FREEZING OF MITOCHONDRIA, MEAT AND LIVER CELLS: OXYGEN CONSUMPTION AFTER SUCCINATE ADDITION

Phung, V. T[1*]., Egelandsdal, B.[1], Sælid, E.[1], Volden, J.[1] and E. Slinde[1,2]

[1]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box5003, N-1432 Aas, Norway

[2]Institute of Marine Research, P.O. Box 1870, Nordnes, N-5817 Bergen, Norway.

[*]Corresponding author (phone: +47-649-66-237; fax: +47-649-65-901; e-mail: vinh.phung@umb.no)

Oxygen consumption is a major factor regarding meat quality and affects color stability and lipid oxidation. Oxygen consumption have been determined in perfused liver and muscle cells and also in isolated mitochondria from the same tissue after succinate addition. Fresh and frozen meat at various pH values have been investigated. Oxygen consumption in both perfused liver cells and liver mitochondria was higher than in muscle cells and mitochondria. pH reduction from 7-5 gave a more pronounced decrease in oxygen consumption than did freezing conditions. Procedures for isolation of mitochondria from liver and *M. masseter* from pork have been established. Permeabilized meat seemed to indicate a higher degree of oxygen consumption at low pH than did isolated mitochondria from the same tissue.

***Index Terms*—perfused cells, respiration, color, electron transport system, myoglobin**

I. INTRODUCTION

Oxygen consumption in meat is important with respect to keeping myoglobin in a reduced state leading to immediate blooming when exposed to oxygen after package opening. Reducing ability, blooming ability and the sustainability of the MbO_2 form are central in quality issues. Mitochondria play a pivotal role in the *post mortem* color stability in meat as an antioxidant (Lass and Sohal 1998). However, knowledge is limited with respect to the importance of mitochondrial oxygen consumption for color stability in fresh and stored meat. As shown by Tang et al., (2005) myoglobin reduction may occur through oxidation of succinate at complex II in the electron transport system. The current theory suggests electron transfer between complex III and IV prior to final reduction of myoglobin on the mitochondrial outer membrane. We have used perfused muscle, liver cells and their isolated mitochondria treated at different pH values and storage conditions relevant to meat production. Oxygen consumption was induced by succinate addition. The main aim was to describe differences in oxygen consumption in the muscle cells and muscle mitochondria and compare the results with liver; the tissue that is most commonly used for respiration studies.

II. MATERIALS AND METHODS

Porcine liver and *M. masseter* muscle of the Norwegian Land Race were obtained on ice from a local abattoir within one hour of slaughter. All chemicals were purchased from Sigma Chemicals Corp. (St. Louis, MO). All chemicals were of analytical grade.

Tissue permeabilization

Muscle and liver pH were measured 2 hrs *post-mortem* with a Beckman-pH meter (Beckman Instrumens Inc., Brea, CA, USA) equipped with a Mettler Toledo Inlab 427 electrode (Mettler-Toledo Gmbh, Urdorf, Switzerland) with temperature adapted calibration. Muscle tissue fibers were separated in relaxing solution containing 15 mM phosphocreatine, 10 mM Ca-EGTA (0.1 mM free calcium), 20 mM imidazole, 20 mM taurine, 6.56 mM $MgCl_2$, 50 mM K-2-(*N*-morpholino)ethanesulfonic acid , 0.5 mM dithiothreitol, and 5.77 mM ATP adjusted to pH 7.1. Liver

tissue were treated equally without the rigorous separation of fibers. The tissues were permeabilized in relaxing solution solution containing 0.052 mg/ml saponin for 30 min.

Isolation of mitochondria

Liver: 10 g of liver was excised and minced in homogenization medium (0.25 M sucrose, 1mM EDTA and 5.0 mM potassium phosphate adjusted to pH 6.0) for mitochondrial isolation. The tissue mince was homogenized at 400 rpm in a glass/teflon Potter Elvehjem and the homogenate centrifugated according to Slinde and Flatmark (1973) for determination of the sedimentation coefficient.

Muscle: Similar conditions as for liver mitochondria were applied to muscle, additionally, after extensive mincing the tissue was digested for 30 min with 0.05% trypsin in PBS solution supplemented with 10 mM EDTA. Following homogenization, cell debris and large amounts of collagen were filtered using medical gauze.

Centrifugation: This was done in a Sorvall RC5-5C at 4°C with swing-out bucket rotor HB-4 (Thermo Scientific, Asheville, NC, USA). Centrifugation and determination of optimal centrifugation speeds for isolation of mitochondria were done according to Slinde and Flatmark (1973).

Storage and pH

Mitochondrial pellets and liver and muscle (40 – 60 g pieces) were frozen at -20°C and flash frozen in liquid nitrogen with subsequent storage in -80°C. Isolated mitochondria were kept concentrated and frozen as pellet. Isolated mitochondria were used in an amount between 0.60 – 3.44 mg of protein/ml. Small pieces of tissue (~5 g) were isolated from the center of the pieces, and subsequent separation yielded 10 – 70 mg of wet weight tissue for respiration measurements.

Tissue samples were thawed in water baths at 37°C and mitochondrial pellets were resuspended in isolation buffer at 4°C. Measurements in oxygen chambers were conducted in respiration medium adjusted with 10 mM KH_2PO_4 or 5.0 M KOH to pH 7.1, 6.5, 6.0, 5.5, and 5.0. Tissues frozen at -20°C was measured the following day (<24h) while tissues at -80°C were measured after 24 - 48hrs.

Respiration measurements

High-resolution respirometry was carried out with Oroboros Oxygraph-2K instruments (Oroboros Instruments, Innsbruck, Austria) as described by Gnaiger (2001). For respiration of permeabilized fibers at 37°C, the oxygen concentrations in the chambers (2 ml) were increased by addition of H_2O_2 and catalase until an O_2 concentration of ~400µM was reached. Respiration of isolated mitochondria were carried out at room temperature and normal oxygen concentrations (250 µM).

The respiration medium were adjusted to pH 7.1, 6.5, 6.0, 5.5, and 5.0 prior to the experiements. The mediums consisted of 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-methanesulfonate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1.0 g/l BSA. Chemicals with pH 7.1 was added to the respiration chambers with stock concentration of 0.1 mM rotenone and 1.0 M succinate. Succinate was adjusted to pH 5.5 and 5.0 to achieve respiration at pH 5.5 and 5.0.

Determination of glutamate dehydrogenase and protein concentrations

Glutamate dehydrogenase (EC 1.4.1.3) was used as marker enzyme for mitochondria and the dehydrogenase activity determined spectrophotometrically at 340 nm by reduction of NADH using glutamate dehydrogenase assay kit from Dialab (Wiener Neudorf, Austria). Protein concentrations were measured by fluorescence-based quantitation using Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA).

III. RESULTS AND DISCUSSION

Glutamate dehydrogenase was used as a marker enzyme to determine the sedimentation properties of liver mitochondria. Based on the theory of convergence of the sediterm at time integrals, low and high speed runs were carried out at time integrals of $5.8 \cdot 10^7$ (3000 rpm) and $3.7 \cdot 10^8$ (7500 rpm), respectively for the isolation of mitochondria (Slinde, Morild et al. 1975).

Oxygen consumption is believed to be an important contributing electron source for the *in vitro* reduction of myoglobin (Tang, Faustman et al. 2005). One method available for elucidating ability to consume oxygen is by measuring oxygen consumption when succinate is administrated to muscle cells. We have looked at oxygen consumption at low temperature and low pH for fresh and frozen meat using succinate induced respiration.

Figure 1a shows the changes in oxygen consumption rate at pH values from 7.1 – 5.0 upon addition of succinate. Oxygen consumption rate for permeabilized liver cells were higher than those obtained for permeabilized muscle cells. Both types of cells revealed decreased rate of oxygen consumption at low pH values. Furthermore, it is interesting to note that the enzyme responsible for reducing myoglobin in bovine, metmyoglobin reductase, was shown to have the highest reductive ability at pH 6.4 (Reddy and Carpenter 1991) where oxygen consumption in the cells was high. Permeabilized tissue succinate dehydrogenase was most active at pH ~6. Isolated liver mitochondria had a higher oxygen consumption when succinate was added than muscle mitochondria (Figure 1b). When pH dropped to 5.0, mitochondria from both types of tissues had a very low respiration.

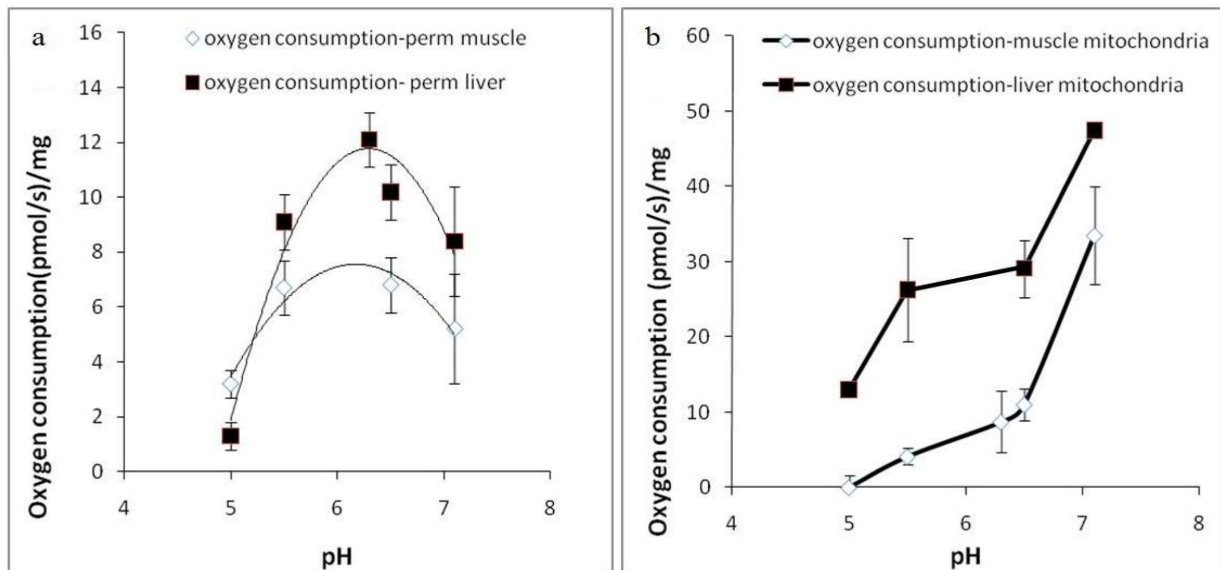

Figure 1. Oxygen consumption rate as a function of pH for permeabilized muscle and liver cells per w.w. mg/ml (panel a) and for isolated muscle and liver mitochondria per [protein] mg/ml (panel b) in the presence of succinate. Error bars indicate standard errors. pH 5.0 and 5.5 (n = 6), pH 6.0, 6.5 and 7.1 (n = 8).

Liver that was frozen did not show reduced oxygen consumption after thawing when examined by permeabilization at pH 7.1 (Figure 2a). Muscle fibers' respiration were seemingly maintained following freezing the tissue both -20ºC or -80ºC at pH 7.1. At pH 5.5 there were obvious effect of freezing upon oxygen consumption of the thawed meat; the meat frozen at -20ºC tended to have the lowest oxygen consuming after thawing. Similar results were obtained for muscle mitochondria and it is apparent that mitochondria at pH 5.5 had a very low oxygen consumption that was strikingly impaired after freezing. Using isolated mitochondria, as the single method for oxygen consumption, would lead to an underestimate of oxygen consumptionn in the *in vitro* situation in meat (Tang, Faustman et al. 2005). Our results indicate that permeabilized meat respire at a higher degree at low pH than what is achieveable in isolated mitochondria of the same tissue.

pH variations affects the enzymatic activity by changing the quaternary structure and the interactions between proteins and swelling of mitochondria by discharging the proton gradient (Wrigglesworth and Packer 1970). Our results show that pH has a stronger effect on mitochondrial respiration than freezing. However, it should be noted

that provided pH changes are reversible (Novelli, Nanni et al. 1962; Wrigglesworth and Packer 1970) the effect might not be as detrimental as freezing. Nonetheless, if not regulated properly cell and mitochondrial lysis will have a propagative effect on pH.

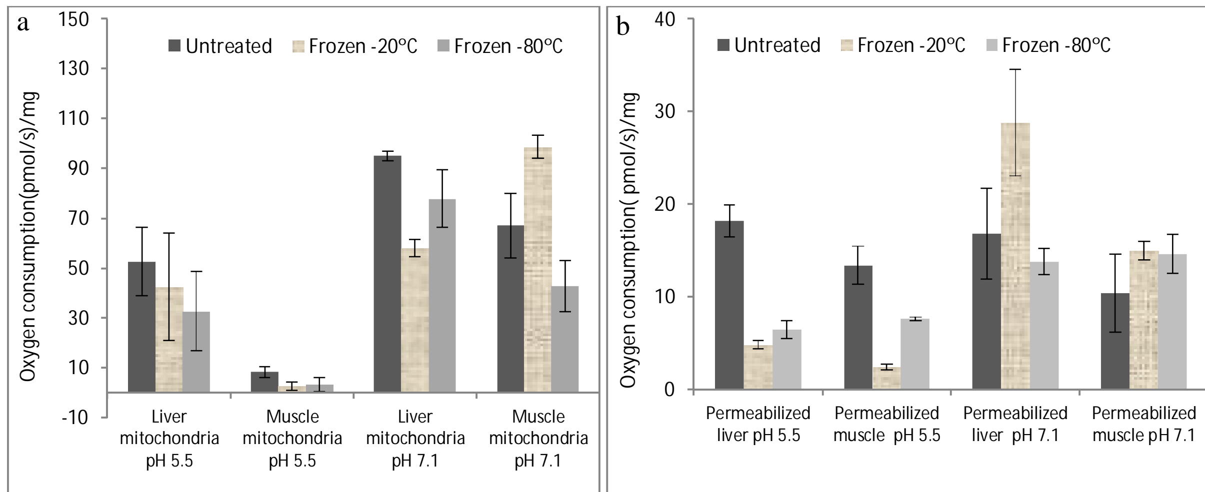

Figure 2. Oxygen consumption rate using two differnt freezing methods for meat and liver before oxygen consumption rate was measured using permeabilized muscle and liver cells w.w. mg/ml (panel a) and for isolated muscle and liver mitochondria [protein] mg/ml (panel b). Error bars indicate standard errors. pH 5.0 and 5.5 (n = 6), pH 6.0, 6.5 and 7.1 (n = 8).

IV. CONCLUSION

Liver and oxidative red muscles are tissues containing high amounts of mitochondria and have traditionally been viewed upon separately because of their different functions. In the presented study we have compared the tissues and their respective mitochondrial population's oxygen consumption after different freezing conditions and at normal and low pH levels.

Oxygen consumption in muscle remained after freezing at -20ºC or -80ºC, provided pH was neutral. Moreover, our results show that pH had the highest impact on meat oxygen consumption compared to freezing. Optimal pH in meat would not only reduce the availability of O_2 as an oxidant but also sustain the mitochondrial electron transport system as a site for myoglobin reduction.

REFERENCES

Gnaiger, E. (2001). Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply, Elsevier Science Bv.

Lass, A. and R. S. Sohal (1998). "Electron transport-linked ubiquinone-dependent recycling of alpha-tocopherol inhibits autooxidation of mitochondrial membranes." Archives of Biochemistry and Biophysics **352**(2): 229-236.

Novelli, A., G. Nanni, et al. (1962). "pH as a controlling factor of shape and function of mitochondria." Experientia **18**(12): 558-&.

Reddy, I. M. and C. E. Carpenter (1991). "Determination of metmyoglobin reductase-activity in bovine skeletal-muscles." Journal of Food Science **56**(5): 1161-1164.

Slinde, E. and T. Flatmark (1973). "Determination of sedimentation coefficients of subcellular particles of rat liver homogenates. A theoretical and experimental approach." Anal Biochem **56**(2): 324-40.

Slinde, E., E. Morild, et al. (1975). "A general and rational approach to the optimal recovery of mitochondria by differential centrifugation in homogenous media." Anal Biochem **66**(1): 151-8.

Tang, J., C. Faustman, et al. (2005). "Mitochondrial reduction of metmyoglobin: dependence on the electron transport chain." J Agric Food Chem **53**(13): 5449-55.

Wrigglesworth, J. M. and L. Packer (1970). "pH-dependent changes in mitochondrial membrane structure." J Bioenerg **1**(1): 33-43.