



## Evaluation of the Activities of Antioxidant Enzyme and Lysosomal Enzymes of the *Longissimus dorsi* Muscle from Hanwoo (Korean Cattle) in Various Freezing Conditions

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### Abstract

This study was conducted to evaluate the activities of antioxidant enzyme (glutathione peroxidase (GSH-Px)) and lysosomal enzymes (alpha-glucopyranosidase (AGP) and beta-N-acetyl-glucosaminidase (BNAG)) of the *longissimus dorsi* (LD) muscle from Hanwoo (Korean cattle) in three freezing conditions. Following freezing at -20, -60, and -196°C (liquid nitrogen), LD samples (48 h *post-slaughter*) were treated as follows: 1) freezing for 14 d, 2) 1 to 4 freeze-thaw cycles (2 d of freezing in each cycle), and 3) refrigeration (4°C) for 7 d after 7 d of freezing. The control was the fresh (non-frozen) LD. Freezing treatment at all temperatures significantly ( $p < 0.05$ ) increased the activities of GSH-Px, AGP, and BNAG. The -196°C freezing had similar effects to the -20°C and -60°C freezing. Higher ( $p < 0.05$ ) enzymes activities were sustained in frozen LD even after 4 freeze-thaw cycles and even for 7 d of refrigeration after freezing. These findings suggest that freezing has remarkable effects on the activities of antioxidant enzyme and lysosomal enzymes of Hanwoo beef in any condition.

**Keywords:** freezing condition, antioxidant enzyme, lysosomal enzyme, Hanwoo beef

### Introduction

Most consumers demand high-quality meat in consideration of their sensory satisfaction, health, and safety. The quality in raw meat is generally evaluated by assessing color, water-holding capacity, texture, palatability, and microbial populations (Harris *et al.*, 2012; Muhlisin *et al.*, 2014; Warren *et al.*, 2008). These quality indicators are directly related to freshness as they deteriorate gradually (or increase, in the case of microbial count) during *post-slaughter* or *post-packaging* distribution. Loss of freshness depends on storage conditions, such as temperature, duration, light, and atmospheric composition (Jayasingh *et al.*, 2001; Martin *et al.*, 2013; Monahan, 2000). High temperature and lengthy storage and periods reduce shelf-life as they promote oxidation and microbial growth (Rogers *et al.*, 2014).

For long-term storage of fresh meat, freezing is widely

acknowledged as better than refrigeration. Freezing storage has many benefits; it inhibits oxidation, delays bacterial replication, and retains flavor, and is particularly useful for long-distance transport (Critchell and Raymond, 1969; Lawrie and Ledward, 2006). Hansen *et al.* (2004) recommended -55°C as the optimal storage temperature, as it entirely suppressed enzymatic and oxidative deterioration. Particularly, in the field of biomedical science, innovative freezing technology, such as ultra-freezing and cryopreservation agents (i.e., liquid nitrogen), are used to conserve the living cells and tissues. However, ice crystals formed by freezing lead to desiccation of cell membrane protein in animal muscles (Love, 1966). This process increases the effluence of water-soluble nutritional components (e.g., vitamins) and the textural deterioration and decreases the water-holding capacity (Cho *et al.*, 2014; Lagerstedt *et al.*, 2008; Pavlov *et al.*, 1994). In other words, freezing causes the decline in the quality of meat.

Biological enzymatic systems, in the organelles of animal muscle cells, such as erythrocytes, mitochondria, and lysosomes, are discharged into the extracellular space by cold shock (Gottesman and Hamm, 1983; Hamm and Gottesman, 1982; Rehbein, 1979). This phenomenon could influence the activities of endogenous enzymes in animal

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muscles and could be used to distinguish between fresh and frozen muscles. Several studies on muscles from farm animals and fish have reported altered activities of cellular enzymes after freezing: ATPase isoenzymes, alpha-glucopyranosidase (AGP), beta-galactosidase, beta-glucuronidase, beta-hydroxyacyl-CoA dehydrogenase, beta-N-acetyl-glucosaminidase (BNAG), esterase-lipase, and malate dehydrogenase (Benjakul *et al.*, 2003; Chen *et al.*, 1988; Duflos *et al.*, 2002; Ellerbroek *et al.*, 1995; Fernández *et al.*, 1999; Hoz *et al.*, 1993; Jeong *et al.*, 2006; Lee *et al.*, 2004; Makri *et al.*, 2007; Toldrá *et al.*, 1991). However, little is known regarding the effects of freezing on the activity of other kinds of cellular enzymes, such as antioxidant enzymes, in animal muscles. Furthermore, Ellerbroek *et al.* (1995) and Toldrá *et al.* (1991) observed remarkably changed activities of AGP and BNAG in the meat frozen in commercial and ultra-freezing temperatures. Jeong *et al.* (2006) found higher mitochondrial enzyme activity in the meat treated in one and two times of freezing-thaw cycles during refrigerated storage compared to fresh meat. However, it is also unknown about lysosomal enzymes (AGP and BNAG) activities of frozen beef in liquid nitrogen storage, freeze-thaw cycles, or refrigerated storage after freezing. The activities of cellular enzymes of fish meat have been examined in these freezing conditions. However, the enzymatic characteristics of muscles from cattle and fish may substantially differ due to differences in enzyme-related metabolism (De Smet, 2012).

Therefore, in this study, we evaluated the activities of antioxidant enzyme (glutathione peroxidase (GSH-Px)) and lysosomal enzymes (AGP and BNAG) of the *longissimus dorsi* muscle in various freezing conditions such as freezing storage, freeze-thaw cycles, and refrigerated storage after freezing, in order to distinguish between fresh Hanwoo (Korean cattle) beef and those frozen at -20, -60, or -196°C.

## Materials and Methods

### Reagents and chemicals

$\beta$ -nicotinamide adenine dinucleotide 2'-phosphate reduced ( $\beta$ -NADPH), ethylenediaminetetraacetic acid (EDTA), glutathione reductase (GSH-R), 4-nitrophenyl- $\alpha$ -D-glucopyranoside, 4-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide, and L-glutathione reduced (GSH) were purchased from Sigma-Aldrich Co. LLC. (USA). Deionized water (DW) was prepared with a Milli-Q Water Purifier (Millipore SAS, France). Liquid nitrogen was supplied by a nearby

gas plant.

### Sample freezing

From a local meat market, fresh ribeyes from Hanwoo steer carcasses (at d 2 *post-slaughter*; quality grade: 1) were purchased. After trimming, the lean meat (*longissimus dorsi*, LD) was cut into about 1 cm thickness and divided into four groups (five replicates per one group) of different freezing temperatures as followings: control, -20, -60, and -196°C. The control was designated as the fresh (non-frozen) beef and immediately used for enzyme activity analysis. The -20°C and -60°C groups were vacuum-packaged with a commercial film (nylon/tie/linear low density polyethylene (LLDPE)/tie/nylon/tie/LLDPE-layered film; FoodSaver pouch, Rollpack Co., Ltd., Korea) using a vacuum packaging machine (CD-120, Webomatic Maschinenfabrik GmbH, Germany) and then stored in a -20°C household refrigerator (DIOS Linear Drive R-S774NH Model, LG Electronics, Korea) and in a -60°C ultra-freezer (OPR-DFU-446CE Model, Operon Co., Ltd., Korea). The -196°C group was stored in a Dewar Flask (29B Model, Karlsruher Glastechnisches Werk-Schieder GmbH, Germany), containing liquid nitrogen, without packaging.

### Experimental design

We analyzed enzymes activities in three different conditions: 1) freezing storage, 2) freeze-thaw cycles, and 3) refrigerated storage after freezing. In the first experiment, LD samples were stored at -20, -60, and -196°C for 14 d. In the second experiment, samples were frozen-thawed for 1 to 4 cycles at the same temperatures. In each cycle, samples were frozen for 2 d. In the third experiment, samples were stored at 4°C (DS-95P Model, Dasol Scientific Co., Ltd., Korea) after 7 d of freezing. The d 2 *post-slaughter* was designated as d 0 of refrigerated storage for the control. Enzyme analysis was performed on triplicates per one sample after thawing at 4°C for 12 h.

### Preparation of meat extracts for analysis

To prepare the aqueous meat extract for GSH-Px analysis, 5 g of ground beef was mixed with 20 mL of ice-cold 50 mM phosphate buffer (pH 7.0) using a homogenizer (T25 Digital Ultra-Turrax, Ika Werke GmbH & Co., KG, Germany) at 13,500 rpm for 30 s and centrifuged at 2°C /1,000 g (Renner *et al.*, 1996; Avanti J-E Centrifuge, Beckman Coulter, Inc., USA) for 15 min. Then, the supernatant was filtered with a Whatman filter paper No. 1 in a 4°C cold room to remove the fat and connective tissue.

Meat extract for AGP and BNAG analysis were prepared by the same way with 0.1 M sodium citrate buffer (pH 4.5).

#### Analysis of glutathione peroxidase activity

GSH-Px (E.C. 1.11.1.6) activity was analyzed with the protocol reported previously by Flohé and Günzler (1984). Briefly, 100  $\mu$ L of extract was gently mixed with enzyme medium (1 mM  $\text{NaN}_3$ -1 mM EDTA-1 mM GSH-0.15 mM NADPH-0.5 units/mL GSH-R-0.15 mM  $\text{H}_2\text{O}_2$  in phosphate buffer (pH 7.0); final concentration) and then incubated at 25°C for 5 min. Immediately after adding 0.15 mM  $\text{H}_2\text{O}_2$  (final concentration), the decrease rate of absorbance value was measured at 340 nm (ProteomeLab DU-800 UV/Vis spectrophotometer, Beckman Coulter, Inc., USA) at 25°C. One unit of GSH-Px activity was designated as the amount of meat extract required to scavenge one  $\mu$ mol  $\text{H}_2\text{O}_2$  per min at pH 7.0/25°C.

#### Measurement of alpha-glucopyranosidase activity

AGP (E.C. 3.2.1.20) activity was measured with the process established by Benjakul and Bauer (2000). Before incubating at 37°C for 1 h, 1 mL of extract was mixed with 0.5 mL of 1 M NaCl and 1 mL of 4.2 mM 4-nitrophenyl  $\alpha$ -D-glucopyranoside. To stop the reaction, 1 mL of 0.3 M KOH was added and then absorbance value was spectrophotometrically determined at 405 nm. The positive control contained 1 mL of sodium citrate buffer instead of sample extract. The negative control was prepared by mixing with KOH before adding 4-nitrophenyl  $\alpha$ -D-glucopyranoside. One unit of AGP activity was designated as the amount of meat extract required to liberate one nmol  $\rho$ -nitrophenol per min at pH 4.5/37°C.

#### Measurement of beta-N-acetyl-glucosaminidase activity

BNAG (E.C. 3.2.1.30) activity was measured according to the method described by Benjakul and Bauer (2000). One milliliter of extract was mixed with 0.5 mL of 0.6 M KCl and 1 mL of 0.6 mM 4-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide and then incubated at 37°C for 15 min. The reaction was stopped with addition of 0.3 M KOH (1 mL) and then absorbance value was monitored at 405 nm. The positive control included sodium citrate buffer. The negative control was prepared by mixing with KOH before adding 4-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide. One unit of BNAG activity was designated as the amount of meat extract required to liberate one nmol  $\rho$ -nitrophenol per min at pH 4.5/37°C.

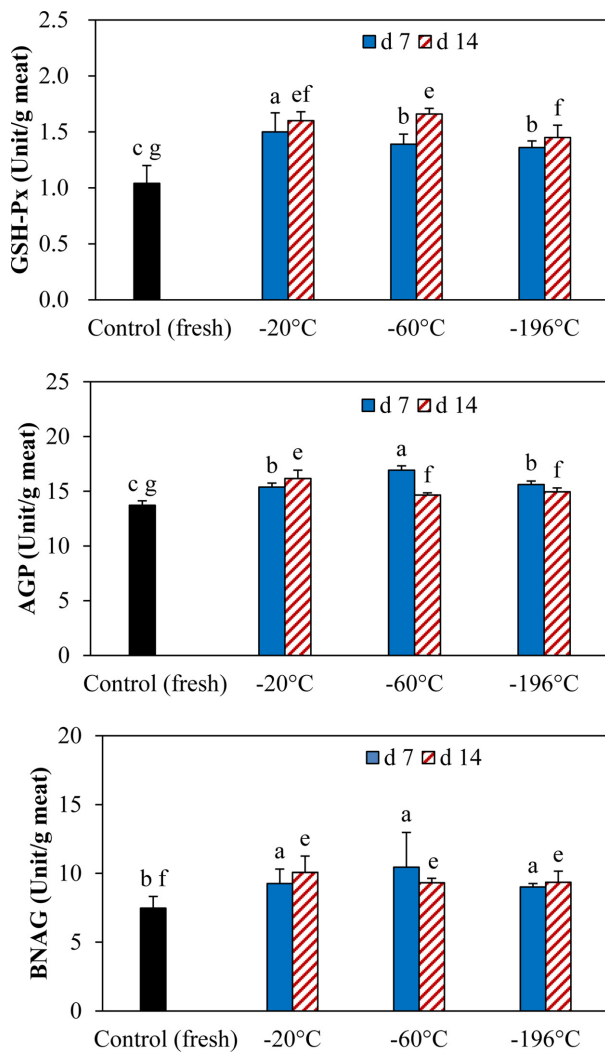
#### Statistical analysis

All data were presented as means $\pm$ standard deviations. Statistical analysis was carried out using the SPSS (2011) program. Differences in mean values among freezing conditions were assessed by an analysis of variance coupled with the Duncan's multiple range test. Significances were identified as  $p < 0.05$ .

## Results and Discussion

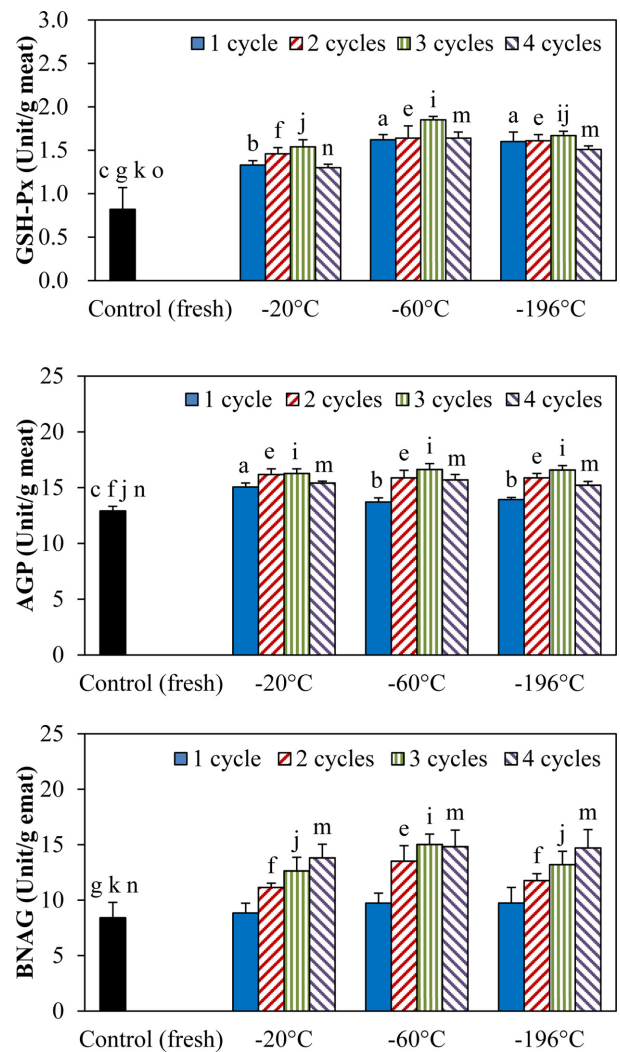
#### Freezing storage

The activities of GSH-Px, AGP, and BNAG of LD from Hanwoo during freezing storage at various temperatures are represented in Fig. 1. The GSH-Px has been often described as a one of major enzymatic antioxidants in several livestock meat (Hernández *et al.*, 2004; Lee *et al.*, 1996; Mercier *et al.*, 2004; Petron *et al.*, 2007; Terevinto *et al.*, 2010). Using the reducing mechanism connected with GSH, this enzyme protects cellular tissues against oxidative stress induced by  $\text{H}_2\text{O}_2$  or polyunsaturated fatty acid hydroperoxides (Brigelius-Flohé and Maiorino, 2013). During 14 d of freezing storage, the LD frozen at -20, -60, and -196°C exhibited significantly ( $p < 0.05$ ) higher GSH-Px activity versus the control. On d 7 and 14 of freezing, the -20°C- and -60°C-frozen LD exhibited the highest GSH-Px activity among three frozen LD. The AGP and BNAG generally exist in subcellular organelle (lysosome) (Rehbein, 1979) and are typically found in a variety of fish meat (Benjakul and Bauer, 2000; Benjakul *et al.*, 2003; Makri *et al.*, 2007; Nilsson and Ekstrand, 1994; Shimomura *et al.*, 1987) but finitely in land animal meat (Ellerbroek *et al.*, 1995; Toldrá *et al.*, 1991). In buffered muscle system, they commonly liberated  $\rho$ -nitrophenol from artificially added 4-nitrophenyl  $\alpha$ -D-glucopyranoside and 4-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide (Benjakul and Bauer, 2000). In this study, when AGP and BNAG activities of LD samples were implemented using these methodologies, the LD frozen at -20, -60, and -196°C indicated higher ( $p < 0.05$ ) those than the control for 14 d of freezing. On d 7 and 14, the -60°C- and -20°C-frozen LD had higher ( $p < 0.05$ ) AGP activity than other frozen LD. There were no differences in BNAG activity among all frozen LD. Our findings are consistent with Chaivechakarn *et al.* (2012), who found increased GSH-Px activity in frozen mammalian sperm. Similarly, Toldrá *et al.* (1991) and Ellerbroek *et al.* (1995) observed AGP and BNAG activities, determined by the APIZYM kit, differed between fresh and frozen meat. In our results, there were not distinct differences in enzymes activities among



**Fig. 1.** The activities of glutathione peroxidase (GSH-Px), alpha-glucopyranosidase (AGP), and beta-N-acetylglucosaminidase (BNAG) in *longissimus dorsi* (LD) muscle in Hanwoo (Korean cattle) during freezing storage. These data present means±standard deviations. <sup>a-c</sup>Different letters indicate significant differences among the control (fresh LD) and 7 d-frozen LD ( $p < 0.05$ ). <sup>e-g</sup>Different letters indicate significant differences among the control and 14 d-frozen LD ( $p < 0.05$ ).

freezing temperatures due to different pattern during storage times. Similar results have been reported in other studies on pork of Toldrá *et al.* (1991) and on fish meat of Fernández *et al.* (1999). Particularly, Fernández *et al.* (1999) explained this variance should not be regarded as an effect of freezing temperature (-10 to -196°C). Toldrá *et al.* (1991) concluded freezing temperature (-10 to -60°C) had no independent effect, considering result of statistics at  $p < 0.01$ , despite much higher activity of cellular enzyme in some frozen meat (-13°C). The increased activities of enzymes in frozen meat are due to the efflu-



**Fig. 2.** The activities of glutathione peroxidase (GSH-Px), alpha-glucopyranosidase (AGP), and beta-N-acetylglucosaminidase (BNAG) in *longissimus dorsi* (LD) muscle in Hanwoo (Korean cattle) after freeze-thaw cycles. These data present means±standard deviations. <sup>e-g</sup>Different letters indicate significant differences among the control and frozen LD after 2 freeze-thaw cycles ( $p < 0.05$ ). <sup>i-k</sup>Different letters indicate significant differences among the control and frozen LD after 3 freeze-thaw cycles ( $p < 0.05$ ). <sup>m-o</sup>Different letters indicate significant differences among the control and frozen LD after 4 freeze-thaw cycles ( $p < 0.05$ ).

ence of intracellular enzymes by the breakdown of cell membrane with formed ice crystals (Gottesman and Hamm, 1983; Hamm and Gottesman, 1982).

#### Freeze-thaw cycles

Fig. 2 shows the activities of GSH-Px, AGP, and BNAG of Hanwoo LD after freeze-thaw cycles. Even after 1 to 4 freeze-thaw cycles, the LD frozen at -20, -60, and -196°C exhibited higher ( $p < 0.05$ ) GSH-Px activity versus the

control. The LD frozen at  $-60$  and  $-196^{\circ}\text{C}$  had higher GSH-Px activity than  $-20^{\circ}\text{C}$ -frozen LD. AGP activity of three frozen LD was also higher ( $p<0.05$ ) than that of the control during 4 freeze-thaw cycles. Only after 1 freeze-thaw cycle, the LD frozen at  $-60$  and  $-196^{\circ}\text{C}$  exhibited lower ( $p<0.05$ ) activity than  $-20^{\circ}\text{C}$ -frozen LD. BNAG activity of all frozen LD was higher ( $p<0.05$ ) than that of the control after 2 freeze-thaw cycles. The  $-60^{\circ}\text{C}$ -frozen LD had higher ( $p<0.05$ ) BNAG activity than other frozen LD only after 1 and 2 freeze-thaw cycles. Our results are in agreement with Benjakul and Bauer (2000), who reported increased AGP and BNAG activities in fish meat after 1 to 5 freeze-thaw cycles. Jeong *et al.* (2006) also described increased mitochondrial enzyme activity in

pork after 1 to 2 freeze-thaw cycles. Similar increase in this study could be due to discharge of intracellular fluids including enzymes from muscle cells broken by freeze-thaw cycles (Benjakul and Bauer, 2000; Makri *et al.*, 2007; Nilsson and Ekstrand, 1993).

### Refrigerated storage after freezing

During refrigerated storage after freezing, the activities of GSH-Px, AGP, and BNAG of Hanwoo LD are indicated in Fig. 3. Even during 7 d of refrigeration after freezing, GSH-Px activity was higher ( $p<0.05$ ) in the LD frozen at  $-20$ ,  $-60$ , and  $-196^{\circ}\text{C}$  than in the control. The  $-20^{\circ}\text{C}$ -frozen LD exhibited higher ( $p<0.05$ ) GSH-Px activity than other frozen LD only on d 0 of storage after freezing. Similarly to the result of GSH-Px activity, AGP and BNAG activities were higher ( $p<0.05$ ) in three frozen LD than the control for 7 d of storage after freezing. On 7 d of storage, the  $-196^{\circ}\text{C}$ -frozen LD had higher ( $p<0.05$ ) AGP activity than other frozen LD. However, no differences were observed in BNAG activity among all frozen LD. Similarly, Pavlov *et al.* (1994) described higher mitochondrial enzyme activity in fish meat stored in crushed ice after freezing. Jeong *et al.* (2006) also noted higher activity of same enzyme in pork during refrigerated storage after freezing.

### Conclusion

Freezing enhanced the activities of antioxidant enzyme (GSH-Px) and lysosomal enzymes (AGP and BNAG) in LD from Hanwoo, regardless of freezing temperature. Frozen LD maintained higher activities of these enzymes than the fresh LD even after multiple freeze-thaw cycles and even during refrigerated storage after freezing. Although extremely low temperature generally keeps cell tissues more stable, super-low freezing (liquid nitrogen,  $-196^{\circ}\text{C}$ ) did not have differential effects on enzymes activities in comparison to a commercial freezing ( $-20^{\circ}\text{C}$ ) and ultra-freezing ( $-60^{\circ}\text{C}$ ). Further investigation on more sensitive enzymes is needed to clarify enzymatic differences among freezing conditions in frozen meat.

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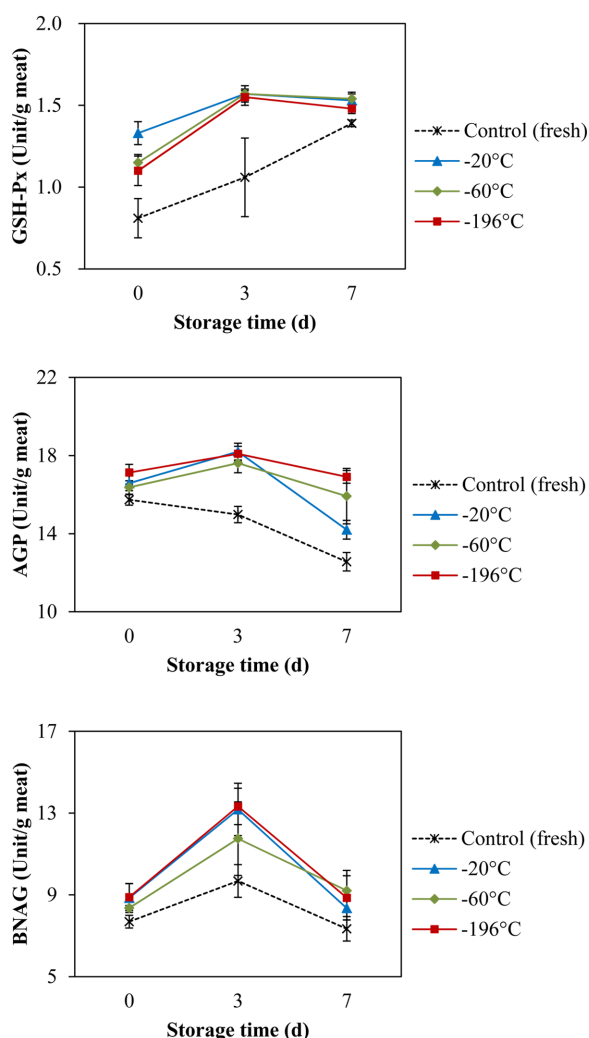


Fig. 3. The activities of glutathione peroxidase (GSH-Px), alpha-glucopyranosidase (AGP), and beta-N-acetylglucosaminidase (BNAG) in longissimus dorsi muscle in Hanwoo (Korean cattle) during refrigerated storage after freezing. These data present means  $\pm$  standard deviations.

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