MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol 10, No 2, Juni 2016, p 39-47

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.10.2.1

Cloning, Expression and Functional Characterization of Autoactivated Human Prethrombin-2 Synthetic Gene by Using *Pichia pastoris* SMD1168 as a Host

TOTO SUBROTO[1]*, WULAN PERTIWI[1], MUHAMMAD FADHLILLAH[1], KHOMAINI HASAN[2,4], OGI BUDIANTORO[1], SUTARYA ENUS[3], AND SOETIJOSO SOEMITRO[1]*

[1]*Biochemistry Laboratory, Department of Chemistry, Universitas Padjadjaran, Bandung 45363, Indonesia;*
[2]*Biosciences and Biotechnology Research Center, Institut Teknologi Bandung, Bandung 40132Indonesia;*
[3]*National Eye Center, Rumah Sakit Mata Cicendo, Bandung, Indonesia;*
[4]*Biochemistry Laboratory, Faculty of Medicine, Universitas Jenderal Achmad Yani, Cimahi-Bandung, Indonesia*

Prethrombin-2 is a thrombin precursor that has important role in blood coagulation. It is the smallest precursor which is activated into thrombin by FXa prior to coagulation process. However, as a commercial theurapetic protein in fibrin sealant component, prethrombin-2 must be activated by ecarin before used. Thus, the production process of this protein needs further purification. In order to eliminate ecarin activation step and to increase production efficiency, we designed, cloned, and expressed the recombinant autoactivated human prethrombin-2 in *Pichia pastoris* SMD1168. The variant was designed with 4 mutations, E40A, D47A, G48P, and E52A, based on the result of a previous study. The synthetic variant gene was first optimized to conform with *P. pastoris* codon preference. The optimized synthetic gene was cloned in pD912 plasmid using X*ho*I and S*ac*II restriction enzymes. The transformed *P. pastoris* was selected on agar plate supplemented with 1000 µg mL^{-1} Zeocin as a selection marker. This study showed that autoactivated prethrombin-2 was succesfully expressed extracellularly by *P. pastoris* SMD1168. The activity of recombinant autoactivated prethrombin-2 using a chromogenic substrate S-2238 was 0.540 unit mg^{-1}. Taken together, these results demonstrated that autoactivated human prethrombin-2 was successfully produced extracellularly in *P. pastoris*.

Key words: autoactivated human prethrombin-2, extracellular expression, *Pichia pastoris* SMD1168

Pretrombin-2 adalah prekursor trombin yang berperan penting selama proses koagulasi darah. Prekursor ini harus diubah menjadi trombin oleh FXa sebelum terlibat dalam proses koagulasi. Namun, sebagai protein terapetik komersial dalam komponen lem fibrin, pretrombin-2 harus diaktivasi menggunakan ekarin. Dengan demikian, proses produksi protein tersebut memerlukan proses pemurnian lebih lanjut. Untuk menghilangkan tahap aktivasi menggunakan ekarin dan untuk meningkatkan efisiensi produksi, kami telah merancang, mengklon, dan mengekspresikan pretrombin-2 manusia terotoaktivasi dalam *Pichia pastoris* SMD1168 sebagai sel inang. Jenis pretrombin-2 ini dirancang dengan 4 mutasi, E40A, D47A, G48P, dan E52A, sesuai dengan penelitian yang telah ada sebelumnya. Gen sintetik telah dioptimasi agar sesuai dengan preferensi kodon *P. pastoris.* Gen sintetik yang telah dioptimasi selanjutnya dikonstruksi ke dalam plasmid pD912 dengan sisi pemotongan enzim restriksi *Xho*I dan *Sac*II. Transforman *P. pastoris* diseleksi pada media agar yang mengandung 1000 µg mL^{-1} Zeocin sebagai marka seleksi. Hasil ekspresi protein menunjukkan bahwa pretrombin-2 terotoaktivasi telah berhasil diekspresikan secara ekstraselular oleh *P. pastoris* SMD1168 dengan aktivitas pretrombin-2 terhadap substrat kromogenik S-2238 mencapai 0,540 unit mg^{-1}. Hasil penelitian ini menunjukkan bahwa pretrombin-2 manusia terotoakativasi telah berhasil diproduksi secara ekstraselular di dalam *P. pastoris.*

Kata kunci: ekspresi ekstraselular, pretrombin-2 manusia terotoaktivasi, *Pichia pastoris* SMD1168

Thrombin is an important proteolytic enzyme in blood coagulation. This enzyme has important roles during coagulation process as it lies in the middle of both pro- and anti-coagulant pathways. Interestingly, it can act on various type of substrates involved in clot formation or platelet activation. It is generated from zymogen prothrombin and prethrombin-2. Prethrombin-2 is activated via proteolytic cleavage at R15 by FXa and separation of the A chain and catalytic B chain (Haynes *et al*. 2012; Pozzi *et al*. 2011; Pozzi *et al*. 2013).

Due to its unique characteristics, prethrombin-2 has tremendous clinical applications. It can be applied as clinical anti-coagulant or as a wound healing accelerator (Yonemura *et al*. 2004). Specifically, it can be implemented in eye surgery as fibrin sealant (Enus *et al.* 2011). However, thrombin applied for clinical purposes is usually isolated from human or bovine plasma that such preparation raises the risk of contamination with infectious agents from the sources (Yonemura *et al*. 2004), xenogenic immune response, cross reaction leading to unstop bleeding or

*Corresponding author; Phone: +62-22-7794391, Email:t.subroto@unpad.ac.id

anaphylaxis (Stricker *et al*. 1988; Zehnder *et al*. 1990; Streiff *et al*. 2002; Dorion *et al.* 1998; Tadokoro *et al*. 1991; Wai *et al.* 2003).

To date, there is an effort to express a recombinant human prethrombin-2 (rhPT2) hosted by microorganisms, such *Escherichia coli* for commercial purposes (Choi *et al.* 1989; So *et al*. 1992; Silaban *et al.* 2014). However, activation of rhPT2 into thrombin via proteolytic cleavage of FXa is replaced by ecarin, the snake venom protease isolated from *Echis carinatus* (Jonebring *et al*. 2012). The activated rhPT2 needs further purification to remove ecarin, hence decreasing the production efficiency.

Previous important study by Pozzi *et al*. (2013) had successfully constructed mutant human prethrombin-2 that converts into thrombin without proteolytic cleavage. The replacement of four key residues within the activation domain could spontaneously convert into active thrombin. Interestingly, the activated product was functionally and structurally equivalent to the wild type. Therefore, further removal of ecarin was unnecessary for this mutant.

In this study, we directed our efforts towards developing the extracellular expression of autoactivated prethrombin-2 (by mutating 4 amino acids) using methanolic *Pichia pastoris* SMD1168.

MATERIALS AND METHODS

Designing the Optimized Autoactivated Prethrombin-2 Codon Based on *Pichia pastoris* codon Preference. A synthetic gene of autoactivated prethrombin-2 (auto-Trm) was designed based on the prethrombin-2 amino acid and nucleotide sequences of *Homo sapiens* coagulation factor II (thrombin) (F2), which is available in GenBank (Accession number NM_000506.3). The codon preference library of *P. pastoris* is available from Codon Usage Database (http://www.kazusa.or.jp/codon/). Codon analysis and optimization was performed by online software Graphical Codon Usage Analyzer (GCUA) (http://gcua.schoedl.de/).

Yeast Transformation. The auto-Trm synthetic gene was purchased from DNA2.0 (New York). The gene was re-optimized by DNA2.0 using Gene Designer software without changing the amino acid sequence and was inserted into pD912 plasmid (Fig1). *Pme*I-digested pD912 was inserted into *P. pastoris* SMD1168, a protease A-deficient strain, as a host cell by using Eppendorf Multiporator (catalogue number 4309000019). The transformed cell was grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) gel medium containing Zeocin as a selection marker.

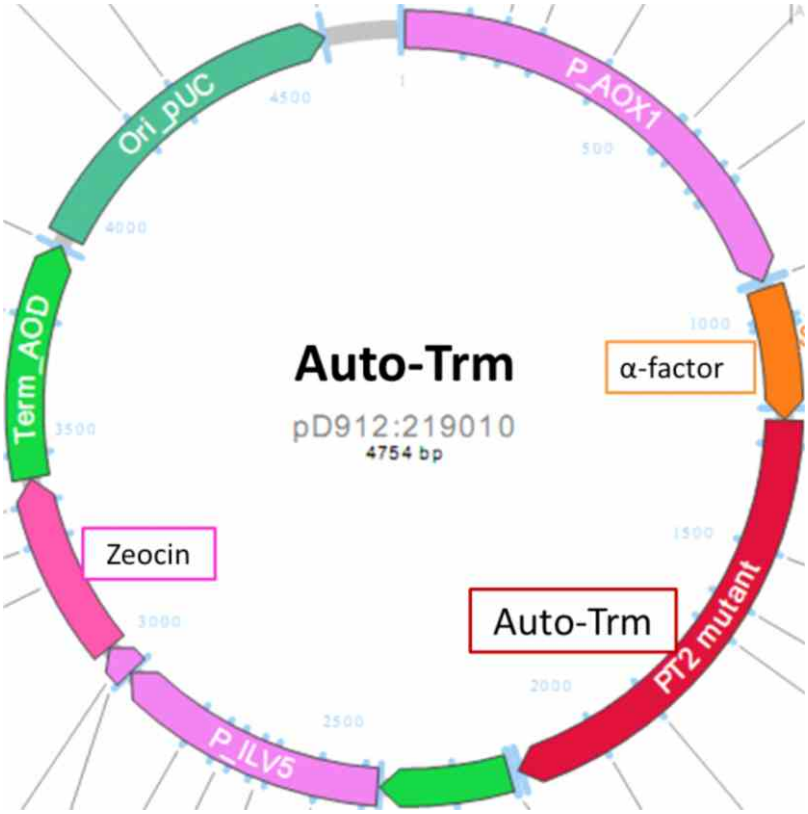

Fig 1 Auto-Trm synthetic gene in pD912 plasmid. Auto-Trm synthetic gene is located as PT2 mutant (red background) in plasmid.

Expression of Recombinant Autoactivated prethrombin-2.

a. Growth Analysis. Culture of transformed *P. pastoris* SMD1168 was grown in BMGY medium (1% yeast extract, 2% peptone, 0.1 M potassium phosphate, pH 6.0, 1.34% YNB, 4 × 10^{-5} % biotin, and 1% glycerol). Sampling was performed every 8 h. Optical density of each sample was measured at λ_{600}.

b. Expression of Recombinant Autoactivated Prethrombin-2. A single colony of the recombinant *P. pastoris* was inoculated from the stock plate into 2,5 mL YPD medium and was incubated overnight at room temperature with 250 rpm shaking. The culture was transferred into 247.5 mL fresh BMGH medium and was grown until the culture reached OD_{600} of 11. The cell pellet was harvested for induction by centrifugation at 6 000 ×g for 5 min at room temperature. The pellet was resuspended in BMMH medium (medium with methanol instead of glycerol) using one-tenth volume of the original BMGH culture. The final methanol concentration was adjusted to 1.5% every 24 h. One mL of culture sample was collected and characterized by SDS-PAGE.

Activity Measurement of Recombinant Autoactivated Prethrombin-2. The activity of auto-Trm was determined by measuring the hydrolysis of a chromogenic substrate S-2238 at OD_{405} (Yonemura *et al*., 2009). Human thrombin was used as standard with dilution to 0.1, 0.2, 0.4, 0.5, 0.8, 1.0, and 2.0 unit mL^{-1} using the same buffer. Human thrombin, ecarin and chromogenic substrate are available commercially from Sigma-Aldrich, Singapore.

RESULTS

Designing of the Optimized Autoactivated Thrombin Codon Based on *P. pastoris* Preference Codon. In this study, prethrombin-2 gene was used as the template to design the autoactivated thrombin (auto-Trm). The prethrombin-2 sequence (GenBank, Accession number NM_000506.3) is shown in Fig 2. The relative adaptiveness of the sequence was examined against *P. pastoris* codon preferences. The relative adaptiveness analysis of human prethrombin-2 codon sequence showed that some amino acids were not compatible with *P. pastoris* codon preferences (Fig 3). The relative adaptiveness of some human codons encoding human prethrombin-2 amino acids was less than 50%, i.e. A (GCA), G (GGC, GGG), I (ata), L (CTG, CTT, CTC), P (CCC, CCG), R (AGG, CGA, CGC, CGG), S (agc, TCG), T (ACG), and V (GTG) while the relative adaptiveness of human codons was more than 50% but less than 100%, i.e. A (GCC, GCA), C (TGC), D (GAC), E (GAG), F (TTC), G (GGA) H (CAC, I (ATC), K (AAA), N (AAT), P (CCT), Q (CAG), S (TCC, TCA, AGT), T (ACC, ACA), V (GTC), and Y (TAT). These codons were optimized based on the *P. pastoris* codon preferences to reach 100% relative adaptiveness (Fig 4).

Four point mutations were made to generate auto-Trm. Based on the study of Pozzi *et al.* (2013), the points of mutation were E (GAA) TO A (GCT), D (GAT) TO A (GCT), G (GGT) TO P (CCC), and E (GAA) to A (gct) as shown in Fig 5 (typed in bold and red). The amino acids sequence after mutation to generate auto-Trm is shown in Fig 6 and the *P. pastoris*-optimized auto-Trm codons is shown in Fig 7.

Expression and Activity Measurement of Autoactivated Prethrombin-2. In this study, the first induction time to express auto-Trm was determined based on the *P. pastoris* SMD1168 growth curve (Fig 8). Induction by methanol was done when OD_{600} reached 11 and it was on 28th hour when the culture was about to be in the stationary phase. Auto-Trm was expressed and secreted as a recombinant protein from *P. pastoris* SMD1168 at room temperature (Fig 9).

The expressed auto-Trm gave thin band in SDS-PAGE result and it had not been further purified, yet the activity of auto-Trm was measured to ensure its manner. The activity measurement of auto-Trm was performed by adding chromogenic substrate S-2238 and the absorbance was measured at 405 nm wavelength (A_{405}). 20 µL auto-Trm sample from 10 mL stock was set into

TATSEYQTFFNPRTFGSGEADCGLRPLFEKKSLEDKTERELLESYIDG
RIVEGSDAEIGMSPWQVMLFRKSPQELLCGASLISDRWVLTAAHCLL
YPPWDKNFTENDLLVRIGKHSRTRYERNIEKISMLEKIYIHPRYNWR
ENLDRDIALMKLKKPVAFSDYIHPVCLPDRETAASLLQAGYKGRVT
GWGNLKETWTANVGKGQPSVLQVVNLPIVERPVCKDSTRIRITDN
MFCAGYKPDEGKRGDACEGDSGGPFVMKSPFNNRWYQMGIVSWG
EGCDRDGKYGFYTHVFRLKKWIQKVIDQFGE (308 amino acids)

Fig 2 The prethrombin-2 amino acids sequence. The sequence was taken from GenBank (Accession number NM_000506.3).

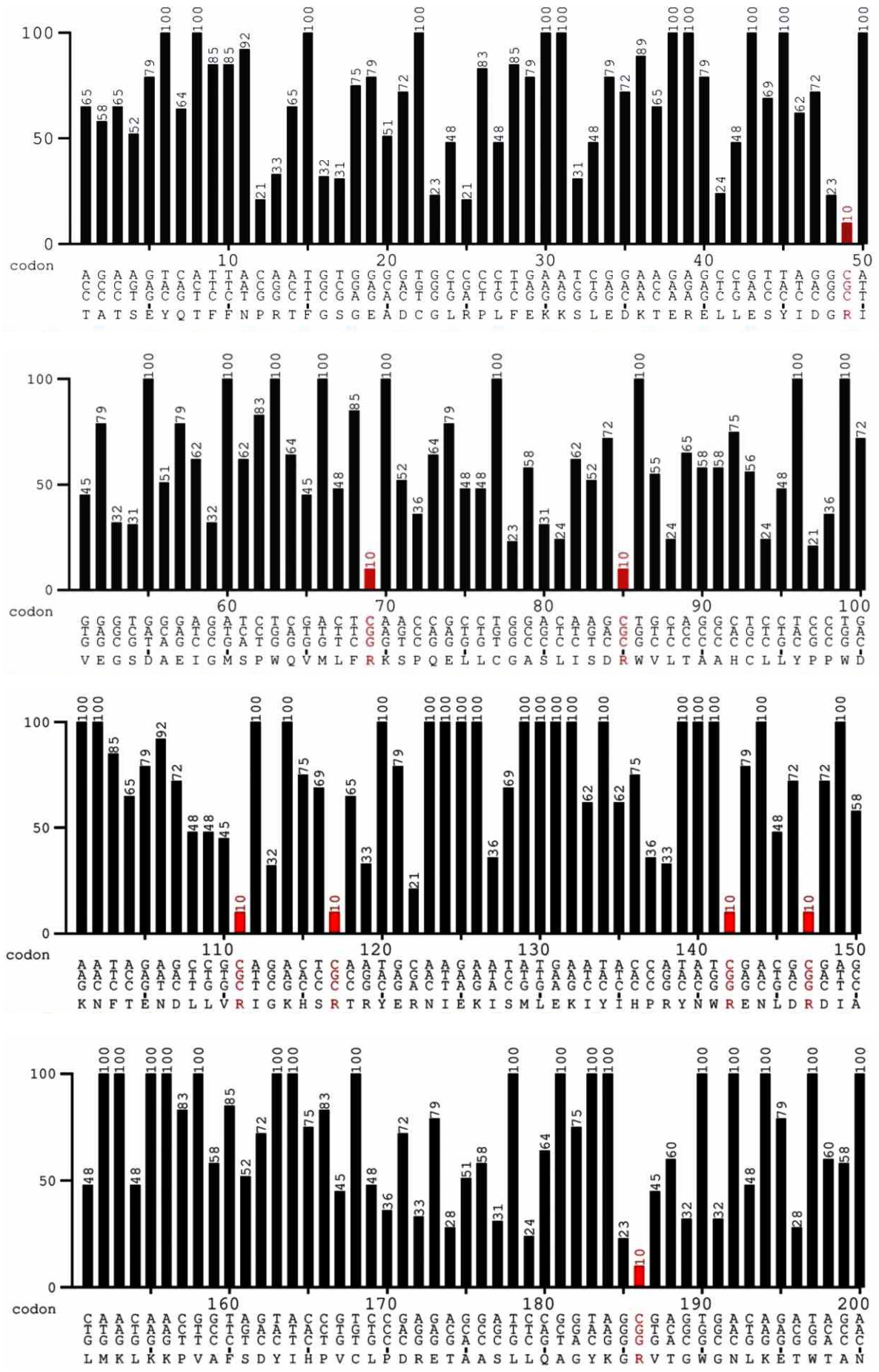

Fig 3 Relative adaptiveness of prethrombin-2 codons to *Pichia pastoris* codon preferences.

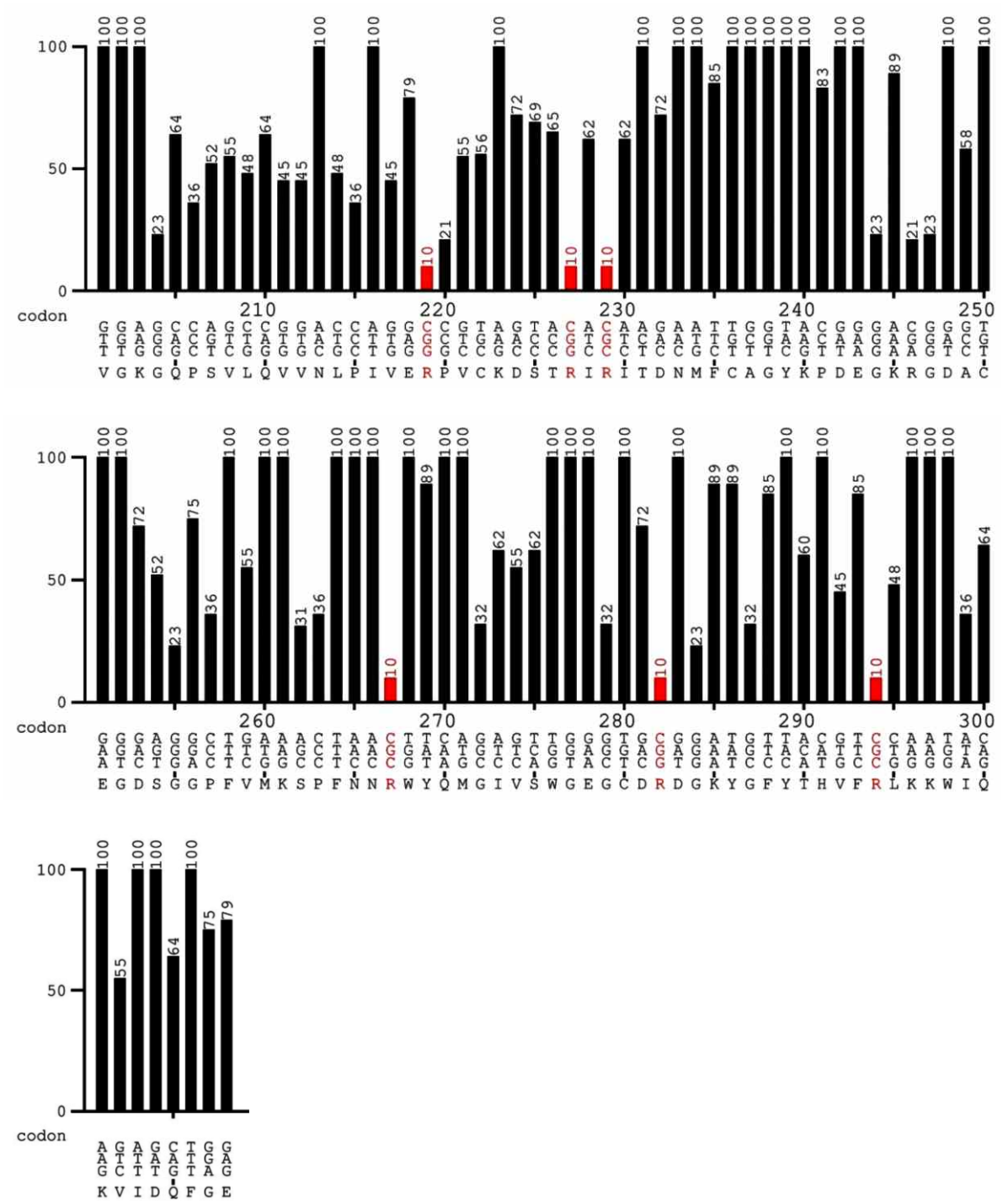

Fig 3 Relative adaptiveness of prethrombin-2 codons to *Pichia pastoris* codon preferences.

reaction. The value of A_{405} was 0,131 (Fig 10) and was plotted into linear regression equation of thrombin standard (y = 0,3357x - 0,0502) to give the activity value. The activity of auto-Trm was 0.540 unit mg^{-1}.

DISCUSSION

In blood, prethrombin-2 was activated by FXa. As a substitute of FXa, snake venom metalloprotease ecarin can be used to activate prethrombin. Autoactivation of prethrombin-2 proposed a new method for thrombin production that eliminates the need of activators such as ecarin and would reduce the production cost of thrombin (Pozzi *et al.* 2013).

Most of the amino acids are encoded by more than one codon. Codon preference varies among species, resulting codon bias that might become a hindrance in the expression of heterologous proteins. Codon optimization tends to replace rare codon with optimum codon and affects translation rate that could impact the structure and the function of the protein (Angov *et al.* 2008). Also, the increase of the heterologous protein

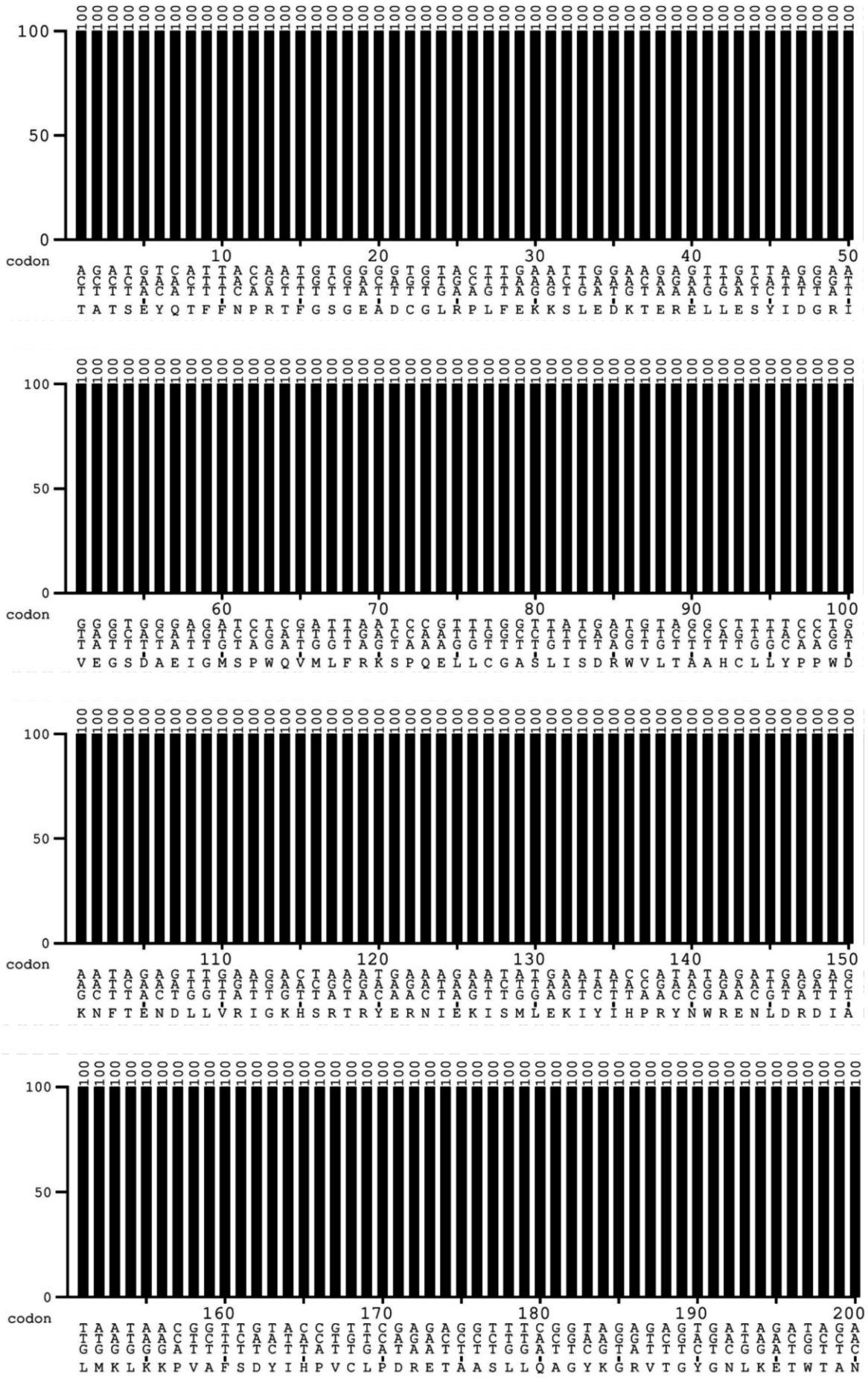

Fig 4 Relative adaptiveness of optimized prethrombin-2 codons to *Pichia pastoris* codon preferences.

TATSEYQTFFNPRTFGSGEADCGLRPLFEKKSLEDKTER
ELLESYI**DG**RIV**E**GSDAEIGMSPWQVMLFRKSPQELLC
GASLISDRWVLTAAHCLLYPPWDKNFTENDLLVRIGKH
SRTRYERNIEKISMLEKIYIHPRYNWRENLDRDIALMKL
KKPVAFSDYIHPVCLPDRETAASLLQAGYKGRVTGWG
NLKETWTANVGKGQPSVLQVVNLPIVERPVCKDSTRI
RITDNMFCAGYKPDEGKRGDACEGDSGGPFVMKSPFN
NRWYQMGIVSWGEGCDRDGKYGFYTHVFRLKKWIQ
KVIDQFGE (308 amino acids)

Fig 5 Four point mutations in prethrombin-2 amino acids sequence (typed in bold and red).

TATSEYQTFFNPRTFGSGEADCGLRPLFEKKSLEDKTER
ALLESYI**AP**RIV**A**GSDAEIGMSPWQVMLFRKSPQELLC
GASLISDRWVLTAAHCLLYPPWDKNFTENDLLVRIGKH
SRTRYERNIEKISMLEKIYIHPRYNWRENLDRDIALMKL
KKPVAFSDYIHPVCLPDRETAASLLQAGYKGRVTGWG
NLKETWTANVGKGQPSVLQVVNLPIVERPVCKDSTRI
RITDNMFCAGYKPDEGKRGDACEGDSGGPFVMKSPFN
NRWYQMGIVSWGEGCDRDGKYGFYTHVFRLKKWIQ
KVIDQFGE (308 amino acids)

Fig 6 Auto-Trm amino acids sequence (mutations were typed in bold and red).

5'ACTGCTACTTCTGAATACCAAACTTTTTTTAACCCAAGAACTTTTGGTTCTG
GTGAAGCTGATTGTGGTTTGAGACCATTGTTTGAAAAGAAGTCTTTGGAAGA
TAAGACTGAAAGA**GCT**TTGTTGGAATCTTACATT**GCTCCA**AGAATTGTT**GCT**
GGTTCTGATGCTGAAATTGGTATGTCTCCATGGCAAGTTATGTTGTTTAGAAA
GTCTCCACAAGAATTGTTGTGTGGTGCTTCTTTGATTTCTGATAGATGGGTTTT
GACTGCTGCTCATTGTTTGTTGTACCCACCATGGGATAAGAACTTTACTGAAA
ACGATTTGTTGGTTAGAATTGGTAAGCATTCTAGAACTAGATACGAAAGAAAC
ATTGAAAAGATTTCTATGTTGGAAAAGATTTACATTCATCCAAGATACAACTG
GAGAGAAAACTTGGATAGAGATATTGCTTTGATGAAGTTGAAGAAGCCAGTT
GCTTTTTCTGATTACATTCATCCAGTTTGTTTGCCAGATAGAGAAACTGCTGCT
TCTTTGTTGCAAGCTGGTTACAAGGGTAGAGTTACTGGTTGGGGTAACTTGA
AGGAAACTTGGACTGCTAACGTTGGTAAGGGTCAACCATCTGTTTTGCAAGT
TGTTAACTTGCCAATTGTTGAAAGACCAGTTTGTAAGGATTCTACTAGAATTA
GAATTACTGATAACATGTTTTGTGCTGGTTACAAGCCAGATGAAGGTAAGAG
AGGTGATGCTTGTGAAGGTGATTCTGGTGGTCCATTTGTTATGAAGTCTCCAT
TTAACAACAGATGGTACCAAATGGGTATTGTTTCTTGGGGTGAAGGTTGTGAT
AGAGATGGTAAGTACGGTTTTTACACTCATGTTTTTAGATTGAAGAAGTGGAT
TCAAAAGGTTATTGATCAATTTGGTGAA3'(924 Nucleotides)

Fig 7 Optimized auto-Trm codons (mutations were typed in bold and red).

expression by optimizing codon usage has been reported previously (Welch*et al*. 2009). The earliest precursor to generate thrombin is prothrombin, which has five domains; Gla, Kringle 1 (K1), Kringle 2 (K2), A and B. The last two domains are the protease units. Other prothrombin derived-precursors are meizothrombin, prethrombin-1 and prethrombin-2. Among the precursors, the latest is the smallest. Active protease thrombin can be generated by cleaving R320 (based on prothrombin numbering) that separate the A and B chains, yet both domains are kept attached as one unit protease by a disulfide bond (Pozzi *et al*. 2013).

Despite the availability of many *P. pastoris* strains that might have become the host, SMD1168 (a protease A-deficient strain) was chosen to prevent auto-Trm degradation by protease during expression process.

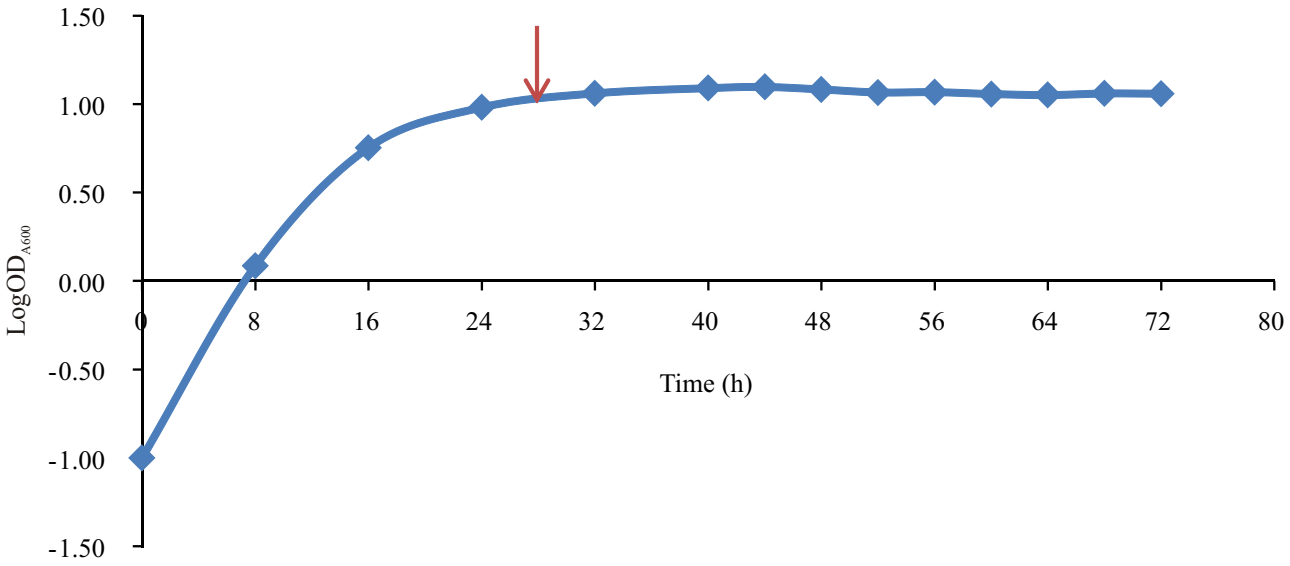

Fig 8 Growth curve of *P. pastoris* SMD1168. Sampling was performed every 8 h.

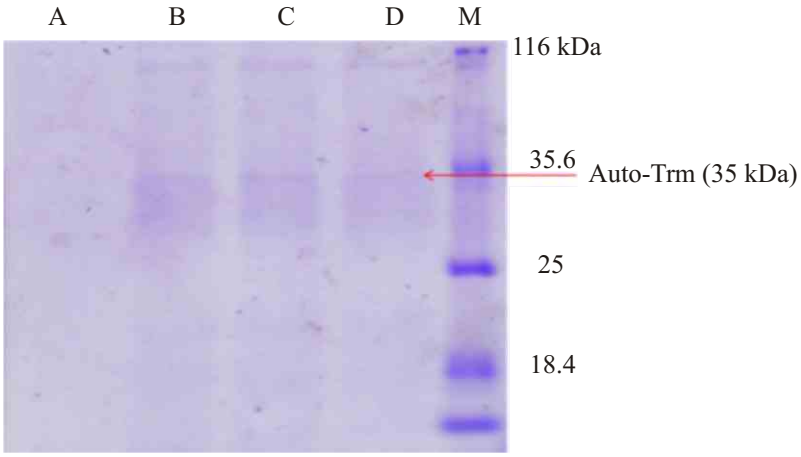

Fig 9 SDS-PAGE of expressed auto-Trm (pointed by ←). (A) 0 h, (B) 24 h, (C) 48 h, (D) 72 h, (M) protein marker. Although the auto-Trm gave thin band but it showed activity in hydrolising chromogenic substrate S-2238.

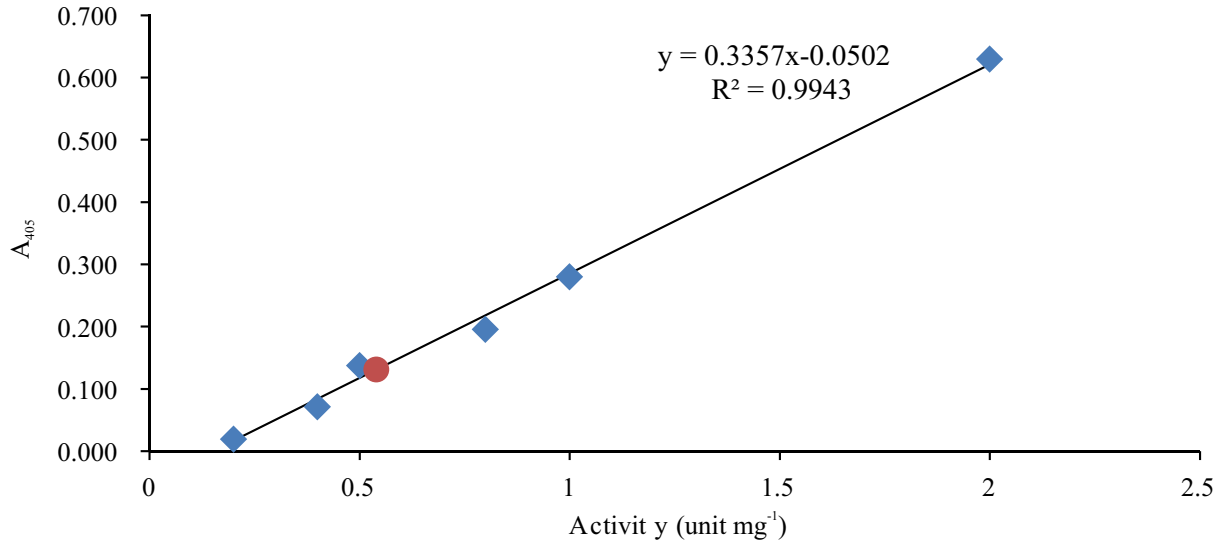

Fig 10 Auto-Trm activity (●) A405 was measured after auto-Trm was reacted with chromogenic substrate S-2238 and was plotted into linear regression of thrombin standard (0.2; 0.4; 0.5; 0.8; 1.0; 2.0 unit mg^{-1}) (◆).

SMD1168 had previously been used to produce recombinant fibrinogen (rFibrinogen). Fibrinogen expressed from SMD1168 was succesfully maintained during fermentation and was biologically active (Tojo *et al.* 2008). In this research, SMD1168 can be used to express and secrete autoactivated prethrombin-2. Despite showing only a thin band in the SDS-PAGE result, it had proteolytic activity. Thus, this result gives way to further development of the autoactivated prethrombin-2 production in optimized condition to increase its productivity. As conclusion, autoactivated thrombin was succesfully expressed extracellularly in *P. pastoris* SMD1168 from synthetic gene and the activity was 0.540 unit mg^{-1}.

ACKNOWLEDGMENTS

We would like to thank the Ministry of Research, Technology, and Higher Education for funding this research through Program Insentif Riset SINas 2015.

REFERENCES

Angov E, Hillier CJ, Kincaid RL, Lyon JA. 2008. Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host. Plos ONE 3. doi:10.1371/journal.pone.0002189.

Choi EH, Kim YJ, Kim JM, Hong HJ, Han MH, Kim J. 1989. Cloning and expression of human prethrombin 2 cDNA in *Escherichia coli*. Korean Biochem J. 22(2):154-160.

Dorion RP, Hamati HF, Landis B, Frey C, Heydt D, Carey D. 1998. Risk and clinical significance of developing antibodies induced by topical thrombin preparations. Arch Pathol Lab. Med. 122(10):887-894.

Enus S, Natadisastra G, Shahib MN, Sulaeman R. 2011. Peran lem fibrin otologus pada penempelan tandur konjungtiva bulbi mata kelinci terhadap ekspresi gen fibronektin dan integrin. [The role of autologous fibrin glue on the attachment of graft conjunctiva bulbi rabbit eye on the expression of fibrinection and integrin gene] MKB. 43(4):183-188. doi:10.15395/mkb.v43n4.67.

Haynes LM, Bouchard BA, Tracy PB, Mann KG. 2012. Prothrombin activation by platelet-associated prothrombinase proceeds through the prethrombin-2 pathway via a concerted mechanism. J Biol Chem. 287(46):38647-55. doi:10.1074/jbc.M112.407791.

Jonebring A, Lange U, Bucha E, Deinum J, Elg M, Lövgren A. 2012. Expression and characterization of recombinant ecarin. Protein J. 31(5):353-8. doi:10.1007/s10930-012-9409-6.

Ortel TL, Mercer MC, Thames EH, Moore KD, Law son JH. 2001. Immunologic impact and clinical outcomes after surgical exposure to bovine thrombin. Ann. Surg. 233(1):88-96. doi:10.1097/00000658-200101000-00014.

Pozzi N, Chen Z, Zapata F, Niu W, Barranco-Medina S, Pelc LA, Di Cera E. 2013. Autoactivation of thrombin precursors. J Biol Chem. 288(16):11601-10. doi:10.1074/jbc.M113.451542.

Pozzi N, Chen Z, Zapata F, Pelc LA, Barranco-Medina S, Di Cera E. 2011. Crystal structures of prethrombin-2 reveal alternative conformations under identical solution conditions and the mechanism of zymogen activation. Biochemistry. 50(47):10195-202. doi:10.1021/bi2015019.

Silaban S, Maksum IP, Ghaffar S, Hasan K, Enus S, Subroto, T, Soemitro S. 2014. Codon optimization and chaperone assisted solubilization of recombinant human prethrombin-2 expressed in *Escherichia coli*. Microbiol Indones. 8(4):177-182. doi: 10.5454/mi.8.4.5.

So IS, Lee S, Kim SW, Hahm KS, Kim J. 1992. Purification and activation of recombinant human pre thrombin-2 produced in *E. coli*. Korean Biochem J. 25:60-65

Streiff MB, Ness PM. 2002. Acquired FV inhibitors: a needless iatrogenic complication of bovine thrombin exposure. Transfusion 42(1):18-26. doi:10.1046/j.1537-2995.2002.00011.x.

Stricker RB, Lane PK, Leffert JD, Rodgers GM, Shu man MA, Corash L. 1988. Development of antithrombin antibodies following surgery in patients with pros thetic cardiac valves. Blood 72(4):1375-1380.

Tadokoro K, Ohtoshi T, Takafuji S, Nakajima K, Suzuki S, Yamamoto K, Ito K, Miyamoto T, Muranaka M. 1991. Topical thrombin-induced IgE-mediated anaphylaxis: RAST analysis and skin test studies. J Allergy Clin Immunol. 88(4):620-629. doi:10.1016/0091-6749(91)90156-I.

Tojo N, Miyagi I, Miura M, Ohi H. 2008. Recombinant human fibrinogen expressed in the yeast *Pichia pastoris* was assembled and biologically active. Prot Exp Purif. 59: 289-296. doi:10.1016/j.pep.2008.02.010.

Wai Y, Tsui V, Peng Z, Richardson R, Oreopoulos D, Tarlo SM. 2003. Anaphylaxis from topical bovine thrombin (Thrombostat) during haemodialysis and evaluation of sensitization among a dialysis population. Clin Exp Allergy 33:1730-1734. doi:10.1111/j.1365-2222.2003.01806.x.

Welch M, Villalobos A, Gustafsson C, Minshull J. 2009. You're one in a googol: optimizing genes for protein expression. J. R. Soc. Interface 6:S467-S476. doi:10.1098/rsif.2008.0520.focus.

Yonemura H, Imamura T, Soejima K, Nakahara Y, Morikawa W, Ushio Y, Kamachi Y, Nakatake H, Sugawara K, Nakagaki T, Nozaki C. 2004. Preparation of recombinant alpha-thrombin: high-level expression of recombinant human prethrombin-2 and its activation by recombinant ecarin. J Biochem. 135(5):577-82. doi:10.1093/jb/mvh070.

Zehnder JL, Leung LL. 1990. Development of antibod ies to thrombin and factor V with recurrent bleeding in a patient exposed to topical bovine thrombin. Blood 76:2011-2016.