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On the Oxidation of Myoglobin to Metmyoglobin During the Storage of Chilled Beef.

D.A. Ledward, Food Science Laboratories, Department of Applied Biochemistry and Nutrition, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough, Leicestershire, England.

Summary

The present study was undertaken to try and determine the factors involved in the formation of the undesirable, brown metmyoglobin during the aerobic storage of fresh beef. Lactobacillus 58 and Pseudomonas 1482 at levels below about $10^8/cm^2$ had no effect on the formation of metmyoglobin during the aerobic storage of beef at 1°C. At higher (spoilage) levels of Pseudomonas oxygen depletion at the meat surface caused increased formation of metmyoglobin and reduced myoglobin. Microbacterium 22 gave slight but significant increases in metmyoglobin concentration at contamination levels above about $10^4/cm^2$.

As bacterial contamination cannot explain the wide differences in metmyoglobin forming abilities found between muscles studies were made of the metmyoglobin reducing abilities of muscles under both aerobic and anaerobic conditions.

The aerobic reduction of metmyoglobin in beef slices (2.0 ± 0.5 mm. thick) formed by storage at low O_2 concentrations, was highly correlated ($r = -0.94$, $P < 0.001$) with the accumulation of metmyoglobin in slices of 15 ± 1 mm. thick, during aerobic storage at 1°C. A positive correlation ($P < 0.05$) was found between the haematin content of muscles and their metmyoglobin forming abilities. No significant correlation was found between the metmyoglobin reducing activity of anaerobically stored minced beef following ferricyanide oxidation (MRA) and the accumulation of metmyoglobin during aerobic storage

at 1°C. It is suggested that differences in the aerobic reducing abilities between muscles is the main factor affecting the formation of metmyoglobin in beef and possible reasons why MRA fails to measure this true reducing ability are discussed.

Introduction

It is well known that during the storage of fresh beef in oxygen containing atmospheres oxidation of oxymyoglobin (red) to metmyoglobin (brown) occurs. This undesirable colour change will attain increased significance as the size of the meat portion decreases. Thus, if half or quarter carcases are held in chilled storage any surface discolouration will be relatively unimportant after butchering for retail consumption. Limitations on storage space often make this method of storage and distribution uneconomical and there is an increasing tendency to age and distribute beef in boneless, retail size cuts. Under these conditions surface discolourations are of major importance.

The autoxidation of myoglobin has been extensively studied in aqueous solution (Brooks, 1931, 1935, George et al., 1952a, 1952b, Brown et al., 1969) and the effect of such factors as oxygen concentration, pH and ionic strength evaluated. However, these detailed studies can not be applied indiscriminately to the behaviour of the haemoproteins in meat, as, even in post-rigor meat, a system is available that can bring about the reduction of metmyoglobin to one of the reduced forms i.e. oxymyoglobin or reduced myoglobin (purple) (Stewart et al., 1965). Thus, at 1°C the concentration of metmyoglobin in whole beef muscles remains virtually constant from 5 to 28 days (Ledward, 1970, 1971). However, this concentration varies from muscle to muscle and the pseudoequilibrium lasts for much shorter periods in thin (2 mm.) slices (Ledward, 1971).

Over the last decade much effort has been directed into attempting to elucidate the metmyoglobin reducing mechanism(s) in meat. Stewart et al., (1965) have described a technique for measuring the metmyoglobin reducing activity (MRA) of anaerobically stored minced meat following ferricyanide oxidation. Ledward and Macfarlane (1971) found that aerobic reduction of

metmyoglobin occurred in meat slices (3 mm. thick) in which high initial metmyoglobin concentrations had been induced by storage in atmospheres containing about 1% oxygen (Ledward, 1970).

The present work was undertaken to see if MRA and/or the aerobic reducing ability (ARA) of beef muscles correlated with the amount of metmyoglobin formed on the surface of beef muscles stored at 1°C. A preliminary note on some of these observations has already been published (Ledward, 1972).

It is possible that the formation of different levels of metmyoglobin in different muscles is dependent, in part, on different levels and types of bacterial flora. Thus it was felt desirable to study the effect of the common meat spoilage organisms, at various levels of contamination, on the formation of metmyoglobin at 1°C.

Experimental

Preparation of the Samples

The *semitendinosus* muscle and highly pigmented central portion of the *biceps femoris* were removed from a beef carcase immediately after slaughter and their outside surfaces flamed. After 3-6 days storage at 1 ± 1°C, 16 slices of thickness 2.0 ± 0.5 mm. (Kaess, 1961) were cut from each muscle and stored in 1% O_2/99% N_2 (Ledward, 1970). Two slices (15 ± 1 mm. thick) were wrapped in sterile polyethylene film (0.0015 in.) and stored in air. All storage was in the dark at 1 ± 1°C and care was taken to avoid bacterial contamination.

Other *semitendinosus* muscles were aseptically sliced to portions 15 ± 1 mm. thick prior to inoculation using the velveteen pad technique (Shaw & Nicol, 1969). Different dilutions of the pure bacterial suspensions were impressed on different slices to give a range of contamination levels.

The food spoilage organisms used were Pseudomonas 1482, Microbacterium 22 and Lactobacillus 58 where the numbers used are the culture collection numbers of the CSIRO Meat Research Laboratory, Brisbane, Australia. The properties of the cultures have been described elsewhere (Shaw and Nicol, 1969). The slices were stored in air for 7 to 14 days at $1 \pm 1^{\circ}C$ and relative humidity 99.3% (Ledward, 1970).

Determination of the equilibrium metmyoglobin concentration

After 7 days storage the concentration of metmyoglobin in the thick (15 mm.) sterile slices used for comparison with the reducing ability was determined as the mean of 16 readings on 2 slices (Ledward, 1970). Metmyoglobin concentrations are expressed as the percentage of the total haem pigments. In the contaminated samples 4 readings on each slice were taken and averaged after 1 cm^2 of the surface had been removed to enable the level of contamination to be assessed.

Determination of bacterial load

The number of viable organisms on 1 cm^2 of muscle was determined by spreading 0.1 ml. of an appropriate dilution on the surface of tryptose phytone yeast extract agar (TPY) and incubating for four days at $20^{\circ}C$.

Determination of Aerobic Reducing Ability (ARA)

After 24 hr. storage in $1\%O_2$ the metmyoglobin concentration was determined in a randomised sample of eight of the thin (2 mm.) slices. The metmyoglobin concentration in the other eight samples was determined after a further 24 hr. in air at $1^{\circ}C$. Each determination was the mean of 32 readings. Thin, 2mm., slices were used to ensure O_2 penetration throughout the sample. As differences occured in the metmyoglobin concentration of different muscles after 24 hr. in $1\%O_2$, ARA was defined as

$$\frac{\text{observed decrease in metmyoglobin conc.}}{\text{initial metmyoglobin conc.}} \times 100$$

Only muscles having metmyoglobin concentrations of 50-70% after 24 hr. in 1%O_2 were used (11 out of 16 muscles from 8 animals).

Determination of MRA, total haematin concentration and pH.

After 10-13 days storage the MRA was measured as the percentage of metmyoglobin reduced 1 hr. after ferricyanide addition (Stewart et al., 1965). Duplicate samples from the interior of the muscle were used. During this storage period no significant change in MRA occurred.

Total haematin concentration of the muscles was determined by the method of Hornsey (1956). pH was measured with a combined probe electrode on the whole muscles.

Results and Discussion

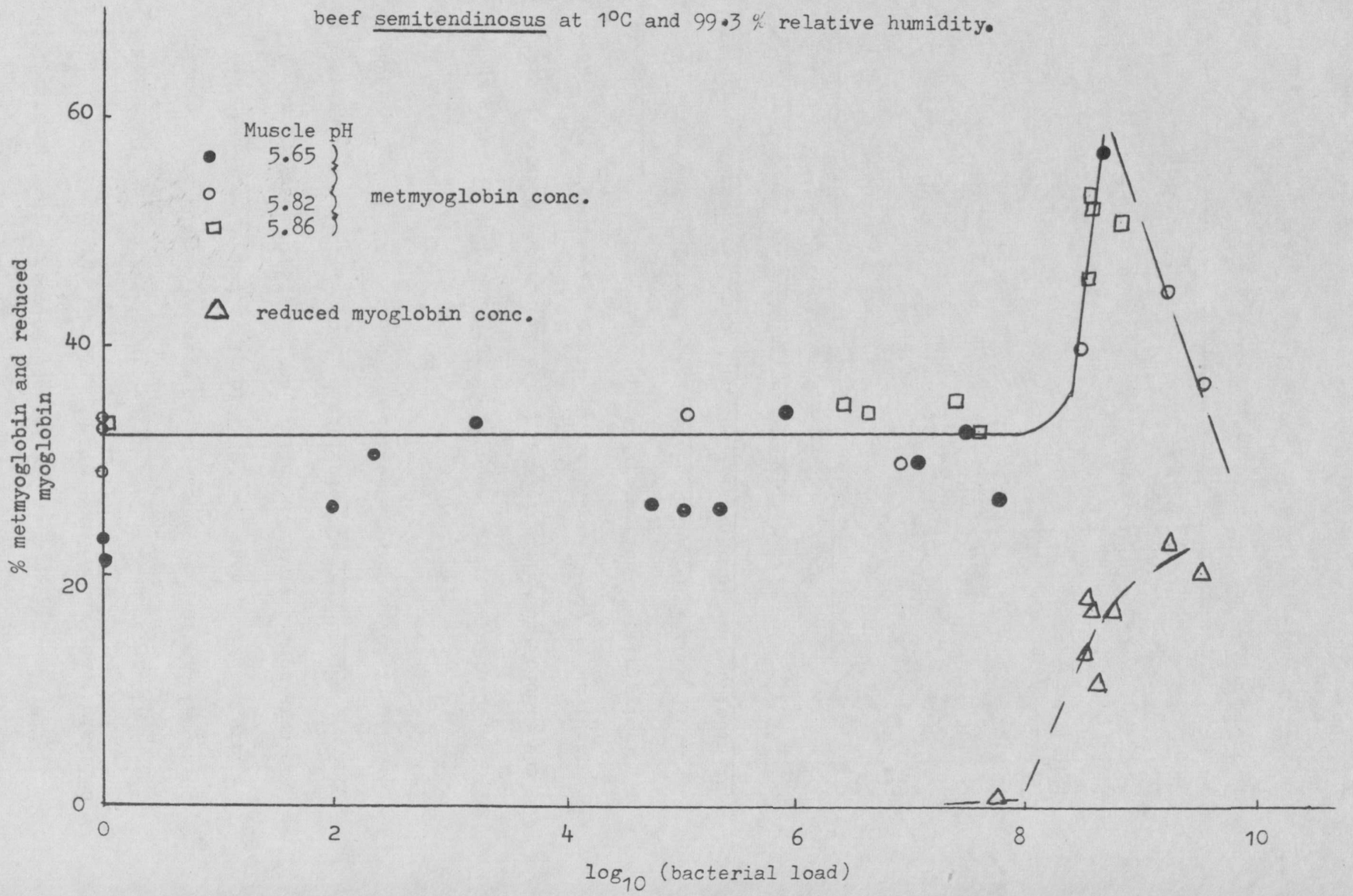
Effect of Bacterial Contamination

During air storage at chiller temperatures spoilage is usually by Pseudomonas sp. (Haines, 1933) and in Figure 1 the relationship between Pseudomonas 1482 surface concentration and metmyoglobin formation is shown.

The similarity between this relationship and that between O_2 concentration and metmyoglobin concentration (Ledward, 1970) would appear to confirm the suggestion of Butler et al., (1953) and Robach and Costilow (1961) that high levels of contamination with Pseudomonas cause increased metmyoglobin formation in fresh meat due to O_2 depletion at the surface as a result of O_2 utilisation by the bacteria.

It is seen that increased metmyoglobin formation occurred only at levels of about $10^8/cm^2$, i.e. spoilage levels. At higher levels of contamination anaerobic reduction of metmyoglobin to the purple reduced myoglobin occurred (Fig. 1.). If the O_2 supply to the meat surface is limited by the packaging conditions then increased metmyoglobin formation may occur at lower, pre-spoilage Pseudomonas populations resulting in

Fig. 1. Effect of Pseudomonas 1482 contamination on myoglobin oxidation in beef semitendinosus at 1°C and 99.3 % relative humidity.



apparent differences between muscles due to different bacteria levels. Butler et al., (1953) found the increase due to Pseudomonas contamination occurred at about $10^7/cm^2$ on steaks stored in MSAT 80. However, the large differences found between muscles in this study and others (Ledward, 1970, 1971) was at Pseudomonas levels of $< 10^4/cm^2$ when stored in air, cryovac L-film or polythene and all these films have similar, or greater, O_2 permeabilities than MSAT 80. Thus, differences in Pseudomonas contamination levels will not explain the differences observed in metmyoglobin levels between muscles.

The storage life of fresh beef can be extended by storage in CO_2 enriched atmospheres when Lactobacillus and Microbacterium become the predominant spoilage orgamisms. Robach and Costilow (1961) found that Lactobacillus plantarum had no effect on the formation of metmyoglobin and this observation was confirmed with Lactobacillus 58 (Table 1).

Table 1. Effect of Lactobacillus 58 on the formation of metmyoglobin in beef at 1°C and 99.3% relative humidity.

Bacterial load/cm^2	% metmyoglobin
$< 10^2$	37.5
1.5×10^3	32.5
3.2×10^4	29.3
7×10^4	29.5
6.5×10^5	38.7
1.9×10^7	36.2
2.1×10^8	39.5

Microbacterium 22, at levels greater than about $10^5/cm^2$, was found to cause a slight, but significant, increase in the concentration of metmyoglobin (Table 2). However, the maximum increase was only about 10% of the total surface haem pigments and it appears as a sharp increase

at a contamination level of between $10^4/cm^2$ and $10^5/cm^2$. (Table 2).

Table 2. Effect of Microbacterium 22 on the formation of metmyoglobin in beef at 1°C and 99.3% relative humidity.

Muscle 1 (pH = 5.95)		Muscle 2 (pH = 5.62)	
Bacterial load/cm^2	% Metmyoglobin	Bacterial load/cm^2	% Metmyoglobin
< 10	21.1	< 10	29.5
< 10	17.3	2.8×10^4	31.5
50	12.0	3.6×10^6	37.8
5.5×10^2	26.0	1.6×10^6	38.5
1.1×10^4	13.3	3.1×10^7	42.8
5×10^4	24.5	2.0×10^8	35.5
5×10^5	30.8	7.1×10^8	35.0
2.9×10^6	31.0	9.3×10^8	37.0
2.1×10^7	36.0		
9.1×10^8	32.0		
$\sim 10^9$	30.0		
$\sim 10^9$	38.0		

Thus, although differences in the type and amount of bacterial contamination on fresh beef may lead to slight differences in metmyoglobin concentration it would not appear to be a major factor in determining the level developed in different muscles during storage.

Relationships between equilibrium metmyoglobin concentration and ARA and MRA

In Table 3 the values found for the equilibrium metmyoglobin concentrations, ARA and MRA of the semitendinosus and biceps femoris muscles from 8 animals are summarised.

Table 3. Values found for the aerobic reducing ability (ARA) of post rigor muscle slices (2.0 ± 0.5 mm, thick), the concentration of metmyoglobin on 15 ± 1 mm. slices after 7 days storage in air and the anaerobic metmyoglobin reducing activity of minced muscle following ferricyanide oxidation (MRA).

Muscle	ARA %	Eqiv. metmyo-globin %	MRA %
Biceps femoris	10.0	66.0	66.0
"	16.4	52.5	73.0
"	15.3	44.2	80.5
"	16.0	43.4	90.0
"	18.2	43.3	26.5
"	21.4	34.8	56.8
"	19.3	32.9	39.5
"	26.4	17.7	75.0
Semitendinosus	20.6	30.5	82.4
"	23.3	19.8	61.0
"	26.1	25.0	92.0
"	N.D	21.0	100
"	N.D	22.5	100
"	N.D	14.2	32.0
"	N.D	18.7	38.0
"	N.D	19.4	80.0

ARA measured as the percentage reduction in 24 hr.

MRA measured as the percentage reduction in 1 hr.

It is seen from Table 3 that the formation of metmyoglobin on the

surface of fresh beef is highly correlated ($r = -0.94$; $P < 0.001$) with ARA. However, there is no significant correlation between the formation of metmyoglobin and MRA ($r = +0.22$).

As ARA was determined in the presence of O_2 it must represent the resultant of a reductive and oxidative mechanism. Thus, the high negative correlation between ARA and metmyoglobin concentration would arise if the differences in metmyoglobin formation between muscles originated from differences in rates of oxidation and /or reduction. In solution the rate of autoxidation of bovine myoglobin in air is about 0.004 hr^{-1} at pH 5.5 and 1°C (Brown et al., 1969). If this rate of oxidation occurs in fresh beef then in samples with 50% to 70% metmyoglobin a further 4.5 to 2.8% will form by autoxidation. In meat of pH > 5.5 less autoxidation will occur. Thus, unless the oxidation is catalysed in meat, differences in rates of oxidation will not explain the wide differences in ARA observed between muscles (Table 3).

However, positive correlations were found between the hematin concentration (haematin) of the muscle and its ability to form metmyoglobin during storage. Separate regressions for the biceps femoris and semitendinosus fitted the data better than one overall regression. The linear regressions, which were both significant at the 5% level, were % metmyoglobin = 1.44 + 4.5 (haematin). For the biceps femoris, where (haematin) was in the range 7 to 12 $mg.g^{-1}$ of wet tissue and

$$\% \text{ metmyoglobin} = 13.9 + 1.2 \text{ (haematin)}$$

for the semitendinosus, where (haematin) was in the range 2.5 to 9 $mg.g^{-1}$ wet tissue.

If catalysis of the oxidation is responsible for the differences in ARA (and metmyoglobin formation) then the positive correlation between (haematin) and metmyoglobin formation would necessitate the ratio of

catalyst : haematin to increase with increasing (haematin). This seems unlikely and so some, or all of the differences in metmyoglobin formation are probably due to differences in the effective reducing mechanism. The correlation between (haematin) and metmyoglobin formation would then reflect the occurrence of decreased pigment to reductant(s) ratios as (haematin) increased.

The lack of correlation between the MRA and metmyoglobin formation agrees with the observation that increasing the MRA of meat by the addition of glutamate had no significant effect on the accumulation of metmyoglobin (Saleh and Watts, 1968). However, Hutchins et al., (1967) found meat of high MRA tended to form less metmyoglobin during 4 days storage ($r = 0.44$ on 30 samples) while Greene (1969) stated that enzymically active samples browned more readily during storage. These results suggest that either MRA technique does not accurately measure the activity of the enzymic reduction or that the effective aerobic reduction does not utilise this mechanism. If the postulated mechanism (Saleh and Watts, 1968) is correct then the lack of correlation may be due to any of the difficulties outlined by Watts et al., (1966) attributable to the use of ferricyanide (or any chemical oxidant) in the study of enzymic reducing activity and/or differences due to ferrocyanide catalysis (Hegesh and Avron, 1967) and/or O_2 inhibition (utilisation) (Saleh and Watts, 1968). Even differences induced by the mincing and preparation of the samples may occur. However, if as suggested by Saleh and Watts (1968), O_2 completely inhibits the MRA then the aerobic reducing mechanism would utilise a completely different mechanism, e.g. Brown and Snyder (1969) have described a non-enzymic metmyoglobin reducing mechanism that could operate in vivo, while Hultquist and Passion (1971) have suggested that methaemoglobin reduction in blood may be dependent on the amount of

cytochrome b_5 in the cell. Perhaps further insight into the effective metmyoglobin reducing system in meat could be obtained by a study of the reduction under other conditions, e.g. the anaerobic reduction of metmyoglobin in beef slices and mince following storage in 1%O_2.

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Ueber den Oxydationsvorgang von Myoglobin in Metmyoglobin während der Lagerung von Chilled Beef (bei gekühlt gelagertem Rindfleisch)

D.A. Ledward, Food Science Laboratories, Department of applied Biochemistry and Nutrition, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough, Leicestershire England.

Zusammenfassung.

Zweck dieser Forschungsarbeit war der Versuch die Faktoren ans Licht zu bringen, die zur Bildung der braunen unerwünschten Metmyoglobin während der Lagerung von frischem Rindfleisch unter aerobischen Bedingungen beitragen. Lactobacillus 58- und pseudomonas 1482- Zahlen unter 10^8/ cm^2 führen zu keiner Bildung von Metmyoglobin während der aerobischen (unter Luftzutritt) Lagerung von Rindfleisch bei +1°C .Bei höheren Pseudomonas-Zahlen bewirkt der Sauerstoffmangel eine verstärkte Bildung von Metmyoglobin und von reduziertem Myoglobin an die Fleischoberfläche. Microbacterium 22 führt zu einer geringfügigen, doch wahrnehmbaren Zunahme der Metmyoglobinkonzentration bei Keimzahlen über $10^4/cm^2$.

Da die bakterielle Verseuchung die bei Muskeln festgestellten grossen Unterschiede im Vermögen Metmyoglobin zu bilden nicht verklickern kann, wurde die Fähigkeit von Muskeln Metmyoglobin zu reduzieren unter aerobischen(Luftzutritt) sowie anaerobischen (Luftausschluss) Bedingungen eingehend studiert.

Die während der Lagerung bei niedrigen Sauerstoffkonzentrationen stattfindende aerobische Reduktion von Metmyoglobin in 2,0 $\pm$ 0,5 mm starken Rindfleischscheiben ist mit der Bildung von Metmyoglobin in 15 $\pm$ 1 mm starken Scheiben während der aerobischen Lagerung bei +1°C sehr hoch korreliert ($r = -0,94$, $P < 0,001$). Es steht eine positive Korrelation ($P < 0,05$) fest zwischen dem Hämatingehalt und dem Reduktionsvermögen der Muskeln. Es besteht keine signifikante Korrelation zwischem dem Reduktionsvermögen von unter Luftausschluss gelagertem gehackten Rindfleisch nach oxydativer Behandlung mit Ferricyanid (MRA) und der Bildung von Metmyoglobin während der aerobischen Lagerung bei + 1°C. Unterschiede nach Muskeln im Reduktionsvermögen unter Luftzutritt dürfen der ausschlaggebende Faktor zur Bildung von Metmyoglobin bei Rindfleisch sein ; mögliche Erklärungen, weshalb das wirkliche Reduktionsvermögen durch MRA nicht erfasst wird, werden besprochen.

OXYDATION DE LA MYOGLOBINE EN METMYOGLOBINE PENDANT LE STOCKAGE DU BOEUF REFRIGERE

D.A. LEDWARD
ROYAUME UNI

Résumé :

Cette étude a été entreprise pour essayer de déterminer les facteurs intervenant dans la formation indésirable de la metmyoglobine brune pendant le stockage en aérobiose de la viande de boeuf.

Lactobacillus 5 8 et Pseudomonas 1482 à des niveaux inférieurs à 10^{8} cm 2 environ n'ont eu aucun effet sur la formation de metmyoglobine pendant le stockage en aérobie de viande de boeuf à 1° C à des niveaux plus élevés de Pseudomonas la déplétion d'oxygène à la surface de la viande provoquait une formation accrue de metmyoglobine et de myoglobine réduite.

Microbactérium 22 provoqua un accroissement léger mais significatif de metmyoglobine à des niveaux de contamination au dessus de 10^{4} / cm 2 environ.

Comme la contamination bactérienne ne peut expliquer les grandes différences dans la capacité de formation de metmyoglobine trouvée entre les muscles, les études furent réduites sur les possibilités de réduction de la metmyoglobine par des muscles à la fois dans les conditions d'aérobiose et d'anaérobiose.

La réduction aérobique de la metmyoglobine dans les tranches de boeuf d'épaisseur 2.0 $\pm$ 0,5 mm formée au cours d'un stockage à basse concentration d'O_2 était hautement corrélée ($r = -0,94$) à $P < 0,001$ avec l'accumulation de metmyoglobine dans des tranches à 15 $\pm$ / mm d'épaisseur dans les conditions aérobiques à + 1°C. Une corrélation positive ($P < 0,05$) a été trouvée entre la teneur en hématine des muscles et leur capacité de formation de metmyoglobine.

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Aucune corrélation significative n'a été trouvée entre la capacité réductrice de la metmyoglobine de la viande de boeuf hachée et tenue en aerobiose après oxydation au ferrocyanure et l'accumulation de metmyoglobine pendant le stockage aérobique à + 1°C.

L'auteur suggère que les différences dans la capacité réductrice des différents muscles en aérobiose constitue le facteur principal affectant la formation de metmyoglobine dans la viande de boeuf.

Les raisons possibles pour lesquelles la capacité réductrice de la metmyoglobine ne mesure pas le pouvoir réducteur réel du muscle sont discutées.

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