Mutation of cysteine residues increases heterologous expression of peach expansin in the methylotrophic yeast *Pichia pastoris*

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Abstract: The study of Carbohydrate-Active enZymes (CAZymes) associated with plant cell wall metabolism is important for elucidating the developmental mechanisms of plants and also for the utilization of plants as a biomass resource. The use of recombinant proteins is common in this context, but heterologous expression of plant proteins is particularly difficult, in part because the presence of many cysteine residues promotes denaturation, aggregation and/or protein misfolding. In this study, we evaluated two phenotypes of methylotrophic yeast *Pichia pastoris* as expression hosts for expansin from peach (*Prunus persica* (L.) Batsch, *PpEXP1*), which is one of the most challenging targets for heterologous expression. cDNAs encoding wild-type expansin (*PpEXP1_WT*) and a mutant in which all cysteine residues were replaced with serine (*PpEXP1_CS*) were each inserted into expression vectors, and the protein expression levels were compared. The total amount of secreted protein in *PpEXP1_WT* culture was approximately twice that of *PpEXP1_CS*. However, the amounts of recombinant expansin were 0.58 and 4.3 mg l⁻¹, corresponding to 0.18% and 2.37% of total expressed protein, respectively. This 13-fold increase in production of the mutant in *P. pastoris* indicates that the replacement of cysteine residues stabilizes recombinant *PpEXP1*.

**Key words:** CAZymes, cysteine, expansin, heterologous expression, *P. pastoris*.

**Introduction**

The cell walls of land plants have a complex structure, consisting of a primary cell wall built around the plasma membrane and subsequently reinforced by a secondary cell wall. The primary cell wall is associated with cell formation, and consists mainly of polysaccharides and proteoglycans (Burton et al. 2010; Somerville et al. 2004). Polysaccharide regions are formed from cellulose, hemicellulose, and pectin. The cellulose is mainly crystalline cellulose consisting of β-1,4-glucan, whereas hemicellulose contains many kinds of structural polysaccharides, including mannans, xyloglucans, xylans, and galactans. Hemicellulose serves to crosslink cellulose to provide greater wall strength and flexibility. Pectin is composed of homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II). The main chain of HG is α-1,4-linked GaLA, and most of the GaLA residues are methylated. RG-I is composed of a repeating [α-1,4-GaLA-α-1,2-Rha-] main chain and side chains such as galactans and arabinans. RG-II has very complex structure, with a main chain of α-1,4-bound GaLA, and many kinds of side chains (Burton et al. 2010; Somerville et al. 2004). The secondary cell wall is formed to thicken the inner primary cell wall after cell elongation, and is composed of cellulose, hemicellulose, and lignin. Lignin is a phenolic high polymer, poorly soluble in water and resistant to microbial enzymes, thereby contributing to chemical resistance and physiological strength (Cosgrove and Jarvis 2012).

Plants construct and reconstruct these walls using many kinds of enzymes, known as carbohydrate active enzymes (CAZymes, http://www.cazy.org, Lombard et al. 2014), to form and/or degrade covalent links or interactions between the various polymers. Thus, in order to understand the structure, role, and biosynthesis or remodeling of the cell wall, it is necessary to characterize the CAZymes. Furthermore, plant cell walls represent the most abundant carbon source on earth, and
an understanding of CAZymes is essential for utilization of this sustainable resource. Therefore, substantial amounts of plant enzymes are required for research purposes. However, purification of the native enzymes from plants is usually inefficient, and consequently heterologous protein expression has attracted great interest. There are many types of expression host, such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*, and insect cells. In this study, we focused on the methylotrophic yeast *P. pastoris*, which has similar molecular, genetic, and biochemical features to higher eukaryotes (Cereghino and Cregg 2000). The production level of recombinant proteins in *P. pastoris* is generally high, often reaching g l<sup>-1</sup> quantities in the culture medium (Higgins and Cregg 2007), although plant proteins represent challenging targets, because their high contents of cysteine residues can result in denaturation, aggregation and/or protein misfolding (Nielsen et al. 2018). Some strategies are available to overcome this problem. For example, in *E. coli*, co-expression of disulfide isomerase increased soluble protein production (Liu et al. 2005; Yuan et al. 2004). Also, replacement of cysteine residues with serine increased productivity in an *S. cerevisiae* expression system (Ito et al. 2011).

Many kinds of *P. pastoris* host cells are available, with different genotypes, nutritional requirements, levels of resistance to methanol, and deletion of protease to prevent the degradation of recombinant protein. Strain SMD1168H is one of major protease-deficient strains (Ahmad et al. 2014; Higgins and Cregg 2007). *P. pastoris* is methylotrophic yeast, but a methanol utilization positive (Mut<sup>+</sup>) strain can produce excessive amounts of toxic formaldehyde, and therefore a methanol utilization slow (Mut<sup>-</sup>) strain, such as KM71H, whose alcohol oxidase gene (*aox1*) is knocked out (Higgins and Cregg 2007), is often preferred.

Several vectors are commercially available for *Pichia* expression. For example, the pPIC and pGAP series (Thermo Fischer) contain methanol-inducible and constitutive promoters, respectively. pPIC vector carries the alcohol oxidase promoter *AOX1*, while pGAP contains the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter, which is constitutively expressed in the growth phase (Higgins and Cregg 2007). Extra- or intra-cellular production can be achieved through the incorporation or omission of the *Saccharomyces cerevisiae* α-factor sequence, respectively.

Expansin is cell wall localized protein which plays a role in plant cell wall loosening (Cosgrove 2000, 2016). They induce pH-dependent “acid growth” and cell expansion (Li et al. 1993; McQueen-Mason et al. 1992; Shcherban et al. 1995). In plantae, all land plants and...
some algae express many expansin genes in various organs, for instance seeds, leaves, stems, flowers, fruits, pollens, and so on. A number of previous studies have shown that expansins are important for plant growth, development, and maturation, but the molecular mechanism of their function remains unclear (Cosgrove 2015, 2016; Marowa et al. 2016; Nikolaides et al. 2014; Sampedro and Cosgrove 2005; Sharova 2007). Although the amino acid sequences of expansins are similar to those of endoglucanases, which degrade cellulose, their hydrolytic activity and molecular mechanism have not been established (Cosgrove 2000; Yennawar et al. 2006). Therefore, there is a need for recombinant expansins. However, expansins are among the most difficult proteins to produce heterologously (Cosgrove 2015; Kerff et al. 2008; Yennawar et al. 2006), and additionally, it is said to precipitate at concentrations above 10 µg ml⁻¹, especially for α-expansin (Cosgrove 2015). Only one α-expansin, from tomato (Solanum lycopersicon, LeEXP2), has so far been successfully expressed in P. pastoris. Expansins contain many cysteine residues and some of them are predicted not to form disulfide bonds. For instance, fruit-ripening-related α-expansin from peach (Prunus persica, (L.) Batsch, PpEXP1) has eight cysteine residues among the 227 residues of the mature protein (Hayama et al. 2000, 2003, 2006), and based on structure modeling, they localize on the surface (Figure 1). In this study, we aimed to examine whether mutation of all the cysteine residues in PpEXP1 to serine would stabilize the recombinant protein and lead to efficient methanol-induced production of soluble mutant PpEXP1 in P. pastoris.

Materials and methods

Construction of expression systems

The cDNA of wild-type α-expansin from peach fruit (PpEXP1, GenBank accession number is AB029083) was a kind gift from Dr. Hiroko Hayama of the National Agriculture and Food Research Organization and Prof. Megumi Ishimaru of Kindai University. The cDNA fragment encoding PpEXP1 excluding the signal peptide was amplified by means of polymerase chain reaction (PCR) with specific primers (Table 1), and EcoRI and XbaI restriction sites were created at the 5’ and 3’ ends of the open reading frame, respectively. The amplified cDNA fragments were cloned into restriction-digested pGAPZA vector. cDNA encoding a mutant in which all the cysteine residues of PpEXP1 were changed to serine (PpEXP1_CS), and with codons optimized for P. pastoris, was synthesized based on the cDNA sequence of PpEXP1_WT using a commercial service (Genscript, Inc., New Jersey, USA). The synthesized DNA was amplified and cloned into pPICZaA vector (Invitrogen Corporation, Massachusetts, USA) by the in-fusion method, using the primers shown in Table 1.

pGAPZA_A_PpEXP1_WT and pPICZaA_PpEXP1_CS were transformed into E. coli strain TOP10 (Invitrogen), then extracted, and linearized using BglII or Bpu1102I, respectively (TaKaRa Bio Inc., Shiga, Japan). The linearized vectors were transformed into P. pastoris SMD1168H and KM71H, respectively (Invitrogen) by electroporation. After transformation, the cells were plated on YPDS agar plates containing zeocin (100 µg ml⁻¹) and incubated at 30°C for 5 days.

Expression check

The protein expression of colonies on YPDS-zeocin plates was confirmed on a small scale. We selected eight SMD1168H_pGAPZA_A_PpEXP1_WT colonies and eight KM71H_pPICZaA_PpEXP1_CS colonies with zeocin and pre-cultured them overnight at 30°C in 3 ml of YPD medium containing zeocin (final concentration: 50 µg ml⁻¹). One milliliter aliquots of the pre-cultures of SMD1168H_pGAPZA_A_PpEXP1_WT were inoculated in 200 ml of basal salt medium (described in the Supplementary information) and cultured at 30°C for 4 days with shaking at 150 rpm. During incubation, 50% (w/v) glycerol was added every 24 h so that the final concentration was 2% (w/v). In contrast, 1 ml of KM71H_pPICZaA_PpEXP1_CS pre-culture medium was inoculated into 200 ml of YPD medium (described in the Supplementary information) and incubated overnight at 30°C with shaking at 150 rpm. The cultures were centrifuged at 2,000 × g for 10 min. Each supernatant was collected, and the cell pellet was suspended in sterile ultrapure water and centrifuged again. The resulting pellet was resuspended inYP medium (described in the Supplementary information), and transgene expression was induced with methanol (final concentration: 1% v/v). Incubation was carried out at 26.5°C for 4 days with shaking at 150 rpm. During the induction period, methanol was added every 24 h to a final concentration of 1% (w/v).

Each supernatant was collected by centrifugation at 7,000 × g for 10 min after the end of the culture. The total amount of

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Table 1. Macromolecule production information.

| Souce organism | Wild type (WT) | Cysteine to Serine mutant (CS) |
|----------------|---------------|-------------------------------|
| DNA source     | cDNA          | pGAPZaA                       |
| Forward primer | ATGAAATTCGACTATGGAGGAGGATGGGAAG | AGGGGTATCTCTCTGGAGAAAAAGAGATTACGAGTTGTTGTT |
| Reverse primer | GCTCTAGAGCGAATTTGACCCCGAGAAAATG | AGAAAGCTGGCGGCGCGCTTTAAAATTGACCACCTGAAA |
| Ligation method| Restriction enzyme and ligation | pPICZaA |
| Expression vector | pGAPZaA | P. pastoris KM71H |
| Expression host | P. pastoris SMD1168H | P. pastoris KM71H |

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expressed protein was determined by Bradford's method using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, California, USA) and bovine serum albumin as a standard, and expression of the induced protein was confirmed by SDS-PAGE on 15% acrylamide gel.

Production of recombinant protein in a jar fermenter

Recombinant protein was produced in a 5 l scale mini-jar fermenter (TSC-M5L; Takasugi Seisakusho, Tokyo, Japan). A single colony of SMD1168H_pGAPZaA_PpEXP1_WT or KM71H_pPICZaA_PpEXP1_CS was inoculated in 5 ml of YPG medium and cultured overnight at 30°C with stirring at 300 rpm. Three ml of the preculture was added to 21 of basal salt medium containing PTM1 salt adjusted to pH 5.0 and incubated at 27°C. The pH of the medium was fixed at pH 5.0 with 10% ammonium solution. After one and a half days of incubation, 50% (w/v) glycerol was added (glycerol feed; GF) at approximately 20 g per hour to make a total of 180 g. At the end of GF, the supply of 100% glycerol (extra glycerol feed; EGF) or 100% methanol (methanol feed; MF) was started for SMD1168H_pGAPZaA_PpEXP1_WT or KM71H_pPICZaA_PpEXP1_CS, respectively, under regulation by the oxygen controller. The supernatant was collected by centrifuging the culture medium 96 h after the start of EGF for SMD1168H_pGAPZaA_PpEXP1_WT and 72 h after the start of MF for KM71H_pGAPZaA_PpEXP1_CS. Each supernatant was applied to a 100 kDa cut-off ultrafilter (Kvick Lab Cassette, GE Healthcare, Chicago, USA, for PpEXP1_WT) or a 300 kDa cut-off ultrafilter (Pellicon XL Cassette, Merck Millipore KgaA, Darmstadt, Germany, for PpEXP1_CS). The filtrate was concentrated using a 5kDa cut-off ultrafilter (Pellicon XL Cassette, Merck Millipore KgaA). Ultrafiltration was performed on ice, and the crude protein solution was pre-cooled.

Evaluation of expressed protein

The integrity of the constructed vectors was confirmed by gene sequencing. The total amount of expressed protein was determined by Bradford's method. All secreted proteins were visualized by SDS-PAGE on 15% acrylamide gel. Two micrograms of protein was run in each lane. The bands of each recombinant expansin were estimated from the molecular weight, and the percentage of it to total protein was determined by ImageJ (Schneider et al. 2012) based on band intensity.

Results and discussion

Vector construction and expression check

The workflow of this study is illustrated in Figure 2. The two expression vectors were constructed as shown in Figure 3 and their sequences were confirmed. The vectors were linearized with restriction enzymes and transformed into P. pastoris. Stable transformants were selected based on zeocin resistance and their expression was confirmed. All colonies expressed the target protein, and the one with the highest expression level was used for cultivation in the 5 l jar fermenter.

Comparison of production of the two proteins in the jar fermenter

In the culture of PpEXP1_WT, the 50% glycerol feed batch (GF) phase and the 100% extra glycerol feed batch (EGF) phase were initiated at 38 and 45 h after the start of the culture, respectively. The protein concentration increased significantly from the start of the culture until approximately 100 h later. The supernatant was collected after approximately 160 h of incubation (Figure 4A).

In the culture of PpEXP1_CS, GF was initiated at 38 h after the start of the culture, as in the case of WT. The methanol feed (MF) was started at approximately 45 h. Between 50 and 62 h after the start of the cultivation, a sharp drop and then increase of DO value and an increase in pH value occurred. These changes suggest that some of the cells could not tolerate the added methanol and may have lysed. After about 120 h of incubation, the protein concentration stopped increasing, and the DO and pH values increased rapidly. The culture supernatant was collected about 133 h after the start of
the culture (Figure 4B).

The concentration of secreted total protein in each construct was assayed by the Bradford method (plotted with black dots in Figure 4), and the result of SDS-PAGE is shown in Figure 5. The total protein amounts were $916 \pm 148$ mg in the 2.851 culture supernatant of $Pp$EXP1_WT and $438 \pm 43$ mg in the 2.401 culture supernatant of $Pp$EXP1_CS. Thus, the total amount of secreted protein in $Pp$EXP1_WT culture was approximately twice that of $Pp$EXP1_CS. However, the production levels of $Pp$EXP1_WT and $Pp$EXP1_CS were 0.58 and 4.3 mg l$^{-1}$, respectively, and the percentages of recombinant protein with respect to total expressed protein were 0.18% and 2.37% after concentration with the 5 kDa ultrafiltration membrane. Thus, the ratio of target protein $Pp$EXP1_CS to total protein was about 13 times higher than in the case of $Pp$EXP1_WT. Absolutely, this result should be paid attention because of the strains of $P$. pastoris and expression vectors of $Pp$EXP1_WT and $Pp$EXP1_CS were different. Therefore, the increased expression level in the current mutant could not be said simply an effect of the introducing mutation of cysteine to serine. However, our method reported in this report can become one of choices to heterologously expression of hardly expressed protein such as expansin.

Other studies of protein expression in $P$. pastoris

There have been many studies on heterologous protein production using $P$. pastoris. In general, fungal proteins are expressed at a high level in $P$. pastoris without any mutations. In our laboratory, production of endoglucanases ($Pc$Cel45A) and cellobiohydrolase ($Pc$Cel6A) from the basidiomycete $Phanerochaete chrysosporium$, amounted to 1.6 and 4.6 g l$^{-1}$, respectively, using $P$. pastoris strain KM71H in a jar fermenter (Igarashi et al. 2012; Nakamura et al. 2013). However, expression of plant enzymes in $P$. pastoris has resulted in large yield differences, depending on the type of enzyme. For example, $\alpha$-amylase from rice ($Oryza sativa$) and $\alpha$-expansin from tomato were relatively...

Figure 4. Production of recombinant $Pp$EXP1_WT (A) and $Pp$EXP1_CS (B) in a 5 l jar fermenter. GF, EGF, and MF show the timings of the start of 50% glycerol feed, extra glycerol feed, and methanol feed, respectively. Each color and symbols show parameter values. Red open circle is DO (ppm), blue cross is pH, green open triangle is temperature (°C), yellow green cross is rotation speed (per minutes), and black circle is total protein concentration (µg ml$^{-1}$).

Figure 5. SDS-PAGE of $Pp$EXP1_WT (A) and $Pp$EXP1_CS (B). M indicates protein molecular weight markers (pre-stained dual color protein markers, Bio-Rad). Lane 1 is the supernatant of fermentation, lane 2 is the fraction concentrated through a 100 or 300 kDa ultrafiltration membrane, lanes 3 and 4 are flow-through fractions of the 100 or 300 kDa ultrafiltration membrane, lane 5 is concentrated fraction of 5 kDa ultrafiltration membrane, and lanes 6 and 7 (only B) are flow-through fractions of the 5 kDa ultrafiltration membrane. Two micrograms of protein was applied each lane. The arrows show the band of $Pp$EXP1_WT or $Pp$EXP1_CS, respectively.
highly expressed at 340 and 70.9 mg l\(^{-1}\) (Lee et al. 2003; Liu et al. 2014), respectively, while rhamnogalacturonan endo‐lactase from Chilean strawberry (Fragaria chiloensis) and β‐galactosidase from radish (Raphanus sativus) were expressed at levels of only 1.6 and 7 mg l\(^{-1}\), respectively (Kotake et al. 2005; Méndez-Yañez et al. 2020) One of the reasons for the relatively high expression of LeEXP2 (Liu et al. 2014) is probably because that six of the eight cysteine residues correctly formed a disulfide bond and the other two free cysteine residues did not cause abnormal bonds. Kaewthai et al. (2010) cloned nine xyloglucan endo‐transglycosylase/hydrolase (XTH) genes from barley (Hordeum vulgare) and expressed them in \(P. pastoris\) strain SMD1168H. Only five isoforms were secreted as soluble proteins, and only four of them showed enzymatic activity. They did not find any correlation between successful protein production and gene/protein sequence characteristics (Kaewthai et al. 2010).

**Effect of removing cysteine residues**

In the expression of the taste‐modifying protein miraculin from miracle fruit (Synsepalum dulcificum) in \(S. cerevisiae\), the expression level of the mutant containing serine instead of cysteine residues was increased compared to that of the wild‐type protein (Ito et al. 2011). Our results are consistent with this, and therefore it appears that the presence of cysteine residues adversely affects the amount of \(Pp\) EXP1 secreted by \(P. pastoris\). These findings support the idea that replacement of cysteine residues in plant proteins is a promising strategy to increase heterologous expression of eukaryotic proteins. In addition, if the mutation of cysteine to serine results in abnormal protein folding, the protein is expected to be degraded by the quality control system of \(P. pastoris\), which suggests that the protein secreted into the culture supernatant is not abnormal (Iinan et al. 2006; Ito et al. 2011; Saito et al. 2002).

XTHs have approximately the same proportion of cysteine residues as expansin. It is noteworthy that \(Pt\) EG16, which is homologous to XTH from \(Populus trichocarpa\), expressed in \(E. coli\) had no effect on the molecular weight of barley mixed linkage glucan, lichenan, from Icelandic moss, or xyloglucan from tamarind seeds, but these polysaccharides were efficiently degraded by \(Pt\) EG16 in the presence of a reducing agent, 0.25 mM dithiothreitol (DTT, Eklöf et al. 2013). Furthermore, since the “acid growth” activity of expansins extracted from plants has been reported to be promoted in the presence of a reducing agent (Li et al. 1993; McQueen-Mason et al. 1992), \(Pp\) EXP1\_CS is expected to be as good or better than \(Pp\) EXP1\_WT as a function of recombinant expansins. These results imply that the function of a mutated protein would be retained, although further study would be needed to confirm this.

In conclusion, the widely used \(P. pastoris\) heterologous expression system might be applicable even to challenging proteins containing large amounts of cysteine residues if the cysteine residues are removed, e.g., by mutation to serine. If the mutated recombinant proteins retain the functional activity of the wild‐type proteins as expected, this system could be useful to produce large amounts of plant proteins for studies of the developmental mechanisms of plants and also for the utilization of plants as a biomass resource.

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**Author contributions**

All experiments were done by KM. NS advised and supported the fermentation experiments and data analysis. KI supervised the experiments and wrote the manuscript.

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