Peptides 91 (2017) 33–39

Contents lists available at ScienceDirect

Peptides

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

Semi-microbiological synthesis of an active lysinoalanine-bridged analog of glucagon-like-peptide-1

Anneke Kuipers[a], Louwe de Vries[a], Marcel P. de Vries[b,c], Rick Rink[a], Tjibbe Bosma[a], Gert N. Moll[a,d,*]

[a] Lanthio Pharma, a MorphoSys AG company, 9727 DL Groningen, The Netherlands
[b] Mass Spectrometry Core Facility, Department of Pharmacy, University of Groningen, 9713 AV Groningen, The Netherlands
[c] Department of Pediatrics, University Medical Center Groningen, University of Groningen, 9713 GZ Groningen, The Netherlands
[d] Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 11 October 2016
Received in revised form 3 March 2017
Accepted 7 March 2017
Available online 12 March 2017

Keywords:
Glucagon like peptide-1
Lanthipeptide
Nisin
Lysine
Dehydroalanine

ABSTRACT

Some modified glucagon-like-peptide-1 (GLP-1) analogs are highly important for treating type 2 diabetes. Here we investigated whether GLP-1 analogs expressed in *Lactococcus lactis* could be substrates for modification and export by the nisin dehydratase and transporter enzyme. Subsequently we introduced a lysinoalanine by coupling a formed dehydroalanine with a lysine and investigated the structure and activity of the formed lysinoalanine-bridged GLP-1 analog. Our data show: (i) GLP-1 fused to the nisin leader peptide is very well exported via the nisin transporter NisT, (ii) production of leader-GLP-1 via NisT is higher than via the SEC system, (iii) leader-GLP-1 exported via NisT was more efficiently dehydrated by the nisin dehydratase NisB than when exported via the SEC system, (iv) individual serines and threonines in GLP-1 are dehydrated by NisB to a significantly different extent, (v) an introduced Ser30 is well dehydrated and can be coupled to Lys34 to form a lysinoalanine-bridged GLP-1 analog, (vi) a lysinoalanine(30-34) variant's conformation shifts in the presence of 25% trifluoroethanol towards a higher alpha helix content than observed for wild type GLP-1 under identical condition, (vii) a lysinoalanine(30-34) GLP-1 variant has retained significant activity. Taken together the data extend knowledge on the substrate specificities of NisT and NisB and their combined activity relative to export via the Sec system, and demonstrate that introducing a lysinoalanine bridge is an option for modifying therapeutic peptides.

1. Introduction

GLP-1 is highly important for treating type 2 diabetes mellitus [7]. It is released as a response to glucose administration and to ingestion of meals. GLP-1 stimulates insulin gene expression, insulin secretion by the pancreatic β-cells, and inhibits glucagon secretion by the α-cells without conferring hypoglycemia [19,40]. In addition GLP-1 improves cardiovascular parameters [4] and promotes satiety. Interestingly, peripherally administered [Ser8]GLP-1 and the endogenous GLP-1 contribute to the regulation of feeding [17]. GLP-1 can act on vagal afferent neurons and does not need to penetrate the CNS [23].

GLP-1 is mainly produced in the L-cells of the intestine. Two active forms of GLP-1 are present, GLP-1-(7-36)-NH_2 and GLP-1-(7-37). Both hormones regulate glucose metabolism with comparable efficacy [31,42]. In addition a C-terminal fragment GLP-1(28-36) amide seems to have therapeutic activity in diabetes [28,41,44].

GLP-1 is *in vivo* broken down by dipeptidyl peptidase IV (DPP-IV), which cleaves the first two amino acids yielding the antagonist GLP-1-(9-36) [20,22]. It contains also six cleavage sites for neutral endopeptidase 24.11 (NEP) [14]. Modified GLP-1 analogs, and analogs of the related DPP-IV-resistant exenatide, have been developed that have prolonged half life. Liraglutide is a GLP-1 variant to whose Lys26 a 16C acyl chain is linked which allows binding to albumin. Albiglutide comprises two GLP-1 molecules covalently bound to albumin. Dulaglutide is composed of GLP-1 fused to an immunoglobulin G [33].

Bacterial production of therapeutic peptides can be combined with posttranslational modifications exerted by bacterial enzymes. Here we investigated whether GLP-1 analogs expressed in *Lac-*

* Corresponding author at: Lanthio Pharma, a MorphoSys AG company, Rozenburglaan 13B, 9727 DL Groningen, The Netherlands. Tel.: +31 50 3050247.
E-mail address: moll@lanthiopharma.com (G.N. Moll).

http://dx.doi.org/10.1016/j.peptides.2017.03.004
0196-9781/

tococcus lactis could be substrates for modification and export by the nisin dehydratase and transporter enzyme. Nisin is produced by some *L. lactis* strains. Its precursor peptide comprises an N-terminal leaderpeptide which leads to the interaction with the modification and transporter enzymes. The dehydratase NisB dehydrates serines and threonines in the core peptide. The cyclase NisC catalyzes the coupling of the formed dehydroamino acids to cysteines yielding nisin's pentacyclic structure. The transporter NisT exports the modified precursor out of *L. lactis*. By genetically fusing the leaderpeptide to angiotensin-(1-7), vasopressin, adrenocorticotropic hormone, an inhibitor of tripeptidyl peptidase II, enkephalin, luteïnizing hormone-releasing hormone and bombesin these could also be exported via NisT [2,21,25]. However, an azurin analog, which is larger than the above mentioned peptides, was less efficiently transported via NisT [27]. Unmodified GLP-1 has been exported out of *L. lactis* via the general secretion system Sec [1]. NisB-catalyzed dehydration of serines and threonines can partly be predicted based on guidelines concerning only the directly flanking amino acids [34]. We here investigated whether GLP-1 analogs, larger than the above mentioned other peptides, could be modified and transported via the nisin modification and transporter enzymes and compared this to NisB-mediated modification followed by export via the SEC system. Making use of dehydroalanine-containing GLP-1 as starting point we installed a lysinoalanine bridge and performed structural and activity measurements.

2. Materials and methods

2.1. Peptides

The control peptide GLP-1-(7-36)NH_2 was purchased from Sigma-Aldrich, the lactam GLP-1 variant, LP1-19 [29], was synthesized by Pepscan, Lelystad, NL. All other peptides were produced by *L. lactis* (Fig. 1) using the two plasmid system [34].

2.2. Molecular cloning

The producing *L. lactis* strain NZ9000 harbors two plasmids, a pIL253 plasmid encoding the *nisBTC* genes behind the inducible nisin promoter and a pNZ8048 derived plasmid encoding the substrate peptide, C-terminally fused to the nisin leader (Table 1). Although no cyclase activity was required in the here described experiments, the *nisC* gene was kept included on the pIL3BTC plasmid since NisBTC has been demonstrated to form *in vivo* a functional membrane-associated enzyme complex [18,36]. The encoding sequences for the different GLP-1 analogs were introduced by means of PCR [26]. Amplifications were performed with Phusion DNA polymerase (Finnzymes, Finland) and PCR fragments were self-ligated with T4 ligase (Roche). The ligations were transformed to competent *L. lactis* cells [12] via electroporation using a Bio-Rad gene pulser (BioRad, Richmond, CA). Transformed cells were plated out on M17 broth [39] containing 0.5% glucose, 1.5% agar and the antibiotics chloramphenicol (5 μg/mL) and erythromycin (5 μg/mL). Plasmid DNA of the transformants was sequenced by BaseClear (Leiden).

2.3. Production, isolation and purification of the peptides with dehydroamino acids

L. lactis was grown overnight at 30 °C in M17 broth [39] supplemented with 0.5% glucose and chloramphenicol (5 μg/mL) and erythromycin (5 μg/mL). The overnight culture was 100-fold diluted in minimal medium [15] supplemented with glucose, chloramphenicol (5 μg/mL) and erythromycin (5 μg/mL). For induction supernatant of the nisin-producing strain NZ9700 (1:1000) was administered and the culture was grown further for 24 h at 30 °C. Cell-free supernatant from the resulting cultures was equilibrated with 1 volume 100 mM lactic acid and acidified with HCl to pH 2.5. Peptides were bound to a 5 mL HiTrap SP column (GE Healthcare), washed with 50 mM lactate buffer pH 4 and eluted with 50 mM lactate buffer containing 1 M NaCl and 6 M Urea. The eluted fraction was desalted by passage over a PD-10 gel filtration column (GE Healthcare). For the isolation from 10 L, whole cultures were equilibrated as described above and a streamline 25 column (GE Healthcare) filled with 150 mL streamline SP agarose was used. The same buffers as mentioned above were used for washing and eluting the loaded peptides. The eluted peptides were precipitated with 10% TCA, the pellet was washed with acetone and dissolved in 10% ACN/0.1% TFA and applied on a C18 HiBar 250 × 25 column (LiChrospher) using a HP 1050 HPLC system. To cleave off the nisin leaderpeptide, desalted and purified peptides were incubated overnight at room temperature with Factor Xa (New England Biolabs) in 78 mM Tris, 10 mM NaCl, 2 mM $CaCl_2$ pH 8.0. It should be noted that in the substrate peptide no IEGR site is present. However, Factor Xa efficiently cleaves here after ASPR, consistent with its known broad substrate specificity [13]. The liberated peptides of interest were further purified using a semi prep C12 RP 250 × 10.00 column (Phenomenex) on a HP 1050 HPLC system. Analyses of modified peptides samples were performed with a JASCO LC-1500 HPLC system using a C12 RP 250 × 4.60 column (Phenomenex). All HPLC runs were performed using a gradient of 10%–90% ACN in 0.1% TFA. The purified GLP-1 analogs were quantified by HPLC analysis by comparing the A280 area of the peak with the A280 area of a known amount of the GLP-1 receptor agonist, GLP-1-(7-36)NH_2.

2.4. Lysinoalanine introduction

Lysinoalanines were introduced in dehydroalanine-containing GLP-1 analogs, by incubating in 0.25% ammonia for 18 h at RT which induced coupling of dehydroalanine to the epsilon NH_2-group of a lysine [3]. The peptides were subsequently once more purified by HLPC. Trypsin (Sigma) was used to establish the localization of the lysinoalanine. Approximately 1 nmol peptide was treated with 1 μg trypsin for 60 min to 90 min at 37 °C. Digestions were analyzed by mass spectrometry.

2.5. Mass spectrometry

The produced modified peptides and peptide fragments obtained after digestion with Factor Xa and/or trypsin were separated and purified by HPLC. Mass spectra were recorded with a Voyager DE PRO Maldi TOF mass spectrometer (Applied Biosystems). In order to obtain high sensitivity an external calibration was applied. HPLC-MS/MS was performed as follows. For separation, a Thermo/Dionex UltiMate 3000 UHPLC was fitted with a Phenomenex Kinetix 100 mm, 2.6u, EVO, C18, 100 Å, column with a diameter of 2.1. The mobile phase composition was (A) Acetonitril, (B) 2% Formic acid and (C) mQ water. The column temperature was maintained at 40 °C. The LC system was coupled to a Thermo Q-Exactive mass spectrometer for analysis using the HESI source in positive-ion mode and maintained at 3.5 kV and 425 °C. The gradient began at 15:5:80 of A:B:C with a flow of 0.25 mL/min and kept constant for 1 min. The gradient was changed to 50:5:45 of A:B:C over a course of 29 min. In 0.1 min the gradient was changed to 80:5:15 of A:B:C and kept constant for 5 min. In 0.1 min the gradient was returned to the starting condition. The total LC run time was 40 min. Data were acquired in full scan mode from m/z 500 to 1500 at a resolution 70,000 at m/z 200 to select the triply charged ions for DDA at a AGC target of 2e5 and at a resolution of 17,500 in profile mode. A stepped NCE of 25–30 was used. Instrument control