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Research paper

Improved peroral delivery of glucagon-like peptide-1 by site-specific biotin modification: Design, preparation, and biological evaluation

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Abstract

Peptide oral delivery is still a significant challenge because of two major impediments, low absorption efficiency and instability in gastrointestinal tract. The aim of this study was to design, prepare, and evaluate an intestine enzyme-resistant, superior intestine absorptive, and biologically preserved glucagon-like peptide-1 (GLP-1) derivative using the site-specific modification of biotin, which can take advantage of the carrier-mediated active intestine transport. Two series of site-specific Lys^{34}- and $Lys^{26,34}$–biotin–GLP-1 derivatives were prepared, and their intestine membrane permeabilities, proteolytic stabilities against the intestine enzymes, and bioactivities were then evaluated. Especially, $Lys^{26,34}$–biotin–GLP-1 was found to have the most promising scores: (i) it displayed a 5.6-fold higher Caco-2 cell monolayer permeability than GLP-1; (ii) it showed an 8.5- and 3.5-fold longer half-life than GLP-1 in rat intestine fluid and homogenate, respectively; and interestingly (iii) it had a well-preserved insulinotropic activity (94.5% vs. GLP-1) in the rat islets. Finally, $Lys^{26,34}$–biotin–GLP-1 showed a 9-fold higher oral hypoglycemic efficacy (25.3%) than native GLP-1 (2.7%) ($P < 0.005$) after direct peroral administration into type 2 diabetic db/db mice. This study highlights the oral hypoglycemic potential of site-specific $Lys^{26,34}$-biotinylated GLP-1, and this orally available analogue would find a role in the treatment of type 2 diabetes.

Keywords: Glucagon-like peptide-1; Oral peptide delivery; Biotin; Site-specific modification; Oral hypoglycemic efficacy; Type 2 diabetes

1. Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from enteroendocrine L-cells of the intestine in response to orally ingested nutrients [1]. GLP-1 is attracting considerable attention on account of its beneficial effects in treating diabetes. GLP-1 normalizes the postprandial blood glucose elevation by stimulating insulin secretion and suppressing glucagon secretion [1,2]. GLP-1 also plays a significant role in improving pancreatic β-cell proliferation [3]. Moreover, GLP-1 can be used as a safe agent owing to its glucose-dependent action mechanism that can prevent hypoglycemia shock [1,3]. These advantages have made GLP-1 a promising anti-diabetic candidate.

Despite recent advances in peptide/protein delivery technologies, the administrations of peptide/protein are still dependent on parenteral injections, which cause poor patient compliance due to pain and frequent dosing. Especially, most patients with diabetes need to self-administer at least two or more injections of insulin per day for glycemic control [4]. Therefore, a non-invasive anti-diabetic treatment through non-parenteral routes, e.g. oral, intranasal, and pulmonary, has been considered to alleviate the patients' discomfort [4–6]. Of these, oral administration is considered to be the best choice due to its maximum convenience [4,7].

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Although a variety of oral anti-diabetic chemical agents are being used clinically, anti-diabetic peptides such as GLP-1 are still unavailable for oral administration. Peptides are subjected to severe proteolysis by gastrointestinal (GI)/brush-border peptidases when delivered orally [8,9]. Minor peptide fractions, which barely avoid proteolytic degradation, rarely permeate the intestinal membrane owing to their large molecular sizes and hydrophilicities along with the tight building structure of the membrane [4,8,10]. These two major barriers are viewed as being responsible for the disappointing therapeutic efficacies of orally delivered peptides. Therefore, dual improvements in the proteolytic stability and intestine absorption are essential for achieving successful oral peptide delivery [11,12].

The aim of this study was to design, prepare, and evaluate an intestine enzyme-resistant, superior intestine absorptive, and biologically preserved GLP-1 derivative for improved peroral delivery. In an attempt to achieve this, peptide surface modification with biotin (vitamin H), which actively traverses the intestine membrane via sodium-dependent multivitamin transport (SMVT), was chosen as a promising approach. Vitamin transporters have been reported to facilitate the intestinal peptide uptake [13,14], and recently the bio-formulations using this transport system have shown the promising results [15,16]. On the basis of this, in this study, the site-specific modification strategy was introduced, and various evaluations for site-specific biotinylated GLP-1 derivatives depending on the number and site of modification were accomplished to optimize their peroral potentials.

2. Materials and methods

2.1. Materials

Glucagon-like peptide-1 (GLP-1, 7–36 amide) was purchased from Bachem (Torrance, CA, USA). Biotin *N*-hydroxysuccinimide ester (Biotin-NHS) was obtained from Sigma (St. Louis, MO, USA). GLP-1 radio-immunoassay (RIA) and insulin enzyme immunoassay (EIA) kits were acquired from Linco Research Inc. (St. Charles, MO, USA) and Mercodia (Uppsala, Sweden), respectively. Male C57BL/6 db/db mice (7–8 weeks old) were supplied by the Korea Research Institute of Bioscience and Biotechnology, Daejon, Korea). Unless otherwise specified, all other reagents used were of the highest quality commercially available.

2.2. Preparation of biotinylated GLP-1s

Two types of biotinylated GLP-1s (Lys^{34}- and $Lys^{26,34}$–biotin–GLP-1s) were prepared by isolation from a biotinylated GLP-1 mixture (Fig. 1). Briefly, a portion (10 mg) of GLP-1 was mixed with an equimolar amount of biotin-NHS in 2.5 ml of a 0.3% triethylamine/dimethyl sulfoxide solution at room temperature for 60 min. The reaction mixture was subjected to reversed-phase high-performance liquid chromatography (RP-HPLC) on a CAPCELL PAK C18 column (250 × 4.6, 5 μm, Shiseido Co. Ltd., Japan) at ambient temperature. Gradient elution was carried out at a flow-rate of 1.0 ml/min with solvent A (0.1% trifluoroacetic acid (TFA) in deionized water) and

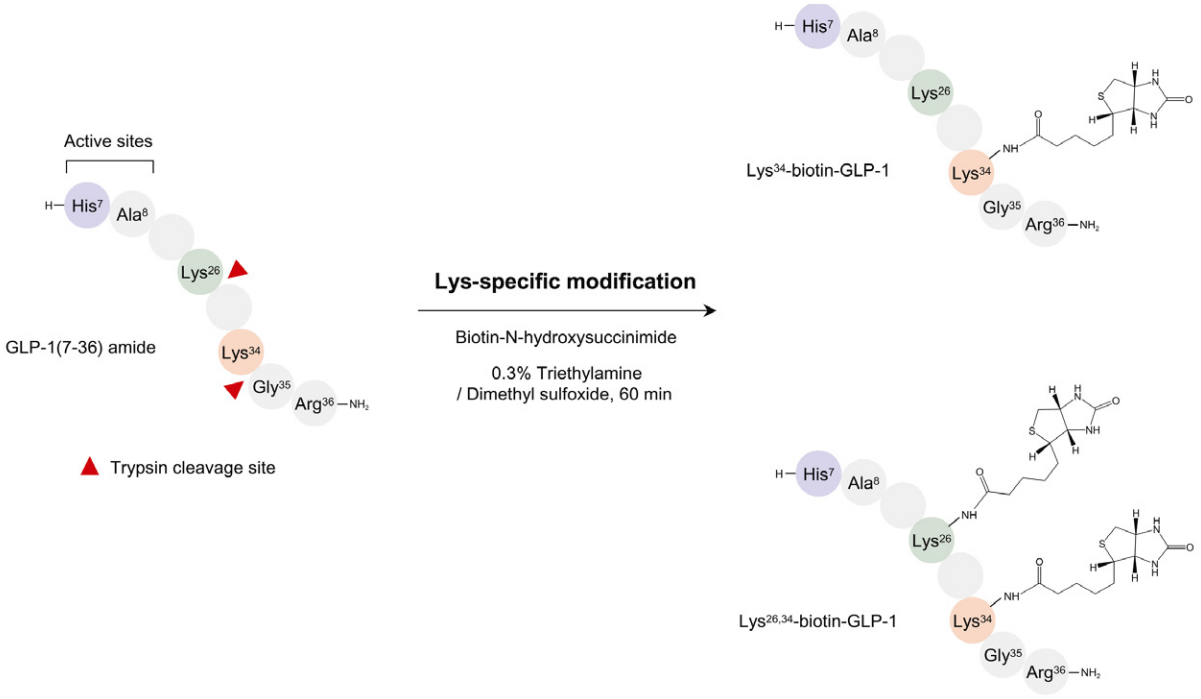

Fig. 1. Reaction scheme of GLP-1 biotinylation and primary structure of biotinylated GLP-1s. GLP-1 was site-specifically modified using biotin-NHS at its Lys^{26} or Lys^{34}-amine. Triangles show the cleavable sites by trypsin.

solvent B (0.1% TFA in acetonitrile), using a 37% B to 40% B linear gradient over a 25 min period. The eluates were monitored at a wavelength of 215 nm. The fractions were collected separately, dried under nitrogen, and stored in PBS (10 mM, pH 7.4) at 4 °C until needed.

2.3. Characterization of biotinylated GLP-1s

Biotinylated GLP-1s and their conjugation sites were identified using a slight modification of a MALDI-TOF MS method described elsewhere [17,18]. Briefly, a 5 μl aliquot of a lysyl endoproteinase Lys-C solution (10 μg/ml in 50 mM Tris–HCl, pH 8.5) was added to 10 μl of the biotinylated GLP-1s (100 μg/ml) dissolved in the same buffer solution. Digestion was allowed to continue at 37 °C for 30 min. Each sample was subjected to MALDI-TOF MS (Voyager-RP Biospectrometry Workstation, PerSeptive Biosystems, Cambridge, MA).

2.4. Investigation of permeabilities of biotinylated GLP-1s

The permeabilities of biotinylated GLP-1s were examined employing a method reported elsewhere [19] using human colon adenocarcinoma Caco-2 cells (clones: C2BBe1, passage number: 28–31; American Type Culture Collection, Rockville, MD). Briefly, the cells were grown until the reached 90% confluence and seeded onto the collagen-coated polycarbonated Transwell® membrane inserts (0.4 μm pore size, 1.13 cm^2 area; Corning Costar, Cambridge, MA) at a density of 1.25×10^5 cells/well. The cell monolayer inserts were used when the transepithelial electrical resistance (TEER) values reached 350–500 $\Omega \times cm^2$ (after a culture period of 18–21 days). After washing the cell monolayer with the transport medium (pH 7.4, HBSS containing 10 mM Hepes and 25 mM glucose), 0.5 ml of the transport medium containing the biotinylated GLP-1s (5 μM) and 1.5 ml of the drug-free transport medium were added to the apical and basolateral sides, respectively. In particular, peptide adsorption to the transwell plate was prevented by exposing the basolateral side of the inserts to a bovine serum albumin solution (10 mg/ml) for 10 min followed by washing three times with the transport medium before the transport experiment. The inserts were moved to the adjoining wells containing the same volume of fresh medium every 15 min for 1 h. The concentration of the basolateral side solution was determined using a commercially active GLP-1 RIA kit (Linco). The transport profiles of GLP-1s from basolateral to the apical side were examined using the same procedure. The apparent permeability coefficients (P_{app}) were calculated using the following equation: P_{app} (cm/s) $= dQ/dt/(A \times C_0)$, where dQ/dt is the transport rate, A is the surface area of the monolayer membrane, and C_0 is the initial concentration of the samples in the apical side. The transepithelial transport of radiolabeled [^{3}H]mannitol (DuPont NEN, Boston, MA) served as an additional control for Caco-2 cell monolayer integrity, and the monolayers having a P_{app} of 1.0–3.0×10^{-6} cm/s for [^{3}H]mannitol were considered to be tight enough for the transport experiment.

2.5. Evaluation of intestine enzyme stabilities of biotinylated GLP-1s

The proteolytic stabilities of the biotinylated GLP-1s were evaluated in the rat intestine fluid and homogenate using a slight modification of a method described elsewhere [19,20]. The intestine fluid and homogenate were obtained from a 24 h-pre-fasted SD rat. The intestine fluid was collected by flushing the intestinal part (from the duodenum to end of the jejunum) with 5 ml of 25 mM phosphate buffer (pH 6.4) and centrifuged 12,000 rpm for 10 min. It was finally prepared by diluting the supernatant 1:10 with the same buffer to slow the rapid breakdown of native GLP-1 by decreasing the enzyme activity. The homogenate was prepared from the supernatant of the homogenized intestine segment (the same part), of which the inner side had been rinsed five times again with a saline solution. A portion (25 μl) of either a GLP-1 or biotinylated GLP-1s solution (200 μg/ml each) was mixed with the same volume of the enzyme solutions (pre-incubated at 37 °C for 15 min), and incubated at 37 °C. At predetermined times, the incubations were stopped by adding 200 μl of 1% TFA/DW and ice-cold methanol for the fluid and homogenate, respectively. The resulting mixtures were centrifuged at 12,000 rpm for 5 min, and the residual amounts in supernatants were analyzed by RP-HPLC. The degradation half-lives obtained from the time vs. residual amount curves were calculated assuming first-order kinetics.

2.6. Assessment of bioactivities of biotinylated GLP-1s

The biological activities of GLP-1s after biotinylation were assessed both in vitro and in vivo using the methods described elsewhere [18,21,22]. First, the in vitro activity was evaluated using an isolated rat pancreatic islet. Briefly, a male Sprague–Dawley (SD) rat pancreas was inflated by injecting a cold Hank's balanced buffered salt solution (HBSS, pH 7.4, Sigma) containing 1.5 mg/ml type V collagenase (Sigma). The isolated islets were purified by centrifugation with a stepwise Ficoll (Amersham Biosciences AB, Uppsala, Sweden) gradient and maintained with a RPMI 1640 culture medium (Sigma) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin–streptomycin (Gibco) at 37 °C. After 2 days, 20 islets were incubated for 2 h in 2 ml of Krebs-Ringer bicarbonate–HEPES in the same atmosphere at 37 °C. The insulin concentrations released after stimulating the biotinylated GLP-1s were determined using a rat insulin EIA kit (Mercodia) ($n = 6$ each). Second, the in vivo activity was evaluated using an oral glucose tolerance test (OGTT) in type 2 diabetic db/db mice (7–8 weeks old). Briefly, the mice, which had been fasted for 18 h, received an intraperitoneal injection of biotinylated GLP-1s (10 nmol/kg) 30 min before orally administering a 1.0 g/kg dose of

glucose ($n = 7$ each). At predetermined times, the blood glucose levels were determined using a one-touch blood glucose meter (ACCU-CHEK® Sensor, Roche Diagnostics Corp., USA). The total hypoglycemic degree (%, vs. saline group) was calculated as follows: $(AUC_{saline,\,0-180min} - AUC_{test,\,0-180min})/AUC_{saline,\,0-180min}] \times 100$. In both studies, saline and GLP-1 were used as the control groups.

2.7. Evaluation of oral hypoglycemic efficacies of biotinylated GLP-1s

The hypoglycemic efficacies of GLP-1 and $Lys^{26,34}$–biotin–GLP-1 in db/db mice (7–8 weeks old) through the peroral route were assessed using an OGTT. The mice fasted for 18 h received consecutive oral administrations of 0.1 ml of a $NaHCO_3$ solution (3%), as a gastric neutralizer and a 0.1 ml sample solution of GLP-1s (each 15 nmol/mice) containing 50% propylene glycol at 5 min intervals. After 30 min, 1.0 g/kg dose of glucose (0.2 ml) was again administered orally to each mouse ($n = 9$ each). The other experimental conditions were the same.

3. Results

3.1. Preparation and characterization of biotinylated GLP-1s

GLP-1 modification with biotin-NHS resulted in a mixture of biotinylated GLP-1 isomers, as shown in the RP-HPLC chromatogram in Fig. 2. The MALDI-TOF mass spectra show that the first, third, and fourth peaks correspond to GLP-1 (calc.: 3298.6), mono- (calc.: 3541.9), and di-biotinylated GLP-1s (calc.: 3784.2), respectively (Fig. 3a). In order to identify their modification sites, the fractions were then subjected to Lys-C enzyme digestion followed by mass spectrometric analysis (Fig. 3b). The observed masses for the Lys-C digested first peak (unreacted GLP-1) were 1005.8 and 2099.6, which corresponded to the Glu^{27}-Lys^{34} and His^{7}-Lys^{26} fragments, respectively. The masses for the third peak were 1463.1 and 2099.4, which corresponded to the Glu^{27}-Lys^{34}-biotin-Arg^{36} and His^{7}-Lys^{26}fragments, respectively. The mass for the fourth peak was 3784.6, which corresponded to His^{7}-Lys^{26}-biotin-Lys^{34}-biotin-Arg^{36}, showing no cleavage of the Lys-amines caused by the strong resistance by biotin-modification. Subsequently, the third and fourth peaks were determined to be Lys^{34}- and $Lys^{26,34}$–biotin–GLP-1s, respectively. The purity of each GLP-1 analog was >98.5% at a concentration of 2.5 mg/ml.

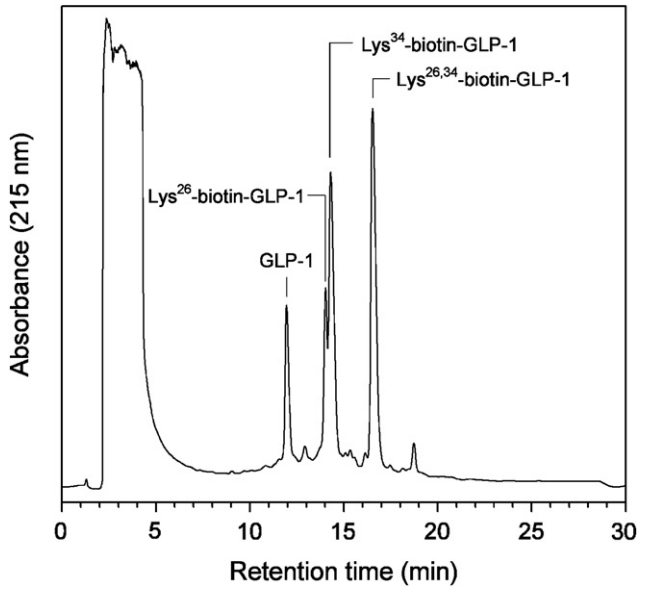

Fig. 2. Reversed phase-HPLC chromatographic separation profile of the biotinylated GLP-1 reaction mixture.

3.2. Permeabilities of biotinylated GLP-1s across Caco-2 cell monolayer

The permeabilities of GLP-1 and biotinylated GLP-1s were examined using a Caco-2 cell monolayer transport system, which has been reported to best mimic the intestine permeabilities across the human intestinal epithelial membrane [19,23]. As shown in Fig. 4, the level of GLP-1 permeation was increased significantly by biotinylation. The cumulative amounts of Lys^{34}- and $Lys^{26,34}$–biotin–GLP-1s at 60 min were 5.7 ± 0.6 and 8.6 ± 0.9 ng, which were 3.6 and 5.4 times higher than that of GLP-1 (1.6 ± 0.5 ng). In particular, di-biotinylation at both Lys-amines of GLP-1 significantly improved its permeability through Caco-2 cells. The apparent permeability coefficients (P_{app}) of $Lys^{26,34}$–biotin–GLP-1s in the apical to basolateral direction were $2.93 \pm 0.35 \times 10^{-7}$ cm/s, which was 5.6 and 1.7 times higher than those of GLP-1 ($0.52 \pm 0.17 \times 10^{-7}$ cm/s) and Lys^{34}–biotin–GLP-1 ($1.78 \pm 0.32 \times 10^{-7}$ cm/s), respectively.

3.3. Stabilities of biotinylated GLP-1s against intestine enzymes

The stabilities of the biotinylated GLP-1s were examined in rat intestine fluid and homogenate. GLP-1 was rapidly degraded in these enzyme systems due to the presence of high concentrations of various digestive and brush-border enzymes, with half-lives of 0.51 ± 0.02 and 0.79 ± 0.01 min, respectively (Fig. 5). In contrast, the biotinylated GLP-1s showed significantly greater resistance to the enzymes. Lys^{34}–biotin–GLP-1 was found to have 2.3- and 1.7-fold longer half-lives than those of GLP-1, respectively. In particular, $Lys^{26,34}$–biotin–GLP-1 showed 8.5- and 3.5-times longer half-lives ($t_{1/2}$: 4.34 ± 0.21 and 2.77 ± 0.05 min) than GLP-1.

3.4. Bioactivities of biotinylated GLP-1s

The in vitro insulinotropic activities of biotinylated GLP-1s were evaluated in isolated pancreatic islets. As

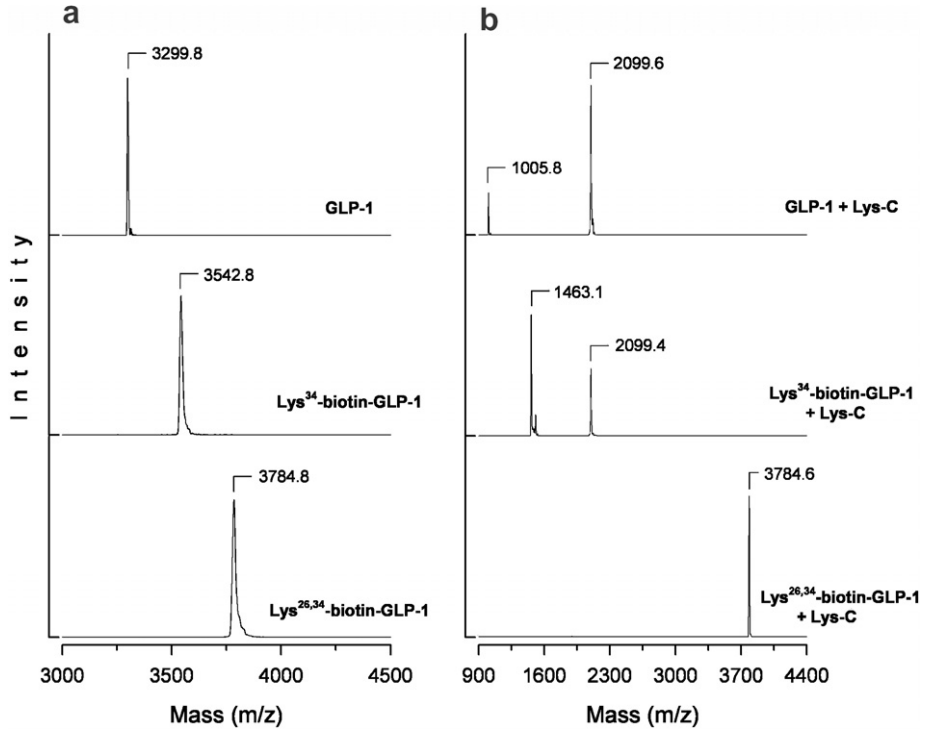

Fig. 3. MALDI-TOF mass spectra of (a) GLP-1 and biotinylated GLP-1s ($Lys^{26/34}$–biotin–GLP-1s) and (b) their Lys-C digested fragments.

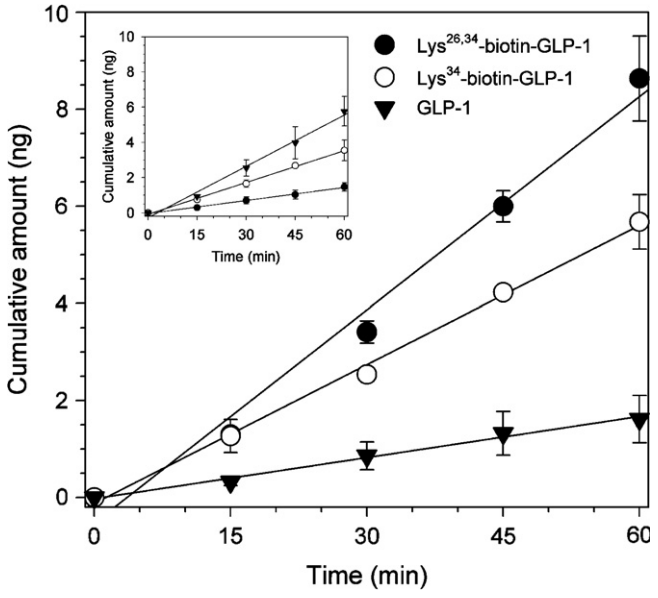

Fig. 4. Permeation profiles of GLP-1 and biotinylated GLP-1s in a Caco-2 cell monolayer transport system (the apical to basolateral side). (Inset) Permeation profiles from the basolateral to apical side. Data are presented as means ± SDs of four individual tests.

shown in Fig. 6a and Table 1, the biotinylated GLP-1s had almost the same insulinotropic activities as the native GLP-1. At a glucose concentration of 16.8 mM, both the potency (EC_{50}; nM) and efficacy (E_{max}; pM/islet/h) of the GLP-1 and the biotinylated GLP-1s were similar (EC_{50}: 3.64–3.85; E_{max}: 244.9 ± 14.2–262.8 ± 13.8). At a glucose concentration of 5.5 mM, the insulin levels of each GLP-1 group (10 nM) were in the range of 14.4 ± 0.1–15.4 ± 1.6 pM/islet/h, which is similar to that of the saline group ($P > 0.64$). In particular, despite all the Lys-modifications, $Lys^{26,34}$–biotin–GLP-1 was shown to have approximately 94.5% biological potency remaining. Separately, the in vivo hypoglycemic potencies of biotinylated GLP-1s were evaluated in type 2 diabetic db/db mice. Similar to the in vitro activity result, GLP-1 and biotinylated GLP-1s had almost the same potency (Fig. 6b). The total hypoglycemic degrees ranged from 29.5 ± 7.1% to 30.6 ± 3.0%, which were not significantly different ($P > 0.49$).

3.5. Oral hypoglycemic efficacies of biotinylated GLP-1s

In contrast to the intraperitoneal efficacy results, $Lys^{26,34}$–biotin–GLP-1 had a remarkably higher oral hypoglycemic efficacy in the db/db mice than the native GLP-1. As shown in Fig. 7, GLP-1 showed negligible hypoglycemic efficacy (total hypoglycemic degree; %), which is similar to the saline group. On the other hand, the total hypoglycemic degree of $Lys^{26,34}$–biotin–GLP-1 (25.3 ± 6.6%) was much higher than that of the native GLP-1 (2.7 ± 12.1%) ($P < 0.005$).

4. Discussion

Oral peptide/protein delivery is still a significant challenge. Although many attempts have been made to use enzyme inhibitors or absorption enhancers, most results are rather disappointing [12,24]. A variety of oral formula-

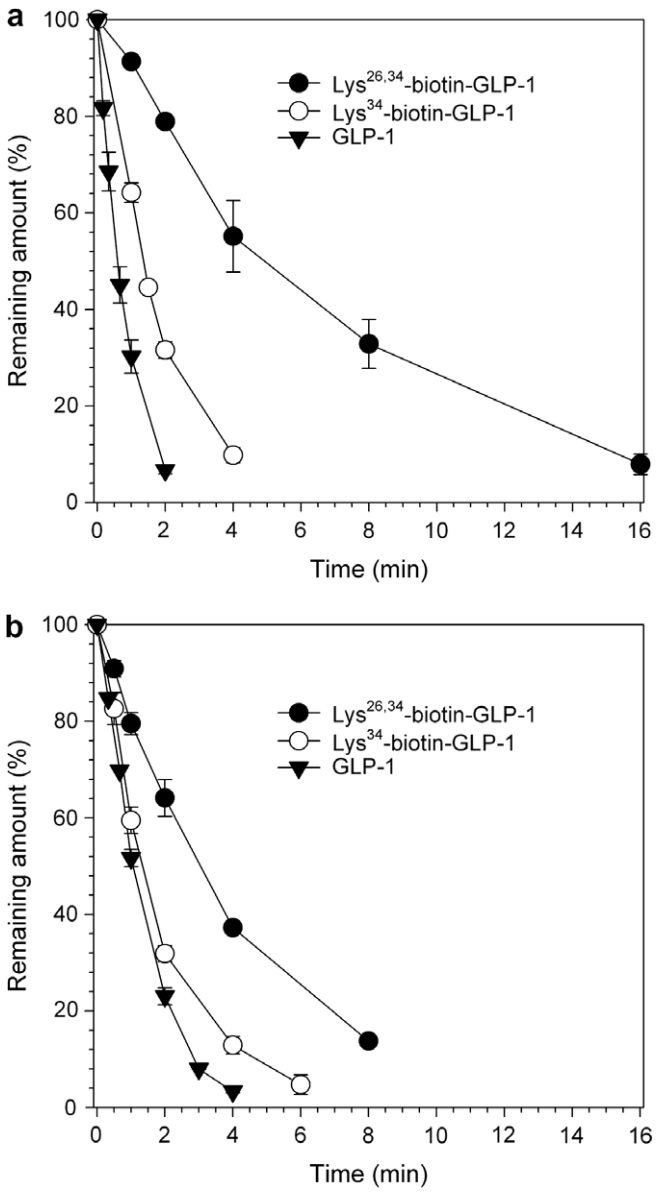

Fig. 5. Degradation profiles of GLP-1 and biotinylated GLP-1s incubated with (a) rat intestine fluid and (b) rat intestine homogenate. Data are presented as means ± SDs of three determinations.

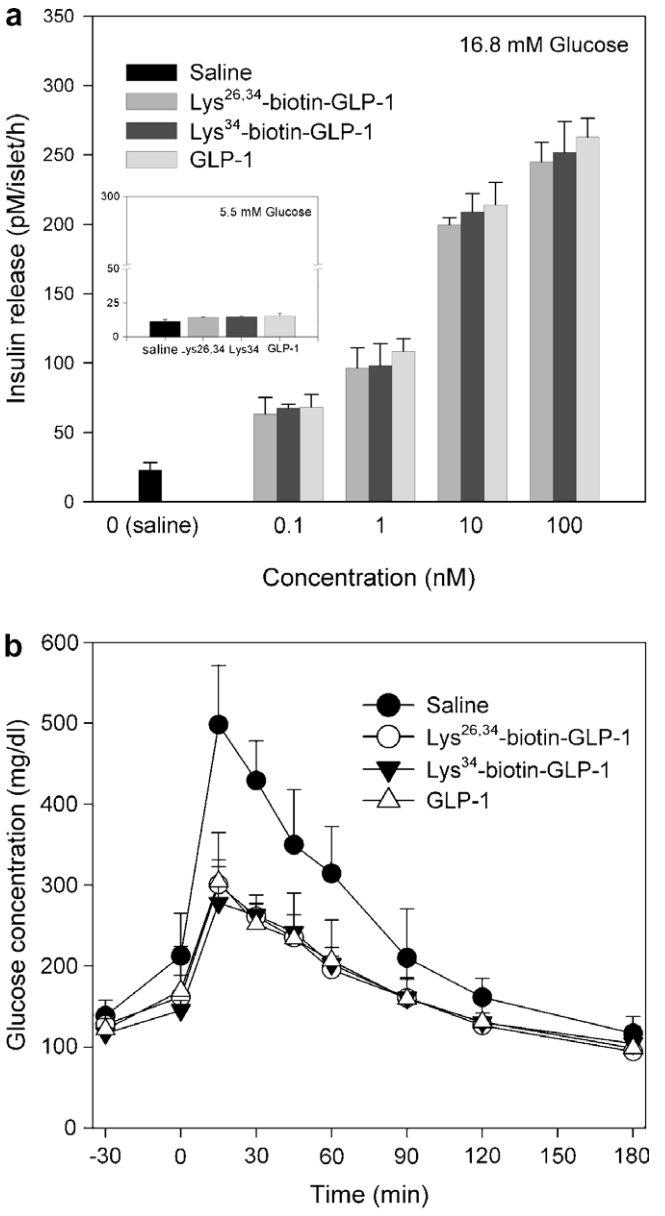

Fig. 6. Biological activity profiles of GLP-1 and biotinylated GLP-1s. (a) Insulinotropic profiles of GLP-1s in isolated rat pancreatic islet at 16.8 mM glucose (Inset: 5.5 mM glucose) ($n = 6$ each group, treatment). (b) Intraperitoneal hypoglycemic profiles of GLP-1 and biotinylated GLP-1s in type 2 diabetic db/db mice ($n = 7$ each). The significance was determined using a Student's t-test.

tions such as nanoparticles, emulsion, micelle, and liposome have shown only limited success [24]. In contrast, the preclinical/clinical results of strategies to deliver peptides orally using chemical modification, which alters their physicochemical properties, have been promising [25–27]. In this study, an attempt was made to confer orally available optimized properties upon the GLP-1 peptide through the chemical modification with biotin.

For the successful GLP-1 oral delivery, the major aim was to achieve a synergistic effect of simultaneously improving the intestine membrane permeability and intestine enzyme resistance of GLP-1 through a single chemical modification. First, for the former property, an attempt was made to apply the endogenous intestinal uptake pathway for biotin. Biotin is a water-soluble vitamin that is taken up actively across the intestine brush-border membrane via sodium-dependent multivitamin transport (SMVT) [28]. Through this process, the oral absorption potentials of several vitamin-modified peptides have been improved [29–31]. Second, a site-specific modification approach was used to increase the intestine enzyme resistance. Among various digestive intestine enzymes, special focus was placed on trypsin, a pancreatic peptidase that cleaves the carboxyl sides of lysine (Lys) or arginine (Arg) of peptides. This is because trypsin not only exerts the strongest digestive activity but is also present in the intestine area at high concentrations [11]. Fortunately,

Table 1
In vitro/in vivo evaluation results of GLP-1 and site-specific biotinylated GLP-1s

Group/evaluations	Insulinotropic activity		Caco-2 cell monolayer permeability		Intestine enzyme stability		Hypoglycemic efficacy (total hypoglycemic degree, %)	
	Potency: EC_{50}[a] (nM)	Efficacy: E_{max}[b] (pM/islet/h)	Permeation coefficient (P_{app}, cm/s, $\times 10^{-7}$)	Total permeated amount (ng)	Intestine fluid ($t_{1/2}$[c], min)	Intestine homogenate ($t_{1/2}$, min)	Intraperitoneal route	Peroral route
GLP-1	3.64 ± 0.25	262.8 ± 13.8	0.52 ± 0.17	1.6 ± 0.5	0.51 ± 0.02	0.79 ± 0.01	29.5 ± 7.1	2.7 ± 12.1
Lys^{34}–biotin–GLP-1	3.82 ± 0.32	251.9 ± 22.0	1.78 ± 0.32	5.7 ± 0.6	1.17 ± 0.08	1.34 ± 0.07	30.0 ± 7.2	–
$Lys^{26,34}$–biotin–GLP-1	3.85 ± 0.21	244.9 ± 14.2	2.93 ± 0.35	8.6 ± 0.9	4.34 ± 0.21	2.77 ± 0.05	30.6 ± 3.0	25.3 ± 6.6

[a] Effective concentration to reach 50% maximum efficacy response.
[b] Maximum efficacy level.
[c] Time required for the initial amount to reduce by 50%.

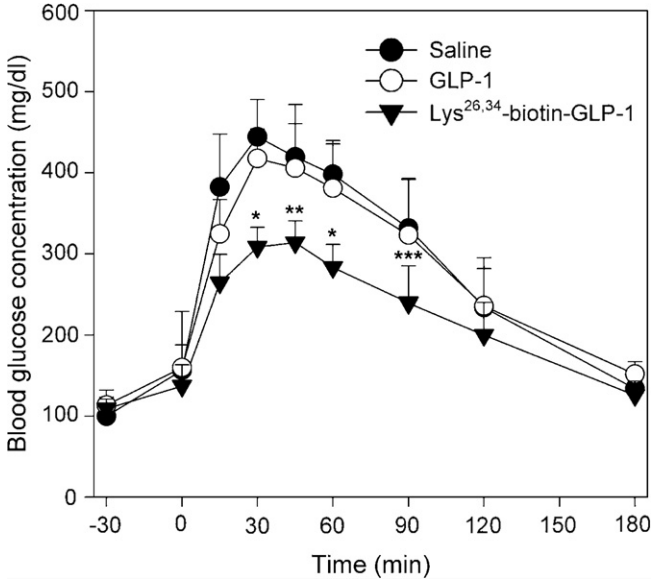

Fig. 7. Oral hypoglycemic efficacy profiles of GLP-1 and $Lys^{26,34}$–biotin–GLP -1 in db/db mice ($n = 9$ each). The significances was determined using a Student's t-test ($^{*}P < 0.007$ over GLP-1; $^{**}P < 0.01$ over GLP-1; and $^{***}P < 0.05$ over GLP-1).

GLP-1 has only two Lys at the 26/34 position, and no available Arg. Therefore, these two Lys amines were selected as the target biotinylation sites to prevent the trypsin activity. Since the Lys^{34} of GLP-1 is less sensitive to bioactivity loss by chemical modification than Lys^{26}, Lys^{34} was considered to be the first choice for biotinylation [18,21,22]. Consequently, two series of biotinylated GLP-1 were designated as Lys^{34}- and $Lys^{26,34}$–biotin–GLP-1s.

A Lys-amine specific reaction was employed to prepare these site-specific biotinylated GLP-1s [16]. Because of the clear difference in pK_a between the α- and ε-amine, biotin substitution to GLP-1 using a TEA/DMSO solvent was shown to greatly prefer both Lys-amines over the N-terminus amine (His^7). Therefore, the GLP-1 biotinylation resulted in Lys^{34}- and $Lys^{26,34}$–biotin–GLP-1s primarily (Fig. 2). The biotinylated GLP-1s were confirmed to have homogeneous biotin-target sites with high purity (>98.5%) using RP-HPLC and lysyl endoproteinase digestion followed by MALDI-TOF mass spectrometry (Fig. 3).

A human intestine epithelial Caco-2 cell monolayer was used to evaluate the permeabilities of the biotinylated GLP-1s. This monolayer was reported to reflect the human intestine brush-border barrier with a biotin-uptake system (SMVT) [23,32]. In this optimized evaluation system, the biotinylated GLP-1s were found to have significantly higher permeabilities. In particular, $Lys^{26,34}$–biotin–GLP-1 had a 5.6- and 1.7-fold higher P_{app} value (apical to basolateral side) than GLP-1 and Lys^{34}–biotin–GLP-1, respectively. This enhanced permeation appears to be due to the active transport mechanism via SMVT because the P_{app} value (apical to basolateral side, A → B) of $Lys^{26,34}$–biotin–GLP-1 was approximately 1.5 times greater than that of the reverse process (basolateral to apical side, B → A). However, a single active transport typically shows a >2.5-time greater A → B transport than B → A. Therefore, the possibility of another transport mechanism, simple passive diffusion, was considered because the biotin modification elevated hydrophobicity of GLP-1, as shown in Fig. 2. Similarly, a previous study showed that deoxycholic acid–heparin conjugates pass through the intestine not only through a bile acid–carrier interaction but simple diffusion on account of their increased hydrophobicity [33]. Furthermore, biotin was reported to traverse the rat intestine membrane through a separate transport mode of either carrier mediation (<10 μM) or simple diffusion (>10 μM) [34]. Taken together, a mixed transport process of both carrier mediation and passive diffusion seems to be responsible for this enhanced permeation of biotinylated GLP-1s.

Two enzyme pools (rat intestine fluid and homogenate) were used to best mimic in vivo intestine enzyme environment. The former was designed as a digestive enzyme system, and the latter as a brush-border enzyme system. In both systems, $Lys^{26,34}$–biotin–GLP-1 had significantly higher stability than the other GLP-1 species. The rat intestine fluid contains various pancreatic peptidases such as trypsin, chymotrypsin, carboxypeptidase, and elastase. Of these, GLP-1 fragment analysis using MALDI-TOF MS revealed the trypsin activity to be strongest (data not shown). Therefore, $Lys^{26,34}$–biotin–GLP-1, which is protected at both Lys-amines, showed greater resistance than either Lys^{34}–biotin–GLP-1 or GLP-1. Also, $Lys^{26,34}$–biotin–GLP-1 exhibited higher resistance to the intestine homogenate mimicking the brush-border enzymes. This enhanced enzyme resistance might be another critical factor for the better oral GLP-1 efficacy.

The next major concern is associated with the GLP-1 bioactivity after biotinylation. The peptide bioactivity can be seriously impaired by covalent chemical modifications [35]. Therefore, in order to ensure good in vivo efficacy, it is essential to have a strategy to minimize the bioactivity loss. It has been reported that the N-terminal region of GLP-1 is essential for its biological action [36] while both Lys^{26}- and Lys^{34}-amines of GLP-1 are weakly involved in its biological action [37,38]. Hence, the modification at these Lys amines is considered to be a good strategy for preserving the GLP-1 activity. Indeed, the modification at both amines had little negative impact on the GLP-1 activity. Especially, $Lys^{26,34}$–biotin–GLP-1 was as effective as the other GLP-1 derivatives in exerting insulinotropic action on islet cells ($P > 0.64$, 16.8 mM glucose). Importantly, similar to the native GLP-1 property, $Lys^{26,34}$–biotin–GLP-1 was shown to elicit negligible insulin release at the lower glucose concentration (5.5 mM), indicating that this derivative still retains the glucose-dependent insulin release action (Fig. 6a: inset). Also, this derivative showed similar hypoglycemic potency in db/db mice to the other GLP-1 species ($P > 0.49$). Consequently, $Lys^{26,34}$–biotin–GLP-1 retained 94.5 and 103.7% of the whole GLP-1 bioactivity in vitro/in vivo, respectively. This finding shows that $Lys^{26,34}$–biotin–GLP-1 can be used as a potential replacement for native GLP-1.

The oral hypoglycemic efficacy of $Lys^{26,34}$–biotin–GLP-1, chosen as a promising candidate through a series of in vitro/in vivo evaluations (Table 1), was examined in type 2 diabetic db/db mice. Since various peptidases are distributed in the GI tract at high concentrations, an attempt was made to attenuate the tremendous enzyme activity. Especially, when delivered perorally, peptides first encounter with pepsin, which is active in the harsh acidic condition (~pH 1.2) of stomach. Therefore, a gastric neutralizer (sodium bicarbonate) to temporarily increase the stomach pH is effective to reduce the pepsin activity. In this respect, this treatment can elevate the fraction of GLP-1s to reach the intestine, which mimics a formulation of enteric coating able to bypass the stomach environment. Moreover, GLP-1 samples were formulated with 50% propylene glycol to decrease the enzyme exposure in the GI tract. Under this optimized condition, $Lys^{26,34}$–biotin–GLP-1 exhibited a 9-fold higher oral hypoglycemic efficacy (25.3%) than native GLP-1 (2.7%), ($P < 0.005$). This evident improvement appears to be the result of the combined effects of: (i) increased permeability, (ii) increased enzyme resistance, and (iii) preserved bioactivity by $Lys^{26,34}$-biotinylation. Therefore, the in vivo result demonstrates the pharmacological utility of biotin in the peroral delivery of anti-diabetic peptides such as GLP-1.

In conclusion, the current findings suggest that $Lys^{26,34}$–biotin–GLP-1 is a good oral type 2 anti-diabetic agent on account of the aforementioned advantages over GLP-1. Further studies on detailed oral formulations would offer even better improvements.

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