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A Novel Potential Signal Peptide Sequence and Overexpression of ER-Resident Chaperones Enhance Heterologous Protein Secretion in Thermotolerant Methylotrophic Yeast *Ogataea thermomethanolica*

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Abstract The thermotolerant methylotrophic yeast *Ogataea thermomethanolica* is a host for heterologous protein expression via secretion to the culture medium. Efficient secretion is a major bottleneck for heterologous protein production in this strain. To improve protein secretion, we explored whether the use of a native signal peptide sequence for directing heterologous protein secretion and overexpression of native ER-resident chaperone genes could improve heterologous protein secretion in *O. thermomethanolica*. We cloned and characterized genes encoding α-mating factor (Otα-MF) and ER-resident chaperones *OtBiP*, *OtCNE1*, and *OtPDI*. The pre and pre-pro sequences of Otα-MF were shown to promote higher secretion of heterologous endoxylanase comparing with the classical pre-pro sequence of *Saccharomyces cerevisiae*. However, in the case of heterologous glycosylated phytase, only

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the Otα-MF pre-pro sequence significantly enhanced protein secretion. The effect of chaperone overexpression on heterologous protein secretion was tested in cotransformant cells of *O. thermomethanolica*. Overexpression of ER-resident chaperones improved protein secretion depending on heterologous protein. Overexpression of *OtBiP*, *OtCNE1*, and *OtPDI* significantly increased unglycosylated endoxylanase secretion at both 30 and 37 °C while only *OtBiP* overexpression enhanced glycosylated phytase secretion at 30 °C. These observations suggested the possibility to improve heterologous protein secretion in *O. thermomethanolica*.

Keywords Methylotrophic thermotolerant yeast · *Ogataea thermomethanolica* · Molecular chaperones · Secretion signal sequence

Introduction

Yeast is widely exploited as a cell factory for a heterologous protein production. It grows rapidly and reaches high cell density in defined medium. Yeast also has an efficient protein secretion system which is suitable for large-scale production and downstream processes. Unlike prokaryotes, yeast has a repertoire of eukaryotic posttranslational modifications. Therefore, yeast has been widely used as host for commercial production of many recombinant proteins, in particular those requiring secretion and posttranslational modification [1]. Secretion of some recombinant proteins can be rather low, and strategies for improving secretion in yeast include engineering of the protein secretion pathway, exploitation of strong promoters, increasing gene dosage, and optimization of culture condition [2]. Among these strategies, engineering of the early secretory traffic pathway from the cytosol to the endoplasmic reticulum (ER) by modification of signal peptide sequence and engineering of protein folding and quality control in ER was found to be the most critical for protein secretion in yeast [3, 4].

In general, the protein secretion pathway in yeast starts from recognition of a signal peptide sequence of nascent peptide by the signal recognition particle (SRP). Newly synthesized peptides are translocated co- or posttranslationally into the ER lumen through the Sec61 translocon [3]. The signal peptide sequence is typically composed of 20–30 amino acids which can be divided to three domains, namely a basic N-domain, a 7–13 residue hydrophobic H-domain, and a slightly polar C-domain [5]. The protein targeting specificity is mainly conferred by the hydrophobic core of the signal peptide sequence. Although many types of signal peptide sequences have been developed for each yeast secretory system, α-mating factor (α-MF) pre-pro signal sequence of yeast *Saccharomyces cerevisiae* is most commonly used [6]. The α-MF pre-pro sequence of *S. cerevisiae* consists of a 19 amino acid pre-sequence followed by a pro-sequence of 70 residues containing three consensus *N*-linked glycosylation sites, a dibasic Kex2 endopeptidase (KR), and Ste13 dipeptidyl aminopeptidase (EAEA) processing sites. Processing of α-MF pre-pro sequence occurs in three steps. First, the peptide is translocated into the ER where the pre-sequence is removed by signal peptidases. Then, the pro-sequence is cleaved by Kex2 endopeptidase between the KR residues. Finally, Ste13 protein removes the EA repeats at the N terminus of peptide in the Golgi [7]. Several strategies have been applied to improve the secretory potential of the α-MF signal sequence. These include codon optimization [8], directed evolution [9], insertion of spacers and deletion mutagenesis [10], and optimization of the Kex2 P1′ site [11].

Besides protein translocation into the ER, protein folding in the ER plays the central role in the secretory process and is a rate-limiting step for the secretion of heterologous proteins. After translocation into the ER, the nascent polypeptide may undergo one or several posttranslational modifications controlled by several chaperones including assisted protein folding, disulfide-bond formation, and glycosylation. Nascent polypeptides are bound by the ER-resident chaperone binding protein (BiP or Kar2; a member of the Hsp70 family) and folded to native structures, whereas nascent glycoproteins are bound by the ER chaperone calnexin (encoded by *CNE1*) to undergo their correct folding and *N*-glycan processing. In addition, correct disulfide bond formation of many proteins is mediated by protein disulfide isomerases (PDI). The correctly folded protein is then targeted to the Golgi network via a transported vesicle for further posttranslational modifications. The secretory protein is sorted by a specialized vesicle that fuses with the plasma membrane for its release outside of the cell [2]. Recently, it has become evident that upon introduction of heterologous genes into the host cell, heterologous proteins are often misfolded and retained in the cytoplasm or ER as a result of suboptimal folding conditions, e.g., limitation of molecular chaperones. The accumulation of aberrant proteins causes ER stress that leads to attenuation of protein synthesis and reduced protein secretion [12]. To overcome this bottleneck, overexpression of ER-resident chaperones such as BiP/Kar2p, PDI, and CNE1 have been found to enhance recombinant protein secretion in yeasts [13–18].

Thermotolerant methylotrophic yeast *Ogataea thermomethanolica* (formerly identified as *Pichia thermomethanolica* and previously isolated in Thailand [19]) has recently emerged as a new potential host strain for heterologous protein production [20–22]. *O. thermomethanolica* can produce heterologous protein across a broad temperature range (30–42 °C). This property should be useful for large-scale fermentation since it should allow for flexible cultivation conditions without strict temperature regulation. In addition, fermentation processes conducted at elevated temperatures will reduce cooling cost [21, 22]. However, a major drawback of this host system is that the expression level of heterologous protein is still not as high as that obtained from the *Komagataella* (*Pichia*) *pastoris* system [23].

To improve protein secretion in *O. thermomethanolica*, this study focused on engineering early secretion pathway from cytosol to ER and increasing the levels of ER-resident chaperones. The secretion signal of *O. thermomethanolica* α-MF was isolated and employed for heterologous protein secretion. Our results showed that the endogenous *O. thermomethanolica* α-MF signal peptide could direct secretion of heterologous enzymes efficiently into the medium. Three major ER-resident molecular chaperone genes were isolated from *O. thermomethanolica* (*OtBiP*, *OtPDI*, *OtCNE1*) and overexpressed together with heterologous reporter proteins. Overexpression of *OtBiP*, *OtCNE1*, and *OtPDI* genes were found to improve the secretion of heterologous protein in *O. thermomethanolica*, especially for unglycosylated protein.

Materials and Methods

Strains and Plasmids

O. thermomethanolica BCC16875 was obtained from the BIOTEC Culture Collection [BCC, Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand]. It was grown at 30 °C on yeast peptone dextrose (YPD) medium.

Escherichia coli DH5α was employed for all plasmid propagation and manipulation and selected on LB supplemented with zeocin (25 μg/mL) or kanamycin (25 μg/mL). Transformant yeast cells were selected on YPD agar supplemented with zeocin (100 μg/mL) or geneticin (100 μg/mL). Plasmids pGAPZA and pGAPZαA (Invitrogen) were used for construction of plasmids pOtGAPZA and pOtGAPZαA harboring the native GAP promoter of *O. thermomethanolica* [21]. Plasmid pOtGAPZNeo harboring neomycin resistant cassette was used for expression of target ER-resident chaperone genes. Endoxylanase and phytase encoding genes were obtained from plasmid pPICZαA-rXylB [24] and pPICZαA-rPhyA [23], respectively. Plasmids pOtGAPZα-Xyl and pOtGAPZα-Phy were constructed for extracellular expression of endoxylanase and phytase, respectively.

Construction of Expression Vectors Harboring Novel α-MF Signal Sequence of *O. thermomethanolica*

The putative α-MF gene of *O. thermomethanolica* was identified from its genome sequencing (unpublished data) by analyzing for a repeating KREA/EA pattern within the encoded peptide sequence. Among the hits, only one putative α-MF of *O. thermomethanolica* was identified. Signal peptide search within the *O. thermomethanolica* α-MF putative protein sequence was conducted using the SignalP4.1 [25] and Pro1.0 servers [26]. *N*- and *O*-glycosylation sites were predicted by NetNGlyc 1.0 and NetOGlyc 4.0 Servers [27]. The signal peptide-encoding pre (Otαpre, 54 bp) and pre-pro sequences (Otα, 114 bp) of *O. thermomethanolica* α-MF were amplified by PCR using primers Otpre-NspV-Fw, Otpre-EcoRI-Rv and Otα-NspV-Fw, Otα-EcoRI-Rv, respectively (Supplementary data, Fig. S1, Table S1). The Otαpre and Otα DNA amplicons were digested with *Nsp*V and *Eco*RI restriction enzymes and subcloned into pOtGAPZαA replacing the pre-pro sequence of *S. cerevisiae* (Scα). The resulting plasmids pOtGAPZOtαpre and pOtGAPZOtα were used for expression of endoxylanase and phytase. Endoxylanase (Xyl) and phytase (Phy) genes were amplified from pPICZαA-rXylB and pPICZαA-rPhyA using primers Xyl-EcoRI-Fw/Xyl-XbaI-Rv and Phy-EcoRI-Fw/Phy-XbaI-Rv, respectively (Supplementary data, Table S1). The resulting plasmids pOtGAPZOtαpre-Xyl, pOtGAPZOtα-Xyl, pOtGAPZOtαpre-Phy, and pOtGAPZOtα-Phy were used for expression of enzymes. The vectors harboring endoxylanase and phytase genes with Scα and without the signal sequence were similarly constructed. The resulting constructs pOtGAPZαA-Xyl, pOtGAPZαA-Phy, pOtGAPZ-Xyl, and pOtGAPZ-Phy were used as a control.

Cloning of ER-Resident Chaperone Encoding Genes from *O. thermomethanolica*

ER-resident chaperone encoding genes from *O. thermomethanolica* (*OtBiP*, *OtCNE1*, *OtPDI*) were obtained by PCR walking before its genome sequencing has been analyzed. To identify those genes, a set of degenerate primers was designed based on the conserved regions of genes encoding *BiP*, *CNE1*, and *PDI* from various yeasts (Supplementary data, Table S1). PCR amplification of partial genes was performed using genomic DNA (gDNA) of *O. thermomethanolica* as a template. PCR products were gel purified and ligated to pTZ57R/T vector (Fermentas, Lithuania). Recombinant plasmids were extracted and DNA insertion was verified by Sanger sequencing (Macrogen, Korea). In order to obtain upstream and/or downstream sequences of target genes, genome walking was done with a GenomeWalker Universal kit according to the manufacturer's instructions (Clontech, Takara Bio Inc., Japan). PCR walks were performed using the outer adaptor primer from the kit (AP1)

together with gene-specific primers designed from the partial sequence of each gene from degenerate-primed PCR (Supplementary data, Table S1). The products of these reactions were used as templates for nested PCR using the nested adaptor primer from the kit (AP2) and a nested gene-specific primer for each gene walk (Supplementary data, Table S1). The major PCR band obtained from each walk was gel purified and ligated into pTZ57R/T. Full-length gene sequences were obtained by automated DNA sequencing (Macrogen, Korea).

Construction of the Plasmids for Expression of ER-Resident Chaperone Genes and Recombinant Enzymes in *O. thermomethanolica*

For intracellular expression of ER-resident chaperone genes (*OtBiP*, *OtCNE1*, *OtPDI*) in *O. thermomethanolica*, the pOtGAPZA plasmid harboring the endogenous *O. thermomethanolica* GAP promoter [21] was used. The zeocin (*Sb Ble*) resistance cassette of the plasmid was replaced by a neomycin (*Neo*) resistant cassette (accession no. AAD47089) via the *Nco*I and *EcoR*V sites. This selectable marker expresses in *E. coli* and yeast under the control of the EM7 and TEF1 promoters, respectively. The resulting plasmid pOtGAPZNeo was used for cloning of target ER-resident chaperone genes that were amplified by PCR using gene-specific primers (Supplementary data, Table S1). The resulting recombinant plasmids named as pOtGAPNeo: BiP, pOtGAPNeo: CNE1, and pOtGAPNeo: PDI were selected on LB agar supplemented with 25 μg/mL kanamycin and further confirmed by automated DNA sequencing (Macrogen, Korea). Positive plasmids were used for transformation into *O. thermomethanolica* by electroporation [20] and selected on YPD agar supplemented with 100 μg/mL geneticin (G418). Specific integration of expression plasmid at OtGAP promoter was verified by colony PCR with 5′-OtGAP-promoter flanking (OtGAP-Flk) primer and 3′-reverse primer for each gene (Supplementary data, Table S1).

To construct expression plasmids for extracellular expression of endoxylanase and phytase under the control of the OtGAP promoter, the endoxylanse and phytase genes were sub-cloned from plasmids pPICZαA-rXylB [24] and pPICZαA-rPhyA [23], respectively. The gene fragments were digested with *Eco*RI and *Xba*I and then ligated with the pOtGAPZα vector. The sequences of the resulting plasmids (pOtGAPZα-Xyl and pOtGAPZα-Phy) were verified by automated DNA sequencing. *O. thermomethanolica* strains overexpressing ER-resident chaperone were transformed with pOtGAPZα-Xyl and pOtGAPZα-Phy and selected on YPD agar supplemented with 100 μg/mL zeocin. Specific integration of expression plasmid at the OtGAP promoter was verified by colony PCR with 5′-OtGAP-promoter flanking (OtGAP-Flk) primer and 3′-reverse primer of each gene (Supplementary data, Table S1).

Expression of Recombinant Enzymes in *O. thermomethanolica*

To analyze the extracellular expression of endoxylanase or phytase, a single colony of the recombinant yeast was inoculated into 5 mL of YPD and incubated at 30 °C overnight with 250 rpm shaking. A starter culture was transferred to 50 mL of YPD medium with an initial OD_{600} of 0.2 and continually cultivated at 30 °C with 250 rpm shaking. After an appropriate incubation, aliquots of cell culture were harvested and subjected to protein analysis by SDS-PAGE and assayed for enzymatic activity. The enzymatic hydrolysis activity of endoxylanase was measured by the 3,5-dinitrosalicyclic acid (DNS) method [28] using xylan as a substrate under the optimal condition described previously [24]. The activity of phytase was determined

by the color formation between molybdate and the released inorganic orthophosphate from phytate [29] under the optimal condition described previously [23]. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol/min of reducing sugar or inorganic phosphate.

Quantitative RT-PCR Analysis of *CNE1*, *PDI*, and Target Enzyme Genes

Total RNA of *O. thermomethanolica* cells was extracted by enzymatic lysis (RNeasy Midi Kit; Qiagen, Germany). RNA was quantified by absorbance (A_{260}) and reverse transcription was performed using Improm-II™ Reverse Transcription System (Promega) with 1 μg total RNA and 0.5 μg Oligo-dT primer, which yielded 20 μL of cDNA. Quantitative PCR was carried out in a total volume of 20 μl containing 5 μL of diluted cDNA, 0.3 μM of forward and reverse primers (Supplementary data, Table S1), and 10 μL of 2× SYBR Green master mix (Kapabiosystems, USA). Each sample was analyzed in triplicate whereas a non-template control of each pair of primers was included. For examination of the relative changes in mRNA levels, the actin gene was used as a reference.

Western Blot Analysis of Recombinant BiP

For Western blot analysis of BiP, total protein was extracted as previously reported [30] and resolved in 10 % SDS-PAGE. After eletroblotting onto a polyvinylidene difluoride membrane (Biorad), target protein was detected with specific BiP antibody (anti-HDEL) purchased from Santa Cruz Biotechnology at a 1:2000 dilution. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) was used as a secondary antibody at a 1:5000 dilution. After washing for three times with PBST, the membrane was incubated in the detection solution of SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, USA) and autoradiographed as described by the manufacturer. For examination of the relative changes in BiP levels, the reference actin was probed with primary antibody against eukaryotic actin (Santa Cruz Biotechnology).

Results

Identification of the Novel α-MF Protein of *O. thermomethanolica*

The most widely used signal peptide sequence for recombinant protein secretion in yeasts is the α-MF pre-pro sequence of *S. cerevisiae*. Recently, this signal sequence was successfully employed for heterologous protein secretion in *O. thermomethanolica* [20–22]. We hypothesized that *O. thermomethanolica* has an α-MF homolog and that the signal sequence of this protein could direct efficient secretion of heterologous protein in *O. thermomethanolica*. We identified one protein sequence (179 amino acids) containing KREA/EA repeats and a putative mature protein (R/GWGWHGVSRNEAIF, Fig. 1). This protein was compared with α-MF homologs in other yeasts, including *S. cerevisiae*, *K. pastoris*, *Nakaseomyces delphensis*, *Candida albicans*, *Eremothecium cymbalariae*, and *Hansenula polymorpha*. Yeast α-MFs are composed of 2–9 identical copies of a mature peptide with 13–14 amino acids in length. Interestingly, the mature peptide sequence repeats are preceded by a 20-residue pro sequence in *O. thermomethanolica* that is substantially shorter than the 60–100 amino acid long pro

(A) *Saccharomyces cerevisiae*

MRFPSIFTAVLFAASSALA*APVNTTTEDETAQIPAEAVIGYLDLEGDFDVAVLPFSNSTNNGLLFINTTIA SIAAKEEGVSLD*KREAEAWHWLQLKPGQPMYKREAEAEAWHWLQLKPGQPMYKREADAEAWHWLQLKPGQP MYKREADAEAWHWLQLKPGQPMY

(B) *Komagataella pastoris*

MKSLILNIISVTLAITSTAA*SAPVESIFANQPDSSLTDTNDGVGVGMSTIKEEDFGKHFVENQILDEAVIM SLKLRKGVNLFFLDDIGLATELIGNKIAQIEAIDLSERLAQSWTNIRKNRLFG*KREAEAEAEAEAFRWRNN EKNQPFGKREAEAEAEAEAEAEAEAFRWRNNEKNQPFGKREAEAEAEAEAEAFRWRNNEKNQPFG KREAEAEAEAEAFRWRNNEKNQPFGKREADAEAEAEAFRWRNNEKNQPFGKREAEAEAEAEAFRWRNNE KNQPFGKREAEAEAEAEAEAFRWRNNEKNQPFGKREAEAEAEAEAFRWRNNEKNQPFGKREADAEAEAE AEAFRWRNNEKNQPFGKREASIDTGTDDGAYWSWRKNSVLERQ

(C) *Nakaseomyces delphensis*

MKFSKILIAAASILTAVQA*APVENVDDSAQVPEEAIIGYIDFEGASDVAILPFSNSTDSGLMFVNTTIYNE ATTAVEGESVE*KREAKWHWLSVRPGQPIYKREAEAEAKWHWLSVRPGQPIYKREAEAEAKWHWLSVRPGQP IYKREAEADAEARWHWLSVRPGQPIY

(D) *Candida albicans*

MKFSLTLLTATIATIVAA*APAQYTGQAIDSNQVVEIPESAVEAYFSIDDELTPVFGEIDNKPVILIVNGTI LTSGANNE*KREAKSKGGFRLTNFGYFEPGKRDANADAGFRLTNFGYFEPGKRDANAEAGFRLTNFGYFEPG K

(E) *Eremothecium cymbalariae*

MKFYNILSVASIASLVFA*APVSVNDAKEIAATFPQEALLGFLDLTDAENIVILSLVDEEKSGIALVNKTIW ATARSEQAAGIS*KRDADAWHWLRFDRGQPIHKRSADAVADAWHWLRFDRGQPIHKRSADAVADAWHWLRFD RGQPIHKRSADAVADAWHWLRFDRGQPIHK

(F) *Hansenula polymorpha*

MIFNRAAFYAMILAVAYAAPIAEAEA*EPLPEANALP*GWGWHRVNRNEVIFKREASPDAEAEAGWGWHRVSR NEVIFKREAESS

(G) *Ogataea thermomethanolica*

MKFNTTLLLSVLLSVSYA*APVALAEAEAVAAAGADAEAR*WGWHGVSRNEAIFKREAEPEAEPEAKPEARWG WHGVSRNEAIFKREADAEADAEANAEAQPEADAEAQPEAQPEAQAEAGWGWHGVSRNEAIFKREAEAGPDA DAQPDAQADAQPEARWGWHGVSRNEAIFKREPEPESS

Fig. 1 Comparison of α-mating factor proteins of yeasts. **a** *Saccharomyces cerevisiae* S288c, **b** *Komagataella pastoris* CBS7435, **c** *Nakaseomyces delphensis* CBS2170, **d** *Candida albicans* P75016, **e** *Eremothecium cymbalariae* DBVPG#7215, **f** *Hansenula polymorpha* DL-1, and **g** *Ogataea thermomethanolica* BCC16875. Sequences for comparison were obtained from GenBank: *S. cerevisiae* (accession no. P01149); *K. pastoris* (accession no. CCA38209); *Nakaseomyces delphensis* (accession no. AAO25615); *Candida albicans* (accession no. KHC75818); *Eremothecium cymbalariae* (accession no. XP_003644796); *H. polymorpha* (accession no. ESX02024). Mature α-mating factor peptide (*underlined*), KREA/EA repeats and its homolog (*gray*), pre sequence (*bold*), and pro sequence (*italic*)

sequence in other species. The *O. thermomethanolica* pro sequence lacks an identifiable Kex2p processing site (KR) at the C terminus. However, a putative Ste13p processing site

(DAEA) was observed. In *O. thermomethanolica* α-MF, four serine residues (S46, S76, S126, S164) are putative *O*-glycosylation sites.

Secretion of Recombinant Proteins Using Pre and Pre-Pro Signal Sequence of *O. thermomethanolica*

Previously, it was shown that the α-MF pre-pro sequence of *S. cerevisiae* drives secretion of heterologous proteins in *O. thermomethanolica* efficiently [20–22]. This was expected since this sequence is functional as a secretion signal in a variety of yeasts [10]. We hypothesized that the native α-MF signal sequence of *O. thermomethanolica* would function similarly as a signal sequence for protein secretion, and perhaps function even more efficiently than *S. cerevisiae* α-MF pre-pro sequence for driving heterologous protein secretion in *O. thermomethanolica.* To test this hypothesis, we constructed recombinant vectors harboring endoxylanase and phytase coding sequences fused in frame with pre and pre-pro sequence (Fig. 2). The plasmids were transformed into *O. thermomethanolica* and transformants were screened for the secretion of these enzymes. The putative pre and pre-pro sequences of *O. thermomethanolica* α-MF drive secretion of recombinant endoxylanase (~20 kDa) about 1.7-fold more efficiently than the *S. cerevisiae* pre-pro sequence (Fig. 3a). For glycosylated phytase (~100 kDa) secretion, about 2-fold higher expression was observed in the transformant harboring the pre-pro sequence of *O. thermomethanolica*, whereas no significant difference was observed for the transformant harboring the *O. thermomethanolica* pre sequence (Fig. 3b).

Cloning and Characterization of Novel ER Resident Chaperone Genes from *O. thermomethanolica*

Full-length sequences of *O. thermomethanolica BiP*, *CNE1*, and *PDI* encoding homologous genes (*OtBiP*, *OtCNE1*, and *OtPDI*) were isolated by genome walking. For the *OtBiP* gene, the putative coding sequence of 2001 bp that showed high similarity to the *BiP* gene of other

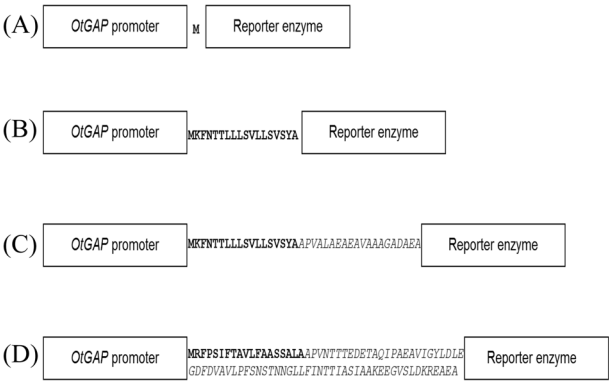

Fig. 2 The constructs developed for evaluate the *O. thermomethanolica* signal sequence properties under the regulatory control of *OtGAP* promoter. Reporter enzymes are endoxylanase and phytase. The signal sequence is designed with single amino acid code. **a** The negative control vector with mature reporter enzyme without any signal sequence. Methionine was presented at N terminus of reporter enzyme. **b** The mature reporter enzyme with native pre signal sequence of *O. thermomethanolica*. **c** The mature reporter enzyme with native pre-pro signal sequence of *O. thermomethanolica*. **d** The positive control vector with mature reporter enzyme fused to native pre-pro signal sequence of *S. cerevisiae*. Pre sequence (*bold*) and pro sequence (*italic*)

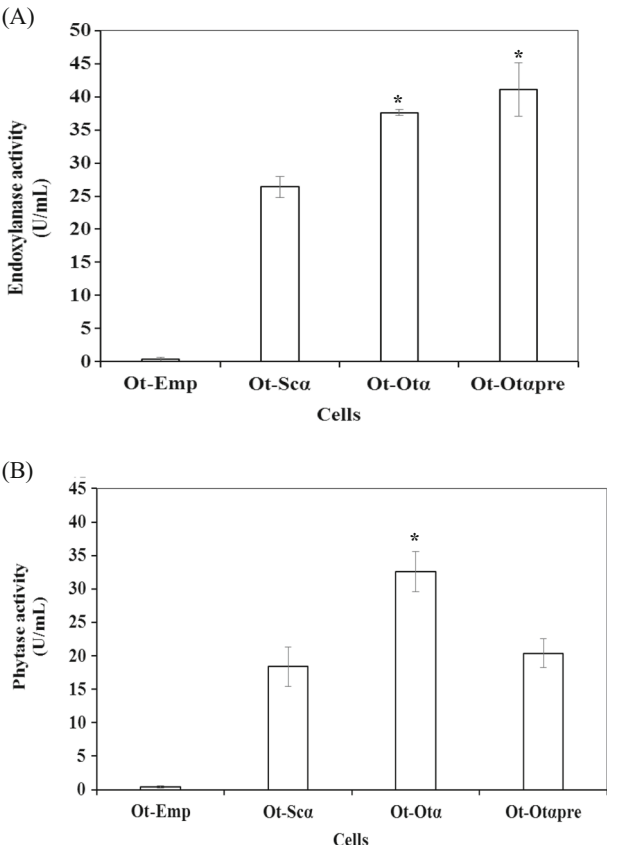

Fig. 3 Enzyme activity of α-MF secretion signal strains. Selected α-MF signal sequences [pre-pro sequence of *S. cerevisiae* (Scα), pre-pro sequence of *O. thermomethanolica* (Otα), pre sequence of *O. thermomethanolica* (Otαpre)] were fused to reporter enzyme endoxylanase (**a**) and phytase (**b**). Emp was the control vector with mature endoxylanase and phytase sequence without any signal sequence. *O. thermomethanolica* strains harboring the recombinant signal peptide were cultivated on YPD medium and harvested after 24 h. Significant improvement of secretion comparing with Scα was indicated by an *asterisk* (*P* value <0.05) from *t* test

yeasts was identified. Its deduced ORF of 666 amino acids was most similar to BiP of *Ogataea parapolymorpha* DL-1 (accession no. EFW95375) with 92 % identity (Supplementary data, Fig. S2). It also showed 79 % identity to BiP of *K. pastoris* (accession no. XP_002491027). BLASTp result of OtBiP protein demonstrated HSP70 conserved domain (data not shown). Moreover, the protein contains a C-terminal-HDEL sequence which is a typical ER-retention signal. BiP belongs to Hsp70 chaperones that have been reported to assist the proteins folding. Hsp70 activity is ATP dependent and Hsp70 proteins are made up of two regions: the amino terminus is the ATPase domain and the carboxyl terminus is the substrate binding region [31].

The putative coding sequence of 1671 bp was identified for *OtCNE1* gene. The deduced amino acid sequence of this ORF (556 amino acids) displayed a high sequence identity (73 %) to a calnexin-like protein of *O. parapolymorpha* DL-1 (accession no. EFW96881) and 60 % identity to calnexin of *K. pastoris* (accession no. XP_002491218) (Supplementary data, Fig. S3). BLASTp analysis of OtCNE1 demonstrated a calreticulin conserved domain (data not shown). Calnexin and calreticulin are calcium-binding molecular chaperones that localize to the ER. They promote correct folding of proteins and target misfolded proteins for degradation [32].

In addition, a putative *OtPDI* gene (1590 bp) was identified. Its deduced ORF of 529 amino acids has highest similarity to the PDI of *O. parapolymorpha* DL-1 (accession no. EFW96462)

with 74 % identity (Supplementary data, Fig. S4). A typical ER-retention signal (HDEL) sequence was found at the C terminus. OtPDI also contains four thioredoxin domains, which are found among eukaryotic protein disulfide isomerases that are retained in the ER [33]. Based on these results, we concluded that the gene isolated encodes the *O. thermomethanolica* homolog of the ER-resident chaperones (*BiP*, *CNE1*, and *PDI*).

Expression of Heterologous Enzymes in *O. thermomethanolica* Overexpressing ER-Resident Chaperone

To prove that heterologous ER-resident chaperone genes can improve the secretion efficiency in *O. thermomethanolica*, *O. thermomethanolica* overexpressing ER-resident chaperone genes were co-expressed with two target enzymes, namely unglycosylated endoxylanase and glycosylated phytase, under the control of the *OtGAP* promoter. Transformants of each construct which showed equal transcription level of target enzyme that was analyzed by quantitative RT-PCR were selected to analyze for protein secretion.

Overexpression of ER-chaperone genes did not affect the growth of recombinant strain compared with the control strain (Supplementary data, Fig. S5). After 72 h of cultivation at 30 °C, endoxylanase was enhanced in yeast harboring recombinant ER-chaperone genes. Overexpression of *OtBiP*, *OtCNE1*, and *OtPDI* significantly increased the yield of endoxylanase 1.4-, 1.2-, and 1.5-fold compared with the control *O. thermomethanolica* that

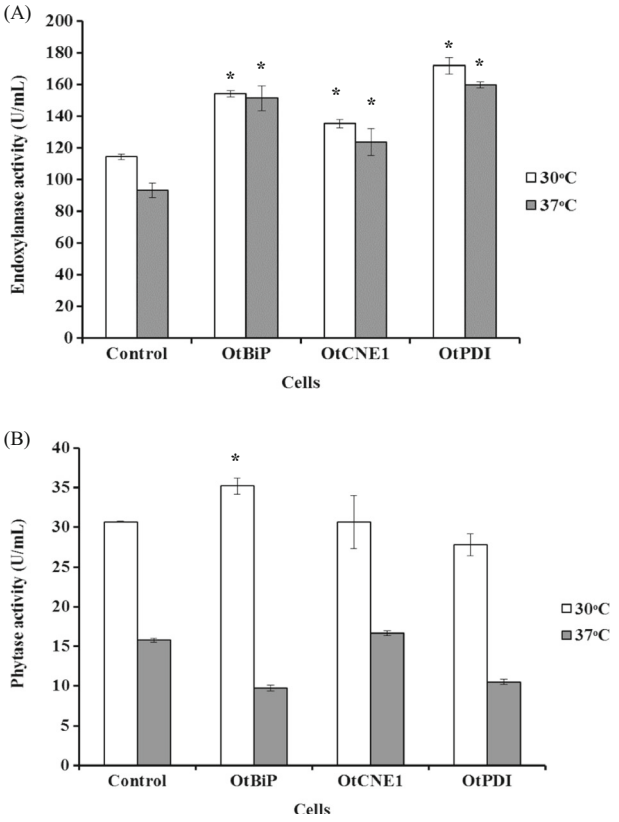

Fig. 4 Enzyme secretion analysis. Endoxylanase (**a**) and phytase (**b**) activity. In the transformants, *S. cerevisiae* α-MF pre-pro sequence was used. Transformant yeasts were expressed in YPD medium at 30 and 37 °C for 72 h. Significant improvement of secretion comparing with the control strain was indicated by an *asterisk* (*P* value <0.05) determined by *t* test

did not overexpress molecular chaperone. For phytase secretion at 30 °C, only the *OtBiP* showed a significant 1.2-fold increase in phytase secretion compared with the control strain (Fig. 4). The efficiency of protein secretion in *O. thermomethanolica* that co-expressed with target enzyme was also investigated at a higher temperature (37 °C). For endoxylanase secretion, overexpression of *OtBiP*, *OtCNE1*, and *OtPDI* significantly increased the yield of endoxylanase 1.6-, 1.3-, and 1.7-fold compared with the control strain, respectively. In contrast, phytase secretion at 37 °C did not improve by overexpression of these ER chaperones (Fig. 4).

To confirm that this secretion improvement was associated with overexpression of ER-resident chaperone genes, expression level of chaperone genes in recombinant *O. thermomethanolica* co-expressing target enzymes was confirmed by quantitative RT-PCR or Western blot. For endoxylanase secretion, all transformants showed a higher level of recombinant chaperone gene at both 30 and 37 °C comparing with the control strain (Supplementary data, Fig. S6). This result suggests that improvement of endoxylanase secretion was associated with the expression of ER chaperones. Although expression levels of ER chaperone gene in transformant co-expressing phytase was higher than the control strain (Supplementary data, Fig. S6), this expression level may not be sufficient to have a significant effect on secretion of glycosylated phytase in *O. thermomethanolica* excluding the overexpression of OtBiP at 30 °C (Fig. 4). This result indicated that overexpression of recombinant ER-resident chaperone genes in *O. thermomethanolica* was accomplished and these chaperones should play a role in secretion of target enzyme in *O. thermomethanolica*, especially for unglycosylated endoxylanase.

Discussion

O. thermomethanolica BCC16875 is a thermotolerant methylotrophic yeast that was isolated from soil in Southern Thailand [19]. Recently, it has been studied and reported as a new alternative potential host for heterologous protein production [20–22]. One caveat concerning the use of this strain as a host for protein production is that the level of heterologous protein expression is not as high as in *P. pastoris* [23]. In order to improve the efficiency of heterologous protein secretion, we explored whether using a native *O. thermomethanolica* signal sequence for directing extracellular secretion and co-expression of native *O. thermomethanolica* ER-resident molecular chaperones could increase heterologous protein secretion efficiency. We identified a candidate α-MF of *O. thermomethanolica* that contains four repeats of the mature α-MF peptide (R/GWGWHGVSRNEAIF, Fig. 1). Kex2p recognition sites (KR) adjacent to the mature α-MF peptide repeats and a putative cleavage site for removal of pre sequence were identified. However, no Kex2p recognition site was found for removal of the pro sequence. We suggest that Ste13p is responsible for pro sequence removal since a putative Ste13p recognition site (DAEA) was detected at the C terminus of the pro peptide. In order to evaluate whether these pre and pre-pro sequences function well for secreting heterologous proteins in *O. thermomethanolica*, recombinant yeasts having endoxylanase and phytase coding sequence fused in frame with these signal sequences were constructed and protein secretion was compared to that having classical pre-pro sequence derived from *S. cerevisiae*.

Secretion of *S. cerevisiae* α-MF requires posttranslational translocation [34]. In this process, heterologous fusion proteins with the α-MF signal sequence should be fully translated

before ER translocation. The pre sequence of α-MF is believed to be important and thought to form an alpha helix which functions to bind the signal recognition particle (SRP) required for entry into the ER. Deletion of entire pre sequence resulted in completed suppression of protein secretion in *K. pastoris* [10]. Similar to most signal sequences, the pre sequence of *O. thermomethanolica* α-MF also consists of a basic amino-terminus, a hydrophobic core, and a carboxy terminal polar region ending with a cleavage site for signal peptidase. This pre sequence was able to promote secretion of proteins in *O. thermomethanolica*, and may function more efficiently than the pre-pro sequence of *S. cerevisiae*, as shown for unglycosylated endoxylanase.

Although the pro sequence of *O. thermomethanolica* was shorter than that found in *S. cerevisiae*, they share a generally hydrophobic region and short stretches of charged or polar amino acids. As most of yeast α-MF precursors are glycosylated and α-MF of *O. thermomethanolica* was predicted as glycosylated protein, this pro sequence should play an important role for secretion of glycosylated protein. In our experiment, the pro sequence of *O. thermomethanolica* was found to enhance the secretion of enzyme phytase which is large and glycosylated. This result is in good agreement with the previous reports where the pro sequence was found to enhance posttranslational translocation of glycosylated proteins into the ER. Deletion of entire pro sequence is deleterious for secretion of glycosylated horseradish peroxidase secretion in *K. pastoris* [10]. In addition, enhancement of translocation was also observed in the case of superfolder GFP. GFP fused in frame with pre sequence was translocated less efficiently than pre-pro sequence [35]. From these results, we propose that pre-pro sequence of *O. thermomethanolica* α-MF presumably directs the same posttranslational process as in other yeasts and the pro sequence plays a vital role in secretion efficiency, especially for glycosylated protein.

Overexpression of heterologous protein can overwhelm the cellular machinery involved in proper protein folding and assembly, leading to accumulation of misfolded or unfolded proteins in the ER [12]. To overcome this obstacle in *O. thermomethanolica*, overexpression of ER-resident chaperones (*BiP*, *CNE1*, *PDI*) was explored based on our finding that these genes are highly upregulated in *O. thermomethanolica* after ER stress induction (data not shown). *O. thermomethanolica* homologs of three major ER-resident chaperone genes namely *OtBiP*, *OtCNE1*, and *OtPDI* were isolated and expressed. The ability of these chaperones to enhance heterologous protein secretion efficiency was tested using two proteins, unglycosylated endoxylanase and glycosylated phytase. Based on amino acid sequences, cysteine residue was not identified in endoxylanase. Thus, endoxylanase should not have any disulfide bond in its mature structure. For phytase, it should have disulfide bonds as ten cysteine residues were identified. The secretion of unglycosylated and non-disulfide bonded endoxylanase was enhanced in all chaperone-overexpressing transformant at 30 and 37 °C. This finding is consistent with a previous report showing that overexpression of *H. polymorpha CNE1* increased expression of an unglycosylated human serum albumin [16]. Although the natural substrates of CNE1 are glycosylated proteins, there are some reports that calnexin can function as a classical molecular chaperone [16, 36, 37]. In addition, overexpression of PDI which helps the correct disulfide bond formation of proteins was also found to enhance secretion of a non-disulfide bonded protein in yeast which suggests that PDI may be acting in a chaperone-like capacity [38].

In contrast to endoxylanase, chaperone overexpression has little effect on the secretion of glycosylated phytase, in which *OtBiP* overexpression increased yield by about 20 % compared with the control strain when expressed at 30 °C. At 37 °C, chaperone overexpression appeared

to have a negative impact on heterologous phytase secretion, in particular *OtBiP* and *OtPDI*. From our results, it appears that chaperone overexpression can have varied effects on heterologous protein secretion in *O. thermomethanolica* that are dependent on the nature of the heterologous protein and its expression conditions. Chaperone overexpression has been reported to have widely ranging effects on heterologous protein secretion in other yeast expression systems. For example, the secretion of several proteins in *S. cerevisiae* yeast is unaffected when *BiP* is overexpressed [38–40]. Overexpression of *BiP* negatively affects the secretion of glucose oxidase in thermotolerant yeast *Hansenula polymorpha* [41]. In addition, the secretion of human serum albumin is not improved by co-expression of *CNE1* in fission yeast *Schizosaccharomyces pombe* [42]. Overexpression of single chaperones thus may not be sufficient to have a significant effect on protein secretion. Consistent improvements in heterologous protein secretion require simultaneous activation of multiple genes in the secretory pathway by controlling expression of global transcription factors [43–45]. This strategy should be applied for improvement of protein secretion in thermotolerant *O. thermomethanolica* once the required regulatory factors have been identified.

In conclusion, this work has shown the improvement of protein secretion in *O. thermomethanolica* by utilizing its native secretion signal sequence and engineering protein folding in the ER. The α-MF signal sequence of *O. thermomethanolica* has the ability to secrete fungal enzymes and the efficiency is significantly higher than that of the *S. cerevisiae* α-MF signal sequence. The overexpression of native molecular chaperones enhance target protein secretion especially for unglycosylated endoxylanase in both normal and high temperature that would be of benefit for improving secretion of heterologous proteins in this thermotolerant strain.

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