Short running head

Lipase inhibitor in young barley leaf extract

Title

Proteinous pancreatic lipase inhibitor is responsible for the antiobesity effect of young barley (*Hordeum vulgare* L.) leaf extract

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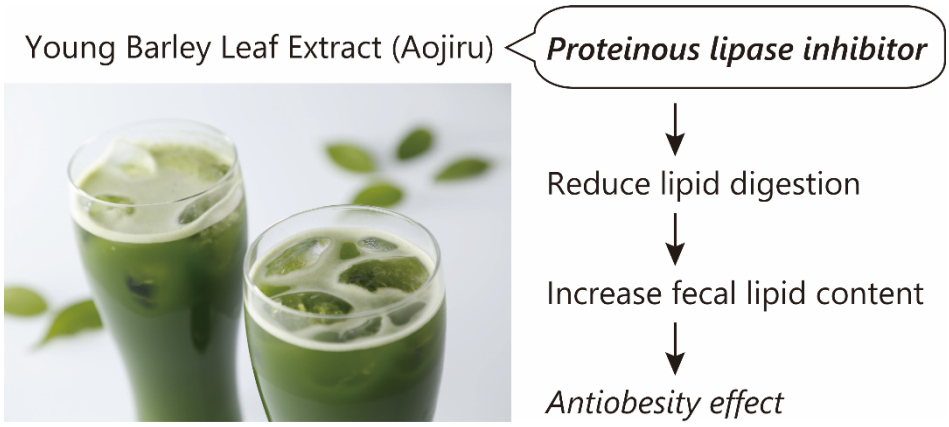
Graphical Abstract

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



Abstract

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

Keywords

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

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

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

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

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

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

Materials and methods

General

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

Young barley leaf extract (YB) powder

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

Animal experiment

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

Analysis of fecal lipid content

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

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

Pancreatic lipase activity assay

Pancreatic lipase inhibitory activity was assessed according to a previously reported method (Ruangaram and Kato 2020). Briefly, a glyceryl trioleate emulsion [200 μL, prepared by sonicating a mixture of L-α-phosphatidylcholine (20 mg), triolein (32 mg), and sodium taurocholate (10 mg) in 9 mL of Tris-HCl buffer (13 mM Tris, pH 8.0) with 150 mM NaCl and 1.3 mM $CaCl_2$] and the sample (100 μL in water) were mixed and preincubated. A porcine pancreatic lipase solution (0.15 mg/mL, Sigma-Aldrich Co., 100 μL) was added, and the mixture was incubated for 15 min at 37°C. To the mixture, 1 M aqueous HCl (40 μL) and hexane (600 μL) were added and mixed. The hexane layer was dried, and the residue was dissolved in DMSO (100 μL). Oleic acid in the solution was quantified using the LabAssay™ NEFA kit to determine the inhibitory activity. Cetilistat (5 μM) was used as the positive control.

Purification of pancreatic lipase inhibitory protein

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

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

Preparation of recombinant protein

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

Statistics

Experiments are repeated at least twice, and representative data is expressed as mean ± standard 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

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

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

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

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

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

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

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

Conclusion

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

Authors' contributions

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

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Data Availability Statement

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

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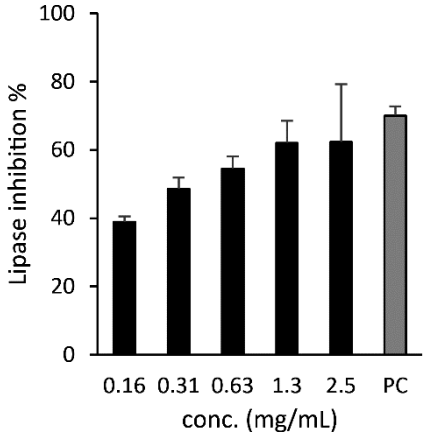
294 Figures



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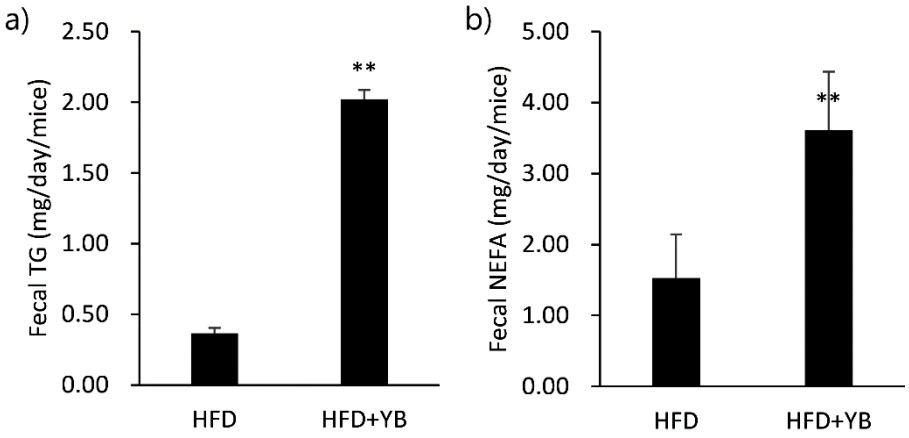
296 Fig. 1 Lipase inhibitory activity of young barley leaf extract (YB)

297 Positive control (PC): 2 μg/mL (5 μM) cetilistat. Bars represent mean± standard deviation (SD), n=4.



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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± standard deviation (SD), n=4, **$p < 0.001$

304 (Student's *t*-test)

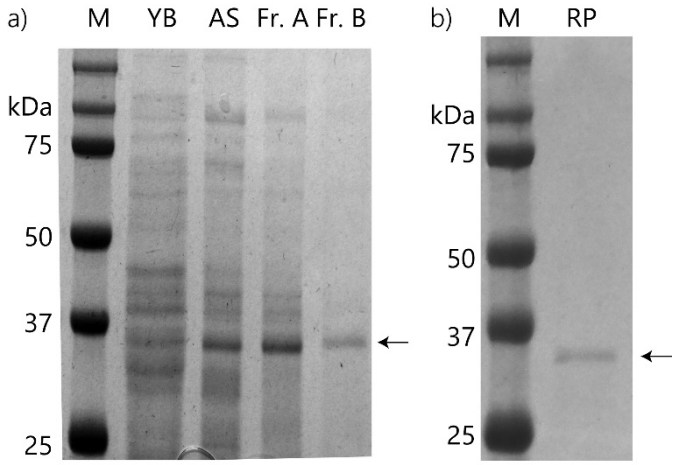

Fig. 3 SDS–PAGE analysis

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

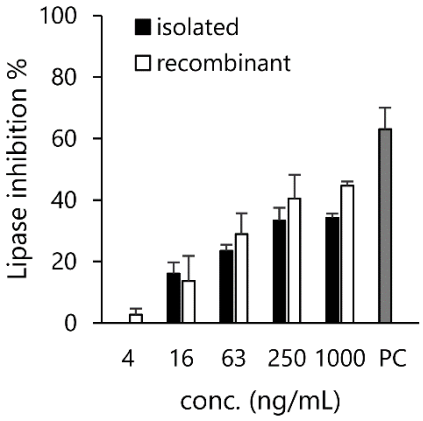

Fig. 4 Lipase inhibitory activity of the isolated protein from YB and the recombinant A0A287NGA7 protein

Positive control (PC): 2 μg/mL (5 μM) cetilistat. Bars represent mean± standard deviation (SD), n=4.

Supplementary data link

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