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Effects on the Qualities of Proteolysis to Beef by Non−coating and Coating Protease Treatment

Kwang-Il Kim, Sang-Yoon Lee, Soo-Jin Kim, Jae-Hee Seo, Joong-Kyu Lee, Jung-Kue Shin[1], Hyung-Yong Cho[2], and Mi-Jung Choi*

Department of Bioresources and Food Science, Konkuk University, Seoul 05029, Korea
[1]Department of Korean Cuisine, JeonJu University, JeonJu 55069, Korea
[2]Department of Food Science and Biotechnology, CHA University, Seongnam 13496, Korea

Abstract

This study was performed to improve the techniques used for tenderizing red meat as elderly food. Beef meat was immersed in liposome encapsulated enzyme solution and the effect of protease encapsulation on the beef properties was analyzed. The protease encapsulation properties were analyzed according to the size distribution and enzymatic activity. After enzyme reaction on the beef, the chemical properties of the meat such as pH, water holding capacity, shear rate, lipid oxidation and total volatile basic nitrogen (TVB-N) were analyzed. The pH of the beef increased during the reaction and coating protease (CP) was higher than non-coating protease (NCP). Total color differences were increased remarkably after 36 h and generally, the difference in CP was relatively lower than in NCP. WHC was significantly decreased within 24 h, and no effect from the protease coating was observed. Protease activity was significantly increased within 48 h and no differences in the enzyme coating were observed. The TVB-N value of NCP was increased within 24 h while CP was sustained for up to 36 h. The TVB-N value of protease treated meat increased after 36 h and no effect from the protease coating was detected. Consequently, liposome encapsulated protease was found to have similar properties as non-coated protease. Application of liposome seems to be an interesting option for injecting various functional materials without changing the properties of meat.

Keywords: liposome, protease, shear force, softening, beef

Introduction

Physiological dysfunctions in elderly people can be characterized by a reduction of appetite due to changes in the taste threshold and the reduction of digestion and absorption capacity (Zaden *et al*., 2015). Poor mastication and dysphasia are some of the most common problems and can lead to malnutrition in elderly people. A strong relationship between dysphagia and the negative health outcomes of malnutrition and pneumonia in elderly adults has been reported (Sura *et al*., 2012). The incidence rate of protein deficiency in the elderly is higher than in younger adults because of the inefficient protein metabolism of elderly individuals. Therefore, older adults require more protein than younger adults (Bauer *et al*., 2013; Brownie,

2006; Zaden *et al*., 2015) and a high protein diet improves body function and the quality of life in these individuals (Wolfe *et al*., 2008). Although a variety of elderly dietary guidelines have been developed, the elderly are still limited with regards to food selection. Moreover, Maitre *et al*. (2014) reported that eating difficulties have a stronger impact on malnutrition than the choice of food and this malnutrition can have several adverse effects on the body. Beef has a high nutritional value and plays an important role in the human body. Additionally, the nutritive components of beef are easy to digest and the proteins in beef have high biological value (Bruce *et al*., 1994). However, beef also contains a high quantity of fat (15%), with 30% of the fat comprised of saturated fatty acids. Although beef fat seems to contribute to a softer texture and marbling is an important factor for high quality classification of beef (USDA, http://www.ams.usda.gov/), beef fat is also related to several chronic diseases such as cardiovascular disease, heart disease, diabetes and cancers (Department of Health, 1994; Committee on Medical Aspects of Food

*Corresponding author: Mi-Jung Choi, Department of Bioresources and Food Science, Konkuk University, Seoul 05029, Korea. Tel: +82-2-450-3048, Fax: +82-2-450-3726, E-mail: choimj@konkuk.ac.kr

Policy, 1984). Consequently, the development of meat tenderization techniques seems to not only produce economically value-added products but also to produce more health oriented food. Mechanical tenderization (Savell *et al.*, 1977), moisture enhancement technology (Robbins *et al.*, 2002), injection of chemical ingredients such as sodium tripolyphosphate, sodium chloride (McGee *et al.*, 2003) and degradation using enzymes (Ashie *et al.*, 2002) are currently applied as tendering techniques. The enzymatic degradation technique involves increasing the tenderness of meat by the degradation of muscle fibers using exogenous enzymes. However, enzymatic degradation results in an undesirable flavor and texture due to broad substrate specificity and the breakdown of major muscle proteins (Ashie *et al.*, 2002; McKeith *et al.*, 1994; Pietrasik and Shand, 2011). Therefore, control of enzyme activity seems necessary to improve the degradation of meat texture and flavour. Nanoparticles are often used to control enzyme activity (Khoshnevisan *et al.*, 2011) by immobilized coating membrane. Liposomes are one of the encapsulation system which are vesicles consisted with phospholipid bilayers membrane (Hsieh *et al.*, 2002; Keller, 2001). The phospholipid bilayer membrane structure of liposomes guarantees to carry hydrophilic and hydrophobic components simultaneously (Hsieh *et al.*, 2002; Keller, 2001) within inner vesicle space and between bilayers membrane. Here, we prepared liposome with nano-scale particles to encapsulate enzyme as hydrophilic material.

This study was performed to develop softening methods for beef to create products that elderly people can easily masticate and swallow. The beef was treated with enzyme encapsulated liposome solution and the chemical properties were compared with non-encapsulated enzyme treated beef.

Materials and Methods

Materials

Eyes of round beef (Australia) were obtained from a commercial market (48 h postmortem, pH 5.7-5.9). All the visible fat and connective tissue was trimmed off, and the beef was cut into a rectangular form ($3\times3\times1$ cm, 12 ± 1 g) parallel to the direction of the muscle fiber. Protease (protease from *Aspergillus oryzae*, 500 U/g, Synonym: Flavourzyme® 500L) was purchased from Sigma Aldrich (USA). The soybean lecithin (Lipoid® S75, 68-73% phosphatidyl choline, 7-10% phosphatidyl ethanolamine) used as a coating agent was provided by Lipoid GmbH (Switzerland). All other chemicals used were of analytical grade.

Liposome preparation and sample treatment

To prepare liposome containing enzyme, 2% (w/v) protease and 2% (w/v) lecithin were dissolved in distilled/deionized water, respectively and then mixed at 1:1 (v:v). Liposomes were produced using a two stage homogenization procedure to obtain small droplets. Pre-liposomes were created with a high speed homogenizer (IKAT25 digital ULTRA-TURRAX®, Germany) at 12,000 rpm for 3 min. Then, the coarse liposomes were treated with an ultrasonificator at 200 W for 3 min. Using the above procedures, liposomes containing 1% protease with fine droplets were produced.

Beef ($3\times3\times1$ cm, 12 ± 1 g) was soaked in distilled/deionized water (Control), non-coated protease (protease 1%, NCP) or coated protease (liposome containing protease 1%, CP) at 4ºC for 48 h. Every 12 h, the beef was sampled and the physicochemical properties were analyzed.

Analysis of particle size and zeta potential

The average particle size, size distribution and zeta potential of the enzymes and liposomes were determined by dynamic light scattering using a Zetasizer Nano-ZS90 (Malvern Instruments, UK). All of the samples were properly diluted with distilled water prior to each measurement to avoid multiple light scattering effects. The particle size was indicated by cumulates mean (z-average) diameter and the size distribution was indicated by the polydispersity index (PdI). Since zeta potential is directly related to the electrophoretic mobility of the particles, the analyzer calculated the zeta potential from the measured velocity. All measurements were taken in triplicate.

pH

The pH of the treated (NCP and CP) and control samples were measured with a pH meter (S-220, Mettler-Toledo Co., Switzerland). Samples (5 g) from 3 meat strips were homogenized in 45 mL distilled water for 1 min and the pH of the sample homogenate was determined in triplicate.

Color

The color of the beef surface was determined using a color reader (CR-10, Konica Minolta Sensing Inc., Japan) calibrated with a white standard plate (L*=97.83, a*=0.43 and b*=+1.98). The CIE L*, a* and b* values were determined as indicators of lightness, redness, and yellowness, respectively. The total color difference (ΔE) between the control and the protease treated sample was numerically calculated with the following equation (eq. 1).

$$E = \sqrt{\Delta L*^2 + \Delta a*^2 + \Delta b*^2} \qquad (1)$$

Water holding capacity (WHC)

The WHC of the beef was determined using the method described by Hong *et al*. (2012). Approximately 1 g of beef (Ms) was placed in a 15 mL test tube along with gauze as an absorbent and centrifuged at 1,500×g for 10 min at 4ºC. The beef pellet was carefully removed from the tube, and the tube was weighed (M$_1$) then weighed again (M$_2$) after drying at 105ºC for 24 h. The WHC of the beef was calculated from the following equation (eq. 2).

$$\text{WHC(\%)} = \left(1 - \frac{M_1 M_2}{M_S}\right) \times 100 \qquad (2)$$

Shear force measurement

The shear force of the treated (NCP and CP) and the control beef was determined in quintuplicate using a texture analyzer (CT3, Brookfield Co., USA) equipped with a v-type plain probe. The conditions used for texture analysis were as follows: compression type, 10 kg force load cell, test speed of 2.5 mm/s, target deformation of 30%, and trigger load of 300 g. The test was repeated at least 10 times. The maximum peak force (kg) was used as an indicator of texture.

Protease activity

Protease activity was determined by Sigma's Non-specific protease activity assay (Sigma Aldrich, USA) with minor modifications. The beef was immersed in an enzyme-substrate complex solution and protease activity was measured every 12 h. The immersion solutions (0.5 mL) were incubated at 37ºC for 10 min and mixed with 2 mL of TCA reagent to stop the reaction. The samples were filtered using polyethersulfon syringe filters (0.45 μm), and the filtrates (1 mL) were mixed with 5 mL of 0.4 M Na$_2$CO$_3$ and 1 mL of 0.66 N Folin & Ciocalteu phenol reagent. The mixtures were incubated at 37ºC for 30 min and then the absorbance of the samples was measured by a spectrophotometer at a wavelength of 660 nm. For the standard curve, L-tyrosine served as the enzyme-substrate complex solution preceded by the same procedures as above.

Total volatile basic nitrogen (TVB-N)

The total volatile basic nitrogen (TVB-N) of the beef samples was measured by micro-diffusion analysis (Conway, 1933). Conway reagent was prepared by dissolving 2.5 mL of 0.066% (w/v) methyl red, 2.5 mL of 0.066% (w/v) bromocresol green, 5 g of H$_3$BO$_3$, and 100 mL of ethanol in 350 mL of distilled/deionized water. The samples (10 g) were homogenized at 17,000 rpm for 2 min with 30 mL distilled water. The homogenate was massed up to 100 mL and filtered using Whatman No 2 filter paper. The filtrate (1 mL) was mixed with 1 mL of saturated K$_2$CO$_3$ (60 g K$_2$CO$_3$ in 50 mL distilled/deionized water) as a releasing agent, and 1 mL of conway reagent was used as the trapping reagent. The dish was incubated at 37ºC for 2 h, and titrated with 0.02 N of H$_2$SO$_4$ until the color of the Conway reagent changed to red. As a blank, 5% (w/v) TCA was used, and TVB-N was calculated as follows (eq. 3):

$$\text{TVB-N (mg\%)} = 0.28 \times (\text{VS} - \text{VB}) \times \text{F} \times \text{D} \times 100 / \text{S} \qquad (3)$$

Where VS, VB F, D, and S indicate the titration volumes (mL) of the blank and the sample, titer of 0.2 N H$_2$SO$_4$, dilution factor, and sample amount, respectively.

Thiobarbituric acid-reactive substanced (TBARs)

The thiobarbituric acid-reactive substances (TBARs) in the beef were evaluated using the lipid extraction method described by Pikul *et al*. (1989) with minor modifications. The samples (3 g) were homogenized in 50 mL 20% trichloroacetic acid-2 M phosphoric acid in the presence of 50 μL of 7.2% (v/v) butylated hydroxyl anisole (BHA) in ethanol. After filtering the homogenized sample, 1 mL of the filtrate was mixed with 2 mL TBA/TCA solution (30 mM TBA in 15% TCA). The mixture was placed in a water bath, kept for 15 min at 90°C, cooled, and centrifuged at 2,000×g for 15 min. After cooling at an ambient temperature, the absorbance of the sample was measured at 530 nm. The TBARS level was determined using a standard curve pre-estimated using tetraethoxypropane and calculated based on the amount of malonaldehyde (MA) equivalents (mg MA/kg sample).

Statistical analysis

All reported values are the average of three (or more) experiments. Analysis of variance and Duncan's test were carried out at the 95% confidence level ($p<0.05$) using the SPSS 20.0 software (SPSS, 2011) to determine significant differences in the results.

Results and Discussion

Particle size and zeta potential of enzyme

Particle size, poly dispersity index (PDI) and zeta potential were measured for analysis of the particle properties.
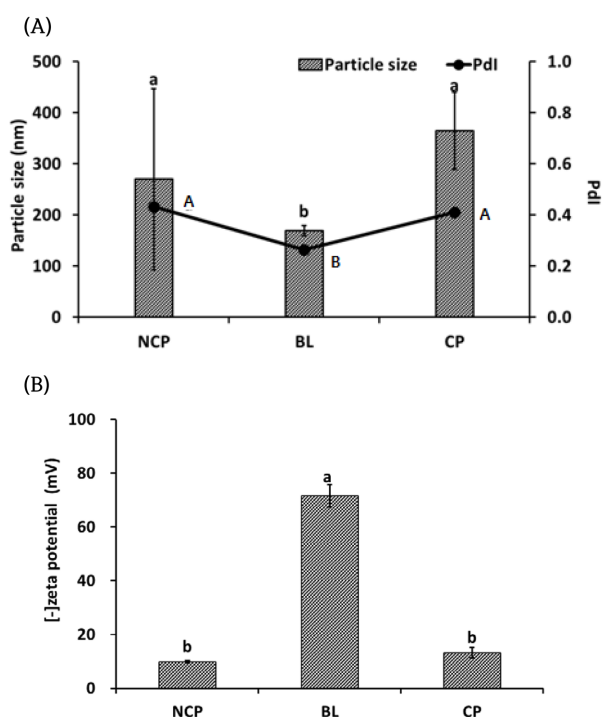
(A)

(B)

Fig. 1. The particle size and poly dispersity index (A) and zeta potential (B) of protease, blank liposome and protease coated liposome. [A,B]Means with different capital letters are significantly different ($p<0.05$) polydispersity when comparing reaction time in the same treatment type. [a,b]Means with different small letters are significantly different ($p<0.05$) particle size when comparing treatment type in each time. NCP: non-coating protease, BL: blank liposome, CP: coating protease. (A) bar: particle size, line/spot: polydispersity index.

Non-coated protease (NCP), Blank liposome (BL) and liposome coated protease (CP) are shown in Fig. 1. The size of the protease was 270±177 nm, and that of the blank liposome (BL) was 169±10 nm. After encapsulation of the protease, the particle size became 365±76 nm, which was significantly larger ($p<0.05$) then BL. NCP had a wide size distribution range (0.43±0.08) while BL had a relatively uniform size (0.26±0.04), However, CP also showed a wide distribution (0.41±0.07) similar to free protease but there was no significant difference ($p<0.05$) between NCP and CP. The zeta potential of NCP was -9.8±0.4 mV and that of BL was -72±4.1 mV. The zeta potential of CP was -13±1.9 mV and there was no significant difference ($p<0.05$) from that of NCP. From our results, the net surface charge of CP and NCP was negative although their surface components are different. It was supposed that the carboxyl groups of NCP are negatively charged the low H^+ concentration (pH>isoelectric point) because of carboxyl group such as protein properties.
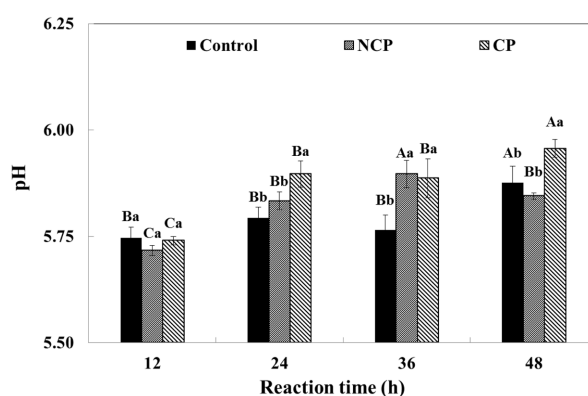

Fig. 2. The effects of enzyme coating on the pH of the enzyme treated beef. [A-C]Means with different capital letters are significantly different ($p<0.05$) when comparing reaction time in the same treatment type. [a,b]Means with different small letters are significantly different ($p<0.05$) when comparing treatment type in each time. NCP: non-coating protease, CP: coating protease.

pH change of meat

The pH of the beef immersed solution was affected by the type of protease treatment applied as well as the reaction time (Fig. 2). The pH of the control, CP or NCP ranged from 5.72-5.75 at the start and there was no significant difference between the treatments. However, the pH significantly increased with reaction time ($p<0.05$). In general, both NCP (pH 5.72-5.85) and CP (pH 5.74-5.95) had high pH values although CP had higher pH than NCP through most of the reaction. NCP had the highest value (pH 5.90±0.03) at 36 h and subsequently decreased slightly, while CP continuously increased for 48 h. It seems that the increase in the pH of the beef was a result of the proteolysis. In the present study, endo-type and exo-type mixed proteases were used and the peptide bonds within the protein chain were disrupted. Structural changes were simultaneously manifested and basic residues of muscle protein may have been exposed (Berardo *et al*., 2015; Ma and Ledward, 2004). Consequently, hydrolysis of the muscle proteins was evidenced by an increase in pH.

Color changes

The color properties of beef are shown in Table 1. The beef became darker as the reaction time increased and NCP was relatively darker than CP. The redness of the beef showed no remarkable differences with reaction time or protease treatment. Total color differences in the beef increased slightly within 24 h and increased significantly from 36 h to 48 h. In general, the total color differences appeared to be larger in the control and NCP whereas CP

Table 1. Effects of the non-coated enzyme and coated enzyme treatment with liposome on the CIE color of beef

Treatment sample	Treatment time (h)	L*(Lightness)	a*(Redness)	b*(Yellowness)	Total color difference (E)	
DW[1]	0	2.91 ± 2.91	0.87 ± 0.87	0.54 ±0.54		
	12	2.25 ± 1.47	0.78 ± 2.71	0.44 ±0.25	12.10	±1.20
	24	3.29 ± 0.72	2.74 ± 1.25	0.95 ±0.23	6.52	±1.47
	36	2.08 ± 3.02	0.40 ±1.44	1.15 ±1.22	9.53	±2.20
	48	1.11 ± 0.29	1.55 ±0.44	1.22 ±0.39	13.89	±2.92
NCP[2]	0	2.91 ± 2.91	0.87 ±0.87	0.54 ±0.54		
	12	1.55 ± 3.72	0.45 ±0.49	0.35 ±0.39	9.91	±2.04
	24	0.19 ± 0.67	1.59 ±1.25	1.08 ±0.81	11.33	±3.42
	36	2.74 ± 1.72	1.76 ±3.40	0.71 ±1.11	7.45	±2.75
	48	0.41 ± 4.29	0.92 ±1.29	0.46 ±1.05	17.76	±4.12
CP[3]	0	2.91 ± 2.91	0.87 ±0.87	0.54 ±0.54		
	12	2.45 ± 1.31	3.84 ±1.82	2.14 ±1.84	6.58	±1.74
	24	1.85 ± 2.04	0.78 ±0.82	0.08 ±0.91	8.76	±1.32
	36	1.01 ± 2.06	0.53 ±4.31	0.46 ±2.19	4.50	±3.32
	48	1.62 ± 1.19	1.74 ±3.22	0.28 ±1.05	9.42	±2.85

[1]DW: distilled/deionized water
[2]NCP: non-coating protease
[3]CP: coating protease

was less affected by the overall reaction time.

Water holding capacity (WHC)

The WHC of the beef decreased with increasing reaction time (Fig. 3). The WHC of the control was 79.39±2.77% at 12 h and decreased to 75.71±0.97% within 48 h while that of CP decreased from 79.16±2.17 to 75.86±1.93%. The control showed no significant difference ($p<0.05$) from CP. NCP and CP were not significantly different ($p<0.05$) from the control in the first 12 h, but after 24 h, the WHC of CP and NCP significantly decreased ($p<0.05$) as the reaction time increased. In general, the WHC of meat was closely related to the muscle pH, and the higher the pH, the higher the WHC of the meat. Meanwhile, the WHC of the beef was decreased by protease treatment, though the partially hydrolyzed beef had higher pH. It is likely that the opposite relationship between pH and WHC was induced by protein degradation (Berardo *et al.*, 2015). Myosin plays a key role in both maintaining the muscle structure and in providing water-binding properties. Upon proteolysis, myosin was partially hydrolyzed, indicating that the muscle had lost its structural integrity, thus losing a larger amount of moisture during centrifugation than the intact muscle (Huff-Lonergan, and Lonergan, 2005). It is possible that the protein hydrolysates had a highly soluble nature. The hydrolyzed peptides were readily resolved in sarcoplasm, and were therefore removed with the exudates during centrifugation. Consequently, the results indicated that the enzymatic hydrolysis resulted in lower WHC.
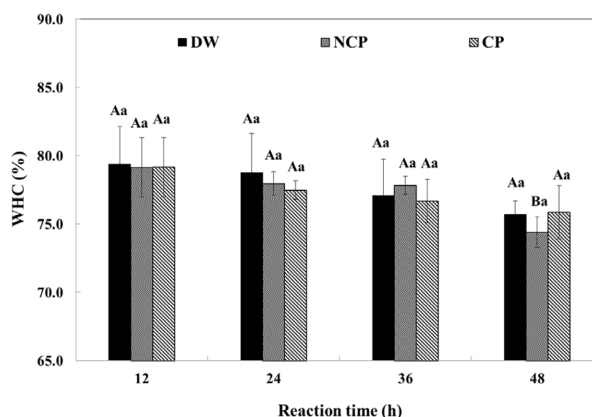

Fig. 3. The effects of enzyme coating on the water holding capacity (WHC) of the enzyme treated beef. [A,B]Means with different capital letters are significantly different ($p<0.05$) when comparing reaction time in the same treatment type. [a,b]Means no significantly different ($p<0.05$) when comparing treatment type in each time. NCP: non-coating protease, CP: coating protease. The WHC of raw meat was 82.33±4.01%.

Shear force

The protease-mediated partial hydrolysis of beef resulted in tenderization of the beef (Fig. 4). The shear force of the control ranged from 2.1-2.4 kg while that of NCP and CP was significantly lower than the control ($p<0.05$) from 24 h. The control showed no significant differences ($p<0.05$) during 48 h of storage, whereas the shear force of the protease treated samples significantly decreased from 2752± 537 kg to 1595±421 kg (NCP), or from 3042±593 kg to
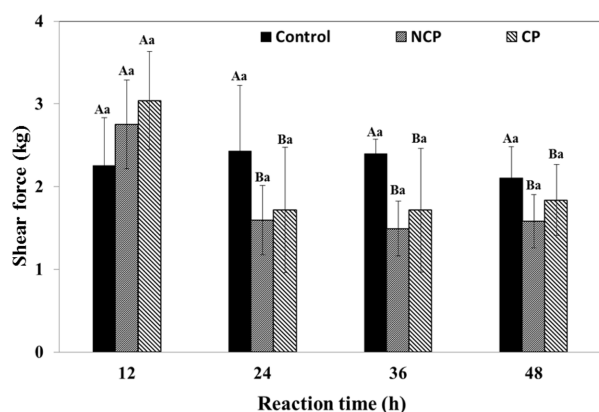
Fig. 4. The effects of enzyme coating on the shear force of the enzyme treated beef. [A,B]Means with different capital letters are significantly different ($p<0.05$) when comparing reaction time in the same treatment type. [a,b]Means no significantly different ($p<0.05$) when comparing treatment type in each time. NCP: non-coating protease, CP: coating protease.

Fig. 5. The effects of enzyme coating on the enzyme activity value of the enzyme treated beef. [A-D]Means with different capital letters are significantly different ($p<0.05$) when comparing reaction time in the same treatment type. [a,b]Means with different small letters are significantly different ($p<0.05$) when comparing treatment type in each time. NCP: non-coating protease, CP: coating protease.

1716±758 kg (CP) within 24 h. After the first 24 h, the shear force did not change till the end of the reaction. Although NCP had a slightly lower shear force than CP, the difference was not significant. The results indicated that protease treatment was effective for tenderizing beef within 24 h regardless of coating and no differences were observed between CP and NCP.

Protease activity

The protease activity of NCP and CP after immersion of the beef was compared (Fig. 5). Tyrosine was not detected in the control initially, but appeared to have increased slightly to 0.04 ppm after 48 h. The NCP or CP contained 0.12 ppm of tyrosine at the beginning, and, over time, these amounts increased to 0.18±0.04 ppm (NCP) and 0.09± 0.03 ppm (CP), respectively. The NCP had a significantly high quantity (0.21±0.03 ppm, $p<0.05$) at 24 h, but the amount of tyrosine at NCP and CP had the same amount after 24 h.

TVB-N and TBAR$_S$ values

Freshness of meat is usually determined by the amount of total volatile nitrogen (TVB-N) and lipid oxidation (TBARs). The TVB-N and TBARs of the protease immersed meat were measured as a function of time and protease coating. The TVB-N of the immersed beef is shown in Fig. 6. All beef samples showed a significant increase in TVB-N during incubation. The protease treatments had higher TVB-N than the control. During the first 24 h, NCP
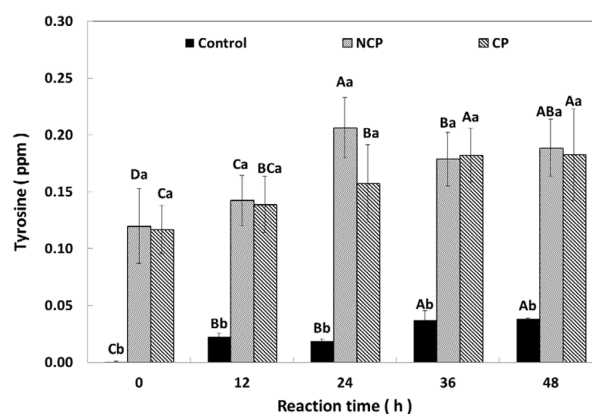

Fig. 6. The effects of enzyme coating on the total volatile basic nitrogen (TVB-N) contents of the enzyme treated beef. [A,B]Means with different capital letters are significantly different ($p<0.05$) when comparing reaction time in the same treatment type. [a,b]Means with different small letters are significantly different ($p<0.05$) when comparing treatment type in each time. NCP: non-coating protease, CP: coating protease. The TVB-N value of raw meat was 11.4 mg%.
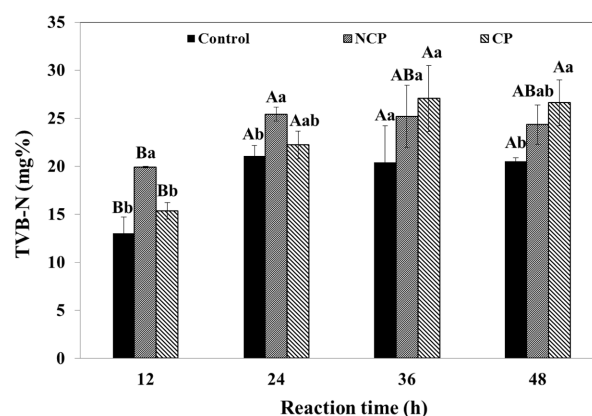
had a significantly higher value (19.89-25.42%, $p<0.05$) than the control (13.01-21.04%) and CP (15.38-22.22%). However, the values changed after 36 h of reaction, with CP showing significantly higher values (27.06±3.44%, $p<0.05$) than the control (20.40±3.83%) and NCP (25.17± 3.25%). The TVB-N of the control and NCP increased during the first 24 h and subsequently stabilized, whereas the TVB-N of CP continued to increase for 36 h. The TVB-
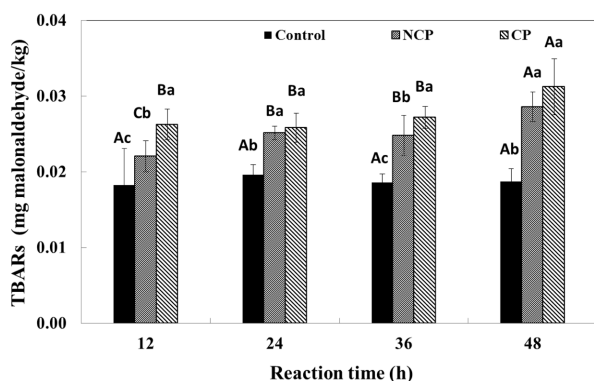
Fig. 7. The effects of enzyme coating on the thiobarbituric acid reactive substances (TBARs) value of the enzyme treated beef. [A-C]Means with different capital letters are significantly different ($p<0.05$) when comparing reaction time in the same treatment type. [a,b]Means with different small letters are significantly different ($p<0.05$) when comparing treatment type in each time. NCP: non-coating protease, CP: coating protease. Raw value was 0.0036±0.0018 mg malonaldehyde/kg.

N of the beef was related to protein hydrolysis. The result is likely because NCP was more efficient at hydrolyzing muscle proteins. The TVB-N of CP probably continued to increase due to the release of protease.

The lipid oxidation of the immersed beef was measured by TBARs analysis and the results are shown in Fig. 7. During the reaction, the TBARs of the protease treatments tended to increase slightly, with significant differences ($p<0.05$). The TBARs values of the control showed no difference (0.018-0.020 mg MA/kg) over 48 h whereas both protease treatments increased significantly ($p<0.05$), indicating that protease treatment increases the lipid oxidation of beef. For the NCP treatment, significant increase in TBARs was observed after 24 h, compared to after 48 h for the CP treatment. The TBARs value of CP was significantly higher (0.026±0.002 mg MA/kg, $p<0.05$) than those of the control and NCP after 12 h. However, it showed no difference until 36 h and then significantly increased at 48 h (0.031±0.004 mg MA/kg, $p<0.05$). In contrast, the TBARs value of NCP was 0.022±0.002 mg MA/kg at 12 h and significantly increased ($p<0.05$) at 24 h and also at 48 h. The CP treatment had a higher value, but the rates of CP and NCP increase after 36 h were not different. The higher value of the CP treatment seems to be due to the lipid composition in the liposome coating. Protease immersion seems to affect lipid oxidation, but coating the protease seems to have no effect. Nonetheless, the TBARs range for the control and the treatments was 0.018-0.031

mg MA/kg, which is less than 1 mg MA/kg and has no effect on the taste or flavor of meat. Actually, the relationship between proteolysis and lipid oxidation was unclear. It is possible that the CP treatment used phospholipids as the coating material, which would account for the higher TBARs value. (Cruz-Romero *et al.*, 2008). However, the high TBARs of NCP compared to the control in the present study was not fully understood. It was presumed that the higher TBARS of the protease treatments was a result of the applied enzyme preparation.

In both TBARs and TVB-N, excessively high reaction time was unfavorable to the quality of the beef. Hence, other strategies such as reaction in cold temperatures or increasing the amount of protease are required to obtain proteolysis activity in the beef, which warrants further investigation.

Conclusion

The effect of liposome coating of protease on beef tenderization was analyzed. Encapsulated protease was compared with non-coated protease according to the size distribution and enzymatic activity. Protease-reacted beef was analyzed to determine the pH, water holding capacity, shear rate, TVB-N and TBARs. Generally, the pH of the beef increased during the reaction and the pH of CP was higher than that of NCP. The total color difference remarkably increased after 36 h. The total difference for CP was relatively lower than that for NCP. WHC significantly decreased within 24 h, but there was no difference between NCP and CP. Protease activity significantly increased over 48 h and no effect was observed from the presence of a protease coating. The TVB-N value of NCP increased over 24 h while that of CP increased after 36 h. The TVB-N value of the protease treatments increased after 36 h and no significant difference between CP and NCP was observed. Consequently, liposome encapsulated protease shows similar properties as NCP. Application of liposome appears to be an interesting option for injecting various functional materials without changing the properties of meat.

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