# THE COMBINED EFFECTS OF FREEZING AND THAWING ON THE ULTRASTRUCTURE OF A SMALL SAMPLE OF NORMAL PORK

Ngapo, T.M., Babare, I.H., Murphy, K.L., Reynolds, J. and Mawson, R.F.
Australian Food Industry Science Centre, Private Bag 16, Werribee, Victoria 3030, AUSTRALIA

## SUMMARY

Samples of porcine m. biceps femoris were subjected to six freezing and three thawing rates. Cryo-scanning electron microscopy was used to study the ultrastructure of the meat. Cavities created after sublimation of the ice crystals were quantitatively analysed using an image analysis software package. Cavity cross sectional areas were used as an estimate of ice crystal areas and were measured perpendicular to the fibres. Significant differences were observed in the means of the upper quartile of areas between samples thawed within 16 h of freezing and samples stored for 4 weeks prior to thawing. Recrystallisation during storage explains these differences. After thawing, the muscle structure appeared to have largely recovered from the gross distortion of the muscle cells observed in the frozen state at all rates of freezing and thawing.

## INTRODUCTION

There is an extensive literature about the effects of freezing rate on changes in physical, chemical and sensory properties of meat. Some of this work has been devoted to histological investigations, and has related ice crystal damage to other deleterious effects of freezing and frozen storage, such as drip loss. Many of these studies have illustrated the distortion of the tissue ultrastructure in the frozen state. In contrast, there are few reports about the effects of thawing rates on meat characteristics, in particular, the ultrastructure of thawed meat.

In an investigation of the effects of frozen storage on the ultrastructure of bovine muscle, Carroll et al (1981) using scanning electron microscopy (SEM) observed that samples frozen at -18°C and stored for 5 days, 4, 12 and 26 weeks showed good preservation of structure with little apparent change from the untreated sample. These samples were cut (18 to 20 mm thick) perpendicular to the longitudinal axis, wrapped in freezer paper and frozen in a stationary air freezer at -18°C (about 4 h to freeze). Thawing occurred at room temperature and took 3.5 h for the centre of the sample to reach 4°C. Tissue fixation was used as sample preparation for SEM.

The aim of the present study is to investigate the effects of combinations of six freezing and three thawing rates on the ultrastructure of meat in a model system using a cryo-SEM technique employed by Payne et al (1994). This method allows examination of the cavities created by sublimation of ice crystals without chemical fixation of the meat sample, thus reducing possible artefacts.

## MATERIALS AND METHODS

**Materials.** Meat was obtained from a local abattoir where a 1:1 ratio of male to female pigs are slaughtered (22-24 weeks old) derived from crossbreeds of Landrace, Large White and Duroc. Porcine m. biceps femoris was obtained from hind quarters of carcasses stored for 24 h at 4°C after slaughter. Meat was stored at 4°C for up to a further 48 hours before use. The pH was measured in triplicate by grinding meat (approx. 2 g) in a mortar and pestle with deionised water (10 ml). Only meat with pH in the range 5.4 to 6.0 was used.

**Freezing/Thawing.** Sample preparation was conducted at 4°C. Six samples (approx. 70 mm length x 10 mm diam.) were cut from the centre of the muscle with fibres parallel to the length of the sample. The samples were placed in aluminium holders (80 mm length x 10 mm i.d., 20 x 20 mm outer cross sectional area in two longitudinal pieces) and plugged with expanded polyurethane foam (5 mm length) at either end. The holders, firmly held together with rubber rings, were placed into latex sheaths. Four samples had copper constantan thermocouples inserted longitudinally into their centres. Two further thermocouples were placed in the coolant and another held at room temperature. The thermocouples were connected as differential inputs to a PCI-20303T Termination Panel which was part of an Intelligent Instrumentation Visual Designer PCI-20,000 system. The samples were placed vertically in a B.Braun Frigomix S cooling bath with a B.Braun Thermomix UB attachment.

Freezing and thawing velocities were defined as the characteristic times ($t_c$) to traverse the temperature range from -1 to -7°C, or the reverse, respectively. Freezing $t_c$ of 12, 30, 60, 120, 240 and >900 min and thawing $t_c$ of 12, 60 and 180 min were used. Temperature was changed manually every 1.0°C for samples with a $t_c$ of 12 min, every 0.5°C for samples with $t_c$ from 30 to 240 min and for the samples with $t_c$ of >900 min, every 0.2°C to -3.6°C where the temperature was held for approx. 15 h, then changed every 0.2°C to -7°C. For all samples after freezing to -7°C at a specific rate, the temperatures were then lowered to -20°C in 15 min. After 1 h at -20°C, three samples were transferred to a freezer at -18°C, removed from the holders, wrapped in cryovac barrier bags and stored for 4 weeks. The remaining samples were held for up to 15 h prior to raising the temperature from -20 to -7°C (approx. 15 min), thawed at the specified rate and held at 2°C for 1 min. Each freezing time was investigated using one muscle from one animal. Experiments were conducted in two replicates, carried out in a randomised block within each replicate. Sections of meat (10 mm length x 10 mm diam.) were cut from fresh, thawed and 4 week stored thawed samples and plunged into liquid $N_2$. After removal from the $N_2$, samples were stored for up to 7 days at -70°C until 1 h prior to SEM when the samples were transferred to a freezer at -18°C.

**SEM.** SEM was conducted using a Cambridge Instruments Stereoscan 90 scanning electron microscope fitted with an Oxford Instruments CT-1000A cryostage. Images were captured using an Image Slave Software Package. Sample preparation was conducted at -18°C. Cores (3 mm diam.) were removed from the meat samples and inserted into a hole (3 mm diam.) drilled in the centre of a cryomicroscopy stub. The stubs were then held in a polystyrene cup over liquid $N_2$ for up to 2 h before plunging into liquid $N_2$. The meat sample was freeze fractured perpendicular to the fibre direction with a scalpel blade, mounted on the cryostage and heated to -60°C until the ice had sublimed. The tissue surface was examined with an accelerating voltage of 2.5 kV and at a constant working distance of 7 mm.

**Data Translation and Statistical Analysis**. Quantitative data was obtained from the images using a Data Translation Global Lab Image Software Package. Image analysis was conducted on two stubs from each freeze, thaw and storage combination. At least 150 cavities were measured for each image. These were sorted by cross sectional area into classes of 0.5 x $10^{-4}$ mm$^2$ (for example, from 1 x $10^{-4}$ to 2 x $10^{-4}$ mm$^2$, 2 x $10^{-4}$ to 3 x $10^{-4}$ mm$^2$) and the means of the lower and upper quartiles and overall medians were calculated for each image. The closest approximation to zero that could be obtained at the magnifications of the images was 0.05 x $10^{-4}$ mm$^2$.

## RESULTS AND DISCUSSION

It has been suggested that the thawing process probably does greater damage to meat than freezing (Judge et al, 1989). The same

principles of the mechanisms of freezing apply to thawing as described by Meryman (1956). However, there is one major practical difference; in general, the positions of the high and low diffusivity phases are reversed. In freezing heat is removed from the internal low diffusivity material and rapidly removed through a good conductor. In thawing initially heat is readily distributed throughout the internal high diffusivity solid, but as thawing proceeds, the heat is provided to the thawing boundary through an increasing layer of poorly conducting melt. This means that in freezing, the boundary moves rapidly, advancing well into the specimen before equilibration of the interior takes place, if at all. On thawing, heat is rapidly distributed through the high diffusivity solid, which equilibrates throughout at the melting point, almost before any melting has taken place. The melting boundary then proceeds into the specimen at a much reduced rate compared with that of freezing. A small sample size was used in the present work and a lag between the temperature at the centre of the meat and the coolant was not evident. Therefore, there was no evidence of a temperature gradient in the sample during the thawing process suggesting that recrystallisation due to a melting front is unlikely. However, recrystallisation while holding the sample at temperatures below the freezing point will occur.

Wirth et al (1979) suggested that by using a small sample, a slow thaw rate results in reduced drip loss due to the partial absorption of exudate by the muscular fibrils. A consequence of this theory is that the ultrastructure of the sample partially recovers from the grossly distorted structure observed in the frozen state (Ngapo et al, 1997). In the present experiment, greater than 90% of the cavities observed had areas between 0.05 to 1.00 x $10^{-4}$ $mm^2$. The presence of these small cavities in the fresh samples suggests that they are probably a consequence of the preparative procedure of the sample for microscopy, a result of freezing in liquid $N_2$. The cavities observed in the frozen state (Ngapo et al, 1997) are 10 fold larger in area, than those observed after thawing in the present study for similar freezing regimes. In the frozen state, more than 80% of the crystals had areas between 0.05 to 1.00 x $10^{-3}$ $mm^2$. Micrographs of typical images in the fresh, frozen and thawed states are given in Figures 1a-c. The distortion of the meat ultrastructure and recovery from this state after thawing are evident in these images.

A least significant difference test on the means in the upper quartile of the areas of the cavities showed that there was a significant difference between the samples that were thawed within 24 h (0.1794 x $10^{-4}$ $mm^2$) and the samples that were stored for 4 weeks (0.2071 x $10^{-4}$ $mm^2$; l.s.d of 0.0223). A typical 4 week sample is shown in Figure 1d. During storage, preferential growth (recrystallisation) in the solid state of large crystals at the expense of smaller ones occurs (Meryman, 1956). This results primarily from surface energy differences between large and small crystals and from differences in free energy due to internal strain. The recrystallisation rate increases as the temperature of the sample increases. While crystal growth could explain the differences between these values, this is complicated by the mean of the upper quartile of the fresh samples (0.1869 x $10^{-4}$ $mm^2$) which is intermediate between the two frozen-thawed values and not significantly different from either. Extensive statistical analysis is being undertaken on the results obtained from these images.

## CONCLUSIONS

After thawing, much recovery of the muscle ultrastructure, from the distorted frozen state, is evident using this cryo-SEM technique. However, observations were only made in cross sections perpendicular to the fibre direction. Actual breakage of cells is probably better seen using transmission electron microscopy and would complement the present work.

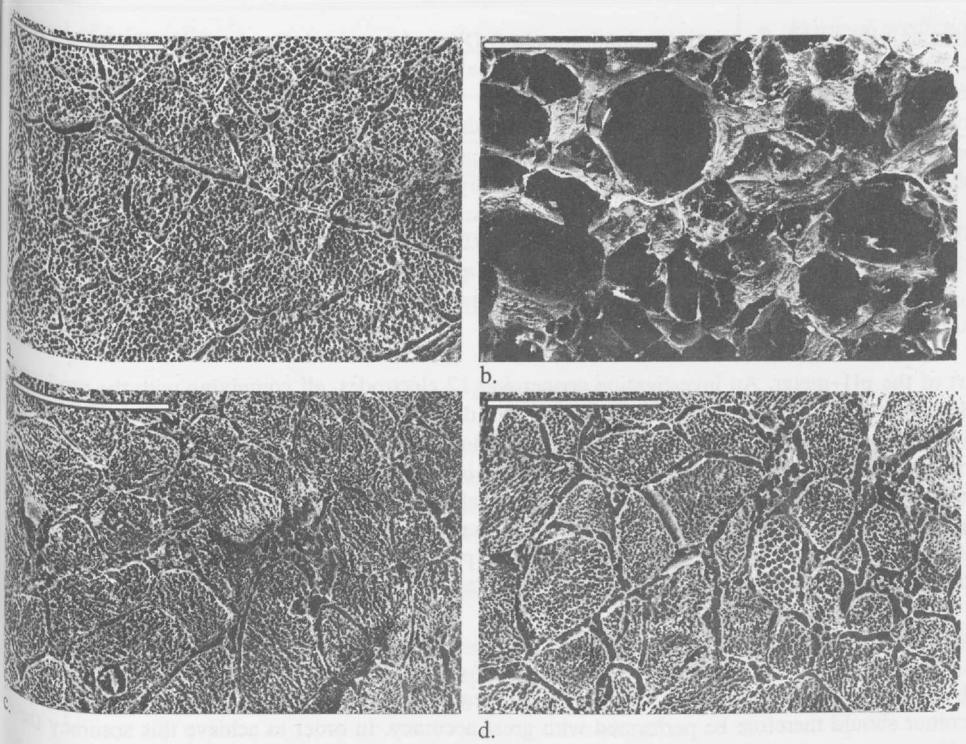


**Figure 1.** A typical example of the effects of freezing and thawing on a small sample of pork. (a) fresh, (b) freezing $t_c$ of 60 min, not thawed (Ngapo et al, 1997), (c) freezing $t_c$ of 60 min, stored up to 15 h, then thawing $t_c$ of 12 min, (d) freezing $t_c$ of 60 min, stored 4 weeks, then thawing $t_c$ of 12 min. Scale bar = 200 μm.

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