Verification of the mechanism of lactate on metmyoglobin-reducing activity in vitro

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Introduction

Lactate is a single natural ingredient that can address both safety and quality issues. On one hand, lactates exhibit antimicrobial properties against nonpathogenic (Brewer *et al.* 1991) and pathogenic (Miller & Acuff, 1994) microflora. On the other hand, the properties of lactate to maintain almost constant the pH of beef meat and avoid its oxidation have been also recognized (Sallam & Samejima 2004). It is well known that lactate promotes color stability of fresh meat although its mechanism is not completely understood. Two different causes have been reported in the literature. First, Mancini *et al.* (2008) demonstrated that sodium lactate limits *in vitro* oxidation of equine oxymyoglobin and therefore metmyoglobin formation. The authors suggest that lactate alters equine oxymyoglobin redox stability. Second, it has been suggested that lactate enhancement improves metmyoglobin reducing activity (MRA) via lactate dehydrogenase (LDH). Watts *et al.* (1966) hypothesized that since postrigor meat contains both lactate and LDH, hydrogen may be donated from lactate to nicotinamide adenine dinucleotide (NAD^+) and subsequent NADH production by LDH could be coupled with MMb reduction. Kim *et al.* (2006) tested this hypothesis when they determined that nonenzymatic metmyoglobin reduction occurred in the lactate-LDH system with NAD^+, but that exclusion of NAD^+, L-lactic acid, or LDH eliminated the MMb reduction. They, consequently, proposed that the lactate-LDH system in post-mortem muscle can replenish NADH by the reduction of NAD^+, and that a NADH-dependent reducing system, either enzymatic or non-enzymatic, can reduce metmyoglobin (Fig. 1). However, in their in vitro experiment, they tested MMb reduction under non-enzymatic conditions in a lactate-LDH system using purified LDH. Since MMb reduction in meat occurs primarily through an enzymatic pathway with a NADH-dependent MMb reductase (Arihara *et al.* 1989), characterizing the mechanism by which the lactate-LDH system can couple with metmyoglobin reductase extracted from a bovine muscle *in vitro* would provide greater insight into understanding the effects of lactate on meat color. Kim *et al.* (2006) used a pure lactic acid salt to evaluate the lactate-LDH system in an *in vitro* study, but this did not realistically simulate the incorporation of lactate into meat tissues in an injection solution. Thus, testing lactate used as industry standard and comparing different kinds of lactate salt forms such as sodium lactate and potassium lactate on MMb reduction *in vitro* would provide valuable insight into the underlying mechanism of lactate color stabilization in enhanced beef. Therefore, the objectives of this study were to determine the effects of the lactate-LDH system on MMb reduction through the enzymatic MRA pathway and to evaluate different lactate salts on MMb reduction via the lactate-LDH system in vitro.

Materials and methods

Metmyoglobin reductase extracts were obtained from freeze stored (-20°C) beef *m. triceps brachi* (shoulder clod) as described by Reddy and Carpenter (1991) with slight modifications. 5g of chopped muscle was homogenized in 25mL phosphate buffer (2.0 mM, pH 7.0) by using an Ultra turrax® homogenizer at 13500 rpm (around 2-3 minutes). The homogenate was centrifuged at 35,000g for 30 min at 4°C, and the supernatant was filtered using a 0.45 µm micropore® membrane, in order to remove fat layer. Oxyhemoproteins were oxidized with an excess of $K_3Fe(CN)_6$ and the solution was dialysed (10,000 MW cut-off membrane) at 4°C with three 1L per hour changes of 2mM phosphate buffer pH 7.0, in order to remove the excess of ferricyanide. The solution was centrifuged at 15,000 g for 20 min at 4ºC. The volume of the supernatant was adjusted to 25mL with 2.0mM phosphate buffer pH 7.0. MRA of the extracts was measured spectrophotometrically by adding reactants in various combinations to 10mm path length polystyrene cuvettes with 1.1 mL final reaction volume under aerobic conditions. The EDTA, $K_4Fe(CN)_6$, NAD^+ and Lactate solutions were prepared in 30mM

phosphate buffer pH 7.0. The reaction was initiated by addition of NAD^+ and lactate. One unit of MRA was defined as the quantity which would reduce one nanomole of metmyoglobin per minute per gram of muscle, during the initial linear phase of the assay, using a difference in molar absorptivity of 12,000 l mol^{-1} cm^{-1} at 580 nm. The assay was conducted at 30°C. Each assay was made on the same day in triplicate for each extract and each set of conditions. Statistical analyses were performed using Statistica (version 7.0). Least square means were generated and significant difference ($P<0.05$) between groups were determined using the ANOVA or MANOVA.

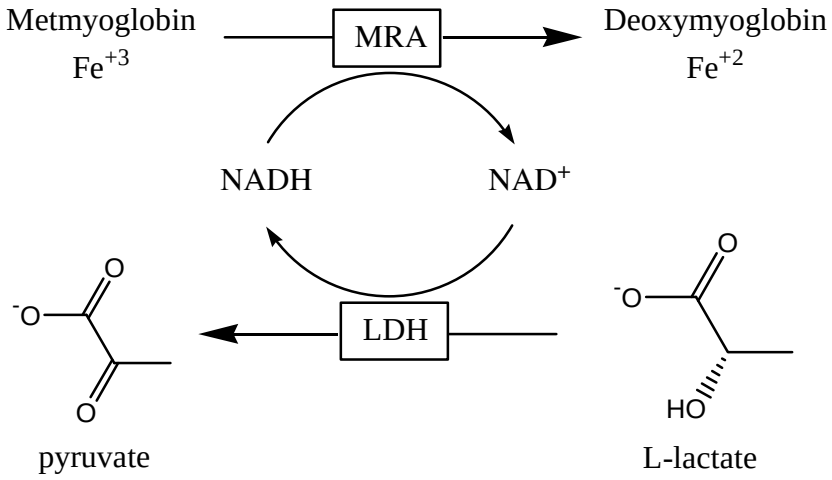

Figure 1. Mechanism of the lactate-LDH system for generating NADH for MRA proposed by Kim *et al.* (2006).

Results and discussion

Kim *et al.* (2006) reported nonenzymatic reduction of equine metmyoglobin in a lactate-LDH-NAD^+ system. In our study, we observed effective reduction of horse MMb by an *m. triceps brach*i extract after addition of NAD^+ and sodium L-lactate to the assay mixture. In the absence of the meat extract, NAD^+ or L-lactate, reduction of MMb didn't take place (Table 1). The role of LDH in the MMb reduction system is confirmed when no MRA is observed by addition of oxalate, a known LDH inhibitor. MMb was neither reduced when sodium L-lactate was replaced by sodium D-Lactate due to the selective interaction of LDH with L-lactate.

Increasing the amount of enzyme extract resulted in a non-linear increased rate of reduction ($P<0.05$; 0.15, 0.30 and 0.40 mL of enzyme extract afforded an activity of 16.50, 31.78 and 37.89 nmol MMb/min/g, respectively). After addition of NAD^+ and L-lactate to the standard reduction mixture a lag period of at least 5 minutes is observed before MMb reduction takes place. A decrease in the volume of extract increased the lag period up to 12 minutes. The extract concentration may affect the NADH formation rate or other unknown processes involved in the reduction of the metmyoglobin from lactate and NAD^+.

NAD^+ concentration is directly related to meat-color stability because NAD^+, a source of oxidized substrate decreases rapidly in post-mortem muscles. Saleh *et al.* (1968) reported higher MMb reduction by NAD^+ addition to ground beef samples. Kim *et al.* (2006) demonstrated that non-enzymatic reduction of MMb in the lactate-LDH system is NAD^+ concentration dependent. We also observed a non-linear increase of MRA at increasing amounts of NAD^+ until a saturation level of 6.5 mM ($P<0.05$; 2.5, 5.0, 6.5 and 8.0 mM of NAD^+ afforded an activity of 22.9, 32.7, 35.4 and 34.5 nmol MMb/min/g, respectively).

Increasing amounts of NaL resulted in an increased rate of reduction ($P<0.05$; 25, 50, 75, 100 and 200 mM of NaL afforded an activity of 3.3, 14.0, 22.9, 29.9 and 41.9 nmol MMb/min/g, respectively). No significant differences ($P>0.05$) were observed in MRA when MMb was reduced by addition of potassium L-lactate instead of NaL to the assay confirming that MRA is influenced by the lactate salt anion and not by the lactate salt cation.

Table 1. Enzymatic reduction of Equine Metmyoglobin with Lactate at 30°C

Solution components								Activity
MetMb (0.1mM)	Extract (0.3mL)	$K_4Fe(CN)_6$ (3mM)	EDTA (5mM)	NAD^+ (6.5mM)	L-Lactate (200mM)	D-Lactate (200mM)	Oxalate (200mM)	(nmoles/min/g)
+	+	+	+	+	+	-	-	40.9
+	-	+	+	+	+	-	-	0
+	+	+	+	-	+	-	-	0
+	+	+	+	+	-	-	-	0
+	+	+	+	+	-	+	-	0
+	+	+	+	+	-	-	+	0

[a]Subtances present (+) or absent (-) in the assay mixture. [b]Bovine extract was freshly prepared from a freeze stored (-20°C) beef *m. triceps brach*i.

The results of this research suggest that the lactate-LDH system is valid a mechanism involving enzymatic MMb reduction *in vitro*. Commercial lactate salts (NaL and KL) can react with NAD^+ and bovine muscle extracts to replenish NADH, which is subsequently coupled with bovine MMb reductase to catalyze MRA *in vitro*. Future research should focus on determining the efficacy of the lactate-LDH system of actual muscle injection enhancement for different muscles and/or different species (beef, pork, or lamb) on meat color stability and MRA.

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