Biologicals 48 (2017) 82–91

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

# Microbial expression of Exendin-4 analog and its efficacy in mice model

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ARTICLE INFO

*Article history:*
Received 21 December 2016
Received in revised form
9 May 2017
Accepted 16 May 2017
Available online 26 May 2017

*Keywords:*
Diabetes mellitus
Exendin-4
Exenatide
Hyperglycemia
Insulin

ABSTRACT

Exendin-4 is a GLP 1 agonist incretin-mimetic peptide hormone comprising 39 amino acids. Exenatide (Byetta®) is a chemically synthesized version of Exendin-4 with an additional C-terminal amidation. Exenatide acts as a GLP-1 receptor agonist. This paper illustrates the method adopted for cloning, fermentation and purification of recombinant Exendin-4 analog expressed in *Escherichia coli*. The biologically expressed analog was extensively characterized using different orthogonal methods to confirm their biological activity and physicochemical properties. It was observed that the expressed analog showed comparable functional properties as that of Byetta® irrespective of their modes of development. Further, *in vivo* efficacy of the recombinant Exendin-4 analog was studied in Oral Glucose Tolerance Test (OGTT) in mice models. Byetta® and Exendin-4 analog treated groups showed comparable glucose lowering activity in the OGTT model.


## 1. Introduction

Diabetes has reached an epidemic status in many developing countries. Prevalence of diabetes is estimated to double by 2030 [1]. The global drug market for Type 2 diabetes mellitus (T2DM) treatments is set to grow by 66% over the next seven years [2]. With an overall market share of 58%, the US dominates the market for Type II diabetes with sales revenue of $16.4 billion for generic and branded drugs.

Among the different available therapies for Type II Diabetes, incretin mimetic such as DPP-4 inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists dominates the non-insulin Type 2 diabetes space [3,4]. GLP-1 receptor agonists are estimated to grow at a rate faster than any available therapies with expected CAGR (Compound Annual Growth Rate) of 17% [5].

Exendin-4 is a GLP-1 receptor agonist belonging to the class of incretin mimetic; it exhibits anti-hyperglycemic actions in a manner similar to endogenous GLP-1 [6–13]. Exenatide (marketed under the trade name Byetta®) is the first synthetic incretin mimetic to be approved by FDA in 2005 for the treatment of Type II Diabetes. Exenatide is used as an adjunct with insulin/metformin/thiazolidinediones to improve glycemic control in T2DM patients. Byetta® is amidated at C terminal end and it is suggested that amidation plays a role in efficacy of the molecule.

The main mechanisms of Exendin-4 includes: i) stimulation of glucose dependent insulin secretion, ii) suppression of inappropriately elevated glucagon secretion, which is known to be elevated in Type II diabetes, and iii) slowing down gastric emptying [7]. Additionally, Exendin-4 does not impair the normal glucagon response and other hormone responses to hypoglycemia and is resistant to breakdown by dipeptidyl peptidase-4 and therefore has a much longer half-life than endogenous GLP-1.

In our study, we have developed a new method for expressing Exendin-4 in a microbial system. The methods of cloning, expression, purification and analytical characterization of non-amidated Exendin-4 analog expressed in *Escherichia coli* [14] are described

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http://dx.doi.org/10.1016/j.biologicals.2017.05.002
1045-1056/

herein. In addition, the paper also discusses the comparative *in vivo* efficacy of proposed non amidated product with that of Byetta® in mice. Data from the *in vivo* glucose lowering activity suggested that both the molecules have similar functional activity and efficacy irrespective of their C-terminal amidation.

## 2. Materials and methods

### 2.1. Cloning of Exendin-4

The *E. coli* strain DH10B (Invitrogen, USA) was used for the transformation of the synthesized DNA. For expression analysis, the *E. coli* strain BL21 (DE3) (Invitrogen, USA) was used. The plasmid pET32a(+) (Novagen, USA) was used for the expression of the synthesized gene in *E. coli*. The protein sequence of exendin-4 was obtained from FDA document and codon optimized for optimum expression in *E.coli*. The synthetic gene containing Exendin-4 gene along with enterokinase site and restriction enzymes was synthesized by GeneArt, Germany and was cloned into pMA-RQ vector. The plasmid pET32a(+) and the Exendin-4 containing pMA-RQ vector were digested with *KpnI* and *XhoI*, ligated, and transformed into *E. coli* DH10B strain. The recombinant plasmids were confirmed by restriction analysis and the gene sequence encoding Exendin-4 was confirmed by DNA sequencing. These recombinant plasmids were later transformed into *E. coli* BL21 (DE3) strain for expression studies.

### 2.2. Shake flask experiments

Exendin-4 is a small peptide with a mass of 4.18 kDa and an isoelectric point (pI) of 4.70. In order to ease the purification process and also to reduce proteolytic effects, Exendin-4 was expressed as a fusion protein containing thioredoxin as a solubility tag to express in soluble form and His-tag as an affinity tag [15,16]. The molecular weight of the fusion peptide was estimated to be approx. 21.1 kDa.

Chemically defined medium (CDM) was used as a basal media for all the shake flask and fermenter experiments. CDM contains 1.7 g/L of citric acid, 13.5 g/L of $KH_2PO_4$, 4.0 g/L of $(NH_4)_2HPO_4$, 1.2 g/L of $MgSO_4 \cdot 7H_2O$ supplemented with 100 mg/L of Thiamine HCl and 50 mg/L of ampicillin and 1 mL/L of trace elements (refer Table 1 **for Trace element composition**). CDM and different carbon sources (glucose and glycerol) were autoclaved at 121 °C for 20 min. *E. coli* BL21 (DE3) strain was used for the expression of fusion protein.

The bacterial strain was grown at 37 °C at 220 rpm using shaker incubator (New Brunswick™ Innova®44) until the absorbance of the culture (600 nm) reached 2, thereafter 1 mM IPTG was added for induction of fusion protein expression. After 3 h of induction, the bacterial culture was harvested and the pellet was subjected to small scale lysis using 1% SDS. Lysis was carried out for 30 min using the sonicator. Subsequently, samples were spun down at 7378 × g for 10 min and the soluble fraction (supernatant) was analyzed in SDS-PAGE to quantify the fusion protein. The fusion protein was quantified using UVP software (UVP, UK) where BSA was used as a standard.

**Table 1**
Composition of trace elements used in the current study.

| Component | Trace elements (g/100 ml) |
|---|---|
| Iron (II) Chloride hexa hydrate | 2.7 |
| Zinc Chloride tetra hydrate | 0.2 |
| Cobaltous chloride | 0.2 |
| Sodium molybdate | 0.2 |
| Calcium chloride di-hydrate | 0.1 |
| Copper (II) Sulphate penta hydrate | 0.1 |
| Boric acid | 0.05 |
| Hydrochloric acid | 10 mL |

### 2.3. Fed batch fermentation conditions

Seed and fermenter media contains CDM basal media and 2% (w/v) glucose was used as a carbon source (Refer shake flask experiment for CDM composition). 400 mL of seed media was inoculated with the *E. coli* BL21 (DE3) strain and incubated at 37 °C, 220 rpm in the shaker incubator (New Brunswick™ Innova®44). After 10 h of incubation, the entire volume of seed culture was transferred into the reactor to start the fermentation. The starting volume for batch fermentation was 4.0 L (Biostat B-DCU II, 5 L vessel, Sartorius). Bioreactor temperature was set at 37 °C and initial stirrer speed was set at 300 rpm. Dissolved oxygen ($pO_2$) was maintained at 30% using the $pO_2$ probe (Hamilton). When the $pO_2$ level dropped below 30% saturation, stirrer speed was increased stepwise upto 800 rpm, followed by sparging of air/pure oxygen to maintain the oxygen level saturation. The pH was maintained at 7.0 using the pH sensor (Hamilton probe) and 25% v/v $NH_3$ base solution. The residual glucose concentration in the fermenter was analyzed at different time points. For glucose estimation, bacterial cultures were spun down at 6721 × g for 5 min. The supernatant was analyzed for glucose concentration using Cedex Bio - Biochemical analyzer from Roche. Once the residual glucose level reached 1% (w/v) in the fermenter, this indicated the end of batch fermentation and feed additions were initiated based on the availability of glucose. Feed addition was initiated at the growth phase and was added manually in a controlled manner, such that the residual glucose concentration was maintained between 1 and 2% (w/v) in the fermenter. When the culture absorbance reached approximately 45 optical density (measured at 600 nm), induction was initiated by adding 1 mM IPTG to the culture (induction at different culture absorbance were used, data not shown). This indicated the end of the growth phase. During induction phase (typically lasts for upto 4 h), two different modes of feeding, bolus and pH stat modes were followed to check for fusion protein expression. After 4 h of induction phase, the culture was harvested and centrifuged at 6721 × g for 20 min. The pellet was lysed and analyzed for soluble fraction using SDS-PAGE analysis. BSA was used as standard for fusion protein quantification. Quantification was carried out using the UVP software. Cell pellet was further subjected to downstream purification and analytical characterization.

### 2.4. Purification of Exendin-4

The downstream process for obtaining purified Exendin-4 broadly consists of three chromatography steps along with incorporation of Tangential Flow Filtration (TFF) and final Ultrafiltration and Diafiltration (UF/DF).

Capture of the fusion protein containing His Tag was carried out using Immobilized Metal ion Affinity (IMAC) chromatography with Ni affinity variant. The Ni Sepaharose resin used as an affinity matrix was procured from GE, Sweden. The resin was packed in XK26/40 column [17]. Tangential Flow Filtration for buffer exchange was carried out for capturing fusion protein before subjecting it to enzyme cleavage assay.

As mentioned earlier, recombinant Enterokinase (EK) was procured from Hangzhou Junfeng Bioengineering Co Ltd, China. 2 units of EK were added per 1 absorbance of the fusion protein (measured at 280 nm) in combination with $CaCl_2$ to a final concentration of 2 mM to cleave the Exendin-4 from fusion tag. Fusion protein sample was incubated with EK at 24–26 °C for 8–10 h. After