Cloning of 1– Fructan: Fructan Fructosyltransferase Gene and Expression of Recombinant 1– Fructan: Fructan Fructosyltransferase in Yeast

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Abstract

Fructosyltransferases (FTases) were important enzymes for fructo-oligosaccharides (FOS) synthesis. Recently, FOS was considered as a potential prebiotic in food industry. In particularly, FOS with short chains was used as a new alternative sweetener. The production of recombinant 1- fructan: fructan fructosyltransferase (1- FFT) in yeast system was conducted in this research. The 1- fructan: fructan fructosyltransferase gene (1- *fft)* cloned from 105 days old tuber of Kaentawan (*Helianthus tuberosus* L.) was sub cloned to expression vector, pPICZαB by adding *Pst*I and *Sac*II sites. The cloned gene was successfully transformed to yeast *Pichia pastoris* X-33 by lithium chloride transformation method. The yeast transformant; *P. pastoris* X- 33 PF1 showed the ability to produce recombinant 1- FFT. The enzyme activity at 3.57 and 3.33 unit/L was determined in cell and culture medium, respectively. It was also found that FOS was synthesized when recombinant 1- FFT was incubated with 1- kestose and synthesized FOS as substrates. The synthesis of FOS was not detected when sucrose was used as substrate of recombinant 1- FFT.

Keywords : *Recombinant 1- FFT, Helianthus tuberosus L., Pichia pastoris X-33, Fructooligosaccharides*

1. Introduction

Fructooligosaccharides (FOS), fructose polymers with a degree of polymerization (DP) in range of 2-10, are major fructan highly stored in underground organs of many *Asteracea*e species (Vijn and Smeekens, 1999). Among them, Jerusalem artichoke (*Helianthus tuberosus* L.), domesticated plant in temperate regions

which accumulated high contents of FOS with average DP of 6-10 (16% dry weight) and it is considered as a good candidate for the industrial production of FOS (Steinbüchel and Rhee, 2005). According to health promoting properties of FOS, it is classified as a prebiotic and widely supplemented in various food products. The short chain FOS (DP2-4) was not digested in the human small intestine,

but fermented in colon where the growth of probiotics such as *Lactobacillus* and *Bifidobacteria* were stimulated (Roberfoid *et al.*, 1993). It is synthesized by the action of different fructosyltransferases (FTases) located in vacuole of plant cells. Edelman and Jefford have proposed model of fructan synthesis by concerted action of two FTases, 1- sucrose: sucrose fructosyltransferase (1- SST, EC:2.4.1.99) initiated reaction by catalyzing transfer of a fructosyl residue from sucrose to another sucrose molecule, resulting in the formation of 1-kestose (GF$_2$) and 1- fructan: fructan fructosyltransferase (1- FFT, EC: 2.4.1.100) functioned to elongate the fructose chains in afterward (Edelman and Jefford, 1968). This model was approved by Koops and Jonker (1994; 1996). It was leaded to the alternative synthesis of fructan with expected degree of polymerization under *in vitro* condition which was controllable. Due to the quality and quantity of FOS derived from naturally grown fructan accumulated plants were varied upon plant species, developmental stages, harvest time and methods and effected by environmental impacts, therefore the synthesis of FOS by means of enzyme reaction under *in vitro* instead of natural synthesis by plant should be considered. Jerusalem artichoke which was named in Thai as Kaentawan was imported and improved to grow in Thailand where is located in subtropical region (Judprasong *et al.*, 2011). Most grown varieties still accumulated high contents of FOS in range of 20.8-23.3 g/100g dry weight (Judprasong *et al.*, 2011; Tanjor *et al.*, 2012). This might be considered to either use as FOS or FTases source for further applications in FOS production for supplying in various functional foods. Many studies have focused on purification and characterization of fructan synthesizing enzymes from natural grown Jerusalem artichoke (Praznik *et al.*, 1990; Lüscher *et al.*, 1993; 1996; Koops and Jonker, 1994) to prove the function of enzymes in FOS synthesis. In addition, genes encoded for 1- SST and 1- FFT of this plant have also been cloned and functionally analyzed by transferring genes to petunia plant (van der Meer *et al.*, 1998) which provides more evidences to clarify the mechanism of fructan metabolites in Jerusalem artichoke. However the establishment of transgenic plant for producing recombinant enzyme has a limitation in term of time consuming. The production of recombinant FTases from several fructan plant species by methylotrophic yeast, *Pichia pastoris* have successfully reported (Lüscher *et al.*, 2000; Van den Ende *et al.*, 2006; Ueno *et al.*, 2011). In case of Kaentawan, the production of recombinant FTases is rare. Therefore, the cloning of *1-fft* (accession no. AJ009756.1) from Kaentawan and sub cloning to express in yeast system were conducted in this study. This investigation might be an alternative approach for FOS production by using recombinant 1-FFT from Kaentawan.

2. Materials and Methods

2.1 Plant materials

The 105 days old tuber of Kaentawan grown in green house of Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University, Nakhon Pathom, Thailand was harvested and kept at -70°C until use.

2.2 Total RNA extraction and RT- PCR

Total RNA was extracted from 105 days old tubers using TRI reagent according to manufacturer's instructions (Molecular Research Center, Inc.) and the first strand cDNA was synthesized with RevertAid™M- MuLV Reverse Transcriptase (Fermentas, Lithuania) and oligo (dT)$_{18}$ primer (Fermentas, Lithuania).

2.3 Cloning of *1-fft* and sequencing

The cDNA of *1- fft* was amplified by PCR using primer pairs of 1-*fft*_F (5'ATgCAAACCCCTgAACCC3') and 1-*fft*_R (5'gCCggTAATTAAAgggTA3') which were designed basing on the sequence of *Helianthus tuberosus 1-fft* gene (accession no. AJ009756.1) (Pan *et al*., 2009). The PCR consisted of an initial 5 min denaturation step (95°C) followed by 30 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 2 min and then a final step at 72°C for 10 min. PCR products were purified from agarose gel using the HiYield™ Gel/ PCR DNA Fragments Extraction Kit (RBC Bioscience, Germany) and sub cloned into pGEM®-T Easy Vector System (Promega, USA). The recombined vectors were transformed into *E. coli* DH5α. The positive clones were identified by blue/white selection and colony PCR. Plasmids containing desired inserts were purified using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen, USA). Inserted plasmid was sequenced by the Automated DNA Sequencer, ABI Prism 3100–Avant Genetic Analyzer, Applied Biosystems. The homology search of nucleotide sequences were analyzed using Blastn program via NCBI.

2.4 Construction of gene expression vectors for recombinant enzymes production in yeast system

To construct expression plasmid named pPIC_1-*fft*, a *Pst*I and *Sac*II restriction sites were also introduced to the 5' and 3' end of the mature protein, in respectively by PCR. The primers pair; *Pst*I_1-*fft*F (5'CAg<u>CTgCAg</u>CAAAC-CCCTgAACCCTTTA3') and *Sac*II_1-*fft*R (5'CAg<u>CCgCggg</u>CC-ggTAATTAAAAg-ggTA3') was used to amplify gene following these conditions: an initial 5 min denaturation step (95°C) followed by 30 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 2 min and then a final step at 72°C for 10 min.

The amplified products were purified and then ligated to the pPICZαB (Invitrogen, USA) in frame with the α-factor from *Saccharomyces cerevisiae*. The resulting pPIC_1fft plasmid was transformed to *E. coli* JM109. The genes cassette of positive transformants were sequenced to analyze the orientation of genes and isolated for further transform to yeast cells.

2.5 *Pichia pastoris* transformation and small-scale expression

P. pastoris strains X-33 was transformed with linearized pPIC_1-fft plasmid, following the lithium chloride transformation protocol, and plated on selective YPD/zeocin plates (protocol and recipes provided by the manufacturer). As a control, the same yeast strains were transformed with the parent pPICZαB plasmid. The integration of gene on yeast chromosome was analyzed by PCR primed with AOX1F (5'gACTggTTCCAATT-

gAC-AAgC3') and AOX1R primer (5'gCAAAT-ggCATTCTgACATCC3').

Single colony of yeast transformants were inoculated on fresh YPD/Zeocin plates. For a small-scale expression, the newly grown colonies were inoculated in 20 ml of pre-culture medium (BMGY). After 18 h of incubation on rotary shaker with 250 rpm at 30°C, the cells (OD_{600}=1 at final cell concentration) were switched to 50 ml of induction medium (BMMY) and incubated at the same conditions. Methanol was replaced every 24 h to maintain a final concentration of 2% for 5 day. The culture broth was centrifuged when fermentation finished. The supernatant was concentrated by ultra-filtration (VivaSpin concentrators 30,000 NMWC, VivaScience Ltd, Lincoln, UK). 15µl of crude recombinant enzyme was either incubated with 50 mM of 1-kestose or synthesized FOS in 100 mM potassium phosphate buffer (pH 5.4) with final volume of reaction at 30 µl. The synthesized reaction was done at 34°C for 6 and 12 h. The FOS produced was analyzed by HPLC. The 1-FFT activity was assayed from the production of nystose. One unit of 1-FFT activity was defined as the amount of enzyme producing 1 µmole of nystose from 1-kestose in 1 min under the conditions described above.

2.6 Determination of FOS

The samples were filtered before inject into HPLC. The separation and detection was performed by carbohydrate column coupled with refractive index (RI) detector. The deionized water was used as mobile phase with flow rate of 0.4 ml min^{-1}. The temperature of column was controlled at 45°C. Glucose, fructose, sucrose, 1-kestose (GF2), nystose (GF3), and inulin from chicory root were used as the standards. Chromatographic peaks were identified by comparing sample retention times with those of known standards.

3. Results

3.1 Cloning of *1-fft* and sequence analysis

As shown in Figure 1, the amplified product of PCR about 1,900 bp in size was obtained when 1-*fft*_F and 1-*fft*_R primers were used.

The amplified PCR product was eluted and purified from agarose gel and further sequenced. The cloned *1-fft* composed of 1,851 bp. The homology search of gene sequence was analyzed by the Blastn program via NCBI. The cloned *1-fft* showed 99% similarity to *Helianthus tuberosus 1-fft* gene (accession no. AJ009756.1).
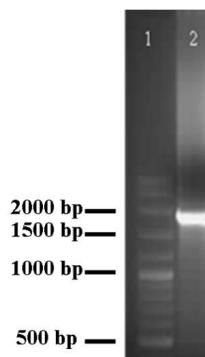

Figure 1. Analysis of the PCR product from cDNA of 105 days old tuber of Kaentawan primed with 1-*fft*_F and 1-*fft*_R primers on 1% (w/v) agarose gel electrophoresis.
Lane 1: DNA marker (VC 100 bp plus)
Lane 2: PCR product from cDNA of 105 days old tuber

3.2 Construction of gene expression vectors for recombinant enzymes production in yeast system

To produce recombinant 1-FFT from Kaentawan by yeast system, sub cloning open reading frame (ORF) of *1-fft*

to expression vector (pPICZαB) was conducted. The appropriated restriction sites (*Pst*I and *Sac*II) were introduced to ORF of genes by PCR. The amplified PCR product was cut and ligated to pPICZαB at *Pst*I and *Sac*II site.
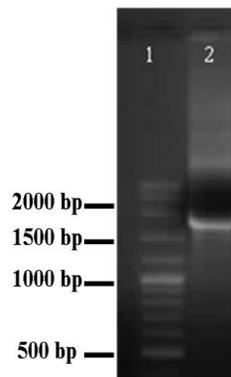

Figure 2. Analysis of the amplified PCR product from positive transformant harboring pPICZαB with *1-fft* on 1% (w/v) agarose gel electrophoresis.
Lane 1: DNA marker (VC 100 bp plus)
Lane 2: PCR product of pPIC_1fft plasmid

The ligated products were then transformed to *E. coli* cells. The positive transformant clones harboring *1-fft* as shown in Figure 2 were chosen for further sequencing. The results showed the correct orientation of gene in pPICZαB. This expression vector containing ORF of *1-fft* was further transformed to yeast cells for recombinant enzymes production.

3.3 Selection of yeast transformants

The linearized pPIC_1fft plasmid was transformed to *P. pastoris* X-33 by lithium chloride transformation method. The yeast transformants were selected on YPD medium containing 100 µg/ml zeocin. The genomic DNA of yeast colonies grown

on YPD/zeocin medium was isolated for analysis of the integration of *1-fft* gene on yeast chromosome by PCR method. As shown in Figure 3, the transformant named PF1 (Lane 3) gave three amplified PCR products of about 2300, 2000 and 500 bp while the other transformants named PF2 (Lane 4), PF3 (Lane 5) and PF4 (Lane 6) showed only one amplified PCR product of about 2000 bp. When compared with that of control transformant (empty pPICZαB plasmid) which showing two amplified PCR products of 2000 and 500 bp. In addition *1-fft* gene was only amplified in PF1 (data not shown). It may indicate that only PF1 transformants may harbor *1-fft* gene.
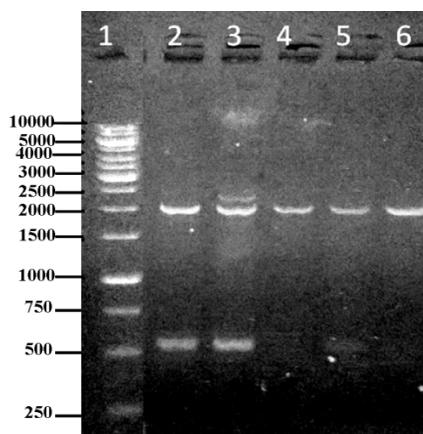
Figure 3. Analysis of the PCR products from genomic DNA of yeast transformants containing *1-fft* primed with AOX1F and AOX1R on 1% (w/v) agarose gel electrophoresis.
Lane 1: DNA marker (1kb ladder, Fermentas)
Lane 2: Transformant with empty pPICZαB plasmid
Lane 3-6: Transformant PF1, PF2, PF3 and PF4 in respectively

3.4 Small-scale expression

To analyze the expression of *1-fft* gene in yeast system, 4 transformants (PF1-4) and control transformant were grown in BMGY medium and induced to produce enzyme in BMMY medium for 3 days. As shown in Table 1, the transformant named PF1 has 1-FFT activity in cells and medium as 3.57 and 3.33 Unit/L, respectively while others showed very low activity of enzyme. This preliminary result indicated that the 1-FFT recombinant enzyme was successfully produced in *P. pastoris* X-33 (PF1 transformant). The HPLC chromatogram as shown in Figure 4 suggested that nystose was produced when incubated enzyme with 1-kestose as substrate.

Table 1. 1- FFT activity in yeast transformants

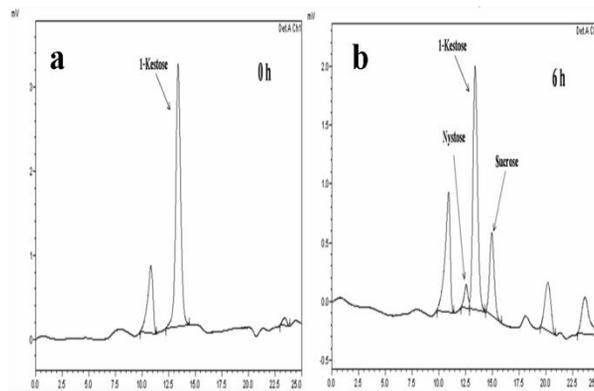| Transformants		1- FFT activity (U/L)
Control	cell	0.00
	medium	0.00
PF1	cell	3.57
	medium	3.33
PF2	cell	1.27
	medium	0.93
PF3	cell	2.38
	medium	0.00
PF4	cell	2.98
	medium	0.00

Figure 4. Analysis of product from recombinant 1-FFT activity of yeast transformants when 1-kestose was used as substrate at 0 (a) and 6 h (b) of incubation.

Additionally, the crude enzyme from PF1 also produced nystose when synthesized FOS was used as substrate whereas there was no any nystose produced when sucrose was used as substrate (Figure 5)
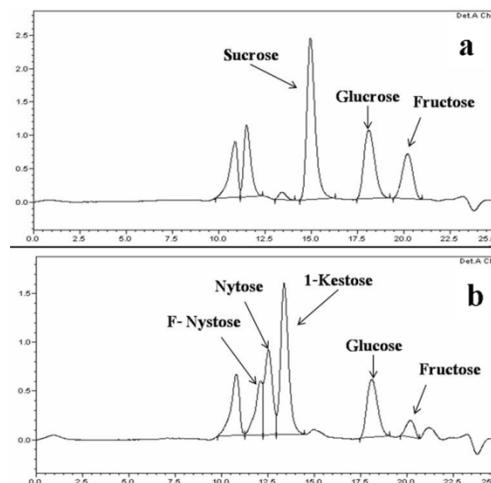

Figure 5. Analysis of product from recombinant 1-FFT activity of PF1 yeast transformants when sucrose (a) and synthesized FOS (b) was used as substrate at 24 h of incubation.

4. Discussions

The nucleotide sequence of cloned gene from 105 days old tuber of Kaentawan showed 99% similarity to *Helianthus tuberosus 1-fft* gene (Accession no. AJ009756.1) in GenBank indicated that *1-fft* was successfully isolated and cloned from Kaentawan. In addition, the sequence of pPIC_1fft plasmid isolated from *E. coli* transformant showed the correct orientation of genes in pPICZαB suggested that this expression vectors containing ORF of *1-fft*.

According to the 4 yeast transformants (PF1-4) gave different size and pattern of PCR products when compared with yeast

transformant containing empty pPICZαB, therefore they were chosen for further analysis of recombinant enzyme expression. The analysis of recombinant 1-FFT activity was analyzed in small-scale expression. Only PF1 transformant showed enzyme activity in both cell and medium fractions. Although, the yeast *P. pastoris* is widely used for expression of foreign proteins due to vectors that are designed to integrate into the *Pichia* chromosome enable stable expression, but transformation efficiencies are very low (Wu and Letchworth, 2004). From the enzyme assay of PF1 with 1- kestose as substrate, fructosyl transferred activity to synthesize nystose was detected in culture medium indicated the secretion of enzyme by function of signal peptide (α- factor) in front of ORF of ligated gene. The enzyme activity detected in cell fraction was almost same as that of activity detected in culture medium. This may suggest the less proper function of signal peptide. When crude recombinant enzyme from PF1 was incubated with sucrose for 24 h (Figure 5a), there was not any nystose produced. In addition, the synthesis of nystose and FOS with longer DP when synthesized FOS was used as substrate (Figure 5b) confirmed that this recombinant enzyme is 1- FFT, not 1- SST. The results showed that recombinant enzyme of *1- fft* was successfully produced in *P. pastoris* X-33 (PF1). The functional analysis of FTases from tall fescue (Lüscher *et al*., 2000), *Echinops ritro* (Van den Ende *et al*., 2006) and edible burdock (Ueno *et al*., 2011) in heterologous system (*P. pastoris*) have successfully reported. In case of Kaentawan, the production of recombinant FTases is rare. This investigation might be an alternative approach for FOS production by using recombinant 1-FFT from Kaentawan. To increase the specific activity of enzyme, purification steps should be performed.

5. Conclusion

The 1-FFT recombinant enzyme was successfully produced in *P. pastoris* X-33. It may benefit for fructooligosaccharides (FOS) synthesis by means of enzyme synthesis.

6. Acknowledgement

7. References

(1)   Edelman J, Jefford TG. The mechanisim of fructosan metabolism in higher plants as exemplified in *Helianthus tuberosus*. New Phytol. 1968; 67: 517-531.

(2)   Judprasong K, Tanjor S, Puwastien P, Sungpuang P, Investigation of Thai plants for potential sources of inulin-type fructans. J Food Comp and Anal. 2011; 24: 642-649.

(3)   Koops AJ, Jonker HH, Purification and characterization of the enzymes of fructan biosynthesis and *Helianthus tuberosus* Colombia I. Fructan: fructan fructosyltransferase. J Exp Bot. 1994; 45: 1623-1631.

(4) Koops AJ, Jonker HH, Purification and characterization of the enzymes of fructan biosynthesis and *Helianthus tuberosus* Colombia II. Purification of sucrose: sucrose 1- fructosyltransferase and reconstitution of fructan synthesis *in vitro* with purified sucrose: sucrose 1-fructosyltransferase and fructan: fructan 1- fructosyltransferase. Plant Physiol. 1996; 110: 1167-1175..

(5) Lüscher M, Frehner M, Nosberger, J, Purification and characterization of fructan: fructan fructosyl transferase from Jerusalem artichoke (*Helianthus tuberosus* L.). New Phytol. 1993; 123: 717-724.

(6) Lüscher M, Erdin C, Sprenger N, Hochstrasser U, Boller T, Wiemken A, Inulin synthesis by a combination of purified fructosyltransferases from tubers of *Helianthus tuberosus*. FEBS. 1996; 385: 39-42.

(7) Lüscher M, Hochstrasser U, Vogel G, Aeschbacher R, Galati V, Nelson CJ, Boller T, Wiemken A, Cloning and functional analysis of sucrose: sucrose 1- fructosyltransferase from tall fescue. Plant Physiol. 2000; 124: 1217-1227.

(8) Pan W, Sunayama Y, Nagata Y, Taniguchi M, Takano M, Inoue E, Tamagake H, Anzai H, Cloning of a cDNA encoding the sucrose: sucrose 1- fructosyltransferase (1- SST) from yacon and its expression in transgenic rice. Biotechnol&Biotechnol. 2009; 23(4): 1479-1484.

(9) Praznik W, Beck RHF, Spies Th, Isolation and characterization of sucrose:sucrose 1^F-β-D-fructosyltransferase from tubers of *Helianthus tuberosus* L. Agri. Biol. Chem. 1990; 54(9): 2429-2431.

(10) Robertfroid MB, Gibson GR, Delzenne N, The biochemistry of oligofructose, a nondigestible fiber: an approach to caloric value. *Nutr* Rev. 1993; 51: 137-146.

(11) Steinbüchel A, Rhee SK, "Polysaccharides and polyamides in the food industry. Properties, production, and patents." eds. Franck A, Leenheer LD, Wiley-VCH Verlag GmbH&Co, Weinheim. 2005.

(12) Tanjor S, Judprasong K, Chaito C, Jogloy S, Inulin and fructooligosac-charides in different varieties of Jerusalem artichoke (*Helianthus tuberosus* L.). KKU Res. J. 2012; 17(11): 25-34.

(13) Ueno K, Ishiguro Y, Yoshida M, Onodera S, Shiomi N, Cloning and functional characterization of a fructan 1-exohydrolase (1-FEH) in edible burdock (*Arctium lappa* L.). Chemistry Central J. 2011; 5: 16.

(14) van der Meer IM, Koops AJ, Hakkert C, van Tunen AJ, Cloning of the fructan biosynthesis pathway of Jerusalem artichoke. The Plant J. 1998; 15(4): 489-500.

(15)   Van den Ende W, Clerens S, Vergauwen R, Boogaerts D, Le Roy K, Arckens L, Van Laere A, Cloning and functional analysis of a high DP fructan:fructan 1-fructosyl transferase from *Echinops ritro* (Asteraceae): comparison of the native and recombinant enzymes. J Exp Bot. 2006; 57(4): 775-789.

(16)   Wu s, Letchworth GJ, High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. Drug Dicovery and Genome Tech. 2004; 36(1): 152-154.

(17)   Vijn I, Smeekens S, Fructan: More than a reserve carbohydrate. Plant Physiol. 1999; 120: 351-359.