



Title	Proteinous pancreatic lipase inhibitor is responsible for the antiobesity effect of young barley (<i>Hordeum vulgare</i> L.) leaf extract
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1 Short running head

2 Lipase inhibitor in young barley leaf extract

3 Title

4 Proteinous pancreatic lipase inhibitor is responsible for the antiobesity effect of young barley

5 (*Hordeum vulgare* L.) leaf extract

6

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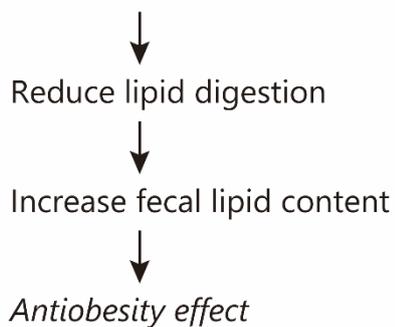
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17 **Graphical Abstract**

18 Proteinous pancreatic lipase inhibitor in young barley leaf extract was identified as an active
19 component responsible for the antiobesity effect.

Young Barley Leaf Extract (Aojiru)

Proteinous lipase inhibitor



20

21

22

23 **Abstract**

24 Young barley leaves (*Hordeum vulgare* L.) have various health effects and are employed as an
25 ingredient in the production of health-promoting foods. Promoting antiobesity is one such health
26 effect; however, the mechanism and bioactive compounds are unclear. In this research, young barley
27 leaf extract (YB) was demonstrated to possess pancreatic lipase inhibitory activity. The addition of
28 YB to a high-fat diet in mice increased fecal lipid content, indicating reduced absorption of lipids as
29 the mechanism underlying antiobesity effect. The investigation of bioactive compounds in YB resulted
30 in the identification of fructose–bisphosphate aldolase as a proteinous lipase inhibitor. Maximum
31 inhibition of the protein was 45%, but inhibition was displayed at a concentration as low as 16 ng/mL,
32 which is a characteristic inhibition compared with other reported proteinous lipase inhibitors.

33

34 **Keywords**

35 Barley, *Hordeum vulgare*, obesity, proteinous lipase inhibitor

36

37 Barley (*Hordeum vulgare* L.) is a major cereal grain cultivated globally. In addition to the
38 use of seeds as a food source, young leaves are known for their health effect and have been employed
39 as a traditional medication and an ingredient for the production of health-promoting foods (Ikeguchi
40 et al. 2004). The health effects of young barley leaf extract (YB) discussed in recent reports include
41 modification of intestinal flora (Ikeguchi et al. 2005; Sasaki et al. 2019), an immunostimulatory effect
42 in the intestine (Kim et al. 2017), suppression of postprandial blood glucose levels (Takano et al. 2013),
43 an antidepressant-like effect (Yamaura et al. 2012), a hypolipidemic effect in a rabbit model of
44 atherosclerosis (Yu et al. 2002), and an antiobesity effect (Minoshima et al. 2017), demonstrating the
45 value of this material in correcting health conditions.

46 YB contains various compounds, majorly including dietary fibers, proteins, polyphenols,
47 vitamins, and minerals (Hagiwara, Hagiwara and Ueyama 2001). Some of these compounds clearly or
48 suggestively account for the health effects of young barley extract, with dietary fibers considered to
49 affect the gut and polyphenols considered to affect other systems (Yu et al. 2002; Kim et al. 2017).
50 However, not all health effects of YB are explained by the specific compounds or have a known
51 mechanism and those responsible for the antiobesity effect remains unclear (Minoshima et al. 2017).

52 Obesity is a global health concern, and treatment methods have been widely studied. Various
53 medications, mainly targeting the digestion of lipids or acting through the suppression of appetite,
54 have been developed (Pilitsi et al. 2019). However, these treatments are generally employed for
55 patients with severe obesity because side-effects accompany medications. Much more of the
56 population has relatively mild obesity or is at risk of developing obesity. Therefore, in addition to
57 medication, studies on obesity should also focus on its prevention, and functional foods are an effective
58 methodology for this purpose.

59 As described above, research on the antiobesity effect of YB is of value for obesity
60 prevention. Revealing the mechanistic aspects and bioactive compounds of this food crop is essential
61 to understand its function further and enhance its application in the prevention of obesity. In this study,
62 the effect of YB in preventing obesity was demonstrated to occur through suppressed digestion and
63 absorption of lipids using a combination of an *in vitro* pancreatic lipase inhibition test and *in vivo* fecal

64 lipid excretion evaluation. Accordingly, the compound responsible for pancreatic lipase inhibition was
65 isolated, analyzed, and determined to be a proteinous inhibitor.

66

67 **Materials and methods**

68 **General**

69 Commercially available chemicals were purchased from Fujifilm Wako Pure Chemical Co. (Osaka,
70 Japan) unless otherwise stated. Absorbances were determined using a Synergy™ MX microplate
71 reader (BioTek Instruments, Inc., Winooski, VT, USA). All experiments were repeated at least twice,
72 and representative data are shown in the figures.

73

74 **Young barley leaf extract (YB) powder**

75 The powder was prepared according to a previous report (Minoshima et al. 2017). Briefly, barley
76 (*Hordeum vulgare* L. (Poaceae) sub.) was cultivated and harvested in Nanporo Town, Hokkaido, Japan.
77 Fresh leaves were washed and compressed. The extract was powdered by freeze-drying.

78

79 **Animal experiment**

80 The experiment was performed with approval (No. 19-0163) from the Institutional Animal Care and
81 Use Committee, Hokkaido University, following National University Corporation Hokkaido
82 University Regulations on Animal Experimentation. C57BL/6 male mice (13 weeks old) were housed
83 in an air-conditioned room at 23 ± 2 °C with a light period from 8:00 am to 8:00 pm. The mice were
84 categorized into two groups (n = 4) and fed a high-fat diet (Research Diet Inc., D12492) with or
85 without YB (6% w/w). After feeding for a week for conditioning, feces from one day were collected.
86 The experimental groups were swapped, and the experiment was repeated.

87

88 **Analysis of fecal lipid content**

89 The feces (100 mg) were powdered and immersed in 4 mL of chloroform/methanol (2:1, v/v) for 24 h
90 with agitation. The suspension was filtered and centrifuged at 12,000 ×g for 15 min. The supernatant

91 was evaporated with a centrifugal evaporator, and the residue was dissolved in 200 μ L of 2-propanol,
92 followed by sonication for 1 h. The lipid content in the resulting solution was quantified using the
93 LabAssay™ triglyceride kit to estimate the triglyceride content in the feces and the LabAssay™ NEFA
94 kit to estimate the NEFA content in the feces.

95

96 **Pancreatic lipase activity assay**

97 Pancreatic lipase inhibitory activity was assessed according to a previously reported method
98 (Ruangaram and Kato 2020). Briefly, a glyceryl trioleate emulsion [200 μ L, prepared by sonicating a
99 mixture of L- α -phosphatidylcholine (20 mg), triolein (32 mg), and sodium taurocholate (10 mg) in 9
100 mL of Tris-HCl buffer (13 mM Tris, pH 8.0) with 150 mM NaCl and 1.3 mM CaCl₂] and the sample
101 (100 μ L in water) were mixed and preincubated. A porcine pancreatic lipase solution (0.15 mg/mL,
102 Sigma-Aldrich Co., 100 μ L) was added, and the mixture was incubated for 15 min at 37°C. To the
103 mixture, 1 M aqueous HCl (40 μ L) and hexane (600 μ L) were added and mixed. The hexane layer was
104 dried, and the residue was dissolved in DMSO (100 μ L). Oleic acid in the solution was quantified
105 using the LabAssay™ NEFA kit to determine the inhibitory activity. Cetilistat (5 μ M) was used as the
106 positive control.

107

108 **Purification of pancreatic lipase inhibitory protein**

109 YB (4.5 g) was immersed in water (45 mL) and subjected to shaking for 24 h at room temperature (rt).
110 The suspension was centrifuged at 10,000 \times g for 10 min at rt, and the supernatant was frozen. The
111 frozen supernatant was thawed and again centrifuged at 10,000 \times g for 10 min. The supernatant was
112 recovered, and saturated aqueous ammonium sulfate was added up to 20% ammonium sulfate
113 saturation. The solution was allowed to settle for 10–16 h at 4°C and centrifuged at 10,000 \times g for 10
114 min to obtain the 20% ammonium sulfate precipitate. The steps were repeated to obtain 30%, 40%,
115 and 50% ammonium sulfate precipitates. Next, the 40% ammonium sulfate precipitate was dissolved
116 in Tris-HCl buffer (13 mM, pH8.0), passed through a HiTrap desalting column (Cytiva Tokyo, Japan),
117 and absorbed onto a HiTrap Q XL column (Cytiva Tokyo, Japan). The column was washed with the

118 Tris-HCl buffer, eluted using a gradient increase of sodium chloride up to 1.0 M within 15 min, and
119 further eluted for 5 min with 1.0 M sodium chloride. The fractions were collected, desalted, and
120 concentrated using a Vivaspin Turbo 15 (10,000MWCO, Sartorius Japan, Tokyo, Japan), followed by
121 analysis via SDS-PAGE and evaluation for lipase inhibitory activity. The proteinous lipase inhibitor
122 of YB was obtained from the 1.0 M sodium chloride eluted fraction. The protein was subjected to
123 SDS-PAGE and stained using EzStain AQua (Atto Co., Tokyo, Japan). The band corresponding to 34
124 kDa was analyzed using nanoLC-MS/MS (JPROteomics, Sendai, Japan). The obtained peptide
125 sequence was subjected to a MASCOT search to identify the candidate protein A0A287NGA7 (see
126 supplementary information).

127

128 **Preparation of recombinant protein**

129 The sequence of A0A287NGA7 was adjusted for expression in *Escherichia coli* and synthesized and
130 cloned into a pET19b plasmid vector (see supplementary information). The plasmid was transformed
131 into BL21(DE3) competent cells and cultivated. Isopropyl- β -D(-)-thiogalactopyranoside (IPTG, 1.0
132 mM) was added to induce the production of the protein. The cells were recovered by centrifugation
133 and lysed using the CelLytic™ B Cell Lysis Reagent (Sigma-Aldrich) containing lysozyme, benzonase,
134 and protease inhibitors. The lysate was centrifuged at 14,000 g for 10 min, and the precipitate was
135 washed and dissolved in 6 M guanidine Tris-HCl buffer (13 mM, pH 8.0, with 0.5 M sodium chloride).
136 The solution was diluted in refolding buffer (50 mM Tris, 0.4 M guanidine, 0.4 M arginine, 0.2 M
137 sodium chloride, 30% glycerol, pH 8.0) and incubated for 4 days at 4°C. The solution was then
138 dialyzed against Tris-HCl buffer (50 mM, 100 mM sodium chloride, pH 8.0). The recovered solution
139 was concentrated using Vivaspin Turbo 15 (10,000 MWCO, Sartorius Japan, Tokyo, Japan) to obtain
140 the recombinant A0A287NGA7 protein.

141

142 **Statistics**

143 Experiments are repeated at least twice, and representative data is expressed as mean \pm standard
144 deviation (SD). Data were analyzed by Student's *t*-test and $p < 0.05$ were considered statistically

145 significant.

146

147 **Results and discussion**

148 In a previous report, the addition of a powdered extract of YB to a high-fat diet efficiently
149 reduced the increase in body weight and accumulation of visceral fat in mice, demonstrating an
150 antiobesity effect (Minoshima et al. 2017). Changes in food consumption and enhanced bowel
151 movements owing to insoluble dietary fiber to decrease the absorption of carbohydrates were excluded
152 as causes by the authors of the study, and improved defense against oxidative stress and enhanced
153 exercise were speculated to be contributing factors. Conversely, digestion and absorption of lipids
154 were not considered. Therefore, as the starting point of this study, the lipase inhibitory activity of YB
155 was evaluated.

156 YB showed 62% inhibition against porcine pancreatic lipase at 1.3 mg/mL (Fig. 1). Higher
157 concentrations of YB showed similar inhibition, indicating that maximal inhibition is around that value.
158 At a lower concentration, 39% inhibition was observed at 0.16 mg/mL, indicating a gradual decrease
159 in inhibitory activity with decreasing concentrations. Inhibition of pancreatic lipase reduces the
160 digestion of lipids in the gut system, thereby reducing the absorption of lipids, which is a major
161 mechanism related to the antiobesity effect of natural compounds (Fu et al. 2016).

162 We presumed from the above result that YB confers its antiobesity effect through decreased
163 digestion and absorption of lipids. For confirmation, C57BL/6 mice were fed a high-fat diet with or
164 without YB, and the fecal lipid content was evaluated (Fig. 2). As presumed, the addition of YB
165 significantly increased both the triglyceride (TG) and non-esterified fatty acid (NEFA) contents in
166 feces, which are similar results to Orlistat (Ahnen *et al.* 2007), indicating that the reduction of lipid
167 absorption is at least in part related to the antiobesity effect of YB. Loose stools or oily spotting, a
168 common side-effect of medicinal lipase inhibitor (Filippatos *et al.* 2008), were not observed in the
169 mice given a high-fat diet with YB which might reflect the milder effect of YB.

170 Subsequently, identification of a lipase inhibitor in YB was attempted. Because several
171 polyphenols possess pancreatic lipase inhibitory activity (de la Garza et al. 2011), our primary focus

172 was on polyphenols contained in YB. However, solvent extraction using organic solvents and the
173 column chromatography method failed to identify polyphenols with lipase inhibitory activity in YB.
174 Thus, the focus was shifted to the proteins in YB because certain classes of proteins inhibit pancreatic
175 lipase (Gargouri et al. 1984b; Tsujita, Matsuura and Okuda 1996; Satouchi et al. 1998). Precipitation
176 of the proteins in YB with 50% ammonium sulfate saturation and an 80% aqueous acetone treatment
177 resulted in obtaining a precipitate with an inhibitory activity comparable with that of YB (ammonium
178 sulfate precipitate: 65% inhibition at 1.25 mg/mL; acetone precipitate: 78% inhibition at 2.5 mg/mL),
179 indicating that the inhibitory compound in YB is a protein. Trypsin treatment of YB also decreased
180 the inhibitory activity to 16% at 2.5 mg/mL, supporting this conclusion.

181 YB was precipitated by the addition of ammonium sulfate, and the precipitate was separated
182 via anion exchange chromatography to recover a fraction exhibiting a single band at 34 kDa in sodium
183 dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 3A) by adjusting the
184 suitable separation method for proteins. Examination of the lipase inhibitory activity of the isolated
185 protein indicated 33% inhibition at 250 ng/mL (Fig. 4). Higher concentrations showed no increase in
186 the activity, and a gradual decrease in inhibitory activity was observed with decreasing concentrations.
187 This characteristic is similar to the activity of YB, suggesting that the isolated protein is responsible
188 for the pancreatic lipase inhibition of YB. The lower maximum activity of the isolated fraction than
189 that of YB might be owing to the contribution of other compounds.

190 The isolated protein was then analyzed by LC-MS/MS. According to the identified peptide
191 sequence (see supplementary information) and molecular mass observed from SDS-PAGE analysis
192 (Fig. 3), a fructose-bisphosphate aldolase (UniProtKB-A0A287NGA7) was identified as the putative
193 protein. A recombinant protein was prepared and compared with the isolated protein to confirm this
194 identification. The SDS-PAGE results (Fig. 3B) and lipase inhibitory activity (Fig. 4) of the
195 recombinant A0A287NGA7 protein were identical to the isolated protein, allowing the conclusion that
196 this protein is the lipase inhibitory compound of YB.

197 Multiple studies have reported on the lipase inhibitory activity of proteins. The most potent
198 proteinous inhibitors are basic proteins. Protamine and histone completely inhibit the enzymatic

199 hydrolysis of triolein at 1 $\mu\text{g}/\text{mL}$ (Tsujita, Matsuura and Okuda 1996). The inhibitory activity of
200 A0A287NGA7 is comparable with that of basic proteins because inhibition is observed between 16
201 and 1000 ng/mL (Fig. 4). However, the maximum inhibition caused by A0A287NGA7 was 45%,
202 showing a clear difference from basic proteins. The lower content of basic amino acid residues in
203 A0A287NGA7 (13%) also distinguishes this protein from basic proteins [protamine (70%) or histone
204 (30%)]. Hydrophobic proteins such as albumin and soybean protein are another class of proteinous
205 pancreatic lipase inhibitors (Gargouri et al. 1984a; Tsujita, Matsuura and Okuda 1996). However, their
206 IC_{50} values are 1.34 and 0.3 mg/mL , much higher than the working concentration of A0A287NGA7,
207 distinguishing A0A287NGA7 from them. Although A0A287NGA7 might be classified as a novel type
208 of proteinous lipase inhibitor, the mechanism of action seems to be similar to basic and hydrophobic
209 proteinous inhibitors, which interact with the emulsified substrate to inhibit the hydrolysis of
210 triglycerides by lipase (Tsujita, Matsuura and Okuda 1996). We observed a diminished inhibition
211 ability of A0A287NGA7 through reduction of the preincubation time of the protein and emulsified
212 substrate (data not shown). Therefore, A0A287NGA7 has distinguishing characteristics compared
213 with other proteinous pancreatic lipase inhibitors, but the mechanistic aspects should be similar to
214 other proteinous inhibitors.

215 Fructose-bisphosphate aldolase is a universally distributed protein involved in the
216 metabolism of glucose and production of glucose both in mammals and plants. The amino acid
217 sequence of A0A287NGA7 has high similarity to the chloroplastic fructose-bisphosphate aldolase
218 produced by other plant species (*Arabidopsis thaliana* 88%, *Oryza sativa* 93%, *Spinacia oleracea*
219 88%, *Zea mays* 94%, and *Glycine max* 88%, according to a BLAST search), suggesting that pancreatic
220 lipase inhibition by this protein is common among plants regardless of species. In contrast, the
221 sequences of fructose-bisphosphate aldolases from mammals have less similarity (*Bos taurus* 54%,
222 *Sus scrofa* 54%, *Gallus* 50%, and *Homo sapiens* 54% identity). This finding is interesting because it
223 may provide an explanation about differences in the digestion of plant-derived and animal-derived
224 food, which may be related to the potential for development of obesity owing to consumption of meat
225 rather than vegetables. Thus, investigation of the protein sequence important for the pancreatic lipase

226 inhibitory activity of A0A287NGA7 is currently underway to pursue this point.

227

228 **Conclusion**

229 The antiobesity effect of YB is revealed to exert through the reduction of lipid absorption
230 due to the inhibition of pancreatic lipase. The lipase inhibitor contained in YB is a proteinous inhibitor
231 determined to be a fructose–bisphosphate aldolase (UniProtKB-A0A287NGA7). There is still an
232 argument how much this protein participates in the effect of YB to increase fecal lipid excretion since
233 a digestion by gut protease or an effect of other components in YB are not investigated. However,
234 these results provide an improved understanding of the antiobesity effect of YB and may enhance the
235 use of YB for the prevention of obesity.

236

237 **Authors' contributions**

238 EK, AT, and AA conducted the research. EK and HS designed the study. EK and AT drafted the
239 manuscript, and all authors revised the manuscript.

240

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243 (JSBBA). We thank Edanz Group (<https://en-author-services.edanz.com/ac>) for editing a draft of this
244 manuscript. Authors declare no conflicts of interests.

245

246 **Data Availability Statement**

247 The data produced from this study are available in the article and in its online supplementary material.

248

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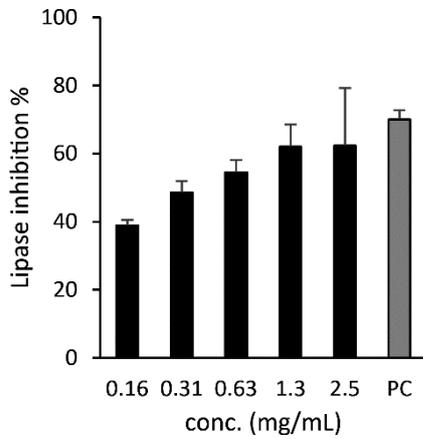
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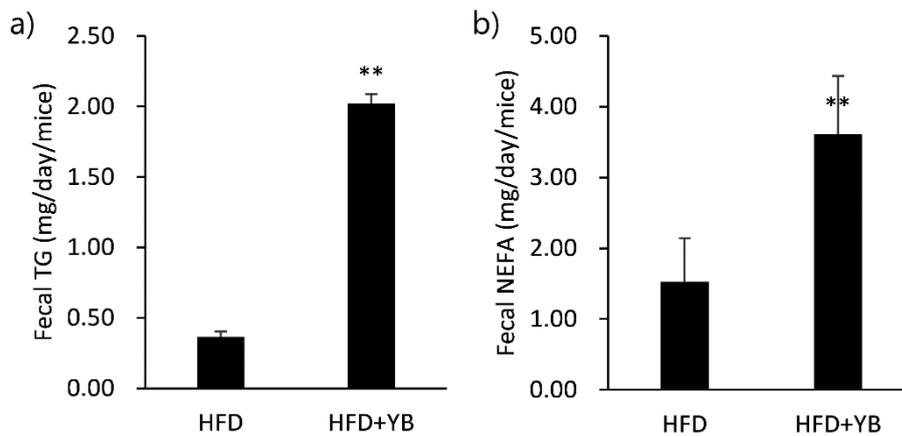
294 Figures



295

296 Fig. 1 Lipase inhibitory activity of young barley leaf extract (YB)

297 Positive control (PC): 2 μ g/mL (5 μ M) cetilistat. Bars represent mean \pm standard deviation (SD), n=4.



298

299 Fig. 2 Fecal lipid content of the mice fed with high-fat diet with/without young barley leaf extract

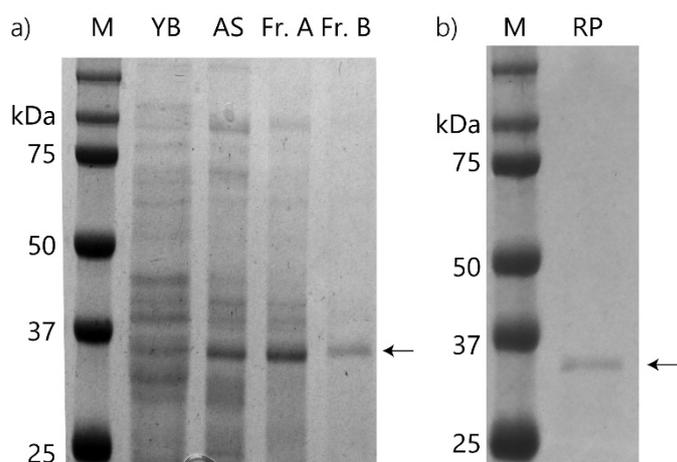
300 (YB)

301 Feces of C57BL/6 male mice fed each diet were collected and examined for a) fecal tryglyceride (TG),

302 b) fecal non-esterified fatty acids (NEFA) content. HFD: high-fat diet group; HFD+YB: high-fat diet

303 + 6% young barley leaf extract. Bars represent mean \pm standard deviation (SD), n=4, **p < 0.001

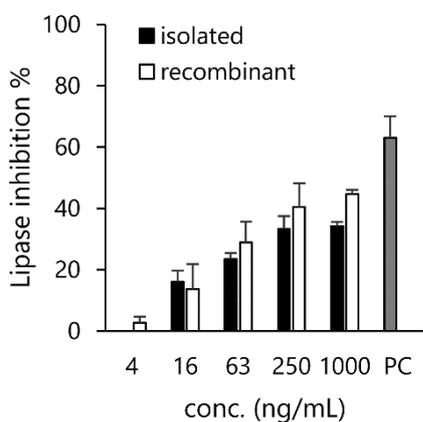
304 (Student's *t*-test)



305

306 Fig. 3 SDS-PAGE analysis

307 Protein samples were separated on 10% acrylamide gel and stained with EzStain AQua (Atto Co.,
 308 Tokyo, Japan). a) Fractions during purification steps of YB. M: marker protein, AS: 40% ammonium
 309 sulfate precipitate, Fr. A: a washout fraction of anion exchange chromatography, Fr. B: a fraction of
 310 anion exchange chromatography showing single band at 34 kDa. b) RP: Recombinant A0A287NGA7
 311 protein. Arrow indicates the bands of isolated or recombinant protein.



312

313 Fig. 4 Lipase inhibitory activity of the isolated protein from YB and the recombinant A0A287NGA7
 314 protein

315 Positive control (PC): 2 μ g/mL (5 μ M) cetilistat. Bars represent mean \pm standard deviation (SD), n=4.

316

317

318 **Supplementary data link**
319 <https://doi.org/10.1093/bbb/zbab096>