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Radical-Scavenging Activity *in vitro* of Lemon Peel Fermented with *Aspergillus saitoi* and Its Suppressive Effect against Exercise-Induced Oxidative Damage in Rat Liver

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We prepared fermented lemon peel (FLP) with high antioxidative activity by fermentation of the lemon peel (LP) with *Aspergillus saitoi*. The antioxidative activity of LP and FLP was examined by the radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) *in vitro*, and the suppressive effect against exercise-induced oxidative damage in rat liver. FLP exhibited higher radical-scavenging activity against DPPH than LP. The administration of LP and FLP prior to exercise significantly suppressed the increases in thiobarbituric acid-reactive substance caused by lipid peroxidation during exhaustive exercise, with FLP having a tendency of higher activity than LP. Moreover, FLP showed a significant suppressive effect of N^{ε}-(hexanonyl)lysine content, a primary oxidative stress marker, which increases due to exhaustive exercise, whereas LP did not. The fermentation of LP with *A. saitoi* was thought to produce a high antioxidative activity. The high antioxidative activity of FLP was assumed to be related to the production of antioxidative flavonoids, as a hydroxyflavanone and aglycones, by the fermentation of lemon peel with *Aspergillus saitoi*.

Keywords: antioxidative activity, *Aspergillus saitoi*, exercise, fermentation, lemon peel, oxidative stress

Lemon (*Citrus limon* BURM. f.) is a popular citrus fruit around the world, and its juice has been used in many kinds of drinks and foods. Lemon peel (LP), which is produced during the manufacturing process of the juice of the lemon fruit, is partially used for foods and food materials such as essential oil and citrus pectin, and is also used in domestic animal food (Park *et al*., 1983; Sinclair, 1984). However, the majority of LP has been disposed of a byproduct, and its effective applications are desired in the field of food processing. We have explored antioxidative flavonoids in LP for the purpose of utilizing them as antioxidants for foods, and isolated eriocitrin (eriodictyol 7-*O*-rutinoside) which has antioxidative activity equal to α-tocopherol, as a standard antioxidant (Miyake *et al*., 1997a). Antioxidants are used as food additives for the prevention of lipid peroxidation and disappearance of food color. They have also been recognized for their suppressive effect against oxidative stress caused by free radicals and reactive oxygen species in disease and aging (Osawa *et al*., 1990). Oxidative stress is considered to play a causal role in the senescence of various organisms, and to increase the accrual of irreparable damage to lipids, proteins and DNA, which may result in disease. Effective antioxidants in food having a suppressive effect against oxidative stress *in vivo* have been explored.

It was reported that eriocitrin showed a significant suppressive effect against oxidative stress caused by diabetic rats (Miyake *et al*., 1998a) and exercise-induced rats (Minato *et al*., 2003), suggesting that it may be useful in preventing oxidative damage. Recently, we reported that hydroxyflavanones of a new potent antioxidant were produced from flavanone glycosides in citrus by the culturing with *Aspergillus saitoi*, such as 8-hydroxyhesperetin from hesperidin and 6- or 8-hydroxynaringenins from naringin (Miyake *et al*., 2003). In this study, the antioxidative activity of the fermented lemon peel (FLP), which was prepared by culturing LP with *A. saitoi*, was examined using a radical scavenging activity *in vitro* and a suppressive effect against exercise-induced oxidative damage *in vivo*. The antioxidative activity of FLP was compared with LP, which is the material before fermentation. Furthermore, the content of antioxidative hydroxyflavanones and flavonoid aglycones in LP and FLP was analyzed to examine their relationship with antioxidative activity.

Materials and Methods

Materials LP was obtained from hand-squeezed lemon fruit produced in California. Eriodictyol, hesperidin, hesperetin, naringenin and narirutin were purchased from Funakoshi, Ltd., Tokyo, Japan. Eriocitrin was prepared by the method of Miyake *et al*. (1997a). 8-Hydroxyhesperetin, 6-hydroxynaringenin and 8-hydroxynaringenin were prepared by the method of Miyake *et al*. (2003). Anti-N^{ε}-(hexanonyl)lysine (HEL) monoclonal antibody prepared as described previously (Kato *et al*., 2000), was obtained from Dr. Y. Kato.

Aspergillus saitoi IAM 2210 was obtained from the Institute

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; ELISA, enzyme-linked immunosorbent assay; FLP, fermented lemon peel; HPLC, high-performance liquid chromatography; HEL, N^{ε}-(hexanonyl) lysine; LP, lemon peel; TBARS, thiobarbituric acid reactive substance.

of Applied Microbiology at Tokyo University (Tokyo). Other reagents were purchased from Wako Chemical, Ltd., Osaka, Japan.

Fermentation of lemon peel with Aspergillus saitoi Potato dextrose broth medium (Difco Laboratories, Detroit, USA) was used as the growth medium for *A. saitoi*. One hundred milliliters of medium was adjusted to pH 5.0 using 0.1 N HCl. After the medium was sterilized at 121°C for 15 min, it was inoculated with 1 ml of *A. saitoi* (2×10^8 spores/ml). The inoculated medium was cultured with a rotary shaker (HRS-19, Shibata Hario Glass Co., Ltd., Tokyo) at a speed of 100 rpm/min at 30°C for 7 days. The growth medium was inoculated into 1.0 kg of LP. It was then incubated and fermented at 30°C for 7 days standing in the dark. FLP was extracted with 3 *l* of methanol at 37°C for 2 days. The extract was centrifuged at 1500×*g* for 30 min and the supernatant was concentrated under reduced pressure. After 100 ml of ethanol was added to the concentrate, it was centrifuged at 6900×*g* for 20 min and the supernatant was evaporated under reduced pressure. The concentrated solution was freeze-dried and the extract of FLP (26.1 g) was obtained. One kilogram of LP was extracted with 3.0 *l* of methanol to the comparative sample for FLP. The extract of LP (27.9 g) was obtained in the same matter of FLP.

Antioxidative assay using radical-scavenging activity against DPPH The sample extracts of LP and FLP were dissolved with ethanol. Two hundred microliters of sample solution was mixed with 800 μl of the 100 mM Tris-HCl buffer (pH 7.4) and then added to 1 ml of 500 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in ethanol. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark, then subjected to high-performance liquid chromatography (HPLC; LC-10A, Shimadzu Co., Ltd., Kyoto) analysis according to the method proposed by Yamaguchi *et al*. (1998). Analyses were performed using a TSKgel Octyl-80Ts column (4.6×150 mm, Tosoh, Ltd., Tokyo) at ambient temperature and a spectrophotometric detector (517 nm) with a mobile phase of methanol/water (70 : 30, v/v) at a flow rate of 1 ml/min. The sample was examined with 1000 ppm, 2500 ppm and 5000 ppm at the final concentration. The DPPH radical-scavenging activity was evaluated from the difference in the decreasing peak area of the DPPH radical detected between the blank and the sample. The results are expressed in %=((the peak area of DPPH in the absence of compound −the peak area of DPPH in the presence of compound)/the peak area of DPPH in the absence of compound)×100.

Determination of components contained in LP and FLP LP and FLP were dissolved in ethanol. The content of flavonoid, amino acid and neutral sugar in the solution was determined by a LC-10A HPLC apparatus (Shimadzu Co., Ltd.) and that of organic acid was determined by a TOA ICA5000 HPLC apparatus (TOA Electronics Ltd., Tokyo). Flavonoid was analyzed by HPLC using a YMC-Pack ODS column (4.6×250 mm, YMC Co., Ltd., Kyoto) at 40°C and a UV detector at 333 nm with a mobile phase of methanol/water containing 5% acetic acid (50 : 50, v/v) at a flow rate of 1 ml/min. The structural formula of flavonoids detected in LP and FLP is shown in Fig. 1. Organic acid was analyzed by HPLC using a PCI-305S column (8.0×300 mm, TOA Co., Ltd.) at 40°C and an electric conduction detector with a mobile phase of 5 mM $HClO_4$ at a flow rate of 1 ml/min. Amino acid was analyzed by HPLC using *o*-phthalaldehyde according to the method proposed by Ishida *et al*. (1981). Neutral sugar was analyzed by HPLC using a YMC-pack Polyamine column II (4.6×250 mm, YMC Co., Ltd.) at 40°C and a refractive index detector with a mobile phase of acetonitrile/water (25 : 75, v/v) at a flow rate of 1 ml/min.

Animals and experimental design Male Wistar rats (10 weeks old) were purchased from Japan SLC, Shizuoka, Japan. Experiments were started between 10 : 00 and 12 : 00 h. All rats were deprived of food at 10 : 00 h. Rats were randomly divided into a control group, an LP group and an FLP group. Rats in each group were subdivided into a sedentary group and an exercise group. One milliliter-dose of the sample solution of 30% (w/w) solution in water was administered intragastrically to rats in

	Substituent				
	5	7	8	3′	4′
Glycosides					
Eriocitrin	OH	*O*-rutinose	H	OH	OH
Naringin	OH	*O*-rhamnoglucose	H	H	OH
Narirutin	OH	*O*-rutinose	H	H	OH
Hesperidin	OH	*O*-rutinose	H	H	OCH_3
Aglycones					
Eriodictyol	OH	OH	H	OH	OH
Naringenin	OH	OH	H	H	OH
Hesperetin	OH	OH	H	H	OCH_3
8-Hydroxyhesperetin	OH	OH	OH	H	OCH_3

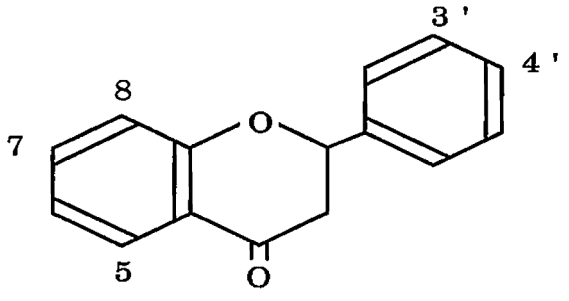

Fig. 1. Structural formula of flavonoids in LP and FLP.

the LP and the FLP groups. The same volume of saline was given to rats in the control group in the same manner. Four hours after administration, the animals in the exercise group were exercised for 35 min according to the method of Armstrong *et al.* (1983) using a motor-driven treadmill designed for mice and rats (model III, Autome Kogyo, Tokyo), by running down a 16° incline at a speed of 20 m/min. The downhill treadmill running was used in the present study (Minato *et al.*, 2003), because it is known as an eccentric exercise in rats (Armstrong *et al.*, 1983; Shimomura *et al.*, 1991). Immediately after running, the rats were anesthetized with pentobarbital (50 mg/kg body weight) and blood was obtained from the inferior vena cava with a syringe. The animals were then killed by exsanguination and the liver was removed, freeze-clamped at liquid nitrogen temperature, and stored at −80°C until analysis. Rats in the sedentary group were treated by the same procedures, and the blood and liver were obtained from the sedentary group of rats within 30 min after treatment of the exercised group.

Measurement of thiobarbituric acid-reactive substance (TBARS) The livers obtained were homogenized in 10 volumes of 1.15% KCl at 4°C. The homogenate was centrifuged at 4500×*g* for 15 min, and the supernatant was obtained for the TBARS measurement. The TBARS concentration of liver was measured as described previously (Yagi, 1976).

Measurement of HEL content by enzyme-linked immunosorbent assay (ELISA) The livers obtained were homogenized in 10 volumes of 50 mM sodium phosphate buffer (pH 7.4) at 4°C. The homogenate was centrifuged at 4500×*g* for 15 min, and the supernatant obtained was used for the following biochemical measurement. The noncompetitive ELISA was performed for measurement of HEL of oxidative stress marker as described previously (Kato *et al.*, 2000). Briefly, 50 μl of sample solution (10 μg protein/ml) was pipetted into the wells and kept at 4°C overnight. The plate was then incubated with the primary antibodies described above. After incubation, the binding of the antibodies to the modified proteins was evaluated by using the anti-mouse/rabbit IgG antibody peroxidase labeled with *o*-phenylenediamine and hydrogen peroxide.

Statistical analyses Values are represented as mean±SE (n=5). Statistical analysis was evaluated by the Fisher's PLSD using Stat View for Windows Ver. 5.0 (SAS Institute Inc., Cary, NC). Differences were considered significant at $p<0.05$.

Results and Discussion

Radical-scavenging activity of LP and FLP against DPPH The antioxidative activity of LP and FLP *in vitro* was examined by using the radical-scavenging activity against DPPH as shown in Fig. 2. The activity of the 1000 ppm for LP and FLP was shown as 8.4% and 25.0%, respectively. The FLP had approximately 3-fold higher activity than LP. The 2500 ppm and 5000 ppm for FLP were also shown to have approximately 3-fold more activity than those of LP. The activity of LP and FLP on this assay system was shown to have a direct correlation to the concentration. The high antioxidative activity of FLP was thought to be caused by the fermentation of LP with *A. saitoi*.

Distribution of compounds contained in LP and FLP The content of flavonoid, amino acid, organic acid and neutral sugar in LP and FLP was determined by HPLC analysis as shown in

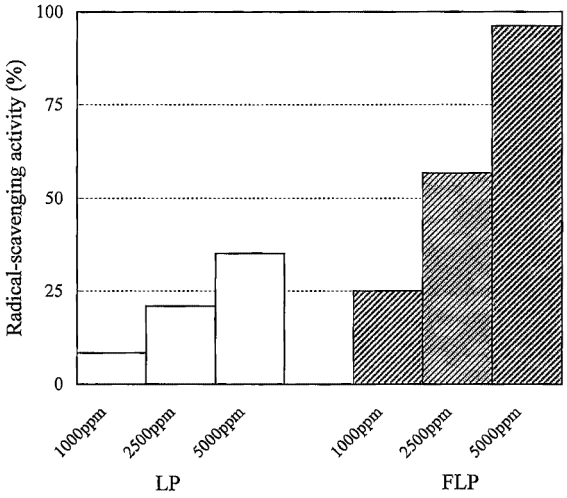

Fig. 2. Radical-scavenging activity of LP and FLP against DPPH.

Table 1. The content of flavonoid, organic acid, amino acid and neutral sugar in LP and FLP.

(1) flavonoid (mg/100 g of sample)

	Eriocitrin	Hesperidin	Narirutin	Naringin	Eriodictyol	Hesperetin	Naringenin	8-Hydroxy-hesperetin	6-Hydroxy-naringenin	8-Hydroxy-naringenin
LP	716.0	213.0	29.7	nd	nd	nd	nd	nd	nd	nd
FLP	113.7	41.9	trace	nd	175.8	41.4	5.1	19.0	nd	nd

(2) organic acid (mg/100 g of sample)

	Citric acid	Maleic acid	Formic acid	Acetic acid	Lactic acid	Quinic acid
LP	239.4	10.5	0.1	0.0	0.0	0.0
FLP	206.8	13.3	4.0	0.0	0.0	0.0

(3) amino acid (mg/100 g of sample)

	Serine	Aspartic acid	Asparagine	Alanine	Glycine	Glutamic acid	Glutamine
LP	16.57	13.56	12.42	6.12	4.90	3.82	0.19
FLP	18.39	2.85	27.99	14.52	10.09	44.63	26.14

(4) neutral sugar (mg/100 g of sample)

	Glucose	Fructose	Rhamnose	Sucrose
LP	550	410	240	170
FLP	20	10	310	10

Table 1. For flavonoids in LP, eriocitrin (eriodictyol 7-*O*-rutinoside) and hesperidin (hesperetin 7-*O*-rutinoside) were abundant, and a small amount of narirutin (naringenin 7-*O*-rutinoside) was also detected. The distribution of flavonoids in LP was consistent with the data in the literature we have reported on the content of flavonoid compounds in lemon peel (Miyake *et al*., 1998b), while their distribution in FLP was shown to be different from that of LP. Eriocitrin, hesperidin and narirutin of flavonoid glycosides in FLP were shown to decrease in comparison with LP, but eriodictyol, hesperetin and naringenin of their aglycones increased. The aglycones in FLP were thought to be produced from flavanone glycosides in LP by the fermentation with *A. saitoi*. We recently reported the production of potent antioxidants for 8-hydroxyhesperetin from hesperidin, and for 6-hydroxynaringenin and 8-hydroxynaringenin from naringin by the fermentation with *A. saitoi* (Miyake *et al*., 2003). In this study, we fermented lemon peel, which contains many flavanone glycosides, with *A. saitoi*. 8-Hydroxyhesperetin was detected in FLP although 6-hydroxynaringenin and 8-hydroxynaringenin were not detected. 8-Hydroxyhesperetin was thought to be produced from hesperidin in lemon peel by the fermentation of *A. saitoi*, and presumed to have a relationship with the high antioxidative activity of FLP. We have reported that the aglycones have a higher antioxidative activity than their flavanone glycosides contained in citrus fruits (Miyake *et al*., 1997b). Therefore, the high antioxidative activity of FLP was thought to relate to the production of 8-hydroxyhesperetin for hydroxyflavanone and of eriodictyol, hesperetin and naringenin for flavonoid aglycones.

The content of amino acid, organic acid and neutral sugar in LP and FLP was examined as shown in Table 1. The content of citric acid was the richest of organic acids in both types of peel. It was thought that citric acid came from the juice into the peel residue on the fruit squeezing process because of its abundance in juice. The distribution of amino acids in FLP was different from that of FP. The content of serine, asparagine, alanine, glycine, glutamic acid and glutamine, but not aspartic acid was higher than that of LP. These amino acids except for aspartic acid were suggested to be produced by the fermentation of *A. saitoi*. Neutral sugars for glucose, fructose and sucrose in FLP, with the exception of rhamnose, were detected in small amounts compared with LP. Those neutral sugars were thought to be consumed by *A. saitoi* during the fermentation, but rhamnose was produced by the decomposition from flavanone glycosides to aglycones. From these results, the content of flavonoid, organic acid, amino acid and neutral sugar in LP was shown to diversify through the fermentation with *A. saitoi*. The high antioxidative activity of FLP was thought to relate to the diversification of the flavonoids in LP.

Suppressive effect against oxidative damage of lipids on exercise-induced rat liver Exercise has expanded in the scope for prevention and/or management of chronic diseases and maintenance of optimal health (Chandrashekhar & Anand, 1991; Singh, 1992). However, it has been reported that strenuous aerobic exercise generates oxygen free radicals of oxidative stress that cause lipid peroxidation of polyunsaturated fatty acids on the bio-membrane (Davies *et al*., 1982; Witt *et al*., 1992; Alessio, 1993). Lipid peroxidation has been generally measured by the TBARS level, which are the reactive products between the aldehydes of the final products of lipid peroxide, and the TBA of a reagent (Yagi, 1976). TBARS levels of liver and skeletal muscle on an exercise-induced rat have been reported to increase (Witt *et al*., 1992). We have reported that eriocitrin, a major flavonoid having antioxidative activity in lemon fruit, suppresses an increase of TBARS levels of liver due to exercise, in the acute exercise-induced rat (Minato *et al*., 2003).

In this study, LP and FLP showed capabilities of antioxidative activity using DPPH radical scavenging assay *in vitro* (Fig. 2); we then evaluated their antioxidative activity *in vivo* by examining for the suppressive effect against oxidative damage of lipid on the exercise-induced rat liver. As shown in Fig. 3, we determined the TBARS levels in liver of the sedentary and exercise-induced rats that were fed the control, LP and FLP diets. The exercise-induced rats for the control diet group showed a significantly higher TBARS level in the liver than the sedentary rats. The data was consistent with respect to the TBARS levels in rat liver increasing with the same levels of exercise (Minato *et al*.,

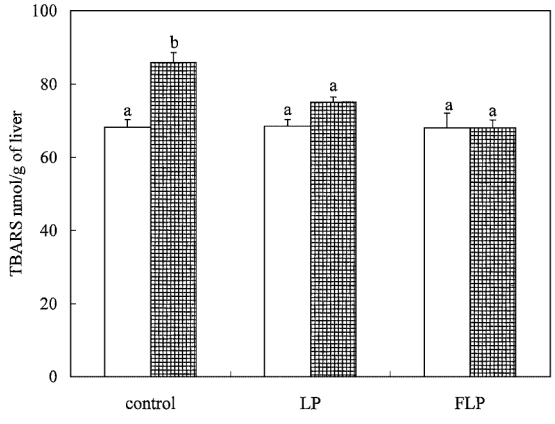

Fig. 3. TBARS levels in liver on the exercise-induced rats administered LP and FLP. Rats were administered LP and FLP prior to exercise and induced exercise; details are given in Materials and Methods. Values are means±SE of 5 rats per group. Values within the same row that do not share a common superscript letter are significantly different at $p<0.05$. □ sedentary, ▦ exercise.

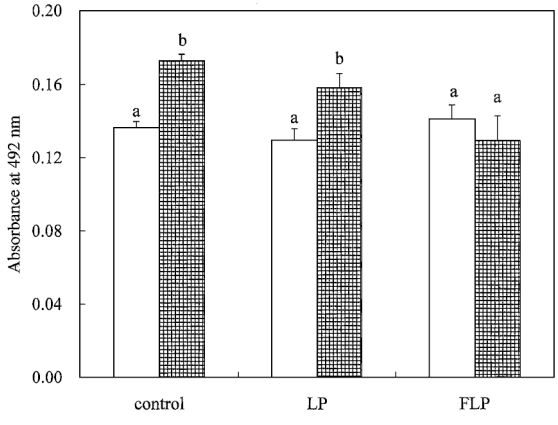

Fig. 4. HEL levels in liver on exercise-induced rats administered LP and FLP. Rats were administered LP and FLP prior to exercise and induced exercise; details are given in Materials and Methods. Values are means±SE of 5 rats per group. Values within the same row that do not share a common superscript letter are significantly different at $p<0.05$. □ sedentary, ▦ exercise.

2003). In this study, LP and FLP diet groups of exercise-induced rats had significantly lower TBARS levels than the control diet, and TBARS did not increase significantly with exercise. Administration of LP and FLP to the exercise-induced rats suppressed lipid peroxidation of rat liver due to exercise. Furthermore, the TBARS levels of exercise-induced rats fed the FLP diet was almost equal to that of the sedentary rats, and was lower than those fed the LP diet although there were not significant differences. Administration of the FLP diet was shown to have a tendency of a high suppressive effect of lipid peroxidation in rat liver compared with the LP diet. It was thought that LP and FLP have a suppressive effect against the oxidative damage of lipid, which is caused by exercise, and that the effect of FLP has a higher tendency than that of LP.

HEL is reported to be a biomarker of oxidative stress for the early stage of lipid peroxidation because it is formed by the reaction between the hydroperoxide caused from linoleic acid and lysine (Kato *et al*., 2000). Therefore, HEL is thought to have a high sensitivity for measuring the oxidative damage of lipids in comparison with TBARS, which are end products of lipid peroxide. In this study, the suppressive effect of LP and FLP against oxidative stress was evaluated by examining HEL levels in liver of exercise-induced rats. As shown in Fig. 4, the sedentary and exercise-induced rats which were fed the control, LP or FLP diet, had HEL levels in liver examined by ELISA using anti-HEL monoclonal antibody. The HEL levels of the control diet group significantly increased after acute exercise. We have reported that HEL levels in liver increased through acute exercise (Minato *et al*., 2003). The oxidative stress induced by exercise is thought to cause the increase of HEL levels in rat liver. However, the FLP diet group was shown to significantly suppress the increase of HEL levels due to exercise. The LP diet group did not show a significant suppressive effect, but showed a suppressive tendency in comparison with the control diet group. It was assumed that the administration of FLP to the exercise-induced rats protected them from early oxidative damage from lipid peroxidation. The high efficacy of FLP on HEL levels was thought to reflect sensitively the high suppressive effect of TBARS formation, which is the end product of lipid peroxidation (Fig. 3)

In this study, the dietary supplementation of LP and FLP was thought to produce the radical scavenging activity *in vitro* and protect against the oxidative stress *in vivo*, because of the suppressive effect against oxidative damage of liver on exercise-induced rats. The fermentation of lemon peel with *A. saitoi* was shown to produce high antioxidative activity, because their effects of FLP were shown to be superior to LP. The high activity of FLP, which was produced from lemon peel by fermentation of *A. saitoi*, was assumed to be connected with the production of flavonoids with high antioxidative activity, 8-hydroxyhesperetin, eriodictyol, hesperetin and naringenin.

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