Title:

Wheat gluten hydrolysate potently stimulates peptide-YY secretion and suppresses food intake in rats

Running Head: Effects protein hydrolysates on gut hormone and appetite

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Wheat gluten hydrolysate potently stimulates peptide-YY secretion and suppresses food intake in rats

Abstract: The study was aimed to compare the satiating effect of various protein hydrolysates in rats and examine the underlying mechanism associated with the satiety hormones. Food intake and portal satiety hormone levels were measured in rats. Enteroendocrine cell-lines were employed to study the direct effect of protein hydrolysates on gut hormone secretions. The results showed that oral preload of wheat gluten hydrolysate (WGH) suppressed food intake greater and longer than other hydrolysates. The portal peptide-YY levels in WGH-treated rats at 2 and 3 hrs were higher than those in control- and lactalbumin hydrolysate (LAH)-treated rats. In a distal enteroendocrine cell model, WGH more potently stimulated glucagon-like peptide-1 secretion than LAH, and the effect was largely enhanced by pepsin/pancreatin digestion of WGH. These results suggest WGH is potent in activating enteroendocrine cells to release satiety hormones leading to the prolonged suppression of food intake.

Keywords: protein hydrolysate; gut satiety hormone; food intake; enteroendocrine cell

Introduction

To explain the complex process of appetite regulation, two different mechanisms are proposed: the long-term regulation of food intake *via* adiposity signals such as leptin and insulin; and short-term regulation from meal to meal, which is coordinated by several anorexigenic and orexigenic hormones secreted by the gastrointestinal tract [1-3]. Satiety is a physiological process that is mediated through a complex system connected with appetite regulation [4,5]. An understanding of the pathways by which foodstuff induces satiety is becoming critical for human health. The link between foodstuff and relevant gut hormones is also critically important.

Cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) are the major gut hormones that induce satiety. CCK is produced by I cells located in the duodenum and upper jejunum. CCK is released mainly in response to dietary protein or its hydrolysates [6-8]. The release of GLP-1 from enteroendocrine L cells in the distal intestine is stimulated by diverse molecules, such as glucose and fatty acids. Recently, it has been reported that protein hydrolysates exerted a marked effect on GLP-1 secretion [9-11]. The PYY-producing cells are mostly co-localized with the GLP-1-producing cells in the distal but not in the proximal gut [12, 13]. Although it has been shown that the release of PYY relies on nutrient ingestion [14-16], the stimulatory mechanism remains largely unknown.

Dietary proteins are considered to have the most satiating effect among macronutrients [17-19], and the mechanism by which proteins exert their satiating effect may include the stimulation of gut hormone signals (CCK, GLP-1, and PYY). However, data on the satiating efficacy with regard to the quality and source of protein are limited and inconsistent [20-22]. The purpose of the present study was to

elucidate the relationship between anorexic gut hormone responses and the satiating effect induced by various protein hydrolysates.

Materials and methods

Materials

Wheat gluten hydrolysate (HyPep 4601, total nitrogen 11%, amino nitrogen >2%, ash <20%, loss on drying <6%, average molecular weight 660, molecular weight distribution; 10.1% for >2000, 57.9% for 500-2000, 32.0% for <500), lactalbumin enzymatic hydrolysate (LAH, total nitrogen 12.9%, average molecular weight 240, molecular weight distribution; 2.1% for >2000, 33.3% for 500-2000, 64.5% for <500), potato protein hydrolysate (peptone from potatoes, PPH, total nitrogen 10.6%, amino nitrogen 5.3%, ash 22%, loss on drying 4.8%, average molecular weight 340 molecular weight distribution; 4.6% for >2000, 28.6% for 500-2000, 66.8% for <500), and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). The soybean protein hydrolysate (Hinute, SPH, average total nitrogen 13.8%, ash 5.5%, loss on drying 5.5%, molecular weight 2500, molecular weight distribution; 59.4% for >2000, 35.8% for 500-2000, 4.8% for <500) was kindly provided by Fuji oil Co., Ltd. (Osaka, Japan). The average molecular weight was determined by the trinitro-benzene-sulfonic acid (TNBS) method using glycine as the standard. The molecular weight distributions of the protein hydrolysates were determined with size-exclusion chromatography in an FPLC system (AKTA explorer 10S, GE Health Sciences, USA) using a Superdex Peptide 10/300 GL column (GE Health Sciences) [23]. Unless otherwise specified, the other materials were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Measurement of food intake after loading of protein hydrolysates in rats

Male Wistar/ST rats (7 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan) and were fed an AIN-93G-based semipurified diet [24]. The diet (3.86 kcal/g) consisted of 250 g/kg casein, 602.5 g/kg sucrose, 50 g/kg soybean oil, 50 g/kg cellulose, 35 g/kg mineral mixture (AIN-93G), 10 g/kg vitamin mixture (AIN-93G), and 2.5 g/kg choline bitartrate. All of the rats were housed individually in a temperature- and humidity-controlled room (22 ± 2°C, 55 ± 5%) under a 12:12-hr light-dark cycle (lights on during 20:30 – 08:30). Rats deprived of food for 12 hrs (20:00 – 08:00) were trained daily for intra-gastric administration using 1-2 ml of water through a feeding tube (Safeed Feeding tube Fr.5, 40 cm; Terumo, Tokyo, Japan) before receiving access to the diet (08:00). After acclimation for 7 days (until the daily food intake became stable), the rats (weighing approximately 250-300 g) received the test solutions through the feeding tube immediately before receiving access to the food. The study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals at Hokkaido University (Approval number: 08-0138).

The satiety effect of protein hydrolysates was evaluated with the crossover study design test (Figures 1, 2A, 2B and 3). Rats were given oral administrations of water (control) and protein hydrolysate in respective order everyday until they receive all of treatments. To examine the consistency of the cross over study results, single parallel administration study was also performed (Figure 2C). The allocation of animals to the experiments was randomized and treatments were performed in a randomized order. Test hydrolysates (WGH, LAH, SPH, or PPH) and intact wheat gluten (WG) were dissolved in deionized water and orally administered at a dose of 0.5-1.5 g/kg BW as indicated in each figure legend. During the experiments, water (6

or 12 ml/kg BW in experiments) was orally administered as a control treatment. The food consumption was measured at 1, 2, 3, 6, and 12 hrs after the administration of the oral solution.

Measurement of plasma gut hormones

The rats were fasted for 12 hrs and were each orally gavaged with WGH, LAH (1.0 g/kg BW), or the control (6 ml/kg BW water) solution through the feeding tube. Afterwards, the rats were respectively allowed to feed for 1, 2, 3, or 6 hrs in experiments. After the designated refeeding time (1, 2, 3, or 6 hrs), portal blood samples (2 ml) were drawn into a syringe containing EDTA (final concentration at 1 mg/ml), aprotinin (final concentration at 0.6 TIU/ml) and DPP-IV inhibitor (final concentration at 50 μM, Millipore, Billerica, MA) under anesthesia with pentobarbital sodium (*i.p.* 50 mg/kg BW, Somnopentyl, Kyoritsu Seiyaku Corporation, Tokyo, Japan), and then euthanized by exsanguination. The plasma was separated from the whole blood and frozen at –80°C until hormone (CCK, GLP-1, PYY) measurements. Hormone measurements were conducted using commercial ELISA kits. In the CCK-33 enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals Inc., Burlingame, CA), the primary antiserum cross-reacts 100% with CCK-33 and 2.36% with non-sulfated CCK (26-33), but does not cross-react with gastrin. The coefficients of the intra- and inter-assay variation are 5-10% and <15%, respectively. The antiserum contained in the active GLP-1 EIA kit (Millipore, Billerica, MA) cross-reacts 100% with both GLP-1 (7-36 amide) and GLP-1 (7-37), and the intra- and inter-assay variation are 6-9% and <13%, respectively. The PYY EIA kit (Yanaihara Institute Inc., Shizuoka, Japan) shows 100% cross-reactivity with PYY (3-36) and 115% with PYY (1-36). The coefficients of intra- and inter-assay variation are 3.1-9.8% and 4.2-14.2%, respectively.

The mimicking gastrointestinal digestion of WGH and LAH by pepsin and pancreatin

WGH or LAH was treated with pepsin (from porcine gastric mucosa, Sigma) and pancreatin (from porcine pancreas, Sigma) for various periods of time, using a slightly modified protocol [25]. Briefly, WGH or LAH was dissolved in 0.02 N H_3PO_4 at a concentration of 50 g/l, and the pH was adjusted to 1.85 using 20 N H_3PO_4. Pepsin was added at 0.5% wt/substrate wt and incubated for 30 or 60 min with shaking at 37°C. The pH of the suspension was then adjusted to 8.2 using $Ca(OH)_2$, and pancreatin was added at 4% wt/substrate wt. The suspension was incubated for 1 or 2 hrs at 37°C, followed by boiling for 20 min to inactivate the enzymes. The suspension was neutralized using 20 N H_3PO_4 and $Ca(OH)_2$. Finally, the soluble fraction was centrifuged, filtered through a filter with 0.45 μm pores, and lyophilized.

Gut hormone secretion study in STC-1 and GLUTag cells

Two murine enteroendocrine cell lines, STC-1 cells (a gift from Dr. D. Hanahan, University of California, San Francisco, CA) [23], and GLUTag cells (a gift from Dr. D. J. Drucker, University of Toronto, Toronto, Canada) [9], were grown in a Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 500 μg /ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. The cells were routinely subcultured by trypsinization upon reaching 80–90% confluency. The cells were grown in 48-well culture plates at a density of 1.25×10^5 cells/well for 2–3 days until they reached 80–90% confluency.

For the CCK secretion study, STC-1 cells were washed three times with HEPES buffer to remove the culture medium and exposed to 100 μl of the buffer containing test hydrolysates (5-10 mg/ml WGH or LAH) for 60 min at 37°C. The

HEPES buffer (pH 7.4) was composed of 140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 0.1% BSA. After the incubation, the supernatants were collected from the 48-well culture plates and centrifuged at 800 × g for 5 min at 4°C to remove the dissociated cells. The supernatant sample was then frozen below -50°C until it was time for CCK concentration measurements, which were performed using the CCK EIA kit (Phoenix Pharmaceuticals Inc., Burlingame, CA). The primary antiserum cross-reacts 100% with the sulfated and non-sulfated CCK (26-33), CCK-33 (porcine), CCK (27-33), caerulein, gastrin-1 (human), and big gastrin-1 (human), and cross-reacts 12.8% with CCK (30-33). Although the primary antibody cross-reacts with gastrin, the EIA kit was used in the present study because gastrin is not expressed at a detectable level in STC-1 cells [26]. The coefficients of intra- and inter-assay variation are 5-10% and <15%, respectively.

For the GLP-1 secretion study, GLUTag cells cultured in 48-well plates were exposed to 80 µl of the test solutions (5 or 10 mg/ml of WGH, LAH, or their hydrolysates dissolved in HEPES buffer), as described above. The GLP-1 concentrations of the collected supernatants were measured using the GLP-1 EIA kit (Yanaihara Institute Inc., Shizuoka, Japan). The primary antiserum cross-reacts 100% with both GLP-1 (7-36) amide and GLP-1 (9-36) amide, and the cross-reactivity for GLP-1 (1-36) amide, GLP-1 (1-37) and GLP-1 (7-37) are 0.3%, <0.1%, and <0.1%, respectively. The coefficients of intra- and inter-assay variation for the rat plasma are 5.36-6.60% and 5.51-18.87%, respectively.

Statistical analysis

The results are presented as the mean ± SEM. In cross-over studies (Figures 1, 2A, 2B and 3), results are expressed as % relative values to the food intake of

individual rats under control treatment. Accordingly, there was no error bar in control treatment. Statistical significance was assessed by one-way ANOVA or two-way repeated measure ANOVA, as described in the figure legends. Significant differences among the mean values were determined using a Tukey-Kramer test. $P < 0.05$ was considered to be statistically significant.

Results

WGH had higher potency for the regulation of food intake in rats

The cumulative food intake was recorded at each respective time point (1, 2, 3, 6, and 12 hrs). Results are expressed as % relative values to the control treatment due to relatively large variances in absolute data. The accumulated food intake values after the control treatment were 2.24 ± 0.16 g at 1 hr, 3.54 ± 0.23 g at 2 hrs, 5.04 ± 0.35 g at 3 hrs, 10.20 ± 0.47 g at 6 hrs, and 18.20 ± 0.52 g at 12 hrs. When the rats were gavaged with the test hydrolysates, they consumed relatively lower amounts of food compared with the control rats (Figure 1). Food intake was notably reduced after an oral preload with WGH after 1 hr of re-feeding. At 2 hrs, all hydrolysate except LAH treatments significantly suppressed food intake; WGH treatment showed the highest reduction in food intake. The total energy intake, including the energy from the preload and diet at 12 hrs, were 70.26 ± 2.00, 62.77± 2.88, 69.38 ± 2.34, 67.76 ±1.79, and 67.54 ±2.78 kcal after control, WGH, LAH, SPH, and PPH treatments respectively ($P = 0.260$, by one-way ANOVA). Because WGH had larger effect than other test hydrolysates, it was adopted for the subsequent studies with LAH as an iso-nitrogenous comparison.

The satiety effect of WGH, LAH, and intact wheat gluten in rats

The dose responses of WGH and LAH on satiety were examined with a cross over design test (Figure 2A and 2B). The accumulated food intake values of the control treatment in cross-over experiment were 4.21 ± 0.24 g at 1 hr, 4.81 ± 0.22 g at 2 hrs, 6.50 ± 0.29 g at 3 hrs, 10.31 ± 0.29 g at 6 hrs, and 19.20 ± 0.33 g at 12 hrs. The result of a two-way repeated measure ANOVA analysis showed a treatment effect by WGH and LAH ($P < 0.0001$). Similarly, an oral preload of WGH at doses of 1.0 and 1.5 g/kg tended to reduce subsequent food intake until 12 hours (Figure 2A). The food intake after treatment with 0.5 g/kg WGH was significantly lower than the control treatment after 1 hr, but the effect disappeared after 2 hrs. A preload of LAH at 1 g/kg tended to reduce food intake only after 1-2 hrs, whereas a 1.5 g/kg dose showed a relatively larger effect (Figure 2B). The total energy intake at 12 hrs was 74.12 ± 1.29, 76.05 ± 1.40, 71.65 ± 1.15, and 73.78 ± 1.35 kcal after preload of the control, 0.5 g/kg WGH, 1.0 g/kg WGH, and 1.5 g/kg WGH, respectively ($P = 0.151$ by one-way ANOVA). The total energy intake at 12 hrs after LAH preload was 73.49 ± 1.75, 72.67 ± 1.09, and 74.96 ± 1.21 kcal after 0.5 g/kg, 1.0 g/kg, and 1.5 g/kg doses, respectively ($P = 0.654$ by one-way ANOVA).

To confirm the consistency of the cross over study result, the rats received a single administration of WGH or LAH (not as a cross-over study) to isolate the effects of repeated administrations of these hydrolysates on food intake. The accumulated food intake values of the control groupwere 3.38 ± 0.39 g at 1 hr, 4.81 ± 0.37 g at 2 hrs, 7.16 ± 0.39 g at 3 hrs, 11.43 ± 0.52 g at 6 hrs, and 20.34 ± 0.45 g at 12 hrs. The accumulated food intake following WGH treatment was significantly reduced for up to 3 hrs (Figure 2C). The food intake decreased significantly only at the first hour after the oral administration of LAH. The total energy intake at 12 hrs was 78.50 ±

1.75, 73.88 ± 3.44, and 82.93 ± 3.48 kcal after control, WGH, and LAH treatments, respectively (P = 0.130, by one-way ANOVA).

Because intact wheat gluten (WG) was insoluble in water, it was administered as a suspension in a volume of 12 ml/kg BW. The values of accumulated food intake were 1.86 ± 0.20 g at 1 hr, 3.17 ± 0.18 g at 2 hrs, 5.28 ± 0.27 g at 3 hrs, 8.91 ± 0.32 g at 6 hrs, and 18.36 ± 0.50 g at 12 hrs following the control treatment. When compared to WG, WGH showed a greater satiety effect (Figure 3). WGH suppressed the food intake from 1 hr to 6 hrs after the treatment, whereas WG only suppressed the food intake during the first hour. The total energy intake at 12 hrs was 70.86 ± 1.93, 68.13 ± 1.45, 70.13 ± 1.43 kcal after the control, WGH, WG treatments, respectively (P = 0.475, by one-way ANOVA).

Gut hormone secretion during food intake with or without protein hydrolysates treatment

The rats treated with WGH and LAH tended to have higher postprandial CCK levels compared to the control treatment at 1 hr after the feeding start (P = 0.040, by one-way ANOVA, Table 1). The plasma CCK level at 6 hrs was significantly higher in the WGH-treated rats compared to the control rats. The portal PYY level was significantly higher in the WGH-treated rats compared to the control-treated rats at 2 hrs and 3 hrs. The plasma GLP-1 level in the WGH-treated rats tended to be higher compared to the control-treated rats at 1 hr and 2 hrs (one-way ANOVA P values were 0.097 at 1 hr and 0.083 at 2 hrs).

The potency of WGH and LAH on gut hormone secretion in enteroendocrine cell lines

To examine the direct effect of WGH and LAH on intestinal enteroendocrine cells, STC-1 cells (as a proximal enteroendocrine cell model) and GLUTag cells (as a

distal enteroendocrine cell model) were exposed to either WGH or LAH solution. As shown in Figure 4A, WGH and LAH (5 and 10 mg/ml) similarly induced a dose-dependent increase in CCK secretion from STC-1 cells. In the GLUTag cells, WGH induced a dose-dependent increase in the GLP-1 concentration in the supernatant. However, GLP-1 release was similar for cells exposed to 5 and 10 mg/ml of LAH and 5 mg/ml of WGH (Figure 4B).

To examine the effect of gastrointestinal digestion on these protein hydrolysates-induced gut hormone secretion, WGH and LAH were treated with pepsin and pancreatin *in vitro* to mimic luminal digestion. Pepsin and pancreatin solution treated for 60 and 2 hrs without substrates had only minor effect on GLP-1 secretion (108.6 ± 5.7%). WGH treated with pepsin (30 or 60 min) and pancreatin (1 or 2 hrs) induced a much higher release of GLP-1 than untreated WGH. A longer treatment with the enzymes led to a higher secretion of GLP-1; the strongest conditions (pepsin for 60 min and pancreatin for 2 hrs) resulted in the highest level of GLP-1 secretion (Figure 5). In the case of LAH, although the treatment with pepsin (30 or 60 min) and pancreatin (1 hr) significantly enhanced its effect on GLP-1 secretion, the extent of enhancement was remarkably lower than that observed with WGH. Moreover, after further digestion of LAH by pancreatin for 2 hrs, the activity returned to the level observed for the untreated LAH.

Discussion

In the present study, we found that WGH, among various protein hydrolysates, had a potent and prolonged effect on suppressing food consumption in rats. Prolonged suppression of food consumption was accompanied with higher postprandial PYY levels in rats treated with a WGH preload. In enteroendocrine cell

311 models, WGH potently stimulated gut hormone secretions such as CCK and GLP-1,
312 and *in vitro* digestion of WGH with pepsin/pancreatin further enhanced its potency.
313 These results demonstrate that the dietary peptide WGH potently and specifically
314 stimulates PYY secretion in rats. The results of the *in vitro* study suggest that WGH
315 could directly stimulate distal enteroendocrine cells even after luminal digestion.

316 A variety of dietary proteins and their hydrolysates possess different
317 potencies for inducing satiety [17-22]. The present results also indicated that a variety
318 of protein hydrolysates differed in affecting satiety in rats, which is consistent with
319 previous reports on the effect of protein source on food intake [27-29]. The oral
320 preload of WGH showed a higher activity on suppressing food intake than the other
321 protein hydrolysates (Figure 1), WGH was further studied for gut hormone secretions
322 in comparison with LAH as an iso-nitrogenous comparison. Although statistical
323 significance was not detected for total energy intake, the reduced energy intake (7.49
324 kcal) with a preload of WGH was apparently larger than the energy provided from the
325 WGH preload (~ 1 kcal/head; 1 g/kg BW = 250 mg/250 g BW).

326 Hydrolysates used in the present study have various nitrogen contents and
327 other components. Because suppressive effects on food intake did not correlate with
328 nitrogen content of these hydrolysates, the differences in the effect would not simply
329 be attributed to the concentration of protein/peptide. Unidentified components might
330 partially be involved in the suppressive effects on food intake, however, results in
331 enteroendocrine cell models suggest that peptides in WGH potently and directly
332 stimulate gut hormone secretions.

333 The effect of WG on food intake suggests that an adequate hydrolysis is
334 critical to prolong the appetite-suppressing effect of gluten peptides (Figure 3). This
335 suggestion is supported by the *in vitro* experiment where the pepsin/pancreatin

digestion of WGH largely enhanced the potency of GLUTag cell activation (Figure 5). The significance of hydrolysis on protein/peptide-induced gut hormone secretion and/or satiety is not consistent in previous studies. A preload of casein and its hydrolysate in rats had a similar effect on meal consumption [30]. In the case of BSA [31] and soybean beta-conglycinin [7], peptic hydrolysis significantly enhanced the potency of CCK release in rats and rat intestinal mucosal cells, respectively. In contrast, a previous report demonstrated that intact proteins are potent to stimulate gut hormone secretion *in vitro* [32]. Such differences may come from different experimental conditions, in addition to different protein sources and their digestibility. In the present study, the higher tendencies of CCK and GLP-1 levels at 1 hr in the WGH- or LAH-preloaded rats (Table 1) imply that the enhanced CCK/GLP-1 secretion by the administration of WGH or LAH contributes to suppress food intake. Furthermore, luminal digestion of wheat gluten hydrolysate might potentiate its appetite-suppressive effect through the stimulation to distal PYY-producing cells. It is well recognized that CCK-producing cells mainly locate in the proximal, and GLP-1/PYY-coproducing cells mainly locate in the distal gut. However, recent immunohistochemical studies [12, 13] have reveled that GLP-1-producing cells also exist in the proximal region without coproducing PYY. The time-related and non-parallel responses of CCK/GLP-1/PYY release after WGH preloading seem congruent with such distribution of enteroendocrine cells. The increase of PYY levels in the WGH-treated group may explain why WGH demonstrated a longer effect on suppressing food intake than LAH. Lower tendency of GLP-1 levels at 2 hrs in the control and LAH groups compared to other time points seemed to reflect biphasic patterns of postprandial GLP-1 secretion [33, 34] due to nutrient delivery in the intestinal lumen and direct/indirect stimulation on GLP-1-producing cells [9, 35].

The suppressive effect of WGH and LAH on food intake seems to be associated with their direct stimulating effect on enteroendocrine cells. STC-1 cells and GLUTag cells produce not only CCK or GLP-1, but also multiple gut hormones [36], as well as native enteroendocrine cells [12, 13]. Although a previous study used STC-1 cells as PYY-producing cells [37], we and other group [36] have not detected PYY in both cell lines. STC-1 cells were derived from murine duodenum and GLUTag cells derived from murine colon. Thus, it maybe no more appropriate to use these cell lines as a CCK-specific or a GLP-1-specific enteroendocrine cell model. When we consider STC-1 cells as a proximal and GLUTag cells as a distal enteroendicrince cell model, WGH appears to be more potent to stimulate distal enteroendocrine cells compared to LAH. This may partly explain the potent effect of WGH on PYY secretion *in vivo*.

In GLUTag cells, WGH induced GLP-1 secretion in a dose-dependent manner, while LAH caused similar and relatively weak GLP-1 secretion with different doses (Figure 4B). The average molecular size of LAH (~200 Da) is smaller than WGH (~600 Da), and FPLC analyses determined that LAH contains a large portion (~50%) of free amino acids; WGH contains only approximately 3% free amino acids. The higher potency of WGH to stimulate GLUTag cells to secret the hormone may be explained by the molecular size of the peptides. Although GLUTag cells are not recognized as PYY-producing cells, the result suggests that WGH directly activated distal enteroendocrine cells including GLP-1/PYY-coproducing cells with a higher potency than LAH. These results provide further data on the stimulation of PYY and GLP-1 secretion by peptones [9-15]. Furthermore, these findings suggest that the highly potent peptides or structures in WGH are liberated by luminal protease digestion after oral administration, which leads to the further release

386 of PYY/GLP-1 secretion from the enteroendocrine L cells. A single active peptide
387 that stimulates PYY/GLP-1 secretion has not been identified. Therefore, further
388 studies are needed to identify the active peptides derived from WGH.
389 There are several limitations in the present study. Dose-dependent effects
390 were not clearly observed after WGH administration (Fig. 2A). It is possible that
391 gastric emptying was immediately reduced by gut hormones secretions (CCK, GLP-1
392 etc.) induced by WGH partly flown in the intestine, so that further delivery of WGH
393 into the intestinal lumen was limited to enhance gut hormone secretions. Although
394 WGH repeatedly reduced food intake in the cross-over (Figures 1 and 3) and single
395 administration study (Figure 2C), the degree of reduction was unstable as observed in
396 Figure 2A. Specific reasons are unclear, but it is necessary to improve the
397 experimental condition for future studies. Furthermore, it will be necessary to
398 compare various WGH sources (not only reagent grade but also food grade) when
399 considering applications in humans for preventing overeating. An unexpected finding
400 was that the CCK concentration, but not the GLP-1 or PYY concentration was higher
401 compared to the control group at 6 hrs after WGH administration (Table 1). This
402 could be explained by the increased food intake after 3 hrs in WGH group (Figure
403 2C).
404 In summary, the orogastric preload of WGH suppressed food intake for a
405 longer period of time than the other protein hydrolysates. WGH had a higher potency
406 to elevate plasma PYY levels *in vivo* and to activate enteroendocrine models to
407 release gut hormones *in vitro*. These results suggest WGH is potent in activating
408 enteroendocrine cells to release satiety hormones leading to the prolonged suppression
409 of food intake.
410

Conflict of interest

No conflict of interest was declared.

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Table 1. The effect of orogastric preload of WGH or LAH on postprandial gut hormone levels in portal vein in rats (pM)

Time	Hormone	Control	WGH	LAH
1 hr	CCK	14.84 ± 2.89	23.50 ± 1.93	23.38 ± 2.51
	GLP-1	20.23 ± 4.28	31.17 ± 3.89	20.24 ± 2.25
	PYY	123.6 ± 27.7	149.4 ± 16.9	120.8 ± 8.7
2 hrs	CCK	13.39 ± 2.55	12.04 ± 2.63	18.21 ± 1.46
	GLP-1	10.08 ± 2.05	19.75 ± 4.40	11.45 ± 1.77
	PYY	131.9 ± 14.2 ^{b}	239.2 ± 31.5 ^{a}	143.4 ± 18.2 ^{b}
3 hrs	CCK	17.78 ± 1.66	18.72 ± 1.33	15.14 ± 1.53
	GLP-1	20.35 ± 2.93	16.84 ± 1.73	16.35 ± 1.58
	PYY	141.6 ± 7.9 ^{b}	193.8 ± 9.7 ^{a}	153.6 ± 15.9 ^{b}
6 hrs	CCK	18.86 ± 0.40 ^{b}	30.72 ± 2.40 ^{a}	21.48 ± 2.35 ^{b}
	GLP-1	30.06 ± 7.00	30.17 ± 9.37	10.86 ± 5.04
	PYY	120.2 ± 16.3	143.2 ± 14.4	134.1 ± 19.5

Portal blood was collected (1, 2, 3 or 6 hrs) after the oral administration of WGH, LAH (1.0 g/kg BW), or water (6 ml/kg BW, as a control); the results are expressed as the mean ± SEM (n = 5-7); superscripts in the same row not sharing the same letters differ significantly ($P < 0.05$ by Tukey-Kramer test).

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Figure 1. The effect of orogastric preload of various protein hydrolysates on food intake in re-fed rats.

The diet was given immediately after the oral administration of wheat gluten hydrolysate (WGH), lactalbumin enzymatic hydrolysate (LAH), soybean protein hydrolysate (SPH) or potato protein hydrolysate (PPH) at a dose of 1.0 g/kg BW. The administration of water (6 ml/kg BW) was the control. The accumulated food intake was measured at 1, 2, 3, 6, and 12 hrs after feeding. The food intake relative to the control (considered to be 100%) is presented. The results are expressed as the mean ± SEM (numbers of rats for water, WGH, LAH, SPH, and PPH treatments are 11, 8, 9, 11, and 10, respectively). The two-way repeated measure ANOVA *P* values are 0.0015, <0.0001, <0.0001 for treatment, time, and treatment × time, respectively. Bars not sharing the same letters differ significantly ($P < 0.05$ by Tukey-Kramer test) at the same time points.

Figure 2. Comparison of satiety effect between WGH and LAH in re-fed rats.

The accumulated food intake was measured after the oral administration of 0.5–1.5 g/kg BW WGH (A) or LAH (B). The food intake relative to the control (considered to be 100%) is presented. The results are expressed as the mean ± SEM (numbers of rats for water, 0.5 g/kg WGH, 1.0 g/kg WGH, 1.5 g/kg WGH, 0.5 g/kg LAH, 1.0 g/kg LAH, 1.5 g/kg LAH treatments are 21, 19, 18, 19, 17, 20, and 20, respectively). The respective two-way repeated measure ANOVA *P* values for WGH (A) are <0.0001, <0.0001, 0.0422 for treatment, time, and treatment × time; the values for LAH (B) are <0.0001, <0.0001, 0.0015 for treatment, time, and treatment × time. (C) The accumulated food intake was measured after a single oral administration of 1.0 g/kg BW WGH or LAH (not cross-over design). The results are expressed as the mean ±

SEM (numbers of rats for water, WGH, and LAH treatments are 8, 5, and 6, respectively). The two-way repeated measure ANOVA *P* values for accumulated food intake are <0.0001, 0.0026, 0.0977 for treatment, time, and treatment × time, respectively. Bars not sharing the same letters differ significantly ($P < 0.05$ by Tukey-Kramer test) at the same time points.

Figure 3. The effect of orogastric preload of WGH or intact wheat gluten on food intake in re-fed rats.

The accumulated food intake was measured after the oral administration of 1.0 g/kg BW WGH or WG (intact wheat gluten). The food intake relative to the control (considered to be 100%) is presented. The results are expressed as the mean ± SEM (numbers of rats for water, WGH, and WG treatments are 20, 19, and 18, respectively). The two-way repeated measure ANOVA P values are <0.0001, 0.0019, 0.1344 for treatment, time, and treatment × time, respectively. Bars not sharing the same letters differ significantly ($P < 0.05$ by Tukey-Kramer test) at the same time points.

Figure 4. CCK and GLP-1 secretion in response to WGH or LAH in enteroendocrine cells.

(A) The CCK levels were measured in the supernatants of STC-1 cells after exposure to the test hydrolysates (WGH or LAH) at 5-10 mg/ml for 1 hr. (B) The GLP-1 levels were measured in the supernatants of GLUTag cells after exposure to the test hydrolysates (WGH or LAH) at 5-10 mg/ml for 1 hr. The level relative to the blank (considered to be 100%) is presented. The results are expressed as the mean ± SEM of

three to seven wells. Bars not sharing the same letters differ significantly ($P < 0.05$ by Tukey-Kramer test).

Figure 5. The effects of *in vitro* digestion of WGH or LAH on GLP-1 secretion in GLUTag cells.

WGH or LAH was treated with pepsin and pancreatin for various time periods indicated below the X-axis. The GLUTag cells were exposed to the digested hydrolysates at 10 mg/ml for 1 hr, and GLP-1 levels relative to the blank (considered to be 100%) are presented. The results are expressed as the mean ± SEM of three to four wells. Bars not sharing the same letters differ significantly ($P < 0.05$ by Tukey-Kramer test).

Figure 1

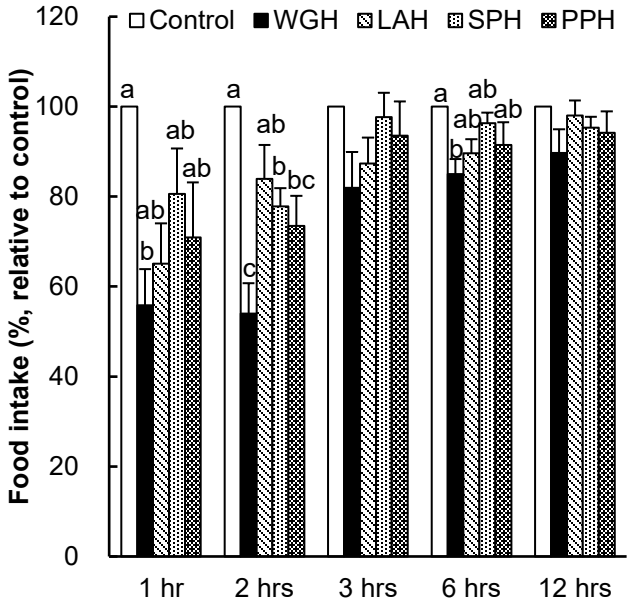

Control
WGH
LAH
SPH
PPH
Food intake (%, relative to control)
120
100
80
60
40
20
0
a
ab
b
ab
ab
a
ab
c
b
bc
a
ab
b
ab
ab
1 hr
2 hrs
3 hrs
6 hrs
12 hrs

Figure 2A

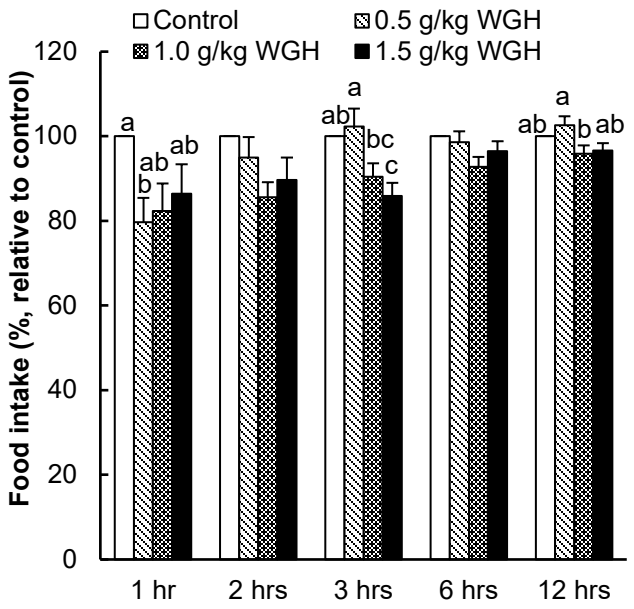

Control
0.5 g/kg WGH
1.0 g/kg WGH
1.5 g/kg WGH
Food intake (%, relative to control)
120
100
80
60
40
20
0
a
ab
b
ab
ab
a
bc
c
ab
a
b
ab
1 hr
2 hrs
3 hrs
6 hrs
12 hrs

Figure 2B

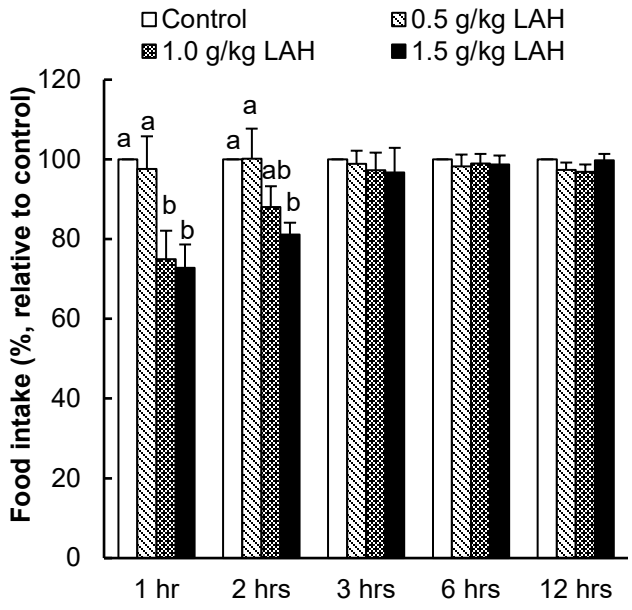

Control
0.5 g/kg LAH
1.0 g/kg LAH
1.5 g/kg LAH
Food intake (%, relative to control)
120
100
80
60
40
20
0
a
a
b
b
a
a
ab
b
1 hr
2 hrs
3 hrs
6 hrs
12 hrs

Figure 2C

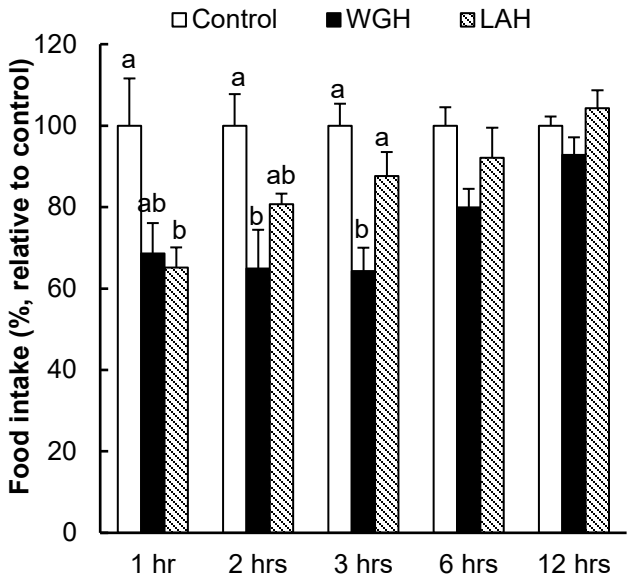

Control
WGH
LAH
Food intake (%, relative to control)
120
100
80
60
40
20
0
a
ab
b
a
b
ab
a
b
a
1 hr
2 hrs
3 hrs
6 hrs
12 hrs

Figure 3

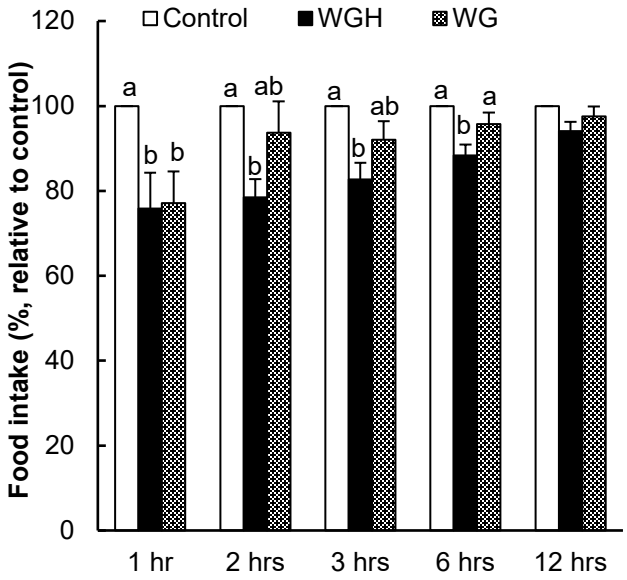

Control
WGH
WG
Food intake (%, relative to control)
120
100
80
60
40
20
0
a
b
b
a
b
ab
a
b
ab
a
b
a
1 hr
2 hrs
3 hrs
6 hrs
12 hrs

Figure 4A

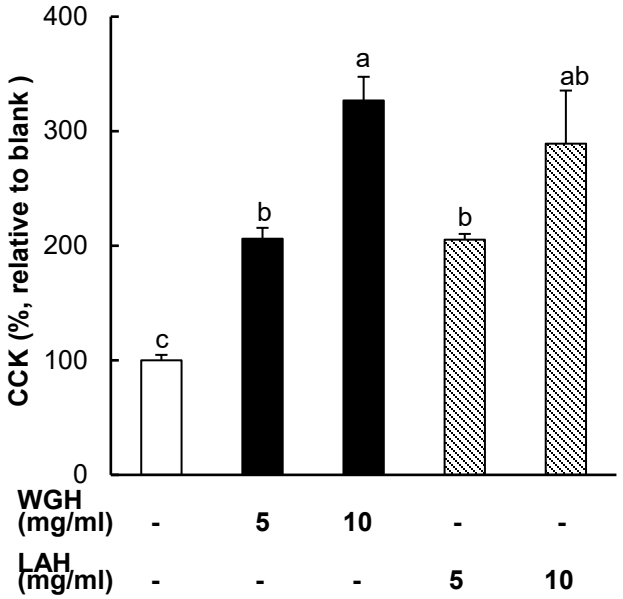

400
300
200
100
0
CCK (%, relative to blank)
c
b
a
b
ab
WGH (mg/ml) - 5 10 - -
LAH (mg/ml) - - - 5 10

Figure 4B

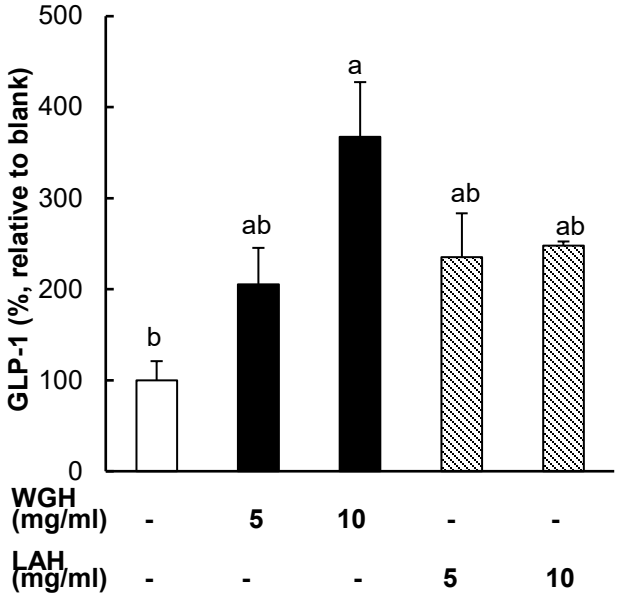
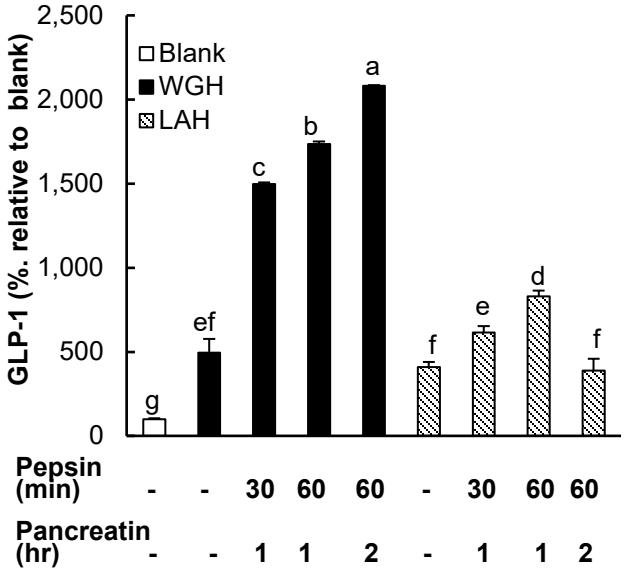

500
400
300
200
100
0
GLP-1 (%, relative to blank)
b
ab
a
ab
ab
WGH (mg/ml) - 5 10 - -
LAH (mg/ml) - - - 5 10

Figure 5


GLP-1 (%. relative to blank)
2,500
2,000
1,500
1,000
500
0
Blank
WGH
LAH
g
ef
c
b
a
f
e
d
f
Pepsin (min) - - 30 60 60 - 30 60 60
Pancreatin (hr) - - 1 1 2 - 1 1 2