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Evaluation of the Mutagenicity Potential of Trace-Rutinosidase Variety of Tartary Buckwheat (*Fagopyrum tataricum* Gaertn.) Using the Ames Test

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Abstract

To ensure the safety of "Manten-Kirari", a non-bitter and trace-rutinosidase variety of Tartary buckwheat, we evaluated its mutagenic activity in a bacterial reverse mutagenicity assay, the Ames test. *Salmonella typhimurium* TA100, TA1535, TA98, TA153, and *Escherichia coli* WP2 *uvrA* were employed as test bacteria. The flour of "Manten-Kirari" was dissolved at 12 - 50,000 μg/mL in DMSO and investigated. The number of revertants did not differ compared to the negative control for all concentrations tested, whereas that in the positive control, the number of revertants was increased with or without metabolic activation for each bacterial strain tested. These results suggested that the flour of the Tartary buckwheat "Manten-Kirari" was not genotoxic.

Keywords

Tartary Buckwheat, Rutin, Rutinosidase, Quercetin, Mutagenicity

1. Introduction

Rutin is a flavonoid and is widely distributed throughout the plant kingdom [1]-[4]. Rutin is reported to have various effects such as strengthening the blood capillaries [5] [6], as an antioxidant [7]-[9] and to have alpha-

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glucosidase inhibitory activities [10]. In addition, the clinical effects of rutin in a double-blind crossover study are investigated and reductions in serum myeloperoxidase and cholesterol levels [11], mucosal symptoms, headache, and tiredness are observed [12]. For these reasons, attention has been focused on buckwheat as a rutin-rich material for food products [13] [14].

Buckwheat is the only known cereal to contain rutin in its seeds. Among cultivated buckwheat species, Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) contains approximately 100-fold greater rutin in the seeds than common buckwheat (*Fagopyrum esculentum* Gaertn.). However, Tartary buckwheat seeds also contain extremely high rutinosidase activity [15]-[17] (**Figure 1**). This activity is sufficient to hydrolyze the rutin present in buckwheat flour (approximately 1% - 2% [w/w]) within a few minutes of the addition of water [15]-[17]. However, the flour of Tartary buckwheat, also known as "bitter buckwheat", is characterized by strong bitterness, thereby limiting its use in food products.

Recently, our research group develops a Tartary buckwheat variety named "Manten-Kirari" [18] [19]. The variety is developed by crossing between trace-rutinosidase line and "Hokkai T8", which is the reading Tartary buckwheat variety in Japan. "Manten-Kirari" flour exhibits rutinosidase activity about two or three orders of magnitude less than that of the common variety of Tartary buckwheat. Therefore, the majority of the rutin in "Manten-Kirari" is not hydrolyzed. As a result, the rutin concentration in foods containing "Manten-Kirari", such as noodle or pound cake, is much higher than doughs made with other varieties, in which almost all the rutin content is hydrolyzed [20]. In addition, the flour and food products lack the characteristic bitterness of other varieties. Therefore, "Manten-Kirari" is a promising ingredient for rutin-rich food products.

Currently, very limited information regarding the safety of Tartary buckwheat or rutin is available. Wilson *et al.* [21] report that intravenous and intraperitoneal injections of 30 to 50 mg/kg in rats and guinea pigs, and intravenous injections of 100 to 200 mg/kg in rabbits, show no deleterious effects. Currently, rutin, such as found in "Manten-Kirari", is not widely consumed in large amounts. Therefore, to ensure the safety of "Manten-Kirari", it is necessary to evaluate its mutagenic activity. In this context, we investigate the mutagenic activity of "Manten-Kirari" flour in a bacterial reverse mutagenicity assay (Ames test).

2. Materials and Methods

2.1. Flour Preparation

Seeds of the Tartary buckwheat variety "Manten-Kirari" (trace rutinosidase variety) were milled using a test mill (Quadrumat® Junior, Brabender® GmbH & Co., Duisburg, Germany) at the flour milling percentage of 63%. The flour was stored at −20˚C until used for experiments.

2.2. Rutin Hydrolysis of "Manten-Kirari" Flour in DMSO

Tartary buckwheat flour (100 mg) and 1.0 mL of DMSO were suspended and incubated at 37˚C for 3 hours. Next, to extract rutin and quercetin from the DMSO-suspended flour, 7.2 mL of methanol and 1.8 mL 0.1% phosphoric acid were added to the mixture and stored at 37˚C for 16 hours. After extraction, the sample was centrifuged at 5000 g, 10 min at 20˚C, the resultant supernatant was analyzed using HPLC [17], rutin and quercetin concentrations were determined. The extracts were filtered through a 0.45 mm filter and applied to HPLC. HPLC was performed on an CAPCELL PAK C18 column (SHISEIDO, Japan) at a flow rate of 1.5 ml/min. Elution gradient program was 0 - 20 min linear gradient from solvent A [methanol-water-phosphoric acid (30:69.7:0.3)] to solvent B [methanol]. A chromatograph was monitored at 360 nm.

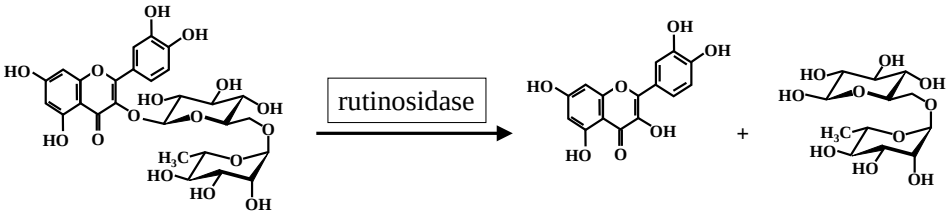

Figure 1. Rutin hydrolysis in Tartary buckwheat seeds.

2.3. Ames Test

Salmonella typhimurium TA100 [22], TA1535 [22], TA98 [23], TA1537 [24], and *Escherichia coli* WP2 *uvrA* were used for the Ames test. Phenotype confirmation, genotype and mutation type detected are shown in **Table 1**. A preliminary experiment was conducted to optimize the test solution. Tartary buckwheat flour did not completely dissolve in any of the following solutions tested: water, acetone, N,N-dimethylformamide, 1,4-dioxisane and 1,4-epoxybutane. Of these, DMSO resulted in the best dispersion condition; therefore, we employed DMSO as the test solution. Tartary buckwheat flour and DMSO were mixed (12 - 50,000 μg flour/mL_DMSO) and subjected to sonication for approximately 2 minutes.

The S9 microsomal fraction was used as a metabolic activation system. As a positive control, B[a]P and 2AA were used in the presence of S9, while AF-2, NaN_3 and ICR-191 were used in the absence of S9. The DMSO was used as a negative control. A standard preincubation assay [25] [26] was performed. In the plate incorporation method, two replicates were conducted per dose group.

In 2 mL of the overlay agar without S9, 0.1 mL of bacterial culture (2.3 - 5.7 × 10^9 bacteria), 0.1 mL of sample solution and 0.5 mL of 100 mM sodium phosphate buffer (pH 7.4) were added and mixed. Bacterial concentration was calculated using optical density. In the S9 added overlay agar, 0.5 mL of S9 mix was substituted for 0.5 mL of the sodium phosphate buffer, and the remainder of the protocol was as for the culture medium without S9 mix. Next, the molten overlay agar was added to the minimum salts agar containing 0.6% (w/v) agar and 0.5% (w/v) NaCl. After allowing the medium to harden, the plates were incubated at 37˚C for 48 h and the number of revertants was recorded. These experiments were performed at BML, Inc. (BML General Laboratory, Kawagoe, Saitama, Japan) under contract from the New Drug Research Center, Inc. (Eniwa, Hokkaido, Japan).

3. Results and Discussion

3.1. Rutin Hydrolysis of "Manten-Kirari" Flour in DMSO

Although the rutinosidase activity in "Manten-Kirari" is two or three orders magnitude less than that of the common variety, some rutinosidase activity remains. Therefore, we investigated the hydrolysis of rutin by trace amounts of rutinosidase in "Manten-Kirari" flour in DMSO. Rutin concentration of DMSO-suspended flour were almost same compared with intact flour (**Figure 2**). In addition, aglycone of rutin quercetin, which is the product of rutinosidase activity, was not increased in DMSO-suspended flour (**Figure 2**). This indicates that the rutin in "Manten-Kirari" was not hydrolyzed in DMSO. Therefore, the extract contained rutin as the major polyphenolic flavonoid.

3.2. Ames Test

To date, there have been few reports dealing with the mutagenic activity of rutin or Tartary buckwheat. Therefore, prior to start detailed examination, we first investigated a range of Tartary buckwheat concentrations, 12 to 50,000 μg/mL, as shown in **Table 2**. The positive controls showed an increase in the number of revertants both with and without S9 compared to the negative control. Also, the S9 mix or sample solution was confirmed as sterile. In the tested samples, colony numbers in "Manten-Kirari" flour did not differ from the negative control at all concentrations with or without metabolic activation for all bacteria tested. Notably, we observed precipitation with Tartary buckwheat concentrations >3130 μg/mL. In response, we used the concentration range 200 - 3130 μg/mL for a detailed examination, and the results are shown in **Table 3**. The Ames test showed no

Table 1. DNA sequence specificity of microbial test strains.

Allele	Strains	DNA target	Revertion event	Reference
*hisG*46	TA100	-G-G-G-	Base-pair substitution	[22]
*hisG*46	TA1535	-G-G-G-	Base-pair substitution	[22]
*trpE*95	wp2 *uraA*	A:T	Base-pair substitution	
*hisD*3052	TA98	-C-G-C-G-C-G-C-G-	Frameshift	[23]
*HisC*3076	TA1537	-C-C-C-C-C-C-(+1 cytosine at run of C's)	Frameshift	[24]

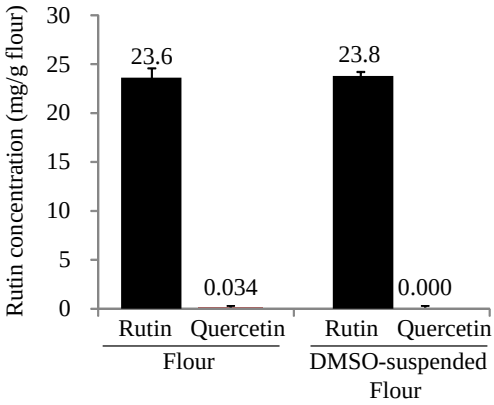

Figure 2. Rutin residual ratio in DMSO-suspended flour. There is no significant difference between flour and DMSO-suspended flour in rutin concent.

Table 2. Consideration of dose level for Tartary buckwheat flour on microbials tested with or without S9.

concentration (μg/ml)	TA100		TA1535		wp2 *uraA*		TA98		TA1537	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Negative controles 0	147	163	14	10	33	35	19	30	17	23
12	170	149	10	13	33	31	18	29	19	21
49	159	147	13	10	33	34	21	31	18	24
200	146	162	15	10	29	30	16	33	16	23
780	156	160	14	11	32	30	18	29	16	24
3130	161	164	11	10	32	31	16	27	16	19
12,500	159	175	11	11	32	31	19	29	15	19
50,000	148	169	10	11	32	28	16	28	14	19
Positive controls	605	949	130	332	143	315	604	225	1820	95
Substance	AF-2	B[a]P	NaN_3	2AA	AF-2	2AA	AF-2	B[a]P	ICR-191	B[a]P

Data are means of two independent experiments.

Table 3. Effect of different dose of Tartary buckwheat flour on microbials tested with or without S9.

Concentration (μg/ml)	TA100		TA1535		wp2 *uraA*		TA98		TA1537	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Negative controles 0	152	162	15	13	33	40	19	29	16	19
200	153	168	14	17	31	42	21	24	14	23
390	166	156	12	14	30	32	18	30	16	25
780	162	169	13	14	34	32	17	22	16	19
1560	166	157	15	16	36	31	20	30	15	21
3130	169	163	16	15	27	35	23	32	15	19
Positive controls	523	947	422	335	158	334	574	226	1710	85
Substance	AF-2	B[a]P	NaN_3	2AA	AF-2	2AA	AF-2	B[a]P	ICR-191	B[a]P

Data are means of two independent experiments.

increase in the number of revertants for each bacterial strain tested with or without S9. In this paper, all experiments were performed in duplicate; therefore, statistical analysis could not be applied. However, the results of "Dose optimization of Tartary buckwheat flour for bacterial mutagenic assessment with or without S9" (**Table 2**) and "Effect of various doses of Tartary buckwheat flour on bacterial mutagenicity with or without S9" (**Table 3**) were almost identical in the number of revertants for each sample. Therefore, although statistical analysis could not be conducted, the results show high reproducibility. From these results, it is suggested that the flour of the Tartary buckwheat variety "Manten-Kirari" does not exhibit genotoxicity. In addition, dough at a dose of 5000 mg flour/ kg is non effect level at acute and subacute test using experimental animals [27]. From these results, "Manten-Kirari" flour should be safe. The concentration of rutin in "Manten-Kirari" flour is about 15 mg/g flour. Among several crops, Tartary buckwheat contains a notably high polyphenol concentration. Some papers have described the effect of polyphenols on mutagenic activity, assessed by the Ames test [28]. Therefore, we hypothesized that rutin may affect the number of revertants in the Ames test. However, in this experiment, "Manten-Kirari" flour did not affect the number of revertants in the Ames test, suggesting that rutin is not mutagenic.

"Manten-Kirari" is a promising Tartary buckwheat variety for use in rutin-rich food products; therefore, the results of our mutagenesis analysis provide important information for optimizing its use in the food industry.

4. Conclusion

Tartary buckwheat flour of "Manten-Kirari" would not have mutagenesis.

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