Antioxidant Activity and Angiotensin I-Converting Enzyme Inhibition by Enzymatic Hydrolysates from Bee Bread

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Enzymatic hydrolysates were prepared from bee bread using three proteases. The antioxidant properties of these hydrolysates were measured using four different methods. These had remarkable antioxidant activity similar or superior to that of 1 mM α-tocopherol. They also had high scavenging activities against active oxygen species as the superoxide anion radical and hydroxyl radicals. Moreover, they showed angiotensin I-converting enzyme inhibitory activities and the activities were similar to those from various fermented foods such as fish sauce, sake, vinegar, cheese, miso, and natto. The present studies reveal that enzymatic hydrolysates from bee bread are of benefit not only for the materials of health food diets, but also for in patients undergoing various diseases such as cancer, cardiovascular diseases, diabetes, and hypertension.

Key words: Bee Bread, Enzymatic Hydrolysates

Introduction

Bee bread is made of pollen, which has been gathered by bees and mixed with its digestive enzymes, carried back to the hive, packed into pellets and preserved with tiny bit of honey and bee wax. This mixture undergoes different chemical processes to the action of different enzymes, microorganisms, moisture and temperature (35–36 °C) and after 2 weeks it is called bee bread. Bee bread is the main source of proteins, lipids, microelements and vitamins and the most nutritious food for bees.

The composition of the bee bread differs slightly from that of pollen. It has higher acidity due to the presence of lactic acid and a larger amount of vitamin K. The quality of lactic acid is six times larger compared to pollen. The higher activity of bee bread causes a good preservation of bee bread due to the inhibition of the growing of molds as well as of microorganisms.

Antioxidants are compounds used in foods to prevent deterioration, rancidity, or discoloration caused by oxidation. As they are substances that can prevent or delay oxidation of a molecule, antioxidants are widely used in food manufacturing to inhibit oxidation.

In the recent publications, we described the functional properties of foods such as honey species, royal jelly, propolis, and honeybee-collected pollen extract (Nagai and Inoue, 2002). It was found that these foods possess the remarkable antioxidant abilities and free radical scavenging abilities. Particularly, as a part of studies on functions in honey species we tried to prepare enzymatic hydrolysates from bee bread. We also characterized these hydrolysates using four different methods, namely antioxidative, superoxide anion radical generated from the xanthine/xanthine oxidase system, DPPH free radical, and hydroxyl radical scavenging tests. Moreover, the study deals with angiotensin I-converting enzyme inhibitory activity which functions to depress hypertension in these hydrolysates.

Experimental

Materials

Fresh bee bread imported from Lithuania (produced by Austeja Ltd.) was obtained from Inoue Yohojo Bee Farm Inc. (Hyogo, Japan) and used in this study.

Pepsin from porcine stomach mucosa (EC 3.4.23.1; 2 × crystallized; 3,085 U/mg protein) was

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purchased from Sigma, USA. Trypsin from porcine pancreas crystallized (EC 3.4.21.4; 4,500 USP trypsin U/mg protein), papain (EC 3.4.22.2; digestive powder; 1:350), linoleic acid, α-tocopherol, ascorbic acid, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), nitroblue tetrazolium salt (NBT), xanthine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, albumin from bovine serum, and 2-thiobarbituric acid (TBA) 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.

Preparation of peptide powder

Pepsin digestion. Dried matters were added and homogenized with 10 volumes of distilled water, and the pH value of the solution was adjusted at 2.0 using HCl. The digestion was started by adding 1.0% pepsin (w/v) at 37 °C. After 48 h, hydrolysis was stopped by boiling for 10 min. The hydrolysate was centrifuged at 30,000 × *g* for 30 min to remove the residue and the supernatants were pooled, dialyzed against distilled water using a dialysis membrane (Wako Chemicals USA, Inc.) for 3 d with a change of solution once a day, and then lyophilized.

Trysin digestion. Dried matters were added and homogenized with 10 volumes of distilled water and the pH value of the solution was adjusted at 7.6 using NaOH. The homogenates were digested with 1.0% trypsin (w/v) at 37 °C. After 48 h, the hydrolysate was boiled for 10 min to inactivate the enzyme and centrifuged at 30,000 × *g* for 30 min. The supernatants were pooled, dialyzed against distilled water for 3 d with change of solution once a day, and the dialysate was lyophilized.

Papain digestion. Dried matters were added and homogenized with 10 volumes of distilled water and the pH value of the solution was adjusted at 7.0 using NaOH. The homogenates were digested with 1.0% papain (w/v) at 37 °C for 48 h, and the hydrolysis was stopped by boiling for 10 min. The hydrolysate was centrifuged at 30,000 × *g* for 30 min to remove the residue. The supernatants were pooled, dialyzed against distilled water for 3 d with a change of solution once a day, and the dialysate was lyophilized.

Protein concentration

The protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Determination of total polyphenolic content

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

Measurment of antioxidation activity

The antioxidant activity was assayed by the method of Nagai and Inoue (2004). A 0.08 ml sample of the solution and 0.21 ml of 0.2 M sodium phosphate buffer (pH 7.0) were mixed with 0.21 ml of 2.5% (w/v) linoleic acid in ethanol. The preoxidation was initiated by the addition of 20.80 μl 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 (Mitsuda *et al.*, 1966) for measuring peroxides by reading the absorbance at 500 nm after coloring 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 control. Distilled water was used as negative control.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity was estimated by the method of Nagai and Inoue (2004). 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 preincubation 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 formazan that was reduced from NBT by superoxide. Ascorbic acid (1 and 5 mM) and α-tocopherol (1 mM) were used as positive control. Distilled water was used as negative control.

DPPH radical scavenging activity

The DPPH radical scavenging activity 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 the 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 control. Distilled water was used as negative control.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by using the method with deoxyribose. 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 a 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 of 0.75 ml of 2.8% trichloroacetic acid and 0.75 ml of 1.0% 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. The hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by the hydroxyl radical (Chung *et al.*, 1997). Ascorbic acid (1 and 5 mM) and α-tocopherol (1 mM) were used as positive control. Distilled water was used as negative control.

Angiotensin I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity assay was performed using a modified version of the method of Cushman and Cheung (1971). 25 μl of sample solution and 75 μl of 0.1 M sodium borate (pH 8.3) containing 5.83 mM hippuryl-L-histidyl-L-leucine and 1.0 M NaCl were preincubated at 37 °C for 5 min, and then incubated with 25 μl of 0.1 M sodium borate buffer (pH 8.3) containing 1 mU ACE and 1.0 M NaCl at 37 °C for 60 min. The reaction was stopped by the addition of 125 μl of 1.0 M HCl. The resulting hippuric acid was extracted with 750 μl of ethyl acetate by mixing for 15 s. After centrifugation at 6,000 rpm for 3 min, 500 μl of the upper layer were transported into a tube and evaporated at 40 °C for 2 h. The hippuric acid was dissolved in 500 μl of distilled water, and the absorbance was measured at 228 nm using a PerkinElmer model Lambda 11 (PerkinElmer, Tokyo, Japan) UV/VIS spectrometer. The IC_{50} value was defined as the protein concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

Statistical analysis

The results were statistically analyzed by analysis of variance (ANOVA) followed by Fisher's PLSD test. Differences were considered significant for $p < 0.05$.

Results

Preparation of enzymatic hydrolysates from bee bread

The enzymatic hydrolysates were prepared from bee bread by digestion of three kinds of enzymes (pepsin, trypsin, and papain). Bee bread was successfully digested and the yields of these hydrolysates were as follows: 10% (pepsin hydrolysate), 10% (trypsin hydrolysate), and 4% (papain hydrolysate) on the dry weight basis, respectively. The total phenolic contents of these hydrolysates were measured and were as follows: 12.8 μg/mg powder (pepsin hydrolysate), 8.6 μg/mg powder (trypsin hydrolysate), and 12.2 μg/mg powder (papain hydrolysate), respectively. On the other hand, the protein contents were as follows: 247.8 μg/mg powder (pepsin hydrolysate), 182.9 μg/mg (trypsin hydrolysate), and 220.2 μg/mg powder (papain hydrolysate), respectively. This suggested that the hydrolysate having a high content of protein tended to a higher total phenolic content.

Antioxidant activity

Antioxidant activities of enzymatic hydrolysates from bee bread were estimated using the linoleic acid model system to evaluate the effect at the initiation stage of lipid peroxidation. Each hydrolysate showed an antioxidative effect and the activity increased with increasing the concentration of the sample (Table I). The hydrolysates for 1 mg/ml showed a lower activity than 1 mM ascorbic acid. For 10 mg/ml the activity of each hydrolysate was higher than that of 1 mM ascorbic acid, but it was lower than that of 5 mM ascorbic acid. The hydrolysates for 10 mg/ml possessed one half activities of 5 mM ascorbic acid. Moreover, the

Table I. Antioxidant activities of enzymatic hydrolysates from bee bread.

	Absorbance at 500 nm												
	Sample												
Time [min]	A	B	C	D	E	F	G	H	I	J	K	L	M
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
50	0.10	0.03	0.01	0.09	0.01	0.00	0.08	0.04	0.01	0.02	0.02	0.01	0.38
100	0.22	0.10	0.03	0.25	0.06	0.02	0.25	0.11	0.00	0.14	0.03	0.03	0.72
200	0.55	0.16	0.02	0.55	0.14	0.02	0.55	0.19	0.01	0.47	0.09	0.03	1.41

(A) 1 mg/ml pepsin hydrolysate; (B) 10 mg/ml pepsin hydrolysate; (C) 100 mg/ml pepsin hydrolysate; (D) 1 mg/ml trypsin hydrolysate; (E) 10 mg/ml trypsin hydrolysate; (F) 100 mg/ml trypsin hydrolysate; (G) 1 mg/ml papain hydrolysate; (H) 10 mg/ml papain hydrolysate; (I) 100 mg/ml papain hydrolysate; (J) 1 mM ascorbic acid; (K) 5 mM ascorbic acid; (L) 1 mM α-tocopherol; (M) control.
Significant differences between the results were determined by ANOVA methodology followed by Fisher's PLSD test. Differences were considered significant for $p < 0.05$.

activities for 100 mg/ml enzymatic hydrolysates were higher than that of 1 mM α-tocopherol. Surprisingly, linoleic acid was hardly peroxidized by adding 100 mg/ml papain hydrolysate. On the other hand, the activity of control was drastically decreased with the passage of time.

Superoxide scavenging activity

Superoxide scavenging activites of enzymatic hydrolysates from bee bread were measured using the xanthine/xanthine oxidase system and the results were indicated as the superoxide productivity. Each hydrolysate had superoxide scavenging activity and these activities tended to increase with an increasing degree of the concentration of the sample (Table II). The activities of 1 mg/ml hydrolysates were similar to that of 1 mM ascorbic acid. For 10 mg/ml the activities of these hydrolysates, except for the trypsin hydrolysate, were higher than that of 1 mM ascorbic acid, but were lower than that of 5 mM ascorbic acid. Trypsin hydrolysate of 10 mg/ml almost scavenged the superoxide anion radical. Moreover, the hydrolysates of 100 mg/ml perfectly scavenged it.

DPPH radical scavenging activity

The DPPH radical scavenging activity was investigated by the passage of time and the results were indicated as relative activity against control. Each hydrolysate showed scavenging activity (Table III). The activities of hydrolysates of 10 mg/ml were higher than that of 0.1 mM ascorbic acid. Except for the papain hydrolysate, the activities of hydrolysates were high, but did not amount to those of 1 mM ascorbic acid and α-tocopherol. Among these hydrolysates, papain was the one and only that scavenged this radical about 50%.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was investigated using the Fenton reaction system and the results were indicated as the inhibition rate. The pepsin and trypsin hydrolysates of 1 mg/ml had no activities againt this radical (Table II). The

Table II. Superoxide anion radical scavenging and hydroxyl radical scavenging activities of enzymatic hydrolysates from bee bread.

	Scavenging activity (%)	
Sample[a]	Superoxide anion radical	Hydroxyl radical
A	17.22	0.00
B	45.50	23.76
C	100.00	80.59
D	10.54	0.00
E	94.86	15.67
F	100.00	72.54
G	11.05	9.12
H	35.48	25.54
I	100.00	86.11
J	14.65	13.15
K	89.85	16.09
L	52.63	67.27

[a] See sample nomenclature in Table I.
Significant differences between the results were determined by ANOVA methodology followed by Fisher's PLSD test. Differences were considered significant for $p < 0.05$.

Table III. DPPH radical scavenging activities of enzymatic hydrolysates from bee bread.

	Relative activity (%)												
	Sample[a]												
Time [min]	A	B	C	D	E	F	G	H	I	J	K	L	M
0	100	100	100	100	100	100	100	100	100	100	100	100	100
1	100	85.6	31.2	97.9	80.7	28.7	96.5	85.0	67.4	94.6	5.3	9.5	99.9
2	99.8	83.0	22.2	97.2	76.8	21.8	96.0	82.9	62.4	94,4	5.3	5.3	98.5
3	99.2	80.9	17.8	96.5	74.3	19.1	95.4	81.3	57.9	94.4	5.3	5.3	98.8
5	98.4	78.2	13.5	95.3	71.8	17.8	94.7	79.3	52.2	94.4	5.3	5.3	99.1
10	97.1	74.4	14.2	92.9	66.3	17.7	95.3	73.9	45.7	94.4	5.3	5.3	98.7

[a] See sample nomenclature in Table I. (J) 0.1 mM ascorbic acid; (K) 1.0 mM ascorbic acid.
Significant differences between the results were determined by ANOVA methodology followed by Fisher's PLSD test. Differences were considered significant for $p < 0.05$.

activity of the papain one of the same concentration was lower than that of 1 mM ascorbic acid. For 10 mg/ml, the activities of these hydrolysates were similar or were slightly higher than that of 5 mM ascorbic acid. Moreover, the activities for 100 mg/ml were higher than that of 1 mM α-tocopherol and these scavenged the radical more than 73–86%.

Angiotensin I-converting enzyme inhibitory activity

Angiotensin I-converting enzyme inhibitory activities of enzymatic hydrolysates from bee bread were measured and the results are indicated in Table IV. The activities of these hydrolysates were as follows: 1.48 mg protein/ml (pepsin hydrolysate), 2.16 mg protein/ml (trypsin hydrolysate), and 5.41 mg protein/ml (papain hydrolysate), respectively.

Table IV. ACE inhibitory activities of enzymatic hydrolysates from bee bread.

Sample species	IC_{50} [mg protein/ml]
Pepsin hydrolysate	1.48
Trypsin hydrolysate	2.16
Papain hydrolysate	5.41

Significant difference ($p < 0.05$).

Discussion

It is known that bee bread contains approximately 20% proteins, 3% lipids, 24–35% carbohydrates, 3% minerals and vitamins. Bee bread is composed of well balanced proteins containing all essential amino acids, the full spectrum of vitamins (C, B_1, B_2, E, H, P, nicotinic acid, folic acid), pantothenic acid, pigments and other biologically active compounds, like enzymes as saccharase, amylase, phosphatases, flavanoids, carotenoids, hormones. Moreover, bee bread contains over 25 different micro and macro elements such as iron, calcium, phosphorus, potassium, copper, zinc, selenium, magnesium.

In our present study, the enzymatic hydrolysates, probably peptides, were easily prepared from bee bread using two gastrointestinal proteases (pepsin and trypsin) and a protein protease (papain). The yields of these hydrolysates were very high and they contained high amounts of proteins and total phenolic compounds. This suggests that the nutritive values of these hydrolysates are high because of high protein concentrations. Moreover, the hydrolysates possessed remarkably antioxidant activities equivalent to that of 1 mM α-tocopherol. In particular, the papain hydrolysate was not almost affected by autoxidation. The hydrolysates also had high scavenging activities against active oxygen species such as the superoxide anion radical and hydroxyl radicals. Bee bread is made of pollen, that has been gathered by bees and mixed with its own digestive enzymes. For that reason, bee bread in itself may be possess an antioxidant activity and scavenging activities against active oxygen species. However, this suggests that it is useful for the present methods to obtain the substances having strong antioxidant activity and scavenging activities against active oxygen species. On the other hand, the enzymatic hydrolysates from bee bread showed angiotensin I-converting enzyme inhibitory activities these activities were

similar to those from various fermented foods such as fish sauce, sake, vinegar, cheese, miso, and natto (Okamoto *et al.*, 1995). This suggests that enzymatic hydrolysates from bee bread possessed the same anti-hypertensive effects as the Maillard reaction products, although it was known that the Maillard reaction products showed the antioxidative activity.

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