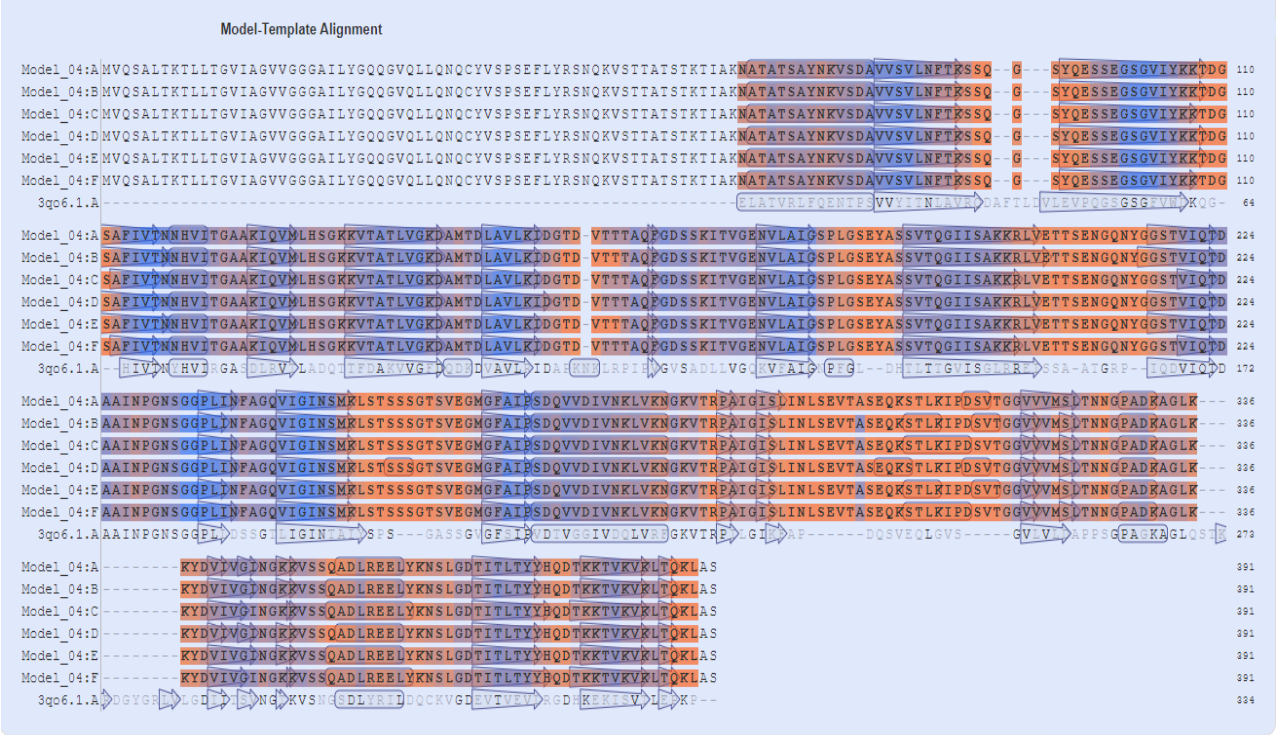

Figure 6. Secondary structure alignment between protease models and template (PDB ID: 3qo6.1.A) using the SWISS-MODEL online server

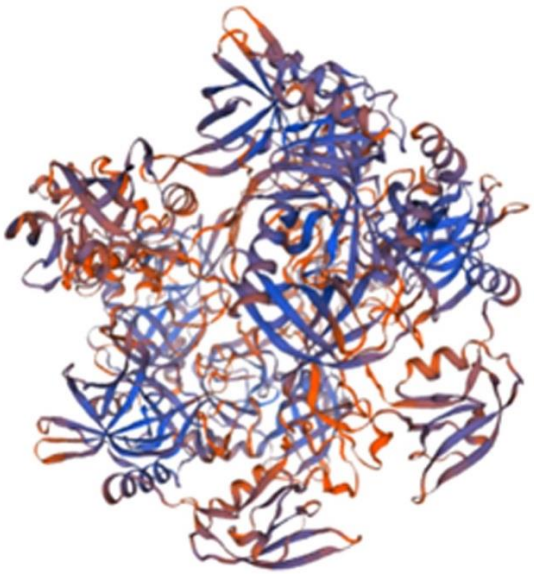

Figure 7. Three-dimensional (3D) prediction model of *L. mesenteroides* K7 protease

In conclusion, characterization of the protease gene from the new *L. mesenteroides* strain K7 bacterium from Kelengkeng fruit revealed a gene fragment length of 1,140 bp with deduced amino acid sequence has 98.60% similarity to *Leuconostoc suionicum* protease. 3D structure modeling of *L. mesenteroides* strain K7 protease obtained identification and prediction results that showed that the level of similarity between target proteins with the templates *Protease Do-like, chloroplastic*, as *Serine protease* and its homolog protein *Serine endoprotease DegS* family is 37.62%.

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