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ORIGINAL RESEARCH PAPER

Expression of a glucagon-like peptide-1 analogue, as a therapeutic agent for type II diabetes, with enhanced bioactivity and increased *N*-terminal homogeneity in *Pichia pastoris*

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Abstract
Objective To improve the bioactivity and increase the *N*-terminal homogeneity of a glucagon-like peptide-1 (GLP-1) analogue expressed in *Pichia pastoris*.
Results The GLP-1 analogue. GGH, consisting of two tandem mutant GLP-1 (GLP-1[A2G]) fused with the *N*-terminus of human serum albumin (HSA), was expressed in *P. pastoris*. We also designed and expressed the novel GLP-1 analogue NGGH, which had a His-tag fused with the *N*-terminus of GGH and an enterokinase (EK) cleavage site at the fusion junction. The His-tag was removed by EK digestion to yield GGH_2, which was subsequently compared with GGH expressed in *P. pastoris*. The purification recovery of GGH_2 was 35 % compared with 23 % for GGH. Furthermore, the bioactivity of GGH_2 was 605 % higher than GGH, and *N*-terminal homogeneity was also improved.
Conclusions A simple method for the preparation of GGH_2 with a cleavable His-tag was developed, and the resultant protein possessed improved bioactivity and *N*-terminal homogeneity.

Keywords *Diabetes mellitus* · Enterokinase · Glucagon-like peptide-1 · His-tag · Homogeneity · Human serum albumin · *Pichia pastoris*

Introduction

Glucagon-like peptide-1 (GLP-1) analogues have potential as therapeutic agents for type II diabetes mellitus (Rosenstock and Stewart 2010). However, GLP-1 is rapidly degraded by dipeptidyl peptidase IV (DPP IV) in vivo, and its half-life is only 2 min in plasma (Chung et al. 2009), although N-chain specific modifications can increase its half-life (Qian 2001). *Pichia pastoris* is an efficient system for heterologous protein expression; however, degradation of secreted proteins can limit the effectiveness of this host. Degradation occurs during secretion, protein folding in the endoplasmic reticulum, and signal transduction (Schroder 2008) and is the result of the activities of signal peptidase and serine, aspartic and extracellular proteases (Zhang et al. 2007). Disruption of two dipeptidyl aminopeptidase family genes reduced

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proteolysis of the *N*-terminus of expressed proteins (Prabha et al. 2009) and disruption of *yps1* similarly decreased protein degradation (Yao et al. 2009). Despite advances in minimizing protein degradation in *P. pastoris,* this complicated process remains poorly understood and there is need for further research.

In an attempt to prevent digestion by DPP IV, we mutated the second amino-acid of GLP-1 to Ala, and a two tandem mutant GLP-1 (GLP-1A_2G) was fused with the *N*-terminal of human serum albumin (HSA) to increase the half-life in serum. Due to its low MW (3.2 kDa), the mutated GLP-1 analogue was rapidly cleared from plasma via glomeruli filtering. A recombinant *P. pastoris* has been engineered to express GGH from the AOX1 promoter (Dou et al. 2008). To improve the homogeneity of the expressed protein, we designed a novel GLP-1 analogue (NGGH) with an *N*-terminal His-tag followed by an enterokinase (EK) cleavage site between the His-tag and the *N*-terminus of GGH. NGGH was purified using two affinity chromatography steps and digested with EK to remove the His-tag. Methods for preparing GGH and NGGH were compared, and the total recovery yield, bioactivity in vitro and in vivo, and *N*-terminal homogeneity were determined.

Materials and methods

Plasmids, strains and reagents

Plasmid strains: Plasmid pPIC9K, *P. pastoris* and *Escherichia coli* DH5α were from our laboratory sample bank. T4 DNA ligase, restriction enzymes, and Pfu DNA polymerase were purchased from ThermoScientific. EK (RP005-250 IU) was purchased from Shanghai Sangon. G25 Sepharose, Ni Sepharose, DEAE Sepharose, and Blue Sepharose were purchased from GE Healthcare. All other reagents were of analytical grade. Diabetes model mice (Kondod K; KK), 8-weeks-old and weighing 22–28 g were purchased from SLAC and fed with standard laboratory chow. Animal experiments were approved by the Animal Ethics committee of Jiangnan University (Wuxi, Jiangsu, China).

Construction of *P. pastoris*/pPIC9K/NGGH expression clones

Using specific primer 1 (CGGAATTCAA AAGA CACCAC CACCATCACC ATGATGACGA TGA CAAGCAC GGTGAAGGTACTTTCAC) and primer 2 (TAAAGCGGCC GCTTATTATA AGCCTAAGG CAG), the NGGH gene fragment was amplified from pPIC9K/GGH, digested using *Eco*RI and *Not*I, and inserted into the multiple cloning site of pPIC9K that had been digested with the same enzymes. The resultant plasmid was transformed into *E. coli* DH5α, and recombinant plasmid was verified by restriction enzyme digestion, further confirmed by gene sequencing using the Shanghai Sangon sequencing service.

The confirmed pPIC9K/NGGH vector was transformed into competent *P. pastoris* GS115 cells using 1.5 kV, 40 μF, and 180 Ω. Recombinant clones were cultured for 3 days on YPDS plates containing 1 mg G418/ml. Potential high-level recombinant clones were selected and further cultured for 4 days on YPDS agar plates containing a higher G418 concentration (2.5 mg/ml). Finally, the highest expressing clones were detected using a trace urinary albumin kit (Min Dian Co., Shanghai). Protein expression was carried out in accordance with Invitrogen guidelines, and high-level protein-secreting clones were confirmed using the genomic PCR with the above primers.

Fed-batch fermentation

Fermentation studies were carried out in 5 l bioreactors (Baoxing Co., Shanghai). A seed culture (200 ml) was inoculated into a 5 l fermenter containing 2.8 l medium (40 g tryptone/l, 20 g yeast extract/l, 20 g glycerol/l, 4.8 g $KH_2PO_4{\cdot}3H_2O$/l, 10.7 g K_2HPO_4/l, 10 g NH_4SO_4/l, 3.4 g yeast nitrogen base/l, pH 6.0). At the start of fermentation, the temperature was set to 30 °C and the pH was maintained at 6 by addition of 30 % (v/v) H_3PO_4 and 2 M KOH. After all glycerol was consumed, the temperature was reduced to 25 °C and methanol was added. The rate of methanol addition was controlled by negative feedback to maintain the dissolved O_2 at 28 %. After every 4 h, the fermentation broth was collected to measure the dry cell weight and protein yield.

SDS-PAGE, western blotting, HPLC analysis and *N*-terminal amino acid detection

SDS-PAGE analysis of the purified samples was performed on 12 % gels. Proteins were transferred onto a nitrocellulose membrane and detected using anti-His antibody (ab154063, Abcam) and anti-HSA antibody (ab31898, Abcam). The immunoreaction was carried out using a horseradish peroxidase color development kit. HPLC analysis was performed as described previously (Kobayashi et al. 2000) and *N*-terminal amino acid detection was carried out by Shanghai Applied Protein Technology using the Edman method.

Bioactivity assay of GGH and NGGH in vitro and in vivo

In vitro bioactivity was assessed using a HEK293/GLP-1R/CRE-fFlu cell line co-transfected with glucagon-like peptide-1 receptor (GLP-1R) and CRE-fFlu (cAMP response element fused to the firefly luciferase gene) (Zhang et al. 2011). Expression of fFlu was upregulated by phosphorylation of CRE via the cAMP/PKA pathway. HEK293/GLP-1R/CRE-fFlu cells were cultured in DMEM with 10 % (v/v) fetal bovine serum in an incubator with 5 % (v/v) CO_2 at 37 °C. Log-phase cells were diluted to 8×10^5 cells/ml and incubated for 12 h in 96-well plates. The culture medium was removed and cells were washed with PBS before the addition of 100 µl of fusion protein (a series of double-dilutions in FBS-free DMEM) and incubation for 4 h. Samples were collected and prepared as described previously (Sigma, luc1). Luciferase activity was measured in 3 s using 560 nm filters.

For in vivo bioactivity measurements, KK mice were randomly divided into three groups and injected with 3 mg/kg of drug including saline (control group), GGH or GGH_2. Blood glucose levels were measured using blood glucose test strips (Abbott, USA) at 0, 20, 60, 80 and 100 min after glucose administration. A sugar tolerance test was conducted as described previously (Dou et al. 2013). All data are displayed as the mean ± SD of triplicates.

Results

Construction of recombinant *P. pastoris*/pPIC9K/NGGH

The NGGH DNA fragment was amplified using PCR and inserted into the pPIC9K vector to generate the recombinant pPIC9K/NGGH. The confirmed pPIC9K/NGGH construct was subsequently linearized using *Sal* I and transformed into competent *P. pastoris* GS115 cells. Recombinant yeast clones with high potential expression levels were cultured on YPDS plates containing a higher G418 concentration (2.5 mg/ml). Nine clones were selected and cultured in flasks containing YPD medium. The highest

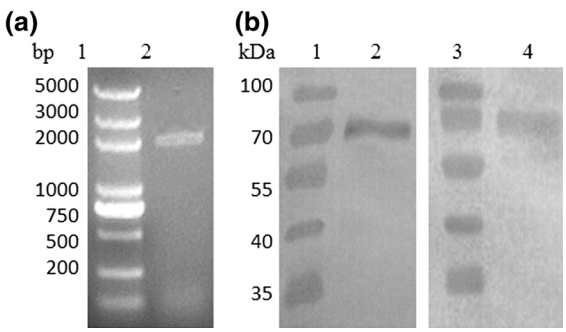

Fig. 1 Confirmation of the recombinant *P. pastoris* clone. **a** Electrophoresis of the PCR product amplified from genomic DNA; *lane 1* molecular weight markers, *lane 2* PCR product for genomic DNA. **b** Western blot of fermentation broth; *lanes 1* and *3* molecular weight markers, *lane 2* Western blot with anti-HSA antibody, *lane 4* Western blot with anti-His antibody

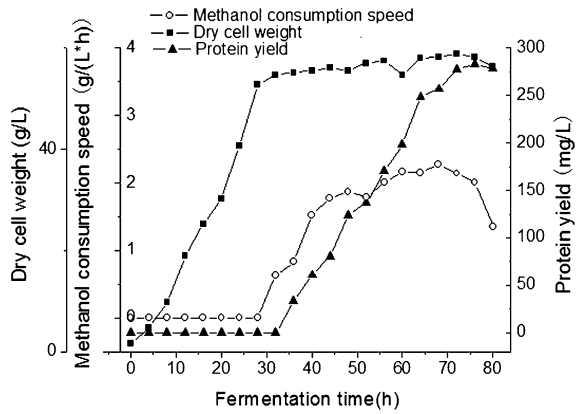

Fig. 2 Summary of the *P. pastoris*/pPIC9K/GGH fermentation process in 5 l bioreactor. Protein concentration, dry cell weight and methanol consumption were determined every 4 h

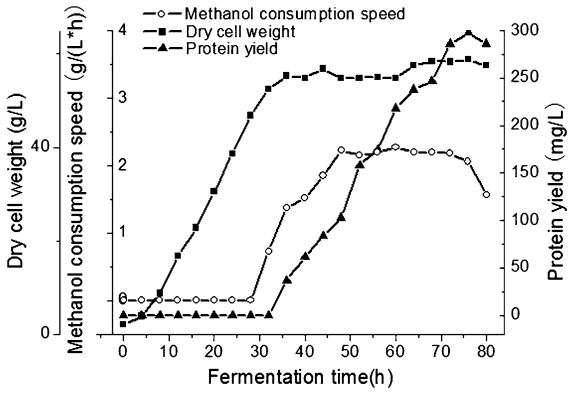

Fig. 3 Summary of the *P. pastoris*/pPIC9K/NGGH fermentation process in 5 l bioreactor. Protein concentration, dry cell weight and methanol consumption were determined every 4 h

yielding clone (63 mg/l) was selected for further studies following confirmation by genomic PCR and western blot analysis. Gel electrophoresis of the amplified PCR products confirmed that the selected clone contained the *NGGH* gene (Fig. 1a). Western blot analysis showed that the expressed protein was recognized specifically by the anti-His-tag and anti-HSA antibodies (Fig. 1b), which confirmed that the expressed protein was the recombinant chimeric NGGH.

Fed-batch fermentation

The *P. pastoris*/pPIC9K/GGH strain used to express the GGH fusion protein was described previously (Dou et al. 2008); however, expression in a bioreactor was not explored in this earlier study. In the present study, fermentation of *P. pastoris*/pPIC9K/GGH in a 5 l bioreactor yielded a dry cell weight of 52.7 g/l after 28 h (Fig. 2). It was evident that all glycerol was consumed by this time, therefore the carbon source was changed to methanol to induce the maximum level of GHH expression, which peaked at 283 mg/l at 76 h following this intervention. Methanol consumption peaked at 2.1 g/(l h) but subsequently decreased to 1.3 g/(l h) at 80 h, along with the protein yield that decreased to 278 mg/l by this time point. The parallel decrease in methanol consumption and protein yield may be due to decreased cell viability, suggesting fermentation should be halted at 76 h.

Fermentation of *P. pastoris*/pPIC9K/NGGH proceeded in a similar manner to *P. pastoris*/pPIC9K/GGH, and protein expression peaked at 298 mg/l at 76 h (Fig. 3).

Purification of GGH and NGGH fusion proteins

The GGH fusion protein was purified using four steps: ultrafiltration, Blue Sepharose affinity chromatography, G25 buffer-exchange chromatography, and DEAE Sepharose ion-exchange chromatography. SDS-PAGE analysis of the final protein sample revealed a single band of ~70 kDa (Fig. 4a), which was consistent with the theoretical molecular weight. The purity of GGH was 95 % (Fig. 4b), and the final recovery yield was 23 % (Table 1).

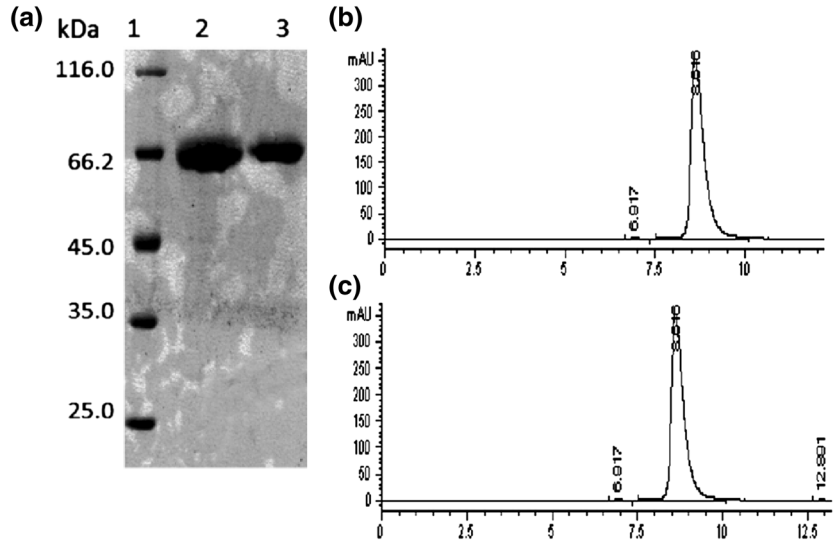

Fig. 4 Protein purity detected by SEC-HPLC and 12 % SDS-PAGE. **a** SDS-PAGE of final purified protein samples; *lane 1* protein molecular weight markers, *lane 2* final purified GGH protein, *lane 3* final purified GGH_2 protein. **b** SEC-HPLC of final purified GGH (purity = 95.1 %). **c** SEC-HPLC of final purified GGH_2 (purity = 95.3 %)

Table 1 Summary of the purification of GGH expressed in *P. pastoris*

Purification step	Volume (ml)	GGH (mg)	Cumulative yield (%)
Supernatant	420	119	100
Ultrafiltration	46	108	90.8
Blue Sepharose	38	63.4	53.3
G25 desalting	48	59.6	50.1
DEAE Sepharose	35	27.1	22.8

The GLP-1 analogue GGH, consisting of two tandem mutant GLP-1 (GLP-1[A2G]) fused with the *N*-terminus of human serum albumin (HSA), was expressed in *P. pastoris*. Cells were removed by centrifugation and the fermentation supernatant was harvested then concentrated by ultrafiltration using a Millipore Flow Filtration System. The concentrated GGH protein was loaded onto a Blue Sepharose column and washed with buffer B (20 mM NaPB, 2 M NaCl, pH 7.2). The buffer B eluted sample was replaced to buffer C (20 mM NaPB, pH 7.2) using G25 Sepharose column and loaded onto DEAE Sepharose. Finally the pure protein was eluted with buffer E (20 mM NaPB, 0.5 M NaCl, pH 7.2). GGH was detected using a urinary albumin kit

Table 2 Summary of the purification of GGH_2 expressed in *P. pastoris*

Purification step	Volume (ml)	GGH_2 (mg)	Cumulative yield (%)
Supernatant	420	125.2	100
Ultrafiltration	48	113.8	90.9
Blue Sepharose	42	64.9	51.9
Ni Sepharose	36	50.9	40.8
G25 desalting	42	48	38.4
EK digestion + Ni Sepharose	45	44.1	35.3

The novel GLP-1 analogue NGGH, which had a His-tag fused with the *N*-terminus of GGH and an enterokinase (EK) cleavage site at the fusion junction. The His-tag was removed by EK digestion to yield GGH_2. Cells were removed by centrifugation and the supernatant was concentrated by ultrafiltration using a Millipore Flow Filtration System. The concentrated NGGH protein was loaded onto the Blue Sepharose column and eluted in buffer B as described above for GGH. We diluted buffer B eluted sample by adding three times volume water. Then loaded onto a Ni Sepharose column and eluted with buffer G (20 mM NaPB, 0.5 M NaCl, 100 mM imidazole, pH 7.2). The buffer G sample was replaced to buffer H (25 mM Tris/HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 7.6) using a G25 Sepharose column. His-tag was removed by enterokinase (1.25 U/mg proteins) for 18 h at 37 °C. The digested sample was re-loaded to Ni Sepharose column and GGH_2 was collected in the flow-through. GGH_2 was detected using a trace urinary albumin kit

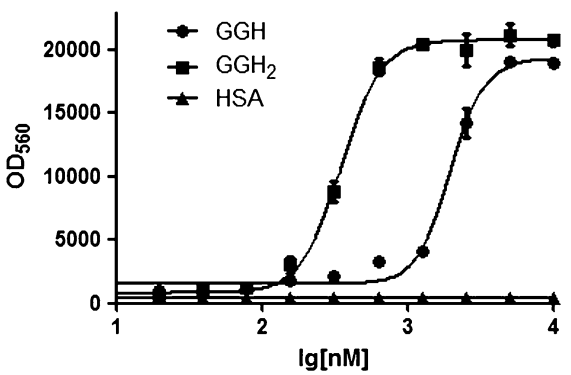

Fig. 5 Bioactivity detected using the HEK293/GLP-1R/CRE-fFlu cell line. The fFlu expression was protortional to the intracellular cAMP concentration. Intracellular luciferase fluorescence emission was measured using the Luciferase Reporter Gene Detection Kit (Sigma, luc1). Data are displayed as the mean ± SD of triplicates

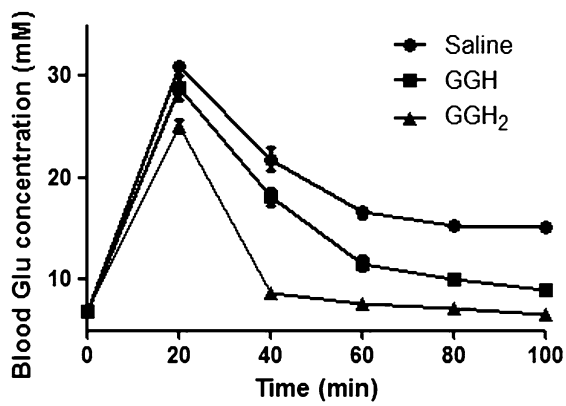

Fig. 6 Glucose-lowering test in Kondod K mice. All three groups were injected 3 mg drug/kg (n = 5). Values are mean ± SEM

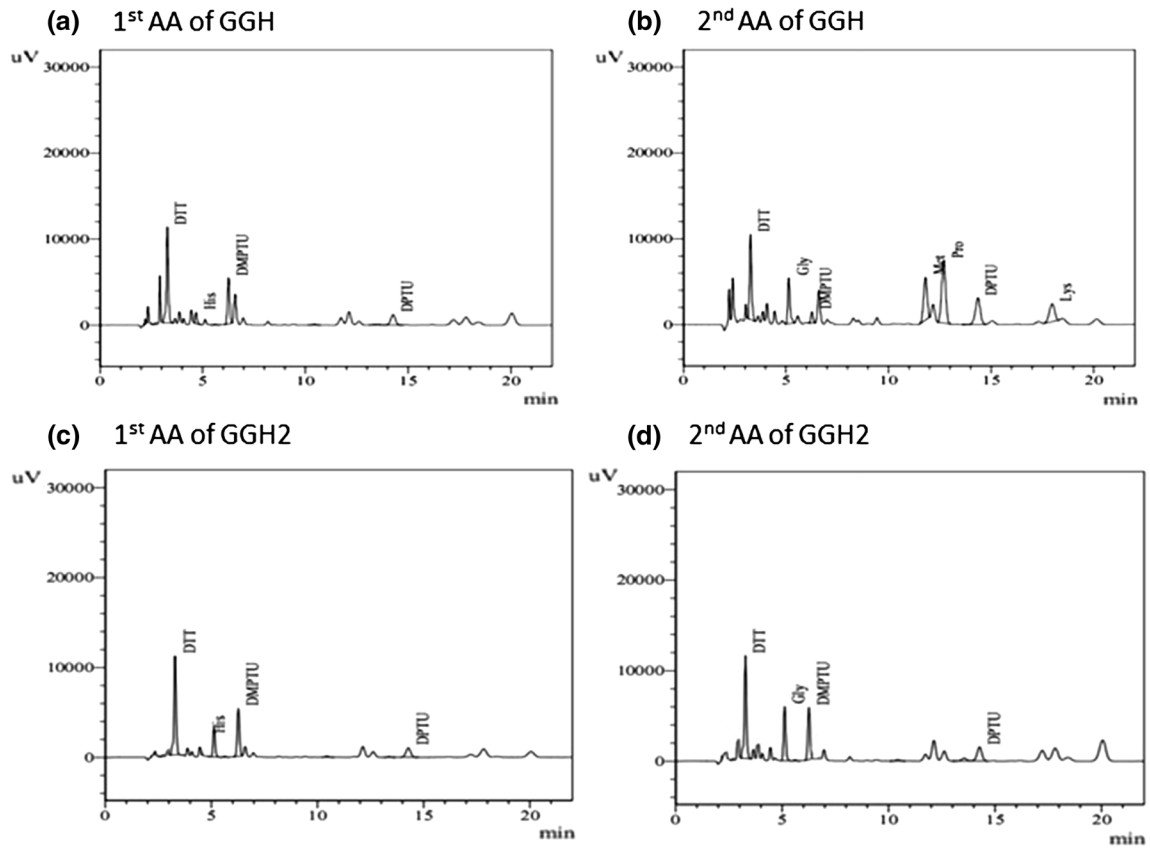

Fig. 7 *N*-Terminal amino acid detected using the Edman method. **a** The first AA of GGH. **b** The second AA of GGH. **c** The first AA of GGH_2. **d** The second AA of GGH_2

NGGH was initially purified by blue Sepharose and Ni affinity chromatographic steps, and the purity of the resultant fusion protein was 95.3 %. Next, a G25 gel filtration column was used to exchange into buffer H in preparation for the EK digest. The NGGH His-tag was removed using EK (1.25 U/mg fusion protein) at 37 °C for 18 h. The tag-free GLP-1 analogue (GGH_2) was separated from the cleaved tag and the his-tagged recombinant bovine EK using a second Ni Sepharose chromatography step. GGH_2 was collected in the flow-through. The final purity and recovery yields were, respectively, 95 % (Fig. 4c) and 35 % (Table 2).

Bioactivity in vitro and in vivo

HEK293/GLP-1R/CRE-fFlu cells were described previously (Zhang et al. 2011). In these cells, GLP-1 analogues increase intracellular cAMP by activating the GLP-1R, and fFlu expression is upregulated via the cAMP/PKA pathway. The results of the present study showed that both GGH and GGH_2 could stimulate fFlu expression, with EC_{50} values of 2021 and 334 nM, respectively (Fig. 5). The bioactivity of the modified GGH_2 was therefore improved by 605 % relative to GGH. The in vivo bioactivity was also improved; GGH_2 controlled blood glucose more effectively than GGH (Fig. 6). Finally, *N*-terminal sequencing showed that the homogeneity of GGH_2 was higher than that of GGH (Fig. 7).

Discussion

Pichia pastoris is an efficient host for heterogeneous expression of proteins. AGGH has been expressed in *Pichia pastorisan* with a protein recovery of 25 % (Dou et al. 2013), while the rhIL2-HSA fusion protein was expressed in the same host with a protein recovery of 12 % (Guan et al. 2013). In these studies, purification of HSA fusion proteins was achieved using

affinity, hydrophobic, and ion-exchange chromatographic steps, however the final protein yields were relatively low. In contrast, in the present study we expressed the novel GGH_2 fusion protein in *P. pastoris* and achieved a final protein yield of 35 % following purification using only two affinity chromatography steps. This method developed in the present work is therefore suitable for large-scale production.

Expression of bioactive peptide drugs using genetic engineering and recombinant protein expression is a more convenient, cost-effective and scalable method than isolating naturally abundant proteins (Li et al. 2012). A novel KGLP-1/HSA fusion protein was expressed previously in *P. pastoris*, however the bioactivity was lower than GLP-1/HSA (Gao et al. 2009). $(GLP\text{-}1A_2G)_2$-HSA (GGH) analogs have also been expressed previously in *P. pastoris* (Dou et al. 2013). The bioactivity of GGH expressed in *P. pastoris* was not very high, possibly due to degradation during protein expression and folding. GLP-1 is a peptide of 30 residues, and a His residue was found to be essential for bioactivity. GLP-1 lacking the His showed no bioactivity in vitro (Underwood et al. 2010). In order to minimize protein degradation during secretion and improve *N*-terminal homogeneity, we incorporated a His-tag at the *N*-terminus of GGH_2 in the present study, which resulted in improved *N*-terminal homogeneity compared with GGH (Fig. 7). This may explain why GGH_2 was 605 % more active than GGH. Additionally, despite a similar protein yield to GGH, the final recovery of GGH_2 was significantly improved. In summary, a simple and efficient method for preparing a GLP-1 analogue with improved bioactivity and *N*-terminal homogeneity was developed.

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