A Proficient Approach to the Production of Therapeutic Glucagon-Like Peptide-1 (GLP-1) in Transgenic Plants

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Abstract: Glucagon-like peptide-1 (GLP-1) is a small peptide hormone with potent insulinotropic activity and represents a promising new therapeutic tool for the treatment of diabetes. Like many other therapeutic peptides, GLP-1 is commonly produced using chemical synthesis methods, but is limited by product quantity and cost. The advent of recombinant DNA technology offers the possibility of producing GLP-1 inexpensively and in vast quantities. In this study, transgenic plants were used as a recombinant expression platform for the production of GLP-1 as a large multimeric protein. A synthetic gene encoding ten sequential tandem repeats of *GLP-1* sequence (*GLP-1x10*) was produced and introduced into tobacco plants. Transcriptional expression of the *GLP1x10* gene in transgenic plants was confirmed by RT-PCR. Western blot analysis showed that the GLP-1x10 protein efficiently accumulated in transgenic plants, with an accumulation level as high as 0.15% of total soluble protein in leaves. Importantly, insulin secretion assays using a mouse pancreatic β cell line (MIN6), showed that plant-derived GLP-1 in its synthetic decamer form, retained its ability to stimulate cellular insulin secretion, although with reduced efficacy. These results demonstrate that transgenic plants are an efficient system for the production of a multimerized recombinant GLP-1. Moreover, transgenic plants synthesizing high levels of GLP-1x10 may prove to be an attractive delivery system for direct oral administration of a novel stable GLP-1 analog in the treatment of patients with Type 2 diabetes.

INTRODUCTION

The gastrointestinal hormone glucagon-like peptide 1 (GLP-1) has recently attracted much attention for its pleiotropic therapeutic effects, especially for its potential to treat Type 2 diabetes [1]. In rats and mice, GLP-1 treatment increases beta cell mass, making it possible to reverse beta cell damage occurring with Type 2 diabetes [2]. Furthermore, GLP-1 treatment decreases glucose levels, stimulates insulin secretion and insulin sensitivity, decreases glucagon secretion, promotes weight loss, delays gastric emptying and limits food intake (appetite suppression) in both humans and animals [3-5]. The insulinotropic activity of GLP-1 is glucose-dependent, thereby lessening though probably not alleviating the risk of hypoglycaemia often associated with sulfonylurea treatment, one of the most commonly used therapies for Type 2 diabetes [6, 7]. The GLP-1 peptide is synthesized in intestinal endocrine L cells by prohormone convertase 1 (PC1)-mediated posttranslational processing of proglucagon and exists in two biologically relevant isoforms: the principal amidated active form of GLP-1 (amino acids 7–36) and the unamidated GLP-1 (7–37) [1]. Chemical synthesis is the most commonly used method for production of therapeutic peptides including GLP-1. However, this method is expensive and produces low yields of active peptide product. The use of recombinant DNA technology offers the possibility to produce these peptides inexpensively and in large quantities.

Currently, yeast and bacteria bioreactors have been generated to produce recombinant GLP-1 [8, 9]. While both microbial systems demonstrate some advantages over traditional chemical synthesis methods for GLP-1 production, conventional cell culture-based expression systems have serious limitations. One such limitation is the ability to scale up recombinant GLP-1 production as these microbial platforms necessitate sophisticated fermentation equipment and facilities, requiring a substantial amount of capital investment. In addition, recombinant GLP-1 accumulated in cell culture-based bioreactors, requires extensive purification prior to use to remove host proteins and other compounds such as bacterial endotoxin. Taken together, the infrastructure needed for fermentation and the protein purification necessary for recombinant product recovery, lead to the high cost of GLP-1 production in microbial-based systems.

Transgenic plants provide a very attractive alternative to conventional microbial systems for recombinant therapeutic protein production [10-12]. Plants as bioreactors permit unlimited scalability, elimination of product contamination by mammalian pathogens, as well as reduced production costs compared to microbial or animal cell-based systems [10, 13]. Eukaryotic plants also permit the modification and processing of many transgenic mammalian therapeutic proteins, often required for biological and/or immunological function. Furthermore, edible transgenic plant tissue offers the possibility of allowing direct oral delivery of plant-derived therapeutic proteins and peptides, eliminating the need for expen-

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sive downstream processing. The expression of recombinant GLP-1 has been reported in transgenic rice [14-16]. Originally, no recombinant protein was detected in rice seeds when GLP-1 was expressed as a monomer fused to the signal peptide of glutelin (a rice storage protein), possibly due to siRNA (small interfering RNA)-mediated gene silencing and/or peptide instability and proteolytic degradation [14]. Subsequently, the accumulation of GLP-1 was demonstrated in rice seeds when it was expressed as a fusion protein with globulin (another rice seed storage protein) [15]. As the biological activity of the globulin-GLP-1 fusion protein was demonstrated only after the physical release of GLP-1 from the fusion protein by *in vitro* enzymatic digestion, it was speculated that the recombinant fusion protein might have reduced GLP-1 biological activity. This reduction in activity may be attributed to the relatively large size of the globulin fusion partner (26 kDa) when compared to GLP-1 (<4 kDa). Recently, GLP-1 was shown to accumulate in rice seed when expressed as a pentamer repeat fused to the signal peptide of rice glutelin or the signal peptide of rice chitinase [16]. However, Western blot analysis showed that the rice seed-derived recombinant protein is smaller in size than that calculated for a synthetic GLP-1 pentamer, leading one to speculate that the engineered plant signal peptide may not be properly removed from the mature protein [16] and as a result, the function of the mature protein could be affected. Furthermore, the biological activity of seed-based recombinant GLP-1 pentamer was not determined in this study.

In the present study, we report an alternative approach for the production of GLP-1 in transgenic tobacco plants. This novel production scheme is based on the expression of GLP-1 as a protein multimer, comprised of ten sequential tandem copies of GLP-1. Here we show that the recombinant protein was stably expressed and accumulated to relatively high levels in transgenic plants. Importantly, plant-derived GLP-1 in its synthetic decamer form remains biological active, although not to the same degree as that of native GLP-1.

MATERIALS AND METHODS

Synthetic Gene and Plasmid Construction

Construction of the synthetic gene encoding ten sequential tandem repeats of *GLP-1* involved the PCR amplification of three separate sets of partially overlapping, complementary oligonucleotide primer pairs. As shown in Fig. (**1A**), three separate DNA fragments designated as Start, Link and Stop were initially generated using various primer pairs amplifying a single 90 bp *GLP-1* open reading frame. The Start fragment was created using engineered forward primer P1 (5'-AATT**CCATGG**GGCATTCTGAGGGAACCTTCA CCTCTGACG-3') and reverse primer P2 (5'-AATTGA ATTCAATTGTCGACCCTTCCCTTCACCACCAAGC-3'). Forward primer P1 contained a 5' *Nco*I restriction site (bold) used as the start codon, while reverse primer P2 contained a 3' *Sal*I (double underline) restriction site for subsequent gene fusion. The Link fragment was obtained using forward primer P3 (5'-AATT**CTCGAG**CATTCTGAGGGAACCTT CACCTCTGACG-3') containing a 5' *Xho*I restriction site (bold) and reverse primer P2. Finally, the Stop fragment was generated using forward primer P3 and reverse primer P4

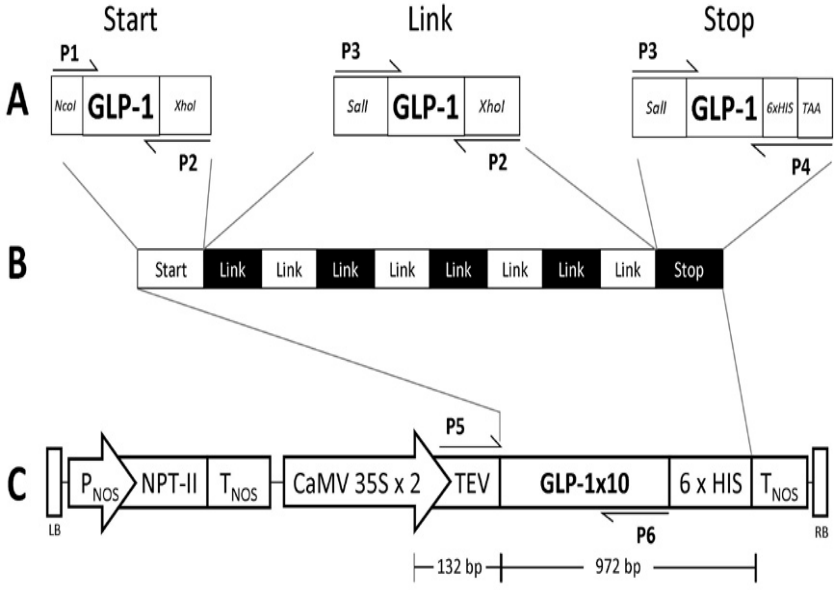

Fig. (1). *Construction of GLP-1x10 and final plant expression vector pALP-GLP-1x10.* (**A**) Three initial DNA fragments (Start, Link, and Stop) initially used to build the *GLP-1* sequence decamer, each containing a single *GLP-1* open reading frame and various 5' and 3' additions: P1 – forward primer containing start codon (ATG), P2 – reverse primer containing *Sal*I restriction site for fusion use, P3 – forward primer containing *Xho*I fusion site, P4 – reverse primer containing 6xHIS tag and stop codon (TAA). (**B**) Final *GLP-1* decamer sequence generated from Start, Link, and Stop fragments. Each Link or Stop fragment was added consecutively to the initial Start fragment using *SalI*/*Xho*I ligation to generate the final product. (**C**) Plant binary expression vector pALP-GLP-1x10: LB – left border region, RB – right border region, P_{NOS} – Nopaline synthase promoter, NPT-II – Neomycin phosphotransferase II marker gene, T_{NOS} – Nopaline synthase terminator, CaMV 35S X 2 – double constitutive cauliflower mosaic virus 35S promoters, TEV – Tobacco etch virus untranslated leader sequence, GLP-1x10 – 10 consecutive repeats of *GLP-1*, 6xHIS – C terminal His purification tag, P5 –TEV 5'UTR specific primer used for PCR, P6 – DNA primer specific for C-terminal sequence of GLP-1x10.

(5'-AATTCTCGAG**TTA**ATGATGATGATGATGATGCC TTCCCTTCACCAACCAAGC-3'). Primer P4 contained 3' 6x His tag (double underline), stop codon (bold) and *Sal*I restriction site (underline) for subsequent gene fusion. All PCR reactions were performed under identical conditions: denature at 94°C for 1 min, anneal at 55°C for 45 s and elongate at 72°C for 35 s, for a total of 35 cycles followed by a final elongation at 72°C for 10 min.

The generation of the *GLP-1* decamer repeat was completed using single step-by-step ligations of the Start, Link and Stop DNA fragments. Initially, one Link fragment was fused to the Start fragment by *Sal*I/*Xho*I ligation to generate a *GLP-1* dimer repeat. When ligated, the compatible ends of the *SalI/Xho*I pair produce a site that is recognized by neither restriction enzyme. A second Link fragment was then ligated to the 3' end of the *GLP-1* dimer repeat using an identical *Sal*I/*Xho*I ligation to generate a *GLP-1* trimer repeat. This step was repeated six more consecutive times until a sequence composed of nine identical *GLP-1* repeats (1 Start fragment + 8 Link fragments) was generated. The final step was composed of ligating the Stop fragment to the 3' end of the final Link fragment via *Sal*I/*Xho*I ligation, generating the final *GLP-1* decamer sequence, designated as *GLP-1x10* (see Fig. **1B**), containing start and stop codons as well as a C-terminal 6xHis tag. The sequence integrity of the synthetic *GLP-1x10* was confirmed by DNA sequencing.

The *GLP-1x10* plant expression cassette was generated by incorporating *GLP-1x10* into plasmid pRTL [17] via *Nco*I and *Xho*I restriction sites. Plasmid pRTL contains an enhanced constitutive cauliflower mosaic virus (CaMV) 35S promoter as well as a tobacco etch virus (TEV) 5'UTL and the 3'UTR sequence of the nopaline synthase (nos) gene. This entire expression cassette was then ligated into the plant binary vector pBI101.1 to generate the final expression plasmid pALP-GLP-1x10 (see Fig. **1C**).

Generation of Transgenic Plants

The plasmid pALP-GLP-1x10 was introduced into *Agrobacterium tumefaciens* strain LBA4404 by the method of triparental mating [13] using helper plasmid pRK4013. Low-alkaloid *Nicotiana tabacum* cv. 81V9 was transformed with *A. tumefaciens* harboring pALP-GLP-1x10 as according to the method of Horsh *et al.* (1985) [18]. Primary transgenic plants were selected on MS medium containing 100 mg/L kanamycin. As regenerated plants matured, they were transferred into a greenhouse and maintained for further analysis.

Screening of Putative Transgenic Plants by PCR and RT-PCR

Total genomic DNA was extracted from individual regenerated tobacco lines using a basic phenol/chloroform extraction protocol [13]. Tobacco DNA was subjected to PCR to confirm integration of *GLP-1x10* to the nuclear genome. The first initial PCR reaction utilized forward primer P3 and reverse primer P4 (see above description) to allow for elongation of all possible repeat lengths of the *GLP-1* decamer. A second PCR reaction using reverse primer P4 along with a forward primer corresponding to the TEV 5' UTL (5'-CGAATCTCAAGCAATCAAGC-3') designated as P5 was used to obtain a single fragment length of the *GLP-1* decamer. Both PCR reactions were subjected to 35 cycles of denature at 95°C for 55 s, anneal at 67°C for 47 s and elongate at 72°C for 1 min with a final elongation at 72°C for 10 min. All PCR samples were subsequently analyzed by electrophoresis on a 7.5% agarose gel.

For RT-PCR analysis, total plant RNA was extracted using a Trizol reagent extraction kit (Invitrogen) according to the manufacturer's instructions. To initiate first strand synthesis reactions, approximately 0.5 μg of oligo$(dT)_{12-18}$ primer was annealed to 5 μg of purified RNA in a total reaction volume of 12 μl for 10 min at 70°C. Generation of cDNA was performed with addition of 20 U of Super-Script™II RNase H Reverse Transcriptase (Invitrogen) subjected to a 50 min incubation at 42°C followed by an additional 15 min incubation at 70°C. Approximately 150 ng of first-strand synthesized cDNA was used as a template for PCR amplification in a 100 μl reaction volume using identical conditions and primers (P4 and P5) outlined for the detection of genome-integrated *GLP-1x10* in transgenic plants. All RT-PCR samples were subsequently analyzed by electrophoresis on a 7.5% agarose gel.

Western Blot Analysis

Plant-derived recombinant GLP-1x10 accumulation was assessed by SDS-PAGE followed by Western blotting. In brief, the uppermost fully expanded leaves of 6-week-old glasshouse-grown tobacco were homogenized in liquid nitrogen and resuspended in cold extraction buffer [25 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.0, 50 mM NaCl, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride, 2 μg/mL aprotinin, 2 μg/mL pepstatin A and 2 μg/mL leupeptin]. Samples were centrifuged for 10 min at 4 °C, and the supernatant collected. Extracted total soluble protein concentration was determined by the method of Bradford (1976) [19] using Bio-Rad reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) (Sigma-Aldrich) as a standard. Proteins were separated on 12.5% SDS-PAGE gels and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Burlington, MA) using a semi-dry transfer method. Commercially available GLP-1 standard (Sigma) was used as a positive control and separated on a 17.5% SDS-PAGE gel before Western blotting. Blots were blocked for 1 h in 5% skim milk-TBS-T [20 mM Tris, 150 mM NaCl, 0.02% Tween 20, pH 7.6] solution and incubated at room temperature for 2 h in a 1:500 dilution (v/v) of mouse anti-GLP-1 primary monoclonal antibody (Santa Cruz Biotechnology) followed by a 1 h incubation in goat anti-mouse secondary antibody conjugated with horseradish peroxidase. The enhanced chemiluminescence (ECL) reaction kit (Amersham Pharmacia Biotech) was used according to the manufacturer's instructions for protein band detection.

Quantification of Plant GLP-1x10 Protein

The amount of accumulated recombinant GLP-1x10 protein was quantified by enzyme linked immunosorbent assay (ELISA) using known amounts of 6xHis-tagged reference protein as a protein standard. Briefly, total protein samples resuspended in sodium bicarbonate (pH 9.6), were coated on a 96-well miccrotiter plate and incubated overnight at 4°C. Wells were washed three times with PBS-T [phosphate buffered saline containing 0.05% Tween-20] and blocked with 3% BSA in PBS-T for 2 hours at room temperature. After

washing with PBS-T, rabbit anti-6xHis monoclonal antibody (Rockland Immunochemicals, Pennsylvania, USA) diluted to 1:5000 was added and plates incubated overnight at 4°C. After PBS-T washes, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) antibody was added and incubated at room temperature for 1 h. Enzyme substrate TMB (R&D Systems, Minneapolis, USA) was added to each well (as per manufacturer's instructions) and incubated at room temperature in the dark for 30 min for color development. The substrate reaction was stopped by addition of 100μL/well stop solution (R&D Systems, Minneapolis, USA). The optical density (OD) value was measured at 450 nm in a microplate reader (Bio-Rad 3550), and converted as a percentage of total extracted protein by reference to an ELISA standard curve constructed with the 6xHis-tagged reference protein.

Purification of His-tagged GLP-1x10

Plant-derived recombinant GLP-1x10 was purified from transgenic tobacco leaf extracts with histidine affinity chromatography using HiTrap Chelating HP columns (GE Healthcare) as according to the manufacturer's protocol. Briefly, uppermost fully expanded leaf samples (5 g) of 6-week-old greenhouse-grown tobacco plants were homogenized in protein extraction buffer [2 mM imidazole, 20 mM Na_2HPO_4, 250 mM NaCl, 50 mM sodium ascorbate, 1% of 0.1 M phenylmethylsulphonylfluoride and 0.1% of 1 mg/mL leupeptin] in a 1:4 (w:v) ratio of sample to buffer. The homogenate was centrifuged at 13,000 x g for 15 min at 4°C. Supernatant was collected and centrifuged again at 13,000 x g for 10 min at 4°C. Approximately 5 mL of supernatant was collected in total and loaded into the HiTrap Chelating HP column and washed with wash buffer [10 mM imidazole, 20 mM Na_2HPO_4, 500 mM NaCl], removing nonspecifically bound endogenous tobacco proteins. The bound plant-derived recombinant GLP-1x10 was eluded from the column using elution buffer [500 mM imidazole, 20 mM Na_2HPO_4, 500 mM NaCl] with 1 mL fractions collected for subsequent analysis. Eluded recombinant GLP-1x10 fractions were dialyzed extensively against PBS and concentrated using a speed vacuum at 4°C.

Biological Activity Assay of Plant-Derived GLP-1x10

The biological activity of plant-derived GLP-1x10 protein was determined by its ability to stimulate insulin secretion from a mouse pancreatic β cell line MIN6 [20] according to the method of Mest *et al.* [21] with minor modifications. In brief, MIN6 cells, a kind gift from Dr. Junichi Miyazaki (Osaka University, Osaka, Japan) [20], were cultured and maintained in Dulbecco's modified Eagle's medium (high glucose) containing 50 μM 2-mercaptoethanol and 10% (v/v) fetal calf serum, 100 U ml^{-1} peniciillin and 0.1 mg ml^{-1} streptomycin at 37°C in an atmosphere of humidified air (95%) and CO_2 (5%). To determine the stimulating effect of plant-derived GLP-1x10 and commercial GLP-1 standard (Sigma) on insulin release, MIN6 cells were seeded into 96-well (flat-bottomed) microtiter plates at a density of 3×10^4 cells per well. After 3 days of incubation, the cells were washed two times with Earle's balanced salt solution (EBSS; Sigma) containing 0.1% BSA (Sigma). After starvation of cells for 1 h in EBSS plus 0.1% BSA the cells were incubated with various concentrations of plant-derived GLP-1 decamer or commercial GLP-1 standard in the presence of either high glucose (10 mM) or low glucose (2mM) for 135 min. Cell culture supernatants were then collected and assayed for insulin concentration using an insulin ELISA kit (Crystal Chem Inc., Illinois, USA) according to the manufacturer's instructions.

RESULTS

Production of Synthetic GLP-1x10 Gene, Plant Expression Vector and Transgenic Plants

Design and construction of the synthetic *GLP-1x10* gene and expression plasmid pALP-GLP-1x10 is described in Materials and Methods. As shown in Fig (**1C**), the expression of *GLP-1x10* was under the control of strong constitutive cauliflower mosaic virus (CaMV) 35S promoters followed by the tobacco etch virus (TEV) 5'UTL, aiding in translation enhancement [17]. The addition of a six histidine tag to the C-terminal of GLP-1x10 was to facilitate purification of the recombinant protein via immobilized metal affinity chromatography (IMAC).

No nicotine and low alkaloid *Nicotiana tobaccum* cultivar 81V9 was transformed with *Agrobacterium* containing plasmid pALP-GLP-1x10 using a standard leaf-disc co-cultivation procedure [18]. More than 25 independent transgenic tobacco lines were produced. There were no morphological differences between transgenic and untransformed control plants. To confirm integration of *GLP-1x10* DNA sequence into the plant nuclear genome, two separate PCR reactions were performed using total genomic DNA extracted from individual transgenic lines. The first PCR was performed using primers P5 (corresponding to the TEV 5′ UTR sequence; see Fig. **1C**) and p6 (corresponding to the C-terminal sequence of GLP-1x10; see Fig. **1C**) to demonstrate the existence of the *GLP-1* repeat, as this primer-pair combination amplifies all forms of *GLP-1,* ranging from monomer band to the DNA band consisting of TEV 5' UTR plus *GLP-1x10.* As expected, evenly spaced multiple DNA bands were generated (Fig. **2A**). The 1.1-kb DNA band (indicated by arrow) represents nucleotide sequences of the TEV 5' UTR (132 bp) and the *GLP-1* decamer (972 bp). The second PCR was performed to ensure that the inserted *GLP-1x10* was intact. Indeed, amplification of transgenic plant DNA using primers P5 and P4 yielded a single DNA product of a expected size of 1.1 kb containing TEV 5' UTR, *GLP-1x10* and the His6 tag (Fig. **2B**).

GLP-1x10 is Actively Transcribed in Transgenic Plants

PCR-positive plants were analyzed at the transcript level by reverse transcriptase-polymerase chain reaction (RT-PCR) using extracted RNA as a template. RT-PCR using primers P4 and P5 revealed the presence of a fragment of expected size (~1.1 kb) (Fig. **3**), suggesting that *GLP-1x10* is actively transcribed in transgenic plants. PCR reactions using extracted plant RNA samples without first-strand cDNA synthesis produced no fragments, eliminating the possibility of DNA contamination of the RNA samples (data not shown).

GLP-1x10 Accumulates in Transgenic Plants

Transgenic plants positive from both PCR and RT-PCR analysis were subjected to SDS-PAGE followed by Western

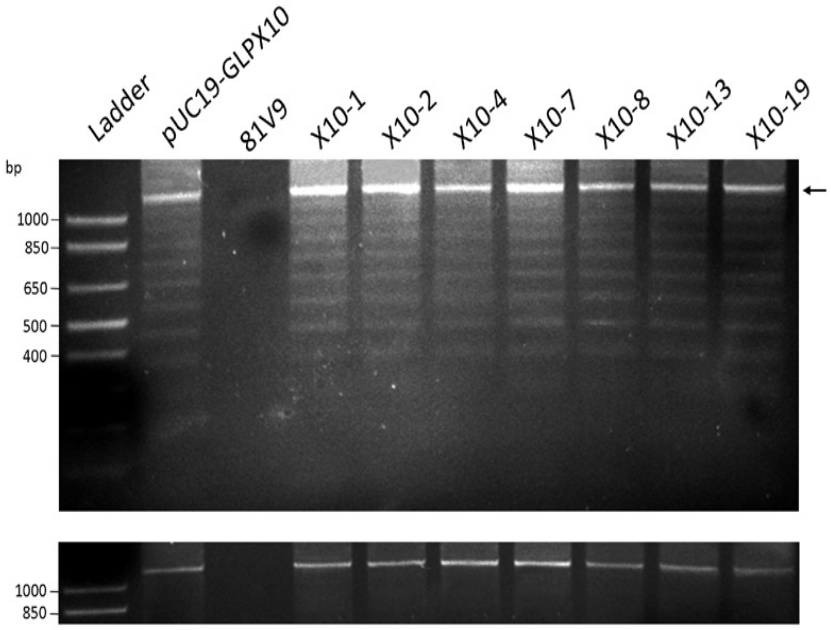

Fig. (2). *PCR analysis of GLP-1x10 transgenic tobacco plants.* Total genomic DNA was extracted from putative transgenic plants and analyzed by PCR for detection of nuclear *GLP-1x10* gene integration. (**A**) Primers P5 and P6 used for PCR generate all ten possible fragment sizes of *GLP-1*, demonstrating the repeat nature of the synthetic gene. The largest 1.1-kb fragment (indicated by arrow) consists of the TEV 5' UTR (132 bp) and the *GLP-1x10* sequence (972 bp). Ladder – DNA fragment size marker, pUC19-GLP-1x10 – positive plasmid DNA control, 81V9 – wild-type tobacco control DNA, T"x" – independent transgenic lines. (**B**) PCR using primers P4 and P5 yields a single 1.1-kb product containing TEV 5'UTR plus *GLP-1x10* and the His6 tag encoding sequence. Numbers on the left indicate the size of DNA ladder.

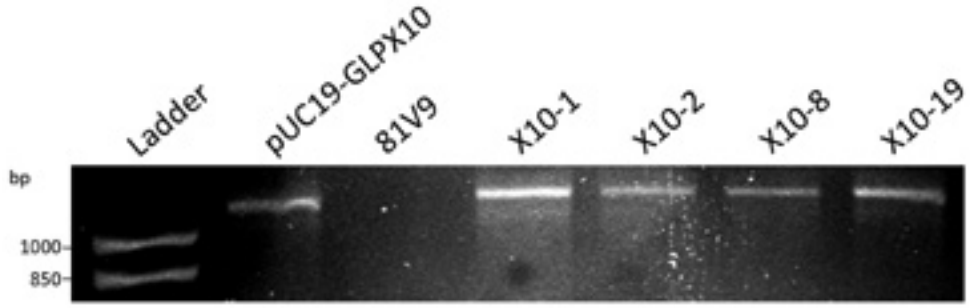

Fig. (3). *RT-PCR analysis of GLP-1x10 transgenic plants.* Total RNA extracted from transgenic plant leaf tissue was analyzed to determine the presence of *GLP-1x10* transcripts by RT-PCR. For RT-PCR, primers P4 and P5 produced a single 1.1-kb DNA product containing TEV 5'UTR plus *GLP-1x10* and the His6 tag encoding sequence. Ladder – DNA fragment size marker, pUC19-GLP-1x10 –positive plasmid DNA control, 81V9 –wild-type tobacco control, T"x" – representative transgenic lines. Numbers on the left indicate the size of DNA ladder.

blotting to verify the presence of accumulated recombinant GLP-1x10 protein. Probing total soluble extracted plant protein with an anti-GLP-1 (Santa Cruz Biotech) monoclonal antibody identified a single ~35 kDa molecular weight band corresponding to that of the expected GLP-1 decamer (Fig. **4B**). No bands were detected from total soluble protein extracted from wild-type 81V9 plants under identical conditions (Fig. **4B**). A commercially available GLP-1 standard (Sigma) was used as a positive control to assess the affinity of the primary antibody (Fig. **4A**). The accumulated amount of GLP-1x10 in transgenic plant extracts was measured with anti-His6 ELISA, using a quantified 6xHis-tagged reference protein as a known standard. Accumulation levels of GLP-1x10 were found variable among individual transgenic lines, with lines T1, T7 and T19 being top producers, accumulating GLP-1x10 at levels ranging from 0.05% to 0.15% of total soluble protein (TSP) (Fig. **5**).

Plant-Derived GLP-1x10 Remains Biologically Active, Although with the Reduction in Activity

One of the main functions of GLP-1 is to stimulate insulin secretion by the pancreatic beta cells in a glucose-dependent manner. Therefore, plant-derived GLP-1x10 was assessed for its ability to stimulate insulin release from a mouse pancreatic β-cell line, MIN6. To this end, GLP-1x10 was first purified by IMAC and checked by Western blotting analysis to confirm its integrity. The purified GLP-1x10 samples or commercial GLP-1 standard at various concentrations was incubated with MIN6 cells in the presence of either high glucose (10 mM) or low glucose (2 mM). As shown in Fig. (**6**), at high glucose level (10 mM), both plant-derived GLP-1x10 and commercial GLP-1 standard produced a concentration-dependent increase in insulin release from MIN6 cells.

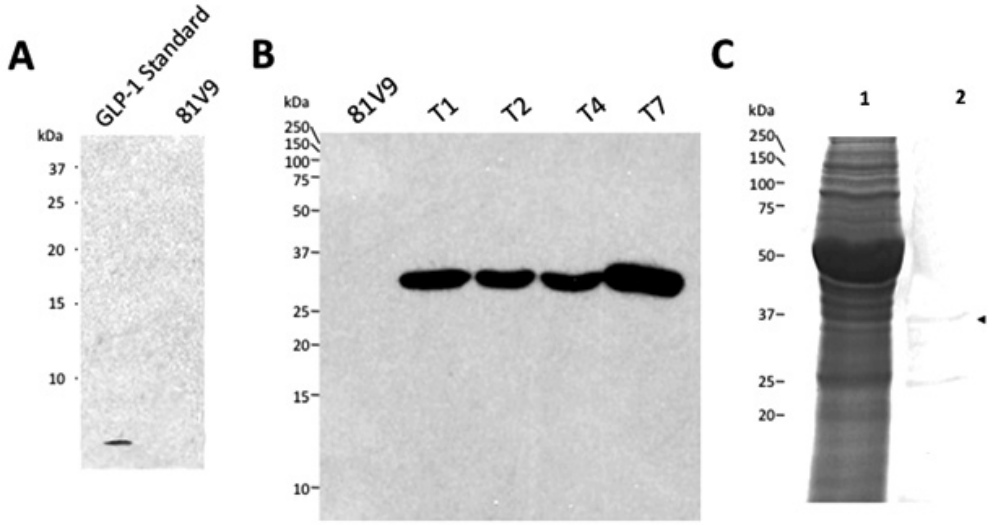

Fig. (4). *Western blot analysis of GLP-1x10 transgenic plants.* (**A**) Commercially available GLP-1 standard (20 µg; Sigma) was separated on a 17.5% SDS-PAGE followed by Western blotting using anti-GLP-1 antibody: 81V9 – total protein from wild-type tobacco control. (**B**) Total plant soluble protein (40 ug) extracted from transgenic tobacco leaf material was fractionated by 12.5% SDS-PAGE followed by Western blotting. Anti-GLP-1 monoclonal antibody detected a single protein band representing that of the expected size of the GLP-1 decamer (~35 kDa): T"x" – representative transgenic lines, 81V9 – negative wild-type tobacco control. (**C**) Visualization of affinity purified GLP-1x10 on 12.5% SDS-PAGE gels by Coomassie blue. Lane1 – total protein extract, lane 2 – GLP-1x10 purified via HiTrap Chelating HP columns (GE HealthCare). The arrow points to the 35-kDa GLP-1x10 protein band. Numbers on the left indicate the positions of protein size markers.

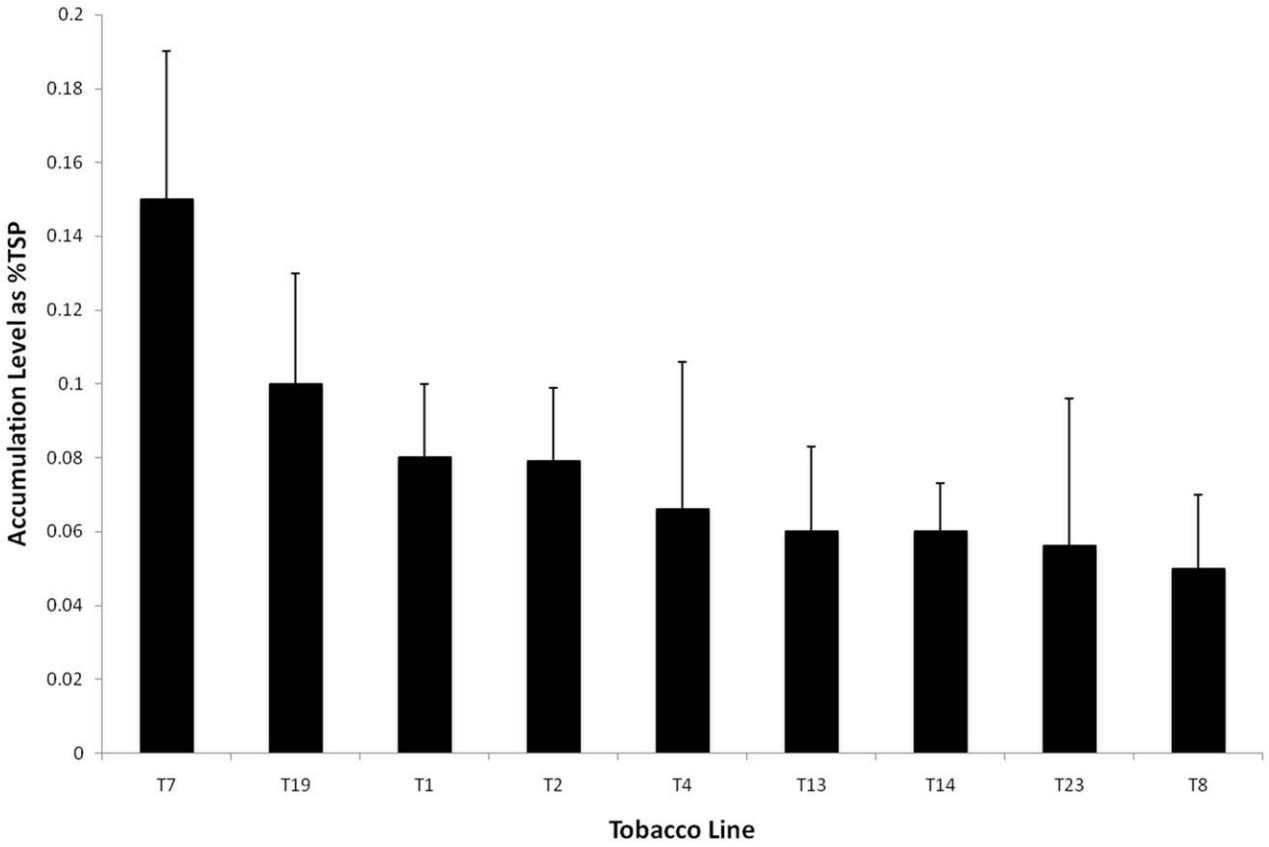

Fig. (5). *ELISA quantification of recombinant GLP-1x10 protein*. The amount of the GLP-1x10 in total soluble protein (TSP) of transgenic tobacco leaf tissues was estimated using anti-His6 ELISA as described in detail in Materials and Methods. The GLP-1x10 protein concentration was expressed as a percentage of TSP. Data shown represent averages of three experiments. The error bars represent the standard deviation: T"x" – individual transgenic tobacco lines.

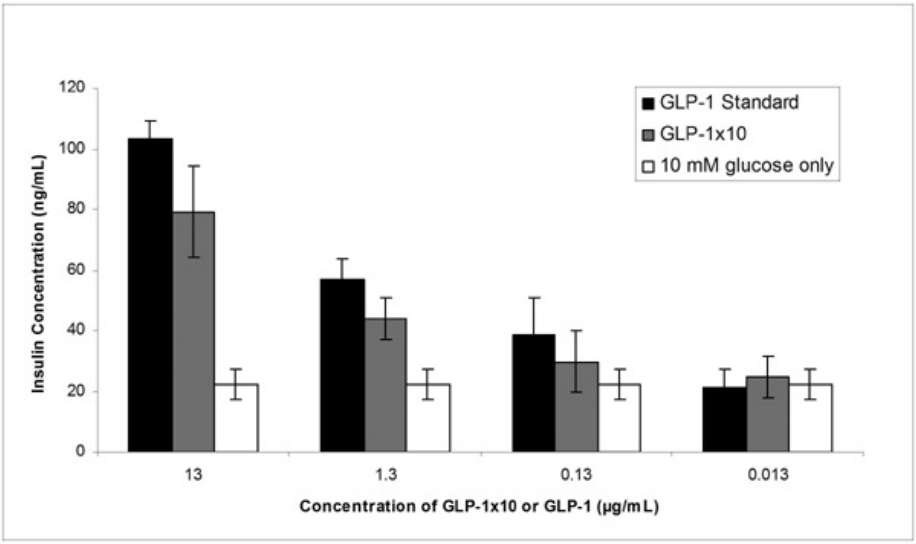

Fig. (6). *Effects of plant-derived GLP-1x10 protein on insulin secretion from MIN6 cells.* MIN6 cells were seeded into 96-well (flat-bottomed) microtiter plates at a density of 3×10^4 cells per well. After 3 days of incubation, the cells were washed two times with Earle's balanced salt solution (EBSS; Sigma) containing 0.1% BSA (Sigma). After starvation of cells for 1 h in EBSS plus 0.1% BSA the cells were incubated with various concentrations of plant-derived GLP-1x10 or commercial GLP-1 standard in the presence of either high glucose (10 mM) or low glucose (2 mM) for 135 min. Cell culture supernatants were then collected and assayed for insulin concentration using an insulin ELISA kit (Crystal Chem Inc., Illinois, USA). The assay was performed in triplicate and repeated once for reproducibility. The data shown are the mean values and standard deviation (error bars) of triplicate analysis.

The treatment with plant-derived GLP-1x10 at a concentration of 13 µg/ml resulted in more than 3-fold increase of insulin concentration in the cell supernatant, compared with 10 mM glucose alone. The increase in insulin release by treatment with commercial GLP-1 is greater, however, than treatment with plant GLP-1x10 at all concentrations tested. Treatment of MIN6 cells with the elute from HiTrap Chelating HP column loaded with total protein extracts from control "81V9" tobacco did not show any stimulatory effect on insulin secretion (data not shown). There was no effect of plant-derived GLP-1x10 on insulin release observed at low glucose (2 mM), although GLP-1 standard still showed insulin-stimulating effect (data not shown). To ensure that the increase in insulin secretion from MIN6 cells by plant-derived GLP-1x10 at high glucose levels (10 mM) was the action of the intact GLP-1x10 decamer protein and not the action of its degradation products, such as the constituent native monomer unit known to be biologically active, cell culture supernatants were subsequently assayed by Western blotting. As shown in Fig. (**7**), a single band was detected, indistinguishable in mobility from the plant GLP-1x10, suggesting that no degradation of GLP-1x10 occurred during incubation with MIN6 cells. Taken together, these results suggest that plant-derived GLP-1x10 remains the bioactivity of GLP-1, although with a reduction when compared with native GLP-1.

DISCUSSION

GLP-1 is a potent insulinotrophic peptide hormone, making it an attractive new drug candidate for the treatment of Type 2 diabetes and related obesity [22-24]. However, the production of small therapeutic peptides such as GLP-1 in quantities sufficient for clinical use is a challenging task. Traditionally, chemical synthesis methods are used for the production of therapeutic peptides [25]. Despite its speed and efficiency, a major limitation of chemical synthesis is its low yield. The use of recombinant DNA technology has the potential to overcome this limitation. Both bacterial and yeast expression systems have proven to be useful for the production of recombinant GLP-1 [8, 9, 26]. However, microbial-based production systems such as bacteria and yeast may not be the system of choice for manufacturing GLP-1 because their production capacity is dependent upon expensive fermentation equipment. In the present study, transgenic plants were tested as an alternative system for the production of recombinant GLP-1. We intended to express *GLP-1* as a large decamer protein (GLP-1x10) as this would increase the stability and thus facilitate the accumulation of GLP-1 in transgenic plants. Western blot analysis using anti-GLP-1 antibody confirmed the expression of a single protein band of the expected size for a GLP-1 decamer (Fig. **4B**). For the purpose of comparison, we have conducted additional experiments to test the feasibility of expressing GLP-1 peptide as a pentamer (GLP-1x5) in transgenic tobacco plants. Unlike the GLP-1 decamer however, no GLP-1 pentamer protein was detected on Western blots containing extracts from GLP-1x5 transformed plants by anti-GLP-1 antibody (data not shown), suggesting that the GLP-1 pentamer may not be as stable as the GLP-1 decamer. It should be mentioned that the mouse anti-GLP-1 primary monoclonal antibody (Santa Cruz Biotechnology) used in the present study showed significant greater affinity for GLP-1x10 than the native GLP-1. Overexposure of a Western blot analysis of native GLP-1 (20 µg) produced only a weak signal (Fig. **4A**). The higher affinity for the GLP-1x10 may suggest that the anti-GLP-1 antibody is capable of binding to multiple sites in GLP-1x10. Yasuda et al. (2006) reported that a GLP-1 pentamer could be accumulated in rice seed when fused to the signal peptide of rice glutelin that directs protein to the endoplasmic reticulum (ER) [16]. However, the rice seed-derived recombinant

protein was found smaller in size than that calculated for a GLP-1 pentamer, likely due to incorrect processing of the engineered signal peptide from the final product. Previously, the recombinant production of GLP-1 in both yeast and bacteria was also achieved by expressing it as large multimers comprised of ten and eight tandem repeats of *GLP-1* sequence respectively [8,9]. Taken together, these results suggest that recombinant expression of small GLP-1 can be optimized by multimerization. This technology has now been increasingly exploited as an effective and reliable tool for achieving efficient mass production of recombinant therapeutic peptides. For example, Tian *et al.* (2007) reported high level expression of an antimicrobial peptide, bovine lactoferricin derivative LfcinB15-W410, in *E. coli* when it was expressed as a tetramer [27]. Kim *et al.* 2008 found that the peptide histonin, also an antimicrobial peptide, was accumulated at high levels in *E. coli* when it was expressed as a peptide multimer [28]. In addition to improving the stability and consequently the accumulation of therapeutic peptides, multimerization may also improve the therapeutic potential of a peptide. Protein/peptide oligomerization may lead to functional advantages such as higher binding strengths, increased immunological potency, and the modification of *in vivo* pharmacokinetic properties [29]. It has been reported that multimerized T cell epitopes increase the immunological potency of soluble peptides and can promote either antigen-specific T cell activation or tolerance through activation-induced cell death *in vitro* at much lower concentrations than the monomeric peptide [30, 31]. Piaggio et al. (2007) demonstrated that intravenous injection of very low-doses of peptides made up of multiple copies of the recognized pancreatic self-antigen epitope (multimerized T cell epitopes) prevented autoimmune diabetes in animal models by inducing dominant tolerance [32].

The accumulation level of plant-derived GLP-1x10 was quantified using ELISA (Fig. **5**). Due to the sequence repeat nature of GLP-1x10, quantification of GLP-1x10 with ELISA using anti-GLP-1 antibody could confuse ELISA results as the anti-GLP-1 monoclonal antibody could theoretically bind to a single GLP-1x10 protein a total of 10 times. This becomes an issue when a commercially available monomeric GLP-1 is used as an ELISA standard, as the anti-GLP-1 antibody could only ever bind the standard once. Indeed, as discussed above, the anti-GLP-1 antibody showed a significantly greater affinity for GLP-1x10 than the native GLP-1. To alleviate this concern, an anti-6xHis-tag antibody was used for ELISA, as the 6xHis-tag was present in only a single sequence motif on each plant-derived GLP-1x10 protein, thereby yielding more accurate results. For this ELISA, a standard curve was generated from a 6xHis-tag-containing reference protein of known quantity. Our ELISA results indicate that plant-derived recombinant GLP-1x10 accumulates up to 0.15% of tobacco plant TSP. This is an encouraging accumulation level, suggesting that the GLP-1 multimer is quite stable in transgenic plants.

Biological activity of the plant-derived recombinant GLP-1x10 was assessed by its ability to enhance glucose-stimulated insulin secretion from a mouse pancreatic β-cell line (MIN6). The MIN6 cell line is derived from a transgenic mouse expressing the large T-antigen of SV40 in pancreatic beta cells and exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets [20]. MIN6 cells have been extensively used as an *in vitro* model to study the mechanism of glucose-stimulated insulin secretion in pancreatic beta cells. Our results indicate that in the presence of high levels of glucose (10 mM), plant-derived GLP-1x10 stimulates insulin secretion in a dose-dependent manner that is similar to that of commercial GLP-1 standard (Fig. **6**). Compared to GLP-1 standard, plant-derived GLP-1x10 showed a reduction in bioactivity. This may be attributed to several factors. First, oligomerization of GLP-1 into a larger molecule may reduce receptor binding affinity due to steric interference. Second, histidine at position 7 (His^7) in GLP-1, as a free N-terminal amino acid, is critical in eliciting biological action [33, 34]. As the N-terminus of GLP-1x10 contains additional methionine (Met) and glycine (Gly) residue serving as part of a translational start site, this could have a negative effect on the bioactivity of the transgenic protein. However, a recent study showed that N-terminal extension of GLP-1 at His^7 by the addition of a lysine residue (Lys) did not affect its biological activity, but instead increased the resistance of GLP-1 to degradation by dipeptidyl peptidase IV (DPP-IV) [35]. It was also found that the substitution of His^7 of the GLP-1 sequence with a phenylalanine (Phe) residue did not lose its biological activity [36]. Third, since GLP-1x10 samples used for assaying glucose-stimulated insulin secretion was only partially purified (Fig. **4C**), the sensitivity of the insulin secretion assay could be compromised by contaminating plant endogenous proteins present in the testing sample. At low levels of glucose (2mM), plant-derived GLP-1x10 failed to show measurable enhancement of insulin release from MIN6 cells, although native GLP-1 still exhibited a stimulatory activity on insulin secretion (data not shown). This may be in part attributed to reduced activity of plant-derived GLP-1x10 relative to native GLP-1. Previously Kitani *et al.* [37] showed that exendin-4, a GLP-1 analogue that mimics the action of endogenous GLP-1 [38], induced insulin secretion from MIN6 cells when stimulated with high levels of glucose (16.7 mM), but did not potentiate insulin secretion under conditions of low glucose (2.8 mM) stimulation. To sure that plant-derived GLP-1x10 remains intact after stimulation of insulin secretion, the supernatant from MIN6 cells incubated with plant-derived GLP-1x10 was further analyzed for the presence of the intact transgenic protein by Western blotting. Western blot analysis showed a single band corresponding to the size of the GLP-1 decamer (Fig. **7**). Taken together, these data suggest that plant-derived GLP-1x10 in its decamer form is biologically active, although with a reduced activity compared to native GLP-1.

In the present study, non-nicotine and low-alkaloid tobacco plants (cultivar '81V9') were used as an expression platform for production of GLP-1x10. Tobacco offers many practical advantages for the large-scale production of recombinant proteins. Tobacco is readily amenable for gene transfer and regeneration. It is a leafy plant, having high biomass yield (up to 100 tonnes of leaf biomass per hectare) and high soluble protein levels. Furthermore, tobacco is neither a food nor feed crop, thus reducing the likelihood of transgenic material contaminating the food or feed chains. One disadvantage of using tobacco as an expression host is the presence of nicotine and other minor alkaloids, which may limit tobacco's therapeutic applications and preclude it from direct oral delivery. Thus, purification of the transgenic protein

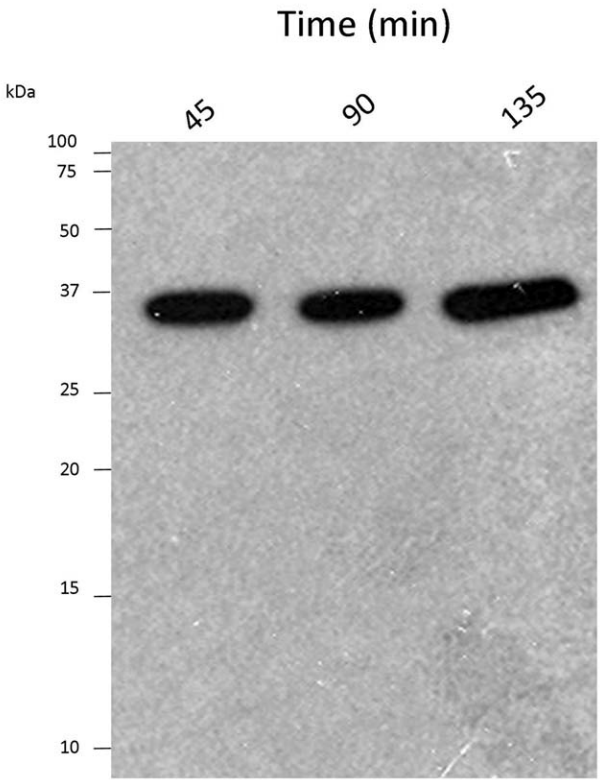

Fig. (7). *Assessment of GLP-1x10 protein integrity during and after the stimulation of insulin secretion by MIN6 cells.* Culture supernatants of MIN6 cells were collected at different incubation times with plant-derived GLP-1x10 and analyzed for possible degradation of the transgenic protein by Western blotting using anti-GLP-1 antibody. A single protein band of the size expected for GLP-1x10 was detected at all three time points, suggesting high stability of the GLP-1x10 protein.

may be required prior to administration [39]. However, many low-alkaloid cultivars of tobacco have been developed for the production of recombinant proteins, such as cultivar '61V9', which makes downstream purification of the target protein easier and less costly, or makes even suitable for direct oral administration of plant tissue or crude protein extracts [40].

In summary, we have developed an alternative approach for the production of GLP-1 in transgenic plants, based on the use of DNA fusion techniques to construct a synthetic gene expressing *GLP-1* as a tandem-repeat decamer. Our data indicate that GLP-1x10 is stably accumulated in plants, and that the plant-derived protein retains biological activity of GLP-1 but with reduced efficacy. To improve the biological activity of plant-derived GLP-1x10, we have recently initiated new experiments designed to leave the N-terminal His of GLP-1x10 as a free amino acid by expressing GLP-1x10 as a C-terminal translational fusion with ubiquitin (UBQ). It has been shown previously that the initial translation products of UBQ fusion genes are accurately and rapidly cleaved *in vivo* by Ubps (UBQ C-terminal hydrolases or deubiquitinating enzymes), a family of sequence specific proteases that release the UBQ monomers [41]. Cleavage occurs irrespective of the amino acid immediately following UBQ, with the exception of Proline (Pro), which is processed inefficiently [41]. Availability of a GLP-1 analog, such as GLP-1x10, with increased plasma stability and low cost will greatly benefit patients with type 2 diabetes.

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