Nanoencapsulation of synergistic combinations of acai berry concentrate to improve antioxidant stability

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Abstract The objectives of this study were to increase the antioxidant activity of acai berry concentrate (Acai) by combining it with various antioxidants to exploit synergistic effects and improve antioxidant stability by nanoencapsulation. Ascorbic acid and trolox were identified as synergistic antioxidants for Acai. The optimal mixing ratio of ascorbic acid (74.64 µg/mL) and trolox (47.88 µg/mL) for synergistic activity in both oxygen radical absorbance capacity (ORAC) and DPPH assays was 1.56:1. A mixture of Acai, ascorbic acid, and trolox at the optimum ratio was nanoencapsulated using chitosan and gum arabic. Nanoparticles exhibited homogenous dispersion with a 230 to 260 nm particle size. During storage, nanoparticles exhibited better antioxidant stability than non-nanoencapsulated antioxidants. These results suggest that mixing Acai with ascorbic acid and trolox or nanoencapsulating this mixture with chitosan and gum arabic are both effective techniques for improving antioxidant activities.

Keywords: antioxidant activity, synergistic effect, nanoencapsulation, acai berry, nanoparticle

Introduction

Reactive oxygen species (ROS) are usually generated by normal intracellular metabolism. External sources including ultraviolet light and ionizing radiation can trigger ROS production (1). Once ROS are produced, homeostasis is achieved via a number of enzymatic and nonenzymatic antioxidant defense systems. However, ROS overproduction can lead to DNA, protein, and lipid damage via oxidative stress. The damage triggered by oxidative stress can impair physiological functions, hasten aging, and increase the risks of cancer and other diseases (1). Oxidative stress damage can be inhibited by antioxidants, which have roles in activating endogenous antioxidant enzymes and directly scavenging ROS (2).

Many studies have suggested that berries have powerful antioxidant activity that is operationalized by reducing radicals through hydrogen donating, metal chelating, and singlet oxygen quenching interactions (3,4). A recent study showed that acai berry possessed noticeable antioxidant activity against superoxide and peroxy radicals, a result attributed to orietin, homoorietin, vitexin, luteolin, chrysoeriol, and quercetin abundance in the berry (5). Our previous study demonstrated that acai berry has the highest polyphenolic content and antioxidant activity for both oxygen radical absorbance capacity (ORAC) and DPPH assays among 10 antioxidant fruit and vegetable concentrates (raspberry, blackberry, blueberry, acai berry, aronia, cranberry, wild berry, spinach, and cabbage) (6).

Unfortunately, natural antioxidants have far lower efficiency than synthetic antioxidants. Synthetic antioxidants are more commonly used because they are readily available and have outstanding activities compared with natural antioxidants (7). However, several synthetic antioxidants have been reported to be carcinogenic and teratogenic (8); thus, the safety and toxicology of these synthetic antioxidants remain controversial (9). Therefore, combinations of synthetic and natural antioxidants may be useful alternatives to minimize toxicity and enhance efficiency.

Several studies have evaluated antioxidant combinations in order to achieve improved efficiency. Indeed, some antioxidants exhibit synergistic activity when used in combination with other antioxidants (7). However, antioxidant combinations may also produce antagonistic or additive effects depending on specific mixture components (10). Hidalgo *et al.* (11) showed an epicatechin and quercetin-3-glucoside combination resulted in highly synergistic effects, while the interaction between myceritin and quercetin resulted in an antagonistic effect. In addition, synergistic antioxidant activity can be influenced not only by mixture components, but also by the mixing ratio, although few studies have evaluated this effect in detail. Thus, further study regarding mixing ratios effects is needed to achieve maximum synergistic antioxidant activity.

Antioxidants easily lose their activity under oxidative conditions that include light, oxygen, high temperature, or abnormal pH. Fortunately, antioxidant stability in oxidative environments can be

improved by encapsulation (12-14). Encapsulation has been applied extensively to protect entrapped antioxidants from oxidative environmental conditions, resulting in improvements in shelf life and bioavailability of encapsulated core material (15).

Previous encapsulation studies have been performed on both macro and micro scales, and nanoencapsulation continues to improve with advances in nanotechnology. Nanoencapsulation technology has the potential to improve solubility and bioavailability and to enable controlled release of bioactive components due to the nanoscale size and large surface area of the nanoparticles (NPs) (16).

Chitosan, a (1,4)-2-amino-2-deoxy- β -D-glucan, has been widely used as a wall material in delivery systems because it is nontoxic, biocompatible, and biodegradable as well as having good mucoadhesive properties and membrane permeability (17). Being the only positively charged natural polysaccharide, chitosan-based capsules can be prepared by ionic gelation via interaction with negative charges (18-20). Encapsulation by ionic gelation of natural polysaccharide has been receiving attention in terms of a mild process without the use of toxic organic solvent and heating process. Gum arabic, a hydrocolloid consisting of D-glucuronic acid, L-rhamnose, D-galactose, and L-arabinose, is a biocompatible and biodegradable polymer with multiple interaction sites and negative charges for interaction with chitosan (21). In addition, gum arabic is widely used in oral and topical pharmaceutical formulations as a suspending, emulsifying, and stabilizing agent (22,23). The interbiopolymer electrostatic interaction between chitosan and gum arabic formed a strong viscoelastic network and provided a more sustained release of encapsulated material in neutral pH than hydrogel with chitosan and tripolyphosphate (24), which is the most extensively used ionic crosslinker for chitosan. Thus, gum arabic may be a suitable wall material for forming NPs with chitosan.

The main objective of this study was to improve the antioxidant activity and stability of acai berry concentrate (Acai) using various combinations of antioxidants and nanoencapsulation. For this purpose, the mixing ratios of antioxidant combinations were optimized for maximum synergistic antioxidant activity, and the nanoencapsulation effect on stability was investigated in terms of physical particle properties and antioxidant activity.

Materials and Methods

Materials Acai was donated by Dong Won F&B (Seoul, Korea). Ascorbic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Daejung (Seoul, Korea) and Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), respectively. Chitosan (Water soluble, 24 cps, 95% deacetylated) and gum arabic were purchased from Kittolife (Seoul, Korea) and ES Ingredients (Seoul, Korea), respectively.

Determination of total phenol content Total phenol content was

determined using the Folin-Ciocalteu method. Briefly, 1 mL of sample was mixed with 1 mL of Folin-Ciocalteu's phenol reagent and 1 mL of a 10% Na_2CO_3 solution. After letting the reaction proceed for 1 h, total phenol content was evaluated by measuring the absorbance at 750 nm. Total phenol content for each sample was expressed in gallic acid equivalents ($\mu\text{mol GAE/g}$).

Oxygen radical absorbance capacity (ORAC) assay The ORAC assay was performed according to the method of Ou *et al.* (25) with some modifications. Briefly, stock solution was prepared by dissolving 0.4 mM fluorescein in 75 mM phosphate buffer (pH 7.5) and stored at 4°C until needed. The working solution was diluted 50,000-fold with a phosphate buffer. Each plate contained 25 μL of the sample mixed with 150 μL of the working solution, which was incubated at 37°C for 15 min. After incubation, 25 μL of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) (80 mg/mL) were added, and fluorescence was kinetically analyzed for 60 min at excitation and emission wavelengths of 485 and 528 nm, respectively. A standard curve was constructed using the net area under the curve (AUC) for various trolox concentrations. The relative ORAC value for each sample was expressed in trolox equivalents ($\mu\text{mol TE/g}$).

DPPH radical scavenging assay DPPH radical scavenging assay was performed according to the method described by Brand-Williams *et al.* (26) with some modifications. DPPH solution was prepared daily. Each reaction mixture contained 180 μL of 0.4 mM DPPH solution and 20 μL of the sample. Antioxidant activity was evaluated by measuring the variation in absorbance at 517 nm after 45 min. Radical scavenging was calculated according to the formula:

$$\text{DPPH radical scavenging effect (\%)} = \frac{A_c - (A_s - A_b)}{A_c} \times 100 \quad (1)$$

where A_c , A_s , and A_b are the absorbance values of the control, sample, and blank, respectively.

Experimental design and data analysis for optimization A two-factor, five-level central composite design was used to optimize the mixing ratio for achieving maximum synergistic activity between ascorbic acid and trolox. The design featured 10 experimental points including fractional 2^2 factorial points, four star points, and one center point. Two preparation conditions were examined as independent variables, ascorbic acid ($\mu\text{g/mL}$, X_1) concentration and trolox ($\mu\text{g/mL}$, X_2) concentration. Based on preliminary experiments, the actual values of each variable were coded at five levels, and the independent variable ranges were also predetermined. Thus, using these parameters, the effects of mixing ratio on synergism were examined. The following equation was used for predicting the optimal mixing point:

$$y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 b_{ij} X_i X_j \quad (2)$$

where b_0 , b_i , b_{ii} , and b_{ij} are regression coefficients for the constant, linear, quadratic, and cross-product, respectively, and X_i and X_j are the independent variables. The Statistical Analysis System (SAS) program 9.3 (SAS Institute Inc., Cary, NC, USA) was used to analyze experimental data. Response surfaces and contour plots were generated using regression models derived from the statistical analyses.

Nanoparticle preparation Antioxidant NPs were obtained by ionic gelation of chitosan with gum arabic anions (24). Chitosan was dissolved in distilled water at a concentration of 0.5 mg/mL. Next, 0.75 mL of Acai, ascorbic acid, and trolox at the maximum synergistic mixing ratio were added to 9 mL of chitosan solution to yield a total volume of 11.25 mL. Finally, the NPs were prepared by combining 3.75 mg of gum arabic with the mixed solution (0.5–1.0 mg/mL) using a magnetic stirrer (1,000 rpm, 10 min, WiseStir MS-MP8, Wids Laboratory Instruments, Wertheim, Germany).

Nanoparticle physicochemical properties NP size, polydispersity index (PDI), and the derived count rate of antioxidant NPs were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK).

Physical and antioxidant properties during accelerated storage NP stability was evaluated under accelerated storage conditions at 60°C in an incubator for four days. NPs were also adjusted to pH 4 and pH 5.5 in order to determine the pH effect on stability. The physicochemical properties and the residual antioxidant activity of the NPs were measured daily, as previously described.

Statistical analysis All numeric values are expressed as the mean ± standard deviation (SD), and experiments were performed at least three times. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (SAS 9.1, SAS Institute Inc.).

Results and Discussion

Effect of ascorbic acid and trolox mixing ratio on synergistic antioxidant activity To identify a combination of antioxidant sources exhibiting synergistic activity with Acai, we first tested the synergic effect between Acai and antioxidants of ascorbic acid, catechin, trolox, and chlorogenic acid, selected through a literature review. A synergistic antioxidant interaction was considered to be present when the observed antioxidant activities were significantly higher than the expected activities derived from individual samples ($p < 0.05$). In this analysis, Acai and the other antioxidant source mixtures were evaluated at a 1:1 ratio, which was determined on the basis of equivalent antioxidant activity. Based on this initial analysis, we found that Acai mixed with ascorbic acid and Acai with trolox had synergistic effects in the DPPH and ORAC assays, respectively (data not shown). On the other hand, Acai mixed with catechin and Acai with chlorogenic acid showed antagonistic effects in both assays. Thus, we utilized mixtures with ascorbic acid and trolox for additional experiments.

We next utilized response surface methodology (RSM) to evaluate the effect of varying ascorbic acid (X_1) and trolox (X_2) amounts on synergistic antioxidant activity in both ORAC (Y_1) and DPPH (Y_2) assays. Synergistic ORAC and DPPH effects, which were determined from 10 experimental runs generated with a central composite design, ranged from -6.63 to 15.64 $\mu\text{mol}/\text{TE}$ and from -0.94 to 3.63% , respectively (Table 1). The maximum synergistic activities in the ORAC and DPPH assays were observed in runs 5 and 8, respectively, and the maximum antagonistic activities in ORAC & DPPH assays were observed in runs 10 and 7, respectively. The mathematical relationship between synergistic antioxidant activity and antioxidant concentration of (X_i), obtained from the regression analysis using Eq. 2, was given by:

$$\begin{aligned} \text{Synergistic ORAC value } (\mu\text{mol}/\text{TE}, Y_1) \\ = -19.7783^{**} + 0.5314X_1^{***} + 0.9403X_2^{***} - 0.0018X_1X_2 \\ - 0.0035X_1^{2***} - 0.0010X_2^{2***} \end{aligned} \quad (3)$$

Table 1. Experimental values for difference activities determined from 10 experimental runs generated by a central composite design

Run Number	Coded variables ¹⁾		Process variables		Synergistic ORAC Value ($\mu\text{mol}/\text{TE}^{2)$	Synergistic DPPH radical scavenging effect (%)
	X_1	X_2	X_1	X_2		
1	-1	-1	30	22.5	8.2158	0.4659
2	1	-1	90	22.5	15.0624	3.2621
3	-1	1	30	67.5	6.8198	2.5741
4	1	1	90	67.5	8.7238	3.1774
5	0	0	60	45	15.6371	2.1433
6	0	0	60	45	15.4511	2.3785
7	-2	0	0	45	3.0483	-0.9439
8	2	0	120	45	4.0738	3.6390
9	0	-2	60	0	-1.4847	-0.6493
10	0	2	60	90	-6.6253	1.5555

¹⁾ X_1 =concentration of ascorbic acid ($\mu\text{g}/\text{mL}$), X_2 =concentration of trolox ($\mu\text{g}/\text{mL}$).

²⁾Trolox equivalents.

Fig. 1. Three-dimensional response surface showing synergistic effects in the ORAC (A) and DPPH (B) assays.

$$\begin{aligned} \text{Synergistic DPPH radical scavenging effect (\%, } Y_2) &= -5.8196^{***} + 0.1094X_1^{**} + 0.1629X_2^{**} - 0.00081X_1X_2 \\ &\quad - 0.0003X_1^2 - 0.0010X_2^2 \end{aligned} \quad (4)$$

where *** and ** indicate significance at $p < 1\%$ and $p < 5\%$, respectively. The analysis of variance of the regression model demonstrated that the models were highly significant and adequate (Y_1 : $p < 0.0073$, $R^2 = 0.9583$, Y_2 : $p < 0.0205$, $R^2 = 0.9290$). Therefore, this result indicated that the model could be used to predict synergistic ORAC and DPPH activities. The linear terms of ascorbic acid concentration (X_1) and trolox concentration (X_2) had the most significant effects on synergistic ORAC and DPPH activities; further, both effects were positive.

The predicted values of synergistic antioxidant activities, obtained from Eq. 3 and 4, were used to generate three-dimensional response surfaces. Figure 1 shows that the synergistic ORAC value increased with intermediate ascorbic acid and trolox concentrations. In addition, these results showed that the synergistic DPPH activity increased with increasing ascorbic acid and trolox concentrations. However, this tendency was not observed for ascorbic acid and trolox concentrations above 80 and 60 $\mu\text{g/mL}$, respectively. Together, these

results suggest that the interaction between ascorbic acid and trolox differs according to the concentration of each. Therefore, it is important to select an optimal antioxidant material ratio in order to achieve a maximum synergistic antioxidant effect.

The effects of ascorbic acid and trolox concentrations on synergistic ORAC and DPPH activities were slightly different. This finding was consistent with observations by Wang *et al.* (10), who demonstrated that the results of ORAC and DPPH assays varied with different adzuki and soy bean combinations. Importantly, these results were probably due to different mechanisms of the ORAC and DPPH assays. Specifically, the ORAC assay is a typical hydrogen atom transfer-based method that measures fluorescein degradation (27), whereas the DPPH assay is a colorimetric assay measuring electron transfer activity (28).

In order to obtain an optimal mixing ratio of ascorbic acid and trolox that would achieve maximum synergistic activity in both the ORAC and DPPH assays, we superimposed the contour plots generated by Eq. 3 and 4. The maximum synergistic activity for both ORAC (Y_1) and DPPH (Y_2) assays was obtained when the ascorbic acid (X_1) and trolox (X_2) ratio was 1.56:1 (X_1 : 74.64 $\mu\text{g/mL}$, X_2 : 47.88 $\mu\text{g/mL}$). The maximum Y_1 and Y_2 values that were predicted using Eq. 3 and 4 for the optimized conditions described above were 16.06 $\mu\text{mol/TE}$ and 3.17%, respectively. The observed Y_1 and Y_2 values for the optimized ascorbic acid and trolox mixture were 17.21 ± 5.10 $\mu\text{mol/TE}$ and $3.29 \pm 1.87\%$, respectively, which corresponded to a 13% and 6% increase, respectively, in relation to the calculated sum of activities of ascorbic acid and trolox. Thus, the experimental values for Y_1 and Y_2 were in close agreement with the predicted values and were within one standard deviation.

Effect of the mixing ratio of Acai, ascorbic acid, and trolox on synergistic antioxidant activity

To evaluate the synergistic antioxidant activity of Acai with the ascorbic acid and trolox mixture of ascorbic acid and trolox, we measured antioxidant activity with varying mixing ratios of Acai and combinations of ascorbic acid and trolox (data not shown). The highest synergistic effects for both the ORAC and DPPH assays were found with a 100:10 (w:w) mixing ratio. As the antioxidant combination ratio increased from 100:10 to 100:25, the synergistic antioxidant activity significantly decreased. Under these conditions, synergistic antioxidant activities in the ORAC and DPPH assays were 17.45 $\mu\text{mol/TE}$ and 6.12%, respectively. Based on these results, a 100:10 mixture was selected for NP formation.

Physical properties of nanoparticles Chitosan-gum arabic NPs entrapping Acai alone (Acai NP) and the optimized concentrations of Acai, ascorbic acid, and trolox (mixed NP) were formed by ionic gelation between the positive chitosan charge and the negative gum arabic charge. The average size and PDI of the NPs are shown in Table 2. As the gum arabic concentration increased, the size of the mixed NPs and Acai NPs decreased significantly ($p < 0.05$). This result can be explained by the chitosan NP cross-linking at higher gum arabic

Table 2. Physicochemical properties of nanoparticles

Nanoparticle	Core material	Wall material		Particle properties	
		Chitosan (mg/mL)	Gum arabic (mg/mL)	Particle size (nm)	Polydispersity index
Mixed NP 0.5	Mix ¹⁾	0.5	0.5	263.4±15.5 ²⁾	0.28±0.01 ^a
Mixed NP 1		0.5	1.0	249.0±16.8 ^b	0.21±0.02 ^c
Acai NP 0.5	Acai berry concentrate	0.5	0.5	242.9±11.4 ^c	0.29±0.01 ^a
Acai NP 1		0.5	1.0	234.1±12.0 ^d	0.23±0.02 ^b

¹⁾Mixture of acai berry concentrate, ascorbic acid, and trolox at the optimal ratio.

²⁾Different letters indicate significant differences ($p<0.05$).

concentrations, induced by higher electro-static interactions, resulting in a more compact NP structure. The size of Acai NPs without addition of ascorbic acid and trolox ranged from 220 to 262 nm. On the other hand, size of the mixed NPs ranged from 230 to 282 nm. Thus, Acai NP size was significantly smaller than the mixed NPs ($p<0.05$). This result may have been due to loading of ascorbic acid and trolox on Acai NPs. Avadi *et al.* (24) also reported that chitosan-gum arabic NP size increased with increasing core material concentration.

In addition, PDI represents particle size distribution (29) and ranges from 0 to 1, where values close to 0 represent a homogeneous dispersion and those above 0.3 indicate high heterogeneity (30). Thus, our results showed that both the Acai NPs and the mixed NPs were homogeneously dispersed, regardless of chitosan and gum arabic concentrations.

Effects of pH and storage on nanoparticle size To evaluate the effects of pH and storage on NP particle size, the pH levels of the nanodispersions were left as prepared (pH 3.7±0.1) or adjusted to pH 4.0 or 5.5. Mixed and Acai NPs were then stored for four days under accelerated conditions, and NP particle size was investigated daily (Fig. 2). The initial size of both Acai NPs and mixed NPs decreased and the derived count rate increased as the solution pH increased. These results were consistent with those reported by López-León *et al.* (31).

Due to an amino group presence on chitosan, the structure of chitosan NPs can be easily changed by varying environmental conditions such as ionic strength and pH (32). For example, in an acidic medium, the amino group has a positive charge, leading to a highly-charged polysaccharide (33). Therefore, chitosan tends to extend due to greater protonation at lower pH, but shrinks under more alkaline conditions (32). Thus, the ionic gelation progress for the formation of chitosan-gum arabic NPs is pH-responsive and can also impact the physicochemical properties.

Changes in the size of the pH-adjusted NP were measured during storage. Most NPs appeared to be stable, and NP size tended to become smaller with increasing storage time, although the size and PDI of mixed NP1 and Acai NP1 increased considerably at pH 5.5 after three days. These data also indicate that NPs prepared with 1 mg/mL of gum arabic are unstable compared to NPs prepared with 0.5 mg/mL of gum arabic. Together, these results suggest that the physical properties of NPs are influenced by pH, and that NPs can

Fig. 2. Nanoparticle size during storage (60°C, original (A), pH 4 (B), and pH 5.5 (C)). Different lower case letters indicate significant differences within samples with the same storage time; different capital letters indicate significant differences as a function of storage time ($p<0.05$).

become unstable in pH-neutral conditions. Thus, it is important to select a suitable wall material concentration for preparing NPs in accordance with the desired pH level.

Fig. 3. ORAC values of nanoparticles during storage (60°C, original (A), pH 4 (B), and pH 5.5 (C)). Different letters indicate significant differences ($p < 0.05$).

pH and storage effects on antioxidant activity NP antioxidant activity was measured to determine pH and storage effects on NP stability (Fig. 3 and 4). We found that the initial antioxidant activities of free compound and NPs decrease significantly with increasing pH level in both ORAC and DPPH assays. The result might be attributed to main active ingredient degradation due to changes in pH ($p < 0.05$). Specifically, phenolic compounds such as those found in Acai, which are the main active ingredients, are easily affected by changes in pH; while they are relatively stable under acidic conditions, they are

Fig. 4. DPPH radical scavenging effect of nanoparticles during storage (60°C, original (A), pH 4 (B), and pH 5.5 (C)). Different letters indicate significant differences ($p < 0.05$).

considerably unstable under alkaline conditions. In this way, phenolic compounds can shift to different chemical forms as a function of pH level, a phenomenon that impacts both their stability and antioxidant activity (34). Xiong *et al.* (35) also evaluated pH effects on the antioxidant activities of black currant anthocyanins, reporting that activity decreased with anthocyanin degradation, resulting in a 30% loss of initial antioxidant activity.

The effects of pH and storage on NP antioxidant stability showed similar patterns in both the ORAC and DPPH assays, regardless of wall material concentration. Antioxidant activities for all samples decreased with increasing storage time. Specifically, non-nanoencapsulated free antioxidant activity exhibited the most dramatic decrease. The antioxidant activities of the nanoencapsulated Acai

and the mixture of Acai, ascorbic acid, and trolox showed significant differences after storage for one day compared to free antioxidants, which were stable up to two days ($p < 0.05$). The antioxidant activities of non-nanoencapsulated free antioxidants were 60 and 75% of those of nanoencapsulated antioxidants after storage for 4 days, as determined using ORAC and DPPH assays. This result was consistent with those reported by Lee *et al.* (13). These data indicate that entrapped antioxidants are protected from the oxidative environment by nanoencapsulation.

As seen from the antioxidant activities in Fig. 3 and 4, Acai NPs prepared with a 1 mg/mL gum arabic concentration rapidly lost their antioxidant activity in pH 5.5 conditions, a result attributed to the physical properties of the NPs. At pH 5.5, the physical properties of the Acai NPs were unstable, exhibiting increasing size with longer storage duration (Fig. 2). Therefore, the physical entrapment of antioxidants within NPs was effective for protecting antioxidant activity from oxidative environments, and the stabilities of the encapsulated antioxidants can be influenced by the physical properties of the NPs. These results indicate that the physical entrapment of Acai as well as an optimized ascorbic acid and trolox combination with chitosan/gum arabic NP could be effective for protecting the antioxidant activities of acai berries.

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