ผลของรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์บริสุทธิ์ที่ผลิตจากยีสต์ *Pichia pastoris* KM17H ต่อการนำเข้ากลูโคสและการแสดงออกของยีนขนส่งน้ำตาลกลูโคส ประเภทที่ 4

นางสาวสวนันท์ ทองหยู

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

Effect of purified recombinant monomeric insulin from *Pichia pastoris* KM17H on glucose uptake and glucose transporter 4 gene expression

Miss Sawanan Thongyoo

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	Effect of purified recombinant monomeric insulin from *Pichia pastoris* KM17H on glucose uptake and glucose transporter 4 gene expression
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สวนันท์ ทองหยู : ผลของรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์บริสุทธิ์ที่ผลิตจากยีสต์ *Pichia pastoris* KM17H ต่อการนำเข้ากลูโคสและการแสดงออกของยีนขนส่งน้ำตาลกลูโคส ประเภทที่ 4 (Effect of purified recombinant monomeric insulin from *Pichia pastoris* KM17H on glucose uptake and glucose transporter 4 gene expression) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ศรินทิพ สุกใส, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.อภิชาติ กาญจนทัต, หน้า.

อินซูลินเป็นฮอร์โมนที่ผลิตจากตับอ่อน มีหน้าที่ควบคุมระดับน้ำตาลในเลือด ซึ่งอินซูลินที่ออกฤทธิ์ได้อยู่ในรูปแบบมอนอเมอร์ที่ประกอบด้วยกรดอะมิโนทั้งหมด 51 ตัว และมีโครงสร้างเป็นเปปไทด์ 2 สาย ปัจจุบันมีการพัฒนาการผลิตอินซูลินโดยอาศัยเทคนิครีคอมบิแนนต์ดีเอนเอในแบคทีเรียและยีสต์ รีคอมบิแนนต์อินซูลินที่ได้จากกระบวนการนี้สามารถถูกทำให้บริสุทธิ์ง่ายกว่าอินซูลินที่สกัดมาจากตับอ่อน งานวิจัยนี้ใช้รีคอมบิแนนต์ยีสต์ *Pichia pastoris* KM71H (TP1) ซึ่งมีชุดควบคุมการแสดงออกของอินซูลินรูปแบบมอนอเมอร์ (MIP) เพื่อผลิตรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์ รีคอมบิแนนต์อินซูลินรูปแบบมอนอเมอร์ถูกเหนี่ยวนำให้มีการแสดงออกด้วย 0.5% เมธานอล ในอาหาร MMH ซึ่งสามารถติดตามการแสดงออกของรีคอมบิแนนต์อินซูลินรูปแบบมอนอเมอร์ด้วยการวิเคราะห์วิธี dot-blot และวิเคราะห์ปริมาณการผลิตด้วยวิธี indirect competitive ELISA พบว่าระดับการแสดงออกของรีคอมบิแนนต์อินซูลินรูปแบบมอนอเมอร์เป็น 16 มิลลิกรัมต่อลิตร น้ำหนักโมเลกุลของอินซูลินรูปแบบมอนอเมอร์ถูกวัดด้วยเครื่องวัดมวลโมเลกุล MALDI-TOF เป็น 5756.95 ดาลตัน ซึ่งรีคอมบิแนนต์อินซูลินรูปแบบมอนอเมอร์ถูกทำให้บริสุทธิ์จากอาหารเลี้ยงเชื้อด้วย 2 ขั้นตอน คือการใช้โครมาโตกราฟประเภท SP sepharose fast flow ในการแลกเปลี่ยนประจุ และการคัดแยกตามขนาดโมเลกุลด้วยการปั่นเหวี่ยงผ่าน Amicon ขนาด 10 กิโลดาลตัน จากนั้นมันถูกเปลี่ยนให้อยู่ในรูปที่ออกฤทธิ์โดยปฏิกิริยาการย่อยด้วยเอนไซม์ทริปซิน รีคอมบิแนนต์อินซูลินรูปแบบมอนอเมอร์ที่ถูกย่อยด้วยทริปซินแล้วถูกนำมาทดสอบการทำงานผ่านการแสดงออกของยีนขนส่งน้ำตาลกลูโคส ประเภทที่ 4 ในเซลล์กล้ามเนื้อหัวใจหนูชนิด H9c2 (2-1) ด้วยวิธี real-time PCR ผลการทดลองแสดงให้เห็นว่า อินซูลินมอนอเมอร์ที่ออกฤทธิ์ (ที่ความข้มข้น 11.20 ไมโครกรัมต่อลิตร) สามารถเหนี่ยวนำการแสดงออกของยีนขนส่งน้ำตาลประเภทที่ 4 ได้ และปริมาณน้ำตาลในอาหารเลี้ยงเซลล์สายพันธุ์ H9c2 (2-1) ลดลงอย่างมีนัยสำคัญ เมื่อมีการเติมอินซูลินมอนอเมอร์ที่ออกฤทธิ์ที่ความข้มข้น 0.70 ถึง 11.20 ไมโครกรัมต่อลิตร

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: RECOMBINANT MONOMERIC INSULIN, PICHIA PASTORIS KM71H, SP-SEPHAROSE FAST FLOW CHROMATOGRAPHY COLUMN, H9C2(2-1) CELL LINE

SAWANAN THONGYOO: Effect of purified recombinant monomeric insulin from *Pichia pastoris* KM17H on glucose uptake and glucose transporter 4 gene expression. ADVISOR: SARINTIP SOOKSAI, Ph.D., CO-ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., pp.

Insulin is a hormone that is produced in pancreas. It is important for regulation of blood sugar level. The active form of insulin is monomer which contains 51 amino acids in two peptide chains. Currently, improvement of insulin production was done by using recombinant DNA technology in bacteria and yeasts. The obtained recombinant insulin is easier to be purified than the pancreatic insulin. This research has been using the recombinant yeast, *Pichia pastoris* KM71H (TP1), which has a cassette of monomeric insulin precursor (MIP) to produce recombinant MIP. The recombinant MIP was induced to be expressed by 0.5% methanol in MMH medium. The recombinant MIP expression was detected and quantitatively determination by dot-blot analysis and indirect competitive ELISA, respectively. The expression level of recombinant MIP was 16 mg/L. The molecular weight of MIP that was determined by MALDI-TOF mass spectra technique is 5756.95 Da. The recombinant MIP was purified from culture broth by two steps, i.e. SP sepharose fast flow cation exchange chromatography and 10 kDa Amicon centrifugal molecular weight cutoff. Afterwards, it was converted to active form by TPCK tryptic hydrolysis. The tryptic digested MIP was used to test the activity through the expression of glucose transporter 4 (*GLUT4*) gene in H9c2 (2-1) rat myocardial cell line by real-time PCR. The results showed that the active MIP (11.20 ug/L) induced the expression of *GLUT4* gene and the glucose in culture medium of H9c2 (2-1) cell line significantly reduced when the cell line was treated with the active MIP at the concentration 0.70 to 11.20 ug/L.

Field of Study: Biotechnology
Academic Year: 2015

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I
INTRODUCTION

Since the discovery of insulin by Banting and Best in 1920s, insulin has been studied extensively in medicine and biochemistry as a drug for treating diabetes. In diabetes, the pancreas doesn't make enough insulin or the body can not respond normally to the insulin that is made. This causes the glucose level in the blood to rise. People with diabetes have high blood glucose, also called high blood sugar or hyperglycemia. Several factors can contribute to hyperglycemia in people with diabetes, including food and physical activity choices, illness, nondiabetes medications. It's important to treat hyperglycemia, because if left untreated, hyperglycemia can damage nerves and blood vessels. In the long term, persistent hyperglycemia, even if not severe, can lead to complications such as heart disease, stroke, kidney disease, blindness, dental disease, and amputations. Other complications of diabetes may include increased susceptibility to other diseases, loss of mobility with aging, depression, and pregnancy problems. No one is certain what starts the processes that cause diabetes, but scientists believe genes and environmental factors interact to cause diabetes in most cases. Most of diabetic patients need insulin injections to reduce blood glucose and avoid complications from hyperglycemia [4, 5].

Initially, insulin was extracted and purified from islets of animal pancreas such as cows and pigs. This production process was difficult and expensive because insulin has to be purified for the oral diabetes medication. So, many researchers improved and developed human insulin as a recombinant protein, using recombinant DNA technology [6]. They synthesized insulin by inserting insulin gene into a suitable expression vector and transform into microorganism such as *Escherichia coli* bacterial cell or yeasts to produce insulin. This method is more responsible and appropriate than extracting and purifying from animal pancreas. Currently, human insulin is produced as recombinant protein in *E. coli* and yeasts, has been used instead of animal insulin in the clinic. *E. coli* has been extensively used as a cellular host for

protein expression, but it always formed inclusion bodies that should be dealt with by denaturing and refolding after fermentation because this simple microorganism lacks the intracellular machinery to secrete protein. Yeast *Saccharomyces cerevisiae, Kluyveromyces lactis* are used to produce human insulin. However, this system has its own limitations, such as difficulty in high-density growth, no powerful and regulated promoters for expression, etc. So the expression level was limited and was not high [7, 8]. Methylotrophic yeast, *Pichia pastoris*, has been reported as a cellular host for the expression of recombinant proteins. Its expression systems offer significant advantages over *E. coli* expression systems for the production of many heterologous eukaryotic proteins, including strongly and highly regulated alcohol oxidase promoter (AOX1 promoter), the stable expression of integrated target gene, high secretory ability, low amount of proteins other than the expression product in the cell culture, the easiness of high-density cell growth, high level production of recombinant proteins and cheap culture medium needed [9].

Several methods for purify secretary recombinant insulin from culture broth were reported such as ion-exchange chromatography, size-exclusion chromatography, adsorption chromatography. CM-Sepharose FF cation exchange chromatography was reported to be the best with 97 % purity indicating the great potential for application in industry. The recombinant insulin production was be able to determine by SDS-PAGE, native-PAGE, dot-blotting, western blotting, UV absorption using spectrophotometry, high performance liquid chromatography (HPLC) mass spectroscopy (MS) or HPLC-MS. Biological activity of recombinant insulin has been demonstrated by several method such as mouse convulsion assay, mouse blood glucose assay or the receptor binding assay. However, it needs to select the suitable technique and considering the factors that convenient for further. Nowadays, many researcher try to improve the property of recombinant insulin action to treat Diabete mullitus patients in the future but there is no the pharmaceutical company produces insulin in Thailand [1-3, 10].

The previous work in molecular genetics of yeast laboratory in the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University has been successfully produced monomeric insulin precursor (MIP) by using recombinant DNA

technology in yeast, *P. papstoris* GS115 (Mut^{+} *his*$^{-}$), *P. papstoris* X33 (Mut^{+}), *P. papstoris* KM71H (Mut^{S}) and *Hansenula polymorpha* strain (WT). It was found that *P. pastoris* KM71H, have the highest expression level of MIP [11].

In this research has studied effect of purified recombinant monomeric insulin (MIP) from *Pichia pastoris* KM17H on glucose uptake and glucose transporter type 4 (*GLUT4*) gene expression. The recombinant MIP expression level was monitored by specific dot-blot analysis and quantitative determined by indirect competitive Enzyme-Linked Immunosorbent Assay (ELISA). The supernatant of P. pastoris KM71H expression culture was purified with SP-Sepharose Fast Flow cation exchange chromatography. Then, the recombinant MIP needs to be converted into monomeric insulin by tryptic hydrolysis and its biological activity was tested by determination of the expression of glucose transporter 4 gene (*GLUT4*) in H9c2 (2-1) cell line by RT-qPCR. These results were used to confirm an action of the obtained recombinant insulin before scale up the production of MIP to the pilot plant.

CHAPTER II
LITERATURE REVIEWS

2.1 Insulin

Insulin is a polypeptide hormone that produces in the islets of Langerhans in pancreas (known as beta cells). It is important for regulating the amount of glucose in the blood. It can keep blood glucose level from getting too high (hyperglycemia) or too low (hypoglycemia).

After food enters the body, it is broken down into sugar in stomach and the sugar enters the bloodstream. Sugar stimulates cells in the pancreas to release insulin. Insulin travels through the blood to other cells in the body and signals them to take up sugar, as indicated in Figure 2.1. If the body has more sugar than it needs, insulin will help to store the sugar into the liver. Therefore, insulin helps balance out blood sugar levels and keeps them in a normal range. As blood sugar levels rise, the pancreas secretes more insulin.

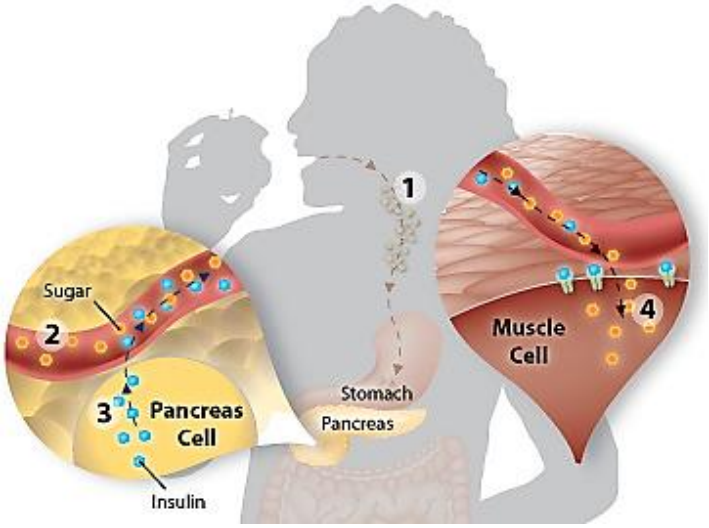


Figure 2.1: Normal blood sugar regulation [12]

Insulin is often described as a “key,” which unlocks the cell to allow sugar to enter the cell and be used for energy. If body does not produce enough insulin or your cells are resistant to the effects of insulin, it may cause hyperglycemia (high blood sugar), which can cause long-term complication such s cardiovascular disease or stroke if the blood sugar levels stay elevated for long periods of time [13, 14].

2.1.1 Discovery of Insulin

In 1920 Frederick Banting, a graduate of the University of Toronto Medical School, became interested in diabetes research. He cooperated with John Macleod, a well-known researcher in the field of carbohydrate metabolism, Charles Best, a fourth-year medical student at the time, and J.P. Collip, a well-known biochemist to study the substance in dog pancreas. These four scientists removed pancreas of dogs. The dogs which removed pancreas were resulting in high blood glucose level, thirsty, more urinated and diabetes. Then, they extracted some substance in the pancreas and named “isletin”. They discovered that the diabetic dog became healthier when it was injected with this extract. So, this was the first evidence to support their hypothesis and they successfully developed the method to extract and purify this substance from the pancreas. Finally, this discovery was presented and this substance was called as insulin in 1922.

In 1923, Banting and Macleod were awarded the Nobel Prize for their discovery of insulin. The primary structure of insulin was determined in 1953 by Frederick Sanger [12, 15].

2.1.2 The structural of insulin

Insulin is composed of two peptide chains referred to A chain and B chain as shown in Figure 2.2. The A and B chains are linked together by two disulfide bonds, and an additional disulfide is formed within the A chain. In most species, the A chain consists of 21 amino acids and the B chain consists of 30 amino acids.

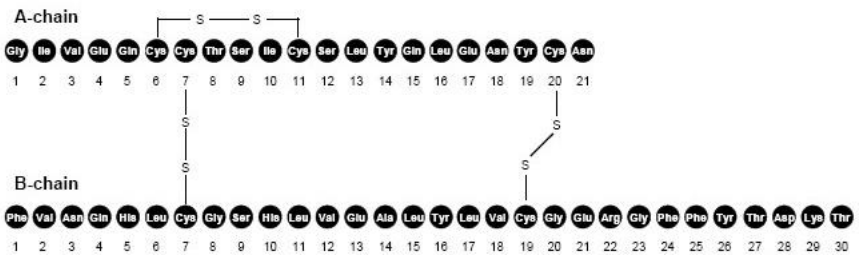


Figure 2.2: The structural of Human insulin

Insulin molecules have a tendency to form dimers in solution due to hydrogen bonding between the C-termini of B chains. Additionally, in the presence of zinc ions, insulin dimers associate into hexamers as shown in Figure 2.3.

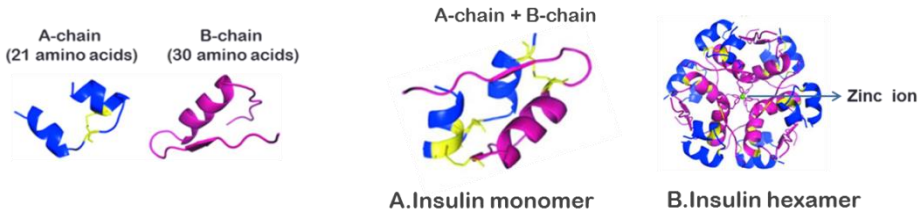


Figure 2.3: Insulin structures. Insulin exists primarily as a monomer at low concentrations (~10^{-6} M) and forms dimers at higher concentrations at neutral pH. At high concentrations and in the presence of zinc ions insulin is produced and stored in the body as a hexamer.

2.2 Diabetes Mullitus

Diabetes, often referred to diabetes mellitus, was described as a group of metabolic diseases in which the person has high blood glucose (blood sugar). Patients with high blood sugar will typically experience frequent urination (polyuria), they will become increasingly thirsty (polydipsia) and hungry (polyphagia) [13, 14].

2.2.1 Type of Diabetes Mullitus

Diabetes mellitus can be divided to two groups as follows :

2.2.1.1 Type 1 Diabetes Mullitus

Type 1 diabetes used to be known as juvenile diabetes or insulin-dependent diabetes, is a chronic condition in which the pancreas produces little or no insulin. Various factors may contribute to type 1 diabetes including genetics and exposure to certain viruses. Although type 1 diabetes usually appears during childhood or adolescence, it also can begin in adults.

2.2.1.2 Type 2 Diabetes Mullitus

Type 2 diabetes used to be known as non-insulin dependent diabetes (NIDDM). People with type 2 diabetes do not respond well or resistant to insulin. They may need insulin to balance blood sugar and prevent long term complications from this disease. Persons with type 2 diabetes may first be treated with oral medications, along with diet and exercise. Since type 2 diabetes is a progressive condition, in long term, they will require insulin to maintain blood sugar levels.

Approximately 90% of diabetes worldwide is type 2. Some people may be able to control their type 2 diabetes symptoms by losing weight, following a healthy diet, doing plenty of exercise, and monitoring their blood glucose levels. However, type 2 diabetes is typically a progressive disease and the patient will probably end up have to take insulin [16, 17].

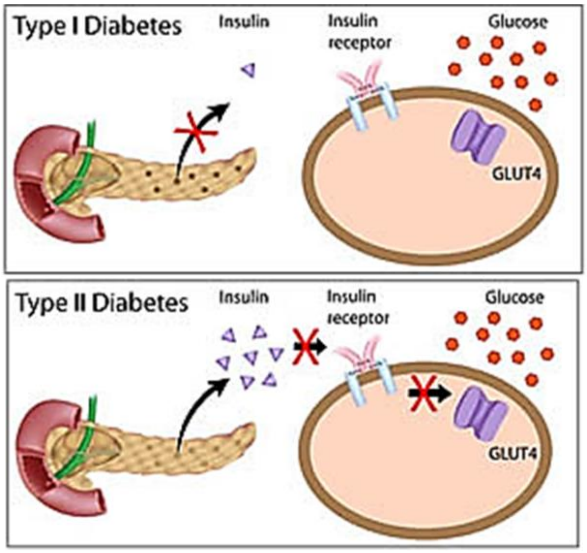


Figure 2.4: The difference between diabetes type 1 and type 2 [13]

2.2.2 Symptoms of Diabetes Mullitus

The most common signs and symptoms of diabetes are:

- Frequent urination
- Disproportionate thirst
- Intense hunger
- Weight gain

- Unusual weight loss
- Increased fatigue
- Irritability
- Blurred vision
- Cuts and bruises don't heal properly or quickly
- More skin and/or yeast infections
- Itchy skin
- Gums are red and/or swollen
- Frequent gum disease/infection
- Sexual dysfunction (men)
- Numbness or tingling, especially in your feet and hands

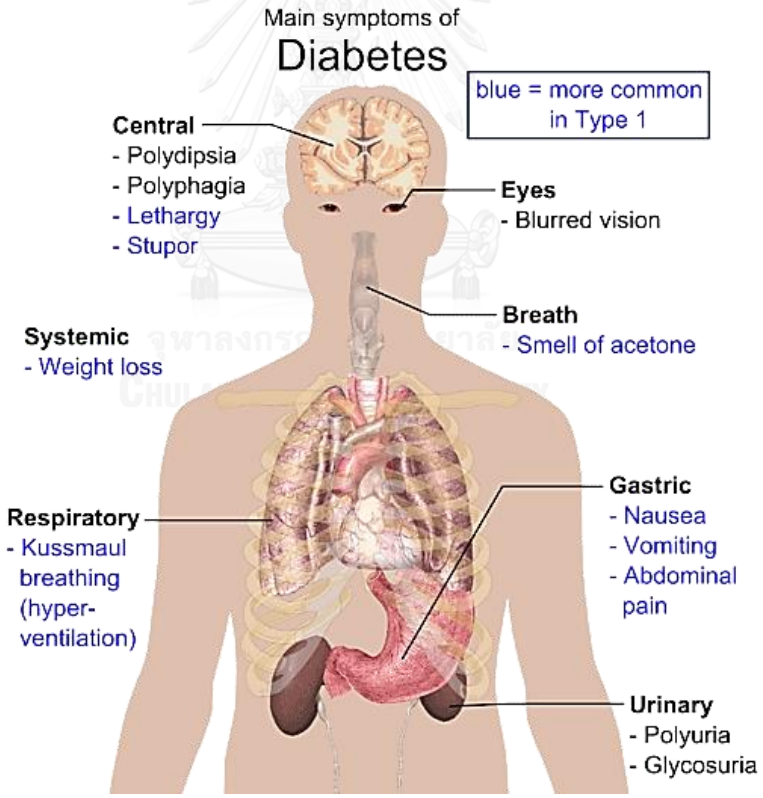


Figure 2.5: Main symptoms of Diabetes Mullitus [16]

2.2.3 Trend of Diabetes Mellitus

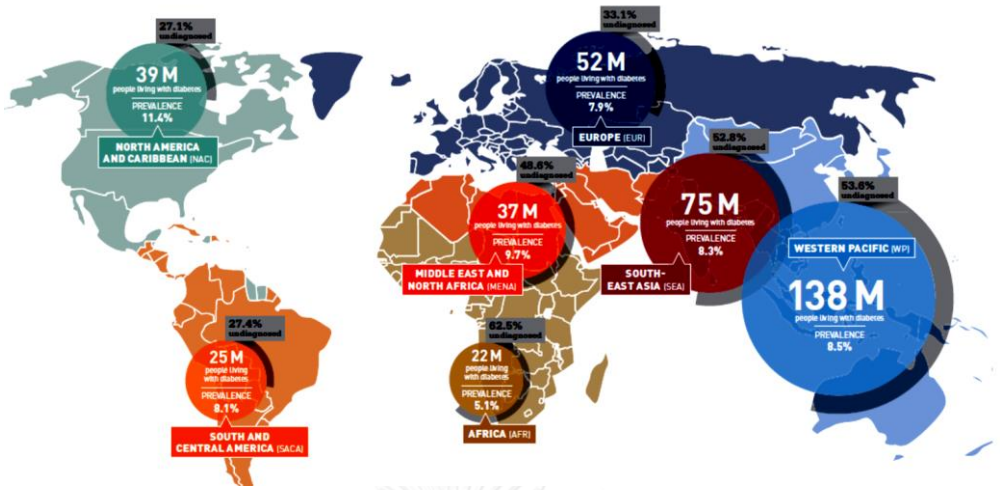


Figure 2.6: The number of diabetes patient in each continent in the world in 2014

In 2014, the International Diabetes Federation (IDF) reported a number of diabetes patient, there are about 387 million diabetes patients in the world and it will be increasing to 592 million people in 2035. Therefore, it is necessary to improve insulin production for supporting an increasing demand of insulin in the treatment of diabetes patients in the world.

2.2.4 Types of insulin

The chart below is the list of injectable insulin types with details about the length of time before insulin reaches the bloodstream and begins to lower blood sugar (onset), the best time period when it lowers blood sugar (peak) and how long that insulin continues to work (duration).

Table 2.1: The lists of injectable insulin types [18]

Type of Insulin	Brand Names	Onset*	Peak*	Duration*
Rapid-Acting	Lispro (Humalog)	15-30 min.	30-90 min	3-5 hours
	Aspart (Novolog)	10-20 min.	40-50 min.	3-5 hours
	Glulisine (Apidra)	20-30 min.	30-90 min.	1-2½ hours
Short-Acting	Regular(R) humulinor novolin	30 min. -1 hour	2-5 hours	5-8 hours
	Velosulin (for use in the insulin pump)	30 min.-1 hour	2-3 hours	2-3 hours

Type of Insulin	Brand Names	Onset*	Peak*	Duration*
Intermediate-Acting	NPH (N)	1-2 hours	4-12 hours	18-24 hours
Long-Acting	Long-acting insulin covers insulin needs for about one full day. This type is often combined, when needed, with rapid- or short-acting insulin.	Insulin glargine (Lantus)	1-1½ hour	No peak time. Insulin is delivered at a steady level. 6-8 hours
		Insulin detemir (Levemir	1-2 hours	
Pre-Mixed*	Humulin 70/30	30 min.	2-4 hours	14-24 hours
	Novolin 70/30	30 min.	2-12 hours	< 24 hours
	Novolog 70/30	10-20 min.	1-4 hours	< 24 hours
	Humulin 50/50	30 min.	2-5 hours	18-24 hours
	Humalog mix 75/25	15 min.	30 min.-2½ hours	16-20 hours

*Premixed insulins combine specific amounts of intermediate-acting and short-acting insulin in one bottle or insulin pen. The numbers following the brand name indicate the percentage of each type of insulin.

2.2.5 Insulin action to treat Diabetes Mullitus

Insulin likes a key that opens up the locks on body's cells so that glucose can get inside. Further, when blood sugar reaches a certain level, the kidneys try to get rid of it through urine, diabetics need to urinate more often. Frequent urination can make you feel tired, thirsty, hungry and start losing weight. In addition, insulin is able to regulate glucose transporter (GLUT) which has shown to enhance insulin sensitivity in an overexpression model.

In mammalian cells, transport of glucose across cellular membranes is mediated by energy coupled by the family of sodium-driven sugar cotransporters (SGLTs) and facilitative mechanisms by the protein family of glucose transporters (GLUTs). SGLT transport is required for absorption and reabsorption of glucose by the body that is uptake of hexoses from food into GI tract and from the urine in the

kidney, respectively. Glucose homeostasis within the body is mainly maintained by the various members of the GLUT protein family.

2.3 Glucose transporter

Glucose transporters (GLUTs) family is a wide group of membrane proteins that facilitate the transport of glucose over a plasma membrane. Because glucose is a vital source of energy for all life, therefore, these transporters are present in all phyla. To date, 12 members of the GLUT have been identified. On the basis of sequence similarities, the GLUT family has been divided into three subclasses as shown in Figure 2.7.

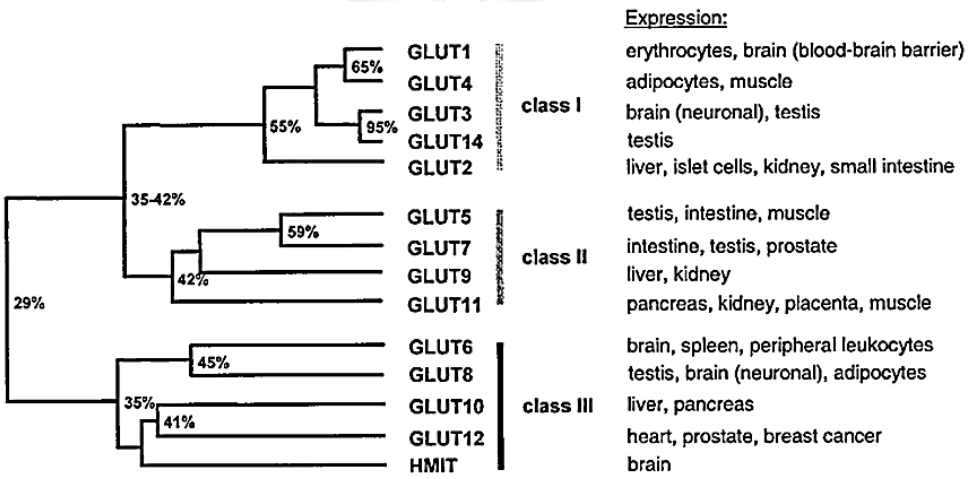


Figure 2.7: Glucose Transporters Family

Each glucose transporter isoform plays a specific role in glucose metabolism determined by its pattern of tissue expression, substrate specificity, transport kinetics, and regulated expression in different physiological conditions.

These transport proteins facilitate the transportation of glucose or fructose from areas of high concentration to areas of lower concentration. Transport activity is dependent upon the sugar concentrations and the number of transport proteins in the outer cell membrane. The delivery of glucose from the blood to the myocardial cells is mainly regulated by *GLUT4* [19-21].

Glucose transporter type 4, also known as GLUT4, is a protein encoded by the *GLUT4* gene that found in adipose tissues and striated muscle (skeletal and

cardiac). Most researchers agree that when insulin is secreted, GLUT4 bind to internal cellular membranes at Golgi apparatus and arrive at the surface membrane which contributes to glucose transport. Insulin enables glucose uptake by adipose tissue and resting skeletal muscle.

As an indicated in Figure 2.8, (1) when Insulin binds to receptor, (2) it initiates the synthesis of glucose transporters (GLUT 4). (3-4) The GLUT 4 transport proteins are integrated into the cell membrane allowing glucose to be transported into the cell.

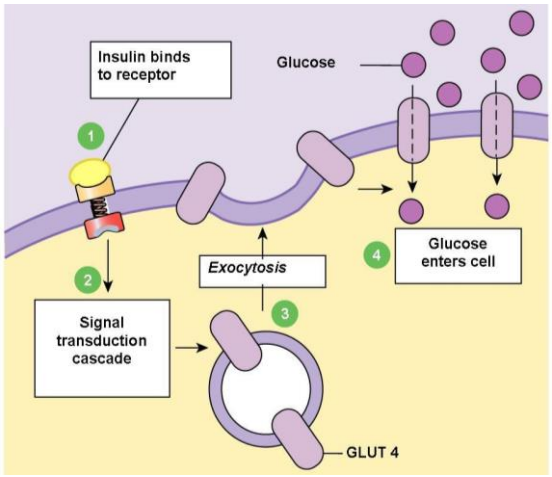


Figure 2.8: Regulation of blood glucose concentrations by insulin and GLUT4 proteins

2.4 Insulin Production

Initially, insulin was extracted and purified from islets of animal pancreases such as cows and pigs as known as bovine and porcine insulin, respectively. The compositions of bovine and porcine insulin are similar to human insulin and their functions are the same. However, a number of patients' immune systems produce antibodies against it, resulting in inflammatory responses at injection sites. Moreover, the production process was difficult and expensive because insulin has to be purified for the diabetes medication. These factors led researchers to consider synthesizing human insulin by inserting insulin gene into a suitable vector and transforming into microorganism such as *E. coli* or yeast. This has been achieved by using recombinant DNA technology. This method is a more reliable and sustainable than extracting and purifying of the animal pancreases.

Recombinant DNA technology has been developed and used to produce human proteins. In 1978, researchers at a Burgeoning Biotechnology Genentech company announced that they had introduced a human insulin gene into *E. coli* bacteria cell, which is a safe strain and then produced the protein. This biotechnology scheme is now in for basic research that helps scientists to understand the human body and doctoral to treat diabetes.

The first recombinant protein production was recombinant human insulin that produced by *E. coli.* In 1982 the first recombinant insulin was produced by Eli Lilly under the brand name Humulin. As indicated in Figure 2.9, a plasmid DNA is extracted from a bacterium and cut with restriction enzyme to form plasmid vector (step 1,2), human insulin gene that was synthesized as a recombinant DNA was inserted into the plasmid (step 3), the recombinant plasmid was transformed into *E. coli* cell (step4), the recombinant bacteria which harbored human insulin gene was cultivated to produce a recombinant human insulin (step 5).

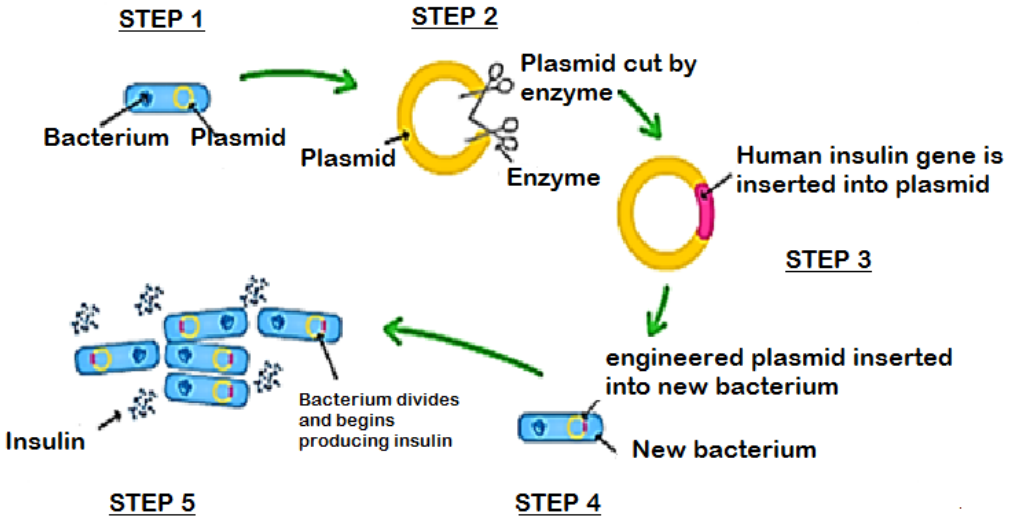


Figure 2.9: Genetics engineering to produce insulin [12]

Human insulin was expressed in *E. coli* as separate chains or proinsulin, but it always formed inclusion bodies that should be dealt with denaturing and refolding after fermentation. Moreover, the broad application of this system with proteins derived from eukaryotic genomes that require post translational modification has been problematic because this microorganism lacks the intracellular machinery to

achieve these outcomes. *E. coli* is therefore not suitable for expression the proteins that contain a high level of disulfide bond.

Yeast was used to minimize such problems, in which the correct folded product with disulfide bridges was synthesized.

Moreover, scientists started to develop new forms of insulin called insulin analogs such as insulin lispro (Humalog) and insulin glargine (Lantus). Analogs have become increasingly popular among prescribers and patients. The scientists improved the analogs properties by tweaking their amino acid sequences in ways that force the body to process them faster or slower than human insulin. These new properties give more options for controlling blood glucose in the diabetics [1, 4].

2.5 Expression of recombinant insulin in *P. pastoris* KM71H

The laboratory of yeast genetics in the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University has been successfully established the recombinant monomeric insulin precursor (MIP) by using recombinant DNA technology in yeast. They synthesized nucleotide sequence of MIP gene [22] was cloned into pPICZαA expression vector . The pPICZαA::MIP plasmid (TP1) was transformed into yeast *P. papstoris* GS115 (Mut^+ his^-), *P. papstoris* X33 (Mut^+), *P. papstoris* KM71H (Mut^S) and *H. polymorpha* strain (WT). It was found that *P. pastoris* KM71H (TP1) has the highest expression level of MIP as shown in Figure 2.10. Therefore, *P. pastoris* KM71H (TP1) has been used for produce recombinant monomeric insulin in this study.

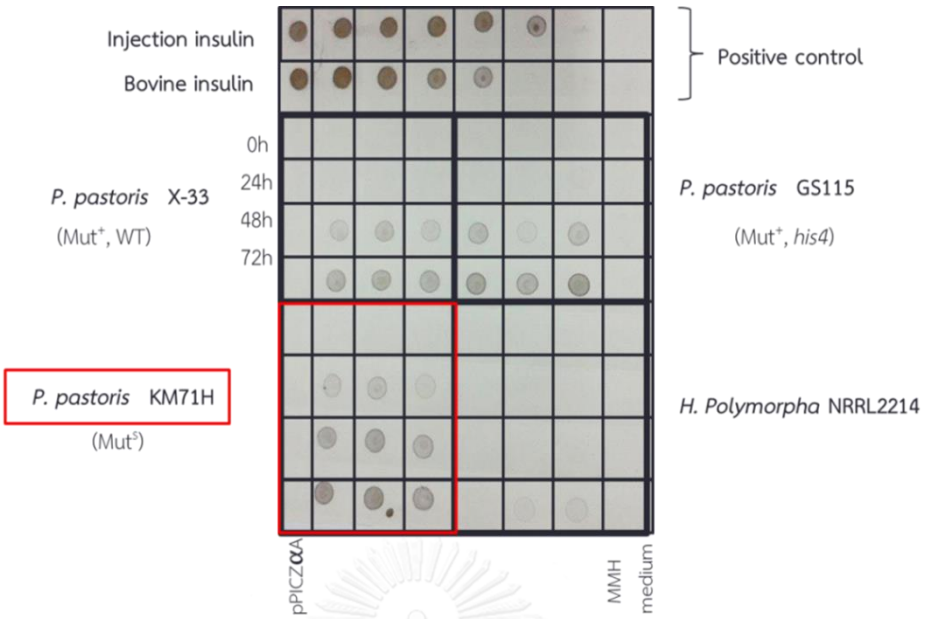


Figure 2.10: Dot-blot analysis of the supernatant of the recombinant *P. pastoris* strains ; X-33, KM71H, GS115 and *H. polymorpha* (NRRL2214) at various cultivation time and the standard insulin (positive control), MMH medium (negative control), the supernatant of the recombinant *P. pastoris* pPICZαA (negative control) [11]

2.5.1 Monomeric insulin precursor (MIP)

Monomeric insulin precursor (MIP) which used in this study was modified and engineered by removing B28-B30 and replacing B27 threonine by lysine (K) by Jin-Guo Ding in 2005. Moreover, between A-chain and B-chain is linked by AAK linker, and K position is a cleavage site of trypsin as shown in Figure 2.11. MIP was designed to express as precursor in *P. pastoris.* By tryptic hydrolysis instead of the tryptic transpeptidation that MIP will become an activate form. So development of monomeric insulin with high activity is very attractive. It is suitable for large scale production from its precursor expressed in yeast and may have benefits for diabetics [22, 23].

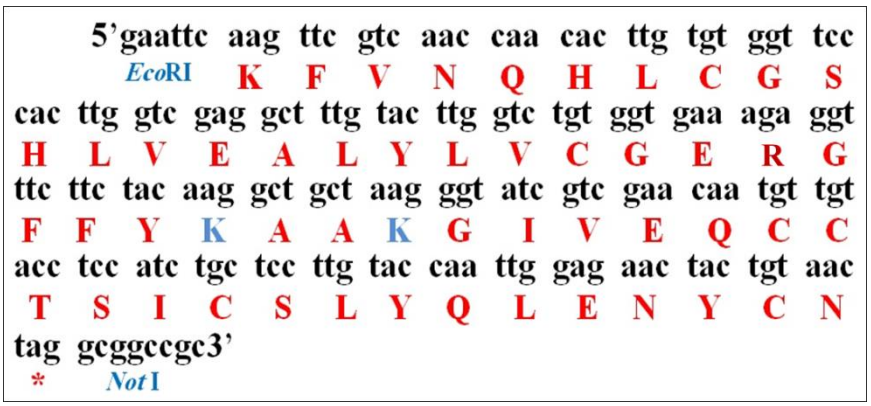


Figure 2.11: Nucleotide sequence and amino acid sequence of MIP gene

2.5.2 Methylotrophic yeast *Pichia pastoris*

Eukaryotic methylotrophic yeasts are able to use methanol as the carbon and energy source. When they are grown on methanol as the sole carbon and energy source, the enzymes involved in methanol metabolism are strongly induced, and the membrane bound organelles, peroxisomes, which contain key enzymes of methanol metabolism, proliferate massively. These features have made the methylotrophic yeasts to be attractive for the production of heterologous proteins and useful model organisms for the study of peroxisome biogenesis and degradation.

P. pastoris is a member of methylotrophic yeasts which is suitable for expressing proteins at a high level through high-density fermentation. Many reasons that made *P. pastoris* to be interested including: [1]

- It has a very strong inducible promoter *AOX1* promoter which induced by methanol.
- The recombinant gene that integrated in *Pichia* genome is stable.
- It has high secretory ability.
- It produces low amount of undesirable proteins.
- It can grow in high-density cell condition and needs cheap culture medium.

Another advantage of *P. pastoris* is that it can use as a model organism as *Saccharomyces cerevisiae*. The two yeast species (*P. pastoris*, *S. cerevisiae*) have simple growth conditions and thus the culturing of *P. pastoris* can be adopted to the labs without special equipment. Moreover, several researches reported that *P.*

pastoris production system has been used in producing many proteins, with an expression level ranging from mg/L to g/L.

2.6 Purification method of insulin production

Nowadays, recombinant insulin from microorganism has been used instead of animal insulin in the clinic because purification method is more convenient and appropriate than extracting and purifying the insulin from animal pancreases. The purification method of recombinant insulin from culture broth has been used column chromatography technique such as ion-exchange chromatography, size-exclusion chromatography, adsorption chromatography, affinity chromatography.

2.6.1 Column Chromatography

Column chromatography is a method used to purify or separate individual proteins, nucleic acids, or small molecules in complex mixtures based on their differing interactions with a stationary phase and a mobile phase. Several reports have demonstrated a simple procedure for purifying the recombinant insulin by liquid chromatography (LC) as shown in Table 2. From these data indicated that CM-Sepharose FF (Cation exchange chromatography) gave the highest percentage of recovery and purity with 97% which is the great potential for application in industry.

Table 2.2: Purification methods of recombinant insulin production from culture medium [1-3]

Type of chromatography column	Purification method	% Recovery	% Purity	Determination and Identification method	Reference
- Ion-exchange chromatography - Size-exclusion chromatography	- Amberite XAD-7 - Sephadex G-50	>80 %	60 %	- Dot-Blotting Method	Wang Y.,et al. (2000)
- Ion-exchange chromatography	- SP sepharose Fast Flow	64%	Not determined	- SDS–PAGE, Western blot analysis - Amino acid sequence analysis - Protein concentration Assay	Chen Z.,et al. (2007)
- Ion-exchange chromatography	- CM-Sepharose Fast Flow	97%	97 %	- Mass spectrometry - HPLC	Xie T., et al. (2008)
- Absorption Chromatography - Affinity chromatography	- Expanded bed absorption - Affinity Chromatography	95%	96 %	- Mass spectrometry	Gurramkonda C. et al, (2010)

2.6.2 Types of column chromatography

2.6.2.1 Ion exchange chromatography

Ion exchange chromatography is the most popular method for the purification of proteins and other charged molecules. . The resin that is coating with either cations or anions is used as stationary phase. In cation exchange chromatography positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. The bound proteins can be eluted either by increasing the ionic strength of the buffer or by adjusting the pH (Figure 2.12).

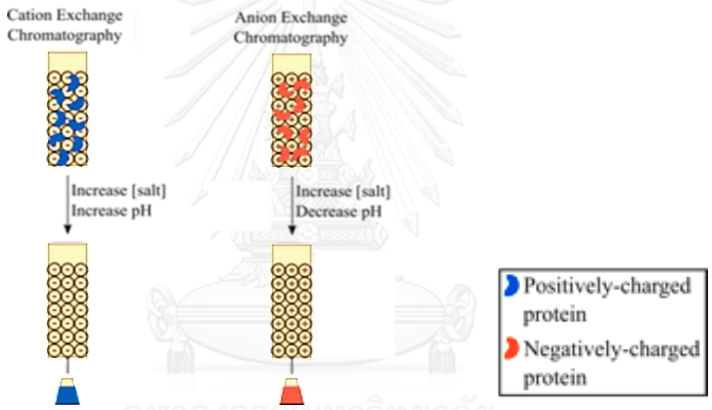


Figure 2.12: Ion exchange chromatography

2.6.2.2 Size-exclusion chromatography

Size-exclusion chromatography is a chromatographic method which the molecules in solution are separated by their molecular weight. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. This method differs from other types of chromatography in that no equilibrium state is established between the solute and the stationary phase. The pore size of a porous gel is designed to allow the large solute molecules to pass through the column. The small molecules, however, permeate the gel, so the smaller molecules will take longer time to get through the column than the large molecules. Thus separation is according to molecular size (Figure 2.13).

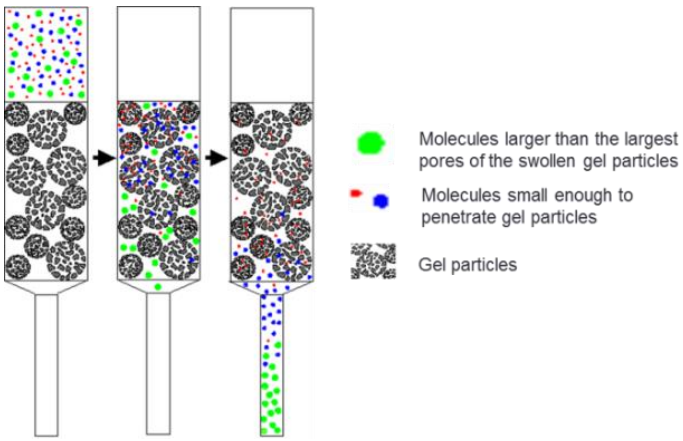


Figure 2.13: Size-exclusion chromatography

2.6.2.3 Affinity chromatography

Affinity chromatography is a method of separating biochemical mixtures based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. It utilizes the specific interaction between specific solute molecule and the immobilized stationary phase.

As indicated in Figure 2.14, the immobilized molecule may be an antigen against the specific antibody, when solute containing a mixture of proteins with the specific antibody passed through the column, only the specific antibody is reacted to the column. This specific antibody will be later extracted by changing the ionic strength or pH [24].

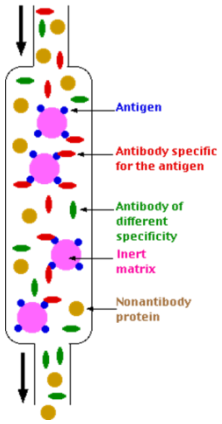


Figure 2.14: Affinity chromatography [25]

2.7 Conversion of recombinant insulin into active form by tryptic hydrolysis

Monomeric insulin precursor (MIP) was shown to be inactive. The active form of insulin could be obtained from MIP through tryptic hydrolysis. In this study, MIP was hydrolyzed by immobilized TPCK – *trypsin* and fractionate by centrifugation.

Trypsin is a member of the serine protease family which consists of a single chain polypeptide of 223 amino acid residues. Trypsin will cleave peptides on the C-terminal of lysine and arginine amino residues. The pH optimum of trypsin is 7 - 9. The rate of hydrolysis is slower if an acidic residue is on either side of the cleavage site and no cleavage occurs if a proline residue is on the carboxyl side of the cleavage site. The trypsin has a wide range of applications including amino acid analysis and protein sequencing studies. Enzymes such as trypsin and chymotrypsin selective in their cleavage of peptide bonds and have become important tools in sequencing studies. Chymotrypsin cleaves peptide bonds in which the carboxyl group is contributed by phenylalanine, tryptophan and tyrosine. The TPCK-trypsin is the modified trypsin that was treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) to inactivate any remaining chymotryptic activity to prevent autolysis [22].

2.8 Identification and determination of recombinant insulin

There are several conventional procedures and techniques to identify and determine the recombinant insulin in the culture broth such as SDS-PAGE, native-PAGE, dot-blotting, western blotting, UV absorption using spectrophotometry, high performance liquid chromatography (HPLC), mass spectroscopy (MS) or HPLC-MS. Moreover, there are simple techniques that quick and easy to detect recombinant insulin are dot-blotting analysis and enzyme-linked immunosorbent assay (ELISA). These techniques are high specificity and high sensitivity for recombinant MIP determination.

2.8.1 Dot-Blot Analysis

Dot-blotting technique is similar to the western blot technique but the advantage of dot-blotting is a simple technique for detecting, analyzing, and

identifying proteins from a large number of samples which the samples do not need to be separated by electrophoresis.

This technique started by slowly dropping samples onto the membrane such as nitrocellulose membrane or polyvinylidene difluoride (PVDF) membrane. Then, the membrane was blocked by soaking in 5% BSA or skim milk in PBS buffer. Anti-insulin was used as the specific primary antibody and used to probe insulin in the samples. Followed by the specific secondary antibody (anti-primary antibody) that was conjugated with enzyme HRP (horse radish peroxidase). The target insulin was visualized by incubating the membrane in the substrate solution (DAB solution) as shown in Figure 2.15. The immunoreactive intensity which visualized on membrane is related to the target protein concentration.

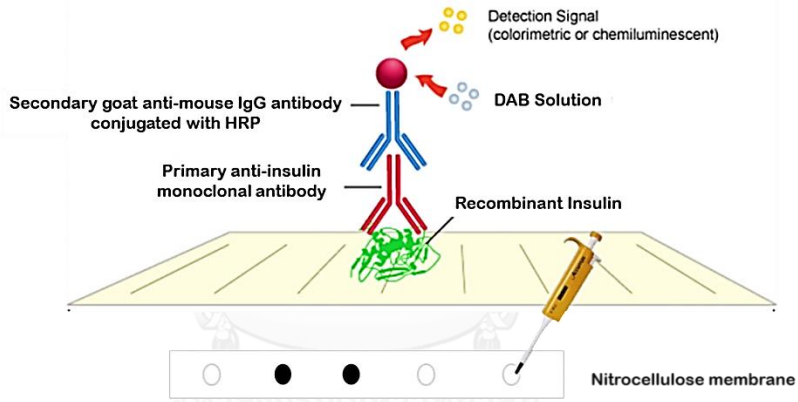


Figure 2.15: Dot blot analysis [19]

2.8.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a plate based assay technique designed for detecting and quantifying substances with high specificity and sensitivity by using the specific antibodies. This technique is typically performed in 96-well (or 384-well) polystyrene plates, which the specific antibody or an antigen must be coated to solid surface.

The ELISA can be divided to 3 groups including;

- Direct ELISA
- Indirect ELISA
- Sandwich ELISA

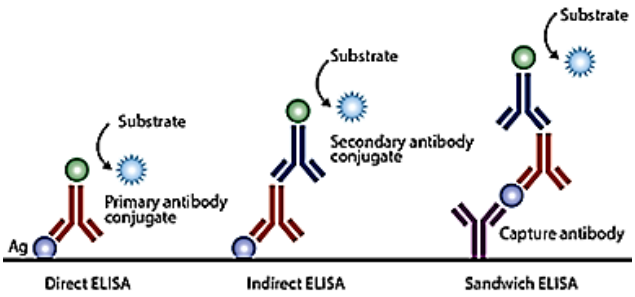


Figure 2.16: The common ELISA formats

2.8.2.1. Direct Enzyme-Linked Immunosorbent Assay

Direct ELISA used only one set of antigens and antibodies. In this method, antigens were fixed to the ELISA plates to react directly with the antibodies which linked HRP enzyme. Then, the enzyme linked antibodies were reacted to external substrate and the color reaction was measured by spectrophotometry.

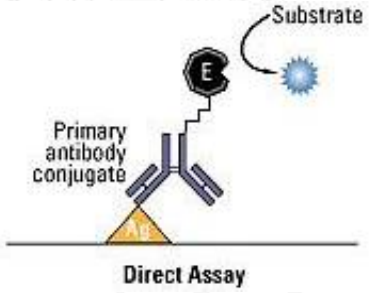


Figure 2.17: Direct Enzyme-Linked Immunosorbent Assay

2.8.2.2 Indirect Enzyme-Linked Immunosorbent Assay

Indirect ELISA is two step ELISA which involves two binding process of primary *antibody and lebeled secondary antibody.* One of the advantages of the indirect ELISA is different primary detection antibodies can be used with a single labeled secondary antibody which is flexible and cost-saving test. The indirect ELISA is usually used in the procedures of antibody determination concentration and the determination of antibody titer.

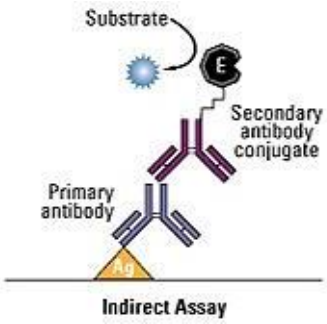


Figure 2.18: Indirect Enzyme-Linked Immunosorbent Assay

Indirect Competitive Enzyme-Linked Immunosorbent Assay was developed to specifically determine the concentration of MIP in this research. The procedures of this technique start by coating and incubating antigen onto an immunological plate. The plate was blocked with blocking buffer and followed by washing. A standard antigen in various concentrations or samples which used as competitor were added. A specific primary antibody (unlabeled-antibody was then added and incubated. The plate was washed so that unbound antibody is removed (the more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence competition). The secondary antibody, specific to the primary antibody was added. The secondary antibody was coupled to an enzyme. A substrate was added and remaining enzyme elicits a chromogenic or fluorescent signal. For competitive ELISA- the higher the original antigen concentration, the weaker the eventual signal. Hence, the antigen is inversely proportional to the fluorescent signal. This technique may has the limitations which are some biomolecules or reagents in the mixture may interfere the binding between target antigen and antibody or antibodies.

2.8.2.3 Sandwich Enzyme-Linked Immunosorbent Assay

Sandwich ELISA is also an indirect ELISA but it is highly efficient in sample antigen detection. The sandwich ELISA quantifies antigens between two layers of antibodies (i.e. capture and detection antibodies) as shown in Figure 2.19. The capture antibody is fixed at the bottom of ELISA plate. The antigen must contain at least two antigenic epitope capable of binding to antibodies, since at least two antibodies act in the antigen. The enzyme-linked antibody, which is the detection

antibody, is usually used as the secondary antibody. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. The advantage of sandwich ELISA is that the sample does not have to be purified before analysis and the assay is high specificity [26, 27].

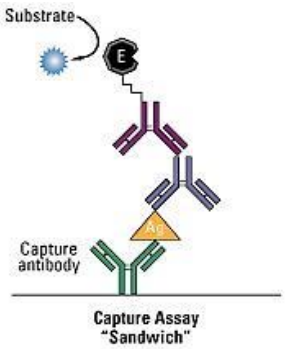


Figure 2.19: Sandwich Enzyme-Linked Immunosorbent Assay

2.9 Biological activity of insulin

Many previous studies that improved production process of recombinant insulin have been tested biological activity of product by several methods such as mouse convulsion assay, mouse blood glucose assay or the receptor binding assay. However, it needs to select the suitable technique and consider the factors that convenient for further study such as time consuming, cost and sensitivity .

This study tests biological activity of recombinant monomeric insulin by transcriptional determination of *glucose transporter 4* (*GLUT4*) in myocardial cell line H9c2 (2-1) by RT-qPCR [21].

The myoblast cell line H9c2 (2-1) is a commercially available myogenic cell line that has been used for biological activity test of recombinant insulin [21, 24]. This cell line derived from embryonic rat heart has been used as an *in vitro* model for both skeletal and cardiac muscle. An interesting feature of this cell line is its ability to differentiate from mono-nucleated myoblasts to myotubes depended upon the reduction of growth factors or serum concentration. Accompanying myotube formation is the expression of myogenic transcription factors, calcium channel proteins. During the differentiation process, cells retain several elements of the

electrical and hormonal signaling pathway of cardiac cells and have therefore become an accepted *in vitro* model to study the effects of ischemia and diabetes on the heart [24].

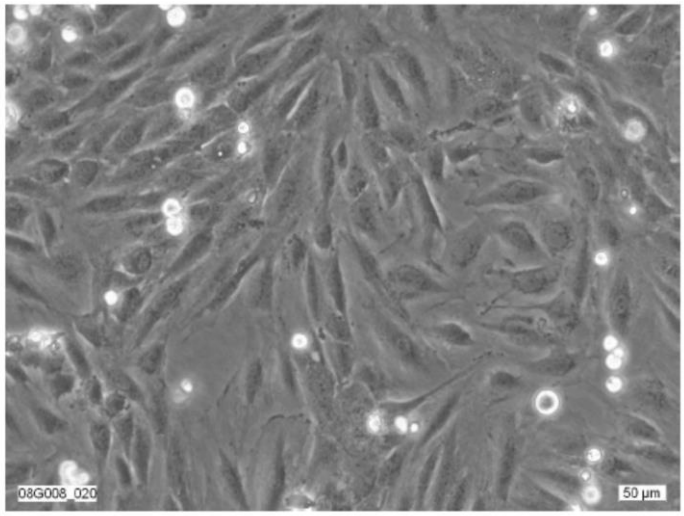


Figure 2.20: Images of Cell Line: H9c2 (2-1)

CHAPTER III

LITERATURE REVIEWS

3.1 Materials

3.1.1 Microorganisms

Pichia pastoris KM71H (TP1) composed of pPICZαA::MIP plasmid were obtained from the molecular genetics of yeast laboratory in the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University [11].

3.1.2 Cell line

H9c2(2-1) rat myocardial cell line was purchased from American Type Culture Collection (ATCC).

3.2 Chemicals and Reagents

Chemicals and Reagents	Company, country
Absolute ethanol	Merck, U.S.A
Absolute methanol	Merck, U.S.A
Bacto™ Peptone powder	Becton, Dickinson and Company, France
Biotin	Fluka, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, U.S.A
Citric acid, anhydrous	Sigma-Aldrich, U.S.A
DAB (3,3'-Diaminobenzidine)	Sigma-Aldrich, U.S.A
di-Potassium hydrogen phosphate	Carlo Erba Reagenti, Italy
di-Sodium hydrogen phosphate	Merck, Germany
Dimethyl sulfoxide (DMSO)	Fluka, Switzerland
Dulbecco's modified Eagle's medium(DMEM)	Gibco, Carlsbad, CA, U.S.A
Fetal calf serum	FBS, Gibco, Mulgrave, Victoria, Australia
Glutaraldehyde 50% in water	Merck, U.S.A.

Chemicals and Reagents	Company, country
Goat anti-Mouse IgG, (H+L) HRP conjugate	Jackson Immuno Research Laboratories Inc., U.S.A
Humalog® (insulin lispro injection)	Lilly, U.S.A
Hydrogen peroxide	Merck, Germany
Insulin from bovine pancreas	Sigma-Aldrich U.S.A
Mixtard® 30 HM Penfill® (Injection insulin)	Novo Nordisk, Denmark
Monoclonal anti-insulin antibody	Sigma Aldrich, U.S.A
Nitrocellulose membrane (NitroBind™)	Life Science Products, Inc., U.S.A
Skim milk	Difco, U.S.A
Sodium chloride	Ajax Chemicals, Australia
Sodium Citrate, Dihydrate	Ajax Chemicals, Australia
TMB (3,3',5,5'-tetramethylbenzidine)	Sigma-Aldrich, U.S.A
Tween-20	Sigma Aldrich, Germany
Yeast nitrogen base powder (w/ ammonium sulfate)	Bio basic, Inc., Canada
Yeast extract powder	Bio Springer, France
Zeocin™	Invitrogen, U.S.A

3.3 Equipment and Supplies

Equipment and Supplies	Company, country
AKTA Protein Purification Systems	GE Healthcare Bio Science, Sweden
Amberlite XAD-7 resin	Sigma-Aldrich, U.S.A
Amicon Ultra-15 Centrifugal Filter (10 kDa)	Merck Millipore., Ltd., Ireland
Autoclave (HV-50)	Hirayama manufacturing Corp., Japan
Autoflex MALDI-TOF-MS	Bruker Daltonics, Leipzig, Germany

Equipment and Supplies	Company, country
Balance (Adventurer™, ARC 120)	Ohaus Corp., U.S.A
Bench-top centrifuge, WiseSpin® (CF-10)	Dihan scientific Co., Ltd., South Korea
Biological safety cabinet (Heal force®, HFsafe-1200)	Shanghai Lishen Scientific equipment Co., Ltd., China
CFX96 Touch Real-Time PCR Detection System	Bio-Rad Laboratories, Inc., USA
Digi Thermopet (NTT-1200)	Tokyo Rikakikai Co., Ltd., Japan
E.Z.N.A.® Tissue culture RNA extraction kit	Omega Bio-Tek, Inc.,U.S.A
Freezer (-20°C) (SF-C697)	Sanyo Commercial Solution, Ltd., Thailand.
Gene Pulser® Cuvette, 0.2 cm.	Bio-Rad Laboratories, Inc., China
High speed micro refrigerated centrifuge (MTX-150)	Tomy Seiko Co., Ltd., Japan
High speed refrigerated centrifuge (6500)	Kubota Corp., Japan
HiTrap™ IEX Selection Kit	GE Healthcare Bio Science, Sweden
Hot plate (PC-101)	Corning, U.S.A
Incubator (MIR 152)	Sanyo Electric Co., Ltd. Japan
MTP 384 ground steel target plate	Bruker Daltonics, Leipzig, Germany
Microplate reader (Multiskan FC, Type 357)	Thermo Fisher Scientific Instruments Co., Ltd., China
MicroPulser™	Bio-Rad, U.S.A
Microwave oven (National®)	Matsushita Electric Industrial Co. Ltd., Japan
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	Thermo Scientific, Canada
Mupid®-EXU Submarine electrophoresis system	Advance Co, Ltd., Japan
NanoDrop 2000 Spectrophotometer	Thermo Scientific, Canada
Pellicon Ultrafiltration Cassettes	Merck Millipore, Germany

Equipment and Supplies	Company, country
pH meter (Accumet® AB15)	Fisher Scientific, Singapore
Refrigerator	Panasonic Appliances Lights Action Alliance Co., Ltd., Thailand
Refrigerated CentriVap concentrator	Labconco, USA
Refrigerated incubator shaker (Innova™ 4330)	New Brunswick Scientific Co., Inc., U.S.A
SA–matrix	Bruker Daltonics, Leipzig, Germany
Tetro cDNA synthesis Kit	Bioline, Inc., U.S.A
Ultrafiltration membrane using a bioreactor system	Amersham biosciences, Sweden
Ultra low refrigerator (MDF 79OAT)	Sanyo Electric Co., Ltd. Japan
Vortex mixer (KMC-1300V)	Vision scientific Co., Ltd., Korea
Water pro plus	Labconco Corp., U.S.A
Whatman® membrane filters nylon (0.45, 0.22 μm)	Pall corporation, U.S.A
YSI 2700 Select Biochemistry Analyzer	YSI incoperate, U.S.A

3.4 Enzymes and Primers

Enzymes and Primers	Company, Country
GLUT4 forward primer (5′ AGCCAGCCTACGCCACCATA 3′, Tm=55.9)	Life Science AP, Inc., U.S.A.
GLUT4 reverse primer (5′ GGACCCATAGCATCCGCAAC 3′, Tm=55.9)	Life Science AP, Inc., U.S.A.
GAPDH forward primer (5′ CGGTGTGAACGGATTTGGCC 3′, Tm=55.9)	Life Science AP, Inc., U.S.A.
GAPDH reverse primer (5’ TCATGGGGGCATCAGCGGAA 3′, Tm=55.9)	Life Science AP, Inc., U.S.A.
Immobilized TPCK-trypsin	Thermo Scientific, USA

3.5 Media

3.5.1 Production medium and induction medium for *P. pastoris* KM71H

YPD Zeocin™ medium (YPD with 100 μg.mL^{-1} of Zeocin™ final concentration) and YPD medium were used for recombinant yeast cultivation and screening. YPG medium was used for cell manipulation in cell production phase. MMH medium with 0.5% methanol was used for MIP induction in an expression phase as described in an appendix A.

3.5.2 Complete growth medium for H9c2(2-1) cell line

The growth medium for H9c2(2-1) cell line culture is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. The medium for biological activity assay of MIP is Dulbecco's Modified Eagle's Medium without D-glucose, Catalog No. F0405 (Biochrom AG, Germany). To make the complete medium, add the following components to the medium: fetal bovine serum to a final concentration of 10%.

3.6 Methods

3.6.1 Cultivation and expression of MIP from *P. pastoris* KM71H

In accordance with Figure 3.1, fresh single colony of recombinant *P. pastoris* KM71H (TP1) was inoculated into 50 ml YPG medium (1% yeast extract, 1% peptone, 1% glycerol) and cultured in a shaking incubator at 250 rpm, 30°C for 24 h. OD_{600} of the culture was adjusted to 5.0±0.2 and used as a starter culture. Ten percent of the starter culture (20 ml) was inoculated into 200 ml YPG medium in 500 ml Erlenmeyer flask (10 flasks) and incubated with shaking at 250 rpm at 30°C for 24 h. Cells were collected by centrifugation at 5,000×g, 4°C for 10 min and resuspended in 200 ml of MMH induction medium in 500 ml baffled flask (10 flasks). All culture samples were incubated with shaking at 250 rpm, 30°C for 96 h and added absolute methanol to a final concentration of 0.5% methanol every 24 h. After 96 h, cells were separated by centrifugation and undissolved material was demounted by filtration using

Whatmann No. 1 filter paper. The supernatant was transferred to a sterile bottle and kept on ice until used [28].

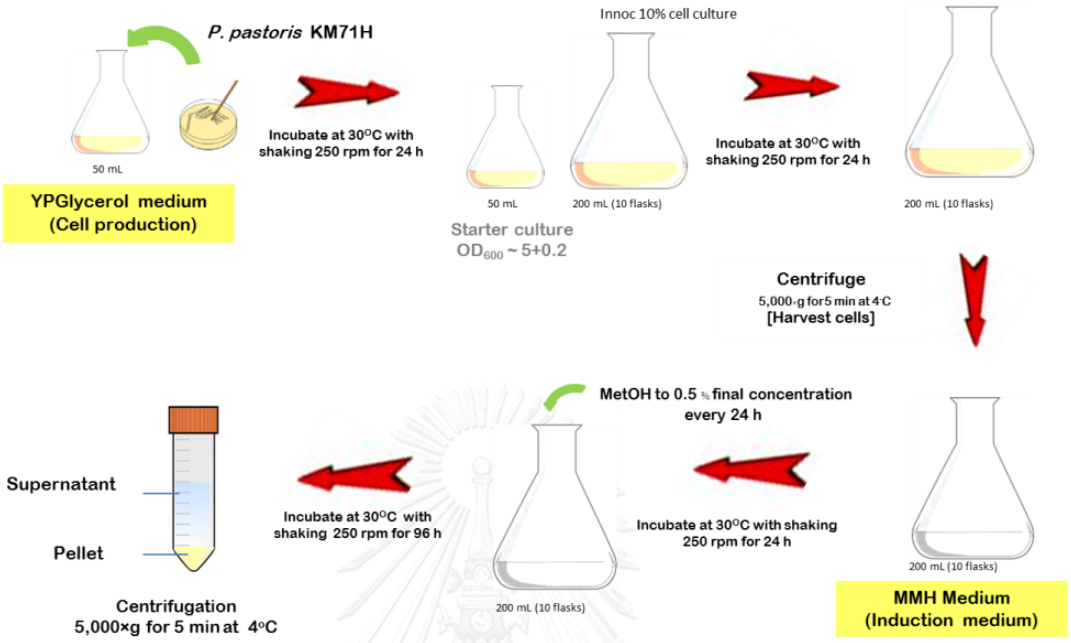


Figure 3.1: Cultivation and expression of MIP from *P. pastoris* KM71H

3.6.2 Determination of molecular weight of MIP by MALDI-TOF

An Autoflex MALDI-TOF-MS (Bruker Daltonics, Leipzig, Germany) was used in the present study for the determination of molecular weight of MIP from culture broth of recombinant *P. pastoris* KM71H (TP1). One microliter of the supernatant was spotted onto the MTP 384 ground steel target plate (Bruker Daltonics), air dried, and subsequently spotted 1 µl of the bead suspension-SA–matrix mixture (1:1, v/v) directly onto the MTP 384 massive aluminium target plate as shown in Figure 3.2. The external calibration of the instrument was done using a standard protein/peptide mixture kit following the instruction of its manufacturer (Bruker Daltonics). Twenty individual spectra were averaged to produce a single mass spectrum in each analysis and the bovine insulin (Sigma-Aldrich, USA) was used as a standard.

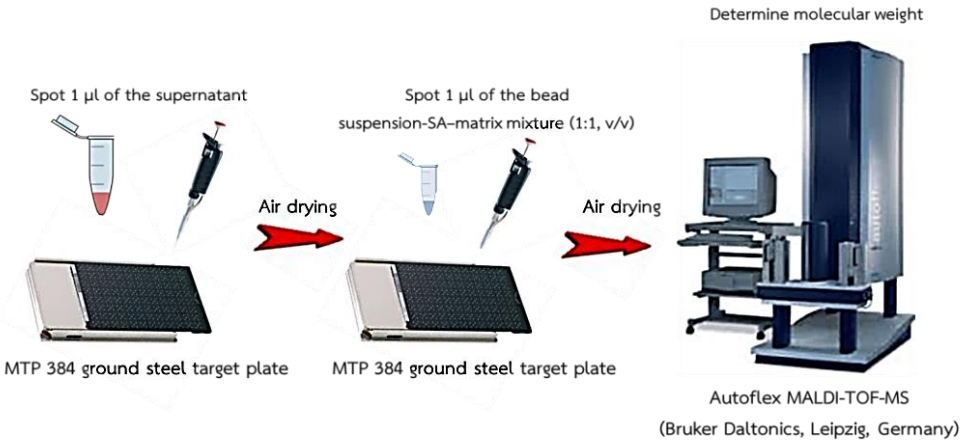


Figure 3.2: Determination of molecular weight of MIP by MALDI-TOF

3.6.3 Monitoring of MIP expression level by dot-blot analysis

Insulin (Mixtrad 30, Novo Nordisk, Denmark) at various concentrations were prepared by serial dilution method was used as an insulin standard. Following Each injection insulin concentrations and the supernatants of culture samples (3 µl) were spotted onto a nitrocellulose membrane. The membrane was dried at 80°C for 5 min and followed by immersing in 0.25% glutaraldehyde for 30 min. The membrane was washed three times with double distilled (DDI) water followed by immersing in blocking buffer, 5% skim milk in PBS buffer. After that, the membrane was incubated at room temperature for 1 h and washed with washing buffer, PBST. Then the membrane was incubated with a monoclonal anti-insulin antibody (Sigma Aldrich, U.S.A.) at the dilution ratio of 1:1,500 at 4°C for overnight or room temp for 2 h. Afterwards, the membrane was washed three times with PBST and incubated in a secondary goat anti-mouse IgG horseradish peroxidase-conjugate (Jackson Immuno Research Laboratories Inc., U.S.A.) at the dilution ratio of 1:1,500 at room temperature for 2 h. Subsequently, the membrane was washed and then visualized by incubation with a substrate solution (0.03% of 3, 3'-diaminobenzidine, 0.03% of H_2O_2, 0.25% of $CoCl_2$ in PBS) for 3-5 min. The immunoreactive spots from samples were compared with the insulin standards [29]. The procedure of this protocol is shown in Figure 3.3.

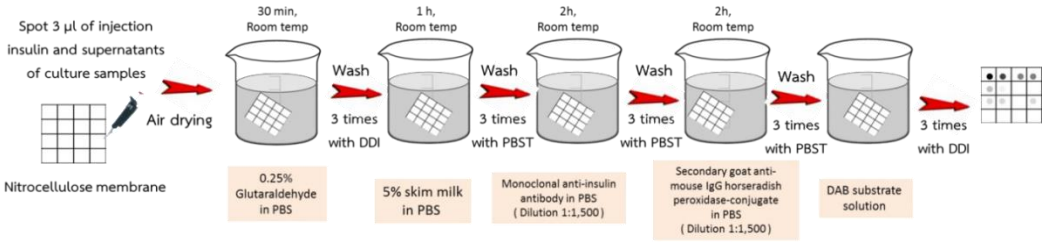


Figure 3.3: Monitoring of MIP expression level by Dot-Blot Analysis

3.6.4 Quantitative determination of MIP by indirect competitive ELISA

Insulin concentration was determined by indirect competitive Enzyme-Linked Immunosorbent Assay (ELISA). A 96 well plate was coated with 100 µl per well of bovine insulin at 5 µg.ml^{-1} and incubated at 4°C for overnight. The plate was washed three times with 300 µl per well of washing buffer (PBST) following by blocking with 300 µl per well of blocking buffer (5% skim milk in PBS buffer) and incubated at 37°C for 1 h. The plate was washed again, after that adding 50 µl per well of samples or insulin standard, and 50 µl of a primary monoclonal anti-insulin antibody at the dilution ratio of 1:10,000. After incubation at 37°C for 2 h and subsequent washing, secondary goat anti-mouse IgG conjugate with horseradish peroxidase was added at the dilution ratio of 1:10,000 (100 µl per well). The plate was incubated at 37°C for 1 h, subsequent to washing and adding of TMB substrate solution (100 µl per well). After incubation in the dark at room temperature for 15 min, the reaction was stopped by adding 100 µl per well of 1 M H_2SO_4. The plate was measured for the optical density at 450 nm and the standard curve of insulin was generated. Standard equation was created from the relationship between standard insulin concentrations and the optical density at 450 nm as shown in Figure 3.4. The standard equation was used for calculation of the insulin concentration in the samples. The procedure of this protocol is shown in Figure 3.4.

Figure 3.4: Quantitative determination of MIP by indirect competitive ELISA

3.6.5 Molecular weight cut-off by ultrafiltration membrane

The supernatants of culture sample were fractioned by ultrafiltration membrane (Amersham biosciences, Sweden) as shown in Figure 3.5. The supernatants were pump through a range of nominal molecular weight cut-off (MWCO) membranes of 30, 10, 5 kDa in order of decreasing pore size. Six fractions were collected from the membrane filtration: retentate from 30 kDa (MW > 30 kDa), retentate from 10 kDa (MW > 10 kDa), retentate from 5 kDa (MW > 5 kDa), permeate from 30 kDa (MW < 30 kDa), permeate from 10 kDa (MW < 10 kDa), permeate from 5 kDa (MW < 5 kDa). All fractions were monitored MIP by Dot-Blot Analysis. Fraction size > 5 kDa was expected to have MIP.

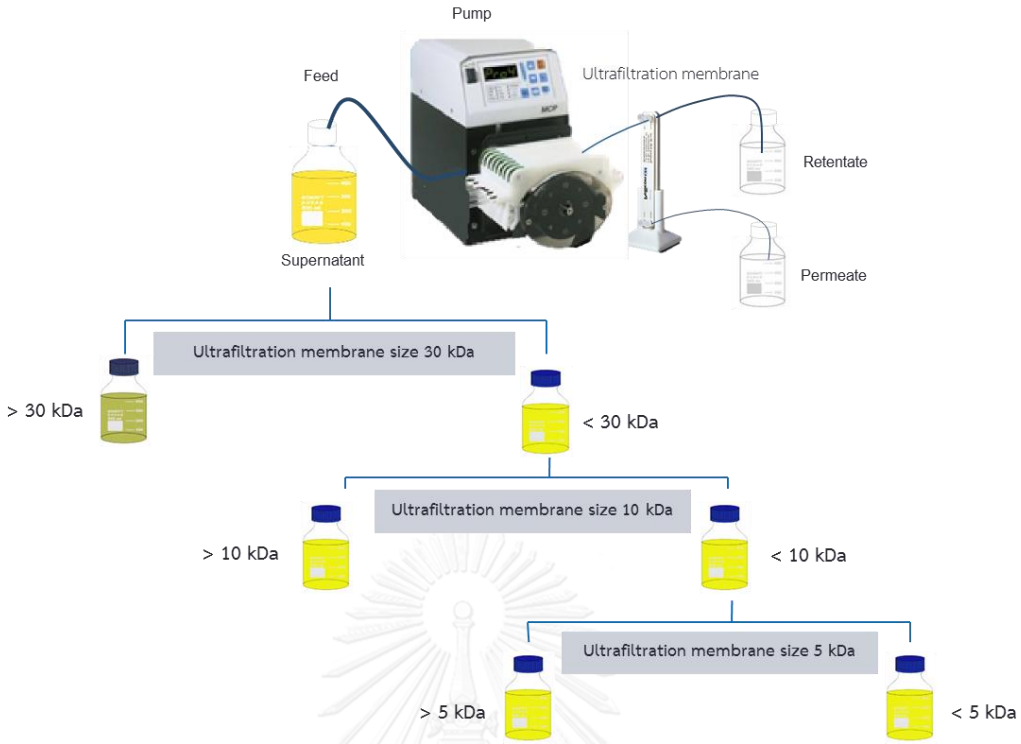


Figure 3.5: Molecular weight cut-off by ultrafiltration membrane

3.6.6 Purification of MIP by amberlite XAD-7 chromatography column

Amberlite XAD-7 resin was prepared by washing in DDI water and soaking in equilibrating buffer (5% acetic acid and 15% ethanol; Ace-EtOH) for overnight before used.

To study binding capacity and binding condition of amberlite XAD-7 resin, bovine insulin standard was prepared by dissolving in 5% acetic acid and 15% ethanol (Ace-EtOH) at three different concentrations 1, 0.5, 0.1 mg/ml. One and half milliliter of each insulin standard was applied on 1 g of XAD-7 resin and incubated with shaking on ice. After incubation, samples were carried out at 1, 6, 12, 24 h and keep on ice for further analysis. The resin was washed with equal volume of washing buffer (5% acetic acid). Adsorbed insulin was eluted from the XAD-7 resin by 45% ethanol (equal volume) and ethanol was removed from the samples by refrigerated CentriVap concentrator (LABCONCO, USA) as shown in Figure 3.6.

To purify MIP by amberlite XAD-7 chromatography column, supernatant was applied on XAD-7 resin and incubated with shaking on ice for overnight. The supernatant and resin was applied into column (36.5x0.5 cm) and purified by following the optimal binding condition of amberite XAD-7.

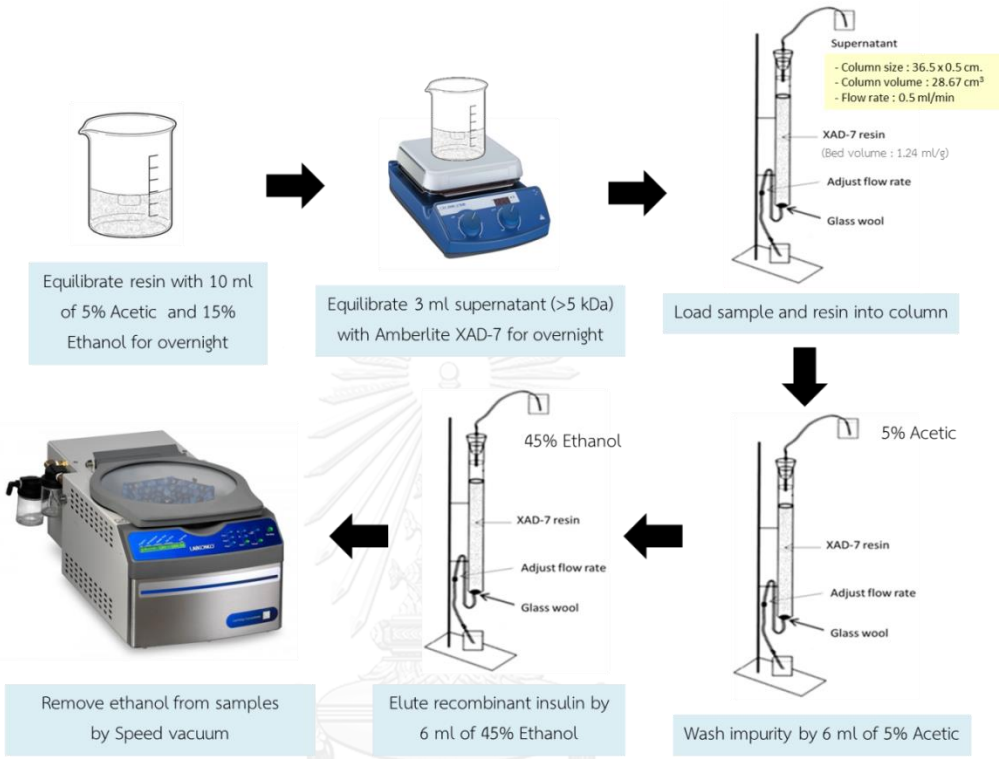


Figure 3.6: Purification of MIP by XAD-7 chromatography column

23.6.7 Purification of MIP by cation exchange chromatography column

After being filtrated supernatant with a filter membrane of 0.45 mm, five milliliter the supernatant was applied on three difference cation exchange chromatography column including; a CM-Sepharose Fast Flow cation exchange chromatography column, SP-Sepharose Fast Flow cation exchange chromatography column, SP-Sepharose XL cation exchange chromatography column [30]. Following Figure 3.7, supernatant was balanced by 50mM citric acid-citrate sodium solution containing 0.01M NaCl, pH 3.0. The MIP was eluted by the eluted solution of 50mM citric acid-citrate sodium solution containing 1.0M NaCl, pH 3.0, with a linear gradient

of the eluted solution (0.01 M–1 M NaCl). The collected eluent containing MIP was collected and kept on ice [1].

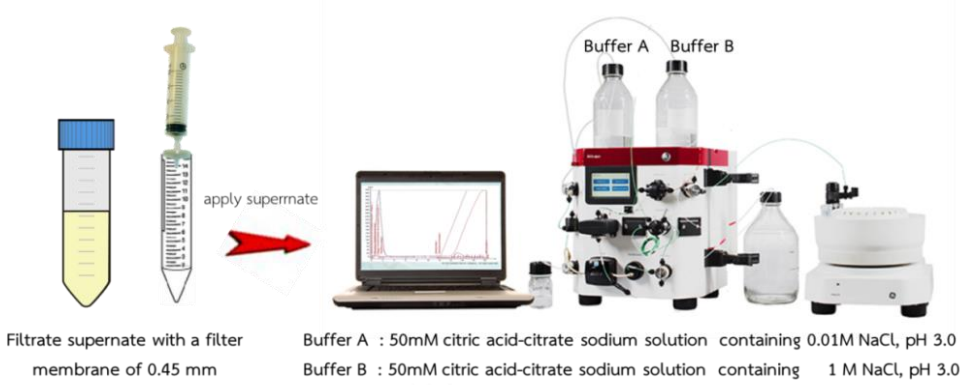


Figure 3.7: Purification of MIP by SP-Sepharose FF chromatography column

3.6.8 Purification of MIP by 10 kDa Amicon Ultra-15 Centrifugal Filter

The purified MIP was added into 10 kDa Amicon Ultra centrifugal tube and spinning in fixed-angle rotor 5,000xg at room temperature for 20 min. Flow through fraction was collected into new centrifuge tube and desalinized in PBS buffer for overnight. The purified MIP was analyzed by 15% native polyacrylamide gel electrophoresis (PAGE) run at pH 8.3 according to the method of Bollag DM [29].

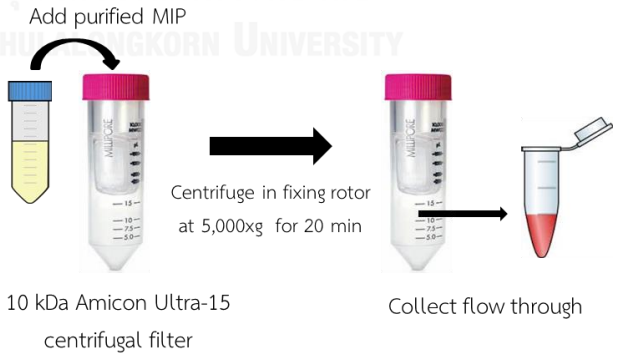


Figure 3.8: Purification of MIP by 10 kDa Amicon Ultra-15 Centrifugal Filter

3.6.9 Conversion of MIP into active form

After desalinized purified MIP in PBS buffer for overnight, immobilized TPCK Trypsin gel (0.10-0.25 mL) was washed with 3 × 500 µL of PBS buffer. Separate the gel from the buffer after each wash by centrifugation. The gel was suspended with ~0.2 mL of PBS buffer. The Immobilized TPCK Trypsin was added to the protein sample (ratio 1 mg MIP: 200 µl enzyme suspension). The reaction mixture was incubated in a rapidly shaking water bath for 2-18 hours at 37°C. The trypsin gel was separated from the digestion mixture by centrifugation at 1,000×g, 25°C for 5 min.

3.6.10 Cell line and culturing

H9c2 (2-1) rat myocardial cell line was obtained from American Type Culture Collection (*ATCC*). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) at 37°C with 5% CO_2. The growth medium was replaced once a week, and cells were passaged once in two weeks [31].

3.6.11 Biological Activity of MIP

H9c2 (2-1) rat myocardial cell line were seeded into cell culture flask at a density of $1x10^6$ cell per flask and were cultured in growth medium at 37°C with 5% CO_2 for overnight. The growth medium was replaced in Dulbecco's modified Eagle's medium without glucose (DMEM, Gibco, Carlsbad, CA, USA) containing 10% fetal calf serum (FBS, Gibco, Mulgrave, Victoria, Australia) and adjusted glucose concentration to 10 mmol/L. Culture H9c2 (2-1) rat myocardial cell line were divided into three groups: control group (No insulin treated), standard insulin group (Humalog® insulin injection) and recombinant monomeric insulin group. According to different group of insulin in culture medium, standard insulin and recombinant monomeric insulin group was further divided into five concentrations: 0.700, 0.525, 0.350, 0.250, 0.175, 0.131 µg/L. These cell line groups were cultured at 37°C with 5% CO_2 for 24 h. The culture medium was collected. H9c2 (2-1) cell line were washed in normal saline and kept at -20°C.

3.6.12 RNA extraction and measurement of *GLUT4* mRNA by RT-qPCR

Total RNA was extracted by using the E.Z.N.A.® Tissue culture RNA extraction kit (Omega Bio-Tek, Inc., U.S.A). The RNA concentration was detected spectrophotometrically by using NanoDrop 2000 Spectrophotometer. The cDNA synthesis was performed with a kit purchased from Tetro cDNA synthesis Kit (Bioline, Inc., USA). The conditions for cDNA synthesis were:

- Up to 5 µg of total RNA
- 1 µl of Primer
- 1 µl of 10mM dNTP mix
- 4 µl of 5x RT Buffer
- 1 µl of RiboSafe RNase Inhibitor
- 1 µl of Tetro Reverse Transcriptase (200u/µl)
- Adjust to 20 µl by DEPC-treated water

The mixture was gently mix by pipetting and incubated at 45°C for 30 minutes. The reaction was terminates by incubating at 85°C for 5 min.

The RT-qPCR reactions for *GLUT4* and *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase) amplification were as follows:

- 5 µl of 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus
- 1 µl of 1x Primer Forward (10 pmol/µl)
- 1 µl pf Primer Reverse (10 pmol/µl)
- 2 µl of DNA template 1-50 ng/µl
- 16 µl of H_2O PCR grade

Use CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc., U.S.A) for PCR. The thermal cycling conditions included an initial denaturation step at 95°C for 30 seconds, 45 cycles at 95°C for 5 seconds, 57°C for 20 seconds and 72°C for 20 seconds. Melting curve analysis was made at the temperatures 95°C for 0 second, 65°C for 15 seconds, and 95°C for 0 second. All experiments were repeated thrice [21, 32, 33].

Forward and reverse primer *GLUT4* and *GAPDH* were as follows:

- GLUT4 forward primer 5′ AGCCAGCCTACGCCACCATA 3′

- GLUT4 reverse primer 5′ GGACCCATAGCATCCGCAAC 3′
- GAPDH forward primer 5′ CGGTGTGAACGGATTTGGCC 3′
- GAPDH reverse primer 5’ TCATGGGGGCATCAGCGGAA 3′

3.6.13 Measurement of Glucose level in culture medium by using YSI 2700 Select Biochemistry Analyzer

Glucose level in culture medium was measured by YSI glucose analyzer. The YSI 2700 Select Biochemistry Analyzer (YSI Inc, Yellow Springs, OH) was used as the reference. The calibration accuracy of the YSI analyzer at the study site was validated by testing glucose standards, which consist of six levels of glucose concentrations as describe in an appendix A [34].

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Cultivation and expression level of MIP from *Pichia pastoris* KM71H

The expression levels of MIP in culture broth of *P. pastoris* KM71H (TP1) at 24, 48, 72 and 96 h were monitored by dot-blot analysis as shown in Figure 4.1. The intensity of each spot that occur on nitrocellulose membrane relate to the amount of insulin that can be interpreted by comparison with standard insulin on the same membrane while the negative control did not show any spot. The result showed that the expression level of MIP in culture broth was increased according to cultivation time and the highest value that was detected at 96 h was between 0.0547 to 0.0273 µg/µL when compared with standard injection insulin (Figure 4.2).

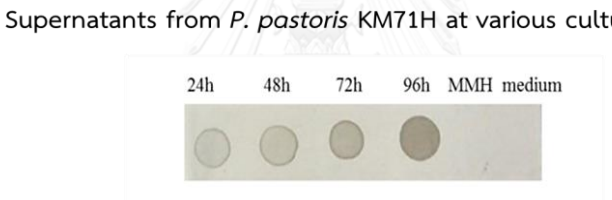


Figure 4.1: Dot-blot analysis of MIP in supernatants from recombinant yeast at various culture times, MMH medium was used as negative control.

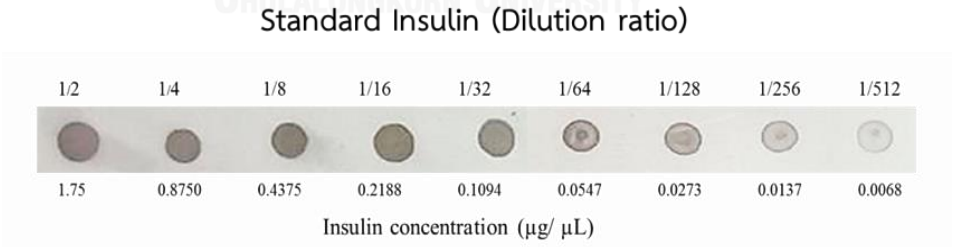


Figure 4 2: Dot-blot of injection insulin (positive control) that generated from two-fold serial dilution from 1/2 to 1/512 (1.75 to 0.0068 mg/ml)

4.2 Mass spectra of MIP by MALDI-TOF

The supernatant from culture broth of the recombinant *P. pastoris* KM71H (TP1) was subjected to Typical MALDI-TOF mass spectrometry for determining the

molecular weight as shown in Figure 4.3. The highest peak was expected to be MIP (*m/z* 5756.951), consistent with the bovine insulin standard as shown in Figure 4.4.

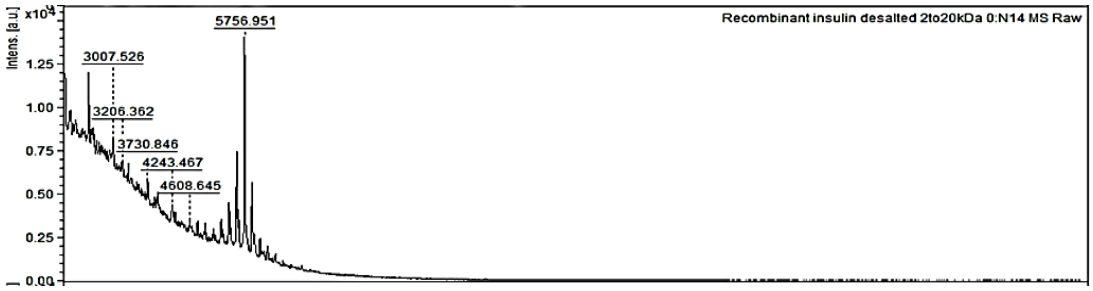


Figure 4.3: Typical MALDI-TOF mass spectra of supernatant of culture sample from *P. pastoris* KM71H (TP1). The highest peak was expected to be MIP (*m/z* 5756.951)

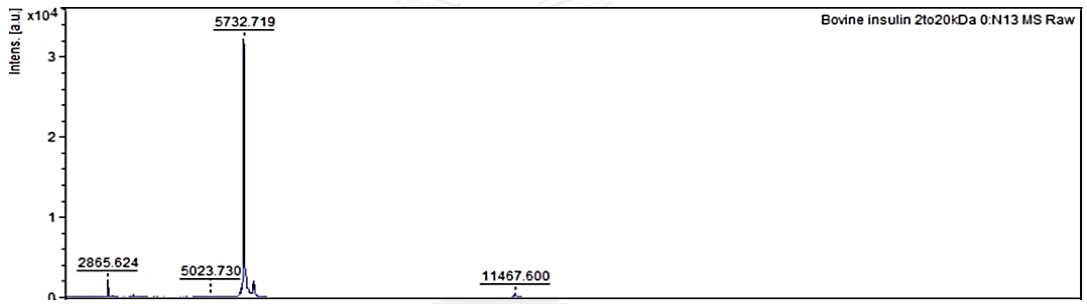


Figure 4.4: Typical MALDI-TOF mass spectra of bovine insulin (SIGMA-ALDRICH, USA). The highest peak was bovine insulin (*m/z* 5732.719).

4.3 Purification of MIP from supernatant

4.3.1 Separation MIP from supernatant by using molecular weight cut-off Ultrafiltration membrane

Ultrafiltration (UF) is a separation technique using membranes to separate extremely high molecular-weight substances, materials, organic and inorganic polymeric molecules in fluid. The primary basis for separation is molecular size. The supernatant of *P. pastoris* KM71H (TP1) was separated and fractionated through a range of nominal molecular weight cut-off (MWCO) membranes of 30, 10, 5 kDa, respectively. Dot-Blot analysis monitored MIP in each fraction. The results showed that the MIP was found mostly in fraction >30 kDa and was found next below in fraction <30 kDa, <10 kDa, >5 kDa and >10 kDa as shown in Figure 4.5. In fact, MIP

should find in fraction >5 kDa only. These unrelated results indicated that ultrafiltration technique was not suitable for separate supernatant in this research. Overall results may be due to other factors such as molecule shape and charge can also interfere in the separation.

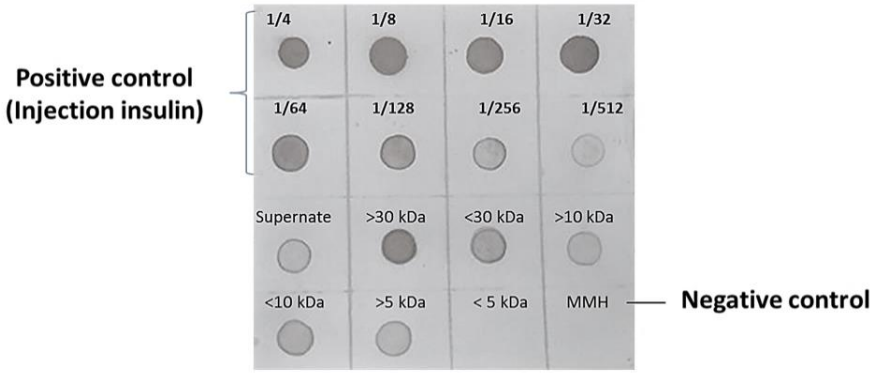


Figure 4.5: Dot-blot analysis of injection insulin dilution ratio from 1/4 to 1/512 (positive control) and separated MIP by using molecular weight cut-off Ultrafiltration membrane. MMH medium was used as negative control.

4.3.2 Purification of MIP by Amberlite XAD-7 chromatography column

Amberlite XAD-7 is a hydrophobic chromatography column. It is efficient to remove pigment and other hydrophilic materials from culture broth. The MIP concentration was quantitative determined by indirect competitive ELISA which is a high sensitivity and high specificity technique. The purified MIP was determined concentration by comparison MIP with standard insulin and calculated using standard equation (see in an appendix D).

For studying binding capacity and binding condition of XAD-7 column to recover the MIP from supernatant of *P. pastoris* KM71H (TP1), results demonstrated that binding capacity of Amberlite XAD-7 resin is minimally 1.45 mg/g resin as shown in Figure 4.6. Incubation time that required for saturated sorption was at least 12 h. In elution step, less than 50% of adsorbed insulin was able to elute from XAD-7 resin as shown in Figure 4.7. Moreover, insulin standard dissolved in Ace pH 3.0 (5% acetic acid) was adsorbed to XAD-7 resin faster and better than that dissolved in Ace-EtOH (5% acetic acid and 15% ethanol). For instance, standard insulin 1.50 mg in Ace was

adsorbed onto Amberlite XAD-7 1.45 mg and was eluted from Amberlite XAD-7 0.64 mg, be equal to 43.92 %.

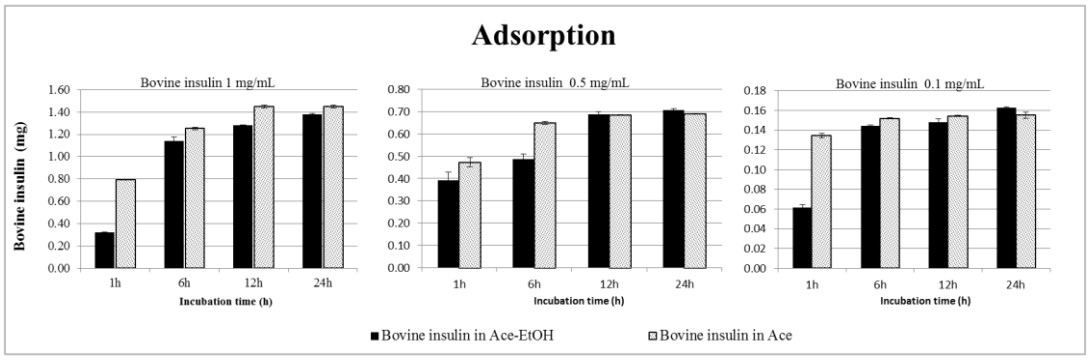


Figure 4.6: ELISA assay of adsorption of bovine insulin standard by Amberlite XAD-7 resin in differ solvents and incubation time. As shown in the left to right, 1, 0.5, 0.1 mg/ml standard insulin dissolve in Ace-EtOH (5% acetic acid and 15% ethanol) and Ace (5% acetic acid) incubate with 1 g of XAD-7 on ice for 1, 6, 12, 24 h, respectively.

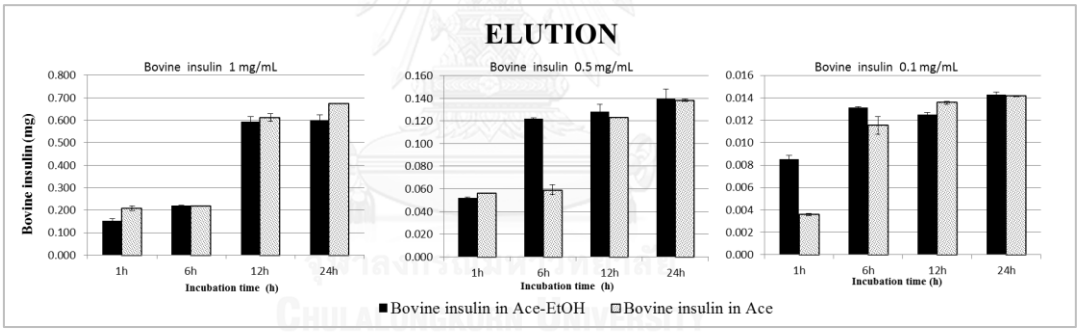


Figure 4.7: ELISA assay of elution of bovine insulin standard from Amberlite XAD-7 resin by using 45 %EtOH. As shown in the left to right, 1, 0.5, 0.1 mg/ml standard insulin from bovine pancreas dissolve in 5% acetic acid and 15% ethanol (■) and 5% acetic acid (□) incubate at 1, 6, 12, 24 h, respectively.

For studying the purification of XAD-7 column to recover the MIP from supernatant of *P. pastoris* KM71H (TP1), 1.50 ml of the supernatant (pH ~3) that containing 45.20 µg MIP was incubated with XAD-7 resin at 0°C for 24 h. The MIP was adsorbed into XAD-7 about 36.80 µg, (be equal to 81.50%). In elution step, adsorbed insulin was eluted from XAD-7 resin by 45% ethanol totally 19.40 µg, (be equal to 52.50%). When concentration of ethanol in elution solution was increased to 60%

and 70%, insulin can be eluted 3.10 and 2.60 µg, be equal to 8.4 and 6.9 %, respectively (Figure 4.8).

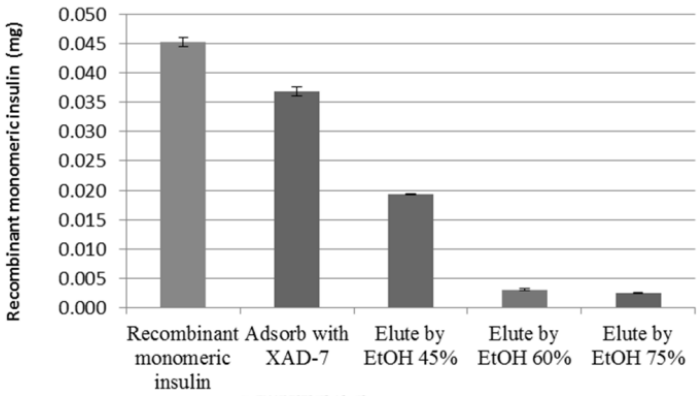


Figure 4.8: ELISA assay of direct adsorbed MIP from supernatant of culture of *P. pastoris* KM71H (TP1) with Amberlite XAD-7

When using XAD-7 column (0.5x35 cm) to recover the MIP from supernatant of *P. pastoris* KM71H (TP1), 25 ml of the supernatant was incubated with XAD-7 resin at 0°C for 24 h. Supernatant with XAD-7 resin was flowed and packed into column. After the XAD-7 resin was completely packed, the supernatant was slowly flowed out at flow rate 0.5 mL/min. Adsorbed MIP was eluted from XAD-7 resin by 45% ethanol. A specific dot-blot procedure was chosen to initial screening of the MIP in each fraction as show in Figure 4.9. The result indicated that MIP flowed out in both flow through fraction and elution fraction.

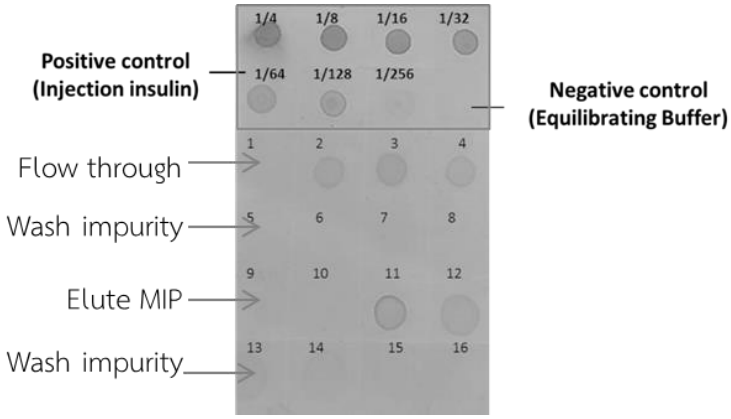


Figure 4.9: Dot-blot analysis of purified MIP by XAD-7 chromatography column

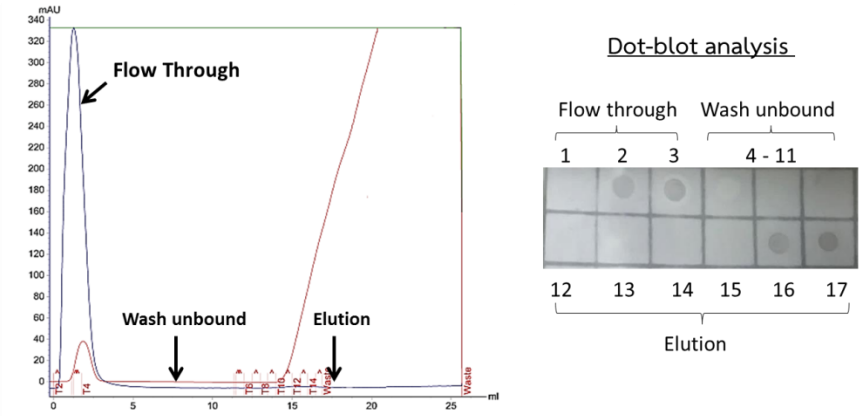
Then, ethanol was removed from the samples by refrigerated CentriVap concentrator (LABCONCO, USA). All fractions were quantitative determined MIP concentration by indirect competitive ELISA. To be doubtful, the result showed that there was no MIP in all fractions. It could assume that MIP was loose in step of removing ethanol or there was some ethanol in sample interfere ELISA detection. From these results shows some disadvantages of using Amberlite XAD-7 chromatography column to purify MIP such as low recovery yield, and ethanol interference. So, this method was not appropriated for purification MIP in this research.

4.3.3 Purification of MIP by HiTrap ion exchange chromatography

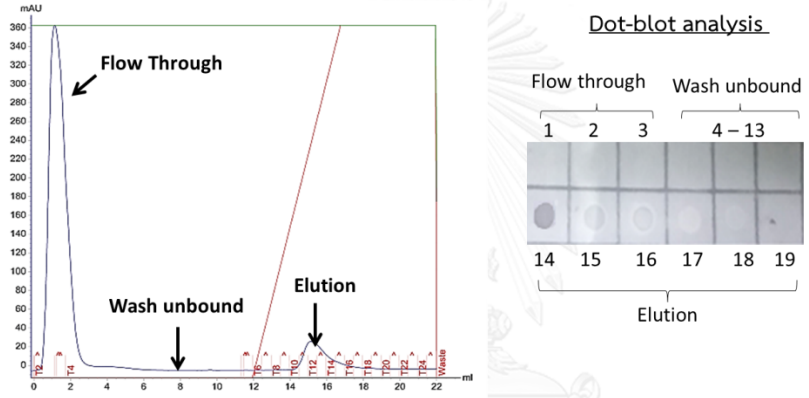
HiTrap ion exchange chromatography (IEX) consists of seven HiTrap columns, prepacked with different Sepharose Fast Flow ion exchange chromatography media. It offers a fast, simple, and convenient way to decide which ion exchanger or ion exchange ligand is best for a given application. Interestingly, SP Sepharose Fast Flow, CM Sepharose Fast Flow and Sepharose XL are cationic exchange in HiTrap IEX that can be used for separate MIP from culture broth in this research. SP Sepharose Fast Flow and CM Sepharose Fast Flow are based on a robust, 6% highly cross-linked beaded agarose matrix with good flow properties and high loading capacities. SP Sepharose XL media have long chains of dextran coupled to a robust, 6% highly crosslinked agarose matrix. The dextran chains increase the exposure of the SP charged groups, which results in higher loading capacity in some applications. SP Sepharose Fast Flow and SP Sepharose XL are strong cationic medium but CM Sepharose Fast Flow is weak cationic medium.

SP Sepharose Fast Flow, CM Sepharose Fast Flow and Sepharose XL were used to purify MIP. Three milliliter of supernatant was applied into column. Linear gradient elution (0.01 – 1 M NaCl) was applied to purify the MIP. The result showed chromatogram profile of MIP containing flow through peak and elution peak obtained in Figure. 4.10. Each fraction peaks were collected and monitored MIP by Dot blot analysis.

A. HiTrap CM sepharose FF



B. HiTrap SP sepharose FF



C. HiTrap SP sepharose XL

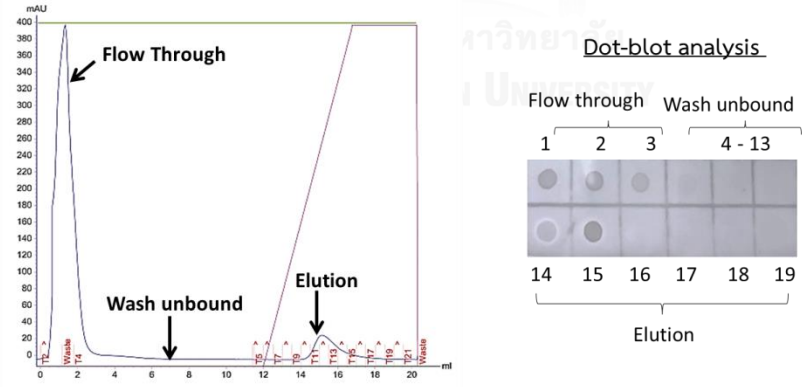


Figure 4.10: Cation-exchange chromatography and Dot-blot analysis for MIP. (A) Chromatogram profile of MIP obtained using Hitrap CM sepharose Fast Flow column chromatography. (B) Chromatogram profile of MIP obtained using Hitrap SP sepharose Fast Flow column chromatography. (C) Chromatogram profile of MIP obtained using Hitrap SP sepharose XL column chromatography.

From the results in Figure 4.10, (A) Chromatogram profile of MIP obtained using Hitrap CM sepharose Fast Flow column chromatography, a single peak was found in flow through. Some of MIP flowed out into flow through fraction and elution fraction. (B) Chromatogram profile of MIP obtained using Hitrap SP sepharose Fast Flow column chromatography. There are two peak of proteins that were detected by UV absorbance 280 nm including flow through peak and elution peak. However, the dot blot analysis of MIP was found only in Elution fraction. (C) Chromatogram profile of MIP obtained using Hitrap SP sepharose XL column chromatography. There are also two peak including flow through peak and elution peak. The dot blot analysis represented MIP in both flow through fraction and. For the best condition, MIP should find in elution fraction only.

Therefore, SP Sepharose Fast Flow column chromatography is the most optimal column to purify MIP from supernatant. SP Sepharose Fast Flow was used to purify MIP with linear gradient elution. Ten milliliter of supernatant was applied into column at a rate of 0.5 mL/min. Chromatogram profile of MIP showed two peak including flow through peak and elution peak (Figure 4.11). A single peak of elution was obtained when the ratio of eluted solution reached 50% and it was quantitative determined MIP by indirect competitive ELISA. The MIP recovery of this purification step reached 90%. The height and peak area of flow through peak and elution peak was reported in Table 4.1.

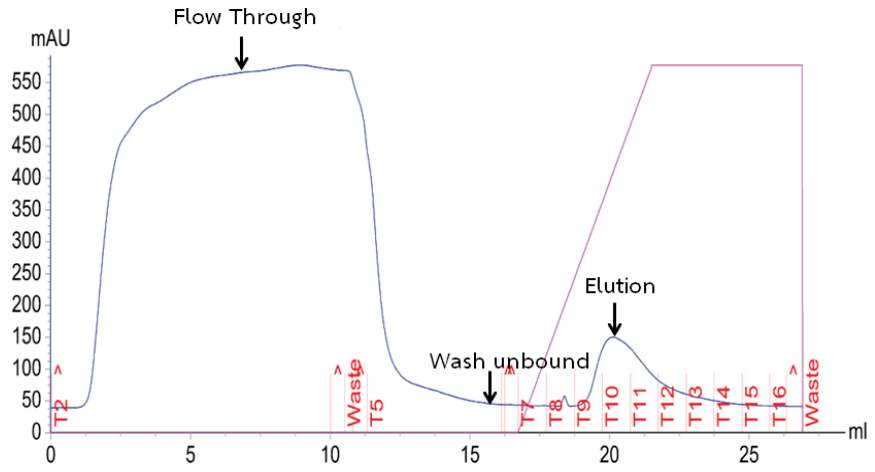


Figure 4.11: Purification of MIP on SP-Sepharose FF cation exchange chromatography column. Balanced by 50 mM citric acid-citrate sodium solution containing 0.01 M NaCl, pH 3.0, the MIP was eluted by 50 mM citric acid-citrate sodium solution containing 1.0 M NaCl, pH 3.0, with a linear gradient of the eluted solution (0–100%) at a rate of 0.5 mL/min

Table 4.1: The height and peak area of flow through peak and elution peak

Peak name	Retention (mL)	Height (mAU)	Area (ml*mAU)
Flow through peak	8.87	535.60	5033.27
Elution peak	20.11	107.90	249.49

According to Table 4.2, MIP and total protein was determined by indirect competitive ELISA and BCA assay Which the concentrated of MIP reached up about 9-folds. This purification step reached 74% recovery protein and 90% recovery MIP. However, the purity of this purification steps was less than 4%. Therefore, it should be further analyzed the impurity by Native polyacrylamide gel electrophoresis (Native-PAGE).

Table 4.2: Total Protein and MIP in flow through peak and elution peak

Sample	Total Protein (µg)	MIP (µg)	MIP : Total protein
Supernatant	8,527.27	39.63	1 : 237
Flow through	5,424.54	1.88	1 : 2,885
Elution	925.09	34.17	1 : 27

A Native polyacrylamide gel electrophoresis (Native-PAGE) stained with coomassie blue in Figure 4.12 showed protein impurity with molecular weight size near 100 kDa in elution fraction (lane 5). Presume that it is the main protein impurity that caused low purity percentage. Therefore, it needed one more step to remove impurity from the elution fraction.

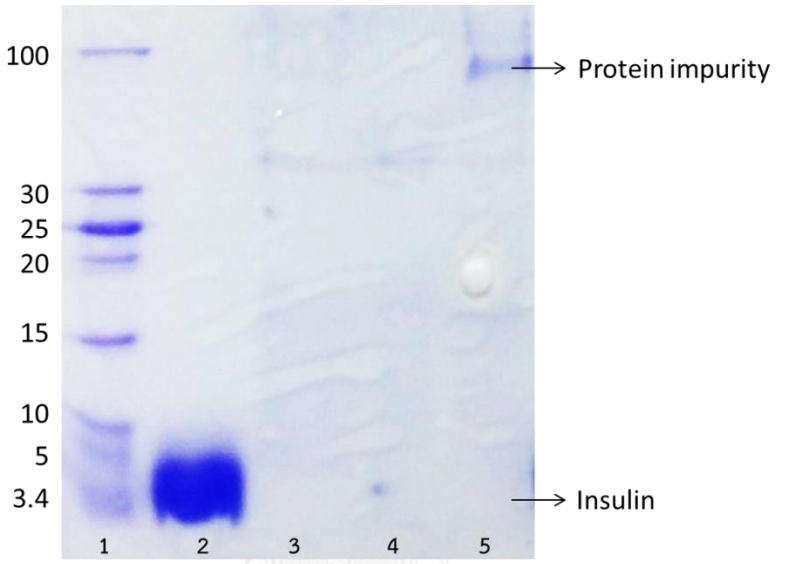


Figure 4.12: 15% Native polyacrylamide gel electrophoresis (Native-PAGE)**.** Lane 1: 5 µl of unstained protein ladder, lane 2: 5 µl of standard insulin (Injection insulin 1 mg/mL), lane 3: 20 µl of Supernatant, lane 4: 20 µl of flow through fraction from SP-sepharose Fast Flow Chromatography column, lane 5: 20 µl of Elute fraction from SP-sepharose Fast Flow Chromatography column.

4.3.4 Seperation of high molecular-weight impurity protein by 10 kDa Amicon ultra-15 centrifugal filter

Amicon Ultra centrifugal filters are ideal for protein separation and concentration. It enables the separation of proteins with a membrane nominal molecular weight limit of 10 kDa with many advantages including high retentate recovery, direct pipettor sample access eliminates processing step to recover concentrate. According to the ELISA and BCA assay, this purification step reached 93% recovery protein and 98% recovery MIP. However, the purity of MIP in this two purification steps was reach up to 27% (Table 4.1).

Table 4.3: Purification of MIP by 10 kDa Amicon Ultra-15 Centrifugal Filter

Sample	MIP (mg)	Total Protein (mg)	MIP : Total protein
MIP from SP FF column	34.17	925.10	1 : 27
Retentate (>10 kDa)	5.86	746.59	1 : 127
Permiate (<10 kDa)	27.95	102.16	1 : 3.6

Sample of separated MIP by 10 kDa Amicon Ultra-15 Centrifugal Filter was analyzed in a Native polyacrylamide gel electrophoresis (Native-PAGE) stained with coomassie blue (Figure 4.13). In the sample of separated MIP (lane 5), MIP band showed correct size while compared with standard insulin band. There was no another band appeared in this lane. Suggest that the main protein impurity was removed. On the other hand, previous reports showed that the purity this band was 27%. That was not related with Native-Page result. Overall unrelated results may be due to two determination method; indirect competitive ELISA and BCA assay detected sample by different position of protein.

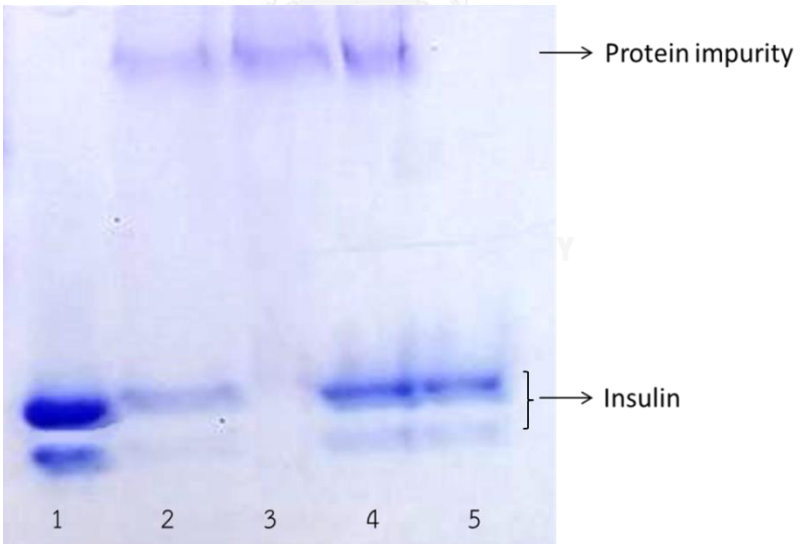


Figure 4.13: 15% Native polyacrylamide gel electrophoresis (Native-PAGE). Lanes1: 10 µl of standard insulin (Injection insulin 1mg/mL), lane 2: 20 µl of supernatant, lane 3: 20 µl of flow through fraction from SP-sepharose Fast Flow Chromatography column, lane 4: 20 µl of Elute fraction from SP-sepharose Fast Flow Chromatography column, lane 5: 20 µl of permeate fraction from 10 kDa Amicon ultra-15 centrifugal filter.

4.4 Biological activity of MIP

H9c2 (2-1) rat myocardial cell line has been used for biological activity test of MIP in this research. Cell line was seeded into cell culture flask at a density of $1x10^6$ cells per flask and cultured in DMEM medium with 10 mM (1.8 g/L) D-glucose which approximates pre-diabetic levels. Cell line was cultured at 37°C with 5% CO_2 for 24 h. The shape of H9c2 (2-1) cell line in all three groups (no insulin treated, treated with standard insulin, treated with MIP) were spindle-shaped flat cells. They were attached to the bottom cell culture flask as shown from light microscopic view in Figure 4.14.

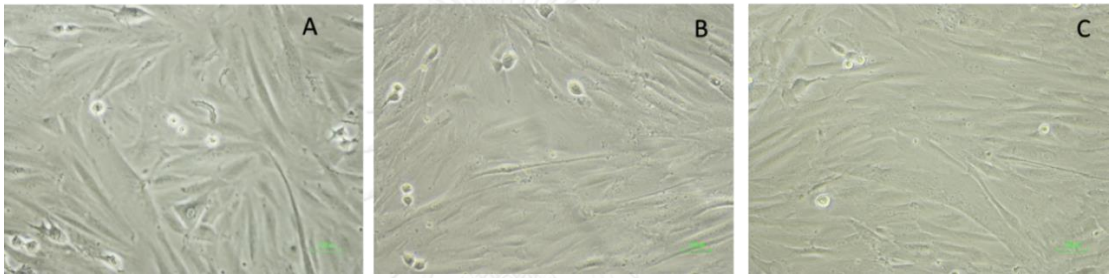


Figure 4.14: Light microscopic view of H9c2 (2-1) in DMEM medium with 10 mmol/L D-Glucose. A: no insulin treated, B: treated with standard insulin 0.700 µg/L (Humalog® insulin injection), B: treated with MIP 0.700 µg/L.

4.4.1 Measurement of glucose level in culture medium by using YSI 2700 Select Biochemistry analyzer and relative quantification of *GLUT4* mRNA by real-time PCR

YSI analyzer was used for measure glucose in this research. At the present, it was used extensively for glucose measurement in clinical, industrial, and research applications especially in diabetes research. Glucose is very soluble and chemically stable in media. Concentration of glucose in this study was approached 10 mmol/L (1.8 g/L) which was pre-diabetic levels. After further cell line cultivation with insulin for 24 h, DMEM medium was collected for measurement of glucose level. It was generally assumed that insulin would increase glucose uptake into tissues. On the other hand, glucose concentration in medium should be decreased.

The measurement of glucose level in culture medium was reported in Table 4.4 and Figure 4.15. Concentration of glucose in DMEM medium approached 1.74 g/L. The concentration of glucose in medium which treated with standard insulin and MIP were became lower than that of no insulin treated. The results indicated that insulin could stimulate the increasing of glucose uptake into tissues.

Table 4.4: Glucose concentration in DMEM medium and culture medium of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.131 to 0.700 μg/L) for 24 h by using no insulin treated culture medium as a control

Sample group	Glucose concentration (g/L)
DMEM medium with 10mmol/L D-Glucose	1.74±0.015
No insulin treated	1.58±0.006
Standard insulin 0.700 μg/L	1.24±0.000
Standard insulin 0.525 μg/L	1.32±0.006
Standard insulin 0.350 μg/L	1.41±0.006
Standard insulin 0.250 μg/L	1.43±0.017
Standard insulin 0.175 μg/L	1.47±0.000
Standard insulin 0.131 μg/L	1.54±0.032
MIP 0.700 μg/L	1.46±0.015
MIP 0.525 μg/L	1.51±0.006
MIP 0.350 μg/L	1.54±0.000
MIP 0.250 μg/L	1.56±0.010
MIP 0.175 μg/L	1.57±0.010
MIP 0.131 μg/L	1.58±0.006

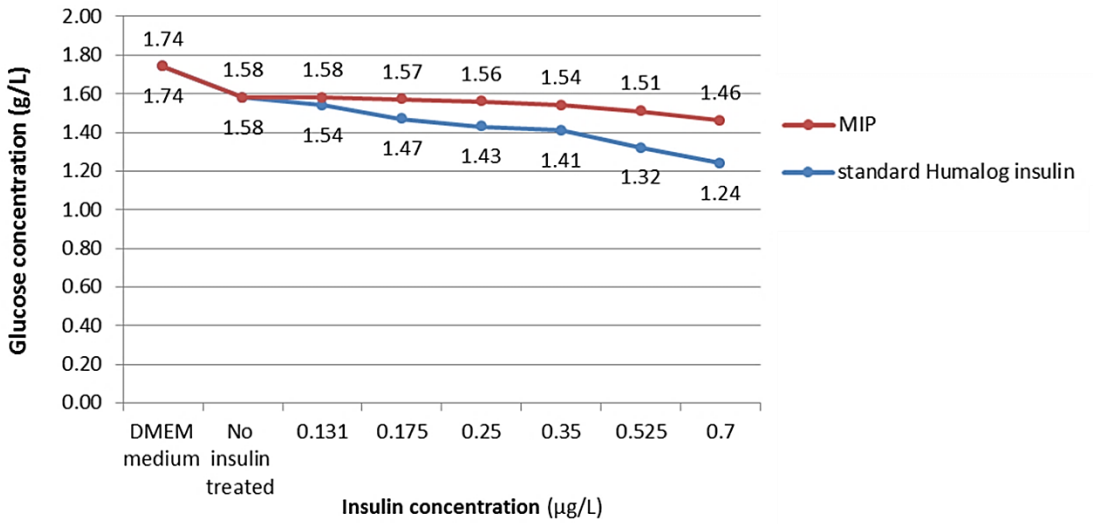


Figure 4.15: Glucose concentration in culture medium of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.131 to 0.700 µg/L) for 24 h by using no insulin treated culture medium as a control

Glucose transporter type 4 (GLUT4) is a protein encoded by the *GLUT4* gene. It is the main glucose transporter activated by insulin in skeletal muscle cells and adipocytes. In human and animals, both insulin and exercise acutely stimulate GLUT4 recruitment from GLUT4 vesicles to the surface membrane which contributes to glucose transport. So, the change of glucose transporter 4 (*GLUT4*) expression could influence glucose uptake in the myocardial cells.

After the measurement of glucose level in culture medium, cell culture which were treated with standard insulin and MIP at concentration 0.700, 0.350, 0.175 µg/L were extracted RNA to study the *GLUT4* mRNA expression. RNA concentration of each sample culture was shown in Table 4.5. Afterwards, the cDNA was synthesized and quantified gene expression by real-time PCR.

Table 4.5: RNA concentration of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.131 to 0.700 µg/L) for 24 h using no insulin treated as a control

Sample	RNA concentration (ng/µL)	A260	A280	A260/280	A260/320
No insulin	144.8	3.621	1.704	2.13	2.30
Std. insulin 0.700 µg/L	305.1	7.628	3.642	2.09	2.17
Std. insulin 0.350 µg/L	606.8	15.170	7.365	2.06	2.06
Std. insulin 0.175 µg/L	586.1	14.651	7.070	2.07	2.06
MIP 0.700 µg/L	593.6	14.841	7.213	2.06	2.11
MIP 0.350 µg/L	578.2	14.454	7.092	2.04	1.98
MIP 0.175 µg/L	456.8	11.421	5.692	2.17	2.17

* Std. insulin = standard insulin

Real-time PCR is a powerful tool to quantify gene expression. The quantitative endpoint for real-time PCR is the threshold cycle (C_T or C_q). The C_q is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold (see in an appendix E). This research reported real time PCR data as a mean C_q and the comparision between each gene expression and regulation threshold of H9c2 (2-1) cell *GLUT4* and *GAPDH* mRNA expressions (Table 4.6, Figure 4.15). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was considered as a housekeeping gene because this gene was often stably and constitutively expressed at high levels in most tissues and cells.

Table 4.6: *GLUT4* and *GAPDH* mRNA expressions of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.131 to 0.700 µg/L) for 24 h using no insulin treated as a control

Target gene	Sample group	Mean C_q	Normalized Expression	Relative Normalized Expression	Compared to Regulation Threshold
GAPDH	No insulin treated	22.30	-	-	No change
GAPDH	Std. insulin 0.700 µg/L	23.58	-	-	No change
GAPDH	Std. insulin 0.350 µg/L	23.07	-	-	No change
GAPDH	Std. insulin 0.175 µg/L	22.43	-	-	No change
GAPDH	MIP 0.700 µg/L	21.41	-	-	No change
GAPDH	MIP 0.350 µg/L	21.57	-	-	No change
GAPDH	MIP 0.175 µg/L	18.71	-	-	No change
GLUT4	No insulin treated	32.39	0.00092	1.00000	No change
GLUT4	Std. insulin 0.700 µg/L	33.26	0.00122	1.32665	No change
GLUT4	Std. insulin 0.350 µg/L	30.76	0.00485	5.28912	Up regulated
GLUT4	Std. insulin 0.175 µg/L	32.82	0.00075	0.81213	No change
GLUT4	MIP 0.700 µg/L	32.61	0.00043	0.46484	No change
GLUT4	MIP 0.350 µg/L	32.50	0.00051	0.55949	No change
GLUT4	MIP 0.175 µg/L	30.00	0.00040	0.43373	No change

*C_q = the threshold cycle, Std. insulin = standard insulin

According to the expression of *GLUT4* mRNA data, *GLUT4* gene is not expressed in mostly samples. Nevertheless, *GLUT4* gene expression up-regulation on relatively sample group of standard insulin at concentration 0.350 µg/L. From *GLUT4* mRNA expression and glucose level in culture medium data could suppose that doze of insulin was too low or time course of gene expression was not be appropriate. So that doze of insulin for treated cell line should be scale up or should study time series gene expression data.

In next experiment, concentration of standard insulin and MIP was raised in range 0.70 µg/L to 1.40, 2.80, 5.60 and 11.20 µg/L. The measurement of glucose level in culture medium was reported in Table 4.7 and Figure 4.16. The concentration of

glucose in medium of H9c2(2-1) which treated with standard insulin and MIP were became lower when compare with glucose concentration in medium which no insulin treated.

Table 4.7: Glucose concentration in DMEM medium and culture medium of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.70 to 11.20 μg/L) for 24 h by using no insulin treated culture medium as a control

Sample group	Glucose concentration (g/L)
DMEM medium with 10mmol/L D-Glucose	1.71±0.01
No insulin treated	1.63±0.01
Standard insulin 11.20 μg/L	1.06±0.01
Standard insulin 5.60 μg/L	1.11±0.00
Standard insulin 2.80 μg/L	1.26±0.00
Standard insulin 1.40 μg/L	1.41±0.01
Standard insulin 0.70 μg/L	1.50±0.00
MIP 11.20 μg/L	1.30±0.00
MIP 5.60 μg/L	1.42±0.04
MIP 2.80 μg/L	1.51±0.01
MIP 1.40 μg/L	1.57±0.01
MIP 0.70 μg/L	1.59±0.01

*Data is shown in mean ± standard deviation.

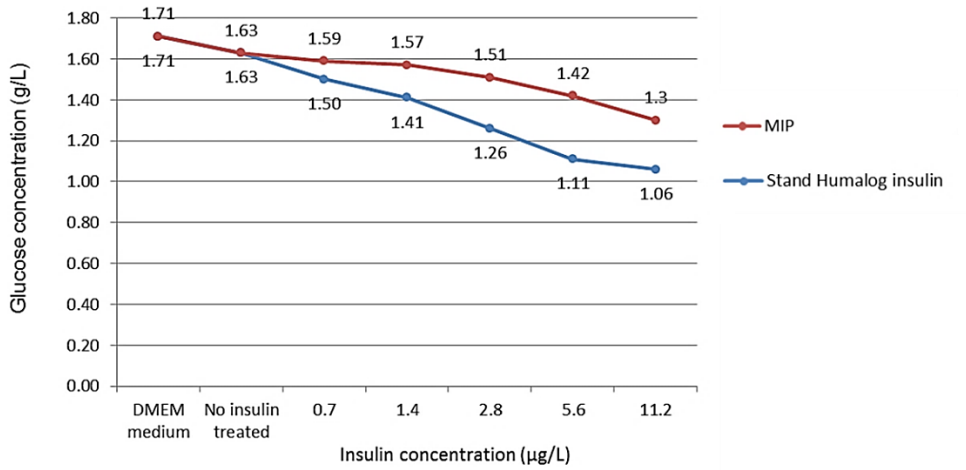


Figure 4.16: Glucose concentration in DMEM medium and culture medium of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.70 to 11.20 µg/L) for 24 h by using no insulin treated culture medium as a control

In accordance with the measurement of glucose level in culture medium results, range of glucose concentration in sample groups of MIP at concentration 11.2, 5.60, 2.80 µg/L were related with that of standard insulin at concentration 2.80, 1.40, 0.70 µg/. Cell cultures which were treated with these six groups were collected to study the *GLUT4* mRNA expression. RNA concentration of each sample culture was shown in Table 4.8.

Table 4.8: RNA concentration of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.70 to 11.20 µg/L) for 24 h using no insulin treated as a control

Sample	RNA concentration (ng/µL)	A260	A280	A260/280	A260/320
No insulin	251.1	6.277	2.919	2.15	2.06
Std. insulin 2.80 µg/L	232.2	5.804	2.831	2.05	2.07
Std. insulin 1.40 µg/L	262.2	6.554	3.185	2.06	1.82
Std. insulin 0.70 µg/L	252.5	6.313	3.068	2.06	1.93
MIP 11.20 µg/L	317.7	7.943	3.828	2.06	1.72
MIP 5.60 µg/L	254.7	6.367	3.103	2.05	2.16
MIP 2.80 µg/L	264.6	6.616	3.210	2.07	2.05

* Std. insulin = standard insulin

After that, cDNA of each sample was synthesized and quantified gene expression by real-time PCR as showed in Table 4.9.

Table 4.9: *GLUT4* and *GAPDH* mRNA expressions of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.70 to 11.20 µg/L) for 24 h using no insulin treated as a control

Target	Sample	Mean C_q	Normalized Expression	Relative Normalized Expression	Compared to Regulation Threshold
GAPDH	No insulin	24.97	-	-	No change
GAPDH	Std. insulin 2.80 µg/L	20.95	-	-	No change
GAPDH	Std. insulin 1.40 µg/L	23.72	-	-	No change
GAPDH	Std. insulin 0.70 µg/L	25.00	-	-	No change
GAPDH	MIP 11.20 µg/L	21.08	-	-	No change
GAPDH	MIP 5.60 µg/L	21.66	-	-	No change
GAPDH	MIP 2.80 µg/L	N/A	-	-	No change
GLUT4	No insulin	38.81	0.00007	1.00	No change
GLUT4	Std. insulin 2.80 µg/L	21.63	0.62187	9175.36	Up regulated
GLUT4	Std. insulin 1.40 µg/L	35.62	0.00026	3.85	No change
GLUT4	Std. insulin 0.70 µg/L	35.82	0.00055	8.11	Up regulated
GLUT4	MIP 11.20 µg/L	21.22	0.90306	13324.15	Up regulated
GLUT4	MIP 5.60 µg/L	37.18	0.00002	0.31	No change
GLUT4	MIP 2.80 µg/L	37.45	-	-	No change

* Cq = the threshold cycle, Std. insulin = standard insulin

According to the decreasing of glucose level data in Table 4.7 and *GLUT4* mRNA expression in Table 4.9, MIP can stimulate glucose uptake by *GLUT4* gene expression. So, time series of *GLUT4* gene expression should be studied.

Due to MIP and standard Humalog® insulin is rapid acting insulin. Humalog has been reported in clinical trials that its action times was often quoted as 3-5 hour

after injection. Therefore, the time course of gene expression was studied in this experiment.

H9c2 (2-1) cell line was cultured repeatedly in same concentration of standard insulin and MIP with previous experiment. Nevertheless, the biological activity was studied at 3, 6, 12 h respectively. The measurement of glucose level in culture medium was reported in Table 4.10 and Figure 4.15. The concentration of glucose in medium which treated with standard insulin and MIP significantly reduced within 3 h after treated. So, cell line after 3 h treated was selected to quantify *GLUT4* mRNA expression

Table 4.10: Glucose concentration in DMEM medium and culture medium of H9c2 (2-1) cell line treated with standard insulin (at 0.70 to 2.80 μg/L) and MIP (at 2.80 to 11.20 μg/L) for 3, 6, 12 h by using no insulin treated culture medium as a control

Incubation Time / Sample	Glucose concentration (g/L)		
	3h	6h	12h
DMEM	1.78±0.01	-	-
No insulin	1.71±0.01	1.68±0.07	1.60±0.01
Std. insulin 2.80 μg/L	1.65±0.01	1.62±0.00	1.56±0.00
Std. insulin 1.40 μg/L	1.68±0.00	1.65±0.00	1.59±0.00
Std. insulin 0.70 μg/L	1.71±0.01	1.65±0.00	1.60±0.01
MIP 11.20 μg/L	1.52±0.01	1.54±0.00	1.48±0.01
MIP 5.60 μg/L	1.62±0.01	1.62±0.01	1.58±0.01
MIP 2.80 μg/L	1.64±0.00	1.68±0.01	1.60±0.00

*Data is show in mean ± standard deviation.

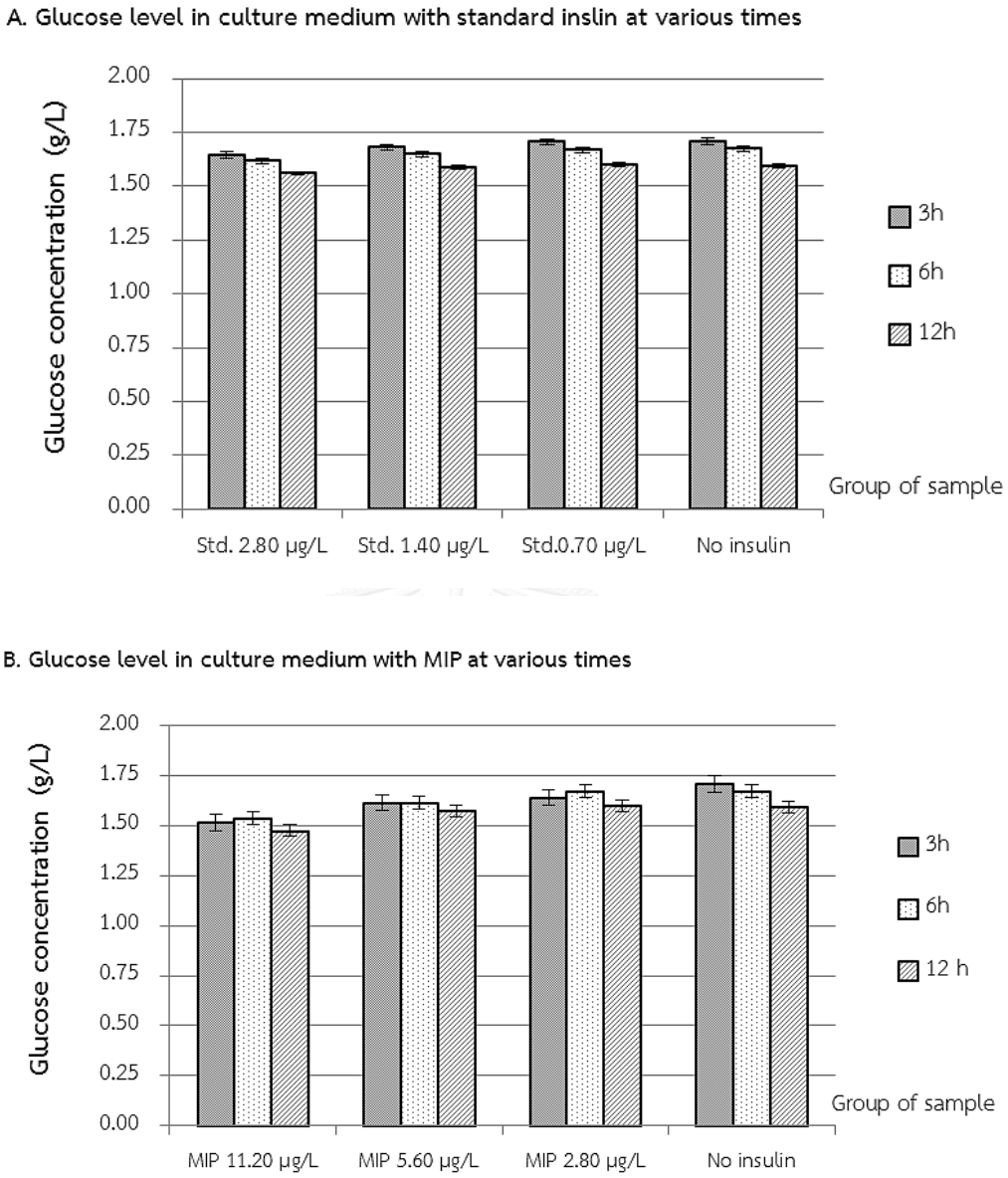


Figure 4.17Glucose concentration in culture medium of H9c2 (2-1) cell line treated with standard insulin (at 0.70 to 2.80 µg/L) (A) and MIP (at 2.80 to 11.20 µg/L) (B) for 3, 6, 12 h by using no insulin treated culture medium as a control

All cell cultures in 3h culture time were collected to study *GLUT4* mRNA expression. RNA concentration of each sample culture was shown in Table 4.11

Table 4.11: RNA concentration of H9c2 (2-1) cell line treated with standard (at 0.70 to 2.80 μg/L) and MIP (at 2.80 to 11.20 μg/L) for 3 h by using no insulin treated as a control

Sample	RNA concentration (ng/μL)	A260	A280	A260/280	A260/320
No insulin	420.8	10.521	1.704	2.06	2.76
Std. insulin 2.80 μg/L	385.1	9.628	3.642	2.11	2.67
Std. insulin 1.40 μg/L	658.1	16.453	7.365	2.16	2.57
Std. insulin 0.70 μg/L	585.8	14.645	7.070	2.13	2.11
MIP 11.20 μg/L	673.7	16.843	7.213	2.08	2.06
MIP 5.60 μg/L	658.2	16.454	7.092	2.05	2.23
MIP 2.80 μg/L	696.8	17.421	5.692	2.19	2.15

* Std. insulin = standard insulin

After that, cDNA of each sample was synthesized and quantified gene expression by real-time PCR as showed in Table 4.12.

Table 4.12: *GLUT4* and *GAPDH* mRNA expressions of H9c2 (2-1) cell line treated with standard insulin (at 0.70 to 2.80 µg/L) and MIP (at 2.80 to 11.20 µg/L) for 3 h by using no insulin treated as a control

Target	Sample	Mean Cq	Normalized Expression	Relative Normalized Expression	Compared to Regulation Threshold
GAPDH	No insulin treated	19.96	-	-	No change
GAPDH	Std. insulin 2.80 µg/L	20.44	-	-	No change
GAPDH	Std. insulin 1.40 µg/L	18.96	-	-	No change
GAPDH	Std. insulin 0.70 µg/L	20.49	-	-	No change
GAPDH	MIP 11.20 µg/L	19.81	-	-	No change
GAPDH	MIP 5.60 µg/L	20.08	-	-	No change
GAPDH	MIP 2.80 µg/L	20.60	-	-	No change
GLUT4	No insulin treated	30.52	0.0007	1.0000	No change
GLUT4	Std. insulin 2.80 µg/L	19.75	1.6120	2433.6031	Up regulated
GLUT4	Std. insulin 1.40 µg/L	30.56	0.0003	0.4853	No change
GLUT4	Std. insulin 0.70 µg/L	30.32	0.0011	1.6571	No change
GLUT4	MIP 11.20 µg/L	20.31	0.7049	1064.2086	Up regulated
GLUT4	MIP 5.60 µg/L	29.47	0.0015	2.2456	No change
GLUT4	MIP 2.80 µg/L	30.45	0.0011	1.6390	No change

* Cq = the threshold cycle, Std. insulin = standard insulin

According to time course of *GLUT4* gene expression by MIP and standard Humalog insulin in this experiment, *GLUT4* expression was induced at 3 h after treated with MIP at concentration 11.20 µg/L like standard Humalog insulin at concentration 2.80 µg/L. Another cell line which was treated with MIP and standard Humalog insulin at lower concentration did not show *GLUT4* expression at 3 h.

CHAPTER V
CONCLUSION AND SUGGESTION

In this work, we reported the expression of recombinant monomeric insulin production (MIP) in *P. pastoris* KM71H (TP1), two steps of the purification of MIP and biological activity of MIP. Expression level of MIP in shake flask showed that the production of the MIP was 16 mg/L. Mass spectrometry assay confirmed that the molecular mass of MIP is 5796.9 Da. The MIP was purified from culture broth by two purification steps. In first purification step, supernatant was loaded onto a SP Sepharose Fast Flow chromatography column resulted in a single elution peak. Nevertheless, a native-PAGE showed an impurity protein with molecular weight size near 100 kDa in elution fraction. So, 10 kDa Amicon Ultra-15 Centrifugal Filter was used as a secondary purification step to remove an impurity protein. According to Elisa assay and BCA assay, the purity of two purification steps was 27%, however the result from a Native-PAGE showed only single band of MIP. Suggest that the main impurity protein was removed. Unrelated results may be due to two determination method; indirect competitive ELISA and BCA assay detected sample by different position of protein. Moreover, two determination methods detected in different concentration range; indirect competitive ELISA detected in rage of insulin concentration 0-10 µg/mL and BCA assay detected in range of total protein concentration 0-250 µg/mL. After that, MIP was hydrolyzed to active form by immobilized TPCK – trypsin.

With the aim to demonstrate effect of purified MIP on glucose uptake and glucose transporter 4 gene expression, the purified MIP was treated in H9c2 (2-1) cell line. After further cell line cultivation with insulin, DMEM medium were collected for measurement of glucose level. The result was generally assumed that MIP could increase glucose uptake into tissues. The increasing of glucose uptake related directly with MIP dose similar to Humalog® insulin used as a positive control. Moreover, the results demonstrate that MIP at hightest concentration in this study (11.20 µg/L) up regulates the expression of *GLUT4* gene at 3 h.

The achievement of insulin in stimulating *GLUT4* expression in L6 Myotubes in early work, high glucose and insulin (25 mmol/L glucose and 763 µg/L insulin) resulted in a 40% increase in basal glucose uptake accompanied at 24 h [35].

Moreover, another previous study also found that insulin levels and glucose level also affected *GLUT4* expression in H9c2 myocardial cells: on the same glucose concentration condition, higher insulin level could increase *GLUT4* expression. And insulin at concentration 0.260 µg/L (7.6 mU/L) could up-regulate *GLUT4* expression. In the same insulin culturing condition: the *GLUT4* mRNA expression in 10 mmol/L glucose was higher on the first day, but lower on the second and the third day [21].

As time-action profile of MIP may vary in different types of insulin or at different concentrations. Furthermore, there are mechanisms that selectively regulate translation or degradation of GLUT4 protein or mRNA was unknown. So, time course of change *GLUT4* expression could not describe clearly in this research

APPENDIX

APPENDIX A

Culture Media

1. Yeast Peptone Dextrose (YPD) agar medium

- 1% Yeast extract
- 2% Peptone
- 2% Dextrose (Glucose)
- 2% Agar (Solid medium)

Sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. In case of YPD with Zeocin™, add Zeocin™ to a final concentration of 100 $\mu g.mL^{-1}$ and store at +4°C in the dark.

2. Yeast Peptone Glycerol (YPG) medium

- 1% Yeast extract
- 2% Peptone
- 1% Glycerol

Sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. Store at +4°C

3. Minimal Methanol Histidine (MMH) medium

- 1.34% YNB (Yeast nitrogen base w/o amino acid w/ ammonium sulfate) *
- 4×10^{-5}% Biotin *
- 0.004% Histidine *
- 0.5% Methanol

* Biotin, Histidine and YNB were dissolved in sterilized double distilled water and sterilized by filtration (pore size 0.22µm, mixed cellulose esters membrane). Store at +4°C.

Stock solution preparation

- 10×YNB; 13.4% YNB w/o amino acid w/ ammonium sulfate, 100 mL
  - YNB W/o amino acid W ammonium sulfate powder 13.4 g
  - Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 µm) and stored at +4°C.

- 500×Biotin; 0.02% Biotin, 50 mL
  - Biotin powder 10 mg
  - Dissolved with sterilized double distilled water and adjust the volume to 50 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 µm) and stored at +4°C.
- 100×Histidine; 0.4% Histidine, 100 mL
  - Histidine powder 0.4 g
  - Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (mixed cellulose esters membrane, pore size 0.22 µm) and stored at +4°C.

- Compositions of MMH medium from stock solution above, for 1 L
  - 10×YNB 100 mL
  - 500×Biotin 2 mL
  - 100×Histidine 10 mL
  - Absolute methanol 5 mL
  - Sterilized double distilled water 883 mL (Autoclaved)

  Aseptically mix the solutions above in a biohazard cabinet.

4. Dulbecco's Modified Eagle's Medium (DMEM) with 10 mmol/L Glucose

(Dulbecco's Modified Eagle's Medium, ATCC Catalog No. 30-2002)

- DMEM 89.9 mL
- Glucose 1 mol/L 100 µL
- Fetal bovine serum 10 mL

APPENDIX B

Chemical Solutions Preparation

1. 50mM citric acid-citrate sodium solution containing 0.01M NaCl, pH 3.0

- Citric acid, anhydrous 8.217 g
- Sodium citrate, dehydrate 2.132 g
- Sodium chloride 0.5844 g
- Double distilled water to 1 L

2. 50mM citric acid-citrate sodium solution containing 1M NaCl, pH 3.0

- Citric acid, anhydrous 8.217 g
- Sodium citrate, dehydrate 2.132 g
- Sodium chloride 58.44 g
- Double distilled water to 1 L

3. 0.01 M Phosphate Buffer Saline (PBS), pH 7.4

- 200 mM Phosphate buffer, pH 7.4 1 L
- Sodium chloride (NaCl) 175.2 g
- Double distilled water 18 L

Stock solution preparation

- Stock solutions, 200 mM each for 1 L
  - Stock A: $NaH_2PO_4.H_2O$ (MW = 137.99 g.mole^{-1}) 27.6 g
  - Stock B: $Na_2HPO_4.12H_2O$ (MW = 358.135 g.mole^{-1}) 71.63 g
  - Each $NaH_2PO_4.H_2O$ and $Na_2HPO_4.12H_2O$ powder was separately dissolved in double distilled water and adjusts the volume to 1 L using volumetric flask. Stored at +4°C.
- 200 mM Sodium phosphate buffer (pH 7.4), 1 L
  - Stock A (774 mL) and B (226 mL) were mixed together and adjust the pH value to 7.4 by titration with 5 M HCl. Store at room temperature.

4. 0.25% Glutaraldehyde (Fixing solution for dot-blot analysis)

- Glutaraldehyde 50% (w/v) in water	0.5	mL
- Double distilled water	99.5	mL

5. 0.05% PBS-Tween 20 or PBST (Washing buffer for dot-blot analysis and ELISA)

- Tween 20	0.5	mL
- 0.01 M Phosphate buffer saline (PBS), pH 7.4	1000	mL

6. DAB solution substrate (Developing solution for dot-blot analysis)

- PBS	20	mL
- DAB	6	mg
- 30 % H_2O_2	20	µL
- 1% $COCl_2$	50	µL

7. 200 mM Potassium citrate buffer for ELISA, pH 4.0

- Stock A: Citric acid monohydrate (MW= 210.14 g.mole^{-1})	10.5	g
- Stock B: Potassium citrate (MW= 324.41 g.mole^{-1})	16.22	g

Each citric acid and potassium citrate powder was dissolved in double distilled water and adjusts the volume to 250 mL using volumetric flask. Stock A (200 mM citric acid) was titrated with stock B (200mM potassium citrate) until the pH value equal to 4.0. The solution was stored at +4°C in the dark.

8. 5% Skim milk in PBS buffer (Blocking buffer for dot-blot analysis and ELISA)

- Skim milk powder	5	g
- 0.01 M Phosphate buffer saline (PBS), pH 7.4	100	mL

9. TMB substrate solution (Developing solution for ELISA)

- TMB	2.5	mg
- DMSO	250	µL
- 200 mM Potassium citrate buffer	10	mL
- 30% H_2O_2	3.5	µL

10. 15% Native-PAGE

- 15% Seperating gel
  - 30 % Acrylamide 2.5 mL
  - 1.5 M Tris (pH 8.8) 1.25 mL
  - 10 % Ammonium persulfate (APS) 150 µL
  - TEMED 10 µL
  - dIH_2O 1.91 mL
- 5% Stacking gel
  - 30 % Acrylamide 0.280 mL
  - 1 M Tris (pH 6.8) 0.200 mL
  - 10 % Ammonium persulfate (APS) 17 µL
  - TEMED 10 µL
  - dIH_2O 1.53 mL

11. Running Buffer for Native-PAGE

Tris-Cl (MW 121) 4.53 g

Glycine (MW 75) 21.6 g

dissolved in double distilled water and adjusts the volume to 1.5 L

(Running buffer should be~ pH 8.3. Do not adjust the pH)

APPENDIX C

Determination of the MIP Expression Level by Dot-Blot Analysis

1. Standard insulin preparation for determination of MIP expression

Insulin from bovine pancreas was dissolved in PBS buffer (pH 7.4) at the concentration of 2 mg/mL and injection insulin (Mixtard® 30 HM Penfill®, 3 $mg.mL^{-1}$) was dissolved in PBS buffer and adjusted the concentration to 1 $mg.mL^{-1}$. Both insulin from bovine pancreas and injection insulin were used as a positive control for dot-blot analysis at various concentrations as shown in Table A.

Insulin from bovine pancreas				Injection insulin			
Final conc. $mg.mL^{-1}$	Stock solution $mg.mL^{-1}$	Volume from stock solution µL	PBS buffer (µL)	Final conc. $mg.mL^{-1}$ *Serial dilution	Stock solution $mg.mL^{-1}$	Volume from stock solution µL	PBS buffer µL
2	2	500	0	1	1	500	0
1	2	500	500	1/2 X	1	500	500
0.5	1	500	500	1/4 X	1/2 X	500	500
0.1	1	100	900	1/8 X	1/4 X	500	500
0.05	0.5	100	900	1/16 X	1/8 X	500	500
0.01	0.1	100	900	1/32 X	1/16 X	500	500
0.0050	0.05	100	900	1/64 X	1/32 X	500	500
0.0025	0.05	50	950	1/128 X	1/64 X	500	500
0.0010	0.01	100	900	1/256 X	1/128 X	500	500
0.0005	0.005	100	900	1/512 X	1/256 X	500	500

Table A : Standard insulin preparation for dot blot analysis

APPENDIX D

Determination of the MIP Concentration by Indirect Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

1. Standard insulin preparation for determination of MIP concentration

Insulin from bovine pancreas was dissolved in PBS buffer (pH 7.4) at the concentration of 1 mg.mL^{-1} which is stock solution. Insulin from bovine pancreas was used as a positive control for quantitative determination of the MIP concentration at various concentrations as shown in Table B.

Insulin standard concentration, µg.mL^{-1}	Stock concentration, µg. µL^{-1}	Use from stock (µL)	MMH medium (µL)
10.00	0.1	100	900
7.50	0.1	75	925
5.00	0.1	50	950
4.00	0.1	40	960
3.00	0.1	30	970
2.00	0.1	20	980
1.00	0.1	10	990
0.80	0.01	80	920
0.60	0.01	60	940
0.40	0.01	40	960
0.20	0.01	20	980
0.10	0.01	10	990
0.075	0.001	75	925
0.050	0.001	50	950
0.025	0.001	25	975
0.000	0	0	1000

Table B: Standard insulin preparation for indirect competitive ELISA

2. Standard graph of standard insulin from bovine pancreas for quantitative determination by indirect competitive ELISA.

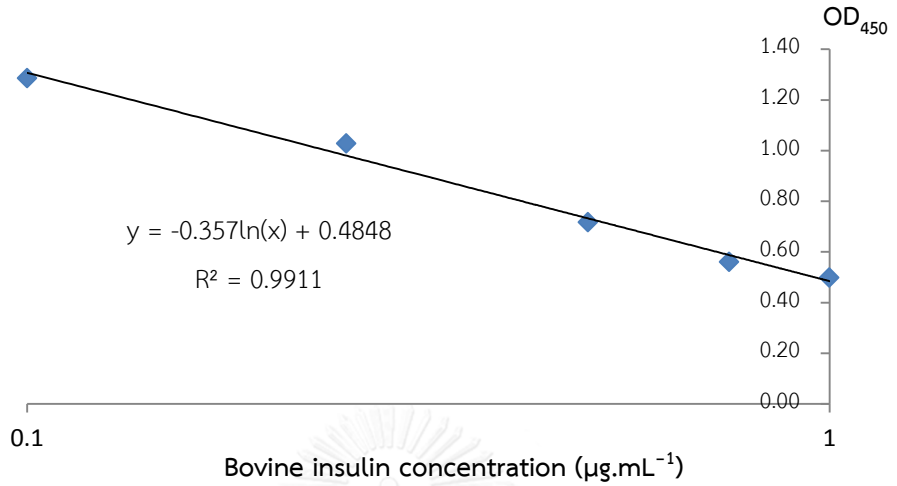


Figure A : Standard graph of standard insulin for calculation of the MIP concentration by indirect competitive ELISA

Standard equation to calculate the MIP concentration;

$$Y = -0.357\ln(X) + 0.4848$$

Calculation the MIP concentration from the standard equation above using Microsoft Excel 2010.

APPENDIX E

Determination of total protein concentration by BCA assay

1. Standard Bovine Serum Albumin (BSA) preparation for determination of total protein concentration by Pierce™ BCA Protein Assay Kit

Use Table A as a guide to prepare a set of protein standards. Dilute the contents of one Bovine Serum Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the samples. Each 1 mL ampule of 2mg/Ml Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table A. There will be sufficiebt volume for three replications of each diluted standard.

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA concentration (µg/mL)
A	700	100 of stock	250
B	400	400 of vial A dilution	125
C	450	300 of vial B dilution	50
D	400	400 of vial C dilution	25
E	400	100 of vial D dilution	5
F	400	0	0 = Blank

2. Standard graph of **standard BSA for quantitative determination by BCA assay.**

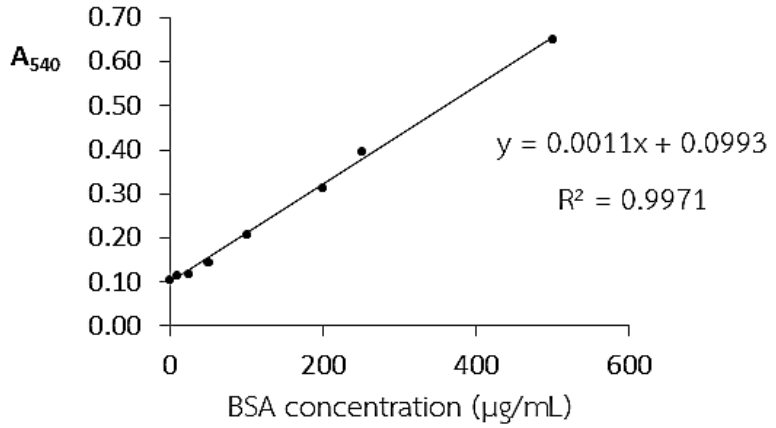


Figure A: Standard graph of standard BSA for calculation of total protein concentration by BCA assay

Standard equation to calculate the MIP concentration;

$$Y = 0.0011X + 0.0993$$

Calculation the MIP concentration from the standard equation above using Microsoft Excel 2010.

APPENDIX F

Analyzing real-time PCR data by the comparative Cq

Real-time PCR (RT-qPCR) is a powerful tool to quantify gene expression. The quantitative endpoint for real-time PCR is the threshold cycle (C_T or C_q). The C_q is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold.

Equation 1 showed the calculate form of $\triangle C_T$.

$\triangle C_T$ = C_T gene of interest- C_T internal control Eq.1

Equation 2 showed the calculate form of relative normalize expression (R). It is the form of the comparison of gene expression between two different samples (sample A and sample B); each sample is related to an internal control gene.

$R = 2^{-\triangle\triangle C_T}$

R = (C_T gene of interest- C_T internal control) Sample A –

(C_T gene of interest- C_T internal control) Sample B Eq.2

1. Relative Quantification of GLUT4 mRNA from H9c2 (2-1) at 24h culture time by Real-time PCR

Target	Sample	Mean Cq	Mean Efficiency Corrected Cq	Normalized Expression	Relative Normalized Expression	Regulation	Compared to Regulation Threshold	P-Value	Exceeds P-Value Threshold
GAPDH	No ins	22.30	22.30				No change		No
GAPDH	No template	33.64	33.64				No change	0.388456	Yes
GAPDH	MIP 0.350 µg/L	21.57	21.57				No change	0.848623	Yes
GAPDH	MIP 0.700 µg/L	21.41	21.41				No change	0.787918	Yes
GAPDH	MIP 0.175 µg/L	18.71	18.71				No change	0.753806	Yes
GAPDH	Standard insulin 0.350 µg/L	23.07	23.07				No change	0.260412	Yes
GAPDH	Standard insulin 0.700 µg/L	23.58	23.58				No change	0.239043	Yes
GAPDH	Standard insulin 0.175 µg/L	22.43	22.43				No change	0.995049	Yes
GLUT4	No insulin	32.39	32.39	0.00092	1.00000	1.00000	No change		No
GLUT4	No template	36.59	36.59	0.12953	141.19818	141.19818	Up regulated		No
GLUT4	MIP 0.350 µg/L	32.50	32.50	0.00051	0.55949	-1.78735	No change	0.021494	Yes
GLUT4	MIP 0.700 µg/L	32.61	32.61	0.00043	0.46484	-2.15126	No change	0.011976	Yes
GLUT4	MIP 0.175 µg/L	30.00	30.00	0.00040	0.43373	-2.30557	No change	0.009447	No
GLUT4	Standard insulin 0.350 µg/L	30.76	30.76	0.00485	5.28912	5.28912	Up regulated	0.000919	No
GLUT4	Standard insulin 0.700 µg/L	33.26	33.26	0.00122	1.32665	1.32665	No change	0.199462	Yes
GLUT4	Standard insulin 0.175 µg/L	32.82	32.82	0.00075	0.81213	-1.23132	No change	0.336396	Yes

2. Relative Quantification of GLUT4 mRNA from H9c2 (2-1) at 3h culture time by Real-time PCR

Target	Sample	Mean Cq	Mean Efficiency Corrected Cq	Normalized Expression	Relative Normalized Expression	Regulation	Compared to Regulation Threshold	P-Value	Exceeds P-Value Threshold
GAPDH	MIP 5.60 µg/L	20.08	20.08				No change	0.944169	Yes
GAPDH	MIP 11.20 µg/L	19.81	19.81				No change	0.827712	Yes
GAPDH	MIP 2.80 µg/L	20.60	20.60				No change	0.953308	Yes
GAPDH	No insulin treated	19.96	19.96				No change		No
GAPDH	Standard insulin 2.80µg/L	20.49	20.49				No change	0.990599	Yes
GAPDH	Standard insulin 5.60 µg/L	18.96	18.96				No change	0.902472	Yes
GAPDH	Standard insulin 11.2 mU/L	20.44	20.44				No change	0.997424	Yes
GLUT4	MIP 5.60 µg/L	29.47	29.47	0.0015	2.2456	2.2456	No change	0.002253	No
GLUT4	MIP 11.20 µg/L	20.31	20.31	0.7049	1064.2086	1064.2086	Up regulated	0.000327	No
GLUT4	MIP 2.80 µg/L	30.45	30.45	0.0011	1.6390	1.6390	No change	0.003130	No
GLUT4	No insulin treated	30.52	30.52	0.0007	1.0000	1.0000	No change		No
GLUT4	Standard insulin 2.80µg/L	30.32	30.32	0.0011	1.6571	1.6571	No change	0.002336	No
GLUT4	Standard insulin 5.60 µg/L	30.56	30.56	0.0003	0.4853	-2.0606	No change	0.005702	No
GLUT4	Standard insulin 11.2 mU/L	19.75	19.75	1.6120	2433.6031	2433.6031	Up regulated	0.001673	No

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