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Expression, purification and biological activity of monomeric insulin precursors from methylotrophic yeasts

Sawanan Thongyoo[a], Tanapati Phakham[a], Nanthika Khongchareonporn[b], Onrapak Reamtong[c], Aphichart Karnchanatat[b], Songchan Phutong[b], Thanaporn Wichai[b], Sajee Noitang[b], Sarintip Sooksai[b,*]

[a] Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[b] The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, 10330, Thailand
[c] Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand

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ABSTRACT

The methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* have been used for the production of recombinant monomeric insulin precursor (MIP). Recombinant plasmids with one, two and four cassettes of the *MIP* gene have been successfully constructed in the pPICZαA expression vector to study the effects of gene copy number on MIP production. The MIP protein can be detected by dot-blot analysis from the culture broth of *P. pastoris* KM71H 24 h after placement in MMH induction medium. The secretion levels of MIP protein in culture broth at 72 h after induction indicated that *P. pastoris* KM71H with one cassette of the *MIP* gene had highest MIP protein levels (4.19 ± 0.96 mg L^{-1}). The transcription levels of the *MIP* gene increased proportionately with copy number. However, the amount of secreted MIP protein showed no correlation. The MIP molecular mass was 5756.951 Da, as confirmed by typical MALDI-TOF mass spectrometry. The MIP protein in culture broth was purified by two steps purification including SP Sepharose Fast Flow chromatography followed by ultrafiltration (10 kDa MW cutoff). The percentage of MIP recovery after the two-step purification was 70%, with a single band in a native-PAGE. The biological activity of tryptic hydrolyzed MIP was determined via the expression of the glucose transporter 4 gene (*GLUT4*) in H9c2 (2-1) cell line by RT-qPCR, and the results demonstrated that the MIP protein can induce glucose uptake and upregulation of *GLUT4* mRNA transcription at 3 h and that this activity was related to Humalog® insulin.

1. Introduction

Insulin is a peptide hormone used to modulate blood glucose in the control of diabetes mellitus. Protein engineering has been utilized to generate insulin variants that exhibit fast-acting control of blood glucose levels as well as other modifications that enhance its utility. In addition to these modifications, adaptations that allow efficient heterologous expression are required for insulin and other recombinant proteins to reach their full clinical and biotechnological potential [1–3]. In the past decade, many recombinant proteins have been produced by yeast for many applications, e.g., cosmetics, pharmaceutical and medical industries. Yeast expression systems have many advantages over prokaryotic systems for recombinant protein production including genetic stability, high rapid growth rate, ability to be grown at high cell density, low cost of required media and very high levels of protein secretion [4–6]. A decade ago, methylotrophic yeasts, which can utilize methanol as a sole carbon source, including *Hansenula polymorpha*, *Pichia pastoris*, *Candida boidinii*, and *P. methanolica,* were used to produce many heterologous proteins [4–6]. *H. polymorpha* and *P. pastoris* are popular host organisms used to study higher eukaryotic gene expression and recombinant protein production [7]. *P. pastoris* has a strongly inducible alcohol oxidase 1 promoter ($AOX1_p$) that can strongly promote target gene expression when cultured in an induction medium containing methanol as an inducer. Methylotrophic yeasts can grow to very high cell densities and have strong and tightly regulated promoters, and the production yields of recombinant proteins are as high as grams per liter [8]. However, there are some factors that could affect recombinant protein production in yeast system including yeast strains, gene dosage,

* Corresponding author.
E-mail addresses: T.sawanan@gmail.com (S. Thongyoo), tanapati.pk@gmail.com (T. Phakham), nanthika.k@chula.ac.th (N. Khongchareonporn), onrapak.rea@mahidol.ac.th (O. Reamtong), Aphichart.k@chula.ac.th (A. Karnchanatat), Songchan.P@chula.ac.th (S. Phutong), thanapornwichai2524@gmail.com (T. Wichai), sajee.n@chula.ac.th (S. Noitang), sarintip.so@chula.ac.th (S. Sooksai).

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expression vector, promoter, secretion signal sequence, translation signals, posttranslational processing in the endoplasmic reticulum (ER) and the Golgi, and fermentation strategies [6,9–11].

In mammalian cells, transport of glucose across cellular membranes is mediated by the family of sodium-driven sugar cotransporters and the protein family of glucose transporters (GLUTs). Glucose transporter type 4, also known as GLUT4, is a protein encoded by the *GLUT4* gene found in adipose tissues and striated muscle (skeletal and cardiac). The role of the GLUT4 glucose transporter is thus a major mediator of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis activated by insulin [12]. Therefore, insulin enables glucose uptake by adipose tissue and resting skeletal muscle. Accordingly, the change in glucose transporter 4 (*GLUT4*) expression could influence glucose uptake in myocardial cells [13,14].

The aim of this study was to enhance production of the monomeric insulin precursor (MIP) by increasing the gene copy number of the *MIP* gene cassettes in pPICZαA expressed in different methylotrophic yeast strains including *P. pastoris* GS115 (Mut^+), *P. pastoris* X33 (Mut^+), *P. pastoris* KM71H (Mut^S) and *H. polymorpha* (WT). Recombinant MIP secretion levels were monitored by specific dot-blot analysis and quantitatively determined by indirect competitive ELISA. Purified MIP protein is required for conversion into monomeric insulin by tryptic hydrolysis, and its biological activity was tested by determining the expression of glucose transporter 4 gene (*GLUT4*) transcription levels in the H9c2 (2-1) cell line by RT-qPCR.

2. Materials and methods

2.1. Microorganisms and plasmid vectors

Escherichia coli TOP10F′ was used for recombinant plasmids construction. Three strains of *P. pastoris* including X-33 (Mut^+, WT), GS115 (Mut^+, His^-) and KM71H (Mut^S, Arg^+) were used as hosts for recombinant MIP production, and pPICZαA was used as an expression vector. All of these were purchased from Invitrogen (EasySelect™ *Pichia* Expression Kit Manual), USA. *Hansenula polymorpha* NRRL2214 was gifted from the Agricultural Research Service Culture Collection, USA. The nucleotide sequence of the monomeric insulin precursor (MIP) as reported by Ding. et al. [1] was synthesized and inserted into Blue Heron's standard pUC vector between *Eco*RI and *Not*I (pUC::MIP) (Blue Heron Biotech, USA).

2.2. Recombinant plasmid construction

The synthetic *MIP* gene was inserted into the pPICZαA expression vector between *Eco*RI and *Not*I restriction sites in frame with the signal sequence to generate the pPICZαA::MIP plasmid, TP1 plasmid. The TP1 plasmid was digested with *Bam*HI and *Bgl*II to obtain the MIP cassette consisting of the *AOX1* promoter, *MIP* gene, and *AOX1* transcription terminator (*5′AOX1-MIP-3′AOX1*, head-to-tail). A purified MIP cassette was inserted into a linearized TP1 plasmid digested by *Bam*HI for generation of TP2 plasmids harboring two MIP cassettes (2xMIP). A TP4 plasmid was constructed by ligation between a linearized TP2 plasmid (digested with *Bam*HI) and two MIP cassettes (digested with *Bam*HI and *Bgl*II). Orientation of the MIP cassettes was confirmed by a restriction fragment length polymorphism (RFLP) analysis using restriction enzyme digestion (*Bam*HI, *Bgl*II, and *Eco*RI), and DNA fragments were analyzed by agarose gel electrophoresis. Each of these recombinant plasmids was transformed into *E. coli* TOP10F′, each recombinant *E. coli* clone was cultivated and plasmids were extracted and purified before transformation into yeasts.

2.3. Yeast transformation

Each recombinant plasmid (uncut plasmid) was transformed into three strains of *P. pastoris* as X-33, GS115 and KM71H and into *H. polymorpha* NRRL2214 by electroporation as suggested in the EasySelect™ *Pichia* Expression Kit Manual (Invitrogen) and modified from DNA-mediated transformation in molecular biology, second edition [15]. Briefly, for yeast competent cell preparation, 5% of overnight culture was inoculated into 50 mL of YPD medium and incubated with shaking at 30 °C, 300 rpm until it reached an OD_{600} of 1. Cells were harvested and resuspended in 10 mL of YPD medium and 2 mL of 1 M HEPES buffer (pH 8.0), and then, 250 μL of 1 M DTT was added and gently mixed. The cell suspension was incubated without shaking at 30 °C for 15 min. Cells were collected by centrifugation and washed 3 times: twice with sterilized ice-cold double distilled water and once with sterilized, ice-cold 1 M D-sorbitol. The cells were harvested by centrifugation and resuspended with 500 μL of sterilized, ice-cold 1 M D-sorbitol.

Eighty microliters of the cell suspension was then aliquoted into 1.5 mL sterilized microfuge tubes and stored on ice. Freshly prepared competent cells (80 μL) were mixed with 5–10 μg of uncut plasmid and transferred to an ice-cold 0.2 cm gap electroporation cuvette. Cells were pulsed with 2.5 kV by a MicroPulser™ Electroporator (Bio-Rad Laboratories Ltd., USA). The cell suspension was plated on YPD plate containing 100 μg mL^{-1} Zeocin™ final concentration and incubated at 30 °C for 2–4 days until colony formation.

2.4. Yeast cultivation and recombinant protein expression

A fresh single colony of each recombinant yeast strain was inoculated in 20 mL of YPG medium (1% yeast extract, 2% peptone, 1% glycerol), and grown at 30 °C overnight on a shaker incubator at 200 rpm. The overnight culture was adjusted to OD_{600} = 1.0 by spectrophotometric measurement and used as a starter culture. Ten percent of starter culture was inoculated into 50 mL of YPG medium and incubated with shaking at 250 rpm at 30 °C for 24 h (cell production phase). Cells were then collected by centrifugation at 2000 × g for 5 min followed by decantation of the supernatant. Next, the cells were resuspended in 50 mL of MMH induction medium (1.34% yeast nitrogen base, without amino acid, with ammonium sulfate), 4×10^{-5}% biotin, 0.004% histidine, and 0.5% methanol in a baffled flask and incubated with shaking at 250 rpm at 30 °C as described in the EasySelect™ *Pichia* Expression Kit Manual. In the induction phase, absolute methanol was added to a final concentration of 0.5% every 24 h to maintain induction. Every 24 h, 6 mL samples of cell culture were taken and centrifuged at 10,000 × g at 4 °C for 5 min. The supernatants were transferred to new sterile microfuge tubes and stored at −20 °C until further assays, and the pH was adjusted to 7.0 using 2.5 M NaOH before the ELISA assay, while the cell pellets were washed twice with TE buffer (pH 8.0) and stored at −20 °C until further use for assays.

2.5. Monitoring of MIP expression level by dot-blot analysis

A specific dot-blot procedure was adapted from Sithigorngul [16] and used to monitor the MIP secretion levels in culture broth. Insulin from bovine pancreas (Sigma Aldrich, USA) and Mixtard® 30 HM (Novo Nordisk, Denmark), which is the active substance in human insulin, were used as positive controls, whereas MMH medium was used as a negative control. Each of the standard insulins at various concentrations and supernatants was spotted onto the same nitrocellulose membrane. The membrane was dried at 60 °C for 5 min before immersion in 0.25% glutaraldehyde for 30 min. The membrane was then washed three times with double-distilled water, followed by immersion in blocking buffer, 5% skim milk in PBS buffer, for 1 h. After incubation and washing with washing buffer, 0.05% Tween 20 in PBS buffer (PBST), the membrane was incubated in a primary mouse monoclonal anti-insulin antibody (Sigma Aldrich, USA) (1:1500) at 4 °C overnight. The membrane was then washed three times with PBST and incubated in a secondary goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Jackson Immuno Research Laboratories Inc., USA)

(1:1500) at room temperature for 2 h. After washing with PBST, the membrane was visualized with a DAB substrate solution (0.03% 3,3′-diaminobenzidine, 0.03% H_2O_2, and 0.25% $CoCl_2$ in PBS) for 5 min. The immunoreactive spot was visually compared with that of standard insulin.

2.6. Quantitative determination of MIP concentration by indirect competitive ELISA

ELISA plates were coated with 100 µL per well of standard bovine insulin (2 µg mL^{-1}) and incubated at 4 °C overnight. Plates were washed three times with washing buffer (PBST) followed by blocking with 300 µL per well of blocking buffer (5% skim milk in PBS buffer) and incubated at 37 °C for 1 h. Plates were then rewashed followed by adding 50 µL per well of samples or standard insulin at various concentrations, which was used as a competitor, and 50 µL of a primary mouse monoclonal anti-insulin antibody (1:10,000) and incubated at 37 °C for 2 h. Plates were washed again, and 100 µL per well of a secondary goat anti-mouse IgG antibody (HRP conjugated) was added (1:10,000) and incubated at 37 °C for 1 h. Plates were washed three times, and TMB substrate solution (100 µL per well) was added, followed by incubation of the plates in the dark at room temperature for 15 min. Then, 100 µL of 1 M H_2SO_4 was added into the wells to stop the reaction. Optical density was measured at 450 nm, and the MIP concentration was calculated from the standard equation generated by the different standard insulin concentrations.

2.7. Determination of molecular mass of MIP by MALDI-TOF

An Autoflex MALDI-TOF-MS (Bruker Daltonics, Germany) was used to determine the molecular mass of the MIP protein in culture broth from recombinant *P. pastoris* KM71H harboring the TP1 plasmid.

2.8. Purification of MIP by ion-exchange chromatography and ultrafiltration

Supernatants were filtered through a 0.45 µm membrane filter before being applied on a SP Sepharose Fast Flow cation exchange column (size 1 mL) (GE Healthcare, USA). Supernatants were exchanged in 50 mM sodium citrate buffer (pH 3.0) containing 0.01 M NaCl. The MIP protein was eluted from the column using linear gradient elution by increasing the concentration of NaCl from 0.01 M to 1 M in a sodium citrate buffer (pH 3.0). Eluted MIP was further purified using ultrafiltration, 10 kDa MW cutoff Amicon centrifugal filter (Merck Millipore Ltd., Ireland) and desalinated in PBS buffer. This process enabled separation of proteins with a nominal membrane molecular weight limit of 10 kDa, with many advantages including high retentate recovery, direct pipettor sample access, and reduced processing steps to recover the concentrated residual. Samples were stored on ice until required for further assays. Total protein was determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA).

2.9. TPCK tryptic hydrolysis

The purified MIP was converted into an active form using immobilized TPCK trypsin (Thermo Scientific, USA) following the manufacturer's protocol. The reaction mixture was incubated in a shaking water bath for 2–18 h at 37 °C. Trypsin gel was separated from the digestion mixture by centrifugation at 1000 × g and 25 °C for 5 min.

2.10. 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, USA) under 37 °C with 5% CO_2 humidified incubator. The growth medium was replaced once a week, and cells were passaged once every two weeks [17].

2.11. Biological activity of MIP

H9c2 (2-1) rat myocardial cell line was seeded at a density of 1×10^6 cells per flask and cultured in growth medium at 37 °C with 5% CO_2 overnight. The growth medium was replaced in Dulbecco's modified Eagle's medium without glucose containing 10% fetal bovine serum (FBS) (Gibco, Australia), and the glucose concentration was adjusted to 10 mM (1.8 g L^{-1}) which approximates prediabetic levels. H9c2 cells were divided into three groups comprising the control group (not insulin treated), standard insulin group (Humalog® insulin for injection), and the recombinant monomeric insulin group. For the standard insulin group and recombinant monomeric insulin group, insulin concentrations were 0.70, 1.40, 2.80, 5.60, and 11.20 µg L^{-1}, while the control group had no insulin added. These cell lines were cultured at 37 °C with 5% CO_2 for 3, 6, 12, and 24 h. The culture medium was collected. H9c2 (2-1) cell lines were washed in normal saline and stored at −20 °C until required for further analysis.

2.12. RNA extraction and mRNA expression levels of MIP and GLUT4 by RT-qPCR

Total RNA of *P. pastoris* was extracted using the E. Z.N.A.® Yeast RNA extraction kit (Omega Bio-Tek, USA), while total RNA from the H9c2 (2-1) cell line was extracted using the E. Z.N.A.® Tissue culture RNA extraction kit (Omega Bio-Tek, USA). RNA concentration was measured using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Canada). cDNA synthesis was performed using a Tetro cDNA Synthesis Kit (Bioline, USA). The RT-qPCR reactions of *MIP*, *ACTIN*, *GLUT4* and *GAPDH* gene expression levels were performed using HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia) with specific primers (Table 1). The reaction was treated as follows: initial denaturation for 15 min at 95 °C, then 40 cycles of denaturation for 15 s at 95 °C, annealing for 20 s at 49 °C for *MIP* and *ACTIN* genes and 57 °C for *GLUT4* and *GAPDH* genes and extension for 20 s at 72 °C.

The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA) was used for mRNA expression analysis [17–19]. The relative amount of mRNA was calculated from the comparative quantification cycle (Cq) value using the *ACTIN* gene as reference and *P. pastoris* (pPICZαA) as a negative control for analysis of *MIP* mRNA expression levels, while *GAPDH* and H9c2 (2-1) nontreated insulin were used as a reference gene and control for *GLUT4* mRNA expression levels [13,17]. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method [18,19].

2.13. Measurement of glucose level using a YSI glucose analyzer

The glucose level in the culture medium was measured by YSI 2700 Select Biochemistry Analyzer (YSI Inc., USA).

Table 1
Oligonucleotides for RT-qPCR.

Oligonucleotides	Nucleotide sequences (5′ to 3′)
MIP forward primer	TTCGTCAACCAACACTTGTG
MIP reverse primer	GTTACAGTAGTTCTCCAATTG
ACTIN forward primer	GGTATTGCTGAGCGTATGCAA
ACTIN reverse primer	CCACCGATCCATACGGAGTACT
GLUT4 forward primer	AGCCAGCCTACGCCACCATA
GLUT4 reverse primer	GGACCCATAGCATCCGCAAC
GAPDH forward primer	CGGTGTGAACGGATTTGGCC
GAPDH reverse primer	TCATGGGGGCATCAGCGGAA

(a)

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CTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGATCA
                                M  R  F  P  S  I  F  T  A  V  L  F
AAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTC
GCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAA
 A  A  S  S  A  L  A  A  P  V  N  T  T  T  E  D  E  T  A  Q
ATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTT
 I  P  A  E  A  V  I  G  Y  S  D  L  E  G  D  F  D  V  A  V
TTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGC
 L  P  F  S  N  S  T  N  N  G  L  L  F  I  N  T  T  I  A  S
ATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTCAAG
 I  A  A  K  E  E  G  V  S  L  E  K  R  E  A  E  A  E  F  K
TTCGTCAACCAACACTTGTGTGGTTCCCACTTGGTCGAGGCTTTGTACTTGGTCTGTGGT
 F  V  N  Q  H  L  C  G  S  H  L  V  E  A  L  Y  L  V  C  G
GAAAGAGGTTTCTTCTACAAGGCTGCTAAGGGTATCGTCGAACAATGTTGTACCTCCATC
 E  R  G  F  F  Y  K  A  A  K  G  I  V  E  Q  C  C  T  S  I
TGCTCCTTGTACCAATTGGAGAACTACTGTAACTAGGCGGCCGCCAGCTTTCTAGAACAA
 C  S  L  Y  Q  L  E  N  Y  C  N  *
AAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGA
GTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGG
TCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGC
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(b)

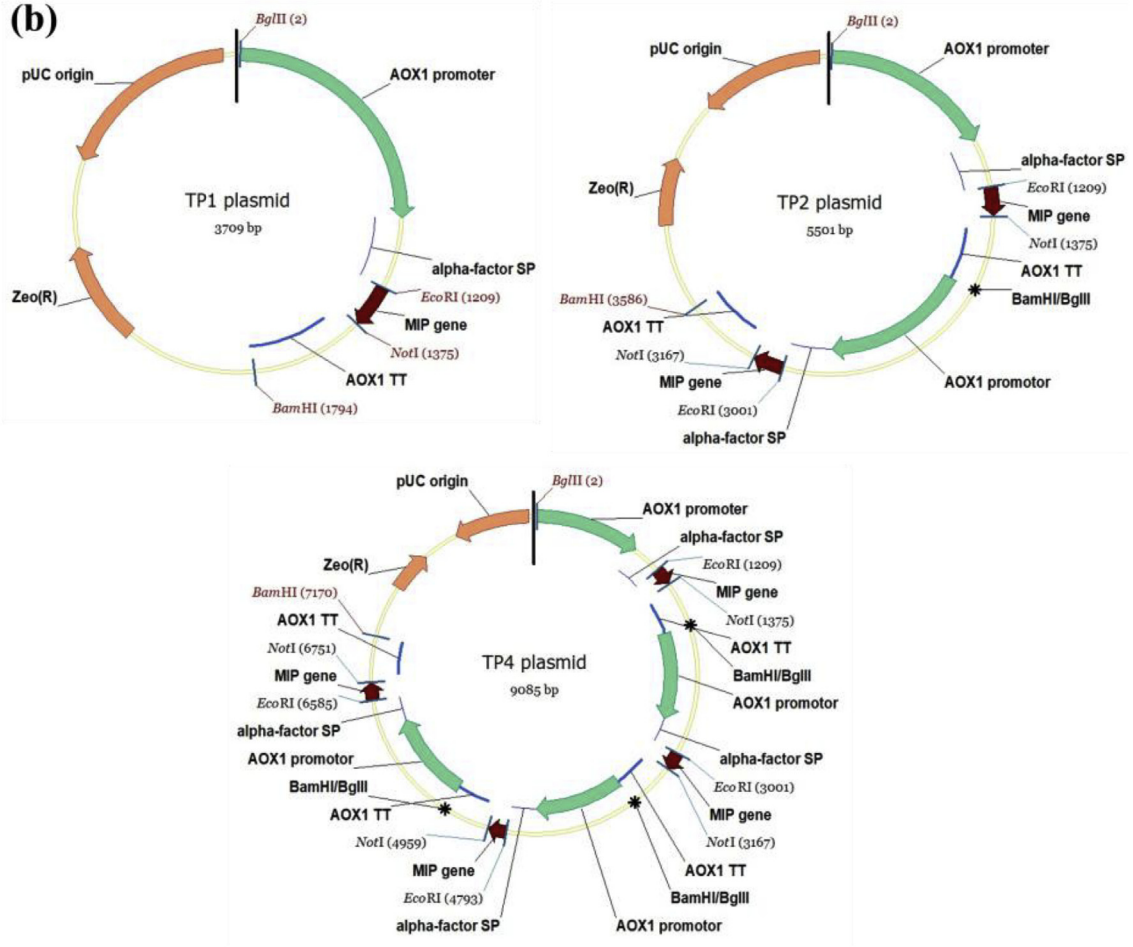

Fig. 1. Amino acid sequence of the MIP cassette and genetic maps of the recombinant plasmids. (a) Nucleotide and amino acid sequences of the MIP gene (bold) including the alpha-factor signal peptide (α-factor SP), restriction sites (underlined) for *Eco*RI and *Not*I and (b) genetic maps of TP1, TP2, and TP4 plasmid harboring one, two and four MIP gene cassettes, respectively.

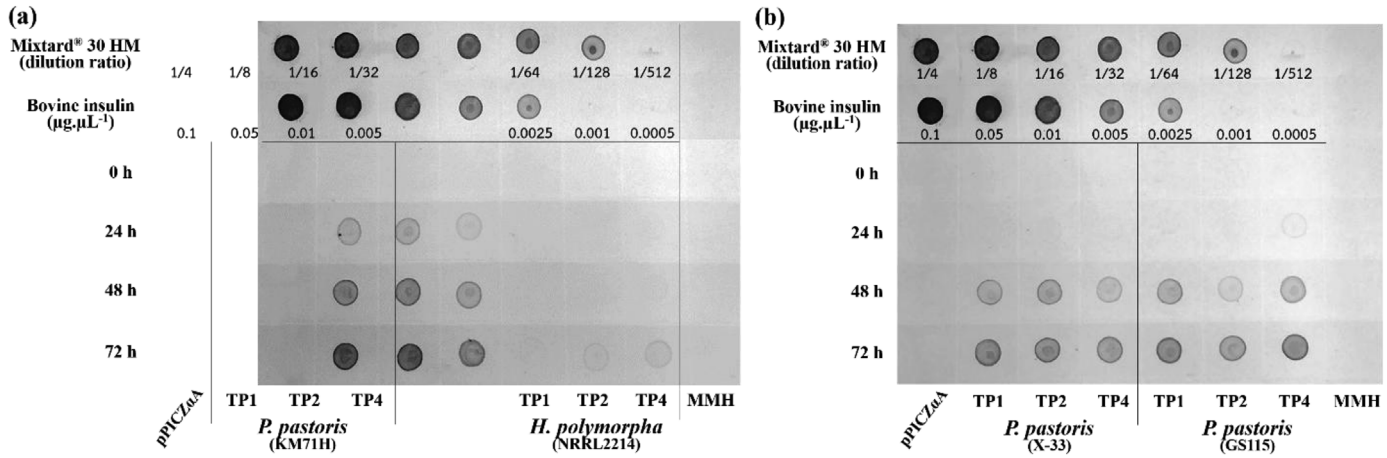

Fig. 2. Dot-blot analysis of the secreted MIP protein in the culture broth at different time points. (a) secreted MIP protein from recombinant *P. pastoris* KM71H and *H. polymorpha* NRRL2214 and (b) secreted MIP protein from recombinant *P. pastoris* X-33 and GS115. The Mixtard® 30 HM and bovine insulin were used as positive controls, while MMH medium and culture broth from *P. pastoris* harboring pPICZαA was used as negative control.

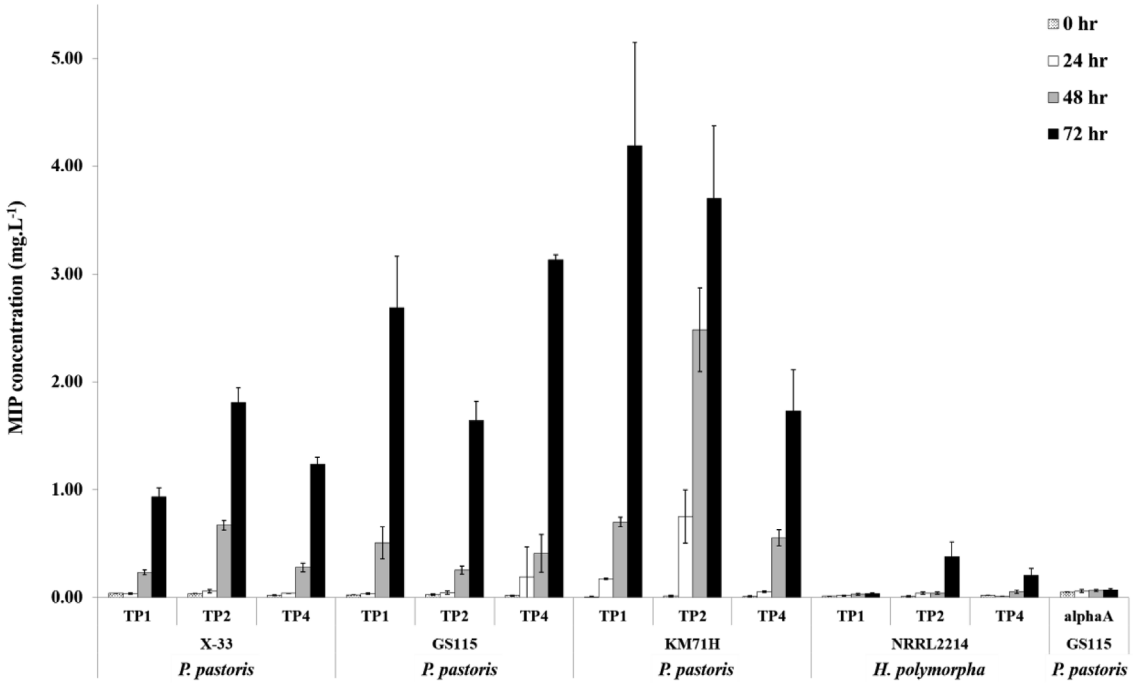

Fig. 3. Quantitative determination of secreted MIP protein in culture broth from recombinant yeasts, *P. pastoris* and *H. polymorpha* harboring pPICZαA, TP1, TP2 and TP4 plasmids at different time points. The results are shown as the mean ± standard deviation, n = 3 ($p < 0.05$).

3. Results and discussion

3.1. Recombinant plasmids

The TP1, TP2, and TP4 plasmids harboring *MIP* gene cassettes were successfully constructed in pPICZαA expression vector. Nucleotide sequence and amino acid sequences of MIP cassette and genetic maps of these recombinant plasmids are shown in Fig. 1. A RFLP analysis was performed to compare the different constructs using suitable restriction enzymes (*Bam*HI, *Bgl*II, and *Eco*RI) and analyzed by agarose gel electrophoresis (data not shown). The results demonstrated that an orientation of *MIP* gene cassette in all of these recombinant plasmids was head-to-tail direction as shown in Fig. 1 (b). Each recombinant plasmid was successfully transformed into yeasts for recombinant MIP production in methylotrophic yeast expression system.

3.2. Effect of yeast strains and gene copy number on MIP secretion

The MIP secretion levels in culture broth from recombinant *P. pastoris* (X-33, GS115 and KM71H) and *H. polymorpha* (NRRL2214) were directly detected by immuno-dot blot assay as shown in Fig. 2. MIP secretion levels in culture broth at different time points were compared with those of standard insulin. The results showed that recombinant *P. pastoris* KM71H strain, Mut^S phenotype strain, was the most efficient strain in secreting the MIP protein in culture broth, correlating with immunoreactive spot detection at 24 h and continuously increasing up to 72 h, while those of other strains (GS115 and X-33) were detected after 48 h after induction by methanol. The lowest MIP secretion level was derived from the recombinant *H. polymorpha* NRRL2214.

The MIP secretion levels in the culture broth were best at 24–72 h, as measured by ELISA. *P. pastoris* KM71H harboring TP1 had the highest MIP concentration (4.19 ± 0.96 mg L^{-1}) at 72 h. The production of MIP did not significantly increase by increasing the gene copy

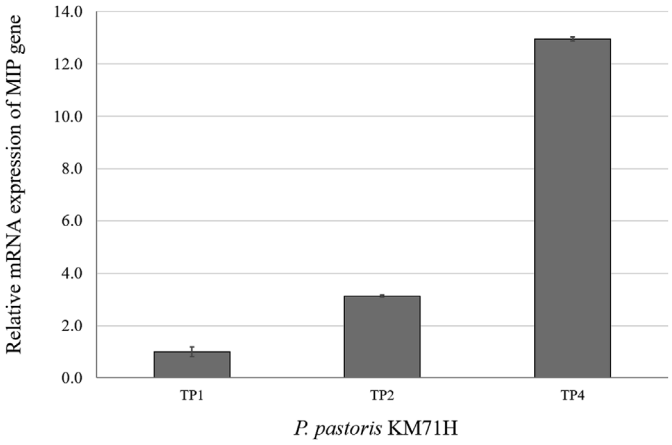

Fig. 4. Relative mRNA expression of *MIP* gene in *P. pastoris* KM71H harboring TP1, TP2 and TP4 plasmids at 24 h after being cultured in MMH induction medium determined by RT-qPCR using *ACTIN* as reference gene and normalized with TP1. Results are shown as the mean ± SEM, n = 3 ($p < 0.05$).

number (Fig. 3). Transcription levels of the *MIP* gene increased according to copy number of MIP cassettes in recombinant *P. pastoris* KM71H harboring TP1, TP2 and TP4 analyzed using RT-qPCR (Fig. 4). Thus, the low secretion ability of hosts harboring different gene copy numbers might relate to the structure of MIP, which consists of three disulfide bonds per molecule and secretory pathway limitations of the host cells. Similar observations were described by Zhu et al. (2009) [11] and Hohenblum et al. (2004) [20]. Secretion of recombinant proteins from yeast cells, membrane translocation, signal peptide, protein folding, disulfide bond formation and posttranslational processing are major bottlenecks. Inan et al. (2006) [21] attempted to increase secretion of a hookworm protein (Na-ASP1) by increasing *Na-ASP1* gene copy number and overexpressing protein disulfide isomerase (PDI) in *P. pastoris*. They found that PDI can improve productivity of the secreted recombinant Na-ASP1 protein in *P. pastoris*. For further studies, this evidence might be useful for the improvement of MIP secretion by increasing disulfide modification of the MIP structure.

3.3. Mass spectra of MIP

Supernatant from culture broth of the recombinant *P. pastoris* KM71H (TP1) was subjected to typical MALDI-TOF mass spectrometry for molecular mass determination. The highest peak was found to be MIP (*m/z* 5756.951), as shown in Fig. 5a, which is consistent with the bovine insulin standard shown in Fig. 5b.

3.4. Purification of MIP

SP Sepharose Fast Flow column chromatography was used to purify secreted MIP protein from culture broth following linear gradient elution procedure. Fig. 6 shows the chromatogram of secreted MIP purification with two peaks of protein including flow-through (fraction 2–12 CV, 10 mL) and elution (fraction 20–22 CV, 2 mL). Native polyacrylamide gel electrophoresis (native-PAGE) stained with Coomassie Blue (Fig. 7) was used for nonreduced protein determination in terms of size in each fraction. In the elution fraction (lane 4), the results showed that the MIP band can be seen at approximately 5 kDa and that it is more concentrated than the secreted MIP protein in the culture broth (lane 1). However, some impurities with a molecular weight at approximately 100 kDa could result from other proteins due to cell debris. Therefore, further purification steps are required to remove impurities from the elution fraction. In this study, ultrafiltration was used to purify the secreted MIP protein after purification by column chromatography. The results showed that a 10 kDa molecular weight cutoff can be used to purify the MIP protein as indicated in Fig. 7 (lane 5). Using a two-step purification procedure, the percentage recovery of MIP was 70% as shown in Table 2.

3.5. Biological activity of MIP

Lui et al. reported that insulin levels affected myocardial cell function by regulating *GLUT4* expression, while changes in *GLUT4*

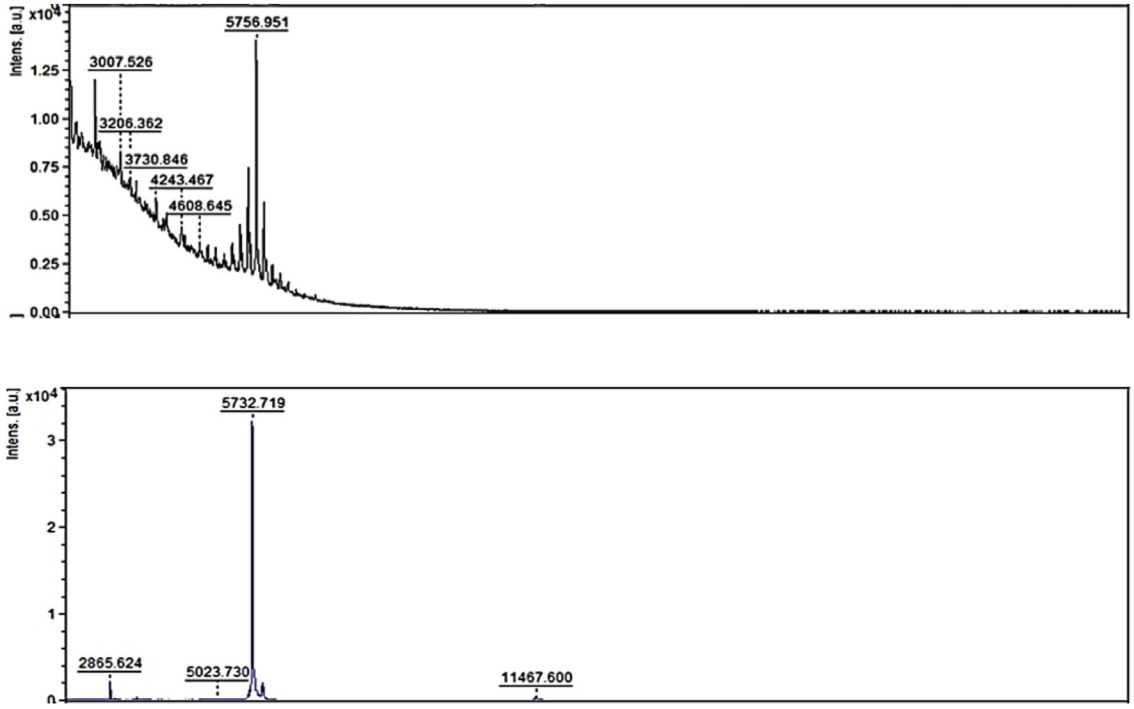

Fig. 5. Typical MALDI-TOF mass spectra analysis of secreted MIP protein. mass spectrometry of (a) secreted MIP in culture broth from *P. pastoris* KM71H harboring TP1 plasmid (*m/z* 5756.951) and (b) bovine insulin (*m/z* 5732.719), Sigma Aldrich, USA.

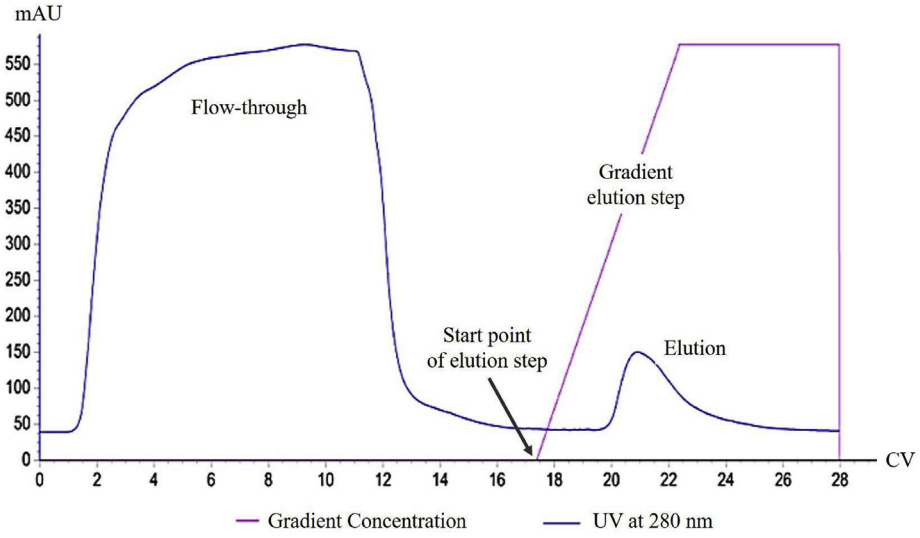

Fig. 6. Chromatogram of secreted MIP purification from culture broth by SP Sepharose Fast Flow chromatography. The MIP protein was eluted from the column using linear gradient elution by increasing the concentration of NaCl from 0.01 M to 1 M in a sodium citrate buffer (pH 3.0). The absorbance of the eluent was monitored at 280 nm.

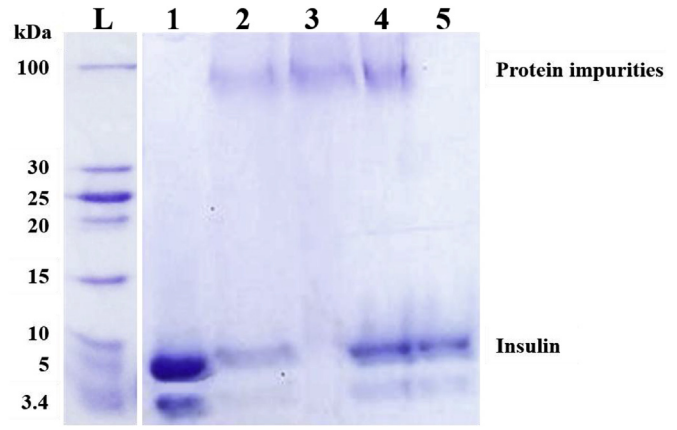

Fig. 7. Native-PAGE analysis of MIP protein. From left to right; L is a PageRuler™ unstained low-range protein ladder (#26632, Thermo Scientific), 1 is a Mixtard® 30 HM at 1 mg mL^{-1}, 2 is a culture broth at 24 h of *P. pastoris* KM71H harboring TP1 plasmid, 3 is a flow-through fraction from SP Sepharose Fast Flow chromatography, 4 is an eluted fraction from SP Sepharose Fast Flow chromatography, and 5 is purified MIP after SP Sepharose Fast Flow chromatography and ultrafiltration using 10 kDa molecular weight cutoff. Each sample was loaded at 20 µL and run in 15% acrylamide gel in Tris/glycine buffer.

Table 2
Protein and MIP content in each purification step using chromatography and ultrafiltration.

Sample[a]	Total protein[b] (µg)	MIP[c] (µg)	% Recovery of MIP
Supernatant	8527.27	39.63	100
Flow-through fraction	5424.54	1.88	–
Eluted fraction	925.09	34.17	86
Purified MIP	102.16	27.95	70

[a] Starting from 10 mL of supernatant.
[b] Total protein was determined by Pierce™ BCA Protein Assay Kit.
[c] MIP was determined by indirect competitive ELISA.

expression influenced glucose uptake in myocardial cells [13]. Measurement of glucose concentration in the culture medium to investigate MIP activity compared with standard insulin (Humalog®), showed that concentration of glucose in a medium of H9c2 (2-1) cells treated with Humalog® insulin and MIP was lower than that of non-insulin-treated cells (control) (Fig. 8). On the other hand, *GLUT4* mRNA transcription levels in H9c2 (2-1) cells were used for the MIP and Humalog® insulin biological activity assay. Clinical trials reported an action time for Humalog® insulin at 3–5 h after injection. The tryptic hydrolyzed MIP acts rapidly, similar to Humalog® insulin; therefore, *GLUT4* gene expression in H9c2 (2-1) cells was analyzed at 3 h after treatment with MIP or Humalog® insulin [22].

RT-qPCR is a powerful tool to quantify gene expression. In this research, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as a reference and the normalized expression of *GLUT4* gene was examined by normalization of the quantification cycle (Cq) values with the *GAPDH* gene. To calculate relative normalization expression, the normalized expression of *GLUT4* gene of non-insulin-treated H9c2 (2-1) cells was set as a control. The results showed that *GLUT4* gene expression was upregulated in H9c2 (2-1) cells treated with 11.20 µg L^{-1} of MIP and 2.80 µg L^{-1} of Humalog® insulin. These results indicated that MIP can induce glucose uptake and *GLUT4* expression of H9c2 (2-1) cells within 3 h, similar to Humalog® insulin. However, the effect of MIP on *GLUT4* gene expression is weaker than that of Humalog® insulin. According to different insulin concentrations in the culture media, *GLUT4* expression could not be detected when treated with lower concentrations of MIP and Humalog® insulin (Fig. 9). This observation may be a result of the low insulin conditions, with most GLUT4 being sequestered in intracellular vesicles in muscle and fat cells. As the vesicles fuse with the plasma membrane, GLUT4 transporters are inserted and become available for transporting glucose, and glucose absorption increases [23].

4. Conclusions

P. pastoris KM71H Mut^S (methanol utilization slow phenotype) strain is superior to *P. pastoris* strains X-33 and GS115, Mut$^+$ (methanol utilization plus phenotype) strains, and *H. polymorpha* NRRL2214 in terms of recombinant protein production. The transcription levels of the *MIP* gene increased in proportion to the copy number of MIP expression cassettes. However, the secreted MIP levels in the culture medium were unrelated to gene copy number. This limitation of a secretory pathway may be a bottleneck for overexpression of high-copy-number recombinants. Purified MIP shows biological activities on H9c2 (2-1) cells, similar to Humalog® insulin in terms of glucose uptake, and upregulates the *GLUT4* gene involved in glucose uptake. Moreover, since time-action profiles may vary in different types of insulin or at different concentrations, expression of the *GLUT4* gene in the H9c2 (2-1) cell line was upregulated by MIP at 3 h. However, the mechanisms associated with the MIP regulation of transcription of *GLUT4* mRNA or function of the GLUT4 protein have to be further studied.

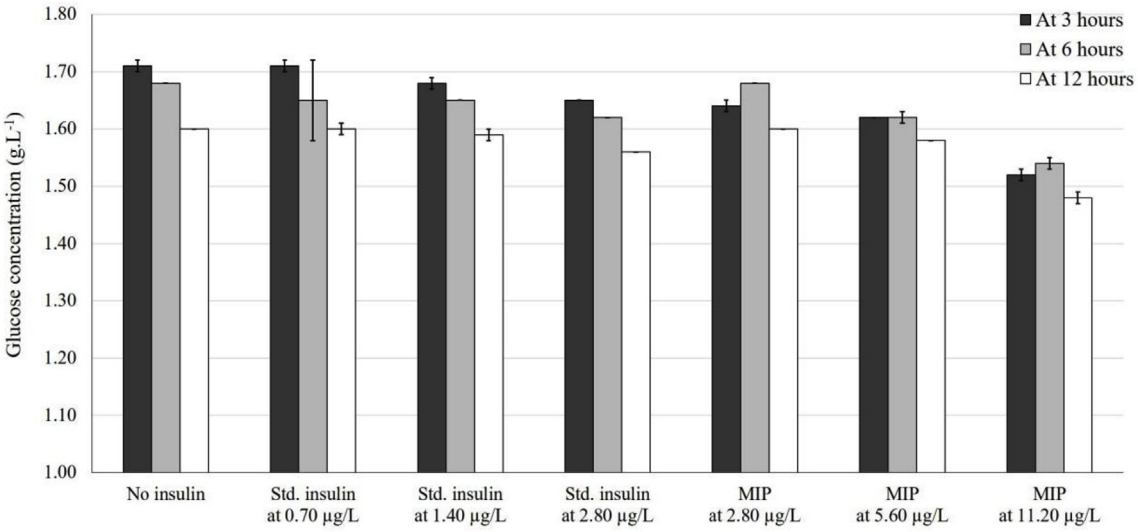

Fig. 8. Quantitative analysis of glucose concentration in culture medium of H9c2 (2-1) cells after treatment with standard insulin (Humalog®) and MIP at various concentrations at different time points. The results are shown as the mean ± SD, n = 3 ($p < 0.05$).

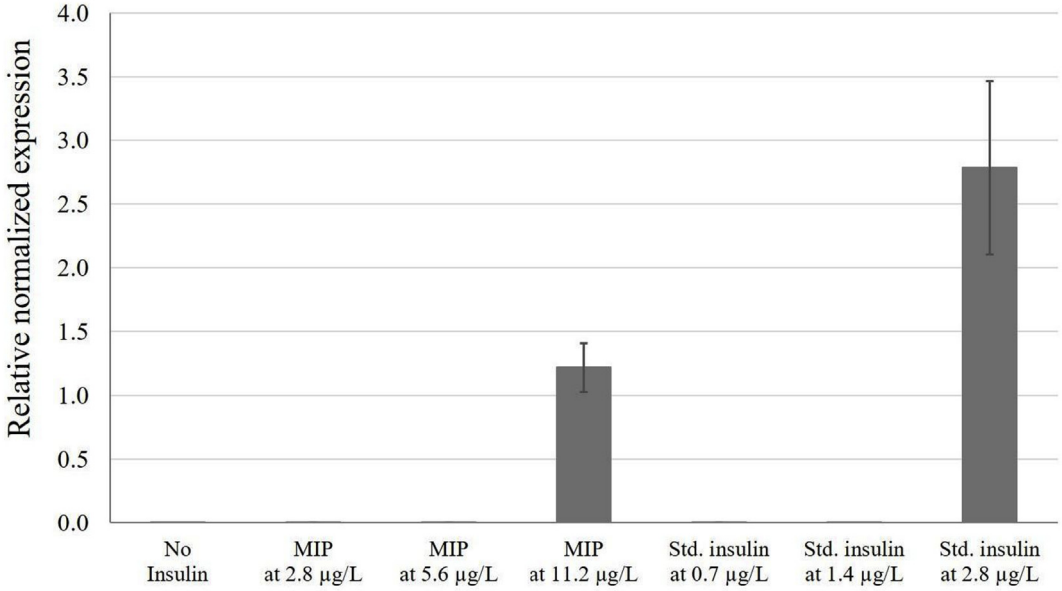

Fig. 9. Relative mRNA expression of the *GLUT4* gene in H9c2 (2-1) cells at 3 h after treatment with standard insulin (Humalog®) and MIP at various concentrations using RT-qPCR. The results are shown as the mean ± SEM, n = 3 ($p < 0.05$).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pep.2018.08.002.

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