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Gamma-irradiation Reduces Allergenicity of Cochineal Extracts

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Abstract - Cochineal extract and carmine are natural red dyes, widely used in the food, cosmetics, and pharmaceutical industries. However, since they are extracted from the female cochineal insects, they are also prone to inducing food allergies such as anaphylaxis and asthma. In this study, cochineal extract solutions were subjected to multiple gamma-irradiation dosages (0, 10, 30, and 50 kGy), and measured the quantitative changes in carminic acid and allergenicity as well as the Hunter color values changes. The carminic acid level of gamma-irradiated cochineal extracts were significantly reduced at higher irradiation dosages. The allergenicity of cochineal extracts was evaluated through a competitive ELISA cross-reacting with a polyclonal antibody to carmine. Gamma-irradiation significantly reduced the allergenicity of cochineal extract as the irradiation dose increased. Additionally, the Hunter color values of gamma-irradiated cochineal extract were reduced, which resulted in darker red color. In conclusion, gamma-irradiation can reduce the allergenicity of cochineal extracts with improving its color.

Key words : Cochineal extract, Carminic acid, Gamma-irradiation, Allergenicity, Color

INTRODUCTION

Cochineal extract is one of the most widely used natural red dyes obtained from dried carcasses of the female cochineal insects (*Dactylopius coccus* Costa), native to Central America. Cochineal extract is a water-soluble food colorant and was used extensively in cosmetics and pharmaceutical industries before the advent of synthetic coloring agents (Stathopoulou *et al*. 2013). Carminic acid, the key coloring ingredient of the cochineal extract consists of an anthraquinone structure linked to a glucose moiety that produces orange, red and purple tones (Lim *et al*. 2014).

Carmine (or Carmine Lake) is a water-insoluble form of carminic acid formed by a metal chelation reaction facilitated by aluminum or calcium ions (Lim *et al*. 2014). The metal chelation imparts the red color to carmine. However, carmine in foods is able to cause initiate allergic reactions in some individuals. Immediate IgE-mediated hypersensitivity reactions (i.e., urticaria, angio-oedema, anaphylaxis and asthma) have been reported to occur following oral exposure to carmine (Beaudouin *et al*. 1995; Wüthrich *et al*. 1997; Acero *et al*. 1998; DiCello *et al*. 1999; Ferrer *et al*. 2005). In many cases, positive skin prick test reactions to carmine seem to occur as immunologic cross-reactions concurrent with reactions to house dust and/or storage mites (Baldwin *et al*. 1997; Liippo and Lammintausta 2009). The acceptable daily intake value of carmine, as stipulated by the Joint FAO/WHO Expert Committee on Food Additives, is 5 $mg \cdot kg^{-1}$ based on bodyweight (JECFA 2001).

Carmine, also called carmine red, E120, is authorized for use in Korea, the United States (FDA 2018), and the Europe-

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an Union (EU 1994) but not in Japan. Carmine and cochineal extract permitted in Korea are prohibited for use in the following food items: Natural foods (e.g., meat, seafood, fruits, vegetables, and their simple-processed derivatives); tea and coffee; red pepper powder; kimchi; and spice products. The permissible level is not established in the Korea Food Additive Code (Lim *et al*. 2014). Furthermore, carmine could be used in food products imported to the Republic of Korea as a non-permitted color additive or illegally added food products to improve the appearance and color by manufactures. Food products may also be labelled as containing cochineal extracts where in reality carmine has been used (Lim *et al*. 2014). Therefore, techniques that render the color of carmine to be more reddish without using the 'lake' method and/or to reduce its allergenicity, may help address the hypersensitivity observed in some consumers. However, since the cochineal extract was demonstrated to be stable to heat, light and oxygen (FAO 2018), other application techniques might need to change the molecules in the cochineal extract.

Gamma-irradiation has recently become one of the best techniques to preserve food with minimal harm to its nutritional and sensory properties (Farkas 2006). Besides the traditional use for sterilization purposes, newer applications of reducing the toxicants or hazardous compounds have been reported with respect to N-nitrosamines (Ahn *et al*. 2002a; Ahn *et al*. 2002b), biogenic amines (Kim *et al*. 2003), and allergenicity of foods (Seo *et al*. 2007; Zhenxing *et al*. 2007; Tammineedi *et al*. 2013). Therefore, the objective of this study was to evaluate the effects of gamma-irradiation on the carminic acid's content, allergenicity and color changes.

MATERIALS AND METHODS

1. Chemicals and Antibodies

A cochineal extract was obtained from ES Food (Gunpo-Si, Kyungki-Do, Korea) and the carminic acid and carmine standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The bovine serum albumin (BSA) conjugated the carmine and polyclonal antibody to carmine was purchased from Cloud-Clone Corp. (Houston, TX, USA).

2. Preparation of Gamma-irradiated Cochineal Extract Solutions

Cochineal extract solutions (100 $mg \cdot mL^{-1}$) were dissolved in deionized distilled water (ddH_2O) and 1 mL samples were tightly capped in Eppendorf tubes and irradiated at 0, 10, 30, and 50 kGy in a ^{60}Co gamma-irradiator (point source AECL, IR-79, MDS Nordion International Co. Ltd., Ottawa, ON, Canada) at the Advanced Radiation Technology Institute, Jeongeup, Korea. The source strength was approximately 8.8 PBq and the dose rate was 10 $kGy \cdot h^{-1}$. The dosimeter was calibrated using the International Atomic Energy Agency (Vienna, Austria) standard. After irradiation, all samples were immediately stored in a refrigerator until analysis.

3. High Performance Liquid Chromatography (HPLC) Analysis

Quantitative changes of carminic acid in the gamma-irradiated cochineal extract (10 $mg \cdot mL^{-1}$) were conducted using an Agilent HPLC 1200 series (Santa Clara, CA, USA) coupled to a photodiode array detector (DAD). All samples were filtered through a membrane filter for HPLC analysis. Chromatographic separation was accomplished with an Eclipse Plus C_{18} column (250 × 4.6 mm, 5 μm, Agilent Technologies, Santa Clara, CA, USA). The injection volume was 20 μL and the wavelength of the DAD was 254 nm. The mobile phase was 0.1% formic acid water (solvent A) and acetonitrile (solvent B). A linear gradient was one that followed at 0 min, A/B = 95/5; at 0~5 min, 80/20; and at 5~24 min, 60/40 with a flow rate of 1 $mL \cdot min^{-1}$. Under these conditions, carminic acid was eluted at 9.2 min.

4. Competitive Inhibition Enzyme-linked Immunosorbent (Ci-ELISA) Assays

Ci-ELISA was performed to determine the changes in the carmine-binding abilities of the gamma-irradiated cochineal extracts solutions. Briefly, polystyrene flat-bottom microtiter plates (Maxisorp, Nunc, Kamstrup, Denmark) were coated with 50 μL of a native BSA conjugated carmine solution (10 $\mu g \cdot mL^{-1}$) in a phosphate buffered saline (PBS), overnight at 4 ± 1°C. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST). To reduce the nonspecific binding, the plates were blocked by incubation for 2 h with 200 μL per well of 2% BSA at 37°C. After a single wash step, 50 μL of a gamma-irradiated cochineal extract solution, serially diluted (50, 25, 12.5, 6.25 $mg \cdot mL^{-1}$) in PBS, was added followed by the addition of 50 μL polyclonal anti-carmine to a final dilution

of 1 : 30000. The plates were incubated for 1 h, and then washed three times with PBST. A 100 μL of goat anti-rabbit IgG solution at a dilution of 1 : 2000 was added to the well, and the plates were incubated for 0.5 h. The wells were then washed, and 50 μL of (3,3′,5,5′-tetramethylbenzidine was added to the color reaction for 30 min before stopping the reaction with 0.1 N HCl (50 $\mu L \cdot well^{-1}$). The absorbance was measured at 450 nm with an ELISA reader.

Absorbance (A_{50}, A_{25}, $A_{12.5}$ and $A_{6.25}$) values were collected at each concentration (50, 25, 12.5, 6.25 $mg \cdot mL^{-1}$). The percentage binding capacity of the proteins attached to the plate was calculated by dividing the absorbance values at each concentration (for example, A_{50}) with the absorbance at 2% BSA (A_{BSA}). The percentage binding of the protein at 50 $mg \cdot mL^{-1}$ concentration was calculated as (A_{50} / A_{BSA}) × 100. A graph was plotted with percentage binding values (4 concentrations = 4 data points = one line) for each irradiation dose. The slope for each line was calculated, and the percentage change in slope was calculated, to indicate the percentage reduction in allergenicity.

5. Color Changes Measurement

The cochineal extract dissolved in ddH_2O (100 $mg \cdot mL^{-1}$) was transferred into a glass cell (CM A-98, 10 mm in width) and measured with a color difference meter (Spectrophotometer CM-5, Konica Minolta, Tokyo, Japan). The instrument was calibrated to standard black and white tiles before analysis. A large-size aperture was used and the measurements were repeated more than three times with consistent results.

6. Statistical Analysis

Statistical significance was calculated using Student's two-tailed *t*-test within the Statistical Package for Social Sciences (SPSS, 22.0 IBM, Armonk, NY). The results were expressed as the mean ± SD and the differences between the means were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

1. Quantitative Changes of Carminic Acid in Cochineal Extract Solution by Gamma-irradiation

The quantitative changes of carminic acid in cochineal extract solutions after gamma-irradiation (0, 10, 30, and 50 kGy) were detected by LC-DAD (Fig. 1(a)). In non-irradiated cochineal extract (10 $mg \cdot mL^{-1}$), the concentration of carminic acid was identified as 694.25 ± 2.2 $\mu g \cdot mL^{-1}$, which indicates the purity of the cochineal extract was about 7%. The reduction of carminic acid was significantly observed in the samples gamma-irradiated with more than 10 kGy ($p < 0.05$). The maximum irradiation dose (50 kGy) decreased the carminic acid concentration to 429.50 ± 2.9 $\mu g \cdot mL^{-1}$, which implies that 38.13% of carminic acid was broken down by gamma-irradiation compared to the non-irradiated control. Previous studies on pigment degradation by gamma-irradiation have reported 65.5% of curcumin (from *curcuma longa* and *curcuma aromatica* extracts dissolved in ethanol) degradation by 30 kGy gamma-irradiation (Kim *et al*. 2006). In addition, 92.6% of cyanidin-3-*O*-glucoside and 92.8% of cyanidin-3-*O*- (6''-malonyl-) glucoside (from centipede grass extract dissolved in methanol) was degraded by 20 kGy gamma-irradiation (Lee *et al*. 2013).

The decrease in the carminic acid peak (retention time t_R = 9.27 min) was concomitant with the appearance of several new peaks (Fig. 1(b)) from 10 kGy gamma-irradiation. Gamma-irradiated cochineal extracts were transformed into several major radiolytic products (t_R = 8.20 min (A); t_R = 8.87 min (B); t_R = 9.02 min (C)). Other minor products were also detected (data not shown). Jung *et al*. (2009) and Byun *et al*. (2014) showed that over 30 kGy gamma-irradiation to genistein resulted in two major and novel radiolysis products among several other known radiolysis products. These products were identified as new compounds. We also observed that three major radiolytic products (A, B and C) gradually increased with gamma-irradiation dose increase (Fig. 1(b)). Further studies are necessary to identify these radiolytic products.

2. Allergenicity Changes of Cochineal Extract Solution by Gamma-irradiation

The allergenicity changes of gamma-irradiated cochineal extracts were determined by competitive inhibition ELISA, which cross-reacted a polyclonal antibody to carmine. In this assay, the degree of allergenicity was measured on the basis of inhibiting the carmine antibody (Ab) binding to the BSA-conjugated carmine-coated plate by competiting with cochineal extract solutions. In Table 1, a lower % value of carmine binding capacity indicates that the test samples

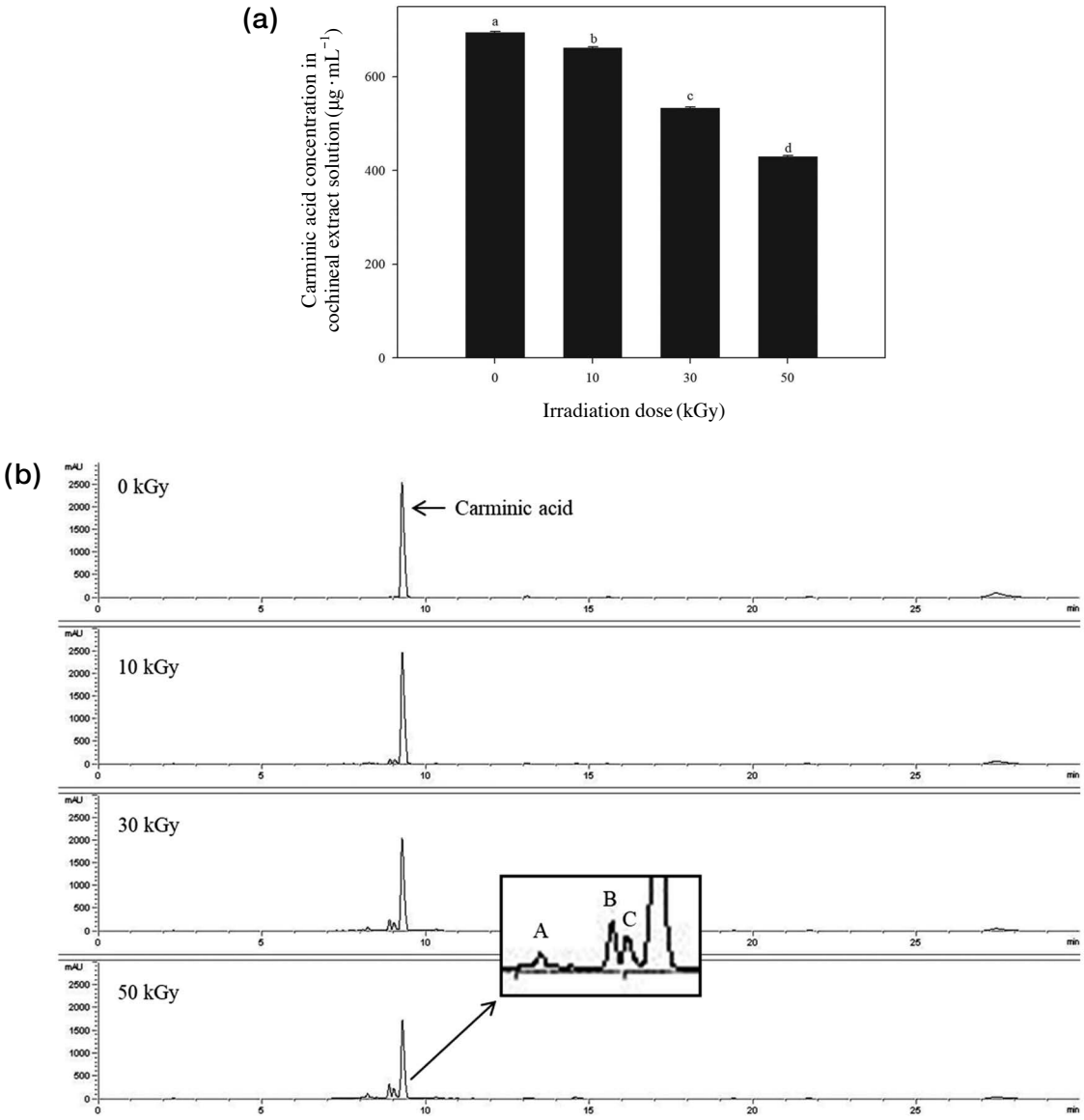

Fig. 1. Effect of gamma-irradiation on carminic acid contents in cochineal extract solutions (10 mg · mL^{-1}). Letters (a-d) are significantly different at $p < 0.05$. Standard error of the mean (SEM, n = 3); (a) Quantitative changes of carminic acid in cochineal extract solutions, (b) HPLC chromatograms of gamma-irradiated cochineal extract solutions.

(non-irradiated cochineal extract solution) and carmine Ab competed to bind with BSA-conjugated carmine. However, gamma-irradiated cochineal extract solutions (50 mg · mL^{-1}) showed a higher % value of carmine binding capacity than the non-irradiated cochineal extract, which implied that the carmine antibody was less likely to recognize the gamma-irradiated cochineal extract than the BSA conjugated carmine. Therefore, gamma-irradiation reduced the allergenicity of the cochineal extract.

The reduction of the binding capacity (implying reduction in the allergenicity) of the carmine Ab to the BSA conjugated carmine was indicated by an increase in the slope of the lines. From our results, the slopes of the curves increased with dose increase, which indicates a reduction of the allergenicity of cochineal extract solutions (Fig. 2). As shown in Table 1, the slope of lines (m) increased as the gamma-irradiation dosage increased. Moreover, based on the percentage change in slope, the reduction of the carmine binding capacity after 10 kGy gamma-irradiation was 89% and it also increased as the irradiation dosage increased. From these results, we concluded that a higher gamma-irradiation dosage led to a greater reduction in allergenicity.

Table 1. Ci-ELISA results showing percent binding capacity of cochineal extract attached to ELISA plate, slope of respective irradiation dose curves, and % change in slope. The % change in slope indicates percent reduction in allergenicity

Irradiation dose (kGy)	% Binding capacity at 50 mg · mL^{-1} concentration[1)]	Slope of curves (m)	% change in slope
0	71.63 ± 2.03^{d}	−9.6271	0
10	87.91 ± 0.19^{c}	−1.0782	89.0
30	95.68 ± 2.57^{b}	1.0296	90.6
50	104.90 ± 2.42^{a}	2.8855	92.0

[1)]Standard error of the mean (n = 3).
[a-d]Means values with different superscripts within column are significantly different ($p < 0.05$).

Table 2. Hunter color values changes of cochineal extract solution (100 mg · mL^{-1} in ddH_2O) by gamma-irradiation[1)]

Irradiation dose (kGy)	Hunter color values			ΔE[2)]
	L*-value	a*-value	b*-value	
0	29.76 ± 0.01^{a}	64.14 ± 0.01^{a}	51.31 ± 0.02^{a}	
10	26.91 ± 0.01^{b}	61.32 ± 0.01^{b}	46.40 ± 0.01^{b}	6.34 ± 0.01
30	23.34 ± 0.02^{c}	57.12 ± 0.02^{c}	40.24 ± 0.04^{c}	14.60 ± 0.02
50	20.26 ± 0.28^{d}	52.48 ± 0.55^{d}	33.90 ± 0.97^{d}	23.01 ± 0.92

[1)]Standard error of the mean (n = 3).
[2)]Overall color difference $((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$
[a-d]Means values with different superscripts within columns are significantly different ($p < 0.05$).

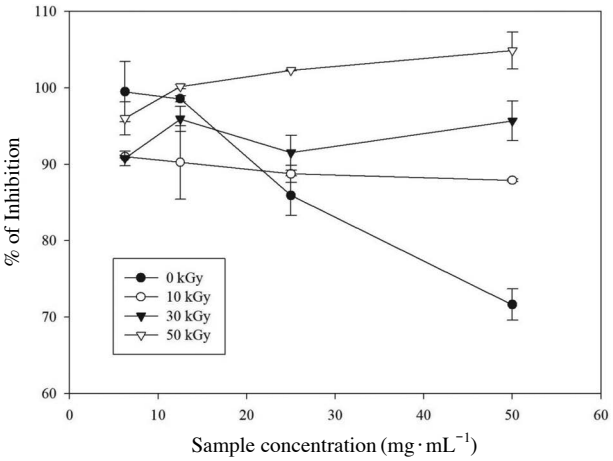

Fig. 2. Effect of gamma-irradiation on carmine binding capacity in cochineal extract solutions using Ci-ELISA.

3. Color Changes of Cochineal Extract Solution by Gamma-irradiation

Although gamma-irradiation reduces the allergenicity of cochineal extracts, the food manufacturing process requires a minimal loss of original color during the irradiation process. Therefore, it is a critical issue to confirm that the color changes of gamma-irradiated cochineal extracts do not result in losing value as a pigment.

The Hunter color value changes of cochineal extract solutions by gamma-irradiation (0, 10, 30, and 50 kGy) are shown in Table 2. The Hunter color L*-value (brightness), a*-value (redness) and b*-value (yellowness) was found to decrease significantly as the gamma-irradiation dose increased, which inferred the cochineal extract solutions got darker after gamma-irradiation. The overall color difference (ΔE) also showed dramatic differences between the non-irradiated and gamma-irradiated samples. The color change of the cochineal extract solutions can be ascertained from Fig. 3. The degrading effect of gamma-irradiation on various pigments have already been reported in green-tea leaves (Jo *et al*. 2003), curcumin (Kim *et al*. 2006) and centipede grass (Lee *et al*. 2013) without adverse changes in biological activities. The degradation of the pigments may be caused by the reaction with reactive molecular species and free radicals such as hydroxyl radical (OH·), hydrogen radical (H·), superoxide anion radical ($O_2^{\cdot -}$), peroxyl radical (HOO·), and hydrogen electrons (e_{aq}^-) released from water during the irradiation process. Although the color changing mechanism of pigments by gamma-irradiation is not elucidatted in detail, our results suggest that the free radicals are capable of crosslinking the chemical bonds of carminic acid, resulting in "lake"-like effect.

In the above study, we identified that gamma-irradiation

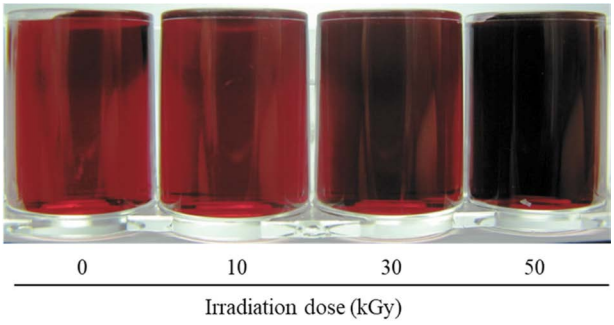

Fig. 3. Color changes of cochineal extract solution (100 mg · mL^{-1}) by gamma-irradiation.

could induce darkening of cochineal extract solutions along with significant reducing of allergenicity. Many studies have reported that irradiation could inhibit the allergenicity of ovalbumin (OVA) (Byun *et al*. 2014), shrimp allergen (Zhenxing *et al*. 2007) and α-casein (Tammineedi *et al*. 2013) by alteration of conformational epitope structures of allergen, which induced by radicals formed during irradiation. In our results, transformation of carminic acid structure by gamma-irradiation (Fig. 1) might induce the reduction in allergenicity. We also observed that higher irradiation dosages resulted in a darker cochineal extract solution, which can be an improvement because this implies a smaller amount of dye may be used to achieve the same red color as before.

CONCLUSION

We conclude that that the above irradiation technique may offer a superior alternative to the food industry, cosmetic industry, pharmaceutical industry, as well as textile industry, which can reduce the amount of pigment usage to achieve same red color of products without allergenicity.

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