



# HOKKAIDO UNIVERSITY

Title	Rice protein hydrolysates stimulate GLP-1 secretion, reduce GLP-1 degradation, and lower the glycemic response in rats
Author(s)	Ishikawa, Yuki; Hira, Tohru; Inoue, Daisuke et al.
Citation	Food & Function, 6(8), 2525-2534 <a href="https://doi.org/10.1039/C4F001054J">https://doi.org/10.1039/C4F001054J</a>
Issue Date	2015
Doc URL	<a href="https://hdl.handle.net/2115/85373">https://hdl.handle.net/2115/85373</a>
Type	journal article
File Information	Food Funct 6_2525.pdf



1 **Title**

2 Rice protein hydrolysates stimulate GLP-1 secretion, reduce GLP-1 degradation, and lower  
3 the glycemic response in rats.

4 **Authors**

5 Yuki Ishikawa<sup>1</sup>, Tohru Hira<sup>2</sup>, Daisuke Inoue<sup>1</sup>, Yukikazu Harada<sup>3</sup>, Hiroyuki Hashimoto<sup>3</sup>, Mikio  
6 Fujii<sup>4</sup>, Motoni Kadowaki<sup>5</sup>, Hiroshi Hara<sup>2</sup>

7 **Affiliations**

8 1. Research Faculty of Agriculture, Hokkaido University, Sapporo, JAPAN

9 2. Graduate School of Agriculture, Hokkaido University, Sapporo, JAPAN

10 3. Basic Research Department , Tsuno Food Industrial Co., Ltd.

11 4. Rice Research Center, Kameda Seika Co., Ltd.

12 5. Graduate School of Science and Technology; Center for Transdisciplinary Research,  
13 Niigata University, Niigata, JAPAN

14

15 **Corresponding author**

16 Tohru Hira

17 Laboratory of Nutritional Biochemistry, Research Faculty of Agriculture, Hokkaido University,  
18 Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

19 Tel & Fax: +81-11-706-2811

20 Email: [hira@chem.agr.hokudai.ac.jp](mailto:hira@chem.agr.hokudai.ac.jp)

21

22 The word count for the entire manuscript (abstract through references): 6520

23 The number of figures: 8

24 The number of tables: 0

25 Online Supporting Materials: 0

26

27 **Abstract**

28 Rice has historically been consumed in Asia as a major source of carbohydrates,  
29 however, little is known regarding the functional roles of rice protein as a dietary factor. In  
30 the present study, we investigated whether peptides derived from rice proteins could  
31 stimulate GLP-1 secretion, and that results in reducing glycemia via the incretin effect in  
32 normal rats. Hydrolysates were prepared from the protein fraction of rice endosperm or rice  
33 bran, and the effects of these hydrolysates on GLP-1 secretion were examined in a murine  
34 enteroendocrine cell line GLUTag. Plasma was collected after oral administration of the rice  
35 protein hydrolysates, under anesthesia, or during glucose tolerance tests in rats. In  
36 anesthetized rats, plasma dipeptidyl peptidase-IV (DPP-IV) activity was measured after  
37 ileal administration of the rice protein hydrolysates.

38 GLP-1 secretion from GLUTag cells was potently stimulated by the rice protein  
39 hydrolysates, especially by the peptic digest of rice endosperm protein (REPH) and that of  
40 rice bran protein (RBPH). Oral administration of REPH or RBPH elevated plasma GLP-1  
41 concentrations, which resulted in the reduction of glycemia under the intraperitoneal  
42 glucose tolerance test. In addition, the plasma DPP-IV activity was attenuated after ileal  
43 administration of REPH or RBPH, which resulted in a higher ratio of intact (active) GLP-1 to  
44 total GLP-1 in the plasma.

45 These results demonstrate that rice proteins exert potent stimulatory effects on GLP-1  
46 secretion, which could contribute to the reduction of postprandial glycemia. The inhibitory  
47 effect of these peptides on the plasma DPP-IV activity may potentiate the incretin effect of  
48 GLP-1.

49

50 **INTRODUCTION**

51 Rice is a staple food throughout Asia, including Japan, and is recognized as a major  
52 source of carbohydrates (energy). However, its consumption in Japan has recently

53 decreased, from 350 g/day in the 1950s to 150 g/day in the 2000s<sup>1</sup>. This decrease is likely  
54 due to the increased consumption of alternative staple foods, such as breads and noodles.  
55 In addition, as excessive consumption of digestible starch can cause glucose intolerance  
56 (which leads to diabetes mellitus), there is a recent trend in Japan towards reduced  
57 consumption of boiled white rice.

58 The incidence of metabolic syndrome is increasing throughout the world, including in the  
59 Asian countries. In Japan, increasing consumption of a Western diet is thought to be the  
60 main cause of the increasing rates of obesity, glucose intolerance, diabetes, dyslipidemia,  
61 and cardiovascular diseases. A positive association between the consumption of white rice  
62 and the risk of type-2 diabetes has been reported<sup>2,3</sup>, although this concept remains  
63 controversial<sup>4</sup>. However, as described above, there were less diabetic patients in 1950-60's  
64 when people consumed much more rice than today. Therefore, it is possible that the  
65 consumption of rice has a beneficial effect on glucose and/or lipid metabolism.

66 Regarding the functional factors present in rice, resistant starch<sup>5</sup> and gamma-oryzanol<sup>6</sup>  
67 reportedly improve glucose homeostasis. Several studies have also demonstrated that  
68 dietary rice protein effectively reduces serum cholesterol levels<sup>7-11</sup>, through the increased  
69 excretion of bile acids or neutral sterols. The protein content in rice is 5–7% by weight, and  
70 this protein consists of glutelin (80%), globulin (4–15%), albumin (1–5%), and prolamin (2–  
71 8%). A recent study showed that dietary rice protein can ameliorate the progression of early  
72 stage diabetic nephropathy in a rat model of diabetes<sup>12</sup>. Although these studies have  
73 investigated the effects of continuous ingestion of rice proteins on lipid metabolism and  
74 kidney function, the acute effects of rice proteins on glucose metabolism are not well  
75 understood.

76 Glucagon-like peptide-1 (GLP-1) is a gut hormone that is produced and released from  
77 enteroendocrine L-cells, which are scattered throughout the intestinal epithelium. The  
78 primary function of GLP-1 is to enhance glucose-induced insulin secretion (the incretin

79 effect), which helps to attenuate postprandial hyperglycemia. GLP-1 also has proliferative  
80 and protective effects on pancreatic beta cells, as well as multiple effects outside the  
81 pancreas including in the liver, adipose tissue, cardiovascular, and central nervous  
82 systems<sup>13</sup>. Recently, GLP-1 receptor agonists and dipeptidyl peptidase-IV (DPP-IV)  
83 inhibitors have been used to treat type 2 diabetes<sup>14,15</sup>. As GLP-1 and glucose-dependent  
84 insulinotropic polypeptide (another incretin) are immediately inactivated by plasma DPP-IV,  
85 inhibitors of DPP-IV can help preserve the physiological activity of both these peptides<sup>16</sup>.

86 Recent studies have revealed that, along with glucose and fatty acids, some of dietary  
87 proteins and peptides have potent stimulatory effects on the secretion of GLP-1. We have  
88 previously reported that a single oral administration of a corn protein zein hydrolysate  
89 (ZeinH) effectively lowered rats' glycemic response by stimulating GLP-1 secretion,  
90 although this results was not observed with meat hydrolysate<sup>17,18</sup>. Whey protein and  
91 glutamine can also induce GLP-1 secretion in animals and humans, although not all dietary  
92 proteins/peptides induce a similar "incretinotropic" effect<sup>18,19</sup>.

93 We conducted the present study to investigate the ability of rice protein hydrolysates to  
94 stimulate GLP-1 secretion *in vitro* and *in vivo*. The hydrolysates were prepared using rice  
95 proteins extracted from the bran and endosperm, and were orally administered respectively  
96 to rats. We also evaluated the effect of the hydrolysates on the glycemic response, under  
97 oral and intraperitoneal glucose loads. Finally, we examined the effect of luminal  
98 administration of the rice protein hydrolysates on plasma DPP-IV activity.

99

## 100 **Materials and Methods**

### 101 **Materials**

102 Cell culture consumables (Dulbecco's modified Eagle's medium, fetal bovine serum,  
103 penicillin-streptomycin, and trypsin-EDTA solution) were purchased from Invitrogen  
104 (Carlsbad, CA, US). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis,

105 MO, US). Other reagents were purchased from Wako (Osaka, Japan), unless specified.

106

### 107 **Preparation of rice protein hydrolysates**

108 Purified rice endosperm protein (REP) was prepared using a previously reported alkali  
109 extraction method<sup>20</sup>. Briefly, milled rice flour was mixed with a 0.2% NaOH solution at room  
110 temperature for 1 h to extract the protein fraction, and the mixture was then centrifuged.  
111 The supernatant was neutralized with 1 M HCl to precipitate the protein fraction; the  
112 collected protein fraction was repeatedly washed with distilled water, and then lyophilized  
113 as REP. The purity of the REP was >90%, as determined using the Kjeldahl method.

114 Purified rice bran protein (RBP) was prepared from commercially available RBP powder  
115 (TSUNO-RBP, TSUNO CO., LTD). The RBP powder was suspended in water, the pH of the  
116 suspension was adjusted to 5.7 using H<sub>2</sub>SO<sub>4</sub>, and the suspension was then stirred for 1 h at  
117 95°C. The precipitated protein was collected by centrifugation, washed with deionized  
118 water and further centrifugation. Lyophilized protein fraction was used as RBP, and its  
119 protein purity was 80%, determined using the Kjeldahl method.

120 Papain digests of REP and RBP were prepared as previously described<sup>19</sup>. In short, 50 g  
121 of rice protein was suspended in deionized water (500 mL), and the pH of the suspension  
122 was adjusted to 7.2 using NaOH. After 250 mg of papain (papain F, Asahi Food and Health  
123 Care, Tokyo, Japan) was added, the suspension was shaken for 60 min at 55°C. The  
124 suspension was then treated in boiling water for 20 min to stop the enzymatic reaction.  
125 After filtration with a 0.45- $\mu$ m filter, the pH was adjusted to 7.0, and the solution was  
126 lyophilized.

127 Pepsin digests of REP and RBP were prepared as follows<sup>21-24</sup>: 50 g of rice protein was  
128 suspended in 0.02N phosphoric acid (1,000 mL), and the pH of the solution was adjusted to  
129 1.85 with 20N phosphoric acid. After the addition of 250 mg pepsin (from porcine gastric  
130 mucosa, Sigma, St. Louis, MO), the suspension was shaken for 30 min or 60 min at 37°C,

131 and then treated in boiling water for 20 min to stop the enzymatic reaction. The pH of the  
132 suspension was adjusted to 7.0 with Ca(OH)<sub>2</sub>. After centrifugation at 3,300 × g, the  
133 supernatant was collected and frozen overnight at –30°C. When the supernatant was  
134 thawed the following day, it was centrifuged again, subjected to filtration (using a 0.45 µm  
135 filter) to remove the calcium phosphate, and finally lyophilized.

136 Papain was chosen because papain-treated Zein (ZeinH) had potent GLP-1 secretory  
137 activity *in vitro* and *in vivo*<sup>19</sup>, and pepsin was chosen to partially mimic gastric digestion.  
138 The degree of hydrolysis (DH) of rice protein hydrolysates was determined by using the  
139 trinitrobenzenesulfonic method<sup>25,26</sup>. The values of DH were as follows: 6.3% for REP  
140 papain digest (60 min), 7.3% for REP pepsin digest (30 min), 8.2% for REP pepsin digest  
141 (60 min), 5.3% for RBP papain digest (60 min), 9.8% for RBP pepsin digest (30 min), and  
142 10.3% for RBP pepsin digest (60 min).

143 Rice protein hydrolysates were analyzed using HPLC system (Waters 2695 system,  
144 Waters, Milford, MA) and an octadecyl silica column (CAPCELL PAK C18 MGII, 5 µm, 4.6  
145 mm x 250 mm, Shiseido Co., Ltd., Tokyo, Japan) with a linear gradient of acetonitrile/water  
146 from 5 to 40% v/v for 45 min, from 40 to 100% v/v for following 20 min (from 45 to 65 min),  
147 and then 100% v/v for 5 min.

148

## 149 **Cell culture**

150 The GLP-1 producing murine cell line GLUTag (a gift from Dr. D. J. Drucker, University of  
151 Toronto, Canada) was cultured in Dulbecco's modified Eagle's medium (Invitrogen, cat. no.  
152 12100-038), which was supplemented with 10% fetal bovine serum, 100 U/mL penicillin,  
153 and 100 µg/mL streptomycin. Cultures were grown in a humidified 5% CO<sub>2</sub> atmosphere at  
154 37°C, and cells were routinely subcultured using trypsinization upon reaching 80–90%  
155 confluency.

156

## 157 **Animals**

158 Male Sprague Dawley (SD) rats (6 weeks old, 150–170 g) were purchased from Japan  
159 SLC (Hamamatsu, Japan). All animals were housed in individual cages, had free access to  
160 water, and received a semipurified AIN-93G diet<sup>27</sup> containing 25% casein. All animal  
161 experiments were performed after an acclimation period (4–7 days) in a  
162 temperature-controlled room that was maintained at  $23 \pm 2^\circ\text{C}$  with a 12-hour light, 12-hour  
163 dark cycle (light period, 8:00 AM to 8:00 PM).

164 This study was approved by the Hokkaido University Animal Committee, and the animals  
165 were maintained in accordance with the Guide for the Care and Use of Laboratory Animals  
166 of Hokkaido University.

167

## 168 **Cell Culture Experiment (Experiment 1)**

### 169 Experiment : GLP-1 Secretion by GLUTag Cells

170 GLUTag cells were grown for 2 days in 48-well culture plates at a density of  $1.25 \times 10^5$   
171 cells/well, until they reached 80–90% confluency. Cells were washed twice with HEPES  
172 buffer (140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 10 mM  
173 D-glucose, 1 mg/mL BSA, pH 7.4) to remove the culture media, and then exposed to the  
174 test agents (60 min at  $37^\circ\text{C}$ ), which were dissolved in the same buffer. In this experiment,  
175 70 mM potassium chloride (KCl) was used as a positive control<sup>19</sup>. Supernatants were  
176 collected from the wells after the exposure, and centrifuged at  $800 \times g$  for 5 min at  $4^\circ\text{C}$  to  
177 remove the remaining cells, then stored at  $-50^\circ\text{C}$  until the GLP-1 concentration was  
178 measured with a commercial enzyme immunoassay kit (Yanaihara Institute, Shizuoka,  
179 Japan). The GLP-1 assay detects intact GLP-1 (7-36) and metabolite GLP-1 (9-36) with  
180 100% and 95.6% cross-reactivity, respectively. Cross-reactivities for GLP-1 (1-37), GLP-1  
181 (1-36) amide, and GLP-1 (7-37) are  $<0.1\%$ ,  $0.3\%$ , and  $<0.1\%$ , respectively. The minimum  
182 detection limit for the assay is 60 pM for GLP-1 (7-36) amide. The intra- and interassay

183 coefficients for this kit in rat plasma are 5.36–6.60% and 5.51–18.87%, respectively.

184

#### 185 **Animal Experiments (Experiments 2-4)**

186 To measure plasma glucose, GLP-1, and insulin, blood samples were collected into a 1.5  
187 mL tube containing aprotinin (final concentration, 500 kIU/mL), heparin (final concentration,  
188 50 IU/mL), and a DPP-IV inhibitor (final concentration, 50  $\mu$ M; DPP4-010; Millipore Co,  
189 Billerica, MA). Plasma was separated by centrifugation at  $2,300 \times g$  for 10 min at 4°C, and  
190 then frozen at –80°C until testing. Plasma glucose concentrations were measured using the  
191 Glucose CII test kit (Wako), insulin was measured using an ELISA kit (AKRIN-010T;  
192 Shibayagi Co, Ltd, Gunma, Japan), and active GLP-1 and total GLP-1 were measured  
193 using the respective ELISA kits (EGLP-35K and EZGLP1T-36K; Millipore Co, Billerica, MA).

194

#### 195 Experiment 2: The effects of oral REPH or RBPH on plasma GLP-1 in rats

196 REPH, RBPH (both, 2 g/kg body weight), or water (12 mL/kg body weight) was orally  
197 administrated to rats by using a feeding tube (5 Fr; Atom Medical Co, Tokyo, Japan). A  
198 blood sample (1 mL) was collected from the portal vein (under isoflurane anesthesia) 15  
199 min after the oral administration. As untreated control, portal blood was collected from rats  
200 without oral administration. The concentration of total GLP-1 in the plasma was measured  
201 as described above.

202

#### 203 Experiment 3: The effects of oral REPH or RBPH on plasma glucose, GLP-1, insulin, and 204 gastric emptying under the oral glucose tolerance test

205 To examine the effects of oral REPH or RBPH on the glycemic response to oral glucose  
206 load, the oral glucose tolerance test (OGTT) was performed in conscious rats. After basal  
207 blood collection (0 min), glucose solution (2 g/kg body weight) or the solution containing  
208 REPH (2 g/kg body weight) or RBPH (2 g/kg body weight) was orally administered (10

209 mL/kg body weight) by using a feeding tube. These test solutions contained acetaminophen  
210 (100 mg/kg body weight) to assess the gastric emptying rate<sup>28,29</sup>. Blood samples (120 µL)  
211 were collected from the tail vein 15, 30, 60, 90, and 120 min after the oral administration,  
212 and the plasma glucose, insulin, and total GLP-1 levels were measured as described above.  
213 The plasma acetaminophen concentration was measured using an acetaminophen  
214 detection kit (01601-96; Kanto Kagaku, Tokyo, Japan).

215

216 Experiment 4: The effects of oral REPH or RBPH on plasma glucose, GLP-1, and insulin  
217 under the intraperitoneal glucose tolerance test

218 The intraperitoneal glucose tolerance test (IPGTT) was used to evaluate the GLP-1–  
219 mediated glycemic control induced by oral REPH or RBPH. After an overnight fast, blood  
220 samples were collected from the tail vein before and after intraperitoneal glucose injection  
221 (–15, 0, 15, 30, 60, 90, and 120 min), and the plasma levels of glucose, insulin, and total  
222 GLP-1 were measured as described above.

223 To examine the dose-response effect for REPH, it was dissolved in deionized water (1 or  
224 2 g/kg body weight), and was administered orally (12 mL/kg body weight) using a feeding  
225 tube. Deionized water was used as a negative control. Fifteen minutes after oral  
226 administration, a blood sample was collected, and then the glucose solution was injected  
227 intraperitoneally (1 g/kg body weight, 0 min).

228 In a separated experiment, the glucose solution was injected intraperitoneally (1 g/kg  
229 body weight, 0 min) immediately after oral administration of REPH (2 g/kg body weight),  
230 RBPH (2 g/kg body weight), or deionized water (12 mL/kg body weight). Blood samples  
231 were then collected as described above.

232

233 Experiment 5: The effects of ileal administration of REPH or RBPH on plasma DPP-IV  
234 activity in the ileal vein of anesthetized rats (in situ experiment)

235 After an overnight fast, rats were anesthetized with sodium pentobarbital (50 mg/kg body  
236 weight, Somnopentyl Injection; Kyoritsu Seiyaku Co, Tokyo, Japan), and a midline  
237 abdominal incision was made. Body temperature was maintained with heating pads during  
238 the experiment. The small tip (6–7 mm) of a polyethylene catheter (SP 10; ID 0.28 mm, OD  
239 0.61 mm; Natsume Seisakusyo, Tokyo, Japan) connected to a silicone catheter (Silascon  
240 no. 00, ID 0.5 mm, OD 1.0 mm; Dow Corning Co.) was inserted into the ileal vein of the  
241 mesenteric tissue, and the ileal lumen was washed with saline. The ligated ileal loop (length,  
242 30 cm) was prepared between 5 and 35 cm proximal to the cecum, and the proximal and  
243 distal ends of the loop were ligated with a silk thread. A basal blood sample (0 min, 50  $\mu$ L)  
244 was drawn from the ileal vein catheter, and the catheter was refilled with sterilized saline  
245 containing heparin (50 IU/mL; Ajinomoto, Tokyo, Japan) between each sampling. Two  
246 milliliters of saline, REPH (250 mg/mL), or RBPH (250 mg/mL) was then administered  
247 directly into the loop. Blood samples (50  $\mu$ L) were collected through the ileal vein catheter  
248 at 15, 30, 45, and 60 min after the ileal administration to measure the plasma activity of  
249 DPP-IV. For active GLP-1 and total GLP-1 measurement, ileal vein blood samples were  
250 collected 60 min after the ileal administration. Blood samples for DPP-IV activity  
251 measurement were drawn into a syringe containing heparin sodium salt (final concentration,  
252 50 IU/mL; Nacalai Tesque, Kyoto, Japan), and samples for active or total GLP-1  
253 measurement were drawn into a syringe containing aprotinin (final concentration, 500  
254 kIU/mL), heparin (final concentration, 50 IU/mL), and DPP-IV inhibitor (final concentration,  
255 50  $\mu$ M). The plasma was separated by centrifugation at  $2,300 \times g$  (10 min at 4°C), and then  
256 frozen until testing.

257 DPP-IV activity was determined based on the rate of hydrolysis of a surrogate substrate  
258 (Gly-Pro-*p*-nitroaniline, Gly-Pro-pNA, Sigma), as previously described<sup>30,31</sup>. Five microliters  
259 of plasma were added to each well of a flat-bottomed 96-well plate, and then 80  $\mu$ L of assay  
260 buffer (0.25 M Tris-HCl buffer, pH 8.0) was added. The reaction was started by adding 80

261  $\mu\text{L}$  of 1.6 mM Gly-Pro-pNA in deionized water. After an incubation at 37°C for 60 min, 40  $\mu\text{L}$   
262 of 1 M acetate (pH 4.2) was added to stop the reaction, and the absorbance at 405 nm was  
263 measured (“absorbance A”) using a microplate reader (infinite 200; Tecan Group Ltd.,  
264 Männedorf, Switzerland). To correct for the influence of hemolysis, a negative control was  
265 also prepared for each plasma sample, and the absorbance at 405 nm was measured  
266 (“absorbance B”) when the plasma added to the assay buffer containing substrate and  
267 acetate after the 60-min incubation. The plasma activity of DPP-IV was defined as the  
268 liberation of pNA from Gly-Pro-pNA, and was calculated by subtracting “absorbance B”  
269 from “absorbance A”. One unit of DPP-IV activity was defined as the liberation of 1  $\mu\text{mol}$  of  
270 pNA in 1 min.

271

## 272 **Statistical analysis**

273 All results were expressed as mean  $\pm$  SEM. Statistical significance was assessed using  
274 one-way or two-way ANOVA to assess the main effects (treatment and time), as well as the  
275 interaction effect (treatment  $\times$  time). The significance of differences between the mean  
276 values was evaluated using Tukey-Kramer’s test, Dunnett’s test, or Student's t-test, as  
277 appropriate and described in figure legends.

278

279

280

281

282

283

284

## 285 **Results**

286 GLUTag cells were exposed to various protein hydrolysates dissolved in HEPES buffer,  
287 and each rice protein hydrolysate was observed to stimulate GLP-1 secretion with varying  
288 potency (Fig. 1A). Among the REP hydrolysates, the papain digest (Papain 60 min) and  
289 pepsin 30 min digest (Pepsin 30 min) had similar potency, although the 60 min pepsin  
290 digest (Pepsin 60 min) had a lesser effect. Among the RBP hydrolysates, the pepsin  
291 digests had higher potency compared to the papain digest. In addition, the 30 min pepsin  
292 digests of REP and RBP induced a relatively higher GLP-1 secretion compared to the  
293 positive control peptide (zein hydrolysate) (Fig. 1B)<sup>19</sup>.

294 As the 30 min pepsin digests of REP and RBP potently stimulated GLP-1 secretion from  
295 GLUTag cells, and their yield was higher than that of papain digestion, we selected the 30  
296 min pepsin digestion for REP and RBP in the subsequent experiments. Each hydrolysate  
297 was designated as REPH and RBPH, respectively.

298 To examine the effect of the rice protein hydrolysates on plasma GLP-1 levels *in vivo*, we  
299 collected portal blood 15 min after oral administration of REPH or RBPH (2 g/kg body  
300 weight). The plasma GLP-1 concentration in REPH-treated rats was significantly higher  
301 than that in control (water-treated) rats (Fig. 3A), and it was also significantly higher than  
302 that in untreated (no oral administration) rats. In a separated experiment, the portal GLP-1  
303 concentration in RBPH-treated rats was significantly higher compared to that in control  
304 (water-treated) rats (Fig. 3B).

305 We next examined whether REPH- or RBPH-stimulated secretion of GLP-1 affected  
306 glycemia via the incretin effect (the main function of GLP-1) under the oral glucose  
307 tolerance test (OGTT). Oral gavage with REPH resulted in attenuation of the glycemic  
308 response to oral glucose load at 15 min (0.5–2.0 g/kg) and 30 min (1.0–2.0 g/kg) (Fig. 4A).  
309 However, no significant difference in the area under the curve (AUC) was observed for  
310 each group (data not shown). The plasma GLP-1 concentration only increased in the 2.0

311 g/kg REPH group, and that was significantly higher from 30 min to 90 min compared to that  
312 in the control group (Fig. 4B). However, the plasma insulin concentration at 15 min was  
313 suppressed by REPH in a dose-dependent manner (Fig. 4C). The plasma insulin  
314 concentration in the 2.0 g/kg group was lowest at 15 min, although this concentration was  
315 higher than the concentrations in the control group at 60 and 90 min. The acetaminophen  
316 test is used to assess the gastric emptying rate, as acetaminophen is absorbed rapidly in  
317 the intestine after emptied from the stomach<sup>28,29</sup>. The change in plasma acetaminophen  
318 concentration was lower in the 1.0 g/kg REPH group (at 15 min and 30 min) and in the 2.0  
319 g/kg REPH group (from 15 min to 90 min) (Fig. 4D), which indicated that gastric emptying  
320 was delayed by oral REPH.

321 The glycemic response during oral glucose load showed a dose-dependent effect, and it  
322 was significantly suppressed by oral RBPH gavage at 15 min (1.0–2.0 g/kg) and 30 min  
323 (0.5–2.0 g/kg) (Fig. 5A), compared with the control treatment. However, the AUC did not  
324 differ for each group (data not shown). The plasma GLP-1 concentration was significantly  
325 increased in the 2.0 g/kg RBPH group (from 30 min to 60 min) compared to the control  
326 treatment. The insulin response at 15 min tended to be reduced by oral RBPH, although  
327 this result was not statistically significant. The changes in plasma acetaminophen levels  
328 were reduced in a dose-dependent manner by RBPH as well as REPH.

329 Although increased GLP-1 and reduced glycemic responses were observed treatment  
330 with oral REPH and RBPH, the involvement of GLP-1 (the incretin effect) was unclear in  
331 OGTT, due to the delayed gastric emptying response (Fig. 4 and 5). Therefore, we  
332 conducted IPGTT to eliminate the possible effect of delayed gastric emptying and luminal  
333 glucose-induced GLP-1 secretion on the glycemic response, as well as to evaluate the  
334 effect of oral REPH or RBPH on GLP-1 secretion (Fig. 6 and 7).

335 Under IPGTT, 2.0 g/kg oral REPH resulted in the attenuation of the glycemia at 15 min  
336 and 30 min (Fig. 6A), although the AUC did not differ for each group (data not shown). The

337 plasma GLP-1 concentrations were increased in a dose-dependent manner by REPH. In  
338 addition, 2.0 g/kg REPH induced continuous elevation of plasma GLP-1 from 15 to 60 min,  
339 and these levels were significantly higher than the levels in the control group (Fig. 6B). Oral  
340 REPH (1.0 g/kg) also resulted in the elevation of plasma GLP-1 at 0 min and 30 min (Fig.  
341 6B). Plasma insulin concentrations tended to be increased by 2.0 g/kg REPH (Fig. 6C).

342 We also conducted IPGTT to examine the effect of oral RBPH on GLP-1 secretion and  
343 glycemic control. In this experiment, RBPH or REPH was orally administered immediately  
344 before the intraperitoneal injection of glucose (0 min), as RBPH failed to reduce the  
345 glycemic response in the preliminary study when it was provided at other time points (e.g.,  
346 15 min or 30 min before the intraperitoneal injection of glucose).

347 Oral administration of RBPH or REPH resulted in the attenuation of the glycemic  
348 response at 30 min, and the potency was similar for both hydrolysates (Fig. 7A). Plasma  
349 GLP-1 concentrations were increased by both hydrolysates in a similar manner, which  
350 lasted until 60 min and significantly higher than control treatment (Fig. 7B). Plasma insulin  
351 concentrations were also significantly increased by RBPH and REPH at 15 min (Fig. 7C).

352 GLP-1 is rapidly inactivated by DPP-IV in the plasma<sup>32</sup>, and a recent study has  
353 demonstrated that peptides derived from rice bran can inhibit DPP-IV activity<sup>33</sup>. Therefore,  
354 we investigated whether luminal REPH or RBPH affected the plasma activity of DPP-IV in  
355 the ileal vein, and thereby prolonged the activity of intact plasma GLP-1. The plasma  
356 activity of DPP-IV in the ileal vein decreased gradually after ileal administration of REPH or  
357 RBPH (Fig. 8A). In the REPH group, the plasma activity of DPP-IV at 30 min was  
358 significantly lower than that in the control group. In the RBPH group, the DPP-IV activity at  
359 45 min was significantly lower than the basal (0 min) activity. We also measured the active  
360 and total GLP-1 concentrations in the ileal vein plasma 60 min after the ileal administration  
361 of REPH or RBPH. Although the total GLP-1 concentration was only significantly elevated  
362 in the RBPH group (Fig. 8B), the concentrations of active GLP-1 were significantly elevated

363 in the REPH and RBPH groups, compared with the control group (Fig. 8C). Because the  
364 incremental degrees in GLP-1 levels for the REPH and RBPH groups (compared to the  
365 control group) were larger for active GLP-1 (~3×) than for total GLP-1 (2–2.5×), we  
366 calculated the ratio of active GLP-1 to total GLP-1 (Fig. 8D). The ratios in the REPH group  
367 (51.4%) and in the RBPH group (54.3%) were approximately 2× higher than that in control  
368 group (28.4%), and this difference was statistically significant.

369

370

## 371 **Discussion**

372 In this study, we investigated whether rice protein-derived dietary peptides could  
373 stimulate GLP-1 secretion both in an enteroendocrine cell line and in rats, and whether this  
374 effect might contribute to the improvement of glycemia. Rice protein hydrolysates (including  
375 REPH and RBPH) potently stimulated GLP-1 secretion from GLUTag cells, and oral  
376 administration of REPH or RBPH resulted in significant elevation of GLP-1 concentrations  
377 in the portal and tail vein plasma. The incretin effect by oral REPH or RBPH was clearly  
378 observed under IPGTT, as well as a continuous elevation of plasma GLP-1. In addition, the  
379 plasma DPP-IV activity was decreased by the ileal administration of REPH or RBPH in  
380 anesthetized rats. This observation was also supported by the higher ratio of intact (active)  
381 GLP-1 to total GLP-1. These results revealed that peptides derived from rice proteins can  
382 stimulate secretion of GLP-1 *in vivo*, which effectively lowers the glycemic response. In  
383 addition, the inhibitory effect of these peptides on the plasma DPP-IV activity may  
384 potentiate the incretin effect of secreted GLP-1.

385 GLUTag cells are widely used as an enteroendocrine cell model to investigate the effect  
386 of various molecules on the secretion of GLP-1, including glucose<sup>34</sup>, fatty acids<sup>35</sup>,  
387 peptides<sup>19</sup> and amino acids<sup>36</sup>. We observed that hydrolysates prepared from REP and RBP,  
388 particularly the 30 min pepsin digests (REPH and RBPH), potently stimulated GLP-1  
389 secretion in GLUTag cells (Fig. 1). Intriguingly, the potency of GLP-1 secretion varied by

390 enzyme (pepsin or papain) and the digestion length (30 min or 60 min) despite the same  
391 origin of rice proteins (REP or RBP). For RBP, the peptide sequence, which is cleaved  
392 preferably by papain, might contribute to the triggering of GLP-1 secretion. The weaker  
393 GLP-1 secretion produced by the 60 min digests in REP hydrolysates (compared to the 30  
394 min digest) suggests that oligo- or larger peptides, rather than small peptides or free amino  
395 acids, might be responsible for this effect. HPLC analysis (Fig. 2) demonstrated both REPH  
396 and RBPH contain so many peptide fragments, which may be due to partial digestion by  
397 relatively short time (30 min) treatment with pepsin. REPH mainly contains hydrophobic  
398 peptides but RBPH contains both hydrophilic and hydrophobic peptides. The specific  
399 structures of the active peptides should be clarified in future studies.

400 Oral administration of REPH or RBPH induced a significant increase in GLP-1  
401 concentration in the portal (Fig. 3) and tail veins (Fig. 4-7) with or without oral glucose, as  
402 demonstrated in experiments 2-4. Under OGTT, both REPH (Fig. 4) and RBPH (Fig. 5)  
403 lowered glycemic response accompanied with decreasing tendency of insulin response.  
404 The glucose-lowering effect under OGTT is likely due to the decrease of glucose absorption  
405 in the small intestine, rather than insulin-dependent glucose uptake into the peripheral  
406 tissues. This interpretation is supported by the ability of REPH and RBPH to suppress  
407 gastric emptying assessed by the acetaminophen test (Fig. 4D and 5D). As GLP-1 is known  
408 to suppress gastric emptying as well as other gut hormones (e.g., cholecystokinin,  
409 serotonin, peptide-YY)<sup>37</sup>, increased GLP-1 secretion might also be involved in the  
410 glucose-lowering effect of REPH and RBPH.

411 IPGTT (Fig. 6-7) is an appropriate method to evaluate the GLP-1 secretion and incretin  
412 effects of orally administered factors, as it avoids various confounding factors, such as  
413 glucose outflow from the stomach, glucose absorption in the small intestine, and luminal  
414 glucose-induced GLP-1 secretion. In contrast to OGTT, oral administration of REPH or  
415 RBPH enhanced insulin response under IPGTT (Fig. 6C and 7C), which is likely due to the

416 increased GLP-1 secretion (Fig. 6B and 7B). Because REPH or RBPH was administered in  
417 the absence of oral (luminal) glucose, the increased GLP-1 secretion should be induced by  
418 luminal rice peptides. In our previous<sup>18</sup> and preliminary study, oral administration of  
419 hydrolysates prepared from meat, soy protein or wheat gluten was not effective to reduce  
420 glycemic response under IPGTT. Thus, the present results clarified that rice peptides can  
421 potently induce GLP-1 secretion, thereby lowering the glycemic response through the  
422 incretin effect.

423 In contrast to REPH (Fig. 6), oral RBPH 15 min before the intraperitoneal glucose  
424 injection failed to attenuate the glycemic response (data not shown), which suggests that  
425 there exists an optimal time for respective dietary peptides to exert their GLP-1-mediated  
426 glucose-lowering effect. This optimal time is likely dependent on the digestibility and  
427 stability of the peptides in the stomach and small intestine. Rice endosperm protein  
428 contains mainly glutelin (an alkaline soluble protein) and prolamin (an alcohol soluble  
429 protein), while rice bran protein contains mainly globulin (a salt soluble protein)<sup>38-41</sup>. These  
430 differences in the protein component of REP and RBP might affect their properties *in vivo*,  
431 although further studies are needed to test this hypothesis.

432 Although we observed that REPH and RBPH reduced the glycemic response in the early  
433 period, the plasma glucose concentrations in the REPH and RBPH groups were slightly  
434 increased in the late period compared to the control group (90 and 120 min). A previous  
435 study has reported that exendin-4 (a GLP-1 receptor agonist) increased plasma glucose  
436 concentrations via the sympathetic nervous system<sup>42</sup>. Similar pathways affected by REPH  
437 or RBPH might be related to the late increase in plasma glucose.

438 DPP-IV is one of serine proteases, and is known to inactivate GLP-1, GLP-2, and  
439 glucose-dependent insulinotropic polypeptide<sup>43</sup>. Recently, oral inhibitors of plasma DPP-IV  
440 have been used to treat type 2 diabetes mellitus. In addition, several studies have reported  
441 that the activity of DPP-IV is reduced by various dietary peptides, including corn zein

442 hydrolysate<sup>17</sup>, whey hydrolysate<sup>44</sup>, casein-derived peptides<sup>45</sup>, and rice bran peptides<sup>33</sup>,  
443 possibly acting as competitive inhibitors. We observed that luminal administration of rice  
444 protein hydrolysates attenuated the plasma DPP-IV activity in anesthetized rats (Fig. 8A).  
445 Because peptides produced from defatted rice bran have potent inhibitory effects on  
446 DPP-IV *in vitro*<sup>33</sup>, it was possible that such peptides were absorbed into the ileal vein, and  
447 inhibited the plasma DPP-IV activity.

448 Moreover, ileal administration of REPH and RBPH both stimulated GLP-1 secretion and  
449 increased the ratio of active GLP-1 to total GLP-1 (Fig. 8B-D), which might be attributed to  
450 the reduced plasma activity of DPP-IV. Dietary peptides, including REPH, RBPH, and corn  
451 zein hydrolysate<sup>17</sup>, may contribute to the reduced postprandial glycemia through their  
452 synergistic effects on GLP-1 secretion and degradation. Glucose and fatty acids are also  
453 known to induce GLP-1 secretion. However, these molecules would not affect the plasma  
454 activity of DPP-IV. The synergistic effects that we observed might be unique to dietary  
455 peptides.

456 Several dietary components, including peptides (whey, casein, zein) and amino acids  
457 (glutamine, ornithine, leucine), have been reported to induce GLP-1 secretion *in vitro* and *in*  
458 *vivo*<sup>19,46-49</sup>. However, limited studies have examined the effects of these peptides and  
459 amino acids on the insulin and glycemic responses together with GLP-1 response.  
460 Furthermore, the effects of ingested dietary factors on DPP-IV have also not been studied.  
461 The synergistic effects that we have suggested require further investigation, particularly  
462 regarding the identification of specific peptide sequences that are responsible for triggering  
463 GLP-1 secretion and reducing the plasma DPP-IV activity.

464 Rice is typically consumed in Asia as a source of carbohydrates rather than a source of  
465 protein, given its low protein content (5-7%). However, our results raise a novel hypothesis  
466 that rice proteins contribute to preventing postprandial hyperglycemia (which is caused by  
467 the easily digested rice starch). To test this hypothesis, future studies must evaluate

468 whether long-term ingestion of rice proteins or peptides improves glucose homeostasis. In  
469 addition, these studies should evaluate rice peptides as functional dietary factors that can  
470 help prevent obesity and glucose intolerance through their synergistic effects.

471 In conclusion, protein hydrolysates prepared from rice endosperm or rice bran potently  
472 stimulated GLP-1 secretion in an enteroendocrine cell model and in normal rats. Oral  
473 administration of the protein hydrolysates effectively reduced the glycemic response by  
474 increasing the levels of GLP-1 and insulin. In addition, luminal administration of these  
475 peptides attenuated the plasma DPP-IV activity, which was reflected by increased ratio of  
476 intact GLP-1 to total GLP-1. These results reveal a novel function of rice peptides as a  
477 dietary factor that can reduce postprandial glycemia through increase in the secretion of  
478 GLP-1 and decrease in plasma DPP-IV activity.

479

#### 480 **Acknowledgement**

481 The present study was supported by a grant for research “Development of fundamental  
482 technology for analysis and evaluation of functional agricultural products and functional  
483 foods” from the Ministry of Agriculture, Forestry and Fishery to M. K.

484

#### 485 **References**

- 486 1. H. Asakura, K. Suzuki, T. Kitahora and T. Morizane, *J Gastroenterol Hepatol*, 2008, **23**,  
487 1794-1801.
- 488 2.A. Nanri, T. Mizoue, M. Noda, Y. Takahashi, M. Kato, M. Inoue, S. Tsugane and J. P. H. C.-b. P.  
489 S. Group, *Am J Clin Nutr*, 2010, **92**, 1468-1477.
- 490 3. E. A. Hu, A. Pan, V. Malik and Q. Sun, *BMJ*, 2012, **344**, e1454.
- 491 4. F. Soriguer, N. Colomo, G. Oliveira, E. García-Fuentes, I. Esteva, M. S. Ruiz de Adana, S.  
492 Morcillo, N. Porrás, S. Valdés and G. Rojo-Martínez, *Clin Nutr*, 2013, **32**, 481-484.
- 493 5. D. L. Topping, M. Fukushima and A. R. Bird, *Proc Nutr Soc*, 2003, **62**, 171-176.

- 494 6. C. Kozuka, K. Yabiku, C. Takayama, M. Matsushita and M. Shimabukuro, *Obes Res Clin Pract*,  
495 2013, **7**, e165-172.
- 496 7. T. Morita, A. Oh-hashii, K. Takei, M. Ikai, S. Kasaoka and S. Kiriyaama, *J Nutr*, 1997, **127**,  
497 470-477.
- 498 8. W. Ni, Y. Tsuda, S. Takashima, H. Sato, M. Sato and K. Imaizumi, *Br J Nutr*, 2003, **90**, 13-20.
- 499 9. L. Yang, T. Kumagai, H. Kawamura, T. Watanabe, M. Kubota, S. Fujimura, R. Watanabe and M.  
500 Kadowaki, *Biosci Biotechnol Biochem*, 2007, **71**, 694-703.
- 501 10. L. Yang and M. Kadowaki, *Ann Nutr Metab*, 2009, **54**, 283-290.
- 502 11. LT. Tong, Y. Fujimoto, N. Shimizu, M. Tsukino, T. Akasaka, Y. Kato, W. Iwamoto, S. Shiratake,  
503 K. Imaizumi, and M. Sato, *Food Chem*, 2012, **132**, 194-200.
- 504 12. M. Kubota, R. Watanabe, H. Kabasawa, N. Iino, A. Saito, T. Kumagai, S. Fujimura and M.  
505 Kadowaki, *Br J Nutr*, 2013, **110**, 1211-1219.
- 506 13. Y. O. Kim and D. Schuppan, *Am J Physiol Gastrointest Liver Physiol*, 2012, **302**, G759-761.
- 507 14. D. J. Drucker, A. Dritselis and P. Kirkpatrick, *Nat Rev Drug Discov*, 2010, **9**, 267-268.
- 508 15. S. Madsbad, U. Kielgast, M. Asmar, C. F. Deacon, S. S. Torekov and J. J. Holst, *Diabetes*  
509 *Obes Metab*, 2011, **13**, 394-407.
- 510 16. Y. M. Cho, Y. Fujita and T. J. Kieffer, *Annu Rev Physiol*, 2014, **76**, 535-559.
- 511 17. T. Mochida, T. Hira and H. Hara, *Endocrinology*, 2010, **151**, 3095-3104.
- 512 18. N. Higuchi, T. Hira, N. Yamada and H. Hara, *Endocrinology*, 2013, **154**, 3089-3098.
- 513 19. T. Hira, T. Mochida, K. Miyashita and H. Hara, *Am J Physiol Gastrointest Liver Physiol*, 2009,  
514 **297**, G663-671.
- 515 20. T. Kumagai, R. Watanabe, M. Saito, T. Watanabe, M. Kubota and M. Kadowaki, *J Nutr Sci*  
516 *Vitaminol (Tokyo)*, 2009, **55**, 170-177.
- 517 21. S. F. Gauthier, C. Vachon, L. Savoie, *J Food Sci*, 1986, **51**, 960-964.
- 518 22. AOAC Official Method 971.09, in *Official Methods of Analysis of AOAC INTERNATIONAL*, ed.  
519 G. W. Latimer. Jr., AOAC International, Gaithersburg, 19th edn, 2012.

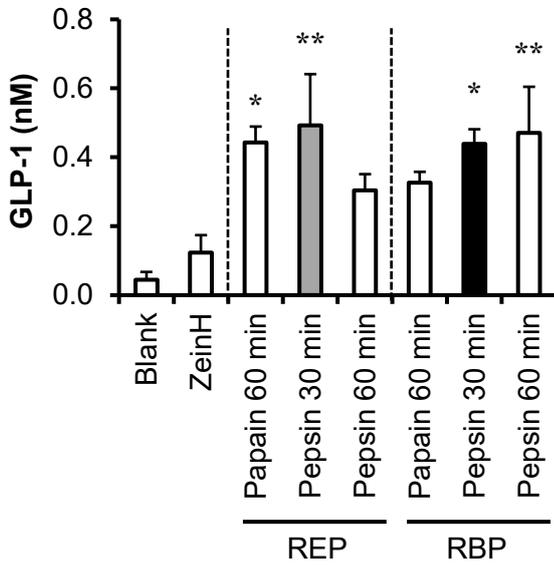
- 520 23. T. Hira, H. Hara, Y. Aoyama, *Biosci Biotechnol Biochem*, 1999, **63**, 1192-1196.
- 521 24. S. Nakajima, T. Hira, H. Hara, *Mol Nutr Food Res*, 2012, **56**, 753-760.
- 522 25. J. Adler-Nissen, *J Agric Food Chem*, 1979, **27**, 1256-1262.
- 523 26. S. M. Rutherfurd, *J AOAC Int*, 2010, **93**, 1515–1522.
- 524 27. P. G. Reeves, F. H. Nielsen and G. C. Fahey, *J Nutr*, 1993, **123**, 1939-1951.
- 525 28. R. C. Heading, J. Nimmo, L. F. Prescott and P. Tothill, *Br J Pharmacol*, 1973, **47**, 415-421.
- 526 29. A. Maida, J. A. Lovshin, L. L. Baggio and D. J. Drucker, *Endocrinology*, 2008, **149**, 5670-5678.
- 527 30. T. Karl, W. T. Chwalisz, D. Wedekind, H. J. Hedrich, T. Hoffmann, R. Jacobs, R. Pabst and S.  
528 von Hörsten, *Regul Pept*, 2003, **115**, 81-90.
- 529 31. G. Flock, L. L. Baggio, C. Longuet and D. J. Drucker, *Diabetes*, 2007, **56**, 3006-3013.
- 530 32. C. F. Deacon, T. E. Hughes and J. J. Holst, *Diabetes*, 1998, **47**, 764-769.
- 531 33. T. Hatanaka, Y. Inoue, J. Arima, Y. Kumagai, H. Usuki, K. Kawakami, M. Kimura and T.  
532 Mukaihara, *Food Chem*, 2012, **134**, 797-802.
- 533 34. F. Reimann and F. M. Gribble, *Diabetes*, 2002, **51**, 2757-2763.
- 534 35. P. L. Brubaker, J. Schloos and D. J. Drucker, *Endocrinology*, 1998, **139**, 4108-4114.
- 535 36. F. Reimann, L. Williams, G. da Silva Xavier, G. A. Rutter and F. M. Gribble, *Diabetologia*,  
536 2004, **47**, 1592-1601.
- 537 37. A. Wettergren, B. Schjoldager, P. E. Mortensen, J. Myhre, J. Christiansen and J. J. Holst, *Dig*  
538 *Dis Sci*, 1993, **38**, 665-673.
- 539 38. H. B. Krishnan and J. A. White, *Plant Physiol*, 1995, **109**, 1491-1495.
- 540 39. M. Wang, N. S. Hettiarachchy, M. Qi, W. Burks and T. Siebenmorgen, *J Agric Food Chem*,  
541 1999, **47**, 411-416.
- 542 40. N. Khan, T. Katsube-Tanaka, S. Iida, T. Yamaguchi, J. Nakano and H. Tsujimoto, *J Agric*  
543 *Food Chem*, 2008, **56**, 4955-4961.
- 544 41. C. Fabian and Y. H. Ju, *Crit Rev Food Sci Nutr*, 2011, **51**, 816-827.

- 545 42. D. Pérez-Tilve, L. González-Matías, B. A. Aulinger, M. Alvarez-Crespo, M. Gil-Lozano, E.  
546 Alvarez, A. M. Andrade-Olivie, M. H. Tschöp, D. A. D'Alessio and F. Mallo, *Am J Physiol*  
547 *Endocrinol Metab*, 2010, **298**, E1088-1096.
- 548 43. D. J. Drucker, *Cell Metab*, 2006, **3**, 153-165.
- 549 44. D. Jakubowicz and O. Froy, *J Nutr Biochem*, 2013, **24**, 1-5.
- 550 45. Uenishi H, Kabuki T, Seto Y, Serizawa A, and Nakajima H, *International Dairy Journal*, 2012,  
551 **22**(1), 24-30.
- 552 46. W. L. Hall, D. J. Millward, S. J. Long and L. M. Morgan, *Br J Nutr*, 2003, **89**, 239-248.
- 553 47. Q. Chen and R. A. Reimer, *Nutrition*, 2009, **25**, 340–349.
- 554 48. D. Samocha-Bonet, O. Wong, E. L. Synnott, N. Piyaratna, A. Douglas, F. M. Gribble, J. J.  
555 Holst, D. J. Chisholm and J. R. Greenfield, *J Nutr*, 2011, **141**, 1233-1238.
- 556 49. M. Oya, T. Kitaguchi, R. Pais, F. Reimann, F. Gribble and T. Tsuboi, *J Biol Chem*, 2013, **288**,  
557 4513-4521.
- 558
- 559

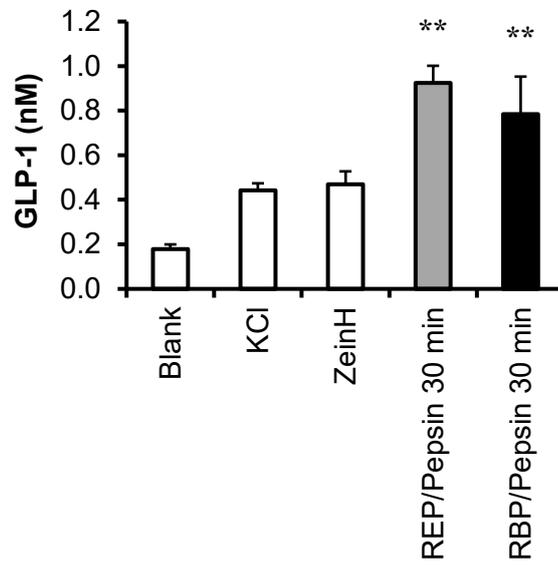
560 **Figures**

561

562 **A**



**B**



563

564

565 **Fig. 1. GLP-1 secretion from GLUTag cells by various protein hydrolysates**

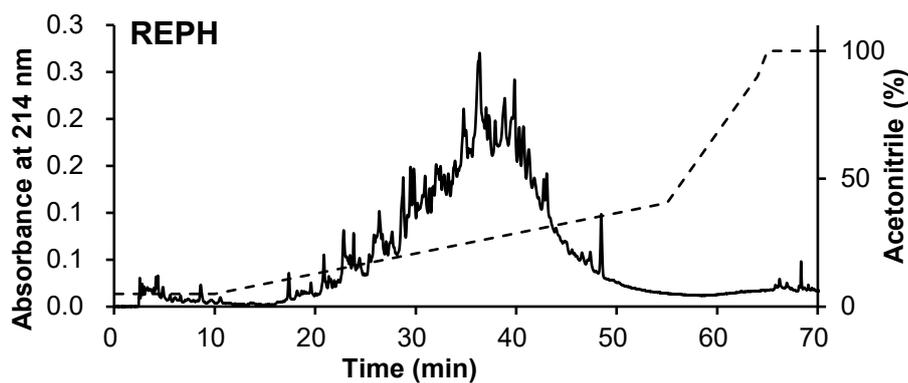
566 GLUTag cells were exposed to various protein hydrolysates (10 mg/mL), or 70 mM KCl  
567 for 60 min. The supernatants were collected and assayed using a GLP-1 enzyme  
568 immunoassay. REP, rice endosperm protein; RBP, rice bran protein; ZeinH, corn zein  
569 hydrolysate. Values are mean  $\pm$  SEM (n = 4). Asterisks (\*) indicate significant differences  
570 compared to the blank treatment (\*P < 0.05, \*\*P < 0.01; Dunnett's test).

571

572

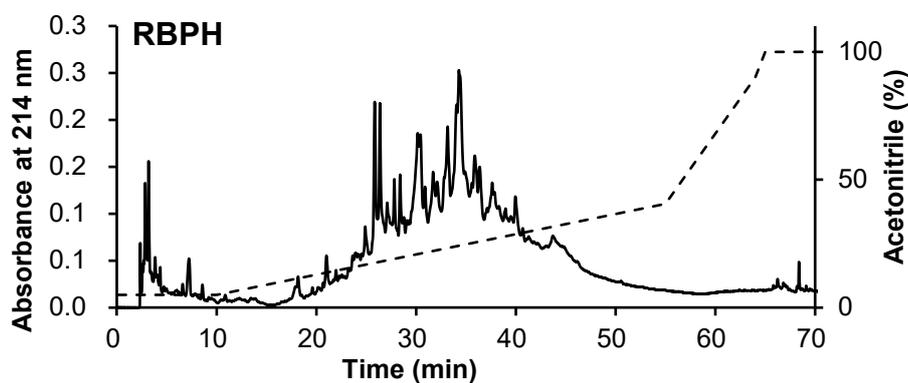
573

574

**A**

575

576

**B**

577

578

579 **Fig. 2. HPLC chromatograms of REPH and PBPH.**

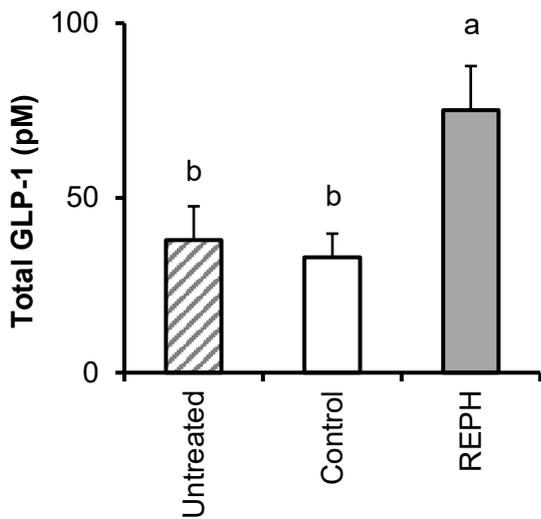
580 REEPH (A) and RBPH (B) were respectively subjected to reverse phase HPLC analysis  
581 using octadecyl silica column with a linear gradient of acetonitrile/water (broken line), and  
582 the optical absorbance at 214 nm (solid line) was monitored.

583

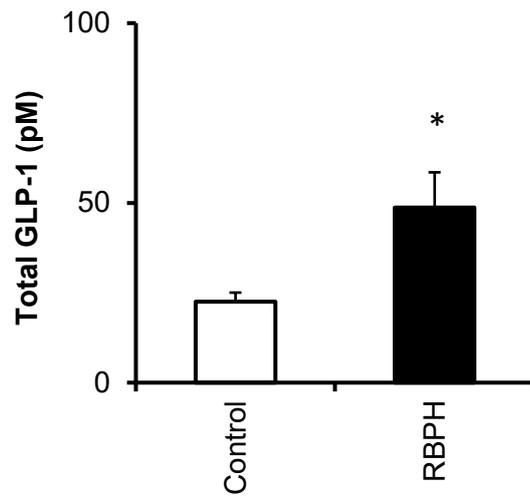
584

585

**A**



**B**



586

587 **Fig. 3. Total GLP-1 concentration in the portal vein 15 min after oral administration of**  
588 **REPH or RBPH**

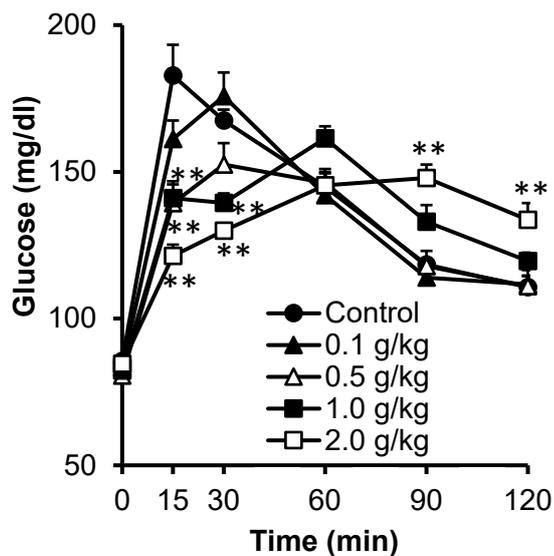
589 Blood samples were collected from portal vein after oral administration of REPH (2 g/kg),  
590 RBPH (2 g/kg), or water (control), and the plasma concentration of total GLP-1 was  
591 measured. As the untreated control, blood samples were collected from rats that did not  
592 receive any oral treatment. Values are mean  $\pm$  SEM (n = 4–6). **(A)** Bars that do not share  
593 the same letter are significantly different ( $P < 0.05$ ; Tukey–Kramer’s test). **(B)** The asterisk  
594 (\*) indicates a significant difference compared to the control treatment (\* $P < 0.05$ ; Student's  
595 t-test).

596

597

598

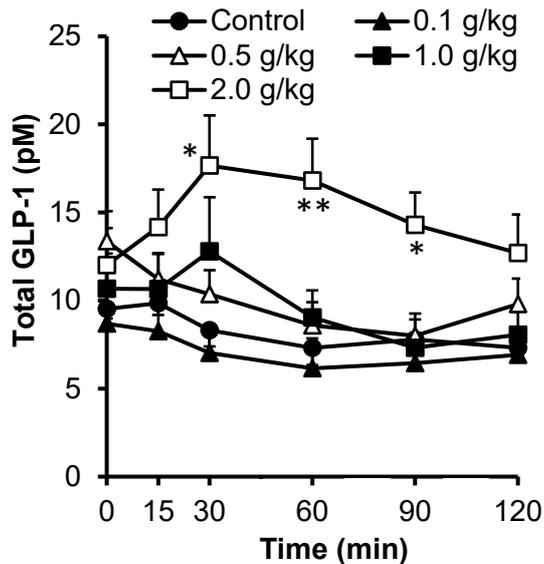
A



599

600

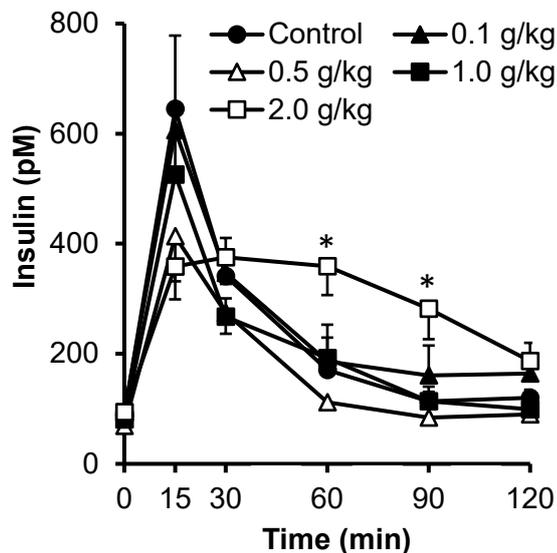
B



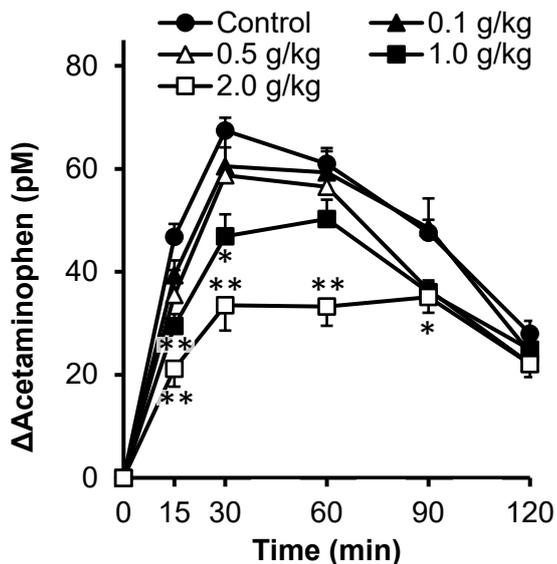
601

602

C



D



603 **Fig. 4. Changes in plasma glucose, total GLP-1, insulin, and acetaminophen after the**  
 604 **oral administration of REPH under the oral glucose tolerance test.**

605 Glucose solution as control (●, 2 g/kg, 10 mL/kg), or the solution containing REPH at 0.1  
 606 g/kg (▲), 0.5 g/kg (△), 1.0 g/kg (■), or 2.0 g/kg (□) was administered orally to rats after an  
 607 overnight fast. Acetaminophen (100 mg/kg) was added to each solution. Blood samples  
 608 were collected from the tail vein before (0 min) and after the oral administration, as  
 609 indicated. Plasma concentrations of glucose, total GLP-1, insulin, and acetaminophen were

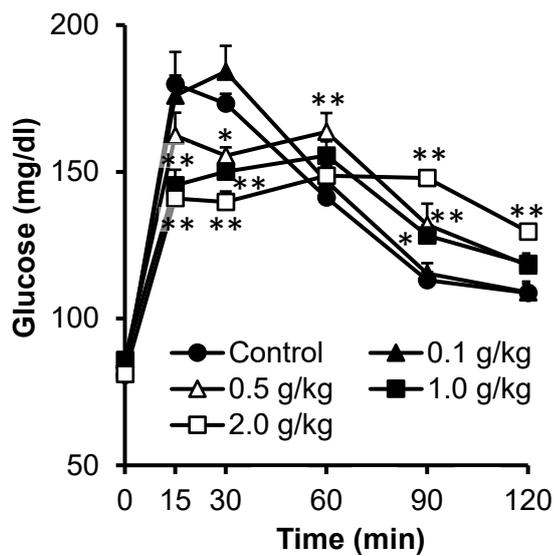
610 measured. The two-way ANOVA P-values were 0.137 (treatment), <0.01 (time), and <0.01  
611 (treatment × time) for glucose (**A**); <0.05 (treatment), <0.01 (time), and <0.01 (treatment ×  
612 time) for total GLP-1 (**B**); 0.133 (treatment), <0.01 (time), and <0.01 (treatment × time) for  
613 insulin (**C**); and <0.01 (treatment), <0.01 (time), and <0.01 (treatment × time) for  
614 acetaminophen (**D**). Values are mean ± SEM (n = 5–6). Asterisks (\*) indicate significant  
615 differences compared to the control treatment at the same time point (\*P < 0.05, \*\*P <  
616 0.01; Dunnett's test).

617

618

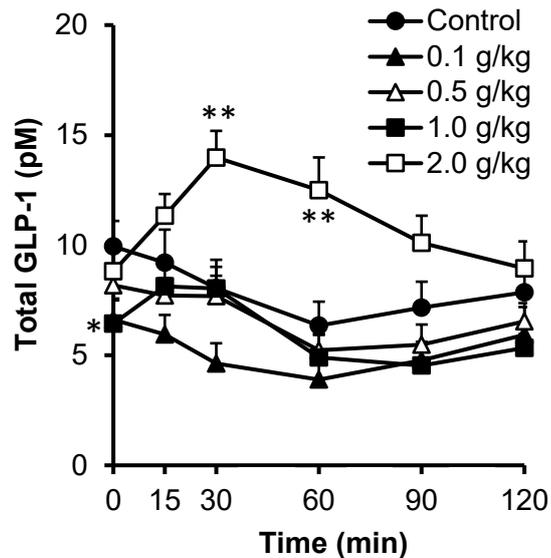
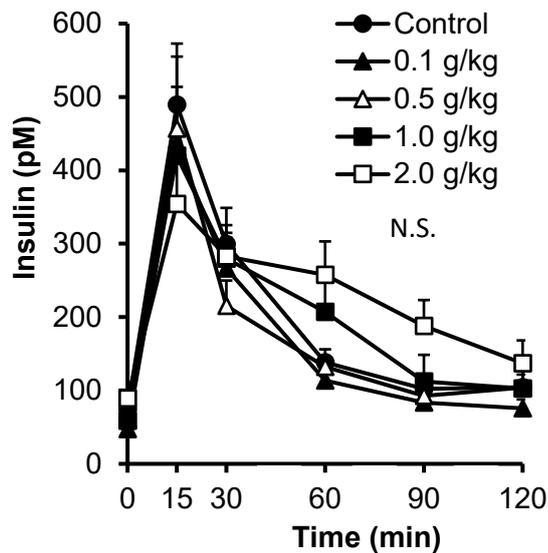
619

620

**A**

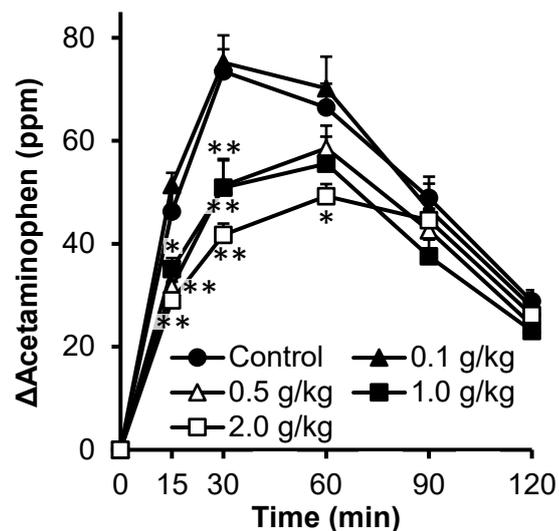
621

622

**B****C**

623

624

**D**

625 **Fig. 5. Changes in plasma glucose, total GLP-1, insulin, and acetaminophen after**  
 626 **oral administration of RBPH under the oral glucose tolerance test.**

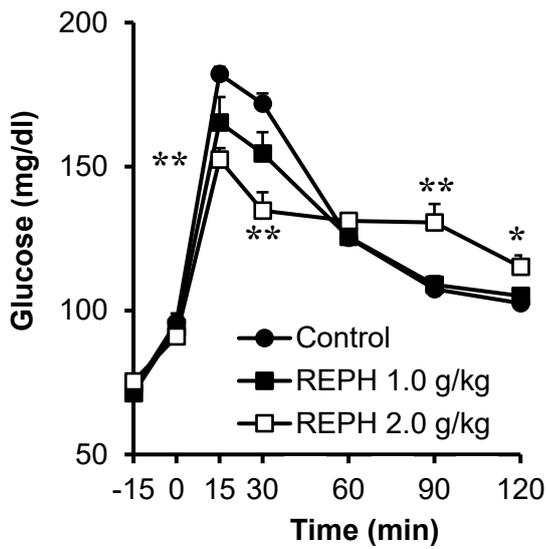
627 Glucose solution as control (●, 2 g/kg, 10 mL/kg), or the solution containing RBPH at 0.1  
 628 g/kg (▲), 0.5 g/kg (△), 1.0 g/kg (■), or 2.0 g/kg (□) was administered orally to rats after an  
 629 overnight fast. Acetaminophen (100 mg/kg) was added to each test solution. Blood  
 630 samples were collected from the tail vein before (0 min) and after the oral administration, as  
 631 indicated. Plasma concentrations of glucose, total GLP-1, insulin, and acetaminophen were

632 measured. The two-way ANOVA P-values for glucose (**A**) were 0.488 (treatment), <0.01  
633 (time), and <0.01 (treatment × time); <0.01 (treatment), <0.01 (time), and <0.01 (treatment  
634 × time) for total GLP-1 (**B**); 0.791 (treatment), <0.01 (time), and 0.269 (treatment × time) for  
635 insulin (**C**); and <0.01 (treatment), <0.01 (time), and <0.01 (treatment × time) for  
636 acetaminophen (**D**). Values are mean ± SEM (n = 6–9). Asterisks (\*) indicate significant  
637 differences compared to the control treatment at the same time point (\*P < 0.05, \*\*P <  
638 0.01; Dunnett's test).

639

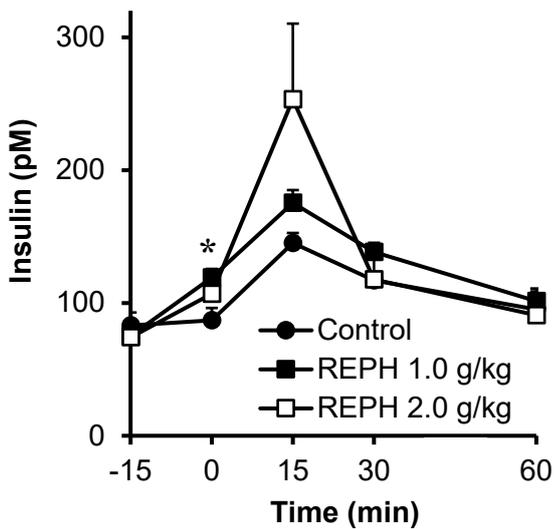
640

641

**A**

642

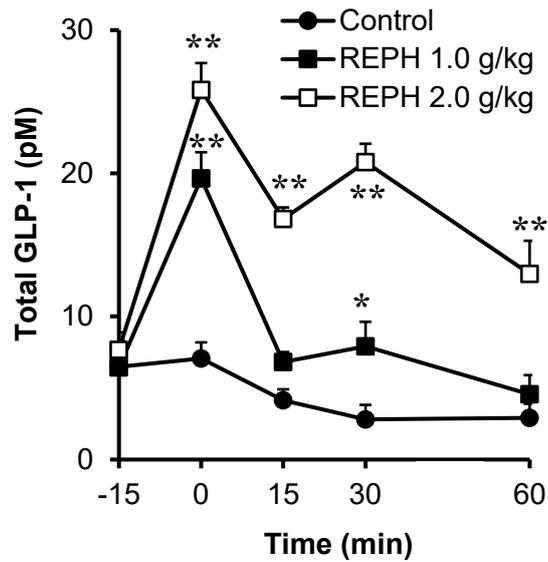
643

**C**

644

645 **Fig. 6. Changes in plasma glucose, total GLP-1, and insulin after oral administration**  
 646 **of REPH under the intraperitoneal glucose tolerance test.**

647 Water as control (●, 12 mL/kg) or REPH at 1.0 g/kg (■) or 2.0 g/kg (□) was administered  
 648 orally 15 min before an intraperitoneal glucose injection (1 g/kg). Blood samples were  
 649 collected from the tail vein before and after the oral administration, and the plasma  
 650 concentrations of glucose, total GLP-1, and insulin were measured. The two-way ANOVA  
 651 P-values for glucose (**A**) were 0.481 (treatment), <0.01 (time), and <0.01 (treatment ×  
 652 time); <0.01 (treatment), <0.01 (time), and <0.01 (treatment × time) for total GLP-1 (**B**); and

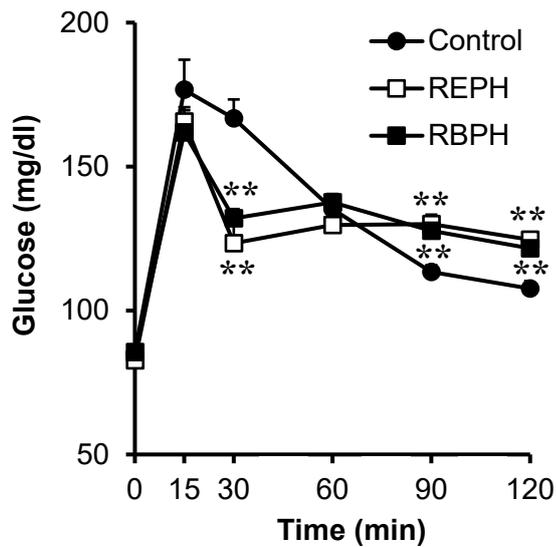
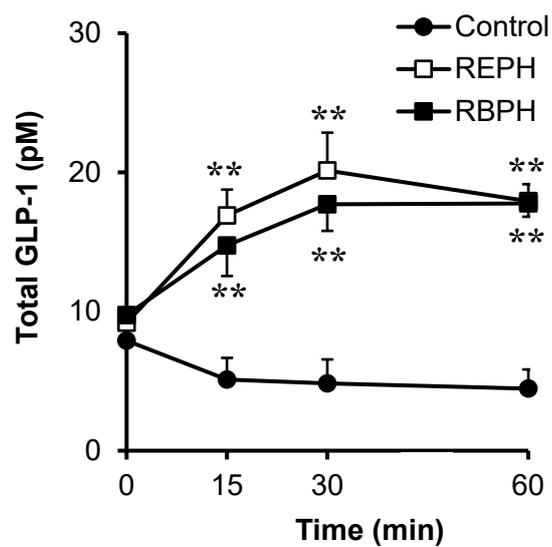
**B**

653 0.273 (treatment), <0.01 (time) and <0.01 (treatment × time) for insulin (**C**). Values are  
654 mean ± SEM (n = 4–6). Asterisks (\*) indicate significant differences compared to the control  
655 treatment at the same time point (\*P < 0.05, \*\*P < 0.01; Dunnett's test).

656

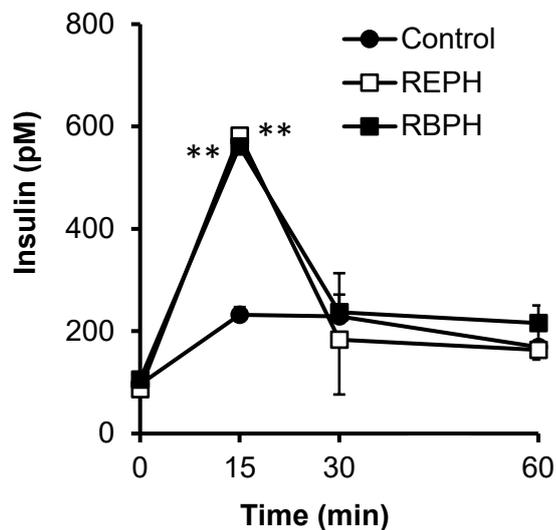
657

658

**A****B**

659

660

**C**

661

662 **Fig. 7. Changes in plasma glucose, total GLP-1, and insulin levels after the oral**663 **administration of rice peptides under IPGTT.**

664 Water as control (●, 12 mL/kg), REPH at 2.0 g/kg (□), or RBPH at 2.0 g/kg (■) was  
 665 administered orally immediately before an intraperitoneal glucose injection (1 g/kg). Blood  
 666 samples were collected from the tail vein before and after the oral administration, and the  
 667 plasma concentrations of glucose, total GLP-1, and insulin were measured. The two-way  
 668 ANOVA P-values for glucose (A) were 0.534 (treatment), <0.01 (time), and <0.01  
 669 (treatment × time); <0.01 (treatment), <0.01 (time), and <0.01 (treatment × time) for total

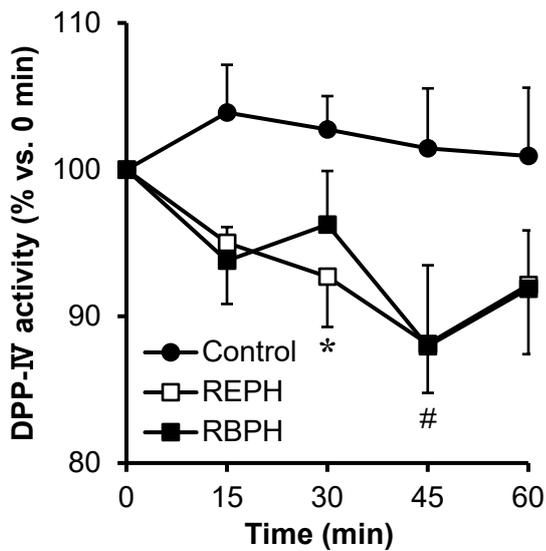
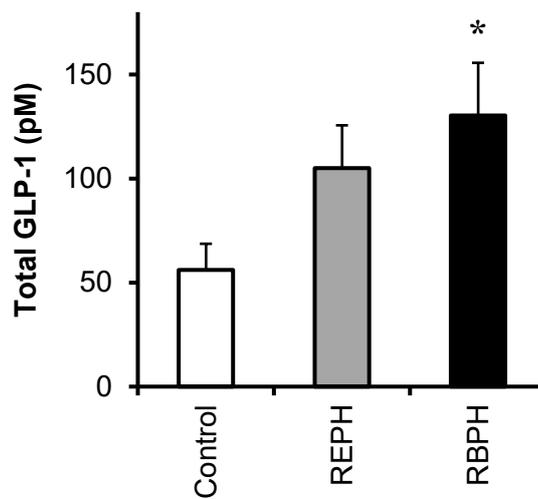
670 GLP-1 (**B**); and 0.118 (treatment), <0.01 (time) and <0.01 (treatment × time) for insulin (**C**).  
671 Values are mean ± SEM (n = 5–6). Asterisks (\*) indicate significant differences compared to  
672 the control treatment at the same time point (\*P < 0.05, \*\*P < 0.01; Dunnett's test).

673

674

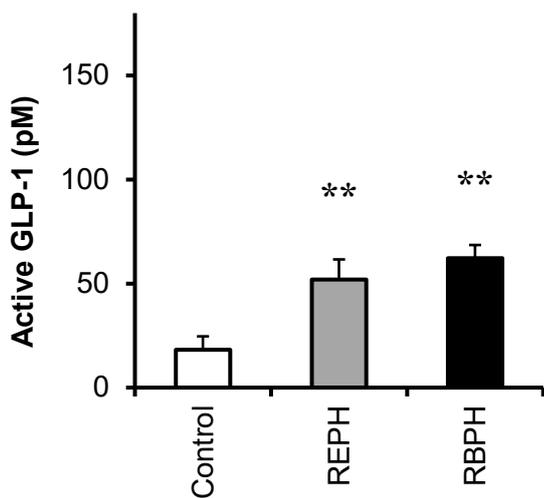
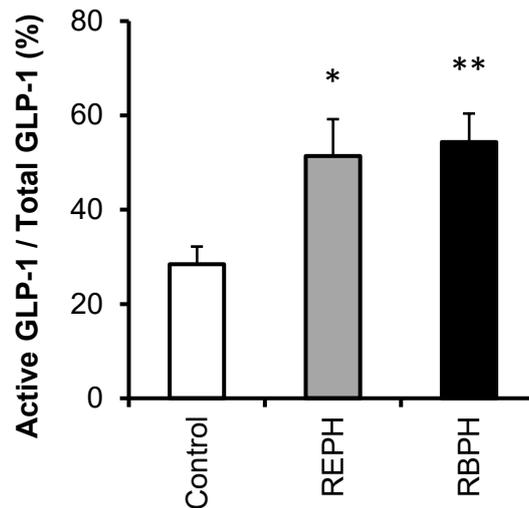
675

676

**A****B**

677

678

**C****D**

679

680

681

682

**Fig. 8. Changes in the plasma DPP-IV activity, GLP-1 levels, and the ratio of active GLP-1 to total GLP-1 in the ileal vein of anesthetized rats after ileal administration of REPH or RBPH.**

683

684

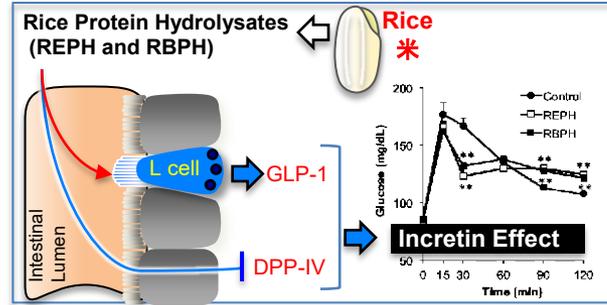
685

686

687

**A:** Saline as control (●, 2 mL/head), REPH (□, 500 mg/2 mL), or RBPH (■, 500 mg/2 mL) was injected into the ligated ileal loop at 0 min. Blood samples were collected through the ileal vein catheter before (0 min) and after (15, 30, 45, and 60 min) the ileal administration of test solutions. **B-D:** Total (**B**) and active (**C**) GLP-1 levels were evaluated 60 min after the ileal administration of REPH or RBPH, as well as the ratio of active GLP-1 to total GLP-1

688 (D). The two-way ANOVA P-values for the plasma activity of DPP-IV (A) were <0.05  
689 (treatment), <0.05 (time), and 0.287 (treatment × time). Values are mean ± SEM (n = 6–9).  
690 Asterisks (\*) indicate significant differences compared to the control treatment (P < 0.05;  
691 Dunnett's test), and hash marks (#) indicate significant differences compared to the 0 min  
692 value (P < 0.05; Dunnett's test).  
693



Single oral administration of rice protein hydrolysates stimulated GLP-1 secretion and reduced glycemic response in awake rats. Luminal REPH and RBPH reduced DPP-IV activity in the mesenteric vein.