

# Search for Compounds Suppressing Intestinal $\alpha$ -Glucosidase Expression in Caco-2 Cells

Kota Noda, Eisuke Kato\* & Jun Kawabata

Graduate School of Agriculture, Hokkaido University, Japan

\*eikato@chem.agr.hokudai.ac.jp

**Abstract:** Diabetes is a chronic disease characterized by elevated blood glucose level. Reducing carbohydrate absorption from the intestinal tract is an effective strategy to control post-meal blood glucose level. Inhibition of intestinal  $\alpha$ -glucosidase, involved in digestion of carbohydrates, is known as an approach to accomplish this. On the other hand, reduction of  $\alpha$ -glucosidase amount is expected to work in the similar manner. However, none of the previous study pursues this approach. A convenient assay was developed to evaluate  $\alpha$ -glucosidase amount employing Caco-2 cells, the intestinal epithelial cell model reported to express  $\alpha$ -glucosidase. Sixty plants were screened and two candidate plants, *Calluna vulgaris* and *Perilla frutescens* var. *crispa* were found to reduce  $\alpha$ -glucosidase expression. *C. vulgaris* extract was subjected to activity guided isolation. Proanthocyanidin was identified as the active principle which was analyzed by thiol decomposition to reveal the components as a mixture of catechin, epicatechin, epigallocatechin, and A type procyanidin dimer. The proanthocyanidin suppressed about 30% of  $\alpha$ -glucosidase amount evaluated through convenient assay, and suppressed bulk of mRNA expression level of sucrase-isomaltase (SI) at 0.125 mg/mL. Several flavan-3-ol monomers were also tested, and epicatechin gallate and epigallocatechin gallate were found to suppress  $\alpha$ -glucosidase amount significantly.

**Keywords:** diabetes,  $\alpha$ -glucosidase, Caco-2 cells, proanthocyanidin

## 1. Introduction

Twelve-percent of global health expenditure is spent on diabetes, which 1 in 11 adults on the earth is estimated as the patient (IDF diabetes atlas 8th Ed., 2017). Diabetes is a chronic disease that occurs when the body cannot produce enough insulin or cannot use insulin effectively. Inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase, the carbohydrate digestive enzymes, is a widely accepted method to prevent or treat diabetes. Treatment with acarbose, the well-known  $\alpha$ -glucosidase inhibitor, before or during the meal effectively decreases post-meal blood glucose level.

On the other hand, reduction of the intestinal  $\alpha$ -glucosidase amount will also suppress post-meal blood glucose level, and may also be effective for treating diabetic patients and people at risk of developing type 2 diabetes. This approach has several advantages, for example, reduction of the intestinal  $\alpha$ -glucosidase amount is an effect toward the intestinal cell, which is assumed to sustain until intestinal cell turnover, or a re-production of  $\alpha$ -glucosidase by the cell, that is at least longer than the  $\alpha$ -glucosidase inhibitors. In addition, the product does not require to take along with a meal.

Some study has shown the possibility to regulate  $\alpha$ -glucosidase expression. A diet rich in carbohydrates leads to intestinal expression of genes related to  $\alpha$ -glucosidase expression (T. Goda et al. 2018). Caco-2 cell, which is frequently used as an intestinal cell model, is also reported to respond to glucose and maltose to change the amount of  $\alpha$ -glucosidase (M. Cheng et al. 2013). Additionally, berberine suppresses mRNA expression of intestinal disaccharides on Caco-2 cell and also in the small intestine of rats (L. Liu et al. 2010). However, not much previous study has focused on reducing the  $\alpha$ -glucosidase amount to treat diabetes up to date. Therefore, a convenient method was developed to evaluate  $\alpha$ -glucosidase amount expressed by Caco-2 cells, and used for searching natural products that suppresses the expression.

## 2. Materials and Methods

### 2.1. Materials

The plant materials used in the study were purchased from Hyakka-Saen Co. Ltd. Caco-2 cell was obtained from Riken Bio Resource Research Center (Tsukuba, Japan). All commercially available chemicals were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan), unless otherwise noted. For the LC-MS analysis, Waters Acquity UPLC system (Water Co., Milford, USA) was combined with LCT-premier (Water Co., Milford, USA). Absorbance was measured by Synergy<sup>TM</sup> MX (Bio-tech Instruments Inc., Winooski, USA) microplate reader.

### 2.2. Methods

#### 2.2.1. Cell culture

Cells were maintained in 10 cm dishes (NIPPON Genetics Co.Ltd) in a standard culture medium at 37°C in a humidified atmosphere of 10% CO<sub>2</sub>. The standard culture medium consisted of Eagle's minimal essential medium supplemented with 20% fetal bovine serum, non-essential amino acids, 100,000 U/L penicillin, 100 mg/L streptomycin, and 50 mg/L gentamycin. For experiments, cells were seeded onto 96 well plate (NIPPON Genetics Co.Ltd). After cells reached 100% confluence (typically 5 days after seeding), cells were grown for further 15 days. Media was replaced every 4 days.

#### 2.2.2. Caco-2 cell assay to measure reduction of $\alpha$ -glucosidase amount

Samples were dissolved in 50% aq. dimethylsulfoxide (DMSO), diluted with standard culture medium, and added to the cells. DMSO concentration was kept below 0.5%, and the same concentration of DMSO was used as a control. After 2 days incubation, the cells were carefully washed by KRPH buffer (20 mM HEPES, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 136 mM NaCl, 4.7 mM KCl, pH 7.4) to completely remove the samples, and 0.1 mM 4-methylumbelliferyl  $\alpha$ -D-glucopyranoside solution was added as a substrate. Fluorescens (Ex365 nm, Em448 nm) of the released aglycon is measured every 5 min for 30 min. Reduction of  $\alpha$ -glucosidase amount was estimated by comparing the reaction rate of the sample treated cells with the control cells.

#### 2.2.3. Extraction and Analysis of mRNA

Cells were washed with phosphatase-buffered saline (PBS), and the total RNA was isolated using ReliaPrep RNA Cell Miniprep System (Promega KK., Tokyo, Japan). The total RNA was reverse transcribed to generate first-strand cDNA using M-MLV Reverse Transcriptase (Promega KK., Tokyo, Japan). Real-time quantitative PCR (RT-qPCR) was performed using Gene Ace SYBR qPCR Mix  $\alpha$  No ROX (Nippon Gene Co., Ltd., Tokyo, Japan) with a Thermal Cycler Dice TP800 (Takara Inc., Shiga, Japan). Relative mRNA expression level for each sample was normalized to that of GAPDH. Following primers were used. SI forward 5'-TCTTCATGAGTTTTATGAGGATACGAAC-3', reverse 5'-TTTGCACCAGATTCATAATCATACC-3'; GAPDH forward 5'-CCTGTTCGACAGTCAGCCG-3', reverse 5'-CGACCAAATCCGTTGACTCC-3' (BL. Williams et al. 2011).

#### 2.2.4. Extraction and Analysis of Protein

Cells were washed with ice cold PBS and lysed in SDS sample buffer supplemented with 0.05 M dithiothreitol. The protein concentration was measured using XL-Bradford (APRO Science Inc., Tokyo, Japan) with bovine serum albumin as the standard. Extracted proteins were denatured by heating at 95°C for 5 min. The prepared protein samples (10  $\mu$ g) were separated using 7.5% (w/v) polyacrylamide gel. Separated proteins were electro-transferred onto PVDF membranes with Trans-blot SD Cell at 15 V for 30 min. The membrane was then blocked with 5% (w/v) skim milk in TBS-T (Tris buffered saline containing 0.1% Tween-20) for 1 h at room temperature. The membrane was subsequently incubated with primary antibody for 2 h at room temperature and then with HRP-conjugated secondary anti-body for 1 h at room temperature. Antibodies used for the immunoblot were goat Sucrase-Isomaltase antibody (dilution 1:100, sc-27605), and anti-goat IgG HRP-linked antibody (1:2000, sc-2020) purchased from Santa Cruz Biotechnology, Inc. (Dallas, USA), or mouse beta-actin antibody (1:1000, #3700), and anti-mouse IgG HRP-linked antibody (1:2000, #7076S)

purchased from Cell Signaling Technology, Inc. (Danvers, USA). The antigen-antibody complexes were then visualized using ImmunoStar® Zeta (FUJIFILM Wako Pure Chemical Co., Osaka, Japan). The luminescence intensity was quantified using Image J software.

### 2.2.5. Isolation

Dried *C. vulgaris* buds (100 g) were extracted with 50% aq. methanol for 24 h. The extract (11.5 g) was dispersed in water and partitioned between ethyl acetate and then between 1-butanol. The 1-butanol layer (2.59 g) was concentrated and the residual aq. solution was separated by DIAION HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) column chromatography with stepwise elution (water, 50% aq. methanol, and methanol). The 50% aq. methanol eluate (1.48 g) was separated by Sephadex LH-20 (GE Healthcare Japan Co., Tokyo, Japan) column chromatography with stepwise elution (20% aq. methanol, 40% aq. methanol, 60% aq. Methanol, 80% aq. methanol, methanol). The 80% aq. methanol fraction was finally purified by HPLC (Inertsil ODS-3, 15-50% methanol aq. with 0.1% TFA 0-90 min) to obtain CV-P.

### 2.2.6. Thiolytic

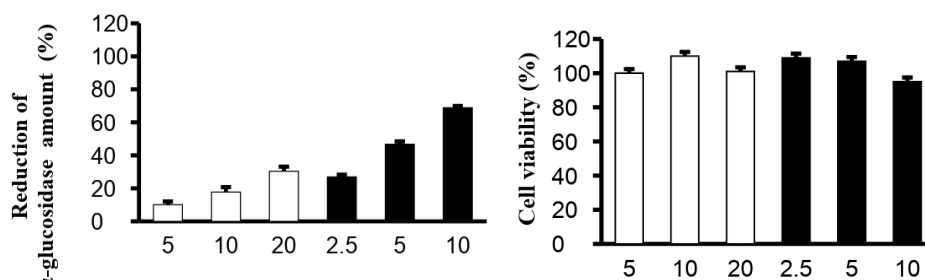
CV-P (1 mg) was dissolved in 0.1 M hydrogen chloride in methanol (183  $\mu$ L), and benzyl mercaptan (5  $\mu$ L) was added. The mixture was stirred for 90 min at 40°C. The reaction mixture was dried and analyzed by HPLC and LC-MS. The peaks were identified by comparing with the retention time of the standards and from the MS spectrum. The ratio of each compound was estimated from the peak area after adjustment using commercial catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate as a standard.

## 3. Result and Discussion

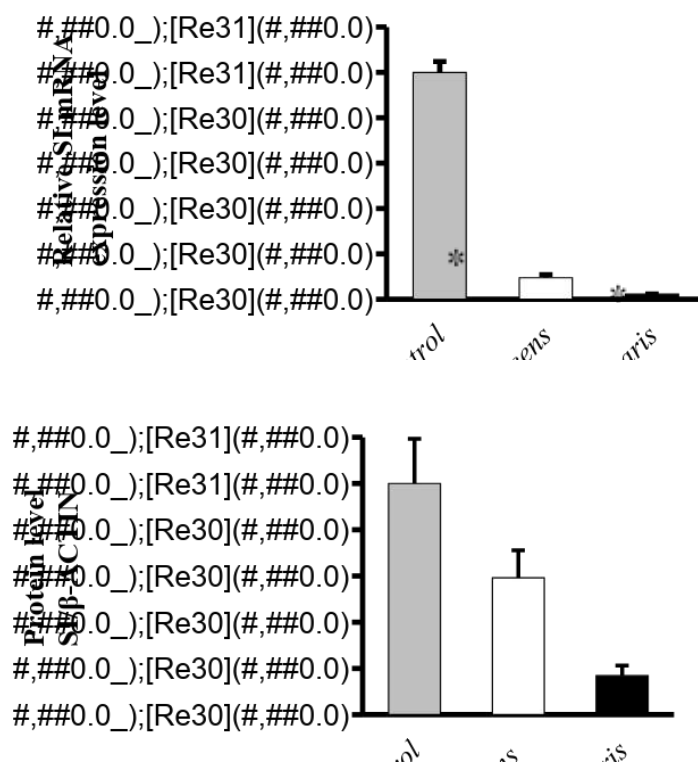
### 3.1. Search for resources that suppress $\alpha$ -glucosidase amount

For the evaluation of resources that suppress  $\alpha$ -glucosidase amount, Caco-2 cell was employed as a model of an intestinal epithelial cell which is reported to express  $\alpha$ -glucosidase when differentiated (Z. Liu et al. 2010). Samples were added to Caco-2 cell and the  $\alpha$ -glucosidase amount expressed by Caco-2 cell was quantitated to evaluate  $\alpha$ -glucosidase amount reducing activity. To enhance the throughput of the screening, the  $\alpha$ -glucosidase amount expressed by Caco-2 cell was monitored from the reaction rate of  $\alpha$ -glucosidase instead of quantitating the protein, since the reaction rate corresponds to the  $\alpha$ -glucosidase amount.

Employing the above method, 60 plants were screened to find *C. vulgaris* and *P. furtescens* as a candidate plants that show effective  $\alpha$ -glucosidase amount reducing activity. These plant extracts suppressed  $\alpha$ -glucosidase amount in a concentration dependent manner without cytotoxicity (Figure 1). To confirm the result,  $\alpha$ -glucosidase mRNA expression and protein level were quantitated. Sucrase-isomaltase (SI), one of the intestinal  $\alpha$ -glucosidase complexes, plays a main role in  $\alpha$ -glucosidase activity of Caco-2 cell. Treating cells with the plant extracts for 2 days significantly reduced Sucrase-isomaltase (SI) at mRNA and protein level (Figure 2).



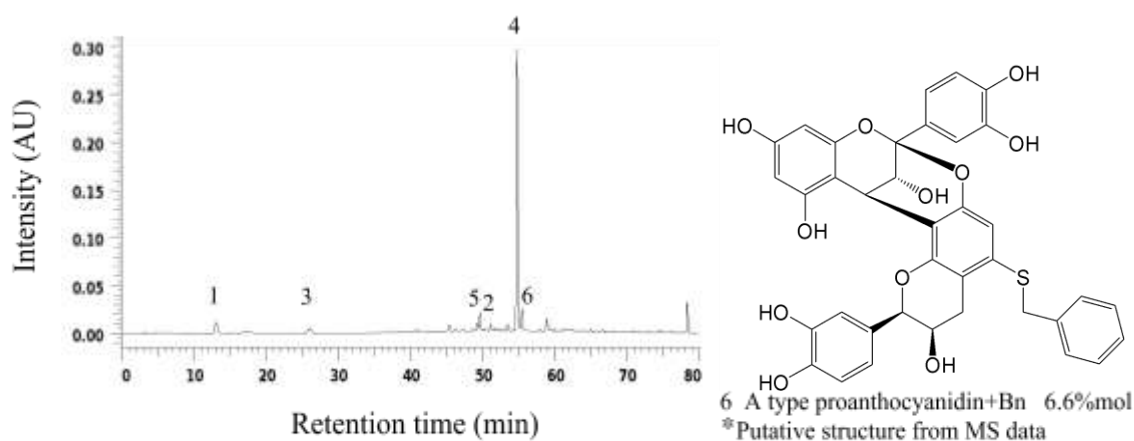
**Figure 1.** *P. crispa* and *C. vulgaris* extracts suppressed  $\alpha$ -glucosidase amount in a dose dependent manner (right). The extracts did not show cytotoxicity (left). Black bar indicates *P. crispa*, the white bar indicates *C. vulgaris*. \* $p < 0.05$  to control.

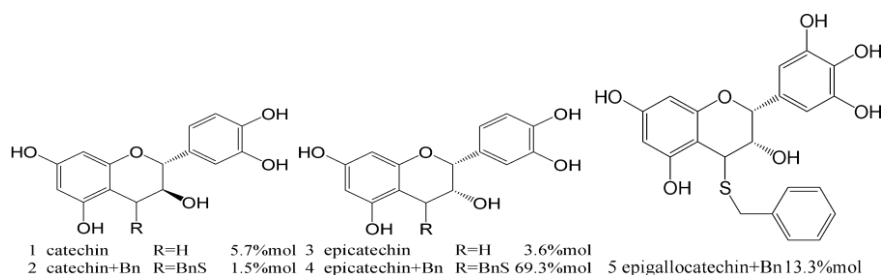


**Figure 2.** *P. crispa* and *C. vulgaris* extracts suppressed  $\alpha$ -glucosidase mRNA expression (left) and protein (right) level. \* $p < 0.05$  to control.

### 3.2. Purification of active principle

*C. vulgaris* extract was subjected to activity guided fractionation to isolate  $\alpha$ -glucosidase amount reducing principle. Chromatographic purification gave the active principle (CV-P) which was identified as a proanthocyanidin from the NMR and HPLC analysis.





**Figure 3.** Thiolysis analysis of CV-P

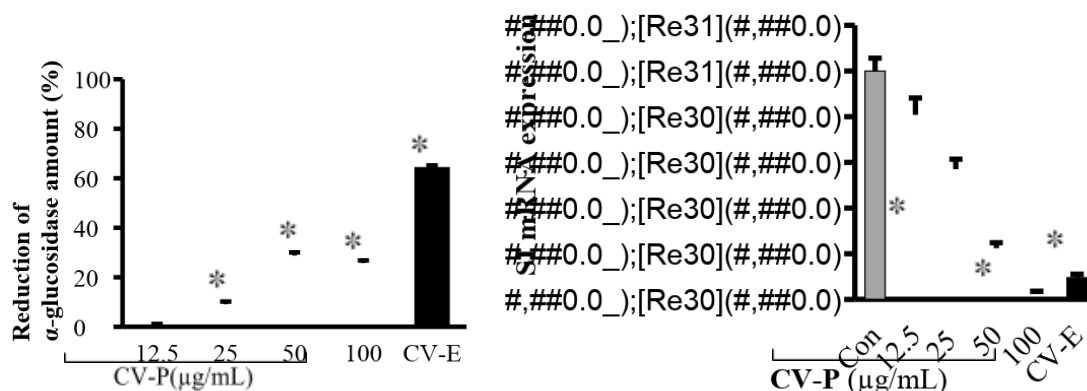
Thiolysis of CV-P was performed to determine the flaven-3-ol units constituting CV-P and estimate the degree of polymerization. After the thiolysis, the reaction mixture was analyzed by LC-MS, and the compounds were identified. CV-P contains catechin, epicatechin, epigallocatechin, and A type procyanidin dimer with mean degree of polymerization estimated to be 10.6 (Figure 3).

### 3.3. Evaluation of the $\alpha$ -glucosidase suppression activity of CV-P

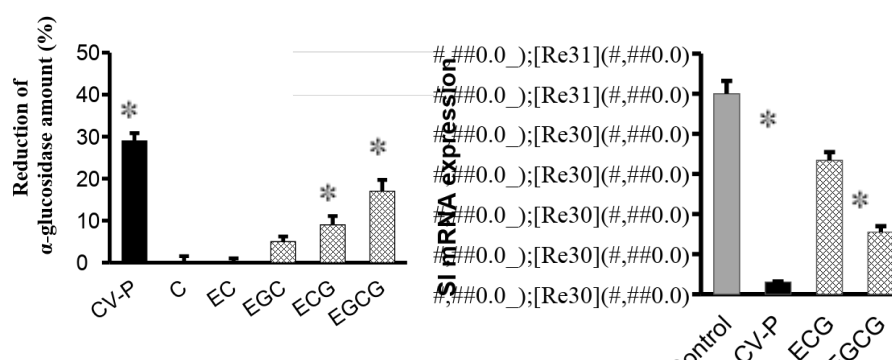
When added to Caco-2 cells, CV-P suppressed  $\alpha$ -glucosidase amount up to 29% compared to control (Figure 4). CV-P also suppressed SI mRNA expression in concentration dependent manner between 25-100  $\mu$ g/mL.

To evaluate if the polymerization of flaven-3-ols is important for the activity, several monomers were also tested for their  $\alpha$ -glucosidase suppression activity (Figure 5). Although monomers of CV-P didn't show significant activity, epicatechin gallate and epigallocatechin gallate were found to suppress  $\alpha$ -glucosidase amount significantly which was also confirmed from decreased mRNA expression (Figure 5).

The effect of proanthocyanidin to prevent the development of hyperglycemia in diabetic obese mice is reported (M. Tomaru *et al.* 2007). Several reports may support the mechanisms to explain this effect. Intake of proanthocyanidin oligomers stimulates glucagon-like peptide-1 and insulin secretion in mice (Y. Yamashita *et al.* 2013). *In vitro* inhibition of dipeptidyl peptidase-4 by proanthocyanidin from grape seed is reported (N. González-Abuín *et al.* 2012). Pancreatic  $\alpha$ -amylase inhibition by proanthocyanidin is also reported (E. Kato *et al.* 2017). The current finding of CV-P to reduce  $\alpha$ -glucosidase amount can be an additional bioactivity to support the value of proanthocyanidin to prevent and treat diabetes.



**Figure 4.** CV-P suppresses  $\alpha$ -glucosidase activity (left) and SI mRNA level (right). \* $p < 0.05$  to control.



**Figure 5.** Catechin gallate body suppresses  $\alpha$ -glucosidase activity (left) and SI mRNA(right). Sample concentration was 100  $\mu$ g/mL. \* $p < 0.05$  to control.

#### 4. Conclusion

Amount of  $\alpha$ -glucosidase was targeted as the novel strategy to treat or prevent diabetes. Proanthocyanidin in *C. vulgaris* (CV-P) was found to reduce  $\alpha$ -glucosidase amount of Caco-2 cells and also suppressed Sucrase-Isomaltase (SI) mRNA gene expression. Epicatechin gallate and epigallocatechin gallate were found to reduce  $\alpha$ -glucosidase amount significantly which was also confirmed by decreasing mRNA expression.

Although the experiments are *in vitro*, these results also suggest that the 2 plants and the compounds found here can be re-evaluated as a new-functional food and/or medicinal resources against diabetes through reducing  $\alpha$ -glucosidase amount.

#### References

- BL. Williams *et al.* (2011) Impaired Carbohydrate Digestion and Transport and Mucosal Dysbiosis in the Intestines of Children with Autism and Gastrointestinal Disturbances, *Plos One*, Volume 6, issue 9, e24585.
- E. Kato *et al.* (2017) *Astilbe thunbergii* reduces postprandial hyperglycemia in a type 2 diabetes rat model via pancreatic alpha-amylase inhibition by highly condensed procyanidins, *Bioscience, Biotechnology, and Biochemistry*, Volume 81, Issue 9, 1699-1705.
- G. Semenza *et al.* (1986) Anchoring and biosynthesis of stalked brush border membrane proteins: glycosidases and peptidases of enterocytes and renal tubuli. *Annual Review of Cell and Developmental Biology*, Volume 2, 255-313.
- L. Liu *et al.* (2010) Berberine suppresses intestinal disaccharidases with beneficial metabolic effects in diabetic states, evidences from in vivo and in vitro study. *Naunyn-Schmied Archives Pharmacology*, 381:371–381
- M. Cheng *et al.* (2013) Different sucrose-isomaltase response of Caco-2 cells to glucose and maltose suggests dietary maltose sensing. *Journal of Clinical Biochemistry and Nutrition*, Volume 54, No. 1, 55-60.
- M. Tomaru *et al.* (2007) Dietary supplementation with cacao liquor proanthocyanidins prevents elevation of blood glucose levels in diabetic obese mice. *Nutrition*, Volume 23, Issue 4, 351-355.
- N. González-Abuín *et al.* (2012) Grape seed-derived procyanidins decrease dipeptidyl-peptidase 4 activity and expression. *Journal of Agricultural and Food Chemistry*, Volume 60, 9055-9061.
- T. Goda *et al.* (2018) Molecular Regulation of Mucosal maltase. *Journal of Pediatric Gastroenterology and Nutrition*, Volume 66, supplement 3, S14-S17.
- Y. Yamashita *et al.* (2013) Cinnamtannin A2, a Tetrameric Procyanidin, Increases GLP-1 and Insulin Secretion in Mice. *Bioscience, Biotechnology, and Biochemistry*, Volume 77, No.4, 888.
- Z. Liu *et al.* (2010) Culture of human intestinal epithelial cell using the dissociating enzyme thermolysin and endothelin-3. *Brazilian Journal of Medical and Biological Research*, Volume 43, No. 5, 451-459.