Identification of multifunctional elongation gene from *Thraustochytrium aureum* ATCC34304 and functional expression of the gene in *Pichia pastoris.*

工學碩士學位 請求論文

*Thraustochytrium aureum* ATCC34304 균주 유래의 다기능 elongation 유전자의 규명과 *Pichia pastoris* 내에서 관련 유전자의 기능적 발현 및 특성 분석

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Identification of multifunctional elongation gene from *Thraustochytrium aureum* ATCC34034 and functional expression of the gene in *Pichia pastoris*
Identification of multifunctional elongation gene from
*Thraustochytrium aureum* ATCC34034 and functional expression of
the gene in *Pichia pastoris*

by

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2008年 2月

主審____________________ (印)

副審____________________ (印)

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ABSTRACT

*Thraustochytrium aureum* contains a high level of polyunsaturated fatty acids (PUFAs). In the present study, the isolation and characterization of fatty acid elongase from *T. aureum* was demonstrated to be associated with the synthesis of not only the C22 PUFAs, but also the C20s. The *TauELO* gene of the elongase contains 825 bp nucleotide ORF, which encodes a protein of 274 amino acids, and it shares high similarity with those of other PUFA elongases. The expression of the *TauELO* gene in *Pichia pastoris* resulted in the production of adrenic acid (ADA, C22:4; n-6) and docosapentaenoic acid (DPA, C22:5; n-3) in cells that were provided with arachidonic acid (AA, C20:4; n-6) eicosapentaenoic acid (EPA, C20:5; n-3), respectively. The conversion of AA and EPA were 31.3% and 33.9% respectively. In addition, the *TauELO* is able to carry out the Δ9-elongation of the C18 PUFAs, such as linoleic acid (LA, C18:2; n-6) and α-linolenic acid (ALA, C18:3; n-3) to eicosadienoic acid (EDA, C20:2; n-6) and eicosatrienoic acid (ETrA, C20:3; n-3), respectively and also able to synthesize eicosenoic acid (C20:1; n-9) from oleic acid (OA, C18:1; n-9), even though the conversion level is low. Further, the *TauELO* was also involved in the elongation of γ-linolenic acid (GLA, C18:3 n-6) to dihomo-γ-linolenic acid (DGLA, C20:3; n-6). The *TauELO* protein was confirmed to have multifunctional activities, such as 5, 6, and 9-elongations, and the elongation of monounsaturated fatty acid.

*Keywords*: docosapentaenoic acid, eicosapentaenoic acid, arachidonic acid, adrenic acid, elongase, polyunsaturated fatty acids, *Thraustochytrium aureum*
국문요약

*Thraustochytrium aureum*은 고농도의 불포화지방산을 함유하고 있다. 이 번 연구에서 *T. aureum*의 불포화지방산 생합성관련 효소인 elongase가 탄소수 22개의 불포화지방산뿐만 아니라 20개의 불포화지방산에도 활성이 있다는 것을 보여주고 있다. *TauELO* elongase 유전자는 약 825개의 염기쌍의 서열로 되어 있으며 이는 274개의 아미노산 서열로 이루어진 단백질을 만들어낸다. 그리고 이는 여타 다른 PUFA elongase 유전자와 상당히 높은 유사도를 보여준다. 이 *TauELO*를 Pichia pastoris GS115 균주에 도입한 재조합 유전체의 발현 실험에서 기질로 사용한 arachidonic acid (AA, C20:4; n-6)와 eicosapentaenoic acid (EPA, C20:5; n-3)는 각각 adrenic acid (ADA, C22:4; n-6)와 docosapentaenoic acid (DPA, C22:5; n-3)로 전환되었다. AA에서 ADA로의 전환율은 31.3%를 보였으며, EPA에서 DPA로의 전환율은 33.9%였다. 뿐만 아니라 외부 기질이 아닌 P.pastoris의 자체 생산 PUFA인 C18 PUFAs 또한 C20 PUFAs로 전환될 수 있었다. 내부기질인 linoleic acid (LA, C18:2; n-6)와 α-linolenic acid (ALA, C18:3; n-3)가 각각 eicosadienoic acid (EDA, C20:2; n-6)와 eicosatrienoic acid (ETrA, C20:3; n-3)로 전환되었다. 이 외에도 매우 낮은 전환율을 보여주었지만 oleic acid (OA, C18:1; n-9) 또한 eicosenoic acid (C20:1; n-9)로 전환될 수 있었다. 마지막으로 외부기질로서 γ-linolenic acid (GLA, C18:3; n-6)를 첨가하였을 때 dihomo-γ-linolenic acid (DGLA, C20:3; n-6)로 전환될 수 있었다. 따라서 앞의 실험 결과에 따라 *TauELO*는 Δ5, Δ6, Δ9 그리고 monounsaturated fatty acid의 elongation의 활성을 모두 보이는 다기능 활성 단백질이라 할 수 있었다.
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I. Introduction

1. A definition of Polyunsaturated fatty acids (PUFAs)

Very long chain polyunsaturated fatty acids (VLCPUFAs) such as arachidonic acid (AA, C20:4Δ5,8,11,14 n-6), eicosapentaenoic acid (EPA, C20:5Δ5,8,11,14,17 n-3) and docosahexaenoic acid (DHA, C22:6Δ4,7,10,13,16,19 n-3) play crucial roles as structural components of membrane phospholipids and as precursors of the eicosanoids of signaling molecules including prostaglandins, thromboxanes, and leukotrienes [1,2] and also have important effects on various physiological processes such as cognitive function and immunosuppressive and anti-inflammatory actions [3]. Recently, the potential roles of polyunsaturated fatty acids (PUFAs) in reducing heart disease and in improving vision sensitivity and reading ability were reported by several investigators [4-6]. Inadequate amounts of PUFAs are produced to meet metabolic demands and must therefore be obtained from dietary sources. Fish have been the major natural sources for the production of PUFAs, but in recent years its production has declined [7,8]. Therefore, there is an interest in obtaining economically important VLCPUFAs from other alternative sustainable sources such as oil seed crops and by implementation of genetically manipulating VLCPUFA biosynthetic pathways.
2. The pathway of PUFAs

The major PUFA product of the n-6 pathway is AA and those of the n-3 pathway are EPA, docosapentaenoic acid (DPA, C22:5Δ7,10,13,16,19) and DHA. Many of the PUFAs are synthesized from essential fatty acids such as linoleic acid (LA, C18:2Δ9,12 n-6) and α-linolenic acid (ALA, C18:3Δ9,12,15 n-3) by an alternating series of desaturation and elongation reactions. These two fatty acids become unsaturated by Δ6-desaturase into γ-linolenic acid (GLA, C18:3Δ6,9,12 n-6) and stearidonic acid (C18:4Δ6,9,12,15 n-3) [9,10], which are elongated by Δ6-elongase to dihomo-γ-linolenic acid (DGLA, C20:3Δ8,11,14 n-6) and eicosatetraenoic acid (C20:4Δ6,9,12 n-3), respectively [11,12]. However, an alternative pathway for the biosynthesis of C20 PUFAs has been demonstrated in some organisms that show lack of Δ6-desaturase and elongase activities. The first step in the route is the elongation of LA and ALA to eicosadienoic acid (EDA, C20:2Δ11,14 n-6) and eicosatrienoic acid (ETrA, C20:3Δ11,14,17 n-3), respectively, by the actions of Δ9-elongase [13,14], followed by a subsequent Δ8-desaturation step to form DGLA and eicosatetraenoic acid, respectively [15]. AA (C20:4; n-6) and EPA (C20:5; n-3) are respectively synthesized from DGLA and eicosatetraenoic acid by a Δ5-desaturase [16,17]. Furthermore, these two fatty acids are converted into adrenic acid (ADA, C22:4Δ7,10,13,16 n-6) and DPA (C22:5; n-3) by the action of an elongase complex (Δ5-elongase) [7]. This DPA is used as a substrate for the further production of DHA. The overall formation of long chain PUFAs (n-6 and n-3 pathways) from their precursors, i.e., LA and ALA, requires at least three elongation and three or more different desaturation steps capable of utilizing the long chain PUFAs as substrates [18].
3. Object

The gene encoding the fatty acid elongase has already been isolated from a few organisms such as *Marchantia polymorpha* L [7], *Mortierella alpina* [11], *Physcomitrella patens* [12], *Pavlova* sp. [19], and *Thalassiosira pseudonana* [20]. Previously, several desaturation enzymes (Δ4, Δ5) have been isolated from Thraustochytrids and their activities for the synthesis of PUFAs were demonstrated [21]. However, there have been no detailed reports on the multifunctional activities of elongase in *Thraustochytrium aureum*. In the present study, a fatty acid elongase gene was isolated from *T. aureum* and expressed heterologously in the yeast *Pichia pastoris* GS115 to investigate multifunctional activities, such as Δ5, Δ6, Δ9, and monounsaturated fatty acid elongation activities.
Π. Experimental procedures

1. Strain and Growth Conditions

*T. aureum* ATCC 34304 was grown in Artificial Sea Water (ASW) medium at 25 ºC for 3 days with shaking at 180 rpm. The cells were then harvested by centrifugation and used for genomic DNA isolation. The genomic DNA was isolated according to the method of Chung *et al.* [22]. The yeast strain *P. pastoris* GS115 was grown in Minimal Glycerol (MGY) media.
2. PCR-based cloning of elongase

The *T. aureum* elongase gene sequence was used to search the NCBI. We designed primers based on the NCBI sequencing data [23]. The forward primer was 5’-aaggatcaccatgggtATGACGAGCAACATGAGC-3’ including the Kozac sequence and the reverse primer was 5’-aagaattcTTAGGCCGACTTGGCCTT-3’ containing *BamHI* and *EcoRI* restriction enzyme sites (underlined), respectively, for cloning. The PCR conditions were: Initial denaturation at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 10 min.

After amplification, the amplified products were fractionated via 0.8% agarose gel electrophoresis, and the DNA bands were purified by using a PCR purification kit (Wizard SV Gel and PCR Clean Up System - Promega, USA) and the purified products were ligated with pPIC3.5 vector (Invitrogen, USA). The recombinant DNA was transferred to *Escherichia coli*(XL1-blue) and amplified through the host cell culture (37 °C, 180 rpm, overnight).
3. Expression of TauELO in P. pastoris GS115

After confirming the PCR products by sequencing, the open reading frame of the 5-elongase gene was subjected to BamHI and EcoRI double digestion and ligated into BamHI and EcoRI sites of the yeast expression vector pPIC3.5 under the control of the inducible promoter AOX1 to generate a recombinant plasmid designated as pPIC/TauELO-1 and TauELO-2. The resulting vector was linearized by BglII. The yeast strain P. pastoris GS115 was transformed with the expression constructs using the electroporation method [24] and the transformants were selected on minimal agar medium plates lacking histidine. Gene sequencing was performed using a Solgent sequencingsystem (Korea).

The transformants were first grown in MGY medium containing 1% glycerol at 28 °C with shaking at 250 rpm for 24 h. The cultured cells were then centrifuged at 3000 rpm, and the supernatant was removed. The cell pellets were transferred to MM medium (Minimal Methanol medium - 1.34% Yeast Nitrogen Base, 0.00004% biotin, 0.5% methanol) for induction. The cells were incubated at 28 °C with shaking at 250 rpm for 24 h. After culturing, the cells were centrifuged at 3000 rpm, the supernatant was removed, and the cell pellets were transferred to MM medium with or without 0.5 mM substrates (AA, n-6; EPA, n-3; GLA, n-6) fatty acids in the presence of 0.1% tergitol, and incubated at 28 °C with shaking at 250 rpm for 48 h. A culture of GS115 containing pPIC3.5 vector was used as a control.
4. Fatty acid analysis

Total fatty acids extracted from the yeast cultures were analyzed by gas chromatography (GC) of methyl esters. Three ml of H$_2$SO$_4$-MeOH (H$_2$SO$_4$ : MeOH= 5:100, v/v) was added to the materials. The resultant solution was then sealed in a 15 ml glass culture tube, which was filled with nitrogen gas, and the samples were heated to 100 ºC for 1 h. After the samples cooled to room temperature, 2 ml of water and 1 ml of hexane were added. The samples were vortexed and centrifuged at 3000 rpm for 10 min. Fatty Acid Methyl Esters (FAMEs) were then extracted in the hexane phase. About 800 µL of the acquired FAMEs was placed into a GC vial and analyzed by GC (Hewlett Packard 6890, USA) equipped with flame-ionized detector (FID) and a DB23 (30 m x 0.25 mm x 0.25 µm, Agilent Technologies, USA) capillary column. Fatty acids were identified by comparison with retention time of specific standards. Further, identified fatty acids were characterized on diethylamide derivative by GC-Mass Spectrometry (GC-MS) using with helium carrier operating at an ionization voltage of 70eV and with scan range of 30 – 520 Da.
III. Results and Discussion

1. Gene isolation & detection of mutant gene

Recently, *T. aureum* has received considerable attention owing to its ability to produce several PUFAs [25]. The present study focuses on identification of the elongase gene, which is involved in the elongation of C20 PUFAs. Using the degenerate primer, the PCR product containing the expected length of gene was obtained. The PCR amplified products were fractionated via agarose gel electrophoresis (Fig. 1) and the PCR product of expected length of gene was cloned into yeast expression vector pPIC3.5 and sequenced. The sequence analysis showed the two amplified PCR products named as *TauELO-1* and *TauELO-2*. In the second clone (*TauELO-2*), the amino acid methionine was changed to threonine in the 165th position. In addition, these two clones were tested for their enzymatic activities in the yeast (*P. pastoris*) to investigate the new elongation functions as well as the improved conversion rate of several elongation reactions. The open reading frame of the *TauELO* gene contains 825 bp lengths and it encodes a protein of 274 amino acids with a calculated molecular mass of 31 kDa (Fig. 2).
Fig. 1. PCR amplified products verified by agarose gel electrophoresis. M, Marker DNA Ladder Lane 1, TauELO-1 Lane 2, TauELO-2.
Fig. 2. Comparison of the amino acid sequence of *TauELO* from *T. aureum* with other elongase. The amino acid sequence of elongase from *T. aureum* (*TauELO*-2), *Thraustochytrium* sp. (*TspELO5*, NCBI accession No. CAJ30843), *M. polymorpha* L (*MpoELO6*, NCBI accession No. AAT85662), *Phaeodactylum tricornutum* (*PtrELO6*, NCBI accession No. ABP49077), *Ostreococcus tauri* (*OtaELO5*, NCBI accession No. AY591336) and *Isochrysis galbana* (*IgaELO9*, NCBI accession No. CAP07470). The conserved histidine and tyrosine boxes are underlined; the 17 amino acid residues conserved among previously reported ELO-like proteins are shaded. #-The amino acid Threonine was substituted instead of Methionine (*TauELO*-2). *TauELO*-1 is similar to the original sequence.
2. Comparison between TaELO and other elongase genes

The deduced amino acid sequence of the cloned elongase was compared with the amino acid sequences of other fatty acid elongases from other microorganisms using the Clustal W program. The predicted amino acid sequence of TauELO shared significant identity of 54% with Thraustochytrium sp. (NCBI accession No. CAJ30843) followed by 35.7% identity with M. polymorpha L (NCBI accession No. AAT85662) (Fig. 2). Similar to other PUFA elongases, this protein contains two conserved motifs (a histidine box - containing three histidine residues, L-H-X-X-H-H and a tyrosine box - containing three tyrosine residues, M-Y-X-Y-Y) as well as 17 invariant amino acids that are conserved in the known elongase. These residues are critical for elongase activity [26]. Homology of the sequence alignment was mainly obtained in the histidine and tyrosine boxes and the areas of the 17 invariant amino acids. In addition, other important characteristics of the TauELO protein were revealed that the fatty acid elongase gene is hydrophobic and contains six transmembrane regions (Fig. 3). Other researchers have also reported that the fatty acid elongase contains six transmembranes [7,11,18,19]. The histidine box is predicted to be located between the 3rd and 4th membranes and the tyrosine box in the 4th membrane (Fig. 3). Qi et al. [27] reported that the histidine box plays an important role in elongase activity and also in desaturase activity [28].
Fig. 3. TMpred – Prediction of transmembrane regions of the TauELO-2 protein coding sequence.
3. Heterologous expression

3.1. Activity of Δ5 elongase

In order to characterize the elongase activity, the TauELO gene was amplified by PCR and inserted into the yeast expression vector pPIC3.5, yielding the plasmids pPIC/TauELO-1 and TauELO-2. The ligated products were transformed into yeast cell *P. pastoris* GS115 under the control of *AOX1* inducible promoter. Similarly, the empty vector was transformed into the yeast as a control. Both clones were grown in MM medium for 24 h. Fig. 4 shows the fatty acids profile of the recombinant *P. pastoris*. The substrates such as AA and EPA being supplemented into the medium, a yeast cell containing TauELO-2, revealed the extra novel fatty acid peaks when compared with the control. This result demonstrates that the fatty acid elongase could convert the AA (C20:4; n-6) and EPA (C20:5 n-3) to ADA (C22:4; n-6) and DPA (C22:5 n-3), respectively (Fig. 4B,D and Table 1,2). The control yeast cultures that were transformed with the empty vector could produce up to C18 PUFAs (C16:0; C16:1; C18:0; C18:1; C18:2; C18:3), but did not produce any C20 PUFAs (Table 1,2 and Fig. 4E), findings that are supported by other researchers [29,30]. This heterologous host is suitable for the transformation of elongase gene for the synthesis of C20 and C22 PUFAs. The percentage of the new fatty acid products ADA and DPA were 2.03% and 3.17% of the total fatty acids (Table 1 and 2). The conversion rate of AA and EPA to ADA and DPA were 31.3% and 33.9%, respectively in the case of TauELO-2 (Table 3). These conversion rates were substantially higher than those of the clone TauELO-1 and the other report [19]. The increased conversion rate of TauELO-2 might be not only due to the high expression levels of the gene in the host yeast cells (*P. pastoris*), but also due to the enhancement of the elongase activity by the particular amino
acid (threonine), because this amino acid residue is present in close to histidine box of the elongase sequence and may be interacted directly with the PUFAs substrate. Ranong et al. [31] had also reported that the mutant amino acid present in close to the histidine rich motif, altered the substrate preferential and beneficial for the production of fatty acids. A similar rate of conversion was obtained in the case of M. polymorphaL [7]. In addition, GC-MS analysis of the new fatty acids verified that two new peaks spectrum were identical to authentic ADA and DPA (data not shown). This ADA (n-6) and DPA (n-3) are used as substrates for the next step, i.e. the final production of DPA (n-6) and DHA (n-3) in marine eukaryotes. Burja et al. [32] reported that the thraustochytrids could produce an abundant amount of omega 3, particularly C20 and C22 PUFAs.
Fig. 4. GC analysis of FAMEs from yeast strain expressing the elongase with exogenous substrates AA and EPA. (A) empty vector with AA, (B) pPIC/TauELO-2 with AA, (C) empty vector with EPA, (D) pPIC/TauELO-2 with EPA, (E) control (empty vector), (F) pPIC/TauELO-2.
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Empty vector with AA</th>
<th>pPIC/TauELO-1 with AA</th>
<th>pPIC/TauELO-2 with AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>12.30 ± 0.19</td>
<td>10.98 ± 0.01</td>
<td>10.31 ± 0.30</td>
</tr>
<tr>
<td>C16:1</td>
<td>2.67 ± 0.01</td>
<td>2.58 ± 0.01</td>
<td>2.52 ± 0.04</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.68 ± 0.21</td>
<td>2.08 ± 0.01</td>
<td>1.88 ± 0.15</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>47.45 ± 1.24</td>
<td>44.34 ± 0.66</td>
<td>45.31 ± 1.29</td>
</tr>
<tr>
<td>C18:2; n-6</td>
<td>22.43 ± 1.42</td>
<td>17.40 ± 0.09</td>
<td>12.66 ± 0.88</td>
</tr>
<tr>
<td>C18:3; n-3</td>
<td>1.60 ± 0.27</td>
<td>0.38 ± 0.08</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td>C20:1 n-9</td>
<td>ND</td>
<td>1.22 ± 0.05</td>
<td>2.12 ± 0.05</td>
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<tr>
<td>C20:2; n-6</td>
<td>ND</td>
<td>9.91 ± 0.00</td>
<td>12.83 ± 0.43</td>
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<td>C20:4 n-6</td>
<td>4.14 ± 0.02</td>
<td>4.98 ± 0.66</td>
<td>4.45 ± 0.23</td>
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<tr>
<td>C20:3 n-3</td>
<td>ND</td>
<td>0.76 ± 0.00</td>
<td>0.73 ± 0.02</td>
</tr>
<tr>
<td>C22:4 n-6</td>
<td>ND</td>
<td>1.25 ± 0.17</td>
<td>2.03 ± 0.04</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD of three independent experiments.
Table 2. Fatty acid profiles of yeast cells containing empty vector and pPIC/TauELO grown in the presence of EPA.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Empty vector with EPA</th>
<th>pPIC/TauELO-1 with EPA</th>
<th>pPIC/TauELO-2 with EPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>12.68 ± 1.47</td>
<td>11.27 ± 0.83</td>
<td>10.90 ± 0.66</td>
</tr>
<tr>
<td>C16:1</td>
<td>3.15 ± 0.04</td>
<td>4.25 ± 0.29</td>
<td>3.84 ± 0.55</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.91 ± 0.51</td>
<td>1.67 ± 0.16</td>
<td>2.06 ± 0.09</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>39.38 ± 2.26</td>
<td>42.71 ± 0.94</td>
<td>40.63 ± 1.09</td>
</tr>
<tr>
<td>C18:2; n-6</td>
<td>23.97 ± 5.00</td>
<td>17.88 ± 1.05</td>
<td>17.33 ± 2.25</td>
</tr>
<tr>
<td>C18:3; n-3</td>
<td>6.31 ± 2.27</td>
<td>2.33 ± 0.42</td>
<td>2.41 ± 0.37</td>
</tr>
<tr>
<td>C20:1 n-9</td>
<td>ND</td>
<td>0.85 ± 0.15</td>
<td>1.10 ± 0.34</td>
</tr>
<tr>
<td>C20:2; n-6</td>
<td>ND</td>
<td>7.64 ± 1.48</td>
<td>7.67 ± 1.13</td>
</tr>
<tr>
<td>C20:3; n-3</td>
<td>ND</td>
<td>1.26 ± 0.24</td>
<td>1.95 ± 0.56</td>
</tr>
<tr>
<td>C20:5; n-3</td>
<td>9.07 ± 6.24</td>
<td>4.42 ± 0.55</td>
<td>5.99 ± 2.00</td>
</tr>
<tr>
<td>C22:5; n-3</td>
<td>ND</td>
<td>1.75 ± 0.25</td>
<td>3.17 ± 1.35</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD of three independent experiments.
Table 3. Substrate conversion rate of TauELO-1 and TauELO-2

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Conversion rate (%)</th>
<th>pPIC/TauELO-1</th>
<th>pPIC/TauELO-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:1 to C20:1 (n-9)</td>
<td></td>
<td>2.20 ± 0.35^b</td>
<td>4.11 ± 1.10^b</td>
</tr>
<tr>
<td>C18:2 to C20:2 (n-6)</td>
<td></td>
<td>28.87 ± 3.96^b</td>
<td>33.22 ± 4.23^b</td>
</tr>
<tr>
<td>C18:3 to C20:3 (n-3)</td>
<td></td>
<td>31.66 ± 4.38^b</td>
<td>49.93 ± 7.19^b</td>
</tr>
<tr>
<td>C18:3 to C20:3 (n-6)</td>
<td></td>
<td>93.00 ± 1.27^a</td>
<td>-</td>
</tr>
<tr>
<td>C18:4 to C20:4 (n-3)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C20:4 to C22:4 (n-6)</td>
<td></td>
<td>20.06 ± 0.10^a</td>
<td>31.32 ± 0.64^a</td>
</tr>
<tr>
<td>C20:5 to C22:5 (n-3)</td>
<td></td>
<td>27.37 ± 1.41^a</td>
<td>33.93 ± 2.96^a</td>
</tr>
</tbody>
</table>

^a - Exogenous, ^b - Endogenous conversion

Each value is the mean ± SD of three independent experiments.
3.2. Activity of Δ9 elongase

We stated above that *P. pastoris* is capable of synthesizing LA and ALA, which are the substrates for Δ9-elongation. The *TauELO-2* gene utilized LA and ALA as endogenous substrates from the yeast cells and it further synthesized EDA (C20:2; n-6) and ETrA (C20:3; n-3) of Δ9-elongation products, respectively (Fig. 4F). These two new fatty acids were further analyzed by GC-MS and identified as C20:2 (n-6) and C20:3 (n-3) (Fig. 5B,D). Although this elongase produced two Δ9-elongation products in both the n-6 and n-3 pathways, the amount of fatty acid present in the n-6 pathway was higher than that of the n-3 pathway, which is the percentage of the new fatty acids EDA and ETrA were accumulated respectively to 7.67 and 1.95% of the total fatty acids (Table 2). However, the conversion rate of LA and ALA to EDA and ETrA was about 33% and 49.9%, respectively (Table 3). To our knowledge, this is the first report of the synthesis of C20 PUFAs (9-elongation products) catalyzed by the same fatty acid elongase. These alternative pathways, LA to EDA and ALA to ETrA, may operate for the biosynthesis of long chain PUFAs. Subsequently, the Δ9-elongation products are desaturated to DGLA (C20:3; n-6) and eicosatetraenoic acid (C20:4; n-3) by the actions of Δ8-desaturase in *Euglena gracilis* [15].
Fig. 5. GC-MS analysis of the novel peaks identified in *P. pastoris* transformed with the recombinant plasmid pPIC/TauELO-2 containing the elongase of *T. aureum*. (A) and (C) authentic EDA and ETrA standards respectively, (B) and (D) comparison of the mass spectra of the novel peaks.
3.3 Elongation of monounsaturated fatty acid

In addition, the elongase has ability to convert the monounsaturated oleic acid (OA, C18:1\(^9\) n-9) into the eicosenoic acid (C20:1\(^{11}\) n-9). The result suggested that the TauELO might also be involved in the elongation of the fatty acid (C18:1\(^9\) n-9). The endogenous conversion rate of OA to eicosenoic acid was only 4.11% (Table 3) and the percentage of eicosenoic acid showed 1.1% of the total fatty acid (Table 2). The additional new peak is represented and confirmed by the pure eicosenoic acid (Fig. 4F). This new fatty acid (Fig. 4F) was not synthesized in the yeast containing the empty vector pPIC3.5 as control (Fig. 4E). The monounsaturated fatty acid produced from OA was further characterized by GC-MS and identified as eicosenoic acid (Fig. 6B). Previously, Leonard et al. [18] have also reported that the recombinant HELO1 was related with the elongation of monounsaturated fatty acid with low level of fatty acid expression.
Fig. 6. GC-MS analysis of the novel peak identified in *P. pastoris* transformed with the recombinant plasmid pPIC/TauELO-2 containing the elongase of *T. aureum*. (A) an authentic eicosenoic acid (C20:1) standard, (B) a comparison of the mass spectra of the novel peak.
3.4. Activity of Δ6 elongase

To determine the substrate specificity of elongation of PUFAs, the recombinant yeast strain pPIC/TauELO-1 was grown in the MM medium supplemented with 0.5 mM of exogenous substrate, GLA. An additional peak was obtained from induced pPIC/TauELO-1 grown in the presence of GLA. The retention time of an additional peak was identical to that of the methyl ester of authentic DGLA standard (Fig. 7B). The percentage of the accumulated fatty acid in the cells was 4.67% (Fig 7B) and its conversion of GLA to DGLA was 93% (Table 3). Further, the GC-MS analysis of the FAMEs confirmed that the spectrum of the peak (Fig. 7D) was identified as that of DGLA standard (Fig. 7C). This finding suggests that the present elongase was involved in the multiple elongation reaction in PUFAs pathway. A similar elongase activity has been reported in the elongation reactions of PUFAs in human [18]. It is interesting to note that the conversion level of all the fatty acids were higher in n-3 series than in n-6 pathway PUFAs.
Fig. 7. GC analysis of FAMEs from recombinant yeast expression Δ6-elongation activity with exogenous substrate GLA (C18:3; n-6) converted into DGLA (C20:3; n-6). (A) empty vector with GLA, (B) pPIC/TauELO-2 with GLA, (C) GC-MS analysis of an authentic DGLA standard, (D) mass spectrum of the novel peak. *The percentage of the fatty acid accumulation was 4.67%.
3.5. **There are three categories based on the substrate specificity of the fatty acids**

Meyer *et al.* [20] classified the PUFA elongases into three categories based on the substrate specificity of the fatty acids. The first group of elongases are involved in the synthesis of saturated and monounsaturated fatty acids. The yeast elongase genes *ScELO1* to *ELO3* are to elongate saturated and monounsaturated fatty acids, but not PUFAs [33,34]. The second group of elongase are specific for a single step in PUFAs synthesis (*M. polymorpha* L – specific to C20 PUFAs) [7] and the final group are related to multi-step synthesis (They involve the synthesis of PUFAs with variable chain lengths). The elongase *TaeELO* falls into the third group, because *TaeELO* can synthesize C20 and C22 PUFAs in multi-steps. The other group of elongases, including *OmELO* – *Oncorhynchus mykiss*, *CiELO* – *Ciona intestinalis* and *XlELO* – *X. laevis*, falls into the third group [20]. The identification of the elongase gene will be important to the production of C22 PUFAs and the cultivation of rich transgenic oil plants for biotechnological applications.
IV. Conclusion

In summary, we investigated TauELO-2, which encodes the fatty acid elongase and found that this enzyme can convert AA (C20:4; n-6) and EPA (C20:5; n-3) to ADA (C22:4; n-6) and DPA (C22:5; n-3), respectively. In addition, this enzyme is also able to synthesize not only the Δ9-elongation products of EDA (n-6), ETrA (n-3) and monounsaturated elongation product eicosenoic acid (C20:1) using LA, ALA and OA as endogeneous substrates but also 6-elongation product of DGLA (n-6) from GLA as exogenous substrate. These results clearly demonstrated that the T. aureum elongase gene encodes an enzyme capable of synthesizing Δ5, Δ6, Δ9 and monounsaturated elongation products.
V. References


33 Toke DA & Martin CE (1996) Isolation and characterization of a gene

감사의 글

대학교를 거쳐 대학원 2년 생활을 마무리가가는 지금까지 9년을 인하대 학교에 몸담아오면서 그 전까지의 20년 인생보다 많은 경험을 했다고 생각합니 다. 철없이 막연하게 생물이 좋아서 생물공학과를 선택해서 이곳으로 오게 되었지만, 이제까지 그 결정을 한 번도 후회한 적은 없었습니다. 다만 마음 한 구석에 항상 두려움은 가지고 있었습니다. 잘 할 수 있을까, 혹은 내가 선택한 분야가 앞으로 잘 되어나갈까 하는 앞으로의 전로에 대한 걱정과 혼란 속에서 가장 먼저 두드린 곳이 생물공학과 대학원, 허병기 교수님 연구실이었습니다. 많이 부족했지만, 또한 연구실 사정이 어려웠지만 교수님께서는 열심히 하겠다는 제 다툤만 들어주셔 훈혜히 승낙해 주셨습니다. 그리고 2년이 지난 지금 수많은 경험과 시행착오를 통해서 많은 것을 배울 수 있었고 지금은 졸업을 앞에 두고 있습니다. 여기까지 오는 데 있어서 교수님의 지도와 격려가 없었다면 지금 이 자리에 서 있지 못했을 것입니다. 항상 학생의 입장에서 모든 걸 배려해주고 지도해주신 교수님께 깊은 감사를 드립니다.

한국어 논문 작성 고지

항상 어떤 일이 끝났을 때는 무엇인가 아쉬움이 남게 되는데, 현재의 제 모습이 별반 다르지 않다는 것을 느끼게 됩니다. 조금만 더 열심히 했더라면 이보다 더 좋은 결과를 얻을 수 있었을텐데 하는 후회와 자기반성을 하게 됩니다. 단지 연구에 있어서만이 아니라 2년 동안 갈등과 생활습관, 인간관계 등에도 최선을 다하지 못한 점은 많이 아쉬움으로 남습니다. 이런 아쉬움을 뒤로 한 채 연구실을 떠나면서 2년 동안 같이 해온 식구들에게 감사의 말을 전하 고 시표됩니다. 먼저 제가 실험실에서 새로운 테마를 잡고 연구를 시작할 수 있도록 기초를 닦는데 도움주신 원호형 감사합니다. 취직하셔서 제가 졸업하는 모습은 보이드리지 못하지만 지금 하시고 계신 일 잘 되시고 목표하신 일 좌 이뤄지길 바랍니다. 그리고 수진 누나. 제가 실험하면서 잘 안되고, 실패만 거듭 할 때 누나의 도움과 격려 덕분에 난관을 잘 해쳐나갈 수 있었습니다. 정말 감

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사드리요. 박사과정 남은 부분 잘 마무리하시길 기원하겠습니까. 그리고 영수형. 졸업하시고 나서도 조언과 지원 아낌없이 보내주신 점 정말 감사드리려요. 실험이만 아니라 인생의 선배로서도 많이 가르쳐 주신 것들 잊지 않고 가슴에 잘 새기겠습니다. 동훈이, 동기이면서 실험실 선배로서 많은 도움을 주고, 후배 셋을 혼자서 이끌기 많이 혼들었으리라 생각합니다. 수고 많았고 열심히 하는 모습 정말 많이 보고 배웠다. 앞으로 남은 과정 무사히 끝내시길 빼계. 그리고 내 동기 동록이랑 은진이, 2년을 같이 하면서 정말 잘 챙기지 못해서 미안하다는 말부터 하고 싶구나. 서로 잘 맞지 않는다고만 생각하고 정작 그것을 고치려고 생각하기보다 고집만 부렸던 것 많이 반성하고 있고. 그래도 마지막까지 함께 돕고, 무사히 졸업해서 모두들 고맙다. 그리고 후배 환희누나, 명주. 어려운 시기에 들어와서 고생하고 있지만, 잘 인내한다면 앞으로 사회에 진출했을 때 지금의 고생이 빛을 볼 수 있을 거라 생각합니다. 특히 명주는 실험적으로 잘 챙겨주지 못해서 미안하구나. 그래도 내가 있기에 실험이를 떠나도 든든함을 느낀다. 이 외에도 지금은 여기 안계시지만 많은 실험적 조언을 아깝없이 주셨던 서정우 박사님, 힘들 때마다 다독어주셨던 지숙이 누나. 그리고 미처 언급하지 못했지만 함께 같이 했던 선배님들, 그리고 후배들 모두에게 감사의 마음을 전합니다. 모두 생물시스템공학 실험실에서의 즐거운 추억을 기억하면서 사회에서 좋은 성과들 내시길 기원합니다.

마지막으로 부족하고 모자란 아들, 그리고 동생이지만 끝까지 밀어주고 지원해주신 어머니, 누나에게 감사드립니다. 그리고 비록 볼 수도 없고 제 모습을 보여드릴 수도 없지만, 어디선가 지켜보고 계실 아버지께도 감사의 마음을 전합니다. 앞으로 사회에 나가서 더욱 성장하는 모습 보여드리겠습니다.