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Original article

Effect of rice bran extract on *in vitro* advanced glycation end product formation

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Abstract

***Objectives*:** In recent years, attention has been paid to the effect of inhibiting advanced glycation end product (AGE) formation in the body for the purposes of anti-aging, health promotion, and lifestyle-disease prevention. In the present study, we evaluated the anti-glycation effects of rice bran extract and the potential of its use as an anti-glycation product.

***Methods*:** The test product used was powdered rice bran provided by Sunbran Co., Ltd. (Tendo, Yamagata, Japan) and its hot water extract contains 37.83 mg/mL as solid content. Using an *in vitro* method with glucose and human serum albumin (HSA), we analyzed the inhibition of the formation of AGEs; specifically fluorescent AGEs, 3-deoxyglucosone (3DG), pentosidine (Pent), and N^{ε}-(carboxymethyl)lysine (CML) by rice bran extract utilizing fluorescence spectroscopy, high-performance liquid chromatography (HPLC), and enzyme linked immunosorbent assay (ELISA). We analyzed the test product's anti-glycation effects by comparing the half maximal inhibitory concentration (IC_{50}) against these glycated products with aminoguanidine, a known inhibitor of glycation.

***Results*:** IC_{50} of fluorescent AGEs (1.3 mg/mL) and 3DG (1.1 mg/mL) by rice bran (hot water extract) were 1.3 mg/mL and 1.1 mg/mL, respectively, 10 times higher than that of aminoguanidine; while IC_{50} against CML (0.38 mg/mL) was 4 times higher for rice bran than that of aminoguanidine. IC_{50} against Pent (3.8 mg/mL) was markedly lower for rice bran since aminoguanidine did not prevent Pent formation in this method.

***Conclusion*:** These results suggest that rice bran extract inhibits glycation, although IC_{50} was greater than that of aminoguanidine, except for Pent, and it may be useful for anti-glycation products.

***KEY WORDS*:** rice bran, advanced glycation end products (AGEs), pentosidine, N^{ε}-(carboxymethyl)lysine (CML), 3-deoxyglucosone

Introduction

Rice bran is known to have a variety of components such as oil, protein, minerals and vitamin B group [1-6)]. Rice bran oil characteristically contains a high ratio of oleic acid in fatty acid constitution and vitamin E, and it is reported to be resistant to oxidation by heating [7)]. However, no information is available regarding the effect of rice bran on glycative stress. The purpose of the present study is to elucidate the effect of the functional food material, a rice bran extract, on the formation of advanced glycation end products (AGEs) in an *in vitro* glycation model [8)] between glucose and human serum albumin (HSA).

Method

Test product

The powdered rice bran "High-Bref ™" (test product) was provided by Sunbran Co. Ltd. (Tendo, Yamagata, Japan). Hot water extract was prepared after adding 15 g test product to 150 mL distilled water followed by boiling for 1 hour at 100°C. The 75 mL extract solution was obtained after centrifugation at 3,000 rpm for 15 minutes and filtration of the supernatant by a Toyo filtration paper No.2 (Toyo Roshi Kaisha, Ltd., Bunkyo-ku, Tokyo, Japan); The extract solution was reserved in a freezer at -20°C until the experiment. The

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5 mL of hot water extract solution contained 37.83 mg/mL as solid content. The solution was diluted with distilled water and adjusted to the concentration of 12.61 mg/mL and 3.78 mg/mL. For the initial study using fluorescent AGE measurement, the ethanol extract was prepared after mixing 15 g test product and 150 mL of 80% ethanol and incubated at 37 °C for 15 hours with stirring (40 rpm/min); The reacted solution was centrifuged at 3,000 rpm for 15 minutes, then the supernatant was obtained. The 5 mL of ethanol extract solution contained 16.50 mg/mL as solid content. The solution was diluted with distilled water and adjusted to concentrations of 5.5 and 1.65 mg/mL. Aminoguanidine [9] (Wako Pure Chemical Industries Co., Ltd., Chuo-ku, Osaka) was used as a positive control.

Fluorescent AGE measurement

AGE-derived fluorescence was measured as reported previously using a glucose/HSA model [7]. Briefly, 100 μL of various concentrations of the test product extract or aminoguanidine in aqueous solution were added to 500 μL 0.1 mol/L phosphate buffered solution (PBS, pH 7.4), 100 μL distilled water, 200 μL 40 mg/mL HSA (Sigma Chemical Co., Ltd; St. Louis, MO, USA), and 100 μL 2.0 mol/L aqueous solution of glucose. Distilled water was then added to make up a total volume of 1.0 mL, and the material was incubated at 60°C for 40 hours (Solution A). Final concentrations were 8 mg/mL HSA and 0.2 mol/L glucose. At the same time, a solution including distilled water, added in lieu of aqueous glucose, was incubated as a blank for each reaction solution (Solution B). Samples prepared without the addition of the test product or aminoguanidine were incubated as positive controls (Solution C). And at the same time, a solution including distilled water, added in lieu of aqueous glucose, was incubated as a blank for each positive control (Solution D). Fluorescent AGEs were measured quantitatively in each sample reaction solution (A, B, C, D) to evaluate inhibitory activity for AGE formation. AGE-derived fluorescence was measured using an ARVO MX 1420 ARVO series Multilabel Counter (Perkin-Elmer Japan Corp.; Hodogaya-ku, Yokohama, Kanagawa, Japan) microplate reader at an excitation wavelength of 370 nm and a fluorescence wavelength of 440 nm. Calibration curves for inhibition of fluorescent AGE formation were constructed by adding individual samples to a reaction solution at three concentrations and calculating the inhibition of AGE formation after the reaction. Inhibition of AGE formation (%) was calculated using the following formula, and half maximal inhibitory concentration (IC_{50}) values were then calculated to represent anti-glycation activity. Results were obtained as the average values of 3 measurements.

Formula: Inhibition of fluorescent AGE formation (%) =
$$(1-(A-B)/(C-D))\times 100$$

3DG measurement

3-deoxyglucosone (3DG) was measured using the Tosoh high-performance liquid chromatography (HPLC) system (Tosoh Corporation, Minato-ku, Tokyo, Japan) as previously reported [10,11]. Samples were prepared from 100 μL of test sample, aminoguanidine or distilled water added to 125 μL of 20 μg/mL 2,3-pentadione (Wako Pure Chemical Industries Ltd.), which was used as an internal standard, and 150 μL of distilled water. The mixture was stirred, then 250 μL of 6.0% perchloric acid (Wako Pure Chemical Industries) was added, stirred, and centrifuged at 12,000 rpm for 10 minutes; 800 μL of the supernatant was added to 1,000 μL of saturated sodium bicarbonate solution (Wako Pure Chemical Industries), stirred; 100 μL of 2,3 diaminonaphthalene labeling reagent (Dojindo Laboratories, Kumamoto, Japan) was added, and the mixture was incubated for 24 hours at room temperature. The HPLC conditions were as follows; Column, YMC-Pack CN, 150 × 4.6 mm I.D column (YMC CO. Ltd., Shimogyo-ku, Kyoto, Japan); eluent, 50 mM phosphoric acid; acetonitrile: methanol = 70:17:13. The flow rate and detection wavelength were 1.0 mL/min and ultraviolet (UV) 268 nm.

Pentosidine measurement

Pentosidine (Pent) was measured by the enzyme-linked immunosorbent assay (ELISA) using a commercial kit "FSK Pentosidine" (Fushimi Pharmaceutical Co., Ltd., Marugame, Kagawa, Japan) as previously reported [10] and the manufacturer's instructions. After adding 50 μL of serum or Pent standard to 20 μL pronase and 80 μL of Tris-HCl buffer, the mixture was incubated at 55°C for 90 minutes, and heated in boiling water for 15 minutes to inactive on the enzyme. These pretreated samples were added to each well and incubated at 37°C for 1 hour after washing. Then, 50 μL of anti-Pent antibody and Pent standard solution or pretreated sample were added to each well and incubated at 37°C for 1 hour after washing. A color development regent containing 0.5 mg/mL of 3,3'5,5'-tetramethylbenzidine (TMB) was added to each well. The reaction was stopped 10 minutes later by adding 100 μL of TMB stop buffer. The absorbance was measured within 10 minutes at 450 nm and 630 nm. The standard curve was obtained by measuring standard Pent solutions.

CML measurement

N^{ε}-(carboxymethyl)lysine (CML) was measured using a CircuLex N^{ε}-(carboxymethyl)lysine ELISA Kit (MBL Co. Ltd., Ina, Nagano, Japan) [10]. Briefly, 30 μL of each reacted sample or CML standard were diluted with 90 μL of sample/standard dilution buffer. Then, 120 μL of anti-CML adduct monoclonal antibody (clone name: MK-5A10) solution was added to each diluted sample, stirred, and 100 μL of each mixture was dispensed into a well of an antigen-coated microplate. The plates were incubated for 1 hour at room temperature, washed with 0.2% Tween-20; then 100 μL of horse radish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) polyclonal antibody solution was dispensed into each well, and further incubated for 1 hour, washed with washing buffer; then 100 μL of tetra-methyl-benzidine solution was added to each well, and the plate was wrapped in aluminum foil and incubated at room temperature for 10 minutes; 100 μL of stop solution was added and the absorbance was measured at dual wavelengths of 450 nm/540 nm using a spectrophotometric microplate reader (SPECTRA MAX 190, Molecular Devices, Chuo-ku, Tokyo, Japan) within 30 minutes. The CML concentration in each sample was calculated from a standard curve of CML standards, and the IC_{50} against CML by test samples was calculated.

Results

Fluorescent AGE measurement

The final concentrations of rice bran hot water extract were 0.378 mg/mL, 1.26 mg/mL and 3.78 mg/mL (***Fig. 1***) and ethanol extract 0.165 mg/mL, 0.550 mg/mL, 1.65 mg/mL and 3.30 mg/mL (***Fig. 2***) in the *in vitro* reaction between glucose and HSA. The test product showed an inhibitory effect on fluorescent AGE formation in the glucose/HSA model; IC_{50} was 1.3 mg/mL in hot water extract and 2.7 mg/mL in ethanol extract. The ethanol extract was less effective than the hot water extract. Aminoguanidine showed IC_{50} of 0.095 mg/mL (***Fig. 3***), which was less than that of the test product.

3DG measurement

Percentage inhibition of rice bran hot water extract on 3DG formation is presented in ***Fig. 4***. The extract dose-dependently inhibited 3DG formation in the glucose/HSA model; IC_{50} was 1.1 mg/mL. Aminoguanidine also showed dose-dependent inhibition on 3DG formation; IC_{50} was 0.12 mg/mL (***Fig. 5***).

Pent measurement

Percentage inhibition of rice bran hot water extract on Pent formation is presented in ***Fig. 6***. The extract showed dose-dependent inhibition on Pent formation; IC_{50} was 3.8 mg/mL in the glucose/HSA model. Aminoguanidine had no effect on Pent formation.

CML measurement

Percentage inhibition of rice bran hot water extract on CML formation is presented in ***Fig. 7***. The extract dose-dependently inhibited CML formation in the glucose/HSA model; IC_{50} was 0.38 mg/mL. Aminoguanidine showed dose-dependent inhibition on CML formation; IC_{50} was 0.12 mg/mL (***Fig. 8***).

Discussion

Rice bran extract contains a variety of active components which are reported to have an anti-oxidative effect [5], and an anti-inflammation effect [12] and improve glucose and lipid metabolism [13-15]. The active components include polyphenols, such as protocatechuic acid, caffeic acid, ferulic acid, gentisic acid which have strong anti-oxidative capacity [5]. Ferulic acid was the dominant phenolic acid in the rice bran samples.

This study added the anti-glycative effect of rice bran extract as an extra value associated to polyphenols. Some reports have shown the anti-glycation effect of ferulic acid [16-19]. Caffeic acid oligomers reportedly prevent AGE formation by inhibiting the increase in 3-DG production [20]. Recently, rice bran was also found to contain quercetin [21,22] which is reported to show an anti-glycative effect [23].

The results of IC_{50} of the rice bran hot water extract showed a marked effect on Pent formation, one-fourth effect on CML formation and one-tenth effect on fluorescent AGE and 3DG formation, when compared with IC_{50} of

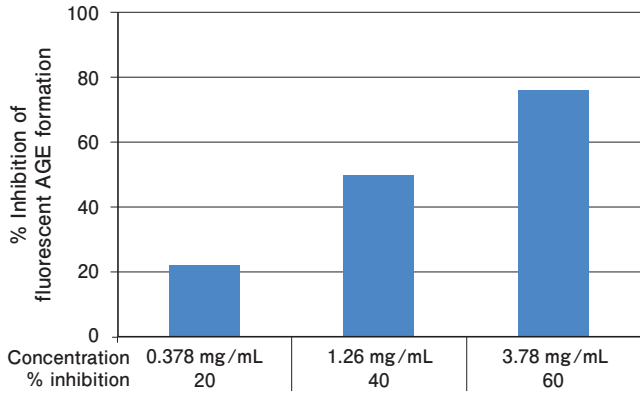

***Fig. 1*: Anti-glycation activity of rice bran hot water extract: Fluorescent AGE formation**

Percent inhibition of fluorescent AGE formation in the glucose/HSA reaction model. Half maximal inhibitory concentration (IC_{50}) = 1.3 mg/mL. AGE, advanced glycation end product; HSA, human serum albumin.

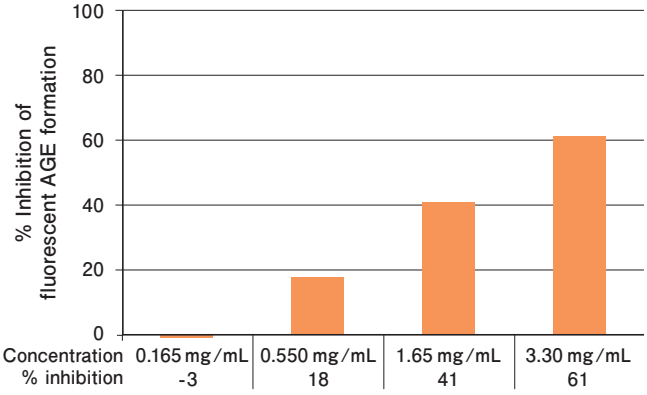

***Fig. 2*: Anti-glycation activity of rice bran ethanol extract: Fluorescent AGE formation**

Percent inhibition of fluorescent AGE formation in the glucose/HSA reaction model. Half maximal inhibitory concentration (IC_{50}) = 2.7 mg/mL. AGE, advanced glycation end product; HSA, human serum albumin.

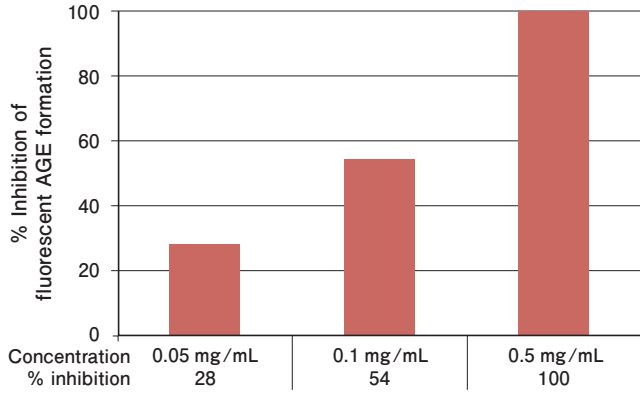

***Fig. 3*: Anti-glycation activity of aminoguanidine: Fluorescent AGE formation**

Percent inhibition of fluorescent AGE formation in the glucose/HSA reaction model. Half maximal inhibitory concentration (IC_{50}) = 0.095 mg/mL. AGE, advanced glycation end product; HSA, human serum albumin.

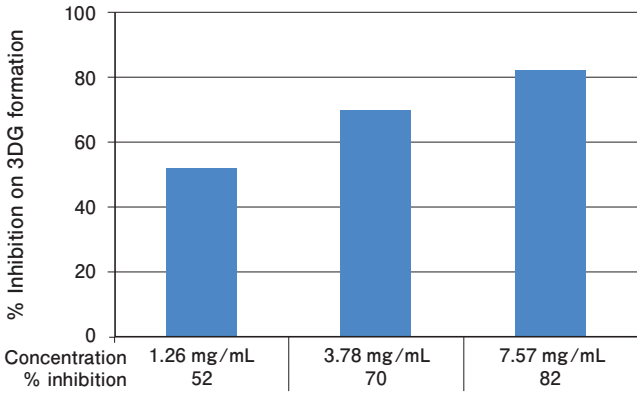

***Fig.4*: Anti-glycation activity of rice bran hot water extract: 3DG formation**

Percent inhibition of 3DG formation in the glucose/HSA reaction model. Half maximal inhibitory concentration (IC_{50}) = 1.1 mg/mL. 3DG, 3-deoxyglucosone; HSA, human serum albumin.

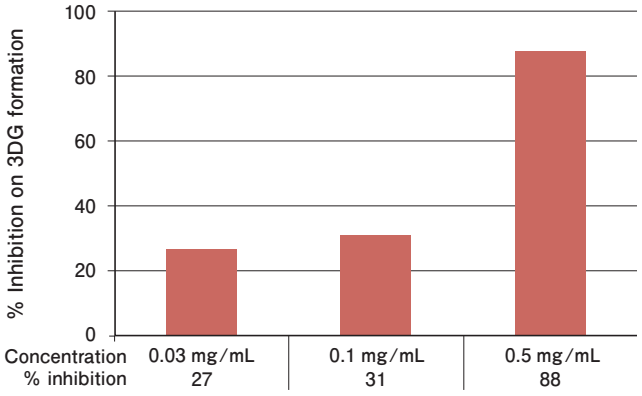

***Fig.5*: Anti-glycation activity of aminoguanidine: 3DG formation**

Percent inhibition of 3DG formation in the glucose/HSA reaction model. Half maximal inhibitory concentration (IC_{50}) = 0.12 mg/mL. 3DG, 3-deoxyglucosone; HSA, human serum albumin.

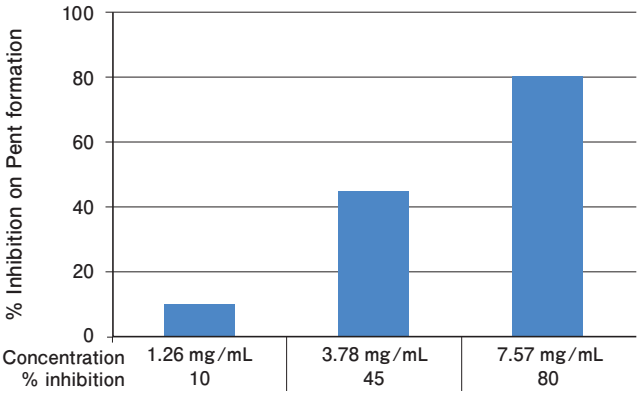

***Fig.6*: Anti-glycation activity of rice bran hot water extract: Pent formation**

Percent inhibition of Pent formation in the glucose/HSA reaction model. Half maximal inhibitory concentration (IC_{50}) = 3.8 mg/mL. Pent, pentosidine; HSA, human serum albumin.

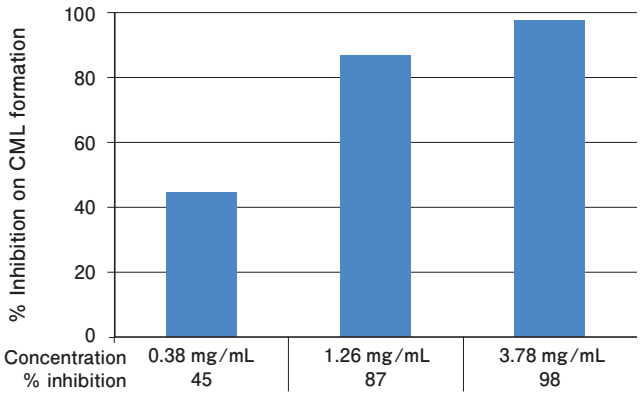

***Fig.7*: Anti-glycation activity of rice bran hot water extract: CML formation**

Percent inhibition of 3DG formation in the glucose/HSA reaction model. Half maximal inhibitory concentration (IC_{50}) = 0.38 mg/mL. CML, N^{ε}-(carboxymethyl)lysine; HSA, human serum albumin.

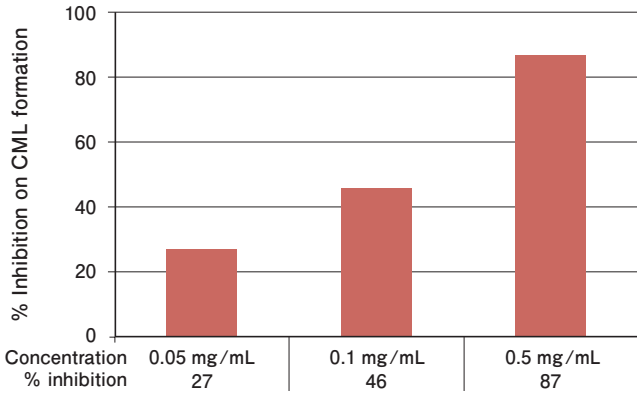

***Fig.8*: Anti-glycation activity of aminoguanidine: CML formation**

Percent inhibition of CML formation in the glucose/HSA reaction model. Half maximal inhibitory concentration (IC_{50}) = 0.12 mg/mL. CML, N^{ε}-(carboxymethyl)lysine; HSA, human serum albumin.

aminoguanidine and rice-related samples [24] as presented in *Table 1*. Rice bran (hot water extract) showed an equal IC_{50} of rice (bran) and much less IC_{50} than that of rice (unpolished and polished), almost ineffective, in the same experimental model [24]. The effect may be due to the contribution of rice bran ingredients, *i.e.*, ferulic acids. The mechanism by which these ferulic acids prevent AGE formation still remains unknown.

An intake of 150 - 300 mg/day of aminoguanidine, a glycation inhibitor, has been shown to have a progression-inhibiting effect on diabetic nephropathy [25], and we previously calculated the daily intake of rice bran needed to obtain anti-glycation activity equivalent to that of aminoguanidine, resulting 4.2 g/day [24]. This amount of rice bran is not difficult to achieve to take orally.

In the present study, the rice bran extract inhibited CML formation more strongly than others, *i.e.*, fluorescent AGEs, 3DG, or pentosidine. In the AGE formation process, oxidative reactions are involved, especially with CML formation [26]. The reason why the effect was marked on CML formation may be explained by the anti-oxidation effects of polyphenols which are rich in rice bran in addition to its anti-glycation effect.

Conclusion

Rice bran hot water extract was confirmed to prevent AGE formation by inhibiting 3DG production in the *in vitro* glucose/HSA model. The mechanism remains unknown. Although IC_{50} of the test product was higher than that of aminoguanidine, except for Pent, it may be useful as an anti-glycation product.

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Conflicts of interest statement

The part of this work was supported by Sunstar Co. Ltd.

Table 1. Comparison of anti-glycation activity in the in vitro glucose / HSA model

No.	Sample name	IC_{50} (mg/mL)			
		Fluorescent AGEs	3DG	Pent	CML
1	Rice bran (hot water extract)	1.3	1.1	3.8	0.38
2	Aminoguanidine	0.095	0.12	NE	0.12
3	Rice (unpolished)*	203.557			
4	Rice (bran)*	1.409			
5	Rice (polished)*	NE			

IC_{50}, half maximal inhibitory concentration; HSA, human serum albumin; AGEs, anti-glycation activity; 3DG, 3-deoxyglucosone; Pent, pentosidine; CML, N^{ε}-(carboxymethyl)lysine; NE, no effect.

*Data of rice-related samples (unpolished, bran, and polished) are from reference 24.

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