

Effect of Nanogrinding on the Pigment and Bioactivity of Djulis (*Chenopodium formosanum* Koidz.)

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ABSTRACT: Betanin is an antioxidant pigment found in djulis, a grain native to Taiwan, and is a natural source food coloring, but the bright red color degrades rapidly if submitted to light, heat, or oxygen. The effects of nanogrinding on the stability of pigments and bioactive components are unknown. In this study, the color characteristics and bioactivity (antioxidant capacity and enzyme activity) of nanoparticle (NP) djulis was compared with those of intact granules (IG) and microparticles (MP). Results showed that the NP samples exhibited the highest betanin content (2.04 mg/g), which was almost twice that of IG. It was observed that nanogrinding resulted in higher pigment extraction efficiency. However, during storage (5–35 °C for 56 days), NP samples showed the most serious pigment degradation, and this color degradation, as expected, had the lowest activation energy. This was more evident when the storage temperature was high. Antioxidant capacities showed the same trends. MP and NP exhibited significantly higher activity of superoxide dismutase-like activity, lactoperoxidase (LPO), and lysozyme than IG. Gel permeation chromatography confirmed the degradation of larger particles during nanogrinding, which might favor enzyme extraction and their activities. Statistical analysis revealed a close relationship between betanin and antioxidant capacity.

KEYWORDS: Betanin, djulis, nanogrinding, bioactivity

INTRODUCTION

Djulis (*Chenopodium formosanum* Koidz.), a cereal plant native to Taiwan, was recently discovered to have many useful properties. Besides the high content of dietary fiber and starch, it also possesses high protein levels and abundant essential amino acids, which make this plant a nutritious food, especially for vegetarians. The whole plant is colorful and traditionally called “ruby of cereals” for its bright red grain color. Betalains are indole-containing pigments composed of red-violet betacyanins and yellow-orange betaxanthins; betanin, a betacyanin, is the main red pigment found in beet root. In previous work, we found that betacyanin was the main source of color and antioxidant capacity in djulis.¹ In addition, betanin was reported to inhibit linoleate peroxidation induced by cytochrome *c* better than either catechin or α -tocopherol.² Purified betanin was more effective than a crude extract for the inhibition of B16F10 melanoma cell proliferation.³

Nanotechnology is one of the important scientific approaches presently studied; particles in the 1–100 nm range have been shown to have different effects than larger particles.⁴ Calcium with a particle size in the nanometer range is reportedly more easily absorbed by rats.⁵ Curcumin nanoparticles show better DPPH scavenging and ferrous iron chelating activities.⁶ However, betanin is very susceptible to heat, oxygen, and pH.^{1,7} A number of questions arise in relation to the effect of nanoprocessing on the characteristics of djulis: (1) What will happen to the pigment after djulis grain is milled into nanoparticle size? (2) Are the color and antioxidant capacities changed compared with the intact grain? (3) Will nanoprocessing affect the stability of pigment or antioxidant enzymes?

Phytochemicals, such as polyphenols and antioxidant enzymes, are also important contributors to biological functions in many plants. In preliminary work, we found high levels of total phenols and superoxide dismutase (SOD)-like enzyme activities in djulis. SOD provides a defense against the superoxide radical and has been used as a natural antioxidant in health foods. SOD enzymes have been reported in both plants and animals.⁸ Lactoperoxidase (LPO) and lysozyme, well-known antibacterial enzymes, were also found in djulis. LPO catalyzes the oxidation of thiocyanate by H_2O_2 and produces hypothiocyanate, which acts as an antibacterial agent. This LPO system naturally occurs in milk and is widely used in foods, cosmetics, and clinical applications. The LPO–SCN– H_2O_2 (LPO) system may inhibit *Streptococcus mutans* and help prevent tooth decay.⁹ LPO also inhibits *Salmonella enteritidis* in lightly processed fruit and vegetable products.¹⁰ Lysozyme, known to be present in chicken egg white and in human tears, may adhere to *S. mutans* through a carbohydrate–protein interaction and can be used as an effective antifungal and antibacterial agent.¹¹ How the activities of these enzymes change after djulis is converted to nanoparticles remains unknown.

The aim of this study is to evaluate the color qualities, antioxidant capacity, and enzyme activity in djulis powder processed to different particle sizes, including nanoparticles. The changes in pigment and antioxidant capacity after storage at 5, 15, 25, and 35 °C for 56 days were also investigated.

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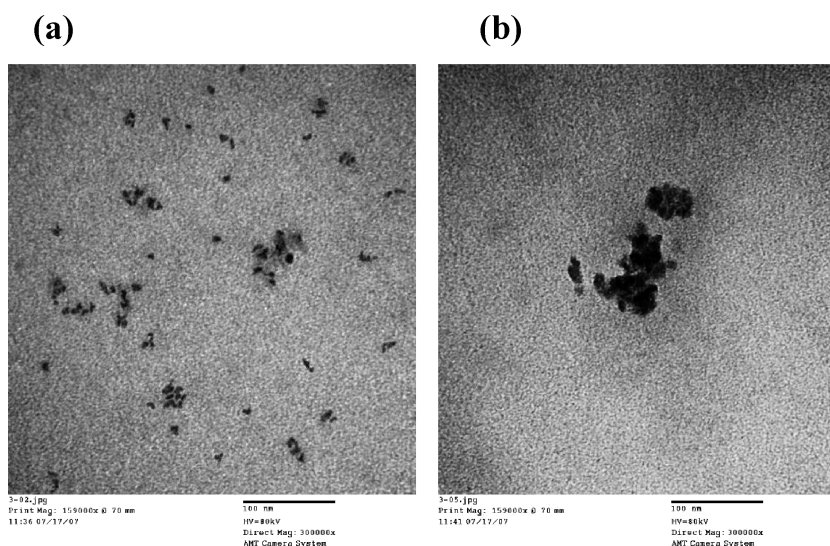


Figure 1. Transmission electron micrographs of djulis with (a) primary particle size and (b) secondary particle size after nanogrinding.

Table 1. Color Qualities and Pigments in Djulis with Different Particle Sizes^a

color quality	IG	MP	NP
Hunter <i>L</i>	69.87 a	59.38 b	58.79 c
Hunter <i>a</i>	39.28 c	40.21 b	43.01 a
Hunter <i>b</i>	10.08 c	15.81 a	14.82 b
hue angle	14.39 c	21.46 a	19.01 ab
chroma	40.55 c	43.21 b	45.49 a
total betacyanin (mg/g)	2.36 c	3.37 b	4.27 a
betanin (mg/g)	1.13 c	1.61 b	2.04 a
isobetanin (mg/g)	0.71 c	1.01 b	1.28 a

^a Values in rows with different letters indicate significant difference ($p < 0.05$).

MATERIALS AND METHODS

Sample Preparation. The djulis for this study was harvested from the National Pingtung University of Science and Technology farm. After air-drying (measured $A_w = 0.6$), the grains were separated as intact granule form (IG) and stored at $-20\text{ }^{\circ}\text{C}$. IG was ground into micro-particles (MP) by using an Ultragrinder (Hosokawa Alpine, Germany). Nanoparticle (NP) powder was prepared by grinding 5 g of MP powder with 500 mL of water in a Nanogrinder (LAB Wet Grinding & Dispersing Mill Batch Type JBM-B035, Just Nanotech Co. Ltd., Taiwan) at $4\text{ }^{\circ}\text{C}$ and 10000 rpm with the 0.1 mm yttria-stabilized zirconia bead (YSZ). Then, the solution was concentrated by using a rotary evaporator at $40\text{ }^{\circ}\text{C}$ (Rotavapor, R-205, Buchi, Switzerland) and freeze-dried (CT-5000D, Panchum Scientific Corp., Kaoshiung, Taiwan) into powder. The IG, MP, and NP powders were stored at 5, 15, 25, and $35\text{ }^{\circ}\text{C}$ for 56 days. Water extracts were prepared by mixing 1 g of each powder sample with 100 mL of distilled water at $4\text{ }^{\circ}\text{C}$ for 24 h, filtered through a Buchner funnel, and then centrifuged ($15652g$) at $4\text{ }^{\circ}\text{C}$ for 30 min. The supernatants were collected as samples for analysis of pigments, LPO, lysozyme activity, activation energy, and antioxidant capacity and were kept at $-20\text{ }^{\circ}\text{C}$ before assay.

Particle Size Analysis and Transmission Electron Microscopy (TEM). The distribution of particle sizes of NP was measured using a Nanometer Particle Analyzer (Dynamic Light Scattering System, Malvern Zetasizer Nano ZS, U.K.). NP samples with different grinding

Table 2. Antioxidant Capacities and Enzyme Activities of Djulis with Different Particle Sizes^a

		particle size		
item		IG	MP	NP
antioxidant capacities	FICA (%)	7.68 b	8.17 b	10.31 a
	DPPH scavenging (%)	46.42 c	60.98 b	65.68 a
	FRAP ($\mu\text{mol/L}$)	517.24 b	699.28 a	713.92 a
phenolic compound	total phenol (mg/g)	15.26 c	23.68 b	26.11 a
	rutin (mg/g)	3.38 c	4.85 b	5.62 a
antioxidant enzyme	lysozyme (U/mg)	267 c	361 b	381 a
	SOD (U/mg)	9.68 c	13.26 a	11.83 b
	LPO (U/mg)	0.21 c	2.96 a	1.82 b

^a Values in rows with different letters indicate significant differences ($p < 0.05$).

times (30, 60, 90, and 120 min) were compared to find the optimum time. TEM (Hitachi H7500) was further used to identify the primary and secondary particle sizes. Primary particle sizes are considered to be the size of individual particles and secondary size the size of aggregates. The size ranges of IG and MP were $1.5\text{--}2.2\text{ mm}$ and $1\text{--}10\text{ }\mu\text{m}$, respectively.

Pigment Measurement¹. *Color.* A Nippon Denshoku color meter (ZE2000, Japan) was used to obtain Hunter *L*, *a*, *b* and color difference of the samples. The higher *L*, *a*, and *b* values indicate higher lightness, red-green color, and yellow-blue color, respectively. The hue, chroma, and color differences were calculated using the following equations:

$$\text{hue : hue angle}_{ab} = \arctan(b/a)$$

$$\text{chroma : chroma}_{ab} = (a^2 + b^2)^{1/2}$$

$$\Delta E = ((L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2)^{1/2}$$

Betacyanin Separation and Quantity Estimation by HPLC. The pigment extracts of djulis samples were subjected to HPLC for further

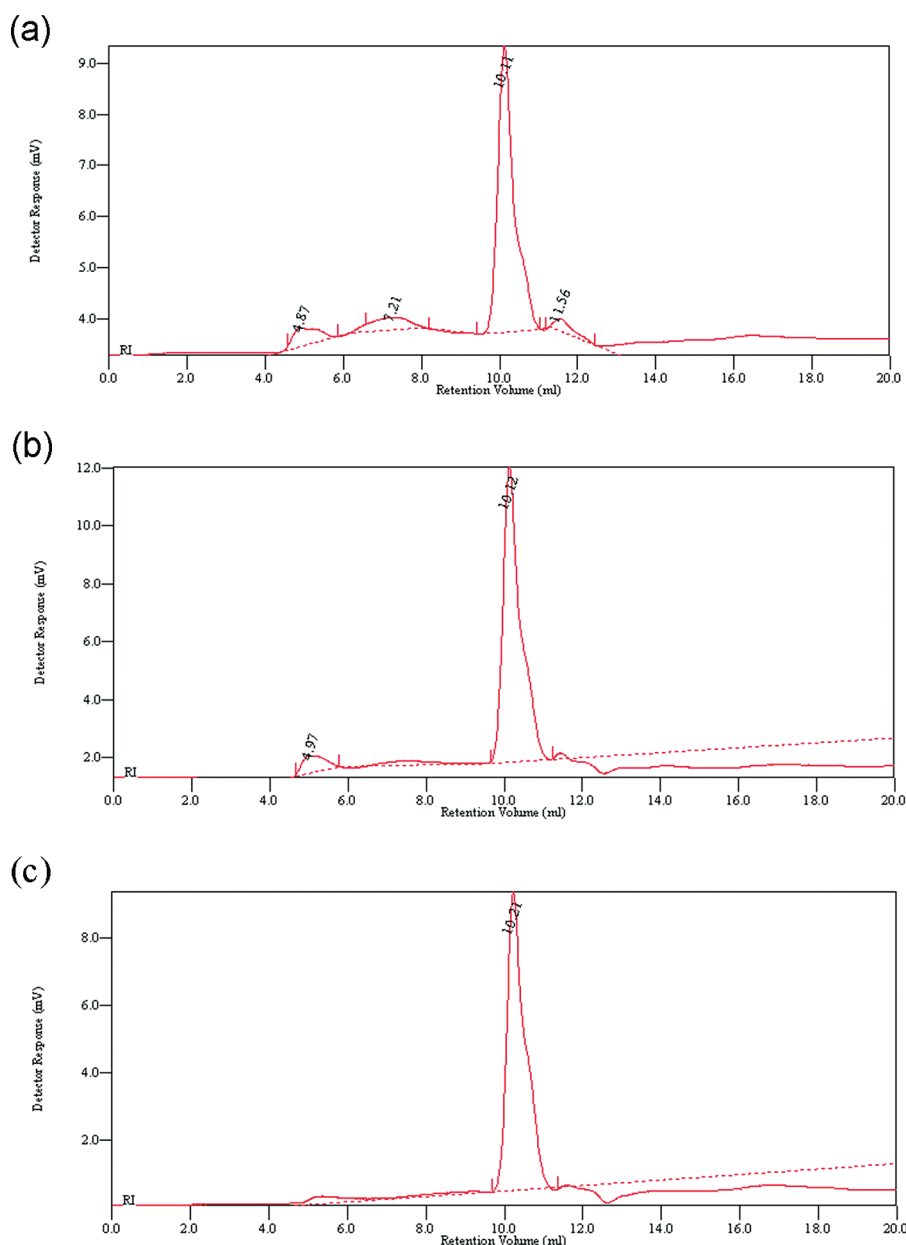


Figure 2. GPC elution profiles of djulis with (a) IG, (b) MP, and (c) NP samples.

separation.¹ The concentration of betanin was estimated by using the extinction coefficient (ϵ) 61600 with Beer's law: $A_{\lambda_{\max}} = 6.16 \times 10^4 \times \text{concentration (M)}$. The total betacyanin pigment was estimated by calculation from the concentration and relative peak areas of betacyanin and other peaks. Pigment retention was calculated by using the total betacyanin before storage as 100%.

Phenolic Compounds Analysis. Total phenolic content was measured spectrophotometrically (U-2001, Hitachi Ltd.) using the modified Folin–Ciocalteu method described by Chang et al.¹² Measurement of phenolic compounds was performed after extraction with 60% methanol at 4 °C for 24 h and filtration. A 20 μ L sample was subjected to HPLC to identify their phenolic compounds according to the method described in Tsai et al.¹³

Gel Permeation Chromatography (GPC) Analysis. The molecular distributions of particles in the water extracts from IG, MP, and NP samples were analyzed using a Waters Breeze model gel permeation chromatograph (Biscotek, USA). DI water was used as

the eluting solvent, and the flow rate was 0.8 mL/min. A hydrogel column (G4000PWXL) was used for molecular weight analyses, and narrow molecular weight polyethylene oxide standards were used for calibration.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity. One milliliter of freshly prepared 1 μ M DPPH solution was added to the sample (4 mL) and maintained at 25 °C for 30 min. The absorbance was measured at 517 nm relative to the control (as 100%), and the percentage of scavenging effect was expressed as $[1 - (A_{517} \text{ of sample}/A_{517} \text{ of control})] \times 100$.¹³

Ferric Reducing Ability of Plasma (FRAP). Freshly prepared FRAP reagent (including acetate buffer, tripyridyltriazine (TPTZ), and FeCl_3) (1.2 mL) was combined with 0.12 mL of distilled water and 0.04 mL of sample. The absorbance of the blue-colored Fe^{2+} –TPTZ complex was recorded at 593 nm after 6 min. The reducing power ($\mu\text{mol/L}$) was calculated from a standard curve constructed using a FeSO_4 solution.¹³

Ferrous Ion Chelating Ability (FICA). The ferrous ion chelating ability of djulis samples was determined using the method of Decker and Welch.¹⁴ Water extract from IG, MP, and NP samples (0.5 mL) was mixed with 1.6 mL of distilled water and 0.05 mL of 2 mM FeCl₂ in a tube. The reaction was initiated by the addition of 0.1 mL of 5 mM ferrozine. The mixtures were incubated at room temperature for 10 min. After incubation, the absorbance was measured at 562 nm. The FICA was calculated as follow: FICA (%) = $[(A_0 - (A_1 - A_2))/A_0] \times 100$, where A_0 was the absorbance of the control, A_1 was the absorbance of the sample, and A_2 was the absorbance of the blank.¹⁴

Activation Energy. Water extracts of IG, MP, and NP were heated at 50, 60, 70, 80, and 90 °C, and their betacyanin contents were measured over time. Plots of the change in betacyanin content with time were used to determine the degradation rates. The activation energy was then calculated by $\ln K = -E_a/RT$, where K = rate constant, R = 1.986, and T = (°C + 273).

Enzyme Assay. Lysozyme Activity. The lysozyme activity in djulis samples was measured by using a method modified from that of Lee and Yang.¹⁵ The water extract of djulis was adjusted to pH 7.5 with 0.5 N HCl and purified by discarding the precipitate. The supernatant was combined with 0.05 M NaCl and 0.05 M Tris-EDTA buffer (pH 8.2) as the crude enzyme. The enzyme solution (0.1 mL) was added to 2 mL of bacterial solution (*Micrococcus lysodeikticus*, 0.3 mg/mL in 0.1 M pH 7.0 phosphate buffer) and mixed well, and the absorbance was measured at 450 nm for 90 s at room temperature. The activity was calculated through the slope of the absorbance at 450 nm time course. A unit of enzyme activity was defined as the enzyme needed to decrease absorbance at 450 nm by 0.001/min. The protein concentration was calculated from a BSA standard curve.

Activity of LPO. Two milliliters of water extracts of IG, MP, or NP was combined with 0.2 mL of ABTS reagent (0.02 M) and 0.2 mL of H₂O₂ (0.01 M), and the absorbance at 412 nm was measured after 5 min. The activity of LPO was calculated by using a standard curve made by using different concentrations of lactoperoxidase ($(0-0.2) \times 10^{-3}$ mg/mL) in phosphate buffer solution (pH 5.5). A unit of enzyme was defined as the amount of enzyme that catalyzes the oxidation of 1 μ mol of ABTS/min.¹⁰

SOD-like Activity¹⁶. Acetone powder was prepared for each sample with 10 times (w/w) the mass of acetone and dissolved in potassium phosphate buffer (50 mM, pH 7.8) in a ratio of 1:5 (w/v) for 12 h at 4 °C. After centrifuging (4 °C, 12000 rpm, 30 min), ammonium sulfate was added to give 50–80% saturation at 4 °C. Precipitated protein was collected and dissolved in phosphate buffer and then subjected to dialysis (UC30-32-100, Viskase Co., Inc., Japan) for 48 h. After centrifuging, 50 μ L of the supernatant was mixed with 3 mL of reagent including riboflavin (2.4×10^{-6} M), methionine (0.01 M), and nitroblue tetrazolium (NBT) (1.67×10^{-4} M). The SOD-like activity was measured by the light-induced NBT/riboflavin assay (A_{560}). One unit of SOD activity was defined as the amount of enzyme that inhibited 50% of the NBT reduction.

Statistical Analysis. Statistical analysis was conducted using SAS statistical software (SAS, 1988). General linear model procedures were used to determine treatment effects, and Duncan's multiple-range tests were used to compare means. All experiments were conducted in triplicate.¹

RESULTS AND DISCUSSION

Particle Size of Djulis after Being Ground into Nano particles. The particle size of NP samples with different grinding times (30, 60, 90, and 120 min) was investigated through their particle size distribution using the Nanometer Particle Analyzer. The particle diameter distribution was focused around 400 nm after 30 min of grinding, 100 nm after 60 min of grinding, and then shifted to 300 nm after 90 or 120 min of grinding (data

Table 3. Changes in Color Qualities and Activation Energy in Djulis with Different Particle Sizes after 56 Days of Storage at Different Temperatures^a

		pigment	Hunter	Δ Hunter	Hunter	Δ	
treatment		retention (%)	<i>a</i>	<i>a</i>	<i>b</i>	Hunter <i>b</i>	ΔE
5 °C	IG	80.13 b	36.35 c	2.93 b	12.85 c	2.77 a	3.71 b
	MP	87.27 a	39.47 a	0.74 c	16.58 a	0.77 c	2.46 c
	NP	79.82 b	38.96 b	4.05 a	16.11 b	1.29 b	6.52 a
15 °C	IG	78.16 b	36.20 b	3.08 b	12.88 c	2.80 a	3.73 b
	MP	83.38 a	38.87 a	1.34 c	16.90 a	1.09 c	2.75 c
	NP	76.44 c	38.69 a	4.32 a	16.24 b	1.42 b	7.26 a
25 °C	IG	74.22 b	35.98 c	3.30 b	13.06 c	2.98 a	4.22 b
	MP	77.92 a	38.43 a	1.79 c	16.95 a	1.14 c	4.15 b
	NP	66.54 c	38.07 a	4.94 a	16.57 b	1.75 b	8.19 a
35 °C	IG	72.25 b	35.50 c	3.78 b	13.15 b	3.07 a	4.64 b
	MP	76.26 a	38.41 a	1.80 c	17.00 a	1.19 c	4.30 c
	NP	63.53 c	37.84 b	5.17 a	16.81 a	1.99 b	8.83 a
				IG	MP	NP	

	IG	MP	NP
activation energy (kcal/mol)	3.05 b	3.84 a	2.37 c

^aValues in columns (rows for activation energy) of each temperature with different letters indicate significant differences ($p < 0.05$).

not shown). The molecular size might increase after long-term (>90 min) grinding. Therefore, 60 min was chosen as the time for NP production in this study. Further observations of the primary and secondary particle sizes were conducted using TEM. As illustrated in Figure 1, the particle sizes of the primary particles (a) and secondary particles (b) of nanoground djulis powder were about 20 and 75 nm, respectively, which confirmed the nanoparticle size of the NP samples used in this study.^{4,5}

Effect of Grinding Methods on the Color and Pigments of Djulis. The color qualities and pigment contents of djulis in IG, MP, and NP are shown in Table 1. The red color pigments (betanin and total betacyanin) increased when the particle size decreased. For example, Hunter *a* and chroma are significantly higher in NP samples. In other words, a more intense red color was observed after nanoprocessing. For the pigments, the total betacyanin and betanin increased from 2.36 and 1.13 mg/g, respectively, in IG to 3.37 and 1.61 mg/g in MP and then to 4.27 and 2.04 mg/g in NP. Furthermore, no change of the λ_{\max} (wavelength of maximum absorbance) or peak ratio (by HPLC) of the pigments in MP or NP samples was observed (data not shown). This suggests that decreasing the particle size by MP and NP grinding might favor the extraction efficiency of the pigments and their coloration without changing the characteristic properties. However, the possible contribution of the extra time in NP grinding should not be ruled out.

Effect of Grinding on Antioxidant Capacities and Enzyme Activities of Djulis. As shown in Table 2, the antioxidant capacity and antioxidant enzyme activities were greatly influenced by nanogrinding. For example, FICA was highest in NP, followed MP and IG (10.48, 6.32, and 5.56%, respectively). This agrees with the report that nanogrinding increases the FICA of curcumin.⁶ Similar trends were observed in free radical scavenging

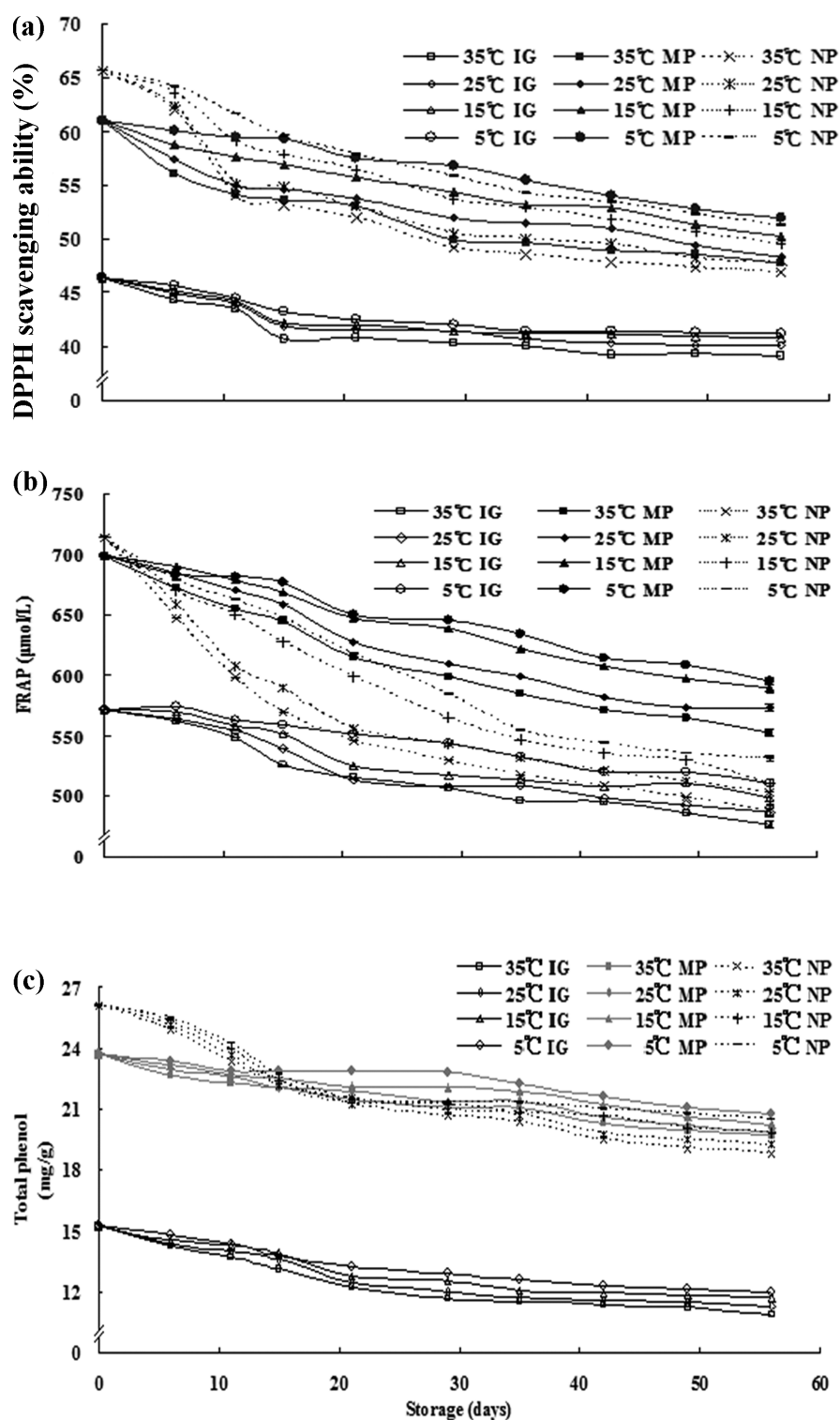


Figure 3. Changes of (a) DPPH scavenging ability, (b) FRAP, and (c) total phenols of djulis powder with different particle sizes during 56 days of storage.

and reducing power. The DPPH scavenging abilities were 46.42, 60.98, and 65.80% in IG, MP, and NP, whereas FRAP values were 517.24, 699.28, and 713.92 $\mu\text{mol/L}$, respectively. Besides the pigments, polyphenols also play an important role in the biological functions. In djulis we found that rutin was the major constituent of polyphenols (about 40% of the total phenolic compounds by HPLC), followed by chlorogenic acid and

catechin (data not shown). We observed that total phenols were highest in NP (26.11 mg/g) when compared with MP (23.68 mg/g) and IG (15.26 mg/g). The content of rutin showed the same pattern. Apparently, the smaller the particle size of djulis, the higher the antioxidant extracted. This suggests that particles with smaller sizes favor higher antioxidant capacity due to more antioxidant ingredients being extracted.⁶

Furthermore, we found that nanogrinding may result in extracts with higher enzyme activities than the original intact granules. For example, the activity of lysozyme in IG djulis was 267 U/mg, whereas MP and NP were 361 and 381 U/mg, respectively. This was confirmed by the distribution of their molecules through the GPC. As shown in Figure 2, four peaks appeared in IG sample with retention volumes (RV) 4.87, 7.21, 10.11, and 11.56 mL, which represented the molecules with molecular weights of 1.25×10^6 , 109258, 2944, and 614 Da, respectively. However, in the MP sample, two peaks with RV 4.97 and 10.12 were observed. In addition, only one peak with RV 10.21 was found in the NP sample. Apparently, the grinding process may favor the presence of molecules with reduced molecular size. The changes of SOD-like and LPO activities showed similar tendencies; however, the activities of both SOD and LPO in the NP samples were less than that in MP samples. It seems that the activities of these enzymes respond differently to grinding stress.

Pigment Stability of Nanoparticled Djulis Powder. Further investigation of the pigment stability of nanoparticle djulis was made of color qualities and activation energy analysis. As shown in Table 3, during 56 days of storage, pigment degradation was more pronounced in NP than in MP or IG samples at all temperature (5, 15, 25, and 35 °C). The most serious losses of pigment and Hunter *a* value (red color) appeared in NP samples and were lowest in MP samples. For example, the pigment retentions at 35 °C after 56 days if storage were 72.25, 76.26, and 63.53%, respectively, in IG, MP, and NP samples. On the other hand, the color difference (ΔE) was highest in the NP samples (8.83) but lowest in the MP samples (4.30). This suggests micro-sized particles had better extraction and stability of pigment. According to Escibano et al., the oxidation of betanin might be mediated by polyphenol oxidase (PPO) and peroxidase (PO).¹⁷ More of these enzymes could be extracted in the samples subjected to nanogrinding. This might be especially true for PPO, which is located mainly in the membrane. In addition, betanidin quinone, betalamic acid, and oxidized cyclo-DOPA 5-*O*- β -D-glucoside polymers were generated during pigment oxidation and led to the discoloration or browning.¹⁸ This explained the highest color difference of NP samples after storage. The pigment in nanoparticles was likely least stable due to their high surface area and susceptibility to oxidation.

Activation energy analysis was used to elucidate the differences in betanin degradation with different particle sizes. Changes in betacyanin contents over time were used to determine the degradation rates for different temperatures, which were then used to calculate the activation energy. The active energies of IG, MP, and NP were 3.05, 3.84, and 2.37 kcal/mol, respectively (Table 3). This suggests that the pigment in NP was more easily degraded, whereas the pigment in MP was the most stable. This is in accordance with the trends in the pigments mentioned earlier.

Stability of Antioxidant Capacity in Nanoparticle Djulis Powder. As shown in Figure 3, NP samples had a more significant drop in antioxidant capacity (both DPPH scavenging and FRAP) in the first 2 weeks than the MP or IG samples. Similar trends were also observed for total phenol contents. Antioxidant capacities were retained at higher levels in IG and MP than in NP samples. For example, there was a 14.40% decrease in DPPH scavenging ability of NP after 56 days of storage at 5 °C, whereas IG had only a 4% decrease. This was more evident when the storage temperature was high. For

Table 4. Correlation Analysis among Color Qualities and Antioxidant Capacities of Djulis with Different Particle Sizes^a

	DPPH scavenging	FRAP	FICA
total betacyanin	0.97****	0.98***	0.94***
Hunter <i>L</i>	−0.70**	−0.75**	−0.69*
Hunter <i>a</i>	0.85***	0.86***	0.95***
Hunter <i>b</i>	0.42	0.67	0.60
chroma	0.74**	0.72**	0.71**
hue angle	0.83**	0.81***	0.82***

^a *, **, ****: significant at 5, 1, and 0.1% levels, respectively.

samples stored at 25 °C, the decreases in DPPH scavenging ability at NP and IG were 19.18 and 7.65%, respectively. Similar trends were found in the FRAP. This suggested the important role of pigment in the performance of antioxidant capacities.^{1,19}

Correlation Analysis. As shown in Table 4, the antioxidant capacities are significantly correlated with pigment concentrations and red color. The correlation coefficients of DPPH scavenging ability and FRAP with total betacyanin were 0.97 and 0.98, respectively, whereas correlations with the Hunter *a* value were 0.85 and 0.86. This confirms the important role of betanin in both the coloring and antioxidant ability of djulis.¹ Similar relations were also found in chroma and hue.

In conclusion, djulis with micro- or nanoparticle size exhibited higher pigment contents, antioxidant capacities, and antioxidant enzyme activities than intact granules. However, the pigment in nanoparticles was more unstable during storage, due to oxidation susceptibility and a lower active energy of pigment degradation.

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Author Contributions

^{||}The contribution of Y.-S.C. is equal to that of the corresponding author.

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