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A Meat-Derived Lactic Acid Bacteria, *Lactobacillus plantarum* IIA, Expresses a Functional Parvulin-Like Protein with Unique Structural Property

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Abstract: The genome sequence of a Lactic Acid Bacterium (LAB) *Lactobacillus plantarum* IIA contains a single gene encoding a parvulin-like protein (Par-LpIIA). This protein belongs to Peptidyl Prolyl *cis-trans* Isomerase (PPIase) family proteins that catalyze a slow *cis-trans* isomerization of *cis* prolyl bond during protein folding. This study aims to provide molecular and biochemical evidences of the existence of Par-LpIIA in *L. plantarum* IIA and have an insight into its structural properties. The result showed that the gene encoding Par-LpIIA was successfully amplified using specific primers yielding a ~900 bp amplicon indicating that the gene indeed exists in its genomic DNA. BLAST analysis confirmed that the protein is a rotamase of parvulin-like protein. Further biochemical analysis demonstrated that cell lysate of *L. plantarum* IIA-1A5 exhibited remarkable PPIase activity towards peptide substrate and ability to accelerate the refolding of RNase T1, with the catalytic efficiency (k_{cat}/K_M) of 1.9 and 0.02 μM^{-1} s^{-1}, respectively. A specific inhibitor clearly inhibited the PPIase activity for parvulin-like protein with IC_{50} of 230 nM confirming that the protein encoded by Par-LpIIA gene is a parvulin-like protein and expressed in an active form. Further, the three-dimensional model of Par-LpIIA showed that this protein consists of two domains of a homolog WW domain and PPIase domain with a unique active site configuration compared to human Pin1. Altogether, we then proposed the possible roles of this protein for *L. plantarum* IIA.

Keywords: Peptidyl Prolyl *cis-trans* Isomerase, *Lactobacillus plantarum*, Parvulin, Active Site, Structural Homology Modelling

Introduction

Peptidyl Prolyl *cis-trans* Isomerase (PPIase) is a group of an enzyme catalyzing a slow *cis-trans* isomerization of the Xaa-Pro peptide bonds during protein folding (Lu *et al*., 1996; Lang *et al*., 1987; Stifani, 2018; Rostam *et al*., 2015; López-Martínez *et al*., 2016). This isomerization is intrinsically slow and regarded as a rate-limiting step of a folding reaction due to the high energy barrier caused by the partial double-bond character of the peptide bond (Bhagavan and Ha, 2015; Fischer and Aumüller, 2003; Chen *et al*., 2012). The involvement of PPIase in protein folding leads to consider this protein as a foldase (folding catalyst). Besides, some PPIase were also known to exhibit chaperone due to the ability to prevent protein aggregation and bind to a folding intermediate protein (Budiman *et al*., 2011).

Parvulin is the third family of PPIase, besides FK506-Binding Proteins (FKBPs), cyclophilin (Chen *et al*., 2018; Matena *et al*., 2018). In contrast to FKBPs and Cyp, Par has no affinity to the immunosuppressant, FK506 or CsA (Matena *et al*., 2018; Tuccinardi and Tizzolio, 2019; Barik, 2006; Romano *et al*., 2004; Maruyama *et al*., 2004). Besides, while FKBPs and Cyp prefer unphosphorylated Xaa residue preceding proline, parvulin specifically catalyzes phosphorylated prolyl bond

(phospho-Ser-Pro or phospho-Thr-Pro (Matena *et al*., 2018; Rostam *et al*., 2015). A prominent parvulin is the mitotic regulator Pin1 (Chen *et al*., 2018; Rostam *et al*., 2015) involved in cell cycle regulation, protein folding disorders such as Alzheimer's or Parkinson's disease (Mueller and Bayer, 2008; Nakatsu *et al*., 2011) and cancer (Lee and Liou, 2018).

Parvulin was found in prokaryote or eukaryote (Rostam *et al*., 2015; Nakatsu *et al*., 2011) and structurally consist of PPIase domain, responsible for catalysis and additional domain(s) which thought to be important for binding to protein substrates/partners (Maruyama *et al*., 2004). While the PPIase domain is structurally conserved among Par family members, the additional domain was found to be more variable. Generally, prokaryotic parvulins have a chaperon-like activity and eukaryotic parvulins have been linked to several aspects of gene regulation and cell cycle progression. The PPIase domain of parvulin is characterized by the presence of conserved amino acids like histidine, isoleucine and leucine (Fanghänel and Fischer, 2004; Lu, 2003; Rippmann *et al*., 2000; Shaw, 2002).

Lactobacillus plantarum IIA is a Gram-positive of Lactic Acid Bacteria (LAB) isolated from beef that displays some probiotic characteristics (Arief *et al*., 2015). Like other LAB, *L. plantarum* IIA is promising for some applications including as a starter for food fermentation or host cells for the production of bioactive compounds. The genome sequence of this bacterium (will be published somewhere else) revealed a single gene encoding a member of the third family of PPIase, parvulin, and designated as Par-LpIIA. The presence of a PPIase member in *L. plantarum* IIA is interesting since it might imply the essential role of this PPIase in the cellular event of Par-LpIIA. Besides, to our knowledge, only a few parvulin members were so far reported with no structural report on this protein. To note, the study on the functionality of PPIase from LAB are limited. Some reports indicated the presence of genes encoding cyclophilin-like and FKBP-like proteins in *L. helveticus* and *L. lactis*, respectively (Broadbent *et al*., 2011; Trémillon *et al*., 2012; Bolotin *et al*., 2001). Further, the *prsA*-like gene of *L. lactis* was also reported to encode a protein with PPIase motif and possibly be involved in the protein maturation and secretion of this bacterium (Drouault *et al*., 2002). Nevertheless, no report so far for the parvulin-like protein in LAB. In this study, we confirmed the evidence of Par-LpIIA existence through molecular and biochemical approaches. The three-dimensional model of Par-LpIIA was also built under structural homology modeling which provides an insight into structural features related to a catalytic property of this protein. To note, this is the first structural report of parvulin-like protein from LAB family.

Methods

Gene Amplification

Genomic DNA of *L. plantarum* IIA was extracted using DNA QIAamp genomic DNA kits (Qiagen, USA) according to manufacturer protocol. The genomic DNA was then used as a template for gene amplification. Amplification was performed using Polymerase Chain Reaction (PCR) with KOD FX Neo PCR kit (Toyobo, Japan) according to the manufacturer's protocol with slight modifications. The primers used for the amplification were 5-ATGAAGAAAAAAATGCGCCTTAAAGTATTATTG G-3 (forward) and 5-TTAATTCGTTGTCGCAAGCTTCTTATAACTATC-3 (reverse). PCR product was then separated under 1% agarose gel and visualized under ethidium bromide staining. The amplicon migrated at expected sized was then excised and extracted using The QIAquick Gel Extraction Kit. The purified DNA was sequenced using the Prism 310 DNA sequencer (Applied Biosystems) using the above primers. All oligonucleotides were synthesized by 1stBase DNA sequence service (Singapore).

DNA and Amino Acid Sequence Analysis

The DNA sequence obtained was subjected to BLAST analysis and translated to amino acid sequence using Expert Protein Analysis System (ExPASy) Translation Tool (https://web.expasy.org/translate/). The deduced amino acid sequence was then used for analysis using Protein Calculator v3.4 (http://protcalc.sourceforge.net) and used for the alignment with other parvulin members, whose the sequences were retrieved from the GeneBank. The alignment was performed using the ClustalW program at EBI (http://www.ebi.ac.uk/clustalw/) and PPIase domain sequence was identified based on the conservation region of PPIase domain of human Pin1, which was identified before (Fanghänel and Fischer, 2004).

PPIase Activity

For the assay, cell lysate of *L. plantarum* IIA-1A5 (CL-LpIIA) was firstly obtained from the sonication of *L. plantarum* IIA followed by ultracentrifugation at 35,000 g for 30 min. Measurements were carried out as described by (Uchida *et al*., 2003) using a WFY(pS)PR-pNA peptide substrate (Bachem, Heidelberg, Germany). Briefly, a 1 mL reaction mixture containing 0.45 $mg.mL^{-1}$ α-chymotrypsin and the protein of CL-LpIIA in 50 mM HEPES and 100 mM NaCl, pH 8.0, was pre-chilled to the measurement temperature (10, 15, 20 or 25°C) and then rapidly mixed into a cuvette containing the substrate WFY(pS)PR-pNA peptide substrate (Bachem, Heidelberg, Germany). The substrate was previously prepared by dissolving at concentration of 5 mM in 470

mM LiCl/trifluoroethanol (Kofron *et al*., 1991) as a stock concentration. The catalytic efficiency (kc_{at}/K_M) was calculated as described previously (Janowski *et al*., 1997). The activity at 10°C was adjusted as 100%. To determine the effect of pH on PPIase activity, the assay was also measured at 10°C in various buffer pH ranging from 2.0 to 12.0. The highest activity was adjusted as 100%.

Inhibition Studies

The WFY(pS)PR-pNA peptide substrate (Bachem, Heidelberg, Germany) was used in the measurements for inhibition studies. The inhibitors used in this experiment were FK506, Cyclosporine (CsA) and juglone (Sigma Aldrich, USA). Stock solutions of the inhibitors were prepared in 50% ethanol. The CL-LpIIA was firstly incubated with one of the inhibitor, at different concentrations, for 15 min at 10°C and then used for the PPIase activity assay as described above. The catalytic efficiency obtained from the assay without any inhibitor was adjusted to 100% activity according to Uchida *et al*. (2003) and Budiman *et al*. (2018).

Substrate Specificity

Substrate specificity was measured by measuring PPIase activity as described above using Suc-Ala-Xaa-Pro-Phe-NH-Np as a substrate obtained from Bachem (Heidelberg, Germany). Xaa represents a variable amino acyl residue in the P1 position of various oligopeptide substrates used for investigation of the substrate specificity. The catalytic efficiency obtained from Suc-Ala-Leu-Pro-Phe-NH-Np was adjusted to 100% activity according to (Uchida *et al*., 2003; Budiman *et al*., 2018).

Catalysis of Protein Folding

The assay was performed according to (Budiman *et al*., 2009; Wojtkiewicz *et al*., 2020; Uchida *et al*., 1999). Briefly, RNase T_1 (16 μM) (Funakoshi Co., Ltd., Tokyo, Japan) was first unfolded by incubating it in 20 mM sodium phosphate (pH 8.0) containing 0.1 mM EDTA and 6.2 M guanidine hydrochloride at 10°C overnight. Refolding was then initiated by diluting this solution 80-fold with 20 mM sodium phosphate (pH 8.0) containing 100 mM NaCl in the presence or absence of the CL-LpIIA. The final concentrations of RNase T_1 was 2 μM. The refolding reaction was monitored by measuring the increase in tryptophan fluorescence with an F-2000 spectrofluorometer (Hitachi High-Technologies Co.). The excitation and emission wavelengths were 295 and 323 nm, respectively and the band width was 10 nm. The refolding curves were analyzed with double exponential fit (Ramm and Pluckthun, 2000). The k_{cat}/K_M values were calculated from the relationship mentioned above, where k_p and k_n represent the first-order rate constants for the faster refolding phase of RNase T_1 in the presence and absence of the enzyme, respectively (Suzuki *et al*., 2004).

Structural Homology Modelling

The amino acid sequence of Par-LpIIA was subjected for comparative homology modelling via SWIS-MODEL Server (Schwede *et al*., 2003), 3DJIGSAW (Bates *et al*., 2001) and PHYRE2 server (Kelley and Sternberg, 2009). Finally, once the 3D structures were generated, model validations were performed. Backbone conformation of all models was evaluated by analysis of Psi/Phi Ramachandran plot using RAMPAGE program (Lovell *et al*., 2003). The overall stereochemical quality of the final developed model for each model was assessed by the program PROCHECK. G-factor was calculated for the developed model using PROCHECK. Environment profile of final developed model was checked using Verify-3D (Structure Evaluation Server).

The best model was then used for structural alignment to identify putative active site residues. Structural alignment of model structure of Par-LpIIA to human Par (PDB ID: 1 nmv) was performed in PyMol (https://pymol.org/2/). Active sites of human Par, which were identified according to (Fanghänel and Fischer, 2004), were manually aligned to corresponding residues model structure of Par-LpIIA. These corresponding residues were then considered as active sites of Par-LpIIA.

Results

Gene Amplification and Analysis

To confirm if the gene encoding Par-LpIIA really exists in the genome of *L. plantarum* IIA, Polymerase Chain Reaction (PCR) was performed using a series of primers designated based on the gene sequence. The amplicon corresponds to the apparent size of 900 bp was successfully obtained (Fig. 1a). Further, DNA sequence of the amplicon is also exactly matched with the sequence obtained from the whole genome sequence (Fig. 1b). This suggested that the genomic DNA of *L. plantarum* IIA indeed harbour a gene encoding Par-LpIIA. This also, to some extent, validated genome assembly, followed by annotation, of this strain.

The full-length DNA sequence of Par-LpIIA encodes a polypeptide of 299 amino acids with a predicted molecular mass of 33.14 kDa and a theoretical isoelectric point of 9.57 (Fig. 2a). This size is considerably higher to that of the average size of the PPIase domain of parvulin members. BLAST analysis of the whole amino acid sequence also displayed high similarities (>90%) to putative PPIase from *Lactobacillus casei* BL23, *Lactobacillus acidophilus* NCFM, *Lactobacillus gasseri* ATCC 33323, *Lactobacillus casei* BL23, *Pediococcus pentosaceus* ATCC 25745, *Lactobacillus plantarum*

WCFS1, *Lactobacillus delbrueckii* subsp. *Bulgaricus* ATCC 1842, *Lactobacillus brevis* ATCC 367, *Lactobacillus reuteri* JCM 1112, *Lactobacillus fermentum* IFO 3956, *Listeria monocytogenes* EGD-e, *Bacillus anthracis* str. 'Ames Ancestor', *Listeria monocytogenes* EGD-e, *Leuconostoc mesenteroides* subsp. *Mesenteroides* ATCC 829. BLAST also revealed that the sequence contains a so-called Rotamase-2 superfamily domain that spans from His150 to Lys232 and was predicted as a PPIase domain. The other residues are predicted to be organized as a non-PPIase domain. Multiple amino acid sequence alignment of Par-LpIIA with other well-studied parvulin is shown in Fig. 2b. The amino acid sequence of Par-LpIIA showed 21.15, 22.22 and 25.58% similarities to human Pin1, Par14 and *E. coli* Parvulin (Eco-Par), respectively. Meanwhile, it shows very high similarity (>99%) to parvulin of *L. casei* and *L. paracesi*.

PPIase Activity, Inhibition and Specificity

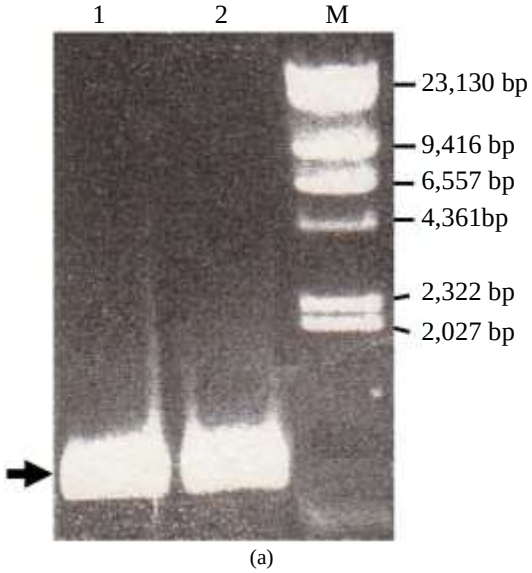
Although it was confirmed that the gene of Par-LpIIA exists, this protein's functionality remains to be confirmed. The functionality refers to the ability of Par-LpIIA to exhibit specific catalytic activity and bind or inhibited by specific inhibitors. In this study, specific PPIase activity of Par-LpIIA in CL-LpIIA was determined using a protease-coupling method, in which the enzyme catalyzes the slow isomerization of Ser-Pro bond of the substrate followed by the releasing of pNA moiety when the substrate was cleaved by chymotrypsin upon the rotation of Ser-Pro bond to *trans* configuration. The result showed that k_{cat}/K_M of apparent PPIase activity of CL-LpIIA was calculated to be 9.8 μM^{-1} s^{-1} (Fig. 3a). Nevertheless, it remains to be confirmed if the activity of CL-LpIIA is originated from Par-LpIIA or other types of isomerase. To confirm, the catalytic activity was also measured in the presence of a specific inhibitor for FKBP (FK506) and Cyclosporine (CsA). None of the inhibitors were able to remarkably inhibit the catalytic activity of the cell lysate (Fig. 3b). Further, when a specific inhibitor of parvulin (juglone) was used in the measurement, it showed a reduction of PPIase activity of CL-LpIIA with an IC_{50} value of about 200 nM (Fig. 3b).

Further, the temperature dependency of the PPIase activity of CL-LpIIA showed that the activity increased as the reaction temperature increased from 10 to 25°C (Fig. 4a). The PPIase activity was not measured at temperatures higher than 30°C, because the rate for spontaneous prolyl isomerization reaction was too high to determine those catalyzed by PPIases accurately. Further, Fig. 4b showed that the optimum pH of PPIase activity of CL-LpIIA was observed at pH 5.0.

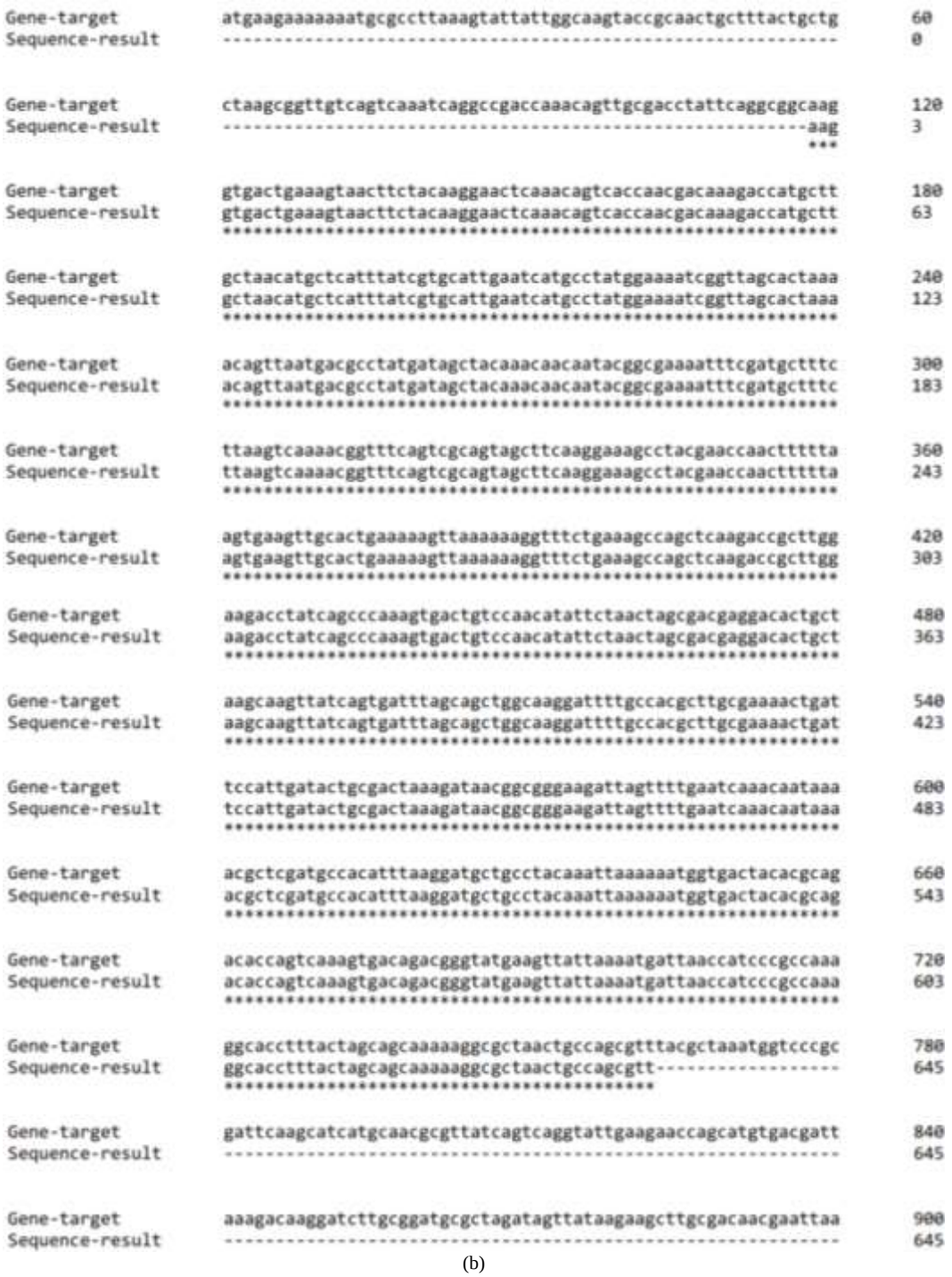
The substrate specificity of CL-LpIIA was shown in Fig. 5. In this study, seven (7) variants of the tetrapeptide substrates were used by replacing Xaa to Leu or Ala or Phe or Gln or Arg or Lys or His. All these residues have differences in polarity and structural bulkiness that might affect the fitting into the binding pocket. Figure 5 showed the preference of Par-LpIIA towards Xaa at P1 position followed the order of Leu>Arg>Gln>Ala>Lys>Phe>His.

Catalysis of Protein Folding

Figure 6 showed the refolding course of RNase T1 in the absence or in the presence of CL-LpIIA. RNase T1 is known to have two prolyl bonds two peptidyl-prolyl bonds (Tyr38-Pro39 and Ser54-Pro55) and its refolding rate is limited by the *cis-trans* isomerization of these bonds (Kiefhaber *et al*., 1990a; 1990b). This experiment was conducted to confirm if the cell lysate of LpIIA, which is assumed to contain Par-LpIIA, is able to accelerate the slow refolding of RNase T1.



(a)



(b)

Fig. 1: (a) Amplification of Par-LpIIA gene. Lane M corresponds to a λ DNA/HindIII Markers, while Lane 1 and 2 correspond to the Polymerase Chain Reaction (PCR) products of the gene from two independent reactions, (b) Comparison between DNA sequence of Par-LpIIA obtained from the sequencing of PCR product (Sequence-result) and that of from whole-genome sequencing (Gene-target)

The ability should refer to the presence of a functional Par-LpIIA as only PPIase protein is able to catalyze the slow refolding rate due to *cis*-prolyl bond isomerization. Figure 6 clearly showed that fluorescence intensity of the refolding of RNase T_1 in the presence of crude extract containing Par-LpIIA reached the maximum intensity faster

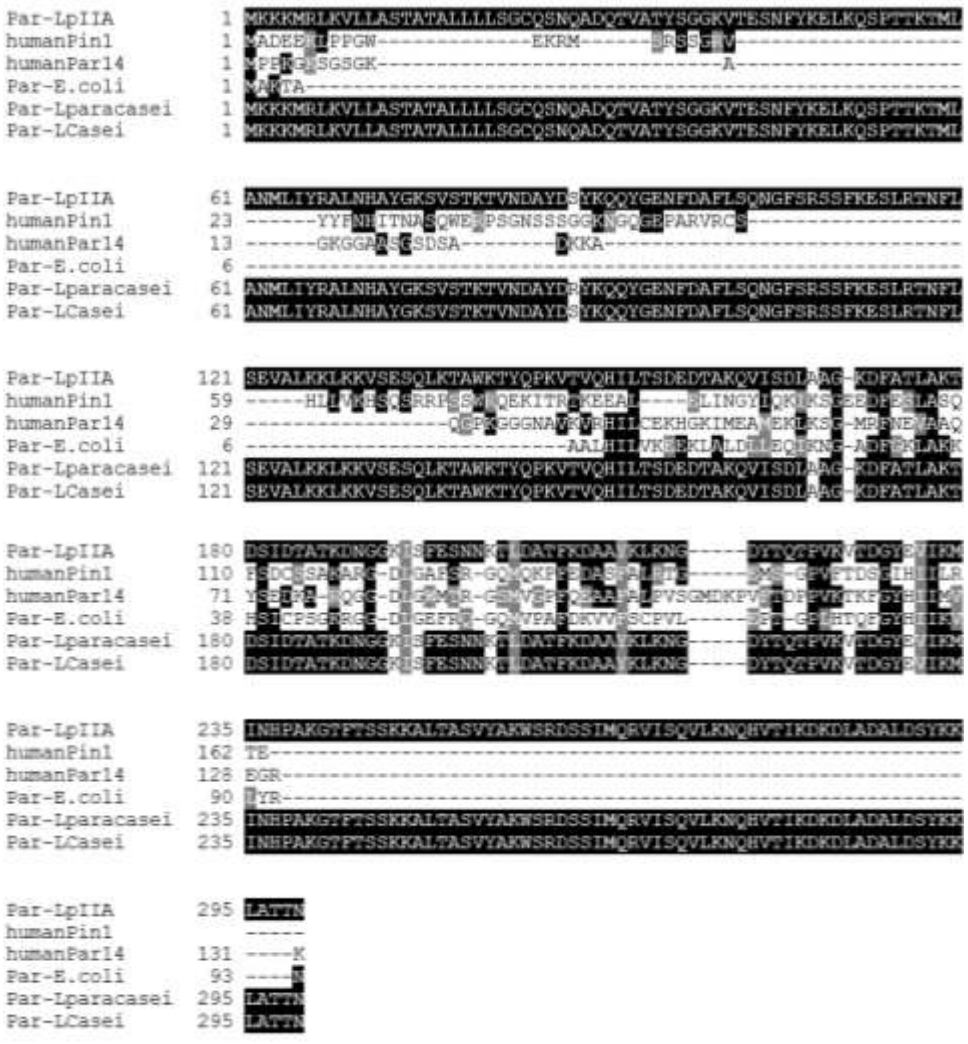
than that of in the absence of the crude extract. This clearly suggested that CL-LpIIA was able to facilitate the catalysis of an RNase T1 refolding course. To note, Fig. 6 also indicated that the catalysis of refolding by CL-LpIIA was a concentration-dependent manner with the calculated k_{cat}/K_M value of 0.02 μM^{-1} s^{-1}.

Three Dimensional Model of Par-LpIIA

Structural homology modeling of Par-LpIIA is unavoidable to obtain a comprehensive understanding of the functional mechanism of this PPIase member. Structural homology modeling under various platforms used in this study yielded 4 models that have similar overall structures with RMSD about 0.51 Å. However, structural validation through Ramachandran Plot revealed that the model from PHYRE2 server platform is more acceptable since it has fewer residues (<1%) in disallowed regions. Noteworthy, the secondary and model structure showed that these residues are located in the flexible regions hence caused the unique steric hindrance properties. The overall main-chain and side-chain parameters, as evaluated by PROCHECK, are all very favorable. No clash between residues of the model has also been identified in the viewer. Further validation under verify-3D also revealed that all residues are reasonably folded as indicated by zero compatibility score. Accordingly, we believed this model is acceptable for further analysis.

```
  1 atgaagaaaaaaatgcgccttaaagtattattggcaagtaccgcaactgctttactgctg 60
  1  M  K  K  K  M  R  L  K  V  L  L  A  S  T  A  T  A  L  L  L  20

 61 ctaagcggttgtcagtcaaatcaggccgaccaaacagttgcgacctattcaggcggcaag 120
 21  L  S  G  C  Q  S  N  Q  A  D  Q  T  V  A  T  Y  S  G  G  K  40

121 gtgactgaaagtaacttctacaaggaactcaaacagtcaccaacgacaaagaccatgctt 180
 41  V  T  E  S  N  F  Y  K  E  L  K  Q  S  P  T  T  K  T  M  L  60

181 gctaacatgctcatttatcgtgcattgaatcatgcctatggaaaatcggttagcactaaa 240
 61  A  N  M  L  I  Y  R  A  L  N  H  A  Y  G  K  S  V  S  T  K  80

241 acagttaatgacgcctatgatagctacaaacaacaatacggcgaaaatttcgatgctttc 300
 81  T  V  N  D  A  Y  D  S  Y  K  Q  Q  Y  G  E  N  F  D  A  F  100

301 ttaagtcaaaacggtttcagtcgcagtagcttcaaggaaagcctacgaaccaacttttta 360
101  L  S  Q  N  G  F  S  R  S  S  F  K  E  S  L  R  T  N  F  L  120

361 agtgaagttgcactgaaaaagttaaaaaaggtttctgaaagccagctcaagaccgcttgg 420
121  S  E  V  A  L  K  K  L  K  K  V  S  E  S  Q  L  K  T  A  W  140

421 aagacctatcagcccaaagtgactgtccaacatattctaactagcgacgaggacactgct 480
141  K  T  Y  Q  P  K  V  T  V  Q  H  I  L  T  S  D  E  D  T  A  160

481 aagcaagttatcagtgatttagcagctggcaaggattttgccacgcttgcgaaaactgat 540
161  K  Q  V  I  S  D  L  A  A  G  K  D  F  A  T  L  A  K  T  D  180

541 tccattgatactgcgactaaagataacggcgggaagattagttttgaatcaaacaataaa 600
181  S  I  D  T  A  T  K  D  N  G  G  K  I  S  F  E  S  N  N  K  200

601 acgctcgatgccacatttaaggatgctgcctacaaattaaaaaatggtgactacacgcag 660
201  T  L  D  A  T  F  K  D  A  A  Y  K  L  K  N  G  D  Y  T  Q  220

661 acaccagtcaaagtgacagacgggtatgaagttattaaaatgattaaccatcccgccaaa 720
221  T  P  V  K  V  T  D  G  Y  E  V  I  K  M  I  N  H  P  A  K  240

721 ggcacctttactagcagcaaaaaggcgctaactgccagcgtttacgctaaatggtcccgc 780
241  G  T  F  T  S  S  K  K  A  L  T  A  S  V  Y  A  K  W  S  R  260

781 gattcaagcatcatgcaacgcgttatcagtcaggtattgaagaaccagcatgtgacgatt 840
261  D  S  S  I  M  Q  R  V  I  S  Q  V  L  K  N  Q  H  V  T  I  280

841 aaagacaaggatcttgcggatgcgctagatagttataagaagcttgcgacaacgaattaa 900
281  K  D  K  D  L  A  D  A  L  D  S  Y  K  K  L  A  T  T  N  *  300
```

(a)



(b)

Fig. 2: (a) The amino acid sequence of Par-LpIIA *trans*lated from its DNA sequence, (b) Pairwise sequence alignment between Par-LpIIA with human parvulin 1 (human Pin1), human Parvulin 14 (human Par14), *E. coli* Parvulin 10 (Par-*E. coli*), *Lactobacillus paracasei* Parvulin (Par-Lparacasei) and *L. casei* Parvulin (Par-Lcasei)

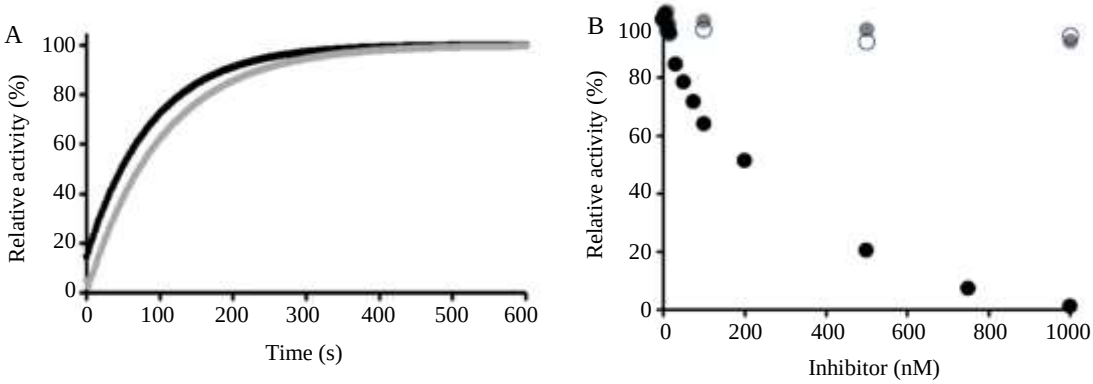


Fig. 3: (a) A representative of the time course of *cis-trans* prolyl bond isomerization of WFY(pS)PR-pNA peptide in the absence (grey solid line) and in the presence (black solid line) of 3.5 nM of CL-LpIIA, (b) The inhibition of apparent PPIase catalytic activity of CL-LpIIA by FK506 (open circle), cyclosporine (gray circle) and juglone (black circle)

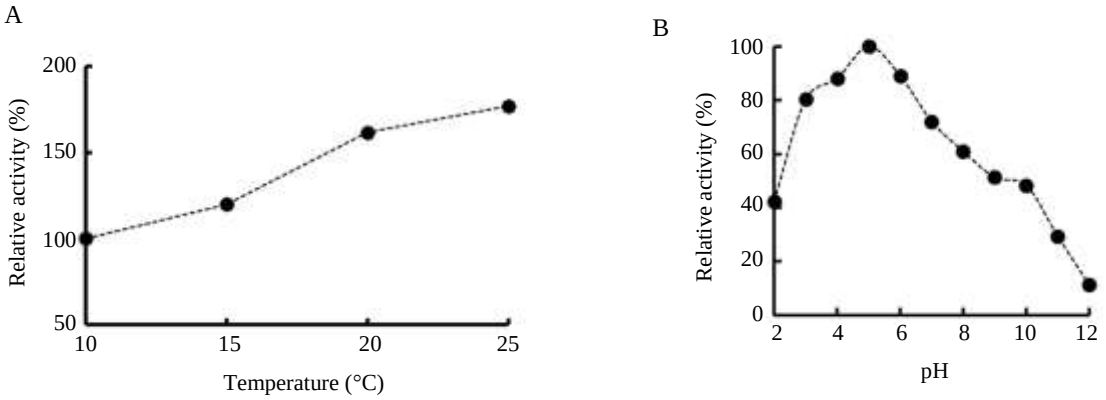


Fig. 4: (a) Temperature and, (b) pH-dependency activity of CL-LpIIA

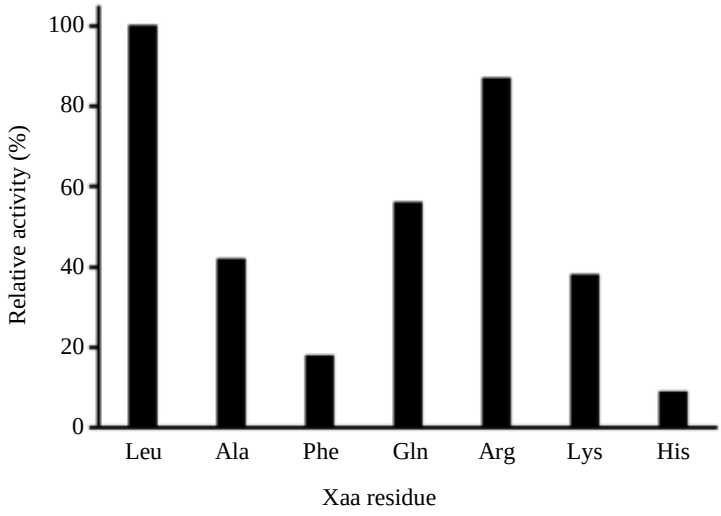


Fig. 5: PPIase catalytic activity of CL-LpIIA towards peptide substrate with the various amino acids at the Xaa position. The activity towards Suc-Ala-Leu-Pro-Phe-pNA was adjusted as 100%

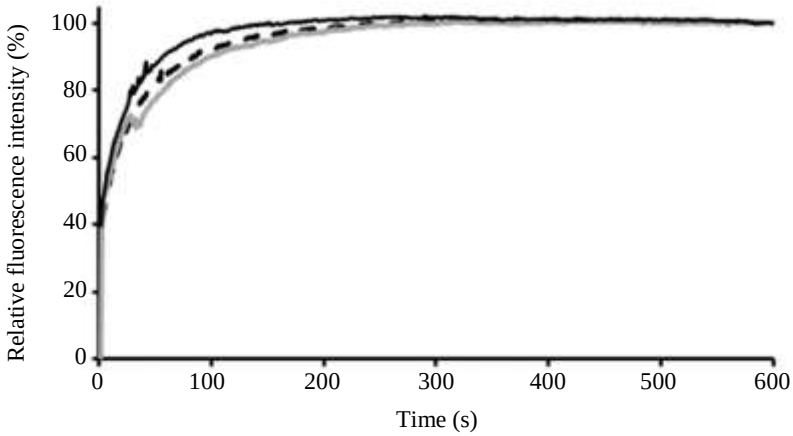
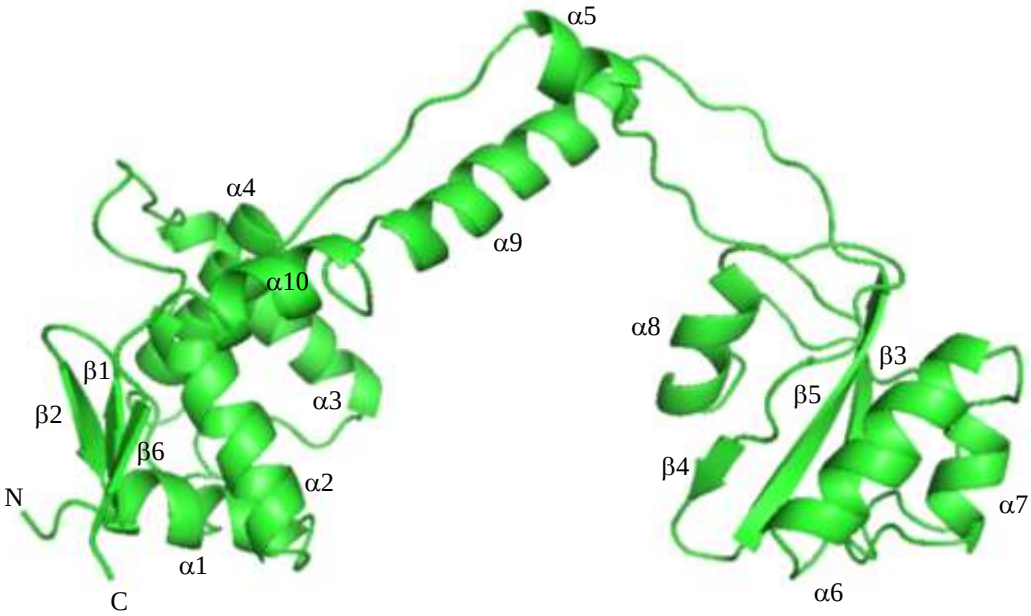
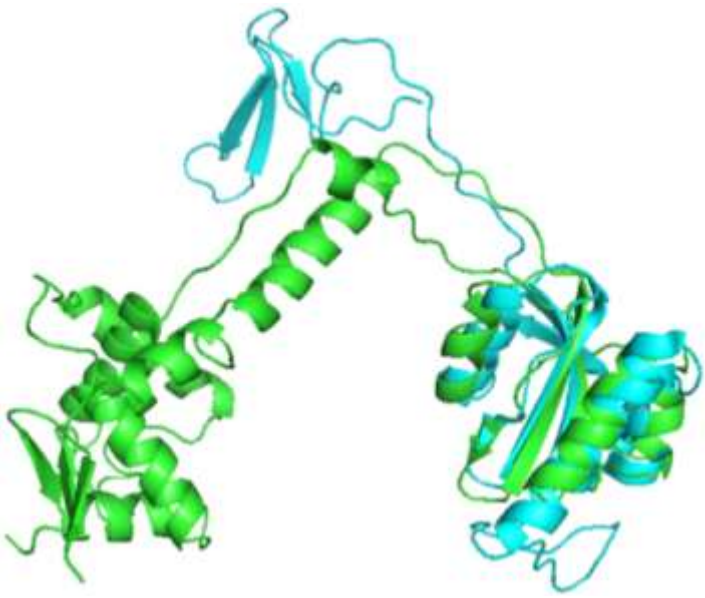


Fig. 6: Refolding course of RNase T_1 in the absence (grey solid line) or in the presence of 1 nM (black dashed line) or 10 nM (black solid line) of CL-LpIIA

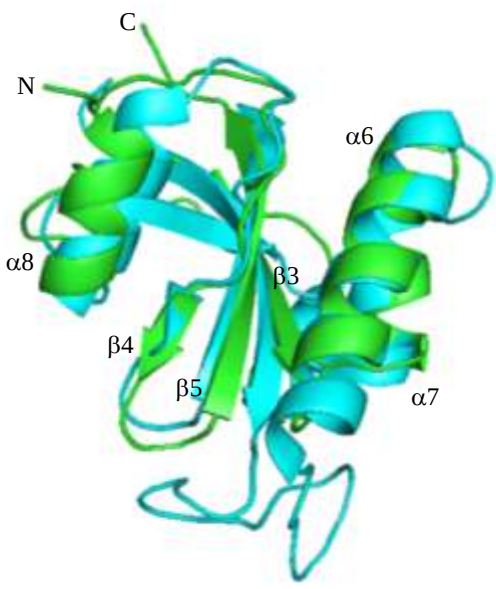
The overall structure of Par-LpIIA indicated that this protein folded into two separated domains of the WW and PPIase domains (Fig. 7a). The WW domain is formed by the residues of Met1-Lys126 and Phe243-Asp282. These residues organized into 3 β-sheets (β1, β2 and β6) and 6 α-helixes (α1, α2, α3, α4, α9 and α10). Meanwhile, the PPIase domain is formed by the residues of Pro145-Thr242 which organized into 3 β-sheets (β3, β4 and β5) and 3 α-helixes (α6, α7 and α8). The structural alignment with well-studied human Pin1 (PDB ID: 1NMV) showed yielded an RMSD of 2.42 Å which suggested that the two proteins were not really similar (Fig. 7b). The differences mainly found in the WW domain, whereby the homolog of WW domain of Par-LpIIA is shown to be remarkably folded into a non-globular shape and has a larger structure than that of Pin1. The difference in this domain might also explain the bigger theoretical size of Par-LpIIA than the other Par members. On the other side, PPIase domain of Par-LpIIA was shown to be similar to that of Pin1 with an RMSD of 0.81 Å (Fig. 7c).



(a)



(b)



(c)

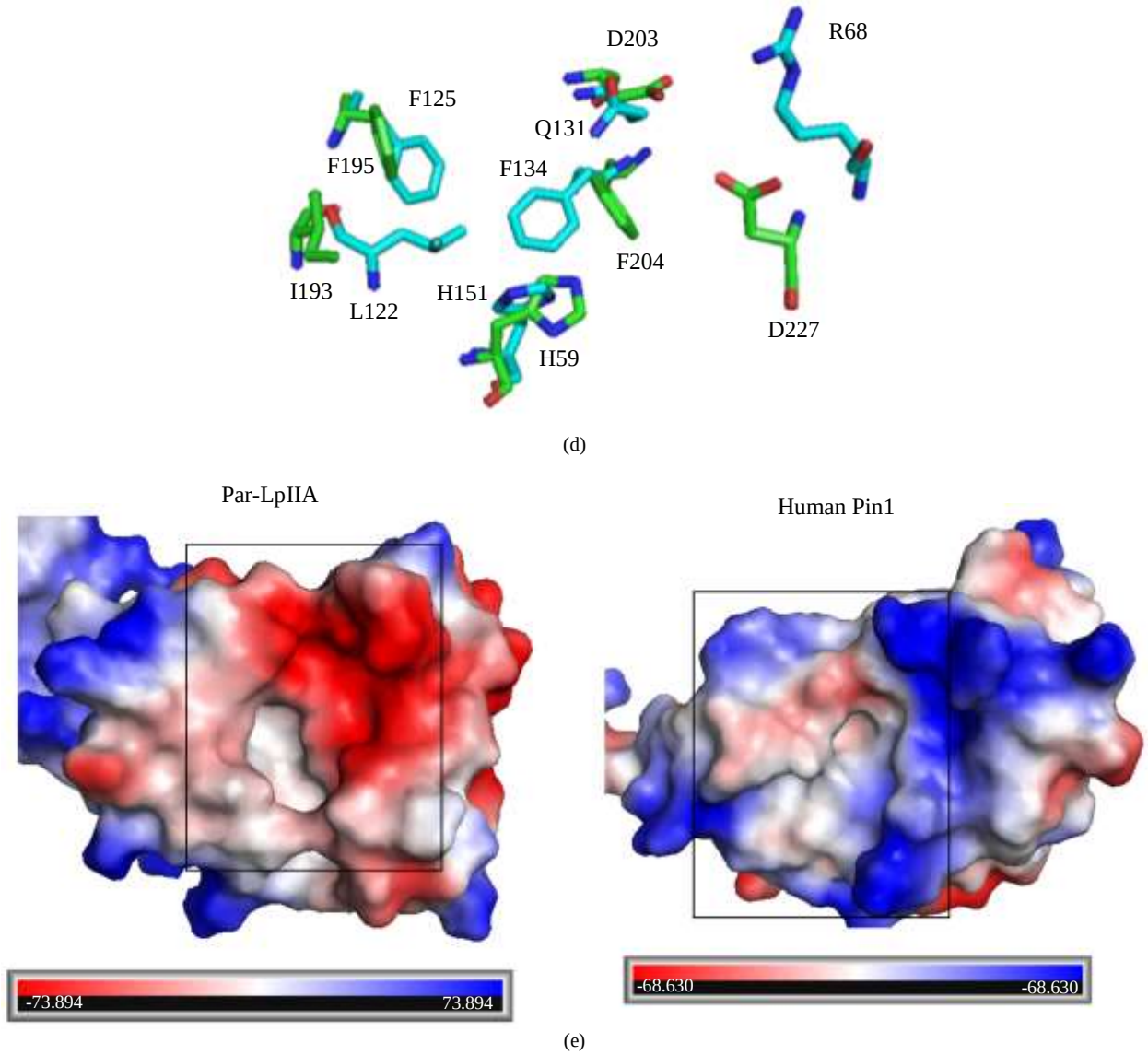


Fig. 7: (a) The best three-dimensional model Par-LpIIA obtained from structural homology modeling, (b) Structural alignment of the full-length structures of Par-LpIIA (green) with human Pin1 (cyan), (c) Structural alignment of PPIase domain of Par-LpIIA (green) with human Pin1 (cyan), (d) Manual structural alignment of active site residues of human Pin1 (cyan) and Par-LpIIA (green), (e) The surface charges of PPIase domain of Par-LpIIA and human Pin1 as rendered by PyMol's Vacuum Electrostatics. The region where the active sites are located indicated by the box. Red, white and blue stand for negative, neutral and positive surface charges, respectively

More interestingly, structural alignment of PPIase domains of human Pin1 and Par-LpIIA (Fig. 7d) active site residues of Par-LpIIA were less conserved. Six putative active sites of Par-LpIIA were identified, including His151, Ile193, Phe195, Asp203 and Phe204 and Asp227. His151, Phe195 and Phe204 were well conserved with His59, Phe125 and Phe134 of human Pin1, respectively. Meanwhile Ile193, Asp203 and Asp227 corresponded to Leu122, Gln131 and Arg68, respectively. Also, surface charge around active sites of Par-LpIIA and human Pin1 are considerably different. The binding pocket of Par-LpIIA is more negatively charged than human Pin1 (Fig. 7e).

Discussion

LAB is considerably a group of extreme bacteria due to their survival capability at a very low pH (Mbye *et al*., 2020). It is widely known that extreme organisms' adaptability to certain physiological and or environmental

stress implied that those organisms are equipped with supporting cellular machinery, including proteins or enzymes (Giordano, 2020). As an example, LAB is known to have a very efficient proton pump allowing the bacteria to maintain their intracellular pH under very low environmental pH (Wang *et al*., 2018). Accordingly, the existence of PPIase family members in this group of bacteria might also imply that protein folding machinery might be influenced by very low environmental pH. Alternatively, this might imply that Par-LpIIA might be functionally required to accelerate the slow isomerization of *cis*-proline containing proteins upon folding after newly synthesized from the ribosome. It was reported previously that pH indeed has an effect on the folding rate of proteins (O'Brien *et al*., 2012; Nemtseva *et al*., 2019). Rami and Udgaonkar (2001) indicated that the folding rate of protein decreased under extreme alkaline or acidic environment. This implied that at very low environmental pH, the folding rate of proteins inside LAB cells is considerably slow and thus required a catalyst for accelerating the rate. In this respect, Par-LpIIA plays important roles, particularly for *cis*-proline containing proteins. This is therefore understandable for *L. plantarum* IIA to have the gene encoding Par-LpIIA

Norgren (2013) highlighted that incorrect gene annotation in whole-genome sequencing may occur due to low-quality genome assembly processes. As a result, some genes that were found in annotation are not existed in genomic DNA, *vice versa*. Figure 1 confirmed that the gene of Par-LpIIA exists inside the genomic DNA of this strain. Further, Par-LpIIA expressed by *L. plantarum* IIA is confirmed to be in an active form and thus can accelerate slow *cis*-proline containing substrate. The PPIase activity against peptide substrate and catalysis against RNase T_1 observed in the cell lysate (cytoplasm) of *L. plantarum* IIA is indeed originated from parvulin-like protein. Alternatively, a parvuline-like protein exists in the cell lysate of *L. plantarum* IIA as a *trans*lation product of Par-LpIIA gene. In addition, this is so far the only parvulin reported from LAB family members.

To note, the result also indicated that the cell lysate containing Par-LpIIA exhibited PPIase activity towards phosphorylated and non-phosphorylated substrates. Fanghänel and Fischer (2004) reported that not all parvulin members were able to catalyze the phosphorylated peptide substrate. For example, human Pin1, was able to exhibit PPIase activity towards phosphorylated peptide substrate, but not for human Par14 and Ec-Par (Uchida *et al*., 1999; Matena *et al*., 2018; Saeed *et al*., 2019). The temperature dependency of PPIase activity of CL-LpIIA is understandable as *L. plantarum* IIA is known to be a mesophilic bacterium (Arief *et al*., 2015). Similarly, a PPIase member from a mesophilic bacterium *E. coli* also increased in the range of 10-25°C (Suzuki *et al*., 2004). Budiman *et al*. (2011) implied that the nature of the organism dictated the temperature dependency of their PPIase activity. A PPIase member of a psychrophilic bacterium *Shewanella* sp. SIB1 had shown to be optimum at 10°C and remarkably lower at higher temperatures (Suzuki *et al*., 2004).

Interestingly, the measurement of the PPIase activity of CL-LpIIA indicated that the activity is dependent on pH, with an optimum pH of 5. This is in good agreement with the previous evidence showing that the isomerization rate of Ser/Thro-Pro segment is highly affected by the environmental pH changes (Schutkowski *et al*., 1998). In general, the most effective catalysis of parvulin was observed at pH values where the phosphorylated side chain is in its anionic form. Noteworthy, the acidic pH optimum of LpIIA was found to be slightly lower than the other reported Par (Fanghänel and Fischer, 2004), which might be due to the nature of *L. plantarum* IIA as a bacterium lives in an acidic environment (Arief *et al*., 2015).

The structural organization of Par-LpIIA, which was organized into two separate domains, apparently followed a common structural feature of parvulin. Yeast parvulin Ess1 and human Pin1 were also organized into two domains, an N-terminal WW domain and a C-terminal PPIase domain and both being important for the in vivo function of these proteins (Kouri *et al*., 2009; Hanes, 2014). While the PPIase domain is responsible for catalytic activity, the WW domain is known to be important for facilitating a protein-protein interactive event and no involvement in the catalysis (non-catalytic domain) (Maruyama *et al*., 2004; Sudol, 1996). As BLAST analysis indicated that His150 to Lys232 which was predicted as PPIase domain, suggested that C-domain of Par-LpIIA is predicted to be a PPIase domain. Meanwhile, the N-domain is predicted to be a homolog of WW-domain. Interestingly, the folding motif analysis using the FastSCOP (http://fastSCOP.life.nctu.edu.tw.) on the homolog WW-domain did not hint any structural motif in the database, which implied that this domain folded into a unique structure.

The presence of a homolog of WW-domain in Par-LpIIA might suggest that this protein might interact with the other proteins in its cellular function. The two domains of Par-LpIIA are expected to be cooperatively functional, whereby a homolog of the WW domain is an anchor region for protein substrates for the catalysis by the PPIase domain. Budiman *et al*. (2011) reported that removing the non-catalytic domain in PPIase abolished the activity against protein substrate due to the lack of an anchoring region. The remarkable acceleration by the cell lysate containing Par-LpIIA might serve as evidence of the cooperativity between these two domains towards the protein substrate. A homolog of the WW domain of

Par-LpIIA might serve as the binding site for the RNase T_1, while the PPIase domain bind to the prolyl bond and catalyze its slow isomerization. This is in good agreement with a proposal of (Budiman *et al*., 2009), in which the non-PPIase domain is important to facilitate an efficient binding to a protein substrate and therefore maximize the ability of PPIase to catalysis the *cis-trans* isomerization. To note, some reports also highlighted the ability of parvulin in accelerating the refolding rate of RNase T_1 (Kale *et al*., 2011; Scholz *et al*., 1997; Uchida *et al*., 1999; Geitner *et al*., 2013; Behrens *et al*., 2001; Vitikainen *et al*., 2004). Nevertheless, (Scholz *et al*., 1997) highlighted that the affinity of parvulin towards RNase T_1 is generally weak and therefore, the catalytic efficiency towards this substrate is 100-fold lower, or more, than that of against a peptide substrate. This explains the finding in this study, whereby the k_{cat}/K_M of CL-LpIIA was extremely lower than that of a WFY(pS)PR-pNA substrate. In addition, the ability of Par-LpIIA to catalyze the phosphorylated peptide substrate (WFY(pS)PR-pNA) might be also due to the presence of a homolog of the WW domain. Fanghänel and Fischer (2004) reported that the WW-domain of human Pin1 was found to interact with phosphoserine (pS) or phosphothreonine (pT) containing peptides or proteins.

Less conservation of active site residues of Par-LpIIA might indicate that this protein might employ a unique catalysis mechanism as compared to human Pin1. Interestingly, His59 is well conserved with His151 of Par-LpIIA. Conservation of His59 was reported in most Par family members, suggesting that this protein employs a covalent catalysis mechanism (Fanghänel and Fischer, 2004; Czajlik *et al*., 2017; Dunyak and Gestwicki, 2017). In human Pin1, the covalent mechanism was explained by the ability of deprotonated His50 to abstract a protein of its adjacent residue (Cys113) to promote a nucleophilic attack to the carbonyl carbon of the prolyl bond. The involvement of Cys residue is known to be a common feature in the catalysis mechanism employed by parvulin (Fanghänel and Fischer, 2004; Dunyak and Gestwicki, 2017).

Interestingly, Cys113 of human Pin1 was not conserved in Par-LpIIA. Besides, no Cys residues were found in the proximity of His151 of Par-LpIIA. Nevertheless, Ser181 is in close distance to His151, which suggested that Par-LpIIA utilizes the nucleophile of Ser181 to attack the carbonyl carbon of the prolyl bond. Similarly, Ser71 of Par of *Arabidopsis thaliana* (At-Pin1) also reported being involved in the catalysis, instead of Cys residue (Fanghänel and Fischer, 2004; Lakhanpal *et al*., 2021). In addition, Cys131 of human Pin1 is also exchanged to Asp74 in human Par14 (Sekerina *et al*., 2000; Matena *et al*., 2018; Thapar, 2015), which suggested that the role of Cys in the catalysis is indeed replaceable. The differences in other active site residues and surface charges might also imply the uniqueness of the catalysis mechanism of Par-LpIIA, which might be related to adaptation to the extreme pH environment of Lp IIA as LAB member. Yet, this assumption remains to be experimentally confirmed. Nevertheless, it is interesting also that the substrate specificity of the CL-LpIIA showed slightly different from that of human Pin1, Eco-Par and human Par14. These differences might be raised due to the unique structural features of Par-LpIIA, particularly in its active sites.

Accordingly, we believe that Par-LpIIA in the cytoplasm is required by *L. plantarum* IIA to support the protein folding events, particularly for the *cis*-proly bond containing proteins. This is good agreement with (Trémillon *et al*., 2012) who proposed PPIase of *L. lactis* as a protein folding helper. A specific feature of Par-LpIIA towards phosphorylated substrate might also imply that this protein is involved in phosphate-mediated cell signaling pathway, including Mitogen-Activated Protein Kinase (MAPK) signaling pathway. Kobatake *et al*. (2017) reported that MAPK is required by LAB to overcome oxidative stress. The cellular function of Par-LpIIA, nevertheless, remain to be experimentally confirmed.

Conclusion

This study clearly confirmed that *L. plantarum* IIA-1A5 expresses Par-LpIIA, a member PPIase family protein under parvulin group. The protein was apparently expressed in the cytoplasm of the cell and generate the ability of *L. plantarum* IIA to exhibit specific PPIase activity towards phosphorylated prolyl bond substrate and was able to catalyze the refolding of a *cis* prolyl bond containing protein (RNase T_1). Further, the structural homology modeling of Par-LpIIA showed the uniqueness of active site configuration at Par-LpIIA might account for the adaptation mechanism of this protein at a low pH environment.

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Author's Contribution

Cahyo Budiman: Conceptualization, conducting most of experiments, data analysis and curation, writing the manuscript and funding acquisition.

Irma Isnafia Arief: Conceptualization, writing manuscript, resources and funding acquisition.

Fernandes Opok: Conducting some experiments, reviewing the manuscript, resources and data analysis.

Muhammad Yusuf: data analysis and writing the manuscript.

All authors read and approved the final manuscript.

Ethics

This manuscript has not been published elsewhere in part or in entirely. The authors declare that there is no conflict of interest.

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