A Dissertation for the Degree of Doctor of Philosophy

Elucidation for mechanism of discoloration of meat induced by cold atmospheric pressure plasma and its application

저온 대기압 플라즈마 처리에 의한 식육의 변색 메커니즘 규명 및 활용

February, 2018

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Overall Summary

Elucidation for mechanism of discoloration of meat induced by cold atmospheric pressure plasma and its application

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The objective of present experiments were 1) to investigate the bactericidal effect of cold atmospheric pressure plasma (APP) on raw chicken meat and to observe quality changes and genotoxicological safety after the APP treatment, 2) to elucidate the mechanism and control the APP-induced green discoloration of raw meat using myoglobin, 3) to confirm the possibility of red color development in pork by APP treatment and to evaluate the physiochemical properties and microbiological safety of pork jerky made with APP as a substitute method of sodium nitrite, and 4) to compare the quality of injected loin ham cured with sodium nitrite and plasma-treated water (PTW).

Experiment I. Evaluation of the microbiological safety, quality changes, and genotoxicity of chicken breast treated with flexible thin-layer atmospheric pressure plasma

The microbiological safety, changes in quality, and genotoxicity of raw chicken breasts treated with flexible thin-layer atmospheric pressure plasma (APP) were investigated. Following 10-min APP treatment, the numbers of *Listeria monocytogenes, Escherichia coli,* and *Salmonella* Typhimurium were reduced by 2.14, 2.73, and 2.71 Log CFU/g, respectively. L^* (lightness) and b^* values (yellowness) increased whereas a^* value (redness) decreased following plasma treatment with increasing exposure duration. Lipid oxidation was unaffected by APP treatment. There was also no significant difference in the texture properties between the APP-treated sample and non-treated control. No genotoxicity was detected in APP-treated chicken breast using the *Salmonella* mutagenicity assay. Therefore, it can be concluded that APP is applicable since it is able to improve microbiological safety with minimal changes in color properties of the chicken breast.

Experiment II. Elucidation of the mechanism of APP-inducted green discoloration of myoglobin

Meat color is an important factor that influences product acceptability by consumers. The most responsible factor for meat color is a myoglobin. Thus, the aim of this experiment was to elucidate the mechanism and control measure of APP-induced green discoloration of myoglobin. Generally, it is known that green-colored pigments derived from myoglobin are in the forms as sulfmyoglobin, choleglobin, verdoheme, nitrihemin or nitrimyoglobin. When myoglobin dissolved in phosphate buffer was exposed to APP for 20 min, L^* and

a^* values were significantly decreased whereas b^* value was increased. In the UV absorption spectrum, APP-treated myoglobin showed absorption peak at 503 and 630 nm, which is not a spectrum of sulfmyoglobin or choleglobin. With evidences that the secondary structure and molecular weight of myoglobin were not changed by APP treatment, we excluded the possibility of the form of verdoheme or nitrihemin in APP-treated myoglobin solution. Nitrite, hydrogen peroxide, and hydroxyl radical were produced in myoglobin solution by APP treatment, which provide a positive environment that nitrimyoglobin could be formed. When 0.1% sodium dithionite, a strong reducing agent, was added to myoglobin solution, green discoloration was slightly prevented after APP treatment. Moreover, addition of 0.5% sodium dithionite in myoglobin solution induced red color, not green color, after APP treatment for 20 min. Red color development in myoglobin was resulted in nitrosomyoglobin formation. Consequently, occurrence of green color in APP-treated myoglobin is due to ntrimyoglobin formation. Addition of sodium dithionite prevents green discoloration and induced red color in myoglobin solution after APP treatment.

Experiment III. Color development, physiochemical properties, and microbiological safety of pork jerky manufactured with APP

Generally, synthetic nitrite including sodium nitrite is used in the production of cured meat products. Nitrites added in meat products reduced to nitric oxide then interact with myoglobin to produce nitrosomyoglobin, which is responsible for characteristic red color of cured meat. Nitrite can also inhibit the lipid oxidation and growth of food-borne pathogens. However, increasing number of consumers avoids synthetic additives for their cured meat products. In persent experiment, possibility of red color development in pork was confirmed with addition of

ascorbic acid and APP treatment. Then, applicability of APP as an alternative to sodium nitrite in pork jerky manufacturing was investigated. Pork marinated with or without sodium nitrite was prepared, and the latter was exposed to APP. As APP-treatment time increased, the a^* value, nitrosoheme pigment content, and residual nitrite content increased, while lipid oxidation decreased (all $P<0.05$). Similar quality properties, particularly color, were observed in jerky applied by APP for 40 min compared to jerky made with sodium nitrite. After inoculation of marinated pork with *Staphylococcus aureus* and *Bacillus cereus*, the amounts of both pathogens in jerky applied by APP for 40 and 60 min were significantly lower than in jerky made with sodium nitrite. Consequently, APP can be applied for manufacturing even potentially safer pork jerky without added sodium nitrite.

Experiment IV. An innovative curing process with plasma-treated water for production of loin ham and for its quality and safety

To extend industrial utilization of APP, plasma treated water (PTW) was investigated as an alternative to synthetic sodium nitrite in loin ham manufacturing. Loin ham is a cured meat product manufactured by injecting brine into raw meat (loin) without chopping, mixing or emulsifying process. For curing of loin ham, two brine solutions made of sodium nitrite were compared against PTW. In comparison to chemical brining PTW resulted in increased a^* value, while allowing to maintain low residual nitrite content and total bacterial counts. No significant differences were found in b^* and L^* value, and lipid oxidation among the treatment groups. Furthermore, the loin ham manufactured using PTW showed no genotoxicity by *Salmonella* mutagenicity assay. Therefore, PTW could be considered as an effective and innovative substitute for synthetic nitrite in cured meat manufacturing without compromising on quality changes.

Keywords: Atmospheric pressure plasma, Bactericidal effect, color, Discoloration, Food-borne pathogen, Meat product, Myoglobin, Nitrimyoglobin, Nitrosomyoglobin, Nitrite

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List of Abbreviations

2-AA	:	2-Aminoanthracene.
4-NQO	:	4-Nitroquinoline-1-oxide
APP	:	Atmospheric pressure plasma
CD	:	Circular dichroism
DBD	:	Dielectric barrier discharge
DDW	:	Deionized distilled water
DW	:	Distilled water
ESI-MS	:	Electrospray ionization-mass
EtOH	:	Ethanol
POV	:	Peroxide value
PTW	:	Plasma treated water
SA	:	Sodium azide
TBARS	:	2-thiobarbituric acid reactive substances.
UV	:	Ultra-violet

CHAPTER I.

General introduction

In these days, consumers have been demanding healthy and safe food, which are existed as natural state as possible. Accordingly, food industry requires a processing technology to produce food labeled “minimal processing", "no preservatives", or "no synthetic additives” while ensuring microbial safety by controlling pathogenic bacteria such as *Salmonella* Typhimurium or *Listeria monocytogenes* (Augustin et al., 2016; Misra & Jo, 2017; Ohlsson, 1994).

Minimal processing technology is a method to prolong shelf life by inactivating food poisoning and spoilage bacteria with the least treatment. Traditional minimal processing technology involves physical methods such as heating, drying, and freezing, as well as chemical methods such as ethylene oxide. However, physical methods induce side-effects in the sensory, nutritional, and functional properties of food. In addition, consumers are unwilling to buy a food treated with chemical methods due to the possibility of chemical residue (Kim et al., 2011; Lee et al., 2017; Moisan et al., 2001). To overcome disadvantages of traditional methods, various non-thermal processing technologies were developed including gamma-irradiation, electron beam-irradiation, and high-pressure processing. These technologies have non-thermal advantages, but also have disadvantages such as expensive construction and operation cost, long processing

times, and requirement for specialized equipment and expert (Brendan & Joseph, 2008; Yun et al., 2010).

Recently, atmospheric pressure plasma (APP) attracts a great deal of attentions as an innovative non-thermal processing technology (Misra et al., 2016; Yong et al., 2014). The term “plasma” was first coined by Tonks and Langmuir (1929) about 70 years ago. Plasma is the fourth state of matter after solid, liquid, and gas as shown in Fig 1. It is well known that 99.99% of all observable matter in the universe is in the plasma state (Pan et al., 2010). In the industry, plasma is usually generated by subjecting a gas (or gases) to steady electric fields. In principle, gas ions are produced by liberating electrons from the gas molecules when the electric field is sufficiently strong. Then, the free electrons accelerated by the electric field and may conflict with other gas molecules. More gas ions and free electrons may be produced and it conflict with further gas molecules and so on. Consequentially, fully or partially ionized gas is generated which called plasma (Balzer et al., 2015; Moisan et al., 2001; Pan et al., 2010).

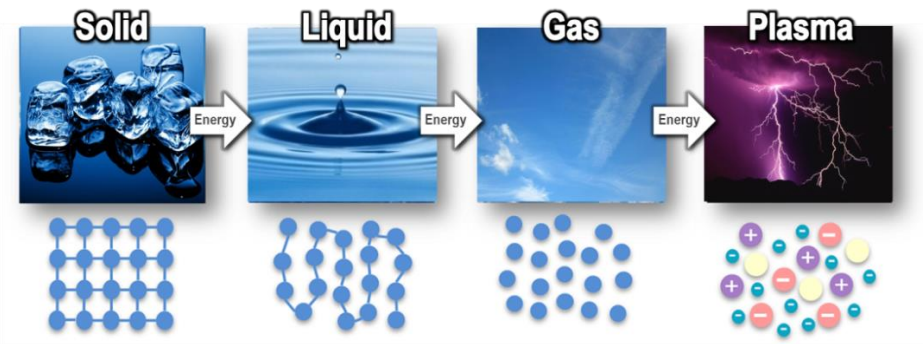

Fig. 1. Plasma called the fourth state of matter (solid-liquid-gas-plasma)

Plasma can be divided into two types based on the operating pressure; low-pressure or atmospheric pressure. When plasma application is carried out in atmospheric pressure, it is called APP (Lee et al., 2017; Misra & Jo, 2017). APP has its advantageous features; (i) bactericidal, virucidal, and fungicidal properties; (ii) high concentration of reactive species, (iii) controllable gas temperature; (iv) inexpensive operation costs compared to other technologies; (v) flexible operation system (Mariotti & Sankaran, 2010; Moisan et al., 2001). Since 2000, APP has been studied in the field of animal-origin food including meat and meat products (Lee et al., 2017; Misra et al., 2016).

In the early stages of APP studies, carrier gas was generally applied to the APP system. According to Rød et al. (2012), ready-to-eat meat product (bresaola) inoculated with *Listeria innocua* was treated with APP using mixture of oxygen and argon gas, and which resulted in a bacterial reduction by 1.6 log CFU/g. With APP treatment using nitrogen and oxygen gas, the number of *Escherichia coli* inoculated on the raw chicken breast was reduced by 1.6 log CFU/g (Yong et al., 2014). Noriega et al. (2011) also reported a 3.30 Log CFU/g reduction in *L. innocua* inoculated on chicken breast after APP treatment using oxygen and helium gas. Additionally, when raw pork loin was exposed to APP which use the helium and mixture of helium and oxygen, the population of *E. coli* was reduced by 0.34 and 0.55 Log CFU/g following a 10 min treatment, respectively (Kim et al., 2013).

In the industry, economical cost of processing technology is important issue. Thus, the operating cost of the carrier gas has to be considered in order to adopt the APP technology in the food industry. An ideal gas for operating APP would be

ambient air (Misra et al., 2014; Yong et al., 2017a). Jayasena et al. (2015) reported 2.04, 2.54, and 2.68 Log CFU/g reductions in *L. monocytogenes*, *E. coli*, and *S.* Typhimurium levels, respectively, in inoculated raw pork after APP treatment for 10 min with an ambient air. In addition, *E. coli* O157:H7, *L. monocytogenes*, and *Aspergillus flavus* populations on the beef jerky were reduced by approximately 2 to 3 Log CFU/g after APP treatment for 10 min using ambient air (Yong et al., 2017a). From the previous studies, antimicrobial effect of APP was also confirmed when ambient air was used as carrier gas.

Up to date, available data reveals that APP could be utilized as an innovative non-thermal processing technology for the improvement of microbial safety of animal-origin foods However, some studies proposed certain limitations of APP in animal-origin foods, such as discoloration of meat color (Misra et al., 2016). Optimal balance between APP treatment conditions to maximize the antimicrobial effects and minimizing the quality deterioration is essential (Lee et al., 2017; Misra & Jo, 2017). Therefore, elucidation of the interaction of APP treatment with the meat quality is highly required.

Meanwhile, APP have been also studying for other purposes besides antimicrobial effect in these days. Park et al. (2013) suggested that use of APP-treated water as a fertilizer in agriculture. APP-treated water successfully improves germination rate of seeds and growth rate of plants, and these results is caused by the production of nitrogen species including nitrite and nitrate in the water.

Nitrite is also a multifunctional compound used in the production of cured meat products. Nitrites added in meat products reduced to nitric oxide then interact

with myoglobin to produce nitroso-myoglobin, which is responsible for characteristic red color of cured meat. Nitrite can also improve the flavour and inhibit the growth of food-borne pathogens, especiallly, *Clostridium botulinum* (Cammack et al., 1999; Zhang et al., 2007). In general, sodium and potassium nitrite, the synthetic nitrite, have been used for years. However, increasing consumers avoid synthetic additives due to the well-being trend. Meat industry continues to find the alternative methods to produce synthetic nitrite-free meats that maintain the quality of cured meat products (Lee et al., 2017; Zhang et al., 2007). If nitrite is produced through APP treatment, the APP can be used as an alternative method to synthetic nitrite.

Therefore, four experiments were conducted 1) to investigate the antimicrobial effect of APP on raw meat and observe quality changes and the genotoxicological safety following different APP exposure times, 2) to elucidate and control the effect of APP on the discoloration of myoglobin for explanation of color changes on APP treated raw meat, 3) to confirm the possibility of red color development in pork after APP treatment and to evaluate the physiochemical properties and microbiological safety of pork jerky made with APP, and 4) to evaluate the quality and microbial safety of injected loin ham cured with plasma treated water (PTW) as a substitute of sodium nitrite.

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CHAPTER II.

Evaluation of the microbiological safety, quality changes, and genotoxicity of chicken breast treated with flexible thin-layer atmospheric pressure plasma

2.1. Introduction

The number of pathogens able to cause food-borne illness is estimated at 31 (Scallan et al., 2011). These food-borne pathogens are often found in retail raw meats; however, the type and amount of existent pathogens differs depending on the type of meat (Zhao et al., 2001). Chicken meat, in particular, is highly susceptible to cross-contamination by enteric pathogens that can cause food-borne illness, such as *Salmonella* and *Listeria*, during slaughter (Noriega et al., 2011). Zhao et al (2001) reported that 38.7% and 4.2% of 212 retail chicken samples were contaminated by *Escherichia coli* and *Salmonella*, respectively. In the case of *Listeria*, the prevalence was 26% (23 of 89 chicken meat samples) (Mayrhofer et al., 2004). Therefore, inactivation of pathogens present in chicken meat is essential for safe consumption.

Plasma, which is an ionized gas or the fourth state of matter, is generated by

the interaction between electromagnetic fields and gas, especially, when the field is sufficiently strong (Heuer et al., 2015). Plasma technology has been studied as a non-thermal sterilization method for improving food safety. The effect of this technology is significant since, unlike thermal sterilization, it does not cause nutritional and quality changes (Jayasena et al., 2015; Kim et al., 2013); in addition, it is more cost-effective and easy to install compared with other non-thermal sterilization technologies such as gamma irradiation, electron-beam, and high pressure (Kim et al., 2013; Yong et al., 2014).

Inactivation of pathogens during plasma discharge is achieved by several factors including the electric field, ultra violet (UV) photons, charged particles, and reactive species such as O, O_3, OH, H_2O_2, NO, or NO_2 (Dobrynin et al., 2009; Guo et al., 2015; Laroussi & Leipold, 2004). A number of studies have established the antimicrobial effect of plasma on meat and meat products (Dirks et al., 2012; Kim et al., 2014; Yong et al., 2014).

Secondary contamination due to processing conditions (staff or equipment) is an unavoidable problem in slaughterhouses (Schwaiger et al., 2012). Therefore, sanitation following packaging is required in order to prevent secondary contamination (Leipold et al., 2011) and quality deterioration; this is thought to be one of the greatest advantages of irradiation or high-pressure treatment. For this reason, researchers have attempted to develop a type of plasma sealed in a bottle (Fröhling et al., 2012), container (Leipold et al., 2011; Yong et al., 2015), or package (Jayasena et al., 2015; Song et al., 2012). A recent study by Jayasena et al. (2015) demonstrated the effect of a sealed package with flexible thin-layer atmospheric pressure plasma (APP) on pork butt and beef loin, thus establishing

an option for using the APP system.

Bacterial inactivation effect of plasma has been tested on chicken meat but following quality changes has not been investigated yet (Dirks et al., 2012; Noriega et al., 2011; Yong et al., 2014). Therefore, the aim of this study was to evaluate the inactivation effect of APP on chicken breasts, generated within a sealed package, and observe changes in quality and the genotoxicological safety using the *Salmonella* mutagenicity assay following different durations of APP exposure.

2.2. Materials and Methods

2.2.1. Sample preparation and sterilization

Raw chicken breasts were purchased from a local market in Seoul, Korea. The samples were cut in to 25 × 25 × 7 mm^3 and divided into two sections. Chicken breast samples in one section was vacuum-packaged and sterilized with irradiation (35 kGy) using a linear electron-beam RF accelerator (10 MeV, 40 kW; EB Tech, Daejeon, Korea) for inoculation test. The other section of chicken breast was treated with APP directly without sterilization step and used to determine the quality properties and toxicological safety.

2.2.2. APP treatment

A flexible package system which is designed for generating APP within the package was prepared using the conductive layer of a commercial, zippered food package (129 × 199 mm^2) as the powered electrode (Jayasena et al., 2015). While the inner patterned electrode was grounded, a bipolar square-waveform voltage of

15 kHz was applied to the outer electrode. Plasma was generated at the surface of the inner electrode at 100 W peak power and 2 W average power. As an operating gas, the atmospheric air was used. After the APP treatment, the respective samples were used for microbial analysis and instrumental color measurement immediately. The other samples were stored under at 4°C until the following day and analysis of other quality properties and toxicological safety.

2.2.3. Inoculation test

2.2.3.1. Preparation of inocula and inoculation

Escherichia coli O157:H7 (KCCM 40406), *Salmonella* Typhimurium (KCTC 1925), and *Listeria monocytogenes* (KCTC 3569) were cultivated in tryptic soy broth (TSB), nutrient broth, and TSB containing 0.6% yeast extract, respectively (Difco Laboratories, Detroit, Michigan, USA), at 37°C for 48 h. Then, the cultures were centrifuged (3000 rpm for 15 min) in a refrigerated centrifuge (UNION 32R, Hanil Science Industrial Co. Ltd., Korea). The obtained pellets were washed twice with sterile saline solution (0.85%) and suspended in sterile saline solution to achieve a viable cell density of approximately 10^8-10^9 CFU/mL. The irradiation-sterilized chicken breast sample (5 g) was inoculated with the 100 μL of test-culture suspensions. Subsequent to spreading the suspensions on the chicken breasts, the meat samples were kept under sterile conditions at room temperature for 10 min to enable attachment of the microorganisms to the samples.

2.2.3.2. Microbial analysis

After APP treatment, inoculated samples were blended with 45 mL of sterile

saline solution (0.85%) using a stomacher BagMixer® 400 (Interscience Co., Saint Nom, France). Then, the samples were decimally diluted using sterile saline solution. The media used for the recording the growth of *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium were typtic soy agar (TSA, Difco Laboratories), TSA containing 0.6% yeast extract (Difco Laboratories), and nutrient agar (Difco Laboratories), respectively. The plates were incubated at 37°C for 48 h. All colonies were counted and the number of microorganisms was expressed as Log CFU/g.

2.2.4. Quality properties

2.2.4.1. Instrumental color measurement

The color of APP-treated and untreated chicken breast samples were measured by a colorimeter (Spectrophotometer, CM-3500d, Konica Minolta Sensing, Inc., Osaka, Japan) using CIE L^*, a^*, and b^* value. The instrument was calibrated with a standard white and black plate before analysis. The color values were monitored using a computerized system controlled by Spectra Magic software (Konica Minolta Sensing, Inc.).

2.2.4.2. Lipid oxidation analysis

Each chicken breast sample (3 g) was homogenized (Ika Laboratory Equipment, Seoul, Korea) with 9 mL of distilled water and 50 μL of 7.2% butylated hydroxytoluene (in ethanol). Then the homogenate (1 mL) was transferred to a 15-mL test tube and mixed with 2 mL of a thiobarbituric acid (20

mmol/L)/trichloroacetic acid (15%) solution. Next, the test tubes were heated in a 90°C water bath for 30 min, cooled in cold water, and centrifuged (Hanil Science Industrial Co. Ltd.) at 2,090 × *g* for 10 min. A spectrophotometer (DU 530; Beckman Instruments Inc., Brea, CA, USA) was used to measure the absorbance of the supernatant at 532 nm. The 2-thiobarbituric acid reactive substances (TBARS) value (mg malondialdehyde/kg sample) was calculated using a standard curve.

2.2.4.3. Texture analysis

APP treated and untreated chicken breasts were minced and separately prepared (4 cm in diameter × 2 cm thickness; approximately 20 g), then cooked to reach an internal temperature of 75°C. Texture of sample was measured by a texture analyzer (TA-XT Plus, Stable Micro Systems Ltd., Surrey, UK) by compressing the centers of the cooked meat samples. A probe (75 mm in diameter) was compressed twice to 75% of their original height at a test speed of 2.00 mm/s and a trigger force of 50 g. The Exponent Lite Texture Analysis software (Stable Micro System Ltd.) was used for texture analysis; hardness, adhesiveness, springiness, cohesiveness, gumminess, and chewiness were recorded. In each treatment combination, three replicate samples were measured.

2.2.5. Mutagenicity assay

The mutagenicity assay was conducted with ethanolic extracts of APP-treated sample for 0 and 10 min. A total of 100 g of each extract was transferred into 900

mL of 70% (v/v) ethanol. The extracts incubated for 8 h at room temperature were filtered by Whatman filter paper No. 4 (Whatman International Ltd., Springfield Mill, Kent, England). Then, 70% ethanol (900 mL) was added again and the procedure was repeated. Ethanol was removed from the samples using a rotary vacuum evaporator (Rotary Vacuum Evaporator N-11 Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The extracts were dried (Freeze dry system, LABCONCO, FreeZone 18, Kansas City, KS, USA,) following freezing and kept at -70°C before use.

The *Salmonella* mutagenicity assay (Ames test) was conducted according to the methods of Ames et al. (1975) and Maron and Ames (1983). *S.* Typhimurium strains TA98 and TA100 were provided by the Korea Institute of Toxicology (KIT, Daejeon, Korea). The strains were purchased from Molecular Toxicology Inc. originally (Boone, NC, USA) and cultured by the KIT in the Korea Research Institute of Chemical Technology (KRICT). Each strain was first tested for its genetic traits including histidine requirement, deep rough (*rfa*) characteristic, UV sensitivity (*uvr*B mutation), and ampicillin or tetracycline-resistance by R-factor prior to use. The strains were inoculated on nutrient broth No. 2 (Oxoid Co., Ltd., Hampshire, England) and cultured at 37°C for 10 h at 200 rpm (Vision Scientific Co., Incheon, Korea) to a cell density of 2×10^9 CFU/mL. The tested sample doses were 1,250, 2,500, and 5,000 μg sample per plate. S9 mix was obtained from the Oriental Yeast Co. Ltd. (Lot No. 0042101, Tokyo, Japan); 5% S9 mix was prepared using the S9 mix fraction and a cofactor (Wako Co., Lot No. 999902, Tokyo, Japan). Treatment concentration was 500 μL/plate.

The positive controls included 4-nitroquinoline-1-oxide (4-NQO, Sigma-Aldrich Inc., St. Louis, MO, USA), 2-Aminoanthracene (2-AA, Sigma-Aldrich

Inc.) dissolved in deionized distilled water (DDW) or dimethylsulfoxide (DMSO, Sigma-Aldrich Inc.), sodium azide (SA, Sigma-Aldrich Inc.). Two plates per concentration were used for the direct plate incorporation method; 100 µL of sample combined with 100 µL of bacterial culture (2×10^9 CFU/mL) and 500 µL of the S9 mixture were added to 2 mL of warm top agar (approximately 45°C) containing 0.5 mM histidine-biotin. This mixture was poured onto a minimal glucose agar plate and allowed to solidify. The plates were incubated for 48 h at 37°C and then the number of revertant colonies was enumerated. The negative control was 100 µL of ethanol. The positive control was 4-NQO and SA (100 µL each) when metabolic activation was not incorporated and 2-AA (100 µL) when metabolic activation was incorporated. Mutagenicity was determined using the method of Maron and Ames (1983) stating that if the number of revertant colonies in a sample is greater than double that in the negative control and it demonstrates a dose-dependent response, it means statistically significant and considered as positive for mutagenicity.

2.2.6. Statistical analysis

Each set of data represents the mean of three replications. One-way analysis of variance was performed with a completely randomized design using the procedure of General Linear Model. Significant differences among mean values were determined using Duncan's multiple comparison test in SAS Release 9.2. (SAS Institute Inc., Cary, NC, USA) with the confidence level of $P<0.05$. Mean values and standard errors of the mean are reported.

2.3. Results and Discussion

2.3.1. Inactivation of foodborne pathogens

The antibacterial effect of APP treatment on chicken breasts has been previously demonstrated (Dirks et al., 2012; Noriega et al., 2011; Yong et al., 2014), although these studies used different types of APP systems. Jayasena et al (2015) inoculated pork butt and beef loin with *L. monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium and treated the meat for 10 min with APP. The number of these pathogens were reduced by 2.04, 2.54, and 2.68 Log CFU/g, respectively, in pork butt samples and by 1.98, 2.57, and 2.58 Log CFU/g, respectively, in beef loin following APP treatment for 10 min. The pathogen reduction rate was higher (3.20, 2.10, and 5.80 Log CFU/g, respectively) in sliced cheddar cheese (Yong et al., 2015). Similar to these previous studies, the results of the current study demonstrate that the number of inoculated pathogens (Table 1) in the chicken breast samples was clearly reduced by APP. As shown in Table 1, the populations of the three pathogens in the chicken breast samples decreased with increasing treatment duration ($P<0.05$); the log-reduction of *L. monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium was 2.14, 2.73, and 2.71 Log CFU/g, respectively, quite similar to the values obtained for other previously tested foods (Jayasena et al., 2015; Yong et al., 2014).

Several plasma agents are generated when plasma is discharged that contribute to the inactivation of microorganisms, including electric field, UV photons, charged particles, and reactive species. However, electric field and UV are not major factors in plasma sterilization. Ma et al. (2008) investigated the effect of electric field on *Staphylococcus aureus* and *E. coli* using a stepwise 60 sec

application of voltage (0-12 kV) until critical voltage was reached (critical voltage; if voltage exceeds the critical point, the influence of the electric field significantly decreased) (Xu & Zhu 1996); however, no significant differences were detected in the inactivation results. UV irradiation is known to trigger the formation of thymine dimers in nucleic acids and can inhibit microorganism growth (Guo et al., 2015). According to Guo et al. (2015), the bactericidal effect of UV generated by plasma alters depending on gas and discharge type. UV has a significant effect only when argon or an oxygen/nitrogen mixture gas is used as the operating gas or when microwave-driven discharge is utilized. When sufficient charged particles accumulate on the surface of microorganisms, they are able to form an electric field and thus alter protein structure This change in protein structure results in the creation of pores in the membrane of microorganisms or inhibition of enzymatic activities (Guo et al., 2015). Ma et al. (2008) demonstrated cytoplasm leakage via pores generated by APP-induced agents by detecting K^+, proteins, and nucleic acids. Reactive species have considered play a crucial role in the inactivation of microorganisms (Jayasena et al., 2015; Lai et al. 2005; Laroussi & Leipold, 2004; Yong et al., 2015). Joshi et al. (2011) reported that reactive species generated by APP cause oxidative DNA damage and membrane lipid peroxidation.

Table 1. Pathogen counts (Log CFU/g) in inoculated chicken breast samples following treatment with atmospheric pressure plasma (APP) for different time

Treatment time (min)	Pathogens (Log CFU/g)		
	Listeria monocytogenes	*Escherichia coli* O157:H7	*Salmonella* Typhimurium
0	5.88[a]	5.84[a]	5.48[a]
2.5	5.34[b]	4.68[b]	4.17[b]
5	4.81[c]	4.02[c]	3.58[c]
7.5	4.37[d]	3.54[d]	3.23[c]
10	3.74[e]	3.11[e]	2.77[d]
SEM[1]	0.034	0.073	0.114

[1]Standard error of the mean (n=15).

[a-e]Different letters within same column differ significantly (P<0.05).

Table 2. Surface color and TBARS values of chicken breasts treated with atmospheric pressure plasma (APP) for different times

Treatment time (min)	Color values			TBARS (mg malondialdehyde /kg meat)
	L^*	a^*	b^*	
0	55.78[d]	-0.21[a]	8.42[b]	0.27
2.5	59.62[d]	-1.93[ab]	6.83[b]	0.28
5	64.61[c]	-2.82[b]	6.00[b]	0.26
7.5	74.22[b]	-3.05[b]	9.43[b]	0.32
10	82.18[a]	-1.31[ab]	14.79[a]	0.34
SEM[1]	1.400	0.695	1.617	0.033

[1]Standard error of the mean (n=15).

[a-d]Different letters within the same column differ significantly (P<0.05).

2.3.2. Surface color

The L^* (Lightness), a^* (redness), b^* (yellowness) values of APP-treated chicken breasts were significantly different compared with the untreated samples (P<0.05) (Table 2). Subsequent to APP treatment, the L^* and b^* values of the chicken breasts

increased with increasing treatment time (P<0.05). In contrast, the a^* value was significantly decreased by APP treatment compared with untreated chicken breasts (P<0.05). Jayasena et al. (2015) reported decreased a^* values and increased b^* values in beef loins treated with APP. A decrease in a^* value was also detected in pork butt samples. Another study also reported that APP-treated pork (*musculus longissimus dorsi*) presented decreased a^* values and increased b^* value (Fröhling et al., 2012). Greenish color of meat can be related with formation of sulfmyoglobin, choleglobin, and verdohaem (Brewer, 2004). If hydrogen sulfide and oxygen react with myoglobin, sulfmyoglobin can be formed (Fröhling et al., 2012). Oxidized porphyrin ring develops green color by forming choleglobin and verdohaem (Brewer, 2004). Fröhling et al. (2012) demonstrated that the green color in APP-treated meat sample is likely to be formed by hydrogen peroxide which is generated during APP treatment.

2.3.3. TBARS values

TBARS values are indicative of the level of lipid oxidation, which is a complex mechanism between unsaturated fatty acids and oxygen molecules (Ladikos & Lougovois, 1990) resulting in extensive quality deterioration in muscle tissue based food (Love & Pearson, 1971). Lipid oxidation is triggered by radicals (Kim et al., 2013), which are a component of APP. Regardless of APP treatment time, APP did not affect lipid oxidation in chicken breasts (Table 2). Kim et al. (2014) obtained similar results for bacon samples treated with atmospheric pressure APP using helium and helium/oxygen as carrier gases. However, Jayasena et al. (2015) reported that lipid oxidation was accelerated by APP in pork butt and beef loin

samples. Thus, TBARS values might be influenced by APP treatment conditions including plasma type, carrier gas, and sample characteristics such as fat content and composition. TBARS value can be increased by an increase of fat content (Jo et al., 1999). Chicken breast has a lower total fat content (%) than beef or pork (*longissimus dorsi* and *semimembranosus* muscles) (Rhee et al., 1996). In addition, the ferric heme pigment is considered to be a critical pro-oxidant in tissue (Love & Pearson, 1971) and TBARS values closely correlate with total pigment and myoglobin content (Rhee & Ziprin, 1987). Chicken breast has lower heme iron content, pigment, and the ultimate metmyoglobin levels compared with pork and beef (Rhee & Ziprin, 1987).

2.3.4. Texture

Cohesiveness was the only texture analysis parameter affected in APP-treated chicken breasts (Table 3). The level of cohesiveness increased with APP exposure time ($P<0.05$). Using APP to inactivate microorganisms did not result in significant changes in any of the texture parameters of the pork butt and beef loin samples (Jayasena et al., 2015). There was no major effect on texture by APP treatment. Irradiation, which is one of the non-thermal sterilization methods, have similarity. Since superoxide radicals, hydrogen peroxide, and singlet molecular oxygen act as main factors in bacterial inactivation (Van Hemmen & Meuling, 1975). Irradiation treatment also have not significant effect on texture in turkey breast roll (Zhu et al., 2004).

Table 3. Texture profile analysis of chicken breasts treated with atmospheric pressure plasma (APP) for different times

Treatment time (min)	Hardness (kg)	Adhesiveness (g/sec)	Springiness (mm)	Cohesiveness (%)	Gumminess (kg)	Chewiness (kg)
0	14.38	-31.02	0.71	0.40[ab]	5.83	1.18
2.5	11.62	-23.67	0.72	0.36[b]	4.37	0.95
5	13.58	-36.92	0.77	0.41[ab]	5.69	1.23
7.5	16.73	-52.04	0.78	0.42[ab]	7.23	1.36
10	17.13	-39.26	0.73	0.46[a]	8.21	1.76
SEM	2.508	10.351	0.035	0.023	1.308	0.281

[1]Standard error of the means (n=15).

[a-b] Different letters within the column differ significantly ($P<0.05$).

Table 4. *Salmonella* mutagenicity assay for chicken breasts treated with atmospheric pressure plasma (APP) for 10 min

Sample	Treat-ment	Dose (μg/plate)	Number of revertant colonies (His+)[a] per plate			
			TA98 (-S9)	TA98 (+S9)	TA100 (-S9)	TA100 (+S9)
Chicken breasts	0	1,250	21±7	32±1	345±44	358±48
		2,500	28±8	33±8	385±18	404±6
		5,000	22±3	28±4	416±25	402±19
	10	1,250	22±5	34±3	317±30	350±34
		2,500	17±2	35±1	340±39	328±40
		5,000	21±5	34±8	322±40	358±31
Negative control	EtOH		20±3	24±5	304±15	356±25
Positive control	4-NQO	0.5	1063±14			
	2-AA[1]	2		2055±95		
	SA	0.5			861±88	
	2-AA[2]	2				2343±112

Abbreviations: EtOH, ethanol; 4-NQO, 4-Nitroquinoline-1-oxide; SA, Sodium azide; 2-AA[1], 2-Aminoanthracene dissolved in DDW; 2-AA[2], 2-Aminoanthracene dissolved in DMSO.

[a]Values are the mean ± SD (P<0.05).

2.3.5. Mutagenicity assay

The results presented in Table 4 indicate that 10 min APP treatment at doses of up to 5,000 μg/plate did not affect mutagenicity. Maron and Ames (1983) determined that when the number of revertant colonies is greater than that in the negative control, the sample is positive for mutagenicity. The number of revertant colonies in our positive control was 20 and 3-fold higher than our experimental samples with the TA98 and TA100 strains, respectively, indicating that the experiment was performed properly. No difference was found between APP-treated and untreated chicken breasts and there was no dose-dependent response. The number of revertants per plate for the APP-treated chicken breasts was the same as the negative control. This confirms the mutagenic safety of APP-treated chicken breast in sealed packages. Lee et al. (2012) also conducted SOS chromotest to confirm the toxicological safety of using atmospheric pressure plasma jet in cooked egg whites and yolks.

2.4. Conclusion

Our findings suggest that the microbial safety of chicken breast can be improved by the newly developed flexible thin layer APP system. APP treatment for 10 min might be suggested as the optimum condition under the present APP system because this condition had the significantly highest log reduction in all three microbial counts. However, further development of this APP system will have to be carried out in order to obtain higher microbial inactivation efficiency and minimum adverse effects on quality qualities.

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CHAPTER III.

Elucidation of the mechanism of APP-inducted green discoloration of myoglobin

3.1. Introduction

Annual occurrence of food-borne illnesses led to considerable attentions to novel non-thermal decontamination technologies, and one of which is an atmospheric pressure plasma (APP) technology. Plasma, produced by electric gas discharges, consists of various reactive species, free radicals, electrons, and ions (Lee et al., 2017; Yong et al., 2015). To date, bactericidal and virucidal effects of APP on meat have been constantly demonstrated. However, some studies reported that APP treatment can cause minor deterioration of meat quality, especially the color (Lee et al., 2017; Misra & Jo 2017). Dielectric barrier discharge plasma using atmospheric air significantly lowered a^* values of raw chicken breast, pork butt, and beef loin, respectively, after 5 min exposure (Jayasena et al, 2015; Lee et al., 2016). Decreased a^* value describes that redness is reduced whereas greenness is induced. Similarly, when indirect plasma system using processed air gas was applied to raw pork (M. longissimus dorsi), greener appearance was shown in plasma-treated sample (Fröhling et al., 2012). Kim et al. (2013) also reported the green discoloration on raw pork loin after plasma treatment with helium gas. In addition, the sensory score for color was significantly lower in plasma-treated raw pork compared to untreated control sample.

Meat color is an important factor more than any other quality attributes because it influences the first impression and purchasing decision of meat. Consumers generally use the meat color as an indicator of freshness or wholesomeness (Schwartz et al., 2008). Around 15% of retail beef are discounted in price due to the discoloration, resulting in an annual revenue loss of 1 billion dollars in the US (Smith et al., 2000). Therefore, in order to use APP in the industry, it is important not only to improve meat safety, but also to maintain the fresh meat color after the APP treatment (Misra & Jo 2017). Thus, green discoloration of meat caused by the APP treatment should be investigated and controlled.

The most responsible factor for meat color is myoglobin, which is a monomeric protein composed of globin protein and heme-group. In the heme-group, iron atom centrally located in porphyrin ring forms six bonds. Four of these bonds coordinate with pyrrole nitrogens in porphyrin ring and one is with the proximal histidine in globin protein. Additionally, the 6th site of iron is available to bind electronegative atoms of various ligands (Brewer, 2004; DeMan, 1999). Color for myoglobin is differentiated depending on the type of the ligands (O2, H2O, NO, CO or etc.) bound to the iron, oxidation state of iron (ferrous or ferric), and integrity of porphyrin ring or globin protein (Seidman et al., 1984). Primary forms of myoglobin found in raw meat are deoxymyoglobin (no ligand; ferric iron; native globin protein), oxymyoglobin (ligand-O2; ferric iron; native globin protein), and metmyoglobin (ligand-H2O; ferrous iron; native globin protein), which appears purplish-red, bright cherish-red, and brownish-red color, respectively (Schwartz et al., 2008).

We hypothesized that APP treatment affected the myoglobin in raw meat, resulting in meat discoloration. Previously, Attri et al (2015) presented the

modification of myoglobin after APP treatment; however, the purpose of the study was not elucidation of meat discoloration induced by APP but identification of biocompatibility of APP in biomedical engineering. A fundamental explanation of the interaction of APP treatment with the myoglobin and consequently appeared color is still insufficient. Therefore, the objective of present study was to elucidate the mechanism of APP-induced green discoloration of myoglobin and its control measure.

3.2. Materials and Methods

3.2.1. Materials and experimental design

3.2.1.1. Experiment I

Horse skeletal muscle myoglobin was purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. The used myoglobin is metmyoglobin which has ferrous iron and no bound oxygen. Also, it possesses no disulfide bridges or free -SH groups in globin protein. Myoglobin (60 mM) was dissolved in distilled water (DW) and 0.4 M sodium phosphate buffer (pH 6.8), respectively. Color and pH changes of myoglobin in DW and phosphate buffer were measured, respectively, after APP treatment. UV-absorption spectra, secondary protein structure, molecular weight, and concentration of nitrite, hydrogen peroxide, and phenol were evaluated with myoglobin solution in phosphate buffer.

3.2.1.2. Experiment II

Horse skeletal muscle myoglobin was also dissolved in the phosphate buffer and added with 0.1 and 0.5% sodium dithionite, respectively. The samples were analyzed for their color and UV-absorption spectra.

3.2.2. APP treatment

An encapsulated APP plasma source was fabricated using a rectangular, parallelepiped plastic container (137 × 104 × 53 mm). All materials in plasma source and plasma generation conditions were the same as those used in our previous study (Yong et al., 2015). Ambient air was used as the carrier gas. Each myoglobin solution (20 mL) was placed in a glass dish at the bottom of the container. Then, it was treated with the APP source for 0, 5, 10, and 20 min, respectively.

3.2.3. Instrumental color measurement

The color of the myoglobin solution was measured using a colorimeter (CR-5, Minolta Camera Co., Osaka, Japan). The instrument was calibrated with a standard black-plate and DW before analysis. The color values were expressed as L∗ (+ brightness, □ darkness), a∗ (+ redness, □ greenness), and b∗ (+ yellowness, □ blueness) values. A more appropriate measure of color was obtained from the chroma (C= $\sqrt{a^2 + b^2}$) and total color difference (ΔE= $\sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$) which were calculated from the L^*, a^*, and b^* values.

3.2.4. pH measurement

The pH of each sample was measured using a pH meter (SevenGo, Mettler-Toledo International Inc., Schwerzenbach, Switzerland).

3.2.5. UV absorbance spectrum

Absorption scans of the myoglobin solution were conducted from 380 to 600 nm at 1 nm increments, using a Model X-ma 3100 spectrophotometer (Human Co., Ltd., Seoul, Korea).

3.2.6. Circular dichroism (CD) spectra

CD spectroscopy was performed with a JASCO J-720 spectropolarimeter, A 1-mm-pathlength cell was used. The reported CD spectra were the average of five scans, and were smoothed by the polynomial curve-fitting program and analyzed. CD data were expressed as molar ellipticity in deg · cm^2 · $dmol^{-1}$.

3.2.7. Electrospray ionization-mass (ESI-MS) spectra

The samples were analyzed in a Triple TOF 5600 Q-TOF LC/MS/MS system (ABSciex, CA) using a Ultimate 3000 RSLC HPLC system (Thermo Fisher Scientific Inc., MA, USA), including a degasser, an auto-sampler, diode array detector and a binary pump. The LC separation was performed on an column (Zorbax, 3.5 um, 300SB-C8, 2.1 × 50 mm, Agilent, PN865750-906) with a mobile phase A (0.1% formic acid in water) and a mobile phase B (0.1% formic acid in acetonitrile). The eluent gradient condition was shown in Table 1. The flow rate

was 0.25 ml/min. The linear gradient was as follows: The auto-sampler was set at 4°C. The injection volume was 1~5 μl. Mass spectra were acquired under positive electrospray ionization (ESI) with an ion spray voltage of 5500 V. The source temperature was 500°C. The curtain gas, ion source gas 1, and ion source gas 2 were 50, 50, and 25 psi, respectively. Two full-scan mass spectra were acquired over an m/z range of 350−1800 on the MS mode. The data were collected using Analyst TF 1.7 software and analyzed using PeakView 2.2., Bio Tool Kit 2.2.0.

Table 1. The eluent gradient condition for analysis of ESI-MS

Time (min)	Eluent A (%) DW w/ 0.1% formic Acid	Eluent B (%) Acetonitrile w/ 0.1% formic Acid
0	95	5
1	95	5
25	0	100
27	0	100
27.5	95	5
30	95	5
33	95	5

3.2.8. Nitrite concentration

Myoglobin solutions were filtered through a 0.2 μm polyvinylidene fluoride syringe filter (Whatman Inc., Maidstone, Kent, UK) and diluted with distilled water (1:200, v/v). Then, nitrite content was measured using an ion-chromatograph (Dionex ICS-3000; Dionex Corporation, Sunnyvale, USA) equipped with a dual eluent generator system, dual chromatography compartments with dual suppressed conductivity detectors, and dual gradient pumps. Samples were analyzed using a guard column, AG 20 (50 × 2.0 mm inner diameter, Dionex Corporation, Sunnyvale, USA) coupled with an IonPac AS20 (250 × 4.0 mm inner diameter, Dionex Corporation, Sunnyvale, USA) analytical column. The flow rate was 1 mL/min. Suppression was achieved using an ASRS URTRA II (4 mm) self-regenerating suppressor, and the injection volume was 25 μL. The analyses were carried out with a gradient elution mode, beginning with 15 mM of potassium hydroxide for 8 min, then 40 mM from 8–18 min, and 15 mM from 19–20 min.

3.2.9. Hydrogen peroxide concentration

In order to analysis the hydrogen peroxide concentration in myoglobin solution, 10 uL of sample was injected to an ion chromatograph ICS5000 (ThermoDionex, USA). Column A (CarboPac_SA10 Analytical, 4×250 mm, Dionex, USA) and column B (CarboPac_SG10 Guard, 4×50 mm, Dionex, USA) was used, and ramped oven temperature was 30°C. 50 mM NaOH was served as the mobile phase at a constant flow rate of 0.6 mL/min.

3.2.10. Statistical analysis

Each set of data represents the mean of three replications. Statistical analysis was performed by one-way analysis of variance (ANOVA) with a completely randomized design using the procedure of General Linear Model. Significant differences between mean values were identified using the Tukey's multiple comparison test in SAS software 9.4. (SAS Institute Inc., Cary, NC, USA) at a significance level of $P<0.05$.

3.3. Results and Discussion

3.3.1. Experiment I

3.3.1.1. Color and pH

When APP treatment time increased, L^* and a^* values significantly decreased in myoglobin in both phosphate buffer and DW (Table 2). The b^* and chroma values of myoglobin in phosphate buffer also decreased with the increase of APP treatment time ($P<0.05$); however, those values of myoglobin in DW only decreased up to 10 min of APP treatment and slightly increased by treatment for 20 min when compared with 10 min ($P<0.05$). ΔE values of myoglobin in phosphate buffer were lower than those of myoglobin in DW. These results indicate that myoglobin in phosphate buffer and that in DW showed yellowish green color and blush green color, respectively, after 20 min of APP treatment. In addition, green discoloration of myoglobin by APP was more pronounced in DW than in phosphate buffer. Visual appearances of myoglobin solutions were shown in Fig. 1.

According to Renerre (1990) and Brewer (2004), porphyrin ring of myoglobin is denatured at pH below 5.0 and which can produce green pigments. Similarly, green color was observed from myoglobin in DW when pH value was reduced to below 5.0 by APP treatment 10 and 20 min (Table 2). It is well known that APP exposure can reduce pH value of liquid due to the generation of acidogenic molecules (e.g, NO, NO2, HNO2, HNO3, H2O2) (Traylor et al., 2011). However, low pH is not the only reason to generate green color in myoglobin. Green color was also observed from myoglobin in phosphate buffer after 20 min of APP treatment, even though pH value of the sample was above 6.0 (Table 2). Because raw meat has its buffering capacity like phosphate buffer, it is difficult to reduce the pH value of raw meat by APP treatment (Jung et al., 2017). Previous studies reported that pH values of APP-treated raw pork were above 5.30 when the sample showed greener appearance (Fröhling et al., 2012; Kim et al., 2013). Therefore, green discoloration of raw meat by APP treatment might be resulted from other reasons than low pH condition.

Myoglobin also appears green color, when it converted to sulfmyoglobin, choleglobin, verdoheme, nitrimyoglobin, and nitrihemin in specific conditions. Sulfmyoglobin is produced by reaction of hydrogen sulfide (H_2S) with deoxymyogloin in vacuum-packaged raw meat. However, if the vacuum-packaged meat is opened, sulfmyoglobin becomes oxygenated to metsulfmyoglobin which appears red color. Sulfmyoglobin is found only on meat having low oxygen concentration ranged about 1~2% (DeMan 1999; Seidman et al., 1984). In the present study, myoglobin solution was exposed to APP in aerobic condition, thus there is a low possibility to be formed sulfmyoglobin. Hydrogen peroxide (H_2O_2) can also cause green discoloration of raw meat by oxidizing

myoglobin to choleglobin. Choleglobin has hydroperoxide (-OOH) attached the 6th coordination site of ferric or ferrous iron. Additionally, further oxidation of choleglobin can open the porphyrin ring (ferrous iron remains) and form green verdoheme (Fox Jr, 1966; Lawrie 2006). Meanwhile, nitrimyoglobin is formed when metmyoglobin is exposed to excess nitrite and a reducing agent at pH values below 7.0. Nitrimyoglobin has been one of concern in meat industry because it arises green discoloration of cured meat product, called 'nitrite-burn' (Bondoc & Timkovich, 1989; Fox Jr & Thomson, 1964). Upon heating and reaction of nitrous acid (HNO_2), nitrimyoglobin can be converted to nitrihemin, one of the green pigments (Schwartz et al., 2008). Unlike sulfmyoglobin, choleglobin, and nitrimyoglobin, globin protein is absent in verdoheme and nitrihemin (Lawrie, 2006).

We hypothesized that the green discoloration of myoglobin by APP treatment is caused by one or more of the above-mentioned green pigments. In the following experiments, myoglobin was used dissolved in the phosphate buffer.

Table 2. Color and pH value of myoglobin in phosphate buffer and DW, respectively, after atmospheric pressure plasma (APP) treatment.

Properties	APP treatment time (min)				SEM[1)]
	0	5	10	20	
Myoglobin in phosphate buffer					
L^*	79.92[a]	79.60[a]	79.10[b]	78.48[c]	0.091
a^*	9.50[a]	7.87[b]	6.58[b]	4.86[c]	0.343
b^*	30.41[a]	29.83[b]	29.77[b]	29.58[c]	0.034
Chroma	31.86[a]	30.85[b]	30.49[c]	29.99[d]	0.033
ΔE	0.00[c]	1.76[b]	3.10[b]	4.94[a]	0.332
pH	6.86[a]	6.85[a]	6.82[ab]	6.80[b]	0.010
Myoglobin in DW					
L^*	62.87[a]	60.32[b]	58.41[c]	57.93[d]	0.015
a^*	18.31[a]	15.83[b]	9.69[c]	0.21[d]	0.015
b^*	42.40[a]	38.86[c]	36.33[d]	39.44[b]	0.016
Chroma	46.19[a]	41.96[b]	37.60[d]	39.44[c]	0.018
ΔE	0.00[d]	5.03[c]	11.46[b]	19.00[a]	0.019
pH	6.43[a]	5.50[b]	4.21[c]	3.39[d]	0.074

[1)]Standard error of the mean (n=12).

[a-d]Values with different letters within the same row differ significantly (P<0.05).

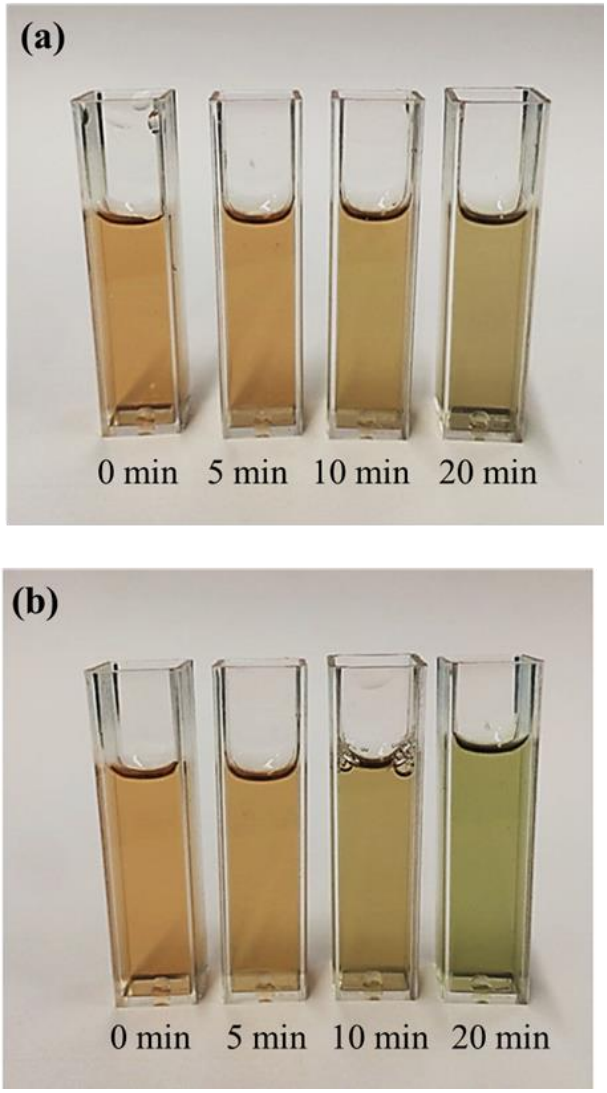

Fig. 1. Visual appearance of myoglobin in phosphate buffer (a) and that in DW (b) after atmospheric pressure plasma (APP) exposure for 0, 5, 10, and 20 min, respectively.

3.3.1.2. UV-absorption spectra

Color appears when electromagnetic radiation, a form of energy, is reflected or emitted by pigments. Accordingly, color is related to the electron structure of the pigment molecule because some of energy can be absorbed by these electrons (Brewer, 2004; Hunt et al., 2012). It is well known that the absorption spectrum varies depending on the state of the heme pigment including myoglobin (Millar et al., 1996). In the UV-absorption spectra of myoglobin solution, absorption peaks at 503 and 630 nm were decreased while that of 590 nm was increased with increasing APP treatment time (Fig. 2).

Sulfmyoglobin and choleglobin has their typical spectrum which absorption maximum at 615 and 628 nm, respectively (Brewer, 2004; DeMan, 1999; Nicol et al., 1970). Because absorption peaks at 615 or 628 nm were not observed in APP-treated myoglobin solution for 20 min, it could be concluded that both sulfmyoglobin and choleglobin were not occurred. Fröhling et al. (2012) and Kim et al. (2013) assumed that hydrogen peroxide generated during APP treatment of raw meat can react with myoglobin and result in choleglobin formation. In fact, 19.71 ppm of hydrogen peroxide was generated in myoglobin solution after APP treatment for 20 min (Fig. 5 (b)). However, choleglobin is not formed in the present study.

UV-absorption spectra of the myoglobin solution showed a typical spectrum of the heme-group which has a maximum absorbance at around 409 nm, called “Soret band”. Depending on chemical state of iron (ferric, ferrous) located in the porphyrin ring, Soret band of heme-group can be shifted to a longer or shorter wavelength (Brewer, 2004; Hunt at al., 2012). In Fig 2, Soret peak of myoglobin solution was not shifted to other wavelength after APP treatment, but the soret

peak is slightly decreased with the increase of APP treatment time. The decreased Soret band means that porphyrin ring in heme-group was partially denatured (Lee & Song, 2002; Onuoha & Rusling, 1995). In order to investigate whether APP affects the porphyrin ring, the protoporphyrin IX dissolved in phosphate buffer was exposed to APP for 0, 5, 10, and 20 min, respectively (Table. 3). Like the discoloration of myoglobin observed earlier, L^*, a^*, b^*, and chroma values decreased while ΔE value increased in protoporphyrin IX solution with the APP treatment time. In other words, green discoloration was also induced in protoporphytin IX solution after APP treatment. Therefroe, it can be inferred that the occurrence of APP-induced green discoloration of myoglobin might be related to the changes in porphyrin ring.

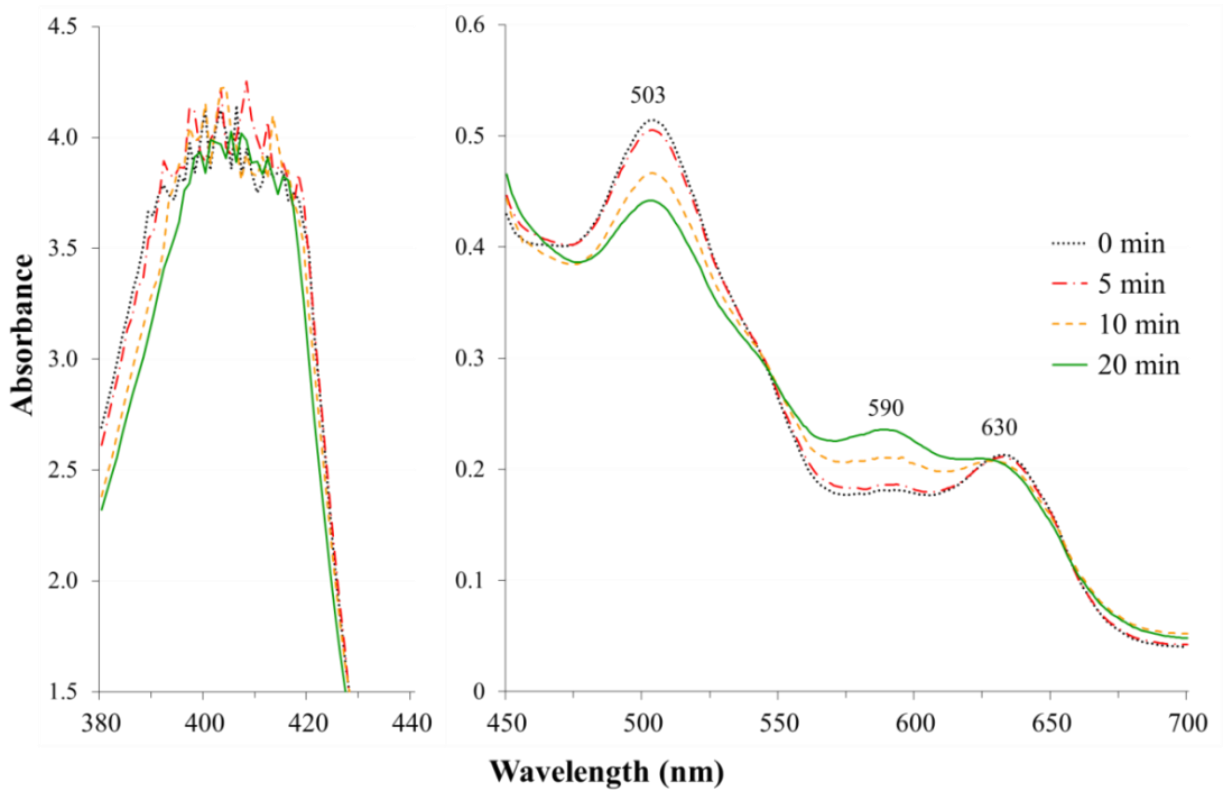

Fig. 2. UV-absorption spectra of myoglobin in phosphate buffer after atmospheric pressure plasma (APP) treatment.

Table 3. Color of protoporphyrin IX in phosphate buffer after atmospheric pressure plasma (APP) treatment.

Properties	APP treatment time (min)				SEM[1)]
	0	5	10	20	
L^*	92.32[d]	93.47[c]	93.78[b]	93.92[a]	0.006
a^*	0.80[a]	0.04[b]	-0.03[c]	-0.10[d]	0.003
b^*	22.58[a]	19.45[b]	18.77[c]	18.55[c]	0.072
Chroma	22.59[a]	19.45[b]	18.77[c]	18.55[c]	0.073
ΔE	0.00[c]	3.41[b]	4.16[a]	4.42[a]	0.066

[1)]Standard error of the mean (n=12).

[a-d]Values with different letters within the same row differ significantly (P<0.05).

3.3.1.3. CD and ESI-MS spectra

Myoglobin has a single chain of 153 amino acids called globin protein, which exist as eight α-helical segments (A–H) enwrapping the heme-group (Brewer, 2004). In Fig 3, the contents of secondary protein structure of myoglobin were 64.2% α-helix, 0.6% β-sheet, 11.9% β-turn, and 17.0% random coli, respectively, and these were not affected by APP exposure up to 20 min. Attri et al. (2015) reported that the structure of α-helix was increased and that of β-sheet was decreased in myoglobin after air plasma treatment. Whereas, the decrease in α-helical structure and increase in β-sheet structure were observed in myoglobin treated with N_2 or argon plasma, respectively (Attri et al., 2015). Accordingly, the

changes in the secondary structure of myoglobin are affected by gas applied to the plasma discharge.

Molecular weight of myoglobin (approximately 16.95 kDa) also did not change after APP treatment up to 20 min, which indicates that myoglobin was not degraded or aggregated by APP treatment (Fig. 4). Verdoheme and nitrihemin, the green pigments, does not have globin protein (Lawrie, 2006). If the globin protein was detached from the heme-group by APP exposure, the molecular weight of myoglobin must be decreased. Thus, the forms of verdoheme and nitrihemin were excluded from the reason of APP-induced green discoloration of myoglobin solution.

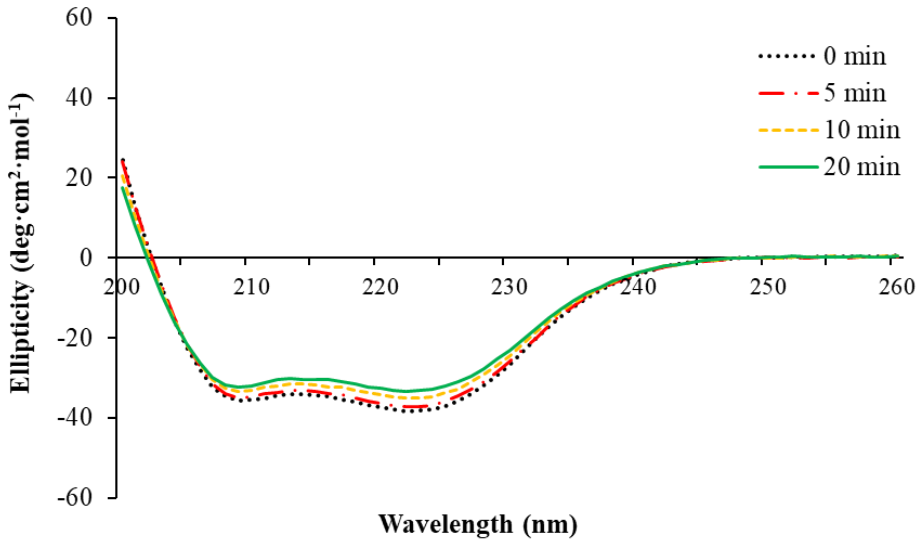

Fig. 3. Circular dichroism spectra analysis of myoglobin in phosphate buffer after atmospheric pressure plasma (APP) treatment

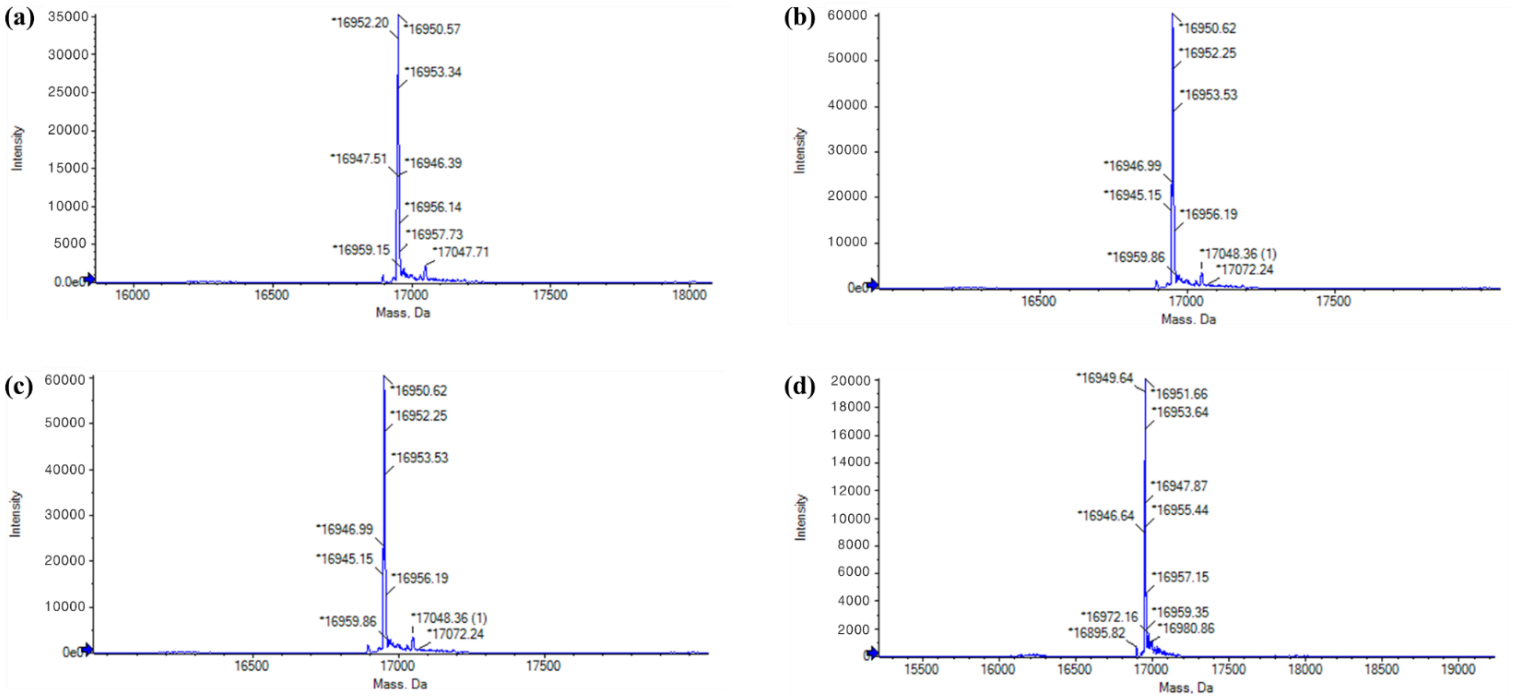

Fig. 4 Electrospray ionization-mass spectra of myoglobin in phosphate buffer after atmospheric pressure plasma (APP) treatment for 0 (a), 5, (b), 10 (c), and 20 min (d).

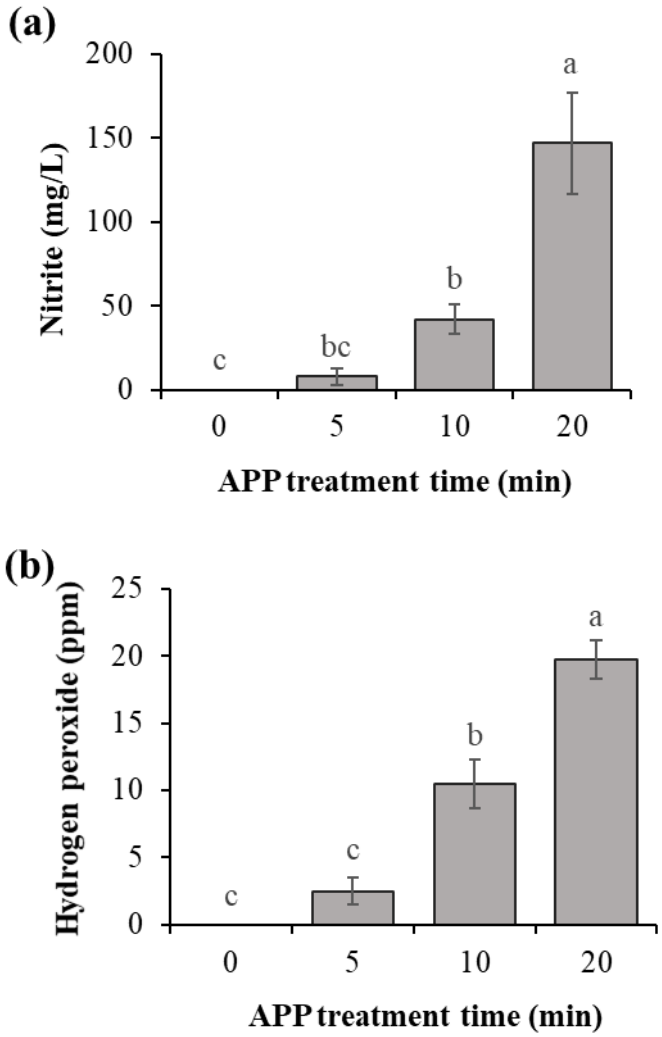

Fig. 5. Nitrite (a), and hydrogen peroxide (b) concentration of myoglobin in phosphate buffer after atmospheric pressure plasma (APP) treatment, respectively. [a-c]Different letters indicate significant differences (P<0.05).

3.3.1.4. Nitrite and hydrogen peroxide concentration

In order to investigate the possibility of nitrimyoglobin formation by APP, nitrite, hydrogen peroxide, and phenol concentration of myoglobin solution were measured. As a result, the concentrations of those compounds were increased with the increase of APP treatment time (Fig. 5). In this experiment, the measured phenol concentration represents the hydroxyl radical (·OH) concentration, because the hydroxyl radicals produced by APP react with the benzene reagent to produce phenol (Al Housari et al., 2010).

Generally, APP discharged with atmospheric air contains various reactive nitrogen species including nitrogen oxide (NO), which can be diffused and dissolved in liquids. Dissolved nitrogen oxides react with oxygen (O_2) present in liquid and generate further nitrogen dioxide (NO_2). Then, reaction of nitrogen dioxide with water molecule forms nitrous acid (HNO_2) and nitric acid (HNO_3) as described by Equations 1-3 (Lee et al., 2017; Machala et al., 2013; Oehmigen et al, 2011).

(1) $NO + O_2 \leftrightarrow 2NO_2$

(2) $NO + NO_2 + H_2O \rightarrow 2HNO_2$

(3) $NO_2 + NO_2 + H_2O \rightarrow HNO_2 + HNO_3$

(4) $HNO_2 \leftrightarrow NO_2^- + H^+$

(5) $HNO_3 \leftrightarrow NO3^- + H^+$

(6) $3NO_2^- + H^+ + H_2O_2 \rightarrow NO_3^- + 2NO + H_3O^+$

As shown in Equations 4 to 5, the nitrous acid (pKa = 2.8~3.2) and nitric acid (pKa = 1.4) can release hydrogen ions, which resulted in acidified liquid.

Subsequently, nitrite oxidized into nitrate in acidic condition (Equation 6). In an alkaline condition, however, nitrite is not oxidized to nitrate and its concentration is maintained (Jung et al., 2015; Machala et al., 2013). The oxidation rate from nitrite to nitrate is dependent on pH of the liquid, thus the rate is remarkably lower in pH 5.80 solution compared to pH 2.85 solution (Braida & Ong, 2000). In Table 2, pH value of myoglobin in phosphate buffer was 6.80 after APP treatment for 20 min.

Excess nitrite addition to metmyoglobin can produce metmyoglobin-nitrite (reddish-brown color). When the latter is treated with an even greater nitrite and nitrous acid, nitrimetmyoglobin is formed which can be converted to nirtimyoglobin (green color) (Fox Jr, & Thomson, 1964; Fox Jr, 1966; Lawrie, 2006). In present study, high concentration of nitrite produced by APP can form nitrimyoglobin from myoglobin solution, which composed of metmyoglobin.

Structure of the nitrimyoglobin revealed that the heme at the 2-vinyl position was nitrated. Thus, the heme-group in nitrimyoglobin has nitrovinyl porphyrin such as 3-(trans-2-nitrovinyl)-2, 7, 12, 18-tetramethyl-8-vinylporphyrin-13, 17-dipropionic acid (Bondoc, & Timkovich, 1989; Yi & Richter-Addo, 2012). In UV absorption spectra of myoglobin solution (Fig. 2), the denaturation of porphyrin ring by APP was shown, and it can be explained by nitration reaction of porphyrin ring due to nitrimyoglobin formation.

Table 4. Color of myoglobin in phosphate buffer added with 0.1, and 0.5% sodium dithionite, respectively, after atmospheric pressure plasma (APP) treatment.

Properties	APP treatment time (min)		SEM[1)]
	0	20	
Myoglobin added with 0.1% sodium dithionite			
L^*	77.09	77.35	0.507
a^*	10.31[a]	7.99[b]	0.304
b^*	34.91	35.12	0.418
Chroma	36.40	36.02	0.360
ΔE	0[b]	2.86[a]	0.224
Myoglobin added with 0.5% sodium dithionite			
L^*	73.89	72.85	0.285
a^*	4.56[b]	11.94[a]	0.237
b^*	29.32	33.43	1.312
Chroma	29.67[b]	35.52[a]	1.173
ΔE	0[b]	8.94[a]	0.310

[1)]Standard error of the mean (n=12).

[a-d]Values with different letters within the same row differ significantly (P<0.05).

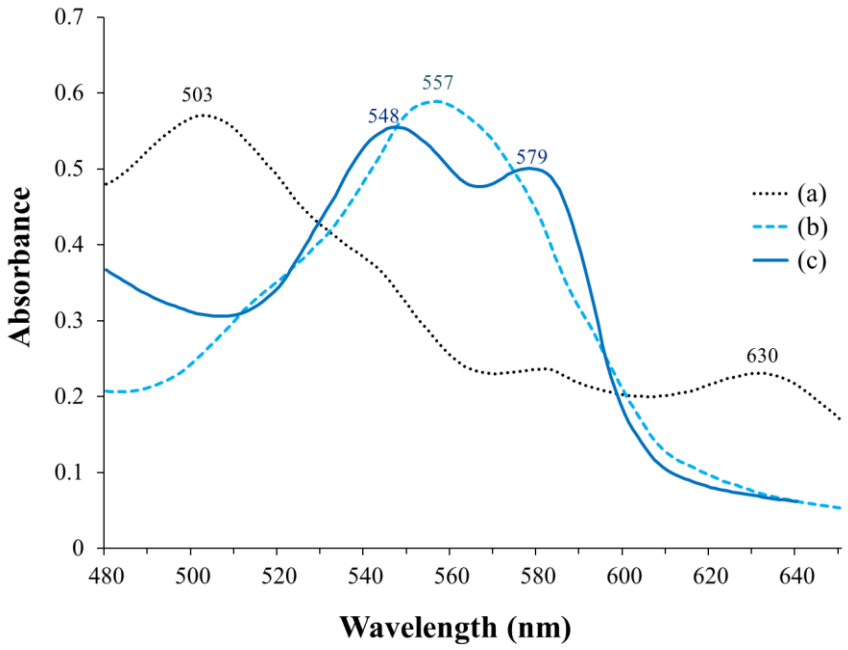

Fig. 6. UV-absorption spectra of myoglobin (a), myoglobin with 0.5% sodium dithionite addition (b), and myoglobin with 0.5% sodium dithionite addition which treated with atmospheric pressure plasma (APP) for 20 min (c). All myoglobin samples were dissolved in phosphate buffer.

3.3.2 Experiment II

3.3.2.1 Color and UV-absorption spectra

In order to control green discoloration of myoglobin by APP, sodium dithionite, one of the strong reducing agent, was added to myoglobin solution followed by APP treatment (Table 4). When APP was treated for 20 min, L^*, b^*, and chroma values were not changed whereas a^* value was decreased by 2.32 in myoglobin solution added with 0.1% sodium dithionite. After 20 min of APP

treatment, ΔE values were 2.86 and 4.94 in myoglobin solution with or without 0.1% sodium dithionite addition, respectively (Tables 2 and 4). Consequently, addition of 0.1% sodium dithionite could reduce the APP-induced green discoloration of myoglobin. In myoglobin solution added with 0.5% sodium dithionite, L^* and b^* values showed no significant differences but a^*, chroma, and ΔE values increased after 20 min of APP treatment. Interestingly, when myoglobin added with 0.5% sodium dithionite was exposed to APP for 20 min, red color was observed instead of the green discoloration.

UV-absorption spectra were measured in order to elucidate the red coloration of myoglobin solution treated with 0.5% sodium dithionite and APP. Myoglobin solution used in the presented study consists of metmyoglobin and it has absorption peak at 503 and 630 nm (Fig. 6 (a)). In myoglobin added with 0.5% sodium dithionite, the absorption peak at 577 nm was shown, which is a typical spectrum of deoxymyoglobin (Fig. 6 (b)) (Millar et al., 1994; Hunt ae al., 2012). Ferrous ion in metmyoglobin might be reduced to ferric iron by strong reducing agent, which resulted in deoxymyoglobin formation (DeMan, 1999; Schwartz et al., 2008). When 0.5% sodium dithionite was added to myoglobin solution followed by APP treatment for 20 min, absorption peaks at 548 and 579 nm were shown (Fig. 6 (c)). This spectrum is in accordance with characteristic absorption spectrum of nitrosomyoglobin (bright red color) Nitrosomyoglobin shows higher absorption peak at 548 nm compared to peak at 579 nm (Millar et al., 1994). Generally, the reaction between deoxymyoglobin and nitric oxide (NO) produces nitrosomyoglobin, also known as nitrosylmyoglobin (DeMan, 1999; Hunt ae al., 2012).

According to Fox Jr and Thomson (1963), when nitrite, metmyoglobin, and hydrogen ion were allowed to stand without the ascorbic acid addition, a green heme pigment was formed which was defined as nitrimyoglobin. By comparison, when nitrite, metmyoglobin, and hydrogen ion were allowed to stand with high concentration of ascorbic acid addition, nitrosomyoglobin was produced due to reducing action. In these regards, Illustrate of effect of atmospheric pressure plasma (APP) and sodium dithionite on metmyolglobn was illustrated in Fig. 7.

During the manufacture of meat products, nitrite is generally added to meat to form nitrosomyoglobin and present desirable bright red color. Consumers do not prefer off-color in raw meat including green or gray and avoid to purchase (DeMan, 1999; Jung et al., 2015). Thus, the mechanism for the formation of nitrosomyoglobin by APP in the present study can be applied in meat industry. Previously, Yong et al. (2018) reported increased redness and nitroso-heme pigment in pork jerky manufactured with ascorbic acid addition and APP treatment.

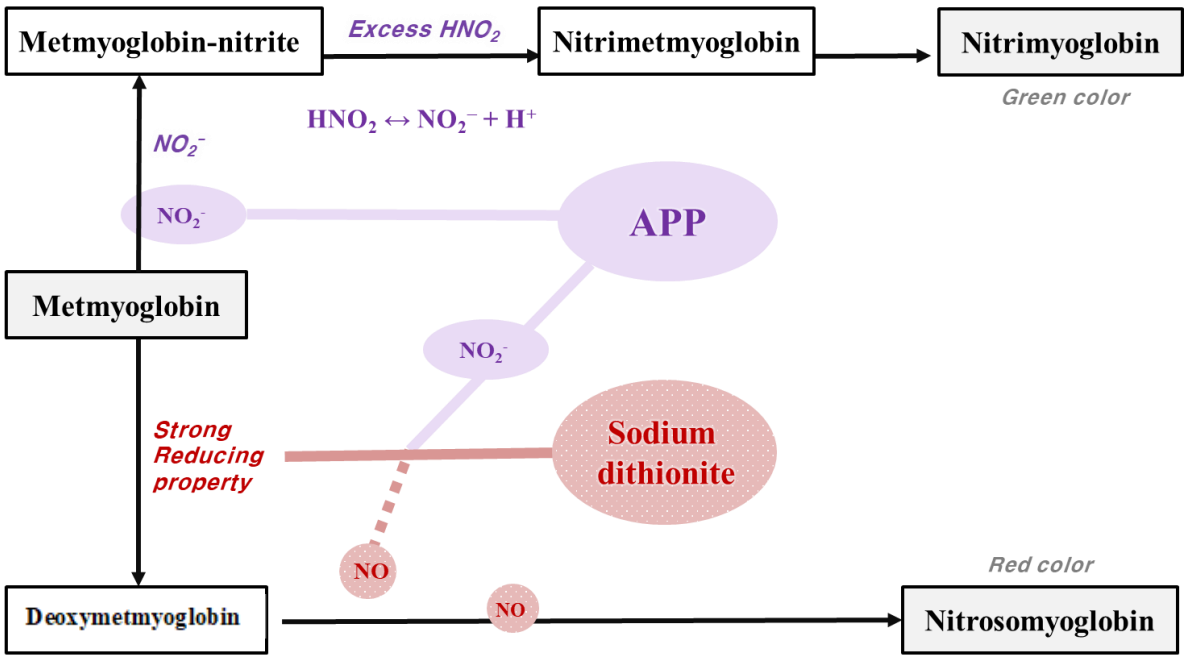

Fig. 7 Illustrate of effect of atmospheric pressure plasma (APP) and sodium dithionite on metmyolglobn.

3.4. Conclusion

The mechanism of APP-induced green discoloration of myoglobin was investigated and its control measure was developed. In general, sulfmyoglobin, choleglobin, verdoheme, nitrihemin and nitrimyoglobin are known as myoglobin-derived green pigment. However, the present study demonstrated that there was no possibility to form sulfmyoglobin, choleglobin, verdoheme, and nitrihemin in myoglobin solution after APP treatment. Nitrite generated by APP in myoglobin solution can be reacted to form nitrimyoglobin. The reducing agent such as sodium dithionite (0.1%) can prevent the APP-induced green discoloration. Moreover, higher concentration (5%) of the reducing agent can induce red color by to the formation of nitrosomyoglobin.

Present study is the first work to reveal the mechanism of APP-induced green discoloration of myoglobin The finding can be applied for acceleration of adaption of new technology, APP, in industry with minimizing barriers.

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CHAPTER IV.

Color development, physiochemical properties, and microbiological safety of pork jerky manufactured with APP

4.1. Introduction

The salt curing of meat is a preservation technology practiced since ancient times. In the 19th century, certain salts (saltpeter, KNO_3) were preferred for the curing process, because their preserving capacities for meat products were recognized to be better than other salts. Later, people realized that the nitrate (NO_3^-) in saltpeter is converted by naturally occurring bacteria to nitrite (NO_2^-), which plays a major role in curing (Akköse et al., 2017; Honikel, 2008). Nitrite prevents lipid oxidation, controls the growth of *Clostridium botulinum*, and produces a red color and unique flavor in meat products. For these reasons, synthetic nitrites, including potassium and sodium nitrites, have been used in meat curing for decades (Alahakoon et al., 2015; Parthasarathy & Bryan, 2012; Pegg et al., 1997).

Meanwhile, today's food industry is confronted with changing market trends, as consumption of foods labeled "natural" and "chemical-free" has been increasing. In response to consumer demand, cured meat producers have begun to use vegetable powders containing high concentrations of nitrates an alternative to

synthetic nitrite, and refer to these products as “naturally cured” (Sebranek et al., 2012). However, using vegetable powder is expensive and time-consuming because it requires incubation with a starter culture to convert nitrate to nitrite. The amount of vegetable powder that can be added to meat products is also limited due to its undesirable flavor (Alahakoon et al., 2015; Jung et al., 2015). Thus, the search for an alternative to nitrite for meat curing continues.

Plasma is an ionized gas made of reactive neutral species, with sufficient energy to break covalent bonds and initiate chemical reactions (Niemira & Gutsol, 2011). Plasma generated under atmospheric pressure is called atmospheric pressure plasma (APP), and this technology has attracted attention as an innovative method of non-thermal sterilization (Misra & Jo, 2017; Yong et al., 2015). Recently, it was reported that APP-treated water contains plenty of reactive species, such as nitrite and nitrate (Machala et al., 2013; Oehmigen et al., 2010). Based on this, our previous studies suggested the possibility of using APP-treated water as a new nitrite source in curing processes, as emulsion-type sausages cured with APP-treated water showed similar color, lipid oxidation, and sensory properties to sausage cured with commercial sodium nitrite (Jung et al., 2015). No mutagenicity and immune toxicity were detected in sausage cured using APP-treated water (Kim et al 2016). However, APP-treated water itself could be considered an additive.

Subsequently, a meat curing system involving direct APP treatment was suggested. When a meat batter composed of pork, water, and sodium chloride was treated with APP during the mixing process, the red color of cured meat developed in the batter without any negative quality changes (Jung et al., 2017). Meanwhile, the number of total aerobic bacteria in the meat batter was not influenced by APP

treatment, even though APP is widely recognized as a non-thermal sterilization technology.

Our hypothesis is that the use of APP in processed meat manufacturing can simultaneously inactivate pathogens and cure the meat. Through this system, potentially safer but added sodium nitrite-free meat products could be produced. Jerky is a typical meat product that is popular in many countries due to its taste, nutrition, and portability (Yong et al., 2017). In the present study, the possibility of red color development was evaluated with ascorbic acid solution and APP treatment. Then, pork jerky was made using APP without the addition of synthetic nitrite sources. The cured color development, physiochemical properties, and microbiological safety were compared between pork jerky made with APP and sodium nitrite. The possible reasons behind the cured color development in the APP pork jerky were also investigated.

4.2. Materials and Methods

4.2.1. Materials and experimental design

4.2.1.1. Experiment I

Raw pork (*Musculus biceps femoris*), sliced to 7-mm thickness, was purchased from a local meat market in Seoul, Korea. Then, the pork slices were cut to 10 × 10 cm^2 and used. To evaluate the color changes of the samples, four different treatments were prepared with the following methods: i) Control, pork without any processing; ii) Plasma, pork was treated with APP for 20 min; iii) Ascorbic acid, pork was immersed in 0.5% ascorbic acid solution for 10 min; iv) Ascorbic acid with plasma, pork was immersed in 0.5% ascorbic acid solution for

10 min, then the solution was removed and treated with APP for 20 min. Fig. 1. shows the experimental design and process of pork jerky production.

In experiment I, each samples were vacuum packaged and cooked in water bath at 85°C for 15 min. After cooling, vacuum-package was opened and the color of the samples were measured.

4.2.1.2. Experiment II

Raw pork slices (7-mm thickness) were cut to 10 × 10 cm^2. Then, the samples were used to analyze the physiochemical properties of jerky made with APP and sodium nitrite. To produce the jerky, two different brines were prepared with the following compositions (w/w, based on raw meat weight): i) Nitrite-free brine: 20% water, 0.15% salt, and 0.03% ascorbic acid; ii) Nitrite-added brine: 20% water, 0.15% salt, 0.03% ascorbic acid, and 0.01% sodium nitrite. Pork slices were marinated in each brine at 4°C for 16 h, removed from the marinades, and placed on dielectric dishes. Pork marinated in nitrite-free brine was exposed to APP for 0, 20, 40, and 60 min. Pork marinated in nitrite brine was kept at room temperature without any other processing during the APP treatment. Then, all samples were placed in a drying oven (DS-510L, Daewonsci Inc., Bucheon, Korea), dried at 75°C for 150 min, 65°C for 90 min, and 55°C for 90 min, and cooled, resulting in APP- and sodium nitrite-cured pork jerkies.

In experiment II, color, nitrosoheme-pigment, residual nitrite content, lipid oxidation value, A_w and shear force of pork jerky were measured. Then, physiochemical properties of brines were evaluated.

4.2.1.3. Experiment III

Prepared raw pork slices (7-mm thickness) were cut to 3 × 3.5 cm^2 for pathogen inoculation tests in order to confirm the bactericidal effects of the APP treatments. Two different brines were prepared as same as Experiment I (nitrite-free brine/nitrite-added brine) and which were used for marination of the pork slices, respectively. Then, bacterium was inoculated onto each marinated pork slices. Pork marinated in nitrite-free brine was exposed to APP for 0, 20, 40, and 60 min whereas pork marinated in nitrite brine was kept without other processing during the APP treatment. All samples were dried and cooled to manufacture APP- and sodium nitrite-cured pork jerkies, respectively.

In experiment III, inoculation test was performed using *Staphylococcus aureus* and *Bacillus cereus*.

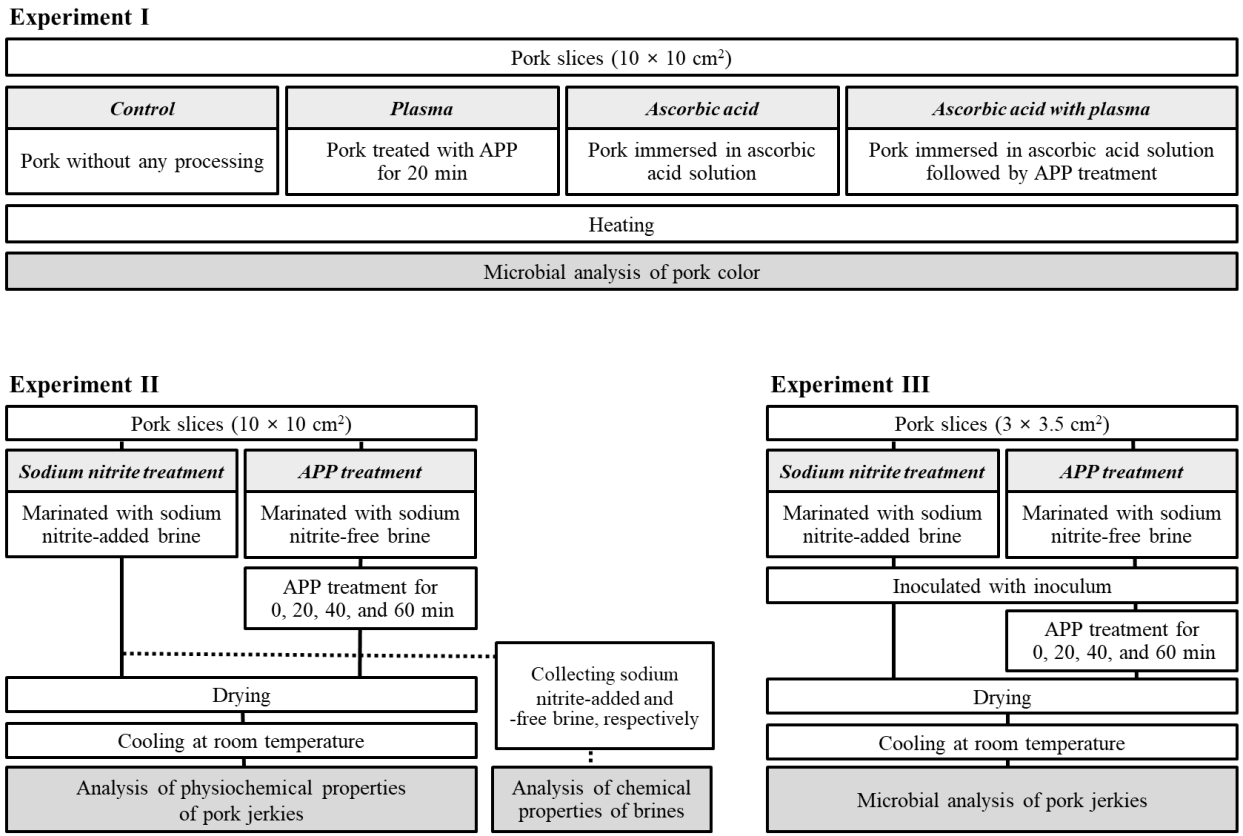

Fig. 1. Experimental design and process of pork jerky production.

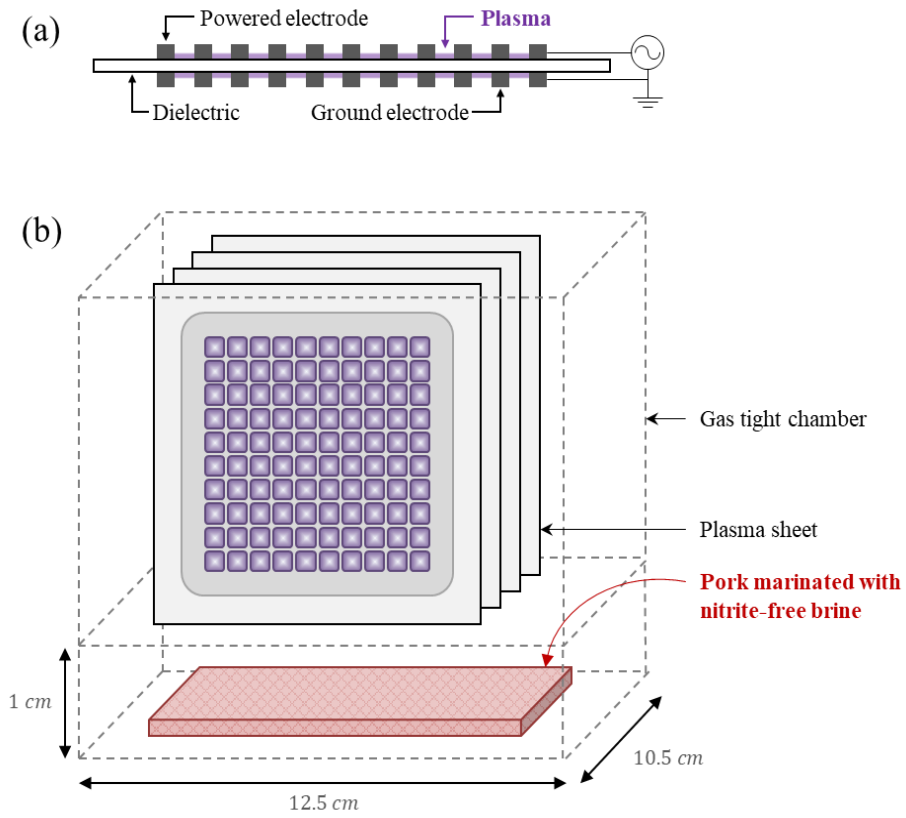

Fig. 2. (a) Detailed illustration of the dielectric barrier discharge (DBD) source, and (b) schematic diagram of the full APP treatment system.

4.2.2. APP treatment

The APP system used in the present study consisted of an array of four dielectric barrier discharge (DBD) sheets and a gas-tight chamber. A detailed configuration of the DBD source can found in Jung et al. (2015). As presented in

Fig. 2(a), two metallic sheets with 3 × 3 mm^2 rounded square patterns were adhered on both sides of a 1 mm thick, 10 × 10 cm^2 alumina plate. One electrode was connected to the power supply, which provided a sinusoidal waveform at a frequency of 4 kHz and a peak-to-peak voltage of 3.8 kV. The other electrode was used as a ground electrode, and air surface discharge was generated at the surface of the electrodes. Plasma was produced in the ambient air without air circulation or supplementation with any particular gas. Fig. 2(b) shows an arrangement of the vertically aligned DBD array and a sample inside the gas-tight chamber. The distance between the sample surface and the DBD array was fixed at 1 cm throughout the experiment.

In experiment II and III, the samples were inverted halfway through APP treatment to expose both sides of the pork samples to APP. Since each side of the samples was exposed to APP for 0, 10, 20, or 30 min, the total APP treatment times were 0, 20, 40, and 60 min, respectively.

4.2.3. Physicochemical properties of pork jerky

4.2.3.1. Instrumental color measurement

Color measurements were performed using a colorimeter (CM-5, Konica Minolta Co., Ltd., Osaka, Japan), using Standard Illuminant D65, a 10° standard observer, and a 30 mm (diameter) measurement area. The color values were expressed as L^* (+ brightness, – darkness), a^* (+ redness, – greenness), and b^* (+ yellowness, – blueness) values. Then, chroma ($C = \sqrt{a^2 + b^2}$) were calculated from the a^* and b^* values.

4.2.3.2. Nitrosoheme-pigment content

Nitrosoheme-pigment content was measured as described by Ahn et al. (2003). The pigments in pork jerky were extracted with an acetone:distilled water (40:6, v/v) solution. The extract was filtered through a Whatman No. 4 filter paper (Whatman PLC., Maidstone, UK) and absorbance was measured at 540 nm using a spectrophotometer (X-ma 3100, Human Co. Ltd., Seoul, Korea). The nitrosoheme-pigment content was expressed as ppm hematin.

4.2.3.3. Residual nitrite content

Residual nitrite in pork jerky samples was measured according to AOAC method no. 973.31 (AOAC, 1990).

4.2.3.4. Lipid oxidation analysis

Lipid oxidation was evaluated by calculating the concentration of malondialdehyde, which is a byproduct of the oxidation of polyunsaturated fatty acids (Misra & Jo, 2017), as a 2-thiobarbituric acid reactive substances (TBARS) value. Each pork jerky sample (5 g) was homogenized with 15 mL of distilled water using a homogenizer (T10 basic). The homogenate (2 mL) was transferred to a test tube and mixed with 4 mL of thiobarbituric acid (0.02 M)/trichloroacetic acid (15%) solution. Then, the test tubes were heated in a water bath at 90ºC for 30 min, cooled, and centrifuged (Continent 512R, Hanil Co., Ltd., Incheon, Korea) at 2,419 × *g* for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (X-ma 3100). The TBARS values were reported as mg malondialdehyde per kg of sample.

4.2.3.5. A_w and shear force

The A_w of the pork jerkies ranged from 0.79–0.82 (Table 2), with no significant differences between samples (P>0.05). During the manufacturing process, it is important to control the A_w of jerky to avoid quality changes and microbial growth during storage (Lee, Alford, Kannan, & Kouakou, 2017). The A_w is also useful for explaining the thermodynamic equilibrium state of jerky (Yang, Hwang, Joo, & Park, 2009).

In dried foods like jerky, texture is an important sensory attribute to consumers and it is highly correlated with Warner Bratzler shear force (Destefanis, Brugiapaglia, Barge, & Dal Molin, 2008; Lee, Alford, Kannan, & Kouakou, 2017). The shear force values of pork jerky displayed no significant differences between treatments (Table 2). According to Lee, Alford, Kannan, and Kouakou (2017), the addition of nitrite does not affect the shear force value of goat meat jerky. Previous studies have also demonstrated that APP treatment does not influence the textural properties of meat and meat products. When raw pork butt and commercial beef jerky were exposed to flexible thin-layer plasma for up to 10 min, the texture parameters of the samples were not affected (Jayasena et al., 2015; Yong et al., 2017). Similarly, Kim, Lee, Choi, and Kim (2014) reported that radio-frequency APP treatment does not influence the shear force value of beef jerky.

4.2.4. Chemical properties of brine

During APP treatment, a small amount of brine (approximately 1.5 mL) remained on the surface of the marinated pork. This nitrite-free brine was collected after APP treatment, as was the nitrite brine before drying (see the

Experiment II in Fig. 1). The nitrite content and pH were analyzed immediately.

The collected brine was filtered through a 0.2-μm polyvinylidene fluoride syringe filter (Whatman PLC) and diluted with distilled water (1:200, v/v). Then, nitrite content was measured using an ion-chromatograph (Dionex ICS-3000; Dionex Corporation, Sunnyvale, USA) equipped with a dual eluent generator system, dual chromatography compartments with dual suppressed conductivity detectors, and dual gradient pumps. Samples were analyzed using a guard column, AG 20 (50 × 2.0 mm inner diameter, Dionex Corporation, Sunnyvale, USA) coupled with an IonPac AS20 (250 × 4.0 mm inner diameter, Dionex Corporation, Sunnyvale, USA) analytical column. The flow rate was 1 mL/min. Suppression was achieved using an ASRS URTRA II (4 mm) self-regenerating suppressor, and the injection volume was 25 μL. The analyses were carried out with a gradient elution mode, beginning with 15 mM of potassium hydroxide for 8 min, then 40 mM from 8–18 min, and 15 mM from 19–20 min.

The pH of each brine was measured using a pH meter (SevenGo, Mettler-Toledo International Inc., Schwerzenbach, Switzerland).

4.2.5. Inoculation test

4.2.5.1. Preparation of inocula and inoculation

S. aureus (KCTC 11764) and *B. cereus* (KCTC 3624) were cultivated in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) at 37°C and nutrient broth (Difco) at 30°C, respectively. After 48 h, the cultures were centrifuged (2,419 × *g* for 15 min) in a refrigerated centrifuge (Continent 512R). The pellets were washed twice with sterile saline and suspended in saline to a final

concentration of 10^8–10^9 CFU/mL. For inoculation tests, each strain (0.1 mL) was inoculated onto marinated pork in the experiment III (see Fig. 1).

4.2.5.2. Microbial analysis

After APP treatment and drying, jerky samples (6 g) were blended with sterile saline (54 mL) for 2 min. Then, appropriate dilutions of the samples were prepared in sterile saline and plated onto selective medium. *S. aureus* were grown in Baird-Parker agar (Difco) containing egg yolk tellurite emulsion (Oxoid, Basingstoke, England), and *B. cereus* were grown in Mannitol egg yolk polymyxin agar (Oxoid) containing egg yolk emulsion (Oxoid) and polymyxin B supplement (Oxoid). *S. aureus* and *B. cereus* plates were incubated for 48 h at 37°C and 30°C, respectively. The results were expressed as log colony-forming units per gram (Log CFU/g).

4.2.6. Statistical analysis

All experimental procedures were repeated in four individual trials. One-way analysis of variance was performed with a completely randomized design using the General Linear Model procedure. Significant differences were identified with the Tukey's multiple-range test using Statistical Analysis System Release 9.4 (SAS Institute Inc., Cary, USA), at a significance level of $P<0.05$.

4.3. Results and Discussion

4.3.1. Experiment I

To confirm the red development of pork, ascorbic acid was added or/and APP was treated (Table 1). No significant difference was found in L^* value among the treatments. Compared to a^* value of control, that of APP and ascorbic acid treatments shows no significant differences ($P>0.05$), while that of ascorbic acid with APP treatment shows higher value ($P<0.05$). b^* value was increased in APP, ascorbic acid, and ascorbic acid with APP treatments compared to control. Visual appearance of the treatments was shown in Fig. 3.

This results demonstrated the possibility that pork jerky can be produced with APP treatment due to the color. In experiment II, pork jerky was manufactured with APP treatment and reason for red color development of pork was investigated.

Table 1. Effect of ascorbic acid addition and atmospheric pressure plasma (APP) treatment on the pork color after cooking.

Treatment[1]	L^* value	a^* value	b^* value
Control	58.37	3.80^{c}	13.12^{a}
APP	59.41	3.24^{bc}	15.07^{b}
Ascorbic acid	56.87	5.01^{b}	15.37^{b}
Ascorbic acid with APP	58.29	9.29^{a}	14.53^{b}
SEM[2]	0.250	0.290	0.715

[1]Control, pork without any processing; APP, pork was treated with APP for 20 min; Ascorbic acid, pork was immersed in 0.5% ascorbic acid solution for 10 min; Ascorbic acid with APP, pork was immersed in 0.5% ascorbic acid solution for 10 min, then the solution was removed and treated with APP for 20 min.

[2]SEM, standard error of the mean (n=12).

[a-d]Different letters within each column indicate significant differences (P<0.05).

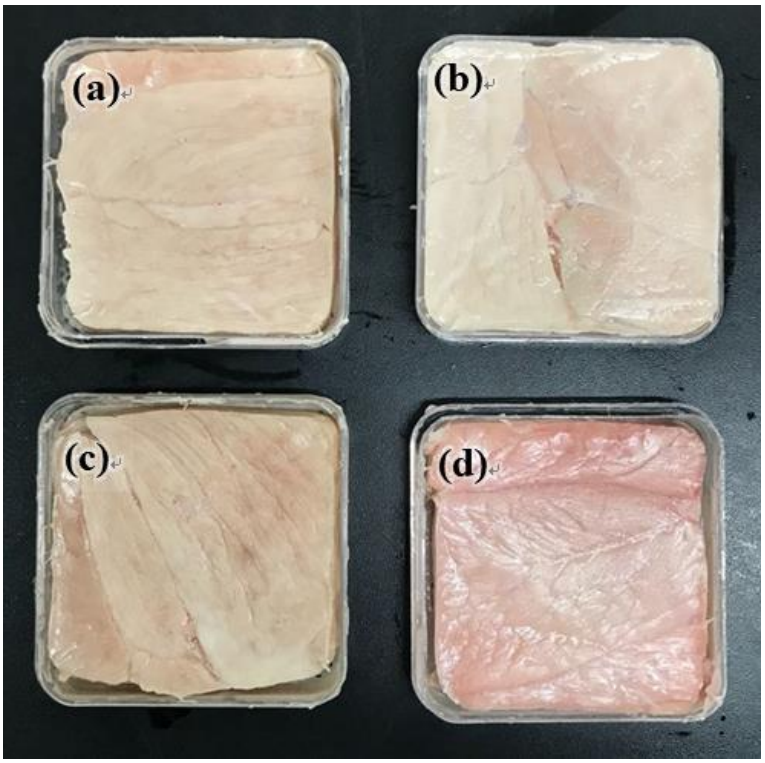

Fig 3. Visual appearance of (a) Control, (b) APP, (c) Ascorbic acid, (d) Ascorbic acid with APP. (Control, pork without any processing; APP, pork was treated with APP for 20 min; Ascorbic acid, pork was immersed in 0.5% ascorbic acid solution for 10 min; Ascorbic acid with APP, pork was immersed in 0.5% ascorbic acid solution for 10 min, then the solution was removed and treated with APP for 20 min).

4.3.2. Experiment II

4.3.2.1. Color and nitrosoheme-pigment

With increasing APP treatment time, the L^*, a^*, and chroma values of the pork jerky gradually increased, whereas b^* decreased (P<0.05, Table 2). In other words, increased APP treatment time made the jerky brighter and more distinctly red, but less yellow. Jerky made with APP for 60 min had the highest a^* value among the treatments, while jerky made with APP for 40 min had a comparable a^* value to (i.e., no significant difference from) jerky made with sodium nitrite.

Development of red (cured) color is important for meat products. Although consumers expect new meat products without synthetic nitrite addition, they also expect the appearance of these products to be similar to conventional meat products (Sebranek et al., 2012). Generally, the red color of meat products results from nitrosoheme-pigments such as nitroso-myoglobin and nitroso-hemochromogen (Honikel, 2008; Pegg, & Shahidi, 2000). When nitrite is added to meat, which usually has a pH of 5.5–6.5, nitrous acid (HNO_2, pKa=3.37) is formed from nitrite. The nitrous acid is in equilibrium with its anhydride (dinitrogen trioxide, N_2O_3) as described as equation 1.

(7) $2HNO_2 \leftrightarrow N_2O_3 + H_2O$

(8) N_2O_3 + ascorbic acid $\leftrightarrow$ 2NO + H_2O + dehydroascorbic acid

(9) NO + myoglobin $\rightarrow$ NO-myoglobin

The presence of endogenous reductants in meat or addition of reductants like ascorbic acid promotes the production of nitric oxide (NO; Equation 2). NO can bind to myoglobin to form nitroso-myoglobin (NO-myoglobin; Equation 3), which is responsible for the distinct red color. With heating, nitroso-myoglobin

decomposes into globin protein and nitroso-hemochromogen, which displays a stable red color (Honikel, 2008; Møller & Skibsted, 2002; Pegg et al., 2000).

We hypothesized that the increased a^* value of pork jerky made with APP treatment resulted from nitrosoheme-pigment formation. Jung et al. (2017) reported that APP treatment, which generates reactive nitrogen species (RNS), resulted in the infusion of nitrite into meat batter composed of pork, water, and salt. Similarly, if nitrogen compounds such as nitrite were produced in pork by APP treatment, there could be a reaction between myoglobin and NO. Consistent with this notion, the nitrosoheme-pigment content of pork jerky made with APP gradually increased with treatment time (Table 3). There was no significant difference in nitrosoheme-pigment content observed between jerky made with APP for 40 min and those made with sodium nitrite. The similar trends suggest that APP increased nitrosoheme-pigment formation, resulting in an increased a^* value.

Meanwhile, previous studies have reported that the a^* values of frozen pork, raw pork loin, and raw pork decreased after treatment with a corona plasma jet using dried air, indirect plasma using processed air, and APP using helium gas, respectively (Choi et al., 2016; Fröhling et al., 2012; Kim, e t al., 2013). The APP system, composed of a DBD source with ambient air, also decreased the a^* values of raw pork butt, even though nitrogen molecular spectra were observed in the emission spectrum of the APP discharge (Jayasena et al., 2015). Our conflicting a^* value results might be due to the pork being marinated instead of raw. In this study, a small amount of brine remained on the marinated pork during APP treatment. This brine was collected after APP treatment (see Fig. 1) and analyzed to further examine why nitrosoheme-pigment formed in the pork jerky made with APP treatment.

4.3.2.2 Physiochemical properties of brine

Consistent with the lack of significant differences in nitrosoheme-pigment content between jerky made with APP for 40 min and with sodium nitrite (Table 3), similar nitrite content was observed in the brine (nitrite-free brine) treated with APP for 40 min and that with sodium nitrite (nitrite-added brine) (Fig. 4 (a)). In addition, the nitrite content of the brine gradually increased with APP treatment time ($P<0.05$). These results indicate that nitrite generated in brine treated by APP may cause nitrosoheme-pigment formation in pork jerky.

In this study, the pH of the brine decreased with increasing APP treatment time (Fig. 4 (b)). Generally, APP treatment results in lower pH in liquid (Oehmigen et al., 2010). When APP treatment was applied to deionized water for 2 h, the pH of the sample decreased from 7 to 2 (Jung et al., 2015). Along with the formation of nitrite in APP-treated water, dissolution of NOx in water leads to a decrease in pH, as described in Equations 3 and 4 (Machala et al., 2013).

(3) $NO_2 + NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$

(4) $NO + NO_2 + H_2O \rightarrow 2NO_2^- + 2H^+$

Table 2. Surface color of pork jerky made with atmospheric pressure plasma (APP) and sodium nitrite

Color parameter	APP treatment time (min)				Sodium nitrite	SEM[1]
	0	20	40	60		
L^* value	45.92^{b}	47.38^{ab}	47.57^{a}	47.66^{a}	47.62^{a}	0.338
a^* value	11.29^{c}	12.51^{c}	14.68^{b}	17.58^{a}	15.29^{b}	0.472
b^* value	16.44^{a}	15.89^{ab}	14.57^{b}	14.26^{b}	14.54^{b}	0.420
Chroma	19.95^{b}	19.58^{b}	21.22^{ab}	22.58^{a}	20.54^{ab}	0.564

[1]SEM, standard error of the mean (n=20).

[a-d]Different letters within each row indicate significant differences (P<0.05).

Table 3. Physiochemical properties of pork jerky made with atmospheric pressure plasma (APP) and sodium nitrite

Physiochemical properties	APP treatment time (min)				Sodium nitrite	SEM[1]
	0	20	40	60		
Nitroso-heme pigment (ppm of hemetain)	1.40^{d}	13.84^{c}	29.92^{b}	40.04^{a}	28.40^{b}	1.728
Residual nitrite (mg/kg)	0.43^{d}	5.56^{c}	18.23^{b}	26.34^{a}	27.27^{a}	1.034
TBARS[2] (mg malondialdehyde/kg)	3.84^{a}	3.65^{ab}	2.84^{bc}	2.27^{c}	2.68^{bc}	0.225
Water activity	0.80	0.82	0.79	0.80	0.82	0.022
Shear force (N)	63.60	65.37	64.78	66.01	65.09	2.401

[1]SEM, standard error of the mean (n=20).

[2]TBARS, 2-thiobarbituric acid reactive substances.

[a-d]Different letters within a low indicate significant difference (P<0.05).

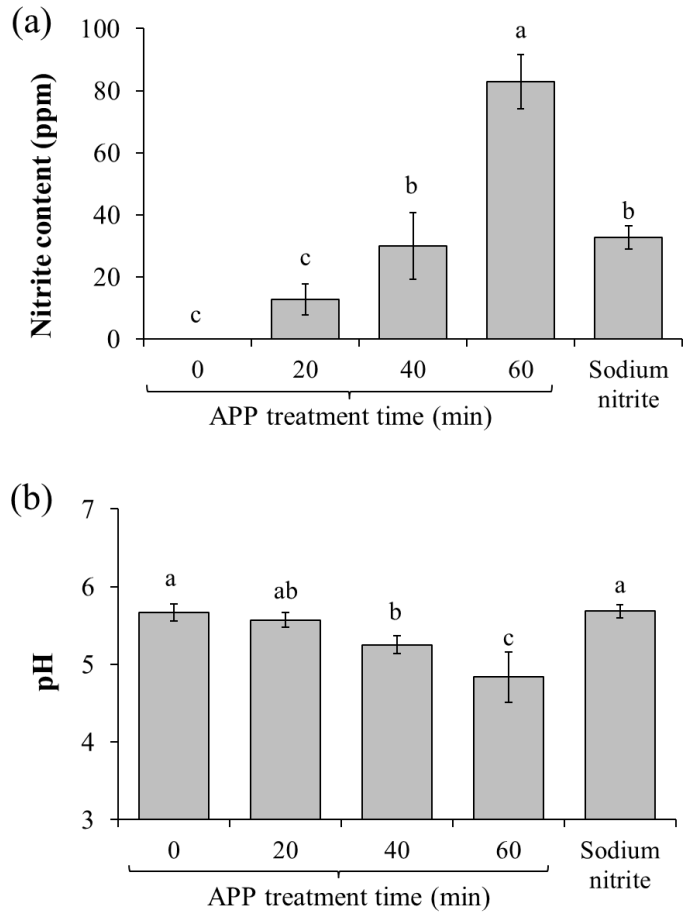

Fig. 4. (a) Nitrite content and (b) pH of the brine surrounding the marinated pork. [a-b]Different letters indicate significant differences (P<0.05). APP, atmospheric pressure plasma.

4.3.2.3. Residual nitrite content

Residual nitrite is the remaining nitrite in the final meat product that has not been converted to other substances. Residual nitrite content in meat products decreases when more nitrite is converted to nitrogen oxide and reacts with

myoglobin to form nitrosoheme-pigment (Alahakoon et al., 2015; Honikel, 2008). Interestingly, while the nitrite content of brine and nitrosoheme-pigment content of pork jerky were similar in APP treatment for 40 min and sodium nitrite treatment, (Table 3, Fig. 4 (a)), the residual nitrite content was significantly lower in pork in APP treatment for 40 min compared to sodium nitrite treatment (Table 3). To identify the reason for this observation, the nitrite content in brine was measured immediately after APP treatment.

Nitrite (pK_a = 3.3) can be progressively oxidized into nitrate under acidic conditions, as shown in equation 5 (Machala et al., 2013; Oehmigen et al., 2010).

(5) $$3NO_2^- + 3H^+ + H_2O \rightarrow 2NO + NO_3^- + H_3O^+$$

Braida and Ong (2000) reported that the rate of decomposition or oxidation of nitrite is dependent on the pH of the solution, with increased rates at low pH. In Fig. 4 (b), the pH of the brines made with APP for 40 min and 60 min were 5.25 and 4.84, respectively, both significantly lower than the pH of brine treated with sodium nitrite (pH=5.68). The decreased pH may explain why the nitrite content of the brine treated with APP for 40 min decreased more quickly, and the increased decomposition rate of nitrite (see Equation 5) would affect the residual nitrite content of pork jerky made with APP. However, further research is required to confirm this phenomenon.

Since the 1950s, laws have been established and authorities have regulated the amount of nitrite that can be used in meat products, as the lethal oral dose of nitrite is 33–250 mg/kg body weight in humans (Honikel, 2008; Schuddeboom, 1993). Thus, the added and residual nitrite contents generally allowed in meat products are 150 and 100 mg/kg, respectively (Honikel, 2008). In this study, the residual nitrite contents of all pork jerkies were below 30 mg/kg (Table 3). However, recent studies have revealed significant therapeutic benefits of nitrite, as a novel therapy

associated with NO insufficiency (Parthasarathy & Bryan, 2012). Using a rationally designed nitrite-enriched dietary supplement has been shown to reduce hyperlipidemia in a clinical trial (Zand et al., 2011).

4.3.2.4. Lipid oxidation

Generally, APP produces free radicals and reactive species that may compromise the functions of fatty acids, inducing lipid oxidation (Misra et al., 2016). When raw pork loin was exposed to APP with helium and oxygen gases, a significant increase in TBARS value was observed (Kim et al., 2013). Choi et al. (2016) reported that the peroxide value (POV) significantly increased in frozen pork upon corona discharge plasma jet treatment. Peroxides are one of the primary byproducts of lipid oxidation. The POV of commercial beef jerky was also significantly increased by 10 min of flexible thin-layer plasma treatment (Yong et al., 2017).

In this study, however, TBARS values decreased as the APP treatment time increased (Table 3). The TBARS value of jerky made with sodium nitrite showed no significant difference with those of jerky made with APP for 20, 40, and 60 min. This result might be attributed to the antioxidant effect of nitrite. The TBARS value was the lowest in the pork jerky made with APP for 60 min, and the brine used in this treatment had the highest nitrite content.

In meat products, nitrous acid derived from nitrite sequentially forms nitrous acid anhydride and NO (Equations 1 and 2). Because NO itself can be easily oxidized to NO_2 by reacting with oxygen, one of the antioxidative actions of nitrite is related to oxygen sequestering (Honikel, 2008). As described in Equation 3, NO can also bind to the iron center of myoglobin, and this reaction can reduce lipid

oxidation by reducing the amount of free iron available to initiate lipid oxidation (Parthasarathy & Bryan, 2012). Furthermore, NO acts as an inhibitor of the lipid peroxidation chain reaction by scavenging lipid peroxyl radicals (Hogg & Kalyanaraman, 1999; Pegg & Shahidi, 2000). Lee et al. (2017) determined that the TBARS values of goat meat jerky cured with and without sodium nitrite were 4.26 and 6.81 mg malondialdehyde/kg, respectively.

4.3.2.5. A_w and shear force

The A_w of the pork jerkies ranged from 0.79–0.82 (Table 3), with no significant differences between samples (P>0.05). During the manufacturing process, it is important to control the A_w of jerky to avoid quality changes and microbial growth during storage (Lee et al., 2017). The A_w is also useful for explaining the thermodynamic equilibrium state of jerky (Yang et al., 2009).

In dried foods like jerky, texture is an important sensory attribute to consumers and it is highly correlated with Warner Bratzler shear force (Destefanis et al., 2008; Lee et al., 2017). The shear force values of pork jerky displayed no significant differences between treatments (Table 3). According to Lee et al. (2017), the addition of nitrite does not affect the shear force value of goat meat jerky. Previous studies have also demonstrated that APP treatment does not influence the textural properties of meat and meat products. When raw pork butt and commercial beef jerky were exposed to flexible thin-layer plasma for up to 10 min, the texture parameters of the samples were not affected (Jayasena et al., 2015; Yong et al., 2017). Similarly, Kim et al. (2014) reported that radio-frequency APP treatment does not influence the shear force value of beef jerky.

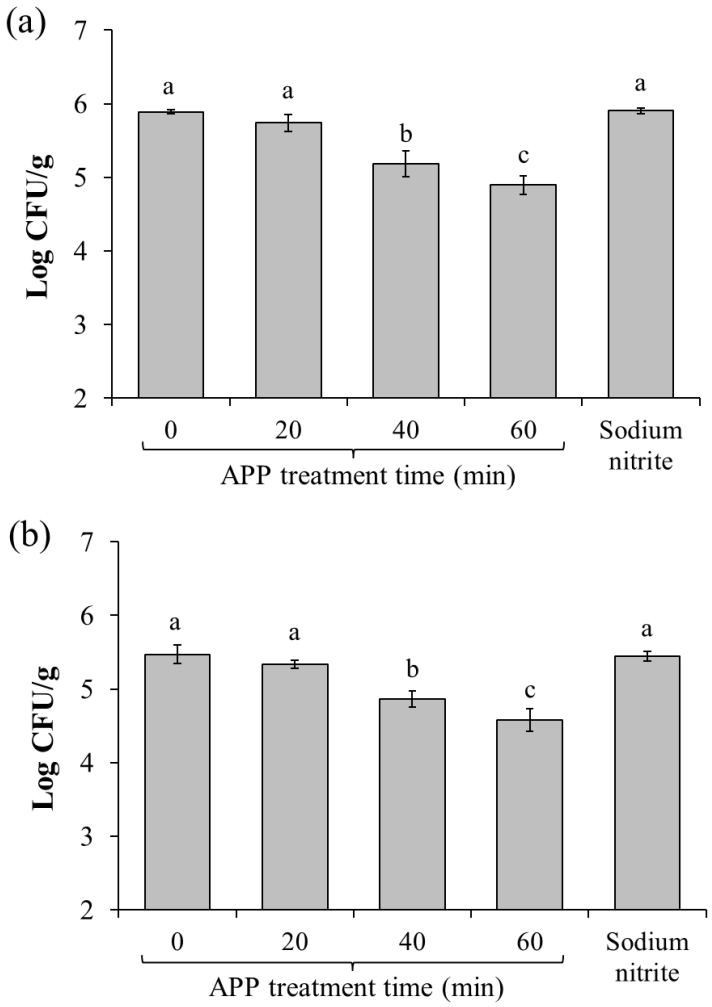

Fig. 5. The number (Log colony-forming units (CFU)/g) of (a) *Staphylococcus aureus* and (b) *Bacillus cereus* on pork jerky made with atmospheric pressure plasma (APP) and sodium nitrite, respectively. [a-b]Different letters indicate significant differences (P<0.05).

4.3.3. Experiment III

4.3.3.1. Inoculation test

During pork jerky manufacturing, bacteria that have relatively low thermal death points are inactivated or destroyed at the drying temperatures. However, both *Staphylococcus* and *Bacillus* are thermoduric bacteria (Walsh et al., 2012), and therefore, *S. aureus* and *B. cereus* were selected to examine the antimicrobial effect of APP in pork jerky manufacturing. The number of *S. aureus* were 5.89, 5.74, 5.18, and 4.90 Log CFU/g, and the number of *B. cereus* were 5.47, 5.33, 4.86, and 4.58 Log CFU/g in pork jerky made with APP for 0, 20, 40, and 60 min, respectively (Fig. 5). Jerky made with sodium nitrite showed a significantly higher number of the pathogens than those made with APP for 40 and 60 min. As the nitrite contents of the jerky made with sodium nitrite and jerky made with APP for 40 min were similar, this suggests that compounds other than nitrite contributed to the antimicrobial effect.

APP is recognized as a novel non-thermal food processing technology that uses energetic, reactive gases to inactivate microbes on various foods. The bactericidal effect of APP is caused by a combination of multiple mechanisms (Niemira, & Gutsol, 2011; Yong et al., 2015), including ROS production. The ROS produced by APP perniciously interacts with vital cellular molecules, such as proteins, enzymes, and DNA. Interaction between ROS and the cell membrane causes the formation of unsaturated fatty acid peroxides and oxidation of amino acids, potentially altering cell membrane function (Misra et al., 2016). Laroussi et al. (2003) reported that APP-initiated cell lysis through membrane disruption causes its antimicrobial effect. However, it should be noted that the bactericidal effect of APP would vary greatly depending on numerous factors, including the plasma

discharge type, operating conditions, choice of gas, humidity, state of the sample, and the microorganisms present (Misra & Jo, 2017).

In this study, the use of APP in pork jerky manufacturing showed the potential for pathogen inactivation. Further studies are required to develop an optimally efficient APP system with increased bactericidal effect.

4.4. Conclusion

APP treatment produced nitrite from the remaining brine on marinated pork, which may affect the surface a^* value and nitrosoheme-pigment content in pork jerky. Jerky made with APP for 40 min showed no differences in L^*, a^*, and b^* values, nitrosoheme-pigment, lipid oxidation, shear force, and A_w compared to jerky made with sodium nitrite (100 ppm of meat weight). In addition, the application of APP improved the microbiological safety of pork jerky. Therefore, APP could be a potentially safer alternative method for pork jerky manufacturing without added sodium nitrite.

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CHAPTER V.

An innovative curing process with plasma-treated water for production of loin ham and for its quality and safety

5.1. Introduction

Plasma-treated water (PTW), which means water subjected to plasma discharge in air, has gained increasing attention in several fields, including disinfectants, preservatives, and fertilizers (Misra et al., 2016; Ma et al., 2015; Park et al., 2013). Various uses of PTW are constantly being suggested owing to its pH and the presence of certain chemical species, especially nitrite (NO_2^-), nitrate (NO_3^-), and hydrogen peroxide (H_2O_2) when N_2 is present in plasma discharge gas (Burlica et al., 2010; Ma et al., 2015; Naïtali et al., 2010; Misra et el., 2016).

In general, nitrite content is decreased while nitrate content is increased in PTW with increasing post-discharge time. This is because subsequent reactions in PTW result in disproportionation of nitrite ($pK_a = 3.3$) into nitrate under acidic conditions: $3NO_2^- + 3H^+ + H_2O \rightarrow 2NO + NO_3^- + H_3O^+$ (Machala et al., 2013; Park et al., 2013). Nonetheless, some studies revealed that nitrite content is maintained during storage when the source liquid is alkaline buffered solution prior to production of PTW (Jung et al., 2015a; Machala et al., 2013; Park et al.,

2013). In other words, PTW with adjusted nitrite content can be produced when needed.

Nitrite, an important chemical species in various industrial areas, is also used in meat products because of the following functions: (i) development of a characteristic red color, (ii) protection against food-poisoning bacteria, including *Clostridium botulinum*, (iii) inhibition of lipid oxidation because of a strong antioxidant activity, and (iv) formation of the characteristic cured meat flavor. Therefore, synthetic nitrites, including sodium or potassium nitrite, have been used as nitrite sources in meat products for decades (Honikel, 2008; Parthasarathy & Bryan, 2012).

Increasing number of consumers is avoiding synthetic additives owing to the growing concern about food additives in recent years. Consequently, meat products cured with a natural nitrite source have attracted much attention in the industry (Alahakoon et al., 2015; Sebranek & Bacus, 2007; Sebranek et al., 2012). Vegetable powders such as celery, lettuce, or beet powders contain 1,500–2,500 mg nitrate/kg. These powders, along with nitrate-reducing bacterial culture, are commercially used in the manufacturing meat products for the functions of nitrite; however, they are not the best alternatives because the incubation steps to reduce nitrate conversion to nitrite are costly and time-consuming (Alahakoon et al., 2015; Sebranek et al., 2012). In addition, vegetable powders cannot be used in injected meat products. Since bacterial culture is not soluble in the brine solution, the culture does not distribute well within meat during the injection process and uncured zones occur in the final product (Sebranek & Bacus, 2007).

As an alternative to synthetic nitrite and natural nitrite sources (vegetable powder), PTW with adjusted nitrite content was suggested for use in emulsion-

type sausage (Jung et al., 2015b). Our previous work proposed that PTW is neither a chemical reagent nor a natural nitrite source but can be classified as purified water containing nitrite (Jung et al., 2015b). Using PTW in meat product is also referred to Misra and Jo (2016) with more details about plasma and food. To extend industrial utilization of PTW, it is necessary to demonstrate suitability of PTW for manufacturing all types of meat products. Compared to that in emulsion-type sausage, emulsifying and mixing processes with meat and other additives are not required in injected meat products. Thus, it is difficult to uniformly cure an injected meat product with good quality (Sebranek & Bacus, 2007). The purpose of the present study was to compare the quality and microbial safety of injected loin ham, cured with sodium nitrite or PTW. In addition, genotoxicological safety of the products was evaluated.

5.2. Materials and Methods

5.2.1. Product manufacture

5.2.1.1. APP treatment

The plasma source used in the experiment consists of a powered electrode, ground electrode, and a dielectric plate between the two electrodes (Fig. 1). All materials in plasma source and plasma generation conditions were same as those used in our previous study (Jung et al., 2015a). A bipolar square-waveform voltage at 15 kHz was applied to the powered electrode while the other electrode was grounded. Then, an ambient air discharge was generated at the surface of the electrode. The distance between the ground electrode and liquid surface was 5 cm. In order to obtain visible emission spectrum of the APP system, optical fiber was used near the plasma discharge and recorded using a spectrometer (MAYA2000 Pro, Ocean Optics, Inc., FL, USA).

5.2.1.2. Preparation of PTW

To produce PTW, distilled water (500 mL, pH 6.5) containing 1% sodium pyrophosphate (w/v) was treated with the APP for 2 h. The sodium pyrophosphate was added to prevent the decrease of pH in PTW because the amount of nitrite ion decreased in acidic PTW. Prepared PTW was used in the next day (approximately 24 h later) to produce loin ham.

The absorption spectra of nitrite and nitrate show two distinct regions (Krishnan & Guha, 1934). Thus, nitrite and nitrate content in PTW was measured by monitoring the absorption in the wavelength range from 270 to 400 nm as described previously (Jung et al., 2015a). UV–visible absorption system consisting of the continuum light source (ISS-UV–VIS, Ocean optics Inc., Florida, USA), spectrometer (MAYA2000 Pro, Ocean optics Inc.), and quartz cuvette (CV-Q-10, Ocean optics Inc.) was used in order to obtain the absorption spectra.

The pH values of PTW was measured using a pH meter (SevenGo, Mettler-Toledo International Inc., Schwerzenbach, Switzerland).

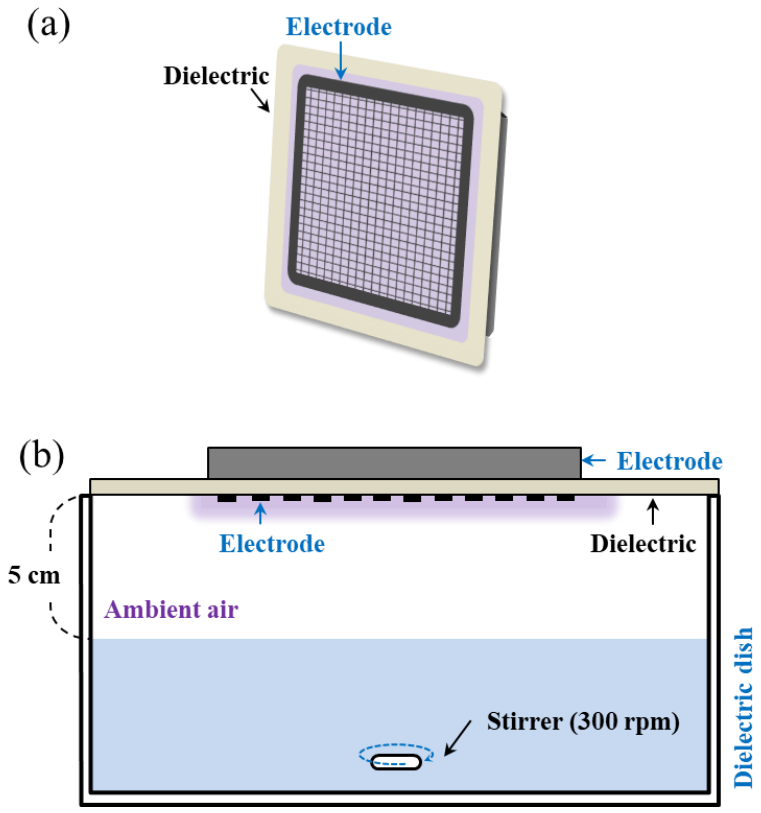

Fig. 1. Schematic drawing of the full experimental system for the generation of PTW (a), and detailed illustration of atmospheric pressure plasma (APP) actuator (b).

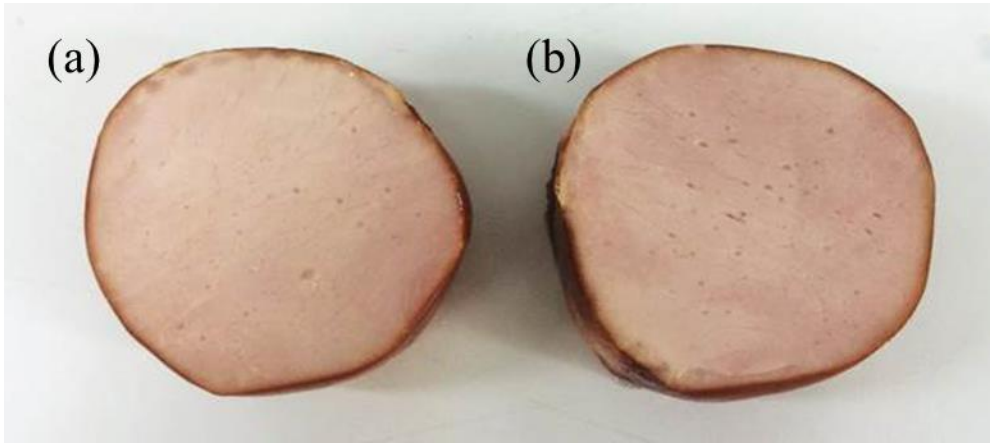

Fig. 2. Visual appearance of loin ham cured with sodium nitrite (a) and PTW (b).

5.2.1.3. Production of loin ham

Loin ham was produced using raw pork loin (*M. Longissimus dorsi*) obtained from a commercial butcher 2 days postmortem. Two brine solutions were designed to obtain the following concentrations of ingredients, % by total injected weight (raw meat + added solution), in the injected meat: (i) sodium nitrite treatment; sodium nitrite 0.01, sodium pyrophosphate 0.20, water 23.02, sodium chloride 1.07, L-ascorbic acid 0.05, beef-flavored seasoning 1.07, white sugar 1.00, egg white 2.15; (ii) PTW treatment; PTW 20.00, water 3.23, sodium chloride 1.07, L-ascorbic acid 0.05, beef-flavored seasoning 1.07, white sugar 1.00, egg white 2.15. The concentration of nitrite ion in both solutions was maintained at 70 mg/kg. A multi-needle brine injector (HPI-236, Hyupjin Machine, Co., Ansan, Korea) was used to inject the brine solution to pork loin. Then, the injected pork loins were tumbled for 48 h at 4 °C and smoked until internal temperature of loin ham reached 70 °C. Visual appearances of final products were shown in Fig. 2. Each

loin ham sample was vacuum-packaged and stored at refrigerator temperature (4 °C). The quality and microbial safety of loin ham samples were analyzed after 0, 1, and 2 weeks of storage, except for nitroso heme-pigment analysis and mutagenicity assay.

5.2.2. Physicochemical properties

5.2.2.1. Instrumental color measurements

Surface color of loin ham were conducted on a colorimeter (CR-5, Minolta Camera Co., Osaka, Japan). The instrument was calibrated with a standard black-and-white plate before analysis. Next, L^*, a^*, and b^* measurements were taken at a random location in each sample. A more appropriate measure of color was obtained from the chroma ($C = \sqrt{a^2 + b^2}$) and hue ($H = \tan^{-1} b/a$) which were calculated from the a^*, and b^* values.

Using the L^* value, lightness or darkness of the sample can be determined where 100 is white, and 0 is black. The a^* value extends from green ($-a$) to red ($+a$) and the b^* value from blue ($-b$) to yellow ($+b$). Chroma (saturation index) refers vivid or dull color and is proportional to its intensity. Hue is an angle in a color wheel which is used to color description. An angle of 0° (or 360°) represents red hue, whereas angles of 90°, 180°, and 270° represent yellow, green, and blue hue, respectively (McGuire, 1992).

5.2.2.2. Absorption spectra of acetone extracts

After manufacturing, the loin ham (10 g) was placed in a brown bottle. Then, acetone (40 mL) and distilled water (3 mL) were added and mixed for 5 min. The

mixture was filtered through a Whatman filter paper No. 1 (Whatman International Ltd., Springfield Mill, Kent, England), and absorption scans of the solution were conducted from 380 to 600 nm at 1-nm increments, using a Model X-ma 3100 spectrophotometer (Human Co., Ltd., Seoul, Korea).

5.2.2.3. Residual nitrite content

This characteristic of loin ham was determined according to AOAC method 973.31 (AOAC, 1995).

5.2.2.4. Lipid oxidation analysis

First, lipid extraction was conducted according to Folch's extraction method (Folch et al., 1957). The extracted lipid sample was placed into a 100-mL Erlenmeyer flask, and we added 35 mL of an acetic acid:chloroform (3:2) mixture and 0.5 mL of a saturated potassium iodide solution. The mixture was kept in the dark for 5 min, after which distilled water (75 mL) was added. The solution was titrated with a 0.005N sodium thiosulfate ($Na_2S_2O_3$) solution, using a 1% starch solution (2.5 mL) as an indicator. The peroxide value (POV) was calculated by means of the following formula:

$$\text{POV (meq/kg)} = [(S - B) \times F \times 0.01] / SW \times 100$$

where S is the titration volume (mL) of 0.005N $Na_2S_2O_3$ in the samples, B is the titration volume (mL) of 0.005N $Na_2S_2O_3$ in the blank, F is the factor of the 0.005N $Na_2S_2O_3$ solution, and SW is the sample weight (g).

5.2.3. Total aerobic bacterial counts

A loin ham sample (5 g) was taken aseptically from each treatment group, transferred to a sterile plastic pouch, and homogenized for 2 min at room temperature with 45 mL of sterile saline, using a stomacher (BagMixer 400, Interscience Ind., St. Nom, France). Appropriate dilutions of the samples were prepared in sterile saline and plated onto tryptic soy agar (Difco Laboratories, Detroit, MI, USA). The agar plates were incubated at 37 °C for 48 h under aerobic conditions. The results were expressed as log numbers of colony-forming units per gram (Log CFU/g).

5.2.4. Mutagenicity assay

This assay was performed on ethanolic extracts of loin ham samples at time point zero (before storage) and PTW, respectively. Loin ham sample (100 g) was chopped and mixed with 900 mL of 70% ethanol for 8 h at 25 °C. The extracts were filtered using Whatman filter paper No. 4 (Whatman International, Ltd.). After that, ethanol was removed from the samples, using a vacuum evaporator (Rotary Vacuum Evaporator N-11 Eyela, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The extracts were lyophilized (Freeze dry system, Labconco, FreeZone 18, Kansas City, KS, USA) after being frozen and were kept in a freezer (−70 °C) before use. On the other hand, PTW was used without further process.

Salmonella mutagenicity assay uses *Salmonella* strains with preexisting mutations that leave the bacteria unable to synthesize the required histidine. Therefore, histidine dependent *Salmonella* Typhimurium strains TA98 (hisD3052/*rfa*/Δ*uvr*B/pKM101) and TA100 (hisG46/*rfa*/Δ*uvr*B/pKM101) were purchased from the Korea Institute of Toxicology KIT, Daejeon, Korea) and used.

When a mutagen is added to the agar plate with the strains, new mutations were allow to bacteria to synthesize histidine and grow in the absence of histidine (Maron & Ames, 1983). The potential mutagenic effects of the loin ham samples were assessed by the Ames test according to Maron and Ames (1983) and Lee et al. (2016).

5.2.5. Statistical analysis

One-way analysis of variance with a completely randomized design was performed using the procedure of the general linear model (GLM). Significance of differences among mean values was determined by Duncan's multiple-comparison tests in the SAS software, Release 9.4 (SAS Institute Inc., Cary, NC, USA), with the confidence level of $P<0.05$. Mean values and standard deviations were presented. All the experimental procedures were conducted in triplicate with two observation numbers.

5.3. Results and discussion

5.3.1. Emission spectrum of APP and chemical properties of PTW

In the APP discharge, NO_g, N_2 (second positive system), and N_2^+ (first negative system) molecular spectra were detected (Fig. 3). The majority of intense peaks were near the ultraviolet (UV) region (300–400 nm) in terms of emission. Hydroxyl radicals were expected to occur in the plasma used here but may not have been detected owing to their short lifetime. Generally, the main radicals present in plasma discharge are nitric oxide (NO·) and hydroxyl radicals (OH·) when ambient air serves as a working gas (Shimmura et al., 199). In these

conditions, the formation of nitrogen oxides and ozone are expected. Then, reactions of these molecules from the plasma with the aqueous liquid can result in generation of nitrites, nitrate, and hydrogen peroxide (Oehmigen et al., 2011). Initial concentration of both nitrite and nitrate in untreated water (1% sodium pyrophosphate in distilled water, w/v) was 0 ppm. After 2 h of APP treatment, the nitrite and nitrate content of PTW reached 782 ppm and 358 ppm, respectively. The pH of PTW was changed from 10.19 to 9.01 after APP treatment for 2 h.

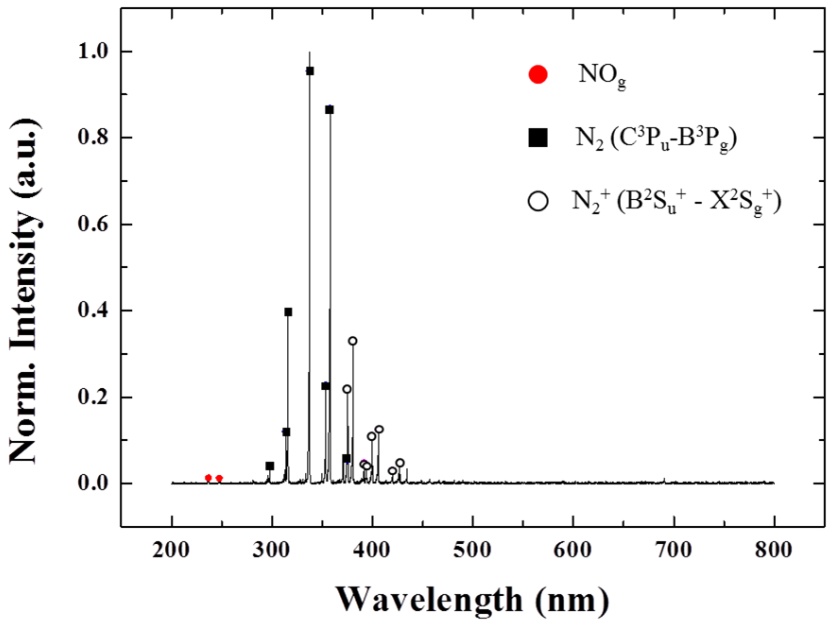

Fig. 3. An emission spectrum of the atmospheric pressure plasma (APP). NO, N_2, and N_2^+ molecular peaks were generated because ambient air was used.

5.3.2. Surface color and nitroso heme-pigment content

Ham cured with PTW showed no significant difference from sodium nitrite-treated ham in the L^*, b^*, and chroma values. Meanwhile, the a^* value was greater and hue angle was lower in the PTW-treated samples compared to those of sodium nitrite-treated ones during storage (Table 1). In other words, the ham manufactured by PTW showed higher redness (associated with high a^* value and low hue angle) compared to that of sodium nitrite.

Generally, cured meat product showed characteristic red color due to the reaction of myoglobin (Honikel, 2008). Myoglobin is the major contributor to meat color and is composed of an iron, heme-group, and globin protein. Iron in myoglobin is ligated with the four nitrogen atoms of the heme group and one nitrogen atom of globin protein. The last, 6^{th}, position of the iron remains available to bind electronegative atoms of various ligands. Therefore, oxygen, carbon monoxide, nitric oxide, and other molecules can bind to the iron in myoglobin, where binding of different ligands affords different meat colors (Alahakoon et al., 2015; Sebranek & Bacus, 2007).

In a meat product, nitrous acid (HNO_2) derived from nitrite can form nitrous acid anhydride, which is in equilibrium with nitric dioxide and nitric oxide. Then, nitric oxide can react with iron in myoglobin and form nitroso-myoglobin, which is responsible for the distinct red cured color (Honikel, 2008; Parthasarathy & Bryan, 2012). Even though the protein moiety of nitroso-myoglobin is denatured by heat treatment, the nitroso-hemochromogen (nitroso heme-pigment) persists and shows a stable red color (Honikel, 2008).

Table 1. Surface color of ham cured with different nitrite sources.

Treatment	Storage (weeks)			SEM[1)]
	0	1	2	
L* **value**				
Sodium nitrite	71.03	71.00	71.08^{y}	0.354
PTW	71.51^{ab}	70.55^{b}	73.23^{ax}	0.549
SEM[2)]	0.466	0.406	0.507	
a* **value**				
Sodium nitrite	6.65^{y}	6.50^{y}	6.35^{y}	0.147
PTW	7.28^{x}	7.40^{x}	7.10^{x}	0.159
SEM[2)]	0.080	0.187	0.171	
b* **value**				
Sodium nitrite	9.64	9.57	9.57	0.092
PTW	9.50	9.64	9.39	0.182
SEM[2)]	0.214	0.110	0.065	
Chroma				
Sodium nitrite	11.71	11.57	11.48	0.127
PTW	11.97	12.15	11.77	0.207
SEM[2)]	0.202	0.182	0.122	
Hue				
Sodium nitrite	55.42^{x}	55.82^{x}	56.47^{x}	0.583
PTW	52.50^{y}	52.51^{y}	52.90^{y}	0.581
SEM[2)]	0.507	0.555	0.672	

[1)]Standard error of the mean (n = 9), [2)](n = 6).

[a–c]Values with different letters within the same row differ significantly ($P < 0.05$).

[x,y]Different letters within the same column indicate that the values differ significantly ($P < 0.05$).

To identify different redness intensity levels, absorption spectra of acetone extracts from different loin ham samples were examined (Fig. 4). Maximal absorption was obtained approximately at 540 and 574 nm, corresponding to the pattern of the nitroso heme-pigment (Parolari et al., 2003). In addition, PTW treatment yielded higher maximal absorbance, which means higher nitroso heme-pigment content in comparison with that yielded by the sodium nitrite treatment. High redness of ham cured with PTW may be due to the nitroso heme-pigment content.

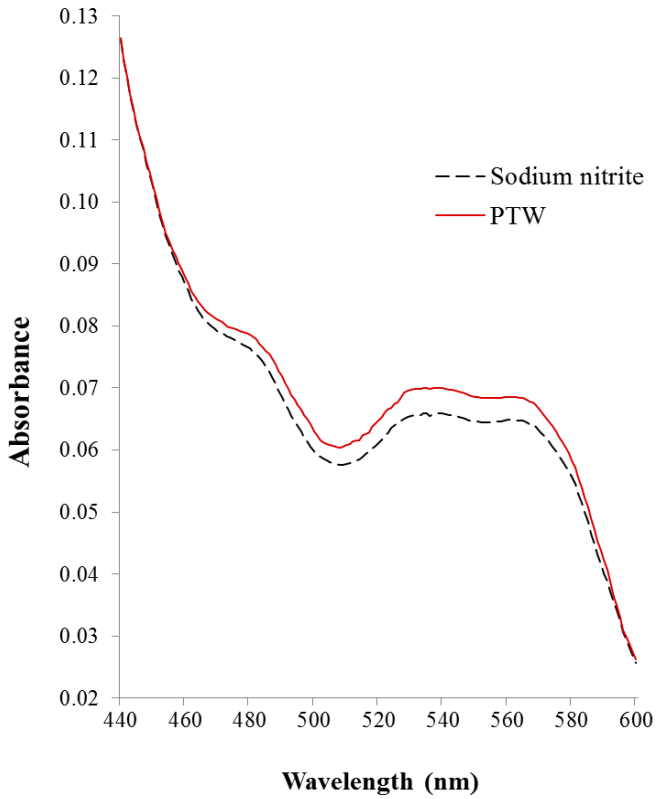

Fig. 4. Absorption spectra of acetone extracts of loin ham after manufacturing.

5.3.3. Residual nitrite content

This parameter was lower in the loin ham made with PTW than in the sodium nitrite-treated ham every week during storage, even though the same amount of nitrite ion was added initially (Table 2). Honikel (2008) reported that residual nitrite content in a meat product decreases when more nitrite is converted to nitrogen oxide (NO). Next, increased amounts of nitrogen oxides react with myoglobin and form more of the nitroso heme-pigment. In the present study, a larger amount of the nitroso heme-pigment was actually produced in the PTW-treated ham compared to that in the sodium nitrite-treated ham (Fig. 3). According to the results, nitrite may be more easily reduced to nitric oxide with PTW treatment than with sodium nitrite treatment. Jung et al. (2015a) reported that either the conversion of nitrite to nitric oxide or the reaction of nitrite with ascorbic acid (one of added reductants) is rapid when added nitrite is dissolved in a solution (PTW) rather than in the solid state (sodium nitrite or celery powder). However, the residual nitrite content was lower in the ham cured with PTW than that with sodium nitrite, although the sodium nitrite was dissolved in water and used as same form as PTW.

Some studies have also shown that irradiation reduces residual nitrite content of meat products (Ahn et al., 2003). Simie (1983) reported that nitrite downregulation by irradiation is due to its reaction with the hydroxyl radical resulting from the radiolysis of water. The hydroxyl radical can also be present in PTW, but it is not known whether it survives until the use in a meat product owing to the short lifetime (approximately 10^{-9} s) (Aikens & Dix, 1991; Burlica et al., 2010). Further in-depth research is necessary to elucidate the exact reason for the lower residual nitrite content in ham samples subjected to PTW treatment.

Residual nitrite content of loin ham samples in both treatment groups decreased after 2 weeks of storage (Table 2). Alahakoon et al. (2015) showed that residual nitrite content in meat products gradually declines during storage because of light- or oxidation-induced fading. Meanwhile, Ahn et al. (2003) demonstrated that residual ascorbic acid converts nitrite, resulting in a decrease in residual nitrite content in a meat product during storage.

The safety of a cured meat product is a significant issue for two reasons that are related to residual nitrite (Honikel, 2008; Sebranek & Bacus, 2007). First, nitrite is an effective antimicrobial agent, particularly for preventing toxin production by *C. botulinum*. After addition of nitrite, nitric oxide can react with iron-sulfur proteins (in bacteria), which are necessary for energy production (Alahakoon, 2015). For this reason, a proper amount of residual nitrite should be maintained in a meat product for antibotulism protection. In contrast, residual nitrite content is a known health risk factor because of potential formation of carcinogenic nitrosamines (Ahn et al., 2003; Pegg & Shahidi, 2008). The nitrosamines can be produced from secondary amines with nitrite in a specific condition such as high temperature (>130 °C) and acidic pH. A number of consumers are interested in lower residual nitrite content, despite the low probability of occurrence of nitrosamines in meat products (Honikel, 2008). In both regards, residual nitrite content should be carefully controlled to ensure product safety.

Table 2. Physicochemical and microbiological properties of loin ham cured with different nitrite sources.

Treatment	Storage (weeks)			SEM[1]
	0	1	2	
Residual nitrite (ppm)				
Sodium nitrite	24.68^{ax}	23.30^{ax}	20.38^{bx}	0.866
PTW	14.96^{ay}	13.93^{aby}	10.36^{by}	0.503
SEM[2]	0.834	0.409	0.801	
Total aerobic bacteria (Log CFU/g)				
Sodium nitrite	4.21^{cx}	6.25^{b}	6.68^{a}	0.055
PTW	3.88^{cy}	6.14^{b}	6.52^{a}	0.047
SEM[2]	0.053	0.039	0.058	
Peroxide value (meq/kg)				
Sodium nitrite	1.17^{b}	1.80^{a}	1.55^{ab}	0.142
PTW	1.27^{b}	1.96^{a}	1.82^{a}	0.090
SEM[2]	0.132	0.102	0.057	

[1]Standard error of the mean (n = 9), [2](n = 6).

[a–c]Values with different letters within the same row differ significantly ($P < 0.05$).

[x,y]Different letters within the same column indicate that the values differ significantly ($P<0.05$).

3.4. Total aerobic bacteria

The mechanisms of interaction between PTW and bacteria are not fully understood. Nonetheless, most authors agree that the bactericidal effects of PTW are predominantly due to hydrogen peroxide, nitrites, nitrates, peroxy-nitrites, and pH changes (Machala et al., 2013). Honikel (2008) hypothesized that among these antimicrobial reagents, long-lived secondary products such as hydrogen peroxide, nitrite, or nitrate are responsible for the extended antimicrobial effects of PTW. Therefore, an alkaline buffered solution was treated by plasma, and the resulting PTW contains high concentrations of nitrite and hydrogen peroxide (Honikel, 2008; Machala et al., 2013). On the other hand, only a small reduction in the number of *Escherichia coli* cells (<0.5 Log CFU) was achieved by means of PTW. Likewise, a weak antimicrobial effect was observed when PTW made from an alkaline solution was applied to *Hafnia alvei* suspension for up to 30 min (Naïtali et al., 2010).

PTW in the present study was also made from an alkaline solution and showed a weak antimicrobial effect. In Table 2, the initial number of total aerobic-bacteria cells in the PTW-treated ham samples was 0.33 Log CFU/g lower in comparison with sodium nitrite-treated samples. At 1 and 2 weeks of storage, no significant differences were observed in the number of total aerobic-bacteria cells between the two treatments.

5.3.5. Lipid oxidation

If free radicals, hydrogen peroxide, or reactive oxygen and nitrogen species exist in PTW used in the present study, they should initiate lipid oxidation in a meat product (Yong et al., 2017). In contrast, no significant differences were

observed in the peroxide value between PTW- and sodium nitrite-treated samples throughout the entire period of storage. After 1 week of storage, peroxide values in both treatment groups increased (Table 2). Peroxide is formed as a primary product during lipid oxidation (Sindelar & Milkowski, 2012).

Lipid oxidation in meat products is prevented by nitric oxide derived from nitrite. This is because nitric oxide can bind to the iron in meat pigments and lower the amount of free iron, which is a potent catalyst of lipid oxidation (Alahakoon et al., 2015; Sindelar & Milkowski, 2012). In the present study, more nitric oxide reacted with myoglobin and formed more of the nitroso heme-pigment (Fig. 3). Nonetheless, the difference in nitroso heme-pigment content may not be sufficient to detect a significant difference in lipid oxidation between the two treatments during 2 weeks of storage. When emulsion-type sausage was cured with PTW, no significant differences in peroxide values were observed in comparison with emulsion-type sausage cured with sodium nitrite during the 28 days of storage (Jung et al., 2015a).

Table 3. *Salmonella* mutagenicity assay for loin ham cured with different nitrite sources.

Treatment	Dose (μg/plate)	Number of revertant colonies (His+) per plate[1)]			
		TA98 (-S9)	TA98 (+S9)	TA100 (-S9)	TA100 (+S9)
Sodium nitrite	188	30±7	39±5	329±47	323±41
	375	22±2	32±6	323±33	365±23
	750	34±3	30±6	385±44	468±4
	1,500	32±4	34±4	361±51	341±15
	3,000	33±6	29±3	341±65	456±50
PTW	188	16±5	23±9	293±85	282±19
	375	24±12	32±4	317±59	329±61
	750	22±4	33±1	338±72	338±40
	1,500	19±6	28±8	291±6	308±6
	3,000	20±3	27±7	332±29	346±13
Negative control[2)]	EtOH	22±3	21±5	294±13	301±25
Positive control[2)]	4-NQO	1108±22			
	2-AA		2214±48		
	SA			902±96	
	2-AA				2423±108

[1)]Values are the mean ± SD (P<0.05).

[2)]EtOH, 70% ethanol; 4-NQO, 4-nitroquinoline-1-oxide; SA, sodium azide; 2-AA, 2-aminoanthracene.

Table 4. *Salmonella* mutagenicity assay for PTW

Sample		Number of revertant colonies (His+) per plate[1)]			
		TA98 (-S9)	TA98 (+S9)	TA100 (-S9)	TA100 (+S9)
PTW		18±5	26±3	204±7	266±25
Negative control	Distilled water	17±4	22±4	323±21	338±33
Positive control[2)]	4-NQO	1063±14			
	2-AA		2055±95		
	SA			861±88	
	2-AA				2343±112

[1)]Values are the mean ± SD (P<0.05).

[2)]4-NQO, 4-nitroquinoline-1-oxide; SA, sodium azide; 2-AA, 2-aminoanthracene.

5.3.6. Mutagenicity assay

The Ames *Salmonella* mutagenicity test is a short-term bacterial reverse mutation assay designed to detect a wide range of chemicals that can generate genetic damage and lead to gene mutations (Kim et al., 2016). In the mutagenicity assay, a sample being tested is positive for mutagenicity when the number of revertant colonies is higher than that in the negative control (McGuire, 1992). As shown in Table 3, the number of revertants per plate for the sodium nitrite and PTW treatments was almost the same as that in the negative control. In other words, loin ham cured with sodium nitrite or PTW at doses of up to 3,000 μg/plate is not mutagenic. The numbers of revertants per plate in positive controls were

20- and 3-fold higher than those in the samples tested, which means that the experiment was performed properly (Maron & Ames, 1983).

PTW used in this study was found to be not genotoxic according to the *Salmonella* mutagenicity assay (Table 4). Addition of PTW to emulsion-type sausage has no mutagenic effect either (Kim et al., 2016). As for immune toxicity, Balb/c mice were given free access to sausage cured with PTW; 32 days later, tumor necrosis factor (TNF)-α levels were evaluated. As a result, a TNF-α value less than 10 μg/mL was detected in mice eating control and treated samples, respectively. This finding indicates that no inflammatory response is triggered in mice consuming sausage cured with PTW (Kim et al., 2016).

5.4. Conclusion

The present study was aimed to see the possibility of PTW as an alternative of synthetic sodium nitrite in processed meat manufacturing such as loin ham. From the results, color-developing capacity is higher and residual nitrite content is lower in the ham treated with PTW than in that treated with sodium nitrite. Genotoxicological safety of the loin ham manufactured with PTW was confirmed by the Ames test. Because there has been no effective substitute for synthetic nitrite in cured meat processing so far, particularly injection type meat products due to solubility, PTW can be considered as a suitable and cost-effective alternative to synthetic nitrite or nitrite-containing vegetable powders for natural curing process.

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CHAPTER VI.

Overall Conclusion

APP can effectively increase the safety of raw meat via pasteurization of pathogenic bacteria, but minimal changes in color properties were observed. Occurrence of green color in APP treated raw meat could be resulted from ntrimyoglobin formation. However, when reducing agent is added, green discoloration can be prevented while desirable red color can be induced after APP treatment. Similarly, when reducing agent (ascorbic acid) was added to pork followed by APP treatment, red color was developed in the sample. Accordingly, pork jerky can be produced with APP treatment as a substitute method of sodium nitrite. Furthermore, plasma treated water (PTW) could be used as an effective and innovative substitute for synthetic nitrite in loin ham manufacturing without compromising on quality changes. Therefore, APP is a promising technology for use in the meat and meat product industry as a non-thermal pasteurization and other technical advantages such as alternative curing method.

Summary in Korean

저온 대기압 플라즈마 처리에 의한 식육의 변색 메커니즘 규명 및 활용

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최근 소비자들은 식품에대한 안전과 건강을 중시하며 가능한 천연 상태의 제품을 요구하고 있다. 즉, 소비자들은 *Salmonella* Typhimurium 등의 병원성 미생물로부터 안전성을 보장하면서도 "최소가공", "합성 첨가물 무첨가"라는 식품 가공기술의 혁신을 요구하고 있고, 이는 식품 산업으로 하여금 새로운 기술 개발을 요구하는 실정이다. 식품의 식중독·부패 방지 및 품질 유지를 위한 기술로서 활발이 연구되고 있는 것은 비가열 가공 기술이다. 실제로 식품 산업에서 이용되고 있는 비가열 가공 기술로 초고압, 감마선, 전자선, X 선. 및 자외선 등이 있다. 하지만, 해당 기술들은 비용이 많이 들거나 소비자 수용성이 낮다는 각각의 단점을 가지고 있다.

비가열 가공기술로서 최근 큰 관심을 받고 있는 것은 대기압 플라즈마 기술이다. 플라즈마란 물질의 제 4 상태로서 고체, 액체, 기체 다음의 이온화된 기체 상태를 의미한다. 경제적 및 기술적

효율성을 극대화시킨 저온 대기압 플라즈마 장치의 개발은 식육을 포함하는 식품의 살균 기술 연구 분야에서 큰 성장을 가져왔다. 하지만, 대기압 플라즈마를 이용한 미생물 제어 연구의 성장에 비해 식육 및 육가공품의 품질 변화에 관한 심도있는 연구는 미미한 실정이다. 특히, 대기압 플라즈마에 의한 육색 변화를 확인, 규명, 활용하는 것은 발표된 바가 없는 매우 희소한 연구분야이다.

따라서 본 연구에서는 대기압 플라즈마 처리에 의한 육색의 변색 기작을 규명하고 이를 활용하는 연구를 다음과 같은 순서로 진행하였다. 1) 생육에 대한 대기압 플라즈마(Atmospheric pressure plasma, APP)의 살균 효과를 조사하고 서로 다른 APP 처리 시간에 따른 생육 품질 변화 및 유전 독성 학적 안전성을 관찰하였으며, 2) APP 처리 후 생육의 녹색화 현상을 설명하기 위하여 APP 처리에 따른 미오글로빈의 변색 원인을 규명하고 제어하고, 3) APP 처리 후 돈육의 적색 발현의 가능성을 확인하고 APP 를 이용하여 돈육포를 제작, 물리 화학적 특성 및 미생물 학적 안전성을 평가하였으며, 마지막으로 4) 합성 아질산 나트륨 대체제로서 플라즈마 처리수(Plasma treated water, PTW)를 이용하여 제작한 등심햄의 품질, 미생물 안전성, 유전 독성학적 안전성 평가를 진행하였다.

실험 1 에서는 APP 를 처리한 닭 가슴살의 미생물학적 안전성, 품질 변화 및 유전독성을 조사하였다. 10 분간의 플라즈마 처리 후, *Listeria monocytogenes*, *Escherichia coli*, 및 *Salmonella* Typhimurium 의 수는 각각 2.14, 2.73 및 2.71 Log CFU/g 감소했다. APP 처리 시간이 증가함에 따라, 육색의 경우 L^*(명도) 및

b^*(+황색/−청색)는 증가한 반면 a^*(+적색/−녹색)는 유의적으로 감소했다. 지질 산패도는 플라즈마 처리에 의해 영향을 받지 않았다. 또한, 대조군과 APP 처리군 사이의 조직감에는 유의적인 차이가 없었다. 플라즈마 처리된 닭 가슴육을 *Salmonella* 변이주를 이용한 유전독성학적 안전성을 확인한 결과 유전독성이 발견되지 않았다. 결론적으로, APP 처리는 닭가슴육의 미미한 육색 변화를 유발하였지만, 다른 품질학적 변화 없이 미생물학적 안전성을 증가시켰으므로 비가열 가공 기술로서 이용 가능성이 확인되었다.

육색은 소비자의 제품 구매에 영향을 미치는 가장 중요한 품질 특성이다. 이러한 육색에 영향을 미치는 가장 중요한 내재적 요인은 미오글로빈으로 알려져 있다. 따라서, 실험 2 에서는 APP 에 의한 식육의 녹색 변이 메커니즘을 규명하기 위하여 APP 가 미오글로빈의 색에 미치는 영향을 규명하고 제어하고자 하였다. 일반적으로, 미오글로빈에서 유도되는 녹색의 헴 색소(heme pigment)로는 sulfmyoglobin, choleglobin, verdoheme, nitrihemin 또는 nitrimyoglobin 등이 있다. 인산 완충액에 용해된 미오글로빈을 APP 에 20 분 동안 노출 시켰을 때, L^* 및 a^* 값은 유의하게 감소하고 b^* 값은 증가하여 미오글로빈의 녹색 변이가 관찰되었다. 자외선 흡수 스펙트럼 측정 결과, APP 처리된 미오글로빈은 503 및 630 nm 에서 흡수 피크를 나타내었다. Sulfmyoglobin 또는 choleglobin 은 전형적인 자외선 흡수 스펙트럽을 보이나, 위 결과는 sulfmyoglobin 과 choleglobin 의 스펙트럼이 아니며 해당 헴 색소들이 형성되지 않았다고 판단된다. APP 처리 후 미오글로빈의 단백질 2 차 구조 및 분자량 또한 변하지 않았으므로, verdoheme 또는 nitrihemin 이

형성되지 않음이 확인되었다. 이후 nitrimyoglobin 의 형성 가능성을 확인하기 위하여 APP 처리된 미오글로빈의 아질산(nitrite), 과산화수소(H_2O_2) 및 히드록실 라디칼(·OH)의 함량을 측정하였다. 해당 화합물들은 APP 처리 시간이 증가함에 따라 그 함량이 증가하여 nitrimyoglobin 이 형성될 수 있는 환경을 제공하였다. 미오글로빈 용액에 강력한 항산화제인 sodium dithionite 을 0.1% 첨가 후 APP 를 처리한 경우, 미오글로빈의 녹색 변이 현상이 줄어들었다. 반면, 미오글로빈 용액에 sodium dithionite 를 0.5% 첨가 후 APP 를 처리한 결과 녹색이 아닌 적색이 확인되었다. 적색으로 변한 미오글로빈의 흡수 스펙트럼으로 측정 결과, nitrosomyoglobin 이 형성 되었음을 관찰하였다. Nitrosomyoglobin 은 염지 육제품의 붉은 색 발현에 관여하는 헴 색소이다. 결과적으로, APP 처리 된 미오글로빈에서 녹색 색상의 발생은 APP 에 의해 발생한 nitrite 에 의한 nitrimyoglobin 형성 때문이며, 항산화제(sodium dithionite)의 첨가는 APP 에 의한 미오글로빈의 녹색 변이를 방지하고 적색을 유도하였다. 본 연구는 APP 에 의한 미오글로빈의 변색 메커니즘을 규명한 최초의 연구로서, APP 에 의한 식육의 품질 변화에 대한 중요한 기초 연구라고 판단된다.

최근 소비자들은 화학적 합성 첨가물인 아질산염(sodium nitrite)을 기피하는 경향을 보이고 있다. 아질산염은 육가공품 내 염지육색의 발현(적색, nitrosomyoglobin 형성), *Clostridium botulinum* 에 대한 정균작용, 육제품의 풍미 향상, 산패취 발생 감소 등의 중요한 역할을 하는 필수적인 첨가제이다. 하지만 육가공 업체들은 소비자들의 요구에 맞춰 합성 아질산염을 천연소재로 대체한 육제품을

개발하고자 노력하고 있다. 실험 3 에서는 먼저, 아스코르빈산 첨가 및 APP 처리에 의한 돈육의 적색 발현 가능성을 확인하였다. 이후, 돈육포 제조공정 중 아질산염의 대안으로 APP 처리의 적용 가능성을 조사했다. 먼저, 아질산염으로 절인 돈육과 아질산염 없이 절인 돈육을 준비하였고, 후자는 APP 를 20, 40, 60 분간 처리하였다. 준비된 두 종류의 돈육은 건조 및 냉각 과정을 거쳐 육포를 제작하였다. 실험 결과, APP 처리 시간이 증가함에 따라, 돈육포의 지질 산패도가 감소하는 반면, a^* 값, nitroso-헴 색소 함량 및 잔류 아질산염 함량이 증가했다. 아질산염을 이용하여 제조한 돈육포와 비교하여 APP 를 40 분간 처리하여 육포에서 유사한 색 및 품질 특성이 관찰되었다. 접종 실험 결과, APP 를 40 및 60 분 동안 처리하여 제조한 돈육포의 *Staphylococcus aureus* 와 *Bacillus cereus* 의 수는 아질산염 첨가군에 비해 유의적으로 낮았다. 따라서, APP 는 아질산염을 첨가하지 않고 잠재적으로 안전한 돈육포를 제조하는 데 적용할 수 있다.

실험 4 에서는 합성 아질산염의 대안으로 대기압 플라즈마 처리 수 (PTW) 의 등심햄 내 적용 가능성을 확인하였다. 이를 위하여 아질산염이 첨가된 염지액과 PTW 를 이용한 염지액을 각각 이용하여 등심 햄을 제조하였다. 아질산염 첨가 등심햄과 비교하여 PTW 첨가 등심햄의 a^* 값은 높았으며 잔류 아질산염 함량이 낮게 나타났다. 저장 0 일차에서 총 호기성 미생물 수는 PTW 첨가 등심햄이 3.88 Log CFU/g 로서 아질산염 첨가 등심햄 보다 0.33 Log CFU/g 낮았다. L^* 과 a^*값은 두 처리군 사이에서 유의적인 차이가 발견되지 않았다. 또한, PTW 첨가 등심햄의 유전독성학적 안전성을 확인하였다. 본 연구

결과, 등심햄 제조 공정에서 PTW 는 합성 아질산염의 효과적이고 혁신적인 대체재로서 이용될 수 있다.

해당 연구들을 통하여 대기압 플라즈마에 의한 육색 변화를 확인, 규명, 활용 하였다. 또한, 대기압 플라즈마를 축산 식품의 살균 기술 및 염지 기술로서 이용 할 수 있는 다양한 가능성을 제시하였다.

주제어: 대기압플라즈마, 미오글로빈, 살균, 식중독균, 식육, 아질산, 안전성, 육가공품, 육색, 변색, Nitrimyoglobin, Nitrosomyoglobin
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