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Secretory Expression of *Saccharomycopsisfibuligera* R64 α-Amylase with native signal peptide in *Pichiapastoris*

Shabarni Gaffar[a,*],Dani Permana[b], Dessy Natalia[c], Toto Subroto[a], and Soetijoso Soemitro[a]

[a]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, PadjadjaranUniversity*
Jl. Raya Bandung-Sumedang Km 21, Jatinangor, 45363, Sumedang,West Java, Indonesia
[b]*Research Center for Chemistry, Indonesian Institute of Science, Bandung, Indonesia*
[c]*Department of Chemistry,* Faculty of Mathematics and Natural Sciences, *InstitutTeknologi Bandung*
Jl. Ganesha 10, Bandung 40133,Indonesia

Abstract

Saccharomycopsisfibuligera R64 α-amylase (Sfamy) can degrade raw starch making it attractive for industrial application. The enzyme has been widely used in the food and textile industries, and recently in the generation of renewable energy. Here we report the secreted expression of Sfamy with native signal peptide in *Pichiapastoris* using the methanol controlled alcohol oxidase (*AOX1*) promoter. The gene for Sfamy with native signal sequence (*WTSfamy*) was amplified from*S.fibuligera* genomic DNA employing PCR method and was cloned using pPICZA expression vector for*Pichiapastoris*. Expression of Sfamy in*P .pastoris*was induced by addition of 0.75% methanol every 24 h. The result showed that Sfamy was secreted by*P.pastoris*to the culture supernatant. The highest activity of Sfamy in the culture supernatant was found at 72 h inductiontime, that was32.29 U/mL. The result showed that Sfamy with native signal sequence was recognize by*P. pastoris*secretion machine, which was confirmed by SDS-PAGE analysis with the molecular weight 54kDa.

*Keywords:*Sfamy; native signal peptide; *Pichia pastoris*

* Corresponding author. Tel.: +6222-7794391; fax: +6222-7794391.
E-mail address: sabarni.ghafar@unpad.ac.id

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1. Introduction

α-Amylase (1,4-α-D-glucanglucano hydrolase, EC3.2.1.1) catalyses the hydrolysis of 1.4-α-glycosidic on the starch. This enzyme together with other amylolytic enzymes involved in conversion of starch into a dextrin, modified starch, oligosaccharides, maltose, and glucose[1]. Amylase was used in industries that hydrolyze starch through enzymatic reactions such as textile, food, detergents, and medicines[2].

S.fibuligera R64 α-amylase (Sfamy) has the potential to be applied in industry because of its ability to degrade raw starch, although cannot absorb raw starch[3]. This enzyme is composed of 494 amino acids with a molecular weight of 54 kDa and contains 20 hydrophobic amino acids at the *N*-terminal, similar to that found in most of the secreted protein precursor[4]. Sfamy have a high homology with *Aspergillusoryzae* α-amylase (Taka-amylase), which consists of three domains, catalytic domains A/B and C domain, containing four disulfide bonds and one glycosylation side[5]. Methylotrophic yeast has been widely used as a host for recombinant protein expression because of its capability of high level expression. *P. pastoris* is suitable for producing eukaryotic proteins with proper folding and post-translational modification. More than 550 heterologous proteins have been successfully synthesized and produced in this yeast[6].

Secretion of heterologous proteins rather than cytoplasmic accumulation is most often the preferred option in Pichia-based production processes. Selection of a signal sequence for secretion of recombinant protein affects the level of protein secretion[7-8]. A number of different signal sequences, including the native signal sequence of heterologous protein, are recognized by the *P. pastoris* with variable secretion levels.

Sfamy is an extracellular enzyme containing *N*-terminal signal peptide that serves for its translocation into the endoplasmic reticulum. The use of Sfamy native signal sequence for secretion in *P. pastoris* expression system needs to be studied to determine the secretion level and to see if Sfamy native signal sequence is recognized by *P. pastoris* secretion machines.

2. Experimental

2.1 Strains, Vectors, and Reagents

S. fibuligera R64 was obtained from Hasan et al. (2008)[3]. *P. pastoris* GS115 (*AOX1*, *his4*) and *Escherichia coli* TOP10F' were both purchased from Invitrogen, whereas pPICZA vector from Invitrogen and pGEMT vector from Promega. Restriction endonucleases, Taq DNA polymerase, and T4 DNAligase were purchased from Fermentas. Oligonucleotide primers for PCR and DNA sequencing were synthesized by Research Biolabs (Singapore). Growth medium were obtained from Pronadisa.

2.2 Construction of Recombinant Plasmid pPICZA-WTSfamy

Genomic DNA was isolated from *S. fibuligera* R64 and used as a template for amplification of *Sfamy* gene with native signal sequence (*WTSfamy*) by PCR method using primer pairs 5' wtsf: 5'-GAGGAGGTACCATGC AAATTTCAAAAGCTG-3' and 3'wtsf :5'GAGGAGGGCCCTGAACAAATGTCAGAAGC-3'. PCR product (1475bp) was subcloned using pGEMT vectors to produce recombinant pGEMT-*WTSfamy* plasmid. Furthermore, pGEMT-*WTSfamy* plasmid was cut with *Apa*I and *Kpn*I, followed by insertion into the same site on pPICZA vector to produce pPICZA-*WTSfamy* plasmid. Nucleotide sequence was verified by DNA sequencing.

2.3 P. pastoris Transformation

P. pastoris transformation was performed by electro-transformation method[9]. Fifty microliters (50 µL) of GS115 competent cell prepared from cultures that growth until log phase were mixed with 1-5 µg of pPICZA-*WTSfamy* recombinant plasmid which has been linearized with *Pme*I. Electro-transformation was performed for ~5 milliseconds with a field strength of 7.5 kV/cm using a BTX electro cell manipulator 600. *P. pastoris* transformants were growth in YPD medium (1% of yeast extract, 2% of peptone, 2% of dextrose, and 2% of bacto agar) with the addition of zeocin 100 µg mL^{-1}, and incubated for 3 days at 30 °C.

2.4 Selection of Mut$^+$/Mut^{s}Transformant

Transformants were grown on MMH medium (minimal methanol histidine medium: 1.34% YNB, 4x10^{-5}% biotin, 0.5% methanol, 0.004% histidine) and MDH medium (minimal dextrose histidine medium: 1.34% YNB, 4x10^{-5}%, biotin, 2% dextrose, 0.004% histidine). Growing cells were analyzed after incubation at 30 °C for 2 days. GS115/His$^+$Mut^s /Albumin and GS115/His$^+$Mut$^+$/β-gal were used as Mut$^+$ and Mut^s controls phenotype (Invitrogen, 2006).

2.5 Expression of Sfamy by P. pastoris

P. pastoris transformant was grown in 10 mL of BMGY medium (buffered glycerol complex medium: 1% of yeast extract, 2% of peptone, 100 mM potassium phosphate pH 6, 1.34% YNB, 4x10^{-5}% biotin, 1% glycerol) with the addition of 2% sorbitol as an additional carbon source[10]. Cells were incubated at 30 °C for 24 h with shaking at 300 g until OD_{600} reached about 8-10. Cells were harvested by centrifugation at 3000 g for 20 min at 25 °C and then resuspended in 25 mL BMMH medium (buffered minimal methanol histidine: 1.34% YNB, 4x10^{-5}% biotin, 40 mg L^{-1} histidine, 100 mM potassium phosphate pH 6 and 0.75% methanol) before incubated for up to 144 h. Induction was done every 24 h by addition of methanol to a final concentration of 0.75% in the medium. Sampling was done every 24 h according to the time of induction and after 144 h, the cells were harvested by centrifugation at 3000 g for 20 min at 4 °C. Culture supernatant was analyzed for α-amylase activity and characterized by SDS-PAGE.

2.6 SDS-PAGE Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed under reduced conditions using 10% running gel and 4% stacking gel. Twenty-five micro liters of transformant *P .pastoris* culture supernatant was analyzed by SDS-PAGE. Protein bands on the gel were detected by Coomassie brilliant blue staining.

2.7 Analysis of Sfamy Activity and Protein Content

Sfamy activity was determined by DNS method[11]. Fifty microliters of sample was added to 325 L of 2% (w/v) soluble starch. 20 mM phosphate buffer was added to achieve the volume of 1 mL. The reaction mixture was incubated for 10 min at 50 °C. A total of 50 mL reaction mixture was added to 50 μL of DNS reagent and boiled for 7 min. Once cooled, samples were diluted 10-times with distillated water. Absorbance was measured at 500 nm. One unit of α-amylase activity was defined as the amount of enzyme that can liberate 1 mol reducing sugar per minute on the reaction conditions.Concentration of protein was analyzed using Bradford method[12]. The results of assay were shown as mean ± SD of data obtained in each experiment performed with three times repetition.

3. Results and Discussion.

3.1 Construction of pPICZA-WTSfamy expression plasmid

PCRprimers were designed based onthe nucleotide sequence of *S. fibuligera* α-amylase gene (*ALP1*) (GenBankX05791)[4].To confirm the species that we used, we perform 18SrDNA *S. fibuligera* R64 gene sequencing. The nucleotide sequence of *S. fibuligera* R64 18SrDNA was stored in Gen Bank (accession number HQ116832). PC Rusing 5'wtsf/3'wtsf primer pairs produced1.47 kb band (Figure 1A) containing *Sfamy* gene with an additional 78 nucleotides encoded signal sequence and also the restriction site of *Kpn*1 at the 5 'end and *Apa*I at the 3' end. PCR products were purified and ligated into pGemTvector and was subcloned in *E.coli* TOP10F'. pGem-*WTSfamy* recombinant plasmid was isolated from white colonies and cut using *Apa*I and *Kpn*1 resulted in two bands, 3.0kb of pGemTand1.47 kb of *WTALP1* (Figure1A). The 1.47 kb fragment was purified from the gel and inserted into the same site on pPICZA vector to generate a pPICZA-*WTSfamy* recombinant plasmid and cloned in *E.coli* TOP10F'. Nucleotide sequence analysis showed that the pPICZA-*WTSfamy* expression plasmid has been successfully constructed (data not shown). Nucleotide sequence of *S. fibuligera* R64 α-amylase gene was stored in Gen Bank (accession number HQ172905). Figure 1B shows a map of expression plasmid.

3.2 P. pastoris transformation and determination of phenotype

pPICZA-*WTSfamy* plasmid was linearized by using *Pme*1 enzymes prior to transformation into *P. pastoris* GS115 (*AOX*, *his4*). All of transformants would have Mut$^+$ phenotype because of the presence of *AOX1* gene[12].

Expression cassette is integrated into the *P. pastoris* genome in the 5 'end of *AOX1* locus by homologous recombination produces the Mut^+ and His^- phenotype. Mut^+ phenotype was confirmed by growing the transformants on MMH and MDH medium. The colonies with Mut^+ phenotype would thrive in both medium, and the colonies with the Mut^s (methanol utilization slow) phenotype would not grow or showed only limited growth on MMH medium (data not shown). The presence of inserts in the genomic DNA of *P. pastoris* transformant was verified by PCR analysisusing 5'AOX and 3'AOX primers (data not shown).

3.3 Sfamy secretion analysis

P. pastoris [*WTSfamy*] transformant was used to express Sfamy. Culture supernatants were collected and α-amylase activity was analyzed every 24 h. GS115 host strain was used as a control. α-Amylase secretion level was increased significantly starting at 48 h and then decreased after 96 h. At 72 h Sfamy activity was optimum at 32.29 U mL^{-1} and the protein concentration was 0,067 mg mL^{-1} (Figure 2A). α-Amylase activity in the GS115 host supernatant was very low (data not shown), while in the pellet the activity was barely detectable (data not shown). The results of SDS-PAGE analysis showed that the size of Sfamy is 54 kDa. The highest secretion was detected at 72 h after induction (Figure 2B), but decrease significantly at 96 h, which might be caused by protease activity.

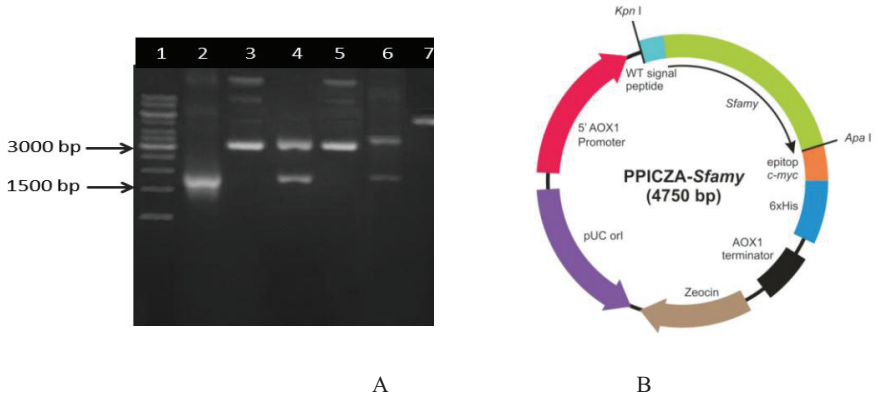

Fig. 1.Construction of pPICZA-*WTSfamy*plasmid. (A) Electrophoresis agarose analysis: (1) 1 kb DNA Ladder, (2) PCR productof *WTSfamy*~1,47 kb, (3) pGemT-*WTSfamy*(~4,47 kb), (4) pGemT-*WTSfamy*/*Kpn*1/*Apa*1 (~3,0 kb and 1,47 kb), (5) pPICZA-*WTSfamy*(~4,8 kb), (6) pPICZA-*WTSfamy*/*Kpn*1/*Apa*1 (~3,3 kb and 1,47 kb), (7) pPICZA-*WTSfamy*/*Pme*1 (~4,8 kb) (B) Map of pPICZA-*WTSfamy*plasmid.

Secretion of heterologous proteins, rather than cytoplasmic accumulation, is most often the preferred option in Pichia-based production processes. The yeast secretory system is, thus, an important engineering target to obtain optimized strains capable of processing and produce a large flux of recombinant protein[14]. The use of native signal sequence of heterologous protein could be used as the first approach to produce extracellular protein in *P. pastoris*.

In this study, we found that Sfamy with native signal peptide was secreted efficiently by *P. pastoris*. There are many factors that can affect the efficiency of recombinant protein production in *P. pastoris*, one of them is a signal peptide[15]. Selection of the signal peptide must be done if the secretion of desired protein is needed. In this research we studied the use of native signal peptide of Sfamy for secretion of Sfamy by using *P. pastoris* expression system. The results showed that the native signal peptide of Sfamy was recognized by *P. pastoris* secretion machines, with resultant of Sfamy active in the culture supernatant.

SDS-PAGE analysis showed that the molecular weight of Sfamy was 54 kDa, as also reported by Hasan *et al*. (2008)[3]. This result indicated that Sfamy was processed efficiently by *P. pastoris*. No other proteins were found in the culture supernatant, indicating that *P. pastoris* do not (or very little) secrete endogenous proteins, therefore it will simplify the purification process. Sfamy's signal peptide contains 20 amino acids located at its *N*-terminal. The signal peptide has recognition site of Kex2 protease (Kex2p), which might be needed in Sfamy maturation. Kex2p recognized a pair of basic amino acid residues and cut at these sites Lys-Arg (KR) and Arg-Arg. This protease is

also present in *P. pastoris*[16]. The presence of protease activity during induction time,reduced the expression level. The length of induction time had an effect on the extent of proteolysis. Longer induction time, causing more and more proteases released from the cells into the culture supernatants. Proteolysis was found to increase over time when the number of viable cells decreased[17]. Protease deficient host is also needed to reduce the effects of proteolysis.

A number of recombinant proteins have also been expressed in *P. pastoris* using the native signal peptide of heterologous protein[18-19]. In other proteins, however, the use of native signal peptide generates a low level of expression. So there is no guarantee whether the native signal peptide will result in a higher secretion, because the results vary widely[20-21].

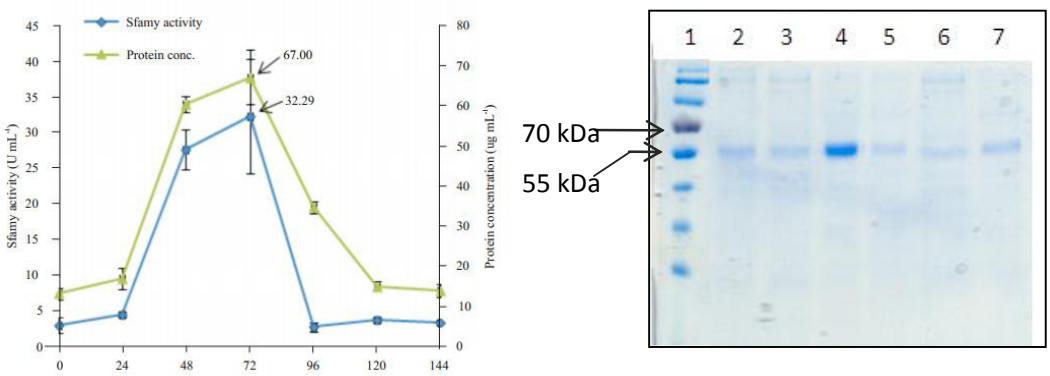

Fig. 2.Sfamy secretion by *P. pastoris* [*WTSfamy*]. A: Graph of Sfamy activity and protein concentration every 24 h induction time. Culture was collected every 24 h and centrifuged to separate the supernantant from the pellet. Sfamyactivity were measured using DNS method, protein concentration were measured by Bradford method; B: SDS-PAGE analysis of culture supernatant every induction time. (1) Protein marker, (2-7) 25 µL of culture supernatant at 24-144 hour induction time.

Expression and secretion of phytohaemagglutinin (PHA) is an example of the use of native signal peptide that produced a secreted protein where the amino terminal was processed and the folding was proper[19]. On the other hand, when PHA was secreted using *S. cerevisiae* α-mating factor signal peptide, the amino terminal was processed incorrectly. However, the use of native signal peptide for PHA secretion resulted low yield secreted protein[19]. Another example is the expression of bacterial thermostable α-amylase in *P. pastoris* using the SUC2 signal peptide of *S. cerevisiae* and the native signal peptide. The experiments showed that both signal peptide produced efficient secretion[7]. In this study we found that the secretion of Sfamy using native signal peptide produced active protein, even though the activity waslow. The pre-pro signal sequence of the *S. cerevisiae* α-mating factor is most often used to induce Sec61p-mediated translocation of the protein into the endoplasmic reticulum of *P. pastoris*. This signal sequence works in most cases, although there have been almost no studies to compare it to other signal sequences[23]. Moreover, the Kex2p/Ste13p-mediated processing of the pro-peptide in this *S. cerevisiae* sequence is often problematic in *Pichia*, resulting in non-native amino acids at the N-terminus of the heterologous protein. The genome sequence of *P. pastoris* reveals a multitude of endogenous signal sequences of *P. pastoris* which may be suited for mediating heterologous protein secretion[14].

To determine the effect of signal peptide selection on the secretion level of Sfamy by *P. pastoris*, further research needs to be performed using other signal sequence commonly used in *P. pastoris*, such as *S. cerevisiae* α-mating factor signal sequence.

Conclusions

Secretion of Sfamy with native signal peptide in *P. pastoris* produced an efficient secretion into the growth medium. Sfamy with native signal peptide was recognized and processed properly by the secretion machinary of *P. pastoris*. *P. pastoris* secreted very little endogenous proteins to culture medium so that the Sfamy purification process would be easier.

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