DARKENING OCCURRENCE IN PROTECTIVE PACKAGED DEBONED TURKEY MEAT: A BIOCHEMICAL, IMMUNOLOGICAL AND HISTOLOGICAL APPROACH

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Background

Our investigation is related to a real problem derived from industrial production in the field of poultry meat: it was found out a case of chromatic alteration associated to male turkey's deboned thighs packaged in protective atmosphere.
As known, the success of an alimentary commercial product, especially concerning meat, is due to many factors, as price, presentation and organoleptic characteristics (i.e. odour, colour, tenderness). Colour, in particular, is directly affected by the conservation period, the muscle type and the packaging technology. Now a day, the packaging in protective atmosphere, using impermeable films allowing the maintenance of high CO_2 and O_2 levels, has become commonly utilised. The CO_2 extends the shelf-life of the product inhibiting micro-organisms' proliferation while the O_2 maintains the primitive meat colour, often intended as the first hygienic indicator by the consumers.
The meat chromatic alteration, excluding additives that can be included during processing, is determined essentially by factors such as the action of microbiological agents which, developing beside a certain threshold, are capable of generating reactive molecules that can interfere with either proteins or lipids. Further significant causes are the ones associated to the tissue oxidative stress, that can be itself related to the consequent alteration of the proteic and lipidic chemical-physical condition.
In our case, since previous microbiological analysis excluded a microbic ethiology, we addressed our efforts to analyse part of the proteic constitution of the tissue and the oxidation state of the lipids.
Fundamentally, there are three proteic factors, responsible for muscles' dye: cytochromes, haemoglobin and myoglobin. The first ones are present in very small quantity, compared with the other two molecular species; haemoglobin concentration dramatically decreases during slaughtering and meat processing, while myoglobin concentration does not change. For this reason we can assume that it plays a primary role in the visible spectrum absorption capacity of the meat. Myoglobin is a 17000 Kd protein which is present in large quantity within the muscle. Its function is related to the storing of the oxygen conveyed by blood, in order to make it disposable when demanded by the muscular fibres requiring energy for contraction. The oxygen binds to the heme-iron of the protein and it is fairly the oxidation state of this atom together with other bound little molecules that imply the various wave-lengths absorption and thus determine the chromatic shades of the whole muscle. Myoglobin is present in different conditions: deoxymyoglobin is the one that keeps 2+ oxidation free iron; it determines the purple shade of the tissue. Oxygen released from blood can bind to iron and form a complex called oxymyoglobin, which doesn't change its own oxidation state. In this case the colour of the meat turns to red as in fresh meat. Further oxidation causes the oxymyoglobin transformation in metmyoglobin (iron 3+) and the darkness of the tissue.

Objectives

After a preliminary statistical evaluation of the darkening process, we have first investigated on the myoglobin's oxidation status in different sites of the samples by a spectrophotometric analysis, also used to calculate myoglobin concentration. We have also performed western immunoblotting and ELISA tests on the proteic fraction, using anti-myoglobin antibodies in order to evince potential differences among the two different methods.
Moreover, with the aim to obtain a better understanding of the phenomenon and formulate the first hypotheses on relationships between causes and effects, we have evaluated the number of α and ε-aminic groups and the content of ABVT, as indicators of decomposition degree. Besides we have investigated the oxidation level of fatty acids with the TBAR analysis.
Finally, we have executed a histological study of the muscle samples to observe the phenomenon from a histopathological point of view. On the same sections, we have carried out a histochemical estimation of thiolic groups, as proteic degradation index.

Methods

Two cycles of experiments have been performed. In each cycle we analysed 20 packaged turkey's thighs: 10 males and 5 females in protective atmosphere and 5 males in stretch. Samples were collected from both normal and darkened areas after pH measurement.
Biochemical analysis:
one gram of muscular tissue was collected and the extracts were prepared by homogenising in 7 ml of buffer (TRIS 10mM, EDTA pH 8 1mM, KCl 80mM) using a "Ultra-turrax t25" homogeniser (3 cycles of 20 sec.), modifying Kranen method. After centrifugation (1600 X g, 15 min.) the supernatant was filtered and then ultra centrifuged (100000 X g, 45 min), after the addition of mono and dibasic potassium (final conc. 3M) to facilitate haemoglobin precipitation. The supernatant was then read by spectrophotometer in a wave-length range from 400 to 700 nm, revealing globins in a different oxidation form from oxymyoglobin. The myoglobin quantitative determination was obtained adding 100 µl of $K_3Fe(CN)_6$ to 900 µl of extracts to obtain cyano-metmyoglobin; reading the sample at 540 nm the concentration can be determined considering $[M] = (A_{540}-A_{700}) \times d/\varepsilon_{540}$, where A_{700} is the sample absorbance at 700nm (turbidity correction), d is the optical path and $\varepsilon_{540\ \tau\eta\varepsilon}$ the molarity extinction coefficient.
The evaluation of α and ε-aminic groups was performed adapting the procedure of Church and the content of ABVT was carried out according to the method published on the U.E. G.U., n° L97 , 29/04/1995.
Lipid oxidation was investigated with the determination of Thiobarbituric Acid Reactive Substances (TBARS), modified with the introduction of an antioxidant (BHA) and by putting a limit to distillation time and to the volume of distillate (50 ml) according to Novelli.
Western-immunoblotting:
10 µl of each sample's proteic extract diluted 1:10 were run with a 12% polyacrylamide in SDS-PAGE. Electrophoresed proteins were then electro-blotted onto a nitrocellulose filters. The filters, after saturation with 2% bovine gelatine, were immunologically revealed by incubation with goat anti myoglobin polyclonal antibodies. Specific enzyme-labelled anti-goat antibodies were then used to bare goat immunoglobulins, with the successive addition of a NBT-BCIP solution.
ELISA:
the same proteic extracts were assayed with goat anti myoglobin polyclonal antibodies by solid phase enzyme immunoassays. Plastic wells were coated with the extracts, saturated with 2% bovine gelatin in PBS and filled with 50 µl of the antibody solution; after overnight incubation, 50 µl of an enzyme labelled anti-goat antibody was added and incubated for two hours at room temperature, followed by the

addition of PNPP fresh solution. Bound antibodies were quantitatively measured by optical density reading, in a automatic micro plates spectrophotometer.

Histology:

muscle samples were collected, fixed in buffered formalin (pH 7.3) and routinely embedded in paraffin. Longitudinal and transversal sections (4µm) were stained with haematoxylin and eosin (HE) for histopathological examination. Thiolic groups were histochemically detected by incubating (3 times /10min) the rehydrated slides in a solution of 0.1% potassium ferricyanide (25 ml) + 1% ferric sulfate (75 ml), according to Chèvremont and Frèderick. Images elaboration was performed with a computerised image analysis system (NIH Image 1.61 Program).

Results and discussions

Darkening process was observed only in male samples packaged in protective atmosphere, with an incidence of about 80% during the whole shelf-life. No consistent pH differences were found among all the samples.

Spectrophotometric analysis showed a sensibly higher concentration of myoglobin in males (average 1,96±0,38 mg/g of tissue) than in females (0,91±0,26 mg/g of tissue). This evidence could be related to the major attitude of males to darkening. Moreover the dark sample's spectra showed a sensible tendency to assume the outline of the metmyoglobin typical ones and, in some cases, to flatten their patterns, as a consequence of a probable advanced proteic degradation. ABVT indexes pointed out slightly higher values in the darkened areas than in the apparently normal ones (64,7±7,6 versus 59,4±6,4 nitrogenous mg/100g of tissue), suggesting a stronger generalised degradation of the tissue associated to the darkening process. This could appear in contrast with the α,ε–amino groups indexes, lower in the dark areas than in the normal ones (6503±350 versus 7143±210 µg equivalent glycin/g of tissue). These data could be explained admitting a quicker further degradation of the molecules carrying α,ε–amino groups deriving from a first proteolysis, that are converted in different kinds of amines. Significativity was obtained by coupled Student's test. The female samples showed always lower values (6184±186 µg equivalent glycin/g of tissue; 43±10,8 nitrogenous mg/100g of tissue) as well as all the stretch packaged samples (6525,5±517 µg equivalent glycin/g of tissue; 43,9±8,4 nitrogenous mg/100g of tissue). A confirm of the advanced state of oxidation and molecules degradation of the dark areas derives from TBAR analysis: very higher values are associated right to the brown zones of the males' muscles (7,4 µg MDA/g of tissue versus 2,6 of the normal areas). As above, protective atmosphere packaged female and stretch packaged samples got low values (< 2 µg MDA/g of tissue).

Samples analysed with western immunoblotting revealed different bands; in particular darkened samples corresponded to bands with a lower intensity than the ones obtained from normal area withdrawals. These evidences revealed a decreasing of myoglobin amount in darkened tissues, confirming the oxidative proteolysis thesis. Contrarily, ELISA showed higher values in darkened samples. This contrast can be explained with the above demonstrated major degradation of the darkened tissue; in fact, the degradation implies a disordered creation of low molecular weight species that could aspecifically interfere with the polyclonal antibodies. This problem is avoided in immunoblotting by the previous electrophoretic run that removes the mentioned molecules without modifying the proteic fraction mobility.

Female samples, according to the lower myoglobin amount, showed a lower intensity band than the male ones, and these results were confirmed by ELISA tests, excluding an experimental artifact.

Histochemical staining for SH groups was significantly stronger in darkened samples than in controls; in particular, the intensity of the reaction appeared to increase from the central to the superficial area of the sections. These results confirm the proteic degradation of the darkened tissues, starting from the external fibres, mainly exposed to the atmosphere.

Histology investigation showed normal samples with regular muscular structure and evident striated fibres (93-100%), while high variability in fibre size and less evident striatures was highlighted in darkened samples. In the latter was also found a higher number of giant cells, oval or round shaped, with homogeneous cytoplasm and a larger cross-section.

Conclusions

Taken together these results suggest a strength link between the darkening phenomenon and the generalised degradation of the muscle's molecules. Such phenomenon seems to be strictly related to the higher presence of oxidative gasses in the protective atmosphere, such as oxygen, able to interfere with the tissue components. In particular, besides fatty acids, myoglobin is strongly involved in the aforesaid process; in fact, its concentration appear to be determinant in the insurgence of tissue darkening. Moreover, the strength of the oxidation process is also demonstrated by the histological alteration that could be related to the damages occurring in the cellular ultrastructures as a consequence of the molecular degradation of the whole tissue.

Pertinent literature

Beccari N., Mazzi V. (1966). *Manuale di Tecnica Microscopica 6° ed., Soc. Ed. Libraria, pagg.* 137 e 230;– Church F.C., Swaisgood H.E., Porter D.H., Catignani G.L. (1983). *J. Dairy Sci.* 66: 1219-1227.– Guidi A., Castigliego L., Benini O., Gaspari P., Gianfaldoni D. (2001). Atti *XI Convegno AIVI*;– Kranen R.W., Van Kuppevelt T.H., Goedhart H.A., Veerkamp C.H., Lambooy E. Veerkamp. J.H. (1999). *Poult. Sci.* 78(3): 467-76;–Marleen B.E., De Grot L., Des Tombe A.L., Van der Laarse W.J. (1988). *The J. Of Histochemistry and Cytochemistry*, 46 (9): 1077–1084;– Novelli E., Zanardi E., Ghiretti G.P., Campanini G., Dazzi G., Madarena G., Chizzolini R. (1988) *Meat Science,* 48 (1): 29–40; – Remignon H., Zanusso J., Gaëlle A., Babilé R. (2000). Occurrence of giant myofibres according to muscle type, pre- or post-rigor state and genetic background in turkeys. *Meat Science,* 56(2000): 337-343;.–Shikama K. (1985). *Experientia* 41(6): 701-706;– Shikama K., Matsuoka A..(1989). *J. Mol. Biol.* 209: 489-491.