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Proximate, Anthocyanin and Oligomeric Proanthocyanidin Compositions of Cowpeas [*Vigna unguiculata* (L.) Walp] Cultivated in Southwest Japan

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ABSTRACT

Cowpea (*Vigna unguiculata*) is one of the traditional ingredients of Japan. The objective of this study was to investigate the nutritional and chemical composition and the biological activities of cowpea grown in Okinawa region, a subtropical area located in south west Japan. This study demonstrated that the protein and dietary fiber content of cowpea grown in the Okinawa region was similar to that of cowpeas grown in other countries. In addition, it was found that the sodium content and anthocyanin composition of cowpeas grown in the Okinawa region was different from that grown in other regions of Japan; however, no significant differences were observed in proanthocyanidin composition and content among the cowpeas grown in different cultivation areas in Japan.

Key words: Anthocyanins, Cowpea, Proanthocyanidin oligomers, Proximate composition.

INTRODUCTION

Cowpea (*Vigna unguiculata*) is a highly nutritious crop that can be grown, where most of the food legumes fail to grow due to heat, drought and salt problem. Cowpeas are grain legumes consumed globally, particularly in tropical and subtropical areas. They are mainly cultivated in Africa, namely, in Nigeria, Niger and Burkina Faso (FAO, 2016). In the Far East Asian countries, such as Korea and Japan, cowpeas are consumed as traditional food ingredients. In Japan, cowpeas are cultivated in various regions, such as in Hokkaido, the northernmost Japanese island and Okinawa, which is located in south–west Japan, both of which are located approximately 2200 km apart. Although the yield of cowpeas is low, the seeds are utilized principally for celebratory meals in Japan, such as rice cakes, zenzai (sweet red bean soup with pieces of rice cake) and sekihan (steamed rice mixed with red beans) (Ohashi *et al*., 2013), similar to azuki beans (*Vigna angularis*), which belong to the same genus. Thus, red cowpeas can be used in a manner similar to that of azuki beans of the same seed coat color and are thus, added to a variety of dishes as an alternative to azuki beans.

Cowpeas are a good source of proteins, carbohydrates, several minerals, and trace metals (Ano and Ubochi, 2008; Sinha *et al*., 2005). Seed coat colors of cowpeas vary and include black, brown, green, red and white colors. Cowpeas contain flavonols and flavonoids (Ojwang *et al*., 2012), especially in cowpeas with coats pigmented by anthocyanins and proanthocyanidins possess various biological properties, including antioxidant (Soobrattee *et al*., 2005), antidiabetic (Key *et al*., 2004) and anti–inflammatory (Ojwang *et al*., 2015) properties. The antioxidant property has attracted attention because overproduction of reactive oxygen/nitrogen species in the human body is associated with aging and pathogenesis of several common diseases (Ames *et al*., 1993).

Although the nutritional and chemical components of cowpeas and their biological activities have been reported in countries other than Japan, there are almost no reports on the proximate, anthocyanin and proanthocyanidin compositions of cowpea grown in Okinawa, a subtropical area. The objective of the present study was to investigate the proximate compositions, the phytochemical profiles (anthocyanins and oligomeric proanthocyanidin complexes), and the antioxidant properties of cowpeas grown in Okinawa.

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MATERIALS AND METHODS

Plant materials

Cowpeas (*Vigna unguiculata*) with black or red seed coats are native varieties grown in Okinawa and were provided

by the Okinawa Prefectural Agricultural Research Center, Okinawa. Cowpeas with red seed coat and azuki beans (*Vigna angularis*) with black or red seed coats that are grown in Hokkaido Island, were obtained from a local market. Grain legumes were stored at -20°C until they were used for proximate analysis. For other analyses, grain legumes were freeze-dried and stored at -80°C until future use.

Proximate composition

Analyses of cowpeas and azuki beans for crude protein, fat, ash and moisture contents were carried out according to the standard methods of the Association of Official Analytical Chemists (1990). The carbohydrate content was obtained from the weight difference using moisture, protein, fat and ash content data. Total dietary fiber (TDF) was determined by rapid enzymatic assay (Asp *et al*., 1983). Sodium in grain legumes was determined by flame photometry. These analyses were done in duplicate and the average values were reported.

Extraction of anthocyanins

Anthocyanins in grain legume sample were extracted with 1% (v/v) hydrochloric acid-methanol as previously described (Orita *et al*., 2019).

Extraction of phenolic compounds

Phenolic compounds in grain legume sample was extracted with an extraction solvent (acetone: water: acetic acid = 70:29.5:0.5, v/v) (Orita *et al*., 2019).

Anthocyanin analysis of grain legume extract

Anthocyanins were analyzed by HPLC equipped with a photodiode array detector according to the method described by Orita *et al.* (2019). Identification was performed by comparing retention times and UV-VIS absorption spectra with commercially available anthocyanin standards. Its content in grain legumes were calculated based on the area of the corresponding peaks, by interpolation based on calibration curves obtained using standards.

Oligomeric proanthocyanidin analysis of grain legume extract

Oligomeric proanthocyanidins were analyzed by HPLC equipped with a fluorescence detector according to the method reported by Obara *et al.* (2016). Identification was performed by comparing retention times with oligomeric proanthocyanidin standards (monomer to heptamer) isolated from apple as described previously (Shoji *et al*., 2006). Its content in grain legumes was calculated based on the area of corresponding peaks, by interpolation based on calibration curves obtained using standards.

Determination of total polyphenol content

Total polyohenol content of grain legume extracts was determined using the Folin-Ciocalteu method (Yoshida *et al.*, 2010). Total polyohenol contents were expressed as gallic acid equivalents (GAE) per gram of dry matter (mg-GAE/g-DM).

Determination of total proanthocyanidin content

Total proanthocyanidin contents of grain legume extracts were determined using the vanillin-sulfuric acid method (Yoshida *et al.*, 2010). Total proanthocyanidin contents were expressed as catechin equivalents (CAE) per gram of dry matter (mg-CAE/g-DM).

DPPH radical scavenging activity assay

The scavenging activity of grain legume extracts against DPPH radicals was measured according to Oki *et al*. (2002), with minor modifications. DPPH radical scavenging activities were expressed as Trolox equivalents (TE) per gram of dry matter (µmol-TE/g-DM).

Hydrophilic oxygen radical absorbance capacity (H–ORAC) assay

The antioxidant capacity of grain legume extracts was measured by hydrophilic oxygen radical absorbance capacity (H-ORAC) assay, as reported by Watanabe *et al.* (2012). H-ORAC values were expressed as Trolox equivalents (TE) per gram of dry matter (µmol-TE/g-DM).

Statistical analysis

Data are reported as means ± standard deviation. All tests were conducted in triplicate. Data were analyzed by a one-way ANOVA followed by a Tukey post-hoc test using the SPSS software (version 22.0 for Windows, SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

One hundred-seed weight and proximate composition of grain legumes

Table 1 summarizes the one hundred-seed weight and proximate composition of cowpeas and azuki beans. Seed size of cowpeas grown in southwest Japan was smaller than that of cowpeas grown in northern Japan. Ajeigbe *et al*. (2008) reported that the one hundred-seed weight of cowpeas grown in Nigeria ranged from 13.2 g to 18.9 g, indicating that the seed size of cowpeas grown in the Okinawa region were either similar or smaller, than those grown in Nigeria. Crude protein and fat content in cowpeas grown in the Okinawa region was almost the same or higher, than that of cowpeas or azuki beans grown in the Hokkaido region, which has the largest yield of grain legumes in Japan. Total dietary fiber content of cowpeas grown in the Okinawa region was almost the same or lower than that in cowpeas grown in the Hokkaido region. It was also demonstrated that the crude protein, fat and total dietary fiber contents in cowpeas grown in Japan were comparable to those in white and red cowpeas grown in Nigeria and India (Ajeigbe *et al*., 2008; Sreerama *et al*., 2012). In general, the seed coats and cell walls of legumes mainly contribute to the high content of fiber, which provides several benefits to human health (Eashwarage *et al*., 2017). This study indicated that black cowpeas are also abundant in dietary fiber, similar to

Table 1: One hundred–seed weight and proximate composition in cowpeas and azuki beans grown in Japan.

Sample	Black cowpea 1	Black cowpea 2	Red cowpea 1	Red cowpea 2	Red cowpea 3	Black azuki bean	Red azuki bean
Seed coat color	Black	Black	Red	Red	Red	Black	Red
Harvest	Okinawa	Okinawa	Okinawa	Okinawa	Hokkaido	Hokkaido	Hokkaido
100–seed weight (g/100 g)	13.1	18.2	8.0	11.8	20.5	18.2	13.7
Moisture (g/100 g)	11.4	11.9	14.1	12.9	11.6	15.1	15.3
Protein (g/100 g)	26.4	26.2	23.8	25.1	17.2	20.4	19.9
Fat (g/100 g)	1.7	2.3	2.0	2.5	1.7	1.7	1.3
Carbohydrate [a](g/100 g)	57.3	56.1	56.8	56.2	65.7	59.1	60.5
Ash (g/100 g)	3.2	3.5	3.3	3.3	3.8	3.7	3.0
Total dietary fiber (g/100 g)	18.3	17.4	23.0	18.8	22.1	20.5	14.7
Sodium (mg/100 g)	8.8	11.9	17.1	20.3	nd	nd	1.5

[a], By difference as 100 – (moisture + protein + ash + fat). Each value is the average of two replications. Abbreviation: nd, not detected.

Table 2: Anthocyanin and oligomeric proanthocyanidin contents (mg/g–DW) in cowpeas and azuki beans grown in Japan.

Sample	Black cowpea 1	Black cowpea 2	Red cowpea 1	Red cowpea 2	Red cowpea 3	Black azuki bean	Red azuki bean
Anthocyanins							
Cyanidin–3–*O*–galactoside	0.07±0.01^{f}	0.09±0.01^{g}	nd	nd	nd	nd	nd
Cyanidin–3–*O*–glucoside	2.38±0.13^{a}	3.11±0.17^{a}	0.01±0.00^{a}	0.03±0.01^{a}	0.07±0.01^{c}	nd	nd
Delphinidin–3–*O*–galactoside	0.23±0.01^{e}	0.27±0.01^{f}	nd	nd	nd	0.07±0.01^{c}	nd
Delphinidin–3–*O*–glucoside	1.69±0.03^{b}	1.94±0.10^{b}	nd	nd	nd	2.03±0.23^{a}	nd
Malvidin–3–*O*–glucoside	1.24±0.05^{c}	1.08±0.06^{d}	0.01±0.00^{a}	0.01±0.00^{a}	0.03±0.02^{c}	0.81±0.07^{b}	0.02±0.00
Pelargonidin–3–*O*–glucoside	nd	nd	nd	nd	0.54±0.02^{a}	nd	nd
Peonidin–3–*O*–glucoside	0.57±0.01^{d}	0.54±0.04^{e}	nd	nd	0.21±0.06^{b}	nd	nd
Petunidin–3–*O*–galactoside[a]	0.52±0.03^{d}	0.54±0.02^{e}	nd	nd	nd	nd	nd
Petunidin–3–*O*–glucoside	1.27±0.02^{c}	1.36±0.07^{c}	nd	nd	nd	1.04±0.11^{b}	0.01±0.00
Total anthocyanins	7.97±0.76	8.93±0.97	0.02±0.00	0.04±0.01	0.85±0.21	3.95±0.41	0.03±0.00
Oligomeric proanthocyanidin							
Monomers	0.21±0.01^{e}	0.18±0.02^{b,c}	0.04±0.00^{e}	0.09±0.01^{c}	0.03±0.00^{f}	nd	0.05±0.00^{d}
Dimers	0.26±0.01^{b,c}	0.30±0.03^{a}	0.14±0.01^{a}	0.24±0.05^{a}	0.08±0.00^{a}	0.01±0.01^{a,b}	0.17±0.00^{a}
Trimers	0.23±0.01^{c,d}	0.20±0.03^{b,c}	0.11±0.01^{b,c}	0.17±0.03^{a,b,c}	0.04±0.00^{e}	0.01±0.00^{b}	0.08±0.00^{c}
Tetramers	0.29±0.00^{b}	0.22±0.01^{b}	0.11±0.01^{b}	0.19±0.03^{a,b}	0.06±0.00^{c}	nd	0.07±0.01^{b}
Pentamers	0.41±0.01^{a}	0.30±0.02^{a}	0.12±0.01^{a}	0.21±0.04^{a,b}	0.05±0.00^{d}	0.02±0.01^{a}	0.09±0.01^{b}
Hexamers	0.25±0.01^{c,d}	0.17±0.01^{c,d}	0.09±0.01^{c}	0.16±0.03^{a,b,c}	0.06±0.01^{b}	0.17±0.01^{a,b}	0.07±0.00^{b}
Heptamers	0.25±0.03^{d}	0.13±0.01^{d}	0.07±0.01^{d}	0.13±0.02^{c}	0.04±0.00^{e}	0.02±0.00^{a}	0.05±0.00^{c}
Total oligomeric proanthocyanins	1.90±0.07	1.50±0.06	0.68±0.03	1.19±0.05	0.36±0.02	0.08±0.01	0.58±0.04

[a], Expressed as petunidin-3-*O*-glucoside equivalent. Results are represented as the mean ± standard deviation (n = 3). Contents are expressed as per gram of dry matter after freeze-drying. Within each row, means followed by different lowercase letters are statistically different ($P < 0.05$). Abbreviation; nd, not detected.

white and red cowpeas cultivated in other countries. Cowpeas grown in the Okinawa region contained more than 8.8 mg sodium/100 g, whereas sodium was not detected in cowpeas grown in the Hokkaido region. According to the standard table of food composition (FCT) of Japan-2015, the sodium content in cowpeas was 1.0 mg/100 g. Therefore, cowpeas grown in the Okinawa region are characterized by the sodium content, and are therefore, distinguished from cowpeas grown in other regions of Japan. The sodium content in plants is known to be directly associated with concentration of sodium chloride in the soil (Taffouo *et al*., 2009). Other studies have reported that leaf nutritional status affects seed trace metal concentrations (Yadav and Choudhary. 2012). The high levels of sodium detected in cowpeas grown in the Okinawa region could be attributed to the close proximity of the cultivation area with the sea.

Anthocyanin and oligomeric proanthocyanidin composition and content in grain legumes

Anthocyanins and proanthocyanidins are two of the predominant phytochemicals in black and red cowpeas. As shown in Table 2, this study demonstrated that black cowpeas grown in southwest contained eight species of anthocyanins, which consisted of five species of the 3–*O*–glucoside and three species of the 3–*O*–galactosides. Mono–glucoside anthocyanins comprise approximately 80% of the total anthocyanins. These results were in agreement with those obtained in United States and Korea (Ojwang *et al*., 2012; Ha *et al.,* 2010). In addition, Ojwang *et al*., (2012) determined the anthocyanin composition of black cowpea, in which delphinidin–3–*O*–glucoside was the most predominant anthocyanin, followed by cyanidin–3–*O*–glucoside, petunidin–3–*O*–glucoside and malvidin–3–*O*–glucoside. In addition, similar results were obtained in our previous study (Orita *et al*., 2019) using black cowpeas grown in regions of northern Japan other than the Hokkaido region. In this study, in both black cowpeas 1 and 2 that were grown in the Okinawa region, the most abundant anthocyanin was cyanidin–3–*O*–glucoside, followed by delphinidin–3–*O*–glucoside, petunidin–3–*O*–glucoside and malvidin–3–*O*–glucoside, the content was significantly different among them. The rank order of anthocyanin compositions was not consistent with the results previously reported. Total anthocyanin content of black cowpeas grown in the Okinawa region was lower, compared to our previous report. The anthocyanin compositions of black cowpeas grown in the Okinawa region and other regions were different, which might be attributed to phenotype, variety, and/or cultivation area.

Grain legumes also contain proanthocyanidins, including polymers of (epi) catechin, (epi) gallocatechin, (epi) afzelechin units, heptamers, or more (Dueñas *et al*., 2003). The cowpeas analyzed in this study contained polymers ranging from monomeric to heptameric proanthocyanidins, as determined by HPLC. Oligomeric proanthocyanidin composition in black cowpea and monomeric proanthocya-nidins were the most predominant proanthocyanidins

Table 3: Total polyphenol content, total proanthocyanidin content, 2, 2–diphenyl–1–picrylhydrazyl radical–scavenging activity, and hydrophilic oxygen radical absorbance capacity in cowpeas and azuki beans grown in Japan.

Sample	Black cowpea 1	Black cowpea 2	Red cowpea 1	Red cowpea 2	Red cowpea 3	Black azuki bean	Red azuki bean
Total polyphenol content (mg–GAE/g–DW)	14.3±0.2^{a}	13.5±0.3^{a}	9.0±0.1^{b,c}	10.1±0.3^{b}	7.9±0.17^{c}	5.7±0.2^{d}	8.3±1.1^{c}
Total proanthocyanidin content (mg–CAE/g–DW)	9.5±1.0^{a}	9.6±0.4^{a}	5.9±0.6^{b,c}	6.2±0.1^{b}	4.5±0.2^{c,d}	1.1±0.3^{d}	5.2±0.6^{b,c,d}
DPPH radical–scavenging activity (mmol–TE/g–DW)	132±1^{a}	126±3^{a}	69±0.1^{c}	77±6^{b}	55±2^{d}	49±1^{d}	64±3^{d}
H–ORAC (mmol–TE/g–DW)	358±5^{a}	331±17^{a}	204±10^{c,d}	261±17^{b}	179±5^{d}	105±10^{e}	226±18^{c}

Results are represented as the mean ± standard deviation (n = 3). Abbreviations: GAE, gallic acid equivalents; CAE, catechin equivalents DPPH, 2,2–diphenyl–α–picrylhydrazyl; TE, trolox equivalents; H–ORAC, Hydrophilic oxygen radical absorbance capacity. Contents are expressed as per gram of dry matter after freeze–drying. Within each column, means followed by different lowercase letters are statistically different (P < 0.05). Total proanthocyanidin content of grain legumes was determined using the vanillin–sulfuric acid method.

(42.0%), followed by dimers (18.0%), trimers (18.3%) and tetramers to heptamers (21.9%) (Gu *et al*., 2004). The results of this study showed that the compositions of tetramers to heptamers were more than 4.6 times higher than those of monomers in black and red cowpeas grown in Japan, indicating that the oligomeric proanthocyanidin composition was significantly different between cowpeas grown in Japan and other countries. Total oligomeric proanthocyanidin contents of black cowpeas grown in the Okinawa region were higher than those of red cowpeas. Total proanthocyanidin content was also determined by the vanillin–sulfuric acid method, which detects monomeric and polymeric proanthocyanidins (Table 3). The ratio of the sum of monomeric to heptameric proanthocyanidins to the total proanthocyanidin contents ranged from 11.5% to 20.0% in cowpeas grown in the Okinawa region. This ratio was higher than the ratio in cowpeas grown in the Hokkaido region (8.0%), suggesting that the proportion of oligomeric proanthocyanidins among total proanthocyanidins in cowpeas grown in southwest Japan is higher than those grown in northern Japan. However, how phenotype/variety affects the composition of proanthocyanidins in cowpea is unknown.

Total phenolic contents and antioxidant properties of grain legumes

Grain legumes are known to be a source of natural antioxidants besides anthocyanins and proanthocyanidins (Amarowicz and Pegg, 2008). In this study, antioxidant activity was evaluated by two methods with different reaction mechanisms; DPPH radical-scavenging activity and hydroxyl radical antioxidant capacity (H-ORAC), whose reaction mechanisms are based on single electron transfer and hydrogen atom transfer, respectively. As shown in Table 3, black cowpeas possessed significantly higher DPPH radical-scavenging activity and H-ORAC, compared to red cowpeas grown in the Okinawa region, accompanied by higher total phenolic content and total proanthocyanidins content. In addition, cowpeas grown in the Okinawa region showed significantly higher H-ORAC, compared to red cowpeas grown in the Hokkaido region. Our previous study (Orita *et al*., 2019) demonstrated that cowpeas grown in northwest regions other than the Hokkaido region showed the highest H-ORAC values among grain legumes commonly consumed in Japan. These values observed in red and black cowpeas were higher than that of other cowpeas in this study. Among rice cultivars, differences in antioxidant activities and phenolic contents due to genetic diversity have been observed (Pathak *et al*., 2017). Thus, the differences in antioxidant properties of cowpeas would not be attributed to the place of cultivation but the type of variety.

CONCLUSION

In the Okinawa region, which is located in a subtropical area of southwest Japan, cowpeas are suitable for planting due to their heat-, drought- and salt-tolerance and have been used as the traditional food for a long time. However, there have been only few studies on the micronutrients, phytochemicals, and antioxidant properties of cowpea. This study demonstrates, for the first time, that native cultivars of cowpeas grown in the Okinawa region as well as in other countries are a rich source of protein and dietary fiber. In addition, the sodium content and anthocyanin composition of cowpeas grown in the Okinawa region were different from those grown in other regions of Japan.

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