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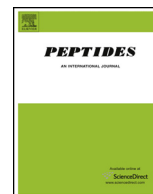
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Semi-microbiological synthesis of an active lysinoalanine-bridged analog of glucagon-like-peptide-1



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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.

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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)-NH₂ 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-*

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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, adrenocorticotrophic hormone, an inhibitor of tripeptidyl peptidase II, enkephalin, luteinizing 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)NH₂ was purchased from Sigma-Aldrich, the lactam GLP-1 variant, LP1-19 [29], was synthesized by Pepsan, 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 CaCl₂ 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)NH₂.

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 NH₂-group of a lysine [3]. The peptides were subsequently once more purified by HPLC. 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, 2.6 µm, EVO, C18, 100 Å, column with a diameter of 2.1. The mobile phase composition was (A) Acetonitrile, (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

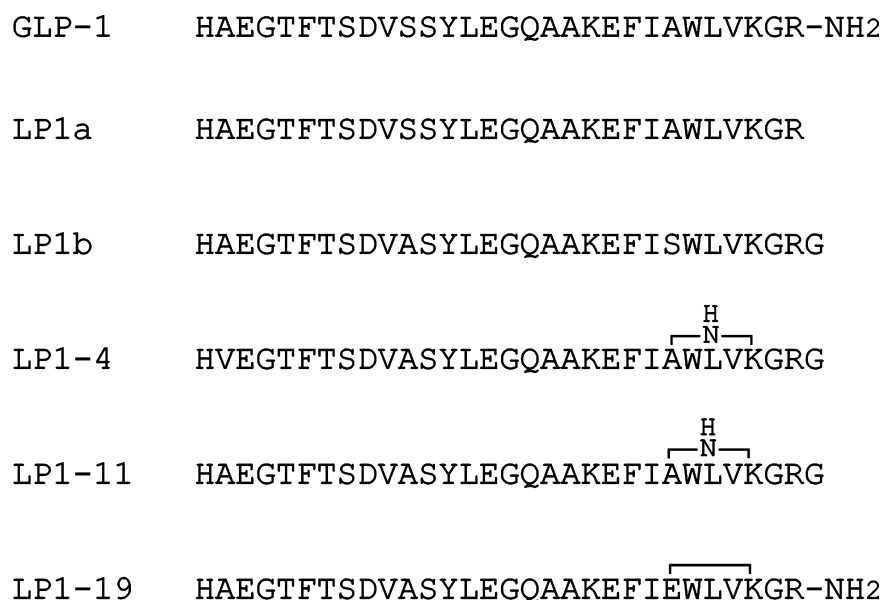


Fig. 1. Peptides used in this study. A fraction of LP1-4 and LP1-11 contains an additional dehydration.

Table 1
Bacterial strains and plasmids.

Strain	Characteristics	Reference
NZ9000	MG1363 derivative; <i>pepN::nisRK⁺</i>	[5]
Plasmids	<i>pIL253-derived plasmids</i>	[38]
pIL3BTC	<i>Pnis</i> + inverted repeat + <i>nisBTC</i> , Cm-r	[34]
pIL5BC	<i>Pnis</i> + <i>nisBC</i> , Em-r	[25]
pNZ8048 derived plasmids	<i>Pnis</i> + encoding sequence for fusion peptide: <i>nisin leaderpeptide::core-peptide</i> , Em-r	[24]
pNZLP1a	leader::HAEGTFTSDVSSYLEGQAAKEFIAWLKGR	This study
pNZLP1b	leader::HAEGTFTSDVASYLEGQAAKEFIASWLKGRG	This study
pNZLP1-4	leader::HVEGTFTSDVASYLEGQAAKEFISWLKGRG	This study
pNG41LP1a	sequence encoding SP _{usp45} :: <i>nisin leader</i> ::LP1a-encoding sequence	This study

and data acquisition and evaluation were performed with Thermo Xcaliber software.

2.6. Circular dichroism spectroscopy

Circular dichroism spectra of 50–150 µg/mL peptide were measured in 5 mM phosphate buffer of pH 7.4 at 23°C on a Jasco J-715 apparatus in a 1 mm cell. Scans were made between 190 nm and 260 nm with 1 nm steps. Increasing concentrations of trifluoroethanol (TFE) were used to observe the step-wise structural change in the peptides. The protein concentrations were determined by measuring the absorption at 280 nm and calculating the concentration using the extinction coefficients 6970 M⁻¹ cm⁻¹. The mean residue ellipticity [θ]mrw (mdeg cm²/dmol⁻¹) was calculated from the measured ellipticity θ using the following formula: [θ]mrw = θ × Mmrw / (10 × c × l); θ = ellipticity (mdeg); Mmrw = mean residue molecular weight (MW/nr. aa's); c = concentration (mg/mL); l = path length (cm). The mean residue ellipticity at 222 nm of -36000 mdeg cm² dmol⁻¹ of polylysine peptide was taken as a measure of 100% α-helix.

2.7. Activity of GLP-1 peptide analogs

Activity was assayed based on the peptide-induced increase in extracellular cAMP using a Rin-5F rat pancreas β cell line [9,37]. Rin5F cells, obtained from the American Type Culture Collection

(ATCC), were grown in 12 well plates as a monolayer culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C in a 5% CO₂ atmosphere. After 4 h of serum free culturing cells were challenged for 10 min with 100 µL of 100 nM of the GLP-1 analogs in triplicate. The cAMP in the medium was measured using the cAMP EIA kit (Enzo Life Science) according to the manufacturer's protocol using synthetic human GLP-1-(7-36)NH₂ and exendin-4-(1-39)NH₂ [6], a lactam(30-34) GLP-1 analog, LP1-19, and the GLP-1 receptor antagonist exendin-9 [10] for control experiments. Activities were measured with and without the antagonist exendin-9-(9-36)NH₂, which was added 10 min prior to the agonist GLP-1 analogs. For dose response curves a DiscoverX cAMP assay was applied. Peptides were dissolved in DPBS buffer, purchased from Biowest, with 0.1% BSA.

3. Results

3.1. *L. lactis* produces GLP-1 via the nisin transporter

Production of leader-GLP-1 out of NisBC-containing *L. lactis* via NisT was significantly higher than via the SEC system. Furthermore, in the presence of NisBC, the extent of dehydration of NisT-exported peptide was higher than of SEC-exported peptide without NisT (Fig. 2). Therefore experiments were continued using the route via NisBTC.

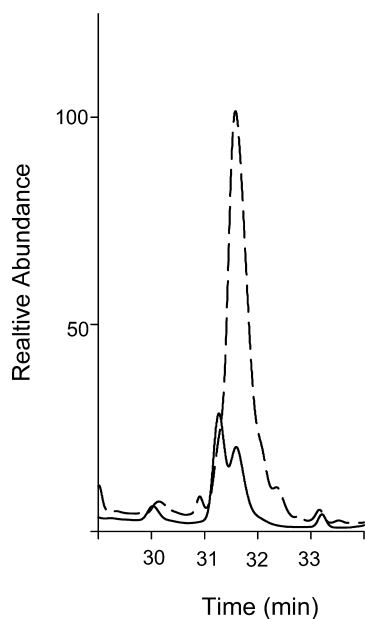


Fig. 2. Both export and dehydration of nisin leader fusion peptides out of NisBC-containing *L. lactis* are more efficient for export via NisT than for export via SEC. The nisin leader::fusion peptides were obtained via TCA precipitation of 20 mL supernatant from the cultures NZ9000 pIL3BTC pNZLP1a (interrupted line) or NZ9000 pIL5BC pNG41LP1a (continuous line) and subsequently applied on a JASCO HPLC system using a C12 column measuring A280. Interrupted line: transport via NisT. The peak corresponds to leader-LP1a mainly 1-fold dehydrated. Continuous line: transport via SEC; the main peak corresponds to leader-unmodified LP1a; the smaller peak corresponds to leader-1-fold dehydrated LP1a. The yield via NisT was 3.7-fold higher than via the SEC system.

3.2. Differential dehydration by the nisin dehydratase

The A30S mutation was introduced aiming at the formation of a Dha30 which would be a suitable starting point for lysinoalanine-formation. NisBTC-exported leader-LP1b was first analyzed by trypsin cleavage, which demonstrated that the C-terminal Δ G35R36G37 fragment EFISWLVK was mostly 1-fold dehydrated, proving dehydration of Ser30, whereas only a small fraction of this fragment had escaped dehydration (Fig. 3).

Subsequently MS/MS analyses were applied to NisBTC-exported LP1b, from which the leaderpeptide had been removed by Factor Xa, which confirmed the efficient dehydration of Ser30 (Table 2). The 1-fold dehydrated peptides contained a Dhb at position 13 or a Dha at position 30. The 2-fold dehydrated fragments contained the Dhb13 and Dha30, which resulted from efficient dehydration of T13 and S30 respectively. The 3-fold and 4-fold dehydrated fragments contained, in addition to Dha30, either Dhb11 and/or Dhb13 and/or Dha14. Taken together, a mixture of peptides with different dehydration patterns was produced in most of which Dha30 was present (Table 2).

3.3. Lysinoalanine introduction

Dehydrobutyrines are much less reactive than dehydroalanines and therefore not suitable for coupling to a lysine. By contrast the Dha30 is more reactive and located close to Lys26 and Lys30. After pH-induced lysinoalanine formation removal of the leader and HPLC purification peptide fragments were analyzed by trypsin cleavage and mass spectrometry to assess whether and where lysinoalanines were formed. A minor fraction with peptide mass of 3050 Da corresponded to two-fold dehydrated Δ (G35R36G37)LP1b, HAEGTFTSDVASYLEGQAAKEFISWLVK, with a lysinoalanine(26–30) in the underlined sequence part. A larger puri-

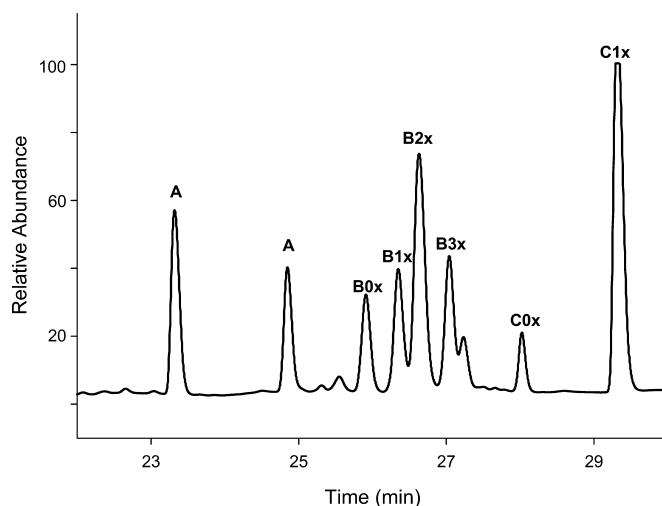


Fig. 3. Proteolytic analysis of a dehydrated leaderpeptide fusion peptide. Peptides from 15 mL induced culture *L. lactis* NZ9000 pIL3BTC pNZLP1b were cleaved by trypsin and analyzed by HPLC using the JASCO system measuring the absorption at 205 nm. Fragments A are nisin leader fragments. Fragment B is the N-terminal part: HAEGTFTSDVASYLEGQAAK (0 \times , 1 \times , 2 \times and 3 \times dehydrated) and fragment C is the C-terminal part EFISWLVK (0 \times and 1 \times dehydrated).

fied peptide fraction yielded, after trypsin treatment, a peptide with a mass of 1218 Da which corresponds exactly with the M+H⁺ mass of the dehydrated fragment EFISWLVKGR corresponding to residues 27–36. Since the fragment EFISWLVK was not observed the lysine in Δ (G37) fragment EFISWLVKGR must be protected by a formed lysinoalanine(30–34) in the underlined sequence part. On the basis of the surface of the HPLC peaks the formation of the lysinoalanine(30–34) was 3.5-fold more efficient than the formation of the lysinoalanine(30–34), and lysinoalanine(30–34) constituted 39.7% of the produced peptides.

3.4. Lysinoalanine(30–34) is compatible with alpha helical structure of GLP-1

CD-spectra were recorded for the peptides GLP-1, LP1-11 and LP1-19. Fig. 4A and B shows the spectra recorded with 0 and 25% TFE, respectively. GLP-1 and LP1-11 show similar spectra in buffer and show a low percentage of α -helix. LP1-19 already contained significant α -helical structure in plain buffer when compared to GLP-1, indicating that the β -lactam ring stabilizes or induces α -helical structure. The presence of TFE induced more α -helical structure in all three peptides. The lysinoalanine bridge was able to induce more α -helix in LP1-11 in the presence of 25% TFE when compared to the natural peptide, GLP-1. In conclusion, the presence of either a lysinoalanine or β -lactam bridge at position 30–34 made the peptide more prone to the α -helix form.

3.5. Activity of lysinoalanine-bridged GLP-1 analogs

The natural linear amidated peptide GLP-1, the 30–34 lysinoalanine-bridged LP1-11 and the control beta lactam-bridged LP1-19 had comparable activity (Fig. 5). The 30–34 lysinoalanine-bridged LP1-4, which has Val8 to confer resistance to DPPIV [11], was less active. These data indicate that the GLP-1 analog LP1-11, which has a S17A mutation and lysinoalanine(30–34), had retained some activity. Pre-incubation with the GLP-1 receptor antagonist exendin-9 caused significant reduction in activity of the active variants, consistent with their action via the GLP-1 receptor.

The activity of the GLP-1 analogs was measured via the induction of cAMP production in Rin-5F rat pancreas β cells relative to that induced by exendin 4. The cAMP level induced by the ago-

Table 2
NisB-dehydrated LP1b variants.

Dehydration pattern	Dehydration	Relative abundance (a.u.)
7 10 15 20 25 30 35		
HAEGTFTSDVASYLEGQAAKEFISWLLVKGRG	0	24
HAEGTFTSDVASYLEGQAAKEFIDhaWLLVKGRG	1	13
HAEGTFDhbSDVASYLEGQAAKEFISWLLVKGRG	1	4
HAEGTFDhbSDVASYLEGQAAKEFIDhaWLLVKGRG	2	38
HAEGDhbFDhbSDVASYLEGQAAKEFIDhaWLLVKGRG	3	9
HAEGDhbFTDhaDVASYLEGQAAKEFIDhaWLLVKGRG	3	10
HAEGDhbFDhbDhaDVASYLEGQAAKEFIDhaWLLVKGRG	4	2

MS/MS analyses were applied to NisBTC-exported LP1b. The sequence ions *b*, *b*+2, *y*, *y*+, *b*+2, *y*, *y*+2 were used to determine which residues were dehydrated. The relative abundance of the triply charged ions in the spectrum was used to indicate the efficiency of dehydration of the determined dehydrated residue. Dha: dehydroalanine, Dhb: dehydrobutyryne, a.u.: arbitrary units.

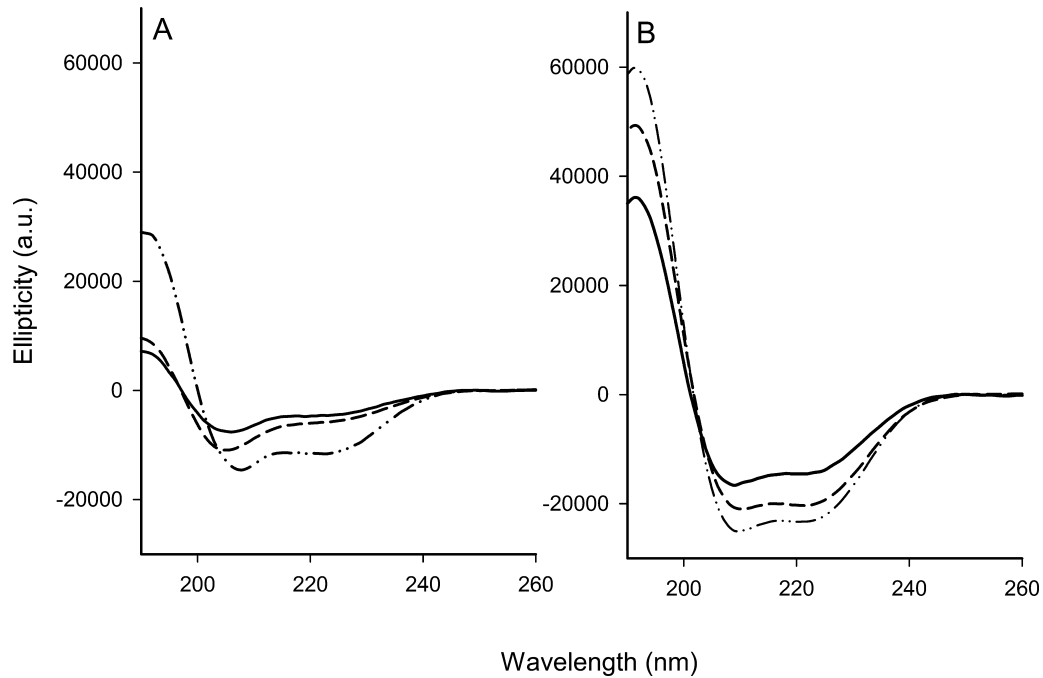


Fig. 4. AB. The lysinoalanine (30–34) in GLP-1 is compatible with alpha helical structure. CD spectra of GLP-1, LP1-11 and LP1-19 spectra in 5 mM phosphate buffer pH 7.4 (A) and in presence of structure-inducing organic solvent 25% TFE (B). Continuous line: GLP-1, interrupted line: LP1-11, strokes/dots: LP1-19.

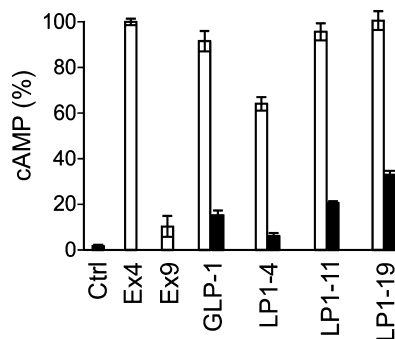


Fig. 5. Activity of lysinoalanine-bridged GLP-1 analogs.

nist exendin4 was set at 100% and the levels induced by the other peptides were expressed as percentage of this value. Ctrl: control medium without a peptide; Ex-4: the GLP-1 receptor agonist exendin4; Ex-9: the antagonist exendin9; the GLP-1 analogs, GLP-1, LP1-4, LP1-11, LP1-19 as depicted in Fig. 1 with (solid bars) or without (open bars) Exendin9 [10].

To accurately compare GLP-1 and LP1-11 with respect to their capacity to induce cAMP dose response curves were obtained (Fig. 6). The two peptides differ in three aspects: LP1-11 has a S17A

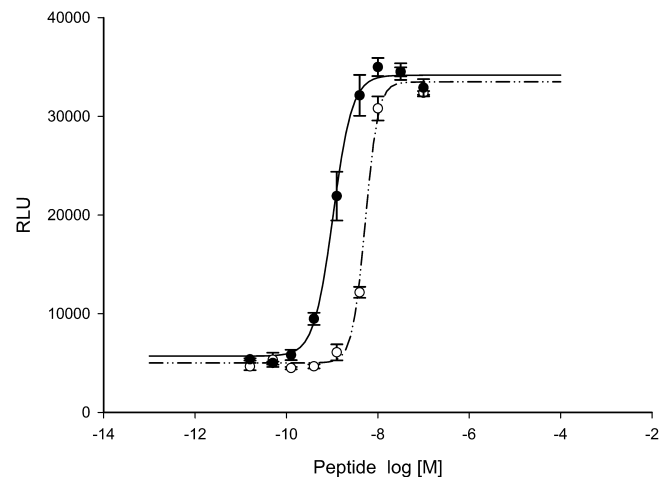


Fig. 6. Dose response curves of GLP-1 and LP1-11.

mutation, a lysinoalanine (30–34) and a C-terminal Gly instead of NH₂. The EC₅₀ values of GLP-1 and LP1-11 were respectively 1.04 nM and 5.4 nM.

cAMP-induction by GLP-1 (filled symbols) and LP1-11 (open symbols) was measured as described under Section 2.

4. Discussion

The number of patients diagnosed with type 2 diabetes mellitus dramatically increases world-wide. This disease is progressive and the decline of β -cell function causes an increasing defect in the insulin response to glucose. The traditionally utilized insulin therapy may cause weight gain and raises the risk of hypoglycemic episodes. As a consequence much effort is invested in developing new effective therapies. Five GLP-1 receptor agonists are presently applied and more are in development [33]. Research is directed at improved receptor interaction by stabilization of the C-terminal alpha helical structure, improving the resistance to breakdown by peptidases, development of specific peptidase inhibitors and improving pharmacokinetics and oral delivery. Lysinoalanines are found in some lantibiotics like cinnamycin [16] duramycin B and duramycin C [43]. Orf7-catalyzed lysinoalanine formation in cinnamycin by coupling of the dehydrated serine at position 6 to the C-terminal lysine has been demonstrated [30]. Neither the substrate specificity of Orf7 nor its functionality in *L. lactis* is known. To the best of our knowledge the introduction of a lysinoalanine is a new method in peptide modification and in particular the use of bacterial engineering to eventually produce lysinoalanine-stabilized peptide therapeutics is new. Here we applied *L. lactis* containing lanthipeptide modification enzymes to produce lysinoalanine-bridged GLP-1 analogs. A lysinoalanine forms a longer bridge from backbone to backbone than a lanthionine. Ahn et al. successfully demonstrated alpha helical structure stabilization in both the N- and C-terminal regions as well as improved receptor activation capability by introduction of multiple beta lactam cross links in GLP-1 [29]. We here demonstrated that also a lysinoalanine(30–34) in GLP-1 is compatible with alpha helical structure. Generally, depending on the type of crosslink and position, intramolecular bridges may strongly reduce or abolish activity. Importantly, lysinoalanine(30–34) retains significant activity. Taken together the data indicate the option of lysinoalanine introduction in the discovery of therapeutic peptide analogs.

Knowledge on the substrate specificities of the nisin dehydratase, NisB, and nisin transporter, NisT, is increasing. NisT can transport short peptides unrelated to nisin [21,34]. Compared to the above-mentioned studies, GLP-1 is a relatively large, surprisingly good substrate of NisT. A systematic study involving amongst others peptide length, charge and hydrophobicity would be desirable to get further insight on the substrate specificity of NisT. In contrast to an azurin fragment [27], which is better transported via the SEC system than via NisT, we here demonstrate that GLP-1 is clearly better transported via NisT than via the SEC system. The data also show that NisBTC-exported GLP-1 is dehydrated to a larger extent than exported via the SEC system in the presence of NisBC without NisT. No association of NisB with the SEC system has been described and therefore SEC-exported peptide may easily escape NisB-mediated dehydration [26]. The structural organization of a membrane-associated NisBTC-complex [18,36] likely favors the efficient modification of substrate peptides prior to export.

In vitro NisB activity has been reconstituted [8] and the structure and mechanism of NisB have been reported [32]. Our studies on NisB-dehydrated GLP-1 provide some specific information on the dehydration of the individual serines and threonines in this peptide. In other peptides threonines generally appear to be better dehydrated than serines and hydrophobicity of at least one of the directly flanking residues of dehydratable serines and threonines favors dehydration [34]. Furthermore glycins and negatively charged flanking amino acids disfavor dehydration. A study with

a small semi-randomized peptide library suggested that dehydration takes place when one or both directly flanking amino acids are hydrophobic, but no dehydration takes place when both directly flanking residues are hydrophilic [35]. The MS/MS analyses of dehydrated GLP-1 here demonstrate that Ser30, which is flanked by two very hydrophobic amino acids, Ile and Trp, is the most frequently dehydrated residue. Surprisingly dehydration occurs of Ser14 which is flanked by a Thr/Abu and an aspartate. On the other hand Ser18 seems to escape dehydration despite the fact that it is preceded by an Ala. Taken together, the hydrophobicity of both the directly flanking amino acids of Ser/Thr plays a role and the extent of dehydration of the individual Ser/Thr positions in the GLP-1 peptide context contributes to the knowledge of the substrate specificity of NisB.

5. Conclusion

We here studied export and modification of GLP-1 via the nisin modification and transporter enzymes out of *L. lactis*. GLP-1 is more efficiently exported via NisT than via the SEC system. Individual serines and threonines in GLP-1 are dehydrated by NisB to a significantly different extent. *L. lactis* containing lanthipeptide modification and transporter enzymes can be exploited in drug discovery for the indirect generation of active lysinoalanine-bridged alpha helix-containing therapeutic peptides.

Conflict of interest statement

The authors confirm that this article content has no conflict of interest.

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