Full Title

Oral administration of corn Zein hydrolysate stimulates GLP-1 and GIP secretion and improves glucose tolerance in male normal rats and Goto-Kakizaki rats

Abbreviated Title: Glycemic control by dietary peptides

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Abstract

We have previously demonstrated that ileal administration of the dietary protein hydrolysate (ZeinH) prepared from corn zein stimulated glucagon-like peptide-1 (GLP-1) secretion and attenuated hyperglycemia in rats. In this study, to examine whether oral administration of ZeinH improves glucose tolerance by stimulating GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) secretions, glucose tolerance tests were performed in normal Sprague–Dawley male rats and diabetic Goto-Kakizai (GK) male rats. The test solution was gavaged before intraperitoneal (i.p.) glucose injection in normal rats or gavaged together with glucose in GK rats. Blood samples were collected from the tail vein or by using the jugular catheter to measure glucose, insulin, GLP-1, and GIP levels. In the intraperitoneal glucose tolerance test, oral administration of ZeinH (2 g/kg) significantly suppressed the glycemic response accompanied with immediate increase in plasma GLP-1 and GIP levels in normal rats. In contrast, oral administration of another dietary peptide, meat hydrolysate, did not elicit a similar effect. The glucose-lowering effect of ZeinH was attenuated by a GLP-1 receptor antagonist or by a GIP receptor antagonist. Furthermore, oral ZeinH induced GLP-1 secretion and reduced glycemic response in GK rats under the oral glucose tolerance test. These results indicate that the oral administration of the dietary peptide ZeinH improves glucose tolerance in normal and diabetic rats by its incretin-releasing activity, namely, the incretinotropic effect.

Introduction

Incretins are gut hormones that enhance glucose-dependent insulin secretion, which is known as the "incretin effect." Two gut hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are recognized incretins. Based on their stimulative effects on glucose-dependent insulin secretion and pancreatic proliferation (1), incretin systems are a convincing target for treating impaired glucose tolerance and type 2 diabetes. Both incretins are immediately

47 degraded and inactivated by dipeptidyl peptidase-IV (DPP-IV) (2); hence, stable GLP-1 analogs and
48 DPP-IV inhibitors are currently used as clinical drugs.

49 Secretion of GIP and GLP-1 is increased in response to nutrient ingestion, especially by glucose and
50 free fatty acids. The molecular mechanisms by which these nutrients induce gut hormone secretion were
51 recently elucidated by the discovery of the sweet taste receptor (T1R2/3) (3-5) and free fatty acid
52 receptors (6).

53 Some dietary proteins and peptides stimulate GLP-1 secretion in animals and humans (7-10).
54 However, the effects of such dietary proteins/peptides on glycemic control and underlying molecular
55 mechanisms are not well studied. We have previously demonstrated that a hydrolysate prepared from
56 zein, a major corn protein, potently stimulated GLP-1 secretion in the murine enteroendocrine cell line
57 GLUTag and in the small intestine of anesthetized rats (11). We also reported that ileal administration
58 of the zein hydrolysate (ZeinH) in conscious rats strongly stimulated GLP-1 secretion, which led to
59 enhanced insulin secretion and attenuation of hyperglycemia (12). Although oral administration of
60 ZeinH attenuated the elevation of glycemia, GLP-1 response was not investigated.

61 The purpose of the present study is to examine whether oral administration of ZeinH increases GLP-
62 1 secretion. We also investigated the secretory response of GIP and the involvement of both incretins
63 in the glucose-lowering effect of orally administered ZeinH under an intraperitoneal glucose tolerance
64 test (IPGTT). It was further examined whether oral administration of ZeinH affects the glycemic
65 response under an oral glucose tolerance test (OGTT) in type 2 diabetic model rats.

66 Because increasing endogenous incretins, especially GLP-1, has great potential to improve glucose
67 tolerance and pancreatic β-cell function, orally available incretin releasers, including dietary proteins
68 or peptides, are considered promising agents for preventing and treating diabetes and obesity.

69

Materials and Methods

Materials

ZeinH was prepared as previously described (11). Briefly, Zein (50 g; Tokyo Chemical Industry, Tokyo, Japan) was suspended in deionized water (500 ml), and the pH was adjusted to pH 7.2. The suspension was shaken for 60 min at 55°C in the presence of papain (250 mg, Papain F; Asahi Food and Health Care, Tokyo, Japan) and then treated in boiling water for 20 min to stop the enzyme reaction. After filtration (0.45 μm pore size) and pH adjustment to 7.0, the filtrate was lyophilized as ZeinH. Whey hydrolysate (WheyH) was prepared from whey protein (Optimum Nutrition, Lindesberg, Sweden) with the same procedure as described above. Meat hydrolysate (MHY) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The peptide content of ZeinH was estimated at 77% by total and free amino acid analysis of ZeinH (79.5% and 2.5%, respectively) (12). WheyH and MHY peptide contents were 77.6% and 80.0%, respectively, as determined by the Lowry's protein assay using bovine serum albumin as a standard protein. ZeinH and MHY had average molecular masses of 1600−1700 Da and less than 1200 Da, respectively (13). Additional chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise specified.

Animals

Male Sprague–Dawley rats (7 weeks old), weighing 210–230 g, and Goto-Kakizaki (GK) rats (6 weeks old), a type 2 diabetic model, weighing 100–160 g were purchased from Japan SLC (Hamamatsu, Japan). All the animals had free access to water and a semipurified diet containing 25% casein based on AIN-93G (14); the rats were housed in individual cages. All animal experiments were performed after an acclimation period (3−7 days) in a temperature-controlled room maintained at 23°C ± 2°C with

93 a 12-h light/dark cycle (08:00−20:00 h, light period).

94 This study has been approved by the Hokkaido University Animal Committee, animals were
95 maintained in accordance with the Guide for the Care and Use of Laboratory Animals of Hokkaido
96 University.

97

98 ***Surgical preparation for in vivo experiments***

99 Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight; Somnopentyl Injection,
100 Kyoritsu Seiyaku Co., Tokyo, Japan). The right jugular vein was exposed, and a silicone catheter with
101 a 0.5-mm internal diameter (ID) and a 1.0-mm outer diameter (OD) (Silascon No. 00; Dow Corning
102 Co., Kanagawa, Japan) was inserted into the vessel and fixed with a thread. The catheter was prefilled
103 with sterilized saline that contained heparin (50 IU/ml; Ajinomoto, Tokyo, Japan).

104 The free ends of the catheter were dorsally exteriorized, which permitted us to conduct the
105 experiment under non-anesthetized and unrestrained conditions. Rats were used for Experiments 2 and
106 4 after a recovery period of 3–4 days. We flushed the jugular catheter with heparinized saline daily to
107 maintain patency.

108

109 ***Intraperitoneal glucose tolerance test (IPGTT)***

110 The glucose solution was administered intraperitoneally to examine the effect of oral peptides on
111 the GLP-1-mediated glycemic control. All IPGTTs in this study were performed in conscious rats.
112 After a 24-h fast, a basal (–15 min) blood sample was collected from the tail vein (Experiments 1 and
113 3) or jugular vein (Experiments 2 and 4). After basal blood collection, test solutions containing ZeinH,
114 MHY, or deionized water (negative control) were administered into the stomach through the mouth
115 using a feeding tube (5 Fr; Atom Medical Co., Tokyo, Japan). Fifteen minutes after oral

116 administration, the blood sample was collected (0 min) and then glucose solution was injected (1
117 g/kg) intraperitoneally. Blood samples were collected from the tail vein or by using the jugular
118 catheter 15, 30, 60, 90, and 120 min after the glucose injection. Plasma was separated by
119 centrifugation at 2500 × *g* for 10 min at 4°C and frozen at –80°C until glucose, incretin, or insulin
120 measurements. Plasma glucose concentrations were measured using the Glucose CII test kit (Wako).
121
122 ***Experiment 1***
123 Effects of oral ZeinH administration on plasma glucose under IPGTT
124 Peptides (MHY or ZeinH, 2 g/kg) and water (negative control) were administered –15 min to SD
125 rats (8 ml/kg body weight). MHY was selected as a dietary peptide that has GLP-1-releasing activity
126 in vitro (15, 16) and in situ (7). Blood samples (80 µl) were collected from the tail vein and
127 transferred into a 1.5-ml tube containing aprotinin (final concentration, 500 kIU/ml; Wako) and
128 heparin (final concentration, 50 IU/ml) at each time point (–15, 0,15,30, 60, 90, and 120 min).
129
130 ***Experiment 2***
131 Effects of oral ZeinH administration on plasma incretins (GLP-1 and GIP) and insulin under IPGTT
132 IPGTT was performed in conscious SD rats with the jugular catheter because a large volume of
133 plasma was required to measure glucose and hormone levels. Peptides (MHY or ZeinH at 2 g/kg) and
134 water were administered at –15 min, and glucose (1 g/kg) was injected intraperitoneally at 0 min, as
135 previously described. Blood samples (300 µl) were drawn from the jugular catheter into a syringe that
136 contained EDTA (final concentration, 1 mg/ml; Dojindo, Kumamoto, Japan), aprotinin (final
137 concentration, 500 kIU/ml) and DPP-IV inhibitor (final concentration, 50 µM; DPP4-010; Millipore
138 Co., Billerica, USA) at each time point (–15, 0, 15, 30, 60, 90, and 120 min). Between each blood

139 sampling, the catheter was refilled with saline containing heparin (50 IU/ml). Insulin in the plasma
140 (20 μl) was measured using an ELISA kit (AKRIN-010T; Shibayagi Co., Ltd., Gunma, Japan); active
141 GLP-1 and total GIP in the plasma (100 μl and 20 μl, respectively) were measured by the respective
142 ELISA kits (EGLP-35K and EZRMGIP-55K; Millipore Co., Billerica, USA).
143
144 ***Experiment 3***
145 Effect of GIP receptor antagonist on ZeinH-attenuated glycemic response (IPGTT)
146 To investigate the involvement of endogenous GIP in reduced glycemic response after oral ZeinH
147 administration, (Pro3)GIP (Phoenix Pharmaceutical, Inc., USA), as a GIP receptor antagonist, was
148 intraperitoneally injected in SD rats, and IPGTT was performed as described above. (Pro3)GIP (25
149 nmol/kg, 25 nmol/ml in saline) was injected immediately after oral ZeinH administration (–15 min)
150 under IPGTT. ZeinH (2 g/kg in 8 ml/kg deionized water) and water (negative control) were administered
151 at –15 min. Blood samples were collected from the tail vein or the jugular vein at the time indicated in
152 the result, and plasma glucose levels were measured.
153
154 ***Experiment 4***
155 Effect of GLP-1 receptor antagonist on plasma insulin after oral ZeinH administration (IPGTT)
156 A GLP-1 receptor antagonist, Exendin (9-39) (Ex9, synthesized by Thermo Fisher Scientific K.K.,
157 Yokohama, Japan) was intraperitoneally injected in SD rats, and IPGTT was performed. Ex9 (80
158 nmol/kg) was added in the glucose solution for intraperitoneal injection. ZeinH (2 g/kg) or water was
159 orally administered at –15 min and then the glucose solution with or without Ex9 was injected
160 intraperitoneally at 0 min. We collected blood samples (300 μl) from the jugular vein from –15 min to
161 60 min, and measured plasma glucose and insulin levels, as described in Experiment 2.

162

163 ***Experiment 5***

164 Effect of in vitro digestion on GLP-1-releasing activity of ZeinH in GLP-1-producing enteroendocrine

165 cell line

166 ZeinH was digested with pepsin and pancreatin to examine whether the GLP-1-releasing potency of

167 ZeinH remains after luminal protease digestion. ZeinH was dissolved in 0.02N H_3PO_4 at a concentration

168 of 50 g/l, and the pH was adjusted to 1.85 by using 20N H_3PO_4. Pepsin (from porcine gastric mucosa,

169 Sigma) was added at 0.5% wt/substrate wt and incubated for 60 min while shaking at 37°C. The pH of

170 the suspension was then adjusted to 8.2 using $Ca(OH)_2$, and pancreatin (4% wt/substrate wt; from

171 porcine pancreas, Sigma) and trypsin (2.5% wt/pancreatin wt; from bovine pancreas, Sigma) were

172 added (17-19). Trypsin was added to sufficiently activate protease zymogens in pancreatin. The mixture

173 was incubated for 120 min at 37°C, followed by boiling for 20 min to inactivate the enzymes. The

174 mixture was neutralized with H_3PO_4 and desalted by centrifugation and filtration (0.45-µm pore size).

175 The soluble fraction was lyophilized as the ZeinH-pepsin/pancreatin digest.

176 To examine the involvement of the peptide fractions of ZeinH in its GLP-1-releasing activity,

177 another in vitro digestion was performed using pronase as the potent protease. Briefly, ZeinH was

178 dissolved in deionized water and the pH was adjusted at 7.0. Pronase (PRONASE® Protease,

179 *Streptomyces griseus*, Calbiochem, Merck KGaA, Darmstadt, Germany) was added to the solution at

180 0.5% wt/substrate wt and incubated for 60 min at 37°C, followed by boiling for 20 min to stop the

181 enzymatic reaction. The solution was lyophilized as ZeinH-pronase digest.

182 The effect of free amino acids on GLP-1 secretion was examined by using the amino acid mixture

183 equivalent to the composition of ZeinH (12). Because Gln and Asn were indistinguishable in the PITC

184 amino acid analysis, the concentrations of Gln (21.3 mg/10 mg ZeinH), Glu (0.4 mg/10 mg ZeinH),

185 Asn (5.8 mg/10 mg ZeinH), and Asp (0.5 mg/10 mg ZeinH) were estimated based on the amino acid
186 sequence of Zein protein (REFSEQ accession number NM_001112418.1). The total amino acid
187 concentration was finally 62 mM in the test solution. To assess the osmotic effect, GLP-1 secretion in
188 response to 31 mM NaCl (added to the HEPES buffer described below) was also examined.

189 GLUTag cells (courtesy of Dr. D.J. Drucker, University of Toronto, Toronto, Canada), a murine
190 GLP-1-producing enteroendocrine cell line, were grown in Dulbecco's modified Eagle's medium
191 (Invitrogen, Cat. No. 12100-038) supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and
192 500 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Cells were routinely subcultured
193 by trypsinization after reaching 80%–90% confluency. GLUTag cells were grown in 48-well culture
194 plates at a density of 1.25×10^5 cells/well for 2 days until they reached 80%–90% confluency. Cells
195 were washed twice with HEPES buffer (140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM $CaCl_2$,
196 1.2 mM $MgCl_2$, and 0.1% bovine serum albumin, pH 7.4) to remove the culture media; the cells were
197 then exposed to test agents that were dissolved in the same buffer for 60 min at 37°C. Supernatants
198 were collected from the wells, centrifuged at 800 × *g* for 5 min at 4°C to remove the remaining cells,
199 and then stored at –50°C until the GLP-1 concentration was measured with a commercial enzyme-
200 immunoassay kit (Yanaihara Institute Inc., Shizuoka, Japan).

201

202 ***Experiment 6***

203 Effects of oral ZeinH on incretin and glycemic response in type 2 diabetic model rats under OGTT

204 To examine the effect of oral ZeinH on type 2 diabetic models under OGTT, we employed GK rats
205 and blood samples were collected by using the jugular catheter as describe above. After basal blood
206 collection (0 min), glucose solution (2 g/kg) as control treatment, or the solution containing ZeinH (2
207 g/kg) or WheyH (2 g/kg) was orally administered. Blood samples (300 μl) were collected from the

208 jugular vein 15, 30, 60, 90, 120 min after the oral administration. Plasma glucose, insulin, active GLP-
209 1, and total GIP levels were measured as described in Experiment 2, and total GLP-1 level was measured
210 using an ELISA kit (EZGLP1T-36K; Millipore Co., Billerica, USA).
211
212 ***Statistical analysis***
213 Results are expressed as means ± standard error of the mean (SEM). Statistical analyses were
214 performed by using JMP Pro version 10.0 (SAS Institute Inc. Cary, NC). Statistical significance was
215 assessed by one-way or two-way ANOVA. Two-way ANOVA analysis was performed to assess the
216 main effects [treatment (Tr), time (Ti)] and the interaction effect [treatment × time (Tr × Ti)]. Significant
217 differences ($p < 0.05$) between mean values were determined by Tukey's test or Dunnett's test as
218 appropriate, and described in figure legends. The primary endpoints of the present study were the
219 significant increment of plasma incretin levels accompanied by the reduction of plasma glucose levels
220 in ZeinH-treated group compared to the control group.
221
222 **Results**
223 ***Experiment 1: Changes in plasma glucose concentrations during IPGTT in conscious rats after oral***
224 ***administration of ZeinH***
225 We first examined the dose-response effect of oral ZeinH administration on plasma glucose
226 concentration under IPGTT in conscious rats (Experiment 1). To observe the effect of luminal ZeinH
227 on GIP/GLP-1 secretion and to avoid the possible involvement of luminal glucose in the secretion, we
228 used IPGTT rather than OGTT. In the case of OGTT, luminal glucose could stimulate GIP/GLP-1
229 secretion, and reduced glycemic response might involve delayed gastric emptying and glucose
230 absorption from the intestinal epithelium, apart from the incretin-releasing effect of ZeinH.

231 Oral administration of test liquids slightly increased plasma glucose concentrations (–15 min to 0
232 min; Fig. 1). Increase in the plasma glucose concentration was significantly lower in ZeinH-preloaded
233 rats (2 g/kg) than that in control rats at 30 min (Fig. 1). Plasma glucose levels similarly decreased in
234 every group after 60 min.
235
236 ***Experiment 2: Changes in plasma glucose, insulin, active GLP-1, and total GIP concentrations***
237 ***during IPGTT after oral administration of ZeinH or MHY***
238 We next examined whether oral ZeinH stimulated incretin and insulin secretion in jugular vein-
239 cannulated rats (Experiment 2). Plasma glucose concentrations (Fig. 2A) in ZeinH-treated rats, but not
240 in MHY-treated rats, were lower than those in control rats 15 min and 30 min after i.p. glucose injection.
241 Plasma insulin concentrations sharply increased at 15 min in every group (Fig. 2B), and ZeinH-treated
242 rats demonstrated higher insulin concentrations than those in the control group and MHY-treated rats at
243 15 min. After peaking at 15 min, insulin concentrations decreased immediately and returned to the basal
244 level at 60 min in every group.
245 The plasma GLP-1 concentration in the control rats remained at a basal concentration throughout the
246 IPGTT. In contrast, GLP-1 concentrations increased after an oral ZeinH administration; the GLP-1 level
247 at 0 min (just before i.p. glucose injection) was significantly higher than that in control rats. The GLP-
248 1 concentration in the MHY group was at an intermediate level between that in the control group and
249 the ZeinH group; this level was not significantly different compared with that of the control group. The
250 total GIP concentrations changed in a manner similar to GLP-1. Plasma GIP concentrations were
251 significantly higher in ZeinH-treated rats (0–60 min) and in MHY-treated rats (0–15 min) than in the
252 control rats. The increment of plasma GIP was significantly larger in ZeinH-treated rats than in MHY-
253 treated rats. These results demonstrate that oral ZeinH stimulates the secretion of both incretins, GLP-

254 1 and GIP, independently of luminal glucose.

255

256 ***Experiment 3: Glycemic responses during IPGTT after oral preload of ZeinH in rats treated with***

257 ***GIP receptor antagonist***

258 In Experiment 3, IPGTT was performed under treatment with the GIP receptor antagonist (Pro3)GIP

259 to examine whether oral ZeinH attenuates plasma glucose increment via the GIP pathway. In this

260 experiment, we collected blood samples until 60 min after i.p. glucose injection because the effect of

261 ZeinH on glycemia was not observed after 60 min in the experiments shown in Figs. 1 and 2. Plasma

262 glucose levels at 30 min in ZeinH/Pro3-treated rats were at intermediate levels between those in

263 Cont/Veh rats and ZeinH/Veh-treated rats, demonstrating partial cancellation of the glucose-lowering

264 effect of ZeinH (Fig. 3A). Treatment with (Pro3)GIP had no significant effect on the glycemic response

265 in control rats that received oral water followed by i.p. glucose injection (Fig. 3B).

266

267 ***Experiment 4: Glycemic and insulin responses during IPGTT after oral administration of ZeinH in***

268 ***rats treated with a GLP-1 receptor antagonist***

269 We examined the effect of the GLP-1 pathway blockage by using the GLP-1 receptor antagonist

270 Exendin-9 (Ex9) on the glycemic and insulin response in jugular vein-cannulated rats (Experiment 4).

271 Consistent with Experiment 2, plasma glucose concentrations in ZeinH-treated rats were significantly

272 lower than those in the control rats at 15 min and 30 min (Fig. 4A), and plasma insulin in ZeinH-treated

273 rats was significantly higher than that in the control rats at 0 min and 15 min (Fig. 4B). The treatment

274 with Ex9 had no effect on the glycemic and insulin responses in the control rats (treated with oral water

275 and i.p. glucose). Treatment with Ex9 attenuated the glucose-lowering effect of ZeinH at 15 min and

276 30 min (Fig. 4A). As expected, Ex9 treatment reduced the plasma insulin (0 to 15 min) in response to

277 the oral ZeinH administration (Fig. 4B).

278

279 ***Experiment 5: Effects of in vitro digestion and amino acids of ZeinH on its GLP-1-releasing activity***

280 ***in enteroendocrine GLUTag cells***

281 We examined the influence of luminal protease (pepsin and pancreatin) digestion on the GLP-1-

282 releasing potency of ZeinH in GLP-1-producing enteroendocrine cell line. Consistent with the findings

283 of our previous study (11), ZeinH significantly increased GLP-1 release into the supernatant of GLUTag

284 cells after 60-min incubation and indicated that ZeinH directly activated GLP-1 secretion in

285 enteroendocrine cells. Pepsin/pancreatin-treated ZeinH also induced a significant increase in GLP-1

286 secretion compared to the blank (control), with slightly lower potency than untreated ZeinH (Fig. 5A).

287 The degree of hydrolysis (DH), determined by using the trinitrobenzenesulfonic method (20, 21), was

288 8.6% for pepsin/pancreatin-treated ZeinH. In the case of casein, DH was 60.6% after the same

289 treatment; this finding confirmed that sufficient digestive condition for general protein was used in the

290 present study, and it suggests that ZeinH has luminal protease-resistant property compared to casein.

291 We used another strong protease (pronase) (22) to examine the involvement of peptide fractions of

292 ZeinH in its GLP-1-releasing activity. DH of ZeinH-pronase digest was 14.5%, suggesting that ZeinH

293 is more sensitive to pronase than pepsin/pancreatin. GLP-1 release in response to pronase-treated ZeinH

294 was largely reduced compared to intact ZeinH (Fig. 5B). The enzymes (pepsin, pancreatin, and pronase)

295 without ZeinH had no effect on GLP-1 secretion in GLUTag cells in the preliminary experiments. The

296 amino acid mixture equivalent to the amino acid composition of ZeinH (12) induced significant

297 increment of GLP-1 concentration in the supernatant (Fig. 5C). The increment was around half of the

298 ZeinH-induced increment. The high osmotic control (HEPES buffer added with 31 mM NaCl) had only

299 a slight effect on GLP-1 secretion.

300

301 ***Experiment 6: Effect of oral ZeinH on glycemic and incretin responses in type 2 diabetic model rats***

302 GK rats are well recognized as one of the best available model of non-obese type 2 diabetes (23, 24).
303 To examine the effect of oral ZeinH under diabetic condition and under the presence of enteral glucose,
304 OGTT was carried out. The hydrolysate of whey protein (WheH) was included as another dietary
305 peptide because whey had been reported to increase GLP-1 secretion in vivo (7-10). Fasting glucose
306 levels were approximately 120 mg/dl, and glucose levels in control rats were drastically increased to
307 more than 300 mg/dl after oral glucose load, indicating the typical glucose intolerance condition of GK
308 rats. Oral administration of ZeinH at 2 g/kg significantly reduced glycemic response in GK rats (Fig.
309 6A), similar to the administration of WheyH. Although the experimental conditions differed, the
310 glucose-lowering effect seemed more apparent than that in normal rats as described in the results above.
311 Insulin levels were immediately increased in all groups, with the highest elevation observed in the
312 ZeinH group (Fig. 6B). Total GLP-1 level was significantly increased 15 min after oral administration
313 of ZeinH (Fig. 6C). Increment of total GLP-1 in the WheyH group was not statistically significant,
314 whereas the control group (oral glucose) showed significant reduction of total GLP-1 after 30 min. Only
315 slight increments of active GLP-1 were observed after oral administration of test solutions, and
316 significant differences were not detected (Fig. 6D). Total GIP levels were immediately increased after
317 the oral glucose load (Fig. 6E). Increments in the total GIP levels were similar in the control and ZeinH
318 groups, whereas the WheyH group showed a smaller increase at 15 min compared to the other groups.

319

320 **Discussion**

321 The objective of this study was to investigate whether oral administration of a dietary peptide, ZeinH,
322 stimulates GLP-1 or GIP secretion and improves glucose tolerance in normal rats. We found that oral

323 administration of ZeinH, but not that of MHY, attenuated the glycemia under IPGTT, associated with
324 enhanced secretions of GLP-1 and GIP. The glucose-lowering effect of ZeinH was reversed by GLP-
325 1/GIP receptor antagonists. Oral ZeinH also induced GLP-1 secretion and reduced glycemic response
326 in diabetic model rats. The results of this study demonstrate that the oral administration of the dietary
327 peptide ZeinH can attenuate hyperglycemia by stimulating incretin secretion in normal and diabetic
328 conditions.
329
330 ***Oral ZeinH suppresses plasma glucose elevation***
331 It has been reported that oleoylethanolamide (25) and alpha-linolenic acid (26) stimulate GLP-1
332 secretion via GPR119 and GPR120, respectively. With regard to dietary proteins, oral administration of
333 whey protein with carbohydrates decreased postprandial glucose level in individuals with type 2
334 diabetes mellitus (27), and whey protein increased GLP-1 secretion under OGTT (9). In contrast, by
335 using IPGTT, we demonstrated a dose-response effect of oral ZeinH in attenuating glycemia, and the
336 effective dose was confirmed at 2 g/kg (Fig. 1). This result clearly indicates that ZeinH improves
337 glucose tolerance regardless of the modification of intestinal glucose absorption. To clarify whether oral
338 administration of ZeinH has the potential to induce incretin secretion independently of luminal glucose,
339 we used IPGTT in this study.
340
341 ***Oral ZeinH stimulates incretins (GLP-1 and GIP) secretion***
342 Oral administration of ZeinH stimulated GLP-1 secretion (Fig. 2C) in conscious rats, as has occurred
343 previously with ileal ZeinH (12). Additionally, we found that GIP secretion was also increased by oral
344 ZeinH (Fig. 2D). These incretin secretions were accompanied with enhanced insulin secretion (Fig. 2B).
345 The incretin-releasing activity had been proposed as 'incretinotropic effect' in previous papers (28, 29);

346 thus, ZeinH can be designated as a potent “incretinotropic” dietary peptide.

347 Plasma GIP and GLP-1 levels peaked 15 min (at 0 min in Fig. 2) after oral administration of ZeinH,
348 indicating that luminal ZeinH—not i.p. glucose—stimulated the secretion of incretins. Enhanced insulin
349 secretion after the glucose injection in ZeinH-treated rats could be responsible for the attenuation of
350 hyperglycemia.

351 Because GIP-producing K cells are primarily located in the upper small intestine, ZeinH flown into
352 the upper small intestine within 15 min might directly stimulate GIP secretion. In contrast, GLP-1-
353 producing L cells are abundant in the distal small intestine and the large intestine. Therefore, oral
354 administration of ZeinH might not reach these regions to directly stimulate L cells within such a short
355 time period. However, we observed that the jejunal lumen was filled with liquid 15 min after oral
356 administration of ZeinH solution in another study, and L cells also exist in the jejunum (11, 30). These
357 observations suggest that GLP-1 was initially released from the jejunal L cells by luminal ZeinH, rather
358 than from the ileal L cells. Other reports documented that GIP activates L cells to release GLP-1 via the
359 vagal nerve pathway (31). Such an indirect pathway might also be responsible for GLP-1 secretion after
360 the administration of oral ZeinH.

361 High GLP-1 levels after 15 min might reflect the release of GLP-1 from ileal L cells. In vitro
362 digestion of ZeinH with pepsin and pancreatin failed to attenuate the GLP-1-releasing ability of ZeinH
363 in GLUTag cells (Fig. 5A). This finding supports the hypothesis that ZeinH or its partially digested
364 products can stimulate L cells directly even after luminal digestion.

365 In contrast to pepsin/pancreatin treatment that was carried out to mimic luminal protease digestion,
366 treatment with pronase, a mixture of potent proteases, which was used for non-specific digestion largely
367 diminished (~50%) the GLP-1-releasing activity of ZeinH (Fig. 5B). This result suggests that peptide
368 fractions in ZeinH are responsible for its GLP-1-releasing activity. Further investigations to identify the

369 active peptide(s) in ZeinH are undergoing in our laboratory. The residual GLP-1 secretion after pronase
370 treatment may be caused by remaining peptide fragments and liberated amino acids. Although the
371 potency was much lower than intact ZeinH, the amino acid mixture reconstituting the total amino acid
372 composition of ZeinH induced GLP-1 secretion (Fig. 5C). Previous reports have documented that amino
373 acids, particularly glutamine, stimulated GLP-1 secretion in vitro (32) and increased plasma GLP-1
374 level in humans (33). Such amino acids liberated from ZeinH might partially contribute to the incretin-
375 releasing effect of ZeinH in the lumen. Even if glucose and fats were included in ZeinH as minor
376 components, such components would require doses similar to ZeinH to trigger significant GLP-1
377 secretion. Previous studies demonstrated that GLP-1 secretion was induced by >300 mg of glucose (7,
378 34) or by a 2.2-kcal (244 mg) dose of lipid emulsion (35) in rats that have similar body weights to those
379 in the present study.
380
381 ***Involvement of incretins***
382 The glucose-lowering effect of ZeinH was attenuated by treatment with a GIP receptor antagonist,
383 (Pro3)GIP (Fig. 3A), and by a GLP-1 receptor antagonist, Ex9 (Fig. 4A). Furthermore, treatment with
384 Ex9 attenuated ZeinH-induced insulin secretion (Fig. 4B). These results demonstrated that GLP-1
385 secretion induced by the administration of oral ZeinH enhanced insulin secretion and resulted in the
386 prevention of hyperglycemia.
387 GIP is also responsible for the prevention of hyperglycemia by enhancing insulin secretion. As
388 previous reports have documented that GIP is involved in the indirect stimulation of GLP-1 secretion
389 via the vagus nerve (31), GIP released from K cells by luminal ZeinH might activate a vagal pathway
390 to trigger GLP-1 secretion from L cells located at distal small intestine. This hypothesis is supported by
391 the results that GLP-1 secretion peaked at 0 min and at same time point with GIP.

392 Incretins have the effect of enhancing insulin secretion and protecting islet β cells, but they also have
393 multiple effects on cardiovascular (36), liver (37), adipose (38) functions, and other systems (39). Stable
394 GLP-1 analogs and DPP-4 inhibitors are already used to treat type 2 diabetes. However, these drugs
395 have potential side effects such as nausea, anorexia, and diarrhea (40). "Incretinotropic" dietary peptides
396 such as ZeinH prepared from corn could have a lower risk of these side effects than incretin-mimetics
397 and incretin-enhancers.
398 Previous studies have demonstrated enhanced incretin secretion by dietary protein/peptides such as
399 whey protein (7-10), casein (41, 42), and meat hydrolysate (7, 15, 16). In some of these studies, meal
400 tolerance tests or OGTTs were employed. Although these experimental methods are relatively
401 physiological compared to the IPGTT used in our study, the effects on incretin secretion and glycemia
402 could involve a combined effect of oral meal or oral glucose. In addition, modified gastric emptying by
403 other gut hormone secretion, such as CCK or serotonin, could affect glycemic responses (43, 44). To
404 our knowledge, it has not been reported that oral dietary peptides induce incretin secretion under IPGTT
405 or intravenous glucose tolerance test. In this study, we demonstrated that single oral administration of
406 dietary peptide ZeinH increased GLP-1 and GIP secretions, which resulted in an improved glucose
407 tolerance without other luminal factors that could enhance incretin secretions.
408
409 ***Oral ZeinH induces GLP-1 secretion and lowers glycemic response in type 2 diabetic model rats***
410 The potent incretin-releasing and glucose-lowering effects of ZeinH led us to apply this peptide to
411 diabetic model rats (GK rats). The glycemic response after oral glucose load was diminished by co-
412 administration of ZeinH (Fig. 6A). Although WheyH had a similar glucose-lowering effect, the
413 insulin response was larger in ZeinH-treated rats than WheyH- and control-treated rats (Fig. 6B). This
414 could be explained by the significant increment of total GLP-1 only by ZeinH administration. The

415 reason why significant increment was not observed in control- and WheyH-treated rats might be the
416 defect of nutrient-sensitive incretin responses in GK rats (45). GIP secretion was significantly lower
417 in the WheyH group than in the control and ZeinH groups. These results indicate that WheyH had less
418 potency to stimulate incretin secretions than ZeinH and that the glucose-lowering effect of WheyH
419 was independent of incretin or insulinotropic effect. Possibly, gastric emptying was strongly inhibited
420 by WheyH, which limited the delivery of glucose into the small intestine. Such an effect might be
421 mediated by CCK or serotonin released from the upper small intestine (43, 44). A recent paper
422 demonstrated that incretin secretory responses to luminal glucose were impaired in GK rats compared
423 to Wistar rats, but responses to lipids were maintained (45). It is interesting to know whether incretin
424 secretory responses to dietary proteins/peptides were impaired in diabetic models. The present result
425 revealed potent GLP-1-releasing potency of ZeinH even in diabetic model rats.
426 In summary, our study demonstrated that the oral administration of a dietary peptide, ZeinH,
427 attenuated hyperglycemia by stimulating GLP-1 and GIP secretions in normal rats. The involvement
428 of increased GLP-1/GIP secretions was determined using GLP-1/GIP receptor antagonists. The GLP-
429 1-releasing activity of ZeinH was maintained in the enteroendocrine cell line even after in vitro
430 digestion with pepsin and pancreatin. Additionally, oral ZeinH effectively reduced the glycemic
431 response under OGTT in type 2 diabetic model rats accompanied with increased GLP-1 and insulin
432 secretions. Our data demonstrate the possibility that the oral administration of dietary peptides such as
433 ZeinH potently stimulates incretin secretions and attenuates postprandial hyperglycemia in normal
434 and diabetic conditions.
435
436

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568

569 **Figure Legends**

570 **Fig. 1. Changes in plasma glucose concentrations during IPGTT in conscious rats after oral**
571 **administration of Zein hydrolysate (ZeinH)**

572 Water as control (open circles, 8 ml/kg) and ZeinH at 1 g/kg (open squares) or 2 g/kg (closed circles)
573 were orally administered 15 min before intraperitoneal (i.p.) glucose injection (1 g/kg). Blood samples
574 were collected from the tail vein before (–15, 0 min) and after (15, 30, 60, 90, and 120 min) glucose
575 injection. Values are displayed as means ±SEM (n = 6). Asterisks (*) indicate significant differences
576 compared to control treatment at the same time points. (Dunnett's test; $p < 0.05$).

577

578 **Fig. 2. Changes in plasma glucose, insulin, active GLP-1, and total GIP concentrations during**
579 **IPGTT after oral administration of ZeinH or meat hydrolysate (MHY)**

580 Water as control (open circles, 8 ml/kg), ZeinH at 2 g/kg (closed circles), or MHY at 2 g/kg (open
581 triangles) were administered orally 15 min before i.p. glucose injection (1 g/kg). Blood samples were
582 collected from the jugular vein before and after the glucose injection. Glucose, insulin, active GLP-1,
583 and total GIP concentrations in the plasma were measured. Two-way ANOVA p values were <0.01 (Tr),
584 <0.01 (Ti), and <0.01 (Tr × Ti) for insulin (B); <0.01 (Tr), 0.15 (Ti) and 0.50 (Tr × Ti) for active GLP-
585 1 (C); <0.01 (Tr), <0.01 (Ti), <0.01 (Tr × Ti) for total GIP (D). Values are displayed as means ± SEM
586 (n = 7–10). Plots at the same time point that do not share the same letter differ significantly between
587 treatments (Tukey's test; $p < 0.05$).

588

589 **Fig. 3. Glycemic responses during IPGTT after oral preload of ZeinH in rats treated with GIP**
590 **receptor antagonist**

591 Water as control (8 ml/kg) or ZeinH at 2 g/kg was administered orally 15 min before i.p. glucose

injection (1 g/kg). A) Cont/Veh (open circles) and ZeinH/Veh (closed circles) groups were intraperitoneally injected with saline, and ZeinH/Pro3 (closed squares) group was injected with (Pro3)GIP (25 nmol/kg) at –15 min. Blood samples were collected from the tail vein before and after the glucose injection. B) Plasma glucose concentrations in response to oral water (–15 min) followed by i.p. glucose injection (1 g/kg, at 0 min) with (open squares) or without (open circles) (Pro3)GIP treatment (25 nmol/kg, at –15 min). Blood samples were collected from the jugular vein before and after the glucose injection. Values are displayed as means ± SEM (n = 4–7). Plots at the same time point that do not share the same letter differ significantly between treatments (Tukey's test; $p < 0.05$).

Fig. 4. Changes in plasma glucose and insulin concentrations during IPGTT after oral administration of ZeinH in rats treated with GLP-1 receptor antagonist

The control groups (open circles or squares) were orally administered with water (8 ml/kg), and the ZeinH groups (closed circles or squares) were orally administered with ZeinH (2 g/kg) 15 min before i.p. glucose injection (1 g/kg). Rats received i.p. injection of glucose solution containing Ex9 (open or closed squares, 80 nmol/kg) or not containing Ex9 (vehicle treatment; open or closed circles). Blood samples were collected from the jugular vein before and after the glucose injection. Glucose (A) and insulin (B) concentrations in the plasma were measured. Two-way ANOVA p values were <0.01 (Tr), <0.01 (Ti) and 0.05 for (Tr × Ti) for insulin (B). Values are displayed as means ± SEM (n = 5–9). Plots at the same time point that do not share the same letter differ significantly between treatments (Tukey's test; $p < 0.05$).

Fig. 5. Effects of in vitro digestion and the amino acid mixture of ZeinH on its GLP-1-releasing activity in enteroendocrine GLUTag cells

615 GLUTag cells were exposed to intact or pepsin/pancreatin-treated ZeinH (A), pronase-treated ZeinH
616 (B) at 10 mg/ml, or the amino acid mixture (C) equivalent to 10 mg/ml of ZeinH for 60 min. KCl (70
617 mM NaCl was replaced with 70 mM KCl in the HEPES buffer) solution was used as positive control
618 that induces GLP-1 secretion via depolarization. The amino acid mixture (AA mix) was prepared to
619 reconstitute the total amino acid composition of 10 mg/ml of ZeinH. NaCl (31 mM) was added to the
620 control buffer to assess the osmotic effect of the amino acid mixture (C). The supernatant was collected,
621 and the GLP-1 concentration was measured. Values are expressed as means ± SEM (n = 4). Plots at the
622 same time point that do not share the same letter differ significantly between treatments (Tukey's test;
623 $p < 0.05$).
624
625 **Fig. 6. Glycemic and incretin responses to oral ZeinH or Whey hydrolysate under OGTT in GK**
626 **rats**
627 Glucose solution (8 ml/kg) as control (open circles, 2 g/kg), glucose solution containing ZeinH at 2
628 g/kg (closed circles), or glucose solution containing WheyH at 2 g/kg (open triangles) were
629 administered orally. Blood samples were collected from the jugular vein before and after the oral
630 administration, and glucose (A), insulin (B), total GLP-1 (C), active GLP-1 (D), and total GIP (E)
631 concentrations in the plasma were measured. Two-way ANOVA p values were 0.02 (Tr), <0.01 (Ti),
632 and 0.02 (Tr × Ti) for insulin (B); <0.01 (Tr), <0.01 (Ti) and 0.07 (Tr × Ti) for total GLP-1 (C); 0.60
633 (Tr), 0.08 (Ti), 0.96 (Tr × Ti) for active GLP-1 (D); 0.38 (Tr), <0.01 (Ti), <0.01 (Tr × Ti) for total GIP
634 (E). Values are displayed as means ± SEM (n = 6–7). Plots with asterisk signs (*) indicate significant
635 differences compared to basal (0 min) values within each treatment (Dunnett's test; $p < 0.05$). Plots at
636 the same time point that do not share the same letter differ significantly between treatments (Tukey's
637 test; $p < 0.05$).

Figure 1

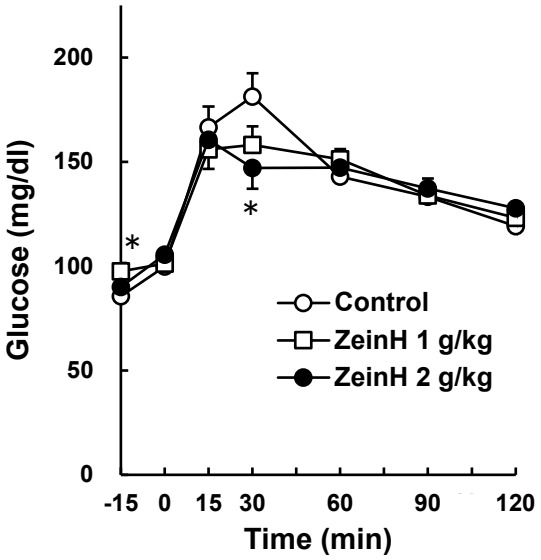

Glucose (mg/dl)
200
150
100
50
0
-15
0
15
30
60
90
120
Time (min)
*
*
Control
ZeinH 1 g/kg
ZeinH 2 g/kg

Figure 2

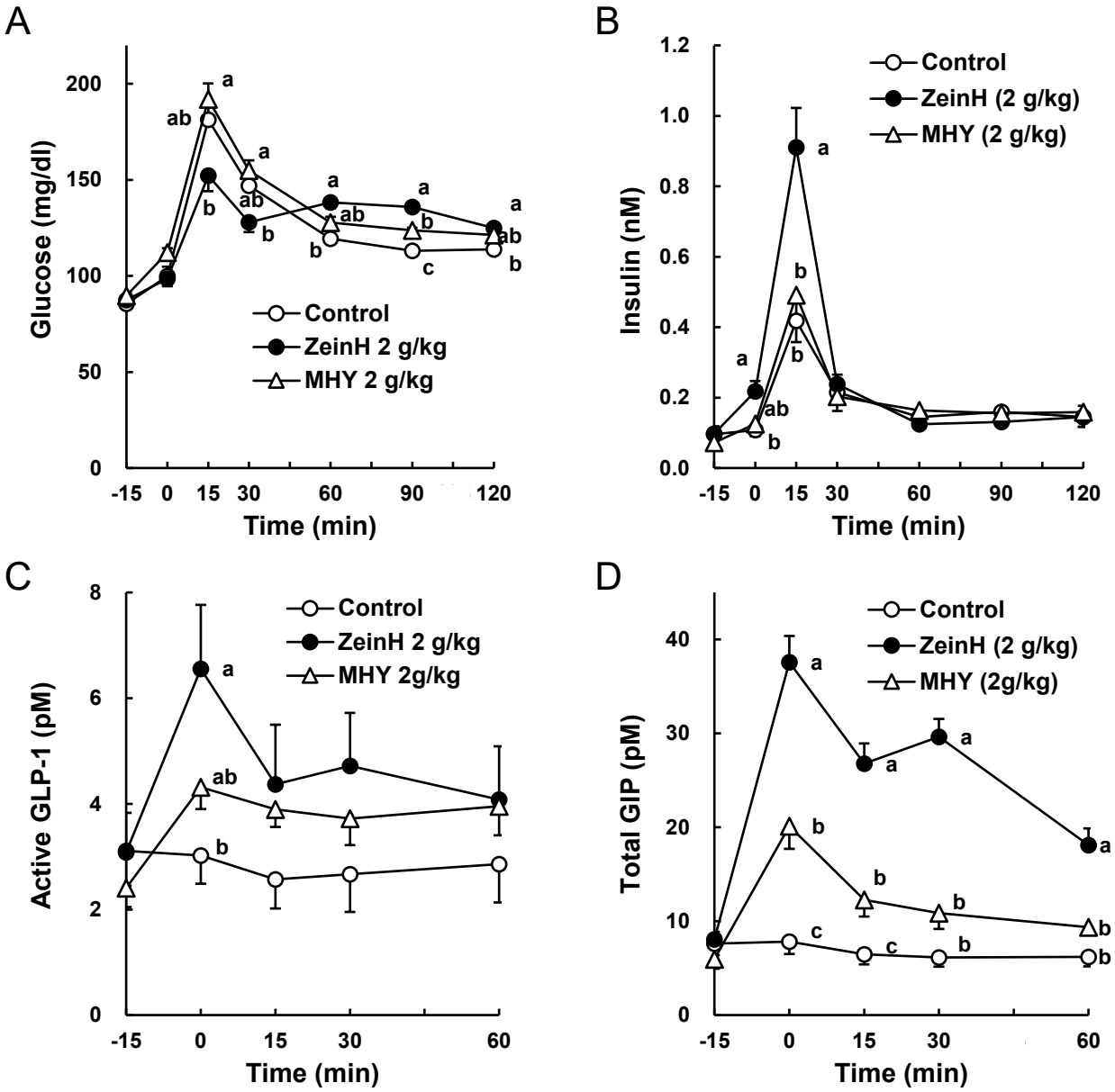
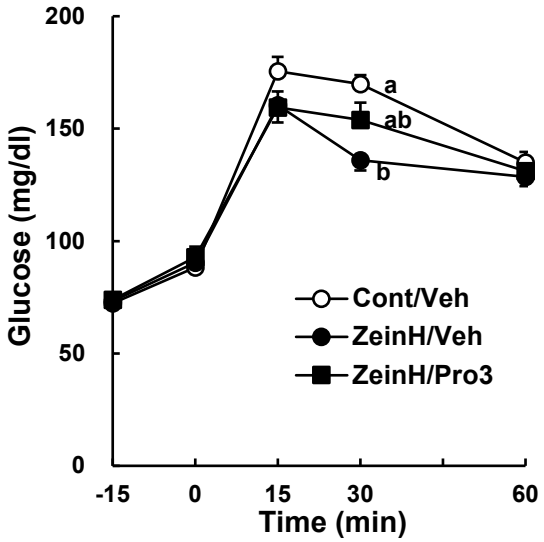

A
Glucose (mg/dl)
Time (min)
Control
ZeinH 2 g/kg
MHY 2 g/kg
B
Insulin (nM)
Time (min)
Control
ZeinH (2 g/kg)
MHY (2 g/kg)
C
Active GLP-1 (pM)
Time (min)
Control
ZeinH 2 g/kg
MHY 2g/kg
D
Total GIP (pM)
Time (min)
Control
ZeinH (2 g/kg)
MHY (2g/kg)

Figure 3

A


200
150
100
50
0
Glucose (mg/dl)
-15
0
15
30
60
Time (min)
a
ab
b
Cont/Veh
ZeinH/Veh
ZeinH/Pro3

B

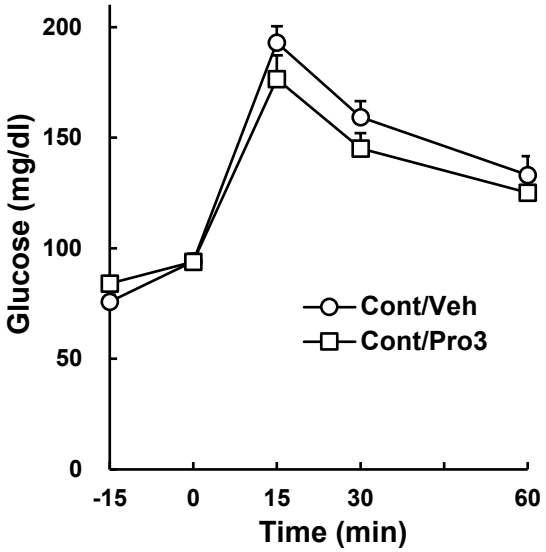

200
150
100
50
0
Glucose (mg/dl)
-15
0
15
30
60
Time (min)
Cont/Veh
Cont/Pro3

Figure 4

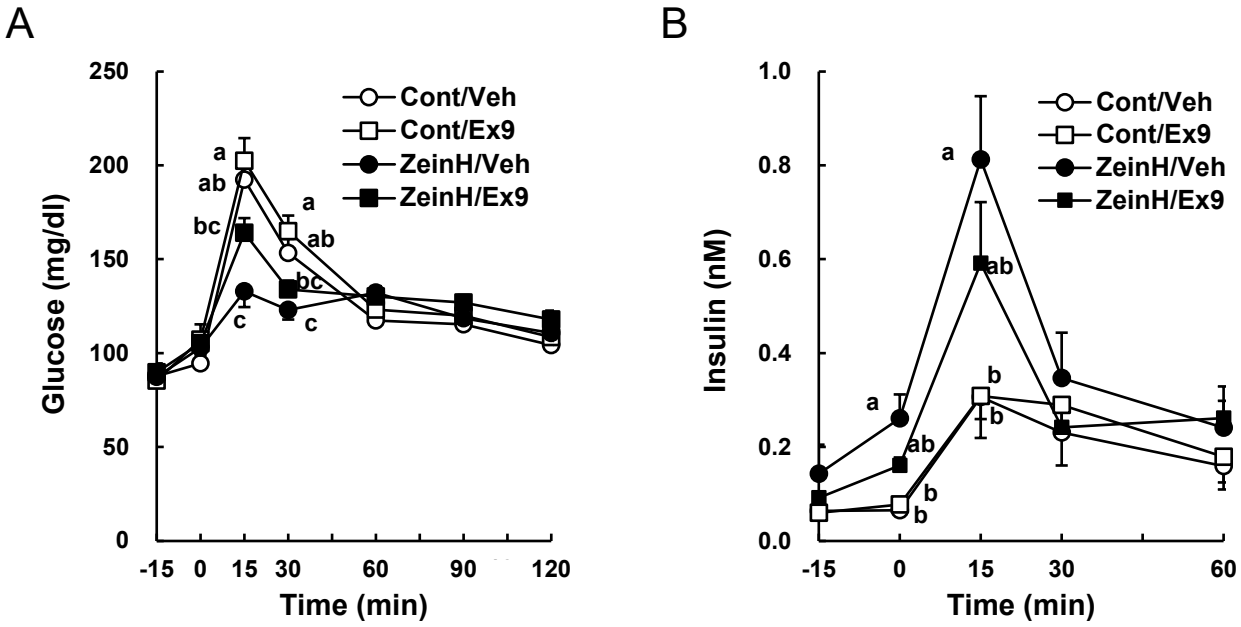

A
Glucose (mg/dl)
250
200
150
100
50
0
-15
0
15
30
60
90
120
Time (min)
Cont/Veh
Cont/Ex9
ZeinH/Veh
ZeinH/Ex9
a
ab
bc
a
ab
bc
c
c
B
Insulin (nM)
1.0
0.8
0.6
0.4
0.2
0.0
-15
0
15
30
60
Time (min)
Cont/Veh
Cont/Ex9
ZeinH/Veh
ZeinH/Ex9
a
ab
a
ab
b
b
b
b

Figure 5

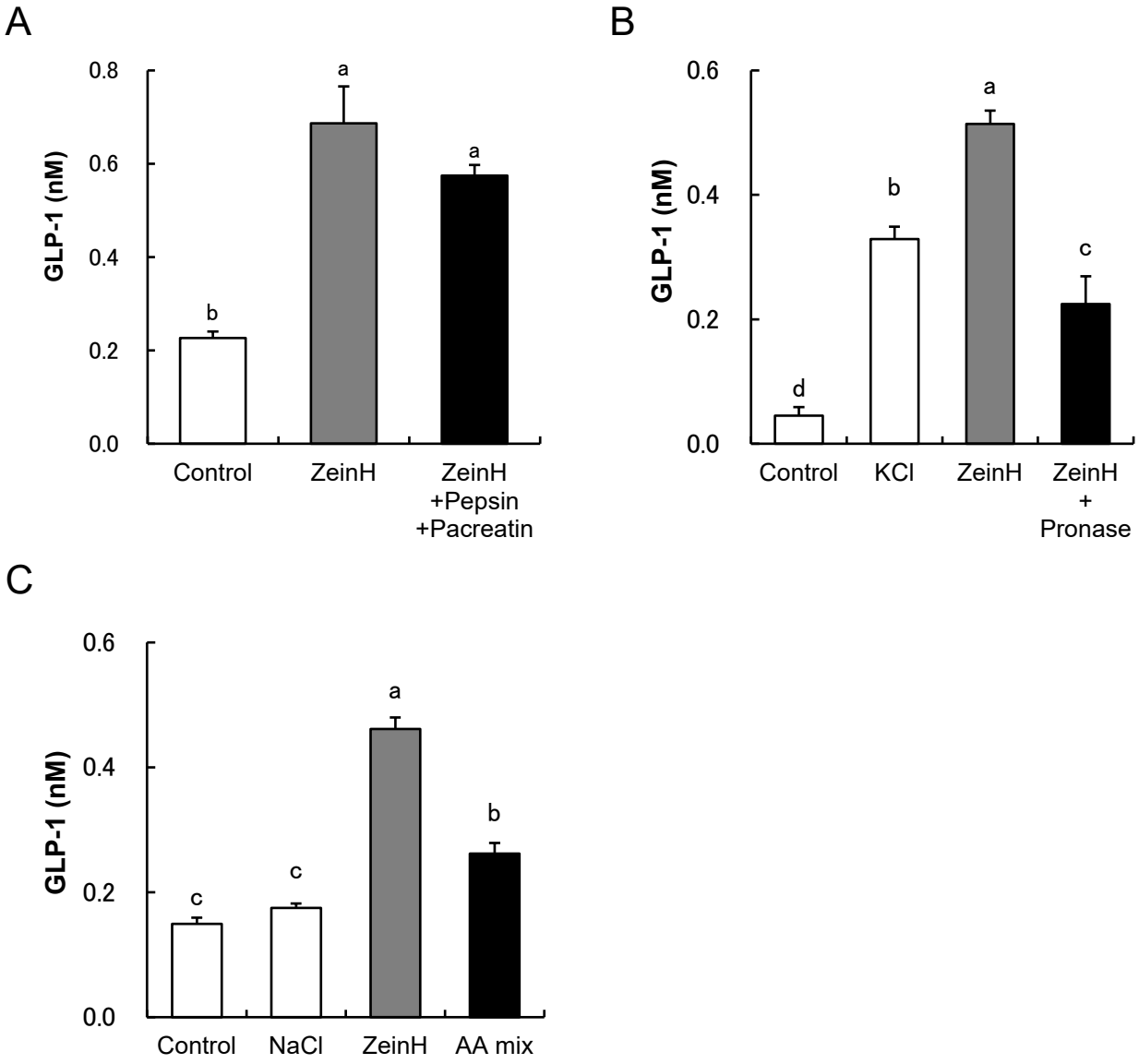
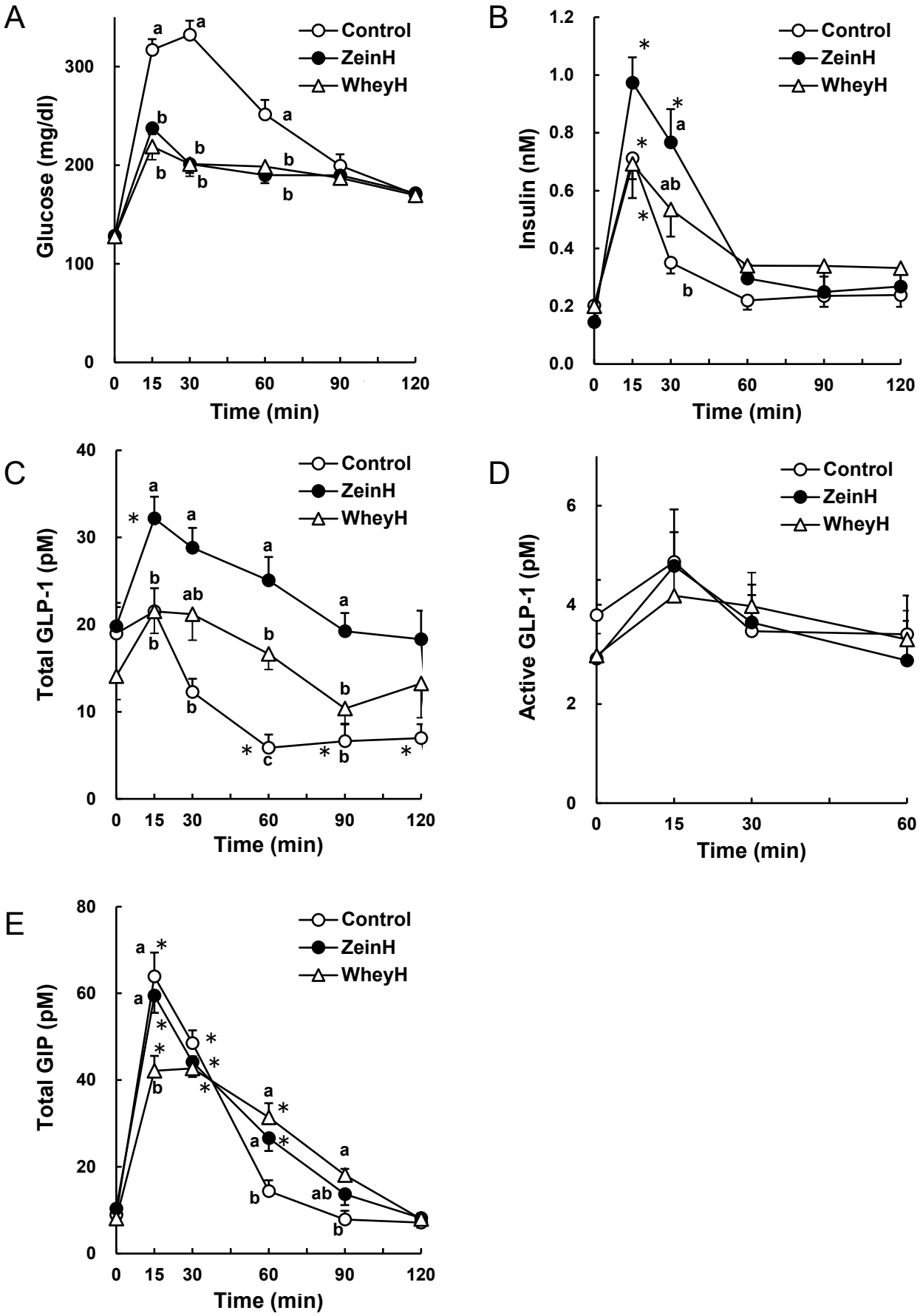

A
GLP-1 (nM)
0.8
0.6
0.4
0.2
0.0
a
a
b
Control
ZeinH
ZeinH
+Pepsin
+Pacreatin
B
GLP-1 (nM)
0.6
0.4
0.2
0.0
a
b
c
d
Control
KCl
ZeinH
ZeinH
+
Pronase
C
GLP-1 (nM)
0.6
0.4
0.2
0.0
a
b
c
c
Control
NaCl
ZeinH
AA mix

Figure 6


A
Glucose (mg/dl)
Time (min)
Control
ZeinH
WheyH
B
Insulin (nM)
Time (min)
Control
ZeinH
WheyH
C
Total GLP-1 (pM)
Time (min)
Control
ZeinH
WheyH
D
Active GLP-1 (pM)
Time (min)
Control
ZeinH
WheyH
E
Total GIP (pM)
Time (min)
Control
ZeinH
WheyH