Original Article

Development of sandwich ELISAs for detecting glucagon-like peptide-1 secretion from intestinal L-cells and their application in STC-1 cells and mice

Kevin Odongo, Ken-yu Hironao, Yoko Yamashita, and Hitoshi Ashida*

Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

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Certain nutrients stimulate glucagon-like peptide-1 (GLP-1) secretion from the intestinal enteroendocrine L-cells, but due to rapid degradation by the DPP-4 enzyme, >90% is converted to inactive metabolite before reaching the target organs via circulation. Plants are a source of potent bioactive compounds that promote endogenous secretion of GLP-1 from L-cells. To search for the effective bioactive compound from a vast library of natural compounds, a reliable and low-cost assay is required considering the high cost of commercial assays. We developed a low-cost sandwich enzyme-linked immunosorbent assays (s-ELISAs) for detecting 'total', 'sensitive active', and 'wide-range active' GLP-1. The s-ELISAs exhibited high sensitivity with measurement ranges between 0.94~240, 0.98~62.5, and 4.8~4,480 pmol/L, respectively. High precision was observed; i.e., CVs within 5% and 20% for intra- and inter-assay variations, respectively, and excellent recovery of exogenous GLP-1 from assay buffer. The developed s-ELISAs had the same performance as the commercial kits and approximately 80% cheaper cost. For their application, cinnamtannin A2-induced GLP-1 secretion was confirmed in STC-1 cells consistent with our previous findings. The s-ELISAs were further validated by measuring plasma GLP-1 level in mice after oral administration of black soy bean seed coat extract containing cinnamtannin A2.

***Key Words*: diabetes, glucagon-like peptide-1, ELISA, plant bioactive compounds, cinnamtannin A2**

Incretin hormones are important for maintaining whole-body nutrient equilibrium as highlighted by their suppression of several metabolic pathologies including obesity and diabetes.[1,2] Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from the intestinal enteroendocrine L-cells in response to the intake of certain nutrients and plays a pivotal role in the maintenance of glucose homeostasis.[2–4] GLP-1 contributes to the regulation of glucose metabolism by stimulating glucose-dependent insulin secretion and promoting pancreatic beta-cell proliferation.[3] In addition, GLP-1 inhibits glucagon release, delays gastric emptying, induces satiety, and reduces food intake in obese humans and animals.[1–5] GLP-1 is a product of differential post-translational cleaving of proglucagon, producing six known isoforms, including GLP-1 (1–37), GLP-1 (7–37), and GLP-1 (9–37), which are further amidated at the COOH-terminal to GLP-1 (1–36 NH_2), GLP-1 (7–36 NH_2), and GLP-1 (9–36 NH_2), respectively, in humans.[6,7] Since the intact (active) form of GLP-1 is highly susceptible to rapid degradation by dipeptidyl peptidase-4 (DPP-4) enzyme, over 90% of the active GLP-1 (7–36 NH_2) converts to its inactive form of GLP-1 (9–36 NH_2), resulting in only about 8% of the active form reaching the target organs via the circulation.[5–7]

Due to the actions of GLP-1, its therapeutic usage is widely recognized as a remedy for type 2 diabetes mellitus (T2DM) patients. Indeed, various GLP-1 agonists and DPP-4 inhibitors have proven effective in diabetes management.[2,3,5] However, it is reported that the bioavailability of GLP-1 analogs is very low due to the rapid degradation of circulating active GLP-1 by DPP-4 enzyme.[5] Moreover, pharmaceutical agents often have side effects. Therefore, much attention has been paid to the search for a safe alternative, such as a potent plant-derived bioactive compound that can promote the endogenous secretion of active GLP-1 from enteroendocrine L-cells, and clarify its secretory mechanisms. To date, certain nutrients, such as glucose, short-chain fatty acids, saccharides, and amino acids, have been reported to directly stimulate GLP-1 secretion from the L-cells.[8–11] However, only a few studies have reported on the plant bioactive compounds. For example, recent papers have shown that curcumin enhanced GLP-1 secretion in GLUTag cells,[12] and cinnamtannin A2—a tetrameric procyanidin, increased GLP-1 and insulin secretion in mice.[13] These previous findings indicate that certain plant bioactive compounds may possess the potential to promote GLP-1 secretion, though their secretory mechanisms are not clear. Plant bioactive compounds are a group of secondary metabolites that are ubiquitously present in many plants and plant-derived foods, possessing health-promoting effects.[14] Based on their diverse chemical structures, more than 8,000 plant bioactive compounds have been identified over the years, creating a vast library of potential natural compounds. As a result, the screening process of plant bioactive compounds is often tedious and costly, in part due to expensive commercial screening assays.

Enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical immunochemistry assay based on the specific bond between the antigen and the antibody.[15,16] Detection of secreted GLP-1 levels relies heavily on commercially available ELISA assays. However, commercial assays present an array of disadvantages. For example, these assays tend to be expensive and money is often tight, particularly in a research setting. This cost is particularly due to the high price of acquisition and storage, resulting from requirements of expensive culture cell media to obtain a specific antibody, refrigerated transport and storage, and labour cost. A conventional commercial GLP-1 ELISA kit measuring total GLP-1 costs approximately United

*To whom correspondence should be addressed.
E-mail: ashida@kobe-u.ac.jp

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States dollar (USD) 760 for one kit with 96 tests ($7.9/test), and USD 702 ($7.3/test) for an active GLP-1 kit, which is expensive considering the extremely large number of plant bioactive compounds available. This hinders how many target compounds can be quantified for GLP-1 secretion, and severely limit the ability to identify the effective compounds. Moreover, commercial GLP-1 ELISA offers a particularly narrow measurement range, usually bellow 0.1 nmol/L for a conventional active ELISA and not exceeding 1 nmol/L for a total ELISA. Even though cell supernatant samples with values that exceeds the range of conventional assays can always be diluted to enter the range, dilution experiments (and increased sample handling) are subject to both pipetting and measurement errors that may cause systematic and accumulative errors and misrepresentation of screening results, thus hindering the ability to effectively analyze samples that vary significantly in concentration. Therefore, to streamline the process to effectively identify effective compounds from the large number of extracts and compound samples available, we developed and validated reliable sandwich ELISA methods, detecting the 'active' and 'total' GLP-1 at a markedly low-cost performance, and offering a wide detection range for the 'active' GLP-1. We assessed their performance by comparing the results with those obtained from a conventional commercial kit. With the developed sandwich ELISAs (s-ELISAs), we confirmed the levels of GLP-1 secreted in the media under stimulation with cinnamtannin A2, a tetrameric procyanidin in intestinal secretin tumor cell line (STC-1) cells, and in plasma of mice following oral administration of black soy bean seed coat extract (BE) and procyanidin-rich black soy bean seed coat extract (PCBE), which contain cinnamtannin A2.

Materials and Methods

Materials. (−)-Epicatechin was purchased from Sigma-Aldrich (St. Louis, MO). BE and PCBE were obtained from Fujicco Co., Ltd. (Kobe, Japan). Procyanidin B2 and C1, and cinnamtannin A2 were prepared as previously described.[17] Pierce 96-Well Polystyrene Plate (Corner Notch) and Plate seal (Adhesive, unsterilized; P96P01N) were purchased from Thermo Scientific Co., Ltd. (Tokyo, Japan) and AS ONE (Osaka, Japan), respectively. 3, 3', 5, 5'-Tetramethylbenzidine (TMB) was from Agilent Technologies Ltd. (Tokyo, Japan). Murine STC-1 was obtained from ATCC (Manassas, VA). High glucose Dulbecco's Modified Eagle's Medium (DMEM), bovine serum albumin (BSA: heat shock fraction; protease-free, fatty acid-free, essentially globulin free), and penicillin were purchased from MilliporeSigma (St. Louis, MO). Streptomycin and fetal bovine serum (FBS) were from MP Biomedicals Inc. (Santa Ana, CA) and BioWest S.A.S. (Nuaillé, France), respectively. Aprotinin solution was purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan). Blocking One and Blocking One-P were from Nacalai Tesque, Inc. (Kyoto, Japan).

Mouse anti-GLP-1 antibody [8G9] (ab26278), anti-GLP-1 antibody [10] (ab121057), anti-GLP-1 antibody [4F3] (ab23472), and HRP-streptavidin solution (ab210901) were purchased from Abcam Co., Ltd. (Hercules, CA). Human GLP-1 (7–36) amide (Cat. 4344-v) was obtained from Peptide Institute, Inc. (Osaka, Japan). The Goat anti-mouse IgG2a antibody (Cat. STAR133) was purchased from Bio-Rad Laboratories Inc. (Hercules, CA). The conjugated antibodies were prepared using the biotin-labeling kit -NH_2 obtained from Dojindo Laboratories Inc. (Kumamoto, Japan), following the manufacturer's protocol. Mouse anti-GLP-1 antibody [10], anti-GLP-1 antibody [4F3], and goat anti-mouse IgG2a antibody was labeled with biotin using this labeling kit. Water for reconstitution was purified through LC-Pak Polisher Filter by using Milli-Q Integral 3/05/10/15 water purification systems. All other chemicals and reagents were of analytical grade available from commercial sources unless otherwise stated.

Development of GLP-1 s-ELISAs. A 96-well plate was coated with 100 µl of capture antibody (Anti-GLP-1 antibody [8G9]), diluted in the coating buffer (0.35 M sodium carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C. This capture antibody is a mouse monoclonal antibody against the C-terminal epitope of the active GLP-1 (7–36 NH_2). The following day, the wells were washed (230 µl/well × 5 times) with Tris-buffered saline (TBS), containing 0.05% (v/v) Tween 20, pH 7.4. The free binding sites were then blocked with 300 µl/well of blocking buffer [Blocking One solution diluted in deionized water (1:4)] for 2 h at room temperature (RT). After blocking, the wells were washed (230 µl/well × 5 times), and 100 µl of serially diluted GLP-1 standard solution [Human GLP-1 (7–36) amide] or L-glutamine (known GLP-1 secretagogue), reconstituted in Krebs-Ringer-Bicarbonate buffer (120 mM NaCl, 5 mM KCl, 22 mM $NaHCO_3$, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4) containing 2% (w/v) BSA, was added to each well and incubated for 2 h at RT. After washing the wells (230 µl/well × 5 times), 100 µl of biotin-conjugated anti-GLP-1 antibody [10] (detection antibody for the sensitive active s-ELISA) or biotin-conjugated anti-GLP-1 antibody [4F3] (detection antibody for the total s-ELISA) was added to each well and incubated for 90 min at RT. Anti-GLP-1 antibody [10] binds to the N-terminal epitope of the active GLP-1 (7–36 NH_2) and was generated from a synthetic peptide corresponding to human GLP-1, amino acid 7–17. On the other hand, the anti-GLP-1 antibody [4F3] binds to the mid-molecular epitope and reacts with all forms of GLP-1, including precursor and GLP-1 (9–37) and GLP-1 (9–36 NH_2) metabolites. Unconjugated anti-GLP-1 antibody [10] was used as the primary detection antibody for the wide-range active s-ELISA and was incubated for 2 h at RT. After incubation, the wells were washed (230 µl/well × 5 times). The wide-range active s-ELISA was further incubated with 100 µl/well of the secondary detection antibody (biotin-labeled goat anti-mouse IgG2a antibody) for 1 h at RT. Next, after the same washing procedure, all the three s-ELISAs were incubated with 100 µl/well of HRP-streptavidin solution for 30 min at RT. After the final washing, the wells were incubated with 100 µl of TMB liquid substrate for 25 min at RT, in a dark cabinet with continuous shaking (~400 rpm). Finally, 100 µl of stop solution (2 M sulfuric acid) was added to each well and the plates were shaken briefly to mix. Absorbance of the developed color was immediately analyzed using a multilabel plate reader (Wallac 1420 ARVO SX-DELFIA; Perkin-Elmer, Boston, MA); detection wavelength at 450 nm with a reference one at 630 nm.

Optimal antibody titration. A checkerboard titration method was used to determine optimal antibody concentrations as previously described.[18] This method can be used to assess two variables at once: e.g., antibody concentration and GLP-1 standard concentration. By running each well with a different ratio of GLP-1 standard [Human GLP-1 (7–36) amide] to antibody, we established not only the optimal concentration of each but also the optimal ratio of concentrations as well.

Validation of the developed s-ELISAs. The developed s-ELISAs were validated according to the guidelines for clinical analytical assays [Clinical and Laboratory Standards Institute (CLSI) guidelines for immunoassays; I/LA23-A, I/LA23-A2, and EP24A] as previously describe.[15,16] The validation of the s-ELISAs according to these recommendations was performed by determining the following method characteristics: sensitivity, specificity, linearity-range (dynamic range), precision, accuracy (including intra- and inter-assay variabilities), and spike recovery.

Cell culture. STC-1 cells were routinely cultured in DMEM supplemented with 4.5 g/L D-glucose, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C under a mixture of 95% (v/v) air and 5% (v/v) CO_2 humidified atmosphere. STC-1 cells between passage numbers 34~50 were

used for all assays. The cells were seeded on the culture plate at a density of 1~2 × 10^6 cells per/well, cultured until confluency (about 48 h), and used in the following experiments.

Cytotoxicity analysis. Cytotoxicity of flavan-3-ols was determined by crystal violet staining as previously described.[19] Briefly, confluent cells on the 96-well plate were treated with flavan-3-ols at the concentrations indicated in each figure, or with vehicle control for 4 h. Treated cells were fixed and stained with 0.2% (w/v) crystal violet in 2% (v/v) ethanol for 10 min. The wells were gently washed several times with Milli-Q purified water, and the stained dye was extracted with 0.5% (w/v) sodium dodecyl sulfate (SDS) in 50% (v/v) ethanol and incubated with continuous shaking (~400 rpm) until completely dissolved. Cell viability was estimated by measuring absorbance at 530 nm using a multilabel plate reader (Wallac 1420 ARVO SX-DELFIA). The relative cell viability was expressed as a percent of the vehicle control-treated cells.

Treatment of STC-1 cells with (–)-epicatechin and procyanidin oligomers. Confluent cells on a 24-well plate were washed twice with Krebs buffer and incubated in the buffer for 1 h. The cells were treated with (−)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 at the indicated concentrations, positive control (10 mM, L-glutamine), and vehicle control [0.1% dimethyl sulfoxide (DMSO)] for 4 h. All culture medium (450 µl) was collected into microtubes containing DPP-4 inhibitor (K597) and aprotinin to a final concentration of 25 and 67.5 µl/ml, respectively, and centrifuged at 900 × *g* for 5 min at 4°C to remove cell debris. The obtained supernatant was stored at −80°C until used for the GLP-1 analysis.

Animal experiment. To examine GLP-1 secretion in animal, we introduced flavan-3-ols-rich food materials, BE and PCBE. Composition of polyphenols in them is shown in Table 1. All animal experiments were approved by the Institutional Animal Care and Use Committee (permission no. 27-05-08) and carried out according to the guidelines for animal experiments at Kobe University. Male C57BL/6J mice (6 weeks old) were obtained from Japan SLC Inc. and were maintained in a temperature-controlled room (22 ± 2°C) with a 12 h-12 h light-dark cycle (lights on at 8:00 am). The mice were allowed free access to tap water and commercial chow [D10012M (AIN-93M base diet, Research Diets, Inc., New Brunswick, NJ)] and were acclimatized for 7 days. To examine the effect of BE and PCBE administration on stimulation of GLP-1 secretion, 15 mice were divided into three groups of five each to receive single oral administration of BE (300 mg/kg body weight), PCBE (300 mg/kg body weight) in water or water alone (5 ml/kg body weight) as a vehicle control. One hour after administration, mice were sacrificed under anesthesia using sevoflurane as an inhalational anesthetic and sodium pentobarbital as an analgesic, and euthanized by exsanguination from cardiac puncture. Blood was collected in tubes containing EDTA (2.5 mM final concentration), DPP-4 inhibitor (2 µl/200 µl) and 100 U/ml of aprotinin. Plasma was then prepared by centrifugation at 3,000 × *g* for 10 min at 4°C and subjected to measurements of GLP-1 using the developed total s-ELISA.

Table 1. Composition of polyphenols in black soybean seed coat extract and procyanidin-rich black soybean seed coat extract

Compounds	BE	PC-rich BE
	% (w/w)	
Total polyphenols	67	85.4
Total flavanols	45.9	80.6
Total isoflavones	0.96	0.69
Cyanidin-3-glucoside	9.2	0.9
Epicatechin	6.2	13.8
Procyanidin	39.7	66.8
Dimer (Procyanidin B2)	6.1	11.8
Trimer (Procyanidin C1)	3.4	7.8
Tetramer (Cinnamtannin A2)	0.5	3.1

BE, black soybean seed coat extract; PC, procyanidin.

GLP-1 secretion assay. The concentration of 'active' and 'total' GLP-1 in the culture medium and in plasma were quantified using the developed sensitive active s-ELISA and total s-ELISA, respectively, according to the protocol described above. We measured both the active [GLP-1 (7–36) amide] and inactive [residues (9–37) and (9–36) amide] forms of GLP-1.

Statistical analysis. Statistical analysis was performed with JMP statistical software ver. 11.2.0 (SAS Institute, Cary, NC). Data are presented as the means ± SE, at least in triplicates. Statistical significance was determined by Dunnett's or Tukey-Kramer's multiple comparison tests as indicated in each figure legend. $P<0.05$ was considered statistically significant.

Results

s-ELISA format and antibodies optimization. The GLP-1 s-ELISAs were developed following direct and indirect sandwich formats. The optimal concentrations and binding ratios of the monoclonal capture antibody, unconjugated, and biotin-labeled primary detection antibodies, biotin-labeled secondary detection antibody, and HRP-streptavidin solution were determined using the checkerboard titration method. The capture antibody concentrations were optimal at 1 µg/ml (1:1,000) for the developed total s-ELISA and sensitive active s-ELISA, respectively, and 0.875 µg/ml (1:1,143) for the wide-range active s-ELISA (Fig. 1A). Using the optimal capture antibody concentrations, the optimal concentrations of the subsequent antibodies in the sandwich ELISA format were established; the HRP-streptavidin solution was optimal at 1:2,000 for all GLP-1 s-ELISAs (Fig. 1B). The biotin-conjugated primary detection antibodies were optimal at 1:2,500 and 1:1,000 for the total s-ELISA (Fig. 2A) and the sensitive active s-ELISA (Fig. 2B), respectively. On the other hand, for the wide-range active GLP-1 s-ELISA, the unconjugated primary detection antibody (Fig. 2C) and the biotin-labeled secondary detection antibody (Fig. 2D) were optimal at 1:8,000 and 1:60,000, respectively.

Sensitivity and specificity of the developed GLP-1 s-ELISAs. Reliable measurement of both intact (active) and total GLP-1 concentrations in peripheral circulation is complex, this is because of low peripheral circulation with physiological ranges between 0~15 pmol/L for active and 5~80 pmol/L for total GLP-1.[20,21] Therefore, assay sensitivity is important. To assess the sensitivity of the developed s-ELISAs, known concentrations of GLP-1 standard [Human GLP-1 (7–36) amide] diluted with assay buffer was measured ($n = 10$) in a single assay. Limits of detection (LOD) and limits of quantification (LOQ) were evaluated based on the signal to noise ratio (S/N) of standard analytes (S) and zero calibrators (N), where; S/N ≥3 and S/N ≥10 were accepted for the LOD and LOQ, respectively.[16] As shown in Table 2, the LOD of the developed s-ELISAs were 1.62, 1.40, and 8.30 pmol/L for the total s-ELISA, sensitive active s-ELISA, and the wide-range active GLP-1 s-ELISA, respectively, while the LOQ were 1.67, 3.22, and 8.44 pmol/L, respectively. As for specificity, the commercial monoclonal antibodies used were affinity purified with high specificity to GLP-1 peptide. The anti-GLP-1 antibody [10] used as the detection antibody in the active GLP-1 s-ELISAs was N-terminal epitope specific and binds to the active GLP-1 (7–37) and GLP-1 (7–36) amide and was generated from a synthetic peptide corresponding to human GLP-1, amino acid 7–17. This antibody showed <0.2% cross-reactivity with GLP-1 (9–36) amide, human gastric inhibitory

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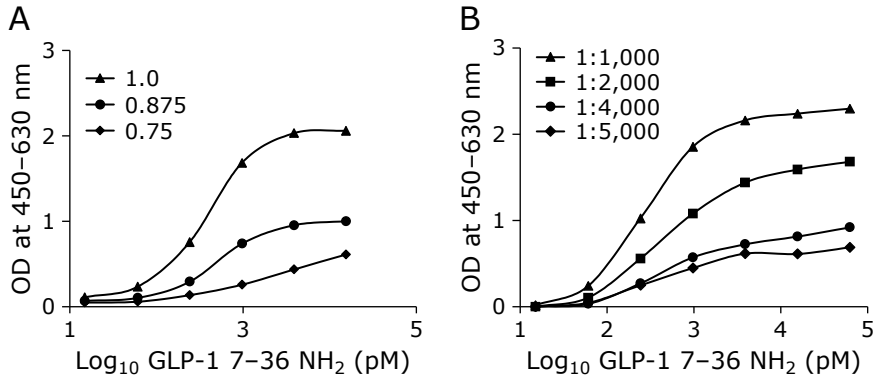

Fig. 1. Titration of the capture antibody and HRP-streptavidin solution for the developed GLP-1 s-ELISAs. The optimal concentrations were determined using the checkerboard titration method as described in Materials and Methods section. (A) Optimal concentrations of the capture antibody. (B) The optimal concentration of streptavidin-HRP solution.

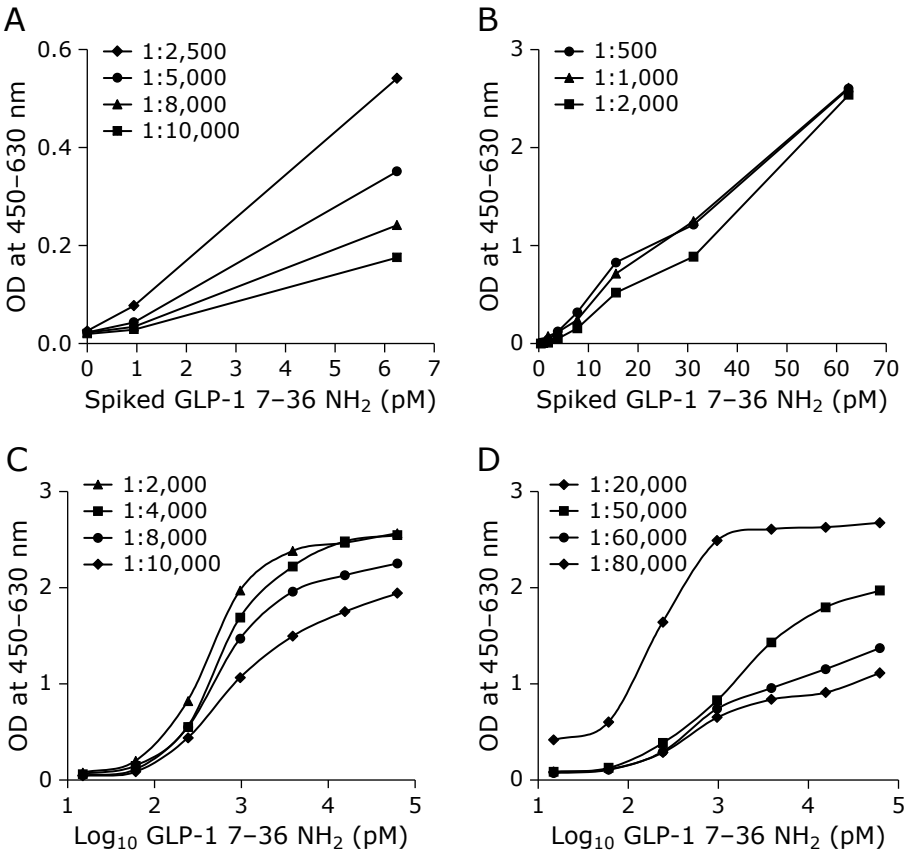

Fig. 2. Titration of the primary and secondary detection antibodies for the three s-ELISAs. Optimal concentrations were determined using the checkerboard titration method as described in Materials and methods section. (A) Biotin-labeled primary detection antibodies for the total s-ELISA. (B) Biotin-labeled primary detection antibodies for the sensitive active s-ELISA. (C) The unconjugated primary detection antibody for wide-range active s-ELISA. (D) Biotin-labeled secondary detection antibody for wide-range active ELISA.

Table 2. Sensitivity, dynamic ranges, and measurement ranges of the developed s-ELISAs

s-ELISA	LOD (pM)	LOQ (pM)	Dynamic range (pM)	Standard range (pM)
Total GLP-1	1.62	1.67	1.62~240	0.94~240
Sensitive active	1.4	3.22	1.40~62.5	0.98~62.5
Wide-range active	8.3	8.44	8.30~4,480	4.8~4,480

LOD, limit of detection; LOQ, limit of quantification.

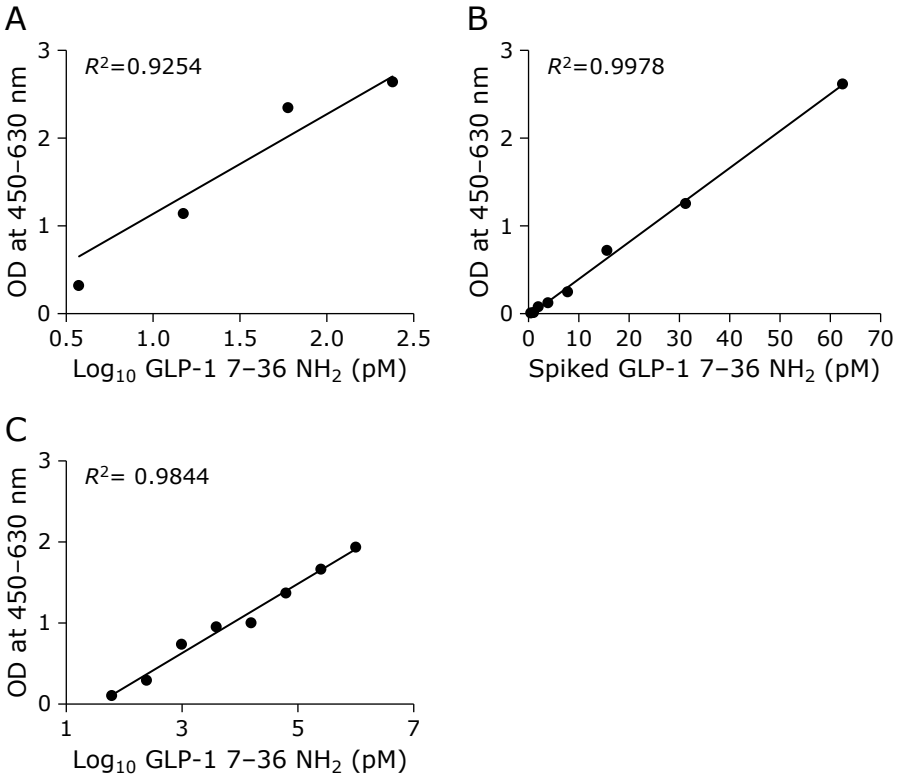

Fig. 3. Calibration curves of GLP-1 standards in assay (Krebs) buffer. Regression graph of GLP-1 standard [Human GLP-1 (7–36) amide] measured in Krebs buffer by the developed s-ELISAs. (A) total s-ELISA. (B) sensitive active s-ELISA. (C) Wide-range active s-ELISA. Points are means from triplicate determinations.

peptide (GIP) and glucagon, and cross-reacted 0.25% with GLP-1 (1–37), and approximately 6% with GLP-2. The anti-GLP-1 antibody [4F3] used as the detection antibody in the total GLP-1 s-ELISAs binds to the mid-molecular epitope and reacts with all forms of GLP-1, including its precursor and GLP-1 (9–37) and GLP-1 (9–36 NH_2) metabolites. The capture antibody, anti-GLP-1 antibody [8G9], was a synthetic peptide corresponding to human GLP-1 aa 1-100, and reacts with the amidated C-terminus of GLP-1 (7–36) coupled to a carrier.

Linearity and measurement ranges of the developed GLP-1 s-ELISAs. To assess linearity and measurement ranges (also termed dynamic range), different concentrations of GLP-1 standard comprising of 0.24, 0.94, 3.75, 15, 60, 240, and 1,000 pmol/L for the total s-ELISA; 0.49, 0.98, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1,000 pmol/L for the sensitive active s-ELISA, and 1.2, 4.6, 18.3, 73.2, 293, 1,185, and 4,740 pmol/L for the wide-range active s-ELISA were prepared in the assay and used. As shown in Table 2, the linearity ranges were between 1.62~240 pmol/L, 1.40~65.5 pmol/L, and 8.3~4,480 pmol/L, respectively, with strong correlation efficiencies (R^2) of 0.9812, 0.9982, and 0.9844, respectively (Fig. 3). GLP-1 measurement ranges were established between 0.94~240, 0.98~62.5, and 4.8~4,480 pmol/L, for the developed total s-ELISA, sensitive active and wide-range active s-ELISAs, respectively (Table 2).

Spike Recovery and precision of the developed GLP-1 s-ELISAs. Precision describes the reproducibility or closeness of various measurements of the same sample using the same method. The precisions of the developed s-ELISAs were determined by conducting a recovery assessment as previously described.[22,23] Briefly, known concentrations (60, 15.6, and 1,170 pmol/L) of GLP-1 standard were spiked into assay buffer and analyzed using the developed s-ELISAs. Each concentration was measured in parallel replicates using the respective s-ELISAs. Intra-assay recoveries were determined by measuring each concentration of GLP-1 standard three to four times in one experiment ($n = 3$), while the inter-assay recoveries were determined by measuring each concentration in four different experiments ($n = 4$). The recoveries were calculated according to the following formula:

$$\% \text{ recovery} = \textit{detected concentration of GLP-1/expected (spiked) concentration} \times 100$$

Whereas, the intra- and inter-assay coefficients of variations (% CV) were derived as follows:

$$\% \text{ CV} = \textit{SD of measurement/mean OD (450 nm–630 nm) of samples} \times 100.$$

As shown in Table 3, the % recoveries of the spiked exogenous GLP-1 peptide ranged from 82.8 ± 9.8~85.6 ± 4.9, 86.1 ± 1.8~114.88 ± 23.9, and 98.8 ± 5.1~119.08 ± 39.2.8 for the total s-ELISA, sensitive active s-ELISA, and wide-range active s-ELISA, respectively. The intra-assay CVs were 5.7%, 2.1%, and 5.2%, respectively, with inter-assay CVs of 11.8% for the total s-ELISA, and 20% for the sensitive active s-ELISA and wide-range active s-ELISA, respectively.

Estimation and comparison of GLP-1 level in the media of STC-1 cells stimulated with L-glutamine using the developed GLP-1 s-ELISAs and commercial kit. To determine the performance of the developed s-ELISAs, STC-1 cells were treated with varying concentrations of L-glutamine (10, 20, and 40 mmol/L) a known positive stimulator for endogenous GLP-1 secretion, or vehicle control (0.1% DMSO) for 4 h. DPP-4 inhibitors and

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Table 3. Recoveries and coefficient of variations (CV) of the developed s-ELISAs

s-ELISA	Spiked GLP1 (pM)	Intra-assay (n = 3)		Inter-assay (n = 4)	
		Recoveries (%)	CV (%)	Recoveries (%)	CV (%)
Total GLP-1	60	82.8 ± 9.8	5.7	85.5 ± 4.9	11.8
Sensitive active	15.6	86.1 ± 1.8	2.1	114.9 ± 23.9	20
Wide-range active	1,170	98.8 ± 5.1	5.2	119.1 ± 39.2	20

Table 4. Measurements of L-glutamine induced GLP-1 levels using the developed s-ELISAs

s-ELISA	Measured GLP1 isoform	L-glutamine (mM)			
		0	10	20	40
		[Secreted GLP-1 (pM)]			
Total GLP-1	All forms*	27.3 ± 1.7[a]	77.5 ± 7.3[b]	73.1 ± 8.2[b]	62.6 ± 2.9[a,b]
Sensitive active	GLP-1 (7–36 NH_2)	9.3 ± 0.1[a]	20.2 ± 0.8[b]	21.8 ± 0.3[b]	18.6 ± 0.6[b]
Wide-range active	GLP-1 (7–36 NH_2)	5.7 ± 0.1[a]	—	17.1 ± 0.2[b]	20.7 ± 0.2[c]

*Measure all forms of GLP-1, including its metabolites GLP-1 (9–36 NH_2) and GLP-1 (9–37). [a,b,c]Values with unlike letters indicate significant differences within given rows, by Tukey-Kramer's multiple comparison test (p<0.05).

Table 5. Comparison of the developed wide-range active s-ELISA and the commercial kit

L-glutamine (mM)	Measured active GLP-1 (pM)	
	Wide-range (active) ELISA	Commercial ELISA (active)
0	18.7 ± 2.5[a]	25.8 ± 5.3[a]
20	56.1 ± 3.0[b]	59.6 ± 1.3[b]
40	68.1 ± 1.1[c]	70.3 ± 3.1[c]

[a,b,c]Values with unlike letters indicate significant difference within given rows, and within columns, by Tukey-Kramer's multiple comparison test (p<0.05).

Table 6. Comparison of the developed sensitive active s-ELISA and the commercial kit

Performance properties	Sensitive active s-ELISA	Commercial ELISA (active)
Specificity	GLP-1 (7–36 NH_2)	GLP-1 (7–36 NH_2)
Standard range (pmol/L)	0.98~62.5	0.474~15.6
Precision (CV within an assay)	within 5%	within 10%
Reproducibility (CV between assay)	within 20%	within 10%
Recovery (%)	86.1~114.9	95.8~103
Linearity (R^2)	0.998	0.998
Cost per 96-well plate (USD)	170	608

CV, coefficient of variations; USD, US dollars.

aprotinin solution were added to the cell culture medium to prevent GLP-1 degradation and inhibit the activities of proteolytic enzymes, respectively. As shown in Table 4, the developed total s-ELISA detected a significant increase in total GLP-1[all forms, including amidated active GLP-1 (7–36 NH_2) and its metabolite, GLP-1 (9–36 NH_2)] at 77.5 ± 7.3, 73.1 ± 8.2, and 62.6 ± 2.9 pmol/L after the cells were treated with 10, 20, and 40 mmol/L of L-glutamine, respectively, compared to 27.3 ± 1.7 pmol/L detected in the vehicle control-treated cells. The sensitive active s-ELISA detected 20.2 ± 0.8, 21.8 ± 0.3, and 18.6 ± 0.6 pmol/L of active GLP-1 after the L-glutamine treatments, respectively, and 9.3 ± 0.1 pmol/L from the vehicle control-treated cells. Similarly, the wide-range active s-ELISA detected 17.1 ± 0.2, 20.7 ± 0.2, and 5.7 ± 0.1 pmol/L of active GLP-1 after the L-glutamine treatments at 20 and 40 mmol/L and vehicle control treatment, respectively. These results indicate that the developed s-ELISAs were capable of measuring both active and total GLP-1 in cultured STC-1 cells.

To compare performance, L-glutamine stimulated active GLP-1 level was measured in cell culture media by both the developed wide-range active s-ELISA and a commercially available kit [LBIS GLP-1 (active) ELISA kit; FUJIFILM Wako Shibayagi Co., Ltd. (Shibukawa, Japan)]. As shown in Table 5, the detected levels of active GLP-1 were the same between the developed s-ELISA and the commercial kit, indicating equal performance. The performance properties of the developed sensitive active s-ELISA compared to commercial (active) kit are shown in Table 6.

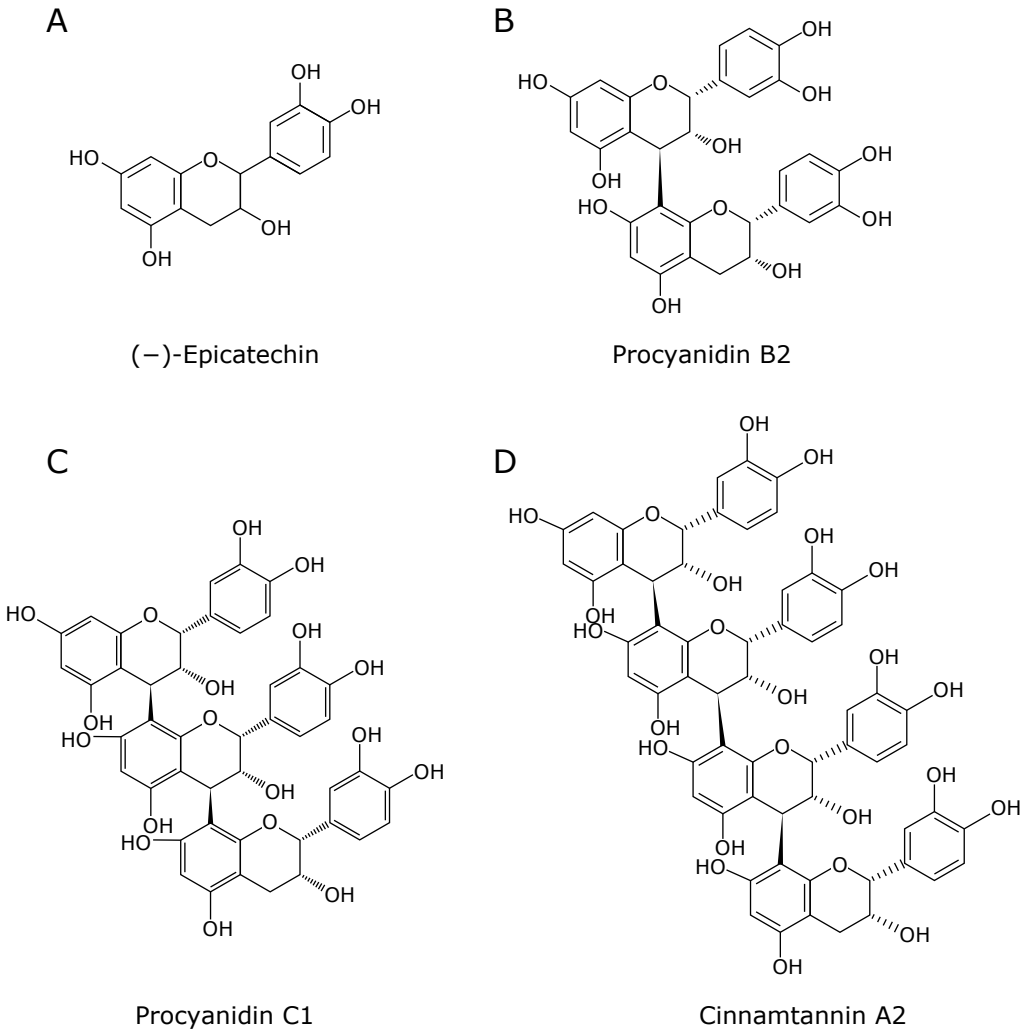

Fig. 4. Chemical structures of the flavan-3-ols used. (A) (–)-epicatechin. (B) Procyanidin B2. (C) Procyanidin C1. (D) Cinnamtannin A2.

Estimation of active and total GLP-1 in STC-1 cells treated with different flavan-3-ols. The application of the developed s-ELISAs was assessed from GLP-1 secretion experiment in STC-1 cells after stimulation with various flavan-3-ols. Flavan-3-ols, in particular procyanidin oligomers, have been reported to possess potential anti-hyperglycemic effects but the molecular mechanisms by which these compounds suppress hyperglycemia are not yet fully understood. Previously, Yamashita *et al.*[13] demonstrated that cinnamtannin A2, a tetrameric procyanidin, increased GLP-1 and insulin levels in the plasma of ICR mice 60 min after oral administration. In addition, a cacao liquor procyanidin-rich extract (CLPr) and its polymerization fraction suppressed postprandial hyperglycemia accompanied by GLP-1 secretion with or without glucose loading conditions in ICR mice.[24] These findings indicated that procyanidins may improve hyperglycemia through the activation of the insulin signaling pathway via GLP-1 secretion. Therefore, we confirmed the influence of flavan-3-ols on stimulation of GLP-1 secretion from STC-1 cells using the developed GLP-1 s-ELISAs. The chemical structures of flavan-3-ols used are shown in Fig. 4. Consistent with the previous *in vivo* results,[13,24] only cinnamtannin A2 promoted both total and active GLP-1 secretion in a dose-dependent manner compared to the vehicle control (Fig. 5A and B). A significant increase in total and active GLP-1 secretion was observed at 0.3 and 30 nM of cinnamtannin A2, respectively. The other flavan-3-ols, namely (−)-epicatechin, procyanidin B2, and procyanidin C1 did not alter the levels of GLP-1 secreted from STC-1 cells (Fig. 5C–H) These compounds did not show cytotoxicity of STC-1 cells up to 10 μM (cinnamtannin A2), 50 μM (procyanidin C1), and 100 μM (procyanidin B2) for 4 h (Fig. 6).

Estimation of total GLP-1 in plasma of mice administered with BE and PCBE. The measurement of GLP-1 is frequently performed in plasma. Therefore, we assessed the application of the developed s-ELISAs in plasma sample matrix using mice after oral administration of BE and PCBE rich in cinnamtannin A2 as described in Materials and Methods section. Using the developed total GLP-1 s-ELISA, significant increase in GLP-1 level was observed in the plasma of mice given PCBE compared to water-given control mice. BE showed an increasing trend in plasma GLP-1 level compared to control without statistical significance (Fig. 7). Taken together, these results indicate that the developed s-ELISAs are capable of measuring GLP-1 secretion from both cultured STC-1 cells and mice plasma sample. The results also confirmed cinnamtannin A2 as an attractive plant bioactive compound for preventing hyperglycemia through increased GLP-1 secretion. Further study is thus needed to elucidate the molecular mechanism of GLP-1 secretion by cinnamtannin A2.

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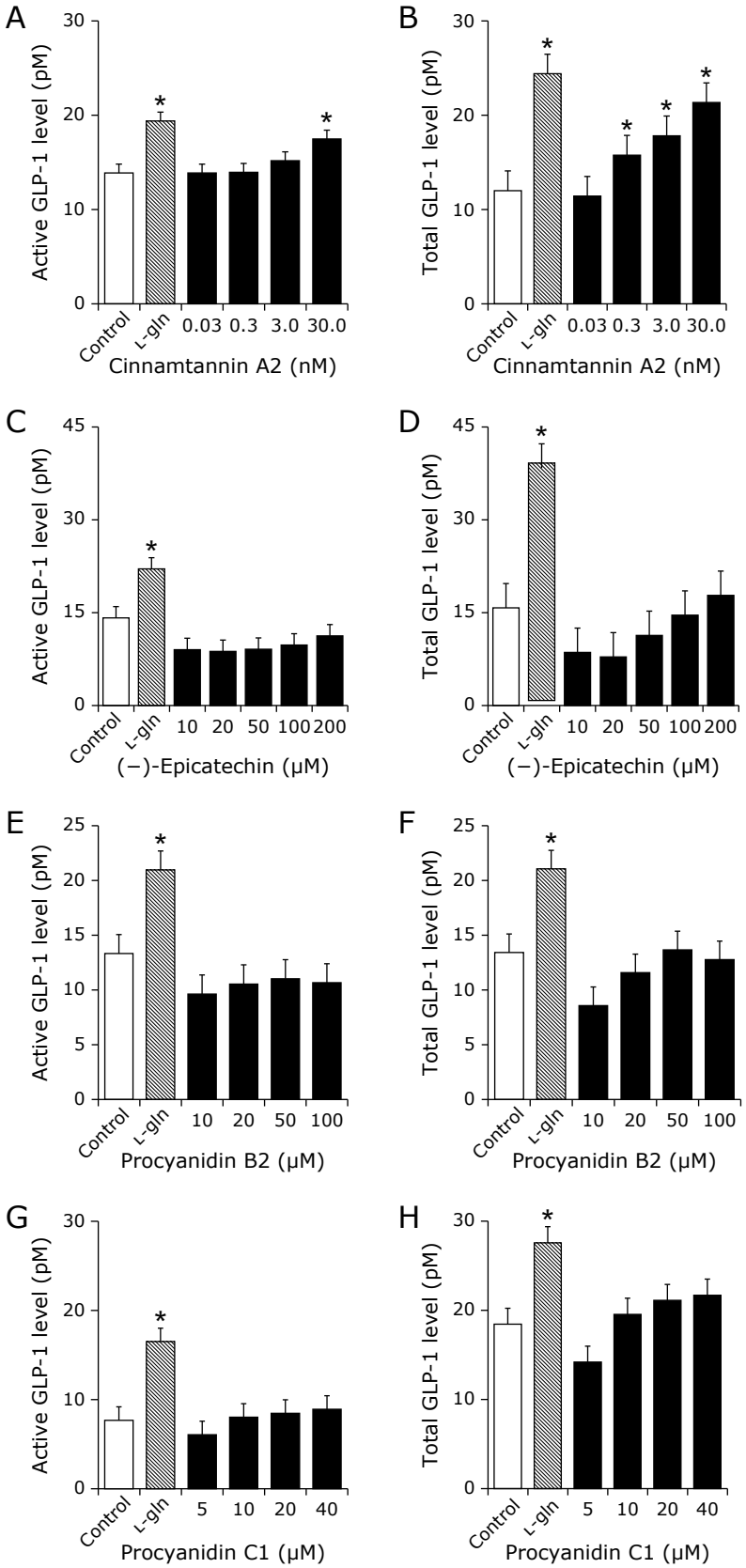

Fig. 5. Cinnamtannin A2 promotes the secretion of GLP-1 from STC-1 cells. STC-1 cells were treated with flavan-3ols at the indicated concentrations. L-glutamine (10 mM) and DMSO (0.1% as final concentration) were used for the positive and vehicle controls, respectively for 4 h. The concentration of 'active' and 'total' GLP-1 in the medium was measured using the developed sensitive active and total s-ELISAs, respectively. (A, B) (–)-epicatechin. (C, D) Procyanidin B2. (E, F) Procyanidin C1. (G, H) Cinnamtannin A2. Data are shown as means ± SE ($n = 3$). *Indicates significant difference from the vehicle control-treated cells ($p<0.05$ by Dunnett's multiple comparison test). All compounds treatment (except cinnamtannin A2) were conducted simultaneously using the same plate and the values of the vehicle control in panels (A), (C), and (E) and that of positive control in panels (B), (D), and (F) were shared, respectively. L-gln, L-glutamine.

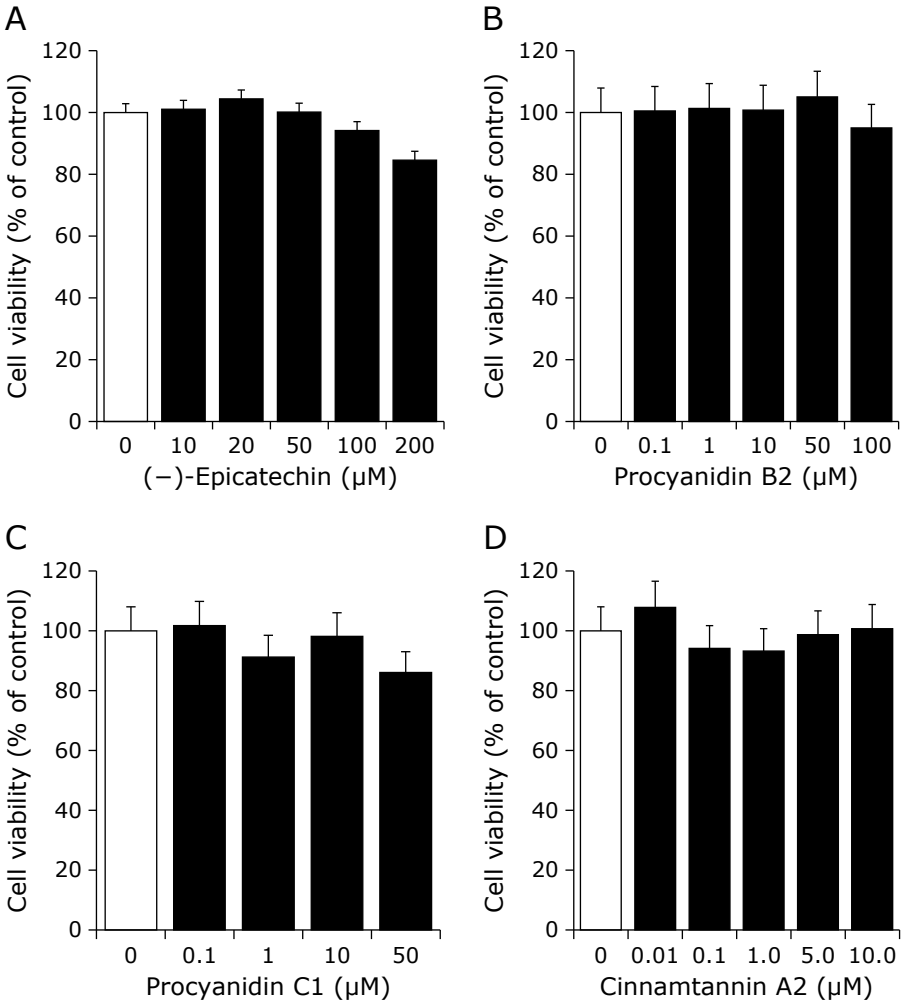

Fig. 6. Cytotoxicity of flavan-3-ols on STC-1 cells by crystal violet staining. (A) (–)-epicatechin (B) Procyanidin B2. (C) Procyanidin C1. (D) cinnamtannin A2. Values are represented as % of control. Data are shown as means ± SE (n = 8) by Dunnett's multiple comparison test (p<0.05).

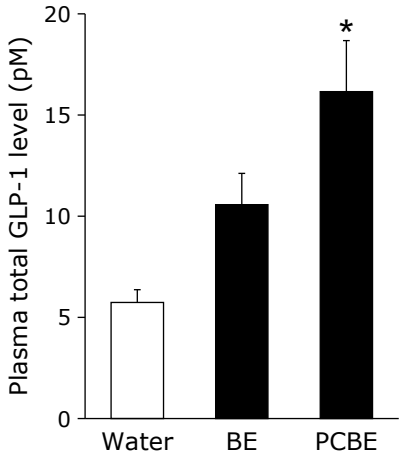

Fig. 7. PCBE increases the secretion of GLP-1 in plasma of mice. Male C57BL/6J mice (6 weeks old) were orally dosed with BE or PCBE in water at 300 mg/kg body weight. Plasma GLP-1 levels were measured 60 min after administration of BE or PCBE using the developed total GLP-1 s-ELISA. Data are shown as means ± SE (n = 5). *Indicates significant difference from the water only-treated group (p<0.05 by Dunnett's multiple comparison test). BE, black soybean seed coat extract; PCBE, procyanidin-rich black soybean seed coat extract.

Discussion

We have developed a cost-effective sandwich GLP-1 ELISAs capable of measuring both total and active GLP-1 in cell supernatants and plasma. Because these s-ELISAs were both accurate and sensitive enough to quantify physiological levels of GLP-1 peptide in cell culture media and plasma, they would be helpful in further understanding the physiology and role of GLP-1 in diabetes, particularly in relation to potent plant bioactive compounds.

GLP-1 have been studied for more than 3 decades, but researchers have noted that accurate and extensive measurement of this incretin hormone is hindered by the high cost of commercially available tools. Commercial GLP-1 ELISA kits, which is the primary measurement tool used in many laboratories, are not only expensive but also present high assay variability which is problematic because of difficulties in precision and accuracy of GLP-1 measurements within and across laboratories. To address the challenges with extensive measurement of GLP-1, we describe here a simple, cost-effective GLP-1 assays specifically developed using commercially available reagents, that can be used as an inhouse tool in laboratories. The developed assays utilize commercially available affinity purified monoclonal antibodies providing equal performance as the commercial product in terms of high sensitivity and specificity, while offering broad

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measurement range and low cost-performance compared to the commercial kits.

These s-ELISAs were validated by demonstrating that they were absolutely capable of measuring GLP-1 levels in real sample conditions. The anti-GLP-1 antibodies used were affinity purified and the detection antibody for the active s-ELISA had less than 0.2% cross reactivity with GLP-1 (9–36) amide, GIP and glucagon, indicating an absolute requirement for the N-terminal epitope of GLP-1 (7–36) amide ensuring for accurate and precise measurement of active GLP-1 molecules. In general, analyte recoveries of an ELISA method are acceptable within 80% and 120%,[22,23,25] while the ELISA method is considered accurate with a CV within 10% for the intra-assay precision and within 20% for inter-assay variations.[16,22,25] The sensitivity (0.94~, 0.98~ pM) and precision (intra- and inter- assay CVs <6% and within 20%, respectively) of the developed s-ELISAs was therefore adequate according to guidelines by the Clinical & Laboratory Standards Institute and as described previously.[16,22,23,25] It is worth noting that the direct s-ELISA in this study (the sensitive active s-ELISA) showed higher sensitivity than the indirect one (wide-range active s-ELISA) contrary to usual observations where indirect sandwich ELISA are more sensitive due to the amplification step. This may be due to antibody affinity. It has been reported that assay sensitivity can be determined by the antibody/antigen interaction rather than the development format. For example, the sensitivity of the kinetic exclusion assay method used to determine solution affinities of protein/protein interactions was typically independent of the assay format employed.[26] Nimmo *et al.*[27] also demonstrated that ELISA performance is influenced by antibody affinity, especially with antigens of low epitope density. In addition, indirect sandwich ELISA has a high risk of cross-reactivity with the antigen, which could cause higher background that can interfere with the sensitivity of this method.

These developed s-ELISAs were further validated by estimating concentration of secreted active and total GLP-1 levels in STC-1 cells supernatants and in plasma of mice after oral administration of BE and PCBE rich in cinnamtannin A2. Consistent with our previous findings of cinnamtannin A2-induced increase in plasma GLP-1 level measured by commercial ELISA,[13] a significant increase in GLP-1 was observed in cinnamtannin A2-treated cells and in mice given PCBE using the developed s-ELISAs. These results indicate that the developed s-ELISAs have strong compatibility and adequacy for GLP-1 measurement in the tested cell supernatant and plasma sample matrix. Compelling evidence shows that GLP-1 concentration in the portal vein is 50~60 pmol/L,[28] and even in peripheral blood its concentration is ~30 pmol/L.[29] The concentration of subcutaneously administered GLP-1 analogue is approximately 100 pmol/L,[30] indicating that the blood levels of GLP-1 does not reach nmol/L concentration, even during treatments. Consistently, even in rats, serum GLP-1 levels do not exceed 100 pmol/L, even in the presence of DPP-4 inhibitors.[31] However, cellular GLP-1 concentration is markedly elevated in the *in vitro* models in comparison with mice and human basal GLP-1 levels. STC-1, GLUTag, and NCI-H716 are the three GLP-1 producing cell lines widely used to study the mechanism of its secretion.[32,33] These cell lines are immortal and derived from carcinomas and considerably differ from native L-cells in terms of the relative amount of secreted peptide.[32] The average concentration (in the media) of active GLP-1 from cultured STC-1 cells stimulated with putative GLP-1 secretagogues was ~250 pmol/L,[33] with the highest concentration reaching approximately ~850 pmol/L in STC-1 cells treated with 50 μM of α-linolenic acid.[34] Commercial GLP-1 ELISA tend to focus on the sensitivity while offering a particularly narrow detection range, usually bellow 100 pmol/L for a conventional active ELISA. The developed wide-range active s-ELISA in this study offered a wide measurement range for active GLP-1 (~4.48 nmol/L), enabling efficient quantification in samples that vary significantly in concentration, particularly in the initial stages of plant extract and compound screening usually conducted *in vitro.*

Finally, the best aspect of the developed s-ELISAs was low-cost performance (all things considered) compared to the commercial assays. The approximated cost (not including rent and labour costs) was USD 170 ($1.77/test), which is 75.9% and 77.6% cheaper compared to the USD 702 ($7.3/test) and 760 ($7.9/test) for the commercial active and total kits, respectively. Because these assays are developed for inhouse application in already established laboratories and not for commercial purposes, the cost analysis did not include other expenses such as salaries, laboratory rent, running costs, taxes etc. which are included in the pricing of commercial assays.

Conclusion

In conclusion, commercial GLP-1 assays have severely limited the ability of researchers to identify effective plant bioactive compounds due to high costs and a particularly narrow detection range. The developed s-ELISAs in the present study are less costly and can effectively quantify both total and active GLP-1 in both cell and plasma samples, with a broad measurement range (~4.48 nmol/L, for active GLP-1). The assays required 100 μl of cell lysate or plasma and completes in 6 h with equal performance as the commercial product at 80% cheaper cost. The application assessments confirmed the potential of plant bioactive compounds, in particular, cinnamtannin A2 as an attractive food factor for preventing hyperglycemia through GLP-1 secretion. Therefore, we envision that the s-ELISAs developed in this study can be effectively utilized as a good tool in extensive search for the plant bioactive compounds with potential 'incretin' effects.

Author Contributions

Study concept and design, YY, HA; acquisition of data, KO, KH; analysis and interpretation of data, KO; drafting of the manuscript, KO; critical revision of the manuscript for important intellectual content, KO, KH, YY, HA; statistical analysis, KO; obtained funding, HA; administrative, technical, or material support, YY, HA; study supervision, HA.

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Abbreviations

BE	black soy bean seed coat extract
BSA	bovine serum albumin
CV	coefficient of variation
CLSI	Clinical and Laboratory Standards Institute
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DPP-4	dipeptidyl peptidase-4
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GIP	gastric inhibitory peptide
GLP-1	glucagon-like peptide-1
LOD	limit of detection
LOQ	limit of quantification
PCBE	procyanidin-rich black soy bean seed coat extract
s-ELISA	sandwich enzyme-linked immunosorbent assay
SDS	sodium dodecyl sulfate
STC-1	intestinal secretin tumor cell line

T2DM	type 2 diabetes mellitus
TMB	3, 3', 5, 5'-tetramethylbenzidine
USD	United States dollar

Conflict of Interest

No potential conflicts of interest were disclosed.

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