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Preparation and the functional properties of water extract and alkaline extract of royal jelly

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Abstract

Water extract (WSR) and an alkaline extract (ASR) were prepared from fresh royal jelly from Chinese bees. The yields were about 8.3 and 6.3% on a dry weight basis, respectively. On SDS–PAGE analysis, the protein patterns of the two extracts were very similar, but not identical. Antioxidant activities, in both extracts, increased, depending on the concentration of the sample. The scavenging activities, against superoxide radical of WSR and ASR were high, and the activities at 100 mg/ml were the same as that of 5 mM ascorbic acid. Although the activities of WSR and ASR, at 50 and 100 mg/ml, did not match that of 1 mM tocopherol, the samples scavenged hydroxyl radical to about 50–60%. This shows that the protein fractions in royal jelly have high antioxidative activity and scavenging ability against active oxygen species. Royal jelly seems applicable in both health food and medicine.
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1. Introduction

The functional food market is growing at a rate of 15–20% per year, and the industry is claimed to be worth $33 billion (Hilliam, 2000). Functional foods—also known as nutraceuticals, designer foods, medicinal foods, therapeutic foods, superfoods, foodiceuticals, and medifoods-are defined as “foods that contain some health-promoting compounds beyond traditional nutrients” (Berner & O’Donnell, 1998). Foods can be modified by addition of phytochemicals, bioactive peptides, polyunsaturated fatty acids, and probiotics and/or prebiotics to become functional (Berner & O’Donnell, 1998). Royal jelly is one of the most attractive ingredients for health foods. It is the exclusive food of the queen honey bee (*Apis mellifera*) larva, and is secreted from the hypopharyngeal and mandibular glands of the worker honey bees mainly between the sixth and twelfth days of their life (Haydak, 1970; Patel, Haydak, & Gochnauer, 1960). Royal jelly has been demonstrated to possess several pharmacological activities in experimental animals, including vasodilative and hypotensive activities (Shinoda et al., 1978), increase in growth rate (Kawamura, 1961), disinfectant action (Yatsunami & Echigo, 1985), antitumor activity (Tamura, Fujii, & Kuboyama, 1987; Townsend, Morgan, & Hazlett, 1959; Townsend et al., 1960; Townsend, Brown, Felauer, & Hazlett, 1961), antihypercholesterolemic activity (Nakajin, Okigama, Yamashita, Akiyama, & Shinoda, 1982), and anti-inflammatory activity (Fujii et al., 1990). Chemical composition analysis has shown that royal jelly consists mainly of proteins, sugars, lipids, vitamins, and free amino acids (Takenaka, 1982; Echigo, Takenaka, & Yatsunami, 1986; Howe, Dimick, & Benton, 1985), together with a large number of such bioactive substances as 10-hydroxyl-2-decenoic acid (Blum, Nova, & Taber, 1959), antibacterial protein (Fujiwara, et al., 1990), a stimulating factor for the development of genital organs in male mice (Kato, Onodera, & Ishijima, 1988) and 350-kDa protein (Watanabe et al., 1998), that stimulates the proliferation of human monocytes. Therefore, royal jelly has been widely used in commercial medical products, health foods and cosmetics in many countries. Although there are many pharmacological reports on royal jelly, there are few about it as an antioxidant. This paper aims to prepare the water extracts and alkaline extracts from royal jelly and to characterize the antioxidant properties (against

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autoxidation) and scavenging properties (against free radicals; such as superoxide anion radical, DPPH radical, and hydroxyl radical). This work should be useful to aid disease prevention.

2. Materials and methods

2.1. Sample

Fresh royal jelly imported from China was obtained from Inoue Yohojo Bee Farm Inc. (Hyogo, Japan) and used in this study.

2.2. Chemicals

Linoleic acid, α-tocopherol, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), nitroblue tetrazolium salt (NBT), xanthine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2-deoxy-D-ribose were purchased from Wako Chemicals Co., Ltd. (Osaka, Japan). Xanthine oxidase from butter milk (XOD; 0.34 U/mg powder) was obtained from Oriental yeast Co., Ltd. (Tokyo, Japan). Other reagents were of analytical grade.

2.3. Preparation of water extract and alkaline extract from royal jelly

Thirty grammes of fresh royal jelly were suspended and extracted with 10 volumes of 10 mM sodium phosphate buffer (pH 7.0) with shaking at 4 °C for 1 day. The extracts were centrifuged at 28000 × *g* for 15 min, and the supernatants were pooled, dialyzed against distilled water, and then lyophilized. The residue was extracted with 100 ml of 0.1 N NaOH at 4 °C for 1 day. The extracts were centrifuged at 28000 × *g* for 15 min, and the supernatants were pooled, adjusted to pH 7.0 with diluted HCl, dialyzed against distilled water, and then lyophilized. The lyophilized powders were used as the sample solution (1, 5, 10, 50, and 100 mg/ml H_2O) for the following tests.

2.4. Determination of protein content

The protein content was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard.

2.5. Determination of total polyphenolic content

The total polyphenolic content was measured by the Folin-Ciocalteu colorimetric method (Slinkard & Singleton, 1977) using catechin as standard and the absorbance was measured at 760 nm.

2.6. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970).

2.7. Effect of autoxidation

The antioxidant activity was assayed by using a linoleic acid model system. Sample solution (0.0833 ml) and 0.208 ml of 0.2 M sodium phosphate buffer (pH 7.0) were mixed with 0.208 ml of 2.5% (w/v) linoleic acid in ethanol. The preoxidation was initiated by the addition of 20.8μl of 0.1 M AAPH and carried out at 37 °C for 200 min in the dark. The degree of oxidization was measured according to the thiocyanate method (Mitsuda, Yasumoto, & Iwai, 1966) for measuring peroxides by reading the absorbance at 500 nm after colouring with $FeCl_2$ and ammonium thiocyanate. A control was performed with linoleic acid but without sample solution. Ascorbic acid (1 and 5 mM) and α-tocopherol (1 mM) were used as positive controls.

2.8. Effect of superoxide anion radical

The effect of superoxide anion radical was evaluated by the method of Nagai, Inoue, Inoue, and Suzuki (2002). This system contained 0.48 ml of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 ml of 3 mM xanthine, 0.02 ml of 3 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 0.02 ml of 0.15% bovine serum albumin, 0.02 ml of 0.75 mM NBT and 0.02 ml of sample solution. After resting at 25 °C for 10 min, the reaction was started by adding 6 mU XOD and carried out at 25 °C for 20 min. After 20 min, the reaction was stopped by adding 0.02 ml of 6 mM CuCl. The absorbance of the reaction mixture was measured at 560 nm and the inhibition rate was calculated by measuring the amount of the formazan that was reduced from NBT by superoxide. Ascorbic acid (1 and 5 mM) and α-tocopherol (1 mM) were used as positive controls.

2.9. Effect of hydroxyl radical

The effect of hydroxyl radical was assayed by using the deoxyribose method. The reaction mixture contained 0.45 ml of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM $FeSO_4$-EDTA, 0.15 ml of 10 mM H_2O_2, 0.525 ml of H_2O, and 0.075 ml of sample solution in an Eppendorf tube. The reaction was started by the addition of H_2O_2. After incubation at 37 °C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% trichloroacetic acid and 0.75 ml of 1.0% of TBA in 50 mM NaOH; the solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at

520 nm. Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical (Chung, Osawa, & Kawakishi, 1997). Ascorbic acid (1 and 5 mM) and α-tocopherol (1 mM) were used as positive control.

2.10. Effect of DPPH radical

The effect of DPPH radical was evaluated by the method of Okada and Okada (1998) with a slight modification. The assay mixture contained 0.3 ml of 1.0 mM DPPH radical solution, 2.4 ml of 99% ethanol, and 0.3 ml of sample solution. The solution was rapidly mixed and this scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. Ascorbic acid (0.1 and 1.0 mM) and α-tocopherol (1 mM) were used as positive controls.

3. Results and discussion

3.1. The protein contents and total phenolic contents of water extract and alkaline extract of royal jelly

The water extract (WSR) and alkaline extract (ASR) of royal jelly were extracted and the yields were about 8.3 and 6.3% on a dry weight basis, respectively. The protein contents and total phenolic contents of these fractions were measured and are shown in Table 1. The protein contents of these fractions were as follows: about 1.15 mg/mg powder (WSR) and 1.38 mg/mg powder (ASR), respectively. Moreover, the total phenolic contents of these fractions were about 21.2 μg/mg powder (WSR) and 22.8 μg/mg powder (ASR), respectively. These quantities of protein and phenolic compounds were very similar to each other. The compositions of each fraction were evaluated by SDS–PAGE using 10% gel (Fig. 1). The patterns of the two protein fractions were very similar to each other, but not identical. In particular, 55 kDa protein was shown in both fractions as the main protein band. This suggests that it is possibly royalisin, a potent antibacterial protein in royal jelly (Fujiwara et al., 1990). On the other hand, 21 and 43 kDa protein bands were found only in the WSR fraction. At present, it is not clear what function these protein bands possess.

Table 1
Protien contents and total phenolic contents on WSR ad ASR of royal jelly

Sample species	Protien contents (mg/mg powder)	Total phenolic contents (μg/mg powder)
WSR	1.15	21.2
ASR	1.38	22.8

3.2. Antioxidant activities of water extract and alkaline extract from royal jelly

The antioxidant effects (on the peroxidation of linoleic acid) were investigated to evaluate the in vitro effects of these fractions at the initiation stage of lipid peroxidation. The sample concentrations used were as follows: 1, 5, 10, 50, and 100 mg/ml. Results are shown in Table 2. The antioxidative activity of the control was suddenly decreased with passage of time. The patterns of 1 and 5 mg/ml WSR and 1 mg/ml ASR were the same as that of the control. The antioxidative activities of WSR (50 and 100 mg/ml) were between those of 1 and 5 mM ascorbic acid. Moreover, the activity of ASR at 50 mg/ml were between those of 1 and 5 mM ascorbic acid. On the other hand, ASR, at 100 mg/ml was more active than 5 mM ascorbic acid, but its activity was lower than that of 1 mM α-tocopherol. The activities of WSR and ASR increased, depending on the concentration of the sample.

3.3. Superoxide-scavenging activity

Superoxide-scavenging activities of WSR and ASR were measured using xanthine–xanthine oxidase system. These results were indicated as the superoxide productivity. As shown in Fig. 2, each sample showed the superoxide-scavenging activity and these activities tended to increase with increasing concentration of the sample. The activities of WSR at 1 mg/ml, and of ASR at 1 and 5 mg/ml, were the same as that of 1 mM ascorbic acid. Moreover, the activities of WSR and

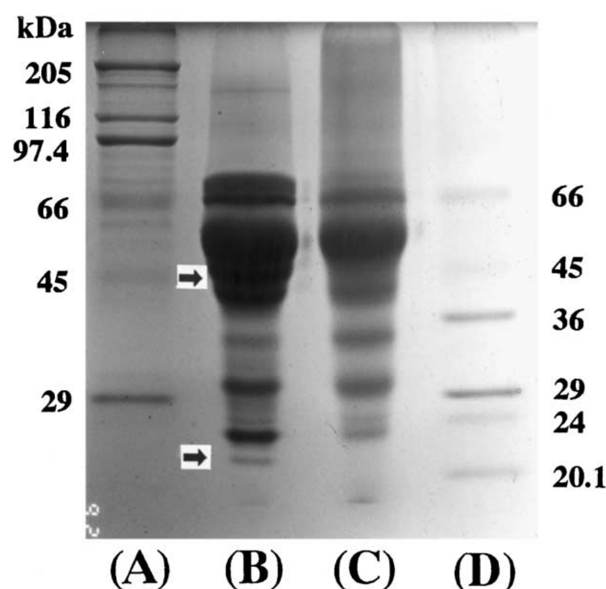

Fig. 1. Electrophoretic patterns of WSR and ASR from royal jelly using 10% sodium dodecyl sulfate-polyacrylamide electrophoresis. (A) High molecular weight standards; (B) WSR; (C) ASR; (D) low molecular weight standards.

ASR, at 100 mg/ml were the same as that of 5 mM ascorbic acid. On the other hand, the activities of WSR at 10 mg/ml, and of ASR at 50 mg/ml, were the same as that of 1 mM α-tocopherol.

3.4. Hydroxyl radical scavenging activity

The scavenging effects, against hydroxyl radical, of WSR and ASR were investigated using the Fenton reaction and these results were indicated as the inhibition rate. Each royal jelly sample showed the hydroxyl radical scavenging activity and activities were increased with increasing concentration of the sample (Fig. 3). The samples (1–10 mg/ml) of both WSR and ASR showed the same activities as 1 and 5 mM ascorbic acid. On the other hand, the activities of WSR and ASR, at 50 and 100 mg/ml, were similar to that of 1 mM α-tocopherol, although the activities of WSR and ASR, at 50 and 100 mg/ml, did not match that of 1 mM α-tocopherol. These samples scavenged hydroxyl radical to about 50–60%.

3.5. DPPH radical scavenging activity

To evaluate the scavenging effect of WSR and ASR, DPPH inhibition was investigated and these results were evaluated as relative activities against control. The activity of WSR was very low and was lower than that of 0.1 mM ascorbic acid, even if the sample at 100 mg/ml was used (data not shown). On the other hand, although the activity of ASR at 1 mg/ml was lower than that of 0.1 mM ascorbic acid, the activities above the concentration of 5 mg/ml were higher than that of 0.1 mM ascorbic acid. These samples scavenged to only about 14% against the DPPH radical (data not shown). The activities of WSR exhibited as intermediate activity between 1 mM α-tocopherol and 0.1 mM ascorbic acid. The activities of royal jelly samples

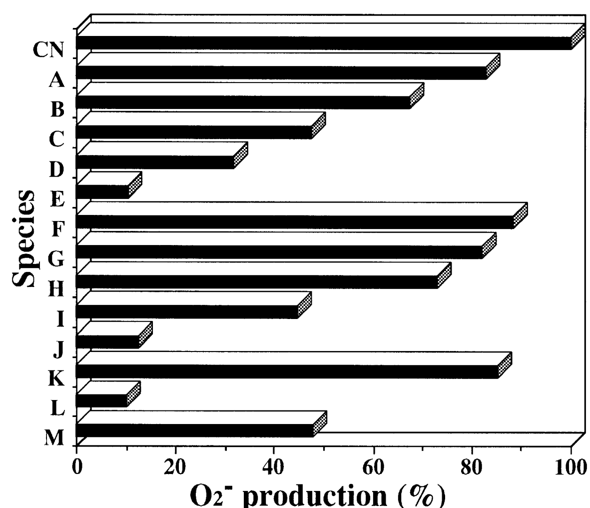

Fig. 2. Scavenging activities of WSR and ASR from royal jelly on the superoxide anion radical in xanthine–xanthine oxidase system by the NBT method. (CN) control; (A) 1 mg/ml WSR; (B) 5 mg/ml WSR; (C) 10 mg/ml WSR; (D) 50 mg/ml WSR; (E) 100 mg/ml WSR; (F) 1 mg/ml ASR; (G) 5 mg/ml ASR; (H) 10 mg/ml ASR; (I) 50 mg/ml ASR; (J) 100 mg/ml ASR; (K) 1 mM ascorbic acid; (L) 5 mM ascorbic acid; (M) 1 mM α-tocopherol.

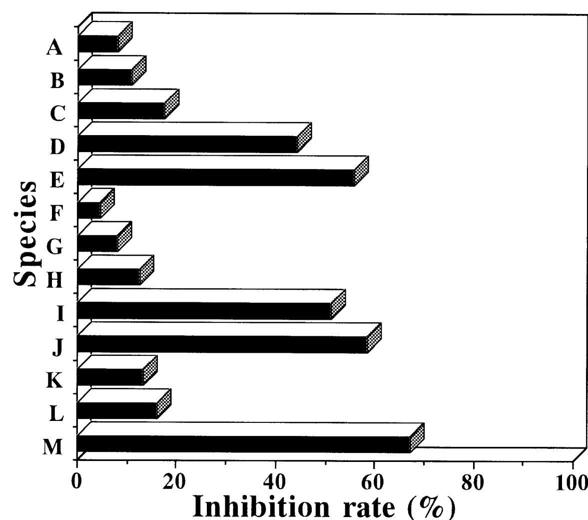

Fig. 3. Hydroxyl radical scavenging activity of WSR and ASR from royal jelly. (A) 1 mg/ml WSR; (B) 5 mg/ml WSR; (C) 10 mg/ml WSR; (D) 50 mg/ml WSR; (E) 100 mg/ml WSR; (F) 1 mg/ml ASR; (G) 5 mg/ml ASR; (H) 10 mg/ml ASR; (I) 50 mg/ml ASR; (J) 100 mg/ml ASR; (K) 1 mM ascorbic acid; (L) 5 mM ascorbic acid; (M) 1 mM α-tocopherol.

Table 2
Antioxidant activities of water extract and alkaline extract from royal jelly

Time (min)	O.D. (500nm)													
	Sample species													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
50	0.213	0.130	0.120	0.045	0.032	0.162	0.068	0.094	0.050	0.000	0.062	0.016	0.006	0.175
100	0.487	0.345	0.276	0.120	0.061	0.410	0.165	0.148	0.063	0.011	0.135	0.032	0.025	0.523
200	1.355	1.066	0.846	0.228	0.150	0.166	0.505	0.480	0.195	0.065	0.469	0.090	0.028	1.409

A: 1 mg/ml WSR; B: 5 mg/ml WSR; C: 10 mg/ml WSR; D: 50 mg/ml WSR; E: 100 mg/ml WSR; F: 1 mg/ml ASR; G: 5 mg/ml ASR; H: 10 mg/ml ASR; I: 50 mg/ml ASR; J: 100 mg/ml ASR; K: 1mM ascorbic acid; M: 1mM tocopherol; N: control.

tended to decrease with decreasing concentration of the sample.

Royal jelly is now being recognized as a healthy food, rich in vitamin B and related components. Chemically, royal jelly comprises water (50–60%), proteins (18%), carbohydrates (15%), lipids (3–6%), mineral salts (1.5%), and vitamins. In particular, it contains some vitamin B and related components as follows: vitamin B_1 (0.006 mg/g), vitamin B_2 (0.006–0.01 mg/g), vitamin B_6 (0.012 mg/g), acetylcholine (1.3 mg/g), pantothenic acid (0.2 mg/g), inositol (0.078–0.15 mg/g), nicotinic acid (0.088 mg/g), and biotin (0.0025–0.003 mg/g). Although royal jelly has been used from ancient times, there are few reports concerning the antioxidative and scavenging properties against active oxygen species. Kimura, Washino, and Yonekura (1995) has reported that a 350 kDa glycoprotein in royal jelly stimulated the proliferation of human monocytes, and that a 55 kDa glycoprotein maintained the high viability of rat liver primary cultured cell (Kimura, Kajiyama, Kanaeda, Izukawa, & Yonekura, 1996) On the other hand, Fujiwara et al. (1990) found a protein having molecular weight about 55 kDa in royal jelly and designated it ‘‘royalisin’’. They have been reported that royalisin possessed selective growth inhibition effects against Gram-positive bacteria, such as *Lactobacillus*, *Bifidobacterium*, and *Leuconostoc* at effective concentrations below 1 μM.

In our present study, it was found that royal jelly had high antioxidative activity and scavenging abilities against radicals such as superoxide and hydroxyl radicals. The fractions (WSR and ASR) used in this study were the dialysates against distilled water. So it was assumed that certain vitamins and related components were not contained in these samples. In fact, these samples contained a large quantity of protein (about 1 mg/mg powder) but a small amount of total phenolic compounds (about 22 μg/mg powder). On SDS–PAGE analysis, it was found that these fractions contained some proteins, but did not have identical protein patterns. There are many studies about antioxidative activity against autoxidation and scavenging abilities against active oxygen species. Up to now, these reports have detailed low molecular weight components, such as vitamins and phenolic compounds. However, our present studies suggest that the protein fractions in royal jelly have high antioxidative activity and scavenging ability. Research is under way to analyze the antioxidant and scavenging compounds of royal jelly and to study protein pattern in detail. At present, consumer demand for natural foods, with the effects of a medicine, has been increasing, and it is therefore desirable to use royal jelly as a food containing naturally occurring compounds. In the future, royal jelly will be used both as a health food and a medicine for its functional properties, such as antioxidative and scavenging activities toward active oxygen species.

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