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Citation	Biochemical and biophysical research communications, 661, 28-33 https://doi.org/10.1016/j.bbrc.2023.04.042
Issue Date	2023-04-16
Doc URL	http://hdl.handle.net/2115/91676
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2 Trp-Tyr is a dipeptide structure that potently stimulates GLP-1 secretion in a murine enteroendocrine
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16

17 **Highlights**

- 18 • Structure-activity relationship in peptide-induced GLP-1 secretion is unknown
- 19 • Dipeptide library was used to identify potent GLP-1-releasing dipeptides
- 20 • Nine of 339 dipeptides had potent GLP-1 releasing activity, with Trp-Tyr (WY) most
- 21 • Addition of Phe to N- or C- terminus of WY further enhanced the potency of WY
- 22 • WY is a specific dipeptide sequence that potently stimulates GLP-1 secretion

23

24 **Abstract**

25 Dietary peptides potently stimulate glucagon-like peptide-1 (GLP-1) secretion, however, the
26 underlying molecular mechanisms, such as structure-activity relationships and sensing mechanisms
27 are only partly elucidated. In this study, we used a dipeptide library to identify dipeptides that potently
28 stimulate GLP-1 release and to clarify the underlying structure-activity relationship.

29 Murine enteroendocrine GLUTag cells were exposed to 339 dipeptides for 60 min, and the
30 concentration of GLP-1 released into the supernatant was measured. Subsequently, selected
31 dipeptides were examined for their reproducibility and dose responsiveness. In addition, we
32 investigated the role of constituent amino acids in the secretion of GLP-1, and whether tripeptides
33 containing the active dipeptide structures maintained their activity.

34 In a concentration range of 1– 5 mg/mL, twelve dipeptides had reproducible and concentration-
35 dependent GLP-1-releasing activity. Among them, nine dipeptides (FY, KF, NI, PM, QL, QY, WF,
36 WN, WY) were novel, with WY exhibiting the most potent activity. The reverse sequences and most
37 free amino acids did not induce GLP-1 secretion, indicating that GLP-1-producing cells recognize
38 the structure of each peptide to induce GLP-1 secretion. However, no apparent similarities were
39 found between the active peptides. A comparison between the six tripeptides composed of F, W,
40 and Y revealed the further potent tripeptides FWY and WYF, than WY.

41 In the present study, a comprehensive analysis revealed nine novel dipeptides with high potential
42 to stimulate GLP-1 secretion. Furthermore, the results indicate that 'WY' is a specific dipeptide
43 sequence that potently stimulates GLP-1 secretion.

45 **Key words**

46 glucagon-like peptide-1, dipeptides, tripeptides, dietary peptides

48 **Introduction**

49 The secretion of gut hormones including cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-
50 1), is stimulated by meal ingestion. Among nutrients, monosaccharides (glucose and fructose), fatty
51 acids, peptides and amino acids induce GLP-1 secretion [1]. GLP-1 is an incretin hormone that
52 enhances glucose-induced insulin secretion. Owing to the incretin effect, GLP-1 receptor agonists
53 (GLP-1RAs) and dipeptidyl peptidase (DPP)-4 inhibitors are widely used in the treatment of type-2
54 diabetes. Besides its incretin effect, GLP-1 has multiple physiological functions including beta cell
55 proliferation/protection, satiety induction, and glucagon secretion suppression [2].

56 Since GLP-1 secretion is stimulated by meal ingestion, researchers have been investigated t gated
57 the positive modulation of GLP-1 secretion through multiple dietary components. Using *in vitro* and *in*
58 *vivo* models, we have demonstrated that various dietary proteins/peptides can enhance GLP-1
59 secretion and subsequently attenuate the *in vivo* glycemic response [3,4,5]. However, the active
60 peptides have not been identified in a majority of the studies. Compared to polysaccharides (e.g.,
61 starch) and triglycerides, dietary proteins can release large quantities of distinct fragments, i.e.,
62 peptides, in the gastrointestinal lumen during the digestion process.

63 Although peptidomic analysis using LC-MS/MS is likely a valuable strategy to comprehensively
64 decode peptide fragment sequences from protein hydrolysate, the number of peptide fragments
65 identified with GLP-1 releasing activity remains limited [6-10]. This includes both natural and synthetic
66 peptides, with less than 30 identified thus far [10,11], as summarized in a supplementary Table (Table
67 S1). The identified fragments do not appear similar in terms of structures or characteristics, such as
68 molecular size and amino acid composition or sequences. Therefore, these peptides might stimulate
69 GLP-1 secretion through mechanisms that are either specific to the respective peptide or based on
70 an unidentified mutual characteristic. Consequently, the structure-activity relationship between
71 peptides and their ability to induce GLP-1 release remains unclear.

72 In the present study, we aimed to identify peptide sequences responsible for stimulating GLP-1
73 secretion, primarily via a dipeptide library containing 336 dipeptides. The library was employed
74 previously to identify ghrelin releasing peptides [12]. GLP-1-releasing activity of dipeptides was
75 evaluated in a murine GLP-1-producing enteroendocrine cell line GLUTag. We further investigated
76 the role of free amino acids that compose active dipeptides, and the role of amino acid sequence in
77 tripeptides that contain active dipeptides identified in the present study.

79 **Materials and methods**

80 Cell culture consumables, such as Dulbecco's modified Eagle's medium (DMEM) and fetal bovine
81 serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The dipeptide library was
82 purchased from AnaSpec (Fremont, CA, USA). Dipeptides GW (Gly-Trp), KA (Lys-Ala), KF (Lys-Phe),
83 PM (Pro-Met), QG (Gln-Gly), QK (Gln-Lys), QL (Gln-Leu), QY (Gln-Tyr), YK (Tyr-Lys), and tripeptides
84 FWY (Phe-Trp-Tyr), FYW (Phe-Tyr-Trp), WFY (Trp-Phe-Tyr), WYF (Trp-Tyr-Phe), YFW (Tyr-Phe-
85 Trp), YWF (Tyr-Trp-Phe) were synthesized by Greiner Bio-One Co. (Tokyo, Japan). NI (Asn-Ile), WY
86 (Trp-Tyr), WN (Trp-Asn) were purchased from AS ONE (Osaka, Japan); FY (Phe-Tyr) from Combi-
87 Blocks, Inc. (San Diego, CA, USA); VY (Val-Tyr) from Watanabe Chemical Industries, Ltd. (Hiroshima,
88 Japan); WF (Trp-Phe) from Bachem AG (Bubendorf, Switzerland); GL (Gly-Leu) from Peptide Institute
89 Inc. (Ibaraki-shi, Japan). Unless otherwise specified, all other reagents were purchased from
90 FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) or Sigma Aldrich (St. Louis, MO, USA).

92 **Cell culture**

93 GLUTag cells (a gift from D. J. Drucker, University of Toronto, Canada) were grown in DMEM (Cat.
94 No. 12100-038) supplemented with 10% FBS, 50 IU/ml penicillin, and 500 µg/ml streptomycin in a
95 humidified 5% CO₂ atmosphere at 37°C. Cells were routinely subcultured by trypsinization upon
96 reaching 80%–90% confluence. Cells at passage numbers 20–40 were used for the experiments.

98 **GLP-1 secretion study**

99 GLUTag cells were seeded at a density of 1.0×10^5 cells/well in a 96- or 48-well plate (depending
100 on the experiment) and grown for 2 days till they reached 80–90% confluency [3]. For the dipeptide
101 library-based assay, a 96-well plate was used since the library contained 1 mg of each peptide.
102 Commercially available dipeptides, FP (Phe-Pro), GL (Gly-Leu), and PL (Pro-Leu) were included in
103 the study. In subsequent studies, 48-well plate was used. Cells were washed twice with HEPES buffer
104 to remove the culture medium and then exposed to the test agents dissolved in HEPES buffer for
105 60 min at 37°C. When hydrophobic peptides were examined, 0.2% DMSO was added to improve their
106 solubility. Neither GLP-1-releasing nor cytotoxic effects were observed under the experimental
107 conditions. The HEPES buffer (pH 7.4) had the following composition: 140 mM NaCl, 4.5 mM KCl,
108 20 mM HEPES, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM D-glucose, and 0.1% BSA. Following incubation,
109 the supernatants were collected and centrifuged at 800 g for 5 min at 4°C to remove the remaining

110 cells. The supernatants were then stored at -30°C for the measurement of GLP-1 using an ELISA
111 kit (GLP-1 ELISA Kit Wako, High Sensitive, FUJIFILM Wako Pure Chemical Corporation, Osaka,
112 Japan).

113

114 Measurement of cytotoxicity

115 The cytotoxic effects on GLUTag cells were determined by measuring the release of lactate
116 dehydrogenase (LDH) from the cells into the supernatant after the cells were exposed to the test
117 agents as described earlier [13]. LDH activity was measured using a commercial kit (Cytotoxicity LDH
118 Assay Kit-WST, Dojindo, Mashiki, Kumamoto, Japan), according to the manufacturer's instructions.
119 Cytotoxicity was calculated as the relative release (%) of LDH after exposure to the test agents
120 compared to the total LDH (100%) released upon treatment with the lysis reagent supplied in the kit.

121

122 Statistical analysis

123 The results are expressed as the mean \pm SEM. Statistical analyses were performed using JMP
124 Pro version 16.0.0 software (SAS Institute, Inc., Cary, NC, USA). Statistically significant difference
125 was determined using one-way analysis of variance (ANOVA) followed by Tukey–Kramer's test or
126 Dunnett's test, as stated in each figure legend. In all analyses, $p < 0.05$ was considered statistically
127 significant.

128

129 **Results and discussion**

130 The potential of stimulating GLP-1 secretion of 339 dipeptides (at 5 mg/mL) was examined. As
131 shown in supplementary figures (Fig. S1A-S), only a limited number of dipeptides increased GLP-1
132 concentrations in the supernatant after exposure to GLUTag cells for 1 hour. In total, 20 dipeptides
133 increased GLP-1 concentrations more than 1.5-fold compared to the control, as summarized in Fig.
134 1. These results indicate that GLUTag cells do not respond broadly to dipeptides, but are able to
135 distinguish each dipeptide, probably based on their structures (sequences).

136

137 To validate their GLP-1 releasing activity, these 20 dipeptides were respectively procured as single
138 peptides, not from the library. Their GLP-1 releasing activity was further investigated on a relatively
139 larger experimental scale (48-well plate) than the library-based study (96-well plate). GL and GW were
140 included because these peptides induced GLP-1 secretion in our preliminary study. Among the 22
141 peptides, 10 peptides at a dose of 5 mg/mL significantly increased GLP-1 concentrations in the
142 supernatant compared to that in the blank control (Fig. 2). Among the 10 positive peptides, WY was
143 observed to demonstrate highest potency to induce GLP-1 secretion. The reason behind the absence
144 of GLP-1 releasing activity in 12 of the 22 peptides in the second study could not be identified. None
145 of these dipeptides demonstrated any cytotoxic effect, as determined by LDH release assay (data not
146 shown).

147

148 The dose-response study further confirmed the GLP-1 releasing activity of 12 dipeptides (Fig. 3).
149 Majority of the dipeptides demonstrated the highest potency at a concentration of 5 mg/mL; however,
150 WF demonstrated higher activity at 2 mg/mL than at 5 mg/mL. This could be the reason that WF failed
151 to induce a significant increase of GLP-1 release at 5 mg/mL (Fig. 2B). Again, WY had the highest
152 potency among the dipeptides at a dose of 5 mg/mL. NI induced significant GLP-1 secretion even at
153 a low dose (1 mg/mL), while further increments in GLP-1 concentration were not observed by
154 increasing the dose (2 and 5 mg/mL). The potency is not very high, but NI is a specific peptide having
155 relatively low threshold to induce GLP-1 secretion.

156

157 The results of study investigating reproductivity and dose response were summarized in Fig. 4.
158 Although N-terminal Trp (WF, WN, WY) and C-terminal Tyr (FY, QY, WY) seems to be partly
159 associated with the GLP-1-releasing activity, there are no apparent mutual characteristics between
160 the 12 active peptides. GL [14], GW [15], KA [6], but not other nine dipeptide sequences, are included
161 in short peptides previously reported to have GLP-1-releasing activity in different cell models. Thus,
162 these nine dipeptides (FY, KF, NI, PM, QL, QY, WF, WN, WY) are newly identified to induce GLP-1
163 release, at least in GLUTag cells.

164 Previously, ghrelin release-modulating dipeptides were identified by using a dipeptide library, in
165 ghrelin-producing MGN3-1 cells [12]. Among 336 peptides, N-terminal Ser-containing dipeptides play
166 a dominant role in stimulating ghrelin secretion. They found Ser-Val (SV) most strongly stimulates,
167 whereas Leu-Ile (LI) suppresses ghrelin secretion. Thus, the peptides identified in the previous study
168 are not similar to the ones identified in the present study, indicating that specific enteroendocrine cells,
169 such as ghrelin-producing and GLP-1-producing cells, equip different peptide-sensing mechanisms.

170

171 Combinations of free amino acids (corresponding to 5 mg/mL of active dipeptides, except for K+A
172 and G+W) did not induce GLP-1 secretion (Fig. 5A), suggesting that free amino acids possibly
173 liberated by extra- or intra-cellular digestion from these peptides are not responsible for the GLP-1-
174 releasing activity of dipeptides. Dipeptides having reversed sequences of the active peptides did not
175 induce GLP-1 secretion in the library-based study (e.g., WY vs YW), supporting the notion that the
176 cells sense the dipeptide structure, instead of the individual constituent amino acids.

177 In additional experiments, the combination of K+A and G+W again induced GLP-1 secretion (Fig.
178 5B and C). However, single amino acids, Ala and Gly, but not Lys and Trp, also induced GLP-1
179 secretion equivalent to the respective combinations (K+A and G+W). These results suggest that Ala
180 [16,17] and Gly [18] are solely able to stimulate GLP-1 secretion via mechanisms distinct from those
181 underlying dipeptide-induced GLP-1 secretion. Some amino acids such as Asn, Gln, Met, Phe, Pro
182 and Trp reportedly stimulate GLP-1 secretion in enteroendocrine cell models [17,19]. The failure of
183 these amino acids to stimulate GLP-1 secretion in the present study could be attributed to differences
184 in experimental conditions.

185

186 Because WY demonstrated potent activity throughout the present study, we further explored the
187 role of the sequence in the GLP-1 releasing activity. We examined the GLP-1 releasing activity of six
188 tripeptides composed of Trp (W), Tyr (Y) and Phe (F). Phe (F) was selected to combine with Trp (W)
189 and Tyr (Y) because three of 12 active dipeptides contained Phe (FY, KF, WF). At a dose of 5 mM (2.6
190 mg/mL), FWY and WYF potently (> 6-folds vs control) induced GLP-1 secretion (Fig. 6A). YWF at 5
191 mM (Fig. 6A) had also an adequately potent effect (4-folds vs control) on stimulating GLP-1 secretion.
192 At 1 mM (0.5 mg/mL), YFW significantly, while FWY slightly increased GLP-1 secretion. We observed
193 no increment in LDH release in the experiment (Fig. 6B). WY at 5 mM (1.85 mg/mL) did not
194 significantly increase the GLP-1 concentration, but the relative increments (1.2-folds) compared to
195 the blank control are within the range expected from the dose (1 and 2 mg/mL)-response study (Fig.
196 3). These results demonstrate that FWY and WYF have potent GLP-1 releasing activity in vitro, and
197 the core sequence "WY" is critical for stimulating GLP-1 secretion. It is very interesting to investigate
198 whether these peptides exert GLP-1 releasing activity in vivo models. Oral administration of WY
199 derived from beta lactoglobulin and related peptides (GTWY, WM, WV, WL) reportedly prevents
200 cognitive decline via suppressing microglial activation [20,21]. Such an effect may partly attribute to
201 the neuroprotective effect of GLP-1 [1,2] whose secretion is induced by WY peptide.

202

203 There are several limitations in the present study. First, some dipeptides and dipeptides
204 containing cysteine were not included due to limited availability. Second, the molecular mechanisms
205 underlying the effect of each or a group of peptides remain unclear. Because a limited number of
206 peptides induced GLP-1 secretion in the present study and oligopeptide transporter 1 (Pept1) is barely
207 detectable in GLUTag cells [22], the dipeptide-induced GLP-1 secretion would not be attributed to
208 Pept1. Third, in vivo effects of the active dipeptides on GLP-1 secretion are unknown. It is assumed
209 that orally administered di- and tri-peptides are unable to stimulate GLP-1-producing cells primarily
210 located in the distal small intestine, because such small peptides would be absorbed or digested in
211 the proximal small intestine. However, longer and slowly digestible peptides containing these
212 sequences may stimulate GLP-1 secretion in the intestine. To address these concerns, further studies
213 are necessary in the future.

214

215 In conclusion, we discovered novel nine dipeptides (FY, KF, NI, PM, QL, QY, WF, WN, and WY)
216 that have GLP-1-releasing activity by the comprehensive analysis using a dipeptide library. Although
217 no apparent mutual characteristics were identified in these active peptides, the WY peptide
218 demonstrated the highest potency. Furthermore, adding Phe residue on either the N- or C-terminal
219 side of WY largely potentiated the GLP-1-releasing activity. These findings provide novel insight into
220 the peptide-sensing mechanisms in enteroendocrine cells and a translational perspective that dietary
221 proteins/peptides or compounds having these novel peptide structures prevent glucose intolerance
222 and/or overeating through effectively stimulate GLP-1 secretion.

223

224 **Acknowledgements**

225 We thank The Tojuro Iijima Foundation for Food Science and Technology, Kieikai Research
226 Foundation, and Urakami Foundation for Food and Food Culture Promotion, for financially supporting
227 this research project.

228

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302
303

304 **Figure Legends**

305 **Fig. 1. GLP-1 secretory response to 339 dipeptides in GLUTag cells**

306 GLUTag cells were exposed to dipeptide solutions (5 mg/mL) for 60 min. Values are expressed as
307 means of GLP-1 levels relative to the blank control (n=3, expect NI). Two of three supernatants
308 collected from NI treated cells had very high optical absorbance (> 3.0) in GLP-1 ELISA. Relative
309 GLP-1 levels higher than 1.5 are highlighted by black background and white letters. Capital letters
310 indicate N- and C-terminal amino acids within the dipeptides, respectively. Gray cells indicate
311 unavailable dipeptides.

312

313 **Fig. 2. GLP-1 secretory response to selected dipeptides in GLUTag cells**

314 GLUTag cells were exposed to dipeptide solutions (5 mg/mL) for 60 min. Values are means \pm SEM
315 expressed as GLP-1 levels relative to the blank control (n=3). Asterisks indicate significant differences
316 compared to the blank control (P<0.05, Dunnett's test).

317

318 **Fig. 3. Dose-responsive effects of dipeptides on GLP-1 secretion**

319 GLUTag cells were exposed to dipeptide solutions (1, 2, 5 mg/mL) for 60 min.
320 Values are means \pm SEM expressed as GLP-1 levels relative to the blank control (n=3). Bars not
321 sharing the same letter indicate significant differences (P<0.05, Tukey's HSD test).

322

323 **Fig. 4. Dipeptides with confirmed GLP-1-releasing effect in GLUTag cells**

324 Dipeptides showing significant increase in GLP-1 secretion effect are highlighted by black cells.

325

326 **Fig. 5. GLP-1 secretory response to various combinations of amino acids composing selected 327 dipeptides, in GLUTag cells**

328 GLUTag cells were exposed to amino acid solutions for 60 min. (A) Each amino acid solution had a
329 concentration of 20 mM. For example, F+Y was prepared to have both of amino acid at 20 mM of Phe
330 (3.3 mg/mL) and Tyr (3.6 mg/mL). (B) Gly at 20 mM, Trp at 20 mM and their mixture. (C) Lys at 20
331 mM, Ala at 20 mM and their mixture. Values are means \pm SEM expressed as GLP-1 levels relative to
332 the blank control (n=3). Asterisks indicate significant differences compared to the blank control
333 (P<0.05, Dunnett's test). Bars not sharing the same alphabet indicate significantly different (P<0.05,
334 Tukey's HSD test).

335

336 **Fig. 6. GLP-1 secretory responses (A) and LDH release to 6 tripeptides composed of Trp (W),**

337 **Tyr (Y), and Phe (F) in GLUTag cells**

338 GLUTag cells were exposed to peptide solutions for 60 min. Tripeptides were examined at 1 mM (0.5
339 mg/mL) and 5 mM (2.6 mg/mL). Values are means \pm SEM expressed as GLP-1 (A) and LDH (B) levels
340 relative to blank control (n=3). Bars not sharing the same alphabet indicate significantly different
341 (P<0.05, Tukey's HSD test).

342

Fig. 1

		N-terminal																			
C-terminal		A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
	A	0.69	1.85	1.03	0.60	0.53	1.28	0.42	1.52	0.70	1.03	0.33	0.58	0.17	0.51	0.38	0.81	0.76	0.66	0.58	A
	D	0.97	0.99	0.97	0.47	0.54	0.72	0.42	0.69	0.90	1.04	0.06	0.19	0.17	0.10	0.25	0.20	0.37	1.01	0.52	D
	E	0.64		0.89	0.56	2.10	0.34	0.84	0.81	0.69	0.96	0.69	0.71	0.23	0.27	0.14	1.08	0.46	0.51	0.29	E
	F	0.57	1.60		0.71	0.78	0.46	0.28	1.66	0.43	0.69	1.07	1.11	1.18	1.22	0.20	0.36	0.57	1.86	0.91	F
	G	0.56	0.97	0.33	0.54	0.92			1.04	0.70	0.81	0.72	0.25	1.68	0.97		0.96	0.69	0.59	0.90	G
	H	0.61	1.23	1.46	0.81	1.41	0.48	0.50	1.04	0.64	1.35	0.78	0.60	0.72	0.92	0.67	0.42	0.56	0.74	0.80	H
	I	0.56		0.45	0.63	1.34	0.49	0.48	0.73	0.69	1.17	7.65	0.65	0.90	0.63	0.26	0.50	0.61	0.47	0.58	I
	K	0.80	1.20	0.34	0.50		1.44	0.65	0.91		0.88	0.45	1.36	1.52	0.67	0.60	0.66	1.16	0.60	15.00	K
	L	1.14	0.72	2.95	0.54	0.97	1.23	0.62	1.15	0.43	0.86	0.99	0.91	1.92	0.40	0.59	1.24	1.01	1.02	0.80	L
	M	0.77	1.42	0.50	0.65	0.66	1.24	0.68	0.93	0.56	0.88	0.72	3.23	1.41	0.31	0.39	0.76	0.84	1.00	0.42	M
	N		2.63	0.54	0.71	0.84	1.00	0.61	0.87	0.79	1.17	0.41	1.41	0.92	0.47	0.47	0.41	0.74	2.47	0.81	N
	P	1.32	1.35	0.93	0.85	0.73	0.72	0.58	1.34			1.03	0.74	0.23	1.19	0.47	0.21	0.62	0.60	0.92	P
	Q	1.02	0.67	0.27	0.47	0.85		0.58	0.71		1.41	0.75	0.48	1.28		0.61	0.42	0.68	0.74	0.95	Q
	R	0.77	0.75	0.50	1.02	1.33	0.41	0.45	1.36		0.86	0.50			0.19	0.35	0.46	0.57	0.75	0.64	R
	S	0.84	1.46	0.35	0.55	1.04	0.97	0.70	0.91		0.94		0.70	0.61	0.92	0.46	0.52	0.80	0.76	1.07	S
	T	0.72	0.93	1.04	0.42	0.67	0.82	0.52	0.93	0.97	0.77	0.29	0.82	0.53	0.18	0.42	0.66	0.65	0.84	1.18	T
	V	0.74	1.87	0.30	0.54	0.46	0.38	0.60	1.11	0.45	0.82	0.35	0.79	0.55	0.36	0.48	0.36		0.86	1.24	V
	W	0.83	0.74	0.33	1.26	0.65	0.87	0.46	0.79	0.47	1.00	0.69	1.32	0.59	0.70	0.86	0.87	0.91	1.34	1.45	W
	Y	0.91	0.41	0.52	2.38	0.97	0.75		1.07	0.67	0.78	0.46	1.39	4.91		1.04	0.14	2.00	3.05	0.82	Y
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y		

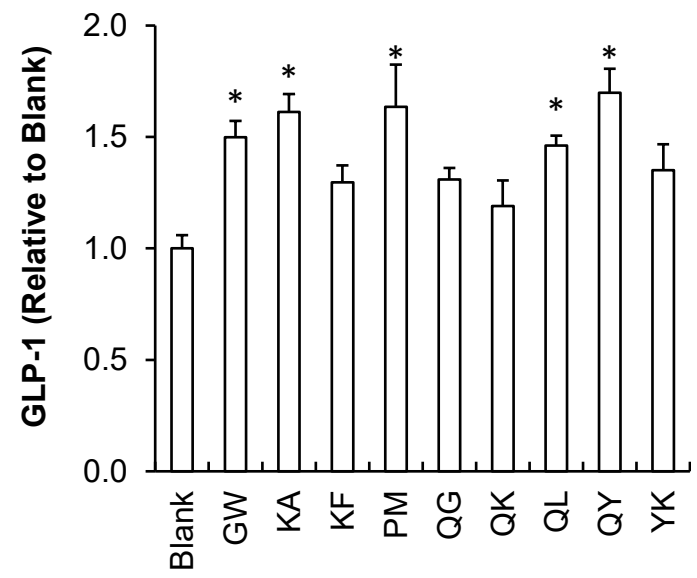
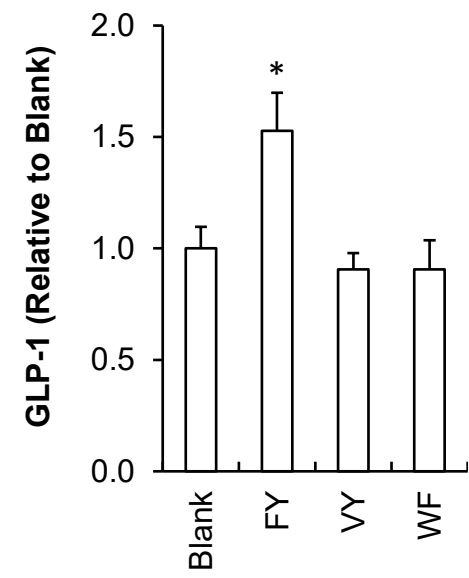
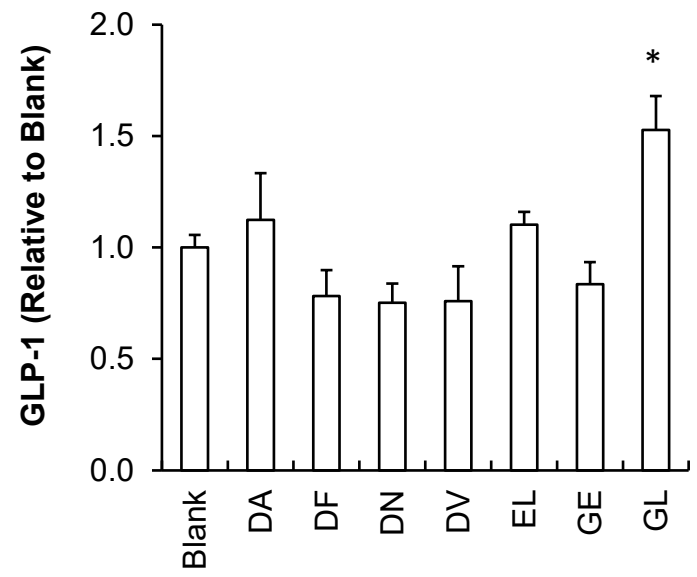
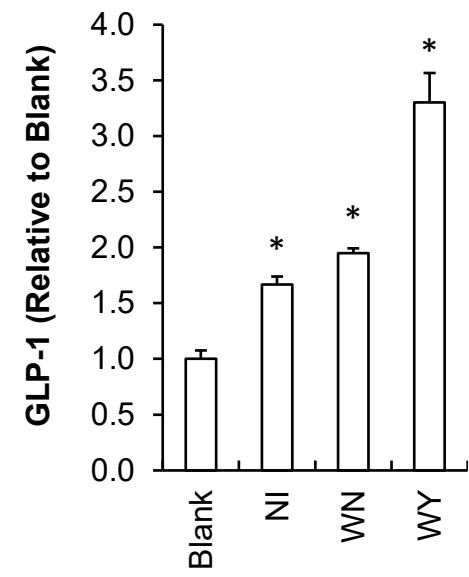
Fig. 2**A****B****C****D**

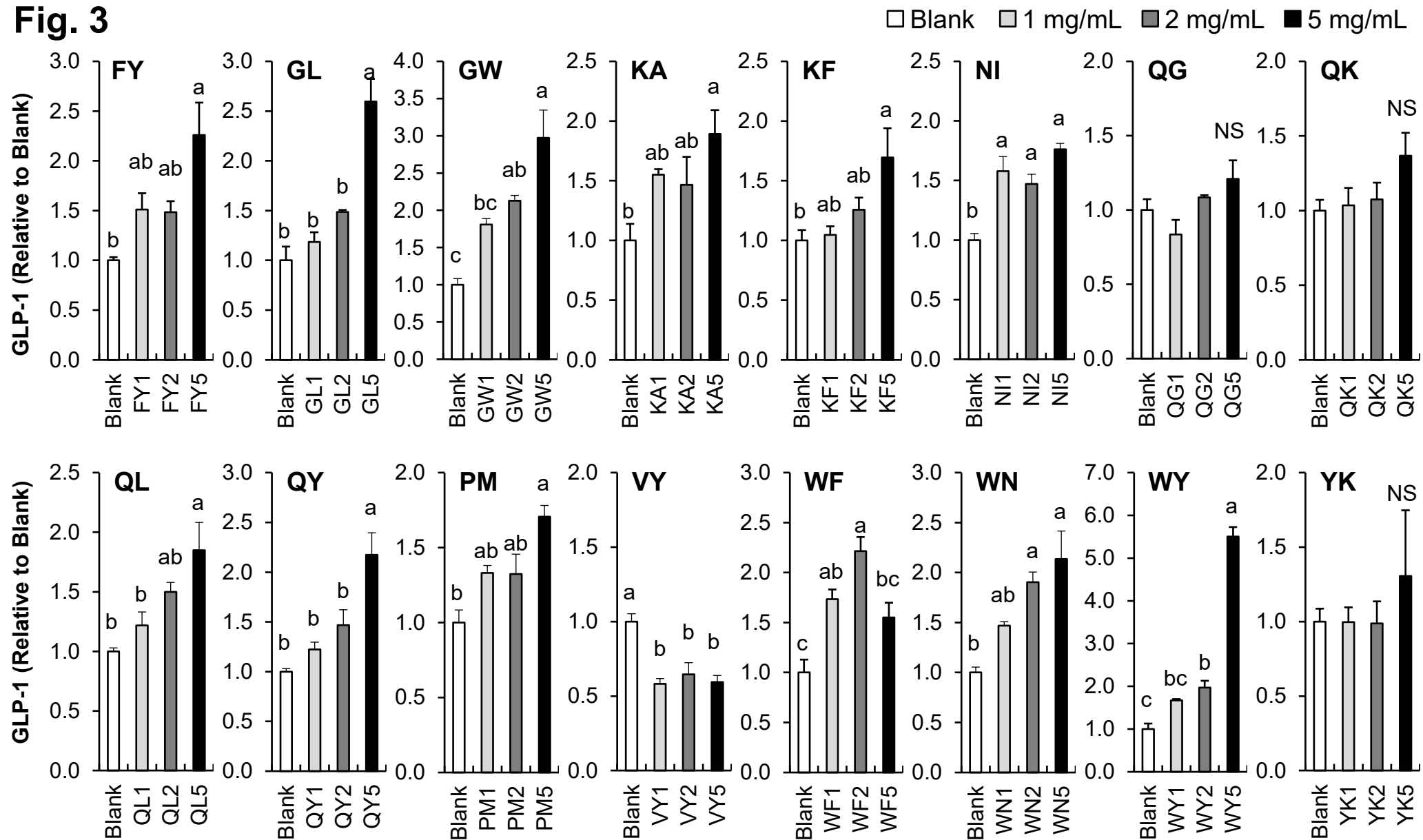
Fig. 3

Fig. 4

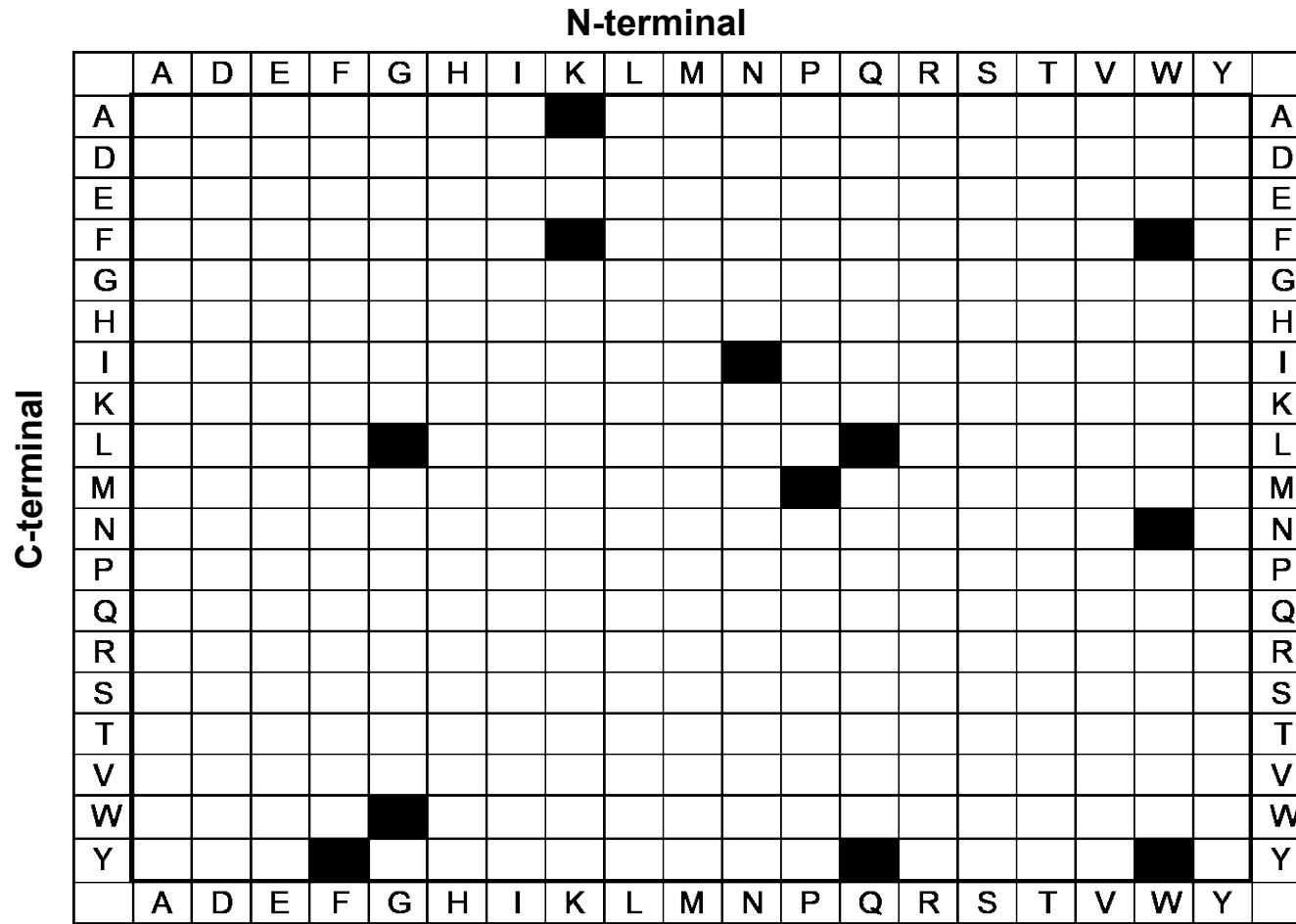
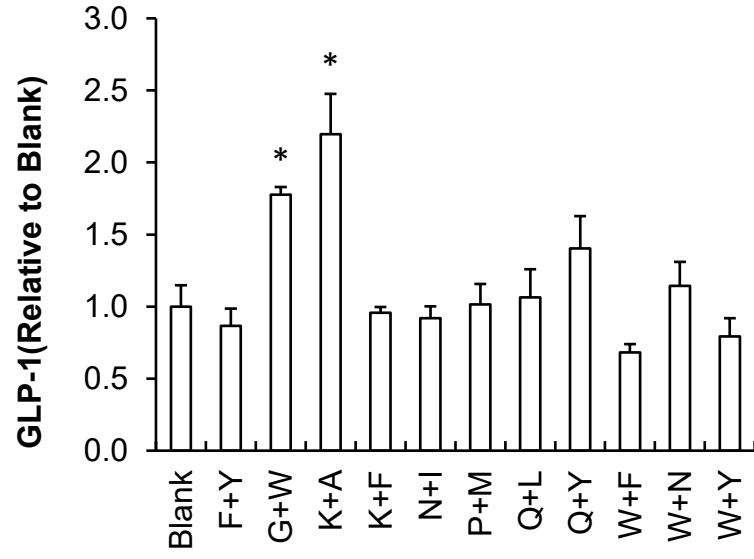
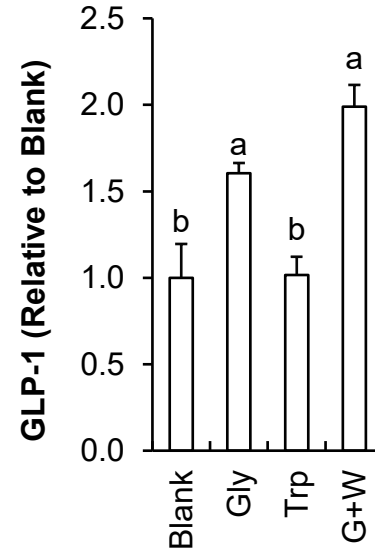


Fig. 5

A



B



C

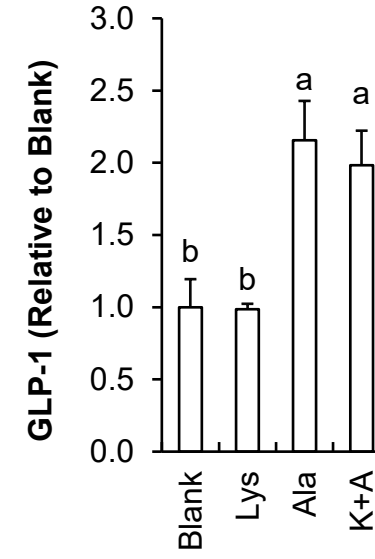
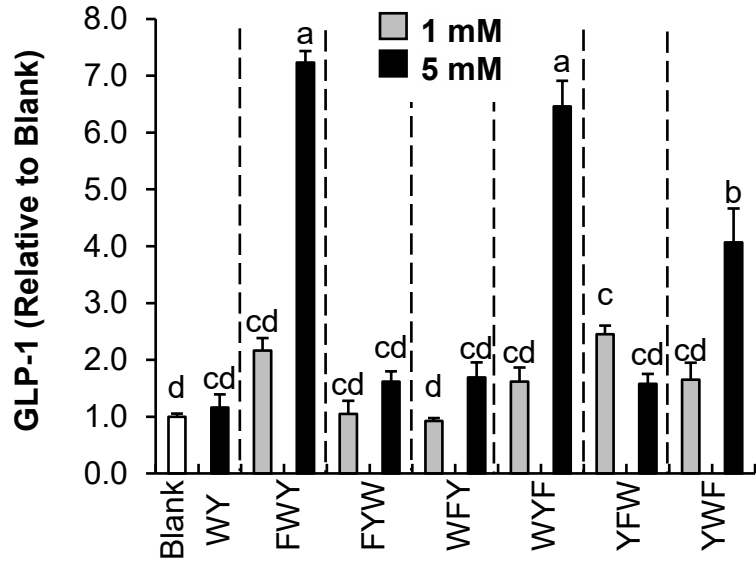


Fig. 6

A



B

