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Structural changes of myoglobin in pressure-treated pork meat probed by resonance Raman spectroscopy

Hainer Wackerbarth [a,*], Uwe Kuhlmann [b], Filip Tintchev [a], Volker Heinz [a], Peter Hildebrandt [b,*]

[a] German Institute of Food Technologies, Prof.-von-Klitzing-Str. 7, D-49610 Quakenbrück, Germany
[b] Technische Universität Berlin, Institut für Chemie, Sekr. PC 14, Straße des 17, Juni D-10623, Germany

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ABSTRACT

Pork meat was pressurised at 600–700 MPa under conditions applicable for non-thermal food preservation and studied by resonance Raman spectroscopy with 413-nm excitation to probe selectively myoglobin, which is the origin of the red colour of meat. The spectra of intact, non-pressurised meat tissue exclusively display the resonance Raman bands of the ferrous deoxy-form of myoglobin whereas upon pressure treatment a new six-coordinated low spin ferrous species is formed (>60%), that is assigned to a bis–histidine complex including the distal histidine 64. This structural change is associated with a shift of the electronic transitions of the haeme and thus affects the colour of the meat. In contrast, solutions containing myoglobin extracted from pressurised and non-pressurised pork meat give rise to resonance Raman spectra characteristic of the ferrous oxy-form of myoglobin, evidently due to the accessibility of the proteins for oxygen in solution. Upon pressure treatment of the extracted myoglobin solution, the oxy-form is partially converted to the met-(like) ferric form implying a pressure-induced oxidation of the haeme. Thus, this structural transition does not only cause a colour change but also may initiate unwanted oxidative side reactions involving further components of meat. Evidently, such effects can be largely avoided when the oxy- to deoxy-myoglobin ratio is kept small prior to pressure treatment.

1. Introduction

As an alternative to thermal processing, meat can be preserved by high pressure treatment, which does not affect flavours and vitamins (Heinz & Knorr, 2002; Töpfl, Mathys, Heinz, & Knorr, 2006). Generally, high pressure processing applied in food industries is based on pressures up to 600 MPa. Such pressures may alter phase transitions of membranes as well as hydrogen bonds and hydrophobic interactions of biomolecules, but do not affect covalent bonds of molecules. Thus, mainly relatively weak intra- and intermolecular interactions related to the secondary, tertiary and quaternary structure of proteins may be perturbed, including interactions between polar groups of the amino acid of the protein and bound water. Such effects have been shown to induce changes in the colour of red muscle from pork and beef even at 5–10 °C (Carlez, Veciana-Nogues, & Cheftel, 1995). However, the underlying molecular processes are not yet fully understood.

In this work we have addressed this issue by focussing on the pressure-response of myoglobin (Mb), which is responsible for the colour in meat. Structural changes of Mb in solution induced by high pressure have been studied by various techniques such as infrared and UV–vis absorption spectroscopy (Cheftel & Culioli, 1997). Alterations of the haeme structure including a change in the spin and coordination state have been monitored by resonance Raman (RR) spectroscopy (Alden et al., 1989). In contrast, possible pressure-induced structural changes of Mb within the intact meat tissue have not yet been analysed. Recently, we have observed a coupling between the oxidative degradation of astaxanthin and reduction of met-myoglobin (metMb) in pressurised smoked salmon tissue using RR spectroscopy (Tintchev et al., 2009). This technique is particularly suitable to probe Mb in tissues since it can selectively probe the vibrational spectrum of the haeme by excitation in resonance with an electronic transition, whereas the remainder of the protein and meat matrix does not contribute to the spectrum. Here we have applied RR spectroscopy to characterise structural changes in the haeme pocket of Mb directly in non-treated and pressurised pork meat. The results, which are compared with those obtained for Mb in solution, contribute to the understanding of the pressure-induced processes of Mb in intact meat tissues and in solution. The spectroscopic studies of meat were complemented by instrumental colour analysis which allows for evaluating the visual appearance and the colour of objects through analysing the reflected and transmitted light.

* Corresponding authors. Fax: +49 54 31183114 (H. Wackerbarth), fax: +49 30 31421122 (P. Hildebrandt).
E-mail addresses: h.wackerbarth@dil-ev.de (H. Wackerbarth), Hildebrandt@chem.tu-berlin.de (P. Hildebrandt).

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2. Material and methods

2.1. Materials

Pork meat from the muscle *Longissmus dorsi* was obtained from a local supermarket and used for RR spectroscopic experiments without and after high pressure treatment. To extract Mb from untreated and pressurised samples, the meat mixed with equal amounts of ice (de-mineralised water) was chopped and then homogenised with an Ultra-Turrax. The meat mash was centrifuged (Cyrofuge 8000, Heraeus) at 35,000g and 3 °C for 15 min and the supernatant was filtered with a (MN 615) filter according to Bünnig and Hamm (1969). This supernatant solution, denoted as "meat extract", was used without further purification for UV–vis absorption and RR spectroscopic measurements.

As a reference, isolated horse heart Mb (Sigma–Aldrich) was measured from aqueous phosphate buffer, pH 7. Addition of sodium dithionite (Merck) to the solution led to the conversion of met-myoglobin (metMb) to deoxy-myoglobin (deoxyMb). Excess dithionate was removed by a Econo-Pac 10DG desalting column (Biorad). In contact with air, deoxyMb bound molecular oxygen to form oxy-myoglobin (oxyMb).

2.2. High pressure treatment

Mb solutions, pork meat extracts, and cylindrically sliced pork meat (d = 32 mm, l = 28 mm) were vacuum-packed and pressurised at 0.1–700 MPa at ambient temperature for up to 10 min. Due to the compression, the temperature increased by ca. 3 °C per 100 MPa (at 700 MPa ca. 21 °C). Fifteen seconds were needed to reach 600 and 700 MPa. The high pressure treatment was carried out in a custom-made lab-scale high pressure system (High Pressure Research Center, Unipress Equipment Division, Sokolowska 29/37, Warsaw, Poland). Maximum design pressure for the system was 1000 MPa at an operating temperature range of −25 to 100 °C. The volume of the sample holder was 0.75 L. A 1:1 mixture of water and propylene glycol (1,2-propanediol) was used as a pressure transmitting medium. Additional experiments were carried out in a pilot plant for the high pressure processing (NC-Hyperbaric, Spain) with a volume of 55 L, allowing pressurisation of 350 kg/h. A pressure of 600 MPa is built up within 3–4 min.

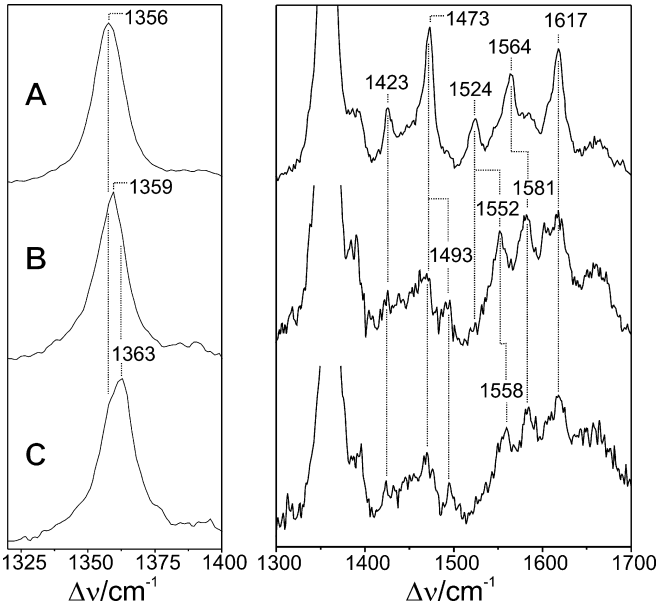

Fig. 1. RR spectra of meat (A) before pressure treatment, and after pressurisation (B) at 600 MPa for 10 min and (C) at 700 MPa for 1 min. The spectra are scaled with respect to the strongest band for a better visualisation of the spectral changes in the ν_4 band region (left panel) and the coordination and spin marker band region (right panel). The spectra were obtained with 3 × 30 s accumulation time and a laser power of 1.3 mW at the sample. Further experimental details are given in the text and Supporting Information.

2.3. Spectroscopic measurements

The absorption spectra were recorded on a Unicam UV2 spectrophotometer with a spectral resolution of 0.5 nm and a Lambda 25 (Perkin Elmer) with a spectral resolution of 1 nm. The path length of the measuring cell was 10 mm and the scan speed 600 nm/min for all experiments.

RR spectra were measured with the 413-nm excitation line of a Kr^+-laser (Coherent Innova 400) using a confocal Raman spectrograph (LabRam Jobin-Yvon/Horiba) equipped with an electronically cooled CCD camera. The spectral resolution was 3 cm^{-1}. The incident laser beam (3 mW) was focused on the sample that was placed in a rotating cell for measurements of proteins in solution and meat extracts. For measurements of meat pieces a stationary sample holder was employed. Under these conditions, laser induced damage of the samples such as photodissociation of oxygen from oxyMb can be ruled out since comparative measurements of meat contained in a rotating device revealed essentially the same results albeit with a lower signal-to-noise ratio.

2.4. Colour measurements

Colour measurements of meat have been performed using a colourimeter CR 110 from Minolta, employing in the specular component mode that includes the diffuse and specular reflection. In this way, the colour can be evaluated independent of the surface structure. Illumination was performed by D65 (standard illuminant defined by the International Commission on Illumination), which is intended to represent average daylight and has a correlated colour temperature of approximately 6500 K. The results are expressed within the $L^*a^*b^*$ colour space which is based on the uniform distribution of colours and is very close to human perception of colour. L^* is the luminance or lightness component, ranging from 0 to 100, and the parameters a^* (from green to red) and b^* (from blue to yellow) are the two chromatic components, which range from −120 to 120 (León, Mery, Pedreschi, & León, 2006).

3. Results and discussion

3.1. Pressure-effects on myoglobin in meat tissue

The red colour of meat is caused by the haeme protein Mb which exhibits a strong electronic absorption band (Soret transition) between 400 and 440 nm, depending on the ligation and redox state. Thus, 413-nm excitation affords intense RR spectra of Mb both from the protein in solution and from meat (Fig. 1, Supporting Information, Fig. S1). The frequencies of the bands in the region between 1300 and 1700 cm^{-1}, specifically the oxidation marker mode ν_4, allows determining the coordination and oxidation state of the haeme iron (Siebert & Hildebrandt, 2007; Spiro, 1985). In the reduced, oxygen-free form, i.e., deoxy-myoglobin (deoxyMb), the frequency is observed at 1356 cm^{-1} whereas in the oxidised oxygen-free form, met-myoglobin (metMb), this mode shifts up to 1370 cm^{-1}. The lower frequency in the ferrous state is attributed to the increase of electron density in the π^*-orbitals that specifically weakens the force constants of the C–N stretching coordinate, the main contribution to the ν_4 mode. Both in deoxyMb and metMb, the haeme iron is in a high spin configuration with His93 serving as the proximal ligand. The distal position remains vacant in the deoxyMb form whereas a water

molecule can coordinate at the sixth coordination site in metMb. These configurations are reflected by the spin- and coordination marker bands above 1450 cm^{-1}, particularly of the mode ν_3, which is found at 1473 and 1481 cm^{-1} in deoxyMb and metMb, respectively. Upon oxygen binding to the ferrous state (oxy-myoglobin – oxyMb), electron density is partially transferred to empty antibonding orbitals of the ligand such that the ν_4 frequency shifts up to 1377 cm^{-1} that is even higher than that of metMb (1370 cm^{-1}, *vide infra*). Also the other marker bands are found at higher frequencies such that, altogether, the analysis of this spectral region allows for an unambiguous identification of the Mb state. Consequently, we conclude that the RR spectrum of meat mainly originates from deoxyMb (Fig. 1). After pressurising, distinct spectral changes are observed in the RR spectrum. The overall RR intensity decreases (Supporting information, Fig. S1), accompanied by drastic changes in the band positions and intensities of the marker bands (Fig. 1). Here a closer inspection of the region between 1420 and 1620 cm^{-1} reveals the intensity decrease of the bands at 1473 (ν_3) and 1564 cm^{-1} (ν_2) which are replaced by bands at 1493 (ν_3) and 1581 cm^{-1} (ν_2). These frequency upshifts indicate the (partial) transition from the five-coordinated high spin (5cHS) deoxyMb to a six-coordinated low spin (6cLS) configuration under preservation of the (ferrous) oxidation state. This conclusion is supported with changes of the ν_4 envelope that shows a slight upshift of the peak maximum from 1356 cm^{-1} to 1359 and 1363 cm^{-1} in spectra of meat previously pressurised at 600 and 700 MPa, respectively. Also the ν_{38} mode at 1524 cm^{-1} (non-pressurised meat) undergoes a distinct upshift to 1552 cm^{-1} (600 MPa) and 1558 cm^{-1} (700 MPa). Only the band at 1617 cm^{-1} originating from the vinyl stretching remains essentially unchanged. Although the relative RR cross-sections of the bands of both Mb species are not known, one may take the vinyl stretching as a reference band for estimating the portion of deoxyMb conversion on the basis of the relative intensity of the 1473-cm^{-1} band. Thus, one obtains that more than 60% of deoxyMb is converted to the 6cLS ferrous Mb upon pressurisation with both 600 and 700 MPa. The nature of the second axial ligand is not clear *a priori*. A possible candidate is His64 which is located in the distal part of the haeme pocket. Indeed, the RR spectral parameters of the non-native 6cLS ferrous Mb form resemble those of the bis–His-coordinated 6cLS ferrous cytochrome *c* (Oellerich, Wackerbarth, & Hildebrandt, 2002). Furthermore, a pressure-induced formation of bis-coordinated haeme in Mb has in fact been previously discussed by Ognumola, Kauzmann, and Zipp (1976).

These changes of the RR spectra are paralleled by colour changes of the meat as determined by instrumental colour analysis. The results reveal that high pressure treatments cause an in-

Table 1
Colour analysis of pork meat at ambient pressure and after high pressure processing.

Pressure	Lightness (L^*)	Redness (a^*)	Yellowness (b^*)
Ambient	56.7 ± 1.4	10.1 ± 0.6	6.0 ± 1.4
6000 bar/10 min	76.6 ± 0.8	3.0 ± 0.4	7.5 ± 0.3

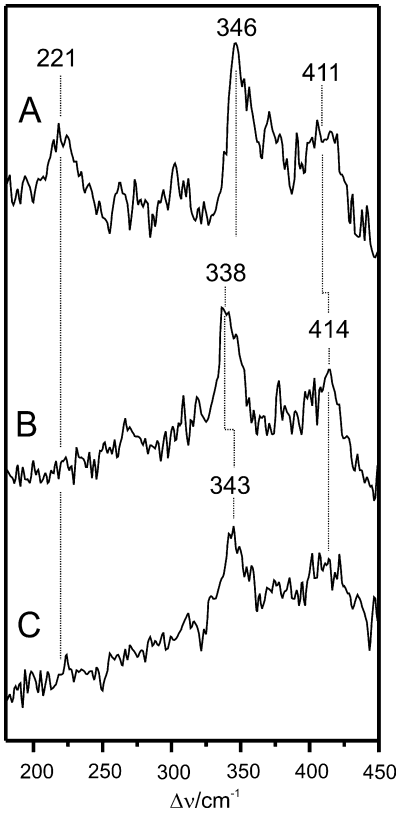

Fig. 2. Low frequency RR spectra of meat (A) before pressure treatment, and after pressurisation for 10 min at (B) 600 MPa and (C) 700 MPa. The spectra are scaled with respect to the strongest band. The spectra were obtained with 3 × 30 s accumulation time and a laser power of 1.3 mW at the sample. Further experimental details are given in the text.

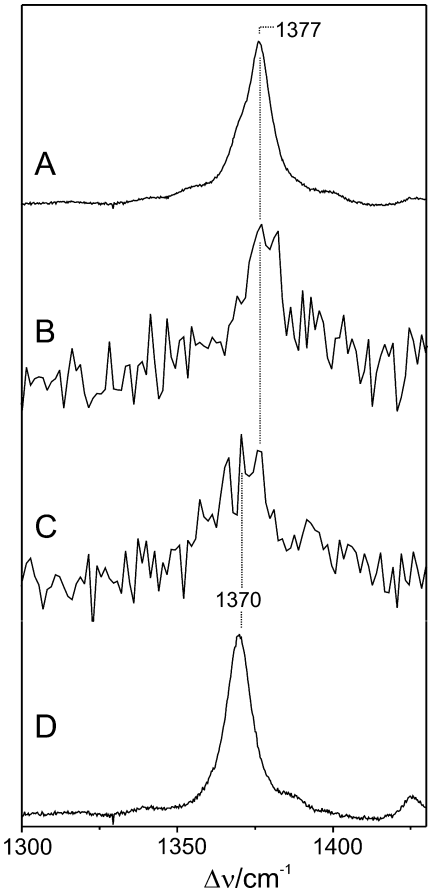

Fig. 3. RR spectra of (A) oxyMb and (D) metMb in solution (pH 7.0), compared with the RR spectra of (B) supernatants obtained from pressurised meat (600 MPa for 10 min) and (C) pressurised supernatants (600 MPa for 10 min) of untreated meat. The spectra are scaled with respect to the strongest band. The accumulation time was 5 × 60 s for the spectra A and D and 3 × 60 s for the spectra B and C. The laser power at the sample was 1 mW for A and D and 2 mW for B and C. Further details are given in the text.

crease in lightness (L^*) and yellowness (b^*) whereas the redness (a^*) of pork meat is decreased (Table 1), evidently related to the intensity decrease of the intensity of the Mb marker bands in the RR spectrum (*vide supra*, Supporting information, Fig. S1).

The pressure-induced conformational transition of Mb is also reflected in the low frequency of the RR spectra of meat (Fig. 2). In the 5cHS deoxyMb, the Fe–N(His) stretching is RR-active and observed at 221 cm^{-1}. After pressurisation of meat to 600 and 700 MPa, this band cannot be detected anymore. This finding further supports the assignment of the 6cLS ferrous Mb form to a bis–His-coordinated species since for such a coordination pattern the Fe–His stretching is replaced by a symmetrical His–Fe–His stretching at distinctly higher frequencies (>300 cm^{-1}) and only very low RR activity upon 413-nm excitation such that it would not be detectable in the present spectra of meat. Further bands in the low frequency region are only slightly affected by pressure treatment and may reflect minor changes in the protein-cofactor interactions associated with the coordination by His64. Note that in previous studies on the isolated deoxyMb, Galkin, Butcher, Tabirian, and Schulte (1997) have shown that the Fe–N(His) stretching mode is a pressure-sensitive marker for the haeme environment up to 180 MPa.

3.2. Pressure-effects on myoglobin solutions

In contrast to the present findings, previous studies indicated a conversion of oxyMb to an (unfolded) ferric form upon exposing extracts of beef meat to pressures higher than 300 MPa (Cheftel & Culioli, 1997). This discrepancy may either result from the different type of meat, i.e., beef vs. pork, or from the different pressure-response of meat extracts and intact meat tissue. We have, therefore, extended the spectroscopic studies to Mb solutions extracted from pork meat after and without pressure treatment. The RR spectrum of the extract solution obtained from pressurised meat is dominated by a band at 1377 cm^{-1} originating from the ν_4 mode of oxyMb (Fig. 3B) as shown by comparison with the reference spectrum of a pure oxyMb solution (Fig. 3A). These findings imply that the distribution between deoxyMb and oxyMb is mainly controlled by the accessibility of the proteins for molecular oxygen, which is significantly higher for the meat extract solutions (oxyMb – Fig. 3B) than for intact pieces of meat tissue (deoxyMb –Fig. 1A). Within the signal-to-noise ratio, extract solutions of non-treated meat afford a spectrum (not shown) that is very similar to that in Fig. 3B. The RR spectroscopic data are in agreement with the UV–vis absorption spectra (Fig. 4) that, as well, indicate oxyMb to be the prevailing Mb state but furthermore reveal small contributions characteristic of metMb in the spectrum of extract solutions of pressure-treated meat. The contribution of this ferric high spin species, which is identified on the basis of the Q-bands at 520 and 549 nm and the blue shift of the Soret band (Fig. 4), as well as of the weak band at 629 nm (Supporting information, Fig. S2), strongly increases upon pressurisation of meat extract solutions whereas the characteristic absorption bands of oxyMb at 542 and 579 nm cannot be detected anymore. This transition is also reflected in the RR spectrum which displays a downshift of the ν_4 band to ca. 1370 cm^{-1} (Fig. 4C), a position very similar to that of metMb (Fig. 4D). Thus, the present spectroscopic data demonstrate the pressure-induced dissociation of oxygen from the haeme and the concomitant oxidation of the central iron. These results are in line with the conclusions drawn by Cheftel and Culioli (1997) inasmuch as the RR spectrum of metMb and a denatured (unfolded) Mb species are likely to be similar.

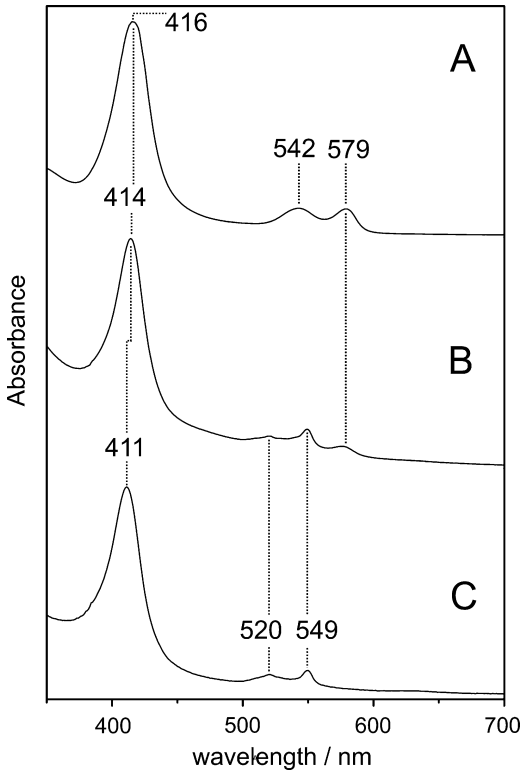

Fig. 4. UV–vis absorption spectra of (A) an extract solution from untreated meat, (B) an extract solution from pressurised meat (600 MPa for 10 min), and (C) an extract solution of untreated meat after pressurisation at 600 MPa for 10 min. The spectra are scaled with respect to the strongest band. Further details are given in the text.

4. Conclusions

The present results strongly suggest that pressure treatment of oxyMb causes the formation of metMb and possibly further denatured ferric Mb species. These ferric Mb forms are not only undesired due to their brownish colour but may also initiate oxidative degradation of other meat components such as lipids. In this respect, the low oxyMb/deoxyMb ratio in meat slices is advantageous since pressure treatment of deoxyMb does not cause a change in the oxidation state, at least not within the intact meat tissue. Although the pressure-induced conversion of deoxyMb to a bis–His coordinated ferrous Mb may as well cause colour changes, it is likely to be chemically inert and does not trigger degradation processes of other meat components.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.01.027. Supporting information includes further figures with RR and UV–vis absorption spectra of meat tissue and extract solutions, respectively.

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