THE EFFECT OF FREEZING RATE ON THE ULTRASTRUCTURE OF A SMALL SAMPLE OF NORMAL PORK

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SUMMARY

Samples of porcine m. biceps femoris were frozen at six freezing rates. Cryo-scanning electron microscopy was used to study the ultrastructure of the meat in the frozen state. Cavities created after sublimation of the ice crystals were quantitatively analysed using an image analysis software package. No significant differences were observed in the medians of the cross sectional areas of the cavities (which estimate the ice crystals areas), measured perpendicular to the fibres, at all six freezing rates. The crystals were ten times larger than those observed in samples frozen in liquid N_2, and grossly distorted the muscle cells. These results are explained in terms of the small sample size used, and therefore lack of temperature gradient during freezing. This system simulates the periphery of a larger piece of meat which has an internal temperature gradient during freezing. Most effects on the ultrastructure will be a result of the larger crystals and extensive statistical analysis is being undertaken on the upper quartiles of the crystal area data.

INTRODUCTION

The freezing of meat has been widely studied as a means to lowering the amount of proteinacious exudate (drip or purge) lost on thawing. The loss of fluid generally reduces the eating quality, binding ability and the weight of meat, factors reducing its monetary value. This reduction in value is of particular interest to the pork industry in the western world where meat is frozen and stored prior to ham production, catering to seasonal consumer demands.

The volume of drip produced on thawing has been extensively related to the rate of freezing, which in turn has been related to the size and location of ice crystals in frozen meat. Reviews of the effects of freezing on food quality, in particular, meat and muscle tissue, are given in the book edited by Jeremiah (1996). Many of the reported histological studies examining the ice crystals formed at different freezing rates have used light and transmission electron microscopy. These have required sample preparation methods such as freeze substitution (for example, Bevilacqua and Zaritzky, 1980) or fixation (such as, Grujic et al, 1993)

This study aims to provide ultrastructural information about ice crystals formed at different freezing rates in a small sample using a cryo-scanning electron microscopy (SEM) technique described 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. Sample preparation was conducted at 4°C. Three 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 expandable polyurethane foam (5 mm length) at either end. The holders, firmly held together with rubber rings, were placed in latex sheaths and secured with cable ties. The samples had copper constantan thermocouples inserted longitudinally into their centres. Two further thermocouples were placed in the coolant and another 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 used to monitor the freezing rate. The samples were placed vertically in a B.Braun Frigomix S cooling bath with a B.Braun Thermomix UB attachment.

Freezing velocity was defined as time to traverse the temperature range from -1 to -7°C, where 80% of the water in meat is frozen. Characteristic freezing times (t_c) of 12, 30, 60, 120, 240 and >900 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 samples with a 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, temperatures were lowered from -7 to -20°C in 15 min. After 1 h at -20°C, samples were transferred to a freezer at -18°C where they were removed from the aluminium holders and wrapped in cryovac barrier bags for up to three days. Each freezing time was investigated using one muscle from one animal. The experiments were conducted in duplicate, using a second set of animals. All experiments were conducted in a random order of freezing rates.

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 cryomicroscope 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 with a scalpel blade perpendicular to the fibre direction, 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. Meat samples from two animals were used for each freezing and storage combination and two stubs of each sample were used for analysis. At least 400 cavities were measured from each image. The cavities were considered equivalent to crystal sizes and were sorted into classes of cross sectional areas of 1×10^{-3} mm^2 (for example, from 1×10^{-3} to 2×10^{-3} mm^2, 2×10^{-3} to 3×10^{-3} mm^2). The median area was determined for each image. The closest approximation to zero that could be obtained, at the magnifications of the images, was 0.05×10^{-3} mm^2. At higher magnifications, no more than 5% of cavities had areas less than this approximation.

RESULTS AND DISCUSSION

No differences were observed in the medians of the numbers of ice crystals formed at different freezing rates. These results are in contrast to the reports of Love (1958a, b) and Love and Haraldsson (1961) who observed a series of changes in the nature of ice crystal formation of the water in the tissue at different freezing rates. These workers defined the freezing rate as the time to freeze from 0 to -5C for layers of cod fillets. It was observed that at a freezing time of 5 min, many small intracellular ice crystals formed which increased in size and

decreased in number until only one intracellular crystal was evident at a time of 50 min. After 75 min, the size of the crystals had increased. This was observed to be the slowest freezing rate at which ice formed intracellularly. At 100 min, small extracellular ice crystals were observed which grew in size and decreased in number as freezing time increased. At greater than 750 min only very large extracellular ice crystals were evident. This work was used to select the freezing rates in the present study.

In the current study, the temperature at the centre of the meat did not lag behind the temperature of the coolant and therefore there was no evidence of a temperature gradient in the meat sample during freezing. Regulation of the size of the crystals by the speed of cooling, according to the relative positions of the curves of nucleation rate and growth velocity versus supercooling, has been suggested by Menegalli and Calvelo (1979) to be limited to samples with no temperature gradients (small pieces of meat) or to the peripheral region of large pieces of meat. Bevilacqua et al (1979) explain that when meat comes into contact with the refrigerating system a substantial supercooling is reached which generates a number of nuclei proportional to it. In the interphase in which the crystals are formed, the equilibrium temperature is soon reached due to a release of the latent heat of crystallisation. As crystal growth elevates the temperature, no further nucleation occurs.

During this nucleation phase, crystal growth and nucleation occur simultaneously. A range of crystal sizes would therefore be expected at the termination of this phase. The continuation of crystal growth beyond this point suggests that there should be few very small crystals, and that the time to reach the final freezing temperature should influence the sizes of crystals. While no differences were observed in the range of crystal areas at the freezing rates investigated here, comparison to frozen in liquid N_2 (Ngapo et al, 1997) show average crystal cross sectional areas 10 fold greater using the slower freezing rates; freezing in N_2 resulted in greater than 90% of the crystal areas between 0.05 to 1.00 x 10^{-4} mm^2 compared to 80% of areas between 0.05 to 1.00 x 10^{-3} mm^2 at the rates used here. The large crystal areas created a gross distortion of the muscle cell structures compared to the structure observed in the samples frozen in liquid N_2. To ensure that this was not an artefact created by the sample preparation for SEM, sublimation was observed as it occurred. No evidence of distortion as a consequence of the preparative procedure was found. The nuclei formed in the samples frozen in liquid N_2 would have had very short growth phases, if any, and it is therefore reasonable to assume that these areas indicate values slightly larger than that of the nuclei. The effective absence of these smaller crystals in the slower frozen samples is an indication that crystal growth has occurred. While the rate difference between freezing in liquid N_2 and freezing in this study result in large differences in crystal areas, it is suggested that the curves of nucleation rate and growth velocities versus supercooling for the freezing rates in the present investigation are not significantly different so as to generate a difference in the sizes of crystals formed at the different freezing rates studied and in the small sample size used.

Menegalli and Calvelo's theory (1979) of dendritic ice formation explains that the different zones of crystal sizes and locations observed in large pieces of meat where a temperature gradient exists, are a consequence of the sample size which leads to different thermal histories according to the zone considered. This explains the different results in the present work and the work of Love (1958a, b) and Love and Haraldsson (1961), who used a much larger sample size than the cylinders of meat used here. In larger samples of tissue, except for a small zone which corresponds to the refrigerated border of the sample (in which nucleation and thermal dendritic growth exist) nuclei grow through cells or cellular dendrites, according to whether the heat extraction is low or high, respectively. Except for at the refrigerated border, only extracellular ice is expected.

The appearance of intracellular ice depends on the magnitude of the supercooling achieved by the refrigerated border. It is generally accepted that intracellular supercooling is lower than that in the interfibre space (Meryman, 1956). This effect, either due to a greater solute concentration in the intracellular fluid, or to the existence of a temperature difference between the interior and the exterior of the fibres, leads to the commencement of the nucleation in the extracellular space. However, if more heat is extracted from the system than that originated by the formation of extracellular nuclei, the temperature falls until the supercooling necessary for nucleation inside the cells is reached. According to these concepts, the appearance of intracellular ice should be expected at low values of the characteristic freezing time. The technique used in the present study could not distinguish between intra- and extracellular locations of ice crystals.

About 80% of the crystal areas are between 0.05 to 1.00 x 10^{-3} mm^2. This large proportion of the crystals equates to an area averaging 21% of the total area of all the crystals in an image. The distortion of the ultrastructure is largely a result of the latter 20% of the crystals. Disregarding the lowest area class, lower and upper quartile values and medians of the remaining distributions were estimated (results not shown). These results showed the emergence of complex patterns and extensive statistical analysis is being undertaken.

CONCLUSIONS

Significant differences in the medians of crystal areas were not evident at the different freezing rates investigated here. These results were attributed to the small sample size used which is suggested to simulate the refrigerated border in a larger piece of meat. As the underlying cause of the ice structure produced in a sample is determined by the process of nucleation that takes place at its border, the study of the conditions that regulate the number of crystals in that zone is of great importance in predicting the ice-fibre configuration in frozen tissues. (Menegalli and Calvelo, 1979). A logical extension of this investigation is the use of a larger sample size that has a thermal gradient across it during freezing thereby simulating industrial conditions. Such a study would provide comparison of results obtained using this SEM method with those reported in literature.

REFERENCES

1. Love, R.M. (1958a). *J. Sci. Food Agric.*, **9**, 257.
2. Love, R.M. (1958b). *J. Sci. Food Agric.*, **9**, 262.
3. Love, R.M. and Haraldsson, S.B. (1961). *J. Sci. Food Agric.*, **12**, 442.
4. Menegalli, F. and Calvelo, A. (1979). *Meat Sci.*, **3**, 179.
5. Ngapo, T.M., Babare, I.H., Murphy, K.L., Reynolds, J. and Mawson, R.F. (1997). Submitted to International Congress of Meat Science and Technology.
6. Payne, S.R., Sandford, D., Harris, A. and Young, O.A. (1994). *Meat Sci.*, **37**, 429.
7. Bevilacqua, A., Zaritzky, N.E. and Calvelo, A. (1979). *J. Fd. Technol.*, **14**, 237.
8. Jeremiah, L.E. (Ed.). (1996). *Freezing Effects on Food Quality.* Marcel Dekker, Inc., New York.
9. Grujic, R., Petrovic, L., Pikula, B. and Amidzic, L. (1993). *Meat Sci.*, **33**, 301.
10. Meryman, H.T. (1956). *Science*, **124**, 515.