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Synopsis of Thesis

Title: Molecular genetic study on fatty acid biosynthesis and desaturation for *Hansenula polymorpha*

(*Hansenula polymorpha*における脂肪酸合成と不飽和化に関する分子遺伝学的研究)

Name of Applicant Juthaporn Sangwallek

Chapter 1: General introduction

Fatty acids are essential components of virtually all cells and fulfill a variety of cellular functions: energy storage, major component of cell membrane and signaling molecules. In mammals, fatty acids particularly polyunsaturated fatty acids (PUFAs) are essential for maintaining health due to their involvement in fever, inflammations, vasodilatation, clotting, pain, neurotransmission, modulation of cholesterol and development of neural, retina and brain functions. Fish and plant oils, as traditional sources of PUFAs, are limited and besides they have large variation in their quality depending on types, climate and geography. To solve the problem of inadequacy and quality, yeast cell oil, which has met both the demand for efficient large scale production and criteria of safety and authenticity of released products, arises as an alternative source to develop for the production of the PUFAs. Although *Saccharomyces cerevisiae* is generally considered to be an excellent model owing to feasible precise genetics, applicability of recombinant DNA technology, and abundant and accessible genetic data, the fatty acid composition of the *S. cerevisiae* is not suitable for use in industrial production of fatty acids due to incapability to produce PUFAs. *Hansenula polymorpha* is therefore chosen for the study because it intrinsically able to produce PUFAs that is the bottleneck in the production of other downstream PUFAs. The objective of this study is to gain more insight in the characteristic of the yeast fatty acid synthase and identification of functional fatty acid desaturase genes for the production of PUFAs in yeast, thus not only allowing us to complement the better understanding in molecular basis of fatty acid biosynthesis in yeast but also giving a gateway to engineer fatty acid biosynthetic pathway and fatty acid desaturation for further biological application.

Chapter 2: Particular domain of yeast fatty acid synthase determining fatty acyl chain length

Yeast fatty acid synthase (Fas) comprises two subunits, α_6 and β_6, encoded by *FAS2* and *FAS1*, respectively. Aiming at understanding feature by which yeast Fas recognizes chain length of fatty acid products from *de novo* biosynthesis, in this chapter, I provide structure-function relationship for yeast Fas activity relative to fatty acid composition. The Fas structure-function relationship was investigated by creating chimeric genes combining *FAS* sequences from *S. cerevisiae* (*ScFAS*) and *H. polymorpha* (*HpFAS*), which mostly produces C16 and C18 fatty acids, respectively. The C16/C18 ratios decreased from 2.2 ± 0.1 in wild-type *S. cerevisiae* to 1.0 ± 0.1, 0.5 ± 0.2 and 0.8 ± 0.1 by replacement of *ScFAS1, ScFAS2*, and *ScFAS1 ScFAS2* with *HpFAS1, HpFAS2*, and *HpFAS1 HpFAS2*, respectively, suggesting that the α, but not β subunits play a major role determining fatty acyl chain length. Replacement of phosphopantetheinyl transferase (PPT) domain with the equivalent region from *HpFAS2* did not affect C16/C18 ratio. Chimeric Fas2 containing half N-terminal ScFas2 and half C-terminal HpFas2 carrying *H. polymorpha*

ketoacyl synthase (KS) and PPT gave a remarkable decrease in C16/C18 ratio (0.6 ± 0.1), indicating that KS plays a major role in determining chain length.

Chapter 3: Cloning and functional analysis of two fatty acid desaturase genes involved in the synthesis of polyunsaturated fatty acids in *Hansenula polymorpha*

In the chapter 3, two fatty acid desaturase genes have been cloned: *HpFAD2* and *HpFAD3* encode *H. polymorpha* Δ12-fatty acid desaturase (HpFad2) and Δ15-fatty acid desaturase (HpFad3), which are responsible for the production of linoleic acid (LA, C18:2, Δ9, Δ12) and α-linolenic acid (ALA, αC18:3, Δ9, Δ12, Δ15), respectively. The open reading frame of the *HpFAD2* and *HpFAD3* genes is 1,215 bp and 1,239 bp, encoding 405 and 413 amino acids, respectively. The putative amino acid sequences of HpFad2 and HpFad3 share more than 60% similarity and three conserved histidine-box motifs with other known yeast Fad homologs. The functional repertoires of both Fads were expanded by gene disruption in *H. polymorpha* and heterologous expression in *S. cerevisiae*, indicating that *HpFAD2* and *HpFAD3* indeed encode Δ12- and Δ15- fatty acid desaturases that function as the only ones responsible for desaturation of oleic acid (C18:1) and linoleic acid (C18:2), respectively, in *H. polymorpha*. As seen in the *S. cerevisiae OLE1* gene, upstream sequences of *HpFAD2* and *HpFAD3* contain a Fatty Acid Regulated (FAR) region and a Low Oxygen Response Element (LORE), which are potentially responsible for unsaturated fatty acid and hypoxia-mediated transcriptional control. I found that the transcription of both genes were up-regulated under hypoxic conditions. However, the transcriptional expression of *HpFAD2* was not controlled by fatty acids, whereas that of *HpFAD3* was repressed by an excess of C18:1, C18:2 and αC18:3. This study indicates that *HpFAD2* may be regulated by post-transcriptional mechanisms, whereas *HpFAD3* may be primarily controlled at a transcriptional level.

Chapter 4: General discussion and conclusion

This study describes structure-function data for yeast Fas activity relative to fatty acid composition to determine feature of yeast Fas that control fatty acyl chain length and, besides, it complements our current knowledge of molecular basis of the *H. polymorpha* fatty acid desaturation system. The KS domain in particular, but not other domains on Fas2, acts as a major determinant of fatty acyl chain length in yeast. The diverse length of fatty acid pools created by HpFas and ScFas are likely not governed by the difference in their catalytic sites on the KS. However, according to clustering of amino acid sequences of the KS from yeast species relative to their C16/C18 ratios, I found that, notably, seventeen amino acid residues in the N-terminal of the KS domain are conserved in the groups. Therefore, I propose that the KS domain especially the N terminal region may be productive targets for engineering synthesis of fatty acid products. This study sheds a way for future researches toward better understanding of the regulation of chain length and desaturation of fatty acids, which will allow us fine-tuning of fatty acid biosynthetic pathway and fatty acid desaturation in response to environmental changes or for further biological application for fatty acid production in yeast.

様式 7

論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

Fatty acids, particularly polyunsaturated fatty acids (PUFAs), are essential for maintaining health due to their involvement in fever, inflammations, vasodilatation, clotting, pain, neurotransmission, modulation of cholesterol and development of neural, retina and brain functions. When traditional sources of PUFAs, fish and plant olis, are limited, it is therefore highly desirable to produce PUFAs from alternative sources. To solve the problem of inadequacy and quality, yeast cell oil, which has met both the demand for efficient large scale production and criteria of safety and authenticity, arises as an alternative source to develop for the production of the PUFAs. The objective of this study is to gain more insight in the characteristic of the yeast fatty acid synthase and identification of functional fatty acid desaturase genes for the production of PUFAs in yeast.

In Chapter 1, Ms. Juthaporn reviewed the previous studies related to production of fatty acids in plants (Napier and Sayanova 2005; Robert et al. 2005), filamentous fungi (Uemura 2012) and indicated the advantages of using yeast as an alternative source for PUFAs production. Although *Saccharomyces cerevisiae* is generally considered to be an excellent model owing to feasible precise genetics, applicability of recombinant DNA technology, and abundant and accessible genetic data, the fatty acid composition of the *S. cerevisiae* is not suitable for use in industrial production of fatty acids due to incapability to produce PUFAs. *Hansenula polymorpha* is therefore chosen for this study because it intrinsically is able to produce PUFAs that is the bottleneck in the production of other downstream PUFAs. Our group has accomplished the basis of molecular genetic studies on fatty acid synthesis (Kaneko et al. 2003; Nakajima 2004), elongation (Prasitchoke et al. 2007) and desaturation (Anamnart et al. 1997; Lu et al. 2000) in *H. polymorpha*. In an effort to complete the whole picture of fatty acid biosynthesis in *H. polymorpha*, in this study, Ms. Juthaporn revealed a more fulfillment of the *de novo* biosynthesis and desaturation of PUFAs in *H. polymorpha* as follows.

Yeast fatty acid synthase (Fas), which functions in *de novo* biosynthesis of fatty acids, comprises two subunits, α_6 and β_6, encoded by *FAS2* and *FAS1*, respectively. Aiming at understanding feature by which yeast Fas recognizes chain length of fatty acid products from *de novo* biosynthesis, in the Chapter 2 of this study, Ms. Juthaporn provided structure-function relationship for yeast Fas activity relative to fatty acid composition. The

Fas structure-function relationship was investigated by creating chimeric genes combining *FAS* sequences from *S. cerevisiae* (*ScFAS*) and *H. polymorpha* (*HpFAS*), which mostly produces C16 and C18 fatty acids, respectively. The C16/C18 ratios decreased from 2.2 ± 0.1 in wild-type *S. cerevisiae* to 1.0 ± 0.1, 0.5 ± 0.2 and 0.8 ± 0.1 by replacement of *ScFAS1*, *ScFAS2*, and *ScFAS1 ScFAS2* with *HpFAS1*, *HpFAS2*, and *HpFAS1 HpFAS2*, respectively, suggesting that the α, but not β subunits play a major role determining fatty acyl chain length. Replacement of phosphopantetheinyl transferase (PPT) domain with the equivalent region from *HpFAS2* did not affect C16/C18 ratio. Chimeric Fas2 containing half N-terminal ScFas2 and half C-terminal HpFas2 carrying *H. polymorpha* ketoacyl synthase (KS) and PPT gave a remarkable decrease in C16/C18 ratio (0.6 ± 0.1), indicating that KS plays a major role in determining chain length.

For better understanding in fatty acid desaturation in *H. polymorpha*, Ms Juthaporn cloned two fatty acid desaturases, *HpFAD2* and *HpFAD3* in Chapter 3. The *HpFAD2* and *HpFAD3* encode *H. polymorpha* Δ12-fatty acid desaturase (HpFad2) and Δ15-fatty acid desaturase (HpFad3), which are responsible for the production of linoleic acid (LA, C18:2, Δ9, Δ12) and α-linolenic acid (ALA, αC18:3, Δ9, Δ12, Δ15), respectively. The open reading frame of the *HpFAD2* and *HpFAD3* genes is 1,215 bp and 1,239 bp, encoding 405 and 413 amino acids, respectively. The putative amino acid sequences of HpFad2 and HpFad3 share more than 60% similarity and three conserved histidine-box motifs with other known yeast Fad homologs. The functional repertoires of both Fads were expanded by gene disruption in *H. polymorpha* and heterologous expression in *S. cerevisiae*, indicating that *HpFAD2* and *HpFAD3* indeed encode Δ12- and Δ15- fatty acid desaturases that function as the only ones responsible for desaturation of oleic acid (C18:1) and linoleic acid (C18:2), respectively, in *H. polymorpha*. As seen in the *S. cerevisiae OLE1* gene, upstream sequences of *HpFAD2* and *HpFAD3* contain a Fatty Acid Regulated (FAR) region and a Low Oxygen Response Element (LORE), which are potentially responsible for unsaturated fatty acid and hypoxia-mediated transcriptional control. Ms. Juthaporn found that the transcription of both genes were up-regulated under hypoxic conditions. However, the transcriptional expression of *HpFAD2* was not controlled by fatty acids, whereas that of *HpFAD3* was repressed by an excess of C18:1, C18:2 and αC18:3. This study indicates that *HpFAD2* may be regulated by post-transcriptional mechanisms, whereas *HpFAD3* may be primarily controlled at a transcriptional level.

In Chapter 4, Ms. Juthaporn summarized a significant breakthrough in this study and proposed a possibility of further experiments to discover more refined knowledge determining fatty acyl chain length from *de novo* biosynthesis and possible regulatory mechanisms on fatty acid desaturation in *H. polymorpha*. Ms. Juthaporn's study describes structure-function data for yeast Fas activity relative to fatty acid composition to determine feature of yeast Fas that control fatty acyl chain length and, besides, it complements our current knowledge of molecular basis of the *H. polymorpha* fatty acid desaturation system. In particular the discovery that the KS domain but not other domains on Fas2 acts as a major determinant of fatty acyl chain length in yeast is excellent. According to clustering of amino acid sequences of the KS from yeast species relative to their C16/C18 ratios and structure modeling, Ms. Juthaporn proposed that seventeen amino acid residues in the N-terminal of the KS domain, especially the residues at the position 1,347 for *S. cerevisiae* or 1,337 for *H. polymorpha*, should be productive targets for engineering synthesis of fatty acid products. In conclusion, Ms. Juthaporn's study sheds a way for future researches toward better understanding of the regulation of chain length and desaturation of fatty acids, which will allow us fine-tuning of fatty acid biosynthetic pathway and fatty acid desaturation for further biological application on fatty acid production in yeast. Judging from these achievements, this dissertation deserves the degree of Doctor of Engineering.