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การเพิ่มผลผลิตรีคอมบิแนนต์อินซูลินโดยการเพิ่มจำนวนชุดของยีนในเมธิลโลโธฟิกยีสต์ (ปีที่2)

Enhancement of recombinant monomeric insulin production in methylotrophic yeasts by increasing copy number of gene (2nd year)

โดย

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บทคัดย่อภาษาไทย

อินซูลินเป็นฮอร์โมนที่ผลิตจากตับอ่อน มีหน้าที่ควบคุมระดับน้ำตาลในเลือด ซึ่งอินซูลินที่ออกฤทธิ์ได้จะอยู่ในรูปแบบของอินซูลินแบบมอนอเมอร์ที่ประกอบด้วยกรดอะมิโนทั้งหมด 51 ตัว และมีโครงสร้างเป็นเปปไทด์ 2 สาย ปัจจุบันมีการพัฒนาการผลิตอินซูลินให้อยู่ในรูปแบบของรีคอมบิแนนต์อินซูลินที่ผลิตโดยแบคทีเรียและยีสต์ เนื่องจากสามารถผลิตและทำบริสุทธิ์ได้ง่ายกว่าการสกัดมาจากตับอ่อนของหมูและวัว และในปัจจุบันประเทศไทยมี จำนวนผู้ป่วยโรคเบาหวานเพิ่มขึ้นทุกปี แต่ยังไม่มีการผลิตอินซูลินภายในประเทศ จึงต้องอาศัยการนำเข้าจากต่างประเทศทั้งสิ้น งานวิจัยนี้จึงสนใจที่จะนำรีคอมบิแนนต์ยีสต์ *Pichia pastoris* สายพันธุ์ KM71H (TP1) ซึ่งมีพลาสมิดที่มีชุดยีนผลิตรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์ (MIP) มาเลี้ยงและเหนี่ยวนำให้มีการผลิตรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์ซึ่งสามารถตรวจติดตามระดับการแสดงออกได้ด้วยวิธี Dot-blot analysis และสามารถวัดปริมาณรีคอมบิแนนต์อินซูลินด้วยวิธี Indirect competitive Elisa นอกจากนี้สามารถตรวจสอบน้ำหนักโมเลกุลของโปรตีนในอาหารเลี้ยงเชื้อได้ด้วยเครื่องวัดมวลโมเลกุล พบว่าอินซูลินที่ผลิตได้มีความเข้มข้น 16 มิลลิกรัม/ลิตร และมีน้ำหนักโมเลกุลประมาณ 5756.95 ดาลตัน งานวิจัยนี้ได้ทำบริสุทธิ์รีคอมบิแนนต์อินซูลินออกจากอาหารเลี้ยงเชื้อโดยใช้สองขั้นตอน ขั้นตอนแรกคือการทำบริสุทธิ์ด้วยโครมาโทกราฟีแบบแลกเปลี่ยนประจุลบชนิด SP Sepharose Fast Flow และขั้นตอนที่สองคือการใช้ Amicon Ultra-15 Centrifugal Filter ขนาด 10 กิโลดาลตัน จากนั้นรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์ถูกเปลี่ยนให้อยู่ในรูปที่ออกฤทธิ์ได้โดยการทำให้เกิดปฏิกิริยา การย่อยสลายด้วยเอนไซม์ทริปซิน นอกจากนี้ ได้ศึกษาผลของรีคอมบิแนนต์อินซูลินแบบมอนอเมอร์บริสุทธิ์ที่ผลิตจากยีสต์ *Pichia pastoris* KM17H ต่อการนำเข้ากลูโคสและการแสดงออกของยีนขนส่งน้ำตาลกลูโคส ประเภทที่ 4 โดยการวัดระดับปริมาณน้ำตาลกลูโคสในอาหารที่ลดลงด้วยเครื่องตรวจวัดระดับน้ำตาลและวัดระดับการผลิตอาร์เอ็นเอนำรหัสของตัวขนส่งน้ำตาลกลูโคสประเภทที่ 4 (GULT 4) ในเซลล์กล้ามเนื้อหัวใจหนูชนิด H9c2 (2-1) ด้วยปฏิกิริยาลูกโซ่พอลิเมอเรสที่สามารถติดตามปริมาณของผลิตภัณฑ์ที่เกิดขึ้น ณ เวลานั้นๆ โดยอาร์เอ็นเอนำรหัสของตัวขนส่งน้ำตาลกลูโคสประเภทที่ 4 มีการแสดงออกและผลิตเพิ่มมากขึ้นเมื่อมีการกระตุ้นจากอินซูลิน พบว่า เมื่อมีการกระตุ้นด้วยรีคอมบิแนนต์อินซูลิน ระดับน้ำตาลกลูโคสในอาหารจะลดลงเมื่อเปรียบเทียบกับเซลล์ที่ไม่ถูกกระตุ้นด้วยอินซูลิน และในเซลล์ที่ถูกกระตุ้นด้วยรีคอมบิแนนต์อินซูลินที่ความเข้นข้น 11.20 มิลลิกรัม/ลิตร มีการเพิ่มการแสดงออกของอาร์เอ็นเอนำรหัสของตัวขนส่งน้ำตาลประเภทที่ 4 ที่ระยะเวลา 3 ชั่วโมง

บทคัดย่อภาษาอังกฤษ (Abstract)

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.

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คำอธิบายสัญลักษณ์และคำย่อที่ใช้ในงานวิจัย (List of Abbreviation)

AOX1	Alcohol oxidase 1
C_T, C_q	Threshold cycle
GLUT	Glucose transporter
GLUT4	Glucose transporter type 4
MIP	Monomeric Insulin Precursor
MMH	Minimal methanol histidine
Mut^+	Methanol utilization plus phenotype
Mut^S	Methanol utilization slow phenotype
PAGE	Polyacrylamide gel electrophoresis
RT-qPCR	Real time Polymerase chain reaction
Tm	Melting temperature (°C)
w/	With
w/o	Without
w/v	Weight by volume
WT	Wild type strain
×g	Multiply by gravitational force (×9.80665 m.s2)
YNB	Yeast nitrogen base
YPD	Yeast extract Peptone Dextrose
YPG	Yeast extract Peptone Glycerol

บทนำ (Introduction)

1.1 ความสำคัญและที่มาของปัญหาที่ทำการวิจัย

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 cannot 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 [1, 2].

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 [3]. 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 [4, 5]. 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 [6].

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 [7].

In this research were used as hosts to study effect of purified recombinant monomeric insulin (MIP) from *P. 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.

1.2 วัตถุประสงค์ของโครงการวิจัย

To purified recombinant monomeric insulin (MIP) from broth culture and study effect of purified MIP from *P. pastoris* KM17H on glucose uptake and glucose transporter type 4 (*GLUT4*) gene expression.

1.3 ขอบเขตของโครงการวิจัย

1.3.1 To cultivate and express MIP from *P. pastoris* KM71Hz

1.3.2 To monitor MIP expression level by Dot-Blot Analysis and determine MIP concentration by Indirect Competitive ELISA

1.3.3 To purify MIP from broth culture

1.3.4 To study effect of purified recombinant monomeric insulin (MIP) from *P. pastoris* KM17H on glucose uptake and glucose transporter type 4 (*GLUT4*) gene expression

1.4 ทฤษฎี สมมติฐาน (ถ้ามี) และกรอบแนวความคิดของโครงการวิจัย

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). The mature insulin molecule is composed of two peptide chains referred to Alpha (A) chain and Beta (B) chain. 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. The chains are linked-together by two disulphide bridges which are between Cys A7 and Cys B7, and between Cys A20 and Cys B19. It has an intrachain disulphide bridge between Cys A6 and Cys A11 creating a loop in the A chain.

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.

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 [8-11].

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 co-transporters (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

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. 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* [12-14].

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.

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 [14].

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 [14, 15]. 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

1.5 วิธีดำเนินการวิจัยโดยสรุป

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

1.5.2 Monitoring and determination of the MIP expression level

1.5.3 Determination of molecular weight of MIP by MALDI-TOF

1.5.4 Purification of MIP by molecular weight cut-off by ultrafiltration membrane or cat-ion exchange chromatography column

1.5.5 Conversion of MIP into active form by TPCK trypsin

1.5.6 Confirmation an action of purified MIP on glucose transporter type 4 (*GLUT4*) gene expression and measurement of glucose level in culture medium

1.6 ประโยชน์ที่คาดว่าจะได้รับ

Able to purify recombinant monomeric insulin (MIP) from culture broth of *Pichia pastoris* KM17H and to confirm an action of the obtained purified MIP from *Pichia pastoris* KM17H on glucose uptake and glucose transporter type 4 (*GLUT4*) gene expression before scale up the production of MIP to the pilot plant.

เนื้อเรื่อง (Main body)

2.1 วิธีดำเนินการวิจัย (Materials and Method)

2.1.1 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 [7].

2.1.2 Cell line

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

2.1.3 Media

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

YPD Zeocin™ medium (YPD with 100 $\mu g.mL^{-1}$ 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.

2.1.3.2 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%.

2.1.4 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 [16].

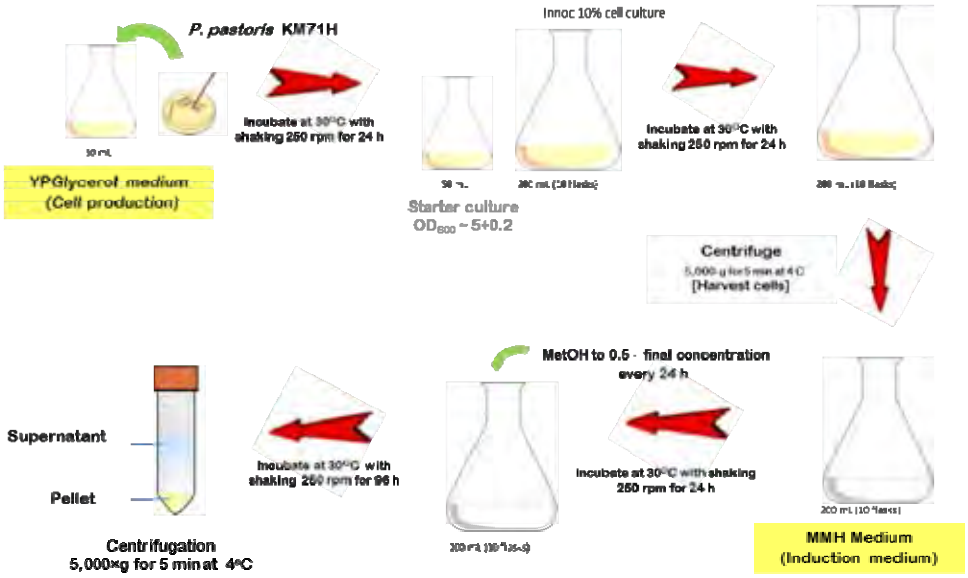


Figure 2.1: Cultivation and expression of MIP from *P. pastoris* KM71H

2.1.5 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.

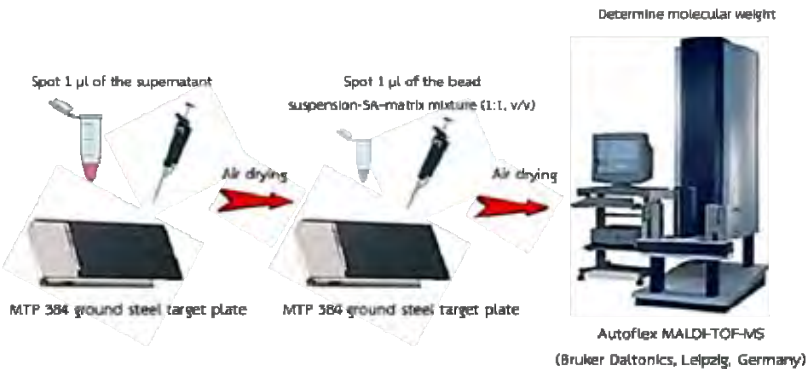


Figure 2.2: Determination of molecular weight of MIP by MALDI-TOF

2.1.6 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 [17]. The procedure of this protocol is shown in Figure 3.3.

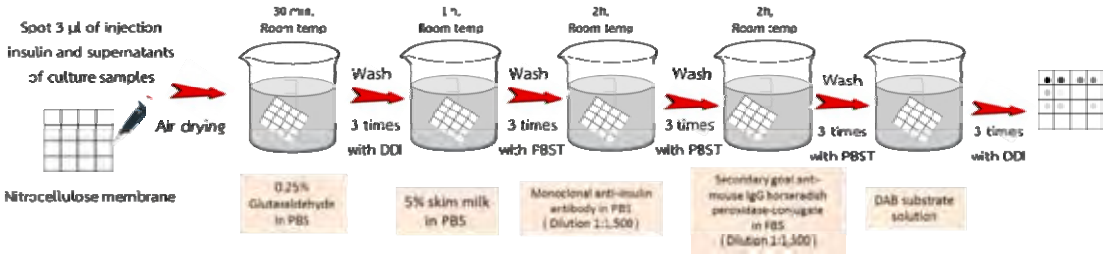


Figure 2.3: Monitoring of MIP expression level by Dot-Blot Analysis

2.1.7 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 $\mu g.ml^{-1}$ 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.

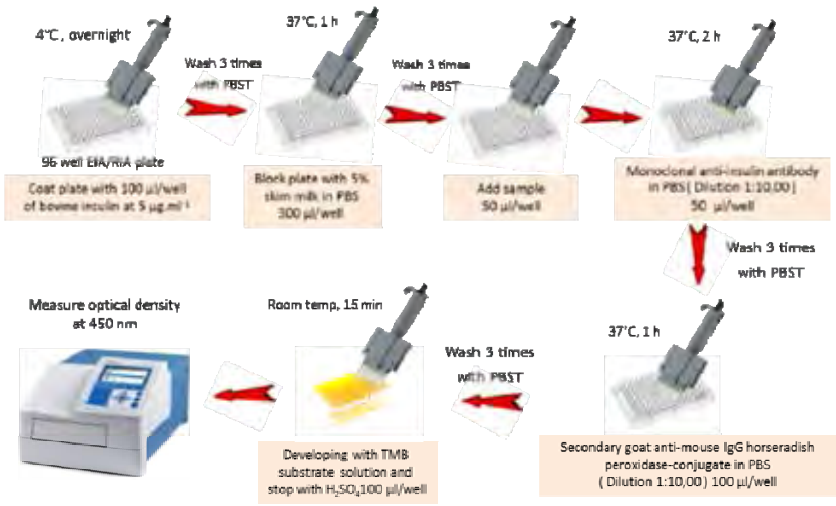


Figure 2.4: Quantitative determination of MIP by indirect competitive ELISA

2.1.8 Purification of MIP from supernatant

2.1.8.1 Separation MIP from supernatant by using molecular weight cut-off 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.

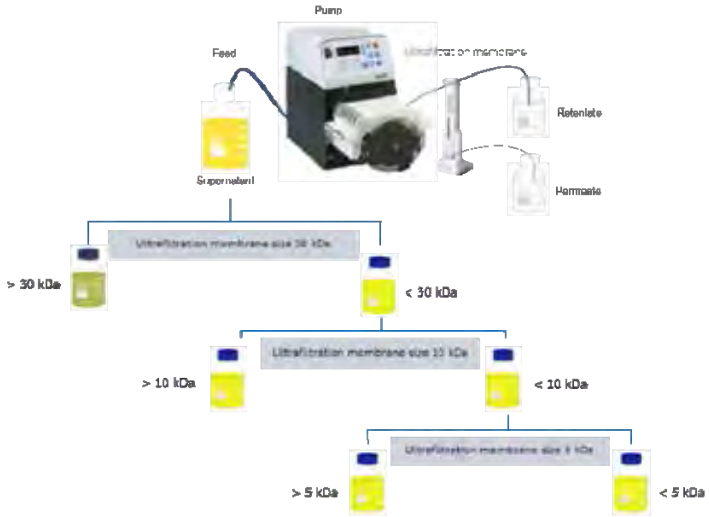


Figure 2.5: Molecular weight cut-off by ultrafiltration membrane

2.1.8.2 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 [18]. 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 [11].

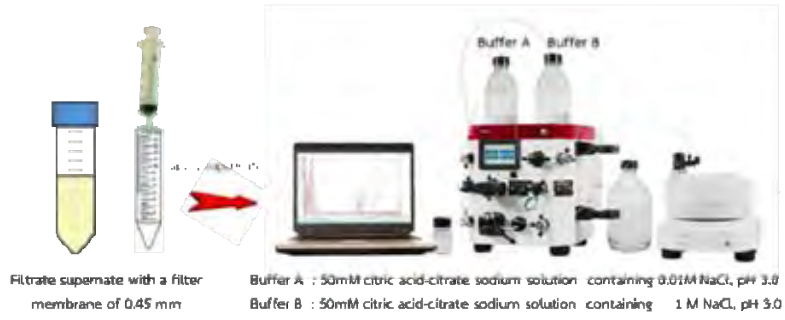


Figure 2.6: Purification of MIP by SP-Sepharose FF chromatography column

2.1.8.3 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 [17].

Figure 2.7: Purification of MIP by 10 kDa Amicon Ultra-15 Centrifugal Filter

2.1.9 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.

2.1.10 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 [19].

2.1.11 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.

2.1.11.1 RNA extraction and measurement of *GLUT4* mRNA by qRT-PCR

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 qRT-PCR 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 [14, 20, 21]**.**

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′

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

Glucose level in culture medium was measured by YSI glucose analyze**r**. 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 [22].

2.2 อภิปรายผล (Results and Discussion)

2.2.1 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 2.9. 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 2.8).

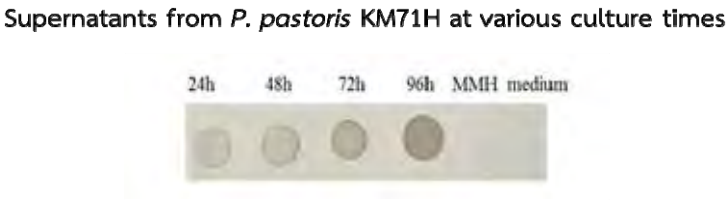


Figure 2.8: Dot-blot analysis of MIP in supernatants from recombinant yeast at various culture times , MMH medium was used as negative control

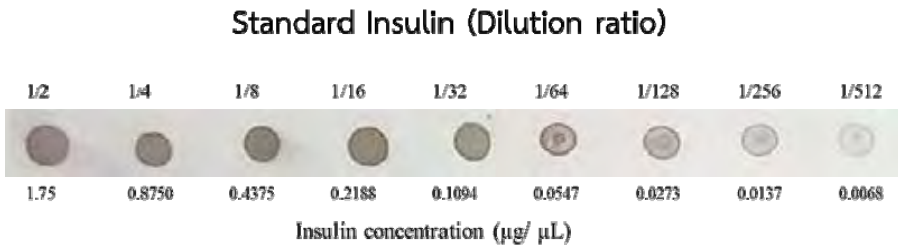


Figure 2.9: Dot-blot of injection insulin (positive control) that generated from two-fold serial dilution from 1/2 to 1/512 (1.75 to 0.0068 mg/ml)

2.2.2 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 2.10. The highest peak was expected to be MIP (*m*/*z* 5756.951), consistent with the bovine insulin standard as shown in Figure 2.11.

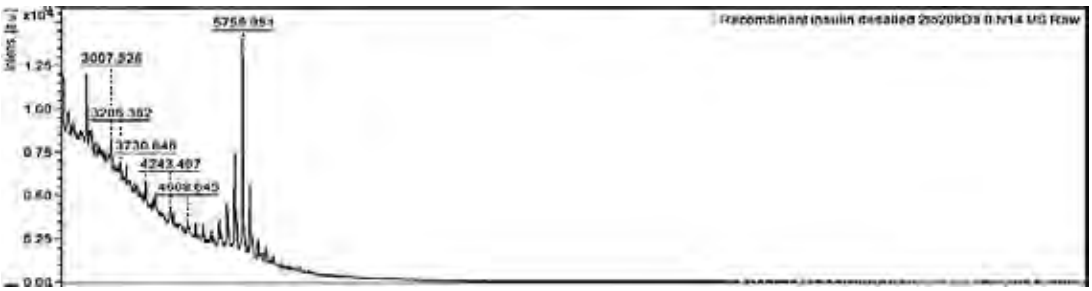


Figure 2.10: Typical MALDI-TOF mass spectra of supernatant of culture sample from *P. pastoris* KM71H (TP1). The highest peak was expected to be MIP (*m*/*z* 5756.951)

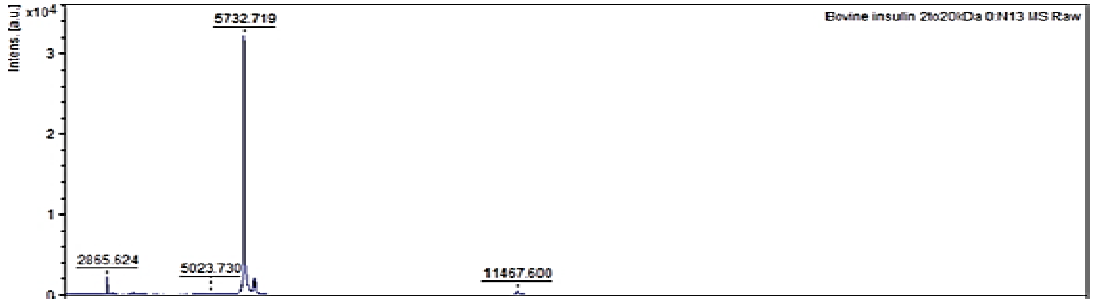


Figure 2.11: Typical MALDI-TOF mass spectra of bovine insulin (SIGMA-ALDRICH, USA). The highest peak was bovine insulin (*m*/*z* 5732.719).

2.2.3 Purification of MIP from supernatant

2.2.3.1 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 2.12. 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.

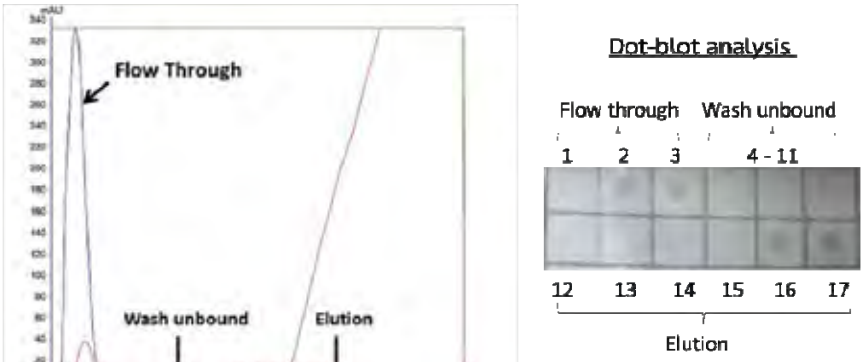
Figure 2.12: Dot-blot analysis of injection insulin dilution ratio from 1/4 to 1/512 (positive control) and separated MIP by using molecular weight cut-off Ultrafiltration membrane. MMH medium was used as negative control.

2.2.3.2 Purification of MIP by HiTrap ion exchange chromatography

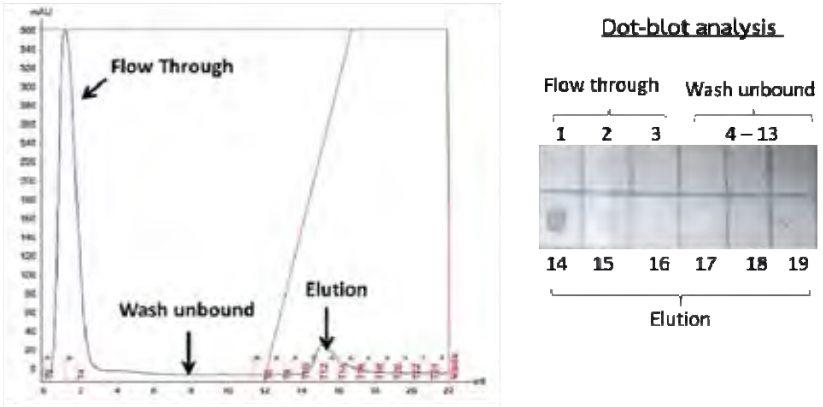
HiTrap ion exchange chromatography (IEX) consists of seven HiTrap columns, prepacked with different Sepharose Fast Flow ion exchange chromatography media. It offers a fast, simple, and convenient way to decide which ion exchanger or ion exchange ligand is best for a given application. Interestingly, 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. 2.13. 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

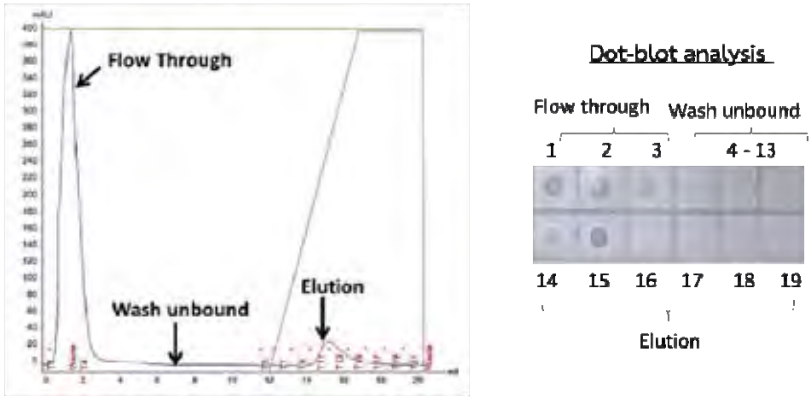


Figure 2.13: Cation-exchange chromatography and Dot-blot analysis for MIP. (A) Chromatogram profile of MIP obtained using Hitrap CM sepharose Fast Flow column chromatography. (B) Chromatogram profile of MIP obtained using Hitrap SP sepharose Fast Flow column chromatography. (C) Chromatogram profile of MIP obtained using Hitrap SP sepharose XL column chromatography.

From the results in Figure 2.13, (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 2.14). 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 2.14.

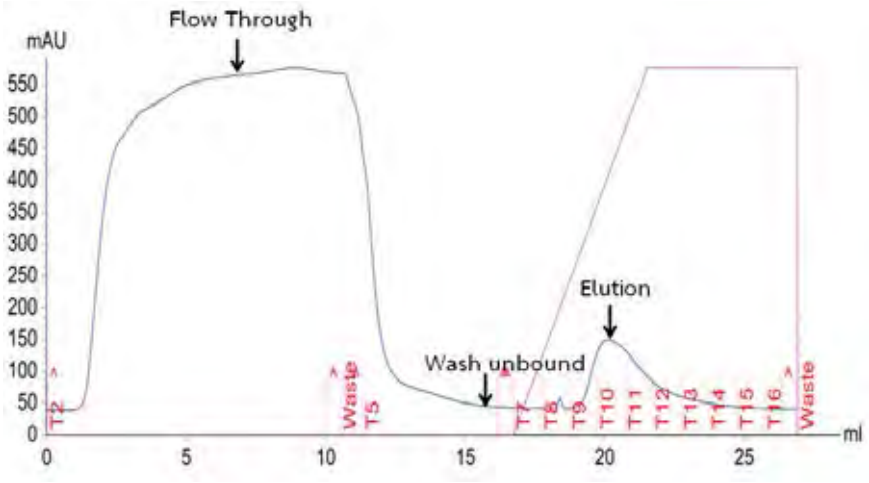


Figure 2.14: Purification of MIP on SP-Sepharose FF cation exchange chromatography column. Balanced by 50 mM citric acid-citrate sodium solution containing 0.01 M NaCl, pH 3.0, the MIP was eluted by 50 mM citric acid-citrate sodium solution containing 1.0 M NaCl, pH 3.0, with a linear gradient of the eluted solution (0–100%) at a rate of 0.5 mL/min

Table 2.1: 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 2.1, 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 2.2: 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 2.15 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.

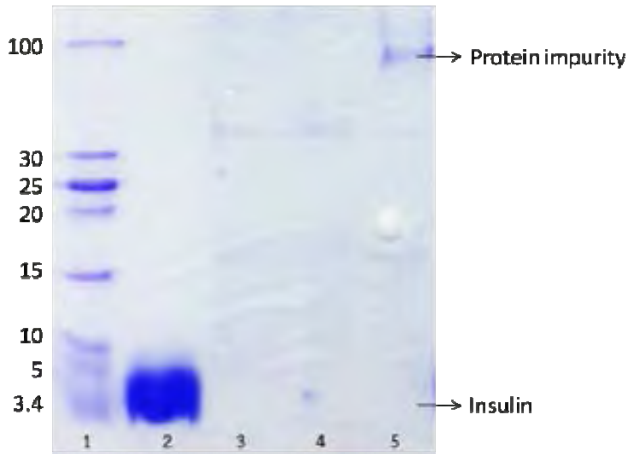


Figure 2.15: 15% Native polyacrylamide gel electrophoresis (Native-PAGE) of supernatant, purified MIP**.** Lane 1: 5 µl of unstained protein ladder, lane 2: 5 µl of standard insulin (Injection insulin 1 mg/mL), lane 3: 20 µl of Supernatant, lane 4: 20 µl of flow through fraction from SP-sepharose Fast Flow Chromatography column, lane 5: 20 µl of Elute fraction from SP-sepharose Fast Flow Chromatography column.

2.2.3.3 Separation 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 2.3).

Table 2.3: 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 2.16). 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.

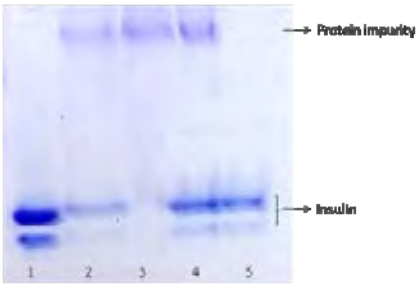


Figure 2.16: 15% Native polyacrylamide gel electrophoresis (Native-PAGE) of supernatant and purified MIP by two purification steps. Lanes1: 10 µl of standard insulin (Injection insulin 1mg/mL), lane 2: 20 µl of supernatant, lane 3: 20 µl of flow through fraction from SP-sepharose Fast Flow Chromatography 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.

2.2.4 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.

2.2.4.1 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 as a pre-

diabetic level. 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 2.4 and Figure 2.17. Concentration of glucose in DMEM medium approached to 1.74 g/L. The concentration of glucose in medium which treated with standard insulin and MIP were become lower than that of no insulin treated. The results indicated that insulin could stimulate the increasing of glucose uptake into tissues.

Table 2.4: Table 2.4: 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	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

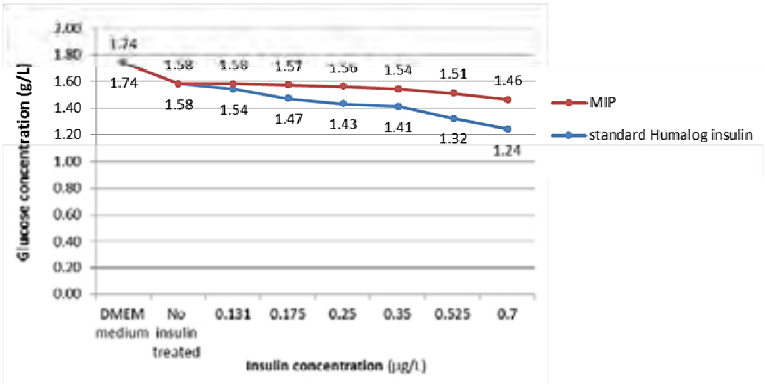


Figure 2.17: Glucose concentration in culture medium of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.131 to 0.700 µg/L) for 24 h by using no insulin treated culture medium as a control

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 2.5. Afterwards, the cDNA was synthesized and quantified gene expression by real-time PCR.

Table 2.5: 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	A260/230
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 2.6). 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 2.6: 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 (Table 2.6), *GLUT4* gene expression up-regulated only on relatively sample 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 2.7 and Figure 2.18. 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 2.7: 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.

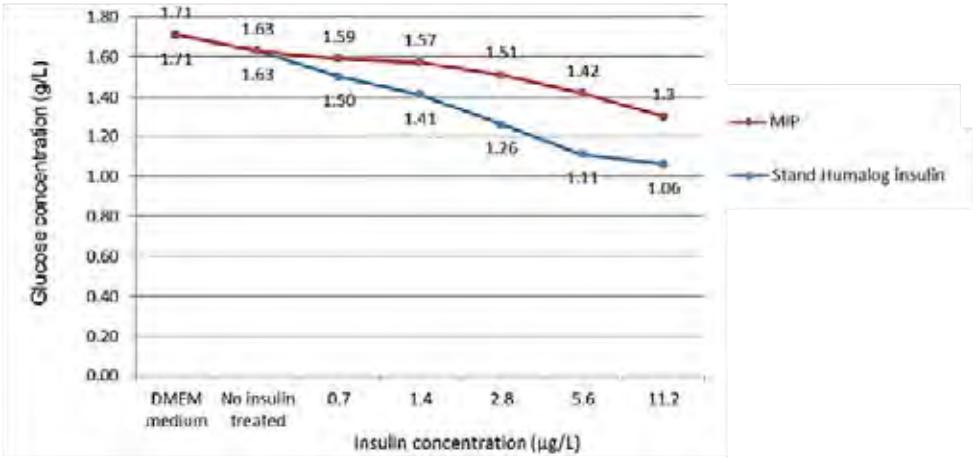


Figure 2.18: Glucose concentration in DMEM medium and culture medium of H9c2 (2-1) cell line treated with standard insulin and MIP (at 0.70 to 11.20 µg/L) for 24 h by using no insulin treated culture medium as a control

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 2.8.

Table 2.8: 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	A260/230
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 2.9.

Table 2.9: 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 2.8 and *GLUT4* mRNA expression in Table 2.9, 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 time 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 2.10 and Figure 2.19. 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 2.10: 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.

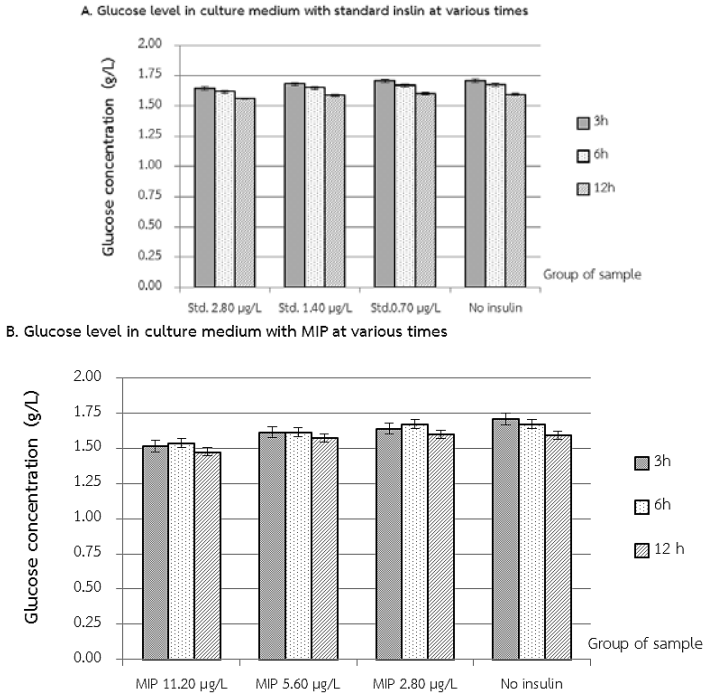


Figure 2.19: Glucose concentration in culture medium of H9c2 (2-1) cell line treated with standard insulin (at 0.70 to 2.80 µg/L) (A) and MIP (at 2.80 to 11.20 µg/L) (B) for 3, 6, 12 h by using no insulin treated culture medium as a control

All cell cultures in 3h culture time were collected to study *GLUT4* mRNA expression. RNA concentration of each sample culture was shown in Table 2.11

Table 2.11: 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	A260/230
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 2.12.

Table 2.12: *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. However, other samples which were treated with lower concentration of MIP (≥ 5.60 µg/L) and standard Humalog insulin (≥ 1.40 µg/L) did not show the expression of *GLUT4* gene.

สรุปและข้อเสนอแนะ (Conclusion and Recommendation)

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 for ... h showed that the production of the MIP was 16 mg/L. Mass spectrometry assay confirmed that MIP was the major product in the culture broth and the molecular mass of MIP is 5796.9 Da. The MIP was purified from culture broth by two purification steps. In first step, supernatant was loaded onto a SP Sepharose Fast Flow chromatography column resulted in a single elution peak. Nevertheless, the result of native-PAGE was showed that there was an impurity protein with molecular weight size near 100 kDa in the 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 the study of Huang and co-workers, high glucose and insulin concentrations (25 mmol/L glucose and 763 µg/L insulin) resulted in a 40% increase in basal glucose uptake accompanied at 24 h [23]. Moreover, Liu and co-workers also found that the concentration of insulin and glucose 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 [14]. Therefore, incubation time that cell was treated with insulin was also important for detection of mRNA expression. Moreover, difference type of insulin is also an important factor that effect directly to the time-action of insulin. However, the mechanisms that regulate transcription of *GLUT4* gene and degradation of *GLUT4* mRNA is unknown. So, time course of *GLUT4* expression and dose of insulin should be further study.

เอกสารอ้างอิง (References)

1. International Diabetes Federation. 2014 [cited 2015 August 20]; Available from: http://www.idf.org/. .
2. Complications of Diabetes Mellitus. 2015 [cited 2015 August 20]; Available from: http://happy32club.com/complications-of-diabetes-mellitus/.
3. Damasceno, L.M., C.J. Huang, and C.A. Batt, Protein secretion in Pichia pastoris and advances in protein production. Applied Microbiology and Biotechnology, 2012. 93(1): p. 31-9.
4. Gellissen, G., et al., New yeast expression platforms based on methylotrophic Hansenula polymorpha and Pichia pastoris and on dimorphic Arxula adeninivorans and Yarrowia lipolytica - a comparison. FEMS Yeast Res, 2005. 5(11): p. 1079-96.
5. Muller, S., et al., Comparison of expression systems in the yeasts Saccharomyces cerevisiae, Hansenula polymorpha, Klyveromyces lactis, Schizosaccharomyces pombe and Yarrowia lipolytica. Cloning of two novel promoters from Yarrowia lipolytica. Yeast, 1998. 14(14): p. 1267-83.
6. Li, P., et al., Expression of recombinant proteins in Pichia pastoris. Applied Biochemistry and Biotechnology, 2007. 142(2): p. 105-24.
7. T., P., Enhancement of recombinant monomeric insulin production in Pichia pastoris strains by increasing copy number of gene., in Program in Biotechnology,. 2014, Chulalongkorn University.: Faculty of Science,.
8. Chase, H.A., Purification of proteins by adsorption chromatography in expanded beds. Trends Biotechnol, 1994. 12(8): p. 296-303.
9. Gurramkonda, C., et al., Application of simple fed-batch technique to high-level secretory production of insulin precursor using Pichia pastoris with subsequent purification and conversion to human insulin. Microbial Cell Factories, 2010. 9: p. 31.
10. Wang, Y., et al., Human insulin from a precursor overexpressed in the methylotrophic yeast Pichia pastoris and a simple procedure for purifying the expression product. Biotechnology and Bioengineering, 2001. 73(1): p. 74-9.
11. Xie, T., et al., Secretory expression of insulin precursor in pichia pastoris and simple procedure for producing recombinant human insulin. Preparative Biochemistry & Biotechnology, 2008. 38(3): p. 308-17.
12. Augustin, R., The protein family of glucose transport facilitators: It's not only about glucose after all. IUBMB Life, 2010. 62(5): p. 315-33.
13. James, D.E., et al., Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. Nature, 1988. 333(6169): p. 183-5.
14. Liu, Q., et al., Effects of glucose and insulin on the H9c2 (2-1) cell proliferation may be mediated through regulating glucose transporter 4 expression. Chinese Medical Journal, 2013. 126(21): p. 4037-42.
15. Sardao, V.A., et al., Vital imaging of H9c2 myoblasts exposed to tert-butylhydroperoxide--characterization of morphological features of cell death. BMC Cell Biology, 2007. 8: p. 11.
16. Bollag, D.M., M.D. Rozycki, and S.J. Edelstein, Protein methods. 2nd ed. 1996, New York: Wiley-Liss. xvi, 415 p.

17. Sithigorngul, P., A.O. Stretton, and C. Cowden, A versatile dot-ELISA method with femtomole sensitivity for detecting small peptides. Journal of Immunologigal Methods, 1991. 141(1): p. 23-32.
18. Ion Exchange Chromatography & Chromatofocusing. 2015 [cited 2015 July 24]; Available from: www.gelifesciences.com/.../Ion%20Exchange%20Chromatography.pdf.
19. Beshay N.M., A.O.S., H9c2 cell line is a valuable in vitro model to study the drug metabolizing enzymes in the heart. Journal of Pharmacological and Toxicological Methods, 2007. 56: p. 317-322.
20. Picard, C., M. Silvy, and J. Gabert, Overview of real-time RT-PCR strategies for quantification of gene rearrangements in the myeloid malignancies. Methods Molecular Medicine, 2006. 125: p. 27-68.
21. Schmittgen, T.D. and K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method. Natural Protocol, 2008. 3(6): p. 1101-8.
22. Astles, J.R., F.A. Sedor, and J.G. Toffaletti, Evaluation of the YSI 2300 glucose analyzer: algorithm-corrected results are accurate and specific. Clinical Biochemistry, 1996. 29(1): p. 27-31.
23. Huang, C., et al., Sustained exposure of L6 myotubes to high glucose and insulin decreases insulin-stimulated GLUT4 translocation but upregulates GLUT4 activity. Diabetes, 2002. 51(7): p. 2090-8.

สิ่งตีพิมพ์เพื่อเผยแพร่ (Proceeding)

Thongyoo S, Phakham T, Karnchanatat A, Khongchareonporn N, Noitang S, Puthong S, Reamtong O, Sooksai S, editors. Recovery of recombinant monomeric insulin from culture broth of *Pichia pastoris* KM71H (TP1) by amberlite XAD-7. The 10TH International Symposium of the Protein Society of Thailand; 2014 July 15-17; Convention Center, Chulabhorn Research Institute. Bangkok

ภาคผนวก (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 1.91mL
- 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, $\mu g.mL^{-1}$	Stock concentration, $\mu g.\ \mu L^{-1}$	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.

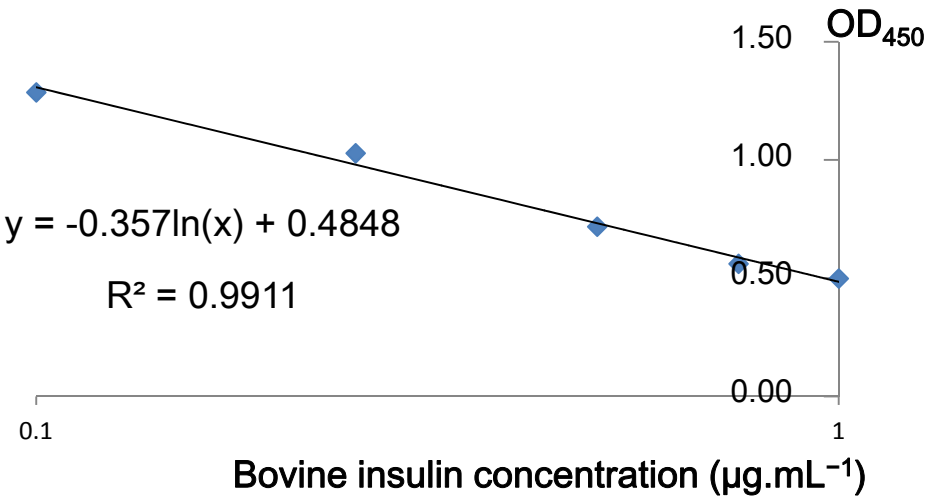


Figure A : Standard graph of standard insulin for calculation of the MIP concentration by indirect competitive ELISA

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.

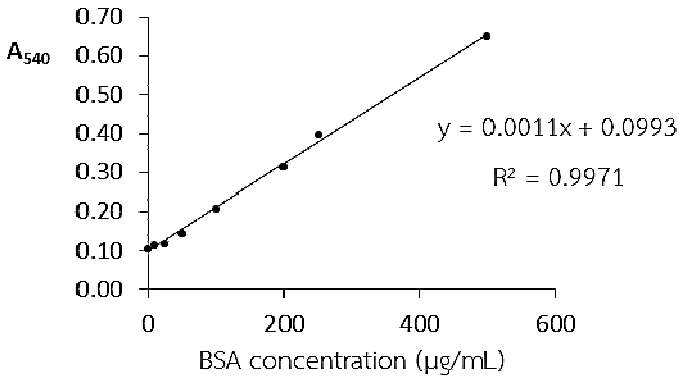


Figure A: Standard graph of standard BSA for calculation of total protein concentration by BCA assay

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	MP 0.350 µg/L	21.57	21.57				No change	0.848623	Yes
GAPDH	MP 0.700 µg/L	21.41	21.41				No change	0.787918	Yes
GAPDH	MP 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	MP 0.350 µg/L	32.50	32.50	0.00051	0.55949	-1.78735	No change	0.021494	Yes
GLUT4	MP 0.700 µg/L	32.61	32.61	0.00043	0.46484	-2.15126	No change	0.011976	Yes
GLUT4	MP 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