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Original article

Suppression of glycated protein cross-linking formation and cross-linking cleavage reaction of edible purple Chrysanthemum flower extract

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Abstract

The accumulation of advanced glycation end products (AGEs) in the body due to glycative stress is a factor in the development and progression of aging and lifestyle-related diseases. In particular, the formation of protein cross-links associated with the formation of AGEs hardens tissues, resulting in the deterioration of biological functions. In this study, we examined the usefulness of the edible purple Chrysanthemum flower from the viewpoint of reducing cross-link formation in the glycated proteins, by examining its inhibitory effect on this cross-link formation, its degradation of glycated protein cross-linking, its inhibition of glycated reaction intermediate formation, its degradation of AGE cross-linking, and its enhancement of oxidized protein hydrolase (OPH) activity. A 50 % ethanol extract (EE) of edible purple chrysanthemum (Chrysanthemum morifolium) flowers was used as a sample. EE inhibited the formation of lysozyme dimer and trimer at sample concentrations of 0.01 to 1.1 mg/mL. EE inhibited the formation of fluorescent AGEs (F-AGEs) and glycation intermediates in a human serum albumin (HSA)-glucose glycation model. This effect was similar to that of aminoguanidine, an inhibitor of glycation reaction. Furthermore, EE enhanced the enzymatic activity of OPH, which is an AGEs degrading agent, by cleaving AGE cross-links using the α-diketone bond of 1-phenyl-1, 2-propanedione (PPD) as a model. The inhibitory effect of EE on the formation of glycated protein cross-links was suggested to be related to the inhibition of the formation of glycation intermediates and AGEs, and the degradation of glycated protein cross-links was suggested to be related to the involvement of various substances in Asteraceae plants and the pigment component of purple chrysanthemum flowers. EE may have an effect on repairing protein dysfunction by degrading and removing cross-linked proteins by glycation. There is a possibility that EE may have the ability to repair protein dysfunction by removing cross-linked proteins through glycation.

***KEY WORDS*:** advanced glycation end products (AGEs), cross-link formation in the glycated protein, cross-link cleavage in the glycated protein, *Chrysanthemum morifolium*

Introduction

The accumulation of advanced glycation end products (AGEs) in the body due to glycative stress is a factor in the development of skin aging, diabetic complications, and osteoporosis [1, 2)]. In particular, the formation of protein cross-links associated with the AGE formation causes tissue hardening, leading to a decline in biological functions. Furthermore, 3-deoxyglucosone (3DG), a glycation intermediate, promotes the formation of protein cross-links [3, 4)]. Inhibition of glycative stress *in vivo* is called anti-glycation. Anti-glycation includes inhibition of postprandial hyperglycemia, inhibition of glycation reactions, and degradation of AGEs. From the viewpoint of glycated-protein cross-link (GPCL) formation, inhibition of postprandial hyperglycemia and inhibition of glycation reaction are preventive measures, while degradation of AGEs is a restorative measure.

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Aminoguanidine (AG) is a substance that inhibits the formation of AGEs [5, 6]. However, AG has side effects, *i.e.*, anemia, liver dysfunction, and vitamin B6 deficiency. On the other hand, inhibitory effects on glycation reactions have been observed in various food materials such as herbal teas [7], vegetables [8], and fruits [9]. *N*-phenacylthiazolium bromide (PTB) has been reported as a substance with GPCL degradation activity [10]. PTB cleaves α-diketone bonds, a type of cross-linkage structure in the glycated protein [11]. The GPCL degradation of has been reported to be inhibited by substances found in plants, including rosmarinic acid [12], ellagitannins [13], and flavonoids [14].

Oxidized protein hydrolase (OPH) is a type of protease found in various tissues, *i.e.*, blood, liver, and skin, and has been reported to degrade AGEs as well as release N-terminal acyl groups of acylated proteins [15]. The activity-enhancing effect of OPH has been observed in many herbal extracts, including Job's tears (*Coix lacryma-jobi var. ma-yuen*), kuma bamboo grass (*Sasa veitchii*), and fenugreek (*Trigonella foenum-graecum*) [16, 17].

There are about 25,000 species of Asteraceae in the world, and they are used as agricultural crops since they can grow in various regions [18]. Lettuce (*Lactuca sativa*), burdock (*Arctium lappa*), and chamomile (*Matricaria recutita*) are typical Asteraceae crops, and their leaves, rhizomes, and flowers are edible. Edible purple chrysanthemum is a variety of Chrysanthemum (*Chrysanthemum morifolium*) and is called "kaki no moto" in Niigata prefecture, "motte no hoka" or "enmei raku" in Yamagata prefecture, and is eaten in a variety of ways such as soaked, namasu, tempura, and soup.

There are two main edible varieties of chrysanthemum flowers: one with reddish-purple petals, such as Enmeiraku, and the other with yellow petals, such as Abokyu. Chrysanthemum flowers have been reported to contain 46 flavonoids and 17 caffeic acid derivatives [19]. Edible purple chrysanthemum flowers also contain pigment components such as delphinidin and cyanidin. It has already been reported that chrysanthemum flowers inhibit AGE production [20, 21], have antioxidant effects [22], and have anti-photoaging effects [23]. In a study in which 11 healthy Japanese women took 150 mg of edible purple chrysanthemum powder per day for 8 weeks, it was found that the material was safe and that the skin elasticity index of the left cheek increased before and after intake [20]. These results verified the usefulness of chrysanthemum flowers in preventing AGE formation. In this study, we examined the usefulness of edible purple chrysanthemum flowers from the viewpoint of preventing and repairing the deterioration of protein function caused by glycation by examining their effects on inhibiting GPCL formation, degrading GPCL, inhibiting glycation reaction intermediate formation, degrading AGE cross-linking, and enhancing OPH activity.

Materials and Methods

1) Reagents

The reagents used were purchased from the following manufacturers: *N*-phenacylthiazolium bromide (PTB), human serum albumins (HSA, lyophilized powder, ≥ agarose gel electrophoresis), and methylglyoxal (40% methylglyoxal solution; MGO) from Sigma-Aldrich Japan (Meguro-ku, Tokyo, Japan). 1-phenyl-1,2-propanedione (PPD), benzoic acid, lysozyme (from Egg White), aminoguanidine hydrochloride (AG), 2,3-diaminonaphthalene 2,3-diaminonaphthalene (DAN) and glyoxal (40% glyoxal solution; GO) were obtained from Fujifilm Wako Pure Chemical Industries (Osaka, Japan). 3-deoxyglucosone (3DG) was obtained from Dojin Chemical Research Institute (Kamimashiki-gun, Kumamoto, Japan). 2-40% Mini-Protean TGX Precast gel is from Bio-Rad (Hercules, California, USA). Acetyl-alanine *p*-nitroanilide (AAPA) is from Bachem (Bubendorf, Switzerland). The oxidized protein hydrolase (OPH) is an acylamino-acid releasing enzyme from Takara Bio (Otsu, Shiga, Japan). Other reagents of special grade or HPLC grade were purchased from Fujifilm Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan) and used.

2) Sample

The sample used was edible purple Chrysanthemum flower extract (EE), which was obtained by 50% ethanol extraction from edible purple chrysanthemum flowers (solid content 22.7%). This sample was provided by Unial (Tokyo, Japan).

3) Inhibition of GPCL formation

The lysozyme-glucose GPCL cross-link model was used for the inhibition of GPCL formation based on previous reports [24, 25]. The reaction solution was prepared by adding 1/10 of the sample solution in 0.1 mol phosphate buffer (pH 7.4) containing 5 mg/mL lysozyme and 0.5 mol/L glucose. The reaction solutions were made by adding all of the phosphate buffer, lysozyme solution, glucose solution, and sample solution; (A), a solution with purified water added instead of the glucose solution in A; (B), a solution with sample dissolution solution added instead of the sample solution in A; (C), a solution with purified water added instead of the glucose solution in C; (D) were prepared and incubated at 60 °C for 40 hours.

After the reaction was completed, the reaction solution was centrifuged using a 3 kDa ultrafiltration membrane (Amicon Ultra-0.5 mL centrifugal filters Ultracel-3K; Merck, Darmstadt, Germany) to remove low-molecular-weight substances. The reaction solution was then subjected to 4-20% polyacrylamide gel electrophoresis (SDS-PAGE). The gel after electrophoresis was stained with CBB Stain One (Nacalai Tesque, Nakagyo-ku, Kyoto, Japan), and the images were captured with Pharos FX System (Bio-Rad), and the intensities of the lysozyme dimer (25.8 kDa) and trimer (40.5 kDa) bands were analyzed with ImageJ (NIH, ImageJ (NIH, Maryland, USA) was used for analysis. AG was used as a positive control substance for the inhibition of glycated protein cross-link formation.

The inhibition rate of protein cross-link formation was calculated based on the following equation.

$$\text{Inhibition rate of protein cross-link formation (\%)} = \{1 - (A - B) / (C - D)\} \times 100$$

A - D ; band intensity of lysozyme dimer or trimer in SDS-PAGE gel staining image of each reaction solution

4) Cleavage of GPCL

The cross-link cleavage action of glycated lysozyme was measured by preparing glycated lysozyme and measuring the degradation rate of the lysozyme dimer by the sample. Glycated lysozyme was prepared by reacting 1 mg/mL lysozyme with 0.1 mol/L phosphate buffer (pH 7.4) containing 0.2 mol/L glucose at 37 °C for 16 hours, and then removing low-molecular-weight substances by ultrafiltration (3 kDa). The reaction solutions were prepared by adding 1/2 volume of the sample solution in 0.05 mmol/L phosphate buffer containing 0.5 mg/mL glycated lysozyme; (A), and by adding the sample dissolution solution instead of the sample solution in A; (B). The reaction solution was then incubated at 37 °C for 16 hours, after which the reaction solution was centrifuged through an ultrafiltration membrane (3 kDa) to remove low-molecular-weight substances. The reaction solution was then subjected to SDS-PAGE in the same manner as described in the previous section, and after staining and imaging the gel, the band intensity of lysozyme dime in the reaction solution was analyzed. PTB was used as a positive control substance for glycated protein cross-link cleavage.

5) Inhibition of glycation reaction

The amount of F-AGEs and glycation intermediates was determined by measuring their concentrations in the reaction solution after incubation. The concentrations of F-AGEs and glycation intermediates were measured in the reaction solution after incubation. AG was used as a positive control substance for the inhibition of glycation reaction, and AGE-derived fluorescence (excitation wavelength: 370 nm/fluorescence wavelength: 440 nm) was measured for F-AGEs according to our previous report[26]. For glycation intermediates, 200 µL of glycation reaction solution was deproteinized with perchloric acid and labeled with DAN under alkaline conditions, and 3DG, GO, and MGO were measured by HPLC according to previous reports[27,28]. The inhibition rate (%) of the formation of F-AGEs and glycation intermediates was calculated according to our previous report[26]. In addition, the 50 % inhibitory concentration (IC_{50}; mg/mL) was calculated from the inhibition rates of three concentrations of the sample solutions[26,29]. The smaller the IC_{50} value, the stronger the inhibitory effect of the glycation reaction.

6) Measurement of AGE cross-link cleavage

The cleavage of the α-diketone bond of PPD by the sample was measured using PPD as a model material for AGE cross-linking, according to previous reports[30,31]. The reaction solution was a mixture of sample solution, 10 mmol/L PPD, and 0.2 mol/L phosphate buffer (pH 7.4) in the ratio of 5 : 1 : 4, and the reaction was carried out at 37 °C for 8 hours. The amount of benzoic acid released was measured under the same HPLC conditions as previously reported[31].

PTB was used as a positive control substance for AGE cross-link cleavage. 1 mol of PPD generates 1 mol of benzoic acid when the α-diketone bond of PPD molecule is cleaved.

$$\text{Cross-linkage cleavage rate (\%)} = \{ (A - B) / C \} \times 100$$

A; amount of benzoic acid in the reaction solution, B; amount of benzoic acid in the sample, C ; amount of PPD used in the reaction

7) OPH activity enhancement

In accordance with our previous report[16], the OPH activity-enhancing effect was determined by incubating 250 µL of the reaction solution (0.1 mol/L Tris-HCl (pH 7.4), 2 mmol/L Acetyl-alanine p-nitroanilide (AAPA), 1 mU/mL OPH, and sample extract) with 1/25 volume of the sample solution at 37 °C for 60 min. Incubated at 37 °C for 60 min, the amount of *p*-nitroanilide (*p*NA) released by OPH degradation of AAPA was measured absorbance at 405 nm (S). The OPH activation rate (%) was calculated by using the following equation, where the amount of *p* NA released during 60 min from the beginning of reaction (R) (0 min) was taken as 100 %.

$$\text{OPH activation rate (\%)} = \{ (S60 - S0) / (R60 - R0) \} \times 100$$

S ; *p*NA concentration of reaction solution with sample solution
R; *p*NA concentration of reference reaction solution
60 ; After 60 minutes
0 ; Immediately after start of reaction (0 min)

Statistical analysis

Measurements were expressed as mean ± standard deviation (SD) of triplicate measurements. Tukey's test or Dunnett's test was used for comparison of measurements. Statistical analysis results were considered significant at a risk rate of less than 5 %.

Results

1) Inhibition of GPCL formation

EE inhibited the formation of lysozyme dimer and trimer in a concentration-dependent manner at sample concentrations of 0.01-1.1 mg/mL (***Table 1***, ***Fig. 1***). 0.1 mg/mL EE and AG inhibited GPCL formation at 37.1 ± 3.4 % (EE), 24.9 ± 8.8 % (AG), and 78.3 ± 8.1 % (EE) and 61.7 ± 14.1 % (AG), respectively. The inhibition rate of GPCL formation by lysozyme dimer was 37.1 ± 3.4 % (EE), 24.9 ± 8.8 % (AG), and by lysozyme trimer was 78.3 ± 8.1 % (EE), 61.7 ± 14.1 % (AG), showing no difference in the effects of EE and AG.

2) Cleavage of GPCL

EE degraded lysozyme dimers at sample concentrations ranging from 11.4 to 56.8 mg/mL (***Table 2***, ***Fig. 2***). 41.2 ± 9.9 % of the protein cross-link cleavage rate of EE at 56.8 mg/mL did not differ from that of PTB at 5 mmol/L (39.6 ± 4.6 %).

3) Inhibition of glycation reaction

EE inhibited the formation of F-AGEs and glycation

intermediates (3DG, GO, and MGO) in the HSA-glucose glycation reaction model (***Table 3***). The IC_{50} values of EE for inhibiting the formation of F-AGEs and glycation intermediates ranged from 0.076 to 0.157 mg/mL. Values of AG ranged from 0.064 to 0.172 mg/mL, which were close to each other.

4) AGE cross-link cleavage

The AGE cross-link cleavage rate of EE increased in a concentration-dependent manner from 0.06 to 5.68 mg/mL (***Fig. 3***). The AGE cross-link cleavage rate of EE at 5.68 mg/mL (14.2 ± 0.02 %) was 0.4 times higher than that of 5 mmol/mL PTB (cleavage rate 40.5 ± 0.2 %, $p < 0.01$).

5) OPH activity enhancement

EE enhanced OPH activity at sample concentrations ranging from 0.9 to 181.6 μg/mL (***Fig. 4***). The concentration of EE at which OPH was most activated was 90.8 μg/mL (173.1 ± 0.6 %).

Table 1. Inhibitory effect of edible purple Chrysanthemum flower extract on cross-linking formation in the lysozyme-glucose reaction model.

Sample	Conc. (mg/mL)	Inhibition ratio (%)	
		Dimer	Trimer
EE	0.01	-0.9 ± 8.8 **	30.4 ± 16.7 *
	0.1	37.1 ± 3.4	78.3 ± 8.1
	1.1	93.0 ± 6.6 **	95.4 ± 7.4 *
AG	0.1	24.9 ± 8.8	61.7 ± 14.1

5 mg/mL lysozyme were incubated at 60 °C for 40 hours. SDS-PAGE was conducted using 2-40 % acrylamide gels. Data are expressed as mean ± standard deviation, n = 3; Dimer, 25.8 kDa; Trimer, 40.5 kDa; *$p < 0.05$, **$p < 0.01$ vs AG by Dunnett test; EE, edible purple Chrysanthemum flower extract; AG, aminoguanidine; Conc., concentration.

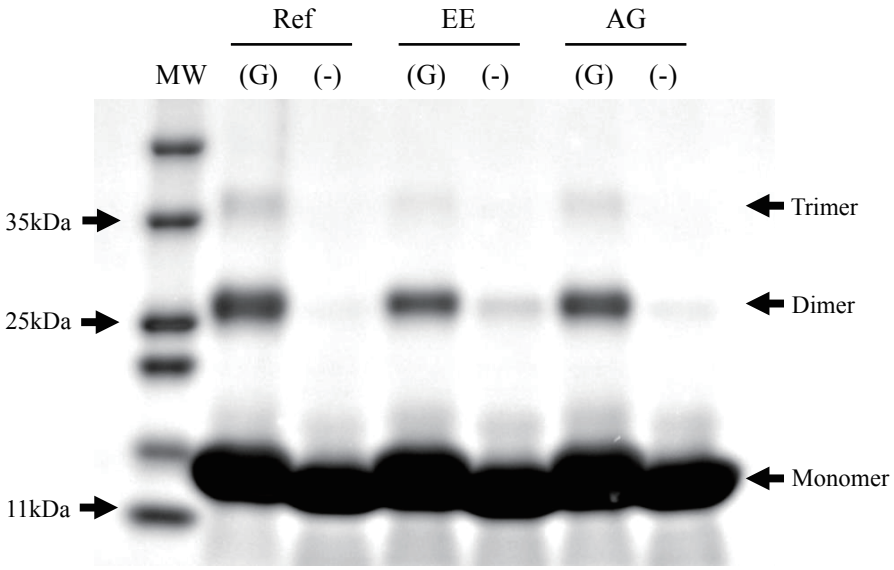

Fig. 1. **Inhibitory effect of edible purple Chrysanthemum flower extract on cross-linking formation in the lysozyme-glucose reaction model.**

5 mg/mL lysozyme were incubated at 60 °C for 40 hours. SDS-PAGE was conducted using 2-40 % acrylamide gels. MW, molecular weight markers; Ref, incubation without EE (with 50 % ethanol); EE, incubation with 0.1 mg/mL edible purple Chrysanthemum flower extract; AG, incubation with 0.1 mg/mL aminoguanidine; (G), incubation with 0.5 mol/L glucose; (-), incubation without glucose.

Table 2. Cleavage effect of edible purple Chrysanthemum flower extract on lysozyme dimer in the glycated lysozyme.

Sample	Conc. (mg/mL)	Cleavage ratio (%)
EE	11.4	-2.9 ± 1.6 **
	37.8	10.2 ± 4.7 **
	56.8	41.2 ± 9.9
PTB	5.0	39.6 ± 4.6

1 mg/mL glycated lysozyme were incubated at 37°C for 16 hours. SDS-PAGE was conducted using 2-40% acrylamide gels. Data are expressed as mean ± standard deviation, n = 3; lysozyme dimer, 25.8kDa; ** $p < 0.01$ vs PTB by Dunnett test; EE, edible purple Chrysanthemum flower extract; PTB, *N*-phenacylthiazolium bromide; Conc., concentration.

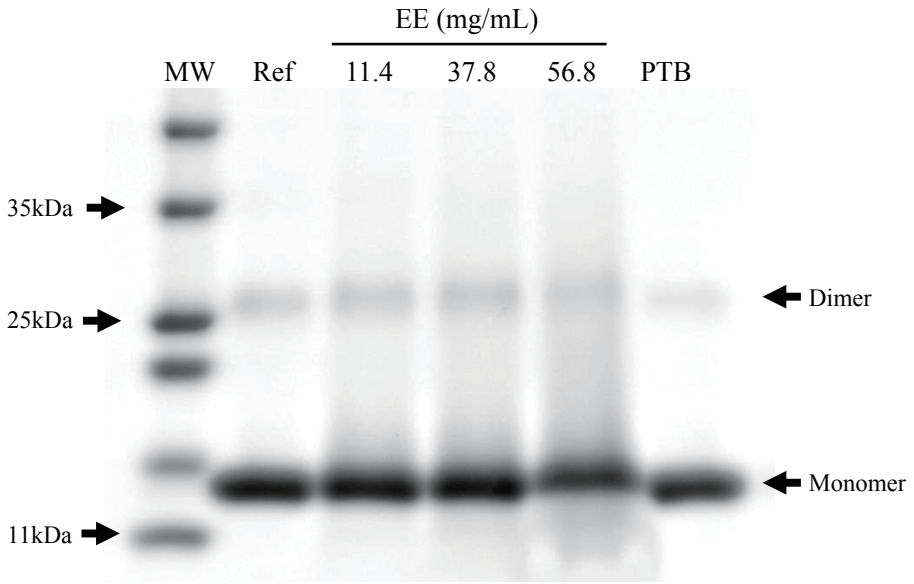

***Fig.2.* Cleavage effect of edible purple Chrysanthemum flower extract on lysozyme dimer in the glycated lysozyme.**

1 mg/mL glycated lysozyme were incubated at 37°C for 16 hours. SDS-PAGE was conducted using 2-40% acrylamide gels. MW, molecular weight markers; Ref, incubation without EE (with 50% ethaol); EE, incubation with edible purple Chrysanthemum flower extract; PTB, incubation with 5 mmol/L *N*-phenacylthiazolium bromide.

Table 3. Inhibitory effect of edible purple Chrysanthemum flower extract on fluorescent AGE in the HSA-glucose glycation model.

Glycation products	Inhibition ratio (%)			IC_{50} (EE) (mg/mL)	IC_{50} (AG) (mg/mL)
	0.01 (mg/mL)	0.1 (mg/mL)	1.1 (mg/mL)		
Fluorescent AGEs	8.9 ± 0.7 **	65.3 ± 0.2 **	99.9 ± 0.3 **	0.076	0.064
3DG	4.0 ± 0.7 **	41.8 ± 0.6 **	86.8 ± 1.0 **	0.157	0.153
GO	-10.1 ± 1.2 **	88.0 ± 0.1 **	94.8 ± 0.1 **	0.081	0.073
MGO	2.8 ± 0.5 **	67.8 ± 1.2 **	85.7 ± 0.9 **	0.101	0.172

Data are expressed as mean ± standard deviation, n = 3; ** $p < 0.01$ vs same glycation products by Tukey's test; IC_{50}, half inhibitory concentration (mg/mL) on the HSA-glucose glycation model; EE, edible purple Chrysanthemum flower extract; AG, aminoguanidine; HSA, human serum albumin; AGE, advanced glycation end product; IC_{50}, half-maximal inhibitory concentration.

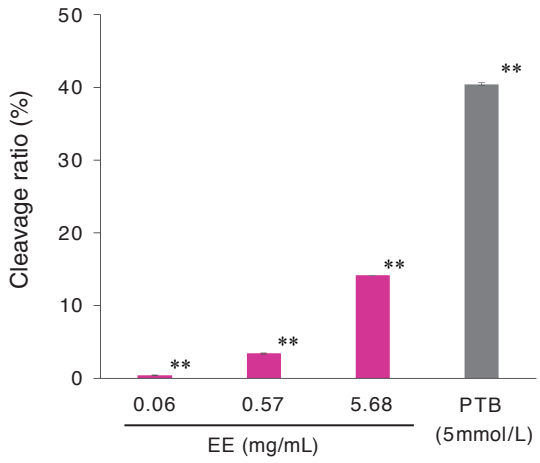

Fig. 3. **Cleavage effect of edible purple Chrysanthemum flower extract on α-diketone bond in the PPD reaction model.**

Data are expressed as mean ± standard deviation, n = 3; ** $p < 0.01$ by Tukey's test; PPD, 1-phenyl-1,2-propanedione; EE, edible purple Chrysanthemum flower extract; PTB, *N*-phenacylthiazolium bromide.

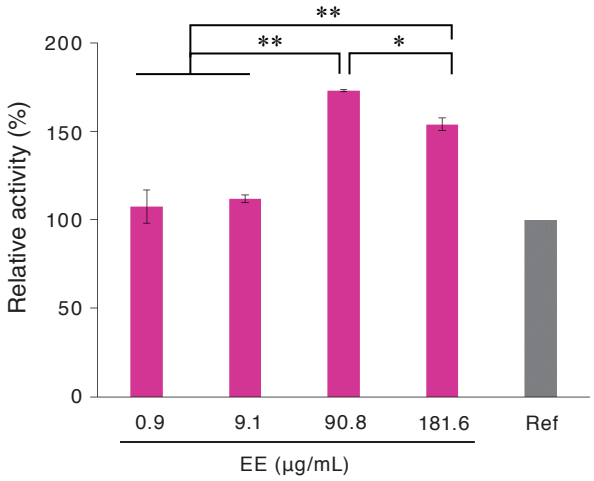

Fig. 4. **OPH activation effect of edible purple Chrysanthemum flower extract.**

Data are expressed as mean ± standard deviation, n = 3; * $p < 0.05$, ** $p < 0.01$ by Tukey's test; EE, edible purple Chrysanthemum flower extract; Ref, water; OPH, oxidized protein hydrolase.

Discussion

1) Inhibition of GPCL formation and glycation reaction

There are many types of AGEs produced *in vivo*, which are classified as fluorescent and non-fluorescent, or cross-linked and non-cross-linked. F-AGEs include argpyrimidine, vesperlysine, and pentosidine. Cross-linked AGEs include pentosidine, crossline, and glucosepane. In addition, dicarbonyls and aldehydes such as 3DG, GO, and MGO are glycation intermediates that promote the formation of many types of AGEs. 3DG has already been reported to be involved in protein cross-link formation[3]. In GPCL formation, 3DG acts as a cross-linker through arginine and lysine residues of lysozyme, ovalbumin, and bovine serum albumin (BSA)[33].

EE inhibited GPCL formation in the lysozyme-glucose glycation reaction model and inhibited the formation of F-AGEs, 3DG, GO, and MGO in the HSA-glucose glycation reaction model. These effects were similar to those of AG, which binds to the dicarbonyl group of glycation intermediates and inhibits protein glycation[34]. EE may have inhibited the formation of F-AGEs and cross-linked AGEs, induced by glycation, by acting similarly to AG. Edible purple chrysanthemum contains luteolin, delphinidin, and chlorogenic acid[20]. Since these substances have both anti-glycation[20] and antioxidant[22] effects, their involvement in the inhibition of GPCL formation in EE was presumed.

2) Cleavage of GPCL and AGE cross-links

GPCL is caused by glucosupanes, α-diketones, and lysine-dihydropyridinium-lysine[11]. PTB has the ability to cleave α-diketone bonds, and this action cleaves GPCL[30]. EE is shown to have cleavage effects both on GPCL and AGE cross-linking in a concentration-dependent manner. AGE cross-link cleavage actions were observed with ellagitannin, cyanidin, delphinidin and their glycosides contained in pomegranate extract. The involvement of the hydroxybenzene structure in these effects has been postulated[31].

Delphinidin, a kind of purple component substance of edible purple chrysanthemum flowers, may have been involved in the GPCL cleavage in EE. Meanwhile, it has been reported that PTB was not involved in the cross-linking of mouse tail tendon collagen[35]. The cross-linking of AGEs using PPD as a model does not necessarily coincide with the cross-linking of proteins. Therefore, it is necessary to verify the GPCL cleavage effect of EE *in vivo*.

3) OPH activity enhancement

The OPH activity-enhancing effect has been observed in many kinds of herbal teas, and its effect has also been reported in those that use leaves and roots as raw materials, such as chicory (Cichorium intybus) and dandelion (Taraxacum sp.)[16]. The enhancement of OPH activity of EE was presumed to be due to the involvement of substances commonly found in Asteraceae plants.

4) Inhibition of GPCL formation and GPCL cleavage potential in edible purple chrysanthemum

Inhibition of glycative stress includes suppression of postprandial hyperglycemia, suppression of AGE formation, and degradation of AGEs. In addition, oxidative stress is a factor that accelerates the formation of AGEs, and antioxidant effects also contribute to the suppression of glycative stress[1]. EE was found to inhibit the formation of F-AGEs, 3DG, GO,

and MGO, as well as inhibit the formation of GPCL. These effects of EE were similar to those of AG, an inhibitor of glycation reaction, and AG has been reported to improve diabetic nephropathy with continuous intake of 150-300 mg per day [16]. Furthermore, delphinidins and cyanidins, which are pigment components of edible purple chrysanthemum, have antioxidant effects [36, 37]. Therefore, the continuous intake of EE may prevent the formation of GPCL by inhibiting the formation of AGEs as well as AG.

The formation of AGE cross-links due to glycative stress is a factor in the reduction of protein elasticity [1]. The accumulation of AGEs therefore increases the risk of bone fractures due to stiffening of bone collagen, reduces range of joint motion, decreases skin elasticity, and causes wrinkle formation and tarseness. Glycated proteins are less likely to be degraded by proteases, thus slowing down tissue metabolism [38].

In a study in which healthy women ingested a mixed herbal extract with both AGE cross-link cleavage actions and OPH activity enhancing effects for 12 consecutive weeks, the blood pentosidine, a cross-linking AGE, and facial wrinkles decreased [39]. In a study of 8 weeks of continuous intake of edible purple chrysanthemum powder, a trend toward improvement in skin elasticity was observed [20]. EE, a 50% ethanol extract of edible purple chrysanthemum powder, showed AGE cross-link cleavage, GPCL cleavage, and OPH activity enhancement. These results suggest that continuous intake of EE may degrade and remove proteins cross-linked by glycation, and prevent and repair protein dysfunction.

Research limitations

The inhibition of GPCL formation and GPCL cleavage by EE in this study were *in vitro* results. Further verification of these benefits when EE is continuously ingested by humans is needed.

Conclusion

EE inhibited the GPCL formation and cleaved GPCL. In addition, EE inhibited the formation of F-AGEs and glycation intermediates, cleaved AGE cross-links, and enhanced OPH activity, suggesting that EE may prevent and repair the deterioration of protein function caused by glycative stress.

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Declaration of Conflict of Interest

Research funding was provided by Unial Corporation for this study.

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