Mini Review: GLP-1 Modification, Development, and Improvement

Fedric Intan Damai*[1], Gracia Christine Lembong Purwanto[1], Andri Wardiana[2], Popi Hadi Wisnuwardhani[2], Dian Fitria Agustiyanti[2], Alfi Taufik Fathurahman[2], and Ratih Asmana Ningrum[2]

[1]Department of Biotechnology, Indonesian International Institute for Life Sciences (i3L), Jl. Pulomas Barat No.Kav. 88, Jakarta 13210, Indonesia
[2]Research Centre for Genetic Engineering, National Research and Innovation Agency (BRIN), Jl. Raya Jakarta Bogor Km. 46, Cibinong, West Java 16911, Indonesia

*Corresponding author: fedricintandamai@gmail.com

Abstract

Diabetes Mellitus Type 2 (DM-2) is the condition where the body comes to be insensitive or even resistant towards insulin, thus resulting in deficient insulin secretion from beta cells in the pancreas. Compared with the available treatments, Glucagon-like peptide 1 (GLP-1) is considered a natural treatment to cure DM-2 due to its characteristic as an incretin hormone, where one of its functions is to improve insulin secretion and enhance beta-cell glucose sensitivity. However, GLP-1 has a limitation, which is a rapid half-life due to active degradation activities in the body. Therefore, many studies have been conducted to develop and improve the pharmacological activity of GLP-1 through structural modification and yield improvement, which are thoroughly reviewed in this paper. Structural modification of GLP-1 covers amino acid substitutions by referring to the GLP-1 analog, Exendin4, to prevent the dipeptidyl peptidase-4 (DPP-4) degradation activity and protein fusion with an additional chain to extend the half-life during administration. The yield improvement at the overexpression of GLP-1 tandem repeats sequences can increase the transcribed genes' yield. The studies show that specific amino acid substitutions and IgG heavy chain constant regions or Fc-based fusion genes successfully enhance the pharmacological activities of GLP-1. At the same time, *Pichia pastoris* expression system managed to yield 219.2 mg/l target protein, where the purified target protein is capable of producing 10× yield of a single GLP analog. Further research could include the utilization of these findings *in vitro* as a GLP-1 analog-based therapeutics to treat DM-2.

Keywords: DM-2, GLP-1, DPP-4, protein expression, optimization strategy

Introduction

Glucagon-like peptide (GLP) is a type of peptide obtained from specific post-translational processing of proglucagon peptide in various organs such as the pancreas, gut, and brain (Müller *et al*., 2019). Generally, there are two types of GLPs: Glucagon-like peptide 1 (GLP-1) and Glucagon-like peptide 2 (GLP-2). GLP-1 is a type of incretin hormone that induces insulin secretion from the gastrointestinal (GI) tract after food intake (Nauck & Meier, 2018). On the other hand, GLP-2 does not induce an incretin effect; however, it is intriguing for its gut and intestinal repair and adaptive growth function (Janssen *et al*., 2013; Montes Castillo *et al*., 2019). GLP-1 is extensively researched due to its promising potential for Diabetes Mellitus type-2 (DM-2) treatment. As an incretin hormone, there are wide varieties of GLP-1 functions in different body organs. However, specifically in the gut and pancreas, GLP-1 is shown to improve insulin secretion and gene expression post-nutrient intake; enhance β-cell glucose sensitivity, neogenesis, and proliferation; inhibit β-cell apoptosis and glucagon secretion; decrease gastric emptying rate as well as increasing natriuresis and diuresis (Ceccarelli *et al*., 2013; Müller *et al*., 2019).

Several limitations of GLP-1 include its rapid half-life, which only could last for approximately 2 minutes in the bloodstream due to a cleavage done by the dipeptidyl peptidase-4 (DPP-4) enzyme. This enzyme is an endogenous peptidase that acts to degrade the peptide bond found in the Ala8 and Glu9, ultimately resulting in the degradation of the GLP-1 protein. This causes only 25 % of the

secreted protein to reach the liver successfully, and in the liver, 40-50 % of it is further degraded, leaving only 10-15 % or less of the secreted GLP-1 protein to enter the systemic circulation via the endocrine pathway (Baggio & Drucker, 2007; Holst, 2007). Several ways to tackle this problem are by creating an analog of the GLP-1 to prevent its degradation by the DPP-4 enzyme. This could be conducted by modifying the GLP-1 structure to enhance its survivability in the bloodstream. Therefore, in this review paper, we focus on reviewing current methods in improving the pharmacokinetics of GLP-1 through structure modification and improving GLP-1 production yield in various expression systems through overexpression of a fusion gene as a potential DM-2 treatment.

Proglucagon Processing

GLP-1 is obtained as a product of proglucagon processing. Due to the various types of proglucagon-derived proteins (PDGP), the expression and processing of each peptide should be controlled and specific to their location. This expression is controlled under a single promoter, the Gcg gene promoter, the same promoter for proglucagon processing in the brain, intestine, and pancreas. However, a number of other transcription factors, such as the paired box protein 2 (Pax2), paired box protein 6 (Pax6), MAF bZIP transcription factor B (MafB), caudal type homeobox 2/3 (Cdx2/3), POU domain transcription factor brain 4 (Brn-4), hepatocyte nuclear factor 3 alpha (HNF3α or also known as Foxa1), hepatocyte nuclear factor 3 beta (HNF3 β or also known as Foxa2), neuronal differentiation factor 1/beta 2 (NeuroD/Beta2), and basic helix-loop-helix transcription factor E47 interaction with the cis-acting element, G1-G4, and others. These transcriptional factors regulate and determine whether they stimulate or inhibit proglucagon expression. After proglucagon is expressed, its cleaving is determined mainly by two enzymes which are the prohormone convertase 1 (PCSK1) and prohormone convertase 2 (PCSK2).

Significance of GLP-1 towards DM-2 and Treatments

DM-2 is one of the predominant diabetes diseases in the world. DM-2 is the condition where the cells in the body become insensitive or resistant towards insulin action, thus resulting in an inadequate insulin secretory. This is because of the inability of beta cells in the pancreas to meet the increased demand for insulin production, or there is a decreased number of insulin receptors. Unlike DM-1 (Diabetes Mellitus Type 1), which is most likely to occur in childhood, DM-2 often occurs in adulthood. Therefore, there are several risk factors for developing DM-2 diseases, such as age, genetic inheritance, and inactive or sedentary lifestyles (Wu *et al*., 2014). The other risk factor is obesity; in most cases, individuals that suffer from DM-2 also experience obesity. The relation relies on the decreased insulin receptors, which are often associated with central obesity (Kim *et al*., 2018). Many approaches have been made to cure DM-2 diseases, including pharmacological treatments in the form of diabetic drugs and therapeutics to treat DM-2 using a non-drug approach.

Pharmacological Treatments for DM-2

Current treatments for DM-2 in the form of pharmacological drugs are progressing significantly to achieve reasonable glycaemic control for the long-term period. Commonly used pharmacological treatments include:
(1) Metformin. Also known as biguanides, an antidiabetic drug generally accepted to be used with no limitation on age. Metformin is considered to have a complicated pathway and mechanism in the body to lower glucose production in the liver and improve the utilization of glucose in the body (Rena *et al*., 2017). In addition, it increases insulin activity by triggering the insulin receptor kinase, activating the GLUT-4, an insulin-dependent transporter (Yan *et al*., 2020). Therefore, it increases glucose uptake into the cells in the body. However, metformin can only function well with increased insulin sensitivity if there is a significant loss of weight, thus appropriate for overweight individuals (Tan *et al*., 2019).

(2) Sulfonylureas. One of the most used oral antidiabetic drugs for the treatment of DM-2. Sulfonylureas bind to the ATP-sensitive potassium channels on the beta-cells in the pancreas, triggering insulin secretion without the presence of glucose (Rendell, 2004). However, sulfonylureas only work well with the presence of residual beta-cells in the pancreas (Tan *et al*., 2019) and depend on individual variability in terms of the drug response (Aquilante, 2010). One of the adverse side effects of sulfonylureas is hypoglycemia.

(3) Insulin Treatment. Insulin treatment by injection is commonly used to treat DM-1 instead of DM-2, where the beta cells can not produce insulin. However, it also can be used to treat DM-2 by oral consumption and gradualist or stage by stage to increase the intensification of the therapy (Swinnen *et al*., 2009). According to Swinnen *et al*. (2019), there is only a slight risk of DM-2 individuals experiencing hypoglycemia effects because of the treatments. However, the insulin administration route needs to be secured by enhancing the pharmacokinetic and pharmacodynamic properties of the insulin treatments by using oral consumption (Tan *et al*., 2019).

(4) DPP-4 Inhibitors. Also known as gliptins is a treatment agent that works with inhibition activity towards the DPP-4 enzyme, which is responsible for the degradation action of incretin hormones, including GLP-1 and GIP (Gastric Inhibitory Polypeptide) (Tan *et al*., 2019). Therefore, the utilization of DPP-4 inhibitors will postpone the degradation process, thus extending the half-life of incretin hormones. The examples of available and approved DPP-4 inhibitors are Vildagliptin which shows favorable responses towards the function of alpha and beta cells in the pancreas (Pan & Wang, 2013), and also Sitagliptin which safety and efficacy are proven and already frequently used in clinical trials in Japan (Iwamoto *et al*., 2010).

Therapeutics for DM-2

Other than the pharmacological treatment approach, some improvement in the therapeutics approach has been developed over the years. The therapeutics aim to reduce the ineffectiveness of using conventional diabetic drugs and reduce the possibility of DM-2 development. The treatment approaches are considered novel due to the requirement of advanced technologies to support the process. Examples of therapeutic approaches include:

(1) Gene therapy. The idea of using gene therapy for Diabetes Mellitus has been developed over the years and suggested to be able to cure the disease. In the application, gene therapy is mainly suggested to be used for DM-1. However, due to the genetic predisposition of DM-2, the gene therapy approach can also be considered a potential treatment. The principle of gene therapy for DM-2 treatment is to limit the potential or even prevent the development of DM-2 in the individual by using genetic engineering techniques (Yue *et al*., 2019). Several target genes contributed to the development and the deterioration of DM-2, including genes that regulate glucose homeostasis (GLUTs or glucose transports, SGLTs, or sodium-glucose co transports, and SIRT 6 or sirtuins 6) (Bae, 2017; Yonamine *et al*., 2017), the gene responsible for insulin secretion or sensitivity (GLP-1, GPCRs or G-protein coupled receptors) (Kwak & Park, 2016; Tasyurek *et al*., 2014), and the gene that responsible for alleviating diabetic-induced complications (CDKN2A/2B and silencing of NLRP3 gene) (Luo *et al*., 2014; VinuÉ *et al*., 2019). The introduction or delivery of gene therapy can be done by using viral techniques such as lentivirus, adenovirus, and AAV (adeno-associated virus), as well as non-viral techniques such as liposomes and naked DNA injection.

(2) Stem cell therapy. One of the novel alternative approaches that aim to replace the pancreatic beta-cells rather than perform pancreas transplantation since it requires donor availability. MSC (Mesenchymal Stem Cells) is one of the most common stem cell therapies used to treat Diabetes Mellitus for both types 1 and 2 due to its immunomodulatory mechanisms by suppressive properties and anti-inflammatory effects (Xiao & Gittes, 2015; Zang *et al*., 2017). The mechanism of MSCs is by regenerating the beta-cells in the pancreas, followed by the partial-suppression activity of Th1 (T-Helper1). Thus, resulting in improved hyperglycemia control.

Gap Between GLP-1 and Current Treatments

The available and most commonly used treatments to treat individuals with DM-2 are varied depending on the feasibility of the production of diabetic drugs, the availability of the treatments or therapy, and the urgency of each individual. Each of the conventional diabetic drugs mentioned has its limitations. However, the adverse effect is generally severe hypoglycemia and other conditions, such as low efficacy and weight gain, due to lacking target specificity and permeability problems (Padhi *et al*., 2020). Moreover, they need a strict dosage level of drug consumption to maintain the glucose level in the body. In addition, several drugs can only be efficiently worked by combining with other drugs, such as antihyperglycemic agents or other treatments (Padhi *et al*., 2020).

Furthermore, diabetic drugs do not perform in treating the decreased malfunction of beta-cells (Kokil *et al*., 2015). The other solution is the therapeutic approach, which offers solutions to those problems. However, the limitation relies on the processes' complexities and the therapy's unaffordable cost range. In addition, most therapy options are still under development, and more extensive studies need to determine future treatments' biosafety and efficacy effectiveness (Tiwari, 2015).

GLP-1 has been viewed as a natural treatment to treat DM-2 disease compared to the available treatments. It plays an important role as one of the incretin hormones that naturally occur in the body. The drawback of GLP-1 relies on the short plasma half-life, which lasts only about 2 minutes. Despite the drawback, the ability of GLP-1 significantly contributes to glucose homeostasis in the body, which includes increased insulin secretion and stimulates satiety. Additionally, the work of GLP-1 heavily relies on the activation of GLP-1r (GLP-1 receptors), which is broadly distributed in the crucial organs and tissues in the body, including the pancreas, lung, heart, kidney, several central nervous systems and adipose, and smooth tissues (Sandoval & D'Alessio, 2015). The activation of GLP-1r stimulates insulin secretion under the glucose-independent condition by generating cAMP, G protein, and protein kinase A activation, while also indirectly promoting several pathways regulated by the beta-cells metabolism to augment the insulin secretion under glucose-dependent conditions (Sandoval & D'Alessio, 2015). Therefore, considering its significant effects, some extensive developments and modifications need to be constructed to improve the performance of GLP-1 in the body to treat DM-2.

Modifications and Therapeutics Developments

Various therapy methods are applicable for GLP-1 delivery, including parenteral, suppositories, oral, buccal, intraperitoneal, and other kinds of administrations (Geigle, Wallrapp & Thoenes, 2009). One of the most common therapy methods of GLP-1 is a subcutaneous injection to avoid degradation by the DPP-4 enzyme (Bellman & Aromataris, 2014). Different types of therapy could require different dosages depending on the delivery method and severity of the disease. For example, a preferable dosage such as in parenteral administration is in the range of 0.1-2,000 µg, where the best amount of dosage would range from 10-100 µg. Burcelin *et al*. (1999) also concluded that 5-10 µg is sufficient for the daily treatment of a diabetic patient. In the suppositories administration, it is required to contain preferably 12 % of the active ingredients. In oral formulations, it is preferable to contain 25-70 % of the active ingredients (Geigle, Wallrapp & Thoenes, 2009). Therefore, the highest yield is essential for ease of production to fulfil the dosage required, However, several drawbacks of these therapy methods are the insufficient GLP-1 quantity for clinical trial usage. Furthermore, the cost required to have a large-scale GLP-1 production is also considerably expensive, especially if the chemical synthesis method is used (Brandsma *et al*., 2009; Xu *et al*., 2017). Due to the limitations mentioned above, several strategies have been implemented to tackle the problem: modifying the GLP-1 structure and increasing the GLP-1 production yield.

Structure Modification

Protein structure is a crucial factor in determining the related biological mechanisms

involved and the specific functions of the protein of interest. According to Hou *et al*. (2007a), the presence of His7 at the N-terminus and the last three C-terminus residues of GLP-1(7-36) are crucial in enhancing insulin secretion and biological activity. This is also confirmed by Gallwitz *et al*. (1990); Mojso, (1992) that the termination of His7 in GLP-1(1-37) resulted in the reduction of affinity activity by 300 fold, and the termination of C-terminal residues caused the binding of GLP-1 was undetectable. In addition, to enhance the understanding of GLP-1 structure and the discovery and study of Exendin4 as a GLP-1 natural analog was developed by several researchers. Exendin4 is a saliva hormone with a 39 amino acid length that is naturally derived from Gila monsters (*Heloderma suspectun*) that was developed by Furman (2007). They found that Exendin4 is approximately 50 % homologous similarity with GLP-1 and suggested that Exendin4 isalso able to contribute to glucose homeostasis in the body. The structural comparison between GLP-1 and Exendin4 shows some differences in amino acid composition that lead to different biological mechanisms involved. The significant difference is the first two amino acid compositions; the GLP-1 is structured with His7 and Ala8 amino acids, while the Exendin4 is structured with His7 and Gly8. The particular structure of GLP-1 (His7 and Ala8) leads to the activity of DPP-4 degradation, which confirmed the short plasma half-life, while the structure of Exendin4 leads to longer plasma life for about 2 hours.

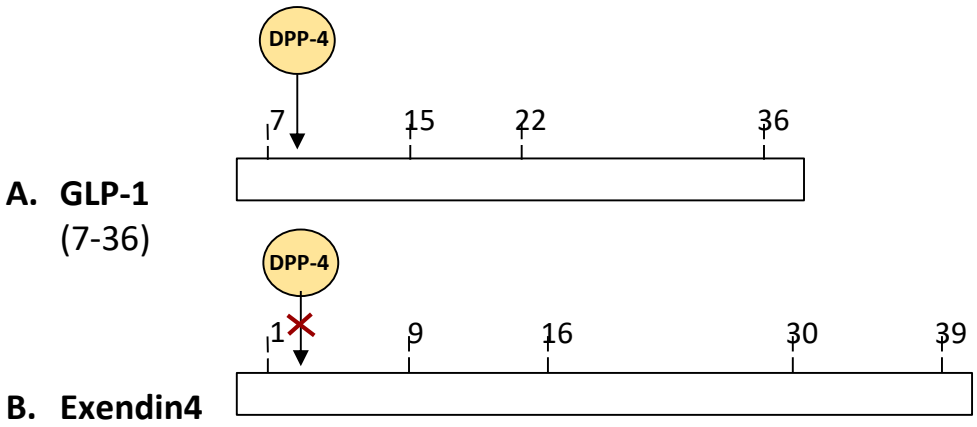

Figure 1. GLP-1 and Exendin4 differences in amino acid composition and structure. Red letters represent the substitution and addition of amino acid composition in Exendin4.

Additionally, Exendin4 shows great activity towards GLP-1r, but not towards secretin receptors. Regardless of the homologous similarity with GLP-1, Exendin4 shares a different affinity towards GLP-1r. Montrose-Rafiadeh *et al*. (1997) conducted an experiment of termination of the first similar eight residues at the N-terminus of GLP-1 and Exendin4. They found Exendin4 maintained high affinity, while GLP-1 affinity was reduced to 370-fold. Donnelly (2012) concluded that different interactions are due to the tolerable degrees of amino acid substitutions and the protein foldings. The other way to increase the affinity bindings is by the addition of side chains or introduction to adding properties by the fusion protein (Manandhar & Ahn, 2014). Because of these findings, the structure modification of GLP-1 can be carried out by doing amino acid substitutions and additional chains to prevent degradation activities and enhance pharmacological properties.

Wang *et al*. (2014) studied GLP-1 and Exendin4 structure modification using the protein fusion approach. Due to the parenteral route of GLP-1 distribution and fast degradation, a fusion protein consisting of the active human GLP-1 and IgG heavy chain constant regions (GLP-1/Fc) was produced to enhance the therapeutic potency and extend its plasma half-life. The study was done by

constructing the cDNA of a heterologous fusion protein consisting of active GLP-1 sequence and IgG sequences derived from human (IgG2) and mouse (IgG1). In addition, the GLP-1 sequences used can be from the GLP-1 active sequence or from GLP-1 fragments that share 70-95 % similarity with the native GLP-1, including Exendin4 and GLP-1(A8G), which has resistant properties towards DPP4 degradation activity (Wang *et al*., 2014). The distribution approaches can be used by direct injection of the protein fusion (GLP-1/Fc) by using liposomes or viral vehicles and through gene transfer by using the combination of non-viral gene therapy and by optimizing electrophoresis methods. Whilst, the dosage varies depending on the needs of the DM-2 individuals. Wang *et al*. (2014) reported that the proper general dosages are 0.02-100 nmol/kg body weight, whereas 0.1-100 µg/kg body weight for the gene therapy method. Thus, considering the process of producing the targeted fusion proteins and to achieve the optimum levels in the body, gene transfer might proceed in a long time.

Wang *et al*. (2014) also conducted an *in vivo* study by transferring the fusion protein gene into the db/db mice. They reported significant differences in insulin secretion by the generation of cAMP activation and protein kinase, meaning that after the transfection of the fusion protein, GLP-1 maintains its native properties. In addition, controlled glucose homeostasis was observed in the treated mice. The role of the heavy chain of IgG from humans and mice is crucial since the fusion protein would be categorized as a long-acting GLP-1 analog and has a low risk of enzyme degradation. Moreover, the Fc-based fusion protein naturally helps to delay the renal clearance by kidneys which extends the plasma half-life (Weir *et al*., 2002). The use of IgG2 from humans is preferable due to its ability to reduce the immune response of the body (Hjelm *et al*., 2006; "Therapeutic antibody classes", 2012). Whilst, Fc-based fusion protein will ease the purification process of the target fusion protein by using Protein G sepharose. The fusion protein (GLP-1/Fc) can treat DM-1 and DM-2 by increasing insulin secretion, improving the overall function of beta-cells by inducing proliferation, and preventing apoptosis activity. However, no significant body weight reductions were observed in the *in vivo* study, which was also confirmed by Turton *et al*. (1996).

Yield Improvement

The approach used to increase GLP-1 yield for large-scale production is through overexpressing a fusion gene construct that contains repeated tandem sequences of GLP-1 (Geigle, Wallrapp & Thoenes, 2009). The fusion gene can contain tandem repeats up to several times, increasing the gene transcribed amount and yield. However, the drawback of this method is, when the tandem repeat is in excess, it could cause substantial stress to the culture due to excessive amounts of foreign protein synthesized, such as in *E. coli* leading to plasmid instability. This also correlates with the type of host used, such as expression systems containing RecA protein capable of forming and expressing higher amounts of plasmid multimer (Saraswat *et al*., 1999). Therefore, the expression system plays a huge role in determining the success of the transformation. This process is conducted in various expression systems including *E. coli*, *P. pastoris*, mammalian cells (e.g. CHO, hTERT-MSC, and HEK293 cells), and transgenic tobacco plant (Brandsma *et al*., 2009; Geigle, Wallrapp & Thoenes, 2009; Hou *et al*., 2007a; Hou *et al*., 2007b). The advantages and disadvantages of each of these expression systems are summarized in Table 1.

Different types of expression systems could also have different factors which determine their recombinant protein expression level. In general, the factors are categorized as genetic and environmental factors. Examples of genetic factors are the promoter strength, ribosomal binding site sequence, the copy number of the plasmid, and the presence of activator and terminator. While the environmental factors could include pH, specific growth rate, medium composition, induction time, temperature, presence of additives such as surfactants to improve the formulation, and others (Hou *et al*., 2007b; Saraswat *et al*., 1999).

As a soluble protein, GLP-1 is available for *E. coli* expression as it does not require complex purification methods (Gecchele *et al*., 2015; Hou *et al*., 2007a). Overexpression of GLP-1 gene in *E. coli* BL21(DE3) strain utilizes the pMDGLP1 vector as the cloning

vector with two restriction enzymes, *Eco*RV and *Sma*I, which cuts in the gene of interest sequence. In their study, the GLP used is the His-GLP-N, which is an analog of GLP-1 where it is mutated at Alanine8, Lysine26, and Lysine34 into Serine8, Glutamine26, and Aspartic Acid34. The mutation is done to ensure that the GLP analog is able to stimulate insulin secretion and resist degradation by the DPP-4 enzyme due to mutation in the DPP-4 target sequence (Hou *et al*., 2007a). His-GLP-N analog is followed by three amino acid sequences, the proline-isoleucine-arginine coded as P-I-R. The sequence is due to the cleavage by trypsin which is done at the peptide bond between the carboxyl group of arginine or lysine and the amino group of the adjacent amino acid (Simpson, 2006). Therefore, a single His-GLP-N analog could be extracted and purified at further steps. Hou *et al*. (2007b) proceeds to construct a plasmid with the fusion gene containing eight tandem repeats. An attempt to create a fusion gene construct containing twelve and sixteen tandem repeats was also made. However, no expression was detected even when varying conditions were applied. The failure in expression was hypothesized to be due to the complexity of the RNA spatial structure, which causes the plasmid to be incapable of conducting translation (Hou *et al*., 2007a).

The cloning vector was further transformed to the expression vector pET-22bG8 with two restriction sites which are the *Sal*I and *Bam*HI, and joined with T4 DNA ligase (Hou *et al*., 2007a). It also contains a robust promoter, T7 lac promoter, induced with IPTG to ease the overexpression of the 32 kDa His-GLP-N analog. T7 promoter is often used in *E. coli* expression due to its robust and precise properties for gene transcription. Several factors were considered in the expression of the His-GLP-N analogs: temperature, induction time, and IPTG solution concentration. Previous studies have shown that lower culture temperature and longer induction time can increase protein expression in *E. coli* BL21(DE)/G8. The optimized condition was found to be at 26°C, induced for 8 hours utilizing 0.4 mmol/L of IPTG. The result was that the system successfully expressed 33.3 % of the target protein as a soluble protein in proportion to the total cell protein (Hou *et al*., 2007a). Hou *et al*. (2007b) also conducted GLP-1 analog expression in *P. pastoris*, where ten tandem repeats of GLP-1 named GLP-M with the molecular weight of 36 kDa were constructed. The GLP-M construct concept used is the same as the His-GLP-N analog, where it is mutated at Alanine8, Lysine26, and Lysine34 into Serine8, Glutamine26, and Aspartic Acid34.

Table 1. Advantages and disadvantages of various expression systems (Brandsma *et al*., 2009; El-Baky & Redwan, 2015; Gecchele *et al*., 2015; Hacker & Balasubramanian, 2016; Hou *et al*., 2007a).

Expression Systems	Advantages	Disadvantages
Escherichia coli	1. Rapid growth rate 2. Cost-effective manufacturing 3. High production yield	1. Difficulties in expressing complex proteins 2. Protease contamination 3. Endotoxin accumulation
Pichia pastoris	1. High production yields, 2. Genetically stable, 3. Secrete recombinant proteins freely into the medium, 4. Inexpensive culture condition, 5. Easily scalable	1. Expensive fermentation equipment 2. Few selectable markers 3. Protease contamination 4. Requires extensive purification
Transgenic plant	1. High production yield 2. Cheap growth condition 3. Easily scalable 4. Able to synthesize complex protein	1. Limited host range 2. Genetically unstable
Mammalian cells	1. Capable of proper post-translational modification and foldings in complex eukaryotic proteins 2. Capable of transient expression	1. Expensive 2. Slow growth rate 3. Low protein yield

P-I-R sequence for trypsin cleavage is also still implemented in their construct. They reported that a single purified M-GLP protein is capable of producing 10× the yield of a single GLP-analogue, indicating the successful expression strategy. Various factors implemented in their study include the optimization of pH; induction time; methanol concentration; and addition of dodecane, oleic acid, and Tween-80 to improve the GLP-1 formulation as also mentioned by Geigle, Wallrapp & Thoenes (2009). The expression of M-GLP in *P. pastoris* was found to be highest at the induction condition of 28°C, pH 7, 230 rpm. The induction time is preferable at 4 days with the medium composition containing 1.5 % methanol concentration, 4 % dodecane, 0.01 % oleic acid, and 0.5 % Tween-80. This condition manages to generate the highest mean yield, up to 219.2 mg/l.

Several species of transgenic plants had been experimented with several times for fusion GLP-1 gene such as on rice seeds and tobacco by Yasuda *et al*. (2005). The first expression of GLP-1 monomer fused with the glutelin promoter sequence on rice seeds was not detected. Yasuda *et al*. (2005) hypothesized that the failure is due to the expression of siRNA or possible plasmid instability and cleavage. The second expression was done by Sugita *et al*. (2015) also on rice seeds, where the GLP-1 gene was fused with the globulin promoter sequence. The GLP-1 gene expression is successfully done; however, the recombinant protein has lower biological activity when tested *in vitro*. This reduction might be due to the size difference between GLP-1 (below 4 kDa) and globulin (26 kDa), which cause GLP-1 biological activity to be disturbed (Sugita *et al*., 2015). A pentamer GLP-1 fused with chitinase as the signal peptide was also constructed in rice seed fused by Yasuda *et al*. (2006). However, when the transformation was verified with Western blot, a faint band with a higher kDa size compared to the supposed GLP transformant size was present on the GLP-1 transformant lane. The larger fusion protein size is suspected to be the unprocessed signal protein that failed to be cleaved during the transformation process due to mutation in the signal peptide gene sequence (Yasuda *et al*., 2006).

While the latest study for GLP-1 fusion gene production was done in transgenic tobacco plants by Brandsma *et al*. (2009). They constructed an expression vector composed of three types of initial DNA fragments, representing the Start, Link, and Stop sequence. The Start sequence is composed of the forward primer containing the start codon, ATG, *Nco*I restriction site, a single GLP-1 ORF sequence and flanked with the *Xho*I restriction site added with the reverse primer coding for *Sal*I restriction site to fuse between the Start and Link by ligating the *Sal*I and *Xho*I restriction site. At the Link sequence, it starts with the *Sal*I restriction site added with the forward primer coding for the *Xho*I restriction site, a single GLP-1 ORF sequence and flanked also with the same *Xho*I restriction site as the Start sequence. This is to enable fusion between every Link sequence with the Start and other Link sequences. The Link sequence will be repeated until 8× and closed with the Stop sequence containing *Sal*I restriction site added with the forward primer containing XhoI fusion site, a single GLP-1 ORF sequence flanked with 6×His tag and a stop codon, TAA. In total, the construct will contain a 10×GLP-ORF sequence flanked with a 6×His tag as the marker. The 10×GLP-1 construct was inserted into the plant binary expression vector, pALP-GLP-1×10. The vector contains double constitutive cauliflower mosaic virus 35S promoters which expresses an untranslated tobacco etch virus (TEV) leading sequence and the GLP-1 fusion gene, combined with the gene coding for neomycin phosphotransferase II marker gene which develops resistance towards several aminoglycoside antibiotics including neomycin (Yenofsky, Fine & Pellow, 1990). The transformation was also done utilizing Agrobacterium-mediated transformation towards no nicotine and low alkaloid *Nicotiana tobaccum* cultivar 81V9 using the standard leaf-disc co-cultivation procedure.

The transformant was verified with PCR utilizing primer for TEV 5'UTR and primer for 10×GLP-1 C terminal sequence. The result showed approximately 1100 base pair DNA bands when containing 132 bp of TEV 5'UTR and 972 bp of 10×GLP-1 fusion gene. The DNA was later quantified with enzyme-linked immunosorbent assay (ELISA) using the known 6×His tag as the protein standard. The accumulation varies between every transgenic line, ranging from 0.05 to 0.15 % of total soluble protein (TSP). The GLP-1 protein was later purified with histidine affinity chromatography and its biological assay was determined *in vitro* in mouse pancreatic MIN6 cell line at low (2

mM) and high (10 mM) glucose concentration. The result shows that in comparison to standard GLP-1, the biological activity is reduced. Several factors affecting its biological activity include: first, the steric interference which reduces the binding affinity of GLP-1 to its receptor, second, the GLP-1 N-terminus which contains additional methionine and glycine is believed to have a negative effect on its bioactivity. Lastly, the partial purification of the protein could also cause reduced insulin secretion assay sensitivity due to contaminating endogenous plant protein. When exposed to the low glucose concentration (2 mM), plant-derived GLP-1 protein is unable to induce insulin secretion which is sufficient for detection, although standard GLP-1 is able to exhibit insulin secretion activity. On the other hand, when 13 μg/mL of GLP-1 is exposed to the high glucose concentration (10 mM), it is able to induce approximately 80 ng/mL insulin secretion, which is lower compared to the standard GLP-1 standard which induced approximately 100 ng/mL insulin secretion (Brandsma *et al*., 2009).

Geigle, Wallrapp & Thoenes (2009) observed the transient expression of monomeric and dimeric GLP-1 constructs in mammalian cells, hTERT-MSC and HEK293. In both monomeric constructs, the active GLP-1 yield is tremendously low in both cells, approximately 1 pM in both constructs. While in the dimeric construct, the expression shows a highly contrasting result with the monomeric construct, where it yields almost 100× and 110× more yield in hTERT-MSC and HEK293 cells respectively. Lastly, when additional four copies of IP2 gene sequence are added to the construct, it does not show any significant increase in the GLP-1 production.

Conclusion

The significance of GLP-1 value as a natural treatment towards DM-2 forges the urge of GLP-1 development and improvement through structure modification and yield improvement. One of the approaches to do structural modification towards native GLP-1 is by executing amino acid substitutions and protein fusion to insert additional chains to enhance the pharmacological properties of GLP-1. The specific amino acid substitutions of the GLP-1 structure which is His7 and Ala8 into His1 and Gly8 that mimic the structure of Exendin4 can avoid the DPP-4 degradation and short plasma half-life, thus enhancing the pharmacological activities in the body. Whilst, the protein fusion approach can be carried out by inserting the additional chain such as IgG heavy chain constant regions or Fc-based fusion protein through gene transfer. The other approach is yield improvement to overexpress the gene construct that involves repeated tandem sequences of GLP-1 in different expression systems. The best construct was found in *P. pastoris*, where it was genetically stable and able to generate a high yield, thus easing the scaling up process. In addition, the purification process is crucial to be optimized to achieve the desired amount of protein target without reducing the biological activity. Future recommendations would include utilization of these findings *in vitro* for a GLP-1 analog based therapeutics to treat DM-2.

References

Arag Aquilante, C. L. (2010). Sulfonylurea pharmacogenomics in Type 2 diabetes: The influence of drug target and diabetes risk polymorphisms. *Expert Review of Cardiovascular Therapy, 8*(3), 359–372. NIH Public Access. https://doi.org/10.1586/erc.09.154

Bae, E. (2017). Sirtuin 6, a possible therapeutic target for type 2 diabetes. *Archives of Pharmacal Research, 40*(12), 1380-1389. https://doi.org/10.1007/s12272-017-0989-8

Baggio, L. L., & Drucker, D. J. (2007). Biology of Incretins: GLP-1 and GIP. *Gastroenterology, 132*(6), 2131–2157. https://doi.org/10.1053/j.gastro.2007.03.054

Bellman, S. & Aromataris, E. (2014). The effectiveness of GLP-1 analogues compared to DPP-4 inhibitors for beta cell function and diabetes related complications among adults with type 2 diabetes: a systematic review protocol. *JBI Database of Systematic Reviews and Implementation Reports, 12*(10), 3–13. https://doi.org/10.11124/jbisrir-2014-1858

Brandsma, M., Wang, X., Diao, H., Kohalmi, S. E., Jevnikar, A. M., & Ma, S. (2009). A Proficient Approach to the Production of Therapeutic Glucagon-Like Peptide-1 (GLP-1) in Transgenic Plants. *The Open Biotechnology Journal, 3*(1), 57–66. https://doi.org/10.2174/1874070700903010057

Burcelin, R., Rolland, E., Dolci, W., Germain, S., Carrel, V., & Thorens, B. (1999). Encapsulated, Genetically Engineered Cells, Secreting Glucagon-like Peptide-1 for the Treatment of

Non-insulin-dependent Diabetes Mellitus. *Annals of the New York Academy of Sciences, 875*(1), 277-285. Doi: 10.1111/j.1749-6632.1999.tb08510.x

Ceccarelli, E., Guarino, E. G., Merlotti, D., Patti, A., Gennari, L., Nuti, R., & Dotta, F. (2013). Beyond glycemic control in diabetes mellitus: Effects of incretin-based therapies on bone metabolism. *Frontiers in Endocrinology, 4*. https://doi.org/10.3389/fendo.2013.00073

Donnelly, D. (2012). The structure and function of the glucagon-like peptide-1 receptor and its ligands. *British Journal of Pharmacology, 166*(1), 27-41. https://doi.org/10.1111/j.1476-5381.2011.01687.x

El-Baky, N. A., & Redwan, E. M. (2015). Therapeutic alpha-interferons protein: structure, production, and biosimilar. *Preparative Biochemistry and Biotechnology, 45*(2), 109-127.

Furman, B. (2007). Exendin-4. *Xpharm: The Comprehensive Pharmacology Reference*, 1-4. https://doi.org/10.1016/b978-008055232-3.61732-5

Gallwitz, B., Schmidt, W. E., Conlon, J. M., & Creutzfeldt, W. (1990). Glucagon-like peptide-1 (7–36) amide: characterization of the domain responsible for binding to its receptor on rat insulinoma RINm5F cells. *Journal of Molecular Endocrinology, 5*(1), 33-39.

Gecchele, E., Merlin, M., Brozzetti, A., Falorni, A., Pezzotti, M., & Avesani, L. (2015). A comparative analysis of recombinant protein expression in different biofactories: bacteria, insect cells and plant systems. *Journal of Visualized Experiments: JoVE*, (97).

Geigle, P., Wallrapp, C., & Thoenes, E. (2009). GLP-1 (Glucagon-like peptide-1) fusion polypeptides with increased peptidase resistance. Europe.

Hacker, D. L., & Balasubramanian, S. (2016). Recombinant protein production from stable mammalian cell lines and pools. *Current Opinion in Structural Biology, 38*, 129-136.

Hjelm, F., Carlsson, F., Getahun, A., & Heyman, B. (2006). Antibody-Mediated Regulation of the Immune Response. Scandinavian Journal Of Immunology, 64(3), 177-184. https://doi.org/10.1111/j.1365-3083.2006.01818.x

Holst, J. J. (2007). The physiology of glucagon-like peptide 1. *Physiological Reviews, 87*(4), 1409-1439. https://doi.org/10.1152/physrev.00034.2006

Hou, J., Yan, R., Ding, D., Yang, L., Wang, C., Wu, Z., Yu, X., Li, W., & Li, M. (2007a). Oral administration of a fusion protein containing eight GLP-1 analogues produced in Escherichia coli BL21(DE3) in streptozotocin-induced diabetic rats. *Biotechnology Letters, 29*(10), 1439–1446. https://doi.org/10.1007/s10529-007-9427-1

Hou, J., Yan, R., Yang, L., Wu, Z., Wang, C., Ding, D., Li, N., Ma, C., & Li, M. (2007b). High-level expression of fusion protein containing 10 tandem repeated GLP-1 analogs in yeast *Pichia pastoris* and its biological activity in a diabetic rat model. *Bioscience, Biotechnology and Biochemistry, 71*(6), 1462–1469. https://doi.org/10.1271/bbb.60694

Iwamoto, Y., Tajima, N., Kadowaki, T., Nonaka, K., Taniguchi, T., Nishii, M., Arjona Ferreira, J. C., & Amatruda, J. M. (2010). Efficacy and safety of sitagliptin monotherapy compared with voglibose in Japanese patients with type 2 diabetes: A randomized, double-blind trial. *Diabetes, Obesity and Metabolism, 12*(7), 613–622. https://doi.org/10.1111/j.1463-1326.2010.01197.x

Janssen, P., Rotondo, A., Mulé, F., & Tack, J. (2013). Review article: A comparison of glucagon-like peptides 1 and 2. *Alimentary Pharmacology and Therapeutics, 37*(1), 1836. https://doi.org/10.1111/apt.12092

Kim, J. W., Luo, J. Z. Q., & Luo, L. (2018). *Bone marrow mesenchymal stem cells as a new therapeutic approach for diabetes mellitus.* In A Roadmap to Nonhematopoietic Stem Cell-Based Therapeutics: From the Bench to the Clinic, 251273. Elsevier. https://doi.org/10.1016/B978-0-12-811920-4.00010-0

Kokil, G., Veedu, R., Ramm, G., Prins, J., & Parekh, H. (2015). Type 2 Diabetes Mellitus: Limitations of Conventional Therapies and Intervention with Nucleic Acid-Based Therapeutics. *Chemical Reviews, 115*(11), 4719-4743. https://doi.org/10.1021/cr5002832

Kwak, S., & Park, K. (2016). Recent progress in genetic and epigenetic research on type 2 diabetes. *Experimental & Molecular Medicine, 48*(3), e220-e220. https://doi.org/10.1038/emm.2016.7

Luo, B., Li, B., Wang, W., Liu, X., Xia, Y., & Zhang, C. et al. (2014). NLRP3 Gene Silencing Ameliorates Diabetic Cardiomyopathy in a Type 2 Diabetes Rat Model. *Plos ONE, 9*(8), e104771. https://doi.org/10.1371/journal.pone.0104771

Swinnen, S., Hoekstra, J., & DeVries, J. (2009). Insulin Therapy for Type 2 Diabetes. *Diabetes Care, 32*(suppl_2), S253-S259. https://doi.org/10.2337/dc09-s318

Manandhar, B., & Ahn, J. (2014). Glucagon-like Peptide-1 (GLP-1) Analogs: Recent Advances, New Possibilities, and Therapeutic Implications. *Journal of Medicinal Chemistry, 58*(3), 1020-1037. https://doi.org/10.1021/jm500810s

Mojsov, S. (1992). Structural requirements for biological activity of glucagon-like peptide-I. *International Journal of Peptide and Protein Research, 40*(3-4), 333-343.

Montes Castillo, M. C., Martínez Ramírez, M. J., Soriano Arroyo, R., Prieto Gomez, I., Segarra Robles, A. B., Garrido-Martínez, M., Santiago-Fernández, P., & Delgado Rodríguez, M. (2019). Glucagon-like peptide 1 and Glucagon-like peptide 2 in relation to osteoporosis in non-diabetic postmenopausal women. *Scientific*

Reports, *9*(1), 1–8. https://doi.org/10.1038/s41598-019-50117-z

Montrose-Rafizadeh, C., Yang, H., Rodgers, B. D., Beday, A., Pritchette, L. A., & Eng, J. (1997). High potency antagonists of the pancreatic glucagon-like peptide-1 receptor. *Journal of Biological Chemistry, 272*(34), 21201-21206.

Müller, T. D., Finan, B., Bloom, S. R., D'Alessio, D., Drucker, D. J., Flatt, P. R., ... & Tschöp, M. H. (2019). Glucagon-like peptide 1 (GLP-1). *Molecular Metabolism, 30,* 72-130. https://doi.org/10.1016/j.molmet.2019.09.010

Nauck, M. A., & Meier, J. J. (2018). Incretin hormones: Their role in health and disease. *Diabetes, Obesity and Metabolism, 20*, 5-21. https://doi.org/10.1111/dom.13129

Padhi, S., Nayak, A., & Behera, A. (2020). Type II diabetes mellitus: a review on recent drug based therapeutics. *Biomedicine & Pharmacotherapy, 131*, 110708. https://doi.org/10.1016/j.biopha.2020.110708

Pan, C., & Wang, X. (2013). Profile of vildagliptin in type 2 diabetes: efficacy, safety, and patient acceptability. *Therapeutics and Clinical Risk Management,* *9*(1), 247-257. https://doi.org/10.2147/TCRM.S30071

Rena, G., Hardie, D. G., & Pearson, E. R. (2017). The mechanisms of action of metformin. *Diabetologia,* *60*(9), 1577. https://doi.org/10.1007/S00125-017-4342-Z

Rendell, M. (2004). The role of sulphonylureas in the management of type 2 diabetes mellitus. Drugs, 64(12), 1339-1358. https://doi.org/10.2165/00003495-200464120-00006

Sandoval, D. & D'Alessio, D. (2015). Physiology of Proglucagon Peptides: Role of Glucagon and GLP-1 in Health and Disease. *Physiological Reviews,* *95*(2), 513-548. https://doi.org/10.1152/physrev.00013.2014

Saraswat, V., Kim, D. Y., Lee, J., & Park, Y. H. (1999). Effect of specific production rate of recombinant protein on multimerization of plasmid vector and gene expression level. *FEMS Microbiology Letters, 179*(2), 367-373.

Simpson, R. J. (2006). *Fragmentation of Protein Using Trypsin.* Cold Spring Harbor Protocols, 2006(28), pdb.prot4550-pdb.prot4550. https://doi.org/10.1101/pdb.prot4550

Sugita, K., Endo-Kasahara, S., Tada, Y., Lijun, Y., Yasuda, H., Hayashi, Y., Jomori, T., Ebinuma, H., & Takaiwa, F. (2005). Genetically modified rice seeds accumulating GLP-1 analogue stimulate insulin secretion from a mouse pancreatic beta-cell line. *FEBS Letters, 579*(5), 1085–1088. https://doi.org/10.1016/j.febslet.2004.12.082

Tan, S. Y., Mei Wong, J. L., Sim, Y. J., Wong, S. S., Mohamed Elhassan, S. A., Tan, S. H., Ling Lim, G. P., Rong Tay, N. W., Annan, N. C., Bhattamisra, S. K., & Candasamy, M. (2019). Type 1 and 2 diabetes mellitus: A review on current treatment approach and gene therapy as potential intervention. *Diabetes and Metabolic Syndrome: Clinical Research and Reviews, 13*(1), 364–372. Elsevier Ltd. https://doi.org/10.1016/j.dsx.2018.10.008

Tasyurek, M., Altunbas, H., Canatan, H., Griffith, T., & Sanlioglu, S. (2014). GLP-1-mediated gene therapy approaches for diabetes treatment. *Expert Reviews In Molecular Medicine, 16.* https://doi.org/10.1017/erm.2014.7

Therapeutic antibody classes. (2012), 197-595. https://doi.org/10.1533/9781908818096.197

Tiwari, P. (2015). Recent Trends in Therapeutic Approaches for Diabetes Management: A Comprehensive Update. *Journal of Diabetes Research,* 1-11. https://doi.org/10.1155/2015/340838

Turton, M. D., O'shea, D., Gunn, I., Beak, S. A., Edwards, C. M. B., Meeran, K., ... & Bloom, S. R. (1996). A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature, 379*(6560), 69-72.

VinuÉ, Á., MartÍnez-HervÁs, S., Herrero-Cervera, A., SÁnchez-GarcÍa, V., AndrÉs-Blasco, I., Piqueras, L., ... & GonzÁlez-Navarro, H. (2019). Changes in CDKN2A/2B expression associate with T-cell phenotype modulation in atherosclerosis and type 2 diabetes mellitus. *Translational Research, 203*, 31-48. https://doi.org/10.1016/j.trsl.2018.08.003

Weir, A. N. C., Nesbitt, A., Chapman, A. P., Antoniw, A. P. P., & Lawson, A. D. G. (2002). Formatting antibody fragments to mediate specific therapeutic functions. *Biochemical Society Transactions, 30*(4), 512-516.

Wu, Y., Ding, Y., Tanaka, Y., & Zhang, W. (2014). Risk Factors Contributing to Type 2 Diabetes and Recent Advances in the Treatment and Prevention. *International Journal of Medical Sciences, 11*(11), 1185. https://doi.org/10.7150/IJMS.10001

Xiao, X. & Gittes, G. (2015). Concise Review: New Insights Into the Role of Macrophages in β-Cell Proliferation. *STEM CELLS Translational Medicine,* *4*(6), 655-658. https://doi.org/10.5966/sctm.2014-0248

Xu, F., Wang, K. Y., Wang, N., Li, G., & Liu, D. (2017). Modified human glucagon-like peptide-1 (GLP-1) produced in E. coli has a long-acting therapeutic effect in type 2 diabetic mice. *PloS One, 12*(7), e0181939.

Yan, Y., L. Kover, K., & V. Moore, W. (2020). *New Insight into Metformin Mechanism of Action and Clinical Application.* In Metformin [Working Title]. IntechOpen. https://doi.org/10.5772/intechopen.91148

Yasuda, H., Tada, Y., Hayashi, Y., Jomori, T., & Takaiwa, F. (2005). Expression of the small peptide GLP-1 in transgenic plants. *Transgenic Research, 14*(5), 677-684.

Yenofsky, R. L., Fine, M., & Pellow, J. W. (1990). A

mutant neomycin phosphotransferase II gene reduces the resistance of transformants to antibiotic selection pressure. *Proceedings of the National Academy of Sciences, 87*(9), 3435-3439.

Yonamine, C., Pinheiro-Machado, E., Michalani, M., Alves-Wagner, A., Esteves, J., Freitas, H., & Machado, U. (2017). Resveratrol Improves Glycemic Control in Type 2 Diabetic Obese Mice by Regulating Glucose Transporter Expression in Skeletal Muscle and Liver. *Molecules, 22*(7), 1180. https://doi.org/10.3390/molecules22071180.

Yue, Z., Zhang, L., Li, C., Chen, Y., Tai, Y., Shen, Y., & Sun, Z. (2019). Advances and potential of gene therapy for type 2 diabetes mellitus. *Biotechnology & Biotechnological Equipment, 33*(1), 1150-1157. https://doi.org/10.1080/13102818.2019.1643783.

Zang, L., Hao, H., Liu, J., Li, Y., Han, W., & Mu, Y. (2017). Mesenchymal stem cell therapy in type 2 diabetes mellitus. *Diabetology & Metabolic Syndrome, 9(*1). https://doi.org/10.1186/s13098-017-0233-1on.