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RESEARCH PAPER

Efficient Expression of Glucagon-like Peptide-1 Analogue with Human Serum Albumin Fusion Protein in *Pichia pastoris* Using the Glyceraldehyde-3-phosphate Dehydrogenase Promoter

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Abstract Glucagon-like peptide-1 (GLP-1) was a potential therapeutic drug for type II diabetes, mainly because of the stimulatory effect on insulin secretion under condition of high blood glucose. We used PCR to obtain a recombination gene, GGH, in which two GLP-1 (GLP-1A_2G) mutants were connected in series and then fused to the N terminal of human serum albumin. The fusion gene was inserted into pGAPZaA plasmid with *Saccharomyces cerevisiae* α-factor secretion signal sequence, and was expressed by the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter. The engineered strain was constructed by integrating the recombinant plasmid pGAPZαA/GGH into the genome of *Pichia pastoris* GS115. Genome PCR and western blot showed that the recombinant *P. pastoris* successfully expressed the fusion protein GGH. The yield of GGH reached 78 mg/L after 72 h fermentation in a flask, using glucose as the optimal carbon source. Fed-batch fermentation was investigated in a 5 L bioreactor, and the expression level of GGH reached 246 mg/L in 52 h. The fusion protein GGH was purified in four steps, and the final purity was 96.1%. The *in vitro* bioactivity of GGH was the same as that expressed in *P. pastoris* by the AOX1 promoter. This study described an efficient way to express GGH fusion protein in *P. pastoris* using GAP promoter, fermentation was easier to control without carbon source change and fermentation time was 20 h less than AOX1 promoter-controlled GGH fermentation.

Keywords: GAP promoter, GGH fusion protein, constitutive expression, *Pichia pastoris*, fed-batch fermentation

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1. Introduction

Glucagon-like peptide-1 (GLP-1) is secreted by gut endocrine L-cells in two major molecular forms, GLP-1 (7-36) amide and GLP-1 (7-37) [1]. GLP-1 stimulates insulin secretion in high intravenous glucose, and does not induce hypoglycemia when intravenous glucose is normal. It also enhances insulin sensitivity, prevents glucagon secretion, controls the feeling of satiety, and inhibits gastric emptying [2,3]. A recombinant GLP-1 analogue has become a potential therapeutic peptide for type II diabetes mellitus. However, the peptide is quickly degraded *in vivo* by dipeptidyl peptidase IV (DPP IV), and its half-life in plasma is only about 5 min [4]. Gao [5] extended GLP-1 by adding a lysine residue at the N-terminal to avoid DPP IV proteolysis, then added human serum albumin (HSA) to the C-terminal of the GLP-1 analogue. The fusion protein was more stable, but the activity was lower. Giguer [6] designed 27 mutations of GLP-1, including N-, C-, and mid-chain modifications, to avoid proteolysis and obtain high bioactivity, the results indicated that specific N- and mid-chain modifications

increased the potency *in vivo*. Even though mutated GLP-1 evaded proteolysis, its half-life in blood was still less then 30 min [6], because its low molecular weight (3.4 kDa) allowed rapid clearance by the kidney.

The yeast *Pichia pastoris* is an efficient host for expressing heterologous proteins [7], of which more then 1000 had been expressed by the end of 2013 according to the National Center for Biotechnology Information. The promoter from the alcohol oxidase 1 (AOX1) gene has been widely used to express foreign genes in *P. pastoris* [8]. Glyceraldehyde-3-phosphate dehydrogenase (GAP) is a key enzyme for glycolysis. The GAP promoter was first found by Waterham [9] in 1997 and had been used to constitutively express heterologous proteins. The process for heterologous protein expression was easy to control, because the whole process used only one carbon source like glucose or glycerol to generate biomass and expressed the heterologous protein. Chen constructed an engineered *P. pastoris* to express goat lactoferrin (GLF) using the GAP promoter, and the yield was as high as 2 mg/mL. The papain-inhibiting property, thermal stability of purified rGLF, and iron-binding behavior did not significantly differ from native GLF [10]. Human chitinase had been expressed in *P. pastoris* with the GAP promoter, and the proteolytic degradation was clearly greater than with the AOX1 promoter expression system [11]. Thus, the GAP promoter expression system in *P. pastoris* was a convenient and cost-effect method for large-scale fermentation [12].

In this study, we expressed the heterologous protein GGH in *P. pastoris* using the GAP promoter. In the GLP-1 mutant, the second amino-acid Gly was mutated to Ala, which avoids DPP IV proteolysis [13]. And the fusion protein gene (named GGH), which was two concatemers of the mutant GLP-1 (GLP-1A_2G) fused to the N-terminal of HSA, was constructed to increase the half-life. In previous study, Dou [13] expressed GGH using the AOX1 promoter in *P. pastoris*. The GGH fusion protein was constitutively expressed with the GAP promoter in *P. pastoris* in this study, and then the activity of the two proteins with different promoters were compared *in vitro*.

2. Materials and Methods

2.1. Plasmid strains and reagents

Restriction enzymes, pfu DNA polymerase, and T4 DNA ligase were from Thermo Scientific. Plasmid pGAPZaA, *Escherichia coli* DH5α and *P. pastoris* were retrieved from our laboratory bank. The Blue Sepharose Fast Flow, G25 Sepharose, DEAE Sepharose Fast Flow and the column were from GE Healthcare. All other reagents were of analytical grade.

2.2. Construction of expression vector pGAPZαA/GGH

The GGH gene fragment was obtained by PCR, using pPIC9K/(Glp-1)$_2$-HSA as the templates and specific primers (primer 1: GCGAATTCCACGGTGAAGGTACTTTCA; primer 2: GAGTGCGGCCGCTTATTATA AGCCTAAG). The gene fragment was digested by *Eco*R I and *Not* I, and then inserted into the multiple cloning site of pGAPZαA (Invitrogen). The constructed plasmid pGAPZαA/GGH was transformed into competent *E. coli* DH5α, then selected on LB agar plates (0.5% yeast extract, 1% tryptone, 0.5% NaCl) with 25 μg/mL zeocin (Invitrogen, Shanghai) incubation for 16 h. The recombinant plasmid pGAPZαA/GGH was obtained and verified by *Eco*R I and *Not* I digestion, and the sequence was confirmed by the Shanghai Sangon sequence service.

2.3. Screening for high-secretion clones and selection of carbon sources

The confirmed recombinant expression vector pGAPZαA/GGH was linearized by *Avr* II, and then transformed into competent *P. pastoris* GS115 cells by electroporation using 1.5 kV, 40 μF, and 180 Ω. Competent *P. pastoris* GS115 cells were prepared using standard methods [14]. Recombinant-positive clones were selected on YPDS plates (2% peptone, 1% yeast extract, 2% glucose, 1.5% agar plus 1 M sorbitol) containing 100 μg/mL zeocin for 3 days. Potential high-level recombinant clones were selected on YPDS agar plates containing a higher zeocin concentration (800 μg/mL) for 3 days. Finally the highest expression clones were detected using a trace urinary albumin kit (Min Dian Co., Shanghai). The protein expression process was carried out by following the Invitrogen guidelines. The high-level secretion clone was confirmed by genomic PCR using the above primers.

The selected clone was cultured in a 50 mL flask with 10 mL YPD medium for 24 h. The culture was then inoculated to an optical density (OD_{600}) of 0.5 to start cell growth and protein expression in different modified YP media (1% (w/v) yeast extract, 2% (w/v) tryptone) supplemented with variety of carbon sources (2% (w/v) glucose, 2.045% (w/v) glycerol, 2.134% (w/v) methanol, 1.727% (w/v) sucrose, 1.838% (w/v) mannitol, or 1.838% (w/v) sorbitol). Samples were collected every 4 h, and the OD_{600} and protein expression measured. All the data were displayed as the mean ± SD of triplicates.

2.4. Fed-batch fermentation

Fermentation studies were carried out in 5 L stirred tank bioreactors (Baoxing Co., Shanghai). One hundred milliliters of seed culture was inoculated into a 5 L fermenter containing 2.4 L modified complex medium (40 g/L tryptone, 20 g/L yeast extract, 20 g/L glucose, 4.8 g/L $KH_2PO_4 \cdot 3H_2O$,

10.7 g/L K_2HPO_4, 10 g/L NH_4SO_4, 3.4 g/L yeast nitrogen base, pH 6.0). At the start of fermentation, the temperature was set at 30°C and then reduced to 25°C at the beginning of the glucose-adding phase. The level of dissolved oxygen was maintained between 20 and 30% by adjusting the agitation and aeration rates. The pH was maintained at 6.0 by addition of NH_4OH or 30% (v/v) H_3PO_4. Fermentation broth was collected at 4 h intervals to measure the glucose level with a biochemical analyzer, glucose was added to maintain the level at 5 g/L and the glucose consumption was calculated.

2.5. Purification of GGH from culture supernatant

After 52 h of fermentation, the broth was collected and centrifuged for 20 min at 8,000 rpm (4°C). The supernatant was then concentrated by ultrafiltration using the Millipore Flow Filtration System followed by purification. The concentrated sample was loaded onto a Blue Sepharose Fast Flow column (1.6 × 18 cm) pre-equilibrated with buffer A (20 mM NaPB, 100 mM NaCl, pH 7.2), washed with buffer B1 (20 mM NaPB, 2 M NaCl, pH 7.2), and then eluted with buffer B2 (1 M arginine, pH 7.2). The B2 eluted sample was further loaded onto a G25 sepharose column (1.6 × 15 cm) eluted with buffer C (20 mM NaPB, pH 7.2). The eluted fraction was further loaded onto a DEAE Sepharose Fast Flow column (1.6 × 12 cm), balanced with buffer D (20 mM NaPB, pH 7.2), and the final pure protein eluted with buffer E (20 mM NaPB, 0.5 M NaCl, pH 7.2). The concentration of GGH fusion protein was determined using a trace urinary albumin kit.

2.6. SDS-PAGE, western blot, and HPLC analysis

SDS–PAGE analysis was performed using 12% gel according to Laemmli's method [15]. The separated proteins were transferred to a nitrocellulose membrane and detected using anti-human GLP-1 monoclonal antibody (ab23468, Abcam) and anti-HSA monoclonal antibody (ab31898, Abcam), each at a dilution of 1:1,000. Immunoreactivity was assessed using chemiluminescent reagent (ECL) as described by the manufacturer. HPLC analysis was performed as described by Kobayashi [16].

2.7. Bioactivity assay of GGH *In vitro*

A stable human embryonic kidney 293 (HEK293) cell line that was co-transfected with glucagon-like peptide-1 receptor (GLP-1R) and CRE-fFlu (cAMP response element connected with firefly luciferase gene) has been constructed [17], and this cell line was used to assess the bioactivity of the GLP-1 analogue. The intracellular cAMP doubled [2] after the GLP-1 analogue activated GLP-1R in the HEK293/GLP-1R/CRE-fFlu cell model. The fFlu expression is upregulated by the phosphorylation of cAMP response protein through the cAMP/PKA pathway. The HEK293/GLP-1R/CRE-fFlu cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in an incubator with 5% CO_2 at 37°C. Log-phase cells were diluted to 8.0×10^5/mL, and then incubated 100 μL in 96-well plates for 12 h. Then the medium was removed, the cells washed with PBS. Then 100 μL of GGH at a series of double-dilutions in FBS-free DMEM was added and incubation continued for 4 h. Then the intracellular firefly luciferase was measured with a luciferase reporter gene detection kit (Sigma, luc1).

The rat beta pancreatic cancer cell (RIN-m5F, ATCC, CRL-11605) is a natural model that expresses GLP-1R. Log-phase cells were diluted to 8.0×10^5/mL, and then incubated in a volume of 300 μL on 24-well plates for 12 h. After removing the medium and washing the cells with PBS, 300 μL of GGH in FBS-free DMEM was added and incubated for 20 min. The intracellular cAMP content was assessed with a cyclic AMP assay kit (R&D, KGE002B). All the data were displayed as the mean ± SD of triplicates.

3. Results and Discussion

3.1. Recombinant strain construction, screening, and characterization

The DNA fragment GGH was obtained by PCR, and inserted into the pGAPZαA in the site of *Eco*R I and *Not* I. The recombinant expression vector was verified by *Eco*R I and *Not* I restriction endonucleases and the resulting DNA fragment was the expected size (Fig. 1B). The DNA sequencing was accorded with the expected sequence (data not shown). The identified recombinant plasmid pGAPzαA-GGH was linearized by *Avr* II and transformed into

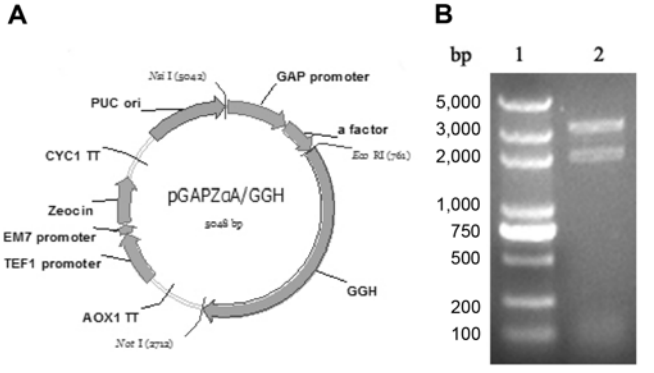

Fig. 1. Construction of the recombinant plasmid pGAPzαA-GGH. (A) Schematic of the recombinant plasmid. (B) Electrophoresis map of the recombinant plasmid with restriction endonucleases; lane 1, molecular weight marker; lane 2, digestion of recombinant plasmid with *Eco*R I and *Not* I.

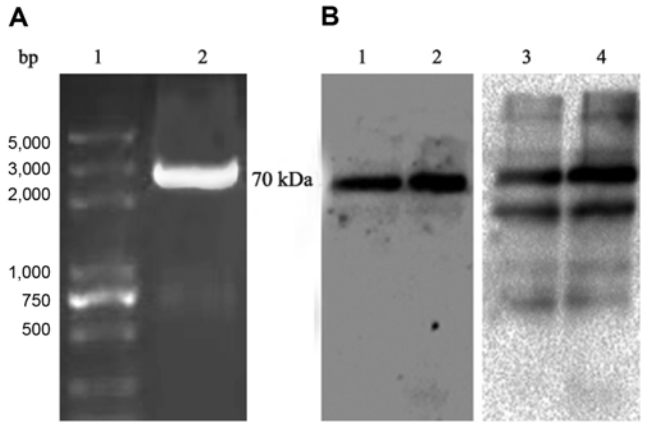

Fig. 2. Confirmation of recombinant *P. pastoris* clones. (A) Electrophoresis of PCR product from genomic DNA PCR; lane 1, molecular weight marker; lane 2, PCR product of genomic DNA. (B) Western blot (WB) of fermentation broth and intracellular protein; lanes 1 and 2, WB of intracellular protein and fermentation protein with anti-HSA antibody; lanes 3 and 4, WB of intracellular protein and fermentation protein with anti-GLP-1 antibody.

P. pastoris GS115 cells by electroporation.

Recombinant yeast clones with high potential expression levels were selected on YPDS plates (YPD plus 1 M sorbitol) containing a high zeocin concentration (800 μg/mL). Eight clones were chosen for expression analysis on YPD medium, and one clone was chosen for further study with the highest yield of 48 mg/L GGH. This clone was confirmed by genomic DNA PCR analysis (Fig. 2A). Western blot analysis showed that the expressed protein was recognized specifically by the anti-GLP-1 and anti-HSA antibodies, indicating that the expressed protein was the recombinant chimeric protein GGH

3.2. Effects of different carbon sources on GGH expression with the GAP promoter

In order to find the best carbon source for growth and expression, we added six different carbon sources (2% (w/v) glucose, 2.045% (w/v) glycerol, 2.134% (w/v) methanol, 1.727% (w/v) sucrose, 1.838% (w/v) mannitol, or 1.838% (w/v) sorbitol) to YP medium. The different weight of each carbon source was to insure the same mole of carbon source. The dry cell weight using glucose and glycerol as carbon sources reached 6.5 g/L and 5.2 g/L after 40 h, respectively, while that using methanol and the other carbon sources was less then 18 (Fig. 3). This result demonstrated that glucose and glycerol are the best carbon sources for *P. pastoris* growth.

The GGH yield in broth reached 78 and 62 mg/L after 72 h fermentation using glucose and glycerol as carbon sources. Due to the limited sensitivity of the Micro Albumin test kit, the yield of GGH with the other carbon sources was not detectable (Fig. 4). These results suggested that glucose was more suitable for GGH expression using the GAP promoter.

3.3. Fed-batch fermentation

To evaluate the feasibility in scaling-up fermentation using the GAP promoter to express GGH in *P. pastoris*, we chose glucose as the sole carbon source in a 5 L bioreactor. The dry cell weight increased to about 41.2 g/L after 32 h fermentation. The rate of glucose consumption reached as high as 2 g/L/h when the cell density was increasing rapidly, then remained at about 0.5 g/L/h after 32 h. The yield of GGH increased in parallel with cell density, and reached a peak of 246 mg/L at 52 h.

Dou [13] had expressed GGH with AOX1 promoter, and

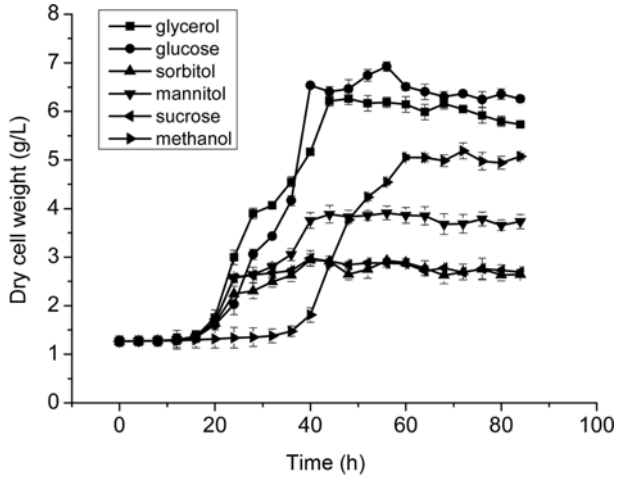

Fig. 3. Growth curves with different carbon sources in flask. Sample was analyzed every 4 h, and baked in 80°C for 16 h to measure dry cell weight.

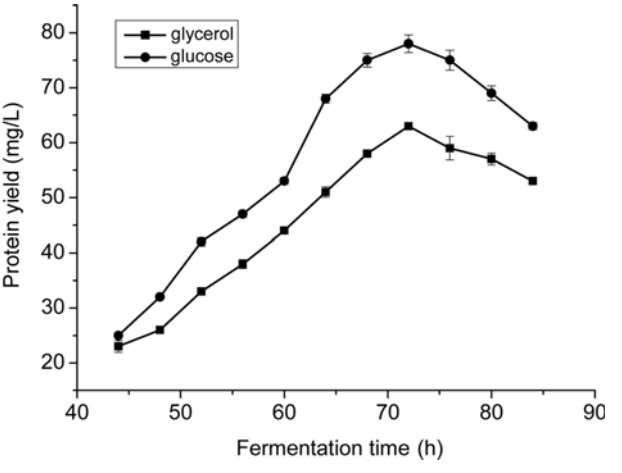

Fig. 4. Protein yield with glycerol and glucose carbon sources in flask. Sample was analyzed every 4 h, and protein concentration was measured by Micro Albumin Test Kit.

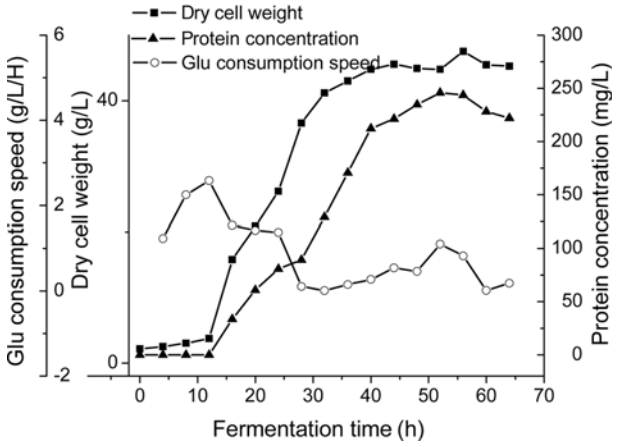

Fig. 5. The process in 5 L fermentation. Dry cell weight, protein concentration, and rate of glucose consumption were measured every 4 h. Dry cell weight was measured by baked in 80°C for 16 h. Protein concentration was measured by Micro Albumin Test Kit. Rate of glucose consumption was measured by glucose decreased speed.

the high yield of GGH reached at 238 mg/L at 72 h. The process of expressing foreign genes with the AOX1 promoter consisted of two phases using two different carbon sources. *P. pastoris* consumed glucose or glycerol to generate biomass in the first phase, and then used methanol as the carbon source in the second phase. All the growth-inhibitory carbon sources like glucose or glycerol should be removed before adding the methanol as inducing carbon. The change of carbon source and the speed of addition of methanol were difficult to control, which led to failure of fermentation [18,19]. What more, methanol was toxic and easily caused environment pollution during fermentation [20]. Due to its toxicity, addition of excessive methanol inhibits the metabolism of *P. pastoris*, induced cell death, and released intracellular proteases that degraded the secretory proteins. When methanol was added less, the metabolism of *P. pastoris* was also inhibited due to the limited carbon source and led to low protein yields. The process of expressing foreign genes with the GAP promoter was convenient because it used glucose alone as the carbon source and fermentation time was 20 h less than AOX1 promoter-controlled GGH expression.

Table 1. Summary of purification process for the fusion protein GGH produced by *P. pastoris* with the GAP promoter

Purification step	Volume (mL)	GGH (mg)	Purity (%)	Cumulative yield (%)
Supernatant	380	86.8	-	100.0
Ultrafiltration	48	73.2	-	84.3
Blue sepharose	32	32.8	79.3	37.8
G25 sepharose	35	29.6	79.3	34.1
DEAE sepharose	28	18.5	96.1	21.3

The protein was purified by 4 steps. Protein concentration was measured by Micro Albumin Test Kit. Protein purity was measured by HPLC. Cumulative protein yield was calculated by total protein recovery.

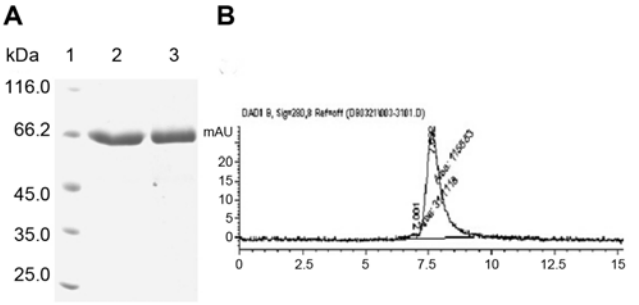

Fig. 6. Protein purity assessed by HPLC and 12% SDS-PAGE. (A) SDS-PAGE of final purified protein; lane 1, protein molecular weight marker, lanes 2 and 3, samples of final purified protein. (B) HPLC of final purified protein, and purity was 96.1%.

3.4. Purification of the GGH fusion protein determination of bioactivity *In vitro*

The recombinant protein was purified from the supernatant in 4 steps: ultrafiltration, blue sepharose affinity chromatography, G25 desalination, and DEAE sepharose ion-exchange chromatography. After the 4 steps, the purity of the recombinant protein reached 96.1%, with a final recovery yield rate of 21.3% (Table 1). The purified protein presented a single band on 12% SDS-PAGE at about 70 kDa (Fig. 6A), which is close to the theoretical molecular weight for this fusion protein. The HPLC analysis (Fig. 6B) confirmed the purity of the GGH protein.

Previously, Dou expressed the GGH fusion protein in *P. pastoris* using the AOX1 promoter [13], we further compared the bioactivity of the purified GGH expressed using

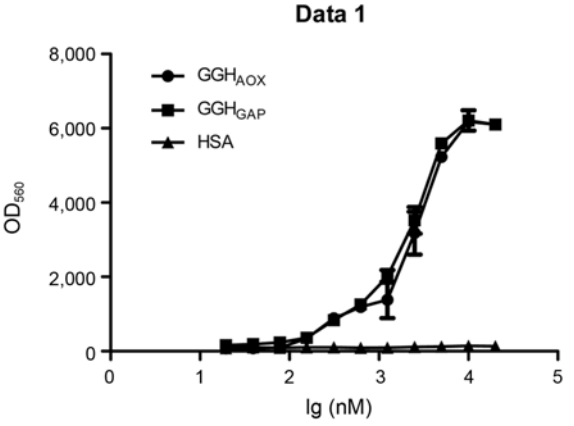

Fig. 7. Bioactivity of GGH in HEK293/GLP-1R/CRE-fFlu cells line. The fFlu expression was in parallel with concentration of intracellular cAMP. The intracellular firefly luciferase was measured by Luciferase Reporter Gene Detection Kit (Sigma, luc1).

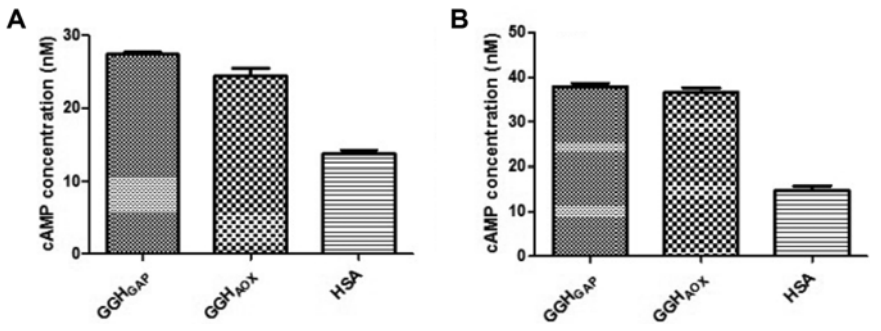

Fig. 8. Bioactivity of GGH in RINm5f cells line. (A) The cAMP concentration in RINm5f cells stimulated by 200 nM GGH_{GAP} or GGH_{AOX}. (B) The cAMP concentration in RINm5f cells stimulated by 2000 nM GGH_{GAP} or GGH_{AOX}. The intracellular cAMP content was assessed with Cyclic AMP Assay Kit (R&D, KGE002B).

AOX1 and GAP promoter. GGH_{GAP} was the GGH protein which was expressed by *P. pastoris* with GAP promoter; while GGH_{AOX} was the GGH protein which was expressed by *P. pastoris* with AOX promoter. EC_{50} of GGH_{GAP} was 2,876 nM and EC_{50} of GGH_{AOX1} was 3,251 nM (Fig. 7). The capacity of both forms of GGH to stimulate expression of the firefly luciferase gene (fLuc) was similar when HSA was used as a negative control. In the native cell (RINm5f) model, the concentration of cAMP was 27.4 ± 0.28 and 24.3 ± 1.1 nM when stimulated by 200 nM GGH_{GAP} and GGH_{AOX1}, while the cAMP concentration was 37.9 ± 0.74 and 36.6 ± 1.0 nM when stimulated by 2,000 nM GGH_{GAP} and GGH_{AOX1}. The cAMP concentration stimulated by GGH in RINm5f showed no significant differences between two fusion proteins (Fig. 8).

4. Conclusion

In this work, we constructed a *P. pastoris* strain that expressed GGH under control of the GAP promoter. Dou [13] had constructed *P. pastoris* with the AOX1 promoter to express the GGH fusion protein. The yield of broth reached 246 mg/L in 52 h using the GAP promoter while the yield was 238 mg/L in 72 h using the AOX1 promoter. The process was convenient and the fermentation time was reduced. The EC_{50} of GGH was 2,876 nM with the GAP promoter and 3,251 nM with the AOX1 promoter. The protein activity and yield were similar to those with the AOX1 promoter. In addition, the fermentation time was less 20 h and the process was more convenient without change the carbon source.

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Nomenclature

GGH : $(GLP\text{-}1A_2G)_2$-HSA
AOX1 : Alcohol oxidase 1
GAP : Glyceraldehyde-3-phosphate dehydrogenase

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