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Original article

Protein cross-linking inhibition effects induced by glycative stress of black galangal, *Kaempferia parviflora* Wall. Ex. Baker (Zingiberaceae)

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Abstract

Accumulation of advanced glycation endproducts (AGEs) due to glycative stress in the body leads to tissue stiffening and physiological dysfunction due to protein cross-link formation, and is one of the factors contributing to aging and lifestyle-related diseases. Suppression of glycative stress includes suppression of postprandial hyperglycemia, suppression of glycation reaction, and degradation and excretion of AGEs. Fluorescent AGEs include both cross-linked and non-cross-linked AGEs. Inhibition of fluorescent AGE formation has been reported in various plant materials, one of which is the ginger plant black galingal (*Kaempferia parviflora* Wall. Ex. Baker; KP), with hydrophilic components and polymethoxy flavonoids (PMF) involved in this effect. On the other hand, there are several types of cross-linked structures of glycated proteins, α-diketone being one of them. The cleavage action of α-diketones is called AGE cross-link cleavage, and phenylpropanedione (1-phenyl-1, 2-propanedione; PPD) has been used as a cross-linking model substance. The rate of AGE cross-link cleavage has been evaluated from the rate of free benzoic acid formation. KP rhizome has also been reported to have AGE cross-link-cleaving activity. There have been only a few reports on the effect of AGE formation inhibition and AGE cross-link cleavage on protein cross-linking by glycation. In this study, we examined the inhibition of glycated-protein cross-linking (IGPC) and cleavage of glycated-protein cross-linking (CGPC) of KP extract, which previously has been shown to have effects of fluorescent AGE formation inhibition and AGE cross-linking cleavage.

KEY WORDS: inhibition of AGE formation, AGE cross-linking, inhibition of glycated-protein cross-linking, glycated-protein cross-linking

Introduction

Accumulation of advanced glycation endproducts (AGEs) due to glycative stress in the body leads to tissue stiffening and physiological dysfunction through the formation of protein cross-links, and is one of the factors that contribute to aging and lifestyle-related diseases. Inhibition of glycative stress in the body is called "anti-glycation" or "glycation care," and includes suppression of postprandial hyperglycemia, suppression of glycation reaction, and degradation and excretion of AGEs[1,2]. Inhibition of fluorescent AGE formation, one of the indicators of glycation care, has already been reported in various plant materials such as vegetables and herbs[3,4]. This effect has also been observed in black galangal (*Kaempferia parviflora* Wall. Ex. Baker; KP), a member of the ginger family (Zingiberaceae)[5,6], and the presence of polymethoxy flavonoid (PMF) and hydrophilic components have been reported[7]. Fluorescent AGEs include various substances such as non-crosslinking argpyrimidine, crosslinking vesperlysine, and crossline[8].

As one of the indicators of AGE degradation action, the cleavage action of α-diketone, a kind of crosslink structure of glycated proteins, is evaluated[9-11]. This is called AGE crosslink cleavage effect. Phenylpropanedione (1-phenyl-1,2-propanedione; PPD) is used as a crosslinking model substance to evaluate the AGE crosslink cleavage effect. The AGE crosslink cleavage rate is calculated from the production rate of benzoic acid, which is liberated by the cleavage of α-diketones by the test substance. Substances that have the AGE crosslink cleavage effect include *N*-phenacylthiazolium bromide (PTB)[9], rosmarinic acid[12], ellagitannins[10], and flavonoids[11] have been reported. This effect has also been reported in the rhizome of KP[13].

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On the other hand, there are only a few reports that have examined the effects of components with AGE formation inhibitory effects on protein cross-link formation and AGE cross-link cleavage effects on glycated proteins [14,15].

In this study, we aimed to evaluate the effects of KP, which has the effect of inhibiting fluorescent AGE formation and cleavage of AGE crosslinks, on the inhibition of glycated-protein cross-linking (IGPC) and cleavage of glycated-protein cross-linking (CGPC).

Materials and Methods

(1) Reagents

Reagents used were purchased from the following manufacturers: *N*-phenacylthiazolium bromide (PTB) from Sigma-Aldrich Japan (Meguro-ku, Tokyo, Japan). Lysozyme hydrochloride (lysozyme derived from egg white) aminoguanidine hydrochloride (AG) is from Fujifilm Wako Pure Chemical Industries, Osaka, Japan. 4-20% Mini-Protean TGX Precast gel is from Bio-Rad (Hercules, California, USA). Other reagents were purchased from Fujifilm Wako Pure Chemical Industries or Nakalay Tesque (Kyoto, Japan).

(2) *Kaempferia parviflora* (KP) rhizome samples and extraction conditions

Dried powder of KP rhizome, which is sold as a dietary supplement product in Japan, was used as the sample; KP powder was provided by Rene Co. The dried powder sample was extracted by mixing 40 mL purified water and 2 g of the powder. The hot water extraction conditions were incubation of the mixture in a water bath set at 80 °C for 60 minutes. The extract was then centrifuged at 2,500 rpm (800 x *g*) for 10 min and further filtered. The resulting solution was used as KP extract (*Kaempferia parviflora* extract; KPE) for verification. The solid concentration of KPE was calculated by placing 5 mL of KPE in an aluminum tray, evaporating it at 120 °C for 2 hours, and weighing the evaporated residue.

(3) Inhibition of glycated-protein cross-linking (IGPC)

The inhibition of glycated-protein cross-linking (IGPC) was performed using the lysozyme-glucose glycated-protein cross-linking model based on previous reports [16,17]. The reaction solution was prepared by adding 1/10 of the sample solution in 0.1 mol/L phosphate buffer (pH 7.4) containing 5 mg/mL lysozyme and 0.5 mol/L glucose. The following four reaction solutions were prepared and incubated at 60 °C for 40 hours; (A) solution with phosphate buffer, lysozyme solution, glucose solution, and each of the sample solutions added, (B) solution with purified water added in place of the glucose solution in A, (C) solution with sample solution added in place of the sample solution in A, and (D) solution with purified water added in place of the glucose solution in C. After completion of the reaction, the reaction solution was centrifugally filtered 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 was stained with CBB Stain One (Nacalai Tesque), and the gel images were imaged with the Pharos FX System (Bio-Rad). The intensities of the monomer (12.4 kDa), dimer (25.8 kDa), and trimer (40.5 kDa) bands of the enzyme were analyzed using ImageJ (NIH, Maryland, USA). AG was used as a positive control substance for inhibition of glycated protein cross-link formation.

The inhibition rate of protein cross-link formation was calculated based on the following formula, as previously reported [14].

Percentage inhibition 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 stained images of each reaction solution

(4) Cleavage of glycated-protein cross-linking (CGPC)

For the cleavage of glycated-protein cross-linking (CGPC), glycated lysozyme was prepared with reference to a previous report [14], and the degradation rate of lysozyme dimer by the sample was measured. 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 and then removing low molecular weight substances by ultrafiltration (3 kDa).

The following two reaction solutions were prepared; (A) A solution in which 1/2 volume of sample solution was added in 0.05 mol/L phosphate buffer containing 0.5 mg/mL glycosylated lysozyme and (B) a solution in which sample lysate was added in place of the sample solution in A. After incubation at 37 °C, the reaction solution was centrifuged through an ultrafiltration membrane (3 kDa) to remove low molecular weight substances. The reaction solution was subjected to SDS-PAGE using the same method as described in the previous section, and the gel was stained after electrophoresis, imaged, and analyzed for band intensities of lysozyme monomer and dimer in the reaction solution. PTB was used as a positive control for glycated protein cross-link cleavage.

The protein cross-link cleavage rate was calculated based on the following formula as previously reported [18].

Percentage of glycated protein cross-link cleavage (%)
$= [1 - \{(D_S/M_S) / (D_R/M_R)\}] \times 100$
S; Lysozyme band intensity at the time of sample addition
R; Band intensity of lysozyme when only sample solution is added
M; monomer band of lysozyme
D; dimer band of lysozyme

Statistical Analysis

Measurements are expressed as mean ± standard deviation. The 50% inhibition concentration (IC_{50}; mg/mL) or 50% effective concentration (EC_{50}; mg/mL) was calculated from the results of the three sample concentrations [19,20]. A smaller IC_{50} or EC_{50} value indicates a stronger effect. Tukey's multiple comparison test was used to compare the measured values. Statistical analysis results were considered significant at a risk rate of less than 5%.

Results

Inhibition of glycoprotein cross-link formation (IGPC)

IGPC showed concentration-dependent inhibition of dimer and trimer formation in both KPE (0.097 – 0.87 mg/mL) and AG (0.10 – 1.00 mg/mL) (***Table 1***). The IC_{50} of IGPC was 3.7-fold smaller for AG than for KPE in dimer and 30.9-fold smaller in trimer, indicating a stronger effect of AG. Comparing IGPCs of trimer and dimer, both KPE and AG showed a stronger inhibitory effect on trimer formation compared to dimer. SDS-PAGE images showed that the monomer band of the glucose-sensitive sample was slightly larger than that of the glucose-free sample in the cathodic side (***Fig. 1***).

Glycated protein cross-link cleavage (CGPC)

CGPC was concentration dependent in KPE (0.1 – 1.0 mg/mL) and PTB (1.4 – 5.7 mg/mL) (***Table 2***). The EC_{50} of CGPC was 1.6-fold smaller in KPE (1.65 mg/mL) than in PTB (2.65 mg/mL), indicating a strong effect. The dimer formation rate of Ref (with water) ranged from 10.3 to 11.9 %, and no trimer formation was observed (***Fig. 2***).

Table 1. Inhibitory effects of KPE and AG on cross-linking formation in the lysozyme-glucose reaction model.

Sample	Conc. (mg/mL)	Inhibition ratio on cross-linking formation (%)			
		Dimer	IC_{50} (mg/mL)	Trimer	IC_{50} (mg/mL)
KPE	0.097	-3.4 ± 4.7	1.26 [1)]	-31.7 ± 10.8	0.51
	0.29	9.7 ± 3.1 *		21.8 ± 0.6 *	
	0.87	45.6 ± 3.9		76.8 ± 1.6	
AG	0.10	30.1 ± 4.3	0.34	72.9 ± 3.8	0.011
	0.30	49.2 ± 3.6 *		88.7 ± 2.8 *	
	1.00	65.9 ± 1.6		98.1 ± 3.3	

1) Extrapolated value, mean ± SD, n = 3; *p < 0.05, Tukey's multiple comparison test; KPE, incubation with *Kaempferia parviflora* rhizome hot water extract; AG, incubation with aminoguanidine; IC_{50}, 50 % inhibition concentration; SD, standard deviation.

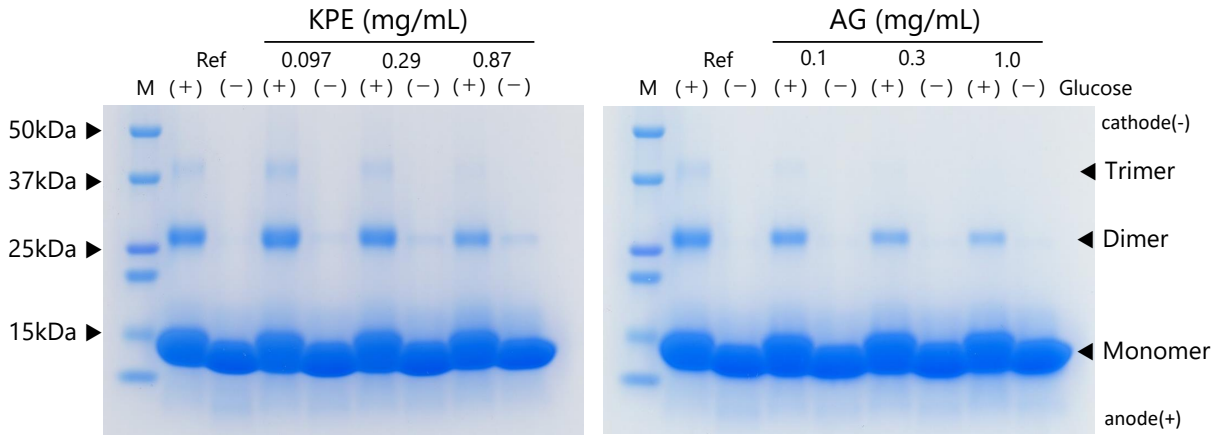

Fig. 1. **Inhibitory effects of KPE and AG on cross-linking formation in the lysozyme-glucose reaction model.**

5 mg/mL lysozyme were incubated at 60 °C for 40 hours. SDS-PAGE was conducted using 4 - 20 % acrylamide gels. M, molecular weight markers; Ref, incubation with water; KPE, incubation with *Kaempferia parviflora* rhizome hot water extract; AG, incubation with aminoguanidine; (+), incubation with 0.5 mol/L glucose; (–), incubation without glucose.

Table 2. Cleavage effects of KPE and PTB on lysozyme dimer in the glycated lysozyme.

Sample	Conc. (mg/mL)	Cleavage (%)		EC_{50} (mg/mL)
KPE	0.1	22.6 ± 7.9	*	1.65 [1)]
	0.3	34.5 ± 6.7		
	1	44.8 ± 4.0		
PTB	1.4	42.8 ± 6.5	*	2.65
	2.8	51.1 ± 9.1		
	5.7	58.4 ± 5.6		

1) Extrapolated value, mean ± SD, n = 3, * $p < 0.05$, Tukey's multiple comparison test; KPE, incubation with *Kaempferia parviflora* rhizome hot water extract; PTB, incubation with *N*-phenacylthiazolium bromide; EC_{50}, 50 % effective concentration; SD, standard deviation.

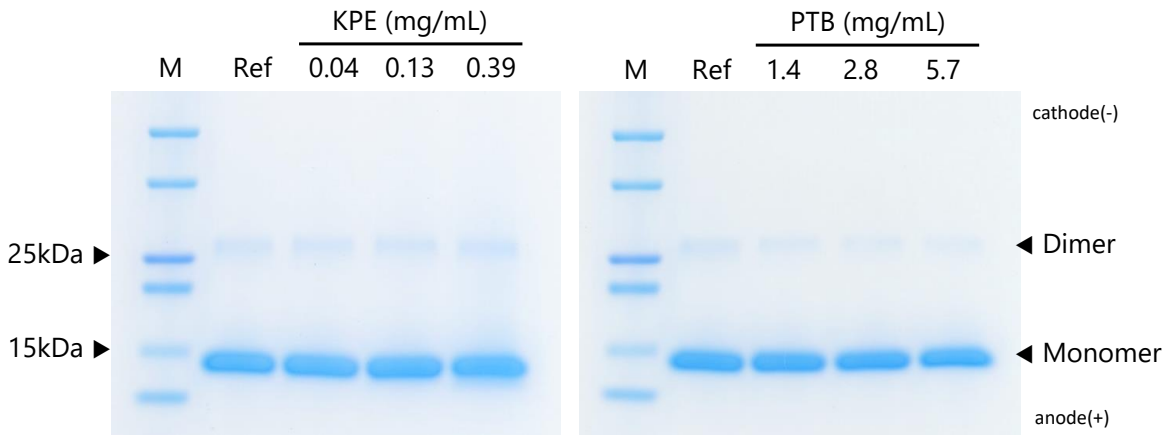

Fig. 2. **Cleavage effects of KPE and PTB on lysozyme dimer in the glycated lysozyme.**

0.5 mg/mL glycated lysozyme were incubated at 37 °C for 16 hours. SDS-PAGE was conducted using 4 - 20 % acrylamide gels. M, molecular weight markers; Ref, incubation with water; KPE, incubation with *Kaempferia parviflora* rhizome hot water extract; PTB, incubation with *N*-phenacylthiazolium bromide.

Discussion

IGPC of KPE

KPE has been shown to inhibit the formation of AGEs (fluorescent AGEs, pentosidine, N^{ε}-(carboxymethyl) lysine; CML) and glycation reaction intermediates (3-deoxyglucosone; 3DG, glyoxal; GO, methylglyoxal; MGO) [13)]. Hydrophilic components of KPE are involved in these effects [7)]. AGEs with crosslinking properties include argpyrimidine, vesperlysine, and crossline [8)]. These are measured as fluorescent AGEs (excitation wavelength: 370 nm, fluorescence wavelength: 440 nm). Pentosidine is also fluorescent (excitation wavelength: 335 nm, fluorescence wavelength: 385 nm) and crosslinkable [21)]. Furthermore, glycation intermediates such as 3DG, GO, and MGO are a type of aldehyde and are involved in the formation of cross-linkable AGEs [22, 23)]. From these facts, it is presumed that the IGPC of KPE, a hot water extract of KP, is related to the inhibitory effect of KPE on the formation of AGEs and glycation reaction intermediates. Protein cross-linking by glycation is different from physiological cross-linking by disulfide bridges and lysyl oxidase, and is called pathological cross-linking because it forms disordered cross-links. The formation of pathological cross-links by glycation of skin and bone collagen is considered to be a factor in the loss of skin elasticity and bone fractures due to the deterioration of bone quality [24, 25)]. IGPC of KPE may function to prevent hardening and functional deterioration caused by protein cross-linking and to inhibit aging in the body.

In the SDS-PAGE image of IGPC, the band width of the monomer was larger in the cathode side in the glucose-added (+) sample than in the glucose-free (–) sample (***Fig. 1***).

The native-PAGE image of aldehyde-induced AGE-modified BSA is shifted toward the positive side, presumably due to a relative negative change in the protein surface charge[26]. In SDS-PAGE, SDS neutralized the protein charge, and the shift of the band was on the cathodic side, suggesting that the difference in the glucose (+) monomer band was due to a different factor than the protein charge change. In the glycation reaction of lysozyme and glucose, not only cross-linked AGEs but also non-cross-linked AGEs such as CML are generated. Therefore, the band shift of monomer may be due to the formation of non-crosslinking AGEs of lysozymes.

CGPC of KPE

The involvement of α-diketones, glucospan, and lysine-dihydropyridinium-lysine[27, 28] in the formation of cross-links by protein glycation has been reported. A hydrophilic version of PTB, agebrium (3-phenacyl-4,5-dimethylthiazolium chloride), has been called an AGE breaker because of its ability to cleave α-diketones[29, 30]. KPE has been reported to cleave α-diketones. However, when KPE and PTB were compared in terms of α-diketone cleavage at 1.42 mg/mL, KPE was only about one-fifth as potent as PTB[13]. The CGPC of KPE was 1.6 times stronger than that of PTB, which was different from the evaluation results of α-diketone cleavage. The reason for this difference may be that the protein cross-links of glycated lysozyme contain many cross-links other than α-diketones, and the protein cross-link-cleaving effect of KPE may have been stronger in cleaving cross-links other than α-diketones.

Proteins cross-linked by glycation are not easily degraded by proteases and are not easily metabolized[31]. Therefore, CGPC may be involved in the reparative action of proteins cross-linked by glycation. On the other hand, the cross-linking structure in which CGPC acts is not clear. In particular, the effect on physiological cross-linking of proteins needs to be verified.

Conclusion

The effect of KPE on the formation of protein cross-links by glycation was verified, and both IGPC and CGPC were observed. In IGPC, the involvement of KPE in the inhibition of crosslinkable AGEs and aldehyde formation was inferred. In CGPC, KPE may be involved in crosslink cleavage action other than α-diketone cleavage as well as in the α-diketone cleaving action of KPE. KPE may function to inhibit glycation-induced protein cross-link formation, prevent stiffening and functional decline, and inhibit the aging process in the body.

Declaration of Conflict of Interest

Research funding was received from Rene Corporation for conducting this study.

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