Faculty of Resource Science and Technology

Isolation and Characterisation of Lipogenesis Inducing Factor in *Enkabang* (*Shorea macrophylla*)

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Bachelor of Science with Honours
Resource Biotechnology
2022

Isolation and Characterisation of Lipogenesis Inducing Factor in *Enkabang* (*Shorea macrophylla*)

Aida Chan Hui Hui

A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours

(Resource Biotechnology)

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Programme of Resource Biotechnology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2022

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Acknowledgements

First and foremost, I would like to express my utmost gratitude to my Final Year Project Supervisor, Dr Chung Hung Hui for his endless guidance, encouragement, valuable advices and insightful knowledge throughout the whole period of my research in his Animal Biotechnology Lab. I believed that his constructive suggestions have helped me throughout writing this thesis and I am truly grateful to have learned from the best.

Secondly, I would like to give my earnest thanks to lecturer Dr Wee Boon Siong (Geochemical Lab), Assoc. Prof. Dr. Samsur bin Mohamad (Ecotoxicology Lab), lab technician Mr. Benedict (GC-MS lab) and lab assistant Encik Iskandar for their kindness in providing the necessary equipment and materials, their cooperation in this project.

Besides that, I would like to extend my sincere thanks especially to the post-graduate students Ivy, Michelle and Cindy for welcoming me warmly to the Animal Biotechnology Lab, assisting me in various forms, giving motivation and being kind, patient and willing to teach me about new knowledge during the period of my project. I would also like to my lab mates Beh and Catherine for assisting me and morally supporting me in writing this thesis. In addition, my deepest thanks too to my good friends Vanessa Rae, Narisa, Andrew, Amos, Farah and Amerull for their endless moral support throughout my degree life in UNIMAS.

Furthermore, a thank you also to my favourite virtual live streamers Astel, Vox and their community for indirectly giving me the mental and emotional strength, and encouragement to keep chasing my dream. And last but most importantly, I would like to express, from the bottom of my heart, thank you to my family for their love, support and believing in the choices I made throughout my life. My family have been a great pillar of motivation to continue fighting for a better life and have always been there to cheer me up when I am struggling since I started my degree. And to my cats for being the comfort I need. Hence, this FYP thesis is dedicated to them.

Isolation and Characterisation of Lipogenesis Inducing Factor in *Enkabang* (*Shorea macrophylla*)

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ABSTRACT

Shorea macrophylla or *Enkabang* is an important feedstock used by Sarawak farmers as finishing feed to induce deposition of intramuscular fats (IMF) in livestock animals. The present study focuses on extracting the potential agonists or potential PPARγ ligands from *Enkabang* and characterise its efficiency in inducing lipogenesis in livestock animals. A total of 218 compounds were successfully identified with GC-MS when extracted with acetone, isopropanol and diethyl ether solvents. Out of 218 potential ligands, approximately 98 of them shown hydrogen bonding to PPARγ model structure and only a selected few have been considered as a possible agonist of PPARγ. Among the 98, the compound that achieved the highest affinity score obtained from molecular docking analysis was -9.1 kcal/mol. Three compounds identified (2H-Benzo[d][1,3]oxazine, 2-[2-(5-methylfuran-2-yl)ethyl]-4,4-diphenyl-1,4-dihydro- compound, ergostane-3,6-dione, 25-(acetyloxy)-5-hydroxy-, (5.alpha.)- compound, and acetamide, N,N'-(methylenebis(2-chloro-4,1-phenylene))bis- compound) extracted with solvent diethyl ether were speculated to have a potential to be PPARγ agonists since it hydrogen bonded to amino acid residues GLN286, ARG288, SER289 and SER342, similar to positive control ROSI. Further research on other lipogenesis-related protein receptors and verification of the three potential agonists in inducing lipogenesis should be conducted due to its great potential as finishing feed in inducing IMF deposition in livestock animals.

Key words: *Shorea macrophylla***,** Lipogenesis, PPARγ, GC-MS, Molecular docking

ABSTRAK

Shorea macrophylla atau *Enkabang* merupakan buah yang kerap diberi oleh penternak Sarawak kepada haiwan ternakan sebagai makanan penyudah bagi memperolehi pemendapan lemak intramuskular (IMF) dalam haiwan ternakan. Objektif kajian memberi fokus dalam mengekstrak agonis berpotensi atau ligan PPARγ berpotensi daripada *Enkabang* dan mengkaji keberkesanan agonis tersebut dalam mengaktifkan proses lipogenesis dalam haiwan ternakan. Sebanyak 218 metabolit sekunder berjaya dikenal pasti dengan GC-MS apabila diekstrak dengan pelarut aseton, isopropanol dan dietil eter. Daripada 218 tersebut, terdapat sejumlah 98 daripadanya menunjukkan ikatan hidrogen kepada struktur model PPARγ dan hanya beberapa yang terpilih telah dianggap sebagai kemungkinan agonis PPARγ. Dalam 98 tersebut, metabolit sekunder yang mencapai skor pertalian tertinggi yang diperoleh daripada analisis dok molekul ialah -9.1 kcal/mol. Didapati bahawa tiga metabolit sekunder (2H-Benzo[d][1,3]oksazin, 2-[2-(5-metilfuran-2-yl)etil]-4,4-difenil-1,4-dihidro- sebatian, ergostan-3,6-dione, 25-(asetiloksi)-5-hidroksi-, (5.alpha.)- sebatian, dan asetamida, N,N'-(methylenebis(2-chloro-4,1-phenylene))bis- sebatian) yang diekstrak dengan pelarut dietel eter mempunyai potensi untuk menjadi agonis PPARγ. Kajian mengenai reseptor protein yang berkaitan dengan proses lipogenesis dan pengesahan tiga agonis yang berpotensi dalam pengaktifan lipogenesis perlu dijalankan secara lanjut kerana mempunyai potensi yang besar sebagai makanan penyudah dalam menyebabkan pemendapan IMF dalam haiwan ternakan.

Kata kunci*: Shorea macrophylla***,** Lipogenesis, PPARγ, GC-MS, Dok molekul

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LIST OF ABBREVIATIONS

ACC	Acetyl-coa carboxylase
ACLY	ATP-citrate lyase
AF-1	Active function region 1
AF-2	Active function region 2
DBD	DNA binding domain
FA	Fatty acids
FABP	Fatty acid binding protein
FAME	Fatty acid methyl ester
FASN	Fatty acid synthase
FTIR	Fourier transform infrared
G6PDH	Glucose-6-phosphate dehydrogenase
GC-MS	Gas Chromatograph-Mass Spectrometry
GRP	Glucose receptor protein
HCLLs	*Hibiscus cannabinus* L. leaves
HPLC	High performance liquid chromatography
IMF	Intramuscular fats
IRP	Insulin receptor protein
KBr	Potassium bromide
LBD	Ligand-binding domain
LPL	Lipoprotein lipase
LXL	Lipid X receptor
MLA	Meat & Livestock Australia
mRNA	Messenger ribonucleic acid
MSA	Meat Standards Australia

O.D.	Optical density
PBS	Phosphate buffer saline
PDB	Protein Data Bank
POP	1,3-dipalmitoyl-2-linoleolyglycerol
POS	1-palmitoyl-2-oleoyl-3-stearoylglycerol
PPAR	Peroxisome proliferator-activated receptor
PPER	PPAR response element
RAS	Renin angiotensin system
ROSI	Rosiglitazone
RXR	Retinoid X receptor
SCD	Stearoyl-CoA desaturase
SFC	Solid fat content
SMILE	Simplified Molecular Input Line Entry System
SOA	1-stearoyl-2-oleoyl-arachidoylglycerol
SOS	1,3-distearoyl-2-oleoylglycerol
SREBP	Sterol regulatory element-binding protein
T2D	Type 2 diabetes mellitus
TG	Triglycerides
TZD	Thiazolidinedione

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CHAPTER 1

INTRODUCTION

1.1 Study Background

Meat is one of the important protein sources that is consumed frequently by the human population. In Malaysia, the commonly consumed meat are beef, lamb, pork meat and poultry meat such as chicken and are usually served in different style of cooking on different occasion. It was forecasted for the year 2021 that approximately 48.7 kilograms of poultry meat would be consumed per Malaysians, followed by pork and beef and veal, each with an estimate of 5.3 kilograms per capita consumption (Hirschmann, 2021). Another source also state that Malaysia have a total production of meat of 1.99 million tonnes in the year 2019, a substantially increase from 162,513 tonnes in the year of 1970 (Knoema, 2021). This indicates that as the Malaysia's population continues to increase, so does the increase in demand for meat.

However, Malaysia's population have significantly increased over the years and the local markets cannot fulfil the demand for high quality meat as the production are either slow or insufficient, in terms of technology or research and development. According to Meat & Livestock Australia, MLA (2020), Malaysians from the middle to high income families favour the good quality imported meat products (particularly meat from grass-fed or grain-fed cattle from Australia). It is also widely used in the higher-end hotels and restaurant due to Malaysia being ranked as second place right after Thailand in the highest number of international tourists in South-East Asia for the year 2019 (The World Bank, 2019). If Australia can produce high quality beef meat, can Malaysia too, particularly Sarawak, follow the footsteps of first world countries and enter the global market with locally produced high quality meat?

The quality of the meat can be determined by using a meat grading system such as Meat Standards Australia (MSA), that grades according to the meats' characteristics (such as marbling, colour, texture, and fat). Marbling (or intramuscular fats; IMF) in meat is an important aspect of determining the meat quality as marbling will bring out the flavour and juiciness of the meat by melting the fats when cooking. And thus, with these grading systems, the Wagyu beef (graded A5 for best quality) with high marbling have become the standard for high quality meat.

The fruit plant *Shorea macrophylla*, commonly known by Sarawak locals as *Enkabang* or 'illipe nut' in English, is a plant endemic to the Borneo Forest. According to Coolen (2013), *S. macrophylla* can be found 90% of the time in Sarawak. Due to its size being relatively bigger (5.5-6.0 × 2.9-3.2cm) compared to other *Enkabang* species (Forest Department Sarawak, 2021), it is easy to forage for the fruit. Besides that, the *Enkabang* fruit is widely used by the locals to make lipsticks, moisturisers, soaps, cooking oil and even as a substitute cocoa butter. This is due to the fruit having a rich fat content; as evidenced by Blicher-Mathiesen (1994), the fat content of the *S. macrophylla* kernel ranges between 45.8–50.0%. It is hypothesised that a certain activator molecule that might be present in the *Enkabang* has an effect on the quality of the meat, as the molecules will induce the lipogenesis process through the activation of peroxisome proliferator-activated receptor gamma (PPARγ) in the IMF of the meats.

Lipogenesis is a process whereby fatty acids, and subsequently the triglycerides, are synthesised in both the liver and adipose tissue. This process can be modulated by activation of PPARγ (Ladeira *et al.*, 2016). Research by Cui *et al.* (2016) proved that the gene expression of PPARγ may have an effect on the IMF content and is involved as a major part on adipocyte differentiation in swine species. A ligand or activator is involved in the activation of PPARγ, which would bind to PPAR response element (PPER) on the gene and express lipogenic enzymes that is involved in the lipogenesis pathway.

1.2 Objectives

The objectives of this research are:

1. To isolate potential lipogenesis inducing factors to PPARγ from *Enkabang* (*Shorea macrophylla)* using Soxhlet extraction method.
2. To conduct molecular analysis of the extracted lipogenesis inducing factor isolated from the *Enkabang* (*Shorea macrophylla)* onto PPARγ model.

CHAPTER 2

LITERATURE REVIEW

2.1 Enkabang

2.1.1 Characteristics

Figure 2.1 *Enkabang* (*Shorea macrophylla*) (Source: Abdul Rahman Adam 2014)

Shorea macrophylla, also mainly known by the locals of Sarawak as the *Enkabang*, is a fruit that is widely used as soaps, cooking oil or alternative cocoa butter by the locals. Its discovery can be dated since 1963 and was found by a botanist named Peter Shaw Ashton (Randi *et al.,* 2019). The *S. macrophylla* can be found 90% of the time in Sarawak, thus making it an endemic plant to the land of Borneo (Coolen, 2013). The *Enkabang* mainly grow on the tree and due to its fast growth nature, the *Enkabang* tree can withstand logging and habitat threats more than the other dipterocarp species (Randi *et al.*, 2019). This species has a size relatively bigger than the other *Shorea* species, with 5.5-6.0 × 2.9-3.2 cm in size (Forest Department Sarawak, 2021), thus it is easy to forage for this fruit on the forest floor. The *Enkabang* is shaped like a flower, five or six petals surrounding a middle part, which is the edible fruit. Before it ripens as shown in Figure 2.1, it has an appearance of a yellow flower. A ripen *Enkabang* will have a brown petal colour and emits a woody smell. When it is peeled off, its edible nut is white-pale yellow in colour, similar to that of a butter.

2.1.2 Biochemical composition

The biochemical composition of the *Enkabang* is mostly lipids. According to Blicher-Mathiesen (1994), it has a relatively high fat profile. Blicher-Mathiesen took *Enkabang* samples from three area, which were Kapit Division, Forest Reserve Plantation from Semengoh Kuching and Agriculture Research Centre. The tables below (Table 2.1, 2.2) were data for the species *S. macrophylla* (*Enkabang*) fats profile extracted and compiled in simplified form from Blicher-Mathiesen's research paper in 1994.

Table 2.1 Fats, glyceride and fatty acid contents of kernel fat of the *Shorea macrophylla* species collected in Sarawak, simplified.

Area	Fats (%)	Glyceride (%)		Fatty acid (%)				
		Triglycerides	Free Fatty Acids	16:0	18:0	18:1	18:2	20:0
Kapit Division	47.2-50.0	95.9-97.5	0.5-2.0	15.6-16.5	46.9-47.5	33.8-35.2	0.6-1.3	1.8
Forest Reserve Plantation, Semengoh Kuching	45.8-49.6	97.9-98.4	0.6-0.7	14.6-15.8	47.6-49.5	33.6-33.7	0.6-0.8	1.6-2.1
Agriculture Research Centre, Semengoh, Kuching	48.4	48.4	0.7	13.7	50.0	34.1	0.7	1.5

(Modified from source: Blicher-Mathiesen 1994, pp. 231-242)

Table 2.2 Triglyceride content of the kernel from the *Shorea macrophylla* species collected in Sarawak, simplified.

Area	Triglyceride (%)						
	Mono-unsaturated					Di-unsaturated (Total)	Others (Total)
	POP	POS	SOS	SOA	Total		
Kapit Division	6.1-6.9	35.6-36.7	46.5-49.6	2.5-3.2	93.3-93.8	5.5-6.2	0.5-0.7
Forest Reserve Plantation, Semengoh Kuching	5.2-6.4	33.5-35.2	50.1-51.2	3.6-4.0	93.5-95.7	4.3-6.2	0.3
Agriculture Research Centre, Semengoh, Kuching	4.8	32.4	52.6	3.0	92.8	6.9	0.3

(Modified from source: Blicher-Mathiesen 1994, pp. 231-242)

Due to its high fat profile, *Enkabang* fats are used fairly in skin treatments such as soaps, moisturisers and also used as a substitute for cooking oil and as substitute butter. Recently, there was a research conducted that are able to justify the use of *Enkabang* fats as plant-based fat formulation to substitute for animal-based lard. The graph (Figure 2.2) shows a downward trendline of the SFC of samples as temperature increases. The abbreviation for the labelling are as followed; CaO: Canola oil, EF-1: 25% of blend, EF-2: 30% of blend, EF-3: 35% of blend, EF-4: 40% blend, EF: *Enkabang* fat. Nur Illiyin *et al.* (2013) proved from their studies that *Enkabang* fats mixed with canola oil had a similar melting point of 24.8-31.2°C and a similar solid fat content (SFC) at a temperature from 25°C to 30°C (Figure 2.2). They deduced from their results stating that the addition of 30-35% of *Enkabang* fats to canola oil will display a better similarity of solid fat content profile to that of lard at a temperature of 25-35°C. Besides that, *Enkabang* nut have been believed to contribute to the taste, tenderness and smooth texture of the *Empurau* fish (Frost & Sullivan, 2015).

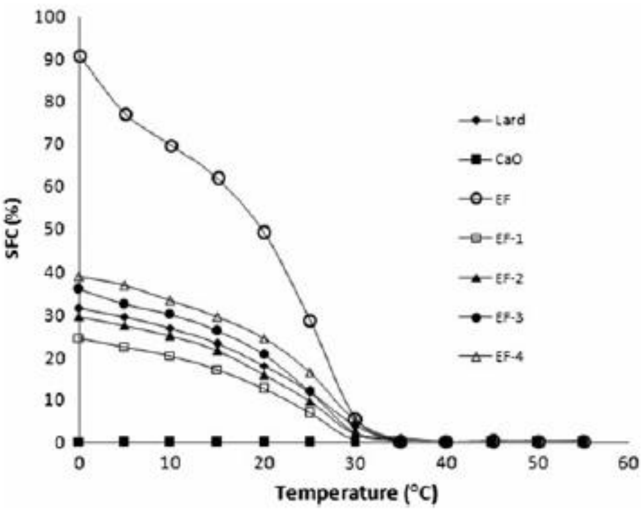

Figure 2.2 Solid fat content profile between lard, Engkabang fat and four different compositions of *Enkabang* fat-canola oil blend. (Source: Nur Illiyin *et al.* 2013)

The *Enkabang* is usually eaten by the fish *Tor tambroides* (*Empurau*) as the trees are usually grow along the riversides. Kamarudin *et al.* (2018) conducted a study to investigate whether the *Enkabang* oil can be served as an alternative dietary lipid source to crude palm oil as fish feed. The factors that were studied were growth performance, body composition and the fatty acid profile of the *Empurau* fish. Even though they were able to demonstrate that feeding the fish with *Enkabang* oil does not negatively influence the growth performance and whole body composition of the fish, its performance are still inferior than that of palm oil. When feed with *Enkabang* oil, the retention of dietary lipids, energy and polyunsaturated fatty acids in the muscle of *Empurau* fish were lower than when being fed with palm oil. Kamarudin *et al.* (2018) reasoned that this low retention value of fatty acids in the muscle of the fish was partly due to the high temperature of the habitat water.

2.1.3 Taxonomy of *Shorea macrophylla*

According to Randi *et al.* (2019), the taxon name for the *Enkabang* is *Shorea macrophylla* (De Vriese) P.S.Ashton from the family group Dipterocarpaceae. The *Enkabang* is categorised as least concern from Jan 2019 to Jan 2021 on the IUCN Red List of Threatened Species.

2.1.4 Factors affecting growth of *Shorea macrophylla* tree and fruit

There are factors that can affect the growth of *S. macrophylla* tree. Fajri and Ruslim (2020) conducted a study to determine the habitat of *S. marcophylla* on Indonesia land, in Tane’ Olen, Malinau District, North Kalimantan Province. They discovered that *S. macrophylla* grows well on lowland near riversides with a flat topography, and soil that are acidic with lower fertility. To add on, it can grow under a suitable microclimate condition such as temperature ranging 24-26.5°C, humidity of 76-87% and only require a low light intensity between 7.25% to 23.46%.

Perumal *et al.* (2017) supported the idea that *S. macrophylla* tree can be repurposed for forest rehabilitation in Sarawak based on their findings after conducting the study at

Sampadi Forest Reserve, Sarawak. The high survivability chance and substantially fast growth rate (Randi *et al.*, 2019) of *S. macrophylla* tree is due to the efficiency of line planting system used at the forest reserve. In addition, the fast growth rate of *S. macrophylla* tree may be due to its good adaptability to compete with other pioneer species for light even though the pioneer plants wasn't near the planting line of *S. macrophylla* tree.

As for the fruit of *S. macrophylla*, it usually ripens in the month of January and February. The germination of *S. macrophylla* seed is fast as it will only take a little bit of sunlight for it to start germinating (Kintamani, 2018). Therefore, it is recommended to gather the fruit as soon as it falls. The tree reaches its fruiting age after 15-30 years and it will take every four to five years for the tree to bear fruit. Since the tree can grow up to 45 meters in height (Randi *et al.*, 2019) and has a hemispherical crown with many branches, a case of low supply of the fruit is lowly plausible.

2.2 Overview of Lipogenesis Mechanism in Vertebrate

Lipogenesis is a process whereby endogenous fatty acids (FA) and subsequently the triglycerides (TG), are synthesised by the breakdown of glucose. It occurs mainly in the liver cells and adipocytes and can be induced by high carbohydrates diet (Kersten, 2001). The FA are stored as energy and will be used when fasting period occurs. Since lipogenesis is commonly associated to obesity in human, therefore, it is a process whereby it cause the synthesis of FA to increase inversely with the development of muscle tissue (Ladeira *et al.*, 2016).

Glucose is the beginning substrate in the lipogenesis process. Glucose will undergo glycolysis process in the cytosol until it successfully converted into acetyl-CoA in mitochondria. Acetyl-CoA will then undergo the citric acid cycle and be converted as energy to be used by the cell. However, when the energy source is relatively high in the cell (in other words, it is not utilised by the cell), acetyl-CoA will be converted into the form of citrate and transported out of the mitochondria via a channel into the cytosol where lipogenesis will occur (Gnoni *et al.,* 2021). Citrate is then broken down into acetyl-CoA and oxaloacetate by ATP-citrate lyase (ACLY) in the cytosol. With the help of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), Hence, acetyl-CoA is the precursor to initiate lipogenesis when energy supply is not utilised and synthesise FA as the final product of the reaction in the cell. Hence, high glucose is the direct cause in the occurrence of lipogenesis in tissues of animals. The Figure 2.3 below is an overall process of lipogenesis occurring in a cell.

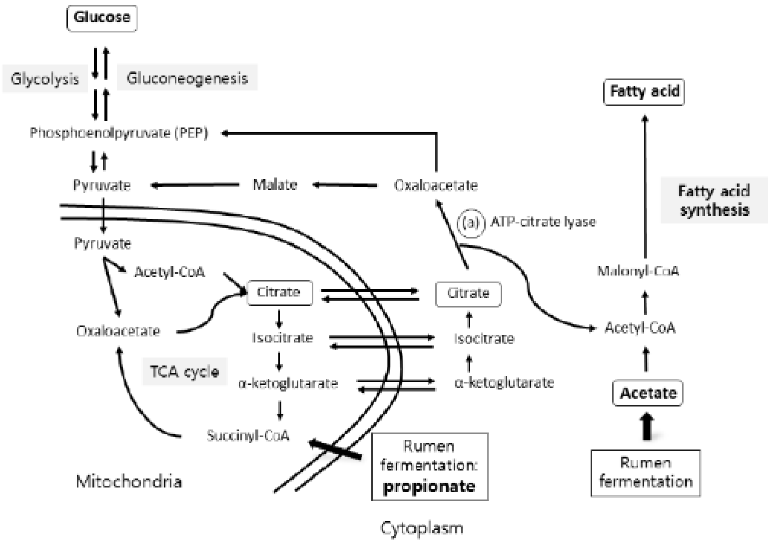

Figure 2.3 Lipogenesis process in a cell. (Source: Park S. J. *et al.* 2018, pp. 1043-1061) CC-BY-NC 4.0

Presence of glucose in the body also induce the expression of lipogenic genes (FASN gene, stearoyl-CoA desaturase [SCD] gene, lipoprotein lipase [LPL] gene, glucose-6-phosphate dehydrogenase [G6PDH] gene; to name a few), which will be transcribed into enzymes involved in the lipogenesis pathway (Poklukar *et al.*, 2020; Stoeckman & Towle, 2002). Stoeckman and Towle (2002) mentioned that sterol regulatory element-binding protein (SREBP) is the key regulator in the expression of lipogenic enzyme.

2.2.1 Lipogenesis aids in marbling

The accumulation of fats at the IMF of meat is generally known as marbling. High level of marbling is a highly valuable characteristic that determine the ability of an animal to accumulate fats in their IMF, particularly meat-producing animals. Fatty acids deposited at the intramuscular fat influences the nutritional quality and flavour of the meat in terms of the meat tenderness and juiciness after cooking. A perfect source of a variety of high quality macronutrients (e.g., proteins and essential amino acids, lipids, minerals, vitamins) is cattle beef. In cattle, fat deposition is not homogenous throughout the body for the duration of its lifespan. According to Pethick *et al.* (2004), the development and deposition of fats begin at the abdominal fat, then intermuscular, followed by the subcutaneous, and lastly at the IMF.

There was a literature that demonstrate that lipogenesis might be involve in the increase of fat accumulation in meat-producing animals. The activities of enzymes such as acetyl-CoA carboxylase, FASN, G6PDH, LPL and malic enzyme were tested by Bonnet *et al.* (2007) in three different cattle. They reported that there was a significant level of G6PDH, FASN, LPL and PPARγ expression activity in intramuscular adipose tissue (IMF). Though, the lipogenic enzyme activity is not as high as that in subcutaneous fat of the three cattle species, it still indicate that there was lipogenesis process involved in marbling of the meat.

There are certain factors that can affect fats deposition at IMF and subsequently influences marbling quality in meat. Factors such as genetic, management and nutritional factors in beef cattle could affect IMF content (Troy *et al.*, 2016). For example, for genetic factors, the ability of marbling is highly heritable in Korean cattle with 0.57-0.67 heritable unit (Park *et al.,* as cited in Park *et al.,* 2018) and Japanese Wagyu with 0.40-0.55 heritable unit (Oyama as cited in Park *et al.*, 2018). Furthermore, for nutritional factor of IMF deposition, a study review by Hocquette *et al.* (2010) mentioned that feeding meat-producing animals with a diet that would increase glucose level significantly in the body after consumption, such as grain (Pethick *et al.*, 2004) will increase the frequency of lipogenesis process occurring in the IMF. Hence, factors like genetic, management and nutritional are important to account for in IMF deposition as it can subsequently affect the final quality of the meat.

2.3 Role of Peroxisome proliferator-activated receptor (PPARγ) receptor in Lipogenesis in Vertebrate

Peroxisome proliferator-activated receptor (PPAR) is involved in many pathways relating to glucose and lipid metabolisms in metabolic tissues, such as the liver and the adipose tissue. There are three isotypes of PPAR, namely PPAR alpha (PPARα), PPAR beta (PPARß) and PPAR gamma (PPARγ). PPARγ will be sole focus in this study to isolate and characterise the ligand activator of PPARγ. The Figure 2.4 below depicts the pathway of a ligand activator of PPARγ to activate lipogenesis process in a cell. Thiazolidinedione (TZD) will be used as an example to demonstrate the general pathway of a PPARγ ligand activator.

There are four steps in the pathway. (1) TZD enters the cell through the plasma membrane and bind to PPARγ. (2) The TZD-PPARγ complex will enter the nucleus and bind to a PPAR response element (PPRE) on DNA. (3) Binding to PPRE caused transcription to occur and expressed target mRNA transported from nucleus into cytoplasm (e.g., lipogenic gene) is translated into target proteins (e.g., lipogenic enzymes). (4) The increase in the amount of lipogenic enzymes produce will induce lipogenesis to occur; simultaneously, IRP and GRP are induced to increase intake of insulin and glucose into the cell.

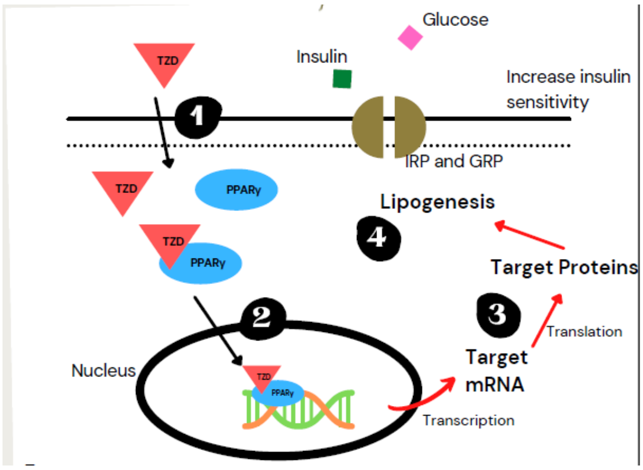

Figure 2.4 Pathway of TZD, a ligand for PPARγ in the induction of lipogenesis process in a cell.

Not shown in Figure 2.4, PPARγ need to heterodimerise with retinoid X receptor (RXR) to be transcriptionally active (Dixon *et al.*, 2021). The ligand activated PPARγ-RXR complex will bind to either the enhancer or promoter region of the 5'-PPRE-3'. The PPARγ structure have six domains, namely the A/B domain that involved in expression of gene specific to PPAR isotypes, the DNA binding domain (DBD; domain C) where PPAR will bind to PPRE at the enhancer or promoter region, the hinge domain (D-domain) that is linking domain C and E, and the E/F domain consisting of the ligand-binding domain (LBD) and active function region 2 (AF-2) (Figure 2.5). The domains are directly responsible to induce transcriptional activity of multiple target gene (lipogenic genes).

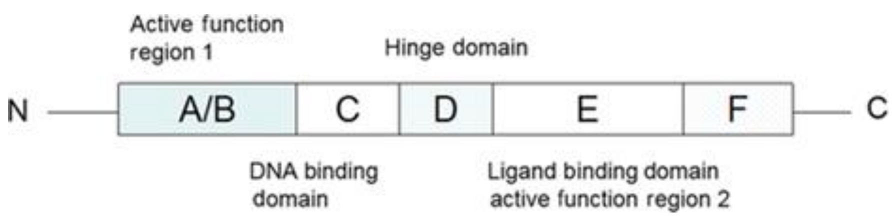

Figure 2.5 Model structure of PPAR. (Source: Liwei *et al.* 2020, pp. 2736-2748)

There are several activators or ligands that can stimulate PPARγ to induce the expression of lipogenic genes in the lipogenesis pathway. Below is the Table 2.3 for PPARs and lipid X receptor (LXL) role in modulation and its associated ligands in metabolic processes at different tissue sites.